The putative oncogene *EEF1A2* and its role in breast and ovarian cancer

Victoria Tomlinson

Thesis submitted for the degree of
Doctor of Philosophy
The University of Edinburgh

2007

Contents

Chapter 2: Materials and Methods

Chapter 3: eEF1A2 is expressed in two-thirds of breast cancers

Chapter 4: eEF1A2 is highly expressed in ovarian clear cell carcinomas

Chapter 5: The mechanism of overexpression of eEF1A2 in ovarian cancer

Chapter 6: The role of eEF1A2 in oncogenesis

Chapter 7: Discussion

Appendix:

Tomlinson, V.A.L, Newbery, H.J., Wray, N.R., Jackson, J., Larionov, A., Miller, W.R., Dixon, J.M. and Abbott, C.M. (2005) Translation elongation factor eEF1A2 is a potential oncoprotein that is overexpressed in two-thirds of breast tumours. *BMC Cancer*, **5**, 113.

Tomlinson VAL, Newbery HJ, Bergmann JH, Boyd J, Scott D, Wray NR, Sellar GC, Gabra H, Graham A, Williams AR, Abbott CM. (2007) Expression of eEF1A2 is associated with clear cell histology in ovarian carcinomas: overexpression of the gene is not dependent on modifications at the EEF1A2 locus. Br J Cancer, **Apr 17**, [Epub ahead of print].

List of Figures

List of tables

Declaration

I declare that I composed this thesis and that all the work contained within it is my own, unless clearly stated otherwise.

Victoria Tomlinson

Acknowledgements

There are so many people who have helped me throughout my PhD. My apologises to anyone I don't mention by name. First of all I would like to thank Dr. Cathy Abbott for her support and guidance over the past three years, I couldn't have asked for a better supervisor. Thank you also to all the Abbott group members past and present including, Jean, Helen, Julia, You-Ying, Permphan, Miriam, and Jan for all your help and some laughs too. Many people in both the Medical Genetics section and the Sir Alastair Currie lab have been extremely kind and helpful over the past three years. I would especially like to thank Ann-Marie and Laura Hyndman for help with the FACS analysis and Kate and Marion for all their help, Dr Alexey Larionov for help with real-time RT-PCR, Ann Doherty for cell culture advice, Dr. Ben Pickard and Jennifer Doig for microscope training, as well as Heather, Rosemary, Susan and Helen for admin support and Stewart Morris and Steve Cass for computing support. Naomi Wray and Andrea Christoforou have given me help with statistics and I am very grateful to them for their help. Finally, I would like to thank Alison Condie for sequencing.

Many people have also provided me with expertise and valuable tumour samples. I would like to thank Professor William R. Miller and Mr Mike Dixon as well as Dr. Alexey Larionov, Dr. Sharon White, Dr. Julliette Jackson, Dr. Jane Macaskil for kindly providing breast tumour samples. Special thanks goes to the pathologists Dr. Alistair Williams and Dr. Dana Faratian for the TMAs, histoscoring and advice – thank you very much. Professor Hani Gabra, Dr. Grant Sellar, Diane Scott and Dr. John Bartlett generously provided me with ovarian cancer samples and helpful advice. I would also like to thank Dr. Catherine Naughton for providing me with RNA and protein samples from a RNA interference assay she carried out.

I would also like to thank all the organisers of the Four Year Wellcome Trust PhD programme. The work was funded by the Wellcome Trust.

A special mention goes to the girls in the PhD office, Jenn, Lorna and Andrea whose friendship made my PhD more enjoyable. Last but definitely not least I would like to thank my mum and dad for all their support over my years at university, and Daren for his encouragement, patience and support throughout my PhD.

Abstract

Eukaryotic elongation factor 1A (eEF1A) is a central component of the translational machinery where it performs the GTP dependent transfer of aminoacyl tRNA to the a-site of the ribosome. There are two variants of eEF1A called eEF1A1 and eEF1A2 that are 92% identical at the amino acid level and encoded at two different loci, 6q14 and 20q13 respectively. eEF1A1 is almost ubiquitously expressed except in skeletal muscle, heart and neurons where it is replaced by eEF1A2 during development.

As well as its role in translation eEF1A1 is thought to perform many additional non-canonical functions. These include cytoskeletal remodelling, apoptosis, susceptibility to transformation and senescence. There is some evidence that eEF1A2 has different properties from eEF1A1. Importantly eEF1A2 has been identified as a putative oncogene and has been shown to be overexpressed at the RNA level in ovarian cancers. Additionally, *EEF1A2* maps to 20q13 and amplifications in this region are common in breast and ovarian cancer. It is not known whether it is the potential non-canonical function(s) of eEF1A2 that mediate its role in oncogenesis or its function in translation and if these differ from eEF1A1. The aim of my PhD was to investigate the expression of eEF1A2 in ovarian and breast cancers and to try to determine the role that eEF1A2 may be performing in cancer.

I investigated the expression of eEF1A2 in breast and ovarian cancers at both the RNA and protein levels. I identified eEF1A2 overexpression in up to two-thirds of breast cancers and moderate to high levels of eEF1A2 expression were associated with oestrogen receptor positive cancers ($p=0.016$). In ovarian cancers eEF1A2 was found to be expressed in up to one-third of cancers and was highly expressed in clear cell carcinomas. The majority of ovarian cancers analysed showed amplification of the *EEF1A2* locus regardless of whether they expressed eEF1A2, suggesting this is not the only mechanism mediating the overexpression of the elongation factor. Mutation analysis of the exonic sequence of *EEF1A2* in low copy number ovarian cancers lead to the identification of only one sequence variant that would not be

predicted to alter the sequence of the protein but may have an effect on post transcriptional regulation of gene expression. Methylation analysis of the 5' CpG island in *EEF1A2* using bisulphite sequencing of DNA from ovarian cancers overexpressing eEF1A2, not expressing eEF1A2 or DNA from normal whole ovary showed no methylation of EEF1A2 from any of the tissues suggesting methylation is not controlling eEF1A2 expression in ovarian cancer.

Immunofluorescence analysis of the sub cellular localisation eEF1A1 and eEF1A2 in breast (MCF-7) and ovarian (PEO1) cancer cell lines suggests that the two isoforms show a similar diffuse cytoplasmic localisation with some concentration in the perinuclear region of the cell. eEF1A1 and eEF1A2 partially co-localise with tubulin in the perinuclear region and with F-actin in cellular protrusions. Finally analysis of eEF1A2 function in the breast cancer cell line MCF-7 using RNA interference of eEF1A2 showed that partial ablation of eEF1A2 does not alter the rate of proliferation, cell cycle distribution or the percentage of cells in apoptosis. Therefore eEF1A2 is frequently overexpressed in breast and ovarian cancers and this overexpression does not appear to be consistently mediated by gene amplification, mutation or methylation. Although the potential role that eEF1A2 is playing in cancer is yet to be determined it appears unlikely to involve alterations in the cell cycle or apoptosis.

Abbreviations and symbols

ALS	amyotrophic lateral sclerosis
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
BrdU	5-bromo-2'-deoxyuridine
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CCC	clear cell carcinoma
CDDP	cisplatin
CGH	comparative genomic hybridisation
CRUK	cancer research UK
CSOC	epithelial ovarian cancer cell line
Ct	cycle threshold
DAB	diaminobenzidene
DAPI	4',6'-Diamino-2-phenylindole dihydrochloride
DCIS	ductal carcinoma *in situ*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
E_2	17β estradiol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediamenetetraacetic acid
EEF1A1	eukaryotic elongation factor 1A1 (human gene)
eEF1A1	eukaryotic elongation factor 1A1 (protein)
Eef1a2	eukaryotic elongation factor 1A2 (mouse gene)
EEF1A2	eukaryotic elongation factor 1A2 (human gene)
eEF1A2	eukaryotic elongation factor 1A2 (protein)
EGF	epidermal growth factor
eIF4E	eukaryotic initiation factor 4E
EOC	epithelial ovarian cancer
ER	oestrogen receptor
ERE	oestrogen response element
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FIGO	International Federation of Gynecology and Obstetrics
FISH	fluorescent *in situ* hybridisation
Fwd	forward
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GC	guanine cytosine
GDP	guanosine diphosphate
GEF	guanine exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
HCL	hydrochloric acid

HNSCC	head and neck squamous cell carcinoma
HOSE	normal ovarian surface epithelial cells
H_2O_2	hydrogen peroxide
HRP	horse radish peroxidase
HSF1	heat shock transcription factor 1
Hrs	hours
HSP	heat shock protein
IDC	invasive ductal carcinoma
IF	immunofluorescence
IFN	Interferon
IHC	immunohistochemistry
kb	kilobase pairs
kDa	kilodaltons
LOH	loss of heterozygosity
mAChR	muscarinic acetylcholine receptor
MMTV	mouse mammary tumor virus long terminal repeat
mRNA	messenger ribonucleic acid
OAS1	2'5'oligoadenylate synthetase
OSE	ovarian surface epithelium
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline Tween
PCR	polymerase chain reaction
PgR	progesterone receptor
Pi	phosphate
Pol II/III	RNA polymerase II/III
Prdx1	peroxiredoxin-1
PTI-1	prostate carcinoma inducing gene 1
PUM1	pumilio homolog 1 (Drosophila)
Rev	reverse
RIPA	radioimmunoprecipitation
Rpm	revolutions per minute
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
rRNA	ribosomal ribonucleic acid
tRNA	transfer ribonucleic acid
$tRNA^{met}$	methionine charged transfer ribonucleic acid
SD	standard deviation
SDS	sodium dodecyl sulphate
siRNA	short interfering ribonucleic acid
shRNA	short hairpin ribonucleic acid
SNP	single nucleotide polymorphism
RT	reverse transcriptase
SOD1	superoxide dismutase
Taq	thermus aquaticus
TBP	TATA box binding protein
TCTP	translationally controlled tumour protein
TMA	tissue micro array

5'TOP	5' terminal oligopyrimidine tract
UTR	untranslated region
WAP	whey acidic protein
wst	wasted
ZPR1	zinc finger protein 1

Chapter 1 Introduction

1.1 General overview

Eukaryotic elongation factor 1A (eEF1A) is involved in the elongation step of protein synthesis where it performs the GTP-dependent transfer of aminoacyl-tRNA to the A-site of the ribosome. In mammals there are two *EEF1A* genes called *EEF1A1* and *EEF1A2*. The proteins encoded by these genes are 92% identical and 98% similar at the amino acid level (Knudsen et al., 1993). However, the two proteins show different expression patterns: eEF1A1 is almost ubiquitously expressed whereas eEF1A2 expression is restricted to the brain, skeletal muscle and heart (Lee et al., 1992). As well as its role in elongation, eEF1A1 is also thought to perform many other non-canonical functions. For instance eEF1A has been shown to bind actin and tubulin and to perform cytoskeletal remodelling (Owen et al., 1992; Shiina et al., 1994). It is unclear whether eEF1A1 and eEF1A2 perform the same non-canonical functions. Interestingly *EEF1A2,* and not *EEF1A1,* has been identified as a potential oncogene in ovarian cancer (Anand et al., 2002). The existence of two eEF1A variants, eEF1A1 and eEF1A2, is often overlooked in the literature and in lower organisms such as *Saccharomyces cerevisiae* there is no eEF1A2 homologue; therefore when the term eEF1A is used, this refers to the yeast eEF1A protein or when a distinction between eEF1A1 and eEF1A2 has not been made in a published article and cannot be deduced from the experimental technique.

1.2 Protein synthesis

Protein synthesis can be divided into three main stages: initiation, elongation and termination. Each stage is controlled by multi-subunit enzyme complexes; initiation being controlled by eukaryotic initiation factors (eIF), elongation being controlled by eukaryotic elongation factors (eEF) and termination involving eukaryotic release factors (eRF).

Initiation

Initiation involves the assembly of the 60S and 40S ribosomal subunits and methionine charged tRNA at the AUG codon of the mRNA. It begins by the binding

of eIF4F to the mRNA. The initiation factor eIF4F is a multimer of eIF4E, eIF4G, eIF4A and eIF4B. eIF4E binds to the 5' methyl-cap upstream of the AUG codon. Secondary structure in the 5'UTR of a mRNA molecule, due to a high GC content, can hinder eIF4E binding. eIF4A is a helicase, eIF4B also binds the mRNA and eIF4G acts as a scaffolding protein that links eIF4A, B and E together. It is thought that binding of eIF4E is the rate limiting step of translation due to the effects of 5'UTR secondary structure. Binding of eIF4E to the 5' methyl-cap of mRNA is negatively regulated by 4EBP1, 2 and 3. The 40S subunit of the ribosome, bound by eIF3, eIF1, eIF1A, eIF2 and $tRNA^{met}$ forming the 43S preintiation complex is brought to the mRNA by the eIF4F multimer. The ribosome is then thought to scan along the mRNA until the start (AUG) codon is located. This occurs by the recognition of the AUG by the initiator methionyl-tRNA, which is located onto the ribosome P-site by eIF2. It is thought that the scanning ribosome is slowed at the sequence surrounding the AUG site due to increased secondary structure in the coding region compared to the 5' UTR. When bound by GTP eIF2 is active and this GTP is hydrolysed to GDP when the initiation is completed. eIF2B is responsible for GTP exchange on eIF2. The initiation factors then dissociate from the 40S ribosome allowing the binding of the 60S ribosomal subunit.

Elongation

Eukaryotic elongation factors 1A1 (eEF1A1) and 1A2 (eEF1A2), as the names suggest, are involved in the elongation stage of protein translation. eEF1A associates with GTP and in this form it binds amino acylated tRNA and targets it to the A-site of the ribosome. Following ribosome binding, but before codon/anticodon recognition occurs, the bound GTP is hydrolysed to GDP and the resultant eEF1A-GDP molecule dissociates from the ribosome. The free eEF1A-GDP then associates with the eEF1B complex (consisting of three subunits α, β and γ), that has GTP exchange activity and acts to exchange GDP bound on eEF1A for GTP. This then allows eEF1A to begin the cycle again by recruiting a new amino acylated tRNA molecule. Finally, the elongation factor eEF2 is required for the translocation of the peptidyl-tRNA from the A-site to the P-site of the ribosome in a GTP dependent manner. A schematic depicting this process can be seen in figure 1.1.

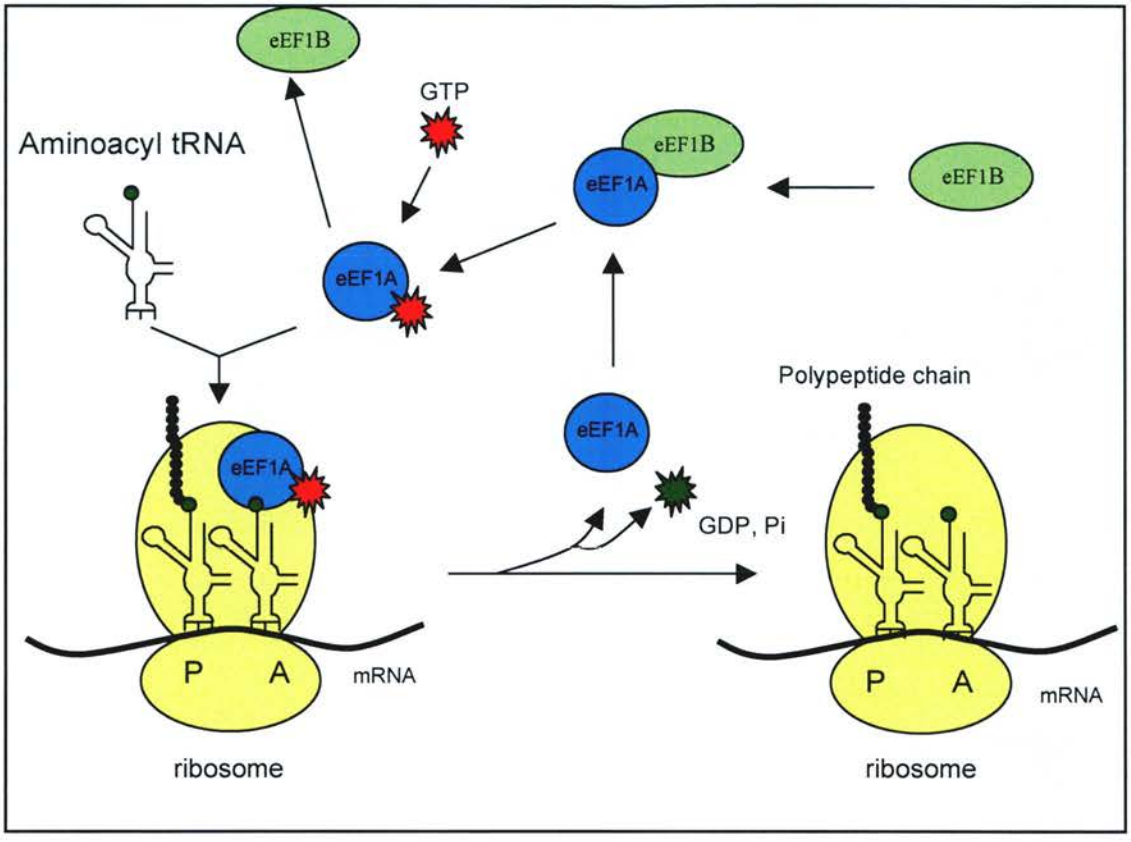


Figure 1.1 Translation elongation, taken from (Abbott and Proud, 2004).

Termination

When a termination codon is present in the A-site of the ribosome the eukaryotic release factor eRF1 recognises this and, in a GTP-dependent manner, the eRF complex promotes the cleavage of the peptidyl-tRNA and release of the ribosome. Mammalian translation is extensively reviewed in (Hershey, 1991) and (Merrick, 1992).

eEF1A1 and eEF1A2 in translation

As the sequence similarity would suggest, the enzymatic activities of eEF1A1 and eEF1A2 in an *in vitro* translation system were found to be similar (Kristensen et al., 1998). However, eEF1A2 displays a higher affinity for GDP than GTP and the opposite is true for eEF1A1 in which the GDP dissociation rate constant is 7 times higher than that of eEF1A2 (Kahns et al., 1998). Kahns *et al.* suggest that this

difference in GDP affinity in eEF1A2 may play a role in one of the non-canonical functions of eEF1A2. For instance, it may reflect the need for eEF1A2 to influence actin binding, as GTP inhibits binding of eEF1A to G-actin (Dharmawardhane et al., 1991). Kahns *et al.* also suggested that the slower rate of GDP dissociation in eEF1A2 could result in an increase in translational fidelity. Mutation of the GTP-binding motifs in *S. cerevisiae* eEF1A, results in an increase in the K_m of eEF1A for GTP and a dramatic reduction in translational fidelity (Carr-Schmid et al., 1999). Therefore the slower rate of GDP dissociation on eEF1A2 could perhaps explain why the expression of eEF1A2 is switched on only in terminally differentiated cell types where translational accuracy would be important for cell viability over a long time period.

Another difference between the two isoforms in protein elongation is that eEF1A2 only interacts weakly with the eEF1B complex in yeast-two hybrid analysis, suggesting this complex is more unstable than the eEF1A1:eEF1B complex and might not function *in vivo* (Mansilla et al., 2002). This suggests that a different factor is involved in nucleotide exchange on eEF1A2. EF1β5a encodes a human tissue-specific isoform of eEF1Bα expressed in brain and skeletal muscle (Pizzuti et al., 1993) but this isoform does not exist in any other species and is unlikely to be a functional guanine nucleotide exchange factor (GEF) (Chambers et al., 2001). The muscarinic acetylcholine receptor (mAChR) M_4, has been demonstrated to accelerate nucleotide exchange on eEF1A2 suggesting this could be the factor responsible for nucleotide exchange on eEF1A2 in the brain (McClatchy et al., 2002). The difference in nucleotide exchange factor for eEF1A2 could be due to the need for specific regulation of translation in the terminally differentiated cell types in which eEF1A2 is expressed.

1.3 The EEF1A genes and proteins

Mammalian *EEF1A1* and *EEF1A2*

There are several *EEF1A*-genes in the mammalian genome but only *EEF1A1* and *EEF1A2* are actively transcribed; the other *EEF1A*-like genes are thought to be retro-pseudogenes originating from *EEF1A1* (Lee et al., 1993b; Lund et al., 1996; Madsen

et al., 1990). *EEF1A1* has been mapped to chromosome 6q14 and *EEF1A2* has been mapped to chromosome 20q13.3 (Lund et al., 1996). *EEF1A2* is 12kb in size, including a 2kb upstream promoter region and is composed of 8 exons and 7 introns. *EEF1A1* is approximately 3.5kb in size and is also composed of 8 exons and 7 introns (Bischoff et al., 2000). The *EEF1A2* promoter contains several potential *cis* regulatory elements including E-boxes, binding sites for the EGR family of proteins and a MEF2 binding site. There is also an Inr element that is thought to mediate transcription initiation (Bischoff et al., 2000). A TATA box has been identified in the promoter region of *EEF1A1* as well as Sp1 and Ap-1 binding sites. The promoter of *EEF1A1* can direct *in vitro* transcription 2-fold more effectively than the adenovirus major late promoter indicating the gene has a very strong promoter (Uetsuki et al., 1989).

The coding regions of *EEF1A1* and *EEF1A2* are very similar (75% identical at the nucleotide level (Knudsen et al., 1993) and the gene structure of the two EEF1A genes is conserved however the 5' and 3'UTRs, introns and upstream promoter regions are very different (Bischoff et al., 2000). The introns of *EEF1A2* are considerably larger than those of *EEF1A1*. The 5' flanking region of *EEF1A2* does not contain a TATA box, which is present in *EEF1A1*, suggesting the regulation of expression is different in these two genes. *EEF1A1* and *EEF1A2* both contain a putative CpG island in the 5' flanking region of the genes that could be involved in the regulation of expression (Ann et al., 1991). The difference in the 5' and 3' UTRs of the eEF1A variants suggests that they may contain different sequence elements involved in RNA processing and stability. Interestingly, *EEF1A1* is a member of the 5' terminal oligopyrimidine tract (5'TOP) gene family. Exon 1 of *EEF1A1* starts with a cytosine nucleotide that is then followed by a stretch of 5 thymidines (Ts) (Uetsuki et al., 1989). Three Ts are necessary in this 5'TOP sequence for high transcriptional activity from the *EEF1A1* promoter and eEF1A1 mRNA has been shown to contain a variable number of Ts (Shibui-Nihei et al., 2003). There is evidence that the 5'TOP sequence is a cell growth dependent *cis*-regulatory element in mammalian mRNA (Kaspar et al., 1992). Many housekeeping genes belong to the 5'TOP gene family including ribosomal protein genes and laminin binding protein

(Kato et al., 1994; Yoshihama et al., 2002). There is no evidence that *EEF1A2* contains a 5' TOP sequence.

The eEF1A1 and eEF1A2 proteins

In a variety of organisms eEF1A belongs to a multigene family and the members of the family are often expressed in a developmental or tissue specific manner. There are three *EEF1A* genes expressed at different developmental stages in *Xenopus laevis* and recent evidence suggests that *Xenopus laevis* also express eEF1A2 (Newbery et al., in preparation). In *Drosophila melanogaster* there are two *EEF1A* genes expressed stage-specifically (Dje et al., 1990; Hovemann et al., 1988). In chicken there was reported to be only one *EEF1A* gene (Wang et al., 1994), however it appears that eEF1A2 is also expressed (Newbery et al., in preparation). In *Saccharomyces cerevisiae* there are two genes TEF1 and TEF2 (Schirmaier and Philippsen, 1984) and in *S. pombe* there are three genes (Mita et al., 1997). These genes again show differential regulation. In *Escherichia coli* there are two EF-Tu genes that show sequence identity.

eEF1A2 was first isolated from rat due to its antigenic similarity to statin, leading to the provisional naming of the protein as S1 (Ann et al., 1991). In humans eEF1A1 and eEF1A2 are 96% similar and 92% identical at the amino acid level, see figure 1.2 (Knudsen et al., 1993). eEF1A1 and eEF1A2 are composed of 462 and 463 amino acids respectively, of which 34 amino acids are different between the two proteins. This degree of similarity in amino acid sequence is perhaps surprising considering there is only a 75% identity in nucleotide sequence between the human *EEF1A1* and *EEF1A2* sequence. This is due to redundancy in the amino acid code. The lack of conservation at the nucleotide level may ensure that recombination between the two genes does not occur.

eEF1A2 peptide sequences from different mammalian species also show a high degree of similarity. The amino acid sequence of rabbit eEF1A2 is identical to human eEF1A2 and has a difference of only one amino acid with mouse and rat eEF1A2 (Lee et al., 1994). The amino acids in rat eEF1A2 that differ from eEF1A1

are totally conserved in mouse and human eEF1A2. This suggests that these amino acid differences confer distinct roles to eEF1A2 and that these functions have been subjected to evolutionary selection (Lee et al., 1994). The differences between the same variants of eEF1A in different species are less than the differences observed between the two variants within the same species.

The functional domains of eEF1A in yeast have been mapped. These include three consensus GTP binding domains (all three of which co-operate to bind GTP) that are highly conserved between eEF1A1 and eEF1A2. This makes the observation by Khans *et al.* of a difference in nucleotide affinity between eEF1A1 and eEF1A2 somewhat surprising. This difference could be due to the amino acid difference at nucleotide 197, which is an asparagine in eEF1A1 and a histidine in eEF1A2 (Kahns et al., 1998). The crystal structure of EF1A·GDPNP from Thermus aquaticus (Song et al., 1999) suggests that the asparagine/histidine residue is close to the guanine nucleotide binding domain. The serine at position 194 forms a hydrogen bond with the guanine ring of the amino acid at position 196 a tryptophan, and stacks onto the guanine base by hydrophobic interaction (Kahns et al., 1998). Therefore a difference at amino acid 197 could also affect nucleotide affinity.

Additionally, regions of eEF1A responsible for actin binding, as well as microtubule interaction domains have been identified in eEF1A from *S. cerevisiae*. It had previously been demonstrated that overexpression of eEF1A in *S. cerevisiae* lead to slow growth, disorganisation of the actin cytoskeleton and accumulation of the cells in G1 (Munshi et al., 2001). Lui *et al.* identified the C-terminal 54 residues as important for eEF1A binding to actin (Liu et al., 2002). Gross and Kinzy used a genetic screen to identify eEF1A mutants with reduced actin bundling activity called N305S and N329S (the regions important for actin bundling are indicated on the protein line up figure 1.2). Expression of these mutants in yeast resulted in an altered actin cytoskeleton but no alteration in translation, thereby separating these two functions of eEF1A (Gross and Kinzy, 2005). The expression of GFP fused, truncated forms of eEF1A from carrot cells in leaf epidermal cells lead to the

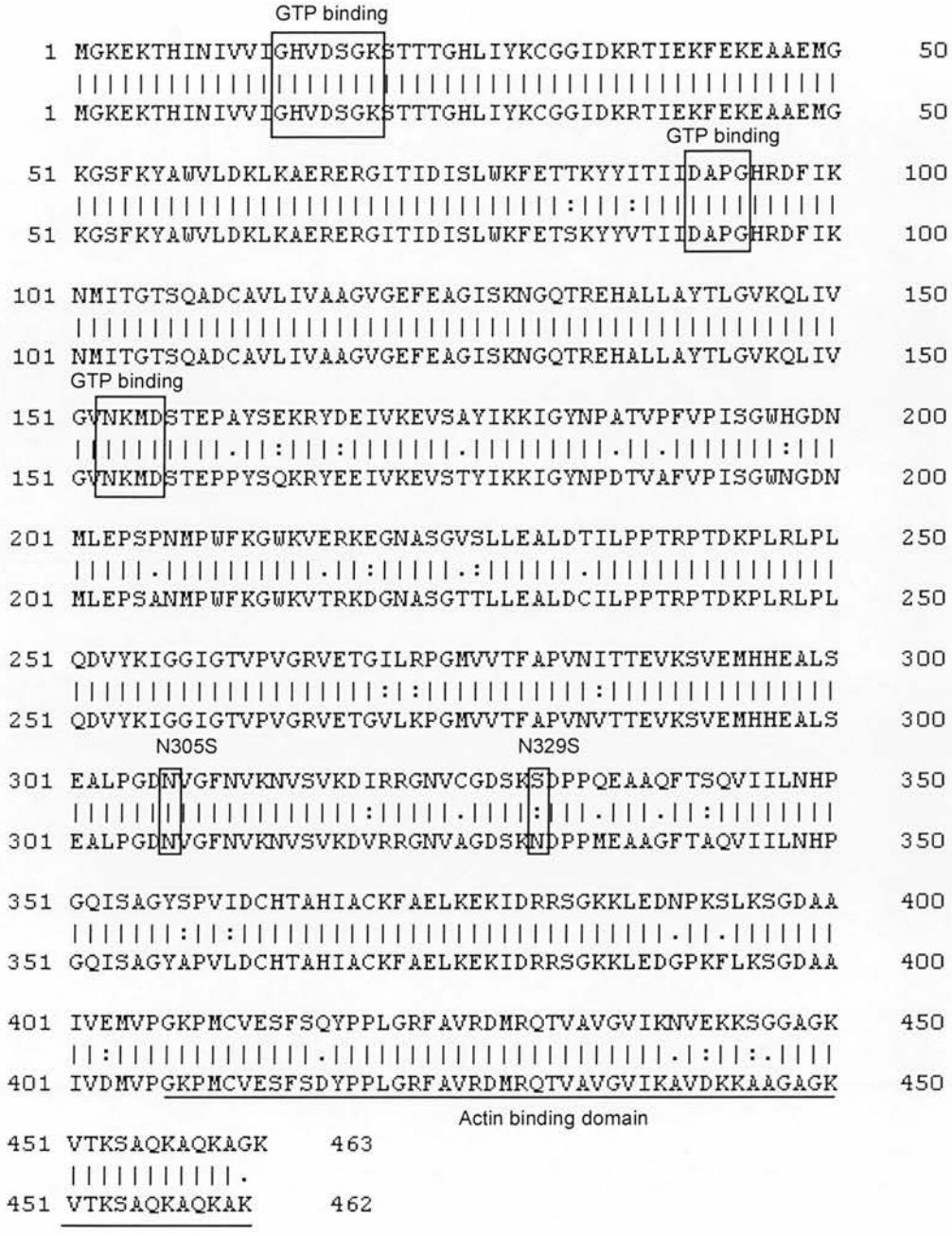


Figure 1.2 Protein line up showing homology between human eEF1A2 (Top line) and eEF1A1 (bottom line). Amino acid identity is indicated by a vertical line, one dot denotes a conservative amino acid difference and two dots denote a semi-conservative amino acid difference. Boxes are shown around the GTP-binding domains showing sequence identity in these regions between eEF1A1 and eEF1A2 (Dever et al., 1987). Mutations in *S. cerevisiae* eEF1A resulting in a loss of actin bundling N305S and N329S (Gross and Kinzy, 2005). Underlined is the actin-binding domain determined by Liu *et al.* (Liu et al., 2002). There is less sequence conservation in the actin binding regions of eEF1A1 and eEF1A2 compared to the regions responsible for GTP-binding.

identification of the third domain of eEF1A (amino acids 318-447) as responsible for interaction with microtubules (Moore and Cyr, 2000).

Post-translational modifications of eEF1A

Seven post-translationally modified residues have been identified on rabbit eEF1A1 using chemical protein sequencing. These modifications were dimethylation of lysine residues 55 and 165, trimethyllysine at residues 36,79 and 318 and the remaining two constituted the addition of ethanolamine to glutamic acid residues 301 and 379 (Dever et al., 1989). Phosphorylation of eEF1A was shown by Davydova *et al.* when mono and polyribosomal fractions of rabbit reticulocytes were incubated with [γ-^{32}P]ATP (Davydova et al., 1984). As well as this, eEF1A1 isolated from rabbit reticulocytes stimulated by phorbol 12-myristate 13-acetate (PMA) has been shown to be phosphorylated by protein kinase C and this phosphorylation increases the activity of eEF1A as measured by poly(U)-directed polyphenylalanine synthesis (Venema et al., 1991a; Venema et al., 1991b).

When the post-translational modifications of rabbit eEF1A2 were compared to those previously reported on eEF1A1 the glycerylphosphorylethanolamine modifications were found to be at the same position as on eEF1A1. Methylation is also found at Lys 55 and Lys 165 but in eEF1A1 these sites are dimethylated whereas in eEF1A2 they are trimethylated (Kahns et al., 1998). It is also possible that the phosphorylation of the two eEF1A variants differs. For example, there are several highly conserved predicted phosphorylation sites in eEF1A2 that are not present in eEF1A1 (Newbery et al., in preparation). Subtle differences in the protein sequences of eEF1A1 and eEF1A2 could result in these differences in post-translational modifications and in turn could contribute to any differences in function, localisation or activity of the variants.

Differential expression of eEF1A1 and eEF1A2

Mammalian elongation factors 1A1 and 1A2 exhibit very different expression patterns. eEF1A1 is near-ubiquitously expressed whereas eEF1A2 expression is restricted to brain, heart and skeletal muscle in rats, mice and humans (Knudsen et al., 1993; Lee et al., 1992; Lee et al., 1995; Lee et al., 1993b). Analysis of

expression of eEF1A2 in the rat brain using *in situ* hybridisation showed it is expressed in the post-mitotic neurons of the cerebral cortex, the motor neurons of the brain stem and the Purkinje neurons in the cerebellum (Lee et al., 1995; Lee et al., 1993a). The expression of the eEF1A variants also appears to be developmentally regulated with eEF1A2 replacing eEF1A1 as the dominant form during late development of the brain, heart and muscle (Lee et al., 1993b). eEF1A2 is the only eEF1A protein expressed in adult mouse muscle and therefore eEF1A1 expression is completely shut off in this tissue (Chambers et al., 1998; Khalyfa et al., 2001). It has been suggested that the rise in eEF1A2 expression and concomitant decrease in eEF1A1 during postnatal development in mice and rat neurons, cardiomyocytes and myocytes is coincidental with the terminal differentiation process (Lee et al., 1995). No mechanism to explain this switching between eEF1A1 and eEF1A2 expression has been identified and it still remains unclear why eEF1A2 is expressed only in long-lasting terminally differentiated cells such as cardiomyocytes, neurons and myofibres. The translational function of the two variants of eEF1A is comparable, and therefore it is reasonable to assume that this switch may occur in order to allow specific cell types to utilize a non-canonical function unique to eEF1A2 or to abolish a non-canonical function specific to eEF1A1. It is also important to note that the above investigations into the expression of the eEF1A variants were carried out at the RNA level using Northern blots and *in situ* hybridisation in rodent and human tissue and therefore the protein level of the variants was not investigated.

1.4 The wasted mouse

One clue to the potentially unique role of eEF1A2 comes from the wasted (*wst*) mouse. This mouse arose spontaneously in the inbred mouse colony HRS/J at the Jackson Laboratories in 1972. These mice are bred as heterozygotes, as these mice are phenotypically normal. The homozygous *wst/wst* mouse shows neurological defects, muscle wasting and immune system abnormalities including a defective response to DNA damage in lymphoid cells. Interestingly, wasted mice are normal until approximately post-natal day 21 at which time they show the characteristic ataxia and tremor phenotype. The mice then lose weight and develop progressive paralysis before dying at round 28 days. Atrophy of the spleen and thymus is also

observed in *wst/wst* mice, resulting in a decrease in the levels of circulating lymphocytes (Shultz et al., 1982).

The wasted phenotype was shown to be caused by a 15.8kb deletion in the *Eef1a2* gene. This mutation is a deletion of the promoter region and first non-coding exon of eEF1A2, resulting in the abolition of transcription of the gene (Chambers et al., 1998). Analysis of the expression of eEF1A2 in wasted and normal control mice indicated that eEF1A1 maintains a normal pattern of expression, whereas eEF1A2 expression is absent in 21-day-old mice. Analysis of eEF1A1 and eEF1A2 expression analysis in wild-type mice between 2 days and 45 days of postnatal development using RT-PCR showed that in skeletal muscle and heart, from 21 days onwards, there is a gradual increase in eEF1A2 levels with a concomitant decrease in eEF1A1 expression. In the brain of mice both isoforms are expressed throughout postnatal development (Chambers et al., 1998). Khalyfa et al also determined the expression pattern of eEF1A1 and eEF1A2 in wild-type (+/+), heterozygous (+/*wst*) and homozygous (*wst/wst*) mice at the protein level using eEF1A1 and eEF1A2 specific antibodies. They observed that in wasted mice a decrease in eEF1A1 is observed in heart, brain and muscle tissues during postnatal development, despite the absence of eEF1A2, and confirmed the loss of eEF1A2 expression in neurons, heart and skeletal muscle (Khalyfa et al., 2001). More recent analysis of expression levels of eEF1A1 and eEF1A2 in the developing wasted and wild-type littermate mouse brain by immunohistochemical and immunofluorescent analysis demonstrated that in wild-type mouse brain eEF1A1 is present in the perikaryon of neurons early in postnatal development and that by day 20 eEF1A1 expression has completely disappeared to be replaced by eEF1A2. In wasted mice this decrease in eEF1A1 expression occurs but the concomitant increase in eEF1A2 is not observed (Pan et al., 2004).

How much of the phenotype of the wasted mouse can be explained by the loss of the translational function of eEF1A2? It is plausible to hypothesize that the wasted muscle phenotype can be attributed to the loss of protein translation in the tissue after the expression of eEF1A1 is switched off. The loss of protein synthesis following

the down-regulation of eEF1A1 in neurons can be used to explain the neurological defects observed in the wasted mouse. Equally, eEF1A2 may be involved in synaptogenesis, as the expression of eEF1A2 coincides with the pattern of synaptogenesis in the rat (Chambers et al., 1998). The mechanism by which ablation of eEF1A2 expression may cause the immune system defects in the wasted mouse is less apparent as expression of eEF1A2 has not been identified in wild-type mouse spleen or thymus. However, there is evidence that the thymus from wasted mice shows an increase in thymocyte apoptosis *in situ* and thymocytes from these mice are more sensitive to apoptotic induction by gamma radiation, heat shock, and dexamethazone. This, the authors suggest, implies that eEF1A2 may be a regulator of apoptosis in thymocytes (Potter et al., 1998).

1.5 Translation and eEF1A in aging and senescence

A decrease in the activity and levels of eEF1A has been associated with the onset of senescence in human fibroblasts (Cavallius et al., 1986). In Drosophila melangaster a similar observation has been made whereby elongation factor 1 (EF1) mRNA and protein levels decrease followed by a decrease in protein synthesis during aging. The rate of initiation and termination in the flies did not decrease during the 70% decrease in protein synthesis, however the rate of elongation declined markedly during aging (Webster and Webster, 1983; Webster and Webster, 1984). In C57BL/6 mice the rate of protein translation is thought to decrease upon aging. The rate of peptide chain elongation, but not initiation, declined substantially in brain, liver, skeletal muscle and kidney in senescent mice compared to young mice (Blazejowski and Webster, 1984). Therefore evidence exists to suggest that a decrease in translation elongation, and eEF1A activity and abundance, could be involved in aging and senescence.

1.6 Translation, eEF1A and cellular proliferation

Insulin is known to stimulate cell cycle progression and rapidly upregulate protein synthesis. Insulin stimulation activates many protein kinases including the multipotential S6 kinase, which has been shown to phosphorylate eEF1α, β, and γ from rabbit reticuloctyes and thereby increase the rate of elongation. Serum-

deprived 3T3-L1 cells showed a 50% reduction in elongation activity compared to serum fed cells and upon insulin administration the elongation activity increased 2-fold (Chang and Traugh, 1997). Phorbol 12-myristate 13-acetate (PMA) is a mitogen and protein elongation activator. PMA activation of cell proliferation results in activation of the protein kinase C family. PKC γ has been shown to phosphorylate eEF1A on threonine 431, as well as the other eEF1 subunits β, and γ, increasing the activity of eEF1 (Venema et al., 1991a). This increase in eEF1 activity is thought to be due to an increase the GDP/GTP exchange activity following phosphorylation by protein kinase C (Peters et al., 1995). Therefore it appears that mitogenic activators upregulate elongation through signalling cascades resulting in the phosphorylation of eEF1A and other eEF1 components.

1.7 The non-canonical functions of eEF1A

As well as its role in translation eEF1A has been shown to perform roles, so called non-canonical functions, in many other diverse cellular processes.

1.7.1 Cytoskeletal remodelling

eEF1A has been shown to bind and bundle actin (Dharmawardhane et al., 1991; Edmonds et al., 1995; Owen et al., 1992) and to sever microtubules (Shiina et al., 1994) and therefore may have a role in the organisation of the cytoskeleton. This interaction with the cytoskeleton may also be important for the compartmentalisation of protein synthesis in the cytoplasm and aminoacyl tRNA channelling (Negrutskii and Deutscher, 1991; Negrutskii et al., 1994). The interaction of eEF1A with the cytoskeleton is further detailed in Chapter 6.

1.7.2 Cell signalling

eEF1A (named PIK-A49 in this assay) can activate phosphatidylinositol 4-kinase in carrot cells (Yang et al., 1993) and phosphatidylinositols regulate many cellular processes including cytoskeletal organisation and membrane trafficking. eEF1A has also been found to interact with the zinc finger protein, ZPR1 by an *in vitro* binding assay using human A431 epidermoid carcinoma cell extract and immobilized GZT-ZPR1 fusion proteins and coimmunoprecipitation. Additionally, immunofluorescent

analysis of subcellular localisation of the proteins in mammalian cells showed that ZPR1 and eEF1A translocated to the nucleus upon mitogenic stimulation with EGF and disruption of this interaction in *S. cerevisiae* leads to the accumulation of the cells in G2/M of the cell cycle (Gangwani et al., 1998). eEF1A has also been identified as a potential binding partner of Akt2 using a GST-Akt2 tail screen of CHOT cell lysates and shown to contain a minimal Akt2 phosphorylation motif, suggesting it could be a substrate of the kinase. This minimal phosphorylation motif, RXRXXT, is located at residues 67-72 of eEF1A and this region is completely conserved between eEF1A1 and eEF1A2 suggesting both variants could be substrates of Akt2. The c-terminal tail of Akt2 was used in the screen to identify binding partners of this isoform and not Akt1 and 3 because this region is an area of variability between the isoforms. β tubulin was also identified as a binding partner of Akt2 in this screen and the authors suggest that eEF1A1, β tubulin and Akt2 may form a complex at sites of cell activity, such as the protrusions of crawling cells (Lau et al., 2006). In a screen for genes whose expression is modulated by blocking the EGFR and Her2 receptors with antibodies on the highly invasive breast cancer cell lines MDA-MB435 and MDA-MB231, eEF1A1 was identified as being down regulated using differential display screening. Talukder *et al.* also observed an increase in eEF1A1 mRNA and protein levels in a variety of cell lines when they were treated with epidermal growth factor (EGF) and heregulin-β1 (HRG) and this activation of eEF1A1 expression by HRG required the SP1 site in the *EEF1A1* promoter and results in promoter histone acetylation (Talukder et al., 2001).

1.7.3 Susceptibility to transformation

Another interesting property of eEF1A1 is that it has been shown to determine the susceptibility of fibroblasts to transformation induced by 3-methylcholanthrene and ultra violet light. Taksuka *et al.* isolated a cDNA clone of eEF1A1 from a BALB/c 3T3 A31 clonal variant that was highly susceptible to transformation. They attributed this effect to eEF1A1 causing the cells to be competent for growth rather than hypersensitive to UV or carcinogens. They also suggested that the ability of eEF1A1 to remodel the actin cytoskeleton may be involved in its ability to increase susceptibility to transformation as fibroblasts showed altered actin organisation.

Additionally, eEF1A1 may simply make these cells growth competent by increasing the rate of translation of genes essential for growth (Tatsuka et al., 1992).

1.7.4 Apoptosis

There is evidence to suggest that eEF1A1 may play a role in apoptosis. Duttaroy *et al.* observed that high levels of eEF1A1 in mouse fibroblasts caused the cells to become pro-apoptotic upon serum starvation. Conversely, when levels of eEF1A1 were low in these cells they were in the mode of anti-apoptosis. Additionally, fibroblasts overexpressing eEF1A1 did not show an altered rate of protein synthesis suggesting that the potential role of eEF1A1 in apoptosis is independent of its function in protein synthesis (Duttaroy et al., 1998). The pro-apoptotic activity of eEF1A1 has also been observed in a rat embryonic heart cell line, where hydrogen peroxide-induced apoptosis resulted in an increase in the level of eEF1A1 and anti-sense eEF1A1 cDNA protected these cells from apoptosis, suggesting eEF1A1 upregulation is required for the execution of apoptosis induced by oxidative stress. This rapid increase in eEF1A1 levels upon hydrogen peroxide treatment was shown to be mediated by an increase in translation and not transcription of eEF1A1. The authors suggest that the 5'TOP sequence in eEF1A1 may play a role in this rapid translational upregulation, by directing the mRNA into an inactive mRNA-ribonucleoprotein pool or into a translationally active polyribosome pool (Chen et al., 2000). A mouse erythroleukemic cell line that expresses temperature-sensitive p53 and goes into apoptosis when cultured at 32°C was used in a screen of genes associated with p53-mediated apoptosis. Expression of eEF1A1 was identified as being upregulated by p53 activation and *EEF1A1* was shown to contain three conserved and functional p53-response elements. They suggest that it could be the microtubule severing activity of eEF1A1 that mediates apoptosis in this model (Kato et al., 1997). More recently, eEF1A1 has been identified as having a role in lipotoxic cell death. Mutant eEF1A1 was identified to confer resistance to palmitate-induced cell death using a random insertional mutagenesis assay in palmitate-sensitive CHO cells. CHO cells containing mutant eEF1A1 were also resistant to hydrogen peroxide-induced cell death. Interestingly, they also observed no change in the rate of protein synthesis in cells containing mutant eEF1A1 and polymerisation of actin

in cells treated with hydrogen peroxide was not observed in eEF1A1 mutant cells suggesting eEF1A1 exerts its role in apoptosis through cytoskeletal remodelling (Borradaile et al., 2006). Therefore, it appears that eEF1A1 is a pro-apoptotic factor *in vitro* and that it could possibly exert this function through its ability to remodel the cytoskeleton rather than any translational function.

1.7.5 Protein degradation

There is evidence to suggest that eEF1A may be involved in the co-translational proteasome-mediated degradation of ubiquitinated cellular proteins in *Saccharomyces cerevisiae* (Chuang et al., 2005). eEF1A1 has also been identified as essential for the degradation of certain N^{α} - acetylated proteins. Gonen *et al.* identified eEF1A to be required for proteolysis of the core nucleosomal histone H2A, actin and α-crystallin. In this function eEF1A1 may be acting as an enzyme, or as a chaperone protein that binds to and changes the conformation of certain proteins rendering them susceptible to degradation (Gonen et al., 1994). The dual role of eEF1A1 in both protein degradation as well as protein synthesis may allow the cell to regulate the overall level of protein present.

The identification of multiple roles of eEF1A1 suggests it may be important in co-ordinating many processes within the cell. This might explain why eEF1A1 is found in such a high excess over other components of the translation machinery.

1.8 The non-canonical functions of eEF1A2

The similarity in amino acid sequence between eEF1A1 and eEF1A2 may suggest that any non-canonical functions exhibited by eEF1A1 would also be performed by eEF1A2. However, the similarity in function in translation between the two proteins suggests that the significance of the developmental switch in expression of the two isoforms could be explained by a difference in the non-canonical roles of eEF1A2. To date not much data exists on the non-canonical functions of eEF1A2 however work by Ruest *et al.* suggests that eEF1A1 and eEF1A2 could in fact play opposing roles in apoptosis. Ruest *et al.* observed the expression of eEF1A1 and eEF1A2 in cultured myoblasts during both muscle differentiation and serum deprivation-induced

apoptosis. They observed an increase in eEF1A2 expression upon differentiation of L6 and C2C12 myoblast cells and that upon serum deprivation differentiated myoblasts decrease the expression of eEF1A2 and increase expression of eEF1A1. Overexpression of eEF1A2 was protective against apoptosis whereas overexpression of eEF1A1 accelerated cell death (and eEF1A1 antisense rescues the cells), suggesting that eEF1A2 is anti-apoptotic and eEF1A1 is pro-apoptotic in myoblasts (Ruest et al., 2002). The pro-apoptotic function of eEF1A1 could be attributed to its microtubule severing ability, which would lead to apoptosis if this occurred in the cells (Wang et al., 1999a). It is unknown whether eEF1A2 is also capable of cytoskeletal remodelling but the regions in eEF1A1 thought to be important for cytoskeletal interactions are not highly conserved in eEF1A2.

Mouse eEF1A2 has also been shown to be protective against oxidative stress-induced apoptosis. In a yeast two-hybrid screen using eEF1A2 as bait, 23 proteins were identified as interactors and peroxiredoxin-1 (Prdx1) had the most hits. Expression of eEF1A2 or Prdx1 in mouse NIH 3T3 fibroblasts conferred partial resistance to oxidative stress-induced apoptosis however co-expression of both proteins lead to substantial resistance. Co-expression of eEF1A2 and Prdx1 also resulted in an elevation in the expression level of Akt especially under oxidative conditions (Chang and Wang, 2006). Akt is a serine/threonine kinase in the phosphatidylinositol 3'-kinase (PI3-K)-Akt signalling pathway. Akt signalling plays a role in the control of cell proliferation and apoptosis and is frequently disrupted in human cancers. Interestingly, Akt has been shown to enhance protein synthesis through phosphorylation of mTOR. mTOR is a serine/threonine kinase that activates ribosomal protein S6 kinase (P70S6K1) and inhibits eukaryotic initiation binding factor 4E-binding protein 1, enhancing the translation of mRNAs containing a 5'TOP sequence and releasing inhibition of translation of mRNAs with a 5' CAP structure. However, recent evidence suggests that mTOR phosphorylation may not effect these translational regulators (Osaki et al., 2004). Other proteins have been identified from the yeast two-hybrid screen as eEF1A2 interactors and these include cytoskeletal proteins, vesicular transport/signalling proteins, and proteins involved in transcription/translation, metabolism and protein maturation (Chang and Wang,

2006). This suggests that eEF1A2 may play a role in diverse cellular functions, as has been suggested for eEF1A1. What is not clear, however, is whether eEF1A1 and eEF1A2 perform analogous or distinct non-canonical functions.

1.9 Breast cancer

Epidemiology, incidence and risk factors

Breast cancer is the most common malignancy in women, worldwide there are 1 million new cases of breast cancer diagnosed each year with approximately 200,000 cases in the United States and 320,000 in Europe (Parkin, 2004). Although the annual incidence of breast cancer is increasing in the United States, improved screening and treatment mean that the mortality rate has declined by 2.3% per year (Stewart et al., 2004). The incidence and mortality rates of breast cancer differ greatly in different geographical locations: low risk locations include the Far East, Africa and South America and high risk areas include North America and Northern Europe (Parkin et al., 1999). Other risk factors for breast cancer include age, with the incidence of breast cancer doubling about every 10 years. Lifetime exposure to endogenous sex hormones has been studied in relation to breast cancer risk. Early menarche (<12 years of age vs. > 14 years of age) and late menopause both increase the risk of breast cancer. Early age at first pregnancy (<20 years of age) and higher parity decrease the risk of breast cancer by 50% compared to nulliparous women. Interestingly in women having their first child at over 35 years of age the risk of developing breast cancer is actually higher than in nulliparous women. Prolonged lactation is protective from breast cancer, giving a 4.3% decrease in the relative risk of breast cancer for every 12 months of breast feeding. Hormone replacement therapy and oral contraceptive use increase breast cancer risk. Alcohol consumption in pre- and postmenopausal women results in an overall risk of 1.6 for developing breast cancer. Postmenopausal obesity is associated with an increase in risk whereas physical exercise is associated with a decrease in risk of breast cancer. In postmenopausal women the main source of oestrogen is from lipid therefore an overweight postmenopausal woman would have a higher levels of circulating oestrogen than slimmer postmenopausal women. Physical exercise can cause amenorrhea and this would therefore decrease the levels of circulating oestrogen and

hence could decrease risk of breast cancer in women who exercise. Other factors that increase breast cancer risk include: high mammographic breast density, a history of benign breast disease and increased bone density. Additionally the risk ratios increase with increasing number of affected first degree relatives demonstrating that family history increases breast cancer risk. For a review of breast cancer risk see (Dumitrescu and Cotarla, 2005).

Low and high penetrance breast cancer susceptibility genes

There are very few high-penetrance breast cancer susceptibility genes that confer a high risk to the development of hereditary breast cancer. These include *BRCA1* and *BRCA2, TP53, ATM, PTEN, NBS1* and *LKB1*. Mutations in these genes account for only 5-10% of breast cancer cases. Hereditary breast cancers are distinct from sporadic cases of the disease because they usually arise in younger individuals and are often multifocal or bilateral. See review by Bennett *et al.* (Bennett et al., 2000)

BRCA1 was first identified by positional cloning in 1994 (Miki et al., 1994) but this gene only showed linkage to about 45% of familial breast cancers. *BRCA2* was then identified by linkage analysis in high risk families not linked to *BRCA1* in 1995 (Wooster et al., 1995; Wooster et al., 1994). Mutations in *BRCA1* and *2* (Breast Cancer 1 and 2) are thought to be involved in 80-90% of all hereditary breast cancers but are infrequently found in sporadic cases of the disease (Futreal et al., 1994; Lancaster et al., 1996). However, decreased expression levels of BRCA1 and BRCA2 have been observed in sporadic breast cancers. LOH at 13q12-13 (*BRCA2* locus) has been observed in approximately one-third of sporadic breast cancers (Cleton-Jansen et al., 1995). BRCA1 expression has been observed to be decreased in sporadic breast cancers (Thompson et al., 1995) and this decrease in expression has been shown to be mediated by promoter methylation in some cases (Dobrovic and Simpfendorfer, 1997). *BRCA1* and *BRCA2* are tumour suppressor genes implicated in many cellular processes including maintenance of genomic stability. BRCA2 is involved in the regulation of RAD51 during homologous recombination in DNA repair. BRCA1 is involved in many aspects of DNA repair such as the transcriptional regulation of genes involved in repair and S-phase and G2/M

checkpoints in response to DNA damage (Gudmundsdottir and Ashworth, 2006; Yoshida and Miki, 2004).

Germline mutations in the tumour suppressor gene *TP53* are found in patients with Li-Fraumeni cancer susceptibility syndrome, which manifests as an increase in susceptibility to breast cancer as well as other types of cancers (Garber et al., 1991). The p53 protein is a transcription factor important in cell cycle arrest, apoptosis, DNA repair and genomic stability, and angiogenesis (Lacroix et al., 2006). Germline mutations in the tumour suppressor gene *PTEN* are commonly found in patients with Cowden syndrome, a disease associated with a 25-50% lifetime risk of breast cancer, but are rarely observed in sporadic breast cancer cases (Ueda et al., 1998). Germline missense mutations in the ataxia-telangiectasia mutated kinase (ATM) protein result in the disease ataxia-telangiectasia (A-T), which among other symptoms including cerebellar ataxia. Patients with A-T are thought to have an 11% risk of developing breast cancer by the age of 50 and 30% by the age of 70 (de Jong et al., 2002). ATM phosphorylates BRCA1 in response to ionising radiation leading to the activation of DNA repair (Yoshida and Miki, 2004).

BRCA1 and *BRCA2* mutations and the rare syndromes associated with breast cancer do not account for all hereditary breast cancers. Low penetrance genetic polymorphisms common within the population acting together with environmental factors are also thought to be important in breast cancer risk. Research on low penetrance breast cancer genes in still in its infancy, however de Jong *et al.* have conducted a pooled analysis with the aim of finding polymorphisms that could be causative in breast cancer or those in linkage disequilibrium with cancer causing variants. Candidate genes include those involved in metabolism of oestrogen or carcinogens, DNA repair and cell signalling (de Jong et al., 2002). Thirty-four polymorphisms in 18 different genes were examined and 13 polymorphisms in 10 genes showed an association with breast cancer. These include polymorphisms in: *HRAS1* (involved in cell growth and differentiation), *GSTM1* (detoxification of xenobiotics), *GSTP1* (detoxification of chemicals), *CYP1B1* (metabolism), *CYP2D6* (drug metabolism), and *VDR* (cell differentiation). Surprisingly little research as

been carried out on combinations of polymorphisms and breast cancer risk (de Jong et al., 2002).

Breast cancer pathology and molecular progression

The historical model of breast cancer progression based on morphological features and epidemiological studies suggested that breast cancer followed a multi-step process in its formation, much like the model proposed by Vogelstein for colon cancer (Vogelstein et al., 1988). The rather simplistic breast cancer progression model argued that normal breast tissue underwent progressive changes to form a hyperplasia with and without atypia, which then developed into an *in situ* carcinoma and onto invasive carcinoma and metastasis. At each step an important genetic alteration is thought to occur giving the cell a clonal selective advantage (Lakhani, 1999; Shackney and Silverman, 2003). This multi-step model is represented in figure 1.3.

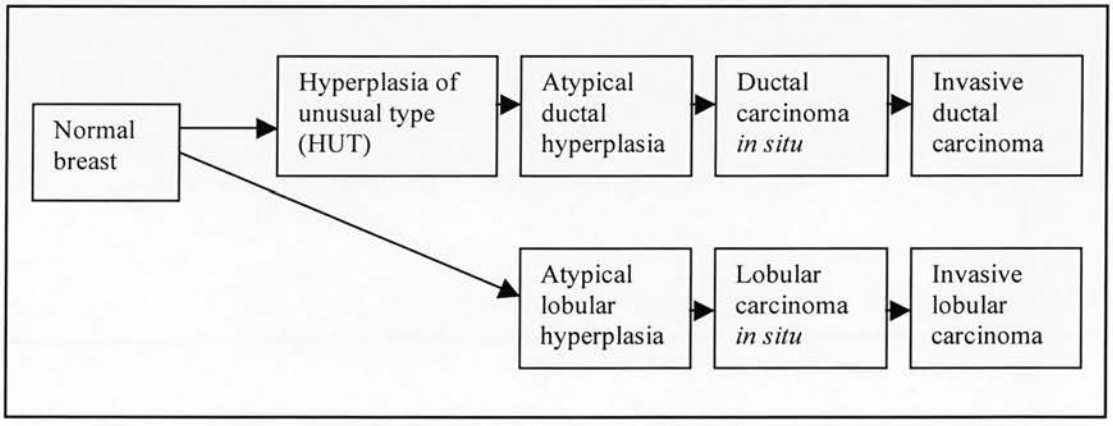


Figure 1.3 Multi-step model of breast cancer progression, redrawn from (Simpson et al., 2005)

Both lobular and ductal carcinomas are thought to arise from terminal duct-lobular units (TDLU) and the terms ductal and lobular simply reflect their different cell morphology. Ductal hyperplasia is characterised by the uneven proliferation of epithelial cells and alterations in nuclei shape and chromatin pattern. There is then a transition from hyperplasia to atypical hyperplasia which is a small clonal outgrowth of low grade malignant epithelium and is associated with an increased risk of breast cancer. Ductal or lobular carcinoma *in situ* formation is the next step upon which

cells acquire cytological characteristics of malignancy but the proliferating cells do not breach the basement membrane but extend the ductal and lobular spaces (Allred and Mohsin, 2000). The detachment from the basement membrane and stromal invasion signifies the progression to an invasive tumour. The majority of invasive breast carcinomas are ductal.

Advances in immunohistochemistry and molecular genetics have changed the multi-step model of breast cancer progression from being perceived as a single pathway to multiple parallel running pathways. This model is based on the identification of distinct molecular genetic features in the invasive tumour being mirrored in pre-invasive lesions of comparable morphology. In the multi-pathway model there are two major arms: one is the development of poorly differentiated ductal carcinoma arising from grade III DCIS with both lesions showing amplifications in 17q12 (the high grade arm) and the other is the development of well differentiated ductal carcinoma from grade I DCIS (low grade arm) with both of these lesions exhibiting a loss at 16q (the locus of the E-cadherin gene). In the arm of the model leading to low grade IDC tumours, as well as harbouring 16q loss, these tumours are usually oestrogen (ER) and progesterone receptor (PgR) positive, HER2 negative, do not express basal markers and have low genetic instability. In the opposing high grade arm tumours frequently express Her2 but not the hormone receptors ER and PgR and show many genetic losses, gains and amplifications including loss of 8p, 11q, 13q and 14q; gains of 1q, 5p, 8q, 17q; and amplifications on 6q22, 8q22, 11q13, 17q12, 17q22-24 and 20q13. Invasive lobular carcinoma (LIC) and the lobular carcinoma *in situ* (LCIS) from which it arises both show loss of 16q and 1q, which is highly similar to the situation observed in well differentiated DCIS. There is however one major difference between LCIS and LIC and low grade IDC/well differentiated DCIS and that is lobular tumours lack E-cadherin expression due to alterations in the *CDH1* gene. A pleomorphic variant of lobular carcinoma (PLC) has recently been identified and differs from classical lobular lesions by being high grade and exhibiting features of apocrine differentiation. PLC appears to have genetic features that overlap between the high and low grade arms of the multiple pathway model.

For reviews of the molecular progression of breast cancer see (Kenemans et al., 2004; Simpson et al., 2005).

Genes commonly involved in breast carcinogenesis

Many genes have been implicated as having a role in sporadic breast cancer. These can be divided into functional groups: oncogenes; tumour suppressor genes; genes involved in apoptosis, invasion, cell adhesion, and angiogenesis; and genes encoding steroid receptors.

ERBB2/neu is an oncogene that is overexpressed in 25-30% of invasive breast cancers, usually due to gene amplification, and this overexpression is associated with poor prognosis (King et al., 1985; Simpson et al., 2005). Her2 is a transmembrane receptor tyrosine kinase and a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. This family also includes Her1, Her3 and Her4 and these receptors are known to have at least 25 ligands including epidermal growth factor (EGF), TGF-α and neuregulin. Epidermal growth factors are known to stimulate cell cycle progression, inhibit apoptosis, and stimulate angiogenesis and metastasis through stimulation of intracellular signalling pathways involving phospholipase C, ras and raf. A ligand that directly binds Her2 has not been identified but Her2 heterodimerises with one of the three other Her proteins and is thereby activated by their ligands (Warren and Landgraf, 2006). Overexpression of *ERBB2* in breast cancer cell lines has been shown to increase the invasive and metastatic properties of the cell (Tan et al., 1997) and in mouse models overexpressing Her2 in mammary epithelium under the control of the mouse mammary tumour virus (MMTV) promoter develop mammary tumours after a long latency (Guy et al., 1992). In contrast, transgenic mice expressing activated Her2 with an activating mutation in the transmembrane domain develop multifocal mammary carcinomas with a significantly shorter latency (Muller et al., 1988). Her2 is the target for Herceptin or Trastuzumab, a humanised monoclonal antibody that binds the extracellular domain of the receptor.

The tumour suppressor gene, *TP53*, appears to play an important role in sporadic breast cancer development and progression. p53 induces or represses the expression of a diverse range of genes involved in cell cycle arrest, DNA repair, apoptosis, angiogenesis and senescence. Mutations tend to be point mutations rendering the protein inactive but stabilised so an increase in the protein that can be identified using IHC. The majority of p53 mutations are found in the DNA-binding domain. (Lacroix et al., 2006; Levine, 1997). It is found to be mutated in approximately 30% of invasive breast carcinomas and is associated with poor prognosis and aggressive tumours (Mazars et al., 1992). Mammary tumours, however, are infrequently observed in $p53^{null}$ mice (Donehower et al., 1992). Overexpression of the oncogenes *ras* or *wnt-1* in p53-deficient mice leads to the development of mammary tumours with aggressive features (Donehower et al., 1995; Hundley et al., 1997). Bitransgenic mice carrying MMTV-*ERBB2* and mutant p53 172Arg-to-His under the control of the WAP promoter develop mammary tumours with a shorter latency than MMTV-*ERBB2* mice, suggesting co-operativity between the two genes (Li et al., 1997a).

In the regulation of apoptosis the anti-apoptotic *BCL2* gene is abnormally expressed in 30-45% of breast cancers. Those genes involved in the alteration of adhesion and invasion important in breast cancer metastasis include N-CAM, integrins, E-cadherin cathespinD and metalloproteinases. Finally, the angiogenesis observed in breast cancer can involve an increase in vascular endothelial growth factor (VEGF) as well as fibroblast growth factor and platelet-derived growth factor (Kenemans et al., 2004; Lerebours and Lidereau, 2002). In addition overexpression of the oestrogen receptor α (ERα) is frequently observed in breast cancer and its role is discussed in Chapter 3: eEF1A2 is overexpressed in two-thirds of breast tumours.

Genomic regions most frequently altered in breast cancers

Gains or losses in chromosomes in cancers are often identified using comparative genomic hybridisation and have been shown to be commonly found within certain cancers and between cancer types. In breast cancer, whole chromosome arm gains are observed at 1q and 8q. As well as whole arm gains in chromosomes 1 and 8,

amplifications are also seen at 1q21, 1q32, 1q41 and 8q24. Other frequently amplified regions of the breast cancer genome include 11q13, 16p11, 17q11.2, 17q24 and 20q13 (Knuutila et al., 1998). 11q13 contains the locus encoding the oncogene *CCND1* and 17q12 contains the *ERBB2* gene however the putative oncogenes in many of these amplified chromosomal regions are yet to be found. Gains on 8q, 17q12 and 20q13 are associated with decreased survival (Isola et al., 1995; Tanner et al., 1995). Regions of loss of heterozygosity (LOH) in breast cancers point to the genetic loci containing tumour suppressor genes. LOH is found at 1p, 3p, 6q, 8p, 9p, 11p, 13q, 16q, 17p in more than 15-20% of breast tumours (Kerangueven et al., 1997).

Subtypes of breast cancers based on distinct gene expression signatures

Large scale gene expression analysis using microarrays has identified the existence of five clinically relevant subgroups of breast cancer based on the expression pattern of over 500 genes in 115 carcinomas and 7 non-malignant tissues. The main group identified was the luminal subtype that expressed genes characteristic of luminal epithelial cells including the oestrogen receptor (ER). This group was itself divided into two subgroups: the luminal A subtype showing the highest expression of ER, oestrogen-regulated protein LIV-1, the transcription factors hepatocyte nuclear factor 3, alpha (HNF3A), X-box binding protein 1 (XBP1) and GATA-binding protein 3 (GATA3). Conversely, the luminal subtype B exhibits lower expression of ER and the oestrogen-regulated genes listed for subtype A and high expression of *GGH*, *LAPTMB4*, *NSEP1* and *CCNE1*. The basal epithelial-like subset of tumours express genes characteristic of basal epithelial cells of the normal breast including *KRT 5* and *KRT17*, *annexin 8*, *CX3CL1* and *TRIM29*. The *ERBB2+* subtype of breast cancer expresses many of the genes in the 17q12 amplicon including *ERBB2*, *GRB7* and *TRAP100*. Finally, a normal like breast tumour group was identified that expresses genes characteristic of the normal breast adipose tissue and other non-epithelial components. The basal-like and *ERBB2+* subtypes have been associated with the shortest survival time. The luminal B subtype also appears to be a clinically distinct group that has a higher propensity to relapse and does not respond to hormone

therapy (Sorlie, 2004; Sorlie et al., 2001; Sorlie et al., 2003). In a recent study by Bergamaschi *et al.* array CGH was used to identify distinct copy number alterations that are associated with these gene expression subtypes. As would be expected the *ERBB2+* subtype showed more frequent amplification at 17q12-q21 (the *ERBB2* locus) than the other subtypes. Luminal A tumours were associated with gain at 1q12-q41 and 16p12-13. Interestingly, Luminal B subtypes showed an association with high level amplification at 20q13 (*EEF1A2* locus) (Bergamaschi et al., 2006) suggesting eEF1A2 expression might be associated with this subtype and its clinicopathological features. Importantly, there are a large number of genes on chromosome 20q13 and a great number of potential oncogenes.

1.10 Ovarian Cancer

Epidemiology, incidence and risk factors

Women have a 1 in 70 lifetime risk of developing ovarian cancer and it is the fifth most common female cancer. Ovarian cancer is the most common cause of death from gynaecological malignancy. In Europe there are 27,000-30,000 cases of ovarian cancer diagnosed each year. The majority of cases of the disease are sporadic, with only 5-10% having a hereditary genetic cause. Due to the asymptomatic nature of ovarian cancer 70% of cases are diagnosed in the advanced FIGO stage III or IV. The overall 5-year survival rate is 40% however this is directly related to the stage of the disease, for example patients with stage I disease have an 89% 5-year survival whereas those with stage IV have a 5-year survival rate of just 11% (Runnebaum and Stickeler, 2001).

Ovarian cancer is more prevalent in the industrialised countries of Northern and Western Europe as well as the United States. In contrast countries in Africa and Asia have a low incidence of the disease. Ethnicity appears to play an important role in ovarian cancer incidence as in the United States white women are at the most risk, African American, Hispanic and Asian American at an intermediate risk while Native American women are at the lowest risk (Runnebaum and Stickeler, 2001).

Dietary factors may play a role in the higher incidence of epithelial ovarian cancer in the western world, as diets high in saturated animal fats and meat have been associated with increased risk. Japanese women have an increased risk of ovarian cancer when they have migrated to the United States suggesting diet may play a role. Obesity may also increase ovarian cancer risk. Asbestos and talcum powder use have also been implicated in conferring an increase in ovarian cancer risk (Runnebaum and Stickeler, 2001).

Reproductive and hormonal factors have also been implicated in epithelial ovarian cancer risk. Pregnancy seems to be protective against ovarian cancer with each pregnancy resulting in a 15-20% reduction in risk. The use of oral contraceptives for 5 years or more decreases the risk by 50% over non-users. Breast feeding also appears to reduce ovarian cancer risk in women while early menarche and late menopause may increase risk but this has not been shown reliably. Tubal ligation and hysterectomy have been shown to decrease ovarian cancer risk by up to 80%. Tubal ligation is thought to prevent the retrograde movement of inflammatory substances to the ovary and therefore this may be the mechanism by which it results in a decrease in ovarian cancer risk. Tubal ligation results in a decrease in ovarian cancer risk of up to 80% suggesting that inflammation may be an important cause of epithelial ovarian cancer. Polycystic ovarian syndrome, pelvic inflammatory disease and endometriosis all appear to increase risk. Prolonged use of hormone replacement therapy also leads to an increase in risk of ovarian cancer of 50% over non-users (Riman et al., 2004).

Pathology of ovarian cancer

Ovarian cancers have three distinct histological and biological types: benign (adenoma), borderline (low malignant potential) and malignant (carcinoma) based on the extent of epithelial proliferation and stromal invasion. The common histological subtypes of epithelial ovarian carcinomas are serous, mucinous, endometrioid and clear cell. Epithelial ovarian cancers (EOC) are thought to arise from the ovarian surface epithelium (OSE). The OSE is a simple epithelium that exhibits some stromal rather than epithelial features. EOC constitutes 90% of ovarian cancers and

the more rare types of ovarian cancer arise from granulosa, stromal and germ cells. Epithelial ovarian cancers are complex histologically, and undergo metaplasia to acquire characteristics of the Mullerian duct-derived epithelium. This is the basis for classification of the tumours, serous resembles the fallopian tube, endometrioid the endometrium and mucinous the endocervix. Serous adenocarcinomas constitute 80% of all epithelial ovarian cancers. The less common clear cell carcinoma has mesonephros-like features. This Mullerian differentiation can be seen as a change in cell shape, E-cadherin, junctional complexes, epithelial membrane antigens, as well as secretory products such as mucins and CA125 (reviewed by (Auersperg et al., 2001) and (Murdoch and McDonnel, 2002)). Currently CA125 is the only marker commonly used in ovarian cancer. It is a reliable marker for the response to or progression on various treatments however it has no role in diagnosis or prognosis (Meyer and Rustin, 2000).

The cell of origin of epithelial ovarian cancers

There is debate over the origin of epithelial ovarian cancer. It is thought that most epithelial ovarian tumours arise from the OSE or from inclusion cysts that form from invaginations of OSE into the ovarian cortex (Feeley and Wells, 2001; Radisavljevic, 1977) . Evidence for this is the observation of atypia in the OSE adjacent to invasive carcinoma; increased inclusion cysts in apparently normal ovaries contralateral to ovarian cancer compared to age-matched controls; OSE adjacent to ovarian carcinoma showing metaplastic and hyperplastic change; and ovaries removed prophylactically from women with a family history of ovarian cancer showing cortical inclusion cysts with abnormalities in the epithelial lining. Additionally, the OSE and the epithelium of the Mullerian ducts have a common developmental origin and the OSE undergoes mullerian metaplasia during adult life. The epithelial lining of inclusion cysts has also been shown to have differentiated to a Mullerian, or to be more exact, serous epithelium and to overexpress p53, an observation frequently made in serous carcinomas (Feeley and Wells, 2001; Fleming et al., 2006).

An alternative hypothesis is that EOC arises from the epithelium of the secondary Mullerian system. This system includes the rete ovarii and paraovarian/paratubule

cysts that represent embryological remnants of the Mullerian ducts. This would mean that the "differentiating up" into Mullerian-like histology would not be necessary in EOC formation and would explain the histological similarity between the subtypes of EOC and Mullerian epithelial tumours. Additionally, Dubeau argues that small benign cysts that are morphologically indistinguishable from ovarian cystadenomas are common in the paratubal/paraovarian area and that ovarian epithelial tumours in rodents arise from dilation of the rete ovarii (Dubeau, 1999). However, it is argued that atypia has not been observed in these secondary Mullerian components and early ovarian carcinoma is commonly observed close to the ovary surface where cortical cysts form (Feeley and Wells, 2001). Additionally there is evidence that the mouse and human OSE can give rise to tumours resembling serous, endometrioid and mucinous-like tumours. Cheng *et al.* stably expressed the *HOX* genes *Hoxa9*, *Hoxa10* and *Hoxa11* in the mouse OSE cell line MOSEC or the human OSE cell line T80H and subsequently injected the cells intraperitoneally into female nude mice. Cells transgenically expressing HOXA9 gave rise to tumours with a serous-like histology, HOXA10 expression gave rise to endometrioid-like tumours and HOXA11 expression induced the formation of mucinous-like tumours suggesting that inappropriate expression of these genes, that are involved in normal patterning of the reproductive tract, can give rise to the distinct histological subtypes seen in EOC (Cheng et al., 2005).

There is also some evidence that endometrioid and clear cell carcinoma could arise from the precursor lesion endometriosis. Endometriosis is common in women in their reproductive years and results from the retrograde implantation of tissue similar to endometrium at sites outside the uterine cavity, most commonly the ovary, during menstruation. Endometrioid and clear cell carcinomas are frequently associated with endometriosis in 28% of endometrioid and 49% of clear cell carcinomas (Russell, 1979). Additionally, Jiang *et al.* observed LOH at candidate ovarian tumour suppressor loci in endometriosis and identified genetic lesions common to endometriosis and the endometrioid or clear cell carcinoma within or adjacent to it (Jiang et al., 1996; Jiang et al., 1998).

Hypotheses of how EOC arises from the OSE

The OSE, from which ovarian cancer is thought to arise, is a single layer of epithelium that covers the ovary. It functions in cyclical ovulatory ruptures and in the exchange of materials between the ovary and peritoneal cavity. Between the OSE and the ovary lies the basement membrane and tunica albuginea (Auersperg et al., 2001). There are differing models on how epithelial ovarian cancers arise from the OSE. The first, formulated by Fathalla in 1971, is called the "incessant ovulation hypothesis". Fathalla suggested that repeated ovulations lead to malignant transformation of the OSE due to repeated rupture and repair introducing genetic aberrations (Fathalla, 1971). The incidence of epithelial ovarian cancer has been increasing over the last century. The number of ovulations a woman experiences has also increased as women have less children lending support for the incessant ovulation hypothesis. Additionally, when the total number of ovulations in women with EOC was calculated and compared to those whom had not had EOC a significant correlation between high number of ovulations and EOC was found (Purdie et al., 2003). Another line of evidence is that in hens, unlike in other animals, EOC does exist and these animals are frequent ovulators (Fredrickson, 1987). Further, epidemiological studies have identified lactation, parity and the oral contraceptive, that all inhibit ovulation, as protective against the development of ovarian cancer (Cramer et al., 1983). However, there is evidence that this model may be too simplistic. For example, the progestin-only contraceptive pill (which does not suppress ovulation) has been shown to be more effective than the combined oral contraceptive against EOC (Risch, 1998). Also women with polycystic ovarian syndrome are at increased risk of EOC despite the anovulatory nature of this disease (Schildkraut et al., 1996).

The second hypothesis is the pituitary gonadotrophin hypothesis that states that excessive gonadotrophin exposure leads to an increase in oestrogen exposure to the OSE and this can lead to malignant transformation (Cramer and Welch, 1983). Gonadotrophins could directly stimulate transformation of the OSE directly or indirectly through the stimulation of oestrogen levels. Pregnancy and oral contraceptive pill use lower levels of pituitary gonadotrophins but during pregnancy

there are high levels of oestrogen and human chorionic gonadotrophin (Risch, 1998). Women with polycystic ovarian syndrome can have high levels of lutenising hormone (LH) compared to follicle stimulating hormone (FSH) and a high risk of developing EOC (Schildkraut et al., 1996). However, breast feeding has been shown to be protective against EOC but during breast feeding there are high levels of circulating FSH. Also low levels of gonadotrophins have been observed in women with EOC (Risch, 1998).

A further hypothesis is called the hormonal hypothesis and states that excess androgen stimulation of the OSE could lead to the development of EOC and that progesterone stimulation of the OSE is protective. Androgens are produced by developing follicles in the ovary and it is proposed that inclusion cysts lined with OSE adjacent to developing follicles may be exposed to this hormone (Risch, 1998). Androgen receptors have been identified on human OSE cells and Mibolerone, a synthetic androgen has been shown to stimulate proliferation of these cells (Edmondson et al., 2002). This effect of androgen administration on human OSE proliferation in culture is not always observed however (Karlan et al., 1995). Patients with polycystic ovary syndrome have high levels of androgens and this may contribute to the increased risk of EOC in these patients (Schildkraut et al., 1996). The oral contraceptive pill has been shown to decrease testosterone production and cause high levels of progestins. Progesterone levels are also very high in pregnant women (Risch, 1998).

The final model is called the inflammation hypothesis. The ovulatory process involves an inflammatory reaction in which there is an infiltration of leukocytes and production of inflammatory cytokines and nitric oxide, vasodilation, DNA repair and tissue remodelling. The genetic damage induced by inflammation may be a factor in the development of EOC from the OSE (Ames et al., 1995). The use of anti-inflammatory medication reduces the risk of colon and breast cancer and exposure to factors causing epithelial inflammation including asbestos, talc, endometriosis and pelvic inflammatory disease have been implicated in causing epithelial ovarian cancer (Ness and Cottreau, 1999).

A model of ovarian cancer disease progression

The pathogenesis of ovarian cancer is unknown and no definitive model exists. Shih and Kurman proposed a dualistic progression model based on genetic and pathological evidence. There are two pathways of progression in this model and this divides ovarian tumours into type I and type II. Type I tumours include low grade serous carcinoma, mucinous carcinoma, endometrioid carcinoma, clear cell carcinomas and malignant Brenner tumours. Type II tumours are moderately and poorly differentiated serous carcinoma (high grade serous carcinoma), malignant mixed mesodermal tumours (carcinosarcomas) and undifferentiated carcinoma. The pathway for the progression of type I tumours has clear precursors called cystadenoma, and atypical proliferative tumour and non-invasive carcinoma (both referred to as borderline). Type I tumours progress slowly and commonly exhibit *KRAS* mutations in serous tumours and *BRAF* mutations in mucinous tumours as well as *β-catenin* and *PTEN* mutations in endometrioid tumours. Type II tumours show a very different progression: they arise directly from the OSE or from inclusion cysts and metastasise early. The type II characteristic molecular alterations are less well known but p53 is frequently mutated in type II tumours. Additionally, high grade serous carcinomas also have been shown to exhibit overexpression of HER2 and Akt2 (Shih Ie and Kurman, 2004). This model is depicted in figure 1.4.

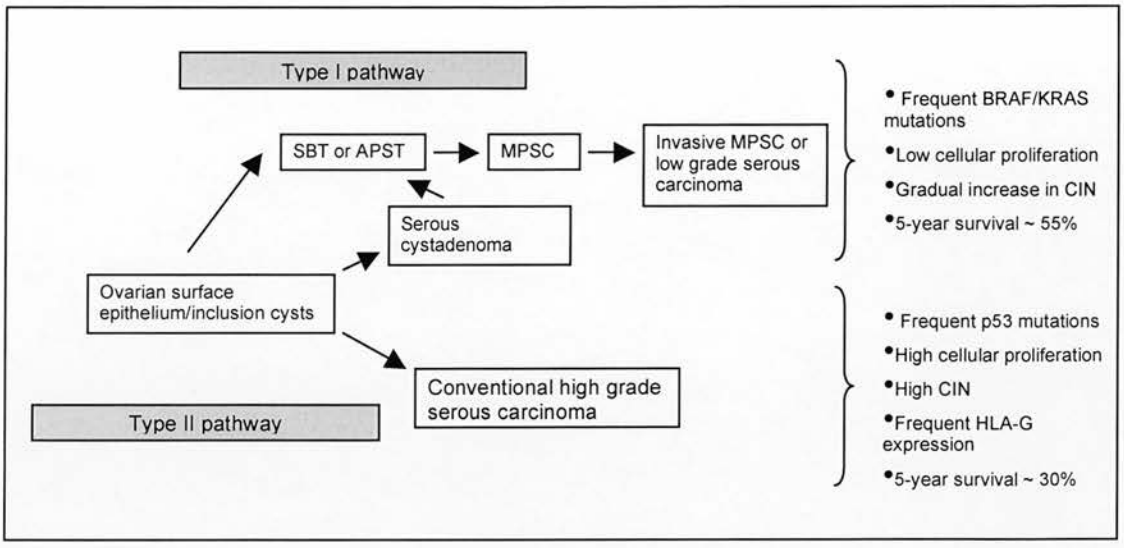


Figure 1.4 Shih and Kurman proposed model of ovarian tumourigenesis, redrawn from (Shih Ie and Kurman, 2004). Abbreviations: MPSC micropapillary serous carcinoma (low grade serous carcinoma), CIN chromosomal instability, HLA-G human leukocyte antigen-G, SBT serous borderline tumour, APST atypical proliferative serous tumour.

The genetics of epithelial ovarian cancer

Familial ovarian cancer

Familial ovarian cancer refers to ovarian cancer associated with highly penetrant autosomal dominant genetic predisposition. There are three clinical types of hereditary ovarian cancer: "site-specific" ovarian cancer, breast and ovarian cancer syndrome and hereditary nonpolyposis colorectal cancer (HNPCC; Lynch II) syndrome. Site-specific and breast and ovarian cancer syndrome are both associated with germline mutations in *BRCA1* and *BRCA2*. HNPCC ovarian cancer syndrome is associated with germline mutations in the DNA mismatch repair (MMR) genes *hMLH1* and *hMSH2*. 10% of ovarian cancers are hereditary and of these 90% harbour germline mutations in the *BRCA* genes, and 10% mutations in MMR genes. Hereditary ovarian cancers are distinct from sporadic forms of the disease in there clincopathologic features. *BRCA1* mutant carriers have a cumulative lifetime risk of ovarian cancer of 40-50%; *BRCA2* mutant carriers have a 20-30% risk. Individuals with HNPCC syndrome have a 12% cumulative lifetime risk of developing ovarian cancer (Prat et al., 2005).

Few low penetrance germline disease polymorphisms have been reliably identified however a SNP in the promoter of the progesterone receptor and an association with risk of developing endometrioid and clear cell carcinoma has been found (Berchuck et al., 2004).

Sporadic ovarian cancer

ERBB2 is amplified in 5-7% of ovarian carcinomas and overexpressed in 17-20% (Mano et al., 2004; Singleton et al., 1994). Increased copy number of *ERBB2* is associated with aggressive disease characteristics and is an independent prognostic factor for overall survival (Lassus et al., 2004). The tumour suppressor gene *TP53* is also found to be inactivated in 30-40% of ovarian carcinomas. Amplification of *c-myc* has been reported in 40% of ovarian cancers (Wang et al., 1999b). Activation of the phosphatidylinositol 3-kinase/Akt pathway has been detected in 30% of ovarian carcinomas and *k-ras* mutations have also been observed in ovarian carcinomas (Mok et al., 1993; Yuan et al., 2000). In a mouse model of induction of ovarian cancer Orsulic *et al* used ovarian cells from transgenic mice expressing the avian receptor TVA and deficient for p53 to show that the addition of any two of the oncogenes *c-myc*, *k-ras* or *Akt* resulted in induction of ovarian tumour formation when the cells were injected into various sites in a recipient mouse (Orsulic et al., 2002). Kusakari *et al.* used immortalised human OSE cells and expressed either *ERBB2* or mutant *Ha-ras* in them, which lead to anchorage independent growth and tumour formation in nude mice suggesting these oncogenes have a role in ovarian cancer (Kusakari et al., 2003).

Specific histological types exhibit specific genetic alterations, as eluded to in the model of ovarian cancer progression proposed by Shih and Kurman (Shih Ie and Kurman, 2004). *K-ras* activation is specifically observed in mucinous tumours including all three histological types: adenomas, borderline and carcinomas, suggesting k-ras is involved in mucinous differentiation. *TP53* mutations are most common in serous carcinoma but not in the benign or borderline types suggesting it is involved in malignant transformation. *TGFβ-2* mutations are mainly found in endometrioid carcinoma. Deleted in Colorectal Cancer (*DCC*), a tumour suppressor

gene, shows a loss of expression in 50% of serous carcinomas but not in the premalignant forms of the tumour. Microsatellite instability is significantly more frequent in endometrioid-type tumours than the other histological subtypes, being observed in 50% of endometrioid cancers (Fujita et al., 2003). Genetic alterations specific to histological subtypes is perhaps to be expected due to their distinct histological and biological characteristics.

1.11 Translation, translational control and cancer

Initiation factor *EIF4E* is involved in the initiation of translation, where it is an mRNA cap binding protein, and has been identified as an oncogene. Overexpression of eIF4E has been shown to transform NIH 3T3 cells and enables them to grow as a tumour when injected into mice (Lazaris-Karatzas et al., 1990). Overexpression of eIF4E has also been observed in breast, colorectal, head and neck, bladder and lung tumours (Berkel et al., 2001; Crew et al., 2000; Haydon et al., 2000; Li et al., 1997b; Rosenwald et al., 2001). The *EIF4E* locus has also been found to be amplified in non-small-cell lung cancers and breast tumours (Balsara et al., 1997; Sorrells et al., 1999). Elevated expression of the initiation factor has been correlated with poor survival in breast cancer (Li et al., 1997b). There is evidence to suggest that eIF4E activates the *Ras* oncogene and may be part of this oncogenic pathway (Lazaris-Karatzas et al., 1992).

Members of the eEF1B protein complex involved in nucleotide exchange on eEF1A have also been implicated in cancer. In a screen for genes specifically upregulated in cadmium transformed BALB/c-3T3 fibroblasts eEF1Bβ was identified and overexpression in NIH 3T3 cells resulted in a transformed phenotype (Joseph et al., 2002). eEF1Bα and β have not been shown to be overexpressed in cancer. eEF1Bγ on the other hand has been shown to be overexpressed at the RNA level in hepatocellular carcinoma, gastric carcinomas, 15% of esophageal carcinomas and 50% of colorectal adenomas (Mimori et al., 1996; Mimori et al., 1995; Shuda et al., 2000). It has not been assessed whether eEF1Bγ is capable of transforming rodent fibroblasts.

The role of eEF1A1 and eEF1A2 in tumourigenesis

There is evidence of overexpression of both eEF1A1 and eEF1A2 in cancers however only *EEF1A2* has been shown to be an oncogene. For a gene to be classified as an oncogene two criteria have to be met: the gene has to be found to be hyper-activated or hyper-expressed in primary human cancers and the gene has to be capable of transforming rodent cells cultured *in vitro*. The rodent cell lines, NIH 3T3 or Rat1, are often used to identify a potential oncogene by overexpressing the protein in them and looking for oncogenic properties. eEF1A2 was shown to have oncogenic properties when expressed under the control of a CMV promoter in NIH 3T3 cells. It enhanced focus formation, allowed anchorage-independent growth and decreased doubling time of the fibroblasts (Anand et al., 2002). *EEF1A2* maps to chromosome 20q13.3, and an increase in copy number at this locus has been identified in breast (Kallioniemi et al., 1994) and ovarian (Suehiro et al., 2000) cancers by comparative genomic hybridisation. In addition, the increased copy number at this region is associated with poor clinical prognosis and is often associated with more aggressive tumours (Isola et al., 1995; Tanner et al., 2000). In a microarray analysis of gene expression in 113 epithelial ovarian carcinomas, upregulation of eEF1A2 was identified in clear cell carcinomas, a particularly poor prognosis ovarian carcinoma (Schwartz et al., 2002). Anand *et al.* found *EEF1A2* to be amplified in 25% of primary ovarian tumours and to be highly expressed, at the RNA level, in 30% of ovarian tumours and ovarian carcinoma cell lines. They also demonstrated that eEF1A2 increased the growth rate of ES-2 ovarian carcinoma cells when xenografted in nude mice (Anand et al., 2002). Kulkarni *et al.* recently showed expression of eEF1A2 in 60% of primary breast cancers and this expression was associated with an increased probability of 20-year survival. Patients with tumours expressing eEF1A2 at moderate to strong level had a cumulative 20-year survival of approximately 75%, whereas only 55% of those patients with tumours showing low to negative expression of eEF1A2 were alive after 20 years (Kulkarni et al., 2006).

Amplification of 20q13 and overexpression of eEF1A2 have been observed in lung cancer as well as breast and ovarian cancers. Zhu *et al.* used array CGH and FISH to identify regions of genomic aberrations in lung adenocarcinoma cell lines and found

frequent gains at 20q13. They then analysed the expression of eEF1A2 by quantitative RT-PCR in normal lung tissue and compared this to its expression in 27 primary lung adenocarcinomas and found eEF1A2 RNA expression was on average 127.9 fold higher in the cancers than in normal lung tissue and was more than 2-fold higher in 70% of the 27 primary lung adenocarcinomas than in normal tissue (Zhu et al., 2006). A second study of DNA copy number, mRNA and protein expression in lung adenocarcinoma cell lines using CGH, transcript microarray and mass spectrometry identified *EEF1A2* as one of only 4 genes in which elevated protein levels correlated with mRNA and DNA copy number. RNA interference of eEF1A2 in these 4 lung adenocarcinoma cell lines resulted in a decrease in proliferation and induction of apoptosis suggesting that eEF1A2 is required for viability and/or growth of these cells. Additionally, immunohistochemical analysis of eEF1A expression in a tissue microarray containing stage I non-small-cell lung cancer showed that the elongation factor was expressed in 28% of these cancers and this expression was associated with short overall survival time, however this analysis was carried out using a non-specific antibody that recognises both eEF1A1 and eEF1A2 (Li et al., 2006). Interestingly, peroxiredoxin-1 (*PrdxI*) was one of the other three genes shown to be amplified and overexpressed in this analysis of lung cancers (Li et al., 2006) suggesting that eEF1A2 and Prdx1 may be playing a synergistic role in resistance to apoptosis perhaps through modulation of Akt, as previously described by Chang *et al.* in mouse fibroblasts (Chang and Wang, 2006).

Amplification at 6q14 (the site of the *EEF1A1* locus) has only been identified in some childhood brain tumours (Shlomit et al., 2000), in fact a loss in this region is more frequently observed in cancer (Verhagen et al., 2002). Overexpression of eEF1A1 has however been identified in breast, lung, prostate and colon cancers (de Wit et al., 2002; Grant et al., 1992; Mohler et al., 2002; Xie et al., 2002). Again overexpression of eEF1A1 in these cancers was often investigated using a commercially available non-specific eEF1A antibody and therefore it is not always clear if it is eEF1A1 or eEF1A2 that is being identified. Furthermore, it is often stated that eEF1A1 has been shown to be more highly expressed in the highly metastatic cell rat mammary adenocarcinoma cell line MTLn3 cells than in the

weakly metastatic MTC cell line (Edmonds et al., 1996). However, this analysis was carried out using an antibody raised against a peptide corresponding to the carboxyl terminus of mouse eEF1A1, which has only one amino acid difference to mouse eEF1A2 and is therefore likely to recognise both variants of eEF1A. It is therefore unclear whether it is eEF1A1 or eEF1A2 that is more highly expressed.

The translationally controlled tumour protein (*TCTP*) gene has been identified as a gene whose expression is down regulated during tumour reversion/suppression and by p53 and Siah-1 activation (Tuynder et al., 2002). eEF1A and eEF1B were identified as TCTP interactors using a yeast-two hybrid screen and interaction between eEF1A and TCTP stabilised eEF1A in its GDP bound form. It is not known whether it is eEF1A1 and eEF1A2 or just one of the 2 elongation factors that interacts with TCTP (Cans et al., 2003). TCTP has been shown to associate with microtubules in a cell cycle dependent manner, to be involved in the progression of cytokinesis and apoptosis. Therefore interaction of eEF1A with TCTP might have a role in cancer.

Shen *et al.* used differential RNA display technology to identify a 2kb transcript they called prostate carcinoma tumour inducing gene 1 (PTI-1) (Shen et al., 1995). This transcript constitutes a 630bp region with 87% sequence similarity to *Mycoplasma hyopneumoniae* 23S rRNA fused to a truncated mutated form of eEF1A and is preferentially expressed in prostate carcinomas but not normal tissue. It was also identified in cell lines derived from breast, lung and colon cancers. *In vitro* translation demonstrated the protein produced from the PTI-1 transcript was 46kDa in size (Shen et al., 1995). PCR to amplify the 5'UTR of PTI-1 in human tumour cell lines suggest that the gene is part of the human genome (Sun et al., 1997). Expression of full length PTI-1, but not the 5' UTR or truncated eEF1A alone, in CREF-Trans 6 cells and injection into nude mice induced tumour formation and this could be reversed by expression of full length anti-sense PTI-1 (Su et al., 1998). Mansilla *et al.* could detect PTI-1 protein expression using anti-eEF1A antibodies in human cell lines overexpressing PTI-1 but not in the LNcap cell line, the cell line from which PTI-1 was originally isolated, suggesting the oncogenicity of PTI-1

could be related to the transcript and not the protein product (Mansilla et al., 2005). The role of an RNA transcript in cancer seems unlikely, however RNA is thought to play a role in cancer. MicroRNAs (miRNAs) are small non-coding RNAs that mediate negative regulation of gene expression at the post-transcriptional level and have been shown to play a role in development, proliferation and apoptosis. MicroRNAs either bind to complementary sequences in the 3'UTR of target mRNAs resulting in endonucleolytic cleavage of the mRNA or the miRNA binds to imperfect complementary sites and interfere with protein synthesis. MicroRNAs are often aberrantly expressed in tumours and are thought to interrupt the cell cycle and interact with signalling oncogenes (Liu et al., 2006). Retroviruses are also thought to play a role in cancers such as leukaemias, breast cancer and skin cancer (Talbot et al., 2004). However there is no evidence that full length coding RNAs, and not the protein, play a role in cancer.

A test of the oncogenic potential of *EEF1A1* in NIH 3T3 cells has not been carried out; this would answer the question of whether both eEF1A variants are capable of transforming rodent fibroblasts.

1.12 Hypothesis: EEF1A2, but not EEF1A1, is an oncogene and the oncogenic properties of eEF1A2 lie in its unique non-canonical functions.

There is evidence that *EEF1A2* is an oncogene but less evidence supporting the role of *EEF1A1* in oncogenesis. The restricted developmental expression of eEF1A2 (Knudsen et al., 1993) and the high evolutionary conservation of the protein (Lee et al., 1994) suggest that eEF1A2 performs functions that are distinct from those performed by eEF1A1. It is not clear whether this difference in eEF1A2 is in its role in translation or whether eEF1A2 exists to perform a non-canonical function that eEF1A1 cannot. The fact that both proteins have been shown to have comparable functions in translation (Kristensen et al., 1998) suggests that it is perhaps a non-canonical function of eEF1A2 that is responsible for its existence in terminally differentiated long-lived cells in mammals and this non-canonical function could perhaps play a role in oncogenesis when eEF1A2 is inappropriately expressed. It is

also possible that overexpression of eEF1A2 results in an increase in the global levels of translation in cancer cells and that this drives the faster proliferation of cancer cells. This is unlikely as the rate limiting step of translation generally considered to occur at initiation (Hershey, 1991) and eEF1A1 is already in vast excess over the other components of the translational machinery, constituting 3-5% of the total cellular protein. Additionally, most cells are thought to be functioning near the maximum rate of elongation (ca. 5 amino acids/ribosome/sec) and therefore large increases in the rate of elongation are not possible (Hershey, 1991). If, in fact, overexpression of eEF1A2 in cells already expressing eEF1A1 does result in an increase in the global rate of translation this may result in the introduction of errors in the protein sequence (overexpression of eEF1A has been linked to increased translational infidelity in *Saccharomyces cerevisiae* (Song et al., 1989)) leading to potentially oncogenic proteins being produced, however it is difficult to see how this would be heritable. It has also been suggested that eEF1A1 and eEF1A2 may differ in the proteins they translate and that this may contribute to the role of eEF1A2 in oncogenesis due to the preferential translation of proteins that enhance oncogenesis, such as those involved in promoting proliferation or inhibiting apoptosis. This is also unlikely as the elongation and termination phases are thought to occur by the same mechanism for all proteins and to be insensitive to mRNA sequence or structure (Hershey, 1991). On the other hand, this is thought to be the mechanism by which *EIF4E* is oncogenic: through the translation of oncogenesis-related mRNAs that contain a specific secondary structure in the 5' UTR such as VEGF, c-myc and Bcl-2 (Graff and Zimmer, 2003).

Despite the amino acid similarity (96%) between eEF1A1 and eEF1A2 (Knudsen et al., 1993) it could be possible that eEF1A2 performs different non-canonical functions to eEF1A1. This could be due to post translational modifications found only on eEF1A2 and not eEF1A1, which alter function. For instance eEF1A2 has predicted phosphorylation sites that are not present in eEF1A1 (Newbery et al., in preparation). eEF1A2 may interact with and remodel the cytoskeleton, as has been demonstrated for eEF1A1 (Gross and Kinzy, 2005), but in a different manner that enhances tumourigenesis is some cell types. The finding that eEF1A2 is protective

against oxidative-stress induced apoptosis through modulation of Akt may also offer a clonal advantage to a cell. In a yeast two-hybrid screen, eEF1A2 was shown to interact with proteins involved in many diverse cellular functions suggesting that an unknown non-canonical function may be behind the oncogenic properties of eEF1A2 (Chang and Wang, 2006).

Therefore I hypothesise that eEF1A2 but not eEF1A1 is an oncoprotein and that it is the potential non-canonical functions of eEF1A2 that mediate its role in cancer.

1.13 Project aims

The aim of my PhD was to investigate the expression of eEF1A2 in breast and ovarian cancers using commercial tissue arrays, as well as locally made arrays and a panel of ovarian tumours for which DNA, RNA and protein were available. This expression analysis data could then be used to look for any clinical associations that could give insight into the potential role of eEF1A2 in these cancers. The mechanism by which eEF1A2 is overexpressed was also to be determined using DNA sequencing, copy number analysis and methylation analysis. The final aim was to use RNA interference in cancer cell lines to investigate the potential role of eEF1A2 in oncogenesis.

Chapter 2: Materials and methods

2.1 Materials

General laboratory chemicals were purchased from Sigma, unless otherwise indicated. All PCR primers were purchased from Invitrogen and cell culture medium was obtained from Invitrogen.

2.1.1 Buffers and solutions

Buffer and solution recipes that are not detailed in Methods are shown in table 2.1.

Table 2.1 *buffers and solutions*

2X Laemmli Loading Buffer	60nM Tris HCL (pH6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol
10X Laemmli Running Buffer	250nM Tris HCL (pH8.3), 1.9M glycine, 10% SDS
Mowiol	7.5g Mowiol 4-88 was mixed with 10ml glycerol in a 250ml flask. 25ml distilled water was added and the flask covered and incubated at room temperature overnight. 50ml of 0.2M Tris HCL pH8.5 was added and the mixture heated in a boiling water bath for 20 minutes. The mixture was allowed to cool before adding DAPI.
PBS (Phosphate Buffered Saline)	1 PBS tablet was dissolved in 200mls of distilled water and autoclaved, pH7.4.
PBS-T (Phosphate Buffered Saline – Tween-20)	As above but also containing 0.1% (v/v) Tween-20 added post autoclaving.
RIPA	150mM NaCl, 1%NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris HCL pH8
TBE (Tris Borate-EDTA)	108g Tris, 55g Boric acid and 9.3g of Na4EDTA in 1L of distilled water, pH8.3
Citric acid	1.05g citric acid per 500mls distilled water, pH to 6.0
Lithium carbonate	5g lithium carbonate per litre of water, pH11.
LB (Luria-Bertani) medium	1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, pH7.0. Medium was autoclaved and allowed to cool to approximately 55°C before antibiotic was added.
LB agar plates	LB was prepared as above but 15g/L agar was added before autoclaving. After cooling to approximately 55°C antibiotic was added and plates poured and allowed to harden.
Low salt LB	10g Tryptone, 5g NaCL, 5g Yeast extract 15g agar if plates, in 1L distilled water, pH 7.5

2.2 Methods

2.2.1 Patient samples

2.2.1.1. Breast cancer samples

Specimens of normal and cancerous breast tumours were obtained with informed consent and local ethical committee approval from patients undergoing surgical treatment at the Lothian University Hospitals NHS Trust.

2.2.1.2 Ovarian cancer samples

Primary ovarian (HOV) tumour material and non-malignant tissues were obtained from patients having undergone gynaecological surgery in the Lothian University Hospitals NHS Trust. Institutional ethical approval was granted for this work by the Lothian University NHS Trust Medicine/Clinical Oncology Research Ethics Subcommittee.

2.2.2 Production and validation of the anti-eEF1A1 and eEF1A2 antibodies

2.2.2.1 Design, generation and characterisation of antibodies

Anti-peptide antibodies were designed in order to specifically recognise each eEF1A variant. Three peptide sequences were selected in regions of the two variants showing amino acid differences (see figure 3.1) these are called 1A1-1, 1A1-2, 1A1-3 and 1A2-1, 1A2-2 and 1A2-3. These peptides were also selected to regions showing no amino acid differences between human and mouse proteins to allow use of the antibodies in the recognition of the eEF1A variants in the two species. Finally the peptides were chosen from regions of the protein thought to be accessible by the antibody based on the structure of the bacterial protein EF-Tu resolved by X-ray crystallography (Song et al., 1999). The peptide were linked to keyhole limpet hemocyanin, KLH, to increase antigenicity of the peptide. All of the antibodies were raised in sheep and 1A2-3 was also raised in rabbit. The serum from each bleed was used to test for the recognition by each antibody of the peptide to which it was raised by ELISA.

The antibodies were purified using ammonium sulphate precipitation and immunoaffinity purification against the peptide to which it was raised. This resulted in the abolition of non-specific bands on Western blots.

2.2.2.2 Validation of eEF1A1 and eEF1A2 antibody specificity

The specificity of the eEF1A1 and eEF1A2 anti-peptide polyclonal antibodies was determined using Western blotting by Helen Newbery. Antibodies 1A2-1, 1A2-2 and 1A2-3 all recognised a band of approximately 50kDa in protein extract from the brain of wild type mice that is known to express both eEF1A variants (Lee et al., 1993). No band was present when extracts from the brain of wasted mice was used in Western blotting and probed with the anti-eEF1A2 antibodies, confirming the specificity of the antibody. In heterozygous wasted mice carrying only one functional *Eef1a2* allele a band of half the intensity to that seen in wild type mice was identified on Western blots.

The specificity of the eEF1A1 antibodies were also determined using Western blot. eEF1A1 is known not to be expressed in mouse skeletal muscle at 25 days (Chambers et al., 1998) and this was used as a negative control. The 1A1-1 and 1A1-3 antibodies recognised a band of approximately 50kDa in all mouse tissues apart from skeletal muscle from 25 day wild type mice. Examples of these Western blots can be found in figure 3.2.

2.2.3 Immunohistochemistry (IHC)

2.2.3.1 Tissue Micro Arrays (TMAs)

A breast tumour histoarray (CB2) and an ovarian cancer tissue array (CJ1) produced by SuperBioChips (AMS Biotechnology) were obtained. An ovarian tumour TMA was produced by Alistair Williams and a breast tumour tissue array was produced by Dana Faratian at the New Royal Infirmary, Edinburgh.

2.2.3.2 IHC on commercial tissue arrays

Formalin fixed, paraffin embedded sections of human normal tissue and tumour tissue were deparaffinized with xylene, rehydrated, treated with picric acid and microwaved in citric acid pH6. Slides were then washed in water and PBS and loaded into a Sequenzer. Slides were blocked in a 1:5 dilution in PBS of sheep/rabbit serum for 30 minutes at room temperature. Primary anti-eEF1A2 rabbit and sheep antibodies were used at a concentration of 1:10 to 1:75 diluted in PBS, for 30 minutes at room temperature and secondary goat anti-rabbit IgG Biotin conjugated antibody (Dako Cytomation) or rabbit anti-goat IgG biotinylated (Dako Cytomation) were used at 1:200 to 1:500, at room temperature for 30 minutes. Slides were incubated with StreptABC complex/HRP (Dako Cytomation) at room temperature for 30 minutes and in diaminobenzidene (Sigma Fast DAB, Sigma) for 2 minutes at room temperature. Finally slides were counterstained in haematoxylin, dehydrated, cleared in xylene and mounted in pertex.

2.2.3.3 IHC Envision method on in-house tissue arrays

An alternative method of IHC was recommended by a Pathologist to decrease the background staining observed using the previous IHC method. This should make the interpretation of the results easier as there should be less background staining. The results using this protocol for IHC and the previous technique should be comparable as the same antibodies are used and controls to ensure specificity are used in both techniques. The kit used for the Envision technique was the ChemMate DAKO EnVision Detection Kit, Peroxidase/DAB, Rabbit/Mouse (DAKO Cytomation). Deparafinization, rehydration and antigen retrieval and were carried out as 2.1. Peroxidase blocking was carried on slides in the Sequenzer using peroxidase blocking solution containing 6% hydrogen peroxide and 10% sodium azide for 5 minutes. Slides were then washed in PBS and blocking carried out as described in Materials and Methods section 2.2.3.2. Primary rabbit anti-eEF1A2 1A2-3 antibody was then added to the slides at a concentration of 1:10, diluted in antibody diluent (DAKO Cytomation), for 30 minutes at room temperature. Slides were then washed in PBS and three drops of ChemMate DAKO EnVision/HRP Rabbit/Mouse secondary antibody (DAKO Cytomation) were added to each slide and incubated for

30 minutes at room temperature. Slides were again washed in PBS then removed from the Sequenzer. The DAB-containing Substrate Working Solution was prepared by mixing 50 parts ChemMate Substrate Buffer with1 part ChemMate DAB + Chromogen (DAKO Cytomation). 1 ml of this solution was added to each slide and incubated for 5 minutes. Slides were then stained with haematoxylin, counterstained with lithium carbonate and dehydrated in alcohol, cleared in xylene and mounted in pertex.

2.2.3.4 Immunohistological scoring methods

Different scoring methods were used depending on the section type being scored. For instance, tissue arrays comprising of multiple, small tissue cores on a single slide were scored as negative, weak, moderate or strong for eEF1A2 expression. This included the breast tumour sections and normal breast sections tissue array (CB2, SuperBioChips, AMS Biotechnology) and the commercial ovarian cancer tissue array (CJ1 produced by SuperBioChips AMS Biotechnology) and in-house ovarian TMA when scored manually. Large single tumour sections were histoscored using a score of 1-3 for staining intensity multiplied by the percentage of tumour tissue staining, giving a maximum score of 300. Blind scoring was carried out by two independent researchers and all ovarian cancer sections were scored by a pathologist. All breast tumour sections were observed by a pathologist for analysis of tissue types.

Automated scoring of the in-house ovarian TMA was carried out by Alistair Williams using a modification of the method of Tolivia *et al.* (Tolivia et al., 2006). Briefly, each core is photographed at 200x magnification, occupying the entire field of view. All cores are photographed without altering the camera or microscope settings. The area of the image occupied by viable carcinoma cells is recorded. The colour range is set by using an unequivocal positive core. The intensity of staining is then measured for each core using Photoshop (Adobe) and the volume adjusted score calculated.

2.2.4 Statistical methods

A two sample t-test (equal variance, unpaired, two-tailed) was used to test for a difference in the average eEF1A2 expression histoscore between the oestrogen receptor negative and oestrogen receptor positive tumours. For breast tumour Quantitative Real-time RT-PCR data, a two-sample t-test allowing for difference in variance between the two samples was used to test the difference between the mean standardised quantity of RNA for the ER-positive and ER-negative groups. Fisher's exact test was used to test for an association between oestrogen receptor positive and p53 wild-type tumours. A one-way, repeated measures ANOVA for 3 correlated samples was used to test for a difference in the percentage of cells in S-phase following RNA interference treatments. P values that were less than or equal to 0.05 were considered significant. To test the inter-rater reliability in histoscoring of the breast TMAs an intra-class correlation coefficient (ICC) was performed. This allows the evaluation of the agreement of measurements made by multiple raters, where the measurements are parametric or interval. Model 2 and individual measurements were selected in the analysis. The coefficient gives a measurement of concordance, where 1 is perfect agreement and 0 is no agreement.

2.2.5 RT-PCR

2.2.5.1 RNA extraction from breast tumour biopsies

Biopsies from normal breast tissue and breast tumour samples obtained from patients undergoing treatment at Royal Infirmary of Edinburgh and Western General Hospital, Edinburgh were snap frozen and stored in liquid nitrogen until RNA extraction.

2.2.5.2 RNA extraction from cell lines

MCF-7 cells transfected with shRNA vectors T1B1 and NEGT1B1 were trypsinised and pelleted at 24 and 48 hours post transfection. The cell pellets were stored at -70^{o}C until use. RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. The RNeasy system involves the use of a silica-based membrane column. The cells or tissue from which the RNA is to be

extracted is lysed and homogenised in a denaturing guanidine-containing buffer, which inactivates any RNase present in order to ensure high yield of RNA. Ethanol is then added to the lysed RNA sample in order to allow binding to the column. Only RNA of over 200 nucleotides binds to the silica-based column therefore enriching for mRNA and excluding rRNA. Contaminants are washed away using wash buffers and finally the RNA is eluted from the column.

2.2.5.3 DNase I treatment of RNA

RNA was treated with DNase I to ensure the removal of any contaminating DNA using DNA-free Kit (Ambion) according to manufacturer's instructions.

2.2.5.4 First strand cDNA synthesis

cDNA was prepared from RNA using the Retroscript Kit (Ambion) following manufacturer's instructions.

2.2.6 Taqman quantitative real-time RT-PCR

2.2.6.1 Taqman assay of eEF1A2 expression in breast and ovarian cancers

TaqMan Assay-on-Demand gene expression pre-designed and optimised primer and probe sets from Applied Biosystems, were used for EEF1A2 (Assay # Hs 00157325 ml) and glyceraldehyde-3-phosphate dehydrogenase quantification (GAPDH; control; Hs 99999905 ml). The sequences of these primers are not supplied by the manufacturer. In a 10µl reaction volume per well of a 394-well plate, 0.5µl of primers, 5µl of TaqMan PCR Master Mix, no AmpErase UNG 10x (Applied Biosystems), and 4.5µl of diluted cDNA were added. Each sample was run in triplicate. Real-time RT-PCR and the quantification of RT-PCR products were performed and the products analyzed using an ABI Prism 7900HT Sequence Detection System, and the appropriate software (SDS3.1) (Applied Biosystems). Minus cDNA and RNA only controls were included to check for contamination of RNA or PCR reagents.

2.2.6.2 Measurement of PCR efficiency

The PCR efficiency was measured by constructing a serial dilution of cDNA and measuring the cycle threshold of the product produced at each dilution of cDNA. If the PCR efficiency is close to 100%, then the amount of product at each dilution will be proportional to the dilution, i.e. there will be one-tenth of the product in a 1:100 dilution of cDNA compared to the 1:10 dilution. The cycle threshold is plotted against the log of the quantity of cDNA and should be a straight line. The slope of the line is equal to 3.3, giving 100% PCR efficiency. A deviation of the slope to more than 3.3 represents a PCR efficiency of less than 100% and a slope of less than 3.3 represents a PCR efficiency of more than 100%. A PCR efficiency of more than 100% simply implies that there is contamination in the PCR resulting in the amount of DNA produced being more than expected from the relative cDNA amount.

2.2.6.3 Real-time RT-PCR standard curve method of analysis

The standard curve method of analysis - with housekeeper and gene of interest amplification in separate tubes - was used for analysis of eEF1A2 mRNA levels in breast and ovarian samples. For analysis of the expression of eEF1A2 in breast cancer and normal breast RNA, a standard curve was created by conducting 4 serial dilutions of the sample F14 from 1:10, 1:50, 1:100, to 1:1000. Primers amplifying both eEF1A2 and GAPDH were used for amplification at each dilution. Results of the analyses were only used if the standard curves had a correlation coefficient of 0.99 or more and a slope of -3.3 (i.e. a PCR efficiency of 100%). The breast tumour RNA samples were run on the same plate at a dilution of 1:10. The average quantity mean of eEF1A2 was then normalised against the average quantity mean for GAPDH for each tumour sample and this value was standardised against the average quantity mean of the two normal breast RNA samples (=1). The standard deviation (S) was calculated using the following equation: $S = (cv)(X)$ where X is the mean normalised quantity and $cv = \sqrt{(cv1 + cv2)}$, where cv1 is the standard deviation of the measurement of eEF1A2 in each triplicate and cv2 is the standard deviation of the measurement of GAPDH in the triplicates. For the analysis of ovarian tumour and cell line eEF1A2 expression, RNA from the ovarian cancer cell line OVCAR3 was used for standard curve construction in the same manner as for the breast cancer

samples. Analysis was also carried out in the same way, however the samples were expressed as normalised and not standardised data as normal ovarian surface epithelium RNA was not available.

2.2.7 SYBR Green method of quantitative real-time PCR

2.2.7.1 *EEF1A2* DNA copy number analysis

2.2.7.1.1 DNA preparation from ovarian cancer tissues and normal human blood

Control normal human DNA was extracted from human blood obtained from the National Blood Transfusion service. DNA was extracted from primary ovarian tumours and normal whole ovary by Diane Scott, Cancer Research Centre, University of Edinburgh.

2.2.7.1.2 Primers used for *EEF1A2* and reference gene amplification

Two sets of primers were designed in the introns of *EEF1A2*, called EEF1A2FR2-3 and EEF1A2FR3-4, to give a product of approximately 100bp. For normalisation of the total amount of DNA present 3 sets of microsatellite primers were used. These were D5S643, D10S586, D11S1315, and have been used for copy number analysis by (Ginzinger et al., 2000). These primers were selected by Ginzinger *et al.* because they amplify regions of chromosomes that have not been shown not to harbour copy number alterations frequently in ovarian cancers. The regions of the chromosomes amplified by the control primers were confirmed to be stable in ovarian cancer by completing a literature search of studies into chromosome copy number in ovarian cancer. As a control for chromosome 20 copy number microsatellite primers on 20p was used, a pericentromeric marker region was not used as it would be difficult to get specific PCR product from this GC-rich repetitive region. The primer information is given in the table 2.2.

Table 2.2 *Sequences of primers used to amplify EEF1A2 and microsatellite loci*

Gene/ microsatellite	Primer name	Sequence (5'-3')	Size of amplified product
EEF1A2	1A2 F2-3	AGAAGGCTCTGGAACTCTGC	93bp
	1A2 R2-3	GGACAAGTGAGGGCAGTACC	
	1A2 F3-4	TGCTCTGGCATCTGAACCAG	102bp
	1A2 R3-4	CCATGTATGAAGGTGTGTCC	
D5S643	D5S643 fwd	TGGGCGACAGAGCCATC	134-138bp
	D5S643 rev	TGTGGTGTGCCATTTATTGACT	
D10S586	D10S586 fwd	TATTATACTCCAGCCGGGG	124-132bp
	D10S586 rev	GGAGACTATTTACTTTGTGTCCTTG	
D11S1315	D11S1315fwd	AAAGGCACAAAAACTAAAACTCTGG	158-162bp
	D11S1315 rev	CCGTCAGTGTGATAAAAGCCAG	

2.2.7.1.3 Primer concentration optimisation for optimisation of PCR efficiency

Standard curves were created using 100ng of normal genomic blood DNA serially diluted 1:2, 1:4 1:18, 1:16 and 1:32. Primers that amplify *EEF1A2* and the three microsatellite markers were used at 50nM, 200nM and 900nM. The concentration of primers giving the optimal PCR efficiency (between 90-110%) was used in the quantitative real-time PCR. The optimised standard curves were repeated three times and the average PCR efficiency for each primer set was used in subsequent analysis.

2.2.7.1.3 Quantitative real-time PCR determination of *EEF1A2* copy number

12.5ul of iQ SYBR Green Supermix (2x) (BioRad), 200nM of forward and reverse primers and 100ng of genomic DNA was used in a 25μl reaction. Each reaction was run in duplicate. For real-time detection a MyiQ Single-Color Real-Time PCR machine was used (BioRad). A melt curve was carried out to confirm specificity of the PCR primers; a sharp peak in fluorescence indicates a single-sized specific product.

The PCR cycling programme used is shown below, collection refers to the cycle and step at which fluorescent signal was detected by the camera and recorded.

PCR cycling programme:

Cycle 1 (1x)	Step 1	95°C	8.5 minutes	
Cycle 2 (40x)	Step 1	95 °C	30 seconds	
	Step 2	62 °C	30 seconds	
	Step 3	72 °C	30 seconds	collection
Cycle 3 (1x)	Step 1	95 °C	1 minute	
Cycle 4 (1x)	Step 1	60 °C	1 minute	
Cycle 5 (80x)	Step 1	60 °C	10 seconds	collection

Increase setpoint temperature in cycle 5 step 1 by 0.5°C for melt curve.

2.2.7.1.4 Analysis of *EEF1A2* copy number

In order to analyse the copy number of EEF1A2 the Plaffl method (Pfaffl, 2001) was used and the mathematical model is represented below:

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct \text{ target (control-treated)}}}{(E_{ref})^{\Delta Ct \text{ ref (control-treated)}}}$$

The copy number of *EEF1A2* was determined for 5 different normal genomic DNA samples and averaged. The amount of EEF1A2 amplified was normalised by each of the amplification at each of the microsatellite loci and the geometric mean of these ratios was taken for each cancer. The final geometric means for each sample were doubled to give a diploid DNA content.

2.2.7.2 Real-time PCR analysis of chromosome 20p copy number analysis

2.2.7.2.1 Primers and optimisation of PCR efficiency

In order to determine the copy number at chromosome 20p two microsatellite primers D20S804 and D20S819 that amplify loci at 20p11.23 and 20p12.3 were used, the primer information is given in table 2.3. The primer concentration at which the PCR efficiency was between 90% and 110% was determined by the method outlined in Materials and Methods section 2.2.7.1.3.

Table 2.3 *Sequences of primers used to amplify microsatellite loci at 20p*

Microsatellite	Primer name	Sequence (5'-3')	Size of amplified product
D20S804	D20S804 fwd	ACTCTGGCTACAAAACACTCTGC	154bp
	D20S804 rev	TCAATGTCACACTCCCAGTAGG	
D20S819	D20S819 fwd	GTTGCCAAAGTCAATAATAGTAAGAAG	173bp
	D20S819 rev	GCTAAATTCTCTTTTACTTTCAGGAAC	

2.2.7.2.2 Real-time PCR protocol

12.5μl of iQ SYBR Green Surpermix (2x) (BioRad), 500nM of forward and reverse primers and 200ng DNA was used in a 25μl reaction and each DNA sample was run in duplicate. The MyiQ Single-Color Real-Time PCR machine was used (BioRad) for PCR detection. Melt curves were run to ensure specificity of PCR product. Normal genomic DNA was again DNA extracted from blood obtained from the National Blood Transfusion Service.

2.2.7.2.3 Analysis of 20p copy number by standard curve method

The amount of D20S804 or D20S819 PCR product was normalised to amount of product from the control microsatellite D5S643 amplification (known to be diploid from previous analysis in all ovarian cancer DNA investigated). For this analysis the standard curve method was used. A standard curve of normal gDNA was constructed by serial dilutions of 1:10, 1:100, 1:1000, 1:10000 and 1:100000. For each primer pair a standard curve was run and analysis was carried out as detailed in Materials and Methods section 2.2.7.1.3. Minus DNA controls were included to check for PCR reagent contamination and again a melt curve analysis was included to check for PCR reagent contamination.

2.2.8 Alterations of oestrogen receptor alpha activity and measurement of eEF1A2 levels

2.2.8.1 Oestrogen receptor alpha RNA interference protocol – Catherine Naughton

RNA interference, estradiol treatment and assessment of oestrogen receptor levels in MCF-7 cells was carried out by Catherine Naughton, Cancer Research Centre, University of Edinburgh.

2.2.8.2 Real-time PCR measurement of eEF1A2 expression

2.2.8.2.1 Primer design and optimisation

Primers used to quantify eEF1A2 expression levels were designed using San Diego Supercomputer Center Biology Workbench 3.2, Primer 3 program and called EEF1A2 left and EEF1A2 right. These were designed to be less than 150bp apart (spanning an intron) in order to reduce the likelihood of the product forming secondary structure that would inflate the SYBR green fluorescent signal. The reference genes used were pumilio homolog 1 (Drosophila) (*PUM1*) taken from (Szabo et al., 2004) and identified as a suitable housekeeping gene in breast cancers and TATA box binding protein (*TBP*) taken from (Dallol et al., 2002). Both of these primers also amplify small regions of the cDNA. Details of the primers are shown in table 2.4. Personal communication with Alexey Larionov suggested that expression of these reference genes were unlikely to be altered by oestrogen treatment of MCF-7 cells. Optimisation was carried out as previously detailed.

Table 2.4 *Sequences of primers used in real-time RT-PCR to amplify eEF1A2, PUM1 and TBP*

Gene	Primer name	Primer sequence (5'-3')	Size of amplified product
EEF1A2	EEF1A2 left	AGGACGTGTACAAGATTGGC	155bp
	EEF1A2 right	CACCTCAGTGGTGATGTTCA	
PUM1	PUM1 fwd	TGAGGTGTGCACCATGAAC	187bp
	PUM1 rev	CAGAATGTGCTTGCCATAGG	
TBP	TBP fwd	TGCACAGGAGCCAAGAGTGAA	132bp
	TBP rev	CACATCACAGCTCCCCACCA	

2.2.8.2.2 Real-time PCR protocol

A standard curve of MCF-7 cells treated with estradiol only was constructed by serial dilutions of cDNA 1:10, 1:100, 1:1000, 1:10000 and 1:100000. All three sets of primers were used at 200nM. 12.5μl of SYBR Green iQ Supermix 2x (BioRad), 200nM of forward and reverse primers and 2μl of cDNA was used in a 25μl reaction. Each reaction was carried out in duplicate. The PCR cycling programme is shown below, collection refers to cycles at which the camera is detecting the fluorescent signal. PCR was carried out using the MyiQ Single-Color Real-Time PCR machine (Biorad). Minus cDNA and RNA only controls were included and melt curve analysis confirmed product specificity.

PCR cycling programme:

Cycle 1 (1x)	Step 1	95°C	3 minutes	
Cycle 2 (40x)	Step 1	95 °C	20 seconds	
	Step 2	60 °C	20 seconds	
	Step 3	72 °C	30 seconds	collection
Cycle 3 (1x)	Step 1	95 °C	1 minute	
Cycle 4 (1x)	Step 1	60 °C	1 minute	
Cycle 5 (80x)	Step 1	60 °C	10 seconds	collection

Increase setpoint temperature in cycle 5 step 1 by 0.5 °C for melt curve.

2.2.8.2.3 Analysis of eEF1A2 expression levels

Data analysis was carried out using the excel macro supplied with the MyiQ Single-Color Real-Time PCR machine (BioRad) based on the method of (Vandesompele et al., 2002).

2.2.9 Determination of OAS1 levels in MCF-7 cells transfected with shRNA vectors using real-time RT-PCR.

2.2.9.1 Primer design

Primers for amplification of OAS1 cDNA were taken from (Bridge et al., 2003) and are shown in table 2.5. GAPDH cDNA levels were used for normalisation and the primer sequences for GAPDH amplification are also listed in table 2.5.

Table 2.5 *Sequences of primers used in real-time RT-PCR to amplify OAS1 and GAPDH*

Gene	Primer name	Primer sequence (5'-3')
OAS1	OAS1 fwd	AGGTGGTAAAGGGTGGCTCC
	OAS1 rev	ACAACCAGGTCAGCGTCAGAT
GAPDH	GAPDH left	CATCAATGGAAATCCCATCAC
	GAPDH right	GGTTTTTCTAGACGGCAGGTC

2.2.9.2 Real-time PCR

For analysis of OAS1 expression levels the SYBR green method of real-time PCR was used as described in Materials and Methods section 2.6.2.2. The annealing temperature was 58°C. Analysis was also carried out as described in Materials and Methods section 2.2.7.2.3.

2.2.10 Western blots

2.2.10.1 Preparation of protein lysates

Protein lysates from RNAi treated cells were prepared from cell pellets frozen at -20°C. Cell pellets were thawed and resuspended in 50-100μl of RIPA buffer and Complete Protease Inhibitor Cocktail (Roche) was added to protect against protein degradation. Cell lysates were then sonicated to break up DNA. Protein lysates from MCF-7 cells treated with siRNA to ERα were obtained at a concentration of 2μg/μl from Catherine Naughton. Protein lysates from cell lines were prepared using previously published protocols (Gilmour et al., 2002); the same method was used for primary tumour samples, but in these cases tissue was initially homogenised in extraction buffer prior to determination of protein content.

2.2.10.2 Protein Quantification

Protein quantification was carried out using the BioRad DC Protein Assay Kit following manufacturer's instructions.

2.2.10.3 Preparation of SDS-PAGE gel and protein loading

The BioRad Mini PROTEAN 3 mini-gel apparatus was used according to manufacturer's instructions. A 12% acrylamide separating SDS-PAGE gel was poured as follows:

30% acrylamide	5.2ml
1.5M Tris HCL pH8.8	4ml
dH_20	6.68ml
20% SDS	80μl
TEMED	10μl
25% AMP	40μl

The separating gel was overlaid with isopropanol to disperse bubbles. The gel was allowed to set for 45 minutes. The isopropanol was removed and the stacking gel prepared as follows:

30% acrylamide	1.45ml
0.5M Tris HCL pH6.8	2.5ml
dH_20	6ml
20% SDS	50μl
TEMED	5μl
25% AMP	50μl

The stacking gel was poured on top of the separating gel and combs inserted into the liquid. This gel was allowed to set for 10 minutes.

The plates were then placed in the gel tank apparatus. The chamber and tank were filled with 1X Laemmeli running buffer. The combs were then removed from the gel and the wells washed out using a pipette. 10μg of protein was mixed with 2X Laemmeli loading buffer and 1μl of 1M DTT/10μl sample. The protein was then boiled for 5 minutes and briefly centrifuged. The protein samples were then loaded into the wells of the SDS-PAGE gel together with a pre-stained SDS-PAGE standard Broad Range (BioRad) marker of protein size. The protein was run at 100V through the stacking gel and then at 150V through the separating gel.

2.2.10.4 Western blotting

The BioRad Mini Trans-Blot Electrophoretic Transfer cell was used for Western blotting. Hybond-P membrane (Amersham Pharmacia Biotech) was first soaked in methanol. Filter paper, sponges and SDS-PAGE gel as well as the Hybond-P membrane were then soaked in a transfer buffer containing the following:

1X Laemmli running buffer	200mls
100% Methanol	200mls
dH_20	600mls

The sponge, filter paper and membrane were then stacked and assembled into the apparatus. The apparatus was filled with the transfer buffer and run at 350mA for one hour at 4°C. After one hour the blots were removed and the non-specific sites on the membrane were blocked in 5% powdered milk in PBS-T overnight at 4 °C.

2.2.10.5 Antibody probing of Western blot membrane

The blots were incubated for two hours at room temperature with primary anti-eEF1A2 rabbit antibody and primary anti-eEF1A1 sheep antibody diluted 1:200 in blocking solution, as well as primary anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal mouse antibody (Chemicon International) diluted 1:10000. Primary rabbit anti-Hsp70 (Hsp72) (Stressgen) was used at a concentration of 1:30000. Blots were then incubated in the appropriate Horse Radish Peroxidase conjugated secondary antibody (Dako Cytomation) at 1:500. Detection was performed using enhanced chemiluminescence detection kit (Amersham Biosciences).

2.2.11 RNA interference

2.2.11.1 Dharmacon pooled oligos

A set of four siGENOME SMARTPOOL human oligos targeted to EEF1A2 (M-003882-00-0010) and a negative control-pool of non-targeting siRNAs (D-001206-13-05) were purchased from Dharmacon.

2.2.11.2 Transfection of cells with siRNA using Oligofectamine (Invitrogen)

24 hours before transfection, $4x10^4$ Hela or MCF-7 cells were plated in a 24 well plate in 500μl of medium. In a transfection tube, 2.5μl of a 20μM stock of oligonucleotide was diluted in 40μl of Optimem (Invitrogen). In a separate tube 2μl of Oligofectamine (Invitrogen) was diluted in 7.5μl of Optimem. These solutions were mixed gently by pipetting. After five minutes the diluted oligonucleotide was combined with the diluted Oligofectamine and mixed by tapping and allowed to stand for 20mins. During this time the medium was removed from the plated cells, the cells washed with PBS, and replaced with 200μl of Optimem. After the 20 minute incubation, 52μl of the complex was added to the appropriate well. After 4 hours the medium in each well was supplemented with 125μl of growth medium containing 30% fetal calf serum. Untransfected and mock transfected controls were included. At 24, 48 and 72 hours post-transfection the cells were trypsinised, pelleted and stored at -20°C or -70°C.

2.2.11.3 shRNA design

The BLOCK-iT U6 RNAi Entry Vector Kit was purchased from Invitrogen. shRNA oligos were designed following manufacturer's instructions, see table 2.6

Table 2.6 *Details of the shRNA constructs targeted to eEF1A2*

Name	Region targeted to	Length	Loop sequence
T1B1	Exon 3	21nts	GAGA
GCGCTACGACGAGATCGTCAA Top strand CACCGCGCTACGACGAGATCGTCAAGAGATTGACGATCTCGTCGTAGCGC Bottom strand AAAAGCGCTACGACGAGATCGTCAATCTCTTGACGATCTCGTCGTAGCGC			
T2B2	Exon 5	24nts	CGAA
AGTGAACATCACCACTGAGGTGAA Top strand CACCGAGTGAACATCACCACTGAGGTGAACGAATTCACCTCAGTGGTGATGTTCAGT Bottom strand AAAAACTGAACATCACCACTGAGGTGAATTCGTTCACCTCAGTGGTGATGTTCACTC			
T3B3	Exon 6	19nts	AAGG
ATCGTGGAGATGGTGCCGG Top strand CACCGATCGTGGAGATGGTGCCGGAAGGCCGGCACCATCTCCACGAT Bottom strand AAAAATCGTGGAGATGGTGCCGGCCTTCCGGCACCATCTCCACGATC			
NEGT1B1	n/a	24nts	CGAA
AAGCATCGAGAGTCCTAGCGATAA Top strand CACCGAAGCATCGAGAGTCCTAGCGATAACGAATTATCGCTAGGACTCTCGATGCTT Bottom strand AAAAAAGCATCGAGAGTCCTAGCGATAATTCGTTATCGCTAGGACTCTCGATGCTTC			
JLTB	Exon 2	20nts	GAGA
GACCATTGAGAAGTTCGAGA Top strand CACCGGACCATTGAGAAGTTCGAGAGAGATCTCGAACTTCTCAATGGTCC Bottom strand AAAAGGACCATTGAGAAGTTCGAGATCTCTCTCGAACTTCTCAATGGTCC			

2.2.11.4 shRNA treatment of cells

MCF-7 and HeLa cells were transfected with the shRNA detailed in Materials and Methods section 2.2.11.3 by two different protocols: lipofectamine and nucleofection. Transfected cells were pelleted at the appropriate time point and frozen at -20°C or -70 °C until Western blot or RT-PCR analysis.

2.2.11.5 RNA interference using Ambion pre-designed siRNAs

The siRNAs used for the ablation of eEF1A1 or eEF1A2 expression are detailed in table 2.7: the Ambion catalogue number of these siRNAs is 16704. The non-targeting siRNA was also supplied by Ambion, catalogue number 4611.

Table 2.7 *Sequences of the Ambion pre-designed siRNAs to eEF1A1 and eEF1A2*

Gene	siRNA name	siRNA ID #	Sequence (sense)	Effective
EEF1A1	1A1-1	2991	GGAUGUCUACAAAAUUGGUTT	Yes
EEF1A1	1A1-2	2898	GGGAUGGAAAGUCACCCGUTT	Yes
EEF1A1	1A1-3	289305	CAUCUAAUUCUGGUUUUACTT	No
EEF1A2	1A2-1	10976	GGUCCAGUGGAAGUUCUUCTT	Yes
EEF1A2	1A2-2	10884	GGACCAUUGAGAAGUUCGATT	Yes
EEF1A2	1A2-3	10789	GGUAUUGACAAAGGACCATT	No

Each of the siRNAs was resuspended to a concentration of 50μM and aliquoted for storage at -70°C.

2.2.12 Transfection protocol

2.2.12.1 Transfection of DNA using Lipofectamine 2000

HeLa cells were plated at a density of $4x10^4$ per well of a 24 well plate 24 hours before transfection. Lipofectamine 2000 (Invitrogen) was used following manufacturer's protocol.

2.2.12.1.1 Transfection of siRNA using Lipofectamine 2000

HeLa cells were plated at a density of $4x10^4$ in 500ml of medium in a 24 well plate. After 24 hours the cells in each well were transfected with 20nM of siRNA with 1μl of Lipofectamine 2000 following manufacturer's protocol (Invitrogen).

2.2.12.2 Transfection using a Nucleofector

A Nucleofector (Amaxa Biosystems) was used for transfections into MCF-7 cells and PEO1 cells. MCF-7 and PEO1 cells were plated 3 days prior to transfection in large T175 flasks. When they reached 70-80% confluency the cells were trypsinised and counted using a Coulter counter (Beckman Coulter). $1x10^6$ cells were then centrifuged at 1000xg for 5 minutes and the pellet resuspended in 100μl of the appropriate nucleofector solution (Amaxa Biosystems). MCF-7 cells were resuspended in solution V and PEO1 cells were resuspended in solution L. 3μg of DNA vector was added to each and the solution transferred to a cuvette. Cells were then electroporated in the nucleofector using the appropriate programme. For MCF-

7 programme P-20 was used, for PEO1 cells programme U-20 was used. The cuvette was removed from the machine immediately following electroporation and 500μl of warmed medium was added to the cells. The cells were then transferred into a well of a six well plate containing 1ml of medium using the pipettes provided. Controls include cells with DNA and nucleofector solution but no electroporation and cells plus solution and electroporation but lacking DNA.

2.2.13 Immunocytochemistry

Transfected cells were washed in warmed 1X PBS, and fixed in 2-4% paraformaldehyde for 10 minutes. Cells were then washed once in PBS containing 0.2% Triton X-100 and once in PBS. Cells were then blocked in PBS containing 5% Bovine Serum Albumin and 0.2% Tween for 30 minutes at room temperature, shaking gently. Cells were then washed once (except secondary only controls) in PBS-T (0.2% Tween-20) and incubated in primary antibodies diluted in PBS-T for one hour at room temperature, shaking gently. Goat anti-V5 (Abcam) was used at a concentration of 1:1000, mouse anti-α-tubulin (Sigma) was used at a concentration of 1:4000 and Alexa Fluor 594 phalloidin (Invitrogen) at 1:400. Cells were then washed twice in PBS-T and incubated in secondary Alexa Fluor conjugated antibodies (Molecular Probes, Invitrogen) for 30 minutes at room temperature. Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 594 donkey anti-sheep IgG were used at a concentration of 1:1000 diluted in PBS-T. Cells were finally washed in PBS-T and mounted on a glass slide in Mowiol (Sigma) containing DAPI and viewed under a fluorescent microscope.

2.2.14 Microscopy

Immunofluorescently stained cells were viewed on an Axioskop 2 fluorescent microscope (Zeiss) and using Smart Capture software. Sections stained by immunohistochemistry were viewed by light microscopy on an Olympus BX51 using DP software (Olympus).

2.2.15 Mutation screening of *EEF1A2*

2.2.15.1 *EEF1A2* exon PCR primer sequences

PCR amplification of each exon of *EEF1A2* was carried out prior to sequencing. In some case two separate primer pairs were used to amplify one exon. The primer sequences are shown in table 2.8, those marked with a * were designed by Helen Newbery.

Table 2.8 *Sequences of primers used to amplify EEF1A2 exons*

Exon of *EEF1A2*	Primer name	Primer sequence (5'-3')
1	Exon 1 fwd	ATTAGGGAGAGCCCCTCAGA
	Exon 1 rev	GAGGTCAGTGGAGAAACCCA
2	2FC	GTCTCCCCACCACCACTG
	2RC	GGAGTTGGGGGTTCCTTCT
3	3F*	CTGTAACAAGCAGCTCGCAC
	2R*	CCCTGCTCACCTGGGATG
4	4F*	GGAGAGGCCTGGAAGTGAG
	4R2*	TTCTCGCTGTAGGCCGGCT
	4F2*	CCTACACGCTGGGTGTGAAG
	4R4*	GGAACCTGCATTTCCCGGG
5	5F2*	AGCAGTACTCCTGGAAGCA
	5R2*	TCTAGGGCAGGCAGAGCT
6	6FB	AGGCTCTGTCCCCGAATATC
	6RB	GACAGTCCTGCTGCTGTCC
7	7F1*	GCAGATTAGCGCCGGCTAC
	7R*	CTGGATCAGCCACAGCCTG
	7F*	CCTCCAGTCAGCACTCCGG
	7R1*	CCAGCTTCTTGCCAGAGCG
8	Exon 8 fwd	TAAGCGTGTTCCGAGGACAT
	Exon 8 rev	TCTCTGCCCTGAGTAGCCAT

2.2.15.2 PCR conditions for amplification of *EEF1A2* exons

The conditions used for each primer set to amplify the *EEF1A2* exons are outlined in table 2.9. In the majority of cases the Taq polymerase used was purchased from Invitrogen however PCR of exons 1 and 8 required the used of DyNazyme EXT DNA polymerase (Finnzymes, NEB), a DNA polymerase specifically designed for use in the amplification of long, difficult to PCR, sequences. Due to the GC rich

sequence around exons 1 and 8 of EEF1A2 it was necessary to design primers that amplified a large fragment that encompassed the exonic sequence.

Table 2.9 *PCR conditions used to amplify EEF1A2 exons*

Exon	Primer names	Annealing temp (°C)	TAQ pol	Additives	No. of cycles	Product size (bp)
1	Exon 1 fwd and rev	60	Dynazyme	10% DMSO	38	993
2	2FC 2RC	60	Invitrogen	Betaine (1.485M)	33	294
3	3F 2R	58	Invitrogen	10% DMSO	33	268
4	4F 4R2 4F2 4R4	54 58	Invitrogen	10% DMSO 10% DMSO	33	258 322
5	5F2 5R2	57	Invitrogen	10% DMSO	33	297
6	6FB 6RB	60	Invitrogen	Betaine (1.485M)	33	391
7	7F1 7R 7F 7R1	58 57	Invitrogen	10% DMSO 10% DMSO	33 33	264 264
8	Exon 8 fwd and rev	60	Dynazyme	10% DMSO	38	807

For PCR using Invitrogen Taq DNA polymerase the following were added to a sterile eppendorf tubes/plates on ice. Appropriate master mixes were used.

10X Invitrogen PCR buffer	2.5μl
dNTPs 10mM	1.0μl
Forward primer 100ng/μl	1.0μl
Reverse primer 100ng/μl	1.0μl
DNA 50ng/μl	4.0μl
DMSO	2.5μl
Taq DNA polymerase 5U/μl	0.25μl
Betaine 5.5M	7.03μl
ddH_2O	Up to 25μl

The PCRs were then placed in a PTC-225 thermal cycler (MJ Research) and the following programme run:

Step 1: 94°C 5 mins
Step 2: 94 °C 30secs
Step 3: Annealing temp 30secs
Step 4: 72 °C 30secs
Repeat steps 2-4 32 more times
Step 5: 72 °C 10mins

The following was added to a sterile PCR tube/plate for PCR amplification of exons 1 and 8 using DyNazyme EXT.

10X DyNazyme EXT reaction buffer	2.5μl
dNTPs 10mM	1.0μl
Forward primer 100ng/μl	1.0μl
Reverse primer 100ng/μl	1.0μl
DNA 50ng/μl	4.0μl
DyNazyme EXT DNA polymerase 1U/μl	1.0μl
DMSO	2.5μl
ddH_2O	Total to 25μl

The following PCR cycling programme was used:

Step 1: 94°C	5 mins
Step 2: 94 °C	30secs
Step 3: 60 °C	30secs
Step 4: 72 °C	1 min
Repeat steps 2-4 37 more times	
Step 5: 72 °C	10mins

Following amplification 5μl of each PCR product was run on a 1% agarose gel to check the size.

2.2.15.3 Agarose gel electrophoresis

PCR products were assessed by agarose gel electrophoresis. 1% to 2% (w/v) agarose solution in 0.5X TBE was heated until dissolved in a microwave. Melted agarose was allowed to cool slightly and 0.003% ethidium bromide was added to the solution. The agarose solution was then poured into a casting tray and combs placed in the liquid. The agarose was then allowed to set and the combs removed. The tray was then placed in an electrophoresis tank and covered in 0.5x TBE. Ready Load 1kb DNA ladder (Invitrogen) was used as a molecular weight marker. PCR products were mixed with 2x loading buffer and added to the wells of the agarose gel alongside the molecular weight marker. The gel was then run at 100V for one hour and viewed under UV light.

2.2.15.4 Sequencing *EEF1A2* PCR products

2.2.15.4.1 Clean up of PCR product

ExoSAP-IT (USB, GE Healthcare Biosciences) was used to clean up the PCR product for sequencing. 2μl of ExoSAP-IT was used per 5μl PCR product and the reaction was incubated at 37°C for 15 minutes and 80°C for 15 minutes.

2.2.15.4.2 Sequencing primer design

Sequencing was either carried out either using the PCR primers used to amplify the exon or using internal primers as indicated in table 2.10. The prefix INT in the

primer name refers to a sequencing primer designed 3' to the PCR primer and the sequence of these is also indicated in table 2.10.

Table 2.10 *Sequences of primers used to sequence EEF1A2*

Exon	PCR primers	Sequencing primers	Sequence of internal primers (5'-3')
1	Exon1 fwd Exon 1 rev	1F1 1R1	TCCGTCTTTGCAGCCCGCGC TCCCTCTTCGGAAGACGGG
2	2FC 2RC	Int2FC 2RC	CACAGAATCACTGCAG
3	3F 2R	Int3F 2R	ACCAGGGACTCCTGGGTCCC
4	4F 4R2 4F2 4R4	4F 4R2 Int4F2 4R4	AGCTCATCGTGGGCGTGAAC
5	5F2 5R2	Int5F2 Int5R2	GGCCTGAGGGTGGGGAG TCCAGCACAGCGCCCTTGC
6	6FB 6RB	Int6FB 6RB	TGCCCCAGCATCCCCTCGG
7	7F1 7R 7F 7R1	Int7F1 7R Int7F Int7F1	GGTCATCGACTGCCACACAG GTGCAGGGGCGCCGGTGAC GGTCATCGACTGCCACACAG
8	Exon 8 fwd Exon 8 rev	8F1 8R3	GCGTGTTCCGAGGACATTCC CAGGGAGTGAAGGATGCTGG

2.2.15.5 Sequencing protocol

Sequencing was carried out using BigDye v3.1 (Applied Biosystems). 6.5μl of PCR product was added to 1μl of BigDye v3.1, 1.5μl of 5x BigDye Buffer (Applied Biosystems), 1μl of sequencing primer and the reaction was made up to 10μl with distilled water in a sterile 96 well plate. The following programme on an MJ Research machines PCR block was carried out:

1x:	96°C	1 min
24x:	96°C	30 sec
	50°C	15 sec
	64°C	4 min
1x	4 °C	HOLD

The sequencing product was then precipitated using ethanol. 2.5μl of 125mM EDTA was added to 10μl sequencing reaction. Following this, 30μl of 100% ethanol was added to each well and the contents mixed by inversion 4 times. The plate was then spun in a sigma centrifuge at 1238xg for 30 minutes. After centrifugation the plate was inverted over tissue paper to remove the ethanol from the wells. To remove any remaining ethanol the plate was spun up-side-down on tissue paper to 700xg. 30μl of 70% ethanol was then added to each well and the plate centrifuged at 1238xg for 15 minutes. The above process of removing the ethanol was repeated and when the wells were entirely dry the plate was submitted for sequencing. Sequencing was carried out at the Wellcome Trust Clinical Research Facility, Western General Hospital and analysed using Chromas version 1.45, Griffith University, Australia.

2.2.16 Bisulphite sequencing

2.2.16.1 Bisulphite conversion of DNA

The first step in bisulphite sequencing is the bisulphite conversion of DNA. This involves the conversion of unmethylated cytosines to uracil so they can be distinguished from methylated cytosines of CpG dinucleotides by sequencing. This was achieved using the EZ DNA Methylation Kit (Zymo Research). 1μg of DNA from ovarian tumours HOV 104, 179, 548 and 557 as well as normal whole ovary DNA 440 and 470 was converted following the manufacturer's protocol.

2.2.16.2 PCR of the 5'CpG island of *EEF1A2*

The 5'CpG island of *EEF1A2* was amplified from the converted DNA extracted from normal and cancerous ovarian tissues by PCR. The primers hF6 and hR4 amplify a 548bp fragment including the 5' 50% of the *EEF1A2* CpG island.

Table 2.11 *Sequences of primers used to amplify the 5'CpG island of EEF1A2*

Primer name	Primer Sequence (5'-3')
hF6	AGGGATTGGAAATTAGTAGATTT
hR4	AAAAAAAATCCACCTATTAA

For PCR the Roche Fast Start Taq DNA polymerase was used as detailed in the table below:

10X FS Bfr/$MgCl_2$ reaction buffer	2.5μl
dNTPs 2.5mM	8.0μl
Forward primer 5μM	3.0μl
Reverse primer 5μM	3.0μl
DNA	3.0μl
FS Taq DNA polymerase 1U/μl	0.2μl
ddH_2O	Total to 25μl

PCR cycling programme:

Step 1	95°C	5 minutes
Step 2	95 °C	30 seconds
Step 3	52 °C	30 seconds
Step 4	72 °C	1 minute 30 seconds
Repeat steps 2-4, 44 times		
Step 5	72 °C	10 minutes
Step 6	10 °C	HOLD

10μl of PCR product was run on a 1% agarose gel to check the product size.

2.2.16.3 TA cloning of bisulphite converted *EEF1A2* PCR products

The *EEF1A2* PCR products were then ligated into the pCR2.1 vector using the TA Cloning Kit (Invitrogen) following manufacturer's protocol. 2μl of PCR product was ligated into the vector and transformed into Chemically Competent TOP10 *E.coli* (Invitrogen). The transformed cells were then plated on LB-agar containing 100μg/ml Kanamycin and 20μg/ml of X-gal (Invitrogen) for blue/white selection and incubated at 37°C overnight. White colonies were picked using a sterile cocktail stick and swirled in sterile water in a 0.5ml Eppendorf tube and streaked onto a reference plate that was then placed at 37 °C. The sterile water containing bacterial cells from positive colonies was heated to 95 °C for 10 minutes to break up the bacteria and release the DNA. Following heating the tubes were centrifuged at 3000rpm for 10 minutes. Colony PCR to identify *EEF1A2* inserts was carried out

and positive colonies were cultured and the pCR2.1 vector containing *EEF1A2* PCR product was extracted using a DNA Miniprep Kit (Qiagen).

2.2.16.4 Colony PCR

PCR was carried out on DNA isolated from bacterial colonies using the following reagents.

10X Invitrogen PCR buffer	2.5μl
dNTPs 10mM	1.0μl
M13 forward 5μM	2.5μl
M13 reverse 5μM	2.5μl
Bacterial DNA	2.0μl
Taq DNA polymerase 5U/μl	0.125μl
ddH_2O	Up to 25μl

PCR cycling programme:

Step1	95°C	2 minutes
Step 2	95 °C	30 seconds
Step 3	50 °C	30 seconds
Step 4	72 °C	2 minutes
Repeat steps 1 to 4, 35 times		
Step 5	72 °C	7 minutes
Step 6	10 °C	HOLD

10μl of PCR product was then run on a 1% agarose gel to check the fragment is the expected 748bp in length.

2.2.16.5 Sequencing of bisulphite converted *EEF1A2* 5'CpG clones

Sequencing was carried out as described in Materials and Methods section 2.2.12.5 using the M13 forward and reverse primers and 2μl of plasmid DNA obtained from DNA Miniprep isolation. Analysis of bisulphite sequence results was carried out using BiQ Analyzer software (Bock et al., 2005).

2.2.17 Cloning

In order to create N-terminal Green Fluorescent Protein (GFP) tagged eEF1A1 and eEF1A2 constructs I used the Gateway cloning system (Invitrogen) to clone the full length cDNA sequences into the destination vector pcDNA-DEST53.

2.2.17.1 Gateway attB flanked PCR primer design

Primers were designed according to manufacturer's instructions to amplify human eEF1A1 cDNA sequence and also incorporate the *att*B sites required for site-specific recombination into vectors. The sequence of the primers can be found in the table below. Human eEF1A2 cDNA sequence in the Gateway entry vector pENTR221 (Ultimate ORF clones) was purchased from Invitrogen and could therefore be cloned directly without the PCR step.

Table 2.12 *Sequences of primers used to clone eEF1A1 by the Gateway method*

cDNA sequence	**Primer name**	**Primer sequence (5'-3')**
eEF1A1	attBntermfwd	GGGGACAAGTTTGTACAAAAAAGCAGGC TTCATGGGAAAGGAAAAGACTCATATCA
	attBntermrev	GGGGACCACTTTGTACAAGAAAGCTGG GTCTCATTTAGCCTTCTGAGCTTTCTGG

2.2.17.2 PCR with attB site containing primers

The full length human eEF1A1 IMAGE clone, accession number BC028674, image ID 4107346 was used to PCR the eEF1A1 insert from. DyNazyme EXT DNA polymerase (Finnzymes, NEB) was used in the following PCR:

10X DyNazyme EXT reaction buffer	5.0μl
dNTPs 10mM	1.0μl
Forward primer 100ng/μl	2.0μl
Reverse primer 100ng/μl	2.0μl
Miniprep IMAGE clone 50ng/μl	5.0μl
DyNazyme EXT DNA polymerase 1U/μl	1.0μl
ddH_2O	Total to 50μl

PCR programme:

Step 1	94°C	5 minutes
Step 2	94 °C	30 seconds
Step 3	60 °C	30seconds
Step 4	72 °C	30 seconds
Repeat steps 2-4 29 times		
Step 5	72 °C	5 minutes

5μl of PCR product was run on an agarose gel to check the size, which should be approximately 1.4kb.

2.2.17.2 BP recombination reaction

In order to clone the EEF1A1 cDNA sequence into the Gateway entry vector pDONR221 a BP reaction was carried out between the *att*B containing PCR product of EEF1A1 and pDONR221, following manufacturer's protocol. This leads to the production of the EEF1A1 cDNA sequence cloned into an entry vector.

2.2.17.4 LR recombination reaction

EEF1A1 was then cloned from the entry vector pDONR221 into the destination vector pcDNA-DEST53 for expression as an N-terminal GFP tagged protein. The human EEF1A2 cDNA was purchased from Invitrogen in the entry vector pENTR221 (Ultimate ORF clones) and cloned into the destination vector pcDNA-DEST53 using the LR recombination reaction. The LR reaction was carried out following manufacturer's protocol. Destination clones were sequenced to check for sequence alterations in the eEF1A1 and eEF1A2 cDNA sequences.

2.2.17.5 Transformation of competent *E.coli* cells

One Shot TOP10 Chemically Competent *E.coli* (Invitrogen) was transformed with plasmid DNA following manufacturer's protocol. The pUC19 control DNA plasmid (Invitrogen) was used as a positive transformation control.

2.2.17.6 Bacterial culture and selection

Transformed TOP10 Chemically Competent *E.coli* cells were plated on Luria-Bertani (LB) agar at 37°C or cultured in liquid LB at 37°C with shaking at 200rpm

containing the appropriate selective antibiotic. Kanamycin was used at 100μg/ml, Ampicillin was used at 50μg/ml and chloramphenicol (diluted in methanol) was used at 25μg/ml and these antibiotics were supplied by Sigma. Zeocin (Invitrogen) selection was carried out in low salt liquid broth at a concentration of 25μg/ml.

2.2.17.7 Plasmid preparation

Plasmids preparations from transformed *E.coli* cells were produced using the Qiagen Miniprep or Maxiprep kits or the ENDOfree maxiprep kit following the manufacturer's protocol.

2.2.18 Maintenance of cell lines

2.2.18.1 Subculturing of cells

All reagents for tissue culture were obtained from Invitrogen. HeLa cells were cultured in DMEM containing 10% fetal calf serum (FCS) and Penicillin/Streptomycin (Pen/Strep). MCF-7 cells were cultured in MEM containing Earle's salts, 10% FCS and Pen/Strep. PEO1 cells were maintained in RPMI 1640 containing 10% FCS and Pen/Strep. Cells were cultured in T75 flasks in 25mls of medium at 37 ^{o}C and 5% CO_2. To passage cells medium was aspirated and 5mls of trypsin:versene (1:1) was added to the cells. Cells were placed at 37 ^{o}C, 5%CO_2 until trypsinised. 5mls of medium was then added to the cells and the cells removed into a 15ml falcon tube. Cells were centrifuged at 1000rpm for 5 minutes and medium was aspirated. Cells were then resuspended in 10mls of medium and 1ml was added to a new T75 flask containing 25ml of fresh medium. Cells were passaged 1:10 approximately twice weekly.

2.2.18.2 Counting cells

Cells were diluted 1:100 in isotone and counted using the Coulter Counter Z series (Beckman Coulter).

2.2.18.3 Cryopreservation of cell lines

Cells in a T75 flask were trypsinised and pelleted as described in 2.15.2. Cells were then resuspended in 6mls of medium supplemented with 5% DMSO and placed into 1ml screw cap cyropreservation vials. These vials were then wrapped in tissue, placed in a polystyrene box and put at -70 °C. After 24 hours at -70 °C the vials were placed in liquid nitrogen.

2.2.19 Assays on cell lines

2.2.19.1 Analysis of the interferon response

MCF-7 cells were plated in a 24 well plate at a density of $4x10^4$ cells per well, 24 hours before transfection. Cells were plated both for analysis of OAS1 expression and cell viability. T1B1 and NEGT1B1 were transfected into the cells using Lipofectamine 2000 (Invitrogen). 0.1μg of plasmid was transfected with 1.25μl of Lipofectamine 2000, 0.2μg was transfected with 3μl of Lipofectamine, 0.4μg was transfected with 6μl of Lipofectamine 2000 and 0.8μg was transfected with 12μl of lipofectamine in serum free medium according to manufacturer's protocol. Untransfected and mock transfected (Lipofectamine 2000 only) controls were included.

2.2.19.2 Cell viability assay

MCF-7 cells transfected with shRNA vectors were trypsinised and pelleted 24, 48 and 72 hours post transfection. Cells from each well were trypsinised, pelleted and resuspended in 50μl of medium. 10μl of resuspended cells were mixed with 10μl of Trypan Blue stain (Invitrogen). 10μl was pipetted between a coverslip and the slide of a haemocytometer and examined under a microscope. The number of cells within the central square was counted and multiplied by x 10^4/ml to give the concentration of cells. Those stained blue were unable to exclude the dye and were therefore classified as dead or unviable.

2.2.19.3 BrdU assay

2.2.19.3.1 5-Bromo-2'-deoxy-uridine (BrdU) incorporation and immunofluorescent staining of cells

Analysis of cell proliferation in MCF-7 cells transfected with siRNA against EEF1A2 was carried out using the 5-Bromo-2'-deoxy-uridine (BrdU) Labelling and Detection Kit I (Roche). MCF-7 cells were plated on 13mm glass coverslips in a 24 well plate and transfected with siRNA as described in Materials and Methods section 2.2.8.2. Each treatment was carried out in duplicate. At 24 and 48 hours post transfection the medium was removed from the cells and replaced with medium containing 10μM BrdU labelling reagent. Cells were then incubated in BrdU at 37°C 5%CO_2 for 30 minutes. Cells were then washed fixed and stained with a primary anti-BrdU and secondary fluorescent conjugated antibody following manufacturer's protocol. Coverslips were finally mounted in Mowiol (Sigma) containing DAPI on glass slides and viewed by fluorescent microscopy.

2.2.19.2.2 Analysis of the percentage of cells in S phase (proliferating)

Two representative fluorescent microscope images of each slide were taken at X40 magnification. Images of cells were then viewed using Adobe Photoshop software (Adobe) and the number of green (BrdU labelled) and blue (DAPI stained) cells were counted.

2.2.19.3 Propidium iodide cell cycle analysis

The following solutions were prepared and the constituent reagents were obtained from Sigma.

Citrate buffer	85.5g sucrose and 11.76g trisodium citrate dissolved in 800ml dH2O 50ml DMSO is added and volume made up to 1000ml with dH20 pH adjusted 7.6
Stock solution	2000mg trisodium citrate 121mg Tris 1044mg spermine tetrahydrochloride 2ml Nonidet P40 dissolved in dH_20 to 2000ml pH7.6
Solution A	15mg trypsin in 500ml stock solution pH7.6
Solution B	250mg trypsin inhibitor and 50mg ribonuclease A in 500ml stock solution pH7.6
Solution C	208mg propidium iodide and 500mg spermine tetrahydrorochloride in 500ml stock solution pH 7.6

MCF-7 cells transfected with shRNA plasmids and plated in 6 well plates were trypsinised 48 hours post transfection in 500μl of trypsin:versene. Following trypsinisation 500μl of medium was added to the cells. One half of the cells were pelleted for protein analysis while the other half was pelleted for cell cycle analysis. MCF-7 cells for cell cycle analysis were resuspended in 50μl of citrate buffer. 225μl of solution A was added and the cells were incubated at room temperature for ten minutes. 162μl of solution B was then added to the cells and a further incubation of ten minutes at room temperature was carried out. Finally 125μl of solution C was added and the cells incubated on ice for 10 minutes. Propidium iodide stained cells were then analysed by flow cytometry. A two-parameter dot-plot of Forward Light Scatter (FLS) vs. Side Scatter (SS) was plotted. A single parameter FL3 histogram plot (area) with a linear x axis was used to visualise the DNA content in the cell population. The voltage of the FL3 laser was adjusted so that the G1 peak was at a mean of 200 on the x axis. No compensation was required as only one fluorochrome (propidium iodide) was used. 10,000 ungated events were collected in total for each sample. Flow cytometry was carried out using a Coulter EPICS XL flow cytometer (Beckman Coulter). Apoptotic, G1, S and G2/M cells were gated using the EXPO ADC analysis software (Beckman Coulter) and the percentage of cells in each region

was recorded. Analysis was carried out on the FL3 histogram data using Multicycle AV for Windows software (Phoenix Flow Systems).

2.2.19.4 Heat shock

In order to test the specificity of the rabbit anti-Hsp70 antibody (Stressgen) HeLa cells were seeded into small flasks and incubated at 37°C, 5% CO_2. Twenty-four hours later the flasks were removed from the incubator and the lids sealed with parafilm. The flasks were then either returned to 37°C or placed in a 40°C or 42°C waterbath for one hour. The flasks were then removed from the waterbath and the parafilm removed from the lids of all the flasks including those at 37°C. The cells were then placed in the 37°C incubator for 2 hours, 7 hours or 24 hours. Following this the cells were pelleted and protein lysates produced and Western blots run to analyse Hsp70 expression. RNA interference was carried out as described in 2.2.11.5 and 2.2.12.1.1 in this chapter. All transfections were carried out in duplicate wells and duplicate plates. Forty-eight hours post-transfection the 24 well plates were removed from the 37°C, 5% CO_2 incubator and sealed using parafilm. One of the duplicate plates was placed at 37°C and the other was heat shocked at 42°C in a waterbath for one hour. The parafilm was removed from the plates and the cells incubated at 37°C, 5% CO_2 for 2 hours to allow protein synthesis. This method is a modified version of the protocol used by Shamovsky *et al.* (Shamovsky et al., 2006).

Chapter 3: eEF1A2 is overexpressed in two-thirds of breast cancers

3.1 Introduction

Oestrogen receptor (ER) positive breast cancers and their treatment

Oestrogens are a group of steroid hormones that perform roles in many processes within the cell, from growth and differentiation to specific organ functions. The classical mechanism of ER activation of transcription of target genes is through ligand-occupied receptor binding to specific elements in target genes. Upon oestrogen binding to the ER in the nucleus the receptor dimerises leading to an activation of its transcriptional activity by binding to the Oestrogen Response Element (ERE), a specific 15 nucleotide inverted palindromic sequence found in the promoter region of certain genes, (see review by (O'Lone et al., 2004). Alternatively, the ER can indirectly activate transcription through association with other DNA-binding transcription factors such as activating protein-1 (AP-1) (Kushner et al., 2000) and Sp1 (Safe, 2001). Both of these mechanisms involve the recruitment of co-activators and components of the basal transcriptional machinery to promote transcription of specific genes. In addition to nuclear ERs there are also plasma membrane associated ERs that mediate gene regulation (Wong et al., 2002). Lastly, the oestrogen receptor can also influence gene expression via the sequestration of common transcriptional components (Speir et al., 2000). Examples of oestrogen-responsive genes include the progesterone receptor (May et al., 1989), pS2 (Brown et al., 1984) and heat shock protein 27000 (Hsp27) (Moretti-Rojas et al., 1988).

There are known to be two ER isoforms, ERα and ERβ. The predominant form of ER in human reproductive tract and mammary tissue is ERα. The incidence of ER positive cancers increased by 344% between 1978 and 2003, with ER receptor positive ductal cancers constituting 80% of all breast cancers in 2003 (Glass et al., 2005). Most tumours that express the ERα are low grade, and those that do not are frequently poorly differentiated (Millis, 1980). ERβ is also expressed in breast cancers, in particular it is found in ERα positive breast cancers, and there are cancer cells that express both of the isoforms simultaneously. ERβ is thought to be

important in regulating oestrogen signalling pathways and its expression is an indicator of good prognosis in breast cancer (Omoto et al., 2002).

Oestrogen receptor positive cancers are good candidates for treatment with endocrine therapy. This can be in the form of anti-oestrogen drugs such as tamoxifen, or aromatase inhibitors. Tamoxifen is thought to work by binding to the ER and rendering it non-functional. Aromatase inhibitors, on the other hand, reduce levels of oestrogen itself. This class of drugs inhibit the biosynthesis of the most potent form of oestrogen, estradiol, from androgens by the cytochrome P450 enzyme complex called aromatase. High levels of this enzyme complex can be found in the ovaries of pre-menopausal women, the placenta of pregnant women and adipose tissue of post-menopausal women, as well as breast tissue and sites surrounding breast tumours. Examples of aromatase inhibitors include Aminoglutethimide, 4-Hydroxyandrostenedione and the third generation aromatase inhibitors Anastrozole, Exemestane and Letrozole, see review (Brueggemeier et al., 2005).

Oestrogen receptors (α and β) are known to bind the following 15bp palindromic sequence in target genes with highest affinity in vitro, where the N represents variable nucleotides (Klein-Hitpass et al., 1986).

-6 -5 -4 -3 -2 -1 +1 +2 +3 +4 +5 +6

5' Pu G G T C A NNN T G A C C Py 3'

There are few characterised EREs in the human and mouse genomes, in fact only three consensus EREs have been identified in human oestrogen-regulated genes: *EBAG9*, *COX7A2L* and *EFP/ZNF147* (Ikeda et al., 2000; Inoue et al., 1993; Watanabe et al., 1998). Near consensus EREs have also been identified in oestrogen responsive genes and these show a base pair diverging from the consensus at one or two nucleotides. It has been shown that some base alterations are better tolerated than others, for instance -5C, -4C and –2A result in a large decrease in ER binding affinity *in vitro*. Symmetrical changes in both arms of the palindrome affect the binding more substantially than non-symmetrical changes. It was also noted that

many natural elements show one change with a small effect on affinity, -6Py+6Pu or -1G/+1C together with another variation. In a genome wide search for near consensus EREs, containing one nucleotide variation or two where one is -6Py+6Pu or -1G/+1C, 71,119 elements were found in the human genome and 65,012 in the mouse genome; the distribution of these EREs was generally consistent with chromosome size. Although many previously characterised EREs are mainly located within 0.5Kb of transcriptional initiation sites, this study found that the ERE was more abundant in the 0 to + 1Kb region of genes in both mouse and human genomes. In general they found a random distribution of EREs with enrichment around transcriptional start sites. EREs are less represented and conserved further from the start site (i.e. -1 to -10Kb) (Bourdeau et al., 2004).

Field cancerization

The concept of "field cancerization" was first proposed for oral cancer by Slaughter *et al.* in 1953 (Slaughter et al., 1953). This concept was proposed to explain the local recurrence of primary tumours (so called second primary tumours) following tumour resection, as well as the observation of multiple primary tumours. Most studies in this field have been done on head and neck squamous cell carcinomas (HNSCC). The model proposed, based on evidence from extensive studies on HNSCC, fits into the model of multistep carcinogenesis. In multistep carcinogenesis the accumulation of genetic mutations leads to the progression of a normal cell into a cancer (Fearon and Vogelstein, 1990). Field cancerization proposes that a stem cell acquires genetic alterations and forms a patch of genetically identical daughter cells. An additional genetic hit that confers a growth advantage to a cell then causes the development of an expanding clone that laterally displaces normal epithelium. The patch of genetically altered cells has now given rise to a field of cells derived from a genetically altered clone. Subsequent genetic hits give rise to sub clones within this field that share a common clonal origin but have divergent genetic alterations. A sub clone then eventually forms an invasive cancer. To summarise, there are two important steps in this model, (1) the emergence of a group of stem cells with superior proliferative capacity from the patch of genetically altered stem cells and (2) the transforming event that leads to the formation of an invasive cancer. Of clinical

importance is the fact that fields may remain after surgery that appear normal histologically but then give rise to local recurrence in the future, see reviews (Braakhuis et al., 2003) and (Garcia et al., 1999).

Common genetic lesions identified in "normal" cells proximal to a cancer include loss of heterozygosity, microsatellite alterations, chromosome instability and mutations in *TP53*, as well as aberrant DNA methylation patterns (Braakhuis et al., 2003). In breast cancer Heaphy *et al.* completed a study of the telomere DNA content and allelic imbalance in normal tissue adjacent to breast tumours. They showed that shortened telomeres and allelic imbalances were present at sites at least 1cm away from the tumour and in a substantial number of the cells present in this apparently normal tissue. Additionally they demonstrated that genomic instability decreased the further away from the tumour the cells were isolated (Heaphy et al., 2006).

Invasion and metastasis in breast cancer

It is well known that particular cancers metastasize to certain sites in the body. Breast cancer shows a distinct metastatic pattern involving the regional lymph nodes, bone marrow, lung and liver. There are two proposed mechanisms mediating this, firstly the cancer cells may differentially survive and proliferate at these sites, or alternatively they are selectively trapped with or without preferential homing (Moore, 2001). In breast cancer, expression of the chemokine receptors CXCR4 and CCR7 on the breast cancer cells and the concomitant expression of their ligands at sites to which these cells metastasize, suggests that the cells are preferentially homing to these sites. Therefore the migration of these breast cancer cells is directed from the primary tumour site through the circulation to the preferential metastatic regions (Muller et al., 2001).

20q amplification and breast cancer

Regions of chromosomes showing frequent alteration in a certain cancers are likely to contain genes important for the genesis of that disease. *EEF1A2* maps to 20q13.3 (Lund et al., 1996) and amplifications in 20q13 are commonly observed in breast

cancers. 20q13 amplification has been associated with more aggressive breast tumours with lymph node involvement (Courjal et al., 1996). Additionally, 20q13 amplification has been associated with high grade breast tumours, aneuploidy and short disease-free survival (Tanner et al., 1995). Cingoz *et al.* identified 20q13 amplification by CGH in breast cancers and found that this amplification was more frequent in ER positive cancers. High level DNA amplifications were mapped to 20q12-qter and 20q13-qter (Cingoz et al., 2003). Amplification at 20q13.2 is frequently observed in breast cancers (Kallioniemi et al., 1994; Tanner et al., 1994; Tanner et al., 1996); this amplicon is proximal to the *EEF1A2* locus.

Evidence that eEF1A2 is involved in breast cancer

There are multiple lines of evidence to suggest that eEF1A2 may be involved in breast cancer. *EEF1A2* maps to 20q13 and amplifications in this region are frequently observed in breast cancer, as detailed above. Furthermore, analysis of expression data in the NCBI SAGE database suggests that eEF1A2 is more highly expressed in breast tumours than in normal breast tissue. Finally, the expression of eEF1A was identified by microarray and immunohistochemical analyses to be more highly expressed at the invasive edge of breast tumours (Zhu et al., 2003). However, the immunohistochemical analysis of breast tumours was carried out using an antibody that recognises both eEF1A1 and eEF1A2 and therefore it is not clear which form of eEF1A may be involved. Helen Newbery developed eEF1A1 and eEF1A2 antibodies (Newbery et al., in preparation) and I have utilised these to investigate the expression of eEF1A2 in breast cancer.

eEF1A1 and eEF1A2 specific antibodies

The specificity of the eEF1A1 and eEF1A2 antibodies was determined by Western blotting of mouse tissue protein lysates. These antibodies are polyclonal anti-peptide antibodies and the mouse peptides to which they were raised are shown in figure 3.1. The Western blots showing specificity of the antibodies is shown in figure 3.2. The eEF1A1 antibodies 1A1-1 and 1A1-3 give a band of approximately 50kDa in adult mouse brain and liver but not in skeletal muscle where only eEF1A2 is expressed. The eEF1A2 antibodies 1A2-1, 1A2-2 and 1A2-3 give a band of approximately

50kDa in adult mouse brain and skeletal muscle but not in the brain of wasted mice, which is null for eEF1A2. These Western blots were produced by Helen Newbery (Helen Newbery, PhD thesis 2002).

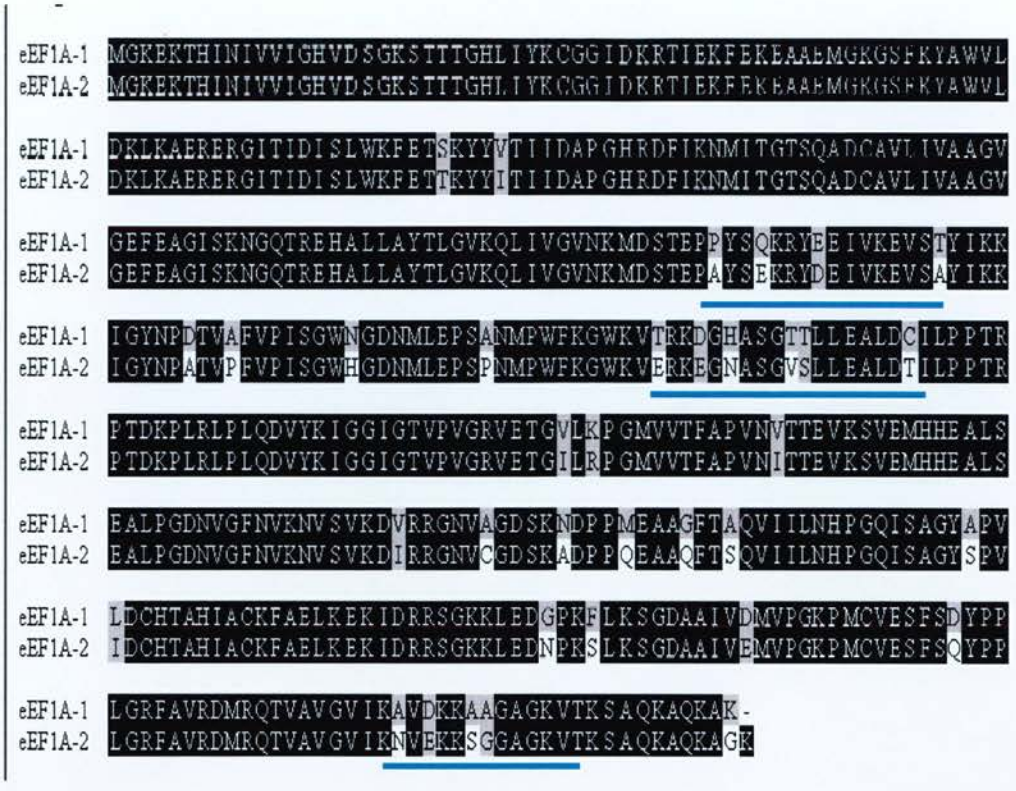


Figure 3.1 eEF1A1 and eEF1A2 protein sequence alignment showing the peptide sequences used to produce eEF1A variant-specific antibodies
The sequences of the peptides used to raise variant-specific antibodies are underlined in green. These regions were chosen because they display amino acid differences between eEF1A1 and eEF1A2 and are conserved between human and mouse eEF1A proteins.

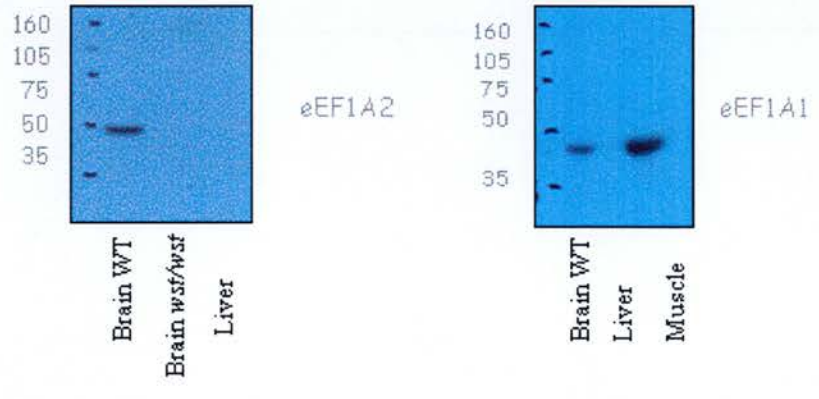


Figure 3.2 Validation of the specificity of the eEF1A1 and eEF1A2 antibodies by Western blot
The eEF1A2 specific antibodies recognise a band at approximately 50kDa in wildtype mouse brain and muscle but not liver. The eEF1A1 specific antibodies recognise a 50kDa band in adult mouse brain and liver but not in skeletal muscle.

3.2 Results

3.2.1 Expression of eEF1A2 in normal breast tissue

Breast cancers are thought to arise from the epithelial cells of the terminal duct-lobular units. It was therefore necessary to first determine the expression of eEF1A2 in these cells in normal breast. I stained seven normal breast sections by immunohistochemistry with two different anti-eEF1A2 antibodies, 1A2-1 and 1A2-3. These antibodies were raised to two different synthetic eEF1A2 peptides, selected from protein regions that show amino acid differences between eEF1A1 and eEF1A2 (Newbery et al., in preparation). The stromal and adipose tissue in normal breast was negative for eEF1A2 expression. Low levels of eEF1A2 expression could be seen in the cytoplasm of the luminal epithelium and no eEF1A2 was present in the myoepithelial cells, shown in figure 3.3. Therefore, eEF1A2 appears to be expressed at negligible levels in the luminal epithelial cells of the normal breast ducts. It is important to note that this normal breast is most likely to have originated from material removed from around a cancer in a patient and is therefore not necessarily completely "normal".

3.2.2 Expression analysis of eEF1A2 in breast cancers by immunohistochemistry

In order to determine whether eEF1A2 is overexpressed in breast cancers a tissue array containing cores from 46 different breast tumours was stained immunohistochemically using the variant-specific anti-eEF1A2 antibody, see figure 3.3. This tissue array was obtained from Super Biochips and detailed clinical information is not available with it. The limited clinical data available with the TMA is represented in table 3.1.

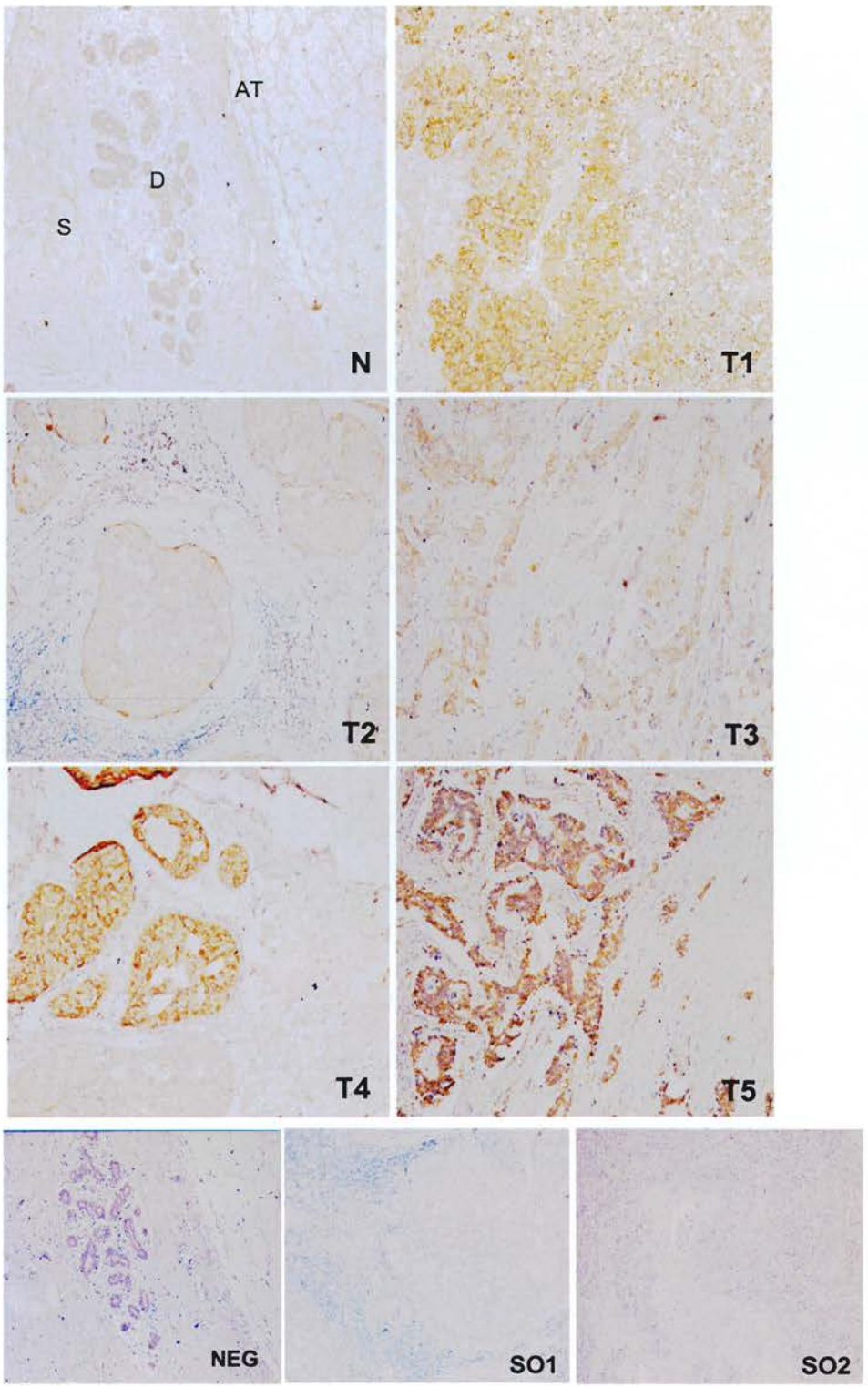


Figure 3.3 Immunohistochemical analysis of eEF1A2 expression in normal breast and breast cancers.

Normal breast (N) shows low levels of staining for eEF1A2 in the ductal epithelium (D). The stroma (S) and adipose tissue (AT) are negative for eEF1A2 expression. T1-T5 show expression of eEF1A2 in the cancerous epithelial cells of five different tumour cores. NEG shows the secondary-only control on a normal breast section and SO1 and SO2 are examples of secondary antibody-only controls, on breast tumours T3 and T1.

Table 3.1 *Clinical data available with the commercial TMA*

Variable		Number of cases
T stage	T1	4
	T2	20
	T3	18
	T4	4
ER	Positive	24
	Negative	22
PgR	Positive	18
	Negative	27
	Unknown	1
p53	Wild-type	29
	Mutant	17
Lymph node	Positive	35
	Negative	11
Histology	Infiltrating ductal carcinoma	31
	Ductal carcinoma *in situ*	2
	Solid papillary	1
	Medullary carcinoma	1
	Signet ring carcinoma	1
	Metastatic carcinoma in lymph node	10
Gender	Female	44
	Male	2
Patient age	Median	47
	Minimum	26
	Maximum	80

The cancerous cells stain cytoplasmically and the stroma and adipose tissue are negative for expression of eEF1A2: the staining on each core is mainly homogeneous. In order to control for heterogeneity in the tumour from which the cores were taken two cores from each tumour were stained with two different antibodies to eEF1A2. The cores were then scored by two researchers and one pathologist for expression level of eEF1A2 using a histoscore method. This method involves scoring the intensity of staining on a level of 1-3, multiplied by the percentage of tumour cells staining at that level, giving a maximum score of 300. Of the 46 cancers, 5 (11%) tumours expressed eEF1A2 strongly, a histoscore of between 200 and 300, and 22 (48%) expressed eEF1A2 at a moderate level, a histoscore of between 100-200. The remaining tumours showed eEF1A2 levels similar to that found in normal breast epithelium. The inter-rater agreement or reliability was tested using the intra-class correlation coefficient (ICC) and this gave

a correlation coefficient of 0.8244, suggesting there is little variation between the scoring of each tumour by the raters. None of the three lobular carcinomas showed expression of eEF1A2. Twenty-two of the 46 cancers were oestrogen receptor negative and 24 were oestrogen receptor positive. Only 4 of the 22 oestrogen receptor negative cancers showed anything more than weak expression of eEF1A2 whereas 18 of the 24 oestrogen receptor positive cancers showed moderate to strong expression of eEF1A2. The oestrogen receptor status was assessed using multiple antibodies by immunohistochemistry (SuperBioChips).

Interestingly in tumour T1 there appears to be some nuclear staining of eEF1A2 as well as cytoplasmic localisation. eEF1A1 has been shown to translocate to the nucleus when bound to ZPR1 in mitogen-stimulated *S. cerevisiae* and human A431 epidermoid cells (Gangwani et al., 1998). The role of ZPR1 has not been determined however eEF1A2 was also shown to bind ZPR1 in a yeast two-hybrid screen (Chang and Wang, 2006) and this may explain the localisation of eEF1A2 in the nucleus of the T1 tumour cancer cells in which a mitogenic signalling pathway could be activated.

3.2.3 Statistical analysis shows eEF1A2 expression is associated with oestrogen receptor-positive cancers

The tissue microarray used in the above analysis was obtained from SuperBioChips. As such, detailed clinical information on each patient was not available with these cores and this is a major drawback of using these readily available tissue microarrays. However, the expression status of the oestrogen and progesterone receptors as well as lymph node status and p53 mutation status was given for each cancer. It appeared that eEF1A2 was preferentially expressed in oestrogen receptor positive cancers and therefore to test the statistical significance of this observation eEF1A2 expression was grouped as [negative and weak], and [moderate and strong] and tested against oestrogen receptor expression status using the Fisher's exact test. This analysis showed an association between oestrogen receptor-positive cancers and cancers showing moderate/strong expression of eEF1A2 ($p=0.016$, Fisher's exact test). There was an apparent association between eEF1A2 expression and p53 status

but this was not statistically significant. This may be due to the significant association between oestrogen receptor positive and wild type p53 cancers (p=0.012, Fisher's exact test). A negative association between mutant p53 and the oestrogen receptor has previously been observed by Levesque *et al.* (Levesque et al., 1994).

In order to look further into the association between oestrogen receptor-positive cancers and moderate to high expression of eEF1A2 we obtained 16 sections from oestrogen-receptor positive cancers of patients at the Western General Hospital. Of these, 13 (81%) showed moderate to high expression of eEF1A2, see figure 3.4. This is higher than the 59% of cores that stained moderate to high for eEF1A2 expression on the commercial tissue array. This is likely to be due to the fact that all of these sections are all from ER-positive tumours. In total, 40 of the 63 breast tumours (63%) examined by immunohistochemistry showed moderate to high expression of eEF1A2.

3.2.4 eEF1A2 expression in a panel of 250 breast cancers using the DAKO Envision immunohistochemical staining technique

In order to extend the analysis of eEF1A2 expression in breast cancers we obtained a larger TMA containing 250 breast cancers. Staining of this in-house TMA with eEF1A2 using the IHC protocol used to stain the commercial TMA resulted in a great deal of background staining when used on this large TMA. In order to increase specificity and decrease background on cancer sections stained with the anti-eEF1A2 antibody I employed a more contemporary immunohistochemistry technique that is standard protocol at the New Royal Infirmary Pathology Department, Edinburgh. This technique involves the use of a different antigen retrieval method and the Envision secondary antibody system from DAKO Cytomation, details of which can be found in Chapter 2: Materials and Methods. This technique utilises an antigen retrieval technique in which the tumour section is pressure cooked in EDTA solution, rather than microwaved in Citric acid, resulting in antigen retrieval taking place at a different pH. The main difference between this and the previous technique is the secondary antibody, which has a HRP labelled polymer conjugated to it. This

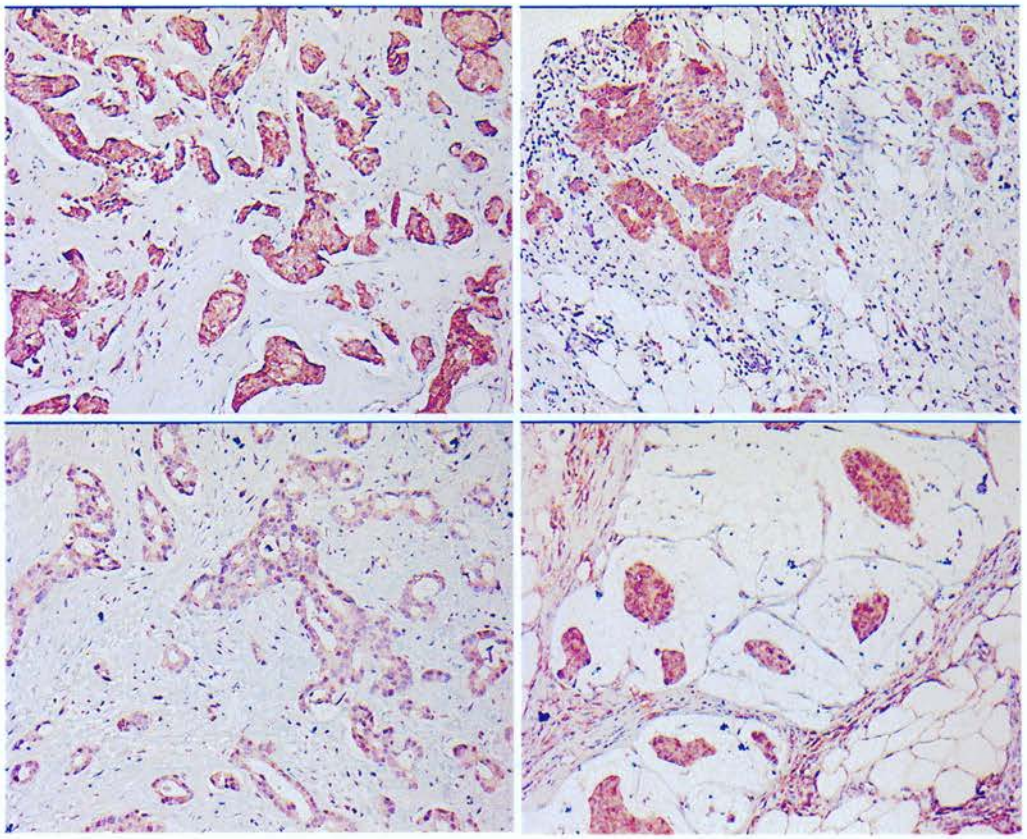


Figure 3.4 Expression of eEF1A2 in oestrogen receptor-positive breast cancers from patients at the Western General Hospital.
The majority of the cancers expressed eEF1A2 at a moderate to high level. The staining was mainly homogeneous on each section.

labelled polymer does not contain avidin or biotin and therefore this reduces non-specific staining. I applied this technique to stain a tissue array containing 250 breast cancer cores with 30 years' of follow-up clinical data. This is a far more powerful tool than the commercial tissue array used in previous expression analysis because of the large amount of clinical data available with it. The clinical data available for the TMA is shown in table 3.2.

Table 3.2 *Clinical data available with the in-house TMA*

Variable		Number of cases
Clinical Size	1	21
	2	62
	3	69
	4	47
	5	29
	Unknown	2
Histology	Ductal	193
	Lobular	27
	Other	10
Grade	1	48
	2	109
	3	71
	unknown	2
ER	0	41
	1	21
	2	156
	unknown	12

The eEF1A2 staining pattern was markedly different from that observed with the previous IHC protocol carried out on the commercial tissue array. The staining was homogeneous within cores as previously noted; however the staining was found to be either very strong or negative and there were not many cancer sections staining at intermediate levels for eEF1A2, see figure 3.5. 201 of these cores were then included for analysis, many cores were excluded on the basis of a lack of tumour present or the core lifting off of the slide and being lost. Two researchers and a pathologist independently scored the staining level using the histoscore method. Of the 201, 10 scored highly (200-300 histoscore) and 14 stained moderately (100-200) for eEF1A2. The inter-rater reliability was tested using the intra-class correlation coefficient (ICC). The ICC for this IHC scoring was 0.7538, suggesting that there was high agreement or concordance between the raters.

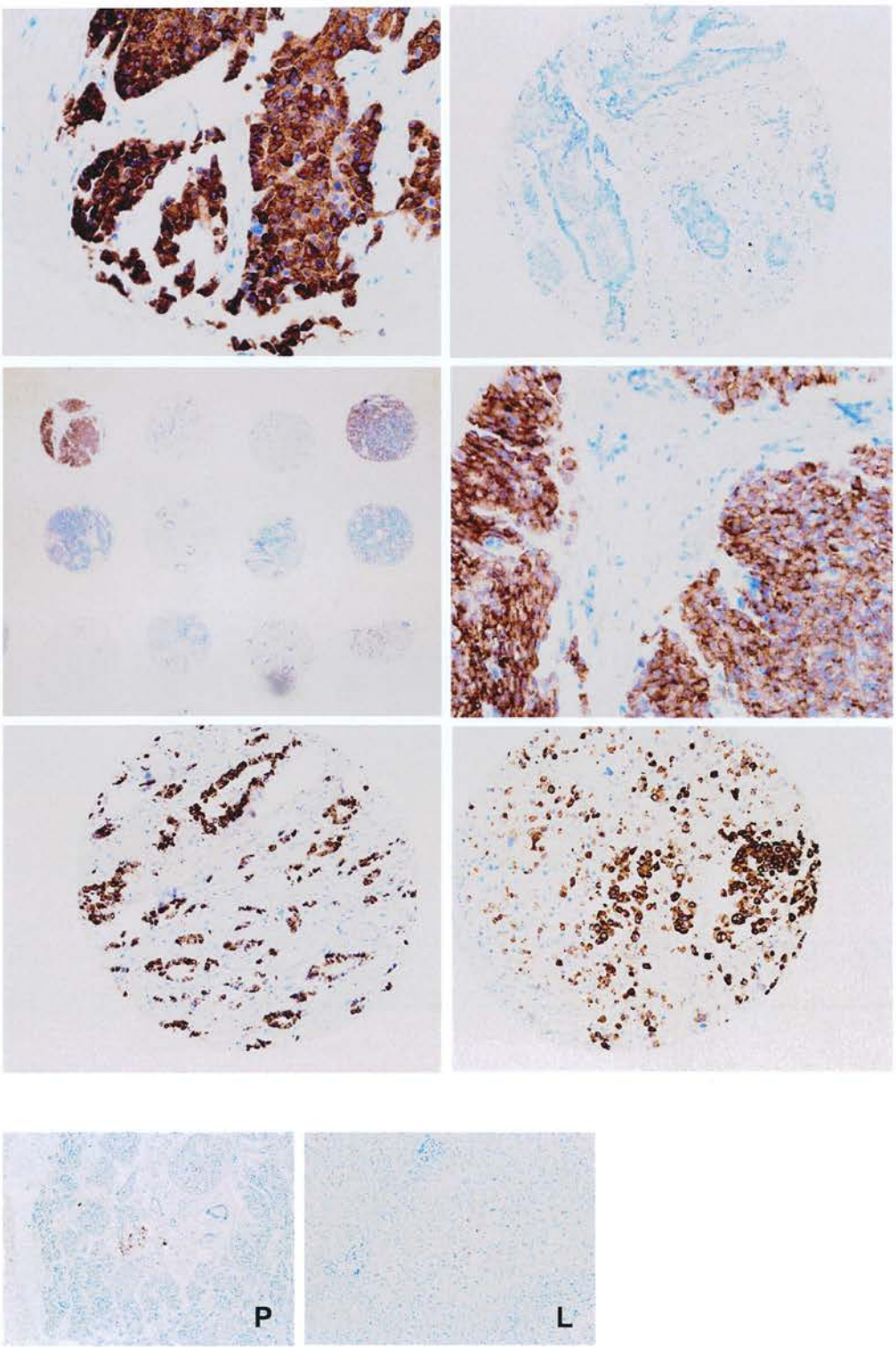


Figure 3.5 eEF1A2 expression in breast cancer cores on a large TMA.
The majority of cores were negative for eEF1A2, however, 11% stained moderately or highly for eEF1A2. In approximately 5% of the cores the expression of eEF1A2 was impressively high (histoscore of 300). Antibody controls show the Islets of Langerhans in the pancreas staining strongly (positive control), and the liver showing no eEF1A2 expression (negative control).

Approximately 5% of the cancers showed high levels of eEF1A2 expression and 7% stained moderately for eEF1A2. This is compared to 11% exhibiting high expression levels of eEF1A2 and 48% exhibiting moderate expression of eEF1A2 in the commercial tissue array. In addition there was no apparent association between eEF1A2 expression and histological type, grade, stage or oestrogen receptor status.

3.2.5 Expression of eEF1A2 at the RNA level in breast cancers

In order to investigate the expression of eEF1A2 at the RNA level we obtained RNA from oestrogen receptor positive (n=21) and negative breast cancers (n=8), as well as normal breast tissue (n=2) and benign breast tumour tissue (n=1) and I conducted quantitative real-time RT-PCR on the samples. These samples were collected and RNA extracted from them by Professor W. Miller and Dr. Alexey Larionov at the Western General Hospital. The RNA was extracted from total tumour tissue and not laser dissected material, however the relative amount of epithelial:stromal components are known. No tumours in which there was less than a 20% epithelial component were used in the analysis. Most cancers showed an epithelial percentage ranging from 40-60%.

I conducted real-time RT-PCR on cDNA samples from the tumours and normal breast using eEF1A2-specific primers and GAPDH primers as a reference gene. These primers were obtained from Applied Biosystems and are pre-designed and optimised, consisting of a primer pair and a FAM-labelled probe.

I confirmed the specificity of the eEF1A2 primers using cDNA obtained from lymphoblastoid cells that are known not to express eEF1A2; no eEF1A2 PCR product formed but a GAPDH transcript was present in this sample. Minus cDNA and RNA only controls were included to check for contamination. In the breast tissue analysed, levels of eEF1A2 were normalised to GAPDH levels to correct for differences in cDNA amounts. The normalised level of eEF1A2 in the two normal breast tissue samples was averaged to give an average expression level in normal breast. Levels of eEF1A2 in all other tumours were then standardised to the average level of eEF1A2 expression in normal breast.

Levels of eEF1A2 were very low in normal breast and benign breast tumour tissue compared to expression in tumours. The majority of breast cancers showed higher levels of eEF1A2 than normal and benign tissue; in fact in the extreme this level was up to 30 times higher than that seen in normal tissue. The expression levels of eEF1A2 in oestrogen receptor-negative cancers compared to the expression in normal breast tissue ranged from 0.01 to 4.53. In oestrogen receptor-positive cancers the eEF1A2 expression levels compared to that in normal breast ranged from 0.1 to 29.35. This is represented in figure 3.6. In oestrogen receptor-negative cancers the average expression level of eEF1A2 was 1.2 times higher than that seen in normal breast. Interestingly, the levels of eEF1A2 were higher in oestrogen receptor-positive breast cancers, showing an average level 8.4 times higher than in normal breast epithelium. This difference in eEF1A2 expression is 7.2 units ($p=0.0087$, t-test, 95% confidence interval 2.0 to 12.5 units), see figure 3.7, where 1 unit is the level of eEF1A2 in normal breast. A comparison of the expression level of eEF1A2 and the malignant component of the breast tumours (where the data is available) is shown in figure 3.8. There does not appear to be a consistently higher expression of eEF1A2 in tumours that also have a high malignant cell content suggesting that the eEF1A2 expression levels measured using this technique are representative of the levels in the tumour and not simply a consequence of the amount of stroma in each sample.

The observation of higher eEF1A2 RNA expression levels in the oestrogen receptor positive breast tumours together with the results of immunohistochemical analysis of breast cancer cores on the commercial tissue array suggests that *EEF1A2* may be an oestrogen-responsive gene or indeed may regulate oestrogen receptor expression by an unknown mechanism.

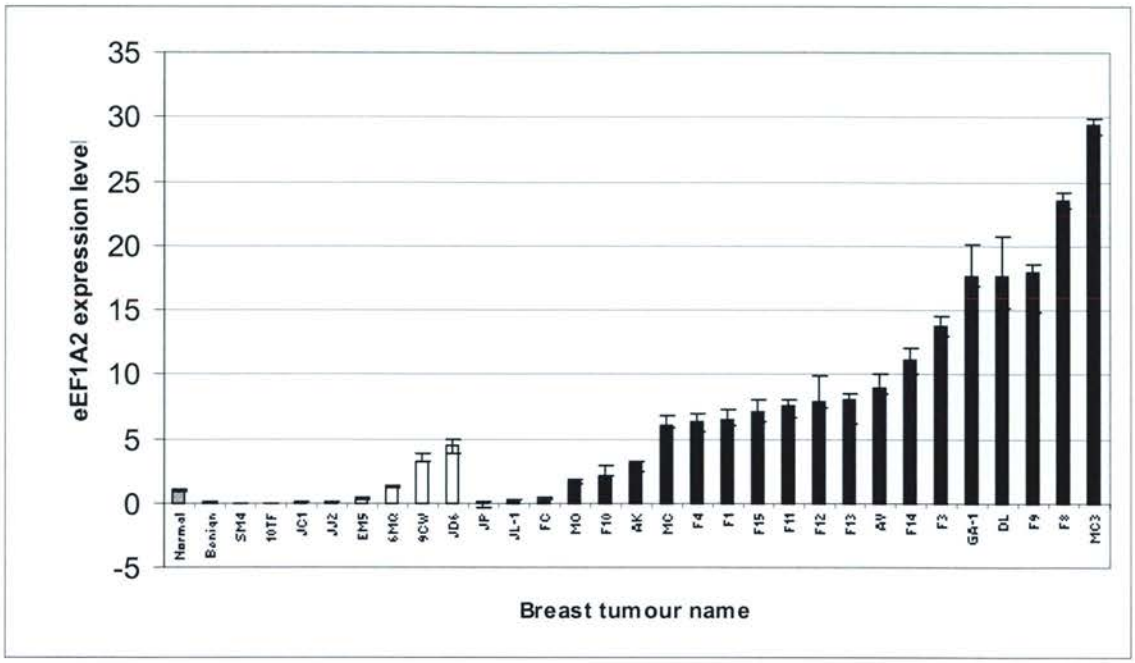


Figure 3.6 Expression of eEF1A2 at the RNA level in oestrogen receptor positive and negative breast cancers, analysed by real-time RT-PCR.
eEF1A2 expression at the RNA level in normal breast and benign breast tissue (grey), oestrogen receptor negative (white) and positive (black) cancers. Expression of eEF1A2 is low in the normal breast tissue and benign breast tumour tissue. The majority of the cancers analysed overexpressed eEF1A2, the highest showing a 30-fold increase in expression compared to normal breast tissue. Error bars represent 1 standard deviation PCR error.

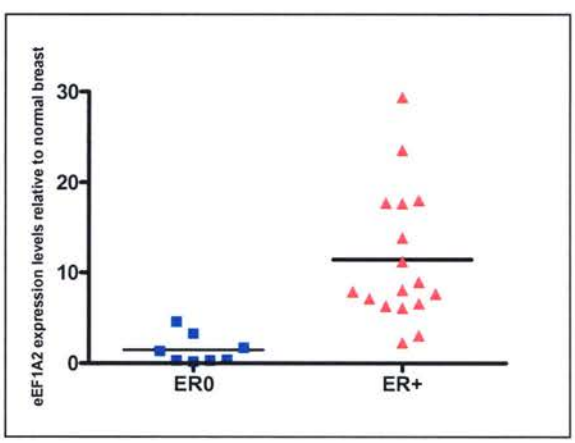
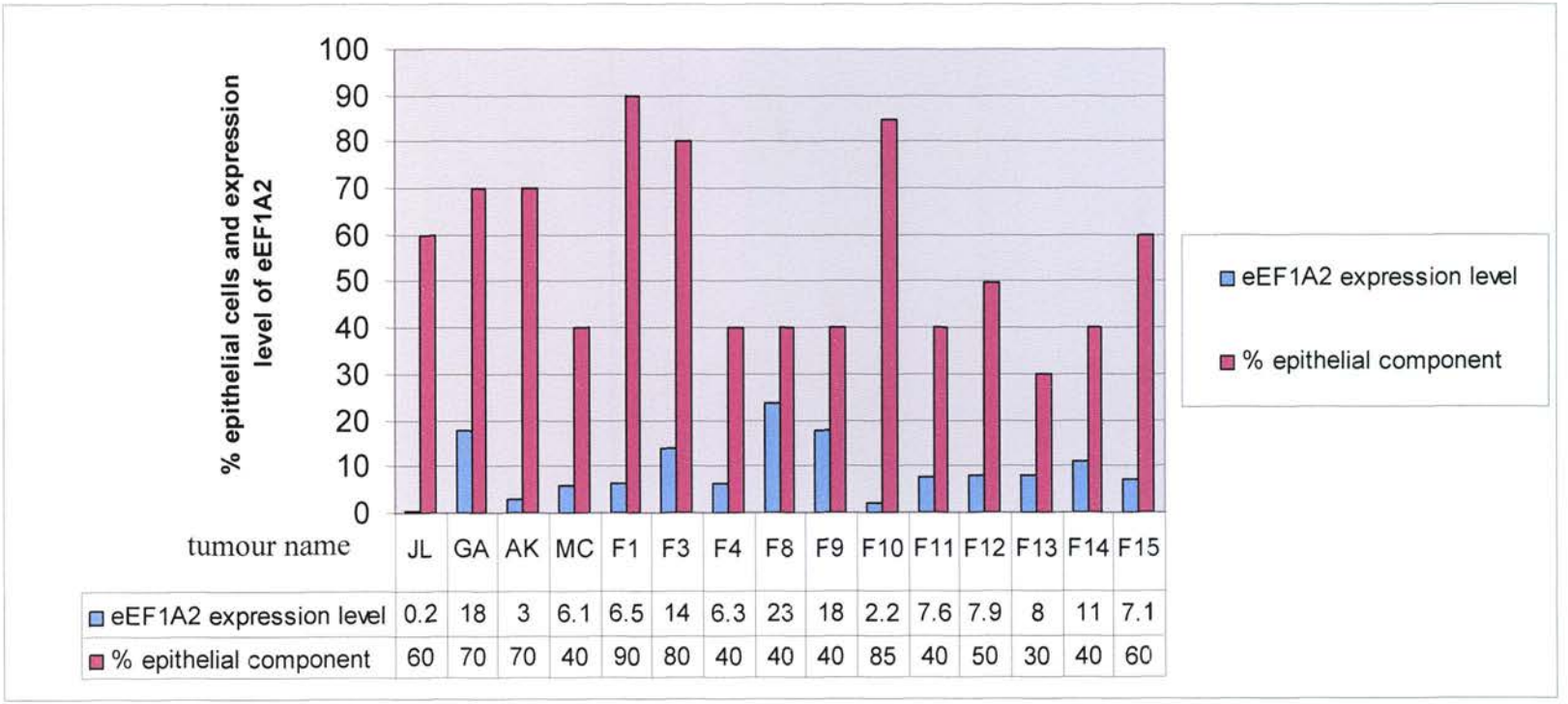


Figure 3.7 Levels of eEF1A2 expression are higher in oestrogen receptor positive (ER+) than negative (ER0) breast cancers.
Horizontal bars show mean eEF1A2 expression level for each group. This difference in eEF1A2 expression is 7.2 units (p=0.0087, t-test, 95% confidence interval 2.0 to12.5 units).



tumour name	JL	GA	AK	MC	F1	F3	F4	F8	F9	F10	F11	F12	F13	F14	F15
eEF1A2 expression level	0.2	18	3	6.1	6.5	14	6.3	23	18	2.2	7.6	7.9	8	11	7.1
% epithelial component	60	70	70	40	90	80	40	40	40	85	40	50	30	40	60

Figure 3.8 A comparison of the eEF1A2 expression levels and epithelial component of breast tumour samples.
There appears to be no relationship between eEF1A2 expression levels and the percentage epithelial cells in the tumours suggesting the expression levels are reflective of the eEF1A2 expression in the tumours and not simply a consequence of the amount of epithelial cells present.

3.2.6 Does the *EEF1A2* promoter region contain an Oestrogen Response Element (ERE)?

The results from analysis of expression levels of eEF1A2 in breast cancers showed that eEF1A2 expression is significantly higher in ER-positive cancers. This in turn suggests that eEF1A2 expression may be switched on by the activation of the oestrogen receptor or by an indirect mechanism resulting from oestrogen receptor overexpression. Hence, *EEF1A2* may be transcriptionally regulated by the oestrogen receptor or may be co-ordinately regulated with the oestrogen-receptor. I completed bioinformatical analysis using the Dragon ERE finder program 2.0 (Bajic et al., 2003) to identify possible EREs in the *EEF1A2* genomic sequence EMBL/Genbank Accession no. AF163763. The sensitivity of the program was set at the default level, which is 83%, and at this level the program predicts one ERE per 13 300nts based on analysis of chromosome 21 (Bajic et al., 2003). The input *EEF1A2* sequence (12kb) includes approximately 2kb upstream of the transcription start site (TSS). The first near consensus ERE identified in *EEF1A2* is on the forward strand at position 11299, located between exons 7 and 8, and is CA-GGTGA-CCC-TGCCC-AG, the divergent nucleotides being -2G and +3G. Two putative sequences were also identified on the reverse strand at positions -6334 and -2680 and were AA-GGGCA-CGG-TGGCC-GG and CA-GGTGG-AGA-TGCCC-TC respectively. None of the putative EREs are in the promoter region of *EEF1A2* on the forward strand and none of the putative ERE sequences in the human *EEF1A2* sequence are conserved in the mouse. The position of the predicted human *EEF1A2* EREs well outside the promoter region of the gene and the lack of conservation of these sequences between human and mouse suggests that they are non-functional.

3.2.7 eEF1A2 levels are not altered by the knockdown of ERα in MCF-7s

To further investigate whether *EEF1A2* is oestrogen responsive we obtained RNA and protein from MCF-7 cell lines that had ERα expression ablated by RNA interference. MCF-7 cells are breast cancer cells that overexpress the oestrogen receptor and are therefore appropriate to use for investigation of oestrogen-responsive genes. This experiment was carried out by Catherine Naughton under the supervision of Simon Langdon at the CRUK Laboratories, Edinburgh University. In

this experiment MCF-7 cells were transfected with 3 different oligos targeting the ERα sequence, called RNAi1 RNAi17 and RNAiERS1, and the levels of the oestrogen receptor measured by Western blot and quantitative real-time RT-PCR. In addition to this, the MCF-7 cells were also first treated with 17β estradiol and then subjected to RNA interference to ERα. Controls include untransfected MCF-7 cells treated and not treated with 17β estradiol, as well as cells transfected with a non-targeting oligo. 48 hours after transfection the cells were harvested for protein and RNA analysis. Catherine Naughton has confirmed knockdown of the oestrogen receptor alpha using this system (see figures 3.9 and 3.11). The highest level of ERα knockdown, at both the RNA and protein level, was seen after 17β estradiol stimulation and subsequent treatment with RNAi17. This experiment could potentially allow us to determine whether *EEF1A2* is an estradiol-responsive gene as opposed to being coordinately regulated with the ERα.

Using Western blot analysis I measured the levels of eEF1A2 in cells subjected to the above treatments. The results are shown in figure 3.9. The levels of eEF1A2 were normalised to the levels of GAPDH using image analysis software. eEF1A2 expression does appear lower in MCF-7 cells treated with siRNAs to ERα, particularly those treated with RNAiERS1. The expression of eEF1A2 in cells treated with non-targeting oligo (Neg) is also lower than untreated cells however. In addition, the expression of eEF1A2 does not increase in MCF-7 cells treated with estradiol compared to untreated cells suggesting that eEF1A2 is not oestrogen responsive.

I also analysed the expression of eEF1A2 at the RNA level using real-time RT-PCR by the SYBR green method. In this assay the reference genes pumilio homolog 1 (PUM1) and TATA box binding protein (TBP) were used. These genes are known not to be altered by changes in oestrogen receptor activity in breast cancers (Alexey Larionov, personal communication). The use of two housekeeping genes results in a more accurate normalisation of the expression level of the gene of interest, in this case *EEF1A2*. RNA only and –cDNA controls were included to rule out the possibility of contamination. Melt curves were used to confirm specificity of the

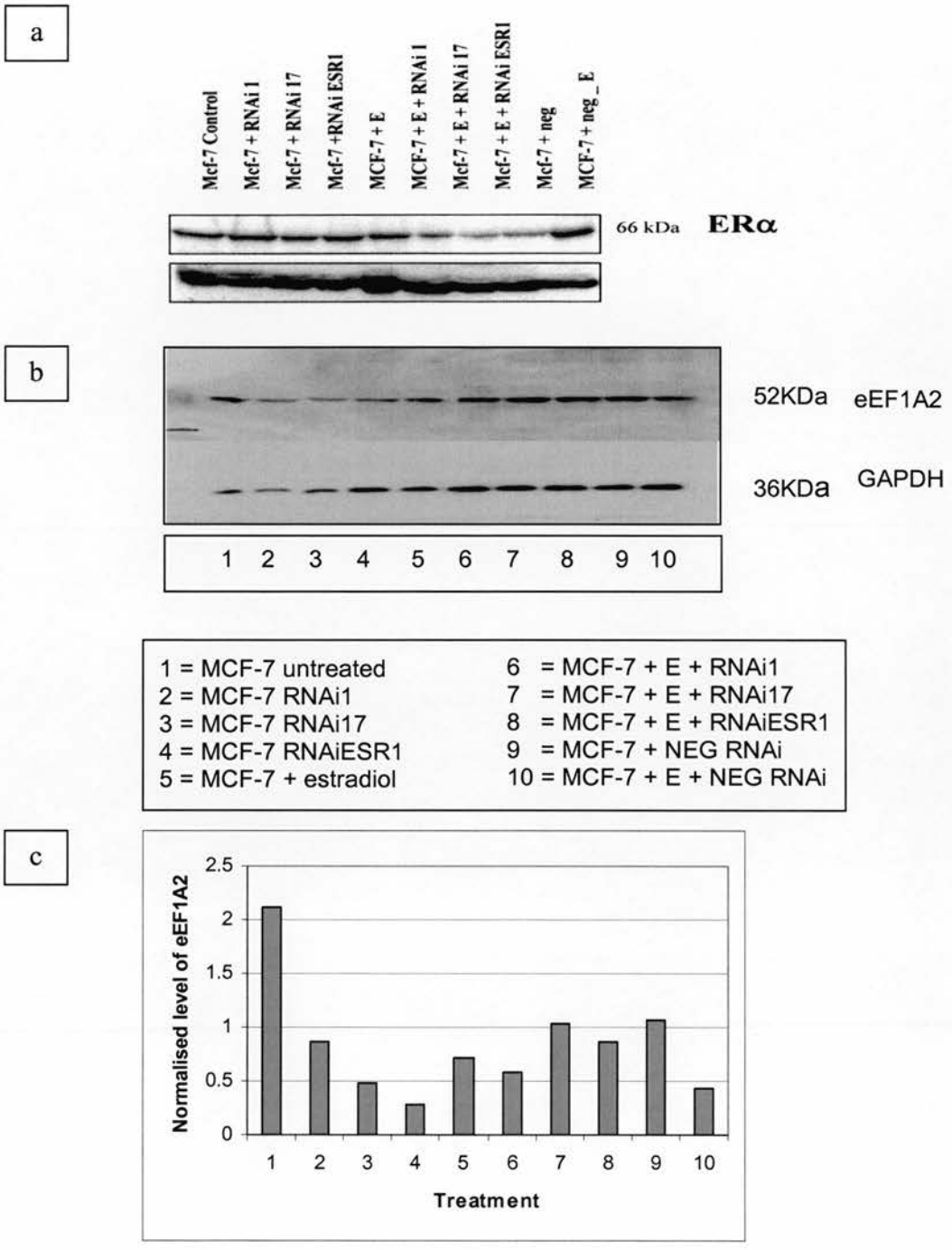


Figure 3.9 Western blot analysis of eEF1A2 protein levels in MCF-7 cells treated with siRNAs against the ERα.

(a) Western blot carried out by Catherine Naughton showing the knockdown of ERα in MCF-7 cells following various treatments (b) Western blot showing eEF1A2 expression in MCF-7 cells treated with siRNAs to ERα and/or treated with estradiol. (c) eEF1A2 expression levels normalised to GAPDH. eEF1A2 protein levels appear to be lower in cells treated with RNAi against the ERα than in untreated cells (treatments 2-4 compared to 1). Treatment of MCF-7 cells with estradiol does not appear to result in an increase in eEF1A2 expression however (treatments 5-8).

PCR products. A standard curve was run for each pair of gene-specific primers, on cDNA from oestrogen-stimulated untreated MCF-7 cells, to determine PCR efficiency, see figure 3.10. Each of the genes did not have the same PCR efficiency and therefore the delta delta Ct method of analysis could not be used. Instead, the results were analysed using the Excel macro available with the real-time PCR machine that uses algorithms detailed in Vandesompele *et al.* (Vandesompele et al., 2002). This algorithm allows the normalisation of a gene using multiple reference genes with different PCR efficiencies. Results of the quantitative analysis of eEF1A2 expression are shown in figure 3.11 as well as results obtained from Catherine Naughton showing the ERα expression at the RNA level. It appears that levels of eEF1A2 actually increase, relative to the level in untreated MCF-7 cells, upon ERα knockdown. Additionally, eEF1A2 expression appears to decrease upon estradiol treatment of MCF-7s. This suggests that eEF1A2 is not positively oestrogen responsive; it even appears in this system to be negatively regulated by ERα at the RNA level. There appears to be some indication that eEF1A2 expression may decrease upon ERα knockdown by RNAi but this is by no means conclusive. Interestingly, ERα levels are only knocked down by the siRNA ERS1 following pre-treatment of the cells with estradiol. This suggests that the siRNA is not effective unless the ERα mRNA levels are high and therefore that the affinity of the siRNA is not high enough to trigger the RNA interference process when there is not much ERα message present.

3.2.8 eEF1A2 expression levels do not decrease in Letrozole treated breast cancers

In order to investigate the apparent association between ER positive tumours and high eEF1A2 expression levels *in vivo* we have obtained sections of tumours from patients that had been given neo-adjuvant hormone therapy with Letrozole (Femara), from Mike Dixon at the Western General Hospital. Letrozole is an aromatase inhibitor; inhibiting the conversion of androgens produced in lipid to oestrogen and is prescribed to patients with ER positive tumours. Therefore post treatment the levels of activated ER should decrease and concomitantly the levels of oestrogen responsive gene expression should decrease. Biopsies were taken before treatment,

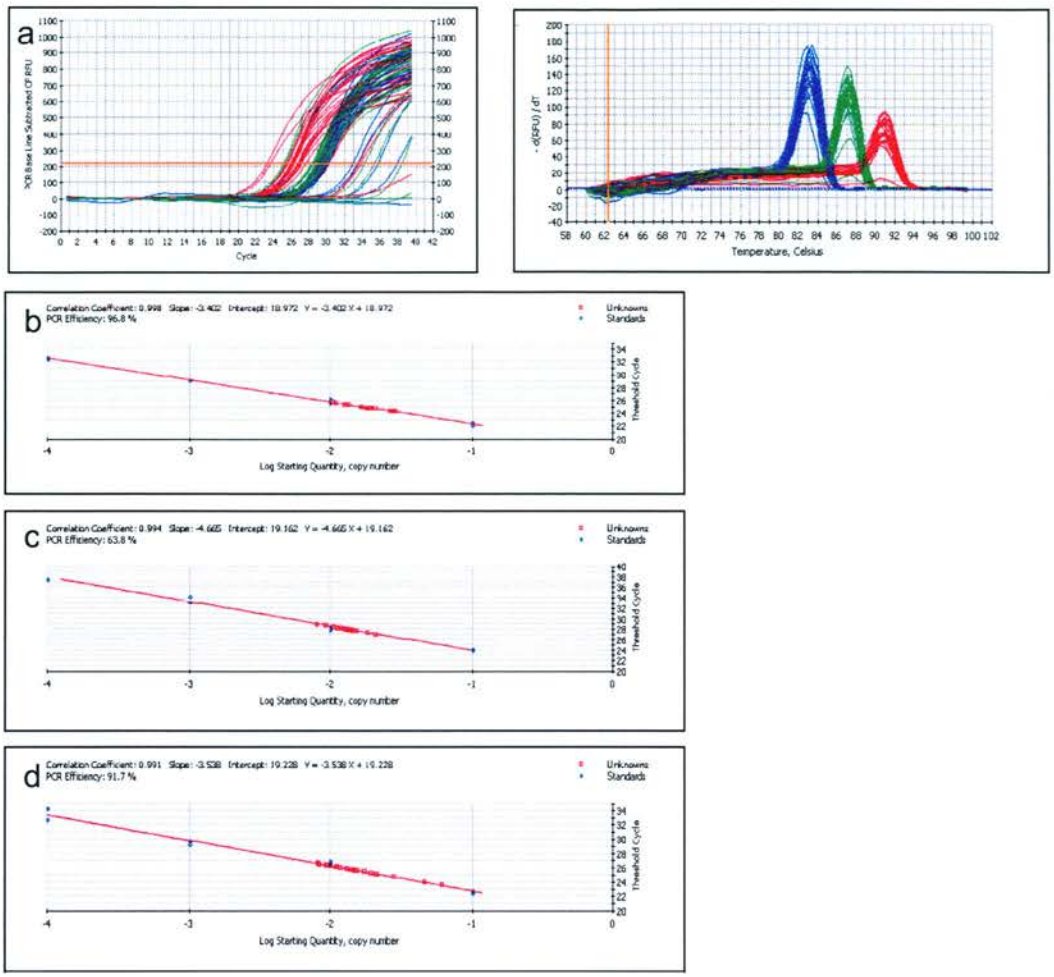
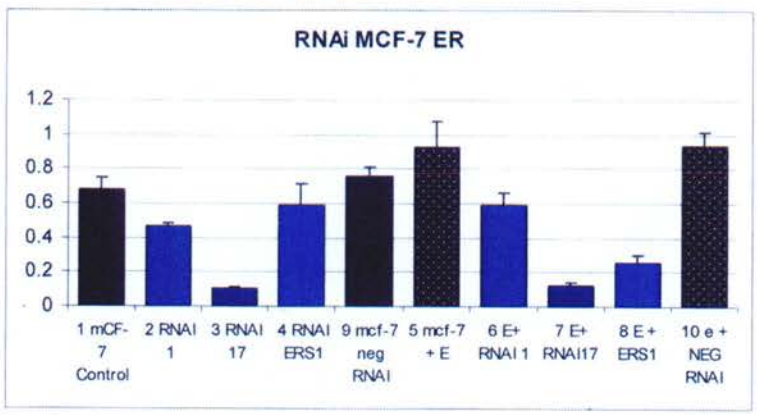


Figure 3.10 Amplification, melt and standard curves for determination of eEF1A2 levels in ERα RNAi-treated MCF-7 cells.
(a) The amplification and melt curves of eEF1A2, TBP and PUM1. Strong specific peaks in the melt curve for each PCR product show the primers anneal specifically. In blue is the eEF1A2 PCR product, in green PUM1 and in red TBP. Standard curves all have a correlation coefficient of more than or equal to 0.99. (b) Standard curve for eEF1A2 PCR, (c) standard curve for PUM1 (d) standard curve TBP product. Blue circles denote the standards, red squares the samples being quantified.

a



b

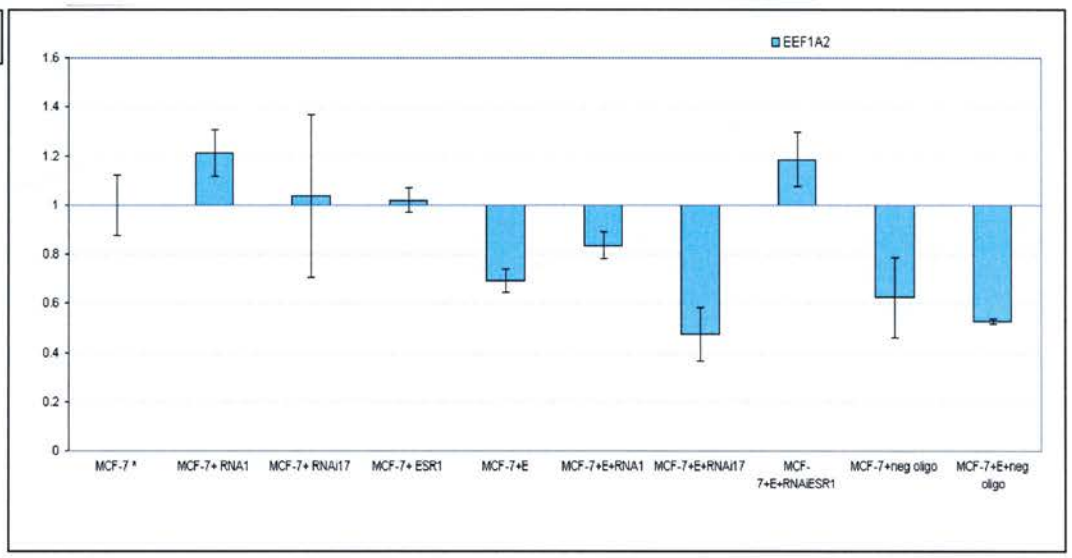


Figure 3.11 eEF1A2 levels in ERα RNAi treated MCF-7 cells.
(a) Real-time RT-PCR quantification of ERα expression levels in MCF-7 cells treated with an ERα targeted siRNA, and/or estradiol - Catherine Naughton. (b) eEF1A2 levels are shown normalised to PUM1 and TBP and standardised to levels in untreated MCF-7 cells (*). Cells in which ERα expression has been ablated show a small increase in eEF1A2 expression compared to controls. Cells treated with estradiol and then subjected to ERα RNAi show a small decrease in eEF1A2 expression. These fluctuations in eEF1A2 levels are not large – no increase or decrease in eEF1A2 levels is greater than approximately 1.2 times the level seen in untreated MCF-7 cells. Levels of eEF1A2 expression do not appear to correlate to the levels of ERα expression.

slides marked with an A, 10-14 days into treatment (B), 3 months into Letrozole treatment (C) (Miller et al., 2006). The sections of biopsies taken during neo-adjuvant treatment and at surgical resection were stained for eEF1A2 expression and secondary antibody only controls were included. Additionally, a tissue array containing normal human liver (negative for eEF1A2) and pancreas (positive for eEF1A2) was also stained with 1A2-3 as antibody controls. Examples of the staining can be seen in figure 3.12.

The sections were examined by a pathologist, Dr Dana Faratian. He observed heterogeneous staining within the cytoplasm of the cancer cell epithelium; eEF1A2 either showed a diffuse cytoplasmic subcellular localisation or was concentrated around the nucleus i.e. in a perinuclear localisation. The expression pattern of eEF1A2 across some sections was also heterogeneous, with some tumours showing areas of cancer expressing high levels of eEF1A2 and concomitantly showing no expression of the elongation factor in another area. It has been previously been reported that eEF1A expression is higher at the leading edge of breast tumours (Zhu et al., 2003) and this may explain the heterogeneity observed in these breast cancer sections. An additional important observation is that in section WB01/12431 normal ductal epithelium is staining positive for eEF1A2; examples of this can be found in figure 3.13. This implies that there may be a field effect causing expression of eEF1A2 and that eEF1A2 expression may be an early event in cancer progression or neoplasia. Alternatively, the expression of eEF1A2 in normal adjacent breast tissue could be induced by the secretion of an unknown factor by the surrounding malignant cells. It would be necessary to obtain DNA from the normal breast tissue in order to determine if there is an underlying genetic aberration in the normal cells causing eEF1A2 expression and therefore the presence of a field effect. Importantly, there appears to be no correlation between stage of aromatase treatment and expression of eEF1A2, adding another argument against *EEF1A2* being an oestrogen responsive gene.

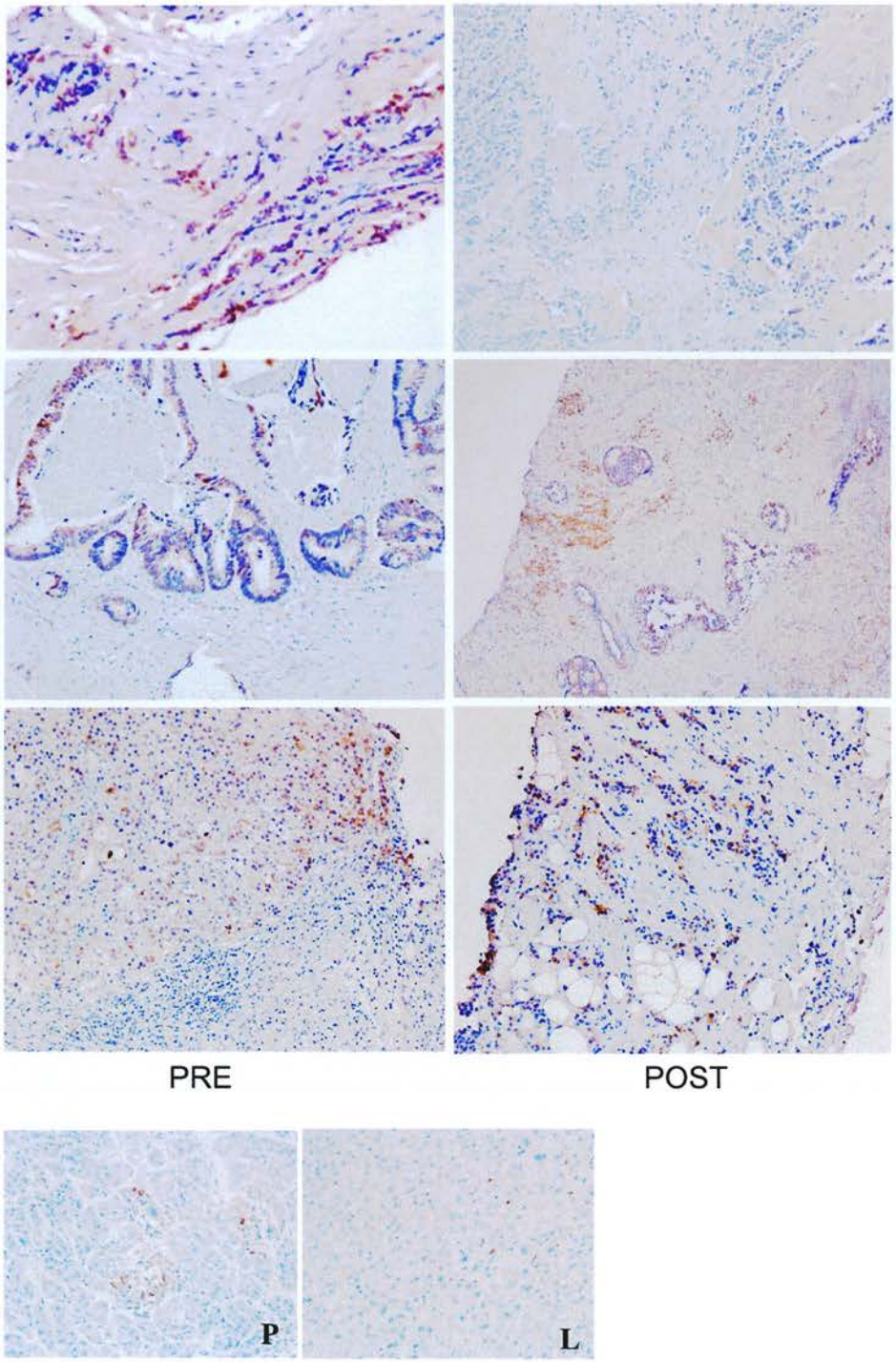


Figure 3.12 Expression of eEF1A2 in breast tumours pre and post Letrozole treatment.
eEF1A2 expression does not appear to decrease, compared to the levels in biopsies taken before Letrozole treatment, in oestrogen receptor positive tumours at 10-14 days, or 3 months into treatment. Positive control for eEF1A2 is the pancreas (P) and the negative control is the liver (L).

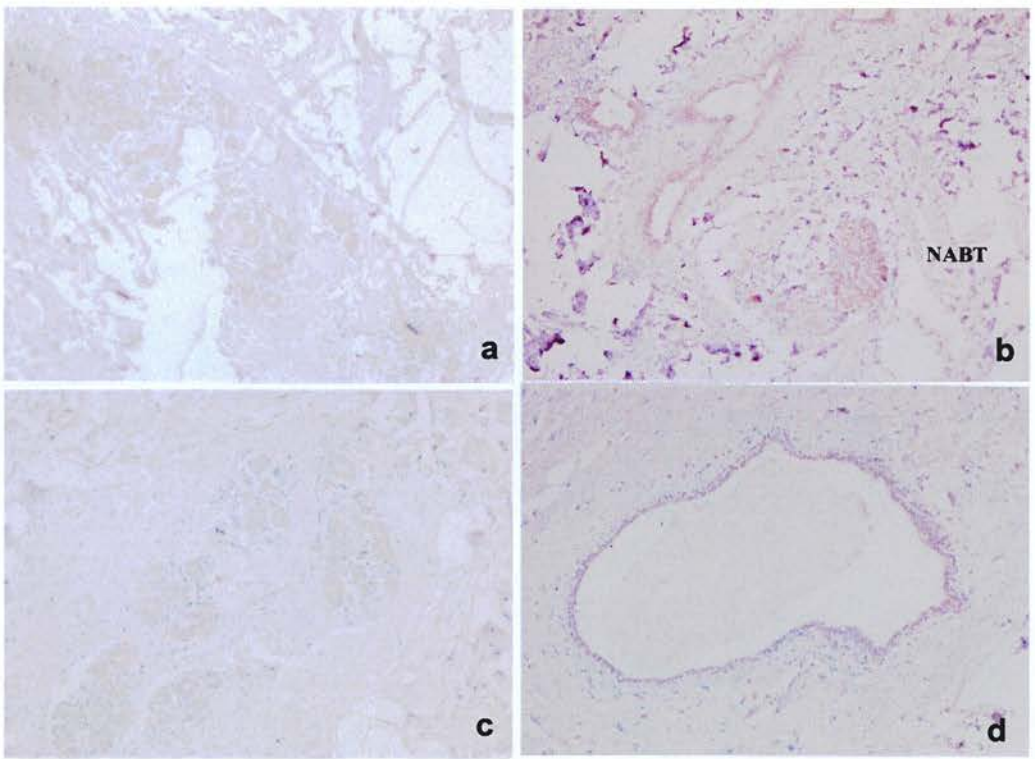


Figure 3.13 Normal adjacent breast tissue (NABT) staining positively for eEF1A2

In section WB01/12431 no invasive breast cancer was present however hyperplastic lesions (d + b) and normal breast tissue can be observed (a-c). eEF1A2 expression is present in both the hyperplastic lesion and the normal adjacent breast tissue suggesting that field cancerization may be at play.

3.2.9 Is eEF1A2 preferentially expressed in lymph node positive cancers?

The heterogeneous expression pattern of eEF1A2 observed in some of the breast tumours examined by immunohistochemistry and the evidence of a higher level of eEF1A at the invading edge (Zhu et al., 2003) lead us to investigate the possibility that eEF1A2 may be more highly expressed in lymph node positive breast tumours, due to a potential role of the protein in invasion. Ten lymph node positive and ten lymph node negative breast tumour sections were stained using the 1A2-3 antibody and the DAKO Envision secondary system. Only one of the ten sections showed uniform staining of eEF1A2. This positive tumour was lymph node positive but due to the small numbers of sections stained nothing could be concluded from this. However, an interesting observation was that in many of these tumours eEF1A2 seemed to be expressed in precursor hyperplasic lesions and Ductal Carcinoma *In Situ* (DCIS): examples of this staining pattern can be found in figure 3.14. In four of the ten tumour sections DCIS staining positively for eEF1A2 was observed by the pathologist, however the grade of DCIS was not assessed and all tumour sections did not contain DCIS. A more thorough analysis of the expression of eEF1A2 in DCIS was not carried out.

This observation of eEF1A2 expression in DCIS implies that eEF1A2 may be expressed early in breast cancer progression and therefore may be important for early events in breast carcinogenesis. It may also suggest that eEF1A2 is important for progression to an invasive phenotype in which the hyperplastic cells breach the basement membrane and begin to invade the surrounding stroma. This is quite nicely illustrated in figure 3.14, where eEF1A2 expression can be seen in cells that have breached the basement membrane into the surrounding stroma. More analysis on a larger set of breast tumours would be required to add validity to these observations. Currently, an array of more than 400 node positive and negative breast tumours is being produced by Dana Faratian at Edinburgh New Royal Infirmary and this tool may further validate the results obtained from the limited investigation carried out here. Additionally, examining the expression of eEF1A2 in a panel of pre-malignant breast lesions would allow us to determine if eEF1A2 expression is common in these early stages of breast cancer.

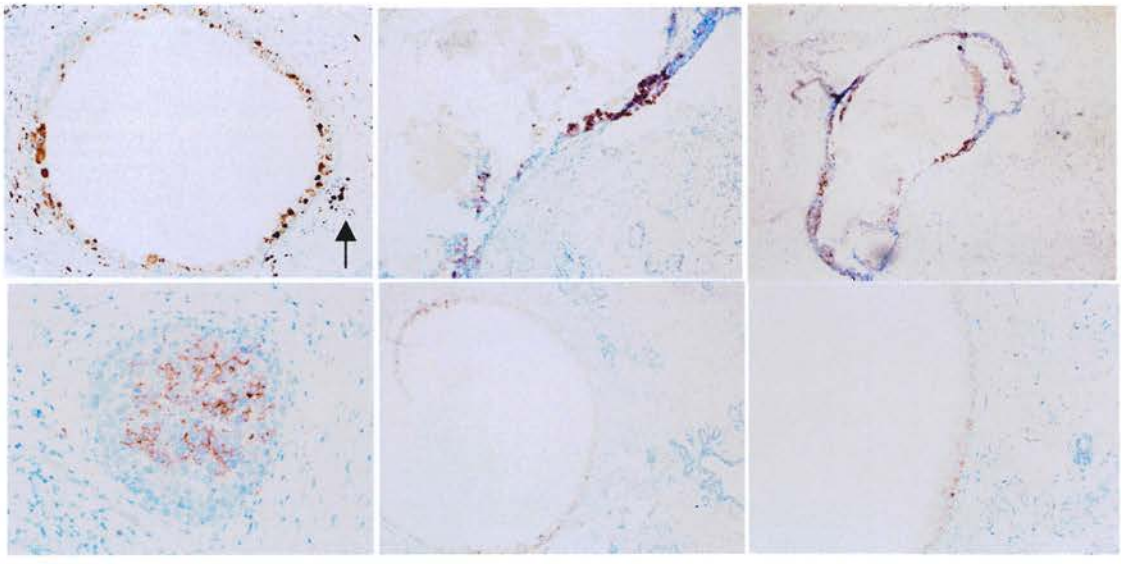


Figure 3.14 Expression of eEF1A2 is often observed in hyperplastic pre-malignant lesions in the breast, such as DCIS.
eEF1A2 expression is frequently found in pre-malignant lesions in the node positive and negative, full face breast tumour sections. This observation suggests that eEF1A2 expression may be an early event in breast carcinogenesis. The arrow denotes eEF1A2 expressing cells that appear to have breached the basement membrane.

3.3 Discussion

Early analysis of eEF1A2 expression levels in breast cancers using commercial tissue arrays and sections from ER positive breast cancer biopsies suggested that the elongation factor was expressed in a large proportion of breast tumours (two-thirds) at a moderate to high level (Tomlinson et al., 2005). Subsequent analysis of eEF1A2 levels in another array containing over 200 cores from breast cancers showed only 11% of the cores stained moderately to strongly for eEF1A2. This difference could be due to the fact that only one tissue array (and therefore one core from each tumour) had been scored with one antibody for the TMA obtained from Dana Faratian whereas two cores from each tumour were stained with two different antibodies on the commercial tissue arrays. The use of two different antibodies on two cores per tumour is likely to give a more accurate representation of eEF1A2 expression in breast cancer as a whole. Additionally, the percentage of tumours staining positive for eEF1A2 on the commercial TMA is more consistent with the number of tumours expressing eEF1A2 at the RNA level. The observed heterogeneity of eEF1A2 in the Letrozole treated and lymph node positive and negative sections would also suggest that multiple cores from each tumour should be stained to get an accurate picture of eEF1A2 expression.

Dana Faratian's TMA not only contained more cores but was stained using a technique (Envision secondary antibody) that is thought to decrease non-specific staining and this was the reason for using this alternative technique. Therefore of the 48% of cores that stained moderately positive for eEF1A2 on the commercial tissue array a percentage may have been exhibiting non-specific staining. However, the specific staining of eEF1A2 in the epithelium but not the stroma acts as an internal control on the commercial TMA and suggests otherwise. Additionally, the discrepancy between the percentages of cores staining positive for eEF1A2 between the two tissue arrays may be due to the different antigen retrieval techniques used. The pressure cooking in EDTA solution method used on the larger array may only lead to partial antigen retrieval and therefore only those cores expressing eEF1A2 at high levels would stain positively.

The clinical and pathological information for the tumours on both the commercial tissue array and the larger in-house array are presented in tables 3.1 and 3.2. For the production of the commercial tissue microarrays the tissues were fixed in 4% buffered neutral formalin for 12 to 24 hours, dehydrated with gradient ethanol, cleared with xylene, and embedded in paraffin. The oestrogen receptor, progesterone receptor and p53 expression was analysed using immunohistochemistry with multiple antibodies. For the in-house TMA the tissues were fixed in formalin, mercuric acid, or Bouin's fixative, the later two being different from the fixation technique used in the production of the commercial TMA. This difference in the method of fixation between the commercial and in-house array could be the reason behind the differences in eEF1A2 expression observed between the two TMAs. The histology of the tumours included on the commercial TMA and the in-house TMA are homogeneous with the majority being ductal and not lobular. Additionally, there is a lower representation of ER positive tumours on the commercial TMA (27/46, 60%) than on the in-house TMA (136/177, 77%), which would be expected to (based on the association of eEF1A2 with ER positive cancers) result in higher percentage of eEF1A2 positive tumours on the in-house TMA than the commercial TMA and not the lower percentage that was observed, suggesting this is not behind the difference in eEF1A2 expression.

A paper published recently by Kulkarni *et al.* suggests that the immunohistochemistry technique used on the commercial tissue array gave a more accurate representation of the actual expression pattern of eEF1A2. They found that 50-60% of primary breast tumours expressed eEF1A2 at a moderate to high level (Kulkarni et al., 2006); a very similar level to that observed in the commercial tissue array. In this array, only primary invasive breast carcinomas were included on the TMA. The majority of the breast cancers on the commercial TMA for which I investigated eEF1A2 expression were also invasive breast carcinomas. The median age of the patients at diagnosis was 61.5 years (higher than the median age of 47 in the cohort of patients on the commercial TMA), with the minimum age of 28.2 years and maximum of 93.5 years. Kulkarni *et al.* used EDTA pH8.0 with heating for antigen retrieval, which is different from the technique I used to immunostain the

commercial TMA: citric acid pH6. Another obvious difference in the staining protocol is the eEF1A2-specific antibody used by Kulkarni *et al.* The peptide used by Kulkarin *et al.* corresponds to the peptide we used to generate the 1A2-2 antibody. I however did not use this antibody in any of the expression analysis because it was less specific than the 1A2-1 and 1A2-3 antibodies. Therefore the immunohistochemical protocols used in my analysis of the eEF1A2 expression on the commercial TMA and Kulkarni *et al.*'s are quite different, however the results seem similar in terms of the proportion of breast cancers expressing moderate to high levels of eEF1A2. The tumours used in my analysis and Kulkarni *et al.*'s appear fairly homogeneous in respect to histology however the proportion of the tumours that are oestrogen receptor positive, lymph node positive and the stage of the tumours is not detailed in the Kulkarni *et al.* manuscript and I am therefore unable to compare these aspects of the different TMAs. The main difference between the staining is that I used two antibodies raised to different regions of the mouse eEF1A2 sequence and I used a different antigen retrieval method.

The expression of eEF1A2 showed a significant association with oestrogen receptor positive breast cancers (Tomlinson et al., 2005). Further investigations into the possibility that *EEF1A2* is an oestrogen responsive gene have not completely resolved the question, however, they do suggest that eEF1A2 is not directly regulated by ERα. The apparent absence of an ERE in the *EEF1A2* promoter region implies the ER does not directly bind to the gene to activate transcription; however the sequence motif of the ERE is not as simple as previously described. For instance the ER is also known to bind to half EREs in multiple copies and ERα can bind extended half site sequences of the orphan nuclear hormone receptor steroidogenic factor 1 (SPREs). Half site binding can also take place in combination with the binding of a nearby Sp1 site (O'Lone et al., 2004). There was an ERE identified in the intron between exons 7 and 8 of *EEF1A2* on the forward strand, and it is possible that the distant EREs may be functional as it has been suggested that EREs upstream of the promoter could be active through the formation of chromatin loops to allow the recruitment of the basal transcription machinery on to the start site (O'Lone et al., 2004). However, the lack of conservation of the predicted ERE sequences between

the human and mouse *EEF1A2* suggests that they are in fact non-functional. At the sensitivity level used (83%), the Dragon ERE program 2.0 is known to predict a putative ERE motif every 13 300nts therefore it would be expected that one such motif would be identified in the 12Kb of *EEF1A2* sequence. The ability of these putative EREs to bind the ER *in vitro* could be tested using CHiP analysis and gel shift assays. It is important to note that the oestrogen receptor can activate the transcription of a gene by mechanisms that do not involve direct interaction with the DNA. As described previously the ER can associate with other DNA binding transcription factors and indirectly mediate transcription (Kushner et al., 2000). Therefore the apparent lack of a functional ERE in *EEF1A2* does not completely rule out the possibility that the ER may regulate its expression.

Further evidence suggesting *EEF1A2* is not oestrogen responsive comes from the results of the evaluation of the protein levels of eEF1A2 in MCF-7 cells in which the ERα has been stimulated by 17β estradiol (E_2) administration for 48 hours and/or ablated by RNA interference. Upon E_2 stimulation of ER-expressing cells the level of an oestrogen-responsive gene would be expected to increase and to decrease upon ER ablation by RNAi; RNA levels of eEF1A2 actually appear to decrease in MCF-7 cells 48 hours after estradiol exposure and increase upon RNAi ablation of the ERα. This even goes as far as to suggest that eEF1A2 is negatively regulated by the ERα. *EEF1A1* on the other hand has been shown to be oestrogen responsive in MCF-7 cells. Charpentier *et al.* looked at global gene expression using SAGE in MCF-7 cells treated with E_2 for 3 hours and 10 hours. They found eEF1A1 to be upregulated 3.2 fold following 3 hours of 17β Estradiol treatment, increasing to 3.5 fold after 10 hours of treatment (Charpentier et al., 2000). Interestingly they did not identify *EEF1A2* in this screen. *EEF1A1* and *EEF1A2* have very similar coding regions but divergent promoter regions, introns, and 5' and 3' UTRs (Knudsen et al., 1993), therefore it is likely that the two genes are differently regulated. *EEF1A1* is also found in the ERGDB database of oestrogen responsive genes (Tang et al., 2004). This database was compiled from a literature review of experimentally identified oestrogen target genes and includes putative ERE sequences identified using the Dragon ERE Finder 2.0 (Bajic et al., 2003). The location of the transcription start

site (TSS) and promoter sequence was determined using FANTOM3 or UCSC's Gene Sorter based on the Refseq ID/GenbankID BC028674. The TSS is listed as position 96624443 on chromosome 6 and two putative EREs were identified; the first at position 681 nucleotides (nts) upstream of TSS, TT-GGTCA-GGC-TGGTC-CT, and the second 3687 nts upstream of the TSS, TT-GGTCT-TGA-TGTCT-CA (Tang et al., 2004). Both of these potential EREs are fairly close to the TSS of *EEF1A1* and therefore are perhaps more likely to be functional than those identified in *EEF1A2*. It has been shown that EREs up to 10Kb upstream from the TSS can be functional (Bourdeau et al., 2004).

There are weaknesses in the way the association of eEF1A2 expression with ER positive cancers was discovered. The commercial tissue array only contained cores from 46 different breast tumours. This is a relatively small number of cores and this may lead to the identification of a false association. Notably there were an approximately equal number of ER positive and negative tumours on the array. The ER association was reproduced when the levels of eEF1A2 mRNA were evaluated by real-time RT-PCR in RNA obtained from a different group of ERα positive and negative breast tumours. This analysis showed that eEF1A2 mRNA levels were significantly higher in the ERα positive group than negative group ($p=0.0087$, t-test, 95% confidence interval 2.0 to 12.5 units). Similarly, this analysis was also carried out on a small number of samples, (n=8 ERα negative cancers, n=21 ERα positive cancers). This was due to the fact that only a limited number of ERα negative tumour biopsies were archived because this tumour type was not relevant to the study for which the samples were originally being collected. Additionally, as eluded to previously, the RNA was extracted from whole tumour samples flash frozen in liquid nitrogen following surgery and therefore contains both stromal and epithelial components. As eEF1A2 is not expressed in stroma, samples with a higher total stromal component will show a lower level of eEF1A2, even if it is expressed at high levels in the epithelial component. There did not however appear to be any correlation between the percentage epithelial content of the tumour samples and the eEF1A2 expression level.

Kuang *et al.* investigated the differential expression of cDNAs between the oestrogen receptor positive cell line MCF-7 and the oestrogen receptor negative cell line MDA-MB-231 and found eEF1A2 was expressed at a 25-fold higher level in MCF-7 cells. However, they did not identify eEF1A2 as being estradiol-responsive (Kuang et al., 1998). This observation together with my results showing eEF1A2 levels do not increase upon estradiol treatment of MCF-7 cells or decrease in tumours post-letrozole treatment, suggests *EEF1A2* may be co-ordinately regulated with the ERα but not oestrogen-responsive. eEF1A2, for instance, may be stimulated by a transcription factor that had its transcription directly activated by the ER. Evidence against eEF1A2 being co-ordinately regulated with the ER comes from Kulkarni *et al.* who did not find an association between eEF1A2 expression in breast cancers and oestrogen receptor positive breast cancers. They did however find that eEF1A2 was expressed almost exclusively in the luminal-like tumours, a tumour type that is known to be mainly ER positive (Kulkarni et al., 2006).

An interesting observation is that eEF1A2 appears to be expressed in some normal breast epithelium surrounding a cancer and was expressed in several hyperplastic lesions and DCIS. In one tumour eEF1A2 expression was observed in both DCIS and surrounding normal breast tissue. These observations suggest that eEF1A2 expression may be an early event in breast carcinogenesis. Interestingly, 20q13 amplification (a potential mechanism of eEF1A2 overexpression) has been identified by FISH and CGH to be present in the early stage of intraductal hyperplasia (Aubele et al., 2002). The inappropriate overexpression of eEF1A2 in adjacent normal breast tissue suggests that genetic alteration, by whatever mechanism it may be, early on in the carcinogenic process has induced eEF1A2 and this would fit into the field cancerization model. Work by Anand *et al.* showing that eEF1A2 is capable to inducing a tumourigenic phenotype in NIH 3T3 cells and accelerated growth of ovarian cells when xenografted into nude mice (Anand et al., 2002), suggests that eEF1A2 expression could confer a growth advantage to the cells in a developing field. An alternative hypothesis is that the cancer cells exert a transforming effect on surrounding cells leading to the induction of expression of gene and this model has been proposed in prostate cancer (Ozen et al., 1997). Nevertheless, field

cancerization has been observed previously in breast cancer and may therefore be an appropriate model to explain the presence of high amounts of eEF1A2 in histologically normal adjacent breast tissue. Additionally, eEF1A2 expression could be induced in the normal adjacent breast tissue by a secreted product from the nearby malignant cells and this is another possible mechanism mediating eEF1A2 upregulation in normal breast.

Another interesting observation in breast tumour sections was the heterogeneity in eEF1A2 staining. This together with the observation by Zhu *et al.* of eEF1A expression being higher at the leading edge of breast tumours (Zhu et al., 2003) suggests that eEF1A2 may be playing a role in tumour invasion and metastasis. In order to attempt to investigate this, the expression of eEF1A2 in a panel of 5 full face lymph node positive, i.e. invasive/metastatic breast tumours, and lymph node negative non-invasive breast tumours was analysed by IHC. I found that only one of the 10 tumours showed expression of eEF1A2 and that there did not appear to be a higher expression of the factor at the edge of the tumour. This suggests that the heterogeneity seen in some breast tumours in the Letrozole study was not due to higher expression at the invading edge. It also suggests that the Zhu *et al.* may have been detecting an increase in expression of eEF1A1 and not eEF1A2 at the leading edge of breast cancers. In order to investigate whether eEF1A2 is more highly expressed in lymph node positive than negative breast tumours it would be necessary to look at a larger cohort of samples. Dana Faratian is currently producing a TMA containing cores from 400 lymph node status known tumours, which would be a very powerful tool for finally drawing a conclusion on this hypothesis.

Another possible method to investigate the potential role of eEF1A2 in invasion and metastasis would be to investigate whether eEF1A2 expression is associated with any of the known markers for invasion and metastasis. These include N-CAM, integrins, E-cadherin, uPA, cathespinD and metalloproteinases, to name but a few. An association could suggest a mechanistic link between the marker and eEF1A2 and could then be further investigated with *in vivo* and *in vitro* studies. One experimental method commonly used to assess the invasive potential of a gene is a matrigel

invasion assay. This involves plating cells in a chamber and adding some form of chemoattractant below the chamber (e.g. FCS). Between the well and the chamber is a synthetic basement membrane through which only invasive cells can travel. The breast cancer cell line MBA-MD-231 is known to be highly invasive and we have shown it to express eEF1A2 (data not shown). Performing RNAi and assaying for changes in invasive potential by this method would allow the assessment of the role eEF1A2 may be playing in invasion.

A recently published paper found that eEF1A2 expression in breast cancer is associated with a significantly increased 20-year survival, meaning eEF1A2 may be a useful prognostic marker. Additionally they found a weak but significant inverse correlation between eEF1A2 expression and p53 and Ki67 leading them to suggest that eEF1A2 drives oncogenesis via a mechanism other than an increase in mitosis (Kulkarni et al., 2006). The suggestion that eEF1A2 expression may be a marker of good prognosis is surprising given it has been shown to exhibit an oncogenic phenotype when overexpressed (Anand et al., 2002). The authors suggest that this may indicate that the potential oncogenic pathway driven by eEF1A2 is less malignant than that induced by other oncogenes such as *ERBB2*. Finally, Kulkarni *et al.* did not find an association between eEF1A2 expression and lymph node involvement suggesting eEF1A2 is not involved in breast cancer metastasis to the lymph nodes (Kulkarni et al., 2006).

In summary, eEF1A2 appears to be expressed in a large proportion of breast cancers (almost two-thirds of tumours cores on one TMA), and this expression is associated with oestrogen receptor positive cancers. *EEF1A2* appears to be co-ordinately regulated with the ER and not oestrogen responsive. Additionally, eEF1A2 does not appear to be more highly expressed at the invading edge of the full face node-positive and negative breast tumours analysed. Interestingly, high eEF1A2 expression was observed in normal adjacent breast tissue, early hyperplastic lesions and DCIS, suggesting eEF1A2 may be important in the early stages of breast carcinogenesis.

Chapter 4: eEF1A2 is highly expressed in ovarian clear cell ovarian carcinomas

4.1 Introduction

Ovarian clear cell carcinomas

Clear cell carcinoma (CCC) is a very distinct subtype of epithelial ovarian carcinoma, often described as having a poor prognosis compared to serous carcinoma (Tammela et al., 1998), partly due to its resistance to platinum-based chemotherapy in advanced stages (Goff et al., 1996). It is a rare form of epithelial ovarian cancer, accounting for only 10% of all cases. CCC is very distinct histologically and is distinguished by the appearance of epithelial nests in a fibromatous mesh. Both CCC and endometrioid carcinomas are thought to arise from the same precursor lesion, endometriosis, and are often found intermixed within the same tumour (Feeley and Wells, 2001). It is known that the expression profiles of endometrioid and clear cell carcinomas differ, for instance CCC show no p53 mutations whereas endometrioid adenocarcinoma show a 63% occurrence (Okuda et al., 2003). CCCs also show a distinct pattern of loss of heterozygosity, exhibiting a prevalence at 1p, 19p and 11q (Okada et al., 2002). Additionally, CCC has been shown to express a unique pattern of cell cycle regulatory molecules compared to the other histological types of ovarian adenocarcinomas, expressing low levels of p53 and cyclin A and showing an increased expression of p21 and cyclin E (Shimizu et al., 1999). Patients with CCC tend to present with stage I disease (48.5%), whereas only 16.6% of patients with serous carcinoma present at this stage (Sugiyama et al., 2000). Important to CCCs resistance to chemotherapy, this histological type has been shown to express glutathione peroxidase 3 (*GPX3*) (Hough et al., 2001). CCCs have also been shown to overexpress the DNA repair genes *ERCC1* and *XPA* more than two-fold higher than the other histological subtypes and this may explain their resistance to DNA damaging agents (Reed et al., 2003). Additionally, high expression levels of Her2 has been shown to associate with ovarian clear cell carcinomas (Rubin et al., 1994). Finally, a microarray study of gene expression in epithelial ovarian cancers showed CCCs expressed the most distinctive gene expression profile of all the histological types. This distinct molecular signature comprised 73 genes that were expressed

two-fold to twenty nine-fold higher in CCC and known to perform roles in the stress response, cell proliferation, differentiation and cell adhesion, to name but a few (Schwartz et al., 2002). Therefore it is apparent that clear cell carcinomas constitute a distinct subtype of ovarian cancer.

Evidence for the role of eEF1A2 in ovarian cancer

eEF1A2 maps to chromosome 20q13.3, and an increase in copy number at this locus has been identified in ovarian cancers (Suehiro et al., 2000). In addition, the increased copy number at this region is associated with poor clinical prognosis and is often associated with more aggressive tumours (Suehiro et al., 2000; Tanner et al., 2000). In a microarray analysis of gene expression in 113 epithelial ovarian carcinomas, upregulation of eEF1A2 has also been identified in clear cell ovarian carcinomas (Schwartz et al., 2002). Anand *et al.* found *EEF1A2* to be amplified in 25% of primary ovarian tumours and to be highly expressed in 30% of ovarian tumours and ovarian carcinoma cell lines. Finally, eEF1A2 has been shown to increase the growth rate of ES-2 ovarian carcinoma cells xenografted in nude mice and to transform NIH 3T3 cells (Anand et al., 2002). Therefore a large amount of data exists to suggest that eEF1A2 may be an oncogene in ovarian cancer.

I therefore sought to investigate the expression of eEF1A2 at both the RNA and protein level (previous analysis has only been carried out at the RNA level), and to look for any clinical associations that may give a clue to the role of the translation factor in ovarian cancer.

4.2 Results

4.2.1 Expression of eEF1A2 in ovarian cancer cell lines

A panel of ovarian cancer cell line protein and RNA samples (plus two leukaemia cell lines) were obtained from Hani Gabra of the Cancer Research Centre, Edinburgh University. The expression of eEF1A2 at the RNA level was determined using quantitative real-time RT-PCR and the results are shown in figure 4.1. Of the 16 cell lines examined 3 did not express eEF1A2 and these were HL60, PEO1 and PEO4. Using Western blot analysis the levels of eEF1A2 protein expression was investigated in these cell lines. The results can be seen in figure 4.2. Of the 14 ovarian cancer cell lines examined only 2 cell lines were negative for eEF1A2 at the RNA and protein level, these were PEO1 and PEO4. The cell line panel also included the leukaemia cell lines, K562 and HL60. Interestingly, the bands corresponding to eEF1A2 on the Western blots are of slightly different sizes in the different cell lines. The eEF1A2 band in PEO14, PEO16 and HeLa cell protein extracts are slightly larger than the bands in the rest of the cell lines. This size difference suggests eEF1A2 may be post translationally modified in these cell lines. Additionally, the size of eEF1A2 in wild type mouse muscle is larger than that seen in any of the cell lines suggesting some difference between the human and mouse protein. This again could be due to post translational modifications in the mouse protein that are not present in the human protein.

It is becoming apparent that most cell lines express eEF1A2, regardless of their tissue of origin. A panel of cell lines were examined for eEF1A2 expression by Western blot and the results are shown in figure 4.3 and table 4.1. Of the ten different cell lines examined only one, HepG2, was negative for eEF1A2 expression. This is a cell line derived from a human hepatocellular carcinoma and it is known that normal liver is negative for eEF1A2 expression.

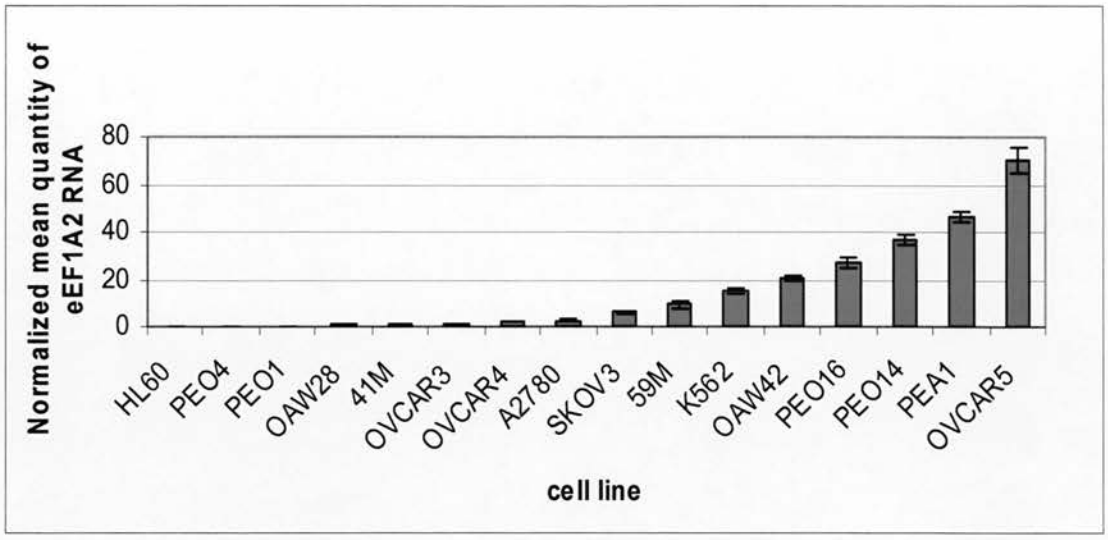


Figure 4.1 Expression of eEF1A2 at the RNA level in ovarian cancer cell lines. Quantitative real-time RT-PCR analysis reveals that the expression of eEF1A2 at the RNA level in ovarian cancer cell lines is similar to expression at the protein level. PEO1 and PEO4 are negative for eEF1A2 at the RNA and protein level. K562 and HL60 are leukaemia cell lines.

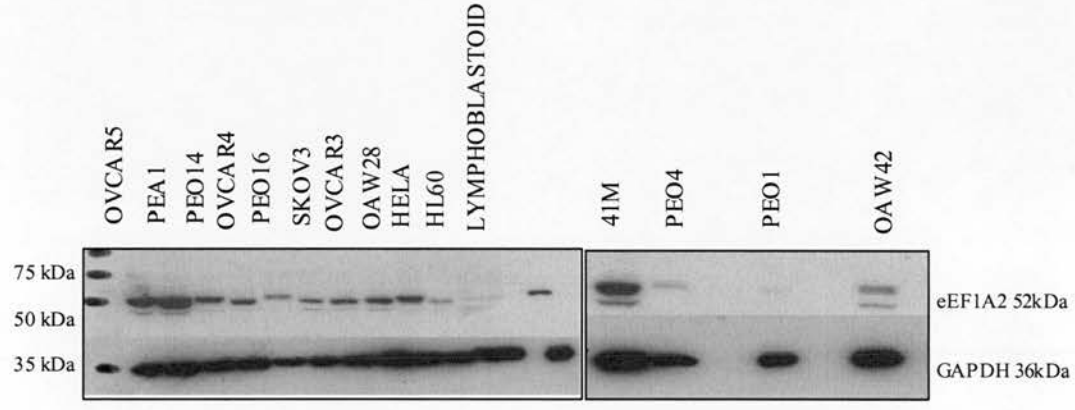


Figure 4.2 Western blot analysis of the expression of eEF1A2 in ovarian cancer cell lines.
HeLa cell and mouse muscle protein extracts are known to express eEF1A2 and act as positive antibody controls and lymphoblastoid is a negative control. PEO14, 16 and HeLa cells show slightly higher bands for eEF1A2 suggesting that protein may be post-translationally modified in these cell lines.

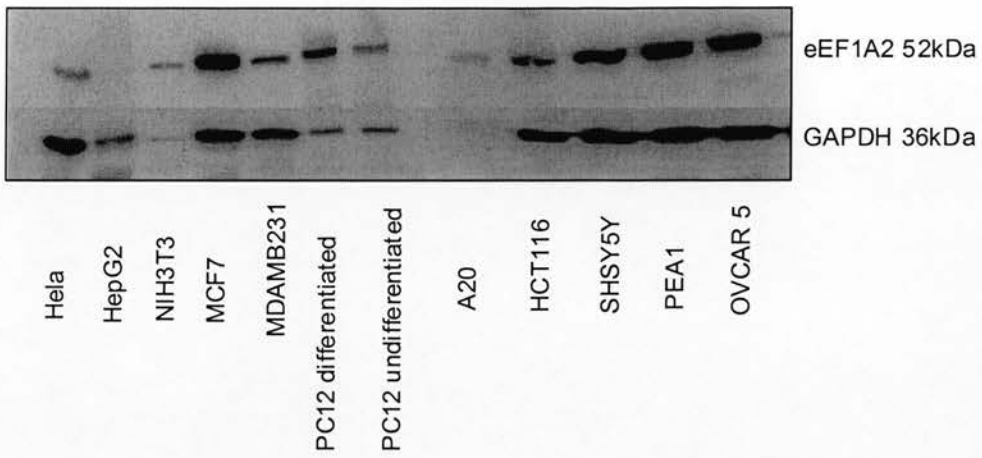


Figure 4.3 eEF1A2 expression in a panel of cell lines derived from a variety of tissues and cancers.
eEF1A2 is commonly expressed in cell lines. The only cell line identified as negative for eEF1A2 expression in this panel was HepG2.

Table 4.1 *Expression of eEF1A2 in a variety of cell lines*

Cell line	Tumour/Tissue type	eEF1A2 expression
HeLa	Cervical carcinoma	Yes
HepG2	Hepatocellular carcinoma	No
MCF-7	Breast carcinoma	Yes
MDA MB 231	Breast carcinoma	Yes
PC12	Phaeochromocytoma (rat)	Yes
HCT116	Colon cancer	Yes
SHSY5Y	Neuroblastoma	Yes

4.2.2 eEF1A2 expression in normal ovary and primary ovarian cancers

Expression of eEF1A2 at the RNA level has previously been examined in an ovarian surface epithelial cell line (Anand et al., 2002) and reported to be negative. In order to test this at the protein level, immunohistochemistry was performed on sections of normal whole ovary with intact ovarian surface epithelium. The results are shown in figure 4.4. eEF1A2 does in fact seem to be expressed at the protein level in the ovarian surface epithelium, contradicting the previous report. Additionally, eEF1A2 is also expressed in lutenized stromal cells in the normal ovary. These cells are found scattered at the edge of the degenerating corpus luteum and are endocrinologically active, secreting androgenic steroids. The novel expression pattern of eEF1A2 in the ovary is interesting and may shed new light on the role the protein is playing in normal cell and organ function.

The expression of eEF1A2 was then examined in a commercial tissue array containing cores from ovarian cancers by immunohistochemistry. Representative images of the cancer cores can be seen in figure 4.5. The sections were analysed by a pathologist and histoscored for expression of eEF1A2. The histoscores for each cancer type were then grouped into negative (0% at 0 intensity), weak (histoscore 1-100), moderate (histoscore 101-200) and strong (201-300) and the results are

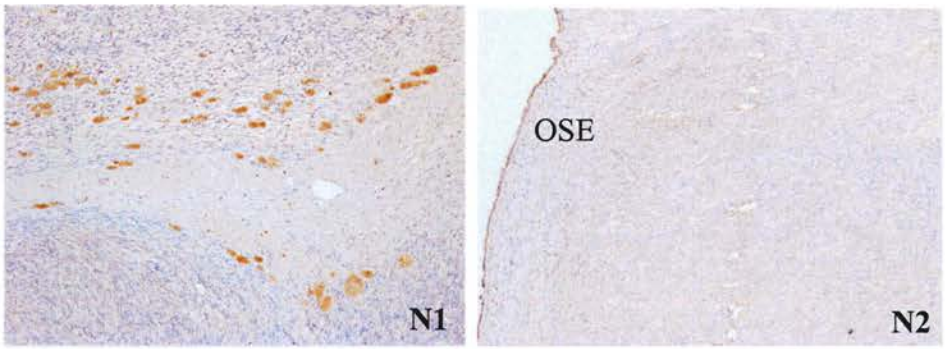


Figure 4.4 eEF1A2 expression in normal ovary
N1 shows expression of eEF1A2 in lutenised stromal cells at the edge of a degenerating corpus luteum. N2 shows the expression of eEF1A2 in the ovarian surface epithelium (OSE).

Figure 4.5 eEF1A2 expression in ovarian cancer (page 124)
HOV304 is a formalin fixed section from ovarian tumour number HOV304, which is a clear cell ovarian cancer and was positive at the RNA and protein level for eEF1A2 expression. T1-T3 are clear cell carcinomas from the commercial tissue array. All the clear cell carcinomas show high eEF1A2 levels in the epithelium and are negative for eEF1A2 in the stroma. T4 is an example of a weak eEF1A2-expressing papillary serous cystadenocarcinoma, T5 is a weak expressing endometrioid carcinoma, and T6 is a weakly expressing mucinous cystadenocarcinoma. This clearly illustrates the much stronger eEF1A2 expression level in clear cell carcinomas compared to the other histological subtypes. C1 is the secondary only control of the tumour T3. N1, N2 and T1-T3 are at x40 magnification. T4-T6 and C1 are at x20 magnification.

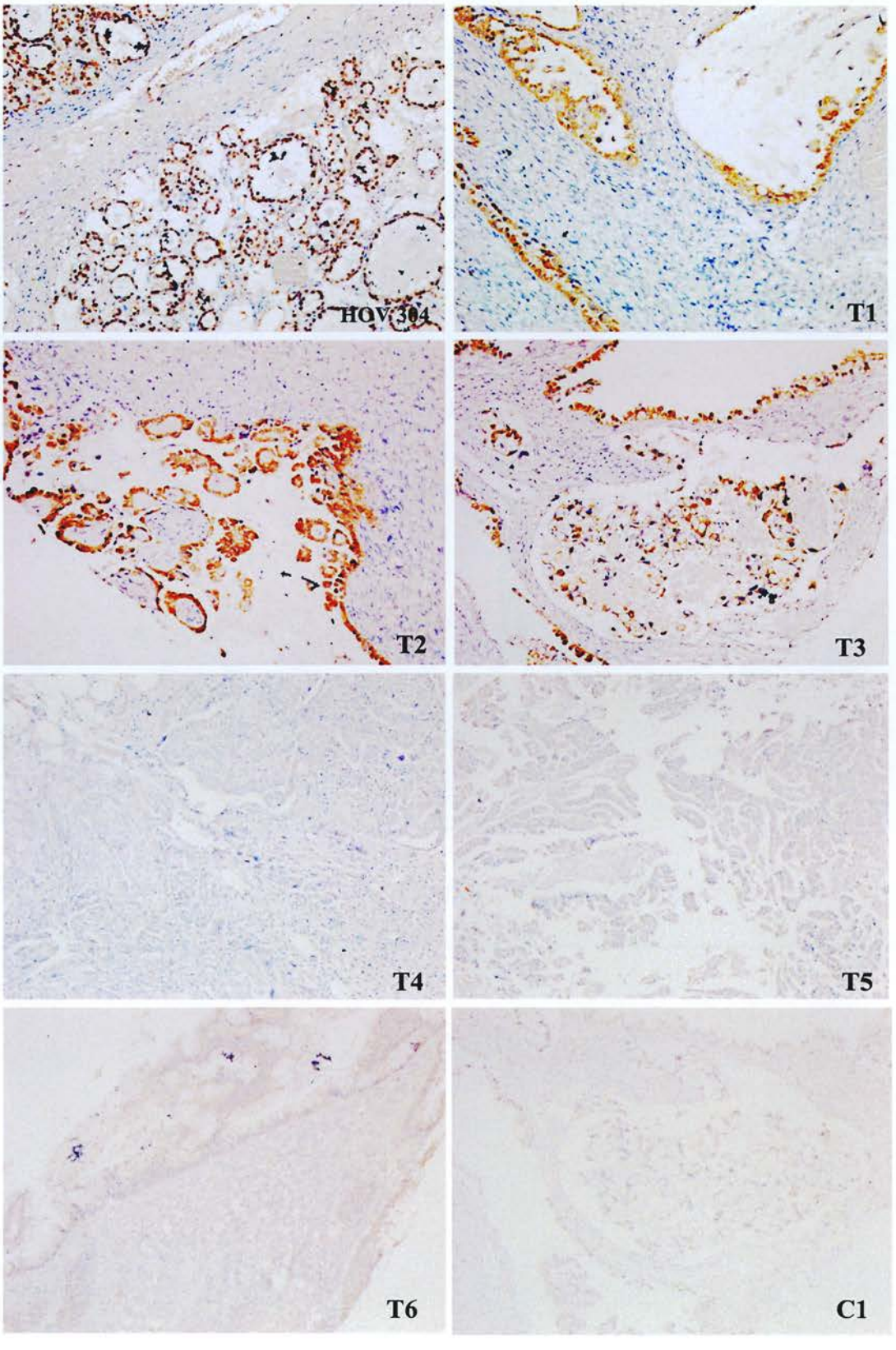


Figure 4.5

represented in table 4.2. A limited amount of information was available with this commercial array: the age of the patient and histological subtype. It appears that there is an association between high expression of eEF1A2 and histological subtype in these cancers.

Table 4.2 *Expression of eEF1A2 in ovarian cancers on a commercial tissue array*

Histological type	Number analysed	Negative	Weak	Moderate	Strong
Clear cell	5	1	1	1	2
Serous	15	3	12	0	0
Endometrioid	5	3	2	0	0
Mucinous	2	0	1	1	0

It is apparent from these results that clear cell carcinomas express eEF1A2 more strongly in comparison to serous, endometrioid and mucinous ovarian cancers; in total 15% of the ovarian cancers represented on this tissue array express eEF1A2 at a moderate to high level but 60% of the clear cell carcinomas express eEF1A2 at a moderate to strong level.

4.2.3 Analysis of eEF1A2 expression at the RNA and protein levels in a panel of ovarian cancers

In order to investigate the expression of eEF1A2 at both the RNA and protein level in ovarian cancers, we obtained a panel of human ovarian cancers (coded HOV) for which RNA, protein and tumour sections were available from most tumours. These ovarian cancer samples represent the common histological subtypes of ovarian cancers. The number of available samples in each histological subtype are listed table 4.3.

Table 4.3 *Clinical data available with the in-house TMA*

Cancer histotype	Total	RNA	Protein	IHC
Clear cell	7	4	5	2
Serous	27	18	26	14
Endometrioid	13	12	11	8
Mucinous	6	2	2	4
Total	**53**	**36**	**44**	**28**

Using real-time RT-PCR analysis the levels of eEF1A2 were assessed in 36 ovarian cancers and the results shown in figure 4.6a, as well as in figure 4.6b that illustrates the low level eEF1A2-expressing tumours more clearly. The majority of the cancer samples showed very low levels of eEF1A2 expression, however, 3 out of the 4 clear cell cancers analysed (75%) had detectable expression of eEF1A2; only 4 of the 18 serous cancers analysed (22%), 5 of the 12 endometrioid cancers (42%) and neither of the mucinous cancers showed expression at the RNA level.

To confirm this result at the protein level, protein samples from many of the corresponding cancers were analysed for eEF1A2 expression by Western blot. The results are shown in figure 4.7. In some cancers, such as HOV77, eEF1A2 was expressed at the RNA but not the protein level when assessed by Western blot suggesting some post-transcriptional mechanism is controlling the expression in these tumours or that a decrease in stability of the RNA or protein results in a lack of detectable protein on the Western blot. Alternatively, Western blot analysis could be too insensitive to detect eEF1A2 expression in these tumours. Four of the five clear cell carcinomas analysed expressed eEF1A2, as well as one serous and two endometrioid tumours. In total 16% of the tumours analysed using Western blot expressed eEF1A2. The results of expression analysis of eEF1A2 at the RNA and proteins levels, as well as the commercial TMA, are shown in table 4.4.

Table 4.4 *eEF1A2 expression at the RNA and protein level in ovarian cancers*

Cancer type	**RNA**		**Protein-Western blot**		**Protein- IHC commercial array**	
	Number analysed	% positive	Number analysed	% positive	Number analysed	% strongly positive
Clear cell	4	75	5	80	5	40
Serous	18	22	23	4	14	0
Endometrioid	12	42	11	18	9	0
Mucinous	2	0	5	0	2	0
Total	**36**		**44**		**30**	

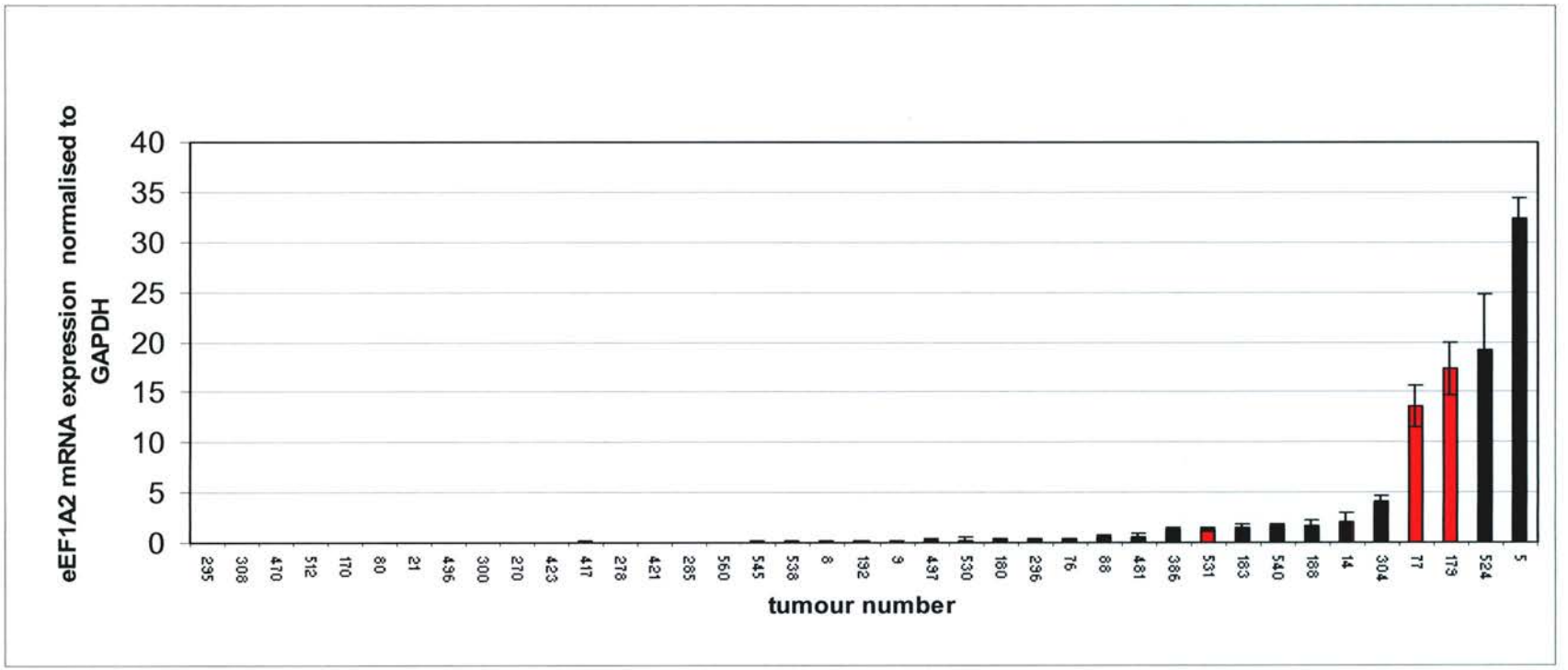


Figure 4.6a Normalised eEF1A2 RNA levels in ovarian cancers

Most ovarian cancers express very low levels of eEF1A2 mRNA. The clear cell carcinomas are shown in red. 3 of the 4 clear cell carcinomas express eEF1A2 at a detectable level.

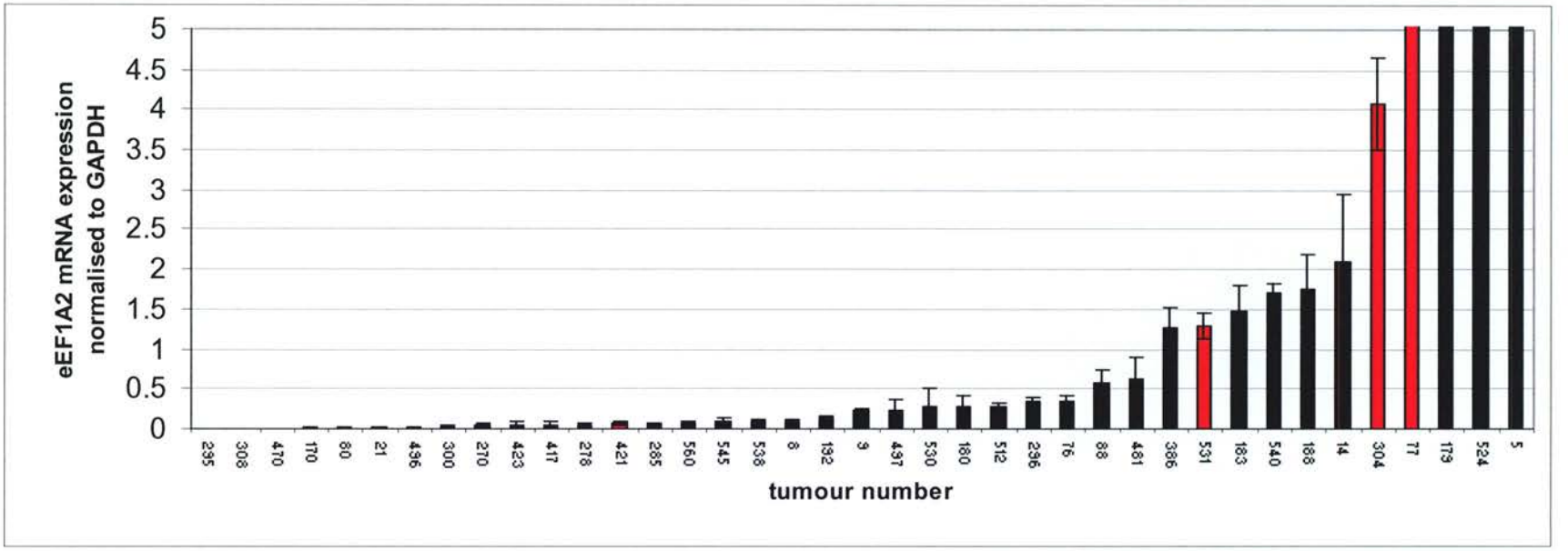


Figure 4.6b Normalised eEF1A2 RNA levels in ovarian cancers showing low level expressors

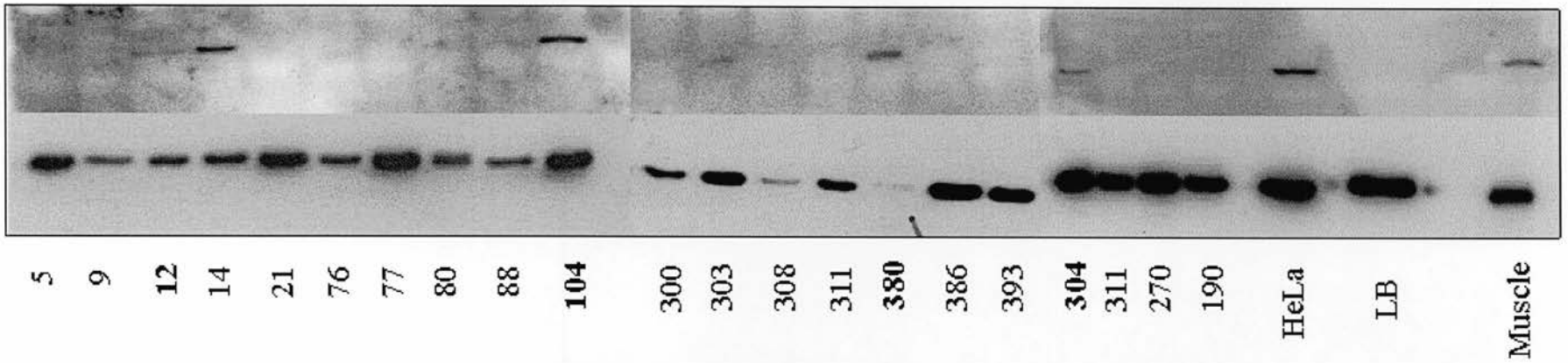


Figure 4.7 eEF1A2 protein expression in a panel of ovarian cancers.
(a) eEF1A2 expression (52kDa) (b) GAPDH (36kDa) loading control. Antibody controls are HeLa and mouse muscle protein and the negative control is lymphoblastoid (LB) protein. A very small number of ovarian cancers express eEF1A2 at the protein level in the HOV panel. These include tumours number 12, 104, 303, 380 and 304. Tumours 12, 104, 380 and 304 are clear cell ovarian carcinomas (HOV number shown in bold). HOV303 is a serous carcinoma and HOV 14 and 179 are endometrioid carcinomas.

It is clear from the table above that eEF1A2 appears to be most highly expressed at the RNA and protein level in clear cell carcinomas. Overall, 33% of the ovarian cancers expressed eEF1A2 at the RNA level and 15% expressed the translation factor at the protein level. Twenty eight of the above tumours had been sectioned and these were also available for eEF1A2 expression analysis by immunohistochemistry. This data is presented in table 4.5.

Table 4.5 *Expression of eEF1A2 in HOV tumour samples for which both Western blotting and IHC data are available*

HOV number	Histotype	Western blot expression of eEF1A2	IHC expression level of eEF1A2	Expression at the RNA level (neg-3)
8	Serous	Negative	Negative	1
9	Endometrioid	Negative	Strong	1
14	Endometrioid	Positive	Negative	2
76	Serous	Negative	Weak	2
88	Endometrioid	Negative	Negative	2
180	Serous	Negative	Weak	1
270	Serous	Negative	Negative	1
278	Serous	Negative	Negative	-
285	Serous	Negative	Negative	-
295	Mucinous	Negative	Negative	Negative
296	Endometrioid	Negative	Moderate	2
300	Endometrioid	Negative	Weak	1
303	Serous	Positive	Strong	-
304	Clear cell	Positive	Strong	3
308	Endometrioid	Negative	Negative	Negative
311	Serous	Negative	Negative	-
380	Clear cell	Positive	Negative	-
386	Serous	Negative	Moderate	2
397	Serous	Negative	Weak	-
432	Unknown	Negative	Negative	-
449	Mucinous	Faint positive	Negative	-
491	Endometrioid	Negative	Negative	-
496	Serous	Negative	Negative	1
512	Mucinous	Negative	Moderate	2
518	Mucinous	Negative	Weak	-
524	Serous	Negative	Strong	3
538	Serous	Negative	Negative	1
545	Serous	Negative	Strong	1
560	Endometrioid	Negative	Negative	1
TOTAL	**29**			

The IHC analysis revealed that many of the cancers that were positive at the RNA level using quantitative RT-PCR but not at the protein level by Western blot, were in fact positive by immunohistochemistry, as shown in table 4.5. RNA levels were classified as negative (no transcript), 1 (<1 standardised amount of eEF1A2 message), 2 (between 1 and 2) and 3 (>2). Tumours HOV9, 296, 386, 389, 397, 512, 518, 524, 545 were positive at the RNA level and by IHC but not on Western blot analysis. Tumours HOV386 and HOV524 express moderate to high levels of eEF1A2 at the RNA level and therefore the negative result by Western blot was perhaps not expected but the positive IHC result suggests that Western blot analysis was not sensitive enough to detect the protein expression in these cancers. An example of the IHC staining of these tumour sections can be found in figure 4.5 (HOV 304). The IHC staining does appear to correlate better with the RNA expression seen in the HOV panel suggesting that it is a more sensitive and effective technique than Western blotting for analysing protein expression in tumours. In total 5 (18%) tumours showed weak eEF1A2 expression, 3 (11%) showed moderate eEF1A2 expression, and 5 (18%) showed strong eEF1A2 expression. Therefore, in total 29% of the HOV ovarian tumours expressed moderate to high levels of eEF1A2 at the protein level when assessed by IHC, 33% express eEF1A2 at the RNA and 15% express eEF1A2 when assessed by Western blot. In addition, 15% of cancers analysed on the commercial TMA expressed eEF1A2 at a moderate to high level.

4.2.4 There is a significant association between high eEF1A2 expression and the clear cell carcinoma histological type of ovarian cancer

There was no apparent association between the grade, stage of the ovarian cancers and eEF1A2 expression, however there did appear to be expression of eEF1A2 in the majority of the clear cell carcinomas investigated. In order to test whether the apparent association between eEF1A2 expression and clear cell carcinomas was statistically significant, a Fisher's exact test was used to test the results from immunohistochemical and Western blot analysis. For the immunohistochemical results a 2x2 contingency table was constructed with clear cell carcinomas and other histological types of ovarian cancer against [weak to negative] expression, and [moderate to strong] expression of eEF1A2; this gave a significant association

between moderate and strong expression of eEF1A2 and CCC, p=0.03, see 2x2 contingency table below.

	Weak and Negative	Moderate and strong
Clear cell carcinomas	2	3
Other histological subtypes	21	1

Western blot data was divided into clear cell carcinomas and other cancer histological types versus positive and negative for eEF1A2 expression; this also gave a highly significant association between eEF1A2 expression and CCCs, p=0.0012, the 2x2 contingency table is shown below.

	Positive	Negative
Clear cell carcinomas	1	4
Other histological subtypes	36	3

4.2.5 Further immunohistochemical analysis of an ovarian cancer tissue array with a high clear cell carcinoma representation

The lack of clinical data available with the commercial ovarian cancer array meant its usefulness in assessing clinical associations with eEF1A2 expression were limited. We therefore obtained a tissue micro array from Alistair Williams, Edinburgh New Royal Infirmary. This in-house array contains cores from 171 different ovarian cancers, 30 of which are clear cell carcinomas, allowing me to confirm or otherwise the association seen in the limited number of samples from the previous analyses.

Four different slides of the TMA (AGOV1-4), i.e. 4 cores from each tumour and one on each slide, were stained with the rabbit anti-eEF1A2 antibody 1A2-3 using the Envision immunohistochemistry protocol. Positive and negative human control tissues were also subjected to the immunohistochemical protocol to confirm antibody specificity and a secondary only control was included.

Representative microscope images of the cores can be found in figure 4.8. The slides were then scored using the histoscore method detailed previously on each slide and an average histoscore for each cancer, from the scores for all four cores, was calculated. The TMA AGOV4 was also examined by the pathologist Alistair Williams and histoscored using an automated method for quantification of DAB staining (method not detailed). The average histoscores obtained by automated analysis are shown in brackets in table 4.6.

Table 4.6 *Average histoscores of eEF1A2 expression by histological subtype on a large tissue microarray. Results in brackets were obtained by automated analysis.*

Histological subtype	Percentage showing more than weak eEF1A2 levels	Average histoscore (max 300)
Clear cell	45% (0.07%)	87.6 (14.5)
Endometrioid	13.3% (0%)	66.2 (5.7)
Serous	40% (0.06%)	73.15 (2.3)
Mucinous	19% (0%)	48.7 (8.2)
Mixed	50% (0%)	103.7 (9.3)
Mixed including clear cell	43% (0.1%)	81 (27)
MMVT	17% (0%)	46.5 (11.5)

The above representation of average histoscores calculated by eye, suggests that although eEF1A2 is more highly expressed on average in clear cell ovarian cancers than in the other main subtypes (apart from mixed for which there are very few tumours), eEF1A2 expression is not present in substantially more CCCs compared to the serous or endometrioid tumours. The histoscores on AGOV4 only, by automated analysis (data in brackets), are all very low but are higher in the clear cell and mixed + clear cell histological types of ovarian cancer.

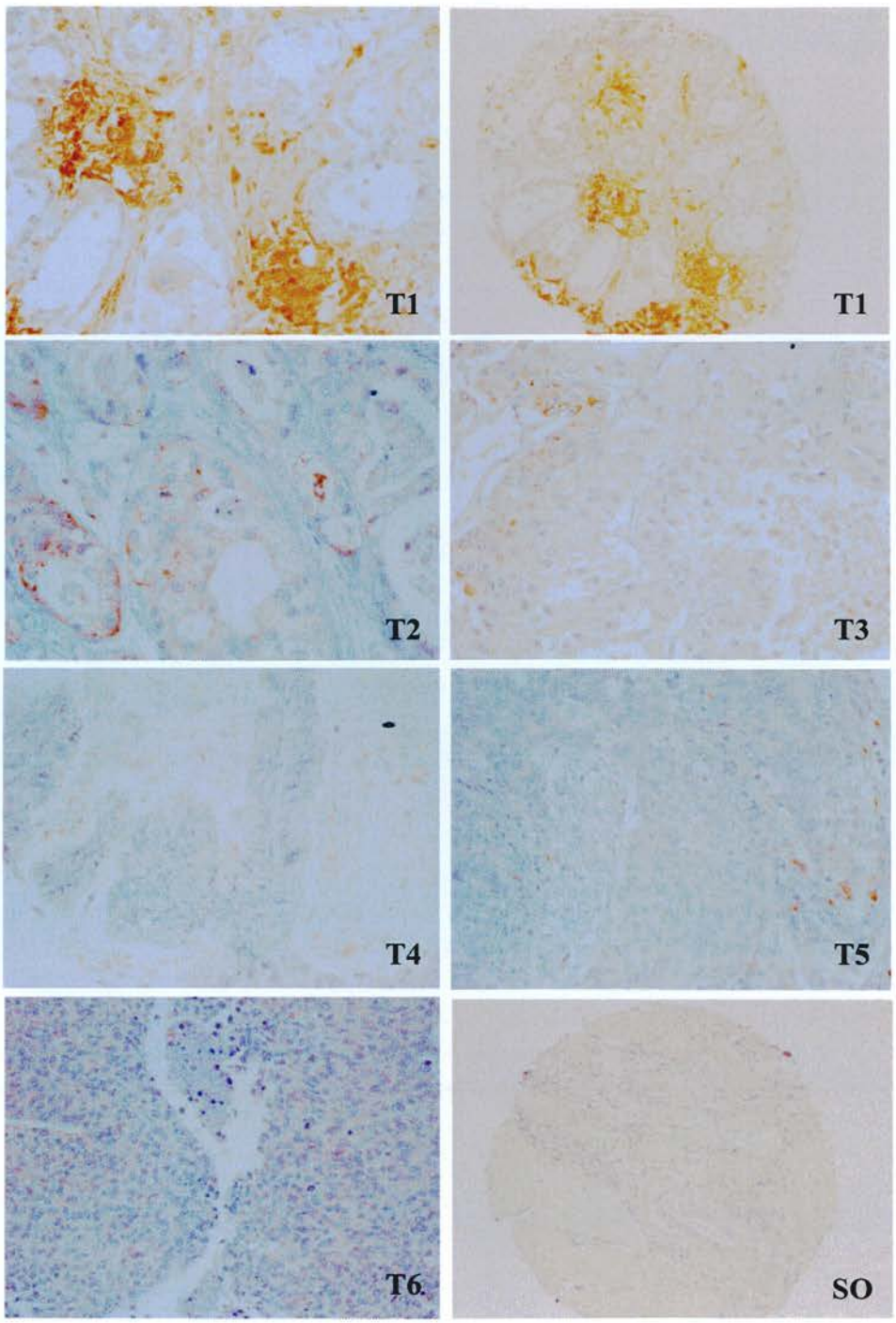


Figure 4.8 Immunohistochemical eEF1A2 staining of epithelial ovarian cancer cores on an in-house tissue micro array.
(T1) A clear cell carcinoma with the highest histoscore for eEF1A2 expression. (T2) clear cell carcinoma T1 at a lower magnification. (T2 and T3) Clear cell carcinomas staining weakly for eEF1A2. (T4) mucinous carcinoma (T5) serous carcinoma (T6) endometrioid carcinoma all showing weak expression of eEF1A2. (SO) Secondary antibody only control on T1. All images are at x40 magnification apart from T1 (right) and SO, which are at x20 magnification.

When the histoscores are subdivided into negative, weak, moderate, and strong for eEF1A2, see table 4.7, clear cell carcinomas are the only histological type that express eEF1A2 strongly. The numbers in each category from automated scoring of AGOV4 are shown in black and manual scoring results are shown in red.

Table 4.7 *The degree of eEF1A2 expression in ovarian histological subtypes. Figures in black were obtained by automated analysis and those in red by eye.*

Histological type	Number analysed		Negative		Weak		Moderate		Strong	
Clear cell	30	30	18	4	10	13	1	10	1	3
Serous	50	52	34	3	16	34	3	15	0	0
Endometrioid	29	28	23	3	6	20	0	3	0	0
Mucinous	9	21	6	1	3	16	0	4	0	0
Mixed	10	10	7	0	3	5	0	5	0	0
Mixed + clear cell	11	13	5	0	5	8	0	5	1	0
MMVT	5	5	2	1	5	3	0	1	0	0

The manual histoscoring technique appears to elevate the scores of the cores into weak and moderate, from the negative classification they were assigned in the automated scoring method. If indeed the automated histoscores are more accurate and thus only 5% of the ovarian cancers express eEF1A2 at a moderate to high level, this is substantially lower than the 15% exhibiting this expression level in the commercial tissue array. In addition only 2 out of the 30 (6%) clear cell carcinoma cores on the in-house TMA showed positive staining for eEF1A2 as opposed to 40% of clear cell tumour cores on the commercial TMA expressing eEF1A2 strongly. This additional evidence does appear to suggest that eEF1A2 is not preferentially expressed in clear cell carcinomas.

In conclusion, an association between eEF1A2 expression and ovarian clear cell carcinomas was found in the analysis of a panel of 57 ovarian cancers by quantitative real-time PCR and Western blot analysis as well as by immunohistochemistry on a commercial tissue array. However, although the highest eEF1A2-expressing ovarian tumours on the large in-house TMA were clear cell carcinomas, the association was no longer apparent.

4.6 Discussion

My observation of eEF1A2 expression in the normal ovarian surface epithelium is contrary to a previous report that the OSE is negative for eEF1A2 (Anand et al., 2002). Anand *et al.* used a normal ovarian epithelial cell line called NOV-61, derived from OSE, to investigate the expression of eEF1A2 at the RNA level by Northern blotting. My immunohistochemical analysis was carried out on whole ovary including intact OSE and therefore is perhaps more representative of the actual expression in the human OSE. Transformation and culturing cells can cause alteration in gene expression and I have shown that the majority of cell lines analysed express eEF1A2, regardless of their tissue of origin. In addition, I observed expression of eEF1A2 in 10 of 12 ovarian cancer cell lines; this is higher than the percentage of positive primary ovarian tumours suggesting transformation induces eEF1A2 expression. In a microarray analysis of genes differentially expressed in cultured normal ovarian surface epithelial cells (HOSE) and epithelial cells from epithelial ovarian cancers (CSOC), eEF1A2 was shown to be expressed 7.3-fold higher in HOSE than CSOC cells (Matei et al., 2002). The expression of eEF1A2 I observed in the OSE was not higher than the expression at the protein level in the epithelial ovarian cancers I analysed – particularly the clear cell histological subtype – but the higher expression in HOSE observed by Matei *et al.* may be due to the culturing of the epithelial cells. In conclusion there is evidence to suggest that eEF1A2 is expressed in normal OSE and it is not clear why Anand *et al.* did not find the normal OSE cell line NOV-61 to be positive for eEF1A2, despite the fact that it was derived from a cell type we have shown to be positive for eEF1A2 and the high frequency of eEF1A2 expression in cultured cells.

The origin of epithelial ovarian cancers is a topic of great debate. One theory is that the majority of epithelial ovarian cancers arise from the ovarian surface epithelium due to incessant ovulation (Fathalla, 1971) or from OSE inclusion cysts in the ovarian stroma (Radisavljevic, 1977). However, a proportion of mucinous tumours are thought to have a germ cell origin and a large percentage of clear cell carcinomas and endometrioid cancers are hypothesised to form from the precursor lesion of endometriosis (Feeley and Wells, 2001). Therefore the OSE may only be a totally

appropriate normal tissue of origin control for serous adenocarcinomas and not other histological types of ovarian cancer. The fact that normal OSE expresses eEF1A2 and two-thirds of ovarian cancers do not suggests that this is not the tissue of origin of many of these cancers or that eEF1A2 expression is down-regulated during malignant progression of the majority of ovarian cancers.

Not only did the majority of ovarian cancer cell lines appear to express eEF1A2 but PEO14 and PEO16 appeared to express a slightly larger protein than the other cell lines. One possible explanation for this size difference is that PEO14 and 16 express a post translationally modified form of eEF1A2. For example there are two predicted phosphorylation sites in eEF1A2 that are highly conserved between mouse, chicken and xenopus and are not present in eEF1A1 (Newbery et al., in preparation) and these sites are shown in figure 4.9 (the sequences were aligned using Clustal W (Thompson et al., 1994)). It is possible that eEF1A2 is phosphorylated in the PEO14 and 16 cell lines and this could potentially alter the function of the protein. Anand *et al.* used Northern blot analysis to detect expression of eEF1A2 in a panel of ovarian cancer cell lines and found only four of the ten cell lines were positive for the translation factor (Anand et al., 2002). Interestingly they showed OVCAR4 to be negative for eEF1A2 whereas my Western blot analysis suggests this cell line does in fact express eEF1A2. The difference between my results and those of Anand *et al.* may be due to the higher sensitivity of real-time RT-PCR and Western blots over Northern blot analysis or differences between sublines of the cell line. Joseph *et al.* examined the expression of 36 translation factors in a variety of cancer cell lines and found that eEF1A2 showed the most significant level of overexpression and was present in 9 of the 10 cell lines analysed (Joseph et al., 2004). This is consistent with the levels of eEF1A2 expression I observed in the cell lines.

eEF1A2 expression was detected in 33% of ovarian cancers at the RNA level and 15% at the protein level by Western blot and 28% by IHC in the HOV panel of tumours, suggesting that Western blotting is not detecting eEF1A2 present in some tumours.

Figure 4.9 Clustal W alignment of eEF1A protein sequences from Human, Mouse, Chicken and Xenopus

The top three sequences are xenopus, mouse and human eEF1A2. The bottom three sequences are human, mouse and chicken eEF1A1. There are very few amino acid differences in eEF1A between species or between the two variants, eEF1A2 and eEF1A1. The predicted phosphorylation sites present in eEF1A2 but not eEF1A1 are shown surrounded by a black box. These regions are highly conserved between species. A star (*) indicates amino acid identity, two dots indicate a conservative amino acid difference, and one dot a semi-conservative substitution.

For instance tumours HOV386 and 524 were positive for eEF1A2 using quantitative real-time RT-PCR and IHC but not using Western blot. In total, of the14 tumours that were positive at the RNA level but negative for protein expression using Western blot, 9 were positive for eEF1A2 when investigated using IHC. This suggests that IHC on tumour sections and not Western blotting should be used to investigate protein expression and that eEF1A2 is expressed only at the RNA level and not at the protein level in only 5 of the tumours that were investigated by IHC. Additionally, approximately the same proportion of the HOV tumours express eEF1A2 at the RNA level (33%) and at the protein level (29%) when assessed by IHC suggesting that IHC gives a more accurate picture of eEF1A2 expression.

A significant association between eEF1A2 expression and clear cell ovarian carcinomas (CCC) was observed when detecting eEF1A2 using Western blots ($p=0.0012$) and immunohistochemistry on the commercial TMA ($p=0.03$). Clear cell carcinomas only constitute approximately 10% of all ovarian cancers but eEF1A2 is expressed in approximately one-third of ovarian cancers, making the observation of an association with CCC surprising. Although the HOV panel contains mostly serous carcinomas, which is representative of the proportion of ovarian cancers that arise in humans, it contains more than 10% clear cell carcinomas (10 of 53, 19%) potentially explaining the identification of the association in the HOV panel analysis.

CCC is a very distinct subtype of epithelial ovarian carcinoma, often described as having a poor prognosis due to its resistance to platinum-based chemotherapy (Goff et al., 1996). The identification of genes differentially expressed in CCC could be important in determining the genetic cause of the poor prognosis of this subtype compared to others. As mentioned previously, eEF1A2 has been identified by microarray analysis to be more highly expressed in CCC compared to other histological subtypes of epithelial ovarian cancer (Schwartz et al., 2002). Although my initial analysis confirmed this observation a caveat to my analysis was that it was carried out on a small number of clear cell carcinomas ($n=10$). The small number of clear cell carcinomas in the ovarian cancer panel and TMA reflects the fact that CCC is rare, accounting for only 10% of all cases of epithelial ovarian carcinoma. The

lack of CCCs in the analysis was overcome by the inclusion of a larger tissue array containing cores from 30 different clear cell carcinomas. In this analysis 45% of the CCCs expressed eEF1A2 at a moderate to strong level when histoscored by eye. This is not substantially different from the proportion of serous (40%) or mixed (50%) ovarian cancers that express eEF1A2 at this level and consequently the significant association between CCC and moderate to strong expression of eEF1A2 has not been replicated in this larger TMA. Nevertheless, 4 of the 5 high eEF1A2-expressing tumours were CCCs suggesting that eEF1A2 is more highly expressed in this histological subtype. In summary, the analysis of a larger number of clear cell carcinomas suggests that eEF1A2 would not in fact be a good biomarker for CCC, as it had first appeared.

The histoscoring of tissue microarray AGOV4 by Alistair Williams using an automated method for quantification of DAB staining did not show good concordance with the average histoscores of multiple cores or the AGOV4 scores obtained by eye. In fact, the majority of the histoscores of eEF1A2 levels generated by automated analysis were negative or low. Only one clear cell tumour and one tumour of mixed histology including clear cell was histoscored as high (201-300) for eEF1A2. Although the detailed protocol for the automated technique is unpublished the analysis involves the subtraction of background staining observed on the secondary-only control from the histoscore for each individual core. The intensity of staining and the volume of tumour exhibiting that staining are measured and the volume adjusted score is produced, in much the same way as is done by eye. The subtraction of background staining was not carried out in the manual histoscoring and may account for some of the differences in histoscores using the different methods. Additionally, the automated technique may not be as sensitive to subtle levels of staining. On the other hand the automated method may give a more accurate picture of the actual level of DAB staining on the tumour cores. Taking the automated histoscores as correct, the percentage of cores that stained moderate to strong for eEF1A2 was 5%, as opposed to 15% in the commercial array. Given that the 15% is more consistent with the results from my RNA (33%) and protein (16%) analysis and with the proportion of eEF1A2-expressing ovarian tumours identified

by Anand *et al.* (Anand et al., 2002) this suggests that the results from the commercial TMA may be more accurate. The in-house TMA was stained using the Envision secondary antibody staining method and this method when used on a breast cancer TMA also resulted in a lower number of eEF1A2-positive tumours compared to the earlier IHC protocol, suggesting that there may be a technical problem with the in-house TMA. It is also possible that a difference in the way the in-house TMA was produced resulted in fewer eEF1A2-positive tumours, such as tissue fixation or storage.

The observation that p53 is rarely mutated in CCC (Okuda et al., 2003) is particularly interesting considering that eEF1A2 expression in breast carcinomas appeared to associate weakly with tumours that did not show mutations in p53. Therefore wild-type p53 may be important to eEF1A2 expression in these breast cancers and clear cell carcinomas. Equally interesting is the profile of abnormalities in DNA sequence copy number in clear cell adenocarcinomas. Increases in copy number that have been identified in more than 20% of CCCs include an increase in copy number of 20q13-qter, the chromosomal location of *EEF1A2*. This amplification of 20q13-qter correlated with increases in 17q25-qter and together the presence of these two amplicons correlated with recurrent disease and non-surviving patients. Amplifications were also observed at various loci on chromosome 8q that occurred more frequently in disease-free, surviving patients. This suggests there are 2 subtypes of CCC, one being associated with increases in 8q and the other with increases in 17q25-qter and 20q13-qter, the latter being poorer prognosis (Suehiro et al., 2000). Therefore, in the CCCs that eEF1A2 isn't expressed there may be a prevalence of 8q amplification and vice versa. Additionally, eEF1A2 may be one of the genes in the 20q13-qter amplicon that is contributing to the more aggressive nature of this subtype of CCC. However, the recent identification of an association between increased 20 year survival and eEF1A2 expression in breast cancers suggests this may not be the case (Kulkarni et al., 2006).

The chemoresistant nature of CCCs distinguishes them from other histological types (Sugiyama et al., 2000). As eEF1A2 may be highly expressed in some CCCs

compared to the other histological types it could be involved in this distinct phenotype. Indeed, glutathione peroxidase 3 is involved in the oxidative stress response and is highly expressed in CCCs and it has been suggested that this contributes to the chemoresistance exhibited by CCCs (Hough et al., 2001), possibly by decreasing the high levels of the free radicals induced by chemotherapy that would otherwise lead to irreversible cell damage and eradication of the tumour by overloading the antioxidant system (Kong and Lillehei, 1998).

On the other hand, oxidative stress has been proposed to decrease the efficacy of chemotherapy. Platinum coordination complexes, the chemotherapeutic drug to which CCCs are commonly resistant, induce high levels of oxidative stress. Oxidative stress during chemotherapy induces lipid peroxidation generating electrophilic aldehydes leading to a decrease in the efficacy of chemotherapy by reducing the rate of cellular proliferation and inhibiting apoptosis. Oxidative stress induces apoptosis by causing DNA damage, but in excessive oxidative stress (as occurs in Platinum-based chemotherapy) caspase activity is inhibited by the binding of electrophilic aldehydes to the active site (Conklin, 2004). eEF1A2 overexpression is thought to be protective against apoptosis induced by hydrogen peroxide (H_2O_2) (a mediator of oxidative stress) (Chen et al., 2000) and even more so in combination with peroxiredoxin-1; this anti-apoptotic effect is thought to be mediated by an increase in the abundance of the pro-survival factor Akt (Chang and Wang, 2006). Therefore eEF1A2 expression in clear cell carcinomas could possibly contribute to chemoresistance by contributing to the inhibition apoptosis in high oxidative stress.

In conclusion, eEF1A2 is expressed in up to one-third of epithelial ovarian carcinomas and this expression is particularly high in clear cell carcinomas compared to other histological subtypes, however the apparent association of moderate to high eEF1A2 expression and clear cell carcinomas was not reproduced with a larger TMA.

Chapter 5: The mechanism of overexpression of eEF1A2 in ovarian cancer

5.1 Introduction

Amplifications at 20q13 in ovarian cancer

Regions of 20q are frequently observed to be amplified in ovarian cancer. It is likely that genes in regions of recurrent imbalance in cancer are important in the pathogenesis of that disease. Many studies have found that it is a region in 20q13.2 that is frequently amplified in ovarian cancer (Tanner et al., 2000), (Diebold et al., 2000); this region does not include *EEF1A2* which is located at 20q13.3. On the other hand other studies have suggested that amplification in ovarian cancer may also involve 20q13.3, and that 20q13.2 and 20q13.3 may in fact be separate amplicons. CGH in 25 malignant ovarian tumours showed that 20q13.2-qter was a frequently observed chromosomal amplification, particularly in advanced stage and high grade tumours and may be associated with disease progression (Sonoda et al., 1997). A study of a small number of clear cell ovarian adenocarcinomas suggested that they can be divided into two subtypes based on recurrent copy number changes; those that show an amplification of 8q and those that harbour amplifications in both 17q25-qter and 20q13-qter. Amplifications at 20q13-ter in these clear cell adenocarcinomas tended to occur more frequently in later stage cancers (stage II-IV) and were significantly higher in high-grade malignant tumours. Importantly they also identified that 20q13-qter amplifications were significantly more frequent in recurrent disease/non-surviving patients (Suehiro et al., 2000). Watanabe *et al.* also identified 20q12-13 to be frequently amplified in ovarian cancer cell lines (Watanabe et al., 2001). The frequent amplification of 20q13-qter in ovarian cancer suggests that this region contains oncogene(s) and *EEF1A2* is a potential candidate; this was recently confirmed by Anand *et al.* who determined that *EEF1A2* is amplified in 25% of ovarian cancers, overexpressed in one-third of ovarian cancers, and capable of transforming NIH 3T3 cells as well as driving tumour formation in mouse xenografts (Anand et al., 2002).

DNA methylation and cancer

Methylation is a heritable epigenetic modification of DNA that acts to regulate the expression of genes. DNA methylation involves the addition of a methyl group to the 5th carbon position on the ring of cytosine within CpG dinucleotides via methyltransferase enzymes (Doerfler, 1983),(Riggs and Jones, 1983). Vertebrate DNA is generally depleted of the dinucleotide CpG, however some genes contain CpG islands, which are regions that show a high frequency of GC compared to the bulk genome. These CpG islands are associated with the 5'ends of housekeeping genes and approximately half of tissue-specific genes. Tissue-specific genes also contain CpG islands at their 3' ends and some genes can contain CpG islands at both the 5' and 3' ends (Gardiner-Garden and Frommer, 1987). CpG islands nearly always encompass gene promoters and exons and the CpGs within islands are generally unmethylated while whose outside islands are generally methylated (Costello and Plass, 2001). *EEF1A2* is a tissue-specific gene and contains putative 5' and 3' CpG islands. It has been shown that in many tissue-specific genes there is no methylation in the normal tissues in which they are expressed. In normal cells methylation is important for the transcriptional repression of imprinted genes (Brannan and Bartolomei, 1999) and X-chromosome inactivation (Migeon, 1994). It has also been shown to be important in the maintenance of chromosomal integrity in gene deficient regions such as pericentromeric heterochromatin and as a defence against mobile genetic elements (Bestor, 1998). For a comprehensive review of DNA methylation patterns see (Bird, 2002).

In cancer, methylation has been widely shown to be abnormal. Tumours in general show a specific alteration in methylation pattern: global DNA demethylation concomitant with local hypermethylation at the 5' regulatory regions of certain genes; the level and frequency of hypomethylation has been shown to increase with tumour progression. This pattern of aberrant methylation has been observed in pre-malignant cells suggesting it is not simply a consequence of the malignant state. It is not known whether genome hypomethylation and localised hypermethylation are mechanistically linked. Hypermethylation can inactivate gene transcription in cancer either by biallelic methylation or methylation accompanied by mutation. Examples

of aberrant hypermethylation leading to gene silencing have been identified in genes involved in many of the processes of malignant progression. These include Retinoblastoma (*RB*), E-cadherin (*CDH1*) and the oestrogen receptor 1 (*ESR1*), see review by (Costello and Plass, 2001).

Global hypomethylation has been suggested to contribute to malignancy by transcriptional activation of oncogenes, activation of latent retrotransposons and chromosomal instability. Hypomethylation and activation of proto-oncogenes is less well studied than hypermethylation in tumour suppressor genes. *C-myc*, *Ha-Ras* and *ERB-A1* have been shown to harbour demethylation at individual CpGs but there is no evidence that this leads to activation of the gene (Kisseljova and Kisseljov, 2005). However, tissue-specific genes with regulatory regions that are methylated in normal tissues have been shown to be demethylated and expressed in tumours. An example of this is the proto-oncogene *HOX11*, which is a transcription factor that is only expressed during embryogenesis. In T-cell acute lymphoblastic leukaemia *HOX11* is expressed and its promoter is unmethylated but in normal T cells the promoter is methylated (Watt et al., 2000). A further example is the tissue-specific gene Synuclein γ, usually expressed in peripheral nervous system, Synuclein γ is highly expressed and the CpG island in exon 1 hypomethylated in breast and ovarian cancer cell lines and primary breast cancers but hypermethylated and not expressed in normal breast cell lines or normal mammary tissue and ovarian surface epithelium (OSE) (Gupta et al., 2003). This could potentially be the case in *EEF1A2*; the CpG island in exon 1 of the gene may be methylated in normal OSE and hypomethylated in ovarian cancer. Hypomethylation is also thought to lead to the expression of cancer-testis antigens called MAGE, GAGE and LAGE, and genes involved in invasion and metastasis, such as uPA, in certain cancers (Kisseljova and Kisseljov, 2005).

The mechanism of overexpression of eEF1A2 in ovarian cancer

EEF1A2 was identified as a potential oncogene in ovarian cancer by Anand *et al.* They observed, using FISH, that an increase in *EEF1A2* copy number was present in 14 of the 53 (25%) of the primary ovarian cancers. Of the primary tumours

expressing eEF1A2 (3 of 11 tumours analysed), one did not show amplification in the gene suggesting another mechanism could be responsible for inappropriate expression of the protein (Anand et al., 2002). The small number of primary ovarian cancers analysed for eEF1A2 expression and mechanism of overexpression, makes it difficult to conclude whether or not gene amplification is the main mechanism by which eEF1A2 overexpression is mediated. I therefore, having completed expression analysis of eEF1A2 in a panel of ovarian cancers, sought to establish the possible mechanism(s) mediating the overexpression of the gene. As described above, overexpression of eEF1A2 is likely to involve DNA amplification but gene mutation and methylation may also play a role.

5.2 Results

5.2.1 A quantitative real-time PCR method for determining the copy number of *EEF1A2* in ovarian cancer

In addition to RNA and protein from the ovarian cancer panel (HOV) there was also DNA available. Using a real-time PCR assay I determined the copy number of *EEF1A2* in the panel of ovarian tumours for which I have previously determined the expression status of eEF1A2. Analysis of copy number at a particular locus is routinely determined using techniques such as FISH or CGH. However, I only had DNA (and not cell lines) from these ovarian tumours in limited amounts available to me and therefore had to use a PCR technique. With larger quantities of DNA available to me I could have also used Southern blotting to assess the copy number of *EEF1A2*.

I began by designing primers in the intron of *EEF1A2* to utilise in the quantification of the copy number of the *EEF1A2* locus. The intronic sequences in *EEF1A2* and *EEF1A1* are, unlike the coding sequence, highly diverged and therefore using primers annealing to this sequence avoids primer cross hybridisation with *EEF1A1* or with any of the *EEF1A1* pseudogenes. For normalisation of total DNA amount in the reaction I used 3 different pairs of microsatellite primers. This technique, although not commonly used for copy number determination, has been used by Ginzinger *et al* (Ginzinger et al., 2000). Ginzinger *et al.* used PCR analysis of copy number at microsatellite loci to determine relative DNA sequence copy number. They used this technique to determine the copy number of *ZNF217* in ovarian cancers and to replicate a study that found an association between an increase in copy number and decreased survival. Microsatellite markers are useful as reference genes because the primers and PCR reaction conditions are well characterised, and they are distributed throughout the genome. I used three pairs of the microsatellite primers used by Ginzinger *et al.* in their human reference pool 2; these amplify regions of the genome thought to be stable in ovarian cancers (Ginzinger et al., 2000). These were D2S385, which amplifies a region of chromosome 2q31, D5S643 that amplifies a region in 5q32 and D11S1315 that amplifies a region of 11p15. Three reference microsatellite markers were used because cancers are genomically unstable and therefore if one of

the three pairs of primers gave a dramatically different amount of product from the others this one could be excluded from the analysis.

In order to assess the validity of the technique the copy number of *EEF1A2* was determined in 5 different DNA samples from human blood obtained from the National Blood Transfusion service. These are expected to have a normal chromosomal content and therefore be diploid for *EEF1A2*. The average copy number of *EEF1A2* in these samples was 2.4. In order to further validate this technique gDNA from blood was spiked with 1000 copies of a human *EEF1A2* P1-derived artificial chromosome (PAC) and analysed for *EEF1A2* copy number in triplicate. The *EEF1A2* copy number is then normalised to the reference locus copy number and the *EEF1A2* copy number in the spiked DNA is standardised to the copy number is the unspiked control DNA. The copy number of the spiked DNA was approximately 1160 (SD+/- 457) times that of the unspiked DNA, see figure 5.1. The standard deviation is large (40% of the mean) suggesting that there was large variation between the replicates and that the measurement is not as accurate as it could be. Perhaps a more effective validation of the method would have been to construct a standard curve of known copy numbers of *EEF1A2* from a human PAC or BAC containing the *EEF1A2* genomic sequence.

I then determined the *EEF1A2* copy number by the above method in ovarian cancers that express eEF1A2 at the RNA and/or protein level, as well as 8 ovarian cancers that do not express eEF1A2 see figure 5.2. The *EEF1A2* copy number was normalised to the copy number of 3 control microsatellite loci thought to be stable in ovarian cancer. *EEF1A2* was amplified at a significant level (more than 2 SDs from normal DNA) in 12 of the 15 (80%) ovarian cancers expressing eEF1A2 at the RNA or protein level. In cancers HOV183, 481 and 179 *EEF1A2* copy number was less than two standard deviations from the copy number in normal blood DNA and therefore there is no evidence of amplification. In those cancers not expressing eEF1A2, all eight had *EEF1A2* copy numbers significantly exceeding that found in normal diploid blood DNA.

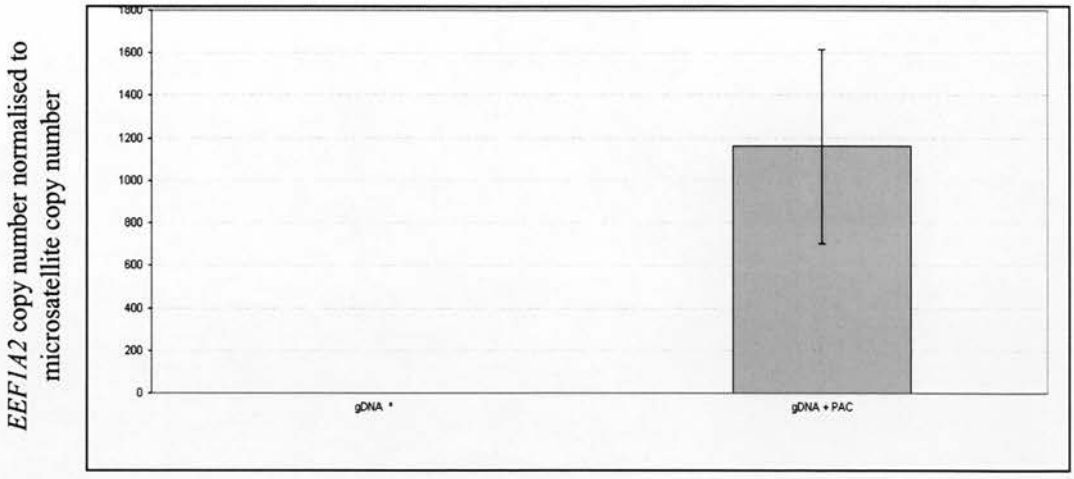


Figure 5.1 Real-time PCR analysis of *EEF1A2* copy number in gDNA and gDNA spiked with an *EEF1A2*-containing PAC.
EEF1A2 copy number was normalised to the copy number of 3 reference microsatellites. The normalised level of *EEF1A2* is 1160 times higher in PAC spiked gDNA than in gDNA alone. This confirms the PCR technique for analysis of *EEF1A2* copy number is effective.

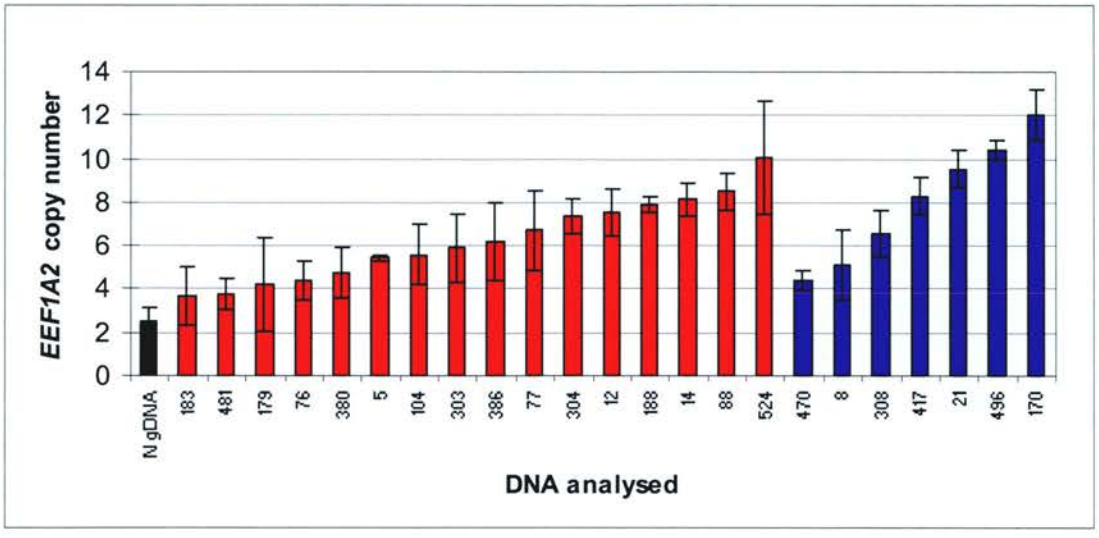


Figure 5.2 *EEF1A2* copy number in ovarian cancers
The mean normalised *EEF1A2* copy number in normal gDNA (black), ovarian cancers expressing eEF1A2 at the RNA and/or protein level (red) and ovarian cancers that do not express eEF1A2 (blue). Error bars show 2 SDs of the mean.

The copy number of *EEF1A2* ranged from low, 3-5, to medium 6-12; none of the cancers showed high copy numbers at this locus. Unexpectedly, the range of copy numbers appears to be very similar between the expressing and non-expressing cancers. In fact it was a non-expressing tumour, HOV170, which showed the greatest *EEF1A2* copy number. The similar pattern of copy number changes between non-expressing and expressing tumours suggests that gene amplification is not the only mechanism mediating the overexpression of eEF1A2 and may even be irrelevant for overexpression.

The level of *EEF1A2* amplification does not appear to be associated with histological subtype, FIGO stage or grade of the ovarian cancers or with recurrent non/surviving patients, see table 5.1.

Table 5.1 *The levels of EEF1A2 amplification and tumour histological subtype, FIGO stage, grade and outcome.*

Copy number	HOV number	Expressing eEF1A2? Y/N	Histological Subtype	FIGO Stage	Grade	Recurrent/non-surviving patient?
3.7	183	Y	Serous	3	2	Y
3.8	481	Y	Endometrioid	3A	3	N
4.2	179	Y	Endometrioid	4	3	Y
4.4	76	N	Serous	3	3	Y
4.4	470	N	Serous	3C	3	N
4.8	380	Y	Clear cell	1A	2	N
5.1	8	N	Serous	3C	3	Y
5.4	5	Y	Endometrioid	1C	3	N
5.6	104	Y	Clear cell	1c		Y
5.9	303	Y	Serous	3C	3	N
6.17	388	N	Serous	4	3	Y
6.5	308	Y	Endometrioid	2C	3	N
6.7	77	N	Clear cell	3B	3	Y
7.3	304	Y	Clear cell	2C		N
7.5	12	Y	Clear cell	1C	2	N
7.9	188	Y	Serous	4	3	Y
8.1	14	Y	Endometrioid	3	3	Y
8.3	417	N	Endometrioid	3C	3	N
8.5	88	Y	Endometrioid	1C	2	Y
9.5	21	N	Serous	3	3	Y
10	524	Y	Serous	3C	3	Y
10.4	496	N	Serous	1C	3	N
12	170	N	Serous	3	3	Y

A comparison of the *EEF1A2* copy number against the expression of eEF1A2 at the RNA level also suggests that there is no correlation between level of amplification and expression, see figure 5.3. For some of the tumours only protein and not RNA was available for expression analysis and therefore these tumours are not represented in this figure.

The real-time PCR technique used for this analysis suggests that 80% of the ovarian cancers analysed have an amplification of *EEF1A2*. Anand *et al.* found using FISH that amplification of 20q13.3 was only present in 25% of ovarian cancers and many other studies suggest that amplification at different regions on chromosome 20q12-13 occur in approximately 50% of ovarian cancers (Sonoda et al., 1997; Tanner et al., 2000). However, Diebold *et al.* have identified a amplification at 20q13.2 in 70% of dissociated nuclei from invasive ovarian carcinoma mainly of the serous type (Diebold et al., 2000). Nevertheless, the majority of studies have shown 20q amplification to be found at a frequency of approximately 50% in ovarian cancers suggesting that either the PCR technique does not give an accurate picture of the *EEF1A2* copy number or that this gene is independently amplified to a higher extent than the surrounding region on chromosome 20.

5.2.2 The increase in *EEF1A2* copy number is not due to chromosome 20 polyploidy

The real-time PCR technique used to determine the copy number of *EEF1A2* does not allow the differentiation between a gene or chromosomal region amplification and a whole chromosome 20 number aberration. In order to determine whether any increase in copy number is due to polyploidy of chromosome 20 a real-time PCR technique was again used. Primers that amplify microsatellite loci on the p arm of chromosome 20, D20S804 and D20S819, were used to determine the amount of 20p present in the cancers and then compared to the copy number of *EEF1A2* determined previously. D20S804 is located at 20p11.23 and D20S819 is located at 20p12.3; these primers amplify regions separated by 10Mb of sequence.

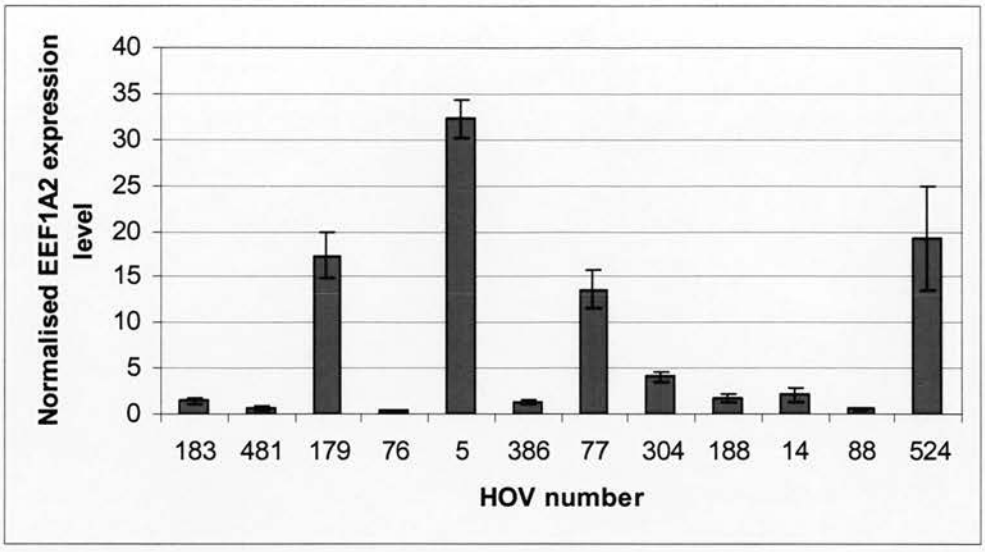


Figure 5.3 eEF1A2 expression at the RNA level in ovarian cancers with increasing copy number.
It is apparent that an increase in *EEF1A2* copy number (left to right) does not result in an increase in the RNA expression level of eEF1A2.

A slightly different real-time PCR amplification and analysis method was used to assess the copy number at the 20p loci from that used to quantify the *EEF1A2* copy number. For normalisation of DNA amount only the microsatellite primer D2S385 was used. The region of chromosome 2p13 that these primers amplify did not show copy number alterations compared to the other reference microsatellite primers in previous analysis and therefore can be used in isolation for normalisation of these same DNA samples in this instance. The PCR efficiency of D20S804 and D20S819 was determined following the previous method. D20S804 was then subsequently chosen for the initial analysis of copy number of 20p.

A standard curve method was used in this analysis and involves the inclusion of a standard curve for each primer pair on every PCR plate. This method can be applied to this experiment because only 2 products are being amplified whereas 4 where being amplified in the *EEF1A2* copy number analysis. The standard quantity mean for each DNA sample was calculated relative to the standard curves. A ratio of the standard quantity mean of D20S819 and D2S385 was then taken. In the majority of the ovarian cancers this ratio was approximately 1, as expected if there are diploid copies at both loci, see figure 5.4. However, ovarian cancers HOV303, 104, 417, 496 and 76 appeared to show losses at 20p12 and HOV 14 appeared to have a gain in this region. I therefore repeated quantitative real-time analysis on these DNA samples using the D20S819 primers. Tumours HOV303 and 104 still showed a loss of 1 copy at this region, suggesting a 20p deletion or a whole chromosome deletion in these cancers. If there is a whole chromosome deletion present in these tumours then the *EEF1A2* locus amplification would be double that quoted in the results. HOV417, 496 and 76 showed diploid copy numbers at the 20p11 locus suggesting that these tumours contain a haploid deletion at a region in 20p12 but not a whole arm or chromosome aberration. Tumour 14 also showed amplification at 20p11 as well as 20p12, suggesting a chromosome 20 duplication or a large amplicon of 10Mb may be present in this tumour. Interestingly, the marker at 20p11 appears to be tetraploid while the marker at 20p12 appears to be hexaploid. This suggests that perhaps there are two separate amplicons within 20p11-12 or that there is whole chromosome number aberration together with localised amplification in one or both

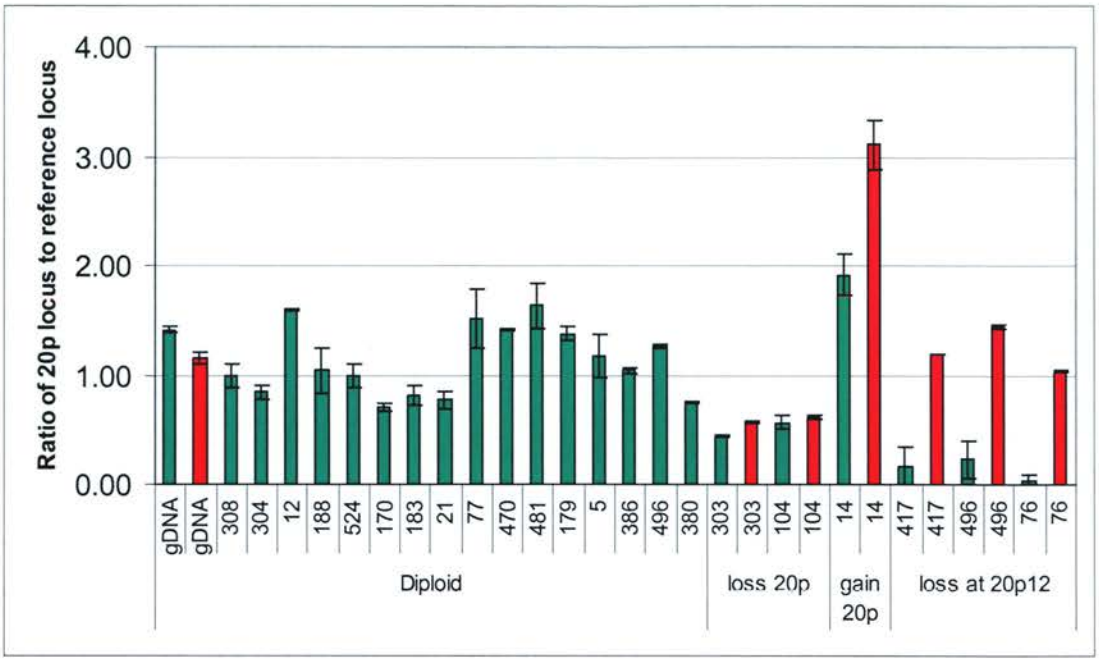


Figure 5.4 Chromosome 20 ploidy analysis using 20p microsatellite primers. 20p copy number in normal genomic blood DNA for both 20p loci, D20S804 and D20S819, is diploid (ratio of 20p locus to reference locus D2S385 = 1). Most ovarian cancers (numbered along the x axis) were also diploid at 20p11 or 20p12, however tumours HOV303 and 104 appear haploid at 20p12-13 and HOV14 shows an amplification at 20p11-12. Loss in region 20p12 only, is seen in tumours 417, 76 and 496. In green is the normalised amount of D20S819 and in red D20S804.

of the regions. Overall, the analysis of copy number at 20p using this method suggests that all of the cancers, apart from HOV14, 303 and 104, are diploid for chromosome 20. Therefore the increase copy numbers at the *EEF1A2* locus must be due to regional amplification in the majority of the tumours. The size and boundaries of the amplified region of 20q13 could be determined by this method if pairs of primers amplifying multiple regions along chromosome 20 were used. Due to time constraints and quantity of DNA limitations I did not carry this out.

5.2.3 Sequencing of *EEF1A2* for the identification of activating mutations

Activating mutations in genes can alter the function of the protein if located in the coding region of the gene or alter splice sites potentially altering splicing in the transcript. In order to determine whether any of the low *EEF1A2* gene copy number ovarian cancers (3-5 copies) that overexpress eEF1A2 have alterations in the gene I sequenced the 8 exons.

Primers were designed to amplify each of the 8 exons of *EEF1A2* by PCR (Newbery PhD thesis, 2002). These primers required different PCR conditions outlined in the methods. Primers designed by myself and those designed by Helen Newbery are indicated in the methods. The amplified PCR fragment of each exon was then sequenced either using the PCR primers or internal sequencing primers, again either designed by myself or Helen Newbery.

Cancers HOV183, 481, 179, 380, 5 and 104 were sequenced (gene copy numbers 3-5) and the sequence compared to the Ensembl published sequence of genomic *EEF1A2* `chromosome:NCBI36:20:61589210:61601549:-1`. To align the sequences I used EMBL-EBI EMBOSS pairwise alignment algorithm. A published common single nucleotide polymorphism (SNP) was identified in exon 3 in cancer 183 base 3780: C/T; however no sequence alterations were identified in the coding exons (2-8) of any of the low *EEF1A2* copy number cancers. All the tumours analysed also contained 2 SNPs in exon 8 at bases 11547: G/C and 11491: T/C.

The only sequence alteration I identified was the following in exon 1 of cancer 183: g. 637G>A substitution, see figure 5.5. This sequence alteration was present in both the forward and reverse sequence and is not listed as a common polymorphism in this gene. Exon 1 is a non coding exon in the 5'UTR of *EEF1A2* and therefore the sequence alteration will have no effect on the amino acid sequence of the protein. I subsequently aligned the 5'UTR regions of *Mus musculus*, *Rattus norvegicus* and *Homo sapien EEF1A2* sequences using EMBL-EBI ClustalW program (Thompson et al., 1994) and this is shown in figure 5.6. It is apparent that the region of the 5'UTR containing the sequence variation in tumour HOV183 is not well conserved between the three species, in fact in rat the nucleotide at this position is an A, suggesting it is not a functionally important region.

5.2.4 Methylation analysis of the *EEF1A2* 5' CpG island by bisulphite sequencing

The above analysis of *EEF1A2* copy number and exon sequence suggests that eEF1A2 overexpression in these ovarian cancers is not fully explained by gene amplification or mutation. Another possible mechanism by which eEF1A2 may be overexpressed is by changes in methylation in the CpG island in the promoter region of the gene. I used the EMBL-EBI EMBOSS CpGPlot/CpGReport/Isochore (Rice et al., 2000) program for analysis of the genomic sequence of *EEF1A2* (AF163763) (Bischoff et al., 2000). This program analyses the genomic sequence and identifies regions of high CpG dinucleotides relative to the bulk genome, called CpG islands. The program parameters were set to an observed/expected ratio of 0.6, a GC content over > 50% and the length of sequence to >200bp. This algorithm then predicted 4 CpG islands in *EEF1A2*, see figure 5.7. The first is found at the 5' end of the gene, spanning across the first exon (nucleotides 1702-2434) and is 733bp in length. The more 3' CpG islands are found in exon 4 (nucleotides 6064-5405) and exon 6 (9334-9537). Importantly, the CpG rich-regions in exon 4 and 6 are considered too small to be CpG islands by current accepted criteria. Finally there is a large CpG island at the very 3' end of the gene (nucleotides 11334-12053) around exon 8. Importantly, this 3' CpG island may be the promoter CpG island of the neighbouring gene *KCNQ2*.

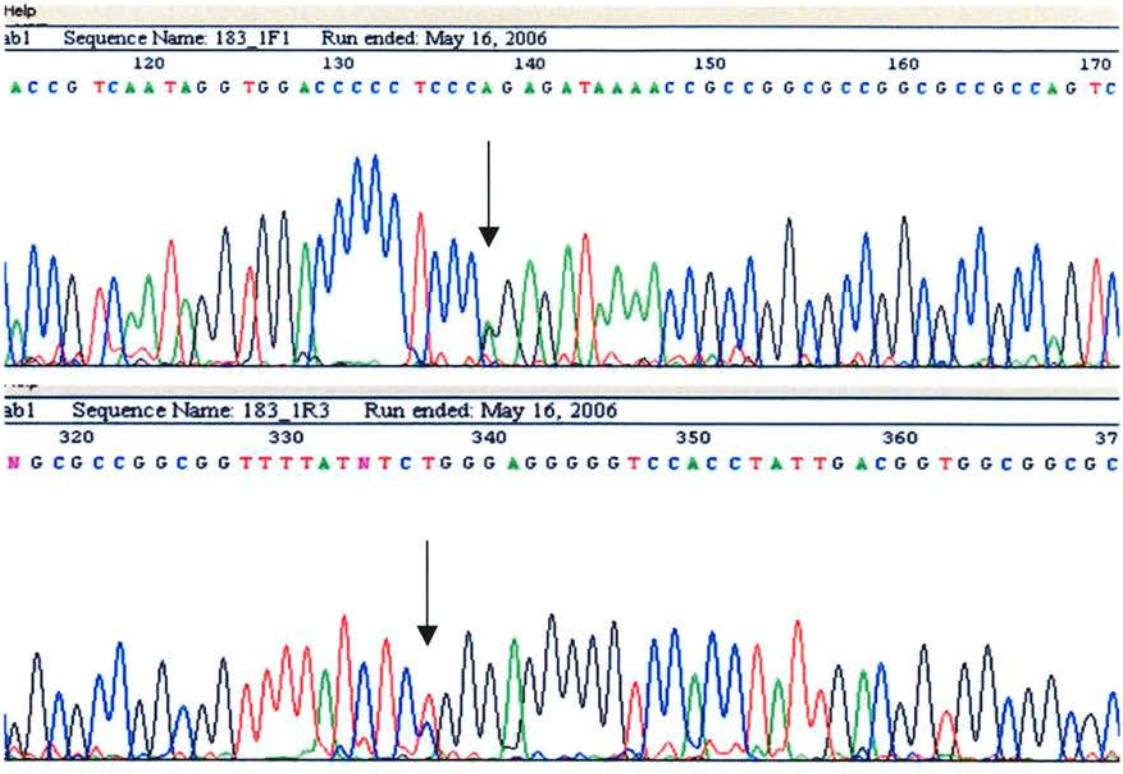


Figure 5.5 Tumour 183 contains a sequence variation in exon 1 of *EEF1A2*
The sequence traces for forward and reverse sequencing products of exon 1 analysis in tumour HOV183. The arrows denote sequence variation g. 637G>A substitution.

Figure 5.6 *EEF1A2* sequence alignment from *Mus musculus, Rattus norvegicus* and *Homo sapiens*, the nucleotide showing sequence variation in HOV183 is not highly conserved.

The top sequence is from *Mus musculus*, the middle sequence is from *Rattus norvegicus* and the bottom sequence from *Homo sapiens*. The nucleotides highlighted in red correspond to the nucleotide showing alteration in HOV183.

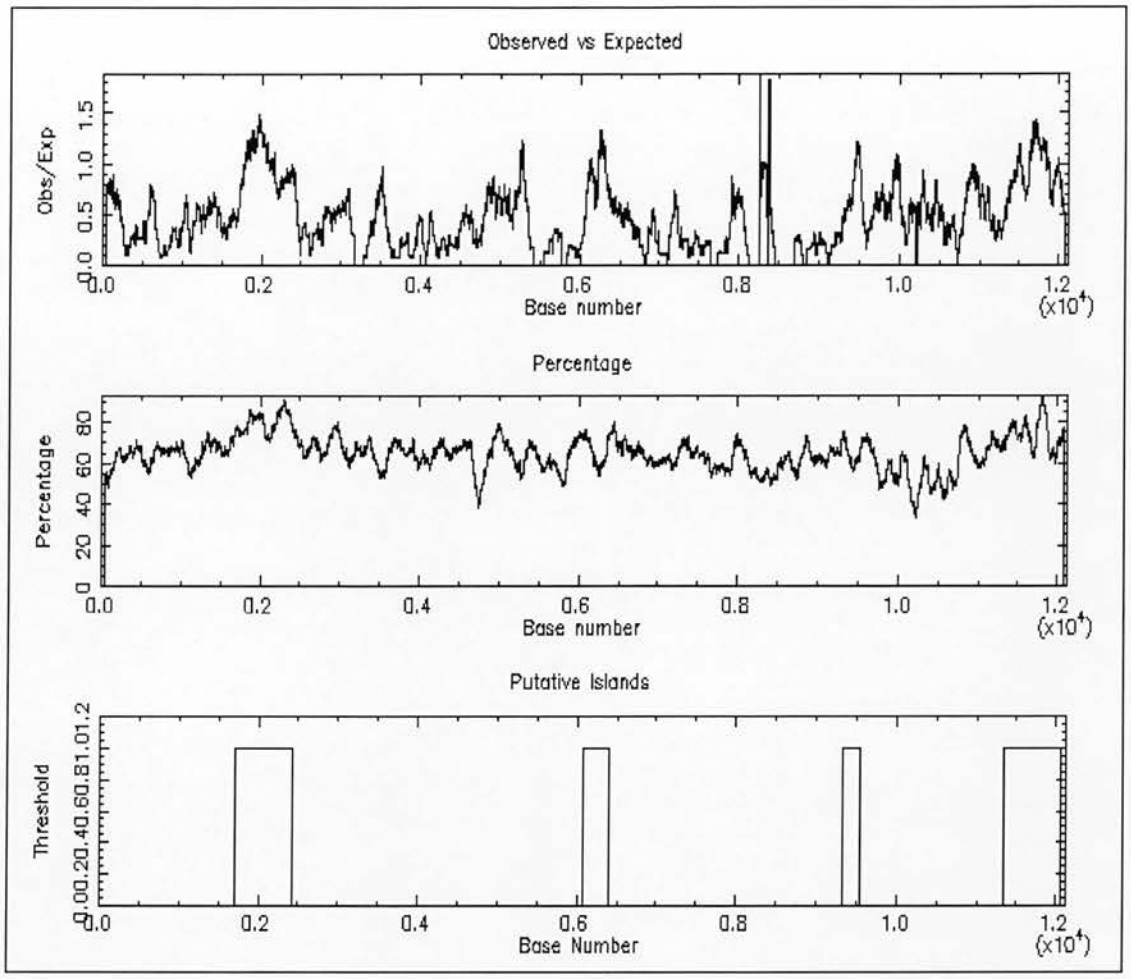


Figure 5.7 Putative CpG islands in *EEF1A2* predicted using EMBL-EBI EMBOSS CpGPlot/CpGReport/Isochore.

EEF1A2 is predicted to contain 4 CpG islands. The 5' CpG island is upstream of the start codon and spans exon 1. The second and third are found within exons 4 and 6 of the gene but are too small to be classified as CpG island by accepted criteria. The final 3' CpG island covers exon 8 but may be a CpG island of the neighbouring gene *KCNQ2*.

The CpG island most likely to be effective in gene expression regulation is the 5' island spanning exon 1 in the promoter region of the gene. Jan Bergmann, an undergraduate student in our lab, set up a bisulphite sequencing method for analysis of the methylation status at this CpG island in *EEF1A2* (see Chapter 2: Materials and Methods) and I subsequently used this protocol for analysis of the methylation status of this predicted island in DNA from ovarian tissues.

I began by selecting two eEF1A2-expressing ovarian cancers, HOVs 179 and 104, two cancers that do not express eEF1A2 HOV470 and 308, and two normal whole ovary DNA samples HOV548 and 440. I then treated the DNA with bisulphite in order to convert all unmethylated cytosine nucleotides to uracil. I subsequently used PCR to amplify a 548bp region of the 5' *EEF1A2* CpG island from each of the converted DNA samples. The primers used for this PCR (designed by Jan Bergmann) amplify the *EEF1A2* sequence from 1547bp to 2095bp. This 548bp region includes approximately 50% of the CpG island – 68 CpG dinucleotides. Subsequently, I used TA cloning and sequencing to analyse the methylation at the CpG dinucleotides in this region of *EEF1A2*.

The results of the bisulphite sequencing were then analysed using the BiQ analyzer program (Bock et al., 2005). This program aligns the sequence of multiple clones with the genomic sequence of interest. It then checks the sequence for converted cytosines and sequencing errors and advises that any sequence with less than 90% converted cytosines and 80% sequence similarity should be excluded from analysis to ensure sequencing errors and incomplete bisulphite conversion do not influence the methylation analysis. Finally the program produces plots of the methylation status at each CpG dinucleotide in the sequence. The results of this analysis can be found in figure 5.8.

The DNA clones from the two ovarian cancers expressing eEF1A2, HOV179 and 104 contained methylated CpGs before the start of the CpG island but then showed no methylation in the CpG island itself. The non-eEF1A2 expressing ovarian tumours showed very little methylation in the CpG dinucleotides before the island or

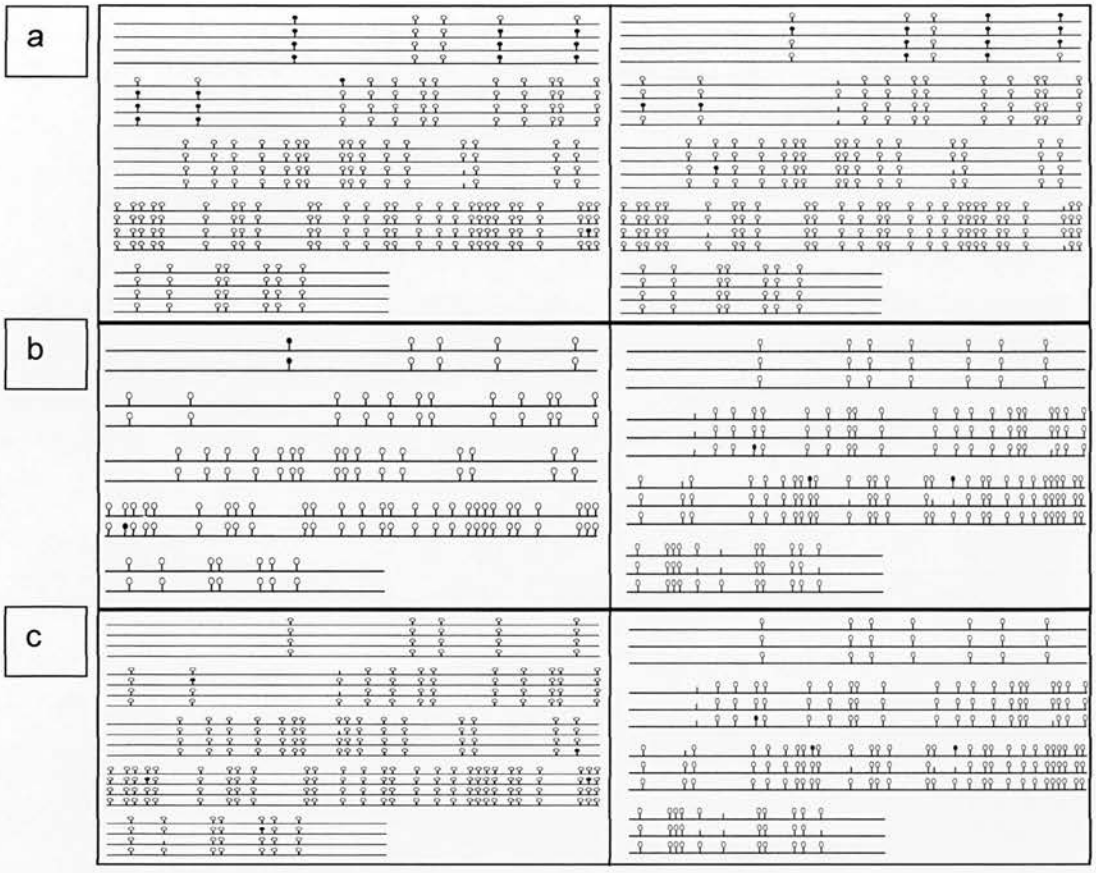


Figure 5.8 Methylation analysis of the *EEF1A2* 5'CpG island by bisulphite sequencing

Above shows lollipop diagrams produced by BiQ Analyzer. Open circles denote unmethylated CpG dinucleotides, while closed circles denote methylated CpG dinucleotides. (a) Tumours 179 and 104 do express eEF1A2 and show some methylation in CpG dinucleotides proceeding the island but no methylation within the CpG island. (b) Tumours 308 and 470 do not express eEF1A2 and there are very few methylated CpGs either within the island or proceeding it. (c) DNA from 2 different normal whole ovaries. There is little methylation present at any CpG dinucleotides within the sequence.

in the island itself, however each clone did have at least one methylated CpG in the CpG island. DNA extracted from normal whole ovary contained no methylated CpGs apart from one clone that had 2 methylated CpGs in the CpG island and one 5' to this region.

Overall, this preliminary analysis on a small number of DNA samples suggests that methylation is highly unlikely to be involved in the regulation of eEF1A2 expression.

5.5 Discussion

Due to the fact that only a small amount of DNA and no cell lines were available from the ovarian cancers for which I did expression analysis it was not possible to use CGH or Southern blot to identify amplifications in *EEF1A2*. An alternative technique that can be used to measure copy number is FISH on tumour tissue. This could have been carried out on the ovarian TMAs and HOV tumour sections however the number of TMAs were limited and a suitable number were not available for optimisation of the protocol. I therefore used a real-time PCR technique adapted from Ginzinger *et al.* (Ginzinger et al., 2000) as DNA from the HOV panel was in plentiful supply. I have shown that this alternative technique allows determination of DNA copy number in tumours, as well as the distinction between chromosome 20 polyploidy and regional amplification. The degree of amplification of *EEF1A2* at 20q13.3 did not appear to correlate with the histological subtype, stage or grade of the tumour or with disease recurrence. This is at odds with previous studies that suggest that 20q13 amplification correlates with late stage, high grade ovarian cancers (Sonoda et al., 1997),(Suehiro et al., 2000).

The majority of the ovarian cancers (80%) that I analysed also appeared to show amplification in *EEF1A2*. However, Anand *et al.* only identified *EEF1A2* amplification in 25% of the ovarian cancers analysed (Anand et al., 2002). This may reflect the differences in the techniques used to determine the copy number of *EEF1A2*. Anand *et al.* used FISH whereas I used quantitative real-time PCR. The main caveat to the use of quantitative PCR is that the technique can introduce variation into the results. PCR variation between gene specific primers and reference gene primers can cause discrepancies in normalisation. This is somewhat controlled for by measuring the PCR efficiency of each primer set and this was done in triplicate. It may also be that real-time PCR is a more sensitive technique than FISH for identifying copy number changes and this has lead to the elevation of the percentage of ovarian cancers showing amplification of *EEF1A2*. The fact that this technique showed amplification at 20p in only one tumour also suggests that the technique is valid and that the apparent amplification in *EEF1A2* is real. Importantly *EEF1A2* appears to be amplified in 80% of ovarian cancers, a frequency far higher

than that suggested by previous studies (Anand et al., 2002), suggesting than although the technique is technically valid, in practice it may not be suitable for measuring gene copy number in cancers. Further validation of the technique is necessary in order to determine if the technique is valid. For instance the use of a cell line in which the *EEF1A2* copy number has been determined by FISH and the real-time PCR technique could be used to determine if the correct copy number is being measured using the PCR technique.

20p11-12 loss was identified in three of the ovarian cancers and loss of 20p11 was present in an additional three of the cancers. Furthermore, tumour HOV14 appeared to show amplification at 20p. Copy number changes at 20p are not a frequent observation in epithelial ovarian cancers however LOH at 20p in serous adenocarcinomas (Okada et al., 2002) and 20p amplification, has been observed in ovarian cancer (Sonoda et al., 1997). Genes immediately surrounding the microsatellite marker at 20p12 include *PLCB1*, *PLCB4* and *PAK5*. Phospholipase C Beta 1 and 4 (*PLCB1/4*) encode different isoforms of a protein that catalyzes the formation of inositol 1,4,5-triphosphate and diacylglycerol from phosphatidylinositol 4,5-biphosphate, these being involved in Akt activation (Osaki et al., 2004). PLCB1 has been identified in the nucleus and nuclear phosphoinositides are thought to be potentially involved in maintaining chromatin in a transcriptionally active conformation. In addition, a decrease in the level of nuclear PLCB1 has been associated with the development of acute myeloid leukaemia (Martelli et al., 2005). p21-activated kinase 5 (*PAK5*), located at 20p12, encodes a serine/threonine protein kinase known to be an effector of Rac1/Cdc42 GTPases. Rac1 and Cdc42 play a role in cytoskeletal regulation and cell motility, and therefore *PAK5* is not a potential tumour suppressor gene but rather a candidate oncogene (Jaffer et al., 2002). A candidate tumour suppressor gene at 20p12.3 is proliferating cell nuclear antigen (*PCNA*). PCNA has been shown to perform a role in eukaryotic DNA replication, where it acts as a sliding clamp for replicative DNA polymerases. Subsequently, PCNA has been identified as playing a role in multiple cellular functions including DNA repair, translesion DNA synthesis, chromatin remodelling and regulation of the

cell cycle (Maga and Hübscher, 2003). The role of PCNA in DNA repair suggests that PCNA is a candidate tumour suppressor gene on chromosome 20p.

A somewhat unexpected observation was that the pattern of *EEF1A2* copy number appears to be the same in both non-eEF1A2 and eEF1A2 expressing tumours. This suggests that copy number alterations are not the only mechanism causing *EEF1A2* expression and that *EEF1A2* amplification does not necessarily lead to increased transcription levels. Additionally, higher *EEF1A2* copy number cancers do not express eEF1A2 at higher RNA levels. The DNA and RNA were, however, extracted from whole ovarian tumour samples and not from microdissected cancer epithelial cells to exclude stromal contamination. This therefore means that the RNA expression levels I obtained may not be an accurate representation of the expression level of eEF1A2 in the malignant cells. Nevertheless, all of the above suggest that gene amplification is not a significant player in determining whether eEF1A2 expression occurs and at what level this expression occurs at in these ovarian cancers. The lack of any apparent relationship between *EEF1A2* copy number and eEF1A2 expression levels could also be due to the fact that the PCR technique may not be giving an accurate copy number measurement.

In order to identify whether mutation in *EEF1A2* may have lead to overexpression of the gene I sequenced all 8 exons of the gene in the low copy number tumours. Only low copy number tumours were sequenced because if only one copy of the gene in a high copy number contained the mutation, the signal from the mutant would be swamped by the other copies of the gene thus making the sequencing difficult to interpret. None of the tumours showed sequence variation in the coding exons or in the non-coding region of exon 8. Tumour HOV183 contained a G>A transversion in exon 1, a non-coding exon that is covered by the 5' CpG island. Interestingly, this tumour showed no amplification in *EEF1A2* and indeed had the lowest *EEF1A2* copy number. It is not clear what significance, if any, this sequence variation has in the expression of eEF1A2. The 5' and 3' UTRs of mRNAs are thought to be important in the post-transcriptional regulation of gene expression. This can include the modulation of translational efficiency, mRNA stability and subcellular

localisation (Mignone et al., 2002). Tumour HOV183 expresses eEF1A2 at the RNA but not the protein level therefore this mutation may be important in post-transcriptional regulation. Nonetheless, the lack of protein expression means this mutation is not important in cancer. Additionally, the lack of species conservation of the sequence surrounding this sequence variation observed in tumour HOV183, also suggests the region may not be an important regulatory region.

The limited analysis of methylation status of the *EEF1A2* 5' CpG island in ovarian cancers that express and do not express eEF1A2 as well as DNA extracted from normal whole ovary, suggests that methylation is not important in the regulation of transcription from this gene; nevertheless the three groups of samples did show subtle differences in methylation. The eEF1A2 expressing cancers, HOV179 and 104, exhibited methylation of CpG dinucleotides upstream of the CpG island but no methylation within the island. This is opposite to the typical situation observed in cancer where there is genome wide hypomethylation concomitant with CpG island hypermethylation, but is typical of the situation observed in normal tissues. The non-expressing tumours, on the other hand, showed little methylation in CpG dinucleotides proceeding the CpG island or in the island itself. One of the clones from normal ovary DNA sample HOV548 exhibited methylation at 3 CpG dinucleotides within the CpG island whereas the other clones from both sample HOV548 and 440 contained few if any methylated CpGs. This suggests that the more highly methylated clone DNA may have originated from a specific cell type in the ovary. Overall, the 5' CpG island of *EEF1A2* does not appear to be methylated in normal ovary, or ovarian cancers expressing and not expressing eEF1A2.

The choice of normal ovary control is a highly debated issue within ovarian cancer research. It is thought that epithelial ovarian cancers arise from the ovarian surface epithelium: a single cell epithelial layer surrounding the ovary (Auersperg et al., 1997). This single cell layer is often destroyed during surgery due to its delicate nature. When the OSE is preserved it is cultured in order to increase the number of cells and therefore the amount of DNA, RNA and protein that can be obtained and this is often used as the normal ovary control in experiments. However, it is possible

that transformation and culturing of the OSE can alter methylation patterns, as is known for other cell types in culture, and therefore is perhaps not a good control for methylation analysis in normal ovary. I used normal whole ovary as a control, and as detailed in chapter 4, eEF1A2 is expressed in lutenised stromal cells as well as the OSE but not in the stroma. The majority of the ovary is composed of stroma and therefore the DNA from whole ovary will mainly have originated from fibroblasts. Hence it is difficult to identify a suitable control for this analysis. However, there is no discernable difference in methylation between eEF1A2 expressing and non-expressing tumours, which acts as an additional control.

The results from analysis of *EEF1A2* copy number, mutation and methylation status analysis suggest that none of these mechanisms are highly significant in mediating the overexpression of eEF1A2; therefore another mechanism could be involved. For example the activation of transcription of *EEF1A2* by a transcription factor aberrantly expressed in cancer could induce eEF1A2 overexpression in some cancers. Alternatively, the predominant mechanism of eEF1A2 overexpression could be gene amplification but this could be being obscured by the PCR technique used in this analysis. Further validation of the real-time PCR technique used to measure *EEF1A2* copy number would be required to determine whether this is the case.

Chapter 6: The role of eEF1A2 in oncogenesis

6.1 Introduction

The non-canonical functions of eEF1A1 and eEF1A2

The eukaryotic elongation factors 1A1 and 1A2 have both been shown to be involved in the elongation step of protein synthesis where they bind and transport aminoacyl-tRNA to the A-site of the ribosome in a GTP dependent manner. In addition to its role in translation elongation, eEF1A1 is thought to perform many other so called non-canonical functions including cytoskeletal binding and remodelling (Dharmawardhane et al., 1991), the susceptibility of rodent fibroblasts to transformation (Tatsuka et al., 1992), and oxidative stress (Chen et al., 2000). eEF1A1 and eEF1A2 are 98% similar and 92% identical at the amino acid level (Lund et al., 1996) and therefore it would be reasonable to assume that the two proteins have similar functions. However, there is evidence that eEF1A1 and eEF1A2 perform different non-canonical functions as there are clear differences in their properties. For example eEF1A1 has been demonstrated to be pro-apoptotic and eEF1A2 anti-apoptotic in serum deprived myotubes in culture (Ruest et al., 2002). Additionally, eEF1A2 but not eEF1A1 has been shown to interact with perioxiredoxin-1 (Prdx1) in mouse brain (Chang and Wang, 2006). Finally, *EEF1A2* has been identified as a putative oncogene (Anand et al., 2002) whereas *EEF1A1* has not, suggesting that eEF1A2 performs different roles to eEF1A1 and that when eEF1A2 is inappropriately expressed this can result in oncogenesis. The fact that both variants of eEF1A are highly evolutionarily conserved and the occurrence of developmental switching from eEF1A1 to eEF1A2 in brain, heart and skeletal muscle in mice (Khalyfa et al., 2001) also suggests that eEF1A1 and eEF1A2 perform distinct functions.

Elongation factor 1A and the cytoskeleton

There is a large amount of evidence to suggest that eEF1A interacts with, and can alter the structure of, the cytoskeleton. A protein of approximately 50kDa (provisionally named ABP-50) was identified as having actin bundling activity and was isolated from the amoebae of *Dictyostelium discoideum* (Demma et al., 1990).

This ABP-50 protein was later identified as elongation factor 1A (Yang et al., 1990). Dharmawardhane *et al.* found that in resting *Dictyostelium discoideum* eEF1A showed a diffuse localisation within the cytosol but upon stimulation of the cells with cAMP eEF1A translocated to filopodia. In resting cells only 10% of eEF1A was shown to be associated with the cytoskeleton. eEF1A also interacts with G-actin or non-filamentous actin and this interaction is inhibited by GTP but not GDP. However the bundling of F-actin was unaffected by guanine nucleotides (Dharmawardhane et al., 1991). eEF1A was also shown to co-localise with poly(A) mRNA at actin filament intersections in human fibroblasts (Bassell et al., 1994). The interaction of eEF1A with F-actin was shown to be pH dependent, unlike the interaction of the elongation factor with aatRNA, and the binding of F-actin and aatRNA was suggested to be mutually exclusive (Liu et al., 1996). Edmonds *et al.* also observed that the eEF1A-induced bundling of F-actin was dependent on pH (Edmonds et al., 1995). Owen *et al.* observed that eEF1A from *Dictyostelium discoideum* cross linked actin with a unique bonding rule that meant actin filaments were rotated at 90° to one another leading to the exclusion of other actin bundling proteins (Owen et al., 1992). Liu *et al.* showed that eEF1A localised with β actin mRNA and F-actin in the protrusions of chicken embryo fibroblasts and that the C-terminal domain was responsible for binding. This implicated eEF1A in β actin mRNA localisation (Liu et al., 2002). When overexpressed in *Saccharomyces cerevisiae,* eEF1A causes reduced budding and altered actin distribution and cellular morphology (Munshi et al., 2001).

eEF1A has also been shown to bind, bundle and stabilise microtubules in plants (Moore and Cyr, 2000) and to associate with the mitotic apparatus of Sea urchin eggs (Ohta et al., 1990). Kuriyama *et al.* also observed an association between eEF1A, the centrosome and mitotic apparatus in dividing sea urchin eggs (Kuriyama et al., 1990). Moore *et al.* also showed that eEF1A from Daucus Carota can modulate the dynamic behaviour of microtubules in a calcium/calmodulin dependent manner (Moore et al., 1998). Direct binding of eEF1A from carrot cells with microtubules has been observed (Durso and Cyr, 1994) and severing of microtubules by eEF1A has been reported (Shiina et al., 1994). Overexpression of eEF1A in fission yeast

resulted in the cells showing elliptic, curved or branched type abnormal morphology and growth defects at high temperatures and this was suggested to be due to eEF1A's ability to alter the actin and tubulin cytoskeleton in yeast (Suda et al., 1999).

The interaction of eEF1A with the cytoskeleton has been suggested to facilitate the spatial and temporal regulation of protein synthesis (Edmonds, 1993). It has been shown that channelling of aatRNA from aatRNA synthetase to eEF1A and then to the ribosome without diffusion in the cytosol occurs during protein synthesis (Negrutskii and Deutscher, 1991). The ability of eEF1A to alter cytoskeletal organisation could directly alter the efficiency of translation because most mRNA is anchored on actin or tubulin filaments (Condeelis, 1995). Additionally, the interaction of eEF1A with the cytoskeleton may be unrelated to its role in translation and could allow regulation of the cytoskeletal structure in, for instance, response to extracellular stimuli.

The role of eEF1A in heat shock and cancer

Heat shock proteins (HSPs) are encoded by distinct gene families and named according to their relative molecular mass (*Mr*), for instance Hsp27, Hsp60 and Hsp 70. The expression of HSPs is mediated by the binding of activated HSF1 (a transcription factor) to specific DNA sequences in their promoter regions. The primary function of HSPs is to bind to unfolded proteins that accumulate in response to cellular stress, such as excessive temperature, and prevent catastrophic protein aggregation and subsequently refold the proteins with the aid of chaperonins. In addition HSPs also inhibit programmed cell death in order to allow time for repair of the proteome (Calderwood et al., 2006). HSPs are expressed in a wide range of cancers and their expression is associated with poor prognosis and resistance to therapy (Ciocca and Calderwood, 2005). This overexpression is mediated by loss of p53 function and by the expression of oncogenes such as *ERBB2* and *c-Myc*. Overexpression of Hsp90 leads to self sufficiency for growth signals in cancer cells by maintaining signalling proteins in an active conformation. Hsp90 also stabilises mutant proteins that arise during oncogenesis such as v-Src, Bcr-Abl and p53. Hsp70 and Hsp27 are involved in the inhibition of programmed cell death. Hsp90 is

essential for telomerase stability and has therefore been implicated in the inhibition of replicative senescence. Hsp70 and Hsp90 stabilise the primary sensor of hypoxia HIF1α, leading to the transcription of factors involved in the proliferation and motility of vascular endothelial cells including vascular endothelial growth factor and nitric oxide synthetase. Finally, tumours overexpressing HSF1 and HSPs show an increased tendency to invade and metastasise by unknown mechanisms, see review by (Calderwood et al., 2006).

HSF1 activation involves trimerisation and acquisition of specific DNA-binding activity. It was widely believed that HSF1 activation occurred as a result of the removal of inhibition imposed by chaperones including Hsp90. However, the fast kinetics of HSF1 activation (Jolly et al., 1999) suggested that this diffusion controlled mechanism was probably not responsible for HSF1 activation (Shamovsky et al., 2006). A recent paper by Shamovsky *et al.* suggests that eEF1A may be essential for HSF1 activation. They identified eEF1A as bound to immobilised-HSF1 after incubation with lysate from heat-shocked BHK-21 or HeLa cells. They also identified a 600 nucleotide non-coding RNA called heat shock RNA-1 (HSR1) as being required for HSF1 activation. The authors suggest that eEF1A can accumulate in cells following heat shock due to translational shut down and cytoskeletal collapse enabling it to partake in HSF1 activation (Shamovsky et al., 2006). Importantly, the methods used in this analysis did not distinguish between eEF1A1 and eEF1A2, or only investigated HSF1 activation with purified eEF1A1, and therefore we do not know if eEF1A2 is also involved in HSF1 activation. Diagram 6.1 depicts the model proposed by Shamovsky *et al.* for HSF1 activation involving eEF1A and HSR1.

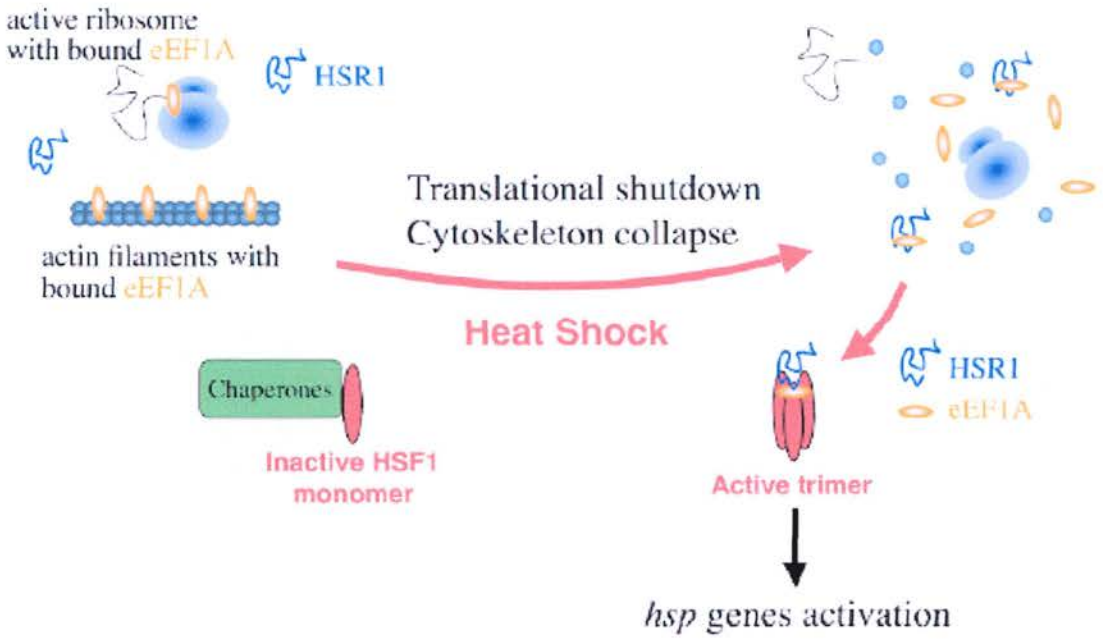


Diagram 6.1 Activation of HSF1 by eEF1A and HSR1 following heat shock, taken from (Shamovsky et al., 2006).

Interestingly, motor neurons have been shown to have an insufficient stress response. Two percent of patients with amyotrophic lateral sclerosis (ALS), characterised by a loss of motor neurons in the brain and spinal cord, have autosomal dominant mutations in Cu/Zn-superoxide dismutase (SOD-1) that is thought to result in structural changes in the protein leading to aggregation (Durham et al., 1997; Rosen et al., 1993). Expression of mutant SOD-1 in motor neurons in culture results in aggregation of the protein and cell death, but the same effect was not seen in nonvunerable cells such as hippocampal neurons expressing mutant SOD-1 (Durham et al., 1997). This suggests that motor neurons are deficient in their heat-shock protein mediated response to mutant SOD-1, resulting in ALS. Batulan *et al.* showed that after heat shock at 42°C for 1 hour, non-neuronal cells in culture expressed Hsp70 but motor neurons in culture did not. Other conditions of heat shock were also tested and the authors concluded that motor neurons did not show activation of a stress response following heat shock. This observation of failure of motor neurons to express Hsp70 was repeated in the motor neurons of mutant SOD-1 transgenic mice and ALS patients. They found that the inability to mount a heat shock response occurred at the level of HSF1 activation (Batulan et al., 2003). Motor neurons do not express eEF1A1 in their cytoplasm (some eEF1A1 expression can be seen in the

nucleus) but do express eEF1A2 cytoplasmically (Newbery et al., in preparation). Therefore, if it is eEF1A1 and not eEF1A2 that is involved in HSF1 activation this may explain why motor neurons are defective in the heat-shock response and consequently implicate eEF1A1 and eEF1A2 in the disease characteristics of ALS in which motor neurons are preferentially vulnerable to disease.

In order to investigate the functions of eEF1A2, how these could differ from eEF1A1, and their potential importance to the role of eEF1A2 in oncogenesis, I used immunofluorescence in cancer cells to investigate the subcellular localisation of eEF1A2 and eEF1A1; any potential difference in localisation could suggest that the isoforms are performing different roles. I also used RNA interference of eEF1A2 in cancer cells to investigate any phenotypic change that could result from eEF1A2 ablation. Finally, I used RNA interference to knockdown eEF1A1 or eEF1A2 in HeLa cells and then subjected the cells to heat shock in order to try to determine if eEF1A2 is also involved in the heat shock response and whether there is compensation by one protein in the absence of another.

6.2 Results

6.2.1 The subcellular localisation of eEF1A in cell lines

Investigation of the subcellular localisation of eEF1A1 and eEF1A2 in cancer cells may give a clue to any differences in function between the proteins and the role that eEF1A2 is potentially playing in oncogenesis. I therefore set about to examine the subcellular localisation of eEF1A2 in the breast cancer cell line MCF-7. This cell line expresses both eEF1A1 and eEF1A2 at high levels, as is common in transformed cell lines.

The first approach used was to attempt to identify the subcellular localisation of endogenous eEF1A1 and eEF1A2 using the isoform-specific antibodies by immunofluorescence (IF). These antibodies were tested for specificity using HeLa and MCF-7 cells, expressing both eEF1A variants, and NIH 3T3 (mouse) and lymphoblastoid cells that only express eEF1A2. A cell line that only expresses eEF1A2 and not eEF1A1 was not available for use as a negative control for eEF1A1 expression and to our knowledge does not exist. A variety of fixation methods were used including ice-cold methanol, 2-4% paraformaldehyde as well as methanol:acetone and all the available antibodies to eEF1A2 (1A2-1, 2 and 3) and eEF1A1 (1A1-1 and 1A1-3) were tested at various concentrations and incubation times, this is represented in table 6.1. All three antibodies to eEF1A2 and both antibodies to eEF1A1 gave a similar staining pattern in the different cell types. This was a faint, diffuse, often granular staining in the cytoplasm of the cells, particularly in the perinuclear region. However, the same faint staining pattern was observed in secondary antibody-only controls. As well as this staining of the eEF1A2 negative mouse fibroblast cell line NIH 3T3 and human lymphoblastoid cell line with the eEF1A2 antibodies showed a similar result to that seen in HeLa and MCF-7 cell lines that are positive for eEF1A2. I therefore concluded that the isoform-specific antibodies, although very effective in Western blotting and immunohistochemistry, are unsuitable for use in immunocytochemistry. The reason for the antibody not working in immunocytochemistry is likely to be the difference in antigen retrieval techniques used for the different applications. Western blotting involves denaturing the protein with detergent and heating, and immunohistochemistry involves heating

Table 6.1 *Method variations attempted for immunofluorescent staining of endogenous eEF1A1 and eEF1A2 in cell lines.*

Cell lines	Hela MCF-7 NIH3T3 Lymphoblastoid
Fixation methods	Ice cold methanol 2% Paraformaldehyde Methanol:acetone
Fixation times	10 minutes room temperature 30 minutes room temperature
Blocking methods	10% serum of animal secondary raised in 3% bovine serum albumin
Antibodies	1A1-1 1A1-3 1A2-1 1A2-2 1A2-3
Primary antibody concentrations	1:10 1:50 1:100
Primary antibody incubation times	30 mins room temperature 1 hour room temperature 2 hours room temperature 4°C overnight
Secondary antibodies	Donkey anti-sheep Alexa 594 Goat anti-rabbit Alexa 488 Biotinylated and Fluorescein avidin D
Secondary antibody concentrations	1:100 1:500 1:1000
Incubation times	30 mins room temperature 1 hour room temperature 2 hours room temperature

in antigen retrieval solutions. On the other hand the antigen retrieval in immunocytochemistry may not be stringent enough to expose the antigen to the antibody. Alternative methods for analysis of eEF1A2 subcellular expression include cell fractionation and Western blotting or *in situ* hybridisation to visualise the subcellular localisation of the eEF1A2 transcript. In order to visualise the subcellular localisation of the eEF1A2 protein I went about obtaining tagged eEF1A2 constructs so that these could be used to determine the subcellular localisation of the protein using antibodies to the tags; in this instance I decided to use V5 and Green Fluorescent Protein (GFP) tags. V5-tagged eEF1A2 was purchased from Invitrogen, this is the full length eEF1A2 cDNA cloned into the pcDNA3.1/GS vector, resulting in the expression of full length eEF1A2 expressed under a CMV promoter and tagged at the C-terminus with the V5 peptide and six polyhistidines. I sequenced the eEF1A2 insert to confirm that the sequence was correct. To determine whether eEF1A2 is expressed from the construct, I transfected PEO1 cells (eEF1A2 negative) with pcDNA3.1/GS-eEF1A2 and used extracted protein from these cells in a Western blot: probing with an anti-V5 antibody and 1A2-1 antibody. Protein lysate from pcDNA3.1/GS-eEF1A2-transfected cells showed a band of approximately 52kDa in size when probed with the anti-eEF1A2 antibody or anti-V5 antibody that was not present in untransfected, mock or empty vector controls, see figure 6.1. Unexpectedly, a band slightly smaller than 52kDa was present in the Western blot probed with the anti-eEF1A2 antibody in protein lysate from PEO1 cells mock transfected, DNA-only controls (DNA added into cells with no transfection) and cells transfected with V5-tagged eEF1A2 or GFP-eEF1A2 but not in untransfected cells. This suggests that a slightly smaller eEF1A2 protein is induced by cellular stress due to transfection or that a low level of eEF1A2 is expressed in PEO1 cells. The later would mean that in cells transfected with V5-tagged eEF1A2 the higher band represents the V5 tagged protein and the lower band the native protein. The V5 tag consists of 14 amino acids and is approximately 5kDa in size. The size difference between the two bands could be determined using a higher resolution molecular weight marker and running the protein on a higher percentage acrylamide gel. Alternatively the smaller bands may be present due to non-specific binding of the antibody to eEF1A1.

I then went on to clone the full length eEF1A2 and eEF1A1 cDNA sequences into the vector pcDNA-DEST53, a Gateway destination vector from Invitrogen, resulting in the expression of an N-terminal Green Fluorescent Protein (GFP) tagged protein in mammalian cells. The Gateway method offers advantages over traditional cloning methods as restriction enzymes are not used. The cDNA is first cloned into an entry vector using the site specific recombination properties of bacteriophage lambda and can be then shuttled between different destination vectors easily to make expression clones. Again, the constructs were sequenced to confirm that there were no sequence alterations in the inserted eEF1A2 and eEF1A1 and Western blotting of protein lysates from transfected PEO1 cells gave a band at approximately 80KDa (GFP is apx 30kDa in size) when probed with the 1A2-1 or 1A1-1 antibodies, see figure 6.1

The V5 and GFP-tagged eEF1A2 expressing constructs were then transiently transfected into MCF-7 cells and PEO1 cells and immunofluorescence used to determine the subcellular localisation of the protein: examples of staining can be seen in figure 6.2, no images from PEO1 cells are included due to poor quality. eEF1A2 tagged with V5 or GFP exhibits a diffuse cytoplasmic localisation with a concentration of expression around the nucleus; this expression pattern fits with its role in translation, which takes place in ribosomes at the endoplasmic reticulum (which has a perinuclear localisation) and in the cytoplasm of the cell. The localisation of eEF1A2 tagged with either V5 or GFP appeared to be similar and no eEF1A2 was located in the nucleus of the MCF-7 cells. Cells transfected with empty pcDNA3.1/GS vector and stained for V5 using the anti-V5 antibody did not show any staining and the secondary only control was also negative.

I then went on to investigate the possibility of co-localisation of eEF1A2 with the cytoskeleton. eEF1A has been shown to associate with the actin cytoskeleton in yeast where it can bind actin filaments (Dharmawardhane et al., 1991; Yang et al., 1990) and can cross-link actin causing the formation of large gel-like structures of actin bundles (Edmonds et al., 1995). In addition eEF1A associates with microtubules in higher plant cells (Durso and Cyr, 1994) and sea urchin embryos

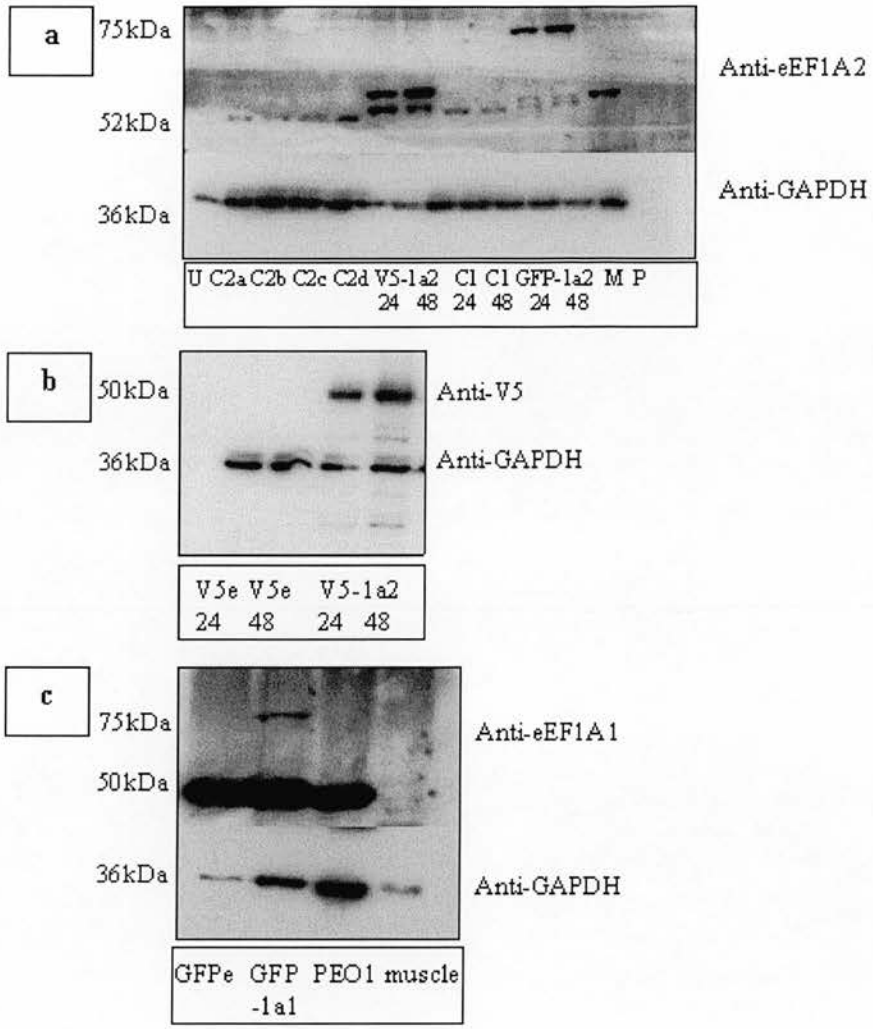


Figure 6.1 Western blot analysis of PEO1 cells transfected with tagged eEF1A2 and eEF1A1 constructs.

(a) Western blot of PEO1 cell lysates from untransfected (U); DNA only controls (C2a) pcDNA3.1/GS, (C2b) pcDNA-DEST53, (C2c) pcDNA3.1/GS-eEF1A2 and (C2d) pcDNA3.1-DEST53-eEF1A2; (V5-1A2) pcDNA3.1/GS-eEF1A2 transfected cells 24 and 48 hours post-transfection; (C1) mock transfection 24 and 48 hours post-transfection; (GFP-1A2) pcDNA-DEST53-eEF1A2 transfected cells 24 and 48 hours post transfection; (M) MCF-7 positive antibody control; (P) PEO1 negative antibody control. GAPDH was used as a loading control. In pcDNA3.1/GS-eEF1A2 a large band corresponding to eEF1A2 is present. A band of approximately 80kDa is present in PEO1 cells transfected with pcDNA3.1-DEST53-eEF1A2. (b) Western blot of protein lysate from PEO1 cells transfected with pcDNA3.1/GS (V5e) and pcDNA3.1/GS-eEF1A2 (V5-1a2) 24 and 48 hours post transfection probed with an anti-V5 antibody. One band at approximately 50kDa is visible in pcDNA3.1/GS-eEF1A2 transfected cells only. (c) Western blot of protein lysate from PEO1 cells transfected with pcDNADEST53-eEF1A1 (GFPe) and pcDNA-DEST53-eEF1A2 (GFP-1a1) and probed with the anti-eEF1A1 antibody. A band of approximately 80kDa is only present in cells transfected with GFP-tagged eEF1A1. PEO1 cell lysate (PEO1) is a positive antibody control and wild type mouse muscle (muscle) a negative antibody control.

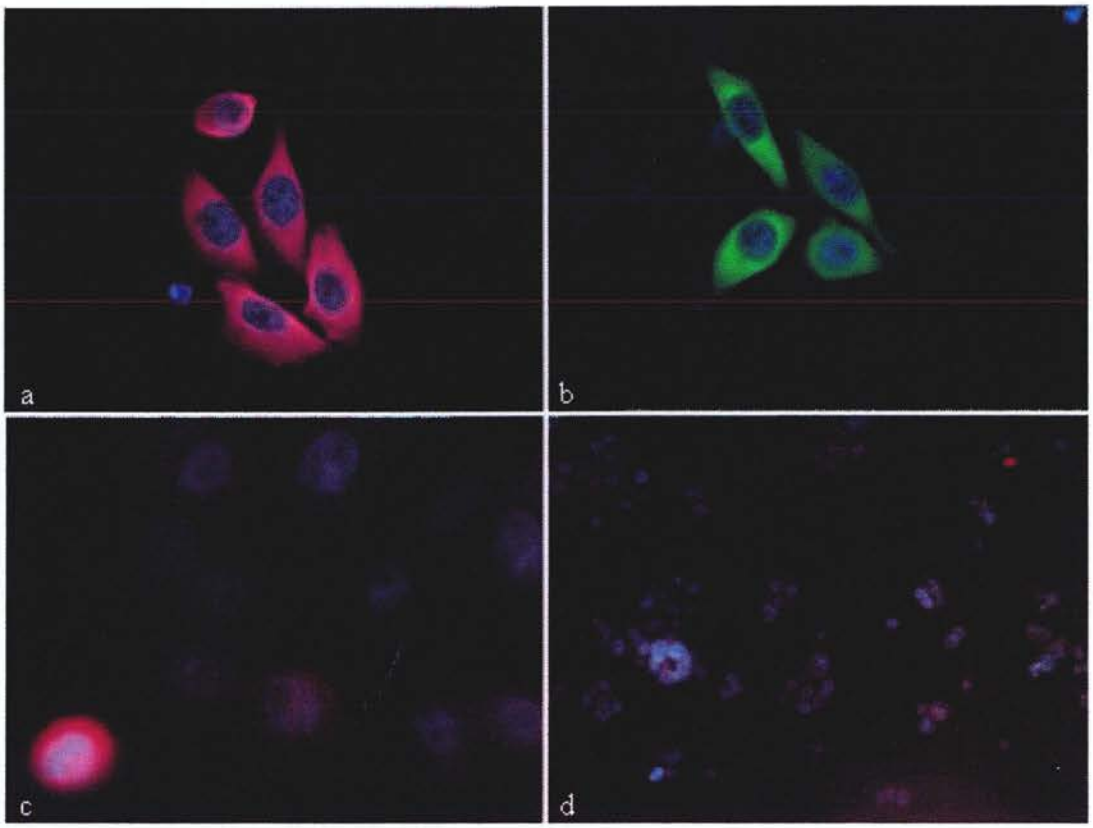


Figure 6.2 Subcellular localisation of eEF1A2 in MCF-7 cells.
(a) MCF-7 cells transfected with V5 tagged eEF1A2 and stained by immunofluorescence with an anti-V5 antibody. (b) MCF-7 cells transfected with GFP-eEF1A2. eEF1A2 tagged with V5 and GFP shows a diffuse cytoplasmic localisation with a concentration of staining around the nucleus. (c) pcDNA3.1/GS empty vector transfected cells stained with the anti-V5 antibody show no staining and (d) secondary Alexa Fluor 594 goat anti-mouse IgG only was also negative. Nuclei are counterstained in DAPI. Images (a), (b) and (c) are at x40 magnification, image (d) is at x10 magnification.

(Hamill et al., 1994) and has been found to possess microtubule-severing activity in Xenopus and fibroblasts (Shiina et al., 1994). It is not known whether both eEF1A1 and eEF1A2 or only eEF1A1 is capable of cytoskeletal binding and remodelling and the localisation of eEF1A2 in relation to the cytoskeleton has not been investigated in human cancer cells. Tubulin was visualised using an anti-α-tubulin antibody, F-actin using fluorescently conjugated Phalloidin and eEF1A2 simultaneously visualised in transfected MCF-7 cells using an anti-V5 antibody and the GFP-eEF1A2 expressing construct. Representative images can be found in figures 6.3. V5-eEF1A2 and GFP-eEF1A2 showed some co-localisation with tubulin, particularly in the perinuclear region of the cells. eEF1A2 overexpressing MCF-7 cells also appeared to show no differences in tubulin morphology when expressing eEF1A2 compared to those transfected with empty vector controls (see figure 6.5) suggesting that under these conditions of forced overexpression, eEF1A2 is not causing cytoskeletal remodelling.

An interesting observation was that V5-tagged eEF1A2 appeared to localise strongly to cellular protrusions in some transfected MCF-7 cells, see figure 6.4. V5-tagged eEF1A2 was found highly concentrated in focal areas at the edges of the cells and these protrusions may be involved in adhesion or motility. This localisation was not as clearly seen in cells transfected with GFP-tagged eEF1A2, however this may be because GFP is a large tag and could disrupt correct subcellular localisation of eEF1A2 when conjugated to it. Alternatively, the localisation of eEF1A2 at the cell periphery in foci may be an artefact of V5-tagging and overexpressing the protein. Nevertheless, F-actin showed strong co-localisation with V5-eEF1A2 in these cellular protrusions and this can be seen in figure 6.4. eEF1A2 therefore also showed partial co-localisation with actin in MCF-7 cells. These F-actin containing protrusions were also identified in cells transfected with empty vector controls and therefore did not seem to be induced by eEF1A2 overexpression in transfected MCF-7 cells. Again the F-actin structure within the cells overexpressing eEF1A2 did not appear to be different from that observed in cells transfected with empty vector, see figure 6.5. The localisation of eEF1A2 in cellular protrusions suggests that eEF1A2 may be involved in cell adhesion or motility, this having obvious implications for the

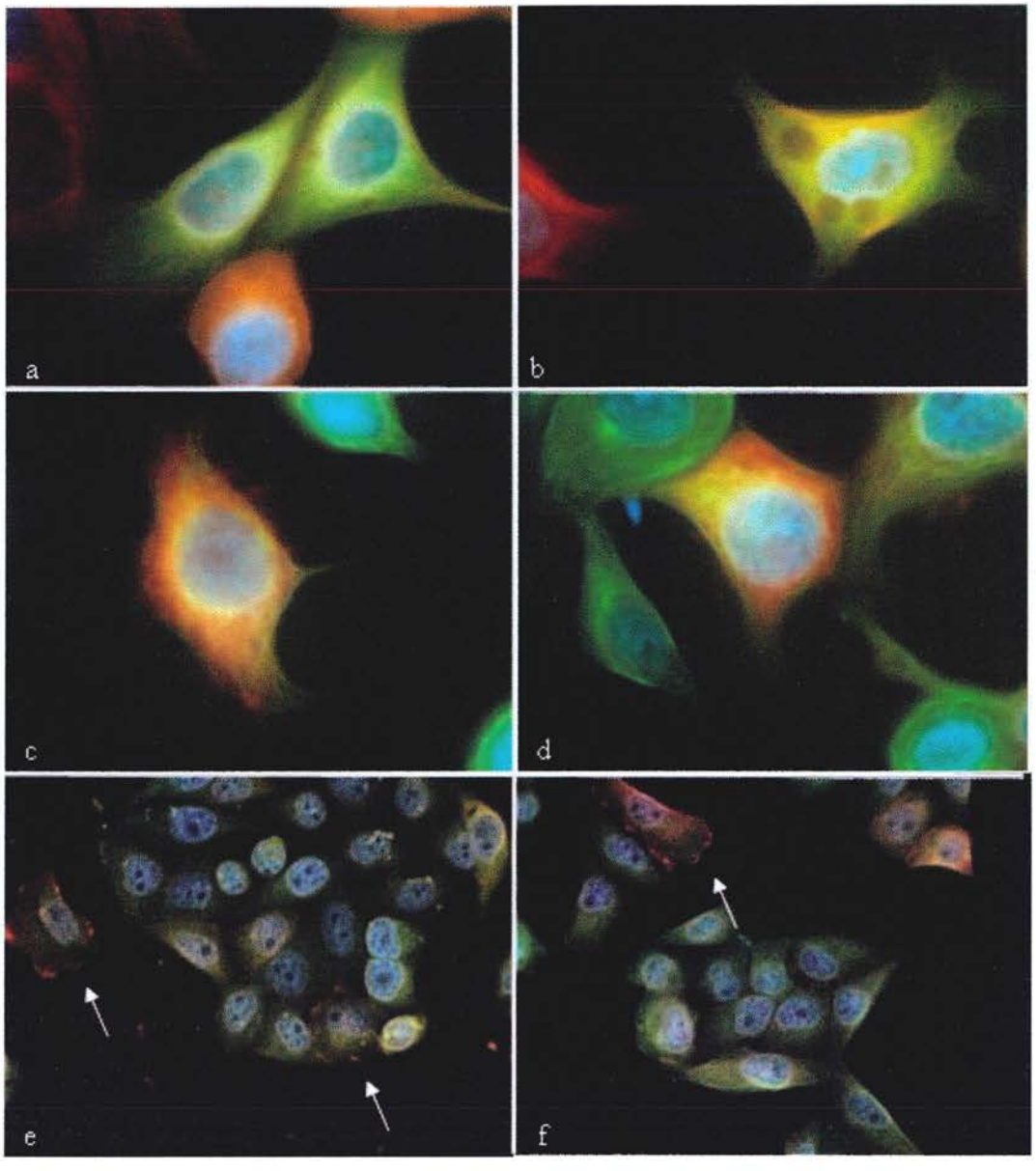


Figure 6.3 Tubulin and eEF1A2 subcellular localisation in MCF-7 cells
(a+b) GFP-eEF1A2 (green) and tubulin (red) show partial co-localisation around the nucleus (yellow). (c+d) V5-eEF1A2 (red) and tubulin (green) show a similar pattern of localisation as GFP-eEF1A2 in MCF-7 cells. (e+f) Interestingly, some cells showed a concentration of V5-tagged eEF1A2 in distinct foci at the edges of the cell (indicated by arrows). DAPI staining of the nucleus is shown in blue. (a-d) are x100 magnification, (e) and (f) x40 magnification.

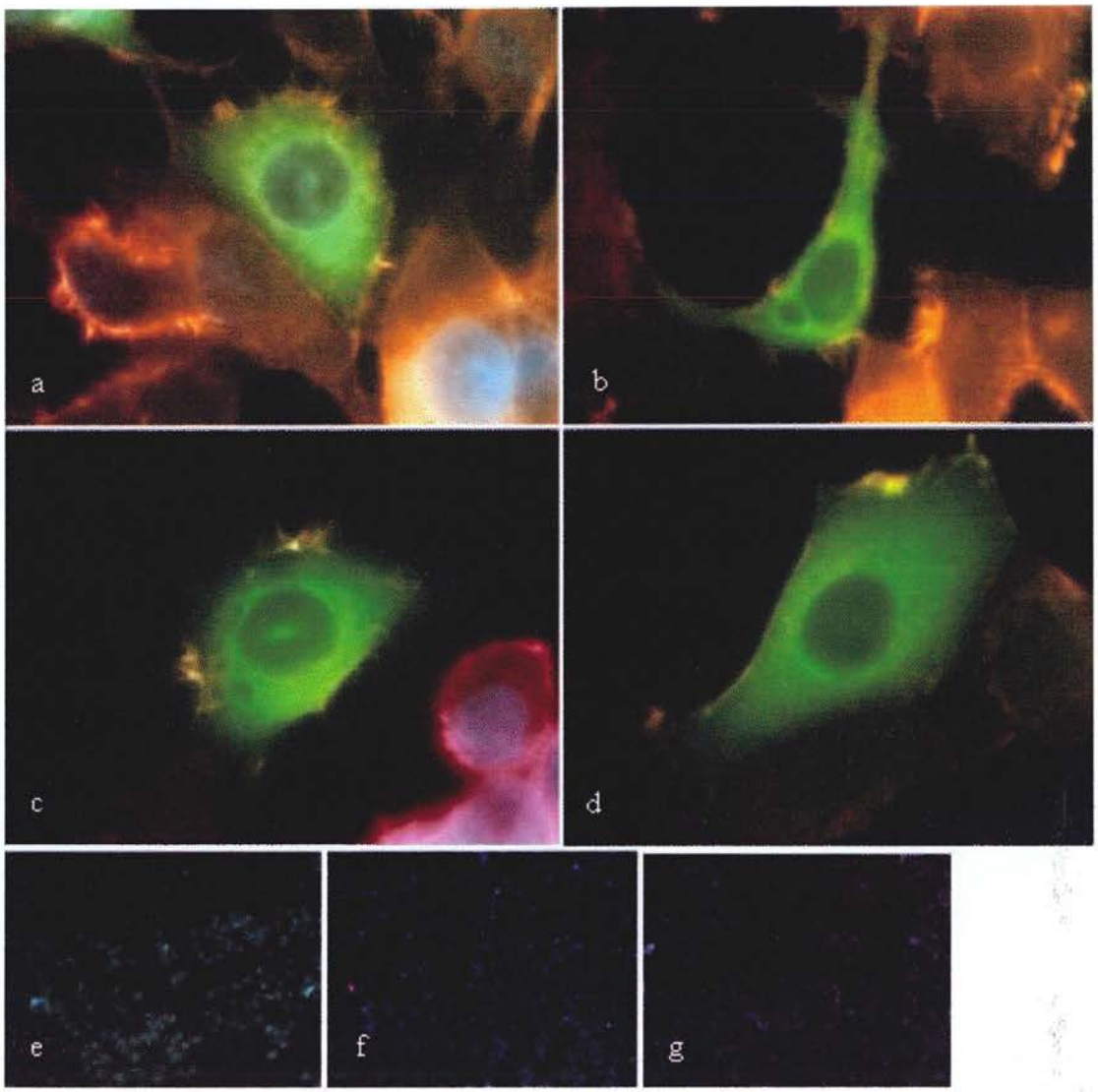


Figure 6.4 eEF1A2 and actin subcellular localisation in MCF-7 cells
(a + b) GFP-eEF1A2 (green) and phalloidin staining of F-actin (red). (c + d) V5-eEF1A2 (green) and Alexa Fluor 594 phalloidin staining of F-actin (red) in MCF-7 cells. Cellular protrusions containing high concentrations of F-actin can be seen at the edges of the cells and some co-localisation between eEF1A2 and F-actin can be observed. Images are at x100 magnification. (e) Secondary antibody Alexa Fluor 594 donkey anti-sheep IgG and Alexa 488 donkey anti-mouse IgG only (f) Alexa Fluor 594 goat anti-mouse IgG only (g) Alexa Fuor 594 donkey anti-sheep IgG only, x10 magnification

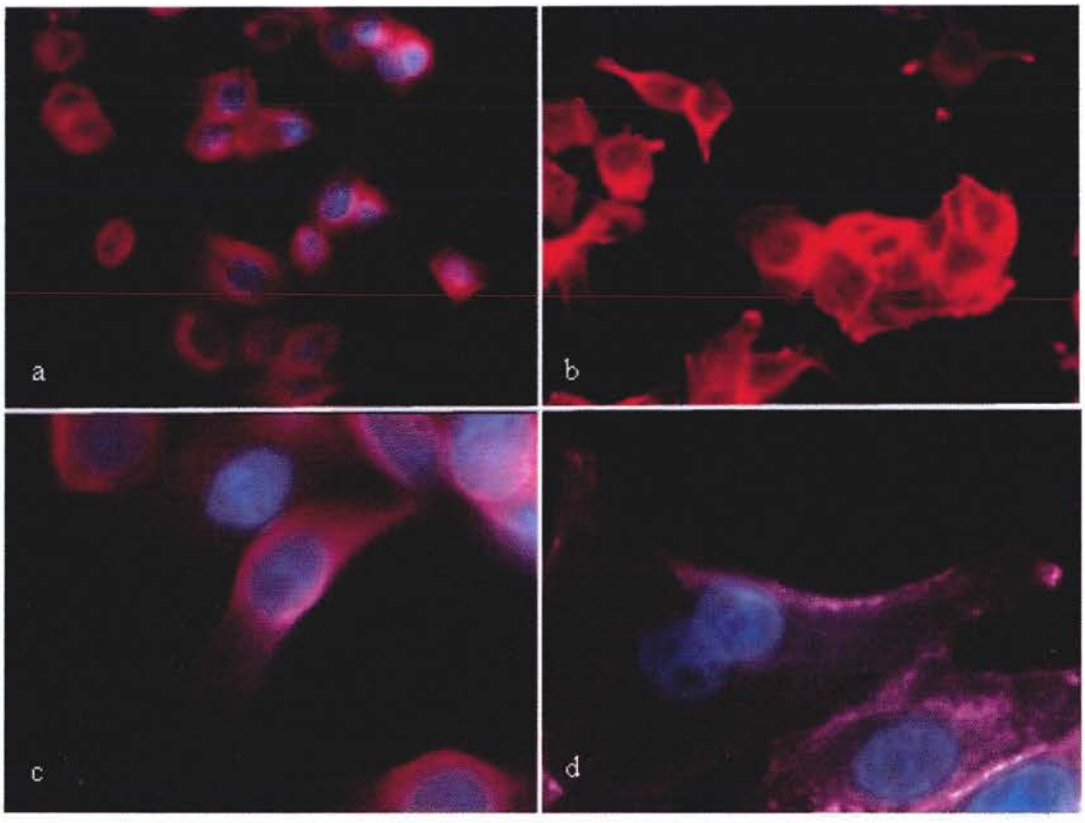


Figure 6.5 Cytoskeletal staining of MCF-7 cells transfected with empty vectors pcDNA3.1/GS and pcDNA-DEST53.
(a) MCF-7 cells transfected with pcDNA3.1/GS and stained with an antibody against tubulin and (b) Alexa Flour 594 phalloidin, x40 magnification (c) MCF-7 cells transfected with pcDNA-DEST53 and stained with an antibody against tubulin and (d) Alexa Fluor 594 phalloidin against F-actin. x100 magnification. The cytoskeletal structure in MCF-7 cells transfected with empty vectors is similar to that seen in MCF-7 cells overexpressing eEF1A2 suggesting that high levels of eEF1A2 expression (as I have observed in some breast tumours) may not alter cytoskeletal structure.

function of eEF1A2 as an oncogene. Alternatively this localisation may allow spatial localisation of protein synthesis. Further work would be required to properly identify the protrusions and characterise the effect of eEF1A2 overexpression on motility and adhesion in various cancer cells. eEF1A1 subcellular localisation was investigated in the ovarian cancer cell line PEO1. The localisation was also visualised in MCF-7 cells however I was unable to obtain satisfactory images at the time of these experiments. GFP-tagged eEF1A1 was transiently transfected into PEO1 cells and viewed under a fluorescent microscope, see figure 6.6. eEF1A1 showed a diffuse cytoplasmic expression pattern similar to the subcellular localisation observed for eEF1A2. No eEF1A1 appeared to localise in the nucleus. Similarly partial co-localisation with the F-actin and tubulin cytoskeleton was observed and although there appeared to be some co-localisation of eEF1A1 with F-actin in cellular protrusions these levels were not as high as observed for eEF1A2. Partial co-localisation with tubulin and F-actin was observed in the perinuclear region of the cell, as seen with eEF1A2. Again the tubulin and F-actin structure within eEF1A1 overexpressing PEO1 cells did not appear to differ from cells expressing only endogenous eEF1A1.

6.2.2 RNA interference of eEF1A2 expression in cancer cell lines: the trials and tribulations

RNA interference (RNAi) can be a powerful tool for ablation of expression of proteins and subsequent determination of the role of a protein within cells both in vitro and in vivo. During RNAi double stranded RNA (dsRNA) is processed into short interfering (si)RNAs of approximately 20 base pairs in length containing 2 nucleotide 3' overhangs by the dsRNA endonuclease DICER. This siRNA then enters the RNA-induced silencing complex (RISC) that mediates the degradation of complementary mRNA transcripts and this step involves Argonaute proteins among others, see review (Filipowicz, 2005).

The identification of *EEF1A2* as a potential oncogene has lead me to attempt to investigate the function of eEF1A2 in cancer cells. Using RNA interference to ablate eEF1A2 expression in cancer cell lines, and subsequent analysis of any resulting

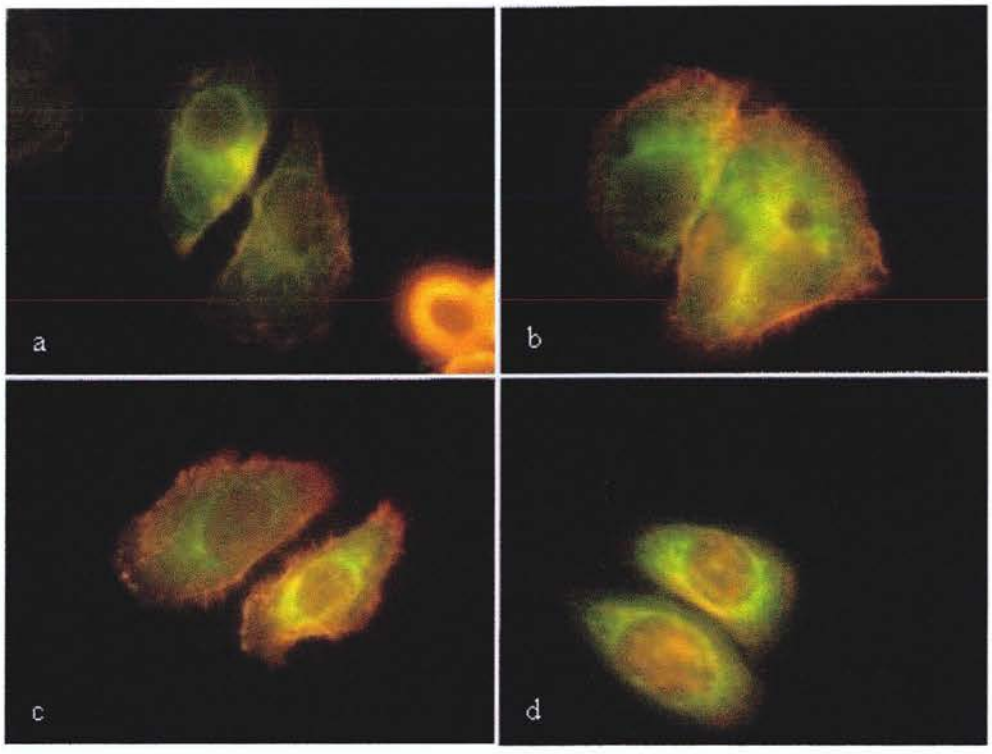


Figure 6.6 GFP-eEF1A1,F-actin and tubulin localisation in PEO1 cells.
(a-c) PEO1 cells transfected with GFP-eEF1A1 (green) and stained with Alexa Fluor 594 phalloidin to F-actin (red) and plated on glass coverslips. Many F-actin rich cellular projections can be seen in these cells. Partial co-localisation between eEF1A1 and F-actin can be seen both in the perinuclear region of the cell and in the cellular protrusions. (d) GFP-eEF1A1 (green) and anti-tubulin (red) in PEO1 cells. Some co-localisation can be observed in the perinuclear region of the cells. x100 magnification.

phenotype, should provide an insight into its function. As all cell lines I have tested that express eEF1A2 also express high levels of eEF1A1, knocking down eEF1A2 in these cells should not significantly reduce the global level of translation. Additionally, it is initiation and not elongation that is thought to be the rate limiting step of translation and eEF1A is in vast excess over other components of the translational machinery. Therefore we propose that knocking down eEF1A2 should not have an effect on the rate of translation. A table summarising the techniques and protocols I used for RNA interference can be found at the end of this chapter in table 6.4.

6.2.2.1 siRNA oligos for ablation of eEF1A2 expression in HeLa and MCF-7 cells

My initial approach to RNA interference (RNAi) of eEF1A2 expression was to use a pool of four siRNAs targeted to the eEF1A2 transcript, produced by Dharmacon, and transiently transfect them in HeLa cells. HeLa cells are derived from a cervical epithelial cancer, are easy to transfect and express both eEF1A1 and eEF1A2 at high levels. They are therefore suitable for this application. Preliminary analysis using these pooled oligos and oligofectamine as a transfection reagent suggested that the technique worked well. eEF1A2 levels, as determined by Western blot, were more than 70% lower in eEF1A2-targeted siRNA transfected cells at 24 and 48 hours post-transfection compared to untransfected and mock transfected cells, see figure 6.7. By 72 hours post-transfection the levels of eEF1A2 were returning to normal. Western blot analysis confirmed that the oligos did not knockdown eEF1A1 and were therefore specific to eEF1A2. I observed a decrease in cell number in eEF1A2 siRNA transfected cells compared to controls including cells transfected with non-targeting siRNA. Non-targeting siRNA was also obtained from Dharmacon and is a siRNA with no known sequence homology to human transcripts that acts as a control for off target effects of siRNA administration. As well as HeLa cells this technique was also applied to MCF-7 cells. MCF-7 is a human breast adenocarcinoma cell line and is therefore potentially a more relevant cell line for the investigation of the role of eEF1A2 in cancer as eEF1A2 is expressed in up to one third of breast cancers. Investigation of eEF1A2 function in ovarian carcinoma cell lines would also be

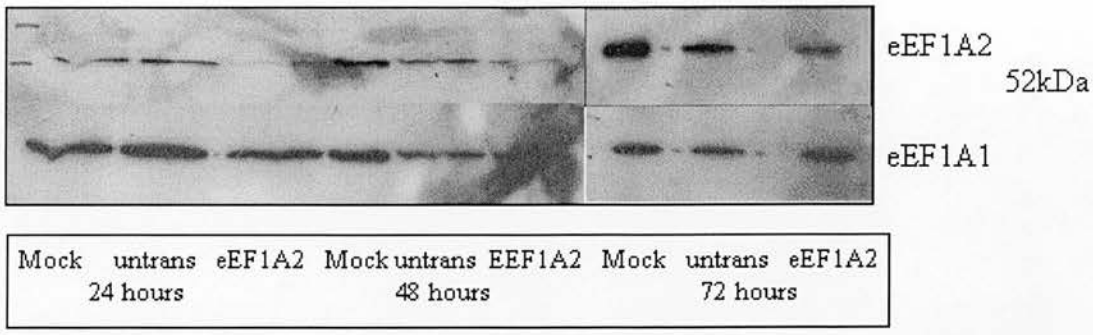


Figure 6.7 eEF1A2 knock down in HeLa cells using pooled siRNAs targeted to eEF1A2

Mock transfected (mock), untransfected (untrans) and cells transfected with siRNAs against eEF1A2 (eEF1A2) were pelleted at 24, 48 and 72 hours post-transfection and the protein analysed by Western blot. By 24 hours post-transfection eEF1A2 levels are negligible in cells treated with eEF1A2 siRNA. eEF1A1 levels are not knocked down showing that the siRNAs are specific to eEF1A2. By 72 hours the level of eEF1A2 is beginning to return to normal.

relevant. Quantification of cell number in subsequent experiments suggested that ablation of eEF1A2 expression in MCF-7 cells resulted in a decreased cell number, see figure 6.8. This suggested that a non-canonical function(s) performed by eEF1A2 may be essential to HeLa and MCF-7 cells in culture, on the assumption that global translation levels are not altered due to the presence of eEF1A1. It could be that MCF-7 expressing decreased amounts of eEF1A2 exhibit a decrease in the rate of proliferation compared to those expressing unaltered levels of eEF1A2 or that they are undergoing apoptosis. It is important to note that the cell count in cells transfected with non-targeting siRNA was variable and on occasion also lower than mock and untransfected cells suggesting that there is toxicity associated with the presence of siRNA in the cell.

Many attempts to replicate eEF1A2 knockdown using pooled siRNA gave very variable results. The ablation level of eEF1A2 expression was not consistently at or above 70% and was often low to zero. Optimisation to increase knockdown of eEF1A2 by alteration of oligo:oligofectamine ratio to 1:3, 1:6 and 1:9, as well as cell plating density from 3x 10^4 to 3.5x10^4 and 4x10^4 per well of a 24 well plate worked to a degree but was also inconsistent.

6.2.2.2 A decrease of eEF1A2 expression in MCF-7 cells does not alter the rate of proliferation of the cells

eEF1A1 has been found to associate with the mitotic apparatus of Sea Urchin eggs and injection of mAb to eEF1A1 blocks the formation of the mitotic spindle (Toriyama et al., 1988) (Ohta et al., 1990); providing evidence that eEF1A1 may have an important role in mitosis. If eEF1A2 has a role in mitosis in mammalian cells this could be important to its function as an oncogene. In rapidly proliferating cells there is also evidence of an upregulation of eEF1A mRNA levels, for instance in cells in culture (Sanders et al., 1992), embryos (Krieg et al., 1989) and in highly proliferating human cancers (Grant et al., 1992). The reason for this is unknown and somewhat puzzling as eEF1A is already found in vast molar excess of the other components of the translational apparatus in normally proliferating cells. However, evidence of overexpression of eEF1A in rapidly proliferating cells does suggest it

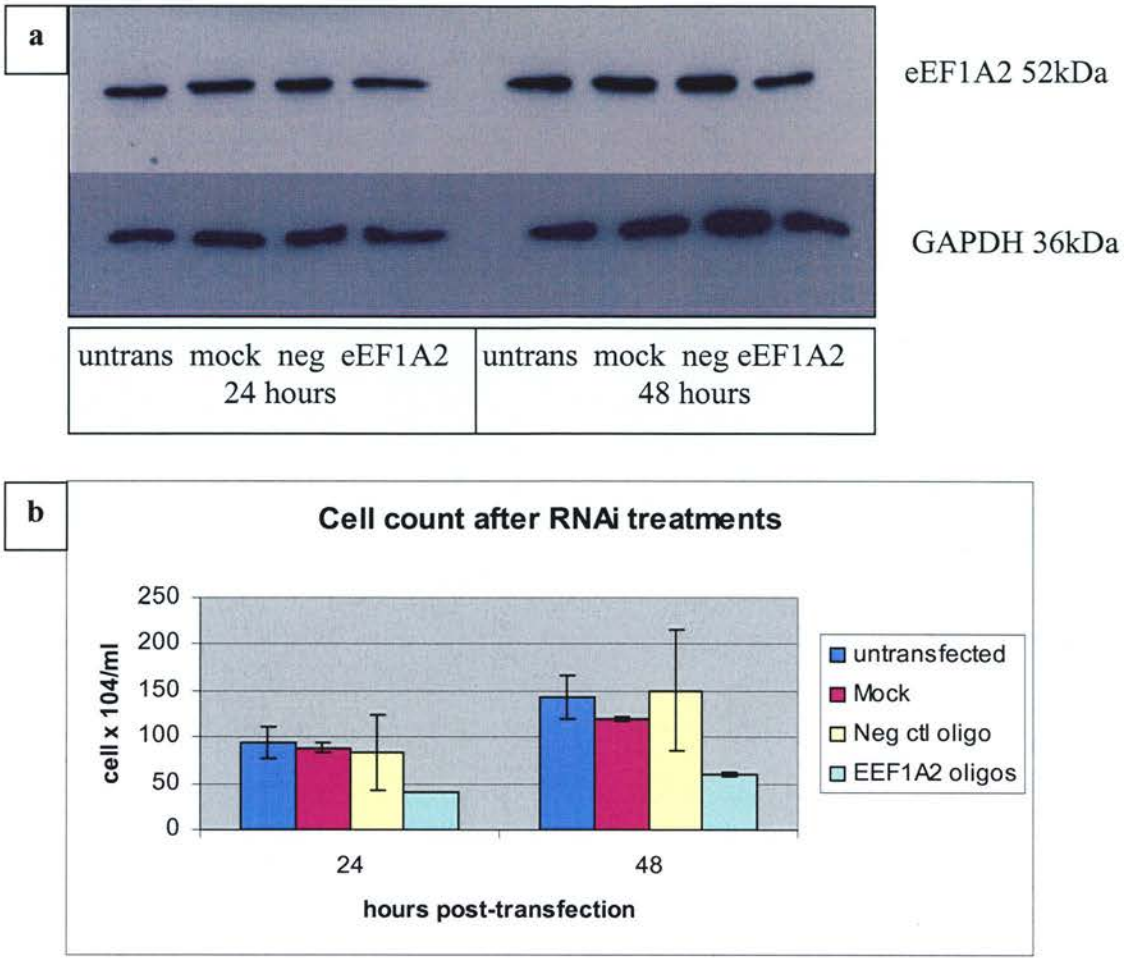


Figure 6.8 Partial eEF1A2 knockdown in MCF-7 cells using eEF1A2 siRNA, and cell counts showing a decrease in cell number in eEF1A2 knockdown cells. (a) Untransfected (untrans), mock transfected (mock), non-targeting oligo (neg), and eEF1A2 targeted siRNA (EEF1A2) treated cells were pelleted at 24 hours and 48 hours post transfection. Western blot analysis shows partial knock down of eEF1A2 at 24 and 48 hours. (b) Cell counts suggest that there is a decrease in cell number in MCF-7s treated with eEF1A2 siRNA compared to controls.

has a role in cell growth and proliferation. Additionally, eEF1A2 overexpression in NIH 3T3 cells decreases their doubling time suggesting that eEF1A2 expression may have a role in cell cycle regulation (Anand et al., 2002). To investigate whether eEF1A2 ablation by RNAi in cancer cells in culture leads to a concomitant decrease in proliferation I conducted RNAi of eEF1A2 in MCF-7 cells and measured their rate of proliferation using a BrdU (5-bromo-2'-deoxyuridine) assay. 5-bromo-2'-deoxyuridine is a thymidine analogue that, when added to the medium of cells in culture, is incorporated into the replicating DNA of cells in S phase. Using a FITC conjugated antibody against BrdU, cells that have incorporated the thymidine analogue into their DNA can be identified by immunofluorescence as having green nuclei. MCF-7 cells were first transfected with eEF1A2 pooled targeted oligos as well as the non-targeted oligo in duplicate and these cells were split between a well and glass coverslips. Controls included untransfected and mock transfected cells. Western blotting of protein lysates from these cells taken at 24, 30 and 48 hours after transfection showed that eEF1A2 levels were lower in cells transfected with EEF1A2-targeted siRNAs compared to controls. In particular at 48 hours post transfection the level of eEF1A2 in eEF1A2-siRNA treated cells was approximately 80% lower than that in controls, see figure 6.9. BrdU staining was carried out on coverslips at 24, 30 and 48 hours post transfection and nuclei were counter stained in DAPI, representative images can be seen in figure 6.10. The number of BrdU positive nuclei (green) and BrdU negative (DAPI positive) nuclei were counted and the results are shown in table 6.2 and figure 6.9. Two counts were performed on each duplicate of each treatment at each time point.

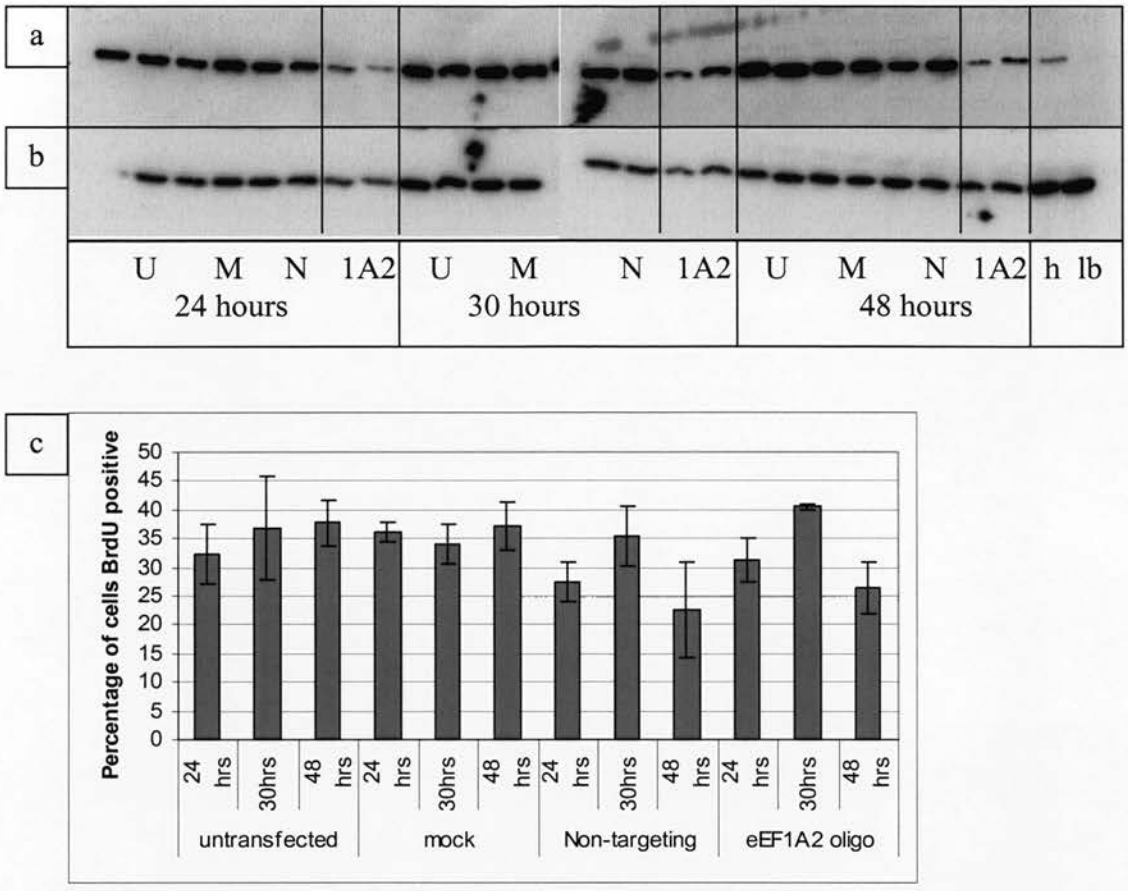


Figure 6.9 Western blot showing eEF1A2 levels in MCF-7 cells treated with pooled siRNA targeting eEF1A2 and the percentage of BrdU positive cells following RNAi treatment.

Western blot showing eEF1A2 protein levels in untransfected (U), mock transfected (M), non-targeting siRNA (N) treated and siRNA to eEF1A2 (1A2) treated MCF-7 cells in duplicate at 24, 30 and 48 hours post-transfection. (a) eEF1A2 levels are knocked down, particularly at 48 hours post transfection. (b) GAPDH has been used as a loading control. HeLa cell lysate (h) acts as a positive eEF1A2 antibody control and lymphoblastoid cells (lb) as a negative control. In boxes are duplicate protein lysates from cells transfected with siRNA targeted to eEF1A2. (c) The percentage of cells in S-phase as determined by BrdU analysis of MCF-7 cells following siRNA treatment and controls. The percentage of BrdU positive cells is not different in cells in which eEF1A2 has been knocked down compared to MCF-7 cells treated with non-targeting oligos.

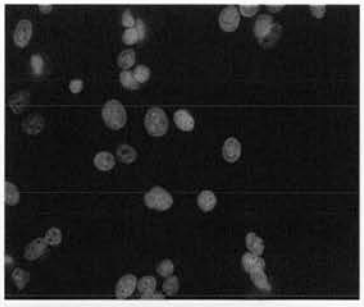
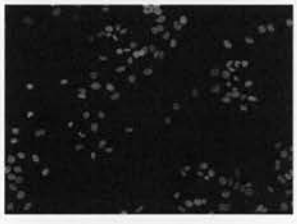
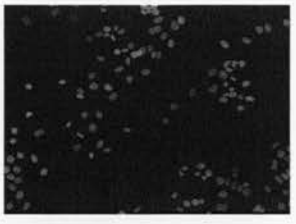


Figure 6.10 BrdU assay on MCF-7 cells treated with siRNA against eEF1A2 and controls.
Left: x 40 magnification image of BrdU stained nuclei (green) and DAPI stained nuclei (blue)
Below: x 20 magnification representative images of BrdU stained cells in untransfected, mock transfected, non-targeting siRNA treated and eEF1A2 siRNA treated MCF-7 cells 24, 30 and 48 hours post treatment

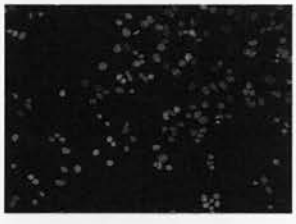


Untransfected 24 hours

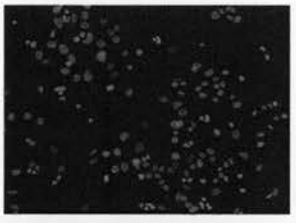


30 hours

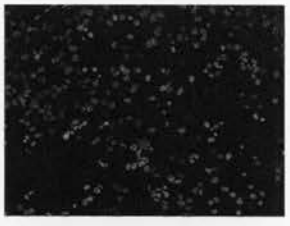
48 hours



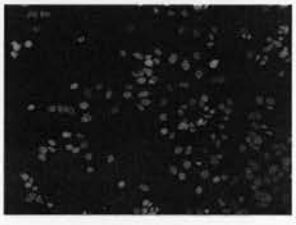
Mock 24 hours



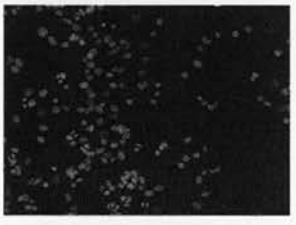
30 hours



48 hours



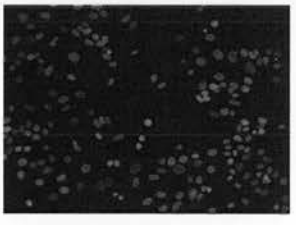
Non-targeted 24 hours



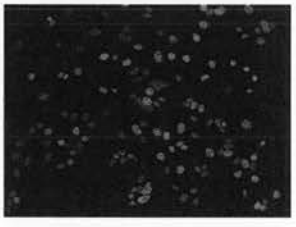
30 hours

48 hours

eEF1A2 siRNA 24 hours



30 hours



48 hours

Table 6.2 *BrdU assay on MCF-7 cells treated with siRNAs against eEF1A2.*

	Time post transfection	Total number of nuclei counted	Average BrdU negative nuclei	Average BrdU positive nuclei	% BrdU positive
Untransfected	24 hours	640	108.75	51.25	32.3
	30 hours	615	98	55.75	36.9
	48 hours	636	99	60	37.7
Mock	24 hours	614	98	55.5	36.1
	30 hours	557	91.5	47.75	34.0
	48 hours	959	151	88.75	37.2
Non-targeting oligo	24 hours	638	116.5	43	27.5
	30 hours	811	130.5	72.25	35.5
	48 hours	715	136.5	42.25	22.7
EEF1A2 targeting oligo	24 hours	471	80.75	37	31.3
	30 hours	676	100.5	68.5	40.5
	48 hours	623	115.25	40.5	26.4

Untransfected and mock transfected MCF-7 cells at 24, 30 and 48 hours after treatment had between 30-40% of cells in S phase or proliferating. MCF-7 cells transfected with non-targeting and eEF1A2-targeted siRNAs showed a similar pattern of proliferation to one another. At 24 and 48 hours approximately 20-30% of the cell population was in S phase and at 30 hrs post transfection 35-40% of the cells were proliferating. These results suggest that transfection of siRNA in MCF-7 cells may decrease their rate of proliferation. There appeared to be no significant difference in the number of proliferating cells in those treated with non-targeted siRNA or siRNA targeting eEF1A2 suggesting that eEF1A2 knockdown (that was particularly high at 48 hours post transfection) does not alter the percentage of cells that enter S phase of the cell cycle. The fact that eEF1A2 expression ablation is not complete might also lead to a lack of phenotypic change in these cells. There was no significant difference in the percentage of cells in S phase when subjected to the different RNA interference treatments ($p=0.25$, One-way repeated measures ANOVA).

An alternative more rapid method that could be used to assay cell growth or proliferation is an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This is a colorimetric assay in which yellow MTT is reduced to

purple formazan in the mitochrondria of living cells and the absorbance is measured at 500-600nm by a spectrophotometer.

The reasons for the variability in knockdown of eEF1A2 using siRNAs might be due to the RNA oligos degrading when in storage or during use. Advice from the manufacturer suggested that some pooled oligos can form a complex when frozen leading to destruction of the RNA. Due to the technique being unreliable I decided to use short hairpin RNAs targeted to the EEF1A2 transcript to ablate eEF1A2 expression instead.

6.2.2.3 Short hairpin (sh)RNAs induce an interferon response in MCF-7s

I designed three different shRNAs targeted to different regions of the eEF1A2 transcript that show low similarity to eEF1A1 in order to ensure specificity. T1B1 was designed to a region of exon 3, T2B2 to a region of exon 5, and T3B3 to a region of exon 6. I also designed a non-targeting oligo (NEGT1B1) that, by Blast searching of human nucleotide database in NCBI, I determined to have no significant homology to any human transcripts. These constructs were cloned into the Invitrogen BLOCK-iT U6 RNAi Entry Vector that allows the expression of shRNA in mammalian cells under the control of the human U6 promoter and subsequently sequence verified the clones. Transient transfection of the shRNA constructs into MCF-7 cells using oligofectamine as a transfection reagent resulted in high levels of cell death in both cells transfected with eEF1A2-targeted and non-targeting shRNAs. Despite their toxicity, constructs T1B1 and T3B3 appeared to be the most effective at ablating eEF1A2 expression as determined by Western blot, see figure 6.11. Stripping and reprobing the western blot with an antibody against eEF1A1 showed that the shRNAs are specific to eEF1A2.

In order to determine whether the toxicity was due to bacterial contaminants in the DNA plasmid maxipreps I produced new maxipreps using endotoxin-free protocol, however this did not decrease the toxicity of the shRNA vectors. dsRNA of less than 30 nucleotides in length is thought to be too short to trigger an interferon response in mammalian cells (Elbashir et al., 2001). However it has been observed that some

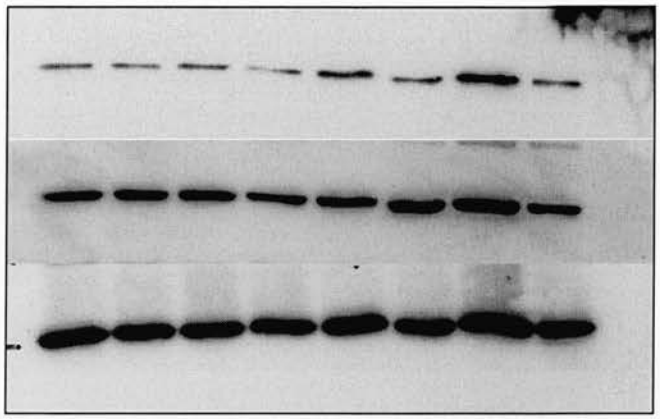


Figure 6.11 RNAi of eEF1A2 using three different shRNA constructs targeted to eEF1A2.

RNAi in MCF-7 cells using shRNA constructs. Protein lysates from untransfected (U) MCF-7 cells, and MCF-7 cells treated with the three eEF1A2 shRNAs T1B1, T2B2 and T3B3 were analysed for eEF1A2 and eEF1A1 expression by Western blotting. At 48 hours post transfection shRNAs T1B1 and T3B3 are effective at ablating eEF1A2 expression. T2B2 does not appear to be functional. T1B1 and T3B3 are specific to eEF1A2, shown by the fact that eEF1A1 levels are not altered in cells treated with these constructs. GAPDH has been used as a loading control.

shRNA vectors are capable of triggering the expression of interferons in mammalian cells. Bridge *et al.* observed that transfection of human lung fibroblasts with shRNAs cloned into lentiviral vectors as well as pSUPER-based plasmid vectors resulted in the induction of many interferon target genes including 2'5'oligoadenylate synthetase (OAS1), a classic interferon target gene. Importantly, Bridge *et al.* did not see an induction of the interferon response in these cells when transfected with chemically synthesised siRNA duplexes corresponding to the shRNAs (Bridge et al., 2003). I set about determining whether the shRNA vectors used in my system were inducing an interferon response in the MCF-7 cells. Firstly, I transfected the cells with 0.1, 0.2, 0.4 and 0.8μgs of eEF1A2-targeted (T1B1) and non-targeting (NEGT1B1) shRNA plasmids and subsequently pelleted the cells for RNA extraction at 24, 48 and 72 hours following transfection. I also quantified the toxicity using a trypan blue dye exclusion assay, results of which can be seen in figure 6.12. Transfection of small amounts of T1B1 and NEGT1B1 plasmids (0.1μg and 0.2 μg) did appear to reduce the toxicity compared to 0.6 μg and 0.8 μg of the plasmids. To determine whether the interferon response has been induced in these cells I conducted quantitative RT-PCR to analyse the levels of OAS1 in cells transfected with T1B1 and JLTB at 24 and 48 hours post-transfection and compared these to levels in untransfected and mock transfected cells. It can be clearly seen in figure 6.12 that by 48 hours post-transfection shRNA plasmids have induced a large up-regulation in the levels of OAS1 (up to 800 times higher than the level in untransfected MCF-7 cells), particularly at higher plasmid concentrations. The eEF1A2 targeted shRNA T1B1 appears to induce higher upregulation of OAS1 compared to the non-targeted shRNA.

In order to attempt to overcome the induction of the interferon response I also used the interferon response-defective cell line HCT 116 (Stojdl et al., 2000). This should have overcome the toxicity of the shRNA as the cells are unable to mount an effective interferon response. Unfortunately eEF1A2 was not knocked down in these cells and I turned to a new technique that could potentially not only overcome the interferon response but that would also allow me to use a more relevant cell line.

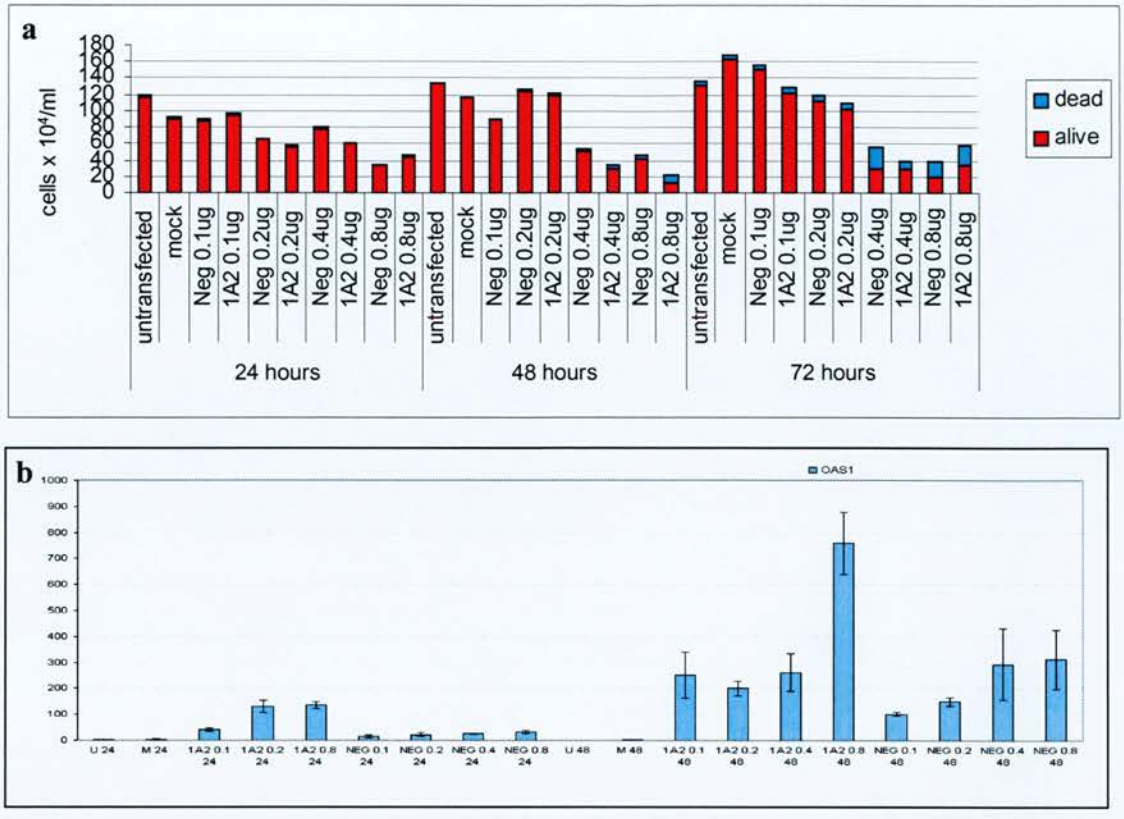


Figure 6.12 Cell viability and expression of OAS1 in cells transfected with shRNA vectors T1B1 and NEGT1B1 using Oligofectamine.

(a) A trypan blue dye exclusion assay was used to assess cell viability in untransfected, mock transfected and 0.1-0.8μg of shRNA plasmid transfected MCF-7 cells, 24, 48 and 72 hours post transfection. Toxicity is particularly apparent in cells transfected with 0.4μg and 0.8μg of plasmid. (b) Quantitative RT-PCR analysis of the levels of OAS1 normalised to GAPDH and standardised to the levels in untransfected cells 24 hours post-plating shows that OAS1 is upregulated in cells transfected with shRNA constructs, particularly by 48 hours post-transfection. OAS1 levels appear highest in cells transfected with the eEF1A2 targeting shRNA construct T1B1.

6.2.2.4 Transfection of shRNAs using a Nucleofector does not induce an interferon response in MCF-7 cells

Nucleofection is an alternative transfection technique that combines electroporation using the Nucleofector with a transfection solution (Amaxa Biosystems). The electroporation settings and transfection solution that give optimal transfection are specific to the cell line and this technique results in the plasmid being delivered directly to the nucleus with high efficiency (up to 80-90% transfection). It is known that long dsRNA within the cytoplasm of mammalian cells trigger the interferon response thereby using the nucleofector system, which targets the plasmid directly to the nucleus, may circumvent the response.

Transfecting the BLOCK-iT RNAi entry vector shRNA clones T1B1 and T3B3, as well as an additional eEF1A2-targeting shRNA JLTB taken from (Kulkarni et al., 2006) by nucleofection into MCF-7 cells resulted in high transfection efficiencies (as measured using a Green Fluorescent Protein vector control) and in many cases a substantial knockdown of eEF1A2 levels, see Figure 6.13. Importantly, there was no evidence of substantial toxicity in cells transfected with any of the shRNA constructs (above which you would expect to see due to nucleofection), suggesting that nucleofection does indeed avoid the triggering of the interferon response. However this technique, as with the pooled siRNA oligos, gave inconsistent results in the degree of eEF1A2 ablation obtained between assays and did not work at all on occasion despite the high transfection efficiencies obtained using nucleofection. This inconsistency meant assaying a phenotype in eEF1A2-knockdown cells was difficult.

6.2.2.5 Partial eEF1A2 ablation in MCF-7 cells does not alter their cell cycle distribution or increase the number of cells in apoptosis

To investigate whether eEF1A2 ablation by RNAi in cancer cells in culture leads to a concomitant change in cell cycle distribution and apoptosis I first conducted RNAi in MCF-7 cells using constructs T1B1 and JLTB which both target eEF1A2, as well as the non-targeting shRNA construct NEGT1B1, in duplicate. Additional controls included untransfected and mock transfected MCF-7 cells. MCF-7s were transfected using a nucleofector and plated in 6-well plates; analysis of transfection levels of a

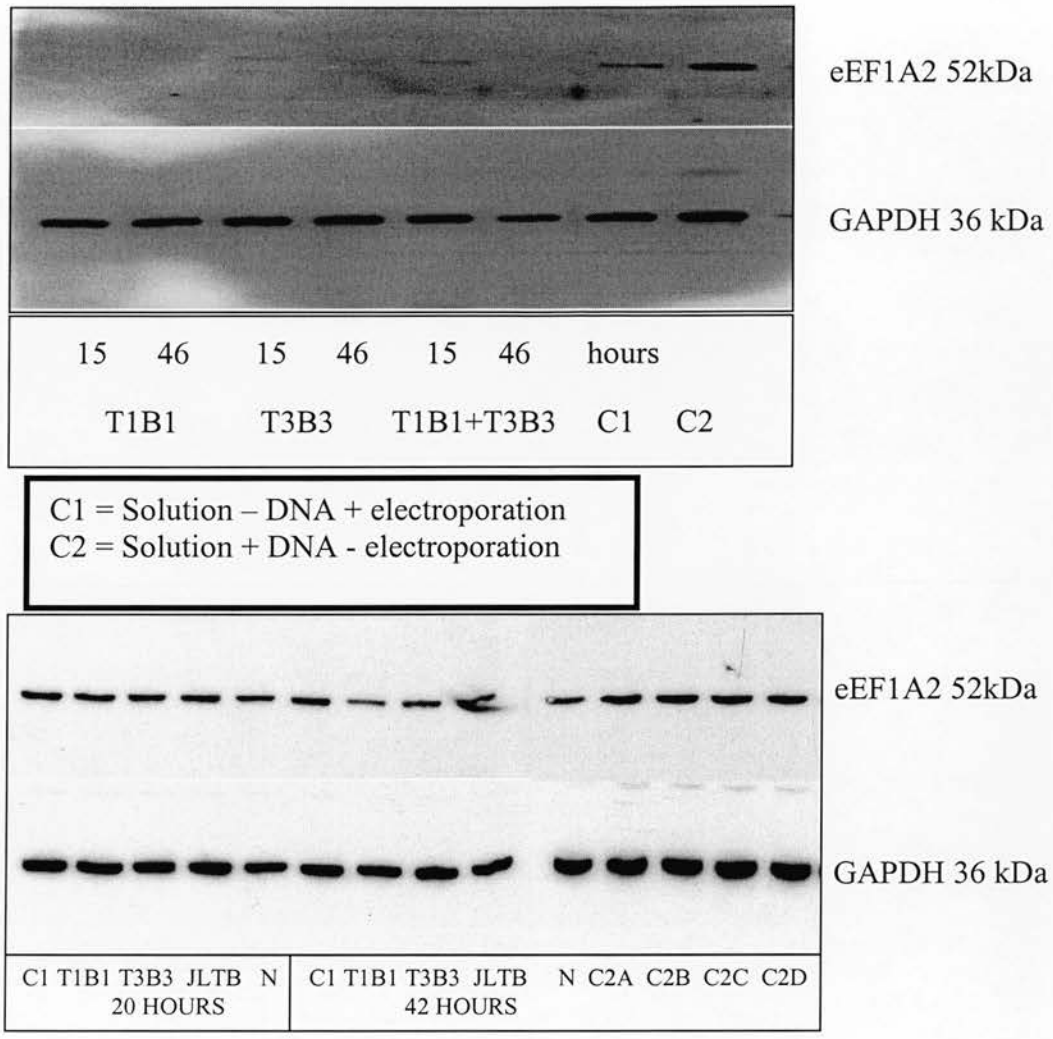


Figure 6.13 Western blots showing knockdown of eEF1A2 in MCF-7 cells using shRNAs T1B1, T3B3 and JLTB transfected by nucleofection.
Top: Knockdown of eEF1A2 is substantial in MCF-7 cells transfected with shRNAs T1B1, T3B3 and co-transfected with both constructs at 15 and 46 hours post transfection. C1 is a control in which cells are mock transfected and C2 is a control in which cells are not transfected but incubated with DNA.
Bottom: Western blot showing eEF1A2 levels in MCF-7 cells transfected with T1B1, T3B3 and JLTB at 20 hours and 42 hours post transfection. Knockdown of eEF1A2 appears partially successful at 42 hours post transfection. N denotes protein lysate from cells transfected with the non-targeting shRNA NEGT1B1. C2A-D are DNA only controls where A is T1B1, B is T3B3, C is JLTB and D is NEGT1B1.

GFP control plasmid by Fluorescent Activated Cell Sorting (FACS) suggested the transfection efficiency was at 80%. Analysis of eEF1A2 protein levels by Western blot showed that eEF1A2 knockdown had occurred in cells transfected with T1B1 and JLTB, figure 6.14. There was an obvious difference in the level of knockdown between duplicates highlighting the inconsistency of the shRNA technique. It appears that at the most, eEF1A2 is at 40-50% of the level in controls in eEF1A2 shRNA transfected cells.

In order to analyse the cell cycle distribution of these cells and to look for any indication of apoptosis I used propidium idodide staining of the cellular DNA and FACS analysis. Propidium iodide intercalates in the major groove of DNA producing a highly fluorescent adduct. Analysis by FACS allows the distinction of cells in cell cycle stages G2/M, S, G1 as well as those in apoptosis, by their DNA content. Cells in G1 have a uniform diploid DNA content and therefore appear as a sharp peak of fluorescence at the lower end of the DNA content scale. Cells in G2/M have twice the normal cell content (4n) and the mean DNA content of this peak is approximately twice that of the G1 peak. Cells in S phase have a range of DNA contents from just above that in G1 to just below that in G2/M and therefore appear as a broader population on the FACS histogram. Cells in the sub G1 population (DNA content less than diploid) have fragmented DNA and therefore represent the apoptotic or necrotic fraction of the cell population. FACS generated histograms showing the DNA content in the experimental cell populations can be seen in figure 6.15. FACS analysis of the cell cycle distribution was more accurately quantified using Multicycle AV, an advanced cell cycle analysis software program from Phoenix Flow Systems. The percentage of cells in G1, S and G2/M as well as apoptosis/necrosis is represented in table 6.3.

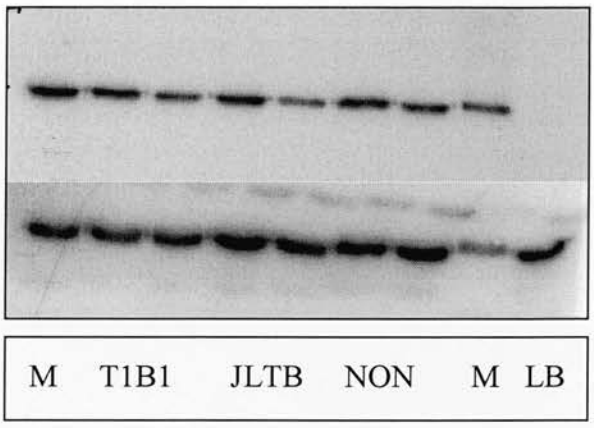


Figure 6.14 eEF1A2 protein levels in MCF-7 cells treated with shRNA constructs targeted to eEF1A2.
MCF-7 cells were mock transfected (M) and transfected with shRNA constructs against eEF1A2 (T1B1 and JLTB) and non-targeting shRNA (NON) by nucleofection and protein samples collected at 48 hours post transfection. eEF1A2 levels have been partially ablated. Protein loading is even as shown by GAPDH quantification. MCF-7 (M) is a positive antibody control and lymphoblastoid (LB) is a negative control.

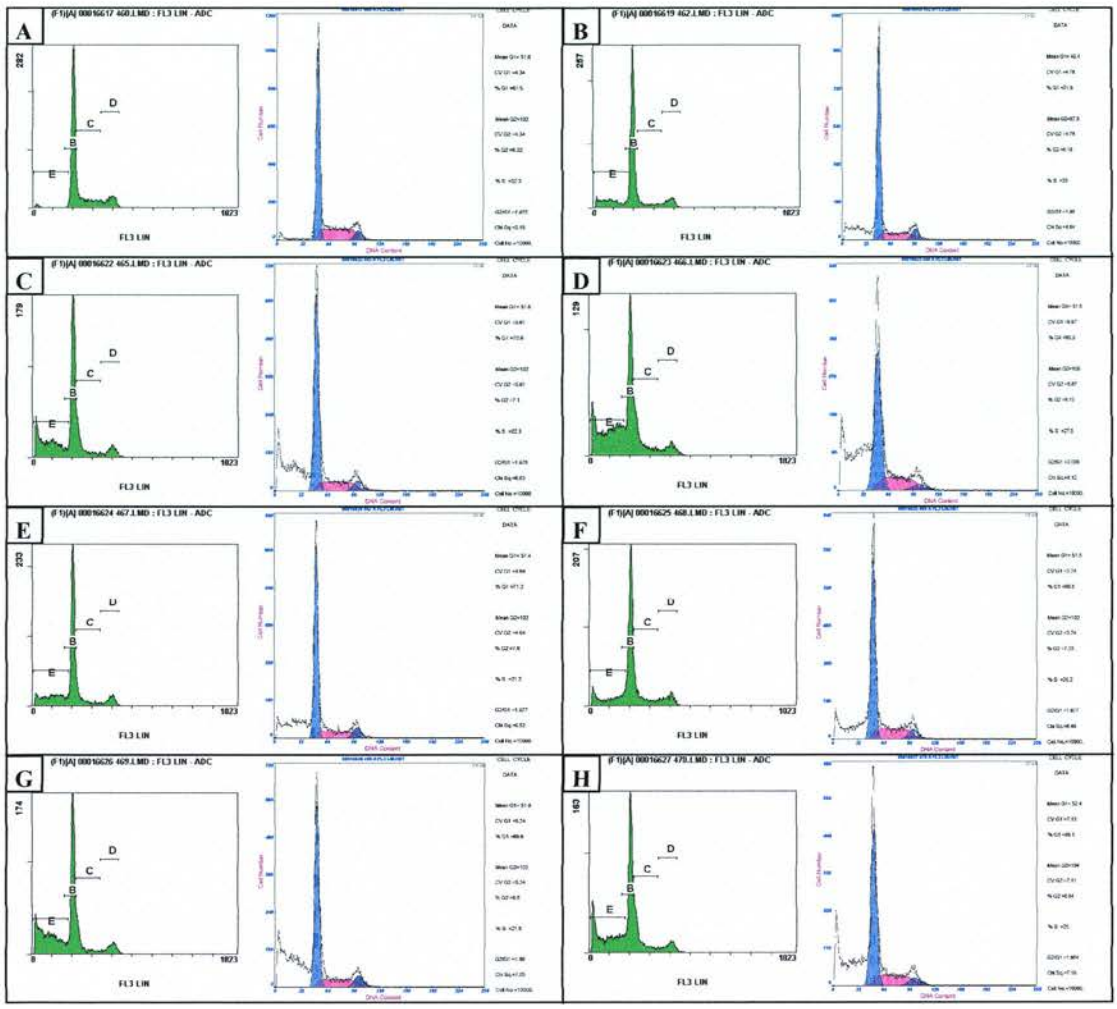


Figure 6.15 Analysis of the cell cycle distribution and apoptosis in MCF-7 cells treated with eEF1A2 targeted shRNA, using propidium iodide staining.
Histograms produced by FACS analysis of cells stained with propidium iodide are shown on the left hand side of each figure. Fraction E are apoptotic/necrotic cells, fraction B are cells in G1, C denotes cells in S phase and D are cells in G2/M. Results from accurate quantification of cell cycle distribution using advanced analysis software are shown on the right hand side of each figure. (A) untransfected MCF-7 cells, (B) Mock transfected, (C) T1B1 eEF1A2 shRNA transfected cells duplicate a and (D) duplicate b, (E) JLTB eEF1A2 shRNA transfected cells duplicate a and (F) duplicate b, (G) non-targeting shRNA transfected cells duplicate a and (H) duplicate b.

Table 6.3 *Cell cycle distribution of RNAi treated MCF-7 cells, (a) and (b) refer to duplicates 1 and 2 of each treatment.*

MCF-7 treatment	Apx % knockdown of eEF1A2	% of cells in G1	% of cells in S phase	% of cells in G2/M	% of cells in apoptosis
Untransfected	0%	58.6	30.7	6.0	4.69
Mock transfected	0%	55.2	15.4	6.3	23.0
T1B1 a	30%	47.3	15.0	4.8	32.8
T1B1 b	50%	40.3	17.0	3.8	38.4
JLTB a	0%	51.9	15.5	5.5	27.0
JLTB b	60%	51.6	20.3	5..7	22.4
NEGT1B1 a	0%	43.1	13.3	5.4	38.1
NEGT1B1 b	0%	45.1	16.6	4.5	33.7

There does not appear to be a significant difference in cell cycle distribution between cells in which eEF1A2 has been knocked down by more than 50% (T1B1b, JLTBb) compared to those in which transfection of eEF1A2-targeting shRNAs has been unsuccessful in knocking down the protein (T1B1a and JLTBb). This suggests that either a decrease in eEF1A2 levels in MCF-7 cells has no effect on their cell cycle distribution or that the level of eEF1A2 knockdown is not high enough to produce a phenotype. Also, it appears that transient transfection of MCF-7 cells using the nucleofector has an effect on cell cycle distribution in MCF-7 cells; decreasing the percentage of cells in S phase and increasing the number of apoptotic cells compared to untransfected MCF-7s. The level of apoptosis in cells transfected with shRNA is approximately 10% higher than that in the mock transfected cell population suggesting that there is some toxicity associated with shRNA expression or the DNA plasmid preparations. Importantly, the JLTB construct appeared less toxic than T1B1 or NEGT1B1 and the reason for this is unknown but could be due to there being less bacterial contamination in the JLTB plasmid preparation compared to the others. In conclusion this analysis is of limited value as transient transfection

appears to have a greater effect on cycle distribution and cell death than partial knockdown of eEF1A2. This may also be the case in some assays of other phenotypes suggesting that transient transfection of shRNAs is not a powerful tool for discerning the non-canonical functions of eEF1A2. Perhaps more useful would be a stable cell line in which shRNA against eEF1A2 is constitutively expressed. Both viral and non-viral vectors are available for this application, however due to time constraints I was unable to utilise this technique.

6.2.3 The role of eEF1A1 and eEF1A2 in heat shock

It was suggested by Shamovsky *et al.* that eEF1A was necessary for HSF1 activation and hence the heat shock response in HeLa cells (Shamovsky et al., 2006). However, the techniques used meant that it was not apparent if eEF1A2 is also involved in HSF1 activation. In order to investigate the potential role of eEF1A1 and eEF1A2 in heat shock I used RNA interference in HeLa cells to ablate the expression of eEF1A1 and eEF1A2 and subsequently heat shocked the cells and assayed for the stress-induced Hsp70 (Hsp72) expression by Western blot. Heat shock protein 70 (Hsp70) family members include: Hsc70 or Hsp73, a constitutively expressed chaperone protein; Hsp70 or Hsp72, a stress-induced protein; BiP or Grp78, that is localised to the endoplasmic reticulum where it is involved in protein folding and can be upregulated in response to stress; and mtHsp70 or Grp75, a mitochondrial Hsp70 (Mayer and Bukau, 2005). In order to assay for the induction of the heat shock response I used an antibody against the stress-induced heat shock protein Hsp70 or Hsp72 (Stressgen).

To ablate the expression of eEF1A1 or eEF1A2 I used pre-designed siRNAs (Ambion). Three siRNAs targeted to eEF1A1 and three siRNAs targeted to eEF1A2 were available and tested in HeLa cells for efficacy. Two of the three sets of siRNAs were very effective at knocking down eEF1A1 and the same was found with the siRNAs against eEF1A2. This is shown in figure 6.16. eEF1A1 has been knocked down to approximately 10% of the level seen in untransfected cells. eEF1A2 knock down by two of the oligos has resulted in almost complete ablation of eEF1A2

expression in the HeLa cells. Therefore these oligos are very effective at knocking down eEF1A1 and eEF1A2.

I then went on to repeat this experiment but to look at the expression of Hsp70 in heat-shocked HeLa cells in which eEF1A1 or eEF1A2 expression had been ablated. Hsp70 expression should be upregulated following heat shock, as described by (Shamovsky et al., 2006). I first tested HeLa cells for the upregulation of Hsp70 by heat shocking them for 1 hour at 40°C or 42°C in a waterbath and allowing 2, 7 or 24 hours post-heat shock for protein production. Control cells were treated in the same manner but kept at 37°C. Protein analysis by Western blot showed up regulation of Hsp70 in heat-shocked HeLa cells compared to controls, see figure 6.16. There was however some expression of Hsp70 in HeLa cells incubated at 37°C.

I then went on to conduct RNAi against eEF1A1 or eEF1A2 in HeLa cells and heat shocked the cells for one hour at 42°C and allowed two hours for protein synthesis before pelleting the cells. The protein lysate from these cells was then run on a Western blot and analysed for the expression of eEF1A1 or eEF1A2, Hsp70 and GAPDH (loading control). The results of this analysis can be seen in figure 6.17. It appears that in HeLa cells with approximately 80% knockdown of eEF1A1 expression and no alteration in eEF1A2 (Western blot not shown), an increase in Hsp70 is still produced following heat shock suggesting that HSF1 activation is still able to proceed. Similarly, in HeLa cells in which the expression of eEF1A2 has been completely ablated but eEF1A1 levels remain unaltered, Hsp70 expression still appears to be upregulated in response to heat-shock however this upregulation is less apparent and was therefore quantified using image analysis software, see figure 6.18.

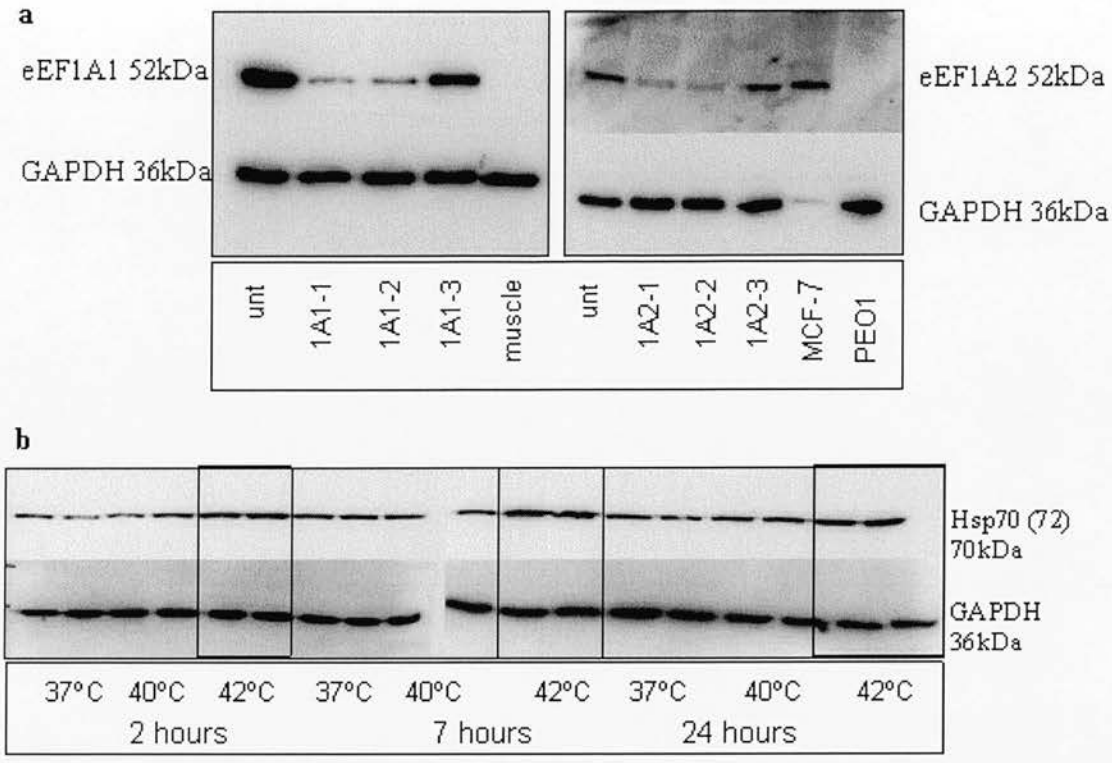


Figure 6.16 Western blot analysis of eEF1A1 and eEF1A2 knockdown using the Ambion pre-designed siRNAs and Hsp70 (72) levels in heat shocked HeLa cells compared to non-heat shocked.

(a) Left: levels of eEF1A1 expression in cells treated with siRNAs 1A1-1 and 1A1-2, but not 1A1-3, is approximately 10% of that in untreated cells. Wild-type mouse muscle (muscle) is a negative eEF1A1 control. Right: eEF1A2 expression levels have been almost completely ablated in HeLa cells treated with siRNAs 1A2-1 and 1A2-2 but not 1A2-3. MCF-7 is a positive eEF1A2 control and PEO1 is a negative eEF1A2 antibody control. (b) HeLa cells were plated in small flasks and the flasks incubated at either 37°C, 40°C or 42°C for one hour. The cells were then allowed to recover for 2 hours, 7 hours or 24 hours, after which time the cells were pelleted for Western blot analysis. Each treatment was carried out in duplicate. Levels of inducible Hsp70 (Hsp72) were analysed using a Hsp70 specific antibody. GAPDH was used as a loading control. It is apparent that levels of Hsp72 increase upon heat shock, the highest increase is seen when the cells are incubated at 42°C. Boxes are shown around duplicates of heat shocked cell lysates.

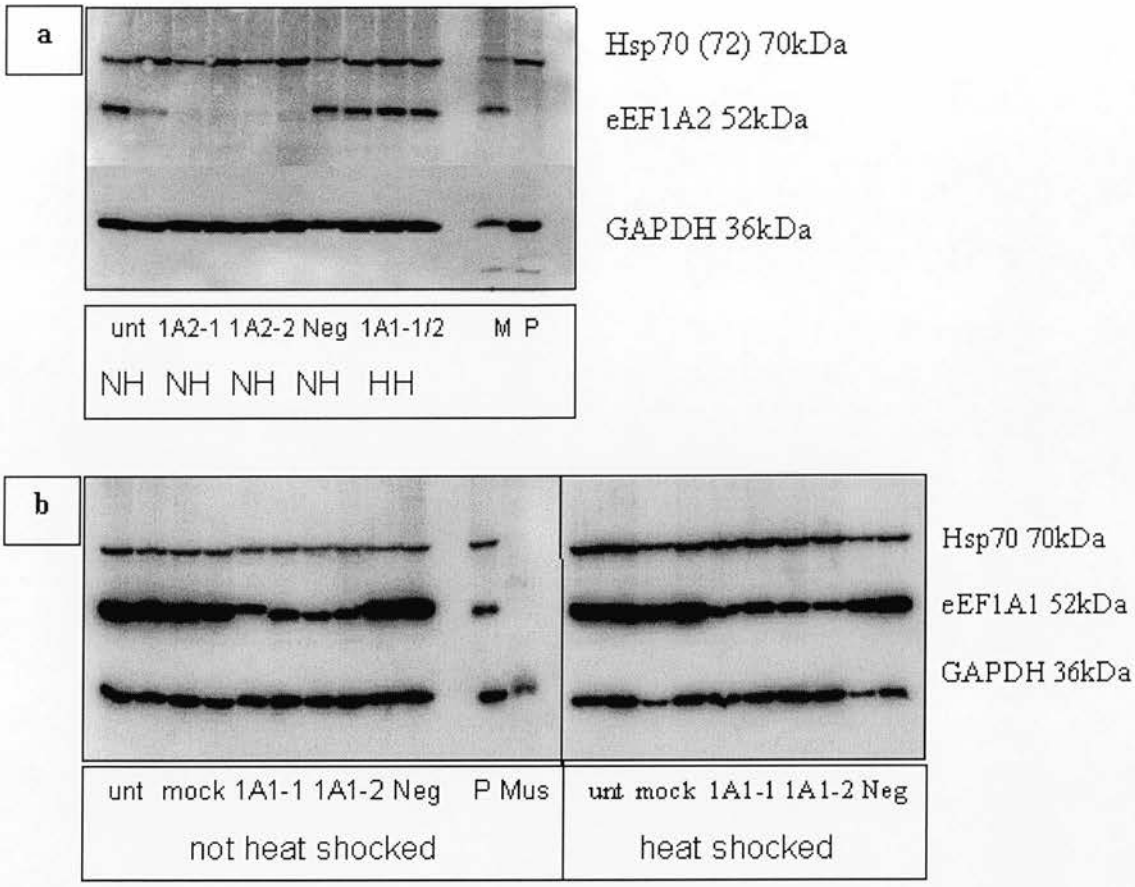


Figure 6.17 Western blot analysis of Hsp70(72) expression levels following RNA interference of eEF1A1 or eEF1A2 and heat shock.
(a) HeLa cells were either untransfected (unt), or transfected with siRNA 1A2-1 or 1A2-2, a negative control siRNA (Neg) and either heat shocked at 42°C (H) or incubated at 37°C (N). Cells in which eEF1A1 has been knocked down (1A1-1 and 1A1-2) are included to show that the 1A1-1 and 1A1-2 siRNAs are specific to eEF1A1. MCF-7 (M) and PEO1 (P) are positive and negative eEF1A2 antibody controls respectively. The increase in Hsp70 expression is still apparent in heat shocked HeLa cells despite the complete absence of eEF1A2 expression, suggesting eEF1A2 is not necessary for HSF1 activation. (b) HeLa cells were untransfected (unt), mock transfected (mock), transfected with two siRNAs targeting eEF1A1 (1A1-1 and 1A1-2) or treated with a non-targeting siRNA (Neg). PEO1 (P) and mouse muscle (mus) are positive and negative eEF1A1 antibody controls. It is apparent that in cells in which eEF1A1 levels have been substantially reduced Hsp70 upregulation in response to hest shock is still occurring. The presence of Hsp70 in HeLa cells incubated at 37°C suggests that the anti-Hsp70 antibody is non-specific or that treatment with siRNA elicits the stress response in these cells, albeit at a lower level.

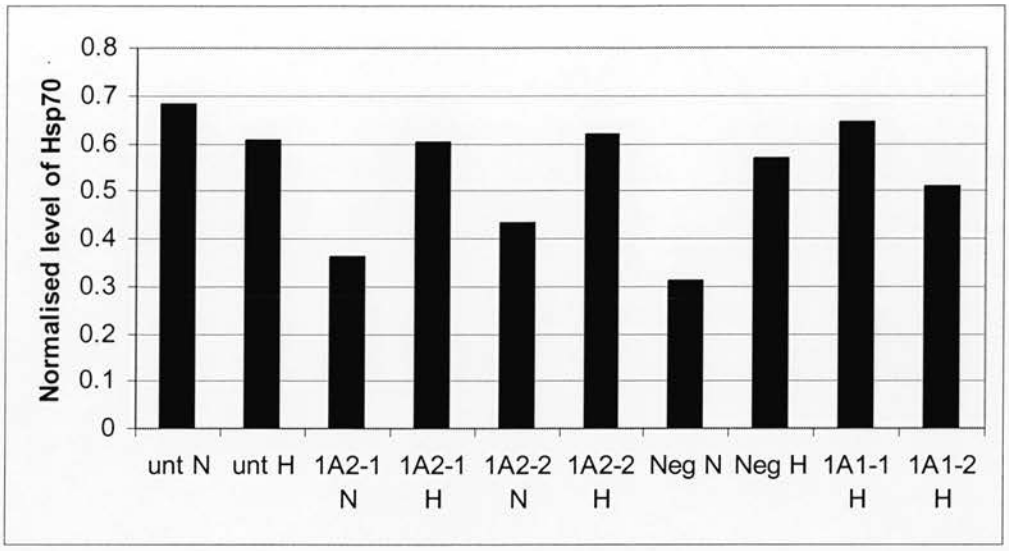


Figure 6.18 Expression levels of Hsp70 normalised to GAPDH.
Levels of Hsp70 protein present in the Western blot shown in figure 6.17a were quantified and normalised to the loading control GAPDH using image analysis software. It is apparent that in HeLa cells in which eEF1A2 expression has been completely ablated (1A2-1 and 1A2-2) there is still an increase in Hsp70 levels in response to heat shock (H) compared to non-heat shocked cells (N).

Table 6.4 *Methods used for RNAi of eEF1A2*

siRNA or shRNA	Cell line	Transfection method	Optimisation	Assays performed	Result from assay?	Highest level of knockdown	Problems
Dharmacon pooled eEF1A2 siRNAs	Hela	Oligofectamine	Cell density RNAse-free technique Altering size of master mix Multiple time points	Western Blot Trypan blue cell viability assay	Cell number in eEF1A2 knockdown cells lower than in controls	70% at 48 hours post-transfection	Oligos did not work consistently
Dharmacon pooled siRNAs	MCF-7	Oligofectamine	Cell density Ratio and amount of oligo and oligofectamine	Western blot Semi-quantitative RT-PCR		60% at protein level 80% at RNA level	Oligos worked inconsistently
3 shRNA constructs targeted to eEF1A2 Invitrogen Block-IT kit U6 RNAi entry vector	MCF-7	Lipofectamine	Cell density Ratio and amount of oligo and lipofectamine Endotoxin-free maxipreps Double transfections	Western blot Cell count BrdU assay Real-time RT-PCR	High amount of cell death including non-targeting shRNA	50% at protein level Constructs 1 + 3 effective	Cell death due to induction of an Interferon response in cells
Re-ordered Dharmacon pooled eEF1A2 siRNA oligos	MCF-7	Oligofectamine siLENTfect	Cell density Ratio and amount of oligo and transfection reagent	Western blot BrdU assay Cell cycle analysis	No difference in BrdU No difference in cell cycle	70%	Knockdown not high when assays performed Inconsistent knockdown

Block-IT U6 RNAi shRNA constructs 1 + 3	HCT116 defective for interferon response	Oligofectamine	Used optimised protocol as determined previously	Western blot	Observed less cell death	0%	Knockdown did not work
Block-IT U6 RNAi shRNA constructs 1 + 3	MCF-7	Nucleofector to overcome interferon response	Electroporation programme – High efficiency P-20 worked better than high viability E-14 Co-transfection of both constructs Transfection efficiency consistently around 60-80%	Western Blot		70-90% at the protein level	Worked inconsistently
Cloned an additional shRNA – JJLTB	MCF-7	Nucleofector programme P-20	Time points after transfection	Western blot		80% western blot – more effective than other shRNAs	
shRNAs 1 and JLTB	MCF-7	Nucleofector programme P-20		Western blot BrdU assay Cell cycle analysis Heat shock protein HSP70 activation	Cell cycle analysis no different HSP70 antibody non-specific	Knockdown up to 70% at protein level	Inconsistent knockdown of eEF1A2 despite high transfection efficiency

shRNAs 1 and JLTB	HeLa	Nucleofector P-20		Western blot	HSP70 antibody shows increased specificity	60%	Inconsistent knockdown of eEF1A2 despite high transfection efficiency
shRNAs 1 and JLTB	MCF-7	Nucleofector P-20 high efficiency – 80%		Western blot cell cycle analysis	No difference between eEF1A2 knockdown and controls	60%	Knockdown may not be sufficiently high to see a phenotype
Cloned shRNA 1 and JLTB into RNAi vector for stable cell line construction							Insufficient time available to complete
Ambion pre-designed siRNA oligos to eEF1A2 and eEF1A1	HeLa	Oligofectamine		Western blot	Oligos that work: 1A2-1 and 2 and 1A1-1 and 2	Levels of knockdown: up to 100% at 48 hours	

6.4 Discussion

Immunofluorescent analysis of eEF1A2 and eEF1A1 subcellular localisation in MCF-7 and PEO1 cells using tagged eEF1A2/eEF1A1 constructs suggests that both isoforms have a diffuse cytoplasmic localisation with a concentration in the perinuclear region of the cell and, in some cases, localisation to F-actin rich cellular protrusions. The localisation of eEF1A in the cytoplasm and perinuclear region fits with its role in translation, where eEF1A may be interacting with ribosomes located on the endoplasmic reticulum or cytoskeletal bound polysomes. In addition to this perinuclear localisation being involved in its role in translation, elongation factor 1A has been implicated in mRNA localisation. Elongation factor 1A was identified as the major component of a complex bound to the 3'UTR of Metallothionein-1 (MT-1), a region that has been identified as important to the perinuclear localisation of the mRNA. Mickleburgh *et al.* hypothesised that eEF1A tethered MT-1 mRNA to the perinuclear cytoskeleton, this being important for subsequent nuclear import of MT-1 protein where it performs roles in G1/S transition and DNA damage (Mickleburgh et al., 2006). Whether this role is performed by eEF1A1 and eEF1A2 or just eEF1A1 is not clear but I have demonstrated that both eEF1A2 and eEF1A1 co-localise with tubulin in the perinuclear region of MCF-7 cells.

Elongation factors 1A2 and 1A1 also show partial co-localisation with F-actin at cellular protrusions in some cells. These cellular protrusions are F-actin rich and have the appearance of fillopodia. Lamellipodia, filopodia and membrane ruffles are involved in cell motility, organisation of membrane domains, phagocytosis and substrate adhesion. Filopodia are composed of actin bundles that extend beyond the edge of the broader flatter lamellipodium and these appear to be present in some of the MCF-7 and PEO1 cells observed. Many proteins have been shown to be important in actin bundling in filopodia including fascin and fimbrin, for a review of lamellipodia see (Small et al., 2002). In cell migration the initial step involves the protrusion of the cell membrane driven by localised actin polymerisation. This can be in response to chemotactic stimuli, such as epidermal growth factor (EGF). Carcinoma cells are thought to move along the extracellular matrix (ECM) by extending pseudopods at the leading edge. These pseudopods are functionally

equivalent to lamellipodia that are observed in cells migrating in 2 dimensional culture experiments. These pseudopods attach to collagen containing fibres through adhesion structures called focal complexes. When migrating through dense extracellular matrix invasive cancer cells extend protrusions with the ability to degrade and remodel the ECM. These actin rich protrusions capable of protein degradative activity have been termed invadopodia and are particularly important in the invasion of cancer cells into blood vessels, for review see (Yamaguchi et al., 2005). Therefore these actin-rich cellular protrusions are pivotal to the invasion and metastasis of carcinomas.

eEF1A has been demonstrated to bind and bundle actin in yeast and, by immunofluorescence, to be found in filopodia and other cortical regions containing actin bundles (Yang et al., 1990). eEF1A2 may also bind and bundle actin in mammalian cells as suggested by its co-localisation with F-actin in what appear to be filopodia. Further experimental analysis would be required to determine whether these actin-rich cellular protrusions are in fact filopodia. For instance as well as F-actin other markers to filopodia include Fascin and VASP, and co-staining of eEF1A2 with these additional markers, could confirm or otherwise the identity of the protrusions. Interestingly in a yeast-two hybrid screen of a mouse brain cDNA library using a GAL4-eEF1A2 fusion bait, eEF1A2 was shown to interact with Fascin (Chang and Wang, 2006) suggesting that eEF1A2 could co-localise with this marker in the filopodia in mammalian cells. Lamellipodia and filopodia are important to cell motility. The potential localisation of eEF1A2 to filopodia in MCF-7 cells suggests that it could be important in motility and hence this could have an implication in the invasion and metastasis of eEF1A2-expressing cancer cells. eEF1A2 was also shown to by yeast two-hybrid analysis to interact with other cytoskeletal proteins including ABP280, Keap1, mRif and RanBPM (Chang and Wang, 2006).

GFP-eEF1A1 also appears to localise to the cellular protrusions in PEO1 cells but to a lesser extent than eEF1A2. This may be due to the N-terminal fusion of GFP to eEF1A1, as eEF1A2 did not show as strong localisation to cellular protrusions when

tagged with GFP but did when tagged with V5. If indeed eEF1A2 and not eEF1A1 is found in filopodia this would clearly suggest that the isoforms perform different roles within cell motility. The observation of eEF1A (eEF1A1 or eEF1A2 not distinguished) at the invading edge of breast tumours does also suggest that eEF1A1 or eEF1A2 or both isoforms may have a role in invasion or motility in cancer cells (Zhu et al., 2003). eEF1A has also been identified to be more highly expressed in rat metastatic breast tumours compared to non-metastatic tumours, suggesting a role in metastasis (Edmonds et al., 1996).

Analysis of the subcellular localisation of eEF1A1 in rat mammary adenocarcinoma cell lines and human fibroblasts has been carried out. In the metastatic rat mammary adenocarcinoma cell line MTLn3, Edmonds *et al.* found eEF1A to have a diffuse cytoplasmic localisation with increased levels in surface projections containing F-actin. They also observed a perinuclear distribution that they attributed to association with ribosomes on the endoplasmic reticulum. Upon stimulation of the cells with epidermal growth factor (EGF), eEF1A was shown to be associated with the F-actin rich zone at the edge of the de novo lamellipods (Edmonds et al., 1996). This is similar to the localisation of V5-eEF1A2 that I observed in MCF-7 cells and to the localisation of GFP-eEF1A1 in some PEO1 cells. This suggests that eEF1A1 has a similar localisation to eEF1A2 and that the presence of the GFP fusion on eEF1A1 is obscuring this localisation so it is only seen in a few cells. In an immunofluorescent study of the localisation of eEF1A in human primary fibroblasts, eEF1A was found to show a diffuse cytoplasmic distribution and unexpectedly a strong nuclear localisation. Interestingly, they did not find any co-localisation of eEF1A with the actin cytoskeleton (Sanders et al., 1996). I also observed a diffuse cytoplasmic localisation of eEF1A1 but I did not observe GFP-eEF1A1 in the nucleus.

EGF stimulation of cells in culture and observation of eEF1A1 and eEF1A2 localisation could also be useful in further investigating the putative localisation of the isoforms in cellular protrusions as it has been demonstrated that in resting *Dictyostelium discoideum* cells eEF1A is associated with G-actin but upon

chemoattractant administration eEF1A binds F-actin (Dharmawardhane et al., 1991). Therefore the amount of eEF1A2 and eEF1A1 that translocates to F-actin upon EGF administration could be compared. Also plating of cells on fibronectin coated coverslips rather than glass coverslips is a more effective substrate for migration of cells and visualising lammelipodia. It is also important to note that the localisation of eEF1A2 (and potentially eEF1A1) at F-actin rich cellular protrusions could be due to the localised synthesis of proteins required for cell motility, adhesion etc… and not due to a potential non-canonical function in F-actin bundling.

In order to further look into the role of eEF1A2 in cancer cells I used RNA interference to attempt to knockdown eEF1A2 expression in HeLa and MCF-7 cells and then look at any resultant phenotype. The initial use of pooled siRNAs targeted to eEF1A2 did result in knockdown of eEF1A2 levels in HeLa and MCF-7 cells, however the technique was problematic. siRNAs appeared effective initially but were ineffective upon subsequent use. This inconsistency suggests that the pooled siRNAs are degrading. Reasons for this may be that the four siRNAs form a complex when frozen that results in degradation. I chose to use shRNA constructs as these are DNA oligos cloned into a vector and therefore not as susceptible to degradation.

This alternative approach to RNAi of using shRNA vectors also uncovered an unexpected problem with the technique, the triggering of the interferon response. Interferons (IFNs) are cytokines that function in host defence against viruses. Double stranded RNA is a replicative intermediate of some viruses and triggers an innate immune response. This leads to the secretion of IFNα and IFNβ that binds to the interferon receptor on the cell surface resulting in activation of a signalling cascade involving Janus tyrosine kinases and resultant phosphorylation of signal-transducing activators of transcription known as STATs and expression of interferon stimulated genes. These IFN stimulated genes include the gene encoding the enzyme 2',5'oligoadenylate synthetase. 2',5'oligoadenylate synthetase polymerizes ATP into a series of $2'-5'$ linked oligomers and this activates endoribonucleic degradation of viral RNA. IFNα and IFNβ also activate the protein kinase PKR that

phosphorylates the eukaryotic protein synthesis initiation factor eIF-2, inhibiting translation and thus perturbing viral replication. These responses to dsRNA are not sequence-specific and can ultimately result in cell growth inhibition and apoptosis (Janeway, 2001).

It has previously been reported that dsRNA of less than 30 nucleotides did not trigger the interferon response and effectively lead to ablation of target gene expression (Elbashir et al., 2001). However, Bridge *et al.* observed triggering of the interferon response in human lung fibroblasts by 21 nucleotide shRNAs in plasmid-based expression vectors as well as lentiviral vectors. They found that increasing vector dose increased the level of interferon response suggesting that the accumulation of unprocessed or aberrantly processed Pol III transcripts may be triggering the IFN response (Bridge et al., 2003). Accumulation of unprocessed dsRNA might be the cause of the induction of the IFN response in my system and decreasing the dose of eEF1A2 and non-targeting shRNA vectors did appear to decrease the level of OAS1 expression. In addition to IFN induction by shRNA vectors Sledz *et al.* have observed that a variety of siRNAs of 21 nucleotides in length can also trigger an IFN response in human cell lines (Sledz et al., 2003). This suggests that the IFN response can be triggered by siRNAs as well as longer unprocessed or aberrantly processed Pol III transcripts produced from shRNA vectors. Targeting of the shRNA vector directly into the nucleus using nucleofection overcame the induction of the IFN response and may be a useful tool in decreasing this off target effect that can be induced in RNA interference.

On an occasion of successful eEF1A2 knockdown using pooled eEF1A2 siRNAs a BrdU assay suggested that 90% ablation of eEF1A2 in MCF-7 cells has no effect on the rate of proliferation of these cells in culture. The evidence behind this investigation was that forced expression of eEF1A2 in NIH 3T3 cells has been shown to decrease their doubling time and when expressed in ES-2 cells and xenografted into nude mice it accelerated growth (Anand et al., 2002). I therefore hypothesised that eEF1A2 ablation in cancer cells in culture might result in a decrease in proliferation and therefore a decrease in BrdU positive cells compared to

those expressing eEF1A2. Transfection of MCF-7 cells with siRNA, including non-targeting oligo, appears to result in a decrease in the number of cells in S phase by 48 hours post transfection. This suggests that there is some toxicity associated with siRNA and that this may be an off target effect of siRNA treatment. The number of cells in which eEF1A2 expression has been ablated in S phase is not significantly lower than that seen in cells treated with non-targeting oligo suggesting eEF1A2 does not perform a role in the proliferation rate of MCF-7 cells. Alternatively eEF1A2 may perform a role in proliferation but because eEF1A2 knockdown, although substantial, is not complete the residual eEF1A2 may be sufficient for there to be no phenotypic change in these cells. Complete knockdown of eEF1A2, as may be achieved using cell lines stably expressing a shRNA construct targeted to eEF1A2, could be used to determine whether complete absence of the elongation factor effects proliferation.

Upon partial knockdown of eEF1A2 in MCF-7 cells using shRNA vectors JLTB and T1B1 the cell cycle distribution and percentage of cells in apoptosis were measured by propidium iodide staining and FACS analysis. Both mock transfection and transfection of targeted and non-targeted shRNA constructs resulted in a decrease of cells in G1, S and G2/M and an increase in the number of apoptotic cells. This increase in apoptosis is to be expected as the nucleofection is known to result in some cell death. This cell death was higher in those cell populations nucleofected with shRNA vectors suggesting their expression harbours some additional toxicity. This toxic effect was less prominent in cells transfected with the JLTB construct perhaps suggesting that this plasmid preparation was cleaner or that the shRNA sequence induced less toxicity. Again there appears to be no difference between in cell cycle distribution in cell populations in which eEF1A2 expression has been partially ablated. Ruest *et al.* observed that in mouse C2C12 and rat L6A Myoblast cell lines, when eEF1A2 expression levels were high and cells were then serum deprived more cells remained viable. However, when eEF1A1 expression was high in these cells more apoptosis was observed. This function of eEF1A2 and eEF1A1 was shown to exert its effect through caspase-3 (Ruest et al., 2002). Therefore, in this system eEF1A2 is anti-apoptotic and eEF1A1 is pro-apoptotic. This suggested

that eEF1A2 may be protective against apoptosis induced by serum starvation. Additionally, eEF1A2 has been shown to be protective against oxidative stress (H_2O_2) induced apoptosis in NIH 3T3 cells (Chang and Wang, 2006). However, knockdown of eEF1A2 does not result in a greater amount of apoptosis in MCF-7s compared to cells expressing high levels of the factor. It may be that this anti-apoptotic role would only be exposed by induction of apoptosis in the MCF-7 cells with H_2O_2 or serum starvation and that eEF1A2 is not required for cell survival in the absence of these stresses. Initial RNAi of eEF1A2 using siRNAs did suggest that knockdown of the protein resulted in a decrease in cell number compared to controls, and it was not clear whether this phenotype was due to a decrease in proliferation or an increase in apoptosis. However, subsequent experiments suggest that eEF1A2 ablation does not result in a decrease in cell number. It may also be that initial RNAi experiments sufficiently knocked down eEF1A2 to levels that produced a phenotype and that subsequent experiments have not been as successful. Again complete ablation of eEF1A2 by stable expression of eEF1A2 shRNA may answer this question but if cell viability is compromised by loss of the factor the use of inducible shRNA constructs may be necessary.

The final siRNAs used in RNA interference of eEF1A1 or eEF1A2 in HeLa cells were effective. These siRNAs were pre-designed by Ambion and consisted of three individual siRNAs targeted to each of eEF1A1 and eEF1A2. Of these three siRNAs, two effectively resulted in the knockdown of eEF1A1 or eEF1A2 when transfected into HeLa cells. These siRNAs are single siRNAs rather then the pooled siRNAs and therefore may be more effective because a complex between different siRNAs cannot form and hence the single siRNAs are not degrading during freezing. Additionally, the individual siRNAs may be more effectively designed than the pooled oligos and this may also make them more effective. There did not appear to be any toxicity associated with transfection of the Ambion siRNAs into HeLa cells suggesting they also do not trigger an interferon response.

HeLa cells in which eEF1A1 or eEF1A2 expression had been ablated appeared to still be capable of HSF1 activation and subsequent Hsp70 upregulation in response to

heat shock. This suggests that one of two things (or indeed both hypotheses) may be true: firstly, that both eEF1A1 and eEF1A2 are capable of participating in HSF1 activation and hence in cells in which one of the variants has been knocked down the other variant is able to compensate. Secondly, it may be the case that eEF1A1 and eEF1A2 are not essential for HSF1 activation, contrary to the suggestion by Shamovsky *et al.* that it is; however this was not conclusively shown (Shamovsky et al., 2006). Also, eEF1A1 knockdown using siRNA only resulted in an approximate 50% reduction in the level of eEF1A1 compared to untreated cells and this remaining eEF1A1 may be sufficient for HSF1 activation to proceed.

The specificity of the Hsp70 antibody is, however, somewhat questionable. In HeLa cells that have not been exposed to cellular stress such as heat shock it would be expected that the stress-induced Hsp70 should not be present but it can be seen from the Western blot that there is Hsp70 present in these cells. Additionally, the eEF1A1 and eEF1A2 antibody controls, protein lysate from MCF-7 and PEO1, but not wild-type mouse muscle, also have high levels of Hsp70 expression. This could be due to the anti-Hsp70 antibody cross reacting with the constitutive Hsc70. Alternatively, the sealing of the plates when incubated at 37°C may induce a stress response in the cells and hence Hsp70 activation. This seems less likely as the antibody detected large amounts of Hsp70 in the control protein lysates, MCF-7 and PEO1, and these cells were not treated in any way that should induce a stress response before protein extraction.

The effectiveness of the Ambion pre-designed siRNAs in inducing eEF1A1 and eEF1A2 knockdown means that this tool could be used for many assays of gene function in different cell lines and should provide valuable information regarding function of these two proteins.

Chapter 7 Discussion

7.1 Evidence for a role of eEF1A2 in breast and ovarian cancers prior to the PhD

Prior to the start of this project a limited amount of research regarding the role of eEF1A2 in cancer had been carried out. Chromosomal arm 20q and the region 20q13 was known to be frequently amplified in breast and ovarian cancers, among other cancers (Courjal et al., 1996). Anand *et al.* identified overexpression of eEF1A2 in approximately one-third of ovarian cancers and found that forced expression of eEF1A2 in NIH 3T3 cells resulted in a transformed phenotype and tumour formation when xenografted into nude mice, suggesting that *EEF1A2* is an oncogene (Anand et al., 2002).

7.2 Summary of results

In breast cancers I observed moderate to high expression of eEF1A2 at the RNA and protein level in up to two-thirds of breast cancers analysed. This moderate to high eEF1A2 expression level appeared to associate with oestrogen receptor (ER) positive cancers ($p=0.016$) however further investigation suggested that *EEF1A2* is not an oestrogen responsive gene. The Letrozole and ERα RNAi in MCF-7 cells did not confirm or otherwise if eEF1A2 is oestrogen responsive and therefore additional more effective experiments would be needed to determine how eEF1A2 expression relates to the oestrogen receptor. In epithelial ovarian cancer eEF1A2 was expressed in 15-30% of tumours analysed and high levels of expression of eEF1A2 were observed in clear cell carcinomas. eEF1A2 overexpression in ovarian cancer does not appear to be mediated by mutation or methylation and is not consistently associated with gene amplification. Another method of evaluation of *EEF1A2* copy number such as FISH would be required to confirm or otherwise the frequency of *EEF1A2* amplification and hence determine the predominant mechanism of overexpression. Partial knock down of eEF1A2 expression in HeLa and MCF-7 cells does not result in a decrease in the percentage of proliferating cells or an alteration in the cell cycle distribution and apoptosis in these cells. Recent evidence suggested that eEF1A was required for HSF1 activation and the expression of heat shock

proteins in response to cellular stress (Shamovsky et al., 2006). The knock down of either eEF1A1 or eEF1A2 in heat shocked HeLa cells using RNA interference does not appear to impede the expression of Hsp70, suggesting that the heat shock response does not depend on either eEF1A1 or eEF1A2 exclusively.

7.3 The role of eEF1A2 in tumourigenesis: evidence from functional studies

RNA interference has not shed much light on the role of eEF1A2 in cancer cells in culture. RNA interference did suggest that eEF1A2 is not involved in the control of proliferation in cancer cells in culture. This is supported by the finding of Kulkurni *et al.* showing eEF1A2 expression correlated negatively with Ki67 in breast cancer, suggesting that eEF1A2 is not associated with mitotic index and hence not involved in proliferation (Kulkarni et al., 2006). The cells in culture in which eEF1A2 had been knocked down (HeLa and MCF-7) were viable and this suggests that eEF1A2 is not required for cell viability and that the levels of eEF1A1 present are sufficient to fulfil the need for protein elongation in the cells. Equally, HeLa cells in which eEF1A1 but not eEF1A2 had been knocked down also appeared viable under the microscope suggesting that the two proteins can perform comparably in protein synthesis however a more detailed assay for cell viability would be required to properly confirm this.

The literature does provide some clues to the role that eEF1A2 could be performing in cancer. For instance as mentioned previously eEF1A2 could be involved in protection of cells from apoptosis induced by oxidative stress (Chang and Wang, 2006). Alternatively it could be that eEF1A2 has a role in actin remodelling and this may be particularly important in cancer cell invasion and metastasis. My observation of eEF1A2 co-localisation with actin at what appear to be filopodia in MCF-7 cells suggests that the elongation factor could be involved in cell migration. The identification of an interaction between eEF1A2 and fascin (an actin bundling protein found in filopodia) in a yeast two-hybrid screen by Chang *et al.* (Chang and Wang, 2006) is consistent with the observation that eEF1A2 is localised to these cellular protrusions. Overexpression of tagged eEF1A1 and eEF1A2 in MCF-7 and PEO1

cells did not seem to result in any alteration in the cytoskeleton of these cells – as has been observed in *Saccharomyces cerevisiae* (Munshi et al., 2001) - perhaps suggesting the actin remodelling properties of eEF1A in yeast are different from mammalian eEF1A. Additionally, overexpression of eEF1A1 or eEF1A2 did not seem to increase the number of cellular protrusions formed compared to cells expressing empty vector. The fact that eEF1A2 expression has been correlated with an increased 20 year survival in breast cancer, suggests that eEF1A2 expression is not associated with aggressive, invasive, metastatic breast cancers (Kulkarni et al., 2006). eEF1A1 has been shown to activate phoshotidylinositol-4-kinase (Yang et al., 1993) and eEF1A2 overexpression has been demonstrated to increase the levels of Akt (Chang and Wang, 2006) and therefore eEF1A2 may regulate tumourigenesis through these pathways. More *in vitro* and *in vivo* assays would be required to determine the role that eEF1A2 could be playing in cancer. It is still not known whether it is the function of eEF1A2 in translation or a potential non-canonical function that is mediating its role in cancer.

7.4 The potential of eEF1A2 as a biomarker or therapeutic target

My expression studies in breast and ovarian cancer suggest that eEF1A2 could be a useful biomarker or therapeutic target in these cancers. More work looking at associations between eEF1A2 expression and clinical features, such as response to chemotherapy or survival time, would better define how eEF1A2 could be a useful marker in breast and ovarian cancer. eEF1A2 could be a possible therapeutic target as eEF1A2 appears to only be exclusively expressed in terminally differentiated cell types that perhaps do not have a high need of protein synthesis compared to tumour cells. However, the phenotype of wasted mice suggests that decreasing levels of eEF1A2 could result in a motor neuron disease-like phenotype.

7.5 Chromosome 20q13 amplification and *EEF1A2*

It is possible that the expression of eEF1A2 I have observed in breast and ovarian cancer is simply a consequence of the gene's location within the 20q amplicon and that other genes at this locus are the important proto-oncogenes. Multiple regions along the q arm of chromosome 20 are amplified, as well as the entire q arm, and

often this amplification is at 20q13.2, which is proximal to the *EEF1A2* locus. Candidate oncogenes in the 20q13 amplicon(s) include *ZNF217*, *AurA*, and *PTK6* and *AIB1*. ZNF217 is a putative transcription factor that has been shown to cause immortalization of human mammary epithelial cells and to increase telomerase activity and stabilise telomere length (Nonet et al., 2001). Aurora kinases belong to a family of mitotic protein kinases involved in the regulation of centrosomal and mitotic activity. Aurora A kinase overexpression in NIH 3T3 cells results in transformation and amplification of the *AurA* gene and overexpression of the protein has been observed in cancer (Ferrari, 2006). 20q13 amplification has been shown to associate with high grade breast tumours showing aneuploidy (Tanner et al., 1995), and overexpression of aurora A kinase has been shown to cause centrosome amplification, chromosomal instability and aneuploidy (Zhou et al., 1998). *PTK6* encodes a non-receptor tyrosine kinase (Brk) and is located close to *EEF1A2* at 20q13.3. It is thought that epidermal growth factor (EGF) stimulates the phosphorylation of paxcillin by Brk leading to activation of Rac1 resulting in the promotion of cell motility and invasion (Chen et al., 2004). This is interesting as 20q13 amplification has been shown to be associated with aggressive breast tumours with lymph node involvement (Courjal et al., 1996), and Brk expression may be involved in conferring this property in 20q13 amplified tumours. Interestingly, Brk has been shown to be expressed in approximately two-thirds of breast tumours (Barker et al., 1997) (similar to my results for eEF1A2) and its expression in mouse fibroblasts and mammary epithelial cells potentates their anchorage independent growth and, in the latter cell type, their response to epidermal growth factor (Kamalati et al., 1996). Amplified in breast cancer 1 (AIB1) is located at 20q12. It is a member of the p160/steroid receptor coactivators family and has been shown to interact with the estrogen receptor and enhance ER-dependent gene expression (Anzick et al., 1997). 20q gain is common in ER positive breast cancers (Cingoz et al., 2003) and AIB1 may be involved in enhancing signalling from the receptor in these breast cancers. In addition, AIB1 amplification has been identified in 25% of sporadic ovarian tumours and may activate oestrogen-dependent growth-promoting pathways in ovarian epithelial cells (Tanner et al., 2000). The expression of multiple genes within the 20q13 amplicon(s) and along the entire 20q arm may act

synergistically, through interplay between oncogenic pathways, to enhance or drive tumourigenesis and *EEF1A2* could be an important player. My finding that eEF1A2 expression levels do not appear to correlate with copy number suggests that eEF1A2 overexpression is not only due to gene amplification.

7.6 Future work

7.6.1 The expression of eEF1A2 in breast cancer

The expression of eEF1A2 in different sets of breast cancers appeared to be different. For instance in the cores of tumours on the commercial tissue array eEF1A2 expression appeared to be moderate to high in more than 60% of the breast cancers. Equally the expression of eEF1A2 was also moderate to high in a large percentage of the oestrogen receptor positive cancers at the RNA level. However, the percentage of tumours on the in-house array that expressed eEF1A2 was substantially lower than that observed in the other breast tumour sets analysed. Unfortunately this discrepancy meant that analysis for clinical correlations was not possible and therefore I was not able to utilise this TMA, for which there is significant clinical data available, to determine whether eEF1A2 expression associated with clinical features. Future work would include further analysis of eEF1A2 expression in more breast tumours and hopefully the identification of clinical associations. One of the most interesting observations of eEF1A2 expression in breast cancers was that eEF1A2 appeared to be expressed in many precursor breast lesions and therefore an array containing examples of hyperplastic lesions and DCIS would be a useful tool for investigating the possibility that eEF1A2 overexpression is an early event in the molecular pathway of breast cancer progression.

7.6.2 The expression of eEF1A2 in ovarian cancer

A similar problem with discrepancies between eEF1A2 expression levels in different panels of ovarian cancer samples also meant that identification of clinical associations was not possible. Initial analysis of eEF1A2 expression in epithelial ovarian tumours on a commercial tissue array and a panel of ovarian cancers for which RNA, DNA and protein was available showed an association between eEF1A2 expression and clear cell carcinomas (CCC). However this association was

apparent but not statistically significant in a study of a larger in-house tissue array for which clinical information was available. Therefore future work would include the analysis of further clear cell carcinomas to confirm or otherwise this association and to optimise eEF1A2 staining by immunohistochemistry. The expression of eEF1A2 in clear cell carcinomas could contribute to the distinct clinical features of this subtype. Particularly interesting is the resistance of clear cell carcinomas to platinum-based chemotherapy. It would be interesting to study the specific role of eEF1A2 in clear cell carcinomas and platinum-based chemotherapy resistance, perhaps using an *in vitro* model. Clear cell carcinomas cells in culture, such as HAC-2 or SMOV-2 cells, could be used in an RNA interference assay in which eEF1A2 expression is knocked down and the resistance to therapy investigated. Cells could be treated with various concentrations of cisplatin (CDDP) and sensitivity determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in cells expressing and not expressing eEF1A2. This protocol has been used by Itamochi *et al.* to investigate the mechanism of Cisplatin resistance in clear cell carcinoma of the ovary (Itamochi et al., 2002). In addition previous *in vitro* studies have demonstrated that CCC cells in culture exhibit resistance to CDDP as well as doxorubicin, cyclophosphamide, and etoposide (Gorai et al., 1995).

7.6.2 The mechanism of eEF1A2 overexpression

The mechanism of overexpression of eEF1A2 in ovarian cancers remains a mystery. It is not clear whether gene amplification plays a role in eEF1A2 expression and further analysis of *EEF1A2* copy number using another technique such as FISH is required to determine this. Mutation and methylation do not appear to mediate eEF1A2 overexpression in the ovarian cancers analysed. Inappropriate expression of a *trans*-acting factor, such as a neuronal specific transcription factor, could play a role in eEF1A2 overexpression and this would be an interesting avenue of investigation. This question could be addressed using a microarray to look for expression of transcription factors in cancers that express eEF1A2. My analyses were carried out on a small number of ovarian cancers and therefore extending the analysis to include more ovarian cancers, particularly for mutation analysis, could perhaps be useful. It would also be interesting to look at the mechanism of eEF1A2

overexpression in other cancers such as breast cancer. It is very possible that the mechanism of overexpression is different in different cancers and even between different subtypes, be they histological or clinical, within cancer of a specific organ. In a chemical screen for methylation-silenced genes in a gastric cancer cell line Yamashita *et al.* identified that eEF1A2 expression was upregulated by greater than 16-fold following 5-aza-dC treatment of the cell line, using an oligonucleotide array. The methylation of the 5' CpG island of *EEF1A2* was examined in other gastric cancer cell lines and primary tumours by methylation-specific PCR and shown to be methylated in 3/6 cell lines but only 1/10 primary gastric cancers (Yamashita et al., 2006). This work suggests that in some tissues and cancers the methylation status of the 5'CpG island of *EEF1A2* could be involved in regulating eEF1A2 expression. In extension to analysis of the methylation status of the 5'CpG island of *EEF1A2* in cancers, investigation in normal human and mouse tissues that express and do not express eEF1A2 would be informative.

7.6.3 The expression and role of eEF1A2 in other cancers

As well as ovarian and breast cancers there is also evidence to suggest that eEF1A2 is involved in other human cancers. For instance it would be interesting to investigate the role of eEF1A2 in lung cancers, particularly because eEF1A expression has been shown to correlate with short overall survival time (Li et al., 2006) and it is not known if it is eEF1A1 or eEF1A2 that shows this association due to the use of a non-specific antibody in immunohistochemical analysis. Comparative genomic hybridisation assays have meant that the identification of single copy gains at 20q or high level amplifications have been identified in many different cancers including bladder cancer (Hurst et al., 2004), pancreatic cancer (Kitoh et al., 2005), oesophageal squamous cell carcinoma (Fujita et al., 2003), and colon cancer (Douglas et al., 2004), to name but a few. Investigating the expression and potential role of eEF1A2 in these cancers could determine if *EEF1A2* is an important oncogene in many different cancers

.

7.6.4 The role of eEF1A2 in oncogenesis

Further analysis of eEF1A2 function using *in vitro* and *in vivo* models is required to elucidate the mechanism by which eEF1A2 could be oncogenic. Stable cell lines expressing eEF1A1 or eEF1A2 targeted shRNAs would perhaps be an effective in vitro model for analysing the differences in function between the two eEF1A proteins. Using constructs with different targeting efficiencies could also prove useful for the analysis of phenotypic change upon subtle changes in eEF1A levels. In order to answer the question of whether eEF1A1 and eEF1A2 are involved in the translation of different mRNAs (I believe this to be unlikely) and if this is contributing to oncogenesis, proteome analysis of cells in which eEF1A1 or eEF1A2 are exclusively expressed could be carried out. Two-dimensional acrylamide gel electrophoresis and mass spectrometry could be utilised to identify those proteins that are potentially differentially expressed. The global level of translation can be measured in cancer cells upon eEF1A1 or eEF1A2 ablation by measuring the rate of poly(U)-directed [^{3}H]polyphenylalanine synthesis. Conversely, the effect of overexpression of either of the eEF1A proteins on the global rate of translation could also be determined. RNA interference could also be used to investigate the potential role of eEF1A in the PI3-K/Akt oncogenic pathway by looking at the levels of activated Akt (using specific antibodies to the phosphorylated protein) in cells expressing and not expressing the eEF1A variants.

The potential role of eEF1A2 in invasion and metastasis could also be investigated using RNA interference in an invasive cell line such as MDA-MB231. The effect of eEF1A2 ablation on the invasive potential of the cell line could be investigated using a matrigel invasion assay. Conversely overexpression of eEF1A2 in a non-invasive cell line such as PEO1, that does not express endogenous eEF1A2, could be used in a matrigel assay to look for increased invasiveness. Additionally, further investigation into the potential localisation of eEF1A1 and eEF1A2 at cellular protrusions would be interesting. A scratch wound assay could be used to look at filopodia formation in cells expressing the GFP tagged eEF1A1 and eEF1A2 and plated on fibronectin. There is also increasing evidence that eEF1A2 is anti-apoptotic and this could be investigated using the eEF1A2 knockdown or overexpressing cell lines to evaluate if

alterations in eEF1A2 levels have an effect on hydrogen peroxide induced apoptosis for instance. The same assays can be applied to cells lines in which eEF1A1 has been knocked down or overexpressed (although we are yet to identify a cell line that does not express endogenous eEF1A1 at high levels) to determine if eEF1A1 and eEF1A2 have different roles in apoptosis.

Further overexpression studies to perhaps determine whether eEF1A1 and eEF1A2 have opposing non-canonical functions in cell lines could be done by creating chimeric eEF1A1/eEF1A2 constructs. For instance the GTP-binding domains are located at the N-terminus of the eEF1A proteins, and this region shows few differences between eEF1A1 and eEF1A2, but the regions thought to be important for cytoskeletal interaction are located at the C-terminus where more differences between the two proteins are located. Therefore engineering eEF1A1 clones with the C-terminus replaced by the C-terminus of eEF1A2, and vice versa, could be informative. For instance the anti-apoptotic function of eEF1A2 could be altered or ablated in the eEF1A2/eEF1A1 chimeric protein. Also using cloning to produce eEF1A1 and eEF1A2 proteins with mutated GTP-binding domains and analysing the ability of these proteins to transform NIH 3T3 cells could potentially be used to identify whether it is the function of eEF1A2 in translation or in other cellular processes that enables it to transform rodent fibroblasts. Importantly, the ability or otherwise of eEF1A1 to transform rodent fibroblasts and to result in tumour formation when xenografted into nude mice has not been investigated and would be important in determining if both eEF1A variants are oncoproteins.

In vivo models of eEF1A2 and its role in cancer could include the overexpression of the translation factor in, for instance, mammary epithelium of the mouse and observation for tumour formation. This could be achieved by the production of a transgenic mouse expressing eEF1A2 under the control of a whey acidic protein promoter (WAP) or mouse mammary tumor virus long terminal repeat (MMTV) promoter to produce a mammary tumour model of eEF1A2 expression (Hutchinson and Muller, 2000). Transgenic mouse models of ovarian cancers have also recently been produced. Connolly *et al.* successfully modelled ovarian cancer in mice by

expressing the potent transforming gene SV40 TAg under the control of the *MISIIR* promoter. Expression of the *MISIIR-TAg* transgene lead to the formation of poorly differentiated carcinomas of the ovary in female mice and these tumours showed characteristics of the human disease such as intraperitoneal dissemination (Connolly et al., 2003). Flesken-Nikitin *et al.* used Cre-*loxP*-mediated gene inactivation by the intrabursal administration of AdCMVCre to model the role of *TP53* and *Rb1* in epitheilial ovarian carcinogenesis (Flesken-Nikitin et al., 2003). Another technique utilised to generate mouse models of ovarian cancer is to produce genetic alterations in mouse or human ovarian surface epithelial cells either in a cell line and then to inoculate the cells into immunocompromised mice subcutaneously, or intraperitoneally (Cheng et al., 2005; Liu et al., 2004; Orsulic et al., 2002). Alternatively, Orsulic *et al.* isolated and cultured mixed ovarian cells from p53 deficient transgenic mice expressing the avian retrovirus receptor TVA and infected them with multiple vectors carrying sequences encoding the oncogenes *c-myc*, *k-ras* and *Akt*. They found that any two of these oncogenes were capable of inducing tumour formation when infected cells were injected into various sites in the animal (Orsulic et al., 2002). Therefore using techniques similar to those described above transgenic mouse models of eEF1A2 in ovarian and breast cancer could be produced.

7.6.5 The role of eEF1A1 and eEF1A2 in heat shock

In HeLa cells in which eEF1A2 expression had been completely ablated using RNA interference it appeared that Hsp70 was still induced following heat shock treatment at 42°C for 1 hour. Likewise, eEF1A1 was knocked down to approximately 20% of the level in untreated HeLa cells and the level of induction of the stress-activated Hsp70 following heat shock appeared equal to that observed in cells expressing eEF1A1 at normal levels. This suggests that either both eEF1A1 and eEF1A2 are capable of HSF1 activation or that the involvement of eEF1A is not essential for HSF1 activation. Alternatively, Hsp70 expression could be induced independently of HSF1, however Shamovsky *et al.* suggest that this is not the case (Shamovsky et al., 2006). The mechanism of HSF1 activation was previously thought to occur spontaneously by the relief of inactivation by bound HSPs. However, the fast HSF1 activation kinetics (Jolly et al., 1999) suggested that this diffusion controlled

mechanism was probably not responsible for HSF1 activation (Shamovsky et al., 2006). Indeed, Shamovsky *et al.* recently showed that HSF1 was activated and that elongation factor 1A and a non-coding RNA, HRS1, were required for this activation. eEF1A purified from rat liver (this is therefore eEF1A1 because liver does not express eEF1A2) together with HSR1, was shown to activate HSF1 indicating that eEF1A1 does perform this function (Shamovsky et al., 2006). My RNA interference has not conclusively shown whether eEF1A2 also is involved in HSF1 activation. In cells in which eEF1A1 expression has been knocked down to approximately 20% of the level seen in untreated cells Hsp70 upregulation appears to occur. This suggests that either eEF1A2 is also capable of performing in the activation of HSF1 or that the residual eEF1A1 in the RNAi-treated cells is sufficient to perform in this process. To rectify this remaining question RNA interference in which stable expression of an effective shRNA targeted to eEF1A1 has resulted in complete ablation of eEF1A1 expression in cell lines also expressing eEF1A2, could be used to test for HSF1 activation in the absence of eEF1A1. Additionally, the use of eEF1A2 purified from mouse skeletal muscle together with HSR1 could be used in an assay of HSF1 activation. More direct assays of HSF1 activation include an electromobility shift assay (EMSA), to determine if HSF1 is bound to DNA. Also the use of a reporter plasmid such as the one used by Shamovsky *et al.*, which consisted of a luciferase reporter fused to an inducible human *HSP70* promoter, could be used to monitor HSF1 activation in cells in which RNA interference has been used to knockdown the expression of eEF1A1.

7.6.6 Elongation factor 1A2 and its interaction with TCTP and Prdx1

The recent interesting observation of an interaction between eEF1A (not clear if eEF1A1 or eEF1A2) and translationally controlled tumour protein (TCTP) has given some clue as to a potential mechanism of eEF1A's role in cancer (Cans et al., 2003). TCTP was shown to be significantly down-regulated during tumour reversion in cell lines selected with Paravirus or stably transfected with SIAH-1, both of which result in the selection of reverted tumour cells. Additionally, down regulation of TCTP using anti-sense cDNA in the human leukaemia cell line U937 resulted in apoptosis (Tuynder et al., 2002). eEF1A and eEF1Bβ were also identified in the yeast-two

hybrid hunt as a TCTP interactor and TCTP was shown to impair GTP/GDP exchange on eEF1A by stabilising the GDP bound form and antagonising eEF1Bβ-mediated exchange (Cans et al., 2003). The first investigation to carry out would be to determine whether it is eEF1A1, eEF1A2 or both that interact with TCTP. This could be done by co-immunoprecipitation experiments in cell lines expressing all three of the proteins using the eEF1A1 and eEF1A2 specific antibodies and an antibody raised to TCTP. Expression analysis of eEF1A2 and TCTP could also be carried out by immunohistochemistry to determine if the two proteins are co-expressed in cancers. Expression of TCTP would have to be analysed at the RNA and proteins levels as its expression is post-translationally regulated. Subsequent *in vitro* analysis using RNA interference could be used to analyse the role of eEF1A2 or eEF1A1 and TCTP proteins in cancer cell lines. This is currently under investigation in the Abbott group by Helen Newbery. Equally, perioxiredoxin-1 (Prdx1) has been identified as an eEF1A2 interactor in a yeast two-hybrid screen (Chang and Wang, 2006) and similar assays could be used to investigate whether these two proteins are co-expressed in cancers and to investigate their potential anti-apoptotic function in cancer cell lines. This yeast two-hybrid screen identified many potential eEF1A2-interactors and investigating these would also be informative (Chang and Wang, 2006).

In conclusion much work still remains to answer the fundamental questions surrounding the potential role of eEF1A2 in cancer. It is important to determine whether both *EEF1A2* and *EEF1A1* are oncogenes or if only *EEF1A2* is an oncogene. Secondly, the mechanism by which eEF1A2 is overexpressed still remains elusive and requires further study. Thirdly, it will be very interesting to determine if it is through its role in translation that eEF1A2 is oncogenic or alternatively if the oncogenesis is mediated by a specific non-canonical function of eEF1A2 that is not performed by eEF1A1.

References

Abbott, C.M. and Proud, C.G. (2004) Translation factors: in sickness and in health. *Trends Biochem Sci*, **29**, 25-31.

Allred, D.C. and Mohsin, S.K. (2000) Biological features of premalignant disease in the human breast. *J Mammary Gland Biol Neoplasia*, **5**, 351-364.

Ames, B.N., Gold, L.S. and Willett, W.C. (1995) The causes and prevention of cancer. *Proc Natl Acad Sci U S A*, **92**, 5258-5265.

Anand, N., Murthy, S., Amann, G., Wernick, M., Porter, L.A., Cukier, I.H., Collins, C., Gray, J.W., Diebold, J., Demetrick, D.J. and Lee, J.M. (2002) Protein elongation factor EEF1A2 is a putative oncogene in ovarian cancer. *Nat Genet*, **31**, 301-305.

Ann, D.K., Moutsatsos, I.K., Nakamura, T., Lin, H.H., Mao, P.L., Lee, M.J., Chin, S., Liem, R.K. and Wang, E. (1991) Isolation and characterization of the rat chromosomal gene for a polypeptide (pS1) antigenically related to statin. *J Biol Chem*, **266**, 10429-10437.

Anzick, S.L., Kononen, J., Walker, R.L., Azorsa, D.O., Tanner, M.M., Meltzer, P. (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science,* **277**, 965-968.

Aubele, M., Werner, M. and Hofler, H. (2002) Genetic alterations in presumptive precursor lesions of breast carcinomas. *Anal Cell Pathol*, **24**, 69-76.

Auersperg, N., Wong, A.S., Choi, K.C., Kang, S.K. and Leung, P.C. (2001) Ovarian surface epithelium: biology, endocrinology, and pathology. *Endocr Rev*, **22**, 255-288.

Bajic, V.B., Tan, S.L., Chong, A., Tang, S., Strom, A., Gustafsson, J.A., Lin, C.Y. and Liu, E.T. (2003) Dragon ERE Finder version 2: A tool for accurate detection and analysis of estrogen response elements in vertebrate genomes. *Nucleic Acids Res*, **31**, 3605-3607.

Balsara, B.R., Sonoda, G., du Manoir, S., Siegfried, J.M., Gabrielson, E. and Testa, J.R. (1997) Comparative genomic hybridization analysis detects frequent, often high-level, overrepresentation of DNA sequences at 3q, 5p, 7p, and 8q in human non-small cell lung carcinomas. *Cancer Res*, **57**, 2116-2120.

Barker, K.T., Jackson, L.E. and Crompton, M.R. (1997) BRK tyrosine kinase expression in a high proportion of human breast carcinomas. *Oncogene*, **15**, 799-805.

Bassell, G.J., Powers, C.M., Taneja, K.L. and Singer, R.H. (1994) Single mRNAs visualized by ultrastructural in situ hybridization are principally localized at actin filament intersections in fibroblasts. *J Cell Biol*, **126**, 863-876.

Batulan, Z., Shinder, G.A., Minotti, S., He, B.P., Doroudchi, M.M., Nalbantoglu, J., Strong, M.J. and Durham, H.D. (2003) High threshold for induction of the stress response in motor neurons is associated with failure to activate HSF1. *J Neurosci*, **23**, 5789-5798.

Bennett, I.C., Gattas, M. and Teh, B.T. (2000) The management of familial breast cancer. *Breast*, **9**, 247-263.

Berchuck, A., Schildkraut, J.M., Wenham, R.M., Calingaert, B., Ali, S., Henriott, A., Halabi, S., Rodriguez, G.C., Gertig, D., Purdie, D.M., Kelemen, L., Spurdle, A.B., Marks, J. and Chenevix-Trench, G. (2004) Progesterone receptor promoter +331A polymorphism is associated with a reduced risk of endometrioid and clear cell ovarian cancers. *Cancer Epidemiol Biomarkers Prev*, **13**, 2141-2147.

Bergamaschi, A., Kim, Y.H., Wang, P., Sorlie, T., Hernandez-Boussard, T., Lonning, P.E., Tibshirani, R., Borresen-Dale, A.L. and Pollack, J.R. (2006) Distinct patterns of DNA copy number alteration are associated with different clinicopathological features and gene-expression subtypes of breast cancer. *Genes Chromosomes Cancer*, **45**, 1033-1040.

Berkel, H.J., Turbat-Herrera, E.A., Shi, R. and de Benedetti, A. (2001) Expression of the translation initiation factor eIF4E in the polyp-cancer sequence in the colon. *Cancer Epidemiol Biomarkers Prev*, **10**, 663-666.

Bestor, T.H. (1998) The host defence function of genomic methylation patterns. *Novartis Found Symp*, **214**, 187-195; discussion 195-189, 228-132.

Bischoff, C., Kahns, S., Lund, A., Jorgensen, H.F., Praestegaard, M., Clark, B.F. and Leffers, H. (2000) The human elongation factor 1 A-2 gene (EEF1A2): complete sequence and characterization of gene structure and promoter activity. *Genomics*, **68**, 63-70.

Blazejowski, C.A. and Webster, G.C. (1984) Effect of age on peptide chain initiation and elongation in preparations from brain, liver, kidney and skeletal muscle of the C57B1/6J mouse. *Mech Ageing Dev*, **25**, 323-333.

Bock, C., Reither, S., Mikeska, T., Paulsen, M., Walter, J. and Lengauer, T. (2005) BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics*, **21**, 4067-4068.

Borradaile, N.M., Buhman, K.K., Listenberger, L.L., Magee, C.J., Morimoto, E.T., Ory, D.S. and Schaffer, J.E. (2006) A critical role for eukaryotic elongation factor 1A-1 in lipotoxic cell death. *Mol Biol Cell*, **17**, 770-778.

Bourdeau, V., Deschenes, J., Metivier, R., Nagai, Y., Nguyen, D., Bretschneider, N., Gannon, F., White, J.H. and Mader, S. (2004) Genome-wide identification of high-affinity estrogen response elements in human and mouse. *Mol Endocrinol*, **18**, 1411-1427.

Braakhuis, B.J., Tabor, M.P., Kummer, J.A., Leemans, C.R. and Brakenhoff, R.H. (2003) A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res*, **63**, 1727-1730.

Brannan, C.I. and Bartolomei, M.S. (1999) Mechanisms of genomic imprinting. *Curr Opin Genet Dev*, **9**, 164-170.

Bridge, A.J., Pebernard, S., Ducraux, A., Nicoulaz, A.L. and Iggo, R. (2003) Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet*, **34**, 263-264.

Brown, A.M., Jeltsch, J.M., Roberts, M. and Chambon, P. (1984) Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7. *Proc Natl Acad Sci U S A*, **81**, 6344-6348.

Brueggemeier, R.W., Hackett, J.C. and Diaz-Cruz, E.S. (2005) Aromatase inhibitors in the treatment of breast cancer. *Endocr Rev*, **26**, 331-345.

Cans, C., Passer, B.J., Shalak, V., Nancy-Portebois, V., Crible, V., Amzallag, N., Allanic, D., Tufino, R., Argentini, M., Moras, D., Fiucci, G., Goud, B., Mirande, M., Amson, R. and Telerman, A. (2003) Translationally controlled tumor protein acts as a guanine nucleotide dissociation inhibitor on the translation elongation factor eEF1A. *Proc Natl Acad Sci U S A*.

Calderwood, S.K., Khaleque, M.A., Sawyer, D.B. and Ciocca, D.R. (2006) Heat shock proteins in cancer: chaperones of tumorigenesis. *Trends Biochem Sci*, **31**, 164-172.

Carr-Schmid, A., Durko, N., Cavallius, J., Merrick, W.C. and Kinzy, T.G. (1999) Mutations in a GTP-binding motif of eukaryotic elongation factor 1A reduce both translational fidelity and the requirement for nucleotide exchange. *J Biol Chem*, **274**, 30297-30302.

Cavallius, J., Rattan, S.I. and Clark, B.F. (1986) Changes in activity and amount of active elongation factor 1 alpha in aging and immortal human fibroblast cultures. *Exp Gerontol*, **21**, 149-157.

Chambers, D.M., Peters, J. and Abbott, C.M. (1998) The lethal mutation of the mouse wasted (wst) is a deletion that abolishes expression of a tissue-specific isoform of translation elongation factor 1alpha, encoded by the Eef1a2 gene. *Proc Natl Acad Sci U S A*, **95**, 4463-4468.

Chambers, D.M., Rouleau, G.A. and Abbott, C.M. (2001) Comparative genomic analysis of genes encoding translation elongation factor 1B(alpha) in human and mouse shows EEF1B1 to be a recent retrotransposition event. *Genomics*, **77**, 145-148.

Chang, R. and Wang, E. (2006) Mouse translation elongation factor eEF1A-2 interacts with Prdx-I to protect cells against apoptotic death induced by oxidative stress. *J Cell Biochem*.

Charpentier, A.H., Bednarek, A.K., Daniel, R.L., Hawkins, K.A., Laflin, K.J., Gaddis, S., MacLeod, M.C. and Aldaz, C.M. (2000) Effects of estrogen on global gene expression: identification of novel targets of estrogen action. *Cancer Res*, **60**, 5977-5983.

Chang, Y.W. and Traugh, J.A. (1997) Phosphorylation of elongation factor 1 and ribosomal protein S6 by multipotential S6 kinase and insulin stimulation of translational elongation. *J Biol Chem*, **272**, 28252-28257.

Chen, E., Proestou, G., Bourbeau, D. and Wang, E. (2000) Rapid up-regulation of peptide elongation factor EF-1alpha protein levels is an immediate early event during oxidative stress-induced apoptosis. *Exp Cell Res*, **259**, 140-148.

Chen, H.Y., Shen, C.H., Tsai, Y.T., Lin, F.C., Huang, Y.P. and Chen, R.H. (2004) Brk activates rac1 and promotes cell migration and invasion by phosphorylating paxillin. *Mol Cell Biol*, **24**, 10558-10572.

Cheng, W., Liu, J., Yoshida, H., Rosen, D. and Naora, H. (2005) Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract. *Nat Med*, **11**, 531-537.

Chuang, S.M., Chen, L., Lambertson, D., Anand, M., Kinzy, T.G. and Madura, K. (2005) Proteasome-mediated degradation of cotranslationally damaged proteins involves translation elongation factor 1A. *Mol Cell Biol*, **25**, 403-413.

Cingoz, S., Altungoz, O., Canda, T., Saydam, S., Aksakoglu, G. and Sakizli, M. (2003) DNA copy number changes detected by comparative genomic hybridization and their association with clinicopathologic parameters in breast tumors. *Cancer Genet Cytogenet*, **145**, 108-114.

Ciocca, D.R. and Calderwood, S.K. (2005) Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell Stress Chaperones*, **10**, 86-103.

Cleton-Jansen, A.M., Collins, N., Lakhani, S.R., Weissenbach, J., Devilee, P., Cornelisse, C.J. and Stratton, M.R. (1995) Loss of heterozygosity in sporadic breast tumours at the BRCA2 locus on chromosome 13q12-q13. *Br J Cancer*, **72**, 1241-1244.

Condeelis, J. (1995) Elongation factor 1 alpha, translation and the cytoskeleton. *Trends Biochem Sci*, **20**, 169-170.

Conklin, K.A. (2004) Chemotherapy-associated oxidative stress: impact on chemotherapeutic effectiveness. *Integr Cancer Ther*, **3**, 294-300.

Connolly, D.C., Bao, R., Nikitin, A.Y., Stephens, K.C., Poole, T.W., Hua, X., Harris, S.S., Vanderhyden, B.C. and Hamilton, T.C. (2003) Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. *Cancer Res*, **63**, 1389-1397.

Costello, J.F. and Plass, C. (2001) Methylation matters. *J Med Genet*, **38**, 285-303.

Courjal, F., Cuny, M., Rodriguez, C., Louason, G., Speiser, P., Katsaros, D., Tanner, M.M., Zeillinger, R. and Theillet, C. (1996) DNA amplifications at 20q13 and MDM2 define distinct subsets of evolved breast and ovarian tumours. *Br J Cancer*, **74**, 1984-1989.

Cramer, D.W., Hutchison, G.B., Welch, W.R., Scully, R.E. and Ryan, K.J. (1983) Determinants of ovarian cancer risk. I. Reproductive experiences and family history. *J Natl Cancer Inst*, **71**, 711-716.

Cramer, D.W. and Welch, W.R. (1983) Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. *J Natl Cancer Inst*, **71**, 717-721.

Crew, J.P., Fuggle, S., Bicknell, R., Cranston, D.W., de Benedetti, A. and Harris, A.L. (2000) Eukaryotic initiation factor-4E in superficial and muscle invasive bladder cancer and its correlation with vascular endothelial growth factor expression and tumour progression. *Br J Cancer*, **82**, 161-166.

Dallol, A., Da Silva, N.F., Viacava, P., Minna, J.D., Bieche, I., Maher, E.R. and Latif, F. (2002) SLIT2, a human homologue of the Drosophila Slit2 gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers. *Cancer Res*, **62**, 5874-5880.

Davydova, E.K., Sitikov, A.S. and Ovchinnikov, L.P. (1984) Phosphorylation of elongation factor 1 in polyribosome fraction of rabbit reticulocytes. *FEBS Lett*, **176**, 401-405.

de Jong, M.M., Nolte, I.M., te Meerman, G.J., van der Graaf, W.T., Oosterwijk, J.C., Kleibeuker, J.H., Schaapveld, M. and de Vries, E.G. (2002) Genes other than BRCA1 and BRCA2 involved in breast cancer susceptibility. *J Med Genet*, **39**, 225-242.

Demma, M., Warren, V., Hock, R., Dharmawardhane, S. and Condeelis, J. (1990) Isolation of an abundant 50,000-dalton actin filament bundling protein from Dictyostelium amoebae. *J Biol Chem*, **265**, 2286-2291.

de Wit, N.J., Burtscher, H.J., Weidle, U.H., Ruiter, D.J. and van Muijen, G.N. (2002) Differentially expressed genes identified in human melanoma cell lines with different metastatic behaviour using high density oligonucleotide arrays. *Melanoma Res*, **12**, 57-69.

Dever, T.E., Costello, C.E., Owens, C.L., Rosenberry, T.L. and Merrick, W.C. (1989) Location of seven post-translational modifications in rabbit elongation factor 1 alpha including dimethyllysine, trimethyllysine, and glycerylphosphorylethanolamine. *J Biol Chem*, **264**, 20518-20525.

Dever, T.E., Glynias, M.J. and Merrick, W.C. (1987) GTP-binding domain: three consensus sequence elements with distinct spacing. *Proc Natl Acad Sci U S A*, **84**, 1814-1818.

Dharmawardhane, S., Demma, M., Yang, F. and Condeelis, J. (1991) Compartmentalization and actin binding properties of ABP-50: the elongation factor-1 alpha of Dictyostelium. *Cell Motil Cytoskeleton*, **20**, 279-288.

Diebold, J., Mosinger, K., Peiro, G., Pannekamp, U., Kaltz, C., Baretton, G.B., Meier, W. and Lohrs, U. (2000) 20q13 and cyclin D1 in ovarian carcinomas. Analysis by fluorescence in situ hybridization. *J Pathol*, **190**, 564-571.

Dje, M.K., Mazabraud, A., Viel, A., le Maire, M., Denis, H., Crawford, E. and Brown, D.D. (1990) Three genes under different developmental control encode elongation factor 1-alpha in Xenopus laevis. *Nucleic Acids Res*, **18**, 3489-3493.

Dobrovic, A. and Simpfendorfer, D. (1997) Methylation of the BRCA1 gene in sporadic breast cancer. *Cancer Res*, **57**, 3347-3350.

Doerfler, W. (1983) DNA methylation and gene activity. *Annu Rev Biochem*, **52**, 93-124.

Donehower, L.A., Godley, L.A., Aldaz, C.M., Pyle, R., Shi, Y.P., Pinkel, D., Gray, J., Bradley, A., Medina, D. and Varmus, H.E. (1995) Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. *Genes Dev*, **9**, 882-895.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S. and Bradley, A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, **356**, 215-221.

Douglas, E.J., Fiegler, H., Rowan, A., Halford, S., Bicknell, D.C., Bodmer, W., Tomlinson, I.P. and Carter, N.P. (2004) Array comparative genomic hybridization analysis of colorectal cancer cell lines and primary carcinomas. *Cancer Res*, **64**, 4817-4825.

Dubeau, L. (1999) The cell of origin of ovarian epithelial tumors and the ovarian surface epithelium dogma: does the emperor have no clothes? *Gynecol Oncol*, **72**, 437-442.

Dumitrescu, R.G. and Cotarla, I. (2005) Understanding breast cancer risk -- where do we stand in 2005? *J Cell Mol Med*, **9**, 208-221.

Durham, H.D., Roy, J., Dong, L. and Figlewicz, D.A. (1997) Aggregation of mutant Cu/Zn superoxide dismutase proteins in a culture model of ALS. *J Neuropathol Exp Neurol*, **56**, 523-530.

Durso, N.A. and Cyr, R.J. (1994) A calmodulin-sensitive interaction between microtubules and a higher plant homolog of elongation factor-1 alpha. *Plant Cell*, **6**, 893-905.

Duttaroy, A., Bourbeau, D., Wang, X.L. and Wang, E. (1998) Apoptosis rate can be accelerated or decelerated by overexpression or reduction of the level of elongation factor-1 alpha. *Exp Cell Res*, **238**, 168-176.

Edmonds, B.T. (1993) ABP50: an actin-binding elongation factor 1 alpha from Dictyostelium discoideum. *J Cell Biochem*, **52**, 134-139.

Edmonds, B.T., Murray, J. and Condeelis, J. (1995) pH regulation of the F-actin binding properties of Dictyostelium elongation factor 1 alpha. *J Biol Chem*, **270**, 15222-15230.

Edmonds, B.T., Wyckoff, J., Yeung, Y.G., Wang, Y., Stanley, E.R., Jones, J., Segall, J. and Condeelis, J. (1996) Elongation factor-1 alpha is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma. *J Cell Sci*, **109 (Pt 11)**, 2705-2714.

Edmondson, R.J., Monaghan, J.M. and Davies, B.R. (2002) The human ovarian surface epithelium is an androgen responsive tissue. *Br J Cancer*, **86**, 879-885.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, **411**, 494-498.

Fathalla, M.F. (1971) Incessant ovulation--a factor in ovarian neoplasia? *Lancet*, **2**, 163.

Fearon, E.R. and Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759-767.

Feeley, K.M. and Wells, M. (2001) Precursor lesions of ovarian epithelial malignancy. *Histopathology*, **38**, 87-95.

Ferrari, S. (2006) Protein kinases controlling the onset of mitosis. *Cell Mol Life Sci*, **63**, 781-795.

Filipowicz, W. (2005) RNAi: the nuts and bolts of the RISC machine. *Cell*, **122**, 17-20.

Fleming, J.S., Beaugie, C.R., Haviv, I., Chenevix-Trench, G. and Tan, O.L. (2006) Incessant ovulation, inflammation and epithelial ovarian carcinogenesis: revisiting old hypotheses. *Mol Cell Endocrinol*, **247**, 4-21.

Flesken-Nikitin, A., Choi, K.C., Eng, J.P., Shmidt, E.N. and Nikitin, A.Y. (2003) Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. *Cancer Res*, **63**, 3459-3463.

Fredrickson, T.N. (1987) Ovarian tumors of the hen. *Environ Health Perspect*, **73**, 35-51.

Fujita, M., Enomoto, T. and Murata, Y. (2003) Genetic alterations in ovarian carcinoma: with specific reference to histological subtypes. *Mol Cell Endocrinol*, **202**, 97-99.

Fujita, Y., Sakakura, C., Shimomura, K., Nakanishi, M., Yasuoka, R., Aragane, H., Hagiwara, A., Abe, T., Inazawa, J. and Yamagishi, H. (2003) Chromosome arm 20q gains and other genomic alterations in esophageal squamous cell carcinoma, as analyzed by comparative genomic hybridization and fluorescence in situ hybridization. *Hepatogastroenterology*, **50**, 1857-1863.

Futreal, P.A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L.M., Haugen-Strano, A., Swensen, J., Miki, Y. and et al. (1994) BRCA1 mutations in primary breast and ovarian carcinomas. *Science*, **266**, 120-122.

Gangwani, L., Mikrut, M., Galcheva-Gargova, Z. and Davis, R.J. (1998) Interaction of ZPR1 with translation elongation factor-1alpha in proliferating cells. *J Cell Biol*, **143**, 1471-1484.

Garber, J.E., Goldstein, A.M., Kantor, A.F., Dreyfus, M.G., Fraumeni, J.F., Jr. and Li, F.P. (1991) Follow-up study of twenty-four families with Li-Fraumeni syndrome. *Cancer Res*, **51**, 6094-6097.

Garcia, S.B., Park, H.S., Novelli, M. and Wright, N.A. (1999) Field cancerization, clonality, and epithelial stem cells: the spread of mutated clones in epithelial sheets. *J Pathol*, **187**, 61-81.

Gardiner-Garden, M. and Frommer, M. (1987) CpG islands in vertebrate genomes. *J Mol Biol*, **196**, 261-282.

Gilmour, L.M., Macleod, K.G., McCaig, A., Sewell, J.M., Gullick, W.J., Smyth, J.F. and Langdon, S.P. (2002) Neuregulin expression, function, and signaling in human ovarian cancer cells. *Clin Cancer Res*, **8**, 3933-3942.

Ginzinger, D.G., Godfrey, T.E., Nigro, J., Moore, D.H., 2nd, Suzuki, S., Pallavicini, M.G., Gray, J.W. and Jensen, R.H. (2000) Measurement of DNA copy number at microsatellite loci using quantitative PCR analysis. *Cancer Res*, **60**, 5405-5409.

Glass, A.G., Lacey, J.V., Hoover, R.N. (2005) The rise in breast cancer incidence , 1960-2003, is largely confined to ER+ tumours. *Proc Amer Assoc Cancer Res.* **46.**

Goff, B.A., Sainz de la Cuesta, R., Muntz, H.G., Fleischhacker, D., Ek, M., Rice, L.W., Nikrui, N., Tamimi, H.K., Cain, J.M., Greer, B.E. and Fuller, A.F., Jr. (1996) Clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy in stage III disease. *Gynecol Oncol*, **60**, 412-417.

Gonen, H., Smith, C.E., Siegel, N.R., Kahana, C., Merrick, W.C., Chakraburtty, K., Schwartz, A.L. and Ciechanover, A. (1994) Protein synthesis elongation factor EF-1 alpha is essential for ubiquitin-dependent degradation of certain N alpha-acetylated proteins and may be substituted for by the bacterial elongation factor EF-Tu. *Proc Natl Acad Sci U S A*, **91**, 7648-7652.

Gorai, I., Nakazawa, T., Miyagi, E., Hirahara, F., Nagashima, Y. and Minaguchi, H. (1995) Establishment and characterization of two human ovarian clear cell adenocarcinoma lines from metastatic lesions with different properties. *Gynecol Oncol*, **57**, 33-46.

Graff, J.R. and Zimmer, S.G. (2003) Translational control and metastatic progression: enhanced activity of the mRNA cap-binding protein eIF-4E selectively enhances translation of metastasis-related mRNAs. *Clin Exp Metastasis*, **20**, 265-273.

Grant, A.G., Flomen, R.M., Tizard, M.L. and Grant, D.A. (1992) Differential screening of a human pancreatic adenocarcinoma lambda gt11 expression library has identified increased transcription of elongation factor EF-1 alpha in tumour cells. *Int J Cancer*, **50**, 740-745.

Gross, S.R. and Kinzy, T.G. (2005) Translation elongation factor 1A is essential for regulation of the actin cytoskeleton and cell morphology. *Nat Struct Mol Biol*, **12**, 772-778.

Gudmundsdottir, K. and Ashworth, A. (2006) The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene*, **25**, 5864-5874.

Gupta, A., Godwin, A.K., Vanderveer, L., Lu, A. and Liu, J. (2003) Hypomethylation of the synuclein gamma gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. *Cancer Res*, **63**, 664-673.

Guy, C.T., Webster, M.A., Schaller, M., Parsons, T.J., Cardiff, R.D. and Muller, W.J. (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A*, **89**, 10578-10582.

Hamill, D., Davis, J., Drawbridge, J. and Suprenant, K.A. (1994) Polyribosome targeting to microtubules: enrichment of specific mRNAs in a reconstituted microtubule preparation from sea urchin embryos. *J Cell Biol*, **127**, 973-984.

Haydon, M.S., Googe, J.D., Sorrells, D.S., Ghali, G.E. and Li, B.D. (2000) Progression of eIF4e gene amplification and overexpression in benign and malignant tumors of the head and neck. *Cancer*, **88**, 2803-2810.

Heaphy, C.M., Bisoffi, M., Fordyce, C.A., Haaland, C.M., Hines, W.C., Joste, N.E. and Griffith, J.K. (2006) Telomere DNA content and allelic imbalance demonstrate field cancerization in histologically normal tissue adjacent to breast tumors. *Int J Cancer*, **119**, 108-116.

Hershey, J.W. (1991) Translational control in mammalian cells. *Annu Rev Biochem*, **60**, 717-755.

Hovemann, B., Richter, S., Walldorf, U. and Cziepluch, C. (1988) Two genes encode related cytoplasmic elongation factors 1 alpha (EF-1 alpha) in Drosophila melanogaster with continuous and stage specific expression. *Nucleic Acids Res*, **16**, 3175-3194.

Hundley, J.E., Koester, S.K., Troyer, D.A., Hilsenbeck, S.G., Subler, M.A. and Windle, J.J. (1997) Increased tumor proliferation and genomic instability without decreased apoptosis in MMTV-ras mice deficient in p53. *Mol Cell Biol*, **17**, 723-731.

Hurst, C.D., Fiegler, H., Carr, P., Williams, S., Carter, N.P. and Knowles, M.A. (2004) High-resolution analysis of genomic copy number alterations in bladder cancer by microarray-based comparative genomic hybridization. *Oncogene*, **23**, 2250-2263.

Hutchinson, J.N. and Muller, W.J. (2000) Transgenic mouse models of human breast cancer. *Oncogene*, **19**, 6130-6137.

Hough, C.D., Cho, K.R., Zonderman, A.B., Schwartz, D.R. and Morin, P.J. (2001) Coordinately up-regulated genes in ovarian cancer. *Cancer Res*, **61**, 3869-3876.

Ikeda, K., Sato, M., Tsutsumi, O., Tsuchiya, F., Tsuneizumi, M., Emi, M., Imoto, I., Inazawa, J., Muramatsu, M. and Inoue, S. (2000) Promoter analysis and chromosomal mapping of human EBAG9 gene. *Biochem Biophys Res Commun*, **273**, 654-660.

Inoue, S., Orimo, A., Hosoi, T., Kondo, S., Toyoshima, H., Kondo, T., Ikegami, A., Ouchi, Y., Orimo, H. and Muramatsu, M. (1993) Genomic binding-site cloning reveals an estrogen-responsive gene that encodes a RING finger protein. *Proc Natl Acad Sci U S A*, **90**, 11117-11121.

Isola, J.J., Kallioniemi, O.P., Chu, L.W., Fuqua, S.A., Hilsenbeck, S.G., Osborne, C.K. and Waldman, F.M. (1995) Genetic aberrations detected by comparative genomic hybridization predict outcome in node-negative breast cancer. *Am J Pathol*, **147**, 905-911.

Itamochi, H., Kigawa, J., Akeshima, R., Sato, S., Kamazawa, S., Takahashi, M., Kanamori, Y., Suzuki, M., Ohwada, M. and Terakawa, N. (2002) Mechanisms of cisplatin resistance in clear cell carcinoma of the ovary. *Oncology*, **62**, 349-353.

Jaffer, Z.M., Chernoff, J. (2002) p21-activated kinases: three more join the Pak. *The Int J of Biochem and Cell Biol.* **34**, 713-717.

Janeway, C.A.T., Paul; Walport, Mark; Shlomchik, Mark. (2001) *Immunobiology*. Garland Science, New York and London:.

Jiang, X., Hitchcock, A., Bryan, E.J., Watson, R.H., Englefield, P., Thomas, E.J. and Campbell, I.G. (1996) Microsatellite analysis of endometriosis reveals loss of heterozygosity at candidate ovarian tumor suppressor gene loci. *Cancer Res*, **56**, 3534-3539.

Jiang, X., Morland, S.J., Hitchcock, A., Thomas, E.J. and Campbell, I.G. (1998) Allelotyping of endometriosis with adjacent ovarian carcinoma reveals evidence of a common lineage. *Cancer Res*, **58**, 1707-1712.

Jolly, C., Usson, Y. and Morimoto, R.I. (1999) Rapid and reversible relocalization of heat shock factor 1 within seconds to nuclear stress granules. *Proc Natl Acad Sci U S A*, **96**, 6769-6774.

Joseph, P., O'Kernick, C.M., Othumpangat, S., Lei, Y.X., Yuan, B.Z. and Ong, T.M. (2004) Expression profile of eukaryotic translation factors in human cancer tissues and cell lines. *Mol Carcinog*, **40**, 171-179.

Joseph, P., Lei, Y.X., Whong, W.Z. and Ong, T.M. (2002) Oncogenic potential of mouse translation elongation factor-1 delta, a novel cadmium-responsive proto-oncogene. *J Biol Chem*, **277**, 6131-6136.

Kahns, S., Lund, A., Kristensen, P., Knudsen, C.R., Clark, B.F., Cavallius, J. and Merrick, W.C. (1998) The elongation factor 1 A-2 isoform from rabbit: cloning of the cDNA and characterization of the protein. *Nucleic Acids Res*, **26**, 1884-1890.

Kallioniemi, A., Kallioniemi, O.P., Piper, J., Tanner, M., Stokke, T., Chen, L., Smith, H.S., Pinkel, D., Gray, J.W. and Waldman, F.M. (1994) Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proc Natl Acad Sci U S A*, **91**, 2156-2160.

Kamalati, T., Jolin, H.E., Mitchell, P.J., Barker, K.T., Jackson, L.E., Dean, C.J., Page, M.J., Gusterson, B.A. and Crompton, M.R. (1996) Brk, a breast tumor-derived non-receptor protein-

tyrosine kinase, sensitizes mammary epithelial cells to epidermal growth factor. *J Biol Chem*, **271**, 30956-30963.

Karlan, B.Y., Jones, J., Greenwald, M. and Lagasse, L.D. (1995) Steroid hormone effects on the proliferation of human ovarian surface epithelium in vitro. *Am J Obstet Gynecol*, **173**, 97-104.

Kaspar, R.L., Kakegawa, T., Cranston, H., Morris, D.R. and White, M.W. (1992) A regulatory cis element and a specific binding factor involved in the mitogenic control of murine ribosomal protein L32 translation. *J Biol Chem*, **267**, 508-514.

Kato, M.V., Sato, H., Nagayoshi, M. and Ikawa, Y. (1997) Upregulation of the elongation factor-1alpha gene by p53 in association with death of an erythroleukemic cell line. *Blood*, **90**, 1373-1378.

Kato, S., Sekine, S., Oh, S.W., Kim, N.S., Umezawa, Y., Abe, N., Yokoyama-Kobayashi, M. and Aoki, T. (1994) Construction of a human full-length cDNA bank. *Gene*, **150**, 243-250.

Kenemans, P., Verstraeten, R.A. and Verheijen, R.H. (2004) Oncogenic pathways in hereditary and sporadic breast cancer. *Maturitas*, **49**, 34-43.

Kerangueven, F., Noguchi, T., Coulier, F., Allione, F., Wargniez, V., Simony-Lafontaine, J., Longy, M., Jacquemier, J., Sobol, H., Eisinger, F. and Birnbaum, D. (1997) Genome-wide search for loss of heterozygosity shows extensive genetic diversity of human breast carcinomas. *Cancer Res*, **57**, 5469-5474.

Khalyfa, A., Bourbeau, D., Chen, E., Petroulakis, E., Pan, J., Xu, S. and Wang, E. (2001) Characterization of elongation factor-1A (eEF1A-1) and eEF1A-2/S1 protein expression in normal and wasted mice. *J Biol Chem*, **276**, 22915-22922.

King, C.R., Kraus, M.H. and Aaronson, S.A. (1985) Amplification of a novel v-erbB-related gene in a human mammary carcinoma. *Science*, **229**, 974-976.

Kisseljova, N.P. and Kisseljov, F.L. (2005) DNA demethylation and carcinogenesis. *Biochemistry (Mosc)*, **70**, 743-752.

Kitoh, H., Ryozawa, S., Harada, T., Kondoh, S., Furuya, T., Kawauchi, S., Oga, A., Okita, K. and Sasaki, K. (2005) Comparative genomic hybridization analysis for pancreatic cancer specimens obtained by endoscopic ultrasonography-guided fine-needle aspiration. *J Gastroenterol*, **40**, 511-517.

Klein-Hitpass, L., Schorpp, M., Wagner, U. and Ryffel, G.U. (1986) An estrogen-responsive element derived from the 5' flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells. *Cell*, **46**, 1053-1061.

Knudsen, S.M., Frydenberg, J., Clark, B.F. and Leffers, H. (1993) Tissue-dependent variation in the expression of elongation factor-1 alpha isoforms: isolation and characterisation of a cDNA encoding a novel variant of human elongation-factor 1 alpha. *Eur J Biochem*, **215**, 549-554.

Knuutila, S., Bjorkqvist, A.M., Autio, K., Tarkkanen, M., Wolf, M., Monni, O., Szymanska, J., Larramendy, M.L., Tapper, J., Pere, H., El-Rifai, W., Hemmer, S., Wasenius, V.M., Vidgren, V. and Zhu, Y. (1998) DNA copy number amplifications in human neoplasms: review of comparative genomic hybridization studies. *Am J Pathol*, **152**, 1107-1123.

Kong, Q. and Lillehei, K.O. (1998) Antioxidant inhibitors for cancer therapy. *Med Hypotheses*, **51**, 405-409.

Krieg, P.A., Varnum, S.M., Wormington, W.M. and Melton, D.A. (1989) The mRNA encoding elongation factor 1-alpha (EF-1 alpha) is a major transcript at the midblastula transition in Xenopus. *Dev Biol*, **133**, 93-100.

Kristensen, P., Lund, A., Clark, B.F., Cavallius, J. and Merrick, W.C. (1998) Purification and characterisation of a tissue specific elongation factor 1 alpha (EF-1 alpha 2) from rabbit muscle. *Biochem Biophys Res Commun*, **245**, 810-814.

Kuang, W.W., Thompson, D.A., Hoch, R.V. and Weigel, R.J. (1998) Differential screening and suppression subtractive hybridization identified genes differentially expressed in an estrogen receptor-positive breast carcinoma cell line. *Nucleic Acids Res*, **26**, 1116-1123.

Kulkarni, G., Turbin, D.A., Amiri, A., Jeganathan, S., Andrade-Navarro, M.A., Wu, T.D., Huntsman, D.G. and Lee, J.M. (2006) Expression of protein elongation factor eEF1A2 predicts favorable outcome in breast cancer. *Breast Cancer Res Treat*.

Kuriyama, R., Savereide, P., Lefebvre, P. and Dasgupta, S. (1990) The predicted amino acid sequence of a centrosphere protein in dividing sea urchin eggs is similar to elongation factor (EF-1 alpha). *J Cell Sci*, **95 (Pt 2)**, 231-236.

Kusakari, T., Kariya, M., Mandai, M., Tsuruta, Y., Hamid, A.A., Fukuhara, K., Nanbu, K., Takakura, K. and Fujii, S. (2003) C-erbB-2 or mutant Ha-ras induced malignant transformation of immortalized human ovarian surface epithelial cells in vitro. *Br J Cancer*, **89**, 2293-2298.

Kushner, P.J., Agard, D.A., Greene, G.L., Scanlan, T.S., Shiau, A.K., Uht, R.M. and Webb, P. (2000) Estrogen receptor pathways to AP-1. *J Steroid Biochem Mol Biol*, **74**, 311-317.

Lacroix, M., Toillon, R.A. and Leclercq, G. (2006) p53 and breast cancer, an update. *Endocr Relat Cancer*, **13**, 293-325.

Lakhani, S.R. (1999) The transition from hyperplasia to invasive carcinoma of the breast. *J Pathol*, **187**, 272-278.

Lancaster, J.M., Wooster, R., Mangion, J., Phelan, C.M., Cochran, C., Gumbs, C., Seal, S., Barfoot, R., Collins, N., Bignell, G., Patel, S., Hamoudi, R., Larsson, C., Wiseman, R.W., Berchuck, A., Iglehart, J.D., Marks, J.R., Ashworth, A., Stratton, M.R. and Futreal, P.A. (1996) BRCA2 mutations in primary breast and ovarian cancers. *Nat Genet*, **13**, 238-240.

Lassus, H., Leminen, A., Vayrynen, A., Cheng, G., Gustafsson, J.A., Isola, J. and Butzow, R. (2004) ERBB2 amplification is superior to protein expression status in predicting patient outcome in serous ovarian carcinoma. *Gynecol Oncol*, **92**, 31-39.

Lau, J., Castelli, L.A., Lin, E.C. and Macaulay, S.L. (2006) Identification of elongation factor 1alpha as a potential associated binding partner for Akt2. *Mol Cell Biochem*, **286**, 17-22.

Lazaris-Karatzas, A., Montine, K.S. and Sonenberg, N. (1990) Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature*, **345**, 544-547.

Lazaris-Karatzas, A., Smith, M.R., Frederickson, R.M., Jaramillo, M.L., Liu, Y.L., Kung, H.F. and Sonenberg, N. (1992) Ras mediates translation initiation factor 4E-induced malignant transformation. *Genes Dev*, **6**, 1631-1642.

Lee, S., Ann, D.K. and Wang, E. (1994) Cloning of human and mouse brain cDNAs coding for S1, the second member of the mammalian elongation factor-1 alpha gene family: analysis of a possible evolutionary pathway. *Biochem Biophys Res Commun*, **203**, 1371-1377.

Lee, S., Francoeur, A.M., Liu, S. and Wang, E. (1992) Tissue-specific expression in mammalian brain, heart, and muscle of S1, a member of the elongation factor-1 alpha gene family. *J Biol Chem*, **267**, 24064-24068.

Lee, S., LeBlanc, A., Duttaroy, A. and Wang, E. (1995) Terminal differentiation-dependent alteration in the expression of translation elongation factor-1 alpha and its sister gene, S1, in neurons. *Exp Cell Res*, **219**, 589-597.

Lee, S., Stollar, E. and Wang, E. (1993a) Localization of S1 and elongation factor-1 alpha mRNA in rat brain and liver by non-radioactive in situ hybridization. *J Histochem Cytochem*, **41**, 1093-1098.

Lee, S., Wolfraim, L.A. and Wang, E. (1993b) Differential expression of S1 and elongation factor-1 alpha during rat development. *J Biol Chem*, **268**, 24453-24459.

Lerebours, F. and Lidereau, R. (2002) Molecular alterations in sporadic breast cancer. *Crit Rev Oncol Hematol*, **44**, 121-141.

Levesque, M.A., Diamandis, E.P., Yu, H. and Sutherland, D.J. (1994) Quantitative analysis of mutant p53 protein in breast tumor cytosols and study of its association with other biochemical prognostic indicators in breast cancer. *Breast Cancer Res Treat*, **30**, 179-195.

Levine, A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323-331.

Li, B., Rosen, J.M., McMenamin-Balano, J., Muller, W.J. and Perkins, A.S. (1997a) neu/ERBB2 cooperates with p53-172H during mammary tumorigenesis in transgenic mice. *Mol Cell Biol*, **17**, 3155-3163.

Li, B.D., Liu, L., Dawson, M. and De Benedetti, A. (1997b) Overexpression of eukaryotic initiation factor 4E (eIF4E) in breast carcinoma. *Cancer*, **79**, 2385-2390.

Li, R., Wang, H., Bekele, B.N., Yin, Z., Caraway, N.P., Katz, R.L., Stass, S.A. and Jiang, F. (2006) Identification of putative oncogenes in lung adenocarcinoma by a comprehensive functional genomic approach. *Oncogene*, **25**, 2628-2635.

Liu, G., Grant, W.M., Persky, D., Latham, V.M., Jr., Singer, R.H. and Condeelis, J. (2002) Interactions of elongation factor 1alpha with F-actin and beta-actin mRNA: implications for anchoring mRNA in cell protrusions. *Mol Biol Cell*, **13**, 579-592.

Liu, G., Tang, J., Edmonds, B.T., Murray, J., Levin, S. and Condeelis, J. (1996) F-actin sequesters elongation factor 1alpha from interaction with aminoacyl-tRNA in a pH-dependent reaction. *J Cell Biol*, **135**, 953-963.

Liu, J., Yang, G., Thompson-Lanza, J.A., Glassman, A., Hayes, K., Patterson, A., Marquez, R.T., Auersperg, N., Yu, Y., Hahn, W.C., Mills, G.B. and Bast, R.C., Jr. (2004) A genetically defined model for human ovarian cancer. *Cancer Res*, **64**, 1655-1663.

Liu, M., Mao, S-Y., Zhu, W-Y. (2006) Impact of tiny miRNAs on cancers. *World J Gastroenterology*, **13(4)**, 497-502.

Lund, A., Knudsen, S.M., Vissing, H., Clark, B. and Tommerup, N. (1996) Assignment of human elongation factor 1alpha genes: EEF1A maps to chromosome 6q14 and EEF1A2 to 20q13.3. *Genomics*, **36**, 359-361.

Madsen, H.O., Poulsen, K., Dahl, O., Clark, B.F. and Hjorth, J.P. (1990) Retropseudogenes constitute the major part of the human elongation factor 1 alpha gene family. *Nucleic Acids Res*, **18**, 1513-1516.

Maga, G. and Hübscher, S. (2003) Proliferating cell nuclear antigen: a dancer with many partners. *J of Cell Sci*, **116**, 3051-3060.

Mano, M.S., Awada, A., Di Leo, A., Durbecq, V., Paesmans, M., Cardoso, F., Larsimont, D. and Piccart, M. (2004) Rates of topoisomerase II-alpha and HER-2 gene amplification and expression in epithelial ovarian carcinoma. *Gynecol Oncol*, **92**, 887-895.

Mansilla, F., Friis, I., Jadidi, M., Nielsen, K.M., Clark, B.F. and Knudsen, C.R. (2002) Mapping the human translation elongation factor eEF1H complex using the yeast two-hybrid system. *Biochem J*, **365**, 669-676.

Mansilla, F., Hansen, L.L., Jakobsen, H., Kjeldgaard, N.O., Clark, B.F. and Knudsen, C.R. (2005) Deconstructing PTI-1: PTI-1 is a truncated, but not mutated, form of translation elongation factor 1A1, eEF1A1. *Biochim Biophys Acta*, **1727**, 116-124.

Martelli, A.M., Follo, M.Y., Evangelisti, C., Fala, F., Fiume, R., Billi, A.M., Cocco, L. (2005) Nuclear inositol lipid metabolism: more than just second messenger generation? *J of Cell Biochem*, **96**, 285-292.

Matei, D., Graeber, T.G., Baldwin, R.L., Karlan, B.Y., Rao, J. and Chang, D.D. (2002) Gene expression in epithelial ovarian carcinoma. *Oncogene*, **21**, 6289-6298.

May, F.E., Johnson, M.D., Wiseman, L.R., Wakeling, A.E., Kastner, P. and Westley, B.R. (1989) Regulation of progesterone receptor mRNA by oestradiol and antioestrogens in breast cancer cell lines. *J Steroid Biochem*, **33**, 1035-1041.

Mayer, M.P. and Bukau, B. (2005) Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci*, **62**, 670-684.

Mazars, R., Spinardi, L., BenCheikh, M., Simony-Lafontaine, J., Jeanteur, P. and Theillet, C. (1992) p53 mutations occur in aggressive breast cancer. *Cancer Res*, **52**, 3918-3923.

McClatchy, D.B., Knudsen, C.R., Clark, B.F., Kahn, R.A., Hall, R.A. and Levey, A.I. (2002) Novel interaction between the M4 muscarinic acetylcholine receptor and elongation factor 1A2. *J Biol Chem*, **277**, 29268-29274.

Merrick, W.C. (1992) Mechanism and regulation of eukaryotic protein synthesis. *Microbiol Rev*, **56**, 291-315.

Meyer, T. and Rustin, G.J. (2000) Role of tumour markers in monitoring epithelial ovarian cancer. *Br J Cancer*, **82**, 1535-1538.

Mickleburgh, I., Chabanon, H., Nury, D., Fan, K., Burtle, B., Chrzanowska-Lightowlers, Z. and Hesketh, J. (2006) Elongation factor 1alpha binds to the region of the metallothionein-1 mRNA implicated in perinuclear localization--importance of an internal stem-loop. *Rna*, **12**, 1397-1407.

Migeon, B.R. (1994) X-chromosome inactivation: molecular mechanisms and genetic consequences. *Trends Genet*, **10**, 230-235.

Mignone, F., Gissi, C., Liuni, S. and Pesole, G. (2002) Untranslated regions of mRNAs. *Genome Biol*, **3**, REVIEWS0004.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M. and Ding, W. (1994) A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science*, **266**, 66-71.

Miller, W.R., White, S., Dixon, J.M., Murray, J., Renshaw, L. and Anderson, T.J. (2006) Proliferation, steroid receptors and clinical/pathological response in breast cancer treated with letrozole. *Br J Cancer*, **94**, 1051-1056.

Millis, R.R. (1980) Correlation of hormone receptors with pathological features in human breast cancer. *Cancer*, **46**, 2869-2871.

Mimori, K., Mori, M., Inoue, H., Ueo, H., Mafune, K., Akiyoshi, T. and Sugimachi, K. (1996) Elongation factor 1 gamma mRNA expression in oesophageal carcinoma. *Gut*, **38**, 66-70.

Mimori, K., Mori, M., Tanaka, S., Akiyoshi, T. and Sugimachi, K. (1995) The overexpression of elongation factor 1 gamma mRNA in gastric carcinoma. *Cancer*, **75**, 1446-1449.

Mita, K., Morimyo, M., Ito, K., Sugaya, K., Ebihara, K., Hongo, E., Higashi, T., Hirayama, Y. and Nakamura, Y. (1997) Comprehensive cloning of Schizosaccharomyces pombe genes encoding translation elongation factors. *Gene*, **187**, 259-266.

Mohler, J.L., Morris, T.L., Ford, O.H., 3rd, Alvey, R.F., Sakamoto, C. and Gregory, C.W. (2002) Identification of differentially expressed genes associated with androgen-independent growth of prostate cancer. *Prostate*, **51**, 247-255.

Mok, S.C., Bell, D.A., Knapp, R.C., Fishbaugh, P.M., Welch, W.R., Muto, M.G., Berkowitz, R.S. and Tsao, S.W. (1993) Mutation of K-ras protooncogene in human ovarian epithelial tumors of borderline malignancy. *Cancer Res*, **53**, 1489-1492.

Moore, M.A. (2001) The role of chemoattraction in cancer metastases. *Bioessays*, **23**, 674-676.

Moore, R.C. and Cyr, R.J. (2000) Association between elongation factor-1alpha and microtubules in vivo is domain dependent and conditional. *Cell Motil Cytoskeleton*, **45**, 279-292.

Moore, R.C., Durso, N.A. and Cyr, R.J. (1998) Elongation factor-1alpha stabilizes microtubules in a calcium/calmodulin-dependent manner. *Cell Motil Cytoskeleton*, **41**, 168-180.

Moretti-Rojas, I., Fuqua, S.A., Montgomery, R.A., 3rd and McGuire, W.L. (1988) A cDNA for the estradiol-regulated 24K protein: control of mRNA levels in MCF-7 cells. *Breast Cancer Res Treat*, **11**, 155-163.

Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M.E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S.N., Barrera, J.L., Mohar, A., Verastegui, E. and Zlotnik, A. (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature*, **410**, 50-56.

Muller, W.J., Sinn, E., Pattengale, P.K., Wallace, R. and Leder, P. (1988) Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell*, **54**, 105-115.

Munshi, R., Kandl, K.A., Carr-Schmid, A., Whitacre, J.L., Adams, A.E. and Kinzy, T.G. (2001) Overexpression of translation elongation factor 1A affects the organization and function of the actin cytoskeleton in yeast. *Genetics*, **157**, 1425-1436.

Murdoch, W.J. and McDonnel, A.C. (2002) Roles of the ovarian surface epithelium in ovulation and carcinogenesis. *Reproduction*, **123**, 743-750.

Negrutskii, B.S. and Deutscher, M.P. (1991) Channeling of aminoacyl-tRNA for protein synthesis in vivo. *Proc Natl Acad Sci U S A*, **88**, 4991-4995.

Negrutskii, B.S., Stapulionis, R. and Deutscher, M.P. (1994) Supramolecular organization of the mammalian translation system. *Proc Natl Acad Sci U S A*, **91**, 964-968.

Ness, R.B. and Cottreau, C. (1999) Possible role of ovarian epithelial inflammation in ovarian cancer. *J Natl Cancer Inst*, **91**, 1459-1467.

Nonet, G.H., Stampfer, M.R., Chin, K., Gray, J.W., Collins, C.C. and Yaswen, P. (2001) The ZNF217 gene amplified in breast cancers promotes immortalization of human mammary epithelial cells. *Cancer Res*, **61**, 1250-1254.

Ohta, K., Toriyama, M., Miyazaki, M., Murofushi, H., Hosoda, S., Endo, S. and Sakai, H. (1990) The mitotic apparatus-associated 51-kDa protein from sea urchin eggs is a GTP-binding protein and is immunologically related to yeast polypeptide elongation factor 1 alpha. *J Biol Chem*, **265**, 3240-3247.

Okada, S., Tsuda, H., Takarabe, T., Yoshikawa, H., Taketani, Y. and Hirohashi, S. (2002) Allelotype analysis of common epithelial ovarian cancers with special reference to comparison between clear cell adenocarcinoma with other histological types. *Jpn J Cancer Res*, **93**, 798-806.

Okuda, T., Otsuka, J., Sekizawa, A., Saito, H., Makino, R., Kushima, M., Farina, A., Kuwano, Y. and Okai, T. (2003) p53 mutations and overexpression affect prognosis of ovarian endometrioid cancer but not clear cell cancer. *Gynecol Oncol*, **88**, 318-325.

O'Lone, R., Frith, M.C., Karlsson, E.K. and Hansen, U. (2004) Genomic targets of nuclear estrogen receptors. *Mol Endocrinol*, **18**, 1859-1875.

Omoto, Y., Kobayashi, S., Inoue, S., Ogawa, S., Toyama, T., Yamashita, H., Muramatsu, M., Gustafsson, J.A. and Iwase, H. (2002) Evaluation of oestrogen receptor beta wild-type and variant protein expression, and relationship with clinicopathological factors in breast cancers. *Eur J Cancer*, **38**, 380-386.

Orsulic, S., Li, Y., Soslow, R.A., Vitale-Cross, L.A., Gutkind, J.S. and Varmus, H.E. (2002) Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. *Cancer Cell*, **1**, 53-62.

Osaki, M., Oshimura, M. and Ito, H. (2004) PI3K-Akt pathway: its functions and alterations in human cancer. *Apoptosis*, **9**, 667-676.

Owen, C.H., DeRosier, D.J. and Condeelis, J. (1992) Actin crosslinking protein EF-1a of Dictyostelium discoideum has a unique bonding rule that allows square-packed bundles. *J Struct Biol*, **109**, 248-254.

Ozen, M., Multani, A.S., Kuniyasu, H., Chung, L.W., von Eschenbach, A.C. and Pathak, S. (1997) Specific histologic and cytogenetic evidence for in vivo malignant transformation of murine host cells by three human prostate cancer cell lines. *Oncol Res*, **9**, 433-438.

Pan, J., Ruest, L.B., Xu, S. and Wang, E. (2004) Immuno-characterization of the switch of peptide elongation factors eEF1A-1/EF-1alpha and eEF1A-2/S1 in the central nervous system during mouse development. *Brain Res Dev Brain Res*, **149**, 1-8.

Parkin, D.M. (2004) International variation. *Oncogene*, **23**, 6329-6340.

Parkin, D.M., Pisani, P. and Ferlay, J. (1999) Global cancer statistics. *CA Cancer J Clin*, **49**, 33-64, 31.

Peters, H.I., Chang, Y.W. and Traugh, J.A. (1995) Phosphorylation of elongation factor 1 (EF-1) by protein kinase C stimulates GDP/GTP-exchange activity. *Eur J Biochem*, **234**, 550-556.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*, **29**, e45.

Pizzuti, A., Gennarelli, M., Novelli, G., Colosimo, A., Lo Cicero, S., Caskey, C.T. and Dallapiccola, B. (1993) Human elongation factor EF-1 beta: cloning and characterization of the EF1 beta 5a gene

and assignment of EF-1 beta isoforms to chromosomes 2,5,15 and X. *Biochem Biophys Res Commun*, **197**, 154-162.

Potter, M., Bernstein, A. and Lee, J.M. (1998) The wst gene regulates multiple forms of thymocyte apoptosis. *Cell Immunol*, **188**, 111-117.

Prat, J., Ribe, A. and Gallardo, A. (2005) Hereditary ovarian cancer. *Hum Pathol*, **36**, 861-870.

Purdie, D.M., Bain, C.J., Siskind, V., Webb, P.M. and Green, A.C. (2003) Ovulation and risk of epithelial ovarian cancer. *Int J Cancer*, **104**, 228-232.

Radisavljevic, S.V. (1977) The pathogenesis of ovarian inclusion cysts and cystomas. *Obstet Gynecol*, **49**, 424-429.

Reed, E., Yu, J.J., Davies, A., Gannon, J. and Armentrout, S.L. (2003) Clear cell tumors have higher mRNA levels of ERCC1 and XPB than other histological types of epithelial ovarian cancer. *Clin Cancer Res*, **9**, 5299-5305.

Rice, P., Longden, I. and Bleasby, A. (2000) EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet*, **16**, 276-277.

Riggs, A.D. and Jones, P.A. (1983) 5-methylcytosine, gene regulation, and cancer. *Adv Cancer Res*, **40**, 1-30.

Riman, T., Nilsson, S. and Persson, I.R. (2004) Review of epidemiological evidence for reproductive and hormonal factors in relation to the risk of epithelial ovarian malignancies. *Acta Obstet Gynecol Scand*, **83**, 783-795.

Risch, H.A. (1998) Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. *J Natl Cancer Inst*, **90**, 1774-1786.

Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X. and et al. (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*, **362**, 59-62.

Rosenwald, I.B., Hutzler, M.J., Wang, S., Savas, L. and Fraire, A.E. (2001) Expression of eukaryotic translation initiation factors 4E and 2alpha is increased frequently in bronchioloalveolar but not in squamous cell carcinomas of the lung. *Cancer*, **92**, 2164-2171.

Rubin, S.C., Finstad, C.L., Federici, M.G., Scheiner, L., Lloyd, K.O. and Hoskins, W.J. (1994) Prevalence and significance of HER-2/neu expression in early epithelial ovarian cancer. *Cancer*, **73**, 1456-1459.

Ruest, L.B., Marcotte, R. and Wang, E. (2002) Peptide elongation factor eEF1A-2/S1 expression in cultured differentiated myotubes and its protective effect against caspase-3-mediated apoptosis. *J Biol Chem*, **277**, 5418-5425.

Runnebaum, I.B. and Stickeler, E. (2001) Epidemiological and molecular aspects of ovarian cancer risk. *J Cancer Res Clin Oncol*, **127**, 73-79.

Russell, P. (1979) The pathological assessment of ovarian neoplasms. I: Introduction to the common 'epithelial' tumours and analysis of benign 'epithelial' tumours. *Pathology*, **11**, 5-26.

Safe, S. (2001) Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. *Vitam Horm*, **62**, 231-252.

Sanders, J., Brandsma, M., Janssen, G.M., Dijk, J. and Moller, W. (1996) Immunofluorescence studies of human fibroblasts demonstrate the presence of the complex of elongation factor-1 beta gamma delta in the endoplasmic reticulum. *J Cell Sci*, **109 (Pt 5)**, 1113-1117.

Sanders, J., Maassen, J.A. and Moller, W. (1992) Elongation factor-1 messenger-RNA levels in cultured cells are high compared to tissue and are not drastically affected further by oncogenic transformation. *Nucleic Acids Res*, **20**, 5907-5910.

Schildkraut, J.M., Schwingl, P.J., Bastos, E., Evanoff, A. and Hughes, C. (1996) Epithelial ovarian cancer risk among women with polycystic ovary syndrome. *Obstet Gynecol*, **88**, 554-559.

Schirmaier, F. and Philippsen, P. (1984) Identification of two genes coding for the translation elongation factor EF-1 alpha of S. cerevisiae. *Embo J*, **3**, 3311-3315.

Schwartz, D.R., Kardia, S.L., Shedden, K.A., Kuick, R., Michailidis, G., Taylor, J.M., Misek, D.E., Wu, R., Zhai, Y., Darrah, D.M., Reed, H., Ellenson, L.H., Giordano, T.J., Fearon, E.R., Hanash, S.M. and Cho, K.R. (2002) Gene expression in ovarian cancer reflects both morphology and biological behavior, distinguishing clear cell from other poor-prognosis ovarian carcinomas. *Cancer Res*, **62**, 4722-4729.

Shackney, S.E. and Silverman, J.F. (2003) Molecular evolutionary patterns in breast cancer. *Adv Anat Pathol*, **10**, 278-290.

Shamovsky, I., Ivannikov, M., Kandel, E.S., Gershon, D. and Nudler, E. (2006) RNA-mediated response to heat shock in mammalian cells. *Nature*, **440**, 556-560.

Shen, R., Su, Z.Z., Olsson, C.A. and Fisher, P.B. (1995) Identification of the human prostatic carcinoma oncogene PTI-1 by rapid expression cloning and differential RNA display. *Proc Natl Acad Sci U S A*, **92**, 6778-6782.

Shibui-Nihei, A., Ohmori, Y., Yoshida, K., Imai, J., Oosuga, I., Iidaka, M., Suzuki, Y., Mizushima-Sugano, J., Yoshitomo-Nakagawa, K. and Sugano, S. (2003) The 5' terminal oligopyrimidine tract of human elongation factor 1A-1 gene functions as a transcriptional initiator and produces a variable number of Us at the transcriptional level. *Gene*, **311**, 137-145.

Shih Ie, M. and Kurman, R.J. (2004) Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. *Am J Pathol*, **164**, 1511-1518.

Shiina, N., Gotoh, Y., Kubomura, N., Iwamatsu, A. and Nishida, E. (1994) Microtubule severing by elongation factor 1 alpha. *Science*, **266**, 282-285.

Shimizu, M., Nikaido, T., Toki, T., Shiozawa, T. and Fujii, S. (1999) Clear cell carcinoma has an expression pattern of cell cycle regulatory molecules that is unique among ovarian adenocarcinomas. *Cancer*, **85**, 669-677.

Shlomit, R., Ayala, A.G., Michal, D., Ninett, A., Frida, S., Boleslaw, G., Gad, B., Gideon, R. and Shlomi, C. (2000) Gains and losses of DNA sequences in childhood brain tumors analyzed by comparative genomic hybridization. *Cancer Genet Cytogenet*, **121**, 67-72.

Shuda, M., Kondoh, N., Tanaka, K., Ryo, A., Wakatsuki, T., Hada, A., Goseki, N., Igari, T., Hatsuse, K., Aihara, T., Horiuchi, S., Shichita, M., Yamamoto, N. and Yamamoto, M. (2000) Enhanced expression of translation factor mRNAs in hepatocellular carcinoma. *Anticancer Res*, **20**, 2489-2494.

Shultz, L.D., Sweet, H.O., Davisson, M.T. and Coman, D.R. (1982) 'Wasted', a new mutant of the mouse with abnormalities characteristic to ataxia telangiectasia. *Nature*, **297**, 402-404.

Simpson, P.T., Reis-Filho, J.S., Gale, T. and Lakhani, S.R. (2005) Molecular evolution of breast cancer. *J Pathol*, **205**, 248-254.

Singleton, T.P., Perrone, T., Oakley, G., Niehans, G.A., Carson, L., Cha, S.S. and Strickler, J.G. (1994) Activation of c-erbB-2 and prognosis in ovarian carcinoma. Comparison with histologic type, grade, and stage. *Cancer*, **73**, 1460-1466.

Slaughter, D.P., Southwick, H.W. and Smejkal, W. (1953) Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer*, **6**, 963-968.

Sledz, C.A., Holko, M., de Veer, M.J., Silverman, R.H. and Williams, B.R. (2003) Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol*, **5**, 834-839.

Small, J.V., Stradal, T., Vignal, E. and Rottner, K. (2002) The lamellipodium: where motility begins. *Trends Cell Biol*, **12**, 112-120.

Song, H., Parsons, M.R., Roswell, S., Leonard, G., Phillips, S.E. (1999) Crystal structure of intact elongation factor EF-Tu from Escherichia coli in GDP conformation at 2.05 A resolution. *J Mol Biol*, **285(3)**, 1245-56.

Song, J.M., Picologlou, S., Grant, C.M., Firoozan, M., Tuite, M.F. and Liebman, S. (1989) Elongation factor EF-1 alpha gene dosage alters translational fidelity in Saccharomyces cerevisiae. *Mol Cell Biol*, **9**, 4571-4575.

Sonoda, G., Palazzo, J., du Manoir, S., Godwin, A.K., Feder, M., Yakushiji, M. and Testa, J.R. (1997) Comparative genomic hybridization detects frequent overrepresentation of chromosomal material from 3q26, 8q24, and 20q13 in human ovarian carcinomas. *Genes Chromosomes Cancer*, **20**, 320-328.

Sorlie, T. (2004) Molecular portraits of breast cancer: tumour subtypes as distinct disease entities. *Eur J Cancer*, **40**, 2667-2675.

Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Thorsen, T., Quist, H., Matese, J.C., Brown, P.O., Botstein, D., Eystein Lonning, P. and Borresen-Dale, A.L. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*, **98**, 10869-10874.

Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J.S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., Demeter, J., Perou, C.M., Lonning, P.E., Brown, P.O., Borresen-Dale, A.L. and Botstein, D. (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A*, **100**, 8418-8423.

Sorrells, D.L., Meschonat, C., Black, D. and Li, B.D. (1999) Pattern of amplification and overexpression of the eukaryotic initiation factor 4E gene in solid tumor. *J Surg Res*, **85**, 37-42.

Speir, E., Yu, Z.X., Takeda, K., Ferrans, V.J. and Cannon, R.O., 3rd. (2000) Competition for p300 regulates transcription by estrogen receptors and nuclear factor-kappaB in human coronary smooth muscle cells. *Circ Res*, **87**, 1006-1011.

Stewart, S.L., King, J.B., Thompson, T.D., Friedman, C. and Wingo, P.A. (2004) Cancer mortality surveillance--United States, 1990-2000. *MMWR Surveill Summ*, **53**, 1-108.

Stojdl, D.F., Lichty, B., Knowles, S., Marius, R., Atkins, H., Sonenberg, N. and Bell, J.C. (2000) Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med*, **6**, 821-825.

Su, Z., Goldstein, N.I. and Fisher, P.B. (1998) Antisense inhibition of the PTI-1 oncogene reverses cancer phenotypes. *Proc Natl Acad Sci U S A*, **95**, 1764-1769.

Suda, M., Fukui, M., Sogabe, Y., Sato, K., Morimatsu, A., Arai, R., Motegi, F., Miyakawa, T., Mabuchi, I. and Hirata, D. (1999) Overproduction of elongation factor 1alpha, an essential translational component, causes aberrant cell morphology by affecting the control of growth polarity in fission yeast. *Genes Cells*, **4**, 517-527.

Suehiro, Y., Sakamoto, M., Umayahara, K., Iwabuchi, H., Sakamoto, H., Tanaka, N., Takeshima, N., Yamauchi, K., Hasumi, K., Akiya, T., Sakunaga, H., Muroya, T., Numa, F., Kato, H., Tenjin, Y. and Sugishita, T. (2000) Genetic aberrations detected by comparative genomic hybridization in ovarian clear cell adenocarcinomas. *Oncology*, **59**, 50-56.

Sugiyama, T., Kamura, T., Kigawa, J., Terakawa, N., Kikuchi, Y., Kita, T., Suzuki, M., Sato, I. and Taguchi, K. (2000) Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. *Cancer*, **88**, 2584-2589.

Sun, Y., Lin, J., Katz, A.E. and Fisher, P.B. (1997) Human prostatic carcinoma oncogene PTI-1 is expressed in human tumor cell lines and prostate carcinoma patient blood samples. *Cancer Res*, **57**, 18-23.

Szabo, A., Perou, C.M., Karaca, M., Perreard, L., Quackenbush, J.F. and Bernard, P.S. (2004) Statistical modeling for selecting housekeeper genes. *Genome Biol*, **5**, R59.

Talbot, S J., Crawford, D H. (2004) Viruses and tumours - an update. *European Journal of Cancer,* **40**, 1998-2005.

Talukder, A.H., Jorgensen, H.F., Mandal, M., Mishra, S.K., Vadlamudi, R.K., Clark, B.F., Mendelsohn, J. and Kumar, R. (2001) Regulation of elongation factor-1alpha expression by growth factors and anti-receptor blocking antibodies. *J Biol Chem*, **276**, 5636-5642.

Tammela, J., Geisler, J.P., Eskew, P.N., Jr. and Geisler, H.E. (1998) Clear cell carcinoma of the ovary: poor prognosis compared to serous carcinoma. *Eur J Gynaecol Oncol*, **19**, 438-440.

Tan, M., Yao, J. and Yu, D. (1997) Overexpression of the c-erbB-2 gene enhanced intrinsic metastasis potential in human breast cancer cells without increasing their transformation abilities. *Cancer Res*, **57**, 1199-1205.

Tang, S., Han, H. and Bajic, V.B. (2004) ERGDB: Estrogen Responsive Genes Database. *Nucleic Acids Res*, **32**, D533-536.

Tanner, M.M., Grenman, S., Koul, A., Johannsson, O., Meltzer, P., Pejovic, T., Borg, A. and Isola, J.J. (2000) Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer. *Clin Cancer Res*, **6**, 1833-1839.

Tanner, M.M., Tirkkonen, M., Kallioniemi, A., Holli, K., Collins, C., Kowbel, D., Gray, J.W., Kallioniemi, O.P. and Isola, J. (1995) Amplification of chromosomal region 20q13 in invasive breast cancer: prognostic implications. *Clin Cancer Res*, **1**, 1455-1461.

Tanner, M.M., Tirkkonen, M., Kallioniemi, A., Isola, J., Kuukasjarvi, T., Collins, C., Kowbel, D., Guan, X.Y., Trent, J., Gray, J.W., Meltzer, P. and Kallioniemi, O.P. (1996) Independent amplification and frequent co-amplification of three nonsyntenic regions on the long arm of chromosome 20 in human breast cancer. *Cancer Res*, **56**, 3441-3445.

Tatsuka, M., Mitsui, H., Wada, M., Nagata, A., Nojima, H. and Okayama, H. (1992) Elongation factor-1 alpha gene determines susceptibility to transformation. *Nature*, **359**, 333-336.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*, **22**, 4673-4680.

Thompson, M.E., Jensen, R.A., Obermiller, P.S., Page, D.L. and Holt, J.T. (1995) Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. *Nat Genet*, **9**, 444-450.

Tolivia, J., Navarro, A., del Valle, E., Perez, C., Ordonez, C. and Martinez, E. (2006) Application of Photoshop and Scion Image analysis to quantification of signals in histochemistry, immunocytochemistry and hybridocytochemistry. *Anal Quant Cytol Histol*, **28**, 43-53.

Tomlinson, V.A., Newbery, H.J., Wray, N.R., Jackson, J., Larionov, A., Miller, W.R., Dixon, J.M. and Abbott, C.M. (2005) Translation elongation factor eEF1A2 is a potential oncoprotein that is overexpressed in two-thirds of breast tumours. *BMC Cancer*, **5**, 113.

Toriyama, M., Ohta, K., Endo, S. and Sakai, H. (1988) 51-kd protein, a component of microtubule-organizing granules in the mitotic apparatus involved in aster formation in vitro. *Cell Motil Cytoskeleton*, **9**, 117-128.

Tuynder, M., Susini, L., Prieur, S., Besse, S., Fiucci, G., Amson, R. and Telerman, A. (2002) Biological models and genes of tumor reversion: cellular reprogramming through tpt1/TCTP and SIAH-1. *Proc Natl Acad Sci U S A*, **99**, 14976-14981.

Ueda, K., Nishijima, M., Inui, H., Watatani, M., Yayoi, E., Okamura, J., Yasutomi, M., Nakamura, Y. and Miyoshi, Y. (1998) Infrequent mutations in the PTEN/MMAC1 gene among primary breast cancers. *Jpn J Cancer Res*, **89**, 17-21.

Uetsuki, T., Naito, A., Nagata, S. and Kaziro, Y. (1989) Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1 alpha. *J Biol Chem*, **264**, 5791-5798.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*, **3**, RESEARCH0034.

Venema, R.C., Peters, H.I. and Traugh, J.A. (1991a) Phosphorylation of elongation factor 1 (EF-1) and valyl-tRNA synthetase by protein kinase C and stimulation of EF-1 activity. *J Biol Chem*, **266**, 12574-12580.

Venema, R.C., Peters, H.I. and Traugh, J.A. (1991b) Phosphorylation of valyl-tRNA synthetase and elongation factor 1 in response to phorbol esters is associated with stimulation of both activities. *J Biol Chem*, **266**, 11993-11998.

Verhagen, P.C., Hermans, K.G., Brok, M.O., van Weerden, W.M., Tilanus, M.G., de Weger, R.A., Boon, T.A. and Trapman, J. (2002) Deletion of chromosomal region 6q14-16 in prostate cancer. *Int J Cancer*, **102**, 142-147.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M. and Bos, J.L. (1988) Genetic alterations during colorectal-tumor development. *N Engl J Med*, **319**, 525-532.

Wang, H., Parent, M. and Morais, R. (1994) Cloning and characterization of a cDNA encoding elongation factor 1 alpha from chicken cells devoid of mitochondrial DNA. *Gene*, **140**, 155-161.

Wang, L.G., Liu, X.M., Kreis, W. and Budman, D.R. (1999a) The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review. *Cancer Chemother Pharmacol*, **44**, 355-361.

Wang, Z.R., Liu, W., Smith, S.T., Parrish, R.S. and Young, S.R. (1999b) c-myc and chromosome 8 centromere studies of ovarian cancer by interphase FISH. *Exp Mol Pathol*, **66**, 140-148.

Watanabe, T., Imoto, I., Kosugi, Y., Ishiwata, I., Inoue, S., Takayama, M., Sato, A. and Inazawa, J. (2001) A novel amplification at 17q21-23 in ovarian cancer cell lines detected by comparative genomic hybridization. *Gynecol Oncol*, **81**, 172-177.

Watanabe, T., Inoue, S., Hiroi, H., Orimo, A., Kawashima, H. and Muramatsu, M. (1998) Isolation of estrogen-responsive genes with a CpG island library. *Mol Cell Biol*, **18**, 442-449.

Watt, P.M., Kumar, R. and Kees, U.R. (2000) Promoter demethylation accompanies reactivation of the HOX11 proto-oncogene in leukemia. *Genes Chromosomes Cancer*, **29**, 371-377.

Warren, C.M. and Landgraf, R. (2006) Signaling through ERBB receptors: multiple layers of diversity and control. *Cell Signal*, **18**, 923-933.

Webster, G.C. and Webster, S.L. (1983) Decline in synthesis of elongation factor one (EF-1) precedes the decreased synthesis of total protein in aging Drosophila melanogaster. *Mech Ageing Dev*, **22**, 121-128.

Webster, G.C. and Webster, S.L. (1984) Specific disappearance of translatable messenger RNA for elongation factor one in aging Drosophila melanogaster. *Mech Ageing Dev*, **24**, 335-342.

Wong, C.W., McNally, C., Nickbarg, E., Komm, B.S. and Cheskis, B.J. (2002) Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci U S A*, **99**, 14783-14788.

Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C. and Micklem, G. (1995) Identification of the breast cancer susceptibility gene BRCA2. *Nature*, **378**, 789-792.

Wooster, R., Neuhausen, S.L., Mangion, J., Quirk, Y., Ford, D., Collins, N., Nguyen, K., Seal, S., Tran, T., Averill, D. and et al. (1994) Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Science*, **265**, 2088-2090.

Xie, D., Jauch, A., Miller, C.W., Bartram, C.R. and Koeffler, H.P. (2002) Discovery of over-expressed genes and genetic alterations in breast cancer cells using a combination of suppression subtractive hybridization, multiplex FISH and comparative genomic hybridization. *Int J Oncol*, **21**, 499-507.

Yamashita, S., Tsujino, Y., Moriguchi, K., Tatematsu, M. and Ushijima, T. (2006) Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2'-deoxycytidine treatment and oligonucleotide microarray. *Cancer Sci*, **97**, 64-71.

Yang, F., Demma, M., Warren, V., Dharmawardhane, S. and Condeelis, J. (1990) Identification of an actin-binding protein from Dictyostelium as elongation factor 1a. *Nature*, **347**, 494-496.

Yang, W., Burkhart, W., Cavallius, J., Merrick, W.C. and Boss, W.F. (1993) Purification and characterization of a phosphatidylinositol 4-kinase activator in carrot cells. *J Biol Chem*, **268**, 392-398.

Yamaguchi, H., Wyckoff, J. and Condeelis, J. (2005) Cell migration in tumors. *Curr Opin Cell Biol*, **17**, 559-564.

Yoshida, K. and Miki, Y. (2004) Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci*, **95**, 866-871.

Yoshihama, M., Uechi, T., Asakawa, S., Kawasaki, K., Kato, S., Higa, S., Maeda, N., Minoshima, S., Tanaka, T., Shimizu, N. and Kenmochi, N. (2002) The human ribosomal protein genes: sequencing and comparative analysis of 73 genes. *Genome Res*, **12**, 379-390.

Yuan, Z.Q., Sun, M., Feldman, R.I., Wang, G., Ma, X., Jiang, C., Coppola, D., Nicosia, S.V. and Cheng, J.Q. (2000) Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene*, **19**, 2324-2330.

Zhou, H., Kuang, J., Zhong, L., Kuo, W.L., Gray, J.W., Sahin, A., Brinkley, B.R., Sen, S. (1998) Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. *Nature Genetics,* **20**, 189-193.

Zhu, G., Reynolds, L., Crnogorac-Jurcevic, T., Gillett, C.E., Dublin, E.A., Marshall, J.F., Barnes, D., D'Arrigo, C., Van Trappen, P.O., Lemoine, N.R. and Hart, I.R. (2003) Combination of microdissection and microarray analysis to identify gene expression changes between differentially located tumour cells in breast cancer. *Oncogene*, **22**, 3742-3748.

Zhu, H., Lam, D.C., Han, K.C., Tin, V.P., Suen, W.S., Wang, E., Lam, W.K., Cai, W.W., Chung, L.P. and Wong, M.P. (2006) High resolution analysis of genomic aberrations by metaphase and array comparative genomic hybridization identifies candidate tumour genes in lung cancer cell lines. *Cancer Lett.*

BMC Cancer

BioMed Central

Research article

Open Access

Translation elongation factor eEF1A2 is a potential oncoprotein that is overexpressed in two-thirds of breast tumours

Victoria AL Tomlinson[1], Helen J Newbery[1], Naomi R Wray[1], Juliette Jackson[2], Alexey Larionov[2], William R Miller[2], J Michael Dixon[2] and Catherine M Abbott*[1]

Address: [1]Medical Genetics, School of Molecular and Clinical Medicine, University of Edinburgh, Molecular Medicine Centre, Western General Hospital, Edinburgh EH4 2XU, UK and [2]Breast Unit Research Group, Western General Hospital, Edinburgh EH4 2XU, UK

Email: Victoria AL Tomlinson - V.A.L.Tomlinson@sms.ed.ac.uk; Helen J Newbery - h.newbery@ed.ac.uk; Naomi R Wray - Naomi.Wray@ed.ac.uk; Juliette Jackson - Juliettemurray@doctors.org.uk; Alexey Larionov - alexey@larionov.co.uk; William R Miller - W.R.Miller@ed.ac.uk; J Michael Dixon - mdixon@staffmail.ed.ac.uk; Catherine M Abbott* - C.Abbott@ed.ac.uk

* Corresponding author

Published: 12 September 2005

BMC Cancer 2005, **5**:113 doi:10.1186/1471-2407-5-113

Received: 16 May 2005
Accepted: 12 September 2005

This article is available from: http://www.biomedcentral.com/1471-2407/5/113

Abstract

Background: The tissue-specific translation elongation factor eEF1A2 was recently shown to be a potential oncogene that is overexpressed in ovarian cancer. Although there is no direct evidence for an involvement of eEF1A2 in breast cancer, the genomic region to which EEF1A2 maps, 20q13, is frequently amplified in breast tumours. We therefore sought to establish whether eEF1A2 expression might be upregulated in breast cancer.

Methods: eEF1A2 is highly similar (98%) to the near-ubiquitously expressed eEF1A1 (formerly known as EF1-α) making analysis with commercial antibodies difficult. We have developed specific anti-eEF1A2 antibodies and used them in immunohistochemical analyses of tumour samples. We report the novel finding that although eEF1A2 is barely detectable in normal breast it is moderately to strongly expressed in two-thirds of breast tumours. This overexpression is strongly associated with estrogen receptor positivity.

Conclusion: eEF1A2 should be considered as a putative oncogene in breast cancer that may be a useful diagnostic marker and therapeutic target for a high proportion of breast tumours. The oncogenicity of eEF1A2 may be related to its role in protein synthesis or to its potential non-canonical functions in cytoskeletal remodelling or apoptosis.

Background

Breast cancer is the most common cancer in females worldwide; there are an estimated 1 million new cases per year [1]. The identification of changes in gene expression in breast tumours relative to normal surrounding tissue is clearly of great importance in terms of prognostic indicators and therapeutic targets.

The translation elongation factor eEF1A2 was first identified as a tissue-specific variant of eEF1A1 (formerly known as EF-1α) in the early 1990s [2,3]. The two forms of eEF1A are encoded by separate loci, but the resulting proteins are 92% identical and 98% similar. Whereas eEF1A1 is widely expressed, eEF1A2 is normally expressed only in neurons and muscle [3-5]. The first specific

evidence implicating eEF1A2 in tumourigenesis came in 2002 when Anand et al [6] showed that eEF1A2 was expressed in 30% of ovarian tumours, but not in normal ovary. The genomic region to which eEF1A2 maps, 20q13, had been known for many years to be amplified in a high proportion of ovarian and breast tumours [7]; [8], but the *EEF1A2* gene maps closer to the telomere than the region previously implicated. Anand et al showed that 14/53 tumours had amplifications of the region surrounding *EEF1A2* [6]. In the same paper, forced expression of eEF1A2 in cells was demonstrated to confer tumourigenic properties on NIH3T3 cells, and to give rise to tumours in xenografted nude mice. Although 20q13 amplification is commonly observed in breast cancer, there has as yet been no evidence for overexpression of eEF1A2 in breast tumours. However, eEF1A1, the widely-expressed isoform, was recently shown to be upregulated in the infiltrating edge of invasive breast tumours compared with the tumour core by microarray analysis of laser microdissected material, confirmed by immunohistochemistry [9]. In this analysis the antibody used was one that detects both eEF1A1 and eEF1A2 with equal intensity, so it is conceivable that eEF1A2 contributes to this pattern of expression.

We have generated antibodies (Newbery et al, in preparation) that allow us to distinguish between the highly related isoforms eEF1A1 (which is expressed in normal breast) and eEF1A2 (which is thought to be expressed only in muscle and neurons). Using these isoform-specific antibodies, we show that eEF1A2 expression is barely detectable in normal human breast tissue, but that the gene is moderately to strongly expressed in 63 % of breast tumours examined. Furthermore, there is a strong correlation between eEF1A2 overexpression and estrogen receptor (ER) positivity.

Methods

Quantitative Real-time Reverse Transcription-PCR (RT-PCR)

Breast cancer samples were obtained in the Edinburgh Breast Unit (Western General Hospital, Edinburgh) with patients' informed consent and ethical committee approval. Biopsies were snap frozen and stored in liquid nitrogen until RNA extraction. Before RNA extraction the frozen tissue was defrosted and stabilized in RNA-later-ICE reagent (Ambion). Total RNA was extracted with RNeasy-mini columns (Qiagen).

Amount and purity of RNA were evaluated by spectrophotometry. RNA integrity was confirmed by agarose gel electrophoresis. Total RNA was isolated from tumour and normal tissue using Qiagen RNeasy kits (Qiagen). RNA was treated with DNase using DNAfree kit (Ambion, Cambridgeshire) and 1 μg was used for RT-PCR using Retroscript kit (Ambion, Cambridgeshire, UK). TaqMan Assay-on-Demand gene expression pre-designed primer and probe sets from Applied Biosystems, Cheshire, UK were used for EEF1A2 (Assay # Hs 00157325 ml) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; control; Hs 99999905 ml). In a 10 μl reaction volume per well of a 394-well plate, 0.5 μl of primers, 5 μl of TaqMan PCR Master Mix, no AmpErase UNG 10×, and 4.5 μl of diluted cDNA were added (Applied Biosystems, Cheshire, UK). Real-time RT-PCR and the quantification of RT-PCR products were performed and the products analyzed using an ABI Prism 7900HT Sequence Detection System, and the appropriate software (SDS3.1) according to the manufacturer's instructions (Applied Biosystems, Cheshire, UK).

Western blots

Protein lysates from cell lines were prepared using previously published protocols [10]. Western blot analyses were carried out using standard protocols. The blots were incubated with primary anti-eEF1A2 rabbit antibody and primary anti-eEF1A1 sheep antibody diluted 1:200 in blocking solution, as well as primary anti-glyceraldehyde-3-phosphate dehydrogenase polyclonal mouse antibody (Chemicon International, Hampshire, UK) diluted 1:10000. Blots were then incubated in the appropriate horse radish peroxidase conjugated secondary antibody (Dako Cytomation, Cambridgeshire, UK) at 1:500. Detection was performed using enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry

Specimens of normal and cancerous tumours were obtained with informed consent and local ethical committee approval from patients undergoing surgical treatment at the Royal Infirmary of Edinburgh and Western General Hospital, Edinburgh. A breast tumour histoarray (CB2) produced by SuperBioChips (AMS Biotechnology, Oxfordshire, UK) was also used. Formalin fixed, paraffin embedded sections of human normal tissue and tumour tissue were deparaffinized with xylene, rehydrated, treated with picric acid and microwaved in citric acid pH6. Slides were blocked in a 1:5 dilution of sheep serum for 30 minutes at room temperature. Primary anti-eEF1A2 rabbit antibodies were used at a concentration of 1:10 diluted in PBS, for 40 minutes at room temperature and secondary goat anti-rabbit IgG biotin conjugated antibody (Dako Cytomation, Cambridgeshire, UK) was used at 1:200 at room temperature for 30 minutes. Slides were incubated with StreptABC complex/HRP (Dako Cytomation, Cambridgeshire, UK) at room temperature for 30 minutes and in diaminobenzidene (Sigma Fast DAB, Sigma, Dorset, UK) for 2 minutes at room temperature. Finally slides

were counterstained in haematoxylin, dehydrated and mounted in pertex.

Immunohistological scoring methods

The breast tumour sections and normal breast sections (CB2, SuperBioChips, AMS Biotechnology, Oxfordshire, UK) were scored as weak, moderate and strong staining for eEF1A2. Weak staining was considered as background since this level of staining is seen in normal tissue. Stromal tissue was negative in all cases. Blind scoring was carried out by two independent researchers. Two slides were analysed, representing different levels within tumours, and each of these was stained with a different antibody to eEF1A2. Almost perfect correlation was seen between the two slides.

Statistical methods

Fisher's exact test was used to test for associations between negative and weak eEF1A2 expressing tumours or moderately and strongly overexpressing tumours with ER positivity. For breast tumour Quantitative Real-time RT-PCR data, a two-sample t-test allowing for difference in variance between the two samples was used to test the difference between the mean standardised quantity of RNA for the ER-positive and ER-negative groups. P values that were less than or equal to 0.05 were considered significant.

Results

Expression analysis in breast tumours

Since 20q13.3 amplification is commonly seen in breast tumours as well as ovarian tumours, and because analysis of the SAGE database at NCBI indicated that eEF1A2 was more highly represented in breast tumours than in normal breast tissue (unpublished observations) we decided to examine eEF1A2 expression in breast tumours at both the RNA and protein level. Initially, we used Western blotting with an anti-eEF1A2 antibody to examine expression in a number of commonly-used cell lines. The anti-eEF1A2 antibody was raised against a peptide that differs significantly between eEF1A1 and eEF1A2 (Newbery et al, in preparation); specificity was confirmed by lack of signal from tissues taken from wasted mice which have a null mutation of eEF1A2 [5]. The majority of transformed cell lines showed high levels of expression of eEF1A2 (Figure 1); in addition, it has previously been shown that NIH3T3 cells do not express eEF1A2 except when they become confluent [2,6]. We therefore chose not to place any emphasis on the analysis of breast cancer cell lines as opposed to primary tumour samples since eEF1A2 expression seems to be a common property of transformed cells, rather than being specific for a tumour type. Instead, we carried out real-time quantitative RT-PCR of RNA samples from breast tumours. The results obtained are shown in Figure 2A. It can be seen that whereas extremely low levels of expression are detected in RNA samples from normal breast and from a benign breast tumour, most malignant tumour samples showed moderate to high (up to 30-fold higher than normal breast) expression levels. On average, the estrogen receptor (ER)-negative tumours showed only 1.2 times higher expression than the normal sample whereas ER-positive tumours had 8.4 times higher expression (Figure 2B). The difference in eEF1A2 expression levels between the estrogen receptor negative tumours and estrogen receptor positive tumours is 7.2 units (P = 0.0087, t-test, 95% confidence interval 2.0 to 12.4 units).

No protein extracts were available from these tumours for Western blot analysis so we then examined expression of eEF1A2 by immunohistochemistry on a commercial tissue array of normal and tumour breast samples using the anti-peptide antibodies described above. The array contained sections from 46 cases of cancer and 7 normal breasts; the results obtained are shown in Figure 3. None of the normal breast sections showed any more than faint staining. No stromal staining was observed and tumour staining within a sample was near-uniform. Of the tumour samples, 5 showed strong expression of eEF1A2 (11%) and 22 showed moderate expression (48%). The remainder appeared to have no more staining than normal breast. None of the three lobular carcinomas on the slide showed any eEF1A2 overexpression. There was no significant correlation between eEF1A2 expression level and tumour grade or lymph node positivity (data not shown). The tumours had all been previously assessed for p53 status; there appeared to be an association between overexpression of eEF1A2 and wild-type p53, but this was not statistically significant. The tendency to association may be a reflection of the significant association between ER positivity and p53 negativity (p = 0.012) in these tumour samples. Only four out of the 22 ER-negative tumours showed staining beyond background levels and none had strong staining. We found a significant association between eEF1A2 overexpression (scored as moderate/strong) and ER positivity (P = 0.016, Fisher's exact test). We then went on to examine 16 tissue sections from ER positive breast tumours obtained from patients at the Western General Hospital. Of these, 13 showed moderate or strong staining with the anti-eEF1A2 antibody. Overall then, 40 out of 63 breast tumours (63%) examined by immunohistochemistry showed significant overexpression of eEF1A2.

Discussion

We have shown that the putative oncogene eEF1A2 is upregulated in a high proportion of breast tumours. This upregulation is considerably more significant in ER-positive tumours. There is little or no detectable expression of eEF1A2 in normal breast tissue. It is not yet known whether this overexpression results from amplification of the *EEF1A2* gene in all cases; in the study of ovarian

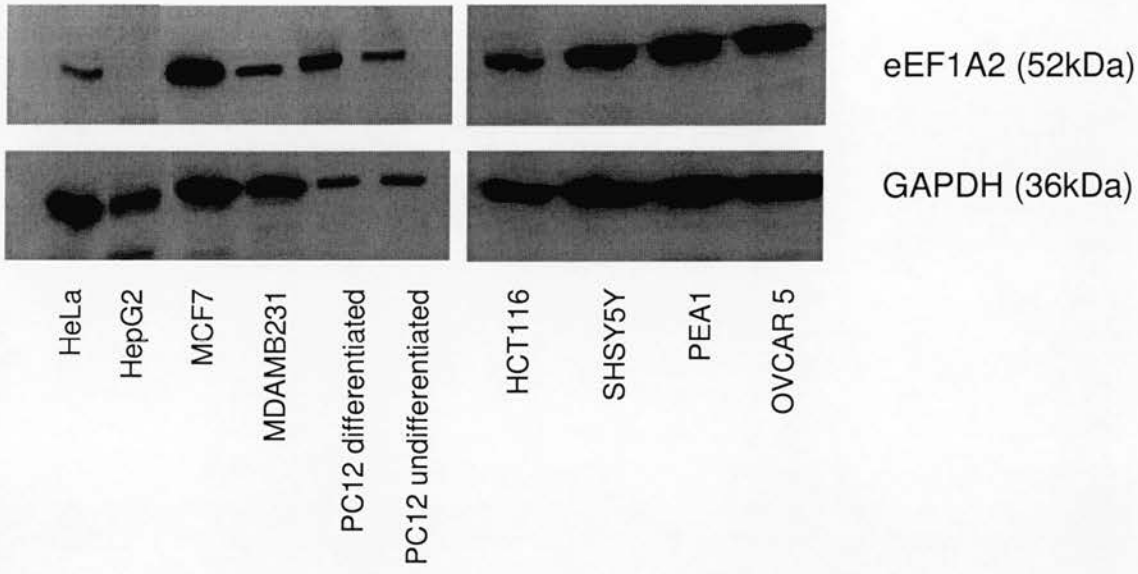


Figure 1
Western blot analysis using an anti-eEF1A2 antibody on a range of cell lines. The loading control is GAPDH.

tumours by Anand et al [6] at least one tumour showed overexpression in the absence of gene amplification, suggesting that there are other mechanisms by which the gene can be upregulated. There is a strong association between ER-positivity and eEF1A2 overexpression which is worthy of further study.

There also appears to be a weak correlation between the absence of p53 mutations and eEF1A2 overexpression. It is possible that eEF1A2 is not upregulated in tumours with p53 mutations because wild-type p53 is required for expression of eEF1A2 in certain cell types; it has been shown that p53 can upregulate expression of eEF1A1 [11], and the p53 binding sites identified in the gene encoding eEF1A1 are shared with that encoding eEF1A2 (unpublished observations). On the other hand it is conceivable that upregulation of eEF1A2 expression rather than p53 mutation is an alternative route for tumours to evade apoptosis in certain cancers.

The basis for the oncogenicity of eEF1A2 is still unclear. We, like Anand et al, have shown that the levels of eEF1A1 in tumours which over-express eEF1A2 are unchanged (data not shown), suggesting that these tumours might have a greater capacity for protein synthesis. However, it has been known for many years that eEF1A is in excess over the other components of the translation elongation apparatus [12], so eEF1A is unlikely to be rate-limiting in protein synthesis. eEF1A1 has been shown to determine the susceptibility of a number of independent cell lines to chemical- and UV-induced transformation [13] and has been identified as an actin binding protein in rat breast tumour cells, where it was found to be more highly expressed in metastatic than non-metastatic cells [14]. It is not yet clear whether these properties are shared with eEF1A2, but the availability of specific antibodies that distinguish between the two isoforms should allow us to shed light on this. One hypothesis is that the non-canonical ("moonlighting") properties of eEF1A1 [15] and eEF1A2 differ so that, for example, the way eEF1A2 interacts with the cytoskeleton might differ from that of eEF1A1 and affect the properties of cells which are expressing high levels of both isoforms. It has been shown, for example, that forced overexpression of eEF1A affects the cytoskeleton in both *S. pombe* and *S. cerevisiae* [16,17]. Alternatively, it has been shown that eEF1A1 and eEF1A2 differ in terms of their response to apoptotic agents [18]; the finding that eEF1A2 is anti-apoptotic, at least in certain conditions, has obvious implications for the possible role of eEF1A2 in tumourigenesis. The observation that eEF1A2 expression is seen in the majority of cell lines, regardless of the tissue of origin, suggests that

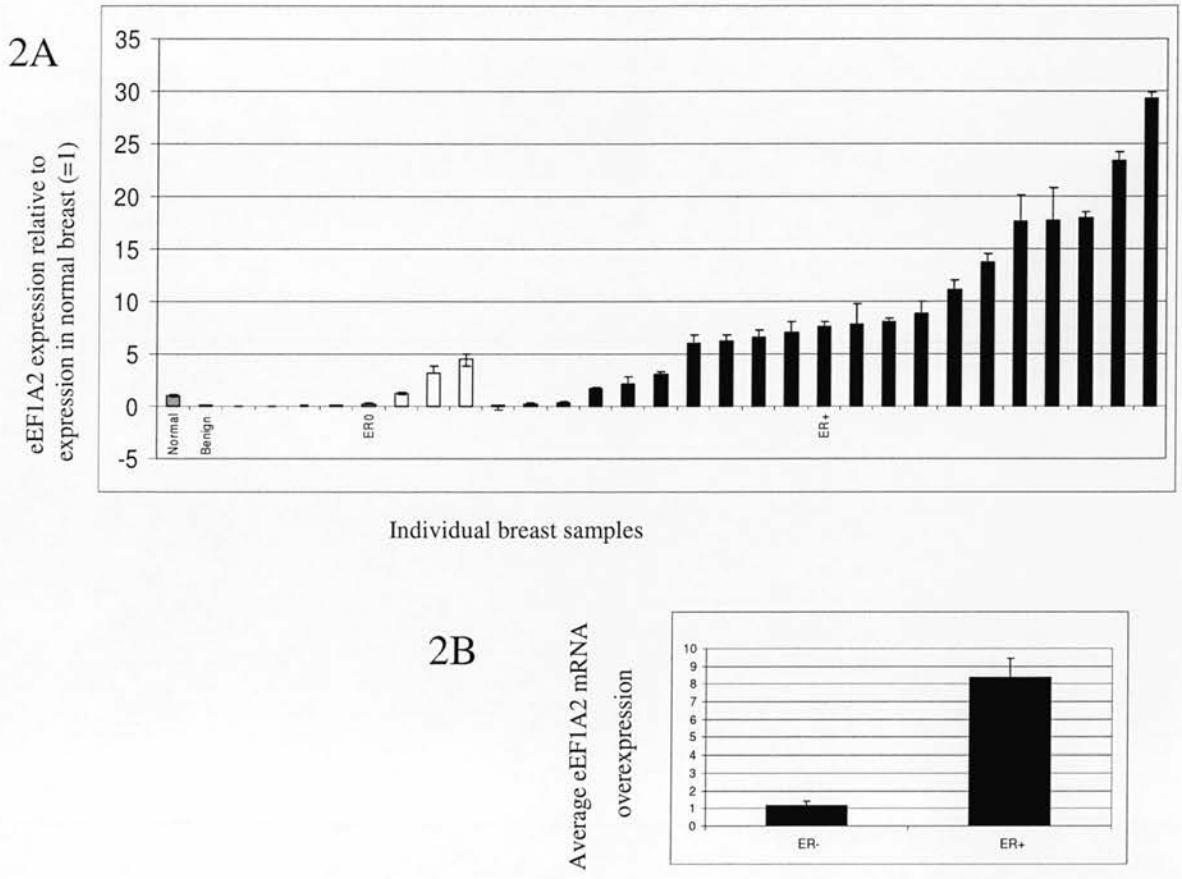


Figure 2
(A) Real-time RT-PCR analysis of RNA from breast tumours. Each block on the × axis represents a different tumour. The amount of eEF1A2 message is shown normalised to GAPDH and expressed relative to the level of expression in the normal breast RNA samples (=1). ER-negative tumours are shown in white, ER-positive tumours are shown in black. The difference in mean expression between ER-positive and ER-negative samples is 7.2 units (p = 0.0087), 95% Confidence Interval 2.0 to 12.4 units. **(B)** Average standardised RNA levels in ER-negative and ER-positive breast tumours. This difference is significant (P = 0.0087, t-test).

eEF1A2 expression may be triggered by the general process of transformation. This idea is strengthened by the fact that most of the few cell lines which do not express eEF1A2 tend to be untransformed, such as NIH3T3 cells (Figure 1).

The presence of increased levels of eEF1A2 in breast tumours may provide a useful new diagnostic marker. Further, eEF1A2 may prove to be a feasible target for therapeutic intervention. It has already been shown that growth-factor mediated eEF1A1 expression can be blocked with anti-EGF antibodies [19]; it would be of interest to examine the response of eEF1A2 to similar antibodies. Investigations into non-canonical functions of eEF1A molecules may shed new light on mechanisms of oncogenicity.

Competing interests

The author(s) declare that they have no competing interests.

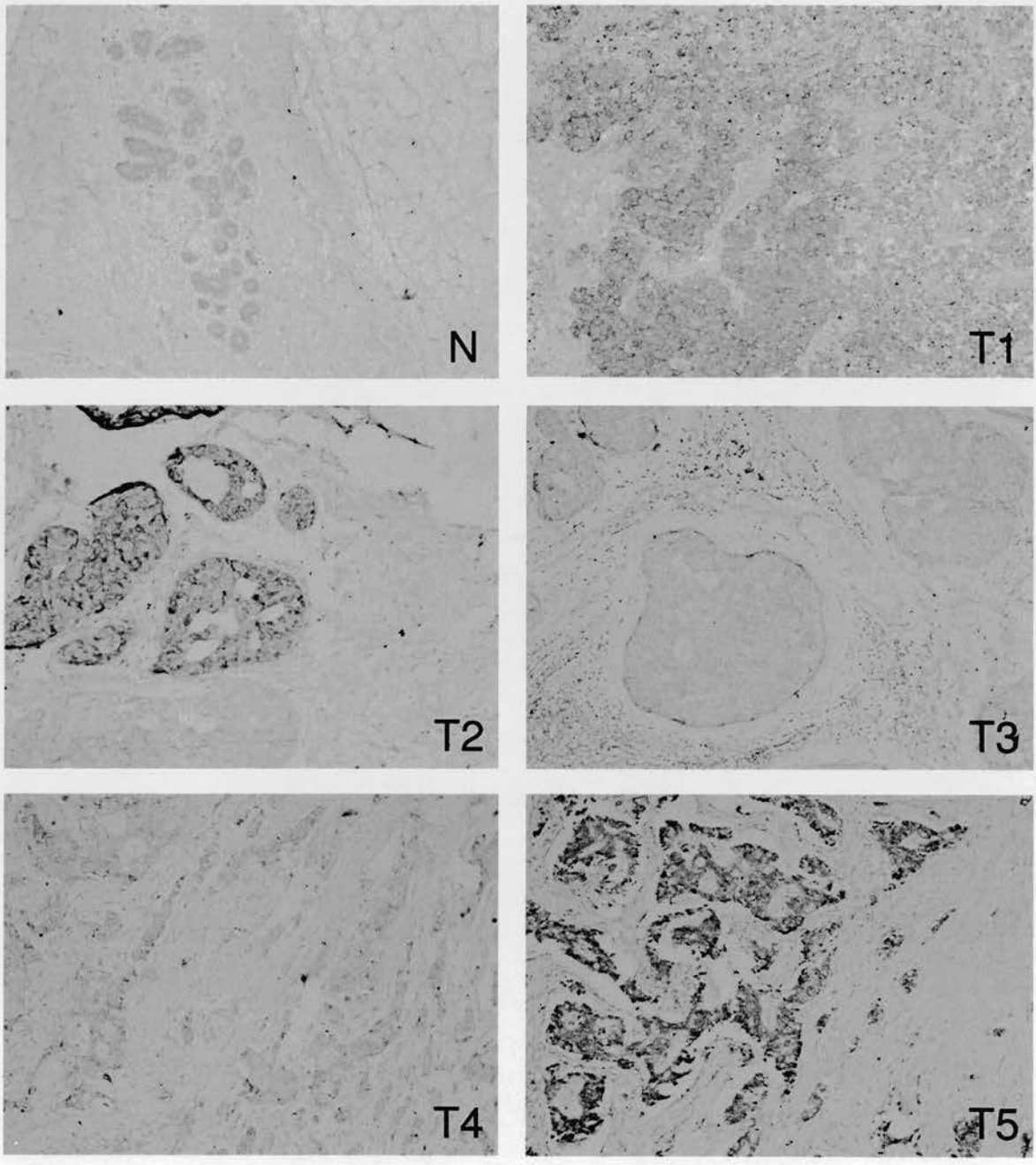


Figure 3
Immunohistochemistry of eEF1A2 in the breast. The panel labelled N shows the antibody staining weakly in a normal breast section. Panels T1 to T5 show breast tumours staining strongly with the anti-eEF1A2 antibody. Magnification ×10.

Authors' contributions

VT carried out the molecular analysis, Western blots and immunohistochemistry. HN isolated and characterised the antibodies. NW carried out the statistical analysis. AL made the RNA. JJ provided some of the tissue sections. WM and JMD collected the patient material. CA conceived the study and wrote the manuscript.

Acknowledgements

We would like to thank Jeremy Thomas for discussion and analysis. This work was funded by Cancer Research UK and the Wellcome Trust.

References

1. Baselga J, Norton L: **Focus on breast cancer.** *Cancer Cell* 2002, **1**:319-322.
2. Ann DK, Moutsatsos IK, Nakamura T, Lin HH, Mao PL, Lee MJ, Chin S, Liem RK, Wang E: **Isolation and characterization of the rat chromosomal gene for a polypeptide (pS1) antigenically related to statin.** *J Biol Chem* 1991, **266**:10429-10437.
3. Knudsen SM, Frydenberg J, Clark BF, Leffers H: **Tissue-dependent variation in the expression of elongation factor-1 alpha isoforms: isolation and characterisation of a cDNA encoding a novel variant of human elongation-factor 1 alpha.** *Eur J Biochem* 1993, **215**:549-554.
4. Lee S, Francoeur AM, Liu S, Wang E: **Tissue-specific expression in mammalian brain, heart, and muscle of S1, a member of the elongation factor-1 alpha gene family.** *J Biol Chem* 1992, **267**:24064-24068.
5. Chambers DM, Peters J, Abbott CM: **The lethal mutation of the mouse wasted (wst) is a deletion that abolishes expression of a tissue-specific isoform of translation elongation factor 1alpha, encoded by the Eef1a2 gene.** *Proc Natl Acad Sci U S A* 1998, **95**:4463-4468.
6. Anand N, Murthy S, Amann G, Wernick M, Porter LA, Cukier IH, Collins C, Gray JW, Diebold J, Demetrick DJ, Lee JM: **Protein elongation factor EEF1A2 is a putative oncogene in ovarian cancer.** *Nat Genet* 2002, **31**:301-305.
7. Kallioniemi A, Kallioniemi OP, Piper J, Tanner M, Stokke T, Chen L, Smith HS, Pinkel D, Gray JW, Waldman FM: **Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization.** *Proc Natl Acad Sci U S A* 1994, **91**:2156-2160.
8. Iwabuchi H, Sakamoto M, Sakunaga H, Ma YY, Carcangiu ML, Pinkel D, Yang-Feng TL, Gray JW: **Genetic analysis of benign, low-grade, and high-grade ovarian tumors.** *Cancer Res* 1995, **55**:6172-6180.
9. Zhu G, Reynolds L, Crnogorac-Jurcevic T, Gillett CE, Dublin EA, Marshall JF, Barnes D, D'Arrigo C, Van Trappen PO, Lemoine NR, Hart IR: **Combination of microdissection and microarray analysis to identify gene expression changes between differentially located tumour cells in breast cancer.** *Oncogene* 2003, **22**:3742-3748.
10. Gilmour LM, Macleod KG, McCaig A, Sewell JM, Gullick WJ, Smyth JF, Langdon SP: **Neuregulin expression, function, and signaling in human ovarian cancer cells.** *Clin Cancer Res* 2002, **8**:3933-3942.
11. Kato MV, Sato H, Nagayoshi M, Ikawa Y: **Upregulation of the elongation factor-1alpha gene by p53 in association with death of an erythroleukemic cell line.** *Blood* 1997, **90**:1373-1378.
12. Slobin LI: **The role of eucaryotic factor Tu in protein synthesis. The measurement of the elongation factor Tu content of rabbit reticulocytes and other mammalian cells by a sensitive radioimmunoassay.** *Eur J Biochem* 1980, **110**:555-563.
13. Tatsuka M, Mitsui H, Wada M, Nagata A, Nojima H, Okayama H: **Elongation factor-1 alpha gene determines susceptibility to transformation.** *Nature* 1992, **359**:333-336.
14. Edmonds BT, Wyckoff J, Yeung YG, Wang Y, Stanley ER, Jones J, Segall J, Condeelis J: **Elongation factor-1 alpha is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma.** *J Cell Sci* 1996, **109 (Pt 11)**:2705-2714.
15. Condeelis J: **Elongation factor 1 alpha, translation and the cytoskeleton.** *Trends Biochem Sci* 1995, **20**:169-170.
16. Munshi R, Kandl KA, Carr-Schmid A, Whitacre JL, Adams AE, Kinzy TG: **Overexpression of translation elongation factor 1A affects the organization and function of the actin cytoskeleton in yeast.** *Genetics* 2001, **157**:1425-1436.
17. Suda M, Fukui M, Sogabe Y, Sato K, Morimatsu A, Arai R, Motegi F, Miyakawa T, Mabuchi I, Hirata D: **Overproduction of elongation factor 1alpha, an essential translational component, causes aberrant cell morphology by affecting the control of growth polarity in fission yeast.** *Genes Cells* 1999, **4**:517-527.
18. Ruest LB, Marcotte R, Wang E: **Peptide elongation factor eEF1A-2/S1 expression in cultured differentiated myotubes and its protective effect against caspase-3-mediated apoptosis.** *J Biol Chem* 2002, **277**:5418-5425.
19. Talukder AH, Jorgensen HF, Mandal M, Mishra SK, Vadlamudi RK, Clark BF, Mendelsohn J, Kumar R: **Regulation of elongation factor-1alpha expression by growth factors and anti-receptor blocking antibodies.** *J Biol Chem* 2001, **276**:5636-5642.

Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2407/5/113/prepub

British Journal of Cancer (2007), 1 – 8

www.bjcancer.com

Full Paper

Expression of eEF1A2 is associated with clear cell histology in ovarian carcinomas: overexpression of the gene is not dependent on modifications at the *EEF1A2* locus

VAL Tomlinson[1], HJ Newbery[1], JH Bergmann[1], J Boyd[1], D Scott[2], NR Wray[1], GC Sellar[2], H Gabra[2,4], A Graham[3], ARW Williams[3] and CM Abbott[1,*]

[1]Medical Genetics, School of Molecular and Clinical Medicine, University of Edinburgh, Molecular Medicine Centre, Western General Hospital, Edinburgh EH4 2XU, UK; [2]Cancer Research UK, Edinburgh Oncology Unit, University of Edinburgh Cancer Research Centre, Edinburgh EH4 2XR, UK; [3]Division of Pathology, University of Edinburgh, Royal Infirmary, Little France, Edinburgh EH4 2XR, UK

The tissue-specific translation elongation factor eEF1A2 is a potential oncogene that is overexpressed in human ovarian cancer. eEF1A2 is highly similar (98%) to the near-ubiquitously expressed eEF1A1 (formerly known as EF1-α) making analysis with commercial antibodies difficult. We wanted to establish the expression pattern of eEF1A2 in ovarian cancer of defined histological subtypes at both the RNA and protein level, and to establish the mechanism for the overexpression of eEF1A2 in tumours. We show that while overexpression of eEF1A2 is seen at both the RNA and protein level in up to 75% of clear cell carcinomas, it occurs at a lower frequency in other histological subtypes. The copy number at the *EEF1A2* locus does not correlate with expression level of the gene, no functional mutations were found, and the gene is unmethylated in both normal and tumour DNA, showing that overexpression is not dependent on genetic or epigenetic modifications at the *EEF1A2* locus. We suggest that the cause of overexpression of eEF1A2 may be the inappropriate expression of a *trans*-acting factor. The oncogenicity of eEF1A2 may be related either to its role in protein synthesis or to potential non-canonical functions.
British Journal of Cancer advance online publication, 17 April 2007; doi:10.1038/sj.bjc.6603748 www.bjcancer.com

Keywords: ovarian tumour; clear cell carcinoma; eEF1A2; translation elongation

Ovarian cancer accounts for 4% of cases of female cancers; it has the highest fatality-to-case ratio of all gynaecological cancers, largely because the vast majority of cases are late-stage at presentation. Tumours arising from the surface epithelium represent the most common form of ovarian cancer. Although a number of molecular mechanisms underlying ovarian tumourigenesis have been identified, a single model of progression has not been described, possibly because of the heterogeneous nature of ovarian carcinoma (Shih Ie and Kurman, 2004). Primary ovarian adenocarcinomas are divided into four common distinct morphological subtypes: serous, mucinous, endometrioid, and clear cell. Although clear-cell tumours are frequently confined to the ovaries at presentation, they are associated with a poor prognosis and are thus treated as high-grade neoplasms (Schwartz *et al*, 2002). The incidence of clear-cell tumours among epithelial ovarian cancers is around 10%. Clear-cell tumours tend to be resistant to platinum-based chemotherapy, giving rise to a poor prognosis (Itamochi *et al*, 2002).

*Correspondence: Dr CM Abbott, E-mail: C.Abbott@ed.ac.uk
[4] *Present address*: Department of Cancer Medicine, Imperial College London, Room 1002 Cancer Centre, Hammersmith Hospital, Du Cane Road, London W12 0HS, UK.
Received 1 December 2006; revised 16 March 2007; accepted 21 March 2007

Translation factor eEF1A2 is a tissue-specific variant of eEF1A1 (previously called EF-1α). While eEF1A1 is almost ubiquitously expressed, expression of eEF1A2 is normally confined to muscles and neurons (Lee *et al*, 1992; Knudsen *et al*, 1993). The gene encoding eEF1A1 is on 6q13 and eEF1A2 on 20q13.3 (Lund *et al*, 1996). The encoded proteins are 92% identical and 98% similar. Whereas lack of eEF1A2 gives rise to the wasted mouse phenotype (Chambers *et al*, 1998), which involves motor neuron degeneration (Newbery *et al*, 2005), inappropriate overexpression has now been linked to cancer. Anand *et al* (2002) showed that eEF1A2, while not normally expressed in ovary, is expressed in 30% of ovarian tumours . The mechanism for the overexpression appeared to be gene amplification in most, but not all, cases. Anand *et al* (2002) were also able to demonstrate that ectopic expression of eEF1A2 in NIH3T3 cells gives rise to colony formation in soft agar and increases growth rate. Furthermore, overexpression in rat fibroblasts enhances focus formation, and eEF1A2-expressing ES-2 ovarian cells and NIH3T3 cells injected into nude mice give rise to tumour formation. There was however no information on which types of ovarian tumours show overexpression of eEF1A2 and expression was assessed at the RNA level only. eEF1A2 has been identified in an expression microarray study as one of a number of genes that are highly expressed (ca. fourfold) in clear cell ovarian tumours than other histological subtypes of ovarian cancer (Schwartz *et al*, 2002). We recently showed that *EEF1A2* is also a potential oncogene in breast tumours (Tomlinson *et al*, 2005).

We have now set out to examine eEF1A2 expression in a panel of ovarian tumours of defined histological subtypes. We have generated and used a panel of antibodies that allow us to distinguish between the highly related variants eEF1A1 (which is expressed in normal breast and ovary) and eEF1A2 (which is thought only to be expressed only in muscle and neurons under normal circumstances) (Newbery *et al*, in preparation). We show in a panel of ovarian tumours that while a high proportion of clear cell carcinomas overexpress eEF1A2, a far smaller proportion of serous, endometrioid and mucinous tumours have high levels of eEF1A2 expression. Furthermore, we show that (1) DNA copy number at the *EEF1A2* locus is unrelated to expression level, (2) there are no activating mutations in the eEF1A2 coding sequence in tumours where there is overexpression in the absence of gene amplification and (3) the methylation status of the *EEF1A2* gene is unrelated to expression level.

MATERIALS AND METHODS

Patient samples: ovarian tumours

Primary ovarian (HOV) tumour material and non-malignant tissues were obtained from patients having undergone gynaecological surgery in the Lothian University Hospitals NHS Trust. Institutional ethical approval was granted for this work by the Lothian University NHS Trust Medicine/Clinical Oncology Research Ethics Subcommittee. Tissue samples were excised and stored in liquid nitrogen. Non-malignant tissue samples were derived from patients who underwent bilateral oophorectomies for suspected malignancy, but were found to have benign histologies; samples were collected from apparently normal contralateral ovaries. Tumours were reviewed by subspecialist gynaecological pathologists, and categorised according to stage and histological type and grade.

Quantitative real-time reverse transcription–PCR (RT–PCR)

RNA was prepared from ovarian samples and cell lines as described previously (Sellar *et al*, 2003). Total RNA was isolated from tumour and normal tissue using Qiagen RNeasy kits (Qiagen, Crawley, UK). RNA was treated with DNase using DNAfree kit (Ambion, Cambridgeshire, UK) and 1 μg was used for RT–PCR using Retroscript kit (Ambion). TaqMan Assay-on-Demand from Applied Biosystems, Cheshire, UK was used for *EEF1A2* (Assay; Hs 00157325ml) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; control; Hs 99999905ml). In a 10 μl reaction volume per well of a 394-well plate, 0.5 μl of primers, 5 μl of TaqMan PCR Master Mix, no AmpErase UNG 10 $\times$, and 4.5 μl of diluted cDNA were added (Applied Biosystems). Real-time RT–PCR and the quantification of RT–PCR products were performed and the products analysed using an ABI Prism 7900HT Sequence Detection System, and the appropriate software (SDS3.1) according to the manufacturer's instructions (Applied Biosystems).

Western blots

Protein lysates from cell lines were prepared using previously published protocols (Gilmour *et al*, 2002); the same method was used for primary tumour samples, but in these cases tissue was initially homogenised in extraction buffer before determination of protein content. Western blot analyses using 10 μg protein were carried out using standard protocols. The blots were incubated with primary anti-eEF1A2 rabbit antibody diluted 1:200 in blocking solution, as well as primary anti-GAPDH polyclonal mouse antibody (Chemicon International, Hampshire, UK) diluted 1:10 000. Blots were then incubated in the appropriate HRP conjugated secondary antibody (Dako Cytomation, Cambridgeshire, UK) at 1:500. Detection was performed using enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry

A computerised search of the archives of the Department of Pathology was used to identify 168 cases of ovarian carcinoma of the common epithelial types. Slides were retrieved and the histological classification reviewed by a gynaecological pathologist. Four representative areas of viable tumour tissue were identified and marked on the slides, and the corresponding areas in the paraffin blocks used as the source of tissue for the tissue microarray (TMA). Tissue cores (0.6 mm diameter) were sampled from each of the four areas, and mounted into separate recipient paraffin blocks by the use of a custom-made instrument (Beecher Instruments, Silver Springs, MD, USA). In the ensuing paraffin array blocks, the tissue cylinders were aligned and marked for identification according to a chart. The recipient TMA blocks were baked at 56°C for 10 min before sectioning, and 3 μm paraffin sections were cut by standard microtomy. A histoarray (CJ1) produced by SuperBioChips (AMS Biotechnology, Oxfordshire, UK) was also used. Formalin fixed, paraffin embedded, sections of human normal tissue, tumour tissue and TMAs were deparaffinised with xylene, rehydrated, treated with picric acid, and microwaved in citric acid at pH 6. Slides were blocked in a 1:5 dilution of sheep serum for 30 min at room temperature. Primary anti-eEF1A2 rabbit antibody was used at a concentration of 1:10 diluted in phosphate-buffered saline (PBS), for 40 min at room temperature and secondary goat anti-rabbit IgG biotin conjugated antibody (Dako Cytomation, Cambridgeshire, UK) was used at 1:200 at room temperature for 30 min. Slides were incubated with StreptABC complex/HRP (Dako Cytomation) at room temperature for 30 min and in diaminobenzidene (Sigma Fast DAB, Sigma, Dorset, UK) for 2 min at room temperature. Alternatively, after the primary antibody step slides were washed in PBS and three drops of ChemMate DAKO EnVision/HRP Rabbit/Mouse secondary antibody (DAKO Cytomation) added to each slide. Slides were incubated for 30 min at room temperature and then washed in PBS. The DAB-containing substrate working solution was prepared by mixing 50 parts ChemMate Substrate Buffer with1 part ChemMate DAB + Chromogen (DAKO Cytomation). One millilitre of this solution was added to each slide and incubated for 5 min. Finally, slides were counterstained in haematoxylin, dehydrated and mounted in pertex.

Immunohistological scoring methods

The ovarian tumour histoarray (CJ1 SuperBioChips AMS Biotechnology, Oxfordshire, UK) and normal ovarian sections were given a histoscore. For each score, the percentages of the tumour tissue (excluding stroma) which stain strongly (3), moderately (2) and faintly (1) were assessed. The Histoscore was calculated by multiplying the percentage of tumour tissue staining by the score in each category, and adding these values to give a maximum of 300. Expression in the TMAs was assessed using a modification of the method of Tolivia *et al* (2006).

Statistical methods

Fisher's exact test was used to test for associations between positive protein expression and tumour subtypes of clear cell carcinomas *vs* all other tumour types combined.

Mutation analysis

For mutation analysis of the *EEF1A2* gene, primer pairs were designed for each exon (see supplementary data). PCR was carried

out using *Taq* polymerase from Invitrogen (Paisley, UK), except in the cases of exons 1 and 8, that required the use of DyNAzyme EXT DNA polymerase (Finnzymes, NEB, Hitchin, UK) with cycling conditions consisting of an initial denaturation step at 94°C for 5 min followed by 32 cycles of 30 s at 94°C, 30 s at the annealing temperature and 30 s at 72°C (60 s when using DyNAzyme). PCR products were then sequenced using BigDye v3.1 (Applied Biosystems) according to the manufacturer's conditions.

Methylation analysis

Methylation analysis was carried out using bisulphite sequencing of a 548 bp region of the *EEF1A2* CpG island. The EZ DNA Methylation Kit (Zymo Research) was used to convert 1 μg of DNA from ovarian tumours HOV 104, 179, 548, and 557 and from normal whole ovary samples 440 and 470 according to the manufacturer's protocol. The resulting DNA was amplified using the following primers: 5′-AGGGATTGGAAATTAGTAGATTT and 5′- AAAAAAAATCCACCTATTAA and Roche Fast Start *Taq* DNA polymerase. Cycling conditions were a 5 min initial denaturation step at 95°C followed by 44 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 90 s. The converted PCR products were cloned into the pCR2.1 vector using the TA Cloning Kit (Invitrogen) following the manufacturer's protocol. Clones (2–5 for each original DNA sample) were sequenced as before and the results analysed using BiQ Analyser software (http://biq-analyser.bioinf.mpi-inf.mpg.de/) (Bock *et al*, 2005).

Quantitative real-time PCR analysis of copy number

For *EEF1A2* DNA copy number analysis two sets of intronic primers were designed (see supplementary data online). Primers designed to amplify microsatellite loci in regions of chromosomes, which are normally stable in ovarian cancers were used for normalisation of total DNA amount. This was based on the method of DNA copy number analysis used by Ginzinger *et al* (2000). For determination of copy number at 20p primers from microsatellite loci on 20p were used and normalised relative to D5S643. Quantitative real-time PCR analysis was carried out using a MyiQ Single Color Real-Time PCR machine (BioRad, Hemel Hempstead, UK) and iQ SYBR Green Supermix (2 ×) (BioRad). Standard curves were conducted on 100 ng DNA extracted from normal blood that was serially diluted five times from 1 : 10 to 1 : 100000. 200 ng of DNA from each tumour was used in a 25 μl reaction. Cycling conditions were an initial 8.5 min denaturing step at 95°C followed by 40 cycles of 95°C for 30 s, and 72°C for 30 s. Fluorescent signal collection was carried out at elongation (72°C). A melt curve was included to confirm primer specificity and minus DNA controls were included to confirm that there was no contamination. Standard curves were used to determine the PCR efficiency for each primer set. Primers were used at 200 nM giving a PCR efficiency of between 90 and 110%. *EEF1A2* copy number was then calculated using the (Pfaffl (2001) method of analysis. The copy number of *EEF1A2* in normal blood DNA was determined using an average value from analysis of five different blood DNA samples. The use of three microsatellite loci for normalisation allowed for the exclusion of outlying values. Copy number at the chromosome 20p microsatellites D20S804 and D20S819 was calculated using the standard curve method of analysis (Applied Biosystems User bulletin #3).

RESULTS

Expression analysis of ovarian tumours

We assessed RNA and protein samples taken from the same group of ovarian tumours, all of which had been assessed for histological subtype. Initially, we carried out real-time RT–PCR to assess the level of expression of eEF1A2 mRNA in the tumour panel. The results are shown in Figures 1A and B and Table 1; whereas three out of the four clear cell tumours analysed (75%) had detectable overexpression of eEF1A2, only four of the 18 serous tumours (22%), five of the 12 endometrioid tumours (42%) and neither of the mucinous tumours were overexpressing. Similar RT–PCR analysis of a panel of ovarian cancer cell lines shows most cell lines to overexpress eEF1A2 compared with a human leukaemic cell line (HL60), with levels as high as 70-fold higher in the most extreme instance (OVCAR5). The results are shown in Figure 1C.

We went on to use anti-peptide antibodies (Newbery *et al*, in preparation) against eEF1A2. The antibodies were raised against synthetic peptides, which were designed to maximise the differences between eEF1A1 and eEF1A2. Western blots are shown in Figure 2A. eEF1A2 protein is expressed at detectable levels in most clear cell tumours (four out of five tumours analysed; 80%), but only one out of 23 serous papillary tumours and two out of 11 endometrioid tumours. None of the five tumours with a mucinous component had detectable eEF1A2 expression. Six of the seven clear cell tumours analysed in total were overexpressing at either the RNA or protein level or both. Unfortunately, only a few tumours had both RNA and protein extracts available; of these, only one (14) was positive at both RNA and protein levels; s 308, 80, 21, and 300 were negative at both RNA and protein level and s 9, 76, 88, 386, 77, and 5 had detectable expression at the RNA level but not at the protein level. In this set of tumours, the expression of eEF1A2 is highly significantly associated with the clear cell carcinoma subtype when compared with all other tumour subtypes combined ($P<0.0012$, Fisher's exact test). The eEF1A2-positive tumours represent less than 16% of our total tumour panel, in contrast to the 26% figure obtained by Anand *et al* (2002). This may simply represent the different frequency of clear cell neoplasms in the two sets of samples, or reflect the higher numbers of tumours that are positive at the RNA level, but which do not express the protein. This may suggest that the mechanism of overexpression of eEF1A2 in the tumours does not necessarily lead to the production of a stable protein.

When we examined expression of eEF1A2 by Western blotting in ovarian cancer cell lines we again found most cell lines to show high levels of expression. The results are shown in Figure 2B. This is in contrast with the results of Anand *et al* (2002), who found overexpression in 4 out of 13 cell lines, but is in agreement with previous results in our lab where we found most transformed cell lines to express eEF1A2 (Tomlinson *et al*, 2005) and the study by Joseph *et al* (2004) showing eEF1A2 expression in nine out of 10 cancer cell lines examined. The size of the eEF1A2 band appears to shift slightly from one sample to the next suggesting the possibility of post-translational modifications that vary between samples.

We then carried out immunohistochemistry. We wanted to extend the Western blot analysis, but also to establish whether there is any detectable expression in any cell type in the normal ovary. Figure 3 shows the results obtained with normal tissue. The only cells that were expressing eEF1A2 were luteinised stromal cells, and the ovarian surface epithelium (OSE); there was no staining in the secondary only control. We then analysed a commercial TMA with 57 analysable tissue cores, and a TMA constructed from local cases of ovarian carcinoma as described above, containing a further 91 analysable cores of a single (i.e. not mixed) histological subtype, 148 in total. It can be seen from Figure 3 and Table 2 that the results are largely consistent with those obtained by Western blotting. For the commercial array, although one serous tumour and one mucinous tumour stained moderately, the only epithelial ovarian tumours to display strong staining with the anti-eEF1A2 antibody are clear cell carcinomas. Moderate or strong expression was seen in 60% of clear cell carcinomas by immunohistochemistry on this array. The association between clear cell carcinomas and moderate or strong expression of eEF1A2, in comparison to the other tumour types,

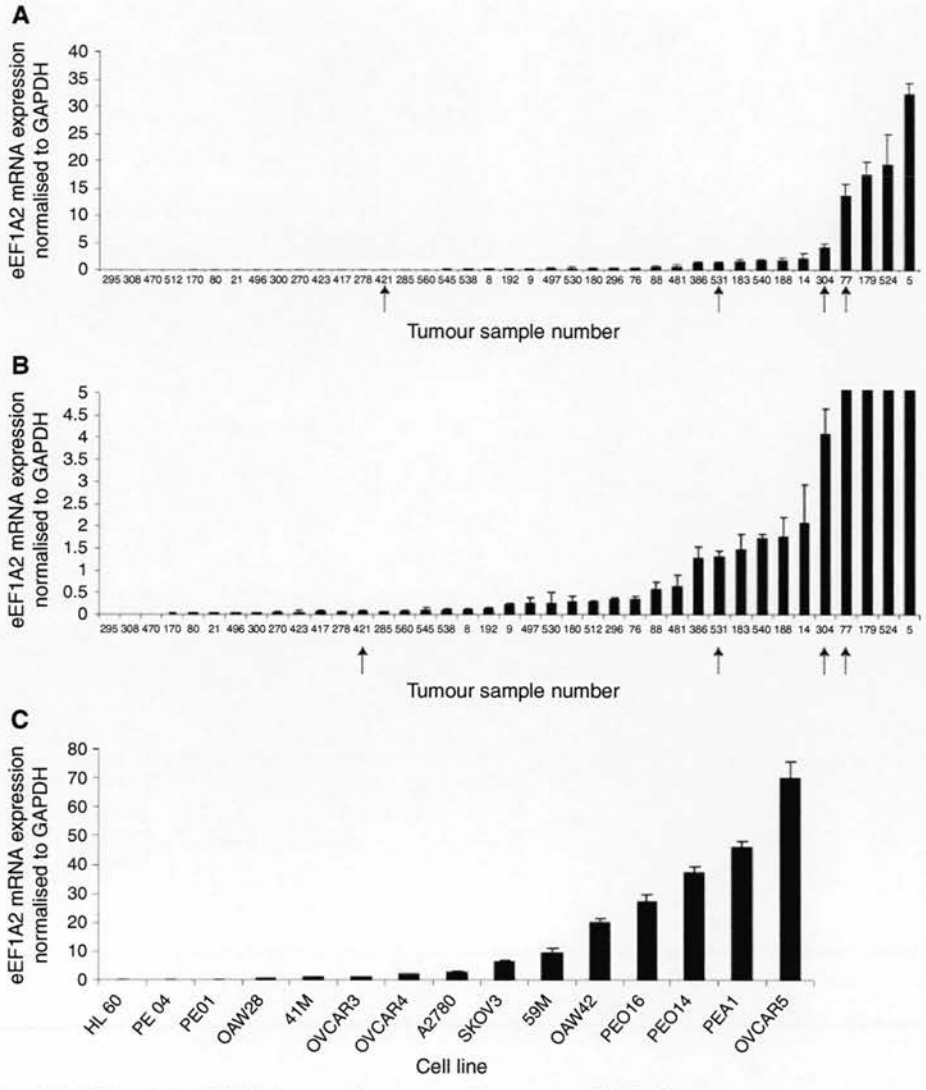


Figure 1 (**A**) Real-time RT–PCR analysis of RNA from ovarian tumours. The amount of eEF1A2 message is shown normalised to GAPDH. Arrows indicate clear cell carcinomas. Samples showing lower levels of expression are shown separately to display small amounts of expression at higher resolution. (**B**) As in (A), but showing the lower part of the scale. (**C**) Real-time RT–PCR analysis of RNA from ovarian cancer cell lines. The amount of eEF1A2 message is shown normalised to GAPDH. The negative control is HL60, a leukaemia cell line.

Table 1 eEF1A2 expression in ovarian tumours

	RNA		Protein-Western	
Tumour type	**Number analysed**	**% positive**	**Number analysed**	**% positive**
Clear cell	4	75	5	60
Serous	18	22	23	4
Endometrioid	12	42	11	18
Mucinous	2	0	5	0

GAPDH = glyceraldehydes-3-phosphate dehydrogenase. Samples are scored as positive at the RNA level if they have a score greater than 0.5 when normalised to GAPDH (see Figure 1A).

was significant ($P = 0.03$, Fisher's exact test). For the in-house array a less strong correlation with histological subtype was seen. Only one tumour fell into the 'strong' category, with a histoscore of 229, and this was a clear cell tumour, but there were far more negatively and weakly staining tumours overall; the total percentage of tumours with moderate or strong histoscores was 5% for this TMA in contrast to the 19% for the commercial TMA. Nevertheless, when the scores were combined, all three of the tumours with a histoscore of >200 were clear cell carcinomas.

DNA analysis of the EEF1A2 locus in ovarian tumours

Anand *et al* (2002) have previously shown that the *EEF1A2* locus is amplified in two out of three tumours they found to be overexpressing eEF1A2, suggesting that amplification of the gene may not be the only mechanism mediating overexpression. We did not have access to cell lines from the tumours we had studied at the RNA and Western level, but did have DNA, so we used a real-time PCR method to estimate the copy number of the *EEF1A2* locus. This was based on the method of Ginzinger *et al* (2000), but control primers were selected from regions that are normally

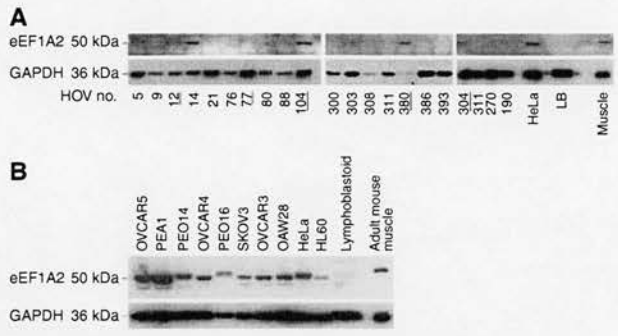


Figure 2 (**A**) Western blot analysis of protein extracts from ovarian tumours. Sample numbers are the same as those shown in Figure 1A. The same blot is shown having been exposed sequentially to eEF1A2 and GAPDH antibodies with a stripping step in between each antibody. The controls are HeLa, which expresses both eEF1A1 and eEF1A2, lymphoblastoid cells (LB) which express only eEF1A1, and adult mouse muscle, which expresses only eEF1A2. Clear-cell tumours are indicated by bold underlined type. (**B**) Western blot analysis of protein extracts from ovarian cancer cell lines. The negative control is a lymphoblastoid cell line and the positive controls are HeLa, which we have previously found to express eEF1A2 at high levels, and mouse muscle.

genomically stable in ovarian cancer; three sets were used in case of between-tumour variation. The copy number of *EEF1A2* was estimated using primers within an intron to avoid amplification of *EEF1A1*. We also estimated copy number at two loci on 20p, to distinguish between amplification of 20q and polyploidy. The results are shown in Figure 4; *EEF1A2* was amplified at a significant level (more than 2s.d. from normal DNA) in 12 of the 15 (80%) ovarian cancers expressing eEF1A2 at the RNA or protein level. Only one of these, tumour HOV12, also had an increase in copy number at 20p. However, all eight of those cancers not expressing eEF1A2 also had copy numbers significantly exceeding that found in normal diploid blood DNA. It is clear that there is no obvious correlation between copy number and gene expression; indeed, a non-expressing tumour, number HOV170, shows the greatest *EEF1A2* copy number. The similar pattern of copy number changes between non-expressing and expressing tumours suggests that gene amplification is not the primary mechanism underlying the overexpression of eEF1A2.

We therefore, went on to establish whether the methylation status of the *EEF1A2* gene differs between ovarian tumours and normal ovarian tissue. We used bisulphite sequencing to compare the methylation profile of 548 bp of the CpG island at the 5′ end of the *EEF1A2* gene in DNA samples from normal ovary, from tumours that do not express eEF1A2 and from overexpressing

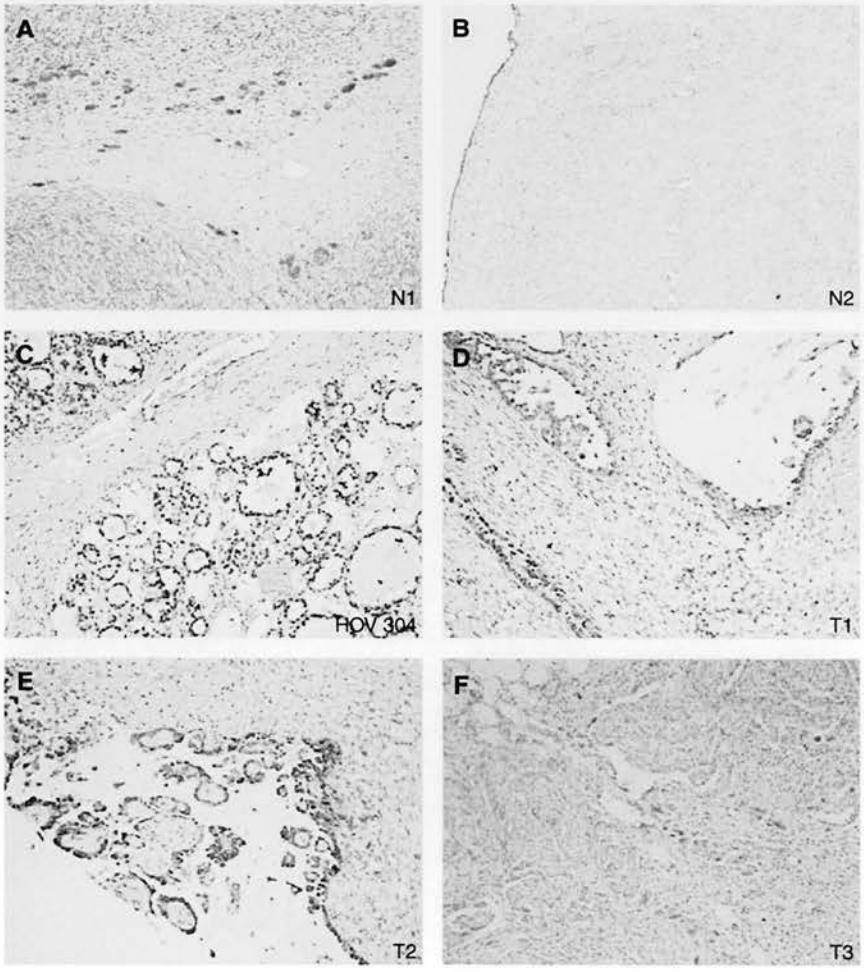


Figure 3 Immunohistochemistry of normal ovary (**A**, **B**) and ovarian cancer (**C**–**F**) sections. Staining in the normal ovary can be seen to be confined to luteinised stromal cells (N1) and low level expression in the ovarian surface epithelium (N2). HOV304 corresponds to one of the clear-cell tumors that was also analysed by RT–PCR and T1 and T2 are clear-cell tumours from the histoarray. T3 is a negative staining tumour. Magnification × 10.

Table 2 Immunohistochemistry for eEF1A2 in different ovarian tumour types using a commercial TMA and an inhouse TMA

Tumor type	Number analysed	Negative	%	Weak	%	Moderate	%	Strong	%
Clear cell	35	19	**54**	10	**29**	3	**9**	3	**9**
Serous	64	35	**54**	26	**40**	3	**5**	0	**0**
Endometrioid	38	29	**76**	9	**24**	0	**0**	0	**0**
Mucinous	11	6	**54**	4	**36**	1	**10**	0	**0**
Total	148	89	**60**	49	**33**	7	**5**	3	**2**

TMA = tissue microarray. A histoscore of 0 is recorded as negative, between 1 and 100 as weak, between 101 and 200 as moderate and over 201 as strong.

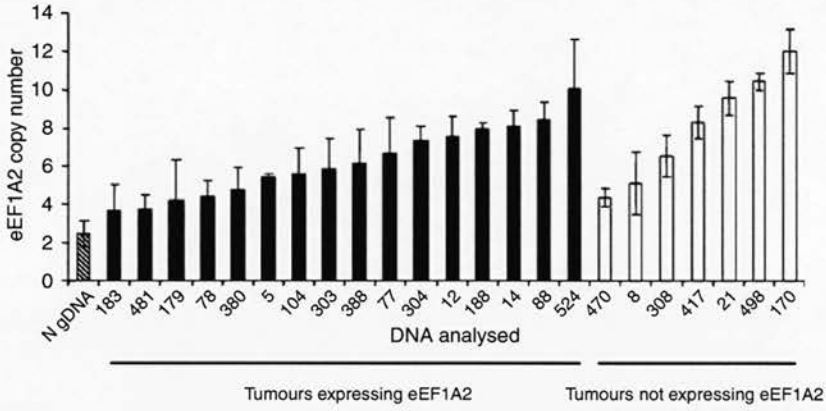


Figure 4 Mean normalised *EEF1A2* copy number in normal gDNA (horizontal stripes), ovarian cancers expressing eEF1A2 at the RNA and/or protein level (black bars), and ovarian cancers that do not express eEF1A2 (white bars). Error bars show 2s.d of the mean.

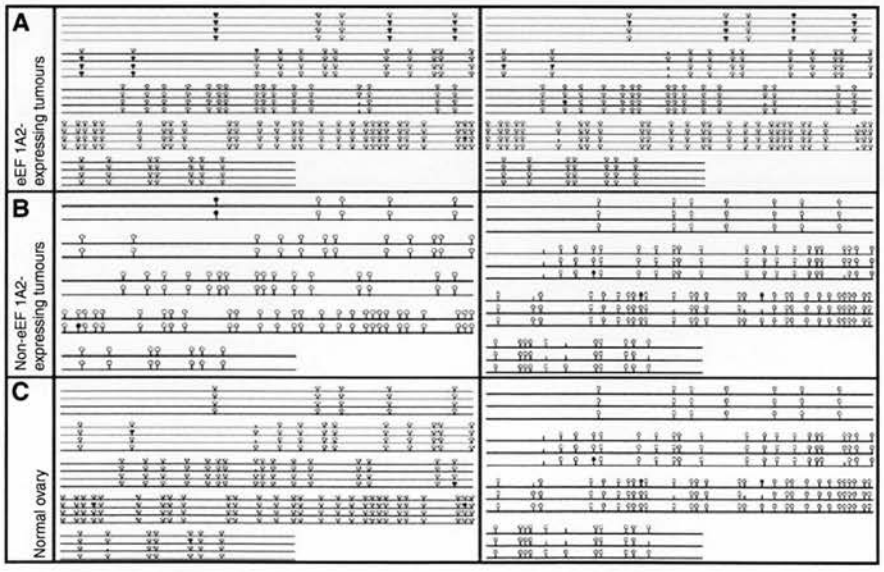


Figure 5 'Lollipop' diagrams produced by BiQ Analyser. Open circles denote unmethylated CpG dinucleotides, while closed circles denote methylated CpG dinucleotides. (**A**) Tumours 179 and 104, which do express eEF1A2 and show some methylation in CpG dinucleotides preceding the island, but no methylation within the CpG island. (**B**) Tumours 308 and 470 which do not express eEF1A2. There are very few methylated CpGs either within the island or preceding it. (**C**) DNA from two different normal whole ovaries. There is little methylation present at any CpG dinucleotide within the sequence.

tumours. It can be seen in Figure 5 that although the CpG dinucleotides immediately 5′ to the CpG island were methylated, the CpG island itself was unmethylated in all cases, suggesting that hypomethylation of the *EEF1A2* gene in tumours is not a mechanism for overexpression.

Finally, we sequenced the coding and untranslated regions of the *EEF1A2* gene in those tumours, which were overexpressing eEF1A2, but which had low copy number (and were thus amenable to analysis by sequencing without the potential complication of sequence differences between gene copies). This group comprised

six tumours in total. All eight exons (including non-coding exon 1 and the 3′UTR in exon 8) were sequenced together with flanking intronic regions spanning splice sites. Apart from two established single nucleotide polymorphisms (SNPs), the only sequence alteration identified was a G to A substitution in exon 1 of tumour 183. This sequence alteration was present on both strands and is not a known SNP; unfortunately DNA from normal tissue from the same individual was not available, so it is not possible to establish whether this is a tumour-specific change. However, given both that exon 1 is noncoding, and that this tumour shows expression of eEF1A2 at the RNA but not the protein level, the mutation is unlikely to be of functional significance in terms of cancer. The mechanism by which eEF1A2 is overexpressed in these tumours thus remains elusive, but it is clearly of interest that overexpression is able to occur in the absence of mutation or demethylation, and that there is no correlation between overexpression and amplification.

DISCUSSION

We have shown that, depending on the detection method used, up to 75% of ovarian clear cell carcinomas show overexpression of eEF1A2 at the protein level. At the RNA level, we find that 33% of ovarian tumours in total overexpress eEF1A2, which is consistent with the 30% figure reported by Anand *et al* (2002). In contrast to the results of Anand *et al* (2002), we found some expression of eEF1A2 in normal ovary, both in the OSE (from which tumours are thought to arise), and in small nests of luteinised stromal cells. We can not detect eEF1A2 expression in the vast majority of ovarian tumours examined, however, which argues against the idea that tumour overexpression is simply a feature of the cells from which the tumours have arisen. Rather, it seems likely that inappropriate expression of eEF1A2 contributes to tumorigenicity, as shown by Anand *et al* (2002).

The finding that eEF1A2 overexpression at the protein level is often associated with clear cell carcinomas is intriguing. Our results are consistent with, and extend, those obtained from microarray analysis by Schwartz *et al* (2002), who identified eEF1A2 as one of a group of genes that are highly expressed in clear cell carcinomas than other ovarian tumours. It is unclear why eEF1A2 overexpression would be found particularly in clear cell tumours. One possibility is that there is a link with p53; we found a weak but not statistically significant association between eEF1A2 expression and p53 wild-type status in breast tumours (Tomlinson *et al*, 2005), and clear cell carcinomas of the ovary normally express p53. Alternatively, Schwartz *et al* (2002) showed a tendency to upregulate stress response genes in ovarian clear-cell tumours, which may be relevant given that eEF1A2 is a known binding partner of peroxiredoxin Prdx1 (Chang and Wang, 2006).

The availability of a specific antibody that recognises eEF1A2, but not eEF1A1, may be of use in a clinical setting once further clinical correlations have been performed.

We have carried out a number of analyses of ovarian tumour DNA to establish the mechanistic basis of the overexpression of eEF1A2. Although amplification of the gene is seen in many tumours there is no correlation between locus amplification and gene expression, suggesting that this cannot be the sole underlying mechanism of overexpression. Amplification was seen just as frequently in tumours that do not express eEF1A2; it would be of interest to examine the expression levels of other genes within this amplicon. We sequenced the *EEF1A2* gene from a number of tumours with low copy number, but failed to find any mutations that could be activating or otherwise lead to overexpression (although it remains a possibility that there are mutations in regulatory regions of the gene in some tumours); similarly, there was no correlation between methylation status and expression. It is clear therefore that overexpression does not depend on genetic or epigenetic changes at the EEF1A2 locus; we suggest that the overexpression may be mediated by the inappropriate expression of a *trans*-acting factor in certain tumours. Microarray assays could be informative in this regard.

It is still unknown why eEF1A2 should have oncogenic properties in tissues in which the closely related eEF1A1 is already expressed at high levels. It has been suggested that a straightforward increase in protein synthesis capacity could be responsible (Thornton *et al*, 2003). It is also possible, however, that the two eEF1A isoforms have subtly (or even substantially) different non-canonical roles. Certainly, it has been demonstrated in myoblasts that while eEF1A1 is pro-apoptotic (Ruest *et al*, 2002), eEF1A2 is anti-apoptotic. Moreover, it has recently been shown that co-expression of eEF1A2 and peroxiredoxin, Prdx1, renders NIH3T3 cells dramatically resistant to apoptotic death induced by exposure to oxidative stress (Chang and Wang, 2006). There are numerous reports of the cytoskeletal modifying properties of eEF1A1 (Condeelis, 1995; Edmonds *et al*, 1996); again, these might differ from eEF1A1 to eEF1A2 and possibly relate to tumour invasion and propensity to metastasize. Further *in vitro* and clinical correlation studies should shed light on this. In the meantime, *eEF1A2* could provide a useful new diagnostic marker for a sub-set of ovarian tumours, and ultimately a possible target for therapy.

ACKNOWLEDGEMENTS

We thank Evangelos Ntougkos and Adrian Bird for helpful discussion and Simon Cooper for help with the figures. This work was funded by Cancer Research UK and the Wellcome Trust. HN is a Research Fellow of the Melville Trust for the Care and Cure of Cancer.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

REFERENCES

Anand N, Murthy S, Amann G, Wernick M, Porter LA, Cukier IH, Collins C, Gray JW, Diebold J, Demetrick DJ, Lee JM (2002) Protein elongation factor EEF1A2 is a putative oncogene in ovarian cancer. *Nat Genet* **31:** 301 – 305

Bock C, Reither S, Mikeska T, Paulsen M, Walter J, Lengauer T (2005) BiQ analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics* **21:** 4067 – 4068

Chambers DM, Peters J, Abbott CM (1998) The lethal mutation of the mouse wasted (wst) is a deletion that abolishes expression of a tissue-specific isoform of translation elongation factor 1alpha, encoded by the Eef1a2 gene. *Proc Natl Acad Sci USA* **95:** 4463 – 4468

Chang R, Wang E (2006) Mouse translation elongation factor eEF1A-2 interacts with Prdx-I to protect cells against apoptotic death induced by oxidative stress. *J Cell Biochem* **100:** 267 – 278

Condeelis J (1995) Elongation factor 1 alpha, translation and the cytoskeleton. *Trends Biochem Sci* **20:** 169 – 170

Edmonds BT, Wyckoff J, Yeung YG, Wang Y, Stanley ER, Jones J, Segall J, Condeelis J (1996) Elongation factor-1 alpha is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma. *J Cell Sci* **109**(Pt 11): 2705 – 2714

Gilmour LM, Macleod KG, McCaig A, Sewell JM, Gullick WJ, Smyth JF, Langdon SP (2002) Neuregulin expression, function, and signaling in human ovarian cancer cells. *Clin Cancer Res* **8:** 3933 – 3942

Ginzinger DG, Godfrey TE, Nigro J, Moore II DH, Suzuki S, Pallavicini MG, Gray JW, Jensen RH (2000) Measurement of DNA copy number at microsatellite loci using quantitative PCR analysis. *Cancer Res* **60:** 5405 – 5409

Itamochi H, Kigawa J, Akeshima R, Sato S, Kamazawa S, Takahashi M, Kanamori Y, Suzuki M, Ohwada M, Terakawa N (2002) Mechanisms of cisplatin resistance in clear cell carcinoma of the ovary. *Oncology* **62:** 349 – 353

Joseph P, O'Kernick CM, Othumpangat S, Lei YX, Yuan BZ, Ong TM (2004) Expression profile of eukaryotic translation factors in human cancer tissues and cell lines. *Mol Carcinogen* **40:** 171 – 179

Knudsen SM, Frydenberg J, Clark BF, Leffers H (1993) Tissue-dependent variation in the expression of elongation factor-1 alpha isoforms: isolation and characterisation of a cDNA encoding a novel variant of human elongation-factor 1 alpha. *Eur J Biochem* **215:** 549 – 554

Lee S, Francoeur AM, Liu S, Wang E (1992) Tissue-specific expression in mammalian brain, heart, and muscle of S1, a member of the elongation factor-1 alpha gene family. *J Biol Chem* **267:** 24064 – 24068

Lund A, Knudsen SM, Vissing H, Clark B, Tommerup N (1996) Assignment of human elongation factor 1alpha genes: EEF1A maps to chromosome 6q14 and EEF1A2 to 20q13.3. *Genomics* **36:** 359 – 361

Newbery HJ, Gillingwater TH, Dharmasaroja P, Peters J, Wharton SB, Thomson D, Ribchester RR, Abbott CM (2005) Progressive loss of motor neuron function in wasted mice: effects of a spontaneous null mutation in the gene for the eEF1 A2 translation factor. *J Neuropathol Exp Neurol* **64:** 295 – 303

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29:** e45

Ruest LB, Marcotte R, Wang E (2002) Peptide elongation factor eEF1A-2/S1 expression in cultured differentiated myotubes and its protective effect against caspase-3-mediated apoptosis. *J Biol Chem* **277:** 5418 – 5425

Schwartz DR, Kardia SL, Shedden KA, Kuick R, Michailidis G, Taylor JM, Misek DE, Wu R, Zhai Y, Darrah DM, Reed H, Ellenson LH, Giordano TJ, Fearon ER, Hanash SM, Cho KR (2002) Gene expression in ovarian cancer reflects both morphology and biological behavior, distinguishing clear cell from other poor-prognosis ovarian carcinomas. *Cancer Res* **62:** 4722 – 4729

Sellar GC, Watt KP, Rabiasz GJ, Stronach EA, Li L, Miller EP, Massie CE, Miller J, Contreras-Moreira B, Scott D, Brown I, Williams AR, Bates PA, Smyth JF, Gabra H (2003) OPCML at 11q25 is epigenetically inactivated and has tumor-suppressor function in epithelial ovarian cancer. *Nat Genet* **34:** 337 – 343

Shih Ie M, Kurman RJ (2004) Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. *Am J Pathol* **164:** 1511 – 1518

Thornton S, Anand N, Purcell D, Lee J (2003) Not just for housekeeping: protein initiation and elongation factors in cell growth and tumorigenesis. *J Mol Med* **81:** 536 – 548

Tolivia J, Navarro A, del Valle E, Perez C, Ordonez C, Martinez E (2006) Application of photoshop and scion image analysis to quantification of signals in histochemistry, immunocytochemistry and hybridocytochemistry. *Anal Quant Cytol Histol* **28:** 43 – 53

Tomlinson VAL, Newbery HJ, Wray NR, Jackson J, Larionov A, Miller WR, Dixon JM, Abbott CM (2005) Translation elongation factor eEF1A2 is a potential oncoprotein that is overexpressed in two-thirds of breast tumours. *BMC Cancer* **5:** 113