p53 DEATH PATHWAYS IN CISPLATIN-DAMAGED PRIMARY MURINE COLONOCYTES AND A POSSIBLE ROLE FOR HEDGEHOG DEVELOPMENTAL GENES IN COLON CARCINOGENESIS

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DECLARATION

I hereby declare that this thesis has been composed by me and has neither been presented nor accepted in any previous application for a degree.

All work presented in this thesis was, unless acknowledged, was carried out by myself.

Anca Oniscu

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Special thanks to Robert Morris for putting up with me as a novice in the labs and teaching me most of the techniques and protocols used in this work. I would also like to thank Dr. Nathalie Sphyris for scientific and critical discussions about p53 and her constant encouragements; Dr. Roberta James and Dr. Scott Bader for helpful debate in the field of Hh signalling pathway and Dr Catherine Payne for help with the RT-PCR experiments. I also thank everyone on the 5^{th} floor laboratory for their cooperation and advice. I would also like to acknowledge Cancer Research UK for funding this research.

Finally, I would like to apologize to my daughter Maria for denying her a lot of precious time and also missing a few milestones of her early childhood. Most of all, I

would like to thank my husband Gabriel for his constant encouragement and support throughout the conception and completion of this thesis.

Last, but not least, I would like to thank my Mom and Dad for pretty much everything. This thesis is a testimony of their dedication and efforts throughout my upbringing.

DEDICATION

I dedicate this thesis to my daughter Maria and husband Gabriel.

ABBREVIATIONS:

List of abbreviations used in the text:

APC	Adenomatous polyposis coli
ATCC	American Type Cell-Culture Collection
BCC	Basal cell carcinoma
BrdU	Bromodeoxyurdine
BSA	Bovine serum albumin
DAB	Diaminobenzidine
DCC	Deleted in colorectal carcinoma
DMEM	Double Eagle Modified Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPC4	Deleted in pancreatic carcinoma 4
ECACC	European Collection of Animal Cancer Cell-lines
EDTA	Disodium ethylenediamine tetra-acetate
FAP	Familial adenomatous polyposis
HCl	Hydrochloric acid
HET	Heterozygous
hMLH1	Human MutL homologue 1
hMSH2	Human *MSH2*
HNPCC	Hereditary non-polyposis colon cancer
HOM	Homozygous
hPMS2	Human postmeiotic segregation increased 2

kb	Kilo-base pair
kDa	KiloDalton
LOH	Loss of heterozygosity
MDM2	Mouse double minute homologue 2
Min	Multiple intestinal neoplasia
MMLV	Moloney-Monkey Leukemia Virus
MMR	Mismatch repair
MSH2	MutS homologue 2
NaCl	Sodium Chloride
NRS	Normal Rabbit Serum
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
Ptch	Patched
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SEM	Standard error of the mean
Shh	Sonic hedgehog
Smoh	Smoothened
SV40	Simian virus 40
TBE	Tris Borate EDTA
TBS	Tris buffered saline
WT	Wild type

ABSTRACT

Colon cancer is one of the most common internal malignancies in the developed world and represents the 2nd cause of cancer death in Western countries. Approximately half of all colorectal cancers show p53 gene mutation, particularly in late stage acting as a progression step. Much of the information in carcinogenesis in the intestine derives from studies based on the small intestine, yet the majority of the mutations leading to cancer occur in the large intestine. To overcome some of these issues, the use a large bowel model and in particular the primary murine colonocytes system was preferred.

The objectives were to better understand the importance of p53 and related proteins in the regulation of growth and responses to DNA damage in these cells. In order to investigate both p53 dependent and independent death pathways we exposed primary colonocytes to cisplatin.

The initial study showed that the role of p53 in preventing entry into S-phase following colonocyte DNA damage appears crucial whereas its role in apoptosis seems redundant. This suggests that p53-mediated apoptosis and growth arrest are neither a major nor a unique protector of colonic epithelial cells against mutation following DNA damage. Furthermore, the nuclear translocation of endogenous p73α in response to DNA damage in primary colonocytes is highly suggestive of a functional pro-apoptotic role for p73α in these cells, within the context of p53-independent apoptosis. Moreover, in the context of p53 deficiency, p73α activation is highly suggestive of its involvement in the p53-independent pathway of apoptosis in these cells.

Consequently, it seemed plausible that other "early" genetic events could affect the colonic epithelium to lead to cancer. Hedgehog developmental pathway is important in controlling normal growth and patterning and has been recently linked to different types of cancer. As cancer appears due to abnormal growth control it was of interest to investigate a possible involvement of Hedgehog genes in colorectal neoplasia.

Therefore, the second part of this thesis focused on investigating the pattern of expression of sonic hedgehog (Shh) signalling pathway in normal colon versus

colonic lesions including hyperplastic polyp, adenoma and adenocarcinoma of the colon. Results showed that Hedgehog signalling pathway members are expressed in normal colonic epithelium with Shh at the top of the crypts and a few basally located cells, patched in the neuroendocrine cells and smoothened at the brush border of superficial epithelium. RT-PCR analysis of laser-microdissected crypts from normal human colon confirmed that mRNAs encoding these proteins were expressed in colonic epithelium. Expression of Shh, Ptch and Smoh was upregulated in hyperplastic polyps, adenomas and adenocarcinomas of the colon and sonic hedgehog expression correlated with an increased expression of the proliferation marker Ki-67 in all lesions examined.

To address whether the Hh signalling pathway is functional in the gut, the effect of Shh on epithelial cells *in vitro* was explored by treating primary murine colonocytes with either Shh peptide or neutralizing anti-Shh antibody. The results illustrated that exogenous Shh promotes cell proliferation in colonocytes while anti-Shh inhibits proliferation, suggesting that sonic hedgehog is required during proliferation of epithelial cells *in vitro*. Therefore, the results suggested that Shh is required during epithelial proliferation in the colon and that there is a possible role for Hh signalling in colonic epithelial tumour progression *in vivo*.

CHAPTER 1

INTRODUCTION

1.1. Colorectal Cancer

1.1.1. Epidemiology and Incidence

Colorectal cancer represents the second most common cause of cancer death in the developed countries [Parker S.L. and Tong T. *et al.*, 1997]. A high rate of cellular turnover combined with environmental factors such as unhealthy diet [Flood A. and Velie E.M. *et al.*, 2002], colonic flora, lack of physical activity and smoking [Le Marchand L. and Wilkens L.R. *et al.*, 1997] give a relatively high rate of neoplasia. Epidemiologic studies indicate that diets high in red meat and animal fat and low in fibres are associated with an increased incidence of colorectal cancer [Giovannucci E. and Rimm E.B. *et al.*, 1994; Potter J.D., 1999]. Approximately 5% of the population develops colorectal cancer and the incidence of colorectal cancer is expected to grow, as more and more people adopt a western style diet and life expectancy increases [Rosenberg S.A. and DeVita V.T. *et al.*, 2001].

1.1.2 Genetic Factors in Colorectal Cancer

Colorectal cancer develops as a series of clinical and histopathologic steps, from a single crypt lesion, aberrant crypt foci to small benign lesions, adenomatous polyps and finally malignant cancer. This progression involves genetic changes in the form of activation of a number of oncogenes and/or inactivation of tumour suppressor genes. Colonic adenomas are recognized as benign lesions but they may give rise to carcinomas. In addition, carcinomas can also arise in patients with inflammatory bowel disease. Adenomas can occur sporadically or as part of a familial syndrome. Genetic inherited influences are best defined in two categories: familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC).

FAP accounts only for 1% of all colorectal carcinomas and HNPCC for approximately 4%. Patients with FAP develop multiple benign tumours in their twenties or thirties. Some of these polyps will become malignant, giving rise to colon carcinomas. FAP is associated with germ-line mutations of the *adenomatous polyposis coli* (*APC*) gene that result in a truncated version of the protein [Nishisho I. and Nakamura Y. *et al.*, 1991]. These mutations are also implicated in breast tumours. In the case of NHPCC, the majority of the patients inherit defects in the *mismatch repair* (*MMR*) genes: *hMSH2, hMLH1, hPMS2.* These tumours are mismatch repair-deficient and therefore genetically unstable and progress faster to carcinomas. In addition, patients with HNPCC exhibit an increased risk of developing cancers of other organs such as uterus, ovary and brain.

The vast majority of colorectal cancers do not have an apparent inherited susceptibility and therefore they are grouped as sporadic cancers. However, many

studies indicate a broader role for inheritance amongst sporadic cancers, as they demonstrated that relatives of patients with sporadic colon cancer also have an increased risk to develop colon cancer or associated cancers [Cannon-Albright L.A. and Skolnick M.H. *et al.*, 1988]. This appears to [Houlston R.S. and Collins A. *et al.*, 1992] suggest that many cases of sporadic colorectal cancer may have an yet undefined genetic background.

1.1.3. Molecular Genetic Changes in Colorectal Cancer

Epidemiological, clinical, pathological and molecular studies have shown that colonic cancer, hereditary or sporadic, grows from pre-existing adenomatous polyps. At least four genetic events at molecular levels seem to be responsible for the progression of colorectal cancer: inactivation of several tumour suppressor genes located on chromosomes 5q, 17p and 18q and the activation of *ras* oncogenes which promote tumour growth.

Adenomas formation begins with the loss of function of the tumour suppressor gene *APC* followed by the activation of the *Kirsten-ras* (*K-ras*) oncogene. Subsequent loss of function of the tumour suppressor genes located on chromosomes 18q (*DCC*, *DPC4* and *JV18-1/MADR2* genes) and 17p (*p53* gene) pushes the adenomatous lesion towards malignant growth (Figure 1).

Carcinogenesis in the colon occurs through two different mechanisms:

- chromosomal instability, when portions of chromosomes are deleted and specific genes lose their function
- microsatellite instability when errors occur in the DNA mismatch repair system.

Chromosomal instability is responsible for approximately 80 - 85% of sporadic colon cancers and all cases of FAP [Piard F. and Martin L. *et al.*, 2002], while the microsatellite instability accounts for approximately 15% of sporadic cancers and most cases of NHPCC [Chung D.C. and Rustgi A.K., 2003].

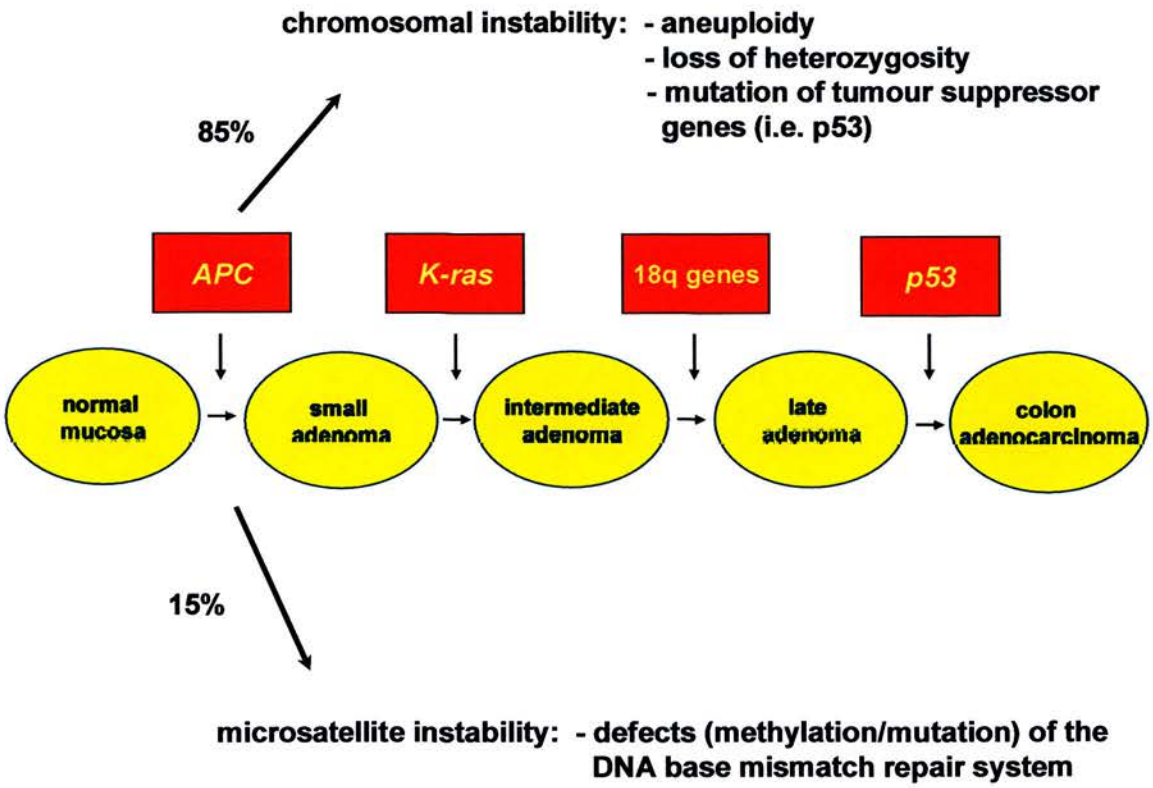


Figure 1: Adenoma to carcinoma sequence, molecular etiology of colorectal cancer: temporal order in which key genes are affected. Normal epithelium seems to become hyperplastic due to age-related methylation. On a hyperplastic mucosa, defects in the APC gene forms a neoplastic process which follows two major pathways: - epigenetic if mismatch repair genes are inactivated - genetic characterized by Ki-ras mutations, p53 mutations

1.1.3.1. Clonal Origin of Colorectal Cancer

Current theories suggest that colorectal cancer develops from a single cell that is altered and acquires proliferative proprieties. This cell has a higher proliferation rate compared to the surrounding normal cells. If a cell form within this population of mutated cells acquires another mutation it creates a sub-clone and continual sequences lead ultimately to a fully developed tumour.

The clonal origin of human cancer was first investigated using techniques based on X-chromosomes inactivation in females using a cloned polymorphic X-chromosomal gene and restriction endonucleases [Vogelstein B. and Fearon E.R. *et al.*, 1985]. Three human tumours were shown to be monoclonal. Another study investigated the monoclonal nature of colorectal cancer using polymorphic markers in 50 tumours, adenomas and carcinomas. All tumours showed monoclonal pattern of x-chromosome inactivation irrespective of the type, familial or sporadic [Fearon E.R. and Hamilton S.R. *et al.*, 1987].

1.1.3.2. Inactivation of Tumour Suppressor Gene APC

The *APC* gene is very important in colon carcinogenesis, being responsible for the progression of the normal colonic epithelium to small adenoma. The gene was found mutated in the germ line of patients with FAP [Nishisho I. and Nakamura Y. *et al.*, 1991]. Subsequently, somatic mutations were detected in both sporadic adenomas and sporadic colon carcinomas [Miyoshi Y. and Nagase H. *et al.*, 1992]. All these studies suggest that the inactivation of the *APC* tumour suppressor gene occurs

frequently in early as well as late stages of colon carcinogenesis, in contrast to mutations of *ras* or *p53* which are detected as the tumours progress.

The role of APC in colon tumourigenesis was also investigated using murine models. Germ line mutations of the murine *Apc,* result in multiple intestinal neoplasia [Su L.K. and Kinzler K.W. *et al.*, 1992]. Mice which are heterozygous for the *Apc* gene gradually develop intestinal tumors in a way that is comparable to that observed in FAP patients [Fodde R. and Edelmann W. *et al.*, 1994]. In addition, inactivation of both murine *Apc* alleles was detected in microscopic lesions of mouse models of colorectal cancer and also in the earliest colonic human lesions, the dysplastic crypts [Levy D.B. and Smith K.J. *et al.*, 1994].

All these mutations result in a truncated protein that is incapable of exerting its function. Studies on colorectal epithelial cells revealed that the wild-type full-length APC protein has also an apoptotic function [Morin P.J. and Vogelstein B. *et al.*, 1996]. Apoptosis is a very important function of the colonic epithelium eliminating damaged cells at the top the crypts, therefore protecting the colon from a neoplastic process.

1.1.3.3. Activation of the *k-ras* Oncogene in Colorectal Cancer

The first leap forward in the study of colonic carcinogenesis was the identification of the *k-ras* gene mutation. *K-ras* activation acts in a transitional stage of colonic carcinogenesis, from a small adenoma to an intermediate adenoma. K-ras gene was found mutated in only 9% of adenomas smaller than 1 cm [Vogelstein B. and Fearon

E.R. *et al.*, 1988], in contrast to 92% cases of hyperplastic polyps [Rommel C. and Hafen E., 1998].

Mutations of this gene result in a gain of function and offer a growth advantage to a small adenoma that has already an inactivated *APC* gene [Yashiro M. and Carethers J.M. *et al.*, 2001]. *K-ras* mutations were also identified in 58% of adenomas larger than 1 cm and 47% of colon carcinoma [Vogelstein B. and Fearon E.R. *et al.*, 1988]. However, other events associated with *k-ras* mutations could also be important in colon tumourigenesis, as many adenomas or colon carcinomas develop in the absence of a *ras* mutation [Vogelstein B. and Fearon E.R. *et al.*, 1988].

1.1.3.4. Loss of Function of Genes on Chromosome 18q

In 1988 Vogelstein *et al.* performed mutational analysis on different stages of colorectal neoplasia and found deletions of a specific region of chromosome 18 in 73% of colon carcinomas, 47% of advanced adenomas and sporadically in earlier-stage adenomas [Vogelstein B. and Fearon E.R. *et al.*, 1988].

Several candidate suppressor genes were identified: *DCC*, *DPC4* and *JV-18/MADR*.

It was difficult to describe a specific function and role for the protein encoded by the *DCC* gene as this is not expressed in colorectal cancers at protein levels. Consistent with its role as tumour suppressor gene, nearly all colorectal cancers fail to express an intact *DCC* transcript, while the transcripts are expressed in normal colonic epithelium [Hedrick L. and Cho K.R. *et al.*, 1994]. Mutational analysis for *DCC* revealed just few mutations or deletions. More evidence for a tumour suppressor role came from a study based on chromosomal transfer that showed the ability of

chromosome 18q to reduce the growth of human cancer cells [Tanaka K. and Oshimura M. *et al.*, 1991].

A study by Thiagalingam *et al.* investigated the incidence of mutations for *DPC4* in 18 colon carcinoma with 18q losses and identified one homozygous deletion, one nonsense mutation and three somatic mutations [Thiagalingam S. and Lengauer C. *et al.*, 1996]. The same tumours were further analyzed for *JV-18/MADR* and displayed only two mutations [Riggins G.J. and Thiagalingam S. *et al.*, 1996].

1.1.3.5. Loss of Function of the Gene Located on Chromosome 17p

p53 gene, located on chromosome 17p, was the first tumour suppressor gene to be identified in colorectal cancers in 1979. Cytogenetic studies revealed that the short arm of chromosome 17 is lost frequently (42-75%) in human colorectal carcinomas [Cunningham J. and Lust J.A. *et al.*, 1992; Lothe R.A. and Fossli T. *et al.*, 1992; Vogelstein B. and Fearon E.R. *et al.*, 1988]. However, multiple mutational analyses for p53 revealed that the gene is inactivated in a higher proportion in late adenomas [Ohue M. and Tomita N. *et al.*, 1994] and only occasionally (about 6-12%) in early benign adenomas [Shaw P. and Tardy S. *et al.*, 1991; Vogelstein B. and Fearon E.R. *et al.*, 1988]. These findings suggested that p53 inactivation occurs late in the adenoma-carcinoma progression. Deletions of chromosome 17p were also found frequently in other human common malignancies such as breast or non-small cell lung cancer and oesophageal carcinomas [Hollstein M. and Sidransky D. *et al.*, 1991].

Initially, p53 was considered a proto-oncogene due to its cooperation with Ha-ras gene in transforming rat embryo fibroblasts into tumourigenic cells [Eliyahu D. and Michalovitz D. *et al.*, 1985]. Further studies crowned the p53 gene as the "the guardian of the genome"[Lane D.P., 1992]. p53 is now considered a tumour suppressor gene due to its growth arrest and apoptotic functions. Wild-type p53 expression is triggered by stress signals (e.g. DNA damage, hypoxia, oncogenes etc.) and produces either G1 arrest to permit the DNA repair machinery to repair the damage, or apoptosis when the damage cannot be repaired. This process of eliminating damaged cells is of great importance as such cells could give rise to neoplastic events.

1.1.3.6. Other Genetic Factors Important in Colonic Tumourigenesis

Gene expression, gene amplification, changes in methylation and modifying loci are factors that could also be important in colorectal carcinogenesis.

The expression of a high number of genes differs in cancer cells compared to normal colon. It is not fully understood whether their modified pattern of expression plays a role in tumour development or it is the effect of the malignant transformation in the colon, but further research will probably answer some of these questions.

Mammalian DNA was discovered to be naturally methylated at 5-cytosine of 5'-CG-3' dinucleotides. Despite many years of research in the field, the role of this DNA methylation is still unclear. Changes in methylation of some genes such as hypermethylation, hypomethylation or *de novo* methylation at previously unmethylated CpG islands could be significant. Experiments on DNA methylation in

cancer suggest that these changes could cause an increased oncogene expression, an alteration in cell-cycle control or silencing of genes important in growth suppression [Issa J.P., 2000]. Altered methylation is also an age-related process as recently demonstrated from studies in ageing normal colonic mucosa [Issa J.P., 1999]. It is believed that the age-related methylation precedes tumourigenesis in the colon. A series of tumour suppressor genes such as *p16* or *THBS1* are inactivated by hypermethylation in sporadic colorectal cancers with microsatellite instability related to hMLH1 methylation [Herman J.G. and Merlo A. *et al.*, 1995; Toyota M. and Ahuja N. *et al.*, 1999].

Epigenetic instability together with chromosomal instability and microsatellite instability are now considered genetic factors required for the clonal evolution of colorectal neoplasia.

1.2. Histopathology of Colorectal Neoplasia

1.2.1. Histology of Normal Colon

The normal colon has a smooth mucosa with crypts that measure approximately 0.5mm in length. The epithelial cells lining the crypt are mostly Goblet cells, with big vacuoles in the cytoplasm, which contain mucin. Mucin secretion flows from the crypt opening in the lumen and also covers the surface of the crypt. The cells facing the colonic lumen are absorptive cells, which absorb water and electrolytes. A small number of endocrine cells are found in the lower portion of the crypt towards muscularis mucosae. Other types of cells found in the crypts are: Paneth cells, enterochromaffin cells, M cells overlaying the lymphoid follicles and rarely T lymphocytes (Figure 2A).

The intestinal epithelial cells are rapidly dividing, and cells are continuously renewed. The process starts at the base of the crypt with cells dividing, migrating and differentiating as they move towards the top and then dying by apoptosis or shedding in the lumen. The cells located at the base of the crypt form the proliferative compartment of the crypt. These cells are undifferentiated stem cells that give rise to daughter cells that differentiate in all the abovementioned cells. This migration takes 3 to 8 days [Eastwood G.L., 1977].

The crypts are surrounded by sheets of myofibroblasts, which sustain their normal morphology and cellular dynamics. Crypts are separated by lamina propria, which consists of connective tissue containing fibroblasts, lymphocytes, macrophages and capillaries.

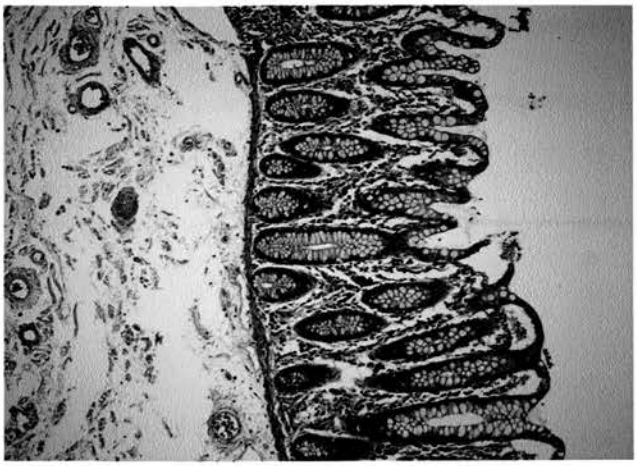


Figure 2A: Section of normal colonic epithelium stained with haematoxylin & eosin.

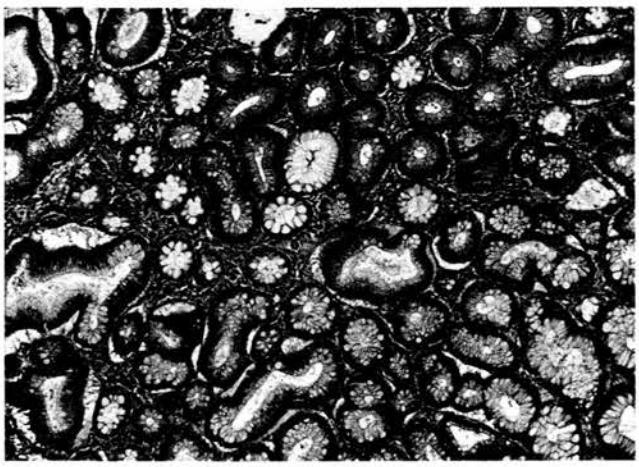


Figure 2B: Section of colonic adenoma stained with haematoxylin & eosin. Arrow indicates a normal crypt surrounded by adenomatous transformed crypts

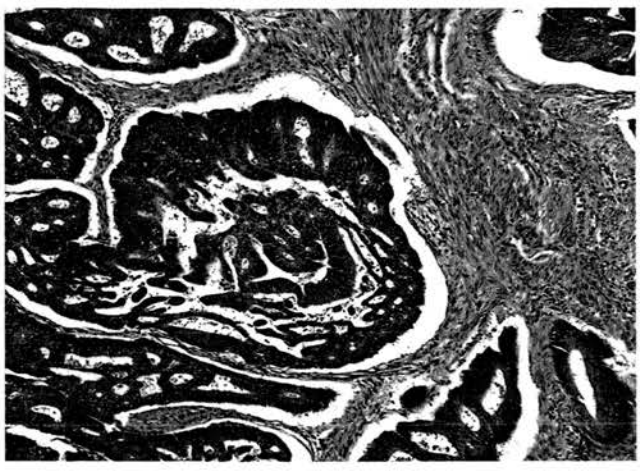


Figure 2C: Section of an invasive colonic adenocarcinoma stained with haematoxylin & eosin.

The other layers of the colonic wall consist of:

- **submucosa** with lymphatics, blood vessels, elastic tissue, nerves and ganglions
- **muscularis propria** with fibres of smooth muscles arranged longitudinally and circularly and collagen fibres, all penetrated by nerves, arterial and venous vessels
- **serosa** which contains blood and lymphatic vessels grouped in a connective tissue

1.2.2. Histopathology of Adenomas

Adenomas represent benign glandular transformation of the normal colon characterized by abnormal proliferation and incapacity to fully differentiate [Kaye G.I. and Fenoglio C.M. *et al.*, 1973]. Adenomas emerge from a single crypt and grow by replacing the surrounding normal epithelium with neoplastic tissue. In contrast to normal crypts, adenomas proliferate at their top and quite often apoptotic cells are seen at their base. Adenomas are formed from partially differentiated Goblet cells and absorptive cells, which are distributed erratically along the crypt (Figure 2B). Some of these cells maintain their propriety of producing mucus but some of them become dystrophic. Multiple mitotic figures (including atypical ones) and hypercromatic nuclei are distributed all over the entire epithelium of the crypt.

Histologically, adenomas could be classified as: tubular, accounting for 68% to 87% in most of the studies, villous adenomas, tubulovillous adenomas, flat adenomas and serrated adenomas.

Tubular adenomas maintain their original architecture but the normal epithelium is replaced by dysplastic cells. Neoplastic tubular adenomas could be investigated using an antibody to Ki-67 that will show abnormal apical proliferation. In contrast villous adenomas contain long crypts with dysplastic epithelium extending to the colonic lumen. Tubulovillous adenomas contain a mixture of villous and tubular structures. Flat adenomas are an alternative of tubular adenomas but their thickness is not exceeding too much the level of surrounding normal epithelium. They usually display a high grade of dysplasia and cellular atypia [Riddell R.H., 1992].

Serrated adenomas constitute less than 0.1% of colorectal adenomas. Histologic examination discloses a pattern reminiscent of the glands in hyperplastic polyps. However, the stratified cells lining the glands appear less mature than those found in the hyperplastic polyps and they are dysplastic.

Adenomas are benign structures but they considered the precursor of colorectal carcinomas.

1.2.3. Histopathology of Carcinomas

Colorectal carcinomas could arise from any type of the previously discussed adenomas that show an increased grade of dysplasia, or in a colon suffering from inflammatory bowel disease. The vast majority of colon cancers are adenocarcinomas. They are moderately to well-differentiated tumours with high mitotic figures and poorly differentiated cells (Figure 2C).

In a large number of cases, the superficial area of the tumour is histologically identical with its deeper part. In some cases however, there is a histological difference between these two parts (residual adenomatous mucosa may be present at the edge of a malignancy, especially in small tumours). Some of the adenocarcinomas are capable to secrete mucin and therefore are named mucinous carcinomas.

Colon carcinomas invade the intestinal layers either expanding or infiltrating. Usually expanding tumours are associated with an inflammatory response, which is absent in infiltrating tumours.

Colonic carcinomas are formed from various cells: Goblet cells, Paneth cells, endocrine cells, melanocytes, trofoblasts, and squamous cells. The proportion of cells differs in different types of carcinoma.

Histologically colon carcinomas are classified as:

- **intra-mucosal carcinomas -** when neoplastic cells invade lamina propria and adjacent crypts through the membrane of the crypt. They could extend to the whole mucosal layer but are limited to the muscularis mucosa. These tumours

are considered invasive, but it is not well defined whether they can metastasize or not.

- **invasive carcinoma** - when the tumour invades the muscularis mucosa and submucosa. The tumour cells spread into the local nodes and have access to the blood vessels, fat, nerves and lymphatics in the submucosa. These tumours often metastasize at distance from the primary location (liver, lungs, bones), due to their access to blood vessels and lymphatics.

In 1932, Dukes proposed a simple method to grade rectal cancer based on invasion into the bowel wall and the involvement of the lymph nodes [Dukes C.E., 1932]:

Stage A - lesions confined to the rectal wall (but not invading serosa)

Stage B - lesions spreading through the muscularis propria to the rectal serosa and beyond, but not to the lymph nodes

Stage C – lesions that metastasize in the regional lymph nodes.

Subsequently, this system was extended to include the colon cancer. This staging system was modified in 1954 when Astler and Coller [Astler V.B. and Coller F.A., 1954] proposed two B and two C stages:

Stage B1 - extension into muscularis propria but the lymph nodes are not involved (Figure 3B1)

Stage B2 – extension through entire wall with uninvolved lymph nodes (Figure 3B2)

Stage C1 – extension through all layers of the wall and involving nodes (Figure 3C1)

Stage C2 – presence of distant metastasis (Figure 3C2).

Figure 3A

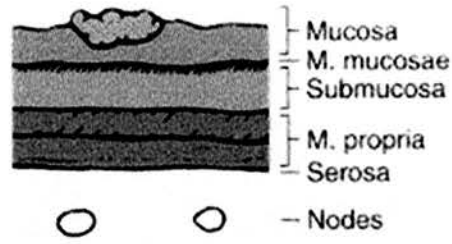
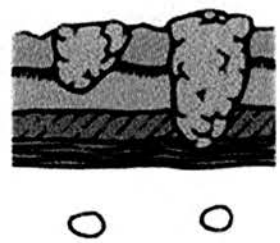
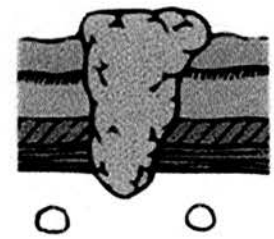


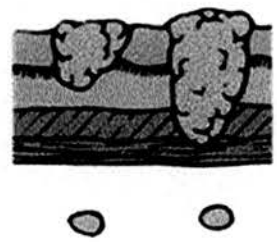
Figure 3B1





3B2

Figure 3C1



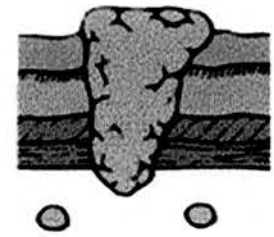
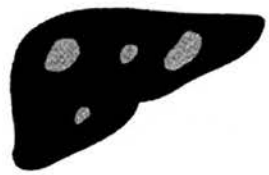


Figure 3C2



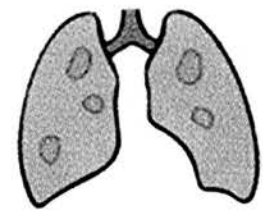


Figure 3: Neoplastic progression from a small lesion confined to the mucosa to invasive carcinoma.

3A - Carcinoma "in situ" *restricted to* ***the basement*** *membrane of the colonic crypts*

3B - Extension into muscularis propria (3B1) or through the serosa (3B2) but the lymph nodes are not involved

3C1- ***Invasion through the muscularis propria*** *or serosa with limph* ***nodes metastasis***

3C2 - ***Distant metastasis: liver, lungs and other sites***

In 1954 Denoix *et al.* [Denoix P.F., 1954] suggested the TNM cancer classification, which was further, adapted by Beahrs [Beahrs O.H., 1984]. It is based on Dukes' classification and also considers the number of invaded lymph nodes and the presence or absence of distant metastases.

The Dukes classification was evaluated in 1994 by Deans *et al.* [Deans G.T. and Heatley M. *et al.*, 1994] and found to be "of greater prognostic value and more reproducible". Both TNM and Dukes's classification are in use today and recommended by different Cancer Authorities.

1.3. p53

1.3.1. The Gene for p53

The p53 gene is the most extensively studied tumour suppressor gene in recent years [Thomas R. and Kaplan L. *et al.*, 1983]. It was isolated in numerous mammalian species: mouse [Oren M. and Levine A.J., 1983], rat [Coulier F. and Imbert J. *et al.*, 1985], frog [Soussi T. and Caron D.F. *et al.*, 1987], monkey [Rigaudy P. and Eckhart W., 1989] and human [Matlashewski G. and Lamb P. *et al.*, 1984]. Using cDNA clone in the analysis of human-mouse hybrid cells, McBride *et al.* [McBride O.W. and Merry D. *et al.*, 1986] located the human p53 gene on chromosome 17p.

The initial research showed that p53 collaborates with Ha-ras oncogene to transform embryo fibroblast and due to this association, it was postulated that p53 is a proto-oncogene [Eliyahu D. and Michalovitz D. *et al.*, 1985]. The p53 protein, probably a mutant form, was found overexpressed in a wide variety of cancer cell-lines and in proliferating untransformed cells. Usually p53 is not expressed or its levels are low in normally growing cells.

Further studies revealed that p53 is activated following genotoxic stresses and its activation has a protective role rather than an oncogenic one. p53 protects from uncontrolled proliferation of damaged cells capable to trigger neoplastic transformation, by inducing cell-cycle arrest and apoptosis in response to a wide variety of cellular stresses [Gottlieb T.M. and Oren M., 1998].

Now, it is well accepted that the inactivation of the p53 gene is a crucial event in carcinogenesis, p53 mutations being the most frequent genetic event in human cancer

[Soussi T. and Dehouche K. *et al.*, 2000]. The loss of p53 function by deletion, mutation or inhibition of its function through various mechanisms [Vogelstein B. and Kinzler K.W., 1992] has been noticed in all types of cancer: colo-rectum, lung, breast, bladder and hepatocellular carcinoma [Hollstein M. and Sidransky D. *et al.*, 1991].

Studies using knockout mice for p53 gene also confirmed the gene as an important tumour suppressor:

- mice with p53 mutations develop tumours early in life [Jacks T. and Remington L. *et al.*, 1994]
- mice deficient for p53 display increased tumourigenesis in response to radiation [Lee J.M. and Abrahamson J.L. *et al.*, 1994] and chemical carcinogens [Harvey M. and McArthur M.J. *et al.*, 1993]
- p53 deficient mice develop spontaneous tumours (sarcomas and lymphomas) early in life [Purdie C.A. and Harrison D.J. *et al.*, 1994] and die because of these tumours when about 10 months old [Donehower L.A. and Harvey M. *et al.*, 1992].

Alterations in the tumour protein p53 occur not only as somatic mutations in human cancers but also as germline mutations in some cancer-prone families. In reviewing medical records and death certificates of 648 childhood rhabdomyosarcoma patients in 1969, Li and Fraumeni identified 4 families in which sibs or cousins had a childhood sarcoma [Li, F. P. and Fraumeni, J. F., 1969]. The spectrum of cancers in the syndrome was shown to include, in addition to breast cancer and soft tissue sarcomas, brain tumours, osteosarcoma, leukaemia, and adrenocortical carcinoma. In 1997, Varley *et al.* [Varley J.M. and Evans D.G.R., 1997] stated that more than 50

families had been identified with Li-Fraumeni syndrome caused by germline tumour protein-53 mutations. More recently, in 2003, Varley stated that nearly 250 independent germline TP53 mutations had been reported [Varley J.M., 2003] and that most were associated with Li-Fraumeni syndrome or Li-Fraumeni-like syndrome and found that the most striking association between germline TP53 mutations and cancer occurred in cases of childhood adrenocortical carcinoma (ADCC). ADCC was identified as a component tumour of Li-Fraumeni syndrome from the earliest studies. p53 has also been implicated in cellular differentiation, development, embryogenesis and all these functions will be discussed in the next section.

1.3.2. p53 Structure and Function

p53 is a 44kDa protein consisting of a N-terminal transactivation domain, a proline-rich domain, a DNA binding domain, a tetramerization domain and a C-terminal domain. MDM2, a regulatory gene for p53, binds to the N-terminal domain resulting in inhibition of p53 transactivation and also in its degradation. Moreover, MDM2 is stimulated by p53-dependent transactivation creating a regulatory feedback loop, which controls the function of p53 under normal conditions. This interaction is disrupted in cellular stresses when p53 activation occurs.

Several roles have been demonstrated for each of the p53 domains:

- The proline-rich domain plays a critical role in the transmission of anti-proliferative signals down-stream of the p53 protein and may link p53 to a direct signal transduction pathway [Walker K.K. and Levine A.J., 1996]. Deletions or mutations within this domain affect both p53 proapototic activity and its ability to transcriptionally activate target genes.
- The DNA binding domain is the host of the majority of the p53 mutations in human tumours. This domain contains the sequence-specific DNA binding activity of the p53 protein (residues 102-292), and their altered structure results in loss of DNA binding and therefore transcriptional activity [Cho Y. and Gorina S. *et al.*, 1994].
- The C-terminal domain is able to bind single stranded DNA, can activate the specific DNA binding domain and induce transcriptional transactivation of p53 providing a signal for DNA damage [Selivanova G. and Iotsova V. *et al.*, 1997].

p53 is usually inactive in normal cells and its half life is very short, 10-20 minutes. When the damage occurs, p53 is stabilised and activated and its function as tumour suppressor is achieved by inducing growth arrest and apoptosis.

In addition to its tumour suppressor activity, p53 performs important functions in normal tissues, being required for cell differentiation and apoptosis in embryogenesis and adult life.

Several studies implicated the p53 protein in the differentiation of hematopoietic cells [Shaulsky G. and Goldfinger N. *et al.*, 1991] [Aloni-Grinstein R. and Schwartz D. *et al.*, 1995], epidermal epithelial cells [Spandau D.F., 1994], cells in the central nervous system [Eizenberg O. and Faber-Elman A. *et al.*, 1996] and different types of cancer cells [Lutzker S.G. and Levine A.J., 1996] [Fagin J.A. and Tang S.H. *et al.*, 1996].

The involvement of p53 during embryogenesis was studied in mouse embryonic cells [Mora P.T. and Chandrasekaran K. *et al.*, 1980] and transgenic mice [Gottlieb E. and Haffner R. *et al.*, 1997]. p53 is expressed in early stages of embryogenesis but not in the late ones and this differential regulation could be associated with a possible role for p53 in cellular proliferation and/or differentiation.

1.3.3. DNA Damage Responses and p53 Activation

p53 is triggered in response to a variety of stress factors: bulky DNA lesions, DNA strand breaks, nucleotide depletion, oncogene activation (c-Myc, E2F-1, ras), hypoxia, hypoglycaemia, UV radiation, ionizing radiation, inappropriate proliferative signals, viral infections [Giaccia A.J. and Kastan M.B., 1998] (Figure 4).

Activation of p53 in response to different stresses is modulated through different mechanisms:

- alteration in the p53 protein half-life acquired by the interaction with MDM2; when MDM2 is not linked with p53, the intracellular levels of p53 protein increase, culminating in p53-dependent apoptosis and growth arrest. Some studies have shown that DNA damage and oncogene activation of p53 are coupled with reduced levels of MDM2-p53 complexes. Hypoxia also increases the p53 protein levels trough hypoxia-inducible factor-1α which is able to bind and stabilize p53 [An W.G. and Kanekal M. *et al.*, 1998].

- phosphorylation of p53 at key residues such as serine 15 or 37 induces conformational changes of the protein that impairs the ability of MDM2 to bind p53 [Shieh S.Y. and Ikeda M. *et al.*, 1997]. The direct contact of p53 with the cellular mdm2 results in degradation of p53.

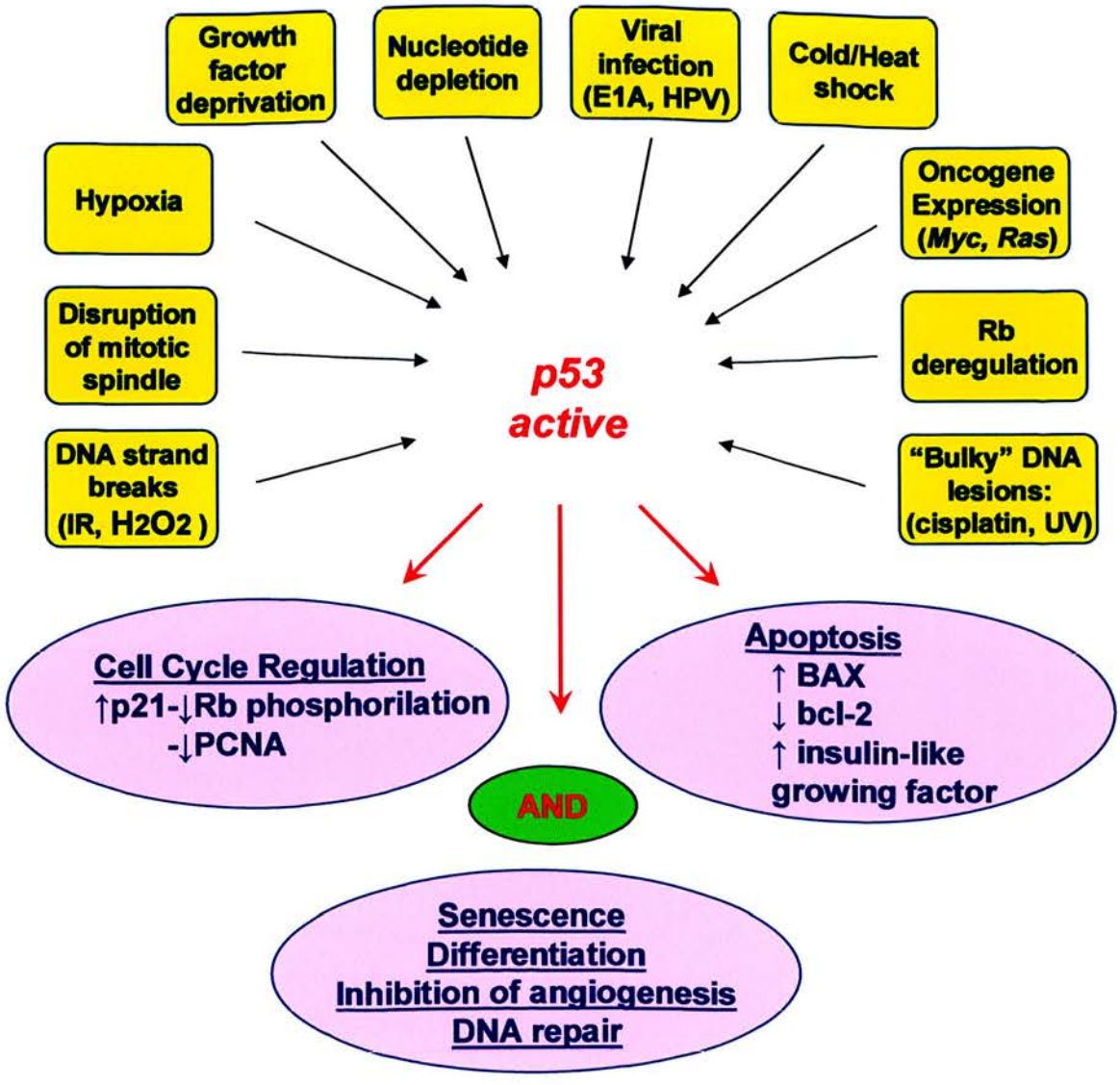


Figure 4: Activation of p53 is triggered by many different stresses including DNA-damaging and non-damaging agents. In response to these factors p53 regulates the expression of downstream genes (p21, BAX, bcl-2) and as a consequence of p53 activation, cells can undergo marked phenotypic changes, ranging from increased DNA repair to senescence, cell-cycle arrest and apoptosis.

- acetylation at the C-terminal domain activates the sequence-specific DNA-binding activity of p53 which subsequently influences the p53 target genes. A recent study showed that the DNA damage enhances p53 activity as a transcription factor through carboxy-terminal acetylation of lysine 382,which, in turn, is directed by amino-terminal phosphorylation [Sakaguchi K. and Herrera J.E. *et al.*, 1998].

- phosphorylation of the serine-392 residue in the human p53 protein (or residue 389 in the murine sequence) also occurs *in vivo* in response to ultraviolet radiation in cells containing the p53 protein. Phosphorylation at this specific residue was not detected in response to γ-radiation or the treatment of cells with etoposide [Lu H. and Taya Y. *et al.*, 1998].

1.3.4. p53 Induced Apoptosis and Growth Arrest

In response to damage factors, p53 is activated and responds by inducing cell-cycle arrest in G1 or G2, promotion of senescence and apoptosis. p53 activates the transcription of responsive genes when bound to particular p53-binding sites.

A major unsolved challenge is what factors are involved in a cell's decision to undergo cell-cycle arrest or apoptosis in response to p53 induction. Several factors have been shown to be important in changing the balance between growth arrest and apoptosis: cell type, the up-stream regulators of p53, the nature of damage and the amount of the activated p53.

Normal diploid fibroblasts undertake G1 cell-cycle arrest in response to gamma radiation induced DNA-damage [Di Leonardo A. and Linke S.P. *et al.*, 1994], while primary murine thymocytes undergo apoptosis following damage by ionizing irradiation [Clarke A.R. and Purdie C.A. *et al.*, 1993].

It seems that up-stream regulators of p53 are also implicated in mediating the p53 activity and different signals may predispose different cells to enter cell-cycle arrest or apoptosis. Ionizing radiation induction of p53 activation is ATM dependent. Irradiated ATM-deficient thymocytes are defective in p53-dependent growth arrest but they still have the capacity to undergo p53-dependent apoptosis [Barlow C. and Brown K.D. *et al.*, 1997], suggesting that ATM could be a factor that modulates cells' decision to undertake apoptosis or growth arrest. Overexpression of the viral oncogene E21 in mouse thymocytes leads to p53-dependent apoptosis and not p53-dependent growth arrest which is normally noticed following irradiation induced damage [Lowe S.W. and Schmitt E.M. *et al.*, 1993].

1.3.4.1. p53 Target Genes Involved in Cell-cycle Arrest Responses

p53 has been shown to be necessary for G1 and G2 growth arrest. The G1 cell cycle arrest is mostly mediated by p21, a cyclin-dependent kinase inhibitor. p21 protein subsequently binds to and inhibits cyclin-dependent kinase activity, therefore blocking cell cycle progression [El Deiry W.S. and Tokino T. *et al.*, 1993]. Other mechanisms are also implicated in G1 cell cycle arrest, as p21 knockout cells are only partially defective in irradiation induced G1 arrest [Brugarolas J. and Chandrasekaran C. *et al.*, 1995].

p53 is involved in G2 arrest following UV damage or injury caused by alkylating agents. This role has been suggested by the fact that GADD45 [Zhan Q. and Antinore M.J. *et al.*, 1999] and p21 [Bunz F. and Dutriaux A. *et al.*, 1998], both p53 target genes, can induce G2 arrest. Following disruption of either p53 or p21, gamma irradiated cells progressed into mitosis only because of a failure of cytokinesis.

1.3.4.2. p53 Target Genes Implicated in Apoptosis

To determine the mechanism by which p53 induces apoptosis, Polyak *et al.* [Polyak K. and Xia Y. *et al.*, 1997] examined in detail 7,202 transcripts induced by p53 expression before the onset of apoptosis. Only 14 transcripts were found markedly increased in p53-expressing cells compared with controls. These genes were named p53-induced genes (PIGs).

Several target genes of p53 take part in apoptotic responses following damage: bax, Fas/APO1, KILLER/DR5 in a caspase dependent manner.

The first p53 target gene involved in apoptosis was bax. p53 induces bax expression but bax is neither required nor sufficient in p53-dependent apoptosis. Bax knockout thymocytes are still capable to undergo apoptosis after gamma irradiation but p53 null thymocytes are severely affected [Knudson C.M. and Tung K.S. *et al.*, 1995]. Fas/APO1 is not required for p53-dependent apoptosis following damage, but it has been shown to delete peripheral T lymphocytes, mediating cytotoxic T-cell apoptosis [Nagata S., 1997].

1.3.5. p53 in Colorectal Cancer

In 1988, Monpezat *el al.* reported loss of p53 allele on chromosome 17 in polypoid colorectal carcinoma [Monpezat J.P. and Delattre O. *et al.*, 1988].

One year later, Baker *et al.* [Baker S.J. and Fearon E.R. *et al.*, 1989] concluded that p53 mutations are involved in colorectal cancer probably by inactivation of the tumour suppressor function of the wild type (wt) p53. Loss of 17p allele and mutations of the p53 gene are common events in human malignancy. Interestingly, when carcinoma and adenoma areas of the same tumour were genetically screened, mutations and loss of allele were detected in the carcinoma but not in the adenoma, suggesting that the two events were linked together and probably occur at the adenoma to carcinoma progression stage [Baker S.J. and Markowitz S. *et al.*, 1990].

Overexpression of p53 by immunohistochemistry using antibodies specific for p53 was demonstrated in 50% of the colorectal cancers examined [Rodrigues N.R. and Rowan A. *et al.*, 1990]. Benign adenomas were all negative for p53 overexpression. This observation was also indicative of an implication of p53 in late stages of colon carcinogenesis as previously mentioned.

In 1992, Vogelstein and Kinzler [Vogelstein B. and Kinzler K.W., 1992] summarized five mechanisms for p53 inactivation and their occurrence in different types of cancer. p53 is capable of activating the expression of adjacent genes that inhibit growth and/or invasion. Deletion of one or both p53 alleles reduces the expression of the protein, decreasing the expression of the growth inhibitory genes. This mechanism is found in occasional tumours of several types. Nonsense or splice site mutations that result in truncation of the protein do not allow oligomerization,

thus resulting in a reduction of p53. This type of mutations are common in lung, oesophagus, and other cancers. A third mechanism, which is seen in colon, brain, lung, breast, skin and bladder cancer involves missense mutations. Their effect is a mutant p53 protein defective in binding DNA in a sequence specific manner and transcriptional activation of downstream target genes. The fourth mechanism by which p53 is involved in carcinogenesis is commonly encountered in cervical cancers, where the expression of the E6 gene of human papilloma-virus results in the functional inactivation of p53 through binding and degradation. The fifth mechanism implicates disruption and alterations of MDM2. MDM2 gene is amplified in the vast majority of human sarcomas [Oliner J.D. and Kinzler K.W. *et al.*, 1992] and the consequent overexpression of MDM2 may interfere with p53 activity. p53 is phosphorylated at serine 15 (MDM2 binding area) in response to DNA damage and this event reduces the interaction of p53 with its negative regulator MDM2 [Shieh S.Y. and Ikeda M. *et al.*, 1997].

Other studies focused on identifying p53 mutations in colorectal cell-lines [Rodrigues N.R. and Rowan A. *et al.*, 1990]. Mutations in the p53 gene were detected in all cell lines examined (although not all were immunopositive for the p53 protein), therefore suggesting that not all mutations could be detected using immunohistochemical tests.

1.4. New Members of the p53 Family Genes

For a long time p53 was believed to be the only member of its family. Recently, two new members of the p53 family were discovered: p73 [Kaghad M. and Bonnet H. *et al.*, 1997] in 1997 and p63, one year later [Yang A. and Kaghad M. *et al.*, 1998]. They are structurally related proteins and although share similar functions such as transcriptional activation, cell-cycle arrest and apoptosis, each of them seem to also play different roles in normal development.

An important subject in oncology research was to investigate whether the new members of the p53 family genes have a tumour suppressor role. Following their discovery, it was believed that they would be involved in tumour suppression, exactly like p53. But, in contrast to *p53*, which is mutated or deleted in approximately 50% of human cancers, it was found that *p73* and *p63* were not inactivated in a large number of cancers. Subsequent studies revealed that both p73 and p63 were actually more involved in neurogenesis, natural immunity and homeostatic control [Yang A. and Kaghad M. *et al.*, 2002] than in tumour suppression.

However, there is a great structural homology between the three proteins, particularly in the N-terminal transactivation domain, DNA binding domain and the oligomerization domain [Chen X., 1999] (Figure 5). p73 and p63 are even capable to activate some of the p53 target genes, like p21, MDM2, GADD45 [Lee C.W. and La Thangue N.B., 1999]. Moreover, when activated, they are also capable of inducing apoptosis, in the same way as p53 [Jost C.A. and Marin M.C. *et al.*, 1997], [Yang A. and Kaghad M. *et al.*, 1998].

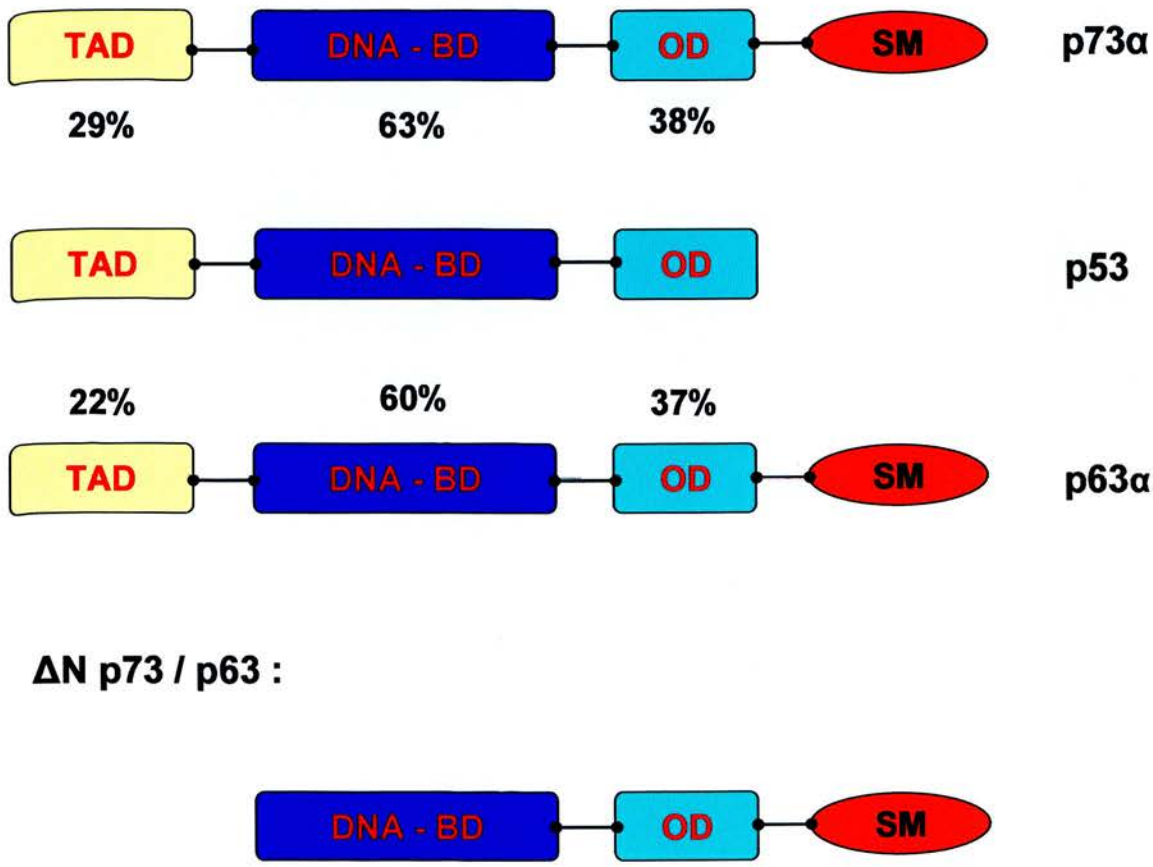


Figure 5: Homology among the p53 family of proteins: p53, p63α and p73α and the percentage of p53-similar residues found in p73 and p63. The ΔN isoforms of both p63 and p73 lack the transactivation domain.

Abbreviations: TAD - transactivation domain

DNA-BD – DNA binding domain

OD – oligomerization domain

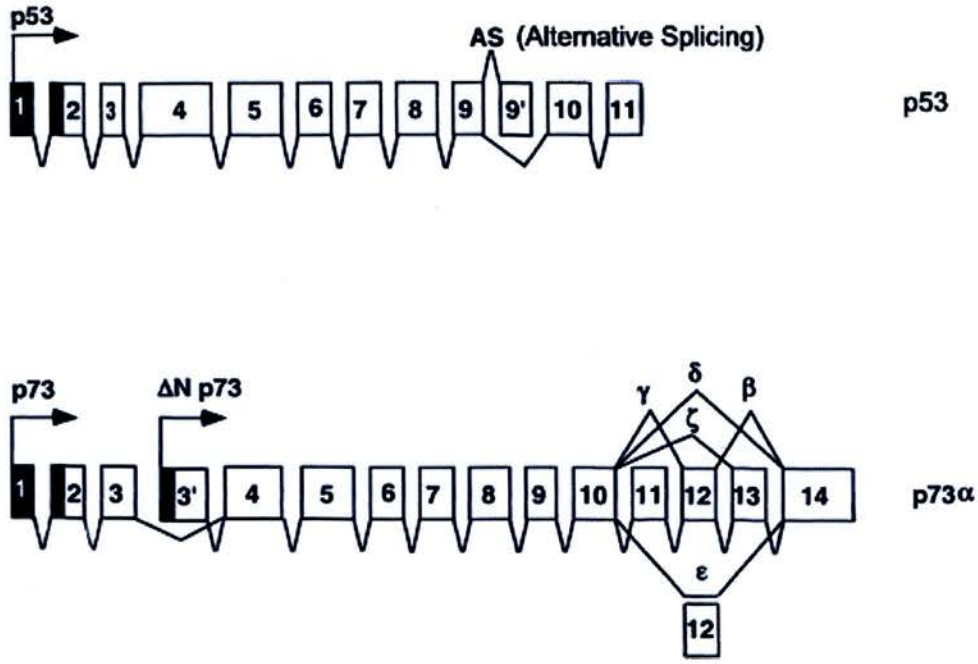
SM – sterile α motif

1.4.1. p73

1.4.1.1. Gene and Protein Structure

p73 protein shares significant homology with p53 in the major functional domains: transactivation domain (30% identical with p53), DNA binding domain (60% identical with p53) and C-terminal oligomerization domain [Marin M.C. and Kaelin W.G., Jr., 2000]. The transactivation domain of p73 interacts with p300 and MDM2 in the same manner as p53. In particular, each of the p53 amino acids implicated in direct sequence-specific DNA binding is preserved in the p73 protein.

The overall genomic organization of the *p73* is similar to that of *p53*, the only difference being the larger size of *p73*, which contains 14 exons compared with only 11 exons in *p53* [Marin M.C. and Kaelin W.G., Jr., 2000]. The *p53* gene encodes only one transcript that yields one protein which is transcriptionally active. In contrast, *p73* produces multiple mRNA transcripts by alternative splicing. *p73* generates two proteins expressed under the control of two independent promoters: the transcriptionally active (TA) p73 generated by the proximal promoter and the N-terminally truncated (ΔN) p73 which is generated by the second promoter located in intron 3 and lacks the transactivation domain [Benard J. and Douc-Rasy S. *et al.*, 2003] (Figure 6). These proteins have opposite activities. The TAp73 isoforms act as transcription factors and induce cell-cycle arrest and apoptosis, whereas the ΔN forms are incapable of inducing gene expression directly and do not trigger cell-cycle arrest and apoptosis. Moreover, the N-terminally truncated forms exhibit dominant-negative effects by controlling the activity of TAp73 and p53, blocking their



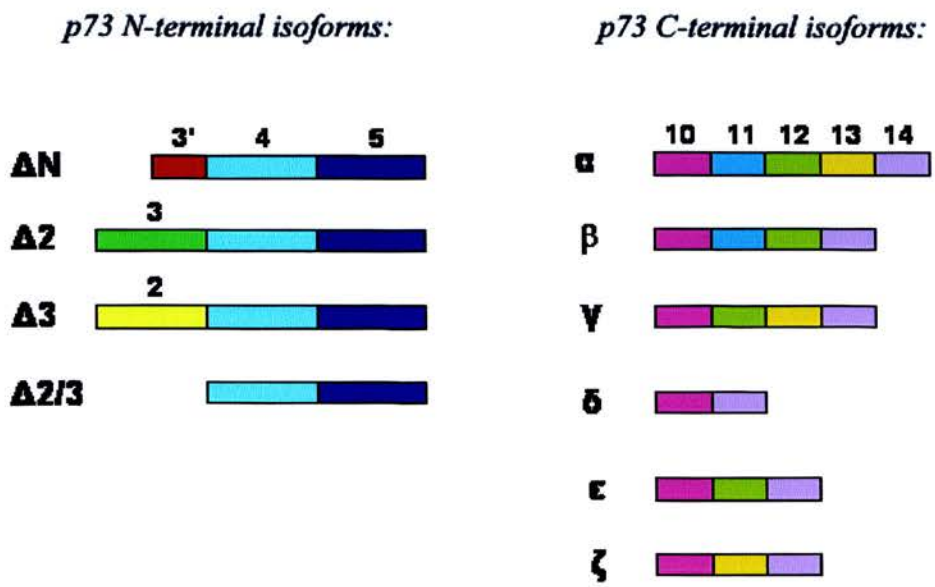


Figure 6: Gene organization of the p53 and p73. Exons are shown as numbers in the boxes and introns as connectors between the exons. p73 generates at least 6 splice variants in contrast to p53 which has only two. p73 C-terminal isoforms are generated by alternative splicing of exons 10 to 14. p73 N-terminal isoforms are transcribed from an alternative promoter identified here as 3'. Apart from this one, recently other N-terminal splice variants have been identified: p73Δ2 and p73Δ2/3 that lack either exon 2 or both exon 2 and 3 [Stiewe T. and Putzer B.M., 2002] and p73Δ3 that lacks exon 3 [Melino G. and DeLaurenzi V. et al., 2002].

transactivation activity and therefore limiting their apoptotic function. Furthermore, the oncogenic variant ΔNp73 is induced by TAp73 and p53, creating a negative feedback loop that regulates the functions of p53 and p73 [Benard J. and Douc-Rasy S. *et al.*, 2003]. Knock out mice for p73 lack both thc apoptotic and the oncogenic form and this may be the reason why they do not develop spontaneous tumours [Yang A. and Sharpe A. *et al.*, 2000].

1.4.1.2. Regulation of p73 Activity

Activation of p53 is usually a result of post-translational modifications such as phosphorylation or acetylation, which are responsible for its stability and activities: cell cycle arrest and apoptosis. p73 activation occurs through similar mechanisms as p53 but novel pathways have also been described.

p73 regulation occurs as a result of interaction with DNA damaging agents, tumour suppressor genes, oncogenes and differentiation signals. Interestingly, even p53 is an upstream regulator for p73, in the same way as p73 is required by p53 to exert its apoptotic functions.

Some DNA damaging agents, such as ionizing radiation and cisplatin, but not UV radiation or actinomycin [Kaghad M. and Bonnet H. *et al.*, 1997], are capable of activating and induce p73. The activation of p73 in response to DNA damage involves post-translational modification rather than transcription. The amount of p73 protein increased in cisplatin treated cells [Gong J.G. and Costanzo A. *et al.*, 1999] and was diminished in cells defective in mismatch repair gene MLH1 or the nuclear enzyme c-Abl tyrosine kinase, suggesting that the molecular mechanism implicated in cisplatin p73 activation involves the MLH1-c-Abl-p73 pathway. c-Abl is also required for p73 phosphorylation in response to ionizing radiation *in vivo* and *in vitro,* as demonstrated by a failure of ionizing radiation to induce apoptosis after disruption of c-Abl-p73 interaction [Yuan Z.M. and Shioya H. *et al.*, 1999]. Another mechanism of p73 activation is the acetylation by acetyltransferase p300 on C-terminal lysine residues [Costanzo A. and Merlo P. *et al.*, 2002]. A non-acetylatable p73 is defective in activating transcription of the p53 gene, but retains the ability to

regulate p21. p300-mediated acetylation of p73 is also proto-oncogene Abl dependent . Therefore, DNA damage-induced acetylation potentiates the apoptotic function of p73 by enhancing the capacity of p73 to selectively activate the transcription of pro-apoptotic target genes.

p73 is also regulated by oncoproteins like E2F1, c-Myc and MDM2. The E2F1 transcription factor, which is deregulated in many human tumours, has the ability to activate p73 by binding to E2F-binding sites (at least 3) in the TAp73 promoter and directly activating p73α and p73β transcription [Irwin M. and Marin M.C. *et al.*, 2000]. The expression of E2F1 causes accumulation of p73 mRNA, therefore increasing the levels of p73 protein. Moreover, disruption of p73 function inhibited E2F-1-induced apoptosis in p53-deficient tumour cells and in p53-/- mouse embryo fibroblasts.

c-Myc can physically interact with and bind p73, but its protein partner MM1 has the ability to remove this interaction, therefore mediating the activation of p73 [Watanabe K. and Ozaki T. *et al.*, 2002].

The MDM2 binding site in p53 is well conserved in the structure of p73. MDM2 binds to p73 resulting in inhibition of p73 binding to transcriptional co-activators p300 and CREB-binding protein [Zeng X. and Li X. *et al.*,]. This binding leads to impaired p73-transriptional activation and defective apoptosis. After binding to p73, MDM2 does not contribute to its ubiquitylation and degradation [Ongkeko W.M. and Wang X.Q. *et al.*, 1999], as it does when it binds p53, highlighting another difference between p53 and p73. This interaction between p73 and MDM2 also affects the sub-cellular localization of p73 and it seems to contribute to its stability [Wang X. and Arooz T. *et al.*, 2001].

Recent studies revealed that hematopoietic and neuronal differentiation influences the expression of p73. mRNA p73 levels were shown to increase in normal and cultured peripheral blood lymphocytes following stimulation with phytohemagglutinin [De Laurenzi V.D. and Catani M.V. *et al.*, 1999]. This stimulation also produced increased p73-dependent apoptosis. Moreover, stimulation of the T-cell receptor with anti-CD3 also leads to increased levels of the p73 protein and apoptosis [Lissy N.A. and Davis P.K. *et al.*, 2000]. Increased levels of the p73 protein and neuronal differentiation specific cell markers were detected in neuroblastoma cells treated with retinoic acid [De L., V and Raschella G. *et al.*, 2000].

Viral oncoproteins have the ability to interact with members of the p53 family, especially p53 and p73. They also have the ability to discriminate between them. If the adenovirus E1B55 , human papilloma virus E6 protein, and simian virus (SV40) T antigen bind to and have the ability to inactivate p53, they do not interact with p73 [Marin M.C. and Jost C.A. *et al.*, 1998]. However, there are viral proteins like the adenovirus E1A protein [Steegenga W.T. and Shvarts A. *et al.*, 1999] and the human T-cell leukaemia virus type 1 Tax protein [Kaida A. and Ariumi Y. *et al.*, 2000] which bind and inactivate both p53 and p73, without any discrimination.

1.4.1.3. Downstream Targets of p73

p73 has been shown to regulate the expression of several genes, some of which are also p53 responsive genes. The regulation of similar p53-responsive promoters causes effects common to those following the activation of p53. For example, *p21, GADD45, cyclin G, IGFBP3, Bax, p53-inducible ribonucleotide reductase 2* genes are involved in cell-cycle control, DNA repair and apoptosis [Nakano K. and Balint E. *et al.*, 2000; Yu J. and Zhang L. *et al.*, 1999; Zhu J. and Jiang J. *et al.*, 1998]. Apart from activating some of these genes, p73 seems to contribute to the apoptotic function of p53, by binding to MDM2 and leading to increased levels of stabilised p53 and consequently apoptosis. In contrast, the truncated forms of p73 have an anti-apoptotic function acquired by inhibition of TAp73 and p53.

Secondly, p73 gene is capable of activating genes that are only weakly induced by p53, like *aquaporin 3* (AQP3) [Zheng X. and Chen X., 2001], a gene important in maintaining cerebral spinal fluid homeostasis. The same authors assumed that the lack of regulation of the above gene could be the reason why p73 knockout mice develop hydrocephalus.

Apart from activating similar p53-responsive genes, p73 has functions that are not shared by p53 such as down-regulation of the expression of *vascular endothelial growth factor gene* (VEGF) [Salimath B. and Marme D. *et al.*, 2000]. In addition, it has a possible role in the control of angiogenesis and possibly in tumour angiogenesis, as the VEGF production is increased following p73 overexpression [Vikhanskaya F. and Bani M.R. *et al.*, 2001]. Another example would be the involvement of p73 in the WNT/β-catenin signalling pathway [Ueda Y. and Hijikata

M. *et al.*, 2001]. p73 activates this pathway but, unlike p53, not through accumulation of β-catenin. The p73 contribution to normal development is made through the ability of p73β to activate the expression of the *cyclin-dependent kinase inhibitor p57(KIP)* and *KvLQT1* genes through mechanisms that again, are not shared by p53 [Blint E. and Phillips A.C. *et al.*, 2002].

1.4.1.4. Role of p73 in Malignancy

p73 maps to the minimal region of 1p36 which is frequently deleted in primary neuroblastomas [Ichimiya S. and Nimura Y. *et al.*, 2001]. Further studies focused on demonstrating that p73 is a tumour suppressor in a classic Knudson's manner. Extensive studies revealed only rare p73 mutations in both cell-lines and primary tumours, including neuroblastomas with 1p36 deletions [Irwin M.S. and Kaelin W.G., Jr., 2001]. There was, though, a significant incidence of loss of heterozygosity in neuroblastoma, lung cancer, gastric cancer, esophageal cancer, ovarian cancer and breast cancer, without a clear confirmation that the remaining allele was silent.

Trying to understand its role in tumourigenesis, researchers were confused about the fact that *p73* encodes two forms with opposite functions: TAp73 tumour suppressor and ΔNp73 oncogene. Additionally, as the same exons of the p73 gene contain the DNA-binding domains of both isoforms, mutations in this region would inactivate both the tumour suppressor and the putative oncogene, and could explain the lack of p73 mutations in cancer [Melino G. and De L., V *et al.*, 2002].

Overexpression of p73 was described in few cancer cell-lines (ovarian, breast , neuroblastoma), in colon cancer [Sun X.F., 2002] and hepatocellular carcinoma

[Tannapfel A. and Wasner M. *et al.*, 1999]. p73 overexpression, without discriminating between its isoforms, was so far associated with poor prognostic factors. Only one study revealed that poor survival rate was associated with overexpression of ΔNp73 in ncuroblastoma [Casciano I. and Mazzocco K. *et al.*, 2002], suggesting that the loss of balance between the TA and ΔN forms is important.

1.5. Sonic Hedgehog Signalling Pathway

It has been long known that Hedgehog (Hh) signalling pathway is crucial for normal development, but in the past few years there has been an increase in the understanding of how developmental genes and Hh in particular, could be involved in tumourigenesis. Recently, it has been hypothesized that tumour suppressor genes deletions or mutations are late events in carcinogenesis and maybe defects or mechanisms that trigger cancer in adult life appear and develop early in embryonic stages. In the past ten years, signalling pathways essential for embryonic development have been found to be abnormally regulated in cancer [Taipale J. and Beachy P.A., 2001].

1.5.1. Sonic Hedgehog in Development and Disease

Few signalling cascades are essential in embryogenesis and one such pathway, Sonic Hedgehog (Shh), is involved in morphogenesis and patterning of many organs. The hedgehog gene was first described in the fruit fly *Drosophila melanogaster.* Unlike drosophila, which has only one hh gene, mammals have three genes, Sonic hedgehog (Shh) [Fang L. and Lee S.W. *et al.*, 1999], Indian hedgehog (Ihh) [Yokomizo A. and Mai M. *et al.*, 1999] and Desert hedgehog (Dhh) [Fang L. and Lee S.W. *et al.*, 1999] that are homologous to the hh gene. Of the three genes, Shh is the most widely expressed. The Hh signalling pathway involves other several genes including *patched, dispatched, smoothened, fused, cubitus interruptus*, most of which are highly conserved between insects and vertebrates. In the mammals, there are two *patched (Ptch1 and Pthc2)* [Hahn H. and Wicking C. *et al.*, 1996; Johnson R.L. and Rothman A.L. *et al.*, 1996] and one *Smoothened* gene *(Smoh)* [Stone D.M. and Hynes M. *et al.*, 1996]. The human equivalent of *cubitus interruptus* is *GLI,* which has three variants, *GLI1*, *GLI2* and *GLI3* [Altaba A., 1999]. Moreover, the functional properties of these genes appear to be highly conserved between species.

The Hh pathway is expressed in the Hensen node, the floorplate of the neural tube, the early gut endoderm, the posterior of the limb buds and throughout the notochord [McMahon A.P. and Ingham P.W. *et al.*, 2003]. It has also been implicated as an important signal in the patterning of the ventral neural tube [Echelard Y. and Epstein D.J. *et al.*, 1993], the anterior-posterior limb axis [Riddle R.D. and Johnson R.L. *et al.*, 1993] and the ventral somites [Johnson R.L. and Laufer E. *et al.*, 1994].

Malformations of several organs have been described in the Shh knockout mice. Morphologic studies in mice homozygous for a disrupted Shh gene [Chiang C. and Litingtung Y. *et al.*, 1996] revealed defects in the maintenance of mid-line structures such as the notochord and floorplate as well as absence of distal limb structures, cyclopia, absence of ventral cell types within the neural tube and absence of the spinal column and most of the ribs. The defects in all tissues extended beyond the normal sites of Shh transcription, confirming that Shh protein acts as an extracellular signal that is required for the normal patterning of several systems. Homozygous Shh null mutant mice were also shown to have foregut defects: oesophageal atresia/stenosis, tracheo-oesophageal fistula and hypoplastic lungs with a single lobe [Litingtung Y. and Lei L. *et al.*, 1998]. The same study showed that the lung mesenchyma displayed increased cell death and decreased cell proliferation, reiterating that the pathway is indeed essential for normal growth and differentiation of oesophagus, trachea, and lungs. Mutations in *Shh* and its signalling components may be involved in foregut defects in humans. Gastrointestinal defects including gut malrotation, reduced smooth muscle, intestinal transformation of the stomach, duodenal stenosis with overgrowth villi, annular pancreas and imperforated anus were also described in homozygous mice for Shh [Ramalho-Santos M. and Melton D.A. *et al.*, 2000].

Sporadic and inherited mutations of the human *Shh* causes holoprosencephaly (HPE) (OMIM 142945) [Belloni E. and Muenke M. *et al.*, 1996], an autosomal dominant condition with features like premaxillary aplasia with midline cleft lip and palate, hypotelorism, sensorineural hearing loss, lack of tooth eruption, single cyclopic eye and cervical cord compression due to stenosis. Holoprosencephaly is also a cause of

prenatal death detected in 1 in 250 abortions [Cohen M.M., Jr., 1989]. Loss of one *Shh* allele is sufficient to cause HPE in humans, whereas both *Shh* alleles need to be lost to produce a similar phenotype in mice [Roessler E. and Belloni E. *et al.*, 1996] [Chiang C. and Litingtung Y. *et al.*, 1996].

Ptch and Smoh genes are also clinically important. Genetic mutations generating a truncated or unstable Ptch protein [Stone D.M. and Hynes M. *et al.*, 1996], or activating mutations of Smoh [Xie J. and Murone M. *et al.*, 1998] are associated with basal cell carcinoma and medulloblastoma. Ptch functions as a tumour suppressor and mutant Smoh as an oncogene. Inactivating mutations of Ptch cause the nevoid basal cell carcinoma syndrome or "Gorlin syndrome", characterized by basal cell nevi with a high potential of malignant transformation, increased risk of medulloblastomas and rhabdomyosarcomas, short metacarpals, dental abnormalities and rib defects.

1.5.2. Hedgehog Signalling Pathway

All hedgehog genes encode molecules that are engaged in short and long range patterning processes during embryogenesis.

To exert its function, Shh undergoes molecular processing in the endoplasmic reticulum, which involves autocatalytic cleavage of the precursor hedgehog protein into two domains: a 19 kDa N-terminal domain (Shh-N) and a 25 kDa C-terminal domain [Marti E. and Bumcrot D.A. *et al.*, 1995]. The N-terminal domain is responsible for the protein signalling activity, whilst the C-terminal domain is required for covalently binding a cholesterol molecule to the N-terminal domain. This anchors the N-terminal domain to the cell membrane, limiting the Shh diffusion from the site of secretion [Porter J.A. and Young K.E. *et al.*, 1996].

Shh-N binds to a 12-transmembrane receptor Patched (Ptch), which normally inhibits the downstream signalling through its binding to a 7-transmembrane receptor, Smoothened (Smoh). When the pathway is activated, Shh-N binds to Ptch, Smoh repression is released and the signalling continues via Gli genes (Figure 7). Ptch further limits the range of Hh action. High levels of Ptch induced by Hh serve to sequester free Hh, therefore creating a barrier to its further movement [Chen Y. and Struhl G., 1996]. The function of Ptch and other inhibitors is to silence the pathway in situations when the active Hh ligand is absent. Therefore, the pathway must be switched off most of the time and active only in situations when its function is required. It is yet unknown whether the Gli proteins are the only mediators of the Hh signalling, but they play a central role in mediating the Hh signals.

1.5.2.1. Targets and mediators of the Gli genes

The Gli family of zinc-finger transcription factors act as important intracellular mediators of Shh signals. In mammals, there are three Gli factors, Gli1, Gli2, Gli3 with Gli1 and Gli 2 acting as activators of many Shh-responsive genes, in contrast to Gli3 which act as negative regulators and suppress the Shh-target genes ([Kato M. and Seki N. *et al.*, 2001]).

As Shh has such a wide range of activities, it was of interest to identify some of the downstream target genes in the pathway. Despite the well-established functions of the Shh signalling in development and disease, only a few regulatory genes have been identified as direct targets of Gli regulation.

One of theses targets, HNF3-beta is an early developmental regulator of notochord and floor plate formation and its expression is controlled by Gli2, through a floor-plate enhancer which has a Gli-binding site [Dufort D. and Schwartz L. *et al.*, 1998] [Sasaki H. and Hui C. *et al.*, 1997].

Gli proteins can also regulate the expression of different Wnt genes, as it has been recently demonstrated [Mullor J.L. and Dahmane N. *et al.*, 2001]. During ventro-posterior morphogenesis in frog Gli2 and Gli3 directly activates Wnt 8 and possibly Wnt11, which in turn will stimulate the β-catenin and the Ca^{2+}/CamKII pathways.

Additional targets of the Shh-Ptch signalling pathway with a particular function in tumorigenesis include Cyclin D and E, FOXM1 and Igf2 [Altaba A. and Stecca B. *et al.*, 2004].

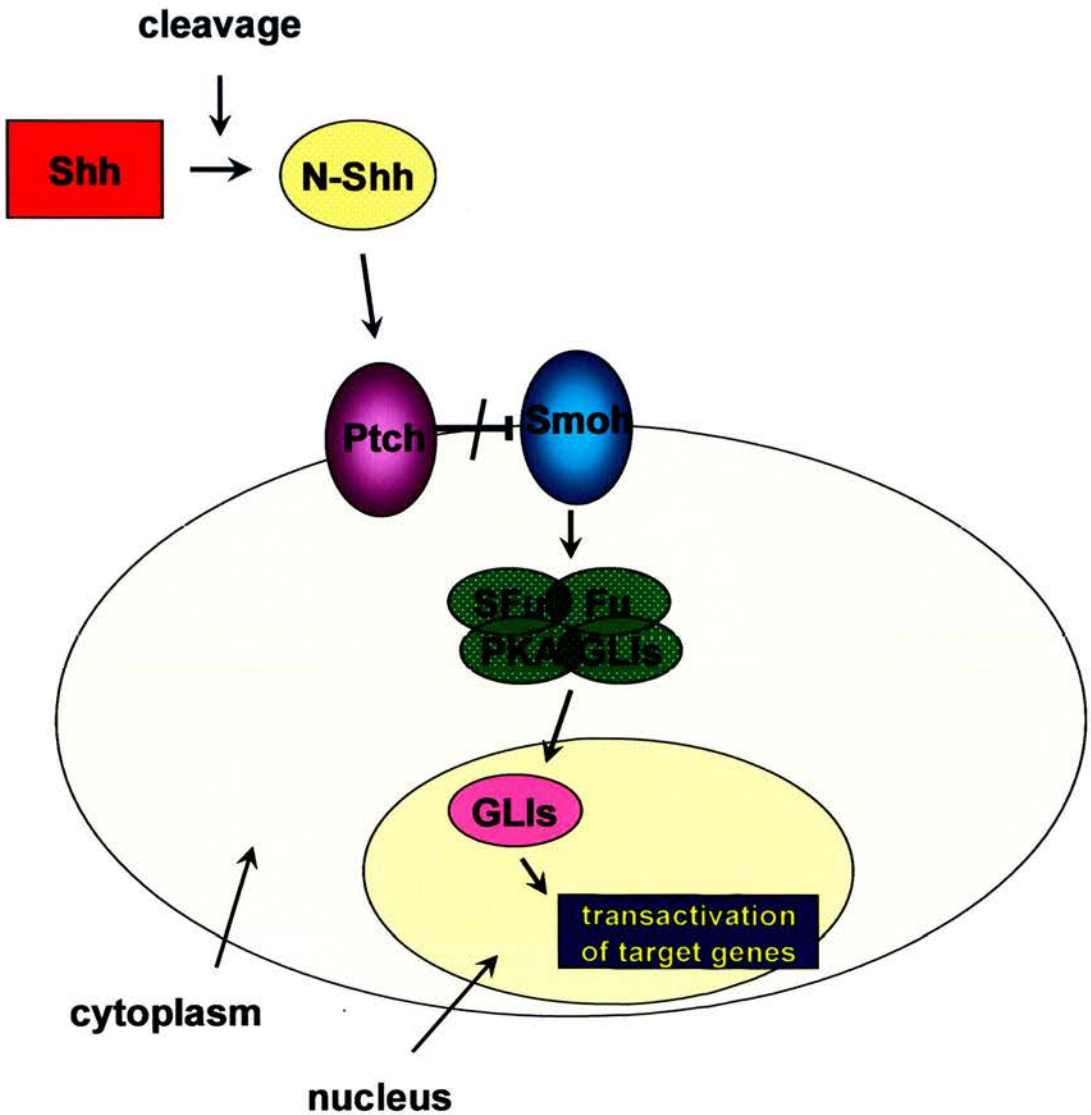


Figure 7: On Shh binding to Ptch, the inhibition Smoh by Ptch is release. Smoh acts on a complex of cytoplasmic factors that includes SFu (Suppressor of fused), Fu (Fused), PKA (protein kinase A), the Gli proteins and other possible components not yet identified. The pathway culminates in the activation of Gli transcription factors and transactivation of target genes.

1.5.3. Sonic Hedgehog Pathway Genes in Tumourigenesis

With the involvement of the Shh receptor Ptch as a tumour suppressor gene responsible for the human nevoid basal cell carcinoma syndrome, the Hedgehog signalling pathway was connected to cancer. It now emerges that constitutive activation of Hedgehog signalling by inactivating mutations in Ptch or activating mutations in Smoh, is required and perhaps sufficient for basal cell carcinoma development.

Indeed, inactivation of Ptch is a major factor in basal cell carcinoma growth with mutation identified in 12 to 40% of sporadic BCCs [Unden A.B. and Zaphiropoulos P.G. *et al.*, 1997] [Wolter M. and Reifenberger J. *et al.*, 1997] [Aszterbaum M. and Rothman A. *et al.*, 1998].

Furthermore, Shh was sufficient to induce BCCs in transgenic mice overexpressing Shh in the skin [Oro A.E. and Higgins K.M. *et al.*, 1997]. These mice developed multiple features of the basal cell nevus syndrome. The mouse BCCs appeared within the first 4 days of skin development, unlike mouse squamous neoplasia where tumours arise 1 to 12 months after oncogene expression. The rapid and frequent appearance of Shh-induced tumours in the mice suggested that disruption of the Shh-Ptch pathway is sufficient to create BCCs. The kinetics of the tumours in these mice were consistent with previous clinical and epidemiologic data, which suggested that BCCs, in contrast to melanomas and squamous carcinomas, lack precursor or intermediate cellular phenotypes.

In addition to its association to BCCs, it seems that the Hh pathway contributes to the formation of a variety of other tumour types including medulloblastoma [Pietsch T.

and Waha A. *et al.*, 1997], squamous cell carcinoma of the oesophagus [Maesawa C. and Tamura G. *et al.*, 1998], transitional cell carcinoma of the bladder [McGarvey T.W. and Maruta Y. *et al.*, 1998] and benign trichoepitheliomas [Vorechovsky I. and Unden A.B. *et al.*, 1997]. Mice heterozygous for Ptch develop smooth muscle tumours [Hahn H. and Wojnowski L. *et al.*, 1998], indicating that the pathway may take part in the pathogenesis of the human rhabdomyosarcoma.

Activating mutations of Smoh have been detected in up to 20% of BCCs [Lam C.W. and Xie J. *et al.*, 1999] as well as in primitive neuroectodermal tumours [Reifenberger J. and Wolter M. *et al.*, 1998].

Very recent work implicates sonic hedgehog in the genesis of pancreatic cancer [Thayer S.P. and Di Magliano M.P. *et al.*, 2003]. The same study showed that Shh is strongly expressed in pancreatic precursor and invasive lesions but is undetectable in human normal ductal epithelium. Additionally, a wide range of digestive tract tumours originating in the oesophagus, stomach, biliary tract and pancreas, but not in the colon, displayed increased hedgehog pathway activity, which was suppressible by cyclopamine, a hedgehog pathway antagonist [Berman D.M. and Karhadkar S.S. *et al.*, 2003].

1.6. AIMS

In the last two decades there have been constant developments in the understanding of the genetics of the colorectal cancer. New genes and mechanisms have been discovered, which have brought us closer to unravelling the complex process of oncogenesis. And yet, there is no complete understating of the function of the newly discovered genes and until human based research can be safely undertaken, murine experimental models will help widening our knowledge.

Consequently, the aims of this thesis are to:

1. Better understand the importance of p53 and related proteins in the regulation of growth and response to DNA damage following cisplatin injury in primary murine colonocyte culture.
2. Investigate p53-dependent and independent death pathways in response to cisplatin DNA damage in primary murine colonocytes.
3. Look at the expression of the Hh signalling pathway at protein and mRNA levels in normal human colon by immunohistochemistry and RT-PCR from laser capture microdissected crypts.
4. Examine the distribution patterns of Shh, Ptch and Smoh proteins in colonic tumourigenesis (hyperplastic polyps, adenomas and colonic adenocarcinomas).
5. Explore a possible functional role for Shh in primary murine epithelial cells *in vitro*.

CHAPTER 2

MATERIALS AND METHODS

2.1. Mice

Wild-type and p53-/- mice [Purdie C.A. and Harrison D.J. *et al.*, 1994] were bred, genotyped and humanely killed between 50 and 75 days of age, before developing spontaneous tumours. The colony was maintained in the Medical Faculty Animal Unit.

BDF-1 mice [Booth C. and Patel S. *et al.*, 1995] were purchased (Charles River-company) and maintained in the Medical Faculty Animal Unit at The University of Edinburgh. All the experiments involving animal tissue were performed in accordance with the animal ethics regulation of the Home Office in the United Kingdom.

2.2. Isolation of Colonic Crypts and Culture of Primary Colonocytes from Adult Wild-Type and p53-/- Mice

The crypt isolation method was successfully adapted from Booth *et al.* (1995). After dissection, colons from 3 mice were removed and placed in HBSS (Invitrogen) supplemented with antibiotics and fungicide to minimise the risk of yeast infections. (Amphotericin, Sigma). Their contents were flushed with HBSS solution using a syringe with a pipette tip. They were then placed in a Petri dish and any mesenchymal tissue was removed using a scalpel. Colons were cut into small pieces and washed vigorously several times in 50ml of HBSS until free from faecal material. After all the excess HBSS was removed the tissue was finely minced to a pulp on a sterile glass dish using two scalpels. The colonic material was then transferred into 30ml of digestion medium. The enzymatic digestion was carried out in a water bath at 37°C with occasional shaking every 20 minutes until the single crypts visible under the microscope were released into the medium. Undigested material was removed by sieving using sterile meshes size 6. The filtrate was transferred to universals and topped up with 2% D-sorbitol medium pre-warmed at 37°C. The tubes were centrifuged at 400rpm to remove contaminants and enzymes and the pellets were washed repeatedly with 2% D-sorbitol medium 4 or 5 times until the supernatant appeared clear. The crypts were then suspended in culture medium and plated onto collagen IV (Sigma) coated sonic-seal slides (Invitrogen) at a density of 800-1000 crypts per ml. The crypts were allowed to settle and attach for 48 hours and subsequently the medium was changed daily with fresh culture medium. The crypts were incubated at 37°C in an atmosphere of 5% CO_2 in air. Experiments were

performed between 3 and 5 days of culture when proliferation and viability was maximal.

2.2.1. Digestion Medium

-DMEM high glucose (4.5g/l) (Invitrogen)

-20μg/ml dispase type II (Calbiochem)

-75U/ml colagenase type IX (Sigma)

-Antibiotics: 100U/ml penicillin (Invitrogen); 30μg/ml streptomycin (Invitrogen) and 25μg/ml gentamicin (Sigma) and 2.5μg/ml amphotericin.

-2.5% Serum (½ Foetal Calf Serum & ½ Foetal Bovine Serum) (both purchased from Invitrogen)

2.2.2. 2% D-sorbitol Medium

- 2mg D-sorbitol powder (Sigma) per 100ml DMEM, sterile filtered

- 2.5% serum (½ Foetal Calf serum & ½ Foetal Bovine serum)

- Antibiotics: 100U/ml penicillin (Invitrogen); 30μg/ml streptomycin (Invitrogen) and 25μg/ml gentamycin (Sigma)

2.2.3. Culture Medium

Medium composition is important for both cell survival and maintenance of murine colonocytes *in-vitro.* The present recipe is adapted from Booth *et al.* (1995). We used low serum content medium to inhibit the growth of fibroblast in culture, therefore minimising the fibroblast contamination. The medium was supplemented with glutamine, insulin/transferrin/selenium, epidermal growth factor important for epithelial cell survival and antibiotics to reduce the possibility of microbial contamination especially important in colonic preparation.

Culture Medium Content:

- DMEM supplemented with Glutamax (L-Glutamine) (Invitrogen)
- 2.5% serum (½ Foetal Calf serum & ½ Foetal Bovine serum)
- Antiobiotics: 100U/ml penicillin (Invitrogen); 30μg/ml streptomycin (Invitrogen) and 25μg/ml gentamycin (Sigma)
- 5ng/ml Epidermal Growth Factor
- Insulin

2.2.4 Collagen Matrix

Primary murine colonocytes were cultured on sonic seal slides (Invitrogen). Cells do not attach normally to normal plastic surface; therefore we applied a collagen IV (Sigma) matrix prior to culture. Slides were kept at 4°C, overnight with collagen IV

(1mg/ml), diluted in acidified DMEM (0.05% acetic acid) and allowed to dry in a tissue culture cabinet. Coated slides were stored at -20°C wrapped in foil and defrosted in a tissue culture cabinet prior to use.

2.2.5. Morphology of Primary Colonocyte Cultures

Immunocytochemistry with monoclonal pan anti-human keratin antibody (DAKO) specific for epithelial cells and monoclonal anti-vimentin antibody (clone V9, Sigma, V6630) specific for fibroblasts, endothelial, lymphoid tissue and melanocytes were performed using a standard avidin-biotin peroxidase staining technique.

Cells were fixed with ice cold methanol for 5 minutes, air-dried for 10 minutes at room temperature and then washed in PBS. Endogenous peroxidase was blocked with 0.5% hydrogen peroxide in methanol. Following Avidin/Biotin block cells were incubated with the corresponding primary antibody diluted in 20% normal rabbit serum 2 hours at room temperature.

Anti-keratin antibody was used at a concentration of 1:50 and anti-vimentin antibody at a concentration of 1:40. Detection was carried out using a rabbit anti-mouse secondary antibody (DAKO) 1:400 in 20% normal rabbit serum, 30 minutes at room temperature, and a Steptavidin/Biotin peroxidase Complex kit (DAKO). A positive signal was detected with diaminobenzidine and haematoxylin was used as nuclear counterstain.

2.3. Cisplatin Treatment of Primary Murine Colonocytes

After 3 days in culture, primary murine colonocytes were exposed to cisplatin (David Bull Laboratories, Warwick, UK). 1ml of the 1mg/ml cisplatin (stock) was diluted in 2.334ml PBS to generate a 1000μM solution that was kept at -20°C and defrosted prior to use. This solution was then diluted further in culture medium to the desired concentration. Untreated cells were fed with culture medium containing similar amounts of PBS as the treated cells. Cells were incubated with the agent for 6, 12 and 24 hours. They were then washed with pre-warmed PBS and fixed as required by the recommended protocols. For immunocytochemistry cells were fixed in either ice cold methanol for 5 minutes or 4% paraformaldehyde 10 minutes at room temperature depending on the recommended method for each antibody. For quantifying apoptosis cells were fixed in modified Bouin's fixative (85% methanol, 5% acetic acid and 10% formalin) at 4°C overnight.

Cells used for RNA isolation were rinsed with pre-warmed PBS and then RNA isolated as described (Chapter 2, Materials and Methods, section 2.10.)

Medium containing cisplatin, the PBS used to rinse the cells and all the plasticware that came into contact with cisplatin were double sealed and disposed by incineration.

2.4. Quantification of Apoptosis

Cells were fixed in modified Bouin's fixative (85% methanol, 5% acetic acid and 10% formalin) at 4°C overnight, the DNA denatured in 5M HCl and the nuclei stained with Schiff reagent (Merck, Glasgow, UK). We used 0.3% light green as counterstain. Apoptosis was quantified using morphological criteria [Bellamy C.O. and Clarke A.R. *et al.*, 1997], with at least 500 cells counted in duplicate and in at least three separate experiments for each time point.

2.5. Proliferation Assay

Primary colonocytes cultures were incubated with 0,01mM 5'-bromo-2'-deoxyuridine (BrdU) (Amersham Pharmacia Biotech) in culture medium for 4 hours to incorporate the BrdU and then fixed in 80% ethanol overnight. DNA was denatured incubating cells with 5M hydrochloric acid (HCl) for 45 minutes. Cells were then washed three times with PBS to remove any acid left on slides. Blocking was performed using a protein free casein block (DAKO) for 30 minutes and then cells were incubated with the primary antibody: rat anti-BrdU IgG-direct peroxidase conjugated (Oxford Biotechnologies) at a concentration of 1:100, diluted in Background Reducing Antibody Diluent (DAKO). Detection was carried out using diaminobenzidine (DAB) according to manufacturer's instruction. Slides were counterstained with haematoxylin, mounted and coversliped. Negative controls omitted the primary antibody.

BrdU incorporation was estimated by counting the positive nuclei in a total of 500 (sufficient to achieve a stable running mean) in duplicate and from randomly selected fields. Experiments were performed at least 3 times with comparable results. The BrdU labelling index is expressed as a percentage.

2.6. Immunocytochemistry for p53, p21, PCNA and p73

2.6.1. Immunocytochemistry for Detection of p53, p21 and PCNA Proteins

The monoclonal antibody against p53 (clone pAB421) was purchased from Calbiochem. This antibody has been previously used in our laboratory to show expression of p53 in cells of mouse origin following DNA damage (irradiation, cisplatin). Primary colonocytes derived from p53-/- mice were used as negative control. Anti-PCNA (clone PC-10) [Al-Nafussi A. and Klys H.S. *et al.*, 1993] and anti-p21 (clone F-5) [Wong N.A. and Mayer N.J. *et al.*, 2003] were both purchased from Santa Cruz Biotechnologies (Santa Cruz). The protocols followed were similar. Immunocytochemistry was carried out on 4% paraformaldehyde fixed cells, permeated with 0.5% Triton in PBS for 20 minutes at room temperature. Endogenous peroxidase was quenched exposing the cells to 1% hydrogen peroxide/distilled water for 20 minutes at room temperature. Slides were then rinsed with distilled water prior to incubation for 20 minutes with protein serum free blocking solution (casein based, DAKO). This solution proved useful in reducing non-specific background. Cells were then incubated with primary antibodies: p53 at a concentration of 1:500, p21 at 1:20 and PCNA at 1:100, all diluted in background reducing antibody diluent (DAKO). Incubation was carried out at 4°C in a humid chamber, overnight. After washing away the primary antibody with PBS, biotinylated rabbit anti-mouse F(ab') (DAKO) 1:400 dilution was applied for 30 minutes. Avidin-biotin peroxidase complex was used as the final detection step. The slides were washed briefly with 0.5% Triton in PBS before incubating with DAB for 1-5 minutes. Staining was monitored under the microscope and if further intensification of staining was

required (usually for the immunodetection of p53), slides were incubated with DAB for an additional 5-10 minutes. Slides were counterstained with haematoxylin, mounted with cedar-wood oil and cover-slipped.

Staining was nuclear for all three antibodies and positive nuclei were counted randomly in at least 500 cells per well, in duplicate and in at least two individual experiments.

2.6.2. Immunocytochemistry for p73 Detection in Primary Colonocytes

Two antibodies against p73 were used to identify the protein in the cells. One of them, rabbit polyclonal anti-p73, clone H-79 (Santa Cruz Biotechnologies) corresponding to amino acids 1-80 mapping at the N-terminus of p73 of human origin and reactive with mouse, rat and human proteins and the other Ab-77 (Abcam) raised in sheep against the first 15 amino-acids of the N-terminus protein. Cells were grown in sonic seal slides as already reported and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Slides were extensively washed in PBS to eliminate the fixative and permeated with 0.5% Triton for 20 minutes afterwards. Following fixation and permeation cells were blocked with 3% PBS-BSA for 1 hour at room temperature followed by incubation with primary antibody diluted in 1% PBS-BSA at 4°C overnight. Rabbit anti-73, H-79 was used at a concentration of 1:100 and Ab-77, raised in sheep at a concentration of 1:300. Next day cells were washed with PBS and incubated with corresponding secondary antibody, Alexa 488-conjugated goat anti-rabbit IgG 1:200 (Molecular Probes) and Alexa 488-conjugated donkey anti-sheep IgG 1:200 (Molecular Probes) for 30 minutes at room temperature in a light proof immunofluorescence box.

The staining was examined and pictures captured using a fluorescent microscope Hamammatsu. Staining intensity was quantified measuring the intensity mean using Image Pro-plus software. Data was transferred to an excel sheet and values plotted into a histogram.

Auto-fluorescence control slides omitted both the primary and secondary antibodies and negative controls just the primary antibodies.

2.6.3. Immunohistochemistry for p73 on Formalin-fixed Paraffin-embedded Mice Skin and Colon Sections

Tail skin and colon from both wt and p53 deficient mice were fixed in formalin for 24 hours and then processed in paraffin blocks. 3μm sections were generated and a peroxidase staining technique used. Sections were de-waxed in xylene and re-hydrated through alcohol steps (100%, 95%, 70% ethanol) and finally water. Heat induced epitope retrieval was performed in a microwave at 1000 Watts for 15 minutes using citrate buffer (Vector). Endogenous peroxidase was quenched by placing slides in 3% hydrogen peroxide diluted in double distilled water. All the remaining steps were performed as already explained (Chapter 2, section 2.6.1.). The primary antibodies were used at similar concentration as for immunocytochemistry: H79 1:100 and Ab77 1:300.

2.7. Maintenance of Cell-lines and Culture Conditions

Cell lines for culture aliquoted and stored in CryoTube (Nunc) were thawed in a water bath at 37°C. The cell suspension was then dispersed into 10ml of the appropriate culture media and spun at 1000rpm for 5 minutes to remove any DMSO used in combination with serum as freezing medium. The medium was then aspirated and cells re-suspended in culture medium and plated out in plasticware, Petri dishes, multi-well plates, flasks or slides depending on the experiments (Greiner or Nunc).

All cell lines were grown at 37°C temperature and in 5% Carbon Dioxide atmosphere.

Cells were split when confluent in culture. Media was removed (except for COLO-320 cells which grow in suspension), cells were washed with pre-warmed PBS and incubated for few minutes with a small amount (enough to cover the cells) of 0.25% Trypsin-EDTA at 37°C. The cells were incubated with trypsin solution until detached completely. Fresh medium was than added to neutralise the trypsin and cells transferred to the appropriate number of culture dishes.

Cell Line and Reference Number	**Tissue**	**Culture Medium**
NIH-3T3 ECACC ref. no: 93061524	Mouse embryonic fibroblasts	DMEM + 10%FBS + antibiotics
COS-7 ECACC ref. no: 87021302	Derived from CV-1, an African Green monkey kidney cell-line SV-40 transformed	DMEM + 10%FBS + antibiotics
NIC-H441 ATCC ref. no: HTB-174	Human lung papillary adenocarcinoma of type II cell origin	RPMI 1640 with 2mM L-glutamine supplemented with non-essential amino-acids + 10% FBS + antibiotics
COLO 320 ECCC ref. no: 87061205	Isolated from human adenocarcinoma from the sigmoid colon	RPMI 1640 + 10% FBS + antibiotics

2.8. Reverse Transcriptase-Polymerase Chain Reaction

Total mRNA was extracted using TRIZOL (Invitrogen) and following the company recommendations. Genomic DNA contamination was removed by treating the total RNA with DNase I (DNAfree kit, Ambion) following the protocol supplied with the product. The total mRNA was then denatured at 95°C for 5 minutes to eliminate any present secondary structures. For cDNA synthesis we used the M-MLV reverse-transcriptase kit (Invitrogen) and generated cDNA from 100ng of total RNA.

Amplification of the region between:

- **p73α exon 11**- forward primer: 5 '-ACA CGG TGG TGT CAA CAA ACT –3'

  exon 14 - reverse primer: 5'-CGG TAC TGG TCA GGG ACC TTC AG-3'

yields a product of 363bp in the case of p73α [Weiss R.H. and Howard L.L., 2001] and/or a product of 270bp in the case of p73β. GENEBANK accession no: Y19234.

For the detection of full-length forms, primers annealing to:

- **p73α exon 3** – forward primer: 5'-GAG CAC CTG TGG AGT TCT CTA GAG-3'

  exon 4 – reverse primer: 5'-GGT ATT GGA AGG GAT GAC AGG CG-3'

were used generating a product of predicted size 297bp as previously reported [Pozniak C.D. and Radinovic S. *et al.*, 2000], GENEBANK accession no: Y19234.

The primers for ΔN-forms, annealing to

- **ΔNp73 exon 3'**- forward primer:5'-ACC ATG CTT TAC GTC GGT GAC CCC-3'

  exon 4- reverse primer: 5'-GAG ACA TGG TGT CGA AGG TGG AGC-3'

generated a product of 187bp [Sayan A.E. and Sayan B.S. *et al.*, 2001], GENEBANK accession no: Y19235.

- **Shh** - forward primer: 5'-GTG AGG CTG CGA GTG ACC G-3'

- reverse primer: 5' –CCT GGT CGT CAG CCG CCA GCA CGC-3'

generated a product of 333bp [Dyer M.A. and Farrington S.M. *et al.*, 2001].

- **PTCH** – forward primer: 5'-CTG CTG CTA TCC ATC AGC GT-3'

- reverse primer: 5'- AAG AAG GAT AAG AGG ACA GC-3'

generated a product of 452bp [Dyer M.A. and Farrington S.M. *et al.*, 2001].

- **PTCH 2** – forward primer: 5'- TGC CTC TCT GGA GGG CTT CC-3'

- reverse primer: 5'-CAG TTC CTC CTG CCA GTG CA-3'

generated a product of 207bp [Dyer M.A. and Farrington S.M. *et al.*, 2001].

- **Gli1** – forward primer: 5'-CAG GGA AGA GAG CAG ACT GA-3'

- reverse primer: 5'- AGC TGA TGC AGC TGA TCC AG-3'

generated a product of 251bp [Dyer M.A. and Farrington S.M. *et al.*, 2001].

- **Ihh** – forward primer: 5'-ACG TGC ATT GCT CTG TCA AGT-3'

- reverse primer: 5' – CTG GAA AGC TCT CAG CCG GTT-3'

generated a product of 221bp [Farrington S.M. and Belaoussoff M. *et al.*, 1997].

Transcripts levels were normalized to

- **GAPDH** - forward primer: 5'-CTT CAC CAC CAT GGA GAA GGC-3'

- reverse primer: 5'-GGC ATG GAC TGT GGT CAT GAG-3'

that generate a product of 237bp.

NIH-3T3 cells of mouse origin were used as species positive control for the RT-PCR experiments.

Thermocycling was as follows:

Primer pair	**Step 1** **Denaturation**	**Step 2** **Anncaling**	**Step 3** **Elongation**
p73α exons 11-14	3 minutes – 94°C	1 minute – 94°C 1 minute – 52°C 1 minute – 72°C	5 minutes – 72°C
p73α exons 3-4	3 minutes – 94°C	1 minute – 94°C 1 minute – 64°C 1 minute – 72°C	5 minutes – 72°C
ΔNp73 exons 3'-4	3 minutes – 94°C	1 minute – 94°C 1 minute – 61°C 1 minute – 72°C	5 minutes – 72°C
GAPDH	3 minutes – 94°C	1 minute – 94°C 1 minute – 55°C 1 minute – 72°C	5 minutes – 72°C
Shh, Ptch, Ptch2, Gli1, Ihh	3 minutes – 94°C	1 minute – 94°C 1 minute – 55°C 1 minute – 72°C	5 minutes – 72°C

PCR products mixed with loading dye were analysed on 2% agarose gel containing 0.5mg/ml ethidium bromide together. 1Kb Plus DNA ladder (Invitrogen) was run simultaneously and the gel was visualised under ultraviolet light. All RT-PCR results have been repeated twice from different batches of RNA.

Agarose Gel Loading Dye (10x):

1.5g Ficoll

0.02g Bromophenol blue

0.02g Xylene Cyanole FF

dH_2O to 10ml

2.9. Colonic Lesions - Samples and Sections

Normal and abnormal human tissue samples were selected from the archives of the Division of Pathology, the University of Edinburgh Medical School. 7 hyperplastic polyps, 20 tubulo-villous adenomas of the colon, 8 adenocarcinomas and 3 normal colon samples (one formalin-fixed paraffin-embedded and two frozen samples) were used. All sections were cut at 3-5 μm except for the frozen sections used for laser capture microdissection that were cut at 8 μm.

Histological Diagnosis

H&E sections of the colonic lesions investigated in Chapter 3 were reviewed and described by Dr. Roger Malcomson and Professor David J. Harrison in the Department of Pathology.

2.10. Immunohistochemistry on Colorectal Lesions

2.10.1. Immunohistochemistry for Detection of Shh, Ptch, Smoh, Ki-67 and Cyclin B1 proteins

For immunohistochemistry we used the following antibodies: goat polyclonal antibodies against Shh (N-19), Ptch (G-19) and Smoh (C-17) all purchased from Santa Cruz Biotechnologies and mouse monoclonal Cyclin B1 (clone V-152,) from DAKO. A mouse monoclonal Ki-67 (clone MIB-1) antibody from DAKO was used to assess the proliferation status of the tumours examined.

The tissue sections used for immunohistochemistry were de-waxed in xylene and re-hydrated through a series of graded alcohols followed by incubation for 20 min in 3% hydrogen peroxide to block endogenous peroxidase. A casein serum free block specific for formalin fixed tissues was then used for 20 minutes as recommended by supplier (DAKO).

Sections were incubated overnight at 4°C with primary antibody at different dilutions

- Shh 1:100
- Ptch 1:80
- Smoh 1:100
- Cyclin B1 1:250
- Ki-67 1:100

Slides were then washed in PBS 3 x 5 minutes and detection carried out using a biotinylated rabbit anti-goat IgG (1:400) and rabbit anti-mouse IgG (1 in 400) secondary antibodies for 30 minutes. Another 30 minutes incubation step was carried

out using StreptAvidin-Biotin HRP Complex (DAKO) and the staining was visualised with diaminobenzidine (DAKO). Sections were counterstained with Mayer's haematoxylin. Negative controls (absence of primary antibody) were run for each set of antibodies (monoclonal and polyclonal) and for each case analysed.

2.10.2. Double Fluorescent Staining for Shh and Ki-67 in a Hyperplastic Polyp

Double immunofluorescence detection for Shh and Ki-67 was performed in a hyperplastic polyp. Both primary and secondary antibodies were diluted in 1% sterile filtered PBS-BSA. Following de-waxing, antigen retrieval and blocking the section was incubated with the first primary antibody mouse anti-Ki-67, 1:100 for 2 hours at room temperature. The slide was washed in PBS and then incubated for 30 minutes at room temperature with the first secondary antibody Alexa 488-conjugated rabbit anti-mouse IgG (Molecular Probes) 1:200. Following extensive washes with PBS, the section was incubated with the second primary antibody goat anti-Shh 1:100 overnight at 4°C in a light proof immunobox. Secondary detection was performed using an Alexa 568-conjugated donkey anti-goat IgG (Molecular Probes) 1:200 for 2 hours at room temperature. DAPI was used as nuclear counterstain. The specimen was examined using a Zeiss laser-scanning confocal microscope.

2.11. Laser Capture Microdissection, RNA Preparation and RT-PCR

Two separate frozen normal colon samples were used to generate 8 μm cryostat sections for laser-capture microdissection. Tissue sections were fixed in 70% alcohol, stained with Mayer's haematoxylin and eosin, de-hydrated in graded alcohols followed by clearing and dehydration in xylene and air dried for 10-15 minutes. Normal colonic crypts (15-20 crypts) were microdissected onto LCM caps (CellPix II, Arcturus) and total mRNA isolated using a phenol-chloroform based method (RNA isolation kit, Ambion). The RNA isolated was DNase I treated (2U per sample of RNA, usually in 0.5ml thin wall tubes) using the DNA free kit from Ambion specially designed to DNase I treat small quantities of RNA from any genomic DNA contamination. The reverse transcriptase reaction was performed using MMLV reverse transcriptase (Life Technologies), Oligo dT_{12-18} (Life Technologies), incubated with RNase inhibitor (Life Technologies) at 37°C for 50 minutes. 5μl of cDNA generated in the RT reaction was used for each 25μl PCR final volume. The oligonucleotides used [Tojo M. and Kiyosawa H. *et al.*, 2002; Tojo M. and Mori T. *et al.*, 1999] were:

Shh - forward primer 706 – 5'-GAA AGC AGA GAA CTC GGT GG-3'

- reverse primer 875- 5'-GGA AAG TGA GGA AGT CGC TG-3'

Shh product size 170bp - GENBANK accession no. L38518

Ptch - forward primer 1092- 5'-TCC CAA GCA AAT GTA CGA GCA-3'

- reverse primer 1238- 5'TGA GTG GAG TTC TGT GCG ACA C-3'

Ptch product size 144bp – GENEBANK accession no. U59464

Smoh - forward primer 615 - 5'-CTG GTA CGA GGA CGT GGA GG-3

- reverse primer 756 - 5'- AGG GTG AAG AGC GTG CAG AG-3'

Smoh product size 140bp – GENEBANK accession no. U84401.

β-microglobulin - forward primer - ACC CCC ACT GAA AAA GAT GA

- reverse primer - ATC TTC AAA CCT CCA TGA TG

β-microglobulin product size 120bp

Thermocycling conditions were as follows:

Primer pair	**Step 1** **Denaturation**	**Step 2** **Annealing**	**Step 3** **Elongation**
Shh	3 minutes – 94°C	1 minute – 94°C 1 minute – 53°C 1 minute – 72°C	5 minutes – 72°C
Ptch	3 minutes – 94°C	1 minute – 94°C 1 minute – 61.6°C 1 minute – 72°C	5 minutes – 72°C
Smoh	3 minutes – 94°C	1 minute – 94°C 1 minute – 64°C 1 minute – 72°C	5 minutes – 72°C
β-microglobulin	3 minutes – 94°C	1 minute – 94°C 1 minute – 55°C 1 minute – 72°C	5 minutes – 72°C

The expression of the housekeeping gene β microglobulin was used as internal semi-quantitative and qualitative control and RNA-cDNA isolated from NIC-H441 cells as primers positive control. The RT-PCR products were run on 6% TBE gels stained with ethidium bromide and visualised under ultraviolet light.

PCR products were sequenced and using ^{33}P Thermosequenase cycle sequencing kits (Amersham) from primers used for PCR amplification (the sequencing experiment was performed in collaboration with Dr. Scott Bader).

2.12. Single-strand Conformation Polymorphism Assay for Shh and Smoh in a Panel of Colorectal Tumours

This experiment was performed in collaboration with Dr. Roberta M. James, with excellent technical assistance from Claire Lomas and Audrey Peter.

The entire coding region of the *Shh* and *Smoh* genes by single-strand conformation polymorphism (SSCP) assay in a panel of colon cancer tumours (60 RER–, microsatellite stable and 20 RER+, microsatellite unstable) was screened as previously described [Bader S. and Walker M. *et al.*, 2003]. Genomic DNA was extracted from tumour and matching normal colon tissues by standard techniques and amplified using primers at 0.4μM in 10μl reactions in the presence of [α-^{33}P]dATP (ICN Biomedicals) under standard conditions. PCR products were analysed on a non-denaturing 0.5× SequaGel MD gels (National Diagnostics) with 10% glycerol and 0.6% TBE for 18 hours at 8W at room temperature. The gels were dried and exposed to autoradiographic film. A total of 12 primer pairs for *Shh* (Table 1) and 21 primer pairs for *Smoh* were designed from genomic sequence (L38518 and AF114819.1 respectively) and were located just inside introns flanking splice acceptor and donor sites of smaller exons, or in overlapping segments in larger exons.

Table 1. Primer pairs used to amplify the 3 exons (including intron/exon boundaries) of the gene *Shh* for SSCP analysis.

exon	forward (5'-3')	reverse (5'-3')
1	CTGGAAGTGTTCGGCTTCTC	TAGGCGCCAGCGGAAGGT
	TCGCTCGGAGTTTCTGGAG	GGCGGAGATGTCTGCTGCTA
	TGCCTCCTCTTCCCGAACC	AAAGCGCAAGAGAGAGCGC
2	AATGGCCTTCCTTGGGTCG	GGAGTCTCTGCACTACGAG
	GACGTGGTGATGTCCACTG	GGCTAACGTGTCCGTCGG
3	GTCGCTGTAGAGCAGCCG	CTCTCGGAACTCAATGCCCT
	CGTCGCGGTCCAGGAAAGT	AACTCAATGCCCTGTCCTC
	TTGCGTTGCTGTTGCTGCC	CTCCAGGTGCTGCCGACG
	GCAGCTGCGAGTACCAGTG	TCATCGAGGAGCACAGCTGG
	AGTGCAGCCAGGAGCGCGT	GCGTGTACGTGGTGGCCGA
	GTCACGCTGTGCACAGCGG	GCGAGCGCCTGCTGCTCA
	AAGAGCAGGTGCGCGGCGG	TGCTGGCGGCGGACGACCA

2.13. Treatment of Primary Colonocytes with Shh Peptide, 5E1 Neutralising Shh Antibody or Isotype Control IgG1 Antibody

Primary murine colonocytes isolated using the method already described (Chapter 2, Materials and Methods, section 2.2.) were treated with exogenous soluble Shh peptide, anti-Shh antibody or isotype control antibody. The mouse recombinant Shh peptide, a 180-residue N-terminal peptide (R&D Systems) was reconstituted in sterile filtered 0.3% PBS-BSA to a final concentration of 25μg/ml. This stock solution was stored at -20°C and added to the culture medium prior to use. The neutralising anti-Shh antibody (clone 5E1) and the isotype control IgG1 antibody were a gift from Dr. Sarah Howie. 5E1 was raised in mouse against the N-terminal region of the Shh protein. They were also stored at -20°C and added to the culture medium before experiments were carried out. The antibodies were both tested by immunoblotting by Dr. Sarah Howie's group.

After 3 days in culture, primary colonocytes were exposed to various concentrations of Shh peptide or neutralising antibody as previously described [Lowrey J.A. and Stewart G.A. *et al.*, 2002]. The isotype control antibody was also used as mentioned [Lowrey J.A. and Stewart G.A. *et al.*, 2002]. The recombinant mouse Shh peptide was added to the cells at a concentration of 0.25μg/ml, 0.5μg/ml and 0.75μg/ml. 5E1 antibody was used at 20 and 50μg/ml and the isotype control antibody at 20μg/ml. Cells were exposed to the above concentration of exogenous Shh peptide, 5E1 or isotype control antibody for 48 or 72 hours in both serum free culture conditions or 2.5 serum culture medium. BrdU was added to the culture medium in concentration of 10mM for 4 hours and then fixed in 80% ethanol at 4°C overnight.

Immunodetection was carried out by immunocytochemistry with a rat anti-BrdU antibody as already detailed in Chapter 2, Materials and Methods, section 2.7. Positive BrdU cells stained with DAB were counted in a total of 1000 cells and in three different experiments for all conditions presented.

2.14. Statistics

Data were analysed using Minitab statistical software version 13. Data are presented in graphs as the mean ± SEM. Comparison of BrdU results was made using analyse of variance (ANOVA) test. Comparison of all other results was made using Mann-Whitney U test. An associated probability (p) value of < 0.05 was considered significant.

CHAPTER 3

p53-DEPENDENT AND INDEPENDENT DEATH PATHWAYS IN CISPLATIN-DAMAGED PRIMARY MURINE COLONOCYTES

3.1. Background

Colonic epithelium is a continually regenerating tissue in which a tightly regulated balance between proliferation and cell death maintains tissue homeostasis. Any perturbation of a proliferating cell compartment can result in tumourigenesis and may cause the abnormalities of epithelium seen in inflammatory bowel disease. Colorectal cancer develops from the pathologic transformation of normal colonic epithelium to an adenomatous polyp and eventually invasive cancer. Escape from the induction of apoptosis is believed to be a critical event in colorectal carcinogenesis. Much of the information on carcinogenesis in the intestine derives from studies utilising cells from the small intestine, yet the majority of the mutations leading to cancer in humans occur in the large intestine. Detailed pioneering work by Potten and others [Booth C. and Potten C.S., 2000; Potten C.S. and Booth C. *et al.*, 1997; Williams E.D. and Lowes A.P. *et al.*, 1992] has elucidated the kinetics of intestinal growth *in-vivo* but primary culture offers a dynamic *in-vitro* model allowing the investigation of growth, apoptosis and response to injury [Booth C. and O'Shea J.A. *et al.*, 1999; Booth C. and Patel S. *et al.*, 1995].

p53 is frequently mutated or deleted in colorectal cancer, particularly in late stage, acting as a progression step [Carder P.J. and Cripps K.J. *et al.*, 1995]. Approximately half of all colorectal cancers show *p53* gene mutations, with higher frequencies occurring in distal colon and rectal tumours and lower frequencies in proximal tumours and those with microsatellite instability or methylator phenotypes [Iacopetta B., 2003].

The role of p53 in tumour suppression is linked to its functions in the regulation of cell-cycle control [Kastan M.B. and Zhan Q. *et al.*, 1992], differentiation [Aloni-Grinstein R. and Schwartz D. *et al.*, 1995], inhibition of angiogenesis [Dameron K.M. and Volpert O.V. *et al.*, 1994], senescence [Atadja P. and Wong H. *et al.*, 1995] and apoptosis following genotoxic stress, nucleotide depletion and oncogene activation [Ryan K.M. and Phillips A.C. *et al.*, 2001]. Following DNA damage, p53 induces expression of $p21^{(WAF1/CIP1)}$, whose induction above basal levels results in binding to cyclin-dependent kinase complexes and their inhibition, thereby blocking cell proliferation [Xiong Y. and Hannon G.J. *et al.*, 1993]. p21 can also bind to proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase δ [Flores-Rozas H. and Kelman Z. *et al.*, 1994; Nakanishi M. and Robetorye R.S. *et al.*, 1995; Waga S. and Hannon G.J. *et al.*, 1994]), blocking PCNA function in DNA replication but not repair. Consequently, p21 can cause G1 arrest and inhibition of DNA replication whilst ensuring potential errors are repaired prior to resumption of cell cycling. p21 appears to be an essential prerequisite for G1 arrest following DNA damage [El Deiry W.S. and Tokino T. *et al.*, 1993], although there may be other unknown mediators of this checkpoint. Thus several studies have reported that homozygous deletion of p21 partially or completely abrogates G1 arrest mediated by p53 [Brugarolas J. and Chandrasekaran C. *et al.*, 1995; Deng C. and Zhang P. *et al.*, 1995; Waldman T. and Kinzler K.W. *et al.*, 1995].

Although the significance of p53 in tumour suppression remains undisputed, it has become apparent that p53-independent mechanisms operate in tandem to ensure fidelity of replication and elimination of rogue cells from the gene pool. It is now recognised that p53 is a member of a gene family encompassing its homologues p73

and p63. p73 shares many similarities with p53, but there are differences in the upstream regulatory signals leading to p73 activation. In p53-/- cells, oncogene activation induces endogenous p73 expression engaging on apoptosis pathway that is p53-independent [Zaika A. and Irwin M. *et al.*, 2001]. Despite the p73 locus (1p36) being on a chromosome region frequently deleted in human cancers, numerous studies on human tumours have failed to detect *p73* gene deletion or the incidence of inactivating mutations [Mai M. and Yakomizo a. *et al.*, 1998; Sunahara M. and Ichimiya S. *et al.*, 1998]. However, the role of p73 in tumourigenesis remains unclear, as silencing of the p73 gene has been detected in neuroblastoma, squamous cell carcinoma and lung cancer [Benard J. and Douc-Rasy S. *et al.*, 2003] suggesting that in the corresponding tissues p73 may assume a tumour suppressor role. Conversely, overexpression of p73 has been reported in a series of malignancies as discussed in the general introduction. Finally, p73 null mice do not display an increased predisposition to spontaneous tumour development [Yang A. and Sharpe A. *et al.*, 2000] although their response to chemical carcinogenesis has yet to be reported.

p73 exists as at least six (α, β, γ, δ, ε, η) full-length transactivation competent (TA) forms which differ at the C-terminus and as N-terminally truncated variants (ΔN) that are believed to be transactivation deficient [Chen X., 1999]. Overexpression of the α and β forms was shown to cause irreversible growth arrest and apoptosis in cells lacking functional p53 [Fang L. and Lee S.W. *et al.*, 1999]. Recently ΔNp73 was identified as the predominant form in the developing brain and sympathetic neurons [Pozniak C.D. and Radinovic S. *et al.*, 2000]. This ΔN-variant assumes a protective role against apoptosis induced by NGF withdrawal, by directly

antagonising the apoptotic functions of p53 [Pozniak C.D. and Radinovic S. *et al.*, 2000] and is capable of suppressing p53 and TAp73-dependent transactivation [Ishimoto O. and Kawahara C. *et al.*, 2002].

In the present study, the colonic crypt isolation technique developed by Booth et al. (1995) [Booth C. and Patel S. *et al.*, 1995] was adapted, to obtain primary colonocytes from adult wt and p53-/- mice. The objectives were to better understand the importance of p53 and related proteins in the regulation of growth and response to DNA damage in these cells. In order to investigate both p53 dependent and independent DNA damage response pathways, primary colonocytes were exposed to cis-diamminedichloroplatinum(II) (cisplatin), a widely used chemotherapeutic agent that cross-links DNA, forming intra- and inter-strand adducts [Jordan P. and Carmo-Fonseca M., 2000].

Why cisplatin?

Cisplatin lesions are detected by components of the mismatch repair machinery leading to futile repair cycles [Mello J.A. and Acharya S. *et al.*, 1996; Vaisman A. and Varchenko M. *et al.*, 1998] that culminate in the induction of apoptosis proceeding via at least two different pathways: one involving p53 and the other mediated by p73 [Jordan P. and Carmo-Fonseca M., 2000]. These pathways have not yet been examined in primary colonocyte cultures.

3.2. Results

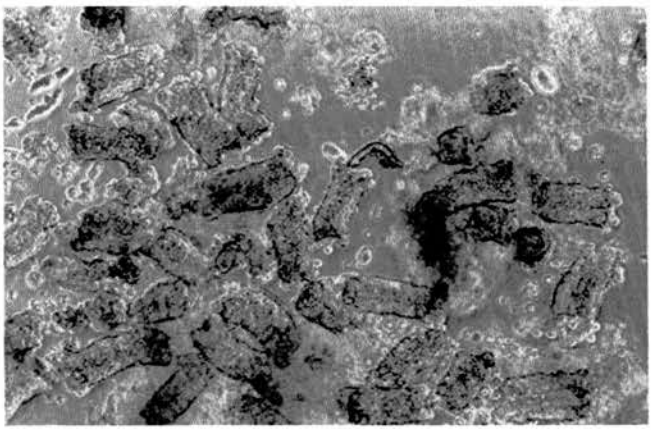
3.2.1. In vitro Culture of Primary Colonocytes

Colonic crypts isolated from adult mouse colons using a combined dispase and collagenase enzymatic method attached to a collagen matrix to spread and grow into colonocytes (Figure 8).

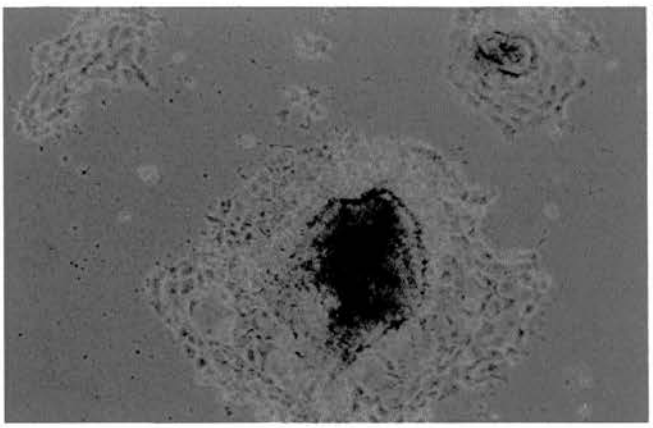
Primary colonocytes survive in culture for a limited period of time (approximately 20-25 days) and have a slow proliferation rate. BrdU positive cells are detected only in the first few days (up to day 10) in culture. BrdU incorporation in cells after 2, 4, 6, 7, 8 and 9 days in culture shows that primary colonocytes are actively proliferating in the first 5 or 6 days in culture (Figure 9). After 7 days in culture just a very small number of cells (roughly 1 or 2 in 10.000 cells) incorporate BrdU, and cells ultimately die after 20 or 25 days in culture.

Therefore, experiments were performed between 3 and 5 days in culture when viability is maximal and count of apoptosis, cell-cycle activity and immunocytochemistry are optimal.

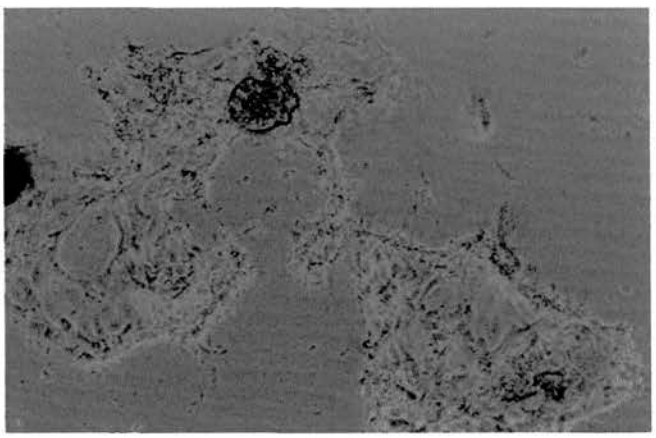
The effect of different culture parameters such as seeding density (densities between 400 and 1000 crypts per well), cell culture plasticware (multi-well slides or plates purchased from different companies), extra cellular matrix (fibronectin, laminin, collagen I, collagen IV or their combination) and growth factor supplements (insulin, EG) were investigated prior to final experiments. These factors had enormous effects on crypt attachment, spreading and morphology of primary colonocytes in culture. The Nunc sonic-seal chamber slides coated with collagen IV proved to be the best



A - freshly isolated
single crypts



B - crypt settled
day 1



C - epithelial cells growing
out from the crypt
day 2

Figure 8: A) Picture of freshly isolated crypts after enzymatic digestion.
B) Crypt settled after 1 day in culture. Epithelial cells proliferate by growing out from the crypt. The initial crypt and its original architecture are hardly visible.
C) Cells start to spread out and tend to become a confluent monolayer.

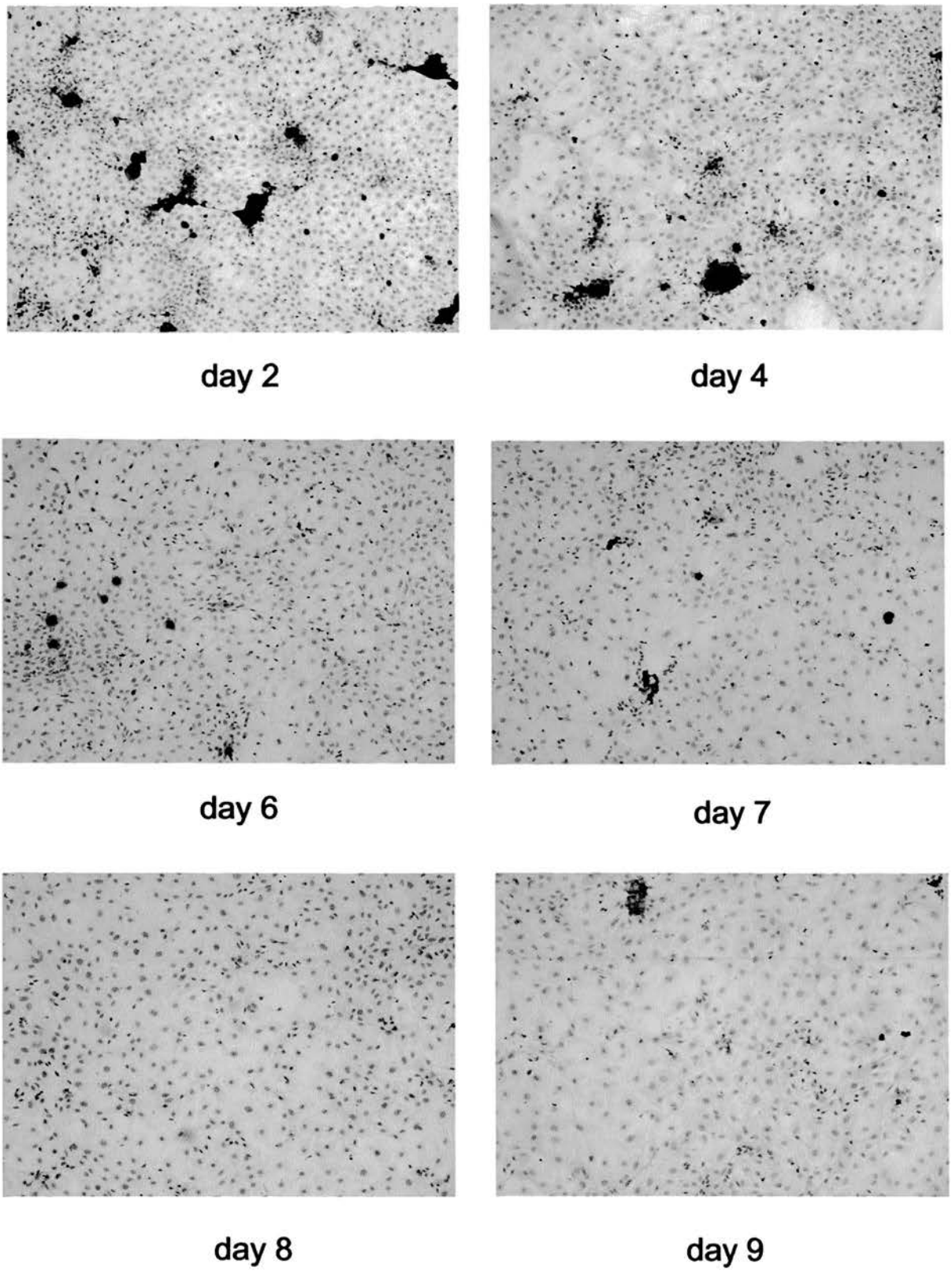
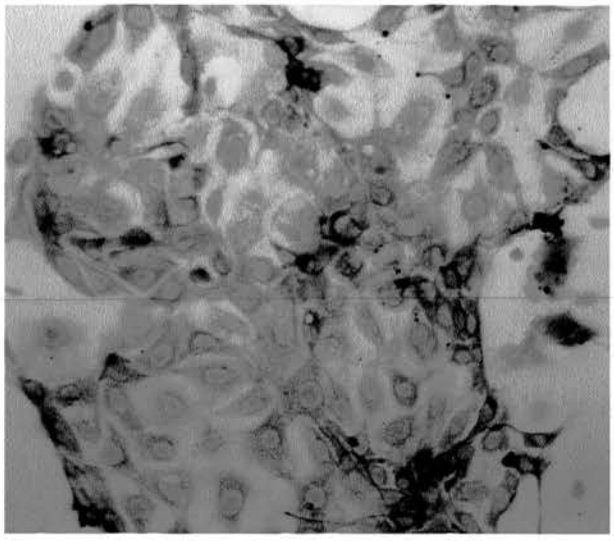


Figure 9: BrdU incorporation assay demonstrating proliferation in wt primary colonocytes over a period of 9 days in culture. Cells were incubated with BrdU for 4 hours and fixed in 80% ethanol. Positive cells were detected with DAB and haematoxylin was used as nuclear counterstain.

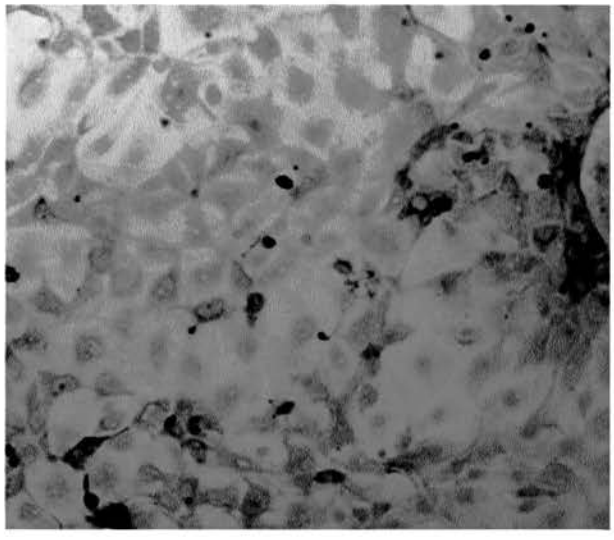
combination that favoured the attachment and survival of the adult mouse colonic crypts. Growth factors like insulin and EGF also proved essential for survival and growth of epithelial cells in culture. We also used low levels of serum in culture medium (2.5%) to inhibit the growth of fibroblasts that usually contaminate primary cultures.

3.2.2. Confirmation of Epithelial Origin of Colonocyte Preparation by Keratin Immunocytochemistry and Evaluation of Culture Purity

The use of a pan-keratin antibody proved that the vast majority of the cells, derived from adult mouse colons were indeed of epithelial origin (Figure 10). Transmission electron micrographs studies carried out by Booth *et al.* (1995) revealed the presence of desmosomes in cells isolated by a similar method, confirming their epithelial nature. Vimentin staining, characteristic of fibroblasts, showed that fibroblast contamination was minimal. Fibroblasts tend to appear in low numbers in culture after 3 or 5 days, when colonocytes are well settled and spread. They occupy the empty spaces in the well. The use of low serum concentration in the culture medium (2.5%) limited their growth.



wt cells
day 3 in culture

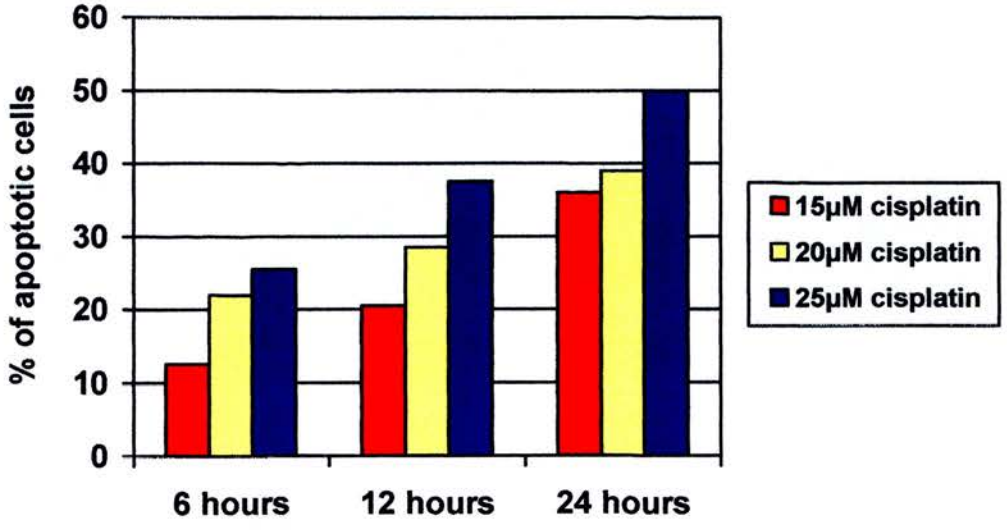


p53-/- cells
day 3 in culture

Figure 10: Keratin expression in wt and p53 deficient primary murine colonocytes, using an anti-pan-keratin antibody. Cells were fixed in cold methanol and detection carried out using a peroxidase techinque. Positive staining is confirmed by brown cytoplasmic staining detected with DAB and haematoxylin was used as nuclear counterstain.

3.2.3. Cisplatin Treatment of Primary Murine Colonocytes

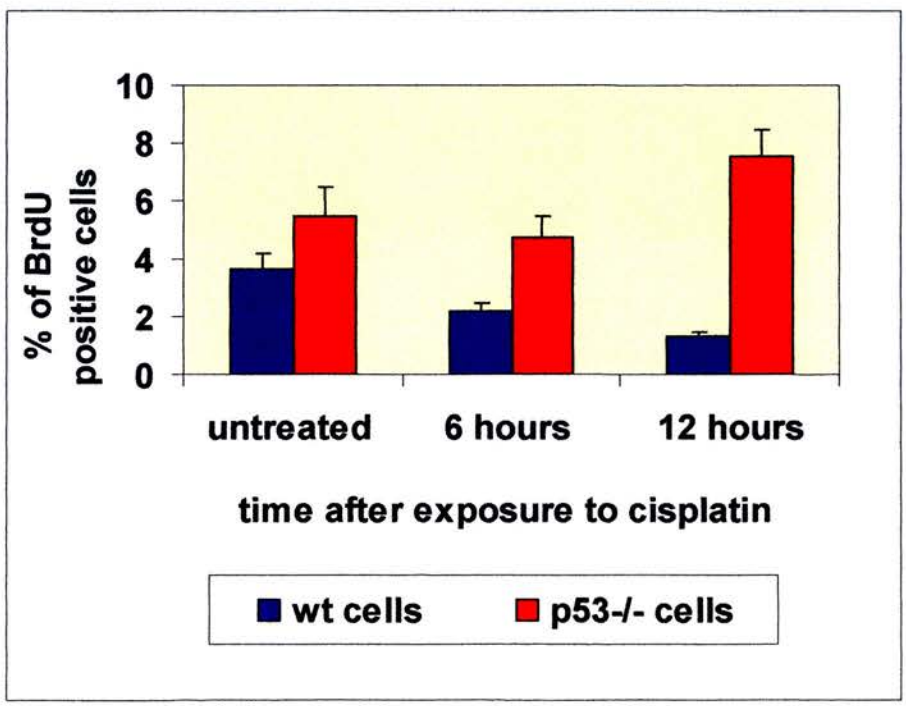
Colonocytes isolated from wt mice were exposed to different concentrations of cisplatin: 15μM, 20μM and 25μM to determine the percentage of cell death. Apoptotic cells were counted on Feulgen stained light green counterstained slides using a morphological criteria [Bellamy C.O. and Clarke A.R. *et al.*, 1997]. Results are presented in the graph below.



As some of the cells die and detach by 24 hours, the percentage of death after 25μM cisplatin is higher in reality. In addition, the cells exposed to high concentrations of cisplatin suffer a greater damage and are therefore less likely to reflect and express damage response proteins. Consequently, 15μM cisplatin was the preferred concentration to be used in the experiments.

3.2.4. p53 Controls Growth in Primary Murine Colonocytes

The percentage of cells in S phase was determined by BrdU incorporation into wt and p53-/- colonocytes under baseline conditions and at 6 and 12 hours after exposure to cisplatin (Figure 11). p53-/- colonocytes exhibit a significantly higher BrdU labelling index, indicating a growth advantage over wt cells under baseline conditions (see graph below). Following cisplatin treatment BrdU incorporation in wt cells was reduced by 12 hours ($p=0.015$, ANOVA) whereas p53-/- colonocytes continued to engage in DNA synthesis suggesting an essential role for p53 in cell-cycle arrest following cisplatin damage in murine primary colonocytes.



BrdU incorporation in untreated and cisplatin-treated wt and p53-/- colonocytes. Results are the average with SEM, and show a higher BrdU incorporation in p53-/- colonocytes regardless of treatment and time-point ($p=0.015$, ANOVA).

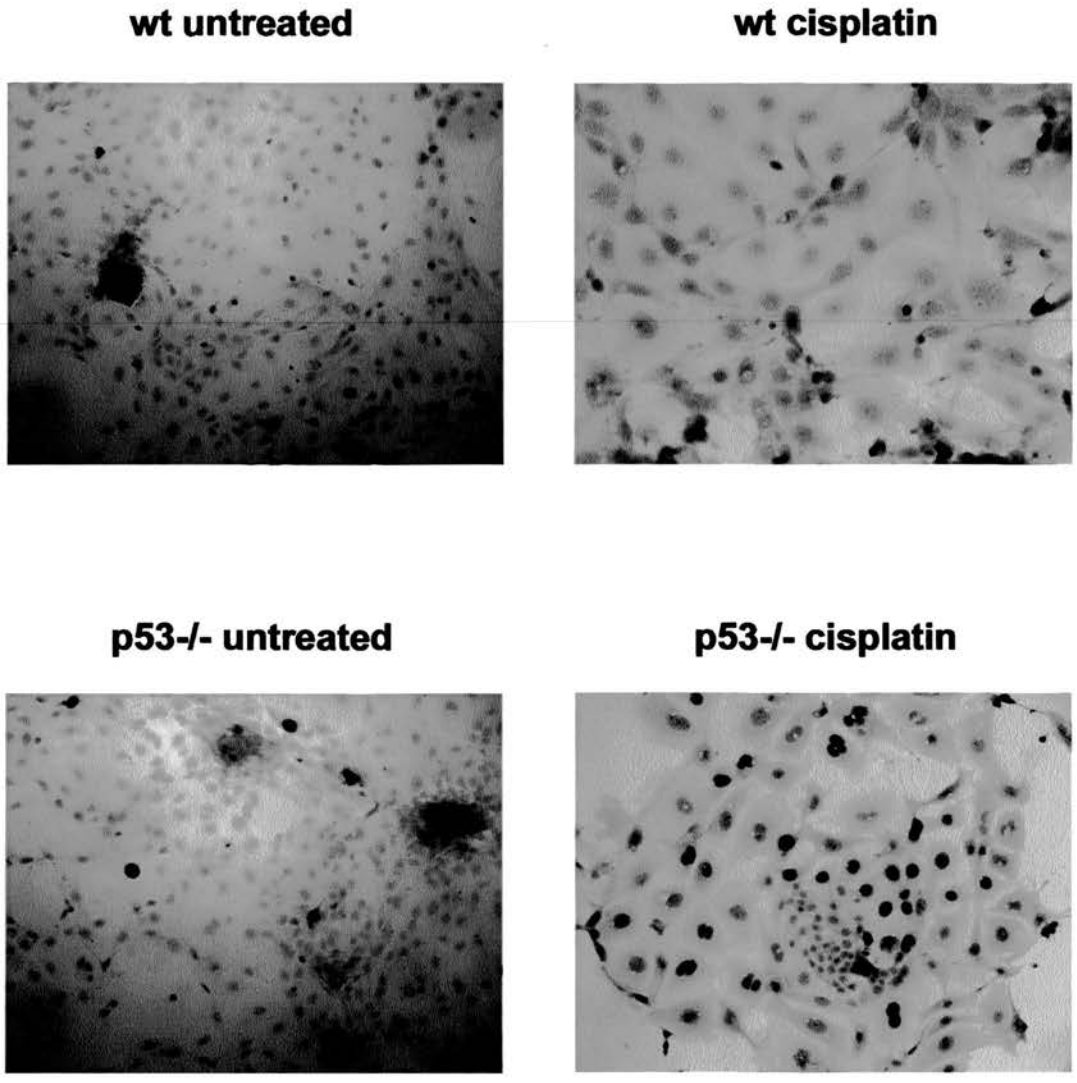
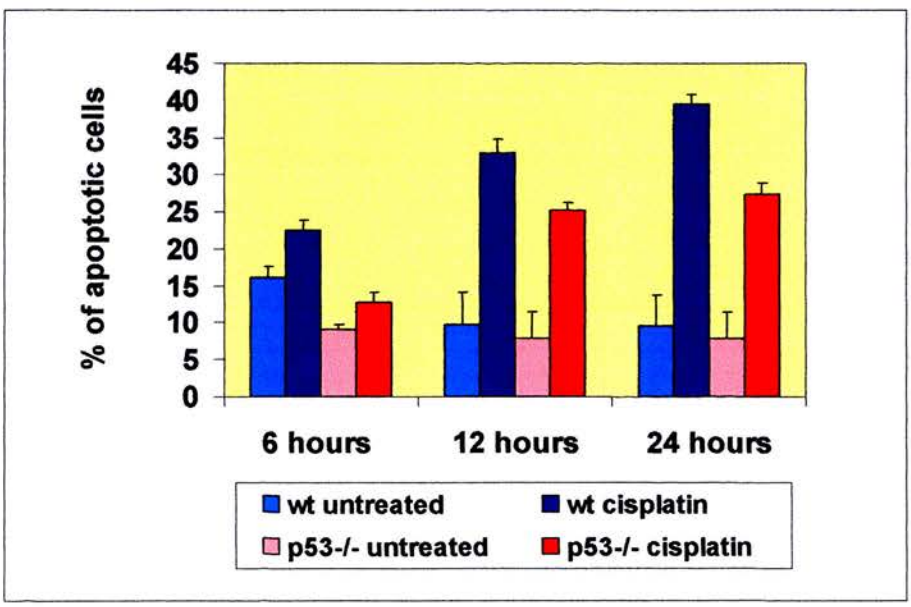


Figure 11: BrdU incorporation as a marker of cells in S-phase (cells in culture were exposed to BrdU just for 4 hours). Following cisplatin treatment the BrdU inrporation decreases in wt cells, whereas p53-/- cells continue to incorporate BrdU into their DNA.

3.2.5. Cisplatin Kills Colonocytes by Apoptosis Irrespective of p53 Status

Apoptosis in colonocyte cultures was monitored at 6, 12 and 24 hours post-cisplatin exposure (Figure 12). At all time points there was a significant increase in the incidence of apoptosis ($p<0.05$, Mann-Whitney U test) between treated cells and their corresponding controls, irrespective of genotype. There were however reduced levels of apoptosis in p53-/- colonocytes compared to wt counterparts (see graph below): whereas 39% of wt cells were apoptotic 24 hours post exposure to cisplatin, only 27% of p53-deficient cells had died by this time. The overall levels of apoptosis at each time point in p53-deficient colonocytes were reduced compared to p53-proficient cells.



Apoptosis in wt and p53-/- colonocytes exposed to cisplatin. Apoptotic nuclei in a total of 500 cells were counted in triplicate and in at least 3 experiments. The figure shows the average percentage of apoptotic cells ± SEM.

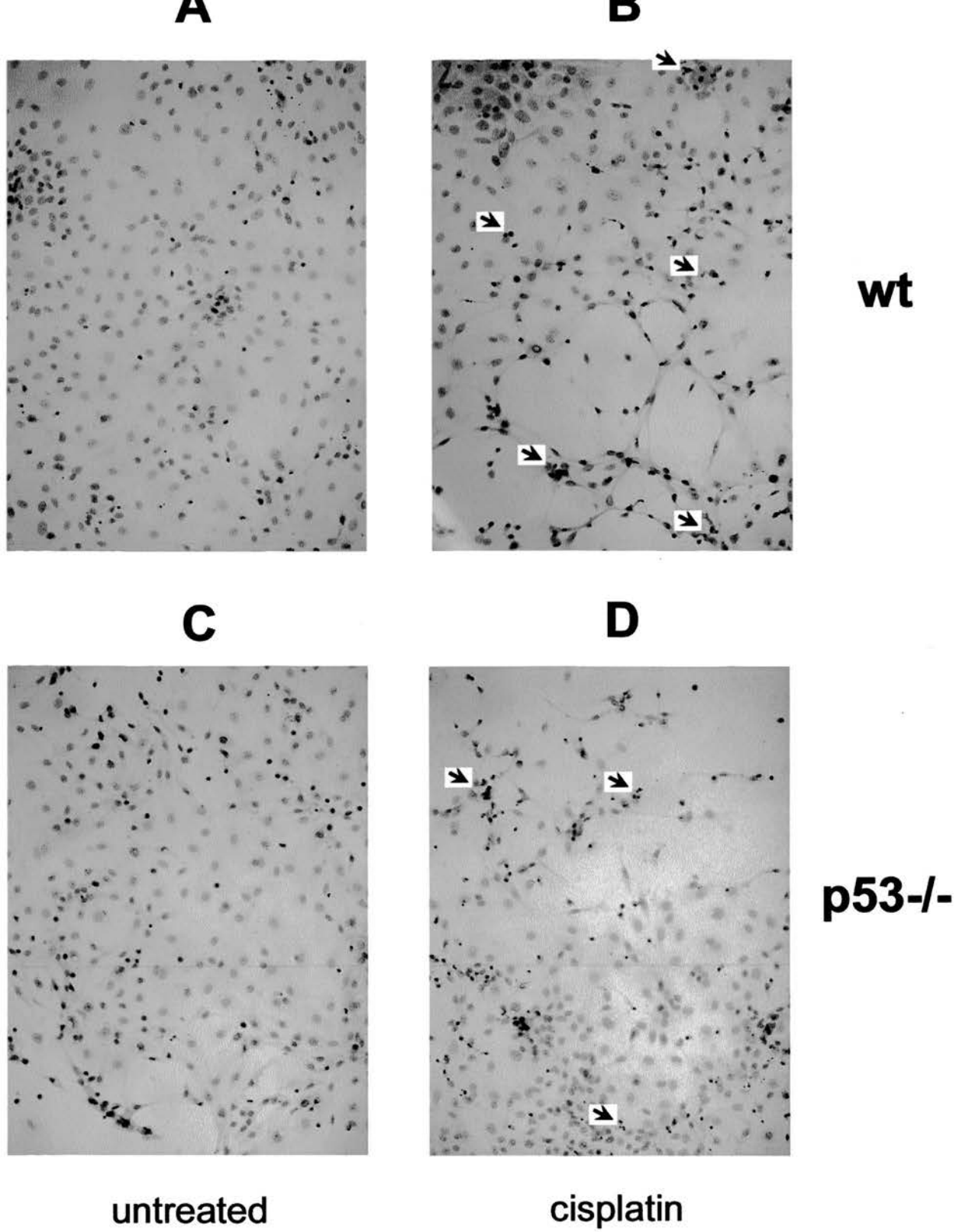
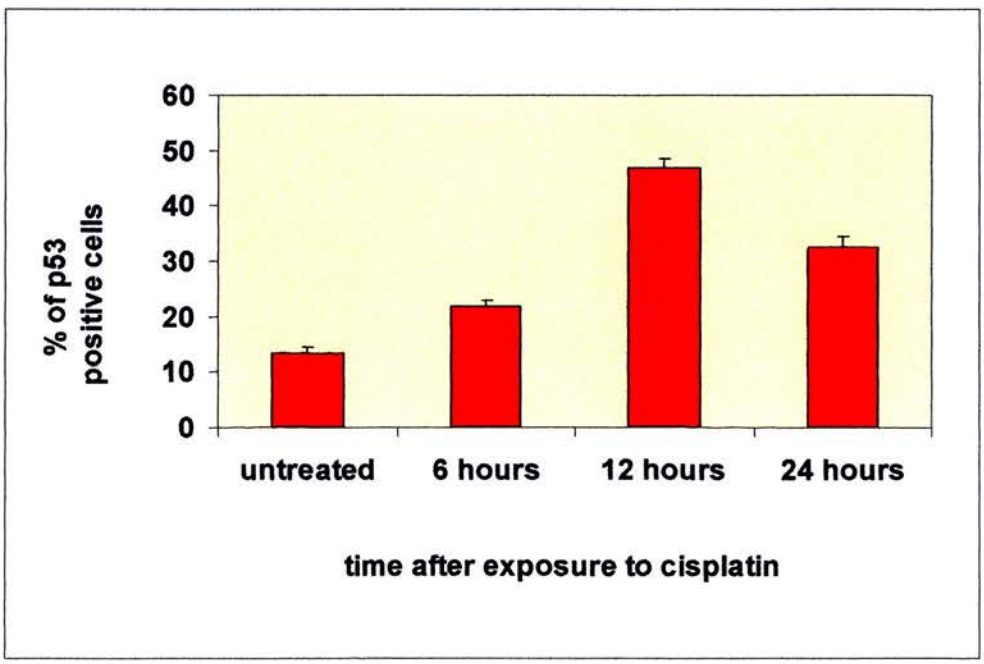


Figure 12: Apoptosis in primary murine colonocytes as detected using Feulgen staining. Light green was used as cytoplasmic counterstain. Increased apoptosis (indicated by the black arrows) is noticed in cisplatin-treated wt (B) and p53-/- (D) cells compared to their untreated couterparts: wt (A) and p53-/- cells (C).

3.2.6. p53 Is Upregulated Following Cisplatin Treatment of Wild-type Colonocytes

The proportion of cells expressing nuclear p53 was determined by immunocytochemistry on 4% paraformaldehyde-fixed untreated and cisplatin-treated colonocytes (Figure 13A and 13B). Although nuclear p53 was detected under baseline conditions, its expression was significantly upregulated after exposure to cisplatin at all time points ($p<0.05$, Mann-Whitney U test) consistent with its stabilization and transcriptional activity (see graph below).



p53 expression in untreated and cisplatin-damaged colonocytes. p53 is expressed under baseline conditions but its expression is significantly upregulated after exposure to cisplatin at all time-points ($p<0.05$, Mann-Whitney U test). The figure shows the average percentage of p53-positive cells in a total of 500 ± SEM.

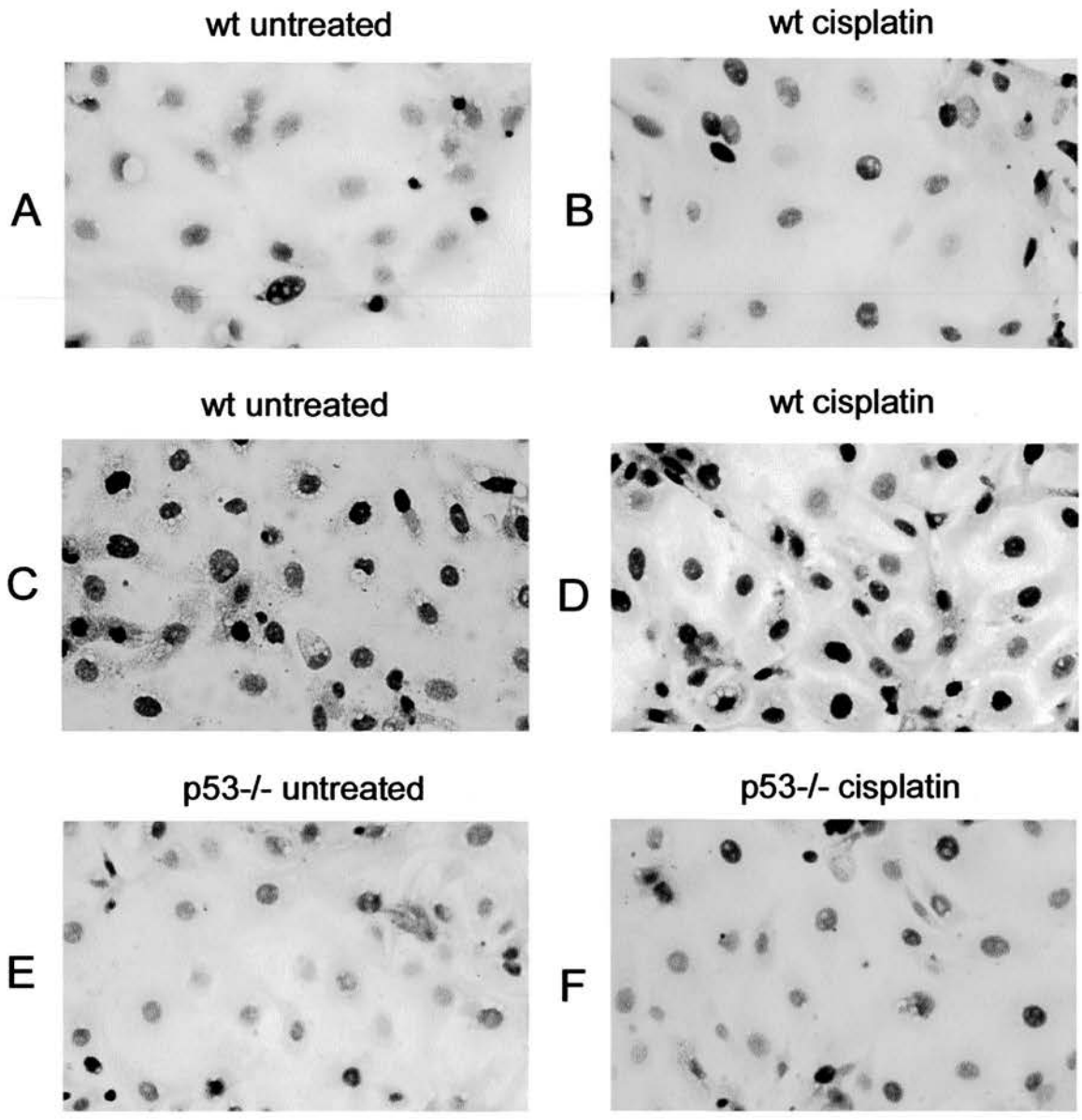
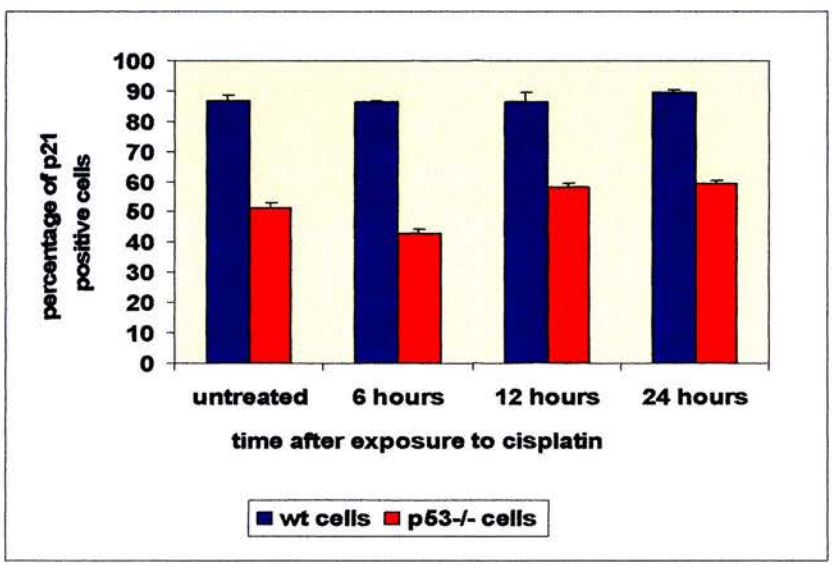


Figure 13: Expression of nuclear p53 and p21 in untreated and cisplatin treated colonocytes detected by immunocytochemistry using an avidin-biotin peroxidase technique. Positive cells were detected with DAB and haematoxylin was used as nuclear counterstain. p53 expression is upregulated following exposure to cisplatin (A and B) whereas p21 levels do not change significantly in cisplatin treated wt cells (C and D) and p53-/- cells (E and F).

3.2.7. p21 and PCNA Are Expressed in Untreated and Cisplatin-Treated Cells

We also investigated levels of the downstream target gene of p53, p21. Remarkably, high levels of nuclear p21 were present in both, wt and p53-/- colonocytes under baseline conditions (Figure 13C, 13E), with no further increase after cisplatin treatment (Figure 13D, 13F), (see graph below). However, the proportion of cells expressing p21 was significantly higher in wt cultures compared to p53-/- cells at all time points ($p<0.034$, Mann-Whitney U test). PCNA, a binding partner of p21 was also present in a high proportion of cells regardless of their p53 status or exposure to cisplatin.



p21 expression in untreated and cisplatin-treated wt and p53-/- colonocytes. The levels of p21 expression were significantly higher in wt when compared to p53-/- cells at all time-points and did not changed following cisplatin treatment. The figure shows the average percentage of p21-positive colonocytes ±SEM.

3.2.8. p73 is Expressed in Wild-type and p53-/- Colonocytes Under Baseline Conditions and It Is Upregulated Following Treatment with Cisplatin but Transcript Levels Remain Unchanged

Some baseline levels of nuclear p73 were detectable by immunofluorescence. Following treatment with cisplatin for 24 hours, there was an increase in the amount of p73 protein in the nucleus as evidenced by a 2-3-fold increase in the intensity signal as measured using Image-Pro Plus software (Figure 14).

The increase is consistent with nuclear translocation of p73. Results were comparable using two different antibodies against p73 (Figure 15A and 15B). Rabbit polyclonal H-79 is raised against the N-terminal 80 amino acids of p73 and could thus not be used to discriminate between full-length forms and ΔN variants of p73. Ab77 was raised against a sequence at the extreme N-terminal 15 amino acids and therefore exclusively identifies full-length p73 and should not cross-react with ΔN-p73. To test whether the constitutive levels of nuclear p73 are a consequence of *in-vitro* culture or whether they reflect expression levels in the colonic epithelium *in situ*, formalin-fixed paraffin-embedded sections were subjected to immunohistochemistry with the abovementioned p73 antibodies. As staining was detected in the basal/parabasal cells of murine skin epithelium [Faridoni-Laurens L. and Bosq J. *et al.*, 2001] the absence of staining in the colonic epithelium is indicative of the fact that p73 is not normally expressed, at least at levels detectable by immunohistochemistry, in the murine large intestine *in situ.*

RT-PCR analysis was employed to investigate the incidence of major p73 forms. We confirmed the presence of full-length p73α (Figure 16A) using primers

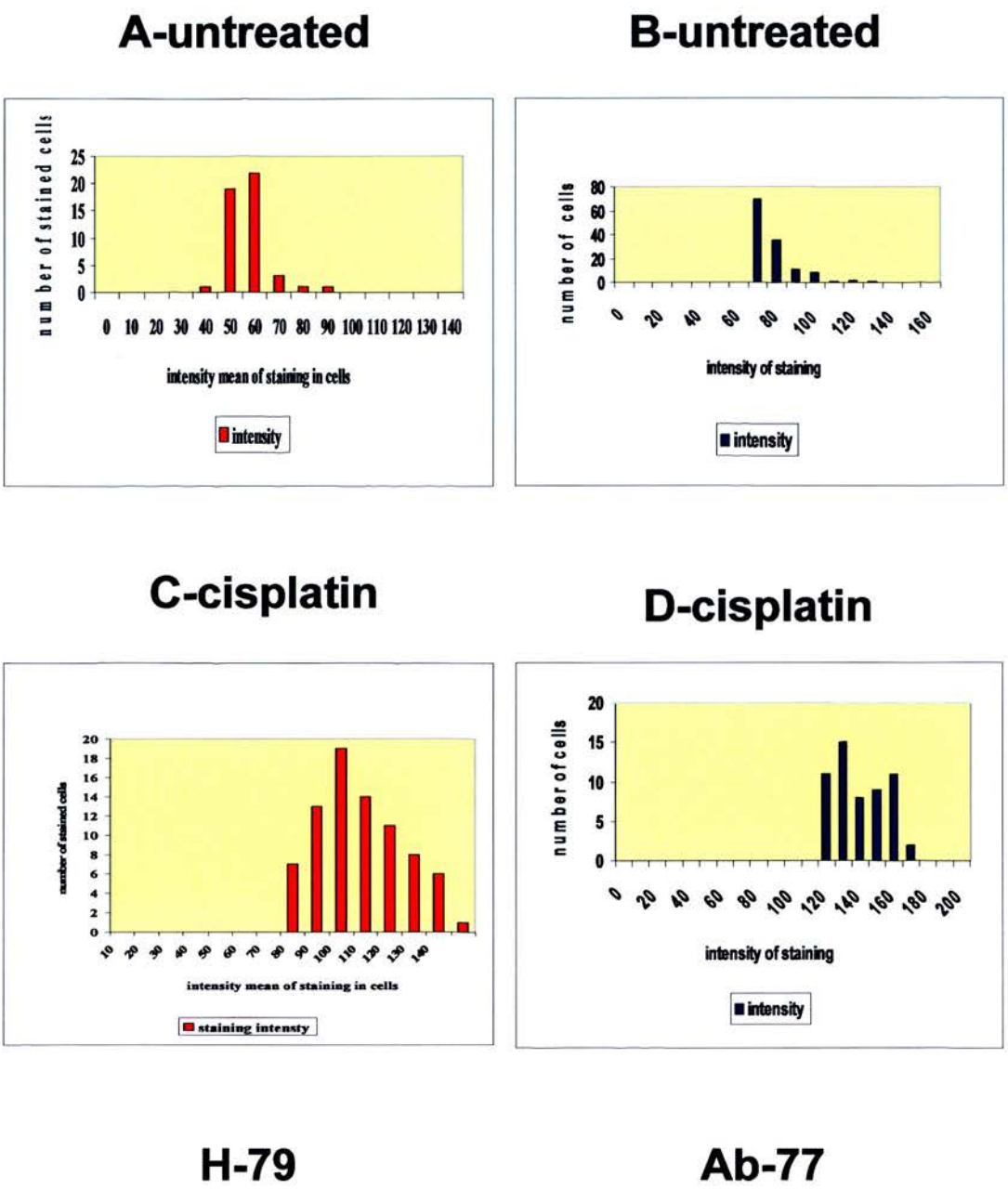


Figure 14: p73 is upregulated in primary colonocytes following treatment with cisplatin as evidenced by a 2-3-fold increase in the amount of p73 protein in the nucleus. The intensity signal was measured using Image-Pro Plus software, in 3 different fields in at least 80 cells. Results were comparable using two different antibodies against p73: rabbit polyclonal H-79 and sheep polyclonal Ab-77

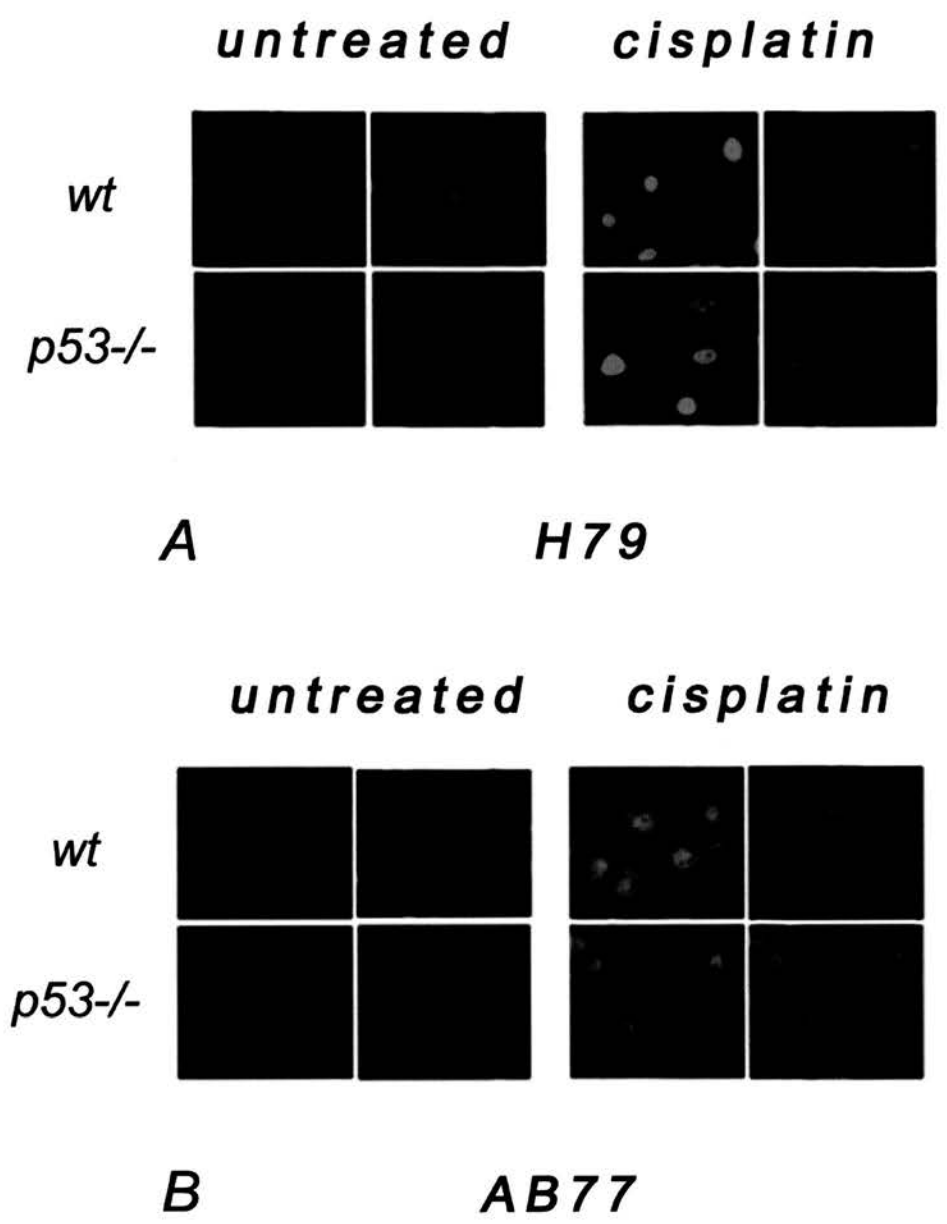


Figure 15: Immunofluorescence detection of nuclear p73 in primary colonocytes using Alexa-488 conjugated secondary antibody. Similar pattern of nuclear accumulation after treatment with cisplatin is manifested in both wt and p53-null cells using two different antibodies: rabbit polyclonal H-79 (A) and sheep polyclonal Ab-77 (B). DAPI was used as nuclear counterstain. The cells shown were exposed to cisplatin for 24 hours. Images were captured using a Hamamatsu chilled CCD camera and Zeiss fluorescent microscope.

for C-terminus, exons 11-14 and N-terminus primers exons 3-4 (Figure 16B). p73α was detected in both wt and p53-/- colonocytes under baseline conditions and levels remained unchanged following exposure to cisplatin, consistent with reports in the literature that p73 stabilization following cisplatin damage does not involve transcriptional upregulation. No products corresponding to p73β, ΔN-p73α (Figure 16C) or ΔN-p73β were detected. The *p73* gene encodes two different proteins that are expressed under the control of two independent promoters, and that have opposite activities: the transcriptionally active full-length p73 identified already in the cells and the amino-terminally truncated ΔNp73. As the truncated protein is induced by TAp73 and p53, we explored its presence in our cells. No evidence for the truncated form was found in our cells. GAPDH RNA primers were used as loading control (Figure 16D).

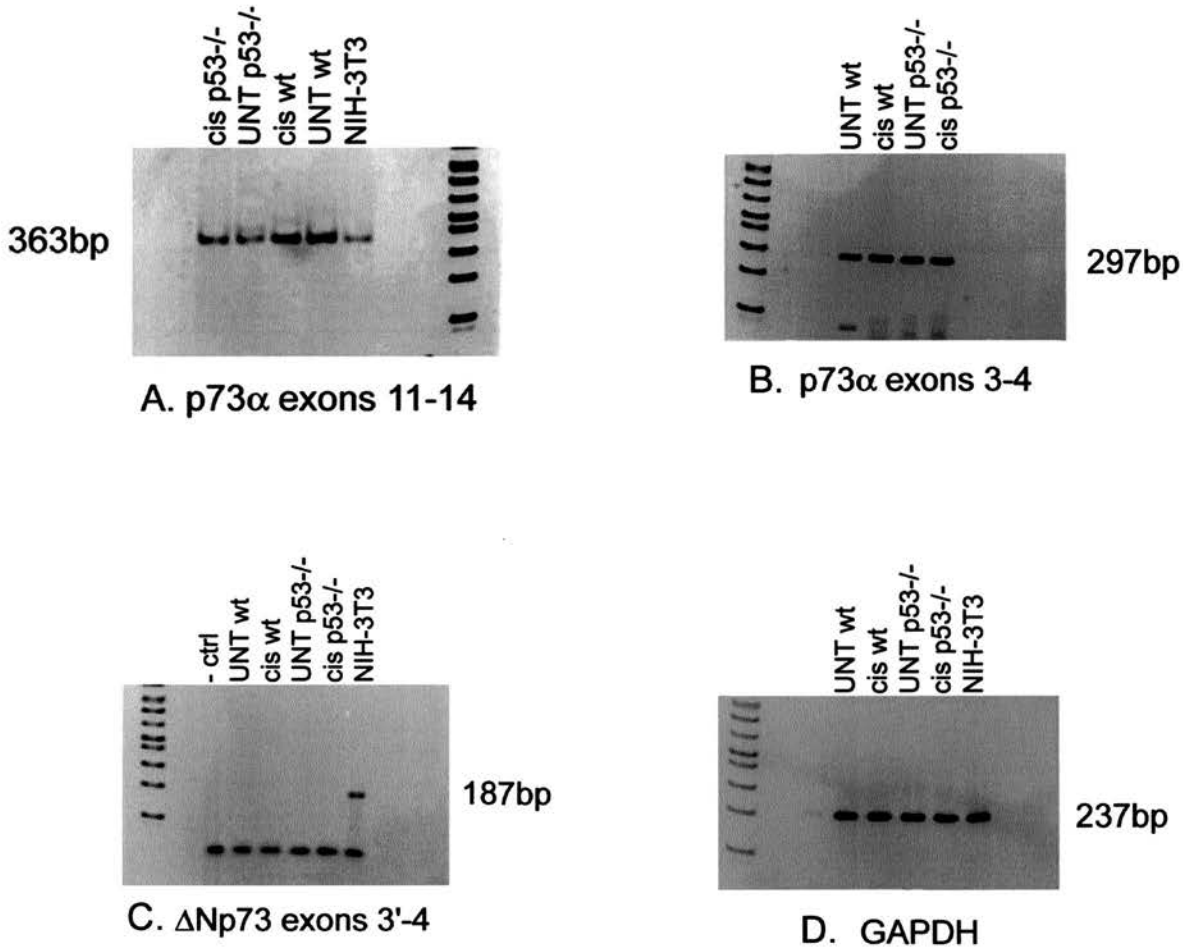


Figure 16: RT-PCR showing p73 expression in wt and p53-null untreated and cisplatin treated colonocytes. A - amplification with primers spanning exons 11-14 in murine primary colonocytes. The primers could also detect the presence of C-terminus p73β but no band of a predicted size was obtained. B - RT-PCR analysis for N-terminus detection of full-length p73 using primers for exon 3-4. C - RT-PCR showing the absence of ΔN-variants in primary colonocytes. NIH-3T3 cDNA proved that the primers recognise a product of a correct size. D - the housekeeping gene GAPDH was used as internal qualitative and semi-quantitative control.

3.3. Discussion

In the present study the role of p53 in the regulation of growth and response to cisplatin injury in primary murine colonocytes was explored. Even though colorectal cancer is resistant to cisplatin treatment, we chose this chemotherapeutic drug to allow the investigation of both p53- dependent and independent pathways following DNA damage.

Under baseline conditions, the proportion of p53-/- colonocytes in S-phase was significantly greater than wt counterparts, suggesting a role for p53 in regulating transition through the cell cycle *in-vitro*. The BrdU incorporation into wt colonocytes decreases following treatment with cisplatin, indicating that wt cells undergo G1 arrest whereas p53-deficient cells continue unperturbed to engage in DNA synthesis, despite sustaining damage to their DNA. Thus, as a first line of defence, wt colonocytes respond to cisplatin damage via a p53-dependent growth arrest.

Both p53-/- and wt cells ultimately died by apoptosis, regardless of the failure of p53-/- colonocytes to enter growth arrest following cisplatin treatment. The time-course of cell death was rapid with approximately 40% of wt cells becoming apoptotic by 24 hours. However, apoptosis levels in p53-deficient cells were reduced. This may be analogous to the delayed second wave of p53-independent apoptosis seen *in-vivo* following irradiation in the small intestine, and to a much lesser extent in the colon [Clarke A.R. and Howard L.A. *et al.*, 1997], suggesting the existence of p53-independent mechanisms of eliminating damaged intestinal cells. Consistent with the proposed role for p53 in mediating the cell cycle arrest and apoptotic responses to cisplatin, the expression of p53 was significantly upregulated

in treated wt cells. However, the levels of its downstream target p21 remained unchanged from the already high baseline. We might predict that these high p21 levels may function to counteract apoptosis. In fact, in the absence of p53, when p21 expression is reduced by 40%, the levels of apoptosis are only reduced. Recently it was shown that Myc recruitment to the p21 promoter prevents bound p53 from activating p21 transcription and thus, by inhibiting p21 expression, Myc influences the decision between life and death favouring induction of apoptosis in colon cancer cells and highlighting the cytoprotective role of p21 [Seoane J. and Le H.V. *et al.*, 2002]. Moreover, HCT116 human colon cancer cells exhibit a G1 arrest following DNA damage by γ-irradiation or chemotherapeutic drugs, whereas isogenic cells, with a targeted inactivation of the $p21^{WAF1/cip1}$ gene [Mahyar-Roemer M. and Roemer K., 2001; Waldman T. and Kinzler K.W. *et al.*, 1995] or cells treated with antisense [Tian H. and Wittmack E.K. *et al.*, 2000], are sensitised to undergo apoptosis. In conditions where apoptosis predominates, p21 is cleaved by caspase 3 and re-localizes to the cytosol enabling apoptosis to proceed [Dotto G.P., 2000]. We did not see a reduction of endogenous p21 or its redistribution to the cytosol preceding the induction of apoptosis as might be predicted from these studies. Given that apoptosis was the ultimate fate of damaged colonocytes, there was no apparent cytoprotective effect of p21, although only the response of p21-null cells would resolve this issue.

Levels of nuclear p21 were high regardless of treatment, time point or genotype. This could be attributed to the stress of *in vitro* culture. The molecular response to perceived hypoxia includes amongst others induction of p21, reiterating the importance of O_2 levels during the culture of primary cells isolated from organs [Roy S. and Khanna S. *et al.*, 2003]. However, elevated amounts of p21 are not

inconsistent with reports that its levels are regulated in cultured cells during the different phases of the cell cycle or indeed with its proposed role in differentiation [Dotto G.P., 2000].

As already discussed p21 binds to PCNA. The levels of PCNA in primary colonocytes were persistently high regardless of cell genotype or cisplatin treatment. One explanation for the high proportion of PCNA positive cells *in vitro*, in stark contrast with *in vivo*, is that it reflects damage or stress to the cells in culture and corresponds to the reparatory function of PCNA. It is also possible that it simply represents a population of cells in G1 consistent with the fact that high levels of Ki-67 nuclear staining were also detected. PCNA is often used as a marker for proliferation but our findings, taken together with the relatively low levels of BrdU incorporation, raise questions as to the suitability of using solely PCNA as a reliable proliferation marker in primary cultures at least.

p53 is not normally implicated in the baseline expression of p21; yet in primary colonocytes the proportion of cells expressing p21 under the normal conditions of *in vitro* culture is halved in the context of p53 deficiency. If we are to accept that growth *in vitro* is indeed stressful for the colonocytes, then it is no surprise that p21 levels are high to counteract this effect. This may be also a limitation of most of the primary culture models, as colleagues in our lab have also detected high levels of p21 and PCNA in hepatocytes and Clara cell cultures (pneumocytes).

p53-/- cells are to a certain extent de-sensitised with regard to these stresses. What is more surprising is the finding that despite high levels of p21, colonocytes are not protected from entering apoptosis as previous studies have suggested. Nevertheless, the significance of the high levels of nuclear p21 in primary cultured colonocytes

remains unclear and cannot be accounted for exclusively by p53-dependent transactivation. Studies of primary colonocytes lacking p73, both p53 and p73 or indeed p21 would yield further insight into this question.

As already discussed, although some p53 is detected under baseline conditions this cannot explain the high percentage of cells expressing p21 nor of course can it account for expression in p53-/- cells. There are numerous pathways culminating in the expression of p21 involving TGFβ, BRCA1, IRF1, p73 and others. Recent reports have placed p73 and p63 as essential downstream mediators of p53-dependent apoptosis [Flores E.R. and Tsai K.Y. *et al.*, 2002]. Therefore, it seems plausible that p73 is implicated in at least certain responses to DNA damage and may indeed act in concert with p53 or, if p53 function is absent, elicit apoptosis by alternative routes [Chen X. and Zheng Y. *et al.*, 2001]. p73 levels are increased and result in apoptosis in human colorectal carcinoma cells treated with cisplatin in a manner independent of their p53 status [Gong J.G. and Costanzo A. *et al.*, 1999]. We therefore hypothesised that p73 may be partly responsible for p21 baseline expression and also p53-independent apoptosis following cisplatin treatment in primary colonocytes.

Given the tissue specificity of p73 expression, we employed RT-PCR analysis to investigate the incidence of major p73 forms in primary colonocytes. RT-PCR confirmed the presence of full-length p73α in both wt and p53-/- colonocytes under baseline conditions and following exposure to cisplatin. No evidence was found for either p73β or the anti-apoptotic form ΔN-p73α/β. By semi-quantitative RT-PCR, the levels of transcript remained unchanged following cisplatin exposure, consistent with reports in the literature that p73 stabilisation following cisplatin damage does

not involve transcriptional upregulation but occurs through post-translational mechanisms involving c-Abl and other modulators of function [Gong J.G. and Costanzo A. *et al.*, 1999].

In support of the RT-PCR data, we detected baseline levels of nuclear p73 by immunofluorescence using two different antibodies raised against p73. Following treatment with cisplatin for 24hrs, there was an increase in the amount of p73 protein in the nucleus as evidenced by a 2-3-fold increase in the intensity signal, consistent with increased nuclear translocation of p73. Therefore it seems possible that p73α cooperates with p53 in primary wt colonocytes as suggested by Flores *et al.* (2002) [Flores E.R. and Tsai K.Y. *et al.*, 2002]. Moreover, in the context of p53 deficiency, p73α activation is highly suggestive of its involvement in the p53-independent pathway of apoptosis manifested in these cells. Presumably p73 α is a weaker effector of apoptosis in the absence of p53, or may require interaction with accessory factors in order that the apoptotic machinery is engaged perhaps accounting for the temporal delay compared to wt cells.

Nuclear accumulation of p73 is not sufficient to induce apoptosis and post-translational modification such as acetylation has been shown to potentiate the ability of p73 to selectively transactivate pro-apoptotic gene targets [Costanzo A. and Merlo P. *et al.*, 2002]. In the present system, further modulation of p73 at activity level, through post-translational modifications and/or association with modulators of function could not be excluded. Given the limited yields of colonocyte extract and the low levels of endogenous p73, we were unable to pursue further analysis of endogenous p73 by immunoblotting or immunoprecipitation. Others [Shimodaira H. and Yoshioka-Yamashita A. *et al.*, 2003] have also reported difficulties in detecting

endogenous p73 in colorectal cell-lines. However, the power of the approach described here is that it enables the study of a specific gene alteration and its effects on the nature of the response elicited compared to normal cells. Several studies have described the transfection of p73 expression plasmids into cancer cell lines for the investigation of p73 modification and interaction with regulatory factors. However, such studies are often confounded by the diversity among cell lines or because they rely on high levels of exogenous protein whilst ignoring the endogenous. Thus, whereas these approaches are undoubtedly fruitful and very likely to yield useful information, we should be reminded of the power of deciphering function in a physiological setting. In the case of p73 this is of particular importance given the tissue specificity of the p73 variants, which have demonstrably different functional consequences. Moreover, the balance of the endogenous full-length and ΔN forms may yet determine physiological outcomes and responses in terms of apoptosis, differentiation and homeostasis.

The emerging evidence of p73 interaction with components of the mismatch repair signalling pathway [Catani M.V. and Costanzo A. *et al.*, 2002], the high incidence of mismatch repair deficiencies in colorectal cancer, the role of p73 as a corroborator of p53 function and also as an independent apoptosis inducer points to a potentially vital role for p73 in coupling damage repair and death in colonocytes.

In the present study it has been shown that the role of p53 in preventing entry into S-phase following colonocyte DNA damage appears indispensable whereas its role in apoptosis seems redundant. Although p53 is indispensable for colonocyte growth arrest, the process itself is unessential. This does raise questions regarding the relative importance of growth arrest as a damage response pathway in a tissue such

as the colon where the ultimate fate of every cell is to die. This strongly suggests that p53-mediated apoptosis and growth arrest are neither a major nor a unique protector of colonic epithelial cells against mutation following DNA damage. Furthermore the nuclear translocation of endogenous p73 α in response to DNA damage in primary murine colonocytes was demonstrated. This is highly suggestive of a functional pro-apoptotic role for p73α in these cells, within the framework of p53-independent apoptosis

One of the major unanswered questions is what governs a cell's choice to undergo growth arrest or apoptosis in response to p53 activation or indeed in the absence of functional p53 [Pietenpol J.A. and Stewart Z.A., 2002] [Vousden K.H. and Lu X., 2002]. The results suggest that cisplatin-injured colonocytes enter p53-dependent growth arrest and apoptosis. In the absence of p53, the failure to enter growth arrest is probably without long-term consequences as alternative death pathways are instigated to eliminate cells harbouring mutations.

CHAPTER 4

ALTERED EXPRESSION OF HEDGEHOG PATHWAY GENES IN HUMAN COLONIC TUMOURS

4.1. Background

The work in the previous chapter suggests that p53-mediated apoptosis and growth arrest are neither a major nor a unique protector of colonic epithelial cells against mutation following DNA damage. Moreover, mutations of p53 are common in colorectal neoplasia but occur late in disease evolution, acting as a progression step.

Cancer represents a deregulation of normal growth in a tissue in which cancer cells escape from the mechanisms that control normal patterning and growth. This is the reason why in the last few years scientists have focused more on investigating a possible role for developmental pathways in carcinogenesis. Hh signalling pathway is such a pathway important in development which controls growth, cellular proliferation during embryogenesis and patterning of various organs including brain, spinal cord, craniofacial structures, lung, teeth, eye, hair and limbs [Wicking C. and McGlinn E., 2001]. It is also plays a role in haematopoiesis [White E. and Prives C., 1999] and members of the pathway have been recently linked to tumour formation (i.e. Ptch and basal carcinoma) [Toftgard R., 2000].

Mutation of the *Shh* gene in humans has been confirmed as the cause of holoprosencephaly (OMIM ref. 236100), while over-expression of Shh in transgenic mouse or transgenic human skin develops BCCs-like tumours within the first few days of skin development [Takada N. and Ozaki T. *et al.*, 1999]. Genetic mutations causing truncation or an unstable PTCH protein are associated with familial (Gorlin's syndrome OMIM ref. 109400) or sporadic basal cell carcinoma [Johnson R.L. and Rothman A.L. *et al.*, 1996]. Medulloblastoma, basal cell carcinoma and meningioma are all tumours linked to Gorlin's syndrome. In addition, *Smoh* mutations have been identified in BCCs and neuroectodermal tumours [Xie J. and Murone M. *et al.*,

1998]. Greig syndrome (OMIM ref. 175700) and Pallister-Hall syndrome (OMIM ref. 146510) result from mutations in GLI3.

Hh signalling proteins interact to maintain equilibrium in the pathway. Repression of Smoh by Ptch in the absence of Hh culminates in cleavage of Gli to a transcriptional suppressor. When Shh binds Ptch, Smoh suppression is relieved preventing cleavage of Gli. This leads to Gli entering the nucleus and activating transcription of the Hh target genes [Johnson R.L. and Scott M.P., 1998; Kalderon D., 2000; McMahon A.P., 2000]. Ptch also limits the action of Shh. High levels of Ptch induced by Shh serve to sequester any free Shh therefore limiting its further diffusion [Chen Y. and Struhl G., 1996].

These findings suggest that mutations that activate the proto-oncogene Smoh or that inactivate the tumour suppressor Ptch may lead to disrupted Hh-Ptch-Smoh balance, the effect being excessive Hedgehog signalling activity that may be important in the pathogenesis of malignant disease.

Shh is important during gastrointestinal tract development. Recent studies have shown that *Shh* is important in gastric gland development and gastric epithelial differentiation in the adult gastrointestinal tract [van den Brink G.R. and Hardwick J.C. *et al.*, 2002]. Therefore, it is important to determine whether the Hh family genes play a similar role in colonic epithelium.

As the Hh pathway is clearly essential for development of the gut, the aim was to examine the expression of the Hh family members at the protein and mRNA levels in normal human colon. Protein expression was also examined in a series of human colonic lesions that included hyperplastic polyps, adenomas and adenocarcinomas. Hyperplastic polyps are considered to be groups of elongated and/or thickened

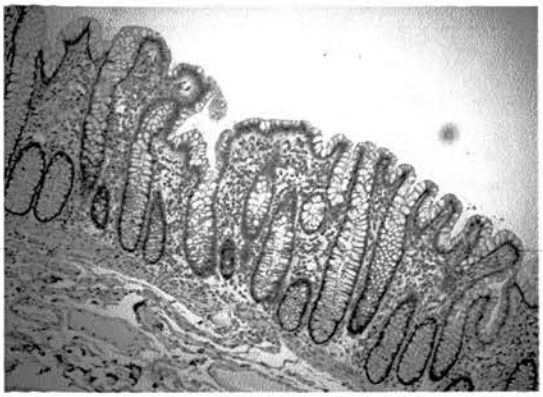
colonic crypts. Some researchers have regarded them as precursors of colorectal carcinoma whereas others as non-neoplastic and unrelated to cancer [Achkar E. and Winawer S.J., 1990]. Consequently, it was of interest to investigate the pattern of expression of Hh genes in these lesions of the colon as well as in more advanced neoplastic tumours such as adenomas and adenocarcinomas.

4.2. Results

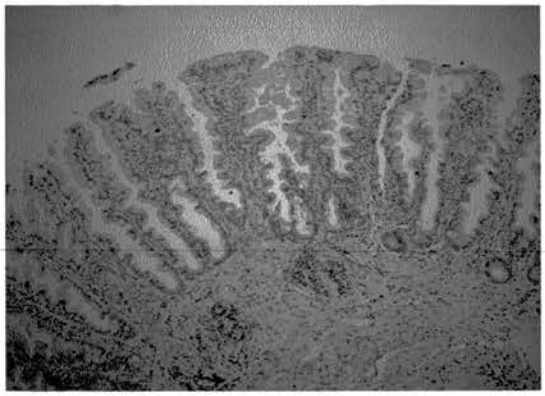
4.2.1. Shh Pathway Members Are Expressed in Normal Colon

Shh, Ptch and Smoh stained in the cytoplasm, while the proliferation marker Ki-67 stained the nuclei. The pattern of expression of the proteins was consistent within each group of cases. No staining was observed when immunodetection was performed in absence of the primary antibody (Figure 17).

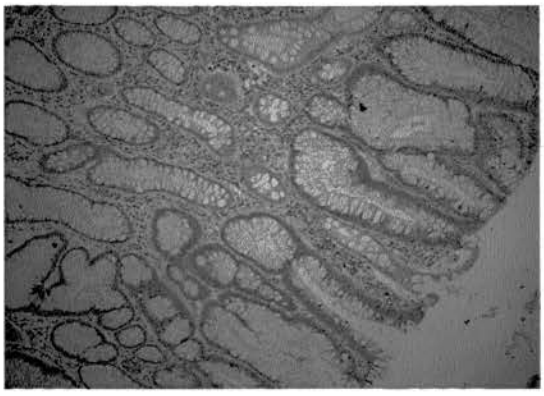
Light positive staining for Shh protein was detected at the top of the normal colonic crypts within the cytoplasm. Few of the epithelial cells (Figure 18) located at the base of the crypts also stained positive for Shh. Ganglion cells of the myenteric plexus found between the circular and longitudinal layers of the muscularis propria expressed Shh, a pattern similar to that previously reported in the mouse [Ramalho-Santos M. and Melton D.A. *et al.*, 2000]. Neuroendocrine cells located basally in the crypts strongly expressed Ptch, consistent with recently published data [Watkins D.N. and Berman D.M. *et al.*, 2003], whereas the rest of the epithelial cells were unstained. Smoh expression was found only at the brush border of superficial colonic epithelium. Ki-67 confirmed that proliferation in normal colon epithelium was located in the basal compartment of the crypts. Cyclin B1 expression was strong and present in the cytoplasm of the majority of the cells, throughout the crypts. Total mRNA isolation, RT-PCR, and sequencing of PCR products from laser microdissected crypts confirmed that mRNAs encoding Hh signalling pathway members are expressed in the epithelium of colonic crypts (Figure 19).



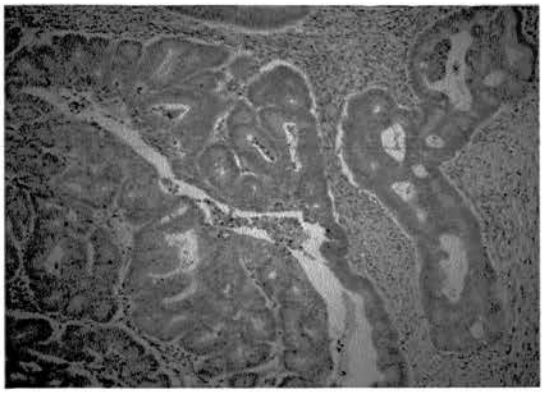
normal colonic epithelium



hyperplastic polyp



adenoma

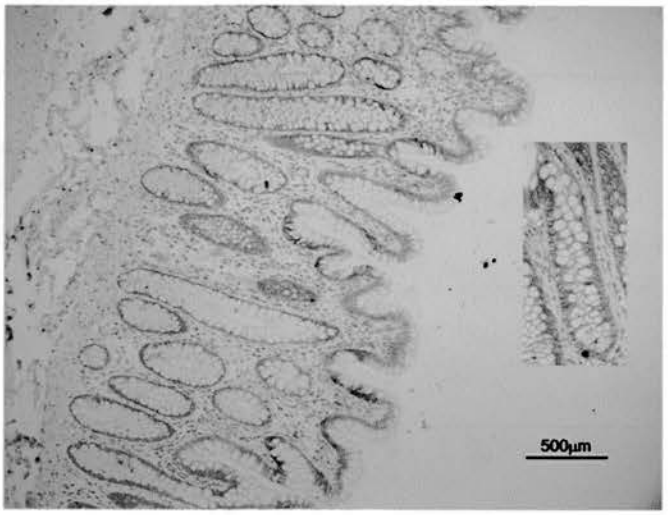


adenocarcinoma

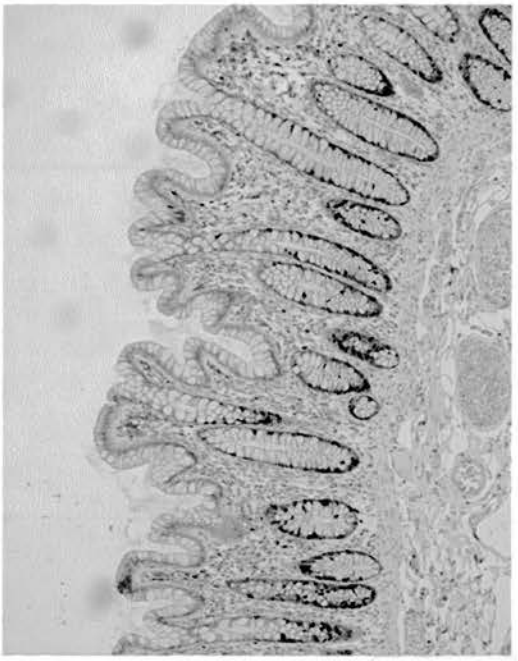
Figure 17: Negative controls for goat anti-shh, anti-ptch and anti-smoh antibodies. No signal was detected when immunodetection omitted the primary antibody.

Figure 18: Micrographs of normal colon stained with SHH PTCH, SMOH and Ki-67. A positive immunohistochemical reaction is indicated by diaminobenzidine (brown staining) and haematoxylin was used a nuclear counterstain. Shh stained slightly the superficial epithelial layer of the colon and few cells located basally, Ptch was detected in the neuroendocrine cells and Smoh stained the brush border of the epithelium.

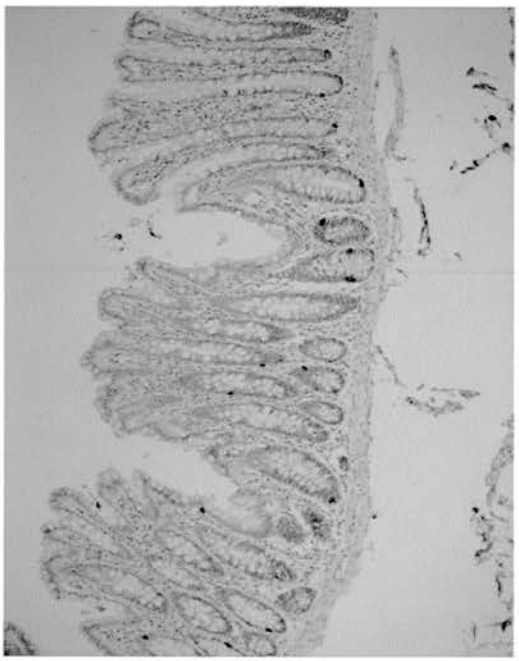
SHH



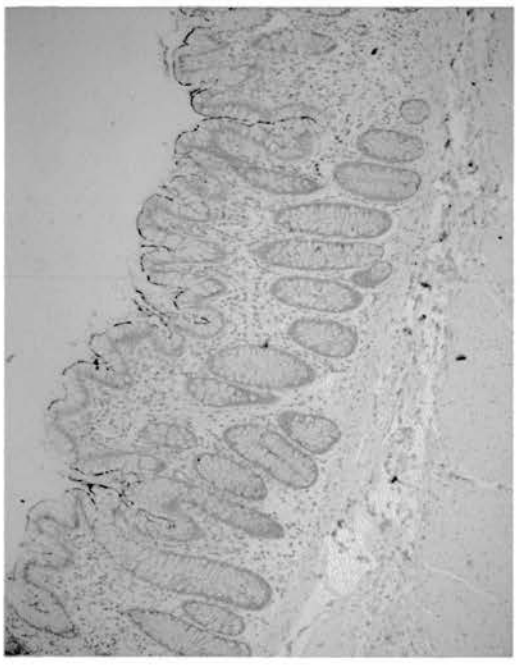
Ki-67



PTCH



SMOH



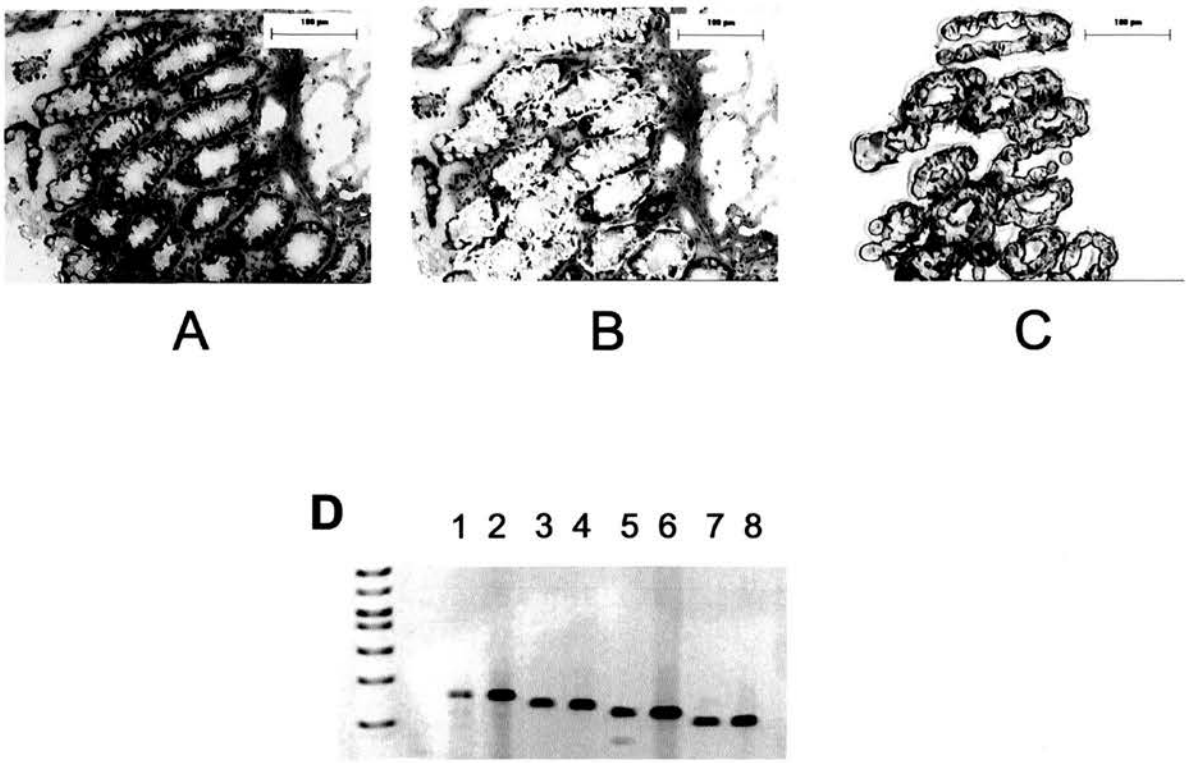


Figure 19: Laser microdissected colonic crypts from frozen normal human colon. A) colonic epithelium before laser microdissection; B) the same section after laser capture microdissection; C) laser microdissected colonic crypts used to generate mRNA. D) RT-PCR analysis for Shh, Ptch and Smoh using mRNA from laser microdissected normal crypts (lanes 1, 3, 5 and 7) and H441 cells (lanes 2, 4, 6 and 8) used as positive control. beta-microglobulin primers were used as an internal qualitative and semi-quantitative control. Lanes 1 and 2: Shh-170bp; Lanes 3 and 4: Ptch-144bp; Lanes 5 and 6: Smoh-140bp; Lanes 7 and 8: β-microglobulin–120bp.

4.2.2. Shh, Ptch and Smoh Are Overexpressed in Hyperplastic Polyps

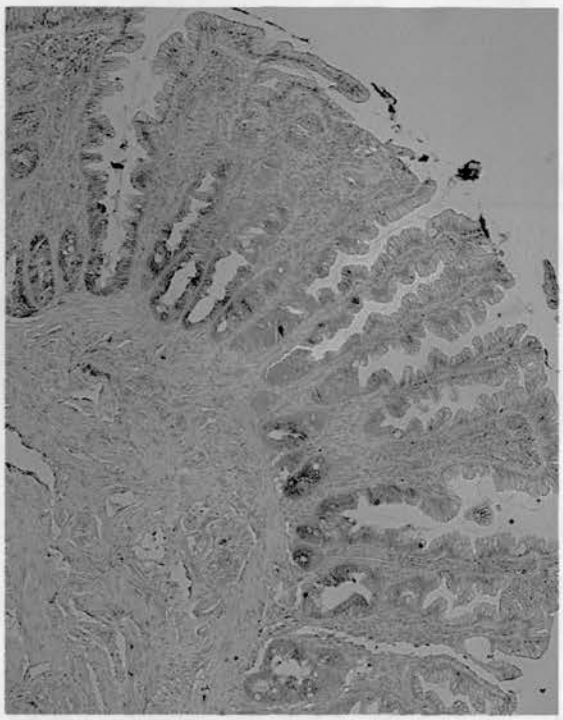
The pattern of Shh expression in hyperplastic polyps contrasts with that described in normal colon epithelium. Strong Shh staining was found at the base of the crypts and became lighter towards the top (Figure 20). Ptch staining was stronger and was present in most of the cells within the crypt with cells located basally also displaying intense staining.

Smoh largely maintained the same pattern of expression as found in normal colon, staining the brush border of superficial epithelium but in addition to that stained the epithelial cells in the crypts. Ki-67 stained a larger number of the nuclei compared to normal. The staining was still located in the basal compartment of the crypts and showed that hyperplastic polyps have a higher proliferation index compared to normal colonic epithelium as expected.

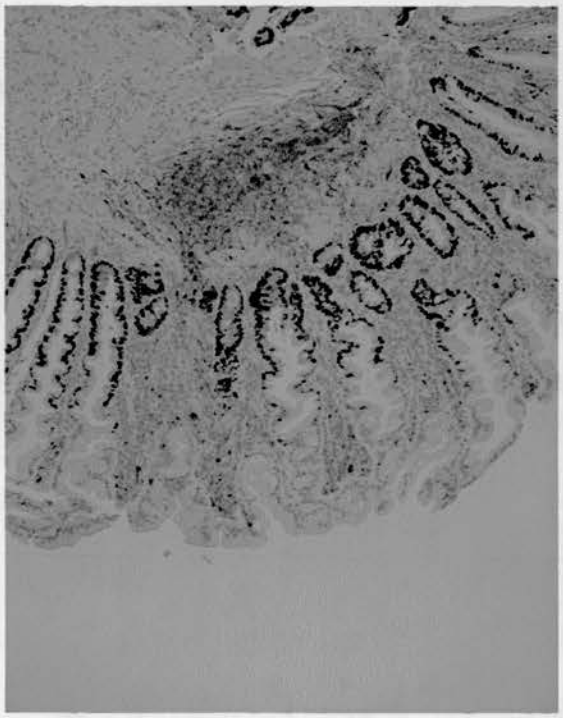
Double fluorescent staining using Shh and Ki-67 also confirmed the pattern of staining detected by immunohistochemistry, with cells located basally expressing both Shh protein and Ki-67 (Figure 21).

Figure 20: Micrographs of a hyperplastic polyp stained with Shh, Ptch, Smoh and Ki-67. A positive immunohistochemical reaction is indicated by DAB (brown staining) and haematoxylin was used a nuclear counterstain. The staining was strong with Shh detected at the base of the crypts and Ptch and Smoh in most of the epithelial cells within the crypts.

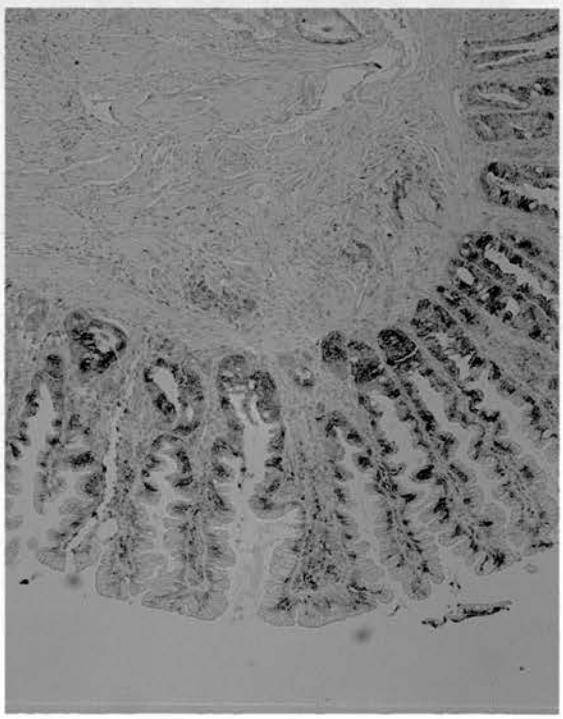
SHH



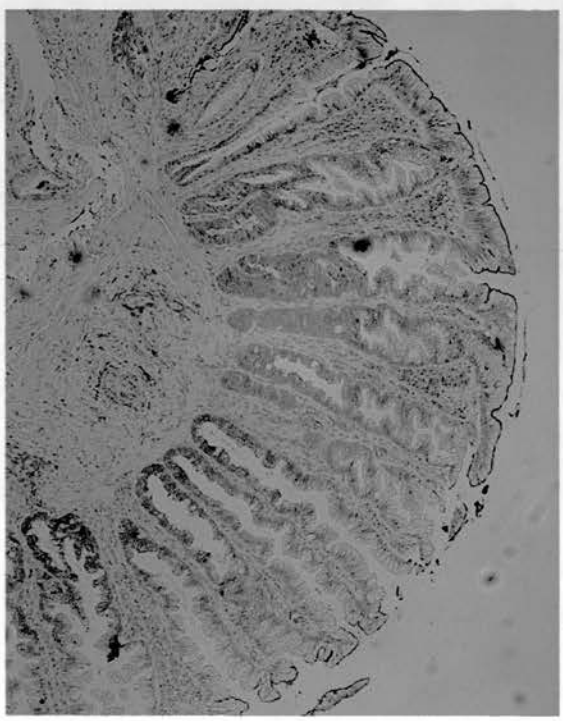
Ki-67



PTCH



SMOH



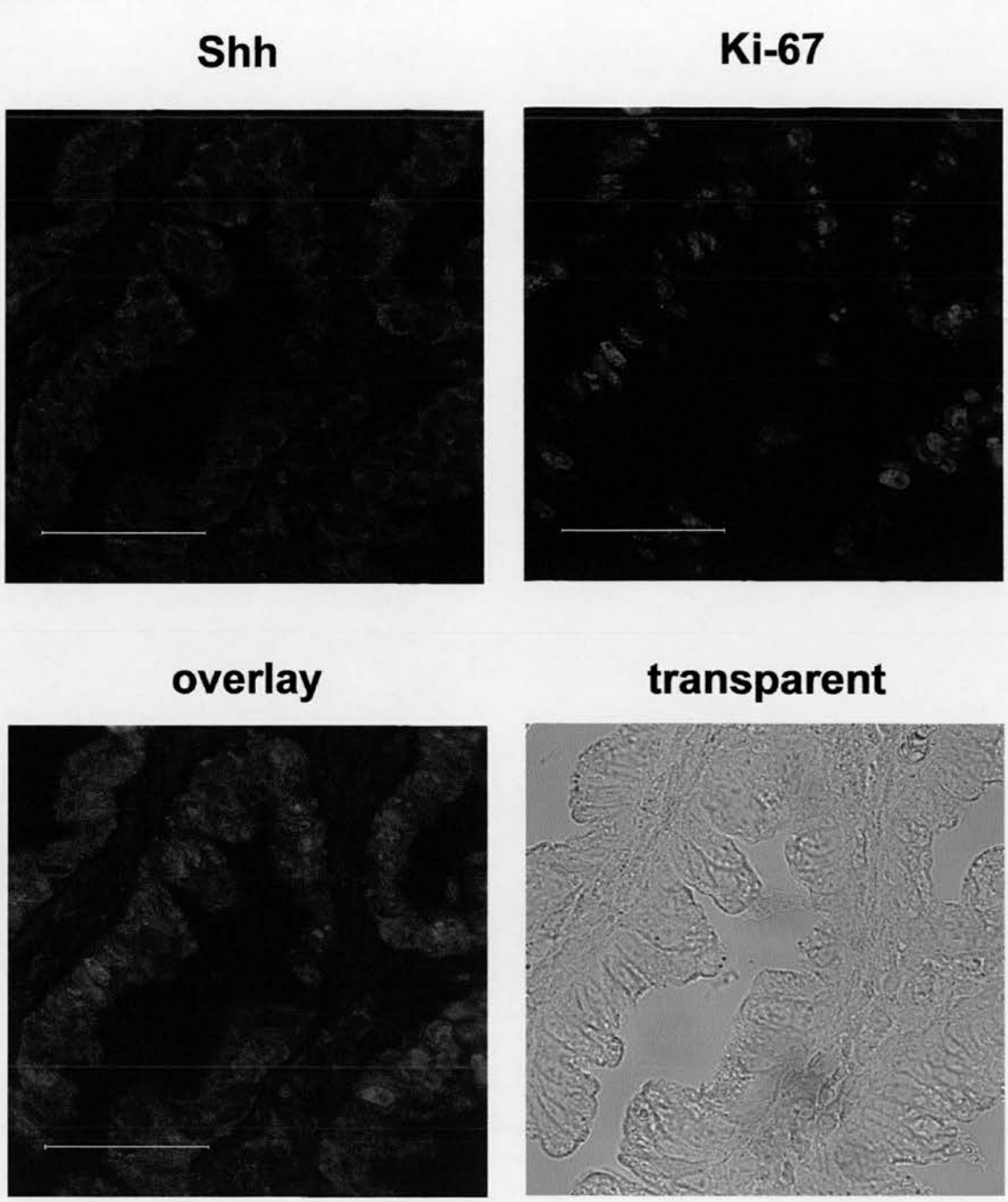


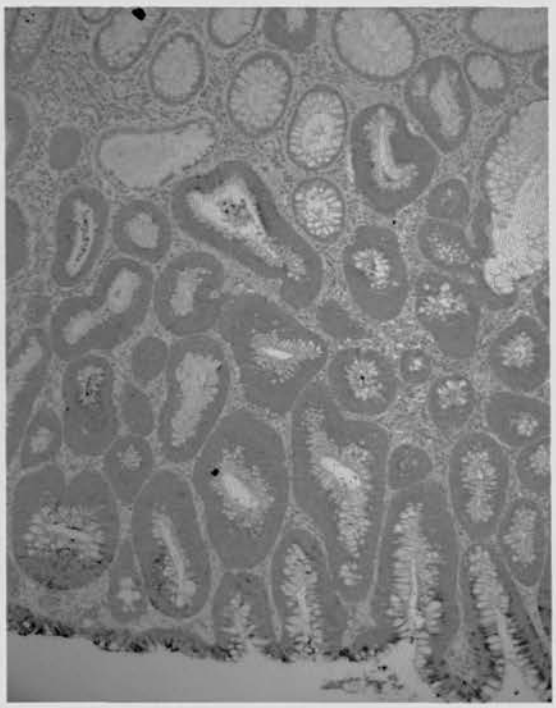
Figure 21: Confocal micrographs (63x) of a hyperplastic polyps immunofluorescently labelled with anti-Ki-67 IgG (Alexa-488 green) and anti-Shh IgG (Alexa-568 red). The overlaid image shows cells located at the base of the crypts displaying positive signals for both proteins.

4.2.3. Hh Pathway Members Are Also Overexpressed in Adenoma and Adenocarcinomas of the Colon

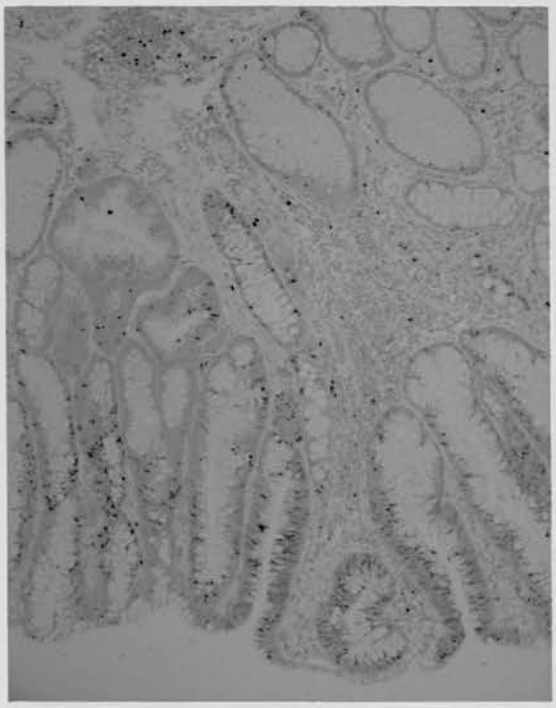
Shh, Ptch and Smoh all showed stronger staining than in normal colon in a large number of malignant crypts. The crypts that stained stronger also displayed multiple mitoses, showed a higher degree of dysplasia and had lost most of their normal characteristics (Figure 22 and 23). The cells in the lamina propria, muscularis mucosa and stroma of invasive carcinoma remained unstained indicating that the expression of the proteins was genuine and specific. Ki-67 confirmed deregulated cell proliferation in adenomatous and malignant crypts with a normal pattern of proliferation within the adjacent, non-neoplastic mucosa. Cyclin B1 expression was more intense where the glands showed a higher rate of mitosis and grade of dysplasia. This pattern of staining appears to reflect that cyclin B1 represents a marker for G2/M phase of the cell-cycle and that most cancer cells actively proliferate.

Figure 22: Micrographs of colonic adenoma stained with Shh, Ptch, Smoh and Ki-67. A positive immunohistochemical reaction is indicated by DAB (brown staining) and haematoxylin was used a nuclear counterstain. Adenomatous crypts stained strong for all three proteins: Shh, Ptch and Smoh. The normal crypts found among the adenomatous ones do not stain suggesting that the expression of the studied proteins is upregulated in colonic adenoma.

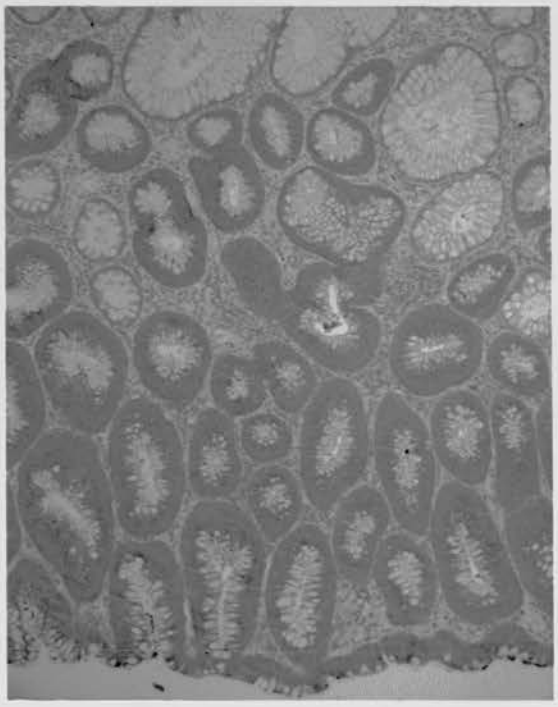
SHH



Ki-67



PTCH



SMOH

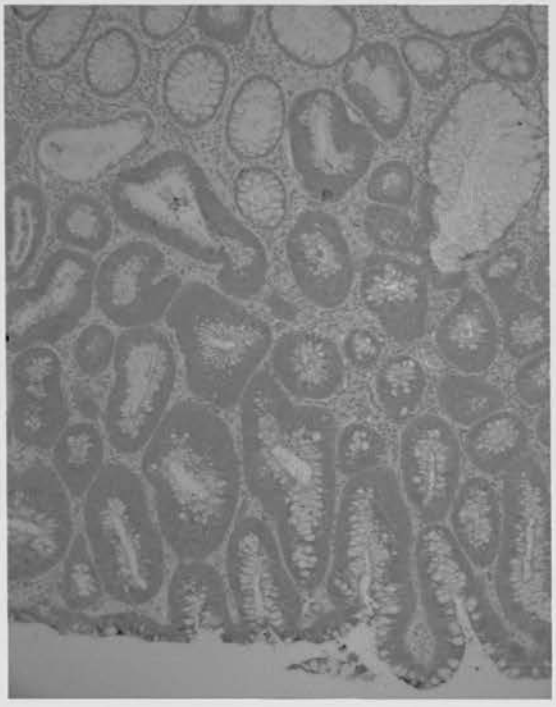
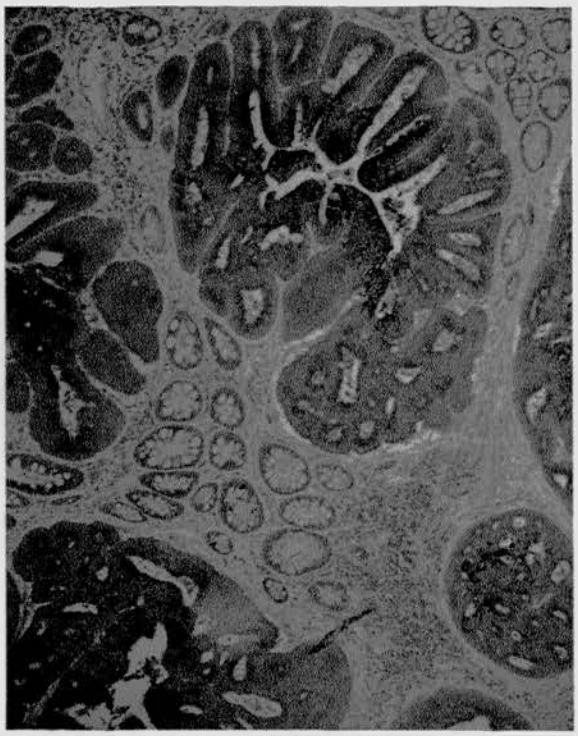
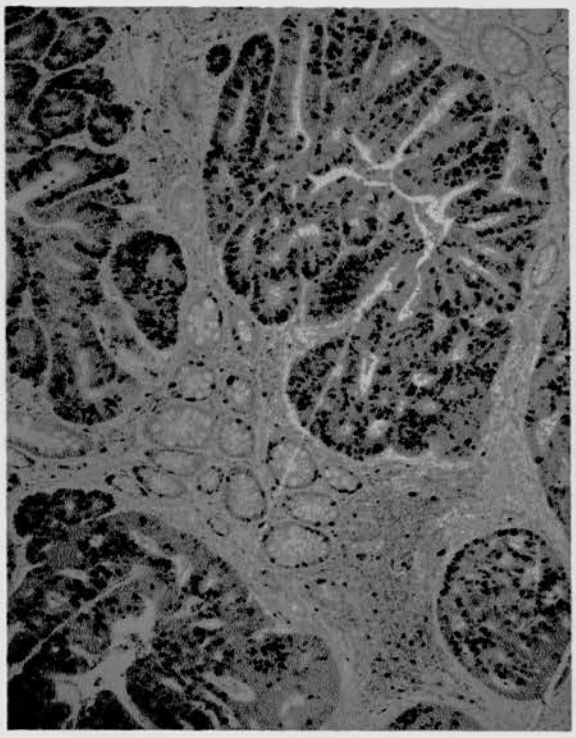


Figure 23: Micrographs of colonic adenocarcinoma stained with Shh Ptch, Smoh and Ki-67. A positive immunohistochemical reaction is indicated by DAB (brown staining) and haematoxylin was used a nuclear counterstain. Strong staining was detected for Shh, Ptch and Smoh in the majority of the malignant crypts.

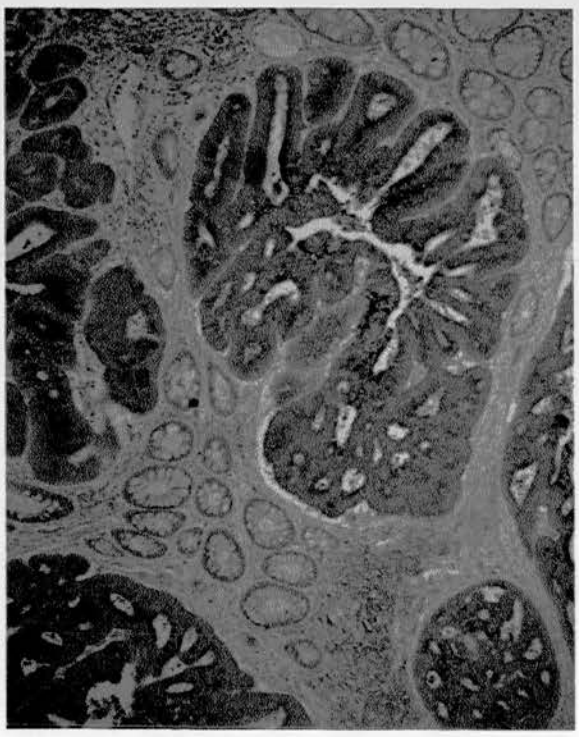
SHH



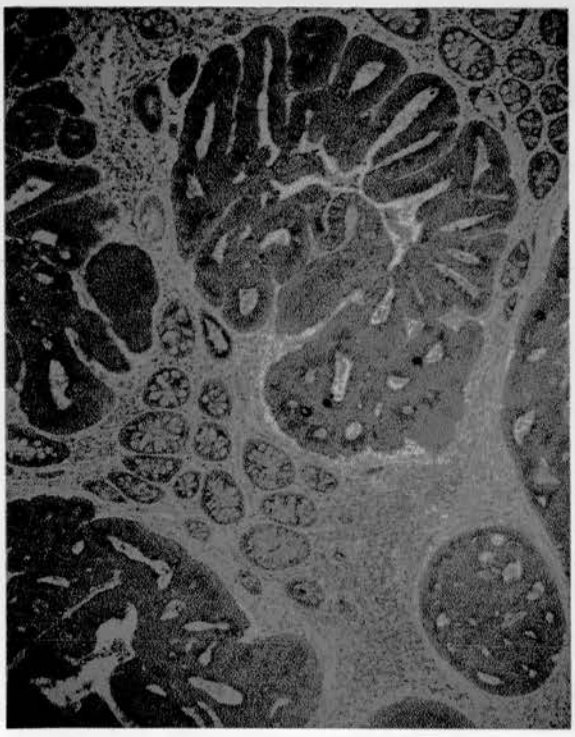
Ki-67



PTCH



SMOH



4.3. Discussion

Shh, Ptch and Smoh are expressed in normal colon and in colorectal neoplasia. Moreover, their protein expression is similar to the distribution of their transcripts in normal human colon.

In the case of hyperplastic polyps, Shh expression was located at the base of the crypts where proliferation was maximal. Similarly, the pattern of expression of Shh correlated with increased expression of the proliferation marker Ki-67 in the adenomatous and malignant crypts of the neoplastic lesions investigated. Ptch and Smoh were also expressed at high levels in the malignant crypts but not all the malignant crypts stained positive.

Shh is important in growth and proliferation in the embryo. Shh has also been shown to promote proliferation when overexpressed in epithelial cells *in vitro* [Kameda T. and Hatakeyama S. *et al.*, 2001]. Consequently, the hypothesis that Shh has a role in epithelial proliferation and possibly in the epithelial colon tumour progression *in vivo* was addressed. Shh was localised in areas of increased cellular proliferation in human hyperplastic polyps and it is also increased in areas of worsening dysplasia in colorectal adenomas as well as in adenocarcinomas.

Therefore, this pattern of expression was suggestive of a role for Shh in epithelial proliferation and possibly progression of epithelial colon tumours *in vivo*.

Ptch and Smoh are central to Shh signal transduction and have both been implicated in human tumourigenesis [Gailani M.R. and Stahle-Backdahl M. *et al.*, 1996; Hahn H. and Wicking C. *et al.*, 1996; Johnson R.L. and Rothman A.L. *et al.*, 1996; Xie J. and Murone M. *et al.*, 1998]. Basal cell carcinoma, medulloblastoma and

rhabdomyosarcoma are all associated with mutations that either activate the proto-oncogene *Smoh* or inactivate the tumour-suppressor *Ptch*. In the cases examined, Ptch and Smoh protein expression was stronger where the lesions displayed a higher malignant grade. Compared with normal colonic tissue, where Ptch expression was detected only in the neuroendocrine cells and Smoh at the brush border of the epithelium, their expression was upregulated in adenoma and carcinoma of the colon. These findings suggest spatially aberrant Hh signalling or epigenetic activation of the genes.

A novel interaction between Ptch and Cyclin B1 has been described [Barnes E.A. and Kong M. *et al.*,]. DNA damage prevents cyclin B1 from entering the nucleus and causes cyclin B1 to shuttle rapidly from the nucleus to cytoplasm thereby delaying mitosis until the damage can be repaired [Toyoshima F. and Moriguchi T. *et al.*, 1998]. Patched could interact with phosphorylated cyclin B1 derivatives and thereby be able to alter their subcellular localisation. As Cyclin B1 is a regulatory subunit of the M-phase proliferating factor (MPF) and Ptch is capable of sequestering MPF to prevent mitotic progression, Ptch may therefore directly inhibit cell proliferation. This inhibitory function supports the notion of Ptch as a tumour-suppressor. Furthermore, overexpression of Shh in transfected cells inhibited the interaction between Ptch and cyclin B1 [Barnes E.A. and Kong M. *et al.*,] strengthening arguments that Shh could have a more direct effect on cell-cycle progression and be involved in tumour growth. As a result, the distribution patterns of Cyclin B1 and Ptch were compared by immunohistochemistry but no significant correlation was found.

A possible interaction between Ptch and p53 has been recently suggested. *Ptch* mutant mice on a p53-null genetic background show a marked increase in the incidence of medulloblastomas [Wetmore C. and Eberhart D.E. *et al.*, 2001]. As many colorectal carcinomas have mutations or deletion of p53, an additional mutation or inactivation of *Ptch* could cause survival and continuous proliferation of cycling cells carrying damaged DNA. Such cells may give rise to tumours.

A possible explanation for the upregulated expression of the Shh proteins noticed in this study would be that the increased expression of Shh is the result of gene mutation or changes in methylation of the *Shh* promoter as opposed to cell-cycle dependent alterations in gene transcription. A panel of human colorectal carcinomas (microsatellite stable and unstable) by single-strand conformation polymorphism for putative mutations in *Shh* and *Smoh* was screened. No mutations were found, suggesting that mutations of these genes do not play a significant role in tumourigenesis of the colon.

In summary, the Hh pathway is an important regulator of body plan patterning, growth and cellular proliferation during embryogenesis, and the results of the present study show that the pathway is expressed in adult colonic tissues. Altered patterns of expression of Hh pathway genes in colonic neoplasia suggest a role in human tumourigenesis.

CHAPTER 5

SONIC HEDGEHOG INDUCES CELL GROWTH AND PROLIFERATION IN PRIMARY MURINE COLONOCYTES

5.1. Background

During embryogenesis, the Hedgehog signalling pathway plays an essential role in the formation of the gastrointestinal tract. Expression of Shh in the gastrointestinal embryonic tract has been described in mouse [Bitgood M.J. and McMahon A.P., 1995], frog [Ryan A.K. and Blumberg B. *et al.*, 1998], chick [Roberts D.J. and Smith D.M. *et al.*, 1998] and human [Marigo V. and Roberts D.J. *et al.*, 1995]. In the murine embryonic gut, expression of Shh has been detected in the endoderm from as early as day 11.5 post coitus, but it changes in the first 3 three postnatal weeks once the crypts and the glandular structures are formed [Bitgood M.J. and McMahon A.P., 1995]. Shh signalling is important for both antero-posterior and radial patterning in the developing murine gut [Ramalho-Santos M. and Melton D.A. *et al.*, 2000] [Sukegawa A. and Narita T. *et al.*, 2000] [Litingtung Y. and Lei L. *et al.*, 1998] but little is known about its roles in the adult gastrointestinal tract. Recently, van den Brink *et al.* [van den Brink G.R. and Hardwick J.C. *et al.*, 2001] reported that Shh is expressed abundantly in the fundic gastric glands of humans and mice at both protein and mRNA level. The same group also revealed that mRNA for Shh was detected in few cells at the base of the crypts in the small and large intestine [van den Brink G.R. and Hardwick J.C. *et al.*, 2002].

In complete concordance with the above research group, results from the previous chapter confirmed that Shh is expressed at protein and mRNA levels in adult normal human colonic epithelium. Moreover, overexpression of the Shh protein and its receptors Ptch and Smoh was detected in colonic lesions including hyperplastic polyps, adenoma and adenocarcinoma. The overexpression of the Shh protein

correlated with increased signal for the proliferation marker Ki-67. Consequently, the hypothesis that Shh plays a role in epithelial proliferation and possibly in epithelial colon tumour progression *in vivo* was addressed. To determine whether Shh may influence cell proliferation in the colon, we used primary murine colonocytes to study the effect of exogenous Shh peptide (Shh-Np) and Shh neutralising antibody (5E1) *in vitro*.

Several studies focused on a possible effect of Shh on cell growth and proliferation. Human blood cells responded to exogenous Shh-N treatment by increased proliferative capacity. Alternatively, cells treated with anti-Shh resulted in maintenance of progenitors and stem cells in an undifferentiated state, reducing the proliferative ability these cells [Bhardwaj G. and Murdoch B. *et al.*, 2001].

In a similar experiment, activated $CD4^+$ T lymphocytes responded with increased/decreased proliferation to external treatment with Shh-N peptide or anti-Shh blocking antibody [Lowrey J.A. and Stewart G.A. *et al.*, 2002], reaffirming that Shh sustains cell proliferation and controls growth.

It appears that the ability of Shh to promote growth is achieved by promoting expression of the cell-cycle regulators Cyclin D and Cyclin E in *Drosophila* [Duman-Scheel M. and Weng L. *et al.*, 2002]. Furthermore, upregulation of murine cyclin D1, D2 and E in response to Hh signalling has also been described in mammalian neuronal precursors [Kenney A.M. and Rowitch D.H., 2000].

5.2. Results

5.2.1. Hh pathway genes are expressed at mRNA levels in primary murine colonocytes

The expression of Hh pathway genes was assessed by RT-PCR analysis using RNA isolated from primary murine colonocytes after 3 days in culture (Figure 24).

All members of the pathway: Shh, Ptch, Ptch2, Gli 1 and Ihh were expressed at mRNA level in actively growing colonocytes.

1 2 3 4 5 6 7

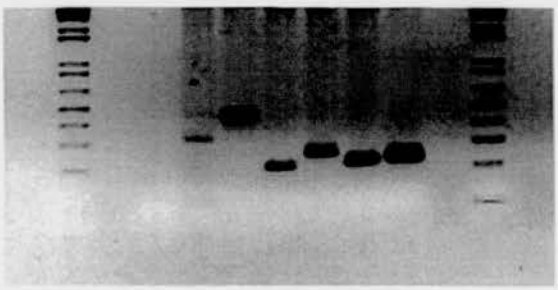


Figure 24: RT-PCR analysis showing expression of murine Shh, Ptch1, Ptch2, Ihh and Gli1 using RNA isolated from primary colonocytes after 3 days in culture.

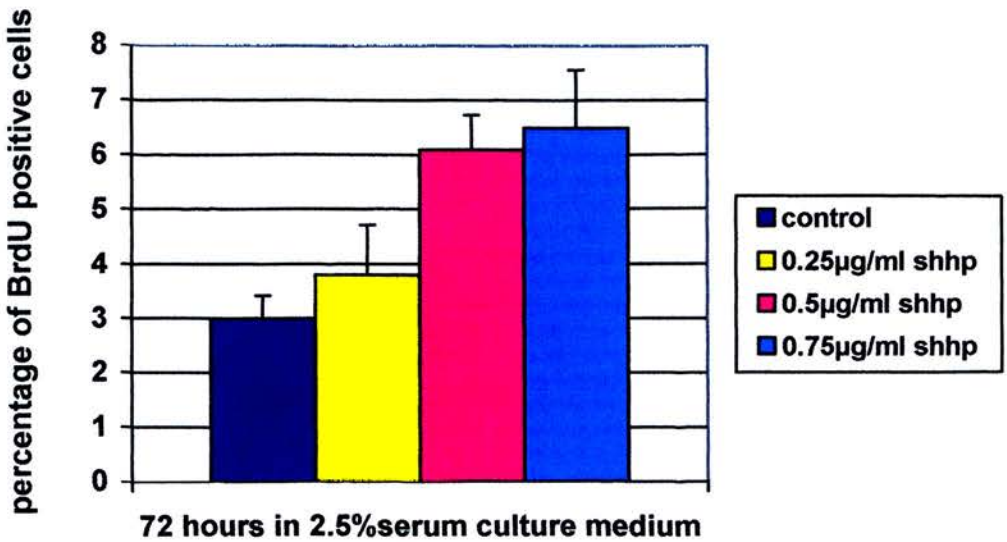
DNA ladder: 1Kb Plus (bands every 100 bp up to 500bp)

Lane 1 - negative control
Lane 2 - Shh: 333bp
Lane 3 - Ptch1: 452bp
Lane 4 - Ptch2: 207bp
Lane 5 - Ihh: 221bp
Lane 6 - Gli1: 251bp
Lane 7 - GAPDH: 237bp

5.2.2. Exogenous Soluble Mouse Shh Peptide Has a Proliferative Effect on Primary Murine Colonocytes

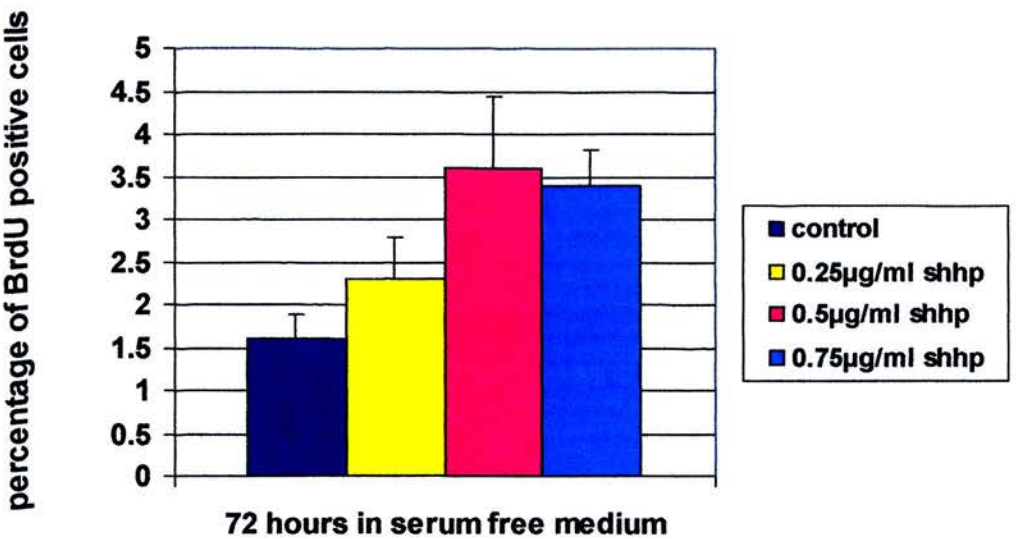
To investigate the possibility that Shh promotes cellular growth primary murine colonocytes were exposed to recombinant 180 amino-acids N-terminus peptide.

Colonic crypts were isolated using the method described in Chapter 2, section 2.2. After 3 days in culture, cells treated with 0.25μg/ml, 0.5μg/ml and 0.75μg/ml of Shh peptide for 72 hours were incubated with BrdU. The percentage of cells in S-phase was quantified by counting the BrdU positive cells. The mouse Shh peptide was reconstituted in 0.3% PBS-BSA, carrier for the soluble protein. Control cells were treated with the same solution. Cells treated with 0.75μg/ml Shh-Np show a statistically significant increase in BrdU incorporation compared to control cells (6.5% vs. 3.0% respectively, p=0.0383, Mann-Whitney U test) (see graph below).



Graph of the percentage cells in S-phase detected by BrdU incorporation in primary murine colonocytes after exposure to exogenous mouse Shh-N recombinant peptide (Shh-Np) for 72 hours in the presence of 2.5% serum.

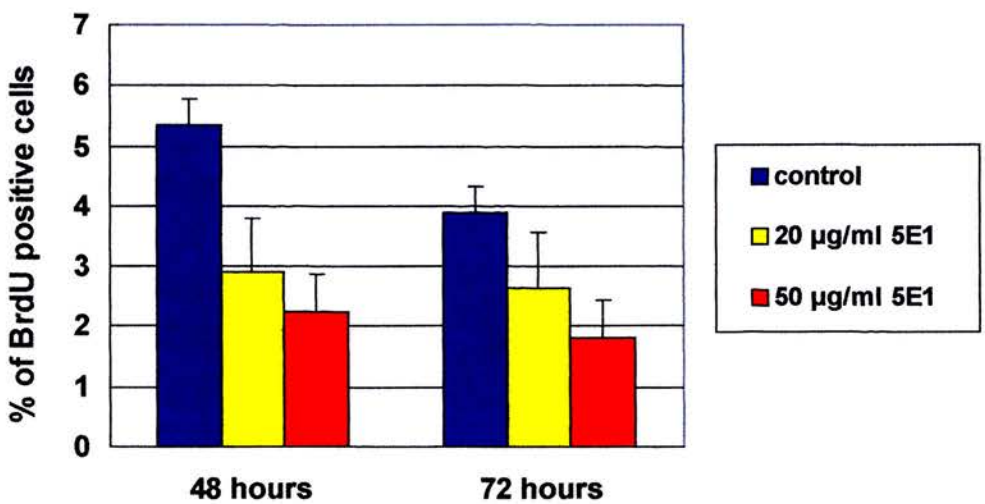
Cells treated with 0.75 μg/ml Shh-Np in the absence of serum show a similar trend in BrdU incorporation compared to control cells (3.4% vs. 1.6% respectively), demonstrating that the proliferative effect of Shh-Np is not due to interaction with serum in culture medium (see graph below).



Graph of the percentage cells in S-phase detected by BrdU incorporation in primary murine colonocytes after exposure to exogenous mouse Shh-N recombinant peptide (Shh-Np) for 72 hours in serum free medium.

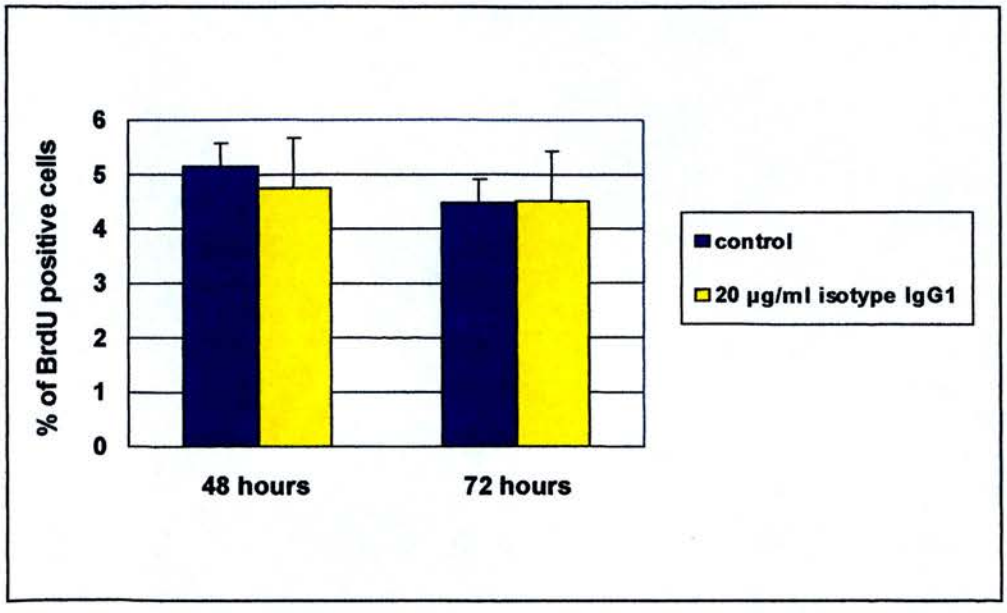
5.2.3. Blocking Endogenous Shh Protein with Mouse Neutralising Anti-Shh Antibody Decreases the Proportion of Cells in S-phase

The mitogenic effect of exogenous Shh in primary colonocytes was similar to that of the anti-Shh treated cells. Colonocytes were exposed to 20μg/ml or 50μg/ml of 5E1 neutralising antibody for 48 and 72 hours. Compared to untreated cells, the mean percentage of BrdU staining cells was significantly reduced after 48 hours in the presence of 20μg/ml (5.35% vs. 2.90% respectively, p=0.0398, Mann-Whitney U test) and 50μg/ml (5.35% vs. 2.25%, p=0.0147, Mann-Whitney U test) of 5E1 (see graph below). After 72 hours a statistically significant difference is seen between untreated cells and cells cultured in the presence of 50μg/ml (3.90% vs. 1.8%, p=0.0152, Mann-Whitney U test).



Graph of the percentage cells in S-phase detected by BrdU incorporation in primary murine colonocytes after exposure for 48 and 72 hours to exogenous anti-Shh antibody (5E1)

No inhibition of S-phase was observed in the presence of the isotype control antibody at previously reported concentrations (see graph below).



Graph of the percentage cells in S-phase detected by BrdU incorporation in primary murine colonocytes after exposure for 48 and 72 hours to the 5E1- isotype control antibody (IgG_1) for 48 and 72 hours.

5.3. Discussion

To investigate whether Shh influences cell proliferation in the colon, primary colonocytes were exposed to the agonist Shh peptide (Shh-Np) and neutralising antibody (5E1). The human colorectal cell-lines SW480 and Colo-320 showed no response to either Shh-Np or 5E1, when analysed by flow cytometry (this experiment was performed in collaboration with Dr. Roberta M. James) (data not shown) Since these cell-lines are transformed to allow clonogenic survival, it is not surprising that they are refractory to external stimuli. Furthermore, Watkins *et al.* [Watkins D.N. and Berman D.M. *et al.*, 2003] reported that a colorectal cell-line, HCT116, showed no growth reduction in response to the drug cyclopamine, which inhibits Hh signalling by binding to Smoh. This suggests that the pathway may not be active in some colorectal cell-lines. Therefore, the use primary murine colonocytes derived from normal adult murine colon was preferred. These cells offer the advantage of being untransformed epithelial cells and more likely to reflect the *in vivo* situation. Furthermore, both exogenous Shh-N peptide and 5E1 blocking antibody have been reactive with human and mouse as reported by several groups [Bhardwaj G. and Murdoch B. *et al.*, 2001] [Lowrey J.A. and Stewart G.A. *et al.*, 2002] [Watkins D.N. and Berman D.M. *et al.*, 2003].

In conclusion, the results in this chapter show that exogenous Shh has a mitogenic effect on primary murine colonocytes. Moreover, uninterrupted blocking of endogenous Shh inhibited proliferation in these cells, suggesting that Shh is required for epithelial cell proliferation *in vitro*.

Cell-cycle is dependent on key components of the cell-cycle including the cyclins and cyclin-dependent kinases (cdk). Shh expression has been reported to associate with increased activity of cdk2 and cdk4 in normal keratinocytes [Fan H. and Khavari P.A., 1999]. Both these kinases have important roles in the G1-S transition of the cell-cycle. Another study showed that Shh exerts his proliferative effects through regulation of the G1-phase cyclins like cyclins D1, D2 and E [Altaba A. and Stecca B. *et al.*, 2004]. Moreover, Shh is capable to promote cell proliferation by activating the M-phase promoting factor. Cyclin B1 is a regulatory subunit of the M-phase proliferating factor as mentioned earlier and could interact with Ptch to prevent cell-cycle progression. Maybe the exogenous Shh-N peptide could disrupt the interaction between Ptch and Cyclin B1 facilitating cell-cycle progression.

Consequently, it would be of interest as a future line of research to investigate possible mechanisms that could be responsible for the ability that Shh has to increase proliferation in primary murine colonocytes.

Two years ago, it has been revealed that cyclopamine, a small steroidal alkaloid, has the capacity to bind Smoothened, therefore inhibiting Hedgehog signalling [Chen J.K. and Taipale J. *et al.*, 2002]. The same study revealed that cyclopamine could reverse the retention of spatially missfolded Smoh in the endoplasmic reticulum, mechanism responsible for the teratogenic and anti-tumour effects of this small molecule.

Inhibition of Hh signalling by cyclopamine induced apoptosis and blocked proliferation in a wide variety of cancer cell-lines in vitro (originating in the brain, oesophagus, stomach, biliary tract, pancreas and lung) [Berman D.M. and Karhadkar S.S. *et al.*, 2003] [Thayer S.P. and Di Magliano M.P. *et al.*, 2003] [Altaba A. and

Stecca B. *et al.*, 2004]. Subsequently, Berman et al. [Berman D.M. and Karhadkar S.S. *et al.*, 2003] showed that cyclopamine inhibits the growth of medulloblastoma mouse allografts. These findings provide a sound basis for therapeutic approach and open a new way of viewing tumorigenesis.

These studies open the way for further investigations using the primary colonocytes *in vitro* model as a bridge for potential studies in a variety of in vivo models for colorectal carcinogenesis.

SUMMARY

The investigations undertaken in this thesis focused on two areas:

1. the importance of p53 and related proteins in the regulation of growth and responses to DNA damage caused by cisplatin.
2. the investigation of expression of developmental Hh signalling proteins in normal colon and colonic neoplastic lesions including hyperplastic polyps, adenomas and adenocarcinomas.

p53 mutations or deletions are common events in colorectal cancer. One might anticipate that the loss of p53 would have an early role in tumourigenesis by allowing mutation to occur through avoidance of normal DNA damage-induced cell cycle arrest, DNA repair or induction of apoptosis mediated by p53. In fact, studies of human disease indicated that p53 loss or mutation is a late stage phenomenon associated with disease progression and a likelihood of invasion leading to metastases. Furthermore, crossing p53 deficient mice with Min mice, which are heterozygous for mutant APC, does not result in an increase in intestinal tumourigenesis in either small or large intestine. Finally, in p53 deficient mice, as in humans with Li Fraumeni syndrome, there is no increase in susceptibility to colorectal cancer. If p53 is the only gene responsible for deletion of damaged cells following damage by apoptosis, then clear differences in frequency of apoptosis after ionizing radiation in p53 wild-type and null epithelium would be expected. In fact, in colon there is little apoptosis in either genotypes and after 24 hours there is a second wave of apoptosis in both genotypes. This strongly suggests that p53 mediated

apoptosis is neither a unique nor a major protector of colonic epithelial cells against mutations following DNA damage.

As much of the information in carcinogenesis in the intestine derives from studies in the small intestine and the majority of the mutations leading to cancer occur in the large intestine, the use of a large bowel model and in particular the primary murine colonocytes system was considered to overcome some of these issues. Primary murine colonocytes isolated from wt and p53 null mice were exposed to cisplatin to better understand the importance of p53 and related proteins in the regulation of growth and responses to DNA damage. The results of the first project in this thesis showed that the role of p53 in preventing entry into S-phase following colonocytes DNA damage appears crucial whereas its role in apoptosis seems redundant. This strongly confirms that p53-mediated apoptosis and growth arrest are indeed neither a major nor a unique protector of colonic epithelial cells against mutation following DNA damage. The baseline expression p21, p53 and p73 could be attributed to the stress of the in vitro conditions, as hypoxia is known to activate both p53 and p73 and this seems to be a limitation of this in vitro model.

Furthermore, the nuclear translocation of endogenous p73α in response to DNA damage in primary colonocytes is highly suggestive of a functional pro-apoptotic role for p73α in these cells, within the context of p53-independent apoptosis. It seems that the effects of p53 are not solely determined by p53 itself but by interaction with its family members, with p73 in particular. It also appears that whereas loss of p53, as a late event in colorectal carcinogenesis, is important in progression, p53 may still have a modulatory role in early tumourigenesis in partnership or competition with variants of p73. As a future research direction, an

investigation of a possible role played by p63, another p53 family member, following treatment with cisplatin in primary murine colonocytes, would be interesting to see if it shares the pro-apoptotic roles with the other members of the family.

The second part of this thesis investigated the roles of developmental Hh genes in colonic tumourigenesis. The expression of Shh, Ptch and Smoh at protein and mRNA levels was examined using immunohistochemistry and RT-PCR from laser captured microdissected colonic crypts.

The novel findings of this study were to show:

1. expression of Sonic Hedgehog developmental genes in normal adult colonic epithelium
2. over-expression of these genes in "pre" and "early" neoplasia as well as in colonic adenocarcinoma .

Previously it was demonstrated that hedgehog genes are important in gut development and function, but they were never shown to be present in adult normal colonic epithelium. These findings suggest that S*hh* dysregulation may be important in tumourigenesis or allowing early changes that facilitate tumourigenesis in colon cancer.

To address whether the Hh signalling pathway is functional in the gut, the effect of Shh on epithelial cells *in vitro* was explored by treating primary murine colonocytes isolated from adult mouse colons, with either Shh peptide or neutralizing anti-Shh antibody. Exogenous Shh promotes cell proliferation in colonocytes while anti-Shh

inhibits proliferation, confirming that Shh is required during proliferation of murine epithelial cells *in vitro*.

These findings indicate that Hh developmental genes play a role in the epithelial proliferation of the colon and possibly in the colon tumour progression *in vivo*. These genes were not found mutated in a set of primary human colorectal tumours but it is possible that it is not mutation of these genes but other events such as methylation and protein overexpression play a role in colon carcinogenesis.

Consequently, more experiments would be necessary to establish a possible role and mechanism by which these genes, or their interconnection with other factors, alter the colonic epithelium to lead to colon cancer.

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APPENDIX

PRESENTATIONS:

1. p53-Dependent and Independent DNA Damage Responses in Primary Murine Colonocytes: a Possible Functional Role for p73α (see page 206).

   Pathological Society of Great Britain and Ireland, 1st-4th July 2003, section: Molecular and Cellular Pathology

2. Altered Expression of Sonic Hedgehog Pathway Genes in Human Colon Tumours (see page 207).

   Pathological Society of Great Britain and Ireland, 1st-4th July 2003, Bristol, UK, section: Gastrointestinal Pathology

3. Expression of Sonic Hedgehog Pathway Genes is Altered in Human Colonic Tumors (see page 208-209).

   American Society of Clinical Pathology, 18th-22nd September 2003, New Orleans, US.

PUBLICATIONS

1. p73α is a candidate effector in the p53-independent apoptosis pathway of cisplatin-damaged primary murine colonocytes.

   Anca Oniscu, Nathalie Sphyris, Robert G. Morris, Scott Bader and David J. Harrison

   Journal of Clinical Pathology 2004, 57:492-498

2. Expression of Sonic Hedgehog Pathway Genes is Altered in Colonic Neoplasia.

   Anca Oniscu, Roberta M. James, Robert G. Morris, Scott Bader, Roger D.G. Malcomson and David J. Harrison

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p53-Dependent and Independent DNA Damage Responses in Primary Murine Colonocytes: a Possible Functional Role for p73α

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p53 is a tumour suppressor gene important in growth regulation, differentiation and apoptosis which is frequently mutated or deleted in colorectal cancer.

We derived primary colonocytes from wild-type (wt) and p53-deficient mice to investigate the effect of p53 status on growth and the p53-dependent and independent pathways following cisplatin-induced DNA damage. Apoptosis, S-phase DNA synthesis and the induction of p53, p21 and p73 expression were monitored over a 24-hour period. As measured by 5'-bromo-2'-deoxyuridine incorporation, p53-null colonocytes displayed a significant growth advantage over wt cells and continued to enter DNA synthesis following cisplatin exposure, suggesting a critical role for p53 in cell-cycle arrest following damage. There was a distinct delayed apoptosis in p53-null cells. Nuclear p21 expression was identified in 87% wt cells compared with 52% in p53-null colonocytes ($p<0.05$). As p73 has been implicated in the transcriptional activation of p21 and the p53-independent apoptosis after cisplatin damage, we investigated its expression. RT-PCR confirmed the presence of full-length p73α. p73 transcript levels remained unchanged whereas the endogenous p73 protein accumulated in the nucleus of cisplatin treated cells.

We therefore propose a pro-apoptotic role for full-length p73α in the absence of p53, and collaboration with p53 in the wt cisplatin-damaged colonocytes.

Altered Expression of Sonic Hedgehog Pathway Genes in Human Colon Tumours

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The Hedgehog signalling pathway is crucial for normal development and patterning of various organs in humans including the gut. Hedgehog proteins are also important in gastric gland development and gastric epithelial differentiation in adults.

This work investigates the presence of the Hedgehog signalling pathway at protein and mRNA level in normal colon and compares the distribution patterns of these genes in a series of colonic lesions: hyperplastic polyps, adenomas and colonic adenocarcinoma.

We report that Hedgehog pathway members are expressed in normal colonic epithelium with sonic hedgehog (SHH) expressed at the top of the crypts, patched (PTCH) in the neuroendocrine cells and smoothened (SMOH) at the brush border of the epithelium. RT-PCR analysis of laser-microdissected crypts from normal human colon confirmed that mRNAs encoding these signalling molecules were expressed in colonic epithelium. Expression of SHH, PTCH and SMOH was upregulated in hyperplastic polyps, adenoma and adenocarcinoma of the colon as detected by immunohistochemistry, suggesting a role for these molecules in human tumorigenesis. SHH expression correlated with an increased expression of the proliferation marker Ki-67 in all lesions examined.

Therefore, we suggest a role for SHH in epithelial proliferation in the colon and possibly in epithelial colon tumour progression in-vivo.

Expression of Sonic Hedgehog Pathway Genes is Altered in Human Colonic Tumours

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Introduction The Hedgehog signalling pathway is crucial for normal development and patterning of various organs in humans including the gut. Hedgehog proteins are also important in gastric gland development and gastric epithelial differentiation in adults. The present work investigates the presence of the Hedgehog signalling pathway at protein and mRNA level in normal colon and compares the distribution patterns of these genes in a series of colonic lesions: hyperplastic polyps, adenomas and colonic adenocarcinoma.

Materials and Methods 7 hyperplastic polyps, 20 tubulo-villous adenomas, 8 adenocarcinomas and 3 normal colon samples were used in the study. Normal colonic crypts were microdissected from 70% ethanol-fixed cryostat sections onto laser-capture microdissection caps and total mRNA isolated using a phenol-chloroform based method. RNA analysis was performed by RT-PCR. For immunohistochemistry we used antibodies against sonic hedgehog (Shh), patched (Ptch), smoothened (Smoh), Cyclin B1 and Ki-67.

Results We report that Hedgehog pathway members are expressed in normal colonic epithelium with Shh expressed at the top of the crypts, Ptch in the neuroendocrine cells and Smoh at the brush border of the epithelium. RT-PCR analysis of laser-microdissected crypts from normal human colon confirmed that mRNAs encoding these signalling molecules were expressed in colonic epithelium. Expression of Shh, Ptch and Smoh was upregulated in hyperplastic polyps, adenoma and adenocarcinoma of the colon as detected by immunohistochemistry, suggesting a role for these genes in human tumorigenesis. Shh expression correlated with an increased expression of the proliferation marker Ki-67 in all lesions examined.

Conclusion We suggest a role for Shh in epithelial proliferation in the colon and possibly in epithelial colon tumour progression *in-vivo*. Further work is underway to determine whether the increased expression of Shh is a result of gene mutation or changes in methylation of the *Shh* promoter as opposed to cell-cycle dependent alterations in gene transcription.

ORIGINAL ARTICLE

p73α is a candidate effector in the p53 independent apoptosis pathway of cisplatin damaged primary murine colonocytes

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See end of article for authors' affiliations

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Aims: Colonocytes were derived from wild-type (wt) and p53 deficient mice to investigate p53 dependent and independent death pathways after cisplatin treatment, and the role of p53 in growth regulation of primary, untransformed epithelial cells.

Methods: Wt and p53 null colonocytes were exposed to cisplatin and DNA synthesis, apoptosis, and p53, p21, and p73 expression were investigated after six, 12, and 24 hours. Major p73 isoforms were identified by reverse transcription polymerase chain reaction (RT-PCR).

Results: Cisplatin treated wt cells exhibited cell cycle arrest, whereas p53 null cells continued to synthesise DNA, although both cell types died. Apoptosis was significantly higher in cisplatin treated wt and p53 null colonocytes than in controls at all timepoints, although apoptosis was lower in cisplatin treated p53 null colonocytes than in wt cells. p53 expression was upregulated in cisplatin treated wt colonocytes. p21 expression was high and remained unchanged in cisplatin treated wt cells, although it was reduced in the absence of p53. p73 was investigated because it could account for p53 independent p21 expression and p53 independent death. RT-PCR detected full length p73α. p73 transcript levels remained unchanged, whereas p73 protein accumulated in the nucleus of cisplatin treated cells, irrespective of genotype.

Conclusions: p53 is essential for cell cycle arrest, but not apoptosis in primary murine colonocytes. Apoptosis is reduced in cisplatin treated p53 null cells. Nuclear accumulation of endogenous p73 after cisplatin treatment suggests a proapoptotic role for p73α in the absence of p53 and collaboration with p53 in wt colonocytes.

Escape from the induction of apoptosis is believed to be a crucial event in colorectal carcinogenesis. Mutations of p53 are common in colorectal neoplasia but occur late in disease evolution, acting as a progression step.[1] Approximately half of all colorectal cancers show p53 gene mutations.[2]

The role of p53 in tumour suppression is linked to its functions in the regulation of cell cycle control,[3] differentiation,[4] inhibition of angiogenesis,[5] senescence,[6] and apoptosis after genotoxic stress, nucleotide depletion, and oncogene activation.[7] After DNA damage, p53 induces the expression of $p21^{(WAF1/CIP1)}$, a cyclin dependent kinase inhibitor that causes cell cycle arrest when overexpressed.[8] p21 can also bind to proliferating cell nuclear antigen (PCNA),[9] blocking its function in DNA replication but not repair.[10] Consequently, p21 can cause G1 arrest and inhibition of DNA replication, ensuring that potential errors are repaired before the resumption of cell cycling.

Although the importance of p53 in tumour suppression remains undisputed, it has become apparent that p53 independent mechanisms operate in tandem to ensure the fidelity of replication and the elimination of rogue cells.

Recently, p73 was identified as a p53 homologue, and exhibits considerable sequence and structural similarities. Similar to p53, p73 can transactivate p21, and induce cell cycle arrest and apoptosis when overexpressed.[11] Moreover, in p53 null cells, oncogene activation induces endogenous p73 expression, engaging an apoptosis pathway that is p53 independent.[12] Despite the p73 locus (1p36) being on a chromosome region frequently deleted in human cancers, studies on human tumours have failed to detect p73 gene deletion or inactivating mutations.[13 14] The role of p73 in tumorigenesis remains unclear, because silencing of the p73 gene has been detected in neuroblastoma, squamous cell carcinoma, and lung cancer,[15] suggesting that p73 may assume a tumour suppressor role. Conversely, overexpression of p73 has been reported in haemopoietic malignancies,[16] ovarian,[17] bladder,[18] and breast cancer.[19] Moreover, p73 null mice do not display an increased predisposition to spontaneous tumour development.[20]

p73 exists as at least six (α, β, γ, δ, ϵ, and η) full length transactivation competent (TA) forms, which differ at the C-terminus, and as N-terminally truncated variants (ΔN).[21] Overexpression of the TA isoforms was shown to cause irreversible growth arrest and apoptosis in cells lacking functional p53.[22] In contrast, the ΔN variants assume a protective role against apoptosis by directly antagonising the apoptotic functions of p53[23] and by suppressing p53 and TAp73 dependent transactivation.[24]

> "Similar to p53, p73 can transactivate p21, and induce cell cycle arrest and apoptosis when overexpressed"

Much of the information on carcinogenesis in the intestine derives from studies in the small intestine or cell lines, yet most of the mutations leading to cancer occur in the large intestine. Pioneering work by Potten and others[25–27] has elucidated the kinetics of intestinal growth in vivo but primary culture offers a dynamic in vitro model that allows

Abbreviations: BrdU, 5′-bromo-2′-deoxyuridine; ΔN, N-terminally truncated variant; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcription polymerase chain reaction; TA, transactivation competent; wt, wild-type

the investigation of growth, apoptosis, and responses to injury.[28 29]

Therefore, we adapted the colonic crypt isolation technique developed by Booth and colleagues[28] to obtain primary colonocytes from adult wt and p53 null mice. Our objectives were to gain a better understanding of the importance of p53 and related proteins in the regulation of growth and response to cisplatin in these cells. Cisplatin crosslinks DNA, forming intrastrand and interstrand adducts. This damage culminates in the induction of apoptosis via at least two different pathways: one involving p53 and the other mediated by p73.[30] These pathways have not yet been examined in primary colonocyte cultures.

MATERIALS AND METHODS

Isolation of colonic crypts and culture of primary colonocytes from adult wt and p53 null mice

Wt and p53 null mice[31] were humanely killed between 50 and 75 days of age. The crypt isolation technique was successfully adapted from Booth *et al*.[28] NIH-3T3 cells were cultured in double modified eagle medium supplemented with 10% fetal bovine serum.

Cisplatin treatment

After three days in culture, cells were exposed to 15μM cisplatin (David Bull Laboratories, Warwick, UK) for six, 12, or 24 hours.

Proliferation assay

Primary colonocyte cultures were incubated with 0.01 mM (BrdU) (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) in culture medium for four hours and fixed in 80% ethanol overnight. Immunodetection of BrdU incorporation was performed using rat anti-BrdU antibody (1/100 dilution) (Oxford Biotechnologies, Oxford, UK) and positive cells were detected using diaminobenzidine. Slides were counterstained with haematoxylin. BrdU incorporation was measured by counting the positive nuclei in a total of 500 cells in duplicate. Experiments were performed at least three times with comparable results.

Measurement of apoptosis

For the determination of apoptosis, cells were fixed in modified Bouin's fixative (85% methanol, 5% acetic acid, and 10% formalin) at 4°C overnight, the DNA denatured in 5M hydrochloric acid, and the nuclei stained with Schiff reagent (Merck, Darnstadt, Germany). We used 0.3% light green as a cytoplasmic counterstain. Apoptosis was measured using morphological criteria,[32] with 500 cells counted in duplicate for each timepoint in three separate experiments.

Immunocytochemistry for p53, p21, and p73

The monoclonal antibody against p53 (clone pAB421) was purchased from Calbiochem and mouse anti-p21 (F-5) and rabbit anti-p73 (H-79) from Santa-Cruz Biotechnologies (Santa Cruz, California, USA). Immunocytochemistry was carried out on 4% paraformaldehyde fixed cells, permeated with 0.5% Triton using an avidin–biotin peroxidase technique, except for p73, which was detected by immunofluorescence using an Alexa-488, conjugated secondary antibody (Molecular Probes, Eugene, Oregon, USA). Negative control samples omitted the primary antibody. In addition, p53 null colonocytes were used as a negative control for immunocytochemistry with anti-p53 antibody. For p21 and p53 we counted at least 500 cells for each individual timepoint in three separate experiments.

p73 immunohistochemistry was also performed on formalin fixed paraffin wax embedded mouse colon and skin sections using a peroxidase technique.

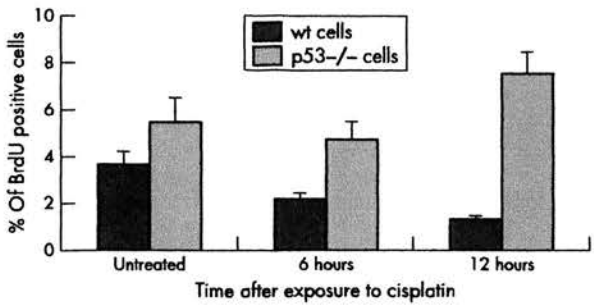

Figure 1 5'-Bromo-2'-deoxyuridine (BrdU) incorporation into untreated and cisplatin treated wild-type (wt) and p53 null colonocytes. Cells were incubated with BrdU for four hours and immunodetection was performed using rat anti-BrdU antibody. Positive cells were detected by immunocytochemistry and a total of 500 cells counted in triplicate in at least three different experiments. Results are mean (SEM), and show a higher BrdU incorporation in p53 null colonocytes, regardless of treatment and timepoint ($p = 0.015$, ANOVA).

Statistics

Data were analysed using Minitab statistical software version 13. Data are presented as the mean (SEM). Comparison of BrdU results was made using ANOVA. Comparison of all other results was made using the Mann-Whitney U test. A p value of < 0.05 was considered significant.

Reverse transcriptase polymerase chain reaction

Total RNA was extracted using TRIZOL (Invitrogen, Breda, the Netherlands). Contaminating genomic DNA was removed by treating the total RNA with DNase I (DNAfree kit; Ambion, Huntingdon, Cambridgeshire, UK) according to the protocol supplied by the manufacturer. For cDNA synthesis we used the M-MLV reverse transcriptase kit (Invitrogen) and generated cDNA from 100 ng of total RNA. Amplification of the region between exons 11 and 14[33] yields a product of 363 bp in the case of p73α and/or a product of 270 bp in the case of p73β. For the detection of full length forms, primers annealing to exons 3–4[23] were used, generating a product of predicted size 297 bp. The primers for ΔN forms, annealing to exons 3–4,[34] generate a product of 187 bp. Transcript values were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (237 bp).[35] NIH-3T3 cells of mouse origin were used as positive controls for the reverse transcription polymerase chain reaction (RT-PCR) experiments.

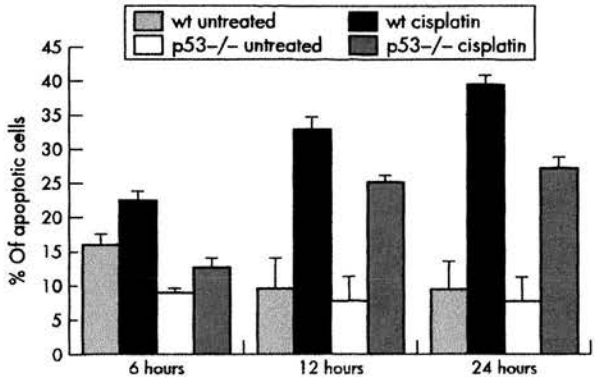

Figure 2 Apoptosis in wild-type (wt) and p53 null colonocytes exposed to cisplatin. The Feulgen stain and a 0.3% light green counterstain were used to assess apoptosis in cisplatin treated cells and their corresponding untreated controls. Apoptotic nuclei in a total of 500 cells were counted in triplicate, in at least three experiments. The mean percentages of apoptotic cells (SEM) are shown.

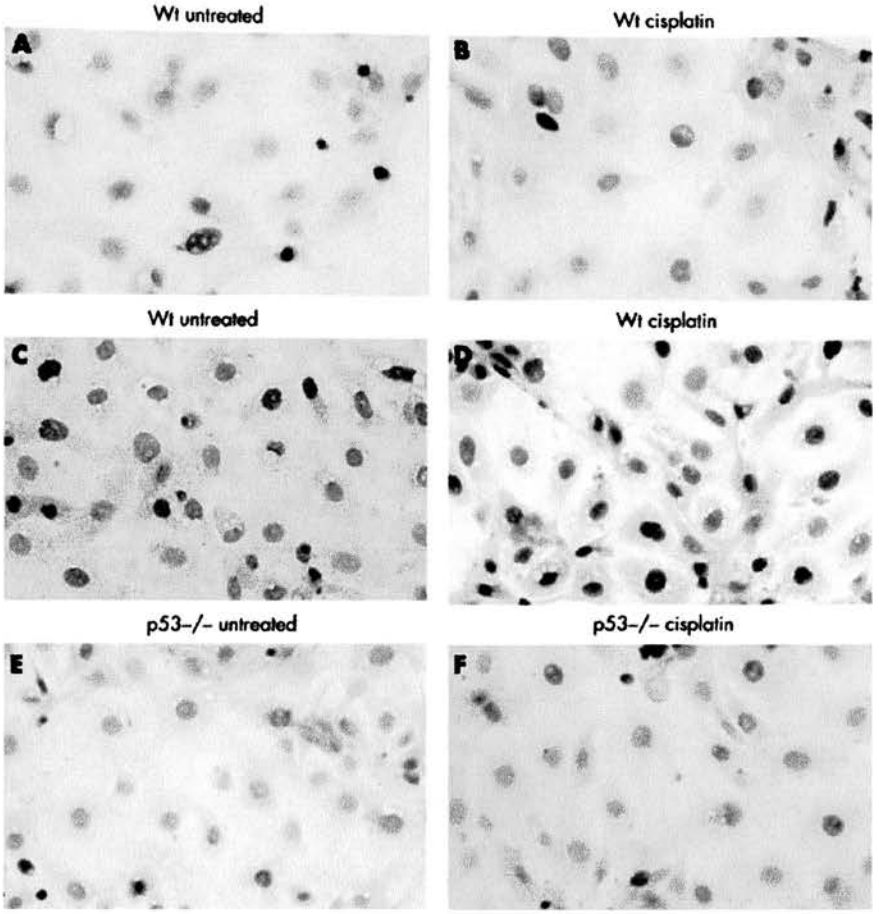

Figure 3 Expression of nuclear p53 and p21 in untreated and cisplatin treated colonocytes detected by immunocytochemistry using an avidin–biotin peroxidase technique. Positive cells were detected with diaminobenzidine and haematoxylin was used as nuclear counterstain. p53 expression is upregulated after exposure to cisplatin (A and B), whereas p21 levels do not change significantly in cisplatin treated wt cells (C and D) and p53 null cells (E and F).

Thermocycling was as follows: three minutes at 93°C, followed by 40 cycles of one minute at 93°C, one minute at 52°C (p73α exons 11–14), 64°C (p73α exons 3–4), 61°C (ΔNp73 exons 3–4), or 55°C (GAPDH), and one minute at 72°C, with a final elongation step of five minutes at 72°C. All RT-PCRs were repeated twice using different batches of RNA.

RESULTS

Confirmation of epithelial origin of colonocyte preparation by keratin immunocytochemistry and evaluation of culture purity

Cells derived from colonic crypts by the method cited were of epithelial origin. These cells were cytokeratin positive. Staining for vimentin, characteristic of fibroblasts, proved that fibroblast contamination was minimal (data not shown). Experiments were performed after three days in culture when viability and counts of apoptosis, cell cycle activity, and immunocytochemistry are optimal.

p53 controls growth in primary murine colonocytes

The proportion of cells in S phase was determined by BrdU incorporation into wt and p53 null colonocytes under baseline conditions and after six and 12 hours of exposure to cisplatin. p53 null colonocytes have a growth advantage over wt cells under baseline conditions, exhibiting a significantly higher BrdU labelling index. After cisplatin treatment, BrdU incorporation into wt cells was reduced by 12 hours ($p = 0.015$, ANOVA), whereas p53 null colonocytes continued to synthesise DNA (fig 1).

Cisplatin kills colonocytes by apoptosis irrespective of p53 status

Apoptosis in colonocyte cultures was monitored six, 12, and 24 hours after exposure to cisplatin. At all timepoints there was a significant increase in the incidence of apoptosis ($p < 0.05$, Mann-Whitney U test) between treated cells and their corresponding controls, irrespective of genotype. However, apoptosis was lower in p53 null colonocytes than in wt cells (fig 2): whereas 39% of wt cells were apoptotic 24 hours after exposure to cisplatin, only 27% of p53 null cells had died at this point ($p < 0.05$, Mann-Whitney U

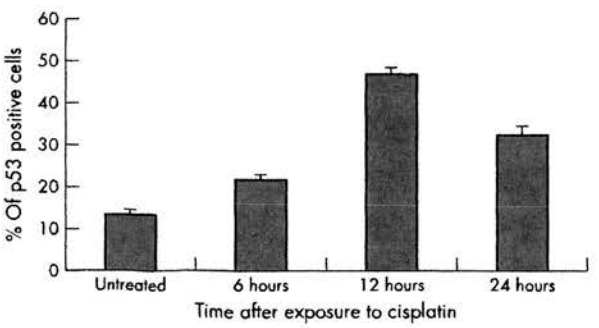

Figure 4 p53 expression in untreated and cisplatin damaged colonocytes. p53 is expressed under baseline conditions but its expression is significantly upregulated after exposure to cisplatin at all timepoints ($p<0.05$, Mann-Whitney U test). The figure shows the mean (SEM) percentage of p53 positive cells in a total of 500.

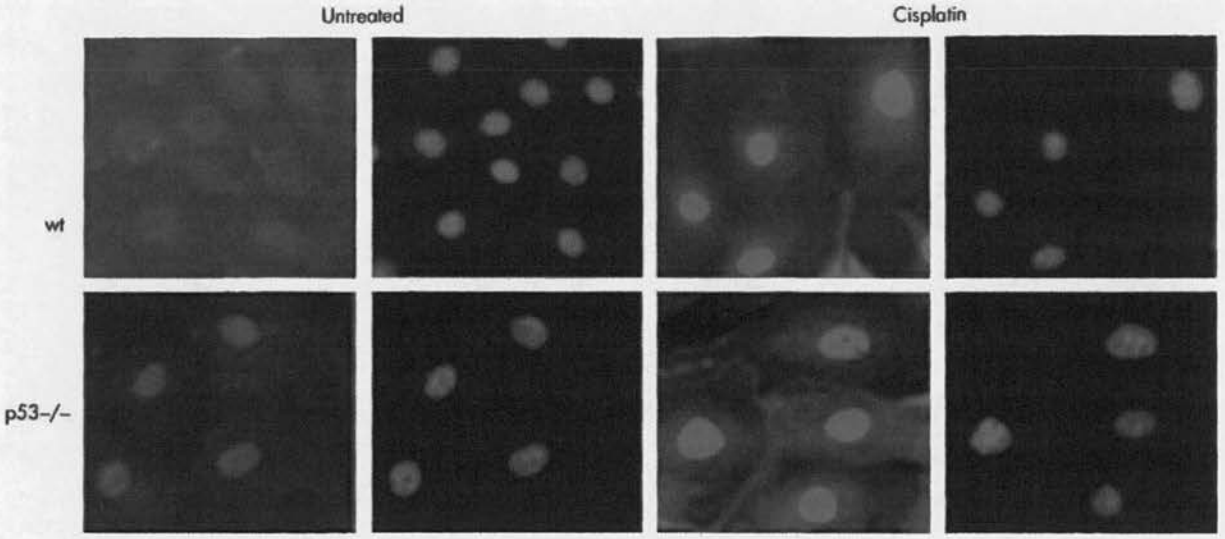

Figure 5 Immunofluorescent detection of nuclear p73 in primary colonocytes using Alexa-488 conjugated secondary antibody. Nuclear accumulation of p73 is seen after treatment with cisplatin in both wild-type (wt) and p53 null cells using the rabbit polyclonal H-79 antibody. DAPI was used as a nuclear counterstain. The cells shown were exposed to cisplatin for 24 hours. Images were captured using a Hamamatsu chilled CCD camera and Zeiss fluorescent microscope.

test). The overall degree of apoptosis was reduced at each timepoint in p53 deficient colonocytes compared with wt cells.

p53 is induced after cisplatin treatment of wt colonocytes

The proportion of cells expressing nuclear p53 was determined by immunocytochemistry on paraformaldehyde fixed, untreated and cisplatin treated colonocytes. Although nuclear p53 was detected under baseline conditions, its expression was significantly increased after exposure to cisplatin (figs 3 and 4) at all timepoints ($p < 0.05$, Mann-Whitney U test), consistent with its stabilisation and transcriptional activity.

p21 is expressed in both untreated and cisplatin treated colonocytes

We also investigated the expression of the p53 target gene, p21. High amounts of nuclear p21 were present in both wt and p53 null colonocytes under baseline conditions, with no further increase after cisplatin treatment (fig 3). Interestingly, in the absence of p53, p21 was still expressed but in significantly lower amounts at all timepoints ($p = 0.034$, Mann-Whitney U test; data not shown).

p73 is expressed in wt and p53 null colonocytes

Low baseline expression of nuclear p73 were detectable by immunofluorescence. However, the intensity of the fluorescent signal increased 24 hours after cisplatin treatment, as measured by means of Image Pro-Plus software, consistent

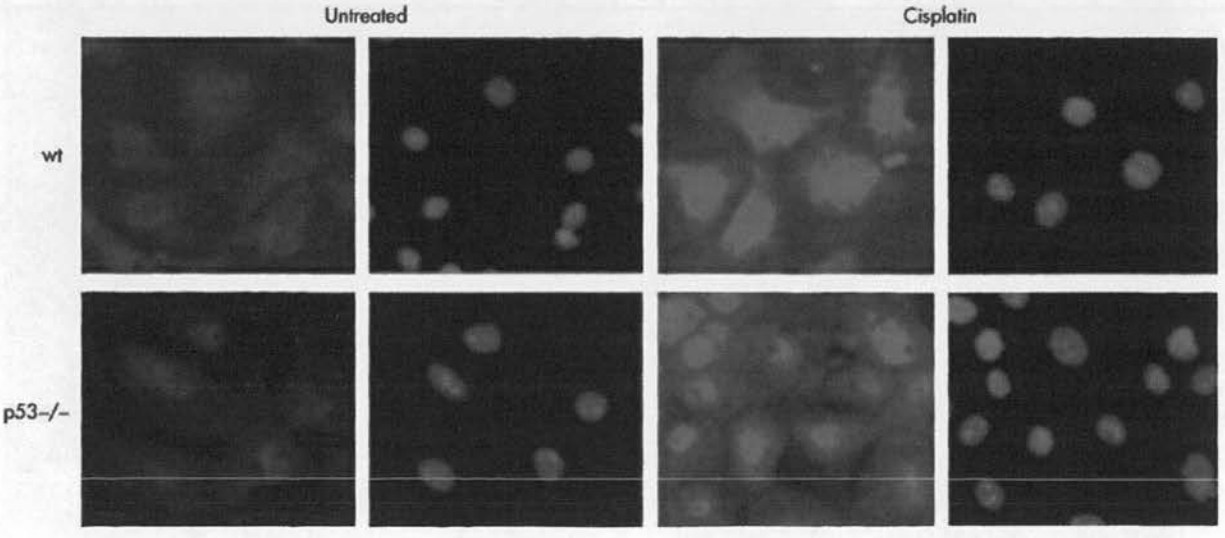

Figure 6 Immunofluorescent detection of nuclear p73 in primary colonocytes using Alexa-488 conjugated secondary antibody. Nuclear accumulation of p73 is seen after treatment with cisplatin in both wild-type (wt) and p53 null cells using the sheep polyclonal Ab77 antibody. DAPI was used as a nuclear counterstain. The cells shown were exposed to cisplatin for 24 hours. Images were captured using a Hamamatsu chilled CCD camera and Zeiss fluorescent microscope.

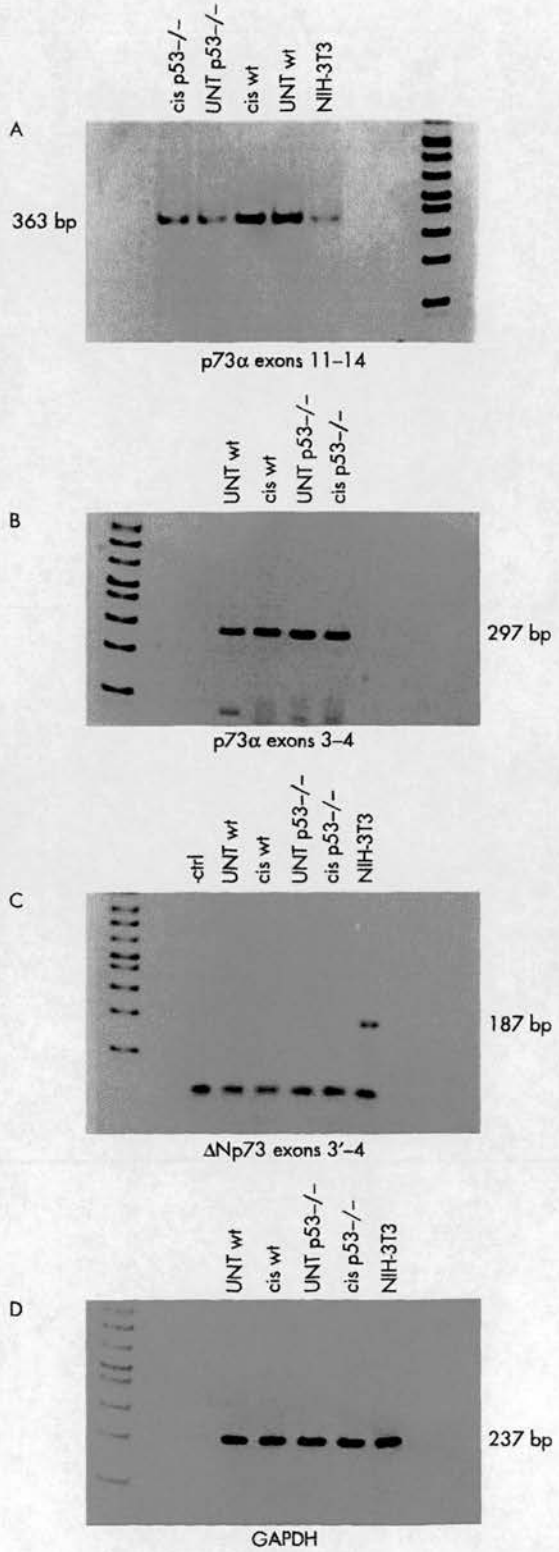

Figure 7 Reverse transcription polymerase chain reaction (RT-PCR) showing p73 expression in wild-type (wt) and p53 null untreated (UNT) and cisplatin (cis) treated colonocytes. (A) Amplification with primers spanning exons 11–14 in murine primary colonocytes. The primers could also detect the presence of the C-terminus of p73β, but no band of the predicted size was obtained. (B) RT-PCR analysis for N-terminus detection of full length p73 using primers for exon 3–4. (C) RT-PCR showing the absence of ΔN variants in primary colonocytes. NIH-3T3 cDNA proved that the primers recognise a product of the correct size. (D) The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal qualitative and semiquantitative control.

with translocation of p73 to the nucleus. Results were comparable using two different antibodies against p73. Rabbit polyclonal H-79 (fig 5) recognises the N-terminal 80 amino acids of p73, and cannot be used to discriminate between full length forms and ΔN variants of p73. Ab77 (fig 6) recognises a sequence at the extreme N-terminal 15 amino acids and therefore exclusively identifies full length p73 and should not crossreact with ΔNp73. To test whether constitutive expression of nuclear p73 is a consequence of in vitro culture or whether it reflects expression in colonic epithelium in situ, formalin fixed, paraffin wax embedded sections were subjected to immunohistochemistry with both of the anti-p73 antibodies. Staining was present in the basal/parabasal cells of murine skin epithelium,[36] but not in the colonic epithelium, indicating that p73 is not normally expressed, at least in amounts detectable by immunohistochemistry, in the murine large intestine in situ.

We also investigated the nature of p73 isoforms in primary colonocytes by RT-PCR and identified the predominant form as full length p73α, present in both wt and p53 null colonocytes with no change during cisplatin treatment (fig 7). No products corresponding to p73β, ΔNp73α, or ΔNp73β were detected.

DISCUSSION

In our study, we investigated the role of p53 in the regulation of growth and the response to cisplatin injury in freshly isolated, non-transformed primary murine colonocytes and found that whereas p53 seemed to regulate cell cycle arrest after cisplatin induced DNA damage, perhaps its role in apoptosis was less important.

Under baseline conditions, the proportion of p53 null colonocytes in S phase was significantly greater than in the wt counterparts, suggesting a role for p53 in regulating the cell cycle in vitro in primary colonocytes. The decreased BrdU incorporation after treatment with cisplatin in wt colonocytes indicates that wt cells undergo G1 arrest, whereas p53 deficient cells continue to enter the phase of DNA synthesis, despite sustaining damage to their DNA. Thus, as a first line of defence, wt colonocytes respond to cisplatin damage via a p53 dependent growth arrest.

Ultimately, many p53 null and wt cisplatin treated cells died by apoptosis. The time course of cell death was rapid, with approximately 40% of wt cells becoming apoptotic by 24 hours. However, cell death in p53 deficient cells was reduced compared with wt colonocytes. This may be analogous to the delayed p53 independent apoptosis seen in vivo after irradiation in the small intestine, and to a much lesser extent in the colon,[37] suggesting the existence of p53 independent mechanisms of eliminating damaged intestinal cells. Consistent with the proposed role for p53 in mediating cell cycle arrest and apoptosis in response to cisplatin, the expression of p53 was significantly upregulated in treated wt cells. Nonetheless, the expression of its downstream target p21 remained unchanged from the already high baseline. We might predict that these high amounts of p21 function to counteract apoptosis.[38] In fact, in the absence of p53, when baseline p21 values are 40% lower, apoptosis is reduced. Expression of nuclear p21 was high regardless of treatment, timepoint, or genotype. This could be attributed to the stress of in vitro culture, which seems to be a limitation of the primary murine colonocyte model. p53 is not normally implicated in the baseline expression of p21, yet in primary colonocytes, the proportion of cells expressing p21 under the normal conditions of in vitro culture is halved in the context of p53 deficiency. If growth in vitro is indeed stressful for colonocytes, then it is not surprising that p21 values are high to counteract this, and that perhaps p53 null cells are to a certain extent desensitised with regard to these stresses.

Take home messages

- **p53 is essential for primary colonocytes to undergo cell cycle arrest but not apoptosis after cisplatin induced damage, suggesting that p53 mediated apoptosis and growth arrest are not the only means by which colonic murine epithelial cells guard against mutation after DNA damage**
- **In the absence of p53, the failure of colonocytes to enter growth arrest probably has no longterm consequences because alternative death pathways are instigated to eliminate cells harbouring mutations**
- **Endogenous p73α was translocated to the nucleus in response to DNA damage in primary murine colonocytes, suggesting a functional proapoptotic role for p73α in p53 deficient cells**

Studies of primary colonocytes lacking p21, both p53 and p21, or p53 and p73 would yield further insight into this question.

> "After treatment with cisplatin for 24 hours, there was an increase in the amount of p73 protein in the nucleus as demonstrated by an increase in signal intensity, consistent with increased nuclear translocation of p73"

We detected constitutive expression of p73 in the nucleus of wt and p53 null cells, and increased translocation of p73 to the nucleus after exposure to cisplatin. This suggests that p73 is responsive to cisplatin induced damage of colonocytes and could contribute to the p53 independent expression of p21 and to apoptosis after DNA damage. We used RT-PCR analysis to investigate the incidence of major p73 isoforms in primary colonocytes and confirmed the presence of full length p73α in both wt and p53 null cells under baseline conditions. Amounts of the transcripts remained unchanged after exposure to cisplatin, consistent with reports in the literature that p73 stabilisation after cisplatin induced damage does not involve transcriptional upregulation.[39] Nuclear expression of p73 detected by means of immunofluorescence was demonstrated using two different antibodies raised against p73. After treatment with cisplatin for 24 hours, there was an increase in the amount of p73 protein in the nucleus as demonstrated by an increase in signal intensity, consistent with increased nuclear translocation of p73. This is in accord with the possibility that p73α cooperates with p53 in wt colonocytes, as has recently been shown in other systems,[40] and that p73α may be a p53 independent pathway of apoptosis in p53 deficient cells. Given the limited yields of colonocyte extract and the low amounts of endogenous p73, we were unable to analyse endogenous p73 by immunoblotting or immunoprecipitation. Several studies have described the transfection of p73 expression plasmids into cancer cell lines for the investigation of p73 modification and interaction with regulatory factors. However, such studies are often confounded by the diversity among cell lines or because they rely on high amounts of exogenous protein and ignore the endogenous protein. Thus, although these approaches are undoubtedly fruitful and very likely to yield useful information, we should be reminded of the power of deciphering function in a physiological setting, such as our primary colonocyte system.

The emerging evidence of the interaction of p73 with components of the mismatch repair signalling pathway,[41 42] the high incidence of mismatch repair deficiencies in colorectal cancer, the role of p73 as a corroborator of p53 function and also as an independent apoptosis inducer point to a potentially vital role for p73 in coupling damage repair and death in colonocytes.

In our study we have shown that p53 is essential for primary colonocytes to undergo cell cycle arrest but not apoptosis after cisplatin induced damage. This suggests that p53 mediated apoptosis and growth arrest are neither a major nor a unique protector of colonic murine epithelial cells against mutation after DNA damage. In the absence of p53, the failure of colonocytes to enter growth arrest probably has no longterm consequences because alternative death pathways are instigated to eliminate cells harbouring mutations. Furthermore, we demonstrated the nuclear translocation of endogenous p73α in response to DNA damage in primary murine colonocytes, which, within the context of p53 independent apoptosis, is highly suggestive of a functional proapoptotic role for p73α in these cells.

ACKNOWLEDGMENTS

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Original Paper

Expression of Sonic hedgehog pathway genes is altered in colonic neoplasia

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Abstract

The Hedgehog (Hh) signalling pathway is crucial for normal development and patterning of numerous human organs including the gut. Hh proteins are also expressed during gastric gland development and gastric epithelial differentiation in adults. Recently, dysregulation of these developmentally important genes has been implicated in cancer, leading to the present study of the expression of Hh signalling proteins in colon cancer. In this study, normal colon and colonic lesions (hyperplastic polyp, adenoma, and colonic adenocarcinoma) were examined by immunohistochemistry using antibodies against Hh signalling molecules: the secreted protein Sonic hedgehog (SHH), its receptor Patched (PTCH), and the PTCH-associated transmembrane protein Smoothened (SMOH). The study shows that Hh signalling pathway members are expressed in normal colonic epithelium. SHH was expressed at the top of the crypts and in a few basally located cells, while PTCH was detected in the neuroendocrine cells and SMOH at the brush border of superficial epithelium. RT-PCR analysis of laser-microdissected crypts from normal human colon confirmed that mRNAs encoding these proteins were expressed in colonic epithelium. Expression of SHH, PTCH, and SMOH was up-regulated in hyperplastic polyps, adenomas, and adenocarcinomas of the colon, and SHH expression correlated with increased expression of the proliferation marker Ki-67 in all lesions examined. To address whether the Hh signalling pathway is functional in the gut, the effect of Shh on epithelial cells *in vitro* was explored by treating primary murine colonocytes with either Shh peptide or neutralizing anti-Shh antibody. The proportion of cells in the S-phase was assessed by bromodeoxyuridine (BrdU) incorporation. It was found that exogenous Shh promotes cell proliferation in colonocytes, while anti-Shh inhibits proliferation, suggesting that Shh is required during proliferation of epithelial cells *in vitro*. It is suggested that SHH is required during epithelial proliferation in the colon and that there is a possible role for Hh signalling in epithelial colon tumour progression *in vivo*.

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Introduction

The *hedgehog* (*hh*) gene was first identified in a screen for genes implicated in the embryonic development of the fruit fly, *Drosophila melanogaster* [1]. *Hh* encodes a secreted protein important in regulating proliferation and establishing cell fate in *Drosophila* [2]. There are three mammalian *hedgehog* homologues, *Sonic hedgehog* (*Shh*) [2], *Indian hedgehog* (*Ihh*) [3], and *Desert hedgehog* (*Dhh*) [2], whose functional properties appear to be highly conserved between organisms. Repression of the transmembrane protein Smoothened (Smoh) [4] by the Hh receptor Patched (Ptch) [5,6] in the absence of Hh culminates in cleavage of Gli [7] to a transcriptional suppressor. When Shh binds Ptch, Smoh suppression is relieved, preventing cleavage of Gli. This leads to Gli entering the nucleus and activating transcription of Hh target genes [8–10]. Ptch also limits the action of Shh, as high levels of Ptch induced by Shh serve to sequester free Shh, therefore limiting its further diffusion [11].

In humans, the Hh signalling pathway is crucial for normal development and patterning of various organs including the brain, spinal cord, craniofacial structures, lung, teeth, eye, hair, and limbs [12]. It also plays a role in haematopoiesis [13] and in tumour formation [14].

Inactivation of the *SHH* gene has been confirmed as the cause of holoprosencephaly (OMIM ref 236 100), while overexpression of *Shh* in transgenic mice resulted in the appearance of basal cell carcinoma (BCC)-like tumours within the first few days of skin development [15]. The latter was mirrored in transgenic human skin [11]. Genetic mutations causing truncation or an unstable PTCH protein are associated with familial (Gorlin's syndrome, OMIM

ref 109400) or sporadic BCC [6]. Medulloblastoma, BCC, and meningioma are all tumours linked to Gorlin's syndrome. In addition, SMOH mutations have been identified in BCCs and neuroectodermal tumours [16]. In summary, mutations that activate the proto-oncogene SMOH or inactivate the tumour suppressor PTCH may lead to activation of the signalling pathway in the absence of Hh, allowing inappropriate transcription of target genes, a factor that may be important in the pathogenesis of malignant disease.

Shh is crucial for gastrointestinal tract development. Homozygous *Shh* knockout ($Shh^{-/-}$) mice show oesophageal atresia/stenosis, tracheoesophageal fistula, and tracheal and lung anomalies, features similar to those observed in humans with foregut defects [17]. Inactivation of the *Shh* gene in mice leads to annular pancreas (OMIM ref 167750) [18], and gut malrotations and imperforate anus (OMIM ref 301800), often associated with annular pancreas in humans, are also a phenotype of $Shh^{-/-}$ mice. In addition, $Shh^{-/-}$ mice also display intestinal transformation of the stomach with overgrowth of the gastric epithelium, and duodenal stenosis caused by hyperplastic villi [18]. Recent studies have shown that *Shh* is involved in gastric gland development and gastric epithelial differentiation in adults [19]. These data led us to address whether the Hh signalling genes play a similar role in colonic epithelium.

As the Hh pathway is clearly essential for development of the gut, our aim was to examine expression of the Hh signalling molecules in normal human colon. Protein expression was also examined in a series of human colonic lesions that included hyperplastic polyps, adenomas, and adenocarcinomas. Some researchers have regarded hyperplastic polyps as precursors of colorectal carcinoma, whereas others view them to be non-neoplastic and unrelated to cancer [20]. Consequently, it was of interest to investigate these lesions as well as more advanced neoplastic tumours such as adenomas and adenocarcinomas.

Materials and methods

All of the samples used in this study were obtained from the archives of the Department of Pathology, the University of Edinburgh. We studied only anonymized tissues, in accordance with the guidance of the local ethics research committee. The investigation did not extend to examination of the individual case records. All animal experiments were carried out in compliance with UK Home Office regulations.

Immunohistochemistry

Tissue samples were selected from the archives of the Division of Pathology, the University of Edinburgh Medical School. Seven hyperplastic polyps, 20 tubulovillous adenomas of the colon, eight adenocarcinomas, and three normal colon samples (formalin-fixed, paraffin-embedded and frozen samples) were used in the study. Sections were serially cut at 3–5 μm.

For immunohistochemistry, we used the following antibodies and dilutions: goat polyclonal antibodies raised against Shh (N-19), 1:100; Ptch (G-19), 1:80; and Smoh (C-17), 1:100 (Santa Cruz Biotechnologies, Inc); and mouse monoclonal Ki-67, 1:100 (clone MIB-1) (DAKO).

Unless otherwise stated, all reagents were from DAKO. Sections were dewaxed in xylene and rehydrated through graded alcohols, followed by incubation for 20 min in 3% hydrogen peroxide to block endogenous peroxidase. A casein serum-free blocking solution specific for formalin-fixed tissues was applied for 20 min and sections were incubated overnight at 4 °C with primary antibodies. Slides were given three 5-min washes in PBS and incubated with either biotinylated rabbit anti-goat IgG (1:400) or rabbit anti-mouse IgG (1:400) secondary antibodies for 30 min before incubating for 30 min in StreptAvidin–Biotin HRP Complex. The antibody complex was visualized with diaminobenzidine. Sections were counterstained with Mayer's haematoxylin. Negative controls (absence of primary antibody) were included for each antibody and for each case analysed.

Laser capture microdissection, RNA preparation, RT-PCR, and sequencing

Eight-micrometre cryostat sections were used for laser capture microdissection. Tissue sections were fixed in 70% alcohol, stained with Mayer's haematoxylin and eosin, dehydrated in graded alcohols, cleared and dehydrated in xylene, and air-dried for 15 min. Normal colonic crypts were microdissected onto LCM caps (CellPix II, Arcturus) and RNA was prepared using an RNA isolation kit (Ambion). cDNA was synthesized from DNase I-treated (2 U; DNA free kit, Ambion) RNA using MMLV reverse transcriptase (Life Technologies) and Oligo dT_{12-18} primer (Life Technologies) in the presence of RNase inhibitor (Life Technologies). Five microlitres of cDNA was amplified in 25 μl PCRs. The primer pairs used [21,22] were *SHH*, forward 706 — 5′-GAA AGC AGA GAA CTC GGT GG-3′ and reverse 875 — 5′-GGA AAG TGA GGA AGT CGC TG-3′, yielding a 170 bp product (GENBANK accession No L38518); *PTCH*, forward 1092 — 5′-TCC CAA GCA AAT GTA CGA GCA-3′ and reverse 1238 — 5′-TGA GTG GAG TTC TGT GCG ACA C-3′, 144 bp (GENBANK accession No U59464); and *SMOH*, forward 615 — 5′-CTG GTA CGA GGA CGT GGA GG-3 and reverse 756 — 5′-AGG GTG AAG AGC GTG CAG AG-3′, 140 bp (GENBANK accession No U84401). The housekeeping gene β-microglobulin (120 bp) was used as an internal semi-quantitative and qualitative control, and cDNA isolated from H441 cells (human pneumocytes type II cell line) as a positive control. RT-PCR products were run on 6% polyacrylamide gels stained

with ethidium bromide and visualized under ultraviolet light. PCR products were sequenced using ^{33}P Thermosequenase cycle sequencing kits (Amersham) from primers used for PCR amplification.

Primary murine colonocyte cultures

Primary mouse colonocytes were used as an *in vitro* assay to address whether the Hh signalling pathway was functional in the gut. BDF-1 mice (Charles River Laboratories) were used to derive primary murine colonocytes as previously described [23]. Recombinant mouse Shh, a 180-residue N-terminal peptide (Shh-Np, R&D Systems), was reconstituted in sterile PBS with 0.3% BSA. The neutralizing mouse anti-Shh antibody (clone 5E1) and the isotype control IgG1 antibody were a gift from Dr Sarah Howie, University of Edinburgh. After 3 days in culture in high glucose, L-glutamine-containing DMEM culture medium (Invitrogen), supplemented with antibiotics [100 U/ml penicillin (Invitrogen), 30 μg/ml streptomycin (Invitrogen), and 25 μg/ml gentamycin (Sigma)], 0.25 U/ml insulin (Calbiochem), and 5 ng/ml EGF (Sigma), colonocytes were incubated for 72 h either with the addition of 2.5% serum or with no serum, in the presence of 0.25, 0.5 or 0.75 μg/ml Shh N-terminal peptide. Control cells were treated with PBS–0.3% BSA. Colonocytes were also grown in the presence of 20 or 50 μg/ml 5E1, or 20 μg/ml IgG1 isotype control antibody as previously reported [24] for 48 and 72 h.

Proliferation assay

After culturing for 48 or 72 h in the presence or absence of exogenous Shh or 5E1, primary murine colonocytes were assayed by BrdU incorporation to determine the proportion of cells in the S-phase. The study was designed having, as models, previously published methods [24–26]. Cells were incubated in the presence of BrdU for 4 h and then fixed in cold 80% ethanol overnight. The incorporated BrdU was detected by immunocytochemistry as previously described [27] using a rat anti-BrdU antibody (1 : 100 dilution) (Immunologicals Direct). Positive nuclei were counted in no less than 500 cells and the experiments were performed in triplicate.

Results

Shh pathway members are expressed in normal colon

SHH, PTCH, and SMOH stained the cytoplasm, while the proliferation marker Ki-67 stained the nuclei. Expression patterns of the proteins were consistent within each group of cases. No staining was observed when secondary detection was performed in the absence of primary antibody. Light positive staining for SHH protein was detected within the cytoplasm at the top of normal colonic crypts (Figure 1A — SHH). A few cells located basally in the crypts stained positive for SHH, as recently reported [19]. Ganglion cells of the myenteric plexus, found between the circular and longitudinal layers of the muscularis propria, expressed SHH, in a pattern •similar to that previously reported in the mouse [18]. Neuroendocrine cells located basally in the crypts expressed PTCH strongly (Figure 1A — PTCH), also consistent with recently published studies in the lung [25], whereas the rest of the epithelial cells were unstained. SMOH expression was found only at the brush border of superficial colonic epithelium (Figure 1A — SMOH). Ki-67 confirmed that proliferation in normal colon epithelium was located in the basal compartment of the crypts (Figure 1A — Ki-67). Total mRNA isolation, RT-PCR, and sequencing of PCR products (data not shown) from laser microdissected crypts confirmed that mRNAs encoding Hh signalling pathway members are expressed in the epithelium of colonic crypts (Figure 2).

SHH, PTCH, and SMOH are expressed in hyperplastic polyps

The pattern of SHH expression in hyperplastic polyps contrasts with that described in normal colon epithelium. Strong SHH staining was found at the base of the crypts and became lighter towards the top (Figure 1B — SHH). The intensity of PTCH staining was strong and present in most of the cells within the crypt, with cells located basally also displaying intense staining (Figure 1B — PTCH). The pattern of SMOH expression was largely maintained compared with normal colon, with SMOH• staining the brush border of superficial epithelium, but in addition the epithelial cells of the crypts were moderately stained (Figure 1B — SMOH). Ki-67 expression remained in the basal compartment of the crypts, although a greater number of nuclei were stained compared with normal colon (Figure 1B — Ki-67), showing that, as expected, hyperplastic polyps have a higher proliferation index than normal colonic epithelium.

Hh pathway members are also expressed in colonic adenoma and adenocarcinoma

SHH, PTCH, and SMOH all showed stronger staining than in normal colon in a large number of adenomatous (Figure 1C) and malignant crypts (Figure 1D). The crypts that stained more strongly also displayed multiple mitoses, showed a higher degree of dysplasia, and had lost most of their normal characteristics. The staining was heterogeneous as not all malignant crypts stained positive for the discussed antibodies. The cells in the lamina propria, muscularis mucosae, and stroma of invasive carcinoma remained unstained, indicating that the expression of the proteins was genuine and specific. Ki-67 confirmed dysregulated cell proliferation at the superficial surface of adenomatous glands in the adenomas examined (Figure 1C) and in

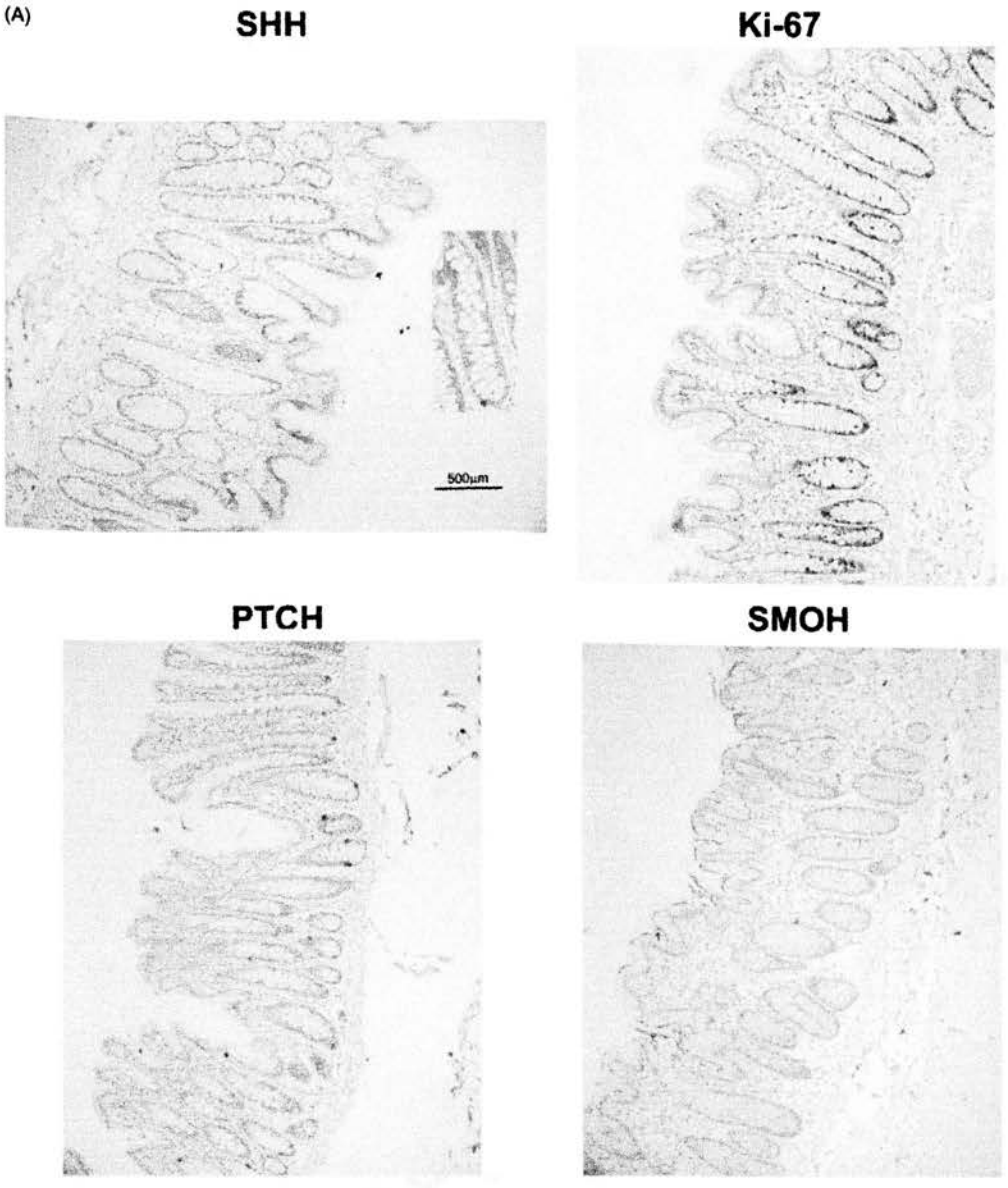

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Figure 1. Micrographs of normal colon (A), hyperplastic polyp (B), colonic adenoma (C), and colonic adenocarcinoma (D) stained with SHH, PTCH, SMOH, and Ki-67. A positive immunohistochemical reaction is indicated by diaminobenzidine (brown staining) and haematoxylin was used a nuclear counterstain. In normal colon, SHH stained lightly the superficial epithelial layer of the colon and few cells located basally; PTCH was detected in the neuroendocrine cells; and SMOH stained the brush border of the epithelium. In hyperplastic polyps, strong staining was detected for SHH at the base of the crypts, and for PTCH and SMOH, the staining was moderate and in most of the epithelial cells within the crypts. Adenomatous crypts stained strongly for all three proteins: SHH, PTCH, and SMOH. Staining was strong and heterogeneous for SHH, PTCH, and SMOH proteins in the majority of the malignant crypts of colonic adenocarcinomas. Ki-67 showed a few positive nuclei at the base of the crypts in normal colonic epithelium; an increased number of positive nuclei, but still located at the base of the crypts in hyperplastic polyps; and a high number of positive cells at the luminal surface of the adenomatous glands. Numerous neoplastic cells stained positive for Ki-67 in the adenocarcinomas examined

the majority of the malignant crypts in adenocarcinomas (Figure 1D), with a normal pattern of proliferation within the adjacent, non-neoplastic mucosa.

Exogenous soluble mouse Shh peptide has a proliferative effect on primary murine colonocytes

To investigate the possibility that Shh may promote proliferation in the gut, primary murine colonocytes were exposed to exogenous mouse Shh recombinant peptide (Shh-Np) for 72 h. The percentage of cells in the S-phase was detected by BrdU incorporation. Cells treated with 0.75 μg/ml Shh-Np showed a statistically significant increase in BrdU incorporation compared with control cells (6.5% versus 3.0%, respectively; $p = 0.0383$, Mann–Whitney U-test) (Figure 3). Cells treated with 0.75 μg/ml Shh-Np in the absence of serum showed a similar trend

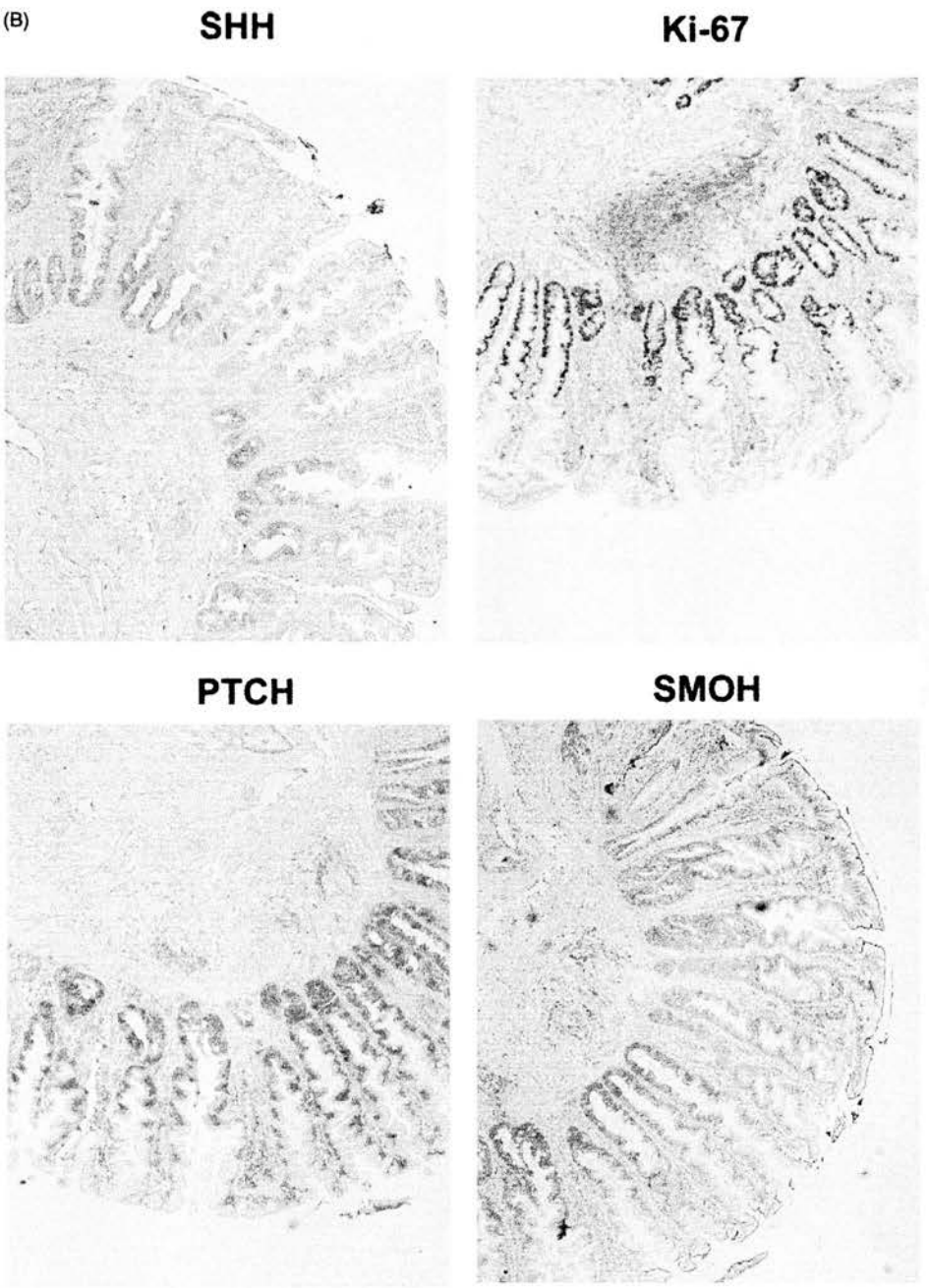

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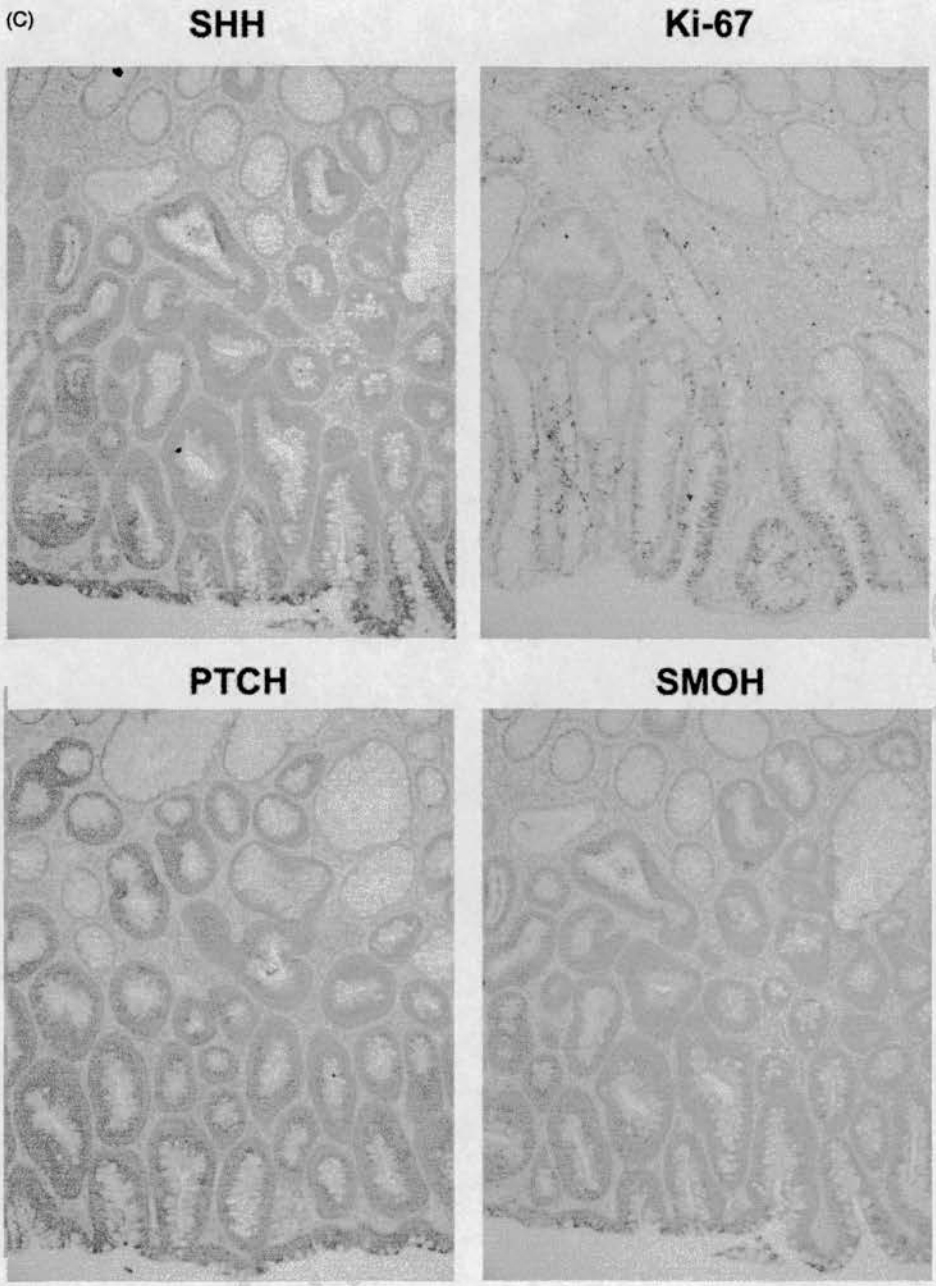
Figure 1. Continued

in BrdU incorporation compared with control cells (3.4% versus 1.6%, respectively), demonstrating that the proliferative effect of Shh-Np was not due to interaction with the serum in culture medium (Figure 4).

Blocking endogenous Shh protein with mouse neutralizing anti-Shh antibody decreases the proportion of cells in the S-phase

To determine whether the Hh signalling pathway is functional in the gut, primary mouse colonocytes were exposed to 20 or 50 μg/ml 5E1 neutralizing antibody for 48 and 72 h. Compared with untreated cells, the mean percentage of BrdU staining cells was significantly reduced after 48 h in the presence of 20 μg/ml 5E1 (5.35% versus 2.90%, respectively; $p = 0.0398$, Mann–Whitney U-test) and 50 μg/ml 5E1 (5.35% versus 2.25%, $p = 0.0147$, Mann–Whitney U-test) (Figure 5). After 72 h, a statistically significant difference was seen between untreated cells and cells cultured in the presence of 50 μg/ml (3.90% versus 1.8%, $p = 0.0152$, Mann–Whitney U-test). No inhibition of the S-phase was observed in the presence of 20 μg/ml isotype control antibody (Figure 5).



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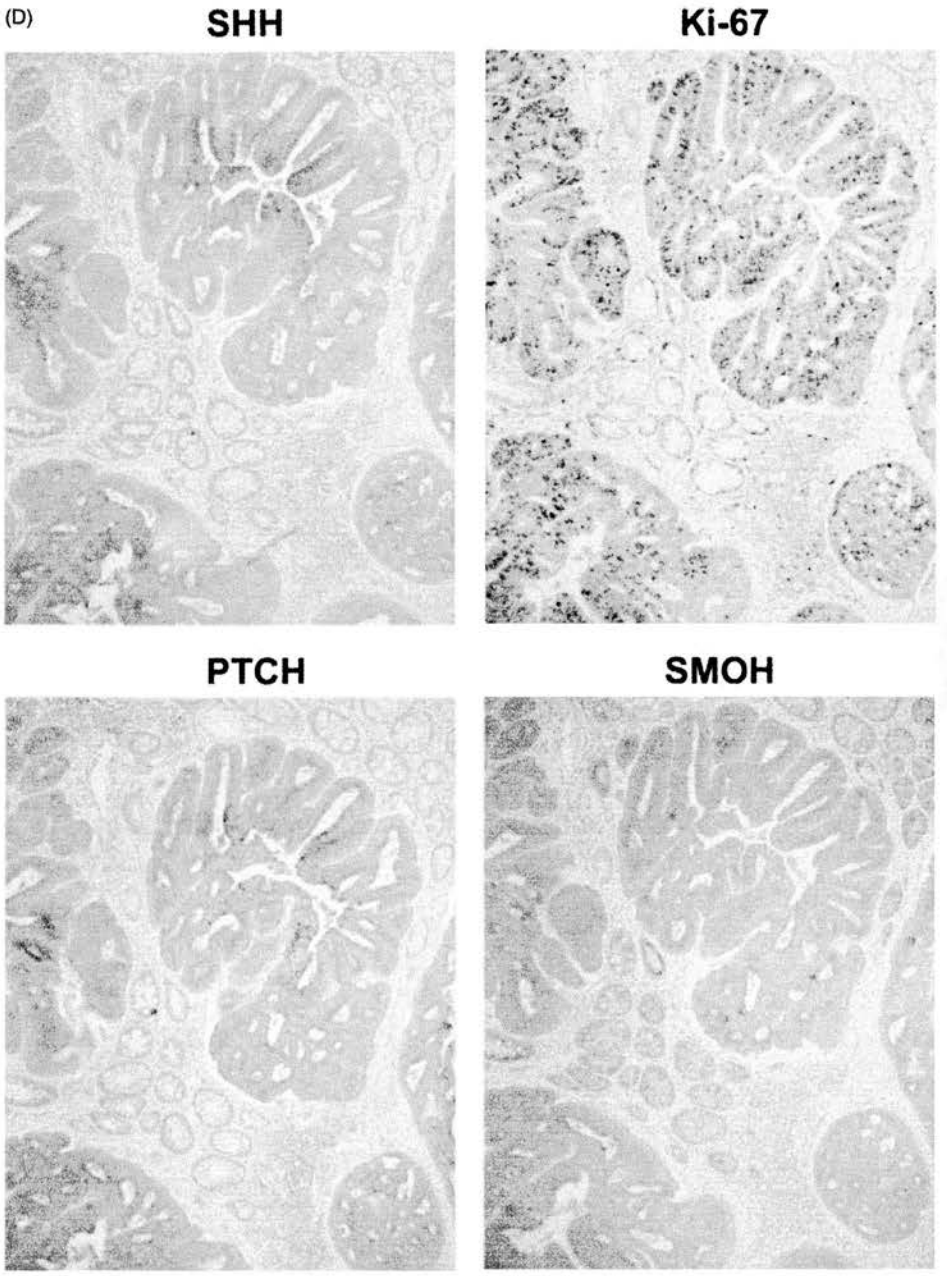
Discussion

SHH, PTCH, and SMOH are expressed in normal colon and in colorectal neoplasia. Moreover, their protein expression is similar to the distribution of their transcripts in normal human colon. In hyperplastic polyps, wild-type SHH protein expression was located at the base of the crypts where proliferation was maximal. SHH is crucial for growth and proliferation in the embryo and has been shown to promote proliferation when overexpressed in epithelial cells *in vitro* [28]. We found that SHH protein localized to areas of increased cellular proliferation in human hyperplastic polyps and that staining was more intense in areas of increased dysplasia in colorectal adenomas and adenocarcinomas. Similarly, expression of SHH correlated with increased expression of the proliferation marker Ki-67 in adenomatous and malignant crypts in the neoplastic lesions. PTCH and SMOH wild-type proteins were also overexpressed in malignant crypts, although not all malignant crypts stained positive for these antibodies, their expression being heterogeneous but of comparable intensity, as can be seen from the figures. A similar observation could also be reflected by our findings regarding expression of Hh pathway genes in colorectal cancer cell lines [29]. Consequently, we addressed the hypothesis that SHH plays a role in epithelial proliferation



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and possibly in epithelial colon tumour progression *in vivo*.

We wished to investigate whether Shh •influences cell proliferation in the colon using the agonist Shh peptide (Shh-Np) and neutralizing antibody (5E1) *in vitro*. The human colorectal cell lines SW480 and Colo-320 showed no response to either Shh-Np or 5E1 when analysed by flow cytometry (data not shown). Since these cell lines are transformed to allow clonogenic survival, it is not surprising that they are refractory to external stimuli. Furthermore, Watkins *et al* [25] reported that several cell lines, including one colorectal (HCT116), showed no growth reduction in response to the drug cyclopamine, which inhibits Hh signalling by binding to Smoh, suggesting that the pathway may not be active in these cell lines. We therefore chose to use primary murine colonocytes, which offer the advantage of being untransformed epithelial cells derived from normal adult tissue and which are more likely to reflect the *in vivo* situation. Our results show that exogenous Shh has a mitogenic effect on primary murine colonocytes and that uninterrupted blocking of endogenous Shh inhibits proliferation, suggesting that Shh is required for epithelial cell proliferation *in vitro*.

PTCH and SMOH are central to Hh signal transduction and are implicated in human tumourigenesis [5,6,16,30]. Basal cell carcinoma, medulloblastoma,

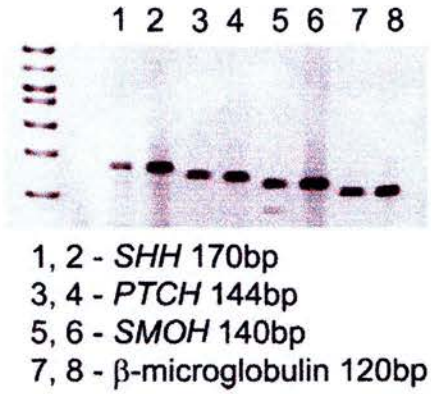

Figure 2. RT-PCR analysis for *SHH, PTCH,* and *SMOH* using mRNA from laser microdissected normal crypts (lanes 1, 3, 5, and 7) and H441 cells (lanes 2, 4, 6, and 8) used as a positive control. β 2-microglobulin primers were used as an internal qualitative and semi-quantitative control. Lanes 1 and 2: Shh — 170 bp;• lanes 3 and 4: Ptch — 144 bp; lanes 5 and 6: Smoh — 140 bp; lanes 7 and 8: β-microglobulin -120 bp

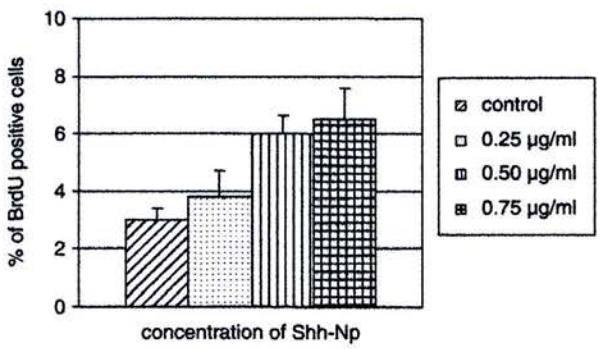

Figure 3. Graph of the percentage of cells in the S-phase detected by BrdU incorporation in primary murine colonocytes after exposure to exogenous mouse Shh-N recombinant peptide (Shh-Np) for 72 h in the presence of 2.5% serum

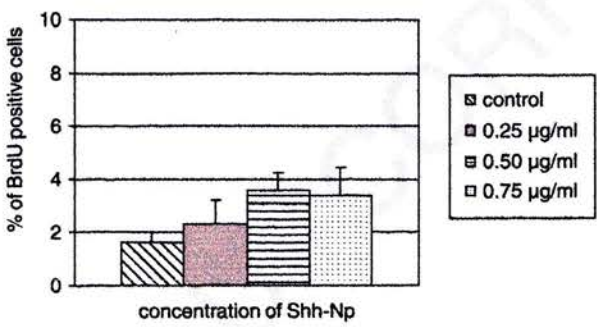

Figure 4. Graph of the percentage of cells in the S-phase detected by BrdU incorporation in primary murine colonocytes after exposure to exogenous mouse Shh-N recombinant peptide (Shh-Np) for 72 h in serum-free medium

and rhabdomyosarcoma are associated with mutations that either activate *SMOH* or inactivate *PTCH*, causing Hh-independent activation of the pathway. PTCH and

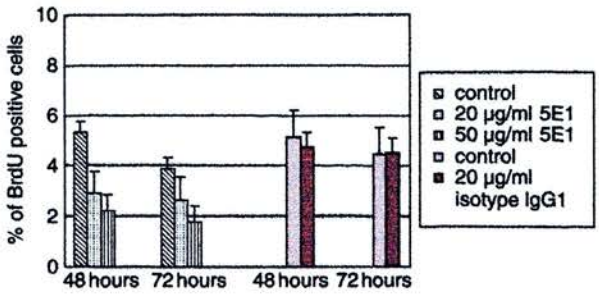

Figure 5. Graph of the percentage of cells in the S-phase detected by BrdU incorporation in primary murine colonocytes after exposure for 48 and 72 h to exogenous anti-Shh antibody (5E1) and exogenous isotype control antibody (IgG_1) for 48 and 72 h

SMOH expression was stronger where the lesions displayed a higher malignant grade. Compared with normal colonic tissue, where we detected expression only in neuroendocrine cells (for PTCH) and at the brush border (for SMOH), expression was up-regulated in adenoma and carcinoma of the colon. These findings suggest spatially aberrant Hh signalling or epigenetic activation of the genes.

Interaction between Ptch and p53 has been recently suggested. *Ptch* mutant mice on a p53-null genetic background show a marked increase in the incidence of medulloblastoma [31]. As many colorectal carcinomas have mutations or deletion of p53, an additional mutation or inactivation of *PTCH* may cause survival and continuous proliferation of cycling cells carrying damaged DNA. Such cells may give rise to tumours.

In summary, the Hh signalling pathway is an important regulator of body plan patterning, growth, and cellular proliferation during embryogenesis, and we have shown that the pathway is expressed in adult colonic tissues. Altered patterns of the expression of Hh pathway genes in colonic neoplasia suggest a role in human tumourigenesis.

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