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DOTTORATO DI RICERCA IN

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CICLO XXV

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Characterization of hERG1 channel role in the regulation of the process of neo-angiogenesis: *in vitro* and *in vivo* studies.

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SUMMARY

Sustained angiogenesis is a critical step in tumour progression; indeed the clinical success of angiogenesis inhibitors has confirmed this paradigm. However, the efficacy of anti-angiogenic therapy has been more challenging than expected, mainly due to intrinsic resistance to anti-angiogenic drugs. Hence the identification of novel mechanisms sustaining tumour angiogenesis, and the development of appropriate pharmacologic targeting of new angiogenesis-related molecules is a major challenge in cancer therapy.

In this thesis, we report a new mechanism that regulates angiogenesis in colorectal cancers (CRC).

The human *ether-à-go-go related gene* (hERG)1 K^+ channel is up-regulated in human colorectal cancer cells and primary samples.

Beyond its canonical role in excitability, hERG1 channel is emerging as a major regulator of intracellular signalling, in tumour cells. This role relies on its ability to assemble with partner proteins, and particularly adhesion receptors of the integrin family. In this thesis, we found that β1 integrin-mediated adhesion to the extracellular matrix (ECM) modulates secretion of vascular endothelial growth factor (VEGF)-A. This effect relies on an intermolecular cooperation between the β1 integrin, hERG1 channels and the p85 subunit of the phosphatidyl inositol-3-kinase (PI3K). Such cooperation is triggered by the ECM and leads to activation of Akt, which in turn increases the HIF-dependent *VEGF-A* transcription. The latter occurs through a mTOR- and FOXO- independent, NF-kB-activated regulation of HIF-1α and HIF-2α.

Moreover, we examined the role of hERG1 in colorectal carcinogenesis *in vivo,* using two mouse models: $Apc^{min/+}$ and Azoxymethane-treated mice.

Colonic polyps of $Apc^{min/+}$ mice over-expressed *m-ERG1* and their formation was reverted by the hERG1 blocker E4031.

Azoxymethane was applied to either hERG1-transgenic mice, which over-express hERG1 in the mucosa of the large intestine, or wild type mice. A significant increase of both mucin-depleted foci and polyps in the colon of hERG1-transgenic mice was detected.

Both the intestine of transgenic mice and colonic polyps of $Apc^{min/+}$ showed an up-regulation of pAkt/VEGF-A and an increased angiogenesis, which were reverted by treatment with E4031, providing evidence that, also *in vivo,* the role of hERG1 in CRC carcinogenesis can be traced back to its role in the signalling pathways which regulate VEGF-A secretion and neo-angiogenesis.

On the whole, this study assigns a relevant role to hERG1 in the process of *in vitro* and *in vivo* colorectal carcinogenesis. hERG1 can be considered a "tumour progression" gene *in vitro*, since a complex comprising integrins and hERG1 channels strongly regulate angiogenesis and tumour progression in CRC cells model, as well as *in vivo*, since the channel strongly cooperates with genetic (loss of the tumour suppressor gene Apc) or environmental (chemical carcinogen) factors in triggering CRC progression in mouse CRC models.

Our results indicate the use of hERG1 blocking drugs devoid of cardiac side effects as a novel anti-angiogenesis treatment of CRC, especially in those cases resistant to anti-VEGF-A drugs.

1. INTRODUCTION

1.1 COLORECTAL CANCER

Colorectal cancer (CRC) is the second cause of cancer-related death, behind lung cancer, in developed countries. It is estimated that 20-30% of CRC have a familiar or hereditary component, but germline mutations of characterized genes have been reported in only about 5% of cases (Rustgi 2007). The two main forms are categorized based on the presence of a more or less large number of adenomatous polyps and are called familiar adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), respectively. In these highly penetrant inherited syndromes, one copy of a gene is constitutionally inactivated in all cells of the individual. The second allele is somatically inactivated in the tumour following the two-hit model of cancer progression originally proposed by Knudson for tumour suppressor genes (Knudson, 2001). FAP is easy to recognize since patients who inherit an APC gene germline mutation present with hundreds or thousands of polyps in their colon. The prevalence of FAP is estimated to be about 1% of all CRC cases (Bisgaard et al., 1994). HNPCC involved in the Lynch syndrome is more frequent than FAP and is caused by germ cell mutations that invalidate the DNA-repair systems. DNA mismatch repair is deficient in 90% of the HNPCC patients.

A large majority of CRCs are sporadic (70%) and are strongly associated with environmental causes, nutrition-related complex disease (e.g. obesity and diabetes mellitus type II), inflammatory situations, ulcerative colitis and Crohn's disease (Terzić et al., 2010). Sporadic cancers can be divided into two groups according to the initiating mechanisms, generally a loss or inactivation of tumour suppressors and constitutive, dominant activation of proto-oncogenes. Tumour progression in the digestive tract is controlled by the lifestyle such as dietary factors, high caloric intake, obesity, sedentary life and tobacco smoking. Sporadic colorectal cancer arises mainly through two distinct pathways: chromosome instability (CIN) and microsatellite

instability (MSI). CIN accounts for 80% of sporadic cases and is connected with loss of heterozygosity (LOH): the initial mutation is the inactivation of the APC tumour suppressor gene followed by clonal accumulation of alterations in additional oncogenes (KRAS, 50% of tumours) and suppressor genes on chromosomes 18 and 17 (DCC; p53 gene, found in 70% of tumours and associated with a shift to a malignant tumour). LOH at a genetic locus can arise by chromosome duplication, subchromosomal rearrangements and loss, somatic recombinations and deletions. MSI occurs in 15-20% of sporadic colorectal cancers; alterations have been found to cluster in genes encoding enzymes involved in the repair of DNA mismatches (in particular MLH1 and MSH2). Each one of these genetic and molecular aberrations leads to the progressive and irreversible loss of the normal control of cell growth and differentiation (Arnold CN. et al., 2005). This in turn ensures in the accomplishment of specific morphological steps which lead the normal colonic mucosa to a true invasive carcinoma, through specific lesions such as aberrant crypt foci (ACF), mucin-depleted foci (MDF), small adenomas, large adenomas with high grade dysplasia (Fig.1) (Weinberg RA. 1989; Vogelstein B. et al., 1988; Ponz de Leon M. and Percesepe A. 2000).

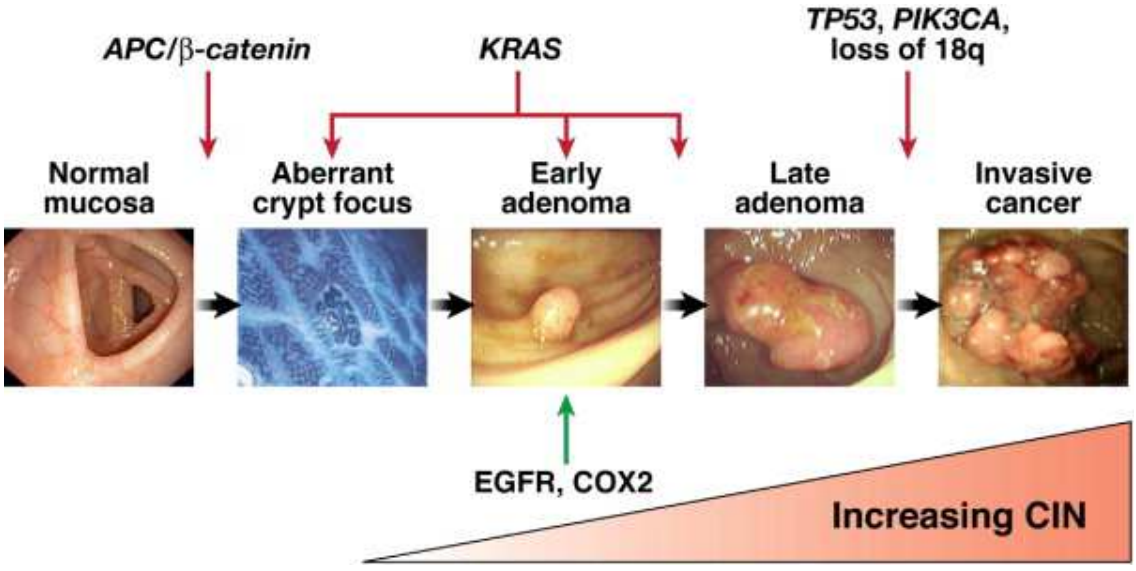

Figure 1: Colorectal adenoma-carcinoma progression sequence. The initial step in colorectal tumourigenesis is the formation of aberrant crypt foci (ACF). Activation of the Wnt signalling pathway can occur at this stage as a result of mutations in the APC gene. Progression to larger adenomas and early carcinomas requires activating mutations of the proto-oncogene KRAS, mutations in TP53, and loss of heterozygosity at chromosome 18q. Mutational activation of

PIK3CA occurs late in the adenoma–carcinoma sequence in a small proportion of colorectal cancers. Chromosomal instability is observed in benign adenomas and increases in tandem with tumour progression.

In the colon mucosa, epithelial stem cell progenitors localized in the stem cell niche at the crypt base are characterized by high proliferation rate and potential to differentiate into several lineages though the dynamic migration of mature epithelial cells to the apical side of the colon crypt (Fig.2).

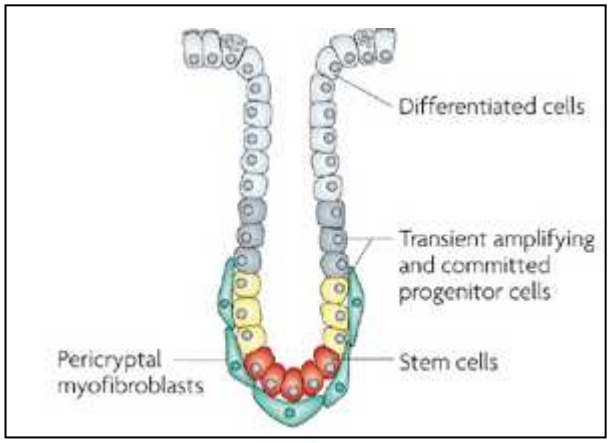

Figure 2: The human colon epithelial crypt unit. Stem cells are housed in the base of the crypts where they communicate with the niche cells-the pericryptal myofibroblasts, which are outside the crypt but communicate by cell signalling. Stem cells feed the transit-amplifying compartment, where most cell production occurs. This portion of the crypt is thought to also house the committed progenitor cells: cells committed to one or more cell lineages.

Epithelial progenitors and their differentiated counterparts in colon crypt units are in direct interface with extracellular matrix (ECM) molecules and growth factors produced in a complementary manner by epithelial crypts and stroma cells. Stem- and progenitor-cell lineages in colon crypts are the target of the neoplastic transformation, due to their continuous active mitosis associated with increased risk of DNA alterations and sequential accumulation of molecular defects inherent to the complex mechanisms of DNA replication and repair and cell division. Several evidences support the existence of a subpopulation of cancer cells with stem cell-like (SCL) phenotypes in solid tumours of the digestive system (Gespach, 2010). The cancer stem cell concept is therefore at the edge between a transient and reversible state and identity of self-renewing pluripotent progenitors that are produced during regeneration, remodelling

and carcinogenesis. Stress conditions linked to digestive processes and environmental pressure, infections, pathogens, carcinogens strongly influence these mechanisms at the proliferation-differentiation interface and maintenance of colon stem cells and progenitors. The tumour stroma microenvironment and the immune system also play a critical role in tumour initiation and the emergence of metastatic cancer cells. Cancer cell spreading and dissemination from primary tumours are influenced by the invasive behaviour of resident tumour myofibroblasts induced by TGFβ (De Wever et al., 2004a,b; Denys et al., 2008).

1.2 THE PROCESS OF ANGIOGENESIS

In vertebrates, the circulatory system is the first functional organ system to arise and is critical in providing adequate oxygen and nutrient delivery to rapidly developing tissues. The vasculature is formed through three main cellular processes: vasculogenesis, angiogenesis and arteriogenesis (Fig.3). Vasculogenesis, the de novo formation of blood vessels, gives rise to the first blood vessels, establishing a primary vascular plexus. Angiogenesis, the growth of blood vessels from pre-existing blood vessels, provides a prominent expansion of the vascular plexus, while arteriogenesis involves an increase in arterial vessel diameter in response to increased blood flow or shear stress. Through these three mechanisms a circulatory system is formed and remodelled into a complex vessel system that mediates a wide range of vital physiological processes including tissue oxygenation, nutrient delivery and waste removal, immune response, temperature regulation, and the maintenance of blood pressure. Accurate coordination of cellular events permits the formation and modification of the vascular system. Moreover, numerous signalling pathways is known to play a pivotal role in activating and modulating these events.

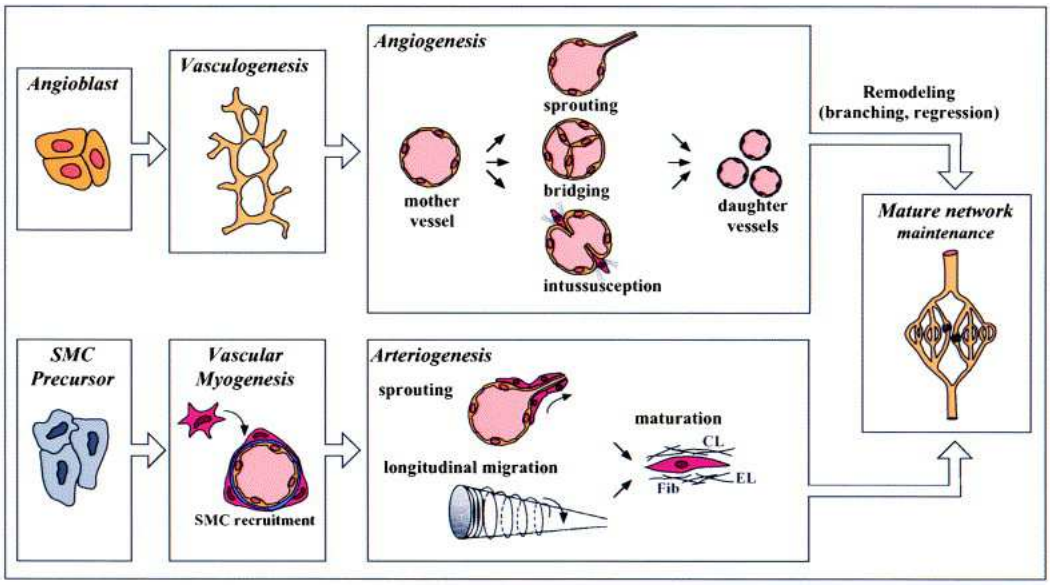

Figure 3: Angioblasts in the embryo assemble in a primitive network (vasculogenesis) that expands and remodels (angiogenesis). Smooth muscle cells (SMCs), originating from SMC precursors, cover endothelial cells during vascular myogenesis and stabilize vessels during arteriogenesis. CL, collagen; EL, elastin; Fib, fibrillin.

1.2.1 Angiogenic factors.

Angiogenesis occurs as a result of interaction between proangiogenic factors and their inhibitors, and between them and the ECM (Salcedo et al., 2005; Turner et al., 2003). Both physiological and pathological angiogenesis are the result of pro-angiogenic factors having an advantage over inhibitors of this process (Salcedo et al., 2005; Moons et al., 2002; Dupuy et al., 2003).

- Activators of angiogenesis include: 1) VEGF: induces and enhances angiogenesis/vasculogenesis, vascular permeability, endothelial cells (EC) proliferation, migration and adhesion of leukocytes; 2) receptors for VEGF: integrate and transmit stimuli; 3) nitric oxide (NO): dilates blood vessels; 4) integrins: α5β1, αvβ3 and αvβ5 (receptors for extracellular matrix (ECM) proteins and macromolecules), they mediate the sprouting blood vessels, are mobilised during EC migration and are involved in intercellular communication (as ECM receptors); 5) transforming growth factor beta 1 (TGFβ1) and its receptors: promotes maturation of blood vessels, deposition of ECM components, induces EC proliferation, differentiation of mesenchymal cells to pericytes; 6) growth factors: acidic fibroblast growth factor

(aFGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), TGFα (Carmeliet and Jain, 2000; Kuo et al., 2011; Zhu et al., 2011; Garcia de la Torre et al., 2002; Turner et al., 2003; Medina et al., 2004), insulin-like growth factor I (IGF-I) (Carmeliet and Jain, 2000; Marek et al., 2001): they induce proliferation of EC and at various stages stimulate angiogenesis; 7) platelet-derived growth factor (PDGF) and its receptors: recruit smooth muscle cells; 8) plasminogen activators (uPA: urokinase plasminogen activator), matrix-metalloproteinases (MMPs), heparinases, chymases, tryptase, cathepsin: rebuild ECM, release and activate growth factors (Carmeliet and Jain, 2000; Garcia de la Torre et al., 2002; Turner et al., 2003; Medina et al., 2004); 9) angiopoietin-1 (Ang-1): prevents apoptosis of EC, promotes vascular sprouting and branching, stabilises vessels and intercellular contacts, inhibits vascular permeability; Tie-2: receptor for Ang-1 and Ang-2 (the soluble form of sTie-2 is their natural inhibitor); angiopoietin-2 (Ang-2): in the presence of VEGF facilitates vascular sprouting (Carmeliet and Jain, 2000; Iribarren et al., 2011; Khoury and Ziyadeh, 2011; Melgar-Lesmes et al., 2008; Garcia de la Torre et al., 2002; Turner et al., 2003; Medina et al., 2004; Carmeliet, 2003; Visconti et al., 2002; Salcedo et al., 2005; Taura et al., 2008); 10) plasminogen activator inhibitor 1 (PAI-1): stabilises new blood vessels (protects against degradation of the provisional ECM located around the new blood vessels) (Carmeliet and Jain, 2000; Garcia de la Torre et al., 2002; Turner et al., 2003; Medina et al., 2004); 11) angiotensin II: induces the VEGF (Yoshiji et al., 2007); 12) monocyte chemotactic protein 1 (MCP-1); 13) chemokines; 14) hypoxia-inducible factor 1 alpha (HIF-1α): induces angiogenesis (Carmeliet and Jain, 2000; Garcia de la Torre et al., 2002; Turner et al., 2003; Medina et al., 2004) and 15) IL-8 (Garcia de la Torre et al., 2002; Turner et al., 2003), IL-1, epidermal growth factor (EGF),prostaglandin (PGE 1, PGE 2, PGF), erythropoietin, histamine, bradykinin, fibrin, heparin (Jośko et al., 2000), tumour necrosis factor alpha (TNFα) (Xiao et al., 2011).

- Angiogenesis inhibitors include: 1) soluble Flt-1 (sFlt-1) and NRP-1 (sNRP-1): binding VEGF and thereby reducing its biological activity; 2) Ang-2: an antagonist of

Ang-1, increases vascular permeability, destabilises the vessel, causes the death of EC; 3) thrombospondin-1 and -2 (TSP-1, -2): inhibits the migration, growth, adhesion of endothelial cells, and shortens their lifespan (inhibits EC proliferation), inhibits the formation of vessel lumen; 4) angiostatin (an internal fragment of plasminogen): inhibits angiogenesis, tumour growth; 5) endostatin (collagen XVIII fragment): inhibits the migration and shortens the lifespan of endothelial cells, increases apoptosis of tumour cells; 6) vasostatin: inhibits the proliferation of EC; 7) platelet factor-4: inhibits the binding of VEGF and bFGF, EC proliferation; 8) tissue inhibitor of metalloproteinases (TIMPs): inhibit the degradation of ECM, EC proliferation, pathological angiogenesis; 9) interferon alpha (IFNα), IFNβ, IFN, inteleukin 4 (IL-4), IL-12, IL-18: inhibit EC proliferation and migration, inhibit bFGF; 10) VE-cadherin (vascular endothelial cadherin), platelet endothelial cell adhesion molecule-1 (PECAM-1): adhesion molecules (including intercellular adhesion), increase the tightness of vessels; 11) claudins, occludin, junctional adhesion molecules (JAM) -1, -2, -3: molecules "tightening" vascular connections, intercellular adhesion, increases vascular integrity; 12) connexins: molecules facilitate intercellular communication; 13) αvβ3 and αvβ5 integrins: shorten lifespan of EC, which is mediated by VEGF and KDR; 14) antithrombin III, LIF (leukaemia inhibitory factor): inhibit the proliferation of EC; 15) an excess of Ang-1: over-sealing blood vessels makes them harder to sprout; 16) PRL: inhibits VEGF and bFGF; 17) osteopontin: interferes with the transmission of the integrin stimuli sprouting (Carmeliet and Jain, 2000; Iribarren et al., 2011; Khoury and Ziyadeh, 2011; Melgar-Lesmes et al., 2008; Turner et al., 2003; Medina et al., 2004; Visconti et al., 2002; Nyberg et al., 2005); 18) somatostatin (Garcia de la Torre et al., 2002; Yarman et al., 2010); and 19) angiostatic steroids (medroxyprogesterone, 2-metoxyoestradiol) (Jośko et al., 2000).

1.2.2 Tumour angiogenesis.

In 1971, Judah Folkman, who became known as the "father of tumour angiogenesis," first emphasized the importance of tumour vascularity for tumour growth (Folkman,

1971). He described how, if a tumour could be stopped from growing its own blood supply, it would wither and die (Fig.4).

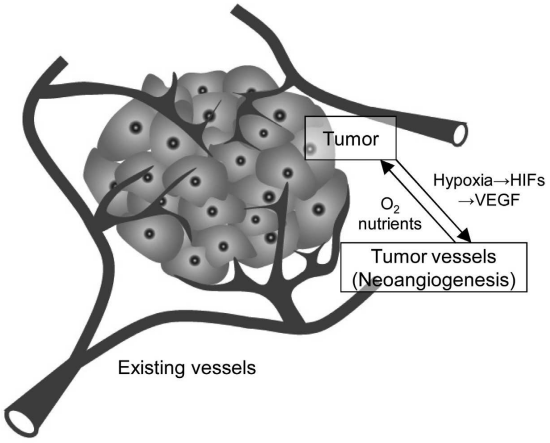

Figure 4: Association between tumour growth and its vascularity. Adult vasculature is stable and rarely proliferates under physiological conditions. However, in cancer, existing vessels again start to grow (neoangiogenesis) in response to hypoxia inducible factor (HIF)-driven VEGF expression in tumours. Newly formed vessels provide oxygen and nutrients to rapidly expanding tumours.

VEGF was first discovered by Senger and colleagues as a vascular permeability factor secreted by a guinea pig tumour cell line (Senger et al., 1983). Various *in vitro* and *in vivo* studies have since uncovered the role of VEGF as a central player in both physiological and pathological angiogenesis (Ferrara, 2009). Pathologically expanding tumours rapidly exhaust the available oxygen supply (when the tumours are 1-2 mm in size) and become hypoxic. The activation of hypoxia-inducible factor (HIF) signalling in hypoxia-sensing cells triggers VEGF expression (Carmeliet et al., 1998, Semenza et al., 1999). VEGF is secreted not only by tumour cells but also by tumour-associated stromal cells (Fukumura et al., 1998). In turn, secreted VEGF stimulates vascular growth into hypoxic tumour tissues to meet the tumour's oxygen requirements. Tumour angiogenesis resembles physiological angiogenesis but presents notable differences (Chung and Ferrara, 2011). A principal difference is that tumour angiogenesis is not self-limited but appears to be a self-perpetuating and persistent process that is not turned off once vessels are formed. Tumour vessels are disorganized compared to normal vessels and display a number of abnormalities, including fragility, reduced number of pericytes, and leakiness with a propensity to produce exudates and to bleed.

These abnormalities have been linked to excess VEGF-A, and thus VEGF neutralization has been reported to somewhat "normalize" the tumour vasculature (Jain, 2005). The tumour cells, particularly melanomas and glioblastomas, may undergo a process of transformation, named "vasculogenic mimicry," such that the tumour cells or the differentiated tumour stem cells acquire phenotypic characteristics of endothelial cells and become components of the vessel wall (Hendrix et al., 2003a,b; Ricci-Vitiani et al., 2010; Wang et al., 2010a). A number of preclinical and clinical studies using inhibitors of VEGF have identified VEGF-independent pathways of tumour angiogenesis, which may account for the poor responses and/or acquired resistance to VEGF neutralization (Shojaei et al., 2007). One such pathway involves the neutrophil-derived proangiogenic factor Bv8 (also known as prokineticin 2), which drives endothelial cell proliferation and migration in the absence of VEGF-A (Shojaei et al., 2007, 2009). Other pathways markedly modulate VEGF expression and/or VEGF-A-induced responses, both physiologically and in cancer, including the Dll4/Notch (Hellström et al., 2007; Noguera-Troise et al., 2006; Ridgway et al., 2006), Angiopoietin-1/Tie2 and Angiopoietin-2/Tie2 (Augustin et al., 2009), platelet-derived growth factors (PDGFs)/PDGF-receptor β (Bergers et al., 2003), and TGFβ1/TGFβ receptor II (Chung and Ferrara, 2011; Fridlender et al., 2009).

1.2.3. PI3K/AKT signalling.

PI3K and AKT may regulate tumour angiogenesis by several downstream targets such as mTOR/p70S6K1 signalling axis, the inhibition of FOXO, the induction of NOS (Emerling et al., 2008; Engelman et al., 2006; Quintero et al., 2006; Wang et al., 2004), and/or the inhibition of GSK-3β. These targets commonly increase HIF-1α expression which induces VEGF transcriptional activation. Inhibition of GSK-3β by the activation of PI3K/AKT can up-regulate HIF-1α expression and increases β-catenin activity, which can enhance HIF-1-mediated transcription through the β-catenin-HIF-1α interaction at the promoter region of HIF-1 target genes (Kaidi et al., 2007; Mottet et al., 2003). In addition, hypoxia is a hallmark of the tumour microenvironment in the fast growth

tumour: hypoxia induces HIF-1α production through the increase of its stability and the activation of ERK1/2 pathway. In some kinds of cancer cells, hypoxia stimulates multiple K-ras effectors and PI3K, which induces VEGF expression in a HIF-1-dependent manner or via PI3K/Rho/ROCK/c-myc pathway (Mizukami et al., 2006; Xue et al., 2006). PI3K can induce VEGF expression through HIF-1, ERK1/2, and NF-κB activation to induce tumour angiogenesis. NF-κB can also stimulate tumour necrosis factor (TNF), CXCL-8, IL-1, and IL-6 to induce VEGF (Amiri and Richmond, 2005; Sparmann and Bar-Sagi, 2004). Growing evidence has shown the key roles of PI3K, AKT, mTOR, and their effectors HIF-1α and VEGF in regulating cancer cell-induced angiogenesis (Fang et al., 2007; Hu et al., 2005; Xia et al., 2006). PI3K/AKT can increase VEGF expression and suppress TSP-1, the endogenous antiangiogenic molecule, in both cancer cells and endothelial cells (Niu et al., 2004; Wen et al., 2001). Furthermore, $AKT1^{-/-}$ mice showed impaired vascular maturation with decreased expression of TSP-1 and TSP-2, while re-expression of TSP-1 and TSP-2 in mice transplanted with wild-type bone marrow is associated with the angiogenic abnormalities in $AKT1^{-/-}$ mice (Chen et al., 2005). Thus, PI3K/AKT signalling pathway induces tumour growth through the over-expression of angiogenic factors and the inhibition of antiangiogenic molecules. Tumour angiogenesis is regulated by the tumour microenvironments composed of tumour cells, vascular endothelial cells, and stromal cells. In addition to cancer cells, the microvascular endothelial cells recruited by the tumour are important for cancer development (Carmeliet and Jain, 2000; Stoeltzing et al., 2006). PI3K/AKT pathway also controls tumour microenvironments, including endothelial cells (Phung et al., 2006; Yuan et al., 2007). PI3K can regulate endothelial migration, proliferation, and survival through the effect of its downstream targets such as NOS, p70S6K1, and FOXO to regulate tumour angiogenesis (Fosbrink et al., 2006; Nakao et al., 2007; Zheng et al., 2008). Class IA PI3Ks regulate vessel integrity during development and tumourigenesis (Yuan et al., 2008). Further analysis of p110 isoforms has demonstrated that p110α is required to control endothelial cell migration and

angiogenesis, and p110α$^{-/-}$ endothelial cells lead to embryonic lethality with severe defects in angiogenic sprouting and vascular remodelling (Graupera et al., 2008; Suzuki et al., 2007). PTEN$^{-/-}$ endothelial cells cause embryonic lethality due to endothelial cell hyperproliferation and impaired vascular remodelling; PTEN$^{+/-}$ endothelial cells enhance postnatal neovascularization and tumour angiogenesis to increase tumour growth (Suzuki et al., 2007). Transgenic expression of Myr-AKT1 in endothelial cells is sufficient to recapitulate the abnormal structural and functional features of tumour blood vessels in non-tumour tissues, likely due to the induction of VEGF-A (Jiang et al., 2000; Phung et al., 2006). Sustained endothelial AKT activation causes enlarged and hyperpermeable blood vessels and its effect can be completely reversed by AKT inhibition or by rapamycin treatment (Phung et al., 2006). The interaction of cancer cells and vascular endothelial cells in the tumour microenvironment affects angiogenesis. In cancer cells, stimuli such as growth factors, insulin, and other hormones activate PI3K/AKT/mTOR/HIF-1α axis, and induce the production of VEGF, which switches angiogenic response and causes endothelial cell activation and permeability increased by PI3K pathway (Nyberg et al., 2008; Stoeltzing et al., 2006). Thus, inhibition of PI3K/AKT/ mTOR pathway is one of the choices in cancer treatment, which is going on under the preclinical and clinical trials. The signalling pathway of PI3K related to tumour growth, metastasis, and angiogenesis is shown in Fig. 5.

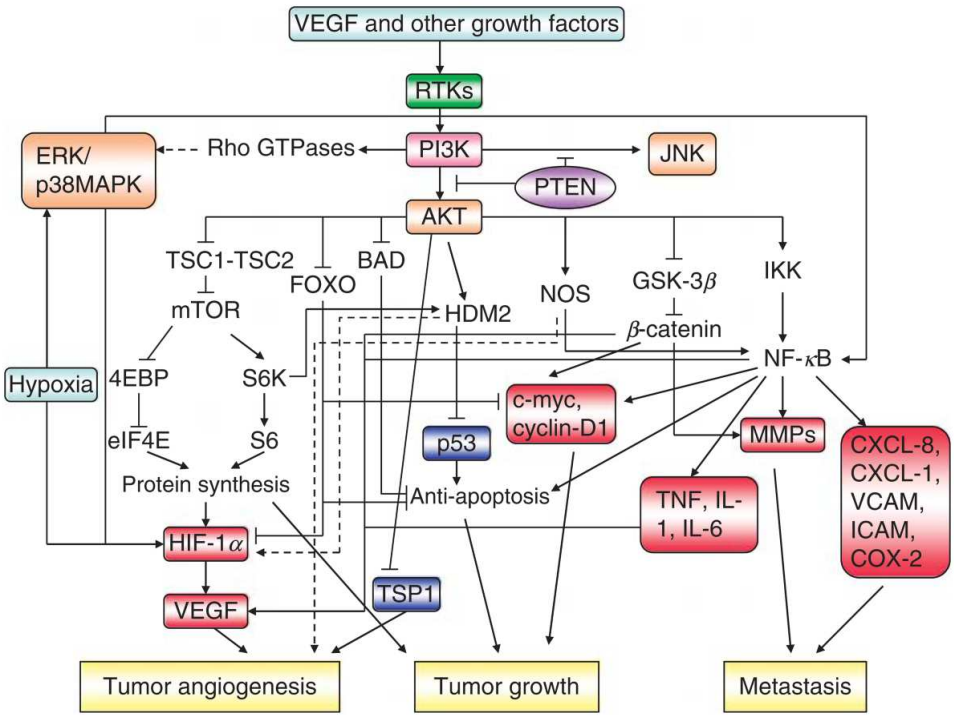

Figure 5: Targets of PI3K and PTEN in regulating tumour growth, metastasis, and angiogenesis.

1.2.4 Therapeutic implications: anti-angiogenesis therapy in solid tumours.

In solid tumours, it is well established that the angiogenic switch from an initial avascular tumour nodule to a rapidly growing, highly vascularised tumour is a critical step in the process of carcinogenesis (Hanahan and Weinberg, 2011). Anti-angiogenic therapy has now become an additional pillar in the treatment options for several cancers. Due to its prominent role in angiogenesis, VEGF has been in the spotlight of research efforts that resulted in the approval of 5 FDA-approved anti-angiogenic drugs targeting the VEGF pathway. The first generation of these drugs is the monoclonal anti-VEGF antibody bevacizumab (Avastin), which was approved as first-line therapy in combination with other chemotherapy in metastatic colorectal cancer (Hurwitz et al., 2004), metastatic breast cancer (Miller et al., 2007), non-small-cell lung cancer (Sandler et al., 2006), metastatic renal cell carcinoma (Escudier et al., 2010; Rini et al., 2010) and as second-line therapy in glioblastoma multiforme (Cohen et al., 2009). The

second-generation FDA-approved broad-spectrum small-molecule receptor TK inhibitors include sunitinib (Sutent), sorafenib (Nexavar), pazopanib (Votrient), and vandetanib (Zactima), which target the VEGF receptors, PDGFRs, and others. Sunitinib, sorafenib, and pazopanib are all approved as monotherapy for metastatic renal cell carcinomas after prolongation of the progression-free survival (PFS) (Escudier et al., 2007; Motzer et al., 2007; Sternberg et al., 2010). Sorafenib is also approved for hepatocellular carcinoma (Llovet et al., 2008). Sunitinib is also approved for imatinib (Glivec)-resistant gastrointestinal stroma tumours (Demetri et al., 2006) and for pancreatic neuroendocrine tumours. Vandetanib is approved for late-stage medullary thyroid cancer (Wells et al., 2010). Notwithstanding these achievements, clinical findings have been less overwhelming than many preclinical results, and antiangiogenic therapy is currently facing several challenges, in particular the intrinsic refractoriness and acquired evasive escape against VEGFR blockers (Bergers and Hanahan, 2008). VEGF-targeted anti-angiogenic therapy prolongs the survival of patients with certain types of tumours by months, but it fails to induce a survival benefit in others (Loges et al., 2010). Surprisingly, bevacizumab elicits better clinical results when combined with chemotherapy, in contrast to receptor TK inhibitors. An increasing number of trials show a transient stabilization of the disease with even tumour regression and a prolonged PFS, but no prolongation of the more important and clinically relevant overall survival (OS) (Miller et al., 2007; Yang et al., 2003; Ebos and Kerbel, 2011). Why prolongation of PFS does not always translate to prolongation of OS in patients undergoing anti-angiogenic therapy remains largely speculative, and several recent overviews have discussed various types of resistance mechanisms (Loges et al., 2010; Ebos and Kerbel, 2011). Another concern, raised by at least some recent preclinical findings in particular experimental conditions, is that VEGF signalling inhibition might inhibit the primary tumour growth, but at the same time also evoke an adaptation in tumour cells to a more metastatic phenotype (Ebos and Kerbel, 2011). Recent preclinical and clinical studies show that a remarkable degree of compensation

by VEGF-independent pro-angiogenic mechanisms occurs, and this compensation works, in particular, in the absence of VEGF signalling. It has also been demonstrated that combining conventional anti-VEGF therapy with blockade of VEGF-independent pro-angiogenesis pathways greatly enhances tumour suppression (Fig.6). For example, blockade of FGFs or angiopoietins enhances and prolongs the anti-angiogenic effects of VEGF blockade (Koh et al., 2010; Casanovas et al., 2005). However, the molecular bases of cancer type-dependent resistance mechanisms against VEGF blockade, especially VEGF-independent pro-angiogenic mechanisms, now need to be clarified. Targeting these mechanisms would greatly enhance the effects and minimize the required doses of VEGF blockers. There is no doubt that effort in this area will yield opportunities to greatly improve anti-angiogenic treatment.

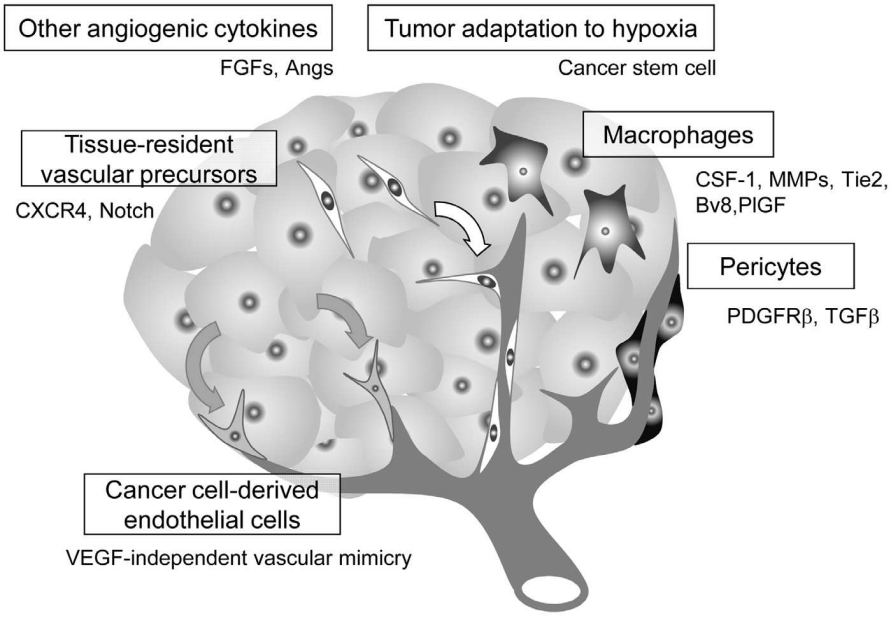

Figure 6: VEGF-independent pro-angiogenic mechanisms: novel targets for anti-angiogenic therapy. VEGF blockade enhances the expression in tumours of other pro-angiogenic cytokines such as fibroblast growth factors (FGFs) and angiopoietins (Angs). Selection of hypoxia-tolerant tumour cells may elicit tumour adaptation to anti-angiogenesis. Some types of perivascular/vessel-associated cells such as macrophages, pericytes, cancer stem-like cells and tissue-resident vascular precursors contribute to tumour angiogenesis and refractoriness to VEGF blockade. Inhibition of macrophage function could be achieved by targeting colony-stimulating factor 1 (CSF-1), matrix metalloproteinases (MMPs), Tie-2, Bv8 or placental growth factor (PlGF). To suppress pericyte recruitment, PDGFRβ or TGFβ signalling could be targeted. The recruitment and function of tissue-resident vascular precursors are mediated by CXCR4 and Notch signalling.

Currently, a new vascular-targeting therapeutic strategy is gaining increasingly more attention. It is well known that tumour blood vessels are highly abnormal in structure and function, characterized by a tortuous, chaotic, and irregular branching network (Figure 7A-B) (Carmeliet and Jain, 2011). In the tumour vasculature, ECs are highly activated, lose their polarity and alignment, and detach from the basement membrane, all resulting in a leaky, fenestrated network that facilitates bleeding and increases the interstitial fluid pressure. Apart from the ECs, the entire vessel wall, including the basement membrane and the covering pericytes, becomes abnormal in most tumours. Tumour ECs are typically covered with fewer and more abnormal pericytes, and their associated basement membrane is only loosely associated and inhomogeneous in structure. It is suspected that this abnormal vasculature impedes the distribution of chemotherapy and oxygen (Carmeliet and Jain, 2011). Traditional anti-angiogenic therapy aims to maximally inhibit angiogenesis and to prune existing tumour vessels; however, this strategy can also increase the risk of aggravating hypoxia and enhancing tumour cell invasiveness. Recent genetic and pharmacological studies have revealed that targeting abnormal tumour vessel function by the induction of vessel normalization can offer alternative options for anti-angiogenic therapy (Figure 7C). Vessel normalization can be achieved by several different approaches, including blockade of VEGF (Carmeliet and Jain, 2011), genetic modulation of the oxygen sensors prolyl hydroxylase domain containing protein 2 (PHD2) (Mazzone et al., 2008), targeting of mechanisms that affect the pericyte coverage and vessel maturation via blockade or genetic loss of PlGF. (Fischer et al., 2007; Van de Veire et al., 2010; Rolny et al., 2011). Vessel normalization could provide a means to increase the responsiveness to chemotherapy, immunotherapy, or radiation, and may contribute to restricting tumour dissemination. Further research will be needed to personalize anti-angiogenic medicine by more optimally matching the pharmacological profile of an anti-angiogenic therapy with correctly selected patients.

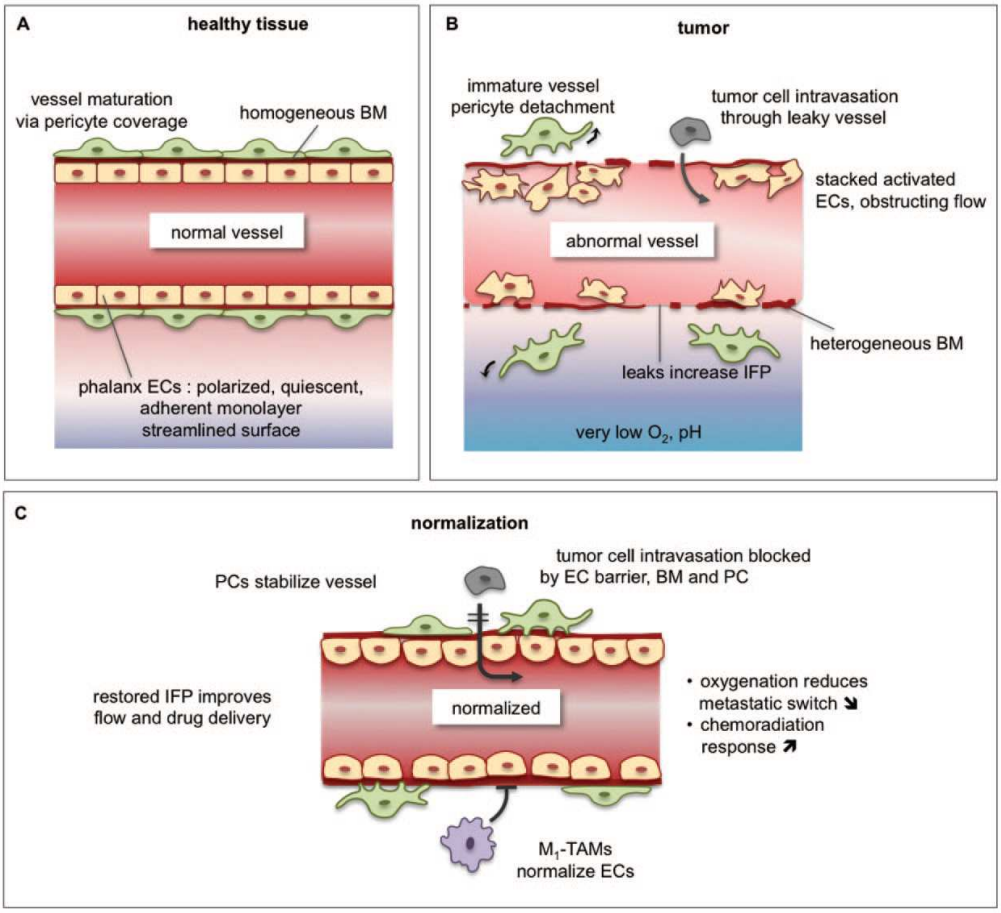

Figure 7: Scheme illustrating a normal blood vessel (A), the abnormalities of a tumour blood vessel (B), and a partially normalized tumour blood vessel (C). BM indicates basement membrane; phalanx ECs, quiescent ECs; IFP, interstitial fluid pressure; PC, pericytes; M1 TAM, M1-polarized tumour-associated macrophages.

1.3 MOUSE MODELS OF CANCER

Cancer is a complex disease in which normal cellular pathways are altered to give rise to the properties leading to cancer, such as inappropriate growth, survival and invasion. While the study of human tumours has yielded many insights into the molecular changes present in cancers, more rigorous testing of hypotheses through experimental manipulation is necessary to better understand which changes are causative, and therefore targetable, and which are secondary. Mouse model systems provide an experimentally tractable mammalian system with a wealth of developed research tools to understand basic mechanisms of cancer and to investigate the factors involved in malignant transformation, invasion and metastasis, as well as for assessment of putative cancer therapies. Cross-species comparison has proven to be powerful in

improving the understanding of a wide variety of human diseases, including cancer (Brown et al., 2008; Kim et al., 2005; Wang and Paigen, 2005; Wang et al., 2005). As mammals, mice share many anatomical, cellular, and molecular traits with humans that are known to have critical functions in cancer, such as an immune system, maternal effects in utero, imprinting of genes, and alternative splicing.

1.3.1 Xenograft models.

The most commonly used mouse transplanted tumour models include subcutaneous (SQ) and orthotopic xenograft models. The ability to grow human tumours cell in immunodeficient mice was established over 30 years ago (Giovanella et al., 1972; Rygaard and Povlsen, 1969; Shimosato et al., 1976). Since that time, the use of xenograft tumours has become an integral part of the drug discovery process. In the age of cytotoxic therapies, where drugs were discovered by their ability to kill tumour cells in cell culture, the analogous endpoint in animal models was whether the growth of tumours was inhibited.

1.3.1.1 Subcutaneous Xenograft Models.

This model uses established human cell lines that can be easily propagated in culture; cells are injected into the subcutaneous (SQ) tissue of immunocompromised mice, commonly athymic nude or SCID mice, which do not reject the species-mismatched cells. Depending upon the number of cells injected, the tumour will develop over 1-8 weeks (or in some instances 1-4 months, or longer) and its assessment can be allowed by simple calliper measurements. Many studies expect that tumours grow for a defined period of time, or to a specific volume (commonly 100-200 mm^3), before starting treatment. There are three different types of response to therapy that can be evaluated: effect on the growth rate of the tumour, effect on tumour shrinkage/regression, and survival, comparing the drug-treated cohort of mice to vehicle-treated controls (Fig.8).

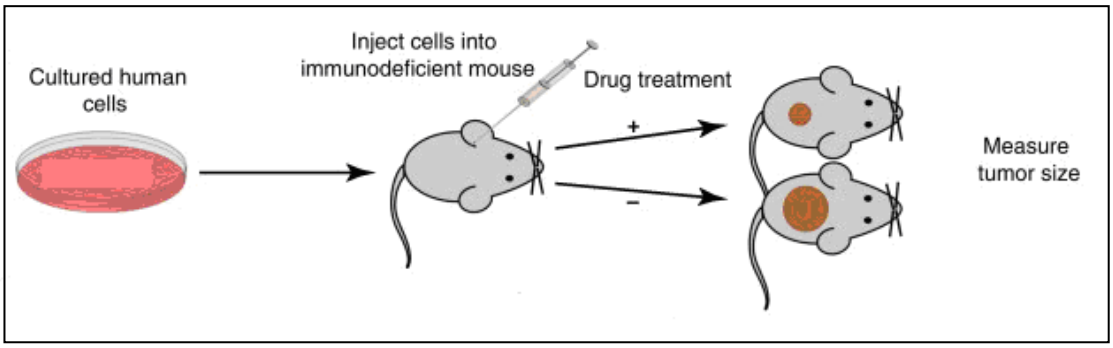

Figure 8: In subcutaneous xenograft studies, cultured human cells are usually injected subcutaneously into immunodeficient mice, which are then treated with the compound of interest for 2–6 weeks, during which time subcutaneous tumours develop. The tumour size in the treatment group is then compared to that in the control group.

However, over the years, many studies paint an unfavourable picture in terms of the use of xenograft SQ tumour models to predict clinical antitumor drug efficacy; in fact, 90% of antineoplastic new chemical entities (NCEs) that evidenced an antitumor efficacy in preclinical xenograft SQ models failed in clinical testing (Von Hoff, 1998).

1.3.1.2 Orthotopic xenograft models.

An essential condition for the use of *in vivo* models is to replicate aspects of tumourigenesis that are not reflected in tissue culture. Stromal cells play a significant role in promoting tumourigenesis, providing fundamental growth factors, nutrients and angiogenesis (Condon, 2005; De Wever and Mareel, 2003; Kim et al., 2005). Although SQ xenografts may recapitulate certain aspects of the tumour-host microenvironment, the latter one is more closely approximated by implanting tumour cells into the organs and anatomical sites from which they originally arose. Moreover, vasculature in SQ tumours may be very different from that obtained by the same tumour cells implanted in an orthotopic location. The capability to metastasize is also influenced by tumour implant site. Experimental models of metastasis can be accomplished by direct inoculation of tumour cells into the target organs or by intravenous or intracardiac injection (Fig.9) (Hoffman, 1999; Manzotti et al., 1993). However these approaches bypass the initial step of local-regional invasion of metastases. A more complete model

of metastasis is accomplished by implantating tumour cells in a primary site and allowing for spontaneous metastasis.

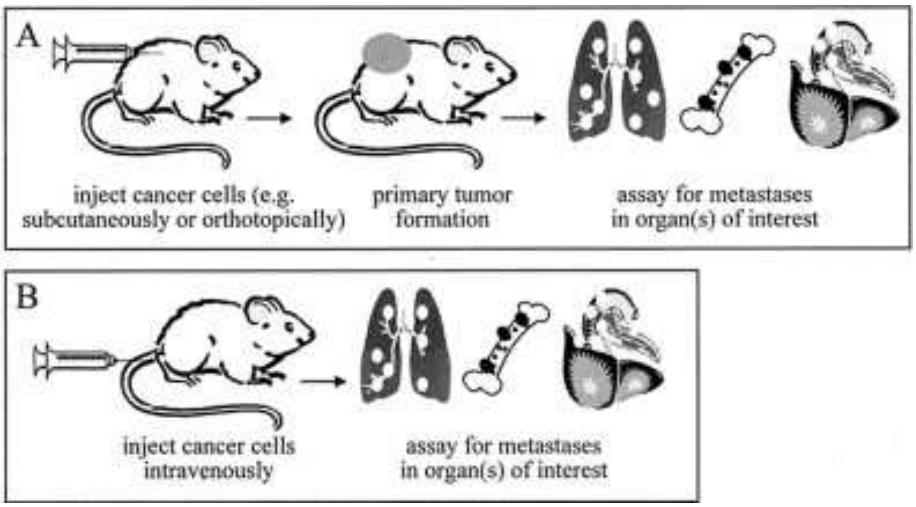

Figure 9: Experimental approaches to *in vivo* study of metastasis. A): for spontaneous metastasis assay cancer cells are injected into mouse, typically subcutaneously or orthotopically, forming primary tumour at injection site. If cancer line is metastatic, primary tumour cells give rise to metastases at secondary site, such as lungs, bone or liver. Mice are assayed for metastases at given time after injection. B): for experimental metastasis assay cancer cells are injected directly into circulation, typically via tail vein or hepatic artery. Mice are examined for metastases at given point after injection. This approach bypasses early steps in metastatic cascade, including primary tumour growth, local invasion and intravasation.

In many cases, orthotopic implantation of tumour cells has been found to enhance metastasis compared to subcutaneous implantation of the same cells (Fidler, 1986; Fidler et al., 1990; Manzotti et al., 1993; Naito et al., 1997a,b; Sthepenson et al., 1992; Waters et al., 1995). The sensitivity of xenografted tumours to therapeutics may also be modulated by their location (Killion et al., 1998). Together, these considerations suggest that orthotopic transplant models may be superior to SQ transplant models in recapitulating many aspects of the tumour-host interactions. However, the major disadvantage of orthotopic models compared to SQ models is the difficulty of following tumour growth. However, the recent development of new magnetic resonance imaging (MRI) and micro-imaging techniques may minimize this problem.

1.3.2 Genetically Engineered Mouse (GEM) models.

With the availability of the complete sequence of the mouse genome, technology to manipulate the mouse genome and well-defined inbred strains, the ability to engineer

mice is achievable. There are two possible strategies to test hypotheses of tumourigenesis:

1.3.2.1 Studying gain of gene function in mouse models.

- *mouse constitutive transgenic models*: gain-of-function studies are often used to study oncogenes in mouse models. Transgenic or knockin animals constitutively over-expressing an oncogene can be used to study how the oncogene drives tumourigenesis *in vivo*. Transgenic mice are created by the pronuclear injection of transgenes directly into fertilized oocytes, followed by implantation into pseudopregant females (Fig.10) (Macleod and Jacks, 1999: Porret et al., 2006).

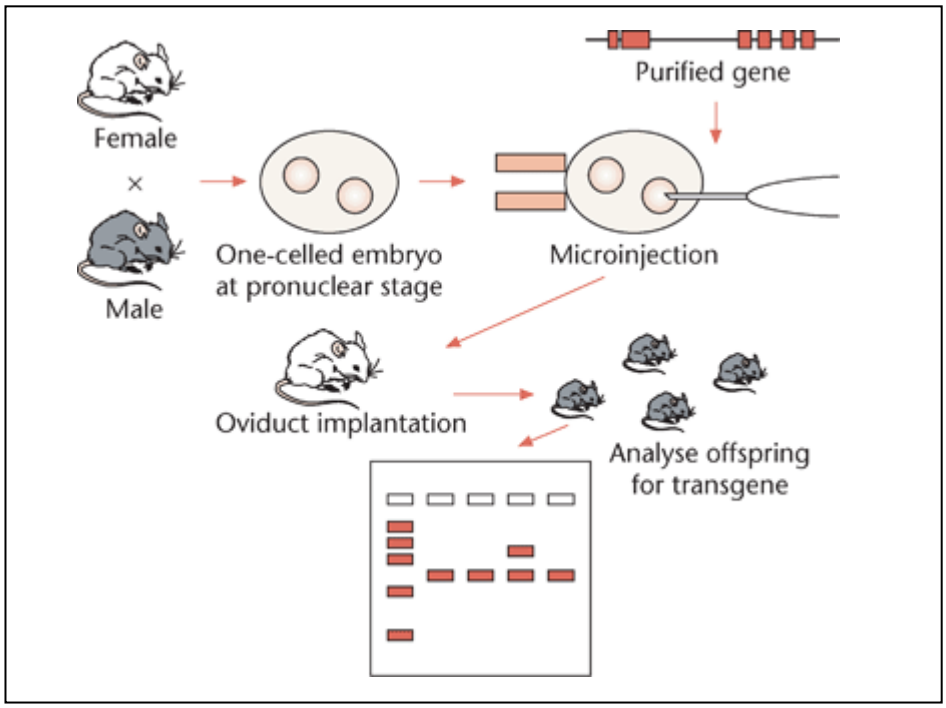

Figure 10: Production of transgenic mice by pro-nuclear microinjection. Female mice are superovulated and mated, and a pronucleus of the fertilized egg is microinjected with DNA. Surviving zygotes are reimplanted and newborn pups are tested for incorporation of new genes.

For this purpose, a plasmid is constructed containing the gene/cDNA of interest placed under the control of a heterologous promoter. For a protein to be expressed, the cDNA must contain a translational start codon (ATG) with an upstream Kozak sequence [GCCGCC (G/A) NN] (Kozak, 1987) for ribosomal recognition of the mRNA start site and an in-frame translation stop codon (UGA, UAG, UAA) for translational termination.

Inclusion of an intron at the 5' or 3' end of the transgene allows splicing of the transgene, which generally results in more stable mRNAs and more efficient RNA translocation from the nucleus to the cytoplasm which typically leads to better transgene expression. Natural introns, such as the simian virus 40 (SV40) intron or the rabbit β-globin intron, as well as artificial introns, can be used. In addition, eukaryotic transcriptional stop signals that include the poly(A)-addition sequence (AAUAAA) are usually positioned at the 3' end of the protein translation sequence. Termination sequences widely used include those from SV40, bovine growth home (BGH) and human growth hormone (HGH) (Sheets et al., 1987; Goodwin and Rottman, 1992; Haruyama et al., 2009). The transgene is randomly incorporated into the genome and usually in the form of concatemers containing multiple copies of the original fragment. The number of integrates transgenes is generally inversely proportional to fragment size. Due to the random nature of transgene integration following pronuclear microinjection, position site-dependent effects may alter transgene expression. These effects may produce transgene silencing, modify the cell and tissue specificity of the transgene or affect overall level of expression. Chromatin-mediated silencing may occur when a transgene integrates into a heterochromatin region while altered expression due to the effects of endogenous enhancers can occur when integration takes place into euchromatic regions. Transgene insertion can also alter the expression of endogenous genes at the integration region. Multiple founders must be screened to confirm adequate and specific expression of the transgene.

1.3.2.2 Studying loss of gene function in mouse models.

- *mouse gene knockouts*: studying the loss of function of genes provides insight into understanding the biological functions for which the protein product is required. Loss-of-function studies most commonly use "knockout" strategies to remove the gene of interest by engineering constitutive or conditional deletions in the gene. For genes that span large genomic regions, deletion of the first few exons encoding the start codon is often sufficient to block transcription or translation into a functional protein product.

However, sometimes an alternative start codon or alternative splicing can lead to a truncated protein product with partial function that can mask the significance of the gene of interest in biological loss-of-function studies. Thus, careful molecular characterization of genetically engineered alleles is important to verify that the function of the gene is truly lost and also that additional inadvertent gene rearrangements or deletions are not present. Conventional knockout vectors contain a positive selectable marker (usually Neo) and a negative selectable marker (TK) (Mansour et al., 1988; Valenzuela et al., 2003). This allows the replacement of specific genes with Neo through homologous recombination between the targeting vector and the cognate sequence in the recipient ES cell genome after the vector is transferred into these cells by electroporation. Only Neo resistant ES cells generated by homologous recombination can grow under selection, and TK is used to eliminate random integration in the presence of gancyclovir (GANC) (Fig.11).

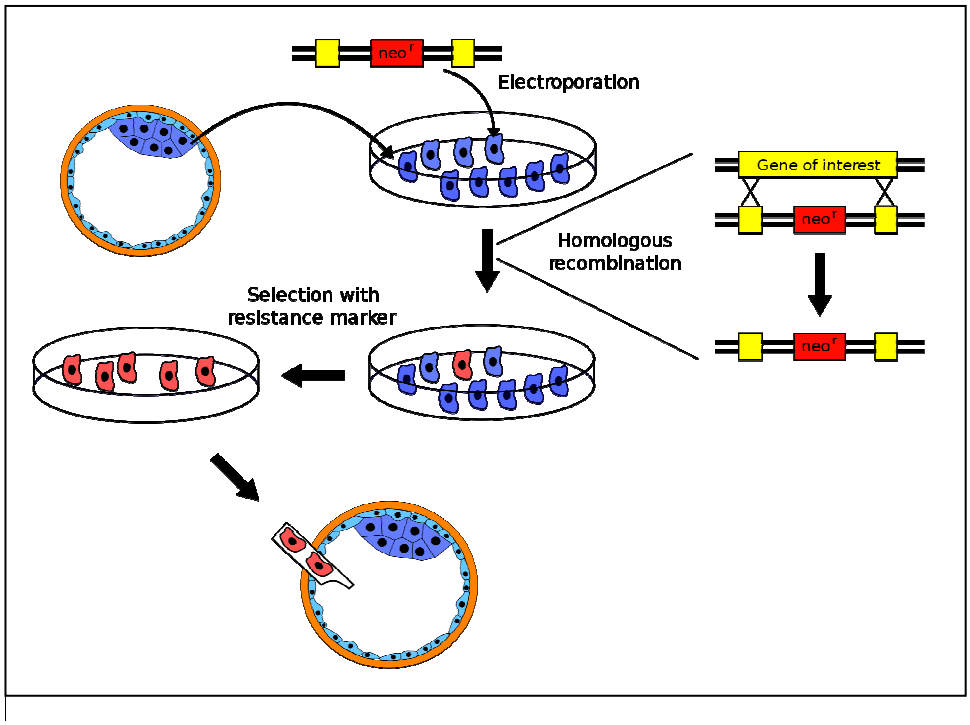

Figure 11: The procedure for making mixed-genotype blastocyst.

DTA (Diphtheria toxin A) is another negative selection marker that is used and was reported to allow more efficient enrichment of targeted clones (Capecchi, 1989). The

DTA protein itself can kill ES cells, and no additional drug is needed in the medium. However, the use of DTA for selection may cause minimal toxicity due to transient expression prior to vector integration (McCarrick et al., 1993; Yagi et al., 1993). After positive-negative selection, the ES cells are injected into mouse blastocysts to produce chimeras. By breeding these chimeras with wild-type mice, heterozygous mice may be produced if the ES cells form the germline in the chimeras. The use of knockout strategies have been critical in understanding cause and effect relationships in cancer development and can be applied to the assessment of many gene classes, including oncogenes, tumour suppressor genes and housekeeping genes. Homozygous deletion studies often lead to embryonic lethality precluding assessment for adult diseases. In many cancer models, however, animals heterozygous for a tumour suppressor knockout allele are susceptible to tumour formation either due to haploinsufficiency or by somatic loss of the wild type allele (Cichowski et al., 1999; Kost-Alimova and Imreh, 2007; Macleod and Jacks, 1999; Reilly et al., 2000; Zheng and Lee, 2002).

- *mouse conditional gene mutations*: with conventional knockouts, loss of a vital gene can often lead to embryonic lethality, severe developmental abnormalities or adult sterility, making it impossible to study the gene in the desired disease context. In addition, ablation of the gene of interest in the entire body does not mimic spontaneous tumourigenesis in humans, where tumours evolve in a wild type environment and the timing of gene loss may be a critical factor in disease development. Sophisticated conditional genetic engineering technology has been developed to create systems where genetic events can be tightly controlled spatially and temporally. Bacteria Cre and yeast FLP enzymes are site-specific recombinases that catalyze specific recombination between defined 34 bp loxP and FRT sites, respectively (Branda and Dymecki, 2004). In the presence of Cre or FLP protein expression, homologous recombination is induced between loxP or FRT sites that flank the gene of interest and are oriented in the same direction, thus recombining out the flanked genetic sequence and deleting the gene of interest. By temporally and spatially controlling expression of

the recombinase, it is then possible to temporally and spatially control deletion of the gene of interest, overcoming interference from developmental abnormalities and lethality (Branda and Dymecki, 2004). Mice carrying the Cre or FLP recombinase under the control of a tissue-specific promoter are crossed with mice carrying the gene of interest flanked by loxP or FRT sites to conditionally knockout the gene in a specific tissue or cell type or at specific times during development (Fig.12).

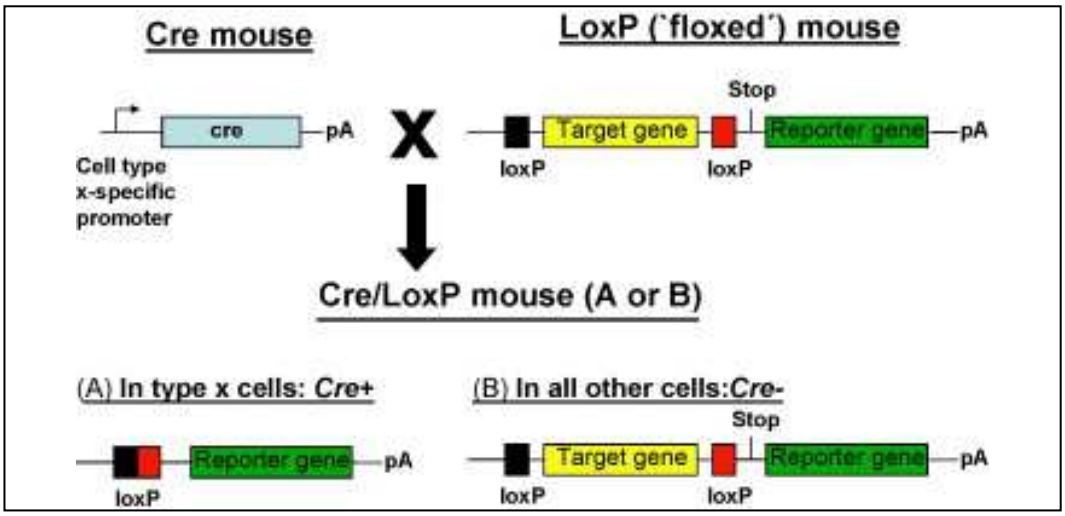

Figure 12: Generation of conditional knockout mice using the Cre/loxP system. Transgenic mice expressing the Cre recombinase under the control of specific promoters are crossed with mice in which the gene of interest is flanked by loxP sites ("Floxed mice", generated by homologous recombination). In addition, to allow a control of the distribution and efficiency of the Cre/loxP system, a reporter gene (e.g. lacZ or EGFP) is inserted, whose expression is prevented in the "floxed mice" by a "stop" sequence, but becomes effective in the "Cre/loxP mice" after recombination.

Multiple types of Cre delivery systems have been developed, such as promoter-driven cell or tissue-specific, viral systems. Viral Cre, in which the *Cre* gene is packaged into adenoviral or lentiviral particles, can be locally delivered topically or by injection to infect cells and create a regional or clonal knockout of cells within a given area (Jackson et al., 2001; Marumoto et al., 2009). Two of the most commonly temporally conditional Cre used systems are the tetracycline-inducible system (Gossen and Bujard, 1992) and the tamoxifen-inducible system (Metzger and Chambon, 2001). The tetracycline-repressor-based system is composed of a transactivator and an effector. The DNA-binding domain of the *Escherichia coli tet*R gene fused to the transactivator domain of the herpes simplex virion protein 16 (VP16) gene (tetR/VP16) makes up the

tetracycline-controlled transactivator (tTA) that can then be driven by tissue-specific promoters (Baron et al., 1997). The tTA binds to the tetracycline operator (tetO) that controls the activity of the human cytomegalovirus promoter driving conditional gene expression, including Cre to generate conditional knockouts. In this Tet-Off system, tTA is bound by tetracycline, or its more stable analogue doxycycline, inhibiting association with the tetO and blocking gene transcription. In the Tet-On system, the tetracycline-repressor has been mutated (rtTA) such that it is only in the proper conformation for association with tetO when it is bound to tetracycline or doxycycline, thus inducing expression of the gene in the presence of drug (Fig.13).

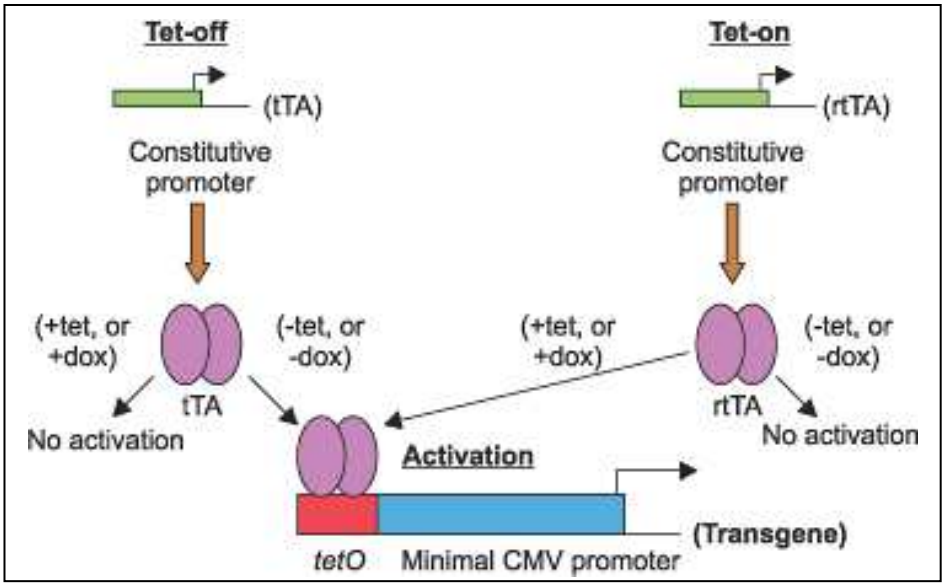

Figure 13: In the "Tet-Off." system, in the absence of tetracycline, tTa binds to the tetracycline operator (tetO), activating transcription of the cDNA. When tetracycline or doxycycline is added to the medium, it binds to tTa, which, in turn, releases the tetO, stopping transcription. In the "Tet-On" system, in the absence of tetracycline, rtTa is free and transcription does not occur. When tetracycline or doxycycline is added to the cultures, it binds to rtTa which in turn binds to the tetO activating transcription of the cDNA.

The tamoxifen-inducible system depends on fusion of the Cre recombinase gene to a mutated ligand-binding domain of the human estrogen receptor (Cre-ER(T)) that is specifically activated by tamoxifen. In the absence of tamoxifen, the ER fusion protein is excluded from the nucleus, but is transported to the nucleus upon binding to tamoxifen when Cre can then recombine DNA. Temporally expression of Cre can be controlled by simply delivering or with holding tamoxifen (Fig.14).

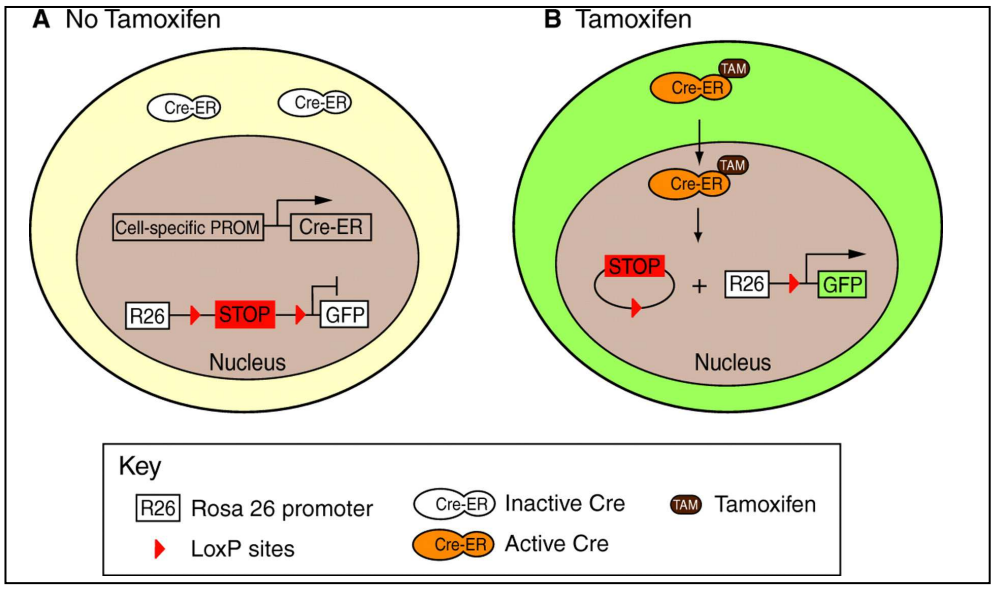

Figure 14: Cre-recombinase-based lineage tracing system. Cre recombinase expression can be spatially restricted by expressing it under the control of a tissue-specific promoter. Temporal restriction is achieved by fusing it to the tamoxifen-responsive hormone-binding domain of the estrogen receptor (Cre-ER^{TAM}). The Cre enzyme is in an inactive state in the absence of the ligand tamoxifen. Once tamoxifen is added, the Cre is active and can translocate to the nucleus. When these Cre constructs are used in conjunction with reporter genes, such as green fluorescent protein (GFP) ubiquitously expressed under the control of the ROSA26 (R26) promoter for example, and placed downstream of a STOP codon flanked by Cre recombinase recognition (loxP) sites, reporter gene expression can be activated in specific cell types at defined time-points. (A) In the absence of tamoxifen, no expression of the reporter gene is observed because of the presence of the stop signal upstream of the reporter gene. (B) When tamoxifen is administered, the Cre is activated and mediates recombination between the loxP sites in cells. As a consequence, the STOP codon is excised and the cells are permanently marked by the reporter gene.

- *mouse models of RNA interference*: loss-of-function studies can use RNA interference (RNAi) to specifically knockdown the expression of target genes post-transcriptionally before the mRNA can be translated into protein (Meister and Tuschl, 2004). Target sequence-specific small interfering RNAs (siRNAs) are short antisense peptides 21-28 nucleotides long. The RNA-induced silencing complex (RISC) recognizes the double-stranded siRNA fragments and cleaves the endogenous complementary messenger RNA (mRNA) that is then rapidly degraded. This system is limited to transient transfection *in vitro*. Short hairpin RNA (shRNA) can be used to cause long-term gene knockdown, both *in vitro* and *in vivo*. The shRNAs are much longer (50-70 nucleotides), made as a single-strand molecule that then forms a short hairpin tertiary structure, folding in on itself to form a stem-loop structure *in vivo*. After transcription and folding, the enzyme Dicer cleaves off the loop laving behind a double-

stranded siRNA molecule that can then be recognized by RISC. The shRNA sequences can be cloned into viral vectors to be stably incorporated into the genome for knockdown of target genes.

1.3.3 Chemically induced tumour mouse models.

Despite several xenograft or genetically defined mouse models of cancer have been exploited for chemopreventive research, they lack the important clinical feature of tumour heterogeneity. On the contrary, chemical carcinogenesis models better reflect the clinical setting because of the diversity and heterogeneous nature of the resulting tumoral lesions. Chemical mouse model provide powerful tools for carcinogen hazard identification and for examining specific chemical-gene interactions allowing a better understanding of the mechanisms of carcinogenesis in a shorter period of time. Therefore, they may be extremely valuable to mimic human cancer for intervention and therapeutic strategies (Gulezian et al., 2000; Maronpot, 2000). Chemical carcinogens are classified as either direct acting (e.g., N-methyl-N-nitrosourea or NMU) or indirect acting (e.g., dimethylhydrazine or DMH; azoxymethane or AOM). They increase cell proliferation and induce the formation of tumours in a variety of organs and tissues, depending on chemical specificity of the carcinogen, the route and time of administration, dose, duration and frequency of administration (Balish et al., 1977). The age, sex, and strain of mice also affects the type of tumour that they develop. A wide variety of chemicals and chemical classes can cause cancer in animals and humans. Most chemical carcinogens are genotoxic, causing DNA damage by reacting with DNA bases. The carcinogens form covalent adducts with DNA in the nucleus and mitochondria. Endogenous carcinogens (which are often reactive oxygen species (ROS) generated as part of normal oxidative metabolism or as a result of the metabolism of xenobiotic compounds, as well as by ultraviolet radiation and gamma radiation) can also cause extensive DNA damage. Metabolic activation of procarcinogens (i.e., carcinogens requiring enzymatic conversion to DNA-reactive intermediates) is generally catalyzed by cytochrome P450 enzymes through oxidation.

More than 100 distinct mammalian P450 enzymes have been identified (Nebert et al., 1991). In addition, there are other enzyme systems involved in carcinogen activation such as peroxidises and certain transferases such as N-acetyltransferase and sulfotransferase (Guengerich, 1992; Eling et al., 1990). Each of these enzymes provides a potential target for modulating carcinogen activation. One common feature of the metabolic activation of all procarcinogens is that their ultimate DNA-reactive carcinogenic species are electrophilic. In addition, many direct-acting carcinogens damage DNA through electrophilic intermediates (Miller and Miller, 1981). Thus, the electrophilicity of the ultimate carcinogenic species serves as a shared intervention target for most chemical carcinogens. The electrophilic metabolites may themselves be ROS and interact with DNA. Oxygen-free radicals may also be involved in a step required for activation of a procarcinogen, and thus the reactions involved in metabolic activation of carcinogens may release ROS that can in turn attack DNA. In addition to the carcinogen-activating enzymes, a series of enzymes (the so-called phase II enzymes) detoxify activated carcinogens, thus preventing their binding to DNA. The induction of the glutathione S-transferases (GSTs) is an important response for the detoxification of xenobiotics. This class of enzymes couples a number of diverse substrates to glutathione to excrete them from the body. GSTs are segregated into three classes based on their sequence homology and specificity for substrates (Ketterer, 1988). Other detoxification enzymes include uridine diphosphate-glucuronosyl transferase, quinone reductase, and the epoxide hydrolases (Oesch et al., 1990; James et al., 1991). The efficiency with which these and other enzymes detoxify carcinogens is a critical factor in determining the carcinogenicity of a particular xenobiotic. The generation of DNA-reactive intermediates by most chemical carcinogens leads to the production of DNA adducts or other types of damage. Normal mammalian cells can efficiently remove DNA damage induced by carcinogens. Cells use different strategies to repair DNA damage, depending on the structure of the damage and its location in the genome. For example, small lesions (such as alkylated

DNA bases) can be repaired by a mechanism termed base excision repair. This process involves removal of the damaged base followed by a “small cut-and-patch” repair involving removal of a few nucleotides. Bulky carcinogen-induced DNA adducts and ultraviolet light photodimers can be repaired through a “large cut-and-patch” mechanism involving a region of approximately 27–29 nucleotides that includes the damaged bases; this is termed nucleotide excision repair (Sancar and Tang, 1993). Cells have also developed a mismatch repair mechanism to correct errors produced during normal DNA replication (Modrich, 1994). If the damage is not repaired, it can lead to genetic mutations: this is the initiation stage of carcinogenesis. The responsiveness of the mutated cells to their microenvironment can be altered and may give them a growth advantage relative to normal cells. In the classic two-stage carcinogenesis system in the mouse skin, a single low dose of 7,12-dimethylbenz[a]antracene (DMBA) causes permanent DNA damage (the initiating event) but does not give rise to tumours over the lifespan of the mouse unless a tumour promoter, such as 12–O-tetradecanoylphorbol-13-acetate (TPA), is repeatedly applied in the same site of the skin (Fig.15) (Slaga, 1984). The tumour promotion stage is characterized by selective clonal expansion of the initiated cells, a result of the altered expression of genes whose products are associated with hyperproliferation, tissue remodelling, and inflammation (Slaga et al., 1980). During tumour progression, preneoplastic cells develop into tumours through a process of clonal expansion that is facilitated by progressive genomic instability and altered gene expression (Pitot, 1989).

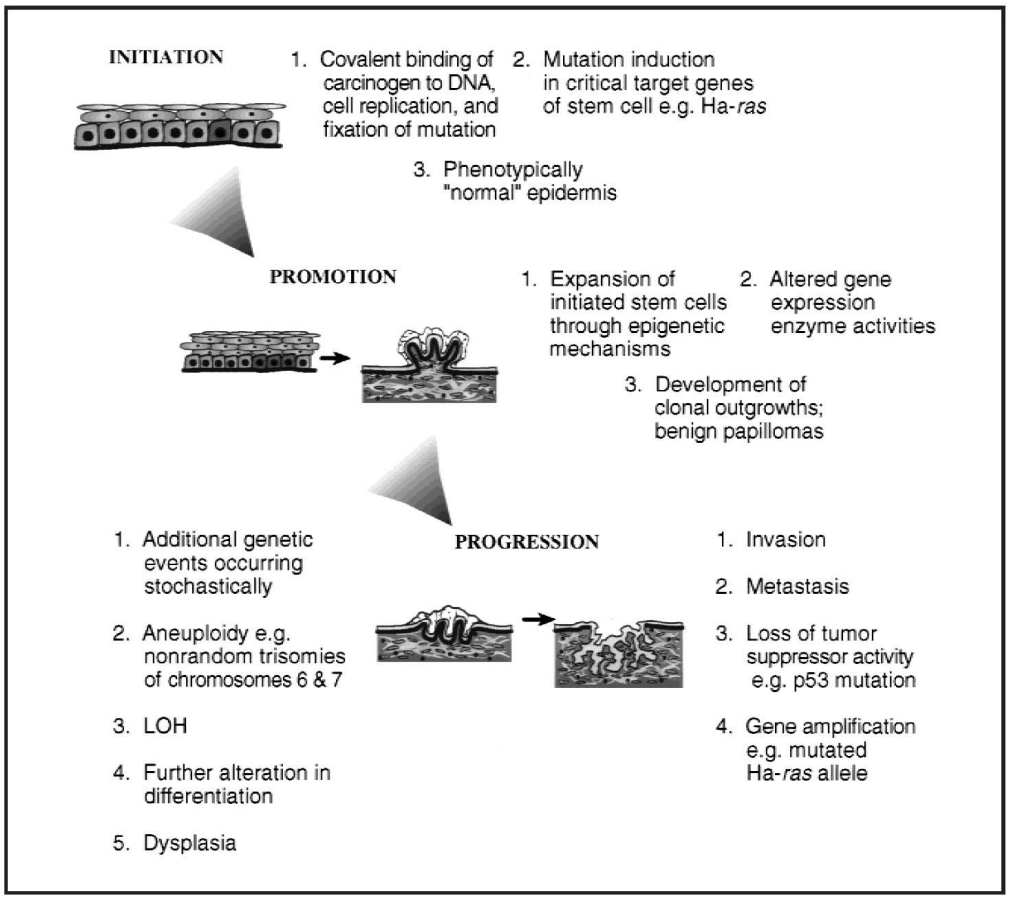

Figure 15: Multistage carcinogenesis in the mouse skin. Schematic presentation of the stages in experimental carcinogenesis, using the mouse skin as an example. The initiation stage is characterized by the generation of a cell that is genetically altered through covalent binding of a carcinogen to DNA and subsequent fixation of a mutation. The epidermis retains a normal appearance at this stage. The promotion stage involves the expansion of initiated stem cells through epigenetic mechanisms, accompanied by alterations in the expression of critical genes that regulate proliferation, inflammation, differentiation, and other processes. Also pictured is the development of clonal outgrowths that result in benign papillomas. The progression stage is characterized first by dysplasia and ultimately by invasion and metastasis, due to additional genetic alterations (such as loss of tumour suppressor function) and progressive genomic instability.

The classic view of experimental carcinogenesis envisages that tumour initiation is followed by tumour promotion and progression in a sequential order but the temporal nature of initiation, promotion, and progression events in the natural world is complex: multiple mutational events are involved in the formation of a tumour (Fearon and Vogelstein, 1990; Sugimura, 1992). Humans are generally exposed to mixtures of agents that can simultaneously act at different stages of the carcinogenesis process and promotional events frequently increase cellular proliferation or decrease apoptosis, influencing subsequent initiation events. It is also increasingly apparent that an individual's genetic background can dramatically affect his or her susceptibility to a

carcinogen (Spitz and Bondy, 1993; Gonzalez, 1995; Drinkwater and Bennett, 1991). Therefore, human carcinogenesis is best characterized as an accumulation of alterations in genes regulating cellular homeostasis, such as oncogenes, tumour suppressor genes, apoptosis regulating genes, and DNA repair genes (Stanley, 1995).

Most chemically induced tumour models used in carcinogenesis research involve high doses of a single genotoxic carcinogen that can induce severe genetic damage, often randomly. The main goal for many of these models, which were generally developed before the identification of cancer-related genes, was to create animals that quickly developed neoplasias to provide investigators with sufficient material in a timely fashion for studies of tumour formation. Although some of the molecular alterations in the commonly used models have been identified, the types of alterations caused by high-dose chemical exposure do not generally reflect the gene environment interactions underlying carcinogenesis in humans. Furthermore, it can be difficult to interpret the activity of preventive compounds in these models, since the effects of an agent on the metabolic activation or detoxification of a high dose of a single chemical may not be mechanistically relevant to typical chronic, low-level human exposures to mixtures of exogenous or endogenous carcinogens.

1.4 MOUSE MODELS OF COLON CANCER

1.4.1 Genetic models.

To understand the precise roles of the different genes that are found to be mutated in colorectal tumours and to understand the nature of gene-environment interactions, over years, many researchers under-took efforts to generate mice that carry mutations in each of several genes implicated in either the initiation or progression of CRC. A variety of genetically altered mice have been generated that serve as models for colon adenoma and cancer. Although they do not have phenotypes that are identical to human diseases, many are extremely useful for investigating the pathogenesis as well

as testing potential preventive and therapeutic agents in preclinical studies. Currently, however, there are no practical models of colon cancer metastasis that progress from endogenous adenomas; development of such models is an important goal for future research.

- *APC mutant mice:* the first mouse that contained a mutation in the *Apc* gene was designated multiple intestinal neoplasia (Min) (Moser et al., 1990). This mouse was obtained in an ENU mutagenesis screen. These mice were found to have a nonsense mutation at the region corresponding to codon 850 of the *Apc* gene. The Min mutation results in a truncated protein of 850 amino acids. $Apc^{min/+}$ heterozygotes are born normally and have a reduced average lifespan of 150 days. These mice can develop more than 100 adenomas in the small intestine depending on the genetic background. Using gene knockout technology in embryonic stem cells, several Apc mutations have been constructed. $Apc^{\Delta 716}$ contains a truncating mutation at codon 716, whereas Apc^{1638N} contains a truncating mutation at codon 1638.(Fodde et al., 1994; Oshima et al., 1995) Similar to $Apc^{min/+}$ mice, both knockout mutants develop polyps mainly in the small intestine. Histologically, all these Apc mutants form polyp adenomas indistinguishable from each other. Interestingly, however, the polyp numbers are very different, even in the same C57BL/6J background. Namely, $Apc^{\Delta 716}$ develops usually ~300 polyps, whereas Apc^{1638N} forms only ~3 polyps. Despite the numerous polyps developing in the small intestine of the $Apc^{\Delta 716}$ as well as in $Apc^{min/+}$ mice, only a few polyps are formed in the colon, although genetic penetrance of the latter phenotype is complete. However, an additional mutation in the *Cdx2* gene in $Apc^{\Delta 716}$ mice reverses the polyp localization, shifting most polyps to the colon as in human FAP. Interestingly, the dramatic increase in the colon polyp number is caused by the increased frequency of Apc LOH due to chromosomal instability. The latter appears to result from the activation of the mTOR pathway and an acceleration of the G1 to S phase transition in the cell cycle (Aoki et al., 2003). These results present a new mechanism for chromosomal instability and suggest a possibility for treatment and prevention of colon

cancer with chromosomal instability. Consistently, introduction of a *BubR1*$^{+/-}$ mutation into Apc$^{min/+}$ mice causes 10 times more colonic tumours, and MEFs derived from compound mice show a higher rate of genomic instability.(Rao et al., 2005) Another Apc knockout strain that has been reported is the Apc$^{\Delta 14}$ mouse line (Colnot et al., 2004). The histopathology of mice of this strain appears essentially the same as in Apc$^{min/+}$ or Apc$^{\Delta 716}$, with differences in only polyp multiplicity. While intestinal polyposis is the most visible phenotype in Apc heterozygous mutant mice, they display a wide variety of additional phenotypes. For example, Apc$^{min/+}$ mice show progressive loss of immature and mature thymocytes from ~80 days of age, with complete regression of the thymus by 120 days (Coletta et al., 2004). They also deplete splenic natural killer cells, immature B cells, and B progenitor cells in the bone marrow due to complete loss of interleukin (IL)-7–dependent B-cell progenitors. Transplantation experiments suggest that an altered bone marrow microenvironment is responsible for the selective lymphocyte depletion in Apc$^{min/+}$ mice (Coletta et al., 2004). Because of the heavy tumour load in the small intestine, most Apc mutant mice die young (4–5 months) due to anaemia and cachexia, and some of them die of intestinal intussusception. It has been reported that Apc$^{min/+}$ mice have perturbations in ammonia metabolism in the liver that appears to be responsible for the mortality (Benhamouche et al., 2006). On the other hand, Apc$^{min/+}$ mice have a 10-fold increase in the level of circulating IL-6, which can cause severe cachexia as exemplified by loss of muscle weight and fat tissues (Baltgalvis et al., 2008). Introduction of an inactivating cyclooxygenase (COX)-2 gene mutation dramatically decreases the polyp number in Apc mutant mice (Taketo, 1998). Likewise, COX-1 mutation also reduces polyp multiplicity (Chulada et al., 2000). Expression of COX-2 protein is found in intestinal polyps of various sizes, and COX-2 is expressed from a very early stage of polyp formation on (Oshima et al., 1996). Introduction of a COX-2 gene (Ptgs2) knockout mutation into the Apc mice causes dramatic reductions in both the number and size of polyps in the compound mutant mice in a mutant gene dosage-dependent manner (Oshima et al., 1996; Oshima et al.,

2001). These results provided the rationale to treat human patients with FAP with COX-2 inhibitors such as celecoxib or rofecoxib, and clinical trials confirmed the results of the animal experiments (Steinbach et al., 2000). Following these experiments, a number of reports have been published in which the Apc mutant mice were dosed with various drugs or drug candidates (Steinbach et al., 2000). The constitutive isozyme COX-1 also has a significant role in polyposis. Introduction of COX-1 mutation into $Apc^{min/+}$ mice reduces the number and size of intestinal polyps by ~80%, a similar effect to that caused by COX-2 mutation (Chulada et al., 2000). In fact, COX-1 and COX-2 cooperate in polyp formation by supplying prostaglandin (PG) E2, which stimulates polyp angiogenesis (Takeda et al., 2003).

- *β-catenin mutant mice*: because APC forms a complex with other proteins that mediate the Wnt signalling pathway, mouse models have been used to determine whether mutations in other components of the complex can also induce polyp formation. Stabilizing mutations in serine/threonine residues of β-catenin have been identified in a subpopulation of human colon tumours that do not carry APC mutations; therefore, conditional mutations that stabilize β-catenin have been specifically expressed in the intestines of mice. When stabilized β-catenin was expressed from the calbindin promoter, mice developed only a few polyps in the small intestine (Romagnolo et al., 1999). By contrast, Cre recombinase expression under the control of cytokeratin 19 (K19) or fatty acid binding protein gene promoters allows expression of a stabilizing β-catenin mutant from a floxed allele and causes the formation of 700–3000 polyps in the small intestine (Harada et al., 1999). These findings confirm the role of Wnt signalling activation in polyp formation and suggest that polyps are initiated from the transient amplifying cells in the proliferative zone where K19 and fatty acid binding protein are expressed at significant levels but calbindin is only scarcely. This conditional system has been used to conditionally express β-catenin in several other organ systems and has provided evidence for a role of Wnt signalling in prostate

tumorigenesis51 and embryonic and immune system development (Lickert et al., 2000; Gounaris et al., 2001; Taketo, 2006).

- *Mismatch repair (MMR) knockout mice:* The MMR system has several functions that are important for maintaining the integrity of mammalian genomes, including the post-replicative repair of base substitution mutations as well as small insertion/deletion mutations (IDLs), the signalling of cell cycle arrest and apoptosis in response to exposure to DNA damaging agents and the suppression of recombination between homologous sequences. The characteristic repair steps of MMR are conserved between prokaryotes and eukaryotes and include the recognition of mismatched bases by MutS proteins and the recruitment of MutL proteins to initiate the subsequent repair steps resulting in the removal and resynthesis of the DNA strand carrying the mutated base(s) (Iyer et al., 2006). The MMR system in eukaryotes is complex and are summarized in Fig.16.

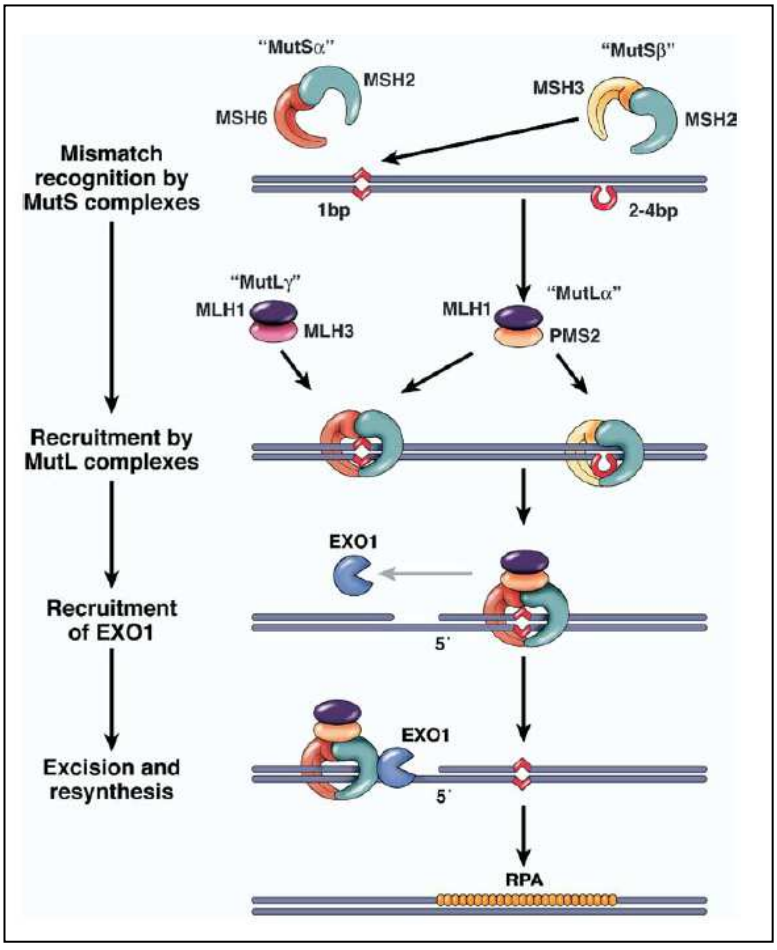

Figure 16: Model for mammalian MMR. MMR is initiated by the recognition of mispaired bases by MutSα and MutSβ complexes that act as sliding clamps. The activation of downstream repair events requires the interaction of MutSα and MutSβwith MutLα. In addition, MutLγ participates in the repair of single-base mismatches and 1–base pair IDLs. MutSα bound to a mismatch recruits EXO1, which initiates mismatch excision from 5' nicks in the template strand. Although the mechanisms that generate 5' and 3' single-stranded nicks is not clear, MutLα contains an endonuclease activity (encoded by PMS2) that can introduce 5' nicks into the template strand carrying the mismatch. Mismatch excision proceeds past the site of the mismatch, and the resulting gap is stabilized by RPA. In mammalian cells, other exonucleases possibly participate in the removal of mispaired bases whose identity remain unknown. Later repair steps require the interaction of MutS and MutL complexes with DNA replication proteins, including proliferating cell nuclear antigen, replication factor C, RPA, and polymerase δ, to coordinate the transfer of information between mismatch recognition and excision/resynthesis. Little is known about the nature of these interactions and the precise composition of the late MMR complexes.

Knockout mice correlate with the associated DNA repair defects and the frequency of MMR mutations found in patients with HNPCC. However, phenotypic differences between MMR knockout mice and patients with HNPCC exist. For example, unlike patients with HNPCC with mutations in *msh2. msh6* and *mlh1*, heterozygous mice carrying the corresponding knockout mutations do not develop early-onset tumours. Homozygous Msh2, Msh6, and Mlh1 knockout mice are cancer prone with a tumour spectrum that includes gastrointestinal cancers, but unlike patients with HNPCC, most mice die prematurely due to aggressive lymphomas (Edelmann and Edelmann, 2004). The differences between humans and MMR knockout mice are likely due to the shorter lifespan of mice, making somatic loss of the wild-type allele and subsequent tumourigenesis unlikely. The recent identification of patients with biallelic mutations in *msh2, msh6, mlh1* and *pms2* revealed that MMR deficiency in humans results in a severely reduced lifespan due to hematologic and other malignancies similar to those of MMR knockout mice (Felton et al., 2007). A comparison of the phenotypes of human and mice indicates that the basic mechanisms of DNA repair and tumour suppression have been conserved; MMR mutant mice have been highly useful in determining how the loss of MMR function results in tumourigenesis. Several Msh2 knockout mice have been generated (de Wind et al., 1995; Reitmair et al., 1996; Smits et al., 2000). Msh2 inactivation results in complete MMR deficiency. As a consequence, $Msh2^{-/-}$ mouse cells are unable to repair single-base mismatches and 1 to 4 base IDLs. The loss of MMR in Msh2-deficient mice causes a severe reduction in survival and a strong cancer

predisposition phenotype. The majority of animals develops T-cell lymphomas and succumbs to these tumours by 6 to 8 months of age. $Msh2^{-/-}$ mice that survive to 6 to 12 months of age develop small intestinal adenomas and invasive adenocarcinomas.. The tumours in these mice display a MSI-high phenotype similar to patients with HNPCC (Reitmair et al., 1996; de Wind et al., 1998); in contrast to $Msh2^{-/-}$ mutant mice, $Msh2^{+/-}$ heterozygous mice display overall normal survival and only a small number of these mice develop similar tumours later in life. $Msh6^{-/-}$ mice display a strong cancer phenotype with a tumour spectrum similar to that of $Msh2^{-/-}$ mice (Edelmann et al., 1997). However, Msh6-deficient mice survive longer (up to 18 months) and the onset of tumour development is delayed compared with Msh2-deficient mice. The longer survival of $Msh6^{-/-}$ mice is the result of the partial repair defect caused by Msh6 inactivation. In Msh6-deficient cells, the repair of base substitution mutations and single-base IDLs is impaired; however, in contrast to Msh2-deficient cells, the repair of 2 to 4 base IDLs is not affected. As a consequence, the mutator phenotype in $Msh6^{-/-}$ mice is characterized predominantly by an accumulation of base substitution mutations rather than frame shift mutations that occur frequently in $Msh2^{-/-}$ mice. Because the repair of 2 base IDLs is not impaired, the tumours that develop in $Msh6^{-/-}$ mice do not display the MSI phenotype that is characteristic of HNPCC tumours. In addition, $Msh6^{-/-}$ mice develop endometrial cancers that are also seen in a significant number of patients with *msh6* mutations (de Wind et al., 1999; Wijnen et al., 1999). In contrast to $Msh2^{-/-}$ and $Msh6^{-/-}$ mice, the disruption of Msh3 in mice does not result in a strong cancer phenotype. Msh3-deficient mice display normal survival and develop gastrointestinal tumours only very late in life and at low incidence (Edelmann et al., 2000). The absence of a significant tumour phenotype is caused by the moderate repair defects conferred by the loss of Msh3 function. Msh3-deficient cell extracts are defective in the repair of 1 to 4 base IDLs but can still efficiently repair single-base substitution mutations. The combined inactivation of Msh6 and Msh3 in mice results in complete MMR deficiency and a strong cancer phenotype similar to $Msh2^{-/-}$ mice (Edelmann et

al., 2000; de Wind et al., 1999). Similar to Msh2, loss of Mlh1 results in complete MMR deficiency and a strong mutator phenotype in the tissues of $Mlh1^{-/-}$ mice. Mlh1-deficient mice have a shortened lifespan (up to 12 months) and display a strong cancer predisposition phenotype that is very similar to that of Msh2-deficient mice. The tumour spectrum of $Mlh1^{-/-}$ mice includes T-cell lymphomas, intestinal adenomas and adenocarcinomas, and skin tumours. The tumours in these mice also display an MSI-high phenotype (Baker et al., 1996; Edelmann et al., 1996; Prolla et al., 1998; Edelmann et al., 1999). $Pms2^{-/-}$ mice also display a strong cancer predisposition phenotype. However, in contrast to Mlh1-, Msh2- or Msh6-deficient mice, Pms2-deficient mice develop lymphomas and sarcomas but not intestinal tumours and these tumours develop later in life (Prolla et al., 1998; Edelmann et al., 1999; Baker et al., 1995). Although the inactivation of Pms2 increased the mutation frequencies at mononucleotide repeat tracts, the increase in mutation frequency was almost 3-fold lower as compared with Mlh1-deficient mice. This difference indicates that Pms2 inactivation, similar to Msh6 inactivation, causes only a partial repair defect in the tissues of mice. Consistent with this notion, *pms2* mutations in human patients are associated with late-onset Lynch syndrome cancers and the overall cancer risk is lower as compared with patients with *msh2* or *mlh1* mutations (Senter et al., 2008). Similar to $Pms2^{-/-}$ mice, the inactivation of Mlh3 results also in a late-onset cancer phenotype (Chen et al., 2005). However, in contrast to $Pms2^{-/-}$ mice, $Mlh3^{-/-}$ mice develop adenomas and adenocarcinomas in their small intestines. In addition, Mlh3-deficient mice develop extra-gastrointestinal tumours, including lymphomas, basal cell carcinoma of the skin, and other tumours. $Mlh3^{-/-}$ mice display increased MSI at mononucleotide repeat sequences in their genomic DNA but to a lesser extent than that seen in $Pms2^{-/-}$ mice. The combined inactivation of both Mlh3 and Pms2 in mice increases the levels of MSI to levels that are similar to those seen in Mlh1-deficient mice. In addition, Mlh3-Pms2 double-deficient mice display severely reduced survival, increased cancer susceptibility, and a tumour spectrum that is comparable to Mlh1-

deficient mice. These results indicate that Pms2 and Mlh3 share overlapping *in vivo* repair and tumour suppressor functions. They also provide an explanation as to why *pms2* and *mlh3* mutations are less frequently found in patients with colorectal cancer than *mlh1* mutations and also suggest that *pms2* and *mlh3* mutations might be more common in patients with late-onset Lynch syndrome-associated cancers. To test the impact of MMR deficiency on Apc-driven intestinal tumourigenesis, mouse lines with homozygous mutations in Msh2, Msh6, Msh6, Mlh1, and Pms2 that also carry heterozygous Apc germline mutations have been constructed. The combination of MMR deficiency with the predisposing Apc mutations in mice limits the tumour development almost exclusively to the intestinal tract and the tumour incidence correlates with the severity of the MMR defects in the different MMR knockout mice. Whereas the loss of Msh2 or Mlh1 function in Apc mutant mice results in a dramatic increase in the number of intestinal tumours, the loss of Msh6 or Pms2 causes a more moderate increase in intestinal tumour numbers and the loss of Msh3 does not increase the tumour load (Edelmann et al., 1999; Reitmair et al., 1996; Baker et al., 1998; Kuraguchi et al., 2001). The increase in tumour number is caused by the accumulation of somatic mutations within the wild-type Apc allele (Smits et al., 2000; Kuraguchi et al., 2001). MMR-defective mice have also been crossed with mice that carry mutations in other tumour suppressor genes considered important for gastrointestinal tumourigenesis. For example, the combination of Msh2 or Msh6 deficiency with p53 deficiency in mice results in accelerated tumourigenesis (Toft et al., 2002; Young et al., 2007). However, the $Msh2^{-/-}/p53^{-/-}$ and $Msh6^{-/-}/p53^{-/-}$ compound mutant mice mainly succumb to T-cell lymphoma. Therefore, it will be necessary to determine the effects of p53 mutation on intestinal tumourigenesis in conditional knockout MMR mice.

1.4.2 Chemically-induced mouse models.

The study of experimental colon carcinogenesis in rodents has had a remarkably long history, dating back almost 80 years. Perhaps the earliest published study of Lorenz et

al. (Lorenz et al., 1941) demonstrated tumourigenesis in the fore stomach and intestine of mice following feeding with the polycyclic aromatic hydrocarbon, methylcholanthrene. Lisco et al. (Lisco et al., 1947) reported that feeding radioactive yttrium to rats induced a high proportion of colon tumours. Walpole et al. (Walpole et al., 1952) reported that white rats given injections of 4-aminodiphenyl and 3,2-dimethyl-4-aminodiphenyl developed colon tumours. However, the most commonly used model for sporadic colorectal cancer (CRC) takes advantage of the organotropism of the colon carcinogens, DMH and AOM. DMH, a metabolic precursor of methylazoxymethanol (MAM), was used in several early studies to induce tumours in rats (Druckrey et al., 1967; Schauer et al., 1969; Thurnherr et al., 1975). Repetitive treatment with this methylating agent was reported to produce colon tumours in rodents that exhibit many of the pathological features associated with the human disease (Haase et al., 1973; Martin et al., 1973; Shamsuddin et al., 1981; Ward, 1974). Thus, DMH has provided cancer researchers with a reproducible experimental system for studying 'sporadic' forms of CRC (LaMont et al., 1978). However, AOM offers advantages over DMH, including enhanced potency and greater stability in dosing solution (Neufert et al., 2007; Papanikolaou et al., 1998). Since then, several thousand studies using AOM have been published (Deschner and Long, 1977; Thurnherr et al., 1973; Toth and Malick, 1976). DMH and its metabolite, AOM, are procarcinogens that require metabolic activation to form DNA-reactive products (Fiala, 1977; Fiala et al., 1991; Weisburger, 1971). Metabolism of these compounds involves multiple xenobiotic-metabolizing enzymes, which proceeds through several N-oxidation and hydroxylation steps, including the formation of MAM following hydroxylation of AOM. The reactive metabolite, MAM, readily yields a methyldiazonium ion, which can alkylate macromolecules in the liver and colon (Fiala, 1977; Fiala et al., 1984; Fiala et al., 1978), including the addition of methyl groups at the O^6 or N^7 position of guanine (O^6-methyl-deoxyguanosine and N^7-methyl-deoxyguanosine) (Fig.17).

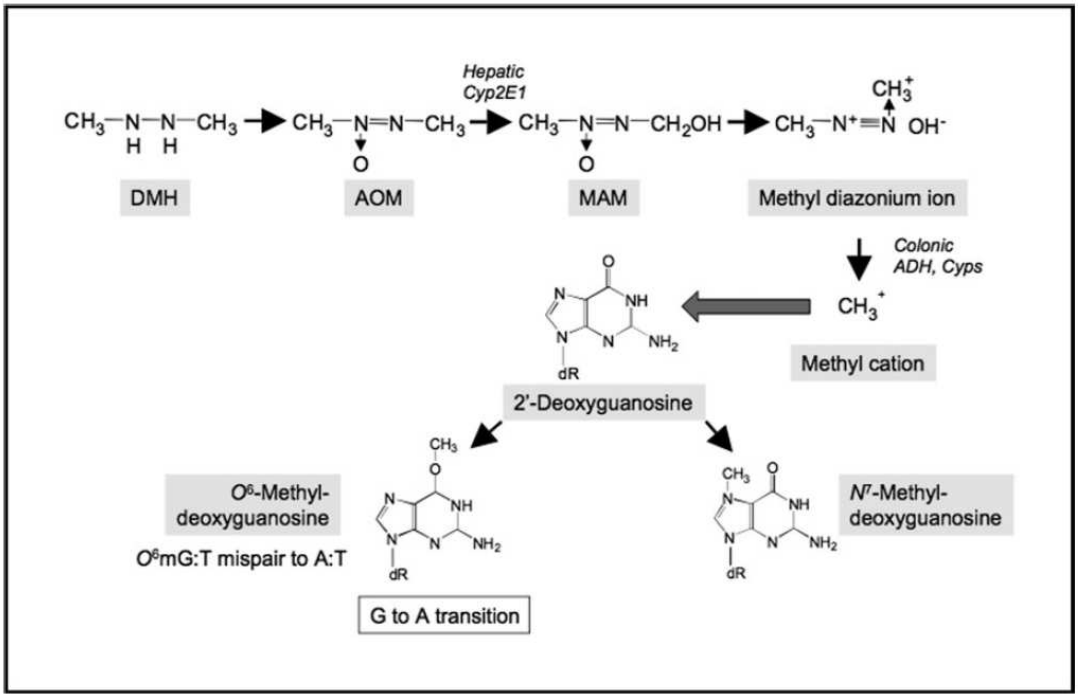

Figure 17: Metabolism of AOM involves multiple transcriptionally regulated xenobiotic-metabolizing enzymes.

MAM is a substrate of the nicotin-amide adenine dinucleotide^{+}-dependent dehydrogenase present in the colon and liver, suggesting that the active metabolite of MAM might be the corresponding aldehyde (Zedeck et al., 1979). A direct role for the alcohol-inducible cytochrome P-450 isoform, CYP2E1, in activation of AOM and MAM has been established (Sohn et al., 2001). These metabolites of CYP2E1 are transported to the colon via the bloodstream. The ability of AOM and DMH to target the colonic mucosa is probably a consequence of the relative stability of the hydroxylated metabolite, MAM (Nagasawa et al., 1972); with a half-life of ~12 h, there is sufficient time for MAM to distribute to the colon (Feinberg and Zedeck, 1980). Further activation of blood-borne metabolites may then proceed via non-P450-dependent mechanisms, including possible oxidation of MAM, directly within the colon (Sohn et al., 2001; Schoental et al., 1973).

1.5 POTASSIUM CHANNELS AND COLON CANCER

Increasing evidence supports the notion that ion channels and transporters control the development of cancer hallmarks in different human cancers (Arcangeli and Becchetti, 2006; Becchetti et al., 2010; Becchetti A and Arcangeli, 2008). In fact, among the genes whose expression is altered during the carcinogenetic process, those encoding ion channels and transporters are acquiring a relevant role in the last years (Arcangeli et al., 2009). They often have pleiotropic effects on the neoplastic cell physiology: for example, by adjusting the membrane potential, they can control the Ca^{+2} flow and then the cell cycle. Their effects on mitosis may also depend on cell volume regulation, usually together with the Cl^- channels. Ion channels are also implicated in more advanced stages of cancer, during which stimulate neovascularisation, mediate cell matrix interactions and regulate cell motility, with ensuing effects on cancer progression (Arcangeli et al., 2009). In particular, K^+ channels of the EAG family, mainly hERG1 (Arcangeli et al., 1995) and EAG-1 (Pardo et al., 1999) were found to be over-expressed in several types of human cancers (Arcangeli et al., 2009; Pardo and Stuhmer, 2008), including CRC (Lastraioli et al., 2004; Ousingsawat et al., 2007; Dolderer et al., 2010; Lastraioli et al., 2012) and represents a negative prognostic factor in non metastatic patients (Lastraioli et al., 2012). Moreover, an up-regulation of oncogenic K^+ ion channels (BK, Elk1 and EAG) was detected in the colon of $Apc^{min/+}$ mice (Ousingsawat et al., 2008). Voltage-gated K^+ channels (VGSC) appear to exert a pleiotropic role in regulating colorectal cancer cell proliferation and progression. The first report was provided by Yao (Yao and Kwan, 1999), who showed that K^+ channel inhibitors reduce cell proliferation in the colon carcinoma cell line DLD-1. Subsequently, different VGKCs were detected in the colon carcinoma cells T84, such as K_v 10.1, K_v 3.4 and K_v 1.5 (Spitzner et al., 2007). Application of channel inhibitors as well as specific small interfering (si) RNAs led to conclude that K_v channels control proliferation, in these cells. Moreover, *K_v 1.3*, *K_v 1.5*, *K_v 3.1*, *K_v 10.1* (Abdul and Hoosein, 2002; Hemmerlein et al., 2006; Ousingsawat et al., 2007), *K_v 11.1* (Lastraioli

et al., 2004) and K_{2p} *9.1* (Kim et al., 2004) transcripts have been detected in primary human samples of colon carcinoma. These results agree with the observation that K_v genes are up-regulated in the colon of mice treated with chemical carcinogens (Ousingsawat et al., 2007). The oncologic relevance turns on the fact that genomic amplification of K_V *10.1* is an independent marker of adverse prognosis (Ousingsawat et al., 2007) and that Lastraioli et al. (Lastraioli et al., 2004) found a high correlation between the level of K_v*11.1* surface expression and carcinoma stage. Moreover, a negative correlation was observed between K_v*11.1* expression and tumour chemosensitivity to doxorubicin (Chen et al., 2005).

Recent work has investigated which genes are mutated at significant frequency, in a subset of human colorectal cancer samples. *Kcnq5 (K_v 7.5)* turned out to be frequently mutated (Sjöblom et al., 2006), whereas *Scn3b* (codifying for the β subunit of the type III VGSC) and *Kctd15* (K^+ channel tetramerisation domain 15) were among the genes synergistically controlled by the mutant *p53* and *Kras* , typical oncogenes of murine and human colon cancers (McMurray et al., 2008).

The K^+ channels commonly found in CRC tumours, are summarized in Table 1.

Table 1: Changes in the expression of potassium ion channels in colorectal cancer.

Ion channel type	Ion channel name	Activity	Changes in colon cancer	Role in colon cancer	References
potassium channel, subfamily K; two-P	KCNK1; KCNK5; KCNK6	inward rectifier activity outward rectifier activity	increased expression		Spitzner et al., 2007
	KCNK9	voltage-gated ion channel activity	genomic amplification	cell proliferation, tumour growth; resistance to hypoxia	Kim et al., 2004
potassium channel tetramerisation domain containing 15	KCTD15	voltage-gated ion channel activity	increased expression		McMurray et al., 2008
Voltage-gated potassium channel: VGKC or K_v	K_v1.3	delayed and outward rectifier activity	increased expression	cell proliferation	Abdul and Hoosein, 2002;.Spitzner et al., 2007; Ousingsawat et al., 2007
	K_v1.5; K_v9.3; K_v10.1; K_vLQT1	delayed rectifier activity	increased expression	cell proliferation cell proliferation oncogenic potential; modulation of cell cycle progression, adverse prognosis cell proliferation	Pardo et al., 1999; Hemmerlein et al., 2006; Spitzner et al., 2007; Ousingsawat et al., 2007;
	K_v 3.1; K_v 3.3/3.4	voltage-gated ion channel activity	increased expression	inhibition of ion transport cell proliferation	Spitzner et al., 2007; Ousingsawat et al., 2007
	K_v 4.1	metal and potassium ion binding	increased expression		Spitzner et al., 2007
	K_v 5.1	potassium ion binding	increased expression		Spitzner et al., 2007
	K_v 7.5	inward rectifier activity	genomic mutation		Sjöblom et al., 2006
	K_v 11.1	delayed rectifier activity	increased expression	cell proliferation, invasiveness, reduced cell differentiation and prognosis, angiogenesis	Lastraioli et al., 2004; Spitzner et al., 2007; Ousingsawat et al., 2007; Lastraioli et al., 2012
member of ETS oncogene family	ELK1	transcription factor activity	increased expression	cell proliferation	Spitzner et al., 2007; Ousingsawat et al., 2007
Inwardly-Rectifying potassium channels: K_{IR}	K_{IR} 6.1	ATP-activated inward rectifier activity	increased expression		Spitzner et al., 2007
potassium large conductance calcium-activated channel	BK	voltage-gated ion channel activity	increased expression	cell proliferation	Ousingsawat et al., 2008; Ge et al., 2012

1.5.1 hERG1 potassium channel.

KCNH2, the gene encoding the human ether a go-go-related gene type 1 (hERG1) K^+ channel, also known as $K_v11.1$, was discovered in 1994 (Warmke and Ganetzky, 1994). Based on the biophysical properties of heterologously expressed channels, it was proposed that hERG1 channels conduct the rapid delayed rectifier K^+ current, I_{Kr} in cardiomyocytes (Sanguinetti et al., 1995; Trudeau et al., 1995). I_{Kr} is one of several K^+ currents that mediate repolarization of vertebrate cardiomyocytes and is distinguished by its disproportionately rapid rate of inactivation compared to activation, its association with an inherited form of arrhythmia, and by its clinically significant pharmacology. A reduction of I_{Kr} caused by long QT syndrome (LQTS)-associated mutations in hERG1 can induce ventricular arrhythmia and cause sudden cardiac death. In noncardiac cells, hERG1 channels primarily contribute to maintenance of the resting potential (Schwarz and Bauer, 2004). In addition to the heart, hERG1 expression has been identified in several regions of the brain (Guasti et al., 2005), tumour cells (Arcangeli, 2005; Bianchi et al., 1998; Guasti et al., 2005), gastro-intestinal smooth muscle myocytes (Farrelly et al., 2003), pancreatic β-cells (Rosati et al., 2000), lactotrophs (Bauer et al., 2003), carotid body cells (Overholt et al., 2000) and the inner ear (Nie et al., 2005).

1.5.1.1 hERG1 structure and accessory subunits.

In humans, hERG1 is located on chromosome 7q35–36, and the coding region comprises 16 exons spanning approximately 34 kb of genomic sequence. The full-length hERG1 subunit (hERG1a) is composed of 1.159 amino acids with a predicted molecular mass of 127 kDa and has six transmembrane domains (S1–S6) (Fig.18).

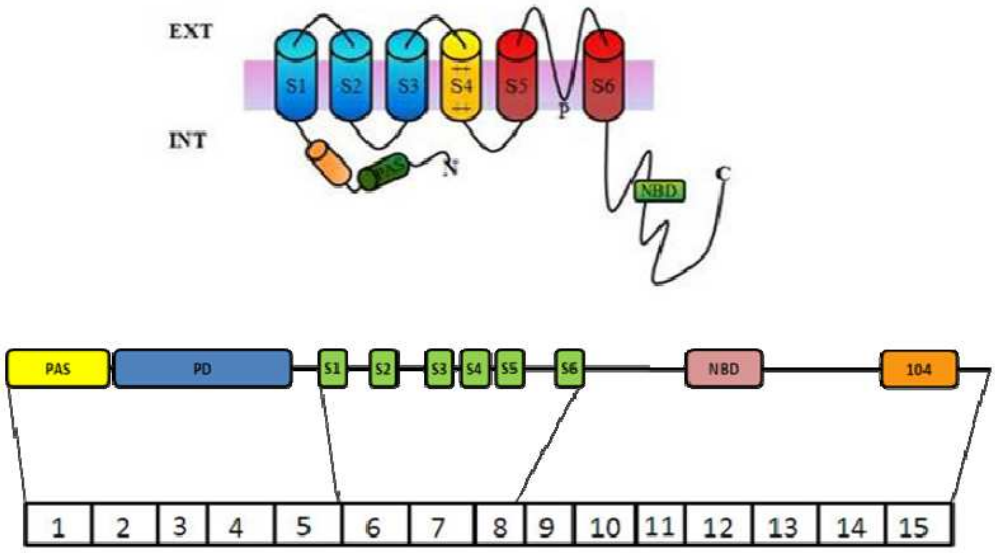

Fig.18: Structure of hERG1 channel.

hERG1a has a long (376 amino acids) N-terminus and residues 1–135 comprise the so-called “eag domain” that was crystallized and found to be the first eukaryotic example of a protein-protein interaction structure called a Per–Arnt–Sim (PAS) domain (Morais et al., 1998). The function of the PAS domain in hERG1a is uncertain; however, LQTS associated mutations in this region disrupt channel trafficking and accelerate the rate of deactivation (Chen et al., 1999; Morais et al., 1998), perhaps by disrupting its interaction with the S4–S5 linker of the channel (Wang et al., 1998). The PAS domain can be phosphorylated (Cayabyab and Schlichter, 2002; Cui et al., 2000) and needs to be properly folded for normal trafficking of the channel complex from the endoplasmic reticulum (ER) to the Golgi and cell surface (Paulussen et al., 2002). An alternatively spliced variant of hERG1 (hERG1b) was isolated from mouse and human heart (Lees-Miller et al., 1997; London et al., 1997) and is composed of 819 amino acids with a predicted molecular mass of 94 kDa (Jones et al., 2004). The N-terminus of hERG1b is only 36 amino acids and lacks the PAS domain but has an “RXR” ER retention signal sequence that prevents its trafficking to the surface membrane unless co-assembled with hERG1a subunits (Phartiyal et al., 2008) (Fig.19).

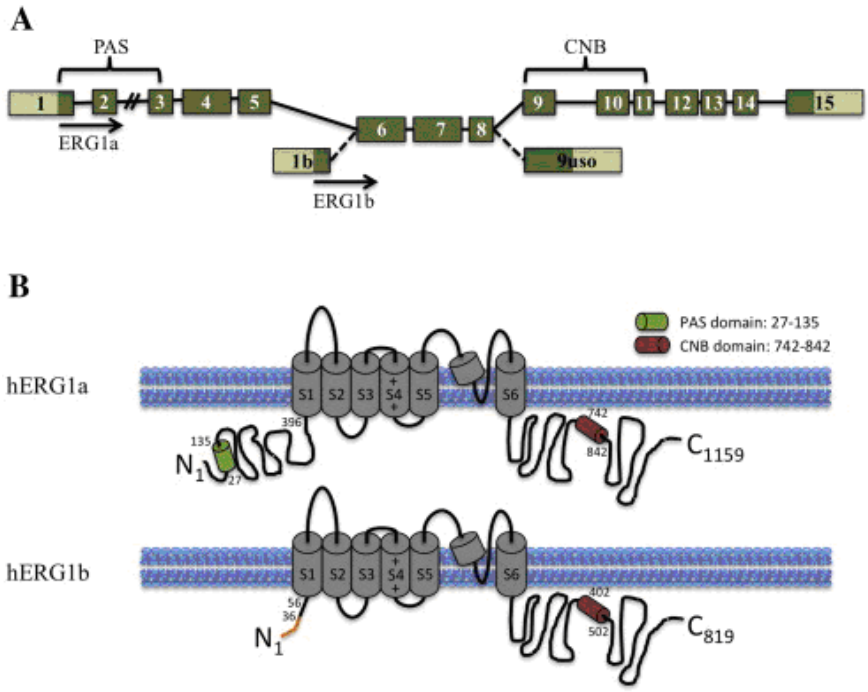

Figure 19: A) Genomic structure of the ether-a-go-go-related gene. hERG1a consists of 15 exons. Dark green is coding areas and light green non-coding. hERG1b has an own initiation site located between exon 5 and 6. B) Protein structure showing hERG1a and hERG1b, hERG1a is the full protein consisting of 1159 amino acids. hERG1b has an alternative 56 amino acids long N-terminus lacking the PAS domain and hERGuso an alternative C-terminus with only half the CNB domain remaining.

In rodents, *m-ERG1a* and *m-ERG1b* has also been shown to co-assemble in the brain (Guasti et al., 2005). The large C-terminus of both hERG1a and hERG1b contains a cyclic nucleotide binding domain (CNBD). Unlike cyclic-nucleotide gated channels, where binding of cyclic adenosine monophosphate (cAMP) to the CNBD is required for channel activation, cAMP has a relatively minor effect on hERG1 channel gating, causing a few millivolts shift in the voltage dependence of channel activation (Cui et al., 2000). Similar to other K_v channels, functional hERG1 channels are tetrameric complexes formed by coassembly of four α-subunits, either hERG1a subunits alone or hERG1a plus hERG1b subunits (Jones et al., 2004; Phartiyal et al., 2007; Sale et al., 2008). In heterologous expression systems, hERG1 proteins can also co-assemble with two ancillary β-subunits, MinK (KCNE1) and MiRP1 (KCNE2) (Abbott et al., 1999; McDonald et al., 1997). The KCNEs are small transmembrane proteins (123–129 amino acids) with a single transmembrane domain. Although MinK most likely functions as the accessory subunit for KCNQ1 to form I_{Ks} channels in the heart (Barhanin et al.,

1996; Sanguinetti et al., 1996), MinK can also modulate hERG1 channel density when over-expressed in heterologous expression systems (Bianchi et al., 1999; McDonald et al., 1997) (Fig.20).

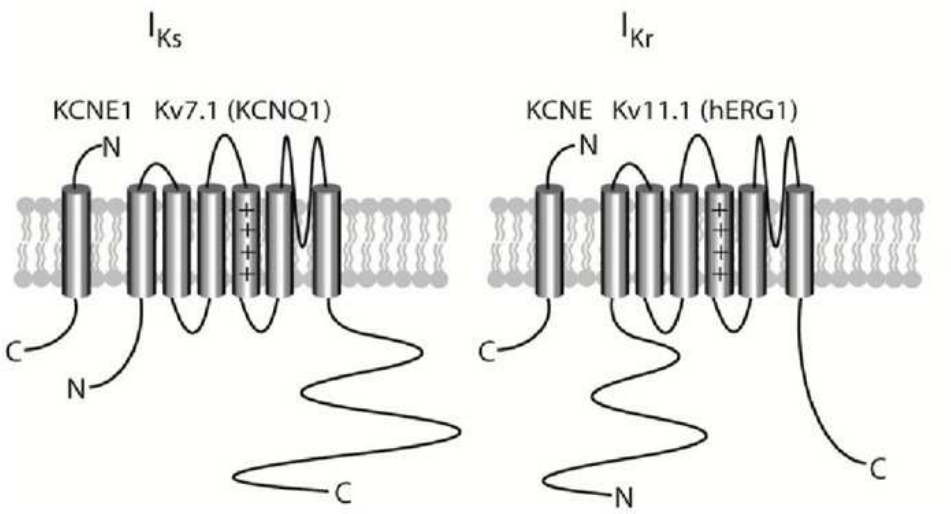

Figure 20: The co-expression of K_v7.1 with KCNE1 recapitulates native IKs (Barhanin et al., 1996; Sanguinetti et al., 1996), which not only plays a pivotal role in repolarising the myocardium but which is also important in transporting potassium across the strial marginal cells in the inner ear (Sunose et al., 1997). The co-assembly of K_v7.1 and KCNE1 results in an increase in single channel conductance, a positive shift in the voltage activation threshold, the slowing of activation and deactivation, and an almost complete absence of inactivation (Splawski et al., 1997); KCNE2/hERG1 expression in heterologous expression systems has been found to provide currents partly resembling native I_{Kr}.

MiRP1 was initially reported to alter the pharmacology and gating kinetics of hERG1 (Abbott et al., 1999), and LQTS-associated mutations in MiRP1 alter hERG1 currents differently from wild-type MiRP1 (Abbott et al., 1999; Gordon et al., 2008; Isbrandt et al., 2002; Lu et al., 2003). However, the consequences and physiological significance of this interaction has been contested (Weerapura et al., 2002), and the effects of MiRP1 in heterologous expression studies are variable (Anantharam et al., 2003; Mazhari et al., 2001; Weerapura et al., 2002). Moreover, it is not clear whether MiRP1 is expressed at high enough levels to affect hERG1 function throughout the heart as physiologically significant levels may be limited to pacemaker cells and Purkinje cells of the conduction system (Pourrier et al., 2003). Most recently, it was reported that interaction of MiRP1 with KCNQ1 may be more physiologically significant than its association with hERG1. Along with MinK, MiRP1 can coassembly with KCNQ1 to form a heteromultimeric channel complex with a net result of decreased I_{Ks} conductance

(Jiang et al., 2009). Moreover, targeted disruption of kcne2 in mice suggests that constitutively active KCNQ1/MiRP1 channels are expressed in thyrocytes and that these channels are required for normal thyroid hormone biosynthesis (Roepke et al., 2009). Altered thyroid function might have a role in LQTS associated with mutations in KCNE2. Linkage of KCNE2 mutations to ventricular arrhythmia and/or sudden cardiac death in a large kindred would go a long way towards substantiating the role of MiRP1 in LQTS.

1.5.1.2 hERG1 potassium channel in cancer.

Various cancer cell lines of epithelial, neuronal, leukemic, and connective tissue origin express hERG1 K^+ channels (Dolderer et al., 2010; Gong et al., 2010; Lastraioli et al., 2004; Shao et al., 2008; Ding et al., 2008; Cherubini et al., 2000; Pillozzi et al., 2002; Smith et al., 2002; Li et al., 2008; Masi et al., 2005; Patt t al., 2004; Chen et al., 2005; Lin et al., 2007; Bianchi et al., 1998; Roy et al., 2008; Wang et al., 2002; Afrasiabi et al., 2010; Crociani et al., 2003; Zhao et al., 2008; Cherubini et al., 2005; D'Amico et al., 2003; Meves, 2001; Pancrazio et al., 1999; Hofmann et al., 2001; Agarwal et al., 2010), whereas corresponding non-cancerous cells and cell lines do not exhibit significant hERG1 protein levels (Rosati et al., 2000; Pond et al., 2000). In corresponding human cancers, hERG1 protein may serve as biomarkers of malignant transition. Furthermore, hERG1 expression is implicated in enhanced cell proliferation, invasiveness, lymph node dissemination, and reduced cell differentiation and prognosis (Shao et al., 2008; Ding et al., 2008). In addition, increased neoangiogenesis, another hallmark of malignant tissue growth, has been reported for glioblastoma where the generation of blood vessels was stimulated by hERG1-dependent secretion of vascular endothelial growth factor (VEGF) (Masi et al., 2005). K^+ channel-dependent hyperpolarization appears to be critical for progression to the S phase. Hyperpolarization evokes Ca^{2+} influx, which is further augmented by calcium-dependent K^+ (K_{Ca}) channels and permits synthesis of mitogenic factors. In addition, hyperpolarization provides the electrical gradient necessary for Na^+-dependent transport of metabolic substrates and ions

across the plasma membrane, which is required for DNA synthesis (Wonderlin and Strobl, 1996). Considering that K^+ channels are involved in cell cycle progression, abundant expression of K^+ channels is expected to cause loss of proliferative control if endogenous pathways fail to block excessively expressed K^+ channels (Wonderlin and Strobl, 1996). Interestingly, the promoter region of the hERG1 gene harbours multiple binding sites for oncoproteins, such as specificity protein 1 and nuclear factor kappa light chain enhancer of activated B-cells, and for the tumour suppressor protein Nkx3.1 (Nk3 homeobox 1) (Lin et al., 2007). It is possible to hypothesize that mutations in oncoproteins constitutively activate hERG1 gene expression, shifting resting membrane potentials of cancerous cells toward more depolarized values and repolarising them at the end of G1 phase, thereby facilitating cell cycle progression and thus leading to cell proliferation. Furthermore, human gastric cancer cells exhibit reduced levels of the regulatory β-subunit KCNE2, leading to hERG1 current increase (Abbott et al., 1999; Roepke et al., 2010). In addition, genetic deletion of KCNE2 is associated with gastric neoplasia and increased nuclear cyclin D1 levels in mice, revealing genetic manipulation of cell proliferation mediated by a hERG1 β-subunit (Roepke et al., 2010).

Therefore, pharmacological intervention using hERG1 antagonists will serve to arrest the cell cycle in the G1 phase. In fact, selective hERG1 channel blockade by E-4031 reduced proliferation in cancerous cell lines (Smith et al., 2002). Cell cycle analysis of FLG29.1 leukaemia cells revealed accumulation of cells in the G1 phase following treatment with hERG1 channel blockers (Pillozzi et al., 2002). hERG1 K^+ channel blockers activate multiple apoptotic pathways. However, evidence for a direct mechanistic link between hERG1 K^+ channels and apoptotic proteins remains sparse to date. hERG1, integrin β1, and FAK form a macromolecular complex in different tumour cells (Arcangeli and Becchetti, 2006). Cell adhesion via integrin β1 causes activation of hERG1, which is essential for direct FAK phosphorylation (Cherubini et al., 2005). FAK is an essential component of integrin signalling and is phosphorylated when cells are

adhered to the extracellular matrix. Thus, it provides a survival signal and prevents apoptosis (Kornberg, 1998). FAK and hERG1 over-expression have independently been related to enhanced dissemination and invasiveness of tumours (Lastraioli et al., 2004; Kornberg, 1998). FAK phosphorylation due to hERG1 activation may explain the ability of malignant cells to circumvent apoptosis once they have lost contact to the extracellular matrix. The abundant expression of hERG1 and FAK might provide crucial survival signals in the absence of cell adhesion, and thus account for increased invasiveness and dissemination of hERG1-positive tumours. In addition, co-localization with hERG1 potassium channels activates the GTPase Rac1 and may contribute to adhesion-dependent modulation of tumour cell motility (Cherubini et al., 2005). The most intriguing perspective of anticancer therapy targeting hERG1 channels is direct blockade of the potassium channel, which is expected to produce anti-proliferative and pro-apoptotic effects that diminish tumour growth and invasiveness. A systematic *in vivo* investigation of chemotherapeutic properties and potential cardiac side effects of hERG1 inhibitors is required. Pro-arrhythmic (Sanguinetti and Tristani-Firouzi, 2006) and cardiotoxic risks of hERG1 inhibitors require careful evaluation (Toyama et al., 2008) when applying these compounds in clinical oncology. Systemic treatment of cancers with hERG1 antagonists may affect cardiac myocytes, resulting in apoptosis and heart failure. In addition, application of hERG1 antagonists may induce QT prolongation and ventricular tachycardia, so optimal suppression of these events will be required. To prevent pro-arrhythmic side effects, short-term drug application may be sufficient to induce apoptosis in tumour cells with minimal effects on cardiac electrophysiology. ECG monitoring should be performed during application of the drug. Cardiomyocyte apoptosis may be circumvented through targeted delivery techniques such as direct injection or trans-arterial drug application. Gene therapy represents an additional therapeutic approach to targeted suppression of hERG1 channel expression in cancers. Different proliferative states of cardiac and tumour cells may render cancerous tissue more susceptible to pro-apoptotic and anti-proliferative stimuli,

reducing the overall risk of heart failure during systemic application of hERG1 antagonists. Feasibility of tumour-selective hERG1-based anticancer therapy will further depend on differential drug effects on cancerous and non-cancerous tissue expressing hERG1 K^+ channels.

2. AIM OF THE STUDY

The aim of the present PhD project is to analyze the *in vitro* and *in vivo* relevance of hERG1 channels during colorectal carcinogenesis. We used CRC cells as well as either $Apc^{min/+}$ mice or AOM-treated mice as a model. Moreover, to analyze the effects of hERG1 over-expression *in vivo,* we produced transgenic (TG) mice, which over-express the *herg1* gene in the intestinal mucosa.

3. MATERIALS AND METHODS

3.1 *IN VITRO* MATERIALS AND METHODS

3.1.1 Cell cultures.

Colorectal cancer (CRC) cell lines HCT116, HCT8, were cultured in RPMI 1640 (Euroclone; Milan, Italy) with 10% Fetal Calf Serum (FCS) (Euroclone Defined; Euroclone; Milan, Italy). HT29 cells were cultured in McCoy's medium with 10% FCS. HCT116 and HT29 cells were kindly provided by Dr. R. Falcioni (Regina Elena Cancer Institute, Roma). HEK-Mock and HEK-hERG1 cells were obtained by transfecting HEK 293 cells with the pEGFP-C1 plasmid (Clontech; Mountain View, CA, USA) and with a pEGFP-C1 hERG1 plasmid (the kind gift of Dr. L. Pardo, Max-Planck-Institut für experimentelle Medizin, Göttingen, Germany). Stable transfectants were selected and maintained in DMEM with 10% FCS (Euroclone, Milan, Italy) and Geneticin (800 mg/ml).

3.1.2 VEGF-A secretion.

Cells were seeded into 24-well cell culture plates at 2 x 10^5 cells/ml in standard culture medium. After twenty-four hours, the medium was removed and 0.5 ml of Optimem (Gibco; Carlsbad CA, USA) was added. After an additional twenty-four-hours incubation, the medium was collected and VEGF-A measured using the DuoSet ELISA Development System (R&D Systems; Wiesbaden, Germany). Cells were recovered and counted to normalize the VEGF-A secretion data. When needed, hERG1-specific inhibitors E4031 or Way 123,398 were added along with Optimem, at a final concentration of 40 µM (as described by Pillozzi et al., 2007), as well as PI3K/Akt pathway inhibitors LY294002 (10 µM, Sigma) or perifosine (20 µM, kindly provided by Dr. A. Martelli, University of Bologna).

3.1.3 Cell transfection.

Cells were cultured as above in 6-well cell culture plates (Corning Costar; Corning NY, USA). Twenty-four hours after plating cells were transfected with 1) KCNH2-siRNAs (44858 anti-*herg1* siRNA1; 290144 anti-*herg1* siRNA2; 44762 anti-*herg1* siRNA3, Ambion; Austin TX, USA) (100 nM final concentration), 2) α-*vegf-a*-siRNA (Hs_VEGF_5, FlexiTube, S102757643, Qiagen) (100 nM final concentration), 3) negative control si-RNA (Silencer Negative control #1, 4611, Ambion) (50 nM final concentration) 4) pcDNA3.1/*hERG1* plasmid (Cherubini et al., 2005) in Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 5 hours, the medium was changed and, after over night incubation, 1 ml Optimem (Gibco; Carlsbad CA, USA) was added. Twenty-four hours later, the supernatant and cells were collected for measurement of VEGF-A secretion and RNA extraction. HT29 cells were transfected with pcDNA3.1/*hERG1* plasmid and collected forty-eight hours after transfection.

For *Akt1*, *Akt2*, *FOXO1A* and *FOXO3* silencing, the following Flexitube siRNA (Qiagen) were used: Hs_AKT1_10 (ID: 2103987, final concentration 5 nM); Hs_AKT2_7 (ID: 2103986, final concentration 25 nM); Hs_FOXO1A_7 (ID: 2103988, final concentration 25 nM); Hs_FOXO3_1 (ID: 2103989, final concentration 25 nM); AllStars negative control siRNA (final concentration 25 nM). Transfection were performed using Hiperfect Transfection Reagent (Qiagen) and following the manufacturer's instructions. 48 hours after transfection, cells were collected and RNA processed.

For hERG1 stable silencing in HCT116 cells, a commercially available TRC shRNA-library (Open Biosystems) was used. The shRNA construct included a hairpin of 21 base pair sense/antisense stem and a 6 base pair loop cloned into the pLKO.1 vector.

Lentivirus viral particles were produced by co-transfecting the second generation lentiviral plasmids and the transfer vector construct into the HEK293T packaging cell line. Stably silenced HCT116 cells (Sh7-5) were obtained with the virus at Multiplicity

Of Infection (MOI) =5. Stable cell lines were selected using puromycin (Fortunato et al., manuscript in preparation).

3.1.4 HIF activity.

The hypoxia responsive element-luciferase reporter gene vector, kindly provided by Dr. A Giaccia (Stanford University School of Medicine, Stanford, USA), was transfected into the various cancer cell lines with Lipofectamine 2000 along with the pRL-CMV plasmid (Promega) for normalization. After 5 hours, the medium was changed, and standard culture medium was added. Twenty-four hours later, some of the cell culture plates were transferred into a hypoxia chamber (Concept 400, Jouan, Milan, Italy) set at 0.1% O_2. After an additional 5 hours of incubation, cells were harvested and firefly and renilla luciferase activities were assayed using the Dual-Luciferase Reporter Assay System (Promega) and employing a Lumat LB 9507 single-tube luminometer (Berthold Technologies).

3.1.5 RNA extraction, reverse transcription, RT-PCR and Real-time Quantitative (RT-qPCR).

RNA extraction and reverse transcription were performed as in Pillozzi et al. (Pillozzi et al., 2002). Amplification of *KCNE1* and *KCNE2* was performed by RT-PCR with 2 µl of cDNA derived from CRC cells and human heart RNA (Ambion®), using a commercially available master mix (Invitrogen). The primer sequences for *KCNE1* were: 5'-TCCATTGGAGGAAGGCATTA-3' forward primer and 5'-CGCTGTGGTGTTAGACAGGA-3' reverse primer and PCR was performed in the following conditions: denaturation at 94°C for 2'min, 35 cycles at 94°C for 30'', 55°C for 1', 72°C for 30'' and a final extension cycle at 72°C for 10'. The primer sequences for *KCNE2* were: 5'-TTGTGTGCAACCCAGAAGAG-3' forward primer and 5'-CTTCCAGCGTCTGTGTGAAA-3' reverse primer and PCR was performed in the following conditions: denaturation at 94°C for 2'min, 35 cycles at 94°C for 30'', 58°C for 1', 72°C for 30'' and a final extension cycle at 72°C for 10'. *hERG1A* and *VEGF-A*

mRNA quantification by RT-qPCR was performed using 2 µl of cDNA using the 7500 Fast Real Time PCR System and the SYBR Green Master Mix Kit (Applied Biosystems; Foster City CA, USA). The *GAPDH* gene was used as a standard reference. The primer sequences for *hERG1A* and *GAPDH* were the same as those used in Cherubini et al. (Cherubini et al., 2000). The primer sequences for *VEGF-A* were the same as those used in Simpson et al. (Simpson et al., 2000).

The relative expression of *hERG1A* and *VEGF-A* was calculated with the comparative threshold cycle method. Standard curves were determined using the FLG29.1 cell line cDNA. HEK 293 sample was used as a calibrator for *hERG1A* expression For *GLUT-1*, *LDHA*, *ANGPTL-4*, *HIF-1α, HIF-2 α,* the following primers were used: *GLUT-1*: 5'-ACCATTGGCTCCGGTATCG-3' forward primer and 5'-GCTCGCTCCACCACAAACA-3' reverse primer; *LDHA*: 5'-ACCAGTTTCCACCATGATT-3' forward primer and 5'-CCCAAAATGCAAGGAACACT-3' reverse primer; *ANGPTL-4*: 5'-GCTGCATGCGTTGCCTC-3' forward primer and 5'-CCCTTGGTCCACGCCTCTA-3' reverse primer; HIF-1α: 5'-GTCGCTTCGGCCAGTGTG-3' forward primer and 5'-GGAAAGGCAAGTCCAGAGGTG-3' reverse primer; HIF-2α: 5'-TTGATGTGGAAACGGATGAA-3' forward primer and 5'-GGAACCTGCTCTTGCTGTTC-3' reverse primeṛ. The relative gene expression was calculated applying the Pfaffl analysis method (Pfaffl et al., 2004).

3.1.6 Protein extraction, immunoprecipitation (IP) and western blot (WB).

Total protein extraction were performed by standard methods using 1X lysis buffer (Cell Signaling Technology) followed by sonication. WBs were carried out as described in Lastraioli et al.(Lastraioli et al., 2004). IP experiments were performed according to Cherubini et al. (Cherubini et al., 2005). For IP, total lysates (1.5–2 mg) were subjected to a pre-clearing step consisting of incubation with protein A-Sepharose 4B beads (for hERG1 IP) or protein G-Agaroses beads (foṛ β1 IP) (Sigma-Aldrich) for two hours at 4°C.

Thereafter, cell lysates were immunoprecipitated with the appropriate antibody (anti-β1 TS2/16, Biolegend: 5 µg antibody/mg protein; anti-hERG1 N135, Enzo Life Science: 5 µl antiserum/1.5 mg protein). Cells were detached using 5 mM EDTA in PBS and incubated under normoxia in standard culture conditions until control cells adhered (30-60 minutes), and then they were seeded on different substrates in the absence or presence of hERG1 (E4031, 40 µM) o̧r β1 (anti-Bv7 (α-β1) blocking antibody 14 µg/ml, kindly provided by Prof. P. Defilippi, University of Turin) inhibitors. For the experiment with the activating monoclonal anti-β1 antibody (TS2/16), cells were resuspended in a solution containing 20 µg/ml of antibody in DMEM + BSA and kept in suspension through continuous low speed stirring for thirty minutes at 37°C.

For hERG1 detection, WBs were decorated with an anti-hERG1 rabbit polyclonal antibody (C54, Enzo Life Science, dilution 1:1000 in 5% BSA in TBS 0.1% Tween). Anti-α-tubulin mouse monoclonal (Sigma, dilution 1:500 in 5% BSA in TBS 0.1% Tween) antibodies were used as loading controls. For KCNE1 detection, the anti-KCNE1 mouse monoclonal antibody (Abcam, dilution 1:500, in 2% skim milk in TBS 0.1% Tween) was used. For KCNE2 detection, the anti-KCNE2 rabbit polyclonal antibody (Alomone, dilution 1:200 in 5% BSA in TBS 0.1% Tween) was used. For pAkt/Akt detection, the anti-Thr 308 pAkt (Santa Cruz, SC-16646, dilution 1:500 in 5% BSA in TBS 0.1% Tween) and anti-total Akt (Santa Cruz, SC-8312, dilution 1:500 in 5% BSA in TBS 0.1% Tween) antibodies were used. For β1 detection, an antị-β1 rabbit polyclonal was used (RM12, Immunological Science, dilution 1:1000 in 5% BSA in TBS 0.1% Tween). For p85 subunit detection, an anti-PI3 kinase p85 antibody was used (Upstate 06-195, dilution 1:2000 in 5% BSA in TBS 0.1% Tween). For p85 phosphorylation, an anti-phosphotyrosine antibody was used (p-Tyr, Santa Cruz Biotechnology, dilution 1:200 in 5% BSA in TBS 0.1% Tween). For HIF-1α and mTOR studies, rabbit anti-HIF-1ą anti-pSK6 and anti-total SK6 antibodies (Cell Signaling Technologies, dilution 1:500 in 5% BSA in TBS 0.1% Tween) were used. Densitometric analysis was carried out using the ImageJ software.

3.1.7 Electrophysiology: solutions.

Extracellular and pipette solutions were prepared as in Masi et al. (Masi et al., 2005). The extracellular solutions were delivered through a 9-hole (0.6-mm) remote-controlled linear positioner with an average response time of 1-2 seconds that was placed near the cell under study.

3.1.8 Patch-clamp recordings and data analysis.

ERG currents were always recorded under conditions of relatively high $[K]_o$ (40 mM) to measure currents under optimal signal-to-noise conditions. Pipette resistances were about 5 MW. Cell capacitance and series resistance errors were compensated (85-90%) before each voltage clamp protocol was run to reduce the voltage errors to less than 5% of the protocol pulse. Currents were recorded at room temperature by means of an Axopatch 1D (Axon Instruments, USA) using a protocol consisting of a 15-s conditioning phase (0 to -70 mV in 10 mV decrements) followed by a 0.5-s eliciting step at -120, as previously described (Masi et al., 2005). pClamp 7 (Axon Instruments) and Origin 6.0 (Microcal Inc, USA) software were routinely used during data acquisition and analysis. The steady-state activation curves were obtained by plotting the normalized peak currents at -120 mV versus the conditioning potential according to Schönherr et al. (Schönherr et al., 1999).

3.2 *IN VIVO* MATERIALS AND METHODS

3.2.1 Construction of hERG1-myc conditional expression vector.

To generate a transgenic mouse model with recombination activated hERG1 expression, we produced a β-actin EGFP hERG1 construct. The vector was assembled in a two step process requiring the production of an intermediate vector constituted by the β-actin minimal promoter plus hERG1-myc6xhis; in the unique HindIII site of this vector we then cloned the floxed EGFP sequence. The complete vector was constituted by the human β-actin promoter, the reporter gene EGFP floxed by two loxP

in the same orientation and the hERG1 cDNA tagged at the C-terminal with the myc-6xhis epitope; a SV40 polyadenilation sequence was used to induce the polyadenilation of both the EGFP and hERG1-myc 6xhis cDNA.

The 4.3 kb human β-actin promoter plus intron and the SV40 polyadenilation sequence were obtained from the β-actin expression plasmid, while the two loxP sequences were from the D11 loxP plasmid. Both plasmids were kindly provided by Dr. S. Aparicio (BC Cancer Agency, Vancouver, British Columbia, Canada).

To produce the hERG1-myc-6xhis chimeric gene a 3.7 Kb hERG1 cDNA was PCR amplified from the plasmid pcDNA 3.1 hERG1 with the F1 primer and the reverse primer R1 to remove the stop codon; removing of the stop codon required the substitution of the last two hERG1 AA (Gly to Ala, Ser to Tyr, respectively). The epitope myc-6xhis was obtained from pcDNA 3.1 (-) frame C plasmid (Invitrogen) by PCR with the F2 and R2 primers. The hERG1 and myc6xhis PCR products were then cut with HindIII/BamHI and BamHI/EcoRI, respectively, and ligated in the HindIII/EcoRI sites of pcDNA3.1 (+). A SV40 polyadenilation sequence obtained by PCR from the β-actin expression plasmid with the F3 and R3 primers and cut with EcoRI/SpeI was then inserted in the EcoRI/XbaI site of the pcDNAhERG1-myc-6xhis plasmid (hERG1-mycS).

The intermediate vector described above was then obtained inserting an HindIII/KpnI hERG1-mycS fragment in the corresponding sites of β-actin expression plasmid.

The EGFP expression cassette was obtained by amplification of EGFP cDNA with the primers F4 and R4 from the pEGFP C1 plasmid (Clontech) (the R4 primer was designed to insert a stop codon in the EGFP cDNA) while its corresponding SV40 polyA sequence was obtained with the F5 and R5 primers. The EGFP and SV40 polyA PCR products were then cut with SpeI/EcoRI and EcoRI/XhoI, respectively, cloned in the SpeI/XhoI sites of pBluescript SK plasmid then retrieved as a SpeI/BamHI fragment and ultimately cloned in the XbaI/BamHI sites of the D11 loxP2 plasmid. In order to destroy a putative splice acceptor consensus sequence in the D11 loxP2 plasmid, the

floxed EGFP was PCR retrieved with the F6 and R6 primers, cut with HindIII and cloned in the HindIII unique site of the intermediate vector so realizing the β-actin EGFP hERG1 construct. Correct orientation of the floxed EGFP was verified cutting the DNA with BamHI. All PCR were performed with the high fidelity proof reading enzyme Hotstart PFU (Stratagene) and the absence of errors in every amplification step was confirmed by DNA sequencing. Primers used to produce the complete vector with information of the restriction enzyme sites inserted and PCR conditions are reported in the table.

Target	**Primers**	**Sequence (5'-3')**	**Inserted restriction sites**	**PCR CONDITIONS (Annealing=A Extension=E)**
HERG1	F1	AAATTAATACGACTCACTATAGGGAG		A:56°C 1' E:72°C 3'30" x10 cycles then for the last 20 cycles E: 72°C 3'30"+10" for cycle
	R1	ACTAACTGCCCGGATCCGAG	BamHI	
myc-6xhis	F2	AGCTCGGGATCCAGCTTACG	BamHI	A: 56°C 1' E: 72°C 1'
	R2	TGGCAACTAGAATTCACAGTCGAG	EcoRI	
SV40 poly A for hERG-myc6xhis	F3	TTCAAGCTTATCGGAATTCAAGTAATGATC		A: 52°C 1' E: 72°C 1'
	R3	AATTCGACTAGTGGTACCGTCTAGAATTG	XbaI, KpnI, SpeI	
EGFP	F4	TCAGATCCACTAGTGCTAC	SpeI	A: 54°C 1 E: 72°C 1
	R4	AGATCTGAATTCTTACTTGTACAG	EcoRI	
SV40 poly A for EGFP-SV40	F5	AAATTCAGAATTCTCGCTAG	EcoRI	A: 50°C 1' E: 72°C 1'
	R5	GGAAACAGCTATGACCATG		
Floxed EGFP-SV40	F6	TAAAACGAGGGCCCGAAGCTTGTAATAC	HindIII	A: 54°C 1' E: 72°C 1'30"
	R6	AATTAACCCTCACTAAAGGGAAC		

3.2.2 Mouse strains and production of transgenic mice.

Fabp-Cre mice were purchased from NCI MMHCC (Saam and Gordon, 1999) $Apc^{min/+}$ mice were obtained from The Jackson Laboratory (stock number 002020).

The 10 kb vector-free XbaI DNA fragment was microinjected into the male pronucleus of fertilized eggs from FVB mice at LIGEMA, University of Florence, Italy, following standard procedures. Transgenic mice were maintained in a heterozygous state in FVB background.

Animals were housed in plastic cages with a wire-mesh providing isolation from the hygienic bed and were kept in temperature-, air-, and light-controlled conditions. They received food and water ad libitum. All experiments involving mice were performed in accordance to the criteria outlined in the Guide for the Care and Use of Laboratory Animals.

3.2.3 PCR genotyping.

$APC^{min/+}$ mice were genotyped by routine PCR assays on genomic tail DNA using a commercially available master mix (Invitrogen). An Apc^{min} nonsense mutation–specific primer (Apc-mutant: 5′-TTCTGAGAAAGACAGAAGTTA-3′), together with a complementary 3′-end primer (Apc-common: 5′-TTCCACTTTGGCATAAGGC-3′), detected the mutant Apc allele (313 bp), which is only present in heterozygous $Apc^{min/+}$ mice. The Apc+ allele–specific primer (Apc-wild-type: 5′-GCCATCCCTTCACGTTAG-3′) with the Apc-common primer detected the wild-type allele (619 bp).

Transgenic mice were genotyped by PCR analysis of genomic tail DNA. Briefly, 0.5 cm of mice tails were digested O/N at 55°C with 100 mg/ml Proteinase K in lysis buffer (50mM TrisHCl pH 8, 50mM EDTA pH8, 1% SDS, 10mM NaCl); after phenol/chlorophorm purification and ethanol precipitation, genomic DNA was resuspended in sterile water. Alternatively, genomic DNA was extracted using a Chelex protocol by incubation of a 0.2 cm of mice tails in 150 µl of 10% (w/v in water) Chelex 100 resin (BIO-RAD) for 4 hours at 56°C and 30 minutes at 98°C. The presence of the

transgene was checked on mouse tail genomic DNA by amplification of a 350 bp fragment spanning the myc-6xhis epitope region with the forward 5'-TTTAAACTTAAGCTGGAGAC-3' and reverse 5'-CTACAGTGCTGTGACCAC-3' primers in the following PCR conditions: denaturation at 94°C for 2 min, 35 cycles at 94°C for 40", 54°C for 1', 72°C for 1 min and a final extension cycle at 72°C for 5 min. Amplification of the control gene, interleukin-2, by PCR was systematically performed on DNA to check the integrity of genomic DNA extracted, using the following primer pair: 5'-CTAGGCCACAGAATTGAAAGATCT-3' and 5'-GTAGGTGGAAATTCTAGCATCATCC-3' and applying the following PCR conditions: denaturation at 94°C for 2 min, 35 cycles at 94°C for 1', 60°C for 1', 72°C for 1 min and a final extension cycle at 72°C for 3 min.

Fabp-Cre and CMV-Cre mice were genotyped by PCR analysis of genomic tail DNA with the following primer pair: 5'-ACCAGCCAGCTATCAACTCG-3' and 5'-TTACATTGGTCCAGCCACC-3', applying the following PCR conditions: denaturation at 94°C for 2 min, 35 cycles at 94°C for 1', 60°C for 1', 72°C for 1 min and a final extension cycle at 72°C for 3 min.

3.2.4 Southern blot analysis.

Southern blot analysis was carried out on 10 µg of genomic tail DNA extracted from mice of each transgenic line. Genomic DNA, digested with Hind III, was transferred to Hybond N+ membrane (GE Healthcare) and tested with a ^{32}P-labeled 1,5 kb probe corresponding to a EGFP fragment, as indicated in A. Transgene copy number was estimated comparing the intensity of DNA band of transgenic animals to a standard of 1, 10 and 50 copies of injected DNA fragment using the ImageJ software.

3.2.5 PCR to detect the readthrough phenomenon.

A readthrough phenomenon was identified by the sequencing of a cDNA fragment, amplified with primer 3: 5'-ACAACCACTACCTGAGCAC-3' and primer 4: 5'-ATGATGGTGTCCAGGAAG-3', spanning from the 3' end of the egfp to the 5' end of

herg1myc of the construct in the following conditions: denaturation at 94°C for 2 min, 35 cycles at 94°C for 40'', 52°C for 1', 72°C for 1 min and a final extension cycle at 72°C for 3 min.

3.2.6 RNA extraction and retrotrascription.

RNA was extracted from different tissues of wild type (WT), hERG1-EGFP^{Floxed}, hERG1-EGFP^{Floxed}-Cre and Apc$^{min/+}$ mice using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was obtained from 1-2 µg of RNA using 200 U reverse transcriptase SuperScript II (Invitrogen, Groningen, The Netherlands), plus 500 µM each of dNTP and 15 ng/µl of random primers, in a 20 µl final reaction volume, for 50 min at 42°C and 15 min a 70°C. cDNA synthesis was monitored by PCR with β-actin primers.

3.2.7 Reverse transcriptase PCR (RT-PCR).

Amplification of *Cre* was performed with 2 µl of cDNA derived from caecum, colon and rectum of hERG1-EGFP^{Floxed}-Cre double transgenic mice and with a specific primer pair: 5'-ACCAGCCAGCTATCAACTCG-3' and 5'-TTACATTGGTCCAGCCACC-3' in the following PCR conditions: denaturation at 94°C for 2'min, 35 cycles at 94°C for 1', 60°C for 1', 72°C for 1 min and a final extension cycle at 72°C for 3' min.

3.2.8 End-point PCR.

hERG1-EGFP^{Floxed}-Cre double transgenic genomic DNA was tested by end-point pcr to verify the presence of Cre mediated recombination in mice. DNA extracted from colon and rectum of WT and hERG1-EGFP^{Floxed}-Cre double transgenic mice was amplified with the 5: 5'-AGGATCAGTCGAAATTCAAG-3' and 4: 5'-ATGATGGTGTCCAGGAAG-3' primers and applying the following PCR conditions: denaturation at 94°C for 2 min, 35 cycles at 94°C for 40", 52°C for 1', 72°C for 1 min and a final extension cycle at 72°C for 5 min. As recombination positive control, tail genomic DNA from hERG1-EGFP^{Floxed}-CMVCre mice was used.

3.2.9 Real-Time Quantitative PCR (RT-qPCR).

herg1, merg1 and *myosin, heavy polypeptide 11, smooth muscle* (*myh11-202)* mRNA quantification by RT-qPCR was performed on 2 µl of cDNA (diluted 1:4) using the 7500 Fast real Time PCR System and the SYBR Green Master Mix Kit (Applied Biosystems; Foster City CA, USA). The *β-actin* gene was used as RT-qPCR reference gene. The primer sequences for *herg1* were: 5'-CTCACCGCCCTGTACTTCAT-3' forward primer and 5'-GCTCCCCAAAGATGTCATTC-3' reverse primer; for *β-actin* were: 5'-GGGGTGTTGAAGGTCTCAAA-3' forward primer and 5'-GATCTGGCACCACACCTTCT-3' reverse primer; for *merg1* were: 5'-GGACCTGCTTACTGCCCTCT-3' forward primer and 5'-GGACGGGCATATAGGTTCAG-3' reverse primer; for *myh11-202* were: 5'-CGACAGGCTAGGGATGAGAG-3' forward primer and 5'-GCTCTCCAAAAGCAGGTCAC-3' reverse primer. The relative gene expression was calculated applying the Pfaffl analysis method (Pfaffl et al., 2004).

3.2.10 Polyp Number assessment and Determination of haematocrit in APC$^{min/+}$ mice.

Intestinal adenomas and colonic polyps were scored for numbers and size (diameter) at different ages. The mice were sacrificed by cervical dislocation and the haematological status of the animals was assessed by haematocrit determinations: blood samples were collected and drawn by capillary force into heparinised haematocrit capillary tubes which were centrifuged and plasma samples were separated. The haematocrit, was determined as the proportion of the volume of the blood sample occupied by the erythrocytes. In addition, the small and large intestines were isolated. The intestinal tract was rinsed with cold PBS using a blunt-end syringe and opened along the longitudinal axis. The small intestine was divided as follows: the duodenum was considered to be a 4-cm segment of small intestine extending from the pyloms. The remainder of the small intestine was divided into three equal-length

segments. These segments were operationally defined as the proximal jejunum, distal jejunum, and ileum. The opened intestine was spread flat between sheets of filter paper and fixed in fresh 4% paraformaldehyde. Paraformaldehyde-fixed intestinal sections were rinsed in deionised water and stained with 0.1% methylene blue and polyps were counted at ×20 magnification under a dissecting microscope. Lesions were categorized as >1 mm or <0.5 mm in diameter size. For histology, segments of intestine and colon were paraffin embedded. For RNA extraction, normal and intestinal tumour tissues were frozen at -80 C.

3.2.11 AOM-treatment.

Two months old mice, 12 TG and 6 controls, maintained in a C57BL6/FVB mixed background, received intraperitoneal injections of AOM (10 mg/kg body weight) once a week for 6 weeks; in addition, 3 control and 6 TG mice were treated with physiologic solution. Three months after the last injection, all animals were killed to evaluate tumourigenesis. The entire gastrointestinal tract was removed for dissection and flushed with PBS to remove intestinal content. The intestine was opened longitudinally and washed extensively with PBS. Colon-rectum was fixed in 4% formaldehyde made in PBS for 24h, after which the tissues were stained with methylene blue (0.1% for 10 minutes). The number of polyps was determined under a dissecting microscope (20x power field).

Aberrant crypt foci (ACF) were determined according to Bird (Bird, 1987). The same methylene blue-stained colons were then restained with high-iron diamine Alcian blue (HID-AB), to identify mucin depleted foci (MDF) as described in Caderni et al., 2003. MDF and ACF were identified at the microscope (400x magnification).

3.2.12 E4031 treatment of $Apc^{min/+}$ and TG mice.

$Apc^{min/+}$ mice received daily for 3 months intraperitoneal (IP) injections of 20 mg/Kg E4031 (TOCRIS) dissolved in sterile water; control $Apc^{min/+}$ mice received buffered saline only. After 3 months, animals were sacrificed by cervical dislocation. The entire

gastrointestinal tract was removed for dissection and flushed with PBS to remove intestinal content. The colon-rectum was opened longitudinally and washed extensively with PBS, fixed in 4% buffered formaldehyde for 24h and then stained with methylene blue. The number of polyps was determined under a dissecting microscope (20x power field).

TG mice received daily for 14 days IP injections of 20 mg/Kg E4031 (TOCRIS) dissolved in sterile water; control mice received buffered saline only. After 14 days, animals were sacrificed by cervical dislocation. The entire gastrointestinal tract was removed for dissection and flushed with PBS to remove intestinal content. The organ was opened longitudinally and washed extensively with PBS, fixed in 4% buffered formaldehyde for 24h and embedded in paraffin. Tissue sections (7-μm) were cut from blocks using a microtome (Leica RM2125/RM2125RT). Immunohistochemistry using anti-VEGF-A antibody was performed to evaluate differences between control and treated TG mice.

3.2.13 Immunohistochemistry (IHC).

IHC was performed on 7-μm sections of tissues fixed in 4% formalin and embedded in paraffin, mounted on positively-charged slides. After dewaxing and blocking endogenous peroxidases, sections were treated with proteinase K (Roche; 5 μg/ml in PBS) and UltraVBlock solution (LabVision) containing 0.1% Triton X-100, and then incubated with the following primary antibodies: anti hERG1 monoclonal antibody (Lastraioli et al., 2012) (dilution 1:200 in PBS-UltraVBlock), anti VEGF-A (Santa Cruz Biotechnology) (dilution 1:100 in PBS-UltraVBlock), anti-CD34 (Cedarlane) (dilution 1:100 in PBS-UltraVBlock) overnight at 4°C, anti pAkt (Santa Cruz Biotechnology) (dilution 1:100 in PBS-UltraVBlock) 1 hour at 37°C. For pAkt detection, antigen retrieval was carried out by heating slides in a microwave for 10 minutes at 600W in citrate buffer (pH 6). For CD34 detection, antigen retrieval was carried out by irradiating the slides in a microwave for twenty minutes at 700 W in citrate buffer (pH 7.8).

Immunostaining was carried out using a commercially available kit (PicTure Plus kit; Zymed). After extensively washing with PBS, colour was developed by incubating the slides with the DAB (3,3′-diamino-benzidine) chromogen solution for 2-5 min or until acceptable colour intensity had been reached. Slides were then counterstained with Mayer's haematoxylin and mounted using Entellus mounting medium. Images were acquired on a Lexica DM 4000B microscope with a Lexica DFC 320 camera using Lexica Win software (Lexica Microsystems; Milan, Italy).

3.2.14 Statistical analysis.

Data obtained from AOM-treated mice were reported as mean ± SEM and analysed by Mann-Whitney U-test. A P-value <0.01 (*) was considered statistically significant.

Data obtained from VEGF-A and pAkt expression were analyzed by Mann-Whitney U-test. A p-value <0.05 and <0.01 were considered statistically significant.

Data obtained from vessel and total vascular area count of WT, TG and $Apc^{min/+}$ mice were reported as mean ± SEM and analyzed by Mann-Whitney U-test. A p-value <0.05 and <0.01 were considered statistically significant.

4. RESULTS AND DISCUSSION

4.1 PART ONE: *IN VITRO* DATA

Beyond its canonical role in excitability, hERG1 is emerging as a major regulator of intracellular signalling, in tumour cells. In different tumours, hERG1 channels can regulate many biological processes such as proliferation, migration, invasiveness and angiogenesis. Such ability is strictly related to the capacity of hERG1 to interact with the β1 integrin subunit forming a macromolecular membrane complex, modulating the signalling pathways which are under integrin control (Arcangeli et al., 1993; Hofmann et al., 2001; Arcangeli and Becchetti, 2006).

4.1.1 β1 integrin and hERG1 channel form a macromolecular complex in CRCs.

By performing IP experiments with anti β1 antibody on primary colorectal tumours (ADK) (Figure 1A), as well as on colon cancer cell lines (HCT116, HCT8, Fig 1B), we confirmed the presence of hERG1/β1 macromolecular complex in CRC cells and primary tumours, as previously reported (Zanieri, PhD thesis). Interestingly, such complex is limited to be exclusively expressed in tumours: in fact, as it is clearly shown in Fig 1A, any detectable band is present in normal human heart, where hERG1 is mainly expressed in physiological conditions (Fig. 1C). Therefore, we can conclude that the hERG1/β1 complex is specifically distributed on tumour cell membrane and it is not detectable in non cancerous tissues.

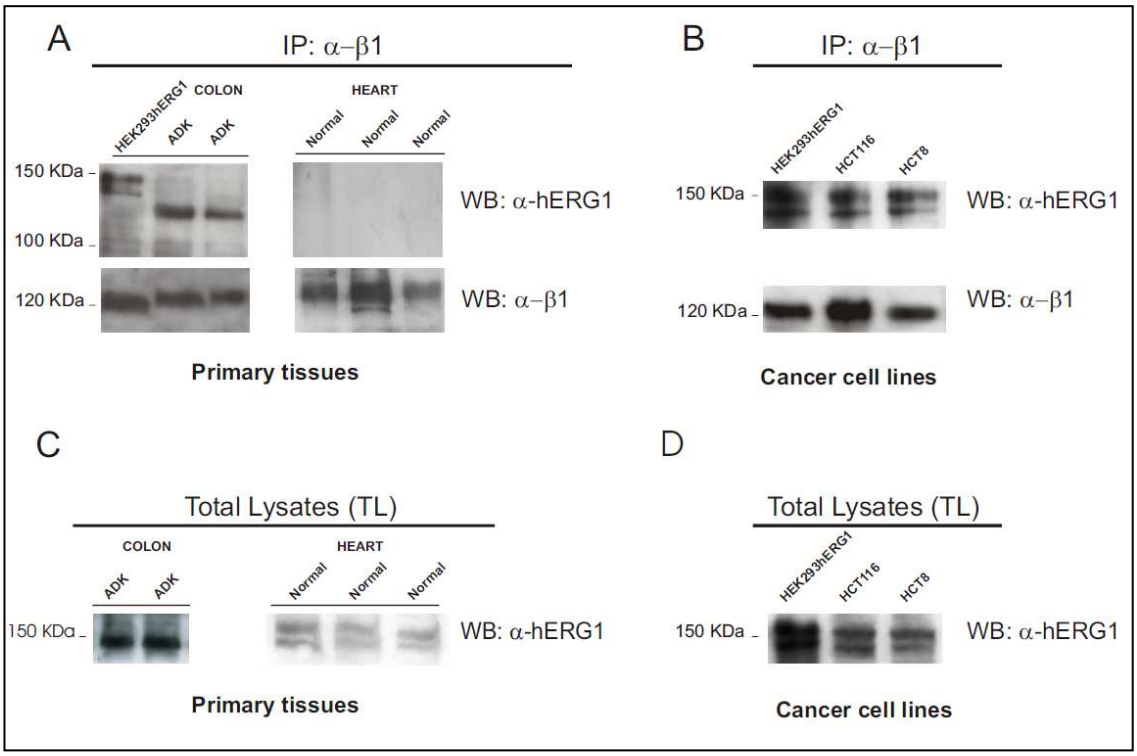

Figure 1: hERG1/β1 interaction in CRC cancer and normal samples A) IP performed on tissue samples derived from primary CRC cancers and normal heart. B) IP on samples derived from colon tumour cell lines. Cell lysates were immunoprecipitated with an α-β1 integrin monoclonal antibody (TS2/16) and the corresponding blots were probed with an α-hERG1 (C54) and an α-β1 integrin (RM12) policlonal antibodies, respectively. Data are representative of at least 2 independent experiments. C) WBs on total lysates derived from primary CRC cancers and normal heart. D) WB experiments on total lysates derived from colon tumour cell lines. Protein extracts were probed using the α-hERG1 policlonal antibody, C54.

It is worth noting that, in heterologous expression systems, as well as in human heart, hERG1 proteins can also co-assemble with two ancillary β-subunits, MinK (KCNE1) and MiRP1 (KCNE2) (Abbott et al., 1999; McDonald et al., 1997), both involved in slowly activation state, playing an important and complex role in ventricular repolarisation. We then analyzed *KCNE1* and *KCNE2* expression in CRC cells and human heart by RT-PCR, and we observed the presence of the two transcripts only in normal cardiac tissues (Fig. 2A and 2B).Similarly, by WB experiments emerged that MinK and MiRP1 proteins were exclusively present in normal human heart and no expression of these β-subunits were found in CRC cells (Fig. 2C and 2D). Such evidences could suggest that, in tumours, the physiological function accomplished by MinK and MiRP1 subunits could be replaced by integrin subunits.

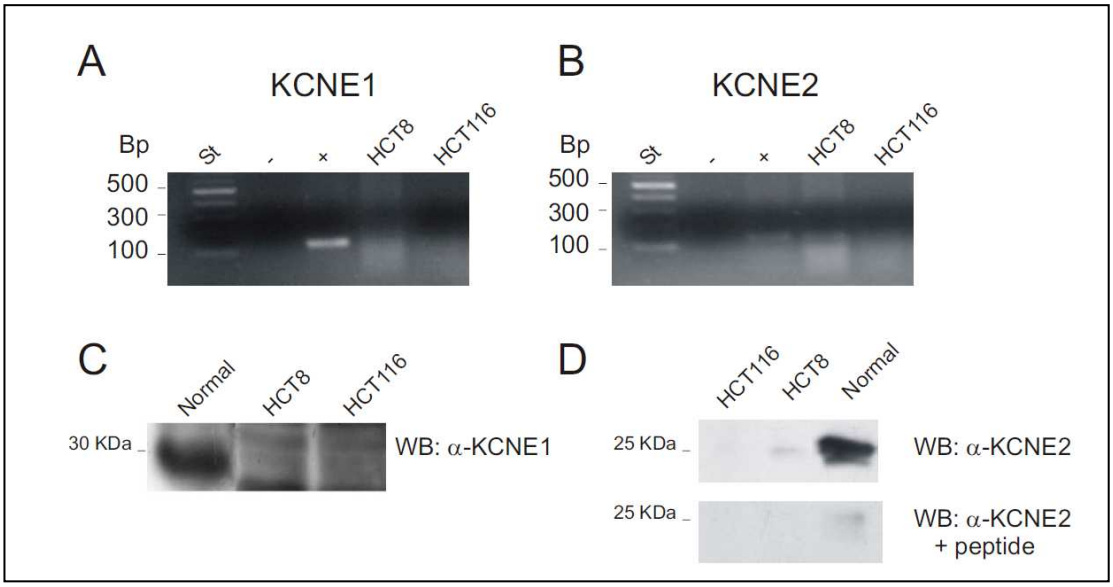

Figure 2: KCNE1 and KCNE2 expression in cancer and normal samples *KCNE1* (A) *and KCNE2* (B) expression in CRC cells and normal heart, by RT-PCR. PCR products were generated through the use of gene-specific primers for *KCNE1* (predicted product size was 120 bp) and *KCNE2* (predicted product size was 120 bp). -: negative control (no template), +: normal human heart cDNA from reference RNA (Ambion®). C) WB experiments on total lysates derived from CRC cells and normal heart, using the α-KCNE1 monoclonal antibody. D) WB experiments on total lysates derived from CRC cells and normal heart, using the α-KCNE2 polyclonal antibody. In the bottom α-KCNE2 antibody was pre-absorbed with peptide.

To deepen this topic and the functional role of the hERG1/β1 complex in tumour cells, we considered 3 CRC cell lines (HCT116, HCT8, HT29) expressing hERG1 at different levels (Fig.3A): in particular, all the tested cells had a significant expression of the β1 integrin and, relative to hERG1 protein, HCT116 displayed the highest expression level, while HT29 the lowest. We then analysed the effects of β1 activation on hERG1 current. By patch-clamp analysis, we observed a significant increase in hERG1 current density, and, even more, of the number of cells displaying detectable hERG1 currents, when the β1 was engaged either by cell adhesion to Fibronectin (FN) or by an anti β1 activating antibody (TS2/16). Data relative to HCT116 cells seeded onto TS2/16 are reported in Fig.3B.

A

Cell line	β1 protein expression (MFI)	hERG1 mRNA expression	hERG1 protein expression	I_{hERG} (pA/pF)	VEGF-A secretion (ng/10^6 cells)	VEGF-A receptors
HCT116	34.4	62.4±19.30	+++	4.66±0.95	7,28±2,54	No
HCT8	57.0	40.58±17.20	++	4.14±1.00	7,86±2,74	No
HT29	20.7	2.89±0.63	-/+	No	ND	ND
HEK 293	34.3	9.96±4.36	-/+	No	2,22±0,17	ND

B

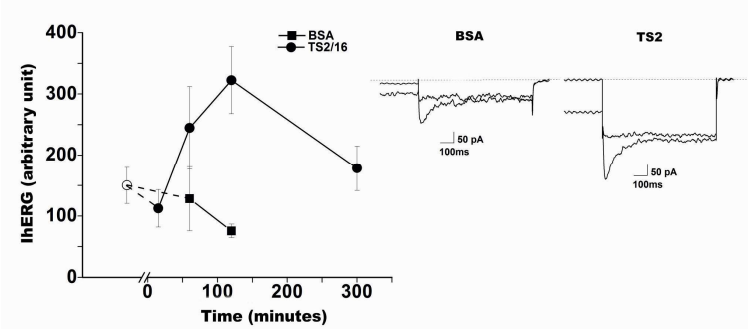

Figure 3: A) Characterization of β1 protein expression, hERG1 mRNA and protein expression, basal levels of secreted VEGF-A as well as the expression of VEGF-A receptors in CRC cells. B) Characterization of hERG1 current in HCT116 cell lines. Effects of β1 stimulation on hERG1 currents. HCT116 were seeded on TS2/16 or BSA coating for different time and then analyzed by patch-clamp (see Material and Methods). Arbitrary units on the y-axis are calculated as channel density (pA/pF) x percentage of hERG1 current positive cells. It clearly emerged that, between one and two hours of TS2/16 stimulation, in HCT116 cells increases non only the hERG1 channel density (see also biophysical traces on the right), but also the number of hERG1 positive cells as compared to BSA stimulation.

4.1.2 The hERG1/β1complex modulates phosphorylation of p85-PI3K and Akt activation.

We further analyzed the signalling pathway downstream to the hERG1/β1complex, first focusing on the role of p85, the regulatory subunit of PI3K. Our immunoprecipitates always contained the p85 subunit of PI3K (Fig.4A and 4B), which was found to be generally phosphorylated (i.e. activated) in CRC cells (Fig. 4C) and phosphorylation was significantly reduced after addition of either E4031, or the Bv7 β1-inhibiting antibody. Instead, treatment with the β1-activating antibody TS2/16 stimulated p85 phosphorylation (Figure 4C).

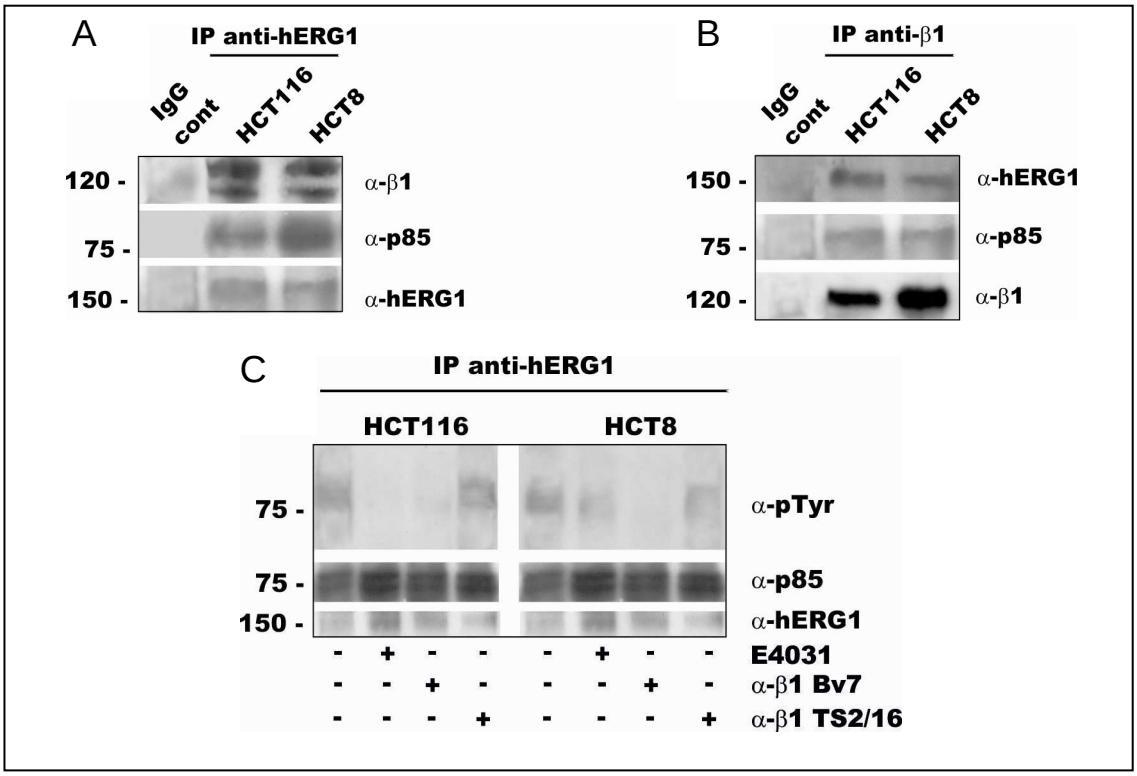

Figure 4: The hERG1/β1 integrin complex modulates phosphorylation of p85-PI3K. A-B) p85 participates in the hERG1/β1 complex. IPs were performed using anti-hERG1 (N135) antibodies (IP anti-hERG1) or anti-β1 (TS2/16) antibodies (IP anti-β1). An anti-PI3 kinase p85 antibody was used to probe WBs. Parallel IP were done using rabbit IgG as negative control. C) The p85 associated with the hERG1/β1 complex is phosphorylated, and its phosphorylation levels are modulated by hERG1 and β1 inhibition (by E4031 and Bv7, respectively) or integrin activation (TS2/16 antibodies).

These results indicate that the p85 subunit of PI3K associates with hERG1, β1 integrin, to constitute a single macromolecular complex and that its phosphorylation depends on integrin activation as well as hERG1 channel activity. Recruitment of p85 has two major consequences: i) it facilitates translocation of PI3K towards its physiological membrane substrates; ii) it determines PI3K phosphorylation, which is strongly dependent on hERG1 activation.

We then tested whether modulation of Akt depended on this mechanism. Treating CRC cells with E4031 decreased Akt activity (Fig. 5A). The dependence of Akt activity on the presence of functional hERG1 was further demonstrated by the observation that its activity was considerably lower in HCT116 cells in which hERG1 was stably silenced (sh 7.5), compared to both Mock-infected HCT116 cells (PLKO), and cells expressing

low levels of hERG1 such as HT29 cells (Fig. 5A). Similarly, Akt nuclear translocation were significantly decreased by either E4031 or Bv7 (Fig.5B).

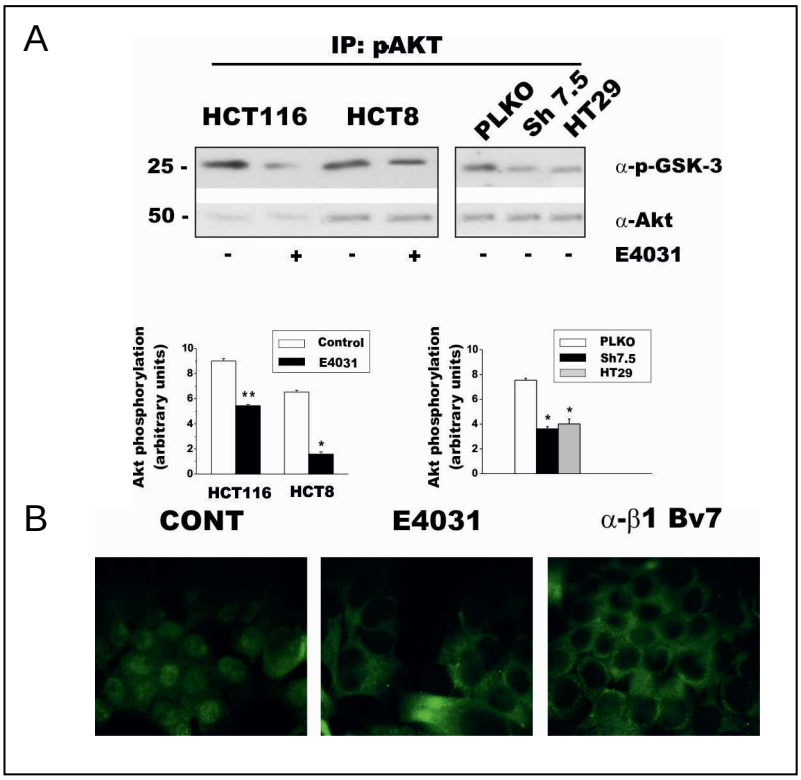

Figure 5: A) Effects of hERG1 inhibition (E4031 blocker) on Akt activity in HCT116, HCT8 and HT29 cell lines, as well as in hERG1 silenced HCT116 cells (Sh 7.5). Akt activity was evaluated using the Akt kinase assay kit (Cell Signaling Technology), following the manufacturer's instructions and immunoprecipitating 500 µg of total lysates. Data were analyzed using ImageJ, and graphs were plotted by Microcal Origin 6.0. * $p<0.05$; ** $p< 0.02$; *** $p< 0.01$ (Student's t test). C) Immunofluorescence (IF) staining using an anti-Thr 308 pAkt (Santa Cruz, SC-16646, dilution 1:500) on control and E4031 (40 µM) or anti-β1 (Bv7) (14 µg/ml) treated HCT116 cells. Treatments were performed for 5 hours. IF protocol was performed as detailed in Cherubini et al., 2005.

Hence, activated hERG1 is a regulator of PI3K, through which it controls the activity and nuclear translocation of Akt. This is the first demonstration of the details of an hERG1-dependent signalling pathway that consists in a complex interaction of membrane and cytosolic proteins organized within a membrane complex. Our data point to hERG1 as a functional hub able to recruit and activate key signalling molecules, thus playing a pivotal role in cancer cell physiology.

4.1.3 Integrin-dependent adhesion and hERG1 activity regulate VEGF-A expression in colon cancer cells.

We then analyzed whether the hERG1/β1 complex was functionally involved in tumour angiogenesis, first determining whether *VEGF-A* expression and VEGF-A secretion depended on both integrin activation and hERG1 channels in CRC cells. As it is shown in Fig. 3A, in the 3 CRC models the highest hERG1 expression corresponded to the highest ability to secrete the VEGF-A protein. Basing on these premises, we tested the effects of altering the hERG1/β1 expression on VEGF-A expression and secretion in these cells. *VEGF-A* expression was significantly reduced after addition of BV7, whereas it was strongly increased by TS2/16 (Fig. 6A). To study the effects of hERG1 inhibition on *VEGF-A* transcription, a mix of three anti-*herg1* (α-*herg1*) siRNAs was applied to HCT116 and HCT8 cells. Moreover, HCT116-sh-hERG1 cells were studied. *VEGF-A* expression was significantly decreased by α-*herg1* siRNAs and in HCT116-sh-hERG1 cells (Fig.6B). A similar inhibition was produced by hERG1 blockers (either E4031 or Way 123,398 (WAY)). Silencing *VEGF-A* through α-*vegf-a* siRNA was taken as control (Fig.6B).

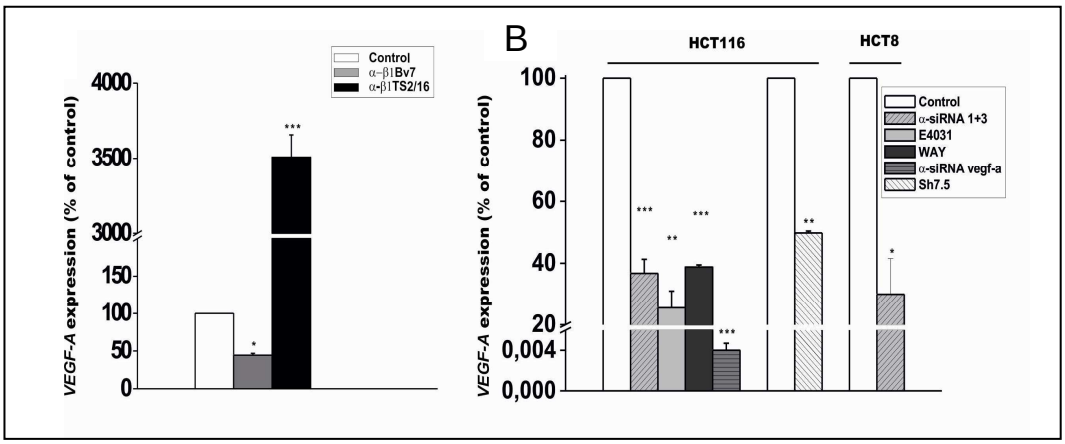

Figure 6: Characterization of the β1/hERG1-dependent signalling pathway able to influence *VEGF-A* expression and secretion in CRCs cells. A) Effect of β1 inhibition or activation on *VEGF-A* expression in HCT116 cells. B) *VEGF-A* expression in HCT116 and HCT8 cells, treated with E4031 or WAY (40 μM), transiently transfected with a mix of α-*herg1* siRNAs 1+3 (HCT116, HCT8) or stably transfected with shRNA 7.5 (HCT116). Controls are represented by cells cultured in standard conditions for the pharmacological treated samples, cells transfected with the pLKO.1 empty vector for shRNA 7.5 and with siRNA negative control for siRNAs 1+3, respectively.

On the whole, both β1 integrin and hERG1 regulate *VEGF-A* expression in CRC cells. Consistently, VEGF-A secretion decreased when hERG1 was inhibited by hERG1 blockers or down-regulated with α-*herg1* siRNAs (Fig. 7). Also in this case, the effect of α-*vegf-a* siRNA was taken as control (see the far right bar in Fig. 7). Blocking hERG1 decreased VEGF-A secretion only in the CRC cell lines (i.e. HCT116 and HCT8) that displayed a substantial channel expression, while it was almost ineffective in HT29 cells, which express low levels of hERG1.

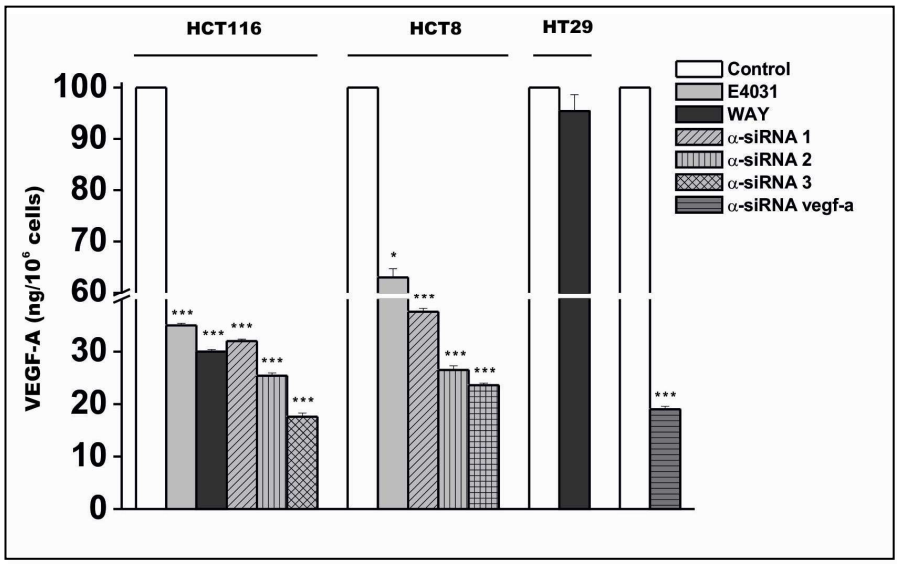

Figure 7: The effect of hERG1 blockers E4031 and Way 123,398 (WAY) and of α-*hERG1* siRNAs on VEGF-A secretion in HCT116 and HCT8 cells. Data are means ± SEM of two-four separate experiments, each carried out in duplicate. *: p<0.05; **: p<0.02; ***: p<0.01 (Student's t test).

4.1.4 The β1/hERG1 complex, through Akt, induces HIF(s) transcriptional activation.

VEGF-A transcription is regulated by the Hypoxia Inducible transcription Factors (HIFs), both HIF-1 and HIF-2 (Forsythe et al., 1996; Imamura et al., 2001), whose α subunits are sensitive to O_2 tension. Increased oncogenic signalling in cancer cells can induce the expression of HIFα subunits through O_2-independent mechanisms that include increased transcription and/or translation of HIFα mRNAs (Pouyssegur et al., 2006.) as well as are modulated by intracellular signals. These signals mainly turn on Akt and its downstream effectors, such as mTOR (Zhang et al., 2011). Hence, we

studied whether and how the β1/hERG1/PI3K-dependent Akt activity could drive HIF activity in CRC cells. We first determined that PI3K/Akt inhibitors (both LY294002, LY (Hu et al., 2000) and perifosine (Kondapaka et al., 2003)) decreased VEGF-A secretion (Fig. 8A, left panel). Consistently, silencing either *Akt1* or *Akt2*, strongly reduced the *VEGF-A* transcript (Fig. 8A, right panel). These effects could be traced back to an Akt-dependent regulation of HIF transcriptional activity: indeed HIF activity (measured through a luciferase assay) was strongly reduced by PI3K/Akt inhibitors (Fig. 4B).

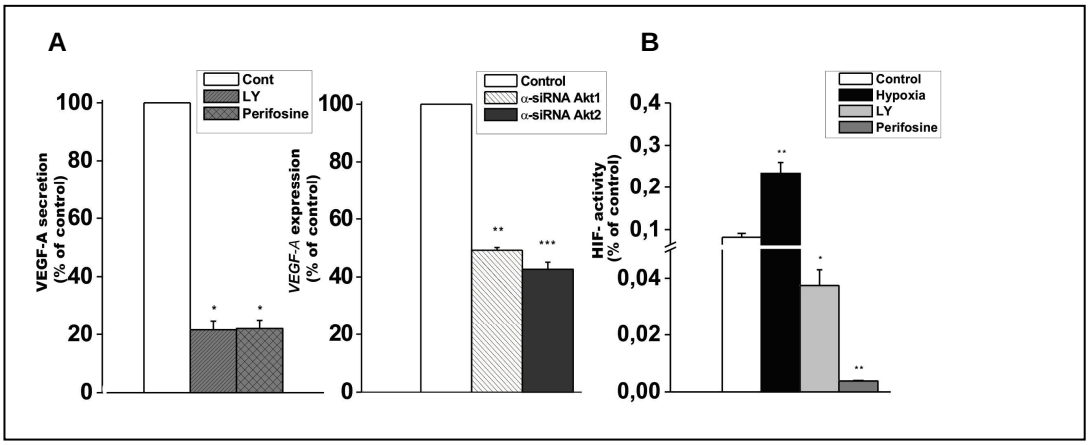

Figure 8: A) The effects of PI3K/Akt inhibitors LY294002 and perifosine on VEGF-A secretion (left panel) and of α-*Akt1* and α-*Akt2* siRNAs on *VEGF-A* expression (right panel). Experiments were performed as described in Materials and Methods. Data are reported as the percentage of control ± SEM of two experiments, each carried out in triplicate. * p<0.05; ** p< 0.02; *** p<0.01 (Student's t test). B) The effects of PI3K/Akt inhibitors LY294002 and perifosine, on HIF-1 transcriptional activity. Briefly, cells were transfected with an HRE-luciferase plasmid along with a Renilla luciferase-CMV control plasmid (see Materials and Methods). Twenty-four hours after transfection, cells were further incubated under normoxia or transferred to hypoxic (0.1% O_2) conditions in the absence or presence of PI3K/Akt inhibitors LY294002 and perifosine. After 5 hours, cells were harvested and luciferase was assayed using the Dual-Luciferase Reporter Assay System (Promega). Hypoxia condition was used as positive control. Data are means ± SEM of three separate experiments. * p<0.05; *** p<0.01 (Student's t test).

We deepened this point by analyzing the transcription of several HIF-dependent genes after either β integrin activation or inhibition (through specific antibodies, TS2/16 or Bv7, respectively), or hERG1 inhibition (through pharmacological blockade or silencing hERG1 in HCT116 cells (HCT116-sh-hERG1 cells)) (Fig. 9A). Both integrin and hERG1 inhibition significantly decreased transcription of the HIF-dependent genes *GLUT-1*, *LDHA*, and of a specific HIF-2 target, *ANGPTL-4* (Imamura et al., 2001). Conversely, integrin activation induced an increased expression of all tested genes. As expected, expression of the tested genes increased in hypoxic condition (Fig. 9A, black

bars), with the exception of *ANGPTL-4*, in agreement with (Imamura et al., 2001). A significant inhibition in the expression levels of HIF(s)-dependent genes was observed after silencing *Akt1* (Fig. 9B). Silencing *Akt2* significantly affected only *GLUT-1* transcription level, suggesting a weaker involvement of Akt2 in the above pathway.

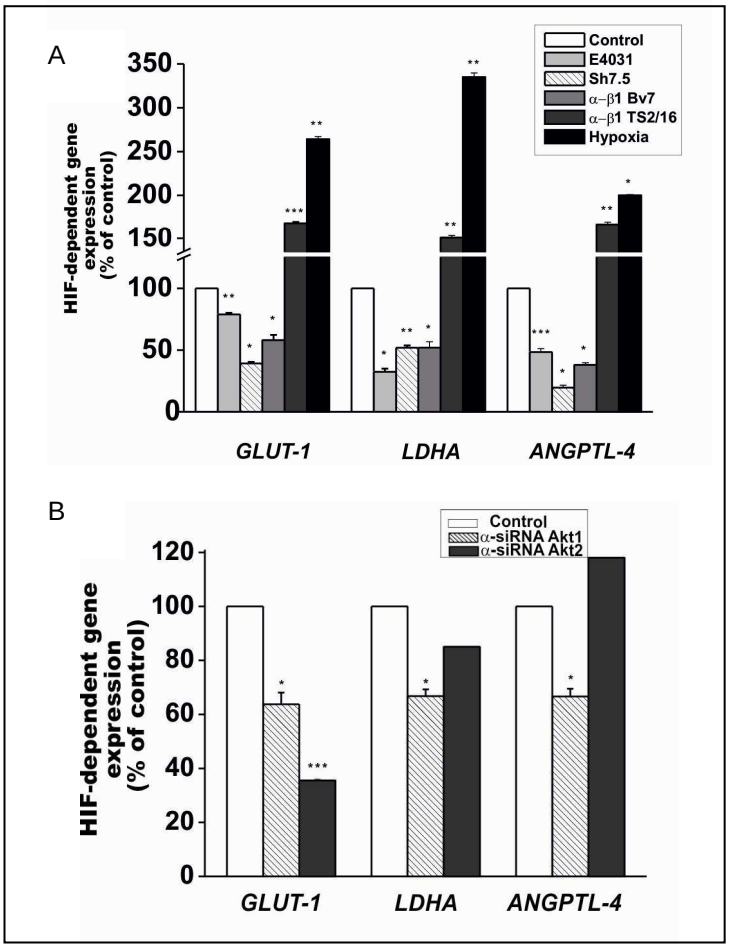

Figure 9: The hERG1/β1 integrin complex up-regulates HIF(s). A) Fold induction of HIF-1 target genes after hERG1 pharmacological and biomolecular inhibition or β1 inhibition and activation. B) Fold induction of HIF-1 target genes after *Akt1/2* silencing. *GLUT1*, glucose transporter 1; *LDHA*, lactate dehydrogenase A; *ANGPTL-4*, angiopoietin-like 4. Data are means ± SEM of three separate experiments, each carried out in duplicate. * $p<0.05$; ** $p<0.02$; *** $p< 0.01$ (Student's t test).

We concluded that both integrins and hERG1 channels activate the expression of several tumour progression genes, besides *VEGF-A*, since they regulate the activity of HIF-1 and HIF-2. This occurs through Akt (mainly Akt1). Because the effects of *Akt* silencing and hERG1/integrin inhibition were not additive, probably these regulators belong to the same signalling pathway, with the integrin and the channel protein being located upstream.

4.1.5 A complex signalling mechanism links the β1/hERG1 complex to HIF(s) in CRC cells.

The β1 integrin- and hERG1-dependent regulation of HIF transcriptional activity was accompanied by regulation of HIF-1 protein level (Fig. 10A). To evaluate if this increase in protein level could be ascribed also to a regulation of the protein synthesis, we analysed the mTOR pathway. As shown in Fig 10B, the up-regulation of HIF-1α did not depend on the regulation of HIF protein synthesis by mTORC1 and mTORC2 complex (Zhang et al., 2011), as neither hERG1 inhibition nor silencing decreased either p70S6K or pT37 phosphorylation.

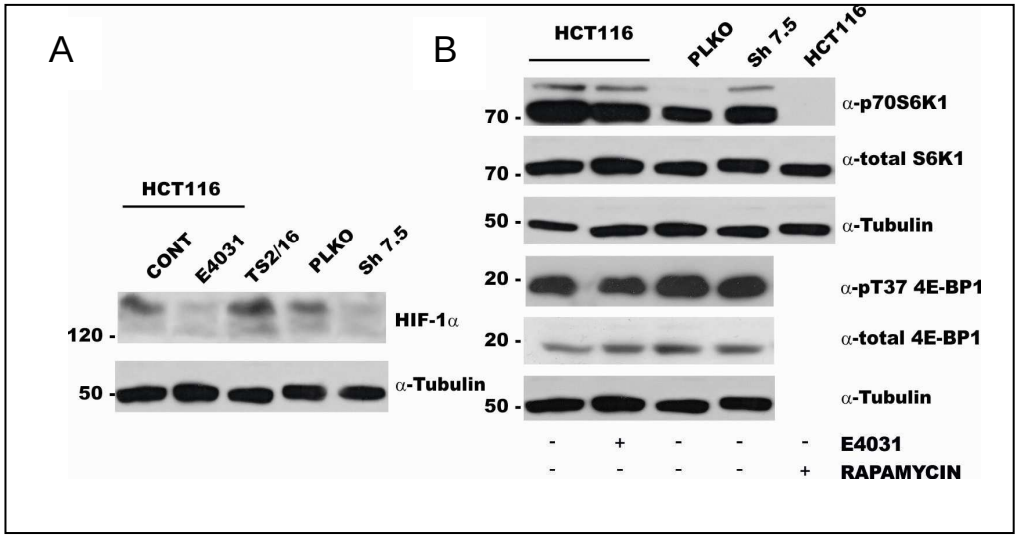

Figure 10: The hERG1/β1 integrin complex up regulates HIF(s) in a mTOR-independent manner. A) The effect of pharmacological and biomolecular hERG1 inhibition on HIF-1α protein. Total lysates and WB was performed as described above. 100 µg of protein extracts were loaded on each well, and WB were blocked using 5% BSA in TBS 0.1% Tween. B) Evaluation of the activation of mTOR signalling pathway components (mTORC-1 and 2) in wild type HCT116 and silenced Sh7.5 cells treated or not with E4031 (40 µM). Rapamycin (100 nM) was applied for 5 h as mTORC-1 internal control.

Otherwise, both silencing *Akt* (mainly *Akt1*) and inhibiting hERG1 significantly decreased the transcription of the two *HIF-α*(s) genes. *HIF-α* transcription was also inhibited by blocking β1 integrin (Fig. 11A). We then checked for transcription factors possibly involved in this pathway. Firstly, we confirmed that such effect did not occur after FOXO 1-3 inhibition using specific siRNA anti-FOXO 1-3. Thus, we can conclude that the hERG1-dependent transcriptional regulation of HIFs did not rely upon a rescue of FOXO(s) inhibition (Fig.11B).

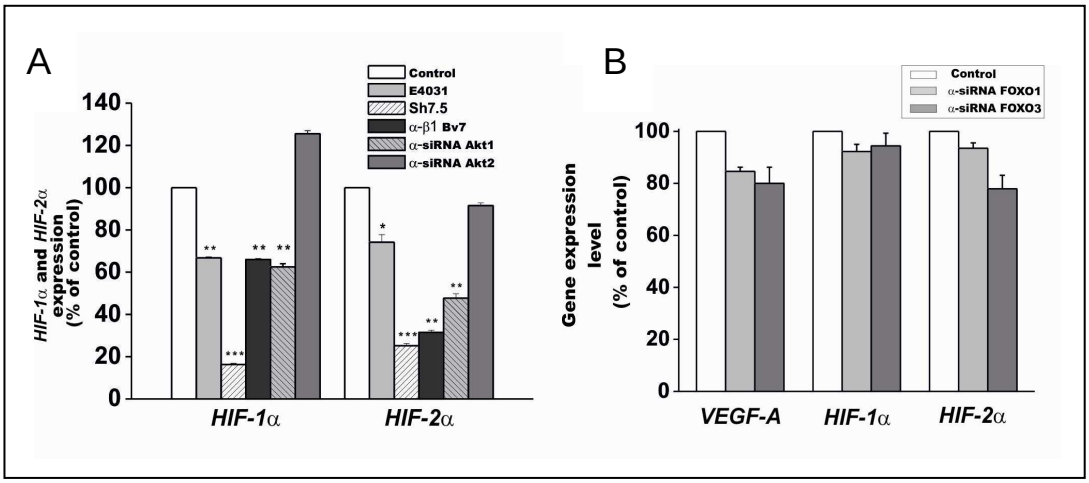

Figure 11: A) Study of hERG1 and Akt involvement in the regulation of *HIF-1α* and *HIF-2α* expression. B) Study of FOXO1A and FOXO3 involvement in the regulation of *VEGF-A*, *HIF-1α* and *HIF-2α* expression. RT-qPCR was performed as described in Materials and Methods. The relative gene expression was calculated applying the Pfaffl analysis method (Pfaffl et al., 2004). wild type HCT116 were indicated as control. α-shRNA 7.5 were normalized to PLKO cells; α-siRNA *Akt1* and α-siRNA *Akt2* as well as α-siRNA FOXO1A and α-siRNA FOXO3 were normalized with the corresponding siRNA negative control. Data are reported as the percentage of control ± SEM of two experiments, each carried out in triplicate. * $p<0.05$; ** $p<0.02$; *** $p< 0.01$ (Student's t test).

We then analysed the nuclear Factor-kB (NF-kB) able to regulate the transcription of HIF-1α (Belaiba et al., 2007; Frede et al., 2006; Nizet and Johnson, 2009; Rius et al., 2008): when HCT116 cells activated by FN were treated with the NF-kB inhibitor IKK inhibitor VII, a significant reduction in the expression of HIF-dependent genes was observed (Fig. 12A). On the whole the NFkB-mediated up-regulation of the HIF genes seems the main regulatory mechanism sustaining the up-regulation of HIF-1α and HIF-2α (and their activation) triggered by integrins and hERG1 (by the β1/hERG1 complex) in CRC cells. Indeed, these results are also confirmed by the differences in NF-kB localization in the presence or not of β1 and hERG1 inhibitors. In Fig. 12B, it is clearly shown how NF-kB translocation induced by β1 activation is significantly decreased by both Bv7 and E4031 treatment.

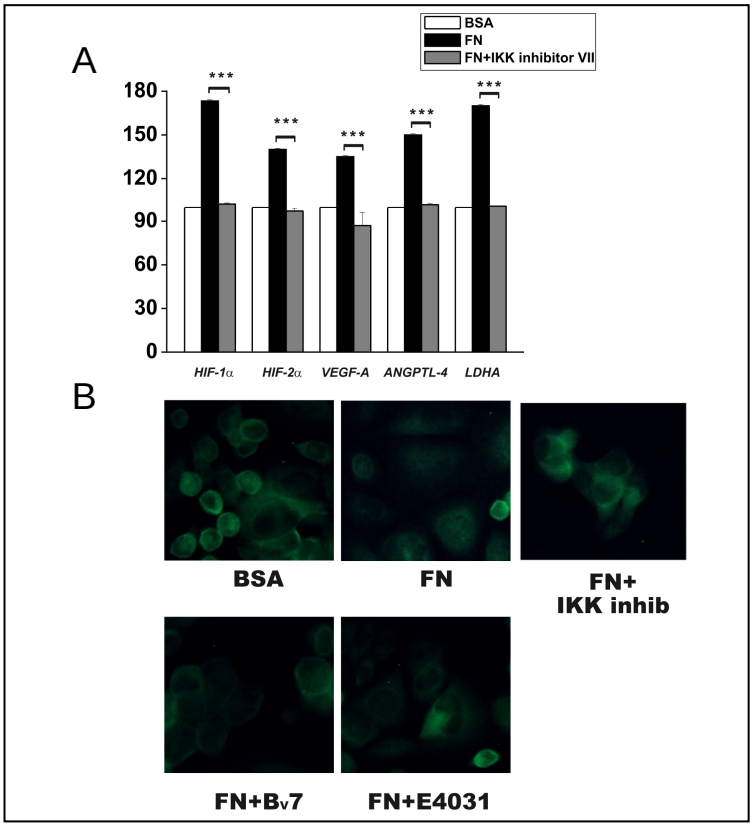

Figure 12: A) Study of NF-kB involvement in the regulation of *HIF1α*, *HIF2α*, *VEGF-A*, *ANGPTL-4* and *LDHA* expression. RT-qPCR was performed as described in Materials and Methods. The relative gene expression was calculated applying the Pfaffl analysis method (Pfaffl et al., 2004). wild type HCT116 were seeded onto BSA or FN coating for four hours in the presence or not of NF-kB inhibitor IKK inhibitor VII (1 µM). Data are means ± SEM of two experiments, each carried out in triplicate. * p<0.05; ** p<0.02; *** p< 0.01 (Student's t test). B) Immunofluorescence (IF) staining using a rabbit polyclonal anti-NF-kB antibody (Santa Cruz, 1:50). wild type HCT116 were seeded onto BSA or FN coating for four hours in the presence or not of NF-kB inhibitor IKK inhibitor VII (1 µM), E4031 (40 µM) or anti-β1 (Bv7) (14 µg/ml). IF protocol was performed as detailed in Cherubini et al., 2005.

Altogether, the role of the β1/hERG1/PI3K complex seems to be the triggering of a signalling pathway which regulates Akt (mainly Akt1), thus leading to activate, in a NF-kB-dependent manner, the transcription of HIF-1α and HIF-2α. This in turn increases the levels of the HIFα subunit, with ensuing substantial increase in transcription of several HIF-dependent, tumour progression, genes, including VEGF-A.

On the whole, the signalling pathway we describe is novel and emphasizes the central role of Akt in the modulation of HIF transcriptional activity in cancer cell, through the intervention of the β1/hERG1/PI3K complex. This modulation is especially important in normoxia, when the oxygen-dependent regulation of HIF-1 does not take place.

4.2 PART TWO: *IN VIVO DATA*

Substantial evidence indicates that cancer can be partially attributed to ion channel malfunction. Numerous studies included hERG1 in the list of ion channels mis/over-expressed in cancer cells, where it plays the role of regulator of tumour cell proliferation and progression (Arcangeli, 2005; Jehle et al., 2011). Here, we analyzed the role of hERG1 in colorectal carcinogenesis *in vivo*, using either genetic ($Apc^{min/+}$ mice) or chemical (AOM-treated) models of CRC. In both models we found a relevant role of hERG1 channels, which could be traced back to a hERG1-dependent control of angiogenesis. Data obtained from this section are under revision (Cancer Medicine).

4.2.1 Role of hERG1 in colonic polyp development of $Apc^{min/+}$ mice.

$Apc^{min/+}$ mice are recognized as a genetically relevant animal model mimicking human intestinal carcinogenesis and have been used extensively for various chemoprevention studies. This strain results from a germ line mutagenesis study with N-ethyl-N-nitrosourea combined with phenotypic screening (Moser et al., 1990). $Apc^{min/+}$ mice develop more than 50 tumours throughout the entire intestinal tract, mainly in small intestine, until they die of bowel obstruction, intestinal bleeding, and severe anaemia at 150 to 170 days of age.

We initially purchased male $Apc^{min/+}$ (C57BL/6J) and female wild-type from the Jackson Laboratory as founders. We sacrificed the animals at different ages. We assessed the haematological status of the animals by haematocrit determinations: animals showed a progressive decrease in haematocrit value until a range of 10% to 20% for moribund mice. This severe chronic anaemia is thought to be the cause of lethality (Fig.13A). Small intestine and colon were fixed in 4% formaldehyde made in PBS for 24h, after which the tissues were stained with methylene blue. The number of lesions in the intestine was counted under a stereomicroscope and the size of each tumour was measured using a calliper (Fig. 13B).The number of colonic tumours is much less than that of small intestine; in fact, the major location of intestinal tumours in $Apc^{min/+}$ mice is

the small intestine. In figure 13C is shown a macroscopic view of the large bowel: we observed a variable number of nodular, polypoid well-vascularised colonic tumours, mainly in the middle and distal colon of male and female Apc$^{Min/+}$ mice.

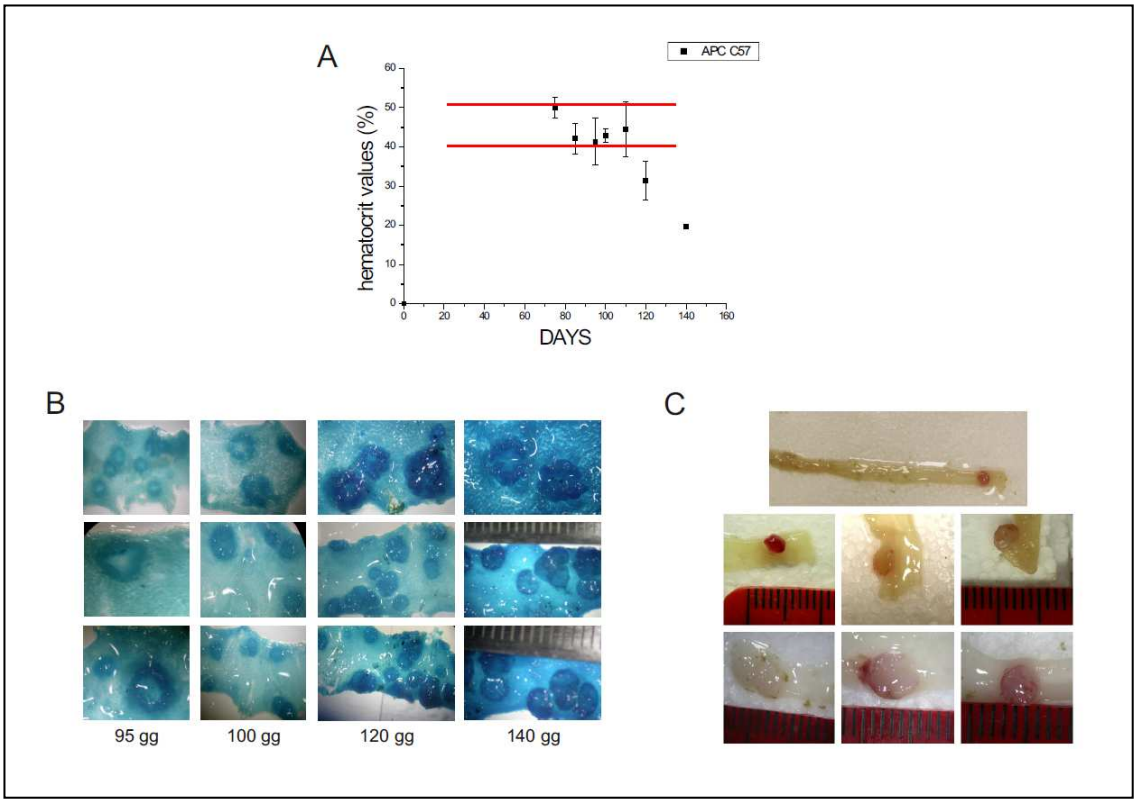

Figure 13: Pathological findings. A) Because Apc$^{min/+}$ mice develop severe anaemia as disease progresses (Hassan and Howell, 2000), haematocrit was measured as an indirect marker for tumour load and disease severity. Values represent percentage proportion of the volume of the blood sample occupied by the erythrocytes and are reported as the mean ± SEM. B) Macroscopic pictures of small intestinal mucosa of APC $^{Min/+}$ mice sacrificed at different ages. C) Macroscopic views of colonic polyps of APC $^{Min/+}$ mice sacrificed at different ages.

Time-course observation of lesions in Apc$^{min/+}$ mice revealed that the incidence and size of lesions gradually increased with time. We fitted temporal distribution of the number of lesions in the small intestine with a generalized sigmoid (ø>1mm) or Gaussian (ø<0,5mm) curve that most closely match the data (Fig.14A and 14B). Overlapping the curves, we identified a temporal window of tumour progression; the intersection point of the curves targets a specific time in which the smaller lesions evolve in larger ones. This well defined time lapse is useful to investigate the underlying molecular aberrations that occur throughout the tumour progression (Fig. 14C).

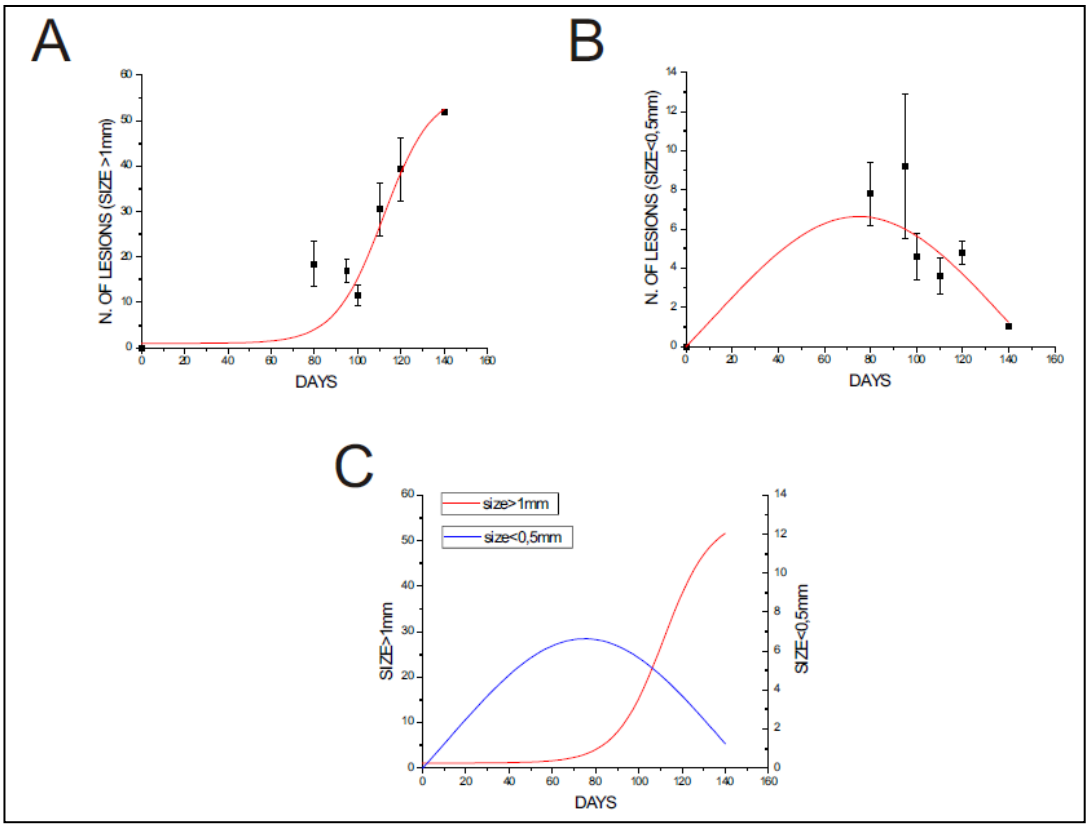

Figure 14: Time-course observation of small intestine lesions in Apc$^{min/+}$ mice. A) The temporal distribution of the number of lesions with a size >1mm in the small intestine were fitted with a generalized sigmoid. R^2 = 0.83. B)) The temporal distribution of the number of lesions with a size <0.5mm in the small intestine were fitted with a generalized Gaussian. R^2 = 0.87. C) The overlap of the curves shows a temporal window of tumour progression; the intersection point of the curves identifies a specific time in which the smaller lesions evolve in larger ones.

Subsequently, based on previous observations (Koehl et al., 2010), indicating that the transcript encoding the murine homologue of the *hERG1* gene, *m-ERG1*, was expressed in the colon of Apc$^{min/+}$ mice, we determined *m-ERG1* expression levels in various tracts of the intestine of such mice, by RT-qPCR.

We found that the colon and rectum of Apc$^{min/+}$ mice showed an increase of *m-ERG1* expression, compared to wild type (WT) mice. Such increase was more evident in colonic and rectal polyps, which spontaneously develop in these mice. Interestingly, *m-ERG1* expression increased along with the size of polyps (Fig.15A). The m-ERG1 protein was also detected by IHC in colonic polyps of Apc$^{min/+}$ mice (Fig.15B). Polyps displayed a high m-ERG1 expression in adenomatous epithelial cells (see arrows in Fig.15B, right panel), while colonic samples of WT mice showed only a faint signal in the stroma (Fig.15B, left panel).

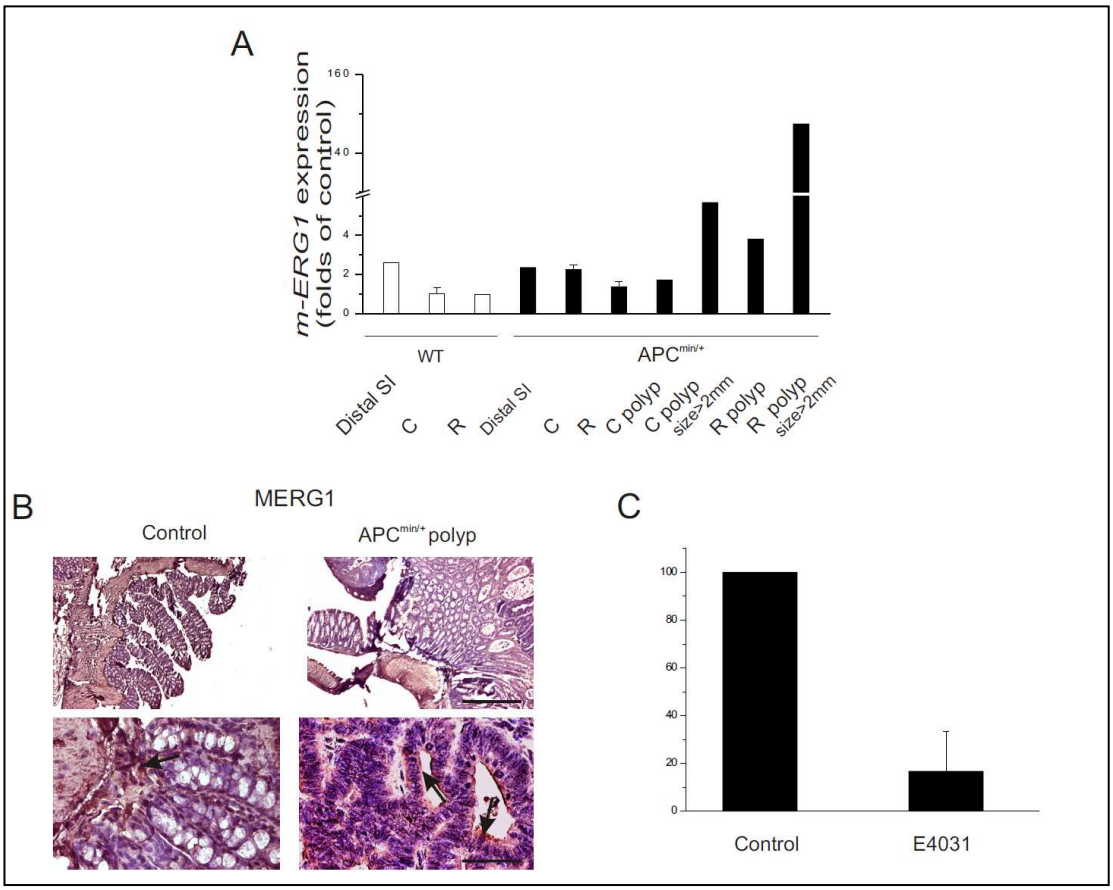

Figure 15: Expression and role of m-ERG1 in Apc$^{min/+}$ mice. A) Analysis of *m-ERG1* expression by Real Time PCR in small and large intestine of WT and Apc$^{min/+}$ mice and in colonic and rectal polyps developed in Apc$^{min/+}$ mice after a normalization for *mouse myosin, heavy polypeptide 11, smooth muscle* (*myh11*), characteristic of myofibroblasts and smooth muscle cells, to detect the only *m-ERG1* epithelial expression (Fortunato A. et al., in press). Distal SI: distal small intestine; C: colon; R: rectum; C polyp: colonic polyp; R polyp: rectal polyp. Data relative to colon and rectum derived from two different experiments, each carried out in triplicate, are reported as the mean ± SEM and were calibrated to the expression levels determined in the rectum of WT mice. Data relative to distal small intestine and polyps derived from a single experiment, carried out in triplicate, are reported as the mean, and were calibrated to the expression levels determined in the rectum of WT mice. B) m-ERG1 expression in control (WT) and Apc$^{min/+}$ polyps was evaluated by IHC. An anti-hERG1 monoclonal antibody was used as detailed in Materials and Methods. Upper panels: 50x magnification, bar 200 µm; lower panels: 400x magnification, bar: 20 µm. C) The number of colonic polyps obtained after E4031 treatment of Apc$^{min/+}$ mice. 4 one month-old Apc$^{min/+}$ mice received daily intraperitoneal injections of E-4031 for three months, while two Apc$^{min/+}$ mice received buffered saline only. After death, the number of polyps that developed in colon of Apc$^{min/+}$ mice was determined under a dissecting microscope (20x power field). Data were expressed as mean ± SEM.

When Apc$^{min/+}$ mice were treated with the specific hERG1 blocker E4031, daily for 3 months, such long-term hERG1 current inhibition produced an impairment in colonic lesion development (Fig.15C). No effects on the number of polyps in the small intestine was observed. Consistently, no over-expression of the m-ERG1 transcript was detected in the small intestine of Apc$^{min/+}$ compared to WT mice (Fig.15A, left most bar).

It is known that the main function of Apc is to degrade cytosolic levels of β-catenin, whose dysregulation is considered a major cause of tumour development. Since previous studies from Carlos Munoz's laboratory showed that β-catenin increased the hERG1 protein levels within the oocyte cell membrane (Munoz et al., 2012), the increased hERG1 channel activity detected in the polyps of $Apc^{min/+}$ mice could be attributed to the over-expression of β-catenin, widely described in this animal model (Yamada and Mori, 2007). It is worth noting that, at difference from what happens in the small intestine, the loss of function of Apc is not sufficient per se to trigger the development of tumours in the colon, where adjunctive genetic events are required for the transition from microadenomas to macroscopic tumours to be accomplished (Jen et al., 1994). Our data could suggest to consider hERG1 as one factor which cooperates with Apc loss to trigger colorectal tumour progression.

4.2.2 Characterization of hERG1 transgenic mice.

To generate a transgenic mouse model conditionally over-expressing *herg1* gene in the gastrointestinal tract we used the RAGE strategy (see Materials and Methods). This strategy consisted in the production of a vector with the human β-actin minimal promoter and the *herg1* cDNA, tagged with the myc epitope and a poly-histidine (His) flag at the protein C-terminal, separated by a floxed stop cassette containing the *EGFP* cDNA followed by 1X SV40 intron/polyA, whose expression is driven by the same promoter (Fig.16A). The *herg1* cDNA is under the control of a promoter for ubiquitous expression, but the transcription should be blocked by the presence of the reporter gene and only the action of Cre recombinase, leading to the excision of the floxed DNA, should allow the activation of *herg1* expression. The vector was microinjected into FVB/N zygotes and four different mice, named 801, 821, 883 and 886, were identified by PCR analysis carried out on tail genomic DNA with primers specific for the hERG1-myc transgene (Fig.16B). A subsequent Southern blot analysis showed a variable transgene copy number in the different founders, ranging from 1 of founder 801 to 14 of founder 821 (Fig.16C). The mice were then mated with FVB/N wild type

animals to obtain four different hERG1-EGFP^{Floxed} transgenic lines, but, due to reproductive problems of 821 founder, only three transgenic lines were expanded.

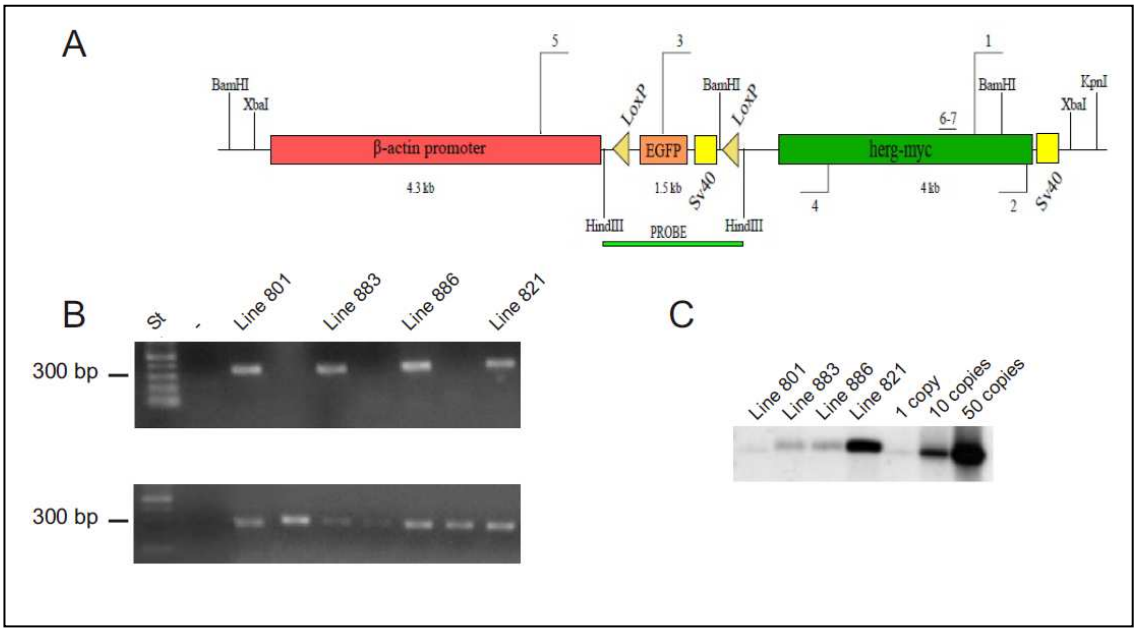

Figure 16: A) schematic representation of hERG1myc conditional expression vector, including location of specific primers (1-7): 1-2 utilized for PCR genotyping specific for the hERG1myc transgene; 3-4 used to detect transcriptional readthrough phenomenon, by amplification of a cDNA fragment spanning from the 3' end of the egfp (primer 3) to the 5' end of herg1myc (primer 4); 5-4 used to detect recombination in double transgenic mice; 6-7: utilized for herg1myc mRNA quantification by RT-qPCR. Restriction sites and the probe used in the Southern blot analysis are indicated. B) Mice were genotyped by PCR analysis; the presence of the transgene was checked on mouse tail genomic DNA by amplification of a 350 bp fragment (upper panel) spanning the myc-6xhis epitope region (as indicated in 1A). Amplification of the control gene, interleukin-2, by PCR (lower panel) was systematically performed on DNA to check the integrity of genomic DNA extracted. C) Southern blot analysis was carried out on 10 µg of genomic tail DNA extracted from mice of each transgenic line. Genomic DNA, digested with Hind III, was transferred to Hybond N^+ membrane (GE Healthcare) and tested with a ^{32}P-labeled 1,5 kb probe corresponding to a EGFP fragment, as indicated in A. Transgene copy number was estimated comparing the intensity of DNA band of transgenic animals to a standard of 1, 10 and 50 copies of injected DNA fragment using the ImageJ software.

To test the correct ability of the used stop cassette to block the transcription of hERG1myc, we controlled, in all the transgenic lines, *herg1* expression in the liver, as control site, by quantitative Real Time PCR analysis with primers specific for *hERG1myc*. Data obtained from this analysis indicated, although at different levels depending on the line, the presence of human ion channel transcript, even in the absence of Cre-mediated recombination (Fig.17A). According to data obtained from Southern Blot, *herg1* resulted more expressed in tissues from line 886, which showed a higher transgene copy number compared to line 801. Moreover, the variable

expression could be due to different integration sites as well as different efficiency of promoter in various organs, as previously reported (Bronson et al., 1996). These data suggested the existence of a transcriptional readthrough phenomenon, which was confirmed by the sequencing of a cDNA fragment (Fig.17B) spanning from the 3' end of the EGFP to the 5' end of hERG1myc of the construct (analysed region amplified with primers 3-4 showed in Fig.16A).

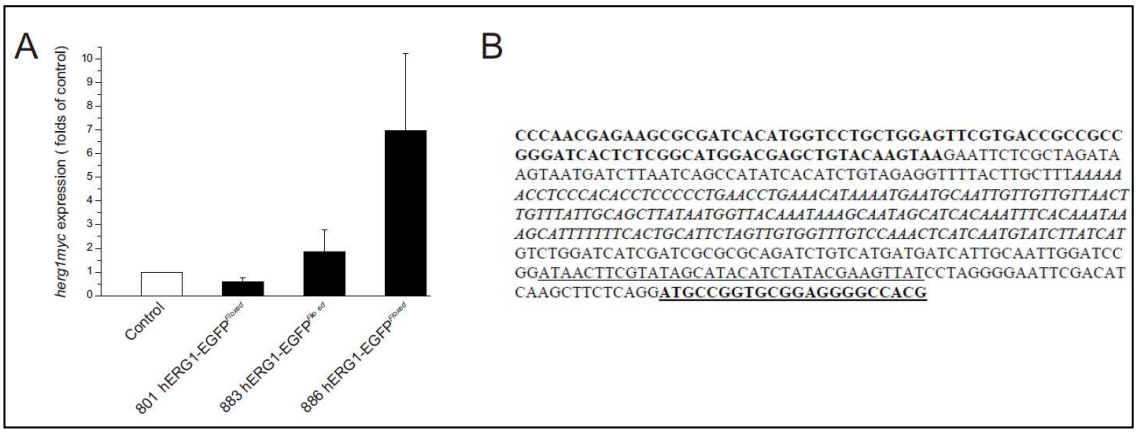

Figure 17: A) hERG1-EGFP^{Floxed} mice and WT mice were sacrificed and livers were analyzed by Real Time PCR to evaluate hERG1myc mRNA. The analysis of *herg1myc* expression levels in liver of WT mice (white bar) and hERG1-EGFP^{Floxed} mice (black bars) respectively from 801, 883 and 886 transgenic line is shown. Data are reported as means ± SEM with normalization to WT liver. B) A readthrough phenomenon was identified by the sequencing of a cDNA fragment, amplified with primers spanning from the 3' end of the egfp to the 5' end of herg1myc of the construct (as indicated in Fig.4A). Bold: egfp sequence; cursive: polyA SV40 sequence; underscored: loxP sequence; bold and underscored: herg1myc sequence.

EGFP^{Floxed} mice of the three lines did not show any apparent phenotype, even at old ages, and presented a normal life span. This put suggested that the ubiquitous expression of the hERG1 protein, produced by the leakiness of the stop cassette, has not grossly deleterious effects.

With the aim of identifying a possible role of hERG1 channel in colorectal carcinogenesis, we tried to obtain an increased expression of hERG1 in the epithelial cells of the large intestine. Despite the readthrough phenomenon, an increase in *herg1* expression could be expected after the cutting off of the stop cassette through genomic recombination. To this purpose, EGFP^{Floxed} mice were mated with Fabp$^{4xat\text{-}132}$Cre (Cre) mice, that express Cre recombinase under the control of the fatty acid-binding protein (Fabp) gene promoter (Saam and Gordon, 1999). The Cre is expressed, in such mice,

mainly, although not exclusively, in the intestinal epithelium. A quantitative Real Time PCR analysis on total RNA extracted from the large intestine of 6 months old EGFP^{Floxed} and hERG1-EGFP^{Floxed}-Cre double transgenic mice (DT) did not show any statistically significative increase of *hERG1myc* transcripts in DT compared to EGFP^{Floxed} mice (Fig.18A), so no further significant increase in hERG1 expression was triggered by Cre. Therefore, hERG1-EGFP^{Floxed} mice, due to the transcriptional control exerted by the β-actin promoter, can be considered to over-express the hERG1 transcript ubiquitously. Both the hERG1 transcript (Fig.18A) and the hERG1 protein (Fig.18B) were over-expressed in either hERG1-EGFP^{Floxed} or hERG1-EGFP^{Floxed}-Cre belonging to different TG lines (801, 883, 886), compared to WT mice. Since EGFP^{Floxed} and hERG1-EGFP^{Floxed}-Cre double transgenic mice resulted over-expressing *herg1* without a relevant difference, both the groups were subsequently analysed indifferently as "transgenic mice" (TG). In TG mice, hERG1 expression was strongly detectable in colonic epithelial cells and not limited to the stroma and myofibroblasts, as occurs in WT mice (Fig. 18B).

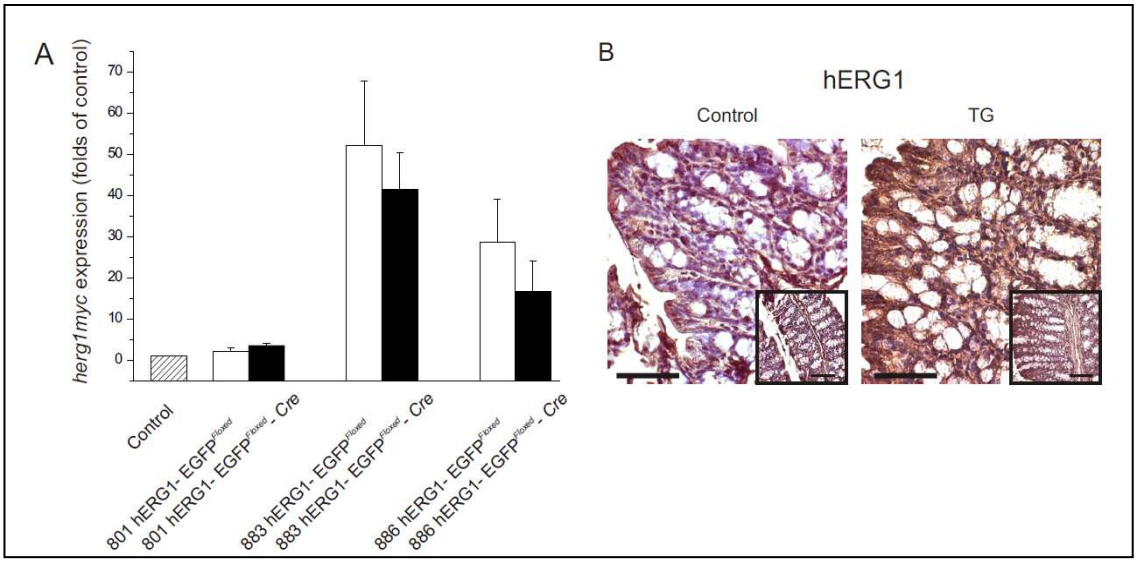

Figure 18: Characterization of hERG1 expression in TG mice. A) Analysis of *hERG1myc* expression by Real Time PCR in colon-rectum of WT mice (left bar), hERG1-EGFP^{Floxed} mice (white bars), and hERG1-EGFP^{Floxed}-Cre mice (black bars) respectively from 801, 883 and 886 transgenic line. Data, each carried out in triplicate, are reported as the mean ± SEM and were calibrated to the expression levels determined in the colon-rectum of WT mice. B) An immunohistochemical analysis was carried out in colon-rectum of control and TG mice, using an anti-hERG1 monoclonal antibody, as detailed in Materials and Methods, to evaluate hERG1 expression and confirm the presence of the transgene. Magnification: 400x, bar: 20 µm; inset: 200x magnification, bar: 50 µm.

A Reverse transcription PCR analysis performed on the RNA extracted from caecum and colon-rectum of hERG1-EGFP^{Floxed}-Cre double transgenic mice showed the presence of the *Cre* transcript (Fig.19A), but a PCR analysis on genomic DNA extracted from the same organs did not individuate the band corresponding to the recombined allele (Fig.19B). On the other hand, the ability of the transgene to be correctly recombined in the presence of Cre protein was verified by PCR analysis on genomic DNA extracted from tails of hERG1-EGFP^{Floxed}-CMVCre double transgenic mice, in which a strong ubiquitous expression of Cre recombinase is present (Fig.19B). This suggests that the lack of an appreciable difference in hERG1myc expression between EGFP^{Floxed} and hERG1-EGFP^{Floxed}-Cre double transgenic mice could be due to the mosaic nature of Cre expression in Fabp-Cre mice (Wong et al., 2000), reducing the number of cells able to recombinate in the intestine and hence, the cells with true over-expression of hERG1myc.

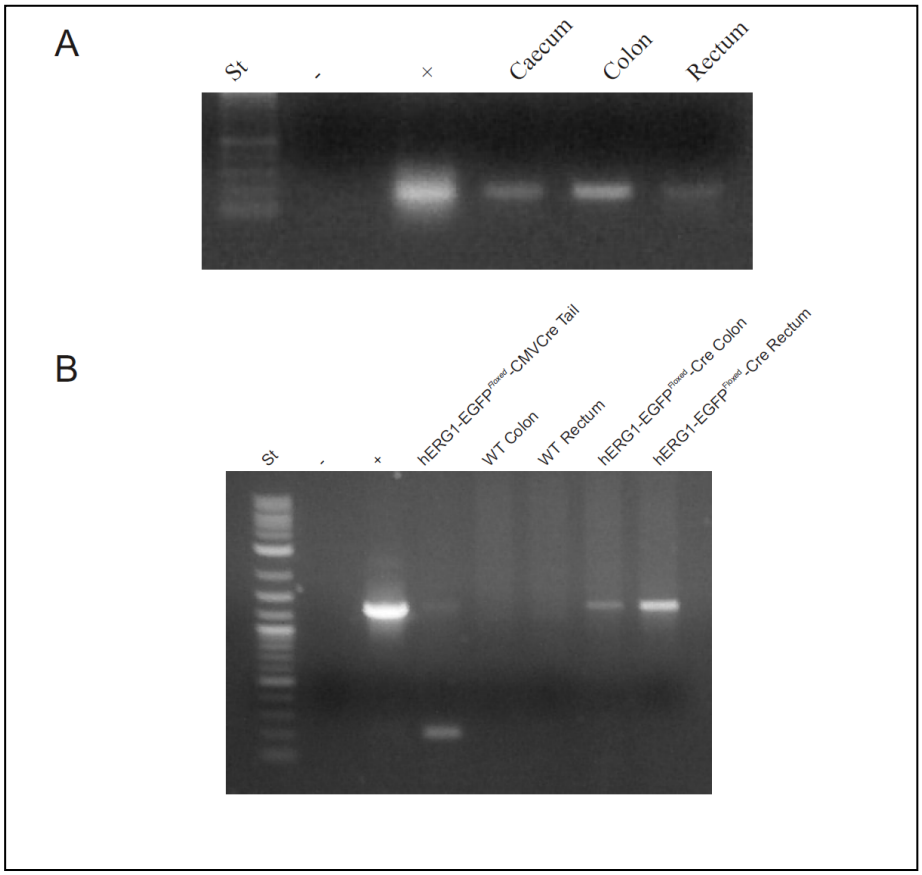

Figure 19: A) Reverse transcription PCR showed mRNA expression of *Cre* in different large intestine segment of hERG1-EGFP^{Floxed} -Cre double transgenic (DT) mice. B). DNA extracted from colon and rectum of WT and hERG1-EGFP^{Floxed}-Cre double transgenic mice was tested by

end-point pcr to verify the presence of Cre mediated recombination in mice. Such end point PCR failed to detect recombination in hERG1-EGFP^{Floxed}-Cre double transgenic mice; on the other hand, PCR analysis performed on genomic DNA extracted from tails of hERG1-EGFP^{Floxed}-CMVCre mice revealed the correct transgene recombination in the presence of Cre.

4.2.3 Effects of AOM treatment in hERG1 TG mice.

The generated TG mice did not show any apparent phenotype, even at old ages, and presented a normal life span. Hence, hERG1 over-expression per se is not life-threatening and does not induce tumour development.

Both TG and WT mice were treated with AOM (or physiologic saline), according to the schedule in Fig.20A, and the occurrence of colonic lesions was analyzed three months after the last injection. The macroscopic inspection of the large intestine of treated mice, revealed, in the colon of AOM-treated TG mice, the presence of polyps, which were, on the contrary, only barely detectable in WT mice (Fig.20B). No lesions were observed in the large intestine of mice injected with physiologic saline. After staining the large intestine with methylene blue, the number of carcinogen-induced aberrant crypt foci (ACF), and, after restaining with high-iron diamine Alcian blue (HID-AB), that of mucin-depleted foci (MDF) were determined. A statistically significant increase in the number of MDF lesions in TG mice compared to WT mice, was detected (fig.20B). The increase in MDF lesions paralleled the increased number of polyps, as evidenced by macroscopic inspection. No significant difference was detected in the number of ACF between TG and WT mice.

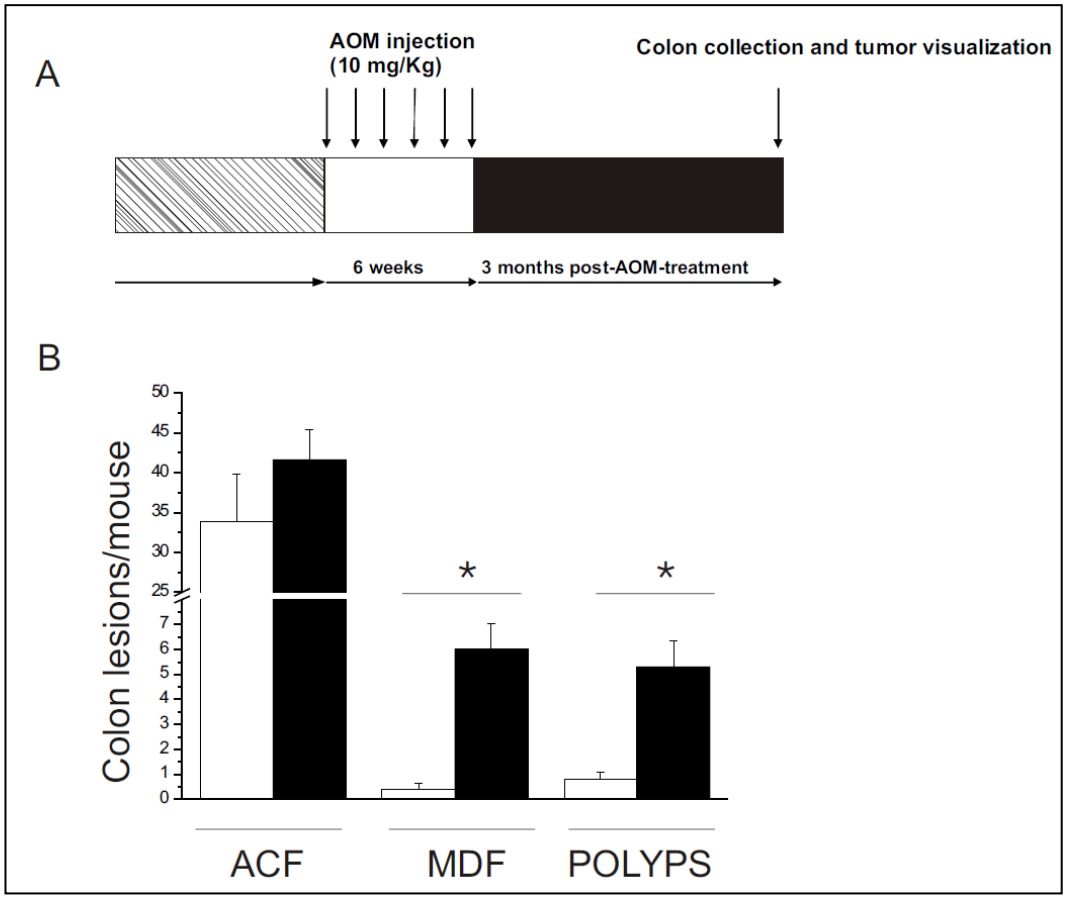

Figure 20: Effect of AOM treatment in hERG1 TG mice. A) Outline of AOM treatment: 6 control mice and 12 TG mice, maintained in a C57Bl6/FVB mixed background, received, at two months after birth, intraperitoneal injections of AOM (10 mg/kg body weight) once a week for 6 weeks and were killed three months after the last injection. B) The number of ACF, MDF and polyps that developed in control (white bars) and TG (black bars) treated mice was determined. Data were expressed as mean ± SEM. Statistical analysis was conducted using the Mann-Whitney U-test (*significantly different with a p-value of <0.01).

On the whole, although, hERG1-TG mice did not develop spontaneous tumour, they displayed an accelerated process of tumourigenesis, when treated with AOM, as witnessed by an increased number of preneoplastic lesions (mainly MDF) and polyps in the colon. It is worth noting that MDF, i.e. dysplastic lesions characterized by a defective mucin production, are considered precursors of CRC both in humans (Fodde and Smits, 2001; Femia et al., 2008; . Yamada and Mori, 2003) and in experimental models (Caderni et al., 2003). Our finding that the concomitant over-expression of hERG1 in the large intestine increases the number of AOM-induced MDF and polyps, strongly indicates that an up-regulation of hERG1 accelerates the process of colorectal carcinogenesis, further stressing the role of hERG1 as a progression gene in CRC.

4.2.4 TG and Apc$^{min/+}$ mice over-express pAkt and VEGF-A in the epithelial lining of the large intestine.

Finally, we tried to decipher whether a common molecular mechanism could underline the effect of hERG1 over-expression in the process of colorectal carcinogenesis, as evidenced in either the genetic (Apc$^{min/+}$) or chemical (AOM-treated) mouse model. As our *in vitro* experimental evidences suggest a role of hERG1 channel in the angiogenesis through induction of PI3K/Akt pathway with subsequent up-regulation of *vegf-a* gene transcription (Crociani et al., in preparation), we analyzed the expression of both pAkt and VEGF-A in the large intestine of TG and in the m-ERG1-expressing polyps of Apc$^{min/+}$ mice. In Figure 21 representative pictures are presented, while semi-quantitative data are reported in the Table 1 and Table 2. A higher expression of both pAkt (Fig 21A) and VEGF-A (Fig 21B) was detected in the proximal colon and rectum of TG compared to WT mice. Similarly and consistent with previous reports (Mora net al., 2004; Spitzner et al., 2008), a clear pAkt (Fig 21E) and VEGF-A (Fig 21F) immunostaining was detected in the polyps of Apc$^{min/+}$ mice with significantly higher levels compared to control mice (Table 1). In both TG mice and Apc$^{min/+}$ polyps, VEGF-A displayed a peculiar expression pattern, different from what observed in control mice. In fact, VEGF-A expression was not limited to the stroma, but was significantly present in the cells of the epithelial lining. The increased expression of VEGF-A in TG mice and Apc$^{min/+}$ polyps, was accompanied by a significant increase in angiogenesis, evaluated as microvessel density and total vascular area measured after staining with an anti CD34 antibody (Fig 21C and Table 2). Control mice showed smaller vessels mainly localized in the muscolaris propria, while transgenic mice and Apc$^{min/+}$ polyps were characterized by larger vessels with a less ordinate distribution.

Finally, we verified whether the up-regulation of VEGF-A in TG mice was directly linked to a higher hERG1 activity and, hence, could be reverted by treatment with the hERG1 blocker E4031. Indeed, treatment of TG mice with E4031 for two weeks, led to a significant decrease of VEGF-A staining (Fig 21D). This indicates that hERG1 channels are not only over-expressed in TG mice, and drive VEGF-A secretion and an increased angiogenesis, but they are active and their activity is more or less directly responsible for the VEGF-A enhanced production observed in these mice. Taken together, data reported in this paper indicate a significant role of hERG1 in

colorectal carcinogenesis *in vivo*, and may further stress the inclusion of hERG1 blockers in the treatment of CRC.

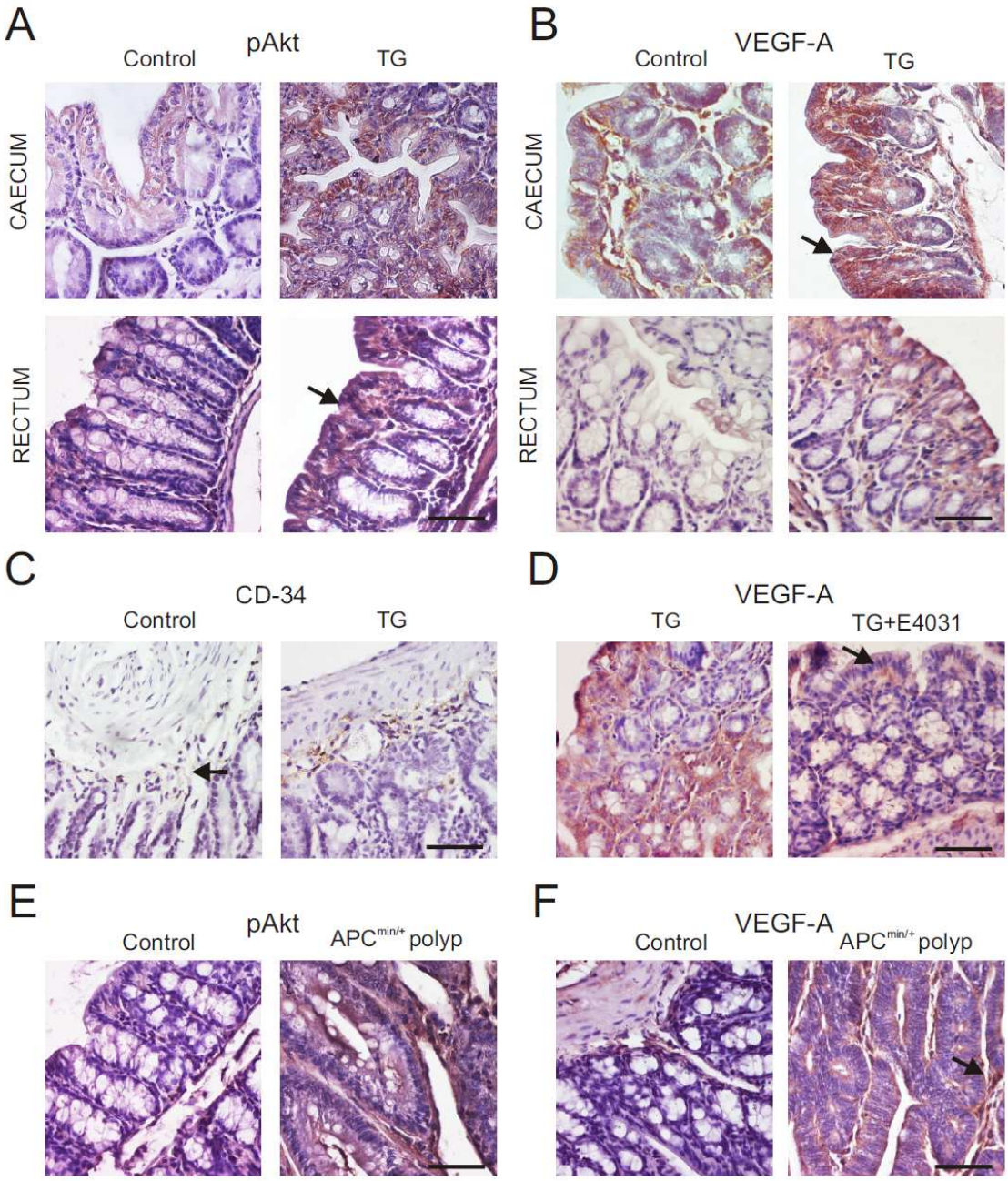

Figure 21: pAkt and VEGF-A expression in hERG1 TG and $Apc^{min/+}$ mice. A) IHC experiments were performed for pAkt in caecum and rectum of control and TG mice. Magnification: 400x, bar: 20 µm. B) Representative pictures of VEGF-A expression for both control and TG mice are reported. In both groups we evaluated VEGF-A expression in the caecum and rectum of animals of different ages (3 and 6 months). Experiments performed on 6 months-old mice were reported, but the same results were obtained with 3 months-old mice. 400x magnification, bar: 20 µm. C) Immunohistochemistry using anti-CD34 monoclonal antibody was performed. Magnification: 400x, bar: 20 µm. D) Representative pictures of immunohistochemistry experiments performed with anti-VEGF-A antibody are shown for both control TG mice and E4031-treated TG mice (addressed as E4031). Magnification: 400x, bar: 20 µm. E-F) Immunohistochemical staining for pAkt (left panels) and VEGF-A (right panels) in control and $Apc^{min/+}$ polyps. Magnification: 400x, bar: 20 µm.

Table 1: VEGF-A and pAkt expression in WT, TG and $Apc^{min/+}$ mice evaluated by the percentage of positively immunostained cells.

	WT mucosa (FVB)		**TG mucosa**		**WT mucosa (C57BL/6)**	**$APC^{min+/-}$ mucosa**	**$APC^{min+/-}$ polyps**
	3 months	***6 months***	***3 months***	***6 months***	***6 months***	***6 months***	***6 months***
VEGF-A	45%	30%	70% n.s.	70% **p <0.01**	1%	5%	60% ***p <0.01;** **°p <0.01**
pAkt	25%	10%	60% **p <0.05**	60% **p <0.05**	10%	10%	30% ***p <0.05;** **°p <0.05**

Positively stained cells were counted in ≥6 randomly selected fields, under 200x magnification. WT: wild type mice; TG: hERG1-transgenic mice; statistical analysis: Mann-Whitney U-test; p: TG mice versus corresponding age-matched WT (FVB) mice; *p: 6 months-old $Apc^{min/+}$ polyps versus 6 months-old WT mucosa (C57BL/6); °p: 6 months-old $Apc^{min/+}$ polyps versus 6 months-old $Apc^{min/+}$ mucosa; ns= non-significant.

Table 2: Vessel and total vascular area count in WT, TG and $Apc^{min/+}$ mice.

	WT mucosa (FVB)		**TG mucosa**		**WT mucosa (C57BL/6)**	**$APC^{min+/-}$ mucosa**	**$APC^{min+/-}$ polyps**
	3 months	***6 months***	***3 months***	***6 months***	***6 months***	***6 months***	***6 months***
Number of vessels	5.6±0.7	3.6±0.8	10.1±1.1 **p <0.01**	8.6±1.6 **p <0.01**	3.9±0.3	4.7±0.6	15.9±3.3 ***p <0.05;** **°p <0.05**
Total vascular area (mm^2/ microscopic field)	3.4±0.6	2±0.4	3.4±0.6 n.s.	5±1.2 **p <0.05**	2.8±0.6	3±0.5	21.6±7.5 ***p <0.01;** **°p <0.01**

Total vascular area was measured, after staining with an anti-CD34 mouse monoclonal antibody, as mm^2 per microscopic field. Data are reported as mean ± SEM (n=12 microscopic fields at 200x magnification) and analysed by the Mann-Whitney U-test WT: wild type mice; TG: hERG1-transgenic mice. p: TG mice versus corresponding age-matched WT (FVB) mice; *p: 6 months-old $Apc^{min/+}$ polyps versus 6 months-old WT mucosa (C57BL/6); °p: 6 months-old $Apc^{min/+}$ polyps versus 6 months-old $Apc^{min/+}$ mucosa. ns= non-significant.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The results presented in this thesis clarified the role of hERG1 in the regulation of the process of colorectal carcinogenesis and neo-angiogenesis *in vitro* and *in vivo,* proving that:

- hERG1 interacts with β1 integrin to form a macromolecular complex on the plasma membrane of colon tumours and colon cancer cells.
- The hERG1/β1 complex is tumour specific, since it is not present in normal heart, in which hERG1 is associated with accessory β-subunits, like MinK (KCNE1) and MiRP1 (KCNE2).
- The hERG1/β1 integrin complex modulates phosphorylation of p85-PI3K and Akt activation.
- Integrin-dependent adhesion and hERG1 activity regulate *VEGF-A* expression and VEGF-A secretion in CRC cells.
- The hERG1/β1 integrin complex regulate VEGF-A up-regulating HIF(s) in a mTOR-independent manner, inducing the activation of HIF(s)-dependent genes.
- nuclear Factor-kB (NF-kB) mediates up-regulation of HIF-1α and HIF-2α triggered by the β1/hERG1 complex in CRC cells.
- Colonic polyps of $Apc^{min/+}$ mice over-expressed *m-ERG1* and their formation was reverted by the hERG1 blocker E4031.
- AOM triggered a higher number of preneoplastic lesions Mucin Depleted Foci and polyps in the colon of transgenic mice, which over-express hERG1, compared to wild type mice, indicating that an up-regulation of hERG1 accelerates the process of colorectal carcinogenesis.
- Both the intestine of transgenic mice and colonic polyps of $Apc^{min/+}$ showed an up-regulation of pAkt/VEGF-A and an increased angiogenesis, which were reverted by treatment with E4031.

In this study, we have shown that the engagement of integrin receptors on the plasma membrane of CRC cells, activate the transcription of several tumour progression genes including the angiogenic factor *VEGF-A*. Pivotal in this mechanism are hERG1 potassium channels, which are activated by and co-assemble with integrins, to form a molecular complex which triggers an intracellular signalling pathway ending to the regulation of HIF transcription factor(s). This complex is exclusively in cancer cells. The potential therapeutical relevance of the data obtained with the CRC cell lines *in vitro* is suggested by our observations in animal models, which allowed to dissect the role of hERG1 *in vivo*. We analyzed the effects of hERG1 over-expression using two mouse models: $Apc^{min/+}$ and Azoxymethane-treated mice. In these models, we provided evidence that i) hERG1 also regulates the expression of VEGF-A and pAkt *in vivo*; ii) this mechanism stimulates neo-angiogenesis.

On the whole, the hERG1 gene can be considered a “tumour progression” gene *in vitro*, since a complex comprising integrins and hERG1 channels strongly regulate angiogenesis and tumour progression in CRC cells model, as well as *in vivo*, since the channel strongly cooperates with genetic (loss of the tumour suppressor gene Apc) or environmental (chemical carcinogen) factors in triggering CRC progression in mouse CRC models.

Therefore, we found, both *in vitro* and *in vivo*, a relevant role of hERG1 channels in colorectal carcinogenesis, which could be traced back to a hERG1-dependent control of angiogenesis, which is impaired by specific hERG1 blockers.

On the whole, data here provided further stress the inclusion of hERG1 blockers in the treatment of CRC.

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UNIVERSITA' DEGLI STUDI DI FIRENZE

Dipartimento di Patologia e Oncologia Sperimentali

DOTTORATO DI RICERCA IN ONCOLOGIA SPERIMENTALE E CLINICA

XXV Ciclo

Dott.ssa Antonella Fiore

La Dott.ssa Antonella Fiore, nata a Crotone, il 07/06/1983, si è laureata in Scienze Biologiche Sanitarie, il 17/12/2008, discutendo una tesi dal titolo "Ruolo del complesso macromolecolare CXCR4/VLA4/hERG1 nella regolazione dei meccanismi di chemoresistenza in cellule di leucemia linfoblastica acuta pediatrica" (relatore: Prof.ssa Annarosa Arcangeli) e riportando la votazione di 110/110 con lode. Dopo la laurea, la Dott.ssa Antonella Fiore ha continuato a svolgere attività di ricerca nel Dipartimento di Patologia e Oncologia Sperimentali, sotto la supervisione della Prof.ssa Annarosa Arcangeli.

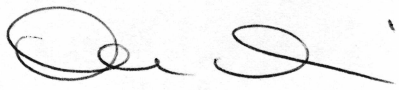
A partire dal Gennaio 2010, la Dott.ssa Antonella Fiore è stata ammessa alla frequenza del programma di Dottorato di Ricerca in Oncologia Sperimentale e Clinica (XXV° Ciclo), continuando la propria attività di ricerca presso il Dipartimento di Patologia e Oncologia Sperimentali, sotto la supervisione della Prof.ssa Annarosa Arcangeli, nell'ambito di un progetto intitolato "Characterization of hERG1 channel role in the regulation of the process of neo-angiogenesis: *in vitro* and *in vivo* studies".

L'attività di ricerca svolta dalla Dr.ssa Antonella Fiore nel corso del triennio di Dottorato ha avuto come oggetto la caratterizzazione del ruolo del canale del potassio hERG1 nella tumorigenesi colorettale e nella regolazione del processo di neo-angiogenesi, mediante studi *in vitro* e *in vivo*.

Questo studio è stato sottomesso alla rivista *Cancer Medicine*. Molti dei risultati di tutti gli studi elencati sono stati anche presentati dalla Dr.ssa Antonella Fiore a congressi nazionali e internazionali.

Durante il triennio di Dottorato, la Dott.ssa Antonella Fiore ha seguito con il massimo impegno il programma didattico stabilito dal Collegio dei Docenti ed ha portato avanti con entusiasmo e determinazione le sue ricerche, dando prova di grande inventiva ed intraprendenza, nonché di notevole elasticità nella elaborazione dei dati sperimentali. Nel corso del triennio, la Dott.ssa Antonella Fiore ha maturato una buona cultura di base ed una vasta esperienza diretta in metodiche sperimentali, cellulari e molecolari, anche molto complesse, alcune delle quali, non in uso nel nostro Dipartimento, sono state da lui indipendentemente messe a punto. La Dott.ssa Antonella Fiore ha tenuto inoltre periodiche relazioni seminariali sull'attività svolta. La curiosità e la tenacia della Dott.ssa Antonella Fiore ne fanno un ricercatore di notevoli potenzialità, in gran parte già ampiamente espresse.

Firenze, 14/12/2012

Prof.ssa Annarosa Arcangeli 

Prof. Persio Dello Sbarba

Università degli Studi di Firenze

Dottorato di Ricerca in
Oncologia Sperimentale e Clinica

A conclusione del corso triennale del XXV° Ciclo del Dottorato di Ricerca in Oncologia Sperimentale e Clinica, il Collegio dei Docenti, facendo propria la relazione presentata dalla Prof.ssa Annarosa Arcangeli, in qualità di *tutor*, circa l'attività di ricerca, l'operosità e l'assiduità della Dott.ssa Antonella Fiore, rilascia con parere unanime il seguente attestato da valere come presentazione del candidato ai fini dell'espletamento dell'esame finale.

La Dott.ssa Antonella Fiore, nata a Crotone il 07/06/1983, si è laureata in Scienze Biologiche Sanitarie il 17/12/2008, discutendo una tesi dal titolo "Ruolo del complesso macromolecolare CXCR4/VLA4/hERG1 nella regolazione dei meccanismi di chemoresistenza in cellule di leucemia linfoblastica acuta pediatrica" (relatore: Prof.ssa Annarosa Arcangeli) e riportando la votazione di 110/110 con lode. Dopo la laurea, la Dott.ssa Antonella Fiore ha continuato a svolgere attività di ricerca nel Dipartimento di Patologia e Oncologia Sperimentali, sotto la supervisione della Prof.ssa Annarosa Arcangeli.

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L'attività di ricerca svolta dalla Dott.ssa Antonella Fiore. nel corso del triennio di Dottorato ha avuto come oggetto la caratterizzazione del ruolo del canale del potassio hERG1 nella tumorigenesi colorettale e nella regolazione del processo di neo-angiogenesi, mediante studi *in vitro* e *in vivo*. In questo settore, la Dott.ssa Antonella Fiore è autore di un articolo che è stato sottomesso alla rivista *Cancer Medicine*. Molti dei risultati di tutti gli studi elencati sono stati anche presentati dalla Dott.ssa Antonella Fiore a congressi nazionali e internazionali.

Durante il corso di dottorato, la Dott.ssa Antonella Fiore ha seguito con il massimo impegno il programma didattico stabilito dal Collegio dei Docenti ed ha portato avanti, con continuità e intelligenza, le ricerche che sono oggetto delle sue pubblicazioni e della sua tesi.

Per quanto sopra, il Collegio dei Docenti unanime ritiene che la Dott.ssa Antonella Fiore, per l'assidua frequenza alle attività didattiche, per l'operosità nel lavoro di ricerca e per i risultati sperimentali ottenuti, possa meritatamente aspirare a conseguire il titolo di Dottore di Ricerca in Oncologia Sperimentale e Clinica.

Firenze, 17/12/2012

per il Collegio dei Docenti
Il Coordinatore del Corso
Prof. Persio Dello Sbarba