The Insulin-like growth factor type 1 receptor and colo-rectal neoplasia : modelling the somatic evolution of cancers

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ABSTRACT

This thesis examines the temporal expression of the Insulin-like growth factor type 1 receptor gene (IGF-1R) in the evolution of human colo-rectal cancer. The IGF-1R is a highly conserved transmembrane receptor tyrosine kinase that is expressed at high levels in embryonic stem cells and in many cancer phenotypes. However, the IGF-1R is expressed at lower levels in some advanced cancers. The reasons for this and the timing of these changes in expression during neoplasia are not understood. Herein these studies examine IGF-1R expression in human colo-rectal neoplasia by means of Northern blotting and Immunohistochemistry validated by tissue and reagent controls and by Western blotting. The studies show that in the normal human colon, adult stem cells in the basal crypt region express high IGF-1R levels which decrease to low levels when these cells migrate to and differentiate in the mid and upper crypt regions. In the aberrant crypt focus, the transformed cells express high IGF-1R levels throughout the crypt axis despite showing varying degrees of differentiation. This pattern of high IGF-1R expression occurring de novo in colo-rectal neoplasia continues with neoplastic progression in adenomatous polyps and cancers. However, reduced IGF-1R expression is seen in some advanced cancer phenotypes – in epithelial-type cancer cells that show a fully polarised morphology in areas with epithelial-mesenchymal type transformation (EMT) and in invasive mesenchymal-type cancer cells that show a loss of cell-basement membrane or cell-cell adhesion after EMT. These morphological changes account for the low levels of IGF-1R expression seen in advanced invasive cancers in the current studies. These studies provide basic insights into how IGF-1R expression in normal cellular development is disrupted at tumour initiation and progression and into how this process might be involved in later stage cancers during EMT and invasion.

DEDICATION

To my parents, Elizabeth and John

ACKNOWLEDGEMENTS

I should like to thank Fouad Habib, Colin McArdle and the Melville Trust for their support over the years.

DECLARATION

I, Andrew Scott Allison hereby declare that the work embodied in this thesis is the result of my own independent investigation. This is in accordance with regulation 3.8.7 of the University of Edinburgh Postgraduate Study Programme.

Andrew Scott Allison

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ABBREVIATIONS

IGF-1R	The insulin-like growth factor type 1 receptor
IGF-2R	The insulin-like growth factor type 2 receptor
EGFR	Epidermal growth factor receptor
FGFR	basic fibroblastic growth factor receptor
PDGFR	Platelet derived growth factor receptor
NGFR	Nerve growthfactor receptor
TGFβRII	Transforming growth factor β type II receptor
RTK	Receptor tyrosine kinase
IRS-1	Insulin receptor-related substrate-1
MAPK	Mitogen activated protein kinase
PI-3K	Phospotidyl inositol-3 kinase
WT-1	Wilms' tumour suppressor
APC	Adenomatous polyposis coli gene
DCC	Deleted in colo-rectal cancer gene
EMT	Epithelial mesenchymal transformation

CHAPTER 1

INTRODUCTION

1.1. Preface

Fifty years ago the first of the modern day oncologists Rupert Willis defined cancer as "an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of normal tissues" (Willis, R.A.,1952). The last thirty years have seen cancer researchers define the disease in terms of gene mutations. The last decade however has seen cancer defined increasingly in terms of the cell signalling network

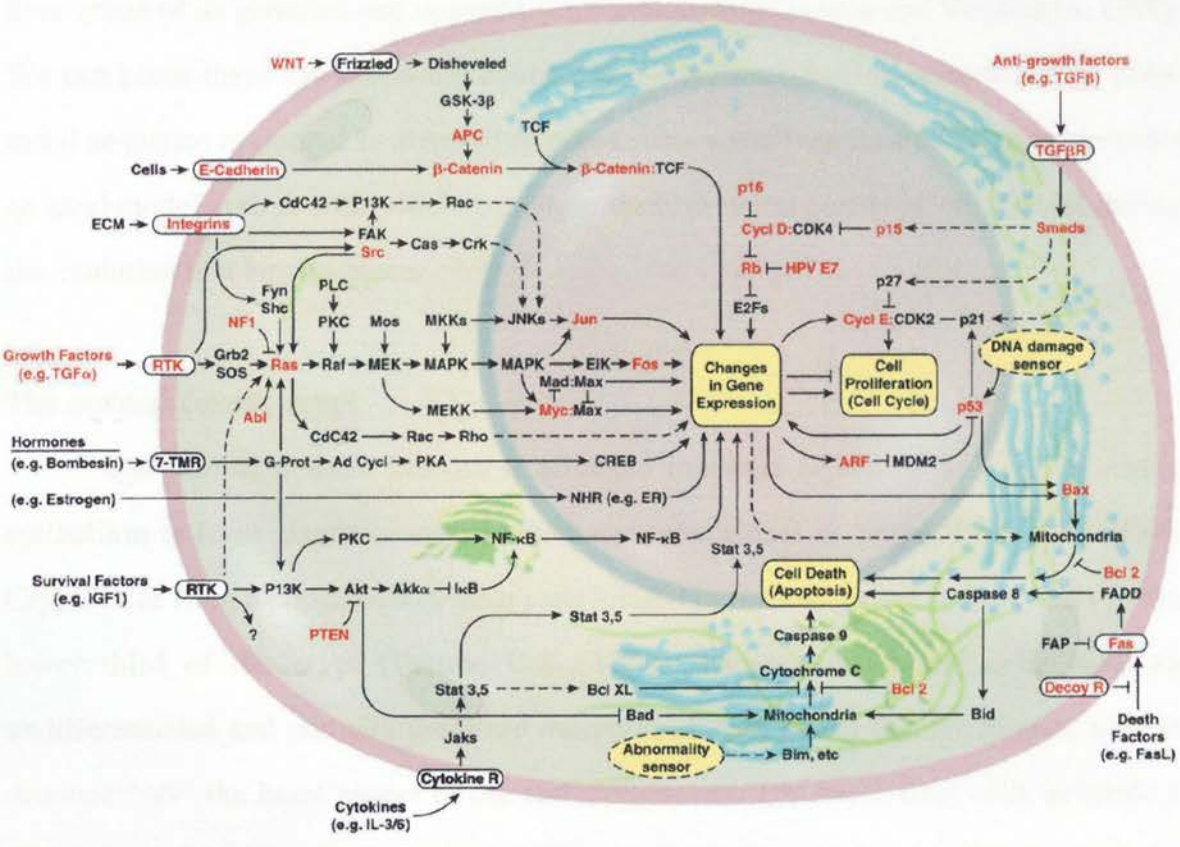
The complexity of cell signalling network is slowly being decoded (Hanahan, 2000)(Figure 1.1). This is a network of interconnected and highly redundant signalling pathways that integrate the cell's extracellular milieu with intracellular function including gene transcription. Put simply, extracellular ligands bind to their cognate cell membrane receptors to activate cytosolic signalling cascades that transduce to the nucleus where they control gene transcription. The resulting changes in gene transcription, although poorly understood, control normal cell behaviours including cell proliferation, cell survival, cell differentiation and cell-cell / cell-substrate adhesion. Components of the signalling network are targeted in the majority of cancers (reviewed Hanahan, 2000; Nurse, 2000). Here, during tumour initiation and progression, changes in gene expression or gene mutations occur in these signalling components to change normal cell growth to the abnormal "uncoordinated growth" of the cancer cell as described by Willis.

This thesis examines one component of the cell signalling network in relation to the "uncoordinated growth" of neoplasia. It examines the expression of the insulin-like growth factor type 1 receptor (IGF-1R) in colo-rectal neoplasia. The IGF-1R is a highly conserved receptor tyrosine kinase which has a central role in the cell's signalling network in the control of cell proliferation, cell survival, cell differentiation and cell-cell / cell-substrate adhesion (summarised in Figure 1.1; reviewed Baserga, 1999, 2000; Valentininis, 2001; Mauro & Surmacz, 2004; LeRoith, 2000; 0'Connor, 2000, 2003). The IGF-1R is also at one of the crossroads of the cancer cell's signalling network and many tumour mutations target components of this signalling network. There is extensive clinical and experimental data to indicate an important role for the IGF-1R in neoplasia of

the breast (Surmacz, 2000), prostate (Djavan, 2001), colon (Giovannucci, 2001), lung (Wu, 2000), pancreas (Korc 1998), liver (Scharf, 2001), ovary (Druckmann, 2002), bladder (Hursting, 2001) and brain (Zumkeller, 1999). Most cancers, including those of the colo-rectum, over express the IGF-1R (Macaulay, V.M., 1992), (Zhang, L., 1997) but there is now evidence to show that the IGF-1R is not as highly expressed in some advanced cancers of the colo-rectum (Nakamura, M., 2004), breast (Schnarr, B., 2000)(Pennisi PA, 2002) and prostate (Hellawell, G., 2002). The reasons for this and the timing of these changes in IGF-1R expression during neoplasia are unknown. This thesis examines these changes in IGF-1R expression during neoplasia, using the colo-rectal polyp-cancer sequence as a model of tumour initiation and progression.

Figure 1.1 The Cell Signalling Network and the IGF-1R

The membrane / cytosolic / nucleus signalling network that integrates the extracellular environment with the nucleus and gene expression. Growth factors (including IGF-1), the extracellular matrix and other components of basement mebrane and cell membrane bind their respective receptors - growth factor receptor tyrosine kinases (including the IGF-1R), E-cadherin, integrins and frizzled. This activates a complex series of interconnected signalling cascades – ras / MAPK, PI3K, APC / β-catenin, JAK /STAT, SMAD. These cascades in turn translocate to the cell nucleus where they control gene transcription to determine cell behaviours such as cell proliferation, cell differentiation, cell adhesion and cell survival.



(Reproduced from Douglas Hanahan, The Hallmarks of Cancer, Cell 2000. 100:57-70)

1.2. A human cancer model : colo-rectal crypt-cancer evolution

Colo-rectal cancer is the third commonest cause of cancer mortality in the western world. In the UK, the incidence of colo-rectal cancer is 35,000 newly diagnosed cases per year whereas in the western world the estimated incidence is at least half a million cases per year.

The classification of the colo-rectal cancer has evolved over the last 70 years from Dukes' pathological description of the disease (Dukes, 1932,1958); to Muto's description of its pathogenesis in the ACF-polyp-cancer sequence (Muto, 1975); to Vogelstein's description of its germline and acquired genetic mutations (Fearon and Vogelstein, 1990). We can place these models in the context of the cell's signalling networks. The colo-rectal sequence of normal to aberrant to adenomatous and then cancerous crypt provides an ideal model system with which to analyse the expression profile of the IGF-1R during the evolution of a human cancer.

The normal colonic crypt

The normal colonic mucosa is arranged in single layers of infolded columnar epithelium to form glands or crypts which are monoclonal in origin (Endo, Y., 1995). Crypts arise from a population of stem cells located in the lowermost 16 or so cells in the lower third of the crypt (Potten, C.S., 1992). These proliferative cells have an undifferentiated and partially polarised morphology with nuclei that are aligned a short distance "off" the basal aspect of the cell membranes. The basal stem cells generate a population of proliferating transit amplifying cells that occupy the middle third of the crypt. These transit amplifying cells remain pluripotent and capable of proliferation for some six or seven cell layers (Potten, C.S., 1992). The cells are partially polarised and sometimes partially differentiated. Thereafter, in the upper third of the crypt the cells fully differentiate to generate mature enterocytes, neuroendocrine cells, Paneth cells and

mucous-secreting goblet cells (Potten, C.S., 1990). These differentiated cells are fully polarised with nuclei that are aligned "on" the basal aspect of the cell membranes. On the luminal surface, these differentiated cells undergo programmed cell death and are shed into the lumen.

The volume of the crypt is determined by: cell cycling rates in the stem and transit amplifying regions; differentiation and programmed cell death rates in the terminal differentiated compartment; and rarely, by apoptosis which can occur in the transit and differentiated compartments. Apoptosis does not appear to occur in the basal stem cells of the colon. This and the pluripotency of transit cells are thought to be important contributory factors in the evolution of colon cancer (Potten, C.S., 1992). This may also explain the reduction in apoptosis seen in colorectal polyps. The stem cell can cycle at between 15 to 22 hours whilst cell migration to the upper differentiated compartment takes about 6 days. In contrast, the latent period between the initiation of an ACF and the emergence of a cancer can be up to 10 to 15 years. Thus initiated tumoural cells must have a life span commensurate with this and be able to avoid migration, differentiation, programmed cell death and shedding from within the crypt. Basal stem cells are therefore the likeliest candidate tumoural progenitors which satisfy these requirements.

The three basic phenotypes in the enterocyte lineage of the normal colonic crypt differ in the expression profiles of many genes. For example, the cell survival gene bcl-2 is expressed in the basal stem compartment (Merritt, A.J., 1995), (Sinicrope, F.A., 1995); some growth response genes such as myc, fos, cyclin D1 and EGFR are expressed in the stem and transit compartments (Chin, L., 1995), (Stopera, S.A., 1992), (Chailler, P. & Menard, D., 1998); whilst some anti-proliferative or differentiative genes such as APC and TGFβRII are expressed in the upper differentiated compartment (Smith, K.J., 1993). Other genes such as E-cadherin appear to be constitutively expressed throughout the length of the crypt (Sellin, 2001). We can therefore infer to a limited degree the characteristics of a gene by its expression profile within the normal colonic crypt.

The aberrant crypt focus : tumour initiation

There is observational evidence that aberrant crypts are the precursors to adenomatous polyps which in turn are the precursors to the majority of colorectal cancers. For example, ACFs were first observed in animals treated with colon carcinogens (Deschner, E.E., 1974) (Tudek, G., 1989) and in humans with colon cancer (Shansuddin, A.K.M., 1981). Also, ACFs and polyps both have the same anatomical and epidemiological distribution as cancers (Ronucci, L., 1991), (Ronucci, L., 1998), (Muto, T., 1975) and carcinoma-in-situ has been seen to arise in ACFs and polyps (Konstantakos, A.K., 1996).

Histologically the aberrant crypt focus exhibits a lack of differentiation in comparison to the normal colonic crypt. Additionally, cell crowding occurs and there may be an increase in nuclear size with heterogeneity and hyperchromasia, features that constitute a dysplastic ACF. The cells are partially polarised with nuclei that are aligned "off" the basal membrane.

The earliest detectable cell kinetic abnormalities at this stage consist of an increased proliferation in the basal and transit amplifying compartments (Lipkin, M., 1988) and a reduction in programmed cell death in the upper differentiated compartment of the crypt (Bedi, A., 1995). The zone of proliferation expands to occupy the entire aberrant crypt (Ronucci, L., 1993), (Otori, K., 1995), (Shpitz, B., 1997). Thymidine labelling studies have shown continued DNA synthesis suggesting that the mechanisms which normally shut off DNA synthesis in the upper two-thirds of the crypt are non functional at this early stage.

Tumour initiation as represented by the ACF exhibits high expression levels of bcl-2 and c-fos (Stopera, S.A., 1992), (Hague, A., 1994). Some mutations are also documented with increased frequency. APC mutations have been documented in up to 4.6% (Smith, A.J., 1994), (Otori, K., 1998) and K-ras mutations in 13% to 50% of ACFs

(Takayama, T., 1998), (Pretlow, T.P., 1993). p53 mutation or expressive changes in p53 have not been found in ACFs (Losi, L., 1996) but decreased expression of p21, a target of p53, has been documented in some dysplastic ACFs (Polyak, K., 1996). This pattern of unchecked expressive change in cell cycle and cell survival genes along with activating mutations in their component pathways continues throughout neoplastic progression.

Adenomas : tumour progression

Continued proliferation in the ACF results in crypt fission, expansion and infolding of epithelial cells to give the characteristic glandular polyp seen in tubular adenomas. Histologically, the cells are tall, thin, crowded and relatively lacking in differentiation. They can have pseudostratified nuclei at different levels giving a "picket fence" appearance. However, the cells retain a partially polarised morphology with the lowermost nuclei aligned "off" the basal membrane. There is a spectrum of cytologic atypia. Some cells can progress to dysplasia and anaplasia but most will remain in orderly palisades. Cell growth control is not entirely abrogated at this stage but whilst some adenomas may spontaneously regress, the majority are thought to inexorably progress, undergoing clonal expansion and further mutations (Bersentes, K., 1997). Proliferation and overgrowth of the underlying and probably normal mesenchyme is also an important component of polyp development. In its advanced form it can result in glandular projections characteristic of villous adenomas. Continued unchecked epithelial cell proliferation in these larger adenomatous and villous polyps will commonly result in progression to the features of cytologic atypia and dysplasia. Here cells become more crowded with larger, hyperchromatic nuclei exhibiting pleomorphism and lesser degrees of polarisation. Tumour progression in adenomas has been represented as involving gene expression changes - including the increased expression of cyclin D1, c-myc and EGFR; and the reduced expression of E-cadherin and TGFβRII (Zhang, T., 1997),(Melhem, M.F.,1992) - arising in concert with multiple gene mutations that include APC, K-ras, DCC and p53 (Powell, S.M., 1992), (Bos, J.L., 1987), (Boughdady, I.S.), (Chen, Y.O., 1999).

Carcinoma-in-situ

At the next stage, cells can pile up upon one another, losing their palisaded architecture but remaining confined to within their basement membrane. This constitutes carcinoma in situ and correlates most closely with aneuploidy and p53 mutations which occur in 50% of CIS (Rodrigues, N.R., 1990).

Cancer

At this stage, the cancer cells exhibit the capacity to invade through the basement membrane. To do so, the cells undergo 2 morphological changes :

1. they loose their differentiated epithelial-type pallisaded alignment and undergo transformation to an undifferentiated mesenchymal-type morphology (EMT – see below);
2. they exhibit motile or invasive behaviour with the loss of cell-cell and cell-basement membrane adhesion and invasion through the basement membrane.

Most of the cells in the cancer specimen however will commonly exhibit a more ordered appearance and maintain a well, moderately or poorly differentiated morphology with the cancer cells maintaining some form of cell-cell or cell-basement membrane contact and confinement. The cells exhibit a high incidence of aneuploidy and p53 mutation at this stage.

Epithelial mesenchymal transformation (EMT)

EMT occurs during the critical stages of embryonic development. An analogous process occurs in the advanced stages of cancer development where cancer cells change from an epithelial to a mesenchymal-type morphology (Reviewed Thiery, 2002). Cancer cells undergoing EMT loose their polarised and palisaded epithelial type appearance and adopt an unpolarised and undifferentiated mesenchymal type appearance. Changes in gene expression such as reduced expression of the epithelial marker E-cadherin occur during EMT in cancer specimens (Frixen, 1991). In vitro, EMT (and in particular "scattering") is dependent on growth factors such as HGF, IGF-1 and –2, FGFs, EGF and TGF-β which can reduce the expression of E-cadherin (Thiery, 2002). The mechanisms

of this are not clear. IGF-1R for example forms a complex with E-cadherin and β-catenin which results in the lysosomal degradation of E-cadherin and the translocation of β-catenin to the nucleus where together with Tcf it induces transcription of genes such as Myc (Morali, 2001). However, the exact mechanism(s) of change in E-cadherin transcription are unknown. Similarly, it is not known if the growth factor receptors themselves (such as the IGF-1R) undergo expressive change during EMT.

As noted above, following EMT, the mesenchymal type cells are not necessarily invasive and can still maintain their cell-cell and cell-basement membrane contacts. That is, poorly differentiated mesenchymal type cancer cells can maintain cell-cell and cell-basement membrane contacts in a manner similar to well and moderately differentiated epithelial type cancer cells. However, because the process of EMT cannot be followed temporally and accurately in cancer specimens, the exact sequence of events during EMT and invasion is difficult to discern. There is commonly considerable morphological overlap between EMT and frankly invasive cells. These processes will be examined in this thesis.

Invasive cancer

Here cancer cells show frank invasion through the basement membrane or blood vessel endothelium or lymphatics. The cells have lost their cell-cell and cell-basement membrane adhesion and show a motile mesenchymal morphology with lamellipodia. They continue to show a reduction in E-cadherin expression and additionally express proteases such as kallikreins (Borongo, 2004).

Metastasis and mesenchymal epithelial transformation (MET)

Once cancer cells have moved via direct invasion, the bloodstream or lymphatics to a distant site or organ, they can form a secondary carcinoma and undergo a reverse process of mesenchymal-epithelial transition (MET). Again, the temporal sequence involved in this process or the changes in gene expression associated with this are not understood.

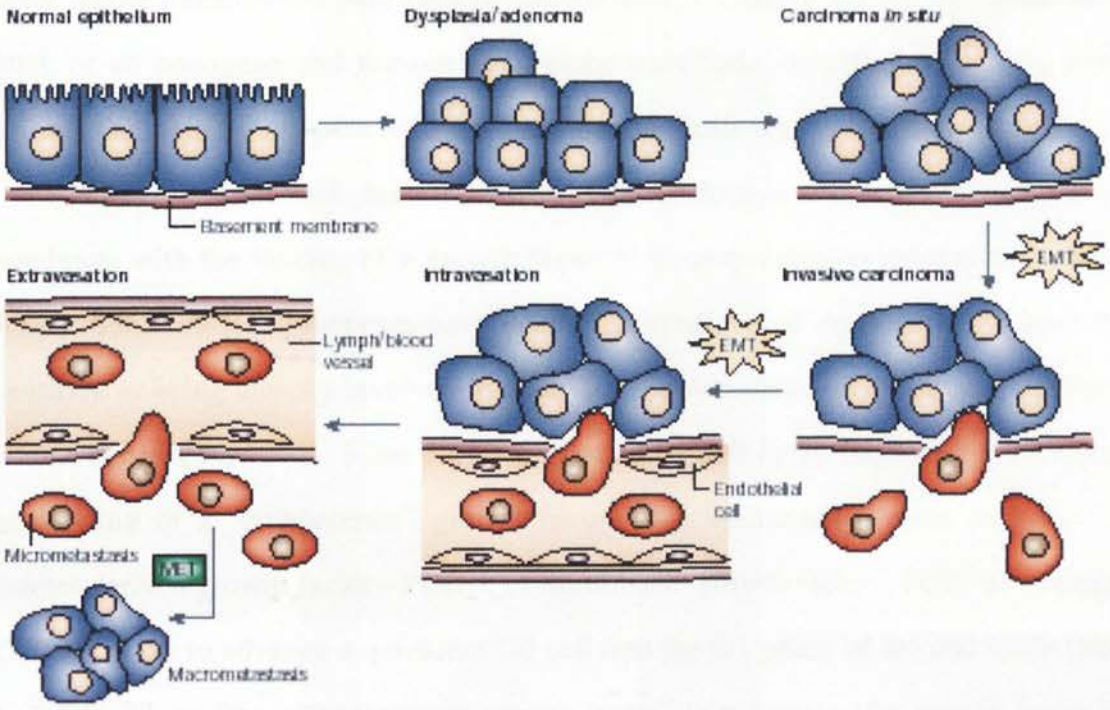
Figure 1.2 Crypt → cancer evolution : normal epithelium → ACF / adenoma → CIS - epithelial cancer → mesenchymal cancer → invasion → metastasis

Normal epithelial cells have a palisaded appearance with cell-cell contact and cell-basement membrane contact. They have a partially or fully polarised morphology (depending on their mitotic state and differentiation) with nuclei aligned either off or on the basal aspect of their membranes respectively.

ACF and adenomatous cells also have a palisaded appearance with cell-cell and cell-basement membrane contact. However their nuclei are partially polarised and are frequently at differing levels to give a "picket fence" appearance.

Cancer cells show varying degrees of differentiation but frequently have "heaped up" highly mitotic cells separated from the basement membrane. Although most of the cells show some form of cell-cell or cell-basement membrane contact, foci exhibit epithelial-mesenchymal transformation (EMT) with loss of these contacts or frank invasion through the basement membrane.

Distant metastases show a reverse process of mesenchymal-epithelial transformation (MET)



(Reproduced from Jean Paul Thiery. Epithelial-Mesenchymal transitions in tumour progression. Nature Reviews Cancer. 2002. 2:442-454.)

1.3. Cell signalling and the IGF-1R

From the foregoing discussion it will be clear that the histopathologist's description of "uncoordinated growth" in the colo-rectal crypt-cancer model might be described in terms of abnormalities in the cell signalling network necessary to cause abnormal cell proliferation, cell differentiation, cell adhesion and cell survival. These are in fact purely arbitrary distinctions as even with our current limited understanding we can see that cell signalling pathways and cell behaviours frequently interconnect and overlap. We will examine next some of these main signalling pathways with particular reference to the IGF-1R and cell behaviours.

Growth factor receptor signalling and the cell cycle

Growth factor receptor signal transduction pathways control the cell cycle and are primary targets for cancer mutations. Programmed differentiation also takes its cues from these pathways and the cell cycle. Of the purported 32,000 genes sequenced in the human genome project, approximately 20% are thought to encode components of these growth factor signal transduction pathways (Blume-Jensen, P., 2001). Of the one hundred and thirty or so oncogene and tumour suppressor mutations identified to date in human cancers, the majority are seen to target these growth factor signalling pathways.

In the normal cell, the control of these pathways starts at the level of cell membrane with the binding of a growth factor to its respective receptor tyrosine kinase (RTK). Some 58 RTK subtypes have been identified and of these some 31 have been identified as being directly involved in cancers through mutational or expressive changes (Blume-Jensen, P., 2001). Some RTKs have specific cell cycle functions. For example, the binding of a "competence" growth factor (e.g. epidermal growth factor - EGF; platelet-derived growth factor - PDGF; or fibroblastic growth factor - FGF) to its cognate RTK is known to advance a quiescent G0 cell into the G1 phase of the cell cycle (Rubin R., 1995). Thereafter, a "progression growth factor" (e.g. insulin-like growth factor type 1 - IGF-1) is required to advance the cell through the restriction point into the synthetic

phase of the cell cycle (see figure 1.3). Although this process is very incompletely understood, it does mean that in practical terms a non-transformed cell is reliant upon at least two growth factors (e.g. PDGF and IGF-1) for optimal cell growth. In contrast, cancer cells display a greatly reduced reliance upon these factors principally, it has been thought, by acquiring a combination of mutations and expressive changes in the pathways described here (see figures 1.1 and 1.4). However, the breadth and scope of those combinations required for cell cycle advancement in individual cancer phenotypes are poorly understood at present.

Binding of a growth factor to its cognate RTK results in a conformational change in the RTK's cytoplasmic domains to recruit a diverse array of signalling substrates (including Src, GAP, Shp2, p85/PI3K, Grb2, PLCg, Crk, Stat, IRS-1 and 14.3.3)(see figure 1.4). Although some substrates recruit to specific receptors (e.g. IRS-1 to IGF-1R; Shc to EGFR and IGF-1R; and p120 to EGFR, PDGFR, VEGFR and NGFR) there is more commonly a considerable overlap between RTKs and their signalling molecules. These signalling molecules recruit second messengers that in turn are integrated into a complex hierarchy of signalling cascades. These can be summarised in terms of six principal pathways which act to control the cell cycle and influence differentiation and which are targeted in the majority of cancers (see figures 1.1 and 1.4) :

1. The ras/raf-1/MAPK pathway - This is activated by EGFR, IGF-1R, PDGFR and Met. This signalling cascade translocates to the cell nucleus where the highly conserved MAPK module targets the transcription of growth response genes such as myc, fos and jun and cyclin D1, necessary for the control of the cell cycle. Ras mutations are amongst the commonest mutations in human cancers whilst MAPK is over expressed in the majority of human tumours including late-stage colorectal tumours (Hoshino, R.,1999).

2. The PI3K/Akt pathway - This is activated by PDGFR, IGF-1R, EGFR, Met, FGFR and E-cadherin. This pathway targets BAD and Bcl-2 (anti-apoptotic regulators), p70 (a ribosomal kinase necessary for translation and the regulation of cyclinD3 in the cell cycle

apparatus) and GSK3 (which phospho-inhibits APC) and can mediate cell cycle and anti-apoptotic controls (Blume-Jensen, P., 2001). The pathway is targeted in many advanced cancers including late-stage colorectal cancers, either through expressive changes or through mutation of Akt-regulating PTEN (Cantley, L.C., 1999).

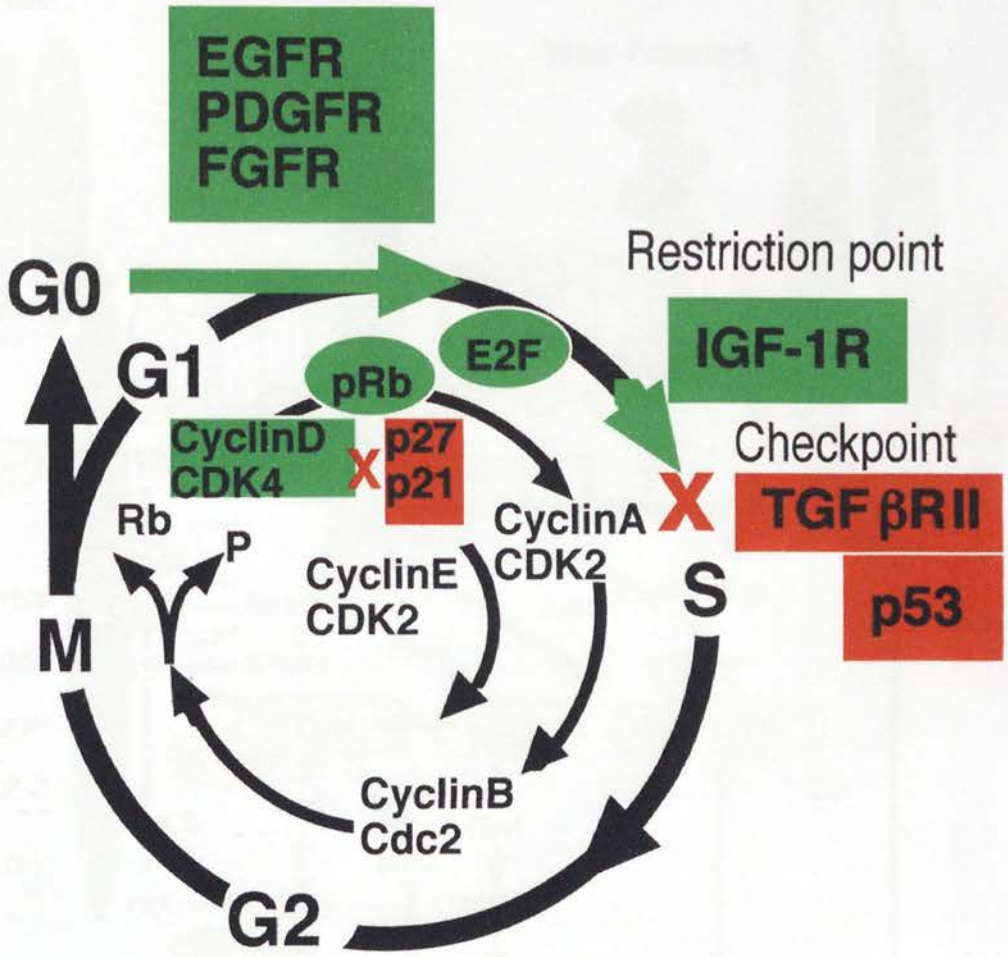
3. The Wnt/APC/β-catenin/Tcf pathway - This pathway modulates the Tcf/LEF family of HMG box transcription factors involved in cell differentiation and components of this pathway are well recognised mutational targets in colon cancer (Hunter, T., 1997). β-catenin also putatively forms a tripartite complex with the IGF-1R and E-cadherin and regulates myc and cyclin D1 expression (Tetsu, O., 1999).

4. The cyclin D1/cdk4/Rb pathway - The above 3 pathways trans activate the cyclins, principally cyclin D1, and thus initiate G1S cell cycle progression through a well defined path (see figures 1.1 and 1.3). One element of the cyclin D1/cdk4/Rb pathway is targeted in the majority of cancers (Sherr, C.J., 1996).

5. The Cadherin pathway - These cell surface receptors modulate cell-matrix adhesion by signalling via the src-family of RTKs (such as IGF-1R) to activate Wnt signalling. Function of this pathway is lost in the majority of epithelial cancers (Christofori, G., 1999). E-cadherin expression is also down regulated during the late polyp-cancer sequence (Ilyas, M.,1997) and in EMT as previously described.

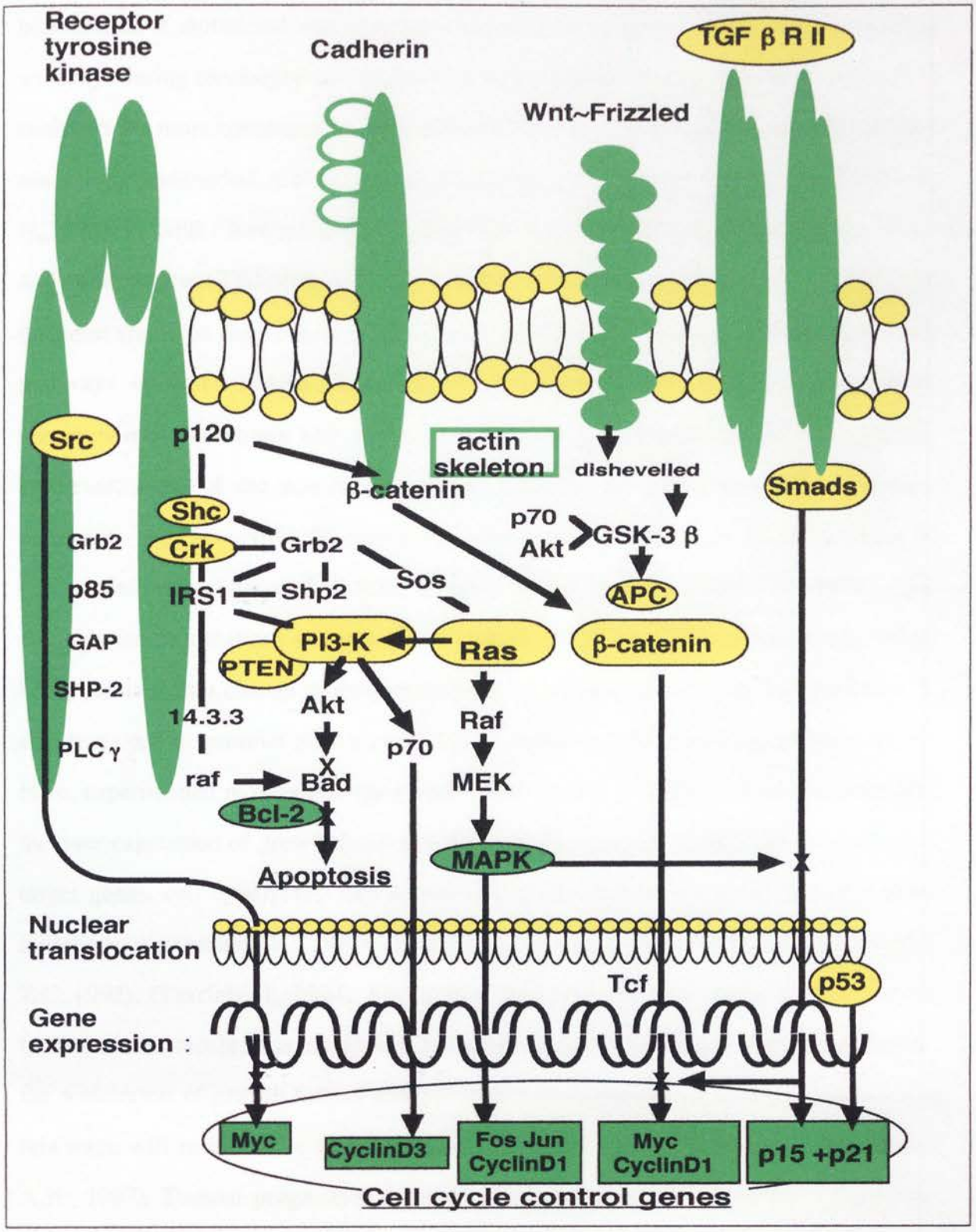
6. The p53 and TGFβRII/SMAD pathways - As important as the growth factor- and anchorage-dependent assembly of cyclin/CDK complexes at the cell cycle restriction point is their TGFbRII- and p53-dependent disassembly at the cell cycle G1S checkpoint (figure 1.3). p53 trans activates or inhibits a vast array of cell cycle and apoptotic genes principal amongst which are the CDK inhibitor p21CIP1/WAF; the growth factor receptors IGF-1R and EGFR; and the pro-apoptotic bcl-2 antagonist Bax (Levine, A.J., 1997). P53 and TGFbRII/SMAD undergo mutational or expressive changes in many late stage colo-rectal tumours (Rodrigues, N.R., 1990),(Zhang, T., 1997).

Figure 1.3 The cell cycle



The cell cycle : mitogenic growth factors (via pathways 1 to 5 above) advance the cell cycle through G0G1 and the G1S restriction point (represented by arrows in green) whilst TGFβRII and p53 (via pathway 6 above) inhibit the cell cycle at the checkpoint (represented by an X in red).

Figure 1.4 RTK / IGF-1R signalling, cell cycle genes and cancer : Genes undergoing mostly expressive change in cancer are in green whilst the commoner oncogenes and tumour suppressors are in yellow.



1.4. Growth factor receptor signalling and colon carcinogenesis

Colorectal cancer studies over the last decade have provided some insights as to how extensive mutational and expressive changes are in these growth factor signalling pathways during carcinogenesis. At the level of the cell membrane, expressive changes in receptors are more commonplace than mutations but the mechanisms responsible for this are poorly understood. Colon cancers frequently over express EGFR, ERB2 (Shirai, H.,1995), PDGFR (Bellone, G.,1997), Met (Liu, C.,1992) and IGF-1R (Freier, S.,1999); and under express TGFβRII (Zhang, T., 1997) and E-cadherin (Ilyas, M., 1997) at different stages in their evolution. Similarly, at the level of the cytoplasmic signalling pathways - ras/raf-1/MAPK, PI3K/Akt, APC/β-catenin/Tcf and TGFβRII/SMAD - there are numerous mutations and expression changes at different stages of neoplasia. However, some of the downstream target genes of the RTKs and their signalling pathways - e.g. fos, cyclin D1 and bcl-2 - are over expressed from the early stages of tumour initiation (Arber, N.,1996), (Shpitz, B.,1999), (Sinicrope, F.A.,1995). The combination of mutations and expressive changes in the signalling pathways required to bring about such a change in gene expression in the early tumour cell are unknown. It also leads one to question what purpose later mutations in the upstream pathways serve? Here, experimental models provide a useful analogy. For example, in transgenic models the over expression of growth factors or their receptors results in the over expression of target genes, cell hyperplasia and disordered differentiation similar to that of tumour initiation (Westermark, B., 1991), (Di Fiore, P.P., 1987), (Li, M.,1992), (Pietrzkowski, Z.C.,1992), (Tsarfaty, L.,1994). On its own however this over expression of growth factors or their receptors is insufficient to confer the fully transformed phenotype. Indeed, the withdrawal of growth factors and cell matrix anchorage or p53/TGFβ stimulation at this stage will result in the terminal differentiation or apoptosis of these cells (Wylie, A.H., 1987). Tumour progression requires additional mutations in the cell's signalling pathways. Ras mutations have long been held up as an example of this, being able to

abrogate the requirement for some growth factor receptors such as EGFR but not for others such as IGF-1R (Falco, J.P.,1988). Equally, many other mutations in the signalling pathways described above are thought to further reduce the cancer cell's dependency on growth factor, cell anchorage and genomic integrity controls necessary for tumour progression. Of note however, in spite of multiple mutations and unlike other RTKs, many cancer phenotypes display a continued dependency on the IGF-1R and its signalling pathways (see below). In light of this, the association of its signalling pathways with oncogene / tumour suppressor mechanisms and its important role in controlling cell cycle entry and differentiation, the IGF-1R is an interesting model gene with which to examine the process of tumourigenesis.

1.5. The IGF system

The insulin-like growth factors (IGF-I, IGF-II), binding proteins (IGFBP1-10+) and receptors (IGF-1R, IGF-2R) comprise a highly conserved endocrine and autocrine signalling system which is utilised by most cell types. The insulin-like growth factor type 1 receptor has been shown to play key roles in embryology (Werner, H., 1989), (Liu, J.P., 1993); cell cycle restriction point progression (Stiles, C.D.,1979), (Baserga, R.,1993); cell survival under stress (Harrington, E.A.,1994), (Valentinis, B.,1999); cell differentiation (Rosenthal, S.,1995), (Jin, S., 2000) and cell adhesion (Mauro, 2004).

Prior studies have shown that embryonic stem cells express high levels of growth factor receptors such as the IGF-1R (Werner, H., 1989)(Macaulay, V.M.,1992). The expression levels of growth factor receptors in adult stem cells has not been examined, primarily because of the difficulty in identifying adult stem cell compartments (the colonic crypt is therefore an excellent model for examining stem cell maturatrion). Most adult differentiated cells express low levels of growth factor receptors but the timing and mechanisms underlying these changes in gene expression are unknown.

A role for the IGF-1R in cancer appears no less important. Many cancers have now been found to over express the IGF-1R or its ligands, IGF-1 and IGF-II (Macaulay, V.M., 1992), (Zhang, L., 1997). There is now extensive clinical and experimental data to indicate an important role for the IGF-1R in neoplasia of the breast (Surmacz, 2000), prostate (Djavan, 2001), colon (Giovannucci, 2001), lung (Wu, 2000), pancreas (Korc 1998), liver (Scharf, 2001), ovary (Druckmann, 2002), bladder (Hursting, 2001) and brain (Zumkeller, 1999). However, there is also evidence to show that the IGF-1R is not as highly expressed in more advanced cancers of the colo-rectum (Nakamura, M., 2004), breast (Schnarr, B., 2000)(Pennisi PA, 2002) and prostate (Hellawell, G., 2002). The reasons for this are unknown but the timing of these expressive changes in the IGF-1R during neoplasia of the colo-rectum are examined in this thesis.

Serum IGF levels have also been correlated with cancer incidences for colon (Klein, I., 1982), (Barzilay, J., 1991), (Ezzat, S., 1991), (Ron, E.,1991), (Ladas, S.D., 1994), (Ma, J., 1999), prostate (Grimberg, A.,1999) and lung cancers (Yu, H., 1999). A role for the IGF-1R is also evident in transgenic models of tumour initiation (Sell, C., 1993),(Christophori, G.,1994), (DeGiovanni, J., 2000). However, the role of the IGF-1R in human tumour initiation and progression has yet to be defined and is examined in this thesis.

1.6. The IGF-1R gene

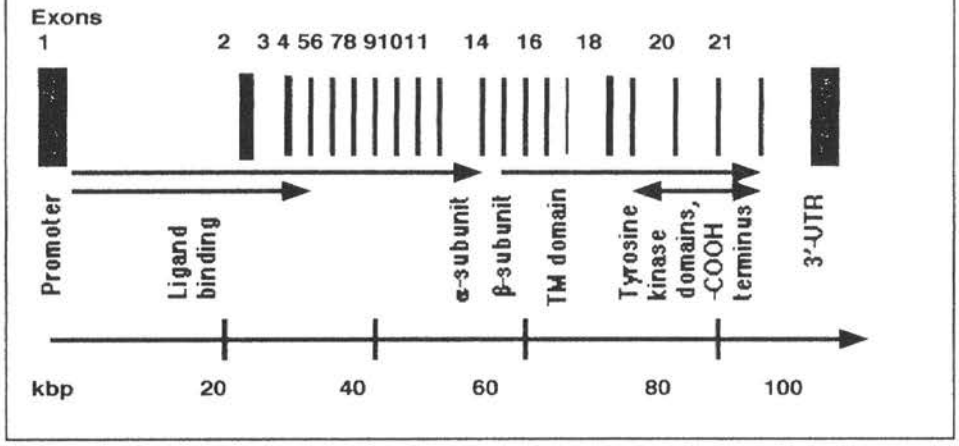
The IGF-1R gene was first cloned in 1986 by Ulrich and colleagues (Ullrich A, 1986). The gene maps to 15q25,26, has 21 exons and is more than 100 kb in length.

Exon 1 has a 1038-bp 5'-UTR with a single initiator (Abott, A.M., 1992). The α-subunit maps to exons 1-11 and the β-subunit maps to exons 12-21. There is a single identified splice-variant at the end of exon 14. Exon 21 has a >840 bp 3'-UTR.

There is considerable exon size and sequence homology with the insulin and insulin-related receptors. Sequence homology varies between 80-95% for exons 16-20 which encode the tyrosine kinase domain of the β-subunit; 48% for exons 1-4 encoding

the cys-rich ligand-binding domain of the α-subunit (which is also homologous in the EGFR and HER2); to 27% in exon 14 which encodes the trans membrane domain in the b-subunit. The C-terminal 108 aa hydrophilic tail of the β-subunit in exon 20 displays the greatest divergence.

Figure 1.5 The IGF-1R gene



1.7. IGF-1R transcripts

Given the sequence homologies between IGF-1R, IR and IRR, it is conceivable that these species might cross-hybridize with antisense IGF-1R probes. This was addressed (Ullrich, A., 1986) by sequential hybridisations of RNA from several tissues with an IGF-1R probe generated from a 2.8 kb EcoRI fragment of the IGF-1R sequence (exon 1-11) and compared with hybridisations with an IR probe. This demonstrated IGF-1R hybridisations with a major transcript at 11kbases and a minor transcript at 7 kbases whilst IR hybridisations were at 10.3, 9.6, 8.5 and 6.7 species in human placenta and were without any evidence of cross-hybridisation in either assay. Also, Southern hybridisations showed single bands after restriction digests so that these species were

derived from a single gene. The IGF-1R minor 7 kb transcripts occurred in placenta but not in other adult tissues.

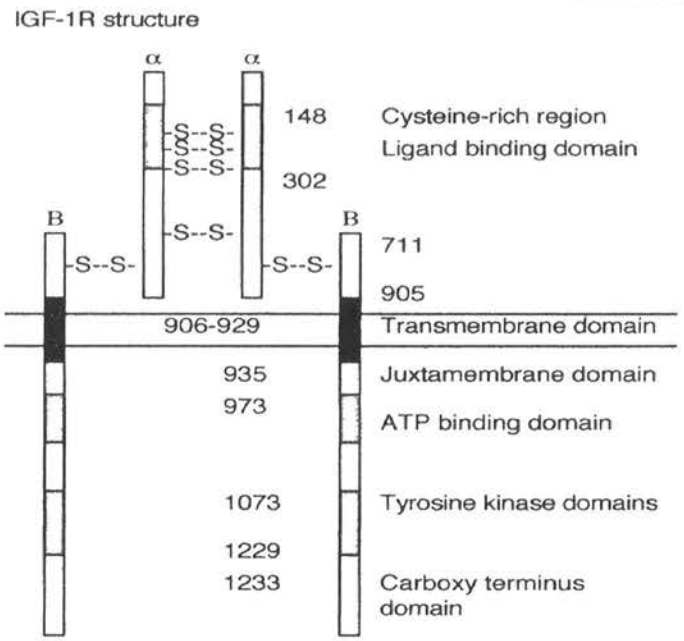
1.8. The IGF-1R peptide

A 4101 nucleotide reading frame translates a 1367 aa NH_2-signal peptide-α-subunit-β-subunit-COOH preproreceptor. Post translational cleavage and glycosylation results in subunits of ~135,000 M_r (α-subunit) and ~90,000 M_r (β-subunit). The IGF-1R is processed to exist as a 350kda $\alpha_2\beta_2$ heterotetramer with the α- and β-subunits attached by disulfide bonds (Grunfield, C.,1985).

The α-subunit region contains a 24-rich cys region from residues 148 to 302, representing the ligand-binding domain.

The β-subunit has an extracellular domain (residues 711-905), a 24 aa hydrophobic sequence (residues 906-929) representing the trans membrane domain, and a 407 aa cytoplasmic domain. Tyrosine kinase domains are located between residues 973 and 1229. A conformational change in these domains on ligand-binding results in ATP-binding at Gly976-Gly981 and lys1003 and the subsequent phosphorylation of residues tyr1131, tyr1135 and tyr1136. Each β-subunit then transphosphorylates the other leading to phosphorylation of tyr950 in the juxtamembrane region and tyr1250, tyr1251 and tyr1316 in the carboxyl-terminal domain.

Figure 1.6 The IGF-1R peptide



1.9. IGF-1R expression

The IGF-1R is expressed to varying degrees in all tissues and cells in culture. Its principal determinants of expression however appear to be :

a. Developmental

High IGF-1R levels are expressed in embryonic stem cells and these decline in the perinatal period to reach their lowest in most differentiated tissues. (Werner, H., 1989), (Chernausek, S.D.,1987). Most differentiated tissues such as the liver express low IGF-1R levels in the adult but some, such as kidney and muscle express higher levels.

b. Proliferation, differentiation and cell survival

IGF-1 receptors have been shown to increase in number between the G1 and S phases of the cell cycle in Burkitt lymphoma cells (Hartman W, 1988); to decrease in number during differentiation in 3T3 fibroblasts, CaCo-2 and HT29 colon carcinoma

cells (Modan-Moses, D.,1998), (Zarrilli, R., 1994), (Garrouste, L., 1997); and to transiently upregulate then undergo downregulation when neuronal cells are progressively exposed to apoptotic cell injury (Roschier, M., 2001). As in the embryonic stem cell, the IGF-1R appears to be an important component of the adult cell's control apparatus.

c. Hormonal regulation

Hormonal regulation also impacts upon IGF-1R expression. For example, physiological concentrations of oestradiol can increase MCF-7 breast cancer cells' IGF-1R expression and growth (Stewart, A.J., 1990) whilst progestins decrease IGF-1R and growth in another breast cancer cell line, T47D (Papa, V., 1991). In SH-SY5Y neuroblastoma cell lines, TPA can increase IGF-1R expression and neurite formation (Ota, A., 1989), whilst ovarian and cartilage cells increase their IGF-1R expression and growth in response to FSH and GH respectively (Hernandez, E.R., 1991), (Isaksson, O.G.P., 1991). These changes may be explained by the hormone-responsive regions of the IGF-1R promoter.

d. Growth factor regulation

PDGF and FGF strongly induce IGF-1R in 3T3 cells (Clemmons, K.R., 1980) and other mesenchyme-derived cells such as vascular smooth muscle cells (Ververis, J.J., 1993). This may reflect the roles of these receptors in the cell cycle as discussed previously. IGF-1 on the other hand can suppress IGF-1R expression in cell cultures including 3T3 fibroblasts, endothelial, lymphoid and thyroid cells (Rosenfield, R.G., 1980), (Rosenfield, R.G.,1982).

1.10. The IGF-1R promoter

The human IGF-1R promoter has been sequenced (Cooke, D.W., 1991) to the extent of its 480 bp 5'-flank and 1038-bp 5'-UTR. Both regions are GC-rich (75% and

68% respectively) with numerous Sp1 binding sites (-CCCGCC-) (Mitchell, P.J., 1989). There are no TATA or CCAAT elements. Instead a single initiator motif (5'-GCCCCCAGTGTGTGGCA-3') is thought to act in concert with the Sp1 sites to direct high levels of basal transcription as previously described elsewhere (Smale, S.T., 1989).

The GC-rich TATA-less promoter of the IGF-1R promoter bears some similarity in this respect to the promoters of housekeeping genes such as the insulin receptor (Seino S, 1989). However, unlike these promoters, it has a single initiator motif more commonly associated with the promoters of developmental and differentiation genes (Biggin, M.D.,1988), (Perkins, K.K.,1988). Additionally, the IGF-1R's long 5'-UTR is a feature shared by genes that regulate cell proliferation such as growth factors and several cellular oncogenes (Kozak, M., 1987).

The IGF-1R promoter exhibits high basal activity in reporter assays (Werner, H., 1992). As IGF-1R gene expression in most adult differentiated cells is low the promoter must be under some form of inhibitory control. In the rat IGF-1R gene, the -494/-331 fragment, which has four Sp1 sites, can have 90% activity. In contrast, the -331/-135 and -135/-26 fragments can each exhibit between 11% and 70% promoter activity depending on the transfected cell type. Thus, promoter activity is the result of combinatorial cis-elements of mostly Sp1 sites acting in the proximal 5'-flanking region. This activity can in turn be enhanced or inhibited by other trans-regulators in the 5'-flanking and 5'-UTR regions. For example, the 5'-flanking region has a potential binding site for the AP-2 transcription factor (5'-CCCCACGC-3') at positions -136 to -129 which may explain the responsiveness of the gene to cAMP (Adashi, E.Y., 1986), (Imagawa, M., 1987), phorbol esters (Ota, A., 1989), (Roesler, W.J., 1988) and FSH (Adashi, E.Y., 1986).

As well as the above cis-regulatory elements, the 5'-flanking region contains several growth factor-responsive regions. The -124 bp region of the 5'-flanking region proximal to the initiator has been found to be a PDGF- and SVLT-responsive element in

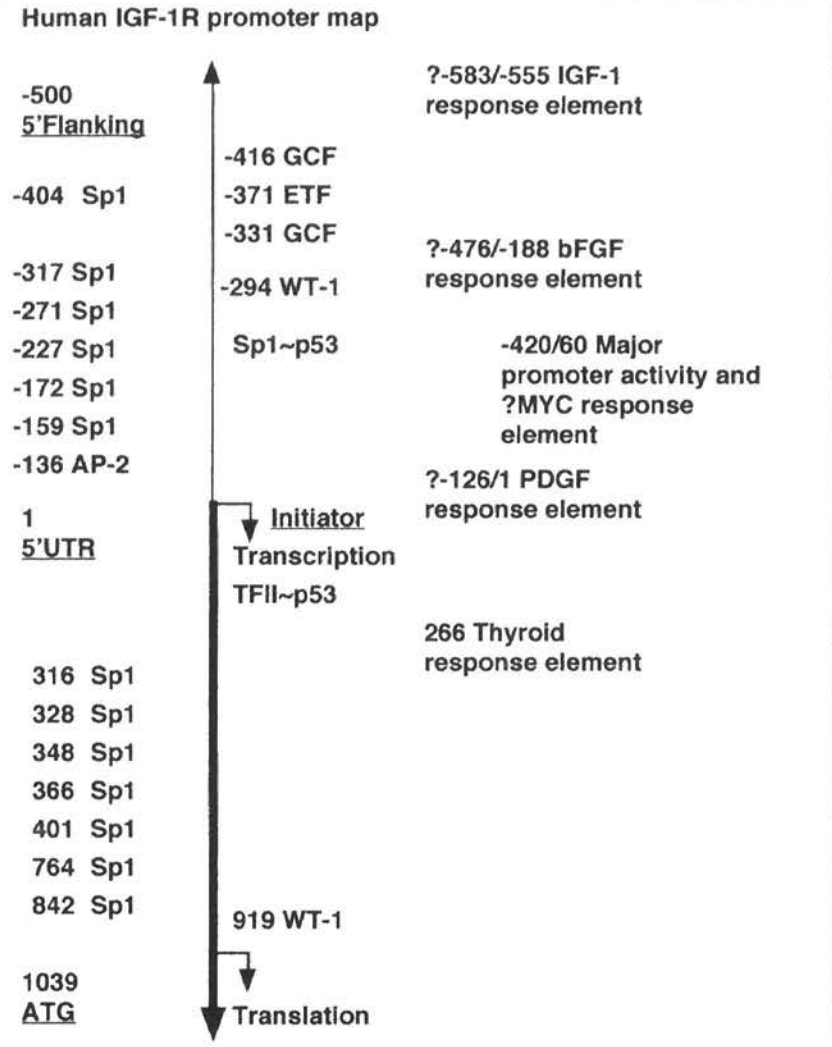
the rat IGF-1R promoter (Rubini, M., 1994). This site can complex E2F, cyclin A and p107 as well as Sp1.

C-myc is induced primarily by PDGF and it is notable that the IGF-1R promoter in the rat but not in the human contains a c-myc E-box domain (Halazonetis, T.D., 1991). Nevertheless, N-myc is able to trans-activate the proximal 5'-flank region at -420/+60 in the human IGF-1R promoter (Chambery, D., 1999) suggesting that some form of direct or indirect response element exists. The myc nuclear phosphoprotein also binds and activates other promoters containing E-box motifs along with cyclins A and E (Jansen-Durr, P., 1993). Its expression closely correlates with those genes expressed during embryogenesis and during proliferation (Mugrauer, G., 1991).

In addition to the above, a putative IGF-I-responsive element homologous to that in the elastin promoter also lies at -583/-555 (Wolfe, B.L., 1993). Although the mechanism of action of the above is not understood, they may represent a means of IGF-1R promoter autocrine feedback control.

The 5'-flank and 5'-UTR are targeted by the tumour suppressors WT-1 and p53. Wt-1 suppresses the promoter by binding the 5'-GCGGGGGCG-3' consensus sequence located at -294/-286 and 919/927 (Werner, H., 1994),(Werner, H., 1993). The WT-1 gene encodes a zinc-finger protein of the Cys_2-His_2 type with homology to the EGR-1 transcription factors (Gashler, A., 1995). It targets and inhibits the promoters of several growth factors and growth factor receptors including PDGF, IGF-II, TGFβ1, EGFR and IGF-1R and is itself induced during epithelial and embryonic stem cell differentiation (Scharnhorst, V.,1997). Wild type p53 can also suppress the IGF-1R promoter, possibly through complexing with and inhibiting the interaction of the TBP component of the TFIID transcription complex (Werner, H., 1996) or by complexing in a similar manner with Sp1 (Ohlsson, C., 1998). Like the myc oncogenes, the WT-1 and p53 may thus represent possible means for tumour cells to over express the IGF-1R.

Figure 1.7 Graphic of the human IGF-1R promoter



1.11. IGF-1R transcription and translation

Although its translative and post-translative control mechanisms are not understood, IGF-1R membrane peptide expression most closely correlates to transcription in all the studies where it has been assessed (Lowe, W.L., 1989); (Ota, A.,1989); (Ohlsson, C., 1998). In keeping with the majority of membrane receptors, most of the synthesised IGF-1R exists in the cytoplasmic pool and the dynamics of its processing between endoplasmic reticulum and membrane are again not understood.

1.12. Ligand binding of the IGF-1R

IGF-1 binds with high affinity the IGF-1R which has 2 to 3 times lower affinity for IGF-II and 100 times lower affinity for insulin (Czech, M.P., 1989). Ligand binding results in a conformational change in the catalytic loop domain of the tyrosine kinase region, ATP binding at Gly976-981/Lys1003 and subsequent phosphorylation of tyr1131, tyr1135 and tyr1136. This leads to autophosphorylation of the juxtamembrane tyr950 and carboxyl-terminal tyr1250,1251,1315 / ser1280-1283 domains. These phosphorylated residues provide docking sites for the primary substrates described above. Phosphorylation of the juxtamembrane domain also causes IGF-1Rs to aggregate and internalise within clathrin-coated micro vesicles. Although this function is not critical for some of the signalling pathways, it is critical for IGF-1R-dependent malignant transformation (Prager, D., 1994).

1.13. IGF-1R signalling

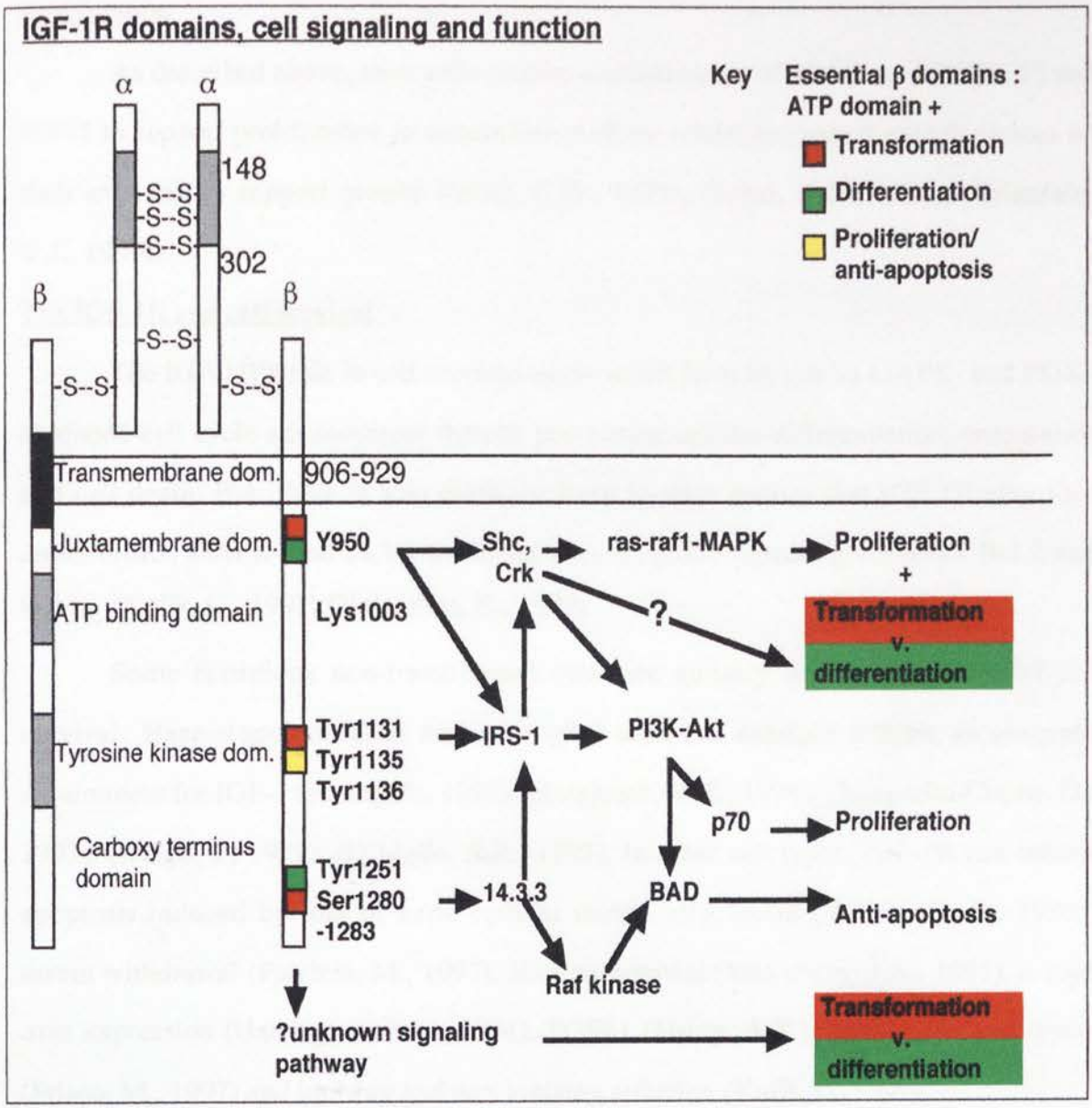
The IGF-1R is a powerful inducer of the classical ras/raf-1/MAPK, PI3K/Akt and β-Catenin/Tcf pathways (as described above). Shc, Crk and several isoforms of IRS bind via their SH2 domains to a NPXpY motif (that includes tyr950) in the juxtamembrane region of the β-subunit (Gustafson, T.A., 1995),(Beitner-Johnson, D., 1995). IRS also binds the tyrosine kinase domain tyr1131-1136. Once bound these proteins become phosphorylated by IGF-1R tyrosine kinases. Consequently, these then bind Grb2 (growth factor receptor-bound protein 2) which then binds mSOS (the mammalian Son of Sevenless)(Pruett, W., 1995), (Tanaka, S., 1994). This then loads GTP onto Ras protein G and hence activates the Ras/Raf/MAPK pathway. IRS also binds the p85 subunit of phosphoinositide 3'-kinase (PI3K) via its SH3 domain and thus can activate the PI3K/Akt pathway (Kulik, G., 1997) whilst other pathways such as β-Catenin/Tcf have been shown to be secondarily recruited through signalling intermediaries such as GSK3β (Playford,

M.P., 2000). Consequently, IGF-1R signal transduction through the MAPK, PI3K and β-Catenin/Tcf cytoplasmic cascades can have diverse effects from the trans activation of growth response genes such as myc, fos, jun and cyclin D1 (Rosenzweig, S.A., 1993), (He T.-C., 1998), (Rosenthal, S.M., 1995), (Tetsu, O.,1999) to the regulation of pro- and anti apoptotic cytosolic substrates such as BAD and Bcl-2 (Datta, S.R., 1997). Although these pathways might explain the proliferative and anti-apoptotic activities of the IGF-1R, they do not appear to fully explain the IGF-1R's role in differentiation and cell transformation. Evidence supporting this has come from mutational analysis.

1.14. IGF-1R mutational analysis

Mutational analysis has shown the domains of the IGF-1R are functionally distinct. The functions of mitogenesis and anti-apoptosis can be shared by the tyrosine kinase domain (Gronborg, M., 1993), (Li, S., 1994) whilst the juxtamembrane and c-terminus domains of the IGF-1R are obligate for cell differentiation and transformation (Surmacz, E., 1995), (Valentinis, B., 1999). An unidentified mechanism and/ or substrate-binding involving the juxtamembrane domain and the sequence 1245-1310 of the c-terminus are required for cell differentiation and transformation. The signal peptide14.3.3 can bind the c-terminus at ser1280,1,2,3 and can also bind to IRS-1 (Craparo, A., 1997). Although it can signal via IRS and BAD, much of its downstream signalling has yet to be elucidated. Whether it can explain the differentiating and transforming functions of the IGF-1R is unknown. Potentially interfering with this part of IGF-1R signalling may have significant effects upon differentiation and cell transformation without affecting cell proliferation.

Figure 1.8 IGF-1R mutational analysis and mitogenesis / apoptosis vs. differentiation / transformation functions



1.15. The IGF-1R and cell function

Proliferation

As described above, most cells require a combination of PDGF (or EGF/FGF) and IGF-1 to support proliferation in serum-free medium whilst individual growth factors on their own fail to support growth (Stiles, C.D., 1979), (Scher, C.D., 1979), (Cristifalo, V.J., 1989).

The IGF-1R and cell survival

The IGF-1R's role in cell survival might result from its role in MAPK- and PI3K-mediated cell cycle advancement thereby preventing cellular differentiation, senescence and cell death. But, there is also evidence from in vitro studies that IGF-1R may also direct PI3K-, MAPK- and 14.3.3-mediated anti-apoptotic signalling via BAD, Bcl-2 and Bcl-x_L (Kulik, G., 1997) (Valentinis, B., 1999)

Some fastidious non-transformed cells are entirely dependent on IGF-1 for survival. Haematopoietic cells and neuroglial cells for example exhibit an absolute requirement for IGF-1 (Huang, S., 1992), (Ratajczak, M.Z., 1994), (Bozyczko-Coyne, D., 1993), (Drago, J., 1991), (D'Mello, S.R., 1993). In other cell types, IGF-1R can inhibit apoptosis induced by loss of extra-cellular matrix attachment (Valentinis, B., 1999), serum withdrawal (Parrizas, M., 1997), IL-3 withdrawal (McCubrey, J.A., 1991), c-myc over expression (Harrington, E.A., 1994), TGFβ1 (Hsing, A.Y., 1996) , p53 activation (Prisco, M., 1997) and ionising and non-ionising radiation (Kulik, G.,1997).

Although cells require PDGF/EGF and IGF-1 for proliferation, G0 cells are largely dependent on a functional IGF-1R for survival in the face of cell stress. For example, whilst IGF-1 can rescue 3T3 cells from c-myc induced apoptosis, PDGF is less effective and EGF is ineffective (Harrington, E.A., 1994). This difference appears more pronounced if the cell has been previously transformed or stressed in some manner. For example, only IGF-1 can acutely prevent apoptosis of BALB/c3T3 cells in serum-free conditions (Tamm, I., 1990).

The IGF-1R and differentiation

Differentiation appears to take some of its cues from the cell cycle and the IGF-1R. IGF-1R signalling via the oncogenic homologues Rb and Crk has been implicated in differentiation (Rosenthal, S.M., 1995), (Jin, S., 2000). In other models of differentiation, including those of the 3T3-L1 preadipocyte (Student, A.K., 1980), L6E9 skeletal myoblasts (Rosenthal, S.M., 1995) and HT29/CaCo-2 colon carcinoma cells (Garrouste, F., 1997)(Augernon, C., 1984), differentiation in some cellular contexts can be induced by IGF-1. In these models, IGF-1 can initially cause confluent G0 growth arrested cells to re-enter G1S and undergo on average a further two rounds of mitosis; a process known as "mitotic clonal expansion" (Bernlohr, D.A., 1985). The resulting DNA replication and chromatin remodelling are thought necessary to render nucleosomal DNA and the appropriate promoter elements accessible to the transcription of differentiation genes. These transcripts and the ensuing events of differentiation are poorly understood (Tontonoz, P., 1994) (Hwang, C.S., 1996). It is recognised however that differentiation is synchronised with a post-clonal expansion downregulation in IGF-1R (Garrouste, F., 1997) as one might expect from the embryology studies. Once differentiated, normal cell lines are unable to re-enter the cell cycle by presumably being unable to re-express the genes (such as the IGF-1R) that are necessary for this. In contrast, transformed cells such as HT29 and Caco-2 cells can, on occasion, re-enter the cell cycle after repassaging from the differentiated state although interestingly they initially loose their ability to survive as inoculates in vivo during the early stages of this process (Augernon, C., 1984). In other words, differentiated tumoural cells can undergo clonal expansion but it may require several rounds of mitosis, if at all, before they can properly re-express cell survival genes such as IGF-1R.

The IGF-1R and cell adhesion

Recent reports have increasingly implicated a role for the IGF-1R in cell adhesion as discussed above (Mauro, 2004). The internalised IGF-1R forms a tripartite complex

with β-catenin and E-cadhern to effectively reduce membrane E-cadherin but also to control gene transcription of myc. The details of this mechanism and some of the other genes controlled have still to be elucidated but it is becoming clear from in vitro and in vivo studies of human colon cancer cell lines and human colon cancers that a reduction in IGF-1R expression predicts a loss of cell adhesion and metastasis repectively (Playford, 2000),(Nakamura, 2004).

The IGF-1R and transformation

It has long been recognised that over expression of growth factor receptors can result in cellular transformation with growth supported solely by the respective ligands. This has been shown to be true for PDGFR (Fantl, W.J., 1989), (Westermark, B., 1991); EGFR (Di Fiore, P.P., 1987), (Pietrzkowski, Z., 1992), (Velu, T.J., 1989); FGFR (Li, M., 1992); HGFR met (Tsarfaty, I., 1994); IGF-1R (Kaleko, M., 1990),(McCubey, J.A., 1991), (Pietrzkowski, Z., 1992); and insulin receptors (Randazzo, P.A., 1990). However, receptor knockout studies reveal some interesting differences between receptor subtypes. IGF-1R knockout cells are completely resistant to transformation by oncogenes: SV40T, Ras, Raf, v-Src and BPV E5 (Sell, C., 1994), (Coppola, D., 1994),(Valentinis, B., 1997) as well as by the over expressed EGFR, EGF, PDGFR, PDGF and insulin receptors (Coppola, D., 1994), (DeAngelis, T., 1995), (Reviewed- Baserga, R.,1995). In contrast, other receptor knockout cells such as those for the EGFR can undergo transformation by say, ras oncogenes (Hansen, L.A., 2000), (Sibilia, M., 2000).

1.16. The IGF-1R and animal models

Transgenic studies provide some clues to the role of the IGF axis in embryology and transformation. Deletion of the IGF-1R in mice by homologous recombination results in embryos which are 45% the size of wild type embryos and are nonviable at birth with defects noted during the later stages of differentiation of the nervous system, skin and bones (Baker, J., 1993), (Liu, J.P., 1993). These knockout cells, although able to grow,

are entirely resistant to transformation. EGFR knockout (-/-) mice in contrast are born with immature epithelium of the gut, liver and pancreas and succumb to necrotising enterocolitis within the first few days of life (Miettinen, P.J., 1995). However, cells derived from these embryos are able to undergo malignant transformation.

Over expression animal studies are very much limited by the promoter and the relevant target tissue. For example, over expression of IGF-1 driven by a bovine keratin promoter in transgenic mice results in tumours of the skin and prostate where the transgene is expressed (DiGiovanni, J., 2000). Over expression of IGF-II driven by the MUP promoter in transgenic mice results in the frequent development of tumours by 18 months of age- notably hepatocellular carcinomas and lymphomas (Rogler, C.E., 1994). Similarly in SV40T-transgenic mice (SV40T is known to trans activate the IGF-1R and IGF-II promoters) islet cell hyper proliferation and islets tumours correlate with IGF-II over expression (Chrisofori, G., 1994). An IGF-1R over expression model has not been developed but all the indications are that the IGF-1R has a perceived role in co-ordinating proliferation and differentiation in the embryo.

1.17. An accounting

The pertinent points from the above discussion can be summarised :

1. The IGF-1R is highly expressed in embryonic stem cells but is expressed at low levels in most (but not all) adult differentiated tissues. Most cancers highly express the IGF-1R but some advanced cancers express comparatively lower levels. The reasons for these changes in IGF-1R expression are unclear. Expressive or mutational changes in p53, WT-1 and myc have been suggested as possible reasons for changes in IGF-1R transcription in some cancers.

2. The IGF-1R signalling cascade is an important determinant of cell cycle G1S progression, cell differentiation, cell survival and cell adhesion. Components of this (and other RTK) signalling cascades are targeted by cancer mutations.

3. IGF-1R functionality is a requisite for experimental cell transformation.

4. The colon crypt-cancer model is ideally suited to examine the temporal expression of genes during the evolution of cancer.

1.18. Prior studies

Ligand binding studies had initially demonstrated the presence of IGF-1 receptors with saturable kinetics and with the correct molecular weight in both normal (Pillion, D.J., 1989), (Rouyer-Fessard, C., 1990) and tumoural colonic mucosa specimens (Guo, Y.-S., 1992). However, the variances arising from the membrane microsomal preparation technique means that direct comparisons between studies for normal and tumoural specimens has not been possible.

Comparison of different ligand binding studies (Guo, Pillion, Rouyer-Fesard)

Scatchard analysis

	Kd(nmol/L)	Bmax (x10^5 sites/cell)
HCT-116+	1.45+/-0.2	1.5+/-0.12
Colo-205+	1.07+/-0.02	1.96+/-0.02
Cancer μsomes+	0.12+/-0.02	0.25+/-0.03
Epithelial μsomes*	1.7	
Placental μsomes*	2.3	
Epithelial μsomes$	8.6+/-1.0	

+,*,$ These assays were from different studies with different microsomal preparation techniques and demonstrated considerable inter-study variability in their Kds for colonic epithelial microsomal preparations.

+(Guo Y-S, Narayan S, Yallampalli C & Singh P., 1992)

*(Pillion D, Haskell JF, Aitchison J, Ganapathy V & Leibach FH., 1989)

$(Rouyer-Fessard C, Gammeltoft S, Laburthe M.,1990)

Also, and significantly, tissue dissection with ligand binding (Laburthe, M., 1988) and autoradiographic mapping (Heinz-Erian, P., 1991) have demonstrated IGF-1R

expression in the basal proliferative crypt as opposed to the upper differentiated crypt in the rat gastrointestinal epithelium.

Ligand binding studies on colon cancer cell lines have also been revealing. Here it has been found that different cell lines express different levels of the IGF-1R (see above). For example, HT-29 cells which have p53 and APC mutations have been found to express lower levels of IGF-1R in comparison to c10 cells which have wild type APC and β-catenin (Playford, M.P., 2000). However, as cancer cell lines frequently accrue diverse mutations during culture and mutational screening of these cell lines is far from complete, such comparisons must be treated with caution. Much more consistent is the finding that differentiation in HT29 and Caco-2 cells is also accompanied by reduced expression of the IGF-1R and a loss of tumourigenicity in vivo (Garrouste, F., 1997), (Remacle-Bonnet, M.M., 1992), (Augernon, C., 1984), (Zarilli, R., 1996). However there are fundamental difficulties in comparing these in vitro studies to human tissues – both show diverse cell heterogeneities and differences in cellular environment and there are practical difficulties in culturing normal enterocytes.

The IGF-1R has been assayed in several tissue-based studies comparing normal and tumoural colonic mucosa. IGF-1R RNA analysis presents difficulties because of the low copy number and high molecular weight of transcripts. Zenilman and Graham (Zenilman, M.E.,1997) assayed IGF-1R mRNA by means of a quantitative PCR but were unable to find any difference between normal and tumoural mucosa given the limitations of the assay. Freier and Raz (Freier, S., 1999) in contrast used RNAse protection assays to determine that IGF-1R and IGF-II were over expressed in 6 colon cancers in comparison to normal epithelium. These studies do not of course examine IGF-1R expression in a cell specific manner and take no account of the heterogeneous nature of tissues. With the development of antibodies to the IGF-1R, Hakam and Coppola (Hakam, A., 1999) found that 8/12 adenomas, 34/36 carcinomas and no normal (differentiated) mucosal specimens stained positively for the IGF-1R. But, how IGF-1R expression might

be related to the processes of tumour initiation and progression was not addressed in this study.

1.19. The hypothesis and aims

Given the foregoing discussion, we are led to ask when and how the IGF-1R is highly expressed in colo-rectal neoplasia ? If high IGF-1R expression is observed at a particular stage in neoplasia, then is this as a consequence of a recognised trans regulator or some other process ?

The primary aim of this thesis is therefore to examine the expression profile of the IGF-1R in the colo-rectal polyp-cancer sequence to elucidate the timing of high IGF-1R expression during early neoplasia and also to elucidate if a change in IGF-1R expression occurs during later stage neoplasia. A secondary aim is to examine the expression of IGF-1R trans regulators to determine if these or the pattern of IGF-1R expression in the crypt-cancer sequence might infer the mechanisms of IGF-1R transcript control.

CHAPTER 2

MATERIALS AND METHODS

2.1. Laboratory facilities

The main body of the experimental work was conducted in three departments of the University of Edinburgh on site at the Western General Hospital, Crewe Road, Edinburgh :

1. The department of Surgical Oncology, growth factor laboratory under the supervision of Dr. Fouad K. Habib and Professor Colin S. McArdle;
2. The department of Molecular Medicine under the supervision of Dr. Karen Chapman and Professor Johnathon Seckle;
3. The department of Pathology under the supervision of Dr. Margaret MacIntyre.

2.2. Materials

Tissue specimens

Fresh colo-rectal resection specimens were obtained from the theatre lists of Mr. Graeme Wilson, Mr. Malcolm Dunlop and Mr. David Hamer Hodges at the Western General Hospital. The specimens were processed fresh in the department of Pathology at the Western General Hospital by Dr. Margaret MacIntyre. Additionally, some fresh and archival specimens were also obtained from Mr. David Bartolo, department of Surgery at the Royal Infirmary and Dr. Hugh Gilmour and Dr. Juan Piris, department of Pathology at the Royal Infirmary of Edinburgh.

General consumables

The majority of chemicals used in preparative work were of molecular biology grade and were purchased from Sigma Aldrich. Molecular biology reagents were almost exclusively purchased from Promega. Nuclease-free Eppendorf tubes were purchased from Sarstedt Ltd., Leicester.

Molecular biology

The plasmid pGEM-T.Easy used to clone the IGF-1R construct was purchased from Promega, UK. The IGF-1R primers were synthesised by Oligonucleotide Synthesis Service, ICRF Clare Hall Laboratories, Herts.

pTri-c-myc and pTri-β-actin (Ambion, Austin, Texas) were purchased from AMS Biotechnology, Witney, Oxfordshire.

pGem-3Z-WT-1 construct was a kind gift from Dr. David Housman, Massachusetts Institute of Technology.

Anti-human IGF-1R α-subunit IgY antibody and a-subunit synthetic peptides (Upstate Biotechnology Inc, Lake Placid, New York) were purchased from TCS Biologicals Ltd., Botolph Claydon, Buckingham. Anti-IGF-1R mouse IgG monoclonal (Oncogene Research Products, Cambridge, Massachusetts) was purchased from Calbiochem-Novabiochem, Beeston, Nottingham.

2.3. Methods - Tissue specimens and preparation

Fresh resective specimens were transported from the operating theatre to the pathology laboratory on water ice where they were processed within half an hour of resection by the pathologist.

Colonic resected specimens were washed, opened and pinned out. Between 1 to 2g of the exophytic luminal aspect of the cancer specimen was taken and washed in ice-cold saline. Separate specimens were then snap frozen and subsequently stored in liquid nitrogen for RNA and protein analysis or placed in 10% formaldehyde for histological analysis or further washed in saline and placed in RPMI supplemented growth medium for primary culture. Rectal cancers were treated separately by harvesting from within the lumen prior to fixation and coronal sectioning of the main specimen. Polyp specimens when pedunculated were taken from the aspect distant to the stalk. Normal colonic mucosa at least 10cm proximal or distal to the tumoural specimen was dissected free from the underlying muscularis mucosa, and then treated similarly to the tumoural specimens.

Archival specimens from the previous 6 months were sourced from the catalogues of the departments of Pathology at the Western General Hospital and the Royal Infirmary of Edinburgh.

2.4. RNA analysis - Isolation of RNA from tissue specimens

Total RNA was isolated from gross tissue specimens using a modification of the acid-guanidium- phenol-chloroform method of Chomczynski and Sacchi (1987). In order to maintain RNA integrity, all solutions and glassware were prepared by treating overnight with 0.05% diethyl pyrocarbonate (DEPC) and then autoclaving for 15 minutes at 15lb/sq.in. on the liquid cycle.

Up to 1g of snap frozen tissue was used as per the following protocol. The frozen tissue was powderised using a Mikro-Dismembrator II (B. Braun Biotech International GmbH, Melsungen, Germany) fitted with a 7ml teflon flask and stainless steel grinding ball, both pre-chilled in liquid nitrogen. The specimen was disrupted at full power for 15 seconds. The resulting powder was then homogenised (Brinkman, Polytron) for 15 seconds and lysed on ice for a further 10 minutes in 12ml of freshly prepared ice-cold GTC extraction buffer in a 50ml Corning centrifuge flask.

GTC buffer: 4M guanidium isothiocyanate
25mM sodium citrate, pH7.0
2% mercaptoethanol

1.2 ml of 2M sodium acetate, pH4, was then added to the crude homogenate and mixed. 12 ml of phenol-chloroform-isoamyl alcohol (PC) were then added, mixed by vortexing and chilled on ice for a further 15 minutes.

PC solution : 10ml water saturated phenol, pH 4
2 ml chloroform-isoamylalcohol (49:1)

The suspension was then centrifuged at 10,000 r.p.m for 20 minutes at 4^{O}C with a swinging bucket rotor. The top aqueous phase was then transferred to Eppendorf tubes

where it was mixed with an equal volume of Isopropanol and incubated overnight at -20°C to precipitate the RNA. This was pelleted by microcentrifugation at 13,000 r.p.m for 20 minutes at 4°C. The pellets were resuspended in 100 µl of GTC buffer, the samples pooled and reprecipitated with another equal volume of Isopropanol for at least 2 hours at –20 °C. The sample was then centrifuged at 13,000 r.p.m. for 10 minutes at 4°C and the RNA pellet then washed twice in 500 µl of 75% ethanol and respun at 13,000 r.p.m at 4°C for 5 minutes. The supernatant was then aspirated and the RNA pellet air dried for 30 minutes at room temperature and then suspended in 100-500 µl of nuclease-free water.

The RNA yield was determined spectrophotometrically by diluting a 5µl RNA sample aliquot in 4.995 ml of DEPC treated water. An OD_{260} of 1=40 µg/ml whilst an OD_{260}/OD_{280} ratio of 2 corresponds to a pure sample. If the ratio was less than 1.7 then the RNA was re-extracted with phenol-chloroform.

2.5. RNA analysis - mRNA purification, reverse transcription and polymerase chain reaction

The basic polymerase chain reaction is an adaptation of that first described by Saiki and is protocolled in the Promega Protocols and Applications Guide (3rd Edition, 1996). Because of the low copy number of the IGF-1R gene in normal colonic epithelium, it became necessary to mRNA purify the samples and employ high cycle numbers, limiting the reproducibility of quantitative PCR.

mRNA purification

A commercial system comprising biotinylated oligo(dT) isolation with streptavadin/paramagnetic capture was utilised (PolyATract mRNA isolation System I, Promega, UK).

2mg of total RNA was made-up to a total volume of 2.43ml in nuclease-free water and heated to 65°C for 10 minutes. To this was added 10µl of biotinylated-oligo(dT) probe and 60µl of 20X SSC (to make a 0.5X SSC solution) and the solution allowed to cool to room temperature. The annealed mixture was then added to 0.5ml of streptavadin-paramagnetic particles previously washed and suspended in 0.5X SSC. This

was incubated at room temperature for 10 minutes and the particles captured then washed four times in 1.5ml of 0.1X SSC. The mRNA was then eluted in nuclease-free water and the concentration and purity determined by spectrophotometry.

Reverse transcription

This protocol for cDNA synthesis is similarly a modification of that described in the Promega Protocols and Applications Guide. 1μg of mRNA was diluted in nuclease-free water up to 5.4μl and heated to 70°C for 5 minutes before cooling on ice and pulsing in a microfuge ready to be loaded into the following reaction mixture:

RT mix : 4μl of 25mM $MgCl_2$ (previously heated to 65 °C)
2μl of 10X reverse transcription buffer (heated to 37 °C)
2μl of 10mM dNTP mix
2μl of rRNasin ribonuclease inhibitor (40 unitsμl-1)
1μl of oligo(dT)15 (0.5μgμl-1)
0.6μl of AMV reverse transcriptase (25unitsμl-1)
(negative control with no RT)
total volume = 20μl

The tube was pulse vortexed and microfuged. First strand cDNA synthesis and amplification proceeded by incubating at 42 °C for 1 hour. The RT was then denatured by heating to 99 °C for 5 minutes and this was followed by 5 minutes on ice.

PCR amplification of IGF-1R cDNA sequences

IGF-1R cDNA was analysed using the following primers locating to and spanning the annotated exons :

Primers : IGF-1Rsense 5'ACC CTT GAT TCT GTT ACT TC3'
IGF-1Ranti-sense AAT TCT TAC AGT GTC TCA TA
Product : 596 base pair, 1063-1659 of IGF-1R (exons 4-8 of α-subunit)

Reactions were performed in a Hybaid thermal reactor using Promega *Tfl* Taq DNA polymerase.

PCR mix : 20µl of cDNA (final [ ]<10ngml^{-1})
10µl of sense primer (50ngml^{-1})(final [ ]=1mM)
10µl of antisense primer
6µl of MgCl2 (final [ ]=1.5mM)
10µl of 10X reaction buffer
2µl of dNTP mix
1µl of Taq DNA polymerase (final [ ]=0.025uml^{-1})
total volume = 100µl

PCR cycles : 40x
Melt 94 ^{o}C, 1minute
Anneal 52 ^{o}C, 1 minute
Extend 72 ^{o}C, 2 minutes

The products were then run on a 2% agarose gel prepared by melting 1.5g of Agarose NA (Pharmacia) in 75ml of 1X TBE. The molten agarose was cooled to ~40 ^{o}C before adding 1µl of 10mgml^{-1} of ethidium bromide and pouring into a sealed mini-gel tray with a 1.5 mm 16 well comb. Once set, this was placed into the submarine mini-gel apparatus (Northumbria Biologicals Ltd.) and covered with 750ml of 1X TBE. 18µl reaction aliquots mixed with 2µl of DNA loading buffer (Sigma) were loaded alongside 20µl of 100bp ladder (Promega) and the gel run at 50 volts for 3-4 hours before photographing under UV transillumination.

5X TBE : 5.4% Tris-base
2.75% boric acid
10mM EDTA, pH 8.0

2.6. RNA analysis - Riboprobe manufacture

Fresh PCR products were TA cloned into the plasmid vector pGEM-T Easy (Promega) as described by Storts and protocolled in the Promega Vector Systems Technical Manual. This vector has single 3'-T overhangs at the insertion site which lies within a β-galactosidase coding region and is flanked by dual transcription / sequencing start sites. Transformants are thus blue/white colour screened. Inserts were screened by restriction analysis and sequencing prior to riboprobe synthesis.

DNA purification from gels

A silica gel based spin recovery kit (Hybaid Recovery TM) was used for the purification of IGF-1R cDNA PCR product from 0.8% low melting point agarose gels. Briefly, the correctly sized PCR product derived from normal epithelium template cDNA was cut from the transilluminated gel with a fresh blade and then heated to 55 ^{O}C for 5 minutes in 400μl of reconstituted silica gel in binding buffer in a spin filter tube (Hybaid Recovery TM). The filter was spun for 30 seconds and then washed twice with dilute ethanol/wash solution before spinning dry. The DNA was then recovered by elution with nuclease-free water and its concentration determined by spectrophotometry

TA cloning of PCR product

Insert-vector ligations were set up as below and run overnight at 4 ^{O}C :

1μl of T4 DNA ligase 10X buffer

1μl of pGEM-T Easy vector (50ng)

10ng of PCR product (~1:1 vector:0.5kb insert ratio)

1μl T4 DNA ligase (3 Weiss units μl^{-1})

nuclease-free water to a total volume of 10μl

T4 DNA ligase buffer : 300mM Tris-HCl, pH 7.8

100mM MgCl2

100mM Dithiothreitol

10mM ATP

Transformations using the vector ligation product

JM109 (Promega) high efficiency competent cells (stored at -70 ^{o}C) were used with selection on LB/ampicillin/IPTG/X-Gal plates. Initially, the competent cells were gently thawed for 5 minutes in an ice bath with gentle mixing by hand. 50µl aliquots of these cells were then transferred into sterile pre-chilled 15ml Falcon tubes and to these were added 2µl of the above ligation reactions. A control tube containing 0.1ng of uncut plasmid instead was also prepared to determine the transformation efficiency. The tubes were gently flicked to mix then placed on ice for 20 minutes. The cells were then heat shocked by placing in a water bath at exactly 42 ^{o}C for 45 seconds and then returned to ice for a further 2 minutes. 950µl of room temperature SOC medium was then added to the tubes which were incubated at 37 ^{o}C for 1.5 hours in a rotary incubator at 150 r.p.m.

100µl of these transfectant cultures were plated onto duplicate LB agar plates previously supplemented with 50µgml-1 of ampicillin and 40µl each of 100mM IPTG and 40mg/ml X-Gal. Once the culture liquid had absorbed, the plates were inverted and incubated for 24-48 hours at 37 ^{o}C after which they were shifted to 4 ^{o}C for 2 hours to allow for proper colour development.

Luria-Bertani medium :	1%	Bacto-Tryptone
	0.5%	Bacto-Yeast extract
	0.17M	NaCl, pH 7.0

SOC medium :	2%	Bacto-Tryptone
	0.5%	Bacto-Yeast extract
	10mM	NaCl
	2.5mM	KCl
	20mM	$MgCl_2$/MgSO4
	20mM	glucose, filter-sterilized, pH 7.0

LB/amp/IPTP/X-Gal plates: To 1L of LB add 15g agar, cool to 50 ^{o}C, add ampicillin to 50µgml^{-1}, IPTG to 0.5mM and X-Gal to 80µgml^{-1}(or add as above) and pour.

Isolation of recombinant plasmid DNA

Transformants were screened with mini-preps and restriction analysis. Once screened, maxi-preps and sequence analysis were performed. All cultures were supplemented with ampicillin to maintain the selection of transformed bacteria. The alkaline cell lysis technique is a modification of that described by Maniatis et al (1989; 1.38-1.39).

The mini-prep protocol is a modification of the method adopted by Birnboim and Doly (1979). A single white colony was inoculated into 5ml of ampicillin-supplemented LB medium in a sterile 15ml Falcon tube using a sterile loop. This was incubated overnight at 37 ^{o}C in a rotary incubator at 225 r.p.m. A satisfactory bloom should achieve an OD 600 of ~600. 1.5 ml of this culture was then pelleted in a centrifuge at 12,000xg with the remaining culture stored at 4^{o}C. The bacterial pellet was then resuspended in 100µl of ice-cold GTE and lysed with 200µl of freshly prepared 0.2M NaOH, 1% SDS with a 5 minute incubation on ice. This lysate was then neutralised with 150µl of ice-cold 5M potassium acetate solution. The resulting flocculate was then centrifuged at 12,000xg for 5minutes and the clear supernatant then taken and treated with 0.5µl of 100µgµl-1 DNase-free RNase A by incubating for 5minutes at room temperature. DNA was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) with a 1minute vortex and a 5minute 12,000xg centrifugation. The upper aqueous phase was transferred to a fresh tube, an equal volume of chloroform:isoamyl alcohol (24:1) added and the tube revortexed and centrifuged. The DNA in this aqueous phase was precipitated with 2.5 volumes of ice-cold 100% ethanol for 5minutes on dry ice. The DNA was pelleted by centrifugation, rinsed with ice-cold 70% ethanol before repelleting and drying in a spinvac. The pellet was then dissolved in nuclease-free water, ready for restriction analysis gel electrophoresis.

GTE : 50mM glucose
25mM Tris-Cl
10mM EDTA, pH8.0

Alkaline SDS :	0.2N NaOH
	1% sodium dodecylsulphate
Potassium acetate solution:	5M K acetate (60mls)
(100 mls)	glacial acetic acid (11.5mls)
	DEPC treated water (28.5mls)

Maxi-preps involved incubating to 15ml those transfectants selected from the mini-prep run and restriction analysis. These cultures were further incubated in 500ml of LB medium with ampicillin in 2L flasks overnight at 225 r.p.m in an incubator-shaker. Satisfactory late-log-phase growths were harvested by pelleting the bacteria in 250ml Nalgene polycarbonate centrifuge pots spun at 6000 r.p.m. and 4 ^{o}C for 5 minutes in a Sorvall GSA rotor. As before, each pellet was resuspended in GTE (12mls) before lysing in alkaline SDS (24mls) for 5 minutes and neutralising in 5M potassium acetate solution (16mls) with all reactions on ice. After a 10 minute 6,000 r.p.m spin, the supernatants were filtered through several layers of gauze into fresh pots then mixed with 0.6 volume of isopropanol (32mls) and left at room temperature for 30 minutes. The mixed precipitate was then pelleted by centrifugation at 10,000 r.p.m. for 3 minutes. This pellet was then dried and resuspended in 2.2ml of Tris-EDTA buffer (10mM Tris, 1mM EDTA, pH8.0).

Plasmid DNA was purified by Cs-gradient centrifugation. 2.7g of CsCl were dissolved in the plasmid preparation (final concentration 1gml-1) along with a 100μl aliquot of 10mgml-1 ethidium bromide. This solution was then placed and sealed in a Beckmann Quickseal tube and spun at 100,000 r.p.m for 4 hours at 22 ^{o}C in a Beckmann Optima Ultracentrifuge TLX with a TLA 100-3 rotor. The lower band of plasmid DNA was taken off and then rebanded as above.

Following this, the ethidium bromide was removed by adding a 0.6 volume of isopropanol and centrifuging the mixture at 1,500 r.p.m. for 3 minutes, to then recover the lower aqueous phase containing the plasmid DNA. This was repeated until the pink stain of ethidium bromide had been removed. The interface between aqueous and organic

phases becomes laterally less distinct and the CsCl can precipitate out but these can be remedied by adding a further 50μl of TE and performing a brief incubation at -20 °C.

The plasmid was then desalted by dialysing for 24 hours against 3 changes of 2L Tris-EDTA buffer, pH 8.0, using Spectra-por molecular porous dialysis membrane with a 3,500dalton cut-off. The plasmid concentration was then determined by spectrophotometry and the solution diluted to a working concentration of 1μgμl^{-1} in Tris-EDTA.

Restriction analysis

A restriction map was generated for the IGF-1R cDNA sequence 1063-1659 (Holyrood server). The plasmid mini-preps were cut with *Eco* RI to yield the insert which was digested with *Ava* II (restriction site G*G(A/T)CC) according to the protocol outlined in Sambrook and Maniatis (1989).

IGF-1R (*Ava* II) :	insert restriction site 1348
(1063-1659)	insert product size 311 + 285 base pairs

Restriction digest:	1μg of plasmid DNA
	1μl of *Eco* RI (10u μl-1)
	1μl of *Ava* II (10u μl-1)
	2μl of 10X Buffer C (10mM Tris-HCl, 10mM MgCl2, 50mM NaCl, 1mM DTT, pH7.9)
	nuclease-free water to a total volume of 20μl

The reactions were run at 37 °C for 1 hour before adding 3μl of loading buffer and analysing by electrophoresis on a 2% agarose gel.

Sequence analysis

Selected transformants were insert-sequenced using an adaptation of the dideoxynucleotide chain termination method of Sanger (1977). Thermo Sequenase

radiolabelled terminator cycle sequencing was employed (Amersham Pharmacia Biotech Inc, Ohio) with resolution on urea-acrylamide gels.

Termination mixes were initially prepared by adding 2µl of dGTP nucleotide master mix (7.5mM of dATP, dCTP, dGTP, dTTP) to 0.5µl aliquots of [α-^{33}P]ddNTP(G,A,T or C)(Redivue, 450mCiml^{-1}). Reaction mixes were then prepared as below:

Reaction mix : 2µl of reaction buffer (260mM Tris-HCl, pH 9.5, 65mM $MgCl_2$)
500ng of plasmid-insert DNA
2pmol of primer - M13 forward primer
(5'-GTTTTCCCAGTCACGACGTTGTA-3')
nuclease-free water to a total volume of 20µl
2µl of thermo sequenase polymerase (4u µl^{-1}) (add last)

4.5µl of the above reaction mix were added to each 2.5µl termination mix aliquot and were then placed in a PCR reactor sub-ambient at 4 ^{O}C then run on the following program:

Melt :	95 ^{O}C for 30 seconds
Anneal :	55 ^{O}C for 30 seconds
Extend :	72 ^{O}C for 60 seconds
Cycles :	X30

4µl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) were then added to the above reactions and the samples heated to 70 ^{O}C for 2 minutes before loading onto a sequencing gel:

sequencing gel:	10mls of 10X TBE buffer
(100mls, 6%)	15mls of 40% acrylamide/bis-acrylamide
	42g of urea
	600µl of 10% ammonium persulphate
	40µl of TEMED
	water to a total volume of 100mls

10X TBE :	108g Tris base
	55g Boric acid
	9.3g Na2EDTA.2H2O
	water to a total volume of 1L

The gel was prepared and mounted on the electrophoresis apparatus (Biorad), the chambers filled with 1X TBE, the water-cooling jacket turned on and the gel pre-run for 30minutes. 3µl aliquots of the samples were then double loaded (ie TCGATCGA) and run at 2kV for approximately 3 hours until the dye-front had migrated about one half the length of the gel whereupon the gel was reloaded and run for a further 3 hours to read more distally. The apparatus was then carefully disassembled and the gel soaked in 5% acetic acid, 15% methanol for 15 minutes then dried on Whatman paper at 80 ^{o}C for 2 hours in a gel drier on vacuum. The gel was then exposed for 48 hours using Kodak Biomax MR film. This was developed in an RP X-Omat automated developer and the sequence read.

The sequenced plasmid~IGF-1R insert read thus :

Plasmid:

2994' TGAATT GTAATACGAC TCACTATA

1' GGGCGAATTG GGCCGACGTC GCATGCTCCC GGCCGCCATC

GCGGGAATTC GATT

Insert (sequence match for IGF-1R exon 4-8 cDNA) :

1063 ACCATTGA TTCTGTTACT TCTGCTCAGA TGCTCCAAGG

1101 ATGCACCATC TTCAAGGGCA ATTTGCTCAT TAACATCCGA

1141 CGGGGGAATA ACATTGCTTC AGAGCTGGAG AACTTCATGG

1181 GGCTCATCGA GGTGGTGACG GGCTACGTGA AGATCCGCCA

1221 TTCTCATGCC TTGGTCTCCT TGTCCTTCCT AAAAAACCTT

1261 CGCCTCATCC TAGGAGAGGA GCAGCTAGAA GGGAATTACT

1301 CCTTCTACGT CCTCGACAAC CAGAACTTGC AGCAACTGTG

1341 GGACTGGGAC CACCGCAACC TGACCATCAA AGCAGGGAAA

1381 ATGTACTTTG CTTTCAA....

This orientated the insert to the plasmid's SP6 promoter for anti-sense transcription. As a preliminary to this, the plasmid was linearised with *Nco* I (having first excluded a similar restriction site in the insert) and it was then gel purified.

Sourcing of other riboprobes

Wilms' tumour suppressor

A 243bp insert spanning exon1 and exon2 of WT-1 in pGEM3Z was a kind gift from David Housman, MIT. This was restriction and sequence analysed prior to linearising with Hind III and transcribing with T7 polymerase.

C-myc and β-actin

Linearised plasmid templates containing c-myc and β-actin cDNA inserts orientated downstream of SP6 and T7 promoters were obtained commercially (Ambion Inc, AMS Biotech., UK). The c-myc transcript encodes nucleotides 1197-1446, Accession V00568, a 250 bp sequence spanning exons 2 and 3. The β-actin transcript encodes nucleotides 704-947, Accession X00351, a 245bp sequence.

2.7. RNA analysis - Northern blotting

Gel electrophoresis

The analysis of total RNA by denaturing formaldehyde gel electrophoresis with Northern transfer and riboprobe detection is an adaptation of that previously described by Maniatis (1989), optimised for high molecular weight transcripts and low copy numbers.

A 1% gel was prepared by melting 0.75g of Agarose NA (Pharmacia Biotech Ltd, Milton Keynes) in 54ml DEPC treated water. This was allowed to cool to 55 ^{o}C before adding 1μl of 10mg/ml ethidium bromide and 7.5ml of MOPS 5X buffer and 13.5ml of 37% formaldehyde (12.3M, pH>4.0).

MOPS 5X buffer: 0.2M 3-[N-morpholino]-2-hydroxypropanesulphonic acid, pH7.0
0.05M sodium acetate
0.005M EDTA, pH8.0
autoclaved, pH5.5-7.0

A 5mm gel was poured into a sealed midi-gel tray with a 1.5mm comb (Northumbria Biologicals Ltd.) having previously cleaned the gel apparatus in weak detergent, 3% hydrogen peroxide and DEPC-treated water.

20μg total RNA samples suspended in 5μl nuclease-free water were mixed with 10μl RNA sample buffer and heated at 65 ^{o}C for 5 minutes before cooling on ice and adding 2μl of RNA loading buffer.

RNA sample buffer: 10ml deionized formamide
3.5ml 37% formaldehyde
2.0ml MOPS 5X buffer

RNA loading buffer: 50% glycerol
1mM EDTA
0.4% bromophenenol blue

The gel tray was was placed in the electrophoresis apparatus and covered to a 1cm depth with 1L of MOPS X1 before pre-running for 10 minutes. The RNA samples were then loaded alongside 5µl of ssRNA ladder (Millennium markers, Ambion, UK) and run at 80V (5V/cm) at 4 ^{O}C for 3 hours until the bromophenol blue had migrated to the bottom of the gel. The gel could then be examined and photographed under UV transillumination.

Northern transfer

An adaptation of Chomczynski's downward alkaline blot technique was employed (Chomczynski, 1992) where the formaldehyde gel was capillary blotted to positively charged nylon filters (BrightStar-Plus, Ambion) under alkaline / 20X SSC elution.

The gel was briefly soaked in DEPC-treated water to remove the formaldehyde before soaking in 0.05N sodium hydroxide for 20 minutes to partially hydrolyse the RNA and aid high molecular weight transfer. The gel was then soaked in 20X SSC for 45 minutes whilst the transfer stack was prepared.

20X SSC: 3M sodium chloride.
0.3M tri-sodium citrate, pH 7.0.

A 5cm stack of paper towels was surmounted by 3 dry pieces of Whatman 3MM filter paper and on top of this were added 2 more pieces of 3MM filter paper trimmed to the size of the gel and pre-wetted in transfer buffer (20X SSC, 0.05N NaOH). The nylon membrane, trimmed to size, was briefly wetted in transfer buffer and placed atop the

filter sheets. The gel was then aligned bottom side up upon the membrane and in turn surmounted by 3 further transfer buffer soaked 3MM filter papers and a sufficient length of wetted 3MM filter paper leading to the buffer reservoir. The gel and apparatus were surrounded by Saran wrap and the whole stack weighted with 150g. Capillary alkaline blotting proceeded for 2 hours at room temperature after which the apparatus was carefully disassembled and transfer confirmed by UV transillumination. The hybridisation filter was then soaked in 5X SSC for 5 minutes, dried at room temperature before cross-linking by baking in a vacuum oven at 80 ^{O}C for one hour.

In vitro transcription and riboprobe hybridisation

Formamide-based hybridisations were performed using riboprobes generated with modified nucleotides (Strip-EZTM RNA, Ambion, UK) to allow effective resolution at sequential rehybridisation with probes for IGF-1R, c-myc (pTRI-c-myc-human, Ambion), WT-1 and β-actin (pTRI-Actin-human, Ambion, UK).

The filters were initially prehybridised in 10ml of preheated hybridisation solution per 100cm2 of membrane in a Hybritube 15 (Gibco BRL) in a rotary hybridisation oven at 65 ^{O}C for 4 hours.

Hybridisation solution :	6.25ml formamide
(for 12.5ml)	3.125ml 20X SSPE
	0.5ml 50X Denhardt's reagent
	0.125ml 10% SDS
	2.5ml DEPC-treated water
	0.125ml denatured salmon testes DNA
	(10mgml^{-1})
20X SSPE :	3M NaCl
	0.23M Monosodium phosphate (NaH_2PO_4)
	25mM EDTA, pH 7.4
	(autoclaved for 20 minutes @ 15lb/sq.in.)

50X Denhardt's : 1% Ficoll
1% polyvinylpyrolidone
1% bovine serum albumin
(0.2 μm filtered)

In vitro transcriptions used an adaptation of the original technique described by Melton and Maniatis (1984) employing a modified CTP nucleotide (Ambion) to generate [α-^{32}P]-labelled riboprobes according to the following protocol :

reaction mix : nuclease-free water to 20μl
0.1μg of linearised plasmid template
2μl of 10X transcription buffer
1μl of 10mM ATP
1μl of 2mM modified CTP
1μl of 10mM GTP
0.5μl of 10mM UTP
5μM of [α-^{32}P]-UTP (Redivue, 450Cimmol-1)
2μl of SP6 or T7 RNA polymerase depending on insert orientation

The reaction was allowed to proceed for 1 hour at 37 ^{o}C before adding 1μl of DNaseI and incubating for a further 15minutes at 37 ^{o}C.

Unincorporated nucleotides were removed by filtration through a Sephadex G-50 column (Pharmacia Biotech, Milton Keynes). RNase-free G-50 columns were equilibrated with 3ml of RNase-free buffer containing 10mM Tris-HCl, pH 7.5 with 1mM EDTA. The transcription product was then added to the gel bed and a further 400μl of buffer added. The purified product was then eluted with a further 400μl of buffer.

The purified radio-labelled RNA probe was heat denatured at 90^{o}C for 10 minutes before adding directly to the hybridisation fluid. Hybridisation proceeded for 18 hours at 65 ^{o}C after which the filter was removed and washed sequentially in increasingly

stringent washing buffer as detailed below whilst monitoring with a handheld counter and a digital scintillation imager.

1. Wash twice with 2X SSC, 0.1% SDS (20ml per 100cm^2) for 10 minutes at room temperature.

2. Wash twice with 0.2X SSC, 0.1% SDS (40ml per 100 cm^2) for 15 minutes at 65 ^{o}C.

3. Wash twice with 0.1X SSC, 0.1% SDS (40ml per 100 cm^2) for 15 minutes at 65 ^{o}C.

The filter was then rinsed in 0.1X SSC, wrapped in Saran wrap and imaged either with the scintillation imager or by exposing Hyperfilm TM-MP film in a Fastrad Autoradiography cassette with an intensifying screen at -70 ^{o}C. After a 12-48 hour exposure the film was developed in an RP X-Omat automated developer. Densitometry readings of the films were performed with the aid of an optical densitometer (Northern Light, Canada).

Stripping and reprobing blots

After exposure, the filters were stripped in probe degradation buffer (Ambion,UK) with 0.1%SDS using 10ml/100cm2 of membrane at 68 ^{o}C for 10 minutes before incubating in 10ml/100cm2 blot reconstitution buffer (Ambion, UK) again at 68 ^{o}C for 10 minutes. The filter was then reimaged and if there remained a high residual signal which was anticipated to interfere with subsequent imaging, the filter was restripped for 15 minutes in 500ml of boiling 0.05X SSC and 0.01M EDTA, pH 8 with 0.1% SDS added when off the boil. The filter was then rinsed in 0.01X SSC before a further exposure and reprobing.

2.8. Protein analysis – Radioligand binding

Membrane microsome preparation

Microsomal preparation from the membrane fraction was an adaptation of the technique of Kuo et al., 1990. Freshly micro dissected normal and tumoural epithelium were minced in lysis buffer with a Brinkman polytron on ice.

Lysis buffer :	0.25M sucrose, 0.15M NaCl, 25mM Tris-HCl,
(1g tissue/10ml)	1% Triton-X, 0.05% Sodium deoxycholate,
	1mM EDTA, 1mM PMSF, 1mM NaF, 1μg/ml aprotinin,
	1μg /ml pepstatin, 1μg/ml leupeptin, 1mM NaF_4 and
	1mM Na_3VO_4, pH 7.4 at 4 ^{O}C

The remaining non-homogenised tissue and nuclear debris were then removed by centrifuging at 3000 r.p.m. for 30 minutes at 4 ^{O}C. The supernatant was then carefully removed and the nuclear and mitochondrial fraction were then pelleted by centrfuge at 13,000g for 30 minutes at 4 ^{O}C. The resulting supernatant was then ultracentrifuged at 100,000g (40,000 r.p.m in a Sorvall OTD-65 Ultracentrifuge) for 60 minutes at 4 ^{O}C to pellet the memrane and remaining high molecular weight fraction. This pellet was then suspended in binding buffer with a Dounce homogeniser and the protein concentration measured (Bradford assay). In each membrane preparation the specimens were treated identically and were homogenised for the same period of time.

Binding buffer :	25 mM TrisHCl, 0.9% NaCl, 10 mM $MgCl_2$,
	2.5 mM EDTA, 0.1% BSA, 10^{-4} M PMSF,
	pH 7.6 at 4 ^{O}C

Radioligand competitive binding assay

Reactions were carried out in triplicate in 75mm plastic tubes which had been precoated with 0.1% BSA. 400μg aliquots of membrane microsome in 400μl binding buffer were incubated for 16 hours at 4 ^{O}C with 0.05mCi of ^{125}I-IGF-1 (Amersham, UK) at a total concentration of 2nM and with or without the addition of unlabelled IGF-1 at varying concentrations (0.1 to 10 nM). Reactions were stopped by the addition of 3 ml ice cold binding buffer, the membranes pelleted by centrifugation at 36,000g for 30 minutes then resuspended and washed with a further 3 ml of binding buffer.The bound radioactivity in the subsequent microsome pellet was measured in a Beckman gamma counter.

2.9. Protein analysis - Immunocytochemistry

Tissue specimens for immunocytochemistry were taken from tumour margins to include both normal and tumoural mucosa. Both frozen sections and paraffin embedded specimens were found to be suitable for IGF-1R analysis, but paraffin embedded ACFs and tissue controls were more widely available in archival stores and so, for uniformity, this method was adopted and is described herein. Briefly, specimens were fixed in PBS-buffered 4% formaldehyde for 2 to 4 hours and then serially dehydrated by soaking for 1 hour in increasing concentrations of ethanol. After soaking in 100% ethanol, the specimens were soaked in 2X changes of xylene for 1 hour each before embedding in paraffin by routine processing with a Bayer VIP processor. The hardened wax blocks were then trimmed for sectioning. 4μm sections were cut on a bench-top microtome and mounted on Superfrost plus slides (Merck) before drying for several hours at 50 ^{O}C in an oven.

Freshly cut specimens were chosen that would allow assessment of normal and tumoural tissue in the same field at histological examination. Dewaxing and rehydration of the selected paraffin-embedded specimen slides in preparation for immunocytochemistry involved a 1 hour incubation at 60 ^{O}C followed by 2X 3minute washes in xylene. The slides were then serially rehydrated with 2X 3minute washes in

100%, 95%, 70% and 30% ethanol followed by 10% PBS then TBS and then finally distilled water.

PBS 1 tablet of Dulbeccos A added to 100mls distilled water

TBS For 1L :6.05g of Trizma base was added to 80mls of distilled water then 3.3mls of concentrated HCl were added. This solution was added to 8.2g of NaCl dissolved in 920mls distilled water. To this was added 1ml of Triton X 100 dissolved in 5mls of warm distilled water. pH 7.6. Stored at 4 ^{o}C.

Antigen retrieval was aided by microwaving the sections for 2X 5 minute cycles at 800 watts in 0.01M sodium citrate, pH6.0, before allowing the sections to stand for 20 minutes in the hot acid. The slides were then washed for 5 minutes in TBS.

Immunodetection employed a chicken polyclonal IgY primary antibody to the human IGF1-R α-subunit (Upstate biotechnology, Lake Placid). This had been produced with a synthetic immunogen [CKYADGTIDIEEVTENPKT] corresponding to residues 642-659 of the α-subunit (Rosenzweig, 1990) with subsequent PEG and ammonium sulphate purification of the IgY. This primary polyclonal was detected with a biotinylated rabbit anti-chicken antibody (Zymed, San Francisco) which in turn was detected using the streptavadin-horseradish peroxidase technique.

The hydrated antigen-unmasked slides initially had their endogenous peroxidase activity blocked by incubating in 3% hydrogen peroxide in distilled water for 15 minutes. The slides were then washed twice in distilled water then TBS, each for 5 minutes. Non-specific binding was then blocked with a 20 minute incubation in 10% rabbit non-immune serum in TBS with 2% bovine serum albumin at room temperature. The serum was then carefully drained from the slides and the specimens ringed with a wax PAP marker pen. The slides were then incubated with 200μl aliquots of 10μgml^{-1} anti-IGF-1R α-subunit polyclonal for 1 hour at room temperature, the optimal conditions having been previously determined by time-dilution experiments. Following this, the slides were washed twice with TBS for 5 minutes. Detection was performed by incubating with

200μl of 1:400 biotinylated rabbit anti-chicken antibody in TBS for 30 minutes. Two further 5 minute washes in TBS were followed by a 30 minute incubation with freshly prepared Streptavadin-horseradish peroxidase complex (Vector Laboratories, Peterborough). The slides were again washed twice in TBS for 5 minutes before developing in 0.06% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, UK) for less than 10 minutes. Following washing in TBS then water, the slides were counter-stained in Mayer's haematoxylin and 0.3% ammonia water before dehydrating in increasing concentrations of ethanol. The slides were finally washed in xylene then mounted with DPX and a coverslip.

Tissue and reagent controls were run synchronously in the above protocol. Hepatocytes are known to express low levels of the IGF-1R (Lemmey, A.B., 1997) whilst renal tubular epithelial cells express high levels of the IGF-1R (Pillion, D.J., 1988) and so paraffin-embedded sections from normal liver and kidney were used as tissue negative and positive controls respectively. Reagent negative controls were prepared by peptide-absorbing the primary antibody with the original immunogenic synthetic peptide corresponding to residues 642 to 659 of the human IGF-1R α-subunit that was used initially as the inoculant in the production of this polyclonal (Upstate Biotechnology). Briefly, the lyophilised a-subunit peptide was rehydrated and serially diluted in TBS to a concentration of 0.2 mgml-1 before storing at -20 ^{o}C. Peptide absorption was performed by incubating the primary antibody with a tenfold excess by weight of the synthetic peptide for 2 hours at room temperature. The resulting immune complexes were then removed by centrifugation at 13,000 Xg for 15 minutes and the supernatant taken and used for the reagent negative primary incubations in the above protocol.

2.10. Protein analysis - Primary cultures and immunocytochemistry

Primary cultures

The technique employed for the primary culture of colonic carcinomas and normal epithelial explants was adapted from that of Paraskeva et al., (1996). The specimens were also examined histologically with IGF-1R immunohistochemistry at the time of primary culture.

Microdissected mucosa was transported in washing medium at 4 ^{o}C and then stringently washed before transferring to and processing aseptically in a laminar flow hood (Microbiological Class II, Howarth Air Engineering Ltd., Farnworth, UK). The specimens were diced with a scalpel to less than 1mm^3 and further stringently washed by pipetting.

Washing medium : RPMI 1640 medium, 5 % fetal calf serum (FCS),
2mM glutamine, 200 U/ml penicillin,
200 μg/ml streptomycin and 50μg/ml gentamicin

The tissue pieces were then suspended in wash medium (500mg in 8ml) to which hyaluronidase at 100U/ml (Sigma) and collagenase at 240U/ml (Sigma) working concentrations were added. The tissues were digested for 12 hours at 37^{o}C in a rotary incubator before centrifuging at 1600g for 3 minutes. The retrieved organoids were washed. These in turn were then retrieved by a combination of gravity sedimentation and differential centrifugation at 160g for 1 minute to aid the removal of fibroblasts. This was repeated until the organoids were microscopically clear of fibrous material. The organoids were then transferred into conditioned growth medium in collagen coated 75cm^2 culture flasks in a humidified incubator (T305GF Assab ; Kebo Assab AB, Solna, Sweden) in an atmosphere of 95% air and 5% CO_2 at 37^{o}C.

Growth medium : As wash medium with 10% FCS,
1mg/ml hydrocortisone succinate, 0.2 U/ml insulin.

Medium was conditioned for 24 hours on 3T3 cells (ATCC no. CCL92) then dialysed and lyophilised to be reconstituted in an initial equivalence in growth medium.

75cm^2 culture flasks were collagen coated by dissolving 50mg type VI human collagen (Sigma) in 50ml 0.1% glacial acetic acid and using a film of this to coat the bottom of the flask. This was then air dried for 4 hours and washed briefly in wash medium before use.

Once a primary culture had been established, organoids were passaged by the addition of 2.5 ml of 2u/ml Dispase (Boehringer Mannheim) in wash medium for 15 to 60 minutes. Once a cell line had been established, passaging was usually possible with 0.1% trypsin/ PBS. Prior to the immunohistochemistry studies, cells were tested for growth on soft agar and cytokeratin staining.

Cell culture immunocytochemistry

Cell lines were plated down on collagen coated 24 well plates for at least 48 hours. Depending on the required degree of confluence or differentiation, the cells were then washed in PBS and fixed in 200µl ice cold paraformaldehyde / acetic acid [4% / 2%] in PBS with 200µl washing medium for 15 minutes. The cells were then washed twice in PBS at room temperature for 15 minutes before their endogenous peroxidase activity was blocked with 200µl of 3% hydrogen peroxide for 5 minutes. After a further wash in PBS, non specific binding was blocked by a 20 minute incubation with 20% non immune rabbit serum / 1% BSA in PBS at room temperature. The blocking solution was then removed and the primary antibody incubation performed with 200µl of 10µg/ml anti-IGF-1R a-subunit polyclonal in 1% BSA in PBS at 4 ^{o}C for 12 hours. Control slides were

incubated with the peptide absorbed antibody. Thereafter, the standard protocol from the tissue histochemistry was followed – namely, detection with a biotinylated rabbit anti-chicken antibody followed by Streptavadin / HRP then developing with DAB and counterstaining with Haematoxylin and 0.3% ammonia water.

2.11. Protein analysis - Western blotting

The low IGF-1R expression in normal colonic tissue necessitated enrichment by immunoprecipitation of the peptide as a prelude to SDS-PAGE and immunodetection. This was adapted from the protocol described by Maniatis (1989).

Protein extraction and immunoprecipitation from snap frozen tissue

The susceptibility of the mature peptide to cleavage during mechanical disruption of frozen tissue and from endogenous proteases required tissue specimens to be homogenised in a Ystrall homogeniser on ice having been briefly thawed in a lysis buffer containing protease inhibitors.

Lysis buffer :	0.25M sucrose, 0.15M NaCl, 25mM Tris-HCl,
(1g tissue/10ml)	1mM EDTA, 1mM PMSF, 1mM NaF, 1μg/ml aprotinin,
	1μg/ml pepstatin, 1μg/ml leupeptin,
	0.05% Sodium deoxycholate, 1mM NaF_4 and
	1mM Na_3VO_4, pH 7.4 at 4 ^{o}C

Remaining non-homogenised tissue and nuclear debris were then removed by centrifuging at 3000 r.p.m. for 30 minutes at 4 ^{o}C. The supernatant was then carefully removed and the membrane and high molecular weight fraction then pelleted from this by ultracentrifugation at 30,000 r.p.m. for 40 minutes at 4 ^{o}C (Sorvall OTD-65 Ultracentrifuge). The pellet was resuspended in approximately 1ml of ice-cold lysis buffer. To reduce non-specific binding during subsequent immuno-separation, this lysate was precleared by incubating with 100μl of 50% Protein G Plus-Agarose slurry

(Oncogene Research Products, Cambridge) in PBS for 10 minutes at 4 ^{o}C. This was then centrifuged at 13,000 Xg for 10 minutes at 4 ^{o}C, the supernatant taken and its protein content determined colourimetrically by Bradford assay.

Briefly, 80µl aliquots of the lysate diluted at least 1/10 in water were added to 20µl of Coomassie Plus reagent (Bio-rad) in a 96 well plate and read at 595nm on a microplate reader (Bio-rad). This was read against a standard scale of BSA in water prepared at concentrations ranging from 40µgml-1 to 1µgml-1.

The lysate was then diluted in PBS to a concentration of 10µgml-1. Separate 500µl aliquots of this lysate were then immunoprecipitated by incubating overnight at 4 ^{o}C on a rotary shaker with 5µg of the following antibodies :

anti-IGF-1R monoclonal antibody (Oncogene Research Products, catalogue no. GR11).

anti-IGF-1R polyclonal antibody (Upstate Biotechnology, catalogue no.06-429).

The immunocomplexes were then captured by adding 100 µl of Protein G Plus-Agarose and incubating overnight at 4 ^{o}C. The chicken polyclonal incubation at this stage required additionally a second-step antibody consisting of 5µg of rabbit anti-chicken antibody (Upstate Biotechnology). The agarose beads were then collected by pulsing in a microfuge for 5 seconds before washing 3 times with ice-cold PBS and collecting in a similar manner. The agarose beads were then resuspended in 50µl of 2X Laemmli sample buffer to dissociate the immune complexes. At this stage the samples could be stored at -20 ^{o}C or boiled for 2 minutes and pulse spun to recover the supernatant ready for SDS-PAGE.

2X Laemmli sample buffer: 100mM Tris-HCl, pH 6.8
20% glycerol
4% SDS
0.2% bromophenol blue

(reducing gels prepared by the addition
of 100mM dithiothreitol before use).

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed in a vertical electrophoresis apparatus with a discontinuous buffer system (Protean II, Bio-Rad). The plates were assembled following cleaning in detergent, distilled water and 100% alcohol. A 7.5% polyacrylamide resolving gel was poured and allowed to polymerise for 20-30 minutes below a shallow layer of isobutanol.

7.5% Polyacrylamide :	16.6ml of water (autoclaved)
Resolving Gel (30ml)	5.4ml of 40% acrylamide/bis-acrylamide
	7.5ml of 1.5M Tris-base, pH 8.8
	0.3ml of 10% SDS
	0.3ml of 10% ammonium persulphate
	12µl TEMED

The isobutanol was removed with a paper towel and the gel's top surface irrigated with distilled water. A 5% stacking gel was then poured onto this surface around a 10 well Teflon comb and allowed to polymerise for a further 20-30 minutes.

5% Polyacrylamide :	7.25ml of water
Stacking Gel (10ml):	1.275ml of 40% acrylamide/bis-acrylamide
	1.25ml of 1M Tris-base, pH6.8
	0.1ml of 10% SDS
	0.1ml of 10% ammonium persulphate
	10µl of TEMED

Once polymerisation was complete, the comb was then removed and the top and bottom gel surfaces irrigated with distilled water. The gel was then placed in the electrophoresis apparatus and the top and bottom reservoirs charged with Tris-glycine buffer.

Tris-glycine buffer (3L) : 25mM Tris-base (9.09g)
250mM glycine (43.23g)
0.1% SDS (3g)
(distilled water to 3L)

The gel was now ready for loading. The protein samples were placed on ice after boiling for 2 minutes, then loaded alongside 15µl of molecular weight markers, range 30-200kdal (Rainbow TM, Amersham). These markers had similarly been mixed 2:1 with 2X Laemmli sample buffer and boiled for 10 minutes prior to loading.

The electrodes were now connected to a Bio-rad power supply on a constant current setting. The water jacket was turned on and the gel run at 20mA until the dye-front had migrated to the interface between the resolving and the stacking gels. The gel was then run at 40mA until the dye-front had migrated off the bottom of the gel. The apparatus was carefully disassembled, ready for gel transfer.

Western blotting of protein to nitrocellulose filters

The SDS-poyacrylamide gel was trimmed and orientated then soaked in 1L of transfer buffer for 30 minutes. During this time a nitrocellulose filter (Hybond C Extra) and 4 pieces of Whatman 3MM paper were cut to the same dimensions as the gel and soaked in transfer buffer.

Transfer buffer (1L) : 48mM Tris-base (5.82g)
39mM glycine (2.93g)
0.037% SDS (3.75mls of 10%)
20% methanol (200ml)
distilled water to 1L, pH9.2.

Electroblotting was performed on a Transblot SD semi-dry transfer cell (Bio-Rad). The electrodes were cleaned with 100% alcohol and 2 pieces of the transfer buffer-soaked 3MM paper were placed on the anode and the nitrocellulose filter positioned atop these. The gel was then briefly soaked in distilled water and then cajoled into position above the filter using a small glass plate or domestic fish slice (Jenners!). The gel was

orientated and aligned, taking care to avoid any air bubbles or short circuits. The 2 final pieces of 3MM paper were aligned above the gel and the cathode placed on top of the stack. The filter was electroblotted at 5V for 16 hours at room temperature. The apparatus was then disassembled, the molecular weight marker lane removed and the filter's orientation marked. Ponceau S staining at this stage showed some non-specific protein carry-over but rarely revealed the specific bands arising from immunoprecipitation.

Blocking and antibody detection of the IGF-1R

The IGF-1R protein was detected using the anti-a-subunit polyclonal employed in the immunocytochemistry protocol. This antibody was effective in detecting the IGF-1R when either it or the monoclonal were used in the primary immunoprecipitation (both antibodies recognise the a-subunit).

The filter was initially washed in 500mls of washing buffer for 10 minutes before blocking in 0.1ml per cm2 of blocking solution for 1 hour.

Washing buffer :	24g of Tris-base
(4L)	35.2g of NaCl
	2.96g of EDTA, pH 7.5
Blocking solution :	5g of skimmed milk
(100ml)	2g of BSA
	washing buffer to 100ml

The filter was then transferred to a heat-seal bag where it was incubated with 1μgml-1 of anti-IGF-1R α-subunit IgY (Upstate Biotechnology) in 0.1ml per cm2 of blocking solution overnight at 4 ^{o}C on a rotary shaker. The filter was then washed twice in water before incubating for 2 hours at room temperature with a 1:1000 dilution of secondary antibody (a rabbit anti-chicken IgY linked to horseradish peroxidase, Upstate) in the same volume of blocking solution as before. The filter was then washed twice with water, then with wash buffer-0.05% Tween 20 for 5 minutes before soaking for 5 minutes each in 5X changes of water. The membrane was then drained and its surface incubated for 1 minute with 0.125mls per cm2 of premixed ECL detection reagents (Amersham). After

this, the membrane was drained of fluid, wrapped in Saran wrap and exposed for 1, 2 and 4 minutes to HyperfilmTM-ECL. The film was then developed in an automated processor and the bands examined both for their molecular weight and optical densitometry.

Pulse labelling and immunoprecipitation / SDS-PAGE

To further increase the sensitivity of Western blotting, primary cultures of colonic cancers and normal epithelial explants were additionally pulse labelled with Met – ^{35}S (Amersham, UK) as a prelude to the immunoprecipitation and SDS-PAGE protocol described above. Here, cell cultures were serum starved for 6 hours before culturing for 12 hours with Met deficient growth medium to which Met – ^{35}S had been added. Thereafter, the cells were lysed in the standard lysis buffer and immunoprecipatation and SDS-PAGE proceeded as per the above protocol. Thereafter, the gel was transferred in 5% acetic acid, 15% methanol to Whatman paper in a gel drier on vacuum at 80 ^{o}C for 2 before exposing on Kodak Biomax MR film.

CHAPTER 3

RESULTS

3.1. PCR mRNA analysis of normal and tumoural colonic mucosa

IGF-1R RNA analysis was initially performed by means of RT.PCR using mRNA extracted from freshly dissected normal and tumoural tissue (2.2.2-2.2.3). The cycling, annealing temperature and Mg concentrations were optimised for the reaction :

Primers :	5'-ACCATTGATTCTGTTACTTC-3'
	5'-TTAAGAATGTCACAGAGTAT-3'
Product :	596 bp, 1063-1659 of IGF-1R cDNA sequence
	(exon 4-8 of α-subunit)
PCR parameters :	40 x (60s @ 94^{0}C, 60s @ 52^{0}C, 120s @ 72^{0}C)
	Tfl Taq polymerase
	1μM primers and c50ng cDNA
	$[MgCl_2]$ = 1.5mM

Total RNA was initially assessed by RT.PCR without success. Subsequent mRNA purification and RT.PCR yielded a weak signal for the 596 bp product for the IGF-1R cDNA. Repeating the assay with total RNA once the PCR had been optimised similarly failed to yield a product. Even with an excess of 2μg mRNA, a relatively low annealing temperature and a high cycle number, the PCR had a low efficacy and yielded a low product signal. In 3/14 reactions there was no detectable IGF-1R signal despite strong HGPRT and IGF-IIR control signals. In those assays that yielded a product, normal

mucosa gave a consistently weak or absent signal whilst cancers could yield strong or absent signals.

Figure 3.1 PCR for IGF-1R and IGF-2R (control) for normal and cancer mucosa.
596 bp IGF-1R PCR product weak / absent signal (arrowed) in normal mucosa (N) and some cancer specimens (C) but stronger in some other cancer specimens (C). 720 bp IGF-2R control present in normal mucosa(N) and cancer (C). RT negative controls (Cx) using cancer specimens for IGF-1R and IGF-2R.

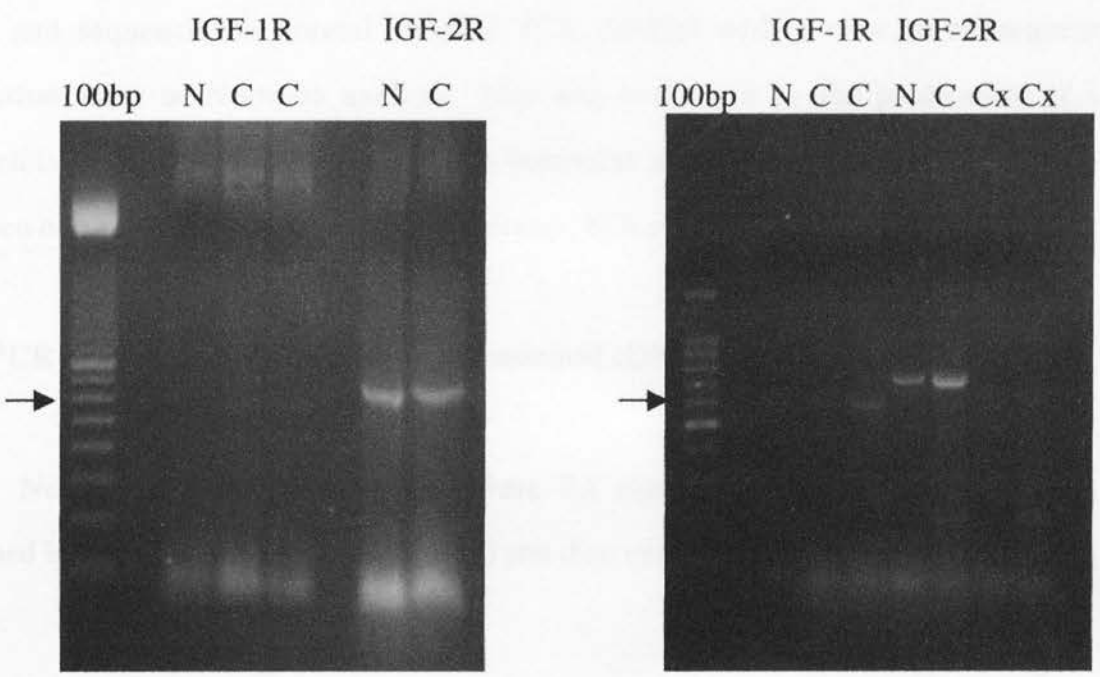


Table 3.1 IGF-1R PCR product signal (2μg mRNA analysed):

	Absent	Weak	Strong
Normal mucosa	2/7	5/7	
Polyp			2/2
Cancer	1/5	2/5	2/5

At this stage, a fresh tissue positive control such as kidney was not readily available. The PCR results were reproduced but the continued inter-specimen variability was suggestive of poor PCR efficacy. Rather than redesign the primers, it was elected to clone and sequence the normal mucosal PCR product with a view to subsequent protection assay or Northern analysis. This was reinforced by the publication of a competitive PCR study which had failed to determine any IGF-1R expression difference between normal and tumoural mucosa (Zenilman, M.E., 1997).

3.2. PCR product analysis from normal mucosal cDNA

Normal mucosal PCR products were TA cloned into the pGEM-TE vector, screened by restriction analysis (Figure 3.2) and then cycle-sequenced (Figures 3.3, 3.4).

Figure 3.2 Restriction analysis screening of plasmid inserts. Lanes 4 and 5 show Ava II digests of different plasmid inserts with predicted products of 311 and 285 base pairs for the correct insert (arrows). Lane 5 shows the correct insert. This plasmid would then be maxiprepped and the insert cycle-sequenced. Lane 4 shows an incorrect insert and this plasmid would be discarded. Controls are uncut plasmids (pl) and plasmids cut with EcoR1.

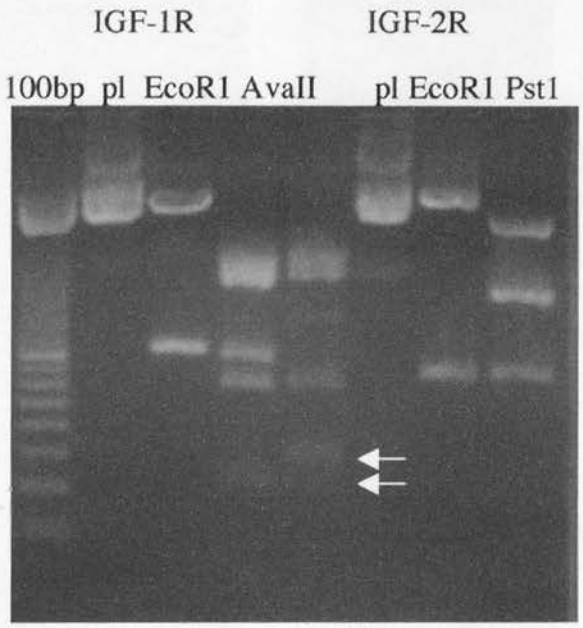


Figure 3.3 Sequencing gel of IGF-1R insert

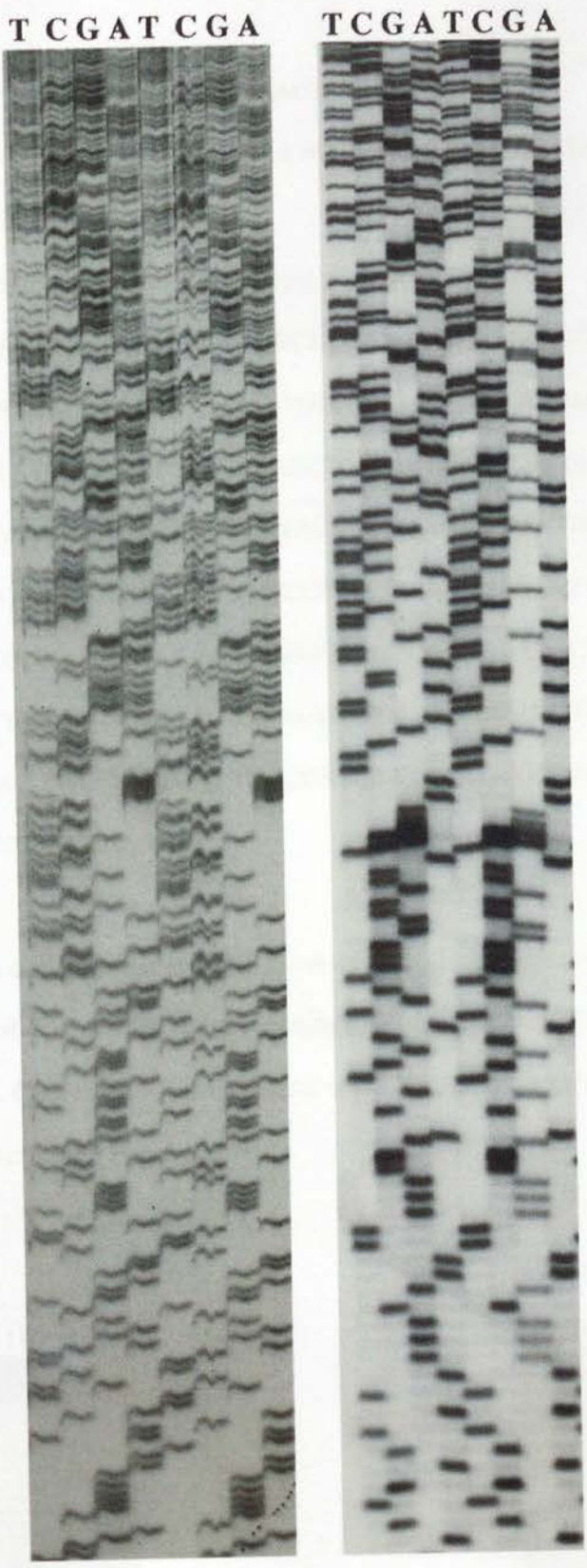


Figure 3.4 Insert sequence :

Plasmid:

2994' TGAATT GTAATACGAC TCACTATA

1' GGGCGAATTG GGCCGACGTC GCATGCTCCC GGCCGCCATC

GCGGGAATTC GATT

Insert (sequence match for IGF-1R exon 4-8 cDNA) :

1063 ACCATTGA TTCTGTTACT TCTGCTCAGA TGCTCCAAGG

1101 ATGCACCATC TTCAAGGGCA ATTTGCTCAT TAACATCCGA

1141 CGGGGGAATA ACATTGCTTC AGAGCTGGAG AACTTCATGG

1181 GGCTCATCGA GGTGGTGACG GGCTACGTGA AGATCCGCCA

1221 TTCTCATGCC TTGGTCTCCT TGTCCTTCCT AAAAAACCTT

1261 CGCCTCATCC TAGGAGAGGA GCAGCTAGAA GGGAATTACT

1301 CCTTCTACGT CCTCGACAAC CAGAACTTGC AGCAACTGTG

1341 GGACTGGGAC CACCGCAACC TGACCATCAA AGCAGGGAAA

1381 ATGTACTTTG CTTTCAA....

Sequencing confirmed the PCR product as IGF-1R exon 4-8 cDNA orientated to the SP6 promoter of pGEM-TE ready for linearising with NcoI (Figure 3.5) before anti-sense transcription. C-myc and WT-1 inserts were similarly sequenced prior to linearising and riboprobe transcription (Figure 3.6).

Figure 3.5

Nco1 cut IGF-1R/ plasmid

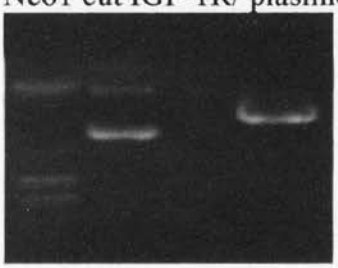
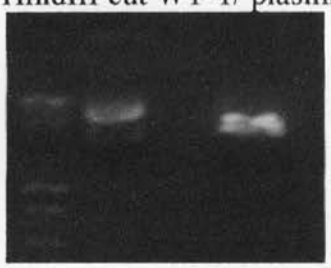


Figure 3.6

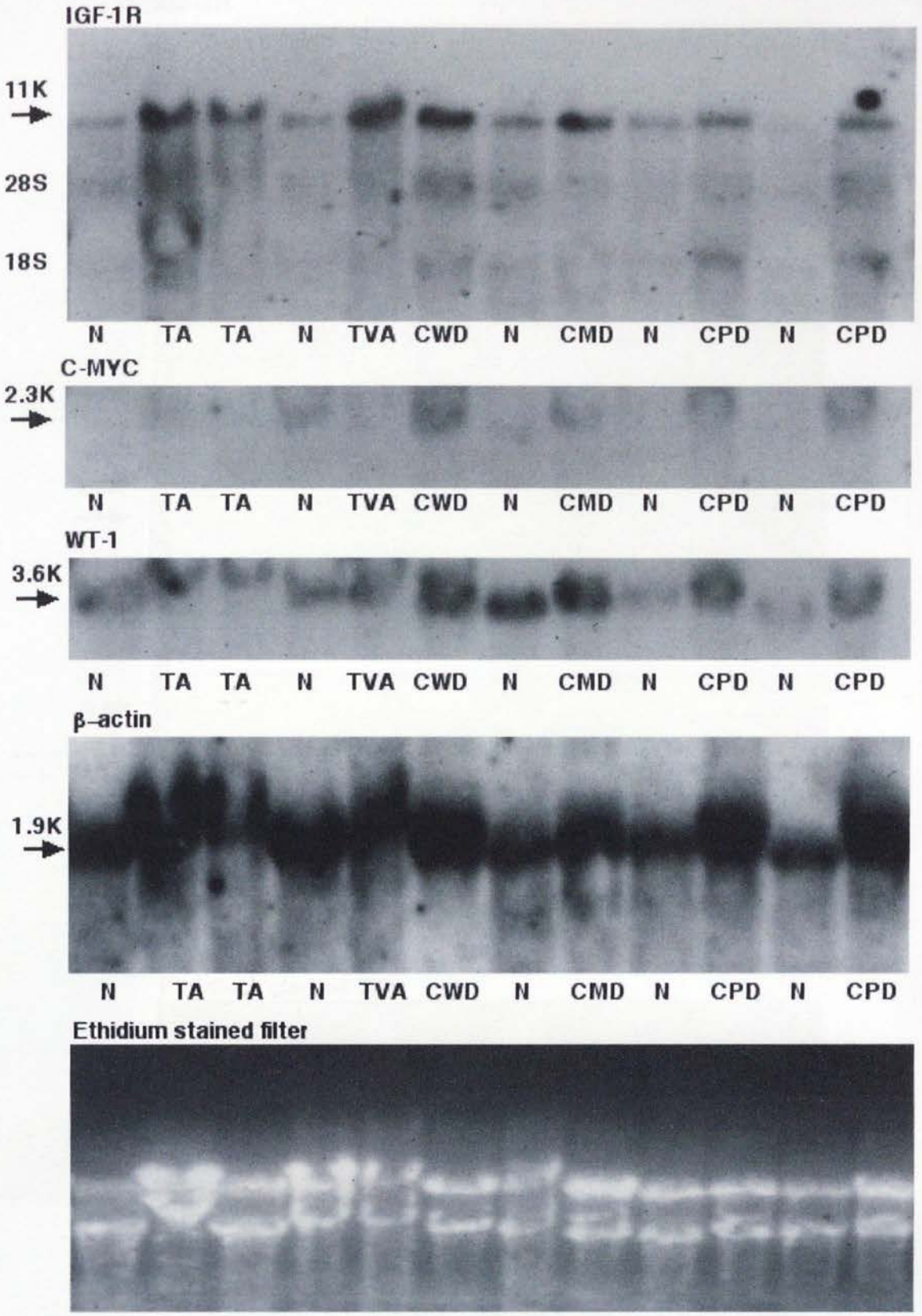
HindIII cut WT-1/ plasmid



3.3. Northern analysis

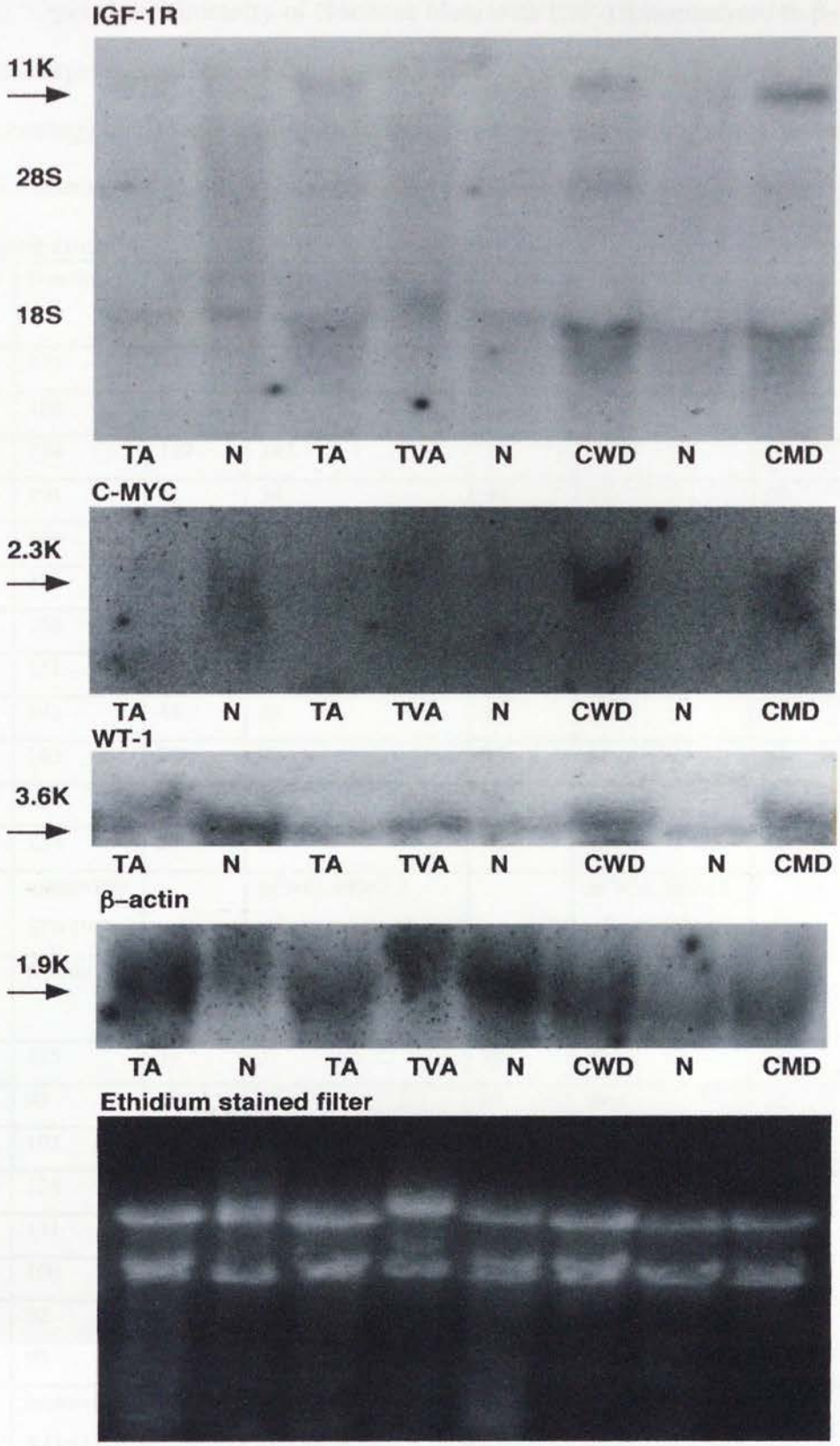
Total RNA Northern blotting with riboprobe analysis was performed in the first instance. This was optimised for the high molecular weight and low copy number of IGF-1R transcripts as detailed (2.2.3.3.1). A pilot assay with total RNA aliquots of between 5 to 20μg from normal and tumoural mucosa was run on a standard formaldehyde gel with hybridisation of the 596 bp IGF-1R riboprobe proceeding for 24 hours at 65^{0}C. RNA integrity was confirmed at each stage. This primary assay determined that a weak 11kb signal, which corresponded with the IGF-1R transcript molecular weight, could in fact be detected by conventional Northern analysis using 20μg aliquots of normal mucosal total RNA. Thereafter, with 20μg aliquots of matched normal and tumoural mucosa total RNA, the hybridisation times were reduced to 12 hours and Strip-EZ product used to facilitate sequential rehybridisations with c-myc, WT-1 and β-actin riboprobes. Optical densitometry was then performed on the autorads to allow normalising and comparison. Additionally, part of each specimen was histologically examined and graded - normal mucosa (N), tubular adenoma (TA), tubulovillous adenoma (TVA), well differentiated cancer (CWD), moderately differentiated cancer (CMD) and poorly differentiated cancer (CPD).

Figure 3.7 Northern blots : IGF-1R, c-myc, WT-1 and β–actin



N = normal mucosa for each specimen; TA = tubular adenomas; TVA = tubulovillous adenomas; CWD, CMD and CPD = well, moderately and poorly differentiated cancers

Figure 3.8 Northern blot :IGF-1R, c-myc, WT-1 and β–actin



N = normal mucosa for each specimen; TA = tubular adenomas; TVA = tubulovillous adenomas; CWD, CMD and CPD = well, moderately and poorly differentiated cancers

Table 3.2. Optical densitometry of Northern blots with IGF-1R normalised to β–actin

Values are expressed as optical densitometry units with a maximun (completely exposed / darkest reading) of 200 and minimum (nonexposed / lightest reading) of 0 for each blot. Means for normal (m^N) and tumours (m^C) and their standard deviations expressed at the foot of each column.

Specimen Fig. 3.7	β-actin	IGF-1R	Norm. IGF-1R	WT-1	Norm WT-1	c-myc	Norm c-myc
N*	173	43	40	27	25	11	10
TA	186	156	133	33	28	23	20
TA	144	129	142	31	34	20	22
N	191	41	34	34	28	29	24
TVA	144	146	161	37	41	20	22
CWD	181	174	153	59	59	68	60
N	156	45	46	61	62	25	25
CMD	151	155	163	62	65	36	40
N	141	44	50	19	21	17	19
CPD	163	72	70	53	52	47	46
N	127	27	34	31	39	18	23
CPD	154	85	88	48	50	54	56
	mean=159 SD=19.9		m^N=41, SD=7.2 m^C=130, SD=37		m^N=35, SD=17 m^C=47, SD=13		m^N=20, SD=6.1 m^C=38, SD=19
Specimen Fig. 3.8	β-actin	IGF-1R	Norm. IGF-1R	WT-1	Norm WT-1	c-myc	Norm c-myc
TA	115	39	37	28	27	20	19
N*	93	18	20	85	99.6	33	39
TA	103	47	50	25	26	21	22
TVA	126	29	25	38	32	31	27
N	141	23	18	30	23	23	18
CWD	108	65	64	79	80	74	75
N	92	19	23	37	44	20	24
CMD	97	84	94	65	73	57	64
	mean=109 S.D.=17.2		m^N=20, SD=2.5 m^C=53, SD=27		m^N=55, SD=39 m^C=48, SD=27		m^N=27, SD=11 m^C=41, SD=26

Figure 3.9 Densitometry of Northern blot (Figure 3.7): ***High levels of IGF-1R RNA expression occur in polyps and well and moderately differentiated cancers but not in the poorly differentiated cancers examined here.*** (95% confidence intervals were calculated for normal and tumoural mucosae as separate sample populations).

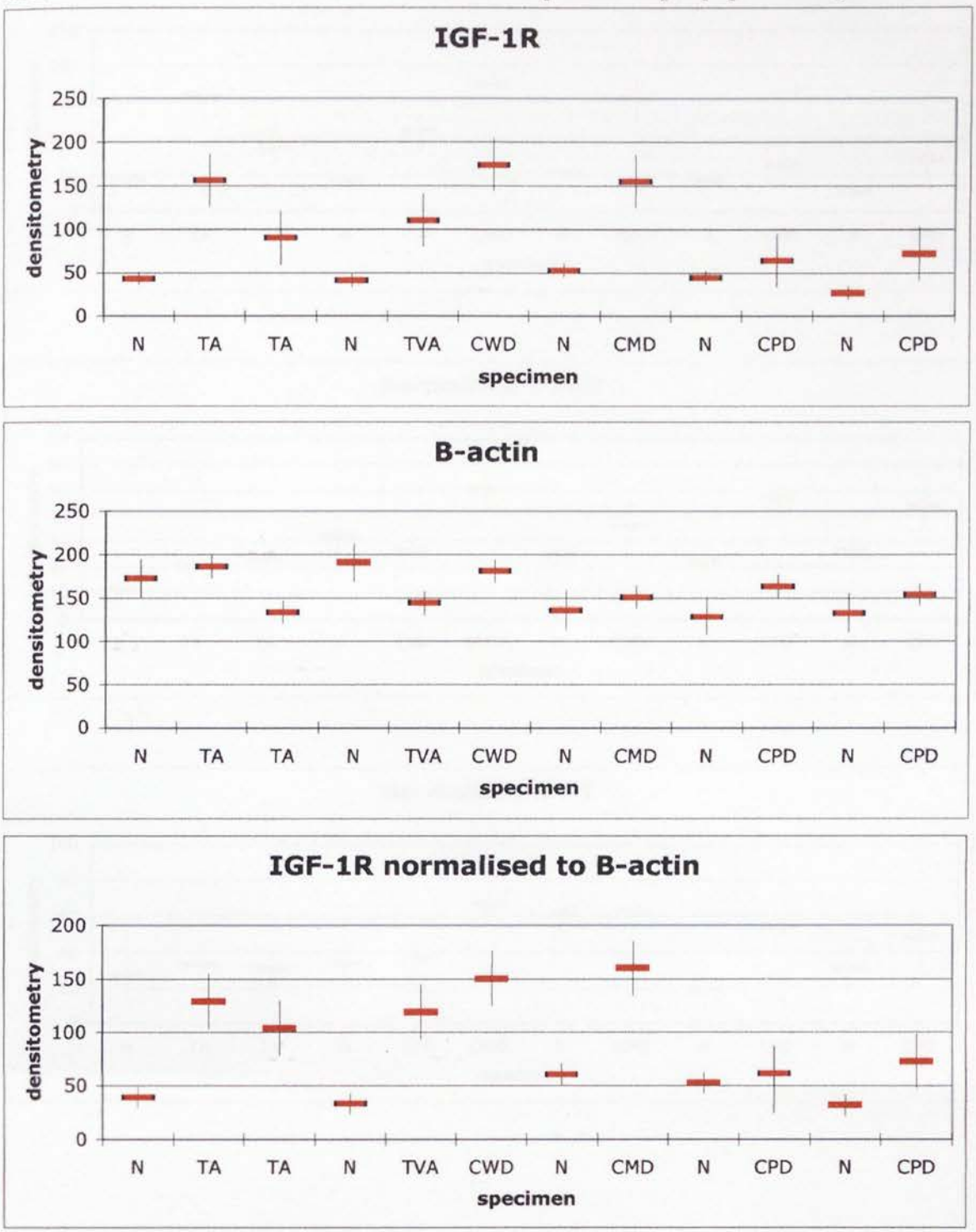
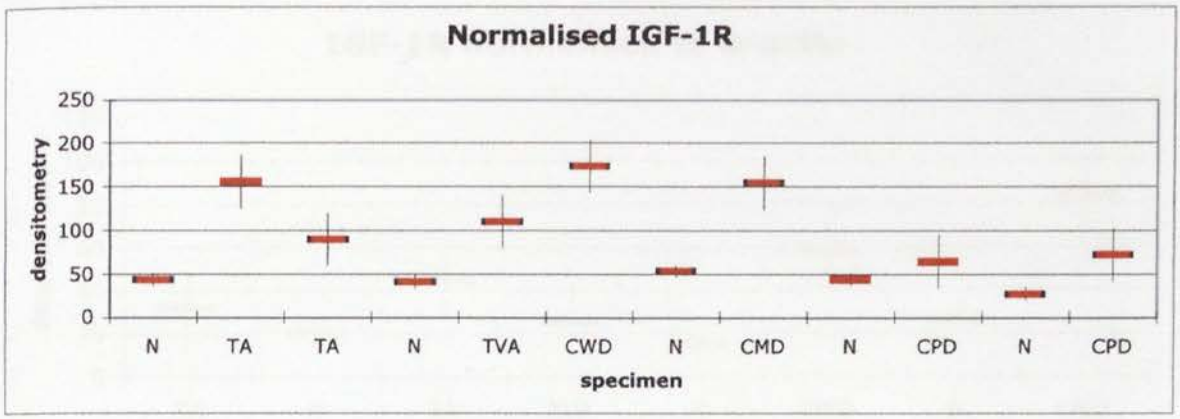
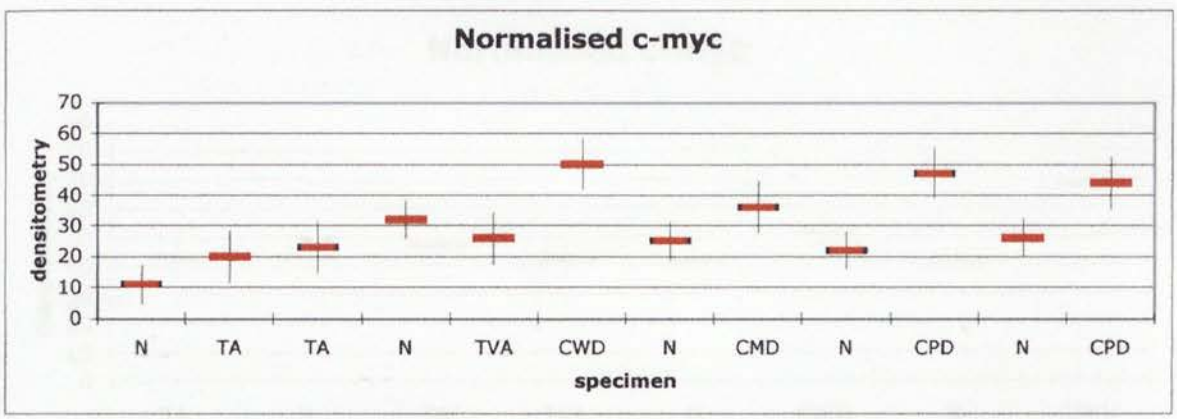


Figure 3.10 Densitometry of Northern blot (Figure 3.7) : ***High IGF-1R expression during colon neoplasia preceeding any expressive change in the trans regulators c-myc and WT-1***





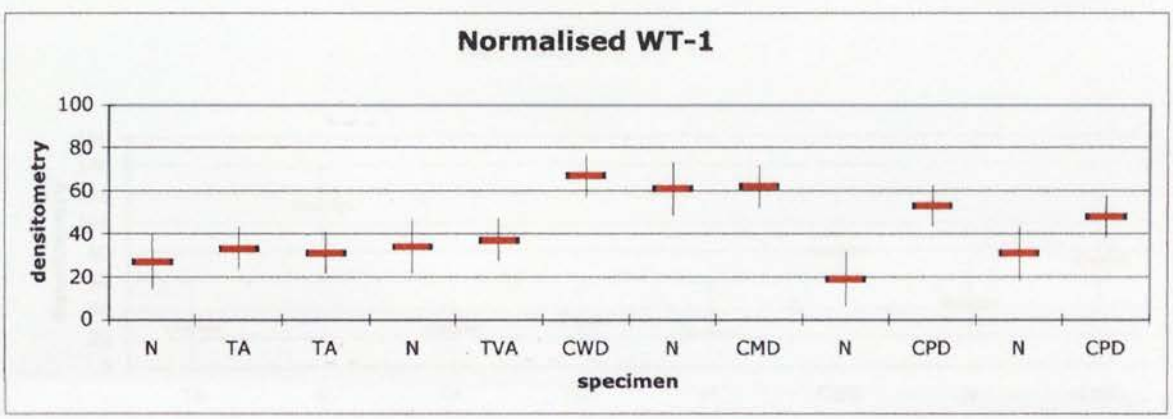
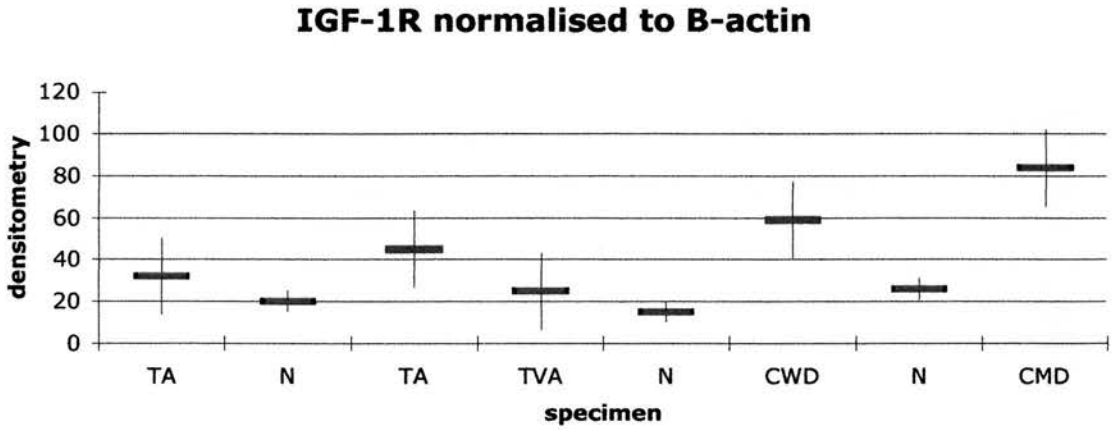
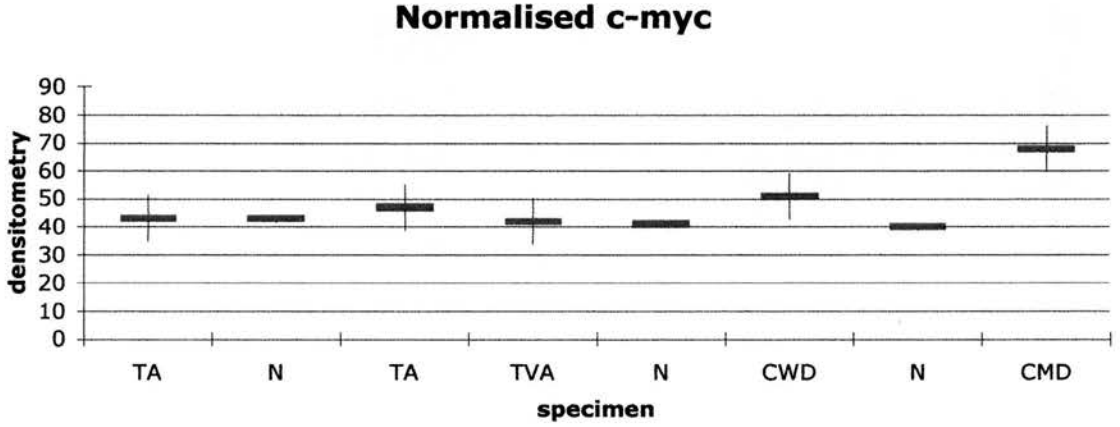
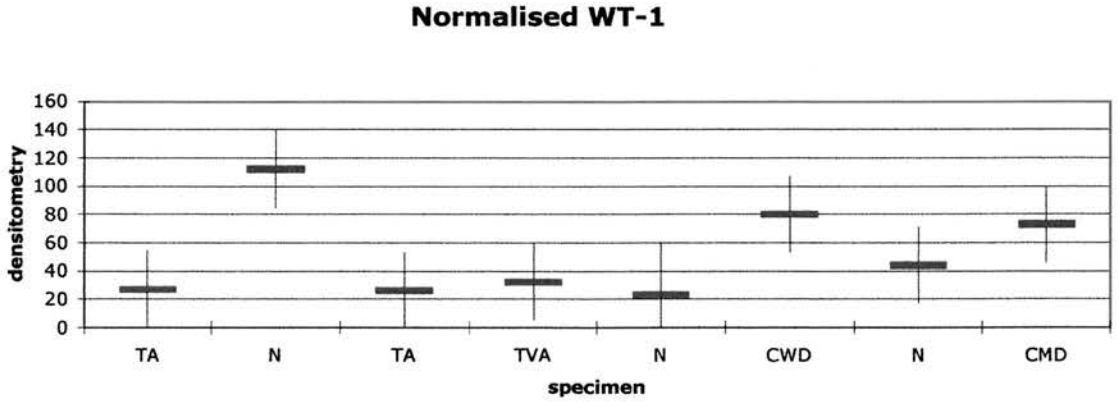


Figure 3.11 Densitometry of Northern blot (Figure 3.8): ***Tumour IGF-1R over expression preceeding any expression changes in c-myc and WT-1.***







The IGF-1R / β-actin normalised densitometry readings from the above Northern blots were combined by assuming equivalences in normal mucosal densitometries and a mean normal mucosal densitometry of integer 1.0. This gave the following values for normal and tumoural populations which can also be represented in the scatter diagram below :

Table 3.3 Statistical analysis of Northern blots: pooled normalised IGF-1R densitometries

1. N	0.98
2. TA	3.23
3. TA	3.45
4. N	0.83
5. TVA	3.91
6. CWD	3.71
7. N	1.12
8. CMD	3.96
9. N	1.21
10. CPD	1.69
11. N	0.83
12. CPD	2.13
13. TA	1.82
14. N	0.99
15. TA	2.46
16. TVA	1.23
17. N	0.88
18. CWD	3.15
19. N	1.13
20. CMD	4.63

Normal :	mean IGF-1R = 1.0 95% confidence interval = ± 0.29

Tumours:	mean IGF-1R = 2.94 95% confidence interval = ± 2.08

The wide range in tumoural densitometries meant that there was some overlap in the 95% confidence intervals for tumoural and normal mucosal specimens. However, when the two poorly differentiated cancers which expressed low IGF-1R levels (lanes 10 and 12 -1.69 and 1.82) and a specimen with degraded RNA (lane 16 – 1.23) are excluded, from the comparison, this results in :

Tumours :	mean IGF-1R = 3.40 95% confidence interval = ± 1.54

The majority of tumoural specimens (excepting some poorly differentiated cancers - see below) expressed significantly higher levels of the IGF-1R than normal mucosal specimens ($p<0.05$). On average, tumoural IGF-1R densitometry was 3.5 times that of normal mucosa.

There was comparatively little inter-specimen variability for IGF-1R expression for the majority of normal mucosal RNA specimens (mean 1.0, range 0.73 to 1.40, n=8). High molecular weight RNA degradation accounted for much of the variance.

The normalised IGF-1R densitometry from matched tumoural specimens in comparison had a much higher mean of 2.94 and wider range of 1.23 to 4.63, n=12. Again, loss of high molecular weight RNA integrity in one specimen (TVA in Figure 3.8) accounted for the low value of 1.23.

The histologic grading of the specimens revealed an interesting pattern when ranked to IGF-1R expression. Although all polyps and most cancers expressed high IGF-1R RNA levels, the poorly differentiated cancers examined displayed a lesser degree of expression albeit at a higher level than normal mucosa. The poorly differentiated cancers with lower levels of IGF-1R expression also exhibited a more invasive morphology and had areas of necrosis in comparison to poorly differentiated cancers with higher levels of IGF-1R expression.

This pattern of high IGF-1R RNA expression in adenomas and most cancers but comparatively lower levels in some poorly differentiated cancers ran somewhat contrary to the pattern envisaged from the reporter assays as discussed in the introduction. The blots were therefore sequentially rehybridised for the IGF-1R trans-regulators c-myc and WT-1.

C-myc expression was found to be higher in all the cancer specimens in keeping with the reports from previous studies (Melhem, M.F.,1992). This result was unaltered when normalised to β-actin. However, there was no correlation between c-myc expression and that of the IGF-1R. In fact, some poorly differentiated cancers expressing high levels of c-myc expressed the lowest IGF-1R levels for tumoural specimens. Moreover, high IGF-1R expression preceded c-myc expression in all of the tubular and tubulovillous adenomas examined. Normalising IGF-1R expression to that of c-myc simply exaggerated the expression profile that was observed in most tumours.

The blots were then examined for expression of the Wilms' tumour suppressor. Although the WT-1 promoter has been found to be hypermethylated in some colorectal cancers (Hiltunen, M.O., 1997), this study demonstrated no significant difference for WT-1 expression between normal and tumoural specimens and certainly no correlation to IGF-1R expression.

In summary, IGF-1R RNA was expressed at high levels in colo-rectal adenoma and cancer specimens in comparison to matched normal mucosa specimens (from the same resected specimen). However, the poorly differentiated cancer specimens examined, although they expressed IGF-1R RNA at levels higher than their matched normal mucosa, they expressed IGF-1R RNA at levels lower than those moderately and well differentiated cancers and polyps examined. This pattern of IGF-1R expression during colo-rectal neoplasia does not seem to be explained by the observed expression profiles of the *trans* acting c-myc and WT-1. Also, it is difficult to envisage that p53 mutation, a comparatively late event in colo-rectal neoplasia (Rodrigues, N.R., 1990) could instead account for the observed early expression of the IGF-1R during the neoplastic process.

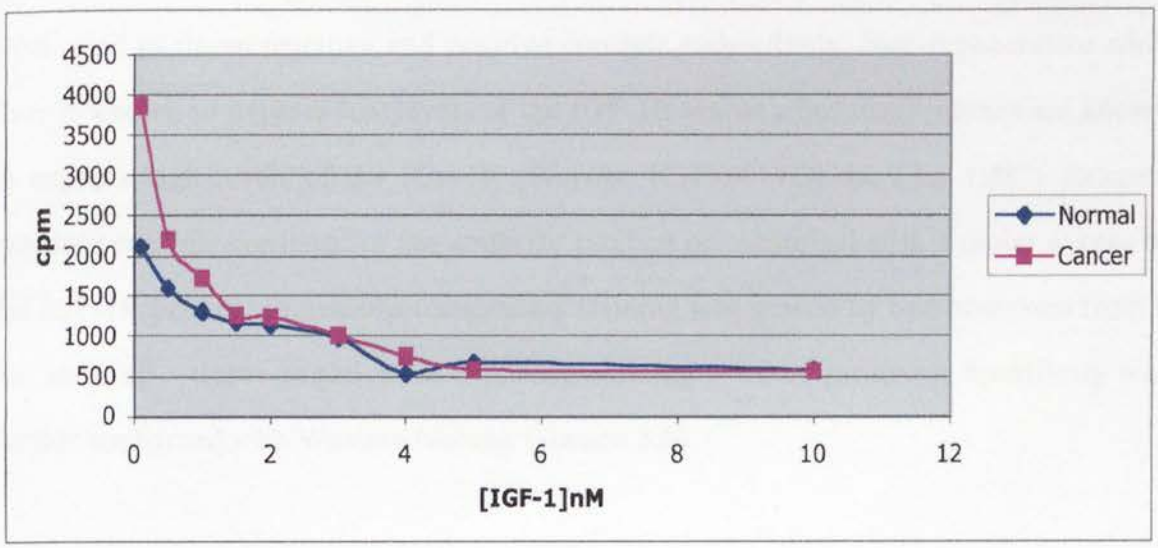
3.4. Radioligand binding analysis

IGF-1R expression at the protein level was next analysed by way of radioligand binding using tissue microsomal preparations of normal and cancer mucosae. However, there were considerable variations noted in the IC_{50} within and between the specimens. The errors inherent in the microsomal preparation technique therefore precluded a meaningful comparison between different tissues and the method was abandoned.

Table 3.4 Radioligand binding : competitive assay. 500ug aliquots of normal and cancer microsomal preparations were incubated with 2nM ^{125}I-IGF-1 with and without increasing concentrations of unlabelled IGF-1 in triplicate to determine the mean specific binding values below (non-specific binding was that which was not displaced by a 100 fold excess of non-labelled IGF-1).

[IGF-1]nM	Normal	Cancer
0.1	2120	3867
0.5	1593	2203
1	1299	1724
1.5	1171	1261
2	1138	1243
3	974	1009
4	523	748
5	670	583
10	578	561

Figure 3.12 Radioligand binding competitive assay : demonstrating IGF-1 specific binding but no detectable difference between normal and cancer microsomal preparations.



3.5. Immunohistochemistry

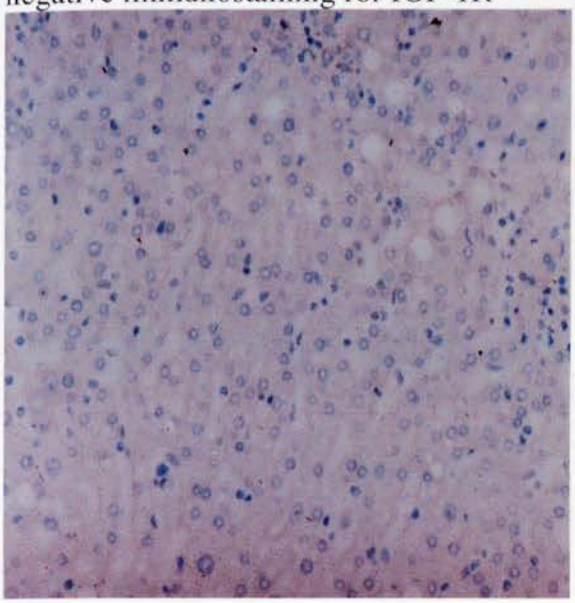
IGF-1R expression in the polyp-cancer sequence was examined at the protein level by means of immunohistochemistry. Archival material was used as this allowed analysis of a greater range of specimens including aberrant crypt foci. Each assay was verified by tissue and reagent positive and negative controls. Liver and kidney specimens were used as tissue negative and positive controls respectively. Non-regenerative adult liver is known to express low levels of the IGF-1R whilst adult renal tubules are known to express high levels of the IGF-1R (Werner, H.,1989) (Ojeda, J.L., 1997). Reagent negative controls consisted of the antibody product pre-absorbed with a molar excess of the IGF-1R peptide. Immunohistochemistry staining was graded by two observers from 0 (no staining – tissue negative) to 5 (strong staining – tissue positive). Specificity was further confirmed with Western blotting (section 3.6).

3.5.1. Immunohistochemistry : controls

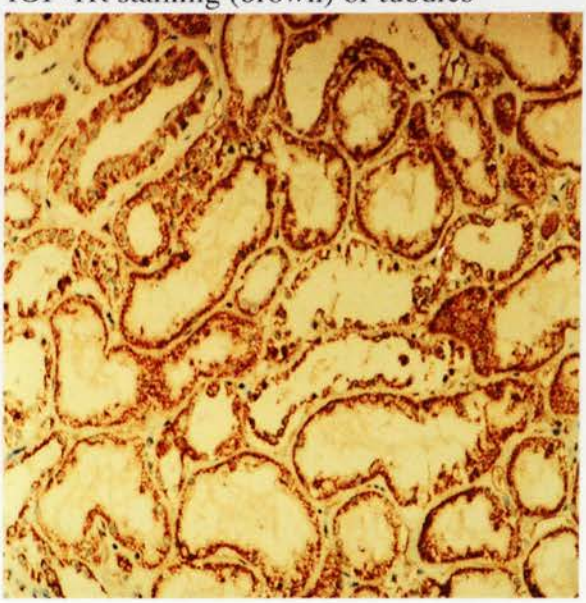
Preliminary assays were used to determine the sensitivity and specificity of immunostaining of the anti-IGF-1R polyclonal for tissue positive (kidney) and tissue negative (liver) controls and reagent-negative (peptide-absorbed) controls. The pattern of immunostaining observed agreed closely with these controls - liver immunostaining was absent (graded 0) and kidney immunostaining was strongly positive (graded 5) (Figure 3.11). Immunostaining occurred in both the membrane and cytoplasmic pools of the tissue positive controls, a pattern in keeping with other growth factor receptors. The reagent negative (peptide absorbed) controls showed no immunostaining of the tissue positive (kidney) controls.

Figure 3.13 Immunohistochemistry : controls

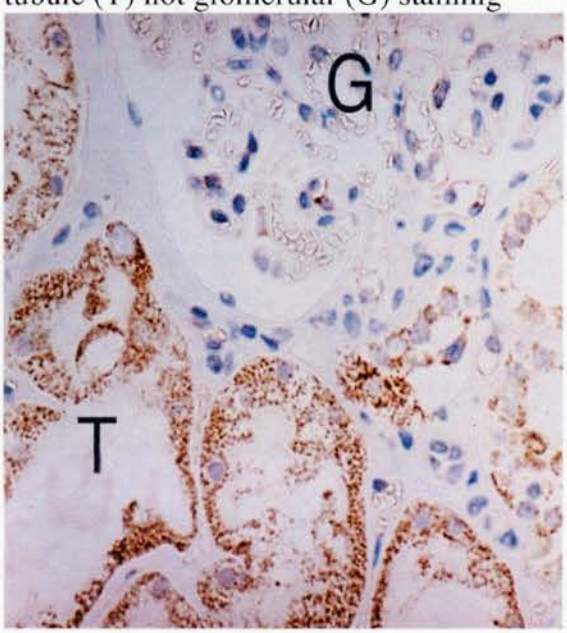
Liver, x40 (tissue –ve control) displaying negative immunostaining for IGF-1R



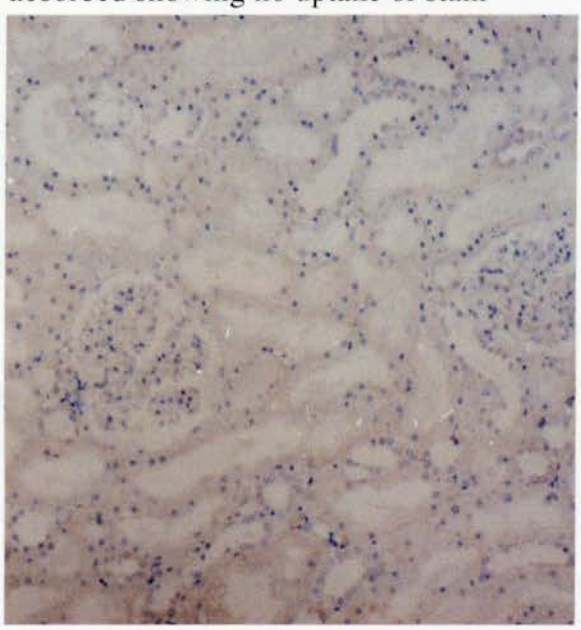
Kidney, x40 (tissue +ve control) showing IGF-1R staining (brown) of tubules



Kidney (tissue +ve) at high power showing tubule (T) not glomerular (G) staining



Kidney (tissue +ve) / reagent –ve peptide absorbed showing no uptake of stain



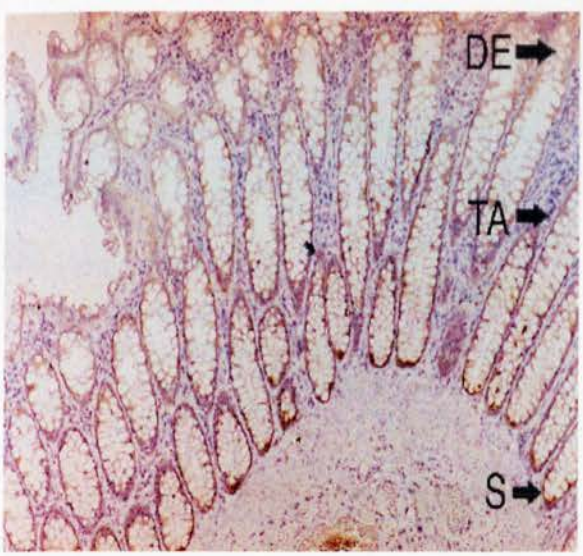
3.5.2. Immunohistochemistry : Normal mucosa

IGF-1R immunostaining of the cells in the normal colonic crypt was found to correlate with the cells' position in the crypt and consequently with their morphology. The cells of the basal colonic crypt immunoexpressed high IGF-1R levels (graded 4/5) in their cytoplasms and membranes whereas the cells of the mid and upper colonic crypt immunoexpressed low IGF-1R levels (graded 0-2) in their cytoplasms and membranes (Figure 3.14). The anatomy and cell morphology of the normal colonic crypt has been described in detail previously (Reviewed- Potten, C., 1990). The basal crypt cells have been characterised as proliferative stem cells whereas the mid and upper crypt cells have been described as transit amplifying (proliferating) and terminally differentiated cells respectively. The cells in these different crypt regions are all aligned on the basement membrane with a pallisaded appearance but have differing morphologies (and differing IGF-1R expressions). The basal crypt cells (with high IGF-1R levels) have a relatively undifferentiated morphology and are partially polarised with nuclei that are aligned a slight distance "off" the basal aspect of the cell membrane (Figure 3.14). In contrast, the cells of the upper crypt (with low IGF-1R levels) are terminally differentiated (columnar, goblet, endocrine and Paneth cells) and are fully polarised with nuclei that are aligned "on" the basal aspect of the cell membrane. The cells of the mid crypt (transit amplifying or proliferating cells) show intermediate degrees of differentiation and polarisation with nuclei that are either "on" or "off" the basal membrane. These cells, regardless of their degree of differentiation or nuclear polarisation, expressed low IGF-1R levels (graded 0-2). The basal crypt stem cells therefore appeared unique in their expression of high IGF-1R levels in the normal colonic crypt. The observed pattern of reduced IGF-1R expression as basal crypt cells migrate to the mid and upper crypts was seen in all of 12 normal mucosa specimens where the entire crypt was visible and in all of 55 normal mucosa specimens where only the upper crypt was visible.

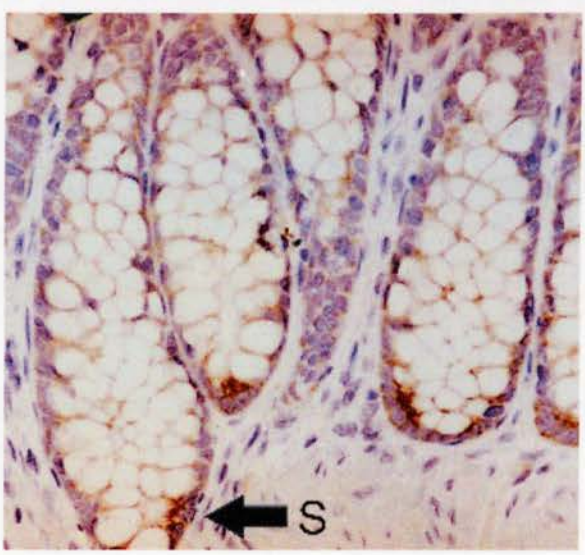
Figure 3.14 IGF-1R immunostaining : the normal colonic crypt

Normal crypt (low power, x40)

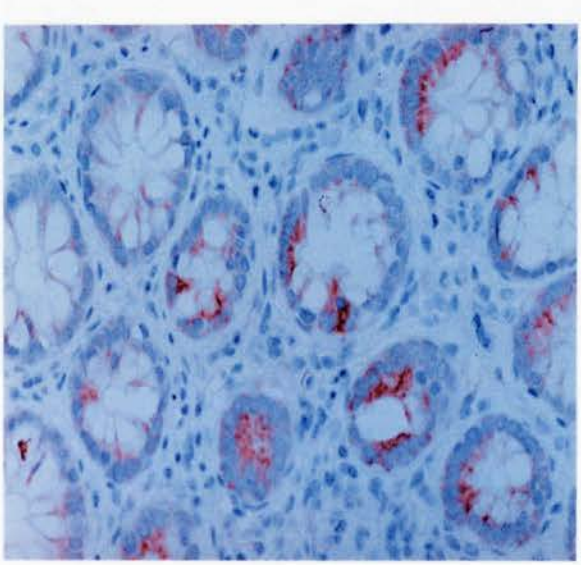
IGF-1R immunostaining (brown) of basal crypt stem cells (S) but not transit (TA) or differentiated (DE) cells



Normal crypt (high power, x200)



Normal basal crypt in transverse section (x200) Staining of stem cell component



Normal control (peptide absorbed, x40)

No stainining of base (ie IGF-1R specific)

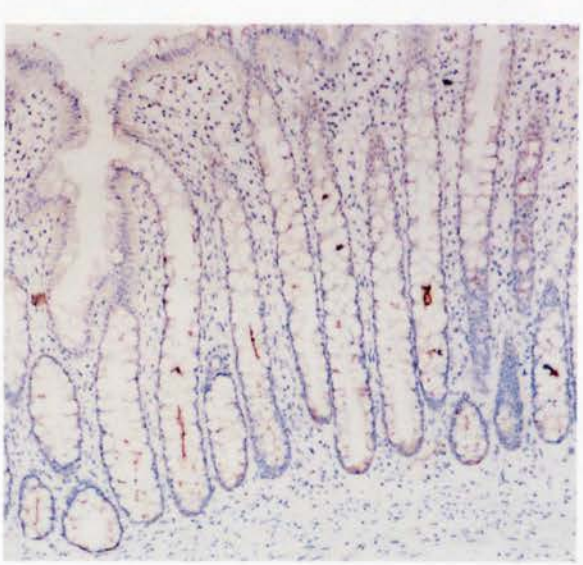
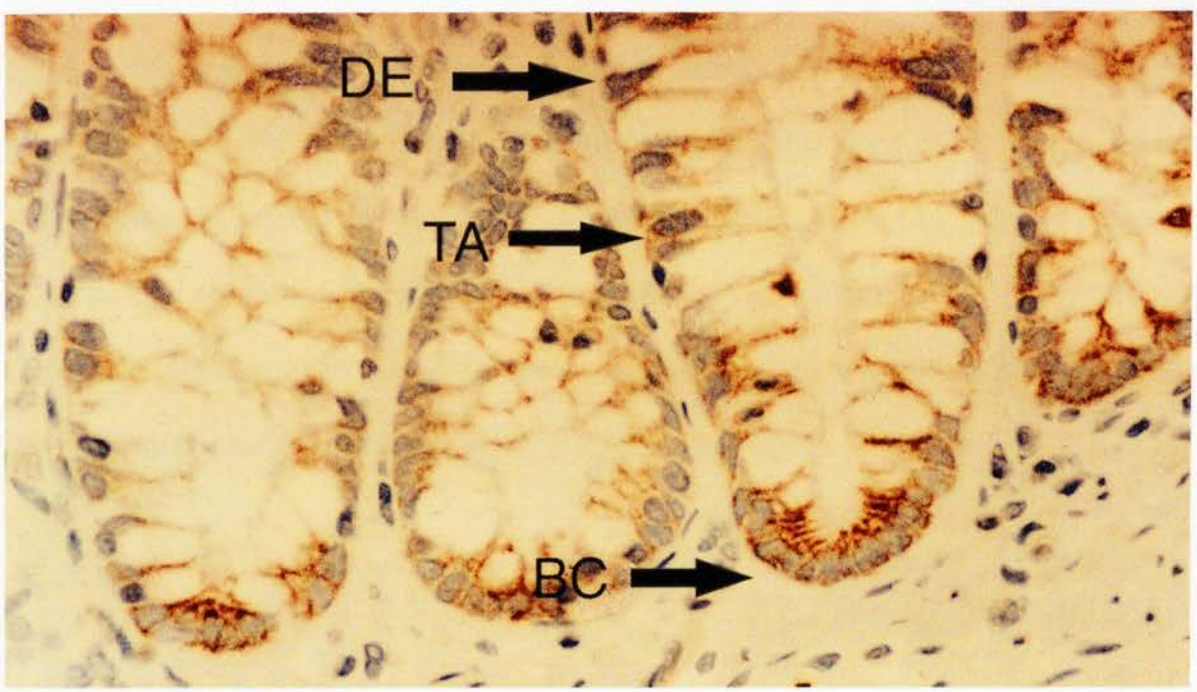


Figure 3.14 IGF-1R immunostaining : the normal colonic crypt

Normal crypt (high power, x200). Basal crypt stem cells (BC) immunoexpress high IGF-1R levels (brown stain). They have an undifferentiated and partially polarised morphology with nuclei positioned "off" the basal membrane. Transit amplifying cells (TA) immunoexpress low IGF-1R levels (low levels of brown staining). They have a more differentiated columnar epithelial morphology and are partially or fully polarised with nuclei positioned "off" or "on" the basal membrane. Differentiated enterocytes (DE) in the upper crypt immunoexpress low IGF-1R levels. They are terminally differentiated and fully polarised.

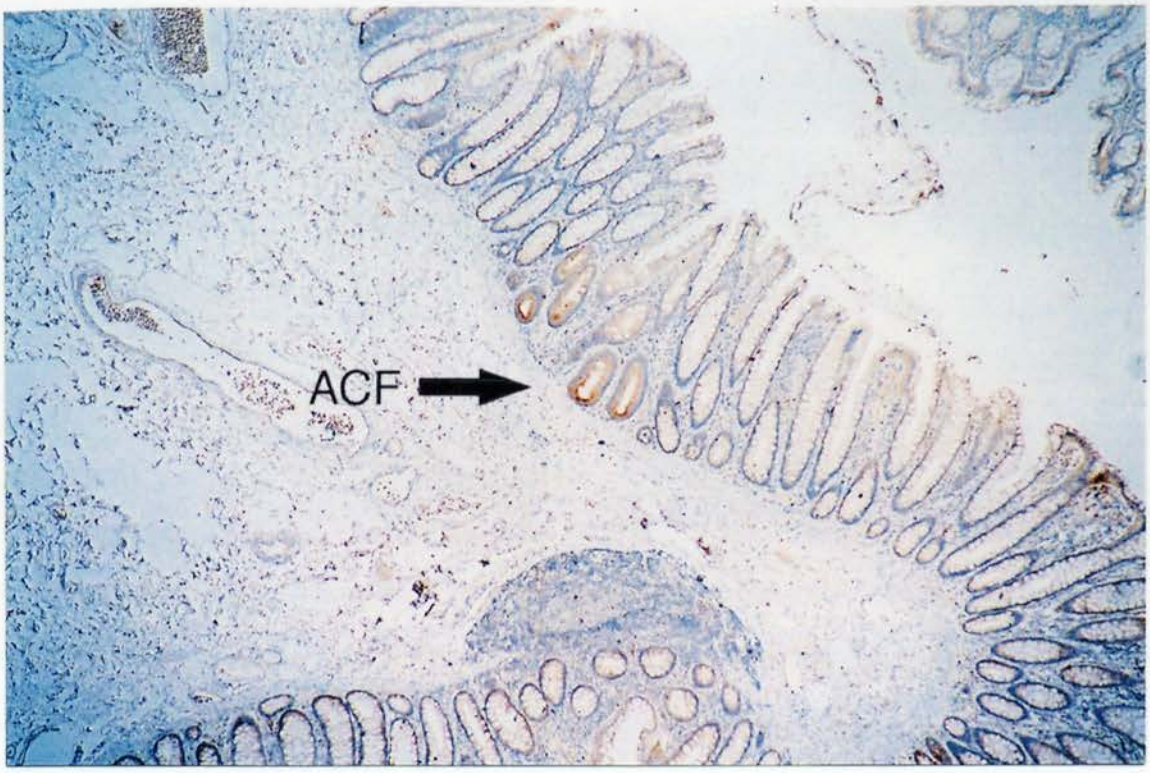


3.5.3. Immunohistochemistry : The aberrant crypt focus

The histologic features of aberrant crypt foci and their likely role as neoplastic precursors have been described previously (McLellan EA, 1991). In this study all 5 of the aberrant crypt foci examined were found to immunoexpress the IGF-1R at high levels throughout the length of their crypt axes (Figure 3.15). This is in contrast to normal colonic crypts where only the basal crypt stem cells exhibited IGF-1R immunostaining with a sharper demarcation between basal and mid crypt regions. There was to a limited degree a gradient in IGF-1R immunoexpression along the aberrant crypt axis with slightly higher immunoexpression at the base (graded 4/5) versus the upper crypt regions (graded 3/4). However, there was no obvious correlation between IGF-1R immunoexpression and the degree of cellular differentiation in the ACF. That is, differentiated cells in the upper ACF immunoexpressed similar IGF-1R levels to undifferentiated cells in the upper ACF. All the cells in the ACF had a partially polarised morphology (like those of the normal crypt basal region) and so the higher IGF-1R immunoexpression might simply reflect the expansion in the zone of proliferation in the ACF as documented in previous studies (McLellan EA, 1991).

Figure 3.15 IGF-1R immunostaining : normal crypts and the aberrant crypt focus

Low power, x40.

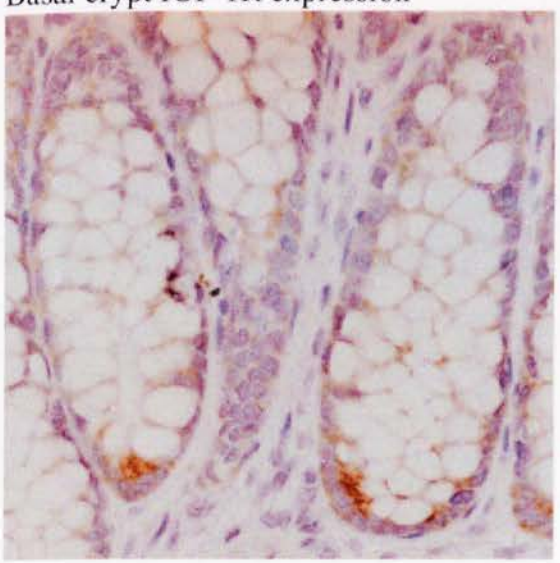


High levels of IGF-1R are noted in the ACF as opposed to the normal crypts.

Figure 3.15 IGF-1R immunostaining : normal crypts and the aberrant crypt focus

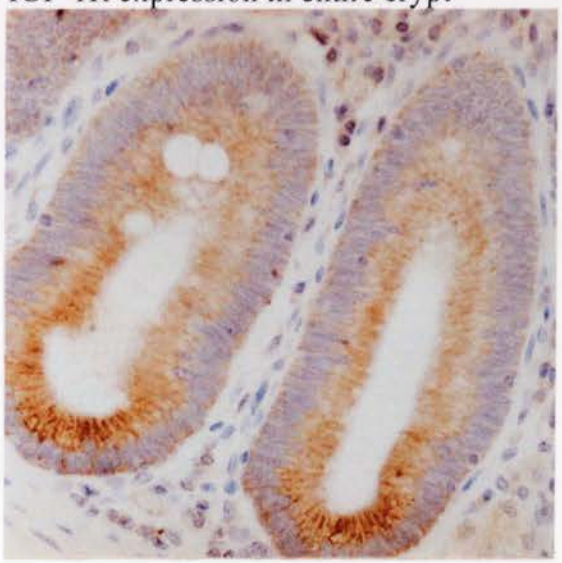
Normal crypt (high power, x200)

Basal crypt IGF-1R expression



Aberrant crypt focus (high power, x200)

IGF-1R expression in entire crypt



Normal colonic crypts show IGF-1R immunostaining confined to the basal crypt region. In comparison, aberrant crypt foci show IGF-1R immunostaining throughout the crypt length albeit with a gradient in immunoexpression to a limited degree.

3.5.4. Immunohistochemistry : colonic polyps

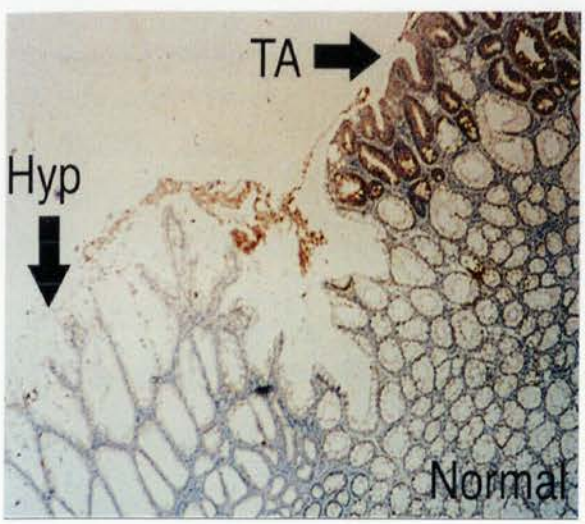
Adenomatous polyps also immunoexpressed high IGF-1R levels. In agreement with the Northern blots, all of the 12 tubular adenomas and all of the 11 tubulovillous adenomas examined were found to immunoexpress high IGF-1R levels (graded 4/5). In the normal mucosa that lay adjacent to these polyps, the normal basal crypt regions immunoexpressed high IGF-1R levels whilst the normal mid and upper crypt regions immunoexpressed low IGF-1R levels. The adenomatous crypts (unlike the aberrant crypts) showed no concentration gradient in IGF-1R immunostaining along the crypt axis. However, like the aberrant crypt cells, the adenomatous crypt cells had a partially polarised morphology. Also, there was uniform IGF-1R immunostaining within and

between adenomatous polyp specimens with no correlation in immunostaining levels to histologic features such as the degree of cellular differentiation, cytoplasm content or dysplasia.

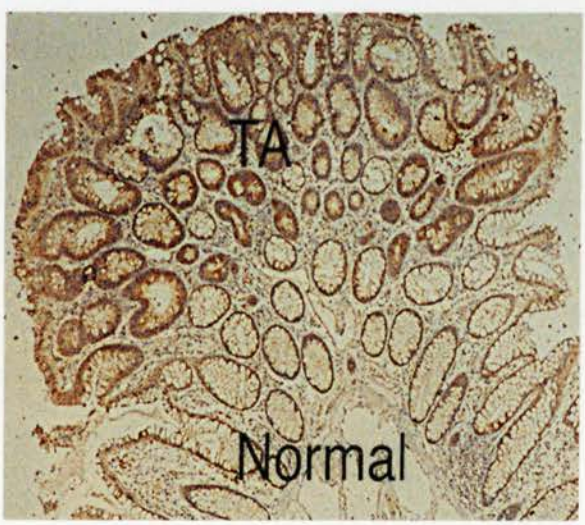
In contrast to aberrant crypt foci and adenomatous polyps, simple hyperplastic polyps did not express the IGF-1R in their mid and upper crypt regions but only in their basal crypt regions in a pattern similar to that found in the normal crypt.

Figure 3.16 IGF-1R immunostaining : adenomatous and hyperplastic polyps

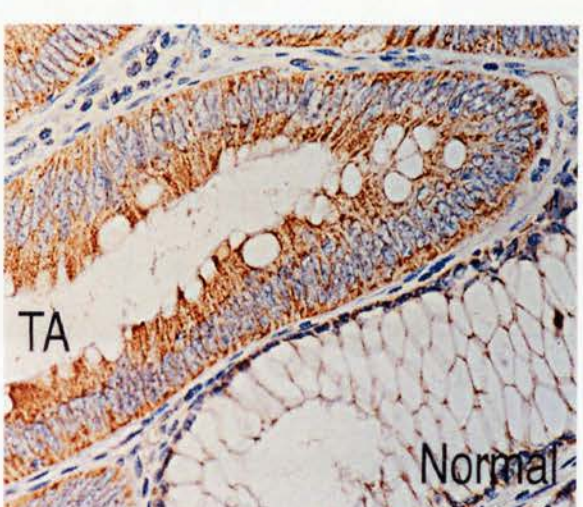
Hyperplastic polyp (Hyp), tubular adenoma (TA) and normal mucosa
Low power, x40
IGF-1R stain in tubular adenoma only



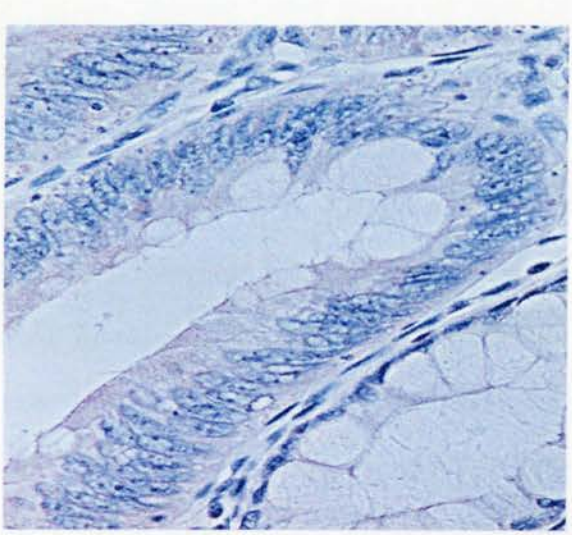
Tubular adenoma (TA) and normal mucosa
Low power, x40
IGF-1R stain in tubular adenoma



Tubular adenoma (TA) and normal crypt at high power, x200 showing high IGF-1R expression in adenomatous crypt



Tubular adenoma and normal crypt peptide absorbed reagent –ve control

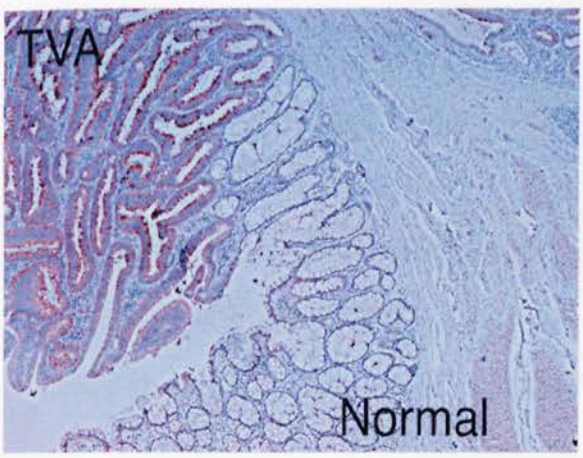


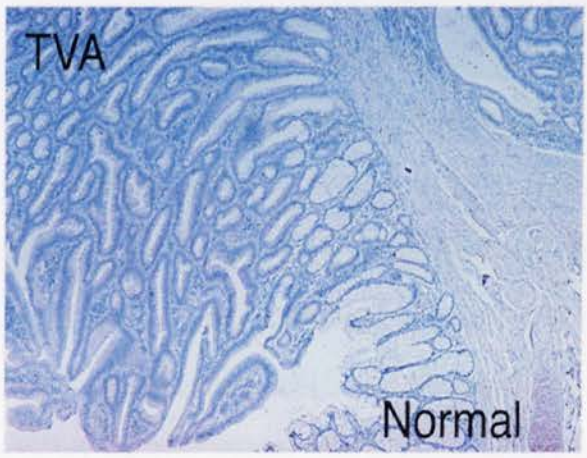
Tubulovillous adenoma (TVA), x40

Showing IGF-1R stain in TVA

Tubulovillous adenoma peptide absorbed

reagent –ve control showing no staining





3.5.5. Immunohistochemistry : colonic carcinomas

IGF-1R immunoexpression occurred at high levels in most but not all colonic cancers where immunoexpression was found to correlate with specific cancer cell morphologies. High levels of IGF-1R immunoexpression (graded 4/5) were found in 6 out of 6 of the well differentiated cancers examined, 7 out of 7 of the moderately differentiated cancers examined and 3 out of 8 of the poorly differentiated cancers examined (Figures 3.17-23). 5 out 8 of the poorly differentiated cancers examined as well as foci in well and moderately differentiated cancers showed lower levels of IGF-1R immunoexpression (graded 0 to 2), and this was found to correlate with two specific cancer cell morphologies:

1. Low levels of IGF-1R immunostaining were observed in highly polarised epithelial-type cancer cells in areas showing morphological features suggestive of epithelial-mesenchymal transformation. In the invasive foci of well and moderately differentiated cancers, there was a transition zone between epithelial and mesenchymal morphologies (suggesting epithelial mesenchymal transformation or EMT). This transition zone contained discrete groups of four or more highly polarised epithelial-type

cancer cells showing low levels of IGF-1R immunoexpression. These epithelial-type cancer cells with low IGF-1R immunoexpression had highly polarised nuclei aligned "on" the basal membrane in contrast to the majority of epithelial-type cancer cells with high IGF-1R immunoexpression which had partially polarised nuclei aligned "off" the basal membrane (Figures 3.21 and 3.23). Basally aligned nuclei were a feature of post-mitotic cells in the normal colonic crypt but in cancerous crypts such cells showed mitotic features such as mitotic bodies. Also, and unlike programmed differentiation in the normal colonic crypt, epithelial-type cancer cells showing basally aligned nuclei appeared to be the precursors of and dedifferentiate into mesenchymal-type cancer cells. This was suggested by the intermediate cancer cell morphologies which lay adjacent to and between these polarised epithelial-type cancer cells and mesenchymal-type cancer cells. Like the mesenchymal-type cells, if these intermediate cell types immunoexpressed low IGF-1R levels they had a disordered morphology with loss of cell-cell contact whereas if they immunoexpressed high IGF-1R levels they had a more ordered morphology with maintained cell-cell contact (see below).

2. Low levels of IGF-1R immunostaining were observed in mesenchymal-type cancer cells with the loss of cell-cell adhesion. In the invasive foci of well, moderately and poorly differentiated cancers (in 5 out of 8 of the poorly differentiated cancers which had a mostly invasive morphology), the cancer cells showed low levels of IGF-1R immunoexpression (graded 1 to 2). This reduction in IGF-1R immunoexpression was observed in invasive mesenchymal-type cancer cells that had lost cell-basement membrane or cell-cell adhesion (Figures 3.18-23). In contrast, adjacent mesenchymal-type cancer cells that maintained cell-basement membrane or cell-cell adhesion maintained high levels of IGF-1R immunoexpression (graded 4 to 5). The mesenchymal-type cells that had lost cell-cell adhesion (and IGF-1R expression) had a higher rate of cell necrosis but were otherwise morphologically similar to the mesenchymal-type cells that maintained cell-cell adhesion (and IGF-1R expression). That is, there were no other

morphologic differences in terms of nuclear pleomorphism, heterochromatin, multiple nucleoli or mitotic bodies between these two cell groups.

Interestingly, a repeating pattern of cell morphology and IGF-1R expression was observed in these cell types at foci which showed the morphological features of EMT and invasion. Discrete groups of four or more mesenchymal-type cancer cells showing cell-cell adhesion and high IGF-1R immunoexpression alternated with adjacent groups of four or more mesenchymal-type cancer cells showing loss of cell-cell adhesion and low IGF-1R expression. This occurred in a cyclical pattern at the invasive foci of cancer specimens. A similar cyclical pattern was observed for epithelial type cancer cells showing basal nucleus polarity. These cyclical patterns in IGF-1R expression suggest that IGF-1R expression can be cell cycle dependent in these unique instances. Notably, this pattern did not occur for other cell types in other specimens. In turn, this cyclical pattern of expression suggests that IGF-1R expression is controlled in part by chromatin remodelling (see discussion).

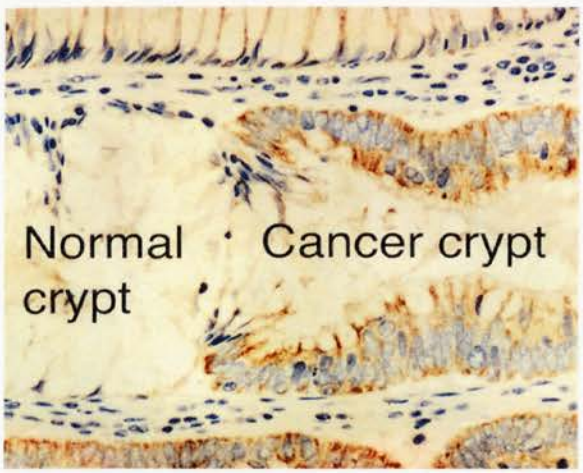
Although cancer cells immunoexpressed low IGF-1R levels during invasion, established metastases (in lymph nodes or in liver) expressed IGF-1R levels in accordance with their morphology (ie they expressed high IGF-1R levels unless showing a highly polarised morphology or loss of cell-cell adhesion). Mesenchymal-epithelial transformation was not seen in the specimens of metastases examined but was discerned in small foci adjacent to areas of EMT in primary cancers where IGF-1R expression was again in accordance with cell morphology.

Uniform immunostaining was observed in all specimens, again in accordance with cell morphology. The above immunostaining findings were verified by internal controls (adjacent normal crypts, both basal and upper crypt regions), by reagent negative controls (peptide absorbed), and by tissue positive and negative controls as before (Figures 3.17-23). Importantly, the immunohistochemistry findings are in agreement with

those of the Nothern analyses where poorly differentiated cancers with a predominantly invasive morphology showed lower levels of IGF-1R immunoexpression.

Figure 3.17 IGF-1R immunostaining : cancer / well differentiated

Well differentiated cancer showing IGF-1R staining in cancer crypt but not normal crypt. High power, x 200



Well differentiated cancer (CWD) peptide absosrbed control.
High power, x 200

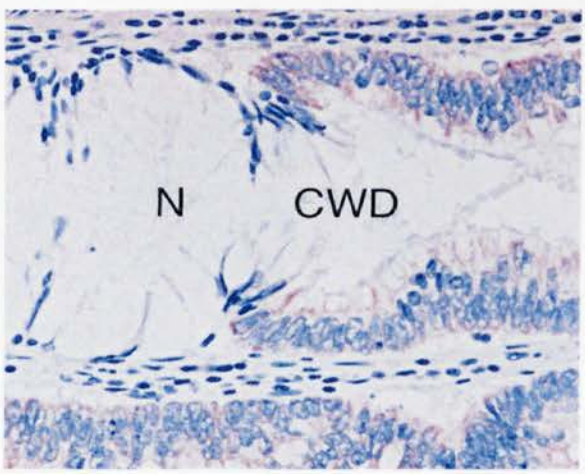
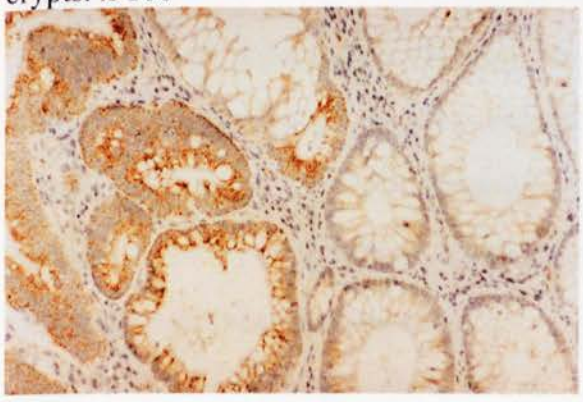
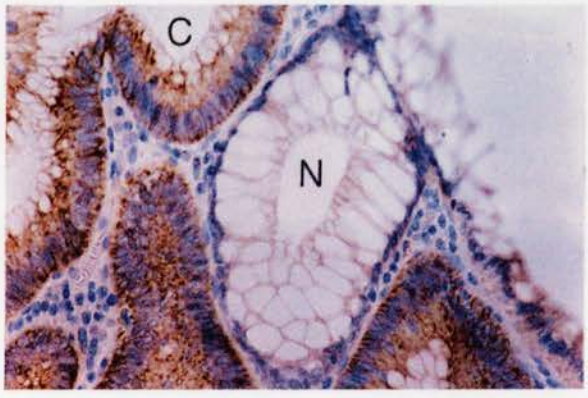


Figure 3.18 IGF-1R immunostaining : cancer / moderately differentiated

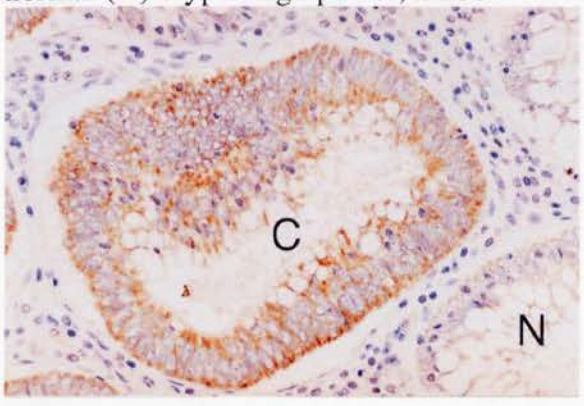
Moderately differentiated cancer and normal crypts. x 100



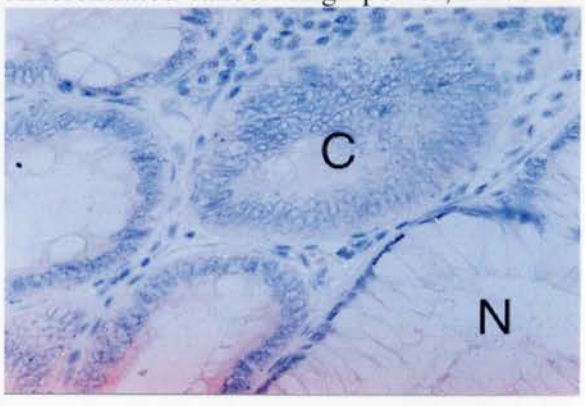
High power, x 200



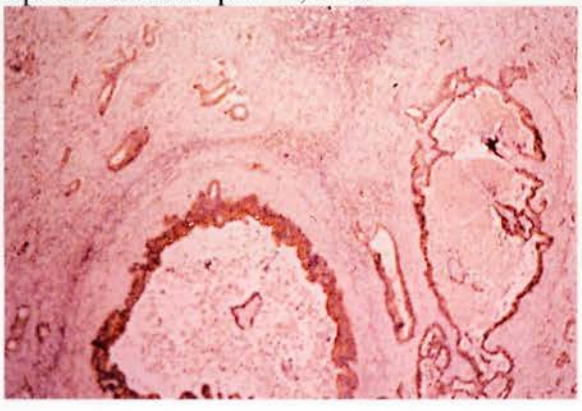
Moderately differentiated (C) and normal (N) crypt. High power, x 200



Peptide absorbed control of moderately differentiated cancer. High power, x 200



Lymph node metastasis from the above Specimen. Low power, x 40



Peptide absorbed control

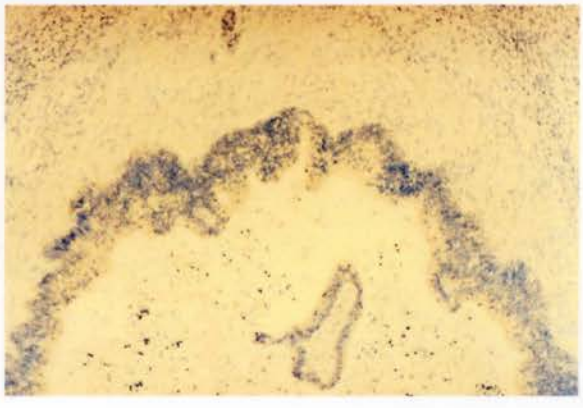


Figure 3.19a IGF-1R immunostaining : cancer / moderately and poorly differentiated. High power, x 200.

The poorly differentiated (CPD) part of the specimen shows low IGF-1R levels and a disordered and invasive morphology. In comparison, the moderately differentiated (CMD) part of the specimen shows high IGF-1R expression and a more ordered "picket fence" morphology with cells aligned on a basement membrane. Apoptotic bodies are arrowed in the CPD part of the specimen but can be seen in both areas where the cells have a more disordered morphology.

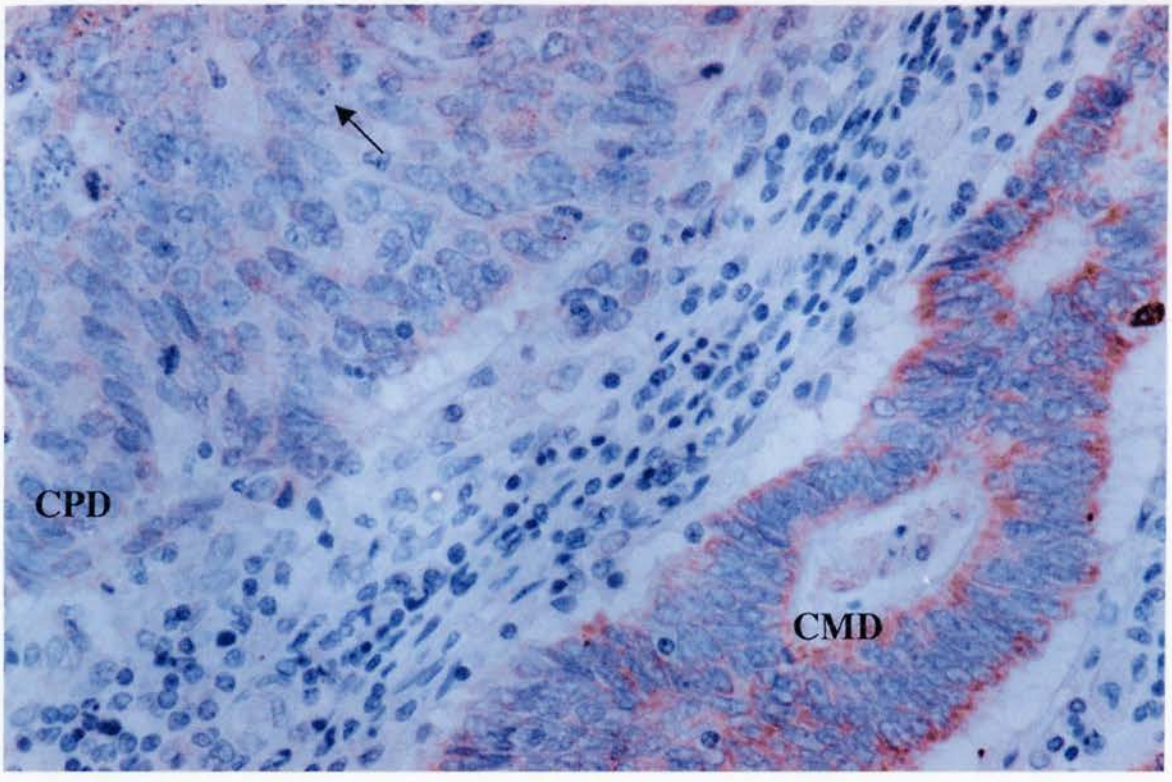


Figure 3.19b IGF-1R immunostaining : normal mucosa and poorly differentiated cancer High power, x 200.

Poorly differentiated cancer (CPD) showing reduced IGF-1R immunoexpression where cells loose their cell-cell adhesion and have a more disordered morphology (b) in comparison to cells showing high IGF-1R immunoexpression where they maintain cell-cell adhesion and have a more ordered morphology (a). The normal upper crypt shows low levels of IGF-1R immunostaining.

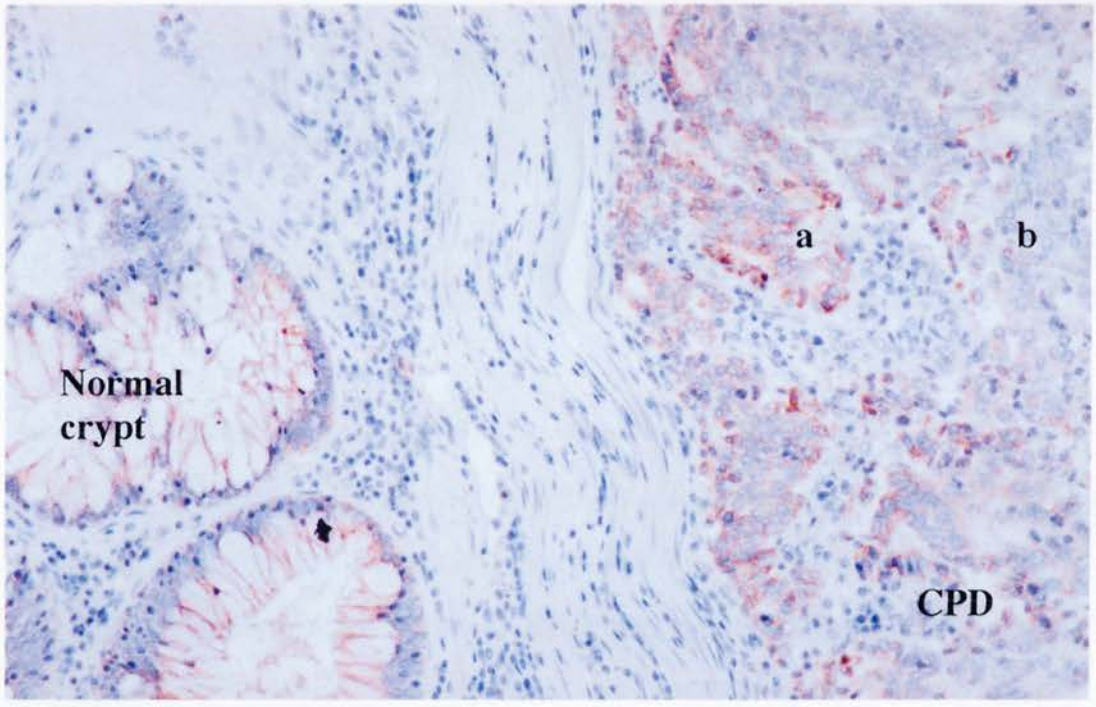
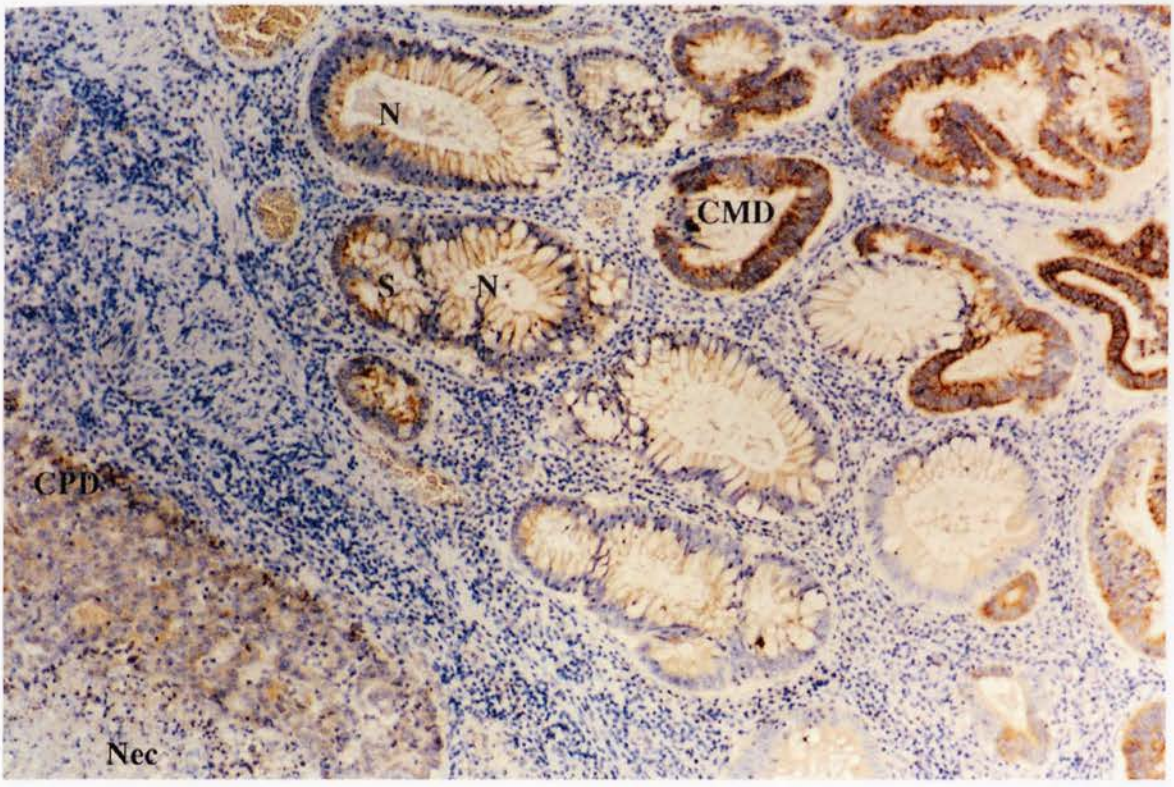


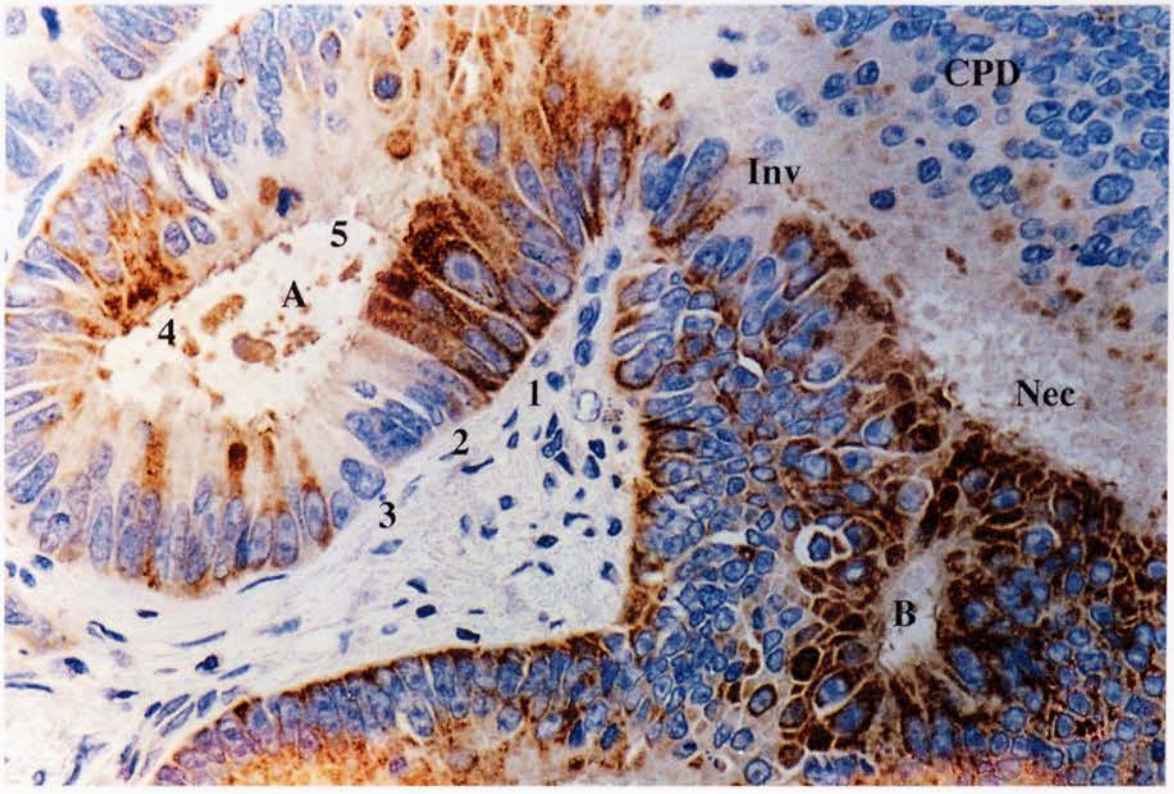
Figure 3.20 IGF-1R immunostaining : cancer differentiation and necrosis. x 100



The above specimen is predominantly a moderately differentiated cancer (CMD) but with areas of poorly differentiated cancer (CPD) and adjacent normal mucosa (N) containing the basal crypt stem cell region (S). There are clear differences in IGF-1R expression according to histologic type and cell morphology. High IGF-1R levels (graded 4,5) are expressed in the moderately differentiated cancer (CMD) and low levels (graded 1/2) are expressed in the poorly differentiated cancer (CPD) which is noted to have an invasive and disordered morphology with areas of necrosis (Nec). The crypts labelled "N" are from the upper differentiated parts of normal colonic crypts. Their cells display normal secretory glandular differentiation and have no significant IGF-1R expression. The crypt labelled "S" represents the lower stem cell containing part of normal crypt. The cells can

be seen to express a high level of the IGF-1R which is reduced upon differentiation to transit amplifying and differentiated enterocytes in the upper crypt (N).

Figure 3.21 IGF-1R immunostaining : variation in morphology and invasion. High power, x 200

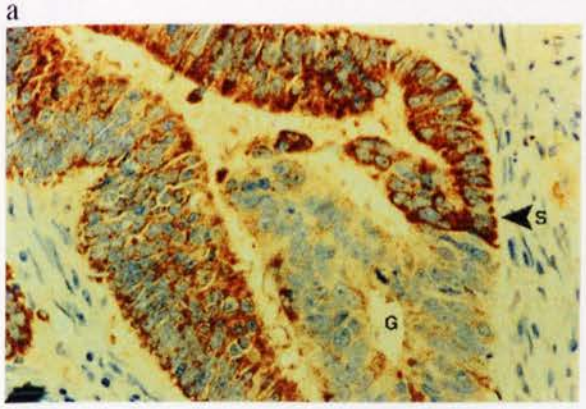


The above slide shows areas with a moderately differentiated cancer with glandular organisation (A), a poorly differentiated cancer with cell-cell contact and a degree of glandular organisation (B) and an invasive poorly differentiated cancer with loss of cell-cell contact and no glandular organisation (CPD). Gland A has a more differentiated and organised morphology with the cells forming a recognisable crypt. However, the cells in this gland show differing morphologies and differing IGF-1R expressions. They show several stages in the transition to basal nucleus polarity in epithelial type cells (1 to 3) and a more invasive morphology in mesenchymal type cells (4 and 5) with concomitant

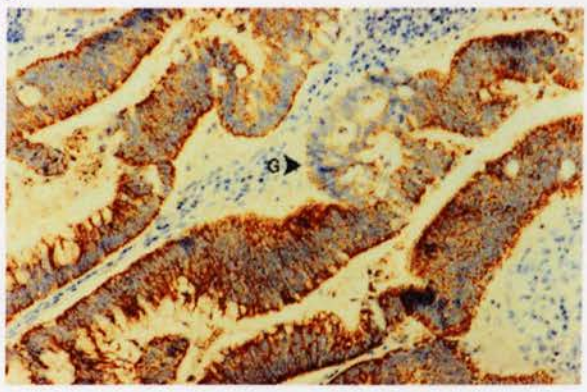
reductions in their IGF-1R expressions. Partially polarised cells with nuclei "off" the basal cytoplasm and a "picket fence" epithelial morphology can be seen to express high levels of the IGF-1R (1). Intermediate cells display an intermediate level of IGF-1R expression (2). Fully polarised cells with nuclei aligned "on" the basal cytoplasm express low levels of the IGF-1R (3). Interestingly, these patterns tend to appear in discrete repeating groups of cells suggesting that this pattern of cell morphology (and IGF-1R expression) is cyclical and therefore related to the cell cycle. Cells in gland A show a morphology suggestive of epithelial mesenchymal transformation. Cells with a partially epithelial / partially mesenchymal morphology that maintain more organised contact with the basement membrane or adjacent cells (in a similar manner to epithelial cells with a "picket fence" pattern) show high levels of IGF-1R immunostaining (4). In contrast, cells with a more mesenchymal morphology that have lost contact with the basement membrane, show low levels of IGF-1R immunostaining (5). In gland B, the cells have a mesenchymal morphology but maintain basement membrane or cell-cell contact and show high levels of IGF-1R immunostaining. At and beyond the point of frank invasion (Inv), the cancer cells have lost cell-cell contact and show low levels of IGF-1R immunostaining (CPD). There is also noted to be cell necrosis (Nec) at this point.

Figure 3.22 IGF-1R immunostaining : cancers and glandular differentiation. x 100

a

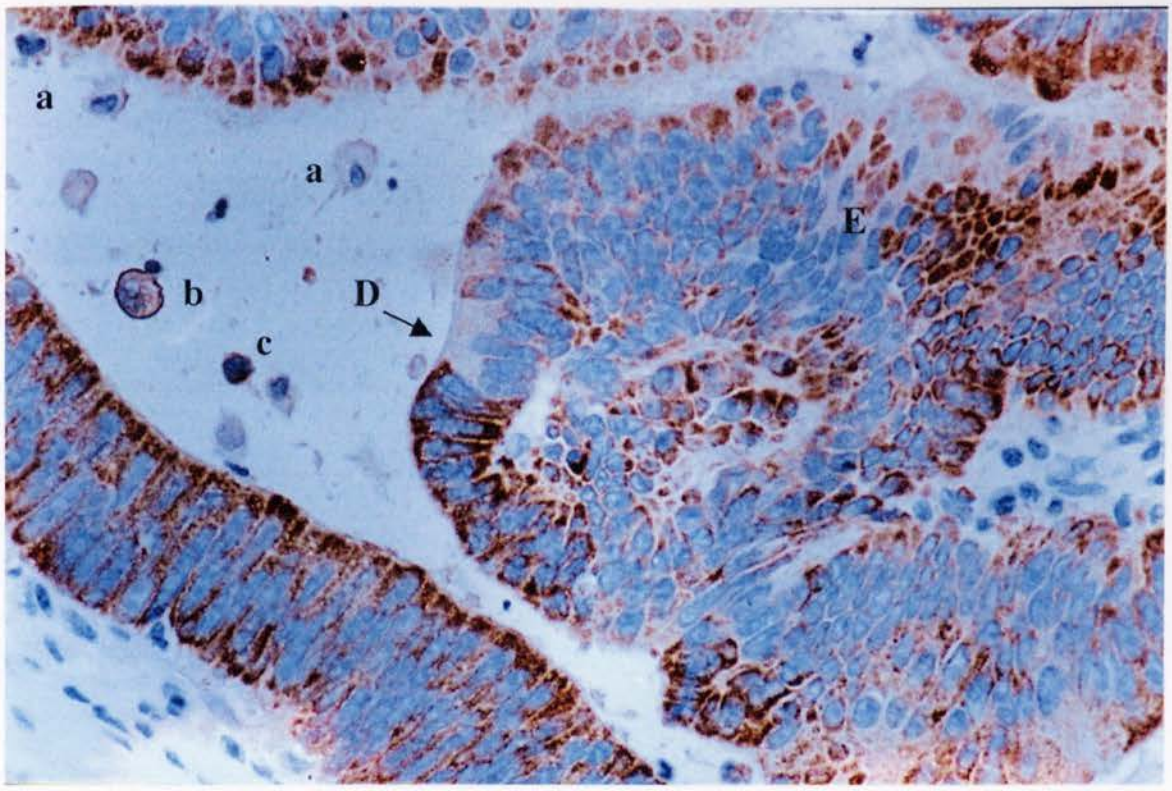


b



The above specimens show also show a similar process of primitive glandular differentiation (G) again with a concomitant reduction in IGF-1R immunoexpression when cancer cells loose their organised "picket fence" type basement membrane or cell-cell contacts. In specimen a, a row of moderately differentiated cancer cells (S) showing "picket fence" morphology with alignment on a basement membrane has formed into a primitive gland (G). This gland exhibits heaped up cells with a disordered morphology and loss of alignment on the basement membrane and a concomitant reduction in IGF-1R immunoexpression. In specimen b, a row of cancer cells has similarly formed a primitive gland (G). In this case, the cells of the gland display also display a disorderd morphology and a low level of IGF-1R expression.

Figure 3.23 IGF-1R immunostaining : variation in morphology and loss of cell adhesion. x 200



The above slide is from a moderately and poorly differentiated cancer showing a predominantly high level of IGF-1R expression. As in Figure 3.21, moderately differentiated cancer cells showing cell-cell / cell-basement membrane alignement in a "picket fence" pattern and poorly differentiated cancer cells showing cell-cell adhesion both show high levels of IGF-1R immunoexpression. However, when epithelial type cancer cells show nuclei aligned on the basal aspect of their cell membranes (D) or when mesenchymal type cancer cells show a loss of cell-cell adhesion (E), then there is a concomitant reduction in IGF-1R immunoexpression. As in Figure 3.21, this was a rare phenomenon and tended to occur in small groups of four or more cells in a repeating pattern rather than in individual cells. This cyclical pattern in IGF-1R expression in areas of EMT suggests that chromatin remodelling is involved in the control of IGF-1R gene expression. It is also notable from the above slide that cells separating from the cancer,

losing their cell-cell contacts and lying free with a motile-type morphology can be seen to immunoexpress low IGF-1R levels. For example, cells labelled "a" in the above specimen have a mesenchymal morphology with lamellipodia and immunoexpress low IGF-1R levels. It is also notable from the above slide that these cells are seen to undergo membrane lysis and nuclear extrusion (b) and cell death (c).

Table 3.5 Summary of tissue immunohistochemistry

Specimen	Number	Average stain	(range)
Normal crypt stem cell	12	4	(3-5)
Normal crypt diff. enterocyte	55	0	(0)
Hyperplastic polyp	7	0	(0)
Aberrant crypt focus	5	4	(3-5)
Tubular adenoma	12	3	(3-5)
Tubulovillous adenoma	11	4	(3-5)
Well differentiated cancer	6	5	(3-5)
Mod. differentiated cancer	7	4	(3-5)
Poorly differentiated cancer	8	2	(0-5)

In summary, the tissue immunohistochemistry results show :

1. in the normal colonic crypt the IGF-1R was expressed at high levels in the basal crypt stem cell region but not in the mid transit amplifying and upper differentiated regions;
2. in the aberrant, adenomatous and cancerous crypts the IGF-1R was expressed at high levels throughout the crypt;
3. in areas of epithelial mesenchymal transformation where epithelial type cancer cells exhibit a fully polarised phenotype and where mesenchymal type cancer

cells loose their cell-basement membrane or cell-cell contacts, the IGF-1R is expressed at low levels.

3.5.7. Immunohistochemistry : primary cultures

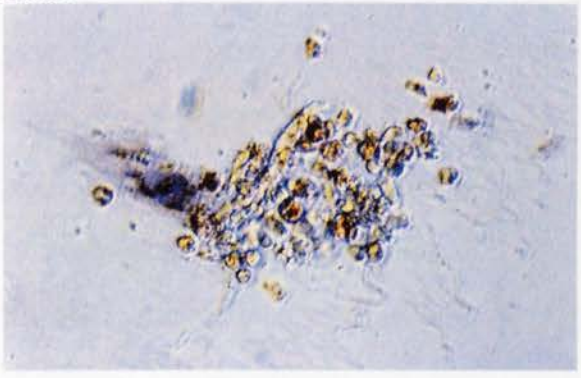
In order to establish if IGF-1R expression correlated with tumour cell morphology in vitro, primary cultures and cell lines were established from human colon cancers. The primary cancers were assayed for IGF-1R immunoexpression and included : 1 well and 1 moderately differentiated cancer (CWD and CMD - both high IGF-1R expression); 2 poorly differentiated cancers with high IGF-1R expression (CPDH); and 2 poorly differentiated cancers with low IGF-1R expression (CPDL). Of these, primary cultures were successful in 4 specimens (CMD, 2XCPDL, 1XCPDH) and cell lines were established from 2 specimens (CMD and CPDL). Cell line CMD exhibited glandular differentiation 48 hours post confluence and became resistant to passageing at this stage. Cell line CPDL in contrast exhibited little differentiation post confluence and was more readily passaged and grown on soft agar (rather than collagen) after a similar period of time. Initially therefore, and even with this limited number, it appeared that the level of IGF-1R expression in the cancer specimen did not correlate directly with cell survival in vitro either as a primary culture or cell line.

A similar pattern of IGF-1R immunostaining was observed in vitro to in vivo. That is, IGF-1R expression was high in tumour explants, sub confluent primary cultures, confluent primary cultures and established cell lines if the primary tumour expressed high IGF-1R levels (and vice versa)(Figure 3.24). However, the findings of basal nucleus polarity during EMT and the loss of cell adhesion with reduced IGF-1R expression were not observed in cultures. However, this was more than likely a simple reflection of the cancer cells' behaviour in culture conditions.

Figure 3.24. IGF-1R immunostaining in vitro

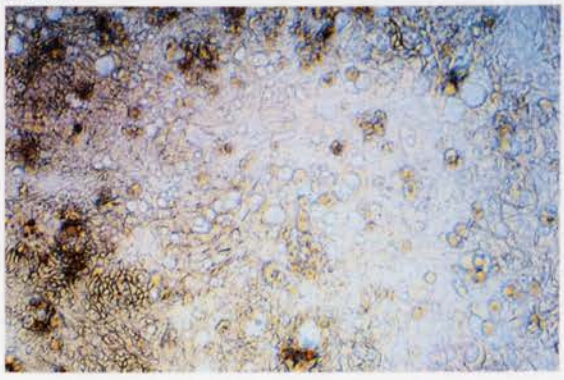
Poorly differentiated cancer (CPDH) explant showing IGF-1R immunostaining.

x 100



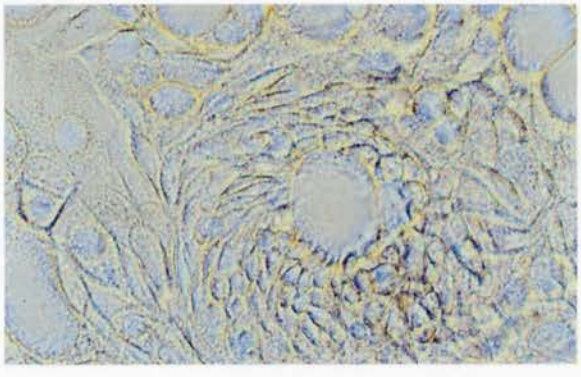
Poorly differentiated confluent primary culture / IGF-1R immunostaining.

x 100

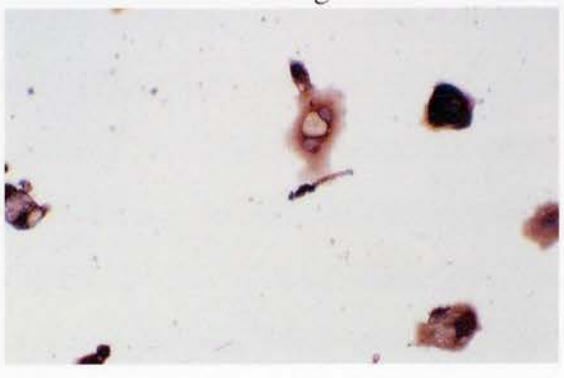


Reagent negative control of CPDH

x 200

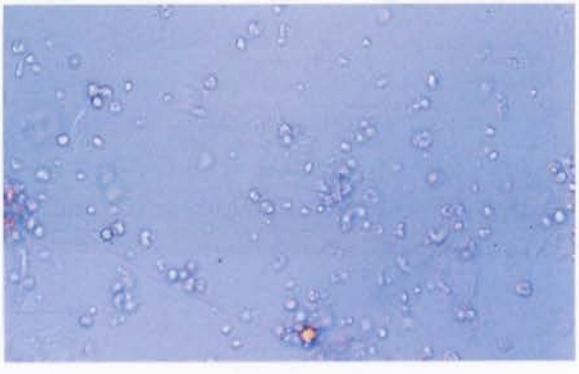


CPDH cell line subconfluent IGF-1R immunostaining. x 200



Reagent negative control of CPDH cell line

x 100



3.6 Western blotting

To confirm the specificity of the IGF-1R polyclonal antibody, Western blotting of normal and tumoural cell lysates were performed. This demonstrated molecular weight specificity, displaying bands at 225kDal. ($\alpha+\beta$ subunits) and 135kDal. (α subunit) under non-reducing conditions for normal and tumoural tissue lysates. Although tumoural tissue appeared to express more of the IGF-1R peptide when equivalences of tumoural and normal protein were examined, the practical difficulties of the immunoprecipitation procedure and the lability of the IGF-1R peptide and the low levels of IGF-1R expression all made verification of this difficult. Importantly however, there were no β-subunit molecular weight variants between normal and colonic tumoural cells as there has been observed between adult and embryonic cells (Alexandrides TK, 1993) and some cancer cell lines (Kellere M, 1990) and human epidermoid carcinoma KB cells (Garolfalo RS 1992). To examine the IGF-1R peptide in the cell culture experiments, the low amount of tissue and the naturally low levels of expression of the receptor presented practical difficulties. In order to increase the sensitivity of the assay, the cell cultures and normal mucosal tissue explants were pulse-chase labelled Met-S35 and immunoprecipitation was performed as previously. Although this technique demonstrated greater sensitivity and a higher IGF-1R level in cancer cell lines, there was poorer specificity with low molecular weight degradation products or carry over.

Figure 3.27 Western blot of normal mucosa (N) and cancer (C)under non-reducing conditions showing weak bands at 225kDal.(α+β) and 135kDal. (α) (arrowed). The cancer specimen also shows some low molecular weight break down / cross reactivity which is absent in the normal mucosa lane.

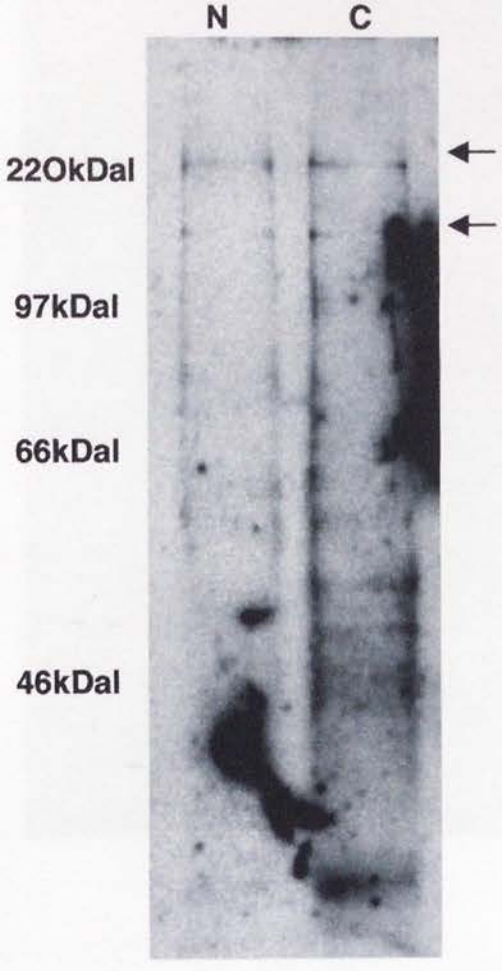
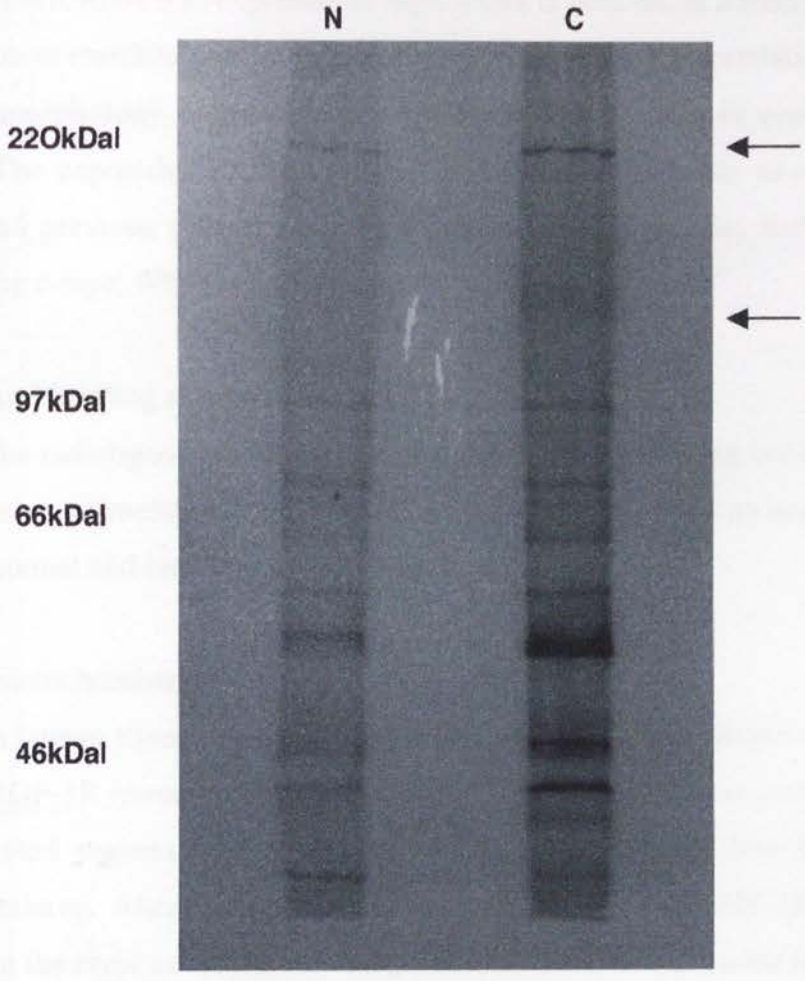


Figure 3.28 Pulse-chase labelled tissue and radio-immunoprecipitation. Band at 225kDal but degraged band at135kDal (arrows) with low mol wt. breakdown products.



3.7. Summary of thesis results :

Northern blotting

IGF-1R RNA was expressed at high levels in colo-rectal adenomas and cancers in comparison to matched normal mucosa. However, poorly differentiated cancers with an invasive morphology expressed lower IGF-1R RNA levels in comparison to other cancers. The expression profile of the IGF-1R does not appear to correlate with this study's and previous studys' findings of the expression profiles and mutations of the *trans* acting c-myc, WT-1 and p53 genes.

Radioligand binding assays

The radioligand binding assays showed saturable kinetics but the errors inherent in the process of membrane microsomal preparation precluded an accurate comparison between normal and tumoural specimens.

Immunohistochemistry

In human tissue, the basal stem cell region of normal colonic crypt showed high levels of IGF-1R immunostaining. In comparison, the mid transit amplifying and upper differentiated regions of the normal colonic crypt showed low levels of IGF-1R immunostaining. Aberrant crypt foci showed high levels of IGF-1R immunostaining throughout the crypt axis, albeit with a gradient in immunoexpression to a limited degree. Adenomatous and cancerous crypts (well, moderately and non-invasive poorly differentiated cancers) showed uniformaly high levels of IGF-1R immunostaining. However, poorly differentiated cancers with an invasive morphology and the invasive foci of well and moderately differentiated cancers showed low levels of IGF-1R immunostaining. Low levels of IGF-1R immunostaining were found to correlate with basal nucleus polarity in epithelial type cancer cells and with loss of cell-cell and cell-basement membrane adhesion in mesenchymal type cancer cells. These morphological features were suggestive of the process of epithelial mesenchymal transformation. Additionally, a cyclical pattern in IGF-1R expression was observed in these foci of cells

which suggests that chromatin remodelling is involved in the control of IGF-1R gene expression.

Primary cultures and cell lines

Tumour explants, primary cultures and cell lines all showed IGF-1R immunostaining. However, it was not possible to observe in vitro basal nucleus polarity or the loss of cell-cell adhesions with the concomitant reduction in IGF-1R expression.

Western blotting

Western blotting showed specificity of the anti-IGF-1R polyclonal for the IGF-1R receptor in the tissue immunohistochemistry. Pulse-chase labelling was required for the culture immunoassays.

CHAPTER 4

DISCUSSION

4.1. Aims and findings

The primary aim of the thesis was to examine the expression profile of the Insulin-like growth factor type 1 receptor in the colo-rectal polyp-cancer sequence to determine when changes in IGF-1R expression occur during tumour initiation or progression. If a change in IGF-1R expression was seen to occur, then a secondary aim was to determine if this correlated with that of its recognised trans regulators (WT-1, c-Myc, p53) or some other process.

The results heretofore show for the first time that colo-rectal tumours express high levels of the IGF-1R de novo at tumour initiation and not during tumour progression. Additionally (and also consequent to the first statement), high IGF-1R expression was not found to correlate with changes in its trans regulators c-myc and WT-1 (or, by inference, p53) during tumour progression. Instead, the study's findings implicate an abnormality of the stem cell differentiation programme during neoplastic initiation in the aberrant crypt focus. This high level of IGF-1R immunoexpression continues with neoplastic progression in adenomas and cancers. Only in advanced invasive cancers was the IGF-1R immunoexpressed at comparatively lower levels. This was found to correlate with two specific cell morphologies in foci that appeared to show EMT and invasion : 1. basal nucleus polarity in epithelial type cancer cells in areas of EMT; 2. loss of cell-cell and cell-basement membrane adhesion in mesenchymal type cancer cells following EMT. Finally, and as we will see, the cyclical pattern of IGF-1R expression in invasive foci suggests that control of expression of the IGF-1R gene in the cancer cell involves chromatin remodelling.

4.2. Controls, validation and significances

4.2.a. Northern blotting

The results of each arm of the study were carefully validated at each stage. In the Northern assays, there was little range in the observed normalised densitometry readings for normal mucosal IGF-1R using total RNA blots. This was despite the low copy number and the high molecular weight of the IGF-1R transcript requiring optimisation of the gel running and transfer. Tumoural specimens exhibited significantly higher densitometry readings for the IGF-1R total RNA blots ($p<0.05$). However, the tumoural specimens exhibited a wider range in that some poorly differentiated cancers had comparatively lower IGF-1R transcripts. This may well account for the low PCR signal observed in some cancers in the preliminary study.

4.2.b. Immunohistochemistry

The immunohistochemistry studies employed tissue positive and negative controls (kidney and liver respectively) along with peptide absorbed reagent negative controls to validate the sensitivity and specificity of each assay. The mixed cytoplasmic and membranous staining observed is similar to that of other growth factor receptors. Much of the receptor exists in the cytoplasmic pool representing post-translational processing of the pre-peptide, turnover of nonactivated membrane-bound receptors or internalisation of the activated membrane-bound receptors. The observed staining intensity showed little variation between histologically similar cells. Importantly, the IGF-1R histo-staining in stem and tumoural cells was significantly higher than that of normal mucosal transit amplifying and terminally differentiated epithelial cells ($p<0.05$). Overall therefore, normal mucosa immunoexpressed lower IGF-1R levels than tumoural mucosa and this was in agreement with the Northern assays (in terms of the fraction of IGF-1R transcripts / total RNA). Tumours immunoexpressed high IGF-1R levels except in invasive cancers and again, this was in agreement with the Northern assays. Additionally, normal mucosa adjacent to tumoural specimens was used as an "internal" positive or negative immunostaining control (depending on the level of the crypt).

The specificity of the anti-IGF-1R antibody was confirmed by peptide-absorbed (reagent negative) controls and by Western blotting. Peptide-absorbed controls showed

an absence of IGF-1R immunostaining for all specimen types. Western blotting of normal mucosa showed specificity for the α and β subunits although some cancer specimens also showed some lower molecular weight products. This may be a result of necrosis within the cancer specimen as well as breakdown of the mature IGF-1R peptide during processing. Certainly, pulse-chase labelling of cell cultures with anti-IGF-1R immunoprecipitation of the lysate showed low molecular weight breakdown products on the Western blot.

4.3. Previous studies and the advances of the current study

The study's findings are in agreement with but are a significant advance over previous studies in this field. Each novel finding in different parts of the polyp-cancer sequence will be discussed in turn:

4.3.a. Normal versus tumoural tissue : gene expression

The only prior study at variance with the finding of IGF-1R over expression in colorectal tumours was that of Zenilman and Graham (Zenilman, M.E., 1997). That study employed a competitive PCR which showed wide variations for all IGF-1R signals when comparing normal and tumoural mucosae. In contrast, the PCR reaction employed in the preliminary study by this author showed a consistently low signal for normal epithelium but a variable signal for cancer epithelium which, with hindsight, may have been due to the low IGF-1R transcripts in some poorly differentiated cancers. The studies of Hakam and Coppola (Hakam, A., 1999), Freier and Raz (Freier, S., 1998) and Weber and Baretton (Weber, M., 2002) have shown by means of immunocytochemistry, RNAse protection assay and RT-PCR respectively that the IGF-1R is over expressed in colo-rectal polyps and cancers in comparison to normal mucosa. High levels of IGF-1R expression in tumoural as opposed to normal tissues have been noted in numerous other studies examining other cancer phenotypes (Reviewed Macaulay, V.M., 1992), (Zhang, L., 1997) and the results of the current study are in agreement with these.

There is now a wealth of data to show that, as a general principle, the growth factor receptor tyrosine kinase families - including ErbB / HER (Roskoski, 2004),

PDGFR / KIT (Fletcher, 2004), Met (Corso, 2005), TGF-βR / Wnt (Mishra, 2005) and VEGFR (Hicklin, 2005) as well as the IGF-1R – are highly expressed in diverse cancer phenotypes in comparison to their normal tissues of origin. However, as stated, the timing of and the reasons for these changes in IGF-1R expression in the context of normal programmed differentiation and the neoplastc initiation and progression have not been explored.

4.3.b. The normal colo-rectal crypt : immunohistochemistry

The study's initial finding of high levels of IGF-1R immunoexpression in the basal crypt stem cell compartment of the normal colo-rectal crypt has not been previously documented. High levels of IGF-1R expression have been documented in all embryonic tissues (embryonic stem cells) and low levels of IGF-1R expression have been documented in the majority of postnatal differentiated tissues (Werner H, 1989), (Chernausek, S.D., 1987). IGF-1R expression during adult stem cell maturation has not been documented because of the inherent difficulty in identifying the stem cell compartment and the stem cell maturation process in most adult tissues. In the colo-rectum where stem cells and stem cell maturation are each compartmentalised in the crypt (reviewed Potten,1990), the expression profile of IGF-1R during stem cell maturation can be examined. IGF-1R expression in the normal colonic crypt has previously only been examined indirectly by means of radioligand binding of IGF-I and -II in rat epithelium (Laburthe, 1988). That study showed an increased concentration of ligand binding at the base of the crypt but the study used autoradiographs and was not able to discern IGF-1R expression according to the cell phenotype. The expression of other growth factor receptors has been documented to a limited degree in the normal colonic crypt using similar methodologies – fibroblast growth factor receptor-3 (Vidrich, 2004) and epidermal growth factor receptor (Menard, 1991). Again, these studies have either examined expression in embryo or in mice using radioligand binding and have not been able to discern cell phenotypes. Therefore, studies examining IGF-1R expression in relation to the cell phenotype in the normal adult colon have to date been lacking.

The reduction in IGF-1R immunoexpression specifically at the stage of basal crypt stem cells migrating into the mid crypt transit amplifying region is an interesting

finding. The proliferating transit amplifying cells in the mid crypt have a relatively undifferentiated and partially polarised morphology and immunoexpress low IGF-1R levels. The proliferating basal crypt stem cells also have an undifferentiated and partially polarised morphology but immunoexpress high IGF-1R levels. IGF-1R expression does not therefore appear to correlate in a simple manner with the proliferative behaviour of cells in the normal colonic crypt. This pattern of IGF-1R expression is in line with that of several other genes. For example (and as discussed in the introduction), the cell survival genes bcl-2 and survivin are expressed in the basal crypt region (Merritt, 1995),(Zhang, 2001) (Sinicrope, F.A., 1995). In contrast, proliferative genes such as such as myc, fos, cyclin D1 and EGFR are expressed in both the basal and mid crypt regions (Chin, L., 1995), (Stopera, S.A., 1992), (Chailler, P. & Menard, D., 1998). Anti-proliferative or differentiative genes such as APC and TGFβRII are expressed in the upper differentiated compartment (Smith, K.J., 1993) whereas other genes such as E-cadherin are constitutively expressed throughout the length of the crypt (Sellin, 2001). Although very limited inferences can be made regarding gene function in this way, it does nevertheless appear that IGF-1R expression in the basal crypt is a property of the stem cell and its behaviours – cell survival, cell adhesion, cell dedifferentiation - and not just a reflection of the proliferative activity of the cells. In line with this, it was also noted that hyperplastic polyps showed a similar pattern of basal crypt IGF-1R expression to normal crypts rather than expression throughout the crypt as was noted in the ACF.

There are also interesting parallels between the current study and cell line models. For example, MacDonald and Bean (MacDonald, R.S., 1993) have shown that the IGF-1R is expressed in increasing concentrations at the crypt base in a cancer cell line model of the colonic crypt. Similarly, induced differentiation in colon cancer cell lines results in reduced expression of the IGF-1R and also in the loss of tumourigenicity (Garrouste, F., 1997), (Remacle-Bonnet, M.M., 1992), (Augernon, C. et al (1984), (Zarilli, R., 1996). All these studies however have used cancer cell lines which are significantly different from nontransformed adult stem cells. Indeed, and unlike transformed cells, normal colon epithelial cells are highly growth factor-, anchorage- and cell contact-dependent and resistant to culture. Whether this reflects the loss of control of IGF-1R expression during programmed differentiation in the transformed cell is unclear.

4.3.c. The aberrant crypt focus / tumour initiation

The stem cell origins of neoplasia have previously been argued (Tomlinson, I.P.M, 1995). However, these arguments are theoretical mathematical ones and are based upon stem cells being able to proliferate beyond a critical number and avoid terminal differentiation so that neoplastic transformation can persist (Loeffler, M., 1991). Evidence for the stem cell origins of neoplasia has been lacking and indeed a "top-down" aetiology has been argued by some (Shih, I.M., 2001). However, in this study, the observed pattern of IGF-1R immunoexpression in the ACF with a residual gradient in immunoexpression representing an expansion of the zone of proliferation, although not conclusive proof, is highly suggestive of the stem cell origins of neoplasia.

How IGF-1R expression might become uncoupled from the stem cell differentiation programme in the ACF is unclear. Indeed, the molecular mechanisms regulating normal stem cell cycling and their commitment to differentiation are poorly understood (Reviewed- Clatworthy, J.P., 2001). Like the IGF-1R, the bcl-2 and fos genes are also highly expressed in colonic crypt stem cells, ACFs and cancers (Merritt, A.J. et al. (1995), (Stopera SA, 1992). Both the bcl-2 and fos genes can be trans activated by the IGF-1R via their CREB motifs (Pugazhenthi, S., 1999), (Li, X.S., 1994). It is unclear if this alone accounts for the high expression of bcl-2 and fos in stem and tumour cells. This is perhaps an over simplification given the complex and highly regulated nature of their promoters. For example, like the IGF-1R, the bcl-2 and fos promoters have multiple cis-elements for AP-2, Sp1, p53 and WT-1 (Wu, Y., 2001),(Miyashita, T., 1994), (Hewitt, S.M., 1995). It is equally difficult to discern how these promoters can be specifically controlled by such ubiquitous trans regulators. Indeed, although Sp1 is ubiquitously expressed in embryonic stem cells (Saffer, J., 1991), its expression is associated with trans activation of the IGF-II ligand rather than the IGF-1R in ras- and polyoma middle T-transfected Caco-2 cell line models (Cadoret, A., 1998). More specific transcription of the bcl-2 and fos genes can be directed by oestrogen receptors which appear to act as co-factors at Sp1 sites in breast cancer cell lines (Dong, L., 1999), (Duan, R., 1999). Whether such combinatorial controls exist at the multiple Sp1 sites in the 5'-flanking and 5'-UTR regions which confer a high basal activity upon the IGF-1R promoter is

unknown. This is possible given that Sp1-IGF-1R promoter interaction is already targeted and inhibited by p53 and BRCA1 (Ohlsson, C., 1998), (Maor, S.B.,2000) and given the responsiveness of the IGF-1R gene to hormones such as oestrogen (Stewart, A.J., 1990), FSH and GH (Hernandez, E.R., 1991),(Isaksson, O.G.P., 1991) and growth factors such as PDGF and FGF (Clemmons, K.R., 1980).

The WT-1-, myc- and p53-response regions in the IGF-1R promoter 5'-UTR (Chambery, D., 1999) (Werner, H., 1994), (Ohlsson, C., 1998) have been cited as potential mechanisms to explain tumoural IGF-1R expression. However, this study has contradicted this attractive hypothesis, placing IGF-1R over expression as an event of tumour initiation, predating any expressive changes in these trans-activators or repressors. Interestingly, IGF-1R levels have in fact been found to be lower in some colon cancer cell lines with APC, β-catenin, E-cadherin and p53 mutations (Playford, M.P., 2000). Although the reasons for this are unknown, it does suggest that regulators other than p53 and myc (a transcriptional target of β-catenin/Tcf) predominate in the IGF-1R transcript control of cancers (Chambery, D., 1999), (Travali, S., 1991), (Werner, H., 1996).

What then are the possible causes of IGF-1R expression in transformed colon crypt cells? Transformed enterocytes in the upper crypt (crypt down morphogenesis) may reacquire stem cell-like expression patterns of genes such as the IGF-1R, bcl-2 and c-fos (Stopera, S.A.,1992),(Sinicrope, F.A., 1995) ,(Shpitz, B., 1999) through mutation of hitherto unrecognised trans-activators or repressors. Alternatively, and in keeping with a stem cell origin to neoplastic initiation, the expression of these candidate genes at stem cell levels in the ACF may reflect a more fundamental abnormality of the stem cell's programmed differentiation occurring at neoplastic initiation. Certainly, identifying how the promoters of genes such as the IGF-1R are controlled could yield insights into the process of tumour initiation.

4.3.d. The ACF-polyp-cancer sequence / tumour progression

Adenomatous and most cancerous crypts showed uniform IGF-1R immunostaining both within and between specimens. The loss of a gradient of IGF-1R immunoexpression (present in the normal crypt and to a lesser degree in the ACF)

suggests that a further change occurs in the control of the IGF-1R promoter after the ACF stage of neoplasia. As noted above, the absence of any variation in the high levels of IGF-1R immunoexpression noted in all polyps and most cancers or the absence of any correlation with c-myc or WT-1 expression, again suggests that the IGF-1R is constitutively expressed de novo in tumours as a result of an abnormality in stem cell programmed differentiation.

Experimental models testify to the cell's survival dependency on the IGF-1R being most acute during cell stress / anchorage independence and during cell transformation (Valentinis, B., 1999), (Resnicoff, M., 1995). The present study also showed this association in human cancers. That is, lower levels of IGF-1R expression in some poorly differentiated colon cancers correlated with higher levels of cell death. However, these cells had also lost their cell-cell and cell-basement membrane anchorage and these factors can equally be implicated in cell death.

The sensitivity of cancer cells to IGF-1R down regulation is poorly understood. As discussed in the introduction, the signalling pathways downstream of the IGF-1R and other growth factor receptors (GFRs) - Ras/Raf/MAPK, PI3K/Akt/Bcl-2/BAD, 14.3.3/raf/BAD, β-catenin/APC/Tcf and TGFβRII/Smad – all undergo expressive or mutational changes in their signalling components in the majority of human cancers (Blume-Jensen, P., 2001), (Schlessinger, J., 2000), (Baserga, R., 1999), (Chow, J.C., 1998), (White, M.F., 1998), (Myers, M.G.,Jnr., 1994) (Kulik, G., 1997),(Furlanetto, R.W., 1997), (Freed, E., 1994), (Playford, M.P., 2000). There is considerable crosstalk and redundancy in these pathways so that "multiple hits" are thought to be required during neoplasia to bypass or reduce the cancer cell's dependency on these normal growth controls. Why the transformed cell should be peculiarly reliant therefore on IGF-1R function is unclear but this bourne out by in vitro studies which show that IGF-1R down regulation causes G0 arrest and differentiation in normal cells but apoptosis in transformed cells (Sell, C., 1994), (D'Ambrosio, C., 1995).

4.3.e. Poorly differentiated cancers : reduced IGF-1R immunoexpression with basal nucleus polarity and the loss of cell adhesion in foci showing the morphological features of EMT.

Cancer cells loose their epithelial-type characteristics as they dedifferentiate during cancer progression. EMT is the final stage in this process. Mesenchymal cells whether normal or cancerous have the ability to migrate through ECM to varying degrees. Revealing the molecular mechanisms involved in this process is therefore of relevance in understanding the pathogenesis of cancer cell invasion and metastasis. Prior studies show that EMT and consequently the malignant phenotype, are accompanied by a decrease in epithelial markers such as E-cadherin and an increase in mesenchymal markers (reviewed Hay ED, 1995; Thiery JP, 2002). Similarly, when mesenchymal cells regain epithelial morphology (MET) they reexpress epithelial markers and adopt a non-invasive behaviour. Of note, this study shows that reduced IGF-1R immunoexpression in foci that show the morphological features of EMT, rather than simply reflecting the loss of an epithelial marker, is specific to two particular cell morphologies or cell behaviours – epithelial cell polarisation and mesenchymal cell loss of adhesion. When the reverse of these processes occur (mesenchymal cells regaining cell adhesion and MET in established metastases) then the IGF-1R is concomitantly re-expressed. Additionally, the cyclical pattern observed in IGF-1R immunoexpression and cell morphology during these processes implicates certain mechanisms in the control of the gene's expression (see below).

The observed reduction in IGF-1R immunoexpression in association with a fully polarised epithelial phenotype in areas of EMT is a novel finding. Although the temporal sequence of EMT cannot be followed on histology slides, intermediate cell morphologies lay between the fully polarised epithelial-type cancer cell and the mesenchymal-type cancer cell suggesting that these were stages in the sequence of EMT. It is interesting to note that this process shows similarities to differentiation in the normal colo-rectal crypt and to experimental models of differentiation (see below). However, in contrast to the normal crypt, fully polarised epithelial-type cancer cells with reduced IGF-1R immunoexpression showed evidence of mitosis (mitotic bodies and clustering of same-cell morphologies suggesting cycling).

The observed reduction in IGF-1R immunoexpression with the loss of cell-cell adhesion in mesenchymal-type cancer cells is another novel finding of the study. This was a consistent finding in foci of EMT. Adjacent mesenchymal cells of similar morphologies that maintained their cell-cell or cell-basement membrane adhesion maintained high levels of IGF-1R immunoexpression. Furthermore, these morphologies alternated in a repeating pattern of four or more cells at invasive foci. Interestingly, these cell behaviours correspond with those of some in vitro models. As was discussed in the introduction, subconfluent 3T3-L1 preadipocytes, L6E9 skeletal myoblasts and HT29 or CaCo-2 colon cancer cells will all normally proliferate and scatter in response to IGF stimulation (Student, A.K., 1980), (Rosenthal, S.M., 1995), (Andre, F., 1999), (Zarilli, R., 1996). However at confluence, these cells down regulate their IGF-1R and IGF-II expression and differentiate (Pommier, G.J., 1992), (Garrouste, F., 1997), (Zarilli, R., 1996). This post confluent differentiation is associated with a permanent loss in the non-cancerous cell lines of the ability to re-express the IGF-1R or re-enter the cell cycle. However, the cancerous cell lines – HT29 and CaCo-2 - are often able to re-express the IGF-1R after undergoing several rounds of mitosis or repassaging, although they lose their ability to survive as inoculates in vivo during this period (Augernon, C., 1984). This result has been reproduced by transfection with an IGF-1R dominant-negative mutant (Jiang, Y., 1999). This resulted in differentiation and the loss of tumourigenicity in the A549 lung carcinoma cell line used. The relevance of these in vitro studies conducted under differing cellular conditions had been unclear. In the current study however we see for the first time that IGF-1R expression in invasive human cancers is a dynamic and modulatable phenomenon that correlates with the loss of cell adhesion during invasion.

In vitro studies have implicated IGF-1R signalling pathways in EMT, cell adhesion and cell motility. For example, IGF-1R activation can result in its co-localisation as a tripartite complex - IGF-1R/IRS-1~β-catenin~E-cadherin – effectively resulting in the down regulation of membranous E-cadherin and the loss of β-catenin / Integrin αvβ5 interaction (Playford, 2000; Morali, 2001; Brooks, 1997). Given that these signalling functions are presumably reliant upon IGF-1R expression, their actions are perhaps counter-intuitive given the findings of the current study and the in vitro studies described above. However, the findings of these signalling studies are entirely dependent

on the cellular context or culture conditions and a much more consistent finding from these studies in terms of cellular function is that over expression of the IGF-1R results in increased cellular adhesion (Mauro, 2002).

The present study also showed that following invasion and metastasis that IGF-1R expression could be reinstated upon regaining cell-cell contact. It would be interesting to learn if this capability varies between cancers or has any correlation with their metastatic potential. Metastases could however express low IGF-1R levels, albeit with high levels of necrosis. Although the immunohistochemistry was unable to discern what effect the loss of cell-cell contact had upon IGF-1R expression in a cancer cell already expressing lower levels of the receptor, it may be that a threshold level of IGF-1R expression is important in this regard. This has been shown to be the case in fibroblast transfection studies (Rubini, M., 1997) and the evidence to date has shown that cancer cells are even more so dependent upon a level of IGF-1R expression for their survival (Valentinis, B., 1999), (Sell, C., 1994), (D'Ambrosio, C., 1995).

4.4. A putative mechanism of IGF-1R transcript control based upon the study's findings.

The observed cyclical pattern of alternating high and low levels of IGF-1R immunoexpression with concomitant changes in cell morphology in foci of EMT and invasion is highly suggestive of regulation of IGF-1R gene expression by chromatin remodelling. This would explain why cancer cells at these foci would have to undergo several rounds of clonal expansion (and thus appear in groups of four or more) before chromatin remodelling 'opened' the IGF-1R promoter to regulation. Epigenetic regulation of transcription is increasingly recognised as an important factor in determining stem cell and cancer cell behaviours. This will be an important area for future research on the IGF-1R.

4.5. A model for the somatic evolution of cancers

Cancer evolution is described in terms of an accumulation throughout disease progression of mutations in multiple pathways to result in abnormalities of the cell cycle,

cell survival and differentiation. How these functions can all go awry at tumour initiation in the aberrant crypt is poorly understood. The present study suggests that some of the gene expression changes in the aberrant crypt might be traced back "upstream" to receptor tyrosine kinases such as the IGF-1R. Yet this is only a small part of the story. Loss of regulatory control in the transformed cell will be dependent upon a host of additional factors- ligand availability, the concomitant activation of other receptor tyrosine kinases and the availability of signalling substrates and their downsteam signalling pathways including ras/raf/MAPK, PI3K/Akt, raf/BAD and β-catenin/Tcf. All these are frequent targets during tumour initiation and progression. With regard to the IGF-1R however, I propose that the findings of this thesis can be encompassed in a simple model of gene expression. Here we see that the expression of genes such as the IGF-1R in the newly initiated cancer cell is a legacy of their stem cell origins. Programmed differentiation of the stem cell involves a coordinated reduction in IGF-1R expression which is somehow disrupted at tumour initiation. Additional mutations in the downstream signalling pathways of genes such as the IGF-1R are clonally selected which further divorce the components of these signalling pathways from control during tumour progression. In late stage cancers, mesenchymal transformation involves a 'dedifferentiation programme' with a sequence of gene expression changes – such as a loss IGF-1R expression – which together confer the properties of the malignant phenotype - such as loss of cell adhesion. These gene expressions can occur in a cyclical manner suggesting that a basic abnormality in chromatin remodelling for candidate genes such as the IGF-1R occurs during neoplasia. Understanding how such candidate genes are controlled in the normal developmental programme may therefore provide further insights into neoplasia.

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