

**Regulation of Fanconi anaemia
complementation group a (Fanca) by
gonadotropin releasing hormone: a role for
Fanca in reproduction.**

Rachel Larder
BSc (Hons), The University of Manchester
MPhil, The Open University

Medical Research Council
Human Reproductive Sciences Unit
Chancellors Building
49 Little France Crescent
Edinburgh
EH16 4SB

Thesis submitted to The University of Edinburgh
for the Degree of Doctor of Philosophy

March 2005

In loving memory
of Gramps.

I declare that all the experiments detailed in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree.

Rachel Larder

March 2005

The work presented in this thesis would not have been possible without the help and support of many people, and to say ‘thanks’ to everyone via this one page would be impossible, so to those whose names are not specifically mentioned here – cheers!

Several people however do require a special mention.....

Firstly I would like to acknowledge my supervisor, Pam Brown, for giving me the opportunity to do a PhD and for all her guidance. To Dimitra, a HUGE ‘Efcharisto’ for help with those pesky cells and evil mutant clones, putting up with my teasing and being a good friend through the dark days – I’m looking forward to your wedding in Athens (ha ha!). I would also like to show my gratitude towards Keith & Derek for help with my MANY computer disasters, Ted in Graphics for assistance with conference posters, Julie for LightCycler tuition and Sheila & Mike in Histology for teaching me how to handle the beast that is the confocal microscope!

Undoubtedly the best bit about the last 3 years has been the opportunity to make some fantastic friends and I would like to say a big thank you to Amanda, Carolyn, Karen, Mhairi, Nancy, Nik, Nina, Paul & Sharon for sharing my love of quizzes, horror movies, Star Trek (O.K only Mhairi there!) and dodgy 80’s music! A special shout out to the ‘dirty’ girls – keep practising the move you never know when it might come in handy! To my two favourite people to drink gin with, an extra acknowledgement: Nancy, thanks for feeding my competitive streak and helping me crush ‘the boys’ at various board games! Sharon, thanks for putting up with my moaning about Lothian buses, and watching all those ‘educational’ hospital dramas with me! You know whenever I visit Marmaris I will always think of you and wonder where MY free drink is! Try not to stalk Will and Roger too much and I promise to keep in touch with more postcards!

A big hug and a kiss to Darren (a.k.a Rockpocket) for going first and passing on the advice even if I didn’t always agree with it! Your love and support has really helped me through the tough times and I will be forever grateful. Lastly thank you to my family – to Sam for not looking too bored when I talked science, to dad for all your encouragement and to mum – your courage and strength over the last two years has been an inspiration to me. Thank you.

Binding of gonadotropin releasing hormone (GnRH) to its receptor on the surface of gonadotroph cells within the anterior pituitary triggers the pulsatile release of the gonadotropin hormones, luteinising hormone and follicle stimulating (LH and FSH) into the blood stream. These hormones then act on their target organs, the gonads, to stimulate and regulate spermatogenesis in males and folliculogenesis in females. LH and FSH are heterodimeric glycoproteins comprised of a common α subunit (α GSU) and a unique beta subunit (LH β or FSH β), each encoded by a single gene. The processes by which GnRH regulates gonadotropin gene transcription are not fully characterised but variation in GnRH pulsatility, differential activation of GnRH receptor and coupling to second messenger signalling pathways, all contribute to give distinct, subunit-specific effects on gene transcription. The identification of transcripts and proteins regulated by GnRH could further characterise the bio-molecular processes by which GnRH controls LH and FSH production.

Previous work performed within the laboratory, using the technique of differential display RT-PCR (DD-RT-PCR), identified that a DNA damage repair gene, *Fanconi Anaemia complementation group a (Fanca)*, is differentially expressed in response to hormone. FANCA is mutated in > 60% of cases of Fanconi anaemia (FA), a genetically heterogeneous autosomal recessive disorder characterised by bone marrow failure, endocrine tissue cancer susceptibility and infertility.

Detailed analysis of *Fanca* mRNA expression using northern blotting, semi-quantitative RT-PCR and quantitative RT-PCR analysis reveals that GnRH induces a rapid and transient increase in *Fanca* mRNA within L β T2 gonadotroph cells. Indirect immunofluorescence and western blotting analysis show that *Fanca* protein is expressed in the cytoplasm and nucleus of L β T2 cells and GnRH induces a transient 2-fold increase in *Fanca* protein levels within both cellular compartments. Furthermore, treatment with inhibitors of nuclear import and export demonstrated that *Fanca* protein actively shuttles between the nucleus and cytoplasm of

gonadotroph cells, via a CRM-1 (yeast chromosome region maintenance protein 1) dependent mechanism.

Transient transfection assays using wildtype and dominant negative, point mutated forms of FANCA reveal that wildtype FANCA protein is required for GnRH induced activity of the α GSU promoter, but not LH β or FSH β promoter activity. The construction of adenoviral vectors expressing wildtype and mutant FANCA proteins and the characterisation of siRNAs to knockdown expression of Fanca in L β T2 cells will allow the confirmation of a novel role for Fanca in gonadotropin gene transcription.

The discovery that GnRH regulates Fanca expression, which in turn regulates GnRH induced α Gsu transcription, provides the first molecular evidence of a role for Fanca in the control of fertility.

Publications in peer reviewed journals

Larder, R., Chang, L., Clinton, M. and Brown, P. (2004) Gonadotropin-releasing hormone regulates expression of the DNA damage repair gene, Fanconi anemia A, in pituitary gonadotroph cells. *Biol Reprod.* **71**(3):828-36.

Oral presentations

Larder, R., Chang, L. and Brown, P. (2002) Gonadotropin releasing hormone regulates expression and cellular distribution of Fanconi Anaemia A. 193rd meeting of the Society for Endocrinology, London, UK.

Poster presentations

Larder, R., Chang, L., Clinton, M. and Brown, P. (2004) Expression of *Fanconi Anaemia a (Fanca)*, a gene implicated in DNA damage repair, is regulated by gonadotropin releasing hormone (GnRH) in L β T2 gonadotroph cells. 37th Annual meeting of the Society for the Study of Reproduction (SSR), Vancouver, Canada.

Larder, R., Karali, D. and Brown, P. (2004) Activation of gonadotropin gene transcription by gonadotropin-releasing hormone requires nuclear localisation of Fanconi Anaemia a (*Fanca*): evidence that *Fanca* acts as a signalling molecule. 12th International Congress of Endocrinology, Lisbon, Portugal.

	Page
Declaration.....	i
Acknowledgements.....	ii
Abstract.....	iii
Publications and presentations relating to this thesis.....	v
Table of Contents.....	vi
List of Figures.....	xii
List of Tables.....	xv
Abbreviations.....	xvi

Chapter One : Literature Review

1.1	The neuroendocrinology of reproduction.....	2
1.1.1	The hypothalamic-pituitary-gonadal axis.....	2
1.1.2	The gonadotropins.....	3
1.1.3	The female reproductive cycle.....	3
1.1.4	Regulation of male fertility.....	7
1.2	Mouse models and human gonadotropin signalling phenotypes.....	8
1.2.1	GnRH signalling mutations.....	8
1.2.2	GnRH receptor mutations.....	8
1.2.3	Common α -subunit mutations.....	9
1.2.4	Specific β -subunit mutations.....	9
1.2.4.1	FSH β -subunit ligand mutations.....	9
1.2.4.2	FSH receptor mutations.....	10
1.2.4.3	LH β -subunit mutations.....	10
1.2.4.4	LH receptor mutations.....	11
1.3	Development of the pituitary-gonadal axis.....	12
1.3.1	The pituitary.....	12
1.3.2	Pituitary gland development.....	14
1.3.3	Transcription factors crucial for pituitary development and patterning.....	14
1.3.3.1	Hesx1/Rpx1.....	14
1.3.3.2	LIM homeodomain transcription factors.....	15
1.3.3.3	Pitx transcription factors.....	15
1.3.3.4	Pit1.....	16
1.3.3.5	Prophet of Pit1.....	16
1.3.4	Factors required for gonadotroph specific gene expression.....	17
1.3.4.1	Steroidogenic factor 1.....	18
1.3.4.2	Early growth response factor 1.....	18
1.4	Gonadotropin releasing hormone.....	21
1.4.1	GnRH decapeptide.....	21
1.4.2	GnRH type I receptor.....	22
1.4.3	GnRH pulse frequency.....	23
1.4.4	GnRH signalling pathways.....	25
1.4.4.1	Initiation of gonadotropin gene transcription via calcium signalling.....	27
1.4.4.2	Initiation of gonadotropin gene transcription via MAPK signalling.....	27
1.5	GnRH and cancer.....	31

1.6	Regulation of gene expression by GnRH.....	32
1.7	Fanconi Anaemia.....	33
1.7.1	Clinical phenotypes of Fanconi Anaemia.....	33
1.7.2	Cellular phenotypes of Fanconi Anaemia.....	34
1.7.3	Genetics of Fanconi Anaemia.....	35
1.7.4	Fanconi Anaemia complementation group A (FANCA).....	35
1.7.4.1	Targeted disruption of the Fanca gene in mice.....	36
1.7.5	Fanconi Anaemia complementation group C (FANCC).....	37
1.7.6	Fanconi Anaemia complementation group G (FANCG).....	38
1.7.7	Fanconi Anaemia complementation group E (FANCE).....	38
1.7.8	Fanconi Anaemia complementation group F (FANCF).....	39
1.7.9	Fanconi Anaemia complementation group D.....	39
1.7.9.1	FANCD2.....	40
1.7.9.2	FANCD1.....	40
1.7.10	Fanconi Anaemia complementation group L (FANCL).....	41
1.7.11	FA protein complex formation.....	41
1.8	Fanconi Anaemia and the HPG axis.....	44
1.9	Aims of this thesis.....	45

Chapter Two : Materials and Methods

2.1	Nucleic acid manipulation.....	47
2.1.1	Solutions.....	47
2.1.2	Primer design and oligonucleotide synthesis.....	48
2.1.3	Reverse transcription-PCR.....	49
2.1.3.1	cDNA synthesis.....	49
2.1.3.2	PCR conditions.....	49
2.1.4	Agarose gel electrophoresis.....	51
2.1.5	Cloning of RT-PCR products.....	51
2.2	Microbiology.....	52
2.2.1	Bacterial solutions.....	52
2.2.2	Transformation of competent cells.....	53
2.3	Nucleic acid analysis.....	54
2.3.1	Solutions.....	54
2.3.2	Small scale DNA preparation.....	55
2.3.3	Preparation of DNA over a caesium chloride gradient.....	55
2.3.4	Spectrophotometrical analysis of nucleic acids.....	57
2.3.5	Restriction digest analysis of plasmid DNA.....	57
2.3.6	Sequencing analysis.....	58
2.3.7	Restriction enzyme digest of plasmid DNA for cloning.....	58
2.3.8	Extraction of DNA from agarose gel fragments.....	59
2.3.9	Ligation of DNA inserts into other vectors.....	59
2.3.10	Southern blotting of RT-PCR products.....	59
2.3.10.1	Probe design to verify Fanca PCR products.....	60
2.3.10.2	Radiolabelling of probes.....	60
2.3.10.3	Hybridisation and washing conditions.....	61
2.3.11	Site-directed mutagenesis of human FANCA.....	61

2.4	Tissue Culture.....	65
2.4.1	Cell lines.....	65
2.4.2	Cell growth conditions.....	65
2.4.3	Solutions.....	65
2.4.4	Resuscitation of cell lines from liquid nitrogen store.....	66
2.4.5	Passaging of cell lines.....	66
2.4.6	Matrigel coating of tissue-culture flasks.....	67
2.4.7	GnRH treatment of cells.....	67
2.4.8	Treatment of L β T2 cells with inhibitor chemicals.....	67
2.5	Transient transfection of DNA into in vitro cultured cells.....	68
2.5.1	Transient transfection of GFP expressing plasmids into cultured cells.....	68
2.5.1.1	Preparation of EGFP transfected cells prior to image analysis.....	68
2.5.1.2	Image analysis of EGFP tagged proteins in mammalian cell culture.....	69
2.5.2	Transient transfection of constructs for reporter gene assays.....	69
2.5.2.1	Transient transfection of L β T2 cells using Fugene.....	70
2.5.2.2	Transient transfection of FSH β promoter constructs.....	70
2.5.2.3	Transient transfection of α T3-1 cells using Geneporter.....	71
2.5.3	Luciferase/ β -galactosidase chemiluminescent reporter gene assay.....	71
2.6	RNA expression analysis.....	72
2.6.1	Solutions.....	72
2.6.2	RNA extraction.....	72
2.6.3	Northern blot analysis.....	72
2.6.3.1	Preparation of northern blot.....	73
2.6.3.2	Probe design for detecting Fanca and Zfp276 transcripts by northern blotting.....	73
2.6.3.3	Hybridisation and washing conditions.....	74
2.6.4	Quantitative RT-PCR.....	74
2.7	Protein expression analysis.....	77
2.7.1	Solutions.....	77
2.7.2	Protein extraction from cells grown in culture.....	79
2.7.2.1	Whole cell extracts.....	79
2.7.2.2	Nuclear and cytoplasmic cell extracts.....	79
2.7.3	Bradford assay of protein extracts.....	80
2.7.4	Preparation of SDS-polyacrylamide gels.....	80
2.7.4.1	SDS-polyacrylamide gel electrophoresis.....	80
2.7.5	Western analysis of protein extracts.....	81
2.7.6	Transfer of proteins to PVDF membrane.....	81
2.7.7	Immuno-blotting.....	82
2.7.8	Quantification of protein expression.....	83
2.7.9	Crude purification of Fanca antisera.....	83
2.7.10	Immunohistochemistry of mammalian cells cultured in vitro.....	84
2.8	'Knockdown' of Fanca gene expression using siRNAs.....	86
2.8.1	siRNA design and production.....	86
2.8.2	Transfection of siRNAs into L β T2 cells using Fugene.....	87
2.8.3	Analysis of Fanca mRNA levels after siRNA treatment.....	88
2.8.4	Analysis of Fanca protein levels after siRNA treatment.....	88
2.9	Construction of recombinant adenoviral vectors expressing FANCA.....	89
2.9.1	Materials and solutions.....	90
2.9.2	Cloning of FANCA into 'donor' vector.....	90

2.9.3	Cloning of FANCA into adenoviral 'acceptor' vector.....	91
2.9.4	Small scale preparation of recombinant adenoviral vectors.....	91
2.9.5	Confirmation of positive clones by PCR.....	92
2.9.6	Large scale preparation of recombinant adenoviral vectors.....	92
2.9.7	Producing recombinant adenovirus.....	93
2.9.7.1	<i>PacI</i> digestion of recombinant plasmid.....	93
2.9.7.2	Transfecting HEK293 cells with recombinant adenoviral DNA.....	93
2.9.7.3	Amplification and preparation of viral stock.....	94
2.9.8	Plaque assay to determine adenoviral titer.....	94

Chapter Three : Regulation of Fanca expression by GnRH

3.1	Introduction.....	97
3.1.1	Background.....	97
3.1.2	Aims of this chapter.....	100
3.2	Results.....	101
3.2.1	Fanca mRNA is expressed in L β T2 cells.....	101
3.2.2	Fanca mRNA is expressed in adult mouse pituitary.....	101
3.2.3	Fanca mRNA is not expressed in immature pituitary gonadotrophs.....	106
3.2.4	Zfp276 mRNA is not regulated by GnRH.....	109
3.2.5	An alternative Zfp276 transcript is expressed in L β T2 cells.....	109
3.2.6	Fanca mRNA levels are regulated by GnRH.....	112
3.2.7	Fanca mRNA levels increase 2-fold in response to GnRH.....	114
3.2.8	Fanca protein levels increase 2-fold in response to GnRH.....	116
3.3	Discussion.....	119
3.3.1	Fanca mRNA is expressed in mature gonadotroph.....	119
3.3.2	An alternative Zfp276 transcript is expressed within L β T2 cells.....	122
3.3.3	Fanca mRNA is regulated by GnRH.....	126
3.3.4	Fanca protein is regulated by GnRH.....	128
3.3.5	Possible role of Fanca in gonadotrophs.....	129
3.3.6	Fanca, GnRH and cancer.....	129
3.3.7	Conclusions.....	130

Chapter Four : Localization of Fanca protein in L β T2 cells

4.1	Introduction.....	132
4.1.1	Background.....	132
4.1.2	Aims of this chapter.....	134
4.2	Results.....	135
4.2.1	Sub-cellular localization of Fanca protein in L β T2 cells.....	135
4.2.2	Western blotting analysis of Fanca sub-cellular localization in L β T2 cells.....	137
4.2.3	Nuclear-cytoplasmic shuttling of Fanca protein in L β T2 cells.....	137
4.2.4	Generation of GFP-tagged mutant FANCA clones.....	142
4.2.5	Western blotting analysis of GFP-tagged FANCA constructs in L β T2 cells.....	143

4.2.6	Expression analysis of GFP-tagged mutant FANCA constructs.....	143
4.3	Discussion.....	148
4.3.1	Fanca protein is expressed in both the cytoplasm and nucleus of L β T2 cells.....	148
4.3.2	Fanca protein shuttles between the nucleus and cytoplasm of L β T2 cells.....	149
4.3.3	Specific mutations affect FANCA sub-cellular localization.....	152
4.3.4	Conclusions.....	155

Chapter Five : Fanca and gonadotropin gene expression

5.1	Introduction.....	157
5.1.1	Background.....	157
5.1.2	Aims of this chapter.....	161
5.2	Results.....	162
5.2.1	Mutating FANCA has no effect on LH β promoter activity.....	162
5.2.2	Mutating FANCA increases FSH β basal promoter activity.....	162
5.2.3	Mutating FANCA obliterates GnRH induced α GSU promoter activity....	163
5.2.4	Design of siRNAs against Fanca.....	167
5.2.5	RT-PCR analysis of Fanca mRNA expression in L β T2 cells treated with siRNAs.....	167
5.2.6	Indirect immunofluorescence of Fanca protein expression in L β T2 cells treated with siRNAs.....	170
5.2.7	Western blotting analysis of Fanca protein expression in L β T2 cells After treatment with siRNAs.....	172
5.2.8	Generation of adenoviral vectors expressing wildtype and mutant FANCA.....	174
5.2.9	Expression analysis of Fanconi Anaemia complex members.....	176
5.2.10	Expression of Fanca and Fancc in α T3-1 cells augments the GnRH induced response of the α GSU promoter.....	178
5.3	Discussion.....	180
5.3.1	FANCA is required for GnRH induced α GSU transcription.....	180
5.3.2	Role of the other FA complex members in activation of α Gsu promoter transcription.....	183
5.3.3	Analysis of gonadotropin levels in FA patients.....	185
5.3.4	Fanca expression can be knocked down in L β T2 cells using siRNAs.....	186
5.3.5	Further studies using FANCA expressing adenoviral vectors.....	189
5.3.6	Conclusions.....	190

Chapter Six : General Discussion and Conclusions

6.1	Introduction.....	192
6.1.1	Regulation of Fanca expression by GnRH.....	192
6.1.2	Effects of knocking out Fanca expression in mice.....	193
6.1.3	Fanca, GnRH and cancer.....	194

6.1.4	Fanca protein expression in gonadotroph cells.....	197
6.1.4.1	Does Fanca act as a molecular scaffold?	198
6.1.4.2	Is Fanca involved in GnRH receptor desensitization?	200
6.1.5	Fanca and gonadotropin subunit transcription.....	202
6.1.6	Role of the FA complex in transcriptional regulation via ubiquitination...	206
6.1.7	FA phenotypes and genotypes.....	209
6.1.8	Understanding the genetic and molecular basis of FA.....	211
6.2	Summary.....	213
Bibliography.....		215

Appendices

I	Vector maps.....	249
II	Academic Sources and commercial suppliers.....	255
III	Publications.....	259

List of Figures

Chapter One : Literature Review

Figure 1.1	Modulation of hormones regulating the reproductive cycle in females.....	5
Figure 1.2	Hormonal regulation of the reproductive cycle in females.....	6
Figure 1.3	Hormonal regulation of testicular function.....	7
Figure 1.4	Cell types within the mammalian anterior pituitary gland.....	13
Figure 1.5	Gradient of transcription factors required for patterning of anterior pituitary cell types.....	17
Figure 1.6	Transcription factors controlling cell type specification during pituitary organogenesis.....	20
Figure 1.7	Amino acid composition of mammalian GnRH.....	22
Figure 1.8	Organisation of the seven transmembrane domains of the human GnRH receptor.....	23
Figure 1.9	Frequency of GnRH pulses during the reproductive cycle in females.....	24
Figure 1.10	Factors involved in signalling through the GnRH receptor to initiate gonadotropin gene transcription.....	26
Figure 1.11	MAPK cascades initiated in L β T2 cells in response to GnRH.....	29
Figure 1.12	Proposed functions of GnRH in tumour progression.....	31
Figure 1.13	FANCA genomic structure.....	36
Figure 1.14	A model of the Fanconi Anaemia pathway.....	43

Chapter Two : Materials and methods

Figure 2.1	Overview of the QuikChange site-directed mutagenesis method.....	63
Figure 2.2	Method of detection of Fanca protein by ECL Plus detection kit.....	82
Figure 2.3	Detection of Fanca protein expression by indirect immunofluorescence....	84
Figure 2.4	Construction of recombinant adenovirus with BD Creator Technology....	89

Chapter Three : Regulation of Fanca expression by GnRH

Figure 3.1	Alignment of the 74 bp clone DD-RT-PCR product and mouse Fanca cDNA nucleotide sequence.....	99
Figure 3.2	Schematic representation of the Fanca locus showing overlap between Fanca and Zfp276.....	102
Figure 3.3	Expression of full-length Fanca and Zfp276 mRNA transcripts in L β T2 cells.....	103
Figure 3.4	Location of northern probes and PCR primers relative to Fanca genomic structure.....	104
Figure 3.5	Fanca <i>in vivo</i> expression analysis.....	105
Figure 3.6	Fanca <i>in vitro</i> expression analysis.....	107
Figure 3.7	Southern blotting analysis of Fanca expression in α T3-1 cells.....	108
Figure 3.8	Semi-quantitative RT-PCR analysis of Zfp276 mRNA expression in L β T2 cells.....	110

Figure 3.9	Alignment of Zfp276 PCR Clone A and Zfp276 PCR Clone B and predicted amino acid sequence.....	111
Figure 3.10	Semi-quantitative RT-PCR analysis of Fanca expression in L β T2 cells...	113
Figure 3.11	Quantitative RT-PCR analysis of Fanca mRNA levels after treatment with GnRH.....	115
Figure 3.12	Western blotting analysis of Fanca protein levels after treatment with GnRH.....	117
Figure 3.13	Fanca mRNA and protein levels increase after treatment with GnRH.....	118
Figure 3.14	Schematic representation of the Fanca locus showing the location of the isolated differential display RT-PCR clone.....	120
Figure 3.15	Addition of 70 bp of genomic sequence disrupts the protein domain structure of Zfp276.....	125

Chapter Four : Localization of Fanca protein in L β T2 cells

Figure 4.1	Detection of sub-cellular localization of Fanca protein in L β T2 cells using indirect immunofluorescence.....	136
Figure 4.2	Detection of sub-cellular localization of Fanca protein in L β T2 cells using western blotting analysis.....	139
Figure 4.3	Western blotting analysis of cytoplasmic Fanca protein levels after treatment with inhibitors and GnRH.....	140
Figure 4.4	Western blotting analysis of nuclear Fanca protein levels after treatment with inhibitors and GnRH.....	141
Figure 4.5	Confirmation of site-directed mutagenesis of GFP-FANCA.....	142
Figure 4.6	Western blotting analysis of GFP-FANCA expression constructs in L β T2 cells.....	145
Figure 4.7	Expression analysis of wildtype and mutant FANCA protein in HeLa cells.....	146
Figure 4.8	Expression analysis of wildtype and mutant FANCA protein in L β T2 cells.....	147

Chapter Five : Fanca and gonadotropin gene expression

Figure 5.1	Transcription factors involved in basal and GnRH activated gonadotropin subunit gene expression.....	159
Figure 5.2	Effects of mutating FANCA on GnRH induced LH β promoter activity in L β T2 cells.....	164
Figure 5.3	Effects of mutating FANCA on GnRH induced FSH β promoter activity in L β T2 cells.....	165
Figure 5.4	Effects of mutating FANCA on GnRH induced α Gsu promoter activity in L β T2 cells.....	166
Figure 5.5	Schematic representation of the Fanca locus showing the location of siRNAs designed from Fanca sequence.....	168
Figure 5.6	Semi-quantitative RT-PCR analysis of Fanca expression in L β T2 cells after transfection with siRNAs.....	169
Figure 5.7	siRNA knockdown of Fanca protein expression in L β T2 cells.....	171

Figure 5.8	Western blotting analysis of siRNA knockdown of Fanca protein expression in L β T2 cells.....	173
Figure 5.9	Vector maps of EGFP-FANCA adenoviral constructs.....	175
Figure 5.10	<i>In vitro</i> expression analysis of FA complex members.....	177
Figure 5.11	Effects of Fanca and Fancc on GnRH induced α Gsu promoter activity in α T3-1 cells.....	179
Figure 5.12	RNAi-mediated gene silencing.....	187

Chapter Six : General Discussion and Conclusions

Figure 6.1	Hypothetical role of GnRH and FANCA in the control of tumour development.....	196
Figure 6.2	Hypothetical role of GnRH in the shuttling of Fanca protein.....	199
Figure 6.3	Hypothetical role of FANCA and SNX5 in recycling of GnRHR.....	202
Figure 6.4	Hypothetical role of FANCA in GnRH induced α Gsu promoter activity...	204
Figure 6.5	Hypothetical role of FANCA in basal FSH β promoter transcription.....	205
Figure 6.6	Hypothetical role of the FA complex in control of gonadotropin gene transcription.....	208
Figure 6.7	Hypothetical consequences of mono-allelic loss of FANCA.....	210

Appendix I

Figure A1.1	Vector map of pCRII-TOPO plasmid.....	250
Figure A1.2	Vector map of pBluescript plasmid.....	251
Figure A1.3	Vector map of pBluescript-FANCA-EcoRV.....	252
Figure A1.4	Vector map of pCEP4-Fanca.....	253
Figure A1.5	Vector map of pEGFP-FANCA(H1110P/Q1128E).....	254

List of Tables

Chapter One : Literature Review

Table 1.1	Frequency of abnormalities seen in Fanconi Anaemia patients.....	34
Table 1.2	Interactions between FA proteins.....	42

Chapter Two : Materials and methods

Table 2.1	DNA sequence of primers used for RT-PCR expression analysis.....	50
Table 2.2	Chemically competent cells.....	53
Table 2.3	DNA sequence of primers used for site-directed mutagenesis of FANCA.....	62
Table 2.4	DNA sequence of FANCA sequencing primers.....	64
Table 2.5	Expression constructs used during the course of this thesis.....	69
Table 2.6	DNA sequence of primers used to generate probes for northern blotting analysis.....	74
Table 2.7	DNA sequence of primers used for LightCycler quantitative RT-PCR.....	75
Table 2.8	Components required to make 100 ml SDS-PAGE acrylamide gel.....	81
Table 2.9	Antisera and conditions used for western blotting analysis of cell extracts.....	83
Table 2.10	DNA sequence of Fanca and scrambled siRNAs.....	87

Chapter Five : Fanca and gonadotropin gene expression

Table 5.1	Transcription factors shown to be involved in basal and/or GnRH transcription.....	158
Table 5.2	Analysis of LH, FSH and testosterone levels in four cases of FA.....	185

A	adenosine
aa	amino acid
ACTH	adrenocorticotrophin hormone
α GSU	alpha gonadotropin subunit
α MSH	alpha melanin-stimulating hormone
AMD	actinomycin D
ANOVA	analysis of variance
AP1	activator protein 1
APS	ammonium persulphate
Arg	arginine
AU	arbitrary units
B2m	beta-2-microglobulin
BLAST	basic local alignment search tool
BMK	BiMC related kinase
BMP	bone morphogenetic protein
bp	base pairs
BRCA1/2	breast cancer susceptibility gene 1/2
BSA	bovine serum albumin
C	cytidine
$^{\circ}$ C	degrees Celsius
C-terminus	carboxy terminus
Ca ²⁺	calcium
cDNA	copy DNA
cfu	colony forming units
CHX	cycloheximide
Ci	Curie
CL	Corpus luteum
cm	centimetres
CMV	cytomegalovirus
CNS	central nervous system
CRH	corticotrophin releasing hormone
CRHR	corticotrophin releasing hormone receptor
CPE	cytopathic effect
ccpm	cherenkov counts per minute
CPHD	combined pituitary hormone deficiency
CRM1	yeast chromosome region maintenance protein 1
CsCl	caesium chloride
DAG	diacylglycerol
DD-RT-PCR	differential display reverse transcriptase polymerase chain reaction
DDT	dithiothreitol
DEB	diepoxybutane
DEPC	diethyl pyrocarbonate

DNA	deoxyribonucleic acid
dNTP	deoxynucleotide phosphate
DMEM	Dulbeccos modified eagles media
dpm	disintergrations per minute
dsRNA	double stranded RNA
E	embryonic day
EDTA	ethylene diaminetetra-acetic acid
EGFP	enhanced green fluorescent protein
Egr1	early growth response factor protein 1
EGTA	ethylene glycolbis (aminoethyl-ether)-tetra-acetic acid
ERK	extracellular signal related kinase
EST	expressed sequence tag
EtOH	ethanol
FA	Fanconi Anaemia
Fanca	Fanconi anaemia complementation group a
Fancc	Fanconi anaemia complementation group c
Fandc1	Fanconi anaemia complementation group d1
Fandc2	Fanconi anaemia complementation group d2
Fance	Fanconi anaemia complementation group e
Fanccf	Fanconi anaemia complementation group f
Fanccg	Fanconi anaemia complementation group g
Fandcl	Fanconi anaemia complementation group l
FCS	fetal calf serum
FSH	follicle stimulating hormone
FSH β	follicle stimulating hormone beta-subunit
FSHR	follicle stimulating hormone receptor
g	grams
G	guanosine
GAP	GnRH associated protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA2	gata binding protein 2
GH	growth hormone
GHRH	growth hormone releasing hormone
GHRHR	growth hormone releasing hormone receptor
GFP	green fluorescent protein
Glu	glutamic acid
Gly	glycine
G-protein	GTP-binding protein
GPCR	G-protein coupled receptor
GnRH	gonadotropin releasing hormone
GnRHR	gonadotropin releasing hormone receptor
GnRH-RE	gonadotropin releasing hormone response element

h	hours
hcG	human chorionic gonadotropin
HCl	hydrochloric acid
HEPES	N-[2-hydroxyethyl]-piperazine-N'-ethane sulphonic acid]
Hesx1	Homeobox gene expressed in ES cells
His	histidine
hnRNP A1	heterogeneous nuclear ribonucleoprotein A1
hpg	hereditary hypogonadism
HPG	hypothalamic-pituitary-gonadal axis
HRP	horseradish peroxidase
IL-6	interleukin-6
IP ₃	inositol 1,4,5-triphosphate
IPTG	isopropyl β-D-thiogalactopyranoside
ITR	inverted tandem repeats
JNK	c-jun NH ₂ -terminal kinase
kb	kilobases
KCl	potassium chloride
kDa	kilodaltons
KH ₂ PO ₄	potassium phosphate
KOH	potassium hydroxide
LacZ	β-galactosidase
LB	Luria Bertani
Leu	leucine
LH	luteinising hormone
LHβ	luteinising hormone beta-subunit
LHR	luteinising hormone receptor
Lhx	LIM homeobox gene
LMB	Leptomycin B
M	molar
mA	milliamps
MAPK	mitogen activated protein kinase
MEN1	multiple endocrine neoplasia type I
MeOH	methanol
mg	milligrams
ml	millilitre
mM	millimolar
MgCl ₂	magnesium chloride
MMC	mitomycin C
MOPS	3N-Morpholino propanesulfonic acid
mRNA	messenger RNA

NaCl	sodium chloride
NaH ₂ PO ₄	sodium phosphate, monobasic
Na ₂ HPO ₄	sodium phosphate, dibasic
NaOH	sodium hydroxide
NES	nuclear export signal
NLS	nuclear localization signal
nM	nanomolar
nt	nucleotide
N-terminal	amino terminal
OD	optical density
OHSS	ovarian hyperstimulation syndrome
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
pfu	plaque forming units
PGBE	pituitary glycoprotein hormone basal element
PHF9	PHD finger protein 9
Pit1	pituitary specific transcription factor 1
Pitx1/2	paired like homeodomain transcription factor 1/2
PKC	protein kinase C
PLC	phospholipase C
Pro	proline
Prop1	prophet of Pit1
PRL	prolactin
PRH	prolactin releasing hormone
PRHR	prolactin releasing hormone receptor
Q-RT-PCR	quantitative RT-PCR
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	revolutions per minute
Rpx	Rathke's pouch homeobox gene
RT-PCR	reverse transcriptase PCR
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
Ser	serine
Sfl	steroidogenic factor 1
SH2/3	Src-homology module 2/3
siRNA	short interfering RNA

SNX5	sorting nexin 5
Sox9	sry-box containing gene 9
T	thymidine
TAE	tris acetate EDTA
TBE	tris borate EDTA
TE	tris EDTA
TEMED	N,N,N,N-Tetramethylethylenediamine
TGS	tris-glycine-SDS
T _m	melting temperature
TSH	thyroid stimulating hormone
TSH β	thyroid stimulating hormone beta subunit
TRH	thyrotropin releasing hormone
TRHR	thyrotropin releasing receptor
Trp	tryptophan
Tyr	tyrosine
U	uridine
Ub	ubiquitin
μ g	micrograms
μ l	microlitre
μ m	micromolar
UTR	un-translated region
UV	ultra violet
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
Zfp276	zinc finger protein 276

A note on nomenclature

To distinguish between different species approved gene names and abbreviations have been used whenever possible and nomenclature protocols have been followed. Human and ovine genes are abbreviated in italicized capitals (*FANCA*, *FSH β*) and proteins in non-italicized capitals (FANCA, FSH). Mouse genes are similarly abbreviated but with a single, leading capital (gene: *Fanca*, protein: Fanca). When a gene or protein is referred to generically, outwith the context of a single species, human nomenclature is used.

Chapter 1

Literature Review

Discovery consists of seeing what everyone else has seen and thinking what no-one else has thought – Albert Szent Gyorgyi.

1 Literature Review

This chapter is split into two sections. Firstly, the current knowledge concerning the regulation of gonadotropin hormone biosynthesis by gonadotropin releasing hormone (GnRH) is discussed. Attention is focused on the development of the hypothalamic-pituitary-gonadal (HPG) axis and the differential biosynthesis of the gonadotropin subunits, within gonadotroph cells of the anterior pituitary. The review then focuses on Fanconi Anaemia, an autosomal recessive disease classified as a DNA damage repair disorder. The major gene mutated in Fanconi Anaemia, *Fanconi Anaemia Complementation group a (Fanca)*, accounts for >60% of cases and was previously identified as a GnRH regulated gene in this laboratory (Chang, 2002). This thesis presents further evidence to show that GnRH regulates *Fanca* expression in gonadotroph cells (Larder et al., 2004) and the possible impact of this regulation on gonadotropin gene transcription.

1.1 The neuroendocrinology of reproduction

1.1.1 The hypothalamic-pituitary-gonadal axis

Together, the hypothalamus, pituitary gland and gonads regulate the development and continuation of reproductive function in mammals. A finely tuned system has evolved in which higher central nervous system (CNS) centres, influenced by both internal and external stimuli, affect in a positive and negative way the secretion of GnRH from the hypothalamus into the pituitary portal circulation. This releasing hormone stimulates the anterior pituitary gland to secrete follicle stimulating hormone (FSH) and luteinising hormone (LH), which, in turn, act on the ovary and testis to promote follicular development and ovulation in females and spermatogenesis in males. FSH and LH also stimulate the gonads to secrete various steroidal and non-steroidal hormones. These target gland hormones then act at the levels of the CNS and/or anterior pituitary to further regulate the system. In this

section, a review of the current knowledge and understanding of the regulation of gonadotropin biosynthesis and secretion is discussed.

1.1.2 *The gonadotropins*

The pituitary gonadotropins are fundamental to endocrine communication along the HPG axis. FSH and LH are heterodimeric glycoproteins comprised of a common α -subunit and a unique β -subunit (Bonsfield, 1994). The α -subunit is also common to the glycoproteins, human chorionic gonadotropin (hCG) and thyroid-stimulating hormone (TSH). Functional heterodimers of LH and FSH are synthesized and secreted into the blood stream in response to hypothalamic GnRH. FSH and LH elicit intracellular signalling pathways by binding to their respective G-protein coupled transmembrane receptors, FSH receptor (FSHR) and LH receptor (LHR) (Minegishi et al., 1990; Minegishi et al., 1991).

1.1.3 *The female reproductive cycle*

LH and FSH control the ovarian cycle in females, which lasts approximately 28 days in humans, and is defined as the interval between successive ovulations. The cycle is split into two phases: prior to ovulation the ovary is in the follicular phase, after ovulation the ovary is in the luteal phase (Figure 1.1). The trophic actions of LH and FSH are dependent on other factors such as inhibins (Mason et al., 1986b; Robertson et al., 1996), activins (Vale et al., 1986; Risbridger and Cancilla, 2000), progesterone and oestrogen (Diczfalusy, 1998). At the beginning of each cycle, an increase in FSH levels leads to increased stimulation of the FSHR on the surface of granulosa cells resulting in the recruitment of multiple follicles to begin pre-ovulatory development. By the mid follicular phase, a rise in oestrogen and inhibin levels precedes a fall in FSH levels and a slow increase in LH levels. Usually, at around day 10 of the cycle, a 'dominant' follicle is selected and a further rise in oestrogen levels is seen (Figure 1.1). This surge in oestrogen levels feeds back positively triggering an increase in the frequency of hypothalamic GnRH pulses (Clarke et al., 1987). This in turn causes the rapid release of LH from a bank of LH containing granules within gonadotroph cells. This LH surge is crucial for reproduction as it

triggers ovulation i.e. the release of the ovum from the mature follicle (Figure 1.1), allowing the cycle to enter the luteal phase. An increase in FSH levels is also seen at the time of ovulation as the increase in LH suppresses synthesis of inhibin (McNeilly, 1988). The ruptured follicle then forms the corpus luteum (CL), and begins to secrete progesterone (Filicori et al., 1984) (See Figure 1.1 and 1.2). Progesterone depresses gonadotropin levels thus preventing the development of any more antral follicles. If, at the end of the luteal phase fertilization of the ovum has not occurred then luteolysis of the CL takes place resulting in the decline of progesterone, oestrogen and inhibin levels. This allows FSH levels to increase again and signals the rescue of pre-antral follicles and repetition of the cycle (Figure 1.1).

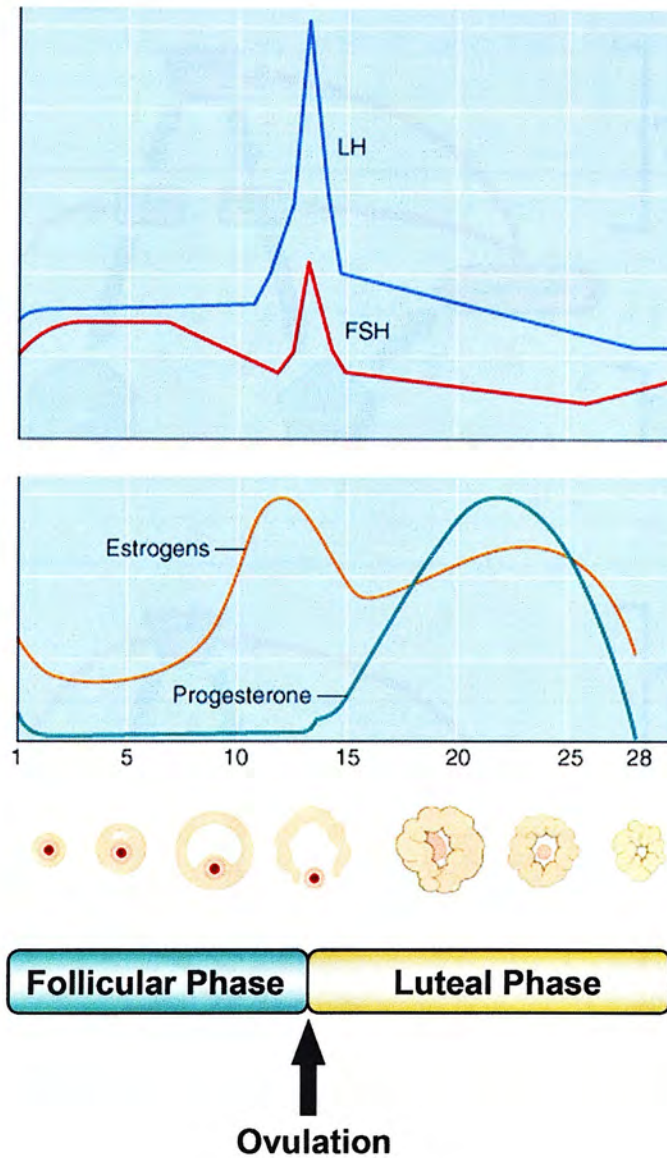


Figure 1.1 Modulation of hormones regulating the reproductive cycle in females.

Relative gonadotropin and gonadal hormone plasma levels are indicated on the graphs. Ovum development throughout the cycle is indicated below the graphs as are the two phases separated by ovulation (arrow). Picture adapted from diagram available at <http://www.colorado.edu/>. LH, luteinising hormone; FSH, follicle stimulating hormone.

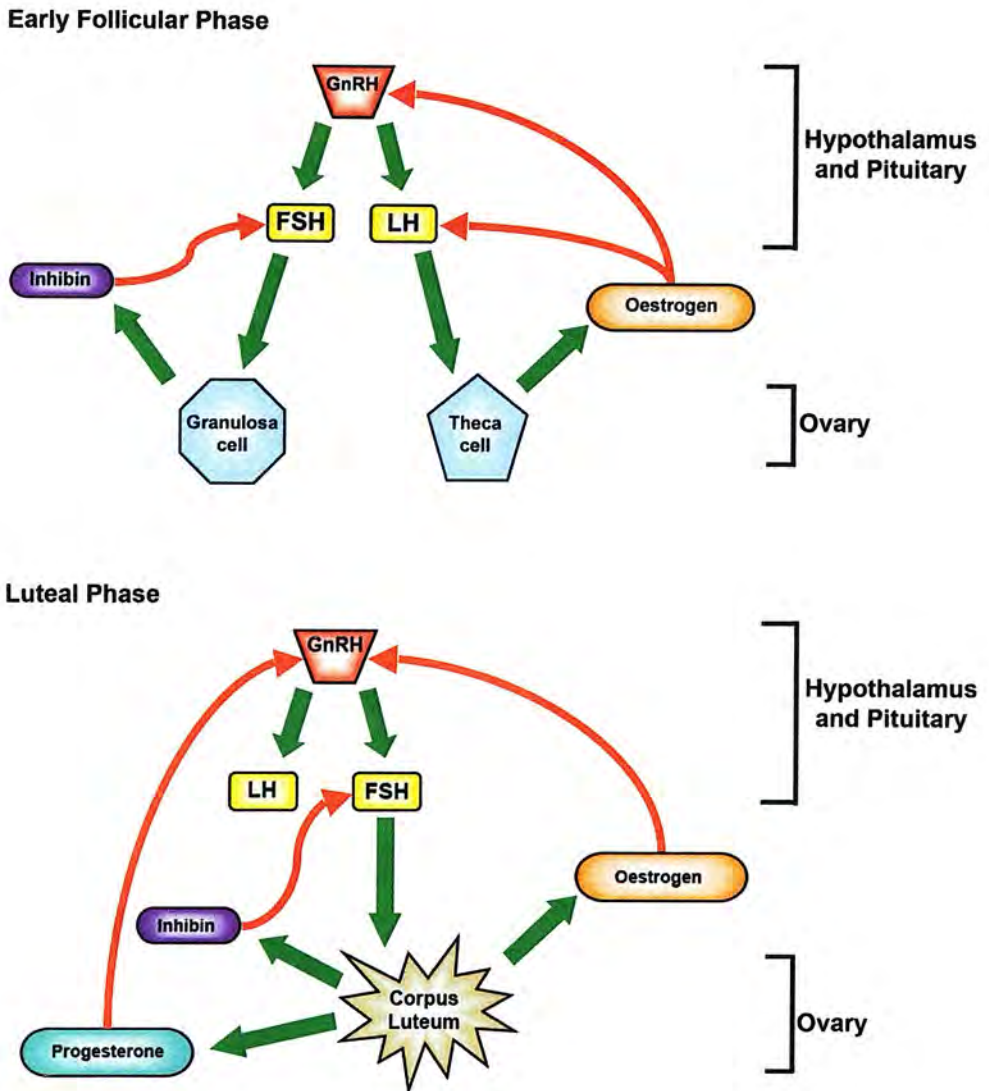


Figure 1.2 Hormonal regulation of the reproductive cycle in females.

Diagram showing hormones involved in the early follicular and luteal phases of the female reproductive cycle. Positive effects and sites of action of hormones produced during the female cycle are shown in green. Negative feedback pathways in operation are shown by red arrows. GnRH, gonadotropin releasing hormone, LH, luteinising hormone, FSH, follicle stimulating hormone.

1.1.4 Regulation of male fertility

The neuroendocrine mechanisms governing testicular function are fundamentally similar to those regulating ovarian function. The main difference being, after puberty, control of gonadal function is continuous rather than cyclical. In males, LH controls testosterone secretion from Leydig cells (Matsumoto et al., 1984) whilst FSH acts on seminiferous tubules to control spermatogenesis (Means et al., 1980). Negative feedback by testosterone and inhibin ensures tight control of LH and FSH production from the pituitary. Testosterone inhibits the pulse frequency of GnRH from the hypothalamus (Matsumoto and Bremner, 1984) as well as acting on the pituitary to decrease GnRH receptor (GnRHR) sensitivity to its ligand. FSH levels are regulated by inhibin secreted from Sertoli cells (Carroll et al., 1989). See Figure 1.3 below.

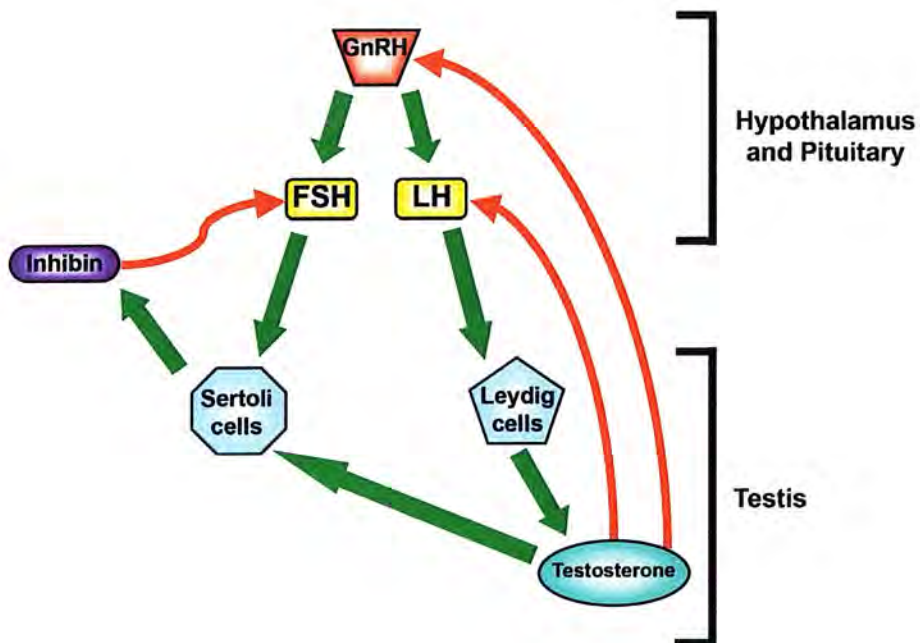


Figure 1.3 Hormonal regulation of testicular function.

Diagram showing hormones involved in male reproduction. Positive effects and sites of action of hormones produced during the control of male reproduction are shown in green. Negative feedback pathways in operation are shown by red arrows. GnRH, gonadotropin releasing hormone; LH, luteinising hormone; FSH, follicle stimulating hormone.

1.2 Mouse models and human gonadotropin signalling phenotypes

Mutations of genes concerned with HPG function are rare, however, they offer the opportunity to understand the mechanisms of reproduction much more clearly. These naturally occurring mutations have been corroborated by genetic manipulation of animal models to allow additional study of the particular phenotypes arising from disruption of the HPG axis.

1.2.1 GnRH signalling mutations

To date, no loss-of-function mutations of the GnRH gene have been described in humans (Weiss et al., 1991). However, in mice, there is a naturally occurring deletion in the *Gnrh* gene resulting in a model of hereditary hypogonadism (*hpg*) (Cattanach et al., 1977; Mason et al., 1986a). Mutant *hpg* mice completely lack GnRH, resulting in severely reduced levels of LH and FSH within the pituitary and consequently infertility and immature gonads. The phenotype can be rescued by administration of GnRH directly, rather than by stimulation of the hypothalamus, indicating that gonadotroph cells in these mice are still responsive to GnRH (Charlton et al., 1983; Lewis et al., 1986). Patients presenting with a similar phenotype to the *hpg* mouse will predictably have mutations in either; genes involved in GnRH releasing neuron migration [Kallmans Syndrome] (Franco et al., 1991; Dode et al., 2003); genes involved in GnRH processing (Seminara et al., 2000) genes hypothesised to be involved in the hypothalamic regulation of GnRH secretion by gonadal steroids (Seminara et al., 2003; Gottsch et al., 2004) or the gene encoding the GnRHR (de Roux and Milgrom, 2001).

1.2.2 GnRH receptor mutations

Several patients with idiopathic hypogonadotropic hypogonadism (IHH) have been shown to carry bi-allelic mutations of the GnRHR gene (de Roux et al., 1997; Layman et al., 1998; de Roux et al., 1999; Pralong et al., 1999; Kottler et al., 2000).

These patients have a broad spectrum of phenotypes varying from partial hypogonadotrophic deficiency with moderately altered levels of gonadotropins and gonadal steroids to complete hypogonadism with the absence of pubertal development (Kottler et al., 1999; Beranova et al., 2001).

1.2.3 Common α -subunit mutations

As hCG is possibly required for embryo implantation in humans, no mutations of α -subunit have been described in man, since they would probably result in embryonic lethality (Licht et al., 2001). However, transgenic mice carrying mutations in α -subunit, and therefore lacking bioactive LH and FSH, have been engineered. Heterozygote mice have normal growth and fertility but homozygote mice are severely growth retarded and infertile. Analysis of these mice shows that whilst neonatal gonads appear normal, adult gonads are pre-pubertal in appearance suggesting that gonadotropins are not necessary for initial programming of the gonads but are required for their continued maturation and adequate function (Kendall et al., 1995).

1.2.4 Specific β -subunit mutations

Unlike α -subunit, naturally occurring mutations in LH β and FSH β subunit ligands and receptors have been described in man.

1.2.4.1 FSH β -subunit ligand mutations

To date, only a few inactivating mutations in the FSH β ligand have been reported (Matthews et al., 1993; Layman et al., 1997; Phillip et al., 1998). In each case the mutation blocks the dimerization of FSH β and α GSU. In women, this results in delayed puberty, primary amenorrhea and no breast development, consistent with the functions of FSH in stimulating follicular development and oocyte maturation. Males with mutations in *FSH β* have small testicles and are azoospermic, suggesting a role for FSH in pubertal development and spermatogenesis. Targeted disruption of the *Fsh β* gene in mice affects female mice more severely than males (Kumar et al.,

1997). Histological examination of females revealed small ovaries that showed a complete failure of follicular development beyond the preantral stage. In male mice much milder phenotypic effects are seen, they have smaller testes with a 75% reduction in epididymal sperm numbers, but they can still reproduce. Studies of mice over-expressing human FSH reveal that males have elevated levels of testosterone and are infertile whilst females develop ovarian cysts (Kumar et al., 1999), a phenotype observed in women with ovarian hyperstimulation syndrome (OHSS). Similarly, mice that can synthesise and secrete FSH, but not LH, are also infertile as males have disrupted spermatogenesis and females do not develop corpus lutea (Allan et al., 2001).

1.2.4.2 FSH receptor mutations

Missense, inactivating mutations of the FSH receptor (FSHR) result in premature ovarian failure and impaired spermatogenesis whilst, activating mutations have been associated with OHSS (Aittomaki et al., 1995; Tapanainen et al., 1997; Smits et al., 2003; Vasseur et al., 2003; Montanelli et al., 2004). *Fshr* gene knockout mice have a similar phenotype to *Fsh β* gene disrupted mice (Abel et al., 2000), thus, FSH activity is required for sexual development and spermatogenesis and is crucial for female reproduction.

1.2.4.3 LH β -subunit mutations

LH β -subunit mutations are extremely rare and to date, no homozygous *LH β* mutations have been described in human females. However, there are two reports of male patients that are homozygous for a single base mutation in the *LH β* gene resulting in a non-functional LH protein. In the first reported case, the proband presented with delayed puberty, low testosterone, arrested spermatogenesis and elevated levels of both FSH and LH (Weiss et al., 1992). In a recently reported second case, a patient presented with similar clinical symptoms to the first case, but whilst FSH levels were elevated, LH levels were undetectable (Valdes-Socin, 2003). The undetectable LH levels in the second patient are due to the single base change disrupting the dimerization of LH β with α GSU. An *Lh β* knockout mouse has yet to

be generated, there is however a mouse line engineered to over-express LH (Risma et al., 1995). Females have enlarged ovaries due to the presence of multiple corpora lutea, cysts and tumours. Further analysis reveals that these mice also have precocious puberty and hyperandrogenemia (Risma et al., 1997), a clinical feature seen in women with elevated levels of LH who have polycystic ovary syndrome [PCOS] (McCartney et al., 2002).

1.2.4.4 LH receptor mutations

Two groups have demonstrated that cases of familial precocious puberty in males, characterised by enlarged testicles by age 4 yr and increased testosterone levels, were caused by single amino acid changes resulting in constitutive activation of the LH receptor (LHR) (Kremer et al., 1993; Shenker et al., 1993). Activating receptor mutations in the female appear to have no obvious phenotype but may impact on the timing of the LH surge in cycling women (Rosenthal et al., 1996). Male patients with inactivating mutations of the LH receptor present with pseudohermaphroditism, female genitalia and un-descended testes which contain no Leydig cells, presumably due to lack of testosterone secretion. (Berthezene et al., 1976; Brown et al., 1978; Eil et al., 1984). In contrast, females present with primary amenorrhea and anovulation (Toledo et al., 1996). Targeted deletion of the *Lhr* gene in mice generated a similar phenotype to that described in humans (Zhang et al., 2001).

In summary, analysis of HPG axis mutations has identified several specific roles for GnRH, LH and FSH. Firstly, GnRH is required for up-regulation of LH and FSH. Secondly, FSH is crucial for the recruitment of follicles within the ovary and is required for sertoli cell function and consequently spermatogenesis in males. Finally, LH controls the secretion of testosterone in males, a factor crucial for spermatogenesis and is required for ovulation in females.

1.3 Development of the pituitary-gonadal axis

In recent years it has been shown that a controlled cascade of temporally and spatially regulated transcription factors are required for the development of specific cell types within the anterior pituitary as well as the initiation and maintenance of production of specialized hormones from these cells. This section will discuss some of the transcription factors crucial for normal anterior pituitary development with particular reference to those required for gonadotroph cell determination and basal expression of gonadotropin genes.

1.3.1 The pituitary

The mature human pituitary gland is comprised of three lobes (anterior, intermediate and posterior). The gland integrates complex feedback mechanisms from the brain, via the hypothalamus, to signal to endocrine organs such as the thyroid, adrenals and gonads, in order to regulate processes such as metabolism, growth and reproduction. The five specialised cell types that comprise the anterior pituitary gland are defined by the hormones they produce and secrete in response to levels of releasing and inhibitory hormones secreted by the hypothalamus. Corticotrophs secrete adrenocorticotrophin (ACTH) to regulate adrenal function; thyrotrophs secrete thyroid-stimulating hormone (TSH) to control thyroid hormone production; somatotrophs secrete growth hormone (GH); lactotrophs secrete prolactin (PRL) to regulate milk production; melanotrophs secrete melanin-stimulating hormone (α MSH) and gonadotrophs secrete luteinising hormone (LH) and follicle stimulating hormone (FSH) to control gonadal function (Dasen and Rosenfeld, 2001). (Figure 1.4). Studies of hormone gene activation in rodents show that the cell types differentiate in a defined temporal order (Simmons et al., 1990; Japon et al., 1994) and arise in distinct regions of the developing gland (Dasen et al., 1999; Kioussi et al., 1999). However this spatial organisation does not appear to be maintained in adulthood as the different cells become dispersed throughout the pituitary (Mikimi, 1992).

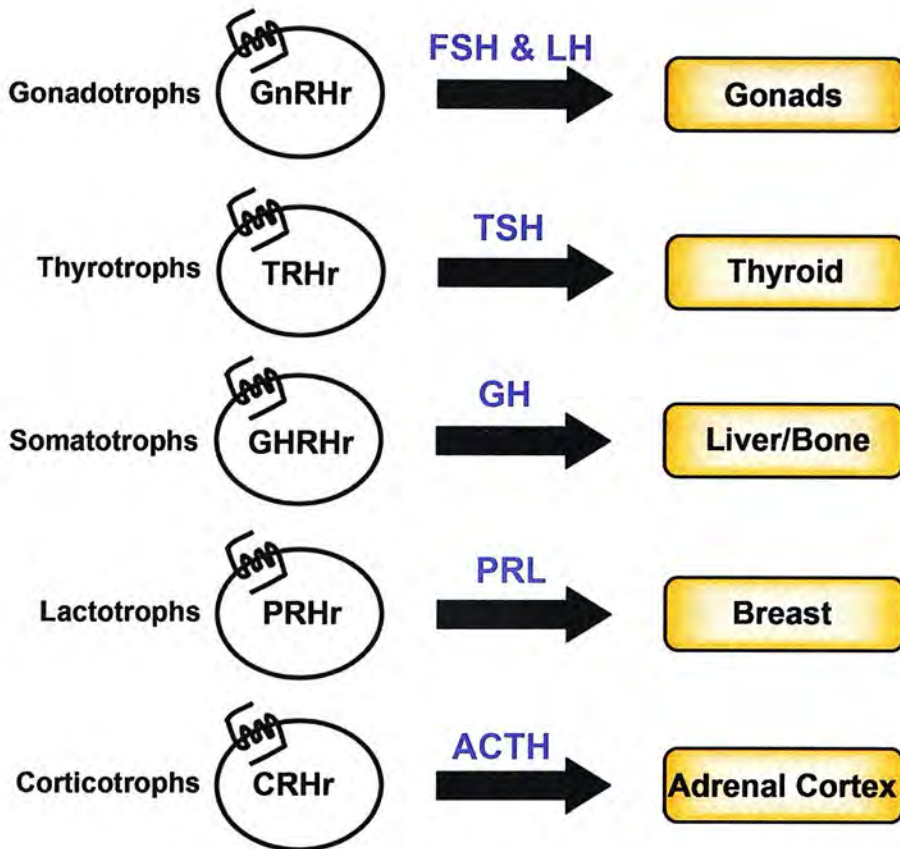


Figure 1.4 Cell types within the mammalian anterior pituitary gland.

Stimulation of specific receptors on the surface of anterior pituitary cells results in the synthesis and secretion of cell specific hormones. The five anterior pituitary hormone secreting cell types, the receptors that allow regulation of pituitary hormone secretion, their hormone products, and the target organs of the hormones are shown. Gonadotropin releasing hormone receptor (GnRHr), FSH (follicle stimulating hormone), LH (luteinising hormone), thyrotropin releasing hormone receptor (TRHr), thyroid stimulating hormone (TSH), growth hormone releasing hormone receptor (GHRHr), growth hormone (GH), prolactin releasing hormone receptor (PRHr), prolactin (PRL), corticotrophin releasing hormone receptor (CRHr), adrenocorticotrophin hormone (ACTH)

1.3.2 Pituitary gland development

The mature pituitary gland is derived from two distinct embryological sources. The anterior gland develops as an invagination of the oral ectoderm forming Rathke's pouch at embryonic stage 9.5 (E9.5) in mouse. At around the same time, the posterior gland forms from an evagination of the neuroectoderm (infundibulum) (Kauffman, 1992). Direct contact between these structures is required for the early phases of pituitary development and it is known that pituitary cell determination occurs in response to the formation of signalling gradients of various transcription factors (Ericson et al., 1998; Takuma et al., 1998; Treier et al., 1998).

1.3.3 Transcription factors crucial for pituitary development and patterning

Several transcription factors have been identified as being crucial for early pituitary development through identification of mutations in humans with pituitary disorders and the engineering of mouse models.

1.3.3.1 *Hesx1/Rpx1*

Homeobox gene expressed in ES cells (Hesx1) is a paired-class homeodomain transcription factor expressed early in mouse embryogenesis in the developing neural plate and later restricted to Rathke's pouch (Thomas et al., 1995). Expression of *Hesx1* is down regulated by E13.5, this coincides with the expression of differentiation markers of the various anterior pituitary cell types such as *pituitary specific transcription factor 1 [Pit1]* (Hermesz et al., 1996) and *Prophet of Pit1 [Prop1]* (Gage et al., 1996a). Persistent mis-expression of *Hesx1* prevents pituitary expansion suggesting that *Hesx1* down-regulation is required for pituitary cell type specification (Gage et al., 1996a). Targeted mutation of the *Hesx1* gene in mice shows that it is essential for the development of the forebrain, eyes and pituitary, which is corroborated by combined pituitary hormone deficiency (CPHD) in humans with *HESX1* mutations (Dattani et al., 1998; Dattani et al., 1999; Thomas et al., 2001). Recently, *Hesx1* has been shown to repress α Gsu and *Lh β* promoters indicating a possible role for *Hesx1* in the inhibition of terminal differentiation markers (Quirk and Brown, 2002).

1.3.3.2 LIM homeodomain transcription factors

Several members of the LIM (Zinc finger binding domain present in *Lin-1*, *Isl-1* and *Mec-3*) homeodomain family, including *Isl1*, *Isl2*, *Lhx2*, *Lhx3* and *Lhx4*, are expressed during pituitary development. *Lhx3* expression is switched on in Rathke's pouch and is maintained in the adult pituitary (Zhadanov et al., 1995). Targeted disruption of the *Lhx3* gene in mice arrests pituitary gland formation at the Rathke's pouch stage in *Lhx3*^{-/-} mice and only corticotrophs can be detected, suggesting that *Lhx3* expression is required for development of the other anterior pituitary cell types (Sheng et al., 1996; Sheng et al., 1997). Human patients homozygous for a point mutation in *LHX3* (Y114C) have growth, reproductive and metabolic disorders consistent with a role for the gene in the differentiation of somatotrophs, gonadotrophs and thyrotrophs (Netchine et al., 2000). This mutation also affects the functional properties of *Lhx3*, as the mutant protein does not activate α *Gsu* or *FSH β* transcription, supporting a role for *Lhx3* in gonadotropin regulation (Howard and Maurer, 2001; West et al., 2004). *Lhx4* is closely related to *Lhx3*, and is expressed during pituitary development. *Lhx4* knockout mice display a lack of pituitary development beyond Rathke's pouch due to a failure to expand the pouch (Raetzman et al., 2002).

1.3.3.3 *Pitx* transcription factors

Paired-like homeodomain transcription factor (PITX) proteins are important for many developmental pathways including pituitary organogenesis (Gage et al., 1999a). *Pitx1* expression precedes Rathke's pouch formation and continues throughout anterior pituitary development in several cell lineages. It is required for α *Gsu* and *Lhx3* expression (Tremblay et al., 1998) and can act synergistically with *Pit1* in somatotrophs and *Steroidogenic factor 1* (*Sf1*) in gonadotrophs to regulate *Prl* and *Lh β* promoters respectively. *Pitx1* null mice have defects in hind limb morphogenesis and have a diminished population of gonadotroph and thyrotroph cells (Szeto et al., 1996; Lanctot et al., 1999; Marcil et al., 2003). This relatively mild pituitary phenotype may be explained by a possible functional redundancy between *Pitx1* and a related paired-homeodomain transcription factor, *Pitx2*.

Expression analysis shows that *Pitx1* and *Pitx2* are co-expressed during mouse pituitary development (Gage and Camper, 1997). In *Pitx2* mutant mice, the gland fails to progress beyond E10.5 resulting in a failure of immature cells to proliferate ventrally and populate the pituitary gland (Gage et al., 1999b; Lin et al., 1999; Suh et al., 2002). *Pitx2* is also hypothesised to be involved in terminal differentiation of pituitary cell types as it can directly increase transcription from the *Fsh β* and *Prl* gene promoters (Amendt et al., 1999; Suszko et al., 2003).

1.3.3.4 *Pit1*

Expression of *Pit1*, a POU-class homeodomain protein, is activated coincident with the down regulation of *Hesx1* expression and is important for development of thyrotrophs, somatotrophs and lactotrophs due to its role in activation of *Tsh β* , *Gh* and *Prl* promoters (Li et al., 1990; Tremblay et al., 2000). *Pit1* expression is excluded from gonadotrophs, but not thyrotrophs, by a high level of *Gata2* expression, which inhibits the expression of *Pit1*, but allows expression of gonadotroph specific transcription factors (Dasen et al., 1999). In dorsal cells, *Gata binding protein 2* (*Gata2*) expression is low therefore *Pit1* expression persists in somatotrophs and lactotrophs precursors. In presumptive thyrotrophs, levels of *Gata2* are insufficient to inhibit *Pit1* expression therefore allowing the emergence of a cell type expressing both *Gata2* and *Pit1*. This is controlled by a *bone morphogenetic protein 2* (*Bmp2*) gradient, which induces the dorsal to ventral *Gata2* expression pattern (See Figure 1.5). If *Pit1* and *Gata2* are disrupted, then this expression gradient is lost and specific cell types fail to arise (Dasen et al., 1999).

1.3.3.5 *Prophet of Pit1*

The role of *Prophet of Pit1* (*Prop1*), a paired-like homeodomain factor, was determined through genetic and phenotypic analysis of the Ames (*df*) mouse. The anterior pituitaries of these mice showed a severe lack of thyrotrophs, somatotrophs and lactotrophs (Gage et al., 1996b), due to an absence of *Pit1* expression, linking *Prop1* to the activation of *Pit1* expression (Sornson et al., 1996). *Prop1* also acts to repress *Hesx1* expression so that pituitary cell-type determination, rather than

proliferation, occurs (Gage et al., 1996a). *Prop1* may also be required for the determination of gonadotroph cells or gonadotropin gene activation as patients with *PRO1* mutations have severe defects in gonadotropin synthesis (Wu et al., 1998). This is probably due to the persistence of *Hesx1* expression within the developing anterior pituitary resulting in the repression of gonadotropin gene expression.



Figure 1.5 Gradient of transcription factors required for patterning of anterior pituitary cell types.

A high level of *Gata2* expression determines the differentiation of gonadotrophs cells (G) due to repression of *Pit1* expression. Expression of *Pit1* determines the differentiation of somatotrophs (S), lactotrophs (L) and corticotrophs (C). Expression of both *Gata2* and *Pit1* determines the differentiation of thyrotrophs (T).

1.3.4 Factors required for gonadotroph specific gene expression

As discussed above, gonadotroph cells arise ventrally within the anterior pituitary. The expression of gonadotroph cell terminal differentiation markers, *LH β* and *FSH β* , is controlled by various transcription factors including *Early growth response-1* (*EGR1*), *SF1* and *PITX1*. Analysis of mice engineered with targeted mutations in these genes reveals a distinct role for these factors in gonadotropin synthesis.

1.3.4.1 Steroidogenic factor 1

Steroidogenic factor 1 (Sf1) is expressed in several endocrine tissues during development and targeted disruption of the *Sf1* gene reveals it is crucial for reproductive development since *Sf1*^{-/-} mice lack gonads and adrenal glands. Males exhibit sex reversal due to a regression of the gonads prior to sexual differentiation (Ingraham et al., 1994; Luo et al., 1995). The lack of adrenal glands causes death soon after birth due to adrenocortical insufficiency but the mice can be rescued by corticosteroid injection. In pituitaries lacking *Sf1* expression, *Tsh*, *Gh* and *Acth* are expressed as normal but there is a marked decrease in the expression of gonadotroph specific markers (Ingraham et al., 1994). This is consistent with a role for *Sf1* in α *Gsu* (Barnhart and Mellon, 1994; Ingraham et al., 1994), *Lh β* (Halvorson et al., 1996; Keri and Nilson, 1996; Brown and McNeilly, 1997; Halvorson et al., 1998; Dorn et al., 1999), *Fsh β* (Brown and McNeilly, 1997) and *GnRHr* (Duval et al., 1997; Ngan et al., 1999) expression. Interestingly, in mice rescued by corticosteroid injection, LH and FSH levels can be restored by treatment with GnRH as lack of *Sf1* does not affect GnRH synthesis but does prevent its secretion (Ikeda et al., 1995). The importance of *Sf1* expression within the developing pituitary was confirmed by tissue-specific targeted deletion of the gene (Zhao et al., 2001). The α *Gsu*-Cre/*loxP* targeted mice lacked *Sf1* expression within the anterior pituitary, but had normal expression in the adrenal cortex and developing gonads. These mice had underdeveloped gonads and markedly reduced levels of LH and FSH demonstrating that locally produced *Sf1* is essential for normal gonadotroph function and gonadotropin gene expression.

1.3.4.2 Early growth response factor 1

Egr1, a zinc finger protein transcription factor, is crucial for gonadotroph and somatotroph cell development. There are two separate reports of mice with a targeted disruption of the *Egr1* gene (Lee et al., 1996; Topilko et al., 1998) and in both cases the mice had an infertility phenotype due to lack of *Lh β* expression. The first report showed that both sexes had lowered levels of *Lh β* expression but only female mice were sterile (Lee et al., 1996). This suggests that either the presence of

an additional regulatory mechanism compensates in males for low levels of LH, or that the gene was only partially inactivated retaining some residual function. A second *Egr1* mutant allele produced a more severe phenotype; in this case both sexes were infertile (Topilko et al., 1998). Further studies of mutant female ovaries identified a role for *Egr1* in ovarian development as well as anterior pituitary development. These phenotypes are consistent with a role for *Egr1* in activation of the *Lhβ* promoter, which contains *Egr1* binding sites that bind *Egr1* and synergise with *Sf1* to transactivate the promoter (Lee et al., 1996).

Figure 1.6 summarises the transcription factors known to determine anterior pituitary cell differentiation with particular reference to gonadotroph cells.

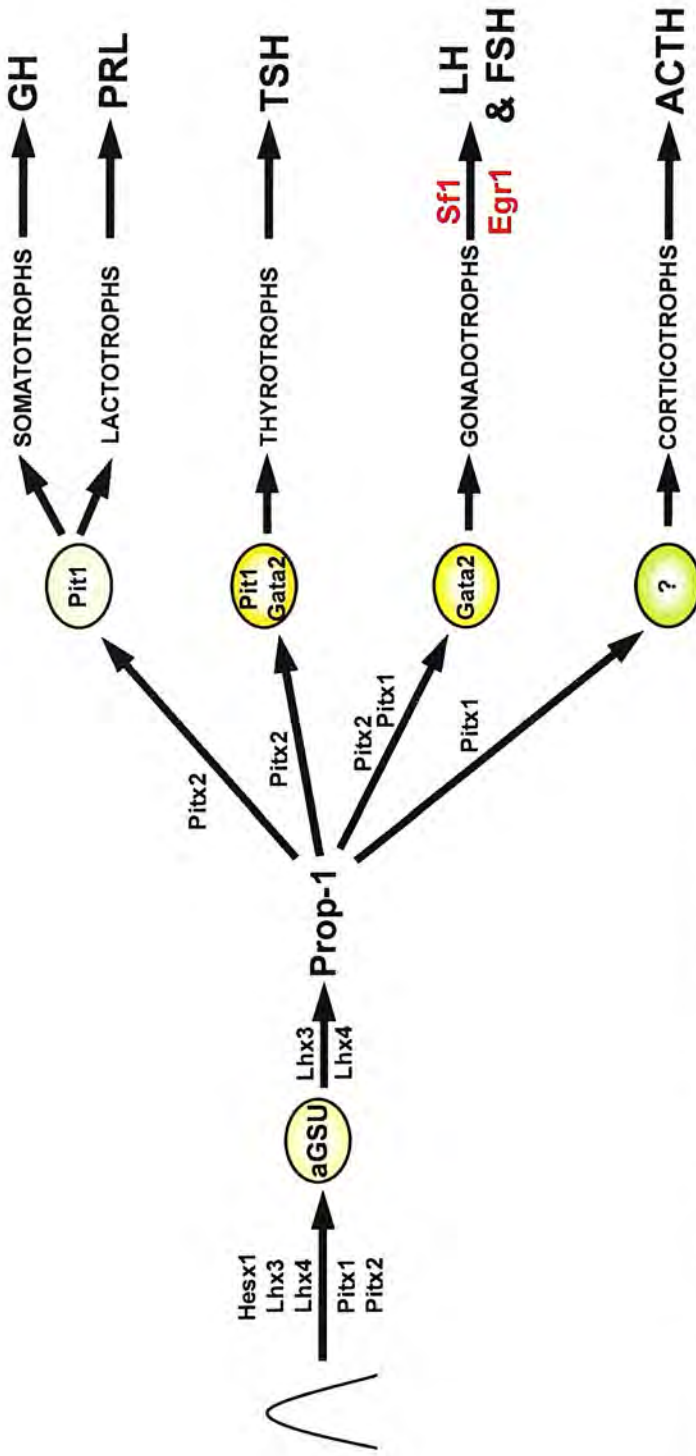


Figure 1.6 Transcription factors controlling cell type specification during pituitary organogenesis.

The expression of various transcription factors guides the development of Rathke's pouch into pre-cursor cells expressing α GSU. A gradient of *Gata2* and *Pit-1* expression determines the development of specific cell types. Expression of *Prop-1* results in activation of *Pit-1* expression and determination of somatotroph and lactotrophs cell lineages. A high level of *Gata2* expression suppresses *Pit-1* expression allowing the development of the gonadotroph cell lineage. Expression of *steroidogenic factor-1* (*Sf-1*) and early growth response-1 (*Egr-1*) activates expression of gonadotroph terminal differentiation markers LH and FSH. Expression of both *Pit-1* and *Gata2* activates transcription of *TSH β* .

1.4 *Gonadotropin releasing hormone*

The hypothalamic decapeptide, GnRH, is the central regulator of the reproductive system. Neurons within the hypothalamus release GnRH into the portal blood system, which transports it to the anterior pituitary gland. Binding of GnRH to its receptor (GnRHR) on the surface of gonadotroph cells stimulates the release of the gonadotropins, LH and FSH, into the blood stream, which carries them to their target organs, the gonads. Although each of the components of the hypothalamic-pituitary-gonadal axis is critical for reproductive function, any abnormality in GnRH synthesis, storage, release or action will result in partial or complete failure of gonadal function as previously described in Section 1.2.1.

1.4.1 *GnRH decapeptide*

There are several GnRH structural variants in vertebrates (King and Millar, 1979), and to date 16 novel GnRH peptides have been identified (Millar, 2002) with at least two, and usually three, forms of GnRH present in most vertebrate species. All GnRH-like peptides are formed from 10 amino acids and are structurally related sharing at least 50% sequence identity, with differences usually existing at amino acids 5-8. In humans two forms of GnRH have been identified, named GnRH-I and II, although in humans there is only one functional GnRH receptor (Morgan et al., 2003). GnRH-I is the prime regulator of the HPG axis in vertebrates and all further reference to GnRH in this thesis refers to GnRH-I unless stated otherwise. The cDNA sequence corresponding to GnRH encodes a 92 amino acid pre-pro-hormone consisting of a 23 amino acid signal peptide, the decapeptide and the 56 amino acid GnRH-associated peptide [GAP] (Seeburg and Adelman, 1984). The amino acid sequence of mammalian GnRH decapeptide is shown in Figure 1.7. GnRH is synthesized in neurosecretory cells within the hypothalamus. From here it is secreted, via GnRH neurons, into the hypophyseal portal vessels which transport it to gonadotroph cells within the anterior pituitary (Fink, 1988). The pulsatile delivery of GnRH, every 15-120 minutes, from the hypothalamus is crucial for the maintenance of gonadotropin gene expression and secretion of LH and FSH. The frequency of

GnRH pulses is highest during the LH surge, triggering ovulation, and lowest during the luteal phase (Clarke and Cummins, 1982; Fink, 1988). GnRH pulse frequency is discussed in more detail in Section 1.4.3.



Figure 1.7 Amino acid composition of mammalian GnRH.

The amino-terminal residues (blue) have a role in receptor activation. Amino acid residues in the middle of the decapeptide (grey) are required for binding to the GnRH receptor. The carboxy-terminal residues (pink) are required for specificity and high affinity binding to GnRH receptor.

1.4.2 GnRH type I receptor

To regulate gonadotropin gene expression, GnRH binds to GnRH type I receptor (GnRHR) located on the surface of gonadotroph cells. The gene for GnRHR has been cloned in various species including mice (Tsutsumi et al., 1992) and humans (Kakar et al., 1992). The 328 amino acid protein belongs to the G-protein coupled receptor (GPCR) family, defined by seven transmembrane domains, however mammalian GnRHR is different from other GPCRs since it lacks a carboxy-terminal cytoplasmic tail. As desensitisation and internalization of GPCRs usually occurs via this C-terminal cytoplasmic tail GnRHRs are resistant to desensitisation and internalise slowly (McArdle et al., 2002). Figure 1.8 shows the organisation of the seven transmembrane domains of the human GnRH receptor.



Figure 1.8 Organisation of the seven transmembrane domains of the human GnRH receptor.

Taken from (Ulloa-Aguirre et al., 2004). Note the lack of a C-terminal receptor tail commonly seen in GPCR's.

1.4.3 GnRH pulse frequency

The frequency and amplitude of GnRH pulses determines the secretion of FSH and LH from the pituitary. Before puberty, in both males and females, GnRH is released sporadically from the hypothalamus resulting in basal plasma levels of LH and FSH. At puberty, in males, GnRH pulse frequency increases to one GnRH pulse per hour, and is maintained at this level throughout adult life (Lincoln et al., 1985). In contrast, when females reach puberty the GnRH pulse frequency varies depending on the stage of the reproductive cycle (Knobil, 1980). After ovulation, the GnRH pulse frequency is at one pulse every 45-60 minutes, this increases to approximately one pulse every 15 minutes immediately prior to the LH surge then returns to one pulse every 60 minutes (Clarke et al., 1987; Moenter et al., 1991). Each GnRH pulse increases the responsiveness of the pituitary to subsequent GnRH pulses. However, continuous exposure of gonadotrophs to GnRH results in maintained occupancy of the receptors and is followed eventually by receptor down regulation and a reduction in pituitary LH and FSH content and secretion (Belchetz et al., 1978). Figure 1.9 shows GnRH pulse frequency throughout the female reproductive cycle.

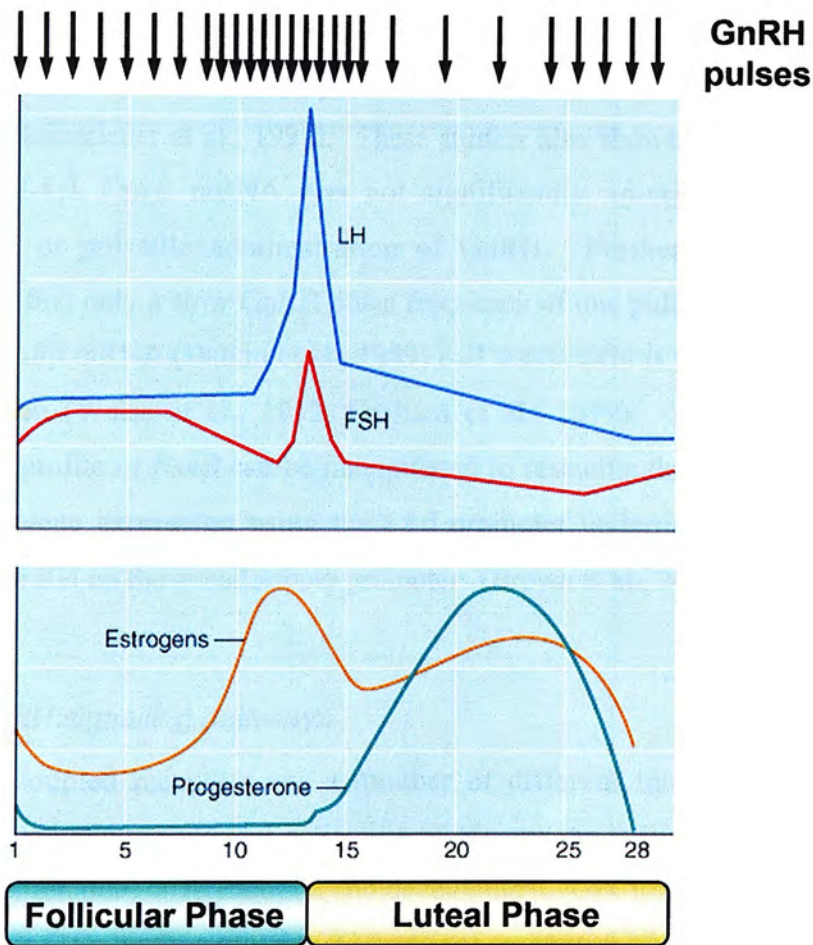


Figure 1.9 Frequency of GnRH pulses during the reproductive cycle in females.

Relative gonadotropin and gonadal hormone plasma levels are indicated on the graphs. GnRH pulses are indicated with an arrow. Picture adapted from diagram available at <http://www.colorado.edu/>. LH, luteinising hormone; FSH, follicle stimulating hormone.

The importance of GnRH pulse frequency and amplitude has been shown in various model systems. In rats, administration of GnRH increases both αGsu and $Lh\beta$ mRNA levels, however, a specific pulse frequency is required to up regulate $Lh\beta$ whereas αGsu mRNA up-regulation occurs in response to either continuous or pulsatile GnRH administration (Papavasiliou et al., 1986; Shupnik, 1990; Weiss et al., 1990; Haisenleder et al., 1991). These studies also showed that, in contrast to αGsu and $Lh\beta$, $Fsh\beta$ mRNA was not significantly up-regulated by either a continuous or pulsatile administration of GnRH. Further research has now determined that only a slow GnRH pulse frequency of one pulse every 120 minutes increases $Fsh\beta$ mRNA (Dalkin et al., 1989) and that activin is also required for this up-regulation (Weiss et al., 1993; Graham et al., 1999). The *in vivo* mRNA expression profile of $Fsh\beta$ can be manipulated to resemble that of $Lh\beta$ by driving $Fsh\beta$ transgene expression using the $Lh\beta$ promoter underlining the differential effects of GnRH on the gonadotropin promoters (Brown et al., 2001).

1.4.4 GnRH signalling pathways

G-protein coupled receptors use a number of different intracellular signalling pathways, including sequential activation of kinases, to initiate cellular processes such as proliferation, differentiation and development. GPCRs transmit their signals primarily via GTP-binding proteins (G-proteins) comprised of an α , β and γ subunit. Upon stimulation, $G\alpha$ dissociates from the $G\beta\gamma$ dimer to allow it to interact with other molecules. G-proteins are classified according to their α -subunit into the following four groups: Gs; Gi/o; Gq/11, and G12/13. Studies of $\alpha T3-1$ gonadotroph cells show that binding of GnRH to its receptor leads to the stimulation of $Gq\alpha$ and/or $G11\alpha$ protein (Shah and Milligan, 1994). Mice lacking $Gq\alpha$ or $G11\alpha$ respond to GnRH stimulation suggesting that either these G proteins are not absolutely necessary or are able to compensate for each other (Stanislaus et al., 1998), studies of double knockout mice would clarify the redundancy of $Gq\alpha$ and $G11\alpha$ in GnRH signalling. Stimulation of $Gq\alpha/G11\alpha$ leads to activation of phospholipase C (PLC) (Hsieh and Martin, 1992) which results in enhanced inositol 1,4,5-triphosphate (IP_3) turnover and production of diacylglycerol (DAG). This, in

turn, leads to the activation of protein kinase C (PKC) and Ca^{2+} mobilization. Downstream signalling to PKC leads to activation of mitogen activated protein kinase (MAPK) signalling cascades which are involved in the regulation of gene expression in various systems, either by activating transcription factors directly or by phosphorylating other protein kinases (Seger and Krebs, 1995). See Figure 1.10.

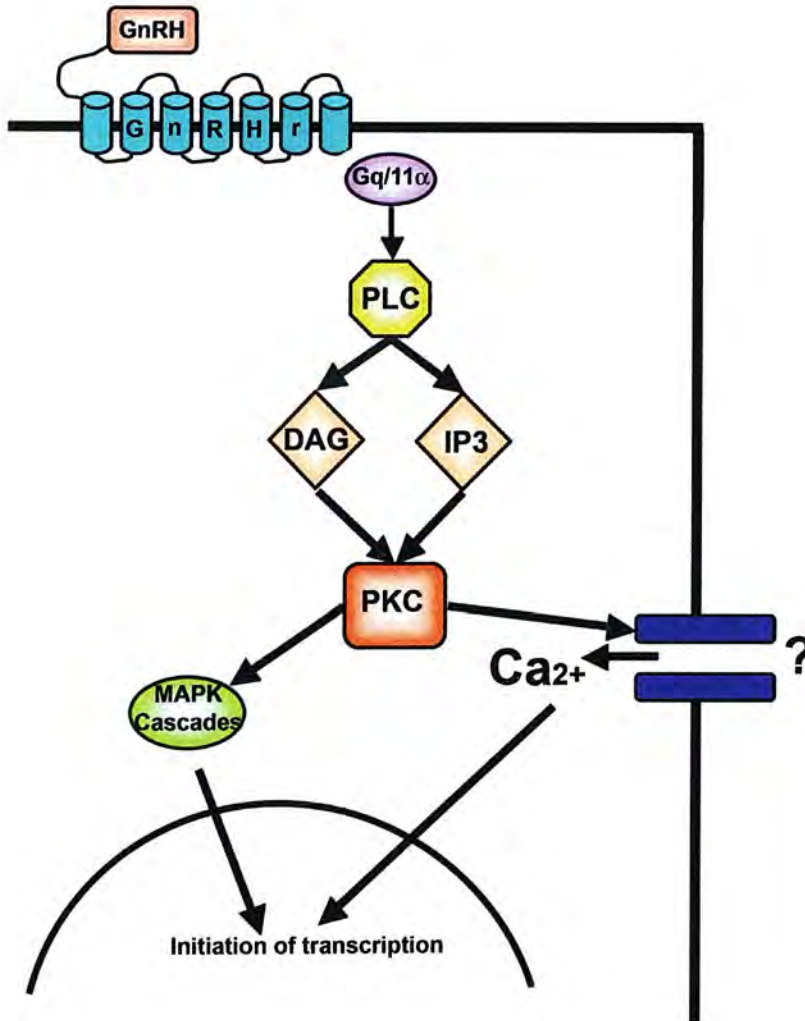


Figure 1.10 Factors involved in signalling through the GnRH receptor to initiate gonadotropin gene transcription.

GnRH binds to and activates the GnRH receptor (GnRHr) releasing the α -subunit of the G protein. This activates phospholipase C (PLC) which results in activation of protein kinase C (PKC) via diacylglycerol (DAG) and inositol phosphate (IP_3). PKC then initiates Ca^{2+} mobilization and MAPK cascades to initiate transcription.

1.4.4.1 Initiation of gonadotropin gene transcription via calcium signalling

Evidence has suggested that an influx in calcium can induce transcription of both α GSU and $LH\beta$ as well as regulating the secretion of α GSU, LH and FSH (Ben-Menahem and Naor, 1994; Stojilkovic and Catt, 1995; Holdstock et al., 1996; Weck et al., 1998). However, current thinking now suggests that the release of calcium from intracellular stores, rather than an influx of extracellular calcium, contributes to the regulation of gonadotropin gene transcription (Mulvaney and Roberson, 2000; Yokoi et al., 2000).

1.4.4.2 Initiation of gonadotropin gene transcription via MAPK signalling

Mitogen activated protein kinase (MAPK) signalling cascades are involved in the regulation of gene expression in various systems and consist of a series of up to five protein kinases that activate each other by phosphorylation. The ability of activated MAPKs to translocate to the nucleus and trigger transcription enables the formation of a signalling pathway from the receptor to the nucleus. To date, four distinct MAPK cascades have been identified (extracellular signal regulated kinase [ERK], c-Jun NH₂-terminal kinase [JNK], p38MAPK and BiMC related kinase [BMK]). GnRH activates them in a hierarchical manner in gonadotroph cell lines (See Section 3.1.1 for a detailed description of these cell lines) with, ERK and JNK activated first (Naor et al., 2000; Liu et al., 2002).

GnRH responsive elements have been mapped to the proximal promoter of α GSU in humans (Kay and Jameson, 1992), cows (Hamernik et al., 1992) and mice (Schoderbek et al., 1993). Elk1, a factor known to be phosphorylated by ERK, has been shown to bind to the GnRH-responsive element at -406 to -399 bp of the α -subunit promoter in α T3-1 cells (Roberson et al., 1995) whilst a recent report shows that ERKs are also involved in basal promoter activity as well as the GnRH-stimulated response (Harris et al., 2003).

GnRH responsive elements have been mapped to the $LH\beta$ promoter of sheep (Brown et al., 1993; McNeilly et al., 1996), rat (Fallest et al., 1995) and cow (Keri et al.,

1994). Induction of *LHβ* subunit transcription is also contentious with two different papers reporting conflicting results on the importance of PKC, ERK, Ca^{2+} and JNK in basal and GnRH-induced transcription (Saunders et al., 1998; Weck et al., 1998). More recently, research into the roles of ERK and JNK in LβT2 cells has further elucidated the roles of MAPK cascades in the control of *LHβ* transcription. One report shows that ERK signalling is PKC and Ca^{2+} dependent in LβT2 cells, but does not control *LHβ* transcription, whereas JNK signalling is involved in *LHβ* transcription but through *c-jun* rather than PKC and/or Ca^{2+} (Yokoi et al., 2000). However, recent results indicate that both ERK and JNK are involved in *LHβ* transcription in response to GnRH (Harris et al., 2002; Liu et al., 2002) (P.Brown, personal communication).

GnRH regulation of *FSHβ* subunit transcription requires PKC, ERK and JNK (Bonfil et al., 2004). JNK directly activates *c-jun* and *c-fos*, which dimerize to form activator-protein-1 (AP-1), a factor that is critical for *FSHβ* promoter activity (Strahl et al., 1997; Strahl et al., 1998; Coss et al., 2004) supporting a role for MAPK cascades in *FSHβ* transcription.

The differences seen in transcriptional regulation of the gonadotropin subunits indicates that differential roles of MAPK cascades in GnRH signalling can partly be attributed to the use of different cell lines, promoters and species and suggests that they may also provide a mechanism to differentially regulate subunit gene expression. Multiple pathways of MAPK activation exist to generate redundancy in signalling and any disrupting cellular changes or extrinsic factors may alter the course by which MAPKs are stimulated and therefore change the transcriptional effects of the MAPK. Figure 1.11 shows a simplified version of the MAP kinase cascades activated in response to GnRH in LβT2 cells.

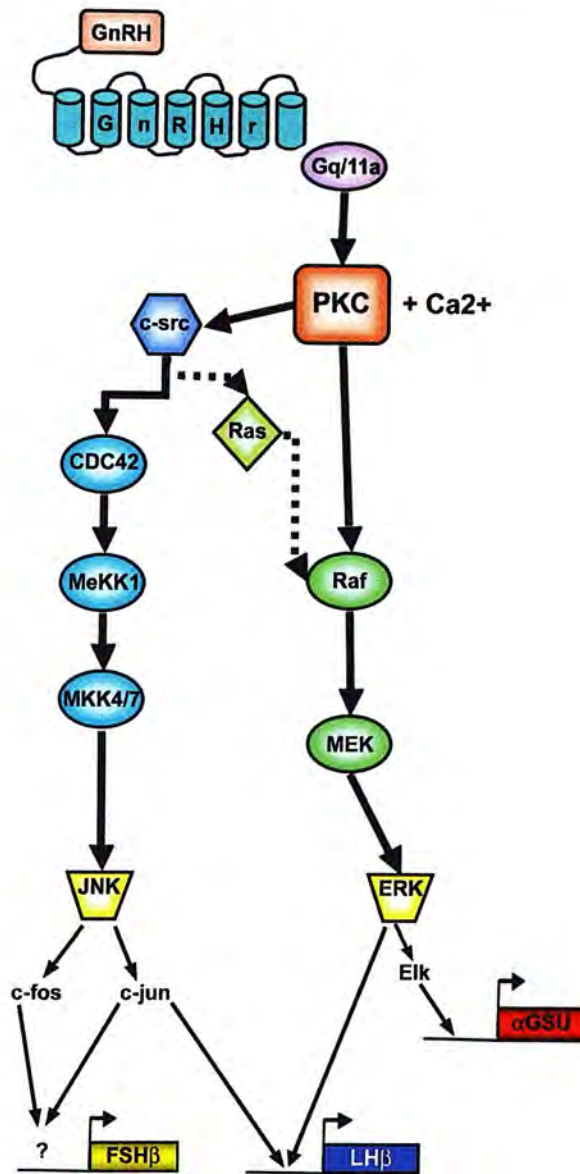


Figure 1.11 MAPK cascades initiated in L β T2 cells in response to GnRH.

GnRH binds to and activates the GnRH receptor (GnRHr) releasing the α -subunit of the coupled G protein. This activates protein kinase C (PKC), which in turn activates various mitogen activated protein kinase (MAPK) cascades. Highest activation is observed via the JNK MAPK cascade family with c-src, CDC42 and MEK kinase 1 (MEKK1) being the main mediators of the GnRH-JNK pathway. JNKs translocate to the nucleus where they phosphorylate and activate the transcription factors c-Jun and c-Fos, which have been shown to regulate LH β and FSH β transcription. GnRH also signals via the ERK MAPK family involving the sequential activation of Raf and MEK. ERK phosphorylates Elk1, a factor that binds to the α GSU promoter to control transcription. Diagram adapted from (Kraus et al., 2001).

This section has described how the generation of a hypothalamic pulse of GnRH and subsequent binding of GnRH to its receptor on the surface of gonadotroph cells results in the activation of distinct MAPK cascades to initiate the biosynthesis and secretion of two disparate, but ultimately synergistic hormones, LH and FSH. The trophic actions of LH and FSH are essential for gonadal function and regulation of the HPG axis, as even a small alteration in the regulated release of these hormones can have a significant impact on fertility.

1.5 GnRH and cancer

In recent years, research has shown that GnRH, and its receptor, are expressed in tissues other than the hypothalamus and pituitary including; the placenta (Kang et al., 2000b), endometrium (Takeuchi et al., 1998), ovary (Peng et al., 1994; Kang et al., 2000a), prostate (Tieva et al., 2001) and testis (Clayton et al., 1980; Bahk et al., 1995). The presence of *GnRH* and *GnRHR* transcripts in cancers of the reproductive tract has also been widely studied including those of the prostate (Limonta et al., 1993; Bahk et al., 1998), endometrium (Imai et al., 1994; Irmer et al., 1994; Chatzaki et al., 1996), breast (Harris et al., 1991; Kakar et al., 1994; Mangia et al., 2002) and ovary (Ohno et al., 1993; Irmer et al., 1995) as well as non-hormonally regulated tumours such as glioblastomas (van Groeninghen et al., 1998) and melanomas (Moretti et al., 2002). Interestingly, in these tumours, activation of GnRH receptors has been shown to down regulate cell proliferation and reduce metastasis (See Figure 1.12), possibly by down regulating the tumour-promoting activity of growth factors (Miller et al., 1985; Keri et al., 1991; Limonta et al., 1992; Emons et al., 1993; Dondi et al., 1994; Yano et al., 1994; Kimura et al., 1999). If so, GnRH could prove a useful tool in the treatment of reproductive cancers.

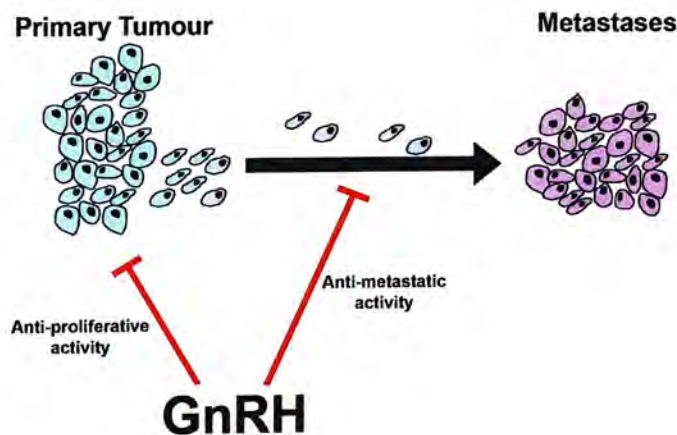


Figure 1.12 Proposed functions of GnRH in tumour progression.

Activation of GnRHR in cancer cells has been shown to decrease the proliferation rate and metastatic capabilities of tumour cells.

1.6 Regulation of gene expression by GnRH

The molecular mechanisms by which GnRH achieves its functions remain unclear. Within gonadotroph cells, a limited number of GnRH target genes have been identified and further characterised. Microarray analysis of untreated and GnRH treated gonadotroph cells has identified a large number of genes (>200) that demonstrated a 2-fold or greater increase or decrease in expression after GnRH treatment (Wurmbach et al., 2001; Kakar et al., 2003). These genes are involved in a wide variety of cellular processes and include transcription factors, ion channel proteins and cytoskeletal proteins, as well as proteins involved in controlling the cell cycle, cell proliferation, apoptosis and signal transduction. Time course experiments identified that several of these genes were highly regulated with elevated levels of expression 1 h after treatment with GnRH, returning to baseline levels 3 h after treatment. These experiments have allowed the identification of many genes that were previously not known to be involved in the regulation of gonadotropin subunit transcription by GnRH and suggest that signalling of GnRH through its receptor may impact on processes other than gonadotropin biosynthesis within gonadotrophs. However, confirmation that these genes are involved in GnRH signalling is required.

1.7 Fanconi Anaemia

Fanconi Anaemia (FA) is a rare, autosomal recessive, chromosomal instability syndrome (OMIM 227650) first described in 1927 by the Swiss paediatrician Guido Fanconi (Fanconi, 1927). The incidence of FA is approximately 1 per 360,000 births with a carrier frequency in the US and Europe calculated at ~1 in 300 persons (Swift, 1971; Schroeder et al., 1976). Founder effects mean that this is increased to ~ 1 in 89 in Ashkenazi Jews (Verlander et al., 1995) and 1 in 83 in the Afrikaans population of South Africa (Rosendorff et al., 1987; Tipping et al., 2001). The disorder has a complex genetic basis and variable phenotype as the same FA mutation within a family can result in a wide variation in clinical symptoms (Alter, 1993; Koc et al., 1999). This section summarises the recent molecular advances that have helped in the understanding of the cellular and clinical phenotypes associated with FA.

1.7.1 Clinical phenotypes of Fanconi Anaemia

FA is a highly heterogeneous syndrome with many associated clinical features. In general, the disease is characterised by bone marrow failure due to a progressive loss of haematopoietic stem cells (Alter, 1998). With the advent of bone marrow transplants, a cure for the haematopoietic aspects of the disease is now possible for some patients. However, this does not alleviate the marked pre-disposition to developing cancer associated with FA (Alter, 1996) including leukaemia and tumours of the digestive system, reproductive tract and liver, which are all commonly seen in older FA patients (Alter, 2003). A wide variety of congenital abnormalities are also associated with FA, the most common of which are listed in Table 1.1.

Abnormality	Frequency (%)
Skeletal	66
Short stature	62
Skin pigmentation	51
Reproductive abnormalities	43
Microcephaly	28
Facial dysmorphism (usually small eyes)	27
Renal and urinary tract defects	24
Mental retardation	9
Cardiac abnormalities	7
No abnormalities	6
Gastrointestinal malformations	4

Table 1.1 Frequency of abnormalities seen in Fanconi Anaemia patients.

Data are percentage of patients (n=838) presenting with the abnormality taken from (Young and Alter, 1994).

1.7.2 Cellular phenotypes of Fanconi Anaemia

FA patients are diagnosed using a lymphoblast assay system that detects spontaneous chromosomal instability and hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC) and diepoxybutane (DEB) (Auerbach and Wolman, 1976; Auerbach, 1993). These factors have led to FA being classified as a DNA repair disorder (Buchwald and Moustacchi, 1998). Studies of lymphoblast cells from FA patients reveal that an increased proportion have a 4n DNA content due to spontaneous arrest and delay during the G2 stage of the cell cycle (Seyschab et al., 1994; Heinrich et al., 1998). The bone marrow defects seen in FA patients suggest that expression of growth factors involved in haematopoiesis could be altered since studies have shown that interleukin-6 (IL6), a cytokine involved in various processes, including B-cell differentiation, is decreased in cells from FA patients (Rosselli et al., 1992; Bagnara et al., 1993).

1.7.3 Genetics of Fanconi Anaemia

Cultured FA cells are sensitive to DNA cross-linking agents and this has been exploited to develop a complementation cloning method. So far eight complementation groups have been discovered (Joenje et al., 1997) indicating the syndrome is genetically heterogeneous. To date, seven FA genes had been cloned: *FANCC* (Strathdee et al., 1992), *FANCA* (Lo Ten Foe et al., 1996), *FANCG* (de Winter et al., 1998), *FANCF* (de Winter et al., 2000b), *FANCE* (de Winter et al., 2000a), *FANCD2* (Timmers et al., 2001) and *FANCL* (Meetei et al., 2003a). FA only occurs if an individual has two abnormal genes from the same complementation group.

1.7.4 Fanconi Anaemia complementation group A (*FANCA*)

FANCA was the second FA gene to be cloned and maps to human chromosome 16q24.3 (Lo Ten Foe et al., 1996). Approximately 65% of all FA patients have mutations in *FANCA* however there does not appear to be a common *FANCA* mutation, instead point mutations and deletions are spread along the gene (Ianzano et al., 1997; Levran et al., 1997; Morgan et al., 1999; Wijker et al., 1999). Murine *Fanca* was cloned in 2000 (van de Vrugt et al., 2000; Wong et al., 2000) and maps to the distal region of mouse chromosome 8, a region syntenic with human 16q. The gene shares 74% nucleotide sequence identity with human *FANCA* and has an ORF of 4365 bp. It is comprised of 43 exons and produces a protein of 1455 amino acids (~160 kD) with 66% identity and 81% similarity to human *FANCA*. Analysis of *Fanca* sequence reveals a bipartite nuclear localization signal (NLS) at the N-terminus, a partial leucine zipper and several regions with weak homology to putative SH2 and SH3 (Src-homology module 2 and 3) domains (See Figure 1.13) suggesting *FANCA* protein can bind DNA and/or other proteins (Alber, 1992; Cohen et al., 1995). These domains are conserved between mouse and human *FANCA* sequence supporting a functional significance for these motifs. Expression of *Fanca* in human *FANCA* deficient lymphoblast cells corrects hypersensitivity to DNA-cross-linking agents, demonstrating functional conservation of the *FANCA* protein between humans and mice. Expression analysis of *Fanca* using *in situ* hybridisation

revealed that *Fanca* is expressed between mouse embryonic day (E) 8.5 and E16 within the developing brain, liver and kidney as well as in the mesenchyme, primarily within cells that will give rise to the soft tissues of the fore and hind-limbs (Abu-Issa et al., 1999). Northern analysis revealed that a predominant 4.5 kb band, corresponding to full length *Fanca*, is expressed from E7.0 and maintained throughout embryogenesis (Abu-Issa et al., 1999). *Fanca* expression is also detected in a wide range of adult tissues (van de Vrugt et al., 2000; Wong et al., 2000).

Interestingly, a novel zinc finger protein, *Zfp276*, is also located at the *Fanca* locus, the 3' untranslated region (UTR) of *Zfp276* overlaps with the last four exons of *Fanca* (Wong et al., 2000). Zinc finger proteins have been shown to be involved in nucleic acid and protein-protein interactions and have been implicated in oncogenesis, limb morphogenesis and proliferation (Ladomery and Dellaire, 2002) however, there are no further reports in the literature that describe a specific role for *Zfp276*.

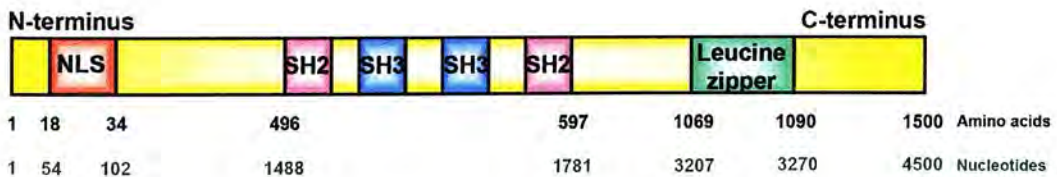


Figure 1.13 FANCA genomic structure.

Bioinformatic analysis of *Fanca* nucleotide and amino acid sequence reveals the presence of a nuclear localization signal (NLS) at the N-terminus of *Fanca* protein. A partial leucine zipper is also predicted at the C-terminus. Several regions showing weak homology to SH2 and SH3 domains are also predicted. Nucleotide positions of the domains are given relative to the start codon.

1.7.4.1 Targeted disruption of the *Fanca* gene in mice

Two transgenic mouse models have been generated with a targeted knockout of the *Fanca* gene. In the first mouse model, exons 4-7 of *Fanca* were replaced with a lacZ-neo construct. *Fanca*^{-/-} mice appeared normal, without obvious congenital malformations or growth retardation although embryonic fibroblasts from the mice

showed increased sensitivity to MMC and had higher levels of chromosomal breakage, as seen in cells from human patients (Cheng et al., 2000). Recently, another mouse model was generated where exons 1-6 of *Fanca* were replaced with a β -galactosidase reporter construct (Wong et al., 2003). These mice displayed a FA-like phenotype, showing growth retardation, microcephaly, craniofacial malformations and tumour formation. In both mouse models the fertility of the knockout mice was severely compromised. Female mice had reduced litter sizes and stopped breeding by 21 weeks of age. Histological examination of the gonads revealed hypogonadism in both sexes with Leydig cell hypoplasia and reduced spermatogenesis in males and few or no follicles in females however, examination of LH and FSH expression within the pituitary glands revealed no difference between mutant and wildtype siblings (Cheng et al., 2000; Wong et al., 2003).

1.7.5 *Fanconi anaemia complementation group C (FANCC)*

FANCC was the first Fanconi Anaemia gene to be cloned and maps to human chromosome 9q22.3 (Strathdee et al., 1992). The cDNA is 4566 bp long with an ORF of 1674 bp comprised of 14 exons and producing a predicted protein of 557 amino acids. Murine *Fancc* maps to mouse chromosome 13 and shares 67% identity with the human gene product (Wevrick et al., 1993a; Wevrick et al., 1993b). Analysis of the protein reveals no known domains or motifs and databases searches reveal no homology with any known proteins. *In situ* hybridisation studies reveal that *Fancc* shares a similar expression pattern during embryogenesis to *Fanca*, but is also expressed in the developing lungs and gut (Wevrick et al., 1993b; Krasnoshtein and Buchwald, 1996). Two transgenic *Fancc* mouse models were generated in order to study the function of the gene in more detail however, as with *Fanca* transgenic mice, *Fancc*^{-/-} mice showed no obvious phenotype other than increased cellular sensitivity to DNA cross-linking agents and compromised fertility (Chen et al., 1996; Whitney et al., 1996). Further examination of gonads from *Fancc*^{-/-} mice and wildtype litter mates revealed that *Fancc* is expressed in the E12.5 developing gonad. A lack of functional *Fancc* results in reduced numbers and slower proliferation of germ cells demonstrating a role for *Fancc* in mitotic proliferation of primordial germ cells (Nadler and Braun, 2000). Furthermore, histological examination of the gonads

showed little or no follicles in female mice and reduced spermatogenesis in males. Co-immunoprecipitation studies have shown that FANCC forms a complex with FANCA (Kupfer et al., 1997a), FANCG (Garcia-Higuera et al., 1999) and FANCF (de Winter et al., 2000c) and interacts strongly with FANCE (Medhurst et al., 2001). Indirect immunofluorescence studies using FANCC antisera localizes FANCC expression to both the cytoplasm and nucleus, though it is principally found in the cytoplasm (Yamashita et al., 1994; Hoatlin et al., 1998). FANCC has also been shown to interact with various proteins including the mitotic cyclin dependent kinase CDC2 (Kupfer et al., 1997b), the molecular chaperone HSP70 (Pang et al., 2001) and the signal transducer and activator of transcription, STAT1 (Pang et al., 2000). Yeast two-hybrid screens identified that a novel BTB/POZ transcriptional repressor protein, (FAZF), interacts with FANCC at amino acids 106-168 (Hoatlin et al., 1999) a region deleted in patients showing a severe FA phenotype (Gillio et al., 1997).

1.7.6 *Fanconi anaemia complementation group G (FANCG)*

The gene mutated in FA patients assigned to complementation group G has been identified as *XRCC9* (de Winter et al., 1998), a gene involved in DNA repair and cell cycle checkpoint control (Busch et al., 1996; Liu et al., 1997). The murine gene shares 80% homology with human *FANCG* and has an ORF of 1872 bp comprised of 14 exons and producing a protein of ~ 69 kD. As with *Fanca* and *Fancc* transgenic mice, targeted disruption of the *Fancc* gene generates mice with viable offspring and no gross abnormalities other than an increased sensitivity to DNA cross-linking agents, decreased fertility and abnormal gonadal histology (Yang et al., 2001). Co-immunoprecipitation studies have shown that FANCG interacts with FANCA and FANCC to form a complex (Waisfisz et al., 1999a). The N-terminus of FANCG (amino acids 1-428) binds FANCA whilst the C-terminus binds FANCC (Kuang et al., 2000).

1.7.7 *Fanconi anaemia complementation group E (FANCE)*

Using genetic linkage analysis, the gene mutated in patients assigned to complementation group E was mapped to human chromosome 6p21-22 (Waisfisz et

al., 1999b). *FANCE* was later cloned by complementation of a *FANCE* deficient cell line using an episomal expression library. Human *FANCE* has an ORF of 1611 bp comprised of 10 exons (de Winter et al., 2000a). The predicted 536 amino acid protein (~60 kD) contains 2 nuclear localization signals (NLS) but lacks any significant homology to other known proteins. Yeast two-hybrid analysis identified that *FANCE* can weakly interact with both *FANCA* and *FANCG* whilst a relatively strong interaction was detected between *FANCE* and *FANCC* (Medhurst et al., 2001). The addition of functional *FANCE* protein to *FANCE*^{-/-} cells restores the nuclear accumulation of *FANCC* and *FANCA*-*FANCC* complex formation (Pace et al., 2002; Taniguchi and D'Andrea, 2002).

1.7.8 Fanconi anaemia complementation group F (*FANCF*)

The gene mutated in FA patients assigned to complementation group F was identified by complementation of FA-F lymphoblasts with an episomal expression library (de Winter et al., 2000b). *FANCF* genomic sequence contains no introns and produces a protein product of 374 amino acids (~42 kD). Bioinformatic analysis predicted a region of homology between *FANCF* and the prokaryotic RNA binding protein ROM (Tomizawa and Som, 1984) suggesting a role for *FANCF* in binding RNA/DNA. *FANCF* is localized predominantly to the nucleus and can be co-precipitated from nuclear extracts using *FANCA*, *FANCC* or *FANCG* antisera (de Winter et al., 2000c).

1.7.9 Fanconi anaemia complementation group D

The FA-D complementation group is distinct from other complementation groups, because, although FA-D patients are phenotypically indistinguishable from other FA subtypes, the FA protein complex assembles correctly in FA-D cells suggesting that the FA-D gene product functions downstream or independently of the FA protein complex (Yamashita et al., 1998). Complementation group D is genetically heterogeneous with patients assigned to the group having mutations in one of two genes, *FANCD1* or *FANCD2*.

1.7.9.1 *FANCD2*

FANCD2 is localized to chromosome 3p22-26 (Whitney et al., 1995; Hejna et al., 2000). It has an ORF of 4358 bp comprising 44 exons, which produces a protein of 1451 amino acids (Timmers et al., 2001). *FANCD2* protein is localized to the nucleus and is present in two isoforms, long (L) and short (S), of 162 and 155 kD respectively. Bioinformatic analysis of *FANCD2* sequence revealed no known functional domains or motifs but, unlike other FA proteins, *FANCD2* homologs are present in *Arabidopsis thaliana*, *Caenorhabditis elegans* and *Drosophila melanogaster* (Timmers et al., 2001; Castillo et al., 2003). In response to DNA damage, *FANCD2*-S is mono-ubiquitinated to form *FANCD2*-L, which then co-localizes with the breast cancer susceptibility protein BRCA1 in nuclear foci (Garcia-Higuera et al., 2001). Cells from other FA complementation groups lack *FANCD2*-L suggesting that the FA complex is required for mono-ubiquitination of *FANCD2*-S (Garcia-Higuera et al., 2001). Targeted disruption of the *Fancd2* gene in mice results in animals with a similar, but more severe, phenotype to that seen in other FA knockouts. *Fancd2*^{-/-} mice show severe hypogonadism, microphthalmia, perinatal lethality and tumour development (Houghtaling et al., 2003). Interestingly, as well as interacting with BRCA1, *FANCD2* has been shown to associate with MENIN (Jin et al., 2003), the protein product of the MEN1 gene that is mutated in multiple endocrine neoplasia type I (Chandrasekharappa et al., 1997).

1.7.9.2 *FANCD1*

As not all FA patients assigned to complementation group D have mutations in *FANCD2*, it was concluded that another gene, now named *FANCD1*, may be mutated in those patients (Timmers et al., 2001). Given that cells from FA patients and *BRCA2*^{-/-} cells share sensitivity to MMC (Patel et al., 1998), and that targeted inactivation of the *Brca2* gene results in viable mice with a FA-like phenotype (McAllister et al., 2002), investigators sequenced the *BRCA1* and *BRCA2* genes from FA-D1 complementation group patients to determine if causal mutations were present. Although no *BRCA1* mutations were detected, bi-allelic mutations in *BRCA2* were observed. Taken together with the evidence that functional

complementation of FA-D1 cells with wild-type *BRCA2* restores MMC resistance these results show that, in fact, *FANCD1* is identical to *BRCA2* (Howlett et al., 2002).

1.7.10 Fanconi anaemia complementation group L (*FANCL*)

Recently, a novel component of the FA protein complex, with ubiquitin ligase activity, has been identified. Several Fanconi anaemia associated polypeptides (FAAPs) of unknown function were immuno-isolated from a complex containing the five core FA proteins (Meetei et al., 2003b). Mass spectrometry identified one of these proteins as PHD finger protein 9 (PHF9) (Meetei et al., 2003a). Plant homeodomain (PHD) fingers confer E3 ubiquitin ligase activity, and indeed PHF9 demonstrated this function (Meetei et al., 2003a). Levels of PHF9 protein were decreased in cells from FA complementation groups A, E and B, and knockdown of PHF9 expression, with a specific small interfering RNA (siRNA), decreased levels of mono-ubiquitinated FANCD2. This suggests that PHF9 is the E3 ubiquitin ligase responsible for FANCD2 mono-ubiquitination and has been named *FANCL*. This new member of the FA complex has been reported to be mutated in an individual with FA previously unassigned to any complementation group (Meetei et al., 2003a). Interestingly, the mouse homolog of PHF9, *Pog*, is mutated in germ-cell deficient (*gcd*) mice, resulting in sub-fertile mice with defective germ cell proliferation (Agoulnik et al., 2002) a similar phenotype to that seen in FA knockout mice (Wong et al., 2003).

1.7.11 FA protein complex formation

Analysis of the FA proteins has shown that they interact with each other. Immunoprecipitation studies demonstrated that FANCA binds both FANCC (Kupfer et al., 1997a) and FANCG (Waisfisz et al., 1999a) and that FANCG is pivotal for the interaction between FANCA and FANCC (Garcia-Higuera et al., 1999). Yeast two-hybrid studies confirmed these findings and demonstrated a strong interaction between FANCA and FANCG, but a weak interaction between FANCA and FANCC, indicating an indirect interaction between FANCA and FANCC, possibly

involving other proteins (Huber et al., 2000; Reuter et al., 2000). The interaction of FANCA with FANCC promotes phosphorylation of FANCA and its biological activity (Naf et al., 1998; Yamashita et al., 1998). The NLS sequence present at the N-terminus of FANCA is crucial for FANCA-FANCG binding (Kruijck et al., 1999). Apart from interacting with FA complex members, FANCA also interacts, via its C-terminus, with Sorting nexin 5 (SNX5) (Otsuki et al., 1999), a molecule belonging to a family of proteins implicated in endocytosis of plasma-membrane receptors and/or the trafficking of proteins from one membrane compartment to another (Worby and Dixon, 2002). Overexpression of SNX5 in cells increases FANCA expression, but the significance of this is unknown (Otsuki et al., 1999). A summary of the interactions between FA family proteins is shown in Table 1.2 below.

	FANCA	FANCC	FANCD1	FANCD2	FANCE	FANCF	FANCG	FANCL
FANCA	-	?	✓	✗	?	✓	✓✓	?
FANCC	?	-	✗	✗	✓	✓✓	✓	?
FANCD1	✓	✗	-	?	✗	✗	✓	?
FANCD2	✗	✗	?	-	✓	✗	✗	?
FANCE	?	✓✓	✗	✓	-	?	?	?
FANCF	✓	✓✓	✗	✗	?	-	✓	?
FANCG	✓✓	✓	✓	✗	?	✓	-	?
FANCL	?	?	?	?	?	?	?	-

Table 1.2 Interactions between FA proteins.

Yeast two-hybrid analysis and co-immunoprecipitation studies have determined which FA proteins interact with each other. ✓ ✓ indicates strong binding, ✓ indicates binding, ✗ indicates no binding, ? indicates that direct binding has not been observed but interaction may be mediated via another protein.

The genes implicated in Fanconi Anaemia appear novel, and therefore unrelated to one another, but the similarity of clinical symptoms and cellular features found in patients with mutations in different genes indicates that they actually interact in a common pathway. A model showing how the proteins cooperate as part of a

common pathway is still being refined as research determines the specific role of this multi-subunit complex in homology-directed DNA damage repair (Figure 1.14).

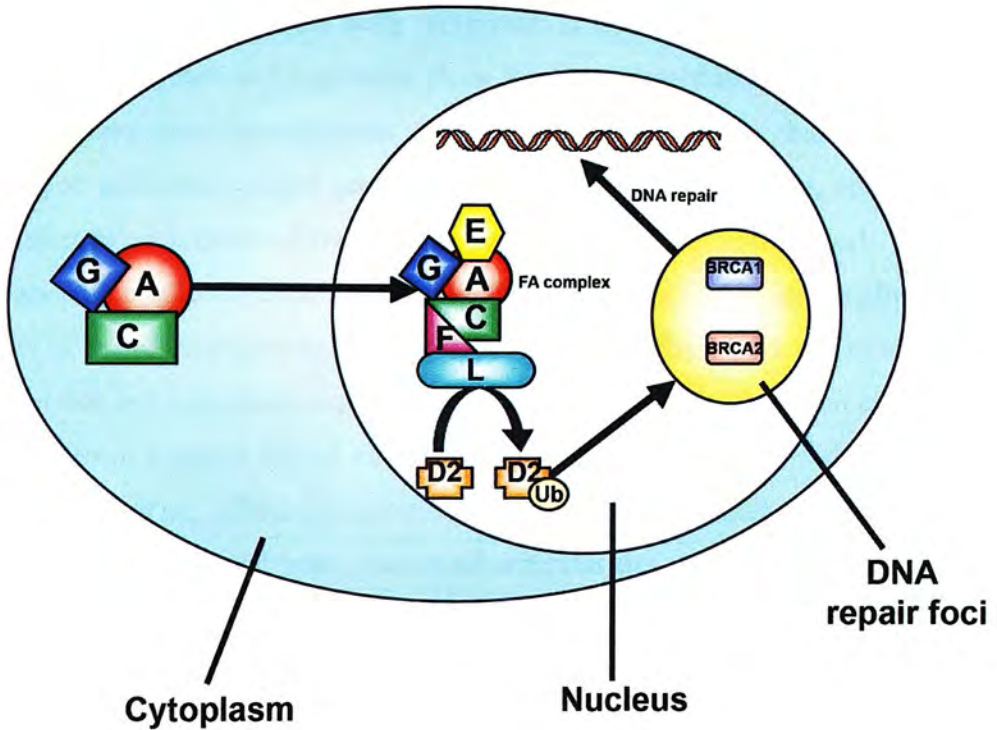


Figure 1.14 A model of the Fanconi anaemia pathway.

Within the cytoplasm, FANCA binds FANCG then FANCC. These proteins then move into the nucleus where FANCF and FANCE bind to form the FA complex. FANCL and this complex are required for the mono-ubiquitination (Ub) of FANCD2. Activated FANCD2 is then translocated to DNA-repair foci containing BRCA2 and FANCD2/BRCA1 where it probably participates in homology directed DNA damage repair. Diagram adapted from (Grompe, 2003).

1.8 Fanconi anaemia and the HPG axis

The FA proteins function together in a common pathway required for repair of DNA damage and thus, bi-allelic mutations in any of the FA genes results in a marked predisposition to cancer. How bone marrow failure, infertility, microcephaly and short stature, are associated with mutations in the FANCA genes has yet to be determined. Studies of FA patients show that a relatively large percentage (43%) have reproductive abnormalities (Young and Alter, 1994), but whether this phenotype indicates a direct role for FANCA genes in reproduction, or occurs as a consequence of defective DNA damage repair has not been established. However, patients with a mild FA phenotype survive to adulthood and provide insights into the effects of the disease on reproductive capability. Fertility in FA males is severely reduced due to hypogonadism and defective spermatogenesis (Bargman et al., 1977), although from a cohort of 115 male FA patients, 4 (3%) fathered children (Alter et al., 1991; Liu et al., 1991). Female FA patients have a high incidence of hypoplastic ovaries, underdeveloped uteri, menstrual irregularities and premature menopause (Alter et al., 1991). However, a relatively high number of female patients become pregnant (15%). An analysis of gonadotropin levels has been reported for a few cases of FA (Berkovitz et al., 1984; Schoof et al., 2000; Massa et al., 2002), all of them had abnormal levels of LH and FSH. No research has established a link between specific mutations in FA genes and an infertility phenotype but the infertility phenotype is faithfully recapitulated in gene-targeted mice lacking *Fanca*, *Fancc*, *Fancg* or *Fancl* (Chen et al., 1996; Whitney et al., 1996; Cheng et al., 2000; Yang et al., 2001; Agoulnik et al., 2002; Wong et al., 2003). A recent paper identified sixty-nine proteins that interacted with FANCA, FANCC or FANCG which were involved in a wide range of cellular processes including transcription, signalling, metabolism and cellular transport (Reuter et al., 2003). Thus FANCA, and the other FA proteins, interact with proteins implicated in a number of diverse and specialized pathways. Elucidating these pathways could explain the wide range of clinical features associated with the disease and be informative when studying the regulation of reproduction.

1.9 Aims of this thesis

The body of research presented in this thesis aimed to address several questions concerning the relationship between GnRH, and *Fanca* and the role of *Fanca* in gonadotropin gene transcription. As the GnRH regulated transcript identified by DD-RT-PCR could correspond to either one of two genes transcribed from the *Fanca* locus, chapter three addresses the issue of whether it is the expression of *Fanca*, *Zfp276* or both genes, which is altered in response to GnRH. Then, having established that it is expression of *Fanca* that is rapidly and transiently up-regulated in L β T2 cells in response to GnRH, chapter four analyses the expression of *Fanca* protein within gonadotroph cells. It addresses whether addition of GnRH promotes a redistribution of *Fanca* protein within L β T2 cells, in a bid to identify a mode of *Fanca* action in these cells, and aims to determine by what specific mechanisms this ‘shuttling’ may be occurring. This chapter also addresses whether the introduction of specific point mutations into the *Fanca* protein can alter the localization pattern of *Fanca* within gonadotroph cells. Finally, chapter five compares the effects of wildtype and mutant *Fanca* proteins on the control of GnRH induced gonadotropin promoter activity to determine whether *Fanca* is an important molecule in the regulation of gonadotropin gene expression. These mutant *FANCA* constructs are genocopies of known FA probands, thus the cumulative aim of the experiments described herein is to provide insight into the potential molecular mechanism of the reproductive phenotype of FA.

Chapter 2

Materials and methods

It is common sense to take a method and try it. If it fails, admit it frankly and try another. But above all, try something – Franklin D. Roosevelt.

2 Materials and Methods

All solutions and buffers were made with deionised distilled water. All eppendorfs, pipette tips and solutions were sterilised by autoclaving before use, unless stated otherwise.

2.1 Nucleic acid manipulation

2.1.1 Solutions

0.5 M EDTA (pH 8.0)

186 g of EDTA (BDH) was dissolved in 800 ml of dH₂O. The pH was adjusted to 8.0 with concentrated NaOH (BDH) then dH₂O was added to 1 litre.

1 M Tris-HCl (pH 7.4/8.0)

121 g of Tris (Roche) was dissolved in 800 ml dH₂O. The pH was adjusted to 7.4 or 8.0 by addition of HCl (BDH) then dH₂O was added to 1 litre.

1 x TE (*10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0*)

500 μ l 1 M Tris-HCl (pH 7.4) and 100 μ l 0.5 M EDTA (pH 8.0) were added to 49.4 ml dH₂O.

Low TE (*10 mM Tris-HCl pH 7.4, 0.1 mM EDTA pH 8.0*)

500 μ l 1 M Tris-HCl (pH 7.4) and 10 μ l 0.5 M EDTA (pH 8.0) were added to 49.4 ml dH₂O.

3 M Sodium acetate (pH 5.2)

408.1 g sodium acetate (Sigma) was dissolved in 800 ml dH₂O. The pH was adjusted to 5.2 with acetic acid (BDH) then dH₂O was added to 1 litre.

50 x TAE (2 M Tris, 50 mM EDTA pH 8.0, 5.71% acetic acid)

242 g Tris, 100 ml 0.5 M EDTA (pH 8.0) and 57.1 ml acetic acid were mixed in 1 litre of dH₂O.

10 x TBE (0.9 M Tris, 0.9 M Orthoboric acid, 40 mM EDTA pH 8.0))

108 g Tris and 55 g orthoboric acid (BDH) were dissolved in 500 ml dH₂O. 80 ml 0.5 M EDTA (pH 8.0) was added and the pH adjusted to 8.3 with concentrated NaOH or orthoboric acid (Roche) then dH₂O added to 1 litre.

1% Agarose gel

1.5 g agarose (Roche) was dissolved in 150 ml of 1 x TAE/TBE buffer by heating in a microwave. The agarose was then allowed to cool to 50°C and 1.5 µl ethidium bromide (Sigma, 10 µg/µl) added before casting the gel.

10 x Loading Buffer (0.025% xylene cyanol, 0.025% bromophenol blue, 40% sucrose)

25 mg xylene cyanol (Sigma), 25 mg bromophenol blue (Sigma) and 4 g sucrose (BDH) were dissolved in 10 ml dH₂O.

2.1.2 Primer design and oligonucleotide synthesis

Primers were designed either from sequences available in GenBank (<http://www.ncbi.nlm.nih.gov>) or Celera (<http://www.celera.com>) databases, or from our own sequence data obtained using vector primers. The major aims when designing primer pairs were to ensure that they were unique to the specific gene and that they had the same or similar annealing temperatures. The annealing or melting temperature (T_m) of a primer can be estimated from its sequence using the formula below:

$$T_m = (2 [A+T] + 4 [G+C]) - 5^\circ\text{C}$$

All primers were commercially synthesized by MWG (<http://www.mwg-biotech.com>) using the 0.01 μmol synthesis scale. Table 2.1 shows primers used to analyze the expression of various genes.

2.1.3 Reverse Transcription-PCR

2.1.3.1 cDNA synthesis

An expression profile for members of the Fanconi Anaemia complex was performed using reverse transcriptase (RT) PCR. First strand cDNA was synthesized by extension with murine reverse transcriptase (RT) and an oligo(dT) primer using RNA harvested from various hormone treated and untreated cell lines. This cDNA was then used as a template for a PCR reaction with gene specific primers to determine the presence or absence of the gene of interest. First strand cDNA was synthesized using the Bulk First-Strand cDNA Synthesis Kit (Amersham Biosciences). Approximately 1 μg of total RNA extracted from cells (as described in Section 2.6.2) was used in each reaction in accordance with manufacturer's instructions. The resulting cDNA was stored at -20°C until required.

2.1.3.2 PCR conditions

Primers were used at a concentration of 100 ng/ μl and 1 μl of each was added to 7 μl dH₂O in an eppendorf. 1 μl of cDNA was added to this and the tube incubated at 95°C for 5 minutes. Tubes were snap cooled on ice then 10 μl of 2x High Fidelity *Taq* Master Mix Buffer 2 (AbGene) added. Unless stated otherwise, thirty-five cycles of PCR amplification were performed as detailed below:

Initial denaturation	94°C	2 minutes	} 35 cycles
Denaturation	94°C	30 seconds	
Specific primer annealing temperature	$X^{\circ}\text{C}$	30 seconds	
Extension	72°C	1min/kb of product	
Final extension	72°C	10 minutes	

Products were analysed on a 1% agarose gel.

	Sense primer (5' → 3')	Position (bp)	Antisense primer (5' → 3')	Position (bp)	Product size	Accession Number
<i>Fanca</i> Exons 7-17	CTGTGTGAGCAGATAGGC	637	CACGCTCGGCAATGTCCC	1617	979 bp	AF178934
<i>Fanca</i> Exons 7-18	CTGTGTGAGCAGATAGGC	637	CCTGAATAATGCTGGCCTCC	1712	1075 bp	AF178934
<i>Fanca</i> Exons 14-18	CAGCATGGTCACTGCGTTCC	1262	CCTGAATAATGCTGGCCTCC	1712	450 bp	AF178934
<i>Fanca</i> Exons 30-32	GTGGTGGAGACCTGGAAGA	2899	CGGCGTAGAACAGCCATG	3110	211 bp	AF178934
<i>Fance</i>	GCAGAAAGCTTTCTGCATGGG	47	CAAAAACCAGCTCTCAAAGG	1190	1143 bp	NM_007985
<i>Fanced2</i>	GGACATTGAGGTTCTCTA	2777	CCAGAAAAGCCTCTCTACA	4148	1371 bp	XM_132796
<i>Fance</i>	GAATGTAGTCCCAGCGAGAT	999	GGCCTTCTTCAGGAAGGT	1596	597 bp	BC039987
<i>Fancef</i>	CACGAGGTCCCTACACAGATGG AGGACATG	-	AGCCTGGAACTGAGAAATCT ACTCTAGCAC	-	228 bp	(Aube et al., 2003)
<i>Fanceg</i>	TGGACCTGTGGAGGAAAAG	49	TCTGCACACTGAGGAGGAAG	49	1586 bp	AF112439
<i>Fancel</i>	CATCTCGGCTCAGCGAAG	83	CCTCTCAGCCACTCATA	83	947 bp	NM_025923
<i>Zfp276</i>	CACCTCCTCTGAGTACTGC	-69	CGTCACCTGCTGAGTTCAAG	1307	1238 bp	AF178935
<i>Gapdh</i>	ATGGTGAAGGTGGGTGTGAACG	47	GCTTCCCGTTGATGACAAAGCT	257	210 bp	M32599

Table 2.1 DNA sequence of primers used for RT-PCR expression analysis.

Position of primers is given in basepairs (bp) relative to the start (ATG) codon.

2.1.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyze DNA fragments after PCR or restriction endonuclease digestion. Typically, 1 x TBE buffer was used for DNA fragments below 1 kb in size whilst 1 x TAE buffer was used in all other situations. The percentage agarose used was typically 1 % (w/v) and the gels were electrophoresed at 10 volts/cm. Ethidium bromide, a powerful carcinogen, which intercalates between DNA bases and fluoresces under UV light, was added at a final concentration of 0.1 ng/ μ l. To visualize the DNA, the gel was transilluminated with UV light (UV transilluminator 2000, BioRad) and gel images captured using a GENEFLASH Imaging System (Sygene Bioimaging).

2.1.5 Cloning of RT-PCR products

To confirm their identity, PCR products were cloned into TOPO-pCRII Vector (Invitrogen) according to manufacturer's instructions then plasmid inserts sequenced as described in Section 2.3.6. Approximately 100 ng of PCR product was mixed with 1 μ l salt solution, 1 μ l TOPO Vector and 2 μ l sterile H₂O. The contents of the tube were mixed gently and incubated at room temperature for 10 minutes and 2 μ l of this mixture was then transformed into 30 μ l electrocompetent TOP10 cells (Invitrogen) as described in Section 2.2.2. The transformation reaction was then plated out onto Luria Bertoni (LB) Agar/Amp plates and incubated at 37°C overnight.

2.2 Microbiology

2.2.1 Bacterial solutions

Luria Bertoni (LB) Broth

25 pellets of LB Broth (Q-Biogene) were dissolved in 1 litre dH₂O then autoclaved.

Luria Bertoni (LB) Agar

31 pellets of LB Agar (Q-Biogene) were dissolved in 1 litre dH₂O then autoclaved.

Ampicillin stock (50 mg/ml)

500 mg ampicillin (Sigma) was dissolved in 10 ml dH₂O then sterilized by filtration through a 0.22-micron filter (Sartorius). This stock solution was aliquoted and stored at -20°C until required. Working concentration was 50 µg/ml

Kanamycin stock (30 mg/ml)

300 mg kanamycin (Sigma) was dissolved in 10 ml dH₂O then sterilized by filtration through a 0.22-micron filter (Sartorius). This stock solution was aliquoted and stored at -20°C until required. Working concentration was 30 µg/ml

Chloramphenicol stock (30 mg/ml)

300 mg chloramphenicol (Sigma) was dissolved in 10 ml 100% EtOH (BDH). This stock solution was aliquoted and stored at -20°C until required. Working concentration was 30 µg/ml.

2.2.2 Transformation of competent cells

Various strains of chemically competent cells (Table 2.2) were used during this project.

Bacterial Strain	Supplier	Genotype	Purpose
TOP10 One Shot Chemically competent cells	Invitrogen	<i>F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str^r) endA1 nupG</i>	Cloning of PCR products
SoloPack Gold supercompetent cells	Stratagene	<i>Tet^r Δ(mcrA)183 (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lacHte [F' proAB lacI^qZΔM15 Tn10 (Tet^r) Amy Cam^r]</i>	Cloning of PCR products
XL10-Gold Ultracompetent cells	Stratagene	<i>Tet^r Δ(mcrA)183 (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lacHte [F' proAB lacI^qZΔM15 Tn10 (Tet^r) Amy Cam^r]</i>	Cloning of mutated inserts
SURE Competent Cells	Stratagene	<i>E14 (McrA') Δ(mcrCB-hsdSMR-mrr)171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuCTN5 (Kan^r) uvrC [F' proAB lacI^qZΔM15 Tn10 (Tet^r)]</i>	Cloning of recombinant adenoviral vectors

Table 2.2 Chemically competent cells.

Genotypes and supplier information for chemically competent cells using during the course of this research.

Cells were thawed on ice (β -mercaptoethanol was added according to manufacturer's instructions for Solopack cells) then 2 μ l ligation reaction added and the tube tapped gently to mix. Cells were incubated on ice for 30 minutes then heat shocked at 42°C for 30 seconds (Solopack cells were heat shocked at 54°C for 60 seconds) before being returned to ice for 2 minutes. A 250 μ l volume of SOC media (GibcoBRL) was added and the cells incubated at 37°C, whilst shaking at 150 rpm, for at least 30 minutes. Between 50 μ l and 250 μ l of the transformation reaction was plated out onto LB-agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

2.3 Nucleic acid analysis

2.3.1 Solutions

DNA maxi-preparation solution P1
(50 mM Glucose, 25 mM Tris-HCl pH
8.0, 10 mM EDTA pH 8.0)

DNA maxi-preparation solution P2
(0.2 M NaOH, 1% SDS)

DNA maxi-preparation solution P3
(3 M KOAc pH 4.8)

Denaturing Solution (1.5 M NaCl, 0.5
M NaOH)

Neutralizing Solution (1.5 M NaCl, 1
M Tris)

20 x SSC (3 M NaCl, 0.3 M sodium
citrate)

10% SDS

1M NaH₂PO₄

9 g glucose, 40 ml 1 M Tris-HCl (pH
8.0) and 20 ml 0.5 M EDTA (pH 8.0)
were mixed together in 1 litre of
dH₂O.

8 g NaOH and 10 g SDS (Roche) were
dissolved in 1 litre of dH₂O.

249 g potassium acetate (Sigma) was
dissolved in 400 ml dH₂O. The pH
was adjusted to 4.8 by adding ~ 500
ml acetic acid then made up to 1 litre
with dH₂O. The solution was stored at
4°C until required.

87.7 g NaCl (BDH) and 20 g NaOH
were dissolved in 1 litre of dH₂O.

87.7 g NaCl and 121 g Tris were
dissolved in 1 litre of dH₂O.

175 g NaCl and 88 g sodium citrate
were dissolved in 1 litre of dH₂O.

100 g SDS was carefully weighed out
in a fume hood then dissolved in 1
litre of dH₂O. This solution was then
filtered using pre-assembled and pre-
sterilized 1 litre filter units (Nalgene).

120 g of NaH₂PO₄ (Sigma) was
dissolved in 1 litre of dH₂O.

1M Na₂HPO₄

142 g of NaH₂PO₄ (Sigma) was dissolved in 1 litre of dH₂O.

1 M NaPO₄ (pH 7.2)

1 M NaH₂PO₄ was added drop wise to 1 M Na₂HPO₄ until the pH reached 7.2.

Modified Church and Gilbert Hybridization Solution (*0.5 M NaPO₄ pH 7.2, 1 % BSA, 15 % formamide, 7% SDS, 2 mM EDTA pH 8.0*)

50 ml of 0.5 M NaPO₄ (pH 7.2); 15 ml deionised formamide (BDH); 35 ml of 10% SDS; 0.4 ml 0.5 M EDTA (pH 8.0) and 1 g bovine serum albumin (BSA, Sigma) were mixed together.

2.3.2 *Small scale DNA preparation*

Single bacterial colonies were picked from a pre-streaked plate and inoculated into 5 ml LB Broth containing the appropriate antibiotic and grown overnight with shaking (150 rpm) at 37°C. The next day plasmid DNA mini-preparations were made using the Boehringer Mannheim High Pure Plasmid Isolation Kit according to manufacturer's instructions. An aliquot was then analysed to determine concentration of plasmid DNA using a GeneQuant spectrometer (Pharmacia Biotech, See Section 2.3.4). Yield was typically 100 ng/ul and plasmid preparations were stored at -20°C until required.

2.3.3 *Preparation of DNA over a caesium chloride gradient*

For large scale preparation of plasmid DNA, 500 ml LB-Broth containing the appropriate antibiotic, was seeded with 1 ml mini-prep culture and grown overnight with shaking at 37°C. The following morning the culture was split between two 250 ml plastic centrifuge tubes and centrifuged in a J2-21 Beckman centrifuge using a JA-14 rotor at 6000 rpm for 10 minutes at 4°C to pellet the bacteria. The supernatant was discarded and the pellet carefully resuspended by gentle shaking in 60 ml of Solution P1. An equal volume of Solution P2 was added whilst gently mixing and

the contents incubated at room temperature for exactly 5 minutes, this was then mixed with 80 ml chilled Solution P3 and the contents incubated on ice for 1 h. The tubes were then centrifuged at 7,000 rpm for 30 minutes. The supernatant was filtered through miracloth (CamLab) to remove any precipitate and split equally over three 250ml centrifuge tubes. To each tube, 0.7 volumes of isopropanol (BDH) was added and the contents gently mixed. The tubes were then centrifuged at 7,000 rpm for 15 minutes at 4°C. The supernatant was discarded and all 3 pellets re-suspended in TE buffer to give a total volume of 8 ml. To purify plasmid DNA from genomic bacterial DNA a caesium chloride (CsCl) gradient was used. A 1 x gradient was prepared by adding 11.82 g CsCl (Roche) to 8 ml DNA solution. To this, 500 µl ethidium bromide solution (10 mg/ml, Sigma) was added and the final volume adjusted to 14 ml with TE buffer. This was centrifuged at 3000 rpm for 10 minutes. The supernatant was removed and split between two 6 ml Ultra centrifugation tubes (Sorvall). The tubes were weighed, balanced against each other and sealed with metal caps. Samples were then centrifuged in a Sorvall Ultra Pro 80 using a TV 1665 upright rotor at 50,000 rpm for at least 16 h at 20°C. After ultra-centrifugation two bands were visible. The lower, thicker band corresponded to plasmid DNA whilst the upper, thin band represented contaminating, genomic bacterial DNA. Bands were visualized using a long wavelength 302 nm UV lamp in a dark room and the plasmid DNA band removed into a falcon tube (Costar) using an 18 gauge needle (Kendal) and syringe (BDPlastipak). To remove the ethidium bromide an equal volume of caesium chloride saturated with isopropanol was added and mixed by shaking. When allowed to settle, two phases appeared and the upper, pink, phase was removed and discarded using a pasteur pipette. TE buffer was added as necessary to ensure the lower phase remained at 2 ml volume. This process was repeated until both phases appeared clear. The bottom phase was then removed into a new tube and 1/10th volume 3 M NaAc (pH 5.2) and 3 volumes of 70% EtOH (BDH) added. This was mixed, then stored at -20°C for at least 1 h. The tube and its contents were warmed to room temperature then centrifuged at 7,000 rpm for 15 minutes. The supernatant was discarded and 500 µl 70% EtOH added. The pellet

and 70% ethanol was transferred to a clean eppendorf tube, the tube was washed out with a further 500 μ l 70% EtOH and this was added to the eppendorf to give a final volume of 1 ml. The DNA pellet was centrifuged at 13,000 rpm for 10 minutes, the supernatant was discarded and the pellet allowed to air-dry before being resuspended in an appropriate amount of TE buffer, usually 900 μ l. Contaminating protein was removed by adding a 1/2 volume of phenol (BDH) to the DNA solution. The contents were briefly mixed and centrifuged for 10 seconds at 13,000 rpm. A 1/2 volume of chloroform (BDH) was added and the contents mixed by shaking. Eppendorfs were then centrifuged at 13,000 rpm for 1 minute. The top aqueous layer was removed into a new eppendorf and a 1/2 volume of chloroform added. This was centrifuged again at 13,000 rpm for 1 minute and the top layer removed into a new eppendorf. A 1/10th volume of 3 M NaAc (pH 5.2) and 3 x volume EtOH was added and the contents mixed by shaking. Eppendorfs were then incubated at -20°C for at least 1 h. Eppendorfs were centrifuged at 13,000 rpm for 10 minutes, the supernatant was discarded and the pellet washed in 150 μ l 70% EtOH by centrifugation at 13,000 rpm for 10 minutes. The supernatant was discarded and the DNA pellet allowed to air-dry before being resuspended in 100 μ l low TE.

2.3.4 Spectrophotometrical analysis of nucleic acids

Quantification of nucleic acids was performed using a GeneQuant spectrophotometer (Pharmacia Biotech). At 260 nm, an OD reading of 1 is equivalent to 50 $\mu\text{g/ml}$ of double stranded DNA or 40 $\mu\text{g/ml}$ of single stranded DNA or RNA. An OD reading at 280 nm indicates the presence of high molecular weight molecules i.e. proteins. A 260 nm:280 nm ratio of 1.7 for DNA or 2.0 for RNA indicates an acceptably pure sample.

2.3.5 Restriction digest analysis of plasmid DNA

All restriction enzymes were purchased from Roche or New England Biolabs (NEB). To identify positive clones, mini-prepped plasmid DNA (See Section 2.3.2) was digested with the appropriate restriction enzyme(s). The cloning site of TOPO-

pCRII (Invitrogen) is flanked by *EcoRI* sites to allow liberation of the cloned PCR insert. A typical restriction enzyme digest contained 10 µl plasmid DNA (~0.8 µg) mixed in an eppendorf with 16 µl dH₂O, 3 µl appropriate enzyme buffer and 1 µl enzyme. Digests were incubated at 37°C for at least 2 h then analysed by running 20 µl of each digest on a 1 % agarose gel in 1 x TAE/TBE buffer.

2.3.6 Sequencing analysis

All sequencing was performed by technical staff at the MRC Human Reproductive Sciences Unit or MRC Human Genetics Unit (Edinburgh). Sequences were analysed and contigs constructed from overlapping DNA sequence using either GeneJockey (Oxford Molecular) or Contig Express (Vector NTI, Informax) computer software. Sequence information was compared against the Genbank databases on the NCBI website (<http://www.ncbi.nlm.nih.gov>) using BLAST (Altschul et al., 1990).

2.3.7 Restriction enzyme digest of plasmid DNA for cloning

In general, when cloning fragments of DNA from one plasmid vector to another, ~50 µg of DNA from each plasmid vector was digested using the appropriate restriction endonucleases. In an eppendorf, 50 µg plasmid was mixed with 10 µl enzyme buffer, and 5 µl enzyme(s) and then made up to 100 µl with dH₂O. Digests were incubated at 37°C for at least 2 h then frozen to inactivate the enzyme. Approximately 2 µl of this reaction was then electrophoresed on an agarose gel to check for complete digestion before proceeding to the next stage. In cases where the vector may be able to re-ligate to itself, digests were then treated with shrimp alkaline phosphatase (SAP, Roche). 40 µl SAP buffer and 30 µl SAP were added to the digested DNA and the final volume increased to 400 µl by addition of dH₂O. Digests were SAP treated at 37°C for at least 2 h and then frozen. All 400 µl of the SAP treated digest was electrophoresed on a 1 % agarose gel at 35 volts/cm overnight. The next morning the appropriate bands were excised from the gel using a scalpel blade and DNA extracted from them as described in Section 2.3.8.

2.3.8 Extraction of DNA from agarose gel fragments

DNA was extracted from agarose gel slices using the GENE CLEAN SPIN Kit (Hybaid) according to manufacturer's instructions. Briefly, 0.3 g agarose was melted in 400 μ l binding buffer, by heating at 55°C for 10 minutes, then centrifuged through a column for 30 seconds. The columns were rinsed with 500 μ l of wash solution for 30 seconds then spun dry. DNA was recovered by addition of 2 x 15 μ l aliquots of elution buffer to the column and 2 μ l of this was electrophoresed on a 1 % agarose gel against a *Lambda/HindIII* DNA marker (Promega) to quantify the amount of DNA present before ligation.

2.3.9 Ligation of DNA inserts into other vectors

After liberation of the DNA insert from its cloning vector, DNA fragments were cloned into the appropriate plasmid vector using compatible overhanging DNA ends and Ready-To-Go ligation tubes (Amersham Biosciences). The amount of insert required was calculated using the following equation:

$$X \text{ ng insert} = \frac{(Y \text{ basepairs of insert})(50 \text{ ng vector})}{\text{base pairs of vector}}$$

To facilitate cloning, the insert to vector ratio was then adjusted to 3:1. The appropriate amounts of insert and vector were mixed together in the ligation tube, with dH₂O up to 20 μ l, and incubated at room temperature for 5 minutes. The ligation was then incubated at 16°C overnight and 2 μ l of this was transformed into 50 μ l competent cells as previously described (See Section 2.2.2).

2.3.10 Southern blotting of RT-PCR products

Southern blotting (Southern, 1975) was used to verify the identity and presence of PCR products. PCR products were electrophoresed on a 1% agarose gel as described in Section 2.1.4. The gel was denatured by washing for 20 minutes in denaturing solution, rinsed twice in dH₂O, then washed for 20 minutes in

neutralizing solution. The gel was blotted on to Hybond-N⁺ (Amersham Biosciences), pre-soaked in 3 x SSC, according to manufacturer's instructions and transferred for 4 h in 20 x SSC. The next morning the membrane was wet in 3 x SSC and the DNA crosslinked by treating for 30 seconds in a SpectroLinker XL-1000 machine. The blot was re-washed in 3 x SSC, excess liquid blotted off, then wrapped in Saran wrap and stored at 4°C until required.

2.3.10.1 Probe design to verify *Fanca* PCR products

For Southern blots to verify *Fanca* PCR products a labeled probe was generated corresponding to exons 14 to 18 of *Fanca* cDNA that had been amplified from L-cell cDNA, cloned into TOPO-pCRII and verified by DNA sequencing. The probe template was liberated from the vector by digestion with *EcoRI*, purified on an agarose gel and labelled as described below.

2.3.10.2 Radiolabelling of probes

All work with radioactive material was performed with appropriate care to ensure no accidental over exposure to the potentially harmful effects of radiation. Probes were labeled with $\alpha^{32}\text{P}$ -dCTP (ICN) using the Rediprime Labelling Kit (Amersham Biosciences) according to manufacturer's instructions. Briefly, 30 ng of probe DNA was mixed with dH₂O to a final volume of 45 μl then boiled for 5 minutes in a waterbath. The DNA was snap-cooled on ice for 5 minutes then added to a Rediprime reaction tube containing the Klenow subunit of *DNA polymerase I*. Contents were mixed gently by tapping and 50 μCi of $\alpha^{32}\text{P}$ -dCTP added. The template was then incubated at 37°C for at least 1 h to allow incorporation of radioactivity. The amount of incorporated activity was measured using a scintillation counter by spotting 2 μl of radioactive probe onto a glass filter disc (Whatmann). Any unincorporated radioactivity was removed by washing 3 times in 5% Trichloroacetic acid/20 mM PPI then once in 70% EtOH. Excess liquid was drained off and the disc placed in a scintillation vial. Counts were read using a Trilux 1450 Microbeta

scintillation counter and the amount of incorporated radioactivity was calculated using the following equation:

$$\text{Specific activity (DPM)/}\mu\text{g} = (\text{CCPMI}) \times (\text{dilution volume}) \times (3) \times (\text{dilution for } 1 \mu\text{g})$$

Probes were used if the specific activity (DPM/ μg) was $> 1 \times 10^9$.

2.3.10.3 Hybridisation and washing conditions

The radiolabelled probe was denatured by addition of 750 μl dH_2O and 250 μl , 2 M NaOH then incubated on ice for 5 minutes and neutralized by addition of 125 μl , 3 M NaAc (pH 5.2). Pre-hybridisation was carried out at 65°C in a Techne hybridisation oven in modified Church and Gilbert Hybridization solution for a minimum of 30 minutes. Denatured, labeled probe was added to fresh Church and Gilbert hybridization solution and the blot incubated overnight at 65°C. The membrane was then washed with 2 x SSC, 0.5% SDS at 65°C for 30 minutes followed by 0.1 x SSC, 0.1% SDS at 50°C for 30 minutes. The blot was sealed in Saran wrap and exposed to X-OMAT (Kodak) x-ray film at -70°C overnight.

2.3.11 Site directed mutagenesis of human FANCA

Mutagenesis was performed using the QuikChange XL Site Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. This kit allows site specific mutation of double-stranded plasmid through the use of mutagenic oligonucleotide primers and *PfuTurbo* DNA polymerase (See Figure 2.1). Mutagenic primers are designed so that both primers contain the desired mutation and anneal to the same DNA sequence on opposite strands of the plasmid. Specific mutagenic primers used to mutate *FANCA* are shown in Table 2.3.

Primer	H1110P point mutation	Position (bp)
Sense	5'-GCGAGCAGTTCTTCC <u>C</u> CTTGGTCAACTCTG-3'	3313-3343
Antisense	5'-CAGAGTTGACCAA <u>G</u> GGGAAGAACTGCTCGC-3'	3343-3313

Primer	Q1128E point mutation	Position(bp)
Sense	5'-GAGGTGCCCTGACA <u>G</u> AGGACATGACTGCCC-3'	3367-3397
Antisense	5'-GGGCAGTGATGTCCT <u>C</u> TGTCAGGGCACCTC-3'	3397-3367

Table 2.3 DNA sequence of primers used for site directed mutagenesis of FANCA.

Mutated bases are highlighted in red. Position of primers (bp) is given relative to ATG of FANCA (Accession N^o NM_000135).

The FANCA/enhanced green fluorescent protein (EGFP) vector was a kind gift from Manuel Buchwald (Toronto, Canada). This vector is large (10 kb) and proved difficult to mutagenise by PCR, so to facilitate easy mutagenesis, a 1.8 kb region containing the sequence to be mutated was sub-cloned into pBluescriptSK⁺ (Stratagene) using *EcoRV* restriction sites (See Section 2.3.7 for protocol. See Appendix I for vector map). This smaller sized, more malleable, vector of ~ 4 kb (pBluSKP-FANCA-EcoRV) was mutagenised, and the inserts then cloned back into the pEGFP/FANCA vector, using *EcoRV* sites, to give a full length, mutated, human FANCA clone tagged with EGFP (pEGFP-FANCA-H1110P/Q1128E).

To perform the PCR mutagenesis the following were mixed together:

5 µl	10 x Reaction Buffer
2 µl	pBluescript/ <i>EcoRV</i> FANCA (10 ng/µl)
1.5 µl	Sense primer (100 ng/µl)
1.5 µl	Antisense primer (100 ng/µl)
1 µl	dNTP mix
3 µl	QuikSolution
39 µl	dH ₂ O
1 µl	<i>Pfu Turbo Taq</i> DNA polymerase

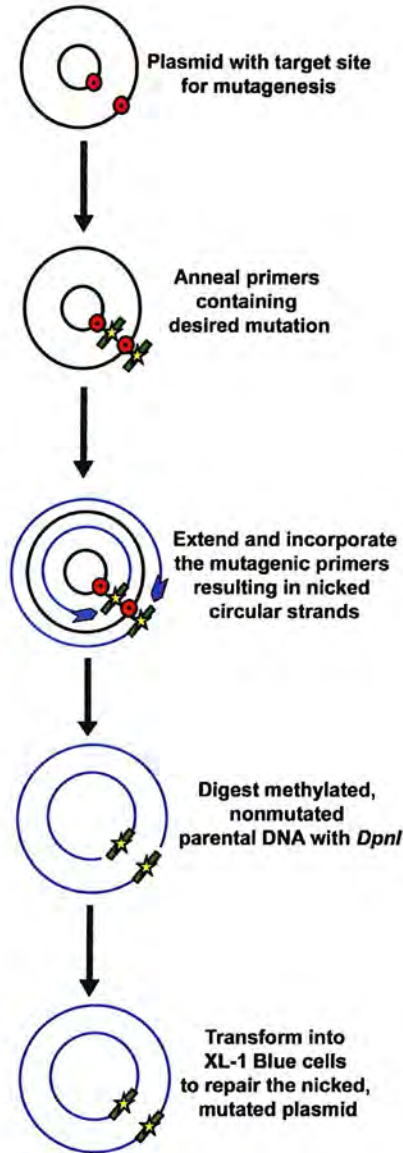


Figure 2.1 Overview of the QuikChange site-directed mutagenesis method.

PCR amplification with oligonucleotide primers containing the desired mutation results in incorporation of the mutation into circular, nicked DNA. Parental, methylated non-mutated DNA is removed by digestion with *DpnI* and nicks in the mutated plasmid are then repaired by transformation into XL-10 Gold competent cells.

18 cycles of PCR amplification were then performed under the following conditions

Initial denaturation	95°C	1 minute	} 18 cycles
Denaturation	95°C	50 seconds	
Specific primer annealing temperature	60°C	50 seconds	
Extension	68°C	7 minutes	
Final extension	68°C	7 minutes	

The PCR reaction was chilled on ice before any methylated, non-mutated parental DNA template was removed by digestion with 1 μ l of *DpnI* (Stratagene) at 37°C for at least 1 h. A 3 μ l aliquot of this reaction was transformed into 45 μ l XL10-Gold competent cells (See Section 2.2.2), plated onto LB-Agar/Amp plates and grown overnight at 37°C. Colonies were mini-prepped as described in Section 2.3.2 then sequenced (see Section 2.3.6) to check for the introduced mutation and ensure that no non-specific mutations had been added. Table 2.4 lists all sequencing primers used.

Primer name	DNA sequence 5' \Rightarrow 3'	Position (bp)
HFAA-1F	CAAGGCCATGTCCGACTCGT	25 – 44
HFAA-2F	ATCCTGCCAGCATGCTGACG	700 – 720
HFAA-3F	TGATGTTATATCGGCACAGG	2039 – 2060
HFAA-4F	GGATTCCTTGTCTTCTGCC	2412 – 2529
HFAA-5F	AGAGGAGCTGCTAATGTAC	3246 – 3265
HFAA-6F	GTCTTCTCTCCTACTTCCAT	4041 – 4060
HFAA-1R	TCAGGAGGCCCTGAAGAAG	810 – 791
HFAA-2R	CTGCTGTCATCCTCATTGTG	2118 – 2099
HFAA-3R	TCAGGAGGCCCTGAAGAAG	3449 – 3430
HFAA-4R	GCACTTGCCAGTATGAAG	3509 – 3490
HFAA-5R	ATGGAAGTAGGAGAGAAGAC	4060 – 4041
HFAA-6R	ACAGCTGATGAAGCCACGTG	5400 – 5381

Table 2.4 DNA sequence of FANCA sequencing primers.

Primers were designed based on FANCA sequence available on Genbank Database (Accession number NM_000135). Position of primers, specified in basepairs (bp) is given relative to the start codon (ATG).

2.4 Tissue Culture

2.4.1 Cell lines

Various cell lines were used during the course of this research. HeLa cells, HEK293 cells and L-cells were purchased from the European Collection of Cell Cultures (ECACC). GH3 cells were a kind gift from Alan McNeilly (Edinburgh). Cell lines derived from developing pituitary gonadotrophs (L β T2 and α T3-1 cells) were a kind gift from Pamela Mellon (San Diego, USA).

2.4.2 Cell growth conditions

L β T2, α T3-1, HeLa and L-cells were grown in DMEM (Sigma) containing 10% Fetal Calf Serum (Sigma) and 0.1% penicillin-streptomycin (Sigma) which will hereafter be referred to as media. L β T2 and α -T31 cells were grown on Matrigel, a basement membrane matrix (See Section 2.4.6). All tissue culture work was carried out under sterile conditions. All media and Phosphate Buffered Saline (PBS, Sigma) were heated to 37°C before use.

2.4.3 Solutions

1 x trypsin

10 ml trypsin (Sigma) was diluted in 100 ml PBS and stored, in aliquots at -20°C until required.

100 μ M GnRH

GnRH (Peninsula) was dissolved in PBS to achieve a concentration of 100 μ M and stored, in aliquots, at -20°C until required. A working concentration of 1 μ M was achieved by diluting 500 μ l GnRH in 50 ml media.

Cycloheximide (10 mg/ml)

100 mg cycloheximide (Calbiochem) was dissolved in 10 ml dH₂O. This stock solution was aliquoted and stored at -20°C until required. Working concentration was 15 µg/ml.

ActinomycinD (1 mg/ml)

5 mg actinomycin D (Calbiochem) was dissolved in 5 ml 95% EtOH. This stock solution was aliquoted and stored at -20°C, protected from light, until required. Working concentration was 5 µg/ml.

2.4.4 Resuscitation of cell lines from liquid nitrogen storage

Cells were removed from liquid nitrogen storage onto ice and thawed rapidly by hand. A 1 ml aliquot of cells was added to 4 ml media and plated in a T-25 cell culture flask (Costar).

2.4.5 Passaging of cell lines

Media was removed from the cells using a 10 ml pipette (Costar) and each flask washed twice with 5 ml of PBS. All PBS was removed and 1.5 ml of 1 x trypsin added per T-75 flask. Flasks were laid flat and incubated at 37°C for 2 minutes, then removed from the incubator and tapped against the hand to dislodge cells from the growing surface. Immediately, 14 ml of media was added to each flask to inactivate the trypsin and using a pasteur pipette, the growing surface was washed with the media then cells were agitated twice to break up any 'clumps' of cells. Flask contents were then pooled and a small aliquot was removed for counting using a haemocytometer (Sigma). Cells were diluted appropriately with media to the required density and plated in new tissue culture flasks.

2.4.6 *Matrigel coating of tissue-culture flasks*

All cell culture plasticware required for growing L β T2 or α T3-1 cells was coated with a 1/30 dilution of a basement membrane matrix (Matrigel, Beckton Dickinson Labware). Concentrated matrigel is a solid at room temperature and liquid at 4°C therefore, before dilution, all apparatus and solutions must be chilled to below 4°C. Matrigel was thawed overnight on ice, then 400 μ l was added to 14.6 ml of pre-chilled PBS. Aliquots were stored at -20°C until required. To coat flasks, Matrigel was pipetted onto the base of the flask or plate, ensuring the whole area was covered. Excess Matrigel was removed and the plasticware allowed to air-dry before plating cells as described.

2.4.7 *GnRH treatment of cells*

α T3-1 and L β T2 cells were grown until 80% confluence in T-75 flasks. To treat cells, media was removed and replaced with fresh media containing GnRH to a final concentration of 1 μ M. After incubation for the appropriate amount of time, cells were harvested for further analysis.

2.4.8 *Treatment of L β T2 cells with inhibitor chemicals*

L β T2 cells were grown until 80% confluence in T-75 flasks. To treat cells, media was removed and replaced with fresh media containing the appropriate inhibitor. Cycloheximide (CHX) was added to a final concentration of 15 μ g/ml, actinomycin D (AMD) was added to a final concentration of 5 μ g/ml and leptomycin B (LMB, Sigma) was added to a final concentration of 2 ng/ml. Flasks were returned to the incubator for 30 minutes then 150 μ l of 100 μ M GnRH was added, to give a final concentration of 1 μ M. Flasks were then returned to the incubator for a further 2 h. Cells were harvested for protein extracts as described in Section 2.7.2.

2.5 Transient transfection of DNA into *in vitro* cultured cells

For L β T2, HeLa and L-cells, Fugene transfection reagent was used at a ratio of 3 μ l of Fugene to 1 μ g DNA. For α T3-1 cells, GenePorter transfection reagent was used at a ratio of 5 μ l of GenePorter to 1 μ g DNA.

2.5.1 Transient transfection of GFP expressing plasmids into cultured cells

Cells to be transfected (L β T2 or HeLa) were plated on 2-well glass slides (Nunc) at a density of 100,000/ml. The following day, 2 h prior to transfection, fresh media was added to the cells. For each well, 1.5 μ g DNA was diluted in 50 μ l Optimem (GibcoBRL) in a 5 ml culture tube (Elkay Laboratory products, UK). Into a separate tube, 4.5 μ l Fugene was diluted in 50 μ l Optimem. The DNA/Optimem mix was pipetted into the Fugene/Optimem mix, thoroughly mixed and incubated at room temperature for 15 minutes. The 100 μ l transfection mix was added drop-wise to each well and the cells returned to the incubator for 48 h.

2.5.1.1 Preparation of EGFP transfected cells prior to image analysis

Localization of EGFP-tagged proteins was determined by comparison of GFP expression with the expression of known nuclear stains. Media was discarded from the transfected cells, and cells were washed twice in PBS. They were then fixed in 1 ml Methanol (MeOH, BDH) at -20°C for 10 minutes. The MeOH was removed and the cells washed again in PBS. At this point the chambers were removed from the top of the slides and a few drops of Topro-3 (Cambridge Biosciences, UK) nuclear stain (1:2000 in PBS) were added to the fixed cells and the slides incubated, in the dark, for 2 minutes. Slides were kept in the dark whilst washing in PBS for 5 minutes then coverslips were mounted using Permafluor mounting medium (Immunotech, Beckman Coulter). Slides were viewed by confocal microscopy and stored, in the dark, at 4°C .

2.5.1.2 Image analysis of EGFP tagged proteins in mammalian cell culture

Analysis of direct immunofluorescence within cells was undertaken using a Zeiss Axiovert 100m microscope and LSM510 scanning module with an oil immersion x40 objective (Zeiss). Single scans and Z-stack analysis were performed at 1024 x 1024 pixels resolution according to manufacturer's instructions. To visualize GFP and Topro-3, fluorescence excitation was performed using the Argon 488 nm and HeNe 633 nm lasers (FITC/Cy5 multitrack).

2.5.2 Transient transfection of constructs for reporter gene assays

Luciferase assays were used to determine the effect a particular protein may have on gene promoter activity. All constructs are detailed in Table 2.5 below. See Section 2.3.7 for information regarding cloning.

Vector Construct	From/constructed by	Size (basepairs)
pBluescript ^{SK+}	Stratagene	2961 bp
pcDNA4HisMax	Invitrogen	5222 bp
pcDNA4HisMax-LacZ	P.Brown	8321 bp
pA3LUC	D.Gordon, Denver	6356 bp
-480 alpha-pA3LUC	P.Brown	6840 bp
-692 LH β -pA3LUC	P.Brown	7038 bp
-4741 FSH β -pXP2LUC	W.Miller	10227 bp
pCEP4	Invitrogen	10188 bp
pCEP4-Fanca	H. van de Vrugt, Amsterdam	14879 bp
pEGFP-C1	Clontech	4700 bp
pEGFP-FANCA	M.Buchwald, Ontario	10205 bp
pEGFP-FANCA-H1110P	R.Larder	10205 bp
pEGFP-FANCA-Q1128E	R.Larder	10205 bp
pCDNA4-HisMaxB	Invitrogen	5259 bp
pREP4-FancC	M.Buchwald, Ontario	12192 bp

Table 2.5 Expression constructs used during the course of this thesis.

For all transfections, other than those analysing *FSH β* promoter activity (See Section 2.5.2.2), cells were plated into 12 well plates (Costar) at a density of 100,000/ml 24 h prior to use. The total plasmid DNA transfected into each well was 2 μ g, 1.5 μ g of this corresponded to the promoter construct, the remainder was

comprised of effector or control pBluescript^{SK+} plasmid. The ratio of gene promoter/reporter construct to effector plasmid DNA was always 12:1. A β -galactosidase (LacZ) reporter construct (pcDNA4HisMax-LacZ) was co-transfected at 100 ng per well to control for transfection efficiency. Each transfection was carried out in triplicate and each experiment repeated at least three times.

2.5.2.1 Transient transfection of L β T2 cells using Fugene

L β T2 cells were transfected using Fugene according to the manufacturer's instructions. At least 2 h prior to transfection, media was removed and fresh media added to the cells. The DNA to be transfected and the Fugene reagent were diluted in Optimem serum free media (GibcoBRL). Optimem was pipetted to give a final transfection volume of 100 μ l per well and was split over two 5ml culture tubes. The appropriate DNA plasmids were added to one tube whilst 3 μ l of Fugene reagent per 1 μ g DNA was added to the other. The diluted DNA was then added to the Fugene/Optimem mix and the two solutions mixed. After incubating at room temperature for 15 minutes, 100 μ l of transfection mix was added drop-wise to each well. The plate was swirled gently to mix and the cells incubated at 37°C for 48 h. Cells were harvested for luciferase assays 6 h post GnRH treatment. (See Section 2.5.3).

2.5.2.2 Transient transfection of FSH β promoter constructs

For analysis of FSH β promoter activity, cells were plated into 6 well plates (Costar) at a density of 150,000/ml 24 h prior to use. As these wells hold 2.5 ml rather than 1 ml, each transfection was performed as for 12 well plates but was scaled up 2.5 times (See Section 2.5.2.1). Approximately 16 h after transfection, cells were serum starved by incubating for 24 h in DMEM containing 0.5% FCS and 0.1% Pen/Strep. The following morning, activin (Sigma) was added to all wells at a final concentration of 25 ng/ μ l whilst GnRH was added to the appropriate wells at a final concentration of 100 nM. After 8 h cells were harvested for luciferase assays as described in Section 2.5.3.

2.5.2.3 *Transient transfection of α T3-1 cells using Geneporter*

α T3-1 cells were transfected using Geneporter according to the manufacturer's instructions. At least 2 h prior to transfection, the media was replaced with Optimem serum free media (GibcoBRL). Transfection reactions were set up as described for Fugene except that 5 μ l of GenePorter was added per 1 μ g DNA. A 100 μ l aliquot of transfection mix was added drop-wise to each well. The plate was swirled gently to mix and the cells incubated at 37°C for 4 h. The Optimem/transfection mix was removed, fresh media added, and the cells were returned to the incubator for a further 48 h then harvested (as described in Section 2.5.3), 6 h post GnRH treatment.

2.5.3 *Luciferase/ β -galactosidase chemiluminescent reporter gene assay*

Luciferase assays for promoter activity were carried out using the Dual Light Assay Kit (Tropix). This kit allows luciferase and Lacz activity to be read from the same sample allowing the luciferase activity to be corrected for the transfection efficiency of the cells. Media was removed from untreated or GnRH treated cells, and the cells were washed 3 times in ice cold PBS. The cells were lysed in 250 μ l lysis buffer (Tropix) containing protease inhibitors (Roche) by shaking at 1000 rpm for 30 minutes. The supernatant from each well was transferred to a 1.5 ml eppendorf tube and centrifuged at 5,000 rpm for 2 minutes at 4°C. A 10 μ l aliquot of each sample was pipetted in duplicate into a white, 96 well plate (Nunc). The luciferase activity was determined using an automatic injection microplate luminometer (FLUOstar Optima, BMG Labtechnologies). After incubation for 30 minutes at room temperature β -galactosidase activity was measured. Luciferase activity was then normalized against the relative amount of β -galactosidase activity in each sample.

2.6 RNA expression analysis

2.6.1 Solutions

Solutions and buffers for protocols involving RNA were made as described in Sections 2.1 and 2.3 except they were pre-treated with 0.1% DEPC (Sigma) overnight, to remove RNases, before autoclaving.

10 x MOPS, pH 7.0 (0.4 M MOPS, pH 7.0, 0.1 M NaAc, 10 mM EDTA)

20.9 g of 3-[N-Morpholino]propanesulfonic acid (MOPS, Sigma) and 2.05 g NaAc were dissolved in 490 ml DEPC dH₂O. To this 10 ml of 0.5M EDTA (pH 8.0) was added

2.6.2 RNA extraction

All reverse transcriptase (RT) PCR was performed using total RNA harvested from cells grown in culture media. RNA is easily degraded by RNases so extreme care was taken during extraction. All equipment and bench areas were cleaned with RNaseZap (Ambion) to remove any enzymes that could degrade RNA. All plasticware was certified RNase free and all solutions were DEPC treated. RNA was extracted by harvesting cells into RNAzol-B (AMS Biotechnology). Cells were washed twice in PBS then removed from the flasks by lysing in 3 ml of RNAzol-B solution for a T-25 flask and 7 ml of RNAzol-B solution for a T-75 flask. RNAzol-B/lysed cell suspensions were stored at -70°C until required. Total RNA was extracted following manufacturer's instructions. Pelleted RNA was resuspended in RNase free water and stored at -70°C until required.

2.6.3 Northern blot analysis

To analyze expression of *Fanca* and *Zfp276* within LβT2 cells, and the effects hormone treatment may have on expression levels, northern blot analysis was performed.

2.6.3.1 Preparation of northern blot

A 1% agarose gel was prepared by boiling 3 g agarose in 30 ml 10 x MOPS mixed with 220 ml DEPC H₂O. Once the gel had cooled to ~50°C, 51 ml formaldehyde was added and the gel cast in a tray that had been thoroughly cleaned with RnaseZap. A 30 µg aliquot of RNA sample was made up to a final volume of 12 µl with DEPC H₂O then adjusted to a final volume of 50 µl by adding 25 µl formamide, 5 µl 10 x MOPS and 8 µl formaldehyde (BDH). This mix was then incubated at 65°C for 10 minutes then chilled on ice and 5 µl 50% (v/v) glycerol/0.1 mg/ml bromophenol blue loading dye added. Samples were loaded onto the gel and electrophoresed overnight at 35 volts/cm in 1 x MOPS buffer. The next day the marker lane, containing L-cell control RNA, was removed and stained in ethidium bromide (5 µg/ml) for 15 minutes. Following de-staining in DEPC H₂O for 15 minutes the RNA was visualized using UV light and photographed to record the distance traveled by 28s and 18s RNA. The rest of the gel was blotted onto Hybond-N⁺ (Amersham Biosciences), pre-wet in 3 x SSC, according to manufacturer's instructions and left to transfer overnight in 20 x SSC. The next morning the blot was crosslinked, as described in Section 2.3.10, then re-washed in 3 x SSC, excess liquid removed and the membrane wrapped in Saran wrap and stored at 4°C until required.

2.6.3.2 Probe design for detecting *Fanca* and *Zfp276* transcripts by northern blotting

To analyze the expression and size of *Fanca* and *Zfp276* mRNA transcripts two probes were designed from mouse *Fanca* cDNA sequence for hybridization to a northern blot containing LβT2 total RNA. A 5' probe corresponding to exons 1-11 of *Fanca* was generated by digestion of pCEP4-*Fanca* (See Appendix I for vector map) with *HindIII* (Roche) and *Asp700* (NEB). This generated three fragments, the smallest 965 bp fragment, corresponding to exons 1-11 of *Fanca*, was gel purified and labelled as previously described in Section 2.3.10.2. As exons 40-43 of *Fanca* overlap with the 3'UTR of *Zfp276*, a probe corresponding to this region should

hybridize to both transcripts. A labelled probe was generated, by amplification of L-cell cDNA, which matched exon 43 of *Fanca* sequence and 1441 – 1949 bp of *Zfp276* cDNA sequence. Table 2.6 shows the DNA sequence of primers used to generate this fragment. This 508 bp PCR product was cloned into TOPO-pCRII and verified by DNA sequencing. The probe template was liberated from the vector by digestion with *EcoRI*, purified on an agarose gel and radiolabelled as described previously.

	Sense primer (5'⇒3')	Antisense primer (5'⇒3')	Product Size	Accession Number
3'probe	CTTGAAGTCAGCAGGT GACG	GCTGAAGGCACTAAGC GTTG	508 bp	AF178935

Table 2.6 DNA sequence of primers used to generate probes for northern blotting analysis.

Sequence of primers used to generate a 3' northern probe corresponding to both *Fanca* and *Zfp276* cDNA sequence.

2.6.3.3 Hybridisation and washing conditions

The probe was prepared and overnight hybridization performed as described in Section 2.3.10.3. The following morning the blot was washed three times with 2 x SSC, 0.1% SDS at room temperature for 30 minutes. The membrane was sealed in Saran wrap and exposed to X-OMAT (Kodak) x-ray film at -70°C for 3 days. To control for even loading, the blot was stripped in 1 litre of freshly boiled 0.5% SDS solution and re-hybridized with an 18s probe using the same hybridization and washing conditions as above.

2.6.4 Quantitative RT-PCR

Quantitative RT-PCR (Q-RT-PCR) was performed using a LightCycler Instrument (Roche) and FastStart DNA Master SYBR Green I (Roche). This is a 'hot start' reaction mix for PCR, containing FastStart *Taq* DNA polymerase and a dye for detection of double-stranded DNA. During each round of amplification the dye binds to the amplified PCR products and the amplicon is then detected by

fluorescence. Quantification of real time PCR is based on calculations taking into account the expression of a house keeping gene, in this case *beta-2-microglobulin* (*B2m*), and the gene of interest (*Fanca*). The internal control gene must be quantifiable over the same number of PCR cycles, expressed at the same levels regardless of any cell treatments and be optimally synthesized at the same primer annealing temperature and magnesium concentration as the gene of interest. *Fanca* and *B2m* reaction conditions were optimized taking these factors into account and primers were designed to amplify a product of between 100 and 200 bp. Prior to using the LightCycler a check for genomic contamination in the sample was performed using these primers in an RT-PCR reaction. If a single band was visible when the product was analysed on a 1% agarose gel then the RNA was free from contamination. Contaminated genomic DNA would generate 2 bands and therefore 2 melt curves and invalidate the results. The specific DNA primer sequences for amplification of *Fanca* and *B2m* are shown in Table 2.7.

Primer	Sense primer	Antisense primer	Size	Position (bp)
<i>Fanca</i> Exons 37-38	5'- GCACTTTGCGTGGAGA GG-3'	5'- CAGGTAGGACGAGAGT AGAC-3'	129 bp	3666 - 3795
<i>B2m</i> Exon 1	5'- ATGGCTCGCTCGGTGA CCCTGGT-3'	5'- TGTTCCGGCTTCCCATT TCC-3'	102 bp	ATG - 102

Table 2.7 DNA sequence of primers used for LightCycler quantitative RT-PCR.

DNA sequence of primers used to amplify *Fanca* and *B2m* for quantification of mRNA levels using the LightCycler. Positions are specified in basepairs (bp) and are given relative to the start codon (ATG) of mouse *Fanca* sequence (Accession N^o AF178934).

The reagents used per glass capillary tube (Roche) are listed below:

5.8 μ l	dH ₂ O
1.2 μ l	MgCl ₂ (4 mM)
1 μ l	Primer Pair Mix (25 μ M each)
1 μ l	LightCycler DNA Master SYBR Green I (Roche)
1 μ l	cDNA

Specific PCR conditions were as follows:

Heat activation of <i>Taq</i> polymerase	95°C	10 minutes	} 60 cycles
Denaturation of DNA	95°C	5 seconds	
Primer annealing	57°C	5 seconds	
Extension	72°C	15 seconds	
Melting curve program	57 - 95°C		

A melt curve analysis is performed to determine the optimum temperature at which to measure fluorescence, which in this case, was determined to be 80°C. This temperature was chosen to ensure that only fluorescence measurements of specific products were taken and fluorescence of non-specific products e.g. primer-dimers was avoided. Any primer-dimers that may have formed during the PCR reaction were denatured at this temperature and could therefore no longer bind SYBR Green I.

A standard curve of *Fanca* and *B2m* expression was determined using serially diluted cDNA made from mouse L-cell or HeLa cell RNA. Results are expressed as arbitrary units of *Fanca* mRNA expression normalized against *B2m* mRNA expression levels and are a mean of three separate experiments performed in duplicate.

2.7 Protein Expression Analysis

2.7.1 Solutions

1 M Tris-HCl (pH 8.0)

121.1 g Tris was dissolved in 800 ml dH₂O. The pH of the solution was adjusted to pH 8.0 by adding ~ 42 ml HCl (BDH) then made up to 1 litre with dH₂O.

1 M Tris-HCl (pH 6.8)

121.1 g Tris was dissolved in 800 ml dH₂O. The pH of the solution was adjusted to pH 6.8 by adding ~ 100 ml HCl then made up to 1 litre with dH₂O.

1 M HEPES (pH 7.6)

0.48 g of HEPES (Calbiochem) was dissolved in 100 ml dH₂O, aliquoted and stored at -20°C until required.

0.5 M EGTA

1.9 g EGTA (BDH) was dissolved in 100 ml dH₂O.

3 M Potassium Chloride

15 g KCl (BDH) was dissolved in 100 ml dH₂O.

10 % Ammonium persulphate (APS)

10 g of APS (Sigma) was dissolved in 100 ml of dH₂O, aliquoted and stored at -20°C until required.

1 M DTT

0.015 g of DTT was dissolved in 100 ml dH₂O, aliquoted and stored at -20°C until required.

Phosphate Buffer Saline (PBS)

8 g NaCl; 800 mg KCl (BDH); 610 mg Na₂HPO₄ and 200 mg KH₂PO₄ (Sigma) were dissolved in dH₂O. The pH was adjusted to pH 7.4 with concentrated NaOH or HCl then made up to 1 litre with dH₂O.

1 x Tris/Glycine/SDS (TGS)

50 ml 10 x TGS (BioRad) was diluted in 450 ml dH₂O.

5 x western loading dye (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 1% β-mercaptoethanol)

1.25 ml 0.5 M Tris-HCl pH 6.8, 2 ml 10% SDS, 1 ml glycerol (BDH) and 1 mg bromophenol blue were mixed together in 5.75 ml dH₂O. the dye was then aliquoted and stored at -20°C until required. Immediately prior to use 10 μl β-mercaptoethanol (Sigma) was added to 1 ml of dye.

Western transfer buffer (1 x TGS, 20% MeOH)

400 ml of 1 x TGS and 100 ml of 100% MeOH were mixed together.

1 x TBST (0.14 M NaCl, 20 mM Tris-HCl pH 7.4, 0.05 % Tween 20)

8 g NaCl; 20 ml, 1M Tris-HCl pH 7.4 and 500 μl Tween 20 (Sigma) were dissolved in 1 litre of dH₂O.

Western blocking buffer (2% milk powder, 1% BSA in 1 x TBST)

2 g milk powder and 1 g BSA were dissolved in 100 ml of 1 x TBST.

Western stripping buffer (2% SDS, 50 mM Tris-HCl pH 6.8, 0.7% β-mercaptoethanol)

2 ml 10% SDS, 4 ml 1.25 M Tris-HCl pH 6.8 and 700 μl β-mercaptoethanol were dissolved in 100 ml dH₂O.

Protein extraction Buffer A (10 mM Hepes pH 7.6, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT)

100 μl 1M HEPES, 33 μl 3 M KCl, 2 μl 0.5 M EDTA, 2 μl 0.5 M EGTA and 10 μl 1 M DTT were diluted in 9.85 ml dH₂O.

Protein extraction Buffer B (20 mM HEPES pH 7.6, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT)	200 μ l 1 M HEPES, 1.33 ml 3 M NaCl, 20 μ l 0.5 M EDTA, 20 μ l 0.5 M EGTA and 10 μ l 1 M DTT were diluted in 8.42 ml dH ₂ O.
--	---

2.7.2 Protein extraction from cells grown in culture

2.7.2.1 Whole cell extracts

Cells were grown in T-75 flasks until confluent. All media was removed and the cells washed twice in chilled PBS. Cells were then scraped into 100 μ l PBS containing protease inhibitor tablets (Roche) and transferred to an eppendorf. After three freeze-thaw cycles on dry-ice, cells were centrifuged at 13,000 rpm for 1 minute to pellet cell debris. The supernatant was aliquoted into new eppendorfs and snap-cooled on dry ice. Extracts were stored at -70°C until required and were not re-frozen once thawed.

2.7.2.2 Nuclear and cytoplasmic cell extracts

Cells to be harvested were grown in T-75 flasks until confluent. All media was removed and the cells washed twice in chilled PBS. Cells were scraped into 1 ml PBS containing protease inhibitor tablets and transferred to an eppendorf. After centrifugation at 1500 rcf for 5 minutes at 4°C , the supernatant was removed and each pellet (representing 1 x T-75 flask) resuspended in 400 μ l chilled Buffer A (See Section 2.7.1). After incubation on ice for 15 minutes, 25 μ l 10% NP40 (Sigma) was added to each eppendorf and cells vortexed for 10 seconds. Tubes were then centrifuged at 13,000 rpm for 45 seconds. The supernatant, representing the cytoplasmic fraction, was removed into new eppendorfs and snap-cooled on dry ice. The remaining pellet was resuspended gently in 25 μ l Buffer B (See Section 2.7.1) and incubated on ice for 15 minutes. Eppendorfs were then centrifuged at 12,000 rpm for 5 minutes at 4°C . Supernatant, representing the nuclear fraction, was transferred into new eppendorfs and snap-cooled on dry ice. Extracts were stored at -70°C until required and were not re-frozen once thawed.

2.7.3 Bradford assay of protein extracts

The concentration of protein extracts was determined using Bradford Assay Dye (BioRad). A standard curve of BSA was used to calculate the amount of protein present in each sample. BSA was diluted in PBS to give a range of protein concentrations from 1 $\mu\text{g}/\mu\text{l}$ to 30 $\mu\text{g}/\mu\text{l}$. To formulate the standard curve, 800 μl of BSA standard was mixed with 200 μl Bradford Assay dye and the OD at 595 λ measured using a WPA Lightwave S2000 UV/Vis Spectrophotometer (SLS, UK). These results were plotted graphically and the linear regression formula of the best fitting line then used to calculate the protein concentration of the samples. To determine levels of protein present in each sample, 5 μl of extract was mixed with 800 μl dH_2O and 200 μl dye and an OD measurement taken.

2.7.4 Preparation of SDS-polyacrylamide gels

All SDS-polyacrylamide (SDS-PAGE) gels were run using a Biorad PROTEAN II cell or a Biorad MINI-PROTEAN III cell. All glass plates, spacers and combs were cleaned thoroughly in detergent before use. After rinsing with dH_2O and EtOH the gel rig was assembled as per manufacturer's instructions.

2.7.4.1 SDS-polyacrylamide gel electrophoresis

In general, SDS-PAGE gels were cast and electrophoresed using a Biorad mini-Protean III gel rig. A 20 ml volume of acrylamide gel mixture was adequate for pouring two mini gels (See Table 2.8). To pour the gel, all components, apart from the SDS and TEMED (Sigma), were mixed together then filtered and de-gassed using a Nalgene filter sterilization unit (Fisher Scientific). The remaining components were added, the gel poured immediately using a pasteur pipette and a clean comb inserted. The gel was left to set at room temperature for at least 30 minutes then the comb was removed and the wells washed out with 1 x TGS running buffer (Biorad). The gel rig was assembled as per manufacturer's instructions and samples loaded as described in Section 2.7.5. Occasionally a Biorad PROTEAN II gel rig was used to pour and electrophorese larger gels (16 cm x 16 cm). The process

was essentially the same as for pouring mini-gels except 100 ml of acrylamide gel mixture was required to pour two large gels and a 3% stacking gel was poured on top of the resolving gel. (5 ml, 30% 37.5:1 acrylamide; 5 ml, 1.25M Tris pH6.8; 39.5 ml dH₂O, 300 µl 10% APS, 500 µl 10% SDS and 25 µl TEMED)

Percentage of resolving gel			
6%	12%	16%	
20 ml	40 ml	53 ml	30% Acrylamide (37.5:1)
25 ml	25 ml	25 ml	1.5M Tris pH 8.8
53.4 ml	40 ml	21 ml	H₂O
600 µl	600 µl	600 µl	10% APS
1 ml	1 ml	1 ml	10% SDS
50 µl	50 µl	50 µl	TEMED
~ 50 – 220 kD	~ 15 – 100 kD	~ 5 – 50 kD	Resolution

Table 2.8 Components required to make 100ml SDS-Page acrylamide gel.

2.7.5 Western analysis of protein extracts

All protein extracts were denatured by boiling for 5 minutes before loading on a SDS-PAGE gel. An appropriate amount of protein extract was mixed with PBS and 5 x western loading dye to make a final loading volume of 25 µl or 40 µl depending on the gel size. In general, 30 µg of whole cell extract and 50 µg of either cytoplasmic or nuclear extract was loaded using duck-billed tips (Anachem). On each gel, at least one well was loaded with Kaleidoscope Protein Marker (BioRad) so the size of protein products could be determined after probing with an antibody. Proteins were electrophoresed in 1 x TGS buffer at 10 volts/cm until they reached the resolving gel, when the voltage was increased to 15 volts/cm. Samples were electrophoresed until the dye front reached the bottom of the glass plates.

2.7.6 Transfer of proteins to PVDF membrane

The gel rig was dismantled, gel plates pulled apart and the gel equilibrated in wet blot transfer buffer for 10 minutes (See Section 2.7.1). Prior to use, PVDF

(Millipore) membrane was rinsed in 100 % MeOH, followed by water then wet transfer buffer to equilibrate the membrane. All blotting was done using a BioRad wet blotter or a BioRad semi-dry blotter according to manufacturer's instructions. Wet blotting was performed overnight at 25 volts, semi-dry blotting was performed for 30 minutes at 10 volts for mini-gels (maximum current 5.5 mA/cm²) or for 1 h at 15 volts for large gels (maximum current 3 mA/cm²). After blotting the PVDF membrane was rinsed in 1 x TBST and blots stored at 4°C in 1 x TBST until ready for probing.

2.7.7 Immuno-blotting

Immunofluorescent detection of protein expression was performed using a secondary antibody conjugated to horseradish peroxidase (HRP) and visualized with an ECL Plus western blotting detection kit (Amersham Biosciences). Figure 2.1 shows a schematic of how the detection system works.

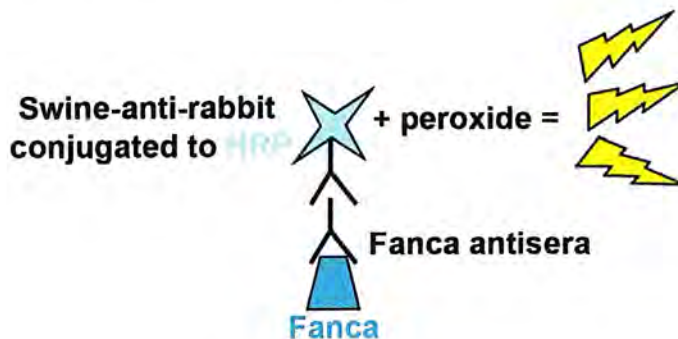


Figure 2.2 Method of detection of Fanca protein by ECL Plus Detection Kit.

All membranes were blocked in 2% milk powder/1% BSA/1 x TBST for at least 2 h at room temperature or at 4°C overnight, with shaking. Blots were then incubated with the appropriate antisera, diluted in blocking solution, for at least 2 h at room temperature with shaking. The anti-serum was recovered and the blots were washed in several changes of 1 x TBST for 30 minutes. Blots were then incubated with the

appropriate HRP conjugated secondary antibody at a 1:50,000 dilution in 1 x TBST for at least 1 h. The blots were then washed in several changes of 1 x TBST for at least 45 minutes. ECL Plus detection reagent was added according to manufacturer's instructions and the blots incubated at room temperature for 5 minutes. Excess solution was dried off and they were sandwiched between 2 sheets of acetate before being exposed to X-AR Kodak x-ray film (Sigma) or scanned using a Typhoon Phosphoimager (Amersham Biosciences) according to manufacturer's instructions. Table 2.8 shows full details of antibodies used and conditions required.

Antibody	Supplier	Info	Dilution	2 ^o antibody
Fanca N-terminus	Frè Arwert	Polyclonal rabbit anti-mouse 1 - 454 aa	1:1500	Swine-anti-rabbit IgG HRP-conjugated (DAKO-Cytomation)
β -Tubulin (H-235) (sc-9104)	Autogen Bioclear (UK)	Polyclonal rabbit anti-human 210 - 444 aa	1:400	Swine-anti-rabbit IgG HRP-conjugated (DAKO-Cytomation)
Sp-1 (sc-59)	Autogen Bioclear (UK)	Polyclonal rabbit anti-rat	1:500	Swine-anti-rabbit IgG HRP-conjugated (DAKO-Cytomation)
GFP	AbCam (ab6673)	Polyclonal goat anti- <i>Aequorea victoria</i> 1 - 246 aa	1:1000	Rabbit-anti-goat IgG HRP-conjugated (Sigma)

Table 2.9 Antisera and conditions used for western blotting analysis of cell extracts.

2.7.8 Quantification of protein expression

Levels of protein expression were determined using the ImageQuant Toolbox computer program (Amersham Biosciences). Results are expressed as arbitrary units of Fanca protein expression, normalized against β -tubulin protein expression levels where appropriate, and are a mean of at least three separate experiments.

2.7.9 Crude purification of Fanca antisera

Rabbit anti-mouse Fanca antiserum, collected from three inoculated rabbits, was provided by Dr. Frè Arwert. To remove fat and serum proteins 30 ml of 60 mM sodium acetate (pH 4.0) was added to 15 ml of antiserum and the solution mixed. To

this, 10.5 ml of caprylic acid (Sigma) was added, a few drops at a time, whilst stirring vigorously at room temperature. The serum protein precipitate was removed by centrifugation at 3,000 rpm for 30 minutes at 4°C and any fat present solidified as a disc on top of the partly purified antiserum. Partly purified antiserum was then filtered through 1 MM Whatmann paper (Fisher Scientific) and dialysed overnight in PBS. Dialysis tubing was prepared by boiling for 10 minutes in 1% sodium hydrogen carbonate/1 mM EDTA/dH₂O; rinsing in water; boiling for 10 minutes in 1 mM EDTA/dH₂O then washing in PBS. After dialysis, the protein concentration of the antisera was determined by Bradford assay and antisera was stored in aliquots at -20°C until required.

2.7.10 Immunohistochemistry of mammalian cells cultured in vitro

Immunohistochemistry was performed on LβT2 cells using antisera against Fanca. Figure 2.3 shows how Fanca protein expression is detected by indirect immunofluorescence.

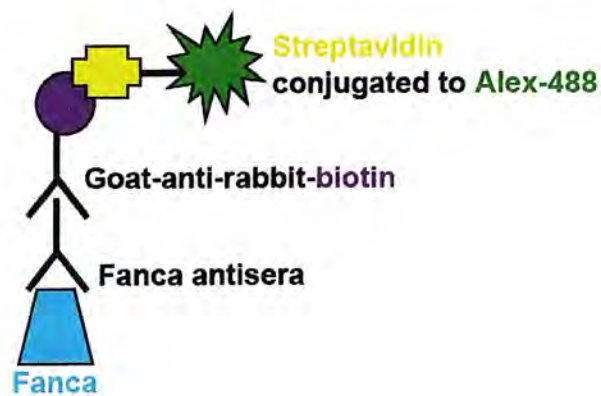


Figure 2.3 Detection of Fanca protein expression by indirect immunofluorescence.

Cells were plated on Matrigel coated 8-well glass slides (Nunc) at a density of 100,000/ml. The next day, media was discarded and the cells were washed twice in PBS. All washes were performed with gentle agitation at room temperature unless

otherwise stated. Cells were fixed in 1 ml MeOH at -20°C for 10 minutes. MeOH was removed and the cells were washed in PBS. Cells were permeabilised by incubating in PBS containing 0.2% NP40 (Sigma), 1% BSA (Sigma) and 10% normal goat serum (Diagnostics Scotland, Edinburgh, UK) for 20 minutes. Two washes in PBS removed any remaining detergent. The cells were then incubated in blocking solution (PBS containing 20% normal goat serum, 5% BSA, 4 drops/ml Avidin (Vector laboratories, California), for 30 minutes. After two washes with PBS, cells were incubated in biotin (Vector laboratories, California) diluted in PBS (4 drops/ml) for 30 minutes. Cells were washed a further two times in PBS then incubated overnight at 4°C in Fanca antisera diluted 1:200 in 20% normal goat serum/5% BSA/PBS. The following day, excess primary antisera was removed by a 5 minute wash in PBS containing 0.05% Tween 20 (Sigma, PBST) followed by a 5 minute wash in PBS. Chambers were removed from the slides and a biotinylated secondary antibody, goat-anti-rabbit-biotin, was applied at a 1:500 dilution in 20% normal goat serum/5% BSA/PBS for 30 minutes at room temperature. Excess secondary antibody was removed by washing for 5 minutes in PBST then 5 minutes in PBS. Slides were maintained in the dark for the remaining treatments. A few drops of a 1:200 dilution of Alexa 488 streptavidin conjugate (Cambridge Biosciences) were applied to each slide and slides were then incubated for 1 h at room temperature. Excess fluorescent probe was removed by washing for 5 minutes in PBST then PBS. Cells were counterstained to visualize nuclei by application of a few drops of Topro-3 (1:2000 in PBS, Cambridge Biosciences) to the top of each slide and incubating for 2 minutes. Slides were then washed in PBS for 5 minutes and coverslips mounted using Permafluor (Immunotech, Beckman Coulter) mounting medium. Cells were viewed by confocal microscopy or stored in the dark at 4°C .

2.8 'Knockdown' of *Fanca* gene expression using siRNAs

RNA interference (RNAi) is the process by which dsRNA silences gene expression by the sequence specific degradation of complementary mRNA (Hannon, 2002). In principle, any gene can be silenced providing a rapid means to assess what consequences loss of specific gene function may have on a cell line or on organ function. Chemically synthesized short double stranded RNA (dsRNA) molecules of 21 nucleotides (nt), commonly known as short interfering RNAs (siRNAs), are used to target mammalian mRNA without eliciting the interferon response associated with introduction of long dsRNAs (Elbashir et al., 2001).

2.8.1 siRNA design and production

Specific siRNAs against mouse *Fanca* sequence (Accession Number: AF178934) were designed using publicly available siRNA design programs on the websites of MWG (<http://www.mwg-biotech.com>); Ambion (<http://www.ambion.com>) and Dharmacon (<http://www.dharmacon.com>). Results from all three programs were analysed and three sequences were chosen that were held in common between all three programs. BLAST analysis was performed on these sequences to ensure that they matched no other transcripts within the database. As a consequence, one sequence was discarded, as it matched sequence on mouse chromosome 2, whilst *Fanca* is located on chromosome 8. The remaining siRNA sequences, meeting all of the match criteria, are shown in Table 2.10.

All siRNAs were synthesized by MWG using the 0.02 μ mole synthesis scale as duplexes in desalted form. They were resuspended in 750 μ l 5 x Dharmacon Universal Buffer (0.1 M KCl, 0.03M HEPES, 1 mM MgCl₂, 0.04 M KOH) to give a final concentration of 20 μ M then aliquoted under RNase free conditions and stored at -70°C until required.

	Fanca-siRNA-A	Fanca-siRNA-B	Scrambled-siRNA
Target Sequence	AAGCGGUGCAGG AUGUUAAGA	AAAGUUCGAGG AGUUACAAUC	AAGAAAUUUGG AAGGCGUCGG
Sense Sequence	GCGGUGCAGGAU GUUAAGAdTdT	AGUUCGAGGAG UUACAAUCdTdT	GAAAUUUGGAA GGCGUCGGdTdT
Antisense Sequence	UCUUAACAUCUCC GCACCGCdTdT	GAUUGUAACUCC UCGAACUdTdT	CCGACGCCUCC AAAUUUCdTdT
Base-pairs (bp)	1644 - 1663	2979 -2999	n/a

Table 2.10 DNA sequence of Fanca and scrambled siRNAs.

Sense and antisense sequence of siRNAs designed against mouse *Fanca*. Position of siRNA sequence, given in basepairs (bp), is relative to the start codon (ATG) for mouse *Fanca* (Accession N^o AF 178934).

2.8.2 Transfection of siRNAs into L β T2 cells using Fugene

L β T2 cells were transfected using Fugene according to the manufacturer's instructions. Cells were plated in T-75 flasks 24 h prior to transfection at 100,000 cells/ml. At least 2 h prior to transfection, media was removed and 5 ml fresh media added to the cells. The siRNA to be transfected and the Fugene reagent were diluted in Optimem serum free media (GibcoBRL). Optimem was pipetted to give a final transfection volume of 500 μ l per flask and was split over two 5ml culture tubes. An appropriate amount of siRNA was added to one tube containing Optimem to achieve a final concentration of either 200 or 400 nM in the T-75 flask. A 3 μ l aliquot of Fugene reagent was used per 1 μ g siRNA and added to a separate tube. The diluted siRNA was added to the Fugene/Optimem mix and the two solutions mixed by gentle tapping. After incubating at room temperature for 15 minutes, 500 μ l transfection mix was added to each T-75 flask to be transfected. Flasks were incubated at 37°C for 24 h then whole cell protein extracts were made, as described in Section 2.7.2.1.

2.8.3 Analysis of *Fanca* mRNA levels after siRNA treatment

Analysis of mRNA expression levels in cells transfected with siRNAs was performed using RT-PCR analysis as described in Section 2.1.3. Table 2.1 details the primers used for amplification of *Fanca*, *Zfp276* and *Gapdh*.

2.8.4 Analysis of *Fanca* protein levels after siRNA treatment

Western blotting of whole cell extracts from untreated cells and cells transfected with siRNA was performed as previously described (See Section 2.7). Blots were probed with *Fanca* antisera then stripped and re-probed with β -tubulin antisera to check for specific knockdown of *Fanca* protein. Results are expressed as arbitrary units of *Fanca* protein expression normalized against β -tubulin protein expression levels and are the mean of at least three separate experiments.

2.9 Construction of recombinant adenoviral vectors expressing *FANCA*

Recent advances in adenoviral technology have made it possible to clone a gene of interest into an adenoviral vector, propagate the virus in HEK293 cells then infect target cells to achieve efficient transfer of the gene and produce high levels of protein expression under standard laboratory conditions (Imperiale and Kochanek, 2004). BD-Adeno-X Expression Systems 2 with BD Creator Technology (Clontech) was used to clone *FANCA* into an adenoviral vector. This system uses *Cre-loxP* site-specific recombination to create recombinant adenoviral vectors. (See Figure 2.4).

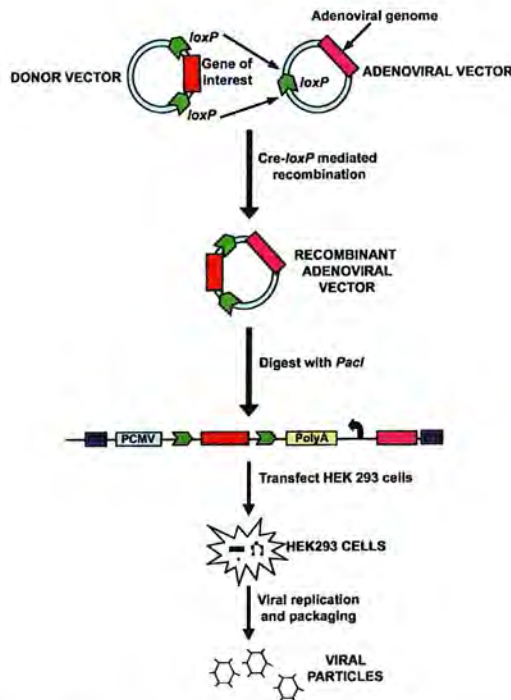


Figure 2.4 Construction of recombinant adenovirus with BD Creator Technology.

Cre-loxP mediated recombination allows the production of a recombinant adenoviral vector containing your gene of interest. Digestion with *PacI* linearizes the DNA and exposes the inverted terminal repeats (ITR) allowing viral replication when transfected into HEK293 cells. Viral particles can then be purified and used to infect target cell lines.

2.9.1 Materials and solutions

Sucrose/Chloramphenicol/LB plates
(7% sucrose, 30 $\mu\text{g}/\text{ml}$
chloramphenicol)

70 g sucrose (BDH) was dissolved in 1 litre of dH₂O. 31 pellets of LB-Agar (Q-Biogene) were added to this and the solution autoclaved. To pour the plates, the agar was melted in the microwave, 1 ml chloramphenicol (30 mg/ml) added and the agar poured into Petri dishes

Buffer 1 (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose)

2.5 ml 1 M Tris-HCl pH 8.0, 2 ml 0.5 M EDTA and 0.9 g glucose were mixed together in 100 ml of dH₂O

Buffer 2 (0.2 M NaOH, 1% SDS)

0.8 g NaOH and 1 g SDS were dissolved in 100 ml dH₂O

Buffer 3 (5 M KOAc pH 4.8)

24.9 g potassium acetate was dissolved in 40 ml dH₂O. The pH was adjusted to 4.8 by adding ~ 50 ml acetic acid then made up to 100 ml with dH₂O

Buffer 4 (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 20 $\mu\text{g}/\text{ml}$ RNase)

500 μl 1 M Tris-HCl pH 8.0 and 100 μl 0.5M EDTA were diluted in 50 ml dH₂O. RNase was added to a final concentration of 20 $\mu\text{g}/\text{ml}$

2.9.2 Cloning FANCA into 'donor' vector

FANCA insert was liberated from pEGFP-FANCA by digestion with *NheI* as described in Section 2.3.7. Donor vector (pDNR-CMV) was linearized by digestion with *XbaI* and SAP treated to prevent self re-ligation. The 6.2 kb FANCA insert with *NheI* overhangs was then ligated into the pDNR-CMV compatible *XbaI* sites to produce pDNR-CMV-FANCA. Ligations were transformed into *E.coli* as described

in Section 2.2.2, plated onto LB-Agar/Amp plates and grown overnight at 37°C. The following day, plasmid DNA was prepared from single colonies as described in 2.9.4 and diagnostic restriction digests performed using *EcoRI* to check for correct orientation of the insert (See Section 2.3.5).

2.9.3 Cloning of FANCA into adenoviral 'acceptor' vector

In an eppendorf, 1 µl pDNR-CMV-FANCA (200 ng/µl), 1 µl *Cre Recombinase* and 18 µl of BD Adeno-X-LP Reaction Mix were mixed together by gentle tapping. After incubating at room temperature for exactly 15 minutes the reaction was stopped by heating to 70°C for 5 minutes and 2 µl was then transformed into SURE competent cells (Stratagene) as described in Section 2.2.2. A 150 µl aliquot of the transformation reaction was plated out onto LB-Agar/sucrose/chloramphenicol plates and the plates incubated at 37°C for 24-32 h.

2.9.4 Small scale preparation of recombinant adenoviral vectors

pLP-Adeno-X is a large vector (34 kb) that is susceptible to damage and rearrangement. Cultures were not left at room temperature or 4°C for >24 h before starting purification of DNA or inoculating a second culture. To ensure strict propagation of recombinant clones, colonies were grown overnight at 37°C in LB containing both ampicillin (50 µg/ml) and chloramphenicol (30 µg/ml). The following day, cultures were centrifuged at 10,000 rpm for 1 minute and the pellet resuspended in 150 µl buffer 1. A 150 µl aliquot of buffer 2 was added, the solution mixed gently and incubated at room temperature for 5 minutes before adding 150 µl of buffer 3, mixing gently and incubating on ice for a further 5 minutes. This mix was then centrifuged at 14,000 rpm for 5 minutes at 4°C and the supernatant removed to a fresh eppendorf tube. To this, 450 µl phenol:chloroform (50:50) was added, the solution carefully mixed and then centrifuged at 14,000 rpm for 5 minutes at 4°C. The top layer was removed to a fresh eppendorf and 1 ml of 100% EtOH added to precipitate the DNA. DNA was pelleted by centrifugation at 14,000 rpm for 10 minutes at 4°C and the pellet was washed in 1 ml of 70% EtOH. The DNA

pellet was dried then resuspended in 20 μ l buffer 4 and incubated at 37°C for 10 minutes. The adenoviral DNA was stored at -20°C until required.

2.9.5 Confirmation of positive clones by PCR

E. coli clones containing recombinant pLP-Adeno-X Viral DNA were identified by PCR using the BD Adeno-X LP Primer Mix (Clontech). A diagnostic PCR fragment of 660 bp indicates the gene fragment has cloned successfully, whilst a 296 bp fragment indicates failure to insert the gene of interest.

To perform the PCR the following components were mixed together:

1 μ l	DNA
1 μ l	BD-Adeno-X LP Primer Mix
8 μ l	dH ₂ O
10 μ l	Abgene High Fidelity Master Mix <i>Taq</i>

30 cycles of PCR amplification were performed under the following conditions:

Initial denaturation	95°C	2 minutes	} 30 cycles
Denaturation	94°C	1 minute	
Specific primer annealing temperature	64°C	1 minute	
Extension	72°C	1 minute	
Final extension	72°C	10 minutes	

PCR products were electrophoresed on a 1% agarose gel and visualised by UV light.

2.9.6 Large scale preparation of recombinant adenoviral vectors

Once a positive clone had been identified by PCR, the clone was amplified by inoculating 100 ml of LB-Broth containing ampicillin (50 μ g/ml) and chloramphenicol (30 μ g/ml) with 2-5 ml of fresh log-phase culture. This culture was incubated at 37°C until it reached log-phase then plasmid purified using the NucleoBond Plasmid Midi Kit (Clontech) according to the manufacturer's instructions (Low copy plasmid purification method). The identity of the purified plasmid was then re-confirmed by PCR as described in Section 2.9.5.

2.9.7 Producing recombinant adenovirus

2.9.7.1 *PacI* digestion of recombinant plasmid

Before the recombinant adenoviral DNA can be packaged, the plasmid must be digested with *PacI* to expose the inverted tandem repeats (ITRs) located at either end of the adenoviral genome. The ITRs contain the origins of adenovirus DNA replication and must be positioned at the ends of the linear adenovirus DNA molecule to allow formation of the replication complex (Tamanoi and Stillman, 1982).

The following components were mixed together in an eppendorf tube and incubated at 37°C for 2 h:

Sterile H ₂ O	20 µl
Recombinant adenoviral plasmid DNA (500 ng/µl)	10 µl
10 x <i>PacI</i> digestion buffer	4 µl
10 x BSA	4 µl
<i>PacI</i> restriction enzyme (1 unit/µl)	2 µl

To this, 60 µl of 1 x TE buffer and 100 µl phenol:chloroform (50:50) were added and the contents gently mixed. Following centrifugation at 14,000 rpm for 5 minutes at 4°C the top, aqueous layer was transferred to a clean eppendorf and 400 µl of 95% EtOH, 1/10th volume of 3 M NaAc (pH 5.2) and 1 µl glycogen (20 mg/ml) added. Contents were mixed gently then centrifuged at 14,000 rpm for 5 minutes at 4°C. The supernatant was removed and the digested DNA pellet washed with 300 µl of 75% EtOH then spun at 14,000 rpm for 2 minutes. The pellet was dried and the DNA resuspended in 10 µl 1 x TE buffer.

2.9.7.2 Transfecting HEK293 cells with recombinant adenoviral DNA

HEK293 cells were plated in T-25 culture flasks (Costar) 24 h prior to transfection at a density of 100,000 cells/ml. Each T-25 flask can be transfected with 10 µl of *PacI* digested adenoviral DNA. At least 2 h prior to transfection, media was removed and

5 ml of fresh media added to the cells. In one 5 ml culture tube, the resuspended *PacI* digested DNA (10 μ l) was diluted in 230 μ l Optimem serum free media whilst in a separate tube 30 μ l of Fugene transfection reagent (6 μ l per μ g DNA) was diluted in 230 μ l Optimem. The DNA/Optimem mix was added to the Fugene/Optimem mix and the two solutions mixed by gently tapping. After incubating at room temperature for 15 minutes, 500 μ l of the transfection was added to a T-25 flask and incubated at 37°C until cytopathic effect (CPE, rounding up of cells and detachment from the plate) was visible.

2.9.7.3 Amplification and preparation of viral stock

Once CPE was visible, a process which may take up to two weeks, amplification was achieved by manually lysing cells to obtain virus then infecting new cells. Cells were transferred to a 15 ml tube (Costar) and centrifuged at 1,500 rpm for 5 minutes at room temperature. The cell pellet was resuspended in 500 μ l sterile PBS and transferred to an eppendorf tube. Cells were lysed by three consecutive freeze-thaw cycles using dry ice. After the third cycle, cell debris was pelleted by centrifugation at 5,000 rpm for 1 min and the lysate transferred to an eppendorf tube. A 250 μ l aliquot of lysate was then used to infect a T-25 flask containing HEK293 cells (100,000/ml) by adding the lysate directly to the medium then incubating as normal, the remaining lysate can be stored at -20°C. CPE should now become evident within one week. When >50% of the infected cells have detached from the growing surface, viral stock was produced by lysing the cells by three consecutive freeze-thaw cycles as before. This primary amplification stock was stored at -20°C until required.

2.9.8 Plaque assay to determine adenoviral titer

HEK293 cells were plated in 6-well plates (Costar) 24 h prior to transfection at a density of 100,000 cells/ml. Using sterile PBS a serial dilution of viral stock was made (1 x 10⁻⁵ to 1 x 10⁻¹⁰). Media was removed from the wells and 250 μ l of diluted virus added to the cells. To control for errors, each viral dilution was added

to 3 wells and the plates gently tipped to spread the virus evenly over the cells. The plates were covered and incubated at 37°C for 1 h to allow the virus to infect the cells, then the virus was removed and 3 ml of 0.5% agarose/PBS solution was added to each well. When the agarose had set, the plates were returned to the incubator for 7 days. To visualize plaque formation, 1 ml of 0.03% neutral red (Sigma)/PBS solution was added to each well and plates incubated at 37°C for 3 h. The stain was then removed and the clear plaques in each well, visible against a pink background, counted. To calculate the viral titer (plaque forming units [pfu]/ml) the following formula is used:

$$\text{pfu/ml} = \frac{\text{\# plaques}}{(\text{dilution factor}) \times (\text{volume of diluted virus added to each well})}$$

This is a quantitative measurement of the biological activity of the recombinant virus.

Chapter 3

Regulation of Fancx expression by GnRH

Science never solves a problem without creating ten more – George Bernard Shaw.

3 Regulation of *Fanca* expression by GnRH

3.1 Introduction

3.1.1 Background

Fertility, and hence the ability to reproduce, is regulated by the action of the gonadotropin hormones luteinising hormone (LH) and follicle stimulating hormone (FSH) on the testes and ovaries. Their biosynthesis and release is controlled by the signalling of GnRH through its cognate receptor on the surface of pituitary gonadotroph cells. As gonadotroph cells only comprise 10-15% of the anterior pituitary, the development of two transformed gonadotroph cell lines, α T3-1 and L β T2 cells (Windle et al., 1990; Turgeon et al., 1996), has provided an enriched source of cell-specific material with which to study GnRH regulation of gonadotropin gene transcription.

Immortalized gonadotroph cell lines were derived from pituitary tumours isolated from transgenic mice carrying fusion genes of the SV40 T-antigen oncogene linked to 5' flanking sequence of either the human *α GSU* or rat *Lh β* genes. As *α GSU* is expressed in gonadotroph and thyrotroph cells of the anterior pituitary, and *LH β* expression is restricted to terminally differentiated gonadotroph cells (Japon et al., 1994), the aim was to develop spatially and temporally specific cell lines. Targeted oncogenesis with the *α GSU* promoter produced a primitive gonadotroph cell line (α T3-1), representative of mouse embryonic day (E) 13.5, that expresses *α Gsu* and *Gnrhr* but not *Lh β* or *Fsh β* (Windle et al., 1990; Alarid et al., 1996). Targeted oncogenesis with the *Lh β* subunit promoter produced a more mature gonadotroph cell line (L β T2), representative of an E16.5 mouse pituitary, that expresses *α Gsu*, *Lh β* , *Fsh β* and *Gnrhr* (Turgeon et al., 1996; Graham et al., 1999).

These cell lines have aided dissection of the GnRH receptor activated intracellular pathways, allowing analysis of the mechanisms involved in gonadotropin synthesis and secretion and the identification of a role for MAPK signalling cascades in GnRH regulation of gonadotropin gene transcription (Naor et al., 2000; Yokoi et al., 2000; Harris et al., 2002; Liu et al., 2002; Bonfil et al., 2004; Coss et al., 2004). To characterise the mechanisms involved in GnRH regulation of gonadotropin subunit transcription, the mouse L β T2 gonadotroph cell line has previously been used as a model to investigate GnRH regulation of gene expression. Differential display (DD) RT-PCR was performed on RNA extracted from untreated and GnRH treated L β T2 cells in order to identify and isolate hormonally regulated transcripts (Chang, 2002).

Differential display involves the linear amplification of partial cDNA sequences from mRNAs by RT-PCR, thus the complexity of the cDNA population is representative of the complexity of the mRNA population from which it was derived (Liang and Pardee, 1992). Unlike other techniques, where success is biased by relative mRNA abundances, differential display detects both abundant and rare mRNAs providing the arbitrary primer sequence used matches the target mRNA. To identify which gonadotroph cell mRNA transcripts were regulated by GnRH, DD-RT-PCR was performed on RNA harvested from L β T2 cells that were either left untreated or treated with one 15 minute pulse of 1 μ M GnRH and harvested for RNA 1, 2 or 4 h later (Chang, 2002). Several hormonally regulated transcripts were identified and one of these showed a rapid increase in expression levels 1 h after GnRH treatment with expression returning to untreated levels after 4 h. This product was isolated, amplified by PCR, cloned, sequenced and matched, using bioinformatic analysis, to exon 43 of *Fanconi anaemia complementation group a (Fanca)*. See Figure 3.1.

	R5 primer	
	AGGACCCTGG	
Fanca cDNA 4264	<u>GTGACCCTGAGGTGAGCAACGCCCTCCGGCAGAGGCAGCAGGCTGACCCGACTTTGACC</u>	4324
	<u>TCTACCAGGAGCCTCAGCTGTTCTGACTGCACCCATCTCAGCGCACCAGCCGGCACCTTT</u>	4384
	CCAGGGAGGA R5(rev) primer	
	<u>GTAATAATTTATGCCAGCAGGACATGGGGCTCTTGTACTACTAGAAAATAGATTACCA</u>	4444
	ATCTCCTTGATTGCTCTAGTTAGGAAAAGAAATATTAATTATTCATAAAAAAAAAAAAAAAAAAAGC	4510bp
	CCTTTTTTTTTTTT PolyA+ site	
	T ₁₂ VC	

Figure 3.1 Alignment of the 74 bp cloned DD-RT-PCR product and mouse *Fanca* cDNA nucleotide sequence.

Bioinformatic line-up depicting the region of homology, shown underlined, between the cloned *Fanca* DD-RT-PCR product and mouse *Fanca* cDNA nucleotide sequence. The likely internal R5 arbitrary primer site is indicated as well as the location of the R5 and T₁₂VC primers that generated the original 216 bp DD-RT-PCR product.

FANCA is mutated in >60% of cases of Fanconi anaemia (FA), a rare, autosomal recessive, chromosomal instability syndrome (OMIM 227650). The disease is classified as a DNA damage repair disorder since patients have a marked predisposition to cancer and suffer from progressive bone marrow failure (Buchwald and Moustacchi, 1998). Murine *Fanca* shares 74% nucleotide sequence identity with human FANCA (van de Vrugt et al., 2000; Wong et al., 2000). At the same time, a novel penta zinc finger protein (*Zfp276*) was also identified at the *Fanca* locus that is transcribed in an antisense orientation and overlaps with *Fanca* (Wong et al., 2000). *Fanca* has an ORF of 4503 bp comprised of 43 exons and encoding a protein of ~160 kD. The function of FANCA protein is largely unknown and the uniqueness of the molecule is reflected by the designation of a new protein domain, Fanconia (PFAM:PF03511), located at the C-terminus of the protein. The discovery, using DD-RT-PCR, that GnRH regulates *Fanca* expression demonstrates the non-user bias of this technique in the identification of genes whose expression has not previously been associated with hormonal regulation. Microarray technology has also been used to identify transcripts regulated by GnRH in LβT2 and αT3-1 cells (Wurmbach

et al., 2001; Yuen et al., 2002). Although *Fanca* was not included on the arrays used in these experiments a rapid and transient increase in transcripts known to be regulated by GnRH, including the transcription factors *early growth factor1* (*Egr1*) and *c-Jun*, was detected over a wide range of GnRH concentrations (1 nM up to 1 μ M) indicating that although no novel transcripts were identified using this approach a detailed profile of transcripts known to be regulated by GnRH was obtained.

3.1.2 Aims of this Chapter

Previous work within the laboratory, using the technique of differential display RT-PCR (DD-RT-PCR), identified several transcripts, within L β T2 gonadotroph cells that were differentially expressed in response to GnRH (Chang, 2002). Bioinformatic analysis revealed that one of these transcripts, which showed a rapid and transient up-regulation in response to GnRH, matched sequence from a region of overlap between two genes, *Fanconi anaemia complementation group a* (*Fanca*) and *penta-zinc finger protein 276* (*Zfp276*), that are transcribed in opposite orientations, from the same locus, on mouse chromosome 8. This chapter aims to address whether it is the expression of *Fanca* or *Zfp276* that is altered within L β T2 gonadotroph cells in response to addition GnRH.

3.2 Results

3.2.1 *Fanca* mRNA is expressed in L β T2 cells

A transcript, corresponding to exon 43 of *Fanconi anaemia complementation group a gene* (*Fanca*), was identified as being regulated by GnRH in L β T2 gonadotroph cells using the technique of differential display (DD) RT-PCR (Chang, 2002). However, following publication of a manuscript detailing an antisense transcript, *Zinc finger protein 276* (*Zfp276*), that overlaps with the 3' end of *Fanca* and could therefore also be the DD-RT-PCR clone, further experimental analysis of the expression of both transcripts within L β T2 cells was required. Figure 3.2 shows a schematic representation of the *Fanca* locus indicating the overlap between the two genes and the location of the DD-RT-PCR clone. To confirm expression of full-length *Fanca* mRNA within L β T2 gonadotroph cells northern blotting analysis was performed. Total RNA was extracted from L β T2 cells, fractionated on a formaldehyde gel, blotted and probed with either 5' or 3' fragments of mouse *Fanca* cDNA as described in Section 2.6.3.2. The 5' *Fanca* probe used corresponded to 1-965 bp of cDNA encoding exons 1-11. The 3' probe used corresponded to exon 43 of *Fanca* cDNA and 1441-1949 bp of *Zfp276* cDNA. Analysis of the blots confirmed that a full-length 4.5 kb transcript, corresponding to *Fanca*, was detected with the 5' probe only (See Figure 3.3). As expected, the 3' probe detected two bands; a large band at 4.5 kb, corresponding to *Fanca* and a smaller, 3.1 kb, transcript corresponding to the reported size of full length *Zfp276* (Wong et al., 2000) (See Figure 3.3).

3.2.2 *Fanca* mRNA is expressed in adult mouse pituitary

In vivo expression of *Fanca* was determined by RT-PCR analysis of adult mouse pituitary tissue. RNA was extracted from the tissue; reverse transcribed and cDNA amplified using gene specific primers corresponding to exons 7 to 18, 14 to 18 or 30 to 32 of mouse *Fanca* cDNA (See Table 2.1 and Figure 3.4). PCR conditions and

RNA integrity were confirmed by RT-PCR of *Glyceraldehyde 3-phosphate dehydrogenase (Gapdh)*. Ethidium bromide staining identified bands at the expected sizes of 1075, 450 and 211 bp whilst DNA sequencing of the cloned PCR products established that *Fanca* is expressed in the pituitary of adult mice (See Figure 3.5).

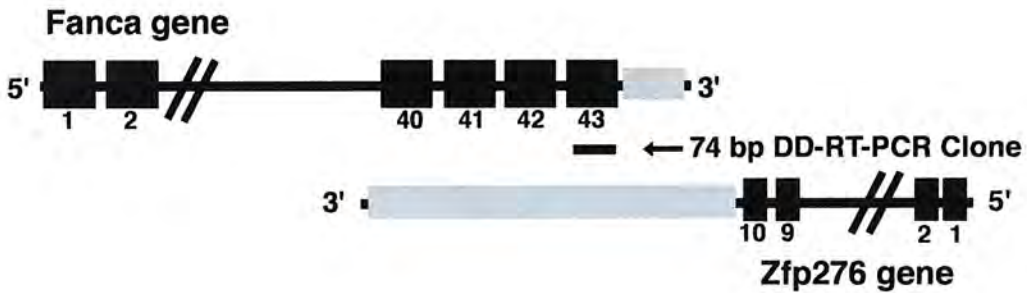


Figure 3.2 Schematic representation of the *Fanca* locus showing overlap between *Fanca* and *Zfp276*.

Exons 40-43 of *Fanca* (black boxes) overlap with the 3' untranslated region (UTR) of *Zfp276* (grey box). The location of the cloned 74 bp DD-RT-PCR clone is indicated with an arrow.

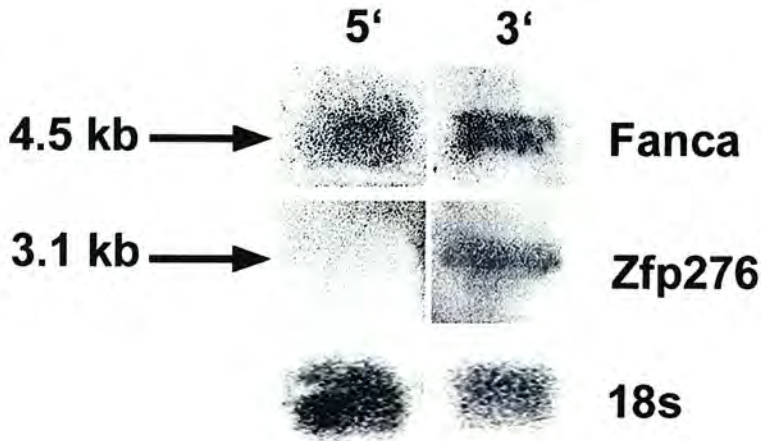


Figure 3.3 Expression of full-length *Fanca* and *Zfp276* mRNA transcripts in L β T2 cells.

Total RNA from L β T2 cells was fractionated on a formaldehyde gel, northern blotted and probed with radiolabelled probes corresponding to either exons 1-11 (5' probe) or exon 43 (3' probe) of mouse *Fanca* cDNA. The 5' probe detected one band at 4.5 kb corresponding to the size of full-length *Fanca* transcript. The 3' probe detected two bands, a *Fanca* transcript at 4.5 kb and a 3.1 kb transcript corresponding to *Zfp276*. The blot was then stripped and re-probed with an 18s probe as a control.

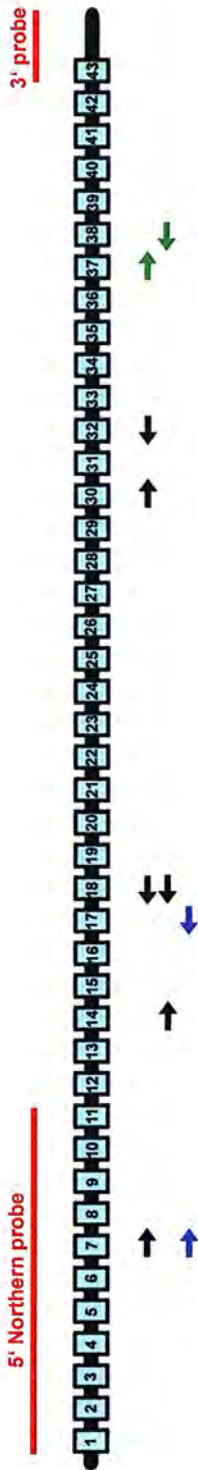


Figure 3.4 Location of northern probes and PCR primers relative to *Fanca* genomic structure.

Exons 1 – 43 are represented as blue boxes. The location of the 5' and 3' probes using during northern blotting experiments are indicated by red lines (See Section 3.2.1). Black arrows indicate the location of PCR primers used for *in vivo* and *in vitro* expression analysis of *Fanca* (See Section 3.2.2 and 3.2.3). Blue arrows indicate the location of PCR primers used for semi-quantitative RT-PCR analysis of *Fanca* expression in L β T2 cells (See Section 3.2.6). Green arrows indicate the location of PCR primers used for LightCycler quantitative RT-PCR analysis (See Section 3.2.7).

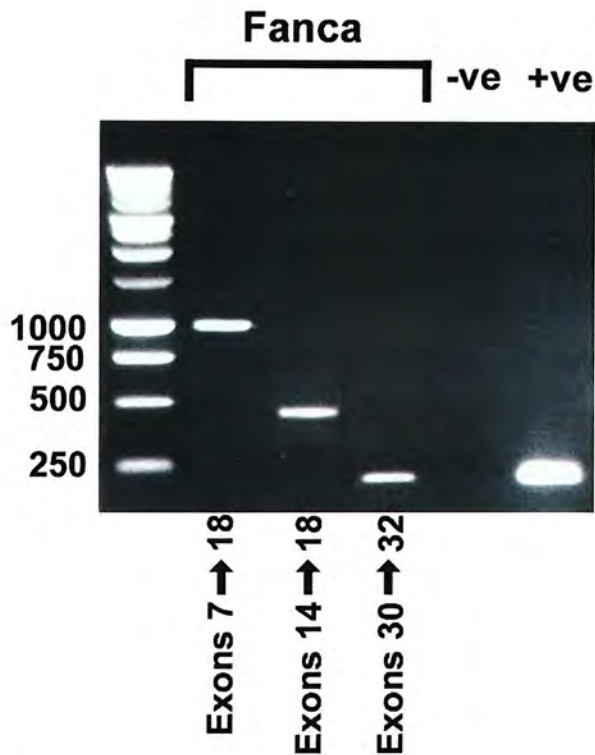


Figure 3.5 *Fanca* in vivo expression analysis.

RT-PCR analysis of RNA extracted from adult mouse pituitary was performed using primers that amplified exons 7-18, 14-18 or 30-32 of mouse *Fanca*. A negative control containing no cDNA was included (-ve). The integrity of pituitary RNA was confirmed by amplification of *Gapdh* (+ve). Ethidium bromide staining identified PCR products of the expected size, which were confirmed as *Fanca* by DNA sequencing.

3.2.3 *Fanca* mRNA is not expressed in immature pituitary gonadotrophs

Having confirmed expression of *Fanca* in adult pituitaries and mature gonadotroph cells, further analysis was carried out to determine if *Fanca* was expressed in α T3-1 cells, an immature gonadotroph cell line. *Fanca* expression was also analysed in a rat somatotroph cell line (GH3 cells); a mouse fibroblast-like cell line (L-cells) and a human cervical cancer cell line (HeLa cells). RNA was extracted from cultured cells; reverse transcribed and cDNA amplified using gene specific primers corresponding to exons 14 to 18 of mouse *Fanca* cDNA (See Table 2.1 and Figure 3.4). PCR conditions and RNA integrity were confirmed by RT-PCR of *Zfp276* using primers designed to *Zfp276* mouse cDNA sequence. As expected, ethidium bromide staining identified DNA bands at the predicted size of 450 bp in L β T2 cells. *Fanca* mRNA expression was also detected in GH3, HeLa and L-cells (Figure 3.6). DNA sequencing of cloned RT-PCR products confirmed amplification of *Fanca* from these cell lines. Interestingly no *Fanca* expression was detectable when amplifying α T3-1 cDNA although *Zfp276* was determined to be present (Figure 3.6). To analyse the lack of *Fanca* expression in α T3-1 cells further, exons 7-18 of mouse *Fanca* cDNA were amplified from L-cell and α T3-1 cDNA, blotted onto Hybond-N⁺ and hybridised with a radiolabelled probe corresponding to exons 1-11 of mouse *Fanca* (as described in Section 2.3.10.1). Exposure of the hybridised blot to x-ray film revealed that a *Fanca* specific band of 1075 bp was amplified from L-cell cDNA only. No *Fanca* expression was detected by Southern blot analysis of α T3-1 amplified cDNA. (See Figure 3.7)

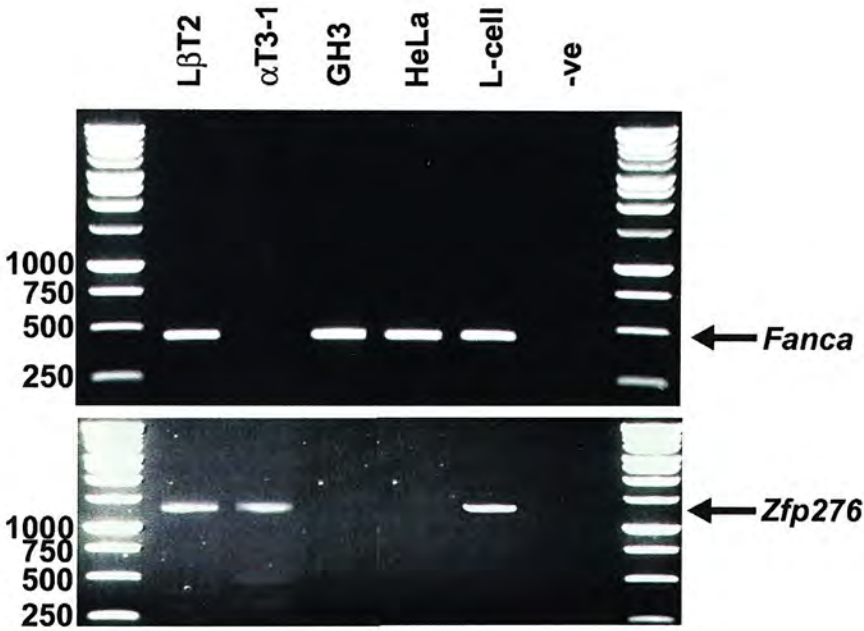


Figure 3.6 *Fanca* in vitro expression analysis.

RNA was extracted from L β T2, α T3-1, GH3, HeLa and L-cells, reverse transcribed and first strand cDNA analysed for expression of mouse *Fanca* using primers corresponding to exons 14-18. Ethidium bromide staining identified an expected DNA band of 450 bp in L β T2, GH3, HeLa and L-cells. No *Fanca* expression was detectable when amplifying α T3-1 cDNA. The integrity of the RNA samples was confirmed by amplification of *Zfp276* using primers matching mouse cDNA sequence. A specific 1238 bp PCR product, corresponding to *Zfp276*, was amplified from L β T2, α T3-1, GH3 and L-cell cDNA.

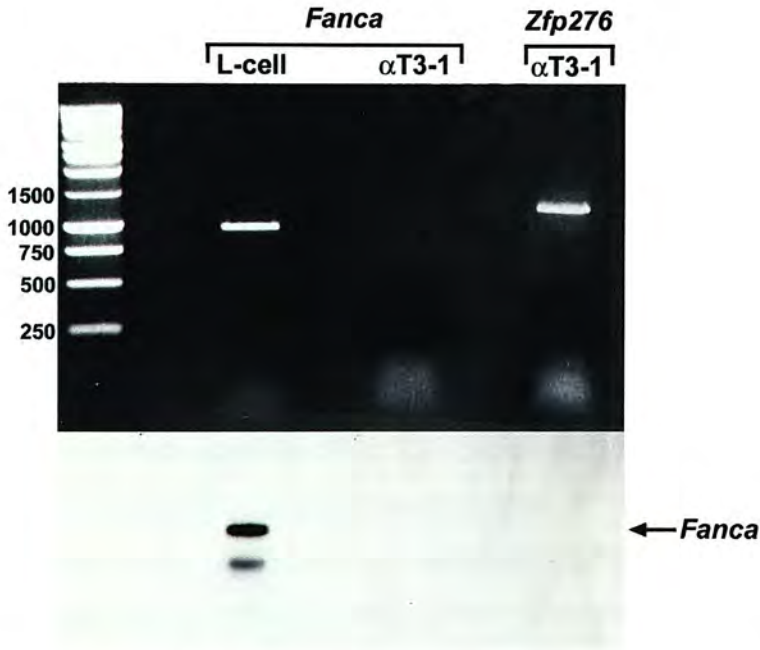


Figure 3.7 Southern blotting analysis of *Fanca* expression in α T3-1 cells.

RT-PCR analysis of RNA extracted from α T3-1 and L-cell was performed using primers that amplified exons 7-18 of mouse *Fanca*. Ethidium bromide staining identified an expected DNA band of 1075 bp from L-cell cDNA. No *Fanca* expression was detectable when amplifying α T3-1 cDNA but the integrity of α T3-1 RNA was confirmed by amplification of mouse *Zfp276*. Southern blotting analysis of the agarose gel with a 5' *Fanca* probe, corresponding to exons 1-11 of mouse *Fanca* cDNA, confirmed that the *Fanca* PCR product was specific.

3.2.4 *Zfp276* mRNA is not regulated by GnRH

Although it appears highly unlikely that the *Zfp276* transcript at the same locus as *Fanca* was the transcript identified by DD-RT-PCR RT-PCR was performed to determine if *Zfp276* mRNA expression levels changed in response to GnRH. RNA was extracted from cultured L β T2 cells that had been left untreated (0 h) or treated with GnRH for 1, 2 or 4 hours. This was reverse transcribed and cDNA amplified using gene specific primers corresponding to full-length mouse *Zfp276*. Ethidium bromide staining identified a DNA band at the expected size of 1238 bp at all time points suggesting no difference in expression levels of *Zfp276* mRNA in untreated and treated cells (Figure 3.8).

3.2.5 An alternative *Zfp276* transcript is expressed in L β T2 cells

The 1238 bp PCR products amplified from L β T2 and L-cell cDNA were cloned into TOPO-pCRII and sequenced to confirm amplification of *Zfp276*. Analysis of the sequence data identified the presence of two amplified *Zfp276* PCR clones (Clone A and Clone B) from both L-cell and L β T2 cDNA. Sequence from Clone A was identical to mouse *Zfp276* nucleotide sequence (Accession N^o AF178935). Clone B was also identical to mouse *Zfp276* sequence but contained a 70 bp insertion of genomic sequence 849 bp downstream of the start codon (See Figure 3.9). Analysis of the predicted protein products of these transcripts revealed that the 70 bp insertion introduces several in-frame premature STOP codons into the predicted *Zfp276* amino acid sequence presumably resulting in a truncated *Zfp276* protein (See Figure 3.9).

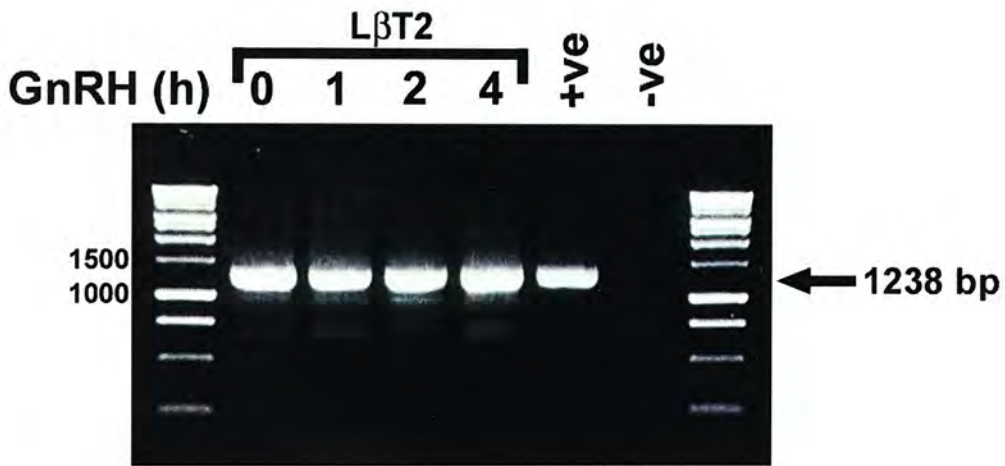


Figure 3.8 RT-PCR analysis of *Zfp276* mRNA expression in L β T2 cells.

L β T2 cells were left untreated (0) or treated with GnRH then harvested 1, 2 or 4 h later. RNA was extracted, reverse transcribed and first strand cDNA analysed for expression of mouse *Zfp276*. Ethidium bromide staining identified an expected DNA band at 1238 bp in all RNA samples. *Zfp276* was also amplified from L-cell cDNA as a positive control (+ve). The 1238 bp *Zfp276* PCR product is indicated with an arrow.

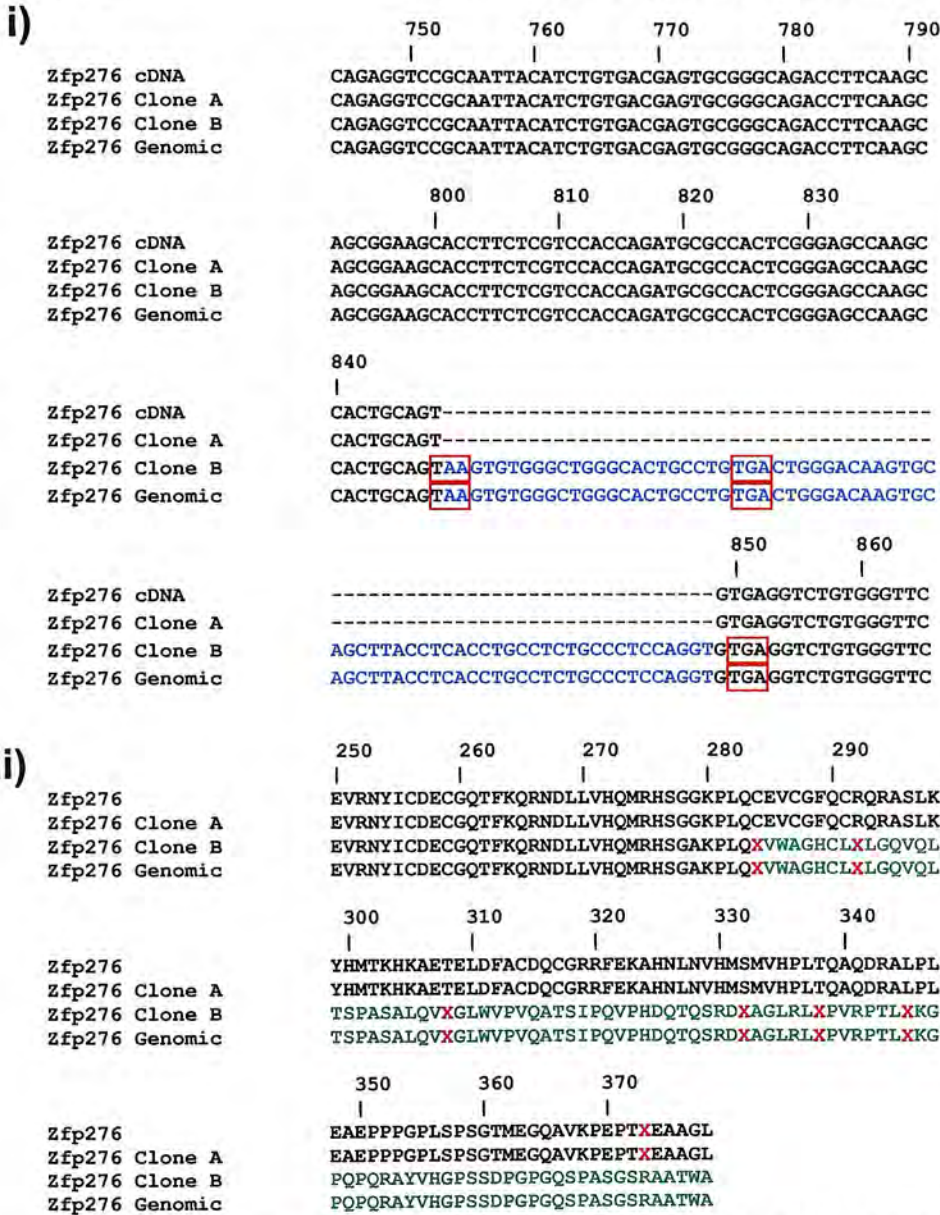


Figure 3.9 Alignment of Zfp276 PCR Clone A and Zfp276 PCR Clone B nucleotide and predicted amino acid sequence.

(i) Bioinformatic line-up of the nucleotide sequence of Zfp276 Clone A and Zfp276 Clone B. The 70 bp insertion of genomic sequence in the Clone B is shown (blue text). The insertion alters the reading frame and results in the introduction of in-frame premature STOP codons (red boxes). Nucleotide positions are given relative to the start codon (ATG). *Zfp276* cDNA sequence is taken from sequence available in the GenBank database (Accession N° AF178935). (ii) Bioinformatic comparison showing the predicted amino acid sequence of the two Zfp276 PCR clones. The altered amino acid sequence of the Clone B is shown (green text). STOP codons are indicated with an X. Clone A encodes a predicted protein of 372 amino acids (aa), Clone B encodes a predicted protein of 283 aa.

3.2.6 *Fanca* mRNA levels are regulated by GnRH

RT-PCR was used to analyse expression of *Fanca* mRNA in untreated and GnRH treated L β T2 cells. RNA was extracted from cultured L β T2 cells that had been left untreated (0 h) or treated with 1 μ M GnRH for 1, 2 or 4 hours. This was reverse transcribed and cDNA amplified, for 30 PCR cycles, using gene specific primers corresponding to exons 7 to 17 of mouse *Fanca* cDNA (See Table 2.1 and Figure 3.4). Ethidium bromide staining identified a DNA band at the expected size of 979 bp in L β T2 cells harvested for RNA after 1 h of GnRH treatment (Figure 3.10) agreeing with DD-RT-PCR results showing that *Fanca* expression increases 1 h after treatment with GnRH (Chang, 2002). Specific amplification of a 979 bp *Fanca* PCR product was confirmed by subsequent Southern blotting analysis, which also identified lower levels of expression in untreated L β T2 cells and a rapid reduction in *Fanca* mRNA expression 2 and 4 h after GnRH treatment (Figure 3.10).

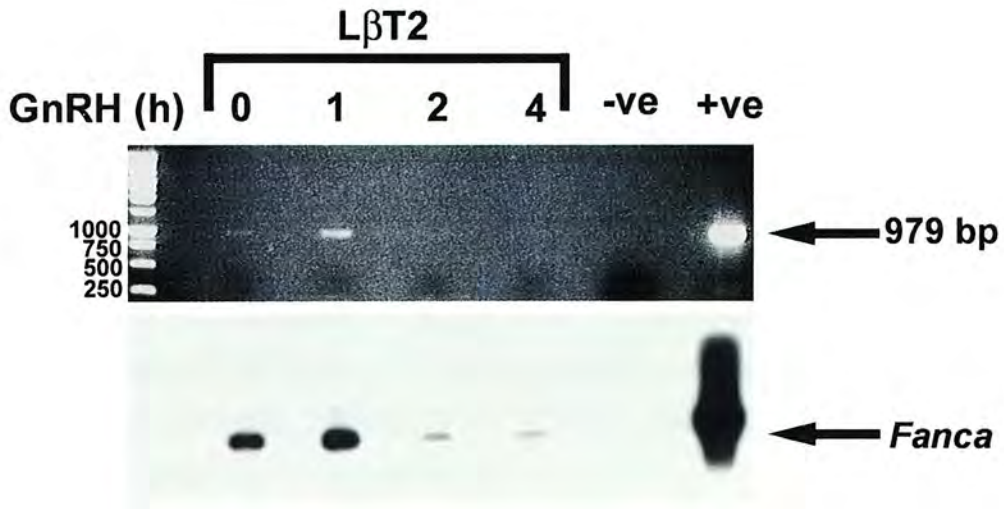


Figure 3.10 RT-PCR analysis of *Fanca* expression in L β T2 cells.

L β T2 cells were left untreated (0) or treated with GnRH then harvested 1, 2 or 4 h later. RNA was extracted, reverse transcribed and first strand cDNA analysed for expression of mouse *Fanca* using primers corresponding to exons 7-17. Ethidium bromide staining identified one PCR product amplified from mRNA harvested 1 h after treatment with GnRH. *Fanca* was amplified from L-cell cDNA as a positive control (+ve). The 979 bp *Fanca* PCR product is indicated with an arrow. Southern blotting analysis of the agarose gel with a 5' *Fanca* probe corresponding to exons 1-11 of mouse *Fanca* cDNA confirmed that the *Fanca* PCR product was specific and present at all time points.

3.2.7 *Fanca* mRNA levels increase 2-fold in response to GnRH

To accurately quantify the GnRH induced increase in *Fanca* mRNA, quantitative RT-PCR was performed on RNA extracted from L β T2 cells that had been left untreated or treated with 1 μ M GnRH then harvested 1, 2 or 4 h later. RNA was reverse transcribed and first strand cDNA analysed using a LightCycler quantitative RT-PCR machine (Roche) and primers corresponding to exons 37-38 of mouse *Fanca* and exon 1 of mouse β -2-microglobulin (*B2m*) (See Table 2.1 and Figure 3.4). *Fanca* mRNA expression levels were measured and normalized against levels of *B2m* mRNA within the same sample. The results were then determined as the fold increase in *Fanca* mRNA levels when compared to levels of *Fanca* mRNA in untreated L β T2 cells (See Figure 3.11). Analysis of the results revealed a significant 2-fold increase in *Fanca* mRNA levels 1 h after treatment with GnRH (ANOVA one-way analysis of variance, $p < 0.05$). *Fanca* mRNA expression had returned to unstimulated levels 4 h after treatment with GnRH corroborating the results seen from the semi-quantitative RT-PCR experiments.

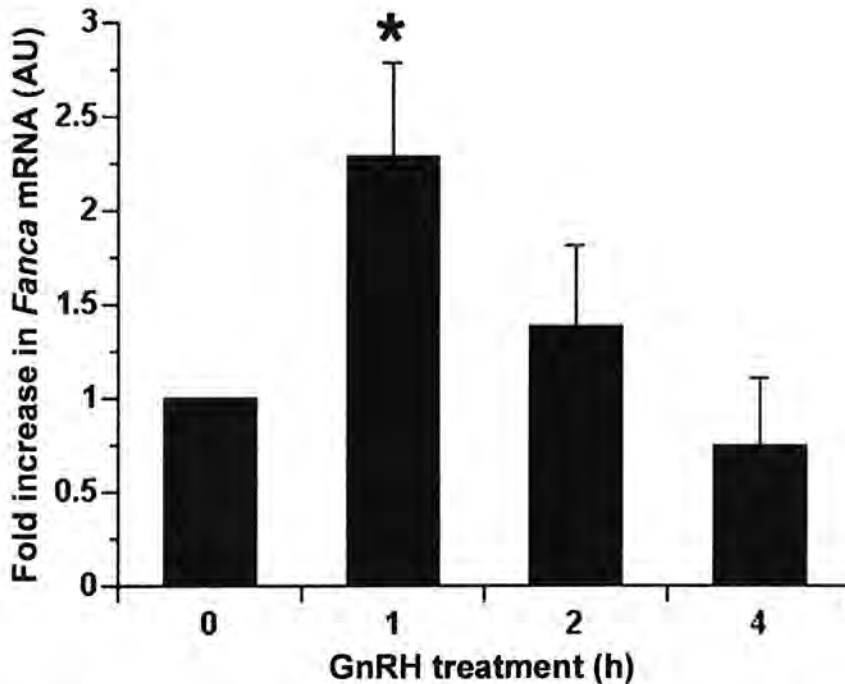


Figure 3.11 Quantitative RT-PCR analysis of *Fanca* mRNA levels after treatment with GnRH.

L β T2 cells were left untreated (0 h) or treated with GnRH then harvested 1, 2 or 4 h later. RNA was extracted, reverse transcribed and first strand cDNA analysed. LightCycler quantitative RT-PCR analysis of *Fanca* mRNA levels detected a consistent 2-fold increase in *Fanca* mRNA harvested 1 h after treatment with GnRH, with expression returning to untreated levels after 4 h of treatment. Results are shown as arbitrary units (AU) of *Fanca* mRNA normalized against levels of β -2-microglobulin (*B2m*). Each experiment was performed in duplicate and repeated three times. Error bars indicate the standard error of the mean. Statistical analysis was performed using ANOVA one-way analysis of variance (* $p < 0.05$).

3.2.8 *Fanca* protein levels increase 2-fold in response to GnRH

The rapid up-regulation of *Fanca* mRNA may not be mirrored by an increase in *Fanca* protein, so western blotting analysis was used to quantify *Fanca* protein levels in L β T2 cells in response to hormone. Whole cell protein extracts were made from untreated L β T2 cells and from L β T2 cells harvested 2, 4 and 6 h after addition of 1 μ M GnRH. Equal amounts of protein extract, as determined by Bradford assay, were then fractionated on a 6% SDS-PAGE gel, blotted and incubated with rabbit anti-mouse *Fanca* antisera specific for amino acids 1-276 [kind gift from Dr. Fré Arwert (Waisfisz et al., 1999)]. A specific protein band at ~160 kD, corresponding to the expected size of *Fanca* protein, was detected in all samples (See Figure 3.12). Blots were stripped, and then re-probed with a rabbit anti-mouse β -tubulin antibody to check for even loading of protein on to the gel (See Figure 3.12). Quantification of protein levels using ImageQuant software and normalization of *Fanca* protein levels against β -tubulin protein levels revealed that a significant 2-fold increase in *Fanca* protein levels occurred after addition of GnRH for 2 h (ANOVA one-way analysis of variance, $p < 0.001$). This 2-fold increase was still evident and highly significant 4 h after treatment with GnRH ($p < 0.001$), but by 6 h, *Fanca* protein levels had begun to return to unstimulated levels. (See Figure 3.12) This shows that the rapid increase in *Fanca* mRNA in response to GnRH is followed by a 2-4 h sustained increase in *Fanca* protein levels (See Figure 3.13).

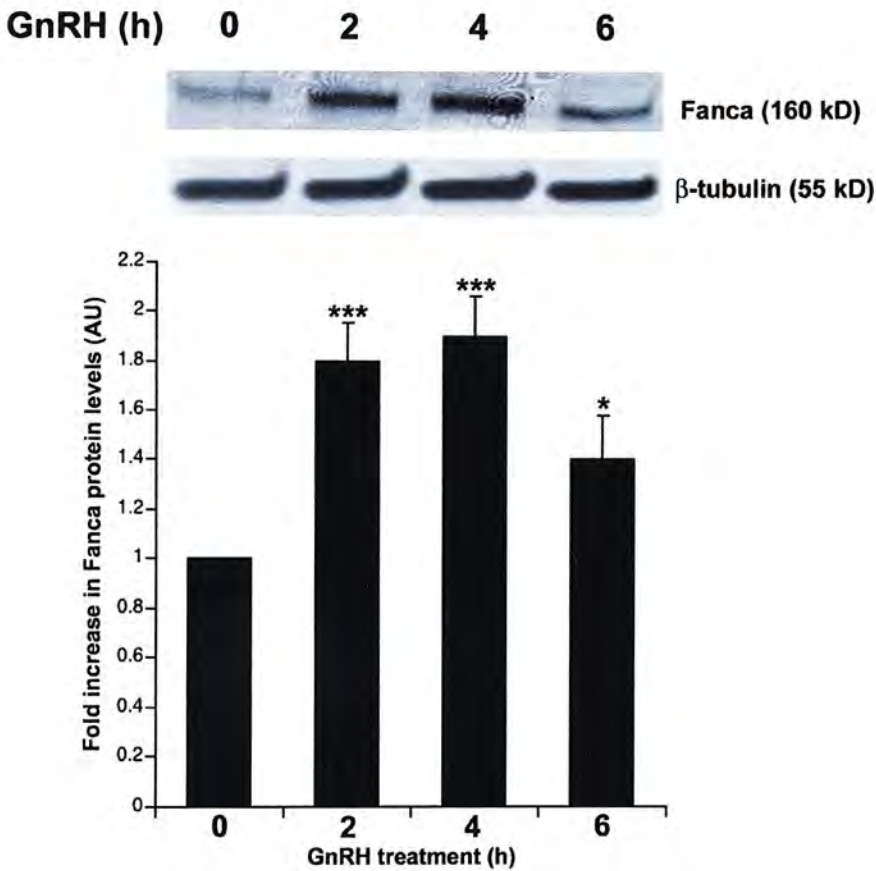


Figure 3.12 Western blotting analysis of Fanca protein levels after treatment with GnRH.

Western blotting analysis of whole cell L β T2 protein extracts either left untreated (0) or harvested 2, 4 or 6 h after treatment with GnRH. Protein was fractionated on an SDS-PAGE gel, blotted and probed with a rabbit anti-mouse Fanca antibody before being stripped and re-probed with a rabbit anti-mouse β -tubulin antibody. Probing with the Fanca antibody detected a protein band at the expected size of ~160 kD. Probing with a β -tubulin antibody gave a protein band at the expected size of ~55 kD. Normalization of Fanca protein levels against β -tubulin levels showed that Fanca protein increased 2-fold 2 h after treatment with GnRH with expression beginning to return to unstimulated levels 6 h after treatment with GnRH. Results shown are the mean of three separate experiments with error bars indicating the standard error of the mean. ANOVA one-way analysis of variance determined that the increase in Fanca protein levels following treatment with hormone was significant when compared against Fanca protein levels in untreated cells: ***, $p < 0.001$; *, $p < 0.05$.

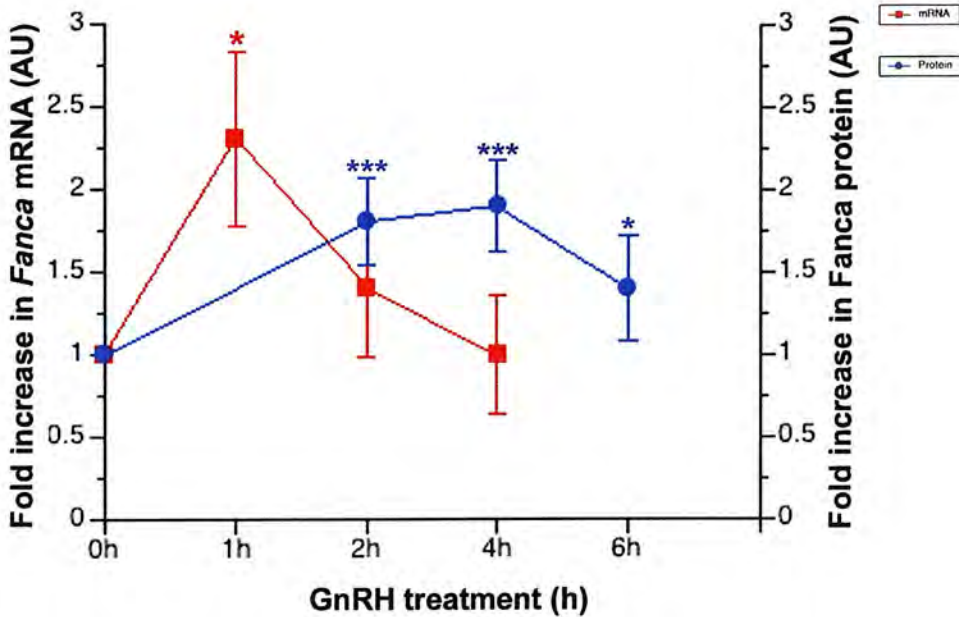


Figure 3.13 *Fanca* mRNA and protein levels increase after treatment with GnRH.

Fanca mRNA levels increase 2-fold 1 h after treatment with GnRH and have returned to untreated levels 4 h after treatment. *Fanca* protein levels also increase after treatment with GnRH, a 2-fold rise is evident 2-4 h after treatment with hormone. Expression levels begin to return to unstimulated levels 6 h after treatment with GnRH. Results are the mean of three separate experiments. Error bars represent the standard error of the mean. ANOVA one-way analysis of variance determined that the increases in *Fanca* mRNA and protein levels in response to hormone were significant: ***, $p < 0.001$, *, $p < 0.05$.

3.3 Discussion

3.3.1 *Fanca* mRNA is expressed in mature gonadotrophs

Previously, differential display (DD) RT-PCR identified that within L β T2 gonadotroph cells, GnRH differentially regulates a transcript corresponding to exon 43 of *Fanconi anaemia complementation group a* (*Fanca*) (Chang, 2002). Since an antisense transcript for *penta-zinc finger protein 276* (*Zfp276*) was recently identified at the same locus as *Fanca* (Wong et al., 2000), with 3' untranslated *Zfp276* sequence actually overlapping with exons 40-43 of *Fanca* (See Figure 3.14), it was conceivable that either *Fanca* or *Zfp276* could be the GnRH regulated transcript identified by differential display. Northern blotting analysis of total RNA from L β T2 cells, using probes designed to either 5' (exons 1-11) or 3' (exon 42-43) *Fanca* cDNA sequence, confirmed expression of both full length *Fanca* (4.5 kb) and *Zfp276* (3.1 kb) transcripts in gonadotroph cells (See Figure 3.3). However, detailed bioinformatic analysis of the likely differential display primer binding sites within *Fanca* and *Zfp276* sequence revealed that the original 216 bp DD-RT-PCR clone corresponded with the amplification of *Fanca*. The mRNA poly A⁺ sequence of *Zfp276*, and therefore likely oligo(dT) anchored priming site, maps a considerable distance downstream from the R5 internal priming site and a lack of any other suitable priming site within 216 bp of the R5 arbitrary primer indicated that, amplification of *Zfp276*, to produce the 216 bp differential display product, was highly unlikely (See Figure 3.14).

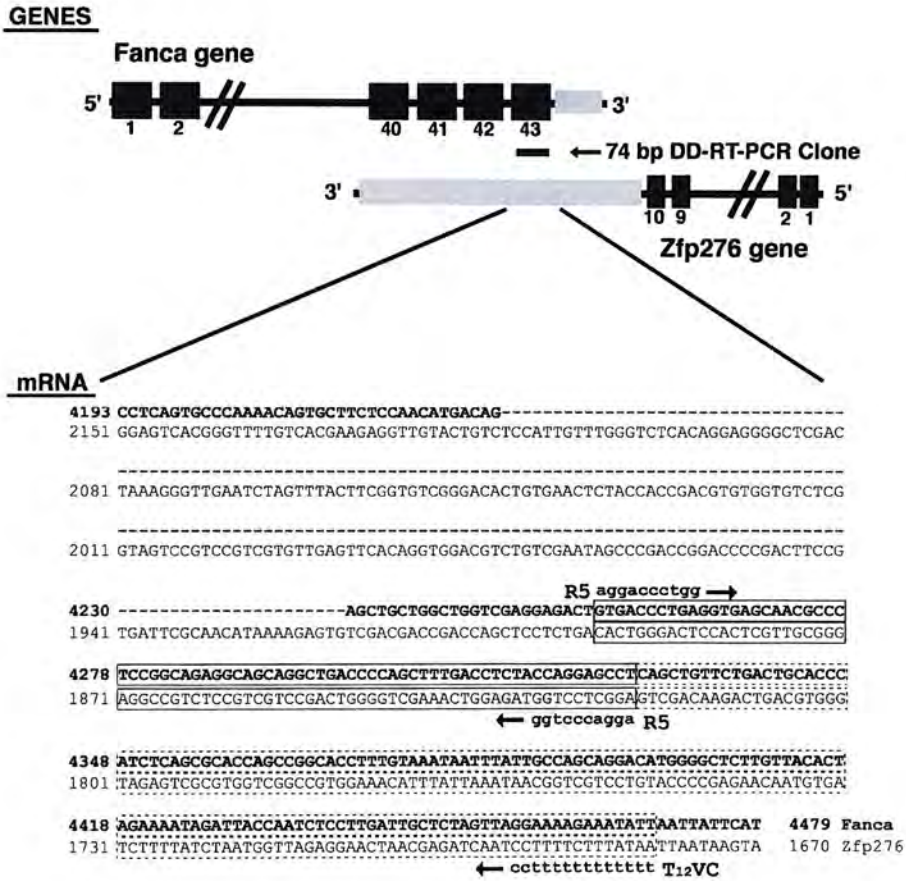


Figure 3.14 Schematic representation of the *Fanca* locus showing the location of the isolated differential display (DD) RT-PCR clone.

Exons 40-43 of *Fanca* (black boxes) overlap with the 3' untranslated region (UTR) of *Zfp276* (grey box). The location of the cloned 74 bp DD-RT-PCR clone is indicated with an arrow. The regions of nucleotide identity between *Fanca* and *Zfp276* mRNA have been aligned beneath the schematic. *Fanca* mRNA sequence is shown in bold type, spliced intronic sequence has been omitted and replaced with a dashed line. *Zfp276* mRNA sequence is in normal typeface. The amplified 74 bp DD-RT-PCR clone has been boxed with a solid line. The original 216 bp DD-RT-PCR clone is indicated as an extension of the boxed region with dashed lines. DD-RT-PCR primers, R5 (arbitrary primers) and T₁₂VC (oligo[dT] anchored primer) are also shown on the corresponding *Fanca* sequence. No suitable priming sites for the T₁₂VC primer were identified by bioinformatic analysis of *Zfp276* 3'UTR sequence.

Previously, expression analysis of *Fanca* using *in situ* hybridisation showed that the transcript is specifically expressed between embryonic day (E) 8.5 and E16 within the developing brain, liver and kidney as well as in the mesenchyme, primarily within cells that will give rise to the soft tissues of the fore and hind-limbs (Abu-Issa et al., 1999). Northern analysis revealed that a predominant 4.5 kb band, corresponding to full length *Fanca*, is expressed from E7.0 and maintained throughout embryogenesis (Abu-Issa et al., 1999). There are conflicting reports detailing the detection of *Fanca* expression in adult mouse tissue by northern blotting analysis. One report states that three *Fanca* transcripts of 4.5, 1.6 and 1.1 kb are expressed in a wide range of adult mouse tissues (Wong et al., 2000), however, another report was unable to detect *Fanca* expression in adult brain, heart, lung or liver (van de Vrugt et al., 2000). This chapter reports that only full length *Fanca* transcript (4.5 kb) is detected in total RNA harvested from L β T2 cells (Figure 3.3). However, as *Fanca* mRNA is expressed at very low levels alternative transcripts may be present and analysis of poly-A⁺ mRNA from L β T2 gonadotroph cells could prove more informative. These findings extend the expression profile of *Fanca* mRNA to include for the first time, L β T2 gonadotroph cells, a specialised endocrine cell line.

To establish that *Fanca* was expressed within the pituitary *in vivo*, RT-PCR analysis was performed on RNA extracted from adult mouse pituitary tissue. Gonadotroph cells only comprise 10-15 % of the adult anterior pituitary, and are not concentrated within a specific area therefore, analysis of the whole gland allows confirmation of *in vivo* expression of *Fanca* within adult mouse pituitary, rather than determining *Fanca* expression within specific cell types of the anterior pituitary. Several small regions of *Fanca* cDNA were amplified by PCR and analysed on an agarose gel to ensure that the 4.5 kb *Fanca* transcript was represented in full. DNA bands corresponding to exons 7-18, 14-18 and 30-32 of murine *Fanca* cDNA were detected by ethidium bromide staining and the specific amplification of *Fanca* confirmed by sequencing of the cloned PCR products (See Figure 3.5). *Fanca* expression was also

analysed in several mammalian cell lines, including a rat somatotroph cell line (GH3), a mouse fibroblast-like cell-line (L-cells) and a human cervical cancer cell line (HeLa cells). This assay failed to detect *Fanca* mRNA in α T3-1 cells by RT-PCR amplification of cDNA (See Figure 3.6 and 3.7). As discussed earlier, α T3-1 cells are an immature, GnRH-responsive gonadotroph cell line derived from E13.5 pituitaries that express α *Gsu* and *Gnrhr*, but not *Lh β* or *Fsh β* subunits (Windle et al., 1990). As *Fanca* is expressed in L β T2 gonadotroph cells and adult pituitary, a lack of detectable *Fanca* mRNA expression within immature α T3-1 cells indicates that the gene may be important for gonadotroph development and/or mature gonadotroph cell functions. Western blotting analysis of α T3-1 protein extracts could be performed to confirm the absence of *Fanca* expression within these immature cells. Furthermore, whole mount *in situ* hybridisation analysing the expression of *Fanca* in mouse pituitaries during various stages of development, and co-localisation of this expression with anterior pituitary cell markers (eg. *Lh β* , *Fsh β* , and *Gh*) could help identify which cell types within the anterior pituitary express *Fanca* as well as providing insight into the onset of *Fanca* expression in gonadotroph cells *in vivo*.

3.3.2 An alternative *Zfp276* transcript is expressed within L β T2 cells

Differential display RT-PCR analysis identified that *Fanca* mRNA is up-regulated in response to GnRH. The isolated differential display clone matched sequence from both *Fanca* and *Zfp276*, two genes located, in opposite orientations, at the *Fanca* locus. However, as discussed earlier, bioinformatic analysis of the cDNA sequence surrounding the DD-RT-PCR clone revealed that *Fanca*, and not *Zfp276*, was the transcript identified from the differential display gel (Figure 3.14). RT-PCR analysis of RNA extracted from untreated and hormone treated L β T2 cells confirmed that *Zfp276* mRNA was expressed at all time points and indicated its expression did not appear to be regulated by GnRH (Figure 3.8). Interestingly, when the amplified PCR products were cloned and sequenced, a previously unreported alternative *Zfp276* transcript was identified in both L β T2 and L-cells.

Murine *Zfp276* cDNA is 3123 bp long with a reported open reading frame of 1118 bp comprising 10 exons and encoding a predicted protein of 372 amino acids (Wong et al., 2000). Bioinformatic analysis of the amino acid sequence predicts the presence of five C₂H₂ zinc finger domains (See Figure 3.15), a nucleic acid binding domain first identified in the *Xenopus* transcription factor TFIIIA (Miller et al., 1985a). These domains are composed of 25-30 amino acid residues including two cysteine and two histidine residues in a C-X₂-C-X₁₂-H-X₃-H type motif. One zinc atom binds to the conserved cysteine and histidine residues to yield a finger-like projection, which interacts with both RNA and DNA (el-Baradi and Pieler, 1991; Ladomery and Dellaire, 2002). Using the Simple Modular Architecture Research Tool (SMART, available at <http://www.smart-embl-heidelberg.de>), analysis also predicted the presence of a possible plant homeodomain (PHD) motif at amino acids 256 to 318 (See Figure 3.15). The function of this C₄HC₃ zinc finger like motif is still not known, however PHD fingers are found in several proteins implicated in chromatin mediated transcriptional regulation including members of the *Drosophila melanogaster* Trithorax and Polycomb group genes, known to regulate transcription of homeotic genes (Gould, 1997). Another characteristic shared by many PHD finger containing proteins is that they function as part of multi-component complexes, as in the case of FANCL (Meetei et al., 2003), raising the possibility that, like the closely related LIM domain, a zinc finger binding domain present in Lin-1, Isl-1 and Mec-3 proteins (Schmeichel and Beckerle, 1998), PHD fingers are involved in protein-protein interactions (Aasland et al., 1995).

The alternative *Zfp276* transcript identified by PCR amplification of LβT2 and L-cell cDNA contains an insertion of 70 bp of genomic sequence 849 bp from the start codon (See Figure 3.9i). This small insertion may explain why this alternative transcript was not detected by northern blotting analysis (Wong et al., 2000). Searches of expressed sequence tag (EST) databases using BLAST (available at <http://www.ncbi.nlm.nih.gov>) revealed that expression of this alternative form of *Zfp276* is seen within the developing pituitary and limb, as well as in the adult brain and

liver. The addition of 70 bp of genomic sequence results in the introduction of an in-frame premature STOP codon (TGA) and, presumably, the production of a 285 amino acid truncated protein product without the last two C₂H₂ zinc-finger domains and a disrupted, putative, PHD domain (See Figure 3.15). Western blotting analysis could be used to determine if this alternative *Zfp276* transcript is translated and if both *Zfp276* proteins (expected sizes 41 and 32 kD) are expressed within the same tissues. Alternatively, *in vitro* translation would identify if the clones encode full-length and/or truncated proteins.

The genomic organisation of *Fanca* and *Zfp276*, and their co-expression within the same tissues, suggests that the transcripts from these genes could potentially form dsRNA. A recent paper suggested that around 8% of the estimated 40,000 human genes have an antisense partner (Yelin et al., 2003). Although the effects of antisense RNAs on the corresponding sense RNAs has not been clearly established, there is evidence to show that they may exert control at various levels of gene expression including transcription, splicing, stability, transport and translation (Knee and Murphy, 1997; Hastings et al., 2000; Li and Murphy, 2000). The recent discovery that dsRNA can trigger post-transcriptional gene silencing through the phenomenon of RNA interference (RNAi) lends further evidence to a role for antisense transcripts in gene regulation (Elbashir et al., 2001). However, detailed experimental analysis would need to be performed to determine if this process was occurring at the *Fanca* locus.

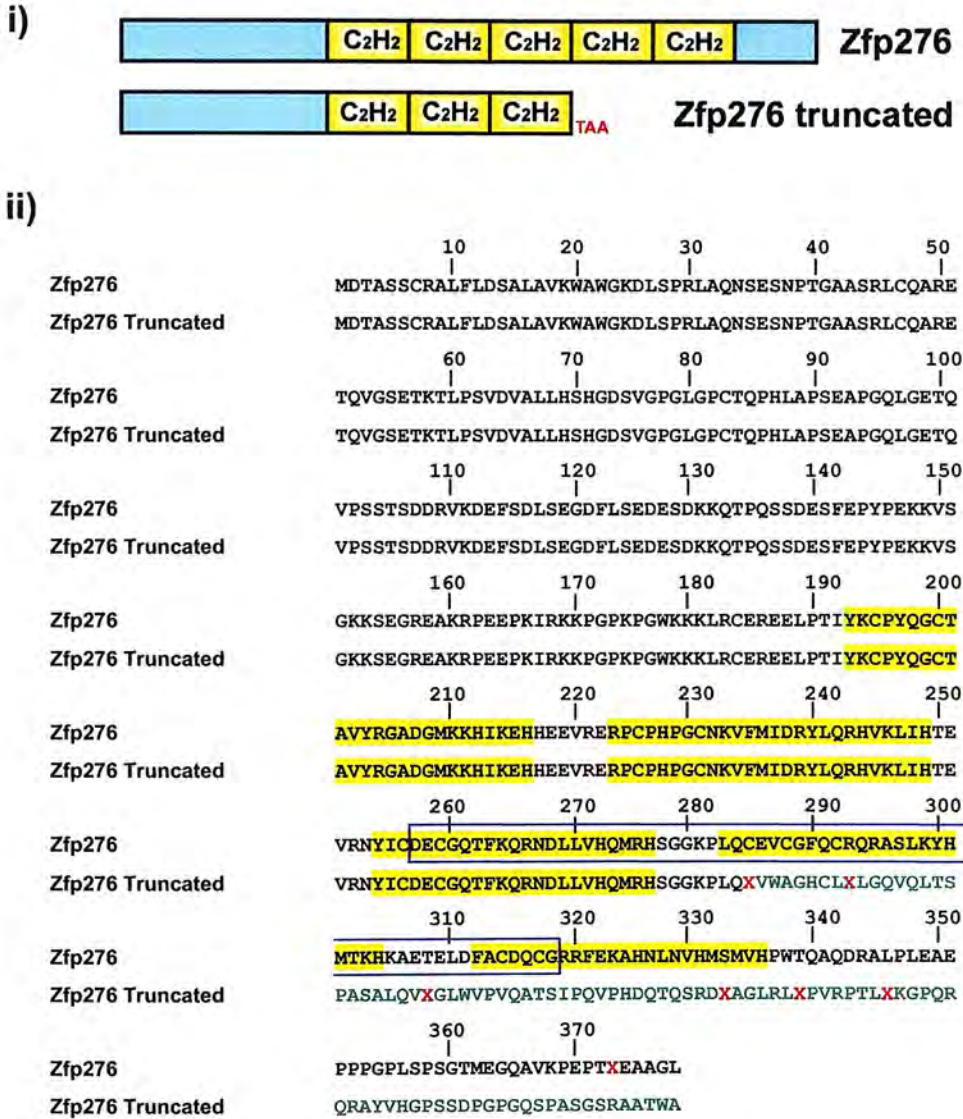


Figure 3.15 Addition of 70 bp of genomic sequence disrupts the protein domain structure of Zfp276.

(i) Schematic to show the domains present within Zfp276 full-length and truncated proteins. Zfp276 contains five C₂H₂ zinc finger domains (yellow boxes). Zfp276 Truncated contains three C₂H₂ zinc finger domains. The last two domains are lost due to the insert of a premature STOP codon (TAA). (ii) Alignment of amino acid sequence of Zfp276 and Zfp276 Truncated. The 5 C₂H₂ zinc finger domains present within Zfp276 sequence are highlighted yellow. The C₄HC₃ PHD domain is indicated by a blue box. Insertion of 70 bp of genomic sequence introduces a premature STOP codon at amino acid 285 removing the last 2 C₂H₂ zinc finger domains and disrupting the PHD domain.

3.3.3 *Fanca* mRNA is regulated by GnRH

GnRH up-regulation of *Fanca* mRNA was first identified by differential display RT-PCR of L β T2 cells that had been treated with one 15 minute pulse of 1 μ M GnRH and harvested for RNA after 1, 2 or 4 h (Chang, 2002). This relatively high concentration of hormone, compared to physiological levels, was chosen because it has been previously shown to stimulate high levels of gonadotropin sub-unit transcription, with a similar response being seen with either 10, 100 or 1000 nM GnRH [P.Brown, personal communication; (Bonfil et al., 2004)]. Southern blotting analysis of RT-PCR products amplified from RNA extracted from untreated and hormone treated L β T2 cells suggested that *Fanca* mRNA levels rose 1 h after treatment with GnRH and returned to unstimulated levels 4 h after treatment (See Figure 3.10). As RT-PCR analysis not quantitative, in order to measure the increase in *Fanca* mRNA levels seen in response to GnRH, quantitative RT-PCR (Q-RT-PCR) was performed on RNA extracted from untreated and GnRH treated L β T2 cells. Q-RT-PCR is an extremely sensitive technique, permitting analysis of gene expression from very small amounts of RNA (Wang et al., 1989). To quantify the changes in *Fanca* mRNA expression in response to GnRH, levels of *Fanca* mRNA were compared against levels of β -2-microglobulin (*B2m*) in L β T2 cells that were either untreated, or had been harvested for RNA after 1, 2 or 4 h of GnRH treatment. *B2m* was chosen as it is detectable in a broad range of tissues and is relatively stably expressed (Bishop et al., 1974). Whilst *B2m* mRNA levels did not vary significantly in response to hormone, *Fanca* mRNA levels increased 2-fold 1 h after treatment with GnRH (See Figure 3.11). Two hours after hormone treatment *Fanca* mRNA levels began to fall and had returned to unstimulated levels by 4 h (See Figure 3.11).

This rapid and transient increase suggests that levels of *Fanca* mRNA are tightly controlled, indicating a role for GnRH in regulating *Fanca* gene transcription. Bioinformatic analysis of the FANCA promoter revealed several putative consensus binding sites for various transcription factors including trans-acting transcription factor 1 (Sp1), early growth response factor 1 (Egr1), adaptor protein complex 2

(AP2) and nuclear factor 1 (NF1) (Ianzano et al., 1997). Both Sp1 and Egr1 are known to regulate *LHB* promoter activity in response to GnRH (Kaiser et al., 1998; Tremblay and Drouin, 1999). Furthermore, *Egr1* transcription has been shown to be up-regulated in response to GnRH (Duan et al., 2002) suggesting that this may be a possible mechanism by which GnRH up-regulates *Fanca* expression. Functional analysis of the *Fanca* promoter, using reporter gene assays, would help determine whether GnRH and *Egr1* are involved in its regulation.

Alternatively, the increase in *Fanca* mRNA levels observed after addition of GnRH may be due to an increase in the stability of *Fanca* mRNA. Interestingly, several hormones, including GnRH, LH and FSH, have been shown to influence mRNA stability of both pituitary and non-pituitary genes regulating their expression at transcriptional, posttranscriptional and posttranslational levels (Staton et al., 2000). The stability of gonadotropin subunit mRNA has also been shown to be regulated by hormones, with α GSU mRNA stability increasing 6.7-fold in the presence of GnRH (Chedrese et al., 1994). Similarly GnRH, in concert with progesterone and oestrogen, stabilises *LHB* mRNA (Park et al., 1996) whilst testosterone has been shown to regulate *FSH β* mRNA stability (Paul et al., 1990). Not surprisingly several hormones influence the stability of their own receptor mRNA via a feedback regulation mechanism. Research has shown that LH acts to destabilise its own receptor mRNA (Lu et al., 1993) whilst, conversely, FSH stabilises its receptor mRNA (Tilly et al., 1992). Transcriptional inhibitors could be used to determine if the increase seen in *Fanca* mRNA after treatment with GnRH is due to an increase in *Fanca* mRNA stability or an increase in transcription. Actinomycin D is widely used to block DNA dependent RNA synthesis and calculate mRNA half-life (Ross, 1995). Cells could be cultured with actinomycin D then harvested at various time points to measure the rate at which *Fanca* mRNA disappears and if this rate changes in response to GnRH. However, as *Fanca* mRNA is expressed at very low levels, and relatively difficult to detect, experiments to measure its degradation to even lower levels could prove difficult.

3.3.4 *Fanca* protein is regulated by GnRH

In order to determine if the increase in *Fanca* mRNA levels was mirrored by an increase in *Fanca* protein levels, western blotting analysis was performed on untreated and GnRH treated L β T2 cells. As *Fanca* mRNA levels were maximally increased 1 h after treatment with GnRH, longer hormone treatments were given to cells harvested for protein extracts to allow sufficient time for translation of *Fanca* mRNA. Results showed that the rapid, transient increase in *Fanca* mRNA levels measured in response to GnRH was followed by a similar, but sustained, increase in *Fanca* protein levels. Measurements of *Fanca* protein expression at 2 and 4 h after treatment with GnRH showed a highly significant 2-fold increase in expression when compared to unstimulated levels. This increase was less significant 6 h after treatment with hormone, with *Fanca* protein expression beginning to return to unstimulated levels (See Figure 3.12). Activation of translation in response to stimuli is a rapid and simple mechanism to increase protein levels within the cell. In concert with the reported increase in α GSU mRNA stability in response to GnRH (Chedrese et al., 1994), significant increases in protein synthetic capacity are seen in α T3-1 gonadotroph cells in response to hormone through a general increase in cap-dependent translation (Sosnowski et al., 2000). As the increase in *Fanca* protein levels in response to GnRH is seen within 2 h of treatment, it seems unlikely that this rapid and transient increase is due to a general augmentation of protein synthesis in these cells in response to GnRH but rather due to the specific regulation of *Fanca* by GnRH. This process could occur through various mechanisms including, an increase in *Fanca* mRNA stability leading to an increase in *Fanca* protein levels; an increase in transcription leading to more *Fanca* mRNA molecules available for translation or; an increased rate of translation of *Fanca* protein.

3.3.5 Possible role of *Fanca* in gonadotrophs

The rapid differential regulation of both *Fanca* mRNA and protein by GnRH in L β T2 gonadotroph cells (Figure 3.13) indicates that *Fanca* may be a component of a rapid response mechanism. This possibility is supported by two recent studies suggesting a potential role for *Fanca* in signal transduction cascades. Yeast two-hybrid screening identified that FANCA interacts with the signalling molecule I κ B kinase-2 (IKK2) which is involved in cellular response to stress and activation of the transcription factor NF- κ B (Mercurio and Manning, 1999), whilst studies of lymphoblast cells show that functional FANCA is required for phosphorylation of JNK and activation of the immediate early response gene *EGR-1* (Pipaon et al., 2004). As GnRH regulates gonadotropin subunit transcription via mitogen activated protein kinase (MAPK) pathways (Naor et al., 2000; Liu et al., 2002), and *Fanca* mRNA is expressed in L β T2 but not α T3-1 cells, regulation of its expression by GnRH could point towards a role for *Fanca* in GnRH initiated MAPK signalling in mature gonadotroph cells. However, much more detailed analysis of GnRH initiated signalling pathways would need to be performed to confirm this theory.

3.3.6 *Fanca*, GnRH and cancer

It is now well established that GnRH and its receptor are expressed, not only in the hypothalamus and pituitary, but also in, both normal and tumorigenic, reproductive tissues (Limonta et al., 2003). In these tumours, activation of GnRH receptors has been shown to down regulate cell proliferation suggesting that GnRH may act as an autocrine negative regulator of tumour growth (Miller et al., 1985b; Limonta et al., 1992; Dondi et al., 1994). The discovery that GnRH up-regulates expression of *Fanca*, a DNA damage repair gene, is exciting as it lends credibility to a link between GnRH, *Fanca* and cancer. Especially given the fact that FA family members have been implicated in ovarian tumorigenesis (Houghtaling et al., 2003; Taniguchi et al., 2003) and the prevalence of endocrinopathies in FA patients (Alter, 2003). Taken together with reports that FA patients have progressive bone marrow

failure due to the loss of haematopoietic stem cells (Alter, 1998); and that GnRH has been shown to suppress haematopoietic stem cell maturation in female mice (Rao et al., 1995); a role for *Fanca*, and other FA family members, in endocrine signalling seems likely and requires further investigation.

3.3.7 Conclusions

This chapter has fully characterised the regulation of *Fanca* expression in L β T2 gonadotroph cells. Quantitative RT-PCR and western blotting analysis have demonstrated that addition of GnRH results in a rapid and transient increase in both *Fanca* mRNA and protein expression. RT-PCR analysis revealed that whilst *Fanca* mRNA expression is observed in L β T2 cells and adult mouse pituitary, no expression is observed in α T3-1 cells, an immature gonadotroph cell line, suggesting a role for *Fanca* in gonadotroph development and maturation, possibly in the regulation of gonadotropin gene expression. Lastly, analysis of the expression of *Zfp276*, the antisense transcript located at the same locus as *Fanca*, revealed for the first time that whilst this gene does not appear to be regulated by GnRH, an alternatively spliced transcript is expressed within L β T2 and L-cells however, further analysis is required to determine the possible function of either of these transcripts in gonadotroph cells.

Chapter 4

Localization of Fanca protein in L β T2 cells

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them – Sir William Bragg.

4 Localization of Fanca protein in L β T2 cells

4.1 Introduction

4.1.1 Background

Eukaryotic cells differ from prokaryotic cells by the presence of intracellular, membrane bound compartments such as the nucleus. Specific mechanisms have evolved to transport proteins translated in the cytoplasm into the nucleus, where genetic information is regulated. This is of particular importance for signal transduction pathways where an extra-cellular signal must be transduced across the plasma membrane and nuclear envelope in order to propagate the signal from the cytosol to the nucleus. Functional molecules communicate continuously between the nucleus and the cytoplasm by transport through the nuclear pore complex (NPC) (Feldherr et al., 1984). Disruption of the nuclear localization of proteins can have serious cellular consequences, such as the initiation and progression of cancer as reported for transformation related protein 53 (p53), adenomatosis polyposis coli (APC) and breast cancer susceptibility 1 (BRCA1) tumour suppressor proteins (Moll et al., 1992; Chen et al., 1995; Zhang et al., 2000).

Small molecules (<50 kD) can diffuse freely in and out of the nucleus through nuclear pores, whereas larger proteins must be actively transported. This process is mediated by specific amino acid sequences, present within the protein to be transported, and referred to as nuclear localization signals (NLS's) and nuclear export signals (NES's). The classical NLS is comprised of a few basic amino acid residues, usually lysines (K) or arginines (R). These occur either in the form of a monopartite NLS with the consensus sequence (K/R)₄₋₆ or as two smaller clusters separated by approximately a dozen amino acids residues (K/R)₂-X₁₀₋₁₂-(K/R)₃ (Dingwall and Laskey, 1991). These NLS sequences are recognised by an NLS receptor comprised of importin α and β , located at the nuclear pore, which regulates

the translocation of cytoplasmic NLS containing proteins into the nucleus (Komeili and O'Shea, 2000). Conversely, NES's are rich in hydrophobic amino acids such as leucines (L) or isoleucines (I). The NES consensus sequence, LX₁₋₃LX₂₋₄LXL, is recognised and bound by the export receptor protein CRM1, the human homolog of yeast chromosome region maintenance protein 1, to direct movement of the NES containing protein to the cytoplasm (Fornerod et al., 1997; Fukuda et al., 1997; Henderson and Eleftheriou, 2000).

FANCA is a relatively large protein (~ 160 kD) that contains a bipartite NLS sequence at amino acids 19-34 (RK-X₁₀-KKQK). Research has shown that the NLS sequence found within FANCA is required, not only for the nuclear localization of FANCA protein (Naf et al., 1998), but also for the direct interaction of FANCA with FANCG (Kruyt et al., 1999; Garcia-Higuera et al., 2000). Interestingly, the C-terminus of FANCA is also required for localization to the nucleus (Lightfoot et al., 1999) and may be required for binding of the NLS motif to importin α/β . Correct localization of FANCA is crucial for nuclear accumulation of the FA complex, comprised of FANCA, FANCC, FANCG, FANCE and FANCF (Joenje and Patel, 2001). Along with the recently identified FANCL protein, this nuclear complex is thought to be required for mono-ubiquitination of FANCD2 which targets the FANCD2 molecule to DNA repair foci containing the DNA damage repair proteins BRCA1 and FANCD1/BRCA2 (See Figure 1.14). This complex appears to be constitutively present and no signals have been identified that lead directly to its assembly, however, bi-allelic mutations in FANCA block complex formation. To date more than 100 different mutations, spread along the length of the *FANCA* gene, have been reported (Lo Ten Foe et al., 1996; Levran et al., 1997; Morgan et al., 1999; Wijker et al., 1999). Most of these involve small insertions or deletions, presumably resulting in null mutations, but >30 mutations are predicted to produce altered proteins with a single amino acid substitution or a small in-frame deletion. A sub-set of these have been studied *in vitro* with particular reference to their cellular localization and ability to reconstitute the FA pathway (Adachi et al., 2002). It is

thought that the variability in phenotypes seen in FA patients is due to variable activation of the FA pathway by these mutations, indicating that nuclear-cytoplasmic transport is a critical component of the FA pathway, however, the transport mechanisms and signals that regulate this process have not been characterized.

4.1.2 Aims of this chapter

Chapter three established that the expression of *Fanca* is rapidly and transiently up-regulated in L β T2 cells in response to GnRH. This chapter analyses the expression of Fanca protein within gonadotroph cells and, in a bid to identify a mode of Fanca action in these cells, asks whether addition of GnRH promotes a redistribution of Fanca protein within L β T2 cells and what specific mechanisms may be involved in this 'shuttling' process. Lastly, the cellular distribution of mutant FANCA proteins within L β T2 cells is also investigated to determine whether the introduction of specific point mutations into the Fanca protein can alter the localization pattern of Fanca protein within gonadotroph cells.

4.2 Results

4.2.1 Sub-cellular localization of Fanca protein in L β T2 cells

The localization of endogenous Fanca protein within L β T2 cells was investigated by indirect immunofluorescence using rabbit anti-mouse Fanca antisera. L β T2 cells, grown on 2-well glass chamber slides, were either left untreated, or treated with 1 μ M GnRH for 2 h before being incubated overnight with rabbit-anti-mouse Fanca antisera specific for amino acids 1-276 [kind gift from Dr. Fré Arwert (Waisfisz et al., 1999)]. The following day, Fanca protein was visualized by incubating with a goat anti-rabbit secondary antibody conjugated to biotin followed by a fluorescent probe conjugated to streptavidin (See Section 2.7.10). Nuclei were then counterstained with a fluorescent dye (Topro-3) and slides mounted to allow detection of fluorescence signal by confocal microscopy. Fluorescence, corresponding to Fanca protein expression, was detected in both the cytoplasm and nucleus of L β T2 cells where it co-localized with Topro-3 staining (See Figure 4.1 a, b and c). Analysis of fluorescence levels measured by Z-stack sectioning through the middle of a L β T2 cell showed that Fanca protein expression was relatively evenly distributed through the cell (See Figure 4.1g). Addition of GnRH had no dramatic detectable effect on Fanca protein localization within L β T2 cell, but, visual examination of Fanca expression in these cells suggested that addition of GnRH increased expression of Fanca, particularly within the cytoplasm (See Figure 4.1 d, e and f). However, Z-stack analysis of the fluorescence levels from a single L β T2 cell revealed that Fanca expression is still detectable in both cellular compartments, as in the case of untreated L β T2 cells (See Figure 4.1h).

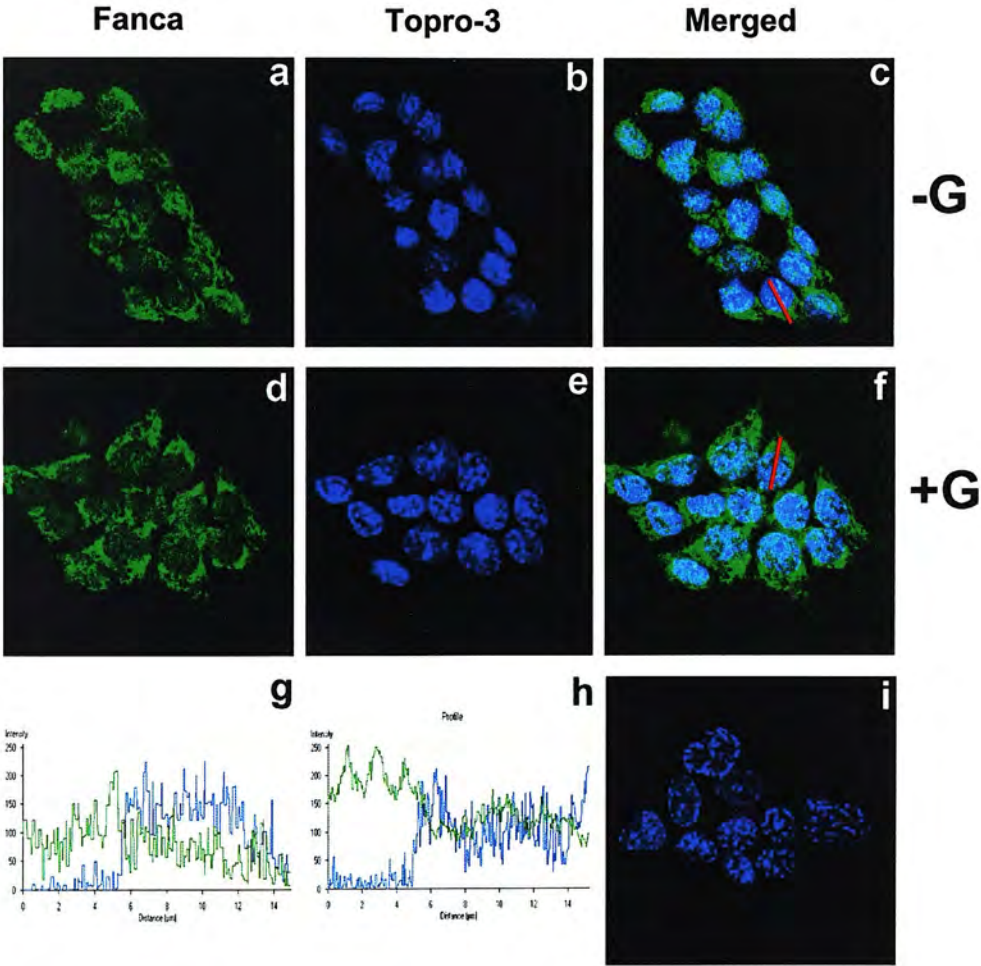


Figure 4.1 Detection of sub-cellular localization of Fanca protein in LβT2 cells using indirect immunofluorescence.

Localization of Fanca protein within LβT2 cells was determined using rabbit anti-mouse Fanca antisera and indirect immunofluorescence. Fanca protein is localized to the cytoplasm and nucleus of LβT2 cells (a). Addition of GnRH does not alter Fanca localization (d). Cell nuclei were identified by staining with Topro-3, a DNA-specific dye (b, e). Fanca protein expression (green) is seen within the cytoplasm and co-localizes with Topro-3 staining (blue) within LβT2 cell nuclei (c, f). Levels of fluorescent signal determined by Z-stack sectioning through a single, untreated LβT2 cell (c, red line) and a GnRH treated cell (f, red line) are represented graphically (g [untreated] and h [GnRH treated]). Negative control, no primary antibody (i).

4.2.2 Western blotting analysis of Fanca sub-cellular localization in L β T2 cells

Having demonstrated that Fanca protein is expressed in both sub-cellular compartments of L β T2 cells, the effects of GnRH on Fanca protein expression within these compartments was investigated using western blotting analysis. Cytoplasmic and nuclear extracts were prepared from untreated L β T2 cells and from L β T2 cells harvested 2 h after addition of GnRH (See Section 2.7.2.2). Equal amounts of protein extracts, as determined by Bradford assay, were fractionated on a 6% SDS-PAGE gel, blotted and incubated with rabbit anti-mouse Fanca antisera. A specific protein band was detected at ~160 kD, corresponding to the expected size of Fanca, in extracts from both cytoplasmic and nuclear compartments (See Figure 4.2i). The veracity of the protein extracts was confirmed by stripping the blots and re-probing with either a rabbit anti-mouse β -tubulin antibody, to confirm cytoplasmic extracts, or a rabbit anti-mouse Sp-1 antibody, to confirm nuclear extracts. Quantification of protein levels using ImageQuant software revealed that a significant 2-fold increase in Fanca protein levels occurred in both the cytoplasmic and nuclear compartments of L β T2 cells after addition of 1 μ M GnRH for 2 h (ANOVA one-way analysis of variance, $p < 0.001$). See Figure 4.2ii.

4.2.3 Nuclear-cytoplasmic shuttling of Fanca protein in L β T2 cells

The observed GnRH induced increase in Fanca protein within L β T2 gonadotroph cells could be due to *de novo* translation with no re-distribution of Fanca protein within the cell. Alternatively, the increase in response to GnRH could be explained by an increase in Fanca protein levels and a further re-distribution of protein between cellular compartments. To investigate these possibilities L β T2 cells were treated with inhibitors of translation, nuclear import and export and western blotting analysis performed on nuclear and cytoplasmic protein extracts. Cells were treated for 30 minutes with cycloheximide (CHX), to block translation; actinomycin D (AMD), to

block nuclear import; or leptomycin B (LMB), to block nuclear export, followed by addition of 1 μ M GnRH for 2 h. Protein extracts were then prepared from both cytoplasmic and nuclear compartments as described in section 2.7.2.2. Equal amounts of protein extract, as determined by Bradford assay, were then fractionated on a 6% SDS-PAGE gel, blotted and incubated with rabbit anti-mouse Fanca antisera. A specific protein band at ~160 kD, corresponding to the expected size of Fanca, was detected in all samples and quantification of Fanca protein levels was performed using ImageQuant software. An approximate 2-fold increase in Fanca protein levels was observed in both cellular compartments after addition of GnRH for 2 h (ANOVA one-way analysis of variance, $p < 0.001$, see Figures 4.3 and 4.4). Treatment of cells with CHX prior to addition of GnRH blocked the 2-fold hormone induced increase in Fanca protein levels in both compartments (Figures 4.3 and 4.4). A significant decrease in Fanca protein levels was observed in L β T2 cytoplasmic extracts made from L β T2 cells after treatment with CHX in conjunction with either AMD or LMB, prior to addition of GnRH, when compared to cytoplasmic Fanca levels seen after addition of hormone alone (ANOVA one-way analysis of variance, $p < 0.01$, see Figure 4.3). The usual 2-fold increase in Fanca protein levels after treatment with GnRH was not detectable in nuclear extracts from cells incubated with CHX and AMD prior to treatment with GnRH. In contrast, Fanca protein levels in nuclear extracts made from L β T2 cells incubated with CHX and LMB prior to GnRH were increased 1.5-fold when compared to Fanca protein levels in untreated cells (ANOVA one-way analysis of variance, $p < 0.01$, see Figure 4.4), and were not significantly different from nuclear Fanca protein levels measured in L β T2 cells treated with GnRH alone. These experiments demonstrate that the GnRH induced increase in Fanca protein is due to *de novo* translation, and importantly they also indicate that Fanca protein is actively transported from the nucleus of L β T2 gonadotroph cells via a CRM1 dependent mechanism.

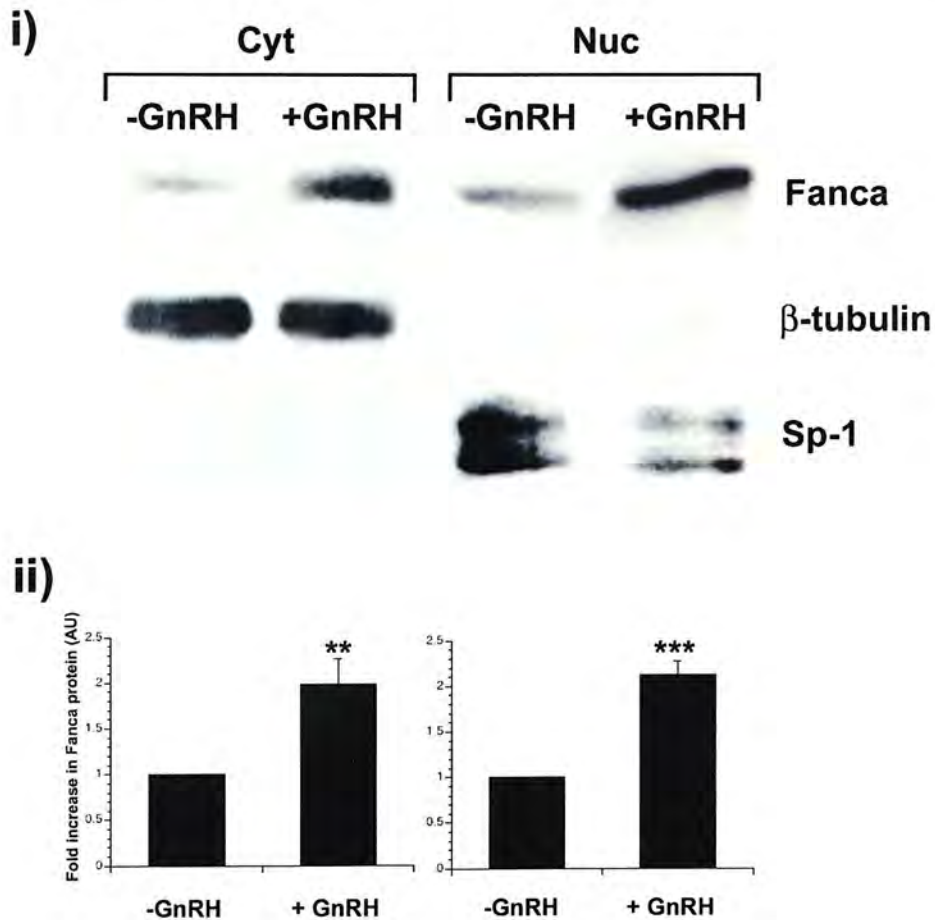


Figure 4.2 Detection of sub-cellular localization of Fanca protein in L β T2 cells using western blotting analysis.

(i) Western blotting of cytoplasmic and nuclear protein extracts from L β T2 cells either left untreated (-GnRH) or harvested 2 h after treatment with GnRH (+GnRH). Protein was fractionated on an SDS-PAGE gel, blotted and probed with rabbit anti-mouse Fanca antisera. A protein band, corresponding to Fanca, was detected at the expected size of ~160 kD in both cytoplasmic (Cyt) and nuclear (Nuc) compartments. Cytoplasmic extracts were confirmed by stripping the blot and re-probing with a β -tubulin antibody. Nuclear extracts were confirmed by stripping the blot and re-probing with an Sp-1 antibody. (ii) Addition of GnRH resulted in a 2-fold increase in Fanca protein levels in both compartments. Results shown are the mean of three separate experiments with error bars indicating the standard error of the mean. ANOVA one-way analysis of variance determined that the increase in Fanca protein levels following treatment with hormone was significant: ** $p < 0.01$; *** $p < 0.001$.

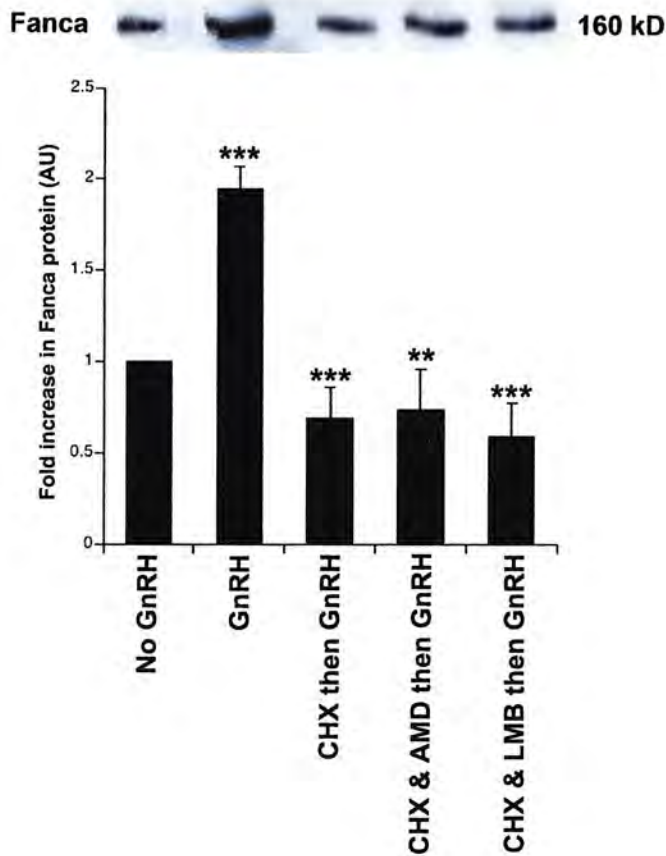


Figure 4.3 Western blotting analysis of cytoplasmic Fanca protein levels after treatment with inhibitors and GnRH.

Western blotting analysis of L β T2 cytoplasmic extracts either left untreated (no GnRH) or harvested after incubation with cycloheximide (CHX), actinomycin D (AMB) or leptomycin B (LMB) prior to treatment with GnRH. Protein was fractionated on a 6% SDS-PAGE gel, blotted and probed with a rabbit anti-mouse Fanca antibody. This detected a protein band at the expected size of ~160 kD as shown on a representative blot. ImageQuant analysis of protein levels showed, as expected, that Fanca protein levels increased 2-fold 2 h after treatment with GnRH (ANOVA one-way analysis of variance ***, $p < 0.001$). Addition of CHX, CHX and AMD or CHX and LMB prior to hormone treatment resulted in no GnRH induced increase in Fanca protein levels within the cytoplasm. Results shown are the mean of three separate experiments with error bars indicating the standard error of the mean. ANOVA one-way analysis of variance determined that addition of inhibitors prior to GnRH significantly repressed the expected 2-fold increase in Fanca protein levels: ***, $p < 0.001$; **, $p < 0.01$).

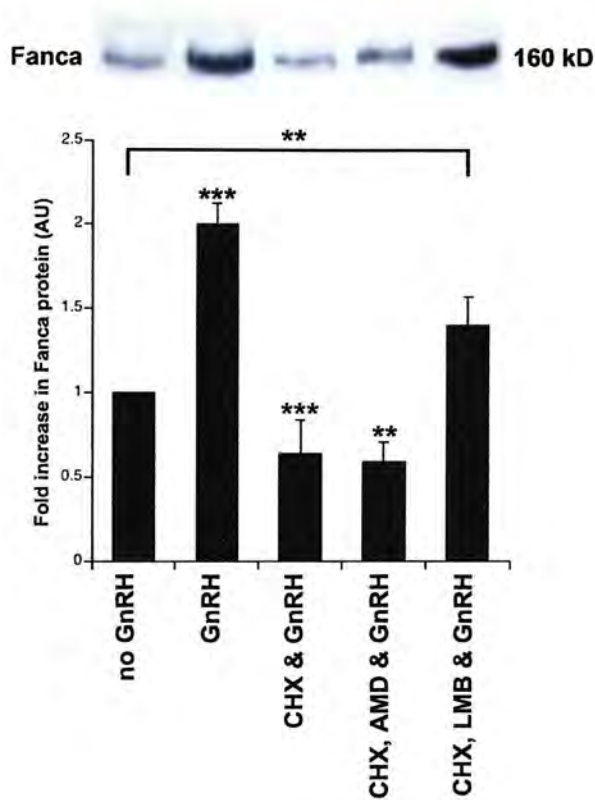


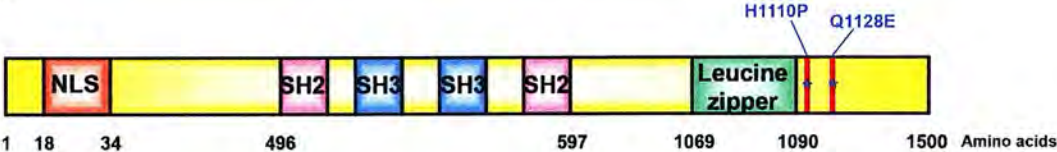
Figure 4.4 Western blotting analysis of nuclear Fanca protein levels after treatment with inhibitors and GnRH.

Western blotting analysis of LβT2 nuclear extracts either left untreated (no GnRH) or harvested after incubation with cycloheximide (CHX), actinomycin D (AMB) or leptomycin B (LMB) prior to treatment with GnRH. Protein was fractionated on a 6% SDS-PAGE gel, blotted and probed with a rabbit anti-mouse Fanca antibody. This detected a protein band at the expected size of ~160 kD as shown on a representative blot. ImageQuant analysis of protein levels showed, as expected, that Fanca protein levels increased 2-fold 2 h after treatment with GnRH (ANOVA one-way analysis of variance ***, $p < 0.001$). Addition of CHX or CHX and AMD prior to hormone treatment resulted in no GnRH induced increase in Fanca protein levels within the nucleus, however Fanca protein levels in nuclear extracts from cells incubated with CHX and LMB prior to hormone treatment increased 1.5-fold when compared against untreated levels (ANOVA one-way analysis of variance **, $p < 0.01$). Results shown are the mean of three separate experiments with error bars indicating the standard error of the mean. ANOVA one-way analysis of variance determined that addition of inhibitors prior to GnRH significantly repressed the expected 2-fold increase in Fanca protein levels: ***, $p < 0.001$; **, $p < 0.01$.

4.2.4 Generation of GFP-tagged mutant FANCA clones

Having determined that Fanca protein is expressed in both the cytoplasm and nucleus of L β T2 cells, the localization of point-mutated forms of FANCA was analysed to determine if a single base change could disrupt the distribution of FANCA protein within L β T2 cells. Site-directed mutagenesis was used to engineer two specific mutations, H1110P and Q1128E (Adachi et al., 2002), into a GFP tagged human FANCA clone (kind gift from Manuel Buchwald) as described in Section 2.3.11. Sequencing analysis verified the production of FANCA clones with a single specific mutated base (See Figure 4.5).

i)



ii)

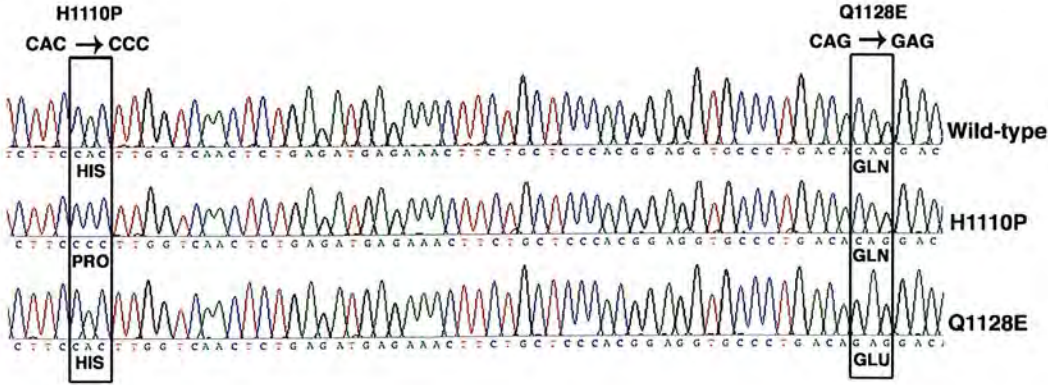


Figure 4.5 Confirmation of site-directed mutagenesis of GFP-FANCA.

Two separate mutations were engineered into GFP-FANCA by site-directed mutagenesis. (i) Position of introduced mutations relative to other FANCA protein features. (ii) Sequencing was performed along the entire length of each clone to ensure that only the specific mutation (H1110P or Q1128E) had been introduced. Electropherograms show the region of GFP-FANCA containing the two introduced mutations and indicate the base-pair change.

4.2.5 Western blotting analysis of GFP-tagged mutant FANCA constructs in L β T2 cells

Since altering the amino acid sequence of a protein can often result in mis-folding and consequently direct the recombinant protein into a lysosomal degradation pathway within the cell, it was important to verify that the engineered wildtype and mutated proteins were expressed in L β T2 cells as fusion proteins and at similar levels. L β T2 cells were transiently transfected for 48 h with pEGFP-FANCA-wildtype, pEGFP-FANCA-H1110P or pEGFP-FANCA-Q1128E plasmid and whole cell protein extracts made. Equal amounts of protein extract, as determined by Bradford assay, were fractionated on a 6% SDS-PAGE gel, blotted and incubated with a goat anti-GFP antibody. A specific protein band at ~187 kD, corresponding to the expected size of GFP tagged full length FANCA, was detected in extracts from cells transfected with GFP plasmids (See Figure 4.6). No band was detected in mock transfected cells (Fugene only). Blots were stripped, and then re-probed with a rabbit anti-mouse β -tubulin antibody as a control (See Figure 4.6).

4.2.6 Expression analysis of GFP-tagged mutant FANCA constructs

Having determined that the GFP-FANCA fusion proteins were intact and expressed in equivalent amounts, confocal microscopy was used to study the location of GFP-tagged wildtype and mutant FANCA proteins in L β T2 and HeLa cells. Cells, grown on 2-well glass chamber slides, were transiently transfected with either GFP-tagged wildtype FANCA or GFP-tagged mutant FANCA (H1110P or Q1128E) plasmid. After 48 h, cells were fixed, counterstained with a DNA-specific dye and confocal microscopy performed to visualize fluorescence levels. Analysis determined that GFP-tagged FANCA-wildtype was expressed in both the cytoplasm and nucleus of HeLa and L β T2 cells (Figures 4.7 and 4.8, panels b and j). Further examination of z-stack scans taken through the middle of a representative cell revealed a predominantly nuclear expression pattern of FANCA in both cell types (Figures 4.7 and 4.8, panels j and n). Replacement of a histidine residue at position 1110 with a

proline (H1110P) resulted in complete restriction of FANCA expression to the cytoplasm of both HeLa and L β T2 cells (Figures 4.7 and 4.8, panels c and k). Replacement of a glutamine residue at position 1128 with a glutamic acid residue (Q1128E) did not alter FANCA expression within HeLa cells (Figure 4.7, panels d and l). Close examination of the z-stack profile of a representative GFP-FANCA-Q1128E transfected HeLa cell (Figure 4.7p) clearly showed a predominantly nuclear staining pattern indistinguishable from GFP-FANCA-wildtype expression (Figure 4.7n) but different from the cytoplasmic and nuclear expression pattern seen with GFP alone (Figure 4.7m). In contrast, analysis of z-stack scans through an L β T2 cell transfected with GFP-FANCA-Q1128E revealed that FANCA protein was highly expressed within both the cytoplasm and nucleus (Figure 4.8l) resembling the expression pattern observed when L β T2 cells were transfected with un-coupled GFP (Figure 4.8m) rather than the expression pattern seen for GFP-FANCA-wildtype (Figure 4.8n).

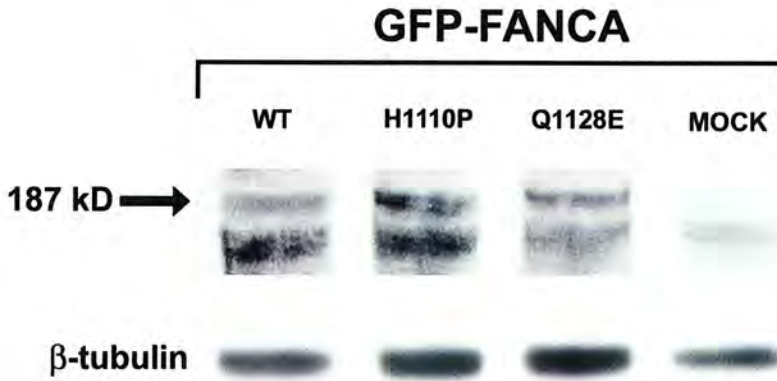


Figure 4.6 Expression analysis of GFP FANCA constructs in LβT2 cells

Western blotting analysis of whole cell protein extracts from LβT2 cells transiently transfected with GFP-FANCA-WT, GFP-FANCA-H1110P or GFP-FANCA-Q1128E plasmid. Protein was fractionated on a 6% SDS-PAGE gel, blotted and probed with a goat anti-GFP antibody before being stripped and re-probed with a rabbit anti-mouse β-tubulin antibody. Probing with a GFP antibody detected a protein band at the expected size of ~187 kD in extracts from cells transfected with GFP plasmid. No band was detected in cells transfected with Fugene alone. Probing with a β-tubulin antibody detected a protein band at the expected size of ~55 kD in all samples.

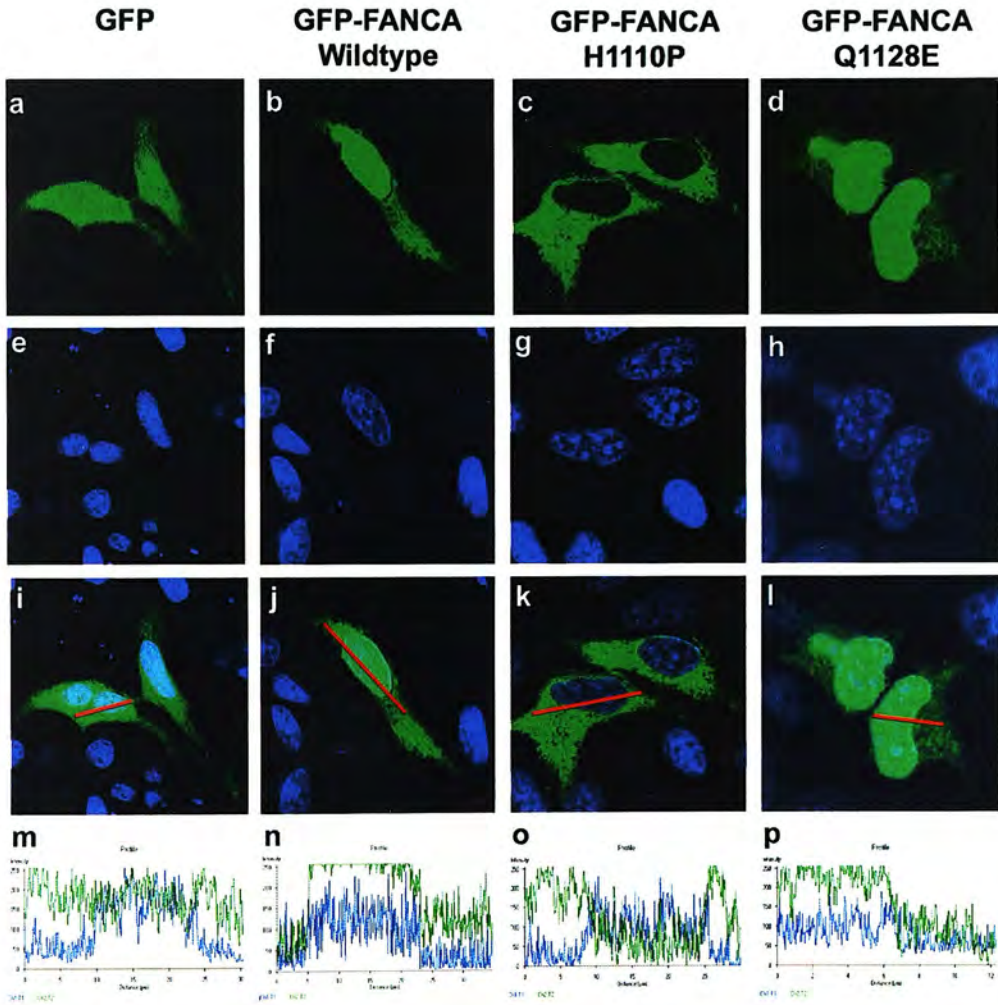


Figure 4.7 Expression analysis of GFP-tagged wildtype and mutant FANCA protein in HeLa cells.

Wildtype and mutant GFP-tagged FANCA clones were transfected into HeLa cells and localization of fluorescence analysed by confocal microscopy. Cell nuclei were identified by staining with Topro-3, a DNA specific dye (e-h). GFP (a,m) and GFP-FANCA (b,j) are expressed in both the cytoplasm and nucleus of HeLa cells with GFP-FANCA expression being predominantly nuclear (b,j). GFP-FANCA-H1110P expression is restricted to the cytoplasm of HeLa cells (c,k). Mutating position 1128 of FANCA (GFP-FANCA-Q1128E) localized protein expression to the nucleus (d,l). Levels of fluorescent signal determined by Z-stack sectioning through the middle of a single cell (red line) are represented graphically for each GFP clone (m-p). Co-localization of GFP expression (green) with nuclear Topro-3 stain (blue) is seen for GFP-FANCA (j,n) and GFP-FANCA-Q1128E (d,p) but not GFP-FANCA-H1110P (c,o).

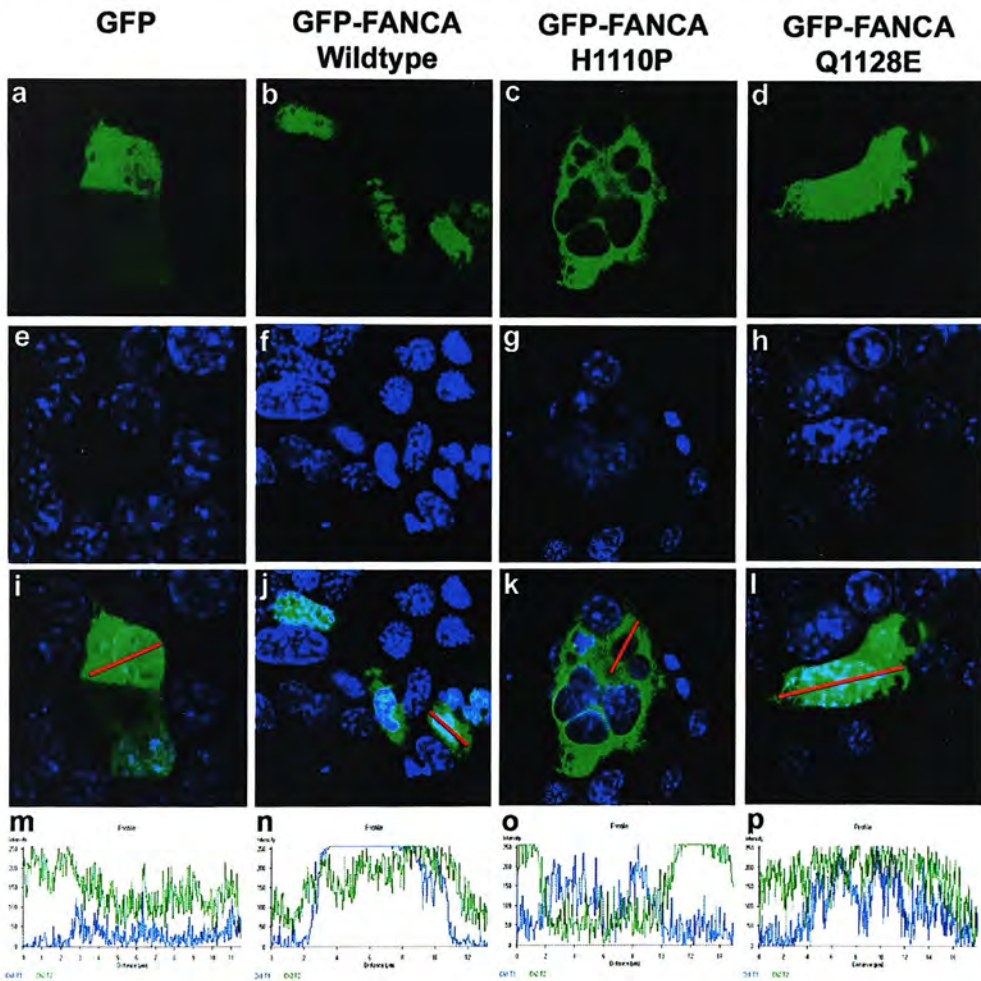


Figure 4.8 Expression analysis of GFP-tagged wildtype and mutant FANCA protein in L β T2 cells.

Wildtype and mutant GFP-tagged FANCA clones were transfected into L β T2 cells and localization of fluorescence analysed by confocal microscopy. Cell nuclei were identified by staining with Topro-3, a DNA specific dye (e-h). GFP (a,i) and GFP-FANCA (b,j) are expressed in both the cytoplasm and nucleus of L β T2 cells with GFP-FANCA expression appearing to be predominantly nuclear (b,j). Mutant GFP-FANCA-H1110P expression is restricted to the cytoplasm of L β T2 cells (c,k). Mutating position 1128 of FANCA (GFP-FANCA-Q1128E) localized protein expression to the nucleus (d,l). Levels of fluorescent signal determined by Z-stack sectioning through the middle of a single cell (red line) are represented graphically for each GFP clone. Co-localization of GFP expression (green) with nuclear Topro-3 stain (blue) is seen for GFP-FANCA (j,n) and GFP-FANCA-Q1128E (l,p) but not GFP-FANCA-H1110P (k,o).

4.3 Discussion

4.3.1 *Fanca* protein is expressed in both the cytoplasm and nucleus of L β T2 cells

There has been much controversy regarding the cellular localization of FANCA protein. Initial reports using a C-terminus GFP tagged FANCA protein stated that FANCA localized to the cytoplasm of HEK293 cells (Kruyt et al., 1997). Further reports analysing the expression of endogenous FANCA protein in HeLa cells, using an antibody to FANCA, demonstrated that the protein is located in both cellular compartments (Kruyt and Youssoufian, 1998). However, more recent analysis of FANCA protein expression in Cos-7, HeLa and NIH-3T3 cells, using an N-terminus GFP tagged FANCA construct, has concluded that FANCA protein is located in both cellular compartments, but is predominantly expressed within the nucleus (Kupfer et al., 1999; Lightfoot et al., 1999; Adachi et al., 2002). With these conflicting reports, and the discovery that *Fanca* is expressed within gonadotroph cells, it was important to establish where *Fanca* protein localizes to in L β T2 cells. Using an antibody to the N-terminus of *Fanca* and indirect immunofluorescence, expression of endogenous *Fanca* protein within untreated and GnRH treated gonadotroph cells was localized to both cellular compartments (Figure 4.1), agreeing with reports detailing endogenous FANCA expression in HeLa cells (Kruyt and Youssoufian, 1998). Western blotting analysis of cytoplasmic and nuclear extracts prepared from untreated and GnRH treated cells confirmed that expression of *Fanca* protein was observed in both the cytoplasm and nucleus of L β T2 cells and revealed that addition of GnRH generated a significant 2-fold increase in *Fanca* protein levels within both cellular compartments (Figure 4.2).

Interestingly, analysis of the expression pattern of GFP-tagged FANCA protein revealed that whilst expression of FANCA is observed within both cellular compartments, GFP-tagged FANCA appears to be localized predominantly to the

nucleus of both HeLa and L β T2 cells (Figure 4.8n). This data agrees with several other reports detailing the expression of GFP-tagged FANCA protein within other cell types (Kupfer et al., 1999; Lightfoot et al., 1999; Adachi et al., 2002) but contrasts with the expression pattern of endogenous Fanca protein within gonadotroph cells (Figure 4.1). This difference may be due to the fact that one method is detecting endogenous protein where as the other is looking at FANCA protein translated from DNA that has been transfected into the cell. As FA proteins are thought to be involved in homologous DNA repair (Thompson et al., 2005), it is conceivable that the process of transfection may trigger DNA damage repair pathways, leading to the nuclear accumulation of FANCA protein. Analysis of endogenous Fanca expression in gonadotroph cells that have been transfected with a LacZ reporter construct would help to determine whether transfecting the cells affects the localization of Fanca protein.

4.3.2 Fanca protein shuttles between the nucleus and cytoplasm of L β T2 cells

Proteins synthesised within the cytoplasm that contain an NLS, such as FANCA, are transported across the nuclear envelope, via pores, to perform their required function within the nucleus. To analyse the rapid and transient increase in Fanca protein levels in response to GnRH (See Chapter 3) and the movement of Fanca protein across the nuclear membrane in L β T2 gonadotroph cells, inhibitors of protein translation, nuclear import and nuclear export were added to L β T2 cells in culture and the location of Fanca protein analysed. Three inhibitors were used: cycloheximide, actinomycin D and leptomycin B.

Cycloheximide (CHX) is an antifungal antibiotic that inhibits protein synthesis in eukaryotes (Obrig et al., 1971). Treating L β T2 cells with CHX prior to addition of GnRH blocked the 2-fold increase in Fanca protein levels usually seen after addition of hormone, in both cytoplasmic (See Figure 4.3) and nuclear (See Figure 4.4)

compartments. This indicates that the rapid increase in Fanca protein levels seen after addition of GnRH is a result of *de novo* translation.

The addition of Actinomycin D (AMD), which is an antibiotic that inhibits DNA dependent RNA synthesis and nuclear import (Pinol-Roma and Dreyfuss, 1992; Kalland et al., 1994) in conjunction with CHX ensured that any observed changes in protein levels within either cellular compartment would be due to the re-distribution of Fanca protein rather than an increase in transcription or translation. Fanca protein levels in both cellular compartments of L β T2 cells treated with CHX and AMD prior to GnRH were not significantly different from levels of Fanca protein in untreated cells but were significantly different from Fanca protein levels in cells treated with GnRH alone (ANOVA one-way analysis of variance, $p < 0.01$, see Figures 4.3 and 4.4). Thus, addition of AMD did not block the nuclear import of Fanca protein in L β T2 cells or an increase in cytoplasmic levels of Fanca protein would have been observed as reported for SRY-box containing gene 9 (Sox9), HIV Type 1 Rev protein and heterogeneous nuclear ribonucleoprotein (hnRNP A1) (Pinol-Roma and Dreyfuss, 1992; Kalland et al., 1994; Gasca et al., 2002). However, mutation of the NLS sequence within FANCA does block nuclear entry (Naf et al., 1998) suggesting that the protein is actively imported. Interestingly, C-terminus amino acids have also been shown to be required for FANCA nuclear localization, as mutation of amino acids at the C-terminus prevents normal nuclear localization, even in the presence of an intact NLS (Lightfoot et al., 1999). Perhaps specific inhibition of the NLS receptor proteins, importin α and β , rather than using a broad spectrum inhibitor such as AMD, would prove informative in determining whether Fanca protein is actively transported into the nucleus of L β T2 cells.

In contrast to AMD, Leptomycin B (LMB) is an antibiotic that inhibits export of certain proteins from the nucleus by disrupting the function of a specific nuclear export pathway controlled by CRM1, a nuclear protein that binds to specific leucine rich nuclear export signal (NES) motifs. Proteins that bind to CRM1 are then

actively exported into the cytoplasm via nuclear pores. LMB disrupts nuclear export of proteins by binding CRM1 and thus inhibiting its interaction with NES containing proteins (Kudo et al., 1997). As before, CHX was added in parallel with LMB to ensure that any observed changes in protein levels within cytoplasmic or nuclear extracts were due to the re-distribution of Fanca protein rather than the presence of newly translated protein. Levels of cytoplasmic Fanca protein in L β T2 cells incubated with CHX and LMB prior to treatment with GnRH were significantly decreased when compared to cells treated with GnRH only ($p < 0.001$, see Figure 4.3). They were also significantly decreased when compared with cytoplasmic Fanca protein levels in untreated cells ($p < 0.05$, see Figure 4.3), indicating that the cytoplasmic pool of Fanca protein had been depleted. Examination of nuclear Fanca protein levels after pre-treatment with CHX and LMB and after addition of GnRH revealed a 1.5-fold increase in Fanca protein levels. This increase in nuclear Fanca protein was not significantly different from the 2-fold increase usually seen in nuclear Fanca levels in response to GnRH (See Figure 4.4), but was significantly different from levels of nuclear Fanca protein measured in untreated cells ($p < 0.01$, see Figure 4.4). Taken together, these results indicate that Fanca protein is exported from the nucleus of L β T2 cells via a specific, CRM1 dependent mechanism since blocking CRM1 mediated nuclear export results in a depletion of cytoplasmic, and an accumulation of nuclear, Fanca protein.

CRM1 dependent protein export from the nucleus requires a leucine/isoleucine rich NES with the consensus sequence (L/I)-X_{1,3}-(L/I)-X_{2,4}-(L/I)-X-(L/I). Analysis of mouse Fanca protein sequence reveals the presence of a CRM1 dependent NES motif at amino acids 1113-1125 (LSRLQEIALDL) that is also relatively well conserved in the human protein (¹⁰¹⁸ISRLQEMVADLEL¹⁰³⁰). Interestingly, a recent report investigated export of FANCA protein from the nucleus of fibroblast cells and hypothesised that this particular sequence was involved (Ferrer, 2003). However, mutational analysis of the putative NES sequence would need to be performed to prove that this motif is required for the export of FANCA protein. Bioinformatic

analysis reveals that the putative NES sequence is located at exons 31 and 32 of human *FANCA* and whilst there are no reports of patients with specific point mutations within this 46 bp region, deletions that include exon 31 have been detected in ~19% of patients assigned to complementation group A (Morgan et al., 1999).

With the discovery that both *Fanca* mRNA and protein levels are differentially regulated in response to GnRH (Chang, 2002; Larder et al., 2004), it is conceivable that GnRH may also be involved in the transport of FANCA protein between the cytoplasm and nucleus of L β T2 cells. Whilst the experiments described in this chapter have shown that *Fanca* is actively transported from the nucleus of L β T2 cells they have not established whether GnRH is playing an active role in this process. However, this issue could be addressed if further western blotting analysis was performed on cytoplasmic and nuclear protein extracts from L β T2 cells treated with LMB and a GnRH antagonist.

4.3.3 *Specific mutations affect FANCA sub-cellular localization*

FANCA point mutations are classified into three groups, group I mutants behave like wild-type FANCA, group II mutants are mildly impaired in their ability to localize to the nucleus whilst the ability of group III mutants to localize to the nucleus and interact with other FANC proteins is severely compromised. Two mutations were chosen from these groups (H1110P and Q1128E) and generated by site-directed mutagenesis. H1110P is classified as a group III mutant as replacement of a histidine with a proline at position 1110 results in restriction of FANCA expression to the cytoplasm and a complete inability to activate the FA pathway. In contrast, Q1128E exhibits normal protein localization and FA pathway activation and as such is classified as a group I mutant. However, patients with this single base change have Fanconi Anaemia indicating other, as yet undefined roles for FANCA within the cell that must be disrupted by this mutation, which was why this particular mutation was chosen for analysis in this study.

Localization of these mutant proteins within HeLa and L β T2 cells was confirmed by tagging FANCA with the green fluorescent protein (GFP), from *Aequorea victoria*. This widely used reporter gene is utilized to track the expression, location and activity of specific proteins of interest within a host cell and is detected after excitation with light at a wavelength of 488 nm (Chalfie et al., 1994). Confocal microscopy analysis of the expression pattern of uncoupled GFP demonstrated localization of GFP within both the cytoplasm and nucleus of HeLa and L β T2 cells (See Figures 4.7 and 4.8, panels a and m). This pattern of protein distribution reflects the small size of GFP (~27 kD), which is capable of passively diffusing across the nuclear envelope after synthesis in the cytoplasm. FANCA protein tagged with GFP had a different expression pattern with the majority of GFP expression, and therefore localization of FANCA, being located within the nucleus, although there was some cytoplasmic expression in both HeLa and L β T2 cells (See Figures 4.6 and 4.7, panels b and j). These findings agree with the expression pattern of GFP-FANCA previously reported after transfection into HeLa, Cos-7 and NIH-3T3 cells (Lightfoot et al., 1999). Expression of a GFP-tagged FANCA construct containing a proline rather than a histidine residue at position 1110 (H1110P) results in no co-localization of GFP and Topro-3 signal within the nucleus, as FANCA-H1110P was completely restricted to the cytoplasm of both HeLa and L β T2 cells (See Figure 4.7 and 4.8, panels c and k). Expression of a GFP-tagged FANCA construct containing a glutamic acid rather than a glutamine at position 1128 (Q1128E) generated a different expression pattern profile within HeLa and L β T2 cells. In HeLa cells this mutant clearly localizes to the nucleus (Figure 4.7 l and p) and gives a similar localization profile to wild-type FANCA (See Figure 4.7, panels j and n). However, in L β T2 cells an even distribution of GFP expression was observed across the cell (Figure 4.8 l and p) as in the case of uncoupled-GFP (Figure 4.8, panels i and m). This cell specific difference indicates that the function of FANCA may differ, or that different FANCA-interacting proteins, are expressed in these cells.

Why a single base change from a histidine to a proline at position 1110 should result in the restriction of FANCA protein expression to the cytoplasm has yet to be determined. This mutation is located outside the putative NLS and NES signal sequences however, it is known that FANCA protein with this single base change fails to interact with FANCC or be phosphorylated, processes that may be required for transport of the FANCA into the nucleus (Kupfer et al., 1999; Adachi et al., 2002). The Q1128E mutation was also studied because this point mutation does not affect the interaction of the mutant FANCA protein with FANCC, FANCG or FANCF (Adachi et al., 2002). Whether this FANCA mutant can still interact with FANCE and FANCL has not been reported but since FANCD2 ubiquitination is restored when FANCA-Q1128E is transfected into FANCA null cells, it seems likely that interactions with other FA proteins are not disrupted (Adachi et al., 2002). FANCA-Q1128E is similar to 4 other reported mutations in this respect; all seem to activate the FA pathway yet patients are reported to have Fanconi Anaemia. The specific phenotype of the patient carrying the Q1128E mutation has not been reported, nor the identification of the FANCA mutation present in the second allele. As FA patients have a wide variety of clinical phenotypes, which are likely to be associated not only with genotype but also genetic background and environmental factors (Alter, 1993; Yamashita and Nakahata, 2001), determination of the relationships between specific mutations and clinical phenotypes would help in the understanding of the molecular basis of genotype-phenotype correlations associated with FA. Further analysis of mRNAs and proteins from patient cells carrying the specific Q1128E mutation may help to determine why this specific, single base change results in Fanconi Anaemia, whilst interactions of Q1128E with other FA and non-FA family members may help determine the pathogenicity of this mutation and the aetiology of Fanconi Anaemia.

4.3.4 Conclusions

This chapter is the first report detailing the localization of Fanca protein within L β T2 gonadotroph cells. Western blotting, indirect immunofluorescence and GFP tagging has been used to study, for the first time, the localization of Fanca protein within L β T2 gonadotroph cells. Studies using indirect immunofluorescence and western blotting analysis show that Fanca protein is localized to both the cytoplasm and nucleus of gonadotroph cells whereas analysis of GFP-tagged FANCA protein indicates that the protein is localized predominantly to the nucleus (See Figures 4.1, 4.2 and 4.8). Preliminary experiments designed to address transport of Fanca between the cytoplasm and nucleus of L β T2 cells have identified that active export of Fanca, via a CRM-1 dependent mechanism, occurs in L β T2 gonadotroph cells. However, as discussed earlier, whether GnRH is involved in shuttling of Fanca protein between sub-cellular compartments requires further investigation. Lastly, a specific, patient-derived, point mutation at amino acid 1110 (FANCA-H1110P) is sufficient to restrict Fanca protein expression to the cytoplasm of HeLa and L β T2 cells (See Figures 4.7 and 4.8, panels k and o). Interestingly, another mutation, FANCA-Q1128E, had a different expression pattern between cell types (See Figures 4.7 and 4.8, panels l and p). Within HeLa cells, a predominantly nuclear expression pattern was observed, as for wildtype FANCA. However, in L β T2 cells, localization of FANCA-Q1128E protein was not predominantly nuclear, since similar levels of protein expression were seen within the cytoplasm, giving a distribution pattern analogous to uncoupled GFP. These results indicate that other factors, specific to gonadotroph cells, may be involved in the correct localization and function of intact FANCA protein within these highly specialised cells.

Chapter 5

Fanca and gonadotropin gene expression

Knowledge is of no value unless you put it into practise – Anton Chekov.

5 Fanca and gonadotropin gene expression

5.1 Introduction

5.1.1 Background

LH and FSH are heterodimeric glycoproteins comprised of a common α -subunit (α GSU) and a unique beta subunit (LH β or FSH β). As each subunit is encoded by a single gene, their transcriptional regulation is complicated especially given that both LH and FSH are produced in, and released from, the same gonadotroph cells (Childs et al., 1987; Liu et al., 1988; Lloyd and Childs, 1988), but with very different patterns of synthesis and secretion, indicating that discrete cellular mechanisms must be in place to control their differential transcription. Regulation of gonadotropin subunit transcription occurs at two levels: basal and GnRH induced. Basal gene expression is switched on during development, whilst activation of the GnRH pulse generator at puberty results in the increased, pulsatile release of GnRH, which stimulates an increase in gonadotropin subunit expression. This process is demonstrated in *hpg* mice, which have basal levels of gonadotropin subunit gene expression, and have functional gonadotrophs, but require injections of GnRH to increase basal levels of subunit expression (Cattanach et al., 1977; Iddon et al., 1980). GnRH induced levels of gonadotropin gene transcription can be decreased to basal levels if GnRH action is blocked, indicating that the regulation of basal and GnRH induced transcription occurs via different mechanisms (McNeilly et al., 1991). The processes by which GnRH regulates gonadotropin gene transcription are not fully characterised, but it is known that variation in GnRH pulsatility is directly involved in the control of subunit specific gene transcription, as demonstrated in L β T2 gonadotroph cells where administration of pulsatile doses of GnRH results in secretion of LH but not FSH (Turgeon et al., 1996). Activation of the GnRH

receptor and coupling to second messenger signalling pathways also contributes to the differential regulation of gonadotropin gene transcription. Analysis of MAPK signalling cascades in gonadotroph cells reveals that signalling through ERK and activation of the MAPK-responsive transcription factor, Elk1 (Roberson et al., 1995), controls α Gsu transcription in response to GnRH. Control of GnRH induced *LH β* subunit transcription has been shown to be regulated by signalling through both ERK and JNK (Harris et al., 2002) and the up-regulation of *Egr1* (Dorn et al., 1999; Tremblay and Drouin, 1999; Wolfe and Call, 1999). Recently, ERK and JNK have also been implicated in GnRH signalling to the *FSH β* promoter via AP-1 sites (Bonfil et al., 2004; Coss et al., 2004). Table 5.1 and Figure 5.1 show some of the transcription factors that have been identified to be important for basal and GnRH induced gonadotropin subunit gene expression, but research is still ongoing to fully characterise how GnRH, steroids and gonadal peptides activate disparate intracellular signalling pathways to transduce specific transcriptional activation of gonadotropin subunit gene expression.

	Basal	GnRH regulated
<i>αGsu</i>	Lhx2/3 (Schoderbek et al., 1992; Roberson et al., 1994; Tremblay et al., 1998)	Elk1 (Roberson et al., 1995)
	Sfl (Barnhart and Mellon, 1994)	
	Gata (Dasen et al., 1999)	
	Pitx1 (Tremblay et al., 1998)	
	USF (Jackson et al., 1993; Jackson et al., 1995)	
<i>LHβ</i>	Sfl (Keri and Nilson, 1996)	Sp1 (Kaiser et al., 1998)
	Pitx1 (Tremblay et al., 1998; Quirk et al., 2001)	Egr1 (Tremblay and Drouin, 1999)
<i>FSHβ</i>	Lhx3 (West et al., 2004)	AP-1 (Strahl et al., 1998; Coss et al., 2004)
	Pitx1 (Zakaria et al., 2002)	

Table 5.1 Transcription factors shown to be involved in basal and/or GnRH regulation of gonadotropin subunit transcription.

Differential display RT-PCR (DD-RT-PCR) has been used to identify and isolate transcripts regulated by GnRH in gonadotroph cells, which may play a role in the differential regulation of gonadotropin gene transcription in response to GnRH. This approach revealed that *Fanconi Anaemia complementation group a (Fanca)* mRNA is differentially expressed in L β T2 gonadotroph cells in response to hormone (Chang, 2002). Data presented in this thesis has illustrated that GnRH tightly regulates the expression of both *Fanca* mRNA and protein in mature gonadotroph cells (See Chapter 3) and that *Fanca* protein is localized predominantly to the nucleus of L β T2 cells (See Chapter 4). *FANCA* is mutated in >60% of patients with Fanconi Anaemia (FA), an autosomal recessive disorder characterised by progressive bone marrow failure and a marked pre-disposition to cancer. A wide range of other symptoms are associated with the disorder including microcephaly, short stature and infertility (Young and Alter, 1994). Transgenic mice with targeted disruption of *Fanca* (Cheng et al., 2000; Wong et al., 2003), *Fancc* (Chen et al., 1996; Whitney et al., 1996) or *Fancg* (Yang et al., 2001) were generated to understand more about the aetiology of the disease, unfortunately neither single or double *Fanca*^{-/-}/*Fancc*^{-/-} gene disrupted mice (Noll et al., 2002) recapitulated the phenotype seen in human patients. Instead, the most striking phenotype in these knockout mice was a dramatic decrease in fertility. As discussed in Section 1.8, studies of post-pubertal FA patients reveal that both males and females are sub-fertile and have hypogonadism (Bargman et al., 1977; Alter et al., 1991; Liu et al., 1991). Gonadotropin serum levels have been measured in a limited number of FA patients and all had abnormal levels of both LH and FSH (Berkovitz et al., 1984; Schoof et al., 2000; Massa et al., 2002). However, abnormal gonadotropin levels may also be explained by disruption of the HPG-axis since the gonadal phenotype in *Fanca* and *Fancc* disrupted mice indicates that the genes are involved in germ cell development and proliferation (Cheng et al., 2000; Nadler and Braun, 2000). Nevertheless, the novel discovery that *Fanca* expression is regulated by GnRH in gonadotroph cells, together with its nuclear localization, suggests that *Fanca* may be involved, either directly or indirectly, in gonadotropin gene transcription.

5.1.2 Aims of this chapter

As previous data presented within this thesis has shown that *Fanca* is expressed within gonadotroph cells and that GnRH regulates the expression of both *Fanca* mRNA and protein, this chapter addresses whether expression of *Fanca* is required for the activation of α *Gsu*, *LH β* and *FSH β* promoters in response to GnRH. This chapter also seeks to determine if other members of the FA complex are expressed within gonadotroph cells and if the innovative technique of RNA interference (RNAi) can be used to knockdown expression of *Fanca* in gonadotroph cells.

5.2 Results

5.2.1 Mutating FANCA has no effect on LH β promoter activity

To assess whether FANCA affects basal or GnRH induced LH β transcription, -692 bp of the ovine LH β gene promoter linked to a luciferase reporter gene (-692LH β -pA3Luc) was co-transfected with vectors expressing either wildtype (pEGFP-FANCA) or mutant FANCA (pEGFP-H1110P-FANCA and pEGFP-Q1128E-FANCA). Cells were left untreated, or treated with 1 μ M GnRH 48 h after transfection and harvested 6 h post GnRH treatment, then luciferase activity was assayed as described in Section 2.5.3. As expected, GnRH significantly up-regulated the activity of the LH β promoter in L β T2 cells (ANOVA one-way analysis of variance, $p < 0.01$, see Figure 5.2). Co-transfection with either wildtype FANCA, FANCA-H1110P or FANCA-Q1128E had no effect on either the basal or GnRH induced activity of the promoter ($p < 0.05$, $p < 0.001$, see Figure 5.2).

5.2.2 Mutating FANCA increases FSH β basal promoter activity

The study the effect of FANCA on basal and GnRH induced FSH β transcription -4741 bp of the ovine FSH β gene promoter linked to a luciferase reporter gene (-4741FSH β -pXP2, kind gift from W.Miller) was co-transfected with vectors expressing either wildtype (pEGFP-FANCA) or mutant FANCA (pEGFP-H1110P-FANCA and pEGFP-Q1128E-FANCA). Cells were left untreated, or treated with 1 μ M GnRH 48 h after transfection and harvested 6 h post GnRH treatment. Co-transfection with wildtype FANCA had no effect on the basal activity of the promoter. However, co-transfection with either FANCA-H1110P or FANCA-Q1128E significantly increased the basal activity of the promoter cells when compared to cells co-transfected with wildtype FANCA ($p < 0.05$, see Figure 5.3). As expected, GnRH significantly up-regulated the activity of the FSH β promoter in

L β T2 cells (ANOVA one-way analysis of variance, $p < 0.001$, see Figure 5.3) and co-transfection with either wildtype FANCA, FANCA-H1110P or FANCA-Q1128E had no effect on the GnRH induced activity of the promoter ($p < 0.001$, see Figure 5.3). These results indicate that nuclear localization of a fully functional FANCA protein may be crucial for basal regulation of *FSH β* promoter activity.

5.2.3 Mutating FANCA obliterates GnRH induced α Gsu promoter activity

To determine whether Fanca effects basal and GnRH induced α Gsu transcription, -480 bp of the α Gsu gene promoter linked to a luciferase reporter gene (-480 α phapA3Luc) was co-transfected with vectors expressing either wildtype (pEGFP-FANCA) or mutant FANCA (pEGFP-H1110P-FANCA and pEGFP-Q1128E-FANCA). This promoter fragment is known to target α Gsu expression to the gonadotroph cells of transgenic mice (Horn et al., 1992) and contains both basal and GnRH responsive regions (Schoderbek et al., 1992). Cells were left untreated, or treated with 1 μ M GnRH 48 h after transfection and harvested 6 h post GnRH treatment. As expected, GnRH significantly up-regulated the activity of the α Gsu promoter in L β T2 cells (ANOVA one-way analysis of variance, $p < 0.01$, see Figure 5.4). Co-transfection with wildtype FANCA had no effect on the GnRH induced activity of the promoter ($p < 0.001$, see Figure 5.4). However, the GnRH induced activity of the promoter after co-transfection with either FANCA-H1110P or FANCA-Q1128E was significantly reduced when compared to the response after co-transfection with wildtype FANCA ($p < 0.05$, see Figure 5.4). These results indicate that nuclear localization of a fully functional FANCA protein is required for up-regulation of α Gsu promoter activity by GnRH.

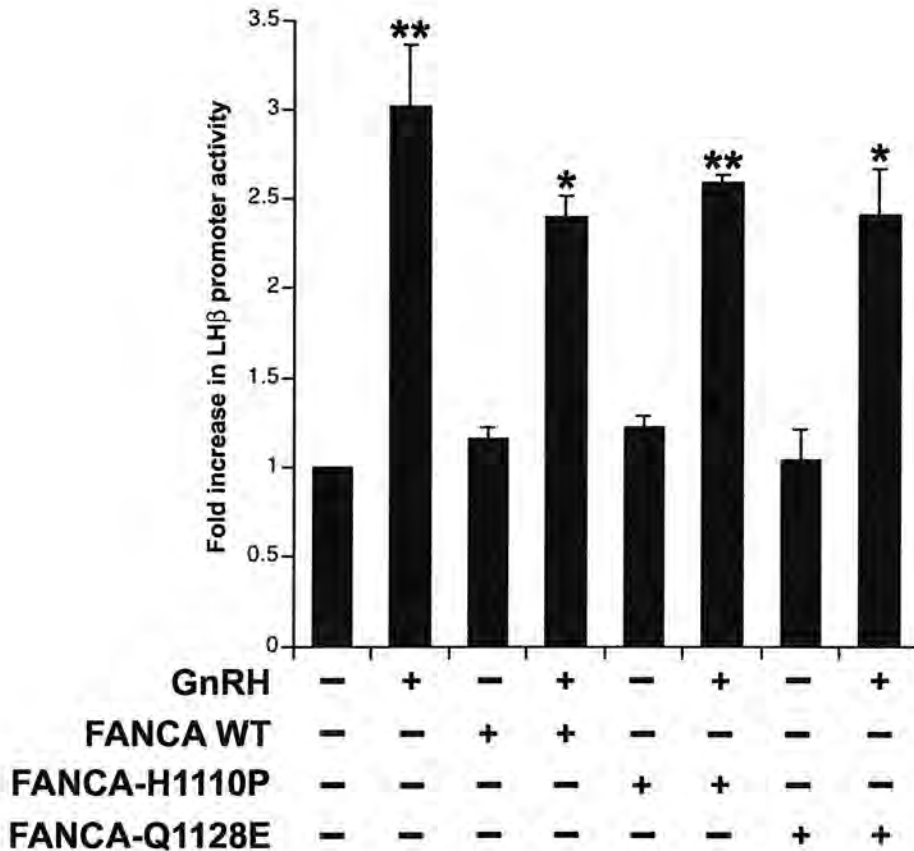


Figure 5.2 Effects of mutating FANCA on GnRH induced LHβ promoter activity in LβT2 cells.

A -692bp *LHβ* promoter construct linked to a luciferase reporter gene, and a control β-galactosidase reporter gene were transfected into LβT2 cells. Where indicated (+), cells were co-transfected with either wildtype (FANCA WT) or mutated FANCA (FANCA-H1110P or FANCA-Q1128E) expression vectors. After 48 h, cells were either left untreated (-) or treated with GnRH (+) before harvesting. The results are expressed as the fold induction of promoter activity and are corrected for transfection efficiency as determined by β-galactosidase expression. Basal promoter activity was not affected by addition of FANCA constructs. Addition of GnRH resulted in a significant 3-fold increase in promoter activity that was not affected when cells were co-transfected with FANCA WT, FANCA-H1110P or FANCA-Q1128E. Results shown are the mean of three separate experiments with error bars indicating the standard error of the mean. Statistical analysis was performed using ANOVA one-way analysis of variance: **, $p < 0.01$; *, $p < 0.05$.

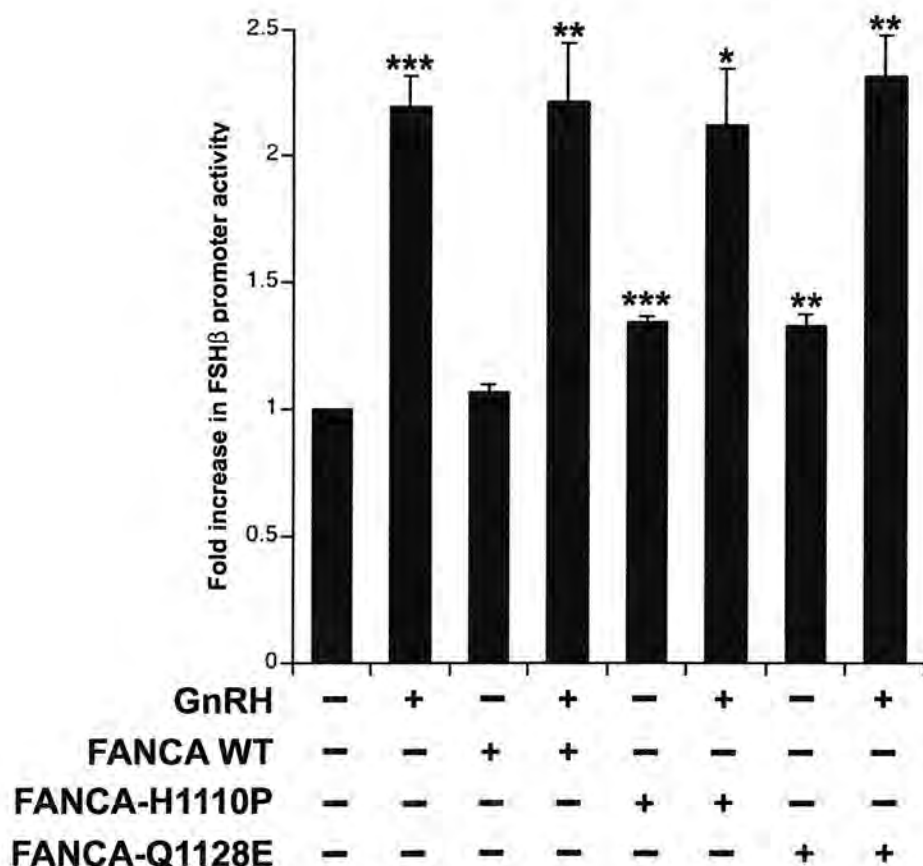


Figure 5.3 Effects of mutating FANCA on GnRH induced FSH β promoter activity in L β T2 cells.

A -4741bp *FSH β* promoter construct linked to a luciferase reporter gene, and a control β -galactosidase reporter gene were transfected into L β T2 cells. Where indicated (+), cells were co-transfected with either wildtype (FANCA WT) or mutated FANCA (FANCA-H1110P or FANCA-Q1128E) expression vectors. After 48 h, cells were either left untreated (-) or treated with GnRH (+) before harvesting. The results are expressed as the fold induction of promoter activity and are corrected for transfection efficiency as determined by β -galactosidase expression. Co-transfection with FANCA-H1110P or FANCA-Q1128E significantly increased basal activity of the FSH β promoter. Addition of GnRH resulted in a significant 2.5-fold increase in promoter activity that was still observed when cells were co-transfected with FANCA WT, FANCA-H1110P or FANCA-Q1128E. Results shown are the mean of three separate experiments with error bars indicating the standard error of the mean. Statistical analysis was performed using ANOVA one-way analysis of variance: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

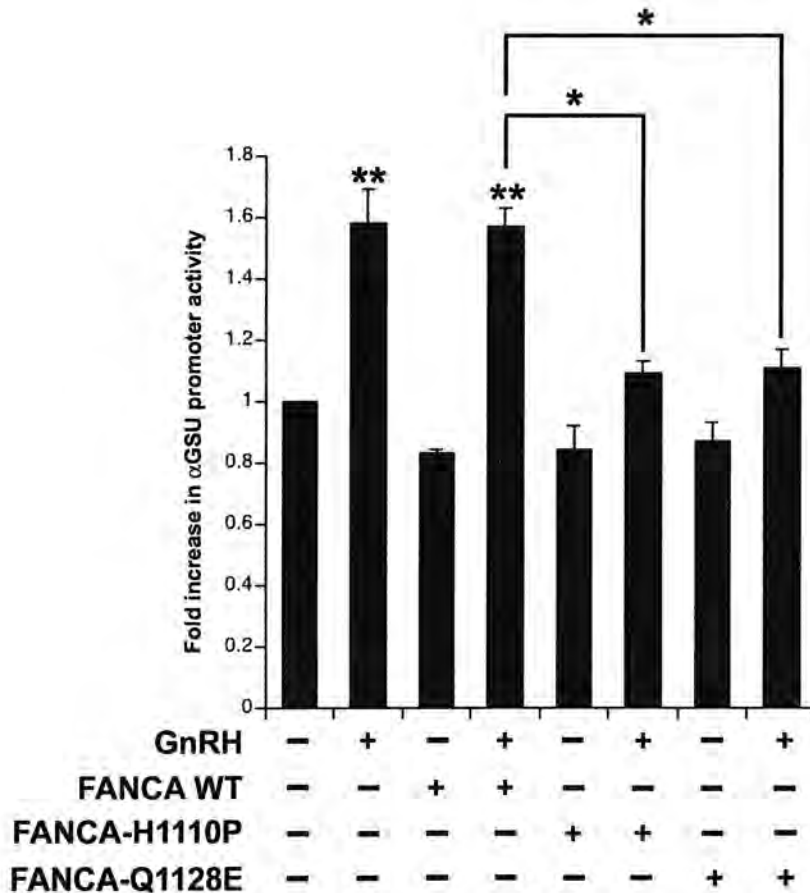


Figure 5.4 Effects of mutating FANCA on GnRH induced α Gsu promoter activity in L β T2 cells.

A -480bp α Gsu promoter construct linked to a luciferase reporter gene, and a control β -galactosidase reporter gene were transfected into L β T2 cells. Where indicated (+), cells were co-transfected with either wildtype (FANCA WT) or mutated FANCA (FANCA-H1110P or FANCA-Q1128E) expression vectors. After 48 h, cells were either left untreated (-) or treated with GnRH (-) before harvesting. The results are expressed as the fold induction of promoter activity and are corrected for transfection efficiency as determined by β -galactosidase expression. Basal promoter activity was not affected by addition of FANCA constructs. Addition of GnRH resulted in a significant 1.6-fold increase in promoter activity that was not affected when cells were co-transfected with FANCA WT. Co-transfection with FANCA-H1110P or FANCA-Q1128E resulted in a significant decrease in GnRH induced increase in promoter activity seen with FANCA WT. Results shown are the mean of three separate experiments with error bars indicating the standard error of the mean. Statistical analysis was performed using ANOVA one-way analysis of variance: **, $p < 0.01$; *, $p < 0.05$.

5.2.4 Design of siRNAs against Fanca

Co-transfection of dominant negative forms of FANCA results in ablation of the GnRH induced response of the α Gsu promoter (See Figure 5.4) and increases basal activity of the *FSH β* promoter (See Figure 5.3) indicating that FANCA is involved in gonadotropin subunit transcription. In order to analyse this in more detail, *Fanca* expression in L β T2 cells was knocked down using the technique of RNA interference (RNAi). Two short interfering RNAs (siRNAs) were designed, specific for murine *Fanca*, as described in Section 2.8 (Fanca-siRNA-A and Fanca-siRNA-B). As a negative control, a scrambled siRNA was also designed that BLAST analysis revealed did not match any known transcripts. Figure 5.5 details the sequence of the designed siRNAs and their target sequence within the *Fanca* gene.

5.2.5 RT-PCR analysis of Fanca mRNA expression in L β T2 cells treated with siRNAs

To ensure that the selected siRNAs specifically knocked down *Fanca* mRNA expression, RT-PCR was performed. L β T2 cells were transfected with 400 nM siRNA (Fanca-siRNA-A, Fanca-siRNA-B or scrambled siRNA), cultured for a further 24 h and RNA extracted. Reverse transcription was performed using an oligo dT primer, as described in Section 2.1.3, and cDNA was amplified using gene specific primers for *Fanca* (exons 14-18), *Zfp276* and *Gapdh* (See Table 2.1). Ethidium bromide staining identified bands at the expected sizes in all samples (See Figure 5.6). However, transfection with specific *Fanca* siRNAs resulted in a highly visible decrease in *Fanca* expression levels when compared against *Fanca* levels in untreated cells or cells treated with a scrambled siRNA. Transfection with specific *Fanca* siRNAs may have had a modest effect on expression levels of *Zfp276* but had no effect on *Gapdh* expression within L β T2 cells.

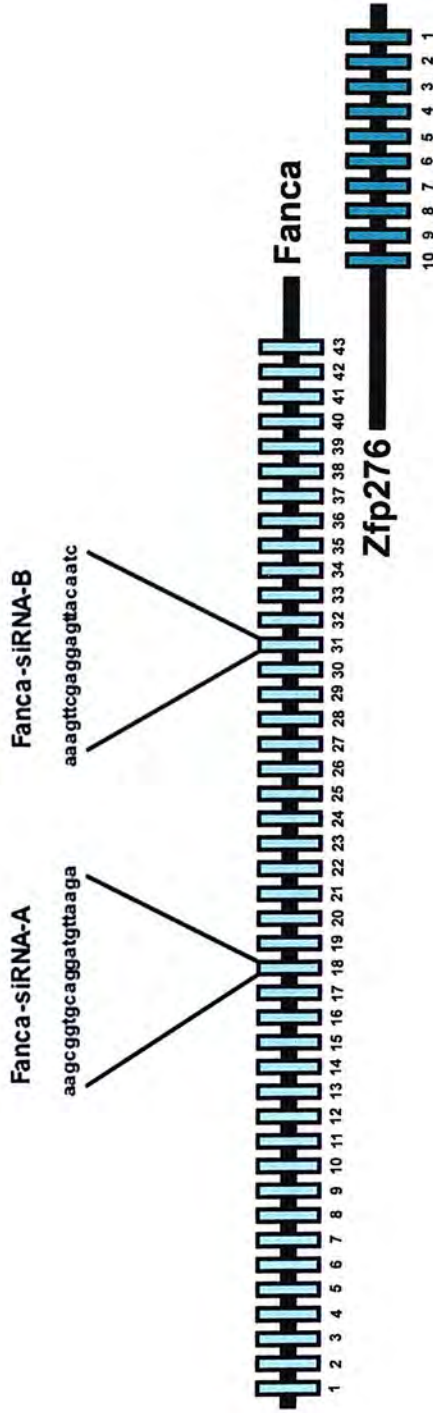


Figure 5.5. Schematic representation of the Fanca locus showing the location of siRNAs designed from Fanca sequence.

Exons are shown as light blue boxes for *Fanca* and green boxes for *Zfp276*. Exons 40-43 of *Fanca* overlap with the 3'UTR of *Zfp276*. The sense strand sequence of siRNAs designed to *Fanca* are indicated. Fanca-siRNA-A is designed from sequence in Exon 18 of *Fanca*. Fanca-siRNA-B is designed from sequence in Exon 31.

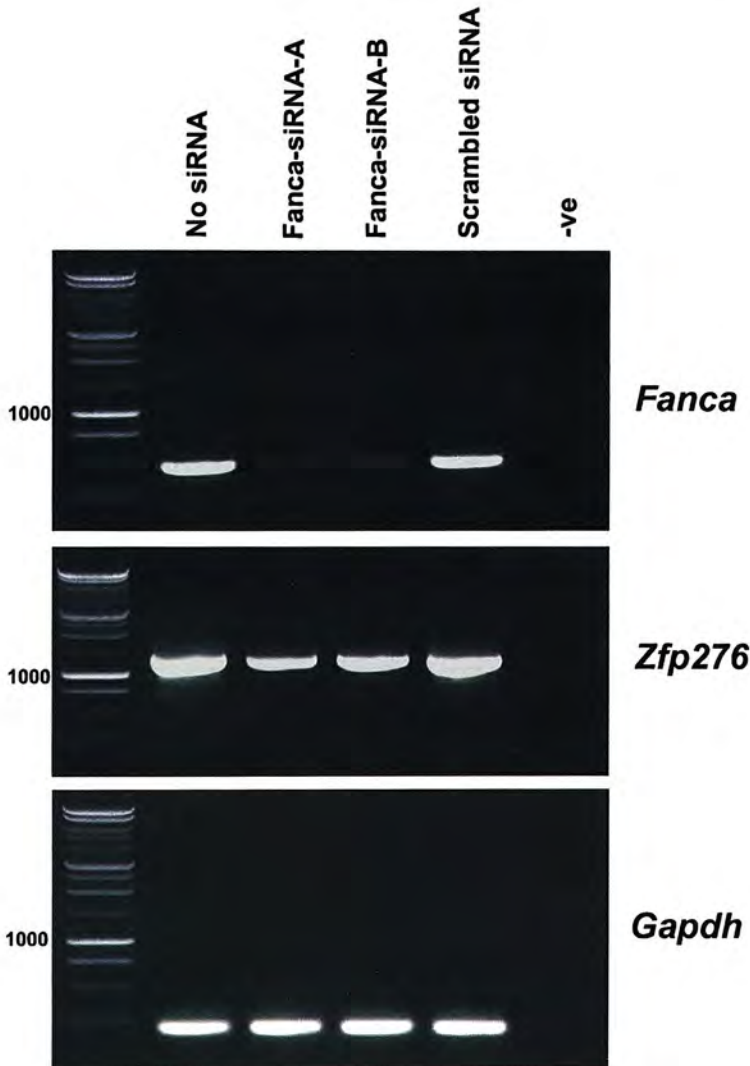


Figure 5.6 RT-PCR analysis of *Fanca* expression in L β T2 cells after transfection with siRNAs.

L β T2 cells were mock transfected (no siRNA) or transfected with an siRNA against *Fanca* (Fanca-siRNA-A or Fanca-siRNA-B) or a scrambled siRNA. After 24 h, RNA was extracted, reverse transcribed and first strand cDNA analysed for expression of *Fanca*, *Zfp276* and *Gapdh*. Ethidium bromide staining identified that all three genes were expressed in all samples tested. Transfection with siRNAs did not alter expression levels of *Gapdh* however, a highly visible decrease in *Fanca* mRNA expression and a modest decrease in *Zfp276* expression could be seen after transfection with *Fanca* specific siRNAs. A negative control containing no cDNA was included (-ve).

5.2.6 Indirect immunofluorescence of Fanca protein expression in L β T2 cells treated with siRNAs

To ensure that the selected siRNAs specifically knocked down Fanca protein expression, indirect immunofluorescence using rabbit anti-mouse Fanca antisera was performed on cells transfected with siRNAs. L β T2 cells, grown on 2-well glass chamber slides, were either left un-transfected, or transfected with 400 nM siRNA. After culturing for a further 48 h, cells were incubated overnight with rabbit-anti-mouse Fanca antisera specific for amino acids 1-276. The following day, Fanca protein was visualized by incubating with a goat anti-rabbit secondary antibody conjugated to biotin followed by a fluorescent probe conjugated to streptavidin (as described in Section 2.7.10). Nuclei were then counterstained with a fluorescent dye (Topro-3) and slides mounted before detection of fluorescence signal by confocal microscopy. L β T2 cells transfected with a non-specific, scrambled siRNA (Figure 5.6, panels j, l) showed similar levels of Fanca protein expression to that seen in un-transfected cells (Figure 5.6, panels a, c). In contrast, cells transfected with Fanca specific siRNAs had visibly decreased levels of Fanca protein expression (See Figure 5.6, panels d, f, g, i).

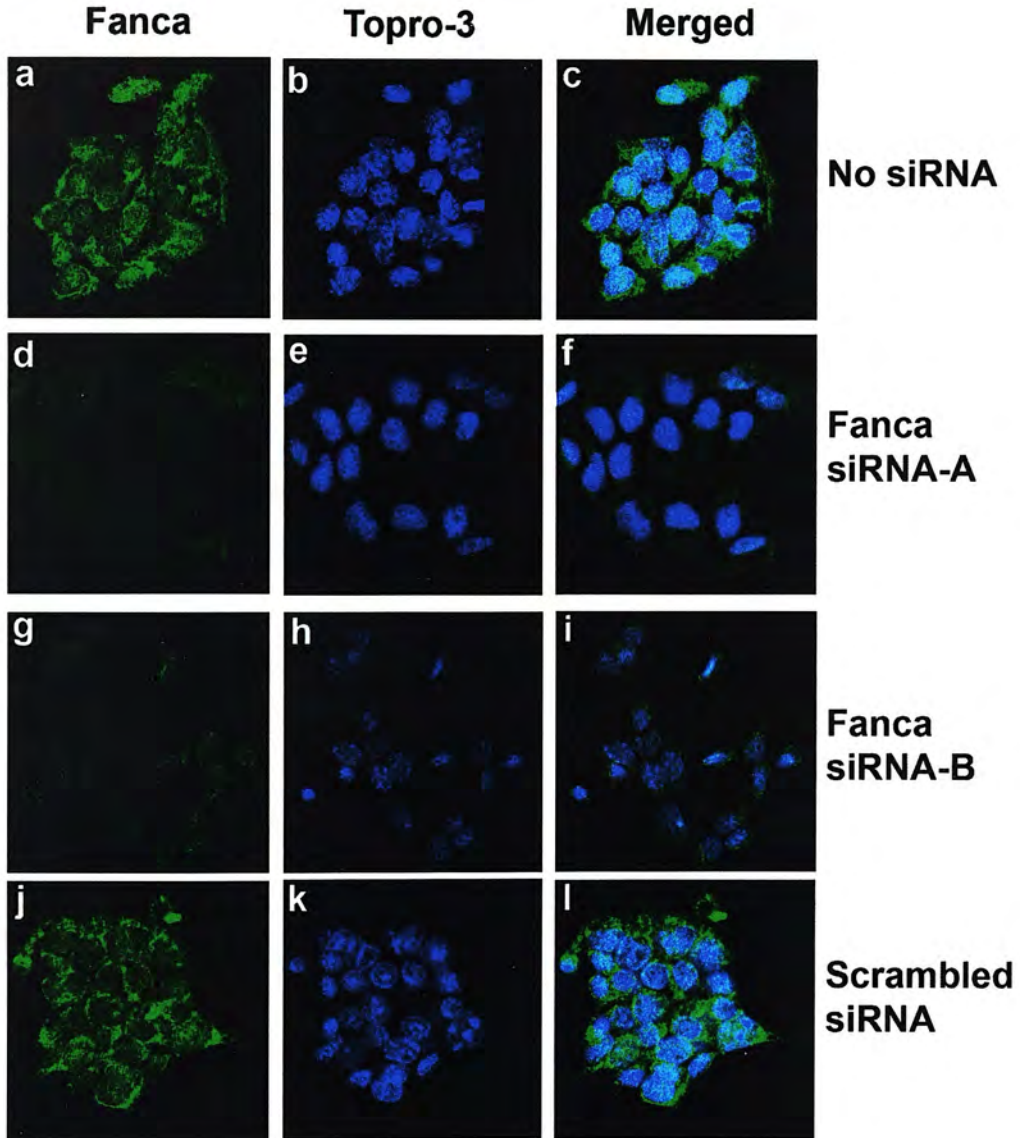


Figure 5.7 siRNA knockdown of Fanca protein expression in L β T2 cells.

Expression of Fanca protein within untreated and siRNA transfected L β T2 cells was determined using rabbit anti-mouse Fanca antisera and indirect immunofluorescence. Cell nuclei were identified by staining with Topro-3, a DNA specific dye (b,e,h,k). Expression of Fanca protein (green) is seen in un-transfected cells (a,c) and cells transfected with a non-specific, scrambled siRNA (j, l). Cells transfected with siRNAs specific to Fanca (Fanca-siRNA-A or B), have decreased levels of Fanca protein expression (d, f, g, i).

5.2.7 Western blotting analysis of Fanca protein expression in L β T2 cells after treatment with siRNAs

The 'knockdown' of endogenous Fanca protein within L β T2 cells was quantified by western blotting analysis. L β T2 cells were transfected with either 200 nM or 400 nM siRNA, cultured for a further 24 h then whole cell protein extracts prepared. Equal amounts of protein extract, as determined by Bradford assay, were then fractionated on a 6% SDS-PAGE gel, blotted and incubated with a rabbit anti-mouse Fanca antisera specific for amino acids 1-276. A specific protein band at ~160 kD, corresponding to the expected size of Fanca protein, was detected in all samples (See Figure 5.8). Blots were stripped, and then re-probed with a rabbit anti-mouse β -tubulin antibody to control for even loading of protein on to the gel (See Figure 5.8). Analysis revealed that Fanca protein levels were only significantly decreased after addition of siRNAs specific to *Fanca* and increasing the concentration of siRNA resulted in a greater decrease in protein expression. Quantification of protein levels using ImageQuant software and normalization of Fanca protein levels against β -tubulin protein levels revealed that Fanca protein expression in L β T2 cells was decreased 19% after treatment with 200nM Fanca-siRNA-A, 15% after treatment with 200 nM Fanca-siRNA-B, 33% after treatment with 400 nM Fanca-siRNA-A and 41% after treatment with 400 nM Fanca-siRNA-B, when compared to levels of expression in untreated L β T2 cells (See Figure 5.8, ANOVA one-way analysis of variance; $p < 0.05$, $p < 0.001$). Transfection of 400 nM of a scrambled siRNA did not significantly alter Fanca protein levels indicating that no non-specific knockdown of Fanca protein had occurred.

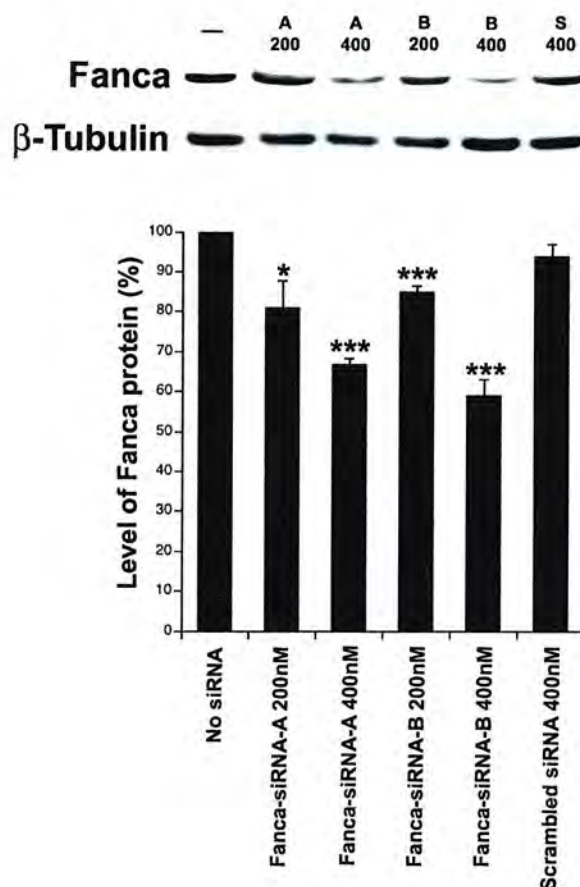


Figure 5.8 Western blotting analysis of siRNA knockdown of Fanca protein expression in L β T2 cells.

Western blotting analysis of whole cell L β T2 protein extracts transfected with siRNAs. Protein was fractionated on an SDS-PAGE gel, blotted and probed with anti-rabbit mouse Fanca antisera before being stripped and re-probed with a rabbit anti-mouse β -tubulin antibody. Probing with the Fanca antibody detected a protein band at the expected size of ~ 160 kD. Probing with a β -tubulin antibody gave a protein band at the expected size of ~ 55 kD. Normalization of Fanca protein levels against β -tubulin levels showed that Fanca protein expression was decreased after transfection with siRNAs specific to mouse Fanca (Fanca-siRNA-A or B). Transfection of a scrambled siRNA did not alter Fanca protein levels. Results show the mean of at least three separate experiments with error bars indicating the standard error of the mean. ANOVA one-way analysis of variance determined treatment with Fanca specific siRNAs significantly decreased Fanca protein levels: *, $p < 0.05$; ***, $p < 0.001$.

5.2.8 Generation of adenoviral vectors expressing wildtype and mutant FANCA

To investigate a link between Fanca protein expression and GnRH induced expression of α Gsu, it was necessary to develop a system that would target all gonadotroph cells. As α Gsu is expressed in >50% of gonadotroph cells (Liu et al., 2002) and L β T2 cells have a low transfection efficiency (P.Brown, personal communication), it is unlikely that transfection of dominant negative forms of FANCA or Fanca siRNAs would have a detectable impact on α Gsu protein expression. However, adenoviral infection of cellular monolayers is extremely efficient, with infection rates of >90% reported (Imperiale and Kochanek, 2004). Thus, to study the effects of over expression of dominant negative forms of FANCA (FANCA-H1110P and FANCA-Q1128E) within gonadotroph cells in more detail, adenoviral expression constructs expressing these proteins were generated as described in Section 2.9. The BD-Adeno-X Expression System 2 with BD Creator Technology (Clontech) was used to clone both wildtype and mutant FANCA inserts into an adenoviral vector using *Cre-loxP* site-specific recombination. The gene of interest is first cloned into a donor vector (pDNR-CMV), then using *Cre Recombinase* the insert is cloned into an adenoviral vector (pLP-Adeno-X) and the resulting virus propagated in HEK293 cells. Target cells (L β T2 cells) are then infected with this virus to achieve efficient transfer of the gene of interest and produce high levels of protein expression under standard laboratory conditions. Vector maps for the three constructs, pLP-Adeno-X-FANCA, pLP-Adeno-X-FANCA-H1110P and pLP-Adeno-X-FANCA-Q1128E are shown in Figure 5.9. Unfortunately, time constraints have meant that virus production has not yet begun but Section 5.3.3 discusses the future experiments that could be performed using this expression system.

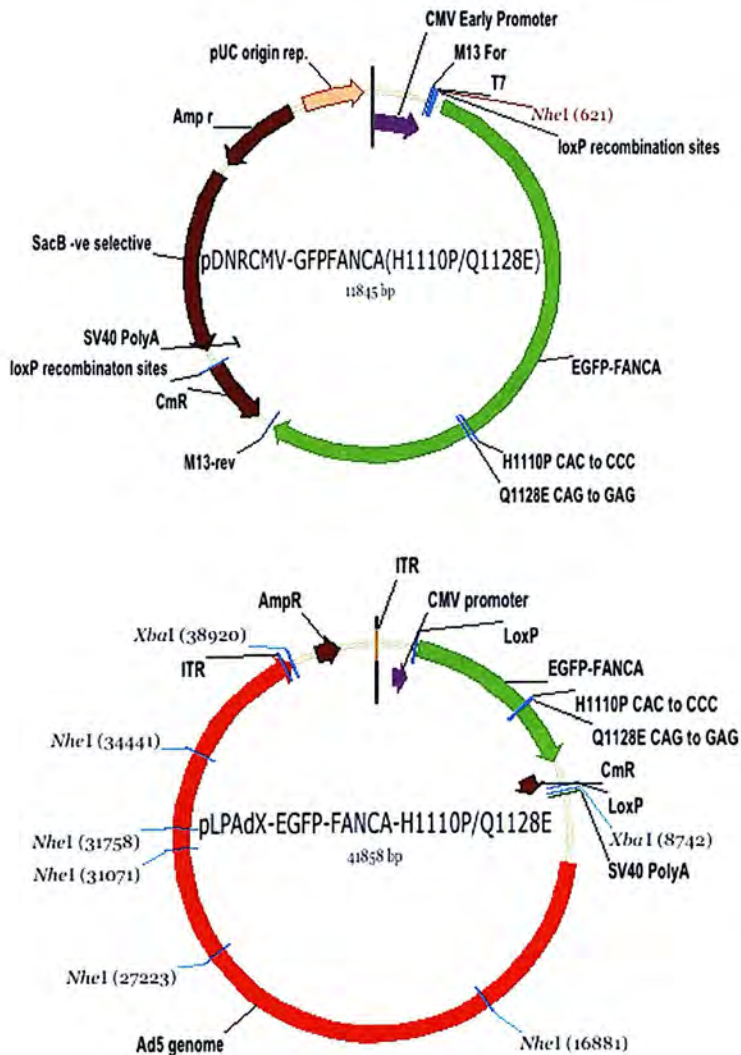


Figure 5.9 Vector maps of EGFP-FANCA adenoviral constructs.

EGFP tagged wildtype and mutant FANCA inserts were cloned into pDNR-CMV Donor vector via *NheI/XbaI* compatible sites. This was then transferred to the adenoviral acceptor vector (pLP-AdenoX) by *Cre-loxP* site specific recombination.

5.2.9 Expression analysis of Fanconi Anaemia complex members

FA proteins form a nuclear complex to regulate the cellular response to DNA damage (See Figure 1.14) (Garcia-Higuera et al., 2001), and defects in any one of the genes involved in the pathway results in an inability to process DNA damage repair effectively (D'Andrea and Grompe, 2003). With the recent discovery that Fanca is expressed within L β T2 gonadotroph cells (Chang, 2002; Larder et al., 2004), RT-PCR analysis was performed to determine if other FA complex members (*Fancc*, *Fancd2*, *Fance*, *Fanccf*, *Fancg* and *Fancl*) are also expressed in L β T2 cells. As a comparison, RNA was also extracted from an immature gonadotroph cell line (α T3-1), a rat somatotroph cell line (GH3) and human (HeLa) and mouse cell lines (L-cell). RNA was extracted, reverse transcribed and cDNA amplified using gene specific primers (See Table 2.1). Ethidium bromide staining was used to identify the presence of amplified DNA at the appropriate sizes for each PCR reaction and DNA sequencing of the cloned PCR products established the correct products had been amplified. *Fancd2* expression was detected in all cell lines tested. *Fanca* and *Fancc* expression was detected in all cell lines tested with the exception of α T3-1 cells. *Fancg* and *Fancl* expression was detected in all cell lines tested with the exception of a rat somatotroph cell line (GH3). *Fance* expression was detected in all cell lines tested with the exception of HeLa cells. *Fanccf* expression was detected in L β T2, α T3-1 and L-cells, but did not amplify from GH3 and HeLa cell cDNA. (See Figure 5.10).

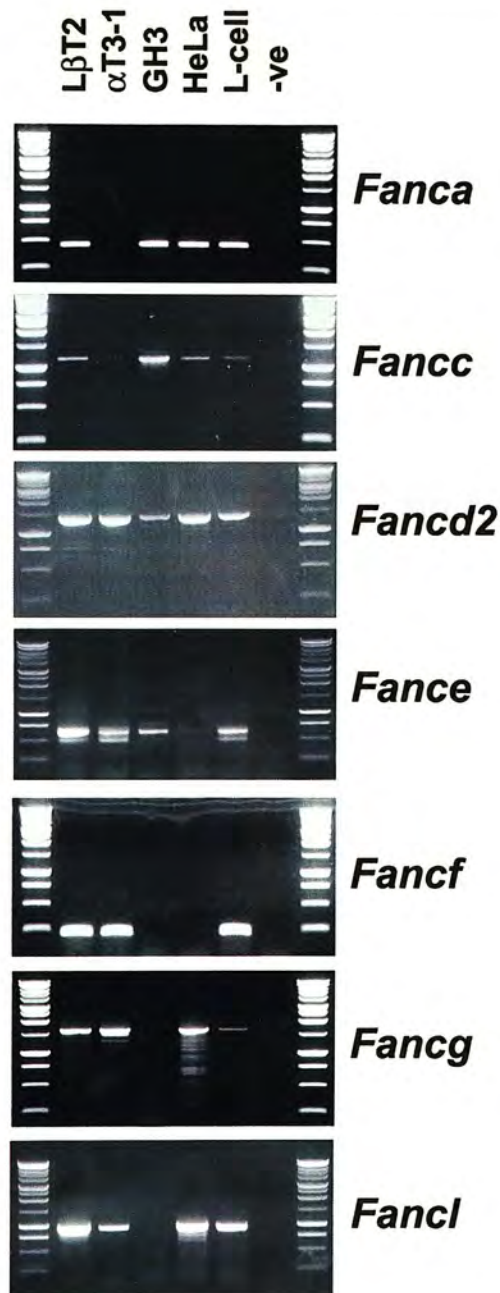


Figure 5.10 *In vitro* expression analysis of FA complex members.

RT-PCR analysis to show the expression profile of various FA family members. RNA was extracted from LβT2, αT3-1, GH3, HeLa and L-cells, reverse transcribed and first strand cDNA analysed for expression of *Fanca*, *Fancc*, *Fancd2*, *Fance*, *Fancl*, *Fancg* and *Fancl* using gene specific primers. A negative control containing no cDNA was included (-ve). Ethidium bromide staining identified PCR products of the expected size. Amplification of specific FA family members was confirmed by DNA sequencing.

5.2.10 Expression of *Fanca* and *Fancc* in α T3-1 cells augments the GnRH induced response of the α Gsu promoter

Expression of dominant negative forms of FANCA has shown that the presence of a fully functional FANCA protein is required for GnRH induction of the α Gsu promoter. As RT-PCR analysis has shown that α T3-1 cells, an immature gonadotroph cell line that expresses α Gsu and *Gnrhr*, express all known FA complex members other than *Fanca* and *Fancc* (See Figure 5.10), experiments were performed to determine if constitutive expression of these proteins within α T3-1 cells would have any affect on GnRH induced activity of the α Gsu promoter. *Fanca* and/or *Fancc* expression vectors were co-transfected with -480 bp of the α Gsu gene promoter linked to a luciferase reporter gene (-480 α -pA3Luc). Cells were left untreated, or treated with 1 μ M GnRH 48 h after transfection and harvested 6 h post GnRH treatment to allow luciferase activity to be assayed. Addition of GnRH resulted in a significant 3.5-fold increase in α Gsu promoter activity (See Figure 5.11, ANOVA one-way analysis of variance, $p < 0.001$). This increase was also observed if cells were co-transfected with either *Fanca* or *Fancc* (See Figure 5.11). However, co-transfection of both *Fanca* and *Fancc* with the α Gsu promoter resulted in augmentation of the GnRH response resulting in a 6.7-fold increase in α Gsu promoter activity, a significant increase when compared to the promoter activity in cells treated with GnRH alone or co-transfected with either *Fanca* or *Fancc* (See Figure 5.11, ANOVA one-way analysis of variance, $p < 0.01$).

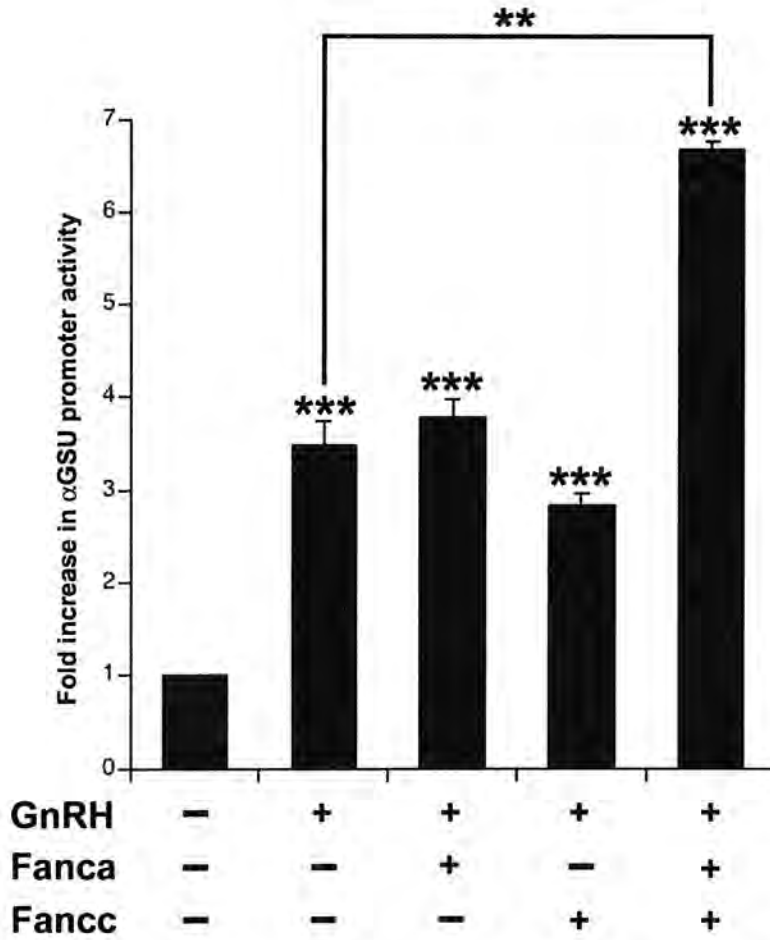


Figure 5.11 Effects of Fanca and Fancc on GnRH induced α Gsu promoter activity in α T3-1 cells.

A -480bp α Gsu promoter construct linked to a luciferase reported gene, and a control β -galactosidase reporter gene were transfected into α T3-1 cells. Where indicated (+), cells were co-transfected with either Fanca or Fancc. After 48 h, cells were either left untreated (-) or treated with GnRH (+) before harvesting. The results are expressed as the fold induction of promoter activity after addition of GnRH and are corrected for transfection efficiency as determined by β -galactosidase expression. Addition of GnRH resulted in a significant 3.5-fold increase in promoter activity. A similar increase was seen when cells were co-transfected with Fanca (3.8-fold) or Fancc (2.7-fold) then treated with GnRH. Co-transfection with both Fanca and Fancc resulted in a 6.7-fold increase in promoter activity upon addition of GnRH. Results shown are the mean of three separate experiments with error bars indicating +/- standard error of the mean. Statistical analysis was performed using ANOVA one-way analysis of variance: ***, p<0.001; **, p<0.01.

5.3 Discussion

5.3.1 *FANCA* is required for gonadotropin subunit gene transcription

Whilst the basal activity of the *LHβ* and *αGsu* promoters were not significantly altered by co-transfection with point-mutated forms of FANCA (See Figures 5.2 and 5.4), a small, but significant, increase in the basal activity of the *FSHβ* promoter was seen after co-transfection with either FANCA-H1110P or FANCA-Q1128E (See Figure 5.3). This suggests that FANCA may act as a specific signalling molecule to regulate basal levels of the *FSHβ* gene. Co-transfection of wildtype and mutant FANCA proteins with *FSHβ* promoter resections could be performed in order to localise the region of the promoter regulated by wildtype FANCA. Treatment with GnRH showed that all three promoters were hormone responsive and co-transfection with wildtype FANCA had no effect on either the basal or GnRH induced activity of the promoters (See Figures 5.2, 5.3 and 5.4). However, whilst the GnRH induced activity of the *LHβ* and *FSHβ* promoters were not significantly affected by co-transfection with point-mutated forms of FANCA, the significant 1.6-fold increase in *αGsu* promoter activity usually observed upon addition of GnRH was no longer measured if mutated forms of FANCA were co-expressed (See Figure 5.4), suggesting that FANCA may act as a specific signalling molecule to differentially regulate GnRH induced gonadotropin gene transcription.

Co-transfection with FANCA-H1110P blocked GnRH induced activity of the -480 bp *αGsu* promoter and increased basal activity of the *FSHβ* promoter. Expression of this mutant form of the protein is restricted to the cytoplasm of LβT2 gonadotroph cells (Chapter 4) and other cellular models (Kupfer et al., 1999; Adachi et al., 2002). However, as Fanca protein is normally localized to both the cytoplasm, and nucleus, of LβT2 cells (Chapter 4), these results, taken alone, suggest that localization of FANCA protein to the nucleus of LβT2 cells is required for control of *αGsu* and

FSH β promoter activity. Wildtype FANCA may be having a direct effect on α *Gsu* and *FSH β* transcription within the nucleus, or could be acting as a carrier molecule to transport a protein required for regulation of gonadotropin promoter activity, into the nucleus. Alternatively, wildtype FANCA may be signalling from its location within the cytoplasm of gonadotroph cells to control gonadotropin gene transcription within the nucleus and mutation of an amino acid at position 1110 disrupts this function. Support for the latter theory was obtained by co-transfecting the gonadotropin promoters with FANCA-Q1128E, which does not have an altered expression pattern as it still localizes to the nucleus of gonadotroph cells. This mutant FANCA protein also compromised GnRH induced activity of the α *Gsu* promoter and increased basal expression of the *FSH β* promoter suggesting that it may not be the ability of FANCA to localize to the nucleus that is crucial for control of α *Gsu* and *FSH β* promoter activity but that it is its capacity to function correctly within the cytoplasm that is important. As with the H1110P mutation, the Q1128E mutation could be altering the ability of FANCA to function as a signalling molecule and as both mutations are at the C-terminus of the protein this suggests that this region of the protein may be important for this process. Recently, yeast two-hybrid studies have shown that FANCA can interact with G-protein beta 2 subunit, a protein involved in signalling via the Ras/Raf pathway (Reuter et al., 2003). This pathway is activated by binding of GnRH to its receptor on the surface of gonadotroph cells and signals downstream to control transcription of the gonadotropin promoters (Benard et al., 2001; Harris et al., 2002; Bonfil et al., 2004). Confirmation of an interaction between Fanca and proteins involved in activation of the Ras/Raf pathway in gonadotroph cells may help determine whether Fanca is acting as a signalling molecule in response to GnRH.

As expression of Fanca protein is localized to the nucleus and recent studies have shown that the FA complex associates with chromatin (Qiao et al., 2001; Mi and Kupfer, 2004; Montes De Oca et al., 2004; Wang et al., 2004), chromatin immunoprecipitation (ChIP) assays could be performed to determine if FANCA is

functioning within the nucleus to control gonadotropin promoter activity by binding directly to the gonadotropin promoters. Yeast two-hybrid analysis also identified that FANCA can interact with several non-FA proteins (Reuter et al., 2003), some of which are involved in transcriptional regulation, suggesting that it may indirectly be involved in gonadotropin promoter regulation via interaction with other proteins, possible as part of a complex.

The evidence that FANCA is involved in both GnRH induced activity of the αGsu promoter and basal activity of the $FSH\beta$ promoter is very interesting. Analysis of transcription factors known to regulate gonadotropin gene transcription reveals that the LIM homeodomain (LIM-HD) proteins, Lhx2, Lhx3 and Lhx4, are involved in αGsu and $FSH\beta$, but not $LH\beta$, transcription (Roberson et al., 1994; Howard and Maurer, 2001; Sloop et al., 2001b; West et al., 2004). LIM-HD proteins have been shown to bind the pituitary glycoprotein hormone basal element (PGBE) of the αGsu promoter (Roberson et al., 1994; Bach et al., 1995; Howard and Maurer, 2001; Sloop et al., 2001a). However, evidence now suggests that within L β T2 cells, Lhx2 is not as essential for basal activation of the αGsu promoter as Pitx1 and Sfl are, but is involved in activation of the promoter in response to GnRH (P. Brown, personal communication). The role of LIM-HD proteins in $FSH\beta$ promoter activity has only recently been established with the identification of six LHX3 binding sites within the human $FSH\beta$ promoter that are required for its basal transcription (West et al., 2004). Whilst it is known that the response of the $FSH\beta$ promoter to activin does not require induction of the $LHX3$ gene, studies of the role of LHX3 in GnRH regulation of the promoter have not been analysed. As LIM-HD proteins are not involved in $LH\beta$ transcription (West et al., 2004), and mutation of FANCA only affects αGsu and $FSH\beta$ promoter activity, it is possible that the LIM-HD proteins and FANCA cooperate to regulate expression of these specific subunits. Yeast two-hybrid analysis of a pituitary cell library using FANCA as bait may help to identify specific proteins, such as the LIM-HD proteins, that interact with FANCA in gonadotroph cells, to activate gonadotropin promoter activity.

5.3.2 Role of other FA complex members in activation of α Gsu and FSH β promoter transcription

To determine whether Fanca may be interacting with other FA complex members to activate gonadotropin promoter activity it was important to first establish whether L β T2 cells express other FA genes. Fanconi Anaemia is a genetically heterogeneous disease with bi-allelic mutations in any one of eight identified genes (*FANCA*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG* and *FANCL*) resulting in the syndrome. With the relatively recent identification of *Fancd2*, *Fance*, *Fancf* and *Fancl*, little information is available regarding their expression profiles. However, northern blotting and *in situ* hybridisation analysis have described the expression patterns of *Fancc* (Wevrick et al., 1993; Krasnoshtein and Buchwald, 1996), *Fancg* (van de Vrugt et al., 2002), *Fancl/Brca2* (Rajan et al., 1997) and *Fanca* (Abu-Issa et al., 1999; van de Vrugt et al., 2000; Wong et al., 2000) in both embryonic and adult mouse tissues.

An RT-PCR screen for FA complex members was undertaken using L β T2 cell RNA (See Figure 5.10). As a comparison, the immature gonadotroph α T3-1 cell-line was also included along with somatotroph GH3 RNA and both human (HeLa) and mouse (L-cell) cell lines. Results revealed that only L β T2 cells expressed the full complement of all the currently identified FA complex members (See Figure 5.10). The lack of detectable *Fancf*, *Fancg* and *Fancl* expression in GH3 cells and *Fance* and *Fancf* expression in HeLa cells probably reflects mis-priming since the PCR primers were optimised for amplification of mouse sequence (See Table 2.1) and may not be ideal for amplifying rat or human cDNA. Western blotting analysis using antibodies against the Fanc proteins could identify if FA proteins are also expressed within gonadotroph cells.

RT-PCR analysis also demonstrated that unlike the other Fanc genes, expression of *Fanca* and *Fancc* is not detectable in α T3-1 cells, an immature, mouse gonadotroph cell line, indicating a possible role for these genes in mature gonadotroph function. To address this further, transient transfection assays were performed in α T3-1 cells to determine if constitutively expressed *Fanca* and *Fancc* would have any effect on GnRH induced activity of the α *Gsu* promoter, which is endogenously expressed in α T3-1 cells. Addition of GnRH resulted in a 3.5-fold increase in the activity of the promoter and this increase was unaffected by co-transfection of either *Fanca* or *Fancc* (See Figure 5.11). However, co-transfection with both *Fanca* and *Fancc* augmented the GnRH response giving a 6.7-fold increase in α *Gsu* promoter activity (See Figure 5.11). This suggests that *Fanca* and *Fancc* can cooperate, possibly with other FA family members, to control GnRH induced activity of the α *Gsu* promoter. This contrasts with the lack of effect of wildtype FANCA on α *Gsu* promoter activity in L β T2 cells (See Figure 5.4), which endogenously express both *Fanca* and *Fancc* (See Figure 5.10). Over expression of both *Fanca* and *Fancc* has not been performed in L β T2 cells so it has not been established whether augmentation of the GnRH induced α *Gsu* promoter, as seen in α T3-1 cells, would also be observed in mature gonadotroph cells. It is possible that both these proteins are required to transport in an additional, potentially regulatory, molecule into the nucleus of gonadotroph cells to activate the α *Gsu* promoter in response to GnRH. Conversely, the complex could be involved in the recruitment and deactivation of a protein that would otherwise repress GnRH induced activation of the α *Gsu* promoter and these hypotheses are discussed in more detail in Chapter Six. The transient transfection of point mutated forms of FANCA with *Fancc* could confirm co-operation between these genes in the control of α *Gsu* expression in α T3-1 cells and could identify whether *Fanca* or *Fancc* is the crucial factor involved in augmentation of the GnRH induced response in α *Gsu* promoter activity seen in these cells.

5.3.3 Analysis of gonadotropin levels in FA patients

Whilst FA patients have been reported with hypergonadotrophic as well as hypogonadotrophic hypogonadism (Cowdell et al., 1955; Stubbe and Prindull, 1975; Aynsley-Green et al., 1978), analysis of gonadotropin levels has only been reported in a few cases of FA (Berkovitz et al., 1984; Schoof et al., 2000; Massa et al., 2002). Although studies of gonadotropin levels in children with FA were not informative, probably due to GnRH stimulation tests being unreliable in children who are pre-pubertal (Kelch et al., 1980), analysis of adult FA patients reveals abnormal levels of LH and FSH in both sexes, with males also having reduced levels of testosterone (See Table 5.1).

	Sex	LH	FSH	T	Mutation	Reference
1	M	Normal	↑ increased	↓ decreased	Not known	(Massa et al., 2002)
2	F	↑ increased	↑ increased	N/A	Not known	(Berkovitz et al., 1984)
3	M	↑ increased	↑ increased	↓ decreased	Not known	(Berkovitz et al., 1984)
4	M	↓ decreased	↓ decreased	↓ decreased	FancD2	(Schoof et al., 2000)

Table 5.2 Analysis of LH, FSH and testosterone levels in four cases of FA.

M: male; F: female; LH: luteinising hormone; FSH: follicle stimulating hormone; T: testosterone.

As negative feedback mechanisms control gonadotropin levels (See Figure 1.2 and 1.3), it is difficult to determine whether the hypogonadism seen in FA patients and *Fanca*^{-/-}, *Fancc*^{-/-} and *Fancg*^{-/-} knockout mice is due to a primary defect involving the pituitary or the gonads as both a diminished amount of functioning gonadal tissue, as a result of defective DNA damage repair, or the abnormal regulation of gonadotropin gene transcription within the pituitary could result in gonadal dysgenesis. Data presented within this chapter has revealed for the first time that the hypogonadism seen in FA patients may be due to abnormal gonadotropin gene transcription within

the pituitary resulting in disruption to the HPG axis. With information concerning gonadotropin levels in FA patients, as well as knowledge concerning the specific mutations in these patients, a picture of the effects of loss of specific FA genes on the regulation of gonadotropin gene transcription could be established.

5.3.4 *Fanca* expression can be 'knocked down' in L β T2 cells using siRNAs

Having established that co-transfection of dominant negative FANCA molecules had a profound effect on basal *FSH β* activity and GnRH induced activity of the *α Gsu* promoter in L β T2 cells, an additional approach, such as the new technique of RNA interference (RNAi), was required to confirm the role of *Fanca* in *α Gsu* gene expression in gonadotroph cells. RNAi is the name of the process by which dsRNA silences gene expression by inducing the sequence specific degradation of complementary mRNA (Hannon, 2002). Figure 5.12 illustrates how RNAi-mediated gene silencing is thought to occur in mammals. Long dsRNAs are processed by Dicer, an evolutionarily conserved enzyme, into short interfering RNAs (siRNAs) of 21-23 nucleotides (nt) with 2-3nt overhangs at the 3' ends and 5' phosphate groups (Bernstein et al., 2001; Elbashir et al., 2001). These siRNAs associate with cellular proteins to form an RNA-induced silencing complex (RISC). This complex contains a helicase to unwind the duplexed siRNA (Nykanen et al., 2001) so the antisense strand of the siRNA can direct the cleavage of a corresponding sense RNA target or the sense strand of an siRNA can direct cleavage of an antisense target (Elbashir et al., 2001; Martinez et al., 2002). (See Figure 5.12). This process has been manipulated in *C.Elegans* to knockdown expression of specific genes (Sharp, 1999), however, introduction of dsRNA molecules longer than 30 bp into mammalian cells provokes the antiviral/interferon response pathway resulting in the global shutdown of protein synthesis (McManus and Sharp, 2002). To bypass this, chemically synthesized short dsRNA molecules of 21-22 nt, matching the target transcript can be transfected into cells to decrease expression of the gene of interest.

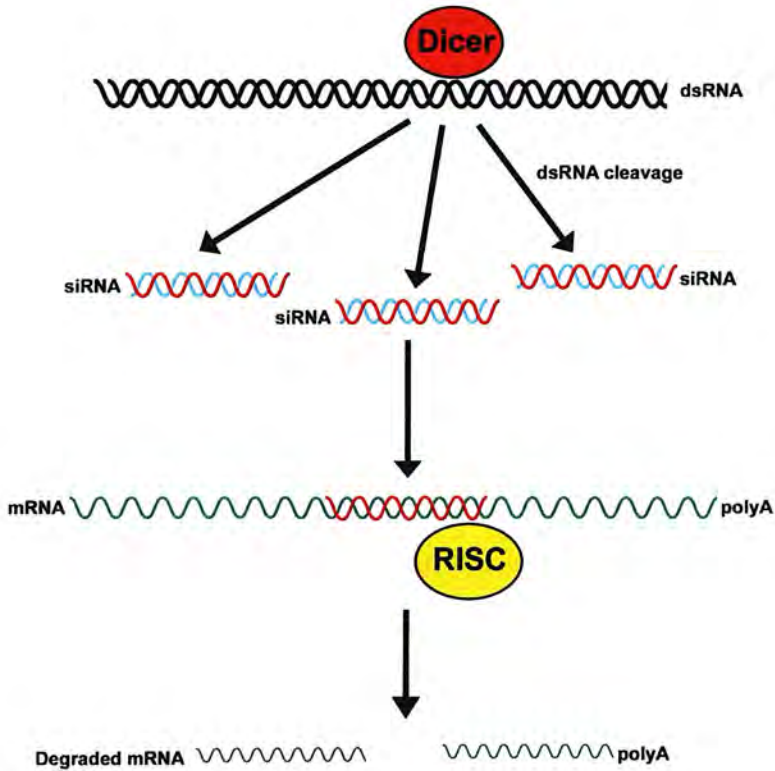


Figure 5.12 RNAi-mediated gene silencing.

Long dsRNAs are cleaved by Dicer to produce short interfering RNAs (siRNAs). These siRNAs then associate with cellular proteins to form an RNA-induced silencing complex (RISC) which unwinds the duplexed siRNA and guides it to the target mRNA for cleavage.

Two siRNAs were designed to knockdown expression of *Fanca* in L β T2 gonadotroph cells and due to the considerations outlined above, a series of experiments were undertaken to verify the siRNAs were specific for *Fanca*. Semi-quantitative RT-PCR was used to analyse *Fanca* mRNA levels in *Fanca* siRNA treated or scrambled siRNA treated cells. *Fanca* mRNA expression was only reduced in cells that had been treated with *Fanca* specific siRNAs (See Figure 5.6). Although *Zfp276* mRNA expression levels appeared to show a modest decrease in L β T2 cells with knocked down expression of *Fanca* (See Figure 5.6), quantitative RT-PCR analysis would need to be performed to fully analyse the effects of *Fanca* knockdown on *Zfp276* expression. For a complete picture of the effects a specific siRNA has on expression of the gene of interest protein levels must also be analysed in siRNA transfected cells thus, *Fanca* protein expression in siRNA treated cells was analysed using an antibody to the N-terminus of *Fanca* and indirect immunofluorescence. In cells treated with either of the *Fanca* specific siRNAs, *Fanca* protein expression was almost undetectable (See Figure 5.7, panels d, f, g, i). Whilst mock transfected cells, and cells transfected with a non-specific scrambled siRNA, showed normal levels of *Fanca* expression (See Figure 5.7, panels a, c, j, l). To quantify the decrease in *Fanca* protein expression after transfection with a siRNA, *Fanca* protein levels were normalized against levels of β -tubulin protein using western blotting analysis. L β T2 cells were transfected with either 200 nM or 400 nM of each siRNA, harvested for protein extracts after 24 h then *Fanca* and β -tubulin protein expression were measured. Increasing the concentration of *Fanca*-siRNA decreased *Fanca* protein expression with the highest concentration giving the greatest reduction (41% for *Fanca*-siRNA-B, See Figure 5.8). Levels of β -tubulin protein expression remained constant (See Figure 5.8) and no significant knockdown of either β -tubulin or *Fanca* protein expression was seen after transfection with 400 nM of a scrambled siRNA (See Figure 5.8) indicating that specific knockdown of *Fanca* had been achieved.

Since siRNAs have been defined as successful when a >90% reduction in protein levels is achieved (Schwarz et al., 2002), the 33% and 41% reduction in Fanca protein expression observed by western blotting analysis after transfection with *Fanca* specific siRNAs (See Figure 5.8) would suggest inefficient knockdown of Fanca protein expression. However, this may be due to two reasons, firstly, Fanca protein may be relatively stably expressed therefore analysis of Fanca protein levels after a longer time period (>24 h) may reveal a greater knockdown in Fanca protein expression. Additionally, as protein extracts for western blotting analysis are made from all the cells in the flask, and not all cells would be transfected with siRNA, it is conceivable that Fanca expression is unaffected in a considerable proportion of cells, which would still be detectable by western blotting analysis. Adenoviral vectors can achieve efficient gene delivery into hard to transfect cell lines by viral infection and could be utilised to achieve better transfection of L β T2 cells with siRNAs. siRNA plasmid expression vectors with selectable markers can enrich for stably transfected cells but repeated passaging of L β T2 cells tends to affect their GnRH responsiveness (P.Brown, personal communication), so this method of siRNA expression would be unsuitable in this cell type. With a specific siRNA (Fanca-siRNA-A or Fanca-siRNA-A) ablating Fanca expression in a large sample of cells, the requirement of Fanca for GnRH induced α Gsu transcription in L β T2 cells, as shown in transient transfection assays, could be confirmed.

5.3.5 Further studies using FANCA expressing adenoviral vectors

To investigate the effects of FANCA on gonadotropin gene expression, adenoviral vectors expressing wildtype and mutant forms of FANCA were constructed (See Figure 5.9). Infectious adenovirus is produced by transfecting *PacI* digested recombinant adenoviral vectors into HEK293 cells. Packaged adenovirus can then be used to infect target cells, such as L β T2 cells, with wildtype and mutant FANCA proteins. This technique gives a high rate of transfection efficiency (~90%) and will prove useful in the study of L β T2 cells. GnRH induced levels of α Gsu mRNA, in

cells infected with dominant negative FANCA adenovirus, could be studied to assess whether wildtype and mutant FANCA do indeed have a role in gonadotropin gene expression. Furthermore, the adenoviral constructs express GFP-tagged FANCA and can be used to study the sub-cellular localization of FANCA in response to GnRH.

5.3.6 Conclusions

This chapter has shown that FANCA may act as a specific signalling molecule to differentially regulate basal and GnRH induced gonadotropin gene transcription. Transient transfection assays have shown that FANCA regulates basal activity of the *FSH β* promoter and GnRH induced activity of the α *Gsu* promoter. Expression of *Fanca* and *Fancc* in immature gonadotroph cells, that do not express these members of the FA complex, results in amplification of the normal GnRH induced response of the α *Gsu* promoter suggesting that *Fanca* cooperates with *Fancc*, and possibly with other FA members, in order to control GnRH induced activity of the α *Gsu* promoter. Data presented in this chapter clearly shows that FANCA is crucial for GnRH regulation of α *Gsu* promoter activity and the control of basal levels of *FSH β* and may explain the molecular basis of the infertility phenotype seen in many FA patients. Whether this phenotype is solely due to the expression of gonadotropin subunits or is also associated with a role for the FA genes in a DNA damage repair pathway requires further investigation. With the development of adenoviral vectors expressing wildtype and mutant FANCA proteins and the characterisation of siRNAs that will specifically knockdown *Fanca* expression the novel role of *Fanca* in gonadotroph cells can be studied in more detail.

Chapter 6

General Discussion and Conclusions

Science is what you know. Philosophy is what you don't know – Bertrand Russell.

6 General Discussion

6.1 Introduction

Previously, differential display RT-PCR (DD-RT-PCR) identified that a transcript corresponding to *Fanconi Anaemia complementation group a (Fanca)*, a gene involved in DNA damage repair, was expressed in gonadotroph cells and regulated by GnRH (Chang, 2002). This thesis presents data that further characterises the effects of GnRH on *Fanca* mRNA and protein expression within gonadotroph cells and has investigated the impact of *Fanca* on gonadotropin subunit gene transcription. This chapter summarises the findings presented in this thesis, discusses the possible roles of this gene in the control of fertility, and the wider implications of these findings in the aetiology of Fanconi Anaemia.

6.1.1 Regulation of *Fanca* expression by GnRH

Detailed investigations of the GnRH regulation of *Fanca* mRNA and protein expression has demonstrated that, within L β T2 gonadotroph cells, both are rapidly and transiently increased in response to GnRH. *Fanca* mRNA increases 2-fold 1 h after addition of GnRH with expression returning to unstimulated levels 4 h after treatment. This increase is mirrored by an increase in *Fanca* protein expression with *Fanca* protein levels increasing 2-fold 2 h after treatment with hormone with expression returning to unstimulated levels by 6 h (Larder et al., 2004).

Mutations in *FANCA* account for >60% of cases of Fanconi Anaemia (FA) (Auerbach et al., 1998). Patients have a mean survival age of 20 years and are affected by a wide range of symptoms other than anaemia, such as short stature, microcephaly and pigmentation abnormalities. In patients that progress through puberty, infertility is a common clinical feature of the disease. FA is genetically heterogeneous with mutations in any one of eight identified genes resulting in FA

(Joenje et al., 1997). These structurally unrelated Fanc proteins interact to form a complex to ensure efficient DNA damage repair and checkpoint control of mitosis within the cell nucleus (D'Andrea and Grompe, 2003). RT-PCR expression analysis of L β T2 cells reveals that all identified members of the FA complex (*Fanca*, *Fancc*, *Fancd2*, *Fance*, *Fancl*, *Fancf*, *Fancg* and *Fancj*) are expressed in mature gonadotroph cells, although so far only *Fanca* has been shown to be regulated by GnRH. Further experiments are required to determine if GnRH also regulates the other members of the complex especially given that a recent paper identified that *Fancc* expression in L β T2 cells is down regulated 1 h after treatment with GnRH (Kakar et al., 2003).

6.1.2 Effects of knocking out *Fanca* expression in mice

Two transgenic mouse lines have been generated with targeted disruption of the *Fanca* gene (Cheng et al., 2000; Wong et al., 2003). In both cases these mice did not recapitulate the severity of the phenotype seen in human FA patients, however, like human FA patients, homozygous offspring from both these transgenic lines had severely reduced fertility. Both lines were hypogonadal with few or no follicles in the ovaries and Leydig cell hypoplasia in the testes. However, the second model did display a broader range of FA-like phenotypes, possibly due to a different genetic background, which included tumour formation, growth retardation, microcephaly, craniofacial malformations and severely reduced fertility (Wong et al., 2003). Furthermore, in this model, female mice developed ovarian cysts, a phenotype associated with elevated LH plasma levels in humans, and a clinical feature seen in some FA patients (Berkovitz et al., 1984; Massa et al., 2002). In the first FA knockout model, whilst α *Gsu* expression was not analysed, immunohistochemical studies of *Lh β* and *Fsh β* expression within the pituitary gland revealed no difference in expression between wildtype and knockout mice (Cheng et al., 2000), and unfortunately, were not investigated in the second model. Since analysis of gonadotropin levels proved inconclusive, pituitaries should be examined further to determine if gonadotroph cell numbers, expression of α *Gsu*, *Lh β* or *Fsh β* and levels

of LH and FSH are altered as a consequence of ablating *Fanca* expression. Data presented in this thesis has shown that wildtype FANCA is required for GnRH induced α *Gsu* transcription, suggesting that α *Gsu* levels within knockout mouse pituitaries may be decreased, which would also cause hypogonadism. Transient transfection assays have shown that FANCA also regulates basal levels of *FSH β* transcription. Mutation of FANCA results in a small, but significant increase in *FSH β* expression. As high concentrations of basal levels of FSH can lead to desensitisation of gonadal cells to the actions of FSH (O'Shaughnessy and Brown, 1978; Gnanaprakasam et al., 1979), a rise in *FSH β* levels, as a result of mutations in *FANCA*, could contribute to the hypogonadism seen in FA patients and knockout mice. Lastly, as a proportion of FA patients present with short stature due to reduced levels of growth hormone (Nordan et al., 1979; Young and Alter, 1994; Massa et al., 2002), and since the second knockout mouse model displayed growth retardation, it would also be interesting to study the development of somatotroph cells and analyse growth hormone levels in these *Fanca*^{-/-} mice to see if *Fanca* is required for the regulation of other pituitary hormones.

6.1.3 *Fanca*, GnRH and cancer

Patients with FA have a marked pre-disposition to cancer with a median age of tumour development at 16 years of age, which contrasts with 68 years of age for the general population (Alter, 2003). The most frequent types of cancer seen in FA patients are aerodigestive (>40%) or reproductive (>15%) (Alter, 2003). The expression of GnRH types I and II, and GnRH receptor type I, has been reported in both normal and cancerous tissues of the reproductive tract (Grundker et al., 2002) and in non-endocrine tumours such as glioblastomas and melanomas (van Groeninghen et al., 1998; Moretti et al., 2002). The activation of GnRH receptors in these tumours down-regulates cell proliferation and reduces the metastatic and invasive capabilities of cancerous cells (Miller et al., 1985; Limonta et al., 1992; Dondi et al., 1994; Moretti et al., 2002; Limonta et al., 2003). Recently it was

demonstrated that the apoptotic effect of GnRH in prostate cancer cells is mediated by JNK signalling (Kraus et al., 2004), and interestingly, GnRH is used as a therapeutic in prostate cancer (Conn and Crowley, 1994) and has been trialled as a second line therapy for advanced ovarian cancer (Balbi et al., 2004). Further investigations could determine if the regulation of Fanca expression by GnRH within gonadotroph cells also occurs within other GnRH receptor expressing tissues. Perhaps the anti-proliferative and anti-metastatic actions of GnRH on cancerous tissues are achieved through an up-regulation in Fanca expression? (See Figure 6.1). Analysis of the levels of Fanca expression in response to GnRH in normal and cancerous tissues could determine whether signalling by GnRH to Fanca contributes to the marked pre-disposition to cancer seen in FA patients with bi-allelic loss of FANCA mutations.

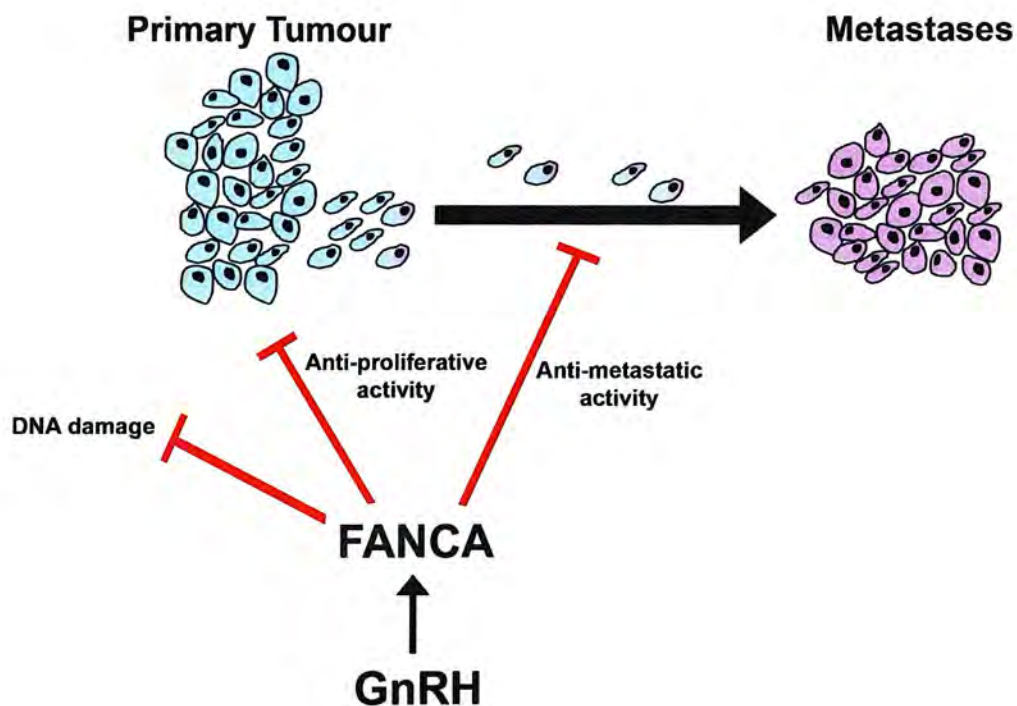


Figure 6.1 Hypothetical role of GnRH and FANCA in the control of tumour development.

Activation of GnRHR in cancer cells decreases the proliferation rate and metastatic capabilities of tumour cells. This process may occur via the up-regulation of FANCA expression in response to GnRH and could be a factor in the marked pre-disposition to cancer seen in FA patients, in addition to the role of FANCA in DNA damage repair.

6.1.4 *Fanca* protein expression in gonadotroph cells

This thesis presents the first report detailing the localization of *Fanca* protein within L β T2 gonadotroph cells. Western blotting analysis and indirect immunofluorescence localized expression of endogenous *Fanca* protein to both cellular compartments and addition of GnRH generated a significant 2-fold increase in *Fanca* protein levels in both the cytoplasm and nucleus. Interestingly, analysis of the expression pattern of FANCA protein tagged with GFP revealed that whilst expression of FANCA is observed within both cellular compartments, GFP-tagged FANCA appears to be localized predominantly to the nucleus of L β T2 cells. However, this difference in localization may be an artefact of the process of transfection resulting in an accumulation of FANCA protein within the nucleus therefore further analysis is required to determine why exogenous and endogenous *Fanca* protein localizes to different areas of the cell. Treatment of gonadotroph cells with inhibitors of nuclear import and export, and subsequent western blotting analysis of protein extracts from these cells, has shown that *Fanca* protein shuttles between the nucleus and cytoplasm of L β T2 gonadotroph cells via a CRM-1 dependent mechanism. Bioinformatic analysis of *Fanca* protein sequence predicted the presence of a putative nuclear export signal (NES) consensus sequence, at the C-terminus of the protein, which has been implicated in the binding of proteins to CRM-1 and their subsequent export to the cytoplasm. This is the first report to describe that *Fanca* protein shuttles between the nucleus and cytoplasm of cells via a specific mechanism. The ability of FANCA protein to translocate to the nucleus has been documented (Naf et al., 1998; Lightfoot et al., 1999) in contrast, movement of FANCA protein from the nucleus to the cytoplasm has not been studied. Mutational analysis of the putative NES sequence identified within *Fanca* would help determine the importance of this region in nuclear export of *Fanca* and could elucidate whether export of *Fanca* is crucial for its role in DNA damage and whether this shuttling is also associated with another role of the protein.

6.1.4.1 Does *Fanca* act as a molecular scaffold?

The localization of key signalling components is highly regulated during signal transduction with many signalling responses rapidly affecting the nuclear localization of various proteins, transcription factors and kinases (Lenormand et al., 1993; Beals et al., 1997; DiDonato et al., 1997; Khokhlatchev et al., 1998). In yeast a molecular scaffold protein, Ste5, is known to shuttle between the nucleus and cytoplasm in response to pheromone (Elion, 2001). Scaffold proteins physically connect various signal transduction components such as receptors, kinases and elements of the cytoskeleton into stable complexes (Pawson and Scott, 1997). In the absence of the yeast mating pheromone factor, Ste5 is localized to the nucleus of the cell. However, in the presence of pheromone, it is translocated to the plasma membrane where it activates a G-protein coupled receptor (GPCR). The receptor then activates the MAPK proteins bound to Ste5 and initiates the signalling mechanisms required to induce morphogenesis. The ability of *Fanca* to bind other proteins is well documented, and bioinformatic analysis of *Fanca* amino acid sequence has identified putative SH2 and SH3 domains, motifs known to be important for the function of scaffold proteins (Pawson, 2004). Taken together, with evidence presented in this thesis that *Fanca* protein shuttles between the nucleus and cytoplasm of gonadotroph cells, and a recent report demonstrating that FANCA interacts with a protein involved in signalling via the Ras/Raf pathway (Reuter et al., 2003), it is conceivable that *Fanca* may be acting as a molecular scaffold protein to elicit the regulation of gonadotropin gene transcription in response to GnRH (See Figure 6.2). However, further experiments are required to determine if GnRH is directly involved in the movement of *Fanca* between cellular compartments. Screening a yeast two-hybrid gonadotroph cell library using *Fanca* as bait could help identify new binding partners for *Fanca* and determine whether this hypothesis is true.

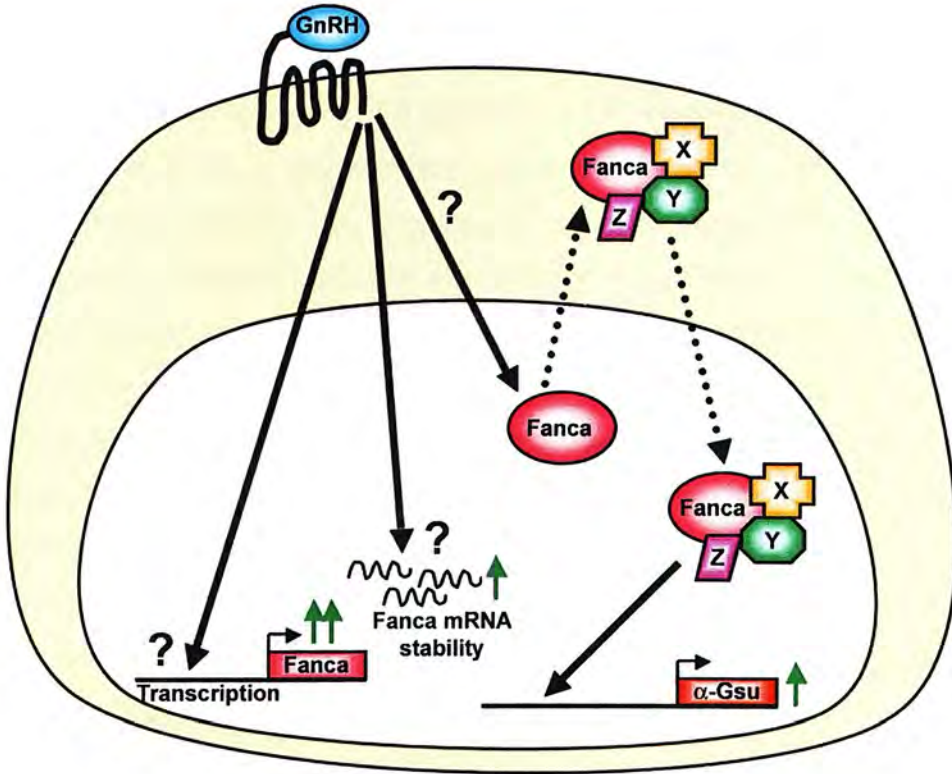


Figure 6.2 Hypothetical role of GnRH in the shuttling of Fanca protein.

In the absence of GnRH, Fanca protein is localised predominantly to the nucleus of L β T2 cells. Binding of GnRH to its receptor on the surface of gonadotroph cells increases Fanca mRNA levels within L β T2 cells, either by increasing Fanca transcription or stabilising Fanca mRNA (black lines). Transient transfection assays have shown that Fanca is required for GnRH induced α Gsu transcription. As in yeast, where pheromone regulates the shuttling of the molecular scaffold protein Ste5, from the nucleus to the cytoplasm, signalling through the GnRH receptor (black line) may regulate shuttling of Fanca between the nucleus and cytoplasm of L β T2 cells (dotted lines). This then allows various signal transduction components (X, Y and Z) to be brought together into a stable complex to regulate α Gsu transcription in response to GnRH.

6.1.4.2 *Is Fanca involved in GnRH receptor desensitization?*

The carboxy-terminal domain of G-protein coupled receptors (GPCRs) has an important role in regulating receptor activity including desensitization of G-protein mediated signalling, recruitment of G-protein independent signalling molecules, receptor internalization, recycling and degradation (Willars et al., 1999; Ferguson, 2001). It is known that binding of β -arrestin to the carboxy-terminal tail of a GPCR leads to the rapid desensitization and internalization of the agonist stimulated receptor. After internalization, receptors can either be sorted into endosomes for recycling back to the cell surface or alternatively may undergo degradation within lysosomes (Koenig and Edwardson, 1997). Non-mammalian type I GnRH receptors, which possess a C-terminal tail, undergo rapid desensitization and internalization (Heding et al., 1998; Pawson et al., 1998; Hislop et al., 2001). Consistent with the requirement of the carboxy-terminal tail for enhanced receptor desensitization and internalization, mammalian type I GnRH receptors, which uniquely lack a C-terminal tail, are desensitized and internalized slowly (Pawson et al., 1998; Willars et al., 1999). Whilst this process does not occur through β -arrestin, there is evidence to suggest that it is mediated via a clathrin dependent mechanism (Vrecl et al., 1998). A large family of cytoplasmic and membrane associated proteins called Sorting Nexins, are known to be involved in GPCR receptor recycling via clathrin coated pits (Worby and Dixon, 2002) and may be involved in the clathrin-dependent internalization of mammalian type I GnRH receptors. Interestingly, yeast two-hybrid analysis has shown that FANCA protein interacts with a member of this family, Sorting nexin 5 (SNX5), although the significance of this has yet to be determined (Otsuki et al., 1999; Reuter et al., 2003). Perhaps FANCA and SNX5 are both involved in rapid recycling of GnRHR to the cell membrane? Or maybe binding of FANCA to SNX5 blocks the internalization and recycling of the receptor resulting in continued signalling by GnRH? (See Figure 6.3). Expression of dominant negative forms of SNX5 and FANCA, and subsequent analysis of GnRHR internalization,

would be required to determine if this process is dependent on either of these proteins.

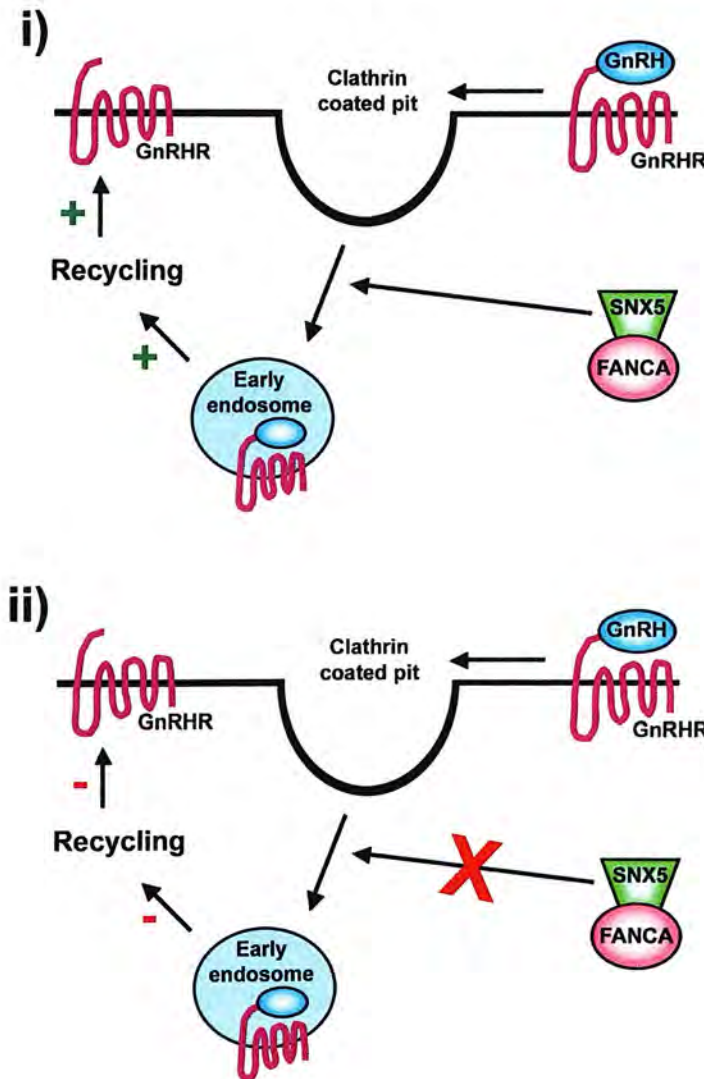


Figure 6.3 Hypothetical role of FANCA and SNX5 in recycling of GnRHR.

Mammalian GnRH receptor (GnRHR) desensitization and internalization occurs via a clathrin dependent pathway. Sorting nexin proteins may also be involved in this pathway. Yeast two-hybrid analysis has shown that FANCA protein interacts with Sorting nexin 5 (SNX5). This interaction may serve to assist (i) or prevent (ii) GnRHR internalization and thus affect gonadotropin subunit gene transcription.

6.1.5 *Fanca* and gonadotropin subunit transcription

The regulation of *Fanca* expression by GnRH, taken together with results showing that *Fanca* protein is expressed in both the cytoplasm and nucleus of gonadotroph cells, suggests that *Fanca* might be involved in the regulation of gonadotropin gene expression. Analysis of the effects of both wildtype and mutant forms of *FANCA* on gonadotropin subunit promoter activity reveals that whilst mutating *FANCA* had no effect on *LHβ* promoter activity, the expression of wildtype *FANCA* was required for GnRH induced activation of the α *Gsu* promoter and basal regulation of the *FSHβ* promoter in LβT2 cells.

This is the first report to show that *FANCA* is implicated in the transcription of genes involved in fertility. However, how *FANCA* regulates these promoters remains to be determined. Analysis of the data presented in this thesis produces several hypotheses to suggest a potential role of *Fanca* in gonadotroph cells. This chapter has already discussed the potential role of *Fanca* as a molecular scaffold and it is possible that *Fanca* may be functioning within the cytoplasm of gonadotroph cells to control signalling, from the GnRHR, to the nucleus in order to activate gonadotropin subunit transcription (See Section 6.1.4.1) and that the C-terminus of the protein is crucial in this process. Alternatively, *Fanca* may be active within the nucleus of LβT2 cells perhaps by binding directly to the gonadotropin promoters in order to regulate their activity. Alternatively, expression of wildtype *Fanca* within the nucleus may be required for binding of another protein involved in gonadotropin transcription. This hypothetical protein could either be involved in the activation of GnRH induced α *Gsu* promoter activity in the presence of *FANCA* or alternatively be prevented from repressing GnRH regulated promoter activity if bound to *FANCA* (See Figure 6.4). A similar situation could be occurring for the basal regulation of *FSHβ* promoter activity. Wildtype *FANCA* could activate a protein that is subsequently required for repression of basal transcription or conversely prevent an

activator from binding the promoter, thus keeping basal levels of expression low (See Figure 6.5).

To date, no FA genes have been directly implicated in transcriptional regulation however, interactions between FANCA and other proteins, such as the transcriptional repressors Inhibitor of NF κ B (I κ B γ) and Fas death domain associated protein (DAXX) are well documented (Reuter et al., 2003). This suggests that it may be the interaction of FANCA with other proteins that controls the activity of the gonadotropin promoters. The two mutant FANCA proteins studied here indicate that specific mutations affecting the C-terminus of the protein severely compromise the activity of the α Gsu promoter in response to GnRH as well as basal regulation of the FSH β promoter. Perhaps this region of FANCA is required for binding of another protein involved in activation of these promoters? It would be informative to study the effects of other FANCA mutations on α Gsu promoter activity to establish if a specific region of FANCA is required for activation of these promoters. Yeast two-hybrid analysis of a gonadotroph cell library using full length FANCA as bait could help identify gonadotroph specific proteins that interact with FANCA and may be involved in α Gsu and/or FSH β transcription in L β T2 cells.

RT-PCR analysis reveals that α T3-1 cells do not express *Fanca* or *Fancc*, however; transient transfection of both these molecules into α T3-1 cells augments the GnRH response of the α Gsu promoter. This suggests that *Fanca* and *Fancc* are cooperating to produce an increase in promoter activity, in response to GnRH, in these cells and this cooperation may also be occurring within L β T2 cells, which endogenously express *Fanca* and *Fancc*. Co-transfection of mutant FANCA and FANCC molecules into both cell types would establish whether expression of both proteins is required for regulation of the α Gsu promoter by GnRH.

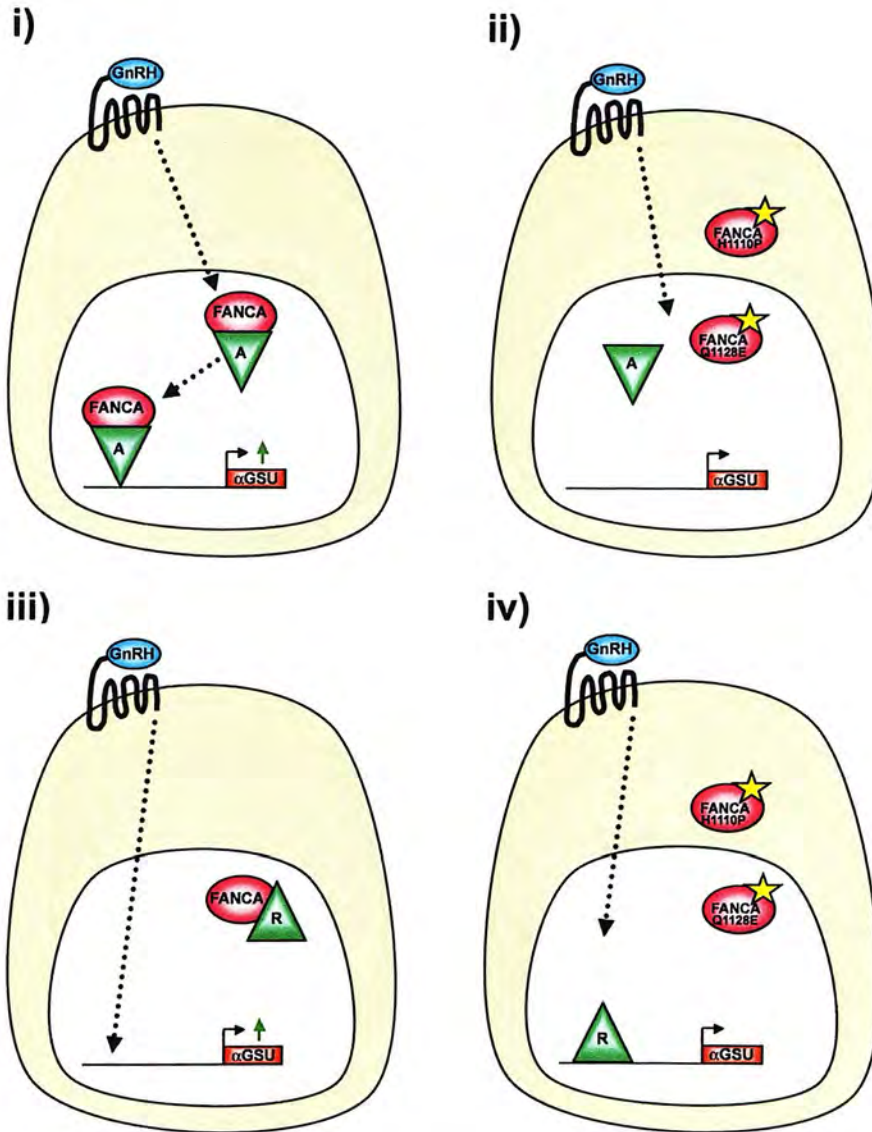


Figure 6.4 Hypothetical role of FANCA in GnRH induced α GSU promoter activity in L β T2 cells.

Wildtype FANCA is required for GnRH induced activity of the α GSU promoter. FANCA may be interacting directly with the promoter to regulate GnRH induced activity or alternatively, interaction of FANCA with either an activator (A) or repressor protein (R) may control promoter activity (i and iii). If FANCA is mutated then this may affect interaction with an activator protein (ii) and prevent GnRH induced activation of the α GSU promoter. Alternatively, mutation may prevent interaction with a repressor protein (iv) allowing the protein to bind the promoter and prevent GnRH induced activation by other factors.

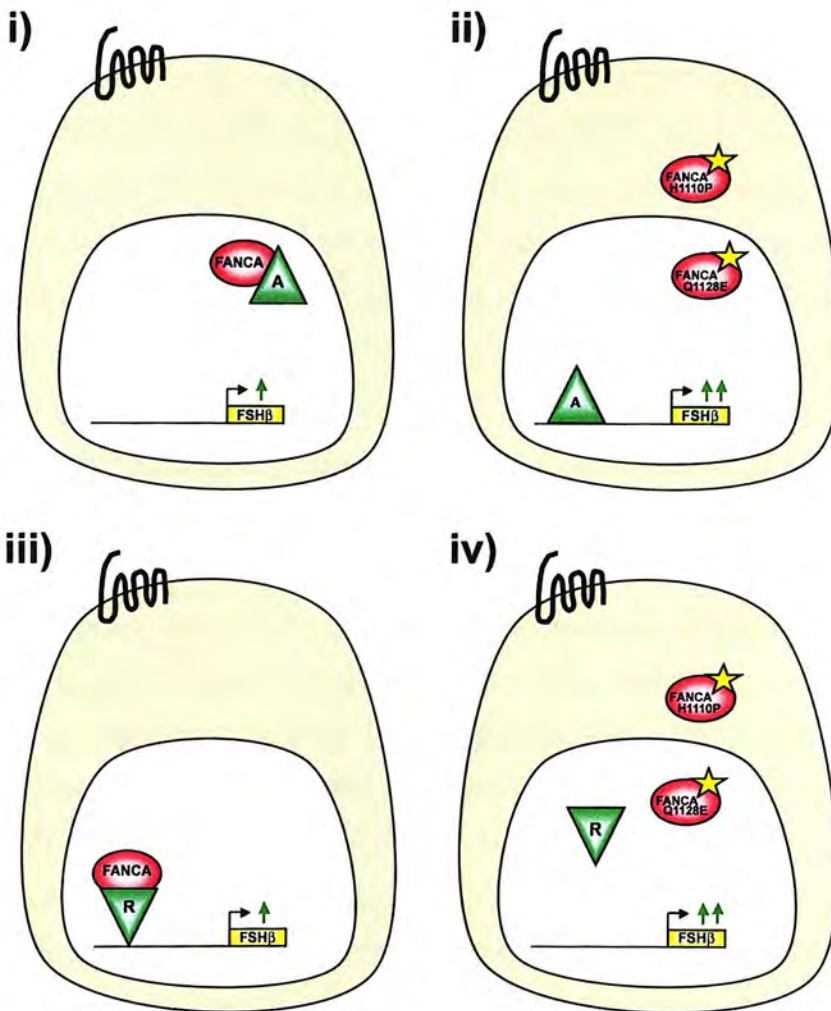


Figure 6.5 Hypothetical role of FANCA in basal $FSH\beta$ promoter transcription in $L\beta T2$ cells.

Wildtype FANCA is required for basal activity of the $FSH\beta$ promoter. FANCA may be interacting directly with the promoter to regulate its activity or alternatively, interaction of FANCA with either an activator (A) or repressor protein (R) may control promoter activity (i and iii). If FANCA is mutated then this may affect interaction with an activator protein (ii) and disrupt correct basal regulation of the $FSH\beta$ promoter. Alternatively, mutation may prevent interaction with a repressor protein (iv) that is controlling basal transcription of the $FSH\beta$ promoter leading to an up-regulation in basal $FSH\beta$ expression.

6.1.6 Role of the FA complex in transcriptional regulation via ubiquitination

Studies to try and elucidate the DNA damage phenotype seen in FA patients have identified that a nuclear, multi-protein complex of five FA proteins (FANCA, C, E, F and G) is required for the mono-ubiquitination of FANCD2 by FANCL, which promotes activated FANCD2 to interact with the DNA damage repair proteins BRCA1 and BRCA2/FANCD1 (D'Andrea and Grompe, 2003; Grompe, 2003). RT-PCR analysis has shown that all the identified *Fanc* genes, including the ubiquitin ligase enzyme *Fancl*, are expressed within L β T2 cells. Ubiquitin is a seventy-six amino acid polypeptide that can be covalently attached to proteins through the hierarchical action of three enzymes termed, ubiquitin-activating enzyme (E1), ubiquitin-conjugation enzyme (E2) and ubiquitin-protein ligase (E3) (Pickart, 2001). The best defined role of ubiquitin is its ability to label a protein with multiple ubiquitins (poly-ubiquitination) and thus target proteins for degradation via the proteasome (Pickart, 2001). Ubiquitin also has another major role in the endocytosis and subsequent trafficking of plasma membrane proteins and this is thought to occur via mono-ubiquitination of the target protein (Raiborg et al., 2003), as in the case of FANCD2 (Garcia-Higuera et al., 2001; Taniguchi et al., 2002). Ubiquitin is also a regulator of transcriptional activation of LIM-HD proteins, ubiquitination regulates binding of Lhx3 to a repressing co-factor rather than an activating one (Ostendorff et al., 2002) and interestingly, Lhx3 has been shown to regulate both α Gsu and *FSH β* gene expression (Roberson et al., 1994; Howard and Maurer, 2001; West et al., 2004). Recent studies have shown that some GPCRs can undergo regulated ubiquitination in response to activation by ligand (Chaturvedi et al., 2001; Petaja-Repo et al., 2001; Cook et al., 2003) although there are no studies analysing agonist-regulated ubiquitination of the GnRH receptor. However, the role of ubiquitin in the regulation of proteins downstream of GnRHR has been analysed in α T3-1 cells. The continued stimulation of the GnRHR results in a dramatic increase in Inositol 1,4,5-triphosphate [Ins(1,4,5)P₃] receptor poly-ubiquitination, leading to the suppression of Ca²⁺ mobilization and consequently, suppression of LH and FSH secretion (McArdle et al., 2002; Wojcikiewicz et al., 2003).

This thesis has shown that, within L β T2 cells, GnRH up-regulates expression of Fanca, one of the FA complex members required for ubiquitination of FANCD2 by FANCL (Garcia-Higuera et al., 2001; Meetei et al., 2004) and that Fanca is involved in gonadotropin subunit transcription. Taken together with the diverse roles of ubiquitin in the regulation of receptor signalling and transcriptional activation it is conceivable that the effects of Fanca on gonadotropin subunit transcription may be mediated through the FA complex and its role in ubiquitination of proteins. Perhaps the FA complex is involved in the ubiquitination and consequent degradation of transcription factors involved in basal *FSH β* transcription, a process that is disrupted by mutation of FANCA resulting in an increase in *FSH β* transcription. Conversely, the FA complex could be involved in the ubiquitination of repressor co-factors of transcription factors required for GnRH induced *α Gsu* transcription. Alternatively, the FA complex may be involved in the ubiquitination of signalling protein pathways initiated in response to GnRHR activation that control gonadotropin subunit transcription. (See Figure 6.6). However, as FANCL was only recently identified as the ligase thought to be responsible for FANCD2 ubiquitination, further studies into the role of this ligase in ubiquitination are required before the role of the FA complex in ubiquitination of proteins involved in GnRH signalling can be determined.

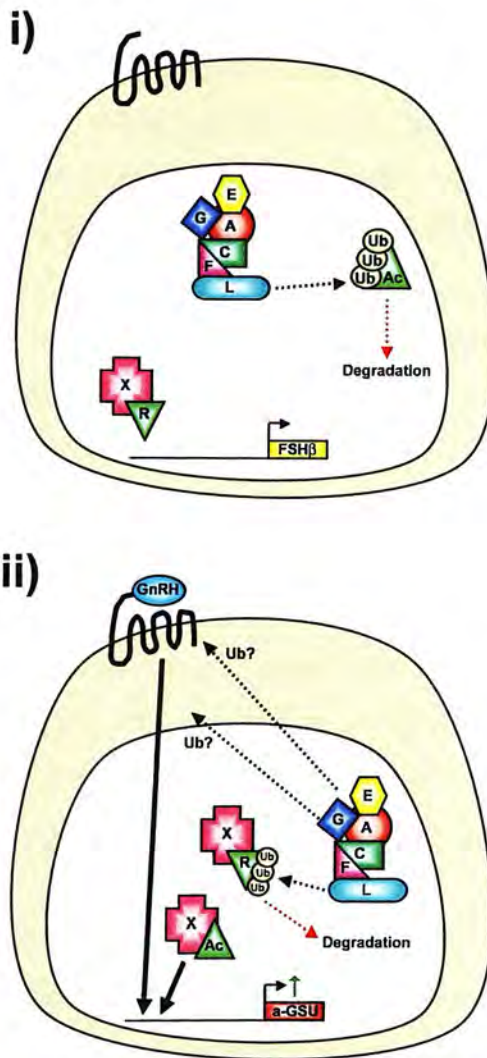


Figure 6.6 Hypothetical role of the FA complex in control of gonadotropin gene transcription.

FANCA (A) forms a complex in the nucleus of cells with other FA proteins (C, G, E, F and L) to ubiquitinate FANCD2. This complex may also regulate gonadotropin transcription via ubiquitination (Ub). Control of *FSHβ* transcription may occur via targeting of an activator protein (Ac) for degradation such that a repressor co-factor (R) can bind transcription factor X thus regulating activation of the promoter (i). Control of GnRH induced activation of α GSU promoter may occur at several levels. Firstly, the FA complex may be involved in ubiquitination of the receptor itself. Secondly it may control signalling from the receptor by ubiquitination of downstream proteins. Lastly it may ubiquitinate a repressor co-factor bound to protein X such that the repressor protein is targeted for degradation and an activating co-factor can bind and regulate promoter activity (ii).

6.1.7 FA genotypes and phenotypes

Data presented within this thesis reveals, for the first time, that FANCA is required for processes other than DNA damage repair, namely gonadotropin gene transcription. This discovery may partly explain the reduced fertility seen in the majority of FA patients. Further analysis using mutated forms of other FA complex members would help to determine whether FANCA is acting independently, or as part of the FA complex, to control gonadotropin gene transcription. Whilst there are reports that some FA patients are fertile (Alter et al., 1991), the specific gene mutations present in these individuals are not known. However, with the huge variability in FA phenotypes, and considering that 30% of FA patients have no abnormalities, it is highly likely that specific FA genes are involved in processes other than DNA damage repair (Tischkowitz and Hodgson, 2003). Therefore, it would be informative to determine which complementation groups these reproductively viable patients were assigned to. If *FANCA* was mutated in any of these patients, analysis of the specific mutations might help to determine which regions of the gene are crucial for α GSU and *FSH β* transcription and which are not.

The realization that *BRCA2* and *FANCD1* are actually the same gene may unravel some of the molecular mechanisms involved in FA. The inheritance of germline mutations that affect one allele of either *BRCA1* or *BRCA2* results in an increased risk of developing breast and ovarian cancer (Ford et al., 1998; Antoniou et al., 2003). Cancers arise in germline mutation carriers that have lost the function of the second *BRCA1* or *BRCA2* allele, due to a further somatic mutation occurring within the breast or ovarian tissue, resulting in tumour development (Collins et al., 1995; Cornelis et al., 1995). However, germline mutations affecting both alleles of *BRCA2* results in Fanconi Anaemia (Howlett et al., 2002) indicating that germline inheritance of mono-allelic or bi-allelic *BRCA2* mutations can generate two very different phenotypes. Perhaps this effect is also seen with mono-allelic or bi-allelic mutation of other FA genes? Given that *BRCA2/FANCD1* confers a pre-disposition to specific cancers when one mutant allele is inherited, and mutations in *BRCA*

genes account for only 40% of familial breast cancer cases (Ford et al., 1998), it is feasible that carriers of other FA gene mutations may also be pre-disposed to developing specific types of cancer. With the evidence presented in this thesis indicating a molecular basis for *FANCA* in fertility, it is conceivable that mono-allelic loss of *FANCA*, or indeed other FA genes, could also impact on fecundity (See Figure 6.7).

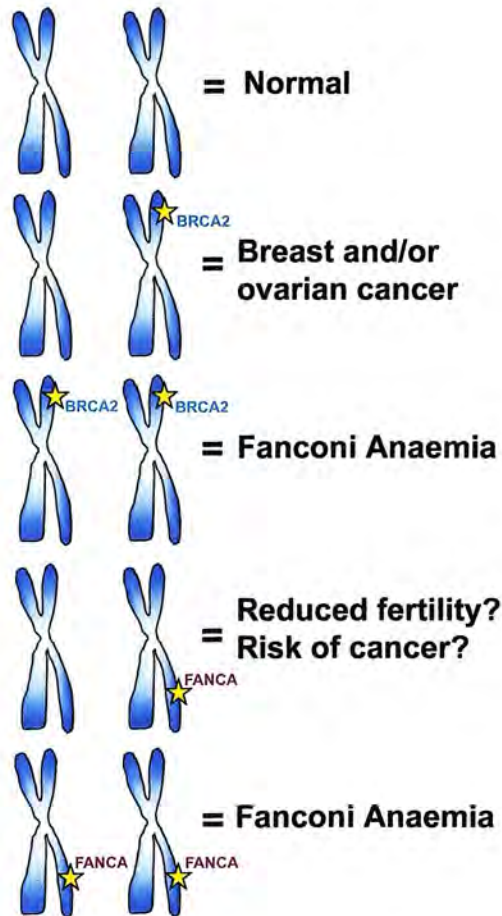


Figure 6.7 Hypothetical consequences of mono-allelic loss of *FANCA*.

BRCA2 and *FANCD1* are identical. Germline mutation affecting one allele of *BRCA2* results in a predisposition to developing breast and/or ovarian cancer. Germline mutations affecting both *BRCA2* alleles results in Fanconi Anaemia. Bi-allelic loss of *FANCA* also results in Fanconi Anaemia. As wildtype *FANCA* is required for GnRH induced α Gsu transcription perhaps mono-allelic loss of *FANCA* results in reduced fertility in carriers of *FANCA* mutations.

6.1.8 Understanding the genetic and molecular basis of FA

The past few years have witnessed a considerable expansion in our understanding of the FA pathway however, much more remains to be elucidated. Unfortunately, the genetic heterogeneity of the disease hinders investigation of its molecular basis. For instance, in other autosomal recessive disorders, such as Cystic Fibrosis (CF), the first stage after diagnosis is to genotype the patient using multiplex PCR techniques (Bradley et al., 1998) and has been of major benefit to both patients and researchers. For the patients this gives a definitive genetic explanation for their illness, allows screening of family members and pre-implantation diagnosis of any future pregnancies. For researchers, it constructs a picture of possible genotype/phenotype correlations and an example of the efficacy of this approach is demonstrated with the discovery that the R117H mutation causes congenital bilateral absence of vas deferens (CBVAD) in CF patients (Bienvenu et al., 1993). Whilst FA patients may be assigned to a complementation group, identification of a specific mutation is rarely performed and unfortunately, ancestral mutations, equivalent to $\Delta F508$ in the *CFTR* (*Cystic fibrosis transmembrane conductance regulator*) gene, have not been identified in any of the FA genes, instead, >100 different mutations have been found in *FANCA* alone (Savino et al., 1997; Tachibana et al., 1999; Wijker et al., 1999). This suggests that, unlike CF, the disease alleles have not evolved by conferring a preferential advantage in the hemizygous state, instead, the *FANCA* locus appears to be a mutational hotspot. Some links between complementation groups and clinical phenotypes have been made. A relatively early onset of anaemia is seen in *FANCG* patients compared with those in groups A and C and *FANCA* null patients have a more severe haematological disease than non-null patients (Faivre et al., 2000). The only report of a specific genotype/phenotype correlation shows that patients with a frameshift mutation in *FANCC* (322delG in exon 1) do not have any of the major skeletal abnormalities usually seen in FA patients, indicating that mutations at the N-terminus of *FANCC* do not alter its role in skeletal development (Yamashita et al., 1996). It would therefore be extremely useful to collect data on the specific mutations and clinical phenotypes of a large cohort of FA patients in order to analyse

phenotype-genotype correlations thereby acquiring more information on the genetic and molecular basis of FA.

6.2 Summary

This thesis presents data characterising the function of *Fanca* in gonadotroph cells and provides the first molecular evidence of a role for *Fanca* in the control of gonadotroph function.

Chapter three analyses in detail the regulation of *Fanca* mRNA and protein expression by GnRH. Semi-quantitative and quantitative RT-PCR techniques reveal, for the first time, that there is a rapid and transient increase in *Fanca* mRNA levels in response to GnRH and western blotting analysis shows that this increase is mirrored by a transient rise in protein levels after treatment with GnRH (Larder et al., 2004).

Chapter four describes *Fanca* protein expression within L β T2 gonadotroph cells. Using western blotting analysis, indirect immunofluorescence and tagging of FANCA with a fluorescent protein (GFP) *Fanca* protein expression was localized to both the cytoplasm and nucleus of L β T2 cells. Treatment of L β T2 cells with an inhibitor of CRM-1 dependent nuclear export prevented the export of *Fanca* protein to the cytoplasm, showing for the first time that *Fanca* protein shuttles between the nucleus and cytoplasm of L β T2 cells. This chapter also characterises the localization of two point-mutated forms of FANCA in L β T2 cells. Substitution of a histidine for a proline at position 1110 (H1110P) restricts FANCA protein expression to the cytoplasm of L β T2 cells whilst substitution of a glycine with a glutamic acid at position 1128 (Q1128E) distributes protein expression across both cellular compartments.

Chapter five analyses the affects of these mutant proteins on gonadotropin subunit transcription using transient transfection assays. Co-transfection of mutant FANCA proteins with α *Gsu*, *LH β* and *FSH β* promoters reveals wildtype FANCA is required for GnRH induction of α *Gsu* promoter activity and also regulates basal levels of the *FSH β* promoter suggesting that FANCA is involved in the differential control of

gonadotropin subunit transcription. Future work to further elucidate the role of Fanca in gonadotroph cells is also discussed in this chapter. The construction of adenoviral vectors expressing wildtype and mutant FANCA proteins and the characterisation of siRNAs that can be used to knockdown expression of Fanca in L β T2 cells to confirm a role for Fanca in gonadotropin gene transcription are also described.

This thesis provides the first molecular evidence of a role for Fanca in the regulation of gonadotroph function and may help in the understanding of both the infertility and cancer development phenotype seen in FA patients. Analysis of the transcriptional regulatory function of Fanca may help identify the specific molecular mechanisms that this gene is required for and may help progress the understanding and treatment of other clinical features of the disease.

Bibliography

Knowledge exists to be imparted – Ralph Waldo Emerson

- Aasland, R., Gibson, T. J., and Stewart, A. F. (1995). The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem Sci* **20**, 56-9.
- Abel, M. H., Wootton, A. N., Wilkins, V., Huhtaniemi, I., Knight, P. G., and Charlton, H. M. (2000). The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. *Endocrinology* **141**, 1795-803.
- Abu-Issa, R., Eichele, G., and Youssoufian, H. (1999). Expression of the Fanconi anemia group A gene (Fanca) during mouse embryogenesis. *Blood* **94**, 818-24.
- Adachi, D., Oda, T., Yagasaki, H., Nakasato, K., Taniguchi, T., D'Andrea, A. D., Asano, S., and Yamashita, T. (2002). Heterogeneous activation of the Fanconi anemia pathway by patient-derived FANCA mutants. *Hum Mol Genet* **11**, 3125-34.
- Agoulnik, A. I., Lu, B., Zhu, Q., Truong, C., Ty, M. T., Arango, N., Chada, K. K., and Bishop, C. E. (2002). A novel gene, Pog, is necessary for primordial germ cell proliferation in the mouse and underlies the germ cell deficient mutation, gcd. *Hum Mol Genet* **11**, 3047-53.
- Aittomaki, K., Lucena, J. L., Pakarinen, P., Sistonen, P., Tapanainen, J., Gromoll, J., Kaskikari, R., Sankila, E. M., Lehvaslaiho, H., Engel, A. R., and et al. (1995). Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. *Cell* **82**, 959-68.
- Alarid, E. T., Windle, J. J., Whyte, D. B., and Mellon, P. L. (1996). Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. *Development* **122**, 3319-29.
- Alber, T. (1992). Structure of the leucine zipper. *Curr Opin Genet Dev* **2**, 205-10.
- Allan, C. M., Haywood, M., Swaraj, S., Spaliviero, J., Koch, A., Jimenez, M., Poutanen, M., Levallet, J., Huhtaniemi, I., Illingworth, P., and Handelsman, D. J. (2001). A novel transgenic model to characterize the specific effects of follicle-stimulating hormone on gonadal physiology in the absence of luteinizing hormone actions. *Endocrinology* **142**, 2213-20.
- Alter, B. P. (1993). Fanconi's anaemia and its variability. *Br J Haematol* **85**, 9-14.
- Alter, B. P. (1996). Fanconi's anemia and malignancies. *Am J Hematol* **53**, 99-110.
- Alter, B. P. (2003). Cancer in Fanconi anemia, 1927-2001. *Cancer* **97**, 425-40.
- Alter, B. P., Frizzera, C. L., Halperin, D. S., Freedman, M. H., Chitkara, U., Alvarez, E., Lynch, L., Adler-Brecher, B., and Auerbach, A. D. (1991). Fanconi's anaemia and pregnancy. *Br J Haematol* **77**, 410-8.
- Alter, B. P., Young, M.S. (1998). The bone marrow failure syndromes. In "Hematology of Infancy and Childhood" (D. G. Nathan, Orkin, S.H., Ed.), pp. 237-335. W.B.Saunders, Philadelphia.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403-10.
- Amendt, B. A., Sutherland, L. B., and Russo, A. F. (1999). Multifunctional role of the Pitx2 homeodomain protein C-terminal tail. *Mol Cell Biol* **19**, 7001-10.

- Antoniou, A., Pharoah, P. D., Narod, S., Risch, H. A., Eyfjord, J. E., Hopper, J. L., Loman, N., Olsson, H., Johannsson, O., Borg, A., Pasini, B., Radice, P., Manoukian, S., Eccles, D. M., Tang, N., Olah, E., Anton-Culver, H., Warner, E., Lubinski, J., Gronwald, J., Gorski, B., Tulinius, H., Thorlacius, S., Eerola, H., Nevanlinna, H., Syrjakoski, K., Kallioniemi, O. P., Thompson, D., Evans, C., Peto, J., Lalloo, F., Evans, D. G., and Easton, D. F. (2003). Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. *Am J Hum Genet* **72**, 1117-30.
- Aube, M., Lafrance, M., Brodeur, I., Delisle, M. C., and Carreau, M. (2003). Fanconi anemia genes are highly expressed in primitive CD34+ hematopoietic cells. *BMC Blood Disord* **3**, 1.
- Auerbach, A., Buchwald, M., and Joenje, H. (1998). Fanconi Anemia. In "Genetic Basis Of Human Cancer", pp. 317-332. McGraw-Hill, New York.
- Auerbach, A. D. (1993). Fanconi anemia diagnosis and the diepoxybutane (DEB) test. *Exp Hematol* **21**, 731-3.
- Auerbach, A. D., and Wolman, S. R. (1976). Susceptibility of Fanconi's anaemia fibroblasts to chromosome damage by carcinogens. *Nature* **261**, 494-6.
- Aynsley-Green, A., Zachmann, M., Werder, E. A., Illig, R., and Prader, A. (1978). Endocrine studies in Fanconi's anaemia. Report of 4 cases. *Arch Dis Child* **53**, 126-31.
- Bach, I., Rhodes, S. J., Pearse, R. V., 2nd, Heinzl, T., Gloss, B., Scully, K. M., Sawchenko, P. E., and Rosenfeld, M. G. (1995). P-Lim, a LIM homeodomain factor, is expressed during pituitary organ and cell commitment and synergizes with Pit-1. *Proc Natl Acad Sci U S A* **92**, 2720-4.
- Bagnara, G. P., Bonsi, L., Strippoli, P., Ramenghi, U., Timeus, F., Bonifazi, F., Bonafe, M., Tonelli, R., Bubola, G., Brizzi, M. F., and et al. (1993). Production of interleukin 6, leukemia inhibitory factor and granulocyte-macrophage colony stimulating factor by peripheral blood mononuclear cells in Fanconi's anemia. *Stem Cells* **11 Suppl 2**, 137-43.
- Bahk, J. Y., Hyun, J. S., Chung, S. H., Lee, H., Kim, M. O., Lee, B. H., and Choi, W. S. (1995). Stage specific identification of the expression of GnRH mRNA and localization of the GnRH receptor in mature rat and adult human testis. *J Urol* **154**, 1958-61.
- Bahk, J. Y., Hyun, J. S., Lee, H., Kim, M. O., Cho, G. J., Lee, B. H., and Choi, W. S. (1998). Expression of gonadotropin-releasing hormone (GnRH) and GnRH receptor mRNA in prostate cancer cells and effect of GnRH on the proliferation of prostate cancer cells. *Urol Res* **26**, 259-64.
- Balbi, G., Piano, L. D., Cardone, A., and Cirelli, G. (2004). Second-line therapy of advanced ovarian cancer with GnRH analogs. *Int J Gynecol Cancer* **14**, 799-803.
- Bargman, G. J., Shahidi, N. T., Gilbert, E. F., and Opitz, J. M. (1977). Studies of malformation syndromes of man XLVII: disappearance of spermatogonia in the Fanconi anemia syndrome. *Eur J Pediatr* **125**, 163-8.
- Barnhart, K. M., and Mellon, P. L. (1994). The orphan nuclear receptor, steroidogenic factor-1, regulates the glycoprotein hormone alpha-subunit gene in pituitary gonadotropes. *Mol Endocrinol* **8**, 878-85.

- Beals, C. R., Clipstone, N. A., Ho, S. N., and Crabtree, G. R. (1997). Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. *Genes Dev* **11**, 824-34.
- Belchetz, P. E., Plant, T. M., Nakai, Y., Keogh, E. J., and Knobil, E. (1978). Hypophysial responses to continuous and intermittent delivery of hypophysial gonadotropin-releasing hormone. *Science* **202**, 631-3.
- Ben-Menahem, D., and Naor, Z. (1994). Regulation of gonadotropin mRNA levels in cultured rat pituitary cells by gonadotropin-releasing hormone (GnRH): role for Ca²⁺ and protein kinase C. *Biochemistry* **33**, 3698-704.
- Benard, O., Naor, Z., and Seger, R. (2001). Role of dynamin, Src, and Ras in the protein kinase C-mediated activation of ERK by gonadotropin-releasing hormone. *J Biol Chem* **276**, 4554-63.
- Beranova, M., Oliveira, L. M., Bedecarrats, G. Y., Schipani, E., Vallejo, M., Ammini, A. C., Quintos, J. B., Hall, J. E., Martin, K. A., Hayes, F. J., Pitteloud, N., Kaiser, U. B., Crowley, W. F., Jr., and Seminara, S. B. (2001). Prevalence, phenotypic spectrum, and modes of inheritance of gonadotropin-releasing hormone receptor mutations in idiopathic hypogonadotropic hypogonadism. *J Clin Endocrinol Metab* **86**, 1580-8.
- Berkovitz, G. D., Zinkham, W. H., and Migeon, C. J. (1984). Gonadal function in two siblings with Fanconi's anemia. *Horm Res* **19**, 137-41.
- Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-6.
- Berthezene, F., Forest, M. G., Grimaud, J. A., Claustrat, B., and Mornex, R. (1976). Leydig-cell agenesis: a cause of male pseudohermaphroditism. *N Engl J Med* **295**, 969-72.
- Bienvenu, T., Beldjord, C., Adjiman, M., and Kaplan, J. C. (1993). Male infertility as the only presenting sign of cystic fibrosis when homozygous for the mild mutation R117H. *J Med Genet* **30**, 797.
- Bishop, J. O., Morton, J. G., Rosbash, M., and Richardson, M. (1974). Three abundance classes in HeLa cell messenger RNA. *Nature* **250**, 199-204.
- Bonfil, D., Chuderland, D., Kraus, S., Shahbazian, D., Friedberg, I., Seger, R., and Naor, Z. (2004). Extracellular signal-regulated kinase, Jun N-terminal kinase, p38, and c-Src are involved in gonadotropin-releasing hormone-stimulated activity of the glycoprotein hormone follicle-stimulating hormone beta-subunit promoter. *Endocrinology* **145**, 2228-44.
- Bonsfield, G. R., Perry, W.M. and Ward, D.N. (1994). Gonadotrophins:chemistry and biosynthesis. In "The Physiology of Reproduction" (E. a. N. Knobil, J., Ed.), pp. 1749-1792. Raven Press, New York.
- Bradley, L. A., Johnson, D. A., Chaparro, C. A., Robertson, N. H., and Ferrie, R. M. (1998). A multiplex ARMS test for 10 cystic fibrosis (CF) mutations: evaluation in a prenatal CF screening program. *Genet Test* **2**, 337-41.
- Brown, D. M., Markland, C., and Dehner, L. P. (1978). Leydig cell hypoplasia: a cause of male pseudohermaphroditism. *J Clin Endocrinol Metab* **46**, 1-7.

- Brown, P., and McNeilly, A. S. (1997). Steroidogenic factor-1 (SF-1) and the regulation of expression of luteinising hormone and follicle stimulating hormone b-subunits in the sheep anterior pituitary in vivo. *Int J Biochem Cell Biol* **29**, 1513-24.
- Brown, P., and McNeilly, A. S. (1999). Transcriptional regulation of pituitary gonadotrophin subunit genes. *Rev Reprod* **4**, 117-24.
- Brown, P., McNeilly, J. R., Evans, J. G., Crawford, G. M., Walker, M., Christian, H. C., and McNeilly, A. S. (2001). Manipulating the in vivo mRNA expression profile of FSH beta to resemble that of LH beta does not promote a concomitant increase in intracellular storage of follicle-stimulating hormone. *J Neuroendocrinol* **13**, 50-62.
- Brown, P., McNeilly, J. R., Wallace, R. M., McNeilly, A. S., and Clark, A. J. (1993). Characterization of the ovine LH beta-subunit gene: the promoter directs gonadotrope-specific expression in transgenic mice. *Mol Cell Endocrinol* **93**, 157-65.
- Buchwald, M., and Moustacchi, E. (1998). Is Fanconi anemia caused by a defect in the processing of DNA damage? *Mutat Res* **408**, 75-90.
- Busch, D. B., Zdzienicka, M. Z., Natarajan, A. T., Jones, N. J., Overkamp, W. J., Collins, A., Mitchell, D. L., Stefanini, M., Botta, E., Albert, R. B., Liu, N., White, D. A., van Gool, A. J., and Thompson, L. H. (1996). A CHO mutant, UV40, that is sensitive to diverse mutagens and represents a new complementation group of mitomycin C sensitivity. *Mutat Res* **363**, 209-21.
- Carroll, R. S., Corrigan, A. Z., Gharib, S. D., Vale, W., and Chin, W. W. (1989). Inhibin, activin, and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels. *Mol Endocrinol* **3**, 1969-76.
- Castillo, V., Cabre, O., Marcos, R., and Surralles, J. (2003). Molecular cloning of the Drosophila Fanconi anaemia gene FANCD2 cDNA. *DNA Repair (Amst)* **2**, 751-8.
- Cattanach, B. M., Iddon, C. A., Charlton, H. M., Chiappa, S. A., and Fink, G. (1977). Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. *Nature* **269**, 338-40.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802-5.
- Chandrasekharappa, S. C., Guru, S. C., Manickam, P., Olufemi, S. E., Collins, F. S., Emmert-Buck, M. R., Debelenko, L. V., Zhuang, Z., Lubensky, I. A., Liotta, L. A., Crabtree, J. S., Wang, Y., Roe, B. A., Weisemann, J., Boguski, M. S., Agarwal, S. K., Kester, M. B., Kim, Y. S., Heppner, C., Dong, Q., Spiegel, A. M., Burns, A. L., and Marx, S. J. (1997). Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science* **276**, 404-7.
- Chang, L. (2002). Molecular characterisation of GnRH regulated factors isolated from gonadotroph cells. In "Medical Research Council - Human Reproductive Sciences Unit", pp. 235. University of Edinburgh, Edinburgh.

- Charlton, H. M., Halpin, D. M., Iddon, C., Rosie, R., Levy, G., McDowell, I. F., Megson, A., Morris, J. F., Bramwell, A., Speight, A., Ward, B. J., Broadhead, J., Davey-Smith, G., and Fink, G. (1983). The effects of daily administration of single and multiple injections of gonadotropin-releasing hormone on pituitary and gonadal function in the hypogonadal (hpg) mouse. *Endocrinology* **113**, 535-44.
- Chaturvedi, K., Bandari, P., Chinen, N., and Howells, R. D. (2001). Proteasome involvement in agonist-induced down-regulation of mu and delta opioid receptors. *J Biol Chem* **276**, 12345-55.
- Chatzaki, E., Bax, C. M., Eidne, K. A., Anderson, L., Grudzinskas, J. G., and Gallagher, C. J. (1996). The expression of gonadotropin-releasing hormone and its receptor in endometrial cancer, and its relevance as an autocrine growth factor. *Cancer Res* **56**, 2059-65.
- Chedrese, P. J., Kay, T. W., and Jameson, J. L. (1994). Gonadotropin-releasing hormone stimulates glycoprotein hormone alpha-subunit messenger ribonucleic acid (mRNA) levels in alpha T3 cells by increasing transcription and mRNA stability. *Endocrinology* **134**, 2475-81.
- Chen, M., Tomkins, D. J., Auerbach, W., McKerlie, C., Youssoufian, H., Liu, L., Gan, O., Carreau, M., Auerbach, A., Groves, T., Guidos, C. J., Freedman, M. H., Cross, J., Percy, D. H., Dick, J. E., Joyner, A. L., and Buchwald, M. (1996). Inactivation of Fac in mice produces inducible chromosomal instability and reduced fertility reminiscent of Fanconi anaemia. *Nat Genet* **12**, 448-51.
- Chen, Y., Chen, C. F., Riley, D. J., Allred, D. C., Chen, P. L., Von Hoff, D., Osborne, C. K., and Lee, W. H. (1995). Aberrant subcellular localization of BRCA1 in breast cancer. *Science* **270**, 789-91.
- Cheng, N. C., van de Vrugt, H. J., van der Valk, M. A., Oostra, A. B., Krimpenfort, P., de Vries, Y., Joenje, H., Berns, A., and Arwert, F. (2000). Mice with a targeted disruption of the Fanconi anemia homolog Fanca. *Hum Mol Genet* **9**, 1805-11.
- Childs, G. V., Unabia, G., Tibolt, R., and Lloyd, J. M. (1987). Cytological factors that support nonparallel secretion of luteinizing hormone and follicle-stimulating hormone during the estrous cycle. *Endocrinology* **121**, 1801-13.
- Clarke, I. J., and Cummins, J. T. (1982). The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in ovariectomized ewes. *Endocrinology* **111**, 1737-9.
- Clarke, I. J., Thomas, G. B., Yao, B., and Cummins, J. T. (1987). GnRH secretion throughout the ovine estrous cycle. *Neuroendocrinology* **46**, 82-8.
- Clayton, R. N., Katikineni, M., Chan, V., Dufau, M. L., and Catt, K. J. (1980). Direct inhibition of testicular function by gonadotropin-releasing hormone: mediation by specific gonadotropin-releasing hormone receptors in interstitial cells. *Proc Natl Acad Sci U S A* **77**, 4459-63.
- Cohen, G. B., Ren, R., and Baltimore, D. (1995). Modular binding domains in signal transduction proteins. *Cell* **80**, 237-48.

- Collins, N., McManus, R., Wooster, R., Mangion, J., Seal, S., Lakhani, S. R., Ormiston, W., Daly, P. A., Ford, D., Easton, D. F., and et al. (1995). Consistent loss of the wild type allele in breast cancers from a family linked to the BRCA2 gene on chromosome 13q12-13. *Oncogene* **10**, 1673-5.
- Conn, P. M., and Crowley, W. F., Jr. (1994). Gonadotropin-releasing hormone and its analogs. *Annu Rev Med* **45**, 391-405.
- Cook, L. B., Zhu, C. C., and Hinkle, P. M. (2003). Thyrotropin-releasing hormone receptor processing: role of ubiquitination and proteasomal degradation. *Mol Endocrinol* **17**, 1777-91.
- Cornelis, R. S., Neuhausen, S. L., Johansson, O., Arason, A., Kelsell, D., Ponder, B. A., Tonin, P., Hamann, U., Lindblom, A., Lalle, P., and et al. (1995). High allele loss rates at 17q12-q21 in breast and ovarian tumors from BRCA1-linked families. The Breast Cancer Linkage Consortium. *Genes Chromosomes Cancer* **13**, 203-10.
- Coss, D., Jacobs, S. B., Bender, C. E., and Mellon, P. L. (2004). A novel AP-1 site is critical for maximal induction of the follicle-stimulating hormone beta gene by gonadotropin-releasing hormone. *J Biol Chem* **279**, 152-62.
- Cowdell, R. H., Phizackerley, P. J., and Pyke, D. A. (1955). Constitutional anemia (Fanconi's syndrome) and leukemia in two brothers. *Blood* **10**, 788-801.
- D'Andrea, A. D., and Grompe, M. (2003). The Fanconi anaemia/BRCA pathway. *Nat Rev Cancer* **3**, 23-34.
- Dalkin, A. C., Haisenleder, D. J., Ortolano, G. A., Ellis, T. R., and Marshall, J. C. (1989). The frequency of gonadotropin-releasing-hormone stimulation differentially regulates gonadotropin subunit messenger ribonucleic acid expression. *Endocrinology* **125**, 917-24.
- Dasen, J. S., O'Connell, S. M., Flynn, S. E., Treier, M., Gleiberman, A. S., Szeto, D. P., Hooshmand, F., Aggarwal, A. K., and Rosenfeld, M. G. (1999). Reciprocal interactions of Pit1 and GATA2 mediate signaling gradient-induced determination of pituitary cell types. *Cell* **97**, 587-98.
- Dasen, J. S., and Rosenfeld, M. G. (2001). Signaling and transcriptional mechanisms in pituitary development. *Annu Rev Neurosci* **24**, 327-55.
- Dattani, M. T., Martinez-Barbera, J. P., Thomas, P. Q., Brickman, J. M., Gupta, R., Martensson, I. L., Toresson, H., Fox, M., Wales, J. K., Hindmarsh, P. C., Krauss, S., Beddington, R. S., and Robinson, I. C. (1998). Mutations in the homeobox gene HESX1/Hesx1 associated with septo-optic dysplasia in human and mouse. *Nat Genet* **19**, 125-33.
- Dattani, M. T., Martinez-Barbera, J. P., Thomas, P. Q., Brickman, J. M., Gupta, R., Wales, J. K., Hindmarsh, P. C., Beddington, R. S., and Robinson, I. C. (1999). HESX1: a novel gene implicated in a familial form of septo-optic dysplasia. *Acta Paediatr Suppl* **88**, 49-54.
- de Roux, N., and Milgrom, E. (2001). Inherited disorders of GnRH and gonadotropin receptors. *Mol Cell Endocrinol* **179**, 83-7.
- de Roux, N., Young, J., Brailly-Tabard, S., Misrahi, M., Milgrom, E., and Schaison, G. (1999). The same molecular defects of the gonadotropin-releasing hormone receptor determine a variable degree of hypogonadism in affected kindred. *J Clin Endocrinol Metab* **84**, 567-72.

- de Roux, N., Young, J., Misrahi, M., Genet, R., Chanson, P., Schaison, G., and Milgrom, E. (1997). A family with hypogonadotropic hypogonadism and mutations in the gonadotropin-releasing hormone receptor. *N Engl J Med* **337**, 1597-602.
- de Winter, J. P., Leveille, F., van Berkel, C. G., Rooimans, M. A., van Der Weel, L., Steltenpool, J., Demuth, I., Morgan, N. V., Alon, N., Bosnoyan-Collins, L., Lightfoot, J., Leegwater, P. A., Waisfisz, Q., Komatsu, K., Arwert, F., Pronk, J. C., Mathew, C. G., Digweed, M., Buchwald, M., and Joenje, H. (2000a). Isolation of a cDNA representing the Fanconi anemia complementation group E gene. *Am J Hum Genet* **67**, 1306-8.
- de Winter, J. P., Rooimans, M. A., van Der Weel, L., van Berkel, C. G., Alon, N., Bosnoyan-Collins, L., de Groot, J., Zhi, Y., Waisfisz, Q., Pronk, J. C., Arwert, F., Mathew, C. G., Scheper, R. J., Hoatlin, M. E., Buchwald, M., and Joenje, H. (2000b). The Fanconi anaemia gene FANCF encodes a novel protein with homology to ROM. *Nat Genet* **24**, 15-6.
- de Winter, J. P., van der Weel, L., de Groot, J., Stone, S., Waisfisz, Q., Arwert, F., Scheper, R. J., Kruijff, F. A., Hoatlin, M. E., and Joenje, H. (2000c). The Fanconi anemia protein FANCF forms a nuclear complex with FANCA, FANCC and FANCG. *Hum Mol Genet* **9**, 2665-74.
- de Winter, J. P., Waisfisz, Q., Rooimans, M. A., van Berkel, C. G., Bosnoyan-Collins, L., Alon, N., Carreau, M., Bender, O., Demuth, I., Schindler, D., Pronk, J. C., Arwert, F., Hoehn, H., Digweed, M., Buchwald, M., and Joenje, H. (1998). The Fanconi anaemia group G gene FANCG is identical with XRCC9. *Nat Genet* **20**, 281-3.
- Diczfalusy, E. a. F., I.S. (1998). The discovery of reproductive steroid hormones and recognition of their physiological roles. In "Estrogens and progesterones in clinical practice" (I. S. Fraser, Jansen, R.P.S., Lobo, R.A. and Whitehead, M.I., Ed.), pp. 3-18. Churchill Livingstone, London.
- DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997). A cytokine-responsive I κ B kinase that activates the transcription factor NF- κ B. *Nature* **388**, 548-54.
- Dingwall, C., and Laskey, R. A. (1991). Nuclear targeting sequences--a consensus? *Trends Biochem Sci* **16**, 478-81.
- Dode, C., Levilliers, J., Dupont, J. M., De Paepe, A., Le Du, N., Soussi-Yanicostas, N., Coimbra, R. S., Delmaghani, S., Compain-Nouaille, S., Baverel, F., Pecheux, C., Le Tessier, D., Cruaud, C., Delpech, M., Speleman, F., Vermeulen, S., Amalfitano, A., Bachelot, Y., Bouchard, P., Cabrol, S., Carel, J. C., Delemarre-van de Waal, H., Goulet-Salmon, B., Kottler, M. L., Richard, O., Sanchez-Franco, F., Saura, R., Young, J., Petit, C., and Hardelin, J. P. (2003). Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. *Nat Genet* **33**, 463-5.
- Dondi, D., Limonta, P., Moretti, R. M., Marelli, M. M., Garattini, E., and Motta, M. (1994). Antiproliferative effects of luteinizing hormone-releasing hormone (LHRH) agonists on human androgen-independent prostate cancer cell line DU 145: evidence for an autocrine-inhibitory LHRH loop. *Cancer Res* **54**, 4091-5.

- Dorn, C., Ou, Q., Svaren, J., Crawford, P. A., and Sadovsky, Y. (1999). Activation of luteinizing hormone beta gene by gonadotropin-releasing hormone requires the synergy of early growth response-1 and steroidogenic factor-1. *J Biol Chem* **274**, 13870-6.
- Duan, W. R., Ito, M., Park, Y., Maizels, E. T., Hunzicker-Dunn, M., and Jameson, J. L. (2002). GnRH regulates early growth response protein 1 transcription through multiple promoter elements. *Mol Endocrinol* **16**, 221-33.
- Duval, D. L., Nelson, S. E., and Clay, C. M. (1997). A binding site for steroidogenic factor-1 is part of a complex enhancer that mediates expression of the murine gonadotropin-releasing hormone receptor gene. *Biol Reprod* **56**, 160-8.
- Eil, C., Austin, R. M., Sesterhenn, I., Dunn, J. F., Cutler, G. B., Jr., and Johnsonbaugh, R. E. (1984). Leydig cell hypoplasia causing male pseudohermaphroditism: diagnosis 13 years after prepubertal castration. *J Clin Endocrinol Metab* **58**, 441-8.
- el-Baradi, T., and Pieler, T. (1991). Zinc finger proteins: what we know and what we would like to know. *Mech Dev* **35**, 155-69.
- Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001a). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494-8.
- Elbashir, S. M., Lendeckel, W., and Tuschl, T. (2001b). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* **15**, 188-200.
- Elion, E. A. (2001). The Ste5p scaffold. *J Cell Sci* **114**, 3967-78.
- Emons, G., Schroder, B., Ortmann, O., Westphalen, S., Schulz, K. D., and Schally, A. V. (1993). High affinity binding and direct antiproliferative effects of luteinizing hormone-releasing hormone analogs in human endometrial cancer cell lines. *J Clin Endocrinol Metab* **77**, 1458-64.
- Ericson, J., Norlin, S., Jessell, T. M., and Edlund, T. (1998). Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. *Development* **125**, 1005-15.
- Faivre, L., Guardiola, P., Lewis, C., Dokal, I., Ebell, W., Zatterale, A., Altay, C., Poole, J., Stones, D., Kwee, M. L., van Weel-Sipman, M., Havenga, C., Morgan, N., de Winter, J., Digweed, M., Savoia, A., Pronk, J., de Ravel, T., Jansen, S., Joenje, H., Gluckman, E., and Mathew, C. G. (2000). Association of complementation group and mutation type with clinical outcome in fanconi anemia. European Fanconi Anemia Research Group. *Blood* **96**, 4064-70.
- Fallest, P. C., Trader, G. L., Darrow, J. M., and Shupnik, M. A. (1995). Regulation of rat luteinizing hormone beta gene expression in transgenic mice by steroids and a gonadotropin-releasing hormone antagonist. *Biol Reprod* **53**, 103-9.
- Fanconi, G. (1927). Familiaere infantile perniziosaartige Anaemie (pernizioeses Blutbild und Konstitution). *Jahrbuch Kinderheild* **117**, 257-280.
- Feldherr, C. M., Kallenbach, E., and Schultz, N. (1984). Movement of a karyophilic protein through the nuclear pores of oocytes. *J Cell Biol* **99**, 2216-22.

- Ferguson, S. S. (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* **53**, 1-24.
- Ferrer, M., Rodriguez, J.A., Spierings, E.A., de Winter, J.P., Giaccone, G. and Kruyt, F.A.E. (2003). Nuclear-cytoplasmic shuttling of Fanca: characterization of three novel nuclear export sequences in Fanca. In "15th Annual Fanconi Anemia Research Fund Scientific Symposium", Houston, Texas, USA.
- Filicori, M., Butler, J. P., and Crowley, W. F., Jr. (1984). Neuroendocrine regulation of the corpus luteum in the human. Evidence for pulsatile progesterone secretion. *J Clin Invest* **73**, 1638-47.
- Fink, G. (1988). Gonadotropin secretion and its control. In "The physiology of reproduction" (E. a. N. Knobil, J., Ed.), pp. 1349-1377. Raven Press, New York.
- Ford, D., Easton, D. F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D. T., Weber, B., Lenoir, G., Chang-Claude, J., Sobol, H., Teare, M. D., Struewing, J., Arason, A., Scherneck, S., Peto, J., Rebbeck, T. R., Tonin, P., Neuhausen, S., Barkardottir, R., Eyfjord, J., Lynch, H., Ponder, B. A., Gayther, S. A., Zelada-Hedman, M., and et al. (1998). Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *Am J Hum Genet* **62**, 676-89.
- Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-60.
- Franco, B., Guioli, S., Pragliola, A., Incerti, B., Bardoni, B., Tonlorenzi, R., Carozzo, R., Maestrini, E., Pieretti, M., Taillon-Miller, P., and et al. (1991). A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. *Nature* **353**, 529-36.
- Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**, 308-11.
- Gage, P. J., Brinkmeier, M. L., Scarlett, L. M., Knapp, L. T., Camper, S. A., and Mahon, K. A. (1996a). The Ames dwarf gene, *df*, is required early in pituitary ontogeny for the extinction of *Rpx* transcription and initiation of lineage-specific cell proliferation. *Mol Endocrinol* **10**, 1570-81.
- Gage, P. J., and Camper, S. A. (1997). Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. *Hum Mol Genet* **6**, 457-64.
- Gage, P. J., Roller, M. L., Saunders, T. L., Scarlett, L. M., and Camper, S. A. (1996b). Anterior pituitary cells defective in the cell-autonomous factor, *df*, undergo cell lineage specification but not expansion. *Development* **122**, 151-60.
- Gage, P. J., Suh, H., and Camper, S. A. (1999a). The bicoid-related *Pitx* gene family in development. *Mamm Genome* **10**, 197-200.
- Gage, P. J., Suh, H., and Camper, S. A. (1999b). Dosage requirement of *Pitx2* for development of multiple organs. *Development* **126**, 4643-51.

- Garcia-Higuera, I., Kuang, Y., Denham, J., and D'Andrea, A. D. (2000). The fanconi anemia proteins FANCA and FANCG stabilize each other and promote the nuclear accumulation of the Fanconi anemia complex. *Blood* **96**, 3224-30.
- Garcia-Higuera, I., Kuang, Y., Naf, D., Wasik, J., and D'Andrea, A. D. (1999). Fanconi anemia proteins FANCA, FANCC, and FANCG/XRCC9 interact in a functional nuclear complex. *Mol Cell Biol* **19**, 4866-73.
- Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M. S., Timmers, C., Hejna, J., Grompe, M., and D'Andrea, A. D. (2001). Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell* **7**, 249-62.
- Gasca, S., Canizares, J., De Santa Barbara, P., Mejean, C., Poulat, F., Berta, P., and Boizet-Bonhoure, B. (2002). A nuclear export signal within the high mobility group domain regulates the nucleocytoplasmic translocation of SOX9 during sexual determination. *Proc Natl Acad Sci U S A* **99**, 11199-204.
- Gillio, A. P., Verlander, P. C., Batish, S. D., Giampietro, P. F., and Auerbach, A. D. (1997). Phenotypic consequences of mutations in the Fanconi anemia FAC gene: an International Fanconi Anemia Registry study. *Blood* **90**, 105-10.
- Gnanaprakasam, M. S., Chen, C. J., Sutherland, J. G., and Bhalla, V. K. (1979). Receptor depletion and replenishment processes: in vivo regulation of gonadotropin receptors by luteinizing hormone, follicle stimulating hormone and ethanol in rat testis. *Biol Reprod* **20**, 991-1000.
- Gottsch, M. L., Cunningham, M. J., Smith, J. T., Popa, S. M., Acohidio, B. V., Crowley, W. F., Seminara, S., Clifton, D. K., and Steiner, R. A. (2004). A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology* **145**, 4073-7.
- Gould, A. (1997). Functions of mammalian Polycomb group and trithorax group related genes. *Curr Opin Genet Dev* **7**, 488-94.
- Graham, K. E., Nusser, K. D., and Low, M. J. (1999). LbetaT2 gonadotroph cells secrete follicle stimulating hormone (FSH) in response to active A. *J Endocrinol* **162**, R1-5.
- Grompe, M. (2003). FancL, as in ligase. *Nature Genetics* **35**, 113-114.
- Grundker, C., Gunthert, A. R., Westphalen, S., and Emons, G. (2002). Biology of the gonadotropin-releasing hormone system in gynecological cancers. *Eur J Endocrinol* **146**, 1-14.
- Haisenleder, D. J., Dalkin, A. C., Ortolano, G. A., Marshall, J. C., and Shupnik, M. A. (1991). A pulsatile gonadotropin-releasing hormone stimulus is required to increase transcription of the gonadotropin subunit genes: evidence for differential regulation of transcription by pulse frequency in vivo. *Endocrinology* **128**, 509-17.
- Halvorson, L. M., Ito, M., Jameson, J. L., and Chin, W. W. (1998). Steroidogenic factor-1 and early growth response protein 1 act through two composite DNA binding sites to regulate luteinizing hormone beta-subunit gene expression. *J Biol Chem* **273**, 14712-20.
- Halvorson, L. M., Kaiser, U. B., and Chin, W. W. (1996). Stimulation of luteinizing hormone beta gene promoter activity by the orphan nuclear receptor, steroidogenic factor-1. *J Biol Chem* **271**, 6645-50.

- Hamernik, D. L., Keri, R. A., Clay, C. M., Clay, J. N., Sherman, G. B., Sawyer, H. R., Jr., Nett, T. M., and Nilson, J. H. (1992). Gonadotrope- and thyrotrope-specific expression of the human and bovine glycoprotein hormone alpha-subunit genes is regulated by distinct cis-acting elements. *Mol Endocrinol* **6**, 1745-55.
- Hannon, G. J. (2002). RNA interference. *Nature* **418**, 244-51.
- Harris, D., Bonfil, D., Chuderland, D., Kraus, S., Seger, R., and Naor, Z. (2002). Activation of MAPK cascades by GnRH: ERK and Jun N-terminal kinase are involved in basal and GnRH-stimulated activity of the glycoprotein hormone LHbeta-subunit promoter. *Endocrinology* **143**, 1018-25.
- Harris, D., Chuderland, D., Bonfil, D., Kraus, S., Seger, R., and Naor, Z. (2003). Extracellular signal-regulated kinase and c-Src, but not Jun N-terminal kinase, are involved in basal and gonadotropin-releasing hormone-stimulated activity of the glycoprotein hormone alpha-subunit promoter. *Endocrinology* **144**, 612-22.
- Harris, N., Dutlow, C., Eidne, K., Dong, K. W., Roberts, J., and Millar, R. (1991). Gonadotropin-releasing hormone gene expression in MDA-MB-231 and ZR-75-1 breast carcinoma cell lines. *Cancer Res* **51**, 2577-81.
- Hastings, M. L., Ingle, H. A., Lazar, M. A., and Munroe, S. H. (2000). Post-transcriptional regulation of thyroid hormone receptor expression by cis-acting sequences and a naturally occurring antisense RNA. *J Biol Chem* **275**, 11507-13.
- Heding, A., Vrecl, M., Bogerd, J., McGregor, A., Sellar, R., Taylor, P. L., and Eidne, K. A. (1998). Gonadotropin-releasing hormone receptors with intracellular carboxyl-terminal tails undergo acute desensitization of total inositol phosphate production and exhibit accelerated internalization kinetics. *J Biol Chem* **273**, 11472-7.
- Heinrich, M. C., Hoatlin, M. E., Zigler, A. J., Silvey, K. V., Bakke, A. C., Keeble, W. W., Zhi, Y., Reifsteck, C. A., Grompe, M., Brown, M. G., Magenis, R. E., Olson, S. B., and Bagby, G. C. (1998). DNA cross-linker-induced G2/M arrest in group C Fanconi anemia lymphoblasts reflects normal checkpoint function. *Blood* **91**, 275-87.
- Hejna, J. A., Timmers, C. D., Reifsteck, C., Bruun, D. A., Lucas, L. W., Jakobs, P. M., Toth-Fejdel, S., Unsworth, N., Clemens, S. L., Garcia, D. K., Naylor, S. L., Thayer, M. J., Olson, S. B., Grompe, M., and Moses, R. E. (2000). Localization of the Fanconi anemia complementation group D gene to a 200-kb region on chromosome 3p25.3. *Am J Hum Genet* **66**, 1540-51.
- Henderson, B. R., and Eleftheriou, A. (2000). A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. *Exp Cell Res* **256**, 213-24.
- Hermesz, E., Mackem, S., and Mahon, K. A. (1996). Rpx: a novel anterior-restricted homeobox gene progressively activated in the prechordal plate, anterior neural plate and Rathke's pouch of the mouse embryo. *Development* **122**, 41-52.

- Hislop, J. N., Everest, H. M., Flynn, A., Harding, T., Uney, J. B., Troskie, B. E., Millar, R. P., and McArdle, C. A. (2001). Differential internalization of mammalian and non-mammalian gonadotropin-releasing hormone receptors. Uncoupling of dynamin-dependent internalization from mitogen-activated protein kinase signaling. *J Biol Chem* **276**, 39685-94.
- Hoatlin, M. E., Christianson, T. A., Keeble, W. W., Hammond, A. T., Zhi, Y., Heinrich, M. C., Tower, P. A., and Bagby, G. C., Jr. (1998). The Fanconi anemia group C gene product is located in both the nucleus and cytoplasm of human cells. *Blood* **91**, 1418-25.
- Hoatlin, M. E., Zhi, Y., Ball, H., Silvey, K., Melnick, A., Stone, S., Arai, S., Hawe, N., Owen, G., Zelent, A., and Licht, J. D. (1999). A novel BTB/POZ transcriptional repressor protein interacts with the Fanconi anemia group C protein and PLZF. *Blood* **94**, 3737-47.
- Holdstock, J. G., Aylwin, S. J., and Burrin, J. M. (1996). Calcium and glycoprotein hormone alpha-subunit gene expression and secretion in alpha T3-1 gonadotropes. *Mol Endocrinol* **10**, 1308-17.
- Horn, F., Windle, J. J., Barnhart, K. M., and Mellon, P. L. (1992). Tissue-specific gene expression in the pituitary: the glycoprotein hormone alpha-subunit gene is regulated by a gonadotrope-specific protein. *Mol Cell Biol* **12**, 2143-53.
- Houghtaling, S., Timmers, C., Noll, M., Finegold, M. J., Jones, S. N., Meyn, M. S., and Grompe, M. (2003). Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice. *Genes Dev*.
- Howard, P. W., and Maurer, R. A. (2001). A point mutation in the LIM domain of Lhx3 reduces activation of the glycoprotein hormone alpha-subunit promoter. *J Biol Chem* **276**, 19020-6.
- Howlett, N. G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., De Die-Smulders, C., Persky, N., Grompe, M., Joenje, H., Pals, G., Ikeda, H., Fox, E. A., and D'Andrea, A. D. (2002). Biallelic inactivation of BRCA2 in Fanconi anemia. *Science* **297**, 606-9.
- Hsieh, K. P., and Martin, T. F. (1992). Thyrotropin-releasing hormone and gonadotropin-releasing hormone receptors activate phospholipase C by coupling to the guanosine triphosphate-binding proteins Gq and G11. *Mol Endocrinol* **6**, 1673-81.
- Huber, P. A., Medhurst, A. L., Youssoufian, H., and Mathew, C. G. (2000). Investigation of Fanconi anemia protein interactions by yeast two-hybrid analysis. *Biochem Biophys Res Commun* **268**, 73-7.
- Ianzano, L., D'Apolito, M., Centra, M., Savino, M., Levrano, O., Auerbach, A. D., Cleton-Jansen, A. M., Doggett, N. A., Pronk, J. C., Tipping, A. J., Gibson, R. A., Mathew, C. G., Whitmore, S. A., Apostolou, S., Callen, D. F., Zelante, L., and Savoia, A. (1997). The genomic organization of the Fanconi anemia group A (FAA) gene. *Genomics* **41**, 309-14.
- Iddon, C. A., Charlton, H. M., and Fink, G. (1980). Gonadotrophin release in hypogonadal and normal mice after electrical stimulation of the median eminence or injection of luteinizing hormone releasing hormone. *J Endocrinol* **85**, 105-10.

- Ikeda, Y., Luo, X., Abbud, R., Nilson, J. H., and Parker, K. L. (1995). The nuclear receptor steroidogenic factor 1 is essential for the formation of the ventromedial hypothalamic nucleus. *Mol Endocrinol* **9**, 478-86.
- Imai, A., Ohno, T., Iida, K., Fuseya, T., Furui, T., and Tamaya, T. (1994). Presence of gonadotropin-releasing hormone receptor and its messenger ribonucleic acid in endometrial carcinoma and endometrium. *Gynecol Oncol* **55**, 144-8.
- Imperiale, M. J., and Kochanek, S. (2004). Adenovirus vectors: biology, design, and production. *Curr Top Microbiol Immunol* **273**, 335-57.
- Ingraham, H. A., Lala, D. S., Ikeda, Y., Luo, X., Shen, W. H., Nachtigal, M. W., Abbud, R., Nilson, J. H., and Parker, K. L. (1994). The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. *Genes Dev* **8**, 2302-12.
- Irmer, G., Burger, C., Muller, R., Ortmann, O., Peter, U., Kakar, S. S., Neill, J. D., Schulz, K. D., and Emons, G. (1995). Expression of the messenger RNAs for luteinizing hormone-releasing hormone (LHRH) and its receptor in human ovarian epithelial carcinoma. *Cancer Res* **55**, 817-22.
- Irmer, G., Burger, C., Ortmann, O., Schulz, K. D., and Emons, G. (1994). Expression of luteinizing hormone releasing hormone and its mRNA in human endometrial cancer cell lines. *J Clin Endocrinol Metab* **79**, 916-9.
- Jackson, S. M., Barnhart, K. M., Mellon, P. L., Gutierrez-Hartmann, A., and Hoeffler, J. P. (1993). Helix-loop-helix proteins are present and differentially expressed in different cell lines from the anterior pituitary. *Mol Cell Endocrinol* **96**, 167-76.
- Jackson, S. M., Gutierrez-Hartmann, A., and Hoeffler, J. P. (1995). Upstream stimulatory factor, a basic-helix-loop-helix-zipper protein, regulates the activity of the alpha-glycoprotein hormone subunit gene in pituitary cells. *Mol Endocrinol* **9**, 278-91.
- Japon, M. A., Rubinstein, M., and Low, M. J. (1994). In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. *J Histochem Cytochem* **42**, 1117-25.
- Jin, S., Mao, H., Schnepf, R. W., Sykes, S. M., Silva, A. C., D'Andrea, A. D., and Hua, X. (2003). Menin associates with FANCD2, a protein involved in repair of DNA damage. *Cancer Res* **63**, 4204-10.
- Joenje, H., Oostra, A. B., Wijker, M., di Summa, F. M., van Berkel, C. G., Rooimans, M. A., Ebell, W., van Weel, M., Pronk, J. C., Buchwald, M., and Arwert, F. (1997). Evidence for at least eight Fanconi anemia genes. *Am J Hum Genet* **61**, 940-4.
- Joenje, H., and Patel, K. J. (2001). The emerging genetic and molecular basis of Fanconi anaemia. *Nat Rev Genet* **2**, 446-57.
- Kaiser, U. B., Sabbagh, E., Chen, M. T., Chin, W. W., and Saunders, B. D. (1998). Sp1 binds to the rat luteinizing hormone beta (LHbeta) gene promoter and mediates gonadotropin-releasing hormone-stimulated expression of the LHbeta subunit gene. *J Biol Chem* **273**, 12943-51.
- Kakar, S. S., Grizzle, W. E., and Neill, J. D. (1994). The nucleotide sequences of human GnRH receptors in breast and ovarian tumors are identical with that found in pituitary. *Mol Cell Endocrinol* **106**, 145-9.

- Kakar, S. S., Musgrove, L. C., Devor, D. C., Sellers, J. C., and Neill, J. D. (1992). Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor. *Biochem Biophys Res Commun* **189**, 289-95.
- Kakar, S. S., Winters, S. J., Zacharias, W., Miller, D. M., and Flynn, S. (2003). Identification of distinct gene expression profiles associated with treatment of LbetaT2 cells with gonadotropin-releasing hormone agonist using microarray analysis. *Gene* **308**, 67-77.
- Kalland, K. H., Szilvay, A. M., Brokstad, K. A., Saetrevik, W., and Haukenes, G. (1994). The human immunodeficiency virus type 1 Rev protein shuttles between the cytoplasm and nuclear compartments. *Mol Cell Biol* **14**, 7436-44.
- Kang, S. K., Choi, K. C., Cheng, K. W., Nathwani, P. S., Auersperg, N., and Leung, P. C. (2000a). Role of gonadotropin-releasing hormone as an autocrine growth factor in human ovarian surface epithelium. *Endocrinology* **141**, 72-80.
- Kang, S. K., Tai, C. J., Cheng, K. W., and Leung, P. C. (2000b). Gonadotropin-releasing hormone activates mitogen-activated protein kinase in human ovarian and placental cells. *Mol Cell Endocrinol* **170**, 143-51.
- Kauffman, M. (1992). "The Atlas Of Mouse Development." Academic Press, San Diego.
- Kay, T. W., and Jameson, J. L. (1992). Identification of a gonadotropin-releasing hormone-responsive region in the glycoprotein hormone alpha-subunit promoter. *Mol Endocrinol* **6**, 1767-73.
- Kelch, R. P., Hopwood, N. J., and Marshall, J. C. (1980). Diagnosis of gonadotropin deficiency in adolescents: limited usefulness of a standard gonadotropin-releasing hormone test in obese boys. *J Pediatr* **97**, 820-4.
- Kendall, S. K., Samuelson, L. C., Saunders, T. L., Wood, R. I., and Camper, S. A. (1995). Targeted disruption of the pituitary glycoprotein hormone alpha-subunit produces hypogonadal and hypothyroid mice. *Genes Dev* **9**, 2007-19.
- Keri, G., Balogh, A., Szoke, B., Teplan, I., and Csuka, O. (1991). Gonadotropin-releasing hormone analogues inhibit cell proliferation and activate signal transduction pathways in MDA-MB-231 human breast cancer cell line. *Tumour Biol* **12**, 61-7.
- Keri, R. A., and Nilson, J. H. (1996). A steroidogenic factor-1 binding site is required for activity of the luteinizing hormone beta subunit promoter in gonadotropes of transgenic mice. *J Biol Chem* **271**, 10782-5.
- Keri, R. A., Wolfe, M. W., Saunders, T. L., Anderson, I., Kendall, S. K., Wagner, T., Yeung, J., Gorski, J., Nett, T. M., Camper, S. A., and et al. (1994). The proximal promoter of the bovine luteinizing hormone beta-subunit gene confers gonadotrope-specific expression and regulation by gonadotropin-releasing hormone, testosterone, and 17 beta-estradiol in transgenic mice. *Mol Endocrinol* **8**, 1807-16.
- Khokhlatchev, A. V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E., and Cobb, M. H. (1998). Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. *Cell* **93**, 605-15.

- Kimura, A., Ohmichi, M., Kurachi, H., Ikegami, H., Hayakawa, J., Tasaka, K., Kanda, Y., Nishio, Y., Jikihara, H., Matsuura, N., and Murata, Y. (1999). Role of mitogen-activated protein kinase/extracellular signal-regulated kinase cascade in gonadotropin-releasing hormone-induced growth inhibition of a human ovarian cancer cell line. *Cancer Res* **59**, 5133-42.
- King, J. A., and Millar, R. P. (1979). Heterogeneity of vertebrate luteinizing hormone-releasing hormone. *Science* **206**, 67-9.
- Kioussi, C., O'Connell, S., St-Onge, L., Treier, M., Gleiberman, A. S., Gruss, P., and Rosenfeld, M. G. (1999). Pax6 is essential for establishing ventral-dorsal cell boundaries in pituitary gland development. *Proc Natl Acad Sci U S A* **96**, 14378-82.
- Knee, R., and Murphy, P. R. (1997). Regulation of gene expression by natural antisense RNA transcripts. *Neurochem Int* **31**, 379-92.
- Knobil, E. (1980). The neuroendocrine control of the menstrual cycle. *Recent Prog Horm Res* **36**, 53-88.
- Koc, A., Pronk, J. C., Alikasifoglu, M., Joenje, H., and Altay, C. (1999). Variable pathogenicity of exon 43del (FAA) in four Fanconi anaemia patients within a consanguineous family. *Br J Haematol* **104**, 127-30.
- Koenig, J. A., and Edwardson, J. M. (1997). Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol Sci* **18**, 276-87.
- Komeili, A., and O'Shea, E. K. (2000). Nuclear transport and transcription. *Curr Opin Cell Biol* **12**, 355-60.
- Kottler, M. L., Chauvin, S., Lahlou, N., Harris, C. E., Johnston, C. J., Lagarde, J. P., Bouchard, P., Farid, N. R., and Counis, R. (2000). A new compound heterozygous mutation of the gonadotropin-releasing hormone receptor (L314X, Q106R) in a woman with complete hypogonadotropic hypogonadism: chronic estrogen administration amplifies the gonadotropin defect. *J Clin Endocrinol Metab* **85**, 3002-8.
- Kottler, M. L., Counis, R., and Bouchard, P. (1999). Mutations of the GnRH receptor gene: a new cause of autosomal-recessive hypogonadotropic hypogonadism. *Arch Med Res* **30**, 481-5.
- Krasnoshtein, F., and Buchwald, M. (1996). Developmental expression of the Fac gene correlates with congenital defects in Fanconi anemia patients. *Hum Mol Genet* **5**, 85-93.
- Kraus, S., Levy, G., Hanoch, T., Naor, Z., and Seger, R. (2004). Gonadotropin-releasing hormone induces apoptosis of prostate cancer cells: role of c-Jun NH2-terminal kinase, protein kinase B, and extracellular signal-regulated kinase pathways. *Cancer Res* **64**, 5736-44.
- Kraus, S., Naor, Z., and Seger, R. (2001). Intracellular signaling pathways mediated by the gonadotropin-releasing hormone (GnRH) receptor. *Arch Med Res* **32**, 499-509.
- Kremer, H., Mariman, E., Otten, B. J., Moll, G. W., Jr., Stoelinga, G. B., Wit, J. M., Jansen, M., Drop, S. L., Faas, B., Ropers, H. H., and et al. (1993). Cosegregation of missense mutations of the luteinizing hormone receptor gene with familial male-limited precocious puberty. *Hum Mol Genet* **2**, 1779-83.

- Kruyt, F. A., Abou-Zahr, F., Mok, H., and Youssoufian, H. (1999). Resistance to mitomycin C requires direct interaction between the Fanconi anemia proteins FANCA and FANCG in the nucleus through an arginine-rich domain. *J Biol Chem* **274**, 34212-8.
- Kruyt, F. A., Waisfisz, Q., Dijkmans, L. M., Hermsen, M. A., Youssoufian, H., Arwert, F., and Joenje, H. (1997). Cytoplasmic localization of a functionally active Fanconi anemia group A-green fluorescent protein chimera in human 293 cells. *Blood* **90**, 3288-95.
- Kruyt, F. A., and Youssoufian, H. (1998). The Fanconi anemia proteins FAA and FAC function in different cellular compartments to protect against cross-linking agent cytotoxicity. *Blood* **92**, 2229-36.
- Kuang, Y., Garcia-Higuera, I., Moran, A., Mondoux, M., Digweed, M., and D'Andrea, A. D. (2000). Carboxy terminal region of the Fanconi anemia protein, FANCG/XRCC9, is required for functional activity. *Blood* **96**, 1625-32.
- Kudo, N., Khochbin, S., Nishi, K., Kitano, K., Yanagida, M., Yoshida, M., and Horinouchi, S. (1997). Molecular cloning and cell cycle-dependent expression of mammalian CRM1, a protein involved in nuclear export of proteins. *J Biol Chem* **272**, 29742-51.
- Kumar, T. R., Palapattu, G., Wang, P., Woodruff, T. K., Boime, I., Byrne, M. C., and Matzuk, M. M. (1999). Transgenic models to study gonadotropin function: the role of follicle-stimulating hormone in gonadal growth and tumorigenesis. *Mol Endocrinol* **13**, 851-65.
- Kumar, T. R., Wang, Y., Lu, N., and Matzuk, M. M. (1997). Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet* **15**, 201-4.
- Kupfer, G., Naf, D., Garcia-Higuera, I., Wasik, J., Cheng, A., Yamashita, T., Tipping, A., Morgan, N., Mathew, C. G., and D'Andrea, A. D. (1999). A patient-derived mutant form of the Fanconi anemia protein, FANCA, is defective in nuclear accumulation. *Exp Hematol* **27**, 587-93.
- Kupfer, G. M., Naf, D., Suliman, A., Pulsipher, M., and D'Andrea, A. D. (1997a). The Fanconi anaemia proteins, FAA and FAC, interact to form a nuclear complex. *Nat Genet* **17**, 487-90.
- Kupfer, G. M., Yamashita, T., Naf, D., Suliman, A., Asano, S., and D'Andrea, A. D. (1997b). The Fanconi anemia polypeptide, FAC, binds to the cyclin-dependent kinase, cdc2. *Blood* **90**, 1047-54.
- Ladomery, M., and Dellaire, G. (2002). Multifunctional zinc finger proteins in development and disease. *Ann Hum Genet* **66**, 331-42.
- Lanctot, C., Moreau, A., Chamberland, M., Tremblay, M. L., and Drouin, J. (1999). Hindlimb patterning and mandible development require the Ptx1 gene. *Development* **126**, 1805-10.
- Larder, R., Chang, L., Clinton, M., and Brown, P. (2004). Gonadotropin-Releasing Hormone Regulates Expression of the DNA Damage Repair Gene, Fanconi anemia A, in Pituitary Gonadotroph Cells. *Biol Reprod* **71**, 828-36.

- Layman, L. C., Cohen, D. P., Jin, M., Xie, J., Li, Z., Reindollar, R. H., Bolbolan, S., Bick, D. P., Sherins, R. R., Duck, L. W., Musgrove, L. C., Sellers, J. C., and Neill, J. D. (1998). Mutations in gonadotropin-releasing hormone receptor gene cause hypogonadotropic hypogonadism. *Nat Genet* **18**, 14-5.
- Layman, L. C., Lee, E. J., Peak, D. B., Namnoum, A. B., Vu, K. V., van Lingen, B. L., Gray, M. R., McDonough, P. G., Reindollar, R. H., and Jameson, J. L. (1997). Delayed puberty and hypogonadism caused by mutations in the follicle-stimulating hormone beta-subunit gene. *N Engl J Med* **337**, 607-11.
- Lee, S. L., Sadovsky, Y., Swirnoff, A. H., Polish, J. A., Goda, P., Gavrulina, G., and Milbrandt, J. (1996). Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). *Science* **273**, 1219-21.
- Lenormand, P., Sardet, C., Pages, G., L'Allemain, G., Brunet, A., and Pouyssegur, J. (1993). Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. *J Cell Biol* **122**, 1079-88.
- Levrin, O., Erlich, T., Magdalena, N., Gregory, J. J., Batish, S. D., Verlander, P. C., and Auerbach, A. D. (1997). Sequence variation in the Fanconi anemia gene FAA. *Proc Natl Acad Sci U S A* **94**, 13051-6.
- Lewis, C. E., Megson, A., Morris, J. F., and Charlton, H. M. (1986). Multiple injections of LH-releasing hormone into hypogonadal (hpg) mice induce the appearance of two morphologically distinct populations of gonadotrophs. *J Endocrinol* **111**, 483-93.
- Li, A. W., and Murphy, P. R. (2000). Expression of alternatively spliced FGF-2 antisense RNA transcripts in the central nervous system: regulation of FGF-2 mRNA translation. *Mol Cell Endocrinol* **170**, 233-42.
- Li, S., Crenshaw, E. B., 3rd, Rawson, E. J., Simmons, D. M., Swanson, L. W., and Rosenfeld, M. G. (1990). Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature* **347**, 528-33.
- Liang, P., and Pardee, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-71.
- Licht, P., Russu, V., and Wildt, L. (2001). On the role of human chorionic gonadotropin (hCG) in the embryo-endometrial microenvironment: implications for differentiation and implantation. *Semin Reprod Med* **19**, 37-47.
- Lightfoot, J., Alon, N., Bosnoyan-Collins, L., and Buchwald, M. (1999). Characterization of regions functional in the nuclear localization of the Fanconi anemia group A protein. *Hum Mol Genet* **8**, 1007-15.
- Limonta, P., Dondi, D., Moretti, R. M., Fermo, D., Garattini, E., and Motta, M. (1993). Expression of luteinizing hormone-releasing hormone mRNA in the human prostatic cancer cell line LNCaP. *J Clin Endocrinol Metab* **76**, 797-800.
- Limonta, P., Dondi, D., Moretti, R. M., Maggi, R., and Motta, M. (1992). Antiproliferative effects of luteinizing hormone-releasing hormone agonists on the human prostatic cancer cell line LNCaP. *J Clin Endocrinol Metab* **75**, 207-12.

- Limonta, P., Moretti, R. M., Marelli, M. M., and Motta, M. (2003). The biology of gonadotropin hormone-releasing hormone: role in the control of tumor growth and progression in humans. *Front Neuroendocrinol* **24**, 279-95.
- Lin, C. R., Kiuoussi, C., O'Connell, S., Briata, P., Szeto, D., Liu, F., Izipisua-Belmonte, J. C., and Rosenfeld, M. G. (1999). Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. *Nature* **401**, 279-82.
- Lincoln, D. W., Fraser, H. M., Lincoln, G. A., Martin, G. B., and McNeilly, A. S. (1985). Hypothalamic pulse generators. *Recent Prog Horm Res* **41**, 369-419.
- Liu, F., Austin, D. A., Mellon, P. L., Olefsky, J. M., and Webster, N. J. (2002). GnRH activates ERK1/2 leading to the induction of c-fos and LHbeta protein expression in LbetaT2 cells. *Mol Endocrinol* **16**, 419-34.
- Liu, J. M., Auerbach, A. D., and Young, N. S. (1991). Fanconi anemia presenting unexpectedly in an adult kindred with no dysmorphic features. *Am J Med* **91**, 555-7.
- Liu, N., Lamerdin, J. E., Tucker, J. D., Zhou, Z. Q., Walter, C. A., Albala, J. S., Busch, D. B., and Thompson, L. H. (1997). The human XRCC9 gene corrects chromosomal instability and mutagen sensitivities in CHO UV40 cells. *Proc Natl Acad Sci U S A* **94**, 9232-7.
- Liu, Y. C., Kato, Y., Inoue, K., Tanaka, S., and Kurosumi, K. (1988). Co-localization of LH beta and FSH beta mRNAs in the porcine anterior pituitary by in situ hybridization with biotinylated probes. *Biochem Biophys Res Commun* **154**, 80-4.
- Lloyd, J. M., and Childs, G. V. (1988). Differential storage and release of luteinizing hormone and follicle-releasing hormone from individual gonadotropes separated by centrifugal elutriation. *Endocrinology* **122**, 1282-90.
- Lo Ten Foe, J. R., Roomians, M. A., Bosnoyan-Collins, L., Alon, N., Wijker, M., Parker, L., Lightfoot, J., Carreau, M., Callen, D. F., Savoia, A., Cheng, N. C., van Berkel, C. G., Strunk, M. H., Gille, J. J., Pals, G., Kruyt, F. A., Pronk, J. C., Arwert, F., Buchwald, M., and Joenje, H. (1996). Expression cloning of a cDNA for the major Fanconi anaemia gene, FAA. *Nat Genet* **14**, 320-3.
- Lu, D. L., Peegel, H., Mosier, S. M., and Menon, K. M. (1993). Loss of lutropin/human choriogonadotropin receptor messenger ribonucleic acid during ligand-induced down-regulation occurs post transcriptionally. *Endocrinology* **132**, 235-40.
- Luo, X., Ikeda, Y., Lala, D. S., Baity, L. A., Meade, J. C., and Parker, K. L. (1995). A cell-specific nuclear receptor plays essential roles in adrenal and gonadal development. *Endocr Res* **21**, 517-24.
- Mangia, A., Tommasi, S., Reshkin, S. J., Simone, G., Stea, B., Schittulli, F., and Paradiso, A. (2002). Gonadotropin releasing hormone receptor expression in primary breast cancer: comparison of immunohistochemical, radioligand and Western blot analyses. *Oncol Rep* **9**, 1127-32.
- Marcil, A., Dumontier, E., Chamberland, M., Camper, S. A., and Drouin, J. (2003). Pitx1 and Pitx2 are required for development of hindlimb buds. *Development* **130**, 45-55.

- Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R., and Tuschl, T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**, 563-74.
- Mason, A. J., Hayflick, J. S., Zoeller, R. T., Young, W. S., 3rd, Phillips, H. S., Nikolics, K., and Seeburg, P. H. (1986a). A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. *Science* **234**, 1366-71.
- Mason, A. J., Niall, H. D., and Seeburg, P. H. (1986b). Structure of two human ovarian inhibins. *Biochem Biophys Res Commun* **135**, 957-64.
- Massa, G. G., Heinrichs, C., Vamos, E., and Van Vliet, G. (2002). Hypergonadotropic hypogonadism in a boy with Fanconi anemia with growth hormone deficiency and pituitary stalk interruption. *J Pediatr* **140**, 277.
- Matsumoto, A. M., and Bremner, W. J. (1984). Modulation of pulsatile gonadotropin secretion by testosterone in man. *J Clin Endocrinol Metab* **58**, 609-14.
- Matsumoto, A. M., Paulsen, C. A., and Bremner, W. J. (1984). Stimulation of sperm production by human luteinizing hormone in gonadotropin-suppressed normal men. *J Clin Endocrinol Metab* **59**, 882-7.
- Matthews, C. H., Borgato, S., Beck-Peccoz, P., Adams, M., Tone, Y., Gambino, G., Casagrande, S., Tedeschini, G., Benedetti, A., and Chatterjee, V. K. (1993). Primary amenorrhoea and infertility due to a mutation in the beta-subunit of follicle-stimulating hormone. *Nat Genet* **5**, 83-6.
- McAllister, K. A., Bennett, L. M., Houle, C. D., Ward, T., Malphurs, J., Collins, N. K., Cachafeiro, C., Haseman, J., Goulding, E. H., Bunch, D., Eddy, E. M., Davis, B. J., and Wiseman, R. W. (2002). Cancer susceptibility of mice with a homozygous deletion in the COOH-terminal domain of the Brca2 gene. *Cancer Res* **62**, 990-4.
- McArdle, C. A., Franklin, J., Green, L., and Hislop, J. N. (2002a). The gonadotrophin-releasing hormone receptor: signalling, cycling and desensitisation. *Arch Physiol Biochem* **110**, 113-22.
- McArdle, C. A., Franklin, J., Green, L., and Hislop, J. N. (2002b). Signalling, cycling and desensitisation of gonadotrophin-releasing hormone receptors. *J Endocrinol* **173**, 1-11.
- McCartney, C. R., Eagleson, C. A., and Marshall, J. C. (2002). Regulation of gonadotropin secretion: implications for polycystic ovary syndrome. *Semin Reprod Med* **20**, 317-26.
- McManus, M. T., and Sharp, P. A. (2002). Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet* **3**, 737-47.
- McNeilly, A. S. (1988). The control of FSH secretion. *Acta Endocrinol Suppl (Copenh)* **288**, 31-40.
- McNeilly, J. R., Brown, P., Clark, A. J., and McNeilly, A. S. (1991). Gonadotrophin-releasing hormone modulation of gonadotrophins in the ewe: evidence for differential effects on gene expression and hormone secretion. *J Mol Endocrinol* **7**, 35-43.
- McNeilly, J. R., Brown, P., Mullins, J., Clark, A. J., and McNeilly, A. S. (1996). Characterization of the ovine LH beta-subunit gene: the promoter is regulated by GnRH and gonadal steroids in transgenic mice. *J Endocrinol* **151**, 481-9.

- Means, A. R., Dedman, J. R., Tash, J. S., Tindall, D. J., van Sickle, M., and Welsh, M. J. (1980). Regulation of the testis sertoli cell by follicle stimulating hormone. *Annu Rev Physiol* **42**, 59-70.
- Medhurst, A. L., Huber, P. A., Waisfisz, Q., de Winter, J. P., and Mathew, C. G. (2001). Direct interactions of the five known Fanconi anaemia proteins suggest a common functional pathway. *Hum Mol Genet* **10**, 423-9.
- Meetei, A. R., de Winter, J. P., Medhurst, A. L., Wallisch, M., Waisfisz, Q., van de Vrugt, H. J., Oostra, A. B., Yan, Z., Ling, C., Bishop, C. E., Hoatlin, M. E., Joenje, H., and Wang, W. (2003a). A novel ubiquitin ligase is deficient in Fanconi anemia. *Nat Genet* **35**, 165-70.
- Meetei, A. R., Sechi, S., Wallisch, M., Yang, D., Young, M. K., Joenje, H., Hoatlin, M. E., and Wang, W. (2003b). A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. *Mol Cell Biol* **23**, 3417-26.
- Meetei, A. R., Yan, Z., and Wang, W. (2004). FANCL Replaces BRCA1 as the Likely Ubiquitin Ligase Responsible for FANCD2 Monoubiquitination. *Cell Cycle* **3**, 179-81.
- Mercurio, F., and Manning, A. M. (1999). NF-kappaB as a primary regulator of the stress response. *Oncogene* **18**, 6163-71.
- Mi, J., and Kupfer, G. M. (2004). The Fanconi Anemia Core Complex Associates with Chromatin During S Phase. *Blood*.
- Mikimi, S. (1992). "Hypophysis." Springer-Verlag, New York.
- Millar, R. P. (2002). Gonadotropin releasing hormones and their receptors. In "Molecular Biology in Clinical Reproductive Medicine" (B. C. J. M. Fauser, Ed.), pp. 199-224. Parthenon.
- Miller, J., McLachlan, A. D., and Klug, A. (1985a). Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. *Embo J* **4**, 1609-14.
- Miller, W. R., Scott, W. N., Morris, R., Fraser, H. M., and Sharpe, R. M. (1985b). Growth of human breast cancer cells inhibited by a luteinizing hormone-releasing hormone agonist. *Nature* **313**, 231-3.
- Minegishi, T., Nakamura, K., Takakura, Y., Ibuki, Y., Igarashi, M., and Minegishi, T. (1991). Cloning and sequencing of human FSH receptor cDNA. *Biochem Biophys Res Commun* **175**, 1125-30.
- Minegishi, T., Nakamura, K., Takakura, Y., Miyamoto, K., Hasegawa, Y., Ibuki, Y., Igarashi, M., and Minegishi, T. (1990). Cloning and sequencing of human LH/hCG receptor cDNA. *Biochem Biophys Res Commun* **172**, 1049-54.
- Moenter, S. M., Caraty, A., Locatelli, A., and Karsch, F. J. (1991). Pattern of gonadotropin-releasing hormone (GnRH) secretion leading up to ovulation in the ewe: existence of a preovulatory GnRH surge. *Endocrinology* **129**, 1175-82.
- Moll, U. M., Riou, G., and Levine, A. J. (1992). Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. *Proc Natl Acad Sci U S A* **89**, 7262-6.
- Montanelli, L., Delbaere, A., Di Carlo, C., Nappi, C., Smits, G., Vassart, G., and Costagliola, S. (2004). A mutation in the follicle-stimulating hormone receptor as a cause of familial spontaneous ovarian hyperstimulation syndrome. *J Clin Endocrinol Metab* **89**, 1255-8.

- Montes De Oca, R., Andreassen, P. R., Margossian, S. P., Gregory, R. G., Taniguchi, T., Wang, X., Houghtaling, S., Grompe, M., and D'Andrea, A. D. (2004). Regulated interaction of the Fanconi Anemia protein, FANCD2, with chromatin. *Blood*.
- Moretti, R. M., Montagnani Marelli, M., Van Groeninghen, J. C., and Limonta, P. (2002). Locally expressed LHRH receptors mediate the oncostatic and antimetastatic activity of LHRH agonists on melanoma cells. *J Clin Endocrinol Metab* **87**, 3791-7.
- Morgan, K., Conklin, D., Pawson, A. J., Sellar, R., Ott, T. R., and Millar, R. P. (2003). A transcriptionally active human type II gonadotropin-releasing hormone receptor gene homolog overlaps two genes in the antisense orientation on chromosome 1q.12. *Endocrinology* **144**, 423-36.
- Morgan, N. V., Tipping, A. J., Joenje, H., and Mathew, C. G. (1999). High frequency of large intragenic deletions in the Fanconi anemia group A gene. *Am J Hum Genet* **65**, 1330-41.
- Mulvaney, J. M., and Roberson, M. S. (2000). Divergent signaling pathways requiring discrete calcium signals mediate concurrent activation of two mitogen-activated protein kinases by gonadotropin-releasing hormone. *J Biol Chem* **275**, 14182-9.
- Nadler, J. J., and Braun, R. E. (2000). Fanconi anemia complementation group C is required for proliferation of murine primordial germ cells. *Genesis* **27**, 117-23.
- Naf, D., Kupfer, G. M., Suliman, A., Lambert, K., and D'Andrea, A. D. (1998). Functional activity of the fanconi anemia protein FAA requires FAC binding and nuclear localization. *Mol Cell Biol* **18**, 5952-60.
- Naor, Z., Benard, O., and Seger, R. (2000). Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. *Trends Endocrinol Metab* **11**, 91-9.
- Netchine, I., Sobrier, M. L., Krude, H., Schnabel, D., Maghnie, M., Marcos, E., Duriez, B., Cacheux, V., Moers, A., Goossens, M., Gruters, A., and Amselem, S. (2000). Mutations in LHX3 result in a new syndrome revealed by combined pituitary hormone deficiency. *Nat Genet* **25**, 182-6.
- Ngan, E. S., Cheng, P. K., Leung, P. C., and Chow, B. K. (1999). Steroidogenic factor-1 interacts with a gonadotrope-specific element within the first exon of the human gonadotropin-releasing hormone receptor gene to mediate gonadotrope-specific expression. *Endocrinology* **140**, 2452-62.
- Noll, M., Battaile, K. P., Bateman, R., Lax, T. P., Rathbun, K., Reifsteck, C., Bagby, G., Finegold, M., Olson, S., and Grompe, M. (2002). Fanconi anemia group A and C double-mutant mice: functional evidence for a multi-protein Fanconi anemia complex. *Exp Hematol* **30**, 679-88.
- Nordan, U. Z., Humbert, J. R., MacGillivray, M. H., and Fitzpatrick, J. E. (1979). Fanconi's anemia with growth hormone deficiency. *Am J Dis Child* **133**, 291-3.
- Nykanen, A., Haley, B., and Zamore, P. D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309-21.

- O'Shaughnessy, P. J., and Brown, P. S. (1978). Reduction in FSH receptors in the rat testis by injection of homologous hormone. *Mol Cell Endocrinol* **12**, 9-15.
- Obrig, T. G., Culp, W. J., McKeehan, W. L., and Hardesty, B. (1971). The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. *J Biol Chem* **246**, 174-81.
- Ohno, T., Imai, A., Furui, T., Takahashi, K., and Tamaya, T. (1993). Presence of gonadotropin-releasing hormone and its messenger ribonucleic acid in human ovarian epithelial carcinoma. *Am J Obstet Gynecol* **169**, 605-10.
- Ostendorff, H. P., Peirano, R. I., Peters, M. A., Schluter, A., Bossenz, M., Scheffner, M., and Bach, I. (2002). Ubiquitination-dependent cofactor exchange on LIM homeodomain transcription factors. *Nature* **416**, 99-103.
- Otsuki, T., Kajigaya, S., Ozawa, K., and Liu, J. M. (1999). SNX5, a new member of the sorting nexin family, binds to the Fanconi anemia complementation group A protein. *Biochem Biophys Res Commun* **265**, 630-5.
- Pace, P., Johnson, M., Tan, W. M., Mosedale, G., Sng, C., Hoatlin, M., de Winter, J., Joenje, H., Gergely, F., and Patel, K. J. (2002). FANCE: the link between Fanconi anaemia complex assembly and activity. *Embo J* **21**, 3414-23.
- Pang, Q., Fagerlie, S., Christianson, T. A., Keeble, W., Faulkner, G., Diaz, J., Rathbun, R. K., and Bagby, G. C. (2000). The Fanconi anemia protein FANCC binds to and facilitates the activation of STAT1 by gamma interferon and hematopoietic growth factors. *Mol Cell Biol* **20**, 4724-35.
- Pang, Q., Keeble, W., Christianson, T. A., Faulkner, G. R., and Bagby, G. C. (2001). FANCC interacts with Hsp70 to protect hematopoietic cells from IFN-gamma/TNF-alpha-mediated cytotoxicity. *Embo J* **20**, 4478-89.
- Papavasiliou, S. S., Zmeili, S., Khoury, S., Landefeld, T. D., Chin, W. W., and Marshall, J. C. (1986). Gonadotropin-releasing hormone differentially regulates expression of the genes for luteinizing hormone alpha and beta subunits in male rats. *Proc Natl Acad Sci U S A* **83**, 4026-9.
- Park, D., Cheon, M., Kim, C., Kim, K., and Ryu, K. (1996). Progesterone together with estradiol promotes luteinizing hormone beta-subunit mRNA stability in rat pituitary cells cultured in vitro. *Eur J Endocrinol* **134**, 236-42.
- Patel, K. J., Yu, V. P., Lee, H., Corcoran, A., Thistlethwaite, F. C., Evans, M. J., Colledge, W. H., Friedman, L. S., Ponder, B. A., and Venkitaraman, A. R. (1998). Involvement of Brca2 in DNA repair. *Mol Cell* **1**, 347-57.
- Paul, S. J., Ortolano, G. A., Haisenleder, D. J., Stewart, J. M., Shupnik, M. A., and Marshall, J. C. (1990). Gonadotropin subunit messenger RNA concentrations after blockade of gonadotropin-releasing hormone action: testosterone selectively increases follicle-stimulating hormone beta-subunit messenger RNA by posttranscriptional mechanisms. *Mol Endocrinol* **4**, 1943-55.
- Pawson, A. J., Katz, A., Sun, Y. M., Lopes, J., Illing, N., Millar, R. P., and Davidson, J. S. (1998). Contrasting internalization kinetics of human and chicken gonadotropin-releasing hormone receptors mediated by C-terminal tail. *J Endocrinol* **156**, R9-12.
- Pawson, T., Gish, G.D. and Nash, P. (2004). The SH2 domain: a prototype for protein interaction modules. In "Molecular protein domains" (G. Cesareni, Gimona, M., Sudol, M. and Yaffe, M., Ed.), pp. 5-36. Wiley.

- Pawson, T., and Scott, J. D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278**, 2075-80.
- Peng, C., Fan, N. C., Ligier, M., Vaananen, J., and Leung, P. C. (1994). Expression and regulation of gonadotropin-releasing hormone (GnRH) and GnRH receptor messenger ribonucleic acids in human granulosa-luteal cells. *Endocrinology* **135**, 1740-6.
- Petaja-Repo, U. E., Hogue, M., Laperriere, A., Bhalla, S., Walker, P., and Bouvier, M. (2001). Newly synthesized human delta opioid receptors retained in the endoplasmic reticulum are retrotranslocated to the cytosol, deglycosylated, ubiquitinated, and degraded by the proteasome. *J Biol Chem* **276**, 4416-23.
- Phillip, M., Arbelle, J. E., Segev, Y., and Parvari, R. (1998). Male hypogonadism due to a mutation in the gene for the beta-subunit of follicle-stimulating hormone. *N Engl J Med* **338**, 1729-32.
- Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70**, 503-33.
- Pinol-Roma, S., and Dreyfuss, G. (1992). Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature* **355**, 730-2.
- Pipaon, C., Casado, J. A., Bueren, J. A., and Fernandez-Luna, J. L. (2004). Jun N-terminal kinase activity and early growth-response factor-1 gene expression are down-regulated in Fanconi anemia group A lymphoblasts. *Blood* **103**, 128-32.
- Pralong, F. P., Gomez, F., Castillo, E., Cotecchia, S., Abuin, L., Aubert, M. L., Portmann, L., and Gaillard, R. C. (1999). Complete hypogonadotropic hypogonadism associated with a novel inactivating mutation of the gonadotropin-releasing hormone receptor. *J Clin Endocrinol Metab* **84**, 3811-6.
- Qiao, F., Moss, A., and Kupfer, G. M. (2001). Fanconi anemia proteins localize to chromatin and the nuclear matrix in a DNA damage- and cell cycle-regulated manner. *J Biol Chem* **276**, 23391-6.
- Quirk, C. C., Lozada, K. L., Keri, R. A., and Nilson, J. H. (2001). A single Pitx1 binding site is essential for activity of the LHbeta promoter in transgenic mice. *Mol Endocrinol* **15**, 734-46.
- Quirk, J., and Brown, P. (2002). Hesx1 homeodomain protein represses transcription as a monomer and antagonises transactivation of specific sites as a homodimer. *J Mol Endocrinol* **28**, 193-205.
- Raetzman, L. T., Ward, R., and Camper, S. A. (2002). Lhx4 and Prop1 are required for cell survival and expansion of the pituitary primordia. *Development* **129**, 4229-39.
- Raiborg, C., Rusten, T. E., and Stenmark, H. (2003). Protein sorting into multivesicular endosomes. *Curr Opin Cell Biol* **15**, 446-55.
- Rajan, J. V., Marquis, S. T., Gardner, H. P., and Chodosh, L. A. (1997). Developmental expression of Brca2 colocalizes with Brca1 and is associated with proliferation and differentiation in multiple tissues. *Dev Biol* **184**, 385-401.
- Rao, L. V., Cleveland, R. P., Kimmel, R. J., and Ataya, K. M. (1995). Hematopoietic stem cell antigen-1 (Sca-1) expression in different lymphoid tissues of female mice treated with GnRH agonist. *Am J Reprod Immunol* **34**, 257-66.

- Reuter, T., Herterich, S., Bernhard, O., Hoehn, H., and Gross, H. J. (2000). Strong FANCA/FANCG but weak FANCA/FANCC interaction in the yeast 2-hybrid system. *Blood* **95**, 719-20.
- Reuter, T. Y., Medhurst, A. L., Waisfisz, Q., Zhi, Y., Herterich, S., Hoehn, H., Gross, H. J., Joenje, H., Hoatlin, M. E., Mathew, C. G., and Huber, P. A. (2003). Yeast two-hybrid screens imply involvement of Fanconi anemia proteins in transcription regulation, cell signaling, oxidative metabolism, and cellular transport. *Exp Cell Res* **289**, 211-21.
- Risbridger, G. P., and Cancilla, B. (2000). Role of activins in the male reproductive tract. *Rev Reprod* **5**, 99-104.
- Risma, K. A., Clay, C. M., Nett, T. M., Wagner, T., Yun, J., and Nilson, J. H. (1995). Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumors. *Proc Natl Acad Sci U S A* **92**, 1322-6.
- Risma, K. A., Hirshfield, A. N., and Nilson, J. H. (1997). Elevated luteinizing hormone in prepubertal transgenic mice causes hyperandrogenemia, precocious puberty, and substantial ovarian pathology. *Endocrinology* **138**, 3540-7.
- Roberson, M. S., Misra-Press, A., Laurance, M. E., Stork, P. J., and Maurer, R. A. (1995). A role for mitogen-activated protein kinase in mediating activation of the glycoprotein hormone alpha-subunit promoter by gonadotropin-releasing hormone. *Mol Cell Biol* **15**, 3531-9.
- Roberson, M. S., Schoderbek, W. E., Tremml, G., and Maurer, R. A. (1994). Activation of the glycoprotein hormone alpha-subunit promoter by a LIM-homeodomain transcription factor. *Mol Cell Biol* **14**, 2985-93.
- Robertson, D., Burger, H. G., Sullivan, J., Cahir, N., Groome, N., Poncelet, E., Franchimont, P., Woodruff, T., and Mather, J. P. (1996). Biological and immunological characterization of inhibin forms in human plasma. *J Clin Endocrinol Metab* **81**, 669-76.
- Rosendorff, J., Bernstein, R., Macdougall, L., and Jenkins, T. (1987). Fanconi anemia: another disease of unusually high prevalence in the Afrikaans population of South Africa. *Am J Med Genet* **27**, 793-7.
- Rosenthal, I. M., Refetoff, S., Rich, B., Barnes, R. B., Sunthornthepvarakul, T., Parma, J., and Rosenfield, R. L. (1996). Response to challenge with gonadotropin-releasing hormone agonist in a mother and her two sons with a constitutively activating mutation of the luteinizing hormone receptor--a clinical research center study. *J Clin Endocrinol Metab* **81**, 3802-6.
- Ross, J. (1995). mRNA stability in mammalian cells. *Microbiol Rev* **59**, 423-50.
- Rosselli, F., Sanceau, J., Wietzerbin, J., and Moustacchi, E. (1992). Abnormal lymphokine production: a novel feature of the genetic disease Fanconi anemia. I. Involvement of interleukin-6. *Hum Genet* **89**, 42-8.
- Saunders, B. D., Sabbagh, E., Chin, W. W., and Kaiser, U. B. (1998). Differential use of signal transduction pathways in the gonadotropin-releasing hormone-mediated regulation of gonadotropin subunit gene expression. *Endocrinology* **139**, 1835-43.

- Savino, M., Ianzano, L., Strippoli, P., Ramenghi, U., Arslanian, A., Bagnara, G. P., Joenje, H., Zelante, L., and Savoia, A. (1997). Mutations of the Fanconi anemia group A gene (FAA) in Italian patients. *Am J Hum Genet* **61**, 1246-53.
- Schmeichel, K. L., and Beckerle, M. C. (1998). LIM domains of cysteine-rich protein 1 (CRP1) are essential for its zyxin-binding function. *Biochem J* **331** (Pt 3), 885-92.
- Schoderbek, W. E., Kim, K. E., Ridgway, E. C., Mellon, P. L., and Maurer, R. A. (1992). Analysis of DNA sequences required for pituitary-specific expression of the glycoprotein hormone alpha-subunit gene. *Mol Endocrinol* **6**, 893-903.
- Schoderbek, W. E., Roberson, M. S., and Maurer, R. A. (1993). Two different DNA elements mediate gonadotropin releasing hormone effects on expression of the glycoprotein hormone alpha-subunit gene. *J Biol Chem* **268**, 3903-10.
- Schoof, E., Beck, J. D., Joenje, H., and Doerr, H. G. (2000). Growth hormone deficiency in one of two siblings with Fanconi's anaemia complementation group FA-D. *Growth Horm IGF Res* **10**, 290-4.
- Schroeder, T. M., Tilgen, D., Kruger, J., and Vogel, F. (1976). Formal genetics of Fanconi's anemia. *Hum Genet* **32**, 257-88.
- Schwarz, D. S., Hutvagner, G., Haley, B., and Zamore, P. D. (2002). Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. *Mol Cell* **10**, 537-48.
- Seeburg, P. H., and Adelman, J. P. (1984). Characterization of cDNA for precursor of human luteinizing hormone releasing hormone. *Nature* **311**, 666-8.
- Seger, R., and Krebs, E. G. (1995). The MAPK signaling cascade. *Faseb J* **9**, 726-35.
- Seminara, S. B., Messenger, S., Chatzidaki, E. E., Thresher, R. R., Acierno, J. S., Jr., Shagoury, J. K., Bo-Abbas, Y., Kuohung, W., Schwinof, K. M., Hendrick, A. G., Zahn, D., Dixon, J., Kaiser, U. B., Slaugenhaupt, S. A., Gusella, J. F., O'Rahilly, S., Carlton, M. B., Crowley, W. F., Jr., Aparicio, S. A., and Colledge, W. H. (2003). The GPR54 gene as a regulator of puberty. *N Engl J Med* **349**, 1614-27.
- Seminara, S. B., Oliveira, L. M., Beranova, M., Hayes, F. J., and Crowley, W. F., Jr. (2000). Genetics of hypogonadotropic hypogonadism. *J Endocrinol Invest* **23**, 560-5.
- Seyschab, H., Bretzel, G., Friedl, R., Schindler, D., Sun, Y., and Hoehn, H. (1994). Modulation of the spontaneous G2 phase blockage in Fanconi anemia cells by caffeine: differences from cells arrested by X-irradiation. *Mutat Res* **308**, 149-57.
- Shah, B. H., and Milligan, G. (1994). The gonadotrophin-releasing hormone receptor of alpha T3-1 pituitary cells regulates cellular levels of both of the phosphoinositidase C-linked G proteins, Gq alpha and G11 alpha, equally. *Mol Pharmacol* **46**, 1-7.
- Sharp, P. A. (1999). RNAi and double-strand RNA. *Genes Dev* **13**, 139-41.
- Sheng, H. Z., Moriyama, K., Yamashita, T., Li, H., Potter, S. S., Mahon, K. A., and Westphal, H. (1997). Multistep control of pituitary organogenesis. *Science* **278**, 1809-12.

- Sheng, H. Z., Zhadanov, A. B., Mosinger, B., Jr., Fujii, T., Bertuzzi, S., Grinberg, A., Lee, E. J., Huang, S. P., Mahon, K. A., and Westphal, H. (1996). Specification of pituitary cell lineages by the LIM homeobox gene *Lhx3*. *Science* **272**, 1004-7.
- Shenker, A., Laue, L., Kosugi, S., Merendino, J. J., Jr., Minegishi, T., and Cutler, G. B., Jr. (1993). A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature* **365**, 652-4.
- Shupnik, M. A. (1990). Effects of gonadotropin-releasing hormone on rat gonadotropin gene transcription in vitro: requirement for pulsatile administration for luteinizing hormone-beta gene stimulation. *Mol Endocrinol* **4**, 1444-50.
- Simmons, D. M., Voss, J. W., Ingraham, H. A., Holloway, J. M., Broide, R. S., Rosenfeld, M. G., and Swanson, L. W. (1990). Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. *Genes Dev* **4**, 695-711.
- Sloop, K. W., Dwyer, C. J., and Rhodes, S. J. (2001a). An isoform-specific inhibitory domain regulates the LHX3 LIM homeodomain factor holoprotein and the production of a functional alternate translation form. *J Biol Chem* **276**, 36311-9.
- Sloop, K. W., Parker, G. E., Hanna, K. R., Wright, H. A., and Rhodes, S. J. (2001b). LHX3 transcription factor mutations associated with combined pituitary hormone deficiency impair the activation of pituitary target genes. *Gene* **265**, 61-9.
- Smits, G., Olatunbosun, O., Delbaere, A., Pierson, R., Vassart, G., and Costagliola, S. (2003). Ovarian hyperstimulation syndrome due to a mutation in the follicle-stimulating hormone receptor. *N Engl J Med* **349**, 760-6.
- Sornson, M. W., Wu, W., Dasen, J. S., Flynn, S. E., Norman, D. J., O'Connell, S. M., Gukovsky, I., Carriere, C., Ryan, A. K., Miller, A. P., Zuo, L., Gleiberman, A. S., Andersen, B., Beamer, W. G., and Rosenfeld, M. G. (1996). Pituitary lineage determination by the Prophet of Pit-1 homeodomain factor defective in Ames dwarfism. *Nature* **384**, 327-33.
- Sosnowski, R., Mellon, P. L., and Lawson, M. A. (2000). Activation of translation in pituitary gonadotrope cells by gonadotropin-releasing hormone. *Mol Endocrinol* **14**, 1811-9.
- Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503-17.
- Stanislaus, D., Janovick, J. A., Ji, T., Wilkie, T. M., Offermanns, S., and Conn, P. M. (1998). Gonadotropin and gonadal steroid release in response to a gonadotropin-releasing hormone agonist in Gqalpha and G11alpha knockout mice. *Endocrinology* **139**, 2710-7.
- Staton, J. M., Thomson, A. M., and Leedman, P. J. (2000). Hormonal regulation of mRNA stability and RNA-protein interactions in the pituitary. *J Mol Endocrinol* **25**, 17-34.
- Stojilkovic, S. S., and Catt, K. J. (1995). Novel aspects of GnRH-induced intracellular signaling and secretion in pituitary gonadotrophs. *J Neuroendocrinol* **7**, 739-57.

- Strahl, B. D., Huang, H. J., Pedersen, N. R., Wu, J. C., Ghosh, B. R., and Miller, W. L. (1997). Two proximal activating protein-1-binding sites are sufficient to stimulate transcription of the ovine follicle-stimulating hormone-beta gene. *Endocrinology* **138**, 2621-31.
- Strahl, B. D., Huang, H. J., Sebastian, J., Ghosh, B. R., and Miller, W. L. (1998). Transcriptional activation of the ovine follicle-stimulating hormone beta-subunit gene by gonadotropin-releasing hormone: involvement of two activating protein-1-binding sites and protein kinase C. *Endocrinology* **139**, 4455-65.
- Strathdee, C. A., Gavish, H., Shannon, W. R., and Buchwald, M. (1992). Cloning of cDNAs for Fanconi's anaemia by functional complementation. *Nature* **356**, 763-7.
- Stubbe, P., and Prindull, G. (1975). Fanconi's anemia. II. Are multiple endocrine insufficiencies a substantial part of the disease? *Acta Paediatr Scand* **64**, 790-4.
- Suh, H., Gage, P. J., Drouin, J., and Camper, S. A. (2002). Pitx2 is required at multiple stages of pituitary organogenesis: pituitary primordium formation and cell specification. *Development* **129**, 329-37.
- Suszko, M. I., Lo, D. J., Suh, H., Camper, S. A., and Woodruff, T. K. (2003). Regulation of the rat follicle-stimulating hormone beta-subunit promoter by activin. *Mol Endocrinol* **17**, 318-32.
- Swift, M. (1971). Fanconi's anaemia in the genetics of neoplasia. *Nature* **230**, 370-3.
- Szeto, D. P., Ryan, A. K., O'Connell, S. M., and Rosenfeld, M. G. (1996). P-OTX: a PIT-1-interacting homeodomain factor expressed during anterior pituitary gland development. *Proc Natl Acad Sci U S A* **93**, 7706-10.
- Tachibana, A., Kato, T., Ejima, Y., Yamada, T., Shimizu, T., Yang, L., Tsunematsu, Y., and Sasaki, M. S. (1999). The FANCA gene in Japanese Fanconi anemia: reports of eight novel mutations and analysis of sequence variability. *Hum Mutat* **13**, 237-44.
- Takeuchi, S., Futamura, N., Minoura, H., and Toyoda, N. (1998). Possible direct effect of gonadotropin releasing hormone on human endometrium and decidua. *Life Sci* **62**, 1187-94.
- Takuma, N., Sheng, H. Z., Furuta, Y., Ward, J. M., Sharma, K., Hogan, B. L., Pfaff, S. L., Westphal, H., Kimura, S., and Mahon, K. A. (1998). Formation of Rathke's pouch requires dual induction from the diencephalon. *Development* **125**, 4835-40.
- Tamanoi, F., and Stillman, B. W. (1982). Function of adenovirus terminal protein in the initiation of DNA replication. *Proc Natl Acad Sci U S A* **79**, 2221-5.
- Taniguchi, T., and D'Andrea, A. D. (2002). The Fanconi anemia protein, FANCE, promotes the nuclear accumulation of FANCC. *Blood* **100**, 2457-62.
- Taniguchi, T., Garcia-Higuera, I., Andreassen, P. R., Gregory, R. C., Grompe, M., and D'Andrea, A. D. (2002). S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. *Blood* **100**, 2414-20.
- Taniguchi, T., Tischkowitz, M., Ameziane, N., Hodgson, S. V., Mathew, C. G., Joenje, H., Mok, S. C., and D'Andrea, A. D. (2003). Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. *Nat Med* **9**, 568-74.

- Tapanainen, J. S., Aittomaki, K., Min, J., Vaskivuo, T., and Huhtaniemi, I. T. (1997). Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility. *Nat Genet* **15**, 205-6.
- Thomas, P. Q., Dattani, M. T., Brickman, J. M., McNay, D., Warne, G., Zacharin, M., Cameron, F., Hurst, J., Woods, K., Dunger, D., Stanhope, R., Forrest, S., Robinson, I. C., and Beddington, R. S. (2001). Heterozygous HESX1 mutations associated with isolated congenital pituitary hypoplasia and septo-optic dysplasia. *Hum Mol Genet* **10**, 39-45.
- Thomas, P. Q., Johnson, B. V., Rathjen, J., and Rathjen, P. D. (1995). Sequence, genomic organization, and expression of the novel homeobox gene *Hesx1*. *J Biol Chem* **270**, 3869-75.
- Thompson, L. H., Hinz, J. M., Yamada, N. A., and Jones, N. J. (2005). How Fanconi anemia proteins promote the four Rs: Replication, recombination, repair, and recovery. *Environ Mol Mutagen*.
- Tieva, A., Stattin, P., Wikstrom, P., Bergh, A., and Damber, J. E. (2001). Gonadotropin-releasing hormone receptor expression in the human prostate. *Prostate* **47**, 276-84.
- Tilly, J. L., LaPolt, P. S., and Hsueh, A. J. (1992). Hormonal regulation of follicle-stimulating hormone receptor messenger ribonucleic acid levels in cultured rat granulosa cells. *Endocrinology* **130**, 1296-302.
- Timmers, C., Taniguchi, T., Hejna, J., Reifsteck, C., Lucas, L., Bruun, D., Thayer, M., Cox, B., Olson, S., D'Andrea, A. D., Moses, R., and Grompe, M. (2001). Positional cloning of a novel Fanconi anemia gene, FANCD2. *Mol Cell* **7**, 241-8.
- Tipping, A. J., Pearson, T., Morgan, N. V., Gibson, R. A., Kuyt, L. P., Havenga, C., Gluckman, E., Joenje, H., de Ravel, T., Jansen, S., and Mathew, C. G. (2001). Molecular and genealogical evidence for a founder effect in Fanconi anemia families of the Afrikaner population of South Africa. *Proc Natl Acad Sci U S A* **98**, 5734-9.
- Tischkowitz, M. D., and Hodgson, S. V. (2003). Fanconi anaemia. *J Med Genet* **40**, 1-10.
- Toledo, S. P., Brunner, H. G., Kraaij, R., Post, M., Dahia, P. L., Hayashida, C. Y., and Kremer, H. T. A. P. (1996). An inactivating mutation of the luteinizing hormone receptor causes amenorrhea in a 46,XX female. *J Clin Endocrinol Metab* **81**, 3850-4.
- Tomizawa, J., and Som, T. (1984). Control of ColE1 plasmid replication: enhancement of binding of RNA I to the primer transcript by the Rom protein. *Cell* **38**, 871-8.
- Topilko, P., Schneider-Maunoury, S., Levi, G., Trembleau, A., Gourdji, D., Driancourt, M. A., Rao, C. V., and Charnay, P. (1998). Multiple pituitary and ovarian defects in Krox-24 (NGFI-A, Egr-1)-targeted mice. *Mol Endocrinol* **12**, 107-22.
- Treier, M., Gleiberman, A. S., O'Connell, S. M., Szeto, D. P., McMahon, J. A., McMahon, A. P., and Rosenfeld, M. G. (1998). Multistep signaling requirements for pituitary organogenesis in vivo. *Genes Dev* **12**, 1691-704.

- Tremblay, J. J., and Drouin, J. (1999). Egr-1 is a downstream effector of GnRH and synergizes by direct interaction with Ptx1 and SF-1 to enhance luteinizing hormone beta gene transcription. *Mol Cell Biol* **19**, 2567-76.
- Tremblay, J. J., Goodyer, C. G., and Drouin, J. (2000). Transcriptional properties of Ptx1 and Ptx2 isoforms. *Neuroendocrinology* **71**, 277-86.
- Tremblay, J. J., Lanctot, C., and Drouin, J. (1998). The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit1 and is an upstream regulator of the Lim-homeodomain gene Lim3/Lhx3. *Mol Endocrinol* **12**, 428-41.
- Tsutsumi, M., Zhou, W., Millar, R. P., Mellon, P. L., Roberts, J. L., Flanagan, C. A., Dong, K., Gillo, B., and Sealfon, S. C. (1992). Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. *Mol Endocrinol* **6**, 1163-9.
- Turgeon, J. L., Kimura, Y., Waring, D. W., and Mellon, P. L. (1996). Steroid and pulsatile gonadotropin-releasing hormone (GnRH) regulation of luteinizing hormone and GnRH receptor in a novel gonadotrope cell line. *Mol Endocrinol* **10**, 439-50.
- Ulloa-Aguirre, A., Janovick, J. A., Leanos-Miranda, A., and Conn, P. M. (2004). Misrouted cell surface GnRH receptors as a disease aetiology for congenital isolated hypogonadotrophic hypogonadism. *Hum Reprod Update* **10**, 177-92.
- Valdes-Socin, H., Salvi, R., Pralong, F., Galliard, R. and Beckers, A. (2003). 12th International Congress of Endocrinology, Lisbon, Portugal.
- Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., and Spiess, J. (1986). Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* **321**, 776-9.
- van de Vrugt, H. J., Cheng, N. C., de Vries, Y., Rooimans, M. A., de Groot, J., Schepers, R. J., Zhi, Y., Hoatlin, M. E., Joenje, H., and Arwert, F. (2000). Cloning and characterization of murine fanconi anemia group A gene: Fanca protein is expressed in lymphoid tissues, testis, and ovary. *Mamm Genome* **11**, 326-31.
- van de Vrugt, H. J., Koomen, M., Berns, M. A., de Vries, Y., Rooimans, M. A., van der Weel, L., Blom, E., de Groot, J., Schepers, R. J., Stone, S., Hoatlin, M. E., Cheng, N. C., Joenje, H., and Arwert, F. (2002). Characterization, expression and complex formation of the murine Fanconi anaemia gene product Fancg. *Genes Cells* **7**, 333-42.
- van Groeninghen, J. C., Kiesel, L., Winkler, D., and Zwirner, M. (1998). Effects of luteinising-hormone-releasing hormone on nervous-system tumours. *Lancet* **352**, 372-3.
- Vasseur, C., Rodien, P., Beau, I., Desroches, A., Gerard, C., de Poncheville, L., Chaplot, S., Savagner, F., Croue, A., Mathieu, E., Lahlou, N., Descamps, P., and Misrahi, M. (2003). A chorionic gonadotropin-sensitive mutation in the follicle-stimulating hormone receptor as a cause of familial gestational spontaneous ovarian hyperstimulation syndrome. *N Engl J Med* **349**, 753-9.
- Verlander, P. C., Kaporis, A., Liu, Q., Zhang, Q., Seligsohn, U., and Auerbach, A. D. (1995). Carrier frequency of the IVS4 + 4 A-->T mutation of the Fanconi anemia gene FAC in the Ashkenazi Jewish population. *Blood* **86**, 4034-8.

- Vrecl, M., Anderson, L., Hanyaloglu, A., McGregor, A. M., Groarke, A. D., Milligan, G., Taylor, P. L., and Eidne, K. A. (1998). Agonist-induced endocytosis and recycling of the gonadotropin-releasing hormone receptor: effect of beta-arrestin on internalization kinetics. *Mol Endocrinol* **12**, 1818-29.
- Waisfisz, Q., de Winter, J. P., Kruyt, F. A., de Groot, J., van der Weel, L., Dijkmans, L. M., Zhi, Y., Arwert, F., Scheper, R. J., Youssoufian, H., Hoatlin, M. E., and Joenje, H. (1999a). A physical complex of the Fanconi anemia proteins FANCG/XRCC9 and FANCA. *Proc Natl Acad Sci U S A* **96**, 10320-5.
- Waisfisz, Q., Saar, K., Morgan, N. V., Altay, C., Leegwater, P. A., de Winter, J. P., Komatsu, K., Evans, G. R., Wegner, R. D., Reis, A., Joenje, H., Arwert, F., Mathew, C. G., Pronk, J. C., and Digweed, M. (1999b). The Fanconi anemia group E gene, FANCE, maps to chromosome 6p. *Am J Hum Genet* **64**, 1400-5.
- Wang, A. M., Doyle, M. V., and Mark, D. F. (1989). Quantitation of mRNA by the polymerase chain reaction. *Proc Natl Acad Sci U S A* **86**, 9717-21.
- Wang, X., Andreassen, P. R., and D'Andrea, A. D. (2004). Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. *Mol Cell Biol* **24**, 5850-62.
- Weck, J., Fallest, P. C., Pitt, L. K., and Shupnik, M. A. (1998). Differential gonadotropin-releasing hormone stimulation of rat luteinizing hormone subunit gene transcription by calcium influx and mitogen-activated protein kinase-signaling pathways. *Mol Endocrinol* **12**, 451-7.
- Weiss, J., Adams, E., Whitcomb, R. W., Crowley, W. F., Jr., and Jameson, J. L. (1991). Normal sequence of the gonadotropin-releasing hormone gene in patients with idiopathic hypogonadotropic hypogonadism. *Biol Reprod* **45**, 743-7.
- Weiss, J., Axelrod, L., Whitcomb, R. W., Harris, P. E., Crowley, W. F., and Jameson, J. L. (1992). Hypogonadism caused by a single amino acid substitution in the beta subunit of luteinizing hormone. *N Engl J Med* **326**, 179-83.
- Weiss, J., Crowley, W. F., Jr., Halvorson, L. M., and Jameson, J. L. (1993). Perfusion of rat pituitary cells with gonadotropin-releasing hormone, activin, and inhibin reveals distinct effects on gonadotropin gene expression and secretion. *Endocrinology* **132**, 2307-11.
- Weiss, J., Jameson, J. L., Burrin, J. M., and Crowley, W. F., Jr. (1990). Divergent responses of gonadotropin subunit messenger RNAs to continuous versus pulsatile gonadotropin-releasing hormone in vitro. *Mol Endocrinol* **4**, 557-64.
- West, B. E., Parker, G. E., Savage, J. J., Kiratipranon, P., Toomey, K. S., Beach, L. R., Colvin, S. C., Sloop, K. W., and Rhodes, S. J. (2004). Regulation of the Follicle-Stimulating Hormone Beta Gene by the LHX3 LIM-Homeodomain Transcription Factor. *Endocrinology*.
- Wevrick, R., Barker, J. E., Nadeau, J. H., Szpirer, C., and Buchwald, M. (1993a). Mapping of the murine and rat Facc genes and assessment of flexed-tail as a candidate mouse homolog of Fanconi anemia group C. *Mamm Genome* **4**, 440-4.

- Wevrick, R., Clarke, C. A., and Buchwald, M. (1993b). Cloning and analysis of the murine Fanconi anemia group C cDNA. *Hum Mol Genet* **2**, 655-62.
- Whitney, M., Thayer, M., Reifsteck, C., Olson, S., Smith, L., Jakobs, P. M., Leach, R., Naylor, S., Joenje, H., and Grompe, M. (1995). Microcell mediated chromosome transfer maps the Fanconi anaemia group D gene to chromosome 3p. *Nat Genet* **11**, 341-3.
- Whitney, M. A., Royle, G., Low, M. J., Kelly, M. A., Axthelm, M. K., Reifsteck, C., Olson, S., Braun, R. E., Heinrich, M. C., Rathbun, R. K., Bagby, G. C., and Grompe, M. (1996). Germ cell defects and hematopoietic hypersensitivity to gamma-interferon in mice with a targeted disruption of the Fanconi anemia C gene. *Blood* **88**, 49-58.
- Wijker, M., Morgan, N. V., Herterich, S., van Berkel, C. G., Tipping, A. J., Gross, H. J., Gille, J. J., Pals, G., Savino, M., Altay, C., Mohan, S., Dokal, I., Cavenagh, J., Marsh, J., van Weel, M., Ortega, J. J., Schuler, D., Samochatova, E., Karwacki, M., Bekassy, A. N., Abecasis, M., Ebell, W., Kwee, M. L., de Ravel, T., Mathew, C. G., and et al. (1999). Heterogeneous spectrum of mutations in the Fanconi anaemia group A gene. *Eur J Hum Genet* **7**, 52-9.
- Willars, G. B., Heding, A., Vrecl, M., Sellar, R., Blumenrohr, M., Nahorski, S. R., and Eidne, K. A. (1999). Lack of a C-terminal tail in the mammalian gonadotropin-releasing hormone receptor confers resistance to agonist-dependent phosphorylation and rapid desensitization. *J Biol Chem* **274**, 30146-53.
- Windle, J. J., Weiner, R. L., and Mellon, P. L. (1990). Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. *Mol Endocrinol* **4**, 597-603.
- Wojcikiewicz, R. J., Xu, Q., Webster, J. M., Alzayady, K., and Gao, C. (2003). Ubiquitination and proteasomal degradation of endogenous and exogenous inositol 1,4,5-trisphosphate receptors in alpha T3-1 anterior pituitary cells. *J Biol Chem* **278**, 940-7.
- Wolfe, M. W., and Call, G. B. (1999). Early growth response protein 1 binds to the luteinizing hormone-beta promoter and mediates gonadotropin-releasing hormone-stimulated gene expression. *Mol Endocrinol* **13**, 752-63.
- Wong, J. C., Alon, N., McKerlie, C., Huang, J. R., Meyn, M. S., and Buchwald, M. (2003). Targeted disruption of exons 1 to 6 of the Fanconi Anemia group A gene leads to growth retardation, strain-specific microphthalmia, meiotic defects and primordial germ cell hypoplasia. *Hum Mol Genet* **12**, 2063-76.
- Wong, J. C., Alon, N., Norga, K., Kruyt, F. A., Youssoufian, H., and Buchwald, M. (2000). Cloning and analysis of the mouse Fanconi anemia group A cDNA and an overlapping penta zinc finger cDNA. *Genomics* **67**, 273-83.
- Worby, C. A., and Dixon, J. E. (2002). Sorting out the cellular functions of sorting nexins. *Nat Rev Mol Cell Biol* **3**, 919-31.
- Wu, W., Cogan, J. D., Pfaffle, R. W., Dasen, J. S., Frisch, H., O'Connell, S. M., Flynn, S. E., Brown, M. R., Mullis, P. E., Parks, J. S., Phillips, J. A., 3rd, and Rosenfeld, M. G. (1998). Mutations in PROP1 cause familial combined pituitary hormone deficiency. *Nat Genet* **18**, 147-9.

- Wurmbach, E., Yuen, T., Ebersole, B. J., and Sealfon, S. C. (2001). Gonadotropin-releasing hormone receptor-coupled gene network organization. *J Biol Chem* **276**, 47195-201.
- Yamashita, T., Barber, D. L., Zhu, Y., Wu, N., and D'Andrea, A. D. (1994). The Fanconi anemia polypeptide FACC is localized to the cytoplasm. *Proc Natl Acad Sci U S A* **91**, 6712-6.
- Yamashita, T., Kupfer, G. M., Naf, D., Suliman, A., Joenje, H., Asano, S., and D'Andrea, A. D. (1998). The fanconi anemia pathway requires FAA phosphorylation and FAA/FAC nuclear accumulation. *Proc Natl Acad Sci U S A* **95**, 13085-90.
- Yamashita, T., and Nakahata, T. (2001). Current knowledge on the pathophysiology of Fanconi anemia: from genes to phenotypes. *Int J Hematol* **74**, 33-41.
- Yamashita, T., Wu, N., Kupfer, G., Corless, C., Joenje, H., Grompe, M., and D'Andrea, A. D. (1996). Clinical variability of Fanconi anemia (type C) results from expression of an amino terminal truncated Fanconi anemia complementation group C polypeptide with partial activity. *Blood* **87**, 4424-32.
- Yang, Y., Kuang, Y., De Oca, R. M., Hays, T., Moreau, L., Lu, N., Seed, B., and D'Andrea, A. D. (2001). Targeted disruption of the murine Fanconi anemia gene, *Fancg/Xrcc9*. *Blood* **98**, 3435-40.
- Yano, T., Pinski, J., Radulovic, S., and Schally, A. V. (1994). Inhibition of human epithelial ovarian cancer cell growth in vitro by agonistic and antagonistic analogues of luteinizing hormone-releasing hormone. *Proc Natl Acad Sci U S A* **91**, 1701-5.
- Yelin, R., Dahary, D., Sorek, R., Levanon, E. Y., Goldstein, O., Shoshan, A., Diber, A., Biton, S., Tamir, Y., Khosravi, R., Nemzer, S., Pinner, E., Walach, S., Bernstein, J., Savitsky, K., and Rotman, G. (2003). Widespread occurrence of antisense transcription in the human genome. *Nat Biotechnol* **21**, 379-86.
- Yokoi, T., Ohmichi, M., Tasaka, K., Kimura, A., Kanda, Y., Hayakawa, J., Tahara, M., Hisamoto, K., Kurachi, H., and Murata, Y. (2000). Activation of the luteinizing hormone beta promoter by gonadotropin-releasing hormone requires c-Jun NH2-terminal protein kinase. *J Biol Chem* **275**, 21639-47.
- Young, N. S., and Alter, B. P. (1994). Clinical features of Fanconi's Anemia. In "Aplastic Anemia Acquired and Inherited", pp. 275-309. W.B.Saunders, Philadelphia.
- Yuen, T., Wurmbach, E., Ebersole, B. J., Ruf, F., Pfeffer, R. L., and Sealfon, S. C. (2002). Coupling of GnRH concentration and the GnRH receptor-activated gene program. *Mol Endocrinol* **16**, 1145-53.
- Zakaria, M. M., Jeong, K. H., Lacza, C., and Kaiser, U. B. (2002). Pituitary homeobox 1 activates the rat FSHbeta (rFSHbeta) gene through both direct and indirect interactions with the rFSHbeta gene promoter. *Mol Endocrinol* **16**, 1840-52.
- Zhadanov, A. B., Bertuzzi, S., Taira, M., Dawid, I. B., and Westphal, H. (1995). Expression pattern of the murine LIM class homeobox gene *Lhx3* in subsets of neural and neuroendocrine tissues. *Dev Dyn* **202**, 354-64.

- Zhang, F., White, R. L., and Neufeld, K. L. (2000). Phosphorylation near nuclear localization signal regulates nuclear import of adenomatous polyposis coli protein. *Proc Natl Acad Sci U S A* **97**, 12577-82.
- Zhang, F. P., Poutanen, M., Wilbertz, J., and Huhtaniemi, I. (2001). Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. *Mol Endocrinol* **15**, 172-83.
- Zhao, L., Bakke, M., and Parker, K. L. (2001). Pituitary-specific knockout of steroidogenic factor 1. *Mol Cell Endocrinol* **185**, 27-32.

Appendix I

Vector Maps

This appendix contains vector maps detailing some of the vectors used during the course of this thesis.

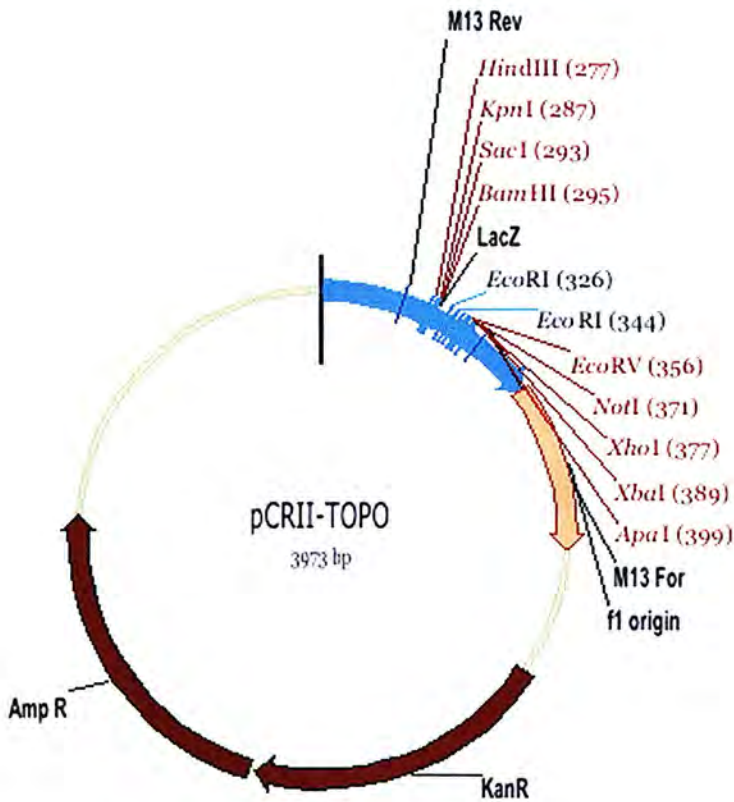


Figure A1.1 Vector map of pCRII-TOPO plasmid.

Features of pCRII-TOPO cloning vector used for cloning of PCR products. Restriction sites are labelled. Kan^R, Kanamycin resistance; Amp^R, Ampicillin resistance; LacZ, β -galactosidase; M13For, M13 Forward primer binding site; M13Rev, M13 Reverse primer binding site.

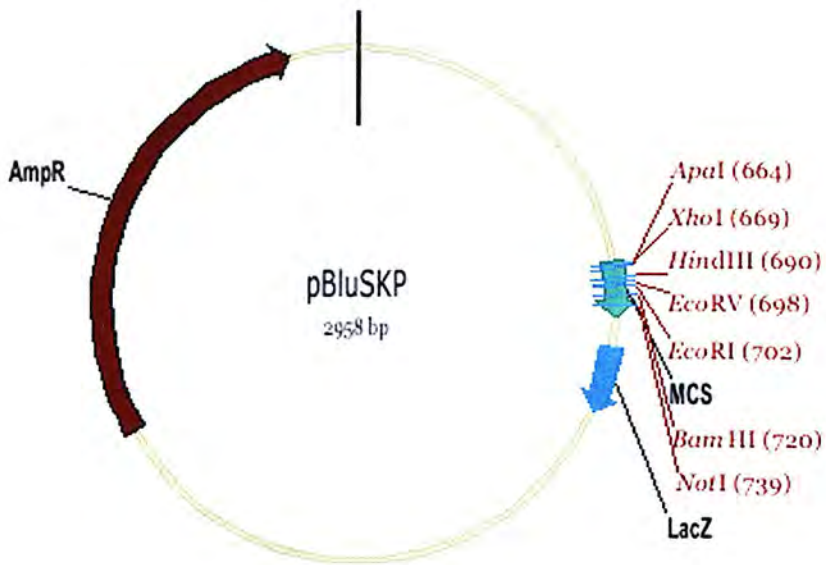


Figure A1.2 Vector map of pBluescript plasmid.

Features of pBluescript-SK⁺ cloning vector. Restriction sites are labelled. AmpR, Ampicillin resistance; LacZ, β -galactosidase; MCS, multiple cloning site.

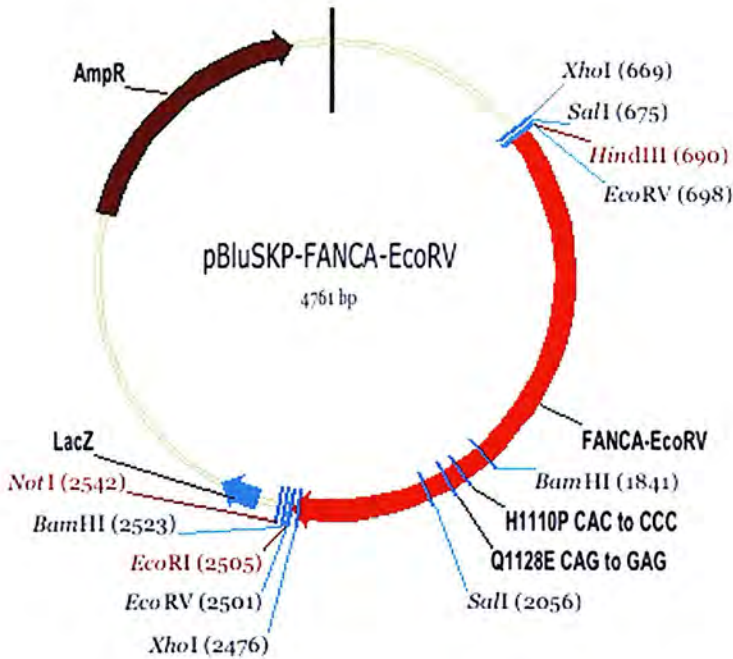


Figure A1.3 Vector map of pBluescript-FANCA-EcoRV.

A 1803 bp fragment was cloned out of pEGFP-FANCA by *EcoRV* digestion. This was ligated into pBluescript plasmid that had also been digested with *EcoRV* restriction endonuclease. This plasmid was then used as a template in site-directed mutagenesis PCR reactions in order to introduce the point mutations H1110P and Q1128E (See Section 2.3.11).

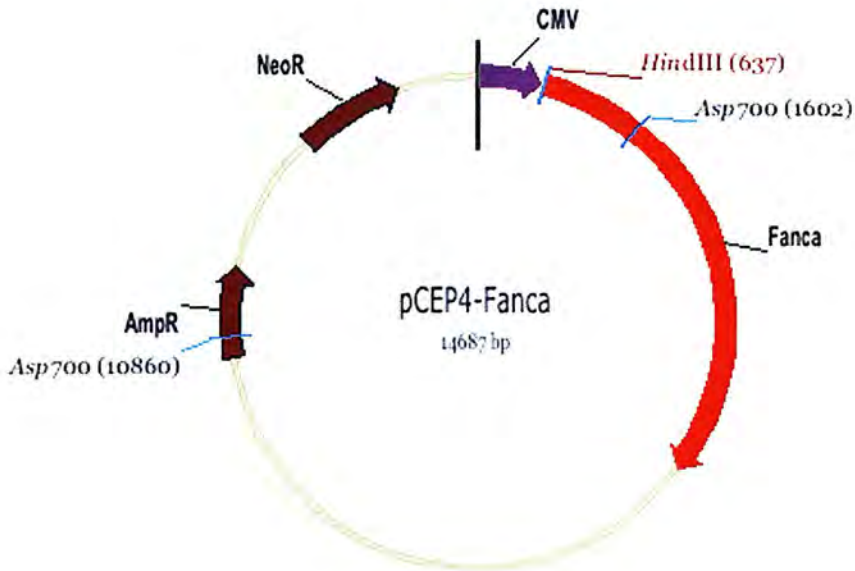


Figure A1.4 Vector map of pCEP4-Fanca.

To obtain a 965 bp probe for hybridization to a northern blot, pCEP4-Fanca was digested with *HindIII* and *Asp700* restriction endonucleases. AmpR, Ampicillin resistance; NeoR, Neomycin resistance; CMV, cytomegalovirus promoter.

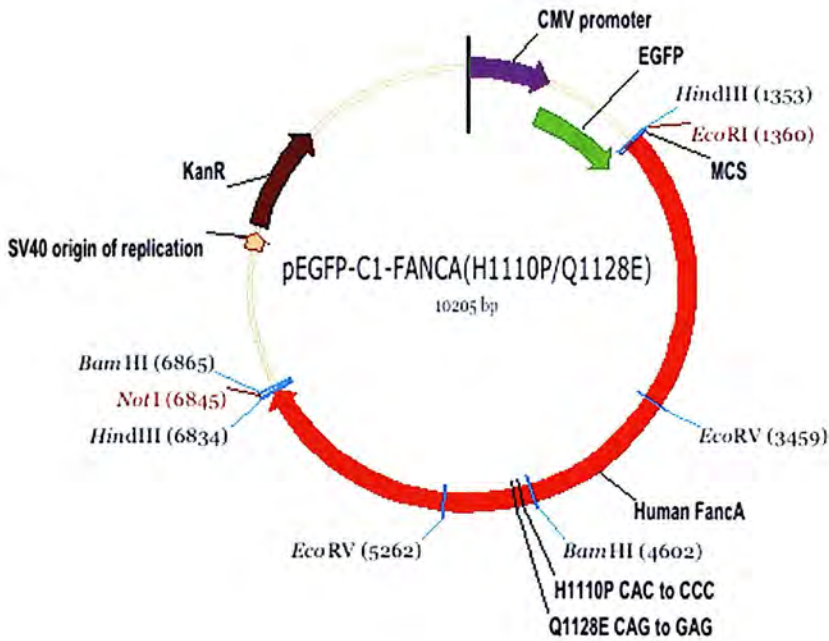


Figure A1.5 Vector map of pEGFP-C1-FANCA(H1110P/Q1128E).

Position of the point mutations (H1110P or Q1128E) introduced by site directed mutagenesis are indicated. Restriction sites are labelled. KanR, Kanamycin resistance; MCS, multiple cloning site; EGFP, enhancer green fluorescent protein; CMV, cytomegalovirus promoter.

Appendix II

Academic sources and suppliers

This section contains a list of the commercial suppliers used during the course of this thesis as well as the addresses of the academic sources used.

Academic Sources

Dr. F.Arwert

Department of Clinical Genetics and Human Genetics
VU University Medical Center
Van der Boechorststraat 7
1081 BT Amsterdam
The Netherlands

Professor M.Buchwald

Program in Genetics and Genomic Biology
Research Institute
Hospital for Sick Children
Toronto, Ontario M5G 1X8
Canada

Dr. P.Brown

MRC Human Reproductive Sciences Unit
Chancellors Building
49 Little France Crescent
Edinburgh
EH16 4SB

Professor A.McNeilly

MRC Human Reproductive Sciences Unit
Chancellors Building
49 Little France Crescent
Edinburgh
EH16 4SB

Professor P.Mellon

Reproductive Medicine
University of California, San Diego
9500 Gilman Drive
La Jolla, CA 92093-0674, USA

Professor W.Miller

Department of Molecular and Structural Biochemistry
 Box 7622
 North Carolina State University
 Raleigh, NC 27695-7622, USA

Commercial suppliers

AbCam	Cambridge	UK
ABGene	Surrey	UK
Ambion	Huntington	UK
Amersham Biosciences	Little Chalfont, Buckinghamshire	UK
AMS Biotech	Abingdon	UK
Anachem	Luton	UK
Applied Biosystems	Warrington	UK
Autogen Bioclear	Calne, Wiltshire	UK
BDH	Poole	UK
BDPlastipak (Fisher Scientific)	Loughborough	UK
Becton Dickinson Laboratories	New Jersey	USA
Biorad	Hertfordshire	UK
BMG Labtechnologies	Aylesbury	UK
Calbiochem	Nottingham	UK
CamLab	Cambridge	UK
Cambridge Biosciences	Cambridge	UK
Clontech	Oxford	UK
Diagnostics Scotland	Edinburgh	UK
ECACC	Salisbury, Wiltshire	UK
Fisher Scientific	Loughborough	UK
GiboBRL Life Technologies	Paisley	UK
Hybaid	Basingstoke	UK
ICN	Eshwage	Germany
Immunotech	High Wycombe	UK
Informax	Bethesda	USA
Invitrogen	Paisley	UK
Kendal (Fisher Scientific)	Loughborough	UK
Millipore	Watford	UK
MWG-Biotech	Milton Keynes	UK
Nalgene	Hereford	UK
Nunc	Hereford	UK
New England Biolabs	Hitchin, Hertfordshire	UK

Academic sources and suppliers

Novagen	Nottingham	UK
Oxford Molecular (Accelrys)	Cambridge	UK
Peninsula	Belmont, California	USA
Pharmacia Biotech (Amersham Biosciences)	Little Chalfont, Buckinghamshire	UK
Promega	Southampton	UK
Q-Biogene	Cambridge	UK
Qiagen	Crawley	UK
Roche	Lewes	UK
Sartorius	Epsom	UK
Sigma	Gillingham	UK
SLS	Nottingham	UK
Stratagene	La Jolla, California	USA
Whatmann	Brentford	UK
Vector Laboratories	Burlingame, California	USA
Zeiss	Welwyn Garden City	UK

Appendix III
Publications

Gonadotropin-Releasing Hormone Regulates Expression of the DNA Damage Repair Gene, *Fanconi anemia A*, in Pituitary Gonadotroph Cells¹

Rachel Larder,⁴ Lynda Chang,^{3,4} Michael Clinton,⁵ and Pamela Brown^{2,4}

Human Reproductive Sciences Unit,⁴ Centre for Reproductive Biology, The University of Edinburgh Chancellors Building, Edinburgh EH16 4SB, United Kingdom
Roslin Institute,⁵ Midlothian EH25 9PS, United Kingdom

ABSTRACT

Gonadal function is critically dependant on regulated secretion of the gonadotropin hormones from anterior pituitary gonadotroph cells. Gonadotropin biosynthesis and release is triggered by the binding of hypothalamic GnRH to GnRH receptor expressed on the gonadotroph cell surface. The repertoire of regulatory molecules involved in this process are still being defined. We used the mouse LβT2 gonadotroph cell line, which expresses both gonadotropin hormones, as a model to investigate GnRH regulation of gene expression and differential display reverse transcription-polymerase chain reaction (RT-PCR) to identify and isolate hormonally induced changes. This approach identified *Fanconi anemia a* (*Fanca*), a gene implicated in DNA damage repair, as a differentially expressed transcript. Mutations in *Fanca* account for the majority of cases of Fanconi anemia (FA), a recessively inherited disease identified by congenital defects, bone marrow failure, infertility, and cancer susceptibility. We confirmed expression and hormonal regulation of *Fanca* mRNA by quantitative RT-PCR, which showed that GnRH induced a rapid, transient increase in *Fanca* mRNA. *Fanca* protein was also acutely upregulated after GnRH treatment of LβT2 cells. In addition, *Fanca* gene expression was confined to mature pituitary gonadotrophs and adult mouse pituitary and was not expressed in the immature αT3-1 gonadotroph cell line. Thus, this study extends the expression profile of *Fanca* into a highly specialized endocrine cell and demonstrates hormonal regulation of expression of the *Fanca* locus. We suggest that this regulatory mechanism may have a crucial role in the GnRH-response mechanism of mature gonadotrophs and perhaps the etiology of FA.

anterior pituitary, gene regulation, gonadotropin-releasing hormone, mechanisms of hormone action, pituitary

INTRODUCTION

Reproduction requires regulated pulsatile release of the gonadotropin hormones, LH and FSH, from the gonadotroph cells of the anterior pituitary to stimulate gonadal function. The gonadotropin hormones are heterodimeric proteins, comprised of a common α subunit (αGSU) and a

hormone-specific β subunit. Their biosynthesis and release is triggered by the binding of decapeptide GnRH to its cognate receptor (GnRHr), this stimulates second messenger signaling pathways, which, coupled with GnRHr number and turnover, combine to differentially regulate hormone biosynthesis [1–3]. Indeed, the pattern of GnRH administration is crucial for upregulating the mRNA levels of the constituent αGSU and β subunits that comprise LH and FSH, both in vivo [4, 5] and in vitro [6, 7].

Gonadotroph cell lines recapitulate the embryonic anterior pituitary temporal expression profile of gonadotropin genes [8–10]. Furthermore, because gonadotroph cells only comprise 10–15% of the cells in the anterior pituitary, cell lines afford greater manipulation of treatment conditions and provide a source of enriched cell-specific material. In this study, we used LβT2 cells, an immortalized gonadotroph cell line that is GnRH responsive, expresses GnRHr and all three gonadotropin subunits [9, 11, 12]. In addition, the second messenger signaling pathways required to transduce the GnRH signal are starting to be elucidated in these cells [13–16]. Thus, because LβT2 cells express all the features associated with mature pituitary gonadotrophs, they are a suitable cell model for investigating regulation of gene expression by GnRH.

To identify and isolate new and novel mRNAs that are differentially regulated by GnRH in gonadotrophs, which may be important for gonadotropin hormone biosynthesis, differential display (DD) reverse transcription-polymerase chain reaction (RT-PCR) [17] was performed on RNA extracted from untreated and GnRH-treated LβT2 cells. This approach identified that, among others, expression of *Fanconi anemia a* (*Fanca*) mRNA, was altered in response to GnRH treatment. *Fanca* is a member of a protein complex required for genome homeostasis [18, 19] and mutations in *Fanca* account for >60% of cases of Fanconi anemia (FA), an autosomal recessive inherited disorder. However, the pleiotropic phenotype of FA patients indicates that other cellular functions may also depend on the FA complex [20].

Therefore, we decided to investigate the expression profile of *Fanca* mRNA in detail and report that GnRH acutely upregulates the expression of both *Fanca* mRNA and protein. This suggests that *Fanca* may have a regulatory role in gonadotroph cells, and this is the first report of distinct hormonal regulation of this gene.

MATERIALS AND METHODS

Cell Culture

LβT2 and αT3-1 cells (obtained from P. Mellon, San Diego, CA) were cultured in DMEM (Sigma, Dorset, UK) supplemented with 10% FCS (Sigma) and 1% penicillin/streptomycin (Sigma). All gonadotroph cell culture plasticware was coated with a 1:30 dilution of Matrigel (Becton Dick-

¹R.L. and L.C. were supported by M.R.C. Ph.D. research studentships during the course of this work, and these two authors made equal contributions to this work.

²Correspondence. FAX: 44 0 131 242 6231;

e-mail: p.brown@hrsu.mrc.ac.uk

³Current Address: Department of Oncology, Beatson Institute, Glasgow, UK.

Received: 2 April 2004.

First decision: 20 April 2004.

Accepted: 27 April 2004.

© 2004 by the Society for the Study of Reproduction, Inc.
ISSN: 0006-3363. <http://www.biolreprod.org>

TABLE 1. Differential display (DD) RT-PCR random 10-mer primers.

DD-RT-PCR random 10-mer primer	DNA sequence
TK	5'-CTTGATTGCC-3'
A1	5'-ACAGAGCACA-3'
A2	5'-ACGTATCCAG-3'
MAX1	5'-GAGCATATCC-3'
MAX2	5'-CACAGCTTGC-3'
MAX3	5'-CCACAGAGTA-3'
R21	5'-AGTCAGCCAC-3'
R5	5'-AGGACCCCTGG-3'

inon Labware, Oxford, UK) in PBS (Sigma). Cells were treated with 1 μ M of native GnRH (Peninsula, St. Helens, UK) for 15 min. This concentration is known to stimulate high levels of LH and FSH β subunit gene expression [21, 22], with an interpulse interval of 75 min, for either 3 or 6 pulses; fresh media was added and the cells harvested or cells were treated with one 15-min pulse and harvested 1, 2, or 4 h post-GnRH treatment into RNAzol B (AMS Biotechnology, Abingdon, UK) and stored at -70°C . Mouse L-cells and HeLa cells (ECACC, CAMR, Porton Down, UK) were passaged in growth media as described above, and all cells were grown in a humidified 5% CO_2 atmosphere at 37°C .

Differential Display RT-PCR

RNA was extracted as per the manufacturer's protocol and differential display was performed as described [17], with further modifications [23]. Briefly, cDNA was generated using a First Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Little Chalfont, UK) and degenerate oligo(dT) primers (primers have 12 thymidine bases and a combination of two random bases TTTTTTTTTTTVN where V and N = A, G, or C). The oligo(dT) primers were pooled into 3 \times 24 μM mixes; T₁₂VA, T₁₂VC, and T₁₂VG were used to prime first-strand cDNA subpopulations. DD-RT-PCR was performed using oligo(dT) and random 10-mer primers (Sigma-Genosys, Pampisford, UK), which are listed in Table 1. The 20- μl reaction mix contained 5 μl of cDNA (pipetted from a 1:131- μl dilution of the first-strand reaction), 0.5 μM random primer, 3 mM MgCl_2 , 2.4 μM T₁₂VC, 2 μM dNTP's (Amersham Pharmacia Biotech), 1 μl ³⁵S-dATP (1000 Ci/mmol, Amersham Pharmacia Biotech), 0.3 μl AGS Gold *Taq* polymerase, 2 μl each reaction buffer and enhancer (Hybaid, Ashford, UK), and 1.2 μl H₂O. The PCR reaction conditions were as described [23]. A 4- μl aliquot was loaded on a 6% acrylamide gel (HR-1000; Beckman Coulter UK Ltd, High Wycombe, UK) and electrophoresed on a GenomylLR DNA analyzer (Beckman Coulter) at 2700 V for 2 h 15 min at 50°C . The gel was transferred to 3MM paper (Whatman, Fisher Scientific, Loughborough, UK) dried, and bands were visualized using BiomaxMR autoradiographic film (Amersham Pharmacia Biotech), excised and rehydrated in 150 μl low TE (10 mM Tris HCl, 0.1 mM EDTA, pH 7.4). The DNA was eluted at 100°C , ethanol precipitated, and resuspended in 10 μl of low TE. To facilitate subcloning, this was combined with a 40- μl reaction mix (3 mM MgCl_2 , 0.3 μl AGS Gold, 4 μl buffer, 4 μl Enhancer, 0.8 mM dNTP, 1.25 μM T₁₂VN, 1.25 μM random primer,

and 11 μl H₂O) and amplified by PCR before splitting into four 10- μl aliquots and reamplified. The *Fanca* fragment was cloned into pT7-Blue (Novagen, CN Biosciences Ltd., Beeston, UK) using the Perfectly Blunt cloning kit (Novagen) and sequenced.

Northern blotting, Semiquantitative and Quantitative RT-PCR

Total RNA (40 μg) was fractionated, Northern blotted, and probed, then quantified as described [24]. Radiolabeled probes used corresponded to either the 5' region (exons 1-11) or the 3' region (exons 42-43) of *Fanca* cDNA.

RT-PCR was performed by reverse transcribing 1 μg RNA using First Strand cDNA synthesis kit (Amersham Pharmacia Biotech) and 1 μl was added to a 25- μl reaction containing 2 μM upstream and downstream primers and 10 μl Extensor High Fidelity Master Mix Buffer 2 (AB Gene, Epsom, UK). Standard PCR conditions were used and were identical for *Fanca* and *penta zinc-finger protein 276* (*Zfp276*). For details of primers and PCR fragments, see Table 2. PCR products were verified by sequencing individual clones after ligation into TA-cloning vectors (Invitrogen, UK) using ABI big dye terminator reagents (ABI, Warrington, UK). DNA fragments were visualized on an ethidium bromide-stained agarose gel, which for *Fanca* was Southern blotted and probed with a radiolabeled probe (exons 7-17) before exposing to x-ray film.

Quantitative RT-PCR was performed for *Fanca* mRNA using a LightCycler Instrument (Roche Diagnostics, Lewes, East Sussex, UK), FastStart DNA Master SYBR Green I (Roche Diagnostics), and mouse *beta-2-microglobulin* (*B2m*) as an internal control. Specific primers are shown in Table 2. Each LightCycler reaction consisted of 5.8 μl dH₂O, 1.2 μl MgCl_2 (4 mM), 1 μl Primer Pair Mix (25 μM each), 1 μl LightCycler DNA Master SYBR Green I (Roche Diagnostics), and 1 μl cDNA. The LightCycler program used for *Fanca* and *B2m* real-time PCR was as follows: one cycle of 95°C for 10 min, 60 cycles of 95°C for 5 sec, 57°C for 5 sec, and 72°C for 15 sec, and a melting curve program (57 - 95°C). A standard curve of *Fanca* and *B2m* expression was determined using serially diluted cDNA made from mouse L-cell RNA. Results are expressed as arbitrary units of *Fanca* mRNA expression normalized against *B2m* mRNA expression levels and are a mean of three separate experiments performed in duplicate.

Western Blotting Analysis

LBT2 cells were left untreated or were treated with GnRH and harvested 2, 4, or 6 h later. Whole-cell extracts were prepared by washing the cell monolayer in ice-cold PBS with Complete (Roche Diagnostics) protease inhibitors, before scraping into a 1.5-ml centrifuge tube, which was frozen on dry ice. Total cellular protein was liberated by three rapid freeze-thaw cycles, and cellular debris was cleared by centrifugation at 4°C for 5 min at 20000 \times g. Protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad, Hemel Hempstead, UK), 50 μg of whole-cell extract was boiled in 1 \times loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% Bromophenol Blue, 1% β -mercaptoethanol), fractionated on a 6% SDS-PAGE gel, and electroblotted overnight at 25 V onto Immobilon-P (Millipore, Watford, UK) in 1 \times TGS/20% methanol (TGS: 25 mM Tris, pH 8.8, 250 mM glycine, 0.1% SDS). Blots were probed as described [5] with anti-mouse *Fanca* antisera raised

TABLE 2. Specific primers were used in PCR reactions to amplify first strand *Fanca*, *Zfp276*, and *B-2-microglobulin* cDNA.

Specific primers*	DNA sequence	Location	PCR product (base pairs)
<i>Fanca</i> s	5'-CTGTGTGAGCAGATAGGC-3'	Exon 7 (640 bp)	979
<i>Fanca</i> as	5'-TCACGCCTCGGCAATGTCCC-3'	Exon 17 (1619 bp)	
<i>Fanca</i> s	5'-CAGCATGGTCACTGCGTTC-3'	Exon 14 (1263 bp)	450
<i>Fanca</i> as	5'-CCTGAATATGCTGGCCFCCA-3'	Exon 18 (1713 bp)	
<i>Fanca</i> s	5'-GTGGTGGAGACCTGGAAGA-3'	Exon 30 (2900 bp)	211
<i>Fanca</i> as	5'-CGGCGTAGAACAGCCATG-3'	Exon 32 (3111 bp)	
<i>Fanca</i> s	5'-GCACTTTGCGTGGAGAGG-3'	Exon 37 (3666 bp)	129
<i>Fanca</i> as	5'-CAGGTAGGACGAGAGTAGAC-3'	Exon 38 (3795 bp)	
<i>Zfp276</i> s	5'-CACTGTCTCTGAGTACTGC-3'	5' ATG 68 bp	1238
<i>Zfp276</i> as	5'-CGTCACCTGCTGAGTCAAG-3'	3' ATG 1170 bp	
<i>B2m</i> s	5'-ATGGCTCGCTCGGTGACCCCTGGT-3'	Exon 1 (ATC)	102
<i>B2m</i> as	5'-TGTTCGGCTCCCATTCCTCC-3'	Exon 1 (102 bp)	

* Primer annealing sites are expressed relative to the translational start site (ATG) in *Fanca*, *Zfp276*, and *B-2-microglobulin* (*B2m*). s, Sense; as, antisense; bp, base pairs.

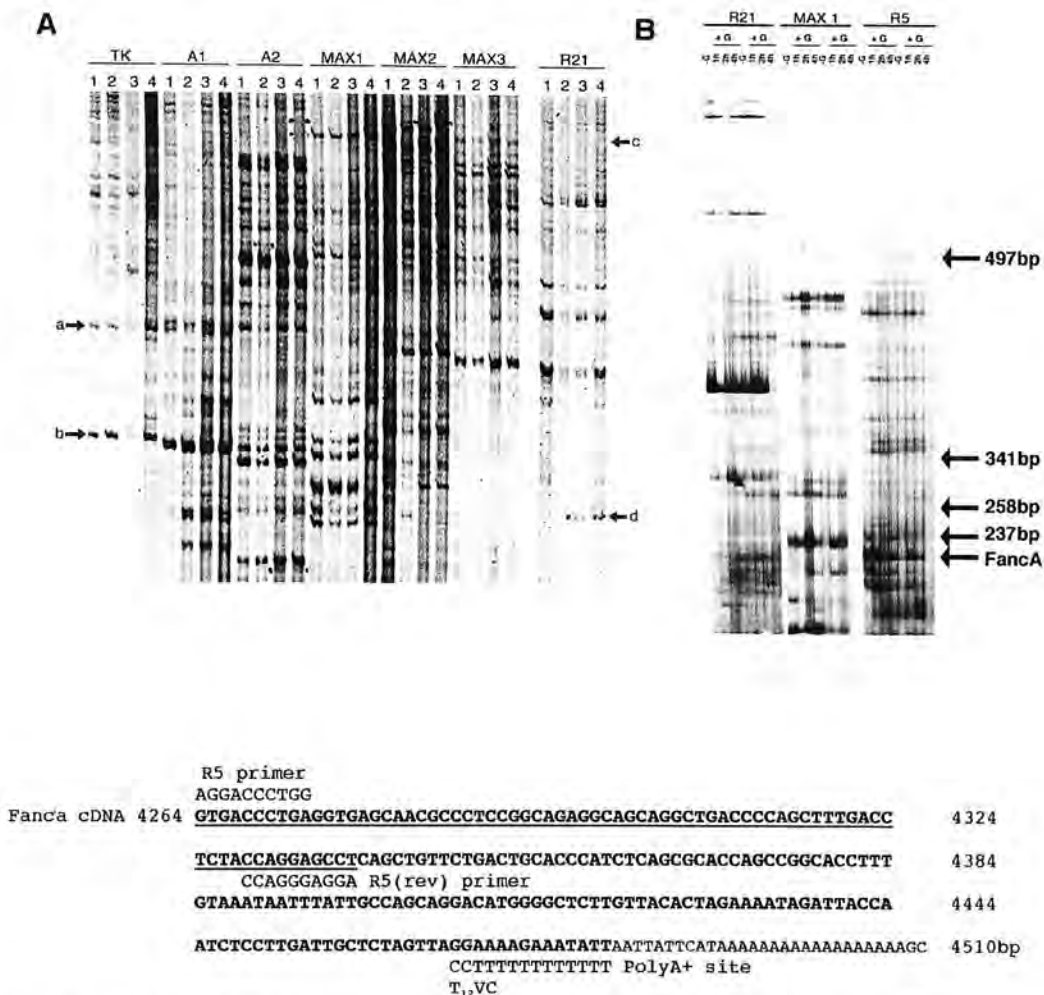


FIG. 1. Differential display RT-PCR analysis of GnRH-regulated transcripts isolated from LBT2 cells. A) Matrigel basement membrane was excluded as an inducer of gene expression in LBT2 gonadotroph cells while GnRH was shown to upregulate transcripts. Cells were grown without Matrigel (1) or were cultured on Matrigel (2, 3, and 4) and treated with 3 (3) or 6 (4) 15-min pulses of GnRH with an interpulse interval of 75 min. RNA was extracted and subjected to differential display (DD) RT-PCR using primers stated. Matrigel did not alter transcript expression (arrows a and b), while numerous transcripts altered after GnRH treatment (arrows c and d). B) In two separate experiments, LBT2 cells were left untreated (-G) or were treated with 1 × 15-min pulse of GnRH (+G), RNA was harvested (-G, 1, 2, and 4 h) and subjected to DD-RT-PCR. First-strand cDNA was generated using the T₁₂VC downstream primer, then amplified by PCR using primers R21, MAX1, or R5. Arrow denotes location of *Fanca*. The location of DNA size markers, indicated as bp, are also shown. C) Bioinformatic line-up depicting the region of homology, shown underlined, between the cloned *Fanca* DD-RT-PCR product and mouse *Fanca* cDNA nucleotide sequence, and the likely internal R5 priming site. The location of DD-RT-PCR primers R5 and T₁₂VC that generated the original 216-bp DD-RT-PCR product are also indicated.

to amino acids 1–271 (kind gift from Dr. Fre Arwert, VU University Medical Center, Amsterdam, The Netherlands) at a concentration of 1:1500, then stripped in 2% SDS, 50 mM Tris, pH 6.8, 0.7% β-mercaptoethanol at 55°C for 30 min before washing and reprobing with anti-mouse β-tubulin antisera at a concentration of 1:500 (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Bioinformatics and Statistics

All bioinformatic comparisons were made using Wisconsin Package Version 10.3 (Accelrys Inc., San Diego, CA) housed on the Medical Research Council's Human Genome Mapping Project server (MRC, HGMP, Hinxton, UK). Statistical differences were determined using one-way analysis of variance (ANOVA), with $P < 0.05$ deemed significant.

RESULTS

Differential Display RT-PCR Analysis of GnRH-Treated LBT2 Cells

LBT2 cells were grown in vitro on Matrigel basement membrane, which contains collagen, other extracellular basement membrane proteins, growth factors, metalloproteinases, and factors that promote cell adherence. These factors may upregulate gene expression independent of GnRH, so to test this, two different treatment regimes were designed. In regime 1, cells were grown on uncoated or Matrigel-coated flasks, while for regime 2, cells were grown on Matrigel and left untreated or treated with GnRH. RNA was isolated and subjected to DD-RT-PCR analysis

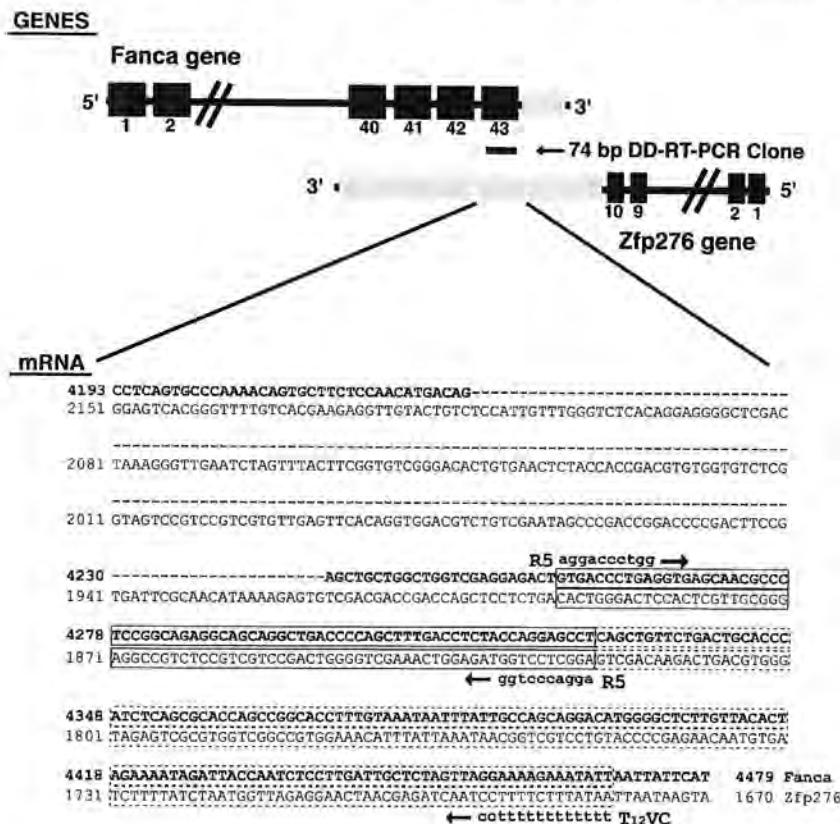


FIG. 2. Schematic representation of the *Fanca* locus showing the location of the differential display RT-PCR clone. Exons 40–43 of *Fanca*, shown as black boxes, overlap with the 3' untranslated region (UTR) of *Zfp276*, shown in grey. The location of the cloned 74-bp differential display (DD) RT-PCR clone is indicated. Beneath the schematic, the regions of nucleotide identity between *Fanca* mRNA and *Zfp276* mRNA have been aligned, *Fanca* mRNA sequence is shown in bold type, and spliced intronic sequence has been omitted and replaced with a dashed line, with *Zfp276* mRNA in normal typeface. The amplified 74-bp DD-RT-PCR clone has been boxed with a solid line, and the original 216-bp DD-RT-PCR clone is indicated as an extension of the boxed region with dashed lines. The amplification primers R5 and T₁₂VC are also shown on the corresponding *Fanca* sequence. No suitable priming sites were identified by bioinformatic analysis for the T₁₂VC-anchored primer on the *Zfp276* 3' UTR sequence.

(Fig. 1A). There was no visible alteration in DD-RT-PCR-generated transcripts between cells grown on uncoated or Matrigel-coated flasks, while addition of GnRH clearly affected transcript levels. This treatment regime has previously been used to regulate expression and trigger pulsatile release of LH from these cells [9]. However, we found that a short 15-min pulse of GnRH induced rapid changes in gene expression (Fig. 1B). The identified 216-base pair (bp) cDNA was isolated, subjected to a further two rounds of PCR amplification, cloned, and sequenced. Bioinformatic analysis of the 74-bp DNA sequence obtained produced an identical match to exon 43 of *Fanca*. The difference in size between the identified 216-bp DD-RT-PCR product and the amplified 74-bp cDNA clone was due to internal priming during the second round of PCR amplification, and the two sets of priming sites are shown in Figure 1C. This was a common feature (unpublished observation) due to the inherent redundancy of the PCR amplification and may reflect the poor processivity of some *Taq* polymerases and preferential cloning of small DNA fragments. The 216-bp DD-RT-PCR product visible on the gel was consistent with priming of *Fanca* mRNA by a T₁₂VC-anchored primer (Fig. 1C). Bioinformatics also highlighted that the 74-bp cDNA clone obtained could possibly correspond to *penta-zinc finger protein 276* (*Zfp276*) because *Zfp276* mRNA is encoded on the opposite DNA strand from *Fanca* and overlaps the *Fanca* gene locus at the 3' end [25]. The orientation of these genes and region of DNA overlap between them is indicated in Figure 2. We concluded that *Zfp276* was unlikely to be the original DD-RT-PCR clone because the mRNA polyA⁺ addition site and likely T₁₂VC priming site maps a considerable distance downstream and there

was no suitable T₁₂VC annealing site within 200 bp of immediate flanking DNA sequence. However, because two transcripts, *Fanca* and *Zfp276*, matched the cloned DD-RT-PCR DNA fragment, additional expression analysis was needed to confirm that *Fanca* was differentially expressed.

Fanca mRNA Is Differentially Expressed in LBT2 Cells

Northern blotting of total RNA extracted from GnRH-treated LBT2 cells and probing with 5' and 3' fragments of mouse *Fanca* cDNA (Fig. 3A), confirmed that a full-length 4.5-kilobase (kb) transcript was expressed. The 5' *Fanca* probe corresponded to 1–965 bp of the cDNA, which encodes exons 1–11. The 3' *Fanca* probe corresponded to 4231–4500 bp of the mouse cDNA that encodes exons 42–43 and should cross-hybridize with the *Zfp276* transcript that overlaps this coding region. Indeed, a large 4.5-kb *Fanca* transcript and a small 3.1-kb transcript corresponding to the reported size of *Zfp276* mRNA [25] were detected. The Northern blot was stripped and reprobated with a ribosomal probe specific for 18s, which was used to control for loading (Fig. 3A).

Although nonquantitative, there appeared to be no difference in expression levels of *Zfp276* mRNA amplified by RT-PCR in untreated and treated cells (Fig. 3B). Interestingly, semiquantitative RT-PCR amplification of *Fanca* mRNA suggested that *Fanca* expression increased 1 h after GnRH treatment. Specific amplification of a 979-bp *Fanca* PCR product was confirmed by subsequent Southern blotting analysis, which also identified lower levels of expression in untreated, and a rapid reduction 2 and 4 h after hormone treatment (Fig. 3C).

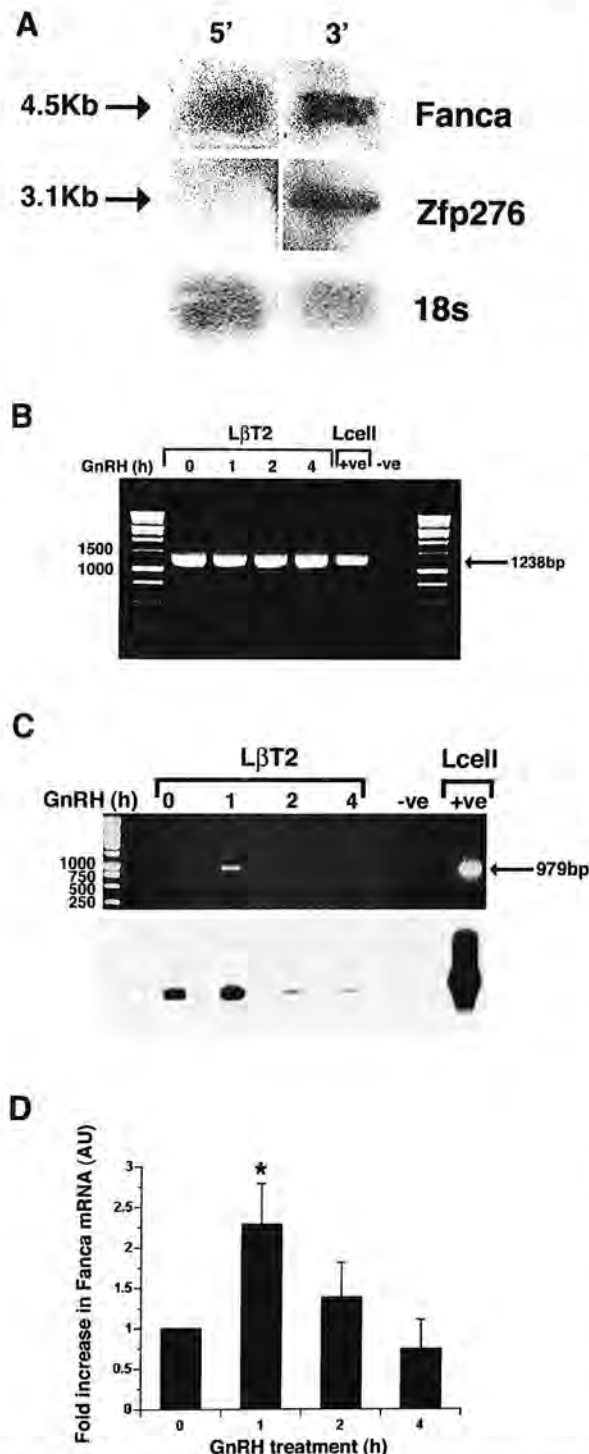


FIG. 3. Characterization and quantification of *Fanca* and *Zfp276* mRNA after GnRH treatment. **A**) Confirmation of expression of full-length 4.5-kb *Fanca* and 3.1-kb *Zfp276* mRNA transcripts in L β T2 cells. Total RNA from L β T2 cells was fractionated on a formaldehyde gel, Northern blotted, and probed with radiolabeled probes corresponding to either the 5' region (exons 1–11) or 3' region (exons 42–43) of *Fanca*. The blot was then stripped and reprobed with an 18s probe. Specific *Fanca*, *Zfp276*, and 18s bands are indicated by arrows. **B**) Semiquantitative RT-PCR analysis of *Zfp276* expression. L β T2 cells were left untreated (0) or treated with 1

Thus, quantitative RT-PCR was used to measure the rapid increase in *Fanca* mRNA after GnRH treatment. In this assay, *Fanca* mRNA clearly increased 2-fold 1 h after GnRH treatment ($P < 0.05$) and returned to unstimulated levels by 4 h (Fig. 3D). Taken together, these results indicate that *Fanca*, but not *Zfp276*, mRNA is acutely regulated by GnRH in L β T2 cells.

Analysis of *Fanca* Protein Expression

The rapid upregulation of *Fanca* mRNA may not be mirrored by an increase in protein, so Western blotting analysis was used to quantify *Fanca* protein levels in L β T2 cell protein extracts. First, protein extracts were made from untreated cells and from cells harvested 2 h after addition of GnRH, and were fractionated on a 6% SDS-PAGE gel, blotted onto polyvinylidene fluoride (PVDF) and incubated with *Fanca* antisera specific for amino acids 1–271 [26]. A specific 160-kDa band, corresponding to the expected size of *Fanca* protein, was detected in untreated and GnRH-treated extracts (Fig. 4A). Thus, the increase in levels of *Fanca* protein was measured over a longer time period following GnRH treatment and quantified (Fig. 4B). Again, *Fanca* protein levels increased 2-fold after addition of GnRH for 2 h ($P < 0.001$). This 2-fold increase was still evident 4 h after treatment with GnRH ($P < 0.001$), but by 6 h, *Fanca* protein levels had begun to fall ($P < 0.05$). This shows that a rapid increase in *Fanca* mRNA (Fig. 3D) is followed by a 2–4-h sustained increase in *Fanca* protein.

In Vivo and Temporal Expression Profile of *Fanca* mRNA

We next determined if *Fanca* was expressed in the pituitary in vivo. Mouse pituitary RNA was extracted, reverse transcribed, and cDNA amplified using specific primers to *Fanca*. DNA bands corresponding to exons 7–18, 14–18, and 30–32 were identified (Fig. 5A). This analysis confirmed pituitary expression of *Fanca* in adult mice. Because L β T2 cells are derived from embryonic Day 16.5 mouse pituitaries, we also investigated if *Fanca* mRNA was expressed in a different immature gonadotroph cell line (Fig. 5B). As expected, *Fanca* was expressed in L β T2 cells, HeLa, and L cells, but no corresponding PCR product was detected in immature precursor α T3-1 gonadotroph cells.

←

× 15-min pulse of GnRH, then harvested 1, 2, and 4 h later, RNA was extracted, and first-strand cDNA made. PCR was performed to amplify full-length *Zfp276* (1238 bp) and the products were visualized on an ethidium bromide-stained agarose gel. One specific 1238-bp PCR product was visible at all time points. L-cell cDNA was included as a positive control. An arrow denotes the 1238-bp PCR product, and DNA size markers are labeled. **C**) Semiquantitative RT-PCR analysis was performed for *Fanca* expression in L β T2 cells that were left untreated (0) or treated with 1 pulse of GnRH; harvested 1, 2, and 4 h later; RNA extracted, and cDNA made. Ethidium bromide staining identified one PCR product amplified from mRNA harvested from the 1-h time point. L-cell cDNA was included as a positive control. An arrow denotes the 979-bp PCR product, and size markers are labeled. Southern blotting analysis of the agarose gel with a 5' *Fanca* probe confirmed that the *Fanca* PCR product was specific and present at all time points. **D**) LightCycler quantitative RT-PCR analysis of *Fanca* mRNA extracted from L β T2 cells either left untreated or treated with GnRH and harvested 1, 2, and 4 h later detected a consistent 2-fold increase in *Fanca* mRNA harvested 1 h after treatment. Results are shown as arbitrary units (AU) of *Fanca* mRNA normalized against the levels of internal control *B-2-microglobulin* (*B2m*) mRNA. This experiment was performed in duplicate and repeated three times. * $P < 0.05$ was determined as being significant by ANOVA one-way analysis of variance.

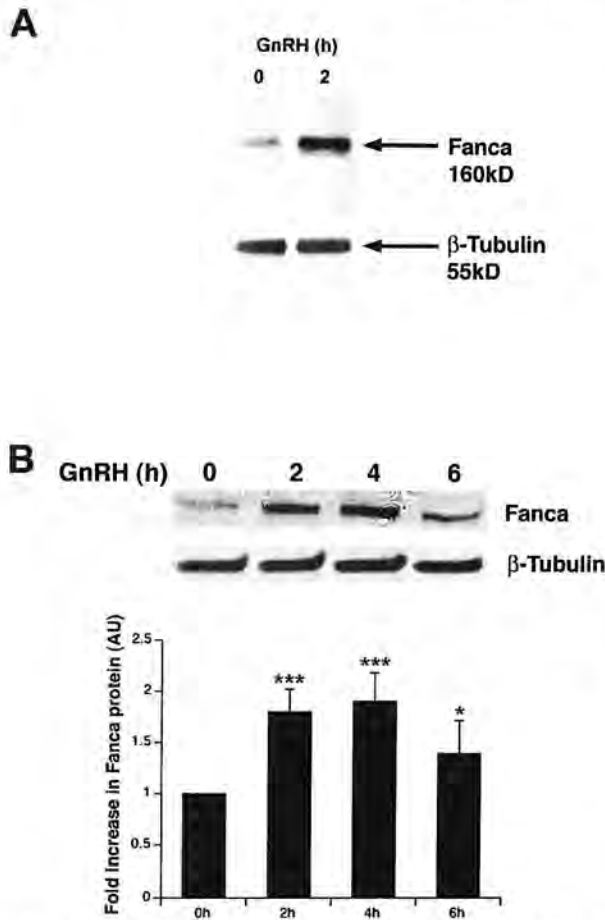


FIG. 4. Western blotting analysis of Fanca protein. **A)** Western blotting analysis of protein extracts from untreated, 0, and extracts harvested 2 h after GnRH treatment identified Fanca protein. Cellular protein was fractionated, transferred to PVDF, and probed with anti-mouse Fanca antisera before being stripped and reprobed with anti-mouse β -tubulin. Arrows indicate the 160-kDa Fanca and 55-kDa β -tubulin proteins. **B)** Western blotting analysis of whole-cell protein extracts from untreated (0) and hormone-treated cells harvested 2, 4, and 6 h later. Blots were probed as above in A. ANOVA one-way analysis of variance determined that the increases in Fanca protein levels after hormone treatment were significant: *** $P < 0.001$; ** $P < 0.001$; * $P < 0.05$.

DISCUSSION

Murine *Fanca* mRNA has a predicted size of 4503 bp, which encodes a 160-kDa protein [27, 28]. *Fanca* mRNA is relatively highly expressed in lymphoid tissues, testes, and ovary in adult mice and is activated as early as Embryonic Day 7, largely in cells of epithelial origin [27, 28]. This is the first report of hormonal regulation of *Fanca* and of *Fanca* expression in a specialized endocrine cell. Although there have been reports of smaller *Fanca* mRNA molecules being expressed in brain [27], none of these were localized to the pituitary, and they may correspond to the recently identified *Zfp276* gene [25], because in our hands, neither Northern nor RT-PCR analysis indicated any variation in size of *Fanca* mRNA in L β T2 cells.

Because DD-RT-PCR amplifies the transcriptome, low-abundance messages are represented with no preselection bias from the user [17, 29]. This approach was particularly suited toward identification and isolation of *Fanca* cDNA.

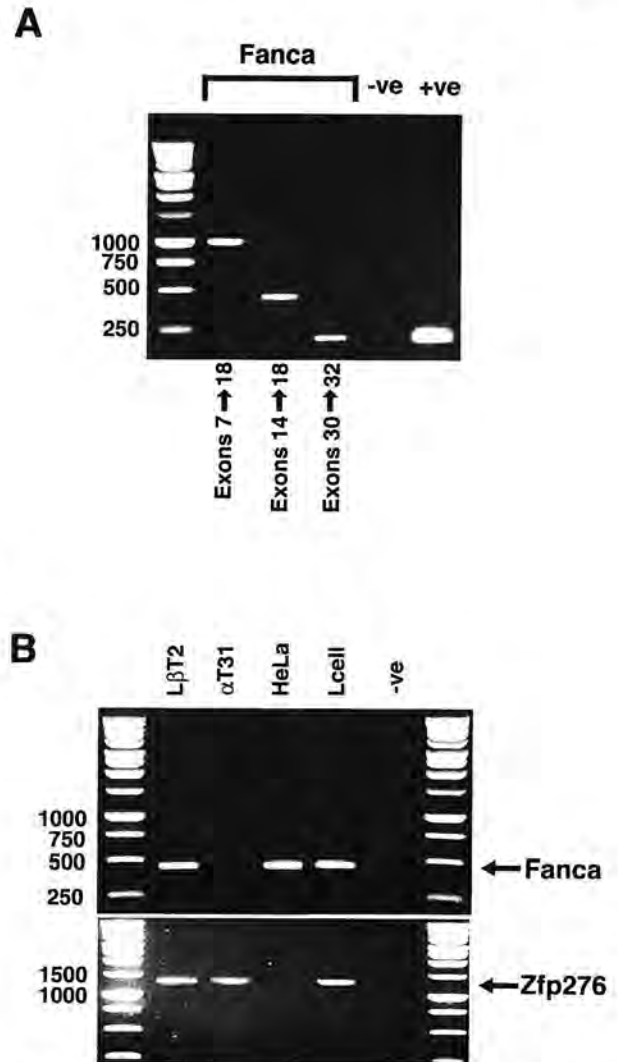


FIG. 5. *Fanca* is expressed in adult mouse pituitary. **A)** RT-PCR analysis of RNA extracted from adult mouse pituitary was performed using primers that amplified exons 7–18, exons 14–18, and exons 30–32 of mouse *Fanca*. Ethidium bromide staining identified PCR products for all regions amplified. A control GAPDH PCR product was also amplified, confirming the integrity of the mouse pituitary cDNA. **B)** RNA was extracted from L β T2, α T3-1, HeLa, and L cells; reverse transcribed; and first-strand cDNA was analyzed by PCR for expression of *Fanca*. The PCR primers corresponded to exons 7–18 of mouse *Fanca* cDNA and ethidium bromide staining identified a 450-bp product in L β T2, HeLa, and L cells. No *Fanca* expression was detectable when amplifying α T3-1 cDNA. In this experiment, expression of *Zfp276* was used as an internal control to check PCR conditions and RNA integrity. A specific 1238-bp PCR product, corresponding to *Zfp276*, was amplified from L β T2, α T3-1, and L cell first-strand cDNA.

Differential GnRH regulation of *Fanca* mRNA was confirmed by a combination of semiquantitative and quantitative RT-PCR. However, the DD-RT-PCR technique does generate high numbers of false positives, and a number of strategies have been suggested to eliminate this [23, 30, 31]. These were taken into account during the design of our experiments and briefly include use of a time course, so differences were easily recognized, RNA extraction and cDNA synthesis was done simultaneously, and samples

from different experiments were electrophoresed in duplicate to ensure repeatability. Thus, we established that, although *Fanca* mRNA was expressed at very low levels in the L β T2 cell line, GnRH regulation was still rapid and transient, with mRNA returning to unstimulated levels after 2 h. In contrast, when using microarray analysis, the design of the cDNA microarray determines which transcripts are identified. Although *Fanca* was omitted from the microarray, rapid transient increases in transcripts were measured in response to GnRH treatment, most corresponded to immediate early gene products that had returned to baseline levels by 3 h post-GnRH treatment, and *early growth factor-1* (*Egr-1*) and *c-Jun* were induced over a wide range of GnRH concentrations (1 nM up to 1 μ M) [32, 33]. Because we and others all measure significant increases in gene expression in L β T2 cells after one treatment with a pharmacological dose of GnRH, this indicates that, although the concentration of GnRH is important, the timing between GnRH pulses is also a critical factor. It is not clear if the same changes in gene expression would be induced with a pulsatile GnRH treatment regime as in the normal physiological state, although our original experiment did address this by using 15-min pulses separated by an interpulse interval of 75 min (Fig. 1A), we found it was not ideally suited to isolation of differentially expressed transcripts and that a shorter time course of induction of gene expression was preferable.

The rapid, transient increase in *Fanca* mRNA levels measured in response to GnRH was followed by a similar, but longer, increase in *Fanca* protein levels. This suggests that levels of *Fanca* mRNA are tightly controlled, and we hypothesize that hormone treatment either increases *Fanca* gene transcription and/or stabilizes *Fanca* mRNA. Measuring steady-state mRNA levels by quantitative RT-PCR does not distinguish between these two possibilities. Our observation that *Fanca* mRNA is highly regulated in a mature GnRH-responsive gonadotroph cell line, an endocrine tissue, may explain why researchers had difficulties in detecting *Fanca* gene expression by in situ hybridization in embryonic mouse testes, but did detect expression in adult testes [27, 28, 34]. In keeping with the rapid, short-lived peak in *Fanca* mRNA, we consistently found that hormonal stimulation also increased levels of *Fanca* protein. *Fanca* protein increased 2 h after treatment with GnRH, but persisted for longer, only starting to decline 6 h after treatment. Because we used whole-cell extracts in this study, we have yet to analyze if this increase in protein also results in a re compartmentalization of *Fanca* within the cell in response to hormone, but prior treatment of cells with cycloheximide, an inhibitor of translation, blocks the GnRH-induced increase in *Fanca* protein levels (unpublished results), suggesting that the increase in *Fanca* protein is indeed due to de novo translation. The hormonally induced increase in *Fanca* protein suggests it may be a component of a rapid response mechanism in these cells. Indeed, the action of *Fanca* in immune cells is upstream of the immediate early response genes [35], suggesting that *Fanca* may act as part of a signal-transduction cascade. We and others have noted that expression of *Fanca* protein is low because detection requires either reasonable amounts of starting material [27] or an enriched population of cells, indicating that relatively small changes in protein expression could have a large impact. Interestingly, expression levels and posttranslational modification are critically important for many proteins involved in signal transduction [36].

The hormonal regulation of expression and temporal pat-

tern of *Fanca* gene activation indicates that this molecule may be important for mature gonadotroph cell function. While the existence of a complementary *Zfp276* transcript complicated our analysis, especially because it was coexpressed in L β T2 cells, there was no clear evidence to indicate that *Zfp276* mRNA was the original DD-RT-PCR fragment or that it was hormonally regulated. Furthermore, although *Zfp276* mRNA was expressed, neither *Fanca* mRNA or protein (data not shown) was detected in α T3-1 cells, which are a GnRH-responsive gonadotroph cell line derived from embryonic Day 13.5 pituitaries, that exclusively express α GSU, but not LH or FSH β subunit [10, 37]. This indicates that *Fanca* gene expression is activated late in pituitary development because L β T2 cells are derived from embryonic Day 16.5 pituitaries, and indeed, we confirmed that *Fanca* was expressed in adult pituitary.

The pleiotropic nature of the FA syndrome, which in humans is an autosomal recessive disorder characterized by bone marrow failure, aplastic anemia, and variable predisposition to cancers of the gynecologic system among other clinical features, including infertility [18, 38, 39], has made it difficult to ascribe particular phenotypic features to mutations in any particular region of *Fanca* or any of the other Fanconi complementation groups [40, 41]. Targeted disruption of *Fanca* in mice has reproduced some of the associated FA phenotypes described above, but the most consistent of these appears to be a severe reduction in fertility [34, 42, 43]. Furthermore, although the pituitary was not examined, the testes were identified as a major site of *Fanca* gene expression, and these mice developed ovarian granulosa cell tumors [34], a phenotype also known to be consistent with elevated plasma levels of pituitary LH [44]. Elevated gonadotropin levels have also been reported in FA patients [45], and there is evidence that FA impacts on reproduction [46, 47]. Taken together, these observations suggest a role for *Fanca* in reproduction, especially gonadal function, but how this impacts on the pituitary is under further investigation.

There may be a link between FA and development of pituitary neoplasms because pituitary tumors develop through various mechanisms [48]. FA is a relatively rare disease, while the incidence of pituitary adenoma within the general population is high (~20%), and gonadotroph cell neoplasms account for ~35% of these [49]. However, a specific subset of pituitary tumors (<2%) are caused by mutations in the *MEN1* gene, which encodes the transcriptional repressor MENIN [50] and interestingly, MENIN has recently been shown to interact with FANCD2, the downstream target of the FA complex [51].

In addition, a number of FA genes have been implicated in ovarian tumorigenesis in man and mouse [52, 53] and *Fanca* is required for gonadal function [34]. GnRH, acting through its receptor, is a key autocrine/paracrine regulator of ovarian and testicular function [54] and has a role in development of GnRH-responsive ovarian, breast, and prostate cancer [55]. This evidence and the prevalence of endocrinopathies in patients support a role for the FA genes in endocrine signaling [20]. Thus, we hypothesize that GnRH regulation of *Fanca* gene expression may be important for the normal endocrine function of the pituitary and possibly reproductive organs.

In conclusion, *Fanca* was identified in and isolated from mouse anterior pituitary gonadotrophs in a DD-RT-PCR screen for transcripts regulated by GnRH. This broadens the expression profile of *Fanca* into highly specialized endocrine tissues and establishes hormonal regulation of the

Fanca locus. The acute hormonal regulation of the molecule indicates that *Fanca* may have a role in mediating GnRH responsiveness in mature gonadotrophs.

ACKNOWLEDGMENTS

We would like to thank Professor Pamela Mellon (San Diego, CA) for supplying L β T2 cells, Dr. Fre Arwert (Amsterdam, The Netherlands) for the generous gift of a mouse *Fanca* cDNA and anti-mouse *Fanca*-specific antisera, Julie Bell for guidance in using the LightCycler, and Dimitra Karali for technical support.

REFERENCES

- Stanislaus D, Pinter JH, Janovick JA, Conn PM. Mechanisms mediating multiple physiological responses to gonadotropin-releasing hormone. *Mol Cell Endocrinol* 1998; 144:1-10.
- Brown P, McNeilly AS. Transcriptional regulation of pituitary gonadotrophin subunit genes. *Rev Reprod* 1999; 4:117-124.
- Shacham S, Harris D, Ben-Shlomo H, Cohen I, Bonfil D, Przedeki F, Lewy H, Ashkenazi IE, Seger R, Naor Z. Mechanism of GnRH receptor signaling on gonadotropin release and gene expression in pituitary gonadotrophs. *Vitam Horm* 2001; 63:63-90.
- Charlton HM, Halpin DM, Iddon C, Rosie R, Levy G, McDowell I, Megson A, Morris J, Bramwell A, Speight A, Ward B, Broadhead J, Davey-Smith G, Fink G. The effects of daily administration of single and multiple injections of gonadotropin releasing hormone on pituitary and gonadal function in the hypogonadal (HPG) mouse. *Endocrinology* 1983; 113:535-544.
- Brown P, McNeilly J, Evans J, Crawford G, Walker M, Christian H, McNeilly A. Manipulating the in vivo mRNA expression profile of FSH β to resemble that of LH β does not promote a concomitant increase in intracellular storage of follicle-stimulating hormone. *J Neuroendocrinol* 2001; 13:50-62.
- Mercer JE, Clements JA, Funder JW, Clarke IJ. Regulation of follicle stimulation hormone beta and common alpha subunit mRNA by gonadotropin releasing hormone and estrogen in the sheep pituitary. *Neuroendocrinology* 1989; 50:321-326.
- Bedecarrats GY, Kaiser UB. Differential regulation of gonadotrophin subunit gene promoter activity by pulsatile gonadotropin-releasing hormone (GnRH) in perfused L β T2 cells: role of GnRH receptor concentration. *Endocrinology* 2003; 144:1802-1811.
- Japon MA, Rubinstein M, Low J. In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. *J Histochem Cytochem* 1994; 42:1117-1125.
- Turgeon JL, Kimura Y, Waring DW, Mellon PL. Steroid and pulsatile gonadotropin-releasing hormone (GnRH) regulation of luteinizing hormone and GnRH receptor in a novel gonadotrope cell line. *Mol Endocrinol* 1996; 10:439-450.
- Windle JJ, Weiner RI, Mellon PL. Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. *Mol Endocrinol* 1990; 4:597-603.
- Graham KE, Nusser KD, Low MJ. L beta T2 gonadotroph cells secrete follicle stimulating hormone (FSH) in response to activin A. *J Endocrinol* 1999; 162:R1-R5.
- Pernasetti F, Vasilyev VV, Rosenberg SB, Bailey JS, Huang HJ, Miller WL, Mellon PL. Cell-specific transcriptional regulation of follicle-stimulating hormone- β by activin and gonadotropin releasing hormone in the L β T2 pituitary gonadotrope cell model. *Endocrinology* 2001; 142:2284-2295.
- Liu F, Austin DA, Mellon PL, Olefsky JM, Webster NJG. GnRH activates ERK1/2 leading to the induction of *c-fos* and LH β protein expression in L β T2 cells. *Mol Endocrinol* 2002; 16:419-434.
- Yokoi T, Ohmichi K, Tasaka K, Kimura A, Kanda Y, Hayakawa J, Tahara M, Hisamoto K, Kurachi H, Murata Y. Activation of the luteinizing hormone beta promoter by gonadotropin-releasing hormone requires c-JUN NH2-terminal protein kinase. *J Biol Chem* 2000; 275:21639-21647.
- Liu F, Usui I, Guojing Evans L, Austin DA, Mellon PL, Olefsky JM, Webster NJG. Involvement of both G $\alpha_{q/11}$ and G α_s proteins in gonadotropin-releasing hormone receptor-mediated signaling in L β T2 cells. *J Biol Chem* 2002; 277:32099-32108.
- Harris D, Bonfil D, Chuderland D, Kraus S, Seger R, Naor Z. Activation of MAPK cascades by GnRH: ERK and Jun N-terminal kinase are involved in basal and GnRH-stimulated activity of the glycoprotein hormone LH β subunit promoter. *Endocrinology* 2002; 143:1018-1025.
- Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 1991; 257:967-971.
- Auerbach AD, Buchwald M, Joenje H. Fanconi anemia. In: *Genetic Basis of Human Cancer*. New York: McGraw-Hill; 1998:317-332.
- Joenje H, Patel KJ. The emerging genetic and molecular basis of Fanconi anemia. *Nat Rev Genet* 2001; 2:446-457.
- Wajnrajch MP, Gertner JM, Huma Z, Popovic J, Lin K, Verlander PC, Batish SD, Giampietro PF, Davis JG, New MI, Auerbach AD. Evaluation of growth and hormonal status in patients referred to the international Fanconi anemia registry. *Pediatrics* 2001; 107:744-754.
- Abbas MM, Evans JJ. Regulation of *c-fos* protein in gonadotrope cells by oxytocin and gonadotropin-releasing hormone. *Neuroendocrinology* 2000; 71:292-300.
- Coss D, Jacobs SB, Bender CE, Mellon PL. A novel AP-1 site is critical for maximal induction of the follicle-stimulating hormone beta gene by gonadotropin-releasing hormone. *J Biol Chem* 2004; 279:152-162.
- Miele G, MacRae L, McBride D, Manson J, Clinton M. Elimination of false positives generated through PCR reamplification of differential display cDNA. *Biotechniques* 1998; 25:138-144.
- Brown P, McNeilly AS. Steroidogenic factor-1 (SF-1) and the regulation of expression of luteinizing hormone and follicle stimulating hormone β -subunits in the sheep anterior pituitary in vivo. *Int J Biochem Cell Biol* 1997; 29:1513-1524.
- Wong JCY, Alon N, Norga K, Kruyt FAE, Youssoufian H, Buchwald M. Cloning and analysis of the mouse Fanconi anemia group A cDNA and an overlapping penta zinc finger cDNA. *Genomics* 2000; 67:273-283.
- Waisfisz Q, De Winter JP, Kruyt FAE, de Groot J, van der Weel L, Dijkmans LM, Arwert F, Scheper RJ, Youssoufian H, Hoatlin ME, Joenje H. A physical complex of the Fanconi anemia proteins FANCG/XRCC9 and FANCA. *Proc Natl Acad Sci U S A* 1999; 96:10320-10325.
- van de Vrugt HJ, Cheng NC, de Vries Y, Rooimans MA, de Groot J, Scheper RJ, Zhi Y, Hoatlin ME, Joenje H, Arwert F. Cloning and characterization of murine Fanconi anemia group A gene: *Fanca* protein is expressed in lymphoid tissues, testes and ovary. *Mamm Genome* 2000; 11:326-331.
- Abu-Issa R, Eichele G, Youssoufian H. Expression of the Fanconi anemia group A gene (*Fanca*) during mouse embryogenesis. *Blood* 1999; 94:818-824.
- Liang P. SAGE genic: a suite with panoramic view of gene expression. *Proc Natl Acad Sci U S A* 2002; 99:11547-11548.
- Liang P. Factors ensuring successful use of differential display. *MethOds* 1998; 16:361-364.
- Luce MJ, Burrows PD. Minimizing false positives in differential display. *Biotechniques* 1998; 24:766-768.
- Wurmbach E, Yuen T, Ebersole BJ, Sealfon SC. Gonadotropin-releasing hormone receptor-coupled gene network. *J Biol Chem* 2001; 276:47195-47201.
- Yuen T, Wurmbach E, Ebersole BJ, Ruf F, Pfeffer RL, Sealfon SC. Coupling of GnRH concentration and the GnRH receptor-activated gene program. *Mol Endocrinol* 2002; 16:1145-1153.
- Wong JCY, Alon N, Mckerlie C, Huang JR, Meyn MS, Buchwald M. Targeted disruption of exons 1 to 6 of the Fanconi anemia group A gene leads to growth retardation, strain-specific microphthalmia, meiotic defects and primordial germ cell hypoplasia. *Hum Mol Genet* 2003; 12:2063-2076.
- Pipaon C, Casado JA, Bueren JA, Fernandez-Luna JL. Jun N-terminal kinase activity and early growth-response factor-1 gene expression are down-regulated in Fanconi anemia group A lymphoblasts. *Blood* 2004; 103:128-132.
- Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Bertram K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001; 22:153-183.
- Alarid ET, Holley S, Hayakawa M, Mellon PL. Discrete stages of anterior pituitary differentiation recapitulated in immortalized cell lines. *Mol Cell Endocrinol* 1998; 140:25-30.
- Alter BP. Cancer in Fanconi anemia, 1927-2001. *Cancer* 2003; 97:425-440.
- Bagby GC. Genetic basis of Fanconi anemia. *Curr Opin Hematol* 2003; 10:68-76.
- Favre L, Guardiola P, Lewis C, Dokal I, Edell W, Zatterale A, Altay

- C, Poole J, Stones D, Kwee ML, van Weel-Sipman M, Havenga C, Morgan N, de Winter J, Digweed M, Savoia A, Pronk J, de Ravel T, Jansen S, Joenje H, Gluckman E, Mathew CG. Association of complementation group and mutation type with clinical outcome in Fanconi anemia. *Blood* 2000; 96:4064–4070.
41. Adachi D, Oda T, Yagasaki H, Nakasato K, Taniguchi T, D'Andrea AD, Asano S, Yamashita T. Heterogeneous activation of the Fanconi anemia pathway by patient-derived FANCA mutants. *Hum Mol Genet* 2002; 25:3125–3134.
 42. Cheng NC, van de Vrugt HJ, van der Valk MA, Oostra AB, Krimpenfort P, de Vries Y, Joenje H, Berns A, Arwert F. Mice with a targeted disruption of the Fanconi anemia homolog *Fanca*. *Hum Mol Genet* 2000; 9:1805–1811.
 43. Rio P, Segovia JC, Hanenberg H, Casado JA, Martinez J, Gottsche K, Cheng NC, van de Vrugt HJ, Arwert F, Joenje H, Bueren JA. In vitro phenotypic correction of hematopoietic progenitors from Fanconi anemia group A knockout mice. *Blood* 2002; 100:2032–2039.
 44. Risma KA, Clay CM, Nett TM, Wagner T, Yun J, Nilson JH. Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumours. *Proc Natl Acad Sci U S A* 1995; 92:1322–1326.
 45. Berkovitz GD, Zinkham WH, Migeon CJ. Gonadal function in two siblings with Fanconi's anemia. *Horm Res* 1984; 19:137–141.
 46. Alter BP, Frizzera CL, Halperin DS, Freedman MH, Chitkara AD, Alvarez E, Lynch L, Adler-Brecher B, Auerbach AD. Fanconi's anemia and pregnancy. *Br J Haematol* 1991; 77:410–418.
 47. Bargman GJ, Shahidi NT, Gilbert EF, Opitz JM. Studies of malformation syndromes of man XLVII: disappearance of spermatogonia in the Fanconi anemia syndrome. *Eur J Pediatr* 1977; 125:169–174.
 48. Melmed S. Mechanisms for pituitary tumorigenesis: the plastic pituitary. *J Clin Invest* 2003; 112:1603–1618.
 49. Asa SL, Ezzat S. The pathogenesis of pituitary tumours. *Nat Rev Cancer* 2002; 2:836–849.
 50. Chandrasekharappa SC, Guru SC, Manickam P, Olufemi SE, Collins FS, Emmert-Buck MR, Debelenko LV, Zhuang Z, Lubensky IA, Liotta LA, Crabtree JS, Wang Y, Roe BA, Weisemann J, Boguski MS, Agarwal SK, Kester MB, Kim YS, Heppner C, Dong Q, Spiegel AM, Burns AL, Marx SJ. Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science* 1997; 276:404–407.
 51. Jin S, Mao H, Schnepf RW, Sykes SM, Silva AC, D'Andrea AD, Hua X. Menin associates with FANCD2, a protein involved in repair of DNA damage. *Cancer Res* 2003; 63:4204–4210.
 52. Houghtaling S, Timmers C, Noll M, Finegold MJ, Jones SN, Meyn MS, Grompe M. Epithelial cancer in Fanconi anemia complementation group D2 (*Fancd2*) knockout mice. *Genes Dev* 2003; 17:2021–2035.
 53. Taniguchi T, Tischkowitz M, Ameziane N, Hodgson SV, Mathew CG, Joenje H, Mok SC, D'Andrea AD. Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. *Nat Med* 2003; 9:568–574.
 54. Kang SK, Choi KC, Yang HS, Leung PC. Potential role of gonadotrophin-releasing hormone (GnRH)-I and GnRH-II in the ovary and ovarian cancer. *Endocr Relat Cancer* 2003; 10:169–177.
 55. Schally AV. LH-RH analogues: I. Their impact on reproductive medicine. *Gynecol Endocrinol* 1999; 13:401–409.