Tumour suppression mediated through DPC4, p53 and APC

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Declaration

This thesis and the work described herein is solely my own work. Where contributions have been made by others this is explicitly stated in the text.

Jan Cullingworth, June 2002

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List of Abbreviations

15% PM	plating medium containing 15% FCS
a.a.	amino acid
AB	avidin/biotin
AIF	apoptosis inducing factor
AP	alkaline phosphatase
AP-1	activator protein 1
APC	adenomatous polyposis coli
ARE	activin response element
ARF	alternative reading frame
ASEF	APC-stimulated guanine nucleotide exchange factor
ATF –2	activated transcription factor 2
BAX	Bcl-associated x protein
BMP	bone morphogenetic protein
BRCA2	breast cancer 2
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
cAMP	3', 5'-cyclic adenosine monophosphate
CBP/p300	cAMP response element binding protein/p300
CCK	choleocystokinin
CDK	cyclin dependent kinase
CKII	casein kinase II
Co-SMAD	common SMAD
Cox-2	cyclo-oxygenase 2
CRD	cysteine rich domain
ddw	double deionised water
DSH	dishevelled
DKK-1	dickkopf 1
DMEM	Dulbecco's modified minimum essential medium
DNA	deoxyribonucleic acid
DPC4	deleted in pancreatic cancer locus 4
DPP	decapentaplegic

DTT	dithiothreitol
E2F	early region 2 factor
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
ENU	ethylnitrosourea
ERK	extracellular regulated kinase
FAP	familial adenomatous polyposis
FAST –1	forkhead activin signal transducer 1
FCS	foetal calf serum
FGF	fibroblast growth factor
FJP	familial juvenile polyposis
FKBP	forkhead-binding protein
Floxed	flanked by *loxP* sites
FRAT –1	frequently rearranged in advanced T cell lymphomas
FRP	frizzled related protein
GADD45	growth and DNA damage 45
GDF	growth and differentiation factor
GDNF	glial derived neurotrophic factor
GSK3β	glycogen synthase kinase 3β
H+E	haematoxylin and eosin
HDLG	homologue of Drosophila discs large
HEPES	N-2-Hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HGF	hepatocyte growth factor
HRP	horseradish peroxidase
IBMX	3-isobutye-1-methyxanthine
IGF	insulin like growth factor
IGFBP	insulin like growth factor binding protein
I –SMAD	inhibitory SMAD
JNK	c-Jun NH_2-terminal kinase
K-RAS	Kirsten RAS
KS	Kolmogorov Smirnov
LEF	lymphoid enhancing factor
LMB	leptomycin B

LOH	loss of heterozygosity
LoxP	locus of crossover P1
LRP6	low-density-lipoprotein-receptor related protein 6
LTBP	latent TGF-β-binding protein
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MDM-2	mouse double minute
MEK	MAP kinase kinase
MH1	Mad homology 1
MH2	Mad homology 2
Min	multiple intestinal neoplasia
MIS	mullerian inhibiting substance
MMP	matrix metalloprotease
MMTV	mouse mammary tumour virus
MOPS	3-[N-morpholino] propane sulphonic acid
MSI	microsatellite instablity
NA	nuclear area
NER	nucleotide excision repair
NES	nuclear export signal
NIS	nuclear import signal
NLS	nuclear localisation signal
NMU	N-Methyl – N – Nitrosourea
NSAID	nonsteroidal anti-inflammatory drug
PAI 1	type I inhibitor of plasminogen activator
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PML	promyelocytic leukaemia
PPARδ	peroxisome proliferator-activated receptor δ
RB	retinoblastoma
RNA	ribonucleic acid
R-SMAD	receptor activated SMAD

RT	room temperature
SAPK	Stress activated protein kinases
SARA	SMAD anchor for receptor activation
SBTI	soya bean trypsin inhibitor
SBE	SMAD binding element
SDS	sodium dodecyl sulphate
Ski	Sloan Kettering avian retrovirus
SMURF	SMAD ubiquitination regulatory factor
SNIP1	SMAD nuclear interacting protein
SnoW	ski related novel gene
SSC	standard saline citrate
TAK-1	TGF-β activated kinase
TBE	tris-borate EDTA
TBS	tris buffered saline
TCA	trichloroacetic acid
TCF	T cell Factor
TFE3	transcription factor E3
TGF-βRI	TGF-β receptor type I
TGF-βRII	TGF-β receptor type II
TGF-β	Transforming growth factor β
TGIF	TGF interacting factor
TIMP	tissue inhibitor of metalloprotease
TNFα	tumour necrosis factor α
TRITC	tetra-methyl rhodamine isothiocyanate
TSG	tumour suppressor gene
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling
VG1	vegetal
WT	wild type

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Bear with each other and forgive whatever grievances you may have against one another. Forgive as the Lord forgave you. And over all these virtues put on love, which binds them all together in perfect unity. Let the peace of Christ rule in your hearts, since as members of one body you were called to peace. And be thankful.

Colossians 3:13-15

Abstract

Mutations in the tumour suppressor genes *SMAD4* (or *DPC4*, deleted in pancreatic cancer locus 4), *APC* (adenomatous polyposis coli) and *p53* have been implicated in pancreatic cancer in humans. This thesis firstly documents the *in vivo* effects of mutations in these genes singly and in combination through spontaneous and carcinogen-induced murine models of pancreatic tumourigenesis. Second, it examines the *in vitro* effects of TGF-β signalling, of which SMAD4 is the central mediator, on murine primary cultured pancreatic acinar cells.

Previously $p53^{-/-}Apc^{Min/+}$ mice have been shown to develop pancreatic tumours. To examine the effect of *Smad4* heterozygous mutation on the development of these tumours, $Smad4^{+/-}$ mutation was introduced into $p53^{-/-}$ and $p53^{-/-}Apc^{Min/+}$ mice. No pancreatic phenotype was found in $p53^{-/-}Smad4^{+/-}$ animals. $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ animals did not exhibit promotion of tumourigenesis in any tissues compared to the $p53^{-/-}Apc^{Min/+}$ mice. Immunohistochemical studies revealed loss of SMAD4 protein within the majority of the lesions arising in $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ animals. Furthermore, microdissection and mutational analysis revealed LOH for *Apc* and *Smad4*.

Treatment of wild-type (WT) $Smad4^{+/-}$, $Apc^{Min/+}$ or $Apc^{Min/+}Smad4^{+/-}$ mice with N-Nitroso-N-Methyl Urea (NMU) resulted in abnormal foci in pancreatic acinar cells, characterised by β-catenin stabilisation. Previously these foci have been shown to be the precursors of pancreatic neoplasia. Only NMU-treated $Apc^{Min/+}Smad4^{+/-}$ mice exhibited a significant increase in abnormal pancreas, which was found to be due to an increased number of abnormal foci rather than increased focus size. A range of foci sizes were analysed, but only smaller abnormal foci were characterised by morphological nuclear atypia. These studies suggest functional co-operation between TGF-β and Wnt signalling pathways in the suppression of pancreatic tumourigenesis in the mouse.

In order to investigate TGF-β signalling in WT murine primary pancreatic acinar cells TGF-β was given to these cells under different conditions. Two main conclusions were drawn from these experiments. First that TGF-β in conjunction with EGF could accentuate acinar cell attachment and motility in the presence of serum. This effect was not associated with changes in cell proliferation. Others working on primary culture of rat hepatocytes have also reported synergy between EGF and TGF-β resulting in increased motility suggesting this interaction may be common among different cell types. Second, in serum free conditions, TGF-β caused reduced BrdU incorporation and increased apoptosis. Immunofluorescent studies, analysed by confocal microscopy, revealed that these cellular behaviours were associated with changes in the cyclin dependent kinase inhibitor, p27, but not p21. Whether these effects are mediated by SMAD4 remains unconfirmed as immunoblotting revealed SMAD4 to be present in the nucleus of these cells in the absence and presence of TGF-β. This investigation into the effect of TGF-β on primary pancreatic acinar cells reflects the multi-functional nature of TGF-β signalling, highlighting interaction between TGF-β and EGF signalling pathways and suggesting a mechanism of TGF-β-induced growth suppression via p27 in these cells.

These studies provide insight into the combined effects of mutation in *p53*, *Apc* and *Smad4* in the development of pancreatic cancer and suggest possible cellular mechanisms through which *Smad4* mutation and disrupted TGF-β signalling could promote pancreatic cancer in humans.

Chapter 1 – Introduction

1.1 Tumourigenesis in the pancreas

Cancers of the pancreas are particularly aggressive and the average survival time after diagnosis is 4-6 months. Strategies for early detection of pancreatic adenocarcinomas have not yet been developed, and most pancreatic adenocarcinomas present with metastatic or locally advanced disease at the time of diagnosis. Chemotherapy and irradiation are largely ineffective and metastatic disease often develops after potential curative surgery (Cohen *et al.*, 1996; Staley *et al.*, 1996; Parker *et al.*, 1997). In the past few years much work has led to a closer understanding of the genetic alterations in adenocarcinomas of the pancreas (Hahn and Schmeigel, 1998), however the molecular events behind this aggressiveness remain largely unknown. Table 1.1 contains a summary of those genes so far thought to be involved in pancreatic tumourigenesis. This work focuses on three tumour suppressor genes (TSG), *deleted in pancreatic cancer locus 4* (*DPC4* or *SMAD4*), *adenomatous polyposis coli* (*APC*) and *p53*. Each of these has been linked with pancreatic cancer and in certain cases with each other. The work contained in this thesis has used mice with mutations in these genes. Throughout the course of this thesis, italics will be used to denote genes and regular font will be used to denote proteins. Human genes will be in capitals however murine genes will have the first letter only capitalised. All protein abbreviations regardless of species will be capitalised.

Table 1.1 - Genes thought to be involved in pancreatic cancer

Gene	Function	Role in pancreatic cancer	Proportion of pancreatic neoplasms with mutation	Chr. locus in human	References
p16	Role in cell cycle control as Cyclin Dependent Kinase inhibitor, present in same pathway as RB.	Inactivation	80-100%	9p21	Caldas *et al.*, 1994; Okamoto *et al.*, 1994; Huang *et al.*, 1996; Schutte *et al.*, 1997
K-RAS	Integration of growth factor signalling into the cell cycle.	Activation	75-90%	12q12	Reviewed in Howe and Conlon, 1997
p53	Cell cycle arrest, apoptosis induction, DNA damage repair, cell senescence and stress responses.	Inactivated	~50%	17p13	Reviewed in Howe and Conlon, 1997
DPC4/ SMAD4	Central mediator of TGF-β family signal transduction.	Inactivation	50%	18q21	Hahn *et al.*, 1996; Schutte *et al.*, 1996
APC	Downstream effector of Wnt signalling pathway, modulator of β-catenin. Also involved in chromosome stability and cytoskeletal organisation.	Inactivation	<40%	5q21	Hahn *et al.*, 1995; Fodde *et al.*, Näthke *et al.*, 1996. Reviewed in Howe and Conlon, 1997
BRCA2	Maintenance of genome integrity.	Inactivation	9.8%	13q12	Goggins *et al.*, 1996; Hahn and Schmeigel, 1998
RB	Cell cycle control. Participates in same pathway as p16.	Inactivation	6-23%	13q12	Huang *et al.*, 1996; Howe and Conlon, 1997

1.2 The pancreas

1.2.1 Physiology of the pancreas

The pancreas is a dual function organ with roles in digestion and maintenance of glucose homeostasis. Thus the pancreas has an exocrine compartment involved in the production and delivery of digestive enzymes to the digestive tract and an endocrine compartment which synthesises and secretes hormones into the bloodstream. The exocrine compartment comprises approximately 95-99% of pancreas and consists of two cell types, the acinar cells (which manufacture digestive enzymes such as proteases, nucleases and amylases) and the duct cells which form a network of ducts that transport the enzymes into the small intestine. The endocrine compartment of the pancreas is found in structures called the islets of Langerhans which are aggregates scattered throughout the exocrine pancreas: they are composed of four cell types: α, β, γ and PP cells. These four cell types are involved in the manufacture of the hormones glucagon, insulin, somatostatin and pancreatic polypeptide respectively. The majority of the cells in the islets of Langerhans are β-cells (reviewed in Ogami and Otsuki, 1998).

1.2.2 Development of the pancreas

In the mouse embryo, the first induction of pancreatic morphogenesis occurs around 9.5 dpc (days post coitum). The dorsal mesenchyme condenses and the underlying endoderm of the foregut evaginates. From this evagination arises first a dorsal epithelial bud followed by a ventral epithelial bud. Through a series of epithelial-mesenchymal interactions the cells in each bud proliferate and begin tissue organisation through the formation of epithelial branches. By 14.5 dpc distinct exocrine acinar and ductal cells are present, and by 18 dpc islets of Langerhans can be found. Finally, the two buds fuse together to form the functional organ (reviewed in Slack, 1995).

1.2.3 Acinar to ductal transdifferentiation in the pancreas

All the cell types present in the pancreas are thought to differentiate from a common pluripotent progenitor cell type. However the existence of a pancreatic stem cell in

adult tissue is still under debate, in humans it has been suggested that the centroacinar cell could be a possible stem cell (Jamieson *et al.*, 1981; Longnecker, 1990). The possible existence of a pancreatic stem cell is of particular interest to this work as cells in the exocrine portion of the pancreas retain the ability to dedifferentiate or transdifferentiate once the organ is fully formed. Most human pancreatic cancers are ductal in composition, however the possibility that a lineage of the acinar cells or a pancreatic stem cell which de- or transdifferentiates into a tumourigenic ductal like cell cannot be ruled out. Most mouse models of pancreatic cancer are acinar in composition (Longnecker *et al.*, 1992), highlighting an intriguing species difference which can be utilised to further investigate this phenomenon. Therefore investigations into transdifferentiation in the pancreas are of particular interest to those investigating pancreatic tumourigenesis in humans.

Previous work using both murine and human primary pancreatic acinar cell cultures has revealed that acinar cells can transdifferentiate under certain conditions and that this differentiation was associated with an increased proliferative capacity. De Lisle *et al.* (1990) investigated the expression of acinar and ductal antigens in acinar cells held in culture for a period of 21 days. Previously they had shown that acinar cells lost their differentiated morphology in culture which coincided with decreased expression of secretory proteins. When the expression of the acinar and ductal antigens was analysed it was found that the acinar cells initially expressed the acinar antigen but that this rapidly decreased by day 7 of culture. Conversely, no ductal antigen was observed until day 5. In culture this ductal antigen expression increased until day 9 when virtually the whole population of cells were positive for the ductal antigen. Day 9 was also the found to be the time when the cells reached their peak of proliferation. Vitally, a stage of dual expression in some cells was reported suggesting that the acinar cells were indeed transdifferentiating. As the cells reached confluency the ductal antigen expression decreased and the acinar antigen was again found to be present and indeed increased to be expressed in 97% of the population. The proliferation rates decreased steadily after confluency was reached. The differences in antigen expression were also found to be correlated with changes in cellular morphology. Expression of the acinar antigen was associated with polarised

cells that had well organised rough endoplasmic reticulum and small apical vesicles. The ductal antigen was associated with cells which were undifferentiated in appearance. This work demonstrated reversible acinar to ductal transdifferentiation, with associated changes in proliferation. Similarly, Hall and Lemoine (1992) published work using human primary acinar cell cultures where acinar cells (as characterised by amylase positivity and keratin 19 and mucin negativity) appeared to transdifferentiate into ductal-like cells (amylase negative and keratin 19 and mucin positive).

1.3 The genetic basis of tumourigenesis

In tumourigenesis, cells which have altered rate-limiting regulatory pathways are positively selected because the mutations have provided the cancerous cell with a growth advantage compared to the surrounding cells. This leads to clonal populations of tumour cells all possessing this selective growth advantage (Rozenblum *et al.*,1997). The ensuing development of neoplasia however involves a serial accumulation of other genetic changes, not just one. The order in which these mutations occur is also important in that for a specific tissue certain gene mutations instigate tumour initiation, in others tumour progression and still others induce invasiveness (Kinzler and Vogelstein, 1996). This has been illustrated through work in colorectal cancer and Figure 1.1 summarises the genes found to be mutated at specific stages in colorectal cancer.

The growth advantage gained by tumour cells reflects changes in signalling pathways in which the products of these mutated genes normally act. Therefore where clonal expansion of a population of cells occurs via multiple genetic alterations, multiple signalling pathways must be affected. As Rozenblum *et al.*, (1997) hypothesise the mutation of one gene in a pathway nullifies the need for another mutation in the same pathway. So coexistence of mutations in different genes in a tumourigenic population of cells implies that these genes are involved in separate tumour suppressor pathways, although communication between pathways must occur. For

example, *p21* is implicated as a downstream target of SMAD4 in TGF-β responsiveness as well as p53 mediated G1/S growth arrest (Ewen *et al.*, 1993).

Figure 1.1 – Summary of genes thought to be involved in colorectal cancer progression.

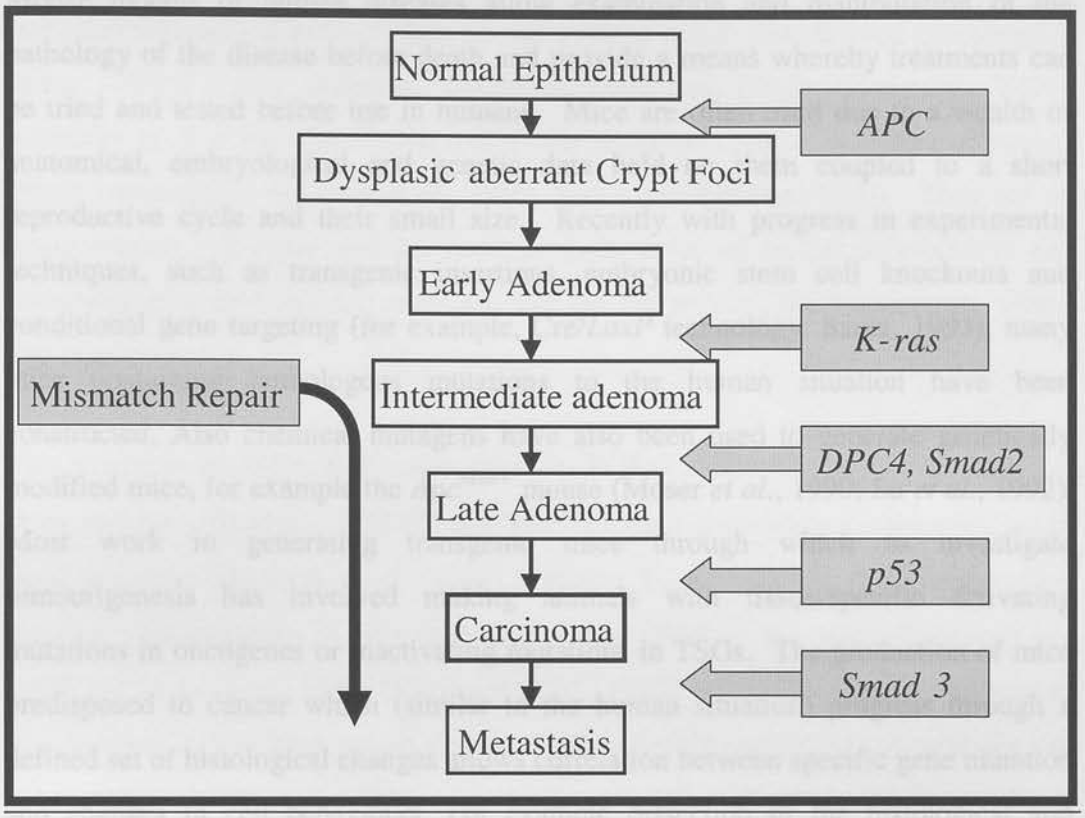


Figure 1.1 – Diagram of histologically identifiable stages in the progression of colorectal cancer. Also shown are genes which, when mutated, have been found to be associated with progression from one stage to the next (Kinzler and Vogelstein, 1996; Zhu *et al.*, 1998).

1.4 Mouse models of tumourigenesis

Animal models are frequently used in the study of cancer and its development. Mouse models of human diseases allow examination and manipulation of the pathology of the disease before death and provide a means whereby treatments can be tried and tested before use in humans. Mice are often used due to a wealth of anatomical, embryological and genetic data held on them coupled to a short reproductive cycle and their small size. Recently with progress in experimental techniques, such as transgenic insertions, embryonic stem cell knockouts and conditional gene targeting (for example, Cre/*LoxP* technology, Sauer, 1993), many mice containing homologous mutations to the human situation have been constructed. Also chemical mutagens have also been used to generate genetically modified mice, for example the $Apc^{Min/+}$ mouse (Moser *et al.*, 1990; Su *et al.*, 1992). Most work in generating transgenic mice through which to investigate tumourigenesis has involved making animals with tissue-specific activating mutations in oncogenes or inactivating mutations in TSGs. The production of mice predisposed to cancer which (similar to the human situation) progress through a defined set of histological changes allows correlation between specific gene mutation and changes in cell behaviours. For example dissection of the histological and genetic changes occurring during pancreatic islet cell tumourigenesis in the RIP-Tag mouse, (in which the SV40 T-antigen is expressed under the control of the rat insulin promoter) has provided information regarding the relative importance of apoptosis, angiogenesis and cell adhesion in tumour development (Hanahan, 1985). For a good review on the utility of mouse models in the study of cancer see Macleod and Jacks, (1999).

1.5 TGF-β superfamily signalling

1.5.1 The TGF-β superfamily

The transforming growth factor-β (TGF-β) superfamily consists of a large number of structurally related growth factors which are remarkably conserved between organisms. Four subfamilies exist which are grouped according to structural or

functional similarity: the TGF-β family; the inhibin/activin family; the decapentaplegic (dpp) and Vegetal (Vg1) related group (DVR group) containing bone morphogenetic proteins (BMP) and growth and differentiation factor (GDF) and other members in none of these groups such as nodal, glial derived neurotrophic factor (GDNF) and mullerian inhibiting substance (MIS) (Kingsley, 1994). Members of this superfamily control a vast range of cellular activities discussed below (reviewed in Barnard *et al.*, 1990; Sporn and Roberts, 1990). They act on many diverse cell types and are fundamentally involved in the development and patterning of a multicellular organism and the maintenance of homeostasis within that organism.

This thesis is primarily concerned with the TGF-β subfamily, of which there are three members, TGF-β1, TGF-β2 and TGF-β3, each possessing a conserved seven exon structure (Derynck *et al.*, 1988). Whilst TGF-β1, 2 and 3 are located on different chromosomes in both man and mouse (Fujii *et al.*, 1986; Barton *et al.*, 1988; ten Dijke *et al.*, 1988; Dickenson *et al.*, 1990) and differentially expressed in development, it has been shown that these three highly conserved polypeptides can have similar activity and potency on cultured cells (Cheifetz *et al.*, 1987; Seyedin *et al.*, 1987; Graycar *et al.*, 1989). Unless otherwise specified the majority of work reviewed here has focused on TGF-β1, however many do not specifically state that they are referring to TGF-β1, thus this thesis simply uses the term TGF-β.

1.5.2 Structure of TGF-β

TGF-β is a dimer consisting of two identical 112 amino acid (a.a.) chains linked by disulphide bonds. The individual chains are secreted as a precursor (390 a.a. in length) containing a hydrophobic NH_2 terminal signal sequence, a glycosylated pro-region (to allow translocation across the endoplasmic reticulum) and a COOH terminal bioactive domain containing 9 cysteines (Massague, 1990). After secretion TGF-β exists in a latent form consisting of a dimer of the two bioactive regions. Whilst the N-terminal signal sequence and pro-region domain has been cleaved off, the proactive domain remains associated with the dimer. A large secretory

glycoprotein called latent TGF-β-binding protein (LTBP) is involved with the TGF-β complex where it functions in the secretion, storage in the extracellular matrix and activation of latent TGF-β (Miyazono *et al.*, 1993; Taipale *et al.*, 1994; Nunes *et al.*, 1997). The dissociation of this complex allows activation of the TGF-β dimer. Once released from the latent complex TGF-β can by bound by various extracellular matrix (ECM) components and certain serum proteins which could be a mechanism of clearance or may function as a reservoir.

1.5.3 Functional consequences of TGF-β signalling on cell behaviour

The TGF-β subfamily has varied and often conflicting functional consequences on cell behaviours depending on cell type and environmental conditions. TGF-β has been shown to be involved in the positive and negative regulation of cell proliferation, cell differentiation, ECM deposition and degradation, cell migration, immunological and inflammatory responses and developmental organisation (Massague *et al.*, 1990; Barnard *et al.*, 1990; see Table 1.2). The actual effect of TGF-β on a particular cell is a result of the integration of many different variables such as cell type, differentiation state, environment (particularly ECM) and the actions of other cytokines. Thus an *in vitro* response may not accurately predict an *in vivo* response to TGF-β signalling. Of particular relevance to this thesis is the function of TGF-β in the control of the cell cycle and of cellular adhesion and matrix interactions.

TABLE 1.2 – The Diverse functional effects of TGF-β

Functional Effect	Mechanism	Example
Cell Proliferation	Increased production of ECM and/or growth factors and their receptors.	Transgenic overexpression of TGF-β in skin leads to stimulation of proliferation. However transgenic overexpression in hyperplastic cells leads to growth inhibition (Cui *et al.*, 1995).
Reverisable G1 growth arrest or cell cycle delay at G1/S transition	Changes in expression and activity of CDKs, Cyclins and CDK inhibitors.	Work using human carcinoma cell lines has shown that a functional SMAD4 is necessary for TGF-β mediated induction of p21 which results in growth inhibition (Grau *et al.*, 1997; Hunt *et al.*, 1998). TGF-β reduces RNA and protein levels of c-Myc (Coffey *et al.*, 1988). This block appears to happen at the level of transcription (Pietenpol *et al.*, 1990).
Immunosuppression	Antiproliferative effect on B and T lymphocytes.	Highly immunogenic tumour cells transfected with TGF-β did not exhibit primary cytolytic T-lymphocyte responses, whereas those not transfected with TGF-β did (Torre-Amione *et al.*, 1990). Also TGF-β has been shown to inhibit TNFα production and downregulate MHC II molecules (Ranges *et al.*, 1987; Czarniecki *et al.*, 1988).
Induction of apoptosis	TGF-β is thought to induce AIF (Apoptosis inducing Factor) in a mechanism involving	TGF-β-induced SMAD3-mediated apoptosis has been shown in lung epithelia cells, by constitutive overexpression of SMAD3 (and to a lesser extent by SMAD2) in the presence of TGF-β. In human gastric cancer cells TGF-β has been shown to induce p53-independent apoptosis (Tamamoto *et al.*, 1996) and in adult and fetal hepatocyte primary cultures TGF-β

	reactive oxygen species. (Langer *et al.*, 1996).	induces apoptosis in a mechanism which involves reactive oxygen species (Sanchez *et al.*, 1996).
Angiogenesis and vascular remodelling	TGF-β has also been shown to be involved in ECM deposition and cellular migration in endothelial and vascular smooth muscle cells.	TGF-β has been shown to induce formation of new blood vessels *in vivo* (Roberts *et al.*, 1986) and some endothelial cell cultures form tubular structures upon stimulation by TGF-β (Majack *et al.*, 1987; Madri *et al.*, 1988).
Control of cell adhesion	Increase in expression and secretion of ECM proteins, decrease in proteolytic proteins which degrade ECM and modification of cell surface adhesion receptors.	TGF-β has been shown in to increase synthesis of fibronectin and collagen I (Ignoz *et al.*, 1987). Inhibitors of matrix degrading enzymes such as PAI-1 and TIMP are upregulated by TGF-β and MMPs are downregulated by TGF-β1 (Laiho *et al.*, 1986; Edwards *et al.*, 1987).
Differentiation	Cells can be both inhibited from, and stimulated into, differentiating by TGF-β.	Intestinal epithelial cells (IEC-6) are stimulated to differentiate by TGF-β, whereas osteoblasts (MC3T3L1) are inhibited from differentiating by TGF-β (Kurokowa *et al.*, 1987; Barnard *et al.*, 1989; Noda and Rodan, 1987; Rosen *et al.*, 1988).

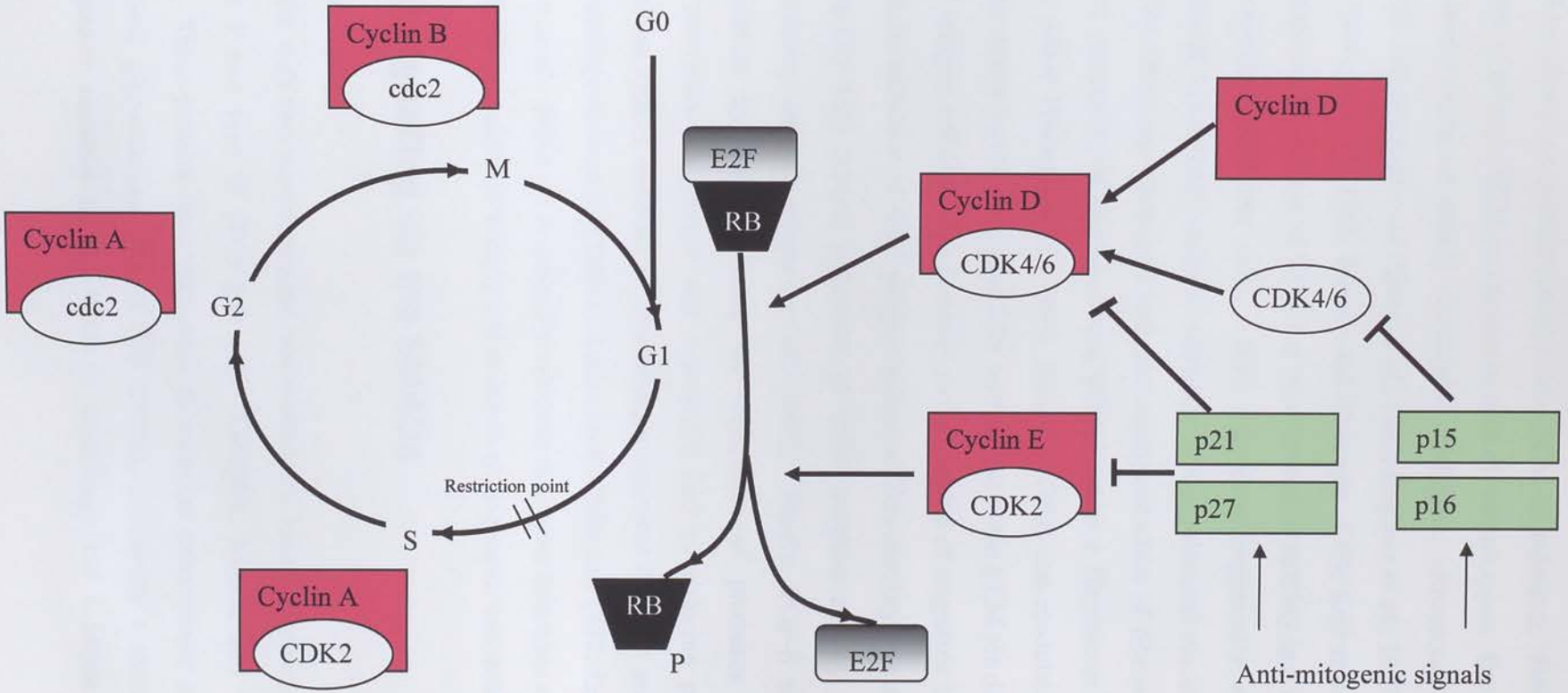
1.5.4 TGF-β signalling - control of the cell cycle

Cell cycle progression is regulated by Cyclins and Cyclin dependent kinases (CDK) which control an ordered series of checkpoints (see Figure 1.2). The action of the CDKs is further modulated by CDK inhibitors. p21 and p27 are structurally related CDK inhibitors which bind and inhibit a variety of CDK/Cyclin complexes. They are involved in the negative control of cell growth and are associated particularly with G1/S phase arrest via interaction with Cyclin E/CDK2 and Cyclin D/CDK4/6 complexes. p21 and p27 are up-regulated by factors such as cell contact, serum deprivation, differentiation and various growth inhibitory cytokines. Their expression is regulated both transcriptionally and by modulation of protein stability. p21 is also induced by p53 in response to DNA damage and its interactions with proliferating nuclear antigen (PCNA) and growth and DNA damage (GADD45) imply that p21 is involved in DNA damage checkpoint control. Nuclear p21 is associated with cell cycle inhibition whereas a C-terminal truncated form of p21 localises to the cytoplasm and inhibits apoptosis (for reviews see Ball, 1997 and Sgambato, 2000).

The induction of growth arrest by TGF-β predominantly occurs at the G1 stage of the cell cycle. Various molecular mechanisms for this growth arrest have been suggested. First, as a result of TGF-β signalling proteins such as c-Myc, CyclinD1 and CDK4 are downregulated (Ewen *et al.*, 1993; Malliri *et al.*, 1996; Sandhu *et al.*, 1997; Ko *et al.*, 1998). Second, TGF-β causes an increase in expression of the CDK inhibitors p21, p27 and p15 (Sandhu *et al.*, 1997; Kamesaki *et al.*, 1998; Hunt *et al.*, 1998; Feng *et al.*, 2000). TGF-β induction of p21 and p15 is linked with SMAD nuclear translocation as well as growth arrest but thus far p27 has not been associated with SMAD-mediated TGF-β signalling (discussed in section 1.6, Grau *et al.*, 1997; Feng *et al.*, 2000). p21 induction by TGF-β has been shown to be p53 independent (Moustakas *et al.*, 1998). Increases in p21 and p27 leads to their binding to the cell cycle regulatory Cyclin D/CDK4 and Cyclin E/CDK2 complexes which decreases the complex activity. Increased p15 causes sequestration of CDK4 and CDK6 removing them from their regulatory Cyclin partners (Sandhu *et al.*, 1997).

Figure 1.2 – Schematic of the cell cycle showing Cyclins (purple), Cyclin dependent kinases (CKDs, white) and Cyclin dependent kinase inhibitors (CKIs, green). Cell cycle progression is regulated by Cyclin and Cyclin dependent kinase (CDK) complexes which control an ordered series of checkpoints. CDK4, when associated with Cyclin D1, phosphorylates RB (retinoblastoma) which then releases the transcription factor, E2F, to initiate the transcription of cell cycle promoting genes. The action of the CDKs is modulated by CDK inhibitors. p15, p16, p21 and p27 are CDK inhibitors which bind and inhibit a variety of CDK/Cyclin complexes. They are involved in the negative control of cell growth and are associated particularly with G1/S phase arrest via interaction with Cyclin E/CDK2 and CyclinD/CDK4/6 complexes. CDK inhibitors are up-regulated by factors such as cell contact, serum deprivation, differentiation and various growth inhibitory cytokines.

Figure 1.2 – Schematic of the cell cycle



1.5.5 TGF-β signalling - control of cellular adhesion and matrix interactions

TGF-β has three effects on cellular adhesion and ECM remodelling. First, TGF-β has been shown to increase ECM production in cells of various types. For example, TGF-β has been shown to induce fibronectin, collagens, vitronectin, tenascin, thrombospondin and proteoglycans (Ignoz *et al.*, 1987; Raghow *et al.*, 1987; Pearson *et al.*, 1988, Penttinen *et al.*, 1988; Bassols and Massague, 1988; Koli *et al.*, 1991;). This TGF-β-induced production of ECM has been related to motility in a migration model of colorectal carcinoma cells. Here TGF-β synergistically stimulated hepatocyte growth factor (HGF)-induced migration of the colorectal carcinoma cell line L-10. This effect was associated with increased production of fibronectin and the synergistic promotion of migration was ablated when a fibronectin blocking antibody was added (Shimao *et al.*, 1999). Second, TGF-β can modulate cellular adhesion to the matrix by influencing cellular recognition of the ECM via differential expression of integrin subunits. For example, the promotion of migration by TGF-β in glioma cells by induction of αvβ5 integrin subunits. This motility was ablated by an RGD (Arg-Gly-Asp) peptide antagonist of αvβ5 integrins and by an αvβ5 integrin neutralising antibody (Platten *et al.*, 2000). Finally, TGF-β suppresses matrix degradation by down-regulating the expression of proteases such as plasminogen activators, collagenase and stromelysin and by inducing proteinase inhibitors such as type 1 inhibitor of plasminogen activator (PAI-1) and Tissue inhibitor of metalloproteinase-1 (TIMP-1) (Laiho *et al.*, 1986 and 1987; Edwards *et al.*, 1987; Kerr *et al.*, 1990). It is generally assumed that these functions of TGF-β are involved in the promotion of tumour cell invasion, angiogenesis and metastasis.

1.6 TGF-β signalling via the SMADs

1.6.1 TGF-β receptors

TGF-β interacts with two transmembrane serine/threonine kinase receptors, TGF-β receptor type I and type II (TGF-βRI and TGF-βRII, 55KDa and 70 KDa respectively). These proteins have three main domains, an extracellular domain, a highly conserved glycine/serine domain (GS domain, containing a characteristic SGSGSG sequence essential for activation of signalling) and a kinase domain

consisting of a serine/threonine protein kinase. Other residues on these proteins include the regulatory T Loop region on TGF-βRII, which when phosphorylated may modulate signalling from the receptor and the L45 loop which appears to be involved in substrate recognition (Massague *et al.*, 1998). TGF-β activates signalling by binding to TGF-βRII (Figure 1.3), followed by a subsequent interaction of the ligand/TGF-βRII complex with TGF-βRI, as such type I receptors can only recognise TGF-β when it is bound to TGF-βRII. The TGF-β dimer, type I and type II receptors therefore form a heterotetramic complex. The type II receptors transphosphorylate the type I receptors on the GS domain which activates the receptor complex (Wrana *et al.*, 1994; Attisano *et al.*, 1996). TGF-βRI is the effector molecule in this receptor complex as mutations which confer constitutive activation on TGF-βRI result in the induction of TGF-β-specific responses (Wieser *et al.*, 1995). It is therefore the activated type I receptor which mediates both the binding of an R-SMAD to the receptor complex and the subsequent phosphorylation of that R-SMAD (Macias-Silva *et al.*, 1996, Zhang *et al.*, 1996). It is thought that TGF-βRII cannot act independently of TGF-βRI, as cells lacking TGF-βRI cannot effect TGF-β responses (Wieser *et al.*, 1993; Brand and Schneider, 1995).

1.6.2 The *Smad* family

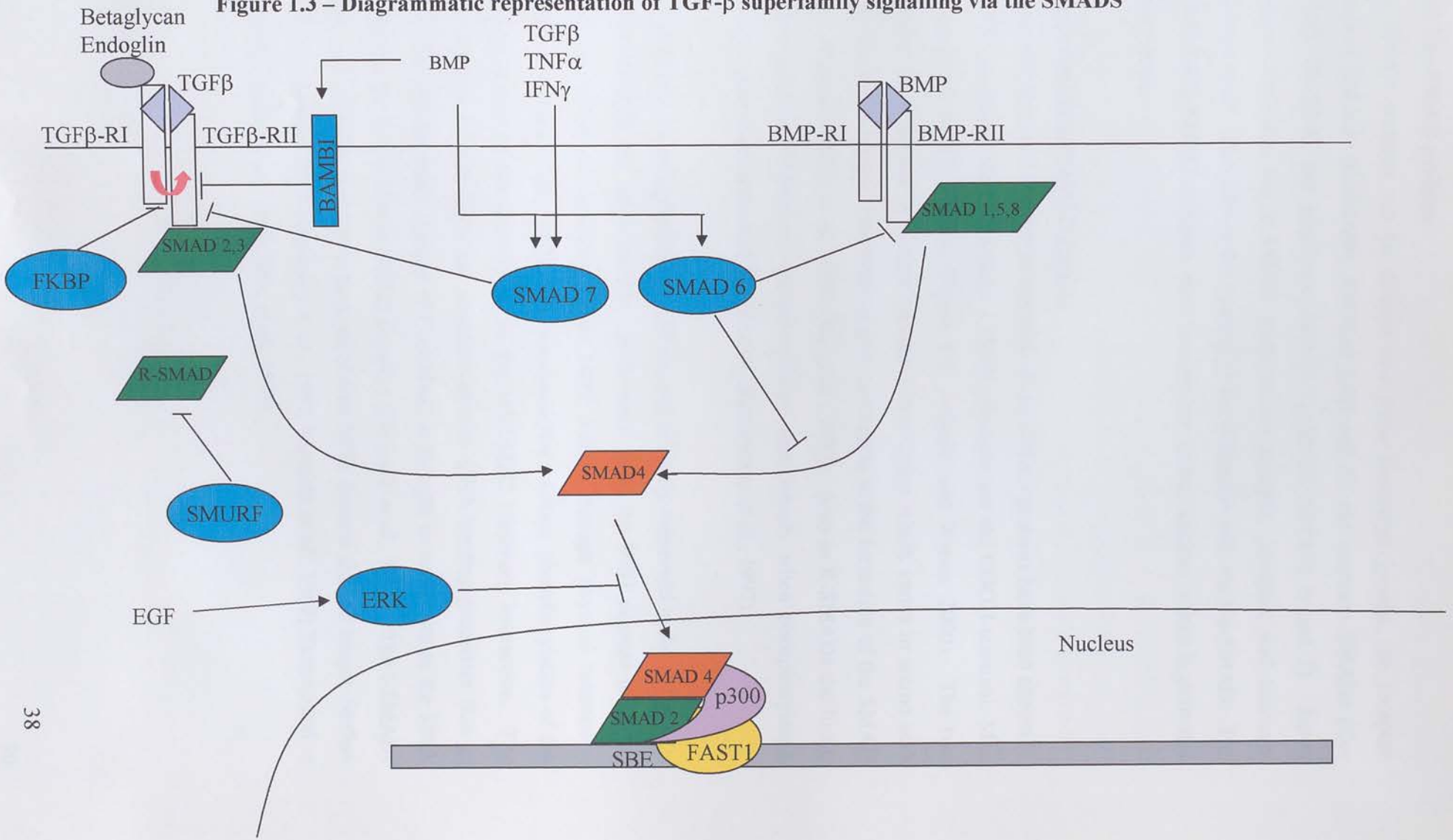
The *Drosophila melanogaster* gene *Mad* (mothers against decapentaplegic) was the first discovered member of the *Smad* Family (Sekelsky *et al.*, 1995; Raftery *et al.*, 1996) and its discovery led to identification of three related genes in *Caenorhabditis elegans* named *sma-2*, *sma-3* and *sma-4* due to the fact that mutation in them caused small body size (Savage *et al.*, 1996). This was closely followed by the discovery of vertebrate homologues which were named *Smads* as a combination of *sma* and *Mad* titles which already existed. The vertebrate *Smads1-8* were then quickly identified by a variety of methods including screening of expressed sequence tag databases and cDNA libraries for *Mad* homologues. As is often the case, work carried out on invertebrates regarding gene function can be utilised when the function of a homologous vertebrate gene is being investigated. MAD proteins had been linked to the TGF-β family of signalling molecules (Hoodless *et al.*, 1996; Graff *et al.*, 1996;

Zhang *et al.*, 1996) and the function of the SMADs was derived from this work on invertebrates revealing that they also participated in the TGF-β signalling pathway (Figure 1.3).

Figure 1.3 - TGF-β activates signalling by binding to TGF-βRII, followed by a subsequent interaction of the ligand/TGF-βRII complex with TGF-βRI. The type II receptors transphosphorylate the type I receptors which activates the receptor complex. The SMAD proteins can be divided into three functional groups, the receptor activated SMADs (R-SMADs, SMADs1,2,3,5 and 8), the common SMADs (Co-SMAD, SMAD4) and inhibitory SMADs (I-SMAD, SMADs 6 and 7). SMADs2 and 3 are activated specifically by TGF-β signalling and SMADs1,5 and 8 by BMP signalling. Upon receptor activation the R-SMAD binds to the receptor complex and becomes activated itself. This allows the binding of the R-SMAD with the Co-SMAD. The R-SMAD/Co-SMAD complex then translocates to the nucleus where it activates transcription. The SBE (Smad Binding Element) is the optimal sequence for R-SMAD/Co-SMAD binding. Transcriptional activation by the SMADs requires additional transcription factors, such as FAST-1(forkhead activin signal transducer) and is greatly modified by transcriptional co-activators (for example p300) and co-repressors (for example Ski and SnoW).

Shown in the diagram are various other regulatory proteins, those which. First, agonists which activate ERK-MAP kinases such as EGF and HGF result in the phosphorylation of R-SMADs that inhibits nuclear translocation of the complex. Second, two cell surface associated proteins, betaglycan and endoglin can bind TGF-β and assist or inhibit ligand binding to the receptor. Third, the SMURF proteins induce ubiquitination receptor activated R-SMADs. Finally, forkhead binding protein (FKBP) acts by inhibiting the phosphorylation of TGF-βRI by TGF-βRII and BAMBI, a truncated type I receptor which disrupts ligand-induced TGF-βRI and TGF-βRII heteromers. Proteins antagonistic to TGF-β signalling are coloured blue.

Figure 1.3 – Diagrammatic representation of TGF-β superfamily signalling via the SMADS



1.6.3 The SMAD proteins

The SMAD proteins can be divided into three functional groups, the receptor activated SMADs (R-SMADs, SMADs1,2,3,5 and 8), the common SMADs (Co-SMAD, SMAD4) and inhibitory SMADs (I-SMAD, SMADs 6 and 7). Upon receptor activation the R-SMAD binds to the receptor complex and becomes activated itself. This allows the binding of the R-SMAD with the Co-SMAD. The R-SMAD/Co-SMAD complex then translocates to the nucleus where it activates transcription.

1.6.3.1 Structure of SMAD proteins

Conserved structural similarities between all the SMAD proteins have been shown in the NH_2-terminal, Mad Homology 1 (MH1) domain and the COOH-terminal, Mad Homology 2 (MH2) domain (Figure 1.4; Attisano and Wrana, 2000). The two domains are separated by a more variable linker region which varies in amino acid sequence and in length. This linker region contributes to the formation of the SMAD homo-oligomers (Hata *et al.*, 1997; Wu *et al.*, 1997). Also in R-SMADs the linker region contains MAP-kinase phosphorylation sites which, when phosphorylated, inhibit nuclear translocation of the SMADs (Kretschmar *et al.*, 1997).

The MH1 domain is approximately 130 a.a. and is highly conserved in the R-SMADs and Co-SMADs but not I-SMADs. In its basal state, the MH1 domain has been shown to inhibit the activities of the MH2 domain through physical interaction (Figure 1.4; Hata *et al.*, 1997). It is presumed that receptor phosphorylation of the SMADs results in the dissociation of the MH1/MH2 inhibitory interaction. The MH1 domain of R-SMADs has sequence-specific DNA binding properties (Liu *et al.*, 1997) and the MH1 domain of Co-SMAD is thought to contribute to the DNA binding of the R-SMAD/Co-SMAD complex (Derynck *et al.*, 1998). The I-SMADs either lack an MH1 domain or have an altered MH1 domain that no longer confers the ability to bind DNA (Imamura *et al.*, 1997; Hayashi *et al.*, 1997; Tsuneizumi *et al.*, 1997; Nakao *et al.*, 1997; Hata *et al.*, 1998).

The C-terminal MH2 domain (about 200 a.a.) contains the receptor phosphorylation sites and physically interacts with type I receptors (Macias–Silva *et al.*, 1996; Kretzschmar *et al.*, 1997). Following phosphorylation it is involved in mediating interaction between SMAD (R-SMADs and Co-SMADs) proteins, nuclear localisation and transcriptional activation (Hata *et al.*, 1997). The MH2 domain also mediates interactions with other transcription factors, coactivators and repressors (Lui *et al.*, 1997; Chen *et al.*, 1997). The MH2 domain is inactive unless an agonist of the pathway is present, however the presence of SMAD4 and isolated MH2 domains of SMAD1 and SMAD2 is sufficient to activate transcription (Lui *et al.*, 1996; 1997). The MH2 domain is therefore the effector domain and the MH1 domain is the regulatory domain.

Figure 1.4 – Protein structure of R-SMADs and Co-SMADs

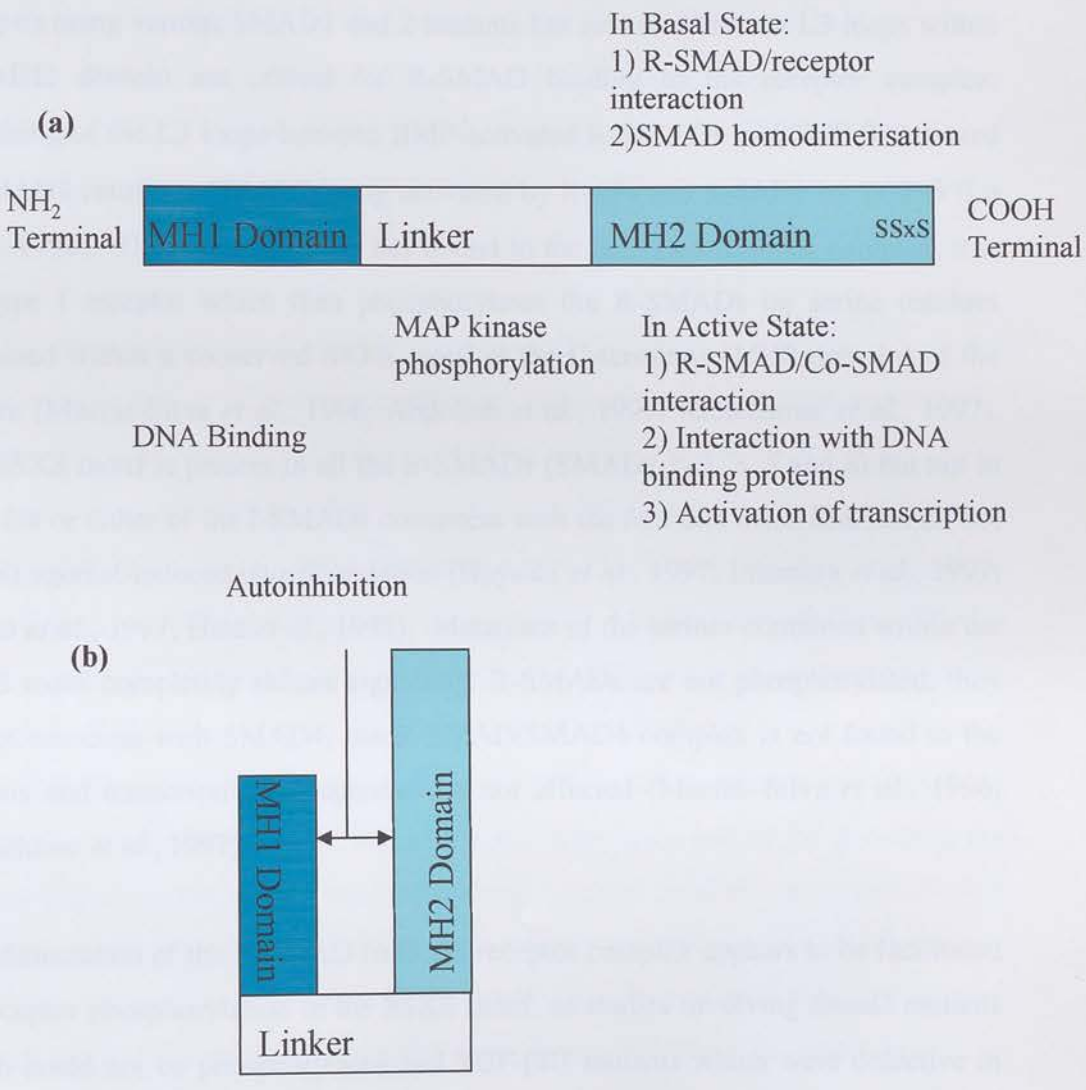


Figure 1.4 – (a) Representation of conserved protein structure of R-SMADs and Co-SMADs, showing functional domains. (b) Physical interaction between the MH1 and MH2 domains is thought to retain the proteins in their basal state.

1.6.3.2 R-SMADs

The transient binding of the R-SMADs, SMAD2 and SMAD3 in the case of TGF-β and activin (Zhang *et al.*, 1996; Graff *et al.*, 1996; Macias-Silva *et al.*, 1996; Baker and Harland, 1997) and SMADs 1, 5 and 8 in the case of BMP signalling (Hoodless *et al.*, 1996; Lui *et al.*, 1996; Kretschmar *et al.*, 1997; Suzuki *et al.*, 1997) to the activated receptor complex allows the phosphorylation of the R-SMAD. Notably SMAD2 mutants which are unable to bind to the receptor activated complex cannot be phosphorylated in response to TGF-β (Macias–Silva *et al.*, 1996; Lui *et al.*, 1997). Analysis using various SMAD1 and 2 mutants has revealed that the L3 loops within the MH2 domain are critical for R-SMAD binding to the receptor complex. Switching of the L3 loops between BMP-activated R-SMAD1 and TGF-β-activated R-SMAD2 results in SMAD2 being activated by BMPs and SMAD1 by TGF-β (Lo *et al.*, 1998). Once the R-SMAD has bound to the activated receptor complex, it is the type I receptor which then phosphorylates the R-SMADs on serine residues contained within a conserved SSXS motif at the C-terminus (MH2 domain) of the protein (Macias-Silva *et al.*, 1996; Abdollah *et al.*, 1997; Kretzchmar *et al.*, 1997). The SSXS motif is present in all the R-SMADs (SMADs 1, 2, 3, 5 and 8) but not in SMAD4 or either of the I-SMADs consistent with the fact that these SMADs do not exhibit agonist-induced phosphorylation (Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997; Hata *et al.*, 1998). Mutations of the serines contained within the SSXS motif completely ablates signalling: R-SMADs are not phosphorylated; they do not associate with SMAD4; the R-SMAD/SMAD4 complex is not found in the nucleus and transcriptional responses are not affected (Macias–Silva *et al.*, 1996; Kretschmar *et al.*, 1997).

The dissociation of the R-SMAD from the receptor complex appears to be facilitated by receptor phosphorylation of the SSXS motif, as studies involving *Smad2* mutants which could not be phosphorylated and TGF-βRI mutants which were defective in their kinase action showed that the R-SMAD remained receptor bound (Macias–Silva *et al.*, 1996; Lui *et al.*, 1997).

1.6.3.3 Co-SMADs

Phosphorylation and activation of the R-SMADs allows heteromeric complex formation with the Co-SMAD, SMAD4, the central mediator on which all TGF-β signalling via the SMADs hinges (Lagna *et al.*, 1996; Wu *et al.*, 1997; Zhang *et al.*, 1997). SMAD4 is a shared partner between both the BMP activated and the TGF-β/activin activated R-SMADs (Lagna *et al.*, 1996; Zhang *et al.*, 1997). This complex of R-SMAD and Co-SMAD has been shown to exist as heterodimers or heterotrimers and interestingly receptor activation can also cause SMAD3 homomers that have DNA binding activity (Kawabata *et al.*, 1998). Again the L3 loop plays an important role here, as mutations in the SMAD4 L3 loop inhibit the association of SMAD4 to SMAD2 (Shi *et al.*, 1997). Thus in the case of R-SMADs the L3 loop mediates interaction with the receptor complex and in the case of the SMAD4 it mediates interaction with the R-SMAD.

1.6.4 Nuclear translocation of SMAD proteins

The MH2 domain of R-SMADs confers an intrinsic nuclear import activity. In SMAD4 defective cells both SMAD1 and SMAD2 were shown to possess the ability to translocate to the nucleus (Lui *et al.*, 1997). SMAD4, however, was shown to only translocate into the nucleus in the presence of SMAD1 and SMAD2 in response to the TGF-β or BMP signalling (Lui *et al.*, 1997). Mostly however these SMADs are retained in the cytoplasm in their basal state to allow prompt response to receptor activation. This retention in the cytoplasm is mediated in part by binding to a protein called SMAD anchor for receptor activation (SARA) which appears to effect three functions: first it anchors the R-SMAD in the cytoplasm; second by occluding the nuclear import signal on the MH2 domain it inhibits nuclear import and third it facilitates binding of the R-SMAD to the activated receptor complex (Tsukazaki *et al.*, 1998). Phosphorylation of the R-SMAD by the activated receptor complex then unmasks the nuclear import signal (Tsukazaki *et al.*, 1998; Xu *et al.*, 2000).

As mentioned above regulation of nuclear translocation occurs by MAP-kinase phosphorylation on PXSP motifs in the linker region of R-SMADs (Kretzschmar *et al.*, 1997). Agonists which activate ERK-MAP kinases such as EGF and HGF

appear to result in the phosphorylation of this linker region that then inhibits nuclear translocation of the complex. This phosphorylation does not however inhibit association of the R-SMAD with the Co-SMAD. Whilst this has been elucidated from work on BMP signalling via SMAD1, it is thought that this regulation may be possible in SMADs2, 3, 5 and 8, as they also contain this MAP kinase phosphorylation site (Kretzschmar *et al.*, 1997;1999).

1.6.5 SMADs as regulators of transcription

Once the R-SMAD/Co-SMAD complex has translocated to the nucleus it then activates transcription. Lui *et al.*, (1996) originally showed that SMADs can activate transcription utilising GAL4-SMAD1 and GAL4-SMAD2 fusion constructs that activated a GAL4 reporter gene. Work with these constructs in SMAD4 deficient cells revealed that SMAD4 was essential to this transcriptional activation. Furthermore the R-SMAD/Co-SMAD complex binds DNA during this transcriptional activation. SMAD4 is itself essential to the transcriptional complex promoting both DNA binding and stabilising the SMAD complex (Lui *et al.*, 1997). The optimal sequence of SMAD3 and SMAD4 binding is a palindromic sequence consisting of two copies of GTCT and its reverse complement AGAC on the opposite DNA strand. Tandem repeats of this sequence confer TGF-β-inducible transcriptional activation (Zawel *et al.*, 1998) and has been termed the SMAD-binding element (SBE). Le Dai *et al.*, (1998) found that mutations in the MH1 domain of SMAD4 affecting DNA binding activity resulted in an inability of SMAD4 to bind to the SBE.

Transcriptional activation by the SMADs is greatly modified by transcriptional co-activators and co-repressors. For example, in response to receptor bound activin, SMAD2/4 has been shown to bind to an activin response element (ARE) in the promoter of the Xenopus *Mix.2* gene. This binding occurs in conjunction with a transcription factor, FAST-1 (forkhead activin signal transducer) that also binds to the ARE of *Mix.2* (Chen *et al.*, 1996; 1997). It has been shown that FAST-1, which resides in the nucleus actually interacts with the MH2 domain of SMAD2 rather than both SMAD proteins. SMAD4 is therefore involved in the transcriptional complex

due to its interaction with SMAD2. (Chen *et al.*, 1997; Lui *et al.*, 1997). However SMAD4 is thought to be vital to transcriptional activation, firstly by promoting the binding of SMAD2/SMAD4/FAST-1 complex to the DNA perhaps by stabilising the complex (MH1 domain), and secondly, by actually activating transcription through its MH2 domain. Cells which express FAST-1 and SMAD2, but not SMAD4 are unable to activate transcription of a reporter gene (Lui *et al.*, 1997). Whilst FAST-1 may only be a DNA binding adaptor, the activity of other transcription factors such as activator protein-1 (AP-1) and Sp1 have been shown to be enhanced by SMAD3 binding (Moustakas and Kardassis, 1998; Zhang *et al.*, 1998).

A second important molecule in SMAD transcription activation is the cAMP response element binding protein/ p300 (CBP/p300). This transcriptional co-activator physically interacts with the MH2 domain of SMAD2 and 3 (Feng *et al.*, 1998, Janknecht *et al.*, 1998, Pouponnot *et al.*, 1998, Topper *et al.*, 1998). In SMAD3 it has been shown that this association is aided by TGF-β-induced phosphorylation of the MH2 domain. A number of pieces of evidence support the hypothesis that CBP/p300 is essential for the transcriptional activity of SMAD3. Deletions within the C-terminal domain of SMAD3 abolish CBP/p300 binding and inhibit transcription. Similarly functional inhibition of CBP/p300 inhibits TGF-β induced transcription via SMAD3/SMAD4. Overexpression of CBP/p300 increases transcriptional activity of SMAD3/SMAD4. SMAD4 is an essential partner in transcriptional coactivation by CBP/p300, as in the absence of SMAD4, CBP/p300 does not act as a coactivator. Again this could be attributed to a role of SMAD4 in stabilising the SMAD3/CBP/p300 complex (Feng *et al.*, 1998). Examples of transcriptional co-repressors include TG-interacting factor (TGIF), c-Ski, SnoN and SMAD nuclear interacting protein (SNIP1) (Wotton *et al.*, 1999; Sun *et al.*, 1999; Stroschein *et al.*, 1999; Akiyoshi *et al.*, 1999; Luo *et al.*, 1999; Kim *et al.*, 2000).

Thus for a given promoter to be responsive to TGF-β-induced SMAD complexes it must first contain a specific SBE that allows direct interaction with the SMAD complex. Second, it must contain a sequence which allows binding of other transcription factors or coactivators that cooperate with the SMAD complex via a

direct or indirect interaction. Therefore whilst many promoters may, for example, bind AP-1, only those with specificity for the SMAD complex would be activated in response to TGF-β signalling. Importantly this model of transcriptional activation by the SMADs also allows a mechanism whereby signalling from two separate pathways may be integrated onto one target gene. Thus stimuli which induces AP-1, such as mitogens or UV irradiation, and TGF-β signalling can converge on a single AP-1 binding/ SBE containing promoter (Zhang *et al.*, 1998). The range of SMAD responses is therefore open to great modification by other signalling pathways which affect the cooperating transcription factors.

1.6.6-Regulation of TGF-β signalling pathway

1.6.6.1-Inhibitory SMADs

The I-SMADs, SMAD6 and SMAD7, have a negative effect on SMAD-mediated TGF-β and BMP signalling. SMAD6 preferentially inhibits BMP signalling and SMAD7 can inhibit both TGF-β and BMP signalling (Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997; Tsuneizumi *et al.*, 1997; Hata *et al.*, 1998). I-SMADs lack the C-terminal SSXS motif (receptor phosphorylation site on the R-SMADs) and the N-terminal region contains only short sequences of MH1 domain homology (Tsuneizumi *et al.*, 1997; Hata *et al.*, 1998). These I-SMADs form part of a negative feedback loop that could regulate both the duration and the intensity of the TGF-β response. Both SMAD6 and SMAD7 respond to multiple signals including TGF-β, Activin and BMP7 (Afrakhet *et al.,* 1998), and appear to act by binding to TGF-βRI and specifically blocking the phosphorylation of the R-SMADs by the activated receptor complex. (Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997). A further mechanism of inhibition by SMAD6 has been shown by its ability to bind SMAD1 and competitively block its interaction with SMAD4 (Hata *et al.*, 1998).

1.6.6.2 Extracellular components and other ligand/receptors

TGF-β ligand can be modulated either via regulation of expression or by binding within an inactive complex with other proteins. Two cell surface associated proteins, betaglycan and endoglin (originally thought to be TGF-βRIII) have been shown to bind TGF-β. However, as these proteins cannot activate signalling, it has been suggested that they act as regulatory components (Cheifetz *et al.*, 1987; Gougos and Letarte 1990; Lopez-Casillas *et al.*, 1991; Wang *et al.*, 1991). Their mechanism of regulation has both positive and negative aspects. The extracellular region of betaglycan, when released, is thought to act as an antagonist to TGF-β signalling by inhibiting binding to receptors (Andres *et al.*, 1989 Lopez-Casillas, 1994). Betaglycan has also been shown to facilitate binding of TGF-β1, 2 and 3 to TGF-βRII and has a particularly high affinity for TGF-β2 (Wang *et al.*, 1991; Lopez-Casillas *et al.*, 1993; Moustakas *et al.*, 1993; Sankar *et al.*, 1995). Endoglin can only bind TGF-β1 and 3. Whilst endoglin/TGF-β/TGF-βRII complexes have been found in monocytes in which endoglin is thought to promote ligand binding, it was also shown that overexpression of endoglin can inhibit TGF-β responses (Lastres *et al.*, 1996). Other proteins which act extracellularly to inhibit ligand binding include Noggin, Caronte and LTBP (Massague, 1998).

1.6.6.3 Intracellular components

Intracellular components which have been shown to modulate TGF-β signalling include extracellular regulated kinase (ERK), which has been linked with both positive and negative regulation of TGF-β signalling (discussed later), SMURF1, which induces ubiquitination of the BMP-activated SMAD1 and SMAD5, and SMURF2, which is thought to act predominantly on TGF-β activated SMAD2 and SMAD3 (Zhu *et al.*, 1999). Other intracellular control elements include forkhead binding protein (FKBP), a negative regulator of receptor signalling, which acts by inhibiting the phosphorylation of TGF-βRI by TGF-βRII (Wang *et al.*, 1994) and BAMBI, a truncated type I receptor which disrupts ligand-induced TGF-βRI and TGF-βRII heteromers (Onichtchouk *et al.*, 1999).

1.6.6.4 Degradation

In addition to the methods described above ubiquitin-mediated degradation of R-SMADs and Co-SMADs is an important method through which TGF-β signalling is restricted. This degradation has been shown to occur both in the cytoplasm via interaction with E3 Ubiquitin ligase SMURF proteins (see above) and of activated R-SMAD/Co-SMAD complexes in the nucleus (Lo *et al.*, 1999). Another method through which degradation modulates the effect of TGF-β signalling is ubiquitination of SMAD partners. For example, the antagonists of TGF-β-induced transcription c-Ski and SnoN have been shown to be degraded in response to TGF-β signalling (Sun *et al.*, 1999), suggesting that removal of these proteins is vital in the initiation of TGF-β induced transcription.

1.6.7 Targets of TGF-β signalling via the SMADs

The functional consequences of TGF-β signalling (outlined in 1.5.3-5) depend largely on the transcriptional regulation of target genes. Table 1.2 outlines known targets of TGF-β signalling.

Table 1.2 – TGF-β target genes involved in regulation of cellular proliferation

TGF-β target genes	Regulation by TGF-β	Reference
TGF-β pathway components		
TGF-β1	Induction	Kim *et al.*, 1990
TGFβ-RI, TGF-βRII	Induction/repression	Kleeff *et al.*, 1998 Piek *et al.*, 1999
Smad3	Repression	Yanagisawa *et al.*, 1998
Smad6	Induction	Afrakhte *et al.*, 1998
Smad7	Induction	Nakao *et al.*, 1997; Afrakhte *et al.*, 1998
Transcriptional regulators		
c-Jun	Induction	Pertavaara *et al.*, 1989
Jun-B	Induction	Pertavaara *et al.*, 1989; Beauchamp *et al.*, 1992
c-Myc	Repression	Pietenpol *et al.*, 1990; Warner *et al.*, 1999
B-Myb	Repression	Satterwhite *et al.*, 1994
c-Fos	Induction	Pertavaara *et al.*, 1989
Fos-B	Induction	Beauchamp *et al.*, 1992
Cell cycle regulators		
p15	Induction	Hannon *et al.*, 1994
p21	Induction	Grau *et al.*, 1997
Cdc25A	Repression	Iavarone and Massague 1997
Cyclin D1	Induction /repression	Kornmann *et al.*, 1999;Ko *et al.*, 1998
Cyclin A	Repression	Satterwhite *et al.*, 1994

Taken from de Caestecker *et al.*, 2000.

1.7 Dysregulation of TGF-β signalling in tumourigenesis

1.7.1-Functional Effects of dysregulation

TGF-β appears to play a dual role in tumourigensis. First, as a tumour suppressor, TGF-β inhibits the proliferation of normal epithelial, endothelial or haematopoietic cells (Sporn and Roberts, 1990; Alexandrow and Moses, 1995). Many tumours exhibit TGF-β unresponsiveness leading to loss of the anti-proliferative effects of TGF-β, this has been linked to tumourigenic progression (Fynan and Reiss, 1993; Markowitz and Roberts, 1996; Polyak, 1996). Various mechanisms for this unresponsiveness are outlined below. Second, TGF-β can also accentuate the malignant phenotype at later stages of tumourigenesis. TGF-β is abundantly expressed in various tumours of epithelial origin in which it functions to suppress immune surveillance, foster tumour invasion and promote angiogenesis and the development of metastasis (Torre-Amione *et al.*, 1990; Fontans *et al.*, 1992; Oft *et al.*, 1998; Akhurst and Balmain, 1999; Yu and Stamenkovic, 2000). Interestingly pancreatic cancers are among those that often over express TGF-β (Friess *et al.*, 1993).

1.7.2 TGF-β as a repressor of tumourigenesis

Work with colon cancer exemplifies this phenomenon. Intestinal crypt cell proliferation is inhibited by TGF-β (Kurokowa *et al.*, 1987) and this inhibition is associated with mid/late G1 growth arrest with downregulation of CyclinD1 (Ko *et al.*, 1995) and blocking of the CDK4 bound RB (Ko *et al.*, 1998). Human colon adenoma cells retain their response to TGF-β, however human colon adenocarcinoma cells partially lose this anti-proliferative response to TGF-β (Manning *et al.*, 1991). Hoosein *et al.* (1989) have shown that responsiveness to TGF-β-induced growth inhibition is linked to the differentiation state of human colon cell lines. This loss of TGF-β-induced growth inhibition is also seen in other epithelial tumour cells including breast (Sun *et al.*, 1994) and pancreatic (Beauchamp

et al., 1990) cells, suggesting that loss of TGF-β-induced growth arrest is not only an important event in cell transformation but also a common event between different carcinomas. Various mechanisms to effect this TGF-β unresponsiveness have been suggested, including inactivation of receptors, R-SMADs or Co-SMADs, overexpression of antagonists of the pathway, for example I-SMADs or even mutations in the SBE.

1.7.2.1 Inactivation of TGF-β receptors

In human disease, mutations in the *TGF-βRII* gene have been shown to make cells resistant to TGF-β leading to increased tumourigenicity (Markowitz and Roberts, 1996). Several groups have reported loss of *TGF-βRII* as an important step in the transformation of epithelial cells (Park *et al.*, 1994; Sun *et al.*, 1994; Freeman *et al.*, 1995). For example, inactivation of *TGF-βRII* has been reported in all microsatellite unstable colorectal carcinomas (about 13% of all colorectal carcinomas) (Markowitz *et al.*, 1995). In microsatellite stable colorectal carcinoma cells however *TGF-βRII* mutations are rare. Wang *et al.*, (1995) found that transfection of TGF-βRII back into receptor negative colorectal carcinoma cells effectively suppressed the tumourigenicity of these cells and restored TGF-β signalling.

Downregulation rather than mutation of TGF-βRII has also been highlighted as a possible mechanism for TGF-β unresponsiveness. Work involving rat intestinal epithelial cells showed that activated H-ras caused a downregulation of TGF-βRII which resulted in insensitivity to TGF-β-induced growth inhibition (Filmus *et al.*, 1992; Zhao and Buick, 1995). Also Zhang *et al.*, (1997) observed that increased Cyclin D1 and CDK4 was associated with downregulation of TGF-βRII in $Apc^{Min/+}$ intestinal adenomas (discussed later).

1.7.2.2 Overexpression of inhibitory SMADs

SMAD6 mRNA overexpression has been seen in pancreatic cancer cells within the tumour mass *in vivo*. Also overexpression of *Smad6* in COLO357 pancreatic cancer cells (TGF-β responsive and SMAD4 active) blocks TGF-β-mediated growth

inhibition and enhances anchorage independent growth in these cells (Kleeff *et al.*, 1999). Further work by Kleeff *et al.*, (1999) has shown that *Smad7* overexpression also enhances tumourigenicity in pancreatic cancer, thus providing another mechanism whereby cells could acquire TGF-β unresponsiveness.

1.7.2.3 Inactivation of R-SMADs

Only two of the R-SMADs have so far been linked to cancer. Inactivating mutations in *SMAD2* (also located at 18q21) have been found in 6% of colon cancers and less frequently in lung cancers (Eppert *et al.*, 1996, Uchida *et al.*, 1996, Riggins *et al.*, 1997). Besides large deletions, screening of tumours and cancer cell lines has revealed that mutations in *SMAD2* are predominantly contained in the MH2 domain, where they disrupt R-SMAD/Co-SMAD binding and affect protein stability (Shi *et al.*, 1997; Eppert *et al.*, 1996; Riggins *et al.*, 1997). Recently it was discovered that *Smad3*$^{-/-}$ mice (unlike *Smad2*$^{-/-}$ or *Smad4*$^{-/-}$ mice) were viable and developed colorectal cancer that had progressed to a metastatic state after 4-6 months (Zhu *et al.*, 1998). However, as of yet *SMAD3* mutation has not been linked to human tumour types (Riggins *et al.*, 1997) though Padgett *et al.* (1998) hypothesise that the likelihood of alterations of other *Smad* genes in other cancer types is quite high.

1.7.2.4 Inactivation of *SMAD4*

Only human pancreatic tumours have been shown to have *SMAD4* significantly (50%) biallelically deleted or existing in a mutated functionally inactive form (Hahn *et al.*, 1996; Schutte *et al.*, 1996). However *SMAD4* is also deleted in other tumour types at a low frequency, for example 20-30% in colorectal cancers, or perhaps totally in a specific subset of colorectal cancers (Riggins *et al.*, 1997 and MacGrogan *et al.*, 1997) and 10% in lung cancer (Schutte *et al.*, 1996; Riggins *et al.*, 1997). Although infrequent, *SMAD4* has also been found to be mutated in breast (Schutte *et al.*, 1996), ovarian (Schutte *et al.*, 1996) head and neck (Kim *et al.*, 1996), prostatic (MacGrogan *et al.*, 1997) oesophageal and gastric cancers (Lei *et al.*, 1996). Again the majority of tumour-associated mutations in *SMAD4* have been found in the MH2 domain however MH1 and linker region mutations have also been reported (Schutte *et al.*, 1996; Jonson *et al.*, 1999). MH1 mutations have been found to increase the

affinity of the MH1 domain for the MH2 domain thus locking the protein in its basal/inactive state (Hata *et al.*, 1997)

Around 5-10% of patients with pancreatic carcinoma have a first degree relative who has had pancreatic cancer. Moskaluk *et al,* (1997) have investigated the possiblity that *SMAD4* is the gene responsible for this familial pancreatic carcinoma. However whereas *p16* (a tumour suppressor) has been highlighted as mutated in 5% of familial pancreatic carcinomas, *SMAD4* mutation does not appear to account for familial pancreatic carcinoma. Functionally inactivating germline mutations of the *SMAD4* locus have been described in families with Familial Juvenile Polyposis (FJP), an autosomal dominant, inherited syndrome associated with harmartomatous polyps and increased risk of gastrointestinal cancer (Howe *et al.*, 1998; Friedl *et al.*, 1999). It is not known if the truncated SMAD4 associated most commonly with FJP has a dominant negative activity on TGF-β signalling, however biallelic inactivation of the *SMAD4* gene is rare.

1.7.3 Does *SMAD4* mutation cause TGF-β unresponsiveness?

Several pieces of evidence support the hypothesis that *SMAD4* mutation causes TGF-β unresponsiveness. First, some TGF-β unresponsive tumour cell lines have also been found to be *SMAD4* null (de Winter *et al.*, 1997; Verbeek *et al.*, 1997) and work using human carcinoma cell lines has shown that a functional *SMAD4* allele is necessary for TGF-β-mediated growth inhibition (Grau *et al.*, 1997; Hunt *et al.*, 1998). Second, *SMAD4* null cell lines are TGF-β unresponsive, as determined by a lack of TGF-β-mediated induction of *3TP-lux* reporter transgene in these cells. The *3TP-lux* reporter transgene contains the TGF-β inducible PAI-1 promoter and a *luciferase* reporter gene. Transfection of these cells with *SMAD4* restores the TGF-β mediated induction of *3TP-lux* (Grau *et al.,* 1997). Finally TGF-β has a known regulatory affect on p21 (a CDK inhibitor) as an inducer of expression and as an activator of this protein leading to cell cycle arrest (Malliri *et al.*, 1996). Loss of SMAD4 correlates to a loss of this TGF-β-induced *p21* expression (Grau *et al.*, 1997).

Whilst this points towards *SMAD4* mutation as a mediator of TGF-β resistance, the fact that other tumour types (in which *SMAD4* deletion has not been implicated) exhibit this resistance highlights the fact that TGF-β unresponsiveness can be achieved through many different mechanisms. It is surprising therefore to note that whilst *SMAD4* mutations are prevalent in pancreatic cancer, *SMAD2* and *TGF-βRII* mutations are not (Vincent *et al.*, 1996; Jonson *et al.*, 1999). It has been suggested that this is because SMAD4 loss of function results in selection for resistance to an endogenous factor other than TGF-β itself (Massague, 1998). TGF-β unresponsiveness in human pancreatic cancers is therefore a complex scenario which is, as yet, poorly understood.

1.7.4 TGF-β as a promoter of tumourigenesis

Whilst much work focuses on TGF-β signalling as a repressor of tumourigenesis, evidence also exists to suggest that it can promote tumourigenesis in a variety of ways (see section 1.7.1). This dual role of TGF-β has been found in a variety of systems. Cui *et al.*, (1996) found these opposing roles of TGF-β in a model system involving mouse keratinocytes. In this system TGF-β inhibited the formation of benign skin tumours. However in tumours which had progressed further, TGF-β overexpression promoted a malignant spindle cell phenotype. Also whilst TGF-β induces growth arrest in well-differentiated primary colon carcinoma cells, it has the opposite effect of stimulating proliferation in poorly differentiated and metastatic colon carcinomas (Schroy *et al.*, 1990; Hafez *et al.*, 1992). Finally, Sheng *et al.*, (1999) exposed RIE-1 non transformed intestinal cells to TGF-β continuously. They found that these cells firstly lost TGF-β responsiveness. Continued exposure after this resulted in morphological changes and cells became resistant to the induction of apoptosis by sodium butyrate. When these cells were implanted into nude mice they caused adenomas. Thus the authors concluded that, through overexpression of TGF-β, the cells had gained a tumourigenic phenotype.

One possible mechanism of TGF-β overexpression was revealed when it was found that transformation of cells with oncogenes such as *Ha-ras* and *v-src* resulted in transcriptional activation of TGF-β (Birchenall-Roberts *et al.*, 1990; Geiser *et al.*, 1991), furthermore this increase in TGF-β did not cause growth inhibition. Oft *et al.*, (1996) reported that *RAS* transformed mammary cells, when treated with TGF-β, became able to invade stroma and obtained a malignant phenotype. This tumourigenic phenotype was associated with upregulation of Cox-2 and downregulation of TGF-βRII.

1.8 The tumour suppressor APC

Familial adenomatous polyposis (FAP), an inherited disorder characterised by the formation of numerous precancerous polyps in the colon (Cannon-Albright *et al.*, 1991), has been shown to be caused by germline mutations in the *APC* gene (Nishisho *et al.*, 1991, Groden *et al.*, 1991, Kinzler *et al.*, 1991). Mutations in the *APC* (adenomatous polyposis coli) tumour suppressor gene have since been found in 80% of spontaneously arising intestinal tumours (Miyoshi *et al.*, 1992). *APC* mutation has also been linked to pancreatic cancer (Horii *et al.*, 1992; McKie *et al.*, 1993). Furthermore $Apc^{Min/+}$ mutant mice exhibited pancreatic preneoplastic lesions which had LOH for *Apc* and stabilised β-catenin (Kongkanuntn *et al.*, 1999) (see below). In the human adult pancreas *APC* is expressed in the ducts, islets and occasionally in the acinar cells (Sieber *et al.*, 2000). In foetal pancreas, however, *APC* is only expressed in the ducts. Whilst this thesis is predominantly concerned with pancreatic tumourigenesis, most of the research into *APC* as a TSG is carried out in the context of intestinal tumourigenesis. As the formation of pancreatic and colorectal cancer has been linked to mutations in many of the same genes (*p53*, *APC*, *SMAD4*, *K-RAS*) work carried out in one tissue provides evidence that may be useful for work being conducted in other tissues.

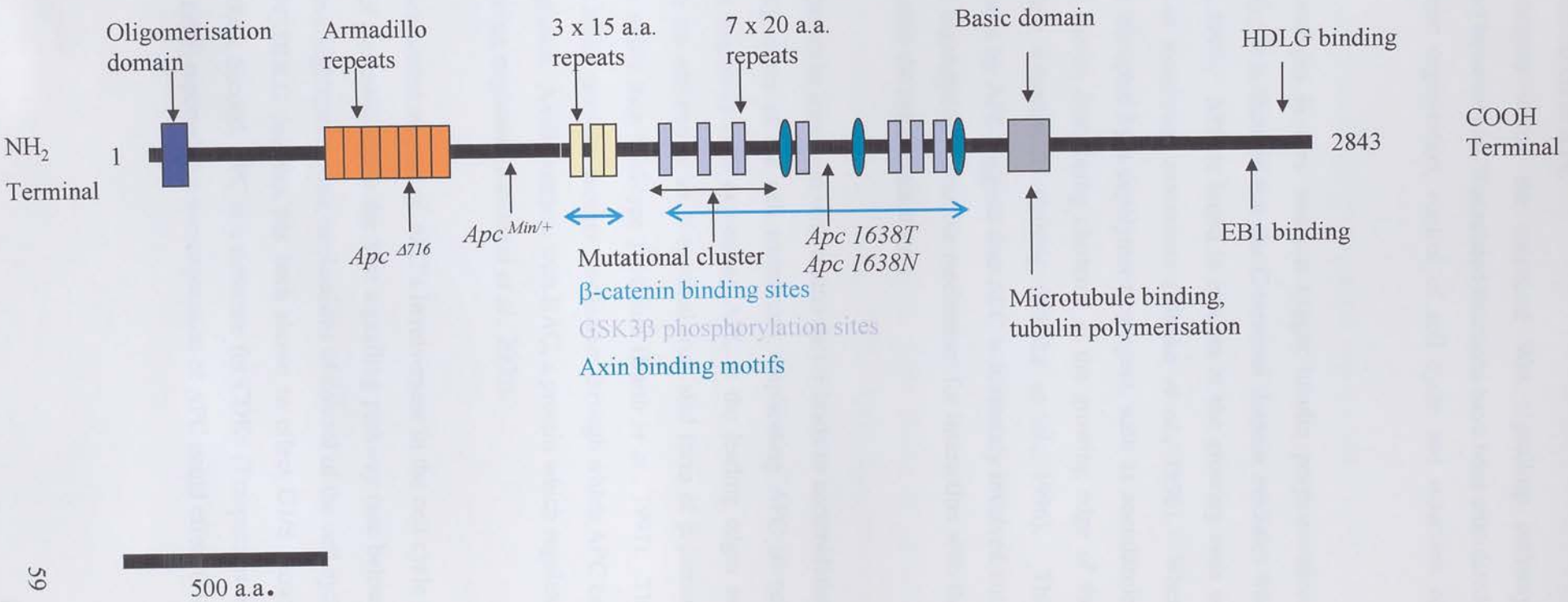
1.8.1 Structure of APC protein

The *APC* gene is situated at 5q21 in humans and 18q21 in mice (Kinzler *et al.*, 1991; Luongo *et al.*, 1993). It contains 16 translated exons, although the actual protein can occur in many forms due alternative splice isoforms (Groden, 1991; Oshima *et al.*, 1993; Santoro and Groden, 1997). The most common *APC* transcript lacks exon 10A and encodes a 2843 a.a. protein. It is this protein which is most commonly studied. The mouse APC protein is a large (approximately 2844 a.a.) multi-domain protein with 90% a.a. homology to human APC (Groden *et al.*, 1991; Kinzler *et al.*, 1991; Su *et al.*, 1992). It contains many motifs which are involved in interactions with other proteins and DNA (see Figure 1.5). Starting from the amino terminus, the first functional domain encountered is the oligomerisation domain containing three heptad repeats which mediate homodimer formation. The armadillo repeats between amino acids 453 and 767 exhibit homology with the *Drosophila* protein armadillo, the homologue of human β-catenin (Hatzfeld, 1999). This area is thought to be involved in modulation of the actin cytoskeleton because it binds to APC-stimulated guanine nucleotide exchange factor (ASEF). The binding of APC to ASEF enhances the interaction of ASEF with a member of the cell adhesion and motility controlling Rho GTPases, RAC. The armadillo repeats are also thought to help mediate protein to protein interactions with β-catenin and perhaps E-cadherin. Also within the armadillo repeats are sites of caspase cleavage. The 15 a.a. repeats have been shown to interact with β-catenin and E-cadherin. The interaction of APC with E-cadherin is thought to be in competition to β-catenin binding (Hulsken *et al.*, 1994). Next are seven 20 a.a. repeats, and these are also thought to possess the ability to bind β-catenin and may mediate its degradation. This region also contains Axin binding sites, a protein involved in the Wnt signalling pathway. Each of these 20 a.a. repeats contains a SXXXS consensus site that is a substrate for glycogen synthase kinase 3β (GSK-3β) phosphorylation. The beginning of these repeats has been found to be a site of many mutations in colorectal cancer and has been named the mutational cluster region. Also within the 20 a.a. repeat regions are DNA binding motifs which mainly bind AT rich DNA sequences (Deka *et al.*, 1999). The basic domain is the site of microtubule association through which APC triggers tubulin polymerisation.

At the C-terminus, APC contains two further DNA interacting sequences. It also contains areas which can interact with two proteins, EB1, a microtubule associated protein and HDLG, a homologue of the *Drosphilia* discs large tumour suppressor. The structure of APC and its functional domains is reviewed in Sieber *et al.*, 2000.

Figure 1.5 – Diagrammatic representation of the protein structure of APC showing various functional domains. Also shown are the locations of the genetic alteration in various *Apc* mutant mice discussed in section 1.9.4. The murine *Apc* gene contains 16 translated exons and is a large (approximately 2844 a.a.) multi-domain protein with 90% a.a. homology to human APC. It contains many motifs which are involved in interactions with other proteins and DNA. These include the oligomerisation domain which mediates homodimer formation and an area which exhibits homology to the *Drosophila* protein armadillo (armadillo repeats) which is though to be involved in modulation of the actin cytoskeleton and mediates interactions with β-catenin. The 15 a.a. repeats and the 20 a.a. repeats interact with β-catenin and E-cadherin. The 20 a.a. repeats are also conatin sites of GSK-3β phosphorylation, Axin binding sites and DNA binding motifs. The beginning of these repeats has been found to be a site of many mutations in colorectal cancer and has been named the mutational cluster region. The basic domain is the site of microtubule association through which APC triggers tubulin polymerisation. APC also contains areas which can interact with two proteins, EB1, a microtubule associated protein and HDLG, a homologue of the *Drosphilia* discs large tumour suppressor.

Figure 1.5 - Protein structure of APC.



1.8.2 Non-Wnt related functions of APC

APC predominantly functions within the canonical Wnt signalling pathway (discussed in detail below) however non-Wnt related functions have been elucidated. These include cytoskeleton organisation, control of cell cycle and induction of apoptosis.

APC can bind to microtubules *in vitro* and can trigger tubulin polymerisation. (Munemitsu *et al.*, 1994). It is thought that the C-terminal domain mediates this interaction (Deka *et al.*, 1998). APC is found in clusters at the growing ends of microtubules contained in membrane extensions (Näthke *et al.*, 1996). When microtubule formation is disrupted by a depolymerising agent, such as nocodazole, the localisation of APC moves from being clustered at the growing edge of the microtubules to a diffuse cytoplasmic staining (Näthke *et al.*, 1996). This stabilisation of microtubules by APC suggests that APC is intimately involved with the microtubule network highlighting a possible mechanism for interaction with the cell cycle, particularly within the cytokinesis stage.

Wounding of cell monolayers or treatment with scatter factor leads to accumulation of APC at the migrating edges of the cell membrane, implicating APC in cell migration. Furthermore migratory cells expressing APC at the leading edges are rendered immobile upon the addition of an N-terminal truncated form of β-catenin which binds APC more stably than wild-type β-catenin (Barth *et al.*, 1997). The interaction of APC with ASEF provides another mechanism through which APC can indirectly affect cell migration. ASEF interacts with RAC, a protein which regulates the actin cytoskeleton during migration (Kawasaki *et al.*, 2000).

The most commonly documented method of APC's involvement in the cell cycle is through the regulation of β-catenin within the Wnt signalling pathway (see below). Some *in vitro* studies have highlighted other mechanisms of control of the cell cycle by APC. First, the APC-HDLG complex has been shown to effect G1/S growth arrest (Ishidate *et al.*, 2000). Second, APC is a substrate for CDKs (Trzepacz *et al.*, 1997) and Baeg *et al.*, (1995) reported that overexpression of APC could effect G1/S

growth arrest via reducing Cyclin/CDK activity. Finally the association of APC with EB1, a protein which localises to the interphase and mitotic microtubules, suggests another mechanism whereby APC could be involved in cell cycle control (Su *et al.*, 1995; Berrueta *et al.*, 1998.

Conflicting results have been obtained from investigations into the role of APC in the control of apoptosis. Overexpression of *APC* in a human colon cancer cell line (HT-29) resulted in loss of cell adhesion and an increase in apoptosis (Morin *et al.*, 1996). Conversely, overexpression of *APC* in normal intestinal epithelia resulted in disordered cell migration but no change in either proliferation or apoptosis (Wong *et al.*, 1996).

1.8.3 APC as a component of the canonical Wnt signalling pathway

The Wnt family of proteins are secreted glycoproteins that have essential roles in development as signalling molecules. In vertebrates there are known to be 19 Wnt ligands which have been categorised according to their ability to promote tumourigenesis (reviewed in Wodarz and Nusse, 1998). Whilst this can occur through various mutational defects in members of the signalling pathway, this thesis is concerned mainly with the regulation of β-catenin by APC and how this regulation is affected in tumourigenesis.

Figure 1.6 - Diagrammatic representation of canonical Wnt signalling pathway showing active and inactive states. Signalling is initiated upon the binding of a Wnt protein to the extracellular domain of a transmembrane receptor called Frizzled. Other proteins which have been found to bind Wnt molecules include a family of secreted proteins called the secreted frizzled related proteins (sFRPs), which are antagonistic to activation of the Wnt signalling pathway. Upon activation of the Wnt/Frizzled receptor complex, the protein Dishevelled (DSH) is hyperphosphorylated by casein kinase II (CKII). Once activated, DSH, via its association with the scaffold protein, Axin, stops GSK-3β from forming a complex with Axin, APC and β-catenin. Thus β-catenin remains unphosphorylated. The unphosphorylated β-catenin escapes degradation as it is not recognised by β-transducin repeat containing protein (β-TRCP, a component of E3 ubiquitin ligase). This leads to increased intracellular β-catenin levels (due to reduced degradation). β-catenin is then translocated into the nucleus where it interacts with LEF/TCF, to activate transcription. A second mechanism of inactivation of GSK-3β has also been found in vertebrates whereby FRAT-1 interacts with GSK-3β thereby inactivating it

In the absence of Wnt ligand, DSH remains inactive and therefore GSK-3β remains active. β-catenin is bound by the APC/Axin complex. This binding of β-catenin to the APC/Axin complex allows the binding of GSK-3β to β-catenin and facilitates the subsequent phosphorylation of β-catenin. This then allows recognition of the phosphorylated β-catenin by β-TRCP and β-catenin is targeted for degradation.

Figure 1.6 - Canonical Wnt signalling pathway

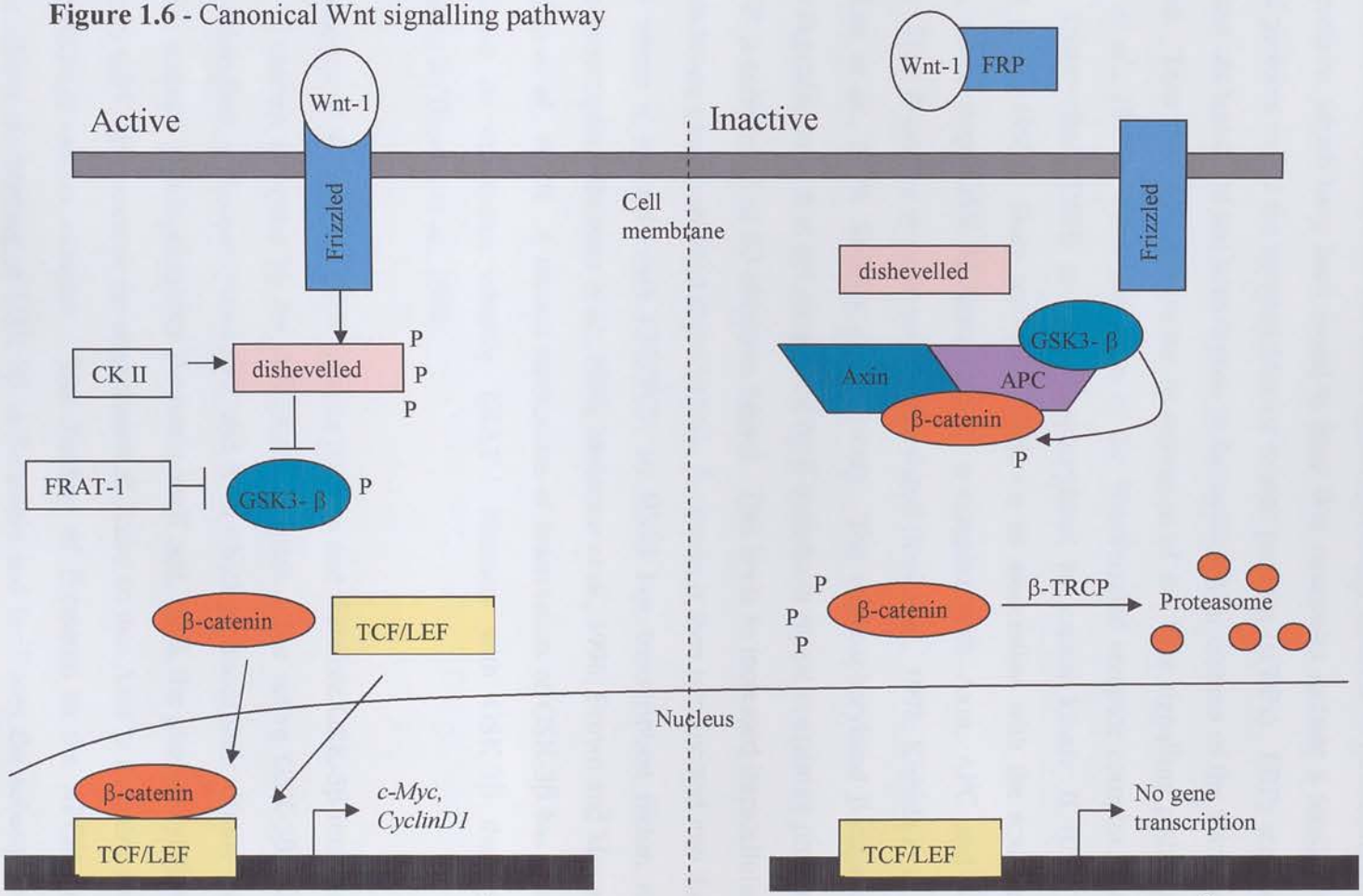


Figure 1.6 shows a simplified overview of the canonical Wnt signalling pathway as outlined below. Signalling is initiated upon the binding of a Wnt protein to the extracellular domain of a transmembrane receptor called Frizzled (Bhanot *et al.*, 1996; Yang-Snyder *et al.*, 1996; He *et al.*, 1997). At least 11 vertebrate *Frizzled* genes have been identified, but the exact ligand specificity remains to be elucidated. Other proteins which have been found to bind Wnt molecules include a family of secreted proteins called the secreted frizzled related proteins (sFRPs). FRPs are not membrane anchored and are homologous to the extracellular domain of the Frizzled receptors. Thus they are antagonistic to activation of the Wnt signalling pathway (Leyns *et al.*, 1997). Upon activation of the Wnt/Frizzled receptor complex, the protein Dishevelled (DSH) is hyperphosphorylated by casein kinase II (CKII) (Willert *et al.*, 1997). Once activated, DSH, via its association with the scaffold protein, Axin, stops GSK-3β from forming a complex with Axin, APC and β-catenin. Thus β-catenin remains unphosphorylated (Itoh *et al.*, 1998, Kishida *et al.*, 1999; Lee *et al.*, 1999; Smalley *et al.*, 1999). The unphosphorylated β-catenin escapes degradation as it is not recognised by β-transducin repeat containing protein (β-TRCP, a component of E3 ubiquitin ligase). This leads to increased intracellular β-catenin levels (due to reduced degradation). β-catenin is then translocated into the nucleus where it interacts with LEF/TCF, an HMG box transcription factor, to activate transcription (Behrens *et al.*, 1996; Molenaar *et al.*, 1996; Brown and Moon, 1998; Hart *et al.*, 1999). A second mechanism of inactivation of GSK-3β has also been found in vertebrates whereby FRAT-1 interacts with GSK-3β thereby inactivating it (Thomas *et al.*, 1999).

In the absence of Wnt ligand, DSH remains inactive and therefore GSK-3β remains active. β-catenin is bound by the APC/Axin complex. The active GSK-3β has various roles: first, it phosphorylates APC and Axin which increases their affinity for β-catenin; second, it phosphorylates β-catenin itself and, third, the phosphorylation of Axin by GSK-3β decreases the degradation of Axin, so that Axin is available for the Axin/APC/β-catenin complex. This binding of β-catenin to the APC/Axin complex allows the binding of GSK-3β to β-catenin and facilitates the subsequent

phosphorylation of β-catenin. This then allows recognition of the phosphorylated β-catenin by β-TRCP and β-catenin is targeted for degradation (Hart *et al.*, 1999; Liu *et al.*, 1999).

Korinek *et al.*, (1997) provided clear evidence supporting this role of APC as a vital regulator of β-catenin. Experiments using $APC^{-/-}$ colon carcinoma cells showed that in the absence of APC, β-catenin was found in complex with the transcription factor TCF in the nucleus. The complex was constitutively active as revealed by activation of a TCF reporter gene. Addition of APC into this system resulted in the breakdown of the β-catenin/TCF complex and the cessation of TCF reporter gene transcription. Thus by preventing β-catenin accumulation, APC is involved in the control of gene transcription.

Targets of β-catenin-mediated signalling include the proto-oncogenes *Cyclin D1* and *c-Myc* (He *et al.*, 1998; Brown and Moon, 1998; Tetsu and McCormick, 1999;). Whilst some TCF/LEF target genes have been identified, many remain unknown. Those TCF/LEF target genes described so far promote proliferation. In support of this $Tcf\text{-}4^{-/-}$ mice have defective proliferation in the stem cell compartment of the small intestine and as a result of this they die shortly after birth (Korinek *et al.*, 1998).

1.9. Dysregulation of the Wnt signalling pathway in tumourigenesis

1.9.1 Evidence of dysregulated Wnt signalling

Wnt promotes the development of tumours in mouse mammary epithelium and *Wnt* genes are activated during MMTV-induced carcinogenesis (Nusse and Varmus 1982; Lee *et al.*, 1995). In human breast lesions *WNT2*, *WNT5a*, *WNT7b* and *WNT10b* exhibit elevated expression patterns (Huguet *et* al., 1994; Lejeune *et al.*, 1995; Dale *et al.*, 1996; Bui *et al.*, 1997) and *in vitro* several different *Wnt* genes transformed epithelial cells (Wong *et al.*, 1994). It appears therefore that Wnt signalling or a

downstream consequence of it is important in mammary gland tumourigenesis and possibly tumourigenesis in other tissues. An important outcome of Wnt signalling is the stabilisation and accumulation of β-catenin.

1.9.2 Mutation of *APC*

Loss of functional APC also leads to β-catenin stabilisation and is thought to be an early event in colorectal tumourigenesis. The smallest detectable intestinal adenomas in $Apc^{\Delta 716/+}$ knockout mice have lost the remaining WT *Apc* allele (Oshima *et al.*, 1995). In humans too LOH for *APC* has been shown in most small colorectal adenomas examined (Kinzler and Vogelstein, 1996). The majority of *APC* mutations are found in the central domain of *APC*, which has therefore been called the mutation cluster region (MCR, see Figure 1.5) (Nagase and Nakamura, 1993). Mutations in the MCR typically result in protein truncations that ablate APC/Axin binding and therefore the subsequent binding of β-catenin to the complex and GSK-3β phosphorylation of β-catenin. Thus mutations in *APC* result in β-catenin stabilisation as is evidenced by high β-catenin levels in colon cancer cells containing mutant *APC* (Munemitsu *et al.*, 1995).

Functionally, loss of APC results in transcriptional changes such as overexpression of *Cyclin D1* or *c-Myc*. Cyclin D1 expression has been found to be increased in 30% of human adenomas and adenocarcinomas in the colon but the actual *Cyclin D1* gene is not amplified (Bartnova *et al.*, 1994; Arber *et al.*, 1996). Cyclin D1 functions in the regulation of the G1 to S phase transition of the cell cycle and has a proliferative effect on cells. It is suggested that overexpression of *Cyclin D1* by β-catenin-mediated transcription could promote tumourigenesis via increased cell proliferation. Similarly He *et al.* (1998) postulate that *APC* mutation within colorectal tumours leads to stabilised β-catenin and overexpression of *c-Myc*. Due to its oncogenic nature and roles in cell growth and differentiation this overexpression of *c-Myc* could promote neoplasia.

1.9.3 Mutation of *β-catenin*

Point or missense mutations in *β-catenin* itself have been found within colon, hepatocellular carcinomas and melanomas (Rubinfeld *et al.*, 1997; Polakis 2000), which predominantly affect the N-terminal serine/threonine residues (Morin *et al.*, 1997). Thus β-catenin remains unbound by the scaffold protein Axin and unphosphorylated by GSK-3β on these serine/threonine substrates. As a consequence of this β-catenin is not targeted for destruction (Aberle *et al.*, 1997; Orford *et al.*, 1997). This mechanism of dysregulated Wnt signalling was confirmed by knock-in mice containing an N-terminal lacking β-catenin. These mice developed adenomas and polyps in the intestine, mammary gland and hair follicles (Harada *et al.*, 1999; Gat *et al.*, 1998).

Work by Sparks *et al.*, (1998) has also highlighted the importance of β-catenin in colorectal tumourigenesis. The authors searched for mutation in genes thought to be involved in the APC/β-catenin/TCF/LEF pathway. Of the human colorectal tumours screened 48% had mutations in the *β-catenin* gene and none of these had mutation in the *APC* gene. The *β-catenin* gene mutations occurred in the early adenomatous stage of colorectal neoplasia, like *APC* mutations. It was also observed that *β-catenin* and *APC* mutations were mutually exclusive. They concluded that in human colorectal tumours oncogenic *β-catenin* mutations can uniquely substitute for APC loss of function in colorectal tumourigenesis. However whilst *APC* and *β-catenin* mutations result in the accumulation of β-catenin and are equally potent regarding transcriptional activation, they appear not to be equally potent in promoting tumourigenesis (Morin *et al.*, 1997). This is reflected in the fact that *β-catenin* mutations tend to be associated with MSI+, small, non invasive adenomas and very rarely with invasive carcinomas. *APC* mutations, whilst present in the small, non-invasive adenomas, are also found to be present in invasive carcinomas (Samowitz *et al.*, 1999). Also *β-catenin* mutations are only found in 25% of colorectal carcinomas whereas A*PC* mutations are found in 80%. Thus it appears that *β-catenin* mutation can substitute for *APC* mutation but that *β-catenin* mutation alone cannot lead to the progression from adenoma to carcinoma.

1.9.4 Mice containing mutations in the *Apc* gene

Much work investigating the tumour suppressive role of APC as part of the Wnt signalling pathway has been carried out on genetically modified mice. Various *Apc* mutant animals have been generated (Figure 1.5). $Apc^{\Delta 716}$ heterozygous knockout mice have been generated by Oshima *et al.*, (1995). These animals have a truncation mutation at codon 716 (affecting the armadillo repeat region of APC) which results in an N-terminal truncated protein and it should be noted that most FAP individuals carry truncation mutations in the N-terminal half of *APC*. These animals develop intestinal adenomas at a high frequency and LOH of *Apc* is seen in these adenomas. Alternative *Apc* mutants are *Apc 1638N* mice which exhibit intestinal adenomas. These animals have an insertion mutation which results in unstable truncated APC mRNA being produced from the mutant allele (Yang *et al.*, 1997; Smits *et al.*, 1998). However, *Apc 1638T*, containing an alteration in the same codon as *Apc 1638N* produces a stable truncated protein. This stable truncated protein still contains some β-catenin binding motifs and notably no intestinal abnormalities are found in these mice (Smits *et al.*, 1999).

1.9.4.1 The *Min* mouse

The *Min* mutation at codon 850 in the mouse *Apc* gene (Moser *et al.*, 1990, Su *et al.*, 1992) results in a truncated protein being produced. This truncated APC protein lacks functional binding sites, including the β-catenin binding sites. It was not caused by gene targeting but by random mutagenesis with ethynitrosourea (ENU), a mutagen which causes point mutations. Mice homozygous for the *Min* mutation die *in utero*, and fail to gastrulate (Moser *et al.*, 1995). Heterozygous mice are viable and exhibit numerous preneoplastic and neoplastic lesions, hence the name multiple intestinal neoplasia (Min). The *Min* mouse also exhibits mammary carcinomas in approximately 10% of female mice. The intestinal lesions were found to have lost the remaining WT copy of *Apc* and progressed to carcinomas (Luongo *et al.*, 1994). Thus due to the presence of *APC* mutations in both the *Min* mouse and FAP patients, the *Min* mouse has been of great use to researchers investigating FAP.

1.10 The tumour suppressor p53

p53 was originally discovered as a protein that co-precipitates with SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine 1979). The finding of high levels of p53 protein in transformed cell lines (De Leo *et al.*, 1979) and that *p53* cDNAs immortalise cells in culture (Jenkins *et al.*, 1984) resulted in *p53* at first being wrongly classified as an oncogene. Subsequently however it was found that this work was being carried out using a mutant *p53* gene and that the WT *p53* gene was actually a tumour suppressor (Hinds *et al.*, 1989). Since then *p53* has been found to be the most commonly mutated gene in human cancer with approximately 50% of all human cancers showing mutation and evidence exists to support the notion that the pathway is dysregulated in all cancers due to loss or upregulation of other members of the pathway (Hollstein *et al.*, 1994). Fundamentally p53 allows cells to respond appropriately to many different kinds of stress, such as DNA damage (caused by ionising radiation, UV irradiation or chemotherapeutics), telomere attrition, oncogene activation, hypoxia, heat shock, abnormal cytokine or growth factor environment. Many of these stress signals are at the heart of the tumourigenic process, thus p53 is a vital tumour suppressor at many stages of tumourigenesis and loss of p53 function promotes tumourigenesis. Individuals with Li-Fraumeni syndrome have been found to have germline mutations in *p53* and display a predisposition to cancer (Malkin *et al.*, 1990; Srivastava *et al.*, 1990).

p53 appears to function in many pathways that when dysregulated are fundamental to tumourigenesis. The roles of p53 include cell cycle regulation, induction of apoptosis in response to DNA damage, DNA repair, cell senescence and stress responses. I will write briefly on three of these functions and their relation to tumour progression, although it is hard to do this subject justice due to the enormous amount of work that has been done on p53.

1.10.1- Structure of p53

The *p53* gene is situated on human chromosome 17 and mouse chromosome 11. It is approximately 20kb in size and contains 11 exons, only 10 of which are translated. Exon 1 encodes a 5' untranslated sequence (Bienz *et al.*, 1984). The p53 protein consists of 393 a.a. and has four main functional domains. The first is N-terminal and is the transactivational domain. This area is also the site of MDM2 and acetyltransferase (such as p300/CBP) binding. Phosphorylation at this region enhances p53 activity by increasing the affinity of p53 for acetyltransferases. Following this is the proline rich domain, that does not modulate the transactivation function of p53 but is necessary in some way to mediate p53-dependent apoptosis (Walker and Levine, 1996; Sakamuro *et al.*, 1997). The central domain is the DNA binding domain. The majority of *p53* mutations in tumours have been found to be within this area of the *p53* gene, thus affecting the sequence-specific binding (Cho *et al.*, 1994; Hollstein *et al.*, 1994). The C-terminal end of the protein is the oligomerisation domain and the p53 protein exists primarily as a tetramer (Friedman *et al.*, 1993). This domain also contains the nuclear localisation and nuclear export signals and is the site of ubiquitination by MDM2. The last 30 a.a. of C-terminal p53 also form an autoinhibitory domain which is involved in the regulation of latent p53 (see Figure 1.7).

Figure 1.7 – Diagrammatic representation of the protein structure of p53 showing major functional domains.

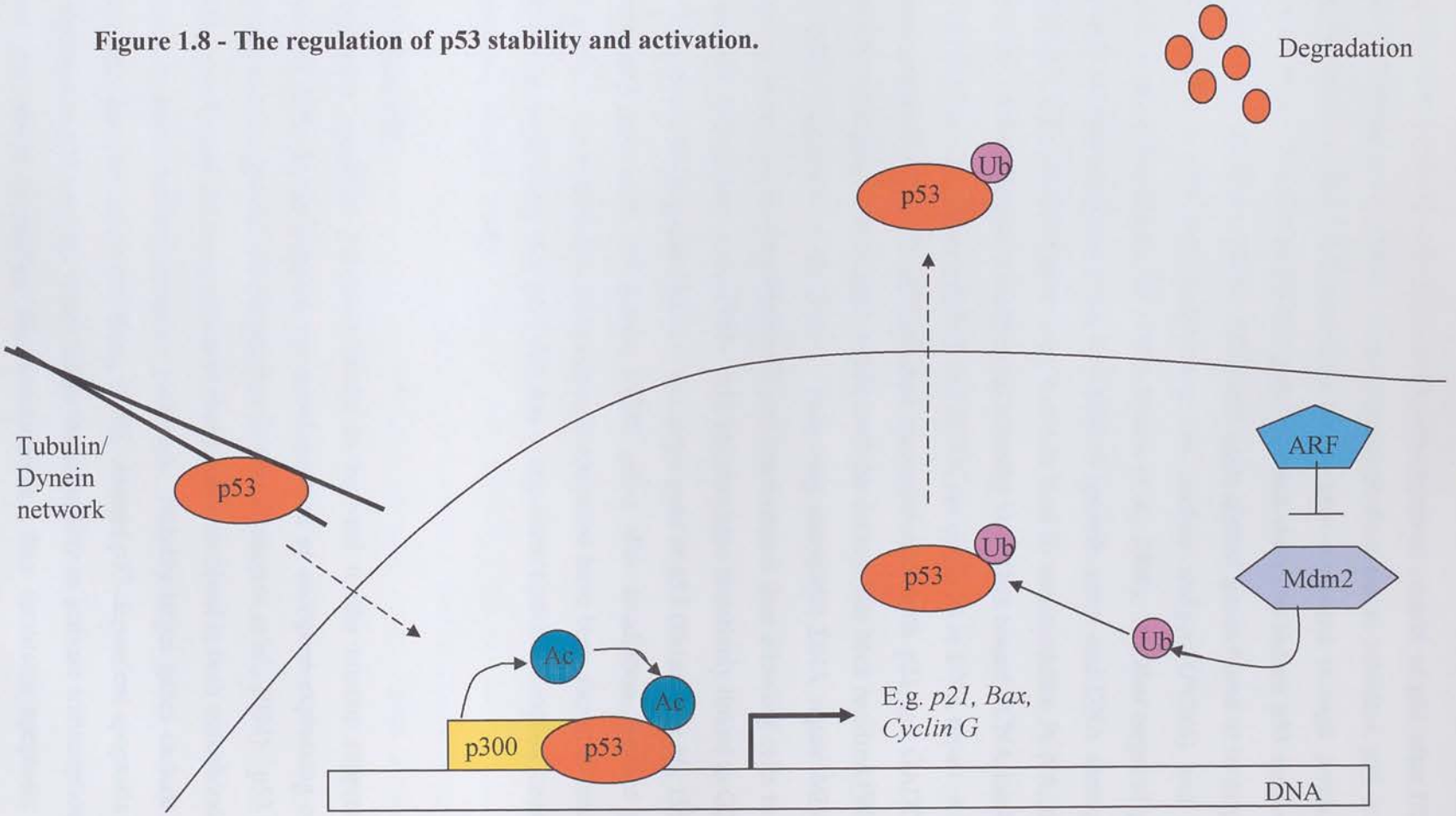
Figure 1.7 - Protein structure of p53

1.10.2 Functions of p53

Primarily p53 is a transcription factor that binds to DNA in a sequence-specific manner to activate or repress transcription. Most of its downstream functions are thought to be mediated by this activation or repression of target genes. Other non-transcriptional functions of p53 are thought to include relocalisation of death receptors to the cell membrane (Owen-Schaub *et al.*, 1995) and a promotion of apoptosis via the relocalisation of p53 to mitochondria (Marchenko *et al.*, 2000). The main cellular responses which are elicited by p53 activation are cell cycle arrest and apoptosis. An overview of p53-mediated signalling in the cell is shown in Figure 1.8.

Figure 1.8 - An outline of some of the mechanisms of p53 stabilisation, activation and localisation. MDM2 can bind p53 and regulates p53 stabilisation through functioning as a ubiquitin ligase. Ubiquitination of p53 on lysine residues in the C-terminus by MDM2 is thought to mediate translocation of p53 from the nucleus into the cytoplasm where it is degraded. MDM2 can also negatively regulate p53 via the inhibition of p53 acetylation, which normally results in amplified p53 activity. ARF functions to induce the stability of p53 by inhibiting the actions of MDM2. To effect this ARF binds MDM2 thus inhibiting ubiquitination of p53 and allowing p53 to accumulate in the nucleus. The nuclear localisation signals of p53 are situated on the C-terminal. These interact with the microtubule network and dynein (a molecular motor) to facilitate the nuclear accumulation of p53 (Giannakakou *et al.*, 2000). Finally, p53 activity is varied by post translational modifications within the N and C-termini of p53. One example is the acetylation of p53 by the transcription factor p300, this augments sequences-specific DNA binding and transcriptional activation.

Figure 1.8 - The regulation of p53 stability and activation.



1.10.2.1 Cell cycle arrest

p53 exerts control of the cell cycle via its transcriptional control of p21 after DNA damage (Waldman *et al.*, 1995). As a Cyclin dependent kinase inhibitor, p21 leads to growth arrest at the G1/S boundary and is also expressed in high levels in senescent cells. Deletion of the *p21* gene has been shown to reduce p53 mediated cell cycle arrest (El Deiry, 1998). Other cell cycle control genes found to be targets of p53 include *Cyclin D1*, *proliferating cell nuclear antigen* (*PCNA*) and *RB* (Osifchin *et al.*, 1994; Chen *et al.*, 1995; Morris *et al.*, 1996). Another target of p53 which acts to induce growth arrest is *GADD45* (growth arrest and DNA damage). Together p21 and GADD45 have been found to bind to and modulate PCNA, thus Smith *et al.*, (1994) suggest a mechanism whereby GADD45 bound PCNA inhibits entry into S phase. As both GADD45 and PCNA are involved in DNA repair, it has also been suggested that this p53-initiated transcription of both p21 and GADD45 allows DNA damaged cells to stop cycling until the damage has been repaired (Waga *et al.*, 1994; Kazantsev *et al.*, 1995). Following successful DNA repair MDM2 (another p53 target gene) then binds p53 and inactivates it thus allowing cells to re-enter the cell cycle (Chen *et al.*, 1994). p53 has also been tentatively linked to G2/M arrest through the finding that *Cyclin G* is a target gene of p53 (Shimizu *et al.*, 1998), however, *p53* null cells still exhibit G2/M arrest after irradiation. Mutant p53 proteins which are defective in transcriptional activation have been shown to initiate growth arrest, suggesting that p53 also has a non-transcriptional function causing growth arrest (Mowat, 1998).

1.10.2.2 Apoptosis

p53-dependent apoptosis has been shown to be vital to the tumour suppressor activities of p53. It can suppress the transformation of oncogene-expressing cells and inhibit tumour growth and progression *in vivo* (Symonds *et al.*, 1994). p53 has been shown to induce the transcription of many genes involved in both mitochondrial and death receptor mediated apoptotic pathways. Notably target genes include the pro-apoptotic *Bax* (Miyashita and Reed, 1995). Altered p53-dependent apoptosis has been reported in *p53* mutants which retain the capability to activate transcription of *p21* but are unable to activate *Bax* transcription. Bax facilitates apoptosis via

depolarisation of mitochondrial membranes, cytochrome c release and activation of caspases. Other target genes which act to effect mitochondrial mediated apoptosis shown to be required for the p53-mediated apoptotic response include *Noxa* which interacts with the anti-apoptotic Bcl2 and p53AIP1 (Oda *et al.*, 2000). p53 also induces the expression of death receptors such as FAS/APO-1, Killer/DR5 and PIDD (Owen-Schaub *et al.*, 1995; Wu *et al.*, 1997; Lin *et al.*, 2000). An apoptotic signal is transmitted upon ligand binding to these death receptors.

Other transcriptional targets of p53 that may act as inducers of apoptosis includes insulin growth factor binding protein 3 (IGF-BP3), which binds insulin growth factor (IGF1) thus preventing binding to IGF1 receptor and inducing apoptosis. In response to oncogene activation (for example, *c-Myc*) IGF1 is thought to repress apoptosis. The Fas/APO-1 receptor is also upregulated by p53. Occupation of the Fas/APO-1 receptor results in apoptosis in T-lymphocytes. This has been suggested as a mechanism tumours use in response to infiltrating cytotoxic T-lymphocytes (Mowat, 1998). finally, work carried out by Polyak *et al.*, (1997) screening expression of p53-activated transcripts that led to apoptosis induction found that p53 transcriptionally induced redox genes. They postulate that this leads to the formation of reactive oxygen species that degrade the mitochondria leading to apoptosis.

1.10.2.3 DNA repair and inhibition of angiogenesis

Chemical and physical agents are constantly subjecting the genome to damage. Failure to repair this damage can lead to gene mutations, which, depending on the gene affected, can lead to changes in cell behaviours such as those found in cancer. Cells have various DNA repair mechanisms including repair by alkytransferases (Pegg *et al.*, 1990), base excision repair (Seeberg *et al.*, 1995), nucleotide excision repair (NER; Satoh *et al.*, 1993; Reardon *et al.*, 1997), mismatch repair (Kolodner, 1995; Modrich and Lahue, 1996) and double strand break repair (Kanaar and Hoeijmakers, 1997). The suggestion that p53 can not only effect changes in the cell cycle and cell survival but also modulate DNA repair has tentative experimental support. First, p53 has been shown to bind NER proteins such as XPB and XPD (Wang *et al.*, 1996). Second, work with $p53^{-/-}$ mice has shown that whilst these

animals are predisposed to cancer, this is increased following exposure to genotoxic chemicals (Tennant *et al.*, 1996), indicating that the cellular functions dysregulated by loss of p53 and DNA damage are related. It is thought that in response to DNA damage p53 functions to initiate cell cycle arrest, allowing the cell to repair its damage. If the damage is too severe, p53 accumulation is thought to then initiate apoptosis.

1.10.3 Control of p53 activity

In normal conditions the short half life of p53 (approximately 15 minutes) means that levels are low (Kamijo *et al.*, 1998). In response to various stimuli, p53 is rapidly induced through mechanisms which do not involve increased transcription of the *p53* gene.

1.10.3.1 Latent p53

p53 is synthesised in a latent, oligomerised form. Phosphorylation of the autoinhibitory domain results in the release of active p53 from the latent form (Hupp *et al.*, 1992). Latent p53 is not protected from MDM2-dependent ubiquitination, however, once activated, it undergoes a conformational change and is less efficiently targeted for protease-mediated degradation (Haupt *et al.*, 1997).

1.10.3.2 MDM2

MDM2 was first identified as a gene amplified in transformed cell lines and human tumours (Fakhararzadeh *et al.*, 1991; Oliner *et al.*, 1992). The finding that MDM2 could bind p53 *in vitro* and *in vivo* and inhibit the sequence-specific binding activity of p53 (Oliner *et al.*, 1992, 1993) led to the discovery that MDM2 negatively regulates p53 activity. It was later found that MDM2 could function as a ubiquitin ligase and thus regulated p53 through the induction of degradation by ubiquitination (Honda *et al.*, 1997; Haupt *et al.*, 1997). A negative feedback model has been suggested since MDM2 is in fact a transcriptional target of p53 (Barak *et al.*, 1993; Zauberman *et al.*, 1995). Ubiquitination of p53 on lysine residues in the C-terminus by MDM2 is also thought to mediate translocation of p53 from the nucleus into the cytoplasm (Geyer *et al.*, 2000). MDM2 can also negatively regulate p53 via the

inhibition of p53 acetylation, which normally results in amplified p53 activity (Kobet *et al.*, 2000). Thus in order for p53 stabilisation in response to a stress factor to occur, MDM2 negative regulation of p53 must be blocked. For example, CHK1 and CHK2 phosphorylate an N-terminal residue which inhibits the interaction of MDM2 with p53 and p53 degradation is prevented (Chehab *et al.*, 2000; Shieh *et al.*, 2000). Unlike $p53^{-/-}$ mice, $Mdm\text{-}2^{-/-}$ mice are embryonic lethal, though when these animals were crossed to $p53^{-/-}$ mice the lethality was rescued (Jones *et al.*, 1995). This suggests that for proper development to occur MDM2-mediated control of p53 functions is vital.

1.10.3.3 ARF

$p19^{ARF}$ has also been found to be a tumour suppressor gene. $p19^{ARF-/-}$ mice have been generated and are predisposed to tumour formation (Sherr and Weber, 2000). ARF functions to induce the stability of p53 by inhibiting the actions of MDM2. To effect this ARF binds MDM2 thus inhibiting ubiquitination of p53 and allowing p53 to accumulate in the nucleus (Zhang *et al.*, 1998; Weber *et al.*, 1999; Tao and Levine, 1999). The expression of ARF is thought to be one of the main ways in which cells respond to abnormal oncogene activation. Thus ARF is readily induced by oncogenes such as *c-Myc*, *RAS*, and *E2F1* and as such p53 is stabilised and can effect its tumour suppressive function (Vousden, 2000). Proteins which can negatively regulate ARF include RAS and JunD (Weitzman *et al.*, 2000; Ries *et al.*, 2000).

1.10.3.4 Control of stability

The phosphorylation of p53 in response to DNA damage has been shown to inhibit the association of p53 with MDM2 thus protecting p53 from degradation (Unger *et al.*, 1999; Bottger *et al.*, 1999; Ashcroft *et al.*, 1999). Phosphorylation of MDM2 is another mechanism whereby MDM2/p53 interactions are reduced and p53 is stabilised (Mayo *et al.*, 1997).

1.10.3.5 Localisation of p53

The function of p53 depends on both its nuclear import and nuclear export. The nuclear localisation signals of p53 are situated on the C-terminal. These interact with

the microtubule network and dynein (a molecular motor) to facilitate the nuclear accumulation of p53 (Giannakakou *et al.*, 2000). As mentioned previously one of the roles of MDM2 is to mediate the nuclear export of p53. This is of particular importance as not only does removal of p53 inhibit its action as a transcriptional activator but it also promotes the ubiquitination of p53. It is suggested that the absence of nuclear p53 in tumours is due to degradation of nuclear p53 by MDM2 (Lu *et al.*, 2000). Conversely, mutations in the C-terminus of p53 have been shown to result in the nuclear accumulation of p53 due to defective MDM2-mediated nuclear export and degradation (Buschmann *et al.*, 2000; Lu *et al.*, 2000).

1.10.3.6 Post translational modifications

One final mechanism of control of p53 activity is post translational modification within the N and C-termini of p53. The effect of these is to augment sequence-specific DNA binding and transcriptional activation. These modifications include phosphorylation, sumoylation and acetylation. An example of this is N-terminal phosphorylation of p53 by p38, a member of the MAP kinase signalling pathway. In response to UV radiation p38 phosphorylates p53 providing a mechanism whereby p53 can respond to the damage caused by the radiation (Huang *et al.*, 1999). Furthermore, PML (promyelocytic leukaemia), a protein induced by stress signals localises with both p53 and p300 leading to N-terminal phosphorylation followed by C-terminal acetylation. Loss of PML is associated with loss of p53 functional responses (Guo *et al.*, 2000; Fogal *et al.*, 2000).

1.11 p53 in tumourigenesis

The effects of loss of p53-mediated activities on tumourigenesis are diverse. Tumour progression can be linked to a loss of negative control and a gain of positive control on growth or by tumour cells responding differently to, for example, factors that would normally promote apoptosis.

1.11.1 Loss of the apoptotic response

Hypoxic conditions are present particularly within large tumours and hypoxia is one of the stimuli which promote p53-dependent apoptosis. Thus when WT p53 is present high levels of apoptosis are observed in the hypoxic areas of tumours, and conversely when tumours have lost p53 function apoptosis levels are not elevated (Graeber *et al.*, 1996). Another example highlighting the importance of p53-dependent apoptosis in the reduction of tumour progression includes an *in vivo* model of choroid plexus tumours. Here a mutant SV40 T antigen which bound and sequestered RB, but not p53, resulted in reduced tumour formation when compared with WT SV40 T antigen. The mutant SV40 T antigen-bound RB still inhibited the proto-oncogene *E2F*. When this experiment was carried out on a *p53* null background the tumour outgrowths from both the mutant SV40 T antigen and the WT SV40 T antigen were aggressive and exhibited attenuated apoptosis (Symonds *et al.*, 1994). Similarly when the experiment was repeated but with $Bax^{-/-}$ mice rather that $p53^{-/-}$ mice, tumour progression rate was increased. On comparison of the apoptotic index in these tumours it was observed that it was reduced by 90% in the $p53^{-/-}$ mice and 50% in the $Bax^{-/-}$ mice (Yin *et al.*, 1997; Mowat, 1998). This highlights the fact that p53 induces apoptosis by more than just inducing the transcription of *Bax* and that whilst p53 is not essential for tumour growth, its loss results in a more aggressive tumour progression due to the loss of p53 dependent apoptosis.

A good human example highlighting the role of p53 inactivation in tumour progression is Wilms' Tumour. Some of these tumours exhibit areas of anaplastic morphology which show loss of p53 and a reduction of apoptosis in comparison with other areas of the tumour. Importantly, Wilms' Tumour patients with these anaplastic areas have a worse prognosis than those without (Bardeesy *et al.*, 1994). It should be pointed out however, that this role in the suppression of carcinogenesis by p53-dependent apoptosis is hard to differentiate from other roles of p53-mediated tumour suppression.

1.11.2 Increased proliferation

Loss of G1 growth arrest usually mediated by p53 may contribute to increased cell proliferation in tumourigenesis. For example, Moll *et al.*, (1996) demonstrated that mutant *p53* present in neuroblastoma-derived cell lines was unable to undergo nuclear translocation following DNA damage. This was coupled with impaired p53-mediated G1 growth arrest. Also, $p53^{-/-}$ murine embryonic fibroblasts *in vitro* exhibit a shorter cell cycle duration and increased proliferation (Harvey *et al.*, 1993).

1.11.3 Increased genomic instability

p53 mutation in human tumours has been associated with nuclear abnormalities such as aneuploidy and genetic instability both *in vivo* and *in vitro* (Blount *et al.*, 1994; Cross *et al.*, 1995). Genomic instability is the fundamental factor in all stages of tumourigenesis. The inability of a cell to repair DNA damage can result in gene mutation, and when this gene mutation occurs in a target gene essential for the regulation of processes such as cell growth, death or DNA repair, the integrity of the genome in that cell is seriously challenged. Thus the mutation rate within cells increases and the accumulation of genetic alterations results in changes in cell behaviours, the appropriate cell cycle check points are circumvented, cells which should die, do not, and abnormal cells survive and proliferate. The end result is the neoplastic transformation of cells.

Two specific types of genomic instability are polyploidy, defined as a state in which nuclei contain multiple copies of a full complement of chromosomes for example 4n or 8n and aneuploidy, defined as a state in which nuclei contain a chromosome complement that is not a multiple of haploid, for example 2n +1 or 4n –3. Polyploidy or aneuploidy can occur via a variety of mechanisms such as mitotic non-disjunction, defects in centrosome organisation, abnormal regulation of cell cycle checkpoints and faulty DNA repair mechanisms. Changes in ploidy have been shown to be associated with the progression of tumourigenesis and the analysis of ploidy within human cancers has been shown to be of great prognostic value. For example, the DNA content of lung adenocarcinomas has been shown to increase in less differentiated more advanced carcinomas, furthermore this abnormal DNA

content as analysed by cytofluorometry was reflected histologically as nuclear atypia (Asamura *et al.*, 1989). Histologically abnormal nuclei could occur for a variety of reasons; due to polyploidy or aneuploidy, because of a diploid cell in a different stage of the cell cycle or due to changes in histones, RNA or chromatin folding.

It is suggested that the fundamental role of p53 is in the maintenance of genome integrity, thus explaining in part the loss of p53 in 50% of all human cancers (Howe and Conlon, 1997). For example, $p53^{-/-}$ cells in culture exhibit increased aneuploidy, gene amplification, point mutations and homologous recombination (Yin *et al.*, 1992; Havre *et al.*, 1995; Fukasawa *et al.*, 1996). Cultured $p53^{-/-}$ murine embryonic fibroblasts have been found to produce multiple centrosomes which resulted in unequal segregation of chromosomes, suggesting a novel mechanism through which loss of p53 promotes genetic instability (Fukasawa *et al.*, 1996). Premalignant thymocytes in $p53^{-/-}$ mice exhibit increased aneuploidy and increased LOH compared to WT counterparts (Guidos *et al.*, 1996; Shao *et al.*, 2000), highlighting the fact that genetic instability early on in tumourigenesis can be initiated by loss of p53 and perhaps even suggesting that loss of p53 could initiate tumourigenesis.

The majority of *p53* mutations in human cancers are missense mutations within the DNA binding domain (Levine, 1997). Thus cells tend to maintain expression of the protein but it lacks functional capabilities. This has led to speculations about whether mutations in the p53 protein not only abrogate its tumour suppressive activity but may indeed have an oncogenic effect on cells. For example the mutant p53His175 (which is altered in conformation rather than in DNA binding) has been shown to contribute to genomic instability resulting in the generation of aneuploid cells and appears to confer resistance of cells to etoposide (Gualberto *et al.*, 1998; Blandino *et al.*, 1999; Murphy *et al.*, 2000). Other mutations in *p53* are simple nulls caused by large deletions, LOH or other mechanisms such as premature degradation. Also loss of function that is associated with *p53* heterozygosity is thought to be due to a dominant negative mutation. Here, the mutant protein subunit drives the p53 tetramer (consisting of mutant and WT subunits) into the mutant conformation in a dominant negative manner.

1.11.4 Increased invasiveness

Whether *p53* mutation promotes invasiveness in tumourigenesis by direct or indirect mechanisms remains unknown. In prostate cancer *p53* mutation is associated with metastatic rather than benign tumours (Bookstein, 1994). Furthermore Kemp *et al.* (1993) showed that chemically-induced skin tumours on $p53^{-/-}$ mice occurred more rapidly and exhibited an increased number of malignant carcinomas compared to those arising on WT mice. Whether this was due to a direct effect of loss of p53 function on the promotion of invasiveness, or whether loss of p53 results in the increased survival of cells with genomic instability and in the accumulation of further mutations, which then act to promote invasiveness, remains unknown.

1.11.5 Increased angiogenesis

The ability to develop new blood supply is a means whereby tumour cells survive and proliferate. Gain of an angiogenic phenotype in tumour cells can be accompanied by loss of p53 function. Three anti-angiogenesis factors, thrombospondin-1, BAI1 and the tissue remodeller matrix metalloprotease 2 (MMP2) have been found to be transcriptionally regulated by p53 (Dameron *et al.*, 1994; Nishimori *et al.*, 1997; Bian and Sun, 1997).

1.11.6 *p53* null transgenic mice

Whilst clinical and *in vitro* studies have highlighted *p53* as a putative tumour suppressor gene, the generation of $p53^{-/-}$ mice provided clear evidence that p53 was in fact a tumour suppressor. These mice die from lymphomas at approximately 6 months but can also develop sarcomas (Purdie *et al.*, 1994; Jacks *et al.*, 1994). $p53^{+/-}$ mice develop osteosarcoma, lymphomas and soft tissue sarcomas, however the *p53* heterozygotes develop these diseases later than $p53^{-/-}$ mice with 50% of animals succumbing to tumours by 18 months. A similar spectrum of tumours is seen in Li-Fraumeni patients (Srivastava *et al.*, 1990) who carry germ line heterozygous mutation in the *p53* gene.

1.12 The integration of signalling pathways

The integration of signalling pathways is vital to allow modulation of cellular behaviour in response to changes in environment. The work detailed in each of the results chapters in this thesis includes investigation into the effect of altering multiple signalling pathways. The following section outlines examples of interaction between TGF-β signalling, Wnt signalling and p53-mediated pathways. In addition, and particularly in relation to chapter 5, an overview of interaction between TGF-β signalling and mitogen activated protein kinase (MAPK) signalling is included.

1.12.1 Interaction of p53 and TGF-β signalling

Several pieces of work confirm interaction between the p53 and TGF-β signalling pathways. CDK4, when associated with Cyclin D1, phosphorylates RB which then releases the transcription factor, E2F, to initiate the transcription of cell cycle promoting genes. Both p53 and TGF-β elicit negative control of the cell cycle by repressing CDK4 translation or transcription and inducing p21, a CDK inhibitor involved in G1 growth arrest (Munger *et al.*, 1992; Li *et al.*, 1994; Datto *et al.*, 1995; Ewen *et al.*, 1995). How these two pathways functionally interact is still under investigation. Induction of p21 by TGF-β has been shown to be p53-independent (Datto *et al.*, 1995), however CDK4 repression by TGF-β has been shown to be p53-dependent (Ewen *et al.*, 1995; Miller *et al.*, 2000). Experiments involving the transfection of murine keratinocytes with mutant *p53* resulted in loss of TGF-β-mediated downregulation of CDK4. Thus CDK levels become abnormally high and cells become resistant to G1 growth arrest (Reiss *et al.*, 1993). Another example of interaction between TGF-β and p53 was revealed when a member of the TGF-β superfamily, *PTGF-β* was shown to be a target gene of p53. Furthermore, PTGF-β can inhibit tumour cell growth through a signalling pathway involving SMAD4 (Tan *et al.*, 2000).

1.12.2 Interaction of p53 and Wnt signalling

Several studies have implied that p53 can result in antagonism of the Wnt signalling pathway. Dickkopf-1, (DKK-1), a protein which specifically inhibits canonical Wnt

signalling by binding to the low-density-lipoprotein-receptor (LRP6) component of the receptor complex (Bafico *et al.*, 2001), has been shown to be transcriptionally induced by p53. Furthermore, p53-induction of DKK-1 occurs in response to DNA damage suggesting that DKK-1 could be mediating p53 tumour suppression by antagonising the Wnt signalling pathway (Glinka *et al.*, 1998; Fedi *et al.*, 1999). β-catenin has also been shown to be downregulated by activated p53 (Sadot *et al.*, 2001). Finally work involving $p53^{-/-}Apc^{Min/+}$ mice has suggested co-operation between *Apc* and *p53* mutation in the promotion of murine pancreatic tumourigenesis (Clarke *et al.*, 1995).

1.12.3 Interaction of TGF-β and Wnt signalling

Studies highlighting a co-operation between the Wnt and TGF-β signalling pathways include work with the *Xenopus* Spemann organiser which showed that activins (TGF-β superfamily member) and Wnts co-operate to control gene transcription (Crease *et al.*, 1998). In *Drosophila*, signals from wingless (Wnt homologue) and DPP (decapentaplegic, TGF-β superfamily member) both coincide on the developmental *vestigial* and *ultrabithorax* genes. Furthermore Labbe *et al.*, (2000) have shown that SMADs and LEF/TCF physically interact to synergistically activate transcription of a *Xenopus* gene (*Xtwn*). They suggest that this synergistic activation will only occur where there is an SBE adjacent to the LEF binding site and that both Wnt and TGF-β can independently regulate LEF target genes (Letamendia *et al.*, 2001). Axin, a negative regulator of the Wnt pathway has been shown to bind SMAD3, facilitate efficient SMAD3 activation by TGF-β receptors and enhance TGF-β transcriptional activity (Furuhashi *et al.*, 2001). Finally, it has been found that SMAD4 can bind β-catenin in a TGF-β-independent manner and that this SMAD4/β-catenin activation results in nuclear translocation of both proteins (Nishita *et al.*, 2000).

1.12.4 Interaction of TGF-β and MAP kinase signalling

TGF-β signalling can be greatly modified by numerous interactions with other signalling pathways. At present it is not clear whether the SMAD proteins are

involved in all aspects of TGF-β signalling. Some aspects of TGF-β signalling could be mediated by a MAP-kinase type pathway. In this model, TGF-β activates TAB-1 which binds to and activates the MAPKKK, TGF-β activated kinasae (TAK1). TAK1 activation in turn induces transcription of, for example, the *PAI-1* promoter by an unknown mechanism (Shibuya *et al.*, 1996; Yamaguchi *et al.*, 1995). It could be the case that this MAP kinase pathway acts independently or it could function in the regulation of SMAD protein phosphorylation.

1.12.4.1 TGF-β and MAPK signalling

The RAS-RAF-ERK/MAPK cascade is involved in regulating cell proliferation in many cell types (for review see Chang and Karin, 2001). Growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF) or hepatocyte growth factor (HGF) bind to corresponding receptor tyrosine kinases, resulting in the adaptor proteins GRB2 and SOS associating with the ligand bound receptor. This is followed by activation of RAS. The GTP-bound RAS then catalyses the translocation of RAF (MAPKKK) to the plasma membrane. RAF is phosphorylated there and in turn phosphorylates the MAP kinase kinase (MAPKK), MEK. MEK then phosphorylates a MAP kinase. There are three subgroups of MAP kinases: extracellular signal-regulated kinases (ERKs), which are stimulated by growth factors and can induced cell proliferation and differentiation; JNK and p38, both of which are involved in separate pathways activated for the most part by cellular stresses, UV irradiation and cytokines. Activated ERKs translocate to the nucleus and stimulate gene expression by triggering transcriptional factors like ELK1 and ATF-2 (Zheng and Guan, 1994; Brunet *et al.*, 1999; Clerk *et al.*, 1999). How TGF-β and the MAP kinase signalling pathway interact is complex with the pathways sometimes co-operating with each other and at other times being antagonist.

1.12.4.2 Antagonist MAPK and TGF-β signalling.

An example of antagonism between the TGF-β and MAPK signalling pathways was demonstrated by Kretzschmar *et al.*, (1997) who showed that the ERK family of MAP kinases phosphorylate SMAD1 in response to EGF and inhibit its nuclear

translocation. Kretzchmar *et al.*, (1997; 1999), have also reported that activated RAS phosphorylates the linker region in SMAD1 and SMAD3, thus opposing the C-terminal phosphorylation by receptors, and thereby inhibiting SMAD/TGF-β transcriptional responses, SMAD nuclear translocation and growth inhibition. Lo *et al.*, (2001) reported SMAD and RAS pathway antagonism at the transcriptional level. They reported that EGF signalling via the RAS-MEK pathway caused the phosphorylation of TGIF, a SMAD transcriptional co-repressor, at two ERK MAP kinase sites. This resulted in TGIF stabilisation and formation of SMAD2/TGIF complex. Work by Giehl *et al.* (2000), demonstrated that TGF-β-induced growth inhibition in a pancreatic carcinoma cell line can be mediated in a SMAD4 independent fashion by the prevention of ERK2 activation, suggesting that the SMADs are not the only target of antagonism by the MAPK pathway. Finally, work with keratinocytes and hepatocytes has shown that TGF-β can antagonise the mitogenic effect of EGF (Russel, 1988, Zendegui *et al.*, 1988).

1.12.4.3 Co-operative MAPK and TGF-β signalling

MAPK and TGF-β signalling have been shown to co-operate both functionally and at a transcriptional level. In TGF-β sensitive untransformed cells, the growth inhibitory activity of TGF-β was associated with MAPK activation (Hartsough and Mulder, 1995). In human breast cancer cells, stimulation with TGF-β caused activation of both ERK2 and SAPK/JNKs (Stress Activated Protein Kinases/c-Jun N-terminal kinases). This activation was directly correlated with growth inhibition in these cells (Frey and Mulder, 1997). Furthermore a dominant negative *RAS* mutant (RasN17) inhibited both the decrease of CDK2 and CyclinA and increase in p21 and p27, usually seen upon TGF-β stimulation, and blocked ERK1 activation (Hartsough *et al.*, 1996, Yue *et al.*, 1998). At a transcriptional level de Caestecker *et al.*, (1998) found that EGF-mediated MEK1 activation resulted in phosphorylation, nuclear translocation and transcriptional activation by SMAD2/4. Also treatment of rat hepatocytes with EGF and TGF-β potentiates SMAD3 transactivational activity. It was shown that this occurs through the physical interaction of c-Jun with SMAD3 and that phosphorylation of c-Jun occurs via a p38 (but not JNK) dependent

mechanism (Peron *et al.*, 2000). Finally, Blanchette *et al.* (2001) demonstrated a parallel stimulation of the SMAD pathway and the p42/p44 MAPK pathway by TGF-β that resulted in increased gene transcription.

Another functional consequence of TGF-β-induced MAP kinase activation includes actin polymerisation and chemotaxis. TGF-β-stimulated activation of p38 in human neutrophils is necessary for TGF-β-induced actin polymerisation and chemotaxis (Hannigan *et al.*, 1998). In a human fibrosarcoma derived line, TGF-β induction of fibronectin expression is dependent on JNK activity and independent of SMAD4 (Hocevar *et al.*, 1999).

1.12.4.4 Altered TGF-β and MAPK interactions in response to oncogenic *RAS*

RAS-activating mitogens (including EGF) have a proliferative effect on normal epithelial cells, and this can be overridden by TGF-β. Cells transformed by oncogenic *RAS* are liberated from the anti-proliferative response to TGF-β. Activating mutations in *K-RAS* occur at a high frequency in pancreatic adenocarcinomas (for review of *K-RAS* mutations in pancreatic cancer see Howe and Conlon, 1997). Besides inactivation of members of TGF-β signalling, other possible mechanisms of release from the TGF-β anti-proliferative response have been suggested in relation to MAPK signalling. First, Saha *et al.* (2001) showed that oncogenic *RAS* inhibited TGF-β-mediated SMAD4 complex formation and transcriptional responses. In parallel reduced levels of endogenous SMAD4 were present due to increased degradation. The addition of proteasome inhibitors, reversal of RAS activation and overexpression of SMAD4 all restored TGF-β-mediated SMAD4 complex formation and transcriptional responses. Second, blockade of SMAD4 activity in transformed keratinocytes containing oncogenic *RAS* leads to hyperactivation of the RAS-dependent ERK signalling pathway (Iglesias *et al.*, 2000). Thus it can be seen that TGF-β and MAPK signalling interact in diverse and sometimes contradictory ways. Furthermore, activation of oncogenic *RAS* is an important mechanism through which TGF-β signalling via the SMADs can be blocked.

1.13 Outline of project

The three TSGs principally discussed here (*DPC4/SMAD4*, *APC* and *p53*) are implicated in the development of neoplasia in the pancreas. SMAD4, as the central mediator of TGF-β signalling, clearly has a tumour suppressive function but the exact mechanism of this in the pancreas remains unknown. APC, via its regulation of cellular β-catenin levels and p53, with its multiple roles including apoptosis, DNA damage repair, genome stability and cell cycle arrest, also act as tumour suppressors in the murine pancreas. The central focus of the work contained in this thesis is to investigate the effect of *Smad4* mutation on pancreatic tumourigenesis. This has been approached by various *in vivo* and *in vitro* methods. First, mice containing combinations of mutations in each of these genes, have been used to analyse the co-operative effect of gene mutation in *p53*, Apc^{Min} and *Smad4* on pancreatic tumourigenesis. Second, the effect of single and combined mutations in each of these genes has been explored in carcinogen-induced pancreatic tumourigenesis. Finally, in an attempt to further elucidate the cellular role of TGF-β signalling through the SMADs specifically in the pancreas, primary culture of murine pancreatic acinar cells has been employed to assess the effect of TGF-β signalling in the normal pancreas.

Chapter 2 – Materials and methods

All solutions and reagents contained in this chapter are detailed in appendix A.

2.1 *In vivo* studies

2.1.1 Transgenic mouse strains

Various mutamt mouse strains were used in these studies. Table 2.1 summarises the animals used, the genetic alteration they contained and the consequences of that alteration.

Table 2.1 – Transgenic mice used

	Alteration	Consequence	Reference
p53$^{-/-}$	Exons 2 – 6 deleted and replaced with a *neo* gene.	Complete absence of p53 protein.	Clark *et al.*, 1993; Purdie *et al.*, 1994
Apc$^{Min/+}$	ENU induced germline mutation (Stop codon at codon 850) in *Apc* allele.	Truncation of APC.	Su *et al.*, 1992; Moser *et al.*, 1990
Smad4$^{+/-}$	Whole of exon 8 and part of exon 9 was replaced by a *neo* gene.	No SMAD4 Protein produced from the targeted allele.	Sirard *et al.*, 1998
β-globin/+	1000 tandem repeats of mouse β-globin coding sequence inserted near telomere of chromosome 3.	No apparent phenotype, inert.	Lo., 1986
Smad4$^{fl/+}$	Exon 1 (containing ATG start codon) flanked by two *LoxP* sites.	No functional consequences until Cre-mediated recombination at *LoxP* sites removes exon 1, then no SMAD4 protein is predicted from this allele	Appendix B

All mice used were maintained in an outbred background (*p53*$^{-/-}$ and *Apc*$^{Min/+}$ animals were predominantly C57BL/6 and the *Smad4*$^{+/-}$ animals were CD1). Single animals

bearing multiple mutations were generated by a series of appropriate crosses. Animals were routinely fed on a standard breeding diet (Harlan), given water *ad libitum* and maintained in non-barrier conditions. Health screening of these animals found them positive for the bacteria Pasteurella spp. and trichomona, an intestinal protozoa.

2.1.2 DNA extraction from mouse tails

Tail biopsies were taken from mice of 3 weeks of age and DNA extracted using the Puregene DNA extraction method (Puregene). This involved lysing tail tips overnight at 37°C in cell lysis solution (Puregene), containing 1μg/ml Proteinase K (Sigma). After samples had been allowed to cool to room temperature (RT), protein precipitation solution (200μl; Puregene) was added and the contents mixed by vortexing and centrifuged at 13,000 rpm (revolutions per minute) for 5 minutes. The supernatant was then removed to a fresh Eppendorf containing 500μl isopropanol to precipitate the DNA. The samples were mixed vigorously and centrifuged for 5 minutes at 13,000 rpm. The supernatant was poured off leaving the DNA pellet, which was left to air-dry overnight. The following day the DNA was resuspended in 500 μl DNA hydration solution (Puregene), aided by vortexing, and incubation at 37°C overnight.

2.1.3 Genotyping of transgenic mice using the polymerase chain reaction (PCR)

PCR to determine the various genotypes was carried out on the extracted DNA samples (2.1.2) in Omnigene Thermal Cyclers (Hybaid) using Gibco PCR kit containing PCR buffer, 1% W1 detergent (used to improve thermostability of enzyme), $MgCl_2$ (50mM) and Taq polymerase (5units/μl: a unit is defined as the amount of Taq polymerase which incorporates 10nmol of dNTPs into acid-precipitable material in 30 minutes at 74°C). Other reagents used were PCR primers (10pM/μl, OSWEL) and dNTP's (50mM, Gibco). Conditions and product sizes are summarised in Tables 2.2 and 2.3.

Table 2.2 – Composition of PCR reaction mixes

Reagent	***p53***	***Apc^{Min}***	***Smad4$^{+/-}$***	***Smad4$^{fl/+}$***
ddw (autoclaved)	27.45µl	37.85µl	25.25µl	41.2µl
PCR buffer	5µl	5µl	5µl	5µl
$MgCl_2$ (50mM)	2µl	2.5µl	2.5µl	2µl
dNTP's (50mM)	0.8µl	0.4µl	1µl	2µl
PCR Primers (10pM/µl)	2.5µl of each	1µl of each	2µl of each	2µl
1% W1 detergent	2.5µl	0	0	0
DMSO	2.5µl	0	0	0
Taq Polymerase (5U/µl)	0.25µl	0.25µl	0.25µl	0.8µl
Genomic DNA	2µl	2µl	2µl	2µl

Table 2.3 – Primers used, thermocycler conditions and products sizes obtained from various polymerase chain reactions

	Primers	Conditions	Product Sizes
p53 Purdie *et al.*, (1994)	5'-GTGGTGGTACCTTATGAGCC 5'-CATCGCCTTCTATCGCCTTC 5'-CAAAGAGCGTTGGGCATGTG	1 X 94°C – 2mins 30 X {94°C – 1min (denaturing), 62°C – 1min (annealing), 72°C – 1 min (extension)} 1 X 72°C – 10 mins	WT – 642bp Targeted – 510bp
Apc^{Min} Luongo *et al.*, (1994)	5'-TCTCGTTCTGAGAAAGACAGAAGCT 5'-TGATACTTCTTCCAAAGCTTTGGCTAT	1 X 94°C – 2mins 35 X {94°C – 1min, 60°C – 1min, 72°C – 1min} 1 X 72°C – 10mins	After Hind III digestion WT-123 bp Targeted-144bp
***Smad4* mutant** Sirard *et al.*, (1998)	5'-CGAAGGGGCCACCAAAGAACG 5'-TACTTTGCCGTGGTGGTGCTC	1 X 94°C – 2mins 30 X {94°C – 1min, 65°C – 1min, 72°C – 1min} 1 X 72°C – 10mins	Targeted allele-650bp
***Smad4* WT** Sirard *et al.*, (1998)	5'-CCTGTGGCCTGCTCTCTTCTC 5'-GGACAGGCAGTGGAGGATAGG	1 X 94°C – 2mins 30 X {94°C – 1min, 65°C – 1min, 72°C – 1min} 1 X 72°C – 10mins	WT allele - 711bp

Smad4 5' *LoxP* site. See appendix B.	5' – ATACTGAGCACTGGTGGT 5'– AAGGCTGAACGGCCCTTC	1 X 95°C – 5mins 30 X {95°C – 1min, 58°C – 1min, 68°C – 1min} 1 X 68°C – 10min	WT – 220bp Targeted allele – 280bp
Smad4 3' *LoxP* site. See appendix B.	5' – GTAGTAAGTCATGCAAGG 5' - CAATTCCAGGTGAGACAA	1 X 95°C – 5mins 30 X {95°C – 1min, 58°C – 1min, 68°C – 1min} 1 X 68°C – 10min	WT – 230bp Targeted allele – 300bp

The Hind III digestion after the Apc^{Min} PCR was carried out by incubation of 17µl of product with 2µl reaction buffer 2 (10x Gibco) and 1µl (20 units) Hind III enzyme (Gibco) for 2 hours at 37ºC.

The products of these reactions were visualised by gel electrophoresis on Tris-borate EDTA (TBE) 2% (or 4% only in case of Apc^{Min}) agarose gels containing ethidium bromide (0.005%) and visualised by UV illumination. This technique of using gel electrophoresis to separate DNA fragments is detailed in Sambrook *et al.* (1989). Agarose gels were submerged in electrophoresis buffer (TBE) and 17µl of samples were loaded into wells after the addition of 3µl of loading buffer containing bromophenol blue. Appropriate DNA ladders (Life Technologies, Boehringer-Manheim) were used to estimate PCR product sizes.

2.1.4 Monitoring of cohorts for illness

Daily monitoring of mice bearing single or multiple genetic mutations was carried out. Table 2.4 details the predominant cause of death and symptoms of illness for mutant mice.

Table 2.4 – Signs of disease in transgenic mice.

	Predominant cause of death	Signs of disease	Other disease signs
$p53^{-/-}$	Lymphoma (occurs at approximately 3 months). (a) Thymic lymphoma (b) Splenic lymphoma	- Hunched appearance, trouble breathing (panting) - Swollen abdomen, hunched, trouble walking.	Sarcoma, acanthoma – visible as external growths.
$Apc^{Min/+}$	Intestinal neoplasia (occurs between 4 – 8 months).	Anaemia (as evidenced by white feet), bloody faeces, hunched appearance.	Mammary acanthomas- visible external growths. Preneoplastic pancreatic lesions.
$Smad4^{+/-}$	Intestinal neoplasia (occurs at 12-24months).	Anaemia, bloody faeces, hunched appearance.	
$p53^{-/-}Apc^{Min/+}$	Intestinal and pancreatic neoplasia, lymphoma.	Hunched appearance, anaemia, rapid weight loss, trouble breathing, and bloated appearance.	
$p53^{-/-}Apc^{Min/+}Smad4^{+/-}$	Intestinal and pancreatic neoplasia, lymphoma.	Hunched, anaemia, rapid weight loss, trouble breathing, bloated appearance.	
$Apc^{Min/+}$ $Smad4^{+/-}$	Intestinal neoplasia.	Anaemia, bloody faeces, hunched appearance.	
$p53^{-/-}Smad4^{+/-}$	Lymphoma. (a) Thymic lymphomas (b) Splenic lymphoma	- Hunched appearance, trouble breathing (panting) - Swollen abdomen, hunched, trouble walking.	Sarcoma, acanthoma – visible as growths externally on animals

2.1.5 Fixation of tissues

At the appropriate time point (in case of NMU studies) or upon signs of illness (for studies of spontaneous neoplasia) animals were injected with BrdU (2.1.7) and culled by cervical dislocation (schedule 1). Dissected organs and tumours were fixed in 4% formalin overnight. Large and small intestine were flushed with water and fixed in methacarn (4 parts methanol, 2 parts chloroform, 1 part glacial acetic acid) for 2 hours before they were removed into 70% ethanol. Following fixation, tissues were dehydrated through a series of 2 X 20 minute incubations in increasing alcohol concentrations (50%, 70%, 85%, 90%, 100%). They were then incubated for 3 X 40 minutes in xylene and 3 X 40 minutes in 56°C wax before being embedded in paraffin wax. 3μm sections on vectabond (Vector Laboratories) coated slides were used for all histological and immunohistochemical staining. 7-10μm sections baked onto plain slides were used for laser microdissection. Haematoxylin and eosin (H+E) staining was carried out routinely for all tissues harvested. Paraffin embedding, sectioning and H+E staining was carried out by the Department of Pathology histology service.

2.1.6 Administration of N-Nitroso-N-Methyl-Urea

33mg N-Methyl-N-Nitroso-Urea (NMU) (Sigma, isopac) was diluted in 5ml sterile PBS (phosphate buffered saline) containing 0.05% acetic acid in a class I hood reserved for work with toxic compounds. Pups weighed on average 3g by day 4. Using an insulin syringe, 25μl of 6.6mg/ml NMU was injected subcutaneously into the flap of skin ('scruff') behind the head of the pups corresponding to a dose of 55μg/g body weight of NMU. Pups were returned to their mothers and held in a class I hood, before the bedding was changed and the animals were returned to experimental holding rooms. Bedding was incinerated. NMU-treated cohorts were weaned as normal and checked daily for signs of illness. Animals which became moribund before the end of the experiment were culled. Littermates were sacrificed at age 65 days after treatment with BrdU (2.1.8). Tissues were harvested as detailed in 2.1.5 and paraffin wax embedded.

2.1.7 Administration of dietary aspirin

Cohorts of NMU-treated and control mutant and WT mice were fed a diet containing 400mg/Kg aspirin (Harlan/Tekad). This level of exposure to aspirin is comparable with the highest doses used in previous studies (Mahmoud *et al.,* 1998, Barnes and Lee, 1998, Williamson *et al.,* 1999, Chiu *et al.,* 2000). At 4 days of age litters were first treated with NMU and then parents and litters were immediately moved onto the aspirin diet until administration of BrdU (2.1.8), culling and harvesting of tissues at age 65 days (2.1.5).

2.1.8 Administration of BrdU intraperitoneally

Animals were injected intraperitoneally with 10ml/kg body weight BrdU (Amersham, final concentration 3mg/100g body weight) 2 hours prior to culling.

2.1.9 Immunohistochemical techniques

All immunohistochemistry was carried out utilising a standard immunohistochemical protocol, however the specific conditions for each individual antibody are detailed in Table 2.5. All immunohistochemistry was carried out on 3μm thick paraffin embedded sections on vectabond-coated slides. As the 3 dimensional structure of proteins within tissues that have been formalin fixed and paraffin wax embedded is altered, some epitopes lose their immunoreactivity. Therefore for some immunohistochemical labelling an antigen retrieval step is needed. To enable antigen retrieval, slides fixed in formalin or methacarn were microwaved in 500ml citrate buffer (10mM, pH 7.6) for three 5 minute incubations (700 Watts). Endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide in ddw (double deionised water or methanol where stated) before incubation in 20% normal serum to block non-specific binding of antibodies. Primary antibodies, diluted in 20% normal serum, were applied at an optimal concentration (determined empirically) for 1 hour at RT or overnight at 4°C. After washing sections were incubated with biotin-conjugated secondary antibody, again diluted in 20% normal serum for 30 minutes. Following further washes, incubation was carried out with avidin-biotin (AB) complex either horseradish peroxidase (HRP) - or alkaline

phosphatase (AP) -conjugated (Vector Laboratories) for 30 minutes at RT. This was an amplification step which results in more HRP or AP enzyme conjugated to each epitope, due to an avidin-biotin complex building up on each biotin molecule from the secondary antibody. Following a final wash, a chromagen was added. Three chromogens were used, DAB and Vector VIP (Vector Laboratories) for HRP-mediated reactions and Vector Blue (Vector Laboratories) for AP-mediated reactions. The Chromogen DAB (3,3 diaminobenzedene) (DAKO) resulted in a brown colour developing, Vector VIP, a burgundy colour and Vector Blue, a blue colour. Slides were counterstained with Harris' haematoxylin and mounted using the non-aqueous pertex in the case of DAB and Vector VIP. For Vector Blue stained sections a methyl green nuclear counterstain (Vector Laboratories) was used and sections were mounted in aqueous mounting medium.

Table 2.6 – Specific conditions for individual immunohistochemical labelling

Protein	Primary Antibody	Normal Serum	Secondary Antibody	Wash	Notes
SMAD4	SC1909, goat polyclonal 1:200 (Santa Cruz)	Goat	Rabbit anti-goat-biotin linked, 1:400	TBS (Tris buffered saline)	H_2O_2 block in methanol
β-catenin	C19920, mouse monoclonal 1:50 (Transduction Labs)	Rabbit	Rabbit anti-mouse biotin linked, 1:400	TBS	
BrdU	MAS250p, Rat monoclonal 1:100 (Harlan)	Rabbit	Rabbit anti-rat-HRP conjugated, 1:100	PBS	Incubation in 5M HCl for 45 minutes RT prior to endogenous peroxidase block. No AB complex needed
β-catenin and BrdU	C19920 mouse monoclonal 1:50 (Transduction Labs) MAS250p, Rat monoclonal 1:100 (Harlan)	Goat	Rabbit anti-mouse biotin linked, 1:400 (for β-catenin) and Goat anti-rat biotin linked, 1:400 (for BrdU)	TBS	Incubation in 5M HCl prior to endogenous peroxidase block. For β-catenin, AB complex AP-linked was used with Vector Blue chromagen. For BrdU, AB complex HRP-linked with DAB chromagen.

2.1.10 *β-Globin* transgene DNA-DNA *in situ* hybridisation and β-catenin immunohistochemistry

To investigate the ploidy status of dysplastic acinar cells in NMU-treated mice (chapter 4), a technique was used whereby *in situ* hybridisation for a *β-globin* transgene on chromosome 3 and β-catenin immunohistochemistry were carried out on paraffin embedded sections. The pancreata to be used for this techniques were fixed in methacarn for 2 hours before being transferred into 100% ethanol. It was vital that pancreata were not exposed to formalin during any stage of the fixation or embedding process as this could have impeded the *in situ* hybridisation reaction. The DNA-DNA *in situ* hybridisation was carried out as detailed in Keighren and West (1993): briefly, dewaxed sections were incubated in 1mM NaOH at 70°C (3 minutes) and prehybridisation solution at 60°C (15 minutes) before incubation with the digoxigenin-labelled *β-globin* probe (in hybridisation solution) overnight at 60°C. After washing the slides were incubated in anti-digoxigenin HRP-linked antibody (Boehringer, 1:100 dilution, 30 minutes). After a further wash, the chromogen DAB was added for 10 minutes. Sections were then held in a humid chamber for a maximum of 4 hours before the β-catenin immunohistochemistry was carried out as detailed above (2.1.9), but with the use of the purple chromogen Vector VIP (Vector Laboratories).

2.1.11 Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) staining

The ApopTag (Intergen) TUNEL kit was used on paraffin embedded sections in accordance with the manufacturer's instructions. In summary, the 3' OH-termini generated by DNA fragmentation were labelled with digoxigenin-conjugated nucleotides by the enzyme TdT. Following the TdT enyzmic step, incorporated nucleotides were detected by an anti digoxigennin peroxidase-linked antibody. The chromogen DAB was then used to visualise positive nuclei. Haematoxylin was used as a nuclear counterstain.

2.1.12 Preparation of whole cell protein extracts

Harvested pancreata were snap frozen in liquid nitrogen. To extract protein, tissue was homogenised in RIPA buffer (with freshly added protease inhibitors; 10μg/ml aprotinin, 1μM pepstatin, 1μM leupeptin, 1mM sodium vanadate, 1mM PMSF) and centrifuged at 13000 rpm. The supernatant was collected, samples were removed for estimation of protein concentration (2.1.13) and the remainder stored in aliquots at –80°C.

2.1.13 Estimation of protein concentration

Nuclear and cytoplasmic extracts (5μl) were diluted with 795μl ddw. 800μl of appropriate protein standards of BSA were also made (1μg/μl - 25μg/μl). 200μl of Biorad assay reagent was added to each of the solutions and incubated at 37°C for 30 minutes. The optical density of the samples was measured at 595nm with a spectrophotometer. A standard curve was generated and protein concentration of experimental samples was estimated to allow equal loading.

2.1.14 Immunoblotting

Immunoblotting was carried out using NOVEX (Invitrogen) Immunoblotting apparatus and reagents. Protein (10-20μg) was added to an equal volume of 1 x sample buffer and 1 x reducing agent (Invitrogen). Samples were boiled for 5 minutes and loaded onto a 14% bis-acrylamide tris-glycine gel. Gels were run at 125 Volts for approximately two hours in running buffer (Invitrogen) before they were blotted onto nitrocellulose membrane in transfer buffer (Invitrogen). Blots were blocked in milk buffer (TBS, 5% non-fat milk, 0.05% Tween) for 45 minutes at RT before primary antibodies to SMAD4 (1:100 dilution, SC1909 Santa Cruz) or β-catenin (1:50, Transduction laboratories) were added overnight at 4°C. Blots were washed and secondary anti-goat HRP-conjugated or anti-mouse HRP-conjugated (1:1000, Santa Cruz) antibodies were added for 30 minutes at RT. Blots were then visualised with ECL (Amersham) and densitometry carried out.

2.1.15 Laser microdissection

7μm formalin-fixed paraffin embedded sections were cut, H+E stained, dried and held in a dehumidified box. Laser microdissection was carried out using the Pix Cell II, Arcturus laser microdissection hardware and software as detailed in the users manual. The appropriate areas of the section were identified and the laser is used to attach tissue to a special Eppendorf lid (Capsure^{TM}). Once all the desired tissue was removed from the section and attached to the lid, the DNA was extracted from the tissue by incubation with microdissection lysis buffer overnight at 37°C. The following day the presence or absence of the *Smad4* or *Apc* WT allele was ascertained by PCR.

2.1.16 PCR analysis of *Smad4* and *Apc* LOH

DNA extracted from microdissected samples was subjected to PCR for the *Smad4* mutant and WT alleles or the Apc^{Min} and WT alleles. Due to small quantities of DNA, a second round (30 cycles of denaturing/annealing/extension) of PCR was carried out on the amplified DNA before samples were run on a 2% or 4% gel (2.1.3). PCR was carried out as detailed in 2.1.3 but high fidelity Taq polymerase was used to minimise the introduction of mutations.

2.1.17 Morphometric analysis and assessment of apoptotic and mitotic index

2.1.17.1 Measurement of area occupied by dysplastic cells and measurement of nuclear area

Areas occupied by dysplastic pancreas were scored from random sections on β-catenin stained slides using a general morphometry (Structure) program on the AxioHOME (Highly Optimised Microscopic Environment) microscope (Zeiss). The areas of all lesions in all genotypes were measured and calculated as a percentage of total area of pancreas on the section. This information was also used to determine the lesion classes (as categorised by area: class I-single cell foci to 0.025mm^2; class II-0.025mm^2 to 0.05mm^2 and class III 0.05mm^2 to 0.1mm^2). Abnormal foci larger than 0.1mm^2 were measured and included in the assessment of total abnormal tissue but excluded from nuclear area studies as they numbered too few for relevant

statistical analysis (2.4). A general morphometry (object) program on the AxioHOME microscope (Zeiss) was also used in the assessment of nuclear area. The areas of 500 acinar cell nuclei per category (foci classes I, II and III, and normal acinar cells from NMU-treated and untreated pancreas) per genotype were scored. The areas were categorised into $10\mu m^2$ stages and subjected to statistical analysis using the Kolmogorov-Smirnov (KS) test (2.4.2).

2.1.17.2 Assessment of apoptosis

Apoptosis was measured both by light microscopy of sections stained using the TUNEL technique (2.1.11) and by standardised morphological criteria (Kerr *et al.*, 1972, Wyllie *et al.*, 1980) on β-catenin stained sections. Apoptotic cells were identified by chromatin condensation, the presence of multiple rounded apoptotic bodies and the shrinking of cytoplasm. Often due to the chromatin condensation nuclear material displays stronger counterstain. A minimum of 1000 cells in sections taken from at least three mice were scored per category (genotype and lesion type or normal pancreas), with a maximum of 100 cells scored in each individual random field. A general morphometry (object) program was used at 400X and 1000X on the AxioHOME microscope (Zeiss). The Mann Whitney U test was used for statistical analysis (2.4.1).

2.1.17.3 Assessment of mitotic index

The mitotic index was measured on the β-catenin and BrdU double-labelled sections. In each genotype, for every lesion type and normal pancreas, 1000 cells in total were scored from a minimum of 3 mice of each genotype, with 100 cells being scored in each lesion. The scoring was carried using a general morphometry (object) program on the AxioHOME microscope (Zeiss). The Mann Whitney U test was used for statistical analysis (2.4.1).

2.2 *In vitro* studies

2.2.1 Preparation of pancreatic acinar cells

The pancreata from three mice were removed immediately post culling, inflated with collagenase (700 units) for 10 minutes (RT) and divided into smaller pieces before shaking (86 rpm) in CO_2-infused collagenase for 10 minutes at 37°C. The collagenase was then replaced, reinfused with CO_2 and pancreata were incubated in the shaking water bath for a further 30 minutes at 37°C. Following digestion with the collagenase pancreata were broken up into acini by gentle pipetting through 3 pipettes of decreasing bore sizes. To ensure preparation of acini which were neither too large or too small, acini were passed through a 150μm^2 gauze and layered onto serum free (SeF) medium containing 4% BSA and spun at 200 rpm for 4 minutes. A final wash in SeF medium removed the final traces of BSA. Yields were estimated by the volume of packed acini, 100µl of packed acini would be resuspended in 24ml of plating medium containing 15% serum (15% PM) and 5ng/ml EGF. Acini were then plated onto collagen IV-coated (Sigma) sonic seal (Nunc) chamber slides (well area 1.54cm^2), 1ml of resuspended acini were seeded gently in each well.

2.2.2 Culture of primary pancreatic acinar cells

Acini were left to attach to the slides in 15% PM containing EGF for the first 3 days of culture and every effort was taken not to disturb slides. After 3 days, attached acinar cells were either placed in 15% PM with EGF for a further 24 hours or placed in Chee's medium containing EGF. To assist with purifying the culture from fibroblasts Chee's medium is arginine free. After at least 24 hours in Chee's medium attached acinar cells were either placed in Waymouth's/HamF12 medium containing EGF or continued in Chee's medium. Medium was changed daily and all experiments were conducted on acini which had been in serum containing PM for up to 4 days or on attached acinar cells which had been cultured for 6-8 days as detailed above. Appendix A details exactly what these media contained.

2.2.3 Culture of NIH3T3 mouse fibroblast cell line

NIH3T3 cells have been shown to contain endogenous SMAD4. These cells were grown to use as a positive control for the SMAD4 immunofluorescence. Frozen stocks (in 10%DMSO, 90% Fetal Bovine serum) were rapidly defrosted, washed in warmed feeding medium (10% FBS, 1% antibiotic and 1% L-glutamine), spun down, resuspended in media (to remove all traces of DMSO) and plated onto small ($25cm^2$) flasks. Experiments were conducted within two passages of defrosting on cells which had been grown on uncoated Sonic Seal chamber slides (Nunc). Cells were fixed with either methanol or paraformaldehyde (2.2.11)

2.2.4 Administration of EGF

EGF (Sigma) was used at final concentration 5ng/ml. A stock solution of 5μg/ml was made up and diluted 1:1000 at use. EGF was made up in sterile PBS containing 5% filtered foetal calf serum or BSA.

2.2.5 Administration of choleocystokinin (CCK)

CCK (Sigma) was used at a final concentration of 10^{-9}M in Chee's medium.

2.2.6 Administration of TGF-β

The concentration of TGF-β (Sigma) to be used was titrated and a final concentration of 4ng/ml was decided upon. Lyophilised TGF-β was rehydrated in filtered 1mM HCl, 0.1% BSA and diluted in the appropriate medium before addition to cells.

2.2.7 Administration of TGF-β1, 2 and 3 neutralising antibody

Studies involving the effect of TGF-β on acini during the first 4 days of culture utilised a TGF-β neutralising antibody (R&D Systems). TGF-β neutralising antibody (1μl/ml in 15% PM) was added directly into wells prior to plating of acini.

2.2.8 Administration of MEK inhibitor

Further studies investigating the effect of TGF-β on acini during the first 4 days of culture utilised the MEK1 inhibitor U0126 (Promega). U0126 was diluted in cell culture tested DMSO to a stock concentration of 1nM and used immediately. Freshly prepared acini were treated with 15pM UO126. This concentration was used as preliminary studies using 5pM, 10pM, 15pM and 40pM of the MEK inhibitor (data not shown) revealed 15pM to be the optimal concentration for the observed effect. As the inhibitor was diluted in DMSO, similar amounts of DMSO were added to cells without U0126 to see whether DMSO alone was causing any effect on the cells.

2.2.9 Treatment with bromodeoxyuridine (BrdU)

BrdU (1:1000 dilution, final concentration 6μg/ml, Amersham) was diluted in culture medium and added to the cells 6 hours prior to fixation.

2.2.10 Treatment with leptomycin B (LMB)

Leptomycin B inhibits the nuclear export of proteins which utilise a CRM-1 binding sequence to exit the nucleus (Kudo *et al.*, 1999). Day 6-9 attached acinar cells were treated with 20ng/ml Leptomycin B for up to 6 hours in the presence and absence of TGF-β (4ng/ml) and fixed in either methanol or paraformaldehyde (2.2.14). Leptomycin B must be kept on ice at all times as when the solution heats up a build up of pressure within the vial can be a hazard.

2.2.11 Fixation of cells

Four methods of fixation were used in the *in vitro* studies. For immunofluorescence cells were either methanol or paraformaldehyde fixed. Methanol fixation involves washing of the cells once in PBS before incubation at -20°C in ice-cold methanol for 5 minutes. After this the methanol was discarded and the cells were left to air dry for at least 30 minutes in a tissue culture hood. Methanol fixed cells were then stored in a -20°C freezer wrapped in foil. For paraformaldehyde fixation, cells were incubated in the paraformaldehyde for 15 minutes RT, before 3 washes in PBS. Cells were

then stored in PBS at 4°C until use. Cells which have received BrdU were washed in PBS and then fixed in 80% ethanol. The cells remained in the 80% ethanol at 4°C until BrdU immunocytochemistry is carried out. Cells intended for Feulgen staining must be fixed in Bouin's fixative (7 parts methanol, 2 parts 40% formaldehyde and 1 part glacial acetic acid). The cells remained in the Bouin's fixative at 4°C until staining was carried out.

2.2.12 Immunofluorescence studies

All immunofluorescence was carried out utilising a general immunofluorescence protocol, however the specifics for each individual immunofluorescence are detailed in Table 2.6 below. Methanol fixed cells (2.2.11) were defrosted before rehydration in PBS for 5 minutes. Paraformaldehyde fixed cells (2.2.11) were pre-incubated in 0.5% Triton in ddw for 10 minutes and washed well in PBS. For both types of fixed cells the same protocol was followed. Non-specific binding was blocked by incubation in 1%BSA/PBS for 1 hour at RT before addition of primary antibody (at an optimal concentration), diluted in 1%BSA/PBS for 1 hour at RT or overnight at 4°C. Cells were then washed 3 X in PBS (5 minutes for each wash). The fluorophore Alexa 488 (Molecular Probes) was utilised. This particular method uses an amplification step whereby cells are incubated with an Alexa 488-conjugated rabbit anti-mouse antibody (diluted in 1%BSA/PBS) for 30 minutes and then incubated with an Alexa 488-conjugated goat anti-rabbit antibody (diluted in 1%BSA/PBS) for 30 minutes. This results in every epitope being labelled with a greater number of fluorophores and thus the immunofluorescent signal is stronger and more sensitive, but also more prone to background problems.

Table 2.6 – Specific conditions used in immunofluorescent labelling.

Protein	Primary Antibody	Secondary antibody (1st Step)	Secondary antibody (2nd step)	Nuclear Counterstain
Smad4	Mouse monoclonal, B8 (Santa Cruz) 1:50	Alexa 488-conjugated rabbit anti-mouse 1:200	Alexa 488-conjugated goat anti-rabbit 1:200	Topro 3
p21	Mouse monoclonal, F5 (Santa Cruz) 1:10	Alexa 488-conjugated rabbit anti-mouse 1:200	Alexa 488-conjugated goat anti-rabbit 1:200	Topro 3
p27	Mouse monoclonal (Sigma) 1:100	Alexa 488-conjugated rabbit anti-mouse 1:200	Alexa 488-conjugated goat anti-rabbit 1:200	Topro 3

2.2.13 Immunocytochemistry for β-catenin

Immunocytochemistry for β-catenin was carried out as detailed in 2.1.9 after methanol fixed cells (2.2.11) were defrosted before rehydration in TBS for 5 minutes (RT).

2.2.14 Immunocytochemistry for BrdU

Immunocytochemistry for BrdU was carried out as detailed in 2.1.9, following a wash in PBS to remove the 80% ethanol in which the cells had been fixed (2.2.11).

2.2.15 Preparation of protein (nuclear and cytoplasmic extracts) for immunoblots

Cells were washed in PBS before they were scraped off in ice cold PBS and spun down. They were then resuspensded in ice-cold lysis buffer containing protease inhibitors (10μg/ml aprotinin, 1μM pepstatin, 1μM leupeptin, 1mM sodium

vanadate, 1mM PMSF, Roche) and 0.5mM DDT added just prior to use. The lysate was vortex mixed before it was passed 5 x through a 25G needle and left on ice for 5 minutes. The lysed cells were spun in a centrifuge in a refrigerated room (4°C) for 5 minutes at 2000 rpm before the supernatant was removed and stored at -80°C (cytosolic fraction). The pellet was resuspended in ice-cold high salt buffer (addition of protease inhibitors listed above and 0.5mM DDT just prior to use), vortex mixed and incubated on ice for 20 minutes. Following this incubation the protein solution was spun at 12,000 rpm for 5 minutes at 4°C. The supernatant (nuclear fraction) was then removed and frozen at -80°C. 5µl samples of both nuclear and cytoplasmic extracts were removed to assay for protein concentration.

2.2.16 Estimation of protein concentration

As detailed in 2.1.13.

2.2.17 Immunoblotting

Immunoblotting with SMAD4 antibody (1:100, B8 Santa Cruz) and β-actin (1:200, Sigma) was carried out as detailed in 2.1.14. As β-actin was a rabbit polyclonal antibody, anti-rabbit HRP-conjugated secondary antibody was used (1:1000, Dako).

2.2.18 Time lapse microscopy

Time lapse microscopy using the Leica QUIPS hardware and software was used to observe acini plated onto collagen IV-coated chamber flasks 24 hours previously. Every 30 minutes for a period of 48 hours a digital image was captured through the microscope. The images were compiled using Adobe Premier software.

2.2.19 TRITC-conjugated phalloidin fluorescence

Phalloidin is a toxin which binds to actin fibres within cells (Dancker *et al.*, 1975). Cells were fixed in paraformaldehyde (2.2.11) overnight prior to permeabilisation for 10 minutes with freshly made up 0.5% Triton. Following washing in TBS and cells were incubated in tetra-methyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (1:100 dilution in TBS) for 1 hour in a fume hood . After this cells were

washed again in TBS and mounted using fluorescent mounting media containing the nuclear stain DAPI (Vector Laboratories).

2.2.20 Feulgen staining

Cells were washed in PBS and fixed in Bouin's fixative (2.2.11). Cells were washed again in PBS before incubation in 5M HCl at RT for 45 minutes. Following a further wash cells were incubated at RT in Schiff's reagent for 1 hour. Following this several washes in ddw were carried out until a strong pink staining developed. 0.01% light green was used as cytoplasmic counterstain before slides were air-dried and mounted with cedarwood oil and coverslips. Apoptotic cells are distinguished from non-apoptotic cells by the presence of strongly stained apoptotic bodies in conjunction with increased light green cytoplasmic staining.

2.2.21 Measurement of BrdU incorporation and apoptosis

Measurement of BrdU incorporation and apoptosis levels was carried out on the AxioHOME microscope using the general morphometry (object) program. For a given experiment each timepoint was carried in duplicate. Within each well 500 cells from 5 - 9 random fields of 200μm^2 were scored, remnants of whole acini containing clumps of cells were not scored. The Mann Whitney U statistical test was used to analyse data (2.4.1).

2.2.22 ^{3}H-thymidine incorporation

Acini were prepared and plated as detailed in 2.2.1 in 15% or 2.5% plating media alone or medium containing EGF (5ng/ml), TGF-β (4ng/ml) or EGF and TGF-β. 24 hours prior to the desired time points (24, 48, 72 and 96 hours), 0.5μCi of ^{3}H-thymidine (Amersham) was added to each well. After the incubation with the ^{3}H-thymidine for 24 hours the cells were washed twice in PBS and 0.5ml of 10% (trichloroacetic acid) TCA was added to each well for 30 minutes at 4°C. Following this incubation, lysed cells were scraped off the slide and transferred onto filter paper using a vacuum flask. A further 2 washes with 10% TCA were carried out again with contents from individual wells being transferred onto the corresponding filter papers. Filter papers were then dried using 100% ethanol and transferred into 3ml of

scintillation fluid. The counts per minute were obtained using a scinti-counter. The experiment was performed in triplicate.

2.3 Image analysis

All images shown were captured digitally and managed in Adobe photoshop. Appropriate contrast, brightness and colour variations were used. Magnification is detailed within individual figure legends.

2.4 Statistical methods (Sokal and Rohlf, 1995)

Three main statistical methods were employed to analyse data within both the *in vivo* and *in vitro* studies. As a normal distribution (a symmetrical bell shaped distribution in which two standard deviations from the mean accounts for 95% of the data set) could not be assumed for any of the data sets non-parametric statistical methods were used. Non-parametric methods are not dependent on a given distribution but will usually work for a wide range of different distributions. They are called non-parametric because their null hypothesis is not concerned with specific parameters but only with the distribution of the variates.

2.4.2 Mann Whitney U test

This statistical test does not compare the actual values in given datasets but rather compares how the values rank in comparison to each other. Thus a similar result would be obtained by comparing dataset (A) e.g. 1,2,3,4,5 with dataset (B) e.g. 1000, 2000, 3000, 4000, 5000 as it would by comparing dataset (A) with dataset (C) 10, 20, 30, 40, 50, even though datasets (B) and (C) are vastly different. The test compares the sum of ranks for each individual dataset, thus if populations are not different from one another the rank sums would be approximately the same. The equivalent parametric 2 samples tests are the t-test and ANOVA (Analysis of Variance). The null hypothesis for the Mann Whitney U test is that the two samples have the same location (i.e. rank-ordered position of individual data values).

2.4.3 Kolmogorov Smirnov (KS) test

The Mann Whitney U test is based on comparisons between the sums of ranks of individual variates, however the non-parametric KS test compares differences between two distributions. The null hypothesis of the KS test is that the two samples are distributed identically; thus the test is sensitive to differences in dispersion, location and skewness. It is based on the relative cumulative frequency distributions of the two samples. Critical values are approximated and compared with the maximum difference between the two cumulative frequency distribution of observed values. A decision can then be made as to whether the maximum difference between two cumulative frequency distribution is significant.

Chapter 3 – Characterisation of *p53*$^{-/-}$*Smad4*$^{+/-}$ and *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice.

3.1 Introduction

3.1.1 Background

The work described in this chapter involves combining targeted mutations in the tumour suppressor genes *p53*, *Apc* and *Smad4*, which have been linked to tumourigenesis either in human or murine pancreas. p53 is a tumour suppressor which functions in cell cycle regulation, induction of apoptosis in response to DNA damage, DNA repair, cell senescence and stress responses. APC is involved in the Wnt signalling pathway where it functions to modulate β-catenin levels. SMAD4 is the central mediator of the TGF-β signalling pathway and, in particular, has been associated with TGF-β-mediated growth arrest. Detailed descriptions of the function of these proteins, the signalling pathways in which they are involved and the effect of mutation in the gene on the promotion of tumourigenesis are contained in chapter 1. Also, possible areas of interaction between these signalling pathways, which may be affected by combining mutations in these genes, are discussed in 1.12. *p53*$^{-/-}$ and *Apc*$^{Min/+}$ mice are described in 1.11.6 and 1.9.4.1 respectively. *Smad4*$^{-/-}$ mice die as embryos, but *Smad4*$^{+/-}$ animals develop multiple gastric and duodenal polyps, which progress to invasive carcinoma (Takaku *et al.*, 1999; Xu *et al.*, 2000).

3.1.2 Compound mutant mice in the study of cancer

In the case of tumourigenesis, whereby progression involves the accumulation of multiple genetic mutations, the ability to combine mutations via the intercrossing of mice is an important tool. Combining multiple mutations within the same mouse also allows researchers to compare the relative importance of different mutations on the different stages of tumour progression. Thus, by highlighting the genetic changes occurring at each stage, scientists can gain valuable information regarding individual gene function and the order in which various genetic events occur (reviewed in Jacks, 1996 and Macleod and Jacks, 1999). For example, Takaku *et al.* (1998) generated

Apc$^{\Delta 716/+}$*Smad4*$^{+/-}$ mice which exhibited an increase in size of intestinal polyps and an increase in frequency of adenocarcinomas compared to *Apc*$^{\Delta 716/+}$ mice. The authors conclude that mutations in *Smad4* play a significant role in the malignant progression of colorectal tumours. Transgenic mice that overexpress Wnt-1 in the mammary gland develop mammary tumours (Kwan *et al.*, 1992). When these animals were crossed to *p53*$^{-/-}$ mice, the mammary tumours arose sooner and grew faster than those in the *p53*$^{+/+}$*Wnt-1* overexpressing transgenic animals (Donehower *et al.*, 1995; Jones *et al.*, 1997). Assessment of apoptosis and proliferation rates found that apoptosis rates were not affected by p53 status however cell proliferation was significantly increased in the *p53*$^{-/-}$*Wnt-1* overexpressing animals compared to the *p53*$^{+/+}$*Wnt-1* animals (Jones *et al.*, 1997). This work highlighted the involvement of p53 in cell cycle control in mammary tumourigenesis.

The *p53*$^{-/-}$*Apc*$^{Min/+}$ mouse is of particular relevance to this thesis. These animals did not exhibit any change in the predisposition towards or the aggression of intestinal neoplasia, as compared to *Apc*$^{Min/+}$mice. However 83% of animals analysed showed pancreatic preneoplastic acinar cell foci and cystic adenocarcinomas which exhibited LOH for *Apc* (Clarke *et al.*, 1995). As *Apc*$^{Min/+}$ animals developed small numbers of preneoplastic acinar foci and *p53*$^{-/-}$ mice did not exhibit any pancreatic abnormalities, this study suggested co-operation between *p53* and *Apc* mutation in the promotion of murine pancreatic tumourigenesis. This work highlighted three things. First, as *p53* deficiency has different effects in different tissues, p53 must have cell type specific roles such that the loss of a particular pathway has greater effect in some tissues than others. This is interesting given that acinar cells in the pancreas normally have low levels of apoptosis. Therefore one would not expect loss of apoptosis to exert such a strong effect. Second, as human pancreatic neoplasia is predominantly within the duct cell compartment, the presence of pancreatic neoplasia in the acinar cell compartment in these mice introduces an interesting species difference. Third, loss of p53 is thought to be a late event in intestinal tumourigenesis, but the phenotype of this mouse suggests that loss of p53-mediated apoptosis does not predispose to intestinal neoplasia.

3.1.3 Questions addressed in this chapter

In humans, *SMAD4* has been found to be biallelically deleted or mutated in 50% of pancreatic ductal adenocarcinomas (Hahn *et al.*, 1996; Schutte *et al.*, 1996). Whilst rare, mutant *APC* has been reported in human pancreatic ductal adenocarcinomas (Horii *et al.*, 1992; McKie *et al.*, 1993). Approximately 50% of human pancreatic ductal adenocarcinomas show inactivation of p53 (reviewed in Howe and Conlon, 1997). These works all refer predominantly to pancreatic ductal tumourigenesis, however the work by Clarke *et al.* (1995) showed that mutations in *p53* and Apc^{Min} predisposed to murine pancreatic acinar tumourigenesis. In fact most rodent models exhibit acinar cell carcinomas following transgenic manipulation or carcinogen treatment (Longnecker, 1992). Whilst acknowledging the discrepancy between acinar and ductal pancreatic tumourigenesis in murine and humans, it appears that similar gene mutations are involved in the promotion of tumourigenesis in both, suggesting that murine models could be informative in the investigation of the genetic changes occurring in both ductal and acinar pancreatic adenocarcinomas in humans. It was hypothesised therefore that *Smad4* mutation may promote pancreatic tumourigenesis in $p53^{-/-}Apc^{Min/+}$ mice. Furthermore, since characterisation of $p53^{-/-}$ $Apc^{Min/+}$ animals by Kongkanuntn *et al.* (1999) led them to suggest that pancreatic adenocarcinomas arising in these mice contained areas of ductal differentiation, the question as to whether *Smad4* mutation would promote ductal differentiation was also addressed. Finally since neither $p53^{-/-}$ nor $Smad4^{+/-}$ mice exhibit pancreatic abnormalities, $p53^{-/-}$ and $Smad4^{+/-}$ mice were intercrossed to determine whether *p53* and *Smad4* mutation would cooperate in the promotion of murine pancreatic tumourigenesis.

3.2 Specific aims

To characterise the pancreatic phenotype of $p53^{-/-}Smad4^{+/-}$ and $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ mice.

To assess whether addition of *Smad4* heterozygosity to $p53^{-/-}Apc^{Min/+}$ mice increases tumourigenicity in the pancreas.

To assess whether addition of *Smad4* heterozygosity to $p53^{-/-}$ mice would predispose to pancreatic tumourigenesis.

To determine whether *Smad4* loss of heterozygosity occurs in pancreatic adenomas.

3.3 The effect of *Smad4* heterozygous mutation on *p53*$^{-/-}$*Apc*$^{Min/+}$ and *p53*$^{-/-}$ mice.

3.3.1 Survival of *p53*$^{-/-}$*Smad4*$^{+/-}$ and *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice

p53 and *Apc* mutation have been shown to cooperate in the development of murine pancreatic neoplasia (Clarke *et al.*, 1995). The present study addresses two issues: first, whether *Smad4* heterozygosity can promote pancreatic tumourigenesis in *p53*$^{-/-}$ *Apc*$^{Min/+}$ mice and second, whether *p53* and *Smad4* mutations can cooperate in the promotion of murine pancreatic neoplasia. A three step intercross breeding strategy was set up to generate *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ and *p53*$^{-/-}$*Smad4*$^{+/-}$ animals. This is outlined in Table 3.1.

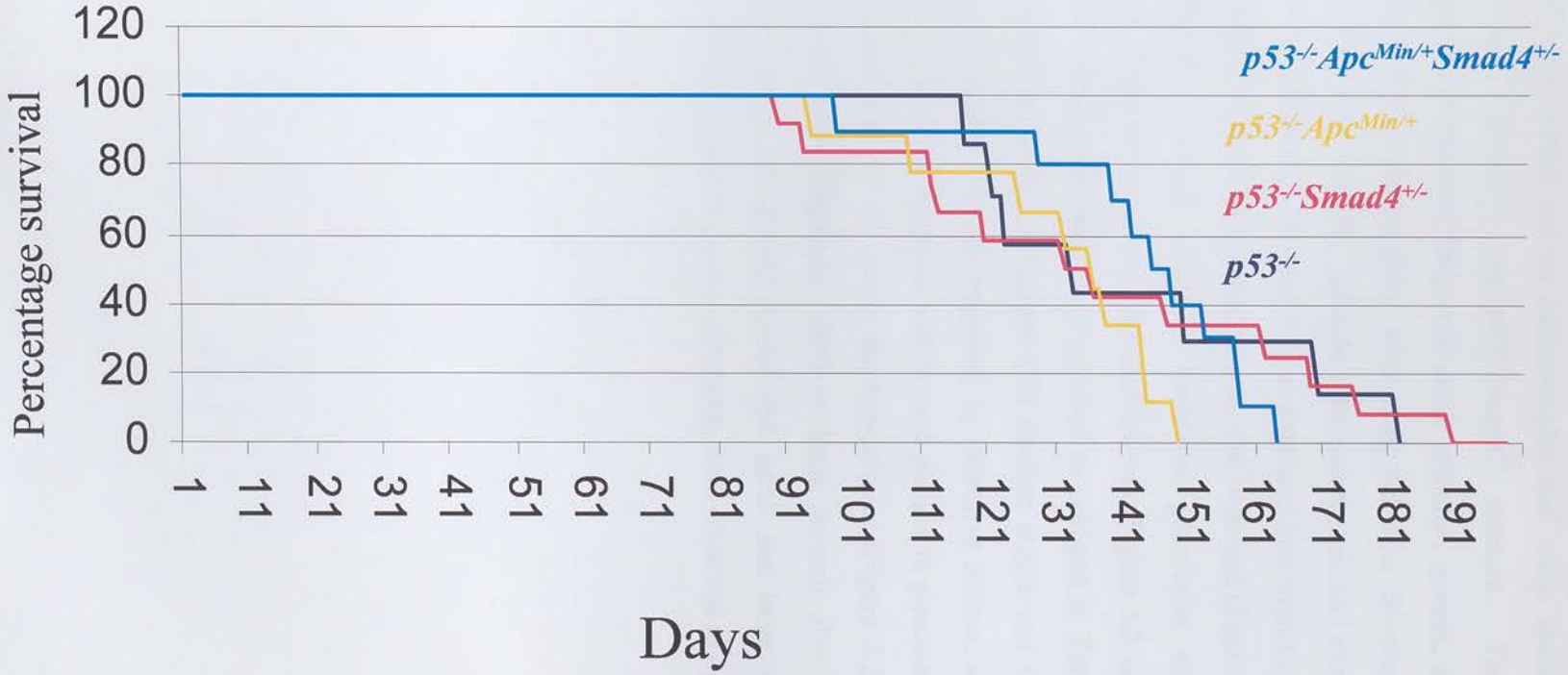
Table 3.1 – 3 step intercross strategy

Mating	Progeny			
(1) *p53*$^{-/-}$*Apc*$^{Min/+}$ X *Smad4*$^{+/-}$	*p53*$^{+/-}$	*p53*$^{+/-}$*Apc*$^{Min/+}$	*p53*$^{+/-}$*Smad4*$^{+/-}$	*p53*$^{+/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$
(2) *p53*$^{+/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ X *p53*$^{+/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$	*p53*$^{+/+}$ *p53*$^{+/-}$ *p53*$^{-/-}$	*p53*$^{+/+}$*Apc*$^{Min/+}$ *p53*$^{+/-}$*Apc*$^{Min/+}$ *p53*$^{-/-}$*Apc*$^{Min/+}$	*p53*$^{+/+}$*Smad4*$^{+/-}$ *p53*$^{+/-}$*Smad4*$^{+/-}$ *p53*$^{-/-}$*Smad4*$^{+/-}$	*p53*$^{+/+}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ *p53*$^{+/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$
(3) *p53*$^{-/-}$*Apc*$^{Min/+}$ X *p53*$^{-/-}$*Smad4*$^{+/-}$	*p53*$^{-/-}$	*p53*$^{-/-}$*Apc*$^{Min/+}$	***p53*$^{-/-}$*Smad4*$^{+/-}$**	***p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$**

Both *p53*$^{-/-}$*Smad4*$^{+/-}$ and *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ animals were found to be viable and cohorts of these animals along with control cohorts (*p53*$^{-/-}$, and *p53*$^{-/-}$*Apc*$^{Min/+}$) were aged. The animals were injected with BrdU, sacrificed and dissected when they became visibly moribund (materials and methods, 2.1.4, 2.1.5 and 2.1.8). *p53* deficiency reduced the life span of both WT and *Smad4*$^{+/-}$ animals, as has been previously shown (Purdie *et al.*, 1994). Death curves were generated to compare the survival of *p53*$^{-/-}$*Smad4*$^{+/-}$ with *p53*$^{-/-}$ mice. It was found that the survival rate of *p53*$^{-/-}$*Smad4*$^{+/-}$ animals was similar to that of *p53*$^{-/-}$ animals (see Figure 3.1). Similarly, no differences were observed in the survival rate of *p53*$^{-/-}$*Apc*$^{Min/+}$ and *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ animals. The majority of the *p53*$^{-/-}$*Apc*$^{Min/+}$ mice, like the *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice, became moribund between 120 and 150 days of age.

Figure 3.1 – Survival rates of mice carrying various mutations for *p53*, Apc^{Min} and *Smad4* limited by the necessity to sacrifice animals showing overt pathological signs. Cohort numbers $p53^{-/-}$ mice - 7; $p53^{-/-}Apc^{Min/+}$ mice - 9; $p53^{-/-}Smad4^{+/-}$ mice - 12 and $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ mice - 12.

Figure 3.1 – Survival rates of various experimental and control mice used in this study.

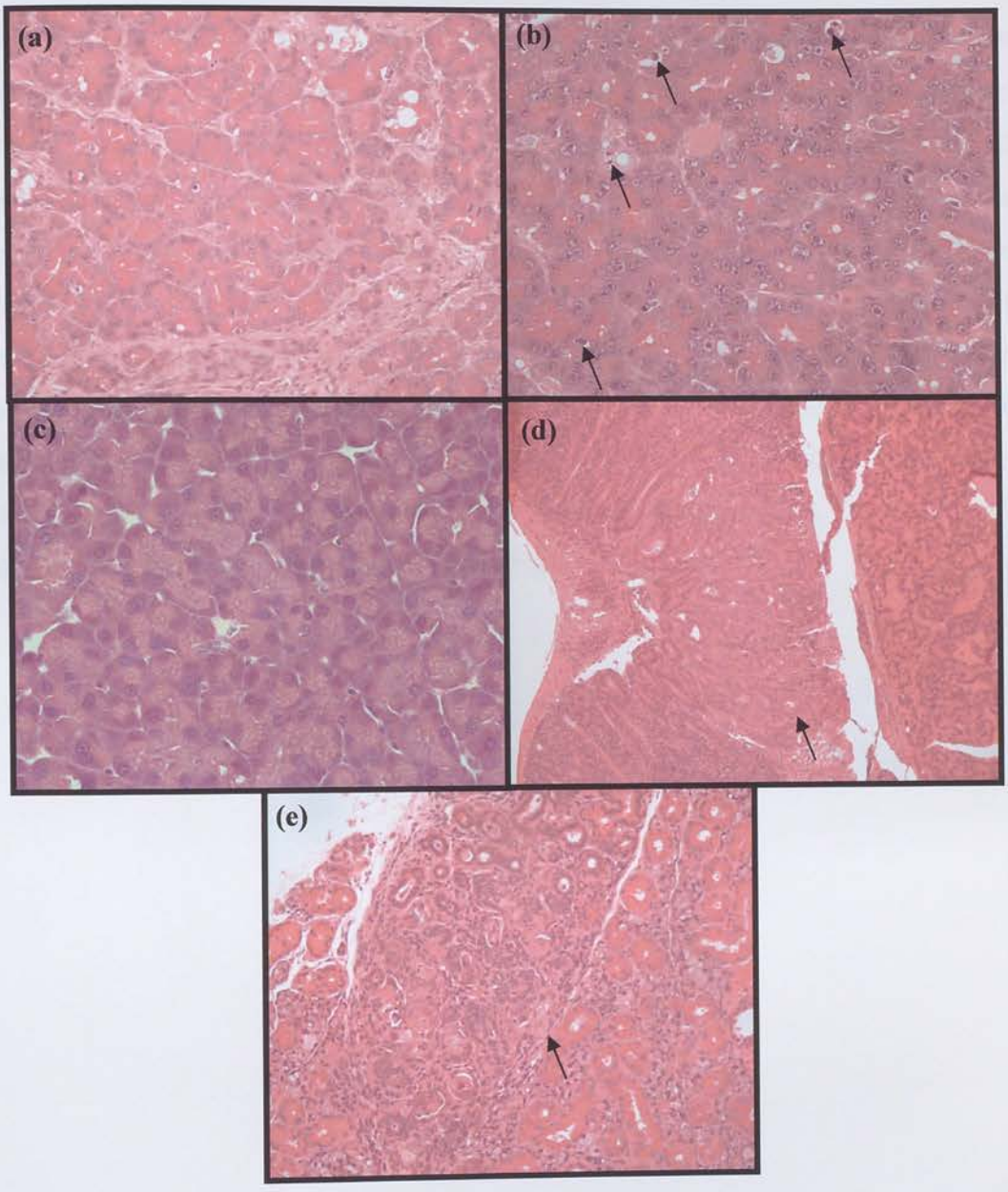


3.3.2 Histological analysis of *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ and *p53*$^{-/-}$Smad$^{+/-}$ animals

Histological analysis was carried out on haematoxylin and eosin stained tissue samples from *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ and *p53*$^{-/-}$*Smad4*$^{+/-}$ animals. The tissues collected and analysed were pancreas, large and small intestine, gonads, mammary gland, spleen, thymus, kidney, salivary gland, liver, lung and skin. Similar to *p53*$^{-/-}$ mice (Purdie *et al.*, 1994), *p53*$^{-/-}$*Smad4*$^{+/-}$ animals exhibited thymic and extra thymic lymphoma (10 out of 10 assessed). All other tissues appeared histologically normal; in particular no pancreatic or intestinal tumourigenesis was observed (Figure 3.2 (c)). The *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ animals exhibited extensive pancreatic acinar cell hyperplasia, adenoma, cystic adenoma and adenocarcinomas (Figure 3.2 (a) and (b) and Figure 3.3 (a-d)). These definitions were ascribed as outlined in Turusov and Mohr, 1994. The hyperplasia was associated with nuclear atypia and the cystic adenomas and adenocarcinomas were accompanied by extensive stroma, suggestive of acute inflammation. Numerous apoptotic bodies were present in pancreatic acinar adenoma and adenocarcinomas but not within the hyperplasias (Figure 3.2 (b)). In addition to the pancreatic tumourigenesis, 100% of these animals also exhibited intestinal single crypt lesions, compound lesions and small and large adenomas (Figure 3.2 (d)). Finally, small salivary gland adenomas were observed in 45% of the animals.

Figure 3.2 – Haemotoxylin and eosin stained tissues from *p53*$^{-/-}$ *Apc*$^{Min/+}$*Smad4*$^{+/-}$ and *p53*$^{-/-}$*Smad4*$^{+/-}$ mice. (a) Pancreatic acinar cystic adenoma from *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ mouse at X200 magnification. (b) Pancreatic acinar cystic adenoma from *p53*$^{-/-}$ *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mouse showing numerous apoptotic cells (arrows) at X400 magnification. (c) Normal pancreatic acinar cells from *p53*$^{-/-}$*Smad4*$^{+/-}$ mice. (d) Arrow identifying adenoma in small intestine *from p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ mouse at X100 magnification. (e) Arrow identifying abnormal focus within salivary gland from *p53*$^{-/-}$ *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mouse at X200 magnification.

Figure3.2 – Representative examples of histology of *p53*$^{-/-}$ *Apc*$^{Min/+}$*Smad4*$^{+/-}$ and *p53*$^{-/-}$*Smad4*$^{+/+}$ mice.

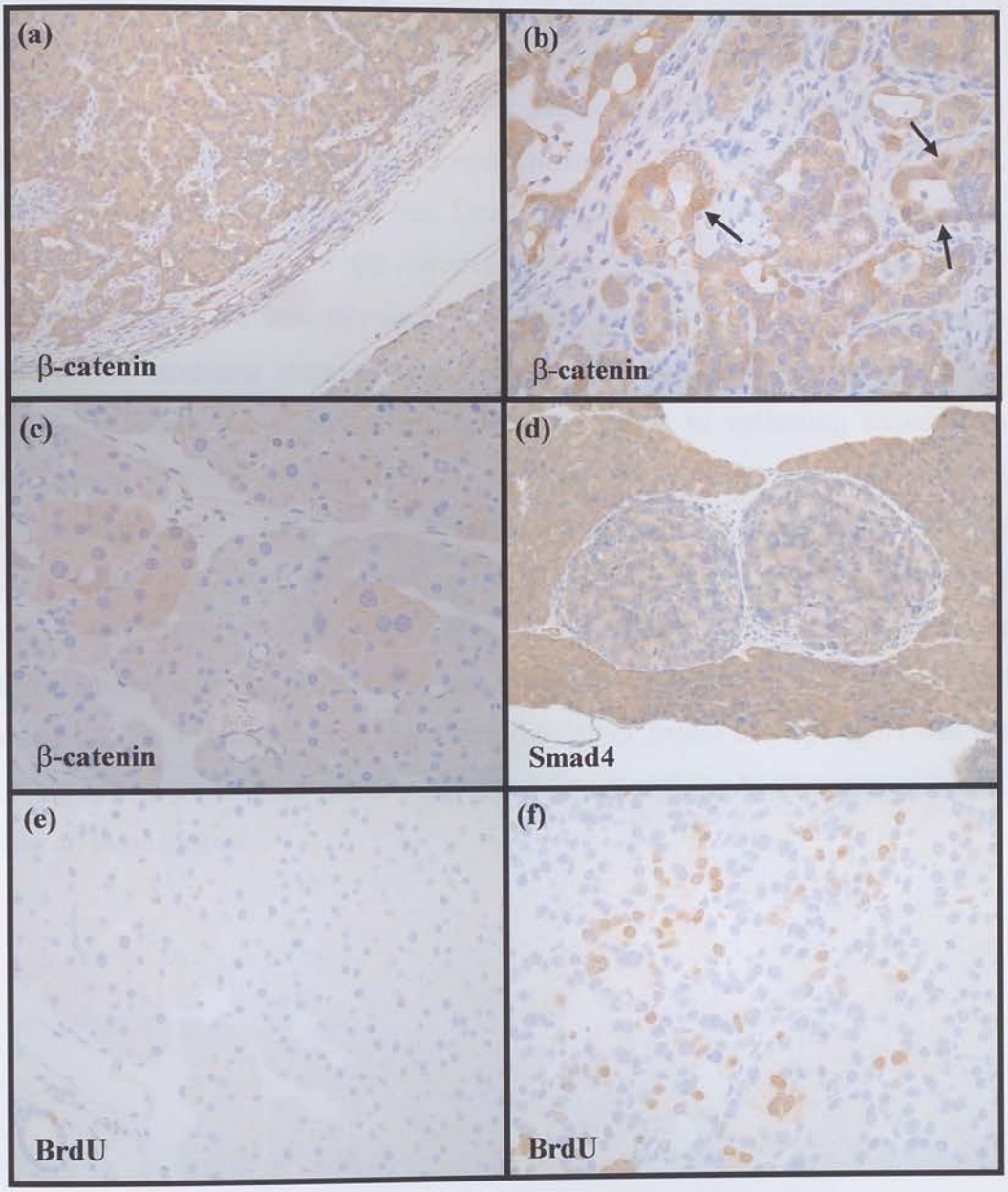


3.3.3 Immunohistochemical analysis of pancreatic lesions

As dysregulated β-catenin has been shown to mark early neoplastic change in the murine pancreas (Kongkanuntn *et al.*, 1999), immunohistochemistry for β-catenin was carried out on pancreas from *p53*$^{-/-}$*Smad4*$^{+/-}$ and *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ animals. Consistent with the histological analysis, no pancreatic dysplasia was found in the pancreas from *p53*$^{-/-}$*Smad4*$^{+/-}$ mice as evidenced by an absence of β-catenin dysregulation. As shown in Figure 3.3 (a-c), immunohistochemistry for β-catenin showed that pancreatic hyperplasia, cystic adenoma and adenocarcinomas all exhibited increased cytoplasmic β-catenin levels. Furthermore, nuclear β-catenin was observed in the cystic adenocarcinomas (Figure 3.3 (b)). Immunohistochemistry for SMAD4 revealed loss of detectable SMAD4 expression within the majority of pancreatic lesions in *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice (Figure 3.3 (d)), suggestive of loss of heterozygosity for *Smad4* within these cells. Finally, immunohistochemical analysis of BrdU incorporation revealed high levels of cell turnover particularly within the adenomas, cystic adenomas and adenocarcinomas, indicative of dysregulated proliferation in these lesions (Figure 3.3 (e) and (f)).

Figure 3.3 – Results of immunohistochemistry for β-catenin, SMAD4 and BrdU on pancreatic abnormal foci and cystic adenomas from *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice. In all cases the chromogen DAB was used with a haematoxylin counterstain. (a)-(c) Immunohistochemistry for β-catenin on (a) pancreatic acinar cystic adenoma at X200 magnification and at (b) X400 magnification (arrows highlight nuclear β-catenin staining) (c) pancreatic acinar abnormal foci exhibiting nuclear atypia. (d) Immunohistochemistry for SMAD4 showing loss of SMAD4 protein within pancreatic abnormal foci at X200 magnification. (e)-(f) Immunohistochemistry for BrdU on (e) normal pancreatic acinar cells and (f) pancreatic acinar cystic adenoma, both at X200 magnification.

Figure 3.3 – Immunohistochemical analysis of *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice.



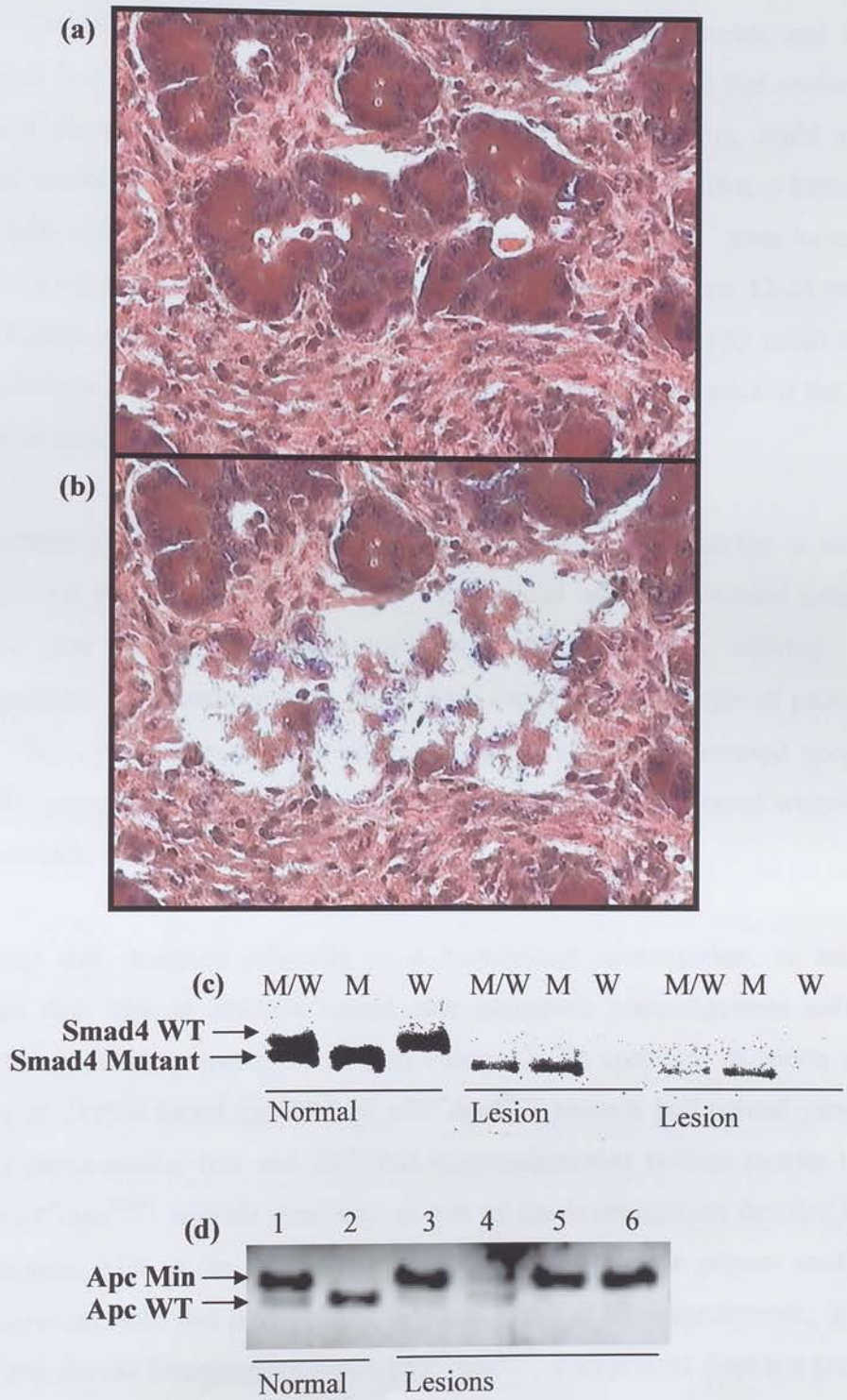
3.3.4 Analysis of LOH for *Smad4* and *Apc* within pancreatic adenomas and adenocarcinomas

Adenocarcinomas arising within $p53^{-/-}Apc^{Min/+}$ and $Apc^{Min/+}$ pancreas exhibited LOH for *Apc* (Clarke *et al.*, 1995; Kongkanuntn *et al.*, 1999). Further, Wagner *et al.* (2001) showed that pancreatic adenocarcinomas arising in $p53^{-/-}$ mice overexpressing TGFα from a transgene under the control of the rat elastase promoter exhibited LOH for *Smad4*. To assess whether loss of SMAD4 immunohistochemical staining within the pancreatic lesions represented LOH for *Smad4* and whether LOH for *Apc* was also a characteristic of pancreatic adenocarcinomas in $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ mice, laser microdissection was carried out. Smaller pancreatic adenomas and hyperplasias had to be eliminated from the investigation as they were only readily visible following β-catenin immunohistochemistry, the process of which was detrimental to the samples rendering them unusable. Laser microdissection was carried out as shown in Figure 3.4 (a-b). This accurate technique of collecting tissue from histological sections allowed the collection of only acinar cells and not the surrounding stroma. PCR was carried out as described in the materials and methods (2.13 and 2.1.16), however, due to the small amounts of DNA extracted from each section, two complete rounds of PCR were carried out. Figure 3.4 (c-d) shows representative examples of PCR reactions which show LOH for both *Smad4* and *Apc* within pancreatic lesions. A detailed investigation of *Smad4* and *Apc* LOH was not carried out, however these results suggest that *Smad4* and *Apc* LOH occurs in at least some of these lesions.

Figure 3.4 – Laser microdissection of pancreastic cystic adenoma from *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ mouse with representative pictures before (a) and after (b) microdissection at X400 magnification. (c) PCR for *Smad4* showing bands for mutant allele (650 bp) and WT allele (711 bp). Three separate reactions were carried out (see materials and methods); 'M/W' with primers for mutant and WT alleles; 'M' with primers only for the mutant allele and 'W' with primers only for the WT allele. Lesions show loss of WT band. (d) PCR for *Apc*^{Min} showing bands for *Min* (144 bp) and WT alleles (123 bp).
Lane 1 - normal tissue from mouse heterozygous for *Apc*^{Min}
Lane 2 - normal tissue from WT mouse
Lane 3 - lesion tissue from mouse heterozygous for *Apc*^{Min}
Lane 4 - lesion tissue from mouse heterozygous for *Apc*^{Min}
Lane 5 - lesion tissue from mouse heterozygous for *Apc*^{Min}
Lane 6 - lesion tissue from mouse heterozygous for *Apc*^{Min}

Lanes 5 and 6 show loss of the WT band.

Figure 3.4 – Microdissection and PCR analysis of pancreatic lesions in *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice.



3.4 Conclusions and discussion

Characterisation of *p53*$^{-/-}$*Smad4*$^{+/-}$ compound mutant mice revealed no differences in survival rate when compared to *p53*$^{-/-}$ single mutants. Histological analysis showed that *p53*$^{-/-}$*Smad4*$^{+/-}$ animals did not exhibit pancreatic tumourigenesis and had a spectrum of lesions similar to that of *p53*$^{-/-}$ mice. It was concluded that neither *p53* mutation or *Smad4* heterozygous mutation singly, or in combination, could initiate pancreatic tumourigenesis within the timescale of this experiment (that is before the animals were overcome with lymphoma). Similarly, whilst *Smad4*$^{+/-}$ mice have been shown to develop intestinal adenomas and adenocarcinomas between 12-24 months of age (Takaku *et al.*, 1999; Xu *et al.*, 2000), the effect of loss of *p53* could not be determined here as the animals became moribund with lymphoma prior to the onset of intestinal tumourigenesis.

Characterisation of *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ animals revealed no difference in survival rate compared to *p53*$^{-/-}$*Apc*$^{Min/+}$ animals. Histological analysis revealed extensive pancreatic and intestinal tumourigenesis and, less commonly, salivary gland tumourigenesis. Increased β-catenin levels were found within all types of pancreatic lesions. The cystic adenomas and adenocarcinomas exhibited increased apoptosis and BrdU incorporation. Finally, LOH for *Smad4* and *Apc* was found within later stage pancreatic lesions.

This study was designed primarily as a histological investigation, to test the hypothesis that loss of SMAD4 would alter pancreatic tumourigenesis either in cellular composition or through a notable change in the spectrum of lesion types. Clarke *et al.* (1995) found that 17% of *p53*$^{-/-}$*Apc*$^{Min/+}$ animals had normal pancreas, 61% had preneoplastic foci and 22% had adenocarcinomas (values similar to the control *p53*$^{-/-}$*Apc*$^{Min/+}$ animals generated as part of the investigations detailed here). In comparison, 22% of the of *p53*$^{-/-}$*Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice in the present study had normal pancreas, 45% had preneoplastic foci and 33% had adenocarcinomas. Thus it appears that *Smad4* heterozygosity on a *p53*$^{-/-}$*Apc*$^{Min/+}$ background does not promote pancreatic tumourigenesis. In retrospect, the design of this study should have

included experiments which could investigate more subtle changes in pancreatic tumourigenesis, such as changes in protein or RNA expression or markers for the differentiation status of pancreatic exocrine cells. Further, the ability to conditionally remove *p53* in the adult pancreas would have allowed the studies to continue beyond the constraints of the lifespan of $p53^{-/-}$ animals.

The question as to ductal versus acinar composition of human and murine pancreatic cancers remains controversial. Given the importance of animal models in the study of cancer, some resolution must be reached. It could be argued that the difference in cell type between the two species is fundamental and therefore murine models can only be of use in the investigation of the rarely occurring human acinar pancreatic carcinoma. Alternatively, perhaps despite the disparities in both genetic make up and cell type, the animal models can still provide information, that upon further investigation in different model systems, could be vital. Experimental examples of this species difference include the fact that activating *K-RAS* mutations are associated with ductal adenocarcinomas in humans (Howe and Conlon, 1997) but not in pancreatic tumourigenesis in the mouse (Terhune *et al.*, 1994; Schmid *et al.*, 1999). The studies presented here showing that $p53^{-/-}Smad4^{+/-}$ mice have normal pancreata demonstrates that *Apc* mutation is fundamentally associated with murine pancreatic tumourigenesis, however this is not the case in the development of human ductal adenocarcinomas (Yashima *et al.*, 1994). Comparison between human pancreatic ductal and acinar carcinomas revealed 9% allelic loss and 9% immunonegativity for DPC4/SMAD4 in ductal carcinomas and 0% allelic loss and 100% immunopositivity for DPC4/SMAD4 in acinar carcinomas (Moore *et al.*, 2001).

A physiological explanation for the human/murine differences could be the absence of the centroacinar cell in the murine pancreas. It has been suggested that human ductal pancreatic adenocarcinomas could be initiated in centroacinar cells which then differentiate to ductal cells (Hall and Lemoine, 1993). Transdifferentiation between acinar and ductal cells has been reported in murine primary cultured acinar cells (Hall and Lemoine, 1992). The question of acinar to ductal transdifferentiation is discussed further in Chapter 5. Also supporting the viewpoint that murine models are useful in

the study of human pancreatic cancer is the involvement of similar gene mutations in murine pancreatic tumourigenesis as in humans, such as *p53*, *p16*, *Smad4*, *ras* and *Rb1* (Howe and Conlon, 1997; Wagner *et al.*, 2001, Quaife *et al.*, 1987). The work by Wagner *et al.* (2001) cited above utilises $p53^{-/-}$ mice which overexpressed TGF-α in the pancreas. The TGF-α overexpressing mice express TGF-α under the control of a rat elastase promoter and exhibited transdifferentiation of acinar cells to duct-like cells, which represent pancreatic premalignant lesions (Sandgren *et al.*, 1990; Wagner *et al.*, 1998).

In humans *Smad4* and *Apc* are located at 18q21 and 5q21 respectivity. In the mouse however these alleles both reside on chromosome 18 approximately 30 centimorgans (cM) apart (Takaku *et al.*, 1998). Therefore mice heterozygous for both *Smad4* and *Apc* could contain the mutations in either the cis or trans conformation. If the mutations reside in the cis conformation then a single event could result in LOH of both these genes. Thus LOH for *Apc* or *Smad4* could simply be an epiphenomenon. In support of this Takaku *et al.* (1998) showed LOH of *Apc* and *Smad4* within $Apc^{\Delta 716/+}Smad4^{+/-}$ (in the cis conformation) mice due to loss of the entire chromosome 18. However if the mutations reside in trans conformation then a single event would only result in LOH of either *Smad4* or *Apc*. Whether the mutations reside in cis or trans confirmation has not been central to this work, however analysis of the intercrossing strategies used suggested that the majority of the compound heterozygous mutations reside in the trans conformation.

3.5 Future work

It would be informative to perform further investigations into pancreatic tumourigenesis in $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ mice. First, it is important to determine whether individual lesions exhibit both *Apc* and *Smad4* LOH or simply for one or the other. This would allow determination of whether LOH for *Apc* or *Smad4* is an epiphenomenon or whether it actively promotes tumourigenesis and would provide information as to whether the mutations are in the cis or trans conformation. By specific microdissection of hyperplastic foci, adenomas, cystic adenomas and

adenocarcinomas and then assessment of LOH for *Apc* and *Smad4* (and perhaps other mutations such as activating *K-ras* mutations) information could be gained as to the order in which certain mutations occur within the progression of tumourigenesis. Third, direct comparision between the mutation status of lesions arising in $p53^{-/-}$ $Apc^{Min/+}$ mice and those in $p53^{-/-}$, Apc^{Min} and *Smad4* heterozygotes could help determine the extent to which LOH for *Smad4* is contributing to tumourigenesis.

Finally, as *Apc* and *Smad4* mutations have been shown to cooperate in intestinal tumourigenesis (Takaku *et al.*, 1998) but *p53* and *Apc* mutations have not (Clarke *et al.*, 1995) it would be of great interest to analyse the intestinal tumours arising in the $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ mice. It is hypothesised that *p53* mutation in conjunction with both $Apc^{Min/+}Smad4^{+/-}$ could result in increased numbers of malignant intestinal tumours.

Chapter 4 – Carcinogen-induced murine pancreatic tumourigenesis: involvement of *p53*, *Apc* and *Smad4* mutation.

4.1 Introduction

4.1.1 p53 deficiency and pancreatic tumourigenesis

As outlined in 1.10.4, loss of *p53* is an extremely important step in tumourigenesis and this gene is inactivated in approximately 50% of human pancreatic cancers (reviewed in Howe and Conlon, 1997). p53 functions primarily to allow cells to respond appropriately to stresses such as DNA damage, oncogene activation and hypoxia. p53 has been associated with DNA repair mechanisms such as nucleotide excision repair, base excision repair and recombinational repair (Wang *et al.*, 1995; Offer *et al.*, 1999; Sturzbecher *et al.*, 1996). Loss of p53 promotes tumourigenesis through mechanisms including loss of G1 checkpoint which normally prevents replication of damaged or fragmented DNA (Ko and Prives, 1996), reduced apoptosis, increased gene amplification and aneuploidy and increased invasiveness of cells. The phenotype of $p53^{-/-}$ mice is discussed in Chapter 1, section 1.11.6.

4.1.2 *Smad4* and *Apc* mutation in murine tumourigenesis

As well as intestinal neoplasia, the $Apc^{Min/+}$ mouse is predisposed to the development of abnormal pancreatic acinar foci characterised by high levels of β-catenin, which in the absence of p53 progress to neoplasia (Clarke *et al.*, 1995; Kongkanuntn *et al.*, 1999). As a component of the Wnt signalling pathway, APC is thought to mediate its tumour suppressive activities predominantly through downregulation of intracellular β-catenin levels (Munemitsu *et al.*, 1995). Loss of functional APC results in increased levels of β-catenin, which upon nuclear translocation mediates transcription of target genes, including *Cyclin D1* and *c-Myc* (He *et al.*, 1998; Tetsu and McCormick, 1999).

Whilst SMAD4 has been linked to tumour suppression-related activities *in vitro*, the relevance to pancreatic neoplasia *in vivo* remains unclear. *Smad4* null embryos die around day E7.5, whereas heterozygous mice survive and develop intestinal polyps by 12 months (Sirard *et al.*, 1998; Yang *et al.*, 1998; Xu *et al.*, 2000; Takaku *et al.*, 1999). Although *Smad4* LOH was reported in later stage intestinal tumours (Miyaki *et al.*, 1999), Xu *et al.* (2000) found that *Smad4* heterozygosity was sufficient to initiate tumourigenesis in the intestine. Mice heterozygous for inactivating mutations in both *Smad4* and *Apc* ($Apc^{\Delta 716}$) developed colorectal carcinomas that were larger in size and more invasive by 20 weeks of age than those found in $Apc^{\Delta 716}$ heterozygotes alone (Takaku *et al.*, 1998). This suggests that *Smad4* mutation plays a significant role in the malignant progression of colorectal tumours, but the precise biological role of *Smad4* mutation in the progression of pancreatic tumourigenesis *in vivo* has yet to be elucidated. Although *Smad4* mutation is linked with 50% of pancreatic tumours in humans, to date no pancreatic phenotype has been reported for any murine transgenic *Smad4* strain.

4.1.3 Cox-2 inhibitors as suppressors of tumourigenesis

Cyclo-oxygenase 2 (Cox-2) is an inducible form of prostaglandin H synthase which is involved in mediating prostaglandin synthesis during inflammation. Cox-2 has been found to be a transcriptional target of the Wnt signalling pathway (Howe *et al.*, 1999; 2001). Cox-2 is overexpressed in colon tumours (Kargman *et al.*, 1995) and in pancreatic tumours (Yip-Scheider *et al.*, 2000). Cox-2 is thought to promote tumourigenesis by making cells refractory to apoptosis or by modulating cell adhesion (Tsujii and Dubois, 1995). Furthermore Cox-2 is thought to increase invasiveness and metastatic potential of human colon cancer cells (Tsujii *et al.*, 1997). Inhibition of Cox-2 by pharmacological agents in $Apc^{\Delta 716}$ knockout mice protects against the development of intestinal tumours (Oshima *et al.*, 1996). In particular much investigation has gone into the action of non-steroidal anti-inflammatory agents (NSAIDs) such as aspirin and sulindac, which inhibit Cox-2 activity (Kune, 2000). Work with $Apc^{Min/+}$ mice found that intestinal tumours arising in these animals overexpress Cox-2 (Williams *et al.*,

1996) and the NSAID, sulindac, was shown to cause regression of these tumours (Giardiello *et al.*, 1996; Chiu *et al.*, 1997). In humans NSAIDs have been found to have a chemotherapeutic effect on the colonic polyps arising in FAP patients, causing the polyps to regress (Giardiello *et al.*, 1995). Furthermore, a 40-50% decrease in the risk of colorectal cancer has been reported in people who continuously take aspirin (Williams *et al.*, 1997).

The mechanism of chemoprevention of intestinal tumours by NSAIDs remains unclear, but is thought to be the induction of apoptosis (Elder *et al.*, 1996). Sulindac decreases β-catenin expression and increases apoptosis in normal intestinal mucosa in $Apc^{Min/+}$ mice and causes at least a 50% reduction of the elevated β-catenin expression seen in intestinal tumours (McEntee *et al.*, 1999). Work by Sheng *et al.* (1999) found a correlation between increased TGF-β expression resulting in an upregulation of Cox-2 and downregulation of TGFβ-RII. In this study non-tumourigenic intestinal epithelial cells (RIE-1) were continuously exposed to TGF-β. The cells first lost responsiveness to TGF-β growth inhibition. Continued exposure after loss of TGF-β responsiveness resulted in the cells acquiring a tumourigenic phenotype associated with Cox-2 upregulation. This tumourigenic phenotype could in part be inhibited by selective pharmacological Cox-2 inhibitors.

4.1.4 The use of a carcinogen to induce murine pancreatic tumourigenesis

$p53^{-/-}$ mice do not exhibit pancreatic lesions, but since *p53* mutation plays an important role in pancreatic tumourigenesis pancreatic tumourigenesis was induced in $p53^{-/-}$ mice using a carcinogen. This served two main purposes. First, it allowed the selective investigation into the effect of $p53^{-/-}$ deficiency *in vivo* before the animals became moribund due to the onset of lymphoma. Second, the carcinogen was used to address the need for additional mutations, which could include the remaining *Smad4* or *Apc* wild-type alleles. It was decided to use the carcinogen N-methyl-N nitrosourea (NMU). NMU is an alkylating agent and a potent mutagen, exposure to which has been shown to

result in pancreatic acinar cell hyperplasia in rats and the development of lymphoma and mammary tumours in mice (Monis *et al.*, 1991; Diamond *et al.*, 1987; Barka, 1982). NMU has also been shown to cause activating *ras* mutations in mice (Corominas *et al.*, 1991). The activation of *K-RAS* is found in most human pancreatic cancers (Almoguera *et al.*, 1988). It was thought that murine pancreatic tumourigenesis was not associated with activating *K-ras* mutation but recent work has shown that this is not the case (Wagner *et al.*, 2001). It could be that activation of *ras* is one of the mechanisms of NMU-induced pancreatic dysplasia pertaining here.

4.1.5 Questions addressed in this study

$p53^{-/-}$ and wild type (WT) control animals were treated with the carcinogen NMU to address the role of *p53* deficiency on pancreatic tumourigenesis. As *p53* mutation has been associated with the genetic instability reported in many cancers, investigations were also carried out to assess the effect of *p53* deficiency on nuclear atypia.

NMU treatment was also utilised to address the role of *Smad4* mutation in the development of pancreatic cancer. The NMU susceptibility of WT mice and mice heterozygous at either or both Apc^{Min} and *Smad4* loci was compared. Given the established synergy between mutant Apc^{Min} and *Smad4* alleles in colorectal cancer (Takaku *et al.*, 1998), I also investigated whether mutations in these genes co-operate in this system.

Two pieces of evidence suggest the possibilty that aspirin may suppress NMU-induced tumourigenesis in the murine pancreas: first, the ability of Cox-2 inhibitors to reduce β-catenin levels and tumour formation in the intestine in $Apc^{Min/+}$ mice; second, the finding by Kongkanuntn *et al.* (1999) that β-catenin stabilisation is a marker of pancreatic preneoplastic abnormal foci in $Apc^{Min/+}$ mice. Therefore the effect of dietary aspirin on NMU-induced pancreatic tumourigenesis was tested in *Smad4* heterozygotes, *Apc* heterozygotes and *Apc* and *Smad4* compound heterozygotes ($Smad4^{+/-}$, $Apc^{Min/+}$ and $Apc^{Min/+}Smad4^{+/-}$ mice).

4.2 Specific aims

To assess the effect of loss of *p53* on pancreatic tumourigenesis in NMU-induced pancreatic dysplasia.

To assess the effect of *Smad4* heterozygosity, *Apc* heterozygosity and combined *Apc* and *Smad4* heterozygosity on NMU-induced pancreatic dysplasia.

To characterise nuclear atypia within pancreatic abnormal foci.

To investigate the chemo-preventative effect of dietary aspirin on NMU-induced pancreatic tumourigenesis in $Smad4^{+/-}$, $Apc^{Min/+}$ and $Apc^{Min/+}Smad4^{+/-}$ mice.

4.3 The effect of *p53* deficiency upon carcinogen-induced pancreatic neoplasia.

4.3.1 Wnt signalling is dysregulated in pancreatic lesions arising in NMU exposed mice

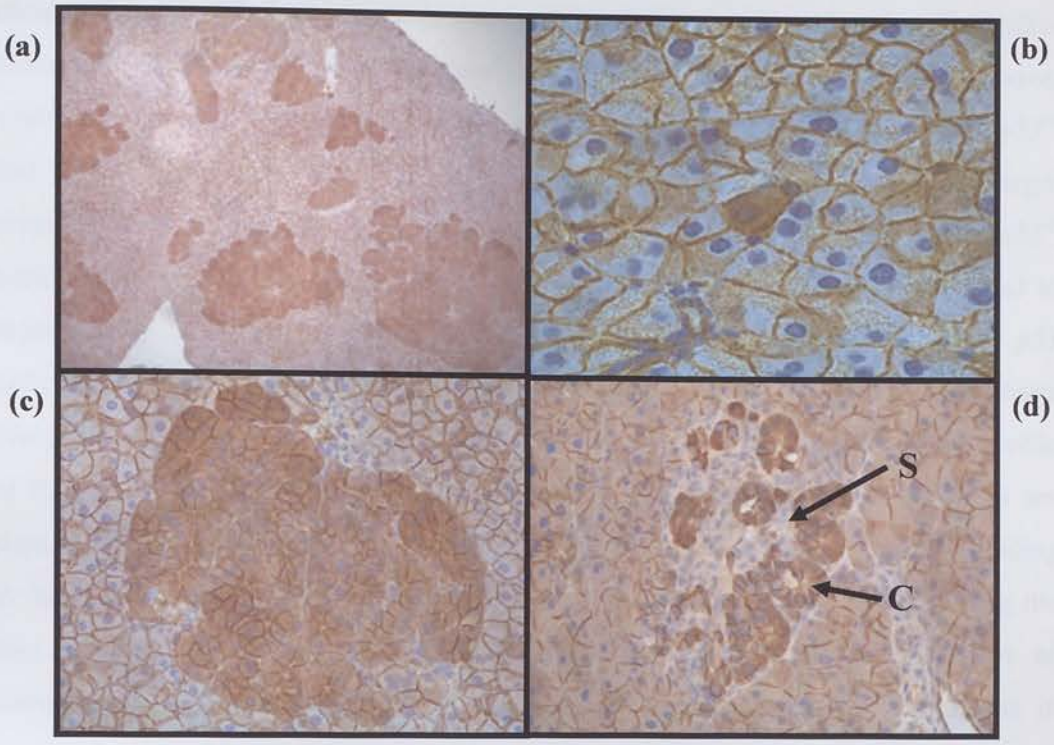
Age-matched cohorts of WT and $p53^{-/-}$ mice which had been subcutaneously injected with NMU at 4 days post partum were killed at 65 days and tissues collected (carried out by Dr A Menke). Histological analysis revealed extensive areas of abnormal acinar cells within the pancreas of NMU-treated WT and $p53^{-/-}$ mice. To identify whether the mechanism underlying the formation of these abnormal foci was similar to that observed in the $Apc^{Min/+}$ mice (Kongkanuntn *et al.*, 1999) these samples were subjected to immunohistochemistry for β–catenin. All morphologically identifiable areas of dysplasia were characterised by increased levels of cytoplasmic β–catenin as shown in Figure 4.1. This suggests that dysregulation of the Wnt signalling pathway is a key event in the development of abnormal pancreatic foci.

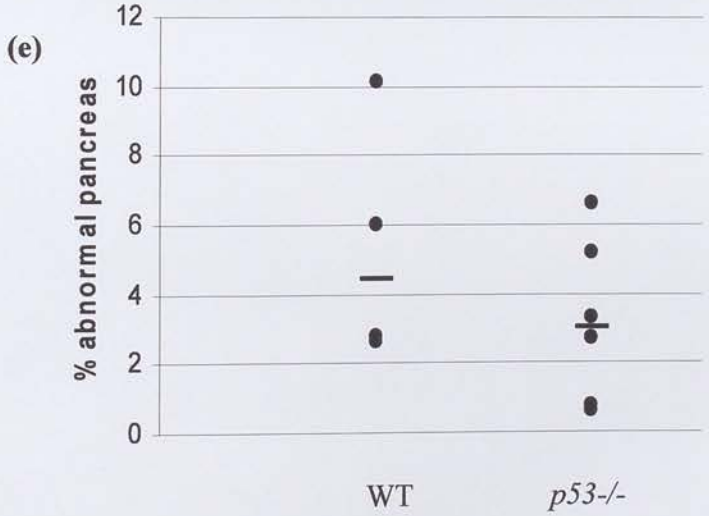
4.3.2 Effect of *p53* deficiency on amount of abnormal pancreatic tissue

To determine if *p53* status influenced the extent of dysplasia, the fact that β-catenin stabilisation marked abnormal foci was exploited to measure the total area occupied by abnormal acinar cell foci on random sections in $p53^{+/+}$ and $p53^{-/-}$ mice. This was then expressed as a percentage of the total area of pancreas in these sections. No significant difference in this percentage was identified between WT and $p53^{-/-}$ mice (Figure 4.1, Mann Whitney U, $p>0.05$). No abnormal foci were observed in the pancreas of control, untreated mice.

Figure 4.1 – Pancreata from NMU-treated WT and *p53*$^{-/-}$ mice immunohistochemically stained for β-catenin. (a) Overview at X40 magnification from NMU-treated *p53*$^{-/-}$ mouse, picture shows several areas of abnormal pancreas as identified by increased β-catenin levels. (b) Single β-catenin stabilised acinar cell at X1000 magnification from NMU-treated *p53*$^{-/-}$ mouse. (c) Focus consisting entirely of acinar cells at X400 magnification from NMU-treated WT mouse. (d) Focus containing acinar and stromal (S) cells and cystic areas (C) at X400 magnification from NMU-treated *p53*$^{-/-}$ mouse. (e) Amount abnormal (β-catenin stabilised) pancreas as measured by area and expressed as percentage of total pancreas in sections from NMU-treated WT and *p53*$^{-/-}$ mice. Data are expressed as a dot plot with median percentage abnormal pancreas shown as a horizontal bar.

Figure 4.1 – Analysis of abnormal acinar foci within the pancreata of NMU-treated WT and *p53*$^{-/-}$ mice.





4.3.3 Effect of *p53* deficiency on size and composition of foci

Preliminary examination of the abnormal foci arising in the $p53^{-/-}$ mice indicated that these differed morphologically from those arising in the treated wild-types. To further characterise this, morphometric analysis was performed on the foci. As stated above, whilst the overall percentage of the pancreas defined as dysplastic was similar in $p53^{-/-}$ and $p53^{+/+}$ mice, three main differences were observed (Figure 4.2). First, larger abnormal foci (classes II and III, see materials and methods) arising in the $p53^{-/-}$ background contained a significantly higher percentage of stromal cells compared to those arising on a WT background (Mann Whitney U, $p<0.05$). Second, many *p53* deficient foci exhibited cystic areas (Figure 4.1(d)). Figure 4.2 shows bar charts comparing the content of lesions in $p53^{-/-}$ and $p53^{+/+}$ mice. Third, analysis of the profile of foci size (as scored by area) shows that in the absence of *p53* abnormal foci are significantly smaller (Mann Whitney U, $p<0.05$), thus *p53* deficiency could be leading to increased abnormal foci initiation. Several hypothesis are suggested regarding the two types of foci. First that the two types represent independent pathways of tumourigenesis that are not related. Second that the acinar-only foci progress in tumourigenesis to become foci containing stroma and cystic areas. Finally, it could be that the focus phenotype of increased stroma and cystic areas reflects an inflammatory response being mounted against the abnormal cells.

Figure 4.2 – (a) Histograms showing average percentage stromal cells (a) and cystic areas (b) within different classes of abnormal foci for a given genotype as scored by area on random sections of immunohistochemically stained for β-catenin. Error bars represent +/- Standard error of mean. (c) The distribution of foci sizes in WT and *p53* null mice is represented in a box plot. A box plot is created as detailed below.

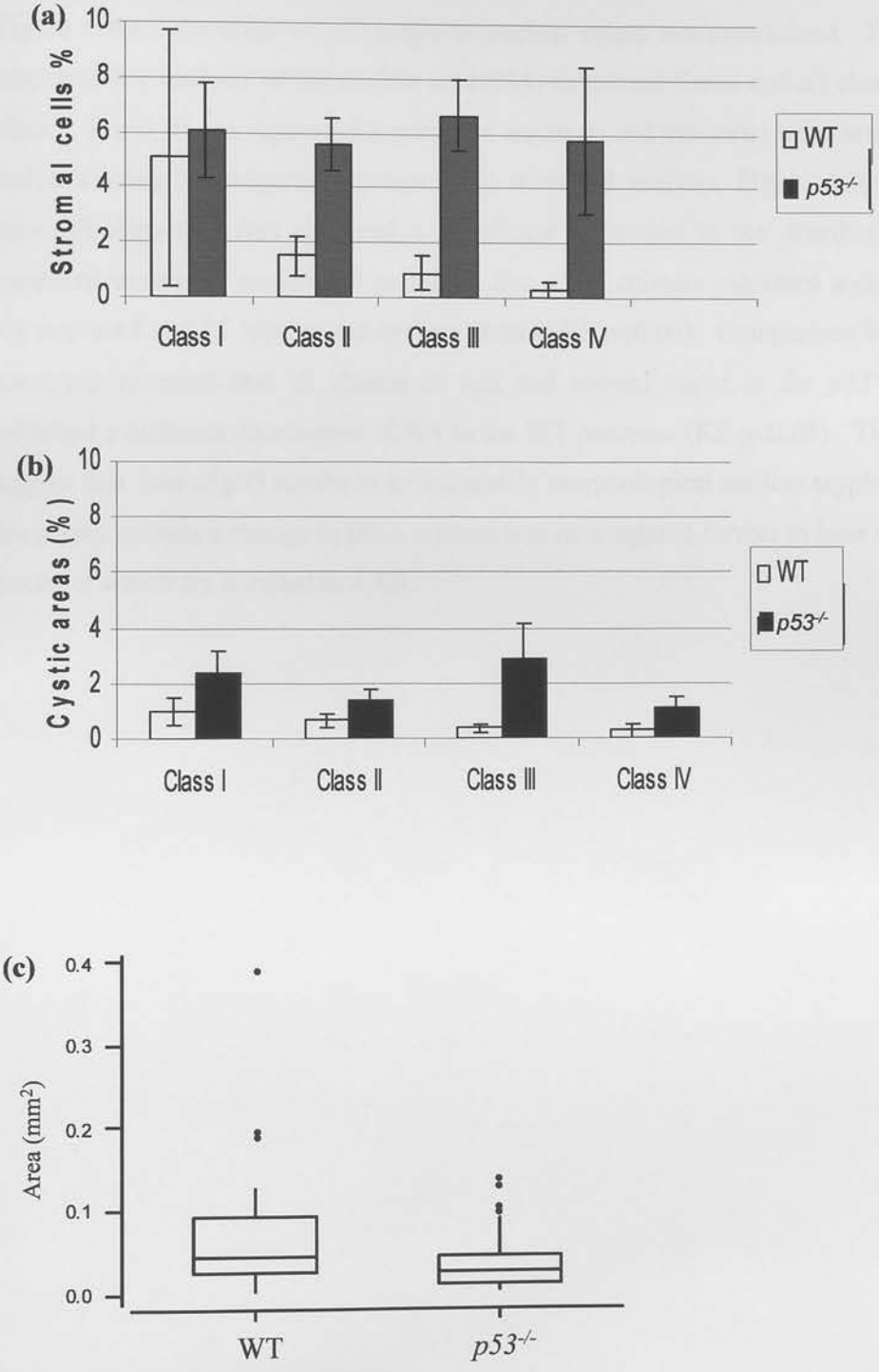
The top and bottom of the rectangular box represent the 1st and 3rd Quartiles (Q1 and Q3) and the line within the rectangle is the median. The vertical whiskers run to the adjacent values in the upper and lower limits. The adjacent values are the lowest and highest observations within their respective limits.

The upper limit is calculated as Q3 + 1.5x the interquartile range (Q3 – Q1).

The lower limit is calculated as Q1 – 1.5 x the interquartile range.

Any observations outside the upper and lower limits are expressed as black dots.

Figure 4.2 – Effect of *p53* deficiency on size and composition of abnormal foci.

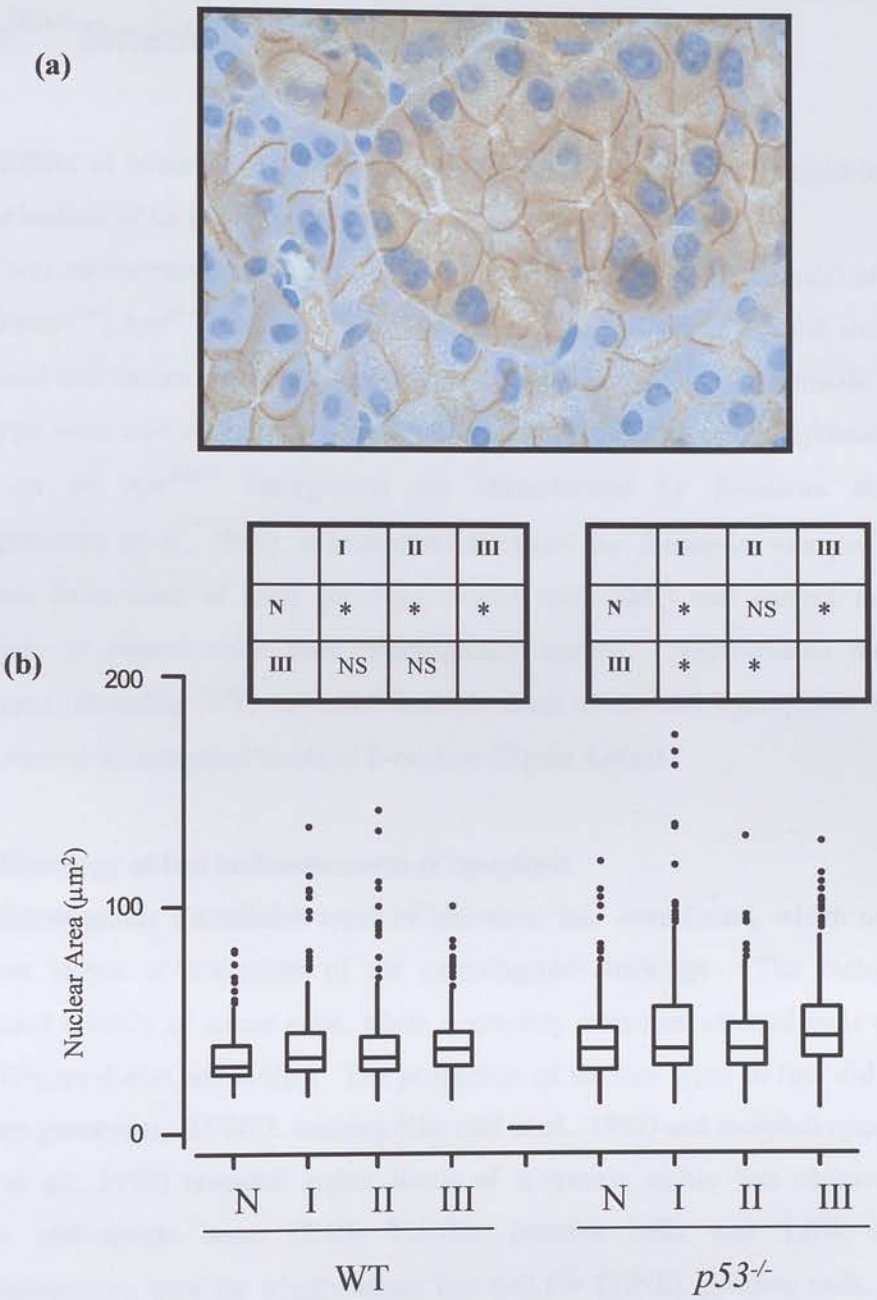


4.3.4 Effect of *p53* deficiency on nuclear atypia within abnormal foci

As *p53* deficiency has been associated with genomic instability and histological examination revealed atypic nuclei consistent with dysplasia within abnormal foci (Figure 4.3(a)), the effect of *p53* status on nuclear atypia was determined. To this end, morphometric analysis of the nuclear area (NA) in normal tissue and all classes of foci (class I, II and III see Figure 4.3 legend and materials and methods) was carried out and analysed using Kolmogorov Smirnov (KS) statistical analysis, Figure 4.3(b). In WT mice, all classes of foci exhibited a significant difference in the distribution of NA compared to normal nuclei (KS $p<0.05$). The $p53^{-/-}$ animals exhibited a difference in NA in class I and III foci but not in class II foci (KS $p<0.05$). Comparison between the genotypes revealed that all classes of foci and normal tissue in the $p53^{-/-}$ pancreas exhibited a different distribution of NA to the WT pancreas (KS $p<0.05$). These results suggest that loss of p53 results in an increase in morphological nuclear atypia. Whether this atypia reflects a change in DNA content was investigated further in later studies, the results of which are detailed in 4.4.6.

Figure 4.3 – (a) Abnormal foci from *p53*$^{-/-}$ mouse stained immunohistochemically for β-catenin showing atypical nuclei within focus. Foci were classified according to size into three classes Class I, < 0.025mm^2; Class II, 0.025 – 0.05mm^2; Class III, 0.05 – 0.01mm^2, foci larger than Class III were excluded from the analysis of nuclear area due to insufficient numbers. (b) Box plot showing distribution of nuclear area within individual classes for WT and *p53*$^{-/-}$ mice. Approximately 500 cells were scored per class from a minimum of 10 lesions, from at least 4 mice. Results of Kolmogorov Smirnov two-sample test (Sokal and Rohlf, 1995) are shown in tables above respective genotype. N denotes NA (nuclear area) in normal tissue; I, NA in Class I abnormal foci; II, Class II; III, Class III. Key for tables: NS, p>0.05 and * p<0.05. Results in a given box correspond to statistical comparison between the associated column title and row title (in bold).

Figure 4.3 – Analysis of nuclear atypia within abnormal foci.



4.4 Effect of NMU on WT, *Smad4*$^{+/-}$, *Apc*$^{Min/+}$ or *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice.

4.4.1 Effect of heterozygosity for *Smad4* and *Apc*^{Min}, singly or in combination, on the formation of focal acinar hyperplasia

NMU was administered subcutaneously to littermate cohorts (6-10 animals) of 4 day old WT, *Smad4*$^{+/-}$, *Apc*$^{Min/+}$ or *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice. At 65 days of age the animals were sacrificed and tissues harvested. Age-matched (non-injected) control animals from each genotype were also examined. Given that pancreatic lesions arising spontaneously in mice on an *Apc*$^{Min/+}$ background are characterised by β-catenin stabilisation (Kongkanuntn *et al.*, 1999), immunohistochemistry for β-catenin was performed on pancreas from mice of each genotype treated with NMU and control mice. The pancreata of control mice were histologically normal. NMU-treated mice of all genotypes (including WT) exhibited multiple focal acinar cell hyperplasia which was characterised by increased levels of β-catenin (Figure 4.4(a)).

4.4.2 Histology of foci and assessment of apoptosis

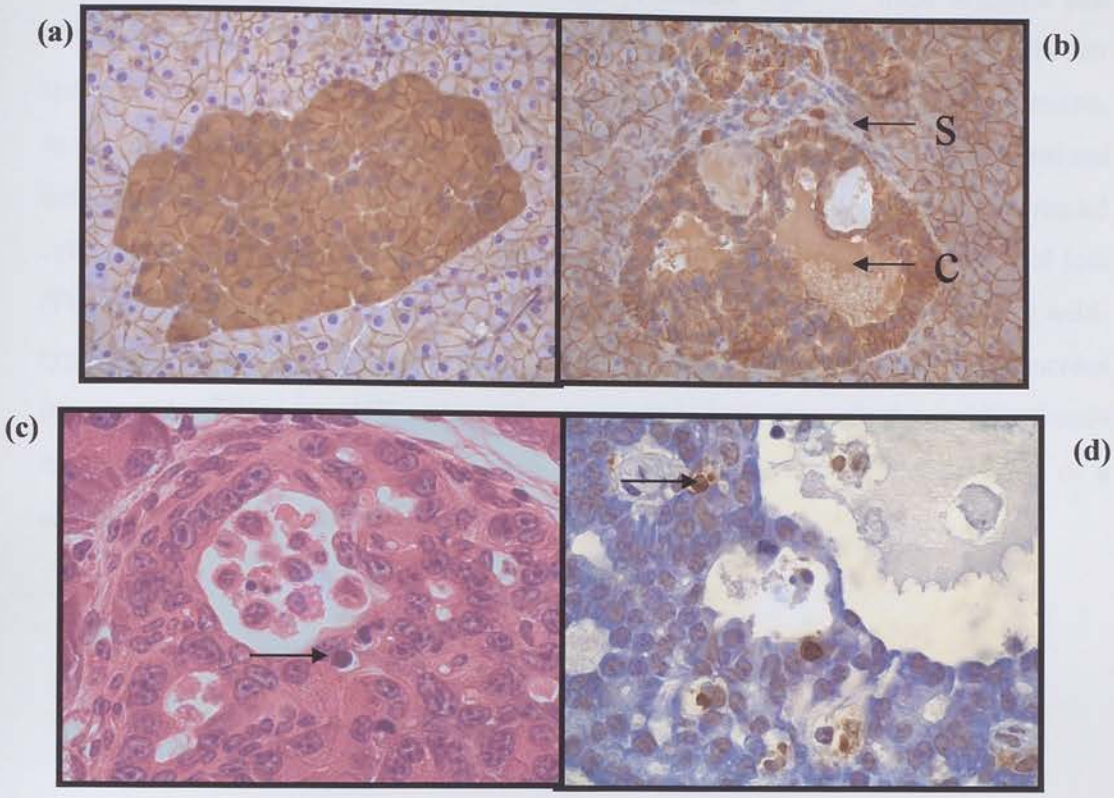
Two histologically identifiable types of abnormal foci were found, which may reflect different stages or endpoints of the carcinogenic challenge. The majority were composed entirely of acinar cells, while a minority contained stromal cells and cystic areas (Figure 4.4(a) and 4.4(b)). The proportion of the two types of foci did not differ between genotypes. TUNEL staining (Gavrieli *et al.*, 1992) and morphological analysis (Kerr *et al.*, 1972) revealed higher levels of apoptosis within foci characterised by stroma and cystic areas (2.4% TUNEL positive cells and 1.0% as scored morphologically) than the wholly acinar foci (<0.1% TUNEL positive cells, and 0.2% as scored morphologically) (Figure 4.4(c) and 4.4(d)). It is possible that the altered content and deregulation of apoptosis in this subset of foci may indicate a progression in tumourigenesis.

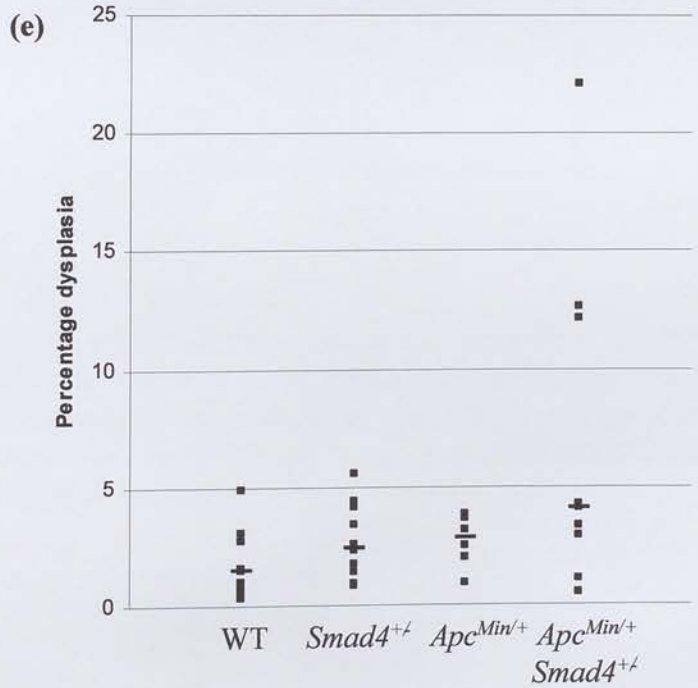
4.4.3 Morphometric analysis of focal acinar hyperplasia in WT, *Smad4*$^{+/-}$, *Apc*$^{Min/+}$ or *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice

Using β-catenin immunohistochemistry, the area of pancreas occupied by abnormal foci and focus size was measured by morphometric analysis. Comparison of abnormal pancreas expressed as a percentage of total pancreas found no differences between *Smad4*$^{+/-}$, *Apc*$^{Min/+}$ *or* WT mice (Mann Whitney U, $p>0.05$), whereas *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice showed a significant increase in abnormal pancreas (Figure 4.4, Mann Whitney U $p<0.05$). Increased abnormal pancreas, only when *Apc* and *Smad4* mutation are present in conjunction, suggests that the pathways these proteins are involved in converge on a common target. This hypothesis is supported by previous work by Nishita *et al.* (2000) and Labbe *et al.* (2000) where interaction of members of the SMAD family with LEF1/TCF and β-catenin was reported.

Figure 4.4 – (a) Abnormal focus of pancreatic acinar cells from NMU-treated WT mouse showing β-catenin stabilisation at X400 magnification. (b) Pancreatic abnormal focus from *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mouse showing β-catenin stabilisation and containing stromal cells (S) and cystic areas (C) at X400 magnification. (c) H and E stained pancreas showing representative example of apoptosis (arrow) as defined by morphological criteria at X1000 magnification. (d) TUNEL labelled foci counterstained with haematoxylin at X1000 magnification. Brown cells (arrow) represent cells positively labelled by the TUNEL method. (e) Amount abnormal (β-catenin stabilised) pancreas as measured by area and expressed as percentage of total pancreas in sections from NMU-treated WT, *Smad4*$^{+/-}$, *Apc*$^{Min/+}$, *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice. Data are expressed as a dot plot with median percentage abnormal pancreas shown as a horizontal bar.

Figure 4.4 - Histology of foci and assessment of apoptosis





4.4.4 SMAD4 expression within abnormal foci

The observed increase in abnormal pancreas in *Smad4*$^{+/-}$*Apc*$^{Min/+}$ mice suggests that SMAD4 may act as a tumour suppressor in normal pancreas. To investigate whether lesions arising in *Apc*$^{Min/+}$*Smad4*$^{+/-}$ pancreas had lost SMAD4 expression, immunohistochemical analysis was carried out. Cells in the majority of foci remained heterozygous for *Smad4* (Figure 4.5(a)) with strong cytoplasmic staining. Decreased cytoplasmic levels of SMAD4 expression were observed only within a minority of foci (Figure 4.5(b) and 4.5(c)) indicating that loss or downregulation of the remaining wild-type *Smad4* allele occurs rarely. Thus it appears that the increase in abnormal pancreas in the *Apc*$^{Min/+}$ *Smad4*$^{+/-}$ mice is associated almost entirely with *Smad4* haploinsufficiency. This suggests that in the presence of other mutations, loss of a single *Smad4* allele could effect a progression in tumourigenesis.

Figure 4.5 – Immunohistochemistry for SMAD4

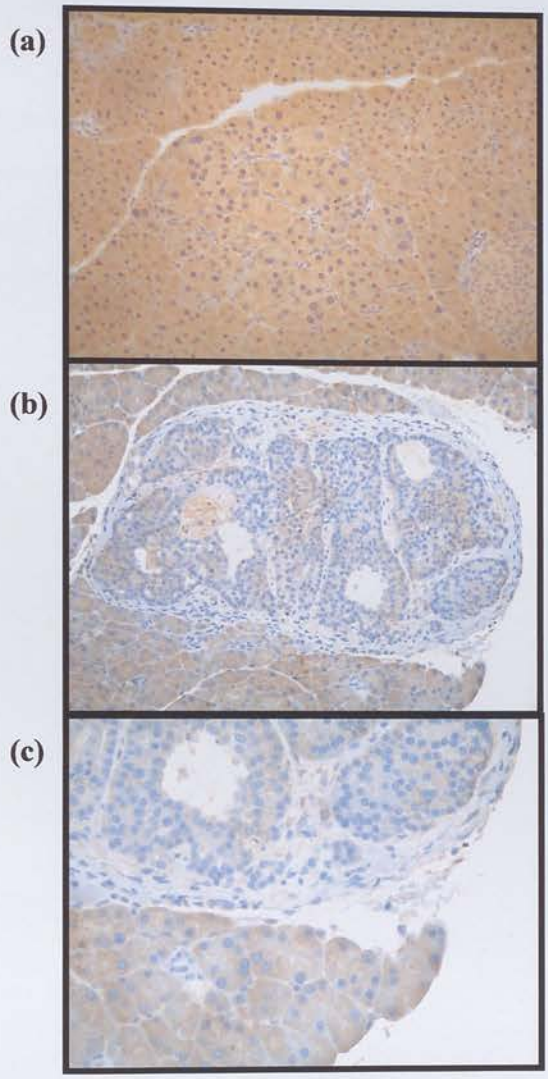


Figure 4.5 – (a) Pancreatic acinar cells from $Apc^{Min/+}Smad4^{+/-}$ mouse exhibiting similar staining for SMAD4 within and outside lesion, at X200 magnification. (b) Reduced cytoplasmic expression of SMAD4 within the lesion from $Apc^{Min/+}Smad4^{+/-}$ mouse at 200X magnification (b), and 400X magnification (c).

4.4.5 Effect of WT, *Smad4*$^{+/-}$, *Apc*$^{Min/+}$ or *Apc*$^{Min/+}$*Smad4*$^{+/-}$ genotypes on size of foci

Statistical analysis comparing focus size between genotypes revealed that abnormal foci arising in *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice were not significantly larger than those in *Smad4*$^{+/-}$, *Apc*$^{Min/+}$ or WT mice (Figure 4.6, Mann Whitney U $p>0.05$). This implies that the increase in abnormal pancreas seen in *Apc*$^{Min/+}$ *Smad4*$^{+/-}$ mice is not due to increased focus size but rather increased incidence. Mutations in both these genes therefore appear to have an additive effect on the initiation but not growth of abnormal foci causing an overall increase in percentage of abnormal pancreas.

Figure 4.6- Morphometric analysis of size of abnormal foci

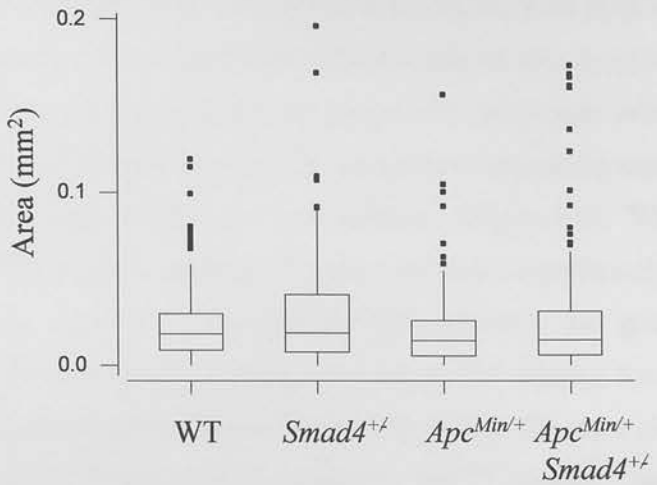


Figure 4.6 - Box plots expressing lesion size in NMU-treated WT, *Smad4*$^{+/-}$,*Apc*$^{Min/+}$ and *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice.

4.4.6 Investigation into nuclear atypia present in abnormal foci from WT, *Smad4*$^{+/-}$, *Apc*$^{Min/+}$ or *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice

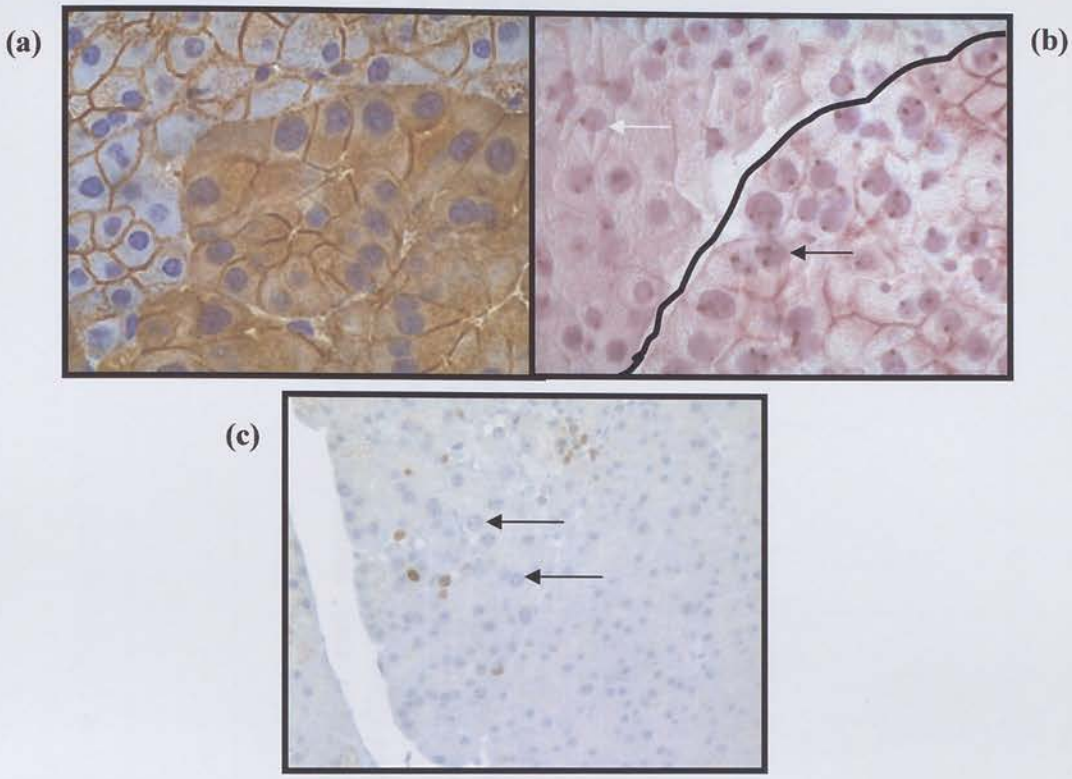
Histological analysis revealed that a subset of abnormal foci in mice of all genotypes examined contained visibly larger nuclei consistent with dysplasia (Figure 4.7(a)). To examine whether *Smad4* mutation played a role in this dysplastic phenotype, nuclear area (NA) was measured in normal acinar cells and acinar cells within abnormal foci (denoted class I, class II and class III according to increasing size, see Figure 4.8 legend and materials and methods) for each genotype (Figure 4.8). While all three classes of lesion in WT, *Smad4*$^{+/-}$ and *Apc*$^{Min/+}$ mice showed a significantly altered distribution of nuclear area compared with controls (KS, $p<0.05$), the greatest increases in NA compared to normal nuclei were observed in the smaller foci, class I and II. The distribution of NA in the larger abnormal foci (class III) more closely resembled that of normal nuclei. Abnormal foci arising in *Apc*$^{Min/+}$*Smad4*$^{+/-}$ pancreas differed in that changes in NA distribution were much less pronounced and only class I abnormal foci exhibited a significant change in NA distribution (KS, $p<0.05$).

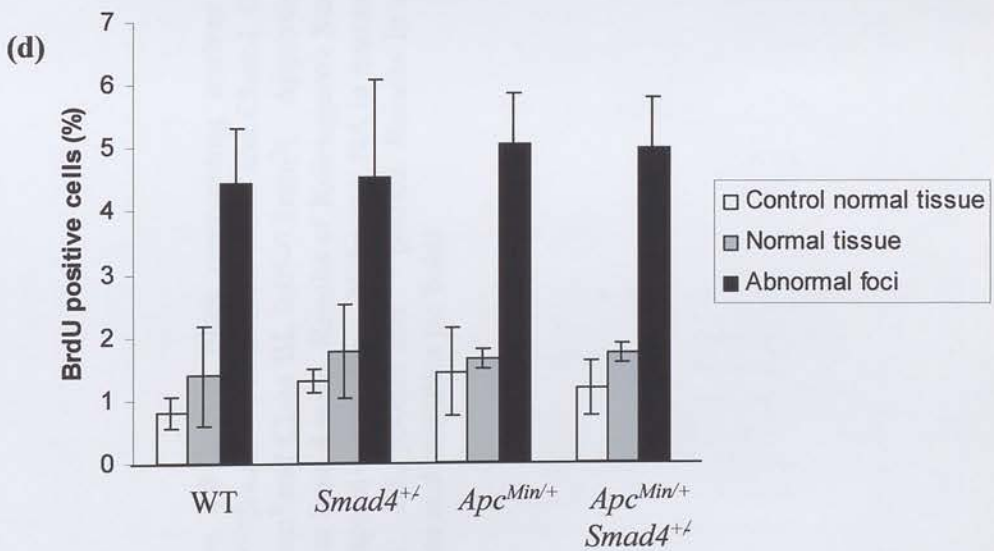
Increased NA has been shown to denote a change in DNA content consistent with genomic instability (Asamura *et al.*, 1989, Suzuki *et al.*, 2000). To investigate whether the nuclear atypia reported above was associated with increased DNA content, NMU was administered, using the same method, to WT animals hemizygous for the *β-globin* transgene *Tg(Hbb-b1)83Clo* present on chromosome 3 and detectable by DNA-DNA *in situ* hybridisation (Keighren and West, 1993). This method, previously used to identify abnormal ploidy (Keighren and West, 1993), provides a visual cue to the ploidy status of a cell. This study utilised a double labelling technique involving DNA-DNA *in situ* labelling of the *β-globin* transgene and β-catenin immunohistochemistry. Increases in the number of *β-globin* hybridisation spots were seen only in abnormal foci (Figure 4.7(b)), suggesting that in this system increased NA is associated with increased DNA content. An increase in the number of *β-globin* hybridisation spots could reflect an increased proportion of cells blocked in the G2 phase of the cell cycle due to increased cell turnover. However, this seems unlikely since *β-globin* staining was not present in

the doublets characteristic of G2 phase (Lawrence *et al.*, 1990). Furthermore, BrdU incorporation studies (Figure 4.7(c) and 4.7(d)) revealed low levels of cell turnover, insufficient to account for the increased NA seen in approximately 40% of cells within abnormal foci. Thus the increase in nuclear area in the abnormal foci is most likely due to polyploidy or aneuploidy affecting chromosome 3.

Figure 4.7– (a) β-catenin stained abnormal focus showing increased nuclear area within the lesion. (b) Double labelling of pancreas from hemizygous *β-globin* transgenic mouse using DNA-DNA *in situ* hybridisation for a *β-globin* transgene (brown punctate staining) and faint β-catenin immunohistochemistry (burgundy). The black line divides dysplastic cells (bottom right) from normal cells (top left). Each *β-globin in situ* hybridisation 'dot' represents 2n. Cells within the lesion exhibit nuclei containing 4n and 6n (arrow). (c) Representative example of BrdU staining within abnormal foci, showing atypical nuclei which are not BrdU positive (arrows). (d) Histogram showing percentage cells incorporating BrdU in non-dysplastic tissue (from uninjected 'control normal tissue' and injected mice 'normal tissue') and abnormal tissue. BrdU was delivered 24 hours prior to culling. After processing, immunohistochemistry for β-catenin and BrdU was carried out to enable scoring in both abnormal and normal pancreas. Error bars express standard error of the mean and in each case sections from a minimum of 3 mice were scored.

Figure 4.7 - Assessment of nuclear atypia





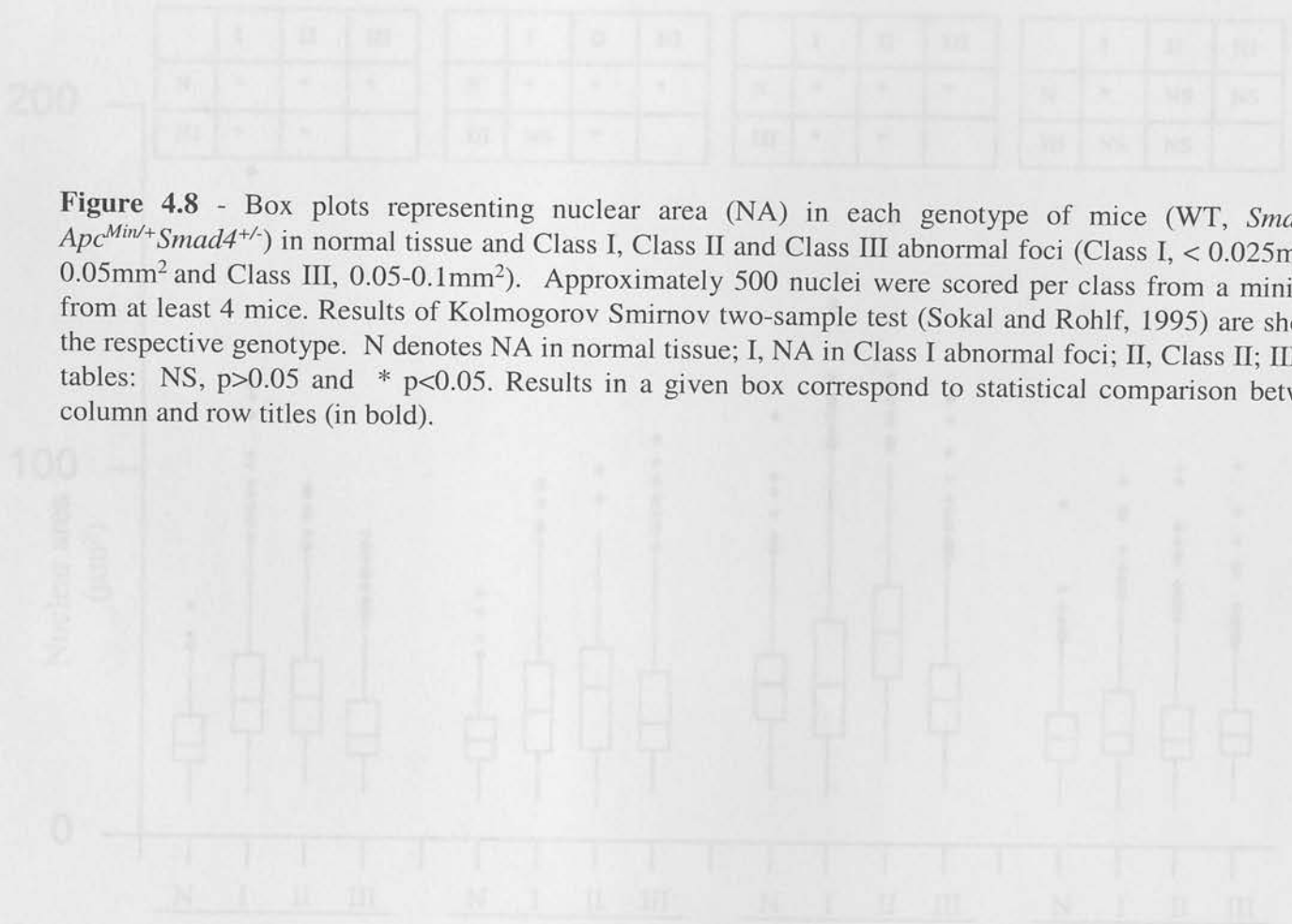
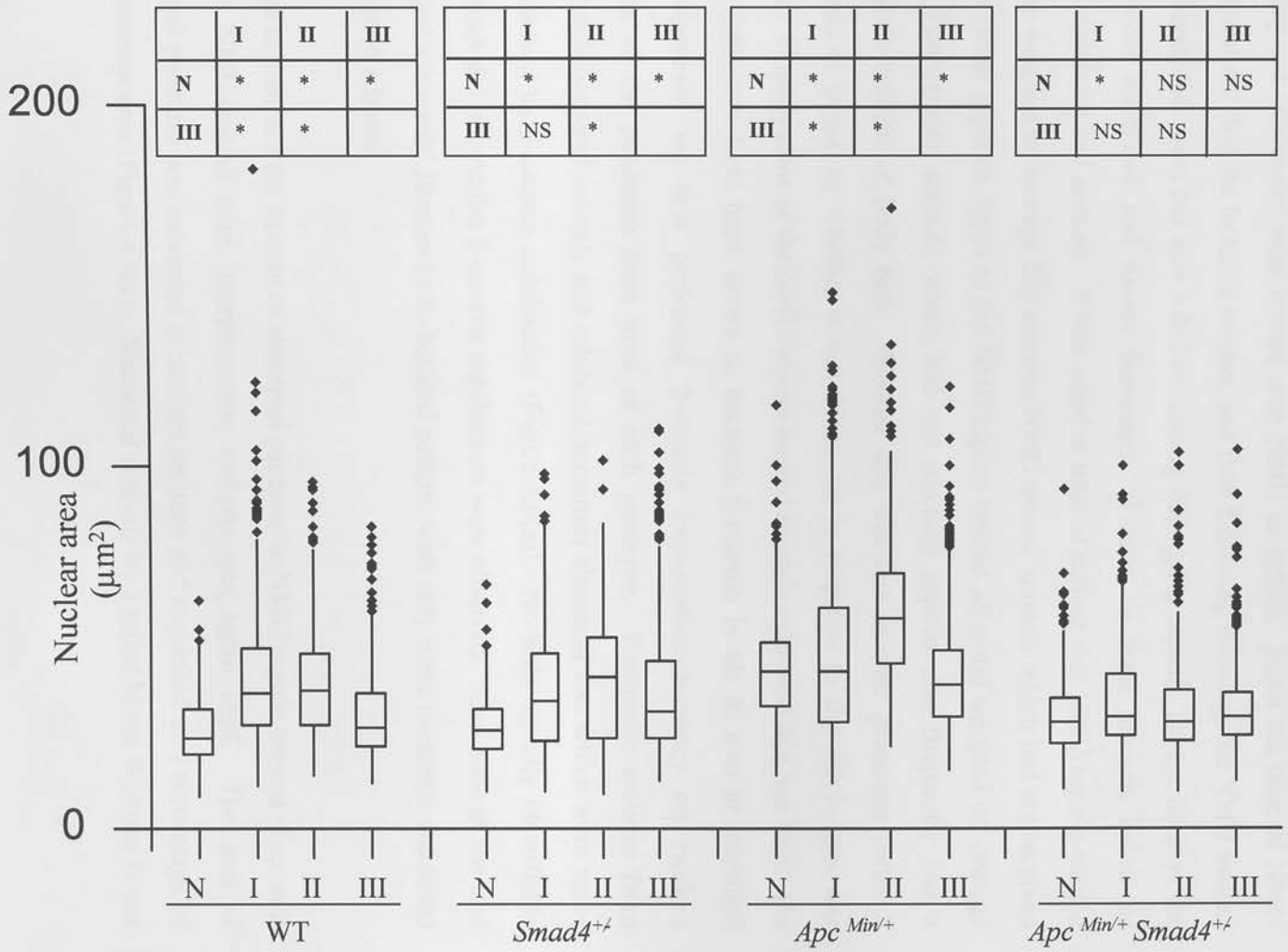


Figure 4.8 - Box plots representing nuclear area (NA) in each genotype of mice (WT, *Smad4*$^{+/-}$, *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$*Smad4*$^{+/-}$) in normal tissue and Class I, Class II and Class III abnormal foci (Class I, < 0.025mm^2, Class II, 0.025-0.05mm^2 and Class III, 0.05-0.1mm^2). Approximately 500 nuclei were scored per class from a minimum of 10 lesions, from at least 4 mice. Results of Kolmogorov Smirnov two-sample test (Sokal and Rohlf, 1995) are shown in tables above the respective genotype. N denotes NA in normal tissue; I, NA in Class I abnormal foci; II, Class II; III, Class III. Key for tables: NS, p>0.05 and * p<0.05. Results in a given box correspond to statistical comparison between the associated column and row titles (in bold).

Figure 4.8 – Morphological analysis of nuclear area and results of Kolmogorov-Smirnov test.



4.5 Effect of dietary aspirin on NMU-treated WT, *Smad4*$^{+/-}$, *Apc*$^{Min/+}$ or *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice.

4.5.1 Effect of dietary aspirin on NMU-induced pancreatic foci

Four day old littermates were treated with NMU as before. From the time of NMU administration, first the lactating mother, and then following weaning, the NMU treated mice themselves were fed with a diet containing 400mg/Kg aspirin. At age 65 days the mice were sacrificed and tissues harvested. I refer to these animals below as NMU/aspirin-treated animals. It was noted at time of culling that NMU/aspirin-treated animals weighed an average 28g whereas NMU-treated animals which had not received aspirin (from separate litters to the NMU/aspirin treated animals) weighed on average 15g. Furthermore animals which had not received aspirin also frequently lost a significant amount of body hair. Whether this was due to over grooming (often a symptom of stress) or whether it had fallen out in response to the carcinogen was unclear. Notably none of the NMU/aspirin-treated animals exhibited this hair loss. As Cox-2 inhibitors have been shown to decrease β-catenin levels *in vivo* in intestinal tumourigenesis, we first performed β-catenin immunohistochemistry on random sections of the pancreata from mice of each genotype. Pancreatic sections from NMU/aspirin-treated animals still exhibited numerous abnormal foci which were again characterised by β-catenin stabilisation (Figure 4.9(a)). No histologically identifiable foci which did not exhibit β-catenin stabilisation were observed. Also none of the foci exhibited a 'mosaic' immunohistochemical pattern, with only some β-catenin stabilised cells within a focus.

In order to compare the amount of abnormal pancreas in NMU/aspirin-treated mice with that in NMU-treated mice, morphometric analysis was again used. The area of abnormal pancreas was measured in random sections and expressed as a percentage of total pancreas area (Figure 4.9(c)). Statistical analysis by 1 tailed Mann Whitney U test

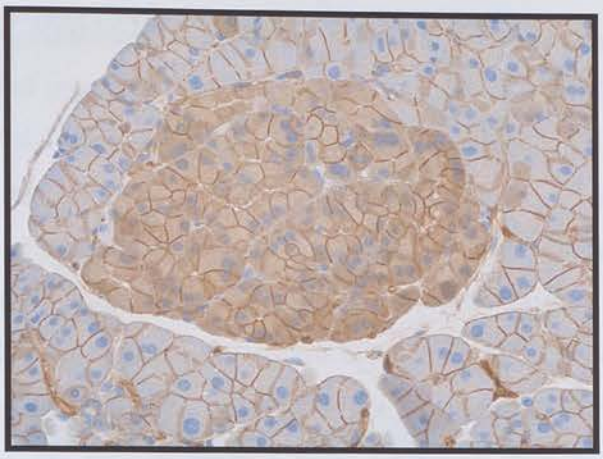
revealed that NMU/aspirin-treated $Apc^{Min/+}$ and $Apc^{Min/+}Smad4^{+/-}$ mice had significantly less abnormal pancreas than animals of the same genotype which had not received aspirin ($p=0.012$ and $p=0.0222$ respectively). Thus it appeared that aspirin treatment was associated with a reduction of abnormal pancreas in Apc^{Min} heterozygotes. In accordance with the previous work on the effect of aspirin on intestinal tumourigenesis this is suggestive of modulation of the Wnt signalling pathway by Cox-2 inhibitors.

Comparison merely within the different genotypes of NMU/aspirin-treated animals revealed no statistical difference in amount of abnormal pancreas. This is in contrast to the findings from the NMU-treated animals which had not received aspirin where $Apc^{Min/+}Smad4^{+/-}$ mice exhibited an increase in abnormal pancreas compared to WT. This suggests a mechanism whereby aspirin negates the additive effect of these mutations.

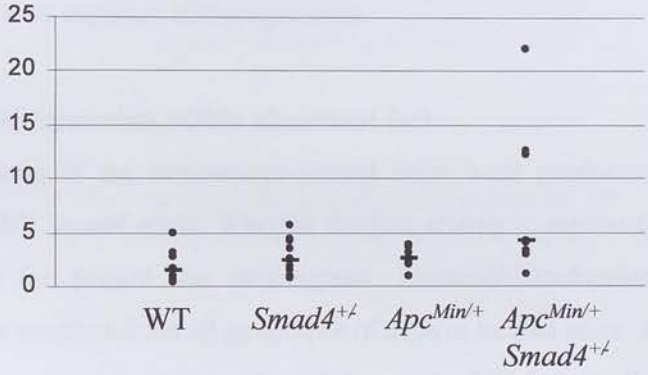
Figure 4.9 – (a) Abnormal foci from an NMU/aspirin-treated $Apc^{Min/+}Smad4^{+/-}$ mouse exhibiting β-catenin stabilisation at X400 magnification. (b) Amount abnormal (β-catenin stabilised) pancreas as measured by area and expressed as percentage of total pancreas in sections from NMU-treated WT, $Smad4^{+/-}$, $Apc^{Min/+}$, $Apc^{Min/+}Smad4^{+/-}$ mice (b), and NMU/aspirin-treated WT, $Smad4^{+/-}$, $Apc^{Min/+}$, $Apc^{Min/+}Smad4^{+/-}$ mice (c). Data are expressed as a dot plot with median percentage abnormal pancreas shown as a horizontal bar.

Figure 4.9 – Analysis of abnormal foci in NMU/aspirin- and NMU-treated mice.

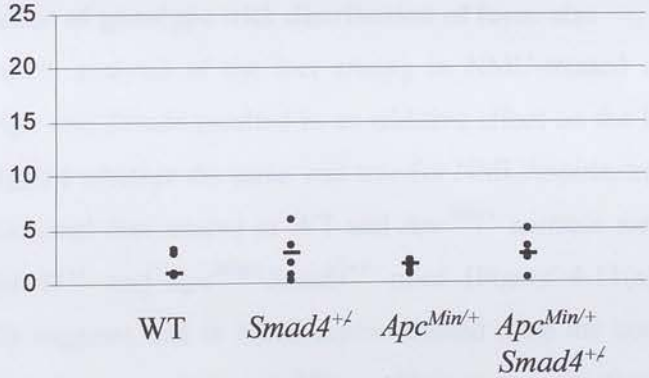
(a)



(b)



(c)



4.5.2 Effect of dietary aspirin on cell composition within abnormal foci

As detailed above, NMU-treated animals exhibited two histologically identifiable types of foci regardless of genotype. Between 10-20% of foci in the NMU-treated animals (no aspirin) exhibited the increased stroma and cystic areas phenotype. Histological analysis of sections of the pancreas from all genotypes of NMU/aspirin-treated mice showed that only foci which were entirely acinar in composition were present. This raises the possibility that aspirin could be exerting an anti-inflammatory response within the pancreas. This would be consistent with the hypothesis that foci containing cystic areas and stromal areas are caused by an inflammatory response which aspirin counteracts. Alternatively, if these foci represent a more advanced stage in tumourigenesis then aspirin appears to suppress this progression.

4.5.3 SMAD4 expression within abnormal foci

The foci arising in the non-aspirin-treated mice were predominantly found to have retained the WT *Smad4* allele. Whether the foci arising in aspirin-treated mice were also heterozygous for *Smad4* was investigated. Immunohistochemistry for SMAD4 was carried out on sections from all genotypes of aspirin treated mice. All foci retained some SMAD4 expression consistent with retention of the WT *Smad4* allele (Figure 4.10).

4.5.4 Association of genotype with distribution of focus size

As morphometric analysis of the foci arising in NMU-treated animals revealed that mutation of *Apc* and *Smad4* resulted in an additive effect on the initiation of abnormal foci, I investigated whether the same was true for NMU/aspirin-treated animals. It was found that abnormal foci arising in WT and $Apc^{Min/+}$ animals were smaller than those arising in $Smad4^{+/-}$ and $Apc^{Min/+}Smad4^{+/-}$ mice (Figure 4.11(a), Mann Whitney U $p<0.05$). This suggests that in NMU/aspirin-treated mice the combination of $Apc^{Min/+}$ and $Smad4^{+/-}$ does not result in an additive effect on the initiation of abnormal foci, in contrast to the findings on mice which had not received aspirin. Thus it appears that aspirin could be causing an inhibitory effect on the initiation of foci in $Apc^{Min/+}Smad4^{+/-}$ animals.

Figure 4.10 – Immunohistochemistry for SMAD4 on NMU/aspirin-treated abnormal pancreas.

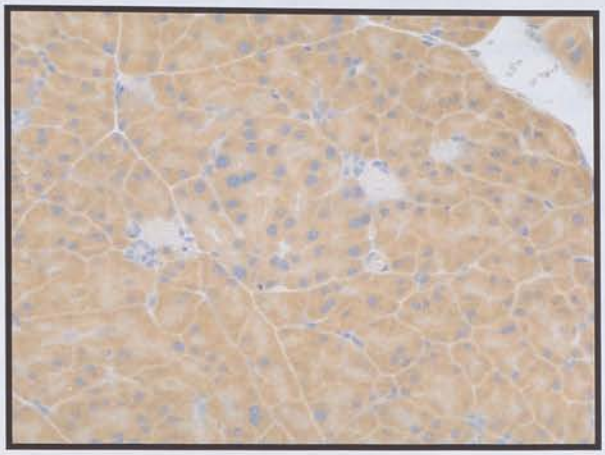


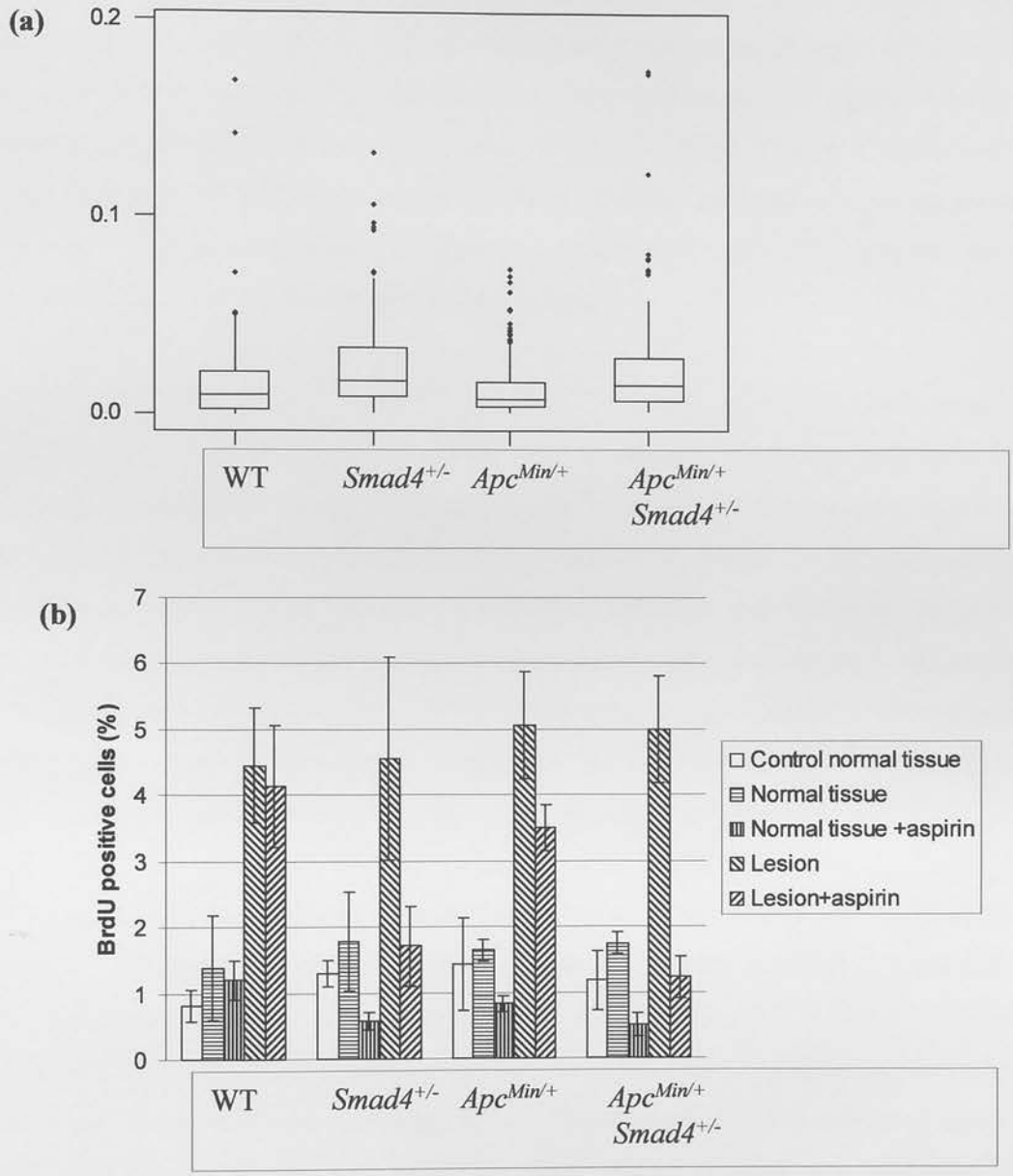
Figure 4.10 – Pancreas from NMU/aspirin-treated $Apc^{Min/+}Smad4^{+/-}$ mouse at X400 magnification showing similar staining within and outside abnormal foci.

4.5.5 Effect of dietary aspirin on cell turnover within abnormal foci

As Cox-2 inhibitors are thought to reduce cell turnover it was hypothesised that the reduction in abnormal foci seen in $Apc^{Min/+}$ and $Apc^{Min/+}Smad4^{+/-}$ mice could be a consequence of reduced cell turnover. To test this, the percentage of cells incorporating BrdU was scored in normal pancreas from control animals (no NMU and no aspirin) and in normal tissue and abnormal foci from NMU/aspirin-treated and NMU-treated mice (Figure 4.11(b)). In WT mice comparison of BrdU incorporation in abnormal foci from NMU/aspirin-treated and NMU-treated mice revealed no difference, however aspirin-treated $Apc^{Min/+}$ and $Apc^{Min/+}Smad4^{+/-}$ mice both exhibited a significant decrease in BrdU incorporation (Mann Whitney U, $p<0.05$). The difference was not significant at the 5% level in $Smad4^{+/-}$ mice (Mann Whitney U, $p=0.108$). Therefore both $Apc^{Min/+}$ and $Apc^{Min/+}Smad4^{+/-}$ mice exhibited a significant decrease in abnormal pancreas after aspirin treatment, suggesting that this is due to reduced cell turnover.

Figure 4.11 – (a) Box plot representing the distribution of foci sizes in NMU/aspirin treated WT, *Smad4*$^{+/-}$, *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$*Smad4*$^{+/-}$ mice. (b) Histogram showing percentage cells incorporating BrdU in non-dysplatic tissue (from uninjected mice 'control normal tissue', NMU-treated and NMU/aspirin-treated mice 'normal tissue' and 'normal tissue+aspirin' respectively) and in abnormal tissue from NMU-treated and NMU/aspirin-treated mice. Error bars express standard error of the mean and in each case sections from a minimum of 3 mice were scored.

Figure 4.11 – Distribution of abnormal foci sizes and analysis of BrdU incorporation studies.



4.6 Conclusions and discussion

In summary, loss of *p53* in an NMU-induced pancreatic tumourigenesis does not appear to increase overall amounts of abnormal pancreas but leads to the development of abnormal foci. Foci arising in $p53^{-/-}$ mice are smaller than those arising in WT mice and have an altered composistion. Furthermore, whilst nuclear atypia is seen in mice of both genotypes it is significantly increased in the $p53^{-/-}$ mice. Thus it appears that the foci arising on a $p53^{-/-}$ background follow a different pathway of tumourigenesis. Whether the foci on the *p53* deficient background reflect a progression of tumourigenesis has not been addressed in this study.

Treatment of WT, $Smad4^{+/-}$, $Apc^{Min/+}$ and $Apc^{Min/+}Smad4^{+/-}$ mice with NMU results in the development of abnormal foci in pancreatic acinar cells which are characterised by β-catenin stabilisation. Only NMU-treated $Apc^{Min/+}Smad4^{+/-}$ mice exhibit a significant increase in amount of abnormal pancreas, associated predominantly with *Smad4* haploinsufficiency. This increase in abnormal pancreas was found to be due to increased number of foci rather than increased focus size. The data also showed that smaller lesions are characterised by morphological nuclear atypia. These studies demonstrate co-operation between *Smad4* and *Apc* mutation and provide a system to investigate the chemopreventative effect of the NSAID, aspirin.

Comparison of the amount of abnormal pancreas in NMU-treated and NMU/aspirin-treated WT, $Smad4^{+/-}$, $Apc^{Min/+}$ and $Apc^{Min/+}Smad4^{+/-}$ mice revealed a reduction in abnormal pancreas in the NMU/aspirin-treated $Apc^{Min/+}$ and $Apc^{Min/+}Smad4^{+/-}$ animals. No obvious reduction of β-catenin levels was observed within the abnormal foci which persisted in the NMU/aspirin-treated mice. However aspirin did appear to effect a reduction in BrdU incorporation within the abnormal foci. Furthermore the altered foci phenotype of increased stroma and cystic areas seen in the NMU-treated mice was completely absent from the NMU/aspirin-treated mice.

β-catenin stabilisation was present in foci arising in NMU-treated WT, $p53^{-/-}$, $Smad4^{+/-}$, $Apc^{Min/+}$ and $Apc^{Min/+}$ $Smad4^{+/-}$ pancreas. As mentioned above, β-catenin stabilisation is also characteristic of preneoplastic pancreatic lesions which arise spontaneously in $Apc^{Min/+}$ mice. This suggests that by whatever mechanism dysregulation of the Wnt pathway is an early event in murine pancreatic acinar cell tumourigenesis. In contrast to this is the finding that APC is dysregulated rarely in human pancreatic ductal cancers (Horii *et al.*, 1992; McKie *et al.*, 1993). This deregulation of the Wnt signalling pathway is either associated more closely with the murine pancreas than in human pancreas, or perhaps other mechanisms of Wnt deregulation other than *APC* mutation occur in the human pancreas. Examples of these other mechanism include mutation of *β-catenin*, reduced levels of the frizzled homologue protein (FRP) and biallelic inactivation of *Axin* (see Figure 1.6, Morin *et al.*, 1997; Zhou *et al.*, 1998; Satoh *et al.*, 2000)

Various studies have suggested possible co-operation between dysregulated Wnt signalling and other mutations in the promotion of tumourigenesis. Interestingly, c-Myc a protein required for G1/S transition and a target of the Wnt signalling pathway, has been shown to co-operate with loss of *p53* to promote the survival of cells with severe DNA damage (Yin *et al.*, 1999). A diploid myeloid cell line was simultaneously depleted of p53 while *c-Myc* was overexpressed, the result of which was spontaneous development of tetraploid cells. The restoration of p53 in the *c-Myc* overexpressing cells resulted in apoptosis. Another study associating *p53* loss and dysregulated Wnt signalling in the promotion of mammary tumourigenesis was published by Donehower *et al.* (1995) (discussed in chapter 3, section 3.12).

NMU-induced pancreatic tumourigenesis in $Smad4^{+/-}$, $Apc^{Min/+}$ or $Apc^{Min/+}Smad4^{+/-}$ mice demonstrated that Apc^{Min} and *Smad4* mutations co-operate in the promotion of tumourigenesis. Takaku *et al.* (1998) analysed intestinal adenomas occurring in $Apc^{\Delta 716/+}Smad4^{+/-}$ mice and found a decrease in polyp number, but an increase in polyp size and frequency of invasive adenocarcinomas when compared to *Apc* heterozygotes.

This work is therefore in contrast to that of Takaku *et al.* and may be indicative of different pathways of tumourigenesis in the pancreas and intestine, affecting different target genes. An example of such tissue-specific differences include SMAD4-independent inactivation of TGF-β signalling (through inactivation of SMAD2 or TGF-β type II receptors), which occurs more often in colorectal tumourigenesis (Markowitz *et al.*, 1995; Takagi *et al.*, 1998). Finally, the absence of pancreatic neoplasia in the $Apc^{\Delta 716/+}Smad4^{+/-}$ mice could be because mutation in genes other than *Apc* or *Smad4* is necessary to initiate pancreatic tumourigenesis. Such genes could be targeted by NMU in this study. In humans, oncogenic *K-RAS* activation is thought to be a prerequisite for pancreatic tumourigenesis (Rozenblum *et al.*, 1997; Almoquera *et al.*, 1988). Notably N-nitroso carcinogens have been shown to induce activating mutation in *K-ras* in mice (Corominas *et al.*, 1991).

It was also found that the co-operative effect of *Apc* and *Smad4* mutation resulting in increased abnormal pancreas was associated with haploinsufficiency for *Smad4* rather than LOH. Others have also reported that haploinsufficiency for *Smad4* is sufficient for the promotion of tumourigenesis in the murine intestine, with loss of the remaining WT allele detected only at later stages of tumour progression (Xu *et al.*, 2000). In $p53^{-/-}$ animals overexpressing TGF-α, LOH for *Smad4* is associated only with late stage pancreatic adenocarcinomas (Wagner *et al.*, 2001). Taken together these works support the hypothesis that haploid insufficiency of *Smad4* may be sufficient for tumour initiation and that loss of even a single allele can have an effect on cell behaviour.

Investigation into the nuclear atypia present in the WT, $Apc^{Min/+}$ and $Smad4^{+/-}$ animals and to a lesser degree in the $Apc^{Min/+}Smad4^{+/-}$ mice revealed that the nuclear atypia present did reflect a change in DNA content of nuclei within abnormal foci. However since the greatest increases in NA were not associated with either the largest lesions (class III) or the most predisposing genotype ($Apc^{Min/+}Smad4^{+/-}$), these results suggest that the processes leading to nuclear atypia and increased focus size may be independent. Although unlikely, it is possible that genomic instability is required in the

less predisposing genotypes for lesion initiation, but is incompatible with continued progression.

The administration of dietary aspirin revealed that in carcinogen-induced pancreatic tumourigenesis aspirin does have a chemopreventative effect. However this effect appears predominantly to relieve the effects of *Apc* mutation rather than *Smad4* mutation. This is evidenced by the fact that reduction in abnormal pancreas following aspirin treatment was associated only with the $Apc^{Min/+}$ and the $Apc^{Min/+}Smad4^{+/-}$ genotypes. In the absence of aspirin, $Apc^{Min/+}$and $Smad4^{+/-}$ mutation in conjunction causes an additive increase in abnormal pancreas, an effect not evidenced when aspirin has been administered. Thus it appears that in this system aspirin somehow modulates the effect of *Apc* mutation. The work here has not provided any evidence suggesting that aspirin can modulate SMAD-mediated TGF-β signalling. Previous work has highlighted a role for NSAIDs in the downregulation of β-catenin levels as one of the molecular mechanism for suppression of tumourigenesis by Cox-2 inhibitors. The immunohistochemical studies of this work revealed no modulation of β-catenin levels.

The results presented here suggest that aspirin mediates its suppression of tumourigenesis through three methods: the reduction of cell turnover; the inhibition of abnormal focus initiation and the suppression of the stromal and cystic areas phenotype. It is unclear whether the weight gain observed in NMU/aspirin-treated animals was symptomatic of, or contributory to, the effects detailed above. The reduction of cell turnover and inhibition of focus initiation in NMU/aspirin-treated mice appears to be associated with the $Apc^{Min/+}$ genotype, suggesting again that these cell behaviours are under the control of the Wnt signalling pathway. Cox-2 is a transcriptional target of Wnt signalling (Howe *et al.*, 1999; 2001) explaining in part the association of tumour suppression by aspirin with *Apc* mutation. However it cannot be ruled out that aspirin is modulating the effect of *Apc* deficiency on some of the non-Wnt related functions of APC (see Chapter 1, section 1.8.2). Others have reported an induction of apoptosis in

response to NSAID treatment (McEntee *et al.*, 1999; Ding *et al.*, 2000), but this was not found in the present study.

Assuming that the altered subset of foci (containing stromal and cystic areas) represent a progression of tumourigenesis, aspirin appears to suppress this progression. This is of particular interest in the context of *Smad4* mutation, as defective TGF-β signalling has been shown to result in neoangiogenesis and fibrosis in the stromal compartment of the pancreas (Bottinger *et al.*, 1997). However this appears not to be the situation here as the effect of aspirin was found in animals of all genotypes. It is suggested that these altered foci represent an inflammatory response amongst those cells, as shown by the infiltrating fibroblasts, cystic areas and increased apoptosis. It could therefore be argued that the fact that aspirin prevents this type of focus supports the idea that the altered composition of these foci is due to an inflammatory response. Cox-2 inhibitors have been shown to decrease intestinal tumourigenesis in $Apc^{Min/+}$ mice, but Cox-2 expression in the murine intestine is restricted to the non-neoplastic stromal cells (Oshima *et al.*, 1996; Shattuck-Brandt *et al.*, 1999). This highlights the fact that Cox-2 is not the only target of NSAIDs in the intestine. For example, He *et al.* (1999) identified a target of Wnt signalling, PPARδ (peroxisome proliferator-activated receptor δ, usually downregulated by APC regulation of Wnt signalling), which was downregulated in the absence of APC by the NSAID and Cox-2 inhibitor, sulindac. It is suggested therefore that this general non-genotype dependent effect of aspirin is due to Cox-2 inhibition and reduction of an inflammatory response, but that Cox-2 inhibition is not the only mechanism whereby aspirin suppresses tumourigenesis.

In summary, therefore, work involving NMU-induced murine pancreatic tumourigenesis revealed that mutation in the tumour suppressors *p53*, *Apc* and *Smad4* each play a role in the promotion of tumourigenesis. *p53* mutation predisposes the animals to the increased initiation of foci associated with an altered pathway of tumourigenesis and nuclear atypia. Work combining *Apc* and *Smad4* mutation revealed a cooperation between these two mutations in the promotion of tumourigenesis. Treatment with

dietary aspirin suppressed tumourigenesis predominantly in mice carrying mutation of the *Apc* gene.

4.7 Future work

As with any carcinogen-induced tumourigenesis the exact mutational status of other genes is largely unknown therefore further work investigating the status of *K-ras* would be informative regarding the actual action of NMU in this system. Also, given the association of *K-RAS* mutation with human pancreatic cancers, if *K-ras* is mutated in this system, it would be informative to investigate the association of spontaneous *K-ras* mutation with the germline mutations present in the animals used here. It has recently been shown that a member of the TGF-β signalling family, PTGF-β, is a target of p53-mediated gene expression and that PTGF-β can induce the suppression of tumour cell growth in a SMAD4-dependent pathway. Thus it would be of interest to assess the effect of combined *p53* and *Smad4* mutation in this system. Finally further investigation into the status of molecular targets of the Wnt signalling pathway (for example *c-Myc*, *Cyclin D1* and *Cox-2*) would provide insight not only into the phenomenon of β-catenin stabilisation, even in NMU-treated WT animals, but also into the possible action of aspirin in the suppression of tumourigenesis.

Chapter 5 – *In vitro* analysis of TGF-β superfamily signalling in the pancreas.

5.1 Introduction

5.1.1 Primary culture of pancreatic acinar cells

An overview of TGF-β signalling under normal and dysregulated conditions is outlined in Chapter 1, section 1.5. Also of particular relevance to this chapter is section 1.2.3 on acinar to ductal transdifferentiation in the pancreas. *In vivo* investigations into acinar to ductal transdifferentiation provide researchers with 'snap shots' of a dynamic process involving a mixed cell population. In addition to this embryonic pancreatic explants have been cultured *in vitro* (Parsa *et al.*, 1980). These have provided a tool whereby researchers could monitor the differentiation process as it occurs but utilised a mixed cell population where it is relatively difficult to distinguish between undifferentiated precursor cells and ductal cells (Richardson and Spooner, 1977). As the real question concerns whether adult acinar or ductal cells can transdifferentiate, the use of embryonic cells is somewhat limited. This study therefore employed a technique whereby acinar cells are isolated from adult mice, thereby allowing the specific investigation of acinar cell differentiation in the adult pancreas. The cells can be monitored continuously and, vitally, the conditions in which the cells are maintained can be defined.

Human pancreatic cancer is predominantly ductal in composition, however focal acinar cell dysplasia has been described in the human pancreas (Longnecker *et al.*, 1980). Whilst murine pancreatic tumourigenesis is predominantly acinar in composition, work with several mouse models has suggested that mutations originating within acinar cells can result in premalignant lesions consisting of duct-like cells. For example, overexpression of TGF-α within acinar cells results in premalignant lesions composed of duct-like cells. Tumour development is accelerated when these animals are crossbred with $p53^{-/-}$ mice (Wagner *et al.*, 2001).

Also, transgenic mice overexpressing *c-Myc* within murine pancreatic acinar cells developed tumours with a ductal phenotype (Sandgren *et al.*, 1991). Third, expression of a dominant negative TGF-βRII in murine pancreatic acinar cells resulted in ductal transformation, neoangiogenesis, fibrosis and adipose replacement of acini (Bottinger *et al.*, 1997). Thus murine acinar cells are of use in the ongoing investigations into the molecular and biological changes that occur in the human pancreas.

Previous work on has shown that the acinar cells of the exocrine pancreas have a low rate of mitosis (Solomon *et al.*, 1981), but that TGF-β can inhibit the growth of primary pancreatic acinar cells (Logsdon *et al.*, 1992). TGF-β has also been shown to inhibit the proliferation of pancreatic duct cells (Bhattaharyya *et al.*, 1995). EGF and CCK are trophic factors for pancreatic acinar cells and both of these factors are thought to act by stimulating the MAPK signalling pathway (Logsdon, 1986; Dabrowski *et al.*, 1997). Simeone *et al.* (2001) demonstrated integration of MAPK and SMAD signalling in primary cultured rat acinar cells upon stimulation of the cells with TGF-β.

5.1.2 Questions addressed in this study

TGF-β has been shown to have conflicting effects in pancreatic tumourigenesis in humans, both promoting and suppressing tumourigenesis. *SMAD4* inactivation is an important step in the development of human pancreatic cancer (Hahn *et al.*, 1996). Work with cell lines has suggested that the biological effect of *Smad4* mutation is indeed loss of responsiveness to TGF-β induced growth arrest (Grau *et al.*, 1997; Villanueva *et al.*, 1998). My work outlined in chapters 3 and 4, and that of others, highlights the fact that murine pancreatic tumourigenesis is also associated with *Smad4* mutation. However the *in vivo* studies described in chapters 3 and 4 did not fully address either the molecular changes occurring following *Smad4* mutation or LOH or the changes in cell behaviours as a consequence of *Smad 4* mutation. As TGF-β can affect a wide variety of cellular responses that are further dependent on cell type, investigation into the intracellular signalling pathways through which TGF-β mediates its cellular actions specifically in normal pancreatic cells is necessary.

Therefore to further investigate the effect of TGF-β signalling on the pancreas, I utilised a primary tissue culture system whereby the pancreas was broken up into its acinar subunits and cultured *in vitro*. In order to assess the biological effect of the *Smad4* LOH seen in pancreas from $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ mice, the effect of TGF-β signalling on pancreatic acinar cells in the presence and absence of SMAD4 was investigated. To this end the effect of TGF-β on WT and $Smad4^{-/-}$ primary murine pancreatic acinar cells was investigated. As $Smad4^{-/-}$ animals die as embryos, I wished to generate $Smad4^{-/-}$ acinar cells *in vitro* by mating $Smad4^{+/-}$ mice with mice carrying a conditional allele of *Smad4* ($Smad4^{fl/+}$ mice). The acinar cells were to be cultured as before and then Cre-*LoxP* technology was to be utilised to remove the remaining conditionally targeted *Smad4* allele. These $Smad4^{-/-}$ cells were then to be used to determine to what extent the effect of TGF-β on murine acinar cells was mediated by SMAD4. For reasons detailed in appendix B I was unable to generate $Smad4^{-/-}$ primary acinar cells and therefore this chapter is entirely concerned with the effect of TGF-β on WT and $Smad4^{+/-}$ acinar cells.

5.2 Specific aims

To investigate the effect of TGF-β on primary murine pancreatic acinar cell culture.

To use Cre-*LoxP* technology to create *Smad4*$^{-/-}$ primary murine acinar cell culture (see appendix B).

To compare the effect of TGF-β administration on *Smad4*$^{-/-}$ acinar cells and WT acinar cells.

5.3 The effect of TGF-β on freshly prepared acini – day 0 – day 4 of culture.

5.3.1 Overview of culture method

Two main areas of investigation into the effect of TGF-β treatment were pursued using primary culture of murine pancreatic acinar cells. First, the effect of TGF-β on freshly prepared acini in serum-containing medium as the cells attached to collagen IV-coated slides (section 5.3) and, second, the effect of TGF-β on cells in extended cultured which had already attached to the cell culture slides maintained in serum free (or low serum) medium (section 5.4). Figure 5.1 details these two stages of culture (early – day 0 to day 4 of culture and late – day 5 onwards) and summarises the techniques used to culture the acinar cells. The culture conditions already established by Dr N Sphyris dictated that acini be plated with 15% foetal calf serum (FCS) on collagen IV-coated Nunc sonicseal slides. Due to variations in the dissociation of the pancreas resulting in clumps containing different numbers of acinar cells it should be noted that in this system seeding densities cannot be accurately controlled. Once in culture these acini, each containing numerous acinar cells, attach to the surface of the chamber slide and gradually the clump of cells begins to flatten out revealing colonies of acinar cells in a non-confluent monolayer. The cells contained within the clumps maintain differentiation features for longer and are difficult to visualise by standard light microscopy. Therefore, for most of the investigations outlined in 5.4 analysis was restricted to monolayer areas.

Figure 5.1 – Schematic diagram of method used to culture murine primary pancreatic acinar cells.

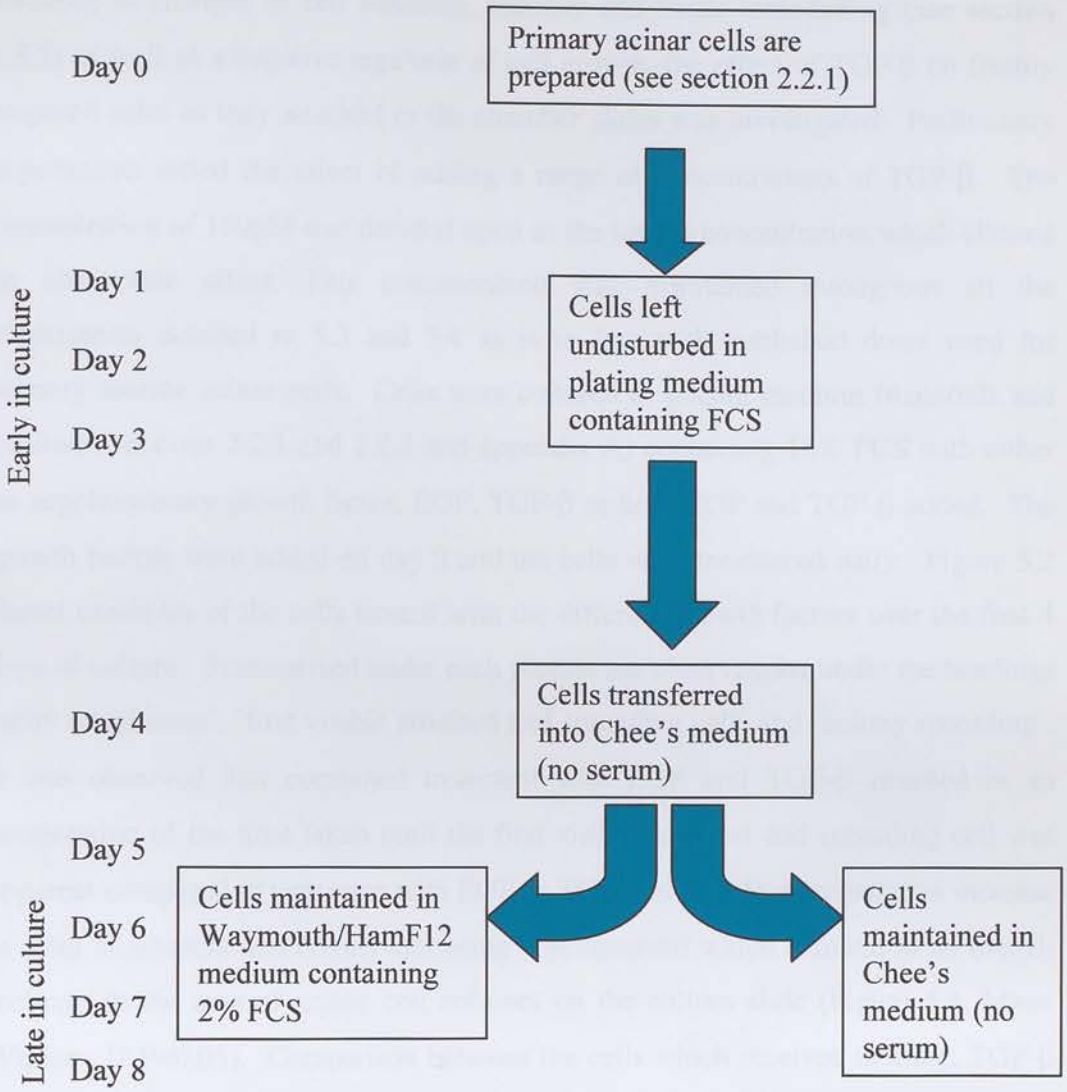


Figure 5.1 – Acinar cells are prepared as detailed in the materials and methods (2.2.1) on day 0. The cells are then left undisturbed in an incubator (37°C, 5% CO_2) until day 4. Regardless of whether the cells will eventually be cultured in Chee's or Waymouth/HamF12 media, the acinar cells spend at least 24hrs in Chee's medium to reduce fibroblast contamination. The cells are then maintained in either Chee's medium or Waymouth/HamF12 medium with the appropriate growth factors. The two main investigations outlined in this chapter are the effect of TGF-β early in culture (day 0 – day 4) and the effect of TGF-β late in culture (day 4 – day 8).

5.3.2 Effect of TGF-β on freshly prepared acini

As TGF-β has been reported to be involved in extracellular matrix deposition resulting in changes in cell adhesion, motility and tissue remodelling (see section 1.5.5) as well as a negative regulator of cell growth, the effect of TGF-β on freshly prepared acini as they attached to the chamber slides was investigated. Preliminary experiments tested the effect of adding a range of concentrations of TGF-β. The concentration of 160pM was decided upon as the lowest concentration which elicited an observable effect. This concentration was maintained throughout all the experiments detailed in 5.3 and 5.4 as is in line with published doses used for primary murine acinar cells. Cells were cultured in plating medium (materials and methods, sections 2.2.1 and 2.2.3 and appendix A) containing 15% FCS with either no supplementary growth factor, EGF, TGF-β or both EGF and TGF-β added. The growth factors were added on day 0 and the cells were monitored daily. Figure 5.2 shows examples of the cells treated with the different growth factors over the first 4 days of culture. Summarised under each picture are observations under the headings 'acini attachment', 'first visible attached and spreading cell' and 'colony spreading'. It was observed that combined treatment with EGF and TGF-β resulted in an acceleration of the time taken until the first visible attached and spreading cell was apparent compared to treatment with EGF or TGF-β alone. Furthermore, an increase in acini attachment and colony spreading was observed which resulted in an overall increase in the area of acinar cell colonies on the culture slide (Figure 5.3, Mann Whitney U $P<0.05$). Comparison between the cells which received nothing, TGF-β or EGF alone revealed that the TGF-β treated cells also exhibited an overall increase in the area of acinar cell colonies (Figure 5.3, Mann Whitney U $p<0.05$).

Figure 5.2 – (a-d) Acini on day 1 of culture grown in 15% plating medium with (a) nothing added, (b) EGF added, (c) TGF-β added and (d) EGF and TGF-β added. (e-h) Acini on day 2 of culture grown in 15% plating medium with (e) nothing added, (f) EGF added, (g) TGF-β added and (h) EGF and TGF-β added. (i-l) Acini on Day 3 of culture grown in 15% plating medium with (i) nothing added, (j) EGF added, (k) TGF-β added and (l) EGF and TGF-β added. (m-p) Acini on Day 4 of culture grown in 15% plating medium with (m) nothing added, (n) EGF added, (o) TGF-β added and (p) EGF and TGF-β added. Images were taken using a digital camera. A calibration slide was used to produce bars which represent 100μm.

Key for annotation:

+/- - little

\+ - some

++ - moderate

+++ - good

++++ - very good

Figure 5.2 – Effect of EGF and TGF-β on newly prepared pancreatic acini.

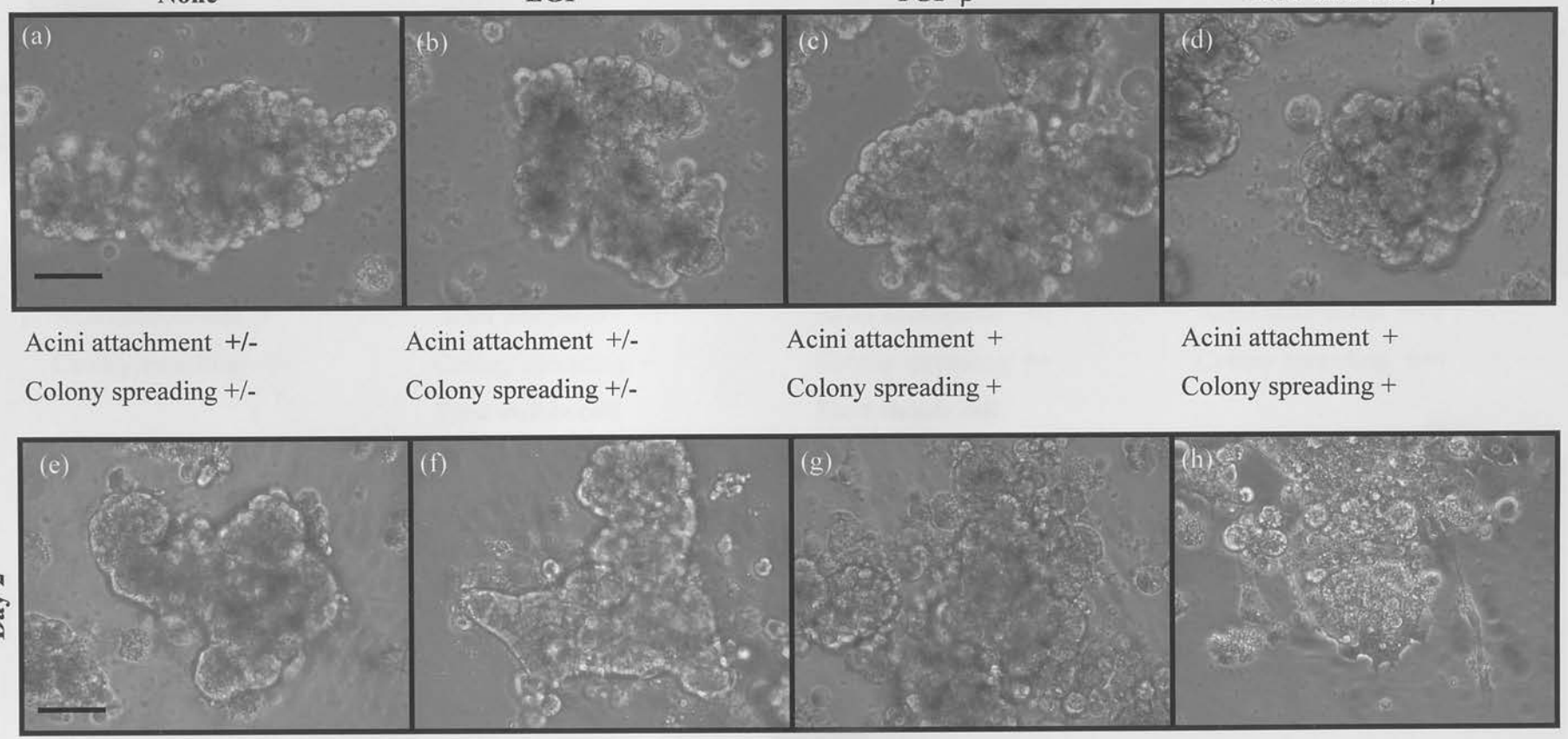
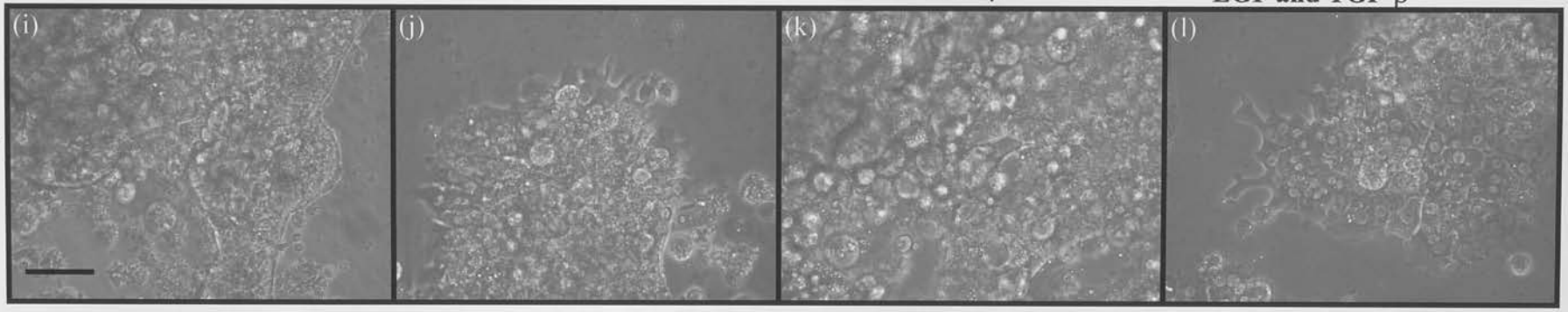


Figure 5.2 – Effect of EGF and TGF-β on newly prepared pancreatic acini.

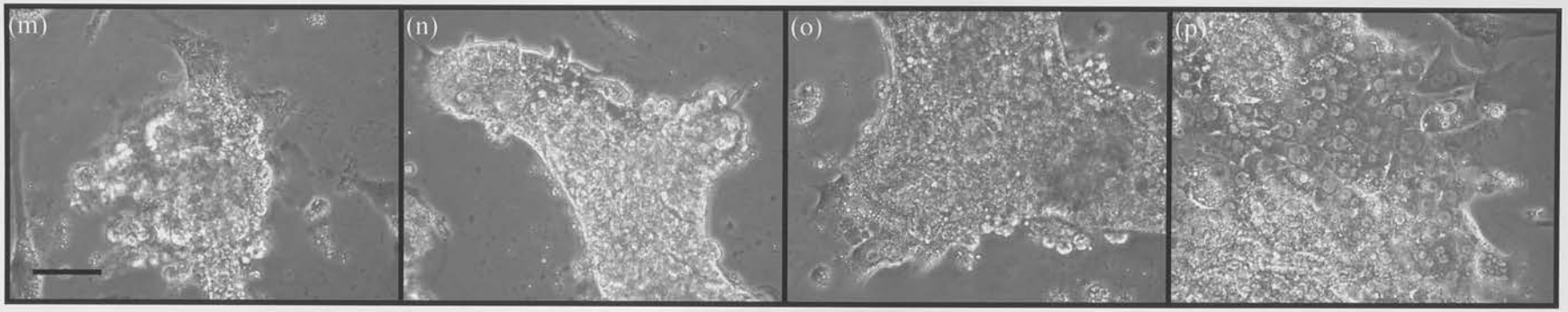


Acini attachment +
Colony spreading +/-

Acini attachment ++
Colony spreading +
First visible cell

Acini attachment ++
Colony spreading ++
First visible cell

Acini attachment +++
Colony spreading +++



Acini attachment +
Colony spreading +
First visible cell

Acini attachment ++
Colony spreading ++

Acini attachment ++
Colony spreading +++

Acini attachment +++
Colony spreading ++++

Figure 5.3 – Area of acinar cell colonies on day 4 of culture after treatment with EGF and TGF-β.

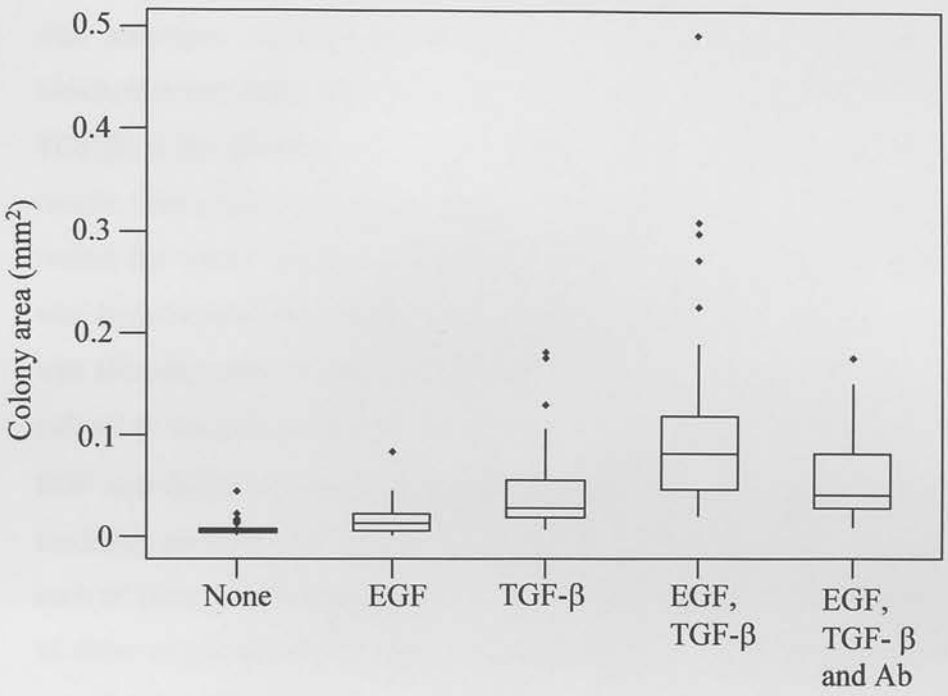


Figure 5.3 – For each condition the area of 40 colonies chosen randomly were measured using the HOME microscope. Cells were grown in plating medium containing 15% FCS with no additional growth factors, EGF, TGF-β, EGF and TGF-β or EGF and TGF-β with neutralising antibody. Following methanol fixation, cells were H+E stained to allow visualisation of colonies under light microscopy.

This surprising result of TGF-β in conjunction with EGF causing increased attachment of acinar cells and increased colony size was reproducible (n=12) and even occurs if the TGF-β is added after 24 hours of culture. A number of hypotheses were suggested to account for this result. First, that the effect was a result of synergy between TGF-β and EGF signalling as has been shown by others (Stoltz and Michalopoulos, 1997 discussed later). Second, that the addition of TGF-β with EGF was somehow causing an increase in proliferation resulting not in increased attachment but rather increased growth of those cells which did attach. Third, that TGF-β, in the presence or absence of EGF, was causing the cells to become more motile, thus acinar cells which attached were more able to move allowing more cells within the whole acinus to contact the chamber slide surface and attach. Fourth, it was hypothesised that TGF-β was inducing extracellular matrix production which was allowing cells to attach more readily to the culture slide surface. Fifth, that the culture of the cells with 15% FCS was indirectly affecting the action of TGF-β and EGF and the observations in Figures 5.2 and 5.3 were not directly due to TGF-β-mediated response but that of growth factors present in the serum. Lastly, whilst each of these possibilities could act in isolation it was also possible that two or more of them might be acting together to produce the increased attachment and colony area that was observed.

5.3.3 Control investigations of TGF-β and EGF treated acini

As the acinar cells which exhibited increased attachment and colony size had been cultured in 15% FCS, I wished first to address how much of this effect was dependent on the various nutrients present in the serum. Comparisons were made between cells cultured in 15% serum and 2.5% serum. As shown in Figure 5.4 (a-d), cells cultured with 2.5% serum containing EGF and TGF-β also exhibit increased attachment and colony size in comparison to those cultured in 2.5% serum with only EGF. Comparison between acini cultured in 15% FCS or 2.5% FCS showed that the acini attach and spread better in 15% serum both in the presence of only EGF or EGF and TGF-β. Thus the effect of combined TGF-β and EGF treatment on acini attachment and growth was evident at lower serum concentrations though to a lesser

degree. This suggested that the effect was not purely a consequence of 15% serum but it was augmented at higher serum concentrations.

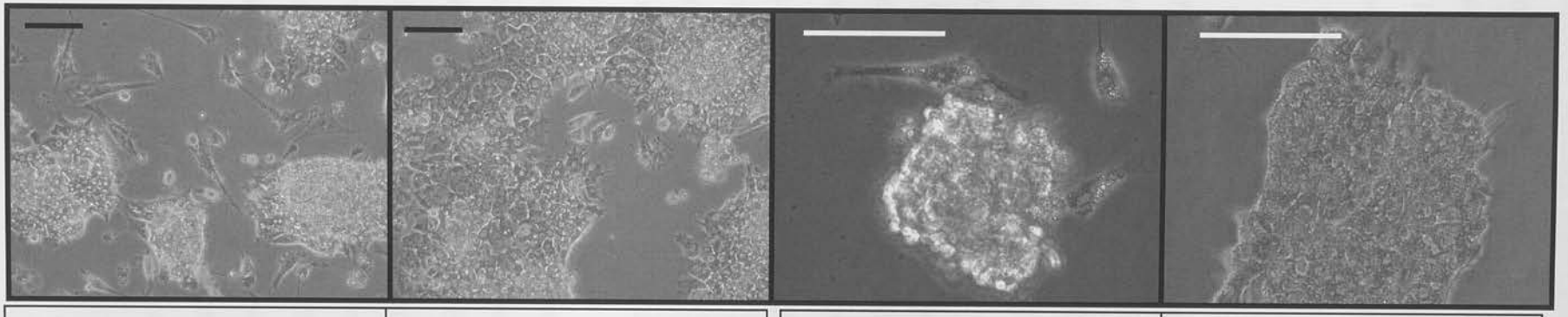
To further investigate how much of the effect was directly attributable to TGF-β rather than factors present in serum, such as insulin, sex hormones or minerals such as iron, calcium and sodium, and to investigate the synergistic effect of TGF-β and EGF on the pancreatic acini three qualitative experiments were carried out. The acini were cultured in the presence of a TGF-β neutralising antibody, a pharmaceutical MEK kinase inhibitor which has been shown previously to block EGF signalling (Learish *et al.*, 2000), or with Choleocystokinin (CCK) instead of EGF. A single addition of TGF-β neutralising antibody to freshly prepared acini in medium containing 15% FCS with EGF and TGF-β resulted in partial inhibition of the increased acini attachment and colony size (Figure 5.4 (h)). Addition of the antibody on day 0, day 1, day 2 and day 3 resulted in a similar partial inhibition of acini attachment and colony size. Measurement of the area of acinar cell colonies on the culture slide showed that the addition of TGF-β neutralising antibody resulted in a significant reduction in colony sizes (Figure 5.3, Mann Whitney U $p<0.05$) compared to acini which had only received EGF and TGF-β. The addition of a MEK kinase inhibitor resulted in complete ablation of acini attachment and cell death (Figure 5.4 (g)).

The hormone and neuropeptide CCK has diverse actions on the pancreas. It leads to hypertrophy and hyperplasia, is a secretagogue, stimulates acinar cells protein synthesis, the expression of proto-oncogenes, and the expression of a subset of digestive enzyme genes and is involved in pancreatic secretion (Kern *et al.*, 1987; Dale *et al.*, 1989; Lu and Logsdon, 1992; Williams and Blevins, 1993). Signalling pathways and mechanisms of these actions are not well defined, however, CCK can activate ERK and JNK in pancreatic acinar cells (Nicke *et al.*, 1999). CCK is another mitogen commonly used in the culture of pancreatic acinar cells (Logsdon, 1986). In order to determine whether this novel effect of TGF-β occurred only in conjunction with EGF or whether it occurred with other growth factors, cells were plated in 15% serum with CCK, TGF-β or CCK and TGF-β. TGF-β in conjunction

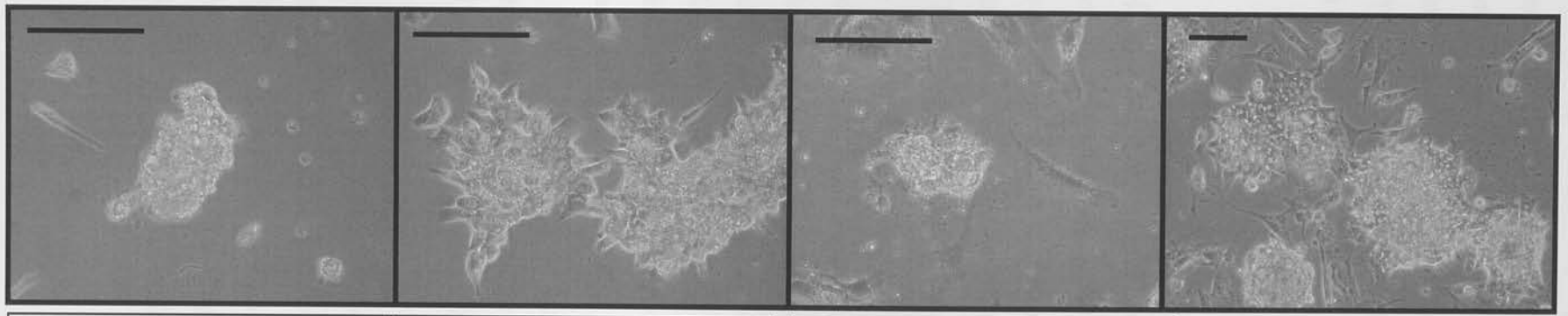
with CCK resulted again in increased acini attachment and colony size compared to acini cultured in CCK or TGF-β alone (Figure 5.4 (e-f)).

Figure 5.4 – Primary pancreatic acinar cells on day 4 cultured in plating medium with 15% or 2.5% FCS containing various growth factors and reagents. Specific conditions are stated on figure and bars represent 100μm.

Figure 5.4 – Comparison of pancreatic acinar cell colonies on day4 of culture after treatment with EGF, CCK, TGF-β, MEK inhibitor (U0126) and TGF-β neutralising antibody.



(a)Cells cultured in plating medium containing 15% FCS and EGF	(b)Cells cultured in plating medium containing 15% FCS, EGF and TGF-β.	(c) Cells cultured in plating medium containing 2.5 % FCS and EGF.	(d) Cells cultured in plating medium containing 2.5% FCS, EGF and TGF-β.



(e) Cells cultured in plating medium containing 15% FCS and CCK	(f) Cells cultured in plating medium containing 15% FCS, CCK and TGF-β.	(g) Cells cultured in plating medium containing 15% FCS, EGF, TGF-β and U0126.	(h) Cells cultured in plating medium containing 15% FCS, EGF, TGF-β and TGF-β neutralising antibody.

Thus from these observations it appears that the effect of TGF-β on pancreatic acini with respect to increased attachment and colony size was not caused by high serum concentration. Experiments with a TGF-β-neutralising antibody supported the hypothesis that TGF-β directly caused the increased attachment and colony size. Whilst EGF and TGF-β appear to act synergistically to promote the effect of TGF-β, it is not associated exclusively with an EGF/TGF-β synergy as CCK and TGF-β treated cells also exhibit increased attachment and colony size, albeit to a lesser extent.

5.3.4 Effect of TGF-β on proliferation

Previous work investigating the effect of TGF-β on primary acinar cells has shown that TGF-β alone causes a 20%-30% reduction in proliferation compared to cells which received no growth factor, when the cells are plated in 2.5% FCS (Logsdon *et al.*, 1992). Whilst contrary to this work, it was investigated whether the increased colony size of acinar cells which had received TGF-β and EGF could be caused by an increase in proliferation. To examine this, ^{3}H thymidine incorporation studies were carried out on acini plated with 15% v/v serum and 2.5% v/v serum containing nothing, EGF, TGF-β or EGF and TGF-β. Cells were incubated in ^{3}H thymidine for 24 hours prior to harvesting on day 2, day 3 and day 4 of culture. Cells undergoing DNA synthesis incorporated the radio-labelled thymidine as well as normal thymidine. Once the unincorporated ^{3}H thymidine was washed away, measurement of the remaining ^{3}H thymidine allowed approximate quantification of the proliferation rate of cells. Results shown in Figure 5.5 revealed no significant increase or decrease of ^{3}H thymidine incorporation in cells grown in either 15% or 2.5% FCS containing media with EGF and TGF-β compared to those which had received EGF or TGF-β only. Comparison between cells treated with either EGF or TGF-β within the different serum concentrations revealed that cells plated in media containing 15% serum with TGF-β did not exhibit a significant reduction in ^{3}H thymidine incorporation (Mann Whitney U $p>0.05$) whereas cells plated in media containing 2.5% serum and TGF-β did (Mann Whitney U $p<0.05$). This highlighted serum concentration as a factor that could protect cells from the antiproliferative

effects of TGF-β. Overall, it was concluded that whilst TGF-β was capable of an antiproliferative effect in these cells, this could be due to the absence of EGF and lower serum concentrations (2.5%). This result therefore reflects the multifunctional nature of TGF-β and highlights again that the actual biological outcome of TGF-β signalling depends on the cell's environment. These results also refuted the hypothesis that the increased attachment and colony size seen in EGF and TGF-β treated acini was a consequence of increased proliferation.

Figure 5.5 – Effect of EGF and TGF-β on ^{3}H-thymidine incorporation

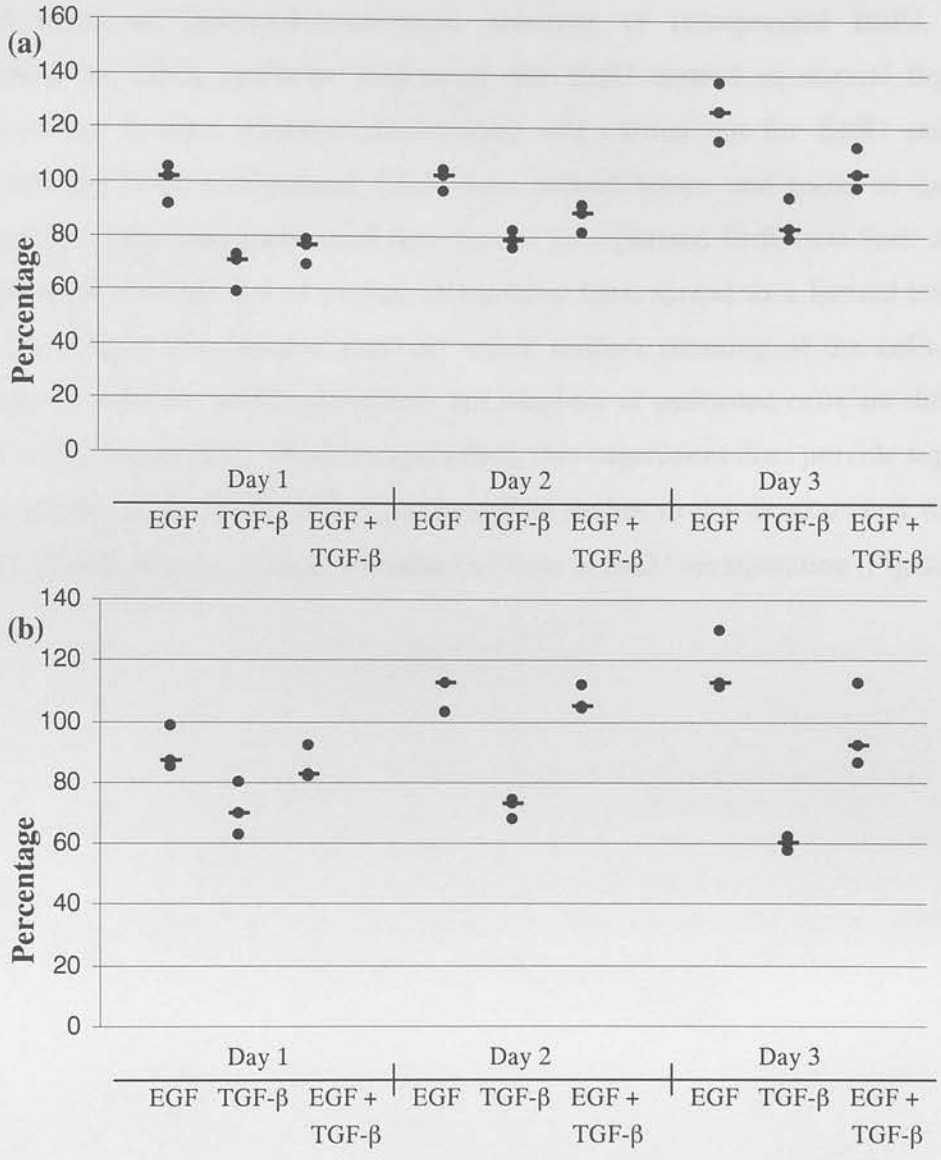


Figure 5.5 – Dot plots showing ^{3}H thymidine incorporation expressed as a percentage of control (no growth factor treatment) in cells which had been grown in plating medium containing (a) 15% FCS and (b) 2.5% FCS. The experiment was conducted ten times however dot plots show representative results from one experiment. Horizontal lines represent median.

BrdU incorporation studies were also conducted on acini grown in plating medium containing 15% FCS with nothing added, EGF, TGF-β or EGF and TGF-β added. BrdU incorporation reflects DNA sythesis similarly to ^{3}H thymidine incorporation but relies on immunohistochemical detection of incorporated BrdU. Cells undergoing DNA synthesis incorporate the BrdU instead of normal thymidine. Following fixation, immunocytochemistry was carried out for BrdU and nuclei containing BrdU-incorporated DNA were stained brown and could be quantified visually. Immunocytochemical detection of incorporated BrdU was least suited to cells days between 0-4 of culture as the acini have spread to a limited extent and retain a three dimensional structure which hinders counting of the cells. BrdU negative cells are readily detectable but numbers of unstained cells are difficult to ascertain (Figure 5.6). Whilst unquantified, this experiment does provide support for the results of the ^{3}H-thymidine incorporation studies in that acini treated with EGF and TGF-β do not exhibit a noticeable increase in BrdU incorporation (Figure 5.6).

Figure 5.6 – Effect of EGF and TGF-β on BrdU incorporation on primary pancreatic acinar cells.

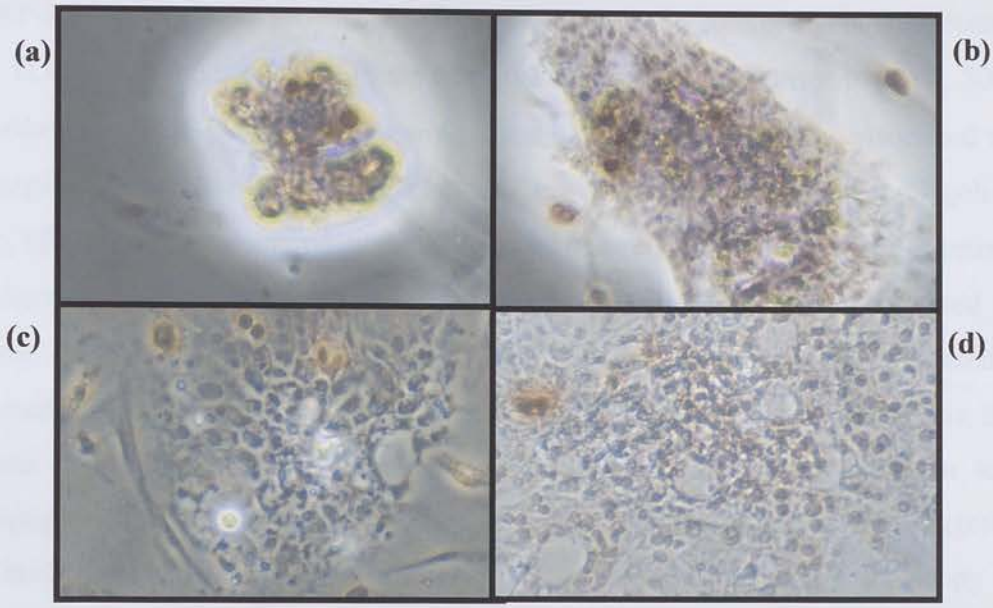


Figure 5.6 – BrdU incorporation on primary pancreatic acinar cells which have been cultured for 4 days in plating medium with 15% FCS with (a) nothing added, (b) EGF added, (c) TGF-β added and (d) EGF and TGF-β added. Nuclei that have incorporated BrdU are brown and images (X200 magnification) were taken under the phase contrast microscope settings to allow clearer visualisation of cells at.

5.3.5 Effect of TGF-β on motility

TGF-β has previously been shown to promote cell motility in various cell types, for example TGF-β induced glioma cell motility via a mechanism involving the increased expression of integrin subunits (Platten *et al.*, 2000). Also activation of RAF-induced invasive growth of MDCK cells grown in collagen gels and secretion of TGF-β was required to maintain this invasive phenotype (Lehmann *et al.*, 2000). Furthermore TGF-β has been shown to cause morphological changes associated with reorganisation of the actin cytoskeleton of primary cultured astrocytes (Gagelin *et al.*, 1995). To determine whether TGF-β was causing increased motility of primary cultured acinar cells two investigations were carried out. First, it was noted that when treated with TGF-β and EGF, cells on the edges of colonies extended numerous, pronounced cytoplasmic processes compared to cells maintained in EGF alone (Figure 5.7). Fluorescent labelling of these cells with Phalloidin toxin conjugated TRITC showed that these processes contained actin filaments suggestive of leading edges (Kislauskis *et al.*, 1997). Second, time-lapse video microscopy was carried out. It was observed in the time-lapse movie that the processes on cells treated with both EGF and TGF-β were more mobile than those on cells treated with EGF only (time-lapse movie on CD 5.1 in appendix D, with annotation in sequential stills in Figure 5.8).

Taken together these studies suggest that combined treatment of TGF-β and EGF does increase the motility of primary cultured acinar cells as evidenced by restructuring of the actin cytoskeleton to produce numerous mobile leading edges.

Figure 5.7 – Primary pancreatic acinar cells (day 4 of culture) with actin filaments fluorescently labelled with TRITC-conjugated phalloidin and countersatined with DAPI (a,b,d and e) and phase contrast images (c and f). (a, b and c): acinar cells which have been grown in plating medium (containing 15% FCS) with EGF only at X100 magnification (a), X200 magnification (b), and X200 magnification (c). (d, e and f): acinar cells which have been grown in plating medium (containing 15% FCS) with EGF and TGF-β showing characteristic cell spreading and prominent processes at X100 magnification (d), X400 magnification (e) and X200 magnification (f).

Figure 5.7 – Fluorescent labelling of actin filaments using TRITC-conjugated phalloidin.

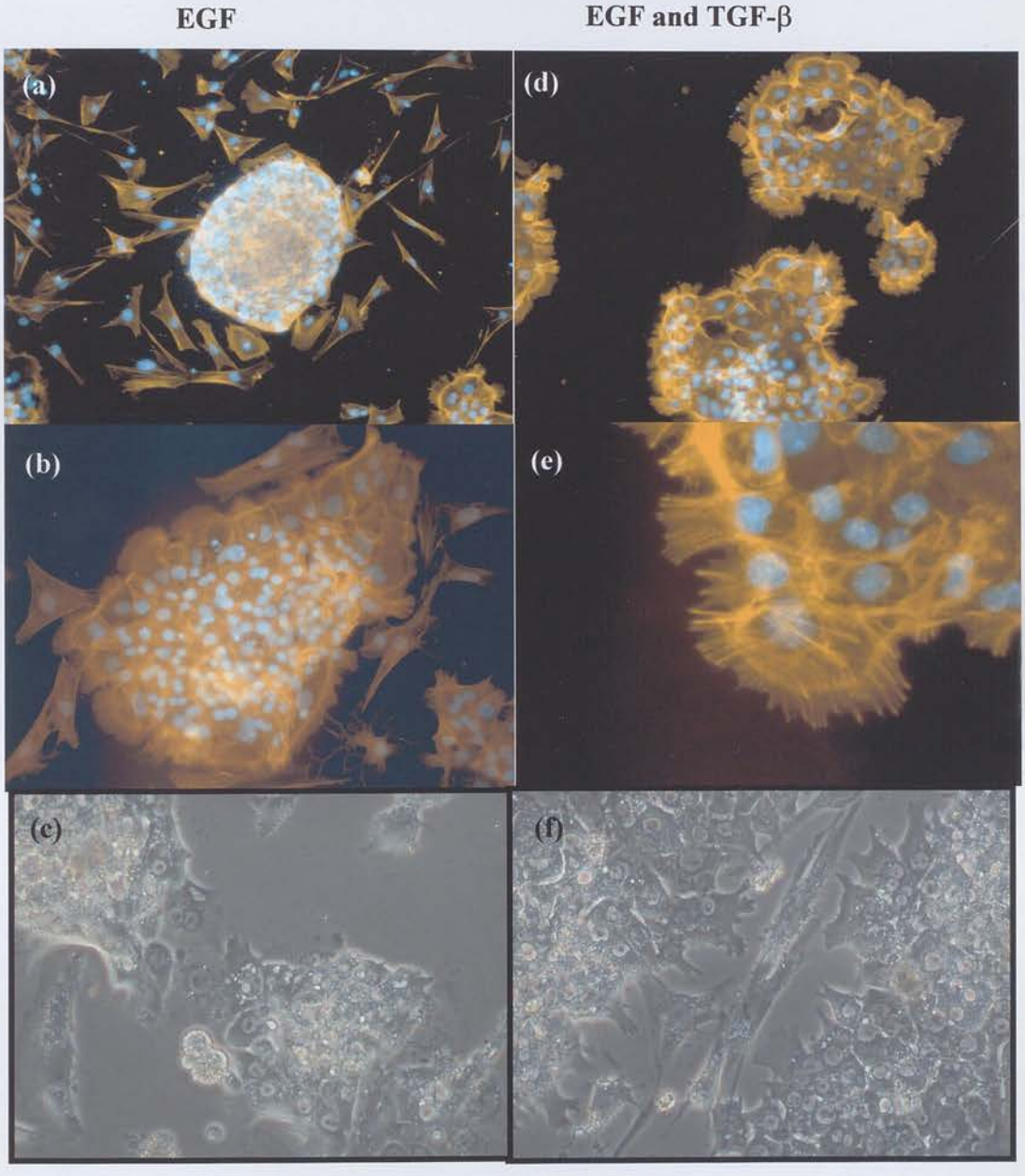


Figure 5.8 –Sequential stills from timelapse video microscopy on CD 5.1.1. Timelapse microscopy was carried out during day 2 and 3 of culture on primary cultured acinar cells treated with (a) EGF or (b) EGF and TGF-β at X400 magnification. Stills in both (a) and (b) show comparable 2 hr stills starting from 0 hrs.

Figure 5.8 – Sequential stills from timelapse video microscopy (a) EGF.

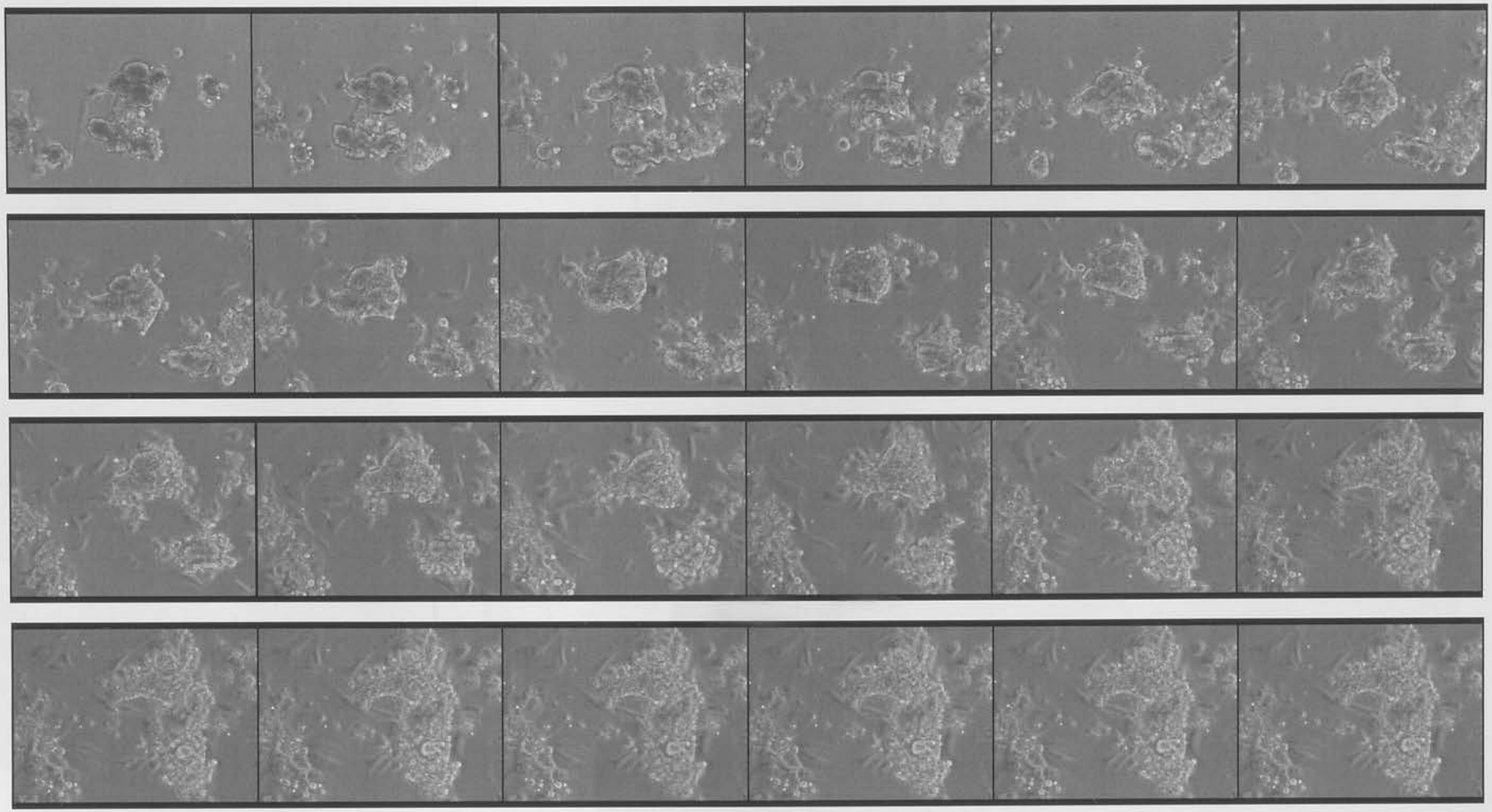
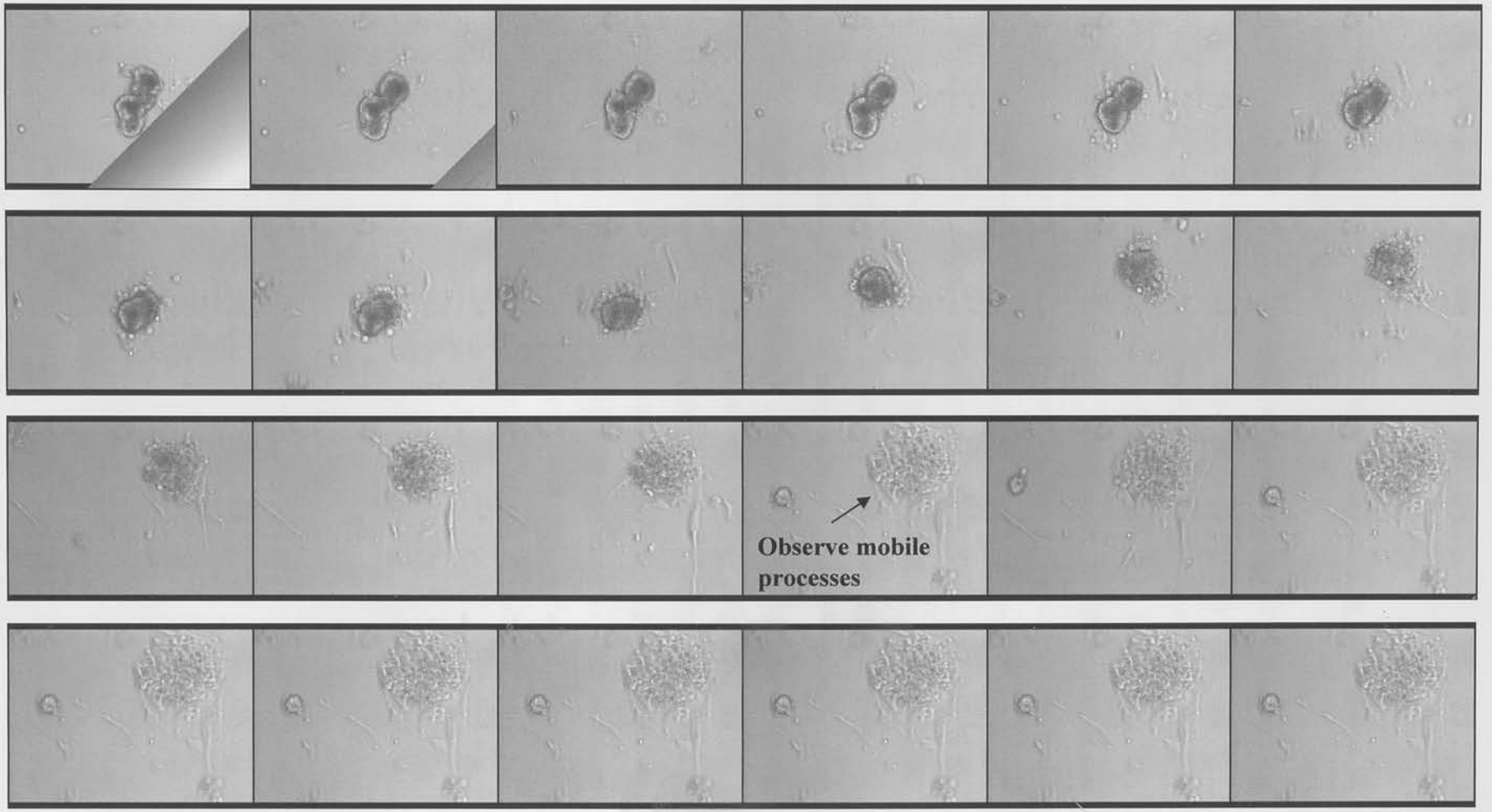


Figure 5.8 - (b) EGF and TGF-β



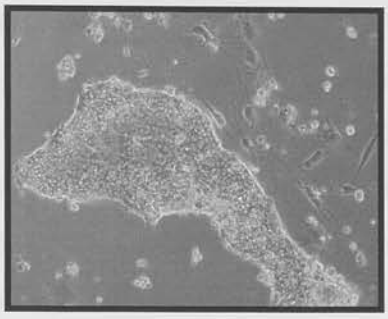
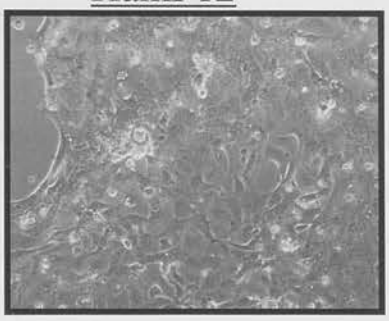
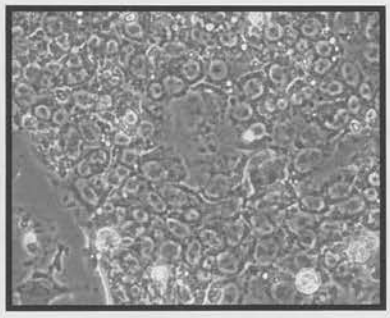
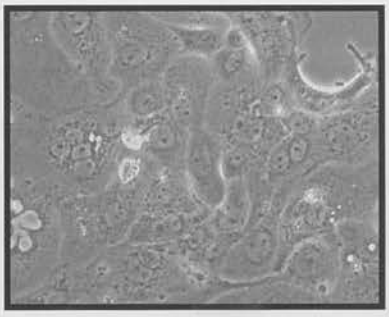
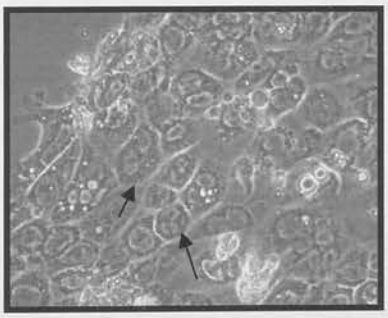
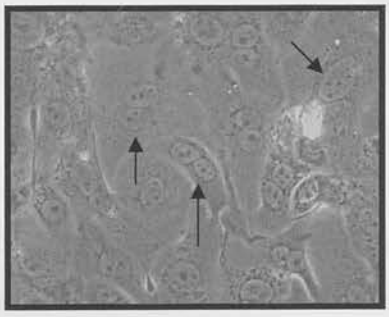
5.4 The Effect of TGF-β on adherent primary cultured acinar cells – day 5 – day 10 of culture.

5.4.1 Morphological differences of primary cultured acinar cells when grown in Chee's or Waymouth/HamF12 media.

As detailed in Figure 5.1 and in the materials and methods, after the acini had been left to attach for approximately 4 days in plating medium containing 15% serum they were transferred into Chee's medium (serum and arginine free but containing ornithine) for 24-48 hours in an attempt to purify the culture from fibroblasts. This method was used because acinar cells express ornithine decarboxylase, thus in the absence of arginine they can utilise ornithine as an arginine precursor. As fibroblasts do not possess ornithine decarboxylase, culturing the cells in arginine free medium starves the fibroblasts of this amino acid and causes them to die. Previous to the adoption of this method at this stage of culture the cells would have been transferred straight into Waymouth/HamF12 media. Upon testing this new method involving the Chee's media it was observed that there were morphological differences between the acinar cells grown in the different media (Dr N Sphyris personal communication). These differences are summarised in Figure 5.9, which includes published work by other researchers also outlined in 1.2.3. As previous work has suggested that acinar cells cultured in Waymouth/HamF12 media transdifferentiate into ductal cells (Hall and Lemoine, 1992; De Lisle and Logsdon, 1990) it was concluded that the Chee's medium allowed cells to retain some of their differentiated acinar cells characteristics (such as the presence of zymogen granules), although the reason for this remains unclear.

Figure 5.9 – Comparison of primary pancreatic acinar cells cultured in Chee's or Waymouth/HamF12 media with EGF. Pictures (a) and (b) were taken at X100 magnification, (c), (d), (e) and (f) were taken at X400 magnification. Antigen expression analysis summary of work by Hall and Lemoine, 1992 and De Lisle and Logsdon, 1990.

Figure 5.9 – Comparison of primary pancreatic acinar cells grown in Chee's or Waymouth/HamF12's culture media with EGF.

	Chee's	Waymouth/ HamF12
Cell spreading	 Acinar cells remain tightly packed in colonies. Do not form monolayer.	 Acinar cells spread to form monolayer.
Cell morphology	 Cells exhibit 'paving stone' morphology.	 Cells exhibit 'tear drop' morphology and flatten out.
Nuclear morphology	 Binuclear cells present.	 Binuclear cells present. Giant nuclei present.
Cell turnover (day 8)	BrdU index - approximately 4%	BrdU index - approximately16%
Antigen expression analysis		Positive for duct-1, cytokeratin 19 and mucin. Negative for amylase.

5.4.2 BrdU incorporation studies on adherent primary acinar cells after treatment with TGF-β

5.4.2.1 Effect of TGF-β on WT acinar cells grown in Chee's medium

As TGF-β has been shown to be a potent inhibitor of growth in many cells of epithelial origin (Roberts and Sporn 1989) the effect of TGF-β (in the presence and absence of EGF) on BrdU incorporation was assessed on primary cultured acinar cells. Acinar cells from WT mice were cultured completely in Chee's medium for 6-7 days using the method summarised in Figure 5.1. TGF-β (160pM) was administered to cells for 6, 12, 18, 24, 30 or 36 hours and 6 hours prior to fixation of the cells BrdU was added to the medium (materials and methods, section, 2.2.9). Cells were stained for BrdU by immunocytochemistry and the BrdU labelling index was measured using the HOME microscope. For each well five random fields of $0.04mm^2$ were scored under a X40 objective, clumps of cells were excluded from the analysis. Whilst overall levels of BrdU incorporation were low, some BrdU incorporation was seen under all conditions examined. Statistical analysis was kindly carried out by Prof M.L. Hooper and involved using a linear model to compare the BrdU index between treatments in order to allow for overall differences in the index between experiments. Comparison between WT cells grown in EGF or EGF and TGF-β for the various timepoints revealed that TGF-β significantly reduced BrdU incorporation at all timepoints ($p<0.05$) except the 24 hours time point ($p>0.05$, Figure 5.10 (a)). As the cells are normally cultured in EGF the effect of EGF and TGF-β compared to EGF alone was the main interest. However, to test whether EGF was protecting cells from complete growth arrest by TGF-β the experiment was repeated on cells which received either nothing or TGF-β for 6, 12, 18, 24 or 30 hours. In the complete absence of EGF, TGF-β resulted in nearly complete inhibition of DNA synthesis. For example, after 18 hours of treatment with TGF-β less than 0.2% of cells were incorporating BrdU (Figure 5.10 (c)). Comparison between the cells which received no growth factor or TGF-β only for different time points showed a reduction in BrdU incorporation at the 18 and 30 hours time points ($p<0.05$). It was therefore concluded that the presence of EGF was

capable therefore of partially protecting cells from the growth inhibitory effects of TGF-β.

5.4.2.2 Effect of TGF-β on *Smad4*$^{+/-}$ cells in Chee's medium

As loss of SMAD4 can lead to the loss of TGF-β mediated growth arrest, it was originally intended to assess the effect of TGF-β on *Smad4*$^{-/-}$ cells. This was not possible (see appendix B). I therefore tested whether *Smad4* heterozygosity had an effect on the ability of TGF-β to elicit a reduction of BrdU incorporation as was seen in WT cells treated with EGF or EGF and TGF-β. The experiment was repeated for 24, 30 and 36 hours time points under the same conditions but with acinar cells cultured from *Smad4*$^{+/-}$ mice (Figure 5.10 (b)). Statistical analysis revealed that TGF-β with EGF exerted a significant reduction in BrdU incorporation on acinar cells at all timepoints ($p<0.05$), compared to cells which received EGF alone. It appears therefore in this system that *Smad4* heterozygosity did not impair the function of TGF-β to exert growth arrest.

5.4.2.3 Effect of TGF-β on WT cells in Waymouth/Ham F12 medium containing 2% FCS

As the cells grown in the Waymouth/Ham F12 media (containing 2% serum) exhibited a different phenotype to those grown in Chee's (no serum) (see Figure 5.9 and section 5.4.1), I investigated firstly whether these 'ductal-like' like cells had a higher BrdU index when treated just with EGF than cells grown in Chee's medium. Analysis showed that the increase in BrdU index in the Waymouth/Ham F12 cells was highly significant at the 6, 12, 18 and 24 hours time points (Figure 5.10 (d) $p<0.05$). Secondly, I determined whether the 'ductal like' Waymouth/HamF12 cells would respond differently to treatment with TGF-β than cells which had retained the more acinar phenotype and exhibited a reduction in BrdU incorporation in response to TGF-β. The Waymouth/HamF12 cells were treated with EGF or EGF and TGF-β for 6, 12, 18 and 24 hours and the BrdU labelling index was measured. Results showed that these cells exhibited a reduction of BrdU incorporation in response to TGF-β and EGF after 12, 18 and 24 hours ($p<0.05$), however even with this reduction, the average percentage of cells incorporating BrdU did not fall below 7%,

a figure still higher than the BrdU labelling index in EGF stimulated cells grown in Chee's media (Figure 5.10 (d) and (a)). Thus it appears that TGF-β can still exert growth inhibitory effects in the 'ductal-like' Waymouth/HamF12 cells.

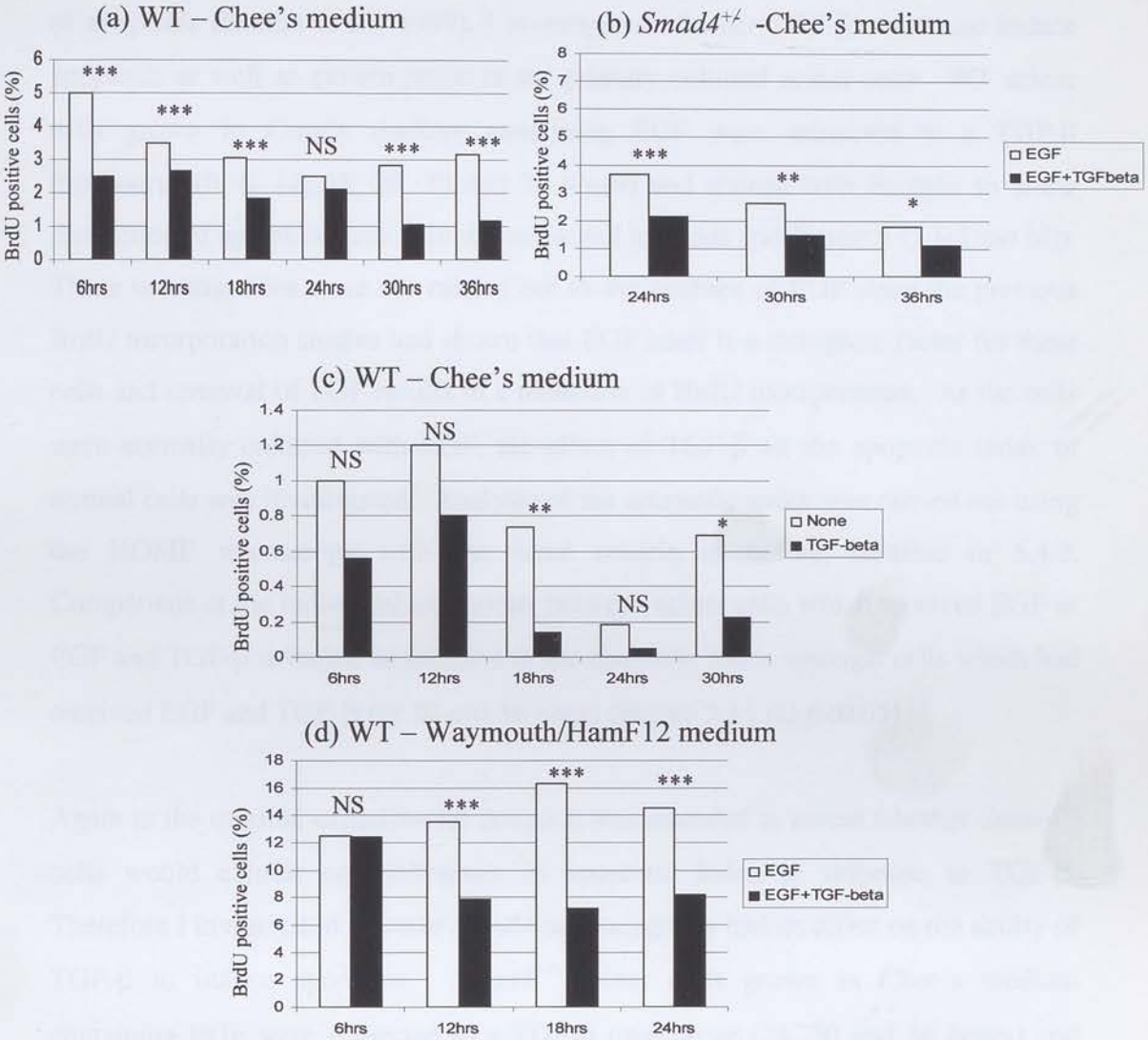
5.4.2.4 Effect of loss of p21 on BrdU index

Since one of the molecular mechanisms of TGF-β-mediated growth arrest is through p21 induction, preliminary investigations assessing the BrdU labelling index in *p21*$^{-/-}$ cells were also carried out. As p21 is a Cdk inhibitor involved in cell cycle regulation it was unsurprising to note that *p21*$^{-/-}$ acinar cells maintained in Chee's medium exhibited a 6-7 fold increase in BrdU labelling index compared to WT cells (Figure 5.10 (e) and (f)). Had time permitted it would have been of great interest to investigate whether *p21*$^{-/-}$ cells were refractory to TGF-β mediated growth arrest.

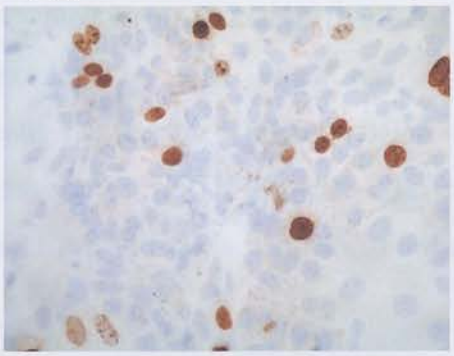
TGF-β therefore causes a decrease in the proportion of S phase WT or *Smad4*$^{+/-}$ cells. In WT cells this effect is evidenced whether the cells have been grown in Chee's or Waymouth/HamF12 media, with or without EGF.

Figure 5.10 – BrdU incorporation studies on the effect of no added growth factor, EGF and/or TGFβ on (a) WT cells cultured in Chee's medium, (b) *Smad4*$^{+/-}$ cells in Chee's medium, (c) WT cells in Chee's medium and (d) WT cells in Waymouth/Ham's medium. Pictures showing BrdU incorporation in (e) *p21*$^{-/-}$ cells cultured in Chee's medium with EGF, X400 magnification and (f) WT cells cultured in Chee's medium with EGF at X400 magnification. Results of statistical analysis comparing between individual timepoints are shown above corresponding bars in histogram. Key: NS, $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Experiment was performed independently three times with at least two replicates in each experiment.

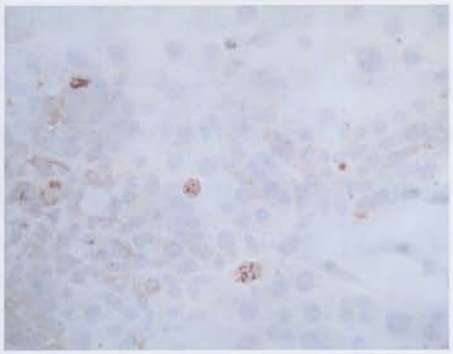
Figure 5.10– BrdU incorporation studies on primary pancreatic acinar cells.



(e) *p21* $^{-/-}$



(f) WT



5.4.3 Assessment of apoptosis of adherent primary acinar cells after treatment with TGF-β

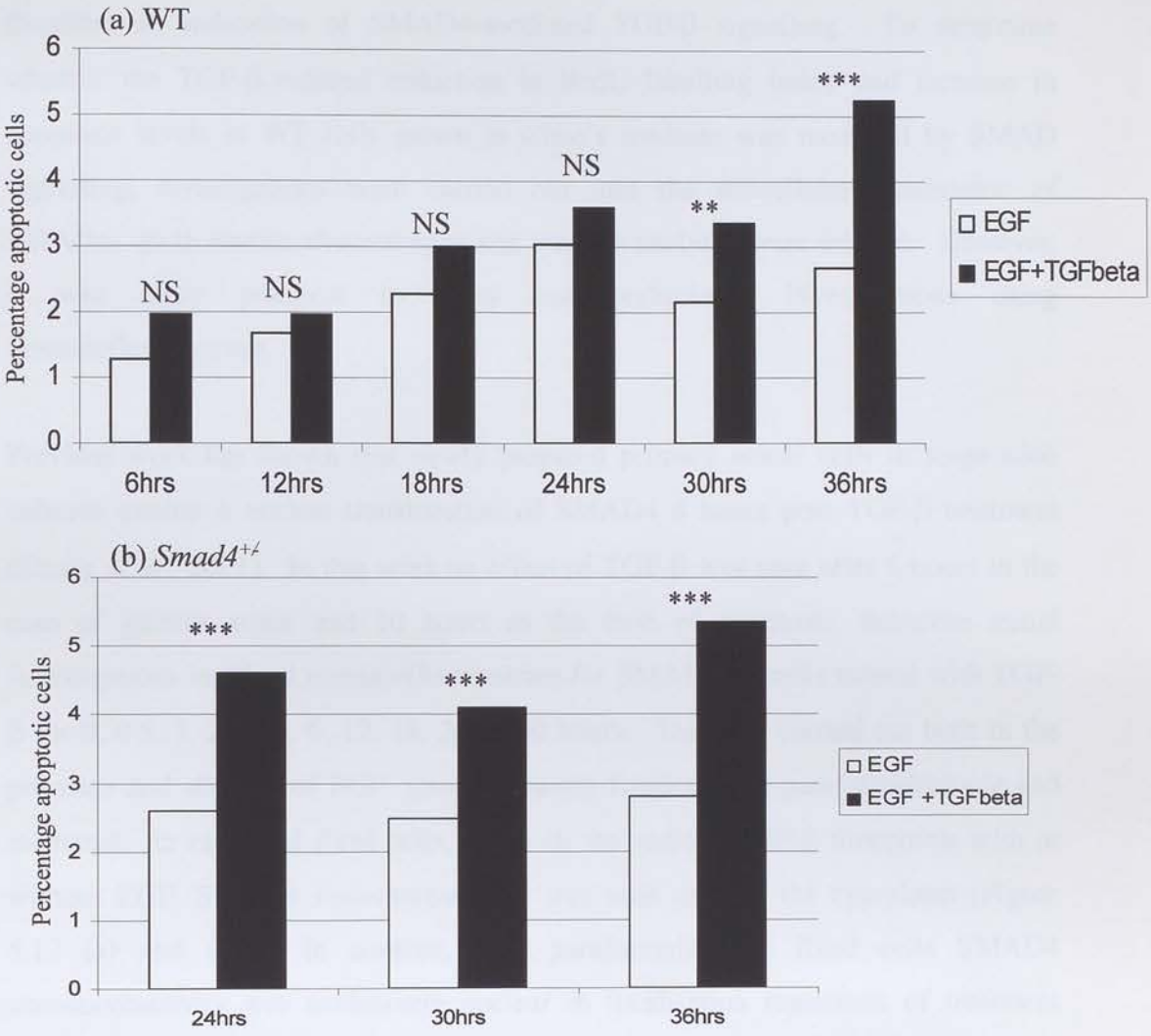
Since TGF-β signalling mediated by SMAD4 has also been linked with an induction of apoptosis (Brodin *et al.*, 1999), I investigated whether TGF-β could also induce apoptosis as well as growth arrest in the primary cultured acinar cells. WT acinar cells grown in Chee's medium containing EGF were subjected to a TGF-β timecourse (0, 6, 12, 18, 24, 30 and 36 hours) and stained with Feulgen to allow distinction of apoptotic cells (See materials and methods and Figure 5.11 (c) and (d)). These investigations were not carried out in the absence of EGF since the previous BrdU incorporation studies had shown that EGF itself is a mitogenic factor for these cells and removal of EGF results in a reduction of BrdU incorporation. As the cells were normally cultured with EGF, the effect of TGF-β on the apoptotic index of normal cells was investigated. Analysis of the apoptotic index was carried out using the HOME microscope with the same criteria as before, detailed in 5.4.2. Comparison at the individual timepoints between acinar cells which received EGF or EGF and TGF-β revealed an increase in the apoptotic index amongst cells which had received EGF and TGF-β for 30 and 36 hours (Figure 5.11 (a) $p<0.05$).

Again in the original experimental design it was intended to assess whether *Smad4*$^{-/-}$ cells would exhibit any difference in apoptotic index in response to TGF-β. Therefore I investigated whether *Smad4* heterozygosity had an effect on the ability of TGF-β to induce apoptosis. *Smad4*$^{+/-}$ acinar cells grown in Chee's medium containing EGF were subjected to a TGF-β timecourse (24, 30 and 36 hours) and Feulgen stained. Analysis of the apoptotic index was carried out with the same criteria as before (5.4.2). Comparison at the individual timepoints between acinar cells which received EGF or EGF and TGF-β revealed an increase in the apoptotic index amongst cells which had received EGF and TGF-β at all timepoints investigated (Figure 5.11 (b) $p<0.05$). It was concluded that even in the presence of the mitogenic EGF, TGF-β causes an increase in apoptosis in both WT and *Smad4*$^{+/-}$ cells grown in Chee's medium. Further work investigating the effect of TGF-β on

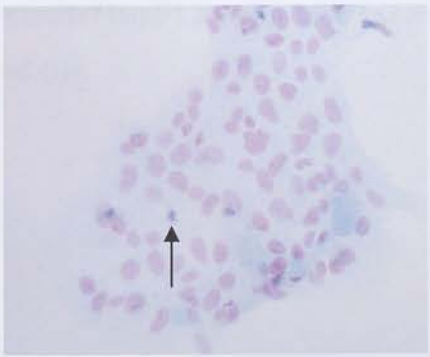
the cells grown in Waymouth/HamF12 medium would be of use in the further characterisation of these 'ductal-like' cells.

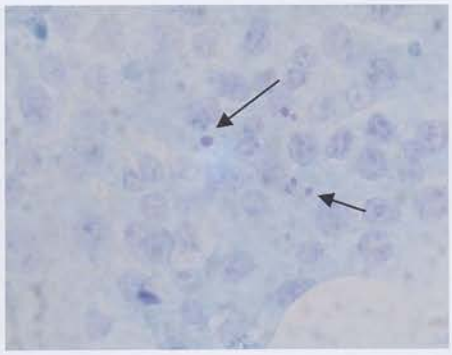
Figure 5.11– Percentage apoptotic cells as analysed by Feulgen staining in (a) WT or (b) *Smad4* $^{+/-}$ primary pancreatic acinar cells cultured in Chee's medium with EGF or EGF and TGF-β. (c) and (d) Representative pictures of Feulgen stained primary acinar cells treated with EGF and TGF-β for 30 hours at (c) X400 magnification and (d) X1000 magnification. Arrows highlight specific examples. Results of statistical analysis comparing between individual timepoints are shown above corresponding bars in histogram. Key: NS, $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Experiment was performed independently three times with at least two replicates in each experiment.

Figure 5.11 – Percentage apoptosis as scored by Feulgen staining on cells cultured in serum free Chee's medium with and without TGF-β.



(c)





(d)

5.4.4 SMAD4 nuclear translocation following TGF-β treatment

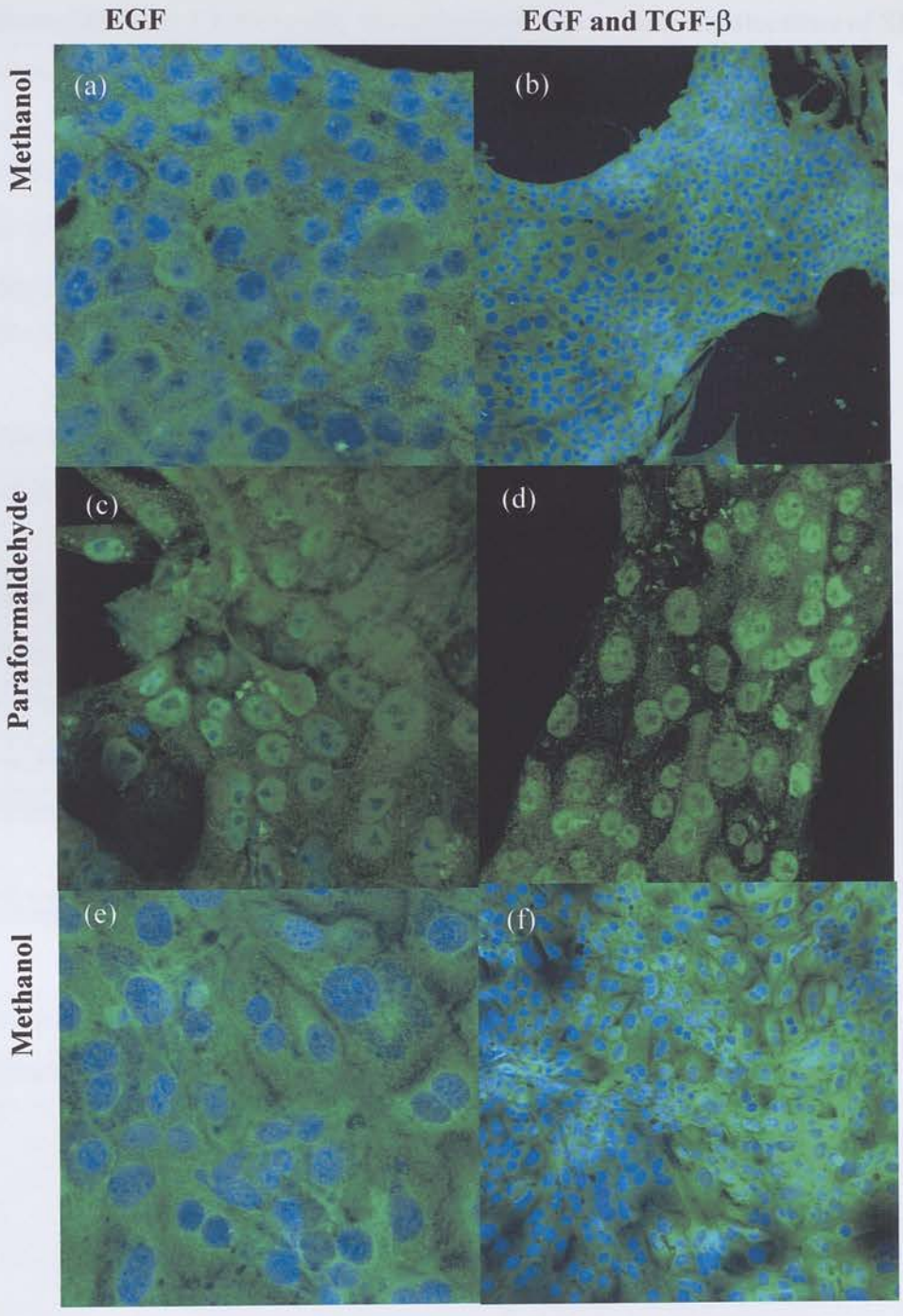
TGF-β can induce growth arrest and apoptosis through SMAD-dependent and independent methods. Nuclear translocation of the R-SMAD/Co-SMAD complex is therefore an indication of SMAD4-mediated TGF-β signalling. To determine whether the TGF-β-induced reduction in BrdU labelling index and increase in apoptosis levels in WT cells grown in Chee's medium was mediated by SMAD signalling, investigations were carried out into the subcellular localisation of SMAD4. Both immunofluorescence and western analyses were utilised. However, it was more practical to carry out preliminary investigations using immunofluorescence.

Previous work has shown that newly prepared primary acinar cells in suspension cultures exhibit a nuclear translocation of SMAD4 4 hours post TGF-β treatment (Zhang *et al.*, 2001). In this work an effect of TGF-β was seen after 6 hours in the case of growth arrest and 30 hours in the case of apoptosis, therefore initial investigations involved immunofluorescence for SMAD4 on cells treated with TGF-β for 0, 0.5, 1, 2, 3, 4, 6, 12, 18, 24 or 30 hours. This was carried out both in the presence and absence of EGF, also comparing fixation with paraformaldehyde and methanol. In methanol fixed cells, under all the various TGF-β timepoints with or without EGF, SMAD4 immunoreactivity was seen only in the cytoplasm (Figure 5.12 (a) and (b)). In contrast, with paraformaldehyde fixed cells SMAD4 immunoreactivity was exclusively nuclear in localisation regardless of treatment (Figure 5.12 (c) and (d)). Methanol fixed cells which had been grown in Waymouth/HamF12 media also exhibited entirely cytoplasmic staining (Figure 5.12 (e) and (f)).

Figure 5.12 – Confocal microscopy of SMAD4 immunofluorescence on primary acinar cells grown in Chee's medium (a-d) and Waymouth/HamF12 medium (e-f) and fixed with methanol or paraformaldehde.

(a) methanol fixed, treated with EGF for 18 hours at X630 magnification (b) methanol fixed, treated with EGF and TGF-β for 18 hours at X200 magnification (c) paraformaldehyde fixed, treated with EGF for 18 hours at X630 magnification (d) paraformaldehyde fixed, treated with EGF and TGF-β for 18 hours at X630 magnification (e) methanol fixed treated with EGF for 18 hours at X630 magnification (f) methanol fixed treated with EGF and TGF-β for 18 hours at X200 magnification. Cells fixed in methanol exhibited entirely cytoplasmic staining for SMAD4 and those fixed in paraformaldehyde exhibited entirely nuclear staining for SMAD4.

Figure 5.12- Comparison of methanol and parafomaldehyde fixation on immunofluorescence for Smad4



In order to assess which fixative was more accurately reflecting the subcellular localisation of SMAD4, a SMAD4 expressing murine cell line, NIH 3T3, was utilised. Previous work with NIH 3T3 cells has shown that treatment with Leptomycin B for 1 hour results in a cytoplasmic to nuclear translocation of SMAD4 (Pierreux *et al.*, 2000). Leptomycin B binds to and inhibits CRM1, a protein involved in the export of a number of proteins from the nucleus (Kudo *et al.*, 1998). NIH 3T3 cells were treated with Leptomycin B (20ng/ml) for 1 hour, with or without TGF-β for 30 minutes and fixed with either paraformaldehyde or methanol (see materials and methods, sections 2.2.10 and 2.2.11). The results are summarised in Table 5.1 and Figure 5.13)

Table 5.1 – Summary of SMAD4 immunofluorescence on NIH3T3 cells

Fixative	**Leptomycin B**	**TGF-β**	**SMAD4 localisation**	**Representative example**
Paraformaldehyde	1hr	30mins	Nuclear	Figure 5.13 (a)
Paraformaldehyde	1hr	-	Nuclear	Figure 5.13 (b)
Paraformaldehyde	-	30mins	Nuclear	Figure 5.13 (c)
Paraformaldehyde	-	-	Nuclear	Figure 5.13 (d)
Methanol	1hr	30mins	Nuclear and cytoplasmic	Figure 5.13 (e)
Methanol	1hr	-	Nuclear and cytoplasmic	Figure 5.13 (f)
Methanol	-	30mins	Cytoplasmic	Figure 5.13 (g)
Methanol	-	-	Cytoplasmic	Figure 5.13 (h)

Figure 5.13 – Confocal microscopy of SMAD4 immunofluorescence of NIH3T3 cells. SMAD4 positive cells are stained green by the alexa 488 fluorophore and the blue to-pro 3 nuclear counterstain was used. All photographs were taken at X630 magnification.

(a) Cells treated with TGF-β for 30 minutes and Leptomycin B (LMB) for 1 hour and fixed with paraformaldehyde. Nuclear staining is observed.

(b) Cells treated with (LMB) for 1 hour and fixed with paraformaldehyde. Nuclear staining is observed.

(c) Cells treated with TGF-β for 30 minutes and fixed with paraformaldehyde. Nuclear staining is observed.

(d) Cells treated with nothing and fixed with paraformaldehyde. Nuclear staining is observed.

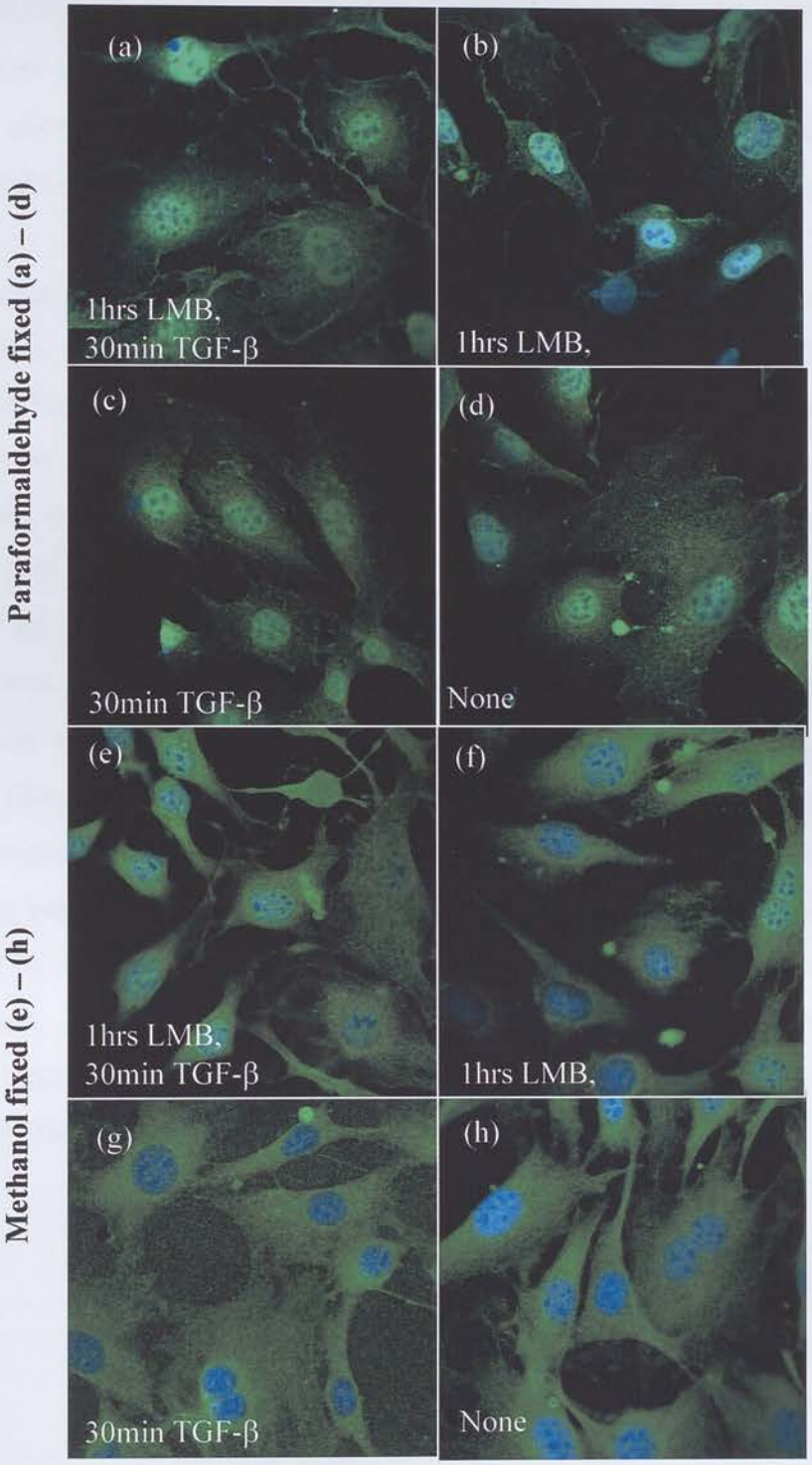
(e) Cells treated with TGF-β for 30 minutes and Leptomycin B (LMB) for 1 hour and fixed with methanol. Nuclear and cytoplasmic staining is observed.

(f) Cells treated with (LMB) for 1 hour and fixed with methanol. Nuclear and cytoplasmic staining is observed.

(g) Cells treated with TGF-β for 30 minutes and fixed with methanol. Cytoplasmic staining is observed.

(h) Cells treated with nothing and fixed with methanol. Cytoplasmic staining is observed.

Figure 5.13 – Immunofluorescence for SMAD4 on NIH3T3 cells



Given the inconclusive results from the SMAD4 immunofluorescence, SMAD4 western immunoblotting was carried out on nuclear and cytoplasmic protein extracts from cells which had received TGF-β for 0 or 4 hours. Figure 5.14 shows two blots with SMAD4 exclusively localised to the nucleus both in cells with received TGF-β and in those that did not. Comparison with the control β-actin blot by densitometry however showed an increase in nuclear SMAD4 protein (as a proportion of β-actin protein) following 4 hours of TGF-β treatment compared to no TGF-β treatment.

Studies were undertaken to investigate the hypothesis that either paraformaldehyde fixation was inappropriate for immunofluorescence with the SMAD4 B8 antibody, due to the fact that no cytoplasmic staining was observed under any conditions, or that SMAD4 was present in the nucleus at all times despite stimulation with TGF-β. The results from the NIH3T3 cells showed that a degree of nuclear staining was possible with methanol fixation. Previous results from the immunofluorescence for SMAD4 on methanol fixed primary acinar cells would therefore suggest that SMAD4 was purely cytoplasmic in response to TGF-β, leading to the conclusion that TGF-β was not signalling through the SMADs in this system. However as the Western blots supported the observation from the paraformaldehyde fixation, it was finally concluded that either SMAD4 was present in the nucleus when the TGF-β signalling pathway was unstimulated or that the SMAD signalling pathway was activated by one of the other TGF-β superfamily members in the primary cultured acinar cells. In either case the results presented here suggest that in the primary cultured acinar cells TGF-β could be signalling via the SMADs to elicit the biological effects observed.

Figure 5.14 – SMAD4 western on nuclear and cytoplasmic protein extracts following TGF-β treatment.

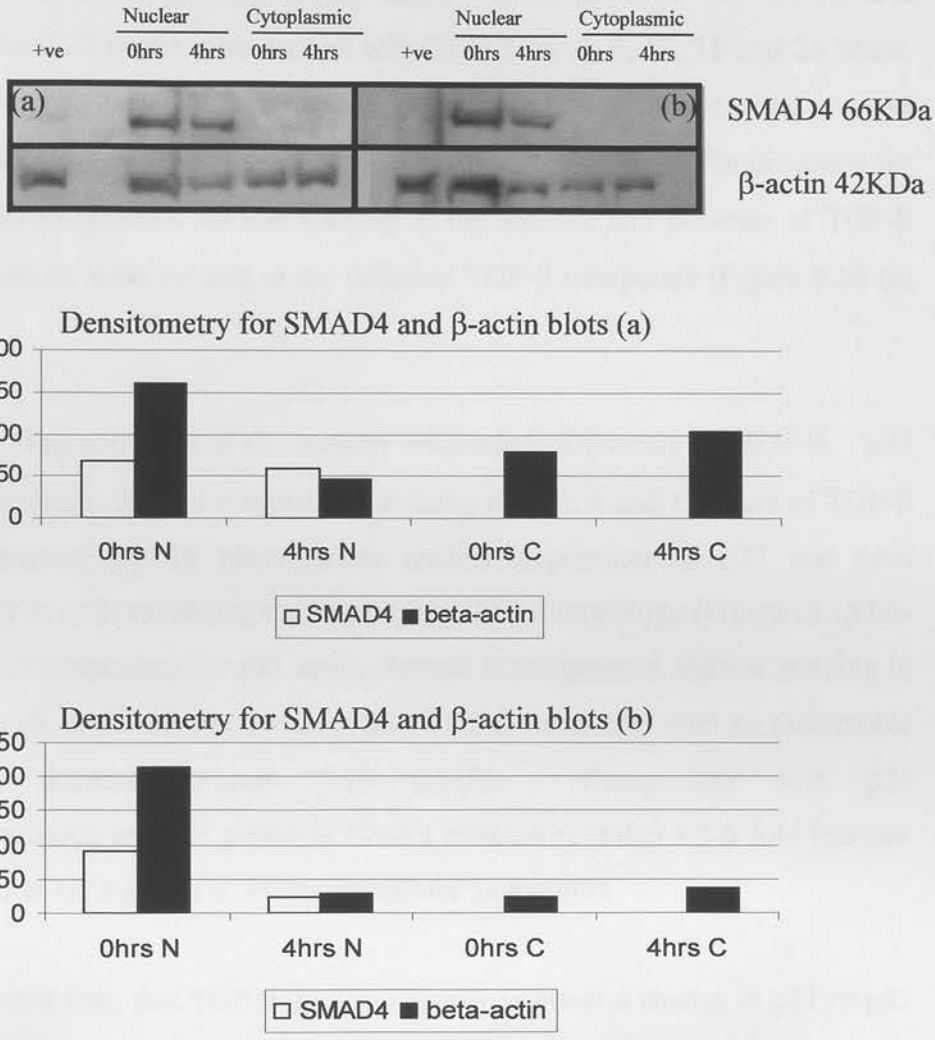


Figure 5.14 – Two representative examples of SMAD4 westerns on nuclear and cytoplasmic protein extracts (10μg loaded) from primary cultured WT acinar cells following TGF-β treatment for 0 or 4 hours. β-actin immunoblotting was carried out on the same blots to control for unequal loading. Densitometry was carried out using the Pro-plus imaging package.

5.4.5 p21 and p27 immunofluorescence on TGF-β-treated cells

As outlined in 1.5.4, p21 and p27 are CDK inhibitors involved in TGF-β-signalling, growth arrest and cell death. They were therefore investigated as possible molecular effectors of the decrease in BrdU incorporation and increase in apoptosis seen in response to TGF-β in primary acinar cells. Cells in both the Chee's and Waymouth/HamF12 media were treated with TGF-β for 0, 6, 12, 18 and 24 hours. In the Chee's medium cells that received TGF-β and those that did not showed cytoplasmic expression of p27 (Figure 5.15 (e) and (f)). Immunofluorescence for p21 showed heterogeneous nuclear staining in the absence and presence of TGF-β and no differences were evident at the different TGF-β timepoints (Figure 5.16 (a) and (b)).

Cells in the Waymouth/HamF12 medium responded differently to TGF-β. p27 immunofluorescence showed cytoplasmic staining after 0, 6 and 12 hours of TGF-β treatment, however by 18 hours, some nuclear expression of p27 was seen predominantly in cells exhibiting the 'ductal-like' like morphology (Figure (5.15 (a)-(d)). Immunofluorescence for p21 again showed heterogeneous nuclear staining in all conditions (0, 6, 12, 18 and 24 hours post TGF-β treatment) with no discernable increase or decrease (Figure 5.16 (c)-(f)). Comparison with p21 immunofluorescence on cells grown in Chee's medium revealed a 5-6 fold increase in nuclear p21 in cells grown in Waymouth/HamF12 medium.

It was concluded first, that TGF-β does not appear to effect a change in p21 or p27 localisation in acinar cells in Chee's medium, however p21 is present in the nucleus and presumably active, although its functional significance is unclear. Second, TGF-β does effect a change in p27 localisation in the 'ductal-like' like cells grown in Waymouth/HamF12 medium. Again p21 is present in these cells and its nuclear localisation suggests it is active, however TGF-β does not appear to effect a change. These differences between p21 and p27 localisation in the Chee's and Waymouth/HamF12 media may reflect differences in differentiation states.

Figure 5.15 – Immunofluorescence for p27 using Alexa 488 fluorophore (green) on acinar cells grown in Chee's or Waymouth/HamF12 media as detailed on figure. (a) cells treated with EGF for 18 hours, methanol fixed at X200 magnification. (b) cells treated with EGF and TGF-β for 18 hours and methanol fixed, X200 magnification. (c) cells treated with EGF for 18 hours, methanol fixed at X400 magnification.(d) cells treated with EGF and TGF-β for 18 hours and methanol fixed, X400 magnification. (e) cells treated with EGF for 18 hours and methanol fixed, X400 magnification. (f) cells treated with EGF and TGF-β for 18 hours and methanol fixed, X400 magnification. (g) cells treated with EGF for 18 hours and paraformaldehyde fixed, X630 magnification. (h) cells treated with EGF and TGF-β for 18 hours and paraformaldehyde fixed, X630 magnification.

Figure 5.15 – Effect of TGF- β on immunofluorescence for p27 on acinar cells grown in Chee's or Waymouth/HamF12's media

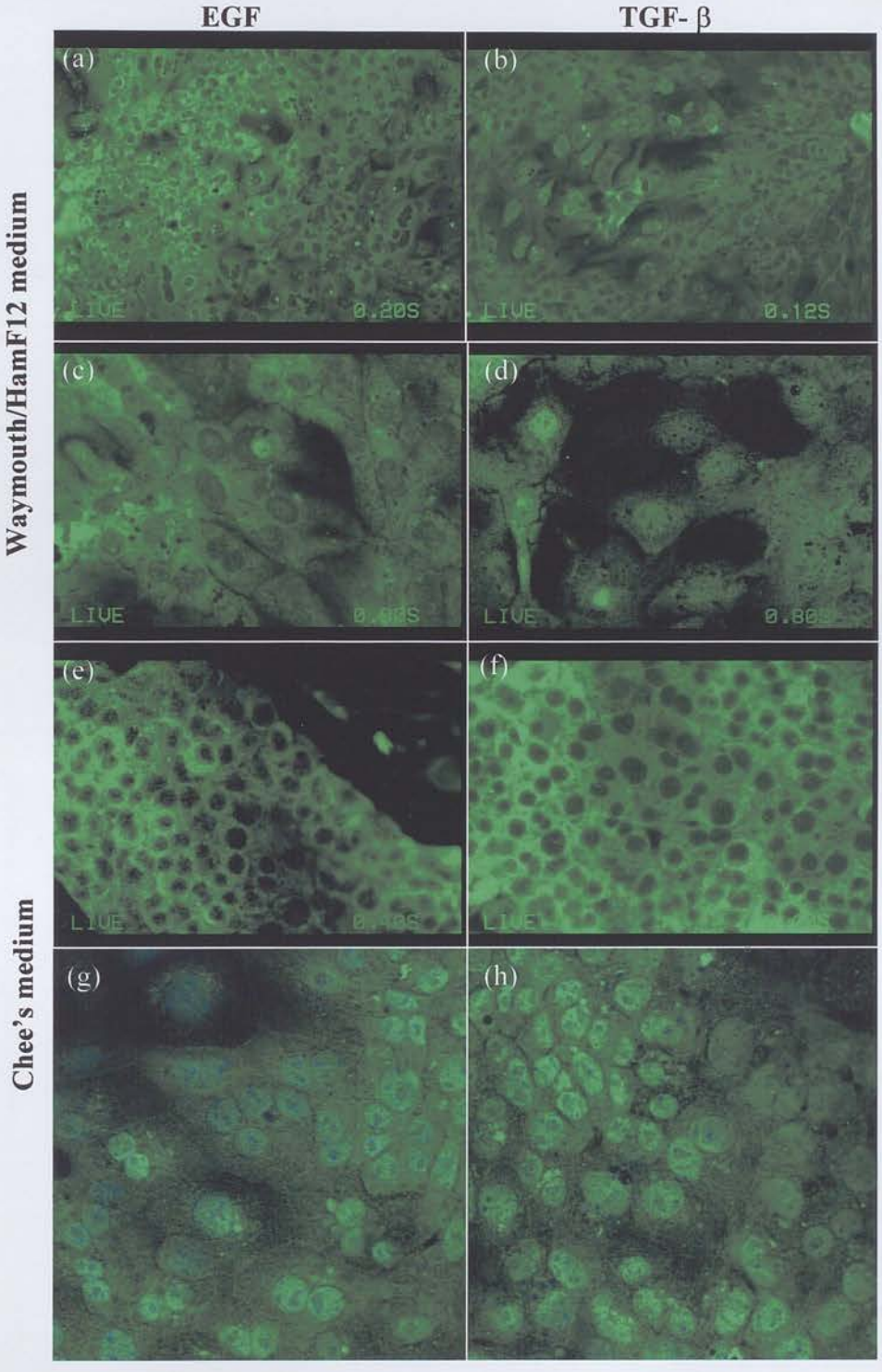
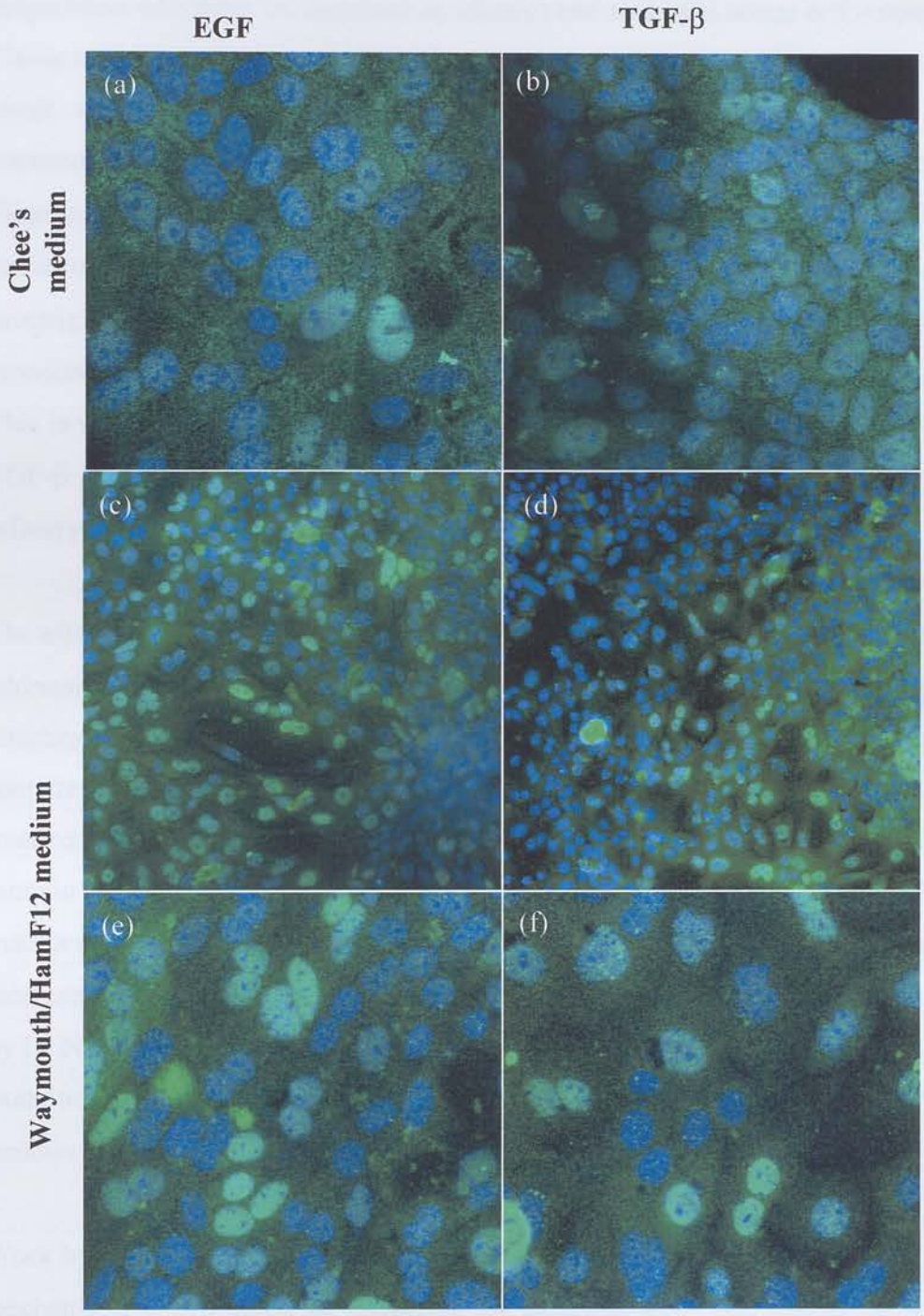


Figure 5.16 – Confocal microscope images of immunofluorescence for p21. The alexa 488 fluorophore (green) was used to visualise p21 positive nuclei on methanol fixed acinar cells grown in Chee's or Waymouth/HamF12 media, as indicated in the figure. Topro3 (blue) was used as a nuclear counterstain. (a), (c) and (e) Cells treated with EGF for 18 hours and (b), (d) and (f) cells treated with EGF and TGF-β for 18 hours. Pictures (c) and (d) were taken at X200 magnification all the others were taken at X630 magnification.

Figure 5.16 – Confocal microscope images of immunofluorescence for p21 on cells grown in Chee's and Waymouth/HamF12 media.



5.4 Conclusions, discussion and future work

In conclusion, investigations revealed a novel effect of TGF-β (early in culture) in conjunction with EGF, of increased attachment and increased acinar cell colony size. Characterisation revealed that this effect was not due to increased proliferation or a result of relatively high serum concentrations (15%), however high serum concentration and the presence of EGF appear to act as survival factors for the cells. Time-lapse video microscopy revealed that EGF and TGF-β treated cells exhibited an increase in mobility compared to EGF treated cells alone. Furthermore, investigation into the actin cytoskeleton of the EGF and TGF-β treated cells revealed pronounced leading edges consistent with the promotion of motility in these cells. This is in accordance with previous work by Boland *et al.* (1996), who showed that TGF-β promoted actin cytoskeleton reorganisation and a migratory phenotype in primary cultured epithelial tracheal cells.

The effect of TGF-β on ECM deposition and acinar cell adhesion molecules was not addressed in these studies. It is hypothesised, however, that the increased attachment, spreading and colony size in primary cultured acinar cells during the first four days of culture could be due to this. The acini have also been cultured on matrices other than collagen IV including fibronectin, collagen I, vitronectin and laminin and whilst the acini attach to a greater or lesser degree on each of these matrices, none of them exhibited the increased spreading, prominent processes and increased colony surface area seen in the TGF-β and EGF treated acini (observations by Dr N. Sphyris, personal communication). It would be of great interest therefore to examine whether various ECM components such as fibronectin, collagen, and laminin are upregulated in response to TGF-β.

Work by various other scientists has provided insights into other possible molecular mechanisms which could be mediating the effect of EGF and TGF-β on primary acinar cells. Lai *et al.* (2000) showed that TGF-β augmented the surface expression of αvβ5 integrin subunits on a murine osteoclast cell line via interaction of the

nuclear Co-SMAD/R-SMAD complex with Sp1, a ubiquitous nuclear transcription factor. The higher levels of integrins resulted in increased adhesion of the cells to vitronectin. Thus investigation into the expression of integrin subunits on EGF and TGF-β treated acini compared to EGF treated acini would provide insight into whether changes in integrin expression are partly responsible for the increased attachment, motility and thus colony size seen in EGF and TGF-β treated acini. Furthermore, RGD (Arg-Gly-Asp) peptides competitively bind integrins thus inhibiting adhesion. These could be of use in continuing investigation into the mechanism behind TGF-β and EGF mediated attachment and motility. As APC has been shown to localise to the edges of migrating epithelial cells (Mimori-Kiyosue *et al.*, 2000) it would be of interest here to investigate the localisation of APC in EGF and TGF-β treated acini.

The question remains as to whether PAI-1 is upregulated in EGF and TGF-β treated acinar cells. PAI-1 is a regulator of ECM homeostasis and cell motility. As mentioned previously TGF-β can induce PAI-1 expression in a SMAD dependent pathway (Datta *et al.*, 2000) and work with TGF-β sensitive renal epithelial cells showed that TGF-β induced both PAI-1 synthesis and cell motility. Use of the MEK inhibitor, PD98059, attenuated this effect of TGF-β on the renal cells showing that MEK was a mediator of TGF-β dependent PAI-1 expression and increased cell motility (Kutz *et al.*, 2001). This work by Kutz *et al.* suggested that molecular interaction of TGF-β and MAPK signalling could be mediating the effect of EGF and TGF-β on primary acinar cells. Preliminary experiments presented here (Figure 5.4 (g)) with the MEK inhibitor, U0126, resulted in complete ablation of attachment of primary acinar cells when they were treated with no growth factor, EGF, TGF-β or EGF and TGF-β. This suggests that the MAPK signalling pathway (of which MEK is a member) is vital in the attachment of primary cultured acini to culture plates, but did not provide evidence that molecular interaction of TGF-β and MAPK signalling was occurring in the EGF and TGF-β treated acinar cells.

Since neither EGF nor TGF-β can alone affect the increase in attachment, motility or colony size it is suggested that synergy is occurring between these two growth factors. Others working on hepatocytes have shown synergy between EGF and TGF-β resulting in a potentiation of SMAD3 transcriptional activation (Peron *et al.*, 2000). Stolz and Michalopoulos (1997) found that TGF-β significantly enhanced the chemotactic mobility of EGF stimulated hepatocytes. Furthermore, the cells only exhibited a decrease in DNA synthesis when TGF-β was added without EGF. Work with fetal hepatocytes showed first, that EGF and TGF-β co-operated to effect terminal maturation which involved the morphological changes consistent with a migratory phenotype and, second, that EGF could suppress TGF-β induced apoptosis in these cells (Sanchez *et al.*, 1998; Fabregat *et al.*, 1996). Finally research into caerulein-induced pancreatitis in rats revealed expression of first TGF-β mRNA and then EGF mRNA, 1 and 5 hours post caerulein infusion respectively, suggesting that both TGF-β and EGF may be needed to stimulate the regeneration of injured pancreas (Konturek *et al.*, 1997). Two hypotheses are therefore suggested, first, that in pancreatic acinar cells co-operative EGF and TGF-β signalling could be effecting changes in mobility, differentiation and the cell cycle consistent with the biological changes that occur in regeneration and wound healing in the pancreas. Second, that EGF and TGF-β act synergistically on primary pancreatic acinar cells to increase the invasive potential of these cells through ECM deposition, increased motility, enhanced expression of adhesion molecules, such as integrins and altered expression of matrix remodellers such as the MMPs and PAI-1. It is suggested that the action of EGF is two-fold, first, to augment the action of TGF-β in promoting cell motility and invasiveness and second, as a survival factor, protecting cells from the growth inhibitory actions of TGF-β. The work with CCK suggests that other growth factors can substitute EGF as a survival factor.

The second study involved the investigation of TGF-β on adherent acinar cells, later on in culture (day 5-10) in predominantly serum free conditions. Results showed TGF-β (in the absence and presence of EGF) decreased BrdU incorporation and increased apoptosis levels in both WT and *Smad4*$^{+/-}$ cells. It was not shown

conclusively that this effect was mediated through SMAD4 or that it was associated with the induction of p21. However results did reveal that this effect could be mediated in part by p27 under certain conditions. TGF-β treatment of acinar cells in the absence of EGF did result in a further decrease in BrdU incorporation compared to cells treated with both EGF and TGF-β. These conclusions are in contrast to previous work which showed that EGF completely inhibited the antiproliferative activity of TGF-β (Fabregat *et al.*, 1996). Lastly whilst these studies did not show that TGF-β-induced growth arrest was p21-dependent, future work could include investigations into the effect of TGF-β treatment on acinar cells from $p21^{-/-}$ mice.

Comparison between cells grown in Chee's medium or Waymouth/HamF12 media showed morphological and biological differences. Cells in Waymouth/HamF12 media exhibited giant nuclei, a 'tear drop' shape and increased spreading leading to a complete monolayer unlike the cells grown in Chee's. These cells had a higher proliferation rate in comparison to the cells grown in Chee's medium but TGF-β treatment still resulted in a significant decrease in BrdU incorporation. Immunofluorescent characterisation of the Waymouth/HamF12 cells showed (with methanol fixation) changes in p27 localisation upon TGF-β treatment. It could be that this nuclear p27 is indicative of cells entering a terminal quiescent state (Alexander and Hinds, 2001), however this is challenged by the fact that BrdU incorporation studies show that these TGF-β-treated cells are still undergoing DNA synthesis. It is suggested that these cells represent the 'ductal-like' cells characterised previously by Hall and Lemoine (1992), De Lisle and Logsdon (1990) and De Lisle *et al.* (1990) (see section 1.2.3).

Finally, investigations into whether the decreased BrdU incorporation and increased apoptosis following TGF-β treatment was associated with a cytoplasmic to nuclear translocation of SMAD4 yielded complex results. The unexpected dependency on fixation techniques as to the immunofluorescent localisation led to SMAD4 either appearing to be entirely cytoplasmic, even following TGF-β treatment (methanol fixation), or SMAD4 appearing to be constitutively nuclear even in the absence of TGF-β (paraformaldehyde fixation). Methanol fixation acts by disrupting the

hydrophobic bonds within proteins, this affects the tertiary structure of proteins but not the secondary structure. Paraformaldehyde fixation acts by forming cross-links between proteins, creating a gel and thus retaining cellular constituents in their *in vivo* relationships (reviewed in Woods and Ellis, 1994). As the integrity of cellular and nuclear membranes is predominantly protected by hydrophobic and hydrophilic interactions this would suggest that paraformaldehyde fixation is most appropriate for the analysis of nuclear proteins. Western blotting on nuclear and cytoplasmic extracts from TGF-β treated and untreated acinar cells showed that SMAD4 was present in the nucleus in both conditions. Densitometry did however suggest a slight increase in nuclear SMAD4 following TGF-β treatment. Work on the nuclear localisation and export signals of SMAD1 and SMAD3 showed that these R-SMADs were constantly shuttling between the cytoplasm and nucleus whether the TGF-β signalling pathway was activated or not. SMAD4, on the other hand, could only translocate to the nucleus when bound to an R-SMAD or when the SMAD4 MH1 domain was altered to represent the nuclear localisation signal (NLS) of the R-SMADs (Xiao *et al.*, 2000; 2001). In contrast to this, work by Pierreux *et al.*, (2000) showed that in the absence of TGF-β, LMB treatment of HaCaT cells resulted in nuclear SMAD4 and cytoplasmic SMAD2 and 3. In the presence of TGF-β, SMAD2 and 3 in addition to SMAD4 were locked in the nucleus by LMB. They suggest, in the absence of TGF-β, that SMAD4 constantly shuttles between the nucleus and cytoplasm and due to a nuclear export signal (NES) within the protein SMAD4 is rapidly and constantly exported from the nucleus. In the presence of TGF-β SMAD2 and 3 are activated, inhibit the SMAD4 NES and can translocate to the nucleus. Thus the complex remains in the nucleus and can activate transcription. Furthermore the authors showed that prolonged TGF-β signalling resulted in the export of SMAD2, SMAD3 and SMAD4 from the nucleus. The results presented in this chapter appear to support the work by Pierreux *et al.* in that SMAD4 appears nuclear in the absence and presence of TGF-β. The process of methanol fixation appears to therefore cause a leaking of SMAD4 protein from the nucleus whereas paraformaldehyde locks it in. It is therefore concluded that immunohistochemical analysis of SMAD4 nuclear translocation is not an appropriate method through which to assess activation of the SMAD signalling pathway by TGF-β. It would be

of great interest to investigate the subcellular localisation of both active and inactive R-SMADs (for example, SMAD2 and phospho-SMAD2 respectively) in response to TGF-β and to explore the status of various downstream targets.

The two studies outlined here highlight interaction between the TGF-β and EGF signalling pathways. The multifunctional nature of TGF-β signalling is also reflected in these studies: first, TGF-β was shown to result in increased attachment, mobility and colony size in freshly prepared acini with no effect on proliferation; second, TGF-β was shown to result in decreased DNA synthesis and increased apoptosis. As outlined in the introduction, in tumourigenesis TGF-β has also been shown to exhibit these divergent behaviours. This system of primary cultured pancreatic acinar cells is therefore an interesting tool through which to further investigate the dual role of TGF-β signalling in the pancreas.

Chapter 6 – General discussion

The central focus of this thesis has been investigation into the effect of *Smad4* mutation on pancreatic tumourigenesis. This was approached via three main methods using mice containing single or compound mutations in *Smad4*, Apc^{Min} and *p53*. First (chapter 3), $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ and $p53^{-/-}Smad4^{+/-}$ animals were generated to investigate whether *Smad4* mutation would promote pancreatic tumourigenesis in the previously described $p53^{-/-}Apc^{Min/+}$ and $p53^{-/-}$ mice (Clarke *et al.*, 1995; Purdie *et al.*, 1994; Jacks *et al.*, 1994). The majority of abnormal pancreatic preneoplastic acinar cell foci, adenomas, cystic adenomas and adenocarcinomas were found to exhibit LOH for *Smad4* and *Apc*. However, *Smad4* heterozygous mutation did not appear to lead to differences in the spectrum of histologically identifiable lesion types in comparison to $p53^{-/-}Apc^{Min/+}$ mice. It was therefore concluded that *Smad4* heterozygous mutation did not lead to a progression in pancreatic tumourigenesis. Analysis of NMU-induced pancreatic tumourigenesis (chapter 4) in singly mutant $Smad4^{+/-}$ and $Apc^{Min/+}$ mice and compound mutant $Apc^{Min/+}Smad4^{+/-}$ mice revealed contrasting results. Here, *Smad4* heterozygosity in conjunction with Apc^{Min} heterozygosity resulted in increased pancreatic dysplasia, whereas *Smad4* or Apc^{Min} heterozygous mutation alone did not. The abnormal pancreatic acinar cell foci arising in the $Apc^{Min/+}Smad4^{+/-}$ mice were not characterised by *Smad4* LOH as revealed by the presence of SMAD4 protein in the majority of abnormal foci.

Speculation regarding this contrasting effect of *Smad4* heterozygous mutation and presence of *Smad4* LOH has led to the suggestion that the results of the experiments in chapter 3 and in chapter 4 represent two distinct pathways in pancreatic tumourigenesis. It is postulated that the first pathway is associated with *Smad4* LOH in pancreatic adenomas and adenocarcinomas from aged cohorts of $p53^{-/-}$ $Apc^{Min/+}Smad4^{+/-}$ mice whereas the second pathway is associated rather with mutation in other genes, for example, mutational targets of NMU, such as *K-ras*. In the latter pathway monoallelic inactivation of *Smad4*, in conjunction with these other hypothesised mutations, elicits an effect on initiation of tumourigenesis. As no

adenocarcinomas were observed in the NMU-induced pancreatic tumourigenesis it is suggested that monoallelic inactivation of *Smad4* in these mice effects the early stages of the tumourigenic process. Conversely, in the aged cohorts of $p53^{-/-}$ $Apc^{Min/+}Smad4^{+/-}$ mice, the absence of these additional mutations could mean that monoallelic inactivation of *Smad4* does not elicit an effect on the early stages of tumour initiation, rather there is a selective pressure towards *Smad4* LOH. Since the $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ mice exhibit large adenomas and adenocarcinomas (absent from the NMU-treated mice), *Smad4* LOH appears to be associated with the later stages of murine pancreatic tumourigenesis. This hypothesis could be tested by detailed investigation into pancreas from $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ mice at earlier time points, before the animals become moribund.

An alternative hypothesis could be that loss of p53 is associated with biallelic inactivation of *Smad4*. NMU has not been shown to cause *p53* mutation therefore it is reasonable to suggest that the lesions arising in the NMU-treated mice have not lost *p53*. Perhaps the genetic instability associated with *p53* deficiency in the aged $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ mice was instrumental in the inactivation of the WT *Smad4* allele. No functional effect of *Smad4* LOH was revealed in the studies contained in this thesis. However, it is logical to suggest that perhaps LOH does elicit a functional effect, but that either the animals became moribund before it was histologically visible, or that the effect is evidenced at a molecular or cellular level that is not reflected in the analyses performed here. Future experiments investigating when the *Smad4* LOH occurs in the $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ mice would help unravel the role that it is playing.

The question as to the relative effect of monoallelic versus biallelic inactivation of *Smad4* is of importance. Work with $Apc^{\Delta 716/+}$ and $Smad4^{+/-}$ compound heterozygotes and $Smad4^{+/-}$ mice (Takaku *et al.*, 1998; Xu *et al.*, 2000, see section 4.1.2) has led to the suggestion that haploid insufficiency of *Smad4* is sufficient for tumour initiation and that biallelic loss of *Smad4* is instrumental in the progression of tumourigenesis. In accordance with this Wagner *et al.* (2001) found *Smad4* LOH to be a characteristic of pancreatic adenocarcinomas in $p53^{-/-}$animals which overexpressed TGFα in the

pancreas. Human pancreatic cancers have been shown to have *SMAD4* biallelically inactivated (either through deletion or mutation) in 50% of adenocarcinomas (Hahn *et al.*, 1996; Schutte *et al.*, 1996). Furthermore it has been shown in human colorectal cancer that *SMAD4* LOH occurs as a late event (Miyaki *et al.*, 1999). Interestingly, however, biallelic inactivation of *SMAD4* is rare in FJP (see 1.7.2.4), rather this inherited syndrome is associated with a heterozygous functionally inactivating mutation of *SMAD4* that results in a truncated SMAD4 protein (Howe *et al.*, 1998; Friedl *et al.*, 1999). Whilst the importance of the biallelic inactivation of *Smad4* observed in the $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ mice is inconclusive and in need of further investigation, the work presented in this thesis does support the concept that *Smad4* LOH is associated with late stage tumours but that haploid insufficiency of *SMAD4* is sufficient for tumour initiation.

The *in vivo* studies outlined in chapter 4 revealed that *Smad4* heterozygous mutation (in conjunction with Apc^{Min} mutation) could exert a functional effect on pancreatic acinar cells. The *in vitro* studies, however, investigating the biological effect of *Smad4* heterozygous mutation in pancreatic acinar cells (chapter 5, section 5.4.2 and 5.4.3) revealed that, like WT cells, $Smad4^{+/-}$ acinar cells were capable of exerting TGF-β-induced decrease in BrdU incorporation and increase in apoptosis. It could therefore be concluded that *Smad4* heterozygous mutation only has a functional effect in conjunction with other mutations. Had time permitted this hypothesis could have been tested via the generation of $Apc^{Min/+}Smad4^{+/-}$ acinar cells. A second hypothesis could be that, functionally, *Smad4* heterozygous mutation effects cellular activities other than cell turnover and apoptosis. However it seems more likely that complete deficiency of *Smad4* rather than haploinsufficiency is required for loss of TGF-β-induced SMAD-mediated cell proliferation and apoptosis. Thus from the *in vitro* experiments it appears that *Smad4* heterozygosity does not elicit a functional effect. Whilst efforts to generate $Smad4^{-/-}$ acinar cells were unsuccessful, future work could involve inactivation of the pathway by other means, for example, transfection of the cells with a dominant negative SMAD4, the preparation of pancreatic acinar cells from $Smad3^{-/-}$ animals or overexpression of *Smad6* or *Smad7* (the I-SMADs). The *in vitro* investigations in chapter 5 revealed the fact that loss of

Smad4 may not only affect cell proliferation and apoptosis but could also effect the invasive potential of pancreatic acinar cells.

The *in vivo* studies also highlighted dysregulation of the Wnt signalling pathway as a common feature in pancreatic acinar tumourigenesis in both the aged cohorts of $p53^{-/-}Apc^{Min/+}Smad4^{+/-}$ (chapter 3) and the NMU-treated animals (chapter 4). This was evidenced by increased levels of β-catenin within abnormal foci, adenomas and adenocarcinomas. The most obvious explanation for this is *Apc* LOH (as previously shown by Kongkanuntn *et al.*, 1999 and Clarke *et al.*, 1995) resulting in loss of APC-mediated degradation of β-catenin. However this effect could also have been the result of mutation in other APC-interacting proteins, for example, *β-catenin* itself, *GSK3β* and *E-cadherin*.

APC has various other non Wnt related functions (see section 1.8.2). In addition to these APC has recently been shown to have a role in the maintenance of chromosome stability (Fodde *et al.*, 2001; Kaplan *et al.*, 2001). APC localises to the kinetochore of metaphase chromosomes, and this localisation is likely to be dependent on the interaction of APC with EB1. *Apc* deficient mouse cells have been shown to have two types of chromosomal abnormalities, quantitative changes such as polyploidy and structural rearrangements causing chromosome translocations (Fodde *et al.*, 2001). Accordingly similar types of chromosomal instability have been found in human colorectal cancers (Thiagalingam *et al.*, 2001). The morphological investigations into nuclear atypia (chapter 4, section 4.4.6) did not find Apc^{Min} heterozygous mutation to be associated with prominent changes in the distribution of nuclear area. However, this association of *Apc* mutation with increased chromosome instability could provide an additional explanation as to why *Apc* mutation promotes pancreatic tumourigenesis. Perhaps in particular it could provide insight into the co-operation between *p53* and *Apc* mutation.

Whilst *Apc* LOH has previously been shown to be a characteristic of early human and murine colorectal tumourigenesis. The work presented here suggests that this is also the case in murine pancreatic tumourigenesis. This appears not to be the case in

human ductal pancreatic tumourigenesis where *APC* is mutated rarely (Horii *et al.*, 1992; McKie *et al.*, 1993). Recently, however, the APC/β-catenin pathway has been found to be mutated in 23% of cases of the rarely occurring human acinar adenocarcinoma (Abraham *et al.*, 2002) suggesting that in humans *APC* mutation is associated with both acinar and ductal tumourigenesis. This raises the possibility that the chemotherapeutic effect of aspirin reported in chapter 4 could be of more relevance to human acinar adenocarcinomas than the more common ductal adenocarcinomas.

The investigations outlined in this thesis have explored the effect of *Smad4* mutation in a variety of situations. It was found that both *Smad4* haploinsufficiency and complete deficiency are characteristics of murine pancreatic tumourigenesis. Also it was shown that the effect of *Smad4* mutation is modulated by the presence of other mutations (such as Apc^{Min}). Furthermore the *in vitro* investigations demonstrated that *Smad4* mutation could have a variety of biological effects depending on the cellular environment and integration of multiple signalling pathways. Whilst the animals used in these experiments may not have been found to provide a model of human pancreatic ductal adenocarcinoma they have provided useful insights into the role of *Smad4* mutation in the pancreas.

Appendix A – Solutions and reagents used

Agarose gel electrophoresis loading buffer

0.1%	Bromophenol blue
0.1M	EDTA
50% v/v	glycerol

4% BSA (Bovin Serum Albumin)

10g	cell culture tested BSA (Sigma)
250ml	Serum free medium (p242)

Filter sterilise before use and store at 4°C.

Collagenase (Lorne Laboratories)

1000units	collagenase
10ml	Serum free media

Aliquot into 700µl aliquots, store at -20°C
Immediately before use dilute one aliquot with a further 9.3ml of serum

DAB Buffer

0.2M	Tris
0.1M	HCl
0.01M	Imidazole

Add 25mg/ml of the chromogen DAB upon use

Denhardt's solution (50X)

0.5g	BSA
0.5g	Polyvinyl pyrollidone
0.5g	Ficoll

Make up in 50ml DDW and store aliquoted in freezer

Dexamethasone (Sigma)

1mg Dexamethasone is dissolved in 1ml absolute EtOH
Add 24.5ml Serum Free medium or DMEM to give 100µM stock.
Use at 100nM (1:1000 dilution)

High Salt Buffer

20mM	HEPES
420mM	NaCl
1.5mM	$MgCl_2$
2mM	Na_2 EDTA

Hybridisation solution for 10 slides(40µl/slide)

10-20µl	labelled probe (20ng/µl)
20µl	Salmon sperm DNA (500µg/ml, stored in TE buffer)
200µl	20% Dextran sulphate
100ul	20X SSC
70µl	DDW

IBMX (3-isobutye-1-methylxanthine, Sigma)

1g	IBMX
8.992ml	1M NaOH

Store in 100µl aliqouts

Invitrogen Sample Buffer (pH 8.5)

10%	Glycerol
141mM	Tris base
106mM	Tris-HCl
2%	LDS
0.51mM	EDTA
0.22mM	SERVA Blue G250
0.175mM	Phenol Red

Invitrogen NuPAGE MOPS SDS running buffer (pH 7.7)

50mM	MOPS
50mM	Tris Base
0.1%	SDS
1mM	EDTA

Invitrogen transfer buffer (pH 7.2)

25mM	Bicene
25mM	Bis-tris (free base)
1mM	EDTA
0.5mM	Chlorobutanol

Lysis Buffer for nuclear and cytoplasmic protein extraction

10mM	HEPES
10mM	$MgCl_2$
10mM	Na Orthovanadate

Microdissection lysis buffer (pH 8.3)

1mg/ml	Proteinase K
1%	Tween
34mM	Tris
0.6mM	EDTA

Paraformaldehyde (pH 7.4)

3.7g	Prill paraformaldehyde
100ml	PBS

Heat at 60°C in fume hood until dissolved
Leave to cool
pH to 7.4
Aliquot and freeze

PBS (phosphate buffered saline)

120mM	NaCl
2.7mM	KCl
10mM	phosphate buffer salts (KH_2PO_4 and $Na_2HPO_4.7H_2O$)

PCR Buffer (10X, Gibco)

200mM	Tris-HCl (pH 8.4)
500mM	KCl

Prehybridisation solution for 10 slides (35µl/slide)

40µl	Denhardt's solution (50X)
180µl	Deionised formamide (Toxic)
120µl	SCC (20X)
20µl	Salmon sperm DNA
40µl	DDW

Primary Cell Culture Media – 100mls

	Serum free medium (SeF)	Plating media with 15% serum (15% PM)	Modified Chee's medium	Waymouth/ HamF12 medium
Fetal Calf Serum (filtered)	-	15ml	-	2ml
DMEM	99ml	83.8ml	-	-
Chee's medium	-		97ml	-
Waymouth's medium	-	-	-	47.9ml
Ham's F12 medium	-	-	-	47.9ml
Antibody/Antimycotic (100X)	1ml	1ml	1ml	1ml
Dexamethasone	-	100µl	100µl	100µl
IBMX (in 1M NaOH)	100µl	100µl	100µl	100µl
L-glutamine	-	-	1ml	-
SBTI (0.2mg/ml)	20ng in 100µl	-	-	-
ITS-X (insulin transferrin selenium X, Sigma)		-	1ml	1ml

Puregene cell lysis solution

100mM	Tris (hydroxymethyl) aminomethane
5mM	EDTA
0.2%	SDS

Puregene protein precipitation solution

9.5M	Ammonium acetate

Puregene DNA hydration solution

100mM	Tris (hydroxymethyl) aminomethane
50mM	EDTA

RIPA Buffer

50mM	Tris HCl pH 7.5
150mM	NaCl
1%	Nonidet p40
0.5%	Sodium deoxycholate
0.1%	SDS (sodium dodecyl sulfate)

SBTI (soya bean trypsin inhibitor) (Sigma) 0.2mg/ml

100mg	SBTI
500ml	DMEM

Filter sterilise

Store at -20°C in aliquots.

SSC (standard saline-citrate) 20X

3M	NaCl
0.3M	trisodium citrate

pH 7-7.4

TBE (tris-borate EDTA)

0.089M	Tris
0.089M	Boric Acid
0.0025M	EDTA

TBS (tris buffered saline) pH 7.4

0.15M	NaCl
0.5M	Tris-HCl

TCA 10% (Highly corrosive)

10g	TCA
100ml	DDW

Appendix B – The generation of *Smad4*$^{-/-}$ acinar cells

B.1 Introduction

B.1.1 Cre-*loxP* technology

The placement of Cre recombination sites (*loxP* sites) into the genome and targeted or controlled expression of Cre recombinase allows researchers to either turn on or off any gene of interest in transgenic or gene modified mice. The use of Cre-l*oxP* technology has allowed researchers to control the tissue specificity and time of inactivation of genes which have been shown to be lethal in a traditional knockout model. Various strategies have now been developed to control the expression of Cre, including the delivery of Cre coding sequence by viral systems. For example, efficient Adenovirus Cre-mediated deletion of *DNA ligase1* and *Rb* floxed alleles has been achieved in primary hepatocytes (Prost *et al.*, 2001).

B.1.2 The generation of *Smad4* Floxed mice

Since *Smad4* homozygous mutation is embryonic lethal (Sirard *et al.*, 1998) a strategy was devised whereby researchers (Dr E.K. Duff and colleagues) would generate a mouse containing a floxed (i.e. flanked by *loxP* sites) *Smad4* allele. It was intended to flox exon 1 of the *Smad4* allele, which contains the ATG start codon. Unlike the *Smad4*$^{-/-}$ mice this animal would be viable, however controlled Cre-mediated recombination would result in the ablation of SMAD4 protein expression in tissues of interest. Figure B.1 shows the targeting strategy employed by Dr Duff. A 3' Southern blot was consistent with the hypothesis that the targeting vector had indeed recombined with the *Smad4* allele. However a 5' Southern blot was not successful because of technical problems. Two PCR reactions were also devised within the targeting vector to show presence of both *LoxP* sites, these are shown in Figures B.2 and B.3.

Figure B.1 – Overall targeting strategy

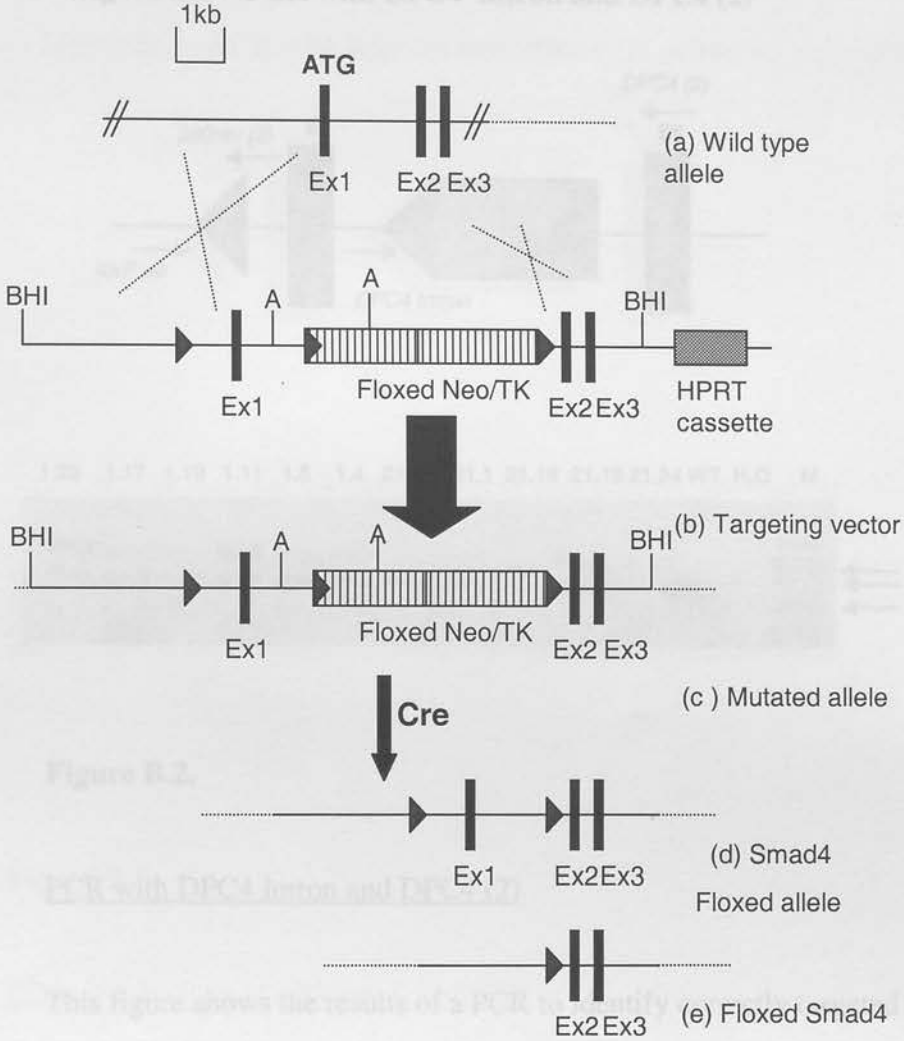


Figure B.1. This figure shows the overall strategy employed to achieve conditional deletion of the *Smad4* allele. To generate the targetting vector a floxed Neomycin/Thymidine Kinase cassette was cloned into a single Bln site approximately 1kb downstream of Exon I and about 1kb upstream from Exon II of the murine Smad4 gene. A single loxP fragment was then cloned into the Nco I site of the 8kb genomic clone, a site determined to be 700bp upstream of Exon I. Finally an HPRT cassette was introduced outside the 3' region of homology. The rational behind this is that if the targeting construct were to integrate randomly (that is not through homologous recombination) then the HPRT cassette would remain present in the DNA and could be selected against using the drug 6-thioguanine (6-TG). The plan is to "flox" the ATG within exon 1 of the *Smad4* locus such that expression of Cre recombinase will result in excision of exon 1 and a resultant null allele.
Figure reproduced by kind permission of Dr E.K. Duff (PhD Thesis, 2000, University of Edinburgh).

Figure B.2 – PCR with DPC4 intron and DPC4 (2)

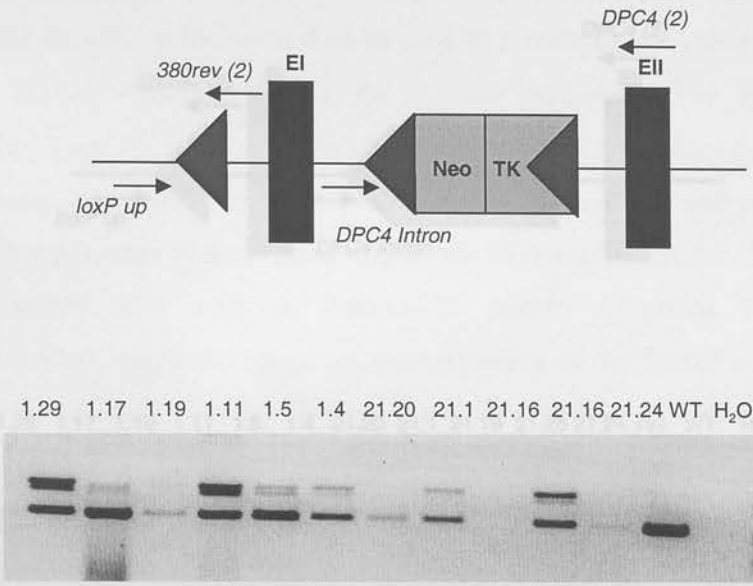


Figure B.2.

PCR with DPC4 Intron and DPC4 (2)

This figure shows the results of a PCR to identify correctly targeted ES cell clones which have lost the floxed Neo/TK cassette as a result of exposure to Cre recombinase. The primers "DPC4 Intron" and "DPC42" are designed to amplify a wild type band of approximately 230bp whereas the presence of a single loxP site remaining after recombination between the two loxP sites at either side of the cassette should increase this band by approximately 70bp to 300bp. Positive clones will therefore show two bands. Figure reproduced by kind permission of Dr E.K. Duff (PhD Thesis, 2000, University of Edinburgh). This PCR was used in conjunction with the one shown in Figure B.3 to genotype the *Smad4* floxed animals.

Figure B.3 - PCR with loxP up and 380rev (2) primers

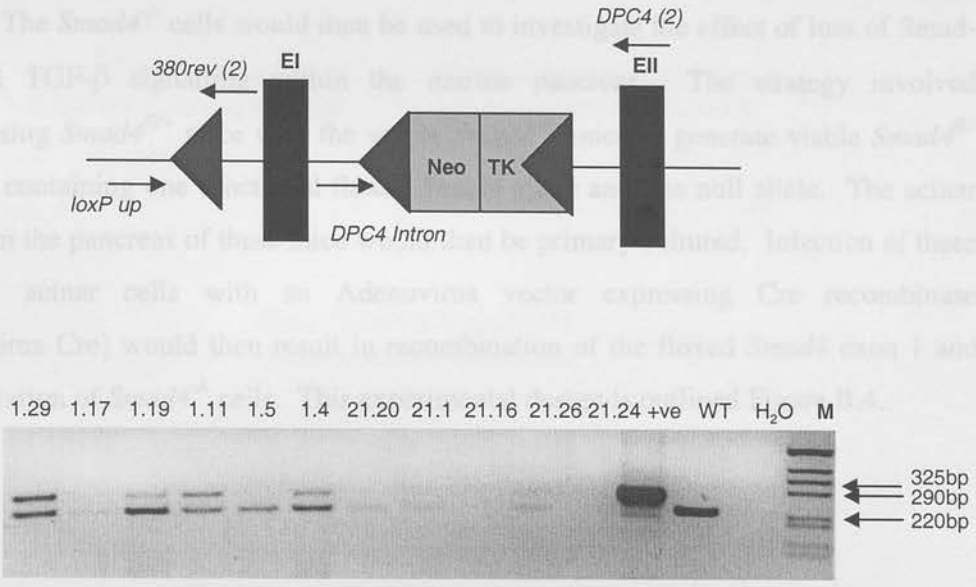


Figure B.3

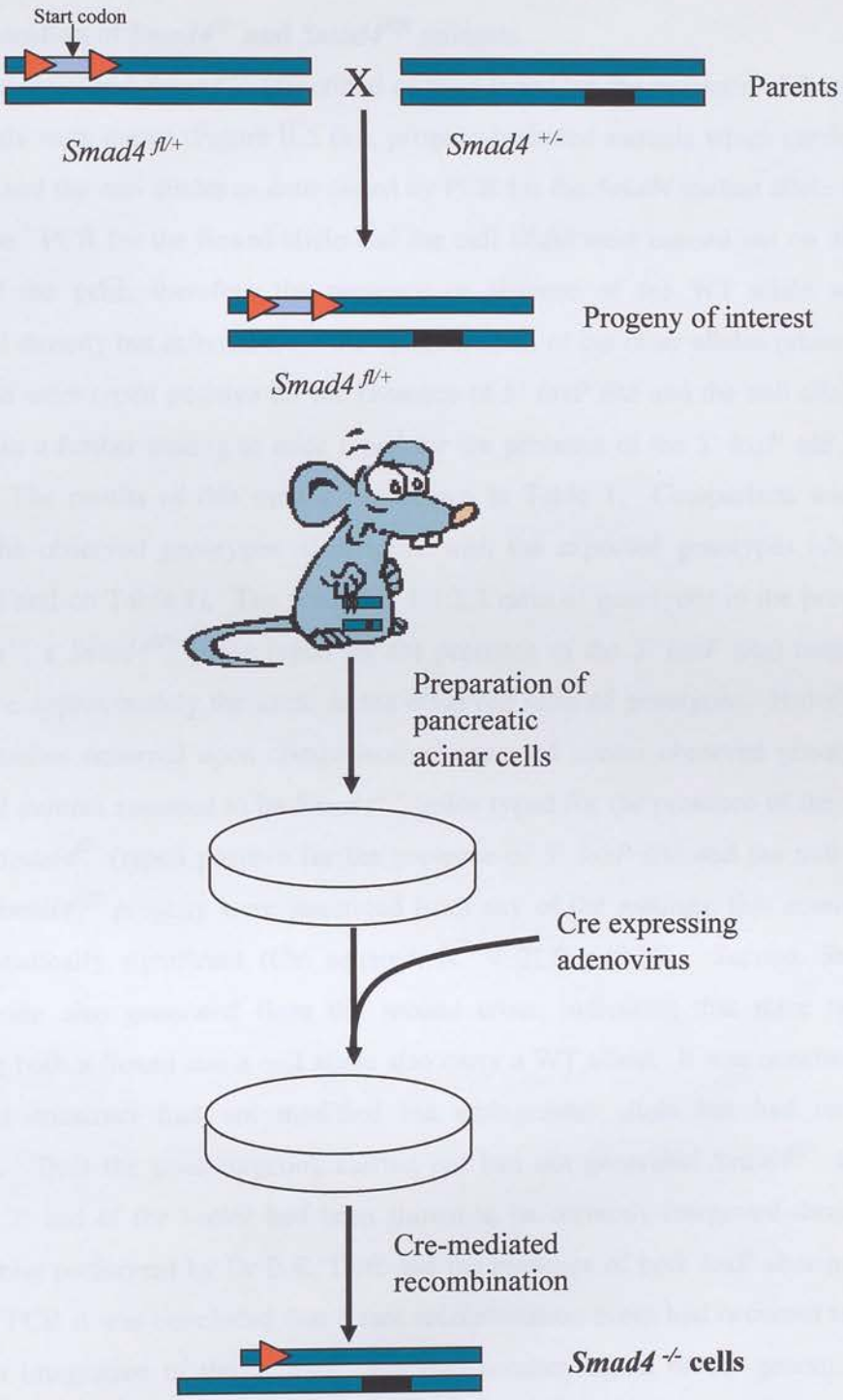
PCR with loxP up and 380rev (2) primers

This figure shows the results of a PCR to detect the 5' *loxP* site within the putative targeted allele. ES cell clones 1 and 21 were tested after exposure to Ganciclovir to remove the floxed Neo/TK. The primers "loxP up" and "380rev (2)" are designed to amplify a band of approximately 220bp with wild type DNA and 280bp when a single loxP site is present. Clones correctly targeted at the 5' end show two bands. Figure reproduced by kind permission from Dr E.K. Duff (PhD Thesis, 2000, University of Edinburgh). This PCR was used in conjunction with the one shown in Figure B.2 to genotype the *Smad4* floxed animals.

B.1.3 Experimental design

A strategy was designed which would allow the generation of *Smad4*$^{-/-}$ acinar cells in culture. The *Smad4*$^{-/-}$ cells would then be used to investigate the effect of loss of Smad-mediated TGF-β signalling within the murine pancreas. The strategy involved intercrossing *Smad4*$^{fl/+}$ mice with the viable *Smad4*$^{+/-}$ mice to generate viable *Smad4*$^{fl/-}$ animals, containing one functional floxed *Smad4* allele and one null allele. The acinar cells from the pancreas of these mice would then be primary cultured. Infection of these *Smad4*$^{fl/-}$ acinar cells with an Adenovirus vector expressing Cre recombinase (Adenovirus Cre) would then result in recombination of the floxed *Smad4* exon 1 and the generation of *Smad4*$^{-/-}$ cells. This experimental design is outlined Figure B.4.

Figure B.4 - Experimental design for generation of *Smad4*$^{-/-}$ acinar cells



B.2 Results and conclusions

B.2.1 Generation of *Smad4*$^{fl/-}$ and *Smad4*$^{fl/fl}$ animals

Initially *Smad4*$^{+/-}$ and *Smad4*$^{fl/+}$ (described as mice typed for the presence of the 5' *loxP* site) animals were mated (Figure B.5 (a)), progeny included animals which carried both the floxed and the null alleles as determined by PCR for the *Smad4* mutant allele and the 5' *loxP* site. PCR for the floxed allele and the null allele were carried out on different regions of the gene, therefore the presence or absence of the WT allele was not determined directly but inferred from the determination of the other alleles present. The mice which were typed positive for the presence of 5' *loxP* site and the null allele were then used in a further mating to mice typed for the presence of the 5' *loxP* site (Figure B.5 (b)). The results of this mating are shown in Table 1. Comparison was made between the observed genotypes of progeny with the expected genotypes (shown in Figure B.5 and on Table 1). The predicted 1:1:1:1 ratio of genotypes in the progeny of the *Smad4*$^{+/-}$ x *Smad4*$^{fl/+}$ (mice typed for the presence of the 5' *loxP* site) mating was found to be approximately the same as the observed ratio of genotypes. However two main anomalies occurred upon comparison of expected versus observed genotypes of progeny of parents assumed to be *Smad4*$^{fl/+}$ (mice typed for the presence of the 5' *loxP* site) and *Smad4*$^{fl/-}$ (typed positive for the presence of 5' *loxP* site and the null allele). First, no *Smad4*$^{fl/fl}$ progeny were generated from any of the matings, this absence was highly statistically significant (Chi squared, $X^2 = 21.9$ $p<0.05$). Second, *Smad4*$^{+/+}$ animals were also generated from the second cross, indicating that mice typed as possessing both a floxed and a null allele also carry a WT allele. It was concluded that the floxed construct had not modified the endogenous allele but had integrated elsewhere. Thus the gene targeting carried out had not generated *Smad4*$^{fl/+}$ animals. Since the 3' end of the vector had been shown to be correctly integrated through the Southern blot performed by Dr E.K. Duff and the presence of both *loxP* sites had been shown by PCR it was concluded that a rare recombination event had occurred resulting in random integration of the 5' *loxP* site into another region of the genome. This phenomenon has been documented by other researchers (Adair *et al.*, 1989)

Figure B.5- Predicted progeny from *Smad4* mutant and Floxed mice matings

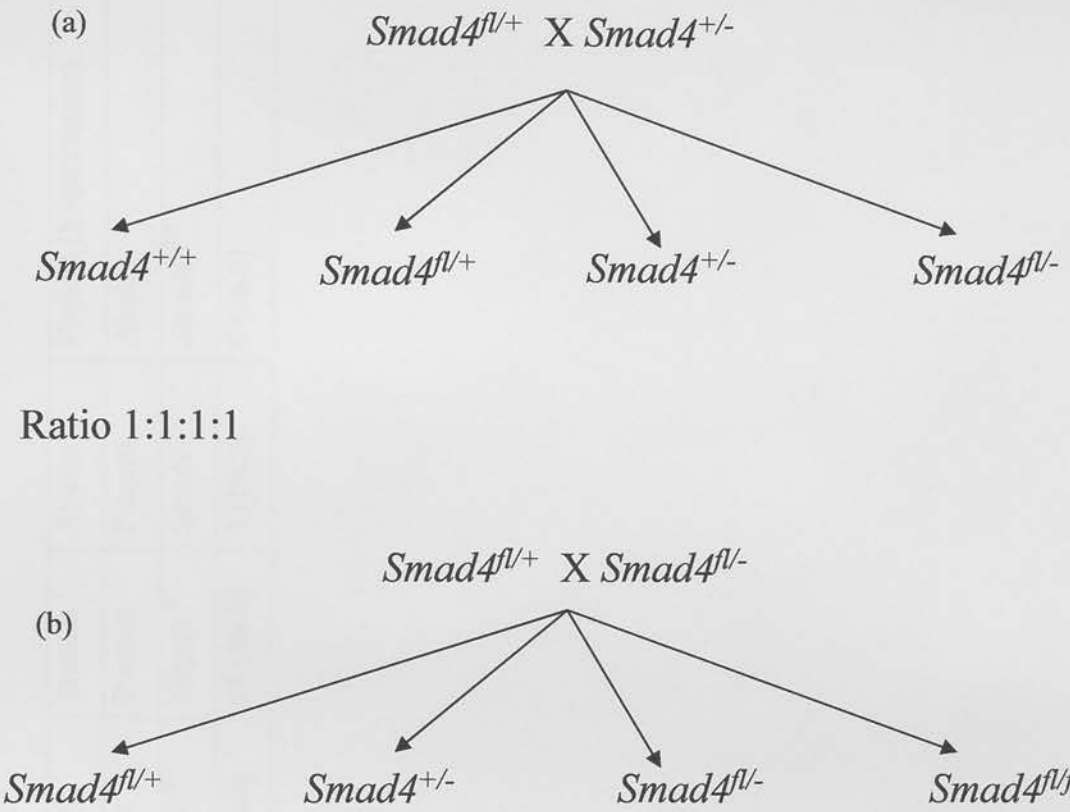


Figure B.5 - Prediction of progeny from *Smad4* mutant mice and floxed mice mating showing expected ratios. Note that predictions were based on the assumption that the targeting event had occurred as predicted.

Table B.1 – Observed and predicted (shown in brackets) outcomes from matings involving *Smad4* floxed mice

	Parent 1	**Parent 2**	**Offspring**				
Floxed allele	present	Present	Absent	Present	Present	Absent	Present (homozygous)
Null allele	present	Absent	Absent	Absent	Present	Present	Absent
Inferred genotype	*Smad4* $^{fl/-}$	*Smad4* $^{fl/+}$	*Smad4* $^{+/+}$	*Smad4* $^{fl/+}$	*Smad4* $^{fl/-}$	*Smad4* $^{+/-}$	*Smad4* $^{fl/fl}$
TOTAL			**12 (0)**	**32 (16.5)**	**15 (16.5)**	**7 (16.5)**	**0 (16.5)**

B.2.2 Investigations into Adenovirus Cre infection efficiency

Preliminary investigations were also carried out on the infection efficiency of primary cultured acinar cells. *R26R* mice contain a *β-galactosidase* gene at the ROSA26 locus which is not expressed due to the presence of a floxed stop cassette containing a number of elements that block expression (Mao *et al.*, 1999). Upon Cre-mediated recombination the stop codon is excised and the β-galactosidase enzyme is produced. When these cells are incubated with a substrate for β-galactosidase the ensuing reaction results in the production of a blue colour. Thus cells which turn blue have been successfully infected with the Adenovirus Cre and undergone Cre-mediated recombination. Acinar cells were prepared from *R26R* mice (a kind gift from Dr Shirley O'Dea) and preliminary optimisation investigations were carried out varying: the number of adenovirus added (2.5×10^4 – 9.0×10^6 plaque forming units or pfu); the incubation time with the adenovirus containing media and the time post-infection before fixation. Figure B.6 shows the result of incubation of these cells with X-gal, the β-galactosidase substrate. Previous work involving infection of primary cultured hepatocytes with Adenovirus Cre found that when cells were incubated with adenovirus for 1 hour and then left for 24 hours, >95% efficiency of infection was obtained (Prost *et al.*, 2001). Results showed that these primary cultured acinar cells exhibited very low infection efficiency (<0.5%) even at high concentrations (9.0×10^6 pfu) when the virus was left on for up to 7 hours. As others have shown that primary cultured acinar cells in suspension can be efficiently infected with 5×10^6 pfu of adenovirus (Padfield *et al.*, 1998), it was concluded that either the Adenovirus Cre stock had expired or that the uptake and expression of Adenovirus Cre in our system was very poor.

Therefore due to the low Adenovirus Cre infection efficiency and the discovery that the *Smad4*$^{fl/+}$ mice had been incorrectly generated, it was decided to abandon the initial plan of generating *Smad4*$^{-/-}$ acinar cells in culture. Investigations were then entirely focused on the effect of TGF-β on WT and *Smad4*$^{+/-}$ acinar cells.

Figure B.6 – Initial optimisation of Adenovirus Cre treated acinar cells containing *Flox-stop LacZ* alleles.

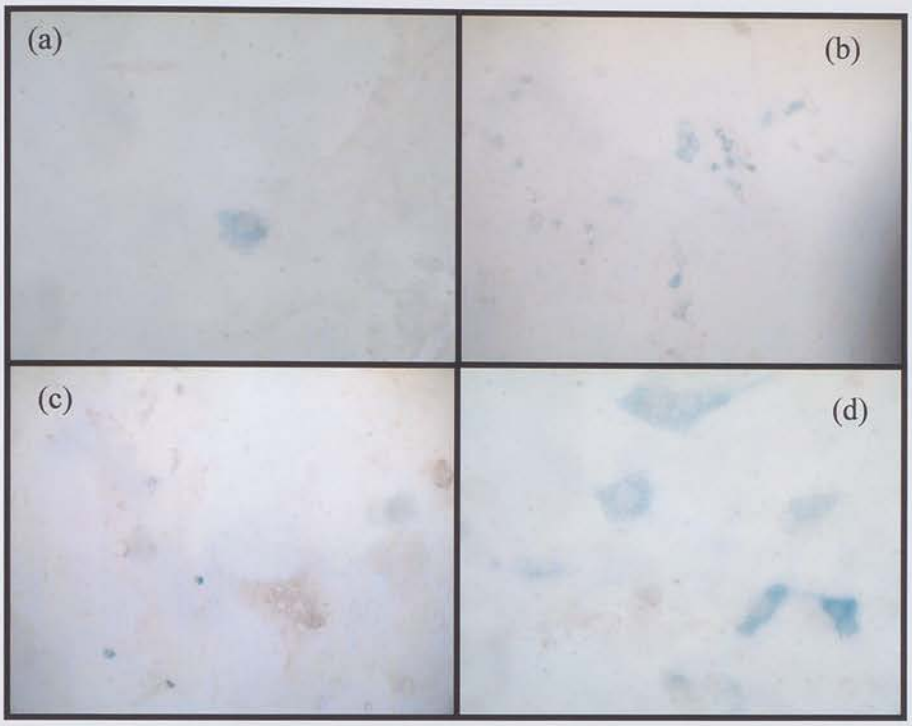


Figure B.6 – Blue staining upon addition of X-Gal substrate on Adenovirus Cre-treated primary cultured acinar cells from *Flox-stop LacZ* mice. (a) Cells were treated for 7 hours with Adenovirus Cre (3.0×10^6 p.f.u) and fixed after 48 hours. X400 magnification (b) Cells were treated for 7 hours with Adenovirus Cre (3.0×10^6 p.f.u) and fixed after 72 hours. X100 magnification (c) Cells were treated for 2 hours with Adenovirus Cre (3.0×10^6 p.f.u) and fixed after 72 hours. X100 magnification (d) Cells were treated for 7 hours with Adenovirus Cre (1.5×10^6 p.f.u) and fixed after 72 hours. X400 magnification.

Appendix C - Publication

Oncogene (2002) 21, 4696–4701

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Carcinogen-induced pancreatic lesions in the mouse: effect of *Smad4* and *Apc* genotypes

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Mutations in the tumour suppressor genes *SMAD4* (*DPC4*, deleted in pancreatic cancer locus 4) and *adenomatous polyposis coli* (*APC*) have been implicated in the development of pancreatic cancer in humans. Treatment of wild-type, $Smad4^{+/-}$, $Apc^{Min/+}$ or $Apc^{Min/+}Smad4^{+/-}$ mice with N-Nitroso-N-Methyl Urea (NMU) results in abnormal foci in pancreatic acinar cells characterized by increased levels of β-catenin. Previously such foci have been shown to be the precursors of pancreatic neoplasia. Interestingly, only NMU-treated $Apc^{Min/+}Smad4^{+/-}$ mice exhibit a significant increase in abnormal pancreas, which was found to be due to increased number of abnormal foci rather than increased focus size. A range of foci sizes were analysed, but only smaller abnormal foci were characterized by morphological nuclear atypia. These studies suggest functional co-operation between TGF-β and Wnt signalling pathways in the suppression of pancreatic tumorigenesis in the mouse.
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Keywords: pancreas; SMAD4; APC; mouse

Genes identified as mutated in pancreatic cancer include *p53*, *p16*, *K-RAS*, *BRCA2*, *APC* and *p15* (Abraham *et al*., 2002; Conlon and Howe, 1997; Hahn and Schmiegel, 1998; Caldas *et al*., 1994; Rozenblum *et al*., 1997). *SMAD4*, also known as *DPC4*, has also been implicated in pancreatic neoplasia (Hahn *et al*., 1996a,b). *SMAD4*, situated on the long arm of human chromosome 18 (its mouse homologue, designated *Smad4*, is on chromosome 8), is part of a family of *SMAD* genes which together work to transduce TGF-β superfamily signalling (Heldin *et al*., 1997). Several pieces of evidence support the assignment of *Smad4* as a tumour suppressor gene (TSG) in the pancreas. First, loss of heterozygosity (LOH) at 18q has been reported in 90% of pancreatic carcinomas (Hahn *et al*, 1996a). Second, 50% of human pancreatic carcinomas are characterized by either homozygous deletion or biallelic mutation of *SMAD4* (Hahn *et al*, 1996a,b; Schutte *et al*., 1996). Third, SMAD4 has been linked to tumour suppression-related activities *in vitro*: Atfi *et al*. (1997) have shown SMAD4-dependent induction of apoptosis via integration of TGF-β and SAPK–JNK pathways and others have shown that functional SMAD4 is necessary for TGF-β-mediated induction of p21 resulting in growth inhibition (Grau *et al*., 1997). Fourth, and perhaps most importantly, SMAD4 has been shown to be the central mediator of TGF-β superfamily signalling, a pathway frequently dysregulated in many cancers including pancreatic cancers (Markowitz and Roberts, 1996; de Caestecker *et al*., 2000).

Adenomatous polyposis coli (*APC*) has been documented as an important TSG in the colon, where germline mutations in *APC* cause Familial Adenomatous Polyposis (FAP) (Polakis, 1997; Groden *et al*., 1991). An ethylnitrosourea-induced germ line mutation within the *Apc* gene produced a mouse model for FAP, the 'Min' mouse ($Apc^{Min/+}$) (Moser *et al*., 1990). As well as intestinal neoplasia, the $Apc^{Min/+}$ mouse is predisposed to the development of abnormal acinar foci characterized by high levels of β-catenin. In the absence of p53, these β-catenin-overexpressing abnormal foci progress to adenoma and adenocarcinoma (Clarke *et al*., 1995; Kongkanuntn *et al*., 1999). APC, a component of the Wnt signalling pathway, is thought to mediate its tumour suppressive activities predominantly through down-regulation of intracellular β-catenin levels (Munemitsu *et al*., 1995). Loss of functional APC results in increased levels of β-catenin, which upon nuclear translocation mediates transcription of target genes, including *cyclin D1* and c-*myc* (He *et al*., 1998; Tetsu and McCormick, 1999).

Whilst SMAD4 has been linked to tumour suppression-related activities *in vitro*, the relevance to pancreatic neoplasia *in vivo* remains unclear. *Smad4* null embryos die around day E7.5, whereas heterozygous mice survive and develop intestinal polyps by 12 months (Sirard *et al*., 1998; Yang *et al*., 1998; Xu *et al*., 2000; Takaku *et al*., 1999). Although *Smad4* LOH

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was reported in later stage intestinal tumours (Miyaki *et al*., 1999), Xu *et al*. (2000) found that heterozygosity in this model was sufficient to initiate tumorigenesis in the intestine. Mice heterozygous for inactivating mutations in both *Smad4* and *Apc* ($Apc^{\Delta 716}$) developed colorectal carcinomas that were larger in size and more invasive by 20 weeks of age than those found in $Apc^{\Delta 716}$ heterozygotes alone (Takaku *et al*., 1998). This suggests that SMAD4 plays a significant role in the malignant progression of colorectal tumours, but the precise biological role of SMAD4 in the progression of pancreatic tumorigenesis *in vivo* remains unclear. Although *SMAD4* mutation is linked with 50% of pancreatic tumours in human, to date no pancreatic phenotype has been reported for any murine transgenic *Smad4* strain.

To address the role of SMAD4 in the development of pancreatic cancer, we therefore compared the susceptibility of wild-type mice and mice heterozygous for either or both the Apc^{Min} and *Smad4* alleles to the carcinogen NMU. NMU is an alkylating agent and a potent mutagen, exposure to which has been shown to result in pancreatic acinar cell hyperplasia in rats and the development of lymphoma and mammary tumours in mice (Monis *et al*., 1991; Diamond *et al*., 1987; Barka, 1982). We therefore utilized NMU to address the need for additional mutation, that could include mutation in the remaining *Smad4* or *Apc* wild-type alleles. Given the established synergy between mutant Apc^{Min} and *Smad4* alleles in colorectal cancer (Takaku *et al*., 1998), we also investigated whether mutations in these genes co-operate in pancreatic tumorigenesis.

NMU was administered subcutaneously to littermate cohorts (6–10 animals) of 4-day-old mice of genotype (i) wild-type, (ii) $Smad4^{+/-}$, (iii) $Apc^{Min/+}$ or (iv) $Apc^{Min/+}Smad4^{+/-}$. At 65 days of age the animals were sacrificed and tissues harvested. Age-matched control animals from each genotype were also examined. Given that pancreatic lesions that arise spontaneously in $Apc^{Min/+}$ mice and are characterized by high β-catenin levls have been shown to be precursors of pancreatic cancer (Clarke *et al*., 1995; Kongkanuntn *et al*., 1999), we performed immunohistochemistry for β-catenin on pancreas from mice of each genotype treated with NMU and control mice. The pancreata of control mice were histologically normal. NMU-treated mice of all genotypes (including WT) exhibited multiple focal acinar cell hyperplasia which was characterized by increased levels of β-catenin (Figure 1a).

Two histologically identifiable types of abnormal foci were found, which we postulate reflect different stages or endpoints of the carcinogenic challenge. The majority were composed entirely of acinar cells, while a minority contained stromal cells and cystic areas (Figure 1a,b). The proportion of the two types of foci did not differ between genotypes. TUNEL staining (Gavrieli *et al*., 1992) and morphological analysis (Kerr *et al*., 1972) of these foci revealed higher levels of apoptosis within foci characterized by stroma and cystic areas (2.4% TUNEL positive cells and 1.0% as scored morphologically) than the wholly acinar foci (<0.1% TUNEL positive cells, and 0.2% as scored

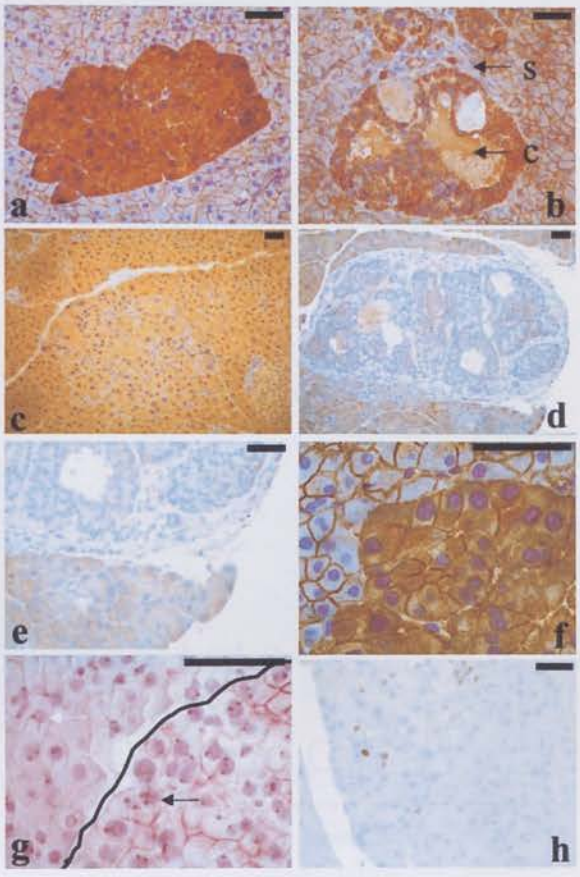

Figure 1 Representative examples of lesion histology and immunohistochemistry. (**a**) Abnormal focus of pancreatic acinar cells from an NMU-treated (50 mg/kg body weight) wild-type mouse showing increased β-catenin level. (**b**) Pancreatic abnormal focus from $Apc^{Min/+}Smad4^{+/-}$ mouse showing increased β-catenin level, containing stromal cells (S) and cystic areas (C). (**c**) SMAD4-stained pancreas from $Apc^{Min/+}Smad4^{+/-}$ mouse showing strong staining within and outside focus. (**d,e**) Pancreatic acinar cells from pancreas of $Apc^{Min/+}Smad4^{+/-}$ mouse exhibiting reduced cytoplasmic expression of SMAD4 within the lesion at low (**d**) and high (**e**) magnification. (**f**) β-catenin expression in abnormal focus showing increased nuclear area within the focus. (**g**) Double-labelling of pancreas from a hemizygous β-globin transgenic mouse using DNA–DNA *in situ* hybridization for β-globin transgene (brown punctuate dots) and faint β-catenin immunohistochemistry (burgundy). The black line divides dysplastic cells (bottom right) from normal cells (top left). White arrow indicates diploid cell containing one β-globin hybridization spot, black arrow indicates polyploid cell containing three β-globin hybridization spots within abnormal focus. (**h**) Representative example of BrdU staining within abnormal focus. Tissues were formalin fixed (methacarn for DNA-DNA *in situ* hybridization), paraffin embedded and sectioned using routine techniques. Immunohistochemistry for β-catenin (Transduction Laboratories, C19220), BrdU (Harlan, MAS250p) and SMAD4 (Santa Cruz, SC 1909) used biotinylated rabbit anti-mouse, rabbit anti-rat or goat anti-rabbit secondary antibody in conjunction with Avidin/Biotin complex before visualization with DAB (brown) or Vector VIP (burgundy). DNA-DNA *in situ* hybridization was performed as detailed in Keighren and West, 1993. Bars represent 50 μm

morphologically). It is possible that the altered content and deregulation of apoptosis in this subset of foci may indicate progression in tumorigenesis. This is of particular interest in the context of *Smad4* mutation, as defective TGFβ signalling has been shown to result in neo-angiogenesis and fibrosis in the stromal compartment of the pancreas (Bottinger *et al.*, 1997).

Using increased levels of β-catenin as a marker for abnormal pancreatic foci, the area of pancreas occupied by abnormal foci and focus size were measured by morphometric analysis. Comparison of abnormal pancreas expressed as a percentage of total pancreas found no differences between $Smad4^{+/-}$, $Apc^{Min/+}$ or *WT* mice (Mann–Whitney U, $P>0.05$), whereas $Apc^{Min/+}$ $Smad4^{+/-}$ mice showed a significant increase in abnormal pancreas (Figure 2a, Mann–Whitney U $P<0.05$). Increased abnormal pancreas only when *Apc* and *Smad4* mutation are present in conjunction suggests that the pathways these proteins are involved in converge on a common target. This hypothesis is supported by previous work by Nishita *et al.* (2000) and Labbe *et al.* (2000) where interaction of members of the Smad family with LEF1/TCF (Lymphoid enhancing binding factor/T cell-specific factor) and β-catenin was reported.

The observed increase in abnormal pancreas in $Smad4^{+/-}Apc^{Min/+}$ mice argues that SMAD4 is acting as a tumour suppressor in normal pancreas. To investigate whether lesions arising in $Apc^{Min/+}$ $Smad4^{+/-}$ pancreas had lost SMAD4 expression, immunohistochemical analysis for SMAD4 was carried out. The majority of foci remained heterozygous for *Smad4* (Figure 1c) with strong cytoplasmic staining. Decreased cytoplasmic levels of SMAD4 expression were observed only within a minority of foci (Figure 1d,e) indicating that loss or down-regulation of the remaining wild-type *Smad4* allele occurs rarely in this model. Thus it appears that the increase in abnormal pancreas in the $Apc^{Min/+}$ $Smad4^{+/-}$ mice is associated almost entirely with *Smad4* heterozygosity. This suggests that in the presence of other mutations loss of a single *Smad4* allele could effect a progression in tumorigenesis.

Statistical analysis comparing focus size between genotypes revealed that abnormal foci arising in $Apc^{Min/+}Smad4^{+/-}$ mice were not significantly larger than those in $Smad4^{+/-}$, $Apc^{Min/+}$ or wild-type mice (Mann–Whitney U $P>0.05$), Figure 2b. This implies that the increased amount of abnormal pancreas seen in $Apc^{Min/+}Smad4^{+/-}$ mice is not due to increased focus size but rather increased incidence. Mutations in both these genes therefore appear to have an additive effect on the initiation but not growth of abnormal foci causing a resultant overall increase in percentage abnormal pancreas. Takaku *et al.* (1998) analysed intestinal adenomas occurring in $Apc^{\Delta 716/+}Smad4^{+/-}$ mice and found a decrease in polyp number, but an increase in polyp size and frequency of invasive adenocarcinomas when compared to $Apc^{\Delta 716}$ heterozygotes. Our work is therefore in contrast to that of Takaku *et al* and may be indicative of different

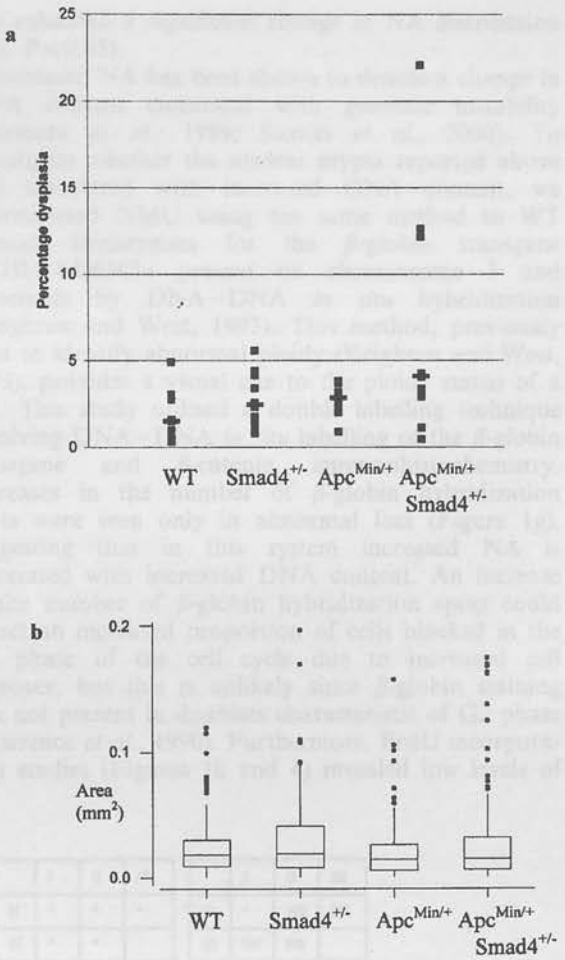

Figure 2 Morphometric analysis of abnormal foci. (**a**) Amount abnormal (with high β-catenin level) pancreas as measured by area and expressed as percentage of total pancreas in sections from NMU-treated WT, $Smad4^{+/-}$, $Apc^{Min/+}$, and $Apc^{Min/+}Smad4^{+/-}$ mice. Data are expressed as a dot plot with median percentage abnormal pancreas shown as a horizontal bar. (**b**) Box plots (Sokal and Rohlf, 1995) expressing size of individual foci in NMU-treated wild type (131), $Smad4^{+/-}$ (172), $Apc^{Min/+}$ (97) and $Apc^{Min/+}Smad4^{+/-}$ (219) mice (bracketed values denote total numbers of foci included in dataset). Box plots express the first (Q1) and third (Q3) quartiles within a given data set by the upper and lower horizontal lines in a rectangular box, inside which is a horizontal line showing the median. The whiskers extend upward and downwards to the highest or lowest observation within the upper (Q3+1.5 X the interquartile range) and lower (Q1−1.5 X interquartile range) limits. Values outside the upper and lower limits are 'outliers' and are shown by individual symbols

pathways of tumorigenesis in the pancreas and intestine, affecting different target genes. An example of such tissue-specific differences include Smad4-independent inactivation of TGF-β signalling (through inactivation of Smad2 or TGF-β type II receptors), which occurs more often in colorectal tumorigenesis (Heldin *et al.*, 1997). Finally, the absence of pancreatic neoplasia in the $Apc^{\Delta 716/+}Smad4^{+/-}$ mice could be because mutation in genes other than *Apc* or *Smad4* is necessary to initiate pancreatic tumorigenesis. Such

genes could be targeted by NMU in our study. In humans, oncogenic *K-RAS* activation and p53 inactivation occur frequently in pancreatic tumorigenesis (Rozenblum *et al*., 1997; Scarpa *et al*., 1993; Almoguera *et al*., 1988). Notably N-nitroso carcinogens have been shown to induce activating mutations in *K-ras* in mice (Corominas *et al*., 1991), but they have not been shown to cause mutational inactivation of *p53* (Kito *et al*., 1996).

Histological analysis revealed that a subset of abnormal foci in all genotypes contained visibly larger nuclei (Figure 1f) consistent with dysplasia. To examine whether SMAD4 played a role in the dysplastic phenotype, we measured nuclear area (NA) in normal acinar cells and acinar cells within abnormal foci (denoted Class I, Class II and Class III according to increasing size, see Figure 3 legend) for each genotype (Figure 3). In wild-type, $Smad4^{+/-}$ and $Apc^{Min/+}$ mice, the distribution of NA in all classes of abnormal foci differed significantly from that of normal nuclei ($P<0.05$, Kolmogorov Smirnov or KS), but the greatest increases in NA compared to normal nuclei were observed in the smaller foci, Class I and II, while the distribution of NA in the larger abnormal foci (Class III) more closely resembled that of normal nuclei. Abnormal foci arising in $Apc^{Min/+}$ $Smad4^{+/-}$ pancreas differed in that changes in NA distribution were much less pronounced and only Class I abnormal foci exhibited a significant change in NA distribution (KS, $P<0.05$).

Increased NA has been shown to denote a change in DNA content consistent with genomic instability (Asamura *et al*., 1989; Suzuki *et al*., 2000). To investigate whether the nuclear atypia reported above was associated with increased DNA content, we administered NMU using the same method to WT animals hemizygous for the β-globin transgene Tg(Hbb-b1)83Clo present on chromosome 3 and detectable by DNA–DNA *in situ* hybridization (Keighren and West, 1993). This method, previously used to identify abnormal ploidy (Keighren and West, 1993), provides a visual cue to the ploidy status of a cell. This study utilised a double labelling technique involving DNA–DNA *in situ* labelling of the β-globin transgene and β-catenin immunohistochemistry. Increases in the number of β-globin hybridization spots were seen only in abnormal foci (Figure 1g), suggesting that in this system increased NA is associated with increased DNA content. An increase in the number of β-globin hybridization spots could reflect an increased proportion of cells blocked in the G2 phase of the cell cycle due to increased cell turnover, but this is unlikely since β-globin staining was not present in doublets characteristic of G2 phase (Lawrence *et al*., 1990). Furthermore, BrdU incorporation studies (Figures 1h and 4) revealed low levels of

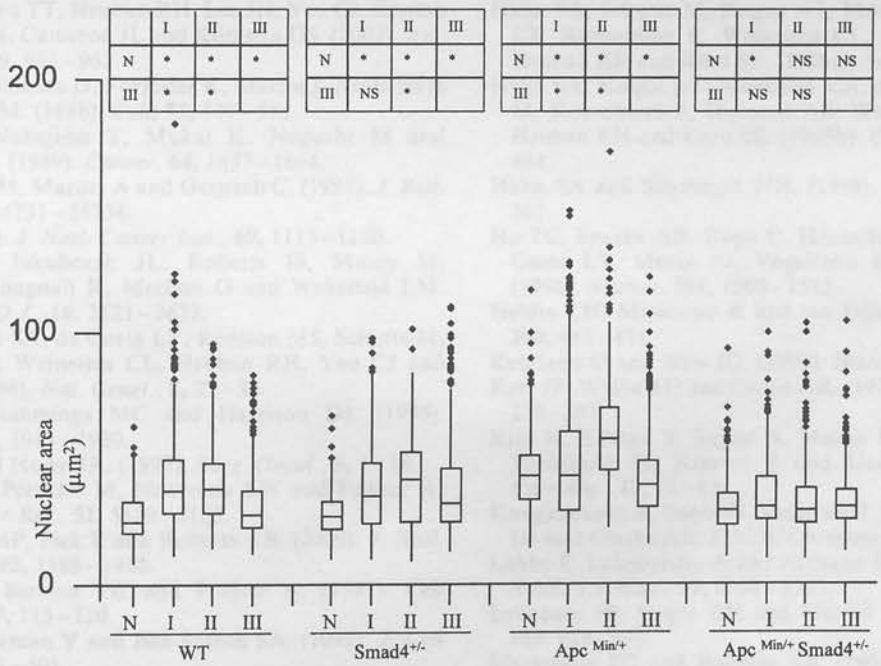

Figure 3 Morphometric analysis of nuclear area. Box plots (see Figure 2 for description) representing NA in each genotype of mice (WT, $Smad4^{+/-}$, $Apc^{Min/+}$ and $Apc^{Min/+}$ $Smad4^{+/-}$) in normal tissue and Class I, II and III abnormal foci (Class I, 0–0.025 mm^2, Class II, 0.025–0.05 mm^2 and Class III, 0.05–0.1 mm^2. Approximately 500 nuclei were scored per class from a minimum of 10 lesions, from at least four mice. Since foci larger than 0.1 mm^2 in each genotype numbered less than 10, they were excluded from the dataset as statistical analysis would be invalid. Results of Kolmogorov Smirnov two-sample test (Sokal and Rohlf, 1995) are shown in tables above respective genotype. N denotes NA in normal tissue; I, NA in Class I abnormal foci; II, Class II; III, Class III. Key for tables: NS, $P>0.05$ and $*P<0.05$

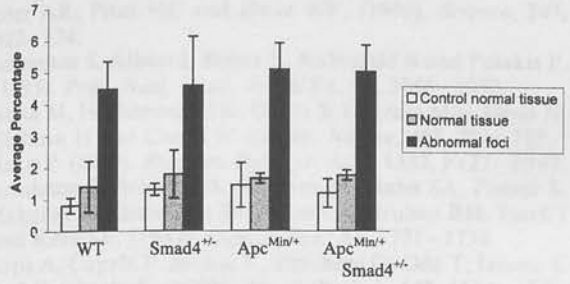

Figure 4 BrdU incorporation in normal and dysplastic pancreas. Histogram showing percentage cells incorporating BrdU in normal (from control and treated mice) and abnormal pancreas tissue. BrdU (Amersham, 3 mg/100 g body weight) was delivered 24 h prior to culling. After processing, immunohistochemistry for β-catenin and BrdU was carried out to enable scoring in both abnormal and normal pancreas. Error bars express standard error of the mean and in each case sections from a minimum of three mice were scored

cell turnover, insufficient to account for increased NA seen in approximately 40% of cells within abnormal foci. Thus the increase in nuclear area in the abnormal foci is most likely due to polyploidy or aneuploidy affecting chromosome 3.

Since the greatest increases in NA were not associated with either the largest lesions (Class III) or the most predisposing genotype ($Apc^{Min/+}$, $Smad4^{+/-}$), these results suggest that the processes leading to nuclear atypia and increased focus size may be independent. Although unlikely, it is possible that genomic instability is required in the less predisposing genotypes for lesion initiation but is incompatible with continued progression.

In conclusion, our results provide *in vivo* evidence from murine models that heterozygous mutation at the *Smad4* locus can predispose toward pancreatic tumorigenesis. Furthermore, since this effect only occurs in conjunction with an *Apc* mutation this suggests that functional co-operation between TGF-β and Wnt signalling pathways may be involved.

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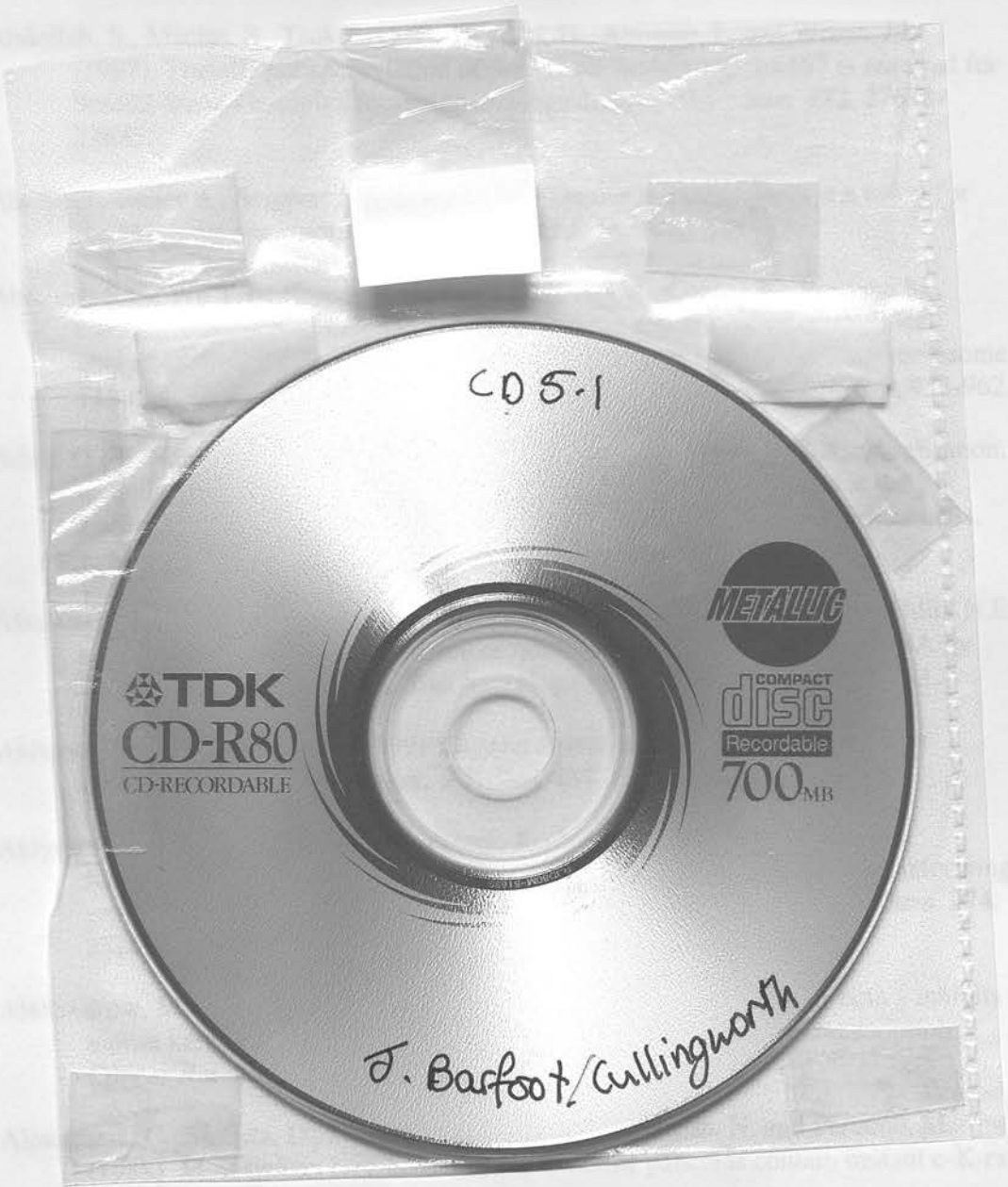
Appendix D – CD 5.1

CD 5.1 – Timelapse movie of acinar cells grown in either EGF or EGF and TGF-β



CD 5.1 - Timelapse video microscopy of cells grown in EGF (first clip) or EGF and TGF-β (second clip, separated by turning page). The acinar colony grown in EGF and TGF-β exhibits mobile processes at the edges of the colony towards the end of the movie. Time between each image is 15 minutes and each individual clip shows a 48 hour period. File is saved in two formats, Timelapse.avi and Timelapse.mov. For viewing on Macs use Quick Time to view the Timelapse.mov file. For view on P.C. use Windows Media Player to view the Timelapse.avi file.

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