Self-renewal molecular mechanisms of colorectal cancer stem cells

TIANHUI PAN[1], JINGHONG XU[2] and YONGLIANG ZHU[1]

[1]Laboratory of Gastroenterology and [2]Department of Pathology, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang 310009, P.R. China

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Abstract. Colorectal cancer stem cells (CCSCs) represent a small fraction of the colorectal cancer cell population that possess self-renewal and multi-lineage differentiation potential and drive tumorigenicity. Self-renewal is essential for the malignant biological behaviors of colorectal cancer stem cells. While the self-renewal molecular mechanisms of colorectal cancer stem cells are not yet fully understood, the aberrant activation of signaling pathways, such as Wnt, Notch, transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP) and Hedgehog-Gli (HH-GLI), specific roles mediated by cell surface markers and micro-environmental factors are involved in the regulation of self-renewal. The elucidation of the molecular mechanisms behind self-renewal may lead to the development of novel targeted interventions for the treatment of colorectal cancer.

Contents

1. Introduction
2. Pathways involved in the self-renewal of CCSCs
3. Molecular surface marker-mediated self-renewal of CCSCs
4. Microenvironment-mediated self-renewal of CCSCs
5. Inflammation factors

1. Introduction

Colorectal cancer (CRC) is the second most common type of cancer and the fourth leading cause of cancer-related mortality worldwide, occupying approximately 9.7% of the total cancer cases and 8.5% of the number of deaths in 2012 (1). CRC is composed of heterogeneous cell populations differing in gene expression and tumorigenicity (2,3). Sporadic CRC and hereditary CRC both originate from the stem cell stage. CRC stem cells (CCSCs) represent a small fraction of the CRC cell population with self-renewal and multi-lineage differentiation potential and the ability to drive tumorigenicity (4).

It is thought that CCSCs originate in three different ways: first, they may be derived from the malignant transformation of normal colorectal stem cells. Colorectal stem cells have the ability to proliferate and self-repair. Gene mutations can further accumulate during their long survival. Evidence has demonstrated that colorectal stem cells become tumorigenic more easily (4-6). Barker *et al* suggested that only Leucine-rich repeat-containing G protein-coupled receptor 5 $(Lgr5)^+$ stem cells, in cooperation with *APC*-deficiency, may lead to colorectal adenoma formation (7). The stem-like $Lgr5^+$ tumor initiating cells located in the base of adenomas are similar to normal stem cells (8). In the initiation process of CRC, normal colorectal stem cells acquire oncogenic mutations through the interaction between internal and external factors. Subsequently, in the evolution of CRC, the heterozygous loss of *APC*, *DCC* and *p53* occurs, accompanied by DNA damage, DNA-repair mutations and altered methylation status (9,10). Second, CCSCs may originate from the dedifferentiation of common cancer cells. Cells with certain differentiation characteristics, such as progenitor cells or mature cells, acquire stemness by dedifferentiation. The successful induction of induced pluripotent stem cells (IPS) has demonstrated that differentiated cells, even in the stage of terminal differentiation, can regain stemness through a reset by certain specific regulation factors. Transducing transcription factor *Oct3/4*, *Sox2*, *c-Myc* and *Klf4* into mouse fibroblast cells can drive cells to dedifferentiate and acquire stemness (6). Schwitalla *et al* indicated that increasing nuclear factor-κB (NF-κB) signaling in intestinal epithelial cells would activate the Wnt signaling pathway, thus eliciting dedifferentiation and promoting tumorigenicity (11). Third, CCSCs may originate from cell malignant transformation through the influence of the micro-environment. The transformation of non-cancer stem cells to cancer stem cells is dependent on transforming growth factor-β (TGF-β) signaling in the micro-environment, and the process is most likely relevant to epithelial-mesenchymal transition (EMT) (12,13). Mani *et al* found that mammary gland cells undergoing EMT by Snail or Twist induction regained stem cell markers and the ability to self-renew (14).

CCSCs are heterogeneous, as they contain various subpopulations or are in different stages of stem cell development (2). B-cell-specific Moloney murine leukemia virus insertion

Correspondence to: Dr Yongliang Zhu, Laboratory of Gastroenterology, The Second Affiliated Hospital of Zhejiang University School of Medicine, 88 Jiefang Road, Hangzhou, Zhejiang 310009, P.R. China
E-mail: drylzhu@163.com

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site 1 $(Bmi1)^+$ quiescent cancer stem cells are insensitive to high-doses of radiation, while $Lgr5^+$ active cancer stem cells have a strong homeostatic regeneration ability (15). If the latter become injured or destroyed, the former can mobilize to transform into an active status. Hence, quiescent cancer stem cells most likely function as a reservoir to maintain the homeostasis of stem cells. The micro-environment dictates the balance between them (15,16). At present, therapy for CRC targets mainly active cells, while quiescent stem cells can escape, leading to relapse and resistance to treatment.

CCSCs are similar to normal adult stem cells as regards biomarkers (Table I). Consequently, three methods have been developed to isolate CCSCs: the first is dependent on cell surface markers. CCSCs can be isolated by FACS based on $CD133^+$ (17,18), $CD44^+CD24^+$ (19), $CD44^+CD58^+$ (20) and $CD166^+$ (21,22). The second is dependent on the characteristic of specific enzymes, such as aldehyde dehydrogenase 1 (ALDH1) (23) and ATP-binding cassette subfamily G member 2 (ABCG2) (24). The third is culturing the cells in serum-free, low-adhesion conditions *in vitro* and enriching suspending colospheres (25). The methods for identifying CCSC properties include evaluating the ability of continuous sphere formation *in vitro*, continuous tumorigenicity in NOD/SCID mice and the similarity of differentiation between xenograft tumors and primary tumors.

The characteristics of CCSCs are regulated by different mechanisms. Self-renewal is a fundamental feature of the malignant biological behaviors of CCSCs. Several pathways, cell surface markers and micro-environmental factors are involved in CCSC self-renewal.

2. Pathways involved in the self-renewal of CCSCs

The aberrant activation of signaling pathways plays important roles in the evolution and progression of CRC. The Wnt, Notch, bone morphogenetic protein (BMP)/TGF-β, Hedgehog-Gli (HH-GLI), epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (MAPK), NF-κB and Akt/mTOR pathways are involved in the self-renewal of CCSCs (Fig. 1).

Wnt pathway. The Wnt pathway is one of the most important pathways in the tumorigenesis and progression of CRC. Over 90% of CRC cases display an over-activation of Wnt signaling (26). According to whether this activation is dependent on transcriptional regulation by transporting β-catenin into the nucleus, the Wnt pathway is divided into the canonical pathway (β-catenin-dependent) and the non-canonical pathway (β-catenin-independent). The canonical pathway mainly consists of extracellular signaling proteins (Wnt1, Wnt3a, Wnt8, etc.), the transmembrane receptor Frizzled (Fzd), co-receptor low-density lipoprotein-related receptor 5/6 (LRP5/6), Dishevelled (Dsh), β-catenin, axis inhibitor (Axin) and the intranuclear transcription factor T-cell factor (TCF)/lymphoid enhancer factor (LEF). In the absence of Wnt, β-catenin interacts with Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase-3β (GSK-3β) to form a destruction complex and is phosphorylated by GSK-3β. Phosphorylated β-catenin recruits ubiquitin E3 β-transducin repeat containing protein (β-TrCP) and is then degraded by the proteasome, thus maintaining cytoplasmic β-catenin at a relatively low level (27-29). With Wnt signaling, the Wnt protein binds to the Fzd-LRP complex and recruits Dsh to the cytomembrane. The induction of phosphorylation by Dsh separates GSK-3β from Axin and inhibits the formation of the Axin-GSK-3β-APC complex, inhibiting the phosphorylation and ubiquitination of β-catenin. Free β-catenin accumulates in the cytoplasm and translocates to the nucleus, targeting LEF and TCF (30). These proteins promote the transcription and expression of downstream targets, such as *c-Myc*, *cyclin D1* and *Axin2* (31). Disrupting the β-catenin/TCF-4 activity of CRC cells induces a rapid G1 arrest and blocks the proliferative compartment in colon crypts from genetic programming. The suppression by *c-Myc* on the promoter of the cell cycle inhibitor p21 plays an important role in this process. Evidence from conditional gene deletion of *c-Myc* suggests that *c-Myc*(-/-) crypt cells are smaller in size and have a slower cycle compared to wild-type cells and *c-Myc*-deficient crypts are prone to loss and can be replaced by *c-Myc*-proficient crypts within weeks (32,33).

In recent years, many other non-classical Wnt proteins have been discovered, establishing a much more complex and precise regulatory network for the canonical Wnt pathway. Yap/TAZ appears to be an important component of the β-catenin destruction complex. In the absence of Wnt, YAP/TAZ recruits β-TrCP to the complex and degrades β-catenin, blocking pathway activity. When the Wnt pathway is activated, YAP/TAZ is released from the complex so that β-catenin can accumulate in the nucleus to stimulate downstream effectors (34). Polycomb group protein Bmi-1 activates Wnt signaling by upregulating the transcription of Wnt factors Wnt3A, Wnt7A, Wnt10A and Wnt4 or by repressing the DKK family. The Wnt downstream target *c-Myc* in turn promotes the transcription and trans-activation of Bmi-1, forming a positive feedback loop (35). Oncogenic transcription factor MYB cooperates with β-catenin to co-stimulate *c-Myc* expression (36).

High Wnt activity can define the CCSC population functionally. CRC cells with high Wnt activity upregulate the expression of the stem cell-associated genes, *Lgr5* and achaete-scute family bHLH transcription factor 2 (*ASCL2*), while ones with low Wnt activity upregulate the expression of the epithelial differentiation-associated genes, mucin 2 (*MUC2*), keratin 20 (*KRT20*) and fatty acid binding protein 2 (*FABP2*) (37). $CD133^+$, $CD24^+CD29^+$ or $CD44^+CD166^+$ cells exhibit high Wnt activity (37). High Wnt activity is associated with high clonogenic cancer stem cell potential, while low Wnt activity is not (37). Similar evidence defines the association between Wnt activity and CRC cell proliferation and EMT (38).

Non-canonical Wnt signaling mainly consists of the Wnt/Ca^{2+} pathway and JNK-mediated planar cell polarity (PCP) pathway. Under different conditions, non-canonical Wnt pathways function synergistically or antagonistically with the canonical Wnt pathway. The Wnt/Ca^{2+} pathway is activated by the interaction between the Wnt-Fzds complex and co-receptor Ror 1/2. They activate phospholipase-C (PLC) to generate 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3), facilitating the accumulation of Ca^{2+} in the cytoplasm. The increase in Ca^{2+} level activates calcinurin (Cn), calmodulin-dependent protein kinase II (CaMKII) and protein

Table I. The comparison of biomarkers between normal stem cells and CCSCs.

	Marker (Ref.)	Property/function
Normal colorectal stem cells	CD29 (140)	Cell adhesion molecule
	DCAMKL-1 (141)	Kinase
	Lgr5 (106)	Component of the Wnt receptor complex
	Msi-1 (142)	RNA-binding protein
	ALDH1 (23)	Detoxifying enzyme
Colorectal cancer stem cells	Bmi1 (143)	A polycomb group protein that regulates gene silencing
	CD24 (144)	Cell adhesion molecule
	CD29 (87)	Cell adhesion molecule
	CD44 (91)	Cell adhesion molecule
	CD49f (145)	Cell adhesion molecule
	CD58 (20)	Cell adhesion molecule
	CD66c (146)	Cell adhesion molecule
	CD133 (18)	Glycoprotein, the most classic marker of CCSCs
	CD166 (21,22)	Cell adhesion molecule
	DCLK1 (147)	Kinase
	EpCAM (22)	Cell adhesion molecule
	Lgr5 (106)	Component of the Wnt receptor complex
	Msi-1 (142)	RNA-binding protein
	OLFM4 (148)	Glycoprotein

CCSCs, colorectal cancer stem cells; Lgr5, Leucine-rich repeat-containing G protein-coupled receptor 5; Msi-1, Musashi-1; ALDH1, aldehyde dehydrogenase 1; DCAMKL-1, doublecortin and CaM kinase-like-1; DCLK1, doublecortin-like kinase 1; EpCAM, epithelial cell adhesion molecule; OLFM4, olfactomedin-4.

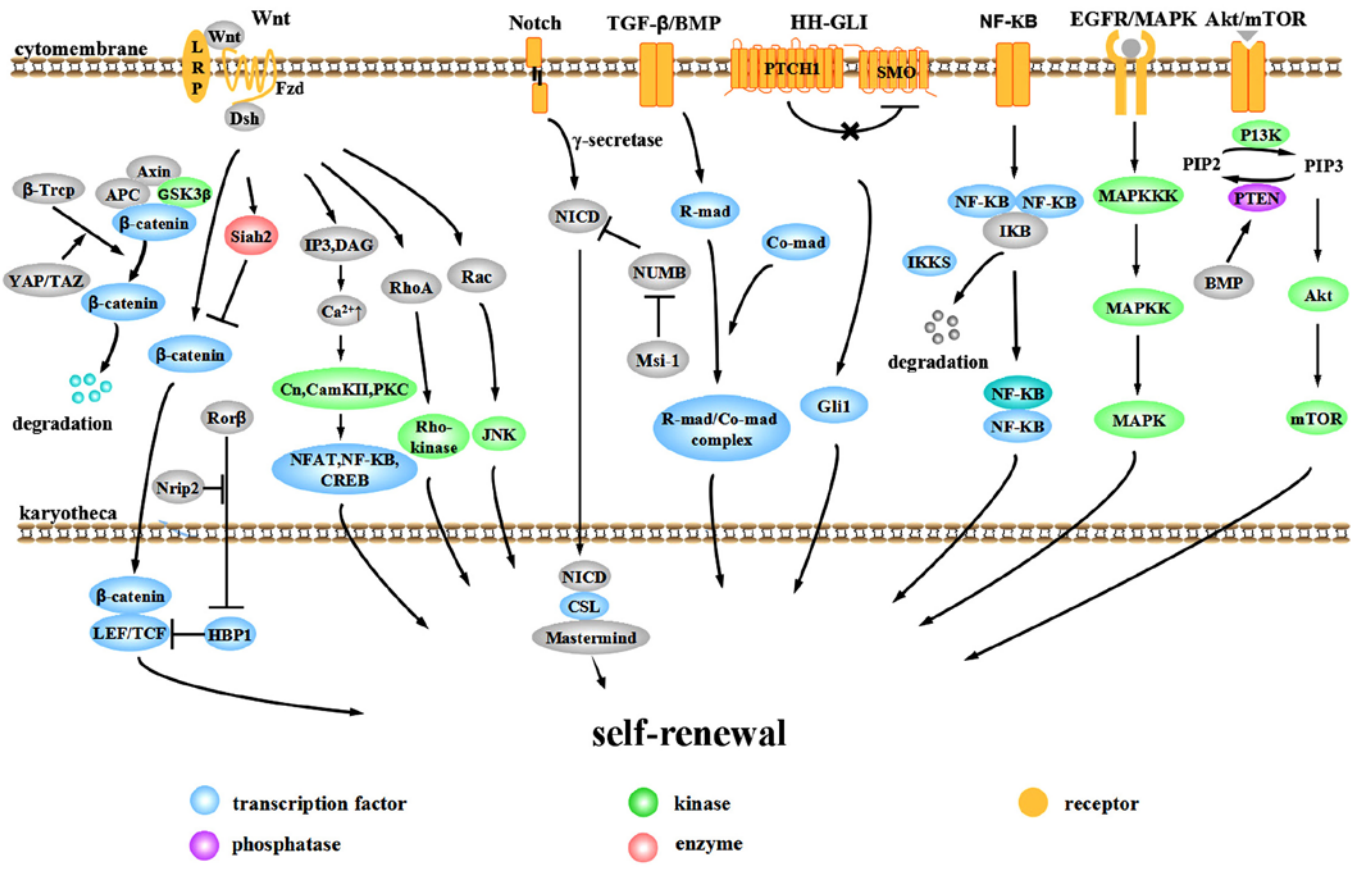

Figure 1. Pathways involved in the self-renewal of colorectal cancer stem cells (CCSCs).

kinase C (PKC), which further activate the transcription factor nuclear factor of activated T-cells (NFAT), NF-κB and cAMP response element binding protein (CREB) (39). In addition, Wnt5/Ror signaling can also generate ubiquitin ligase Siah2, calpain and caudal type homeobox 2 (CDX2). CDX2 acts as a transcription factor to regulate the downstream targets (39). Wnt5a acts differently as a proto-oncogene or tumor suppressor in different tissues. With respect to CRC, MacLeod *et al* found that Wnt5a can generate Siah2 and promote β-catenin phosphorylation and degradation, which inhibit the growth of cancer stem cells (40). PKC can phosphorylate β-catenin independent of GSK-3β to facilitate degradation (41). Moreover, PKC can suppress APC phosphorylation, suggesting that PKC can inhibit colorectal cells from proliferating through the negative regulation of the canonical Wnt pathway by APC (42). The PKCα-dependent phosphorylation of retinoic acid-related orphan nuclear receptor α (RORα) on serine residue 35 can suppress the expression of target proteins of the canonical Wnt/β-catenin pathway (43). CaMKII acts upstream to activate the TAK1-NLK pathway and inhibit the DNA-binding activity of the β-catenin-TCF-4 complex through serine/threonine phosphorylation of TCF-4 (44). The Wnt/PCP pathway is mediated by Wnt (Wnt5a, Wnt11)-Fzd and Dsh. Wnt/PCP plays an important role in regulating tissue polarity and cell motility through the activation of small GTP-binding proteins, including Rac and RhoA, and protein kinases, including c-Jun N-terminal kinase (JNK), Rho-associated kinases and nemo-like kinase (NLK) (45). Van-Gogh-like 2 is an important component of Wnt/PCP, essential in establishing epithelial cell polarity. Van-Gogh-like 2 inhibits CRC through antagonizing the canonical Wnt pathway (46). By contrast, JNK/c-Jun regulates the expression of TCF4 to promote canonical Wnt signaling (47).

We recently found that nuclear receptor-interacting protein 2 (Nrip2) is a novel interactor of the non-canonical Wnt pathway. Nrip2 inhibits the transcription of HMG-box transcription factor 1 (HBP1) through the arrest of retinoic acid-related orphan nuclear receptor β (RORβ) in the cytoplasm and and its subsequent degradation to promote the transcription of the downstream gene, TCF/LEF, a process connected to activating the canonical Wnt signaling pathway (unpublished data).

Notch pathway. Notch can also be divided into canonical and non-canonical pathways. Typical Notch ligands include Delta-like (DLL)1, DLL3, DLL4, jagged (JAG)1 and JAG2 with a Delta-Serrate-Lag 2 (DSL) domain, while atypical ligands include DNER, F3/Contactin and NB-3 without a DSL domain. When the ligand interacts with the Notch1, Notch2, Notch3 or Notch4 receptor, continuous proteolysis is triggered by γ-secretase, releasing the active Notch intracellular domain (NICD). In the canonical Notch pathway, NICD translocates to the nucleus and binds to the transcription factor, CSL. Then CSL-NICD complex is activated by Mastermind family co-activators for the transcriptional activation of targets *HES1* and *HEY1* to suppress differentiation and maintain stemness. Otherwise, NICD binds to nuclear p50 or c-Rel to activate NF-κB activity 9 (non-canonical pathway). Another non-canonical Notch pathway is triggered by an atypical Notch ligand to form the CSL-NICD-Deltex complex, activating MAG transcription and promoting differentiation (48). Which pathway is activated depends on the interaction between ligands and receptors. NUMB regulates intracellular Notch activity in the process of cell division, inhibiting Notch transmission in the cytoplasm (49,50). Musashi-1 (Msi-1), a conservative RNA-binding-protein (RBP), can upregulate Notch activity by inactivating NUMB (51).

In general, an aberrantly activated Notch pathway is oncogenic although anti-oncogenic partly in dermatoma and the cervical uterus (48). Notch activity in the CRC initiating cells is 10- to 30-fold higher than in colon cancer cells. Notch inhibits apoptosis of CCSCs by repressing the cell cycle inhibitor p27. In addition, Notch can maintain self-renewal and inhibit differentiation through repressing secretory cell lineage differentiation targets *MUC2* and atonal homolog 1 (*ATOH1*) (52).

Notch1 and *HES1* are involved in the malignant transformation of the normal colonic mucosa (53). *HES1* increases stemness-related genes in CRC cells and leads to the overexpression of CCSC markers, such as CD133, ABCG2, Nanog and ALDH1. Additionally, *HES1* increases the size of CD133 and stem-like side population cells to enhance self-renewal properties (54). The inhibition of Notch signaling by a γ-secretase inhibitor can inhibit the growth of CRC cells, suggesting a potential therapeutic target for CRC (55).

BMP/TGF-β pathway. The TGF-β family has over 40 members, including the TGF-β and BMP subfamily (56). When the signaling is activated, activated receptors further phosphorylate the intracellular receptor-regulated SMAD (R-Smad). Phosphorylated R-Smad interacts with Co-Smad to create a complex that translocates into the nucleus and plays a role as a transcription factor (57).

The downstream targets of TGF-β are pivotal cell cycle regulation proteins, including p21, p27 and p15. In most situations, their activation leads to growth arrest (58). Mutated inactivation occurs in at least one component of almost every CRC case, from frameshift mutations caused by microsatellite instability to mutations of Smad4 and Smad2 (59-62). Zubeldia *et al* injected colon adenocarcinoma cells pre-treated with TGF-β into the spleens of mice and found that it promoted primary tumor development and liver metastasis (63). TGF-β can promote EMT through inducing EMT-related transcription factors Snail1/2, Twist and zinc finger E-box binding homeobox (ZEB)1/2 and elicit cell dedifferentiation (64). Snail induces interleukin (IL)-8) expression through binding to its E3/E4 E-boxes, maintaining stemness through function of IL-8. ZEB2 activates the PI3K/AKT pathway and induces cell transformation (65). ZEB2 attenuates the expression of phosphatase and tensin homolog (PTEN) through microRNAs, such as miR-181, miR-200b, miR-25 and miR-92a (66).

As regards the BMP signaling pathway, mutations in the BMP receptor BMPR1A and Smad4 lead to juvenile intestinal polyposis and Cowden disease, respectively (56). The down-regulation of BMP3 occurs in 89% of primary CRC cases. The early silencing and frequent silencing of BMP3 is crucial in CRC progression (67). BMP4 is not expressed in CRC, while it exists in differentiated cells. Recombinant BMP4 promotes the differentiation and apoptosis of CCSCs (68). Inhibiting the BMP pathway with Dorsomorphin causes mesenchymal stem cells to acquire epithelial-like traits, including the expression of cytokeratin-18 and E-cadherin. The progress occurs

through the downregulation of Snail, Slug and COX2 to affect cell motility, invasiveness and tumor growth *in vitro* (69). Moreover, in CRC initiation, BMP signaling maintains the balanced control of stem cell self-renewal by inhibiting the Wnt pathway. The zinc-finger transcription factor, GATA binding protein 6 (GATA6) is a crucial regulation factor connecting the Wnt and BMP pathways. Competing with β-catenin/TCF4, GATA6 binds to a distal regulatory region of BMP4, decreases the threshold of the BMP pathway for CCSC expansion and inhibits stem cell differentiation (70).

HH-GLI pathway. It has been demonstrated that the self-renewal ability of CCSCs is dependent on HH-GLI activity *in vivo* (71). HH proteins initiate signaling through binding to transmembrane receptor PATCHED1 (PTCH1) and relieve its inhibition to GPCR-like protein Smoothened (SMO). SMO then localizes to primary cilia and the signaling transduces through several parts to finally mediate the three GLI zinc finger transcription factors, enhancing the GLI1 function and inhibiting GLI repressors. GLI code (the sum of all functions of the three GLI proteins) transforms into an activating state and triggers the expression of target genes such as *PTCH1*, *GLI1*, *HIP*, many of which are components of the HH-GLI pathway. Varnat *et al* found that CRC cells exhibited a high activity of HH-GLI signaling and located active HH-GLI signaling in $SHH^+/PTCH1^+/GLI1^+$ tumor cells rather than the stroma. They inhibited HH-GLI signaling by RNAi or cyclopamine treatment and tested the influence on CRC cell growth, recurrence and metastases. The results suggested that the growth, recurrence and metastases of CRC xenografts required HH-GLI activity and cells with high HH-GLI activity owned stronger EMT potentials. Furthermore, they injected $CD133^+$ cells infected with lentivirus encoding *shSMO* or *shPTCH1* into nude mice, isolated tumor cells after 2-3 weeks and subsequently analyzed the ratio of $CD133^+$ subpopulation to test stem cell behaviors *in vivo*. The results suggested that the self-renewal of CCSCs relied on the direct function of HH-GLI activity *in vivo* (71).

Components of the HH-GLI pathway may also influence CRC progression by connecting to the Wnt pathway. The role is controversial. GLI1 can downregulate the level of β-catenin, the expression of *c-Myc* and proliferation ability (72). However, SMO significantly increases in the intestinal adenoma of Apc(+/Delta716) mice and knockdown of *SMO* in human CRC lines inhibites cell proliferation. In Apc(+/Delta716)SMO(+/-) mice, the number of large polyps decreases, polyp morphology recesses and proliferation of cancer cells reduces. It is further demonstrated the decreased expression of SMO attenuates the β-catenin-dependent transcription instead of HH-responsive GLI-dependent transcription and SMO promotes tumorigenesis through Wnt signaling (73).

NF-κB pathway. The NF-κB pathway regulates immuno-inflammatory responses, cell survival and proliferation, playing an important role in tumor formation. Members of the NF-κB family often display as dipolymers. In a quiescent condition, NF-κB binds to IκB to form a heteromultimer, residing in the cytoplasm in an inactive state. With the stimulation of NF-κB activators, IκB kinase (IKKs) are activated, leading to tripolymer phosphorylation, ubiquitylation of the N-terminus of the IκB protein and degradation. Thus, the NF-κB dipolymer is released and is further activated through post-translational modifications. The NF-κB dipolymer then translocates to the nucleus to interact with κB sequences and regulates transcription (74).

The CCSC self-renewal mechanisms related to NF-κB lie mainly in three areas. The first is related to dedifferentiation. Schwitalla *et al* demonstrated that increasing NF-κB recruits CREB-binding protein (CBP) and helps CBP to bind RelA/p65 to β-catenin, thus activating Wnt and inducing the dedifferentiation of non-cancer stem cells (11). The second is the induction of stem cell gene expression. IκB kinase 2 (IKK2ca) is constitutively activated in intestinal epithelial cells and increases stem-like genes *ASCL2*, olfactomedin 4 (*OLFM4*), Delta-like 1 homolog (*DLK1*) and *Bmi*-1 in intestinal stem cells to drive intestinal tumorigenesis (75). The third is that IKK2ca-expressing intestinal epithelial cells can secrete cytokines and chemokines, induce bone marrow cells, and activate fibroblasts, thus creating a tumor micro-environment (75).

EGFR/MAPK pathway. Epidermal growth factor (EGF) signals are essential for the emergence and maintenance of CCSCs (76). The interaction between EGF and EGFR promotes the dimerization and phosphorylation of receptors, thus activating a downstream signaling cascade involving MAPK, AKT and JAK-STAT (77). The MAPK pathway passes through a three-step phosphorylation to further activate downstream targets (78). EGF promotes the expression of high levels of Musashi-1, Lgr5 and low levels of CK20 by CCSCs, facilitating rapid tumor growth. The activation of EGFR signaling promotes the expression of stem cell-related molecules Notch, Shh, Oct3/4 and Wnt. EGFR inhibitors inhibit CCSC proliferation and induce apoptosis through suppressing EGFR phosphorylation and downstream signaling proteins, such as Akt kinase and ERK kinase (76).

Akt pathway. The Akt cascade regulates cell survival and proliferation in many different tumors (79). Recently, a novel kinase-independent Akt pathway was discovered (80). When the Akt kinase domain is inactivated, Akt can resist a hostile environment through another domain of the Akt protein, which is named PH domain. The process may be related to the regulation of interacting protein partners. The function of PH domain may be influenced by kinase domain to get to a particular sub-cellular compartment and/or interact with specific effector proteins (80). Akt can be a bridge connecting the BMP pathway with Wnt pathway. BMP positively regulates PTEN activity to inhibit Akt pathway. The active serine/threonine kinase in the Akt pathway can activate 14-3-3zeta in the β-catenin complex. 14-3-3zeta contributes to the stabilization and nuclear translocation of β-catenin, thus facilitating CCSC self-renewal by activating Wnt (81).

3. Molecular surface marker-mediated self-renewal of CCSCs

CCSC biomarkers are putative to be related to the CCSC phenotype (Table I). Characteristic cellular surface markers, important components of CCSC biomarkers, are what we focus on in this review. Specific mechanisms of these surface markers to regulate CCSC self-renewal are shown in Fig. 2.

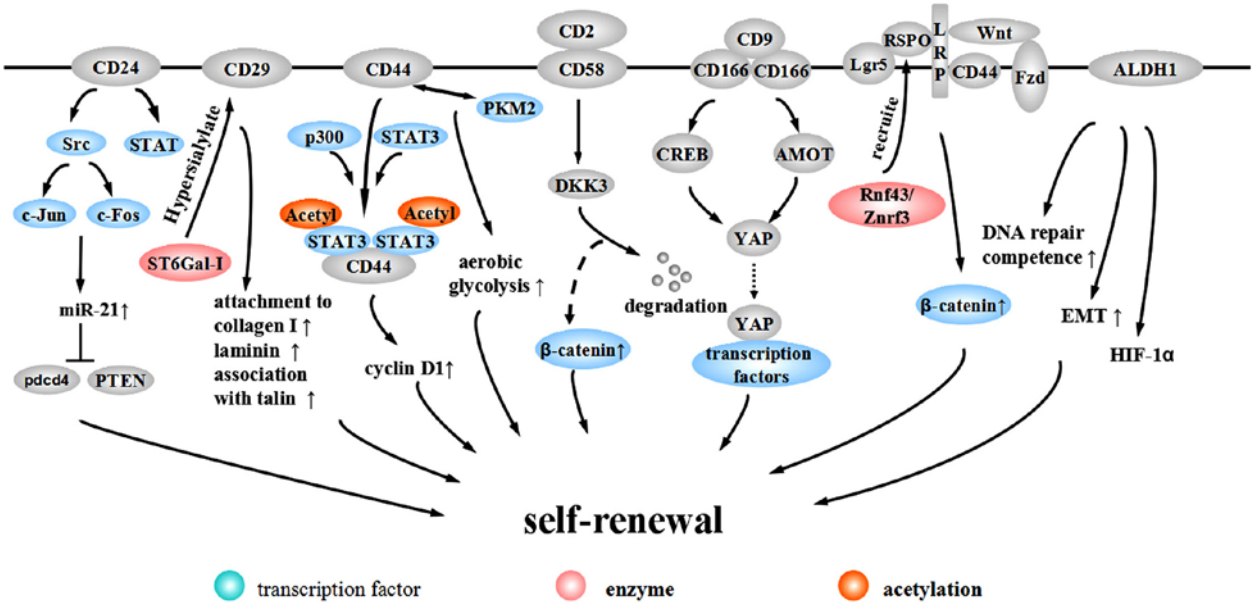

Figure 2. Molecular surface marker-mediated self-renewal of CCSCs.

CD24. CD24 is a heavily glycosylated protein located on the external membrane surface, promoting the renewal of the cell cycle through increasing proliferation and suppressing apoptosis (82). $CD24^+$ cells exhibit CCSC properties, including high chemotherapy resistance, self-renewal ability and tumorigenic potential (83). The promoter of CD24 contains a *TCF/LEF* sequence, suggesting that CD24 is a target of the Wnt pathway (84). In addition, CD24 activates the Src pathway and induces the expression of c-Jun and c-Fos to promote the expression of miR-21, which inhibits Pdcd4 and PTEN to facilitate CRC progression (85). Evidence from gastric cancer suggests that CD24 can maintain cell survival and promote invasion through activating STAT (86).

CD29. CD29, also known as β1-integrin, heterodimerizes with one of 12 possible α subunits (87). β1-integrin is the major integrin protein in normal cells and tumor-associated cells and is thought to play a part in tumor invasion and metastasis. Alteration of expression in integrin β1-subunit significantly correlates with lymph node metastasis and depth of invasion in CRC (88). Song *et al* proposed β1-integrin may induce proliferation and migration through HH-GLI pathway (89).

The involvement of β1-integrin in tumor invasion depends on the process of hypersialylation. Seales *et al* discovered oncogenic ras expressed in 50% of colorectal adenocarcinomas and it upregulated ST6Gal-I, a Golgi glycosyltransferase. The enforced expression of ST6Gal-I in SW48 cells has been shown to induced the sialylation of β1-integrin to upregulate attachment to collagen I and laminin and increase the association with talin, a well-accepted marker for integrin activation, which suggests that β1-integrin alters cell preference for certain extracellular matrix milieus and stimulates cell migration to promote CRC progression (87).

CD44. CD44 is type I transmembrane glycoprotein, comprised of variable spliceosomes, generating different isoforms through inserting one or more exons into a common framework region. These variable isoforms may be a critical process in CRC metastasis (90-92). CD44 expression is restricted to the crypt of the epithelia in the normal intestinal mucosa of APC-mutated mice while it is highly expressed in adenomas and invasive carcinomas (93). Although the specificity of CD44 to CCSCs is under debate, CD44 is useful in isolating CCSCs combined with other surface markers (20,21,94). The mechanism of the participation of CD44 in maintaining stemness has mainly three aspects. First, *CD44* is a Wnt target gene and is involved in the Wnt-related tumorigenesis. At the level of Wnt receptor LRP6, CD44 phosphorylates LRP6 on Wnt stimulation and assists LRP6 in correct membrane localization (95). Second, internalized CD44 interacts with STAT3 and acetyltransferase p300 to form a complex, eliciting STAT3 acetylation and dimerization. The acetylated STAT3 dimer associated with CD44 translocates to the nucleus dependent on a nuclear localization signal (NLS) and binds to the promoter of *cyclin D1*, increasing *cyclin D1* expression and cell proliferation (96). Further research demonstrates that the C terminus of the CD44 molecule is related to increased anoikis resistance in sphere-forming cultures while the N terminus contributes to the interaction with STAT3 and p300. Nuclear CD44/STAT3 signaling promotes the reprogramming of cancer cells, with increasing expression of *SOX2* and *OCT4*. Additionally, CD44 is related to EMT (97). Third, Interaction between CD44 and pyruvate kinase M2 (PKM2) facilitates synthesizing glutathione through the pentose phosphate pathway to resist reactive oxygen species (ROS) accumulation, thus promoting tumor cell aerobic glycolysis to meet the requirements for tumor synthesis demands (98).

CD58. CD58, also known as lymphocyte function-associated antigen-3, is a heavily glycosylated protein on the cell surface and a cell adhesion molecule belonging to the Ig superfamily. CD58 is expressed on the surface of most cells in the hematopoietic system and non-hematopoietic system, including endothelial cells and epithelial cells (20). Colon cancer HT-29

and Caco-2 cells express CD58 at low levels (99). CD58 is a ligand for CD2 and their interaction functions as a specific immunity co-stimulation to promote T cells to secrete CXCL-8, which can promote the self-renewal of CCSCs (100). Our research demonstrated that CD58 promotes the maintenance of self-renewal through activating the Wnt/β-catenin pathway (20). The DKK family is comprised of inhibitors of the canonical Wnt signaling pathway. CD58 interacts with downstream DKK3 and elicits DKK3 degradation to activate the Wnt pathway (20).

ALCAM/CD166. ALCAM/CD166, belonging to the Ig superfamily, is highly expressed in endogenous intestinal stem cells and is related to intercellular adhesion (101,102). CD166 is a target of the Akt pathway regulated by TGF-β and NF-κB p50/p65 (103-105). Whether CD166 is a positive or negative regulator of tumor development is still under debate. Traditional opinions suggest that CD166 is associated with tumor aggression and progression, anti-apoptosis and resistance to autophagy (101,104). CD166 mediates its functions via homophilic (CD166-CD166) or heterophilic (CD166-CD6/9) interactions (102). A recent study indicated that through the PI3K/AKT pathway, CD166 regulates the downstream YAP protein to resist apoptosis, which can be promoted by the interaction between CD9 and CD166. Additionally, the interaction between PI3K/AKT signaling and CD166 can form a positive feedback loop to further promote CD166 expression (105). PI3K/AKT/CD166 regulate the expression and activity of YAP in at least two types of mechanisms. The first utilizes the downstream target CREB to regulate YAP transcription. The second mechanism is the post-transcriptional control of YAP protein stability by suppressing AMOT130 (105).

Lgr5. Lgr5, a G-protein-coupled receptor with a leucine rich repeat, is overexpressed in CRC cells and alters along with CRC progression (32,106). $Lgr5^+$ cells are located in the crypt base and have the potential to generate all of the intestinal epithelial cell lineages, maintaining self-renewal and homeostasis of the intestinal mucosa (107,108). The mechanism of self-renewal regulation by $Lgr5^+$ is through the Wnt/β-catenin pathway. With activated Wnt signaling, $Lgr5^+$ forms a complex with Frizzled/LRP. The complex can bind to Rspondin (RSPO), a Wnt collaborative enhancer, to further enhance Wnt signaling (109). Recently, it has been indicated that after Lgr5 recruits RSPO, RSPO binds to Rnf43/Znrf3, an E3 ligase that ubiquitinates Frizzled to protect Frizzled from degradation and maintain Wnt signaling (110).

ALDH1. ALDH1 is reported to be a potential marker for normal or malignant CCSCs. Immunostaining has demonstrated that normal crypt bases only express a small amount of ALDH1. However, in the transformation from normal epithelia to mutant epithelia, and finally to adenomas, $ALDH1^+$ cells increase in number and distribute more widerly along the crypt axis (23). Patients with inflammatory bowel diseases with atypical hyperplasia express higher levels of ALDH1, while patients with simple inflammation exhibit no increase. This finding suggests that ALDH1 may be a critical point distinguishing atypical hyperplasia from inflammatory hyperplasia and a potential marker for pre-cancerous lesions (111). Evidence from prostate cancer suggests that ALDH1A1 activity is associated with increased DNA repair competence and an induction of EMT. $ALDH1A1^+$ cells express high level of hypoxia inducible factor-1α (HIF-1α). The expression of ALDH1A1 is regulated by the Wnt pathway through β-catenin/TCF-dependent transcription and co-occurs with the expression of β-catenin (112).

4. Microenvironment-mediated self-renewal of CCSCs

The tumor micro-environment represents non-tumor cells and adjacent tissues, including uncontrollable inflammation. The micro-environment experiences pathological change and provides cancer stem cells with soil, contributing to the complexity of CRC biology. The interaction between CCSCs and the micro-environment plays a role in regulating CCSC self-renewal.

Cancer-associated fibroblasts (CAFs). Fibroblasts in the tumor micro-environment are termed CAFs. This cell population is not stationary, but can be transformed from mesenchymal cells, endothelial cells, adipose cells and even cancer epithelial cells. CAFs promote tumor progression and invasion by both mechanical forces and metabolic machinery (113). On the one hand, CAFs induce protease-mediated extracellular matrix remodeling, serving to guide the structures that direct migration (114). On the other hand, CAFs secrete cytokines and growth factors to affect the proliferation, survival, adhesion and migration of cancer cells. The secreted mitogenic factors involve hepatocyte growth factor, EGF family members, chemokine ligand 12, fibroblast growth factors and stanniocalcin-1 (115). CAFs promote invasion. One of the mechanisms is the secretion of matrix metalloproteases or cytokines, such as tumor necrosis factor-α (TNF-α) that promotes EMT. Activated CAFs by TGF-β can trigger GP130/STAT3 signaling to express IL-11, contributing to CRC metastasis (116).

HIF. Anoxia is recognized to promote tumor survival and progression by inducing changes in tumor metabolism, angiogenesis, invasion, metastasis and drug resistance to reduce the clinical prognosis (117,118). Anoxia regulates gene expression through the transcriptional control of HIF-1α and HIF-2α, which can bind to hypoxia response elements (HRE) in the promoters of many genes. The induction of these genes triggers aggressive tumor growth, invasion and metastasis (117,118). HIFs are involved in the canonical Wnt pathway. HIF-1α can increase the transcriptional activity of β-catenin (119). Moreover, Newton *et al* found that anoxia could inhibit *APC* expression through the suppression of the *HRE* in the *APC* promoter by HIF-1α (120). Shay *et al* treated colitis-associated colon cancer with acriflavine, an inhibitor of HIF transcription, and found that it could inhibit the hypoxic induction of M-CSFR and angiogenic factors to inhibit tumor growth and progression (121).

Myeloid-derived suppressor cells (MDSCs). MDSCs are a heterogeneous cell population derived from bone marrow, including immature neutrophils, immature dendritic cells, immature monocytes and early myeloid progenitors (122). The

recruitment from the circulation to the intestinal mucosa is associated with CXCR2 expression on CRC endothelial cells and immunocytes (123). With stimulation by cytokines from the tumor stroma, such as cyclooxygenase 2 and colony-stimulating factor, MDSCs expand and generate arginase 1, ROS, and inducible nitric oxide synthase to inhibit the anticancer function of NK cells and T cells. This process contributes to the escape from immunological surveillance and promotes tumor initiation and progression (123-125). Additionally, MDSCs can secrete vascular endothelial growth factor A and matrix metalloproteinase (MMP)9 to promote angiogenesis (126). Evidence from ovarian cancer suggests that MDSCs can drive the expression of miRNA101 and subsequently suppress repressor gene CtBP2. CtBP2 interacts with nuclear genes, leading to increased stemness, tumorigenesis and metastasis in cancer cells (127).

5. Inflammation factors

Inflammatory mediators and inflammatory effectors are both important components of the tumor micro-environment. They can be derived from the intrinsic pathway, such as the mutation of an oncogene and chromosomal rearrangement, or the extrinsic pathway, such as infection (128). The two pathways activate transcription factors, mainly NF-κB and STAT3, to elicit the release of downstream inflammatory factors, such as IL-1β, IL-6, IL-8, IL-17, IL-23 and TNF-α (128,129). The inflammatory response has a series of tumor facilitating effects, including tumor initiation, survival, malignant transformation, invasion, metastasis, destruction of the adaptive immune response, influencing immune surveillance, and changing chemotherapy resistance (128,129). Activated STAT3 by IL-6 plays an important role in the early stage of colitis-associated CRC. STAT3 upregulates the expression of cell cycle regulators *cyclin D1*, *cyclin D2*, *cyclin B*, proto-oncogene *myc*, and anti-apoptotic genes *Bcl-2* and Bcl-2-like 1 to promote proliferation and survival (130). IL-23 can promote CRC cells to generate TGF-β and induce the process of EMT to facilitate malignant transformation (131). Activated STAT5 by IL-23 with impaired Socs3 expression is associated with the metastasis of CRC (132), and the function of the IL-23/IL-17 axis on CRC initiation and progression has been recently recognized (133). IL-17A promotes the malignant progression of CRC through the activation of ERK, p38 MAPK and NF-κB signaling while it also regulates the production of IL-6 (134). Apart from activating multiple signaling pathways to activate transcription factors, such as STAT3 and β-catenin to regulate stemness (135), IL-8 is related to EMT. In the induction of EMT by Snail, Snail and IL-8 can form a positive feedback loop and increase the expression of stemness genes *Sox2*, *Nanog* and *Oct4* (65). Leukotrienes D4 and prostaglandin E2 can increase the $ALDH^+$ subpopulation and enhance sphere forming and tumor growth (136). The loss of TGF-β can induce the secretion of chemokine CCL9 (CCL15 in humans), which recruits immature myeloid cells that express the CCL9 receptor CCL1. These cells secrete MMP9 and MMP2 to promote CRC invasion and metastasis (137).

The components of the micro-environment are very complex. In addition to the above mentioned, others, such as blood vessels, microorganisms and normal cells, all exert an influence on the micro-environment (113,138). All of these complex components interact with each other to regulate CCSC self-renewal.

Prospectives for CCSC-targeted intervention. Because CCSCs possess a strong resistance to therapy, the clinical effects on CRC are very limited. The reactivation of signaling cascades, enhancement of DNA repair and drug efflux by ABC transportation may be responsible for the resistance (139). Considering that the activation of Wnt is a dominating process in the evolution and progression of CRC (26), the targeted intervention of this pathway should be most reliable. How to block this signaling pathway efficiently and specifically while maintaining normal somatic function is the aim of future study. Additionally, targets on other signaling pathways and micro-environmental factors should be included to achieve the best therapeutic effects.

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