p53 loss and *Kras* mutation in an invasive murine model of Colorectal Cancer

By

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SUMMARY

For many years there have been many excellent mouse models of benign intestinal adenoma though not of later stage invasive disease. In this study we have attempted to generate a number of murine models that closely recapitulate the human disease by manipulating mutations that occur during colorectal cancer (CRC) progression.

The *p53* tumour suppressor gene is commonly mutated in sporadic CRC, though loss alone does not drive intestinal tumorigenesis. When we targeted *p53* deletion to the adult murine intestine in concert with a single mutation in the Adenomatous Polyposis Coli (*Apc*) tumour suppressor gene, this led to an acceleration of tumorigenesis and an increase in the number of invasive tumours and more rarely metastasis. These invasive tumours closely resemble human tumours and importantly had many features of Epithelial to Mesenchymal Transition (EMT). Tumours in this background still formed at relatively long latencies suggesting other genetic events were occurring to drive the progression in the absence of p53.

Up to 50% of colon cancers have a mutation in *KRAS*. Targeting mutant $Kras^{G12D}$ to the intestinal epithelium promotes hyperplasia though not tumorigenesis. In combination with *Apc* mutation there is an acceleration of tumorigenesis and a greater propensity to develop colonic tumours. To test whether *Kras* mutation caused a more marked phenotype in the background of p53 deficiency we generated $VillinCre^{ER+}$ $Apc^{fl/+}$ $p53^{fl/fl}$ $Kras^{G12D/+}$ mice. Remarkably these mice develop invasive and rarely metastatic tumours in as little as 50 days. Moreover even small tumours (less than 1mm) could be invasive.

We believe these mouse models of invasive and metastatic intestinal adenocarcinoma will be an excellent tool to study the invasive and metastatic process *in vivo*. Moreover they should allow us to test the efficacy of drugs aimed to inhibit the invasion process.

OBJECTIVES

The main objective of this thesis was to develop and characterise new murine models of human colorectal cancer (CRC) that recapitulate the human disease in a more related fashion, in particular the spontaneous appearance of tumours and also the progression from benign adenoma to invasive carcinoma and eventually metastasis. In order to achieve this, we wanted to base our models on the three most common mutated genes in CRC: *APC*, *p53* and *KRAS*.

These murine models would help to better understand the biology of CRC, allowing us to improve the treatment and therapies of patients and hopefully contributing to the fight against cancer.

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AUTHOR'S DECLARATION

I declare that the present thesis has been composed entirely by me. The work and results discussed here have been done by me in collaboration with other members of the Sansom lab who are continuously investigating in this field.

The results from the *AhCre*$^{+}$ *Apc p53* mouse model discussed here and the figures 1,2,3 and 4 formed part of a thesis submitted for PhD to the Faculty of Medicine, University of Glasgow on May 2010 by Brendan Doyle and they have been included in this present thesis under his permission due to the importance of this model for the rest of the project.

LIST OF FIGURES

ABBREVIATIONS

ADF	Advanced DMEM/F12
APC	Adenomatous Polyposis Coli
ARF	Alternative Reading Frame
BMP	Bone Morphogenic Protein
BSA	Bovine Serum Albumin
CRC	Colorectal Cancer
Dsh	Dishevelled
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular Signal-Related Kinase
FAP	Familial Adenomatous Polyposis
GDP	Guanosine Diphosphate
GEM	Genetically Engineered Mouse
GSK3	Glycogen Synthase Kinase3
GTP	Guanosine Triphosphate
HGD	High Grade Dysplasia
IHC	Immunohistochemistry
ISC	Intestinal stem cell
LRP	Lipoprotein-Receptor-Related Protein
LOX	Lysyl Oxidase

MAPK	Mitogen-Activated Protein Kinase
MDM2	Murine Double Minute 2
MEK	Mitogen Extracellular Kinase
MIN	Multiple Intestinal Neoplasia
MMHCC	Mouse Models of Human Cancer Consortium
NGS	Normal Goat Serum
PBS	Phosphate Buffered Saline
RT	Room Temperature
TCF	T-Cell Factor

Chapter 1 INTRODUCTION

1.1 Colorectal cancer

Cancer is the leading cause of death worldwide. Among the main common types of cancers, colorectal cancer (CRC) is the third most common type in men and the second in women worldwide and the one with the fourth highest mortality rate. In the United Kingdom, there are 304,200 new cases of cancer diagnosed every year (data from 2008, Globocan), where CRC represents the forth most common type of cancer, and more frequent in women rather than men. Over the past years, the incidence of CRC cases has remained practically the same, however, there has been a slight decrease in mortality, likely due to the improvement in the early diagnosis and treatment. (Ferlay *et al*. Globocan, 2008; IARC, 2010).

The etiological factors that increase the risk and contribute to the development of this disease are very wide and complex. Many dietary factors have been attributed to increased risk of this disease, the main association seems to be the combination of a diet rich in unsaturated fats, high in red meat and low in fibre. Other potential risk factors are more related with lifestyle, such as poor physical activity and alcohol consumption. (Slattery *et al*. 2000). More firmly associated factors are medical conditions such as Crohn's disease or Ulcerative Colitis and a wide spectrum of inherited and somatic conditions such as Familial Adenomatous Polyposis (FAP), Gardner syndrome or Turcot's syndrome. Patients with these syndromes, which all have mutations in *APC*, have double the risk of having CRC in a first-degree relative (Johns *et al*. 2001; Fearon, E.R. 2011).

The molecular genetics of CRC are being intensely investigated at the moment and there are many pathways found to be involved. Grossly, a rectal neoplasia integrates a combination of mutational activation of oncogenes and inactivation of tumour-suppressor genes. Moreover, it seems to be the accumulation of these mutations rather than their chronologic order that determines the grade and characteristics of the tumour (Radtke *et al*. 2005). The three main genes implicated in malignant transformation of CRC are *APC, p53* and *KRAS*, their different roles are described in more detail below.

1.1.1 Role of APC in the Wnt signalling pathway

APC exerts its main effect on CRC through the protein β-catenin, both of them have important roles in the canonical Wnt signalling pathway. This pathway has a main role in controlling cell fate, adhesion and cell polarity during embryonic development; and the majority of the CRC are initiated by mutations and deregulations in this pathway (Behrens, J. 2005).

The Wnt pathway is first activated when a Wnt ligand binds to the seven trans-membrane receptors of the Frizzled family and low-density lipoprotein-receptor-related protein (LRP) co-receptors on the cell surface. This binding activates Dishevelled (Dsh) which prevents the cytoplasmic degradation of β-catenin, through inactivation of Glycogen Synthase Kinase3 (GSK3). β-catenin is then stabilized, accumulates in the cytoplasm, enters the nucleus and binds to the T-Cell Factor (TCF) to promote transcription of Wnt target genes (Radtke *et al*. 2005).

APC forms part of the β-catenin destruction complex, together with the GSK3 and axin/conductin proteins. When this complex is active, β-catenin is phosphorylated by GSK3 and subsequently ubiquitinated and destructed by the proteasome pathway (Aberle *et al*. 1997). In the absence of β-catenin, the transcription of Wnt target genes is therefore suppressed. The loss of APC perturbs the Wnt signalling and allows the accumulation of β-catenin within the cytoplasm and, therefore, the transcription of Wnt signalling target genes in a Wnt-independent way (Sansom *et al*. 2004). *APC* is mutated in the majority of human CRC (Powell *et al*. 1992; Miyoshi *et al*. 1992) and this mutation is generally seen as the gatekeeper in CRC, occurring early in the disease process (Vogelstein *et al*. 1988; Powell *et al*. 1992).

1.1.2 The tumour suppressor gene *p53*

P53, a protein encoded by the gene *TP53*, has been named as the “Guardian of the Genome” due to its broad role in regulating the cell cycle and conserving the stability of the genome by preventing mutations (Strachan *et al*. 1999).

In a normal healthy cell, p53 is produced continuously and is normally associated to the oncoprotein Murine Double Minute (MDM2), a potent inhibitor of p53 that acts through the binding and degradation of the protein, shortening the half-life of p53 (Haupt *et al*. 1997). However, when the cell is stressed by multiple assaults such as DNA damage, irradiation, shortened telomeres, hypoxia or oncogenic activation, p53 develops several

modifications that release it from MDM2 and this way becomes stable and activated. (Maltzman *et al*. 1984; Graeber *et al*. 1994; Junttila *et al*. 2010).

The activation of p53 leads to its translocation to the nucleus resulting in the transcription of several target genes. This can result in three different actions in the cells through three different mechanisms: activation of temporary cell cycle arrest (quiescence), induction of permanent cell cycle arrest (senescence), or triggering of programmed cell death (apoptosis), (Shaw *et al*. 1992; Diller *et al*. 1990).

Within the intestine, p53 induction has clear impact on intestinal cells e.g. induction of apoptosis (Fazeli *et al*. 1997). *p53* is mutated in a large proportion (~50%) of human CRC (Baker *et al*. 1990); however, in contrast to *APC*, *p53* mutations are not common in adenomas but more common in invasive carcinomas; this suggests that *p53* mutation is a later event in tumour progression (Baker *et al*. 1990; Vogelstein *et al*. 1988). Moreover, mutations of *p53* are usually point mutations occurring in the "hot spot" region of the protein and can exert an oncogenic effect beyond simple loss of the normal protein function (Brosh *et al*. 2009).

1.1.3 *KRAS* and the Mitogen-Activated Protein Kinase (MAPK) Signalling

MAPK signalling has an important role in the regulation of cell proliferation and differentiation. This cascade is dependent on multiple intermediates; the most important mediators are RAS and RAF proteins, the intracellular kinases Mitogen Extracellular Kinase (MEK) and Extracellular Signal-Related Kinase (ERK). RAS family consist of numerous proteins from which three of them (K-RAS, H-RAS and N-RAS) are often mutated in human cancers.

RAS is normally associated with the inactive Guanosine Diphosphate (GDP) which is dissociated by the binding of the RTK-Grb2-Sos complex formed in the cell due to different external stimuli. When RAS is freed from its union with GDP, it has affinity to bind to Guanosine Triphosphate (GTP) and activates a downstream signalling cascade from the cell membrane to the nucleus through the activation of different kinases and proteins, the main intermediates of which are RAF, MEK and ERK. (Nandan *et al*. 2011).

RAS oncogene was one of the first to be isolated from cancers and characterized (Malumbres *et al*. 2002). Its mutation causes RAS protein to be permanently bound to GTP and therefore leads to a hyper-activate state of the MAPK signalling pathway (Dhillon *et*

al. 2007). The *KRAS* oncogene is mutated in up to 50% of the colorectal tumours cases (Forrester *et al*. 1987; Bos *et al*. 1989) and *KRAS* mutations have also been detected in apparently normal mucosa in association with nearby carcinomas in some patients with CRC (Minamoto *et al*. 1995).

1.1.4 Role of SRC family kinases in CRC

Increase in SRC kinase activity has been demonstrated in colonic adenomas correlated with malignant potential and poor prognosis (Aligayer *et al*. 2002) and is, in fact, associated with induction of invasive phenotype in colonic tumour cells (Brunton *et al*. 1997). This association is related to its anatomic and biochemical properties; on epithelial cells, SRC family kinases are located at adherent junctions (Calautti *et al*. 1998) and its activity targets numerous membrane proteins, several of them related to cellular growth, survival and adhesion (Leroy *et al*. 2009). One of these proteins is the complex formed by the association of E-cadherin with β-, α- and γ-catenins, which is essential for the establishment of cell adhesions (Calautti *et al*. 1998).

SRC Family Kinases have multiple and different effects on cell adhesion, resulting in cell rounding and detachment (Fincham *et al*. 1998). One of these important mechanisms is based on the catalytic activity of the SRC Family Kinases, which has been proven to be required for the disruption of cell to cell adhesion; while the exact mechanisms are still not clear, it is certain that the phosphorylation of β-catenin by SRC kinases decreases its affinity for E-Cadherin (Owens *et al*. 1999). This fact can be the key for the translocation of β-catenin to the nucleus and its consequent transcription and can be one of the reasons for the higher levels of β-catenin at the invasive edge of the tumours.

1.2 Genetically Engineered Mouse (GEM) models of intestinal cancer

There are many factors and pathways involved in the development of CRC. Extensive research has been done using cell lines derived from colorectal cancer, but there are several factors that can only been studied on live animals. In this aspect, mouse models have a special importance and uses due to the easy genetic manipulation and their biological characteristics of breeding, among others.

1.2.1 The *Apc* mice

Several mouse models of CRC have already been developed; most of them in which the Apc protein is non-functional through the introduction of truncated mutations at different codons of the *Apc* gene. Main examples of these models are the formerly named Multiple Intestinal Neoplasia (MIN) mice (now $Apc^{Min/+}$), $Apc^{\Delta 716/+}$ or the $Apc^{1638N/+}$ mice (Moser *et al*. 1990; Oshima *et al*. 1995; Fodde *et al*. 1994). The result of the *Apc* mutations in these mice is the development of numerous polyps, in both the small and large intestines. The condition resembles human inherited conditions such as the Familial Adenomatous Polyposis (FAP), in which the colonic polyps are usually of a serrated type (sessile adenomas) that, most frequently, do not progress towards a malignant counterpart and only a little percentage of them progress towards CRC. Intestinal adenocarcinomas were only found in the in the $Apc^{1638N/+}$ model and very occasionally in the $Apc^{Min/+}$ mice; these mouse models are, therefore, not adequate for the study of spontaneous and invasive CRC, which comprise 80% of the total cases in humans (Kinzler *et al*. 1996).

Other problem of these models was that the homozygous condition was lethal (Moser *et al*. 1995). The embryological lethality and the poor resemblance with the sporadic human CRC disesase was solved with the creation of the Apc^{580S} mouse model (Shibata *et al*. 1997). In this model, a pair of *loxP* sites are introduced in introns 13 and 14 of the *Apc* gene, the recombination by a *Cre* recombinase induces the deletion of the exon 14 leading to a framshift mutation at codon 580 that results in a non-functional protein. 80 % of the heterozygous Apc^{580S} mice (named from now on as Apc^{fl} mice) developed colorectal adenomas over 3 months after induction. The homozygous condition is not embryologically lethal and 83% of the mice developed colorectal adenomas 4 weeks after induction. This new mouse model allows better study of the spontaneously occurring CRC disease and, through the *Cre-Lox* technology, allowed the mutation to be targeted to specific regions within the intestinal tract (Marjou *et al*. 2004).

1.2.2 Combination of *Apc* and *p53* in a mouse model of CRC

Due to their implication in colorectal cancer development, the combination of these two specific mutations are the key to develop a murine model of human CRC and more specifically its progression to carcinoma and metastasis.

Loss of *p53* is achieved using the insertion of *loxP* sites in the introns 1 and 10 of the *Trp53* gene, deletions of exons 2-10 with *Cre* recombinase results in a null allele (Jonkers *et al*, 2001). The mutant $p53^{R172H}$, a point mutation commonly found in spontaneous

human tumours is created by using *loxP* flanked transcritional STOP cassette into intron 1 of the *p53* gene, the deletion of the STOP cassette using *Cre* recombinase results in the expression of the mutant R172H (Olive *et al*, 2004).

Previous studies have found only little effect of *p53* loss with Apc^{Min} or acute *Apc* loss at early intestinal tumorigenesis *in vivo* (Fazeli *et al*. 1997; Reed *et al*. 2008; Halberg *et al*. 2000). *P53* loss had no effect on crypt size, proliferation or apoptosis following *Apc* deletion, which reflects the fact that p53 expression is only increased in a small number of cells (Reed *et al*. 2008).

1.2.3 Combination of *Apc, p53* and *Kras* in a mouse model of CRC

Several studies have characterized the effect of mutant *Kras* in the colonic epithelium. While early studies with exogenous mutant showed *Kras* to induce growth arrest in primary cells unless accompanied by cooperating oncogenes or coincident loss of functional genes as *p53* (Kamijo *et al*. 1997, Serrano *et al*. 1997), later studies with endogenous mutant in fact demonstrated its contribution to the development of CRC.

Different types of mutations have been studied in order to determine the main mutation that is associated with human cancer. While $Kras^{V12}$ activation does not alter intestinal homeostasis, it neither alters the architecture of the crypt-villus axis and has no or little additional changes after *Apc* loss in early stages of intestinal neoplasia but accelerates tumorigenesis and causes invasion in a later onset (Sansom *et al*. 2006).

In contrast, activation of $Kras^{G12D}$ affects proliferation and differentiation of crypts, promoting hyperplasia in the colonic epithelium by increasing the number of proliferative progenitor cells in the tissue through activation of MEK and ERK pathways, although itself does not cause neoplasia (Haigis *et al*. 2008).

When combined with a mutation in the *Apc* tumour suppressor gene, however, $Kras^{G12D}$ is able to promote tumour progression with a higher dysplastic grade than in those tumours formed with *Apc* loss alone (Haigis *et al*. 2008), accelerating the process of adenoma formation and reducing survival (Luo *et al*. 2011).

Taking these studies into account, Kras mutation is regarded as an important key in the malignant transformation of colonic cancer cells and therefore its study and characterization is needed to be fully understood, even more following *APC* and *p53* mutations; in fact, combination of the three mutations *APC*, *p53* and *KRAS* in cancer samples from human patients have been found in 30-50 % of samples (Wang *et al*. 2007)

and the cooperation of *Kras*^{G12D} and mutant *p53* to initiate and develop oral tumour formation has also been shown (Acin *et al*. 2011).

1.2.4 Other mouse models of CRC: *Myc* and *Arf* mice

c-MYC is a downstream Wnt target that controls proliferation, differentiation and transformation in the adult intestine (Bettess *et al*. 2005). Several studies have determined that approximately 70% of the human colonic tumours have up-regulated *c-myc* expression (Augenlicht *et al*. 1997). Following the fact that wild-type APC has a repressor effect over MYC in physiologic circumstances (He *et al*. 1998), it was demonstrated that after *Apc* deletion, there is a dramatic increase in the levels of MYC in the intestinal epithelium and there is a marked proliferation of cells with elongation of crypts (Athineos *et al*. 2010). Furthermore, recent studies have demonstrated the importance of MYC in an *Apc* deficient background demonstrating that complete deletion of *Myc* totally rescues the *Apc*-driven phenotype and that even a partial deletion (*Myc* heterozygosity) is able to reduce the proliferation of crypts up to 50% (Sansom *et al*. 2007; Athineos *et al*, 2010) when APC is lost.

p19^{ARF} (p14^{ARF} in humans) is a protein encoded by the *CDKN2A/INK4A* locus. This locus has two transcripts that produce two protiens, *p16*^{INK4a} and the Alternative Reading Frame (*ARF*) (Quelle *et al*. 1995). The ARF protein is not detectable in normal cells, however, its levels increase after oncogenic or hyperproliferative signals leading to activation of p53 through the nucleolar localization of MDM2 (Weber *et al*. 1999). The tumour suppressor role of ARF, independent of *p16*^{INK4a}, was confirmed by Kamijo *et al*. in a murine model in which mice lacking *p19*^{ARF} developed tumours early in life (Kamijo *et al*. 1997). However, recent lines of research suggest a role of *ARF* in tumour promotion, although the mechanisms are still not well characterized. Humbey *et al*. demonstrated, in a mouse model of B-cell lymphoma with a mutant or null p53, that silencing of ARF limited the progression of the tumour (Humbey *et al*. 2008). Humbey *et al*. associated this finding with the role of *ARF* in increasing autophagy, a process that is necessary for cell survival. On the other hand, a recent study showed that expression of ARF induces the formation of a Myc/Miz1 complex that leads to the repression of multiple genes involved in cell adhesion and induces apoptosis (Herkert *et al*. 2010). Moreover, in this study they demonstrated that when apoptosis was inhibited the loss of cell-cell adhesion persisted, giving to *ARF* a possible role in tumour progression through an invasive phenotype.

1.3 Treatment of Colorectal Cancer

Treatment of metastatic CRC continues to have limited efficacy in patients, and a large number of drugs aimed to treat the disease have resulted in toxicity or harming of the patient. Due to the multifactorial aspect of cancer, a personalized therapy would be the most effective method to treat the disease. Therefore, knowing the mutations and pathways involved can assist in the endeavour to the identify more effective therapy or even the correct combination of drugs.

Numerous drugs have been developed and tested for specific targets in the process of CRC development and metastasis. Current approaches are very different, some of them are oriented towards targets to activate autoimmunity against colorectal tumoral cells (Chou *et al*. 2012), but the majority are focused on key proteins involved in cancer pathways, such as epidermal growth factor receptor (EGFR) inhibitors or SRC Family Kinases. EGFR inhibitors such as Cetuximab and Panitumumab have been studied in cases of metastatic CRC (Ballestrero *et al*. 2012), however, these inhibitors are not effective when a *Kras* mutation is present (Karapetis *et al*. 2008, Hecht *et al*. 2009).

1.4 Thesis aims

Considering the background information displayed in the introduccion, this thesis presents the work performed in Colorectal cancer research focusing on the development of a murine model that allows the study of this human disease in order to prevent and reduce the incidence of invasive and malignant transformation. Therefore, the main objectives of this work have been as follow:

- The development and phenotypic characterization of murine models with mutations in *Apc*, *p53*, *Kras*, *Myc* and *Arf*; as well as the study of the interactions between these mutations when present concurrently in the same animal model.

- The characterization of the effects of SRC family kinases on the progression to an invasive phenotype of the colonic carcinoma and the preliminary study of potential inhibitors (Dasatinib) in order to develop treatment therapies.

- The development of cell lines extracted directly from tumours formed in the intestine of these genetically engineered mice able to conserve the tumoral phenotype in order to allow further *in vitro* studies.

Chapter 2 MATERIAL AND METHODS

2.1 Genetically Engineered Mouse (GEM) models of colorectal carcinoma

All experiments were performed in accordance with UK Home Office guidelines and local ethical approval. Mice were maintained under non-barrier conditions and fed a standard diet (Harlan) and water *ad libitum*.

Mice were examined twice per week for signs of intestinal tumours, such as paling feet, hunching and weight loss. Upon developing any 2 of these signs the mice were euthanized and underwent a full necropsy. Tumour invasion was assessed by histology.

2.1.1 *AhCre*$^{+}$ cohorts

For the creation of the *AhCre*$^{+}$ cohorts, the following alleles were used: *Apc*^{fl} (Shibata *et al.* 1997), *p53*^{fl} (Jonkers *et al.* 2001) and *p53*^{R172H} (Olive *et al,* 2004). Activation of the relevant allele was induced using the inducible *AhCre* recombinase in which the plasmid p*Ahcre* is inserted in the pronuclei of C57Bl/6 zygotes of mice, *Cre*-mediated recombination is obtained in several epithelial tissues, mainly liver and small intestine. *AhCre* expression activates specific genes and it is induced using 3 intra-peritoneal injections of 80 mg/kg β-naphthoflavone (Sigma, Dorset, UK), separated by 8 hours at ~6 weeks of age, as has been described previously (Ireland *et al*. 2004). C57Bl/6 mice were used for all the *AhCre*$^{+}$ cohorts.

The experimental cohorts were generated by crossing *AhCre*$^{+}$ *Apc*$^{fl/+}$ mice with either *p53*$^{fl/+}$ or *p53*$^{R172H/+}$ mice. The progeny were then interbred to yield the cohorts of *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{+/+}$; *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/+}$; *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$; *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/+}$ and *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/fl}$.

2.1.2 *VillinCre*$^{ER+}$ cohorts

For the creation of *VillinCre*$^{+}$ cohorts, the following alleles were used: *Apc*^{fl} (Shibata *et al.* 1997), *p53*^{fl} (Jonkers *et al.* 2001), *LSL-Kras*$^{G12D/+}$ (Tuveson *et al,* 2004), *Myc*^{fl} (de Alboran *et al,* 2001), and *p19*$^{ARF+/-}$ (Kamijo *et al,* 1997). Activation of the relevant allele was

induced using the inducible *VillinCre* recombinase, in which transgenes carrying the plasmid *VillinCre*^{ER} were injected into the pronuclei of oocytes from C57Bl/6 mice (Marjou *et al*, 2004). *VillinCre*^{ER} expression activates the specific gene and it is induced using a single intra-peritoneal injection of 80mg/kg of tamoxifen (Sigma) at ~60 days of age. C57Bl/6 mice were used for all the *VillinCre*$^{+}$ cohorts.

p53$^{fl/+}$ mice were mated to *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ mice. The progeny was then interbred to yield the cohorts of *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{+/+}$; *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/+}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$.

Conditional *LSL-Kras*$^{G12D/+}$ mice were mated to *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/+}$ mice and the progeny from these crosses was interbred to obtain the cohorts *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$; *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$.

Myc colonies were created by mating *Myc*$^{fl/+}$ mice with *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *LSL-Kras*$^{G12D/+}$ to obtain the following cohorts: *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{+/+}$; *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{fl/+}$; *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *LSL-Kras*$^{G12D/+}$ *Myc*$^{+/+}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *LSL-Kras*$^{G12D/+}$ *Myc*$^{fl/+}$.

Arf colonies were created by mating *p19*$^{ARF+/-}$ mice with *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *LSL-Kras*$^{G12D/+}$ to yield the following cohorts: *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *LSL-Kras*$^{G12D/+}$ *Arf* $^{+/+}$; *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *LSL-Kras*$^{G12D/+}$ *Arf* $^{+/-}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *LSL-Kras*$^{G12D/+}$ *Arf* $^{-/-}$.

2.2 Genotyping of mice

The genotyping of the mice was performed externally by Transnetyx (Cordava, TN).

2.3 Dasatinib treatment

For the Dasatinib experiment, mice from the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *LSL-Kras*$^{G12D/+}$ colony were induced with a single intra-peritoneal injection of tamoxifen (8mg/kg) and were dosed daily 60 days after induction by oral gavage with 10mg/kg Dasatinib (Bristol-Myers Squibb, Princeton, NJ) in 80mmol/L citrate buffer. Untreated controls were induced similarly with a single intra-peritoneal injection of tamoxifen (8mg/kg). A lower dose of tamoxifen (8mg/kg) was used in this dasatinib experiment to slow the development of tumours and therefore obtained better time frame to assess the treatment since the mice induced wih the 80mh/kg dose became ill too abruptly as to be able to treat them

effectively. The mice were monitored regularly and upon developing signs of intestinal tumours were euthanized and underwent a full necropsy.

2.4 Tissue sampling

For the tumorigenesis study, the entire intestine was removed and flushed with water. Both small intestine and colon were mounted 'en face' and fixed overnight in either methacarn (60% methanol, 30% chloroform and 10% glacial acetic acid) or 10% neutral buffered formalin. Lesions were then scored macroscopically and the small intestine and colon were then wound into a roll in 10% neutral buffered formalin.

For the metastatic study, lungs, liver, spleen and lymph nodes were collected and fixed in 10% neutral buffered formalin. All the samples, intestine and organs, were incubated overnight in formalin (long fixation) and subsequently embedded in paraffin, sectioned at 5-10μm and stained with haematoxylin and eosin prior to microscopic analysis.

2.5 Histology

Samples were observed under a light microscope (Olympus BX51). Intestinal neoplasms were characterized and classified according to the human clasiffication of malignant tumours or TNM staging (Edge, S.B., et al. 2010) as follow: non-invasive when the neoplasia was restricted to the mucosa; superficially invasive (T1-T2) when the neoplasia invaded the submucosa and/or infiltrated superficially (less than half of its thickness) the muscularis propria; and deeply invasive (T3) when the neoplasia invaded the full thickness of the muscularis propria. Whenever tumours invaded through the surface of peritoneal organs this was classified as T4. Organs were checked and metastasis, if found, were characterized and classified as metastasis in either regional lymph nodes (N1) or in distant organs (M1).

This classification of tumours in the mice, according with the human classification, was assessed and stablished by the student helped and advised by Dr. Ayala King, Research Fellow at the Beatson Institute for Cancer Research specialising in mouse pathology.

2.6 Immunohistochemistry (IHC)

2.6.1 $p19^{ARF}$

Sections were de-waxed by immersion in xylene and hydrated by serial immersion in 100% ethanol (2 × 1min), 95% ethanol (2×1min), 70% ethanol (2×1min) and distilled

water (2×1min). Antigen retrieval was performed by boiling the slides immersed in a pre-heated Citrate buffer (Thermo scientific) diluted 1/10 with distilled water in a water bath for 20 minutes and then cooling in the solution for 30 minutes at room temperature (RT). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in de-ionized water; the slides were then blocked with 10% normal goat serum (NGS) for 30 minutes at RT. Primary anti-p19^{ARF} antibody (Abcam, ab80 rabbit polyclonal) was applied overnight at 4^{o}C in a concentration of 1/300 in 10% NGS. Secondary anti-rabbit antibody (Vector ABC Kit) was applied the following day for 30 minutes at a concentration of 1/200 in 10% NGS. After washing, signal amplification was performed using the ABC Complex (Vector ABC Kit), applied for 30 minutes. After washing positivity was visualised with DAB and slides were counterstained haematoxylin, prior to being dehydrated in increasing concentrations of ethanol and mounted.

2.6.2 pSrc^{Tyr416}

Sections were de-waxed as described above and antigen retrieval was performed with the same technique using Ethylenediaminetetraacetic acid (EDTA, Thermo scientific) in a solution of 1/10 in distilled water. Endogenous peroxidase activity was blocked as described above and slides were blocked for 30 minutes at RT with 5% normal goat serum (NGS). Primary anti-pSrc ^{Tyr416} antibody (Cell signalling, #2101 rabbit polyclonal) was applied overnight at 4^{o}C in a concentration of 1/200 in 5% NGS. Secondary anti-rabbit antibody (Vector ABC Kit) was applied the following day for 30 minutes at a concentration of 1/200 in 5% NGS. After secondary antibody wash, signal amplification, DAB positivity visualisation and mounting of the slides were performed as described previously.

2.6.3 B-catenin

Sections were de-waxed and rehydrated as described. Peroxidase block was carried out in a solution of 1.5% hydrogen peroxide for 45 minutes. Antigen retrieval was performed in pre-heated Tris EDTA in a boiling water bath for 50 minutes. Slides were then cooled for 1 hour and blocked with 1% BSA for 30 minutes. Slides were incubated with primary mouse anti-β-catenin antibody (Transduction Laboratories, C19220, mouse monoclonal) in a concentration of 1/50 in 1% BSA for 2 hours at RT and then with HRP-labelled polymer (Mouse Envision+ system, Dako) for 1 hour at RT. After washing, positivity was visualised with DAB and slides mounted as described above.

2.7 Cell culture

Cells were extracted from tumours and crypts following the method of primary cell culture described by Sato and Clevers in 2009 (Sato, T., et al. 2009). Cells from murine intestine were purified and cultured in a matrigel 3D culturing system to support its laminin-based growth. Culture media was made up of ADF with 0.25% BSA, 0.01 % L-Glutamine, 0.01 % HEPES and 0.01 % PenStrep (Gibco®). Growth factors were added every other day and entire media was exchanged after 7 days or before if required. Crypts were passaged after 7-10 days by mechanical disruption with a P200 pipette.

Cells were fed every other day with two main growth factors for the intestine, Epidermal Growth Factor (EGF, Prepotech) and Noggin (Prepotech). The receptor for EGF (EGFR) is widely detected in colon cancer cells and its binding strongly stimulates proliferation of the epithelial cancer cells (Dignass *et al*. 2001). Noggin, an antagonist to bone morphogenic protein (BMP) singnaling, is associated with formation of numerous new crypts units, therefore expansion in the crypt number. Due to the *Apc* mutation of these cell lines, there was no need to use R-spondin 1, an APC agonist.

2.7.1 Crypt culture

Mice were culled 3 days after induction with tamoxifen and their small intestine was flushed with cold Phosphate Buffered Saline (PBS) and longitudinally opened. Villi were scraped off using a glass coverslip. The small intestine was cut in small pieces (5mm) and washed around 8 to 10 times with cold PBS and left for 30 minutes at 4° C in a solution with 25 ml of 2mM EDTA in PBS. Crypts were then washed 4 times with cold PBS and the supernatant from the 2^{nd}-4^{th} washes was collected. Crypt fractions were combined and washed with 50 ml of Advanced DMEM/F12 (ADF, GIBCO®) and centrifuged at 1200 rpm during 5 minutes to be collected after that and passed through a 70 µm cell strainer. Crypts were then washed with ADF and centrifuged at 800 rpm for 3 minutes other 3 times to be collected and re-suspended in Growth Factor reduced Matrigel (BD Bioscience) in a 24 well plate. 500 µl of culture media, 25ng of EGF (Prepotech) and 50ng of Noggin, an inhibitor of BMP signaling (Prepotech) were added to each well.

2.7.2 Tumour culture

Mice were culled by a Schedule 1 method upon developing signs of hunching and weight loss; the intestine was flushed with cold PBS and tumours were removed, cut in little pieces and washed 2-3 times in cold PBS to be incubated for 10 min in 5mM EDTA at RT

with vigorous shaking inbetween. After removing the EDTA with 2 additional washes with PBS, adenomas were incubated in 4.5ml 10x Trypsin 2.5% (Gibco®) with 100µl 10x DNase buffer and 100U DNase (Roche) for 30 minutes at 37°C. Tumours were then further dissociated mechanically by pipetting with P1000 and 5ml pipettes and collected in 50ml of ADF after being passed through a 70µm cell strainer. Cells were then centrifuged at 1200 rpm for 5 minutes and washed with ADF twice to plate in Matrigel as described above.

The medium and growth factors used were the same as described for crypt culture. Passage was performed after 7-10 days with the same technique.

Tumours collected for cell culture were only assessed grossly at necropsy, which impairs their classification as adenomas or adenocarcinomas if no obvious invasion was seen grossly.

2.8 Subcutaneous tumour growth in mice

Tumorigenic spheres maintained in culture were taken by mechanical disruption with P200 pipette, washed with ADF and centrifuged at 800 rpm for 3 min. Cells were then re-suspended in 150µl of matrigel and injected subcutaneously in the flank of immunodeficient CD1-nude mice. The number of spheres injected per mouse was ~50.

Mice were monitored three times a week and tumours were measured from 6mm size. When the tumours became ulcerated or bigger than 15mm the mice were culled and the neoplastic mass was isolated and collected in 10 % neutral buffered formalin for histology processing as described. In order to check for possible metastasis, liver, spleen and lungs were also collected.

To characterize the *in-vitro* growing of cells after the allografts, a small piece of the tumour was collected in cold PBS and cultured as described for adenoma culture.

Chapter 3 RESULTS

3.1 *The murine model of combined mutation of Apc and p53 drives intestinal tumorigenenesis*

Previous studies failed to reveal the tumorigenic properties of *p53* loss with deletion of *Apc in vivo*, in these studies p53 had no effect on crypt size, proliferation or apoptosis (Fazeli *et al*, 1997; Reed *et al*, 2008; Halberg *et al*, 2000). All these experiments were performed with rapid models of *APC* such as Apc^{Min}, we believe the use of Apc^{fl} mouse model could overcome this given the longer latency of tumour development in these mice. Having this longer latency and the formation of occasional invasive carcinomas with this model, it is possible to more accurately model the human disease and better understand the transforming properties of *p53* mutations in APC-mediated tumourigenesis.

3.1.1 Loss of a single copy or both *p53* alleles in an *Apc* deficient background accelerates tumorigenesis

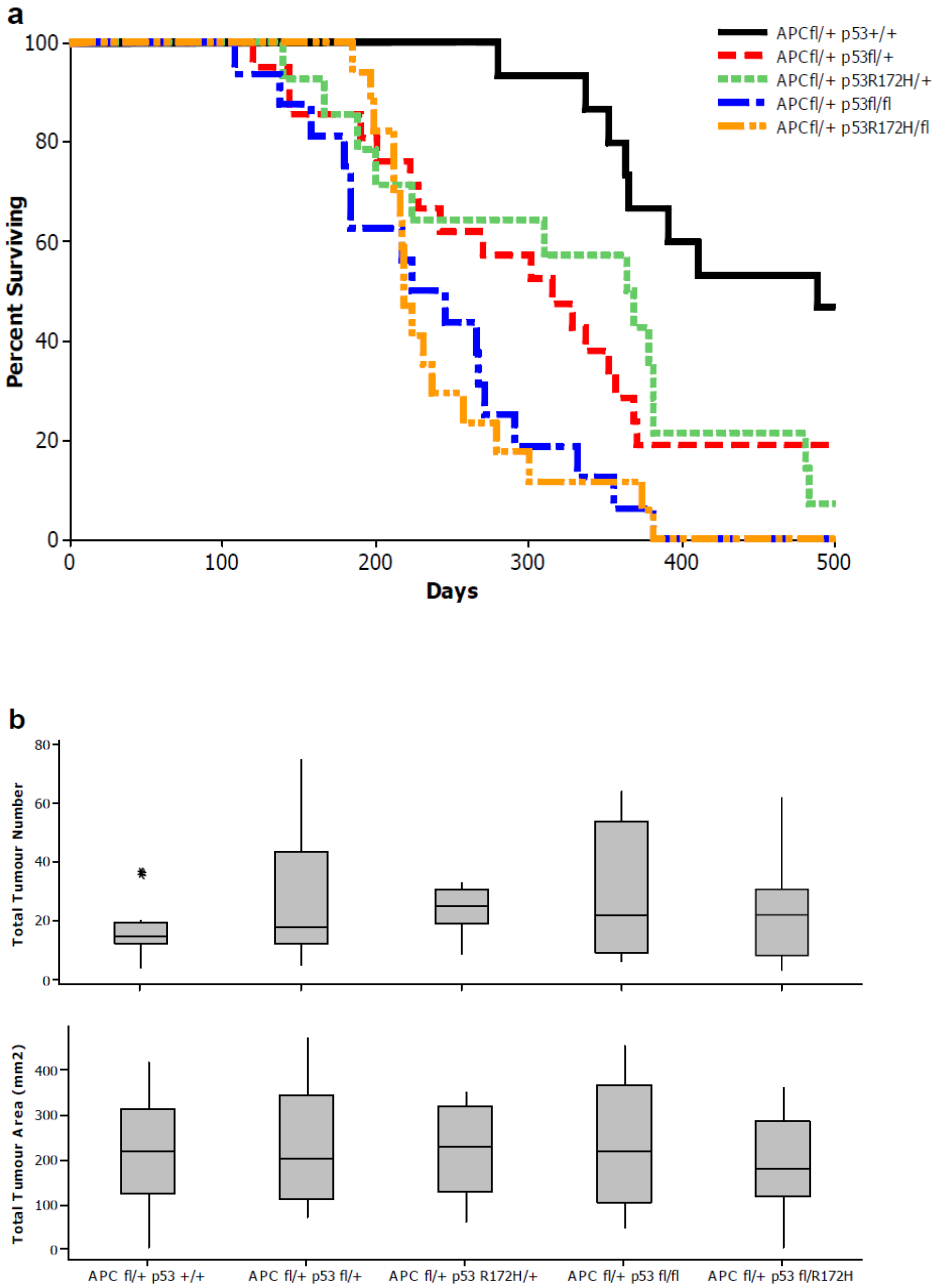
It has been previously shown that deletion of one copy of *Apc* in the adult intestine using the inducible *AhCre* recombinase results in mice developing tumours with long latencies, and which will convert into adenocarcinomas in approximately 25% of mice (Athineos *et al*. 2010).

When a single *p53* allele was lost or mutated ($AhCre^{+}$ $Apc^{fl/+}$ $p53^{fl/+}$ and $AhCre^{+}Apc^{fl/+}$ $p53^{R172H/+}$ cohorts) on this background, there was a significant acceleration in the tumorigenesis compared to parental controls ($AhCre^{+}$ $Apc^{fl/+}$ $p53^{+/+}$) (Log-Rank p=0.008). Deletion of the two copies of *p53* ($AhCre^{+}$ $Apc^{fl/+}$ $p53^{fl/fl}$) or mutation of one of the copies with deletion of the second one ($AhCre^{+}$ $Apc^{fl/+}$ $p53^{R172H/fl}$) led to a remarkable acceleration in tumorigenesis comparing to either the control and heterozygous group (Log-Rank p<0.001). In fact, the median tumour onset was reduced to more than half in $AhCre^{+}$ $Apc^{fl/+}$ $p53^{fl/fl}$ and $AhCre^{+}$ $Apc^{fl/+}$ $p53^{R172H/fl}$ mice compared to controls ($AhCre^{+}$ $Apc^{fl/+}$ $p53^{+/+}$). The survival was no different between the $AhCre^{+}$ $Apc^{fl/+}$ $p53^{fl/+}$ and $AhCre^{+}$ $Apc^{fl/+}$ $p53^{R172H/+}$ cohorts or between the $AhCre^{+}$ $Apc^{fl/+}$ $p53^{fl/fl}$ *and* $AhCre^{+}$ $Apc^{fl/+}$ $p53^{R172H/fl}$ cohorts. There was neither difference in tumour size or number among the different groups, indicating that all the mice were at a similar stage of tumour development when euthanized (Figure 1).

Figure 1: *Apc* driven tumourigenesis is accelerated by *p53* deletion/mutation.

a) Kaplan-Meier survival analysis. There was a significant difference in tumour-free survival between the *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{+/+}$ cohort (n=15) and both *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/+}$ (n=20) and *Apc*$^{fl/+}$ *p53*$^{R172H/+}$ (n=14) cohorts (Log-Rank p=0.008). There was a further increased acceleration in tumorigenesis between *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ (n=15) and the *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/fl}$ (n=18) cohorts and the heterozygote or wild type p53 cohorts (Log-Rank p<0.001). Graph belonging to a PhD thesis presented to the University of Glasgow by Brendan Doyle, 2010.

b) There was no difference in either tumour number or size across the 5 cohorts studied. First graphic shows the total tumour number per mouse in each of the cohorts, (* represents outlying values). Second graphic shows the tumour size per mm^2 in each mouse of the colonies. Graph belonging to a PhD thesis presented to the University of Glasgow by Brendan Doyle, 2010.



3.1.2 Loss of *p53* alone is not sufficient for intestinal tumour development

To evaluate the effect of loss of *p53* alone in tumorigenesis, *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ mice were induced in similar way to the other groups. These mice had longer survival time than *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ mice but none of them developed intestinal tumours. In contrast, the mice developed other type of tumours such as lymphomas, sarcomas or occasional mammary tumours, squamous cell carcinomas and one endometrial carcinoma. This is explainedby low level cre-mediated recombination from the *AhCre* transgene in these tissues. Nonetheless, these results demonstrate that *p53* loss alone is not sufficient to initiate intestinal tumour development and that another event, such as *Apc* loss, is therefore required.

3.1.3 Mutation of a single copy of *p53* is sufficient for the progression to invasive carcinoma

All tumours which developed in the intestines of mice were examined under the microscope for characterization. High grade dysplasia (HGD) and invasive carcinomas were found in all the groups but at very different stages.

33% (5/15) of the *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{+/+}$ mice were identified with HGD and 27% (4/15) with invasive carcinomas. The percentages were similar in the *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/+}$ cohort, in which there was 40% (8/20) of mice with HGD and 25% (5/20) with invasive carcinomas. In contrast, the percentages were increased in the other cohorts. The rates of HGD and invasive carcinomas identified in these cohorts were, respectively, 93% (13/14) and 86% (12/14) in the *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/+}$ cohort, 87% (13/15) and 80% (12/15) in the *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ cohort and 100% (18/18) and 89% (16/18) in the *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/R172H}$ cohort (Figure 2).

When examining the invasive tumours microscopically, there were several differences in the degree of invasion in a similar way to those seen in the human disease. Some tumours showed superficial invasion, equivalent to T1-T2 human tumours. Others invaded all the way through the *muscularis propria* and peritoneum, equivalent to T3-T4 in human CRC. A single *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ mouse was found to harbour a metastasis in a pancreatic lymph node (N1). Apart from the metastasis in the *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ cohort, the rest of the tumours were similar in terms of depth and differentiation in the three cohorts *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/+}$, *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ and *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/fl}$ (Figure 3).

Figure 2: *p53* point mutation promotes high grade dysplasia and an invasive phenotype.

a) Percentage of high grade dysplasia in the tumours. Percentages were higher in mice having either deletions or mutations affecting both copies of *p53* (either *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ or *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/fl}$) than in controls (*AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{+/+}$) or the heterozygous condition (*AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/+}$). The single loss of *p53* function (*AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/+}$) had as well very high degree of high grade dysplasia. Graph belonging to a PhD thesis presented to the University of Glasgow by Brendan Doyle, 2010.

b) Percentage of mice with invasive carcinoma. There was a marked increase in the number of invasive carcinomas developed in mice with deletions or mutations of both copies of *p53* (either *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ or *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/fl}$) or a single loss of *p53* function (*AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/+}$)than controls (*AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{+/+}$) or the heterozygous condition (*AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/+}$). Graph belonging to a PhD thesis presented to the University of Glasgow by Brendan Doyle, 2010.

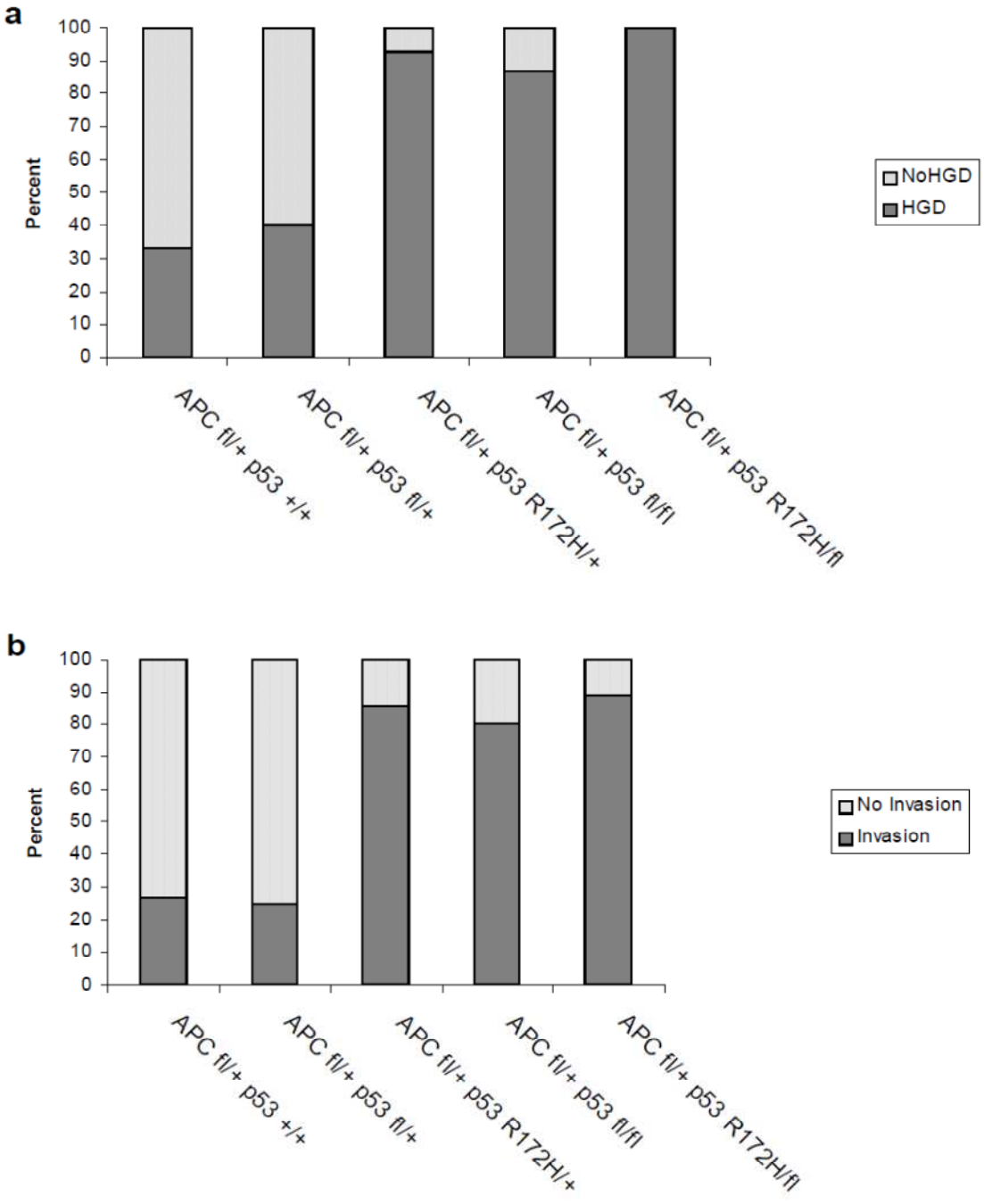


Figure 3: Variation in the invasion of the tumours.

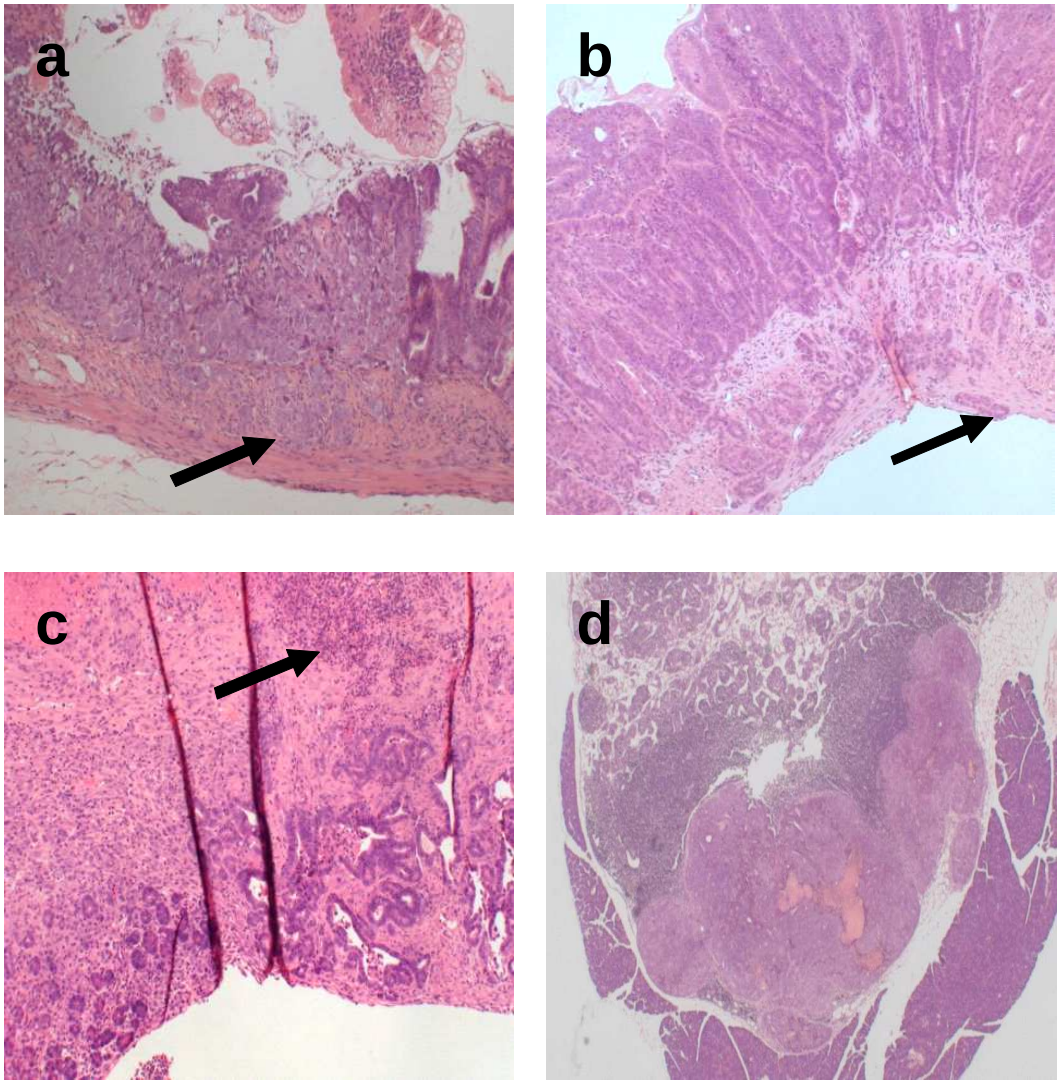
a) Superficial muscle invasion through the inner muscular layer (arrow). T2 stage.

b) Full thickness invasion, extending to the serosa but without perforating it (arrow). T3 stage.

c) Infiltration through the serosa and extension to the peritoneum with associated inflammatory reaction (arrow). T4 stage.

d) Metastatic carcinoma in a pancreatic lymph node. N1 stage.

Pictures belonging to a PhD thesis presented to the University of Glasgow by Brendan Doyle, 2010.



3.1.4 Invasive edge of tumours exhibit EMT changes

Having proven the histologic resemblance between tumours in these mouse models and human CRC, we decided to assess this resemblance at the molecular level. EMT is meant to be a critical step in the development of invasive carcinoma and progression to metastasis. E-cadherin loss and ZEB1 expression are two main markers of EMT (Schmalhofer *et al*. 2009). ZEB1 is a member of the Zinc finger homeobox family whose action represses E-cadherin from transcription and is upregulated at the invasive front of CRC (Spaderna *et al*. 2006).

IHC for ZEB1 and E-cadherin was performed on several invasive tumours, the results correlated with the presumed theory. ZEB1 expression was elevated at the invasive front in a similar manner as E-cadherin expression was decreased (Figure 4a-b).

Furthermore, E-cadherin loss is related with nuclear transcription of β-catenin through activation of WNT signalling pathway (Brabletz *et al*. 2001). To confirm this upregulation of β-catenin at the invasive edge of the tumours, we performed the β-catenin IHC in a subset of invasive tumours. There was a clear positive nuclear staining in cells situated at the invasive edge, whilst the cells at the bulk of the tumour expressed both nuclear and cytoplasmic positive staining (Figure 4c).

These data suggest that EMT actually takes place at the invasive front of these tumours, and that is correlated with β-catenin expression. The similarities between human CRC and these mouse models are therefore strongly supported. The expression of all of these markers was similar in the different cohorts $AhCre^{+}$ $Apc^{fl/+}$ $p53^{R172H/+}$, $AhCre^{+}$ $Apc^{fl/+}$ $p53^{fl/fl}$ and $AhCre^{+}$ $Apc^{fl/+}$ $p53^{R172H/fl}$.

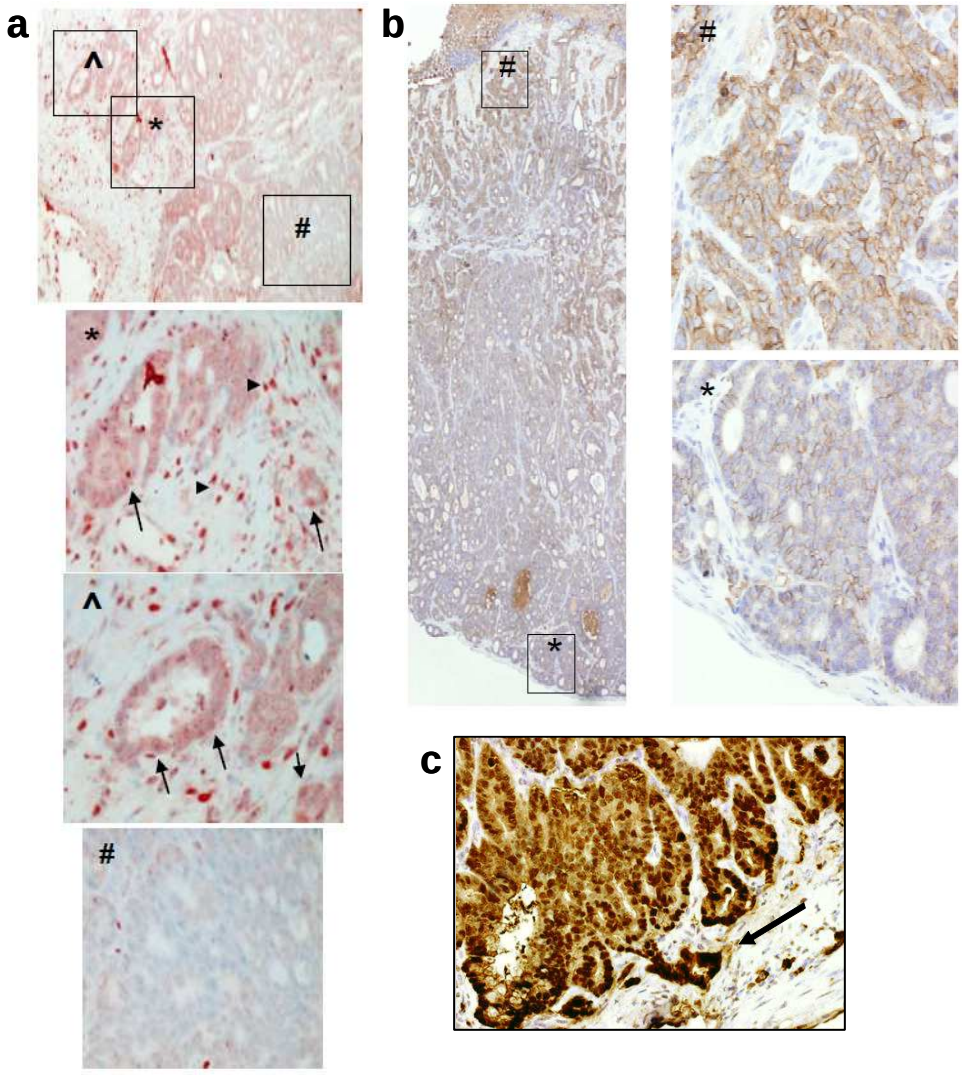
Figure 4: EMT markers at the invasive edge of the tumours.

a) IHC for ZEB1. Its expression is positively stronger (nuclear and cytoplasmic red staining) at the invasive edge of the tumour (^ and *) comparing to the central part of the tumour (#). Arrows depict nuclear staining at the invasive front: the expression of the dissociated cells (arrowheads) is stronger than the cells that still conserve epithelial properties (arrows) as show by red nuclear staining.

b) IHC for E-cadherin. Its expression, predominantly cytoplasmic, is much stronger at the centre of the tumour (#) than at the invasive edge (*).

c) IHC for β-catenin. Its expression is much stronger and predominantly nuclear at the invasive edge (arrow) than at the centre of the tumour, where it is more cytoplasmic.

Pictures belonging to a PhD thesis presented to the University of Glasgow by Brendan Doyle, 2010.



3.2 *Modelling the invasive properties of colorectal cancer in an Apc p53 Kras mouse model*

Considering the interactions among these three main genes, *APC p53* and *KRAS,* in human CRC, we decided to investigate the role of *Kras* mutation in our model to assess whether this can finally reveal the phenotype of *p53* loss. Germline embryonic expression of an endogenous $Kras^{G12D}$ allele is lethal (Tuveson *et al*. 2004). *Cre*-recombinase models for endogenous Kras have been created and used for demonstrating its ability to develop hyperplasia and carcinomas in lung, pancreas, ovaries and myeloproliferative diseases. (Jackson *et al*. 2001; Hingorani *et al*. 2003; Chan *et al*. 2004; Johnson *et al*. 2001; Tuveson *et al*. 2004). Due to *Cre* leakiness we were unable to generate adult $AhCre^{+}$ $Kras^{G12D/+}$ mice so instead we crossed mice to a different intestinal inducible *Cre* recombinase, the $VillinCre^{ER}$, which induces recombination in both the small intestine and colon upon tamoxifen administration.

3.2.1 Additional *p53* deletion or *Kras* mutation reduces the survival of mice deficient for *Apc* loss

To first confirm the impact of *p53* deletion or *Kras* mutation on mice in which one copy of *Apc* was deleted in the adult murine intestine using the $VillinCre^{ER}$ technology, we first created mouse colonies with these different genotypes: $VillinCre^{ER+}$ $Apc^{fl/+}$, $VillinCre^{ER+}$ $Apc^{fl/+}$ $Kras^{G12D/+}$ and $VillinCre^{ER+}$ $Apc^{fl/+}$ $p53^{fl/fl}$.

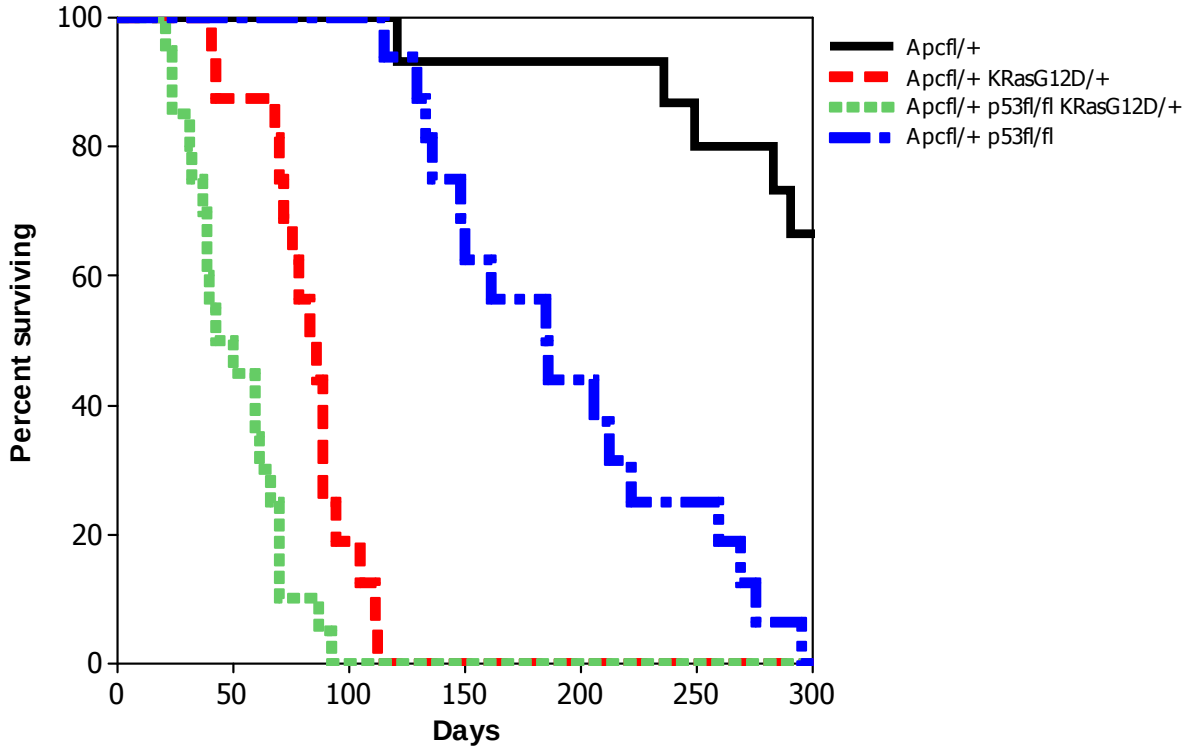
Comparing to mice with only one *Apc* copy deletion ($VillinCre^{ER+}$ $Apc^{fl/+}$) that usually were able to survive an average of 400 days, mice carrying an additional *p53* deletion ($VillinCre^{ER+}$ $Apc^{fl/+}$ $p53^{fl/fl}$) had the survival rate significantly reduced to less than 200 days and mice carrying an additional *Kras* mutation ($VillinCre^{ER+}$ $Apc^{fl/+}$ $Kras^{G12D/+}$) had the survival rate highly reduced to a mean of 100 days (Figure 5). In the case of the $VillinCre^{ER+}$ $Apc^{fl/+}$ $Kras^{G12D/+}$ there was an increased number of tumours formed in the large intestine, predominantly in the colon, which could have contribute to the reduce in the lifespan.

To develop a murine model with alterations in these three main genes (*Apc*, *p53*, *Kras*), the mice were intercrossed to yield the $VillinCre^{ER+}$ $Apc^{fl/+}$ $p53^{fl/fl}$ $Kras^{G12D/+}$ cohort. Mice from this colony had a drastic reduction in the survival rate, being an average of 50 days. The reduction in this survival rates compare with the other three cohorts was significantly different (Figure 5).

Figure 5: Survival time comparing *p53* deletion or *Kras* activation with one copy of *Apc* deleted.

Kaplan-Meier survival curves demonstrating the survival times of *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ (black line), *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ (blue line), *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ (red line) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (green line). There was a significant difference in survival between *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ (n=15) cohorts and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ (n=16) cohorts (Log Rank p=0.002) and a further significant difference between *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ (n=16) cohorts and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ (n=16) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (n=20) cohorts (Log Rank p<0.0001). Survival rate between the cohorts *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ (n=16) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (n=20) was also significant (Log Rank p=0.0002).

Median survival values for each cohort are: 428 days for *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ (black line), 86 days for *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ (red line), 59 days for *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (green line) and 186 days for *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ (blue line).



3.2.2 *Apc p53 Kras* genotype drives early invasive tumour formation

Mice that developed signs of intestinal tumours such as pale feet or pain were culled and examined. Mice that died due to other reasons rather than small intestinal tumours were not included in the study. The tumours developed in the intestine of these mice were classified in terms of number, size and aggressiveness. Total number and size was assessed macroscopically, regardless whether those tumours were invasive or not, which could only by assessed histologicaly.

Tumours formed in the colon in the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ colonies were extremely occasional; on the oher hand, the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ cohort developed higher number of tumours in the colon, for a still unknown reason. Considering this, only tumours within the small intestine are included in this section of total number.

The total tumour number across the three colonies (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$) was not statistically different, except between the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ cohorts (Figure 6a). In terms of tumour burden (tumour area/mouse) and average of tumour size, the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ developed bigger tumours and had higher tumour burden compared with either of the other two cohorts *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$. Neither tumour burden or tumour size was different between the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mice (Figure 6b-c).

When the tumours were examined under the microscope and classified as invasive and non-invasive (adenomas versus adenocarcinomas), there was a significant difference between the percentage of invasive tumours per mouse in the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mice, with almost 24% of invasive tumours; and the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ mice with less than 12% (Figure 6d). Tumours formed in the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ cohort were never invasive, therefore this colony is not included in the invasion graphic (Figure 6d).

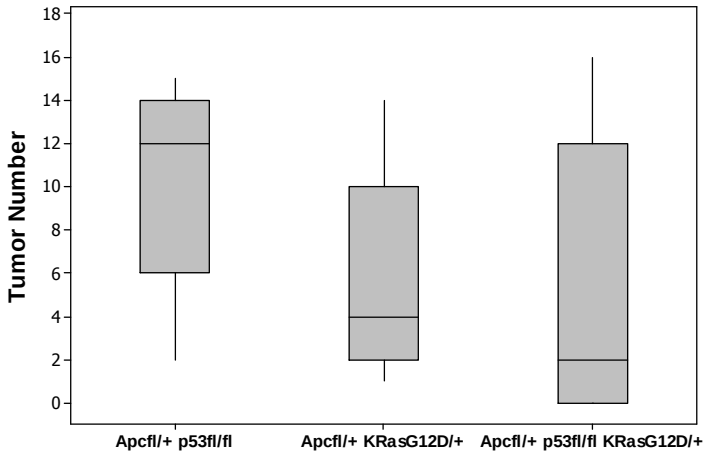
Histologically, tumours from the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mice were identified as invasive even with a total size of as little as 1 mm, which differs from the invasive tumours of the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ colony which were much bigger in size. Metastasis in other organs was only found in one mouse of the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ cohort (Figure 7).

Figure 6: *Apc p53 Kras* mutations drive early invasive tumour formation.

a) There was no significant difference observed in the total tumour number across the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ (n=9) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ (n=5) cohorts (Mann-Whitney test p=0.14) and between the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ (n=5) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (n=15) cohorts (Mann-Whitney test p=0.50)**.** There was significant difference between the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ (n=9) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (n=15) cohorts (Mann-Whitney test p=0.0467)**.**

b) There was significant difference in the tumour burden (tumour area/mouse) between the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ (n=9) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ (n=5) cohorts (Mann-Whitney test p=0.0112) and between the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ (n=9) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (n=15) cohorts (Mann-Whitney test p=0.0008). There was no significant difference between the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ (n=5) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (n=15) cohorts (Mann-Whitney test p=0.1842).

a



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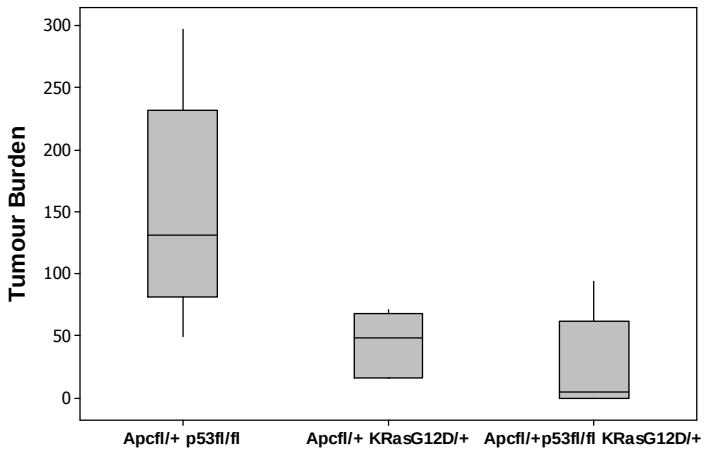
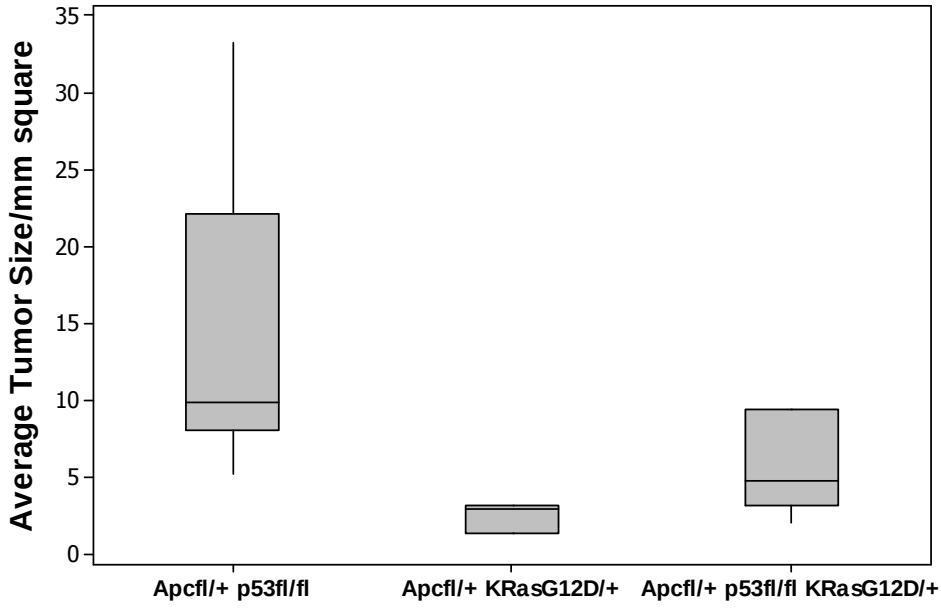


c) *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ mice (n=9) developed significantly bigger tumours as compared to *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ mice (n=3) (Mann-Whitney test p=0.0162) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mice (n=11) (Mann-Whitney test p=0.05). There was no difference in tumour size between the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mice (Mann-whitney test p=00868).

d) Graph showing the percentage of invasive and tumours formed in each mouse of the 2 cohorts. *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mice (n=27) developed significantly more invasive tumours compared to *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ (n=23) mice (Chi-square test p<0.001).

c



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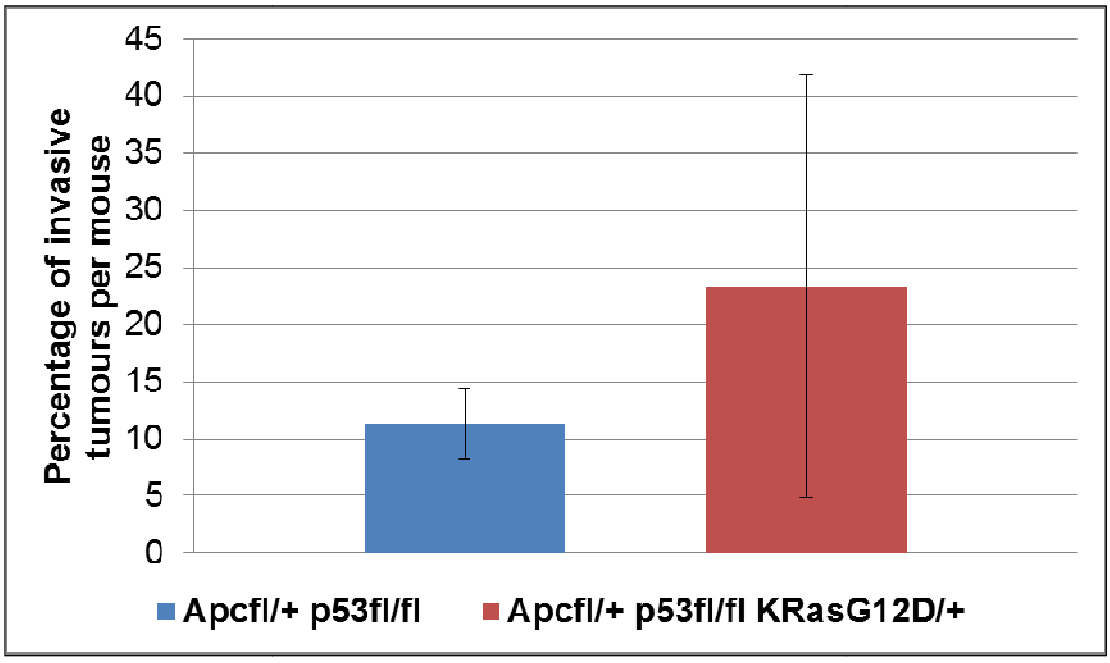
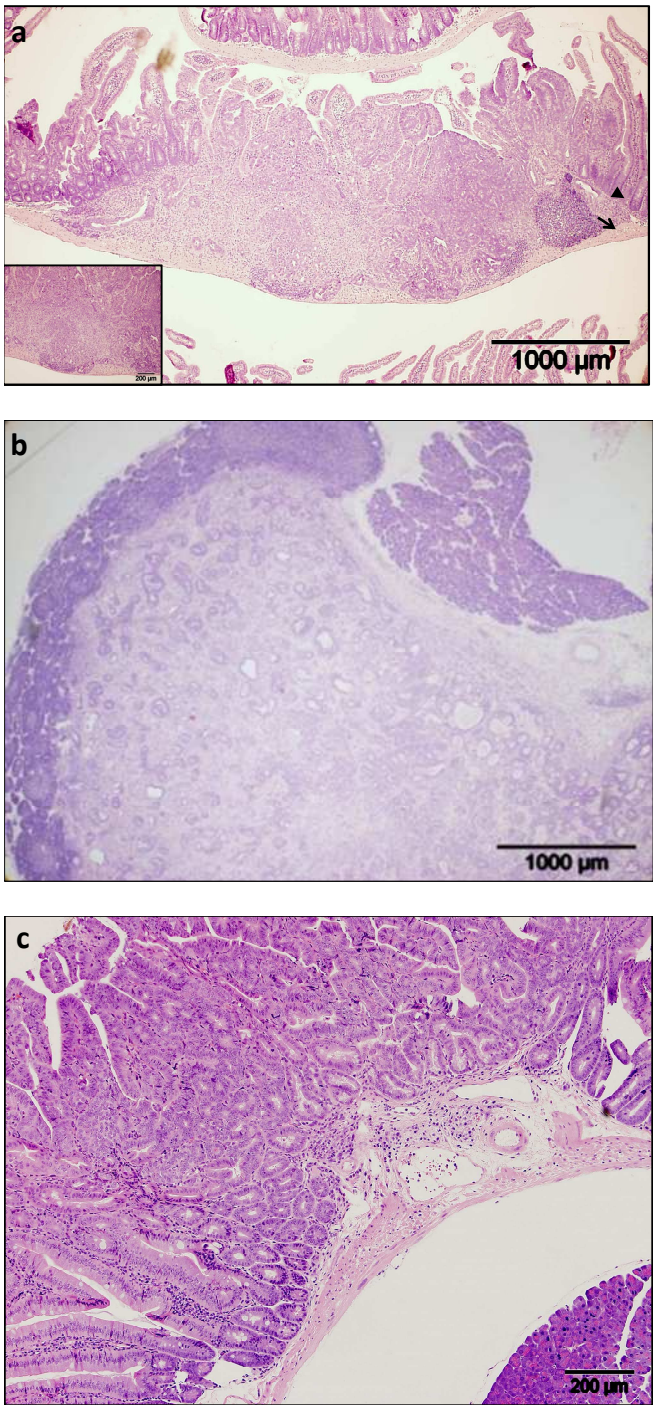


Figure 7: Intestinal adenocarcinoma and metastasis in a *VilinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Kras$^{G12D/+}$* mouse.

a) Primary intestinal adenocarcinoma, note the invasion beyond the mucosa (arrowhead) and through muscularis propria (arrow); inset: higher magnification of the invasive edge. The intestinal adenocarcinomas formed in the *VilinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Kras$^{G12D/+}$* cohort were histologically similar to those few adenocarcinomas formed in the *VilinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$* but smaller in size.

b) Metastasis into a regional lymph node (N1).

c) Example of a non-invasive tumour. Neoplastic cells are restricted to the mucosa.



3.2.3 Invasive edge of tumours showed EMT changes

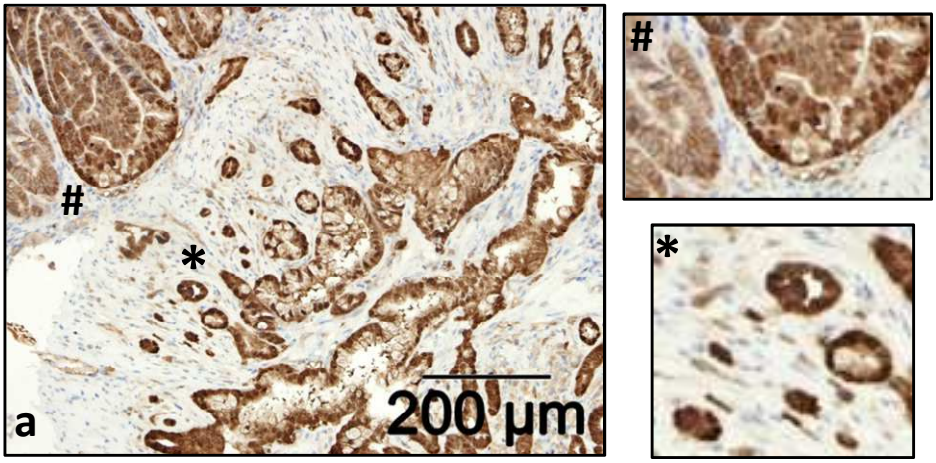
In the previous mouse model with the *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/+}$, *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ or *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/fl}$ cohorts, we demonstrated upregulation of EMT markers at the invasive edge of the tumours. To investigate whether this was still happening in the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mouse model, we examined the expression of a number of genes involved in candidate pathways.

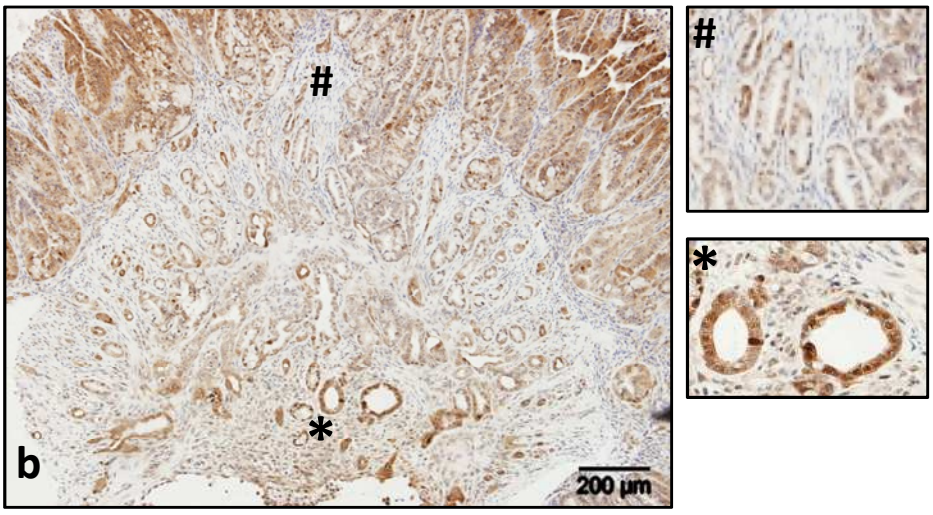
Mutant *Kras* has been previously seen to enhance the canonical Wnt/β-catenin signalling pathway in a mutant *Apc* background (Janssen *et al*. 2006). As with the *AhCre*$^{+}$ *Apc p53* mouse model, in this *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mouse model we saw increased β-catenin levels at the invasive fronts. Moreover there was increased levels of p-ERK at the invasive front (Figure 8), which correlates with earlier *in-vitro* studies where oncogenic *Kras* mutation uncoupled Rho activation from stress fiber formation via a mitogen-activated protein/ERK pathway leading to poor adhesion of these cells and enhancing their motility (Pollock *et al*., 2005).

Figure 8: Immunohistochemical analysis of *VilinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Kras$^{G12D/+}$* tumours.

a) β-catenin is activated at the invasive edge (*) showed by the stronger expression compared to the bulk of the tumour (#).

b) p-Erk is activated at the invasive edge (*), showed by the stronger expression compared to the bulk of the tumour (#).





3.2.4 SRC family kinases are required for nuclear β-catenin at the invasive front

To see whether Src expression was correlated with β-catenin expression in our *VillinCre*$^{ER+}$ mouse models, we first evaluated the expression of Src and its correlation with the high levels of β-catenin previously found. We found high expression of Src at the invasive edge in both of the mouse models, *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mice (Figure 9).

To confirm the association between expression of Src with low E-cadherin and translocation of β-catenin to the nucleus, we performed a short term experiment with a Src inhibitor, Dasatinib. *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mice treated with Dasatinib had lower levels of p-Src, lower levels of nuclear β-catenin and higher levels of E-Cadherin at the invasive front in comparison with the vehicle-treated mice, (Figure 10).

Figure 9: Immunohistochemical analysis of Src expression.

a) Src expression is higher at the invasive edge in a *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ tumour.

b) Src expression is higher at the invasive edge in a *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ tumour.

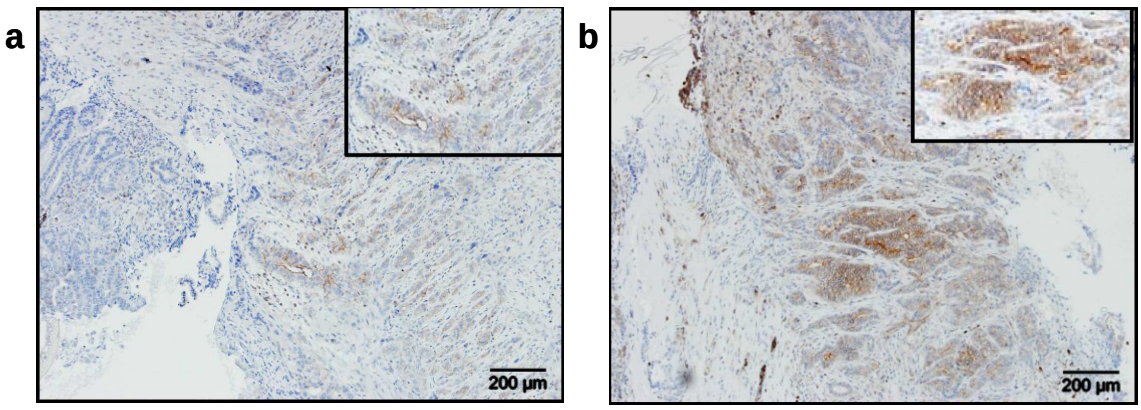
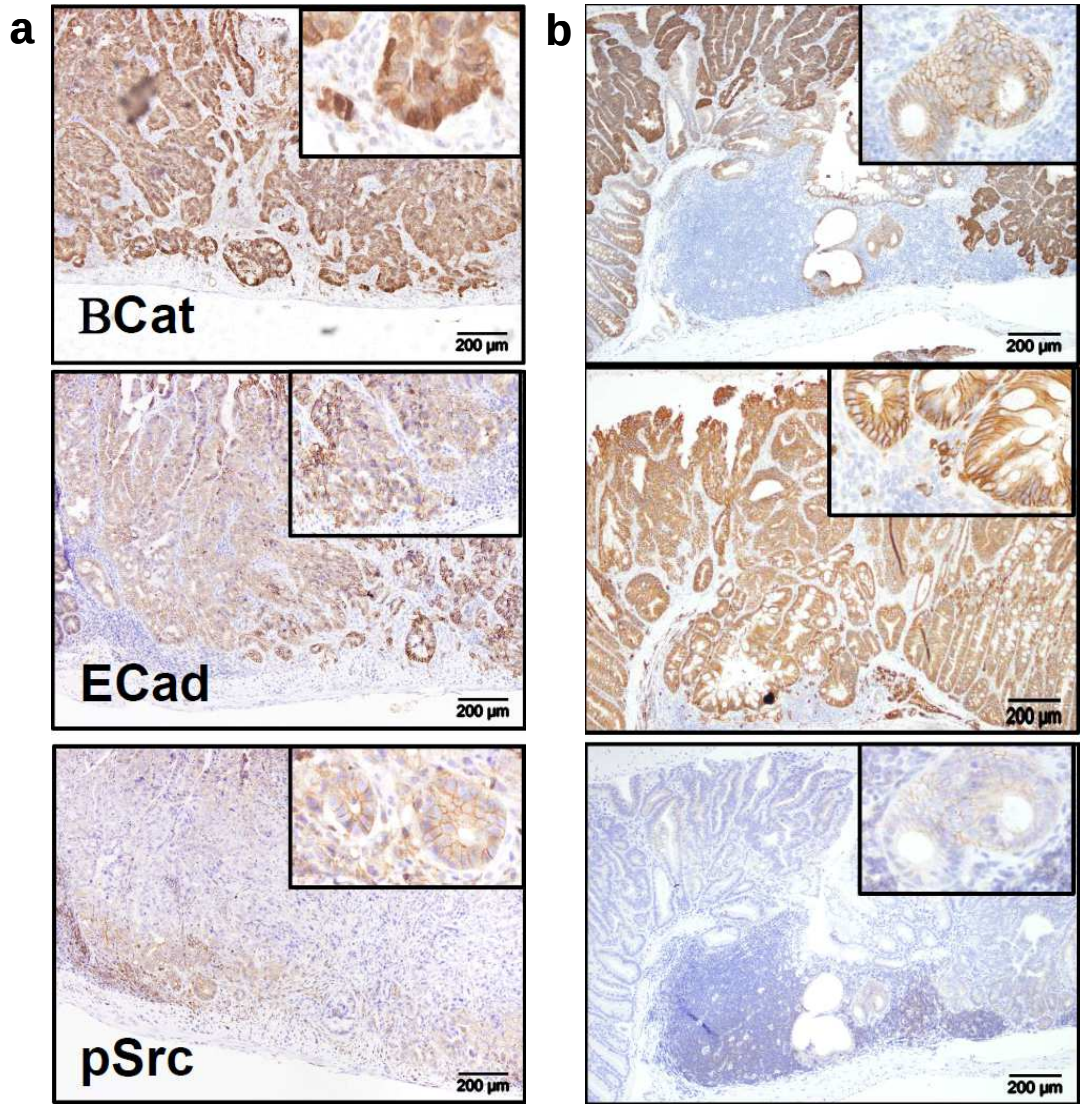


Figure 10: Immunohistochemical analysis of short term invasive tumours.

Tumours from vehicle-treated mice (a) and Dasatinib-treated mice (b).There was reduction of β-Catenin, phospho-Src and restoration of membranous E-Cadherin at the invasive edge after Dasatinib treatment. Images are representative of at least 3 individual tumours.



3.3 Dasatinib treatment

Several Src inhibitors have been studied, such as Saracatinib, Bosutinib or Dasatinib. Dastinib has been tested in pre-clinical studies with good results in other types of cancer such as prostate, breast, gliomas or pancreas (Araujo *et al*. 2010, Morton *et al*. 2010). More specifically, in a study developed at our institution, Dasatinib showed to prevent migration and invasion (and therefore metastasis) in a mouse model of pancreatic cancer (Morton *et al*, 2010). Since in our *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mouse models, we had demonstrated high levels of Src expression at the invasive edge of tumours and the ability of Dasatinib to reduce this Src expression at the invasive fronts; thus we decided to follow the line of experiments being developed at our institution with Dasatinib and see whether it was also applicable to our CRC model.

Given the rapid onset of clinical signs in the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mice, it was not possible to properly test a long term treatment such as Dasatinib. Instead, the dosis of the single intra-peritoneal induction with Tamoxifen was reduced to 8mg/kg, which slowed down the onset of clinical signs to an average of 140 days instead of 50 days with the 80mg/kg induction. In this study, we treated with Dasatinib mice carrying *Apc p53 Kras* mutations (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$) with a dose of 10 mg/kg daily from 60 days post induction until the appearance of clinical signs associated with intestinal tumours. The study was focused on evaluating the effect of the drug on tumorigenesis, life span, invasiveness and metastasis.

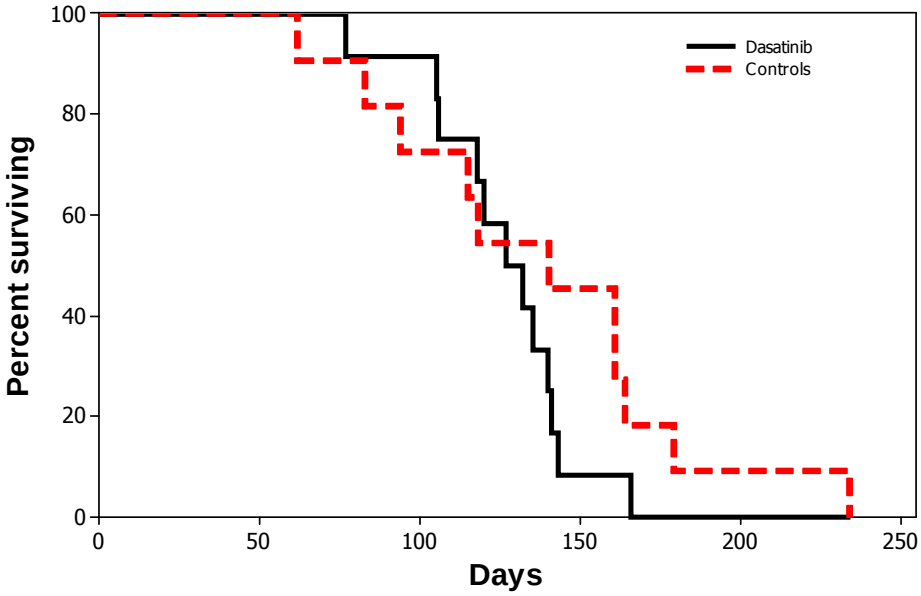
Dasatinib treatment did not increase survival; treated mice became ill at the same time (around 140 days) as the control cohort (Figure 11a). There was neither difference in the tumour number or the average of tumour size between controls and treated mice (Figure 11b-c). However, there was a decrease in the number of invasive tumours formed per mouse, with only 7% of adenocarcinomas in the treated mice against the 44% found in the controls. Histologically, non-invasive intestinal tumours from treated mice tended to be bigger in extension, with a marked hyperplasia of the tunica muscularis, but the neoplastic cells were restricted to the epithelial cell layer without invading into deeper tissues (Figure12).

Figure 11: Tumorigenesis study of Dasatinib-treated *VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Kras$^{G12D/+}$* mice.

a) Kaplan-Meier survival curves demonstrating the survival times of untreated mice (n=12, red line) and Dasatinib-treated mice (n=11, black line). There was no significant difference between the two survival curves (p= 0.224).

b) The total tumour number developed in the small intestine of mice did not differed between the controls (n=22) and Dasatinib-treated mice (n=10). Mann-Whitney test p=0.0721.

a



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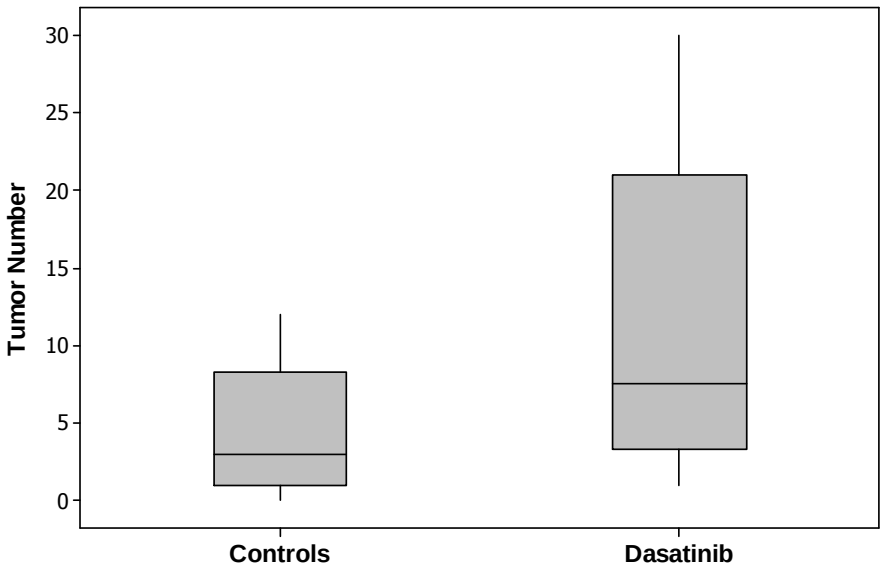
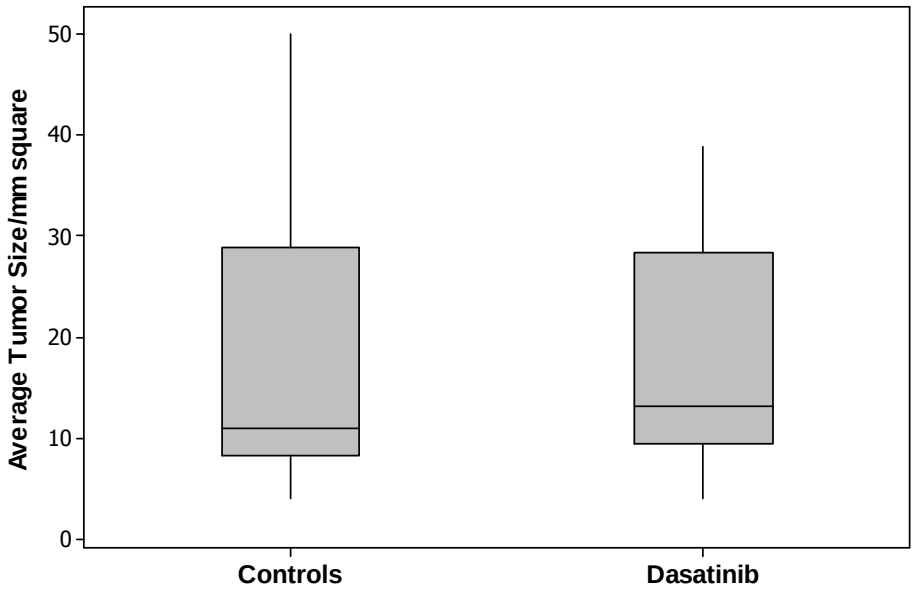


c) Both controls (n=16) and Dasatinib-treated (n=10) mice developed tumours with no significant difference in size. Mann-Whitney test p=0.6730.

d) Dasatinib prevented invasion of tumours. The control mice (n=8) developed significantly more invasive tumours (44% of invasive tumours per mouse) compared with the treated mice (n=6) which had a percentage of 7% invasive tumours per mouse. Chi-square p=0.010.

c



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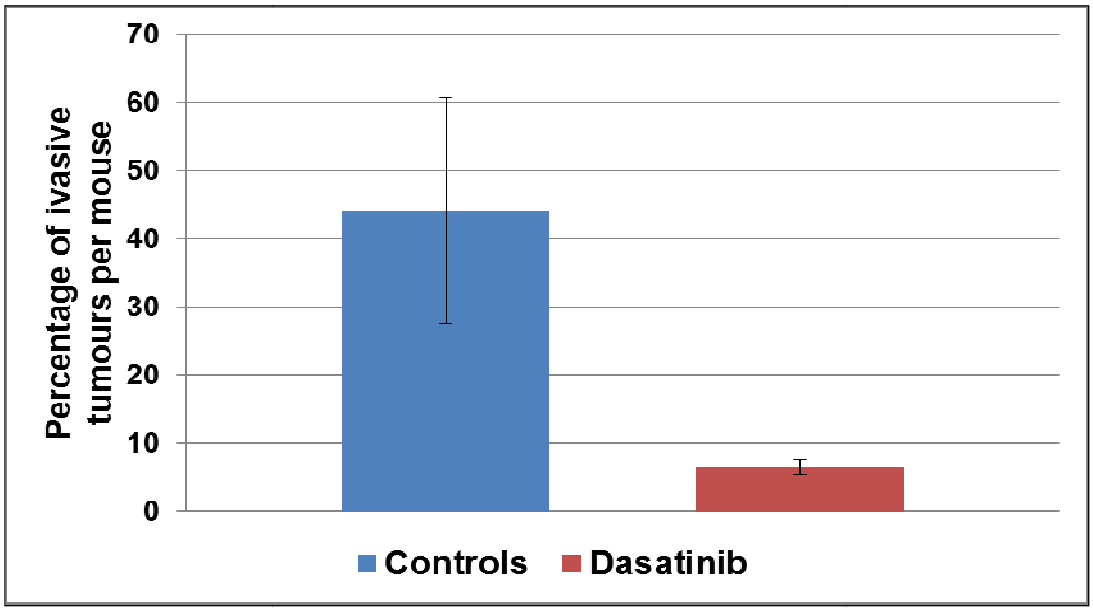
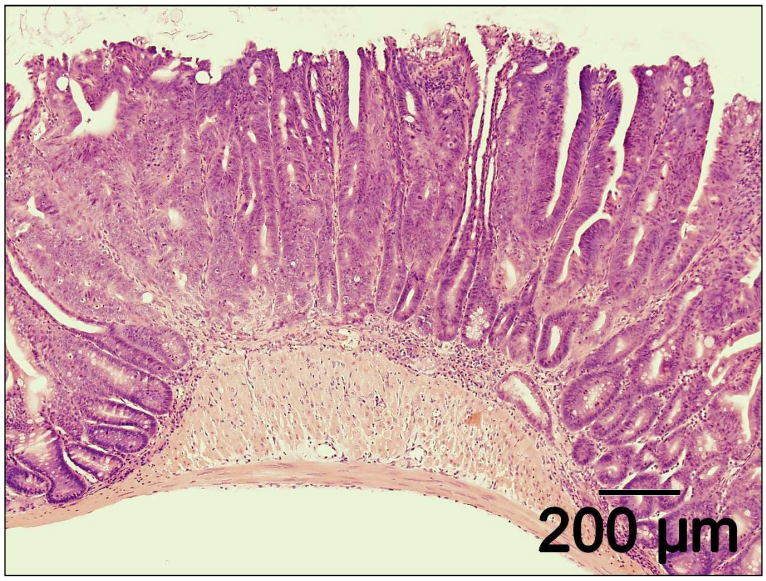


Figure 12: Dasatinib treatment prevents invasion throught the submucosa.

a) Picture showing a non-invasive tumour from the treated-mice. Neoplastic growth is large in extension but it is restricted to the mucosa. There is marked hyperplasia of the tunica muscularis.

b) Picture showing an invasive tumour form the untreated-mice. Neoplastic cells have invade through the tunica muscularis and reached the serosa.

a



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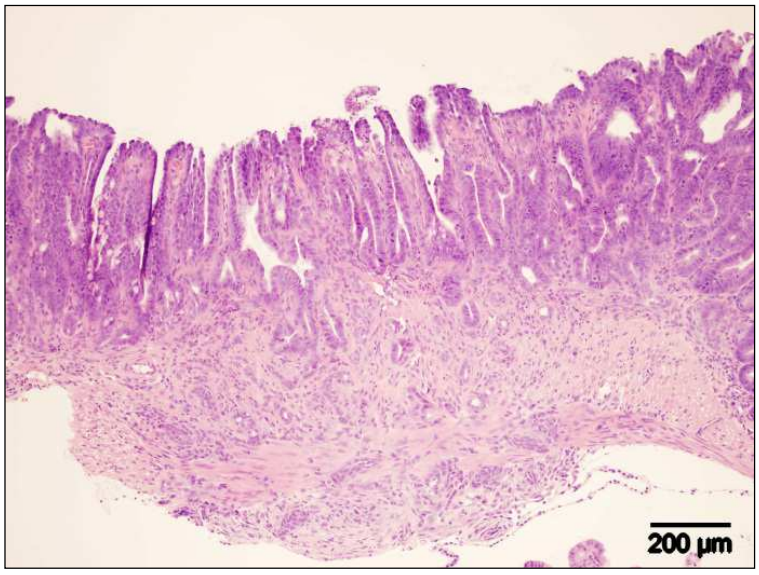


3.4 Growing of tumoral cells *in vitro*

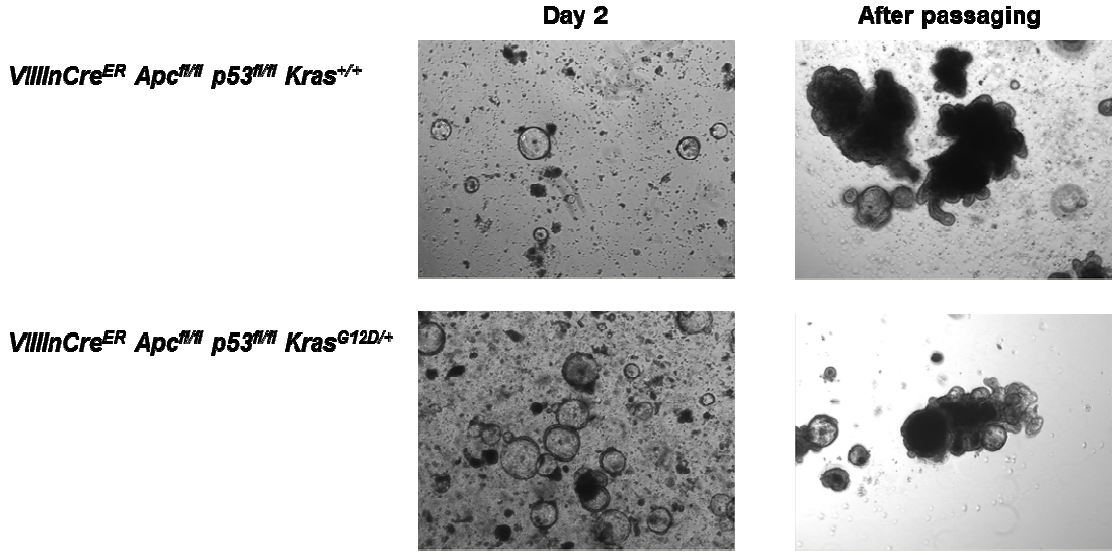
In order to test the tumorigenic capacity of the tumoral cells from the different genotypes and their ability to grow *in vitro,* we performed several cultures of cells coming from four different genotypes: *VillinCre^{ER+} Apc$^{fl/fl}$ p53$^{fl/fl}$*, *VillinCre^{ER+} Apc$^{fl/fl}$ p53$^{fl/fl}$ Kras$^{G12D/+}$*, *VillinCre^{ER+} Apc$^{fl/fl}$ p53$^{R172H/fl}$*, *VillinCre^{ER+} Apc$^{fl/fl}$ p53$^{R172H/fl}$ Kras$^{G12D/+}$*.

All the cultured cells were able to form round spheres within 3 to 4 days. The appearance of the spheres was, as has been described, with a peripheral thick layer of cells and a lumen that becomes filled with dead cells and cellular debris. After the passage, at one week of growth, single isolated cells were able to reconstitute new spheres. In accordance with the literature (Sato *et al*, 2009), the spheres started to bud with the formation of new small crypts adhering to the main sphere, giving the appearance of numerous crypts surrounding a central lumen.

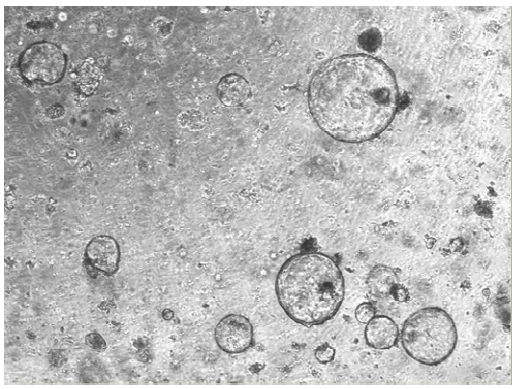
This pattern of growth was similar in both cells extracted from tumours and from normal crypts, with no apparent difference in proliferation, specific quantification ot the pattern and time of growth was not performed. Spheres were segregated and re-plated at least three to four times, as well as frozen and thawed without loosing the characteristics described. No clear difference was recognized in the morphology or pattern of growth between the different genotypes, the pictures shown are representative of the different cohorts in culture and are not comparable to each other due to lack of specific quantification (Figure 13).

Figure 13: Crypt and tumour culture successfully grown in culture media.

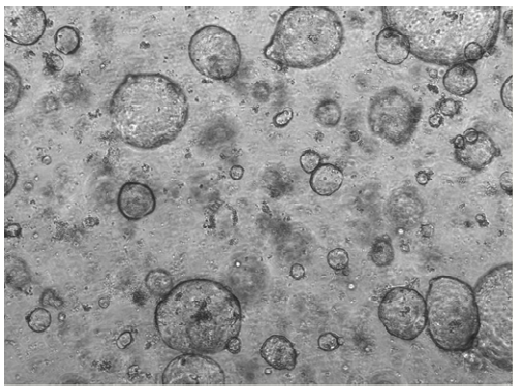
a) Culture from crypts. These pictures show the appearance of the spheres formed by the cells isolated from crypts. As soon as two days of culture, individualized cells were able to form medium size spheres and started to bud around one week of culture or after passaging. The morphology was very similar in the different genotypes.



b) Culture from tumours. These pictures show two examples of spheres formed from tumoral cells isolated from the intestine of the mice. In a similar manner as cells extracted from crypts, as soon as 2 days of culture, big spheres were already formed and were able to bud in a few days. There was no difference in the morphology of the spheres between the genotypes.



VillinCre^{ER} Apc$^{fl/+}$ p53$^{R172H/fl}$ Kras$^{+/+}$ (day 2)



VillinCre^{ER} Apc$^{fl/+}$ p53$^{R172H/fl}$ Kras$^{G12D/+}$ (day 2)

3.5 Subcutaneous tumour growth in allografted recipients

With the cell culture *in vitro*, we demonstrated the capacity of these *VillinCre*$^{ER+}$ *Apc*$^{fl/fl}$ *p53*$^{fl/fl}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/fl}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/fl}$ *p53*$^{R172H/fl}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/fl}$ *p53*$^{R172H/fl}$ *Kras*$^{G12D/+}$neoplastic cells to maintain their tumoral phenotype and grow in culture into spheres recapitulating the original tumour in a matrigel-based matrix. In order to test whether these cells were also able to grow in tissue, we designed a preliminary pilot experiment *in vivo*, we injected a suspension of single cells into the subcutaneous tissue of the flank of a total of 12 immunocompromised mice. Mice were monitored to evaluate the neoplastic growth in their flank and were culled when the nodule become either ulcerated or bigger than 15 mm. Mice were culled between 14 and 71 days after injection, the time of growing for these nodular tumours was no comparable since many mice had to be culled due to ulceration of the area, therefore this data was not comparable.

Mice were injected with cells from the following genotypes: *VillinCre*$^{ER+}$ Apc$^{fl/fl}$ p53$^{fl/fl}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/fl}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/fl}$ *p53*$^{R172H/+}$ *Kras*$^{G12D/+}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$, *VillinCre*$^{ER+}$ Apc$^{fl/+}$ *p53*$^{R172H/+}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/+}$ *Kras*$^{G12D/+}$. All the mice developed subcutaneous growths in their flank. The gross appearance of all the nodules was very similar, consisting in a non-encapsulated, round, white firm mass not attached to the surrounding tissue except in a few cases where the skin became ulcerated and the mass was adhered to the dermis. None of the tumours showed any infiltrative behaviour into the abdominal cavity, however, a few tumours grossly infiltrated the dermis, which was confirmed microscopically. There was no significant difference in the growing pattern among any of the different genotypes (data not shown).

Tumours were collected for histological evaluation. Samples from visceral organs, such as liver, lungs and kidneys, were collected as well to search for possible metastasis. The histological appearance of the subcutaneous tumoral nodules was characterized by irregularly shaped acinar structures lined by a single layer of cuboidal epithelial neoplastic cells; the tumoral cells were moderately dysplastic and mitotic figures were frequently present. Acini contained principally cellular debris within the lumen and were separated and divided by a moderately thick matrix of loose to mildly dense fibrovascular stroma. There were frequent areas of necrosis within the tumours. No metastatic foci were found in any of the organs examined (Figure 14).

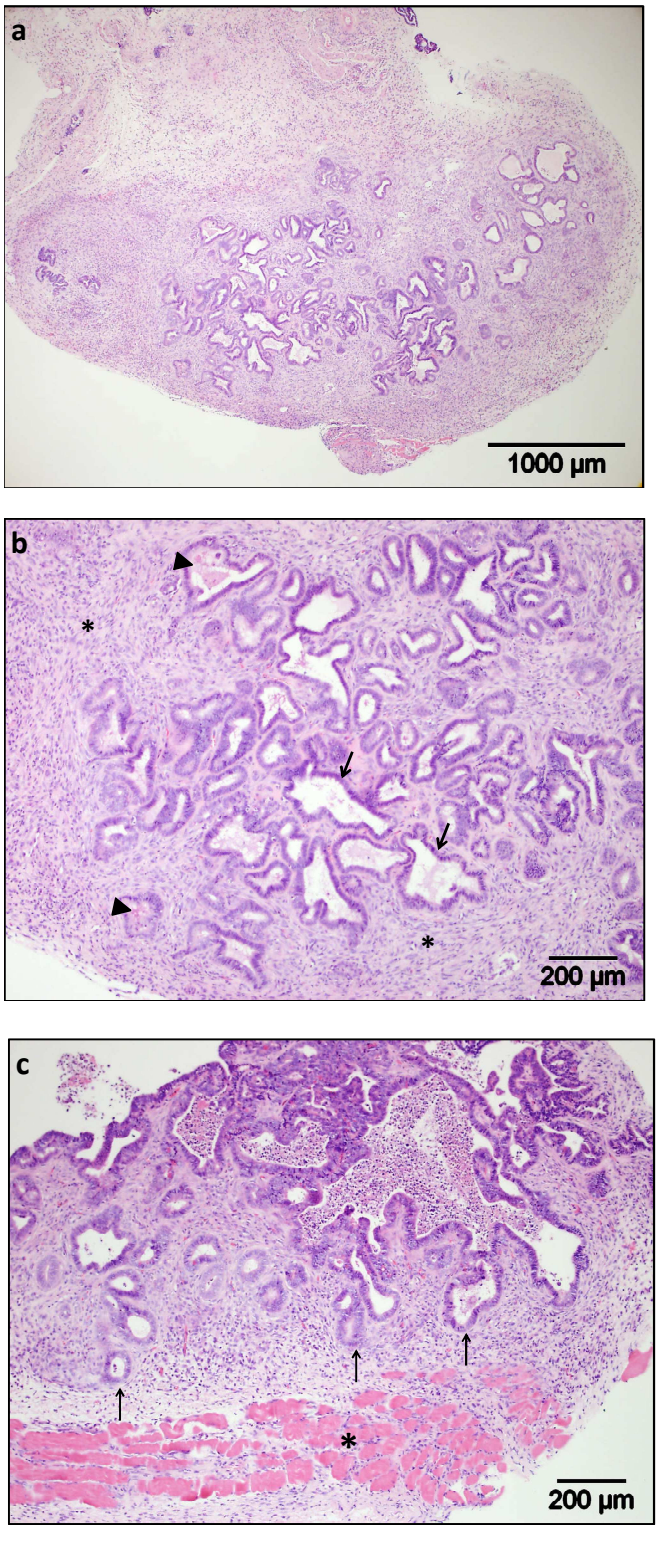
Histology revealed that the cells were able to recapitulate the original tumour phenotype, with proliferating acini and a moderate desmoplastic reaction. In the majority of the samples, the appearance of the tumour was even more aggressive and dysplastic than their original counterparts extracted from the murine intestine. Some of the tumours revealed infiltration into the dermal tissues without reaching the epidermis. There was no difference in the appearance of the neoplasm or the infiltrative behaviour among the different genotypes.

Figure 14: Histology from harvested allografts.

a) Allograft formed with *VillinCre*$^{ER+}$*Apc*$^{fl/+}$ *p53*$^{R172H/+}$ *Kras*$^{+/+}$ genotype cells taken from an intestinal tumours. The majority of the tumours grew in a nodular pattern as shown in this picture.

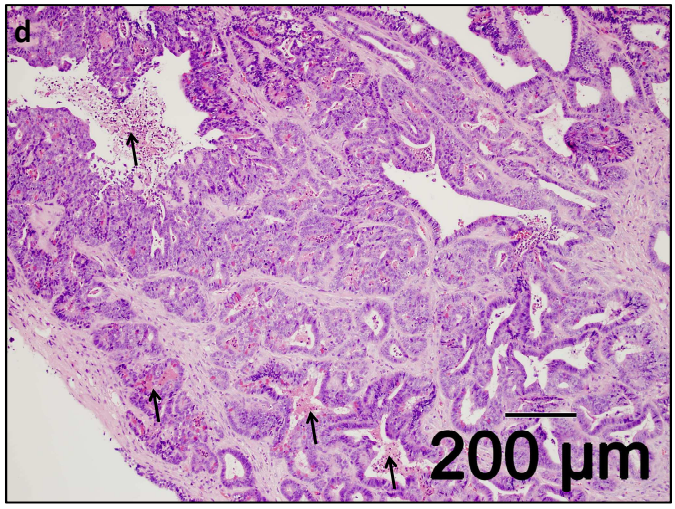
b) Higher magnification of (a). Neoplastic cells were arranged in irregular patterns within variably sized acini (arrows) with either empty or cellular debris-filled lumens (arrowhead). Surrounding the acini there is mesenchymal cell proliferation (*).

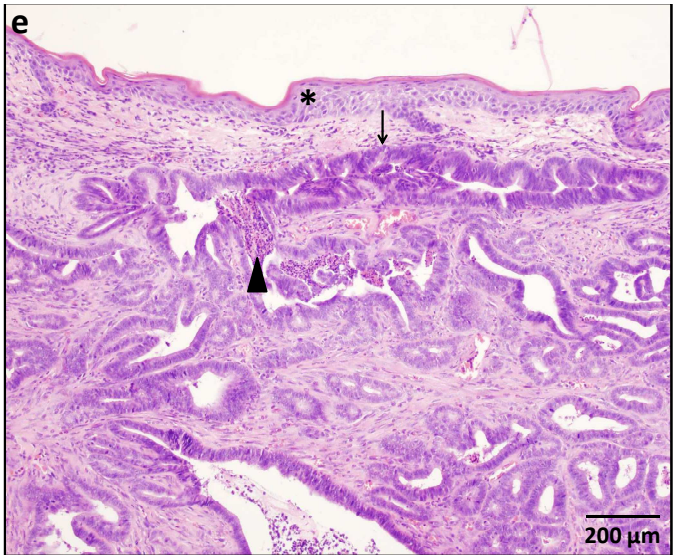
c) Allograft formed with cells taken from crypts of a *VillinCre*$^{ER+}$ *Apc*$^{fl/fl}$ *p53*$^{R172H/+}$ *Kras*$^{G12D/+}$ mouse. An intact muscular layer from the skin (*) is present, neoplatic cells (arrows) are not infiltrating the muscle.

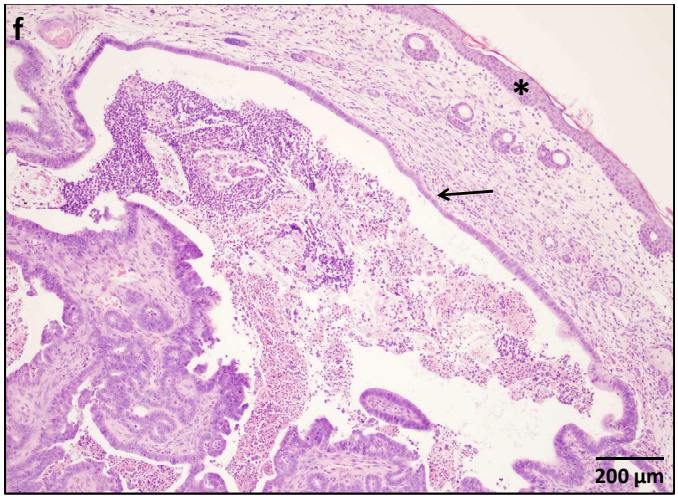


d) Allograft formed with cells taken from crypts of *VillinCre*$^{ER+}$ *Apc*$^{fl/fl}$ *p53*$^{R172H/+}$ *Kras*$^{G12D/+}$ genotype. Note the pleomorphic acinar-like structures formed by the neoplastic cells, in this example many of them are filled with karyorrhetic debris ("dirty necrosis") indicated by the arrows.

e-f) Allografts formed with cells taken from crypts of *VillinCre*$^{ER+}$ *Apc*$^{fl/fl}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ genotype. These are two examples of dermal expansion without invasion into the epidermis (*). Arrows indicate the limit of the neoplastic growth. Arrowhead is indicating a debris-filled lumen.







3.6 Other murine models of intestinal cancer

3.6.1 *Myc* deletion

As *Myc* is one of the Wnt target genes that was overexpressed at the invasive edge of our first *AhCre*$^{+}$ *Apc p53* (*AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{+/+}$; *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/+}$; *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$; *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/+}$ and *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/fl}$) mouse model (data not shown), we wanted to test whether *Myc* deletion would have an effect on the formation of adenocarcinomas. We intercrossed our *VillinCre*$^{ER+}$ *Apc p53 Kras* cohort with mice carrying *loxp*-inducible knockout *Myc* alleles (Baena *et al*. 2005). This yielded the following genotypes for the study: *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{+/+}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{fl/+}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Myc*$^{+/+}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Myc*$^{fl/+}$. Mice were bred and induced with tamoxifen following the same protocol as perviously described.

The survival rates of mice carrying the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{+/+}$ genotype and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{fl/+}$ genotype were, in both cases, around 200 days, which was not significantly different. The difference was neither significant when comparing tumour number and tumour burden between both cohorts (*Apc p53* and *Apc p53 Myc*). After microscopic examination, the percentage of invasive tumours in the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{+/+}$ cohort was approximately 18% while the percentage of invasive tumours in the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{fl/+}$ was slighlty increased (to 21%) but not significantly different (Figure 15).

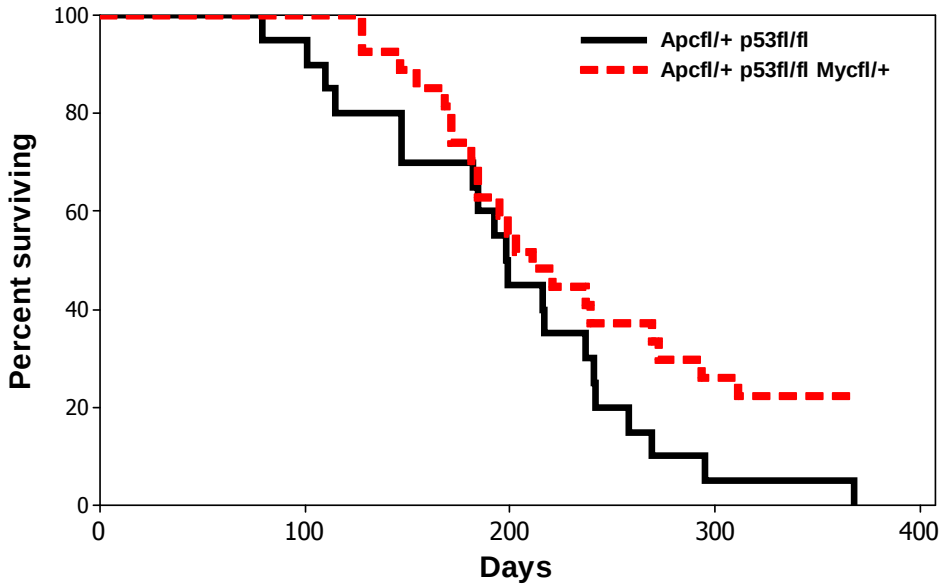
In the *Apc p53 Kras* cohort, the survival rate of *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mice was around 50 days while the surival rate of mice carrying additional *Myc* deletion (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Myc*$^{fl/+}$) was around 60 days; these results were not significantly different. There was no difference when comparing tumour number and tumour burden between both cohorts (*Apc p53 Kras* and *Apc p53 Kras Myc*). After microscopic examination, the percentage of invasive tumours was 14% in the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ cohort while it increased to 21% in the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Myc*$^{fl/+}$ cohort; these percentages were not significantly different (Figure 16).

Figure 15: *Myc* heterozygosity does not decrease formation of invasive tumours in the *VillinCre^{ER+} Apc p53* mouse model.

a) Kaplan-Meier survival curves demonstrating the survival times of *VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Myc$^{+/+}$* (black line, n=20) and *VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Myc$^{fl/+}$* (red line, n=27) cohorts. There was no significant difference in survival between the two cohorts (Log Rank p=0.114).

b) There was no significant difference observed between the total tumour number across the *VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Myc$^{+/+}$* (n=9) and *VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Myc$^{fl/+}$* (n=19) cohorts (Mann-Whitney Test p=0.0858).

a



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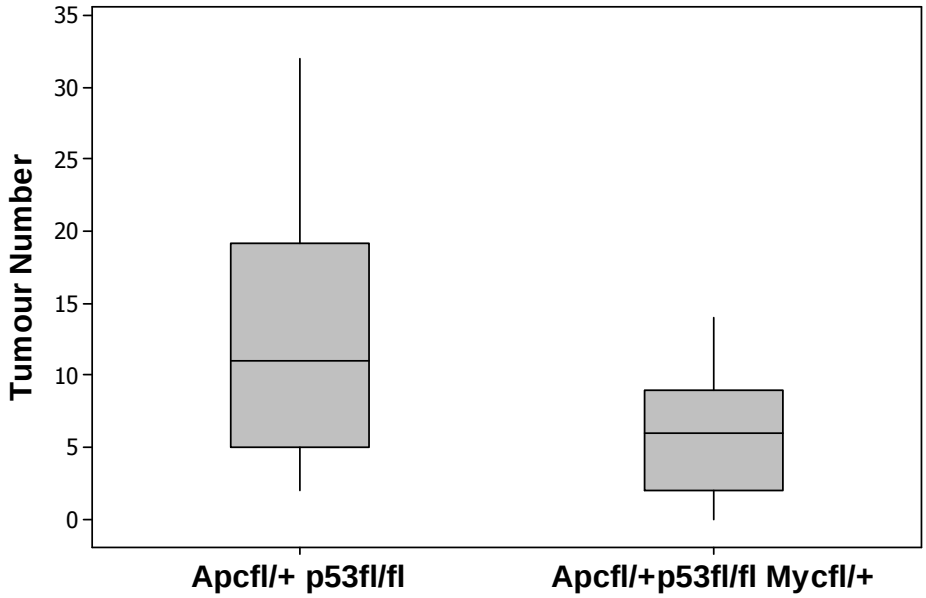
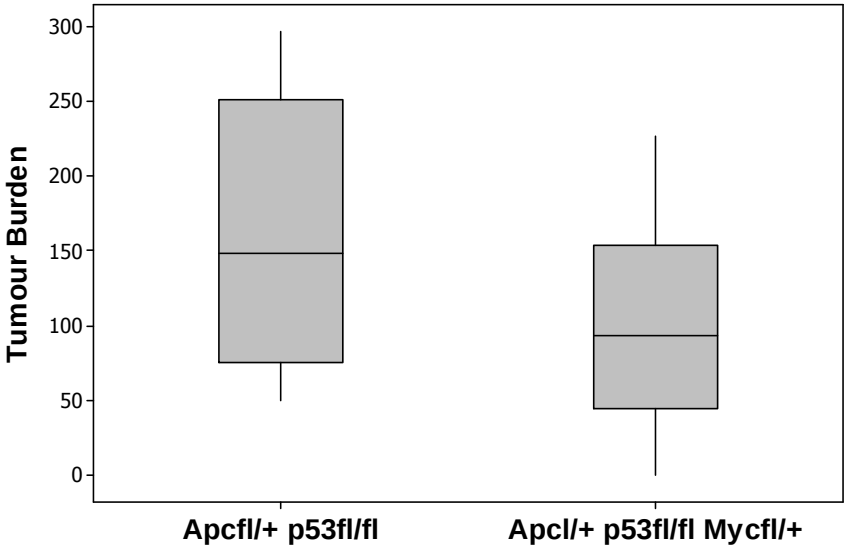


c) There was no significant difference observed between the tumour burden across the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{+/+}$ (n=8) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{fl/+}$ (n=19) cohorts (Mann-Whitney Test p=0.1172).

d) Graph showing the percentage of invasive tumours formed in the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{+/+}$ (n=19) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Myc*$^{fl/+}$ (n=15) mice. There was no significant different across the cohorts (Chi-square test p=0.276).

c



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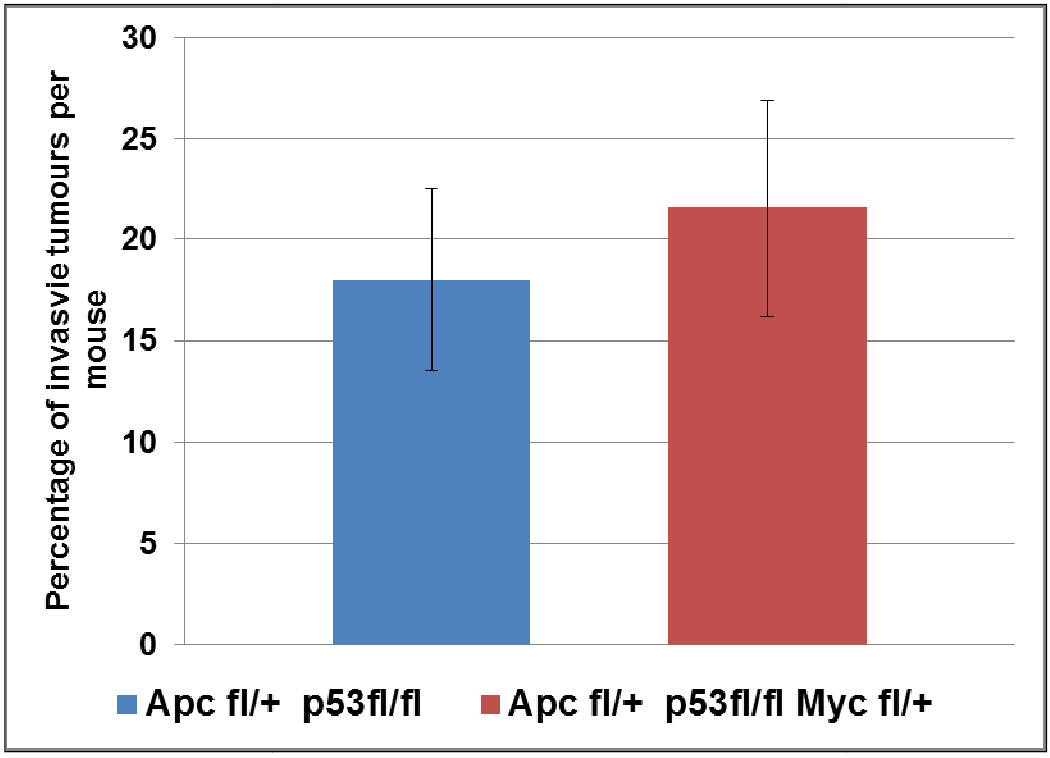
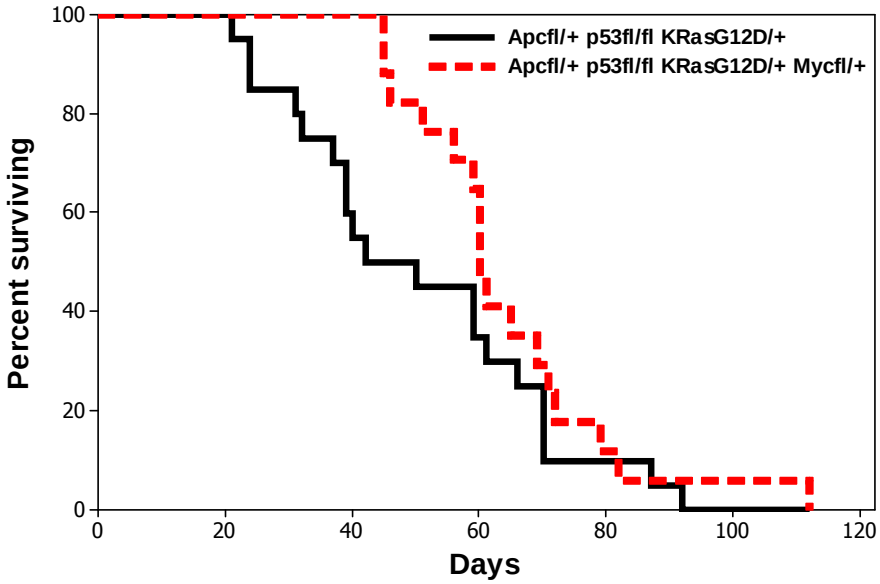


Figure 16: *Myc* heterozygosity does not decrease formation of invasive tumours in the *VillinCre^{ER+} Apc p53 Kras* mouse model.

a) Kaplan-Meier survival curves demonstrating the survival times of *VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Kras$^{G12D/+}$ Myc$^{+/+}$* (black line, n=20) and *VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Kras$^{G12D/+}$ Myc$^{fl/+}$* (red line, n=17) cohorts. There was no significant difference in survival between the two cohorts (Log Rank p=0.177).

b) There was no significant difference observed between the total tumour number across the *VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Kras$^{G12D/+}$ Myc$^{+/+}$* (n=36) and *VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Kras$^{G12D/+}$ Myc$^{fl/+}$* (n=8) cohorts (Mann-Whitney Test p=0.144).

a



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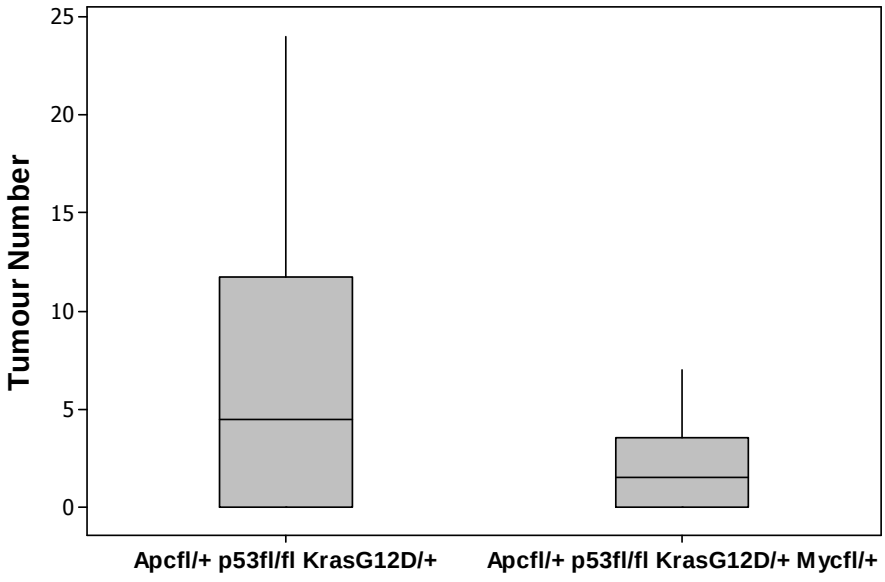
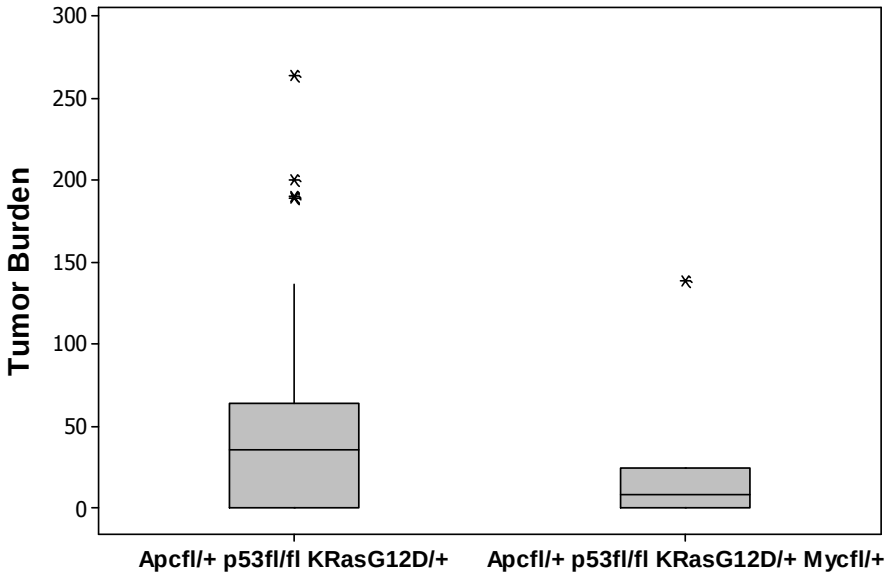


c) There was no significant difference observed between the tumour burden across the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Myc*$^{+/+}$ (n=36) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Myc*$^{fl/+}$ (n=7) cohorts (Mann-Whitney Test p=0.3838).

d) Graph showing the percentage of invasive tumours formed in the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Myc*$^{+/+}$ (n=18) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Myc*$^{fl/+}$ (n=10). There was no significant different across the cohorts (Chi-square test p=0.487).

c



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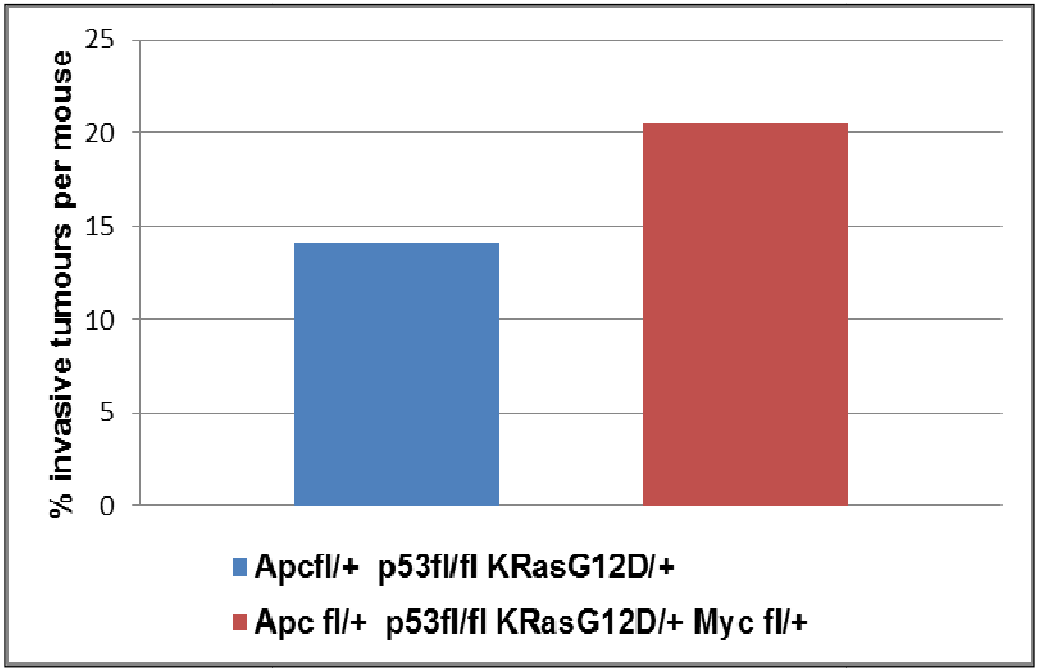


3.6.2 *Arf* deletion

Our *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mouse model of colon cancer easily allows us to further characterize the mechanisms of *Arf* tumour suppression in the absence of the canonical Wnt signalling and loss of *p53* in combination with a *Kras* mutation. Mice lacking *p19*^{ARF} were intercrossed to *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ colonies to create the following cohorts: *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{+/+}$, *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{+/-}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{-/-}$.

The survival rate of the control cohort (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$) was established around 75 days which was very similar to the 80 days of survival in mice carrying deletion of one copy of *Arf* (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{+/-}$). In the case of double deletion of *Arf* (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{-/-}$) the survival rate was reduced to 50 days, which was still not significantly different from the previous two cohorts, the heterozygous condition and the control (Figure 17a).

When comparing the total tumour number there was no significant difference across the three cohorts. Neither was there a difference across the cohorts in the tumour burden of the mice (Figure 17b-c).

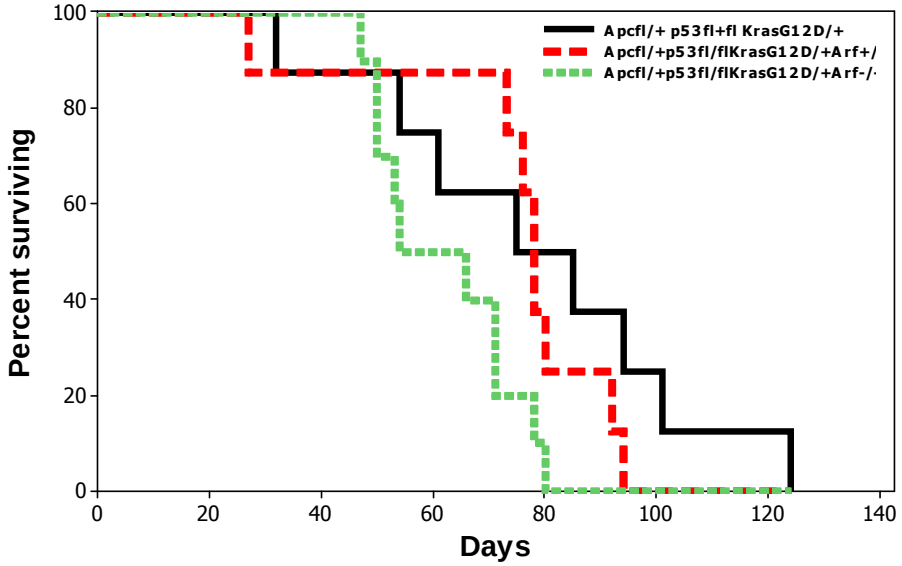
When the tumours were examined under the microscope and classified regarding the invasiveness through the intestinal wall, there was a significant difference between the control mice *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$, in which 14% of the tumours arising from the intestine were invasive; and mice carrying one deletion (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{+/-}$) or double deletion of *Arf* (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{-/-}$), in which the percentages of invasive tumours were approximately 3% and 5.5% respectively (Figure 17d).

Figure 17: Arf deletion reduces the incidence of invasive tumours

a) Kaplan-Meier survival curves demonstrating the survival times. There was no significant difference in survival between the *Arf* wildtype condition *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (n=7) and either the heterozygous *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{+/-}$ (n=8; Log-Rank p=0.72) or the homozygous condition *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{-/-}$ (n=10; Log-Rank p=0.093).

b) There was no significant difference observed in the total tumour number across the cohorts *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (n=14) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{+/-}$ (n=10) (Mann-Whitney Test p=0.960); *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{-/-}$ *(n=9)* (Mann-Whitney Test p=0.669); *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{+/-}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{-/-}$ (Mann-Whitney Test p=0.459).

a



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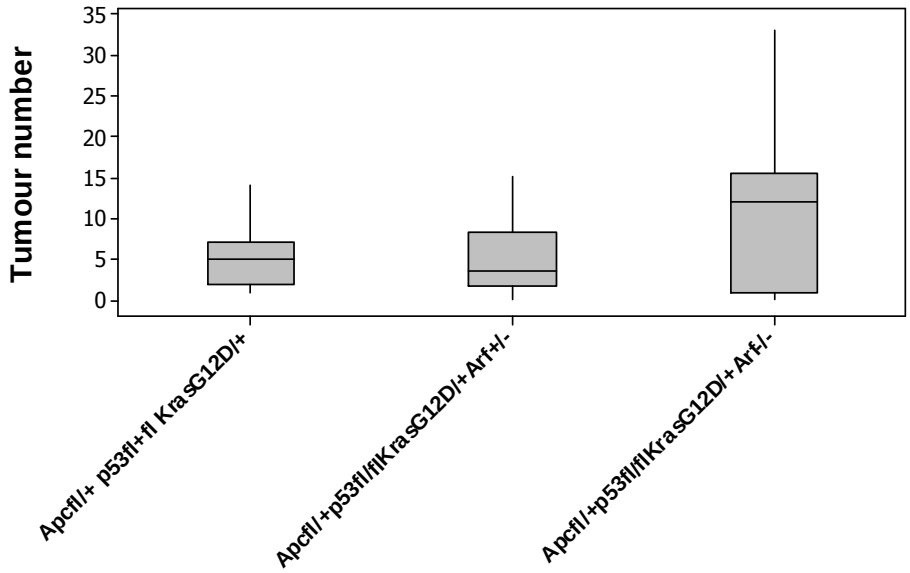
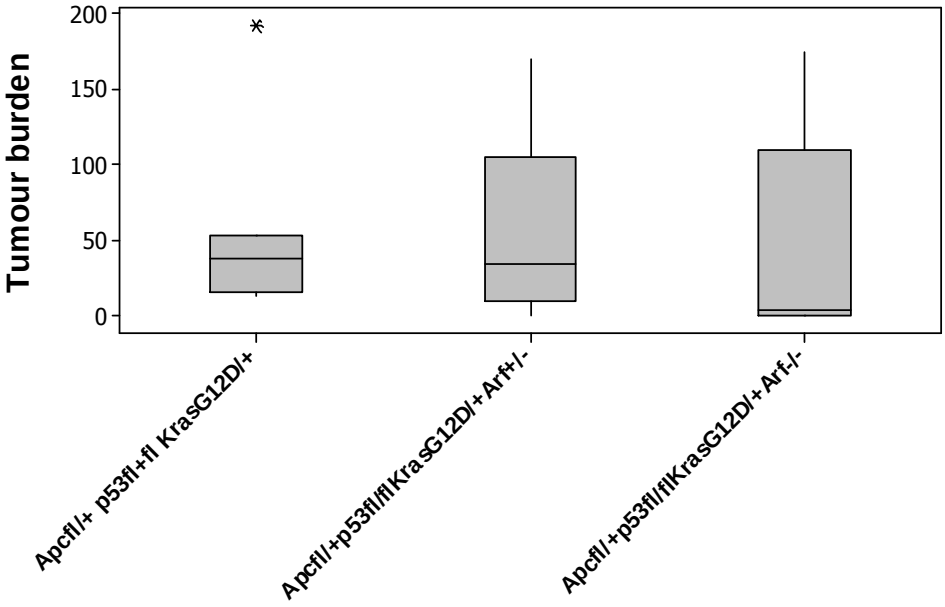


c) There was no significant difference observed in the total tumour burden across the cohorts *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (n=7) and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{+/-}$ (n=9) (Mann-Whitney Test p=0.915); *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{-/-}$ (n=9) (Mann-Whitney Test p=0.243); *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{+/-}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{-/-}$ (Mann-Whitney Test p=0.376).

d) The incidence of invasive tumour formation was decreased from 14.1% in *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ (n=18) cohorts to 2.88 % in *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{+/-}$ cohorts (n=7) and to 5.47 % in *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{-/-}$ (n=12). This decrease was significant (Chi-square test p=0.004).

c



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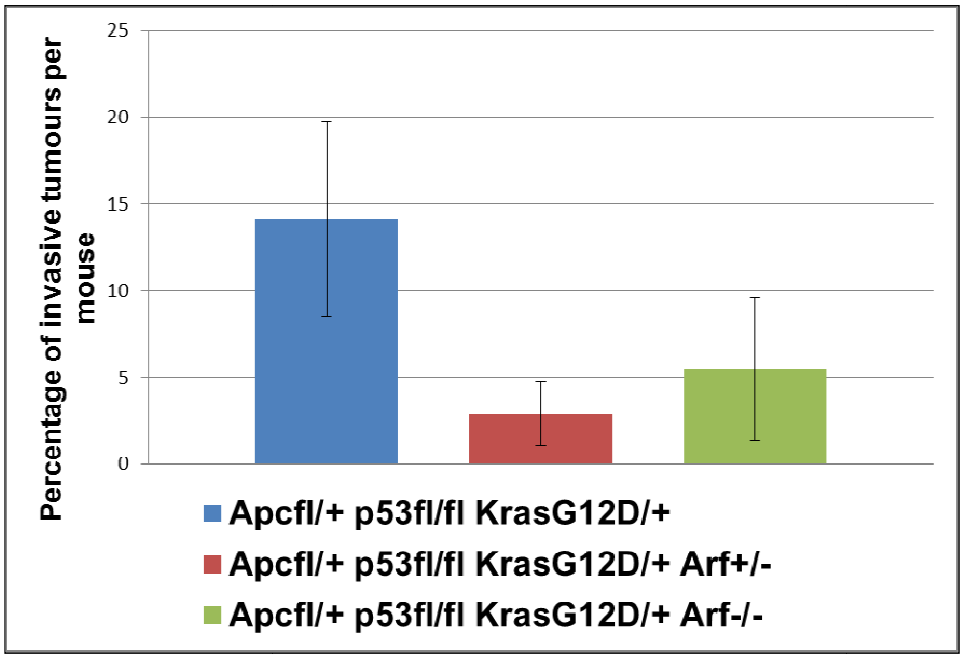

Chapter 4 DISCUSSION

4.1 Intestinal role of *p53* mutation with an *Apc* loss background

Mutations in the *p53* gene are well recognized and categorized as one of the most important events in the development of intestinal cancer. However, its specific effects on the intestine and its relation with other mutated genes in this disease are still unknown.

APC mutations occur early in the CRC process (Vogelstein *et al*. 1988), however, *p53* mutations are considered to occur more frequently in intestinal carcinomas and only rarely in adenomas (Baker *et al*. 1990). This way, *p53* loss or mutation could give advantage in tumour progression after an *APC* mutation has already occurred in the intestine. In previous studies, *p53* loss failed to demonstrate any effect on intestinal tumorigenesis (Fazeli *et al*. 1997). The work of Fazeli and colleagues assessed a model of early stage disease (Apc^{Min}) in which mice become ill within a period between 80 to 210 days of age and only adenoma formation occurred in the intestine. As determined by the study of Baker *et al*, *p53* mutations occur more frequently in intestinal carcinomas than in intestinal adenomas, which suggests *p53* mutation is a late event that occurs near the stage in which an intestinal adenoma develops into its malignant counterpart. Following this idea, we developed a longer-latency mouse model ($AhCre^{+}$ $Apc^{fl/+}$) in which the survival rate of mice was between 300 and 500 days. With this longer latency, an additional *p53* mutation or loss could be of selective advantage in the formation of intestinal adenocarcinomas and will confirm the role of mutations in the *p53* gene in intestinal cancer.

In this $AhCre^{+}Apc^{fl/+}$ mouse model, we obtained a significant acceleration in tumorigenesis with a single allelic *p53* mutation or allele loss ($AhCre^{+}$ $Apc^{fl/+}$ $p53^{fl/+}$, $AhCre^{+}$ $Apc^{fl/+}$ $p53^{R172H/+}$) and the acceleration in tumorigenesis was even more pronounced when a mutant p53 occurred in a p53 loss background ($AhCre^{+}Apc^{fl/+}$ $p53^{fl/fl}$, $AhCre^{+}Apc^{fl/+}$ $p53^{R172H/fl}$) compared to controls ($AhCre^{+}Apc^{fl/+}$ $p53^{+/+}$). These results confirm that loss of *p53* and the R172H mutant have a role in the carcinogenic process in intestinal cancer. There was no difference in tumour number or size across the cohorts, which indicates all the animals were euthanized at the same stage of tumour development.

In order to confirm that the acceleration of tumorigenesis were not due to *Apc* or *p53* mutations alone and that, instead, it was due to the combined effect of both *Apc* and *p53* mutations, we bred mice with double deletion of *p53* in an *Apc* wild type background (*AhCre*$^{+}$ *Apc*$^{+/+}$ *p53*$^{fl/fl}$). None of these mice developed intestinal tumours; these mice became ill due to the occurrence of other non-intestinal tumours, such as lymphoma. With this experiment, we further clarified the collaboration between the deficiency or mutations in these two genes (*Apc* and *p53*) and we established *p53* mutations as an important event in the development of tumorigenesis but not in the intestinal tumour initiation.

The invasion of the tumours seen in the intestine differed between the different cohorts. Only a low percentage of invasive tumours were observed where p53 is wild type (*AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{+/+}$) while there was high percentage of invasiveness in either the cohorts with single *p53* mutation (*AhCre*$^{+}$*Apc*$^{fl/+}$ *p53*$^{R172H/+}$) and the homozygous condition (*AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$) or with a mutation on a p53-deletion background (*AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{R172H/fl}$). There was, however, a similar percentage of invasive tumours in mice with single *p53* loss (*AhCre*$^{+}$*Apc*$^{fl/+}$ *p53*$^{fl/+}$) compare to controls (*AhCre*$^{+}$*Apc*$^{fl/+}$ *p53*$^{+/+}$). These results demonstrate the enhanced oncogenic properties of the mutated p53 protein above single loss of the allele and the similar properties of double deletion and mutation with a concomitant *Apc* deletion. These analyses further confirm previous theories in that *p53* mutations occur late in the development of the disease and at the transition from adenoma to adenocarcinoma formation (Baker *et al*, 1990; Vogelstein *et al*, 1988).

Our *AhCre*$^{+}$ *Apc p53* mouse model recapitulate several aspects of the human disease. Histological grading of invasive tumours from mice resulted in different grades from superficial to deep invasion and metastasis as it is also seen in the human disease following the TNM staging (Edge *et al*, 2010). Even more, when we analyzed the tumours looking for EMT markers, there was no significant difference between the *AhCre*$^{+}$ cohorts *Apc*$^{fl/+}$ *p53*$^{fl/+}$, *Apc*$^{fl/+}$ *p53*$^{R172H/+}$, *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ and *Apc*$^{fl/+}$ *p53*$^{R172H/fl}$. E-cadherin and ZEB1 expression, both markers of EMT changes (Schmalhofer *et* al, 2009), were immunohistochemiscally tested on invasive tumours. There was a decrease of E-cadherin and an increase of ZEB1 at the invasive front of the tumours and this was correlated with the nuclear translocation of β-catenin at the invasive front, manifested as increased intranuclear immunolabelling of this protein. This further characterized the model as an experimental model very similar to the human disesase, even at the molecular level.

Metastasis is the cause of death in at least 50% of cases of CRC and often occurs long before cancer is diagnosed (Hölzel *et al*. 2009). In the *AhCre*$^{+}$ *Apc p53* mouse model, only

one mouse developed metastasis to distant organs despite the aggressive appearance of the tumours examined on histological sections and the confirmed mesenchymal transition of cells at the invasive edges of tumours. Metastasis could have occurred in some other mice but not have been detected by the microscopic examination; this raises the question of whether histology of organs is a good method of detecting distant metastasis since only one microscopic section of the organ is examined. On the other hand, such a wide variety of factors are involved in the process of migration of cells through capillaries and stablishment of neoplastic cells in distant organs that it is probable that more events are involved in the process of metastasis and further studies are needed in order to develop a good metastatic mouse model of CRC.

4.2 Establishing an in vivo model for intestinal cancer with *Kras* mutation and *Apc p53* deletions.

As previously discussed, *RAS* is one of the main genes mutated in human CRC with a frequency of up to 50% of the cases (Forrester *et al*. 1987, Bos *et al*. 1989) and between 30 and 50% in combination with *APC* and *p53* deletions (Wang *et al*. 2007). Previous studies have suggested that mutant $Kras^{G12D}$ accelerates the progression to malignancy of colonic tumours in an *Apc* loss background (Haigis *et al*. 2008). Regarding these previous studies and considering a *p53* mutation as unlikely to be selected for by *Apc* loss alone, we considered *Kras* mutation as the most likely event that will contribute to reveal the phenotype of *p53* loss in an *Apc* loss background.

It was not possible to continue the work on the *AhCre* model since the $Kras^{G12D}$ mutation is lethal in mice during fetal development because of leaky expression from the AhCre transgene. Instead of using *AhCre* , we reproduced the *Apc p53* model with the $VillinCre^{ER}$ transgene, which induces recombination in both the small intestine and colon in the adult mice upon tamoxifen induction. In order to combine *Apc* and *p53* loss with a *Kras* mutation, a *lox stop lox* $Kras^{G12D/+}$ allele was crossed to mice carrying Apc^{fl} and $p53^{fl}$ alleles creating the following colonies: $VillinCre^{ER+}$ $Apc^{fl/+}$, $VillinCre^{ER+}$ $Apc^{fl/+}$ $Kras^{G12D/+}$, $VillinCre^{ER+}$ $Apc^{fl/+}$ $p53^{fl/fl}$ and $VillinCre^{ER+}$ $Apc^{fl/+}$ $p53^{fl/fl}$ $Kras^{G12D/+}$. Survival rate and tumour number (when comparable) were slightly different between the $AhCre^{+}$ $Apc^{fl/+}$ $p53^{fl/fl}$ and the $VillinCre^{ER+}$ $Apc^{fl/+}$ $p53^{fl/fl}$ as it has been exposed in the results. Given this finding, we decided not to compare both cohorts and, instead, create and characterized the whole $VillinCre^{ER+}$ model in order to have as much accurated results as we could.

We first confirmed the effect of *p53* deletion or *Kras* activation alone on mice where one copy of *Apc* was deleted in the adult murine intestine using the *VillinCre*^{ER} technology. As expected, *p53* deletion accelerated tumorigenesis in an *Apc* deficient background reducing the survival curve to less than 200 days; both adenomas and adenocarciomas were formed in the intestine of these mice. These results confirmed the effect of *p53* loss on tumorigenesis when combined with *Apc* loss as we had previously showed in the *AhCre*$^{+}$ *Apc p53* model.

Kras activation (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$) drastically accelerated the tumorigenesis, reducing the survival curve to 100 days, demonstrating the effect of *Kras* activation in intestinal tumorigenesis when combined with *Apc* loss and also suggests *Kras* activation to have more effect in the intestinal tumorigenesis than *p53* deletion alone in an *Apc* loss background. In this *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ model, all the tumours formed in the intestine were numerous adenomas but no adenocarcinoma was found in any of the mice, howevere, there were numerous tumours formed in the colonic epithelium, alhtough any of them were adenocarcinoma. The large total number of tumours, eventhough they were not invasive, is probably the explanation for the reduced survival curve of this *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ cohort; and the fact that tumours in the small intestine (only tissue considered for the graphics) were lower in number than those in the large intestine, explains the low tumour burden for this *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ cohort. With these results we confirmed *Kras* activation has an effect in the tumour progression, but it is not able by its own to induce malignant transformation.

When combining deletions and mutations of the three genes (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$) there was a dramatic acceleration in the tumorigenesis with the survival time being reduced to as little as 50 days. These results highly differ from the controls (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$) and even more with the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ and *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ models which shows that deletions on these three genes have a combined effect during intestinal tumorigenesis and that Kras activation has a clear effect in revealing the mutated *p53* phenotype in an *Apc* deficient background.

With the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ model, both adenomas and adenocarcinomas were found in the intestine of mice, but the size and conversion to adenocarcinoma had become uncoupled as some invasive tumours were less than 1 mm, when all invasive tumours in our previous mouse models (*AhCre*$^{+}$ *Apc p53* and *VillinCre*$^{ER+}$ *Apc p53*) had been much larger, this is reflected in the tumour burden graphic. These small tumours were able to invade through the full thickness of the intestinal wall

and there were even a few metastasis found in distant organs. In fact, the percentage of invasive tumours found in *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mice was much higher (almost 70%) than the percentage of the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ mice (less than 10%), which is reflected in the survival curves. These findings suggest the combined loss of *Apc* and *p53* with *Kras* activation confers the neoplastic cells with increased ability to divide and invade adjacent tissues than with deletions of *p53* or *Kras* activation alone when in an *Apc* loss background.

In the previous *AhCre*$^{+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ model we had proven the similarity between the mouse model and the human disease even at the molecular level through the EMT markers. Immunohistochemical analysis performed on the *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ model showed higher expression of β-catenin at the invasive front; furthermore, there were also increased levels of p-ERK at the invasive front. This is in concordance with previous studies of *Apc Kras* mouse models (Janssen *et al*. 2006) and clarifies the process that leads to a more aggressive behaviour of these tumours and their invasive characteristics. Our results show that intestinal adenocarcinomas achieve invasive capacity through the transition towards a mesenchymal phenotype, in which the combination of these three genes, *Apc*, *p53* and *Kras* is involved. Furthermore this data suggests that the presence of a *Kras* mutation causes a strong selection for a *p53* mutation, both in terms of tumour growth and progression; which is in contrast to *Apc* mutation alone, where there is little immediate selective benefit to losing *p53*.

4.3 SRC family kinases and the conversion to malignant intestinal adenocarcinoma

In the past, CRC has been associated with clear invasive fronts that have an upregulation of nuclear β-catenin and EMT markers and, recently, the expression of EMT markers in cancer stem cells has been associated with high levels of Wnt signaling genes. But for many years, these findings were not consistent with all the cells at the invasive front and no clear effector key for the activation of Wnt signalling pathway has been detected.

One hypothesis concerns the correlation between the high levels of β-catenin at the invasive front with SRC expression, which is overexpressed in many invasive phenotypes (Brunton *et al*. 1997). SRC family kinases activity is involved in the detachment of cells by

disruption of cell-cell adhesions (Calautti *et al*, 1998; Owens *et al*, 2000) and, giving our results, this is possible to occur through the phosphorylation and destabilization of E-Cadherin at adherens junctions. E-Cadherin is a cell-to-cell adhesion molecule to which β-catenin is also attached. A destabilization of E-Cadherin by the explained mechanisms would therefore release β-catenin with its consequent translocation to the nucleus for transcription. This release would explain the higher expression of β-catenin found in cells localised at the invasive front, as these cells are undergoing a higher rate of division and mesenchymal transition. On the other hand, SRC family kinase are directly involved in the stabilization of cell adhesion through the tyrosin phosphorilation of β-catenin and recent studies have demonstrated a direct inhibition of this phosphorilation and therefore decrease in the growth and motility of colorectal cancer cells by using the Src inhibitor Bosutinib (Calautti *et al*, 1998; Coliccia *et al*, 2006; Müller *et al*, 1999).

To prove the relation between SRC expression with β-catenin and E-cadherin expressions at the invasive front, we designed an short-term experiment using one of the inhibitors of Src family kinases: Dasatinib. As expected, tumours from treated mice had lower expression of β-catenin and higher expression of E-cadherin at the invasive front compare with the untreated controls; in addition to lower levels of Src expression. With this experiment we further proved the direct relationalship between activity of SRC family kinases and E-cadherin at adherent junctions and its effects above β-catenin. We could state, therefore, that SRC family kinases are required for the deregulation of Wnt signaling at invasive fronts.

Following this line, given that in this short-term experiment Dasatinib was able to reverse the EMT phenotype through restoration of E-Cadherin levels at the invasive front, we designed a long-term experiment to assess the capacity of Dasatinib to suppress invasive adenocarcinoma formation. Dasatinib clearly reduced the number of invasive adenocarcinoma in treated mice compare with untreated controls, despite not having any effect in reducing the survival time. Dasatinib did not prevent adenoma formation and mice came down due to the burden of numerous intestinal adenomas. In spite of this, Dasatinib should be considered as part of the treatment in patients with high risk of stage II CRC.

4.4 Single cells from harvested tumours were able to reproduce the neoplastic phenotype

We have successfully created a murine model of invasive intestinal cancer, driving some of the pathways and proteins implicated in the process. However, there is still much to know about the molecular keys that can take place in the overall process of intestinal tumorigenesis. There has recently been much interest in the overlap of normal intestinal stem cell (ISC) markers with colorectal cancer stem cell markers. Two recent studies have worked on the prediction of a good or poor prognosis of human CRC based on these ISC markers (Merlos-Suarez *et al*. 2011; de Sousa *et al*. 2011). While one of the studies confers poor prognosis to the high expression of the ISC marker genes, the other attributes the poor prognosis to the methylation of this subset of genes. In any case, both studies agreed in that a Wnt high population marks a colon cancer stem cell population.

In our studies, we have seen high levels of Wnt signaling downstream genes in association with the invasive front of tumours and expression of a number of ISC and EMT markers. Working with isolated tumoral cells would allow further characterization of these protein properties in the CRC. A common difficulty that the studies of Merlos-Suarez and de Sousa could have found is that their experiments were developed from sorted populations of cancer cells and thus the precise expression of these signatures in a cell by cell basis in the tumour is unclear. The design of an experiment using tumoral cells harvested from neoplastic murine intestine to re-grow them in culture plates would hopefully offer the ideal scenario for this study.

We succesfully cultured cells extracted from the intestines of the *VillinCre^{ER+} Apc p53* and *VillinCre^{ER+} Apc p53 Kras mice* (*VillinCre^{ER+} Apc$^{fl/fl}$ p53$^{fl/fl}$*, *VillinCre^{ER+} Apc$^{fl/fl}$ p53$^{fl/fl}$ Kras$^{G12D/+}$*, *VillinCre^{ER+} Apc$^{fl/fl}$ p53$^{R172H/fl}$*, *VillinCre^{ER+} Apc$^{fl/fl}$ p53$^{R172H/fl}$ Kras$^{G12D/+}$*). The cells were able to survive and recreate spheres that recapitulated the characteristics of the intestinal crypts. There was no difference in the growing pattern and characteristics between cells taken from already formed adenomas and cells taken from still non-tumoral, but already induced, crypts. Even more, cells transplanted into subcutaneous tissue of nude mice were able again to reproduce similar adenocarcinomas as seen in the intestine of the transgenic mice; in this case the cells had a very different environment to that of a culture plate but this did not change the tumorigenic capacities.

This clearly indicates the cells conserve the tumour phenotype and are able to form tumours following culture and transplantation, which suggest all cells have tumour initiating capacities.

This culture cell experiment would be valuable for studying the molecular characteristics of tumour growth and will allow elucidation of the role of intestinal stem cells in the tumorigenesis process.

4.5 Role of other candidate genes in CRC

There are quite a few genes that are also known to be implicated in the CRC disease and that needed to be further characterized. Two of these candidate genes are *MYC* and *ARF*.

4.5.1 *Myc* deletions do not modify the *VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$* and *VillinCre^{ER+}Apc$^{fl/+}$ p53$^{fl/fl}$ Kras$^{G12D/+}$* phenotypes

Myc deletions in an *Apc*-deficient background have been observed to rescue the unrestricted intestinal crypt proliferative phenotype of the *Apc* loss, even the heterozygous condition (*Myc$^{fl/+}$*) had an effect in partially reducing the proliferation of crypts (Athineos *et al*. 2010; Sansom *et al*. 2007). In our models, we observed overexpression of c-Myc at the invasive front of intestinal tumours. Given this, a deletion in *Myc* would theoretically reveal an effect in tumour progression and invasion. In the models we created (*VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$* and *VillinCre^{ER+}Apc$^{fl/+}$ p53$^{fl/fl}$ Kras$^{G12D/+}$*) the aditional single deletion of *Myc* (*Myc$^{fl/+}$*) did not increase the survival rate of mice and did not reduce the tumour burden or total tumour number in either the *VillinCre^{ER+} Apc$^{fl/+}$ p53$^{fl/fl}$ Myc$^{fl/+}$* or *VillinCre^{ER+}Apc$^{fl/+}$ p53$^{fl/fl}$ Kras$^{G12D/+}$ Myc$^{fl/+}$* models. Moreover, there was no reduction in the percentage of invasive tumours in the intestine of these mice. While the previous studies were performed in an *Apc Myc* model, the addition of a *p53* mutation in our models might have confered the cells the ability to compensate for the lack of *Myc* and which may explain why we did not observe a reduction in the formation of tumours as expected. Another possible explanation for these results is that, although in the experiment of Athineos *et al*, even a single deletion of *Myc* was able to rescue the *Apc* loss phenotype, our model contains additional double *p53* deletion and *Kras* mutation, which may require a stronger effect from the lack of *Myc*, such as double deletion (*Myc$^{fl/fl}$)* to be able to show the rescue phenotype observed in the experiment of Athineos. Further experiments are

therefore necessary in this field to characterize the effect of *Myc* deletion on an *Apc p53 Kras* background.

4.5.2 *Arf* deletion reduces the incidence of invasive tumours

The ARF gene has been considered as a tumour suppressor gene for many years and its suppressor functions were actually demonstrated in a study of mouse embryogenic stem cells (Kamijo *et al*. 1997). In the past few years there has been an emerging theory that confers *ARF* tumour promoter characteristics (Humbey *et al*. 2008; Herkert *et al*. 2010). When *Arf* was deleted in our experiments, tumour survival rates were not increased in either the heterozygous or homozygous condition (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{fl/+}$ or *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{-/-}$) and there was no difference either in the tumour number or tumour burden across these cohorts. Regarding these results, we can state that, at least in our *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mouse model, deletion of *Arf* in the murine intestine, either single or double, did not confer any advantage for the tumour progression but neither for the prevention of tumour formation. In a study performed by Bennecke *et al*., lack of *Ink4a/Arf* in mice with a *Kras*^{G12D} mutation allowed colorectal tumours to metastasize in the absence of Wnt pathway (Bennecke *et al*, 2010); although this study follows a different pathway than our models (absence of Wnt signalling), it would be interested to test in our model the possible effects of *Arf* deletion in the presence of *Kras* mutation alone (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *Kras*$^{G12D/+}$ *Arf* $^{+/-}$).

When the tumours were examined under the microscope, striking results were obtained. Mice carrying additional single or double deletions in *Arf* (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{fl/+}$ or *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ *Arf* $^{-/-}$) had significantly less numbers of invasive tumours than controls (*VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$). These results strongly support the theory suggested by Herkert et al. who attributed to ARF the creation of the complex *Myc/Miz1* that inhibits genes involved in cell to cell adhesion; in these experiments, the inhibition of the cell to cell adhesion lead to the apoptosis of the cell (Herkert *et al*. 2010). Following this theory, in the absence of apoptotic signals, such as loss of p53 as we have in our *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ mouse model, the inhibition of the cell to cell adhesion genes will not lead to immediate apoptosis but, instead, it would lead to the disgregation of epithelial cells and, therefore, the transition to a mesenchymal phenotype; in this context *Arf* will therefore act as a tumour promoter. Our results confirm that *Arf*, in a *VillinCre*$^{ER+}$ *Apc*$^{fl/+}$ *p53*$^{fl/fl}$ *Kras*$^{G12D/+}$ background, despite not having any apparent effect in the formation of tumours in the intestine, it actually has an effect in the progression of these tumours towards an invasive phenotype, probably

through its involvement in the cell-cell adhesion properties of the cells and the combination with *p53* loss. Further studies will be important to understand the exact mechanisms involved in this process.

FUTURE WORK

This model of colon cancer has been proven extremely useful for the study of later stages of cancer such as invasion through the intestinal wall thickness, progression to mesenchymal phenotype and metastasis to distant organs.

We have, preliminarily, tested the drug Dasatinib, which has been effective in preventing the tumours from invading mainly via impeding the cells to exhibit EMT changes. Since Dasatinib inhibits other Kinases rather than only SRC (Araujo *et al*, 2010), the reduction in invasion observed in our experiment could have been related with the inhibition of oher kinases, not only SRC; the use of other more specific SRC inhibitors, such as Saracatinib, are therefore necessary to perform.

In addition, there are more factors involved in the process of invasion, such as the creation of a proper matrix to facilitate the infiltration of neoplastic cells into adjacent tissues. In this context, lysyl oxidase (LOX) has a main role in catalyzing the cross linking of collagen and elastin in the extracellular matrix (ECM) and it has been directly implicated in promotion of the invasion and metastasis of some solid tumours, including colorectal cancer (Cox *et al*, 2011; Kirschmann *et al*, 2002; Baker *et al*, 2011). Recent studies have linked LOX with the reduced phosphorilation of SRC and there are evindence of abolition of LOX-associated dimensional growth of cells by treatment with Dasatinib (Baker *et al*, 2011). Testing the LOX inhibitor, lox-ab, in our model would yield more information in this field and it may further characterizes the relation with Dasatinib.

Another emerging factor involved in the invasion of cells is the *ARF* tumour suppressor gene. Our results showed a possible role for this gene in the mesenchymal transition and therefore invasion of metastatic cells, this line of study could yield interesting results in the future.

On the other hand, allograft injection of cultured cells from primary tumours in the mice has been seen to be successful in the first instance. Our aim is to develop other better models of metastasis using these cultured cells. The next step then is to inject the cells orthotopically into the intestinal mucosa of mice to assess the ability to invade the tissue and therefore metastasize. With these allografts and orthotopic models, we would then be able to test other drugs whose main effect is to repress the metastatic growth of the

neoplasms. This will also provide a good framework in which further investigate the tumour initiating properties at the molecular level.

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