Chapter 9
Colorectal Cancer Stem Cells

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Abstract Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer deaths in the United States with over 50,000 deaths per year. The sporadic colorectal cancer, which occurs in ~80 % of the patients, is an age-related disease, the incidence of which rises dramatically after 50 years of age. According to stochastic model of sporadic cancer, it was thought that all cancer cells that possess driver mutation(s) will lead to the process of carcinogenesis. However, in recent years, numerous studies have appeared to challenge the stochastic model. It is becoming increasingly accepted that not all, but only a small subpopulation of pluripotent self-renewing tumor cells that are termed as cancer stem cells (CSC) play a determinant role in the development and progression of many malignancies, including colorectal cancer. The focus of this book chapter is to briefly describe the role of cancer stem cells in recurrence of colorectal cancer, which leads to metastasis and remains a major clinical challenge. Although the underlying biochemical and molecular events leading to recurrence of various malignancies are not fully understood, CSCs that have been shown to be resistant to conventional chemotherapy play pivotal role in these processes. While the origin of CSCs is not fully known they are thought to be derived from mutations in normal stem, progenitor or differentiated cells. Despite recent advances in medicine, nearly 50 % of the patients develop recurrence of colon tumor that is highly enriched in CSCs. Unfortunately the conventional chemotherapy has shown limited success in treating recurrent cancer. This underscores the need for development of novel treatment strategies for recurrent colon cancer by targeting CSCs. Attempts are being made to target CSCs utilizing combination of chemotherapy and specific inhibitors of growth factor receptors or signal transduction. In addition, efforts have also been made to utilize non-toxic natural agent(s), either alone or in combination with conventional chemotherapy.

Keywords Colorectal cancer • Stem cells • Cancer metastasis • Drug resistance

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1 An Overview of Colorectal Cancer Biology and Pathology

Colorectal carcinomas are one of the most frequent neoplasms in the Western society. It is the second leading cause of cancer-related deaths in the United States. The American Cancer Society estimates about 140,000 new cases of colorectal cancer in the United States for 2014 with approximately 50,310 deaths due to this disease. Overall, the lifetime risk of developing colorectal cancer is about 1 in 20 (5 %) and can be graded into well, moderately and poorly differentiated lesions. Diet and lifestyle as well as inherited and somatic mutations are the contributing factors in CRC. The progression of colorectal neoplasms involves uncontrolled epithelial cell replication, continuation into formation of adenomas of various dimensions and eventually evolving into malignancy. This process has been termed the polyp-carcinoma sequence and the transformation from the initial events to an invasive carcinoma takes about 8–12 years. In the earliest phases of colorectal tumorigenesis, a disorder of cell replication is initiated in the normal mucosa, which is associated with clusters of enlarged crypts [aberrant crypts] having abnormal proliferative, biochemical and biomolecular characteristics. A well demarcated mass of epithelial dysplasia with uncontrolled crypt cell division is termed as an adenomatous polyp. An adenoma can be considered malignant, when neoplastic cells pass through the muscularis mucosae and infiltrate the submucosa. Hyperplastic polyps, serrated adenomas, flat adenomas, hamartomatous polyps, and inflammatory polyps are some other types of polypoid lesions beside adenomas. Thus colorectal tumors cover a wide range of premalignant and malignant lesions. The pathology of colorectal cancer has been reviewed in details earlier (Ponz de Leon and Di Gregorio 2001).

The focus of this chapter is on the sporadic colorectal cancers, which constitute 80–85 % of CRC and arise as a consequence of progressive genetic and epigenetic alterations that drive the transformation and progression of normal colonic epithelial cells to cancer. Fearon and Vogelstein presented a model for genetic alterations necessary for different stages of colorectal tumorigenesis (Fearon and Vogelstein 1990). According to this model, (a) colorectal cancer arises as result of genetic mutations in tumor suppressor genes and oncogenes; (b) mutations in at least 4–5 genes are required for the formation of a malignant tumor; and (c) total accumulation of changes rather than their sequence is important for the transformation. The molecular and genetic events leading to CRC have been reviewed in details earlier (Fearon 2011). A brief description of the transforming events is provided here. The first and most important mutation in the earliest adenomas leads to truncation of the APC (adenomatous polyposis coli) tumor suppressor protein. According to Kinzler and Vogelstein (Kinzler and Vogelstein 1996), APC plays a gatekeeper role in the normal colorectal epithelial cells maintaining a constant cell number; mutations in this gatekeeper gene lead to a permanent imbalance of cell division over cell death. *APC* encodes a 300 kDa protein that may regulate cell-cell adhesion, cell migration, chromosomal segregation and apoptosis in colonic crypt (Polakis 2007; Aoki and Taketo 2007; Brocardo and Henderson 2008). The best established function of

mutated (inactivated) APC protein is to partner with β-catenin leading to activation and up-regulation of oncogenes such as cyclin D1 and c-myc as well as many other tumor associated genes (Aoki and Taketo 2007; Polakis 2007).

The second most common mutations observed in almost 40 % of colon carcinomas are in the *KRAS* gene (Malumbres and Barbacid 2003). These mutations contribute to colorectal adenoma development, but are not required for the initiation event. Ras proteins regulate several downstream signaling cascades including mitogen activated protein kinase (MAPK) and PI3K pathways. Moreover, mutations in PI3K gene *PIK3CA* were reported in approximately 15–25 % of CRCs (Wood et al. 2007; Samuels et al. 2004). In addition, loss of PTEN expression is observed in about 15–20 % of CRC, although the somatic mutations in *PTEN* are found only in 10 % CRC. The above mutations result in activation of Akt and mTOR pathways, which regulate apoptosis and nutrient availability during cellular growth.

The mutations in *p53* gene are associated with adenoma-carcinoma transition (Baker et al. 1990). The p53 protein regulates the transcription of several proteins such as p21, PUMA, BAX and MDM2 that serve as cell cycle checkpoint, promote apoptosis and restrict angiogenesis. It is suggested that mutation in *p53* facilitates the growth and acquisition of invasive properties in the adenoma cells, which otherwise will be severely limited due to the stresses of rapid tumor growth. *p53* mutations also result in altered miRNA processing (Suzuki et al. 2009).

The other tumor suppressor genes that show mutations in 5–10 % CRCs are *SMAD2* and *SMAD3*. Both of these proteins are regulated by TGF-β-mediated receptor phosphorylation (ten Dijke and Hill 2004; Harradine and Akhurst 2006) and are involved in nuclear translocation of SMAD4 leading to transcriptional control of the target proteins. High copy amplification of the oncogene *CMYC* is observed in approximately 5–10 % (Leary et al. 2008), and a moderate increase is reported in more than 30 % of CRCs. c-Myc protein is a transcription factor and regulates genes that regulate cell cycle progression, survival and metabolism (Eilers and Eisenman 2008; Ruggero 2009). *CMYC* is the key target gene regulated by activation of β-catenin by APC inactivation. In addition, post-trascriptional changes such as methylation and microRNAs also modulate the expression of many proteins (Babashah and Soleimani 2011; Babashah 2014). Role of miRNAs in colon cancer progression has recently been reviewed by us (Yu et al. 2014).

2 Colorectal Carcinogenesis and Colon Cancer Stem Cells

Despite recent advances in medicine, 30–40 % of patients with CRC show tumor recurrence. Although the reason for this is not fully understood, the presence of chemotherapy resistant cancer stem cells (CSCs), which are distinct from the bulk of the cells in the tumor, is thought to be one of the primary cause for tumor recurrence (Dean et al. 2005). Over the last decade, the cancer stem cell model has become increasingly accepted as an explanation for cancer development, spread and recurrence.

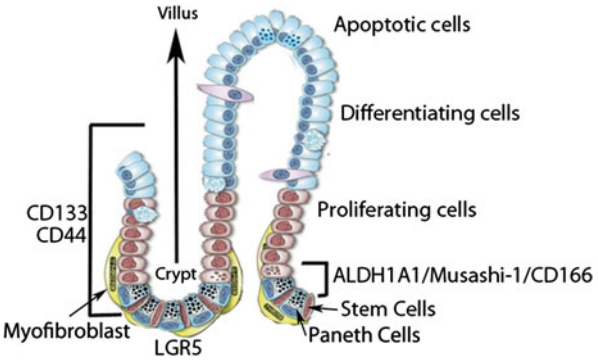

Fig. 9.1 *Schematic diagram showing expression patterns of normal intestinal stem cell andCSC-markers in normal small intestine and colon*. The intestinal epithelium undergoes continous generation and differentiation along the crypt-villus axis. Stem cells residing near the bottom of the crypt give rise to rapidly proliferating progenitor cells, which subsequently differentiate into functional enterocytes (Adapted from Lin et al. Toxins (Basel) 2010)

The CSCs are thought to originate from stem, progenitor or the differentiated cells that have acquired mutations in tumor suppressor genes and/or oncogenes as described above. Like normal stem cells CSCs possess two essential properties: their long-term self-renewal property and their ability to give rise to one or more differentiated cell lineages (pluripotency). In addition, these cells are capable of giving rise to tumor, even when injected in small numbers.

In the normal intestine or colon, stem cells are undifferentiated, multipotent and self-renewable cells that are found towards the bottom of the crypt in the proliferative zone and are also responsible for generating all epithelial cell types along the crypt-villus axis (Fig. 9.1) and maintaining tissue homeostasis and repair. Two types of stem cells have been reported: the LGR5$^+$ crypt base columnar cells (CBCs) and the quiescent DNA label-retaining intestinal stem cells (LRCs) marked by the expression of polycomb group gene Bmi1 (Sangiorgi and Capecchi 2008). Both of these cell types are present in the small intestine, but the presence of LRCs in the colon has not been confirmed. In vitro studies utilizing single LGR5$^+$ CBCs showed organoid formation and crypt domains containing all lineages of the adult intestinal epithelium including enteroendocrine and crypt paneth cells confirming true multipotent nature of these stem cells (Sato et al. 2009). Like LGR5$^+$ CBCs, the Bmi1$^+$LRCs also form spheroids in vitro containing all differentiated epithelial cell types (Yan et al. 2012; Sato et al. 2009). The stem cell characteristics of these cells have been further strengthened by the fact that these cells can restore radiation ablated mouse intestinal epithelium in the total absence of Lgr5$^+$ stem cells (Yan et al. 2012). However, Buczacki et al have identified quiescent LRCs not as the stem cells but rather as partially-differentiated secretory precursors (Buczacki et al. 2013). It is suggested that both these cell types co-exist in the intestinal epithelium, LGR5$^+$ cells comprise the active population of the crypt, whereas Bmi$^+$ cells are quiescent SCs that represent a reserve pool of SCs that replace the LGR5$^+$ cells in case of loss or injury (Medema and Vermeulen 2011; Tian et al. 2011).

Since stem cells proliferate throughout life, they are more susceptible to accumulate oncogenic mutations than differentiated cells with their comparatively short life span. On the other hand, it is also assumed that differentiated cells reacquire stem cell like characteristics by reactivating signaling pathways that are linked to malignant transformation such as the Wnt/β-catenin and Bmi1 pathways or certain Hox genes.

As mentioned above, colorectal cancer results from a series of genetic/epigenetic alterations that transform the normal colonic mucosa into an aberrant phenotype (Markowitz and Bertagnolli 2009; Lampropoulos et al. 2012). The cell that acquires mutation and becomes the first tumor cell is termed the cell of origin. The development of heterogenous tumor from the tumor initiating cell can be explained according to stochastic or CSCs model. The stochastic model suggests that every cell within a tumor is capable of both initiation, propagation and further mutations, whereas CSC model posits that a few cells within the tumor have the potential to propagate the tumor. Increasing evidence is now supporting the latter model or a combination of the two.

Considering that the appearance of CSCs might be one of the initial events in neoplastic transformation in solid tumors as well as in intestinal neoplasia, we investigated the status of CSCs in normal appearing colonic mucosa during aging in patients with adenomatous polyps. Colon CSCs, as evidenced by the expression of CSC markers (CD44, CD166 and Ep-CAM) were observed not only in premalignant adenomatous polyps, but also in normal appearing colonic mucosa, where expression increased with advancing age indicating increased risk of developing colorectal cancer during aging (Patel et al. 2009). Additionally, we found the age-related increase in adenomatous polyps in the colon was associated with increased expression of colon CSC markers (Patel et al. 2009).

3 Identification of Colon Cancer Stem Cells

Identification and isolation of the CSC responsible for tumor initiation and propagation is a huge challenge due to the complexity of their biology and expression of cell surface markers, which differ between tissue types and also other unsolved technical issues. Three methods are usually employed for isolation and characterization of CSCs: (a) isolation based on their drug efflux property by flow cytometric sorting of a side population (SP); (b) colonosphere formation, which are considered as surrogate tumors; (c) sorting on the basis of cell surface markers, which are described in more detail in the following section.

Based on cancer stem cell properties, several investigators identified various membrane and cytoplasmic CRC stem cells markers such as CD133 (O'Brien et al. 2007; Puglisi et al. 2009; Ricci-Vitiani et al. 2007), CD24, CD29, CD44 (Dalerba et al. 2007), CD166 (ALCAM) (Dalerba et al. 2007), EpCAM (ESA) (Ricci-Vitiani et al. 2007), Musashi 1 (Msi-1) (Glazer et al. 2012), Lgr5 (Das et al. 2010), ALDH1 (Todaro et al. 2010). Presence of these proteins has been associated with stem-ness

and generation of tumors reiterating the primary tumor with increased clonogenic ability and multi-lineage potential and has also been associated with tumor stage, differentiation, invasiveness, metastasis formation as well as prognosis (Dick 2008; Reya et al. 2001; Vaiopoulos et al. 2012; Wang and Dick 2005).

CD133 (prominin-1), a transmembrane glycoprotein, was identified as a potential CSC marker for brain tumor (Singh et al. 2004) and several histological variants of tumors, including colon. Later, it was demonstrated that CD133 positive cells from colon metastases formed SCID mice xenografts that resembled the original tumor, whereas CD133-negative cells did not form metastases in mice even when injected in high numbers (O'Brien et al. 2007; Ricci-Vitiani et al. 2007). Both reports point out that the vast majority of CD133 positive cells were not CSC. On the contrary, Shmelelkov et al (Shmelkov et al. 2008) demonstrated that CD133 negative cells had the same tumor initiating capacity as CD133 positive cells and that CD133 was expressed equally in differentiated and undifferentiated cells in the normal human colon. Other investigators also did not observe an enhanced tumor initiating capacity by the presence or absence of CD133 whether the cells were isolated from primary colon tumors or colon cancer cell line (Chu et al. 2009; Feng et al. 2010; Ricci-Vitiani et al. 2007). These differences to form CD133 tumor from primary tumor largely depend on the methodology. However, no functional data are available to date and conflicting results have been reported regarding its role as a true CSC marker (Kemper et al. 2010; Puglisi et al. 2011). In colorectal cancer, CD133 expression is not restricted to rare cell subsets, but it is detectable in a large heterogenous populations of tumor cells, irrespective of their tumorigenicity (Shmelkov et al. 2008). The co-expression on tumor cells of Msi-1, CD44, CD166, and EpCAM molecules has been reported to identify the CSC pool more precisely than CD133 expression alone (Dalerba et al. 2007; Todaro et al. 2008; Vermeulen et al. 2010). Todaro et al. (2010) reported that only tumorigenic $CD133^+$ cells were able to generate colonies organized in crypt-like structure under differentiation conditions on Matrigel. Another study demonstrated that neither over-expression nor loss of CD133 was significantly associated with tumor progression or survival (Langan et al. 2013; Lugli et al. 2010). Muraro et al. (2012) evaluated the correlation of the expression of CD133 or the co-expression of CD166/CD44 or CD24/CD44 with several CSC functional properties but it did not appear to reliably identify CSC populations in established CRC cell lines. Despite the conflicting reports of CRC-SC's to be utilized as clinically relevant biomarkers, CD133 is identified as a potential prognostic marker in a number of cancers (Grosse-Gehling et al. 2013; Ozawa et al. 2014; Pirozzi et al. 2013; Ren et al. 2013; Yamamoto et al. 2014; Yang et al. 2011) and however, little is known about the prognostic value of non-CD133 CRC-SC markers.

CD166 (ALCAM) expression is pathologically correlated with aggressive disease in a variety of cancers and aberrant cell surface CD166 expression is strongly correlated with a shortened survival (Levin et al. 2010; Weichert et al. 2004). It has been reported that loss of membrane CD44, CD166 and EpCAM from normal to early colorectal cancer is linked to tumor progression. This is attributed to loss of their cell adhesion function (Lugli et al. 2010), which is known to be fundamental

Table 9.1 Cancer stem cell marker specific survival in colorectal cancer

Stem cell marker expression	Five year survival rate
High CD133 expression, regardless of CD44 or CD166 expression	44 ± 8.6 % (lowest)
Tumors with high expression of CD44 and/or CD166 regardless of other markers	77 ± 6.1 % (intermediate)
Tumors with low/none expression of all markers	87 ± 9 % (highest)

to initiation of the metastatic process (Woodhouse et al. 1997). A significant increase in CD166 expression in adenomatous glands and an age-dependent increase in CD44 and CD166 expression has been reported suggesting a role for CD44 and CD166 in tumor development from the pre-cancerous state (Patel et al. 2009). Horst et al (Horst et al. 2009) reported colon cancer patients specific survival based on the expression of stem cell markers using the Kaplan-Meier method (Table 9.1).

Msi-1 was also identified as a putative colon SC marker. Most of the Msi-1$^+$ cells were located at the base of human colon, between cell position 1 and 10: a distribution that is believed to maintain the undifferentiated state of SCs (Battelli et al. 2006; Imai et al. 2001; Nakamura et al. 1994; Nishimura et al. 2003). The integrin subunit β1 (CD29) has been reported as a surface marker for the proliferative zone of the human colonic crypt. CD29 is expressed highly in lower third part of the colonic crypt, which harbors stem cells and progenitor cells (Fujimoto et al. 2002).

CD44, a cell surface glycoprotein, first identified as a stem cell marker in breast cancer, is involved in tumor invasiveness, migration and malignant progression to metastases and recently has also been described as a putative colorectal CSC marker (Visvader and Lindeman 2008). More recently, several investigators used lineage-tracking experiments to identify unique markers of normal colon SCs, and identified an orphan G-protein-coupled receptor, Wnt target gene Lrg5 (leucine-rich repeat-containing G protein-coupled receptor 5 (Barker et al. 2007, 2009; Sato et al. 2009; Schepers et al. 2012). There are controversial reports about the role of LRG5 as a tumor suppressor or oncogene in colorectal cancer. Walker et al reported that loss of LRG5 expression increased tumorigenicity and invasion, whereas increased expression of LRG5 inhibited tumorigenicity and clonogenicity (Walker et al. 2011). On the contrary, colorectal cancer patients with high Lrg5 expression were associated with poor prognosis (Han et al. 2015).

Aldehyde dehydrogenase 1 (ALDH1) is a detoxifying enzyme and another potential colon cancer SC marker which is positive with subsets of CD44$^+$ or CD133$^+$ cells and located at the base of the normal crypt. All three markers increased during colon tumor progression to carcinoma (Huang et al. 2009). Increased expression of ALDH1 was associated with poor clinical outcome in colon cancer patients (Goossens-Beumer et al. 2014), whereas Fitzgerald et al. (2014) reported that ALDH1 expression did not increase with progression from normal colon to primary tumors and metastases.

A recent report revealed that high levels of lipid droplets are distinctive marks of CSCs (Tirinato et al. 2014). CRCs exhibited more lipid droplets compared to differentiated tumor or normal epithelial cells (Krahmer et al. 2009; Tirinato et al.

2014). Lipid droplets are dynamic cytosolic lipid strorage organelles. Differential expression of lipid droplets is associated with disease and a possible functional or metabolic link of lipid droplets in CR-CSC is postulated (Bozza and Viola 2010; Farese and Walther 2009). Flow cytometric analysis revealed CD133 expression in lipid droplet containing CRCs and that these CRC-SC retain tumorigenic potential in vivo (Tirinato et al. 2014).

4 Colon Cancer Stem Cells and Metastasis

Tumor recurrence associated with metastasis is by far the biggest clinical challenge associated with cancer. Cancer recurrence and metastasis is dependent on the ability of some cells to detach from the primary location, implant at a separate site and generate secondary tumor. As mentioned earlier, as per the stochastic model, the tumor heterogeneity originates from aberrant mutations within the initial tumor mass as well as due to various micro-environmental influences (Vries et al. 2010; Visvader and Lindeman 2008). Out of the diverse population within the tumor, only selected clones can migrate and form metastasis. On the other hand according to the CSC model, only CSCs can migrate and as a result the metastatic tissue resembles the pattern of original lesion. These cells can undergo further genetic and epigenetic alterations and evolve into new and more malignant CSCs and drive tumor migration and metastasis rather more effectively than the original CSCs (Visvader and Lindeman 2008; Vries et al. 2010). In order for the metastatic process to succeed, a cancer cell should be able to survive under attachment-free conditions, migrate and invade through surrounding stroma, intravasate into the vascular system, survive the rigors of the blood flow, extravasate into an advantageous distant environment, adhere and proliferate. Vast genetic changes including mutations in APC, K-Ras, TP53, PIK3CA, SMAD4 genes and activation of signaling pathways such as Wnt, Notch and Hedgehog, enable these cells to successfully accomplish this intricate process (reviewed in (Rattan et al. 2012)).

Tumor microenvironment plays an important role in helping these stem cells gain tumor-promoting traits (Burness and Sipkins 2010). It has been postulated that a normal intestinal niche can prevent tumor growth even if CSCs are present (Bissell and Labarge 2005). The tumorigenic niche is composed of transformed fibroblasts, recruited myeloid cells, other cell types and extracellular components, which produce many growth factors and cytokines including TGF, HGF, TNF-α, IL-6, EGF and IGF that promote dedifferentiation, carcinogenesis and invasiveness (Medema and Vermeulen 2011; Vermeulen et al. 2010). These extrinsic factors have also been reported convert non-CSCs to CSCs through a process called EMT. In this event simultaneous down-regulation of epithelial phenotype along with enabling of fibroblast-like traits, enhances motility, invasiveness and resistance to apoptosis (Chaffer and Weinberg 2011; Singh and Settleman 2010). Investigations have shown that stimulation of pathways like Wnt, Notch, hypoxia, integrins and PI3K/Akt result in EMT-related changes (reviewed in (Rattan et al. 2012)). Therefore, the

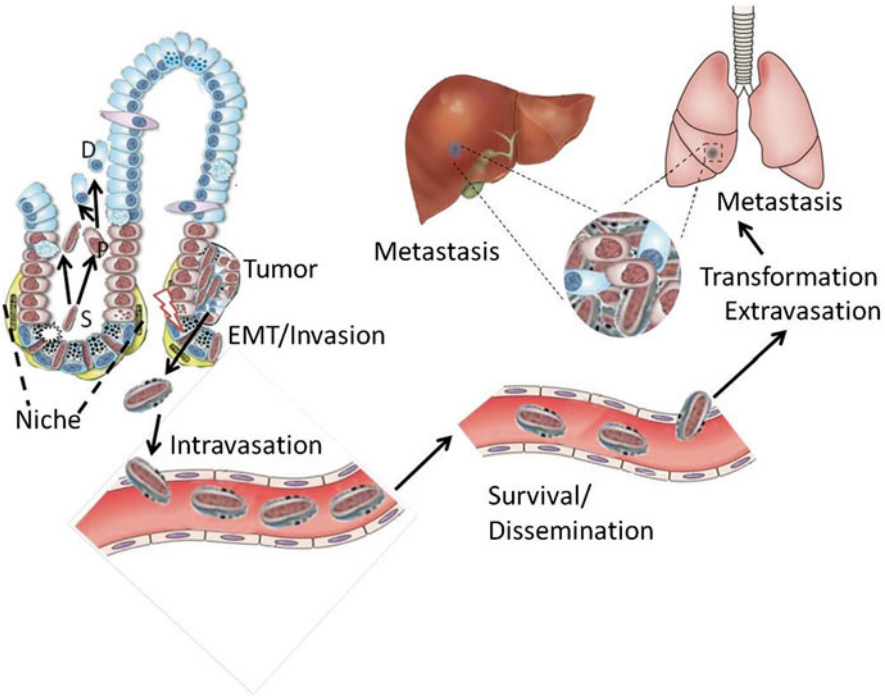

Fig. 9.2 Stem cell model of metastasis. Stem cells (S) give rise to progenitor (P) and differentiated (D) cells in the normal course of events. The CSCs that have undergone EMT due to genetic and epigenetic mutations (lightening bolt) are released from the primary tumor into the blood vessels. The microenvironment or the niche supports this transition. The invaded cells travel to distant locations, where they undergo transformation and form metastasis

circulating tumor cells are expected to have stem-like characteristics. Indeed, presence of stemness markers in the peripheral blood of cancer patients is associated with worse prognosis and recurrence (Iinuma et al. 2011; Gazzaniga et al. 2010). A stem cell model of colon cancer metastasis is shown in Fig. 9.2.

5 Chemoresistance in Colon Cancer Stem Cells

5.1 CSCs Are Self-Renewing Cells with a Low Proliferation Rate

A low rate of multiplication is a hallmark of the somatic stem cells of normal tissues. The presence of quiescent cells with CSC properties has been demonstrated in several tumor systems, using retention of DNA label or lipophilic dye. CSCs can divide to yield a more differentiated cell and a daughter cell that maintains the same properties as the parental cell. This ability of self-renewal in CSCs drives tumor

growth, metastasis and recurrence. Actually, despite their capacity for self-renewal, CSCs are relatively quiescent; that is, they have low proliferative rate and are often not cycling. Indeed, they have been shown to have significantly longer cell cycle times than proliferating non-stem cells. We have reported that the growth of CSCs from colonospheres formed by colon cancer HCT-116 and HT-29 cells remains 30–40 % lower than the corresponding parental cells (non-CSCs) (Kanwar et al. 2010). This is presumably due to the arrest of CSCs at a G0-like cell cycle phase or checkpoint (Paldino et al. 2014). Touil and colleges have recently reported that 5-FU resistant colon cancer cell population expresses a typical cancer stem cell-like phenotype and enter into a reversible quiescent G0 state upon re-exposure to 5-FU (Touil et al. 2014). Quiescent CSC avoid DNA damage induced by chemotherapeutic drugs, because these agents primarily hit cells in the S-phase cycle. Hence, cells in the G0/G1 phase of the cell cycle are thought to be relatively resistant to classical cytotoxic therapy (Stewart et al. 2007). Yan and colleges have reported that Bmi1 and Lgr5 mark two functionally distinct crypt intestinal stem cells (ISCs) in mice. $Lgr5^+$ ISCs are mitotically active ISCs, Bmi1 marks quiescent ISCs that are insensitive to Wnt perturbations, contribute weakly to homeostatic regeneration, and are resistant to high-dose radiation injury. Clonogenic culture of isolated single $Bmi1^+$ ISCs yields long-lived self-renewing spheroids of intestinal epithelium that produce Lgr5-expressing cells, thereby establishing a lineage relationship between these two populations in vitro (Yan et al. 2012). In conclusion, quiescent CSC have enough time to avoid, to reduce and to repair drugs induced DNA damage or cytotoxicity.

5.2 Side-Population and ABC Transporters in CSC Cells

Side population (SP) cells can rapidly efflux lipophilic fluorescent dyes to produce a characteristic profile based on fluorescence-activated flow cytometric analysis. Previous studies demonstrated SP cells in bone marrow from patients with acute myeloid leukemia, suggesting that these cells might be candidate leukemic stem cells. Recent studies have found that many types of cell lines and tissues including colon cancer cell lines to contain SP cells. Studies on testicular stem cells indicate that more than 40 % of the SP (defined in this case as cells that show higher efflux of DNA-binding dye Hoechst 33342) were undifferentiated spermatogonia, while other differentiated fractions were represented by only 0.2 % (Takubo et al. 2008). We have observed 80 % increase in dye exclusion in CSCs which were derived from colonospheres of HCT-116 cells (Kanwar et al. 2010). This was associated with increased expression of ABC transporter protein ABCG2, a member of the superfamily of ATP-binding cassette (ABC) transporters whose primary function is to transport various molecules across the intra- and extra-cellular membranes (Fletcher et al. 2010).

ABCG2, also termed BCRP/MXR/ABCP, was independently cloned from placenta as well as cell lines selected for resistance to mitoxantrone or anthracyclines. An increased expression was also observed in the human colon CSC that were

enriched in chemo-residual and chemoresistant colon cancer cells (Kanwar et al. 2010; Yu et al. 2009, 2013). ABCG2 consists of a nucleotide-binding domain (NBD) at the amino terminus and a transmembrane domain (TMD) at the carboxyl terminus and it is postulated to form a homodimer to perform its biological functions. Over-expression of ABCG2 in cell lines confers resistance to a wide variety of anticancer drugs including mitoxantrone, daunorubicin, doxorubicin, topotecan and epirubicin. The expression of ABCG2 has been implicated in multidrug resistance (MDR) of acute myeloid leukemia and some solid tumors. In addition, ABCG2 can transport several fluorescent dyes or toxins. ABCG2 is found to be expressed in epithelial cells of intestine and colon, liver canaliculi, and renal tubules, where it serves to eliminate the plasma level of orally administered anticancer drugs as well as ingested toxins.

Recently, Xiong et al. (2014) reported isolation of SP cells by fluorescence-activated cell sorting (FACS) from multiple human colon cancer cell lines. Each cell line contains only about 1 % SP cells. These SP cells could differentiate into SP and non-SP cells. SP cells had a higher proliferation potency than non-SP cells. Compared to non-SP, SP cells showed increased mRNA and protein expression of drug export transporters (ABCG2, MDR1), stem cell growth related pluripotency factors (OCT-4, NANOG, SOX-2) and CSC marker (CD44, CD133). Moreover, SP cells were more resistant to chemotherapeutic drug 5-FU and cisplatin and were more invasive and displayed increased tumorigenic ability than their non-SP counterparts. They also exhibit higher expression of drug export transporters such as ABCG2 and lead therapeutic resistance in colon cancer.

5.3 Epithelial to Mesenchymal Transition

There are two major forms of drug resistance: *de novo* or acquired. Patients who are initially refractory to therapy display intrinsic or "de novo" drug resistance. Patients that initially respond to therapy typically relapse as a consequence of "acquired" drug resistance (Singh and Settleman 2010).

Recent studies have reported that CSCs or CSC-like cells are enriched in tumor remnants after chemotherapy. These include glioma, breast cancer, colon cancer and sophisticated CML mouse model (Clevers 2011). Data from our laboratory have demonstrated that although the combination of 5-FU and Oxaliplatin (FUOX) inhibited the growth of human colon cancer HCT-116 or HT-29 cells growth, it led to enrichment of CSC phenotype (Yu et al. 2009). We have now generated FUOX-resistant HCT116 and HT29 cells that exhibit both enrichment of CSCs/CSLCs and elevated levels of microRNA-21 (miR-21). Further, we have demonstrated that miR-21 plays a determinant role in inducing stemness in colon cancer cells (Yu et al. 2009, 2012).

Although the precise mechanism of acquired resistance is unclear, it is suggested that differentiated or undifferentiated cancer cells or CSCs adjust their gene expression profile, which is regulated by CSC niche. A simple example is that the cancer

cells increase their expression of thymidylate synthase (TS) after 5-FU treatment (Peters et al. 2002). A massive adjusting or reprogramming of gene expression leads to epithelial to mesenchymal transition (EMT).

Conventionally, EMT are trans-differentiation programs that are required for tissue morphogenesis during embryonic development. Recent reports indicate that the emergence of CSCs occurs in part as a result of EMT and EMT of tumor cells not only causes increased metastasis, but also contributes to drug resistance. The relationship of EMT, CSC and drug resistance have been summarized by Singh and colleagues (Singh and Settleman 2010).

6 Development of Cancer Stem Cell Targeted Therapies

6.1 CSC Targeted Therapy

One of the characteristics of CSCs is the expression of a distinctive set of surface biomarkers, which has led to the identification of key cellular activities that makes them vulnerable to therapeutic interventions. For instance, cell surface marker expression could be used for antibody-directed therapy to target proteins such as CD133, CD44 or EpCAM (Deonarain et al. 2009). ABCG2, which are ATP-dependent drug efflux pumps, could be targeted by ATP-competitive agents (Kuhnle et al. 2009; Robey et al. 2007). CSC- targeted therapy should be combined with conventional therapeutic agents that can eliminate both differentiated and undifferentiated cancer cells in order to avoid recurrence of tumor due to dedifferentiation of cancer cells into CSCs by the process of EMT.

6.2 Induction of CSCDifferentiation and Treatment

While CSCs are resistant to conventional chemotherapy, differentiated or differentiating cells that form bulk of the tumor, are sensitive to chemotherapy. Therefore, the differentiation induction is one of the therapeutic options proposed to eliminate or functionally antagonize CSCs. This therapeutic strategy consists of forcing CSCs to shift into a terminal epithelial phenotype, losing their self-renewal abilities, and therefore becoming vulnerable to conventional therapies (Paldino et al. 2014).

Recently, few molecular agents including bone morphogenetic protein 4 (BMP4), antisense oligonucleotides (anti-miR-21) and some natural compounds like difluorinated curcumin (CDF) and Omega-3 polyunsaturated fatty acids (ω-3 PUFA) have been proposed to induce differentiation in colon CSCs. BMP4 is able to activate a differentiation program and stimulate apoptosis in colon CSCs, reducing β-catenin activation through inhibition of PI3K/AKT pathway and up-regulation of Wnt-negative regulators. Additional, administration of BMP4 to immune-compromised mice with tumors, which arose from colon CSCs, increased the antitumor effects of

5-fluorouracil and oxaliplatin, confirming that BMP4 might be developed as a therapeutic agent against cancer stem cells in advanced colorectal tumors (Kanwar et al. 2011; Paldino et al. 2014).

MicroRNAs (miRNAs, miRs) are endogenous posttranscriptional modulators that negatively control the expression of their target genes and play an important role in the development and progression of many malignancies, including colorectal carcinoma. In particular, expression of miR-21 is greatly increased in chemotherapy-resistant colon cancer cells that are enriched in undifferentiated CSCs (Yu et al. 2009, 2012). Down-regulation of miR-21 in chemoresistant colon cancer cells by antisense miR-21 induced differentiation, as evidenced by marked increases in cytokeratin-20 (CK-20) expression and alkaline phosphatase activity (Yu et al. 2013). These changes were accompanied by a significant reduction in the expression of colon CSC marker CD44, colonosphere formation, and T-cell factor/lymphoid enhancer factor (TCF/LEF) activity but increased the expression of proapoptotic programmed cell death 4 gene (Yu et al. 2012). Induction of differentiation greatly increased sensitivity of chemoresistant colon cancer cells to the chemotherapeutic agents 5-FU, oxaliplatin and the combination of 5-FU and oxaliplatin (FUOX) (Yu et al. 2013).

Treatment of CSC-enriched chemoresistant colon cancer cells with CDF $^{+}$FUOX showed a higher magnitude growth inhibition by either agent alone. Growth inhibition by CDF and CDF^{+} FUOX in differentiating CR colon cancer cells was associated with reduction in the expression of CD44 and epidermal growth factor receptor (EGFR) (Yu et al. 2013; Kanwar et al. 2011). The observation suggests that down-regulation of miR-21 induces differentiation of CSCs and differentiation enhances susceptibility of CR cancer cells to conventional and nonconventional therapeutic regimen.

More recently, we reported that eicosapentaenoic acid (EPA; one of the ω-3 PUFA) alone was effective, combination of EPA and FUOX was more potent in inhibiting the growth of CSC- enriched chemoresistant colon cancer cells as evidenced by decreased colonosphere formation and increased sphere disintegration as well as suppression of growth of xenografts of CR colon cancer cells in SCID mice, and lastly reduction in proinflammatory metabolites in mice (Vasudevan et al. 2014). In addition, EPA^{+} FUOX increase apoptosis as evidenced by PARP cleavage and resulting reduction in CSC/CSLC population. Furthermore, increased pPTEN, decreased pAkt, normalization of β-catenin expression, localization, and transcriptional activity were observed by EPA^{+} FUOX treatment (Vasudevan et al. 2014). The data suggest multiple signaling pathways are involved in regulation of self-renewal of CSCs.

7 Future Directions

The drugs under development mainly attempt to target signaling pathways involved in the regulation of self-renewal of normal somatic stem cells, such as the Wnt, the Sonic Hedgehog and the Notch pathways. The focus needs to be shifted towards the

development of drugs that would either preferentially block stem cell (and CSC) renewal or drive the stem cells into differentiation, thus closing down the tumor supply line (Zhou et al. 2009; Frank et al. 2010). A thorough understanding of stem cell biology in terms of signaling and proliferation is essential for the development of therapeutic strategies to eliminate them. A major hurdle in achieving a successful therapeutic modality is specificity. Small molecules or chemically modified oligonucleotides such as anti-miR (Stenvang et al. 2012) that target multiple pathways involved in stem cell self-renewal provide an excellent therapeutic modality (Kreso et al. 2014).

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