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# Role of Sphingosine-1-phosphate pathway in intestinal epithelial cells and its involvement in intestinal tumorigenesis

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# 2 INTRODUCTION

## 2.1 THE SPHINGOSINE-1-PHOSPHATE (S1P)

The sphingosine-1-phosphate (S1P) is a pleiotropic and widespread bioactive molecule belonging to the sphingolipid family, a complex group of lipids present in all eukaryotic cells. Previously considered playing only structural functions, sphingolipids are now recognised as key regulators of a myriad of cellular mechanisms and physiological processes [1]. Sphingolipids are amphipathic molecules, constituted by a hydrophobic backbone (a sphingoid long chain base), a fatty acid chain and a hydrophilic region (Figure1).

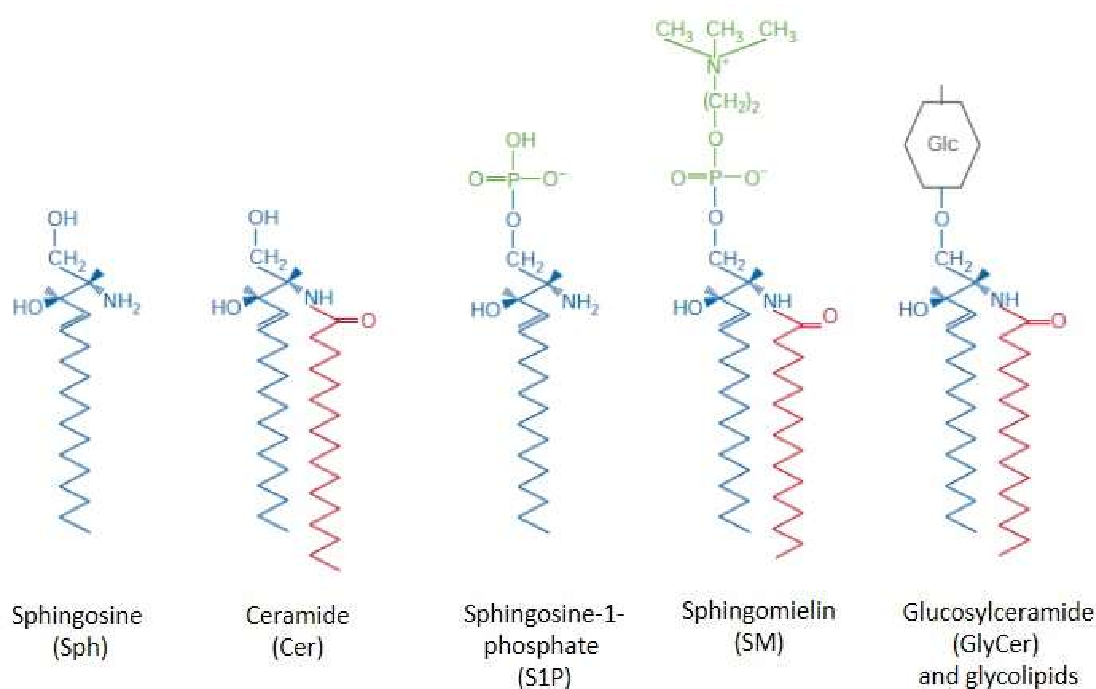



***Figure 1 The structure of sphingolipids.*** *Examples of sphingolipids are represented. The hydrophobic region is constituted by a sphingoid base chain (only the sphingosine as sphingoid base chain is represented in the picture, in blue) which binds a fatty acid chain (in red) and an hydrophilic region (in green) (Modified from Futerman and Hannun, 2004)* [2].

The *de novo* pathway of sphingolipid biosynthesis, highly conserved among all the eukaryotes, initiates in the endoplasmic reticulum (ER) with the condensation of serine

and palmitoyl coenzyme A, catalysed by serine palmitoyltransferase (SPT), and leads to the *de novo* synthesis of ceramide, the hub of the sphingolipid metabolism. In mammalian cells, the newly produced ceramide is usually transported to the Golgi apparatus and the addition of different head groups, during its trafficking, converts ceramide into complex sphingolipids, such as the sphingomyelin (SM) and glucosylceramide (GluCer). Ceramide can also be produced by a stepwise degradation of complex sphingolipids in the lysosomes or by the activation of sphingomyelinase (SMase) at the plasma membrane, which metabolize SM to ceramide. The breakdown of ceramide by a family of ceramidases (CDases) regenerates sphingoid bases, among which, the sphingosine, which could be recycled in the sphingolipid pathway or phosphorylated to generate S1P [2,3] (Figure 2).

S1P is produced inside the cells in different compartments by the phosphorylation of the sphingosine in a reaction catalysed by one of the two known isoforms of the sphingosine kinase (SphK1 and SphK2). The S1P levels are tightly controlled by its fast degradation. S1P can be dephosphorylated back to sphingosine by sphingoid base-specific phosphatases (SPPases) SPP1 and SPP2 or by broad-specific lipid phosphatases known as LPPs. Alternatively, S1P can be irreversibly degraded in ethanolamine phosphate and hexadecenal by S1P lyase, the exit point of the sphingolipid metabolic pathway (Figure 2).

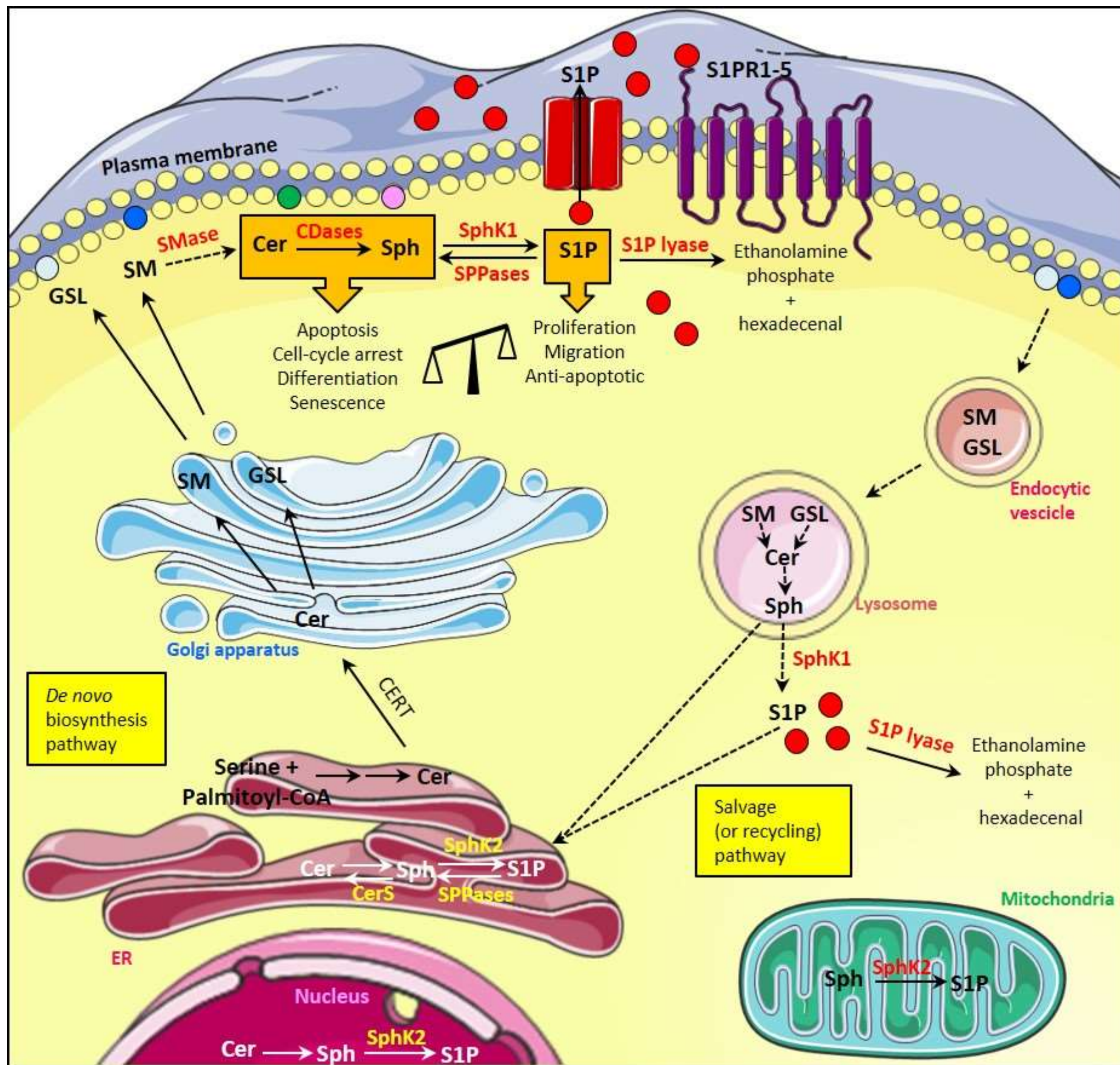



***Figure 2 Sphingolipid metabolic pathway.*** *The de novo biosynthesis (continue arrows) of sphingolipids and salvage (or recycling) pathway (dashed arrows) are depicted in the picture. Ceramide (Cer) synthetized in the ER from the condensation of Serine and Palmitoyl-CoA is transported to the Golgi apparatus. The transport of ceramide from the ER to the Golgi occurs either through the transfer protein CERT or the vescicular transport (VT). Sphingomyelin (SM) and glucosylceramide (GluCer) are synthesized in the latter compartment and then transported to the plasma membrane by vescicular transport. In the inner layer of the plasma membrane the sphingomyelinase (SMase) activated by multiple stress signalling metabolize the SM to Cer. SM and GluCer on plasma membrane are internalized and proceeding through the endosomal pathway are degraded to Cer by specific enzymes in the lysosome compartment. Cer is transformed by ceramidases (CDase) in sphingosine (Sph), which can be recycled to Cer in the ER or phosphorylated to form S1P in different cell compartments. The exit point of the sphingolipid metabolic pathway is the irreversible breakdown of S1P to ethanolamine phosphate and hexadecenal by S1P lyase.The S1P dephosphorylation by S1P phosphatases (SPPases) in ER would be recycled to form Cer (Petti L).*

Among the wide family of sphingolipids, the interest of researchers has been focused on S1P, since the discovery that it regulates cell growth in response to external stimuli [4,5] and suppresses the apoptosis [6]. Actually, a great corpus of studies demonstrated the

involvement of S1P in several physiological and pathological processes, including multiple sclerosis, inflammation, diabetes and cancer [7].

The reason why S1P plays such different roles is due to its dual signalling mechanism. Indeed, S1P not only has different functions inside the cells as second messenger, but it can be transported outside the cell by different members of the ABC transporters, such as ABCC1 and ABCA1 [8,9], and activate five specific cell surface G protein-coupled receptors (S1PR1-5) in autocrine or paracrine manner (the so-called 'inside-out signalling'), activating different downstream signalling pathways [10] .

## 2.2 THE INTRACELLULAR S1P SIGNALLING AND THE 'SPHINGOLIPID RHEOSTAT'

The intracellular signalling functions of S1P have not been completely clarified yet and many effects previously attributed to its role as second messenger, did not completely excluded the involvement of the S1P receptors [1,11].

Studies demonstrating a direct effect of S1P in the regulation of the calcium release from the intracellular stores, for the first time pointed out the function of S1P as a second messenger inside the cell [11,12]. The direct interaction of S1P with intracellular targets has been confirmed by two interesting studies. The first one demonstrated that S1P is an essential co-factor for the activation of NF-kB triggered by TNFα, through the canonical pathway, important for inflammatory and immune processes and anti-apoptotic responses [13]. Afterwards, a recent study revealed the role of S1P in the nucleus. Through its binding to the histone deacetylase 1 and 2 (HDAC1-1) corepressor complex, S1P controls the histone acetylation and influences gene expression [14].

S1P and ceramide are bioactive interconvertible metabolites. The sphingosine produced during the ceramide catabolism could be reversibly phosphorylated to form

S1P and in turn S1P can be dephosphorylated back to sphingosine, that can be recycled for ceramide biosynthesis by ceramide synthase (CerS). The concept of the 'sphingolipid rheostat' was proposed after pioneering studies *in vitro* that demonstrated the involvement of S1P in the cell proliferation induction and in the suppression of the ceramide-induced programmed cell death [4–6,15–17]. These studies suggested that the balance of S1P and ceramide intracellular concentration determines the cell fate decision between growing/surviving or dying [18,19]. However, the 'sphingolipid rheostat' is not the only sphingolipid mechanism that regulates the cell fate. Rather, the balance between conflicting signals due to different sphingolipids, their localization within the cell and the activation of different enzymes and receptor subtypes, could influence cell behaviour ('sphingodynamics' model) [1].

## 2.3 The S1P receptors (S1PRs)

S1P is the ligand for five specific G protein-coupled receptors (GPCRs), called S1P receptors (S1PRs) or endothelial differentiation gene receptors (EDGRs): S1PR1/EDG1, S1PR2/EDG5, S1PR3/EDG3, S1PR4/EDG6 and S1PR5/EDG8 [10]. The S1P binds the S1PR1-3 with high affinity (Kd=8.1nM for S1PR1, kd=27nM for S1PR2 and kd=23nM for S1PR3), whereas it has a lower affinity for S1PR4 and S1PR5 [20]. The S1PR1, S1PR2 and S1PR3 are ubiquitously expressed, whereas S1PR4 and S1PR5 have a narrower expression, respectively in the hematopoietic system and in the central nervous system (CNS). The S1PR1 couples exclusively with the Gi/o alpha subunit of heterotrimeric G proteins and activates the Ras/ERK pathway to enhance proliferation, the phosphatidylinositide 3-kinase (PI3K)/Akt pathway to inhibit apoptosis and the PI3K/Rac pathway to promote cytoskeletal rearrangement and cellular migration. The

S1PR2 and S1PR3 have been reported to be associated to several G proteins. Indeed, they couple with Gi/o and activate Ras/ERK and (PI3K)/Akt pathways. S1PR2-3 also couple to G12/13 enhancing the activation of Rho. These two receptors additionally bind Gq and activate phospholipase C (PLC). Similarly, the S1P4 and S1P5 couple with Gi/o and G12/13 [21] (Figure 3). Since S1PRs are widespread in different cell types and tissues, their specific pattern of expression and their association with different G proteins activate several downstream pathways and determines the regulation of specific cellular mechanisms and physiological processes [21,22].

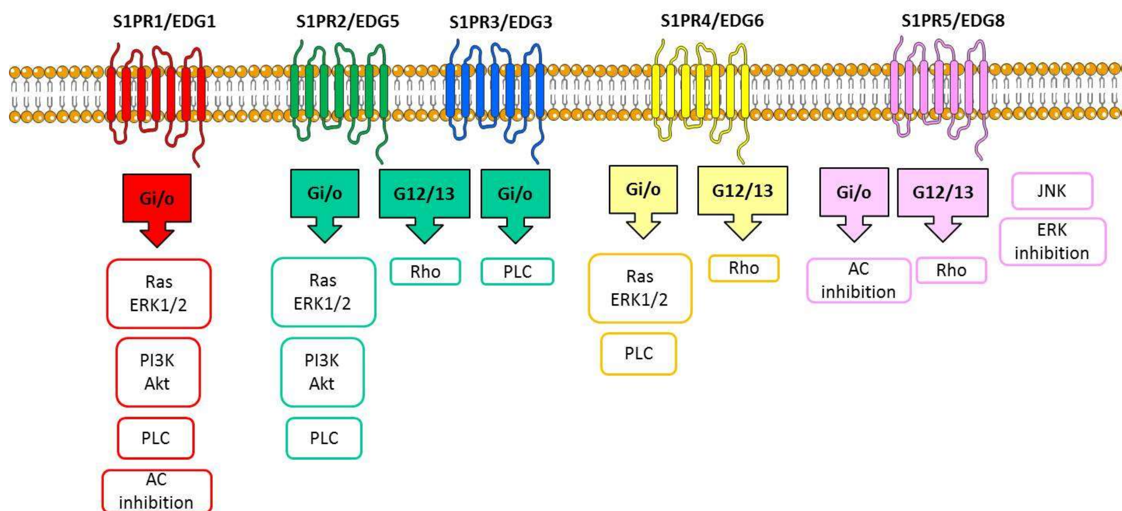



***Figure 3 The S1PR family and downstream pathways.*** *S1P binds five G-protein coupled receptors which are specifically coupled to different G proteins and lead to the activation or inhibition of the indicated downstream signalling pathways. Abbreviations: ERK = extracellular signal-regulated kinase; PI3K = phosphatidylinositol 3-kinase; PLC = phospholipase C; AC = Adenylyl cyclase-cyclic AMP; JNK = Jun aminoterminal kinase (Petti L).*

## 2.4 Sphingosine-1-phosphate receptor 2 (S1PR2)

The S1PR2 is ubiquitously expressed in different cell types and tissues. Therefore, not surprisingly, it has been found involved in multiple physiological and pathological mechanisms. Although mutations of the S1PR2-like gene in zebrafish lead to evident

abnormalities in embryonic development, S1PR2 knock out mice are normal at birth and do not display any relevant anatomical defects in the development [23].

However, important functional roles for S1PR2 have been identified in different tissues and organs, such as the central nervous system (CNS), the auditory and vestibular systems, the immune system, the liver and the kidney.

Phenotypically, the 14% of S1PR2 deficient mice, between three and seven weeks of age, develop lethal spontaneous seizures associated with a higher excitability of the pyramidal cortical neurons [24]. These seizures-prone mice displays impairment of the spatial working memory and increased anxiety, suggesting that S1PR2 plays an essential role in the development and/or maintenance of a proper neuronal excitability [24,25]. In the central nervous system, S1PR2 is thought to be involved also in neurogenesis, since its inhibition enhances the migration of neural progenitor cells toward the brain injury area [26].

The S1PR2 receptor is also fundamental for the auditory and vestibular systems functioning. S1PR2 knockout mice show deafness by one month of age associated with morphological abnormalities of the epithelial barrier layer and vasculature in the stria vascularis. These alterations are possibly due to defects in the vascular tone regulation of the spiral mediolar artery, which directly supplies blood to the capillaries of the stria vascularis [27]. In line with these observations, a previous analysis of regional vascular function in S1PR2 knock out mice revealed an essential physiological contribution of S1PR2 in the maintenance of normal cardiovascular function. The deficiency of S1PR2 resulted in a lower mesenteric and renal vascular resistance and tone and in a decreased responsiveness to vasoconstrictor agents [28]. The first mechanism

hypothesized by the authors involves the vascular smooth muscle cells (VSMCs). The VSMCs abundantly express S1PR2 [29] and have been implicated in different mechanisms, such as migration [30], contraction [31,32] and differentiation, through the regulation of the transcript expression of smooth muscle differentiating genes [33,34].

S1P has been recently identified as the signal that promotes the activation of the satellite cells (SCs), skeletal and muscle stem cells, fundamental to muscle homeostasis, by the repression of cell cycle inhibitors via the S1PR2/STAT3-dependent signalling in response to injuries, thus suggesting a role for S1PR2 in the regulation of the muscle regeneration [35].

Noteworthy, S1PR2 is the receptor for conjugated bile acids (CBA) in the hepatocytes. The CBA/S1PR2 signaling in the liver, through the activation of ERK1/2 and Akt, controls different hepatic metabolic pathways, such as lipid and glucose metabolism and the synthesis of bile acids [36]. The role of S1PR2 in the liver extends to the regulation of hepatocyte regeneration after injury. Indeed, the liver of S1PR2 knockout mice is less fibrotic and increases the hepatic regeneration after treatment with carbon tetrachloride [37].

In addition, the importance of S1PR2 in the metabolism is further evidenced by its role in diabetes. S1PR2 pharmacological blockade and genetic deletion reduce blood levels of glucose, the apoptosis of pancreatic beta cells and protect against the insurgence of STZ-induced diabetes in mice [38].

One of the most severe complication of the diabetes mellitus is the diabetic nephropathy (DN), characterized by kidney hypertrophy, glomerulus and tubular basement membrane thickening, and tubular interstitial fibrosis. The mesangial cells

proliferation and the accumulation of extracellular matrix are both hallmark of the disease [39]. S1PR2 is upregulated in kidney of diabetic rats and in rat mesangial cells under high glucose conditions, increasing the expression of fibronectin through the activation of the MAPK pathways [40].

The role of S1PR2 in the immune system has been reported by studies on mast cells, macrophages and B-cells within the germinal centre (GC). Recent studies report opposing results regarding the role of S1PR2 in the regulation of histamine release/ clearance and in anaphylaxis [41,42]. A vascular gradient of S1P exists between the blood and interstitial fluids and a role of S1PR1 has been demonstrated in directing the chemotaxis of lymphocytes out of lymphoid tissues and toward S1P in blood and lymph [43]. However, S1PR2 has been demonstrated to inhibit the macrophage migration *in vitro* and S1PR2 knock out mice displays a higher macrophage recruitment in a thioglycollate (TG) peritonitis model of acute inflammation [44]. Finally, S1PR2 plays an important role in GC B-cell positioning, since it inhibits GC B cell responses to follicular chemoattractants and maintains the GC homeostasis [45].

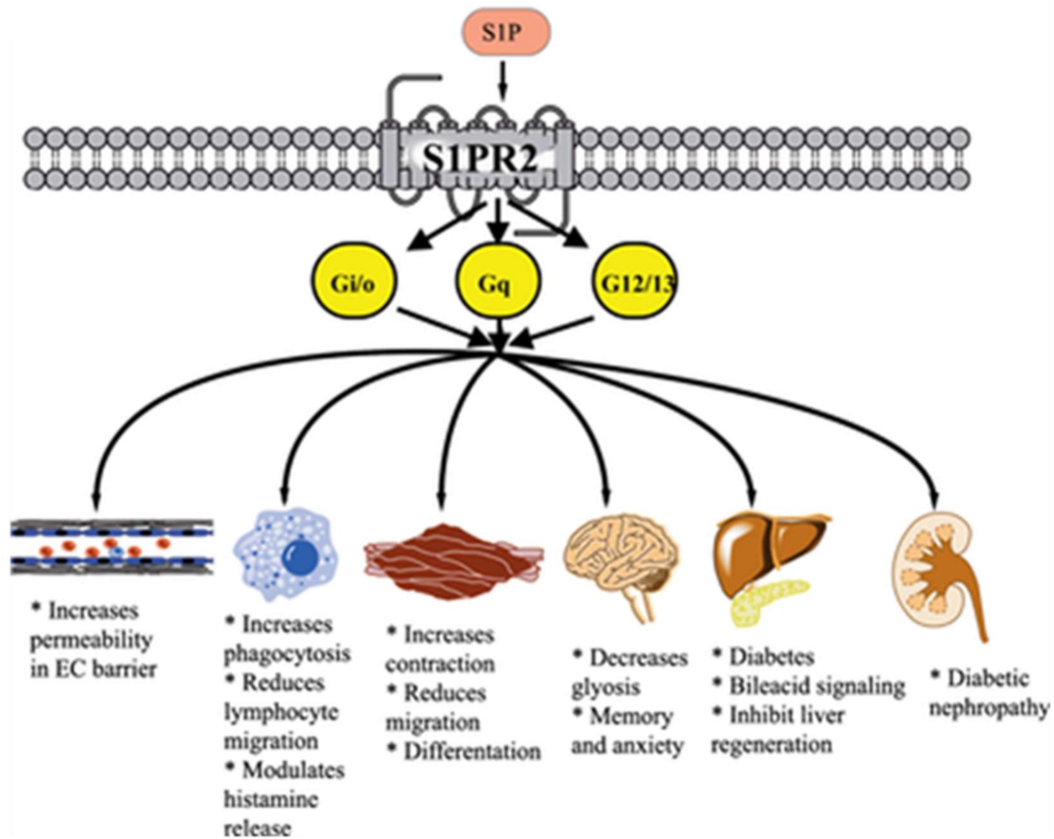



***Figure 4 The S1PR2 mediates multiple physiological and pathological mechanisms.*** *S1PR2 has different functions in the immune system, regulating macrophage and B-cell migration as well as the histamine release and clearance. S1PR2 has multiple physiological roles in the liver, where it mediates the CBA signalling and the cellular regeneration in response to injuries. Moreover it regulates the neuronal excitability and plays different functions in the biology of VSMCs. It is also involved in pathologies, such as the diabetes and diabetic nephropathy (Modified from Adada et al. 2013)* [46].

## 2.5 S1P SIGNALLING IN CANCER

Many evidences demonstrate the role of the S1P pathways in cancer. Indeed, S1P regulates different mechanisms involved in the tumor development and progression as inflammation, angiogenesis, cell growth, survival, apoptosis and migration. According with the ceramide-sphingosine-S1P rheostat model in mammalian cells, the relative amount of these three lipids determines the balance between the pro-growth and antigrowth signals as well as the cell fate. There are many examples showing that the sphingolipid rheostat is involved in cancer cell proliferation, survival, apoptosis and

chemoresistance [47]. Most of the studies are focused on the role of the sphingosine kinases SphK1 and SphK2 that regulate the interconversion of sphingosine into S1P, driving the cell towards a survival fate [47]. The first observation suggesting an oncogenic role of SphK1 in cancer was the transformation of NIH3T3 fibroblasts overexpressing SphK1 [48]. Despite *SphK1* is not defined an oncogene, since no mutations have been reported in cancer yet, a wide spectrum of evidences demonstrates that cancer cells increase the SphK1 expression and depend on it for survival and growth (*non-oncogene addiction*) [49]. Nevertheless, an increased expression of the SphK1 transcript and protein has been detected in different human cancers, such as astrocytoma/glioblastoma multiforme [50,51], lymphomas [52], breast [53], lung [54] and gastrointestinal cancers [55–57] and it is often associated with a poor prognosis and drug resistance [47].

### 2.5.1 S1P IN COLORECTAL CANCER

The earliest studies dealing with the link between the sphingolipids and the colon carcinogenesis demonstrated a protective role of sphingomyelin (SM) against the development and progression of the colorectal cancer [58,59]. However, the wide majority of the latest literature mainly focuses on the role of the S1P regulatory enzyme SphK1. Studies *in vivo* demonstrated that the deletion of SphK1 resulted in a decrease of the intestinal polyp size in the $Apc^{Min/+}$ genetic model [56] and in lower incidence and multiplicity of ACF and colon cancers in the azoxymethane (AOM) murine model of colonic tumorigenesis [57]. Moreover, Sphk1 is significantly up-regulated in human colorectal cancer tissues and in the AOM-induced ACFs and tumors in the murine colon [56,57,60]. The increased activity of SphK1 fuels elevated intracellular

S1P levels that lead to tumor development. However, it is still poor clear which of the S1P receptors mediates these effects. Recently, the SphK1/S1P/S1PR1 axis has come to light as the link between chronic inflammation and colitis-associated cancer. Indeed, the SphK1/S1P/S1PR1 axis has been demonstrated to regulate the production of IL-6 and induce the persistent activation of STAT3 in epithelial cells which consequently leads to malignant transformation in these cells [13,61]. Although these findings involve the S1PR1 in the inflammation-induced colorectal tumorigenesis, overall the function of the S1PRs and the S1P extracellular signaling in the development of colorectal cancer is not well investigated.

## 2.6 THE ROLE OF S1PR2 IN CANCERS

Although S1P regulates the cell growth and apoptosis also through the activation of the S1P receptors, the role of the S1P/S1PRs in cancer development and progression appears mainly associated to cancer cell invasion and motility, lymphocyte trafficking and transactivation of different growth factor-activated receptors [47]. Interestingly, some studies have pointed out the involvement of S1PR2 in the development and progression of different type of cancers. However, its role seems to be not univocal and depends on the cell-type and the tumor type and staging [46]. Most of the papers pointed out the high-level expression of S1PR2 in cell lines derived from human and murine tumors compared with the other S1PRs and support an anti-tumor function of S1PR2 [62–66]. Melanoma cell lines as well as breast cancer cells have been observed to overexpress endogenously S1PR2 and S1PR3, whereas downregulate others S1PRs [64,65,67]. Similarly, glioma cells and human gastric cell lines revealed high levels of S1PR2 and S1PR3 [62,66]. Arikawa and colleagues provided evidences that S1PR2 regulates

migration and invasion capabilities of melanoma cells through the regulation of Rac and RhoA activity [67]. Moreover, *in vitro* studies conducted on human glioblastoma and gastric cell lines revealed that in these tumors S1PR2 can act as a negative regulator of the migration [62,63]. A role of S1PR2 in the regulation of cancer cell proliferation and growth was first provided by studies on a human renal tumor cell line derived from a metastatic Wilms' tumor (WiT49). They demonstrated that the S1P/S1PR2 pathway promotes the expression of the connective tissue growth factor (CTGF), downregulated in advanced Wilms tumors, which inhibits the proliferation of WiT49 cell line. [68] Interestingly, i*n vivo* studies reported that Lewis lung carcinoma and B16 cell lines implanted in S1PR2 knockout mice grow faster and enhanced tumor angiogenesis and vascular maturation has been observed in these mice compared to the wildtype controls [69]. Moreover, S1PR2 knockout mice develop lymphoma at advanced ages with features of a germinal center (GC) derived diffuse large B cell lymphoma (DLBCL) phenotype [70] and mutations affecting putative S1PR2 regulatory sequences have been found in > 25% of human DLBCL [71].

In contrast, the few studies that reported a pro-cancerous role for S1PR2, attribute to S1PR2 a function in the modulation of tumor suppressor proteins, such as  the master suppressor of metastasis Brms1 [46,72].

## 2.7 The role of the S1P/S1PRs signalling in the regulation of the paracellular permeability

Several *in vitro* and *in vivo* evidences demonstrated the role of the S1P/S1PRs signalling in the regulation of endothelial cell permeability in response to enhancing factors or pathological conditions [73–76]. Studies using FTY720-phosphate (FTY720-P), a

high affinity agonist for all the S1PRs (particularly for S1PR1) except for S1PR2, highlighted the role of the receptor S1PR1 as a vascular permeability regulator [73,77]. Latest studies identified also the S1PR2 and S1PR3 as key regulators of endothelial barrier permeability. S1PR2 has been demonstrated to enhance the vascular permeability of endothelial cells *in vitro* through the activation of its downstream effectors Rho, ROCK and PTEN and to mediate the pulmonary vascular permeability also *in vivo* [77]. Moreover, experimental models of lung injury and endothelial permeability demonstrated that S1PR3 contributes to the endothelial barrier disruption [78]. Therefore, the regulation of the endothelial permeability mediated by S1P depends on the balance of the signalling mediated by the S1PRs [77,79]. However, there are few studies dealing with the role of S1P signalling on epithelial barrier function. Nowadays, S1P has been demonstrated to enhance the intestinal epithelial barrier resistance in human colorectal cancer cell line Caco-2 and in porcine- and rat-derived intestinal epithelial cells, by reorganizing and increasing the expression of E-cadherin and occludin [80–82]. However, the involvement of the S1PRs in this mechanism is still unknown.

## 2.8 THE BIOLOGY OF INTESTINAL AND COLONIC EPITHELIUM

The intestinal tract of the vertebrates is a tubal structure constituted by four tissue layers: the mucosa, the submucosa, the muscular layer and the serosa. The epithelium is the innermost constituent of the mucosal layer that lines the lumen and defines the interface between our internal and external environments. It is a simple cell layer, mainly constituted by absorptive enterocytes, columnar cells that have hydrolytic and absorptive functions of luminal contents, with apical microvilli that increase the

absorption surface [83,84]. The principal functions of the small intestinal epithelium are the digestion and absorption of the nutrients. It is organized in crypt-villus units and it is constituted by four major cell type: the enterocyte, the Goblet cells, the enteroendocrine cells (EECs) and the Paneth cells [83]. The Goblet cells produce and secrete mucins, peptides and proteins involved in the formation of the mucus layer, a highly viscous extracellular layer, that provides the first defence line against physical and chemical injury caused by the ingested food, microbes and microbial products [85]. The EECs are distributed through the whole gastrointestinal tract and scattered along the entire crypt-villus axis and represent the 1% of the whole epithelial cell population. They regulate digestive enzyme secretion, metabolism, bowel motility and food intake acting as chemoreceptors that sense the lumen content signals and release molecules which enter the circulation and target epithelial cells and the nervous system [86]. The Paneth cells are highly specialized epithelial cells present at the bottom of the crypt in the small intestine. They synthetize and secrete antimicrobial peptides and regulate the homeostatic balance between the essential colonizing microbiota and the innate immune protection against pathogens [87]. An emerging role of the Paneth cells is to provide the niche signals, essential for the growth and maintenance of intestinal stem cells [88]. The main functions of the colonic epithelium are the absorption of water and salts and to compact stool for the excretion. The fetal colonic epithelium is constituted by transitory wide and flat villi, which in humans are lost at birth. Therefore, the adult colonic epithelium is arranged in crypts associated with a flat luminal surface. The colonic epithelium is constituted by three types of epithelial cells: the enterocytes, alternatively called colonocytes, the enteroendocrine cells and the Goblet cells. The colonocytes are located at the top of the crypts and in the inter-crypt table, the surface

between the crypts, whereas the Goblet cells are located in the midcrypt. Although the colonic epithelium is lacking of Paneth cells, at the base of the crypts there are Paneth-like cells, called deep crypt secretory cells or crypt base goblet cells, with a gene expression profile intermediate between goblet and Paneth cells, whose the functions are still unknown [84].

## 2.9 INTESTINAL STEM CELLS (ISCS) AND EPITHELIAL HOMEOSTASIS

The intestinal and colonic epithelium is daily exposed to injuries and aggression by the luminal contents that induce the death of many epithelial cells. Therefore, in the adult epithelium a rapid and efficient mechanism of self-renewal is essential. Even if the debate over the real identity and position of the ISCs remains still unsolved, the Clevers' group demonstrated that the self-renewal ability of the epithelium is driven by a small population of adult stem cells located at the bottom of the crypts, named crypt base columnar cells (CBCC), able to produce the whole epithelial cell lineages [89]. The CBCCs, also called active stem cells (ASC) since they are highly proliferative, specifically express the leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) marker, the receptor for RSPO1 [90]. The Lgr5+ CBCC are intercalated with Paneth cells in the small intestine, whereas the Lgr5+ stem cells are located at the bottom of the crypt in the colon.  In response to an array of signals produced by the crypt niche, the CBCC rapidly divide to generate progenitors, better known as Transit-Amplifying (TA). TAs rapidly divide four to five times, start migrating upward the crypt-villus axis and differentiate into mitotically-inactive specialized epithelial cells that continue to migrate at the top of the villi (small intestine) or at the surface epithelium (colon), where they finally die and are extruded into the lumen [89] (Figure 5). Complete

epithelial turnover occurs every 3-5 days in the intestine and every 5-7-days in the colon. These mechanisms maintain the balance between cell production and cell death in the gastrointestinal epithelium.

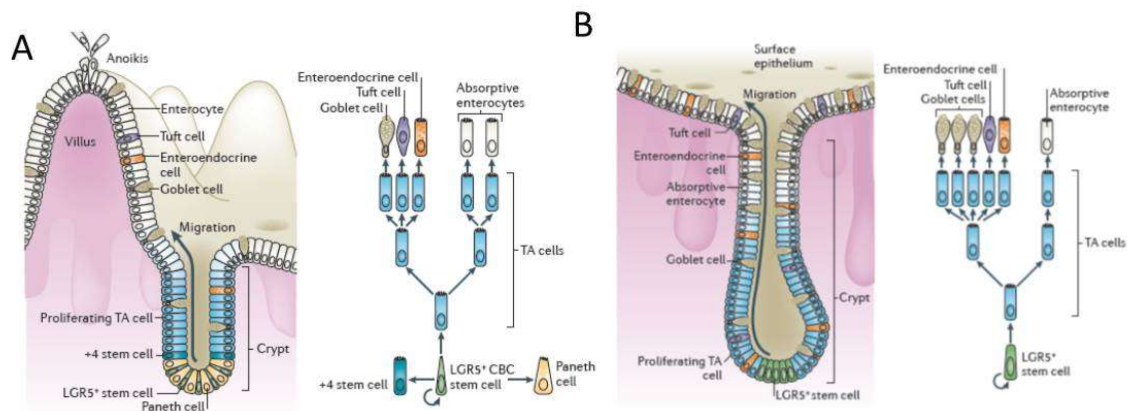



***Figure 5 Epithelial self-renewal.*** *The figure represents the self-renewal mechanisms occurring in intestinal (A) and colonic (B) epithelium and the differentiating hierarchy from Lgr5+ CBC into Transit-Amplifying which while migrate upward the crypt-villus axis or at the top of the crypt, differentiated in enterocytes, enteroendocrine and goblet cells. Adapted from Barker et al., 2014* [89].

## 2.10 SIGNALLING PATHWAYS INVOLVED IN THE SELF-RENEWAL OF THE INTESTINAL EPITHELIUM

The renewal process is spatially organized and several signalling pathways are involved in the spatial and functional regulation of the niche compartment [83]. The Wnt signalling was the first signalling pathway discovered to be involved in the control of the stem cell niche and the main regulator of the intestinal self-renewal. The members of the Wnt family are cysteine-rich secrete glycoproteins. Wnt proteins bind to Frizzled (Fz) and Lrp5/6 receptor complexes triggering the nuclear translocation of β-catenin, through the inhibition of the APC/Gsk-3β/Axin complex that, in absence of Wnts, promotes the ubiquitin-mediated degradation of β-catenin. The nuclear β-catenin associates with the transcription factors of the T-cell factor (TCF) family to promote the transcription of Wnt target genes, which regulate proliferation and differentiation [91].

Several studies demonstrated the role of the Wnt signalling and the downstream TCF-4/β-catenin complex in the control of the proliferation of epithelial cells and in the maintenance of the epithelial stem cell compartment in the small intestine [92–94]. Indeed, the complex TCF-4/β-catenin has been considered the 'master switch' that controls the stemness and differentiation of the intestinal epithelial cells [93]. In the last decade, several studies went deeper into the identification and localization of the Wnt family members in the murine adult intestine and colon. Nowadays, it is known that a subgroup among the Wnt members (Wnt3, Wnt6 and Wnt9b) and their specific receptors of the Frizzled (Fz-5 and Fz-7) and LRP family, are expressed by the crypt epithelial cells and activate the canonical pathway leading to the nuclear translocation of β-catenin and the activation of the TCF-4/β-catenin target genes. However, these studies identified other Wnt family members (Wnt-2b, Wnt4, Wnt-5a and Wnt-5b), expressed in the villus mesenchyme and in the mesenchyme adjacent to the surface epithelium in the colon, activating non-canonical signals that are thought to inhibit proliferation and to induce the epithelial cells differentiation [95,96]. The TGF-β signalling is another pathway involved in the regulation of stemness/differentiation cell fate. The TGF-β superfamily comprises both the TGF-β family members and the bone morphogenetics proteins (BMPs). The BMP/TGF-β pathway is strongly activated in the differentiated epithelial compartment and its inhibition is essential for self-renewal of the ISCs [91,97,98].

## 2.11 DYSREGULATION OF THE EPITHELIAL SELF-RENEWAL MECHANISMS AND CANCER DEVELOPMENT

As previously described, the homeostasis of the adult intestinal and colonic epithelium is determined by a complex interplay of mechanisms regulated by several signalling

pathways. Mutations that dysregulate these signalling pathways have been observed in both hereditary colon cancer syndromes and sporadic colorectal cancer suggesting a link between their role in the physiology and in the intestinal cancer development [83,93].

The familial adenomatous polyposis (FAP) and the hereditary nonpolyposis colorectal cancer (HNPCC) are both autosomal-dominant cancer syndromes [83]. The FAPs are caused by a germline mutation in the *adenomatous polyposis coli (APC)* gene and are characterized by the presence of hundreds to thousand colonic adenomas that develop in colorectal carcinomas (CRCs) in 100% of cases [83,99–101]. These CRCs are characterized by chromosomal instability (CIN) and accumulating mutations in key oncogene and tumor suppressor genes (such as *K-RAS* and *p53*) [83,99]. The *APC* mutations associated with the FAP are non-sense mutations that truncate the amino-terminal β-catenin interacting domain of the APC protein [102]. Mutations at the extreme 5' or 3' of the *APC* locus induce attenuate forms of FAP. The HNPCCs are caused by germline mutations in the members of the DNA mismatch repair (MMR) complex, especially MLH1 and MSH2, and are characterized by microsatellite instability, since the cancers accumulate mutations in the microsatellite alleles. As a consequence, in HNPCC tumors mutations in the β-catenin gene *CTNNB1*, *APC* and *TGFBR2* genes are usually observed [83,99].

Inactivating mutations in *APC* have been observed not only in the familiar cancer syndromes, but also in most of the sporadic cancers [102] and, like mutations in other members of the Wnt pathway, they occur early during the tumor development [103,104]. The Fearon and Volgenstein's model of colorectal carcinogenesis, based on the analysis of the mutations present at different stages of the CRC development, proposes that key genetic alterations in oncogene and tumor suppressor genes,

acquired in a preferred sequence, drive epithelial cells to change their normal behaviour and confer characteristics advantageous to the development of sporadic cancers. In the adenoma-carcinoma progression, activating mutations of the Wnt signalling pathway are early key events and are the only ones found in the aberrant crypt foci (ACFs) [104,105]. Mutations in *RAS* or *B-RAF* genes have been observed in intermediate adenomas [106], whereas genetic alterations inactivating TGFBR2 have been observed at later steps during CRC development and confer additional malignant characteristics to the adenomas. Furthermore, mutations inactivating *P53* are coincident with the progression of colon adenomas to carcinomas [107].

As previously described, the APC protein in complex with GSK3β and Axin binds β-catenin, which is targeted for ubiquitination and destruction by the phosphorylating activity of GSK3β. The active Wnt signalling inhibits GSK3β activity and promotes the nuclear translocation of the β-catenin and the transcription of the Wnt target genes. The APC is expressed as a gradient from the bottom of the crypts, where its expression is nearly negative, to the luminal side in the epithelia of intestinal villi and colorectal crypts where it is highly expressed, opposite to the Wnt gradient [108–110]. In cancer, the truncating mutations in *APC* gene or mutations in other members of the APC/GSK3β/Axin complex lead to the persistent activity of the Wnt signalling through the constitutively formation of the β-catenin/TCF-4 complex in the nucleus, which inhibits the differentiation and forces a progenitor-like phenotype on epithelial cells outside of the crypts, giving rise to aberrant crypt foci (ACFs) [93].

The *Min* (multiple intestinal neoplasia) is a mutant allele of murine APC which encodes a non-sense mutation at the codon 850 [111]. The mice heterozygous for min ($Apc^{Min}$ ) develop a high number of adenomas most of all in the small intestine by 4 weeks of

age and have a reduced lifespan of 150 days [112]. The *Apc*$^{Min/+}$ mice are used as model of FAP and sporadic colorectal cancers [111].

## 2.12 The intestinal epithelial barrier function and regulation

The intestinal and colonic epithelial barrier works as a defence barrier to prevent the passage of intraluminal microorganisms and as a filter that allows the selective translocation of dietary nutrients and electrolytes through transcellular or paracellular pathways. Indeed, the epithelial cell membranes represent the main barrier against hydrophilic solutes except for the presence of specific transporters that allow the translocation of electrolytes, sugars, aminoacids and short chain fatty acids (transcellular permeability). The other pathway used by the epithelium to regulate the translocation of solutes is the paracellular space between adjacent epithelial cells. The paracellular permeability is regulated by three junctional complexes localized along the lateral membrane of epithelial cells: the tight junctions (TJs), the adherent junctions (AJs) and the desmosomes [113,114]. Whereas the AJs and desmosomes are thought to be principally involved in the mechanical linkage of epithelial cells, the main role of the TJs is the functional regulation of the epithelial paracellular permeability between adjacent cells. Indeed, the TJs are multi-protein complexes localized at the border between the apical and lateral membrane regions, forming a continuous belt-like ring around epithelial cells [115]. TJs are constituted by different transmembrane proteins, which interact in the paracellular space with identical (homophylic interaction) or different (heterophylic interaction) proteins on adjacent cell membranes. The intracellular domains of transmembrane proteins interact with PDZ-domain-containing adaptor proteins (as ZO-1) that link the TJ complex to the actin cytoskeleton. The

proteins that constitute the TJs are occludins, claudins and junctional adhesion molecules (JAMs). Occludin is the first TJ protein identified [116]. It is an integral membrane protein with two extracellular loops, a short cytoplasmic N-terminus and a long cytoplasmic C-terminus, which interacts with the ZO-1 protein essential to link occludin to the actin cytoskeleton [115]. The function of the occluding is not completely clear yet. Indeed, although many evidences suggest a role of occludin in the TJ formation and in the regulation of paracellular permeability [117], these mechanisms seem not to depend on this protein [118]. The claudins are a family of transmembrane proteins with four hydrophobic transmembrane domains, two extracellular loops and two N- and C-terminal cytoplasmic domains. The intracellular C-terminal domain binds the PDZ-binding domain proteins (including ZO-1, -2 and -3) which anchor claudins to the actin cytoskeleton. The claudins have been demonstrated to regulate the barrier function, however different claudins have different effects on the paracellular permeability [119,120].

# 3 AIMS

Sphingosine-1-phosphate (S1P) is a pleiotropic bioactive sphingolipid metabolite recognized as a critical regulator of many physiological processes including growth, proliferation, survival, apoptosis, migration and differentiation. The considerable progress made in the last twenty years in understanding cellular S1P functions has revealed its involvement not only in physiological, but also in pathological processes including cancer and inflammation. Indeed, several evidences report high tissue levels of S1P in many cancers influencing survival, proliferation, formation of new vascular vessels and metastatic dissemination [7]. Intracellular S1P levels are finely regulated by the equilibrium between its formation and degradation. When one of these mechanisms, involving sphingosine kinases (SphKs) for the synthesis and S1P lyase (SPL) and S1P phosphatases for the degradation, is altered, the S1P is deregulated. In many cancers, the high levels of S1P correlated with the increased activities of SphKs [121]. On the other hand, S1P gradient plays a crucial role in lymphocyte migration and trafficking [122]. S1P levels are normally higher in the blood and lymph than in tissues. Augmented S1P levels occur during inflammatory conditions, favoring the S1P-mediated recruitment of immune cells from the blood and lymphoid organs to the inflamed tissues. For these aspects, enormous interest has been paid on S1P signaling as a new therapeutic target for cancer and immune disorders. The role of S1P in mediating both cancer and inflammatory processes has pointed out this signaling as the link between two these conditions [13,61]. However, the intra- and extracellular mechanisms underlying this interaction are still poor clear. S1P is both an intracellular second messenger and the ligand of five EDG family G protein-coupled receptors 1-5 (S1PR1-5) through which exerts most of its functions [7]. Several evidences support the

hypothesis that, beside the alteration of SphK activity, there is also a deregulation of S1PR expression underlying tumorigenesis processes. Nevertheless, it is still controversial whether the S1PRs act as pro- or anti-tumor receptors. Among all S1P receptors, S1P1-3 are those ubiquitously expressed and mainly studied. Upon activation of a specific receptor, S1P regulates cell growth, survival, migration, adhesion, angiogenesis, vascular tone and permeability. Interestingly, S1PR2 seems to mediate opposite effects than other receptors. Indeed, whereas S1PR1 and S1PR3 control respectively immune cell trafficking, vascular tone and formation, proliferation and migration, S1PR2 acts inhibiting migration and proliferation. It is likely that the pro- or anti-tumor effects of the S1P/S1PRs activation depends strictly on the receptor involved and the tissue/cellular context [21,22]. This theory could explain the controversial functions of a same S1PR in different tumors [123]. An increasing body of evidence suggests that S1P signalling plays a crucial role in the intestinal tumorigenesis. High levels of the S1P regulatory enzyme, SphK1, were found in colon cancer patients. Studies *in vivo* demonstrated that the deletion of SphK1 resulted in a decrease of the intestinal polyp size in the *Apc*$^{Min/+}$ genetic model [56] and in lower incidence and multiplicity of ACF and colon cancers in the azoxymethane (AOM) murine model of colonic tumorigenesis [57]. However, the function of the S1PRs in this process is still unknown, as well as it is still poor clear whether the dysregulation of SphK1 in colon cancer activates a specific receptor. Recently, the SphK1/S1P/S1PR1 axis has come to light as the link between chronic inflammation and colitis-associated cancer. Indeed, the SphK1/S1P/S1PR1 axis has been demonstrated to regulate the production of IL-6 and to induce the persistent activation of STAT3 in epithelial cells which consequently leads to malignant transformation in these cells [13,61]. Although

these findings involve the S1PR1 in the inflammation-induced colorectal tumorigenesis, so far there are no evidences of S1P receptor distribution on intestinal epithelial cells in healthy and colon cancer tissue. Data obtained from my laboratory revealed an enhancement of epithelial barrier function in presence of S1P [81], suggesting the involvement of S1P receptors on epithelial cells. However, these effects were not inhibited by FTY720, a specific inhibitor with high affinity to S1PR1 and low affinity to S1PR3, thus excluding the role of S1PR1 in the regulation of intestinal barrier and pointing a potential role for other S1P receptors [81]. Nevertheless, the role of S1P signaling and S1P receptors on intestinal epithelial cells remain to be clarified. Given the emerging role of S1P signaling in the intestinal carcinogenesis and the scarcity of knowledge on the role of S1P/S1PRs pathways in the biology and pathophysiology of the intestinal epithelium from which colon cancer derived, the main proposals of my thesis have been:

**Aim.1 To explore the physiological functions of the S1PRs on intestinal epithelial cells**

**Task 1.1 Characterization of the S1P pathway in the intestinal epithelial cells;**

**Task 1.2 Investigation of the physiological functions of the S1PRs on intestinal epithelial cells**

**Aim.2 To explore the role of S1PR2 in intestinal tumorigenesis**

**Task2.1 Analysis of S1PR2 expression in tumour biopsies from human patients**

**Task2.2 Functional characterization of S1PR2 in experimental models of CRC**

# 4 RESULTS

## AIM 1 TO EXPLORE THE PHYSIOLOGICAL FUNCTIONS OF THE S1PRS ON INTESTINAL EPITHELIAL CELLS

## 4.1 CHARACTERIZATION OF S1PRS IN EPITHELIAL CELLS

### 4.1.1 CHARACTERIZATION OF S1PRS IN HUMAN EPITHELIAL CELLS

In order to characterize the S1P/S1PRs pathway in the colonic epithelium, I enrolled 8 patients which underwent colon surgery for colorectal cancer. The surgical samples collected from the healthy portion of the colonic tissues were processed to obtain mucosal biopsies and primary epithelial cells. Since the epithelial cells quickly undergo apoptosis when the contact between neighboring cells is lost, affecting their analysis, the approach I used for the isolation of primary epithelial cells was rapid and consisted in the collection of entire crypts from the mucosa instead of single cells, as reported in Figure 6A.

Since S1PR1, S1PR2 and S1PR3 are ubiquitously expressed, whereas the expression of S1PR4 and S1PR5 is less widespread and mainly confined to the lymphoid and hematopoietic tissue and central nervous system, I focused the analysis on the first three receptors. I analysed the mRNA expression of S1PR1-3 by qRT-PCR both in the mucosa and in primary epithelial cells isolated from colonic biopsies. I observed that S1PR1 transcript levels were higher compared to S1PR2 and S1PR3 in the mucosa, whereas S1PR2 and S1PR3 mRNA were comparable to each other (Figure 6B). However, the analysis of the S1PR mRNA expression in primary human epithelial cells isolated from colonic biopsies revealed a higher level of S1PR2 compared to the S1PR1 and S1PR3 transcripts (Figure 6B). These observations indicated an enrichment of

S1PR2 rather than S1PR1 and S1PR3 in the epithelial compartment. On the contrary, the higher expression of S1PR1 and S1PR3 in the whole mucosal tissue than in the epithelial cells suggested an enrichment of these receptors in non-epithelial compartments (Figure 6B). The immunohistochemistry analysis of mucosal S1PR2 protein, confirmed that the receptor is highly present on epithelial cells indicating the epithelium as the main compartment expressing S1PR2 (Figure 6C).

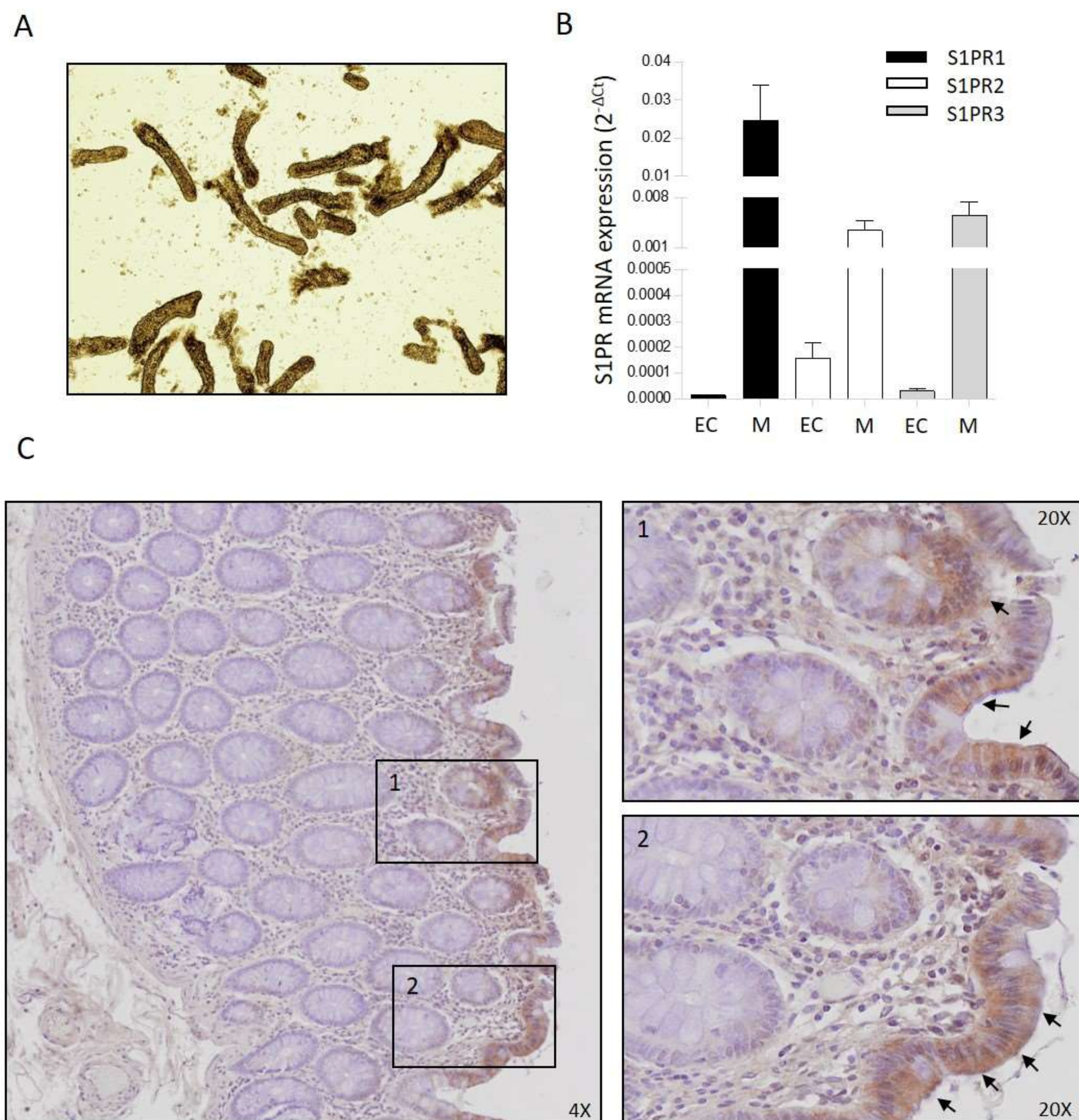



***Figure 6 Characterization of S1PR expression in human colonic epithelial cells and mucosa.*** *(A) Image of human crypts isolated from colonic biopsies. (B) S1PR1-3 mRNA expression in human epithelial cells (EC) (n=4) and mucosal biopsies (M) (n=4) from intestinal specimens of healthy controls by qRT-PCR. (C) Expression of S1PR2 (black arrows) in human colonic tissues by immunohistochemistry. Original magnification is 4X for the panel on the left and 20X for panels 1 and 2 on the right.*

### 4.1.2 CHARACTERIZATION OF S1P RECEPTORS IN MURINE EPITHELIAL CELLS

Since mouse experimental models have been widely used for studying biological and pathological processes triggered by S1P signalling, I decided to characterize the expression of S1PR1-3 also in the murine colonic epithelium. To this end, I isolated primary murine colonic epithelial cells. Then I compared the expression of the three receptors in the epithelium and in the whole tissue. The expression analysis of the transcriptional levels of S1PR1-3 confirmed that also in mouse S1PR2 was the most abundantly S1P receptor expressed in the epithelial cells (Figure 7A). The western blot analysis of S1PR1-3 proteins confirmed a greater abundance of S1PR2 in epithelial cells compared to the relative expression of S1PR1 and S1PR3 in the epithelial compartment. While the levels of S1PR1 in the epithelium appeared very low, the S1PR3 protein was highly present, but its relative expression was lower than S1PR2 (Figure 7B-C). To better localize the S1PR2 expression in the mucosa, I performed an immunohistochemistry staining on colon sections. Interestingly, the staining showed a marked positivity for S1PR2 in the plasmatic membrane and cytoplasm of the epithelial cells localized at the top of the crypts (Figure 7D). The absence of commercially available antibodies for the detection of S1PR1 and S1PR3 by immunohistochemistry, impeded the localization in the mucosa of these receptors. However, altogether these data demonstrated that the S1P/S1PR axis is present on intestinal epithelial cells and that, among the three major S1P receptors, S1PR2 is the most abundant in the healthy epithelium.

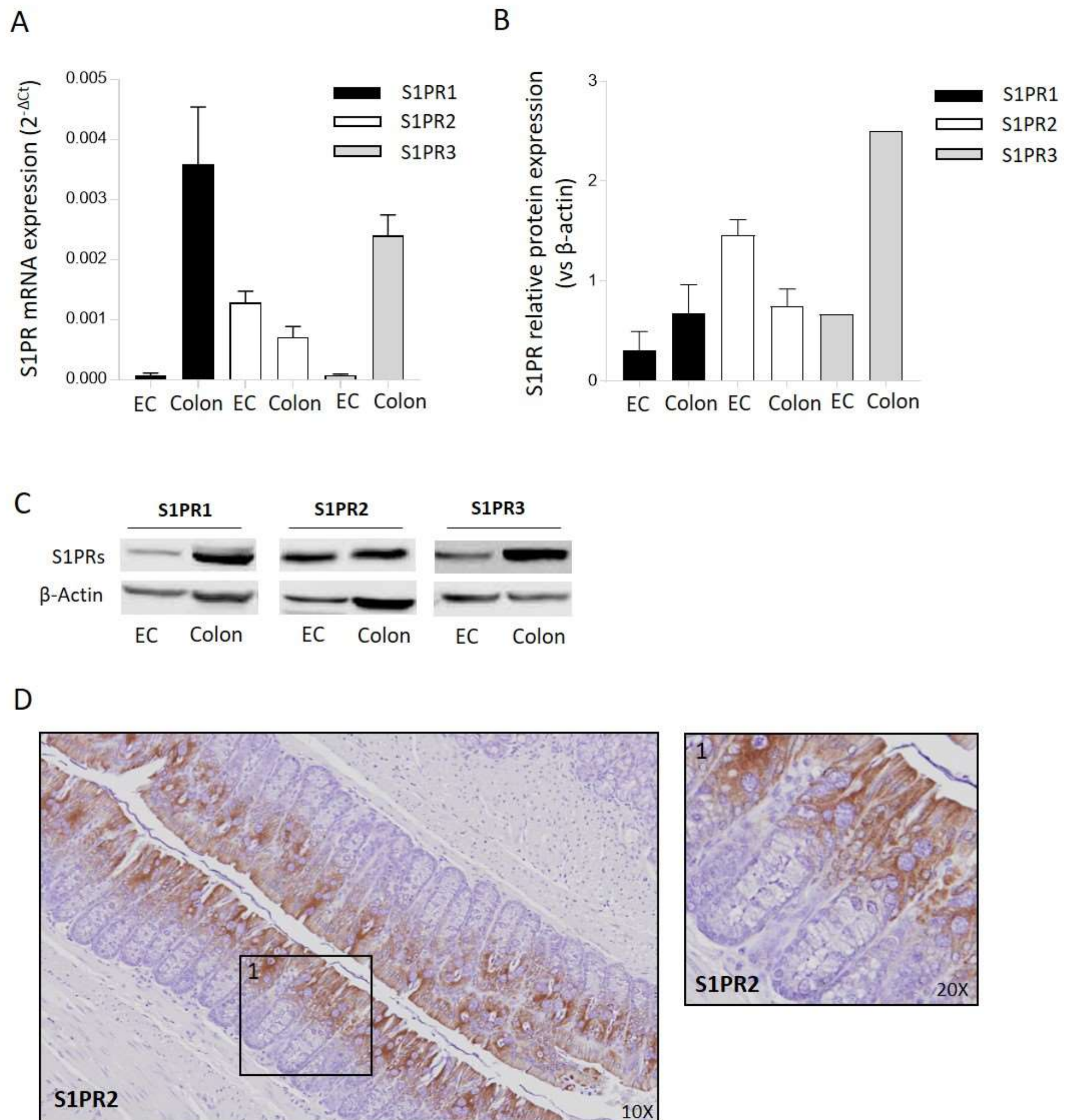



***Figure 7 Characterization of S1PR expression in the murine colonic tissues.*** *(A) S1PR mRNA expression in murine epithelial cells (n=6) and colon (n=4) of wild type mice. (B) Densitometric analysis of S1PR protein expression in colonic epithelial cells and colon relative to β-Actin by Western blot. (C) Representative image of the Western blot. (D) Expression of S1PR2 in normal murine colonic tissue by Immunohistochemistry. Original magnification is 10X for the panel on the left and 20X for panel 1 on the right.*

## 4.2 ANALYSIS OF THE PHYSIOLOGICAL FUNCTIONS OF THE S1PRS ON INTESTINAL EPITHELIAL CELLS

### 4.2.1 ANALYSIS OF THE ROLE OF S1PR2 IN COLONIC EPITHELIAL PERMEABILITY

Given the abundant constitutive expression and unknown functions of S1PR2 in intestinal epithelial cells, I decided to focus my studies on its role in the epithelium. Intestinal epithelial cells constitute a physical barrier against bacterial and dietary antigens and regulate the passive movement of solutes through spaces between adjacent epithelial cells (paracellular permeability). It was shown that the activation of S1PR2 on endothelial cells caused a disruption of endothelial adherens junctions and increased paracellular permeability [77]. I hypothesized therefore that S1PR2 could also be involved in the regulation of the epithelial barrier permeability. To assess this hypothesis, I took advantage of total knockout mice for S1PR2 (s*1pr2*$^{-/-}$) and I determined the colonic permeability *in vivo* on naïve *S1PR2* knock out mice and wild type littermates (s*1pr2*$^{+/+}$) by the Evans Blue assay. This method is based on the *in vivo* perfusion of the colon with the Evans Blue dye, which physiologically does not translocate through the epithelial barrier. In presence of mucosal damage which leads to alteration of epithelial barrier function, the Evans Blue dye penetrates through the mucosa and the amount of the dye translocated into the intestinal wall correlates with the grade of barrier dysfunction. The Evans Blue assay performed on healthy *s1pr2*$^{-/-}$ and *s1pr2*$^{+/+}$ mice did not reveal any macroscopic difference in colonic permeability (Figure 8A). The intestinal barrier function depends on the expression and distribution of an apical junctional complex, which is composed of both tight and subjacent adherens junctions. Therefore, to confirm that the loss of S1PR2 does not affect the intestinal barrier permeability, I analysed the mRNA expression of genes coding for three members of TJs proteins involved in the regulation of the epithelial barrier

permeability, i.e. occludin (*ocln)*, claudin-2 (*cldn-2)* and Zonula occludens-1 (*tjp-1*), in colonic epithelial cells isolated from *s1pr2*$^{-/-}$ and *s1pr2*$^{+/+}$ mice. The analysis of *tjp-1*, *claudin 2* and *occludin* transcripts in the epithelial cells did not reveal any significant difference between the two groups indicating that the complete deletion of S1PR2 does not affect the normal intestinal barrier function (Figure 8B). The intestinal barrier is constituted by heterogeneous cells including differentiated epithelial cells known as goblet cells that participate in the regulation of intestinal epithelial barrier by the production of mucins. In order to explore the role of S1PR2 in secreted barrier function, I analysed the distribution of mucins in naive wildtype and S1PR2 knock out mice performing a combined Alcian blue-PAS staining, which allows to differentiate neutral and acidic mucins. As reported in Figure 8C, no differences were observed in the distribution of the different mucins in the colon of wild type and S1PR2 knockout mice, indicating that S1PR2 in intestinal epithelium does participate in epithelial barrier regulation.

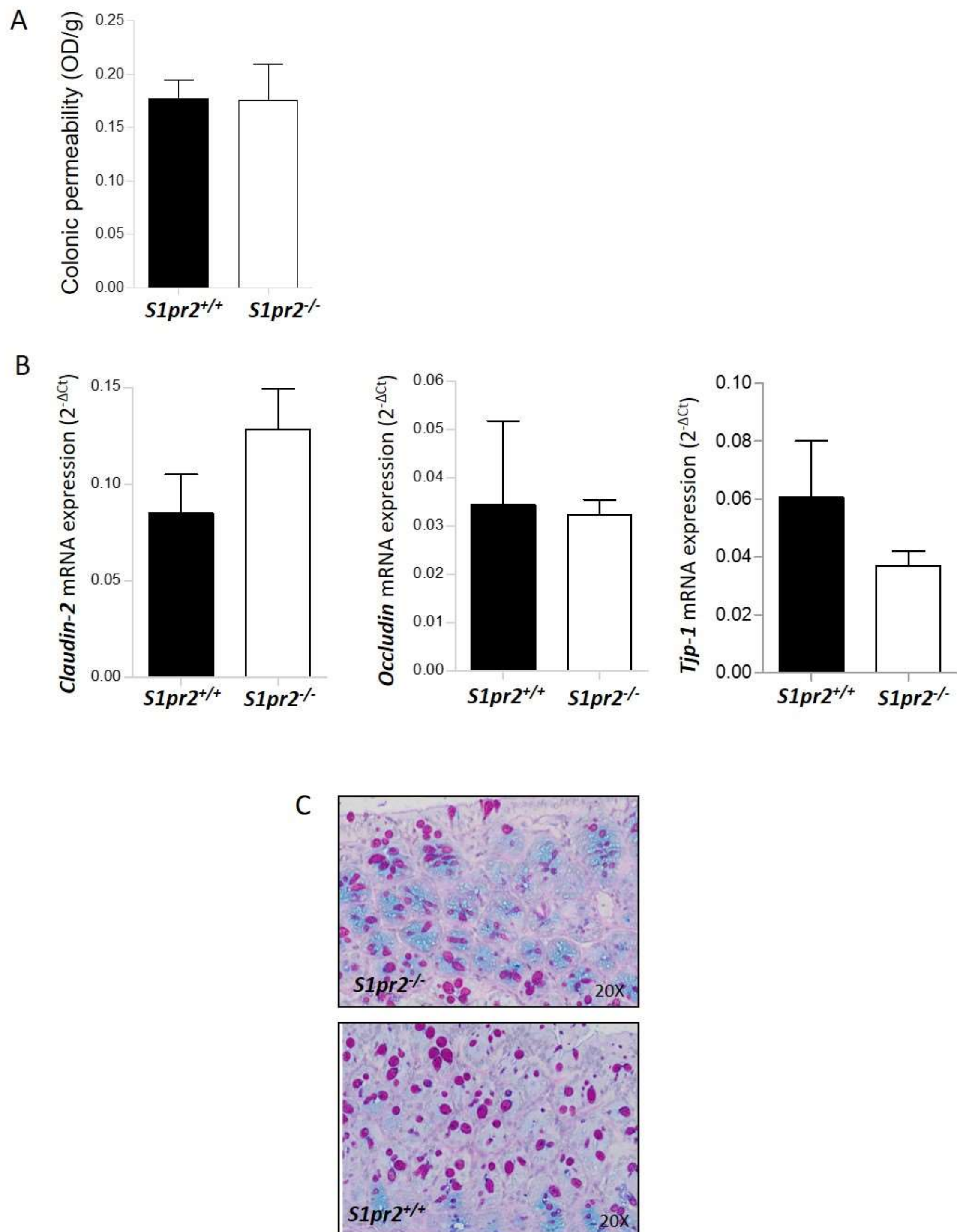



***Figure 8 Analysis of the colonic epithelial barrier permeability in naïve s1pr2$^{-/-}$ mice.*** *(A) Quantification of the basal colonic permeability in s1pr2$^{-/-}$ (n=3) and s1pr2$^{+/+}$ (n=3) mice by the Evans blue assay. (B) qRT-PCR analysis of the TJs mRNA basal expression in colonic epithelial cells isolated from s1pr2$^{-/-}$ (n=5) and s1pr2$^{+/+}$ (n=3) mice. (C) Representative image of the mucin distribution in s1pr2$^{-/-}$ and s1pr2$^{+/+}$ by Alcian blue-PAS staining.*

I further investigated whether the loss of S1PR2 would accelerate the dysregulation of the epithelial barrier permeability occurring under acute inflammation. To this purpose, I assessed the permeation of the Evans Blue dye in *s1pr2*$^{-/-}$ and *s1pr2*$^{+/+}$ littermates after DSS-induced colitis. The status of the disease was assessed daily monitoring body weight loss, bleeding and faecal consistency. After 10 days of 3% DSS administration mice displayed a strong colitis with a compromised epithelial barrier function. I did not observe any difference neither in clinical parameters associated with the colitis (Body weight, DAI, colon length) between *s1pr2*$^{-/-}$ and *s1pr2*$^{+/+}$ mice, nor in the inflammation score (Rachmilewitz score) (Figure 9A), thus indicating no differences in colitis susceptibility between two groups. Moreover, the analysis of the Evans Blue dye permeation did not reveal any significant difference between the two groups further supporting the conclusion that the S1PR2 does not regulate the intestinal epithelial barrier permeability (Figure 9B).

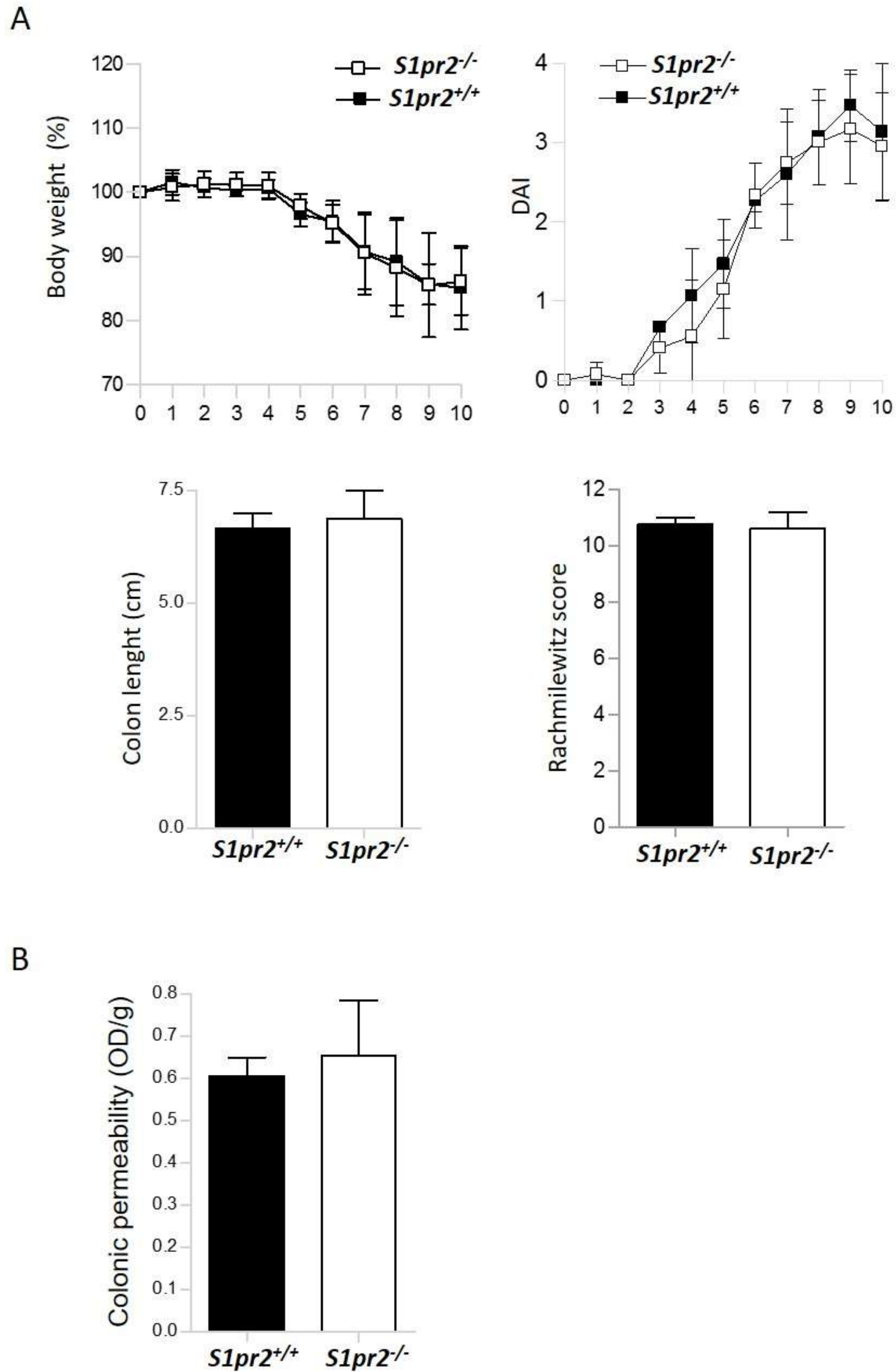
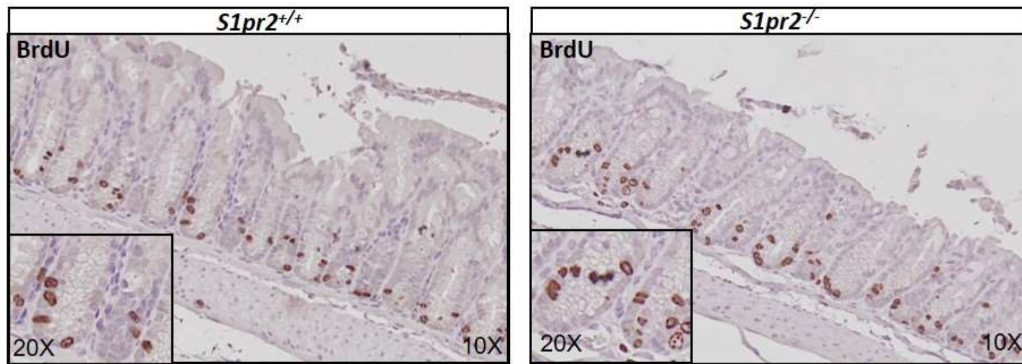



***Figure 9 Analysis of the colonic permeability in s1pr2$^{-/-}$ mice with DSS-induced acute colitis.*** *(A) Clinical parameters associated with acute colitis (percentage of body weight, disease activity index and colon length) and colonic inflammation score (Rachmilewitz score) in s1pr2$^{-/-}$ (n=9) and s1pr2$^{+/+}$ (n=5) mice 10 days after acute colitis induction with 3% DSS provided ad libitum. (B) Colonic epithelial permeability by Evans blue assay in s1pr2$^{-/-}$ (n=3) and s1pr2$^{+/+}$ (n=3) mice 10 days after acute colitis induction.*

### 4.2.2 S1PR2 DEFICIENCY INCREASES THE PROLIFERATION OF COLONIC EPITHELIAL CELLS *IN VIVO*

Since several studies reported that S1PR2 controls the proliferation in different cell types, such as murine embryonic fibroblasts and rat hepatocytes *in vitro* [124,125], I analysed whether S1PR2 could regulate the proliferation also of intestinal epithelial cells. To this end, I evaluated intestinal epithelial cell proliferation *in vivo* in $s1pr2^{-/-}$ mice. The colonic tissues of untreated 8 weeks old female $s1pr2^{-/-}$ *and* $s1pr2^{+/+}$ mice have been collected and formalin-fixed two hours after the intraperitoneally injection of the BrdU (5mg/kg). The BrdU has been detected by immunohistochemistry and the analysis of the proliferation has been performed by counting the average number of BrdU positive cells per 20 crypts in three different areas. Since the presence of regional differences in the proliferation rate between different colonic regions has been reported [126–128], we analysed separately proximal, middle and distal colon. Although the analysis of the whole colon did not show any difference between $s1pr2^{-/-}$ and $s1pr2^{+/+}$ mice, a significant higher proliferation was observed in middle colonic epithelial cells of the $s1pr2^{-/-}$ mice compared with $s1pr2^{+/+}$ mice ($p=0.034$) (Figure 10A-B).

A




B

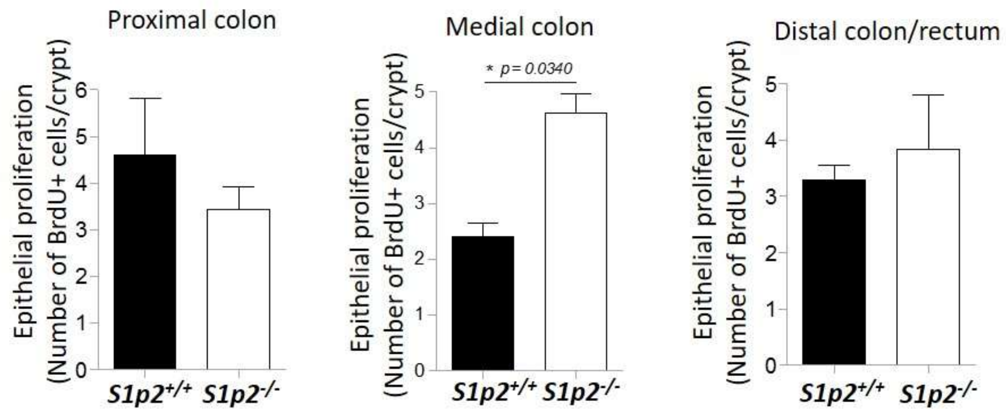



***Figure 10 Analysis of the epithelial proliferation in vivo in s1pr2$^{-/-}$ mice.*** *(A) Representative image of the BrdU positive cells in the epithelium of the medial colon of s1pr2$^{-/-}$ and s1pr2$^{+/+}$ mice. (B) Analysis of the BrdU incorporation in the proximal, medial, and distal colonic/rectal epithelium of s1pr2$^{-/-}$ (n=3) and s1pr2$^{+/+}$ (n=3) mice.*

### 4.2.3 *IN VITRO* INHIBITION OF S1PR2 INDUCES EPITHELIAL CELL PROLIFERATION

Due to the well-known difficulty of propagating long-term primary intestinal epithelial cell cultures, to further investigate the role of the S1PR2 in epithelial cell proliferation *in vitro*, I used the Caco-2 cell line derived by human epithelial colorectal adenocarcinoma. Before assessing the proliferation, I analysed the expression of S1PRs in Caco-2 cell line by RT-PCR, which confirmed the exclusive expression of S1PR2 using primary colonic fibroblasts as positive control for the S1PR expression (Figure 11A). Thus, I cultured Caco-2 cells in presence of different concentrations of the specific S1PR2 inhibitor (JTE013) and/or its ligand S1P and I evaluated cell proliferation after 24 hours by the BrdU incorporation assay. We observed that the treatment with JTE013 in a range of 0.4-0.08 µM significantly increased the normal proliferative activity of epithelial cell after 24 hours (Figure 11B). The choice of the range of the inhibitor concentrations was based on the pharmacological characteristics of the inhibitor (IC50 value=0.017 µM). Although I did not observe changes in the BrdU incorporation, when Caco-2 cells were stimulated with different concentrations of the S1P ($10^{-7}$M-$10^{-5}$M) alone, the treatment with the highest concentration of ligand ($10^{-5}$M) reversed the JTE013-induced increase of the proliferation to basal conditions (Figure 11C). Overall *in vitro* and *in vivo* results demonstrated that the inhibition of the S1PR2 activity increases colonic epithelial cell proliferation and suggested that the S1P/S1PR2 axis is responsible for keeping under control the proliferation in colonic epithelial cells.

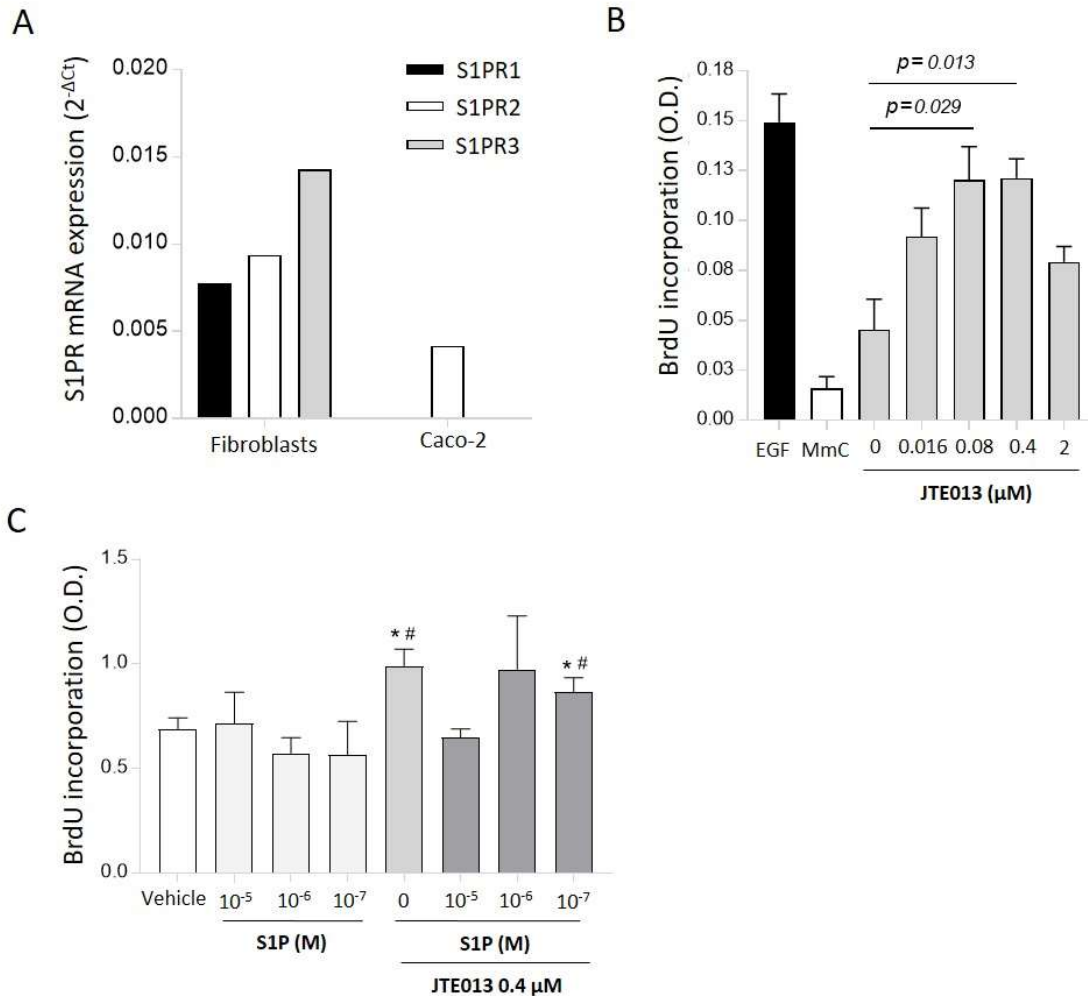



***Figure 11 The S1PR2 inhibition increases the proliferation of Caco-2 epithelial cells in vitro.*** *(A) Analysis of S1PR mRNA expression in Caco-2 cells by qRT-PCR. (B) Brdu incorporation assay in Caco-2 cells after 24-hour treatment with different concentrations of the S1PR2 selective antagonist JTE013 (0.016µM-2µM) and (C) with different concentration of S1P ($10^{-7}$M-$10^{-5}$M) in presence or absence of JTE013 0.4 µM. EGF=epithelial growth factor; Mmc = mitomycin C. Significance versus the vehicle is represented by * (p=0.015 for the JTE013 0.4 µM and p=0.023 for JTE013 0.4 µM + S1P$10^{-7}$M treatments). Significance versus JTE013 0.4 µM + S1P$10^{-5}$M treatment is represented by # (p=0.007 for JTE013 0.4 µM and p=0.009 for JTE013 0.4 µM + S1P$10^{-7}$M treatments).*

## AIM 2 TO EXPLORE THE ROLE OF S1P RECEPTOR 2 IN INTESTINAL TUMORIGENESIS

Considering that, on the one hand the deregulated cell proliferation is a key event that propels the tumour cell expansion and on the other hand the evidences of S1PR2 involvement in the development and progression of different type of cancers [62–64,68,70],

I aimed at studying whether S1PR2 might play a role in the development and progression of the colorectal cancer.

## 4.3 ANALYSIS OF S1PR2 EXPRESSION IN TUMOUR BIOPSIES FROM HUMAN PATIENTS

To address this hypothesis, I firstly characterized the expression of S1PR2 in human colon cancer (CRC) samples. For this purpose, I analyzed adenomas and adenocarcinomas samples collected from patients with CRC from the Humanitas biobank. To explore the role of S1PR2 in the different stages starting from very early stages, we selected T1N0M0 and T2N0M0 adenocarcinomas (based on the Tumor/Node/Metastasis (TNM) classification for colon cancer), whose extension is restricted to mucosa, submucosa and muscolaris propria (T1 and T2) and did not spread nor in lymph nodes (N0), nor in distant organs (M0). Additionally, in order to exclude any interference due to the chemotherapy, I included only specimen from patients without any treatments. As healthy controls, I analyzed colonic biopsies collected from areas surgically resected 10 cm distant from the tumor and therefore not affected by the tumor itself. The S1PR2 expression was analyzed by western blot on protein lysates. As shown in figure 12, the relative expression of S1PR2 protein was significantly lower in both adenocarcinomas T1N0M0 and T2N0M0 compared to normal colonic mucosa ($p=0.02$ and $p=0.04$ respectively). The same trend was also observed analyzing the protein lysates extracted from adenomas.

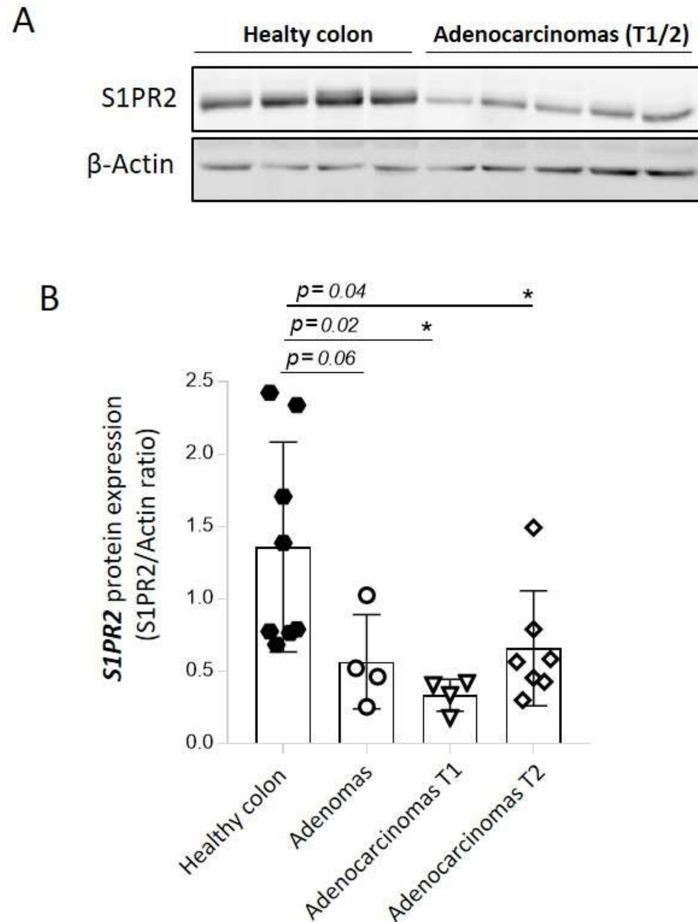



***Figure 12 S1PR2 protein expression in human CRC biopsies and healthy colonic tissues.*** *Characterization of S1PR2 protein expression in human adenomas (n=4), adenocarcinomas T1 (n=4), adenocarcinomas T2 (n=7) and healthy colonic mucosa (n=8) from colonic specimens of patients with CRC by western blot. (A) Representative image of S1PR2 protein expression in four sample of healthy colonic mucosa and five samples of adenocarcinomas. In (B) the quantification of the relative S1PR2 expression by densitometric analysis and normalization on the β-actin expression.*

## 4.4 THE FUNCTIONAL ROLE OF S1PR2 IN EXPERIMENTAL MODELS OF CRC

In order to gain insight into the potential role of S1PR2 in the intestinal tumorigenesis, I took advantage of S1PR2 knockout mice. Indeed, I assessed the development of tumors in *s1pr2*$^{-/-}$ compared to wild type mice employing a model of inflammatory-driven colorectal cancer. Tumorigenesis was induced by a single intraperitoneal injection of the carcinogen Azoxymethane (AOM) (10 mg/kg) followed by four cycle of four days DSS (2.5% *ad libitum*) spaced out by ten days of water. Body weight, faecal

bleeding and consistency were monitored at least twice a week during the experiment. At the day of the sacrifice, the tumours were analysed firstly by colon endoscopy and then the colon was collected for the histological evaluation of the inflammatory score and the number of dysplasia of each type. I did not observe any significant difference neither in the clinical parameters, such as body weight loss, disease activity index (DAI) and colon length, nor in the inflammation score between *s1pr2*$^{-/-}$and *s1pr2*$^{+/+}$ mice (Figure 13), thus confirming previously shown data observed in acute induced colitis and demonstrating that the loss of S1PR2 does not worsen intestinal inflammation.

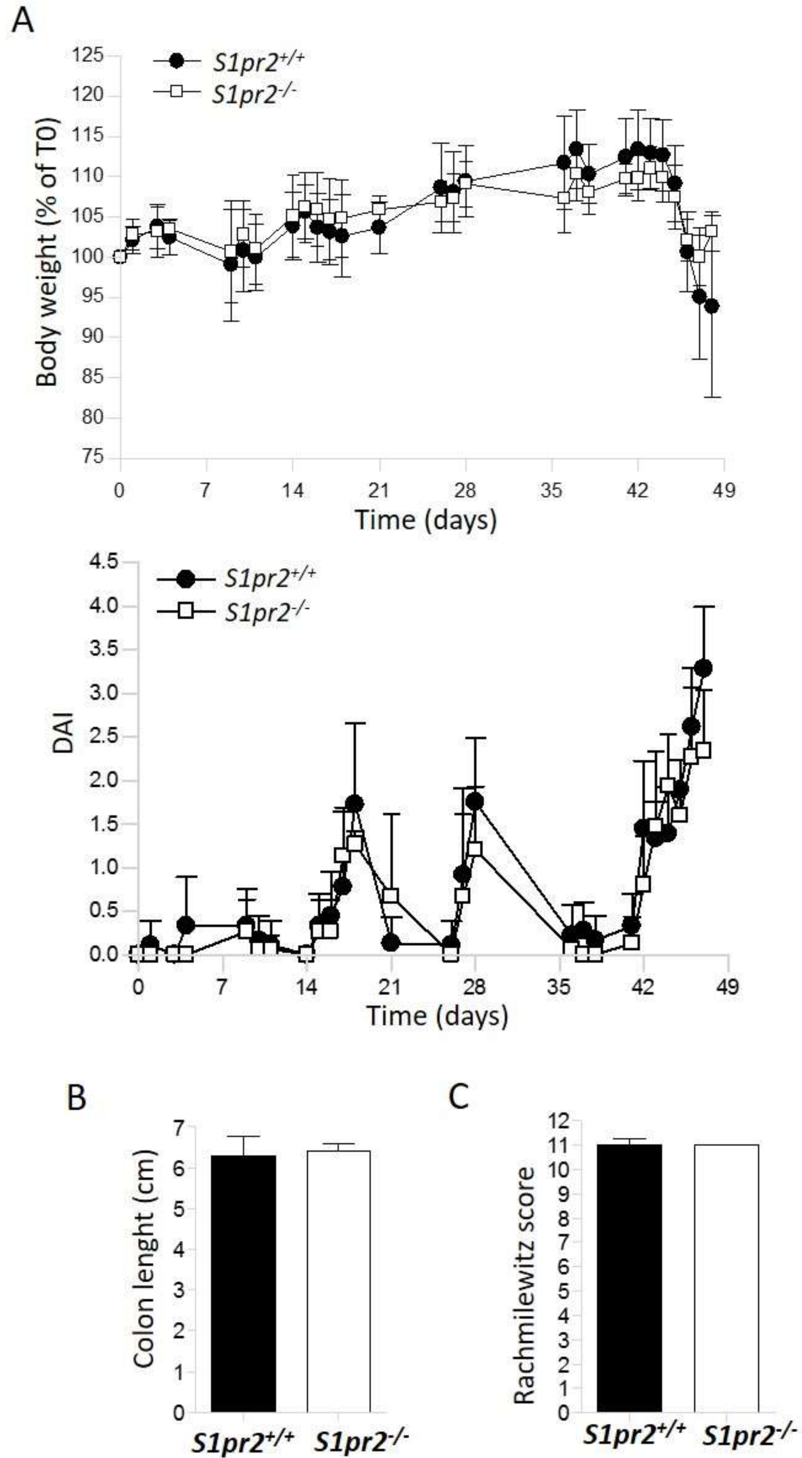



***Figure 13 Clinical parameters and inflammation score in s1pr2$^{-/-}$ and s1pr2$^{+/+}$ mice with inflammation-driven CRC.*** *(A-B) Clinical parameters analysed in s1pr2$^{-/-}$ (n=5) and s1pr2$^{+/+}$ (n=6) mice: percentage of the body weight, DAI and colon length at the day of the sacrifice. (C) Total score of the colonic inflammation by histological analysis (Rachmilewitz score).*

However, the endoscopic analysis of the colorectal region revealed a significant higher count of polyps in *s1pr2*$^{-/-}$ compared to wildtype mice (p=0.027) (Figure 14A). This result was further confirmed by the histological analysis. Indeed, a higher number of colonic adenomas characterized by a high grade of dysplasia (HGA) (p=0.024) (Figure 14B right panel) and larger carcinomas (p=0.0006) (Figure 14B left panel) were found in *s1pr2*$^{-/-}$ mice compared to *s1pr2*$^{+/+}$ mice. Overall these results suggested that S1PR2 exerts an anti-tumor function and that the increased susceptibility to develop colorectal cancer observed in S1PR2 knockout mice was not correlated to the grade of inflammatory response.

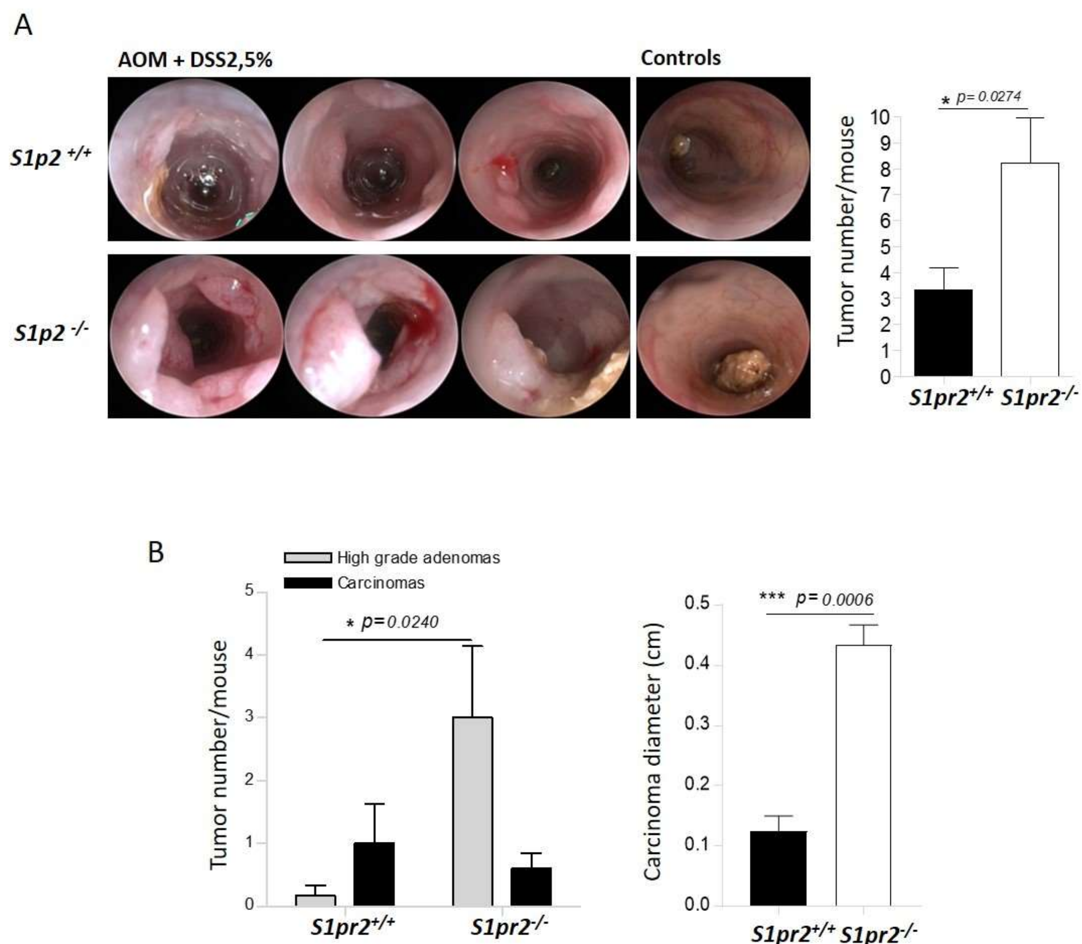



***Figure 14 S1pr2*$^{-/-}$ *mice develop a higher number of dysplasia in AOM/DSS-induced CRC model.*** *(A) Representative images of colorectal endoscopies and endoscopic analysis of the tumor number in s1pr2*$^{-/-}$ *(n=5) and s1pr2*$^{+/+}$ *(n=6) mice. (B) Blinded histological analysis of number and grade of the tumors in*

*$s1pr2^{-/-}$ (n=5) and $s1pr2^{+/+}$ (n=6) mice. The graphs represent the number of colonic tumor per mouse and the size of the carcinomas.*

To further confirm these results, I explored the role of S1PR2 in a genetic model of colorectal cancer, *$Apc^{min}$* mice, which reproduce the spontaneous and inherited mutations occurring early in the colorectal cancer development in human. To this purpose, I crossed *$s1pr2^{-/-}$* mice with *$Apc^{min/+}$* mice and I analysed the number of high and low-grade adenomas (HGA and LGA) and carcinomas (K) in *$Apc^{min/+}$* and *$s1pr2^{+/+}/Apc^{min/+}$* littermates. Colon and small intestine were collected from 21-week-old *$s1pr2^{-/-}$*, when the polyps are established and a single-blinded histological analysis was performed. In accordance with this model, only three out of five *$s1pr2^{+/+}/Apc^{min/+}$* mice showed one or two colonic polyps, whereas all mice developed high number of polyps in the small intestine. By contrast, all *$s1pr2^{-/-}/Apc^{min/+}$* mice developed colonic lesions and the number of polyps per mouse in the *$s1pr2^{-/-}/Apc^{min/+}$* group was significantly higher (2.750 ± 0.9574 average) compared to the *$s1pr2^{+/+}/Apc^{min/+}$* mice (0.8000 ± 0.8367 average) (Figure 15A). Interestingly, the analysis about the number of polyps per mouse at different grade of dysplasia showed that *$s1pr2^{+/+}/Apc^{min/+}$* developed few colonic LGAs (0,4000 ± 0,8944 average), HGAs (0,4000 ± 0,5477 average) and K (0,2000 ± 0,4472 average), whereas *$s1pr2^{-/-}/Apc^{min/+}$* mice did not show any colonic LGAs, few HGAs (0,2500 ± 0,5000 average) and a higher number of carcinomas (2,500 ± 1,291 average) (Figure 15B). By analysis of the tumor size, no difference between colonic HGAs in *$s1pr2^{-/-}/Apc^{min/+}$* mice compared to the *$Apc^{min/+}$* has emerged, whereas the colonic carcinomas of *$s1pr2^{-/-}/Apc^{min/+}$* mice were larger (0.4600 ± 0.1578 cm) than the only one carcinoma observed in *$Apc^{min/+}$* (0.3 cm) (Figure 15C).

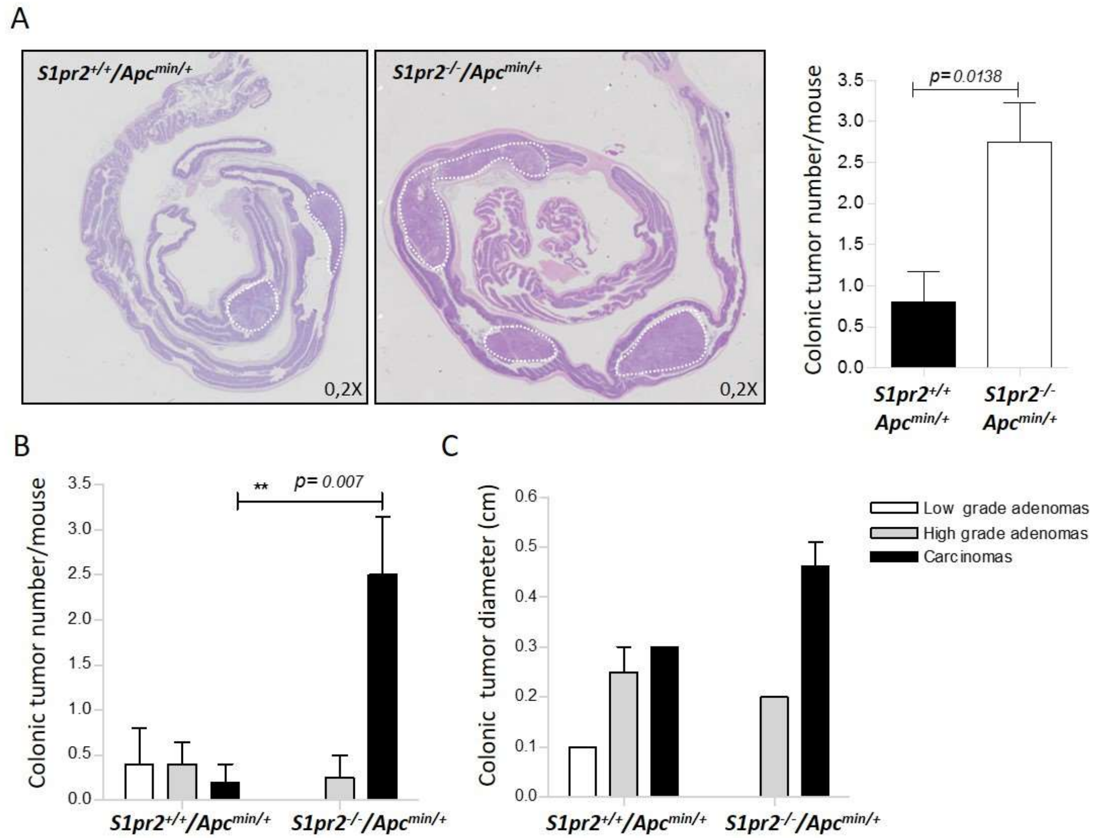



***Figure 15 Characterization of colonic tumors in s1pr2$^{-/-}$/Apc$^{min/+}$ and s1pr2$^{+/+}$/Apc$^{min/+}$ mice.*** *A representative image of colonic tissue of 21-week-old S1PR2$^{-/-}$/Apc$^{min/+}$ and S1PR2$^{+/+}$/Apc$^{min/+}$ mice (HE staining) (A left panel). (A right panel, B and C) The graphs represented the histological analysis of number, grade and size of the tumors in colonic tissues of 21-week-old s1pr2$^{-/-}$/Apc$^{min/+}$ (n=4) and s1pr2$^{+/+}$/Apc$^{min/+}$ (n=5) mice.*

In contrast, no significant difference was observed in the number and size of small intestinal polyps between *s1pr2$^{-/-}$/Apc$^{min/+}$* and *s1pr2$^{+/+}$/Apc$^{min/+}$* mice (Figure 16A-B).

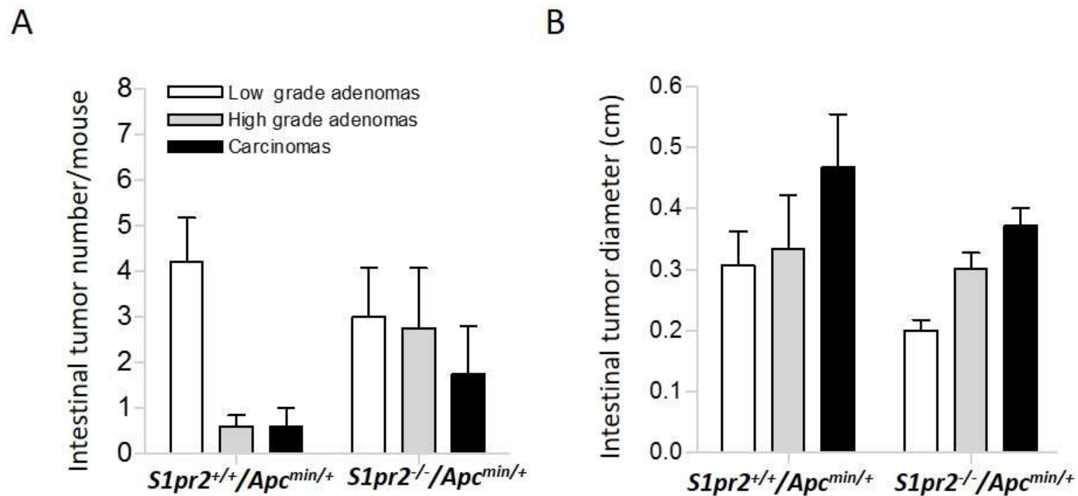



***Figure 16 Analysis of polyps in the small intestine of s1pr2$^{-/-}$/Apc$^{min/+}$ and s1pr2$^{+/+}$/Apc$^{min/+}$ mice.*** *The graphs represent the hystological analysis of tumor number per mouse (A) and size (B) in the small intestine of 21 week-old s1pr2$^{-/-}$/Apc$^{min/+}$ (n=4) and s1pr2$^{+/+}$/Apc$^{min/+}$ (n=5) mice.*

Overall these data demonstrated that *s1pr2$^{-/-}$/Apc$^{min/+}$* mice are more susceptible to develop colonic tumors, corroborating the crucial anti-tumorigenic role of S1PR2 in the intestinal tumor development.

Furthermore, in line with human results, the analysis of S1PR2 in different stage of tumors showed a strong decrease of its expression not only in early-stage adenocarcinomas (Figure 17B), but also in low grade adenomas compared to normal epithelium (Figure 17A). These observations raised further the suspect that S1PR2 down-regulation could be an early event during cancer development.

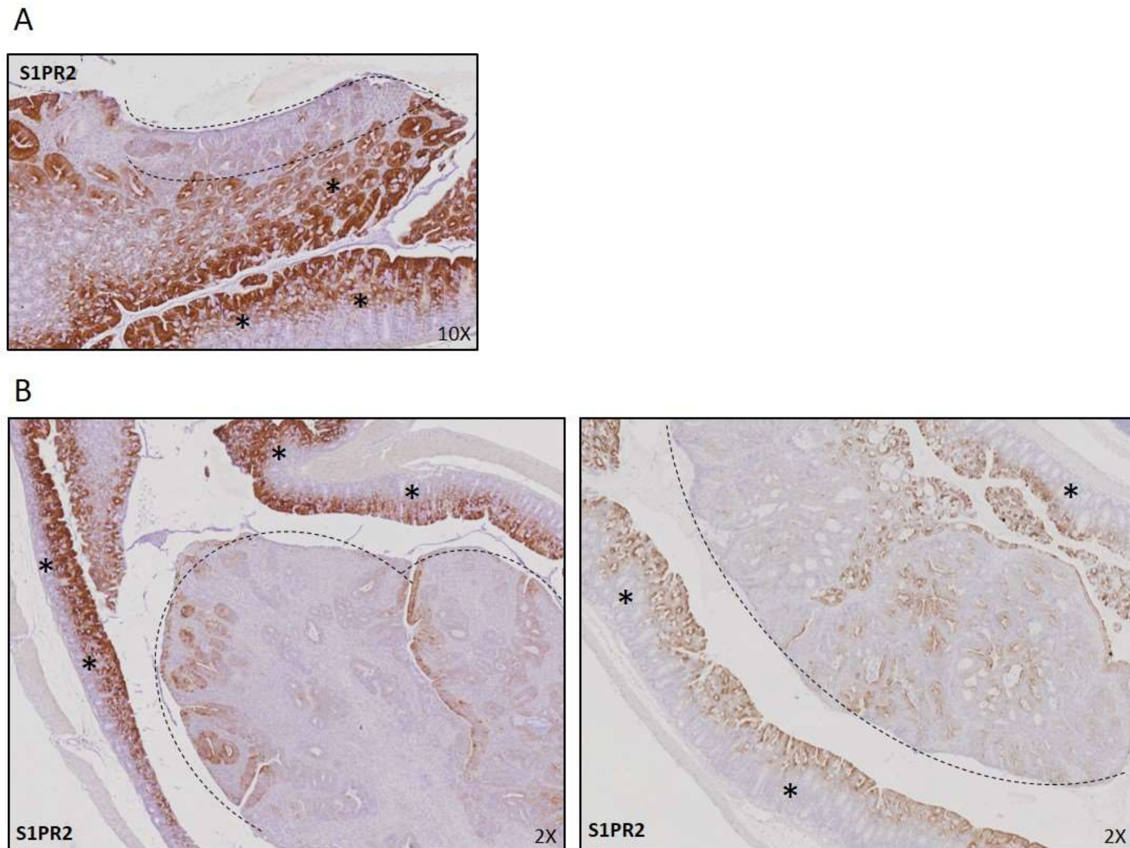



***Figure 17 Expression of S1PR2 in murine colonic adenomas and carcinomas.*** *(A) Representative image of S1PR2 expression in LGA (dashed lines) within colonic tissues of 21 week-old Apc $^{min/+}$ performed by immunohistochemistry. (B) Representative images of S1PR2 expression in early-stage adenocarcinomas (dashed lines) within colonic tissues of 21 week-old Apc $^{min/+}$ mice (left panel) and wild type mice under AOM/DSS treatment (right panel). The asterisks highlight the adjacent areas of normal epithelium.*

## 4.5 Potential mechanisms of S1PR2-mediated colorectal cancer development

### *Role of the S1PR2 in the intestinal renewal and epithelial differentiation in organoids*

The epithelial surface is continually renewed by intestinal epithelial stem cells (IESCs) that reside at the crypt base. However, the exact mechanisms that regulate IESC proliferation, differentiation and functional potential are not completely known. Since the pharmacological inhibition of S1PR2 increases the proliferation of epithelial cells *in vitro* and the expression of S1PR2 was mainly observed in the differentiated epithelial

cells characterized by a low proliferative rate in the upper region of the crypts in the colon and at the top of the crypts and in the villi in the small intestine (Figure 7D-18A), I hypothesized that S1PR2 could act as a master regulator for intestinal epithelial differentiation. To verify this hypothesis, I isolated small intestinal crypts from normal C57BL6/N mice and put them in culture in order to obtain organoids. Organoid display many important functions of the normal intestinal epithelium. They originate from the proliferation and differentiation of Lgr5+ intestinal stem cells located inside of the crypts. Initially IESCs form villus-like cystic structures, then proliferate resulting in outward buds containing also intestinal differentiated cells. The final structure characterized by multiple branches and the localization of the different cell type within the mini-gut epithelium, reflect the villus-crypt axis and the cellular localization *in vivo.* The organoids obtained from *s1pr2*$^{+/+}$ mice were untreated or treated thirty minutes per day with JTE013 at different concentrations (10 µM, 1 µM and 0,01 µM) for three consecutive days, starting from six hours after the plating of the crypts within Matrigel®. After 48 hours, the crypts treated with the higher concentration of the S1PR2 inhibitor formed fewer branched enteroids that were maintained until the complete mini-gut formation (Figure 18B). On the contrary both lower concentrations of JTE013 did not show any difference in the morphology of the organoids compared to the untreated ones (data not shown). These data demonstrate that the inhibition of S1PR2 during the organoid development blocks the complete formation of the branches. The round morphology of organoids raises the possibility that the inhibition of S1PR2 could prevent the epithelial differentiation. To gain insight into this hypothesis, I extracted the mRNA from organoids at day 7 of development after three treatment with 10 µM, 1 µM and 0,01 µM of JTE013 and I assessed the expression of

stem cell and differentiation markers for different lineages. I evaluated the levels of the stemness markers *Olfactomedin-4* (*Olfm4*) and *Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)*. *Olfm4* is highly expressed in the murine intestinal crypt base columnar cells (CBC) and it has been recently emerged as a highly specific marker for stem cells [129]. The *Lgr5* gene encodes for a G-protein coupled receptor for R-spondins, a family of Wnt pathway agonists and it is a component of the Wnt receptor complex. Lgr5 gene is exclusively expressed by cycling columnar cells at the crypt base and Lgr5+ cells generate all epithelial lineages within the intestinal epithelium [90], suggesting Lgr5 being a stem cell marker. Additionally, I evaluated the expression of the intestinal differentiation markers *Lysozyme* (*Lyz*) encoding for the secretory product of Paneth cell; *Mucin 2* (*Muc2*), the glycoprotein secreted specifically by Goblet cells and *Chromogranin A* (*ChrA*), a soluble protein produced by enteroendocrine cells. Interestingly, the analysis showed a strong significant increased expression of both *Olfm4 and* Lgr5 stemness markers under S1PR2 inhibition at the highest concentration, whereas no difference in the expression of differentiation markers was observed between organoids treated with both high and low concentrations of JTE013 or vehicle (Figure 18C). These results suggested that the inhibition of S1PR2 altered the crypt-villus differentiation axis inducing an increase of the relative expression of the stemness markers, thus maintaining the organoids in an undifferentiated status.

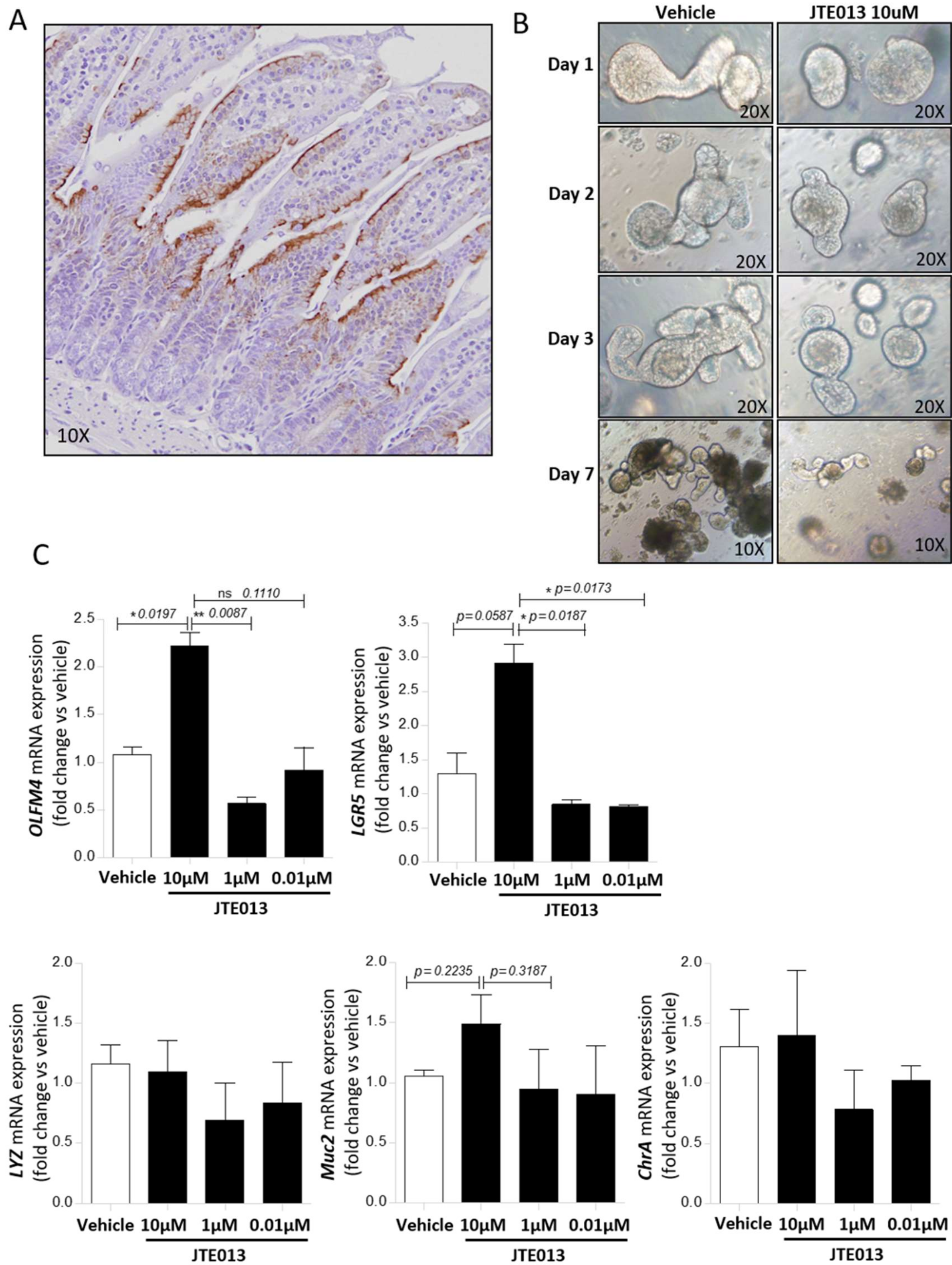



***Figure 18 The in vitro inhibition of S1PR2 increases the expression of the epithelial stem cell markers.*** *(A) Immunohistochemistry for S1PR2 in the small intestine of C57BL6 mice. (B) Organoid development at the day 1, 2 and 3 and 7 treated with 10 µM JTE013. (C) Gene expression analysis of stemness and differentiation epithelial markers at the day 7 of the organoid development in controls and upon treatment with different concentration of JTE013, by qRT-PCR.*

# 5 Discussion

Sphingosine-1-phosphate (S1P) is a ubiquitous lipid-derived metabolite whose synthesis and degradation is finely regulated by sphingosine kinases (SphKs), S1P lyase (SPL) and/or S1P phosphatases. Its widespread physiological roles are well known and studied, covering cell growth, proliferation, survival, apoptosis, migration and differentiation. S1P can act as an intracellular second messenger or by binding to a family of five G protein-coupled receptors, namely sphingosine 1 phosphate receptors (S1PR1-5). Interestingly, S1PR1, S1PR2 and S1PR3 are widely expressed throughout all the tissues, whereas the S1PR4 and S1PR5 expression is restricted to the lymphoid/hematopoietic tissue and the central nervous system, respectively [10]. Although S1P signaling has been extensively studied in the intestine, most at pathological conditions, interestingly only one study reported the expression of S1PRs in the intestinal epithelial cells, showing only the presence of S1PR1 and the absence of S1PR3 in the rat small intestinal epithelial cell line [80]. The present research study covered the gap in the characterization of S1PR1-3 on human and/or murine colonic epithelium. The analysis on the epithelium partition, finely derived from the isolation of the human intestinal crypts from healthy subjects, revealed that among the S1PRs, the S1PR2 was the most abundant. Similarly, the S1PR2 was highly expressed in respect to the other receptors on wild type murine colon-derived epithelial cells, both at the transcript and protein levels and its localization at the colonic epithelium is also confirmed by immunohistochemistry. On the contrary, S1PR1 and S1PR3 were mainly localized in other non-epithelial compartments, since they showed a low expression on epithelial cells and a very high expression in the whole mucosal or colonic tissues. Based on these findings, I focused my studies on the role of S1PR2 in the colonic

epithelium. The gut mucosa is a highly dynamic system characterized by the interplay between enterocyte, tight junction and mucin proteins covering the surface of intestinal epithelium. This organization functionally works as a defence barrier and is fundamental for the intestine physiology. The knowledge of this fine balance, managed by the epithelium compartment, is not fully depicted to date. Few studies have focused on the role of the S1P signalling on the intestinal epithelial cell integrity, reporting the S1P function in controlling epithelial barrier permeability in murine intestinal and human colonic Caco-2 epithelial cell lines *in vitro* [80,81]. However, there are no evidences of which sphingosine receptor is responsible of S1P-mediated effects. My data showed that the epithelial permeability of the colon of *S1pr2*-deficient mice did not display any dysregulation compared to wild type mice in physiological conditions. In fact, the gene expression of the tight junction *claudin-2*, *occludin* and *tjp-1* in the epithelial cells, were not significantly different in absence of S1PR2. Finally, the characterization of the mucin pattern did not show any significant difference between the wild type versus S1PR2 knockout mice. These data have been also confirmed in *in vivo* model of acute inflammation. In fact, the loss of S1PR2 does not significantly increase the epithelial barrier permeability in inflamed gut. These results suggest that S1PR2 is not involved in the S1P signalling-dependent regulation of the epithelial barrier.

The intestinal and colonic epithelium is daily exposed to injuries and aggression by the luminal contents that induce the death of many epithelial cells. Therefore, in the adult epithelium, a rapid and efficient mechanism of self-renewal is essential. The highly proliferative ability of the intestinal epithelium is driven by a small population of cells located at the bottom of the crypts [90]. Several studies reported that the S1P signalling

controls cell growth [4] and only few papers showed the anti-proliferative role of the S1PR2 in different cell lines [37,125]. To date, the role of S1PR2 in the proliferative capability of the intestinal epithelium cells is still unknown. In line with my data on the epithelium, I found a higher expression of *s1pr2* in immortalized human Caco-2 cell line in comparison to *s1pr1* and *s1pr3* and, accordingly with the literature data [37,125], the pharmacological inhibition of S1PR2 increased the proliferation rate of the Caco-2 cell line. These data have been also confirmed in *in vivo* studies in which the BrdU incorporation assay showed an increased proliferation of epithelial cells in the medial colon of S1PR2 knockout mice compared to the wild type. Interestingly, the BrdU positive epithelial cells were located at the crypt bottom where the epithelial stem cells as well as the transit-amplifying (TA) cells are usually localized [130,131]. It has been well accepted that stem cells are responsible of the proliferative activity of the crypts and that these two pathways (proliferation and differentiation) are intimately coupled in the intestinal epithelial cells, with the β-catenin/TCF-4 complex regulating both these processes. Indeed, this complex has been identified as the master switch between proliferating progenitors and differentiated epithelial cells both in healthy and malignant intestinal stem cells [93]. Importantly, I found that S1PR2 was mostly localized at the upper region of both colon and intestinal crypts as well as in the villi, whereas no expression was detected on the stem cell and TA compartment at the base of the crypts. This observation sustains the hypothesis that the receptor has a role during the differentiation of the epithelial cells. The role of S1PR2 in the intestinal epithelium differentiation has been strengthen by using the three-dimensional mini-gut culture system that is a powerful tool to investigate the regenerative properties of intestinal stem cells [91,132]. The pharmacological inhibition of S1PR2, during the early

phases of the organoid development blocked the complete formation of the organoid branches and kept them in a round shape, most probably associated with an undifferentiated phenotype [133]. The higher expression of the two stem cell gene markers *Lgr5* and *Olfm4* in the organoids treated with the S1PR2 inhibitor confirmed, at the gene level, the undifferentiated phenotype observed. This remark points out a novel role for S1PR2 in promoting differentiation of the intestinal epithelial cells as well as promotes this receptor as a master player in the maintenance of the crypt-villus differentiation axis. This results are also proved by the literature data proposing the involvement of S1PR2 in the differentiation of smooth muscle cells [33] and in the regulation of the skeletal muscle regeneration [35].

The primary driving forces behind the proliferation and/or differentiation of epithelial cells in the intestinal crypts is the Wnt/β-catenin/TCF-4 signalling. Indeed, this pathway has been identified as the master switch between proliferating progenitors and differentiated epithelial cells, both in healthy and malignant intestinal stem cells. In fact, the epithelial cells carrying mutations that aberrantly activate the Wnt pathway become unresponsive to physiological signals of differentiation and keep to behave as progenitor stem cells developing thus tumor lesions in surface epithelium [93]. Several studies have revealed the involvement of S1PR2 in the development and progression of different type of cancers. However, its role is not univocal and it depends on the tissue, on the tumour kind and on its staging. Most of the *in vitro* and *in vivo* studies investigated the high-level expression of S1PR2 in cell lines derived from human and murine tumours compared with the other S1PRs and supported the anti-tumour function of the S1PR2 [62,64–67]. The relevance of the S1P pathway in the development of the CRC has emerged in the last years as mediated by the activation of the S1PR1

[134,135]. However, the overall function of the S1PRs in the development of colorectal cancer, in particular of the S1PR2, is not completely understood. In line with our previous data on epithelial cells, we found a higher expression of *s1pr2* compared to s*1pr1* and s*1pr3* transcripts (found to being very low or undetectable) in seven human cancer cell lines derived from colorectal adenocarcinomas and carcinomas (data not shown). These observations advise that S1PR2 is ubiquitously expressed in malignant cells and suggest a role for this receptor in the regulation of the malignant cell properties. A key role of the S1PR2 in the development of colorectal cancer has been confirmed by the analysis of the AOM/DSS-induced tumor development in *S1PR2*-null mice, which display a higher number of high grade adenomas and larger carcinomas compared with wild type mice, whereas no difference in the clinical parameters of inflammation has been observed between *S1PR2* knockout and wild type mice. These observations indicate that the loss of S1PR2 leads to an increased onset and a faster progression of colorectal cancers independently of inflammatory conditions. The analysis of tumors in the $Apc^{min/+}$ genetic model of intestinal and colon cancer further confirmed this hypothesis. Indeed, a higher incidence and size of tumors in the colon of $s1pr2^{-/-}/Apc^{min/+}$ mice compared to $s1pr2^{+/+}/Apc^{min/+}$ controls was also observed. A strong decrease of the S1PR2 expression was observed in adenomas and early staged human carcinomas and also in the murine adenomatous polyps and adenocarcinomas of both AOM/DSS and genetic models, suggesting that S1PR2 down-regulation is an early event required for the development of colorectal cancers. Overall these data displayed for the first time a protective role of the S1PR2 during colon carcinogenesis. In the context of the tumorigenic model, which associates the tumorigenesis with the maintenance of a stem phenotype, I propose that the S1PR2 may contrast the

tumorigenesis by promoting differentiation and anti-proliferative pathways and blocking malignant transformation of epithelial cells (Figure 19).

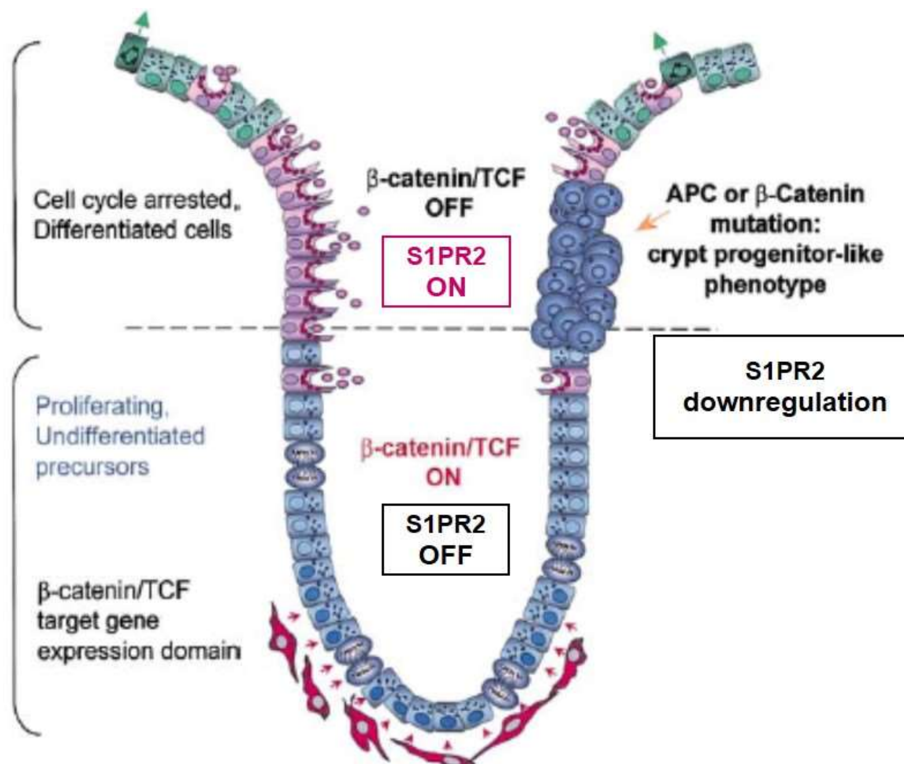



***Figure 19 Model of the role of S1PR2 in the colonic tumorigenesis.*** *Epithelial stem and progenitor cells highly proliferating at the half bottom of the crypts accumulate nuclear β-catenin and express β-catenin/TCF target genes. When they reach the midcrypt the β-catenin/TCF activity is downregulated, the cell cycle is blocked and the cells started to differentiate. Moreover, when mutations that aberrantly activate the Wnt pathway occur, epithelial cells become independent from the physiological signals which control the β-catenin/TCF-4 complex and continue to behave as crypt progenitor cells in the surface epithelium. On the contrary S1PR2 is not expressed by cells at the bottom of the crypts, whereas it is present in the cells at the top of the crypt near the lumen. Since in tumors the expression of S1PR2 is down-regulated, we hypothesize that S1PR2 by blocking the proliferation and inducing the differentiation pathways, may contrast the malignant transformation of the epithelial cells (Adapted from van de wetering, 2002 [93]).*

# 6 MATERIALS AND METHODS

## 6.1 HUMAN TISSUE COLLECTION

I enrolled in the study 16 patients underwent colon surgery for colorectal cancer and after having informed the patients about the goals of my study, I obtained a written consensus. Healthy colonic specimens have been obtained from these patients. The colonic tissues have been processed to obtain mucosal biopsies and isolate epithelial cells. Moreover, I obtained from Humanitas biobank 15 human biopsies of adenomas (n=4) and adenocarcinomas (n=11). Among the adenocarcinoma samples I selected tumors classified as T1N0M0 (n=4) and T2N0M0 (n=7), on the bases of the Tumor/Node/Metastasis (TNM) classification for colon cancer. Both biopsies and epithelial cells have been frozen in dry ice and homogenized for the mRNA and protein extractions. Human studies were approved by the ethics committee of the Istituto Clinico Humanitas (Rozzano, Italy).

## 6.2 MICE AND ANIMAL MODELS

Mice were housed in pathogen-free conditions. The S*1pr2*$^{-/-}$ mice were kindly provided by Dr Richard L. Proia (NIH, Bethesda, MD) [136] and heterozygous mice were bred to generate *S1pr2*$^{-/-}$ and *S1pr2*$^{+/+}$ littermates. *Apc*$^{min/+}$ were purchased from the Jackson Laboratory. *S1pr2*$^{-/-}$ and *Apc*$^{min/+}$ mice were crossbred to obtain *S1pr2*$^{-/-}$/*Apc*$^{min/+}$ and *S1pr2*$^{+/+}$/*Apc*$^{min/+}$ littermates. The genotypes were determined by PCR analysis of genomic DNA extracted from tail biopsies. The primer sequences used for the genotyping of the *S1pr2* mice are: 5'- GCA GTG ACA AAA GCT GCC GAA TGC TG-3', 5'- AGA TGG TGA CCA CGC AGA GCA CGT AG -3' and 5'- TGA CCG CTT CTT CGT GCT TTA

CGG TAT -3' [136]. The primer sequences used for the *Apc*$^{min/+}$ mice genotyping are 5'- GCC ATC CCT TCA CGT TAG -3', 5'- TTC CAC TTT GGC ATA AGG C -3' and 5'- GTG CAA TCC ATC TTG TTC AAT -3'. The acute colitis mouse model was induced in 8-12-week-old female *S1pr2*$^{-/-}$ and *S1pr2*$^{+/+}$ littermates by administration of 3% DSS in drinking water *ad libitum* for ten days. The body weight, stool consistency and faecal bleeding were daily monitored and scored as previously described. The disease activity index (DAI) has been evaluated according to the criteria proposed by Cooper *et al* [137] as the mean of the body weight loss (0=none, 1=1-5%, 2=5-10%, 3=10-15%, 4=>15%), stool consistency (0=normal, 2=loose, 4=diarrhea) and faecal bleeding scores (0=none, 2=occult bleeding, 4=bleeding). At day 10 the Evans blue dye permeation assay has been performed in treated mice or the mice have been immediately sacrificed and the colon collected, fixed overnight in 4% formalin, processed and paraffin embedded. The inflammation-driven colon carcinogenesis model was induced by a single intraperitoneal injection with the Azoxymethane (AOM), 10mg per kg body weight, followed by four cycles of DSS 2.5% in drinking water ad libitum for four days spaced out by ten days. The body weight of the mice, the faecal bleeding and consistency were monitored at least twice a week and the DAI evaluated as previously described. The tumor development has been monitored by endoscopy after the last DSS cycle and the colon has been collected and fixed in 4%. The 21-week-old *S1pr2*$^{-/-}$/*Apc*$^{min/+}$ and *S1pr2*$^{+/+}$/*Apc*$^{min/+}$ littermates have been sacrificed and the colon and intestine collected and fixed in 4% formalin. The fixed colon and intestine have been processed, paraffin embedded, sectioned in 2µm slides and stained with hematoxilin (Dako) and eosin (Diapath) for the single-blinded histological evaluation of the inflammation (Rachmilewitz score) and tumor count, or immunostained. All the procedures involving

mice were carried out with the approval from the ethics committees of the Istituto Clinico Humanitas, conformed to institutional guidelines in agreement with national and international law.

## 6.3 EVANS BLUE DYE PERMEATION ASSAY

The mice have been anesthetized with Ketamin and Xilazine. The abdominal quadrant of peritoneum has been opened in correspondence of the caecum, which together with the proximal region of the colon was completely exposed. A tiny cut in the proximal region of the colon nearby the caecum has been performed and a catheter has been inserted into the lumen and secured with suture. The anus has been exposed and another catheter has been fixed into the anal lumen with suture. The Evans blue solution 0.1% has been perfused into the colonic lumen flowing through the two catheters 1ml/min for five minutes and then washed to remove the excess of Evans blue dye. The mice have been sacrificed and the colon has been collected and let dry overnight. The overnight incubation of the colon with Dimethyl-formamide allow the dissolution of the Evans blue dye into the solution. The amount of the Evans blue into the solution has been quantified as the spectrophotometric optical density (OD) at 620 nm wavelength and the colon permeability evaluated as the ratio between optical density and the weight of the colon (Figure 20).

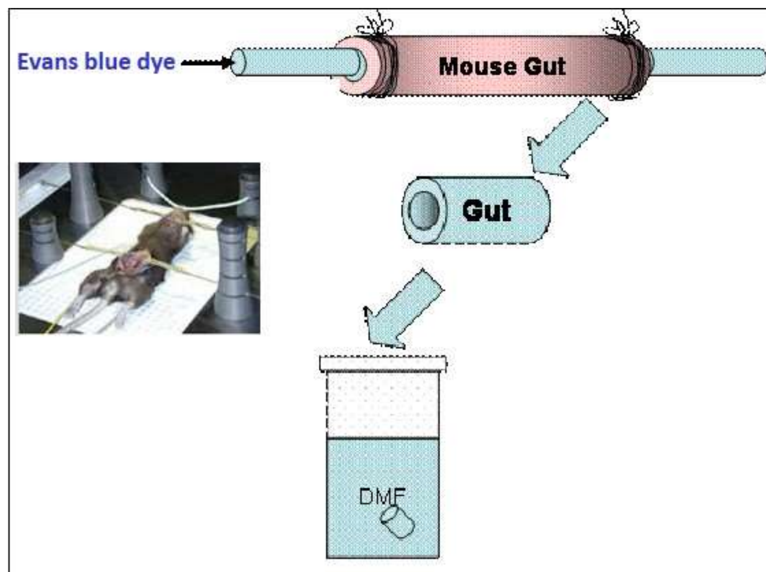



***Figure 20 The Evans Blue dye permeation assay.***
*(Adapted fom Vetrano S)*

## 6.4 ISOLATION OF HUMAN INTESTINAL EPITHELIAL CELLS

The colonic tissues obtained by surgical resection of the colon have been washed with PBS without calcium and magnesium to remove the superficial mucus. The mucosal layer has been isolated from the whole tissue, cut into 1cm pieces and washed in 2mM DTT for 15 minutes to remove the residual mucus. The mucosa was then cut into smaller pieces and incubated in 1Mm EDTA for 30 minutes, to chelate the calcium essential for the epithelial junctions. Then the crypts have been collected by vortexing the tissue and pelleted.

## 6.5 ISOLATION OF MURINE COLONIC EPITHELIAL CELLS

The protocol I used for the isolation of the murine colonic epithelium for mRNA and protein extraction has been proposed by Nik and Carlsson [138]. The colon extracted from the abdominal cavity has been washed flushing the lumen with ice-cold PBS

without calcium and magnesium. The entire colon has been inverted by inserting a thin crochet hook into the lumen, securing the rod end with suture and pulling the rod back (Figure 21A). Then, one end has been closed with suture and the other has been fitted to the tip of an Eppendorf Combitip plus and secured by suture. The tissue has been incubated in ice-cold BD Cell Recovery Solution (BD Biosciences) 20 minutes on ice, inflating with the Combitip plunger every 5 minutes (Figure 21B). Following the incubation, the colon has been rinsed by dipping it in cold PBS and the detached epithelium has been gently recovered with a cell scraper (Figure 21C). The recovered tissue has been centrifuged and the pellet immediately frozen in dry ice.

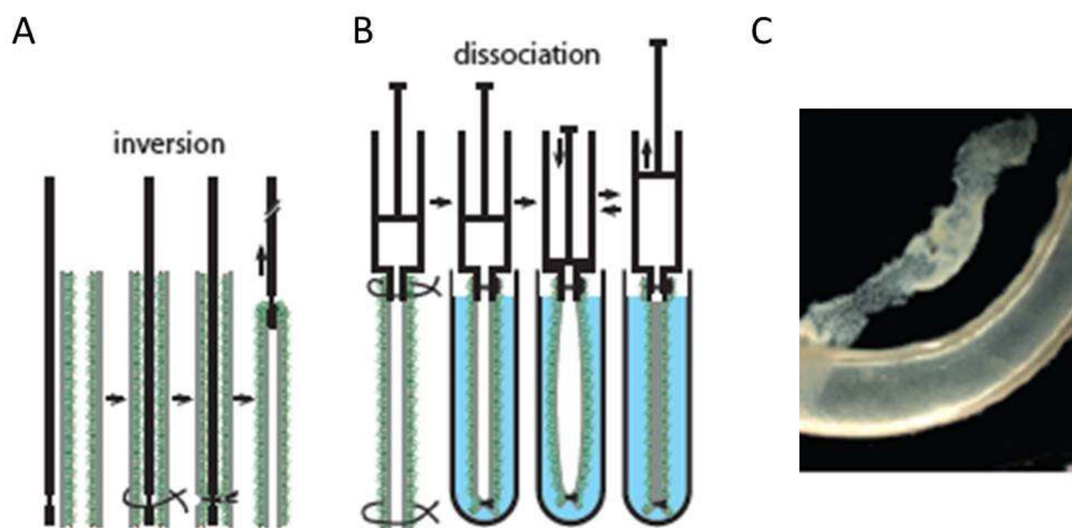



***Figure 21 Isolation of murine colonic epithelial cells.*** *(A) A thin crochet hook has been inserted into the lumen, secured with suture and then pulled back to invert the colon. (B) One end has been closed with suture and the other end has been fitted to the tip of an Eppendorf Combitip plus. The tissue has been incubated in ice-cold BD Cell Recovery Solution and inflated with the Combitip plunger every 5 minutes. (C) Removal of the epithelial sheet after incubation (Modified by Nik and Carlsson, 2013)* [139].

## 6.6 ISOLATION OF MURINE INTESTINAL EPITHELIAL CELLS FOR ENTEROID CULTURE

For the isolation of intestinal crypts, I adapted the protocol provided by Mahe in the Current Protocols in Mouse Biology journal [140]. I extracted the intestine from the abdominal cavity and washed the lumen with ice-cold PBS to remove all the faeces.

The intestine has been opened lengthwise, cut into 2 cm pieces and washed in ice-cold PBS. The tissue has been further cut in smaller pieces and incubated in PBS 2mM EDTA for 30 minutes. Then the crypts have been collected by shaking the tissue and filtering the solution through a 70-µm filter to remove the villus fraction. The crypts isolated have been counted and plated within the Matrigel®. The crypts have been cultured in complete DMEM/F12 medium (B27 supplement, N2 supplement, PenStrep, hepes, Glutamine, 500 ng/ml R-spondin1 (Peprotec), 100 µg/ml Noggin (Peprotec) and 50 ng/ml murine EGF (Peprotec)) and the budding enterospheres completely developed after 7 days.

## 6.7 WESTERN BLOT ANALYSIS

The tissue biopsies were mechanically homogenized in the Lysis Buffer (TrisHCl ph7.4 50mM, EDTA ph8 1mM, NaCl 150mM, 1% Triton, 0.5% Sodium Deoxycholate, 0.1% Sodium dodecyl sulfate) by a short 25-30Hz shaking with a bead in 2-ml tubes, through the bead mill Tissue Lyzer II (Quiagen) and kept on ice for 30 minutes. The murine epithelial cells have been homogenized by disrupting mechanically the pellet by a 18G-needle syringe in the Lysis Buffer. Samples were centrifuged at 12000 g for 20 minutes at 4°C and the protein concentration of the supernatants were evaluated with the DC protein assay kit (Biorad). The protein content from each sample were separated on 10% polyacrylamide gel and transferred to a nitrocellulose membranes (Biorad) on nitrocellulose membranes. The nonspecific binding sites were blocked with 5% milk for one hour. The membranes were incubated with the primary and horseradish peroxidase (HRP)-conjugated secondary antibodies. The immunoreactivity was detected by an enhanced chemiluminescence reaction (ECL) and developed by the

ChemiDoc Imaging System. Primary antibodies used for western blot were anti mouse/human rabbit S1P1 (1:200; LSBio LS-C137385), anti-mouse/human S1PR2 rabbit (1:200; Acris AP01198PU-N), anti mouse/human rabbit S1P3 (1:1000; LS-C138479), and anti-actin C-11 (1:1000 Santa Cruz sc-1615). Secondary antibodies used were anti rabbit HRP and anti goat HRP. The expression is represented as the densiometric analysis of protein relative to the actin.

## 6.8 RNA extraction, inverse transcription and quantitative real time Polymerase Chain Reaction (qRT-PCR)

Total RNA has been extracted from human and murine epithelial cells using the RNeasy® Lipid Tissue kit (Quiagen) according to the manufacturer's protocol. The homogenization step with stainless steel beads (5 mm mean diameter) has been performed to properly disrupt the pellet. The mRNA has been retrotranscribed in cDNA by using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystem) and quantitative real-time PCR has been performed using the Fast SYBR® Green Master Mix (Applied Biosystems) and detected with 7900HT Sequence Detection System (Applied Biosystems). The primer sequences used are reported in the Table 1. The GAPDH gene has been used as housekeeping. The relative mRNA expression has been evaluated as $2^{-\Delta Ct}$.

**Table 1**

| Gene | Forward (5'->3') | Reverse (5'->3') |
|---|---|---|
| *S1pr1* | ATGGTGTCCACTAGCATCCC | CGATGTTCAACTTGCCTGTGTAG |
| *S1pr2* | ATGGGCGGCTTATACTCAGAG | GCGCAGCACAAGATGATGAT |
| *S1pr3* | CCATTGCCATTGAGCGACAC | TTAGCCAGCACATCCCAATCA |
| *S1PR1* | TTCCACCGACCCATGTACTAT | GCGAGGAGACTGAACACGG |
| *S1PR2* | CATCGTCATCCTCTGTTGCG | GCCTGCCAGTAGATCGGAG |
| *S1PR3* | CGGCATCGCTTACAAGGTCAA | GCCACGAACATACTGCCCT |
| *Cldn2* | CAACTGGTGGGCTACATCCTA | CCCTTGGAAAAGCCAACCG |
| *Ocln* | TTGAAAGTCCACCTCCTTACAGA | CCGGATAAAAAGAGTACGCTGG |
| *Tjp1* | GCCGCTAAGAGCACAGCAA | TCCCCACTCTGAAAATGAGGA |
| *Olfm4* | GGAGCGCTTAGAGTACACAG | GGAGCCTCTTCTCATACACAC |
| *Lgr5* | ATGAACAACATCAGTCAGCTAC | CTCCCTTGGGAATGTGTGTC |
| *Lyz* | TCTAAGAATGCCTGTGGGATC | TTGTATGGCTGCAGTGATGTC |
| *Muc2* | ATGCCCACCTCCTCAAAGAC | GTAGTTTCCGTTGGAACAGTGAA |
| *ChgA* | CAGGGACACTATGGAGAAGAGA | -GGTGATTGGGTATTGGTGGCT |
| *Gapdh* | AGG TCG GTG TGA ACG GAT TTG | TGT AGA CCA TGT AGT TGA GGT CA |
| *GAPDH* | CATGAGAAGTATGACAACAGCCT | AGTCCTTCCACGATACCAAAGT |

## 6.9 IMMUNOISTOCHEMISTRY

The colonic and intestinal paraffin embedded tissue slides have been deparaffinized and any antigen retrieval procedure has been performed. The inhibition of the endogenous peroxidase has been performed with hydrogen peroxide 3% and the Rodent Block M (Biocare Medical RBM961) has been used for blocking the endogenous mouse IgG and non-specific background. The primary antibody used for the S1PR2 staining was the anti-mouse/human rabbit S1PR2 antibody (1:200 Acris AP01198PU-N). For the primary antibody detection, the MACH 1 Universal HRP-Polymer (Biocare Medical) and the Betazoid DAB Chromogen Kit (Biocare Medical) have been used. The combination of the alcian blue and the PAS techniques has been used to distinguishing neutral and acid mucins. The Alcian/PAS staining has been performed on colonic tissues fixed in formalin 4% according to the protocol. After deparaffinization the sections are stained with the standard Alcian Blue (pH 2.5) and the Periodic Acid 1%.

## 6.10 Statistical analysis

The Prism (GraphPad) software was used for statistical analysis. Data were analysed with a two-tailed unpaired Student's t-test and the p-value was considered significant when smaller than 0.05.

# 7 Future perspectives

There are a several issues we plan to address to strengthen our findings. Some of them are already on the way to be solved and others have been planned for the next future.

Overall these concerns regard:

1. demonstrating the hypothesis that the S1PR2 protein appear to be expressed in differentiated epithelial cells whereas it is absent in the region of the crypt where usually are located stem and progenitor cells. Indeed, taking advantage of the Lgr5-EGFP-IRES-creERT2 mice, expressing GFP in Lgr5 (a well-known marker of stemness) positive stem cells, I would like to confirm preliminary data which showed a differential expression of the S1PR2 between stem cells and differentiated epithelium (data not shown).
2. Identifying the S1PR2 downstream pathways and genes involved in the protection of the tumor development especially by the investigation on the role of the S1PR2 in the modulation of Wnt/β-catenin gene target.
3. Analysing whether S1P and/or other sphingolipids are involved in the S1PR2 functions in tumour development.
4. Characterizing the expression of S1PR2 in different stage of human tumours.

# 8 CONCLUSION REMARKS

My thesis has brought to light several novelties on the knowledge of S1P signalling and in particular on S1PR2 in intestinal epithelium. In detail:

1. S1PR2 is the most abundant S1P receptor being expressed in the colonic epithelium.
2. S1PR2 is not involved in the mechanism controlling intestinal barrier function;
3. S1PR2 is a crucial player in the proliferation of the colonic epithelial cells and its absence dramatically promotes an increase of the colon tumour onset
4. S1PR2 may have a prominent role into the differentiation of intestinal stem cells acting as a new regulator of epithelial differentiation.

Although further studies are necessary, my results shed light not only on the molecular mechanisms underlying intestinal tumorigenesis, but also point to S1PR2 as a new potential target for the treatment of colon cancer.

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