Molecular Regulation of Intestinal Stem Cells

Review

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Abstract

Our body undergoes a remarkable growth from a single cell to a fully functional organism. Once into adulthood, the organism changes its focus from developing new tissue to maintaining the steady state. The intestine is an astonishing example of continuous tissue renewal in adult mammals. The finding that specialized cells with virtually unlimited self-renewal capacity replenish the intestinal epithelium leads to the identification of adult intestinal stem cells and molecular factors involved in their regulation. An understanding of mechanisms involved in intestinal stem cells can provide us with valuable insights and opportunities to treat disease such as cancer. This review summarizes the findings about intestinal stem cells with a focus on different signaling pathways involved in molecular regulation of these cells.

1. INTRODUCTION

The mammalian intestine lines a very dynamic and vigorous environment, the lumen, where the epithelial lining is exposed to considerable movement which brings a certain level of toxicity with it. Therefore, constant and proper self-renewal of intestinal cells is extremely important in maintaining the mammalian intestine. Under normal circumstances a tight control of the molecular signaling pathways ensures a well established balance between proliferation, terminal differentiation and apoptosis.

Specialized cells, called the intestinal stem cells with unlimited self-renewal capacity are able to replenish the intestinal epithelium. . Stem cells are present in all multicellular organisms and have a remarkable potential to differentiate into specialized cells in the body. Two main features of adult stem cells are multipotency and continuous self-renewal. Thanks to their unlimited division capacity, they act as repair systems in several tissues. Intestine is an example of such a tissue in which unspecialized intestinal stem cells replenish cells of the intestinal epithelium. Given the mentioned properties, stem cells can be used in regenerative medicine. Stem cells' potential as regenerative therapy lies in removing them from their natural niche, transferring them to culture and finally placing them into an environment that is foreign to them. For this reason, it is important and necessary to understand the interaction between the stem cells and their microenvironment, so that their properties can be established and maintained in culture as well.

Recently, several markers of intestinal stem cells have been identified. These molecules mark the proliferative stem cells residing in the stem cell niche at the intestinal crypts. Stem cell behavior depends mainly on signals provided by different signalling pathways.

Wnt signalling is the main pathway involved in regulation of stem cells in the mammalian intestine. Other prominent pathways involved are Notch, Bmp, and Hedgehog signalling pathways. Disruption of one or more of these pathways can lead to developmental defects, disease, even cancer. Identification of these molecular factors and an understanding of mechanisms involved in intestinal stem cells behavior can provide us with valuable insights and opportunities to treat disease such as colorectal cancer.

This review summarizes the findings about the intestinal stem cells with a focus on different

signalling pathways involved in molecular regulation of these cells.

1.1 Structure of the small intestine and the colon

Mammalian intestinal tract is divided into two segments: the small intestine and the colon (Gregorieff *et al.* 2005). Small intestine, which is responsible for digestion of food and uptake of nutrients, follows the stomach and is followed by the large intestine. The small intestine consists of duodenum, jejunum and ileum whereas the large intestine consists of the cecum, colon and the rectum. Cecum connects the ileum of the small intestine with the ascending colon of the large intestine. Descending colon follows the ascending colon and is followed by the rectum. Colon extracts water, salts and some fat soluble vitamins from the solid waste before it leaves the body. A single layer of epithelium forms the inner lining of the intestine. This single layer of epithelium is the most rapidly renewing tissue in human body (Heath *et al.* 1996). Epithelium of the small intestine is completely renewed every 5 days.

Both the small intestine and the colon are composed of three layers: an outer layer, a middle layer and an inner layer. The outer layer, made up of smooth muscle, is responsible for peristaltic movement of food through the tube. The middle layer is made up of connective tissue and harbors the nerves and lymphatic vessels. Inner surface of the small intestine is folded and has fingerlike structures called villi which project into the lumen. This folded structure of the inner layer perfectly fits with this function of the small intestine as it increases the surface area greatly. Biggest difference in the epithelial inner lining of the small intestine and the colon lies in the absence of these villi in the colon. The flat surface of the colon lacks these protruding structures. This difference in the epithelial inner layer is a reflection of both segments' function. However, both segments have invaginations called the crypts. Crypts are the basic functional units of the intestine (Humphries *et al.* 2008). There are millions of crypts in both the small intestine and the colon. These structures drive the entire self renewal process of the intestinal epithelium.

1.2 Cells of the intestinal epithelium

Intestinal epithelium consists of four cell types: enterocytes, goblet cells, enteroendocrine and Paneth cells. Enterocytes are absorptive cells which also secrete hydrolytic enzymes. These cells are found throughout the entire intestine but are highest in number in the duodenum which harbors the longest villi. Goblet cells secrete mucus which makes the passage of stool easier by meeting the need for lubrication along the tube. These cells are mostly found in the duodenum and the colon. Enteroendocrine cells are the rare hormone secreting cells of the intestinal lining. Another type of cell found in the intestinal epithelial layer is the Paneth cell. Paneth cells reside at the bottom of the crypt (fig 1a). They secrete a peptide called human α-defensin that possesses antimicrobial activity, and lyzosomes, indicating Paneth cells' involvement in antimicrobial control in the intestine (Porter *et al.* 2002). Besides their role as defense cells, Paneth cells are also involved in intestinal stem cell maintenance. More about the latter function of Paneth cells will be discussed later.

1.3 Stem cells

Multiple types of mammalian stem cells exist in multicellular organisms. Examples of stem cells are *in vitro* embryonic stem cells, embryonic tissue stem cells and adult stem cells. Whereas pluripotent embryonic stem cells can give rise to all of the cells of an organism, multipotent and self-renewing adult intestinal stem cells have the ability to differentiate into all types of specialized cells of a tissue. Beside the property of multipotency, it is claimed by some that intestinal stem cells have two other characteristics: quiescence and asymmetric cell division (Barker *et al.* 2008), while some suggest that these cells divide symmetrically. They are quiescent because stem cells are believed to divide infrequently. Adult stem cells act as the maintenance and repair systems of the body during the lifespan of an organism. This type of stem cells divide either symmetrically or asymmetrically. The two types of cell divisions have three different outcomes. They can divide asymmetrically and give rise to one stem cell and one progenitor cell. They can also expand stem cell population by following a self-renewing symmetric division pattern. Third possible outcome is the none self-renewing symmetric division where two progenitors are generated. Due to asymmetric cell division intestinal stem

cells give rise to one daughter cell cycling and another daughter cells that becomes the parent stem cell. Rapidly cycling daughter cells are the transient amplifying (TA) cells which eventually terminally differentiate after a couple of cell divisions and finally form the four main cell types of the intestinal epithelium.

Intestinal stem cells are believed to be quiescent in a microenvironment called the stem cell niche in which all the necessary factors for maintaining the stemness is present. Other type of specialized cells in the niche provide stem cells with these necessary factors. Stem cells are anchored to the niche by cell-cell contact. This cell-cell contact ensures that intestinal stem cells remain far from differentiation stimuli while they stay close to self-renewal signals provided by the niche. A comprehensive understanding of the molecular traffic within this niche can provide us with valuable insights and open new avenues for treatment of various diseases.

1.4 Stem cells of the intestine and the crypt

The most rapidly self-renewing tissue in adult mammals is the intestinal epithelium . The epithelium of the small intestine is renewed every 5 days thanks to the invaginations called the crypts. Each crypt is capable of generating about 250 new epithelial cells per day (Barker *et al.* 2010). The finger-like invaginations of the epithelial layer into the underlying connective tissue are believed to house the stem cell population at the bottom in the stem cell niche. Inheritance pattern tracking of genetic markers revealed the existence of self-renewing multipotent stem cells in the crypt (Bjerkens *et al*. 2002). In Bjerkens' study, genetic markers were introduced into single crypt cells via somatic mutations. Another study supporting the existence of self renewing stem cells in the crypts is again an inheritance pattern study of mitochondrial mutations in human intestinal crypts (Taylor *et al*. 2003). Intestinal stem cells produce the highly proliferative transit-amplifying (TA) progenitor cells (fig 1b). TA cells divide rapidly as they migrate upward towards the border of the crypt and the villus. When they reach villi, TA cells differentiate into three of the four types of epithelial cells which are mentioned before: enterocytes, goblet cells and enteroendocrine cells. They leave the crypt as mature epithelial cells in 2 days. These epithelial cells continue moving towards the tip of the villus and finally go through apoptosis and detach from the villus into the lumen. Another type of cell which TA cells differentiate into

is the Paneth cell which does not follow this upward migration. Paneth cells move in the opposite direction towards the bottom of the crypt. These cells have a longer life span of 6-8 weeks. The situation in the colon is similar to that in the small intestine except for Paneth cells. Paneth cells are not present in the colon.

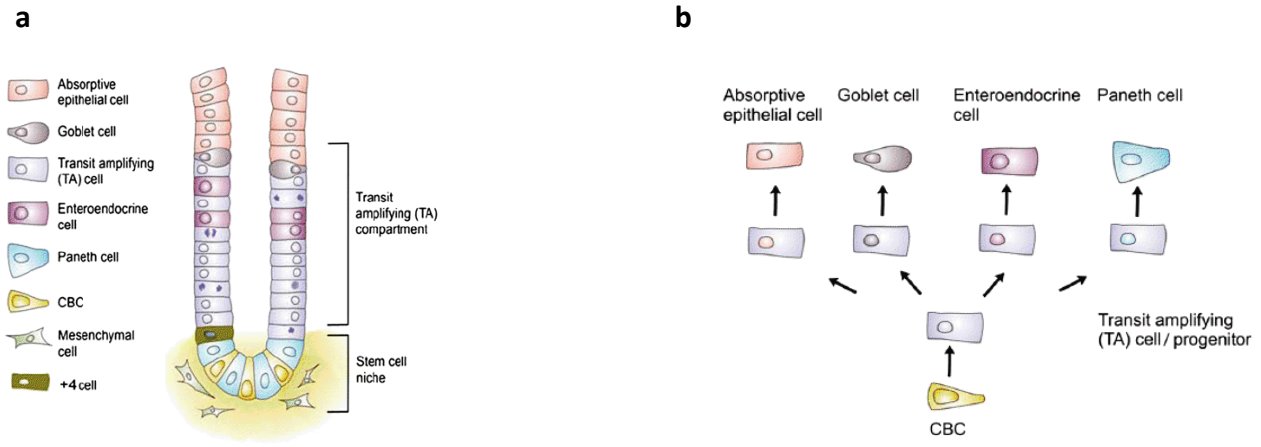

Figure 1. Schematic representation of epithealial cell organization within the intestinal crypt **(a)**, Crypt Base Columnar Cell gives rise Transit Amplifying cells which in turn can differentiate into the four epithelial cell types **(b)**.

Establishing a balance between proliferation, differentiation, migration and cell death is important for renewal and maintenance of intestinal epithelium. The intestinal epithelium and the underlying mesenchyme constantly communicate to regulate proliferation, differentiation, lineage commitment and cell death. It is known for a long time that the crypts harbor the stem cell population. For decades, substantial work is put into definitive identification of stem cells. Two stem cell models exist in the literature: +4 model and the stem cell zone model. According to +4 model, the stem cell candidate resides right above the Paneth cells at position +4 (Potten *et al.* 1974) . Two characteristics of stem cells are believed to be radiosensitivity and label retention. This stem cell candidate possessed both of the characteristics. In an attempt to trace the progeny of dividing cells, thymidine analogues have been used to label dividing cells. Since the DNA is replicated during every cell division, rapid cell division or differentiation of dividing cells lead to eventual depletion of the label. Only when cells that incorporate the thymidine

analogue undergo infrequent divisions or remain quiescent, the label is retained following a long chase. Cell tracking experiments, in which tritiated thymidine is incorporated in the DNA of cells prior to division for labelling purposes, also supported this model (Cairnie *et al.* 1965).

An alternative model, the stem cell zone model, emerged upon identification of a second type of small cycling cells, called the crypt base columnar cells (CBC) between Paneth cells. Using electron microscopy, Leblond discovered that the Paneth cells were not the only cells populating the crypt base (Leblond *et al.* 1974). After H3-Thymidine labeling, surviving CBC cells phagocyctose the dying, radiolabelled CBC cells. The resulting radioactive phagosomes were also observed in the differentiated cells of the 4 major lineages higher above the crypt. According to the stem cell zone model, CBC stem cells are located at the crypt base and give rise to progenitors which leave the niche at position +4 and commit themselves to differentiation into various cell lineages. Differentiated Paneth cells, on the other hand , migrate downwards to the very base of the crypt.

The major challenge in identification of the stem cells was the absence of molecular markers for a long time. This is why research mostly relied on properties such as label-retention and radio-sensitivity are used for identification until recently. Today we have several markers of intestinal stem cells.

2. STEM CELL MARKERS

As it was mentioned before, the main problem in identifying the stem cells was the lack of molecular markers. Since the Wnt signaling pathway drives the cell proliferation in the crypts, targets of the pathway were investigated with the hope of stem cell specific gene identification (Wetering *et al.* 2002).

Multiple candidates are proposed as stem cell markers such as DCAMKL1, P-Pten, mTERT, Musashi1, Bmi1, Wip1 as +4 markers and Lgr5, Prominin1, Ascl2, Musashi1, OLFM4 as CBC markers (Potten *et al.* 2009). Some of these candidates marked the +4 position, therefore it was initially believed that the cells at this position were the true stem cells. This view has changed with the identification of a G protein –coupled receptor which is encoded by the *Lgr5* gene. Expression of *Lgr5* is restricted to the CBC cells located at the crypt base while most of the Wnt target genes are expressed throughout the whole crypt (Barker *et al.* 2007). *Lgr5* was already in the list of about 80 Wnt target genes. *Lgr5* showed a unique expression pattern that was different than other Wnt target genes which were mainly expressed in Paneth cells or TA-cells. *Lgr5* was expressed in the crypt but not in villi. This difference in expression pattern was Barker's motivation to study *Lgr5* in more detail. *In situ* hybridization showed that *Lgr5* is expressed in cycling crypt base columnar cells at the bottom of the crypt of *Apc* mutant mouse small intestine (Barker *et al.* 2007). Barker further analyzed expression of *Lgr5* in detail using a knock-in allele in mice. Knock-in allele was achieved by integrating *LacZ* to the first transmembrane domain from the amino-terminal side. Resulting heterozygous *lgr5-lacZ* mice was used to study expression of Lgr5. Results were the same as in *in situ* hybridization pointing to expression of *Lgr5* in CBC cells at the bottom of crypt of mouse small intestine. Another knock-in allele was generated by integrating enhanced GFP-IRES-creERT2 cassette to the *Lgr5* gene in order to study the stemness of these LGR5+ CBC cells. The Cre recombinase is encoded by the *cre* genes and promotes intracellular recombination of 2 target DNA sequences leading to removal of a specific part of DNA with the purpose of either activating or inactivating a specific gene. Cre expressing mice were crossed to cre reporter lines to genetically label Lgr5 expressing cells at a clonal level. The progeny of CBCs generated all the principle cell types of the intestine over an extended period of time, indicating that Lgr5 expressing progenitors

display both self-renewal and multipotency.

In mouse expressing a tamoxifen- inducible Cre from *Bmi1* locus, *Bmi1* is expressed *in vivo* at +4 position in the crypt (Sangiorgi *et al.* 2008). These *Bmi1* expressing cells near the bottom of the mouse crypt are able to proliferate, self-renew and give rise to the differentiated cells of the intestinal epithelium. Induction of a stable form of β-catenin in these cells lead to formation of adenomas. Sangiorgi's finding that ablation of *Bmi1+* cells leads to loss of crypts in the mouse intestine confirms *Bmi1*'s being an intestinal stem cell marker. Labelling of many more crypts in the duodenum and the first part of the jejunum , the decreasing number of labeled crypts or total absence towards the ileum points out to a descending gradient of *Bmi1* expressing cells in the small intestine. So the *Bmi1* expressing cells are not distributed uniformly suggesting that there may be other stem cell populations in the small intestine.

Expression pattern of cell surface marker CD133 mRNA and protein, known as Prominin1 in mice, was investigated in intestinal crypts (Snippert *et al.* 2009). Snippert and colleagues created knock-in mice in order to visualize and trace Prom1+ cells. This study shows that although Prom1 is used to identify and isolate stem cells , it is not a specific marker of Lgr5+ stem cells, because it marks both intestinal stem cells and TA progenitors.

Although several Intestinal stem cell markers are discovered, identified markers pint to different cells in the crypt. LGR5 points to CBCs whereas BMI1 and TERT point to +4 position cells.

3. MOLECULAR MECHANISMS INVOLVED IN STEM CELL PROLIFERATION

3.1 Wnt signallig pathway

The main pathway regulating the self-renewal of the intestinal epithelium is the Wnt signaling pathway. Loss of Wnt signalling *in vivo* results in destruction of the epithelium due to blockage of cell proliferation. Wnt signalling is essential for cell proliferation and stem cell renewal. Wnt proteins are produced by both the epithelial cells and the underlying mesenchyme (Vermeulen *et al*. 2011). Transduction of Wnt signals to the canonical pathway plays a role in cell fate determination whereas to the non-canonical pathway transducing Wnt signals play a role in cell movement and tissue polarity (Katoh *et al.* 2007). Wnt target cells are activated through interaction of Wnts with the transmembrane proteins Frizzled (Fz) and LRP (Gregorieff *et al.* 2005). Binding of Wnts to Fz and LRP initiates the pathway and eventually leads to formation of the Tcf/ β- catenin complex in the nucleus. Katoh *et al.* also reports that the transduction of canonical Wnt signals to β-catenin signalling pathway is realized through Frizzled family receptors and LRP5/LRP6 co-receptor (Katoh *et al.* 2007).

In the presence of Wnt signaling , β-catenin translocates into the nucleus and forms a complex with the transcriptional repressor TCF transcription factor. This active transcription complex– rescues the target gene enhancers from the repression. In the absence of Wnt signaling, an activated complex made up of Axin, APC and GSK3B binds to β-catenin and ensures targeting of β-catenin for degradation. This proteosomic degradation takes place upon phosphorylation of β-catenin at several serine/threonine residues of the N-terminus as a result of binding of the Axin/APC/GSK3 β complex (fig 2). GSK3β (Glycogen Synthase Kinase 3 β) and CK1α (casein kinase 1alpha) are responsible for phosphorylation of the complex (Katoh *et al.* 2007).

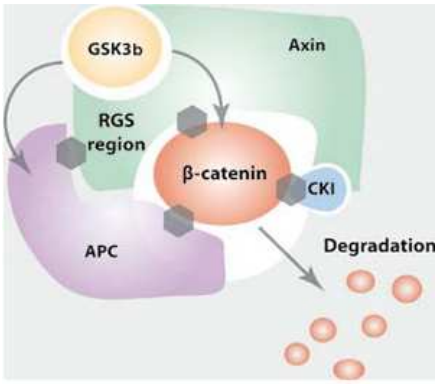

Figure 2. APC/Axin/GSK3β/CK1 complex bound to β-catenin and targets β-catenin for degradation.

In that sense β-catenin/TCF Transcription factor complex acts as an effector of the Wnt signalling pathway(fig 3). There seems to be a Wnt signaling gradient along the crypt-villus axis. As cells migrates away from the crypt base, they start differentiating and do not proliferate anymore. This is an indication of loss of proliferation ability due to migration away from the Wnt signalling gradient.

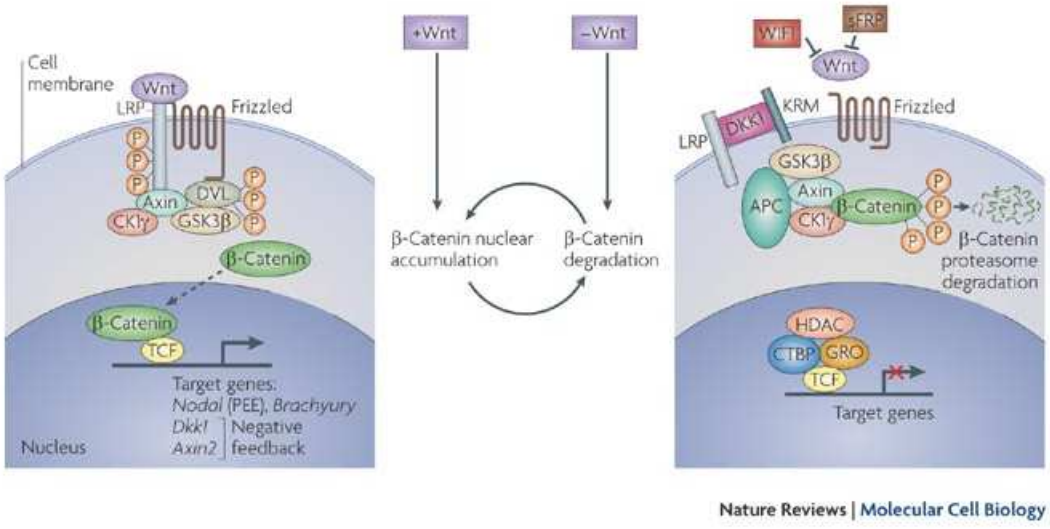

Figure 3. Nuclear accumulation of β-catenin in the presence of Wnt signalling versus β-catenin degradation in the absenceof Wnt signalling (Arnold et al. 2009)

Loss of Tcf-4 expression in the stem cell niche results in cessation of proliferation and entry to premature differentiation (Wong *et al.* 2004; Korinek *et al.* 1998). Effect of loss of function of β-catenin has been investigated in a system where Cre recombinase is applied to

delete β-catenin (Ireland *et al.* 2004). In this study a transgenic line expressing Cre under the control of a cytochrome P450 promoter element is used. This rat cytochrome P450 promoter element is silent under normal circumstances but can be up-regulated when exposed to xenobiotics. Deletion of β-catenin have led to ablation of the crytps, increase in apoptosis, and decrease in goblet cell numbers. Another consequence of loss of β-catenin function is detachment of the absorptive cells of the villus from the villus core. These observations indicate β-catenin's involvement in crypt formation.

During intestinal development, *Lgr5*, one of the targets of Wnt signalling, is a negative regulator of the Wnt signalling pathway (Garcia *et al.* 2009). In attempt to understand the role of LGR5 during intestinal development, Garcia and colleagues used LGR5 null/LacZ-NeoR knock-in mice and X-gal staining. LGR5 knock-out mice displayed a premature differentiation of Paneth cells and an upregulation of the Wnt signalling due to LGR5 deficiency. Whereas *Lgr5* is expressed at the bottom of the intestinal crypts, expression of *Lgr4*, another G-coupled protein receptor, is confined to all of the crypt cells, particularly TA region above the Paneth cells, CBCs, and at low levels to the underlying mesenchyme in homozygous mice with a *LacZ* gene trap knocked in *Lgr4* locus (Mustata *et al.* 2011). Thus Lgr5 is co-expressed with Lgr4 (de Lau *et al.* t). When Lgr4 gene is inactivated, epithelial cell proliferation is decreased by half and differentiation of Paneth cells decrease by 80%. *Ex vivo* culture shows that LGR4 deficient crypts die. This observation indicates that Lgr4 is required for Paneth cell differentiation and stem cell maintenance in postnatal mouse intestinal crypts. Another study shows that conditional deletion of Lgr5 and its homologue Lgr4 results in inhibition of Wnt pathway in the mouse gut leading to impairment of Wnt target gene expression thereby death of intestinal crypts. A green fluorescent protein-ribosome entry site-CreERT2 (GFP-Ires-CreERT2) cassette is integrated in one allelle of Lgr4. The tamoxifen inducable system in which lineage tracing was performed after crossing to the R26R-LacZCre reporter strain showed that Lgr4 is expressed in both short-lived daughter cells and long-lived Lgr5+ stem cells. Lgr4 and Lgr5 play an important role in Wnt dependent intestinal stem cells and their progenitors through incorporation into frizzled/LRP complexes. These orphan transmembrane receptors trigger downstream Wnt signals by binding of R-spondins.

Mutations in *Apc* gene have an activating affect on Wnt signalling resulting in intestinal hyperplasia (Reya *et al.* 2005). Same effect is observed in activating mutations in *Ctnnb1* gene encoding β- catenin. Another *in vivo* study shows a similar effect of ISC expansion when the Wnt activator R-spondin-1 is overexpressed (Kim *et al.* 2005). R-spondin protein family consists of four secreted proteins RSPO1, RSPO2, RSPO3 and RSPO4 (Nam *et al*. 2006) RSPOs are capable of enhancing the Wnt/ β- catenin signalling and thereby stimulating ISC proliferation (Kim *et al.* 2006;Kim *et al.* 2008). R-spondins bind to LGR4 and LGR5 orphan receptors and thereby enhance Wnt dependent LRP6 phosphorylation (Carmon *et al.* 2011) In that sense R-spondins act as ligands of LGR4 and LGR5 and play a role in mediating the Wnt/β-catenin signalling. RSPOs act as antagonists of DKK1, a Wnt target (Kim *et al.* 2008). DKK1 inhibits canonical Wnt signalling because when bound to the transmembrane receptor Kremen1/2, leads to reduction of LRP6-Frizzled-Wnt multimers (Kelly *et al.*). This multimer formation is necessary for Wnt signalling. After DKK1 binds to Kremen1/2, Kremen1/2 forms a complex with LRP6 which in turn prevents LRP6-Frizzled-Wnt multimer formation. As a result, Wnt signaling is downregulated. Kremen1/2 also plays a role in mediating LRP6 degradation as Kremen1/2 makes LRP6 unavailable for Wnt signalling because it removes the LRP6 from the cell surface (Li *et al.* 2010).

The expression of a variety of positive and negative regulators of the pathway, such as Frizzleds, LRP and HSPG, *Axin2*, and TCF/Lef are all controlled by the β-catenin/TCF complex (Clevers *et al.* 2006) . Some of the known canonical Wnt target genes are FGF20, DKK1, WISP1, MYC, CCND1, and Glucagon (GCG) (Katoh *et al.* 2007). Ascl2 transcription factor is a Wnt target and signature of intestinal stem cells and controls stem cell fate (van der Flier *et al.* 2009). Loss of function experiments show disappearance of Lgr5 stem cells whereas transgenic expression induces crypt formation on villi.

Wnt signaling induces Wnt component expression either directly or indirectly (Table 1). This shows that there is a clear feedback control in regulation of Wnt signalling pathway (Logan *et al.* 2004). Below table gives an overview of Wnt target genes (Table 1).

Table 1. Wnt signaling components as Wnt pathway targets (Logan C.Y. et al. 2004)

Target gene	Effect of Wnt signal on target gene expression	Effect of changes in target gene expression on Wnt pathway	Target gene interacts with	Reference
Fz	Down	Inactivate	Wnt	(Muller et al. 1999)
Dfz2	Down	Inactivate	Wnt	(Cadigan et al. 1998)
Dfz3	Up	Activate	Wnt	(Sato et al. 1999)
Fz7	Up	—	Wnt	(Willert et al. 2002)
Arrow/LRP	Down	Inactivate	Wnt	(Wehrli et al. 2000)
Dally (HSPG)	Down	—	Wnt	(Baeg et al. 2001)
Wingful/notum	Up	Inactivate	HSPG?	(Giraldez et al. 2002)
naked	Up	Inactivate	Dsh	(Rousset et al. 2001)
Axin2	Up	Inactivate	β-catenin	(Jho et al. 2002)
β-TCRP	Up	Inactivate	β-catenin	(Spiegelman et al. 2000)
TCF1 (dn)	Up	Inactivate	TCF	(Roose et al. 1999)
LEF1	Down	Activate	β-catenin	(Hovanes et al. 2001)
Nemo	Up	Inactivate (*Drosophila*)	β-catenin/LEF/TCF	(Zeng & Verheyen 2004, Thorpe & Moon 2004)
		Activate (*Zebrafish*)		

3.2 Notch Signalling

Activation of Notch signalling through interaction of the Notch receptors with Notch ligands Delta and Jagged leads to expression of target genes such as Hes genes. Expression of the transcription factor Hes1, one of the many multiple targets of Notch signalling, inactivates target gene expression regulating proliferation and differentiation (fig 4a) (Scoville *et al.* 2008). After binding of the ligands to the receptors, S3 cleavage by presenilin takes place and then S3 cleavage in which the Notch receptor is cleaved by ADAM Metalloprotease. Then the cleaved receptor translocates to the nucleus where it forms a transcriptional complex with RBP-jk and activates the expression of its target genes (fig 5). Mammals have four membrane bound type 1 Notch receptors (Bolos *et al.* 2007).and five ligands are identified in mammals (Delta-1, 3, 4 and Jagged 1, 2 (Pannequin *et al.* 2009). Notch 1 and 2, Delta like 1 and 3, Jagged-1 are all expressed in crypt epithelial cells (Scoville *et al.* 2008). Several knockout mice studies were conducted to

unravel the role of Notch signalling in the intestine. Such an experiment involving RBP-jk conditional knockout mice and use of γ-secretase inhibitor results in increase of secretory cells in the intestine (van Es J. H. *et al.* 2005) (fig 4b&c). A similar result was observed in *Hes1*-deficient mice (Jensen J. *et al.* 2000). Fre and colleagues also conducted a mutational study in which constitutive activation of Notch signaling has been shown to increase proliferation and attrition of secretory cells (Fre S. *et al.* 2005). Radtke and colleagues demonstrated thatgut specific mutant mice lacking both Notch 1 and Notch 2 receptors thereby causing loss of Hes1 expression showed that proliferative crypt progenitors differentiate into secretory goblet cells (Radtke *et al.* 2008).

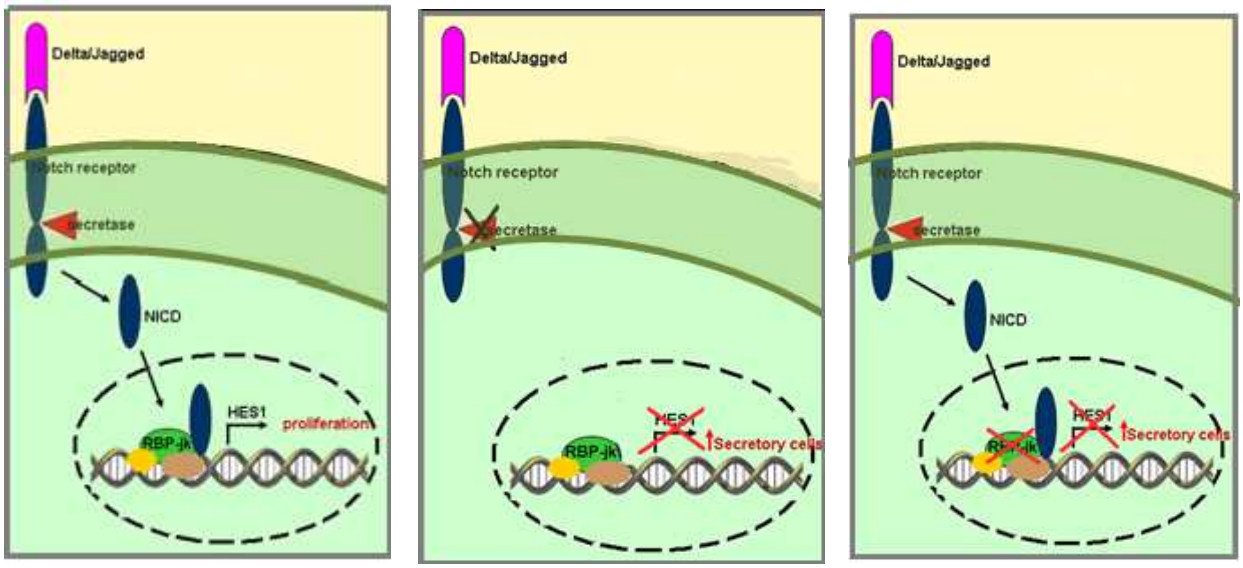

Figure 4 When Notch ligands Delta/Jagged interact with a Notch receptor, γ-secretase cleaves the NICD (Notch Intracellular domain). NICD translocates to the nucleus where it forms a transcriptional complex with RBP-jk. This complex induces expression of Hes which in turn activates expression of target genes resulting in proliferation **(a),** In presence of γ-secretase inhibitors, NICD is not cleaved. Therefore there is no Hes expression taking place. Cells differentiate into secretory cells instead of proliferating **(b)**, RBP-jk knoack-out delivers the same result because Hes expression is not induced and consequently an increase in secretory cells is observed **(c)**

Another finding of Radtke' s research was derepression of CDK inhibitors p27 and p57 of which promoters are occupied with the transcriptional repressor Hes-1 in wild-type progenitor crypt

cells. Thus HES1 represses two CDK inhibitors and plays a role in maintenance of proliferative progenitor crypt cells in the intestine. Cells that differentiate into goblet cells upon inhibition of Notch signaling pathway requires Math 1 transcription factor (van Es *et al.* 2010). Math1 is expressed in secretory progenitor cells and mature cells and is negatively regulated by Notch signaling. When Notch signaling is active, Math1 is repressed in a Hes1 dependent manner. RPB-jk knock-out mice exhibit up-regulation of Math1. Secretory lineages are depleted in *Math1* deficient mice. These findings suggest that Notch signaling is essential for proliferation and its absence results in transition of progenitor cells into secretory goblet cells in a MATH1 dependent fashion. To conclude, Notch signalling pathway is also involved in both cell fate decision and maintenance of progenitor cell proliferation.

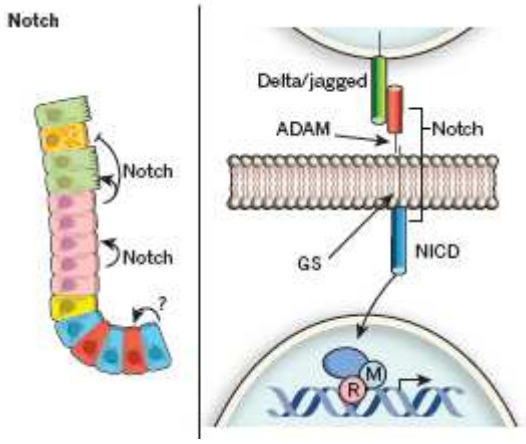

Figure 5 Notch drives proliferation in cooperation with Wnt signalling. Notch target genes are expressed as a result of nuclear translocation of NICD after binding of Notch ligands to their receptor and cleavage of NICD (Vermeulen et al. 2010).

3.3 Bmp signaling

BMPs are known to play a role in limb development, tooth development, primordial germ cell generation and regulation of apoptosis in vertebrates (Cho *et al*. 2001). In the intestine, Bmp signaling pathway is the negative regulator of intestinal stem cell proliferation (Moore *et al.* 2006), as it counteracts Wnt signalling .

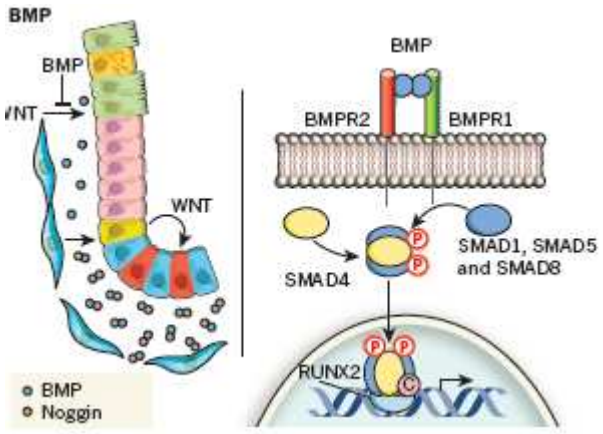

Figure 6 BMPS are produced by the mesenchymal cells. Initiation of Bmp signalling requires heterodimerization of BMPR1 and BMPR2. SMADs are involved in Bmp signalling and required for Bmp target gene expression with the help of RUNX2 cofactor (Vermeulen et al. 2010)

BMPs are expressed by the mesenchymal cells adjacent to ISCs both at the top of the crypt and the bottom (fig 6) (Vermeulen *et al.* 2011; Moore *et al*. 2006). At the top of the crypt, BMPs have a coordinating role in segregation between proliferation and differentiation. BMPs can halt proliferation by inhibiting Wnt signalling. BMP2 and BMP4 are examples of BMPs stopping proliferation at the crypt-villus border, permitting differentiation. Both mice lacking a BMP receptor or overexpressing BMP inhibitor Noggin support this role by leading to hyperproliferation (Moore *et al.* 2006; He *et al*. 2004). Auclair *et al.* 2007 also generated mice lacking Bmpr1a mutant mice lacking BMP receptors by using tissue specific gene ablation. The result was an altered intestinal epithelium and an increased proliferation. SMADs are necessary for Bmp signalling as they are phosphorylated and form a cascade after heterodimerization of of BMPR1 and BMPR2. Phosphorylated SMAD1,5, and 8 associate with SMAD4 (Vermeulen *et al.* 2010), a mediator of TGF-β signaling (Miyaki *et al*. 2003), which in turn drives Bmp target gene expression in the nucleus after translocation. Formation of epithelial cancer is detected in gastrointestinal tract of mice when Smad4- dependent signaling is lost selectively in T cells which shows that these T cells lose the ability to send the right message to their neighboring stromal and epithelial cells (Kim *et al.* 2006). However, epithelial specific deletion of Smad4 gene does not lead to epithelial cancer.

Another observation was that loss of epithelial signaling in Bmpr1a mutant mice has

significant consequences for maturity and terminal differentiation of secretory cells, suggesting an important role for Bmp signaling in epithelial architecture maintenance in the crypt-villus axis. Unlike some other studies in which Bmp signaling was affected in both epithelial and mesenchymal compartments, epithelial loss of Bmp signalling does not lead to de novo crypt (Auclair *et al.* 2007) suggesting a role in maintenance of epithelial cell differentiation, proliferation and cell renewal. On the other hand, formation of de novo crypts in mesenchymal loss of Bmp signaling suggests that this pathway plays a significant role in crypt morphogenesis. At the bottom of the crypt, Noggin binds to BMPs and thereby prevents interaction with receptors leading to block of differentiation initiation.

Based on these findings we conclude that there is a BMP gradient along the crypt- villus axis determining proliferation regulation via Wnt signalling.

3.4 Hedgehog signalling

Hedgehog signalling is mainly involved in patterning, control of smooth muscle cell proliferation and intestinal development. While plenty of data exists on Hedgehog signalling's involvement in intestinal development, this pathway's role in adult intestine is not as clear and pronounced as during development.

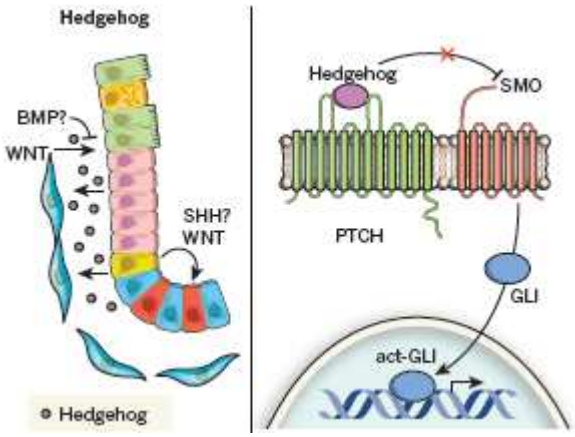

Figure 7 Hedgehogs help epithelium communicate with the mesenchyme. Normally pactched (PTCH) represses smoothened (SMO), however this repression is blocked when hedgehog is bound to the receptor (Vermeulen et al. 2010).

The small intestine of a developing mice restricts expression of Shh and Ihh to the epithelium (Cosnier *et al*. 2006). These two ligands become more concentrated in the region between two villi. The receptors patched 1 and patched 2 are expressed in the mesenchyme. So are the three effectors of hedgehog signaling, Gli 1, Gli 2, and Gli 3 (fig 7). Inhibiting the hedgehog signaling results in absence of villi and highly proliferative intestinal epithelium (Madison *et al*. 2005). Blocking the signalling also leads to an increase in Wnt activation. Cells are not properly differentiated either. Other studies blocking hedgehog signaling either partially, or through unconditional knockouts of Shh and Ihh lead to similar results (Crosnier *et al*. 2006). All these studies show that this signaling between the epithelium and mesenchyme are crucial for villus formation and restricting proliferation to the region between the villi. Conditional deletion of patched, one of the membrane protein through which hedgehog pathway acts, has been shown to not only inhibit Wnt signalling in the small intestine but results in a premature enterocyte differentiation.

The fact that SHH and IHH protein production takes place at highly proliferative regions such as the intervillus pocket and the crypt base (Crosnier *et al*. 2006) suggest that both of these ligands diffuse away from site of production and ensure villus formation at a distance.

4. TISSUE CULTURE SYSTEMS

Study of the intestine mostly depends on *in vivo* methodologies simply because there were not successful *in vitro* approaches until recently. Epithelial cells in primary culture could be maintained up to 10 days (Beaulieu *et al*. 1998). The problem these cultures is that they are difficult to preserve as cells rapidly enter apoptosis after being removed from the basal membrane and the underlying stroma. Although there are 3D organoid cultures allowing proliferation and differentiation of a tissue, they are restricted to embryonic tissues and no organoid culture methodology was applied to the intestine until recently. Ootani and colleagues managed to produce a 3D culture system mimicking the intestinal epithelial growth and differentiation. Mouse neonatal tissue as a starting material can be grown in long-term culture system showing intestinal epithelial expansion as sphere-like organoids with proliferation and multilineage differentiation (Ootani *et al*. 2009). The 3D architecture of the mouse intestine can be preserved for 30 to longer than 350 days. This research also demonstrated that the Wnt antagonist Dkk-1 (Dickkopf-1) inhibits culture growth while a fusion protein between a Wnt agonist and immunoglobin Fc stimulates culture growth. On the other hand, overexpression of secretase inhibitor induces secretory cell differentiation in culture which is consistent with *in vivo* Notch signaling requirement. Lgr5+ and Bmi1+ epithelial cells also expanded *in vitro* upon treatment with the fusion protein. Altogether this study suggests that growth of successful long-term intestinal culture *in vitro* requires presence of both Wnt and Notch signals in the microenvironment . In other words, proliferation and differentiation of an intestinal organoid culture in vitro is possible in a Wnt and Notch dependent ISC niche providing all the necessary signals.

Recently, it has been found that under long term culture conditions single mouse crypts are able to generate villus-like epithelial domains containing all the different cell types of intestinal epithelium (Sato *et al*. 2009). At the same time these crypts go through several crypt fission events. For establishing such a long term culture which can be maintained for longer than 8 months, Sato and colleagues made use of a matrigel containing all the required elements necessary for growth of an intestinal crypt. Previous studies have already shown that crypt growth requires EGF and the Wnt signalling. As Wnt signalling, the Wnt agonist R-spondin 1 is

used to ensure growth. Another element which was added to the matrigel to ensure increase of crypt number was Noggin. Isolated of stem cells result in anoikis because they are detached from the surrounding extracellular environment of the normal tissue. Two extracellular proteins are enriched at the base of intestinal crypts; laminin $\alpha 1$ and $\alpha 2$. Therefore these proteins were also added to the matrigel to further support growth of the crypts. This single lumen is lined with a single layered villus-like epithelium containing all differentiated cells of the intestinal epithelium (fig 8). Similarity of these organoids to freshly isolated colon crypts is confirmed based on microarray expression analysis.

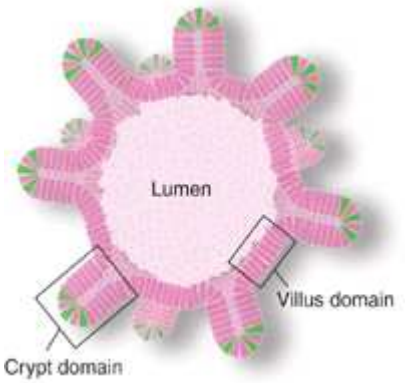

Figure 8 Crypt organoid consisting of a single lumen lined with villus-like epithelium and the surrounding crypt-like domains (Sato et al. 2009)

Sato and colleagues also demonstrated that organoids can be established from single LGR5+ stem cells *in vitro*. These organoids also contained enterocytes, goblets cells, enteroendocrine cells just like organoids derived from whole mouse crypts. Concurrently, Matrigel was provided with a Notch agonist peptide to compensate for loss of cell-to-cell contact due to isolation of single stem cells and thereby loss of Notch signalling which plays an essential role in proliferation of crypts. For the rest, same matrigel- based culture system provided with all the necessary signals such as EGF, the Wnt agonist R-spondin 1, and noggin was used just like for whole cyrpts. Thus it is possible to grow a self organizing, expanding organoid without the surrounding epithelial cellular niche in picture. Finally, this long-term culture system is can be used to generate human organoids. In this sense this culture system can be a very valuable tool not only for regenerative medicine but gene therapy as well. A subsequent mouse study of Sato

and colleagues (Sato *et al.* 2011) shows evidence that Lgr5 stem cells require cell to cell contact with the Paneth cells for maintaining stemness. Intestinal stem cells compete for Paneth cell contact Paneth cells -direct progeny of intestinal stem cells- provide stem cells with niche signalling in the intestine. Besides secreting antimicrobial agents, Paneth cells provide Lgr5 + stem cells with the necessary niche signals. Paneth cells produce CD24+ which is a good marker of paneth cells as mentioned before. CD24+ Paneth cells produce all the signals required for stem cell maintanance in culture. These signals are EGF, TGF-α, Wnt3 and the Notch ligand Dll4. When sorted Lgr5+ stem cells are co-cultured with Paneth cell, organoid formation is enhanced greatly *in vitro*, whereas genetic removal of Paneth cells leads to loss of Lgr5+ stem cells *in vivo*.

Above mentioned long term in vitro culture systems are important because similar cultures can be started from human stem cells.

5. DISCUSSION

In the past century a lot of effort has been made to identify the stem cells of the mammalian intestine and to unravel the molecular cues regulating the mechanisms maintaining proliferation, terminal differentiation, lineage commitment and apoptosis in this tissue. Although the intestinal epithelium is the most rapidly renewing tissue in the mammalian body, stem cells driving the process of cell-renewal have been identified not so long ago.

As mentioned above, two intestinal stem cell models have been proposed. Cycling Lgr5+ CBCs are the active stem cells and LRCs at the +4 position right above the Paneth cells are the quiescent stem cells (Potten *et al.* 1997). Another proposal is that both CBCs and LRCs co-exist in the intestinal crypts as adult intestinal stem cells (Scoville *et al.* 2008; Li *et al.* 2010). Identification of different intestinal stem cells in the crypt has changed the view on monoclonality which suggests that one singe intestinal stem cells generates monoclonal populations (Leblond *et al.* 1974). Existence of more than one type of intestinal stem cells points more to clonality. To what degree these distinct intestinal stem cell candidates interact with each other is not known yet. Although these stem cells candidates have separate functional roles, they can function in a cooperative way. The relationship between these cells requires further research. Another point worth mentioning is that Bmi1 did mark cells at the +4 position and these Bmi1+ cells do give rise to the four epithelial cell types but whether these cells are the quiescent LRCs still needs to be found out.

As we have mentioned earlier, Lgr4 is expressed in all of the cells of the intestinal crypt , obviously including the BMI1+ +4 position cells. It would also be interesting to look at the effect of LGR4 in these LRC cells. Such an investigation could provide us with insights regarding the interdependence of the two distinct intestinal stem cell populations.

Wnt being the most important, all of the in this review mentioned signalling pathways are important in regulation of proliferation, differentiation, and apoptosis. BMP's inhibiting Wnt signaling are negative regulators of ISC proliferation. Obviously BMPs interact with Wnt receptors at the top of the crypt as they ensure differentiation of epithelial cells. When bound to the BMP inhibitor Noggin at the bottom of the crypt, proliferation is not disrupted. Which signals cause the binding of Noggin to BMPs at the bottom of the crypts? Is there some kind of

interaction between Bmp and Hedgehog signalling? More research is required to uncover the cross communication between the signals of different pathways. Therefore it is very important to connect these pathways. Disruption of one pathway can interfere and alter another pathway which in turn may impair the indispensable balance between proliferation , differentiation and apoptosis in the intestinal crypt. Furthermore, disruption of these pathways can lead to developmental defects and have serious consequences such as cancer. For this reason a thorough understanding of the mechanisms involved in these processes has become a necessity given cancer being the epidemic of our century. Identification of the intestinal stem cells together with comprehensive understanding of molecular cues behind regulation of ISCs, the intestinal development and maintenance thereof can help us come up with golden tools to help treat disease and cancer. Establishment of long-term culture systems in which 'miniguts' are created from one single mouse LRG5+ stem cell is one such golden tool that can be applied to human intestinal stem cells. This culture system also opens new avenues for treatment with transplantation.

Researchers have applied two approaches to get a better understanding of molecular regulation of intestinal stem cells: the transplantation approach and the genetic marking approach as defined by Barker et al. (2008). The first approach makes use of molecular markers to identify and enrich the stem cells and then makes use of in vitro cultures and /or transplantation. Genetic marking is more challenging as this second approach must happen at the level of single stem cells and involves genetic modification. Recently established long- term *in vitro* cultures can make a big difference for patients suffering from diseases such as colorectal cancer.

Colorectal cancer (CRC) has become the number one cause of cancer mortality in developed countries in the 21st century (Jemal *et al*. 2006). Colorectal cancer is mostly caused by mutations in Wnt signaling pathway. There are two types of mutations of the Wnt signaling pathway components both of which results in translocation of β-catenin to the nucleus and thereby activating the Wnt target genes (Fodde *et al*. 2007). Either inactivating mutations occur by which suppressor genes APC or Axin are inactivated, or activating mutations occur by which β-catenin oncogene is activated. Genomic rearrangements, deletions or insetions might

eventually lead to activation or in activation of target genes. Loss of control over proliferation due to genetic alterations may result in tumors. Tumor growth might be a consequence of various cellular changes such as losing sensitivity to growth-inhibiting signals, evading apoptosis, limitless replicative potential (Hanahan *et al.* 2000). For example, both Wnt and Notch signallings are abnormally active in CRC (Pannequin *et al.* 2009).

Several molecules that are associated with signalling pathways are found in tumor cells. One such molecule is progastrin. Pannequin and colleagues have shown that depletion of progastrin in CRC cells leads to inhibition of Wnt signaling and an increase in secretory cell differentiation. Several laboratories showed that a big proportion of CRC tumors secrete a gastrin precursor called progastrin which is capable of activating signaling pathways elevating cancer progression ((Pannequin *et al.* 2009; Ottewel *et al.* 2005; Singh *et al.* 2000). Downregulation of progastrin inhibits Notch signaling and as a result the secretory lineage markers Math-1 and Hath-1 expression is restored in CRC cells. This effect is due to a decrease in Jagged-1 expression. When Jagged-1 levels are restored, Tcf 4 activity is activated. his points to presence of a feedback from Notch towards Wnt signaling. Mannequin's findings make progastrin interesting for lessening abnormal activation of Wnt and Notch pathways in CRC cells. These kind of molecules associating with hyperproliferation can be targeted for treatment of cancer as well.

It is believed that there might be Cancer Stem Cells (CSC) and this CSC hypothesis seems to promise a more effective treatment of cancer as it targets the starting point of cancer. However, presence of cancer stem cells is still under investigation.

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