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Stem cells in gastrointestinal cancers

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1. Introduction

The malignant change from normal to cancer stem cells is a hallmark transition in gastrointestinal carcinogenesis. In addition to their ability for self-renewal, stem cells are sufficiently long-lived to acquire the necessary sequential mutations that allow for malignant transformation. Cancer stem cells (CSCs) have the capacity to initiate and maintain tumor growth in several cancers, though only in the past decade have these cells been identified and characterized in hematological malignancies [1–4]. Recent studies have described CSCs in solid tumors including cancer of the breast, prostate, brain, colon, pancreas, and liver [5–21]. However the mechanism underlying the emergence of cancer stem cell formation remains elusive. In GI cancers, signaling pathways such as TGF- β , Wnt, FGFs, and Hedgehog are responsible for gut development and have a potential role in the formation of CSCs. Underlying this hypothesis is the finding that deregulated signaling pathways in gut development, as manifested in rare human cancers as well as genetic mouse studies, are driven by a population of CSCs. Importantly, it was found that the same surface markers used to identify embryonic stem cells are capable of identifying and sorting CSCs as well. These phenomena point to the need for scientists to understand how CSCs become insensitive to inhibitory signals as well as chemotherapeutic agents and are thus permitted to continuously self-renew. Targeting CSCs holds much promise for the development of novel therapeutic agents.

2. Identification of the key genes in cancer stem cells of GI malignancies

2.1. Role of Wnt/ β -Catenin signaling pathway

Mutations in the adenomatous polyposis coli (*APC*) gene, a critical component of the WNT pathway, act to suppress Wnt signaling and result in familial adenomatous polyposis (FAP) syndrome [22]. In the canonical Wnt pathway, the binding of Wnt ligands to the Fz receptors results in activation of the disheveled protein and the subsequent inhibition of glycogen synthase kinase 3β (GSK- 3β). This in turn prevents APC and Axin dependent degradation of β -catenin, leading to its accumulation in the cytosol, where it then translocates to the nucleus and binds to transcription factors that regulate tissue patterning, cell fate, and cell proliferation [23]. The canonical β -catenin signaling pathway plays a central role in modulating the delicate balance between self-renewal and differentiation in several adult stem cell niches including regeneration of the mammary gland, hair follicle, intestinal crypt, and the skin [24,25]. In the

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majority of sporadic colorectal cancer cases, either loss of *APC* function or oncogenic β -catenin mutations seem to be the early events in tumor development. *Apc* 1638N, the chain-termination mutation, results in multiple intestinal tumors in mice [26]. Likewise, mutations in the glycogen synthase kinase 3 β (GSK-3 β) phosphorylation sites of the β -catenin gene are found in 20–30% of human primary hepatocellular carcinoma (HCC) [27], while mutations in the *APC* or *AXIN* genes are found in other HCC populations [28]. These findings point to the canonical Wnt cascade as a critical regulator of stem cells, and highlight the importance of accumulated nuclear β -catenin as a key event in carcinogenesis of GI malignancies [29].

2.2. Role of BMP receptor 1A and TGF β family signaling

The TGF- β family signaling is most prominent at the interface of development and cancer in gut epithelial cells and is a key player in the self-renewal and maintenance of stem cells [30]. TGF- β forms a complex with the serine-threonine kinase receptors type I (T β RI) and II (T β RII). The constitutively active T β RII phosphorylates T β RI, which in turn phosphorylates one of the receptor-activated (R-Smads) [31]. The active R-Smad will heterodimerize with the common mediator Smad, Smad4, and the two translocate to the nucleus where they drive the transcription of target genes. Their activity is modulated by adaptors such as SARA in the case of Smad2 [32], and ELF in the case of Smad3 and Smad4 [33], though the activity of these adaptor proteins also include functional interactions with multiple signal transduction pathways apart from the TGF- β pathway. When TGF- β signaling is disrupted, the imbalance can result in an undifferentiated phenotype which may set the stage for cancer development. Functional breakdown of different TGF- β members is observed throughout the spectrum of GI malignancies. For example, colon cancer involves inactivating mutations in the TGF- β type II (T β RII) receptor. Moreover, intracellular signaling is disrupted in pancreatic carcinoma through the inactivation of Smad4, also known as DPC4 (deleted in pancreatic carcinoma locus 4), which occurs in one-half of pancreatic carcinoma cases [34]. Significant loss of ELF and reduced Smad4 expression are also found in human gastric and colon cancer tissue samples [35,36]. Genetic studies in mice have suggested that loss of TGF- β signaling plays an important role during early tumor development. Mice that are heterozygously null for *smad4* develop gastric polyps that can develop into tumors at a late age. A wide range of GI tumors, including those of the stomach, liver and colon are found in *elf*^{+/-} and *elf*^{+/-}*lsmad4*^{+/-} mutant mice [37]. Forty percent of *elf*^{+/-} mice spontaneously developed hepatocellular carcinoma indicating that functional TGF- β signaling is a critical component in maintaining normal stem cells in GI malignancies.

An important finding in spontaneously developed hepatocellular carcinoma involves the observation that CDK4 and IL6/Stat3 signaling are constitutively activated in this cancer. Down-regulating CDK4 or IL6/Stat3 attenuates hepatocellular carcinoma formation. Since CDK4 and IL6/Stat3 play important roles in liver development and regeneration, regulating CDK4 and IL6/Stat3 signaling by TGF- β may be one of the mechanisms responsible for controlling the signal between self-renewal and differentiation in liver stem cells. Mutations in the TGF- β pathway, resulting in unregulated CDK4 and IL6/Stat3 activation, lead to increased cell self-renewal and decreased differentiation.

2.3. Role of PTEN-Aki pathway

Mutations that affect receptor tyrosine kinase signaling pathways have been found in inherited polyposis syndromes. Mutations in PTEN, a phosphatase that antagonizes PI3 kinase activity, causes Cowden's syndrome which includes hamartomas in the gastrointestinal tract, central nervous system, and skin, as well as tumors of the breast and thyroid gland [38]. Intestinal polyposis, a precancerous neoplasia, results primarily from an abnormal increase in the number of crypts, which contain intestinal stem cells. In PTEN-deficient mice, excess intestinal stem cells initiate *de novo* crypt formation and crypt fission, recapitulating crypt production in fetal

and neonatal intestines [39]. Additionally, PTEN helps control the proliferative rate and the number of intestinal stem cells, and loss of PTEN results in an excess number of intestinal stem cells. It is proposed that the PTEN-Akt pathway probably governs stem cell activation by helping control nuclear localization of the Wnt pathway effector β -catenin. Nuclear localization of β -catenin is considered a key event in the activation of stem cells and is potentiated by Akt phosphorylated β -catenin at Ser552. In addition, Akt phosphorylates GSK-3 β at serine 9, allowing for the accumulation of β -catenin in the nucleus. This process is found to be Smad7-dependent and regulated by TGF- β signaling.

2.4. Crosstalk among Wnt signaling, TGF- β signaling and PTEN-AKT signaling

Wnt signaling, TGF- β signaling, and PTEN-AKT signaling seemingly integrate in the formation of cancer stem cells in GI malignancies (Fig. 1). As mentioned earlier, PTEN-AKT phosphorylates β -catenin and GSK-3 β , β -catenin accumulates in the nucleus, and cells are able to proliferate. This crosstalk between PTEN-Akt and Wnt- β -catenin is Smad7-dependent and TGF- β regulated.

Wnt signaling and TGF- β signaling interact at different levels. E-cadherin accumulation at cell-cell contacts and E-cadherin- β -catenin-dependent epithelial cell-cell adhesion is disrupted in *elf*^{+/-}/*Smad4*^{+/-} mutant gastric epithelial cells, though it is rescued by ectopic expression of full-length *elf*, but not by Smad3 or Smad4 [37]. Smad4 potentiates the activity of transcription factor Lef-1 downstream β -catenin [40]. Axin from the Wnt signaling pathway associates with Smad3 in the cytoplasm and facilitates phosphorylation by T β RI/II, then disassociates when phospho-Smad3 associates with Smad4 [41]. Remarkably, TGF- β induced redistribution of β -catenin is Smad7-dependent [42]. The critical role of Wnt/ β -Catenin, TGF- β family signaling and PTEN-Akt pathways in gut development and GI malignancies already reveal crosstalk possibilities among these pathways which may elucidate the disruption of normal stem cells in GI carcinogenesis, paving the way for future therapeutic development.

3. Isolation of CSCs

Multiple surface markers that are used to identify embryonic stem cells have been used to identify and sort cancer stem cells. CD133 (AC133) is a highly conserved antigen as the human homologue of mouse Prominin-1, which was originally identified as a 5 transmembrane cell surface glycoprotein expressed in a sub-population of CD34⁺ hematopoietic stem and progenitor cells derived from human fetal liver and bone marrow [43]. GD133 is expressed by normal primitive cells of the neural, hematopoietic, epithelial and endothelial lineages. Notably, CD133⁺ cells were found in some types of tumor tissues including tumors associated with AML, brain, ependymoma and prostate [17,18,44]. In glioblastoma as few as 100 CD133⁺ cells were described to be able to produce tumors in immunodeficient mice, whereas 1×10^5 cells from the same tumor without this surface molecule failed to produce a similar outcome [18]. Moreover, CD133 is found to be able to distinguish CSCs from non-CSCs in colon cancer [45,46]. In colon cancer it was found that only 2.5% of tumor cells were in fact CD133⁺, though only this cohort of cells was capable of reproducing the primary tumor in immunodeficient mice upon subcutaneous injection of these cells. Unlike CD133⁻ cells, CD133⁺ colon cancer cells grew exponentially for more than one year *in vitro* as undifferentiated tumor spheres in serum-free medium, maintaining the ability to engraft and reproduce the same morphological and antigenic pattern as the original tumor. CD133⁺ colon cancer cells can differentiate and become CK20⁺, thus losing their ability to be transplanted into SCID mice. Following a similar pattern as observed in mice, CSCs from human colon cancer samples may be isolated on the basis of their ability to initiate human colon cancer after transplantation into NOD/SCID mice. Purification experiments established that all CSCs able to initiate tumor growth were CD133⁺. The ratio of CSCs as a proportion of total tumor cells reveals that there is only one CD133⁺ CSC in 5.7×10^4 unfractionated tumor cells, whereas

there is one CD133⁺ CSC in only 262 CD133 cells, which represents a >200-fold enrichment by using CD133 as a marker. Furthermore, it was observed that CSCs are bidirectional within the CD133⁺ population, as they were both able to maintain themselves as well as to differentiate and re-establish tumor heterogeneity upon serial transplantation.

The fact that regenerative CD117⁺/CD133⁺ hepatic precursor cells are identified in fresh frozen liver samples from patients suffering from massive liver necrosis supports the use of CD133 as a marker for liver progenitor cells [47]. CD133 has been used to isolate CSCs in hepatocellular carcinoma recently [39,48]. From the SMMC-7721 cell line, CD133⁺ cells isolated by MACS manifested high tumorigenicity and clonogenicity as compared with CD133⁻ HCC cells [46].

However, it is not clear that CD133 is sufficient alone in isolating HCC cancer stem cells. While CD133 is used to sort these progenitors from Huh7 cells, CD133⁺ cells were detected in 46.7% of Huh-7 cells [48]. However, when flow cytometry and the DNA-binding dye Hoechst 33342 were used to isolate side population (SP) cells from various human gastrointestinal system cancer cell lines, SP cells were detected only in 0.25% of Huh7 [49]. SP analysis and sorting followed by serial transplantation of the cells into NOD/SCID mice was used to isolate HCC cells with stem cell properties. Only 1×10^3 SP cells were sufficient for tumor formation, whereas an injection of 1×10^6 non-SP cells did not initiate tumors, [Microarray analysis identified a differential] gene expression profile between SP and non-SP cells. It seems two or more markers are necessary to identify CSCs reliably in HCC.

CD44 and CD24 have been used to isolate breast CSCs (Ponti, 2005). A subpopulation of pancreatic cancer cells expressing the cell surface markers CD44, CD24, and epithelial-specific antigen [50] are found to be highly tumorigenic using a xenograft model [51]. Pancreatic cancer cells with the CD44⁺CD24⁺ESA⁺ phenotype (0.2–0.8% of pancreatic cancer cells) have a 100-fold increased tumorigenic potential compared with nontumorigenic cancer cells and 50% of animals injected with as few as 100 CD44⁺CD24⁺ESA⁺ cells formed tumors that were histologically indistinguishable from the human tumors from which they originated. The enhanced ability of CD44⁺CD24⁺ESA⁺ pancreatic cancer cells to form tumors was confirmed by using an orthotopic pancreatic tail injection model. The CD44⁺CD24⁺ESA⁺ pancreatic cancer cells showed the stem cell properties of self-renewal as well as the ability to produce differentiated progeny, with a further increase in the expression of the developmental signaling molecule sonic hedgehog.

GI malignancies are noted for their characteristically high incidence of disease relapse after surgery or chemotherapy as well as resistance to chemotherapy, all of which may be explained by the aggressive phenotype of their CSCs. To effectively combat GI cancers, specific markers for CSCs must be used to target these populations using novel therapeutic agents.

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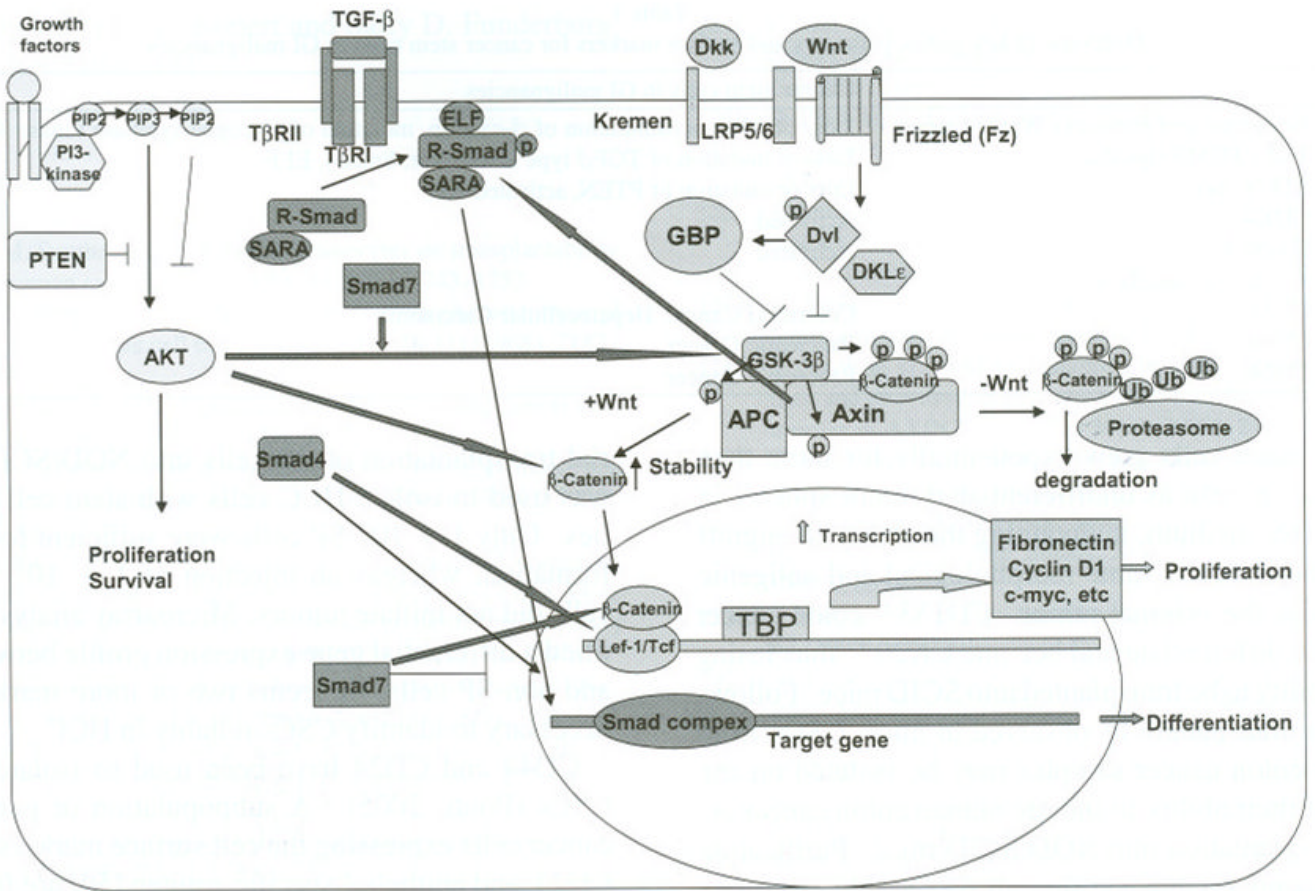


Fig. 1. Crosstalk between Wnt, TGF- β , PTEN-AKT signaling pathway.

Table 1

Overview of key genes, pathways and surface markers for cancer stem cells in GI malignancies

Cancer stem cells in GI malignancies	
<i>Key genes and Pathways</i> Wnt/ β -catenin	Loss of APC, accumulation of β -catenin, mutation of glycogen synthase kinase 3 β
TGF- β /BMP signaling	Loss or mutation of TGF β type II receptor, Smad4, ELF
PTEN-Akt	Loss or mutation of PTEN, activated Akt
CDK4	Activated
IL6/Stat3	Activated
<i>Key surface markers</i>	
CD133	Colorectal Cancer, Hepatocellular Carcinoma
CD44	Pancreatic Cancer
CD24	Pancreatic Cancer