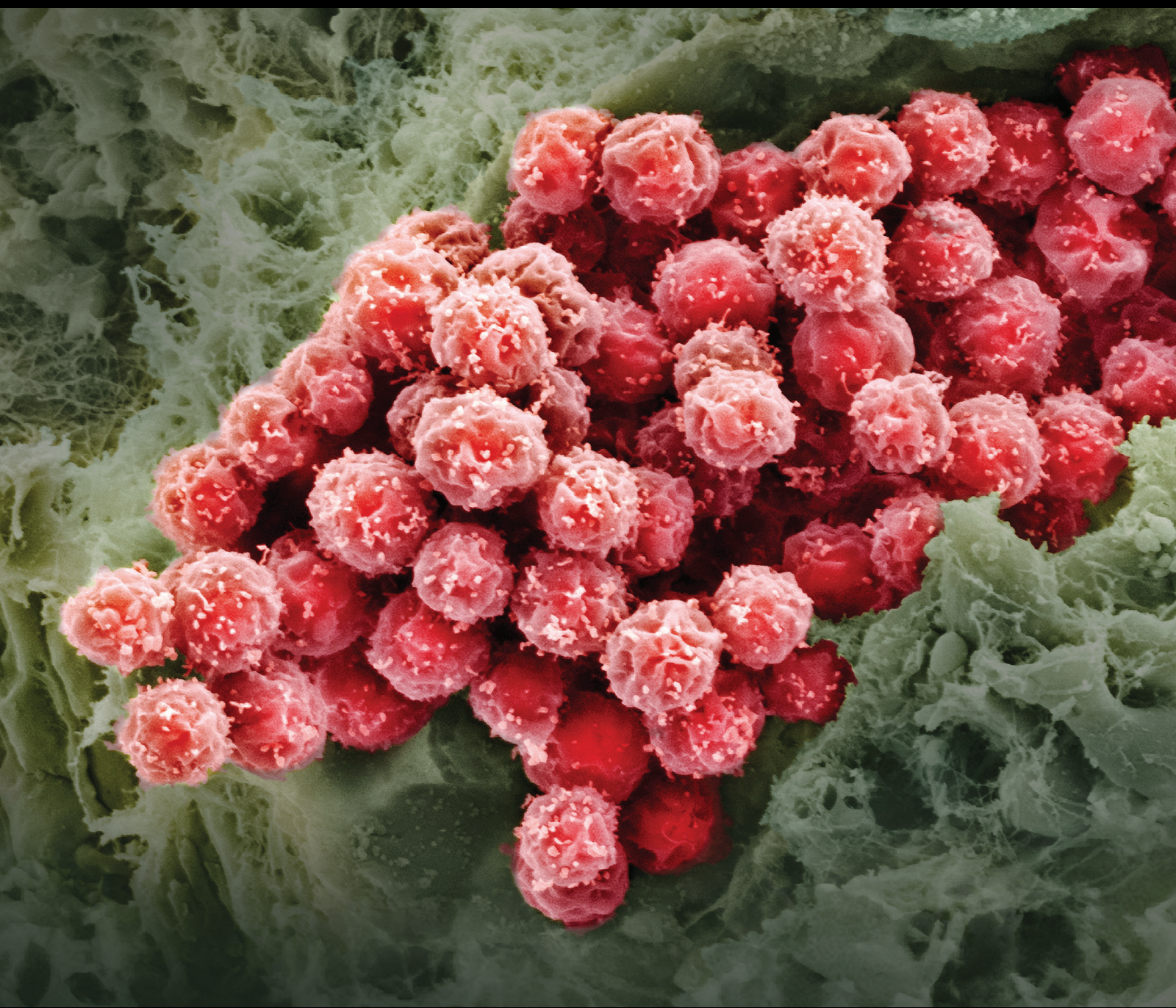
Stem Cells International

Regulation of Stemness in Carcinoma Cells

Lead Guest Editor: Jijun Hao
Guest Editors: Juli Unternaehrer, Xiaojiang Cui, Ninghui Cheng, and Yusuke Oji



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Editorial Board

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https://doi.org/10.1155/2017/7681769

Editorial

Regulation of Stemness in Carcinoma Cells

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Received 16 July 2017; Accepted 16 July 2017; Published 27 July 2017

Over the last decade, the important roles of cancer stem cells (CSCs) in tumor recurrence and metastasis have been increasingly recognized. Significant efforts have been made to understand the mechanisms underlying regulation of CSCs' stemness and biology with the goal of developing effective therapies to target CSCs for cancer treatment.

The purpose of this special issue is to provide readers with a representative outlook of the recent advances in the CSC research field. The topics cover signaling pathways, transcription factors/epigenetics, miRNA, and microenvironment in the regulation of CSC stemness as well as identification of CSC markers.

This special issue publishes seven selected papers regarding the above specific timely topics, and the details are summarized below.

Signaling regulation and epigenetic modification are believed to play key roles in CSCs' stemness and functions. J. Koury et al. focused on three critical evolutionarily conserved signaling pathways (Wnt, Hedgehog, and Notch pathways) and their crosstalk in governing CSCs' fate and summarized therapeutic studies targeting these pathways to eliminate CSCs and improve overall cancer treatment outcomes. In addition, G. M. Kelly and M. I. Gatie reviewed current available knowledge of transcription factors, DNA methylation, and chromatin remodeling in embryonal carcinoma cells (ECCs). Furthermore, the roles of miRNA in ECCs and rhabdomyosarcoma (RMS) have been discussed, respectively, in G. M. Kelly and M. I. Gatie's and A. J. Hron and A. Asakura's articles.

Compelling evidence indicates that tumor microenvironment is a key regulator in maintenance of CSC stemness, invasiveness, and drug resistance. P. M. Aponte and A. Caicedo reviewed the organization of tumor microenvironment components with a focus on mesenchymal stem/stromal cells (MSCs), followed by therapeutic strategies targeting CSCs in tumors. In parallel, E. Y.-T. Lau et al. offered a thorough overview of stromal cells, immune cells, extracellular matrix, tumor stiffness, and hypoxia in the regulation of CSC plasticity and therapeutic resistance.

CSCs normally constitute a very small proportion of total tumor cells. Thus, identifying reliable and specific CSC markers will help develop effective therapies to precisely target and destroy the CSCs. In prostate cancer, numerous markers are postulated to be associated with prostate CSCs; however, the clinical significance of these markers remains largely unproven. In the article titled "Prostate Cancer Stem Cell Markers Drive Progression, Therapeutic Resistance, and Bone Metastasis", K. S. Harris and B. A. Kerr reviewed current prostate CSC markers with functional relevance linked to cancer progression, metastatic colonization and growth, recurrence, or therapeutic resistance. Moreover, X. Song et al. reported that the cells with CD19, CD45, and

CD44 surface markers identified in atrial myxoma were CSC-like cells and may have the capacity for myxoma initiation and progression.

The guest editors hope this special issue provides readers with helpful information of recent advances in CSC research and may stimulate interest in further research in this area.

Acknowledgments

We would like to thank all the authors and reviewers for their excellent contributions.

Jijun Hao
Juli Unternaehrer
Xiaojiang Cui
Ninghui Cheng
Yusuke Oji

Hindawi
Stem Cells International
Volume 2017, Article ID 8629234, 9 pages
https://doi.org/10.1155/2017/8629234

Review Article

Prostate Cancer Stem Cell Markers Drive Progression, Therapeutic Resistance, and Bone Metastasis

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Received 3 January 2017; Revised 29 March 2017; Accepted 3 May 2017; Published 11 June 2017

Academic Editor: Juli Unternaehrer

Metastatic or recurrent tumors are the primary cause of cancer-related death. For prostate cancer, patients diagnosed with local disease have a 99% 5-year survival rate; however, this 5-year survival rate drops to 28% in patients with metastatic disease. This dramatic decline in survival has driven interest in discovering new markers able to identify tumors likely to recur and in developing new methods to prevent metastases from occurring. Biomarker discovery for aggressive tumor cells includes attempts to identify cancer stem cells (CSCs). CSCs are defined as tumor cells capable of self-renewal and regenerating the entire tumor heterogeneity. Thus, it is hypothesized that CSCs may drive primary tumor aggressiveness, metastatic colonization, and therapeutic relapse. The ability to identify these cells in the primary tumor or circulation would provide prognostic information capable of driving prostate cancer treatment decisions. Further, the ability to target these CSCs could prevent tumor metastasis and relapse after therapy allowing for prostate cancer to finally be cured. Here, we will review potential CSC markers and highlight evidence that describes how cells expressing each marker may drive prostate cancer progression, metastatic colonization and growth, tumor recurrence, and resistance to treatment.

1. What Is a Cancer Stem Cell?

Cancer stem cells (CSCs) are tumor cells capable of self-renewal and asymmetric division postulated to drive tumor growth, metastasis, and therapeutic relapse [1–7]. These cells may be a subset of or entirely separate from circulating tumor cells (CTCs), disseminated tumor cells (DTCs), tumor-initiating cells, or tumor progenitor cells [8–11]. It has been reviewed in depth elsewhere that both CTCs and DTCs may contain a subpopulation of cells with CSC characteristics, while tumor-initiating or tumor progenitor cells do not necessarily require self-renewal capacity [6, 12, 13]. CSCs were originally hypothesized in an attempt to explain tumor heterogeneity and both metastatic and recurrent tumor growth. Under this hypothesis, only one or a small number of CSCs are needed to recapitulate the tumor and its initial heterogeneity. In addition, multiple studies have demonstrated that these CSCs were more resistant to most chemotherapeutics and radiation and thus may survive initial treatment [3, 5, 14]. This survival ability means that these CSCs could drive recurrence and cancer progression after therapy and targeting these CSCs would improve cancer treatment resulting in increasing numbers of cured patients.

A controversy in the field surrounds whether or not a CSC can be considered a true stem cell. Stem cells in normal adult tissues maintain tissue integrity and are essential for repair. These normal stem cells are capable of self-renewal with asymmetric division such that the progenitor cells required for tissue heterogeneity are produced while maintaining the stem cell population. Depending on the tissue, some CSCs have the ability to enter the cell cycle; however, most are found to have a quiescent phenotype and are characterized as a dormant reserve pool of cells for regeneration [15, 16]. Confusion also arose over whether CSCs had developed from normal stem cells. Current consensus is that CSCs are not necessarily malignant or transformed normal stem

cells and can arise from a variety of cell sources [1, 17]. While CSCs express some genes and cell surface proteins associated with normal stem cells, their division and ability to differentiate are significantly different [6, 18]. This difference was highlighted in a study suggesting that CSCs had the ability to reconstitute and self-renew differentiated carcinoma [19]. To further demonstrate pluripotency, self-renewal capacity is measured by clonogenic assays and serial in vivo tumor initiation or limiting dilution experiments are designed to examine whether a population could regenerate an entire tumor and thus be considered a CSC [2, 4, 12, 15]. In order to differentiate tumor-initiating cells from CSCs, repeated tumor-initiating xenografts are required [9, 15]. Additional studies for self-renewal examine the formation of prostaspheres, which represent three-dimensional tumor progenitor structures [7]. In concert, expression of the stem cell markers *Oct3/4*, *Sox2*, *Klf4*, *Nanog*, and *c-Myc* in CSCs are often used to examine stemness [20–22]. Several of these stem cell markers were upregulated in prostate cancer when compared to prostatitis or benign hyperplasia [23]. The stemness gene markers, *Sox2* and *Nanog* in particular, may be considered markers of CSCs on their own; however, for the purposes of this review, they are part of the stemness gene expression profile that is used to identify potential stem-like cells that may comprise a CSC population if proven capable of serial tumor initiation.

2. A Multitude of Markers

A variety of markers are postulated to identify prostate CSCs; however, the clinical significance of these markers remains largely unproven (Table 1). In part, the high number of proposed markers for CSCs is due to the heterogeneity of prostate tumors [24] and additional research is need to parse out the cellular origin of the CSCs. A majority of the proposed CSC markers are cell surface proteins, which have the added benefit of being able to separate out and enrich the CSC population; however, a number of intracellular proteins have also been postulated to function as CSC markers (Table 1) [18]. Initial CSC marker identification was largely based upon immunohistochemistry of primary and metastatic tumors. For example, Trop2, CD133, and $\alpha_2\beta_1$ integrin positive cells were increased in prostate cancer containing Gleason grade 4 regions compared with benign tissue and localized in the epithelium as single cells or small clusters, which may represent CSC niches [25]. In addition to localization within tissues, individual CSCs were identified by separating out cell populations from dissociated primary tumors or cancer cell lines as well as by examining markers on cells displaying stem-like phenotypes. A limitation of the field has been the reliance on prostate cancer cell lines in the identification of CSC markers especially for those involved in metastasis and in therapeutic resistance. Thus, additional research is needed using patient-derived metastatic and resistant tumors. Due to the difficulty of obtaining metastatic or recurrent biopsies, multiple studies have focused on the ability to isolate and quantify CSCs in a "liquid biopsy" using the whole blood of patients. This test would be less invasive and could provide more prognostic information [26, 27]. CSCs enriched from patient whole blood would be representative of a CTC subpopulation but may still miss the CSCs developing in the metastatic niche or after therapy. In general, a great deal of research is needed to truly define the markers of prostate CSCs involved in all steps of human disease progression.

While single markers are often used, multiple markers could create a signature capable of reliably isolating and quantifying CSCs. For example, $CD133^+$ $CD44^+$ $ABCG2^+$ $CD24^-$ cells were concentrated in spheroids derived from medium-scored (5-6) Gleason patient biopsies, when surgical intervention is most effective [28], whereas $EZH2^+$ E-$cadherin^+$ cells in primary tumors were associated with disease recurrence based on tissue microarrays from 259 patients with clinically localized prostate cancer [29]. Additionally, $ALDH^{hi}$ $CD44^+$ $\alpha_2\beta_1^+$ cells increased with castration resistance in mice implanted with prostate cancer cell line xenografts and, when isolated from patients, displayed potential self-renewal capacity based on colony and spheroid formation [30]. In fact, $CD44^+$ $\alpha_2\beta_1^{hi}$ $CD133^+$ cells isolated from 40 patients did not correlate with Gleason scores, but were capable of self-renewal, as shown by second generation colony formation, displayed a basal phenotype, and were predicted to be CSCs [31]. Unfortunately, the use of multiple markers greatly decreases the numbers of cells isolated from prostate cancer patients making additional characterization more difficult. As new methods to reliably propagate CSCs are discovered, the use of multiple markers to study CSCs will become more viable. Despite these difficulties in identification and culture, the ongoing interest in CSCs is driven by their potential roles in tumor progression, metastasis, and lack of response to therapy. In this review, we examine potential prostate CSC markers with functional relevance linked to cancer progression, metastatic colonization and growth, recurrence, or therapeutic resistance.

3. Cancer Stem Cells Drive Prostate Cancer Progression

CSCs may make up <1% of the primary tumor and yet are postulated to drive continued tumor progression in the face of hypoxia and other assaults [4, 5]. In response to hypoxia, nutrient deficiency, and oxidative stress, CSCs displayed altered gene expression allowing them to become more mobile, invasive, and resistant to additional stress. In order to invade locally and then metastasize, CSCs are predicted to have undergone epithelial-mesenchymal transition (EMT) and the transition to mesenchymal marker expression is often one measurement of prostate cancer progression. Markers for EMT include increased N-cadherin and vimentin, in addition to decreased E-cadherin, epithelial cell adhesion molecule (EpCAM), and other epithelial cell markers, which includes both cytokeratins and zonula occludens-1. These changes during EMT and in response to stressors greatly alter the surface and intracellular proteins that may be expressed by CSCs. When these migrating CSCs have entered the patient circulation, they are referred to as circulating tumor cells (CTCs). A subset of the CTCs, which survive through the circulation, may become metastatic cells. While EpCAM is regularly utilized to detect cancer cells in

Table 1: Reported markers for prostate cancer stem cells.

Marker name	Effects	References
Extracellular markers		
CD117/c-kit	Tumor progression	[37]
	Metastatic colonization and growth	[60, 61]
	Recurrence and therapeutic resistance	[37]
CD133	Tumor progression	[25, 95, 96]
	Self-renewal capacity	[31, 95, 97, 98]
	Stemness gene expression	[23, 99]
CD44	Tumor progression	[96, 100]
	Self-renewal capacity	[30, 31, 40, 41, 97, 101]
	Stemness gene expression	[41, 99]
	Metastatic colonization and growth	[102]
$\alpha_2\beta_1$ integrin	Tumor progression	[25, 50, 63]
	Self-renewal capacity	[30, 31]
	Recurrence and therapeutic resistance	[63]
α_6 integrin	Tumor progression	[63]
	Self-renewal capacity	[101, 103]
	Recurrence and therapeutic resistance	[63]
CXCR4	Tumor progression	[88, 104]
	Self-renewal capacity	[70]
	Metastatic colonization and growth	[58, 62]
	Recurrence and therapeutic resistance	[62, 70]
E-cadherin	Stemness gene expression	[20, 36]
	Metastatic colonization and growth	[65]
	Therapeutic resistance	[29]
EpCAM	Tumor progression	[33, 59, 71]
	Metastatic colonization and growth	[59, 90]
	Recurrence and therapeutic resistance	[33, 71, 72]
Cytokeratin 5	Tumor progression	[67]
	Self-renewal capacity	[103]
PSA^{lo}	Tumor progression	[38]
	Self-renewal capacity	[38]
	Stemness gene expression	[38]
	Recurrence and therapeutic resistance	[100]
ABCG2	Recurrence and therapeutic resistance	[49, 73, 75]
Trop2	Tumor progression	[25, 105]
	Self-renewal capacity	[91, 92, 101, 103]
AR variant 7	Recurrence and therapeutic resistance	[79, 81, 82]
CD166/ ALCAM	Tumor progression	[39, 106]
	Self-renewal capacity	[39]
	Recurrence and therapeutic resistance	[39]
Intracellular markers		
ALDH1	Tumor progression	[47, 48, 51, 100, 107]
	Self-renewal capacity	[30, 47, 107]
	Stemness gene expression	[49]
	Recurrence and therapeutic resistance	[47, 49, 100]
TG2	Tumor progression	[77]
	Recurrence and therapeutic resistance	[77, 78]
EZH2	Tumor progression	[50, 96]
	Stemness gene expression	[108]
	Metastatic colonization and growth	[51, 68]
	Recurrence and therapeutic resistance	[29, 50, 69]

EZH2: enhancer of zeste homolog 2; ALDH1: aldehyde dehydrogenase 1; ABCG2: ATP-binding cassette G2; PSA: prostate-specific antigen; TG2: transglutaminase 2. Self-renewal capacity includes sphere formation, colony formation, clonogenic assays, and limiting dilution assays. Stemness gene expression includes *Sox2*, *Oct3/4*, *Nanog*, *c-myc*, and/or *Klf4*.

the circulation of prostate cancer patients, the requirement of cells to undergo EMT prior to metastasis suggested that neither EpCAM nor E-cadherin would be expressed on CSCs [10, 32]. The widely used and FDA-approved CellSearch™ system is based on EpCAM positivity and multiple studies have demonstrated that the numbers of circulating $EpCAM^+$ cells increased with prostate cancer progression. In a study comparing 15 healthy controls with 20 locally advanced, 40 metastatic castration resistance, or 15 taxane-refractory prostate cancer patients, the CellSearch system was used to enumerate EpCAM positive CTCs and demonstrated that metastatic patients had more CTCs in their circulation compared with normal controls and locally advanced patients [33]. Another study used transgenic mice to label prostate cancer cells as either epithelial, undergoing EMT, or mesenchymal like. This study reported that cells partially underwent EMT, expressed both EpCAM and vimentin, and were increasingly capable of self-renewal as demonstrated by sphere formation assays and progenitor Lin^- $Sca1^+$ $CD49f^{hi}$ counts when compared with cells either completely epithelial or mesenchymal [34]. Perplexingly, it was found that E-cadherin knockdown stimulated EMT in prostate cancer PC3 cell line spheres and xenografts [35], while E-cadherin expression was associated with stemness gene expression and sphere formation in DU145 and PC3 cell lines [20, 36]. Therefore, continued research is needed to understand

whether EMT and stemness gene expression are linked in CSCs or are present in separate populations of CSCs.

Multiple nonepithelial surface and intracellular markers are associated with cancer progression. In a preclinical study of 115 patients' primary tumors and CTCs, $CD117^+$ cells were higher in patients with high-grade tumors (T3 staged or Gleason 8+) in comparison with low-grade tumors (Gleason 6-7 or T2 staged) and xenograft tumors expressing CD117 were larger with increased angiogenesis [37]. However, CD133 was found increased in high-grade, Gleason 8+ primary tumors, but could not be measured in the circulation [37]. Actually, PSA^{lo} and $CD166^+$ cells were also increased with tumor grade in prostate cancer patients (43 patients for PSA^{lo} and 112 patients for $CD166^+$) and demonstrated increased sphere formation [38, 39], indicating a possible self-renewal capacity. Using cell lines, CD44 expressing LNCaP and DU145 cells were more invasive through matrigel, expressed EMT and stemness markers, exhibited self-renewal capacity, and were more tumorigenic in xenografts [40, 41]. Furthermore, in prostate cancer cores from 73 patients, CD44 expressing cells were also positive for chromogranin A, a neuroendocrine cell marker [42]. This supports new evidence that CSCs may include neuroendocrine cells, which are terminally differentiated and resistant to common therapies. Both prostate CSCs and neuroendocrine cells in primary tumors are androgen independent and have lost androgen receptor (AR) as well as PSA expression. It has been shown that some neuroendocrine cells express stemness markers and may have undergone EMT. These neuroendocrine cells may represent a potential subpopulation of CSCs that drive castration resistant prostate cancer progression [43–46]. This possibility requires additional research to understand the relationship between CSCs and neuroendocrine prostate cancer. For example, in prostate cancer tumor microarrays, ALDH1 expression was increased in cancerous tissue compared to that in benign tissue and was associated with AR positivity and neuroendocrine marker expression [47]. When cells were isolated from more than 100 patient prostate specimens, ALDH1 expression was higher in cancerous tissue compared with that in benign hyperplasia [48]. ALDH1 expression predicted poor clinical outcomes and drives stemness markers, while additional intracellular markers, including EZH2, have increased prevalence in higher grade cancer sections [47, 49–51]. While a number of markers are associated with progression of the primary tumor and may be relevant for prognosis, the greatest need is in uncovering markers to characterize CSCs driving tumor escape and to identify patients likely to experience metastases.

4. Cancer Stem Cells Control Colonization and Metastatic Growth

Approximately 3.2×10^6 cells/g tissue are shed from tumors daily; however, only <0.01% develop into metastases [10, 52]. Shed tumor cells are predicted to comprise 1 cell out of 10^5 to 10^7 leukocytes in the bloodstream [53]. While in the circulation, these cells are called circulating tumor cells (CTCs) and when in the metastatic niche, disseminated tumor cells (DTCs). The ability of these cells to enter the circulation and survive requires EMT to have occurred as described above [54]. However, all CTCs and DTCs may not be capable of forming micro- or macrometastases, as many cells remain dormant within the metastatic tissue and many do not survive the shear stresses, oxygen tension changes, and other dangers of the circulation. Growth of the metastatic tumor and recapitulation of the primary tumor heterogeneity in a secondary site is driven by CSCs [55, 56]. Asymmetric division of CSCs allows for the maintenance of the CSC population as well as expansion of cells representing the full spectrum of the original heterogenic tumor. Several markers associated with tumor progression and therapeutic resistance can identify CTCs and can be found on DTCs in patients' bone metastases. Primary tumor expression of CXCR4 (in 57 patients or in 35 patients in a second study), EpCAM (in 90 primary tumor and 16 metastatic tumors), and EZH2 (in 146 patients) were associated with increased distant metastasis and local recurrence during patient follow-up [51, 57–59] indicating that these markers may drive metastasis. However, since staining was only in the primary tumors, these markers have not been implicated directly in metastatic colonization. CD117 and CXCR4 staining, however, was increased in patient bone metastatic tumors over levels seen in the primary tumor [60–62]. This metastatic staining indicates that CD117 and CXCR4 likely either drive the colonization of metastatic cells, the growth of metastatic tumors, or possibly escape from dormancy. One study examining DTCs measured the percentage α_6 integrin or α_2 integrin expressing cells in the white blood cells extracted from the bone marrow. These two integrin markers were increased with tumor progression from localized T1-T2 tumors (44 patients) to hormone-refractory metastatic tumors (28 patients) and were associated with decreased metastasis progression free survival [63]. In a study of 53 patients, CTC enumeration using both the CellSearch method and the AdnaTest kit isolating $EpCAM^+$ and $HER2^+$ cells demonstrated that $EpCAM^+$ cell numbers in the circulation correlated with the presence of metastases [64]. Interestingly, E-cadherin expression was associated with bone metastasis in 109 patients, but not soft tissue metastasis in 56 patients [65], suggesting that mesenchymal-epithelial transition (a reversion from EMT) may be occurring, which is required for escape from the dormancy normally associated with bone metastatic growth. Nonetheless, the mechanisms of bone colonization, dormancy, and subsequent reactivation remain to be elucidated and will need to be confirmed using human samples. It is this transition from micro- to macrometastases or escape from dormancy that drives prostate cancer recurrence. We postulate that CSCs will play an important role in these processes.

5. Cancer Stems Cells in Recurrence and Resistance to Treatment

After radical prostatectomy, radiation, cryotherapy, chemotherapy, or other treatments, CSCs remaining in the tissue or in the circulation may induce the development of recurrent or metastatic tumors. As most cancer therapies cause

DNA damage in rapidly dividing cells or target hormonal or signaling pathways, they may not affect CSCs which are functionally different from the bulk tumor cells [66]. CSC markers, such as EZH2, PSA^{lo}, and CD117, expressed in the primary tumor were predictive for biochemical recurrence (rising PSA in the circulation) after radical prostatectomy [37, 38, 50]. $CD117^+$ CTC numbers, in particular, remained high 3 months after radical prostatectomy in the circulation of 12 patients who experienced biochemical recurrence 6–18 months later [37], indicating that CSCs in the circulation may be used to predict therapeutic failure earlier. In another study of 50 patients with CTCs measured before and after androgen deprivation therapy by the CellSearch apparatus, it was shown that CTC levels were associated with rising PSA and decreased progression free survival [64]. In addition, staining of potential CTCs from 27 patients with metastatic castration-resistant prostate cancer demonstrated that patients with neuroendocrine prostate cancer have significantly different CTCs with lower AR and cytokeratin expression and smaller size [67]. In murine models, CD166 was upregulated in prostates after castration [39]; while in human tissues samples, EZH2 was increased in hormone refractory metastatic tissues and was associated with decreased failure-free survival [68, 69]. Overall, these data indicate that the numbers of CSCs in primary tumors or the patient circulation can be used to identify patients likely to experience a recurrence and for whom more aggressive treatment is warranted.

In addition, CSCs are postulated to be resistant to chemotherapeutics and radiation. Surface markers, even those with no known biological function, were associated with resistance to several chemotherapeutics. For example, specific inhibition of CXCR4 with AMD3100 resensitized DU145 and PC3 prostate cancer cells to the chemotherapeutic docetaxel [62, 70], while knockdown of EpCAM in multiple prostate cancer cell lines enhanced radiosensitivity and chemosensitivity to docetaxel, paclitaxel, and doxorubicin [71]. Thus, these cells may be driving therapeutic relapse. Not to mention, CSC surface markers, including EpCAM, were measureable in salvage prostatectomy tissue from patients with recurrence after radiotherapy [72], implying that these cells may drive recurrence due to radiation resistance as well. Intracellular CSC markers in particular can directly control therapeutic resistance. ATP-binding cassette (ABC) transporters including ABCG2 drive CSC resistance to multiple drugs including taxanes, tyrosine kinase inhibitors, topoisomerase inhibitors, and antimetabolites [49, 73–75]. The ability of this transporter protein to efflux drugs in cell lines also results in removal of Hoechst 33342 leading to most $ABCG2^+$ cells initially being called "side population" cells based on flow cytometry [4, 76]. These ABCG2 expressing and other side population cells are able to efflux most drugs and prevent the desired effects of treatment. Another mechanism of therapeutic resistance is based on metabolic changes. For example, ALDH1 expression induces metabolism of chemotherapeutic agents and reduces radiosensitivity in prostate cancer cell lines [49]. Finally, CSC markers, such as TG2, were increased after androgen deprivation therapy in prostate cancer patient samples and the associated androgen-resistance in prostate cancer cell lines [77, 78] suggesting that they may be involved in the loss of androgen sensitivity and relapse. In a study of 62 patients treated with enzalutamide or abiraterone, $EpCAM^+$ CTCs demonstrated upregulation of the AR variant 7 and decreased progression free survival [79]. In a separate study of 161 patients treated with enzalutamide or abiraterone, men with AR variant 7^+ CTCs had worse overall survival compared to men with AR variant 7 negative CTCs [80, 81]. Further, another study reported that AR variant 7 expressing CSCs increased following treatment with enzalutamide and the presence of this variant led to tumor growth during androgen deprivation therapy [82, 83]. Additional evidence also indicates that AR expression might be induced in CSCs after treatment and progression to castrate resistance [24, 84, 85]. Thus, both extracellular and intracellular CSC markers may induce resistance to treatment and are prime targets for attempts to sensitize CSCs to therapy.

6. The Future of Cancer Stem Cells

The interest in identifying CSC markers rests in the hope of developing therapies that specifically target the CSC population. If CSCs can be precisely identified and destroyed, the expectation is that then conventional treatments will be effective on the non-CSC population and tumors will be eradicated. Potential methods for targeting CSCs include drugs inhibiting CSC-specific signaling pathways, methods to induce differentiation or a loss of stemness, compounds targeting alterations in CSC metabolism, and immunotherapy directed at CSC markers [18, 55]. One proposed CSC targeting drug is derived from a cruciferous vegetable metabolite called BR-DIM that could be administered prior to radical prostatectomy. In cell culture studies, BR-DIM inhibited self-renewal ability of CSCs and decreased EZH2 expression [86], suggesting that this treatment may induce CSC terminal differentiation and prevent therapeutic resistance. In addition, new small molecule inhibitors are under development which are capable of targeting signaling pathways and transcription factors prevalent in CSCs but not normal cells including Stat3 [87]. Further possible pathways of interest include Akt activation and Erk signaling [70, 88], which may be upregulated in CSCs in comparison with the bulk tumor population and responsible for the enhanced CSC survival. Other signaling pathways associated with stemness are also potential targets for inhibition. The Wnt, TGF-β, Hedgehog, and Notch pathways in particular drive CSC self-renewal capabilities and are inhibited by several drugs being tested clinically [18, 89]. In addition to these CSC-targeted interventions, combination therapies could also target the bulk tumor cells, hypoxia responses, or angiogenesis in concert. Several stem cell markers including CXCR4 and CD117 were associated with increased angiogenesis and escape from tumor hypoxia [37, 88]. These data indicate that additional combination therapies with antiangiogenic or antihypoxia inducible factor-1α treatments may have improved efficacy over a single therapy. Combined therapies may also target the tumor microenvironment. Disrupting the CSC niche and preventing interaction between CSCs and the

extracellular matrix could also prevent survival signaling. Altering the interplay between CSCs and cancer-associated fibroblasts, tumor-associated macrophages, or the adaptive immune system are another area for additional research [18]. A final area of directed therapy would be using the CSC markers as immune targets. In one study, using EpCAM as a chimeric antigen receptor to induce T cell targeting of $EpCAM^+$ tumor cells resulted in inhibition of PC3M tumor growth leading to increased murine survival [90]. Continued examination of CSC markers is needed as some markers may identify both normal stem cells and CSCs, such as Trop2 and α_6 integrin [91, 92], causing adverse events in clinical trials. Continuing research is also focused on novel methods to isolate, identify, and enrich for CSCs, particularly using CTCs collected in a liquid biopsy. Several groups are developing microfluidic chips using either CSC markers, cell size, or electromagnetic changes to isolate and quantify CTCs [93, 94]. The development of these devices requires knowledge of either the markers for use in enrichment or understanding of the physical property differences between CSCs and non-stem-like CTCs or other blood cells. Additional CSC marker identification and refinement are required for the development of new screening and enumeration methods as well as for the eventual development of prostate CSC-based therapeutics aimed at preventing tumor progression, therapeutic resistance, and bone metastasis.

Abbreviations

ABC: ATP-binding cassette
ALDH1: Aldehyde dehydrogenase 1
AR: Androgen receptor
CSC: Cancer stem cell
CTC: Circulating tumor cell
DTC: Disseminated tumor cell
EMT: Epithelial-mesenchymal transition
EpCAM: Epithelial cell adhesion molecule
EZH2: Enhancer of zeste homolog 2
PSA: Prostate-specific antigen.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Mr. Koran S. Harris was supported by an NC A&T NIH/NIGMS MARC U*STAR Grant (T34 GM083980-08) and the DOD PCRP NC Summer Research Program (W81XWH-16-1-0351). Dr. Bethany A. Kerr was supported by a NIH/NCI Pathway to Independence Award (R00 CA175291).

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Hindawi
Stem Cells International
Volume 2017, Article ID 2480375, 10 pages
https://doi.org/10.1155/2017/2480375

Review Article

An Examination of the Role of Transcriptional and Posttranscriptional Regulation in Rhabdomyosarcoma

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Received 12 January 2017; Revised 1 April 2017; Accepted 18 April 2017; Published 30 May 2017

Academic Editor: Ninghui Cheng

Rhabdomyosarcoma (RMS) is an aggressive family of soft tissue tumors that most commonly manifests in children. RMS variants express several skeletal muscle markers, suggesting myogenic stem or progenitor cell origin of RMS. In this review, the roles of both recently identified and well-established microRNAs in RMS are discussed and summarized in a succinct, tabulated format. Additionally, the subtypes of RMS are reviewed along with the involvement of basic helix-loop-helix (bHLH) proteins, Pax proteins, and microRNAs in normal and pathologic myogenesis. Finally, the current and potential future treatment options for RMS are outlined.

1. Introduction

Rhabdomyosarcoma (RMS) is an aggressive and malignant form of pediatric cancer developed from myogenic cell lineages, as evidenced by expression of MyoD and desmin. The key to our current understanding of RMS is the role of tissue-specific transcription factors including MyoD, Pax family of proteins, tissue-specific microRNAs (miRNAs), and molecular mechanisms for cell cycle regulation and differentiation governed by these factors.

MyoD is a positively regulating bHLH myogenic regulatory factor (MRF) that acts as a critical control point in conjunction with enhancer box- (E-box-) binding partners and other MRFs including Myf5 and myogenin to commit mesoderm cells to a skeletal muscle lineage [1]. During development and repair, high MyoD expression acts to repress cell renewal, to promote terminal differentiation, and to induce apoptosis [1]. In conjunction with other MRFs, MyoD acts to oppose the role of proliferation-inducing transcription factors including Pax3 and Pax7.

The Pax family of proteins plays an essential role in muscle stem cell maintenance and proliferation. Pax proteins play a nonpeaceful role in fusion protein-positive cases of RMS, where they are thought to contribute in part to its malignant phenotype [2–6]. Together, MyoD and Pax proteins are drivers of the myogenic program and are regulated by multiple factors including miRNAs.

miRNAs are small, noncoding RNAs that are vital to myogenesis and eukaryotic organisms in general due to their ability to posttranscriptionally modify target mRNA [6]. miRNAs function via base pairing with complementary sequences within mRNA molecules. They achieve their silencing effect through a combination of mRNA strand cleavage, reduced translational efficiency in the ribosome, and destabilization of mRNA through poly(A) tail shortening. The effect that the miRNA has on the target mRNA is largely dictated by sequence complementarity, with higher sequence complementarity leading to cleavage of the mRNA and low complementarity leading to reduced translational efficiency [4, 7].

In RMS cells and supportive tissues, key regulatory miRNAs have been disrupted, perhaps partially as a consequence of excessive negative bHLH/E-protein-binding events. Some of these key regulatory miRNAs that have been disrupted include miR-26, miR-27, miR-29, miR-133, miR-181, miR-203, miR-206, miR-214, and miR-378, among others.

Throughout this article, the roles of bHLHs, E-proteins, Pax proteins, and miRNAs in the pathophysiology of RMS are reviewed. Additionally, chromosomal and histological differences between the two major variants are outlined. Finally, current and potential future therapeutic approaches to RMS are explored.

2. Rhabdomyosarcoma (RMS)

With nearly 200 new cases being diagnosed yearly in the United States and accounting for 6–8% of all pediatric tumors, RMS is the third most common form of muscle tumor. It is known as a cancer of adolescence due to the majority of new cases being diagnosed in children at or below 14 years of age. More than 50% of new cases occur in children at or below the age of 5, with another, smaller incidence peak in early adolescence [3, 8].

RMS is currently subdivided into embryonal and alveolar variants; each having its own distinct histological, molecular, and genetic markers. Embryonal RMS is the most common form of RMS, with approximately two thirds of all diagnosed RMS cases falling under this category [3]. Embryonal RMS consists of two subtypes, including botryoid RMS and leiomyosarcoma. Histologically, botryoid RMS is denoted by its namesake "grape-like" cell clusters and a dense tumor cell layer under an epithelium (cambium layer) [3]. The leiomyosarcoma form of embryonal RMS often shows up as elongated spindle cells in a storiform pattern [3]. Most embryonal tumors are characterized by their close resemblance to developing skeletal muscle. Additionally, embryonal tumors often display abnormal myoblasts, called rhabdomyoblasts, that have oblong shapes with elliptical nuclei and bland chromatin. Genetically, embryonal RMS is characterized by the loss of heterozygosity at the 11p15 locus, a region of chromosome 11 harboring the insulin-like growth factor 2 (IGF2) gene and is associated with the loss of maternal and copying of paternal chromosomal materials [3]. Alveolar RMS tissue is characterized by the appearance of small, round, densely packed cells that are arranged in such a manner that they resemble pulmonary alveoli, with an empty space in the center of the cluster. There is also a solid variant, which belongs to the alveolar variant, but does not have the characteristic empty space in the middle of the cluster [3]. The solid variant of alveolar RMS can make it difficult to tell the difference between embryonal and alveolar RMS through histology alone. However, alveolar RMS cells often tend to be larger, with centrally located nuclei and less cytoplasm than cells of the embryonal RMS variant [3]. Prognostically, embryonal RMS variants are associated with a limited stage disease and a favorable outcome. On the other hand, alveolar RMS variants are linked with a less favorable prognosis [9]. Currently, few effective, targeted treatment options exist for RMS; however, research is being done to determine potential future treatment options.

3. Chromosomal Translocations and Fusion Proteins in RMS

In terms of molecular and genetic markers of embryonal and alveolar rhabdomyosarcoma, 80–90% of alveolar RMS cases have chromosomal translocations of the DNA-binding domain of PAX3 or PAX7 at 2q35 to the transactivation domain of the FOXO1 gene at t(2;13) (q35;q14) or t(1;13) (p36;q14), respectively [3, 10–14]. This typically results in the formation of a fusion protein between PAX3 or PAX7 and FOXO1 in alveolar RMS, although PAX7-FOXO1 fusion is much less common and less potent than the PAX3-FOXO1 fusion protein form [3, 14]. Both members of the paired box type homeobox transcription factor family, Pax3 and Pax7, are involved in neurogenesis, cardiogenesis, melanoma cell pathophysiology, and myogenesis during development. Pax3 gene mutant mice have shown the essential roles of Pax3 in several developmental systems including embryonic myogenesis and muscle satellite cell differentiation by regulating gene expression of cMET. cMET is a hepatocyte growth factor/scatter factor (HGF/SF) receptor required for myogenic progenitor cell migration, with Bcl-2 and Bcl-xl serving antiapoptotic functions [13, 15, 16]. In contrast, Pax7 is required for specification of muscle satellite cells and myogenic stem cells and essential for postnatal muscle growth and regeneration [17, 18]. FOXO1 is a member of the forkhead/HNF-3 transcription factor family. The chimeric protein of PAX3-FOXO1 is a more potent transcriptional activator than wild-type Pax3. Ectopic expression of the chimeric gene converts fibroblasts to myogenic cells by the activation of multiple muscle-specific genes [19, 20]. These observations indicate that the overexpression of growth factors such as IGF2 or the activation of Pax genes may result in RMS.

4. bHLH/E-Protein Heterodimers in RMS

An increasingly relevant family of proteins to developmental biology, the bHLH family of transcription factors, has gained considerable attention, especially in myogenesis-related research. bHLH proteins including MyoD, Myf5, and musculin (MSC)/MyoR are vital for the regulation of the differentiation program that takes place in skeletal muscle cells [21]. They act through direct binding to promoters upstream of target gene sequences, as well as through heterodimer formation with E-proteins [22]. Depending on the characteristics of the bHLH protein that eventually binds the E-box through either of these mechanisms, myogenesis can either be initiated or inhibited [23]. Based on the effects of the bHLH protein, it can be classified as a negative bHLH or a positive bHLH. Positive bHLHs, such as MyoD and Myf5, upregulate target sequences, whereas negative bHLHs, such as MSC, downregulate them. Contrary to the roles of proliferation-inducing transcription factors such as PAX3 and PAX7, MyoD acts to end the proliferative phase and

Table 1: Deregulated miRNAs, their roles, targets, and expression in both alveolar and embryonic RMS.

Name	miRNA level in RMS relative to normal human myoblasts		Target genes in RMS	Function	Reference
	Alveolar	Embryonal			
miR-1	Down	Up	CCND2, cMET, PAX3	Tumor suppressor	[35, 36]
miR-24	Down	Down	—	—	[76]
miR-26a	Down	Down	Ezh2	Tumor suppressor	[36, 37, 76]
miR-27a	Down	Down	PAX3	Tumor suppressor	[36, 76]
miR-29	Down	Down	CCND2, PAX3, CCND2	Tumor suppressor	[36, 41]
miR-133a	Down	Down	TPM4	Tumor suppressor	[36, 76, 77]
miR-133b	Down	Down	—	Tumor suppressor	[36, 75]
miR-181	Down	Down	HOXA11	Tumor suppressor	[36, 42]
miR-183	Up	—	EGR1, PTEN	Oncogene	[36, 77]
miR-203	Down	Down	p63, LIF	Tumor suppressor	[43, 76]
miR-206	Down	Down	CCND2, cMET, PAX3	Tumor suppressor	[35, 75, 77]
miR-214	Down	Down	N-RAS	Tumor suppressor	[38, 44]
miR-301	Up	Up	—	Oncogene	[76]
miR-378a	Down	Down	IGF1R	Tumor suppressor	[45]
miR-450b	Down	Down	ENOX2, PAX9	Tumor suppressor	[44, 78]
miR-485	Up	—	NF-YB	Oncogene	[79]

begin the differentiation into skeletal muscle. One study of interest by Tapscott et al. found that in a genome-wide binding comparison of MyoD in normal human myogenic cells versus RMS cells, MyoD bound to the same areas in both cell types. However, MyoD exhibited poor binding at a subset of myogenic genes often underexpressed in RMS cells, including RUNX1, MEF2C, JDP2, and NFIC. Further, when these genes were re-expressed, myogenesis was rescued [24].

In normal tissue, MyoD can bind either directly to E-boxes upstream of target sequences or through dimerization with a full-length E-protein to these same sites. The ultimate binding of MyoD:E-protein heterodimers to the E-box in normal tissue is also regulated through competitive inhibition with negative bHLHs that are present in varying amounts during different stages of differentiation. In normal tissue, the level of competition between MyoD and negative bHLHs for E-proteins is relatively low compared to the RMS model [25]. Current research suggests that negative bHLHs, such as MSC, found in RMS tissue compete with MyoD and other positive bHLHs to a much greater extent for binding with E-proteins. Subsequently, affected cells remain in a stage between muscle precursors and terminally differentiated skeletal muscle. Proteins that are competing for binding with full-length E-proteins include negative bHLHs, such as MSC, and the splice form of the full-length E-protein, E2A-2/5. These two influences act synergistically through different mechanisms to ultimately decrease the transcription of genes that are key to the process of myogenic differentiation. Negative bHLH transcription factors, such as the myogenic inhibitory factor MSC, compete with MyoD for dimerization with full-length E2A proteins. When MSC:E2A heterodimers form, they bind to the E-box upstream of target sequences and downregulate downstream regions including MyoD gene. This maintains a tissue form intermediate between proliferating muscle precursors and fully differentiated skeletal muscle. Additionally, MSC likely plays an opposing role to MyoD, as it shows substantial overlap in binding when analyzed through genome-wide studies [26].

The splice form of the E2A protein, the E2A-2/5 splice variant, also competes with positive and negative bHLHs alike for binding with the full-length E2A protein. In recent in vitro studies, gel shift assays were used to determine the binding potential of the E2A-2/5 splice form and the full-length E2A protein. Based on the results of the study, E2A-2/5 splice forms have the potential to bind full-length E2A proteins in in vitro gel shift assays [25]. In vivo, it is thought that the E2A-2/5 splice variant competes with positive and negative bHLHs for binding with the full-length E2A protein. The resulting E2A:E2A-2/5 heterodimers likely do not bind the e-box; instead, the E2A-2/5 protein acts to sequester full-length E2A proteins that are present in the cell so that the upregulation of target regions is unable to occur because of the diminished amounts of E2A:MyoD heterodimers. Current research has uncovered that MyoD:E2A heterodimer levels are lower and are antagonized by negative bHLHs and E2A-2/5 splice forms to a greater extent than in normal tissues [25]. Taken together with Pax fusion proteins and microRNA dysregulation, this molecular mechanism likely contributes to the pathophysiology of RMS.

5. Posttranscriptional Control in RMS through Muscle-Specific miRNAs (myomiRs) (Table 1)

Beginning with the discovery of the first canonical miRNA in *C. elegans*, lin-4, miRNA function in eukaryotes has become an increasingly important and relevant topic for researchers [27–29]. Within the RMS disease field, miRNAs have gained

new attention not only as important contributors to the disease but also as potential therapeutic targets. miRNAs have no protein product and are encoded by specific sequences downstream of promoters. When activated, the miRNA sequence is transcribed then processed initially in the nucleus by the RNase III enzyme Drosha, which removes the 5′ cap and poly(A) tail [30, 31]. Afterwards, the pre-miRNA is passed out of the nucleus into the cytoplasm, where further processing by dicer enzymes converts the pre-miRNA into the final miRNA molecule [32, 33]. This molecule then incorporates into an RNA-induced silencing complex (RISC) with another protein which aids in binding to target mRNAs [34]. Depending on sequence consensus between the miRNA and the target region of the mRNA, the mRNA will either be degraded (high consensus) or translationally inhibited due to the RISC present on the mRNA (low consensus) [7]. This mechanism is especially important because it provides ways in which the cell can control protein production posttranscriptionally, which allows multilayered regulation of gene expression. Depending on tissue type, various miRNAs are more abundant than others. In the case of skeletal muscle-specific miRNAs (myomiRs), miR-1, miR-206, and miR-133a are common, with each playing regulatory roles integral to myogenesis. Myogenic dysfunction in RMS tissues is exacerbated by deregulated miRNA levels, which have in many cases been found to be lower than in adjacent skeletal muscle tissue. At low levels, miRNAs have less of a repressive effect on their target genes, opening tissue up to potential problems including cancer.

Perhaps the most studied myomiR is miR-206. MiR-206 is currently known to target cMet, which is a proto-oncogene receptor overexpressed in a variety of cancers, including RMS. cMet levels in RMS tissue have been found to be inversely related to miR-1/206 levels, and various studies utilizing this knowledge have shown that MET is a key target for the anticancer effects of miR-1/miR-206 [35]. This leads to the possibility that restoration of miR-1/miR-206 to normal physiological levels may provide therapeutic potential for RMS. Indeed, this potential has been tested in mice with xenografted, lentivirus-infected RD cells, an RMS cell line, expressing either miR-1, miR-206, or the negative control. Transient transfection of miR-1/206 into cultured RD cells led to a significant decrease in cell growth and migration. Additional findings from this study revealed that the differences in tumor volume were apparent between miR-1/206-expressing tumor cells and the negative control, with miR-1/206-expressing tumor cells displaying growth delay in comparison with the negative control [35].

miRNAs that are predominantly expressed in other tissue types also play a role in RMS. Among these, miR-26, miR-27, miR-29, and miR-181 play roles in myogenesis and have all been shown to be deregulated in RMS [36]. miR-26a has been shown to have a positive effect on myogenesis by targeting the histone methyltransferase enhancer of zeste homolog 2 (Ezh2) [37, 38]. Ezh2 is an enzyme in humans that aids in maintaining closed chromatin structures that prevent the transcription of key developmental genes. It performs this role through the trimethylation of lysine 27 of histone 3, resulting in chromatin condensation and thus transcriptional repression of target genes. Acting through this mechanism, Ezh2 inhibits myogenesis by repressing late-stage muscle-specific genes such as muscle creatine kinase (MCK) and myosin heavy chain (MHC) [39, 40].

Another crucial myomiR that is currently undergoing scientific studies is miR-29, which is regulated by NF-κB acting through YY1 and the polycomb group. In many muscle tumors, including RMS, miR-29 has been shown to be downregulated in part due to an elevation in NF-κB and YY1, leading to a decrease in likelihood that the cell will undergo differentiation [36]. Wang et al. also showed that in immunocompromised mice with RH30 tumors, injection of miR-29b-expressing virus intratumorally resulted in tumors that displayed slower growth. Between eight days postinjection and the experimental end point, the average size of the control tumor was 1.9 times larger than the miR-29b tumor [41].

Another important group of miRNAs in RMS pathology is the miR-181a/miR-181b gene cluster. During normal myogenesis, the homeobox gene HoxA11 initially inhibits myogenesis. In order for myogenesis to occur, this gene must be downregulated. The miR-181a/miR-181b gene cluster is able to do just that by inhibiting the expression of HoxA11, which allows for terminal differentiation to occur. In most cases of RMS, miR-181 is downregulated and is unable to exert a repressive role on HoxA11, which effectively prevents RMS cells from differentiating [42].

As more is learned about the various miRNAs that contribute to the RMS phenotype, epigenetic miRNA control mechanisms are being examined. One such miRNA in which epigenetic controls are at work is miR-203. miR-203 directly targets p63 and leukemia inhibitory factor (LIF) in RMS cells. Targeting of these factors then promotes myogenic differentiation via the inhibition of the Notch and JAK/STAT pathway, respectively. In both RMS biopsies and various RMS cell lines, miR-203 was found to be downregulated due to promoter hypermethylation. Interestingly, miR-203 function was found to be restored after exposure to DNA-demethylation agents. Further, this led to a reduction in migration and proliferation as well as the promotion of terminal myogenic differentiation [43].

miR-214 has also been shown to be downregulated in human RMS cell lines. miR-214 exerts its suppressive role in mouse embryonic fibroblasts (MEFs) by suppressing their proliferation. After the introduction to RD cells, it was shown to have a repressive effect on tumor cell growth and culture colony formation and a stimulatory effect on myogenic differentiation, apoptosis, and xenograft tumorigenesis. miR-214 was shown to exert its inhibitory effects on the proto-oncogene N-ras. In MEF *miR-214*$^{-/-}$ cells, N-ras was found to be elevated. Additionally, in control cells, forced expression of N-ras from cDNA lacking a 3′-untranslated region neutralized the antiproliferative and promyogenic activities of miR-214 [44].

One final miRNA of interest is miR-378. Like many of the miRNAs described thus far, it has been found to be downregulated in RMS cells. In one study by Megiorni et al., the expression level of 685 miRNAs was investigated via a deep-sequencing approach, where miRNA expression across

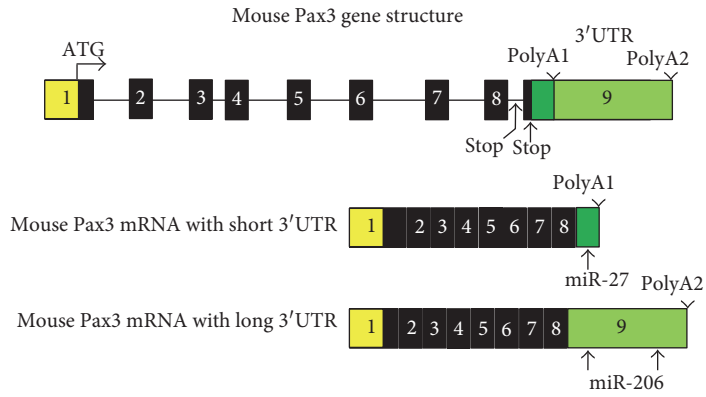

Figure 1: Pax3 3′UTR contains microRNA-binding sites. Mouse Pax3 gene and mRNA structures. Numbered boxes denote each exon. White boxes denote the 5′UTR and the shorter 3′UTR. Black boxes denote coding regions. There are 2 stop codons and 2 polyA signal sequences (polyA1 and polyA2) in mouse Pax3 gene, leading alternative polyadenylation. The right side white box denotes the shorter 3′UTR containing miR-27-binding site. The gray box denotes the longer 3′UTR containing two miR-1-/miR-206-binding sites.

various RMS cell lines was investigated. In their study, they found that miR-387 was, on average, downregulated and that it may function as a tumor suppressor in RMS. Further, they posited that restoration of miR-387 expression could provide therapeutic benefits [45].

6. miRNA-Mediated Pax3 Regulation in RMS and Muscle Stem Cell Maintenance

Pax3 expression is subject to posttranscriptional regulation, and timely downregulation of Pax3 expression is crucial for myogenic differentiation. Recent work demonstrates that Pax3 expression is regulated by multiple stages, including ubiquitination-mediated protein degradation, Staufen 1-mediated mRNA decay, and miR-27b-mediated translational inhibition [46–48]. During embryonic myogenesis, both types of miR-27 (miR-27a and miR-27b) target the 3′UTR of PAX3, an important transcription factor for myoblast proliferation, in order to downregulate PAX3 expression. This leads to a shift from PAX3-positive cells to myogenin-positive cells, indicating a transition from a predominantly proliferative state to differentiation. We have recently demonstrated that MyoD negatively regulates Pax3 gene expression through the action of miRNAs. Because Pax3 functions as a cell fate determination factor and for maintenance of the undifferentiated state in muscle and melanocyte stem cells, downregulation of Pax3 is essential for terminal differentiation, which is also accompanied by apoptosis. We also noticed that Pax3 is a survival factor that transcriptionally activates the antiapoptotic genes Bcl-2 and Bcl-xL [16]. Therefore, negative regulation of Pax3 expression by MyoD-regulated miRNAs is a critical point for MyoD-dependent apoptosis in myoblasts. Experiments from gene knockout mice demonstrate that Pax3 functions as a survival factor during embryogenesis [49–51]. It has been reported that Pax3 positively regulates Bcl-xL gene expression by binding to the 5′-flanking region of the Bcl-xL gene [52]. Previously, screening of binding proteins for the 1 kb Bcl-2 promoter identified 43 different transcription factors including Pax3 [53]. We demonstrate that Pax3 positively regulates Bcl-2 gene expression via the 5′-flanking region of this gene, strongly indicating that Pax3 functions as an antiapoptotic factor by transcriptionally upregulating Bcl-2 and Bcl-xL gene expression. Pax3 also facilitates the malignant progression of RMS and melanomas [54–56]. Overexpression of MyoD or inhibition of Pax3 by miRNAs may induce apoptosis in RMS and neuroblastoma cells, which may provide a novel anticancer therapy for associated tumors [2, 5, 57, 58].

Adult skeletal muscle possesses extraordinary regeneration capabilities. After exercise or muscle injury, large numbers of new muscle fibers are normally formed within a week because of expansion and differentiation of muscle satellite cells [59]. Satellite cells are a small population of myogenic stem cells for muscle regeneration which are normally mitotically quiescent. Following injury, satellite cells initiate proliferation to produce myogenic precursor cells, or myoblasts, to mediate the regeneration of muscle [60–62]. The myoblasts undergo multiple rounds of cell division prior to terminal differentiation and formation of multinucleated myotubes by cell fusion. Pax3 together with expression of Pax7 and downregulation of MyoD is detected in a subset of satellite cells and potentially important for muscle stem cell maintenance and self-renewal [46, 63–66]. For mouse Pax3, there are two putative polyA signal sequences in the 3′UTR. Both proximal (polyA1) and distal (polyA2) polyA signal sequences were indeed used for transcription of Pax3 mRNAs with the shorter and longer 3′UTRs, respectively (Figure 1). The shorter 3′ UTR contains a miR-27-binding site, and the longer 3′ UTR contains both putative miR-1- and miR-206-binding sites [16, 48, 67]. In contrast, the human Pax3 gene only contains the polyA2 sequence, and thus, the human Pax3 mRNA contains the longer 3′UTR with the two putative miR-1-/miR-206-binding sites [68, 69]. Recent work showed that quiescent satellite cells (QSCs) express high levels of Pax3 and miR-206 [67]. In these QSCs, Pax3 transcripts possess shorter 3′UTRs that render them resistant to suppression by miR-206, which is important in maintaining muscle stem cell status in skeletal muscle. These results suggest alternative polyA signals in circumventing miRNA-mediated regulation

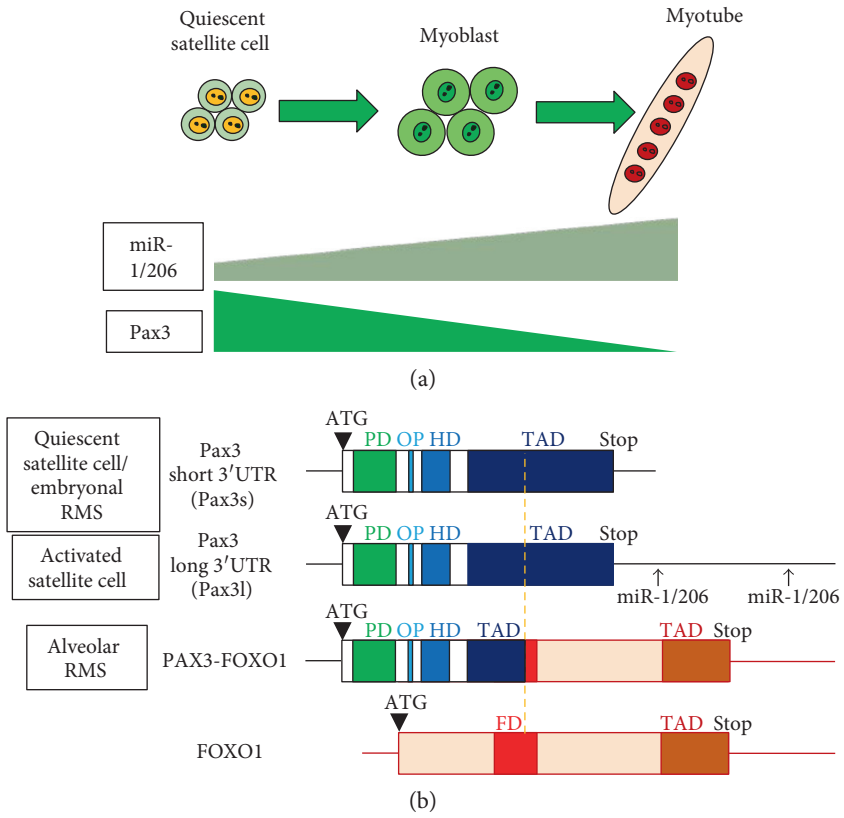

Figure 2: Truncation and loss of Pax3 3′UTR during muscle stem cell self-renewal and RMS progression. (a) Schematic model of Pax3 and miR-1/206 expression during muscle stem cell self-renewal and activation. (b) Mouse Pax3 mRNA structures with short and long 3′UTRs and human RMS-derived PAX3-FOXO1 fusion gene.

of muscle stem cell function including stem cell self-renewal and maintenance.

Both miR-1 and miR-206 expressions are downregulated in RMS compared to normal skeletal muscle but still much higher than nonmuscle tissues, supporting the myogenic origin of RMS. In alveolar RMS, the chromosomal translocation-generated PAX3-FOXO1 fusion protein is a superactive transcription factor due to the activation domain of FOXO1 and thus promotes RMS proliferation and progression. In addition, PAX3-FOXO1 fusion gene lost Pax3-3′UTR due to the translocation as shown in Figure 2. Therefore, PAX3-FOXO1 fusion gene is no longer the target of miR-1/206, which may lead to an increased expression level of this fusion gene. In embryonal RMS, Pax3 is not associated with chromosomal translocation, but there are Pax3 3′UTR abnormalities including shorter transcript variants lacking miR-1/206-binding sites [70], escaping the miR-1/206-mediated Pax3 gene suppression as seen in the QSCs (Figure 2). Therefore, there are common molecular mechanisms in Pax3 gene regulation in both muscle stem cell self-renewal and RMS progression.

7. Therapies and Approaches

Like many cancers, RMS can carry a dismal prognosis, especially in cases where the alveolar variant is displayed. Treatment options that currently exist include surgical removal of affected tissues, chemotherapy, radiation, or these treatments in combination [71, 72]. In some cases of RMS, surgical excision may be recommended. This is an effective treatment option in cases where the cancer has not metastasized to other tissues. Often, large portions of affected tissue can be resected; however, microscopic margins may remain. Tumor resection, followed by a combination of intensive chemotherapy and radiation, can help to suppress and kill unresected portions [73, 74]. Although current treatment options are effective in some cases, they continue to be a nonideal treatment option for patients with RMS. With ongoing research into the molecular mechanisms at place in RMS, more advanced and effective treatment options for RMS may begin to emerge.

By researching the roles of bHLH transcription factors in myogenesis along with the regulatory roles of miRNAs, more effective treatment methods for RMS can be elucidated. Common to all forms of RMS is that the tissue is in an intermediate state between muscle precursor cells and terminally differentiated muscle. This leaves determining a potential treatment option square in the lap of developmental biologists and stem cell researchers, specifically those studying diseases of skeletal muscle. One common idea among many stem cell researchers is that it may be possible to coax the RMS tissues to differentiate into muscle fibers, thus losing their tumorigenic and metastatic potential [73, 74].

One promising method for coaxing differentiation of these muscle precursor-like cells is to use RNA interference methods, such as miRNAs and siRNAs, to force differentiation to occur. This could be put into practice by introducing a miRNA or siRNA that posttranscriptionally modulates MSC mRNA so that it does not have the chance to compete with MyoD and Myf5 for E-protein dimerization, which might ultimately lead to increased transcription of MyoD target genes, thus inducing myogenic differentiation with subsequent loss of proliferative capacity. An important area that needs further research before RNA interference methods could be used on human patients would to be to determine what genes a certain miRNA represses in addition to the target gene, as most miRNAs lack the specificity of siRNAs. Another option for inducing terminal differentiation would be to use gene therapy to insert another gene for the MyoD protein into RMS patients. This would theoretically cause a twofold increase in the amount of MyoD that is present in the cell, leading to increased competition with negative bHLHs such as MSC. This would also lead to increased competition with inhibitory E2A splice forms such as E2A-2/5. Yet another treatment option might involve using protein therapies to induce differentiation. Proteins could be used for treatment of RMS in multiple ways, either as negative bHLH-binding proteins or as supplements to the existing positive bHLHs that are present in the cell. One example of how this therapy could be used would be to introduce a protein into the RMS patient that binds to MSC and/or other negative bHLHs in RMS tissues and renders them inactive and unable to bind to full-length E2A proteins, allowing for MyoD to have a more profound effect in these tissues.

miR-206, as described earlier, has been shown to inhibit human rhabdomyosarcoma growth in xenotransplanted mice by promoting tumor differentiation [75]. Similarly, miR-29b, also described earlier in this article, was shown to slow tumor growth in immunocompromised mice with RH30 tumors. Between an eight-day postinjection and the experimental end point, the average size of the control tumor was 1.9 times larger than the miR-29b tumor [41]. Based on results of these studies and others in this article, translation of these therapies into clinical trials may have some merit after safety evaluation and delivery verification.

As seen throughout this review, experiments in xenotransplanted mice with microRNAs have shown slowed tumor growth and increased differentiation of cells from an arrested myoblast phase state. The combination of current and past research in this field has led to a climate in which discovering new treatments may be just around the corner. However, even as the scientific community continues to discover new molecular targets, it is important to keep in mind that further challenges still exist in finding therapeutic options, including identifying reliable and reproducible delivery methods and evaluating safety and efficacy in human patients.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by the NIH R01 (1R01AR062142) and NIH R21 (1R21AR070319).

Abbreviations

bHLH: Basic helix-loop-helix
E-protein: E-box-binding protein
EZH2: Enhancer of zeste homolog 2
FOXO1: Forkhead box protein O1
HoxA11: Homeobox A11
miR: MicroRNA
miRNA: MicroRNA
MSC: Musculin
myomiR: Myofiber microRNA
PAX: Paired box
RMS: Rhabdomyosarcoma
siRNA: Short interfering RNA.

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Hindawi
Stem Cells International
Volume 2017, Article ID 5619472, 17 pages
https://doi.org/10.1155/2017/5619472

Review Article

Stemness in Cancer: Stem Cells, Cancer Stem Cells, and Their Microenvironment

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Received 9 December 2016; Revised 31 January 2017; Accepted 19 February 2017; Published 4 April 2017

Academic Editor: Xiaojiang Cui

Stemness combines the ability of a cell to perpetuate its lineage, to give rise to differentiated cells, and to interact with its environment to maintain a balance between quiescence, proliferation, and regeneration. While adult Stem Cells display these properties when participating in tissue homeostasis, Cancer Stem Cells (CSCs) behave as their malignant equivalents. CSCs display stemness in various circumstances, including the sustaining of cancer progression, and the interaction with their environment in search for key survival factors. As a result, CSCs can recurrently persist after therapy. In order to understand how the concept of stemness applies to cancer, this review will explore properties shared between normal and malignant Stem Cells. First, we provide an overview of properties of normal adult Stem Cells. We thereafter elaborate on how these features operate in CSCs. We then review the organization of microenvironment components, which enables CSCs hosting. We subsequently discuss Mesenchymal Stem/Stromal Cells (MSCs), which, although their stemness properties are limited, represent essential components of the Stem Cell niche and tumor microenvironment. We next provide insights of the therapeutic strategies targeting Stem Cell properties in tumors and the use of state-of-the-art techniques in future research. Increasing our knowledge of the CSCs microenvironment is key to identifying new therapeutic solutions.

1. Introduction

Cancer is a major cause of death worldwide [1, 2]. While the incidence of infectious diseases has significantly declined over the last several decades, overall incidence of solid tumors and leukemia has shown to be increasing [3]. Longer average life span, accumulation of genetic mutations, and permissive microenvironment are key factors promoting cancer progression [4, 5]. Most therapies include the use of strong cytotoxic molecules to target specific unregulated factors to eventually affect cell proliferation and survival of the tumor [6]. Due to its fast replication capacity and constant mutations, cancer adapts to aggressive environments and can persist after therapeutic management. Stemness of cancer cells is a key feature for cancer progression and in many cases the source of its survival [7–12]. Understanding the development and acquisition of resistance in cancer cells may therefore provide opportunities for more effective therapies.

Stem Cells (SCs) have the capacity to self-renew and give rise to progeny capable of differentiating into diverse cell types [13]. SCs cannot survive either outside their environment or in the absence of specific factors and cytokines [14, 15]. Interestingly, the environment and/or specific stimuli can promote the emergence of new SCs, as cells in general maintain the ability to dedifferentiate and return to a primitive state of development [16–18]. Such capacities are comprised in the term stemness and correspond to cells devoid of differentiation marks [19, 20].

Malignant cells develop all aspects of stemness, fail to sustain tissue homeostasis, and, contrary to the physiological role of adult SCs, sustain the progression of cancer disease [8]. Stemness features common of SCs and cancer cells provide the building blocks for cancer maintenance and survival, from self-renewal and differentiation potential to the organization of stemness supporting microenvironments [5, 9, 21]. Thus, Cancer Stem Cells (CSCs) are a small population of cells within tumors holding stemness properties that sustain cancer progression, such as enhanced capacities for self-renewal cloning, growing, metastasizing, homing, and reproliferating. CSCs show remarkable organizing capacities as they can educate neighboring cells to provide nutrients and collaborate in the elusion from the immune system, creating an environment favorable for tumor growth. CSCs give rise to heterogeneous cell populations, often with a high plasticity potential [10, 22], high resistance to stressful factors within the tumor microenvironment (such as low oxygen or nutrient levels) or to the induction of cell death by chemotherapeutic agents [11, 23], and quiescence as a common response [12, 24].

In order to understand how we can take advantage of stemness to develop applications in the field of oncology, this review will discuss the most relevant known stemness features shared by adult SCs and CSCs in normal tissues and tumors, from the origin and progression to the outcome. As stemness involves the organization of a microenvironment that protects normal SCs (Stem Cells) niche or CSCs (the Tumor Microenvironment, TME) we will present the most common companions of cancer cells and their interactions within the TME. Among such neighbors of SCs and CSCs, Mesenchymal Stem/Stromal Cells (MSCs) are the main contributors to the maintenance of stemness, as they provide support to the niche and the TME during stress and generate an immune-privileged regulatory microenvironment [25, 26]. Therefore, we will provide insights into the particular contribution of MSCs to cancer. As cancer cells are continually readapting to conventional therapies, current research is constantly evolving to generate new approaches to effectively target their progression. Many of these therapeutic procedures show an increasing trend towards personalization. They aim to affect the hallmarks of cancer development and, in particular, the stemness elements affecting specific patients. Therefore, the current understanding of the mechanisms underlying stemness in tumors will be covered in this review, in the context of new therapies potentially targeting the organized TME.

2. Adult Stem Cell Characteristics

All tissues in the body organize their functions around cellular communities essentially conforming microenvironments, where SCs play a key role in the general homeostasis. Through well-regulated asymmetric cell divisions, SCs provide the progenitors that will in turn generate specialized daughter cells responsible for maintaining organ functions and replacing wear-and-tear cell losses [27]. At the same time, SCs are able to self-renew with the purpose of regulating their numbers under both physiological and abnormal conditions [28]. Adult (nonembryonic) SCs all have by definition some degree of differentiation potential. Therefore, adult SCs have the capacity to produce several differentiated lineages, a differentiation potential restricted to multipotency. Adult SCs are located in specialized microenvironments that provide support and cues that instruct them to maintain themselves and self-renew as required by local cell dynamics in specific tissues [29–32]. Although located in multiple different niches in several tissues of the body, SCs share common features such as self-renewal capacity, undifferentiated state combined with differentiating potential, long cell cycling, genome repair abilities, and microenvironmental protection by the niche itself when under attack from a wide range of insults [27]. The term stemness condenses all key properties of SCs, defined by specific patterns of gene expression or epigenetic status within the context of the tissue where they reside. In the skin, a well-characterized tissue in terms of SC activity, epidermal SCs (interfollicular keratinocyte progenitor cells) express β1-integrin while their progenitors do not [33]. In the small intestine SCs express specific sets of stemness determining genes [34]. The characterization of SC's specific gene product profiles can be conveniently used to track them along their cellular dynamics in specific body systems [35]. In most tissues, SCs are located at the top of hierarchical organizations collectively called Stem Cell Systems (SCSs). Thus, almost every major organ in the body has at least one SCSs. Even organs long-held as not prone to regeneration are now appearing to show SCs activity under certain conditions. For instance, the long-held dogma of global terminal differentiation in adult neurons has been strongly challenged [36].

SCSs consist of (1) basal, (2) transit-amplifying, and (3) differentiation compartments [27]. Figure 1 summarizes the structure of SCSs and some possible ways of transformation into a Tumor Stem Cell System. The basal compartment is where SCs reside. That compartment, including its immediate surroundings, corresponds to the SCs niche where other cells, Extracellular Matrix (ECM), and factors like oxygen levels and physical forces contribute to the maintenance and survival of SCs [29]. Cellular components of this niche include local elements (very often, cells of mesenchymal origin) or immune cells recruited to the site [25, 37, 38].

The direct SCs progeny or transit-amplifying cells occupy the transit-amplifying compartment. These transient-in-nature cells have a shorter cell cycle than their mother SCs and they therefore rapidly divide to produce daughter cells that "amplify" the next compartment (differentiation compartment), where terminally differentiated cells that perform normal tissue/organ functions dwell [27]. Transit-amplifying cells, also called progenitor cells (or progenitors), are morphologically similar to their SCs ancestors but show different sets of markers that define their differentiation commitment. However, under certain circumstances they dedifferentiate and contribute to the SCs pool. An example of such a dedifferentiation process occurs in the seminiferous epithelium in the testis, where spermatogenesis, the Spermatogonial Stem Cells- (SSCs-) dependent system, generates sperm [39]. In this well-characterized SCs system, SSCs divide asymmetrically to produce differentiated daughter cells that, through mitosis (transit-amplifying activity), generate clones that remain connected through intercellular bridges [40].

Table 1: Comparison of traits of normal Stem Cells and Cancer Stem Cell biology.

Trait	Normal Stem Cells	Cancer Stem Cells
Self-renewal	High capacity [27, 28]	High capacity [10, 64, 70, 71]
Cell cycle duration	Long. Tissue-regulated generation of transit amplifying progenitors [28]	Redundant self-renewal pathways become activated. Pathological self-renewal balance over differentiation [72]
Genome repair abilities	Yes [73, 74]	Altered (constant generation of new mutations and epigenetic profiles to generate clones with strong adaption capacity to aggressive environments) [46, 73]. Hypoxia mediated cell cycle lengthening and DNA repair [75, 76]. Shorter cell cycle contribution [12]
Microenvironmental protection by niche from noxious agents	Yes [77]	Yes [11, 23]
Location at hierarchy	Basal compartment [27]	Basal compartment [72]
Transit amplifying compartment	Progenitor cells have short cycles to generate enough numbers of normal differentiated cells [47, 78]	It seems to be present as the basis for rapid growth of tumors. Progenitor cells have short cycles [10]
Plasticity	Can go back and forth between differentiation and dedifferentiation states [79]	Epithelial mesenchymal transition and self-renewal acquisition [10, 80, 81] Dedifferentiation and mutation accumulation in committed cells [22]

Any exogenous process interfering with intercellular bridge integrity (i.e., irradiation) will produce individual single undifferentiated SCs, reversing the differentiation process back to the SCs level [41, 42]. These initial observations were more recently corroborated through in vivo experiments and functional tests for SCs capacity [43]. SCSs are thus tightly regulated cellular hierarchies where SCs activity, modulated by the niche, follows a proper balance between self-renewal and differentiation in order to maintain normal organ activity.

3. Analogous Features of CSCs and Adult SCs

There is growing evidence that cancer disease follows SCSs organization where cancer cells or CSCs generate a comparable hierarchical structure within tumors (Table 1). The Theory of CSCs is a modern derivation of the Embryonic Rest Theory of cancer. This Theory states that vestiges of embryonic tissue would remain in adult postnatal organs while holding the capacity to pathologically unbalance the surrounding tissues (Field Theory), therefore leading to a situation in which the remnant embryonic tissues start proliferating into a tumor mass whose cells are similar to the embryonic cells of origin [44]. The existence of teratoma tumors supports the Embryonic Rest Theory, since embryonic Primordial Germ Cells (PGCs) give rise to this kind of tumor in adult-age locations which are spatially associated with their prenatal migration path into the genital ridge, where either testes or ovaries eventually develop [45].

Several other models proposed to explain the origin of cancer cells (chemical carcinogenesis, infections, mutations, and epigenetic changes) are likely to involve dedifferentiated cells with SCs properties. Consequently, many cancers could arise from the maturation arrest of adult SCs in different tissues [44, 46]. The origins of CSCs are traceable with techniques previously used to uncover unipotent or multipotent SCSs under normal physiological conditions [47]. Blanpain (2013) [33] traced tumor initiation back to SCs in several known SCSs through the use of recombinant Cre-Loxp technologies. It is now possible to conditionally express oncogenes or delete tumor suppressor genes through the targeted activation of Cre recombinase expression in solid tumors in order to trace their cellular origin to one precise cell [33]. In some tumors in which progenitors appeared to be the initiating tumor cells, a dedifferentiation process generating primitive CSC that feed the cellular hierarchy has been found [22, 46].

Currently, a fundamental question in cancer biology is whether there is order within the chaos inside cancer masses. Tumors in and of themselves are very complex biological entities; they are heterogeneous aggregations of cells disorganized to the point of chaos. Within a disorder that seems to prevail, remnants of an orderly arrangement of normal tissues become apparent after careful analysis. Thus, knowledge about the origin of cancer cells becomes crucial to understand cell heterogeneity in cancer. In a Stochastic Model of Cancer Cell Dynamics, mutations giving rise to cells with unrestricted division capacities occur at random. Transformed clones suffer successive mutations along their descendant lines in branching patterns [10]. The high mutation rates found in tumors increase the likelihood of developing clones adapted to the tremendous selection pressures present at the tumor site (i.e., local chemotherapeutic agents, radiation, ROS, and immune attack).

A more recent model of cancer, the CSCs Model, covers issues not completely explained by the Stochastic Theory, such as tumor recurrence after treatment. The CSCs Model states that surviving, transformed subclones that form part of tumors have SCs properties that allow them to drive tumor

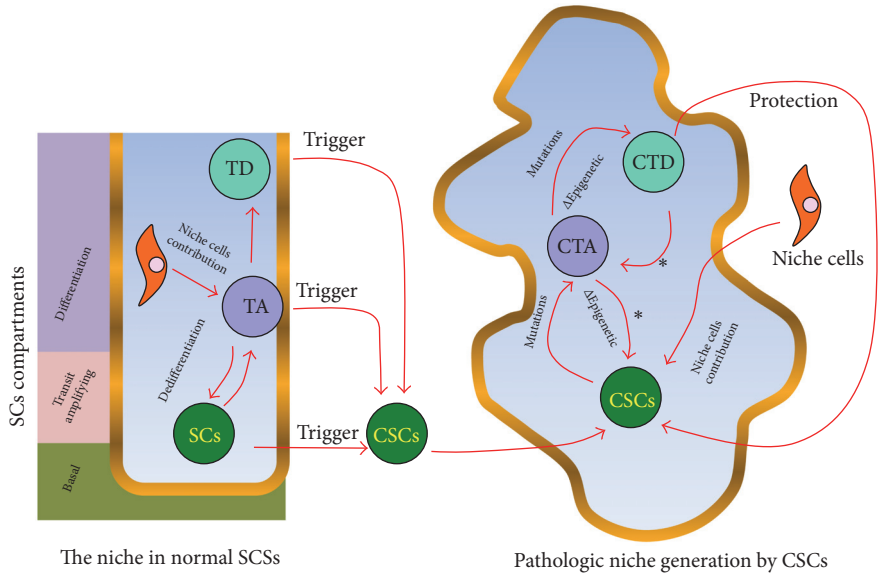

Figure 1: The origin of Cancer Stem Cells (CSCs) and Stem Cells (SCs) involvement in the generation of pathological cell hierarchies in tumors. In normal Stem Cell Systems, SCs located at the basal compartment generate committed progenitors (through asymmetrical divisions) which become spatially relocated to the transit-amplifying (TA) compartment. There, progenitors actively divide to produce differentiated daughter cells that carry on the normal physiology of the organ. Under physiological emergencies associated with SC loss, TA cells can dedifferentiate to reload the SC pool. Certain stressful triggers (i.e., chronic inflammation, ROS accumulation, and aging) can promote the transformation of cells in the system and generate CSCs or cancer initiating cells. CSCs remodel the niche and produce a pathological cancer microenvironment and associated hierarchy (pathological Stem Cell System) that resembles the original normal Stem Cell Systems (SCSs). The tumor is a very heterogeneous entity with cells that have accumulated mutations and epigenetic profile changes to secure CSCs survival and thriving. Features typical of SCSs such as niche support, SCs stemness, and dedifferentiation paths (∗) remain in the tumor environment. SCs = Stem Cell; TA = transit-amplifying progenitor; TD = terminally differentiated cell; CSC = Cancer Stem Cell; CTA = cancer transit-amplifying progenitor; CTD = cancer terminally differentiated cell.

progression. The CSCs Model is unidirectional in that SCs-like cells may generate progenitor daughter cells (transit-amplifying cells) that in turn divide to produce differentiated (nontumorigenic) cells. Cellular heterogeneity within tumors depends on factors including the already mentioned branching mutation patterns and on cues from the TME. Thus, the TME can contribute to cell transformation (Figure 1).

Similarly, as in normal SCSs, there is growing evidence that indicates progenitor cell pools within tumors revert back to CSCs by several means such as Epithelial Mesenchymal Transition (EMT) [70, 94]. EMT and plasticity are related processes that are associated with cancer progression. Multiple potential cell fate paths among the pool of progenitors and CSCs add a high degree of complexity to the cell dynamics of the cancer model. Plasticity has been termed "dynamic stemness" in this context [10]. Thus, plasticity, usually mediated by microenvironmental signals, is another very important mean for gaining excessive SCs self-renewal properties in tumor environments. Many of the mutations found in tumors are involved in the activation of self-renewal pathways in one way or another [70, 94]. In cancer, cells' multiple self-renewal pathways can not only be enhanced but also become continuously activated in ways that are only subtly different from the self-renewal pathways of normal tissues [72]. This self-renewal program activation forms an integral part of CSCs stemness, actively promoting tumor progression and metastasis by generating a high cell turnover and production of progenitors. Thus, a pathogenic self-renewal over differentiation balance in tumors further aggravates the process of mutation accumulation (Figure 2).

Another cause of cellular heterogeneity in tumors studied in recent times is epigenetics. Tumor complexity and plasticity can hide a hierarchical organization within the TME in part because of altered epigenetic profiles that may adopt mutation phenotypes. Deoxyribonucleic Acid (DNA) methylation changes and chromatin remodeling have been detected in many types of cancer [46, 95]. Overall, DNA methylation is enhanced, causing many differentiation genes to shut down [96]. The Polycomb group of proteins is one of the epigenetic regulators in cancer and SCs. The Polycomb Repressive Complexes (PRCs), active in binding to the CpG-rich promoters of genes controlling development and differentiation in embryonic SCs, are involved in the transcriptional repression [96]. The inhibition of one of such complex, PRC2, is being explored as a new cancer-treating therapy because its deletion has been shown to inhibit tumor progression [97]. However, since different types of cancer have different genetic and epigenetics profiles, the ablation of PRC2 could also cause cancer cells to become more aggressive by reinforcing their phenotype [98]. When tumor suppressor or differentiation genes are altered through the abnormal activation of epigenetic mechanisms, more resources are

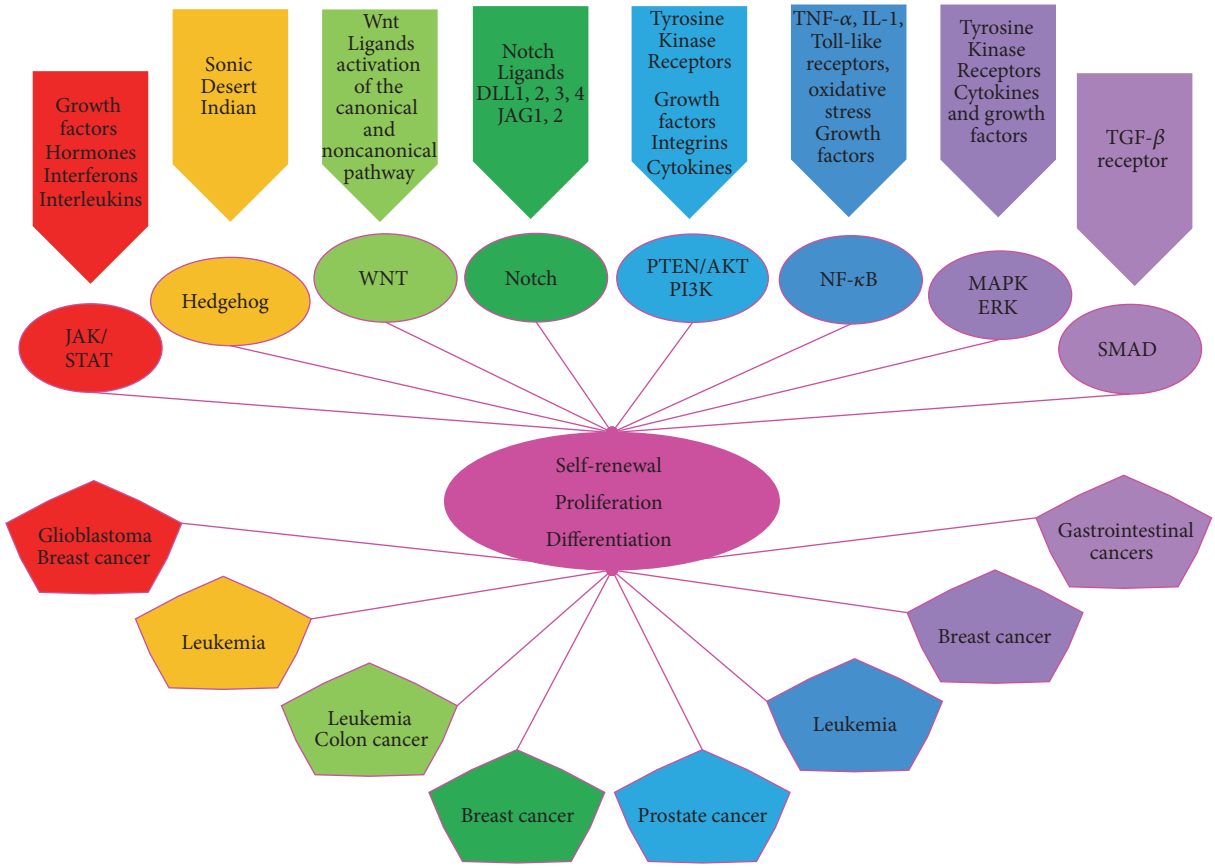

Figure 2: Common signaling pathways between Stem Cells (SCs) and Cancer Stem Cells (CSCs) [48]. CSCs share common signaling pathways, like the JAK/STAT, Hedgehog, Wnt, Notch, PTEN/AKT/PI3K, NF-κB, MAPK/ERK, and SMAD. These SCs mechanisms are altered in CSCs and are characteristic of the cancer types mentioned. The JAK/STAT pathway (Janus kinase/signal transducer and activator of transcription) is mainly involved in glioblastoma development and breast CSCs [49–52]. The Hedgehog pathways have effects on the patterning of the embryo but play a crucial role in the induction of myelogenous leukemia. Blocking of the Hedgehog pathway decreases the quantity of CSCs in leukemia, then representing an important target for cancer therapy [53]. The Wnt pathway is an important regulator of SCs and CSCs regarding self-renewal, being perturbed in colon cancer and leukemia [54–56]. The Notch pathway is involved in the development of breast tissue as a regulator of cell fate and differentiation. An excess in the activation of Notch could determine the aggressiveness of breast cancer [55, 57–59]. The phosphatase and tensin homolog (PTEN)/protein kinase B (PKB or AKT)/phosphatidylinositide 3-kinase (P13K) signaling is a key regulator of self-renewal and maintenance of SCs and CSCs with an important role in the emergence of CSCs in prostate cancer [51, 60]. The NF-κB pathway is crucial for leukemic cells survival and its inhibition affects CSCs development in breast cancer [61]. It has been seen that the increase of neural stem cell (NSC) proliferation is caused by the activation of NF-κB, through the TNF-α signal transduction pathway, but its aberrant regulation could lead to CSCs development in glioblastomas [62, 63]. Blocking the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) results in the growth inhibition of breast cancer and the emergence of CSCs, sensitizing cancer cells to chemotherapy [64–66]. Gastrointestinal SCs can be perturbed, changing their plasticity and differentiation potential by generating an aberrant response to TGF-β affecting the SMAD pathway and generating CSCs [67]. The hepatocellular carcinoma is an aggressive form of cancer in which the TGF-β, Notch, and Wnt are deregulated, also having consequences in the SMAD proteins and changing SCs renewal, differentiation, and survival patterns [68, 69]. In adult and CSCs systems all the mentioned pathways are common and conserved in the control of SCs renewal, proliferation, and differentiation.

added to the toolkit that allows tumor survival and evolution within the TME [46].

4. Development of the CSCs Population in the TME

As previously mentioned, cancer is the product of cells deviating from normal tissue regulation mechanisms, due to the accumulation of oncogenic mutations with survival advantages over other cells [99]. During carcinogenesis any cell type is prone to malignant transformation depending on the degree of accumulation of nononcogenic or oncogenic mutations [21, 100]. Normal SCs, progenitor cells, or differentiated cells can escape regulation and become a CSCs, a cancer progenitor cell, or a poorly regulated differentiated cell [101] (Figure 1). Tumor Initiating Cells (TICs) can be traced back to CSCs, but CSCs are not always the cell of origin of the fittest clones in cancer [31]. The great variety of mutations within a tumor give rise to the many different phenotypes and

Table 2: Comparison of traits of normal Stem Cells and Cancer Stem Cell (CSCs) niches.

Trait	Normal Stem Cells	Cancer Stem Cells
Niche element: mesenchymal cells	Contribute with nutritional and stem-cell-fate factors [82]	Tumors educate the surrounding cells to provide nutrients, although highly resistant to lack of nutrients [83, 84]
Niche element: immune system cells	Modulate local environment for immune protection of SCs (immune suppression = immune-sanctuaries) [85]	Modulate local environment for immune protection of CSCs (immune suppression = immune-sanctuaries) [86]
Niche element: extracellular matrix	The matrix signals to SCs for fate regulation, promoting stemness and physiological maintenance [87]	Cancers cells produce large quantities of metalloproteinases, enzymes that degrade and remodel the ECM, thus promoting invasion and tumor angiogenesis [88]
Niche element: oxygen	Oxygen supply by blood vessels [89, 90]	Highly resistant to lack of oxygen [91], common in specific regions of tumors
Resistance to environmental stress	Highly resistant to cell death by noxious agents [92]	Highly resistant to lack of nutrients. Highly resistant to cell death by chemotherapy agents. Multidrug Resistance (MDR) pumps that extrude toxic compounds ATP-binding cassettes transporters (abc) [93] Quiescence activation through the ability to perceive stress [12, 24]

variations in plasticity properties [102]. A poorly regulated cell can transform into a cancer progenitor cell leading to an expression profile similar to that of SCs, but there is no direct evidence that a poorly regulated cell could eventually become CSCs (even if it passes through all the transformation stages) [23, 103, 104]. Hematopoietic bone marrow represents a good example of how cancer can arise from the accumulation of mutations due to high tissue turnover [105]. Hematopoietic Stem Cells (HSCs) constantly accumulate DNA damage due to physiological stress produced by infections or persistent blood loss, contributing to age-related tissue degeneration and malignant transformation [100, 104]. SCs' constant transition out of dormancy and the subsequent continuous proliferation of their progeny can lead to the development of CSCs [106]. In several types of human leukemia, mutant HSCs prevail over normal HSCs [106]. This unbalance has been associated with mutations that increase the activity of Ras signaling, which in turn favors the presence of cancerous HSC clones in the niche [106].

The organization of the tumor can be mediated by CSCs cytokines which induce changes in the cells that make up the TME and thus generate a cancer niche [107]. The presence and proliferation of CSCs or TICs stimulate the organization of the TME, providing the tumor with more heterogeneity and fueling its aggressiveness [108]. Very actively self-renewing and Long-Term TICs (LT-TICs) have been observed in colon cancer from the first stages of development. Additionally, it has been observed that they are able to maintain tumor progression in murine xenotransplants [109]. Tumor Transient Amplifying Cells (T-TACs) with less self-renewal capacity/metastatic potential and Delayed-Contributing TICs (DC-TICs) do not become activated in primary tumors but contribute to the advance of the disease only after transplantation [110]. These cells with different self-renewal capacities and nontumorigenic progeny generate the necessary genetic heterogeneity within tumors that allows for the existence of clones that withstand chemotherapy [81]. Other cells, like MSCs and macrophages, can be attracted to the tumor site and fuel the tumor with prosurvival factors that promote the cancer development [26]. Through this process, multipotent MSCs cells support the stemness of tumor tissues in the same way that they would function during normal SCSs homeostasis. MSCs are able to react, for example, to Interleukin-1 (IL-1) secreted by cancer cells and produce Prostaglandin E_2 (PGE_2) [111]. In normal SCs niches, PGE_2 regulates the amplification of multipotent progenitors and therefore represents a key factor in the homeostasis of the HSCs during stress [112, 113]. In the HSCs niche, MSCs contribute to the maintenance of normal HSCs as well as the organization of the CSCs microenvironment [114]. Thus, MSCs are key players in the maintenance of the stemness in both adult SCs and CSCs microenvironments, helping them to survive physiological stress and therapies [83, 115, 116].

Inside the TME, distinct cancer clones struggle to endure environmental pressures (e.g., lack of nutrients, oxygen, and immune surveillance). The hierarchical organization of cells created by selective processes benefits the establishment and survival of CSCs [23, 117]. Cells surrounding the CSCs niche cooperate to create a microenvironment that shares several common features with normal SCs niches (Table 2). Both types of SCs niches, normal and tumoral, set the stage for complex interactions with hematopoietic cells, endothelial cells, fibroblasts, MSCs, soluble signaling elements, and the ECM to create an immune-privileged environment, [118]. As cancer cells approach a SCs phenotype they become more resistant to chemotherapy and assume the top position of the TME hierarchy. The analysis of high burden (advanced-stage metastatic disease) versus low burden (early-stage metastatic disease) patient-derived triple-negative (Estrogen Receptor Negative, ER^-, Progesterone Receptor Negative, PR^-, and Human Epidermal Growth Factor Receptor-2 Negative, $HER2^-$) Breast Cancer Cells (BCCs) showed that low burden metastatic cells are more similar to SCs, expressing genes like *Cyclin Dependent Kinase Inhibitor 1B (CDKN1B), serine/threonine-protein kinase Chk1 (CHEK1), Transforming Growth Factor Beta Receptor 3 (TGFβR3),* and *Transforming*

Growth Factor Beta Receptor 2 (TGFβR2) and a quiescent phenotype with the capacity to initiate tumors when xenografted in mice [119].

5. Comparison between the Tumor Microenvironment and the Stem Cell Niche

Both the SCs niche and the TME are rich and complex environments that combine cellular and noncellular components to sustain stemness [120]. As mentioned before and similar to the normal SCs niches, the TME is made of a mix of cells (i.e., hematopoietic cells, endothelial cells, fibroblasts, and MSCs) together with noncellular components (i.e., nutrients, growth factors) that all together sustain the survival of the CSCs and cancer progression [23, 84, 121, 122]. The ECM is an important noncellular component of both SCs niches and the TME, playing different roles in each of them. The TME is a three-dimensional network mainly composed of collagens, glycoproteins, and proteoglycans, elastin, fibronectin, laminins, and other structural molecules [123]. SCs depend on the ECM architecture as a scaffold to grow and differentiate, but instability and stiffness of the ECM in the TME promote cancer development [124, 125]. Matrix-degrading enzymes secreted by normal and cancer cells can remodel the ECM. In the first case, the degradation of the normal ECM is key for tissue growth and development while in cancer it constitutes one of the first metastatic steps [125–127]. Cancers cells produce large quantities of metalloproteinases, enzymes that degrade the ECM, thereby contributing to the instability of tissue architecture and promoting invasion and tumor angiogenesis [88, 128].

TME cells of hematopoietic origin belong to two groups: (a) those coming from lymphoid lineages and (b) those from myeloid lineages. T-cells, B-cells, and NK-cells (Natural Killer Cells) are able to substantially inhibit tumor progression but in time they can be educated by the TME to help cancer cells survive and escape from immune surveillance [84]. CD4+ T helper and CD8+ Cytotoxic T Lymphocytes (CTL) are part of the main elements of the tumor microenvironment. Th1 Lymphocytes produce Interferon Gamma (IFN-γ), Tumor Necrosis Factor Alpha (TNF-α), and Interleukin-2 (IL-2), which are all essential for tumor rejection. Th1 can collaborate with Th17 to produce IFN-γ and Interleukin-17 (IL-17) which in turn recruit antitumor CTLs [86]. In normal SCSs and TMEs, MSCs interact with normal adult SCs and CSCs and induce them to secrete immunoregulatory cytokines such as Interleukin-10 (IL-10) and TGF-β, which force CD4+ T-cells to become anti-inflammatory [129–131]. Moreover, Th2 cells' interaction with the TME and particularly with MSCs inhibits immune rejection of the tumor (through the production of Interleukin-4, IL-4, Interleukin-5, IL-5, and Interleukin-13, IL-13) and promotes the presence of immunosuppressive type 2 macrophages [132–135]. Overall, MSCs present in the SCs niche and in the TME exert their immune regulatory profile, a feature common in both microenvironments [136–138].

Emerging only from the TME, fibroblasts modified through crosstalk with cancer cells become Cancer Associated Fibroblasts (CAFs) which provide growth factors, chemokines, and ECM-modifying metalloproteases and promote local tumor invasion [121]. CAFs have a spindle shape, express α-smooth muscle actin (α-SMA), and lose their normal cytokine expression profile, thereby attaining superior migratory, proliferative, and phagocytic capacities [139]. Additionally, cancer cells secrete factors that suppress the antitumoral control of the stroma. For instance, it has been observed that melanoma cancer cells secrete Platelet-Derived Growth Factor-BB (PDGF-BB) and TGF-β. These factors promote the transformation of fibroblasts, inducing them to express low levels of Pigment Epithelium-Derived Factor (PEDF), which in turn has been demonstrated to have anticancer properties [140]. Furthermore, it has been shown that a CAFs conditioned medium, cultured with prostate cancer cells, promotes their survival in the presence of gemcitabine, a potent chemotherapeutic agent, or after radiotherapy [139, 141].

Myofibroblasts and adipocytes, also part of the cellular stromal/endothelial cell population, are responsible for creating a tumor-permissive niche [83, 86]. Myofibroblasts, which may be present in prostate cancer, can also be educated by cytokines from cancer cells to be protumorigenic, similar to CAFs [142]. Pericytes, cells with properties similar to MSCs, are able to support tumor progression and chemotherapy survival after contact with cancer cells [83, 143, 144].

As previously mentioned, a common feature of both SCs niche and the TME is the presence of MSCs [26, 136] which are key players in cancer survival and the organization of the TME [145]. Therefore, the dialogue between MSCs and cancer cells is important to understanding other aspects of stemness in tumors. This is true in terms of the biology of CSCs, but also in terms of the participation of multipotent cells like MSCs that reside in the microenvironment or are attracted to it to promote tumor progression [146–148].

6. MSCs and Cancer Cells

MSCs were first identified about half a century ago when they were isolated from bone marrow and identified by their (1) capacity to adhere to plastic surfaces, (2) high potential to proliferate, (3) capacity for osteogenic, adipogenic, or chondrogenic differentiation, (4) cell surface markers such as CD105, CD73, and CD90, and (5) lack of CD45, CD34, and CD14, among others [149–151]. Although MSCs are named stem/stromal cells, they show limited stemness features which are highly variable depending on their origin, donor age, proliferation limitations, and time of isolation, even when in the presence of specific growth factors during their maintenance in vitro [152–154]. Stemness properties and the lack of a comprehensive classification of MSCs generate great controversy as they are not by definition SCs, but yet show multipotent properties [155, 156]. Even though more research is required to understand the stemness properties of MSCs, their role in providing support not only to SCs in normal niches but also to pathologic CSCs is well-documented [122, 136]. The relationship between MSCs and the TME of CSCs is essential to maintain Cancer Stemness. Current evidence indicates that MSCs and cancer

cells establish a complex partnership with strong implications for tumor progression and resistance to therapy. Normally, MSCs become physiologically attracted to sites of inflammation, where they demonstrate immunomodulatory capacities while helping tissues to heal. The TME constitutes a unique site of inflammation where MSCs are able to home. Thus, TME hijacks MSCs and integrates them into the functioning of the cancer stroma in order to stimulate tumor growth and induce angiogenesis, immune evasion, and resistance to chemotherapy [145]. Cancer cells interact with MSCs, thereby leading to changes in MSCs' phenotype and inducing them to adopt features of CAFs such as the expression of α-*SMA, FSP1 (Fibroblast-Specific Protein),* or *FAP (Fibroblast-Activated Protein)* [157] or, depending on the cancer type, MSCs can further differentiate [114]. As an example, MSCs undergo osteoblastogenesis due to the secretion of Fibroblast Growth Factor 9 (FGF9) by bone metastatic prostate cancer 3 (PC-3) carcinoma cells, which results in the osteopetrotic phenotype of MSCs in prostate cancer [158, 159].

MSCs are able to increase cancer cell proliferation/survival and induce tumor metastasis [160–164]. They can also promote tissue disorganization and the EMT for Michigan Cancer Foundation-7 (MCF-7) breast carcinoma cells through cell-to-cell interactions and the secretion of paracrine factors such as TGF-β [165]. The rapid proliferation rate of cancer cells predisposes them to having an increased sensitivity to endogenous sources of DNA damage such as Reactive Oxygen Species (ROS), which negatively affect their survival. In such cases, MSCs reduce intracellular ROS in cancer cells in organs such as the lung through the secretion of substances that uncouple oxidative phosphorylation and direct metabolism towards glycolysis, like stanniocalcin-1 (STC1) [166]. Furthermore, MSCs contribute to cancer cells' resistance to therapeutic treatments [167]. This finding represented a major breakthrough with important clinical consequences, as cancer resistance to therapy is one of the major flaws of current cancer treatments. The role of MSCs in contributing to cancer treatment resistance has been demonstrated in Chronic Myeloid Leukemia (CML) cells [168]. An important mechanism of this process is the secretion of interleukins with dual roles in physiological conditions and cancer disease. For instance, Interleukin-7 (IL-7) plays an important role in the regulation of normal precursor T cell and B cell development. However, recent evidence shows that IL-7 promotes DNA synthesis in leukemia cells. Zhang and colleagues [169] identified a source of IL-7 in MSCs that secretes high levels of this cytokine to protect leukemic cells against apoptosis induced by Imatinib or Gleevec, a potent tyrosine kinase inhibitor. Of particular interest was another report showing that fatty acids produced by MSCs help cancer cells to survive after platinum-based chemotherapy (Cisplatin) [170]. Two unique fatty acids secreted by activated MSCs, 12-Oxo-5,8,10-Heptadecatrienoic Acid (KHT) and Hexadeca-4,7,10,13-Tetraenoic Acid, were shown to be responsible for the cancer cells' acquired resistance to Cisplatin treatment. Indeed, blocking the MSC release of these PIFAs (Platinum-Induced Polyunsaturated Fatty Acids), by targeting thromboxane synthase or cyclooxygenase-1, restored the sensitivity of Lewis lung carcinoma cells to chemotherapy in vivo [170].

The immunosuppressive properties of MSCs and their ability to attract immune cells are important partners in the promotion of tumor progression. MSCs are able to effectively inhibit the proliferation of T-cells, B-cells, NK, and dendritic cells because of their production and secretion of molecules such as TGF-β, PGE2, and indoleamine-pyrrole 2,3-dioxygenase (IDO) [171, 172]. Furthermore, Tumor Necrosis Factor Alfa (TNF-α) activates MSCs to secrete chemokine (C-C motif) ligand 5 (CCL5), C-C chemokine receptor type 2 (CCR2), and the interleukin 8 receptor, beta (CXCR2) ligands that in turn recruit CXCR2+ neutrophils into the tumor. The interactions between CXCR2+ neutrophils and cancer cells enhance the expression of their metastatic genes, thus activating this cancer-spreading mechanism [167]. MSCs' contribution to cancer survival is very similar to their role in tissue regeneration and maintenance of normal SCs niches [136, 173]. Among other cancer survival strategies, the TME promotes the generation of CSCs which in turn can actively educate their surroundings through interactions with other SCs (like MSCs) to guarantee self-renewal states and to give rise to the subsequent production of aggressive cells [169, 174]. Thus, MSCs' stemness-related properties, plasticity, and the ability to sustain tissue repair should be further studied in order to effectively target their cancer-supporting pathways in the TME.

7. Pharmacological Targeting of the Microenvironment and Cancer Stem Cells

We long ago stopped conceiving of cancer as a homogenous population of cells with a broken connection to body homeostasis. We have seen through this review that cancer is far from being a chaotic system and rather shows an organized structure, independent progression dynamics, and tremendous adaptability to environmental pressure, all factors that make full clinical remission difficult to achieve. The many tumor-promoting properties that CSCs show during cancer development establish them as pivotal as therapeutic targets in oncology. CSCs develop DNA repairing mechanisms more rapidly than their normal neighbors develop and display prosurvival factors that inhibit induced apoptotic cell death induced by chemotherapeutic agents [93]. Moreover, CSCs maintain an undifferentiated state that arms them with the ability and plasticity to survive environmental stress [93]. One way to achieve this is through Multidrug Resistance (MDR) pumps that CSCs use to extrude amphiphilic chemotherapeutic compounds like Taxanes and Anthracyclines [175]. Quiescence, another SC property key for cancer survival, allows the tumor to survive chemotherapy designed to target rapidly dividing cells [24].

Tumors tend to increase their overall volumes during their growth phase, which restricts proper vascularization and causes their centers to have low oxygen concentrations. Hypoxia in the TME promotes the generation and survival of CSCs [176]. Hypoxia activates the secretion of the Hypoxia-Inducible Factors- (HIF-) 1α and HIF-2α that can activate the expression of AlkB Homolog 5 (ALKBH5), an m^6A demethylase enzyme reported to increase *NANOG* demethylation,

thereby facilitating NANOG production. NANOG is a potent inducer of pluripotency, which contributes to the generation of CSCs [176]. The production of Carbonic Anhydrase IX (CAIX), another cancer expressed protein, is induced by hypoxia. CAIX regulates cellular pH while simultaneously promoting cancer cell survival and invasion [177]. Blocking the downstream activation of proteins induced by hypoxia (like ALKBH5, CAIX, and NANOG) has been shown to inhibit CSC expansion in the tumor site, decreasing the probabilities for tumor relapse after therapy [169, 174, 177].

The understanding of these mechanisms and the way in which SC properties in CSCs evolved in tumors in response to therapy is inspiring new effective strategies in combination with classical approaches. In an interesting recent work, Bartosh and colleagues [178] observed that BCCs internalized and degraded MSCs. In 3D coculture systems, MSCs surround BCCs, promote the formation of cancer spheroids, and then become phagocytosed by BCCs in a process mediated by Rho kinases. The engulfing of MSCs by BCCs promotes dormancy and the activation of prosurvival factors in the tumor, which is indeed a characteristic of CSCs [178]. The internalization of MSCs or its exosomes (vesicles between 40 nm and 100 nm) promotes quiescence in BBCs, favoring dormancy and relapse after the application of therapies targeting rapidly cycling cells [179].

Learning how to interrupt the interaction of MSCs with cancer cells and with the TME signaling will dramatically improve the efficiency of current chemotherapeutic options. For instance, Regorafenib or Stivarga, apart from being an oncogenic multikinase inhibitor, is also potent repressor of MSCs expression of *vascular endothelial growth factor receptors 1–3 (VEGFR1–3), Receptor Tyrosine Kinase (TIE2), PDGFR-β*, and *Fibroblast Growth Factor Receptor 1 (FGFR1)*. Thus, the use of Regorafenib suppresses the influence of MSCs over the TME, inhibiting cancer progression [180]. Platinum-based chemotherapy drugs inhibit DNA repair and synthesis in proliferative cells, making them one of the most frequently used therapeutic options to treat aggressive tumors such as those that appear in ovarian cancer [181]. Despite the fact that platinum-based drugs have achieved clinical remission with an absence of cancer disease signs or symptoms, more than half of the treated patients suffered a relapse and showed resistance to the therapy [57]. The presence of chemoresistant CSCs is the main cause of therapeutic failure. Several signaling pathways (Figure 2), such as Notch, Wnt/β-Catenin, and Hedgehog, play important roles in the maintenance of somatic SCs and have also been involved in CSCs' self-renewal, proliferation, and survival in the face of DNA damaging agents [182]. The successful targeting of the Notch3 pathway in mouse models affects the presence and survival of CSCs in ovarian and breast cancers, increasing the cancer sensitivity to platinum-based therapies like Cisplatin, opening new strategies to counteract the relapse of the disease [57, 93, 182].

Adenosine triphosphate- (ATP-) Binding Cassette Transporters (ABC) play an important role in cell survival as they are able to pump out toxic compounds across the cell membrane [175]. The use of CDy1 dye represents a fast and simple method to stain live SCs. Hawley and colleagues [183] reported that Multiple Myeloma (MM) cells positive for the CDy1 dye, with a SC-like gene expression signature, have an increased expression of the P-glycoprotein, a member of the ABC superfamily. The MM CDy1+ cells were resistant to the proteasome inhibitor carfilzomib, which is used in combination with other drugs like lenalidomide and dexamethasone (KRd treatment) [183, 184]. KRd treatment has been shown to promote progression-free survival but did not change a poor prognosis in patients with relapsed MM [184]. The expression of P-glycoprotein is linked to the Hedgehog pathway and it has been observed that new therapeutic drugs, such as vismodegib, show promise in sensitizing MM cells to other therapeutic drugs [183, 185].

Metastasis has been associated with CSCs that migrate from the tumor site and establish themselves in a new niche where they give rise to differentiated cancer cells. It has been shown by single cell analysis that early-stage metastatic cells possess a SCs-like expression pattern [119, 186]. These metastatic CSCs are able to resist chemotherapy through quiescence and a SC program for survival, one example of which is the Leukemia Stem Cells (LSC) in Acute Myeloid Leukemia (AML) which, as well as HSC, have mutual capacities for self-renewal and quiescence [187]. miR-126 is able to control self-renewal and quiescence in both HSCs and LSCs by the activity of P13K/AKT/MTOR, but miR-126 shows opposite outcomes. The overexpression of miR-126 in Leukemic cells enhances self-renewal and quiescence while its knockdown in normal HSCs initiates the same process. These characteristics and its knockdown of miR-126 make it a promising candidate for therapy [187]. Having the knowledge of how CSC pathways are regulated in relation to cell differentiation, renewal, and quiescence opens the possibility of targeting CSCs specific pathways without affecting normal cells [188], (Figure 2).

The continuous treatment of cancer with chemotherapeutic agents that target a single specific cell mechanism tends to facilitate the generation of resistance or in the best of cases the increase of progression-free survival, but not the cure of the disease. It is becoming more clear that targeting the SC properties employed by CSCs to self-renew, generate plasticity, survive toxicity, and/or disrupt the communication between cancer and its microenvironment could have great impact on patient remission [189]. New pharmacological combinations of compounds already available on the market to target diseases other than cancer could have a significant impact on hindering tumor progression. For instance, the use of metformin, commonly used to treat type II diabetes, in combination with 5-fluorouracil, epirubicin, and cyclophosphamide (FEC), greatly affects CSCs' ATP production, thereby impairing the cell repair mechanisms of DNA damage induced by FEC [190].

8. Future Perspectives in Research

The CSC field has rapidly grown in the last 20 years, generating from around 2,500 publications/year in the early 2000s to more than 5,000 in 2015 alone. This rapid progression has provided a much better understanding of SC biology [191]. Specific topics, such as the behavior of SCs in adult tissues and their mechanisms of activation, capacity for long-term

self-renewal, and differentiation were key to comprehend the cellular heterogeneity of the TME, the presence of CSCs, and cancer resistance to therapy [100, 192]. From now on, the use of techniques like lineage tracing, single cell analysis, and organoid culture alone or combined will represent important tools to gain new insights into the complexity of CSCs biology and eventually to test new pharmacological compounds to target Cancer Stemness.

Cancer cells behave oddly within tumors and even cancer cell lines have shown to be heterogeneous in their proliferative potential in culture [192, 193]; this fact represents a challenge in terms of knowing the exact cellular origin of cancer. Lineage tracing using Cre-dependent marker systems, for example, takes advantage of a reporter gene to track the destiny of a cell or lineage of cells. SCs, CSCs, and other cell progeny occupying the niche are traceable with techniques that allow a better understanding of the factors activating their proliferation, differentiation, or quiescence. Lineage tracing is a fundamental tool to observe how modifications of cytokine response and their downstream signaling cascades affect cells individually, providing them with enhanced survival capacities or not. Factors such as TGF-β and mutations of its receptor induce changes in intestinal SCs that could lead to carcinogenesis. By using lineage tracing, Liskay and his team [194] observed that the transformation of TGFβR2 or its loss increased intestinal SC survival but altered their proliferation, suggesting that TGF-β response and sensitivity are determining factors in the sequence of events that lead to tissue transformation and cancer [194]. With the same approach, Corey and colleagues [195] demonstrated that tumor endothelial cells, while helping in the organization of blood vessels derived from a common precursor that tends to disappear, give rise to different subclones as the tumor evolves [195]. Untangling the dynamics of CSC behavior in tumors by lineage tracing will definitely open ways for a better understanding of cell transformation that usually leads to aggressive types of cancers, with the condition that similar pathways are shared by different tumors and that these mechanisms can be therapeutically targeted.

Single cell analysis by RNA-seq provides information about how a precise gene signature in SCs or CSCs determines their potential to resist stress, quiescence, proliferation, and differentiation [196, 197]. It has been hypothesized that metastasis is produced by tumor cells with unique SC properties. Using single cells analysis, Lawson and colleagues [119] observed that rare cancer cells with a SC-like gene expression profile (overexpression of *CDKN1B, CHEK1, TGFBR3,* and *TGFβ-2*) are more efficient in metastasizing and homing to other distant tissues [119]. Besides the genetic signature of a cell, the influence of epigenetic profiles on the generation of somatic mutations that lead to carcinogenesis is an important question in the understanding of cancer progression that will definitely require more research. Sunyaev and his team showed in 2015 [198] by comparing cell-type-epigenomic characteristics and mutations between diverse tumor cells that chromatin accessibility and replication timing are better predictors of their capacity to generate more mutations than the mutation signature itself. Interestingly, Sunyaev's group determined that the original cancer cell could be identified its epigenetic profile and by the distribution of mutations on its genome [198]. The identification of the cancer cell of origin by techniques like single cell analysis and lineage tracing will allow us to more clearly elucidate the dynamic of the TME heterogeneity, the presence of cancer cells progenitors, and predictions of the response of tumor cells to treatments [196].

Techniques involving mimicking the 3D cell-to-cell interactions and contact with the matrix are crucial to gaining insights into many aspects of cancer development and the generation of the CSC niche. Cells grown in 3D matrices have different gene signatures and show a better capacity to resist chemotherapeutic agents [199, 200]. The development of 3D organoid culture systems will help to understand how cell hierarchies emerge from original CSCs. Most 2D coculture systems fail to reproduce the conditions for cell-matrix interactions which are essential for processes like hypoxia generation (crucial for the CSCs phenotype), induction of the EMT, and metastasis [91, 201, 202]. BCCs interacting with fibroblasts in 3D show an enhanced invasion and secretion of metalloproteinase- (MMP-) 2 and survival cytokines [203]. Moreover, 3D culture models have been instrumental for the successful maintenance of cells that would otherwise die in 2D, such as glioblastoma cells. Interestingly, Hubert and colleagues [91] showed that organoids established from different regions of tumors from glioblastoma patients developed a fast proliferative region, a hypoxic core composed by non-stem senescent cells and quiescent CSCs. In addition, non-stem cancer cells were sensitive to radiotherapy while CSCs in the core were radio-resistant [91].

In summary, 2D cancer models and monoculture in vitro are not sufficient to address the way in which CSC niche organize and how CSCs persist after therapy. The development of 3D culture systems is fundamental to the study of the inherent heterogeneity of tumors, the details of cancer origin, and, with the use of lineage tracing and single cell analysis, how the niche evolves. Understanding which factors stimulate the persistence and division of CSCs in 3D models, their gene expression signature, and their mutational and epigenetic profiles will undoubtedly lay a firm foundation to develop better therapeutic target specific compounds for this cell population. As a foundation in the development of personalized medicine, the high throughput screening of organoids isolated from Patient-Derived Cells (PDCs) is providing important information about the most appropriate drug strategies for the treatment of cancer [204–206]. The systematic study of CSCs' behavior and education of their niche through organoid culture, lineage tracing, single cell analysis, and bioinformatics will be instrumental to comprehend and target cancer development and persistence after therapy.

9. Conclusions

Stemness is part of the normal repertoire of the genetic program of every cell and is very active during the first stages of development of any organism that reaches adulthood. CSCs use their stemness properties to perpetuate their lineage and survive stress and chemotherapy. The understanding

of these mechanisms, first in normal adult SCs and then in CSCs in the context of their niche, is key to develop better therapeutic approaches. Stemness in cancer cannot be self-sustained. As in normal niches, it requires the configuration of complex cell-to-cell and matrix interactions generating the heterogeneity needed by the TME to maintain tumor progression. Comprehending the successive genetic and epigenetic changes in cancer cells to become a CSC or how CSCs thrive is currently allowing the development of applied knowledge based on targeting Cancer Stemness properties and reinforcing the present challenge to develop new preventive and healing strategies.

The TME as a heterogeneous mix of cells and noncellular components contributing to cancer progression should be considered an important element when novel cancer therapies are designed. Of particular relevance are MSCs that reside or are attracted to the TME and have the potential to foster cancer growth and generate immunoregulation using similar mechanisms as those observed in the normal SC niche [25]. MSCs and fibroblasts can change when faced with different types of cancer cells or conditioned mediums, generating CAFs [145]. New therapeutic approaches must be developed to target the interaction between cancer cells, MSCs, and fibroblasts as this process is linked to metastasis [167]. All TME stemness-related properties and genetic and epigenetic modifications in CSCs can be used as therapeutic targets, but they cannot be approached independently because compensatory mechanisms are activated that promote cancer survival [207, 208].

Finally, the understanding of the stemness properties shared by adult SCs and CSCs and their niches bring light to the fundamental question of how the TME organize and promote cancer progression and survival. New challenges include the tracking of the origin of CSCs and their progenitors as well as the quest to understand ways they educate other cells in the TME to help them grow and thrive in different types of cancer and in a wide range of patients. For these purposes, the use of high throughput assays for lineage tracing, single cell analysis, and organoid culture will find a place in novel research strategies. The combination of these techniques will hopefully elucidate the essential mechanisms for the maintenance of Cancer Stemness and will be instrumental in the design of more effective and personalized therapeutic approaches.

Conflicts of Interest

The authors declare no conflict of interests.

Acknowledgments

The authors would like to thank the School of Medicine, the College of Biological Sciences, and the Chancelor's and Medicine Grant support team of the Universidad San Francisco de Quito for their support in this research endeavor. They would also like to thank Jonathan Guillemot and Caley Mikesell for their support.

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Hindawi
Stem Cells International
Volume 2017, Article ID 3684178, 20 pages
https://doi.org/10.1155/2017/3684178

Review Article

Mechanisms Regulating Stemness and Differentiation in Embryonal Carcinoma Cells

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Received 30 October 2016; Revised 10 January 2017; Accepted 8 February 2017; Published 8 March 2017

Academic Editor: Jijun Hao

Just over ten years have passed since the seminal Takahashi-Yamanaka paper, and while most attention nowadays is on induced, embryonic, and cancer stem cells, much of the pioneering work arose from studies with embryonal carcinoma cells (ECCs) derived from teratocarcinomas. This original work was broad in scope, but eventually led the way for us to focus on the components involved in the gene regulation of stemness and differentiation. As the name implies, ECCs are malignant in nature, yet maintain the ability to differentiate into the 3 germ layers and extraembryonic tissues, as well as behave normally when reintroduced into a healthy blastocyst. Retinoic acid signaling has been thoroughly interrogated in ECCs, especially in the F9 and P19 murine cell models, and while we have touched on this aspect, this review purposely highlights how some key transcription factors regulate pluripotency and cell stemness prior to this signaling. Another major focus is on the epigenetic regulation of ECCs and stem cells, and, towards that end, this review closes on what we see as a new frontier in combating aging and human disease, namely, how cellular metabolism shapes the epigenetic landscape and hence the pluripotency of all stem cells.

1. Introduction

We have just celebrated the 10th anniversary of the Takahashi-Yamanaka report on induced pluripotent stem cells, where introducing four transcription factors (Oct4, Sox2, Klf4, and c-Myc) was sufficient to reprogram fibroblasts towards pluripotent stem cells [1]. Although this work is a milestone in itself, paving the way for research into furthering our understanding of development and disease [2, 3], we must be reminded that most of the investigations into embryonic stem cells (ESCs) and cancer stem cells (CSCs) were preceded by those that focused on teratomas and teratocarcinomas [4–10]. The history is attention-grabbing, as over the last two thousand years teratomas have been attributed to everything from lucky omens, consorting with demons and the devil, participating in inappropriate sexual behavior, and incomplete twinning [5, 11]. Depending on the source, we know the word is derived from the Greek *terato(s)* [12], *teras* [13], or *teraton* [14] meaning monster and *oma* from onkoma or swelling [15] and was first reported in the mid-1860s by Rudolf Virchow [16]. Teratomas, which are benign germ cell tumors that contain cells derived from one or more of the three germ layers, develop spontaneously in the testes of the 129 family of inbred mouse strains, or they can be induced in adult mice when the genital ridges of embryos or early embryos themselves are ectopically transplanted into the testes or kidney [17, 18]. How teratomas develop has been the topic of much debate and is well beyond the scope of this review. However, we would be remiss if we did not note the recent findings that Cyclin D1, a target of canonical Wnt/β-catenin signaling, plays a key role in predisposing germ cells to switch their developmental potential to form

teratomas containing somatic tissues [19]. These teratomas represent "an intersection of pluripotency, differentiation and cancer biology" [20]. Teratocarcinomas contain early embryo-like cells called embryonal carcinoma cells (ECCs) that share three distinct features: (1) they are malignant; (2) they can differentiate into any of the three germ layers or extraembryonic tissue; and (3) they can develop normally when injected into the blastocyst [21, 22]. Although ECCs cells can be propagated following transfer of individual cells [23], the ability to culture them in vitro and their loss of "multipotentiality" [24] set the stage for the studies that followed. Pioneering work by Ralph Brinster, Richard Gardner, Michael McBurney, Beatrice Mintz, Virginia Papaioannou, and many others recognized the importance of ECCs, and their ability as noted by François Jacob, to adopt a normal fate when injected into host mouse blastocysts [25, 26]. In those early days, many were not fully aware that the attributes of these in vitro model systems would be so instrumental in contributing to studies that delved into trying to understand how ESCs and CSCs remain in a pluripotent state and how intrinsic and extrinsic factors reverse the ability of these cells to self-renew to allow them to differentiate into new lineages. In fact, the suggestion that the genetics of ECCs would uncover genes involved in stem cell self-renewal and pluripotency [27] only serves to underscore the importance of ECC lines. These lines have been and continue to be studied extensively [28–31], and although similarities and differences exist between them, as well as between ECCs and those representative of ESCs and CSCs, this review will focus almost exclusively on two ECC lines from mouse (F9 and P19) and one from human (NTERA-2) and how various pathways influence their pluripotency state. In light of the considerable number of studies generated using these lines, especially in regard to differentiation, which warrants its own review and has been presented in part for P19 cells [32], we have purposely concentrated our efforts to highlight what has been learned about self-renewal and pluripotency from ECCs, and in some cases how these studies have extended to ESCs and CSCs.

2. Embryonal Carcinoma Cells

The utility of ECCs as a proxy for the study of early mammalian development and neoplasia was recognized long before we began asking questions regarding pluripotency and self-renewal [4, 33, 34]. Not only were these early studies instrumental in uncovering many of the in vivo mechanisms that govern development [35], but also they led to the widely accepted theory on the process of cancer development [31].

2.1. F9 Teratocarcinoma Cells. F9 teratocarcinoma cells, one widely used mouse ECC line developed from another teratocarcinoma [36], give rise to tumors consisting almost exclusively of undifferentiated cells [21, 37, 38]. F9 cells exhibit a pseudodiploid karyotype composed of 38 acrocentric and 1 metacentric chromosomes, and a G1 and S phase of approximately 8 hours [37]. Once considered nullipotent, as they have lost the ability to differentiate spontaneously [37], studies would later reveal that F9 cells are capable of differentiating into extraembryonic endoderm-like cells [39, 40], and evidence would indicate that they share many characteristics of ESCs [41]. Subsequent studies reported that F9 cells can be induced by all-*trans* retinoic acid (RA) [42], a natural derivative of vitamin A (retinol), thus setting the stage for a plethora of studies to follow [43].

2.2. P19 Cells. P19 cells, another mouse ECC line, were derived from a 7.5-day *post coitum* embryo that was transplanted into the testis of an adult mouse [44–46]. These cells, which represent a population at a later stage of development than the F9 cells [38], are pluripotent and resemble epiblast stem cells. P19 cells have a male euploid karyotype (40 and XY), and much like F9 cells are considered nullipotent [47]. P19 cells can differentiate into neurons, glial cells, and fibroblasts when treated with RA or into skeletal and cardiac muscle when treated with DMSO [32, 48–52]. While many studies have shed light on the similarities between F9 and P19 cells, details would eventually emerge to indicate that differences in gene regulation allow them to break from pluripotency and differentiate [53–55]. For instance, F9 cells have greater reprogramming capabilities than P19 cells, and this is probably due to differences in the levels of the master pluripotency gene *Sox2*.

2.3. NTERA-2 Cells. While most studies with ECC lines have focused on those of mouse origin, the NTERA-2 cell line is a human ECC line first established in the 1980s from a testicular teratocarcinoma from a 22-year-old Caucasian male [56]. NTERA-2 cells exhibit a hypotriploid karyotype with a modal chromosome number of 63 [57]. NTERA-2 cells, like mouse ECCs, respond to RA and differentiate towards a neural lineage [5, 58–67]. Furthermore, NTERA-2 cells differentiate into nonneural epithelial cells when treated with bone morphogenic protein-2 (BMP-2), whereas 27X-1 cells, another human ECC line, differentiate into extraembryonic endoderm when exposed to BMP-2 or RA [68, 69]. It is obvious that differences within ESCs [70] and ECC lines exist (Table 1); however, one unifying concept is in their ability to respond to RA, which leads to a loss of pluripotency factors resulting in differentiation towards certain lineages.

3. Retinoic Acid: Lessons from the Inducer

RA is a potent teratogen and an important endogenous regulator of proper and extraembryonic endoderm [71]. RA signaling has many diverse roles in the differentiation of ECCs [72, 73], most often leading to extraembryonic endoderm-like lineages, which in itself has led to the derivation of the extraembryonic-like ECC lines PYS2 and END2 cell [74]. RA-induced differentiation is accompanied by changes in gene expression in F9 cells [75–80], P19 cells [80, 81], and NTERA-2 cells [82]. Genes on this exhaustive list include *c-Myc* [83, 84] and *Int-1* (later renamed *Wnt1*), which have become the focus of many subsequent cancer-related studies. In the case of *c-Myc* in P19 cells, its expression following RA treatment follows two transient increases at 3 h and 48 h, and then it drops below basal levels by 144 h [83]. In contrast, *c-Myc* expression in F9 cells declines with RA-induced

Table 1: Key features of ECC lines.

	F9 cells	P19 cells	NTERA-2 cells	References
Origin	Mouse	Mouse	Human	[37, 38, 57]
Colony morphology	Compact	Flat	Flat	[37, 38, 57]
Global methylation	Hypomethylated	Hypomethylated	Hypermethylated	[234, 248, 255]
Stemness genes	*Oct4, Sox2, Nanog, Klf4, Rex1, c-Myc*	*Oct4, Sox2, Nanog*	*Oct4, Sox2, Nanog, Dnmt3b, Fgf4, Rex1, Dppa5*	[58, 75–77, 81, 82]
LIF requirement	No	No	No	[122, 149]
RA-responsiveness	Yes	Yes	Yes	[37, 38, 59, 72]
Reprogramming efficiency	High	Low	Low	[41]
Teratoma formation	High	High	High	[22, 26]
Chimera contribution	High	High	No data available	[22, 26]
X chromosome status	X:0	Male	Male	[37, 38, 60]

differentiation [78] comparable to ESCs [85]. We now know c-*Myc* is downstream of the Wnt targetome [86], but it was the discovery of *Wnt1* itself that was exciting to many in the scientific community as it linked *Drosophila* embryogenesis and the Wingless protein to protooncogenes and cancer [87–89]. Later reports have highlighted *Wnt1* and other *Wnt* genes expression during RA-induced differentiation in P19 cells [90–92], F9 cells [78, 93, 94], and NTERA-2 cells [95, 96]. These early discoveries led to the assembly of complex cell signaling pathways and gene networks linked to ECC differentiation, and these were to be the platform that many have since used to identify the crosstalk and autoregulatory loops that exist within and between ECCs and ESCs [97–103]. It is interesting that while many of these studies revealed that RA must repress certain genes during differentiation, little discussion at the time linked these genes to self-renewal and stemness in ECCs. In fact, despite the irony that stemness genes including *c-Myc* [83, 104–107], *Oct3/4* [108–110], and *Sox2* [111–113] had already been identified in ECCs, their specific roles in self-renewal and pluripotency would not be elucidated until later [27, 114]. Meanwhile, studies showing expression of genes such as *K-fgf* and *Hst-1* [115–117], *TGFα*, and *LAMIN A/C* [117–121] provided the framework that gene activity was sufficient and necessary to keep ECCs in the pluripotent state. Two genes linked to stemness and pluripotency are *Rex1* (*Zfp42*), encoding a zinc finger transcription factor, and *Ccnd1*, encoding Cyclin D1. *Rex1* expression is not detected in undifferentiated P19 cells [122–124]; however, it is induced when *Nanog* is overexpressed [122, 125]. Similarly, Nanog controls the expression of *Ccnd1*, as seen when *Nanog* is depleted or overexpressed in P19 cells [125] and by retinoids in NTERA-2 cells, which promotes the ubiquitination and degradation of Cyclin D1 [67]. In addition to these studies and others involving *c-Myc* [104, 126–130], *Oct4* [110, 131–133], and *Sox2* [111, 112], further evidence that pluripotency genes must be developmentally regulated in the early embryo came from reports that *Nanog* [134, 135] and *Foxm1* [136] are downregulated in P19 and F9 cells in response to RA treatment. Foxm1, a member of the Forkhead box of transcription factors, is an interesting example as we now know it plays pivotal roles in cell proliferation, differentiation, and self-renewal and acts downstream of canonical Wnt/*β*-catenin signaling [137, 138]. This downregulation of *Oct4*, *Nanog*, and *Sox2* is also seen in NTERA-2 cells treated with RA [139], which together with reports on the effects of deregulating *c-Myc* expression [140] and *ZNF536,* encoding a novel zinc finger protein [141] in F9 and P19 cells, respectively, underscores the importance of activating, regulating, and maintaining self-renewal and pluripotency genes in the undifferentiated state. Thus, while much of the focus was initially on ways to differentiate ECCs, subsequent efforts were underway to identify pluripotency factors that would attenuate differentiation, thereby maintaining stemness.

4. Pluripotency Factors and Signaling Crosstalk

4.1. Leukemia Inhibiting Factor (LIF). Differentiating inhibiting activity (DIA)/leukemia inhibiting factor (LIF) produced by feeder cells or medium conditioned by Buffalo rat liver cells can block differentiation and promote stemness [124, 142–144]. Interestingly, ECCs harbor components responsible for LIF signaling [145, 146] and can secrete factors that support self-renewal of ESCs, but, unlike ESCs, they can maintain pluripotency in the absence of LIF or feeder layers [124, 147]. LIF belongs to the IL-6 family of cytokines, which contain IL-11, oncostatin M, ciliary neurotrophic factor, and others [148], all of which can signal through the JAK/STAT3 (Janus Kinase/Signal Transducer and Activator of Transcription) pathway. LIF activation in P19 cells [149, 150] blocks endodermal and mesodermal differentiation [114] and potentiates RA-induced neural differentiation [144, 151]. LIF has no apparent effect on NTERA-2 cells [114], and although there was early debate as to whether or not it had an effect on F9 cells [152, 153], recent evidence indicates that it blocks the ability of F9 cells to differentiate towards an extraembryonic lineage when induced by RA [154]. Moreover, STAT3 in F9 cells is regulated by Src-homology protein tyrosine

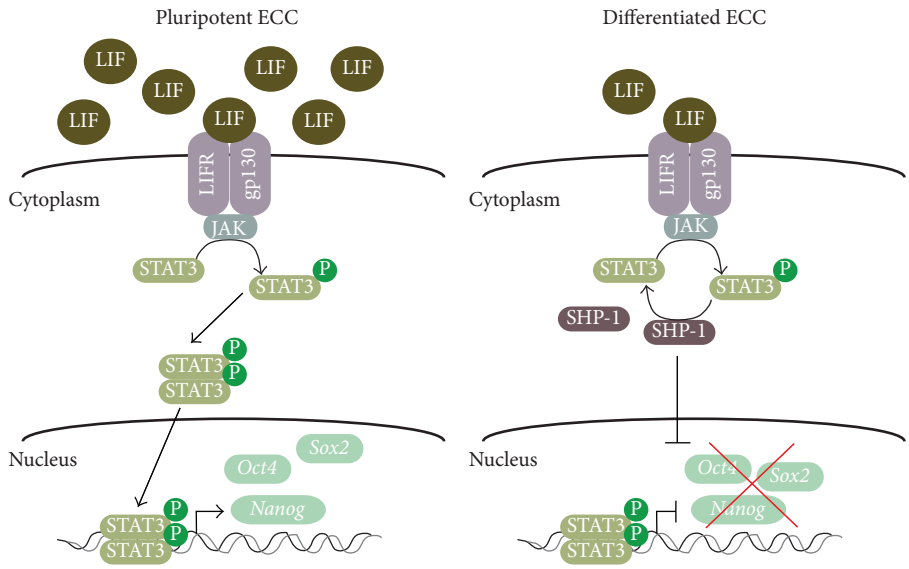

Figure 1: LIF signaling maintains ECCs pluripotency. In pluripotent ECC lines, LIF binds to the LIF receptor (LIFR) and gp130 recruiting Janus Kinase (JAK), which in turn phosphorylates and activates Signal Transducer and Activator of Transcription 3 (STAT3). Phosphorylated STAT3 homodimerizes and translocates to the nucleus promoting the expression of pluripotency genes encoding OCT4, SOX2, and NANOG. During differentiation, LIF levels decline substantially leading to decreased phosphorylated STAT3, which is augmented by the tyrosine-specific protein phosphatase SHP-1. The decline in active STAT3 reduces the expression of pluripotency genes.

phosphatase-1 (SHP-1), leading to its dephosphorylation and subsequent decrease in *Nanog* expression, which, as noted by the authors, restricts the expansion of the epiblast at implantation (Figure 1) [155].

4.2. Nanog. The homeodomain transcription factor Nanog plays an essential role in maintaining stem cell pluripotency and self-renewal. The *Nanog* promoter is well characterized, and although it is known to contain Oct4 and Sox2 binding sites, the early studies were contradictory with the report that Oct4 acts alone to induce *Nanog* expression [156], while another noted that both Oct4 and Sox2 were required [157]. *Nanog* overexpression in F9 cells maintains them in the undifferentiated state, as evident by the upregulation of *Oct4* and *SSEA-1* and downregulation of markers of differentiation including *Gata-6*, *Gata-4*, *Hnf1β*, and *LamininB1* [135]. The *Nanog* promoter contains one negative and two positive *cis*-regulatory elements that are active in F9 and ES cells, but only one positive element is active in P19 cells [134]. This example not only underscores the complexity of Nanog regulation, but also highlights the differences that exist between ECC lines. Negative feedback loops that regulate ESC pluripotency involving Oct4, FoxD3, and Nanog are known [158]. Furthermore, the presence of one of these loops whereby the *Nanog* promoter is negatively regulated by its own ectopic expression in ECCs would indicate that the pathway is even more complex than first thought [159]. Similarities in the pathway controlling Nanog expression exist between F9 and P19 cells, but because their Nanog levels differ, as well as Sox2, so too does their pluripotency state [41, 134]. Nevertheless, Nanog in ECCs is regulated by a Sox2 : Oct4 ratio [159] as well as the interplay between *Oct4* and *Rex1*, which are both involved in the maintenance of self-renewal downstream of Nanog [125].

4.3. Oct4. The crosstalk and feedback within proteins encoded by self-renewal and pluripotency genes are highlighted by the regulation of Oct4, considered as the master regulator of totipotency [160]. Oct4 has many roles in gene regulation, and its own positive and negative regulation is the topic of many studies. For instance, Nspc1, a polycomb protein and a transcriptional repressor that is highly expressed in undifferentiated P19 cells, directly activates the *Oct4* promoter [161], which in itself gets negatively regulated following RA treatment [78, 108, 162–165]. Once *Oct4* is transcribed and translated in F9 cells, *Rex1* expression is upregulated [166, 167], and similarly, in P19 cells, the *Rex1* promoter is activated by either Oct4 or Sox2 when Nanog is overexpressed [122]. The *Rex1* promoter is also activated in differentiated P19 cells when Oct4 is overexpressed [166]. However, while it may seem contradictory, high levels of Oct4 downregulate *Rex1* expression in F9 cells [166], which again highlights the importance of the cellular environment and context.

4.4. Wnt Signaling and miRNAs. Although studies indicate that Wnt signaling suppresses pluripotency and promotes differentiation, *Oct4* overexpression in P19 cells suppresses canonical Wnt signaling [168]. Nevertheless, canonical Wnt signaling is linked to Oct4 and pluripotency as evident by the fact that the downregulation of *Oct4* expression occurs when T-cell factor 3, Tcf3, serving as a transcriptional repressor in the absence of β-catenin, is overexpressed in F9 cells (Figure 2) [169]. This supports what was described earlier where Wnt is induced and Axin, a negative regulator of

Table 2: Trend of select miRNAs in P19 and NTERA-2 cells.

miRNA name	P19		NTERA-2		References
	P	D	P	D	
miR-9	−	+	−	+	[183]
miR-124	−	+	−	+	[178, 179, 183]
miR-125	−	+	=	=	[179]
miR-302	+	−	+	−	[179, 180, 183]
Let-7	−	+	−	+	[181]

P: pluripotent ECCs; D: differentiated ECCs
+: increase, −: decrease, and =: no change in levels.

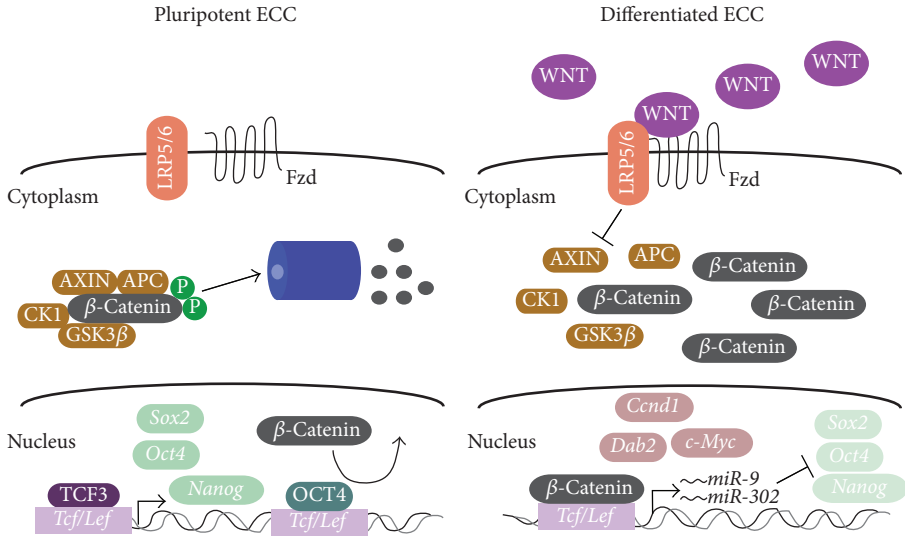

Figure 2: The dual role of WNT/β-catenin signaling ECCs stemness and differentiation. In the absence of WNT ligand, β-catenin is phosphorylated and degraded by the proteasome; subsequently, TCF3, a transcriptional repressor of WNT target genes, translocates to the nucleus and upregulates pluripotency genes. TCF3-dependent upregulation of OCT4 allosterically binds to TCF/LEF, preventing β-catenin from binding, which attenuates WNT signaling and differentiation. In the presence of WNT, AXIN is downregulated leading to the dissociation of the destruction complex, which results in the accumulation of β-catenin in the cytoplasm and subsequent translocation to the nucleus where it binds to TCF/LEF proteins. As a result, WNT target genes including *Dab2*, *Ccnd1*, and *c-Myc* are upregulated leading to cell differentiation. β-catenin-TCF/LEF interactions also result in the increase in *miR-9* and *miR-302* expression, which in turn downregulate the expression of pluripotency genes.

canonical Wnt signaling, declines in RA-treated F9 and P19 cells [88, 91–93, 170]. Another level impacting pluripotency and stemness involves microRNAs (miRNAs), which together with Oct4, Sox2, and Nanog are regulated positively by Wnt signaling (Figure 2) [171]. In one case this regulation involves the *mir-302* gene, which encodes a cluster of 5 microRNAs (miRNAs) that are highly expressed in undifferentiated NTERA-2 cells and P19 cells [172, 173]. Oct4 can bind directly to *miR-302* and upregulate its expression [171], while canonical Wnt signaling regulates *mir-302* expression involving 3 TCF/LEF binding sites. In the latter, knocking down β-catenin leads to decreased expression of *mir-302*, whereas knocking down Tcf3 produces the opposite effect [174], which promotes the expression of pluripotency genes in F9 and P19 cells [154, 175]. Other miRNAs play a role in regulating stemness and differentiation of mouse and human ECCs (Table 2), including *miR-9,* whose expression not only increases with differentiation, but also serves to repress *Sox2* in NTERA-2 cells [176]. Other examples include *miR-124,* where elevated levels in P19 cells promote neuronal differentiation by suppressing *Ezh2*, a histone methyltransferase, which represses genes involved in neurogenesis [177]. While many miRNAs are known to impact the ability of ECCs to remain pluripotent [172, 177–182], other modes of regulation, including that by PI3K/AKT signaling, can influence pluripotency and stemness.

4.5. PI3K/AKT Signaling. The PI3K/AKT signaling pathway is well known to have a key role in cell growth, proliferation, metabolism, and survival [183]. This list can be added to as RA and the negative regulation of the *Oct4* promoter are accompanied by an increase in AKT signaling in F9 cells [184] and the suppression of PTEN in P19 cells [185]. Furthermore, the phosphorylation of Oct4 and Klf4 by AKT leads to their degradation by the ubiquitin-proteosome system, which promotes the loss of pluripotency in F9 cells [186]. Although RA was previously described as an inducer of differentiation, its presence promotes AKT-dependent

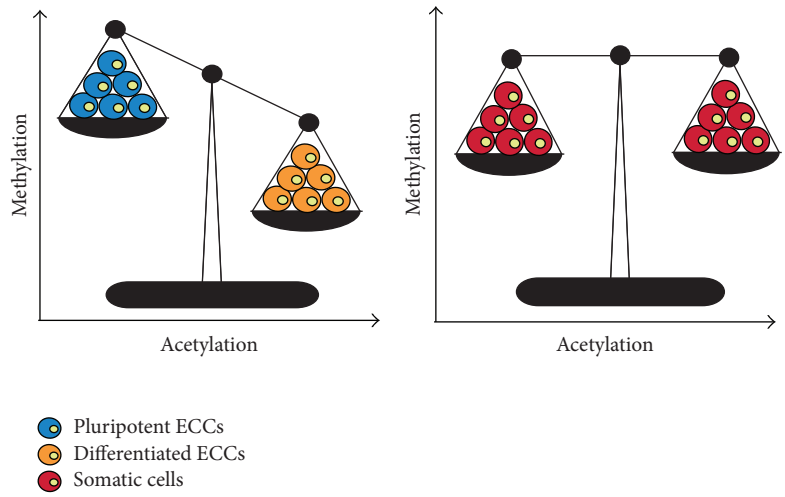

Figure 3: Global methylation and acetylation pattern of ECC lines during stemness and differentiation. Pluripotent ECCs are methylated and deacetylated yielding a low expression profile of differentiation markers. The dimerization of Jun Dimerization Protein 2 (JDP2) results in the recruitment of Histone Deacetylase 3 (HDAC3), which interacts and inhibits Histone Acetylases (HATs) from activating differentiation genes. Pluripotency is lost when DNA methyltransferase 1 *(Dnmt1)* expression and activity is reduced, resulting in the demethylation of differentiation-inducing genes including *Vimentin*, *Laminin B1, Collagen IV,* and *Endo A*. Similarly, HDAC activity is reduced as the p300 complex displaces the JDP2/HDAC3 complex, resulting in the recruitment of HATs and initiation of c-Jun transcription leading to differentiation.

phosphorylation of the chromatin remodeler SATB1, which binds to SOX2 thereby preventing it from associating with Oct4 to maintain pluripotency. In addition, and contrary to what was described earlier [186], active AKT signaling in P19 cells induces a transient increase in *Nanog* expression [187], which seems counterintuitive because AKT inhibits pluripotency markers. However, it is more complex than this as Oct4 in ECCs can bind to the human *AKT1* promoter, and this is dependent on its phosphorylation state controlled by AKT itself [188]. In fact, these authors found that the stabilization of Oct4 through AKT phosphorylation not only promotes its dissociation from the *AKT1* promoter, but also facilitates its interaction with Sox2 to upregulate *Nanog* expression. Thus, PI3K/AKT signaling is another means by which pluripotency is dictated in ECCs. However, other mechanisms downstream of signaling pathways such as epigenetic modifications also play a role in regulating stemness and differentiation [189].

5. Epigenetic Modifications

Understanding how global changes in gene expression are required to maintain ECCs in a pluripotent state has been a daunting task, one that is nearly eclipsed when considering the roles epigenetic modifiers and chromatin remodelers have on regulating these genes. The complexity imposed by these control mechanisms, especially as they relate to ESC pluripotency and differentiation, is evident by the many recent reviews and their historical account of the field [190–200]. While many epigenetic modifications exist, we will focus on the most common: DNA methylation and histone modifications by acetylation, which intricately link pluripotency genes and microRNAs in germ cell tumor development [201–208] to cancers and other diseases [209–215]. The molecular details of how these modifications occur and the effects imparted by these changes are presented elsewhere [193, 216–219].

5.1. DNA Methylation. Methylation of the fifth cytosine of CpG islands on the promoter of various genes is conserved in Eukaryotes [220]. Global methylation patterns in somatic cells are relatively stable and well characterized (Figure 3); however, DNA methylation or demethylation is tightly regulated and highly dynamic during embryo development [221, 222]. While multiple studies have highlighted the importance of DNA methylation in the maintenance of stemness and differentiation in ESCs [223–233], some of the first reports were with ECCs.

5.1.1. Methylation, Stemness, and Differentiation. These studies used 5-Azacytidine, which can induce differentiation of various ECCs by inhibiting DNA methyltransferases (DNMTs), though surprisingly not in F9 cells [234–237]. We have seen that F9 cells treated with a DNMT inhibitor do not differentiate and undergo apoptosis instead (unpublished data), which corroborates an earlier report [238]. Therefore, global demethylation in F9 cells is not sufficient to induce differentiation, even though it results in a demethylation profile like that seen following RA treatment [236, 239–241]; however, gene-specific demethylation pattern might vary. The methylation status of any gene can be misinterpreted, as in the case of those involved in the maintenance of pluripotency in F9 cells, which are hypomethylated and therefore transcriptionally active, while those induced with RA, including *Vimentin* [242], *Laminin B1* [243], *Collagen IV* [244], and *Endo B* [245], are hypermethylated making them inactive. Furthermore, the promoter of the *Thrombomodulin*, a marker of differentiation, gene shows a similar methylation pattern regardless of the differentiation status of the F9

cells. Thus, other mechanisms such as chromatin remodeling may also play a role in gene expression [246]. We know P19 cells treated with RA differentiate towards a neuronal lineage [49, 51], and this is marked by global demethylation [247], which is similar to what is seen in F9 cells treated with RA [239]. A reduction in DNMT protein levels and activity leads to reduced global and gene-specific methylation involved in differentiation seen in other ECCs [237, 239, 248, 249]. Specifically in P19 cells, the AP-1 regulatory site in the *Dnmt1* promoter is heavily methylated in the undifferentiated state [250]. When challenged by 5-Azacytidine, CpG islands upstream of the AP-1 site are demethylated leading to the recruitment of the Jun/Fos complex leading to expression of *Dnmt1*, which in turn methylates those same sites leading to transcriptional repression [250]. In fact, the methylation of regulatory regions in the *Dnmt1* promoter acts in a feedback mechanism, as sensors for the methylation capacity of the cell [250]. While downstream changes to methylation profiles have been linked extensively to pluripotency potential, much less attention has been given to upstream regulators. Early work has emphasized the importance of RAS and its downstream effectors [54, 251] on the role in global and site-specific demethylation by inducing the phosphorylation and activation of c-JUN, which binds to the *Dnmt1* promoter, inducing its expression and leading to differentiation [252]. These examples of consistent methylation trends, differences in DNMT activity, and activation of signaling pathways reveal the complexity underpinning the maintenance of stemness and differentiation in ECCs. Although much has been learned regarding the influence of DNA methylation on genes involved in differentiation, we know that similar mechanisms are in place for self-renewal. For instance, Oct4 and Nanog promote pluripotency in ESCs and ECCs, but it is important to note that the expression of these genes is regulated by methylation. The *Nanog* promoter in undifferentiated NTERA-2 cells is methylated 200 bp upstream of Oct4/Sox2 binding domains [253]. Similarly, the methylation status of the *c-Myc* promoter in F9 cells dictates the levels of the protein, which are high in the undifferentiated state but fall precipitously due to RA treatment [130]. This is recapitulated in human ESCs where high c-Myc levels maintain pluripotency in the absence of LIF/STAT3 [254]. Undifferentiated P19 cells maintain high levels of *Oct4* expression by promoting a low methylation profile on the *Oct4* locus [255, 256]. Taken together, these studies would indicate that the ratio of demethylation-to-methylation dictates the expression of pluripotency genes in ECCs, and thus the maintenance of stemness [255, 256]. If so, this role of methylation status could account for the heterogeneity in pluripotency as others and we have noted in ECCs populations.

5.2. RA Signaling and Methylation. While overwhelming evidence was presented earlier that RA induces ECCs differentiation, it is necessary to devote a brief description on how RA and retinoid synthesis and transport to their RAR/RXR are linked to the methylation status of promoters. During differentiation, the cellular RA binding protein 1 (CRABP-1) binds to RA and delivers it to RAR/RXR sites on the DNA of target genes [257]. RA induces CRABP-1 in P19 and F9 cells, yet the methylation pattern of its promoter remains unchanged, even in the presence of 5-Azacytidine, which does not induce *CRABP-1* expression [258]. Conversely, a methylated Histone H2B variant (TH2B) transfected into F9 cells gets demethylated, signifying that there is active histone gene expression in the undifferentiated state [259]. The *Th2b* gene, like many other housekeeping genes, maintains a low methylation profile, which is largely due to the protection of CpG islands imparted by the SP1 transcription factor [260, 261]. Whereas DNA methylation can play a role in transcriptional repression, the activation of the *H-2K* gene during differentiation is associated with hypermethylation, and this is evident from studies with 5-Azacytidine, which attenuates *H-2K* expression in F9 cells [262]. Since DNA methylation is not easily reversed, gene regulation may be better controlled temporally by the more labile and reversible modifications made to histones.

5.3. Chromatin Remodeling. Eukaryotic DNA with all its modifications is folded into nucleosomes, which are made up of histone octamers. Since resolving the nucleosome structure [263, 264], many histone modifications have been discovered [265–268]. These discoveries have provided us with a great understanding of how changes to chromatin availability directly or indirectly regulate gene expression. Although histone modifications by methylation are known to play a role in chromatin availability [269], we will focus on acetylation modifications in ECCs. In one example, the modification involves histone acetylation of lysine residues via histone acetyl-transferases (HATs), which weaken DNA-histone interactions allowing several proteins to dock and initiate transcription.

5.3.1. Histone Modifications, Stemness, and Differentiation. P19 cells maintained in an undifferentiated state have high levels of nonacetylated histones (Figure 3), but this declines with RA induction [270, 271]. A similar situation occurs in NTERA-2 cells, where regulatory regions of *Oct4* and *Nanog* in differentiated cells are hypoacetylated, leading to the closed chromatin conformation and reduced expression [272]. The expressions of *Oct4* and *Nanog* are influenced by the knockdown of the *Brahma related gene*, *Brg1*, encoding a protein present in Brg-containing Switch/Sucrose NonFermentable (SWI/SNF) complexes [273]. In P19 cells, the involvement of Brg1 with the promyelocytic leukemia protein maintains an open chromatin conformation of the *Oct4* gene [274], whereas RA induces silencing and chromatin remodeling by receptor-interacting protein 140 [72, 274]. This silencing and remodeling displaces Brg1 for the Brahma- (Brm-) containing SWI/SNF complex on both the *Oct4* and *Nanog* promoters, thus silencing transcription [275]. Although comparable conditions exist in ESCs [276] this is not a universal phenomenon as histone acetylation near the *Nestin* locus accompanies RA-induced differentiation in P19 cells [277]. It is interesting to note that $RAR^{-/-}$ F9 cells exhibit increased expression of *Slc38a* and *Stmn2*, which is normally associated with differentiated F9 cells due to hyperacetylation, suggesting that RA signaling might have a role in regulating histone

modification [278]. Modifications by histone deacetylases (HDACs) are also intricately linked to RA signaling. HDACs antagonize HATs, as they remove acetyl groups from lysine residues, a modification that restores their positive charge and leads to chromatin stability that is largely associated with transcriptional repression. Jun Dimerization Protein 2 (JDP2) maintains stemness in F9 cells by serving as a transcription factor not only to recruit HDAC3, but also to have it interact and inhibit HATs [279]. During RA treatment, however, the p300 complex displaces JDP2/HDAC3 leading to acetylation, initiating c-Jun transcription and differentiation [280, 281]. Other examples of this regulation are seen with HDAC3 in P19 cells, which inhibits the autoactivation of the neuronal transcription factor NeuroD [270], or in studies using HDAC inhibitors, which show reduced *Nanog* expression in undifferentiated P19 and F9 cells, and *Esrrb*, *Klf2,* and *Rex1* in ESCs [282]. Differentiated ECCs treated with an HDAC inhibitor and showing elevated pluripotency markers would suggest that HDAC activity could be largely regulated by the stemness state of a cell [282]. These reports and many others document the importance of HDACs in regulating chromatin availability, but the fact they also physically interact with pluripotency markers including Sox2 [282], Oct4 [283], and Nanog [284] reminds us of their involvement at other levels. In addition to the control conveyed directly by HDACs, chromatin remodeling of pluripotency genes is facilitated by other factors. For instance, in RA-induced differentiation of P19 cells, *Nanog* repression is the result of Foxa1, a member of the forkhead/winged-helix gene family induced by RA, to recruit the transcriptional corepressor Grg3, which belongs to the Gro/TLE/Grg family [285]. This potentiates subsequent recruitment of HDACs, together with Foxa1, to deacetylate histone 3 and repress the *Nanog* locus [285]. This type of multiplex control is not just reserved for *Nanog*. In differentiated P19 cells, *Oct4* expression initially increases, and in cooperation with histone H3 acetylation it induces *Meis1a* expression, which recruits HDAC1 directly to the *Oct4* promoter to subsequently reduce its activity [286]. Examples of this complex interplay between HDACs and other factors are well documented from ESC studies [198, 287, 288], and it would be of interest to investigate whether similar mechanisms are in place in ECCs.

5.4. RA Signaling and Histone Modification. Since HDAC deacetylation of key lysine residues on the regulatory regions of the RAR and RXR receptors would lead to their transcriptional repression, it is easy to envision given the network of genes regulated by RA how important this mechanism is to maintaining pluripotency. Butyrate inhibits histone deacetylation and promotes reversible morphological changes to F9 cells, but it does not induce differentiation [289]. Surprisingly, in the presence of cycloheximide, transcript and protein levels of differentiation markers were sustained in butyrate treated F9 cells [290]. Trichostatin A is another HDAC inhibitor that cannot induce P19 cell differentiation by itself but is able to when cells are cotreated with RA [291]. Like butyrate, treatment of Trichostatin A alone induces apoptosis in P19 cells, whereas cotreatment with RA induces RAR/RXR-induced transcription via histone acetylation [291]. In this study, the authors postulate that histone acetylation alone is not sufficient to induce differentiation, but it nevertheless primes ECCs for these events. Although the RAR/RXR response to acetylation plays a limited role during butyrate and Trichostatin A treatment, it more importantly suggests that other mechanisms are likely involved in the maintenance of stemness [292]. For example, CDK-associated Cullin 1 (CAC1), affects RA-induced differentiation by directly binding to RARα, inhibiting its transcriptional activity in P19 cells [293, 294]. In this position, CAC1 recruits HDAC2, which deacetylates RAR2 to promote pluripotency [293]. While we have provided some examples of how epigenetic modifications influence gene regulation, we did not cover the source of cofactors required to induce such changes. The idea that epigenetic regulation is energetically demanding is often overlooked, and for that reason we will complete our discussion by addressing the fact that many of the cofactors involved are provided by cellular metabolism. Thus, the epigenome is intricately linked to the metabolome [295, 296], and any changes to one would be expected to have direct consequences on the other.

6. Cellular Metabolism

We begin this section with some of the earliest discussions to spotlight the contribution of the mitochondria and metabolism to pluripotency and stemness [296–299]. Mitochondria have many roles including its most well-known role, to produce ATP, and obligatory production of reactive oxygen species as the by-product of cellular metabolism. In addition, mitochondrial metabolism is linked to calcium signaling and apoptosis, all of which play a part in pluripotency in ESCs [300–303]. During oxidative phosphorylation (OXPHOS) one molecule of glucose generates 38 molecules of ATP, which in comparison to glycolysis generates 2 ATP molecules. Although glycolysis is inherently less efficient than OXPHOS, it does support and promote high cellular proliferation during embryonic development [304, 305], cancer initiation and progression [306, 307], and neurodegenerative disease states [308, 309]. A balance between glycolysis and OXPHOS metabolism has been considered as a "rheostat" for stem cell fate [310], as these processes that generate and utilize metabolites have an impact on changes in epigenetic modifications and cell signaling networks governing the equilibrium between pluripotency and differentiation [311–314]. The majority of ESCs tend to transition towards OXPHOS with differentiation [315, 316], although this is not universal [304] as evident in iPSCs derived from fibroblasts that revert to glycolysis [317]. These findings and others only serve to strengthen the argument that the metabolic state of a cell must be considered when discussing stemness and pluripotency, and we are reminded that some of the seminal studies that led to this account were first reported in ECCs in the 1990s.

One such study examined Phosphofructokinase (PFK), a glycolytic enzyme that catalyzes the phosphorylation of fructose-6-phosphate to fructose 1,6-bisphosphate in the presence of ATP. Van Erp and colleagues (1990) reported that undifferentiated P19 cells show preference to *Pfk-L* expression, encoding an isoform that is more sensitive to

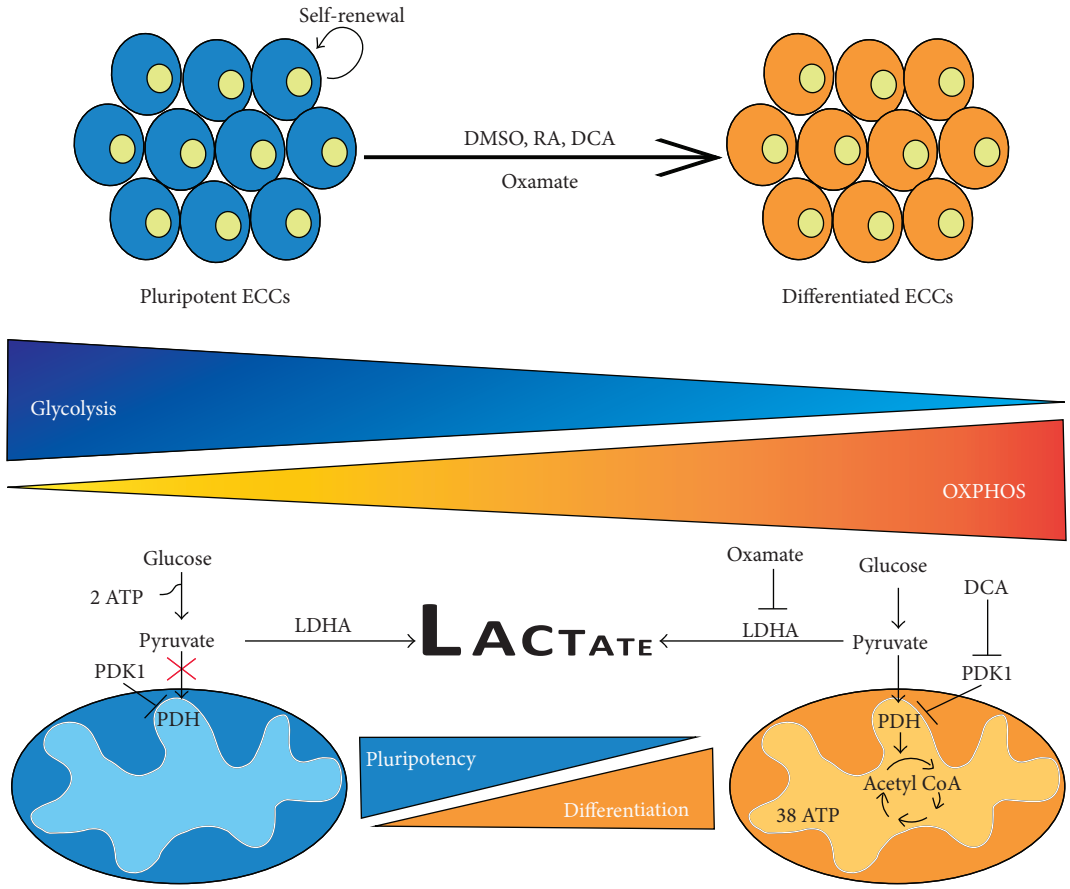

Figure 4: General overview of ECCs differentiation and the metabolic transition associated with the loss of stemness. ECCs can be differentiated towards neuronal and extraembryonic endoderm in the presence of retinoic acid, or cardiomyocytes in the presence of DMSO. With the differentiation process, others and we have observed a metabolic transition from glycolytic metabolism towards oxidative phosphorylation. Moreover, we can induce F9 cell differentiation towards an extraembryonic lineage using dichloroacetate (DCA), a pyruvate dehydrogenase kinase (PDK) inhibitor, or oxamate, a lactate dehydrogenase A (LDHA) inhibitor, which would indicate that the metabolic status of a cell determines whether it will remain pluripotent or if it will differentiate towards a specific lineage.

activation by fructose 2,6-bisphosphate, and thus increasing the glycolytic rate [318]. Similar results were reported in highly proliferative cells for lactate dehydrogenase A (LDHA), which converts pyruvate to lactate [319] and pyruvate dehydrogenase kinase (PDK), which by phosphorylating and blocking the pyruvate dehydrogenase (PDH) complex inhibits the conversion of pyruvate to acetyl Co-A in the mitochondria [320]. Undifferentiated P19 cells have a strong glycolytic profile, which is correlated with high levels of Oct4, Sox2, and Nanog [320]. Similarly, we have found that F9 cells maintained in the undifferentiated state have high levels of LDHA and PDK1 (manuscript in preparation) (Figure 4). This parallels the increased lactate and pyruvate production seen in differentiating P19 cells [321]. The fate of pyruvate in these cells, however, differs significantly between the undifferentiated and differentiated cells, as when cells are grown in the presence of galactose, pyruvate is shuttled to the mitochondria and this is associated with reduced stemness [320]. It is interesting to note that undifferentiated P19 cells have fewer mitochondrial proteins [322] compared to their differentiated counterparts, even though both have similar mitochondrial DNA copy number [320]. This may not be a general phenomenon for all ECCs, as we have seen the opposite in F9 cells (manuscript in preparation). Similar mitochondrial DNA copy number between the undifferentiated and differentiated state is likely offset by mitochondrial activity, highly prevalent in differentiated ECCs, and substrate availability, which would fuel these mitochondria. What is perplexing is that P19 cells induced to form cardiomyocytes initially have low mitochondrial DNA content and ATP levels during the early stages of differentiation, but both eventually return to basal levels [323]. That these changes are not expected to occur during differentiation only reinforces the idea of the complex, nonuniversal nature and specificity of cellular metabolism in maintaining stemness or promoting differentiation. We have shown that F9 cells differentiate when OXPHOS metabolism is promoted (Figure 4), and this parallels what was reported earlier for P19 cells [320]. Likewise, while many have shown that the differentiation of ECCs is accompanied by a shift in the metabolic profile towards OXPHOS [320–322], it remains to be determined if this metabolic transition precedes the differentiation process

or is the result of it. If we are to champion one or the other, it would probably be the former given the evidence that mouse embryonic fibroblasts reprogrammed using the Yamanaka factors transition to glycolytic metabolism prior to the induction of pluripotency [324]. This phenomenon, if general, would indicate that metabolism might be the master regulator of pluripotency and stemness and not just their slave in preventing differentiation.

7. Conclusion

Using ECCs as our platform we have highlighted some of the dramatic interplay that must exist between key genes and their regulators for these cells to remain pluripotent and to self-renew. Extrapolating many of these events to ESCs and CSCs in vivo has been fruitful in many cases, but as we have noted on several occasions, there are fundamental differences that preclude striking a unifying model. Other means of regulation are known to play key roles in ECC self-renewal and differentiation, including modifications imparted by reactive oxygen species (ROS) [325, 326], as well as the influence of ROS homeostasis in cellular programming [300, 327], cell cycle control as demonstrated in ESCs [328–333], as well as long noncoding RNAs in these cells [334–337]. In fact, signaling through Ca^{2+} channels in ECCs has also been reported to be involved in regulation [338]. Changes to the epigenome and chromatin remodeling, however, have garnered much attention. Together, we expect that in the future a better understanding of how these changes contribute to self-renewal and pluripotency in development will only serve to elucidate a major question on the minds of everyone—why do we get cancer?

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

Gregory M. Kelly would like to thank the Natural Sciences and Engineering Research Council of Canada for funding. The authors also acknowledge the Faculty of Graduate and Postgraduate Studies, Western University, for support to Mohamed I. Gatie. Gregory M. Kelly would like to dedicate his part of the review to Dr. Iskra Sarafov for her constant reminders that a single view through the telescope captures much more than several views through the microscope. Mohamed I. Gatie would like to dedicate his portion of the review to Nesrine Cheikh, and both authors would especially like to thank Tina Nicole Cuthbert for her feedback on this manuscript.

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Hindawi
Stem Cells International
Volume 2017, Article ID 2925869, 10 pages
https://doi.org/10.1155/2017/2925869

Review Article

Targeting Signaling Pathways in Cancer Stem Cells for Cancer Treatment

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Received 2 December 2016; Revised 10 February 2017; Accepted 13 February 2017; Published 5 March 2017

Academic Editor: Shimon Slavin

The Wnt, Hedgehog, and Notch pathways are inherent signaling pathways in normal embryogenesis, development, and hemostasis. However, dysfunctions of these pathways are evident in multiple tumor types and malignancies. Specifically, aberrant activation of these pathways is implicated in modulation of cancer stem cells (CSCs), a small subset of cancer cells capable of self-renewal and differentiation into heterogeneous tumor cells. The CSCs are accountable for tumor initiation, growth, and recurrence. In this review, we focus on roles of Wnt, Hedgehog, and Notch pathways in CSCs' stemness and functions and summarize therapeutic studies targeting these pathways to eliminate CSCs and improve overall cancer treatment outcomes.

1. Introduction

Cancer stem cells (CSCs) are a small subset of cancer cells with the capability of self-renewal and differentiation into heterogeneous tumor cells, and they have been believed to be responsible for tumor initiation, growth, and recurrence. The first population of CSCs was identified in human acute myeloid leukemia (AML), where they displayed strong tumorigenic ability in an in vivo mouse model [1, 2]. Subsequently, many laboratories across the globe have been able to capture and propagate CSCs from a variety of human tumors including brain cancer, melanoma and breast cancer, liver cancer, pancreatic cancer, colon cancer, and prostate cancer [3–9]. As CSCs can survive traditional cancer therapies and result in tumor recurrence and drug resistance [10–12], eradication of CSCs in tumors may represent an effective anticancer therapeutic strategy. Towards this goal, significant efforts have been made to explore the signaling mechanisms underlying CSCs' self-renewal and differentiation, as well as development of regimens targeting the CSCs. In this review, we focus on three key evolutionarily conserved CSC signaling pathways (Wnt, Hedgehog, and Notch pathways) and therapeutic strategies disrupting CSCs' stemness and functions by modulating these pathways.

2. Signaling Pathways in CSCs

In the past, multiple CSC models have been proposed for tumor heterogeneity including the classical CSC unidirectional differentiation model and the plastic CSC bidirectional dedifferentiation model [13, 14] (Figure 1). In the classical CSC unidirectional differentiation model, CSCs differentiate to non-CSC tumor cells that are unable to move back up the hierarchy to acquire CSC-like activity; however, in the plastic CSC bidirectional dedifferentiation model, non-CSC tumor cells can undergo a dedifferentiation process and acquire CSC-like properties, presumably through epithelial-mesenchymal transition (EMT) in carcinoma [15–19]. Nevertheless, in either CSC model, Wnt, Hedgehog, and Notch pathways are considered important CSCs' regulators.

2.1. Canonical Wnt Signaling Pathway. Canonical Wnt pathway, in which Wnt ligands signal through β-catenin for their biological functions, is a critical evolutionarily conserved

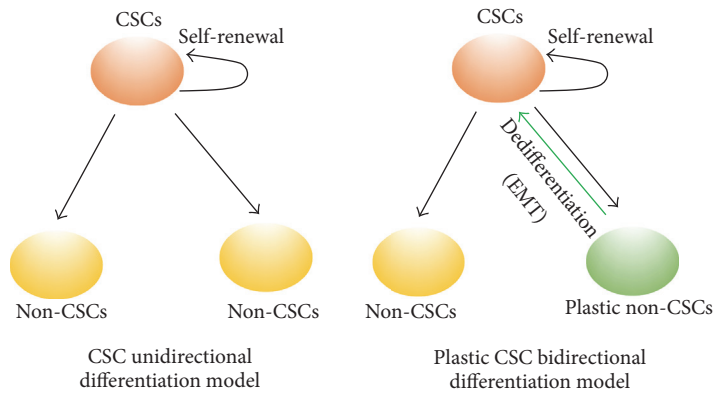

Figure 1: A schematic outlining the classical CSC unidirectional differentiation model and the plastic CSC bidirectional dedifferentiation model. In the unidirectional differentiation model, CSCs preexist in the tumor environment and solely self-renew or differentiate into the non-CSC tumor cells. However, in the plastic CSC bidirectional model, the plastic non-CSC tumor cells can dedifferentiate to acquire a CSC phenotype via epithelial-mesenchymal transition (EMT).

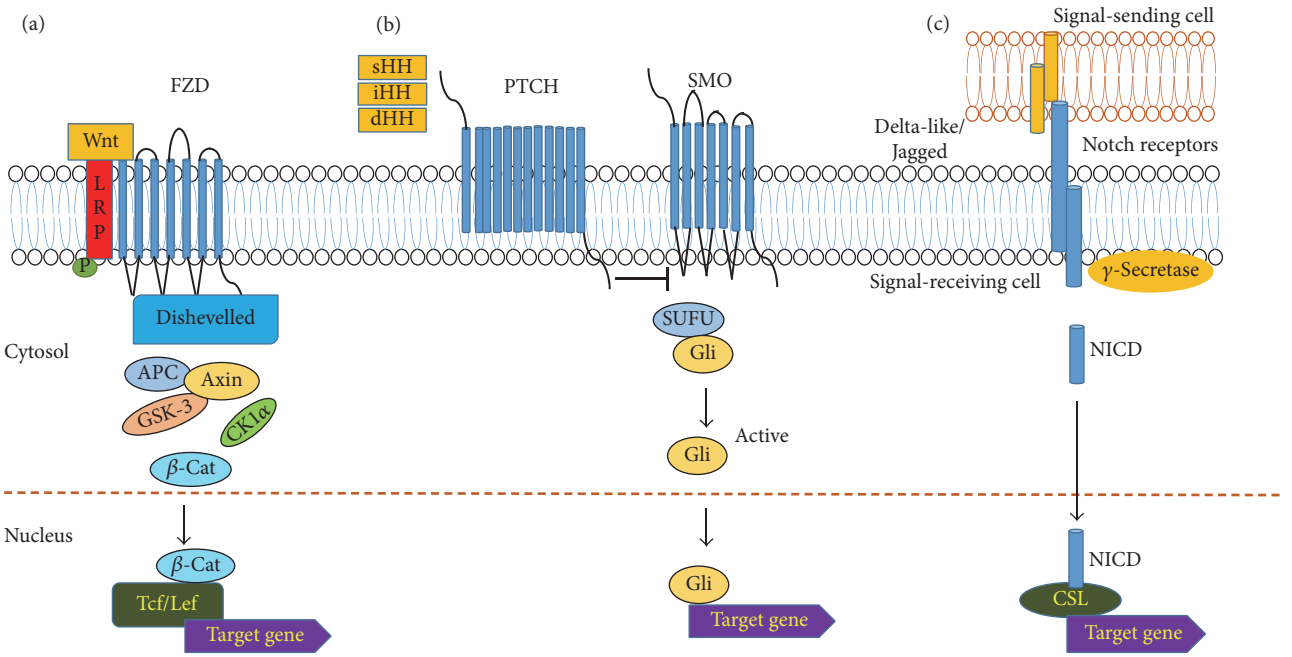

Figure 2: Wnt, Hedgehog, and Notch canonical signaling pathways. (a) In the canonical Wnt signaling pathway, when Wnt ligand binds to FZD and LRP receptors, the β-catenin destruction complex (Axin, GSK3, APC, and CK1) is decomposed, and active β-catenin accumulates and translocates to the nucleus for target gene transcription. (b) In the canonical Hedgehog pathway, when ligand binds to PTCH, the inhibitory effect of PTCH to SMO is removed. Subsequently GLI is activated and translocates into nucleus for target gene transcription. (c) In the canonical Notch pathway, delta-like or Jagged ligand binds to Notch receptor and a series of extracellular and intracellular cleavages occur, and NICD translocates to the nucleus to regulate target gene transcription. For the detailed pathway information, please refer to the text.

pathway in embryonic development and tissue homeostasis [20]. In the absence of Wnt ligands, the cytoplasmic β-catenin is phosphorylated for proteasome-dependent degradation by a "destruction complex" consisting of axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), and casein kinase Iα (CKIα) [21]. However, in the presence of the Wnt ligands, the signaling is activated through the ligands binding to the seven-transmembrane receptor Frizzled (FZD) and the single-membrane-spanning low-density receptor-related protein 5/6 (LRP5/6). FZD then recruits the intracellular protein dishevelled (Dvl), which subsequently sequesters Axin and GSK3β from the cytoplasm to the cellular membrane resulting in decomposition of the "destruction complex" [22]. Consequently, the active unphosphorylated β-catenin accumulates and translocates into the nucleus to regulate target gene expression (Figure 2(a)).

2.1.1. Abnormal Wnt Signaling Activation and CSCs. Abnormal activation of Wnt signaling has been implicated in the regulation of a plethora of CSC types including colorectal

Table 1: Small molecule/biological therapeutics targeting the Wnt pathway.

Molecule	Function	Phase/clinical trials	Cancer type	NCT Number
PRI-724	Dishevelled inhibitor	Phase 1/2	Pancreatic cancer, acute myeloid leukemia, and colon cancer	NCT02413853 NCT01606579 NCT01764477
LGK974	Porcupine inhibitor	Phase 1	Melanoma, breast cancer, and pancreatic adenocarcinoma	NCT01351103
Vantictumab (OMP18R5)	Anti-Frizzled 7 receptor	Phase 1b	HER2 negative breast cancer and pancreatic cancer	NCT01345201
Ipafricept (OMP-54F28)	Fc-Frizzled 8 receptor	Phase 1a/1b	Pancreatic cancer and ovarian cancer	NCT02092363 NCT02050178

The clinical trial information was accessed via https://clinicaltrials.gov with National Clinical TrialNumber (NCT Number).

cancer, breast cancer, hematologic cancer, skin cancer, and lung cancer [23–27]. For instance, in colorectal cancer, Wnt signaling deregulation is often associated with mutations in APC and β-catenin genes [28]. Mutations in APC have been found in around 80% of all human colon tumors, and these mutations inactivate APC function resulting in Wnt signaling activation by preventing β-catenin phosphorylation and subsequent β-catenin degradation [29, 30]. In addition, β-catenin oncogenic mutations have been reported in approximately 10% of colorectal cancer patients, and these missense or deletion mutations are located at β-catenin sites where GSK3β normally phosphorylates β-catenin, leading to stable β-catenin translocation into the nucleus for Wnt activation [22, 31]. Abnormal activation of Wnt signaling disrupts the normal growth and differentiation of colonic crypt stem cells, resulting in a colorectal CSC phenotype by upregulating expression of target genes such as *c-myc* and cyclin D [22, 32]. Moreover, in a recent comparative analysis of signaling pathways between the CD^{44+}/CD^{133+} colorectal CSCs and CD^{44-}/CD^{133-} cancer cells, Wnt pathway was shown to be highly associated with CD^{44+}/CD^{133+} colorectal CSCs [33].

In addition to colorectal CSCs, Wnt signaling is also involved in other types of cancer CSCs. For example, in an elegant study of squamous cell carcinomas, canonical Wnt signaling activation was shown to be critical in tumorigenesis of CD^{34+} bulge CSCs, and ablation of the β-catenin gene resulted in depletion of CD^{34+} CSCs and complete tumor regression in mice [27]. In addition, β-catenin-deficient tumor cells devoid of the CD^{34+} CSCs were unable to propagate secondary tumors, and conversely tamoxifen-induced expression of a nondegradable β-catenin in the skin sufficiently expanded the bulge CSC's population [27].

Moreover, dedifferentiation through EMT is a critical step for non-CSC tumor cells to acquire CSC-like properties as defined by the plastic CSC bidirectional model. Wnt signaling plays an important role in this cancer cell dedifferentiation. One study showed that experimental knockdown of CD146 can dedifferentiate colorectal cancer cells to acquire a stem cell phenotype through inhibiting GSK-3β which in turn promoted nuclear translocation of β-catenin for Wnt signaling activation [34]. Therefore, modifying Wnt signaling may be essential in the pursuit to curb colorectal cancer, specifically colorectal cancer stem cells.

2.1.2. Therapeutic Agents Targeting Wnt Signaling. As Wnt signaling activation is implicated in CSC's self-renewal, tumorigenesis, and cancer cell dedifferentiation into CSCs, targeting CSCs by inhibiting the Wnt signaling may be a promising therapeutic approach for cancer. Recently numerous Wnt signaling inhibitors, including biological agents and small molecule agents, have been developed [35]. However, to date, no Wnt signaling inhibitors have been approved for clinical usage. The majority of Wnt inhibitors have been evaluated preclinically, and the readers can refer to our recent review paper in this area [35]. Here we summarize ongoing clinical trials of Wnt inhibitors (Table 1). For instance, one clinical trial of a Wnt inhibitor is PRI-724 which inhibits the Wnt signaling by specifically binding to downstream CREB-binding protein. PRI-724 was previously shown to induce apoptosis of colon carcinoma cells and exhibit antitumor activity in the mouse xenograft models of colon cancer [36]. In the phase I trial, 18 patients were treated showing favorable toxicity profiles with only one dose-limiting toxicity of grade 3 of reversible hyperbilirubinemia [37]. An impending phase II trial for PRI-724 is almost underway involving mFOLFOX6/Bevacizumab with or without PRI-724. The focus of the study is to target patients with Stage IV metastatic colorectal cancer (NCT Number: NCT02413853). In addition, Porcupine is a membrane bound O-acyltransferase (MBOAT) specific to Wnt posttranslational acylation, which is required for subsequent Wnt secretion, and loss of Porcupine can lead to Wnt signaling inhibition [38, 39]. In addition, a specific small molecular Porcupine inhibitor LGK974 was identified in a luciferase-based cell screening and was efficacious in targeting Wnt signaling in multiple tumor models including murine and rat mechanistic breast cancer models and a human head and neck squamous cell carcinoma model [40]. Recently, a phase I, open label dose escalation trial of the LGK974 has been initiated to treat a variety of malignancies including melanoma, breast cancer, and pancreatic adenocarcinoma (NCT Number: NCT01351103). Other than small molecules, several biologic therapeutic agents targeting the Wnt pathway

have entered clinical trials as well (Table 1). For instance, OMP18R5 (also known as Vantictumab), a fully humanized monoclonal antibody that targets FZD receptor, has recently concluded an open label phase 1 dose escalation study for solid tumors [41, 42] (NCT Number: NCT01345201). Another biologic therapeutic agent OMP-54F28, a fusion protein that binds Wnt ligands and prevents them from binding to FZD receptors, was recently developed. In phase I trial of OMP-54F28, minimum doses at 0.5 mg/kg ranging up towards 10 mg/kg were administered intravenously once every 3 weeks in patients with solid tumors (NCT Number: NCT01608867). Although this clinical trial has recently been completed, the results have not been announced publicly [41, 42]. Moreover, another two phase 1b trials are underway involving OMP-54F28 and the chemotherapy drug Paclitaxel to treat ovarian and Stage IV pancreatic cancer (NCT Numbers: NCT02092363, NCT02050178).

2.2. Hedgehog Signaling. HH signaling is essential in a wide variety of cellular and molecular processes during embryogenesis, development, and adult tissue homeostasis [43, 44]. Three Hedgehog homologues, Sonic Hedgehog (sHH), Indian Hedgehog (iHH), and Desert Hedgehog (dHH), have been well studied in mammals [45, 46]. In the absence of HH ligands, a cell-surface transmembrane protein Patched (PTCH) inhibits the transmembrane protein Smoothened (SMO), and full length GLI proteins are then proteolytically processed to generate the repressor GLI^R to suppress HH signaling target gene expression (Figure 2). However, when extracellular HH ligands bind to PTCH, PTCH's inhibitory influence on SMO is removed, and activation of SMO results in nuclear translocation of GLI and induction of HH signaling target gene transcription [45, 47].

2.2.1. Abnormal HH Signaling Activation and CSCs. Aberrant activation of the HH pathway in CSC's regulation and maintenance has been reported in numerous cancer types including glioblastoma, lung squamous cell carcinoma, breast cancer, pancreatic adenocarcinoma, myeloma, and chronic myeloid leukemia (CML) [48–53]. In multiple myeloma CSCs, it was shown that SMO and Gli1 were highly expressed in comparison to non-CSCs, and activation of HH signaling by HH ligands promoted multiple myeloma CSC's expansion, whereas inhibition of the HH signaling markedly blocked CSC's clonal expansion [54]. The data supports that HH signaling promotes multiple myeloma CSC's functions. Moreover, higher activation of HH signaling was also observed in CSCs of human lung squamous cell carcinoma and glioma as compared to bulk tumor cells, further supporting aberrant HH signaling activation's critical role for CSC self-renewal and regulation [49, 53]. In another study, Zhao et al. reported that HH pathway activation was also involved in maintenance of CML CSCs [52]. In a murine CML model of study, deletion of SMO significantly reduced the CML CSCs, and conversely overexpression of SMO in a SMO-deficient mouse CML model dramatically enhanced CML CSCs 4-fold and significantly increased CML progression [52]. In addition, inhibition of HH signaling by SMO antagonist cyclopamine reduced a glioblastoma stem cells population [50], and similar findings have been observed in colon CSCs, pancreatic CSCs, prostate CSCs, and lung CSCs [55–58].

In the plastic CSC bidirectional dedifferentiation model, HH signaling plays an important role during the EMT process to acquire stem cell-like phenotypes. For instance, Gli1 was shown to correlate with markers of EMT and highly express in the claudin-low breast CSCs, and knockdown of Gli1 resulted in reduced claudin-low breast CSC's viability, motility, clonogenicity, and self-renewal as well as tumor growth in orthotopic xenografts [59]. Recently, Wang et al. have demonstrated that HH pathway and EMT are active in pancreatic cancer cells-derived tumorspheres that exhibit CSC properties, and inhibition of HH signaling by SMO knockdown blocks the self-renewal, EMT, invasion, chemoresistance, and tumorigenesis of pancreatic CSCs [60].

2.2.2. Therapeutic Agents Targeting HH Signaling. As evidenced above, HH signaling plays a critical role in CSC's self-renewal and regulation, and inhibition of the HH pathway disrupts CSC's stemness and induces CSC's differentiation which are desirable for cancer treatment. In the past, numerous HH pathway inhibitors have been developed. For a complete review of HH signaling inhibitors, please refer to the recent reviews [61–63]. Here we only summarize the HH signaling inhibitors either approved by FDA or under clinical trials (Table 2). Vismodegib developed by Genentech is the first HH signaling inhibitor approved by FDA. Vismodegib targets SMO for HH signaling inhibition and has been used to treat metastatic basal cell carcinoma. The initial phase I trial of Vismodegib showed that 18 out of 33 enrolled patients with locally advanced or metastatic tumors had a response to Vismodegib. Among the remaining 15 patients, 11 had stable disease for up to 10.8 months and 4 had progressive disease. No dose-limiting toxic effects or grade 5 adverse events were seen in the trial and reported toxicities were mild with common side effects of mild loss of taste, hair loss, weight loss, and hyponatremia [64, 65]. Currently, clinical trials of Vismodegib as a monotherapy or in combination with other therapeutic drugs are ongoing for various cancers including medulloblastoma, small cell lung cancer, metastatic pancreatic cancer, metastatic prostate cancer, intracranial meningioma, recurrent glioblastoma, and acute myeloid leukemia (NCT Numbers: NCT00833417, NCT01201915, NCT00739661, and NCT01088815) [61]. In 2015, a new SMO inhibitor, Sonidegib, was approved by the FDA to treat adult patients with locally advanced BCC. In the dose escalation phase I trial, maximum tolerated doses of 800 mg daily and 250 mg twice daily were established [66]. Grade 1/2 adverse effects were apparent, consisting of nausea, anorexia, vomiting, muscle spasms, fatigue, and alopecia and grade 3/4 adverse effects were weight loss, hyperbilirubinemia, myalgia, fatigue, and dizziness (NCT Number: NCT01529450) [66]. A phase II study demonstrated that Sonidegib sustained tumor responses in patients with advanced BCC after a 12-month follow-up [67]. Currently several phase I/II trials of Sonidegib to treat other solid tumors and hematological malignancies are still underway (NCT Numbers: NCT02195973, NCT01487785) [61]. Moreover, a few of HH signaling inhibitors that are actively being

Table 2: Small molecule/biological therapeutics targeting the Hedgehog pathway.

Molecule	Function	Phase/clinical trials	Cancer type	NCT Number
GDC-0449 (Vismodegib derivative)	SMO inhibitor	Phase 2	Basal cell carcinoma, ovarian cancer, metastatic pancreatic cancer, medulloblastoma, small cell lung cancer, metastatic prostate cancer, glioblastoma, and acute myeloid leukemia	NCT00833417 NCT01201915 NCT00739661 NCT01088815
Genistein	Downregulate Gli1	Phase 1/2	Colorectal cancer	NCT01985763
Sonidegib (LDE225)	SMO inhibitor	Phase 1/2	Prostate cancer, ovarian cancer, pancreatic cancer, and basal cell carcinoma	NCT02195973 NCT01487785 NCT01529450
5E1	Prevent HH ligand-Patched binding	Preclinical	Prostate cancer	N/A
Glasdegib (PF-04449913)	SMO inhibitor	Phase 1b/2	Acute myeloid leukemia, chronic myelomonocytic leukemia	NCT01841333 NCT01286467

The clinical trial information was accessed via https://clinicaltrials.gov with National Clinical TrialNumber (NCT Number).

tested in clinical trials include additional SMO inhibitors (Saridegib, BMS-833923, Glasdegib, and PF-5274857) and GLI Inhibitor (arsenic trioxide). Other than small molecule inhibitors, a monoclonal antibody, 5E1, blocks binding of all three mammalian HH ligands to PTCH for HH signaling inhibition [68, 69]; however, this antibody has not entered clinical trials yet.

2.3. Canonical Notch Signaling. Canonical Notch signaling is another essential evolutionarily conserved pathway in development and adult tissue homeostasis [70]. Notch signaling is activated when the extracellular domain of Notch transmembrane receptor binds to Notch ligands and subsequently induces proteolytic cleavage and release of the intracellular domain (Notch ICD or NICD) of Notch. The Notch ICD then translocates to the nucleus where it interacts with a CBF1/Suppressor of Hairless/LAG-1 (CSL) family DNA-binding protein and regulates the expression of target genes including those pertinent to CSC self-renewal such as Survivin, Myc Nanog, Oct-4, and Sox2 (Figure 2) [71–76]. In mammals, four Notch receptors (Notch 1–Notch 4) and five ligands (Jagged 1 and 2 and Delta-like 1, 3, and 4) have been identified [77].

2.3.1. Abnormal Notch Signaling Activation and CSCs. Abnormal activation of Notch signaling plays a pivotal role in the CSCs of breast cancer, pancreatic cancer, and glioblastoma. For instance, Barnawi et al. reported that fascin (an actin-bundling protein) effectively regulates breast CSCs at least partially through Notch pathway [78]. Fascin knockdown significantly reduced breast stem cell-like phenotype (downregulation of stem cell pluripotent genes such as Oct4, Nanog, Sox2, and Klf4), and the cells became less competent in forming colonies and tumorspheres. Conversely, activation of Notch signaling induced the relevant downstream targets predominantly in the fascin-positive cells, and fascin-positive CSCs showed stronger tumorigenesis [78]. In another study, immunohistochemical analysis of 115 breast tumor tissues from primary lesions was performed, and results showed that Notch positive tissues were significantly associated with a CSC marker aldehyde dehydrogenase 1 family member A1 levels [79]. Very recently, Choy et al. reported that Notch 3 signaled constitutively in a panel of basal breast cancer cell lines and in more than one-third of breast basal tumors [80].

Moreover, the important role of Notch signaling was also demonstrated in several other types of CSCs. In a study of patient-derived pancreatic CSCs, Notch ligands Notch 1, Notch 3, Jag1, Jag2, and Notch target gene Hes1 were found to be highly expressed in the pancreatic CSCs, and an inhibitor of γ-secretase (an important protease mediating Notch signaling by releasing the Notch ICD) significantly decreased the CSC's subpopulation and tumorsphere formation [81]. Moreover, activation of Notch signaling by delta/Serrate/Lag-2 peptide or inhibition of the signaling by knockdown of Hes1 enhanced or decreased pancreatic CSC's tumorsphere formation, respectively [81]. In addition, Notch signaling dysregulation has also been recognized in glioblastoma CSCs [82]. It was found that Protein Kinase C Iota (PKCi) was highly expressed in glioblastoma patient-derived CSCs, and silencing PKCi resulted in apoptosis and reduction of proliferation of the glioblastoma CSCs in vitro and tumor growth in vivo in a xenograft mouse model [83]. Gene expression profiling of PKCi-silenced glioblastoma CSCs revealed a novel role of the Notch signaling pathway in PKCi mediated glioblastoma CSC's survival [83]. In addition to its important roles in CSCs, Notch signaling is also involved in EMT to promote cancer cell acquisition of a stem-like phenotype and drug resistance. For instance, prostate cancer cells undergoing EMT displayed stem-like cell features characterized by increased expression of Notch 1 and other pluripotent genes such as Sox2, Nanog, Oct4, and Lin28 [84].

2.3.2. Therapeutic Agents Targeting Notch Signaling. Therapeutics targeting the Notch pathway mostly consist of γ-secretase inhibitors and anti-DLL4 antibodies (Table 3). Inhibition of the Notch pathway via γ-secretase inhibitors prevents

Table 3: Small molecule/biological therapeutics targeting the Notch pathway.

Molecule	Function	Phase/clinical trials	Cancer type	NCT Number
RO4929097	Gamma secretase inhibitor	Phase 2	Breast cancer, ovarian cancer, renal cell carcinoma	NCT01131234
LY900009	Gamma secretase inhibitor	Phase 1	Advanced solid tumor or lymphoma	NCT01158404
PF-03084014	Gamma secretase inhibitor	Phase 1/2	T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma	NCT00878189 NCT01981551
Enoticumab	Anti-DLL4 antibody	Phase 1	Advanced solid tumors and ovarian cancer	NCT00871559
Demcizumab	Anti-DLL4 antibody	Phase 1b/2	Advanced solid tumors, pancreatic cancer, ovarian cancer, and non-small cell lung cancer	NCT02722954 NCT01189968 NCT01189929
Tarextumab	Anti-Notch 2/3	Phase 1b/2	Solid tumors, Stage IV pancreatic cancer, and Stage IV small cell lung cancer	NCT01277146 NCT01647828 NCT01859741

The clinical trial information was accessed via https://clinicaltrials.gov with National Clinical TrialNumber (NCT Number).

Notch receptor cleavage at the cell surface, thus blocking activation of self-renewal target genes. In a preclinical study, a γ-secretase inhibitor RO4929097 significantly suppressed Notch target genes Hes1, Hey1, and HeyL [85]. Several phase I and phase II studies have been conducted in hopes of synergistically utilizing RO4929097 with other agents for cancer treatment. For instance, in a completed phase I trial, RO4929097 and Cediranib Maleate were used in tandem to determine the phase II dose and safety profile of RO4929097 in solid tumors (NCT Number: NCT01131234), and the clinical trial data shall be announced soon. Another γ-secretase inhibitor is LY900009, developed by Eli Lilly, which is in phase I for patients with advanced cancer including leiomyosarcoma and ovarian cancer [86]. A third γ-secretase inhibitor (PF-003084014) was developed by Pfizer, and it is progressing in its phase I trials in patients with T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma [87]. In addition to γ-secretase inhibitors, another category of Notch pathway molecules is monoclonal antibodies that target DLL4 (Delta-like ligand 4) to prevent ligand binding. Enoticumab (REGN421) is an anti-DLL4 antibody that has been used to target advanced solid tumors with overexpression of DLL4 (such as ovarian cancer) [88]. In 2015, a recommended phase II dose of 4 mg/kg every 3 weeks or 3 mg/kg every 2 weeks administered intravenously was established based on PK profiles in patients diagnosed with ovarian, colon, or breast cancer. [89]. Another anti-DLL4 monoclonal antibody developed by OncoMed Pharmaceuticals and Celgene is Demcizumab, which has recently completed a phase I dose escalation clinical trial as well. In this study, Demcizumab was well tolerated at doses $\leq$5 mg with disease stabilization and tumor size decreases when administered weekly. The side effects of Demcizumab include hypertension and an increased risk of congestive heart failure in prolonged drug administration (NCT Number: NCT02722954) [90].

3. Crosstalk among Pathways and Combination Treatments

Many pathways do not act as isolated units but rather often interact with other pathways as a biological network during development and homeostasis. Crosstalk among Wnt, HH, Notch, and other pathways have been reported in cancer and CSCs [91]. For instance, in a colorectal cancer study, progastrin secreted by colorectal tumors was shown to activate Wnt signaling and result in expression of Wnt target genes including Jagged-1, one Notch ligand. Upregulation of Jagged-1 induces Notch signaling which in turn may further elevate β-catenin activity of progastrin-driven Wnt and Notch signaling in colorectal cancer cells [92]. Similarly, in breast CSCs, Mel-18 was reported as a negative regulator of breast CSC's self-renewal. Knockdown of Mel-18 increased Wnt signaling, which subsequently upregulated Wnt target gene jagged-1's expression, leading to activation of the Notch pathway for CSC's self-renewal [93]. In addition, HH signaling can crosstalk with both Wnt and Notch pathways as well. In gastric cancer cells, HH signaling was shown to suppress Wnt signaling through the soluble frizzled-related protein 1 (sFrP1), a target gene of HH signaling capable of modulating Wnt pathway by directly binding to Wnt ligands [94]. In another study of glioblastoma cells and patient specimens, Notch signaling inhibition was shown to downregulate its target gene Hes1 which in turn upregulates GLI transcription in the HH pathway [95].

Complex signaling networks are known to contribute to the cellular diversity of stem cells during embryogenesis and tissue homeostasis and may play essential roles in the cancer and CSC's biology. In recent years, significant efforts have been made to develop combination therapies to target multiple signaling pathways for cancer treatments. For instance, a recent study demonstrated that combination inhibition of both Notch and HH signaling depleted the CSC

subpopulation cells in a prostate cancer model [96]. In addition, a clinical trial of combination of HH pathway inhibitor Vismodegib and Notch signaling inhibitor RO4929097 has been conducted in patients with advanced breast and sarcoma. In another recent study, Sharma et al. showed that combination treatment with HH signaling inhibitor NVP-LDE225 and pI3/mTOR/Akt signaling inhibitor NVP-BEZ235 inhibited self-renewal capacity of pancreatic CSCs by suppressing the expression of pluripotency maintaining factors Nanog, Oct-4, Sox-2, and c-Myc and transcription of GLI [97].

4. Conclusions

Since the first identification of CSCs in leukemia, the important roles of CSCs in cancer progression, metastasis, and relapse as well as drug resistance have been increasingly recognized. Eradication of CSCs by targeting the key signaling pathways underlying CSC's stemness and function represents a promising approach in cancer treatment. In this review, we mainly summarized the three critical evolutionarily conserved pathways (Wnt, HH, and Notch signaling) in CSCs and potential therapies targeting these pathways for cancer treatment. To date, numerous agents have been developed to specifically target each of these pathways for cancer treatments. Nevertheless, it has been recognized that the signaling pathways may interact with each other as a coordinated network to regulate CSC stemness and functions. Therefore, understanding the crosstalk among the signaling pathways in CSC regulation is critical for the development of therapies targeting CSCs.

Competing Interests

The authors declare no competing financial interests.

Acknowledgments

This work was supported by the seed fund of College of Veterinary Medicine and intramural grant at Western University of Health Sciences to Jijun Hao and the National Natural Science Foundation of China (nos. 81272444 and 81472744 to Li Zhong).

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Hindawi
Stem Cells International
Volume 2017, Article ID 3714190, 11 pages
https://doi.org/10.1155/2017/3714190

Review Article

Cancer Stem Cells and Their Microenvironment: Biology and Therapeutic Implications

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Received 9 January 2017; Accepted 9 February 2017; Published 26 February 2017

Academic Editor: Xiaojiang Cui

Tumor consists of heterogeneous cancer cells including cancer stem cells (CSCs) that can terminally differentiate into tumor bulk. Normal stem cells in normal organs regulate self-renewal within a stem cell niche. Likewise, accumulating evidence has also suggested that CSCs are maintained extrinsically within the tumor microenvironment, which includes both cellular and physical factors. Here, we review the significance of stromal cells, immune cells, extracellular matrix, tumor stiffness, and hypoxia in regulation of CSC plasticity and therapeutic resistance. With a better understanding of how CSC interacts with its niche, we are able to identify potential therapeutic targets for the development of more effective treatments against cancer.

1. Introduction

Cancer exists as a heterogeneous population, with different cancer cells showing distinct phenotypic and functional properties, leading to the limitation of therapeutic efficacy and treatment outcomes. In fact, the discovery of the "Cancer Stem Cell (CSC)/Tumor-Initiating Cell (T-IC)" theory provides an alternative explanation for the failure of existing therapies. Although the idea of CSCs was proposed over a decade ago, the existence of CSCs has been identified in various types of cancer by taking the advantage of available cell surface markers in the last 10 years. In this model, cancer cells are organized in a hierarchy with cancer stem cells (CSCs)/Tumor-Initiating Cell (T-IC) located at the apex [1]. The new concept of CSCs is based on the idea that stem cells are present in cancer tissue, like in normal tissues, and are part of the hierarchy of cells. In other words, just as there are normal stem cells in normal tissues, CSCs are found in tumor tissues. Although the origin of CSCs remains controversial, there is increasing evidence to support that CSCs arise by either mutation from normal stem/progenitor cells or deregulation of genetic programs regulating these cells. These acquired mutations allow normal stem cells to transform from their quiescent and tightly regulated phenotype to constitutively activated ones. This model proposes that CSCs, which share some similar functional properties with normal stem cells, possess the ability to self-renew and initiate tumor formation and generate additional differentiated progenies that compose the heterogeneous tumor bulk. Furthermore, mounting evidence has shown that CSCs are protected by multiple resistance mechanisms, leading to tumor metastasis, therapeutic resistance, and recurrence. Therefore, CSC-targeting therapies represent a promising strategy for the long-term cure of the disease.

And in theory, stem/progenitor cells represent the natural target of tumorigenic mutations since they are possibly the only cells that have the longevity and are endowed with the appropriate capabilities to accumulate the required number of mutations needed to disrupt intrinsic mechanism regulating normal cell proliferation and differentiation [2, 3].

In a normal organ, stem cells reside in a "stem cell niche," a specific microenvironment that plays a key role in regulating stem cell maintenance and self-renewal by secreting various paracrine factors or by direct cell-cell

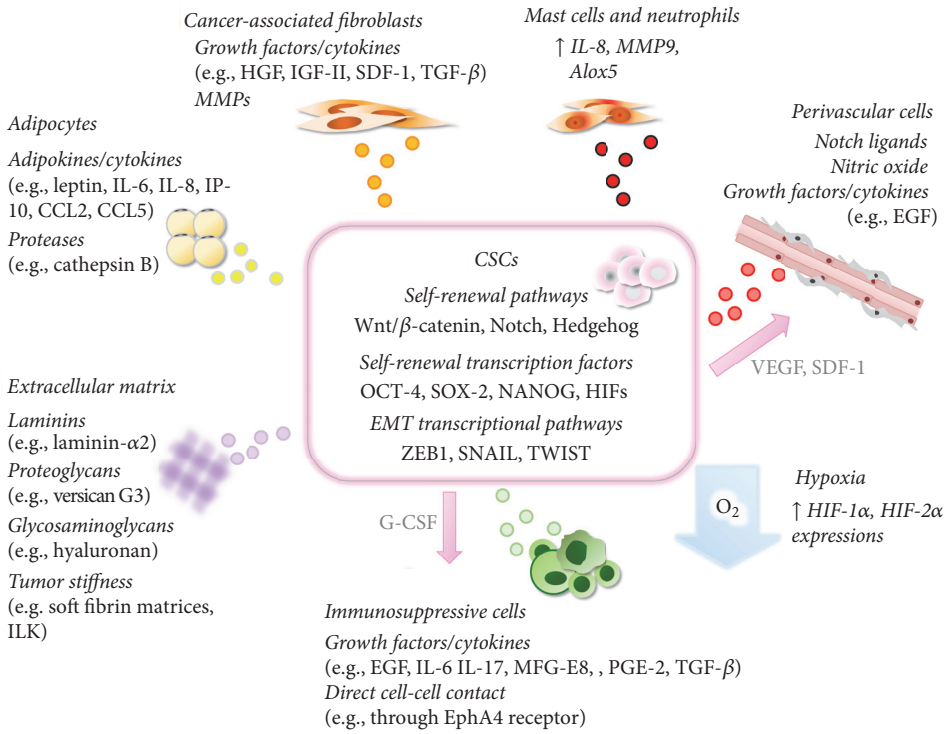

Figure 1: The cross talks between CSCs and their niches. CSCs reside in their habitats, which are specific microenvironments within the tumor consisting of CAFs, masts cells, neutrophils, perivascular cells, adipocytes, ECM, and immune-suppressive cells, as well as hypoxia. By providing various paracrine factors or via direct cell-cell contact, these niches play a crucial role in maintaining CSC plasticity by regulating pathways or transcription factors involved in self-renewal or EMT process. Reciprocally, CSCs can actively recruit some of these niche components to create a microenvironment that is favorable for its survival. For example, CSCs can secrete VEGF and SDF-1 to recruit perivascular cells or produce G-CSF to recruit MDSCs into the tumor microenvironment.

contact that interferes with self-renewal and differentiation pathways. A similar concept applies to CSCs in which a cancer-specific "cancer stem cell niche" is also present and interactions with this niche are essential for maintaining the CSC population. Tumor specific microenvironments comprise stromal cells, immune cells, networks of cytokines and growth factors, hypoxic regions, and the extracellular matrix (ECM) (Figure 1). These environmental factors collectively maintain the stemness of CSCs through altering self-renewal pathways, such as the Wnt/β-catenin, Notch, and Hedgehog pathways, or by interrupting the master transcriptional regulators that sustain embryonic stem cell self-renewal, such as NANOG, OCT-4, and SOX-2 [2, 3]. Furthermore, extensive evidence has revealed that cancers do not strictly follow the CSC model and the actual CSC model is more complex and flexible. Given a specific environmental stimulus, certain cancer cells exhibit plasticity, enabling these cells to reversibly convert from differentiated to a stem-like state through dedifferentiation processes, such as the epithelial-to-mesenchymal transition (EMT) [4]. Considering the essential role of the tumor microenvironment in regulating the CSC phenotype, this review will focus on the recent findings on the molecular mechanisms involved in the cross talk between CSCs and their niches which contributes to maintaining the CSC population.

2. Stromal Cells

2.1. Cancer-Associated Fibroblasts. Cancer-associated fibroblasts (CAFs) are the major components of the tumor stroma [10, 11]. Recent studies have revealed that CAFs are a heterogeneous population, most of which acquire the activated phenotype with increased contractile force, proliferative activity, and enhanced secretion of ECM, proteases, and growth factors. CAFs emerge from multiple origins that widely vary among different cancer types. Several studies have shown that cancer cells could actually secrete signaling molecules, such as basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), and interleukin- (IL-) 6 to "educate" resting fibroblasts to become CAFs [12–15], and in turn, CAFs promote tumor growth and sustain the stemness property of CSCs in a paracrine manner. Through the secretion of hepatocyte growth factor (HGF), CAFs from colon cancer were demonstrated to support CSC properties through the induction of Wnt/β-catenin signaling [16]. More interestingly, the paracrine activation of Wnt/β-catenin signaling by CAFs could restore the stem-like features of non-CSCs, thereby expanding the pool of these cells. Using conditioned media from CAFs, we showed that CAFs from liver cancer promote cancer stemness through the noncanonical induction of the

Notch signaling effector HEY-1 mediated by HGF [17]. A recent study also demonstrated that CAFs in lung cancer induce the expression of the NANOG transcription network through paracrine insulin-like growth factor II (IGF-II)/IGF-1R signaling [18]. EMT is the process where cancer cells acquire a mesenchymal trait and become more invasive and metastatic. Cancer cells that have undergone EMT typically acquire an increased stemness property because some of the EMT-mediating transcription factors, such as Snail and ZEB1, are essential for self-renewal. Several studies have also shown that the activation of EMT could induce the generation of the CSC population [19, 20]. In prostate cancer, CAFs can elicit EMT and increase the stemness properties of cancer cells through the secretion of MMPs [13]. Furthermore, CAFs from breast cancer have been reported to promote the EMT of cancer cells via the secretion of stromal-derived factor 1 (SDF-1) and TGF-β1 [21, 22], providing additional support, suggesting that CAFs play a crucial role in promoting cancer stemness.

2.2. Adipocytes. Obesity is a well-recognized risk factor of several common human malignancies, including breast cancer, colon cancer, and liver cancer [23]. In addition to its epidemic significance, emerging studies have uncovered the functional role of adipose tissues in carcinogenesis and cancer progression, particularly in cancers with adipose tissue constituting a major part of the tumor microenvironment. Adipose tissue primarily comprises adipocytes and a variety of cells that make up the stromal vascular fraction. In addition to its lipid storage function, adipocytes can actively secrete multiple adipokines and cytokines, such as leptin, adiponectin, IL-6, MCP-1, and TNF-α, during excessive adiposity [24]. In addition to its role in lipid homeostasis, many of these adipokines and cytokines are proinflammatory, which attract the infiltration of inflammatory cells, particularly macrophages, causing chronic inflammation to promote cancer growth and metastasis. Furthermore, some of these adipocyte-secreted adipokines/cytokines were directly involved in regulating CSCs. In breast cancer, the expression of leptin receptor is highly upregulated in tumor tissue, particularly in the CSC subpopulation, as driven by the self-renewal associated transcription factors OCT-4 and SOX-2. The secretion of leptin by adipocytes activates the STAT3 signaling in CSCs and induces the expression of OCT-4 and SOX-2, in turn stimulating the expression of leptin receptor, which maintains a self-reinforcing signaling cascade to expand the CSC population and promote tumor growth [25]. Another study showed that the coculture of adipocytes and breast cancer cells stimulates the production of various cytokines that promote cancer stemness through the Src/SOX-2/miR-302b signaling pathway [26]. In prostate cancer, where obesity is associated with a more aggressive phenotype, adipocytes produce cathepsin B (CTSB) upon coculture with prostate cancer cells to support the self-renewal of CSCs [27]. Adipocytes from colorectal cancer are also demonstrated to enhance cancer stemness, and their oncogenic function can be impaired by grape seed extract, a well proven agent with anticolorectal cancer activity, through inducing the "browning" of adipocytes [28].

2.3. Perivascular Cells. Angiogenesis is essential for tumor growth and metastasis. With the excessive production of proangiogenic factors by cancer cells, tumors typically develop disorganized and rich blood vessel networks to meet the high demand on oxygen and nutrients required for tumor outgrowth. CSCs promote tumor angiogenesis. For example, in brain, skin, pancreatic, and liver cancer, the $CD133^+$ CSC populations produce higher levels of proangiogenic factors, such as vascular endothelial growth factor (VEGF) and SDF-1, recruit more endothelial cells, and stimulate more tube formation compared with their differentiated $CD133^-$ counterparts [29–31]. Intriguingly, glioblastoma stem cells, which reside in the perivascular niche, undergo differentiation to generate vascular pericytes and endothelial cells to expand tumor vascularization [32, 33]. Indeed, a mean of approximately 60% of endothelial cells in glioblastoma are derived from neoplastic cells [33]. In turn, CSCs reside in close proximity to the perivascular niche, which provides functional support. Strong evidence suggests that vascular endothelial cells play a key role in maintaining CSCs. In the context of glioblastoma, endothelial cells provide Notch ligands to neighboring CSCs, activating Notch signaling and promoting CSCs self-renewal [34]. In another study, perivascular endothelial cells were demonstrated to activate Notch signaling in glioma stem cells through another soluble factor, nitric oxide [35]. A similar observation was also made in colon cancer, suggesting that endothelial cells secrete the Notch ligand Jagged-1 to promote colon CSC phenotype [36]. A recent study on head and neck cancer also highlighted a role for endothelial cells in regulating CSCs, in which endothelial cells were shown to secrete epidermal growth factor (EGF) to induce EMT and promote cancer stemness [37]. Together, these findings reveal an intriguing reciprocal interaction between CSCs and perivascular cells.

2.4. CSCs and Immune Evasion. Tumor immune escape is a fundamental step for tumor development and the major reason for the failure in cancer immunotherapy. Cancer cells evade the infiltration and the cytotoxic function of natural killer (NK) T cells and $CD8^+$ cytotoxic T cells through various strategies, including the active attraction of immune-suppressive cells, production of immune-suppressive factors, and the activation of "immune checkpoints" that induce anergy or apoptosis in T lymphocytes to downmodulate immune functions [38, 39]. Several studies have revealed that the activation of prosurvival pathways, such as PI3K/AKT, in CSCs not only facilitates escape from conventional chemotherapies but also confers immune evasion [40]. The expression of MHC-I and MHC-II proteins, required for recognition by T lymphocytes to elicit immune responses, is also downregulated in CSCs [41]. In head and neck cancer, the programmed death-ligand 1 (PD-L1), which binds to the programmed death 1 (PD-1) receptor on T cells to suppress its function, is selectively expressed on $CD44^+$ CSCs [42]. Furthermore, it has been well documented that CSCs actively recruit immune-suppressive cells into the tumor microenvironment. In addition to functions in modulating immune cells, these tumor-associated immune-suppressive cells, which mainly include tumor-associated macrophages,

myeloid-derived suppressor cells (MDSCs), T-regulatory (Treg) cells, and NK cells, have been widely demonstrated to support CSCs through multiple pathways.

2.5. Tumor-Associated Macrophages. Macrophages are classified into M1- and M2-polarized subtypes. The M1-subtype secretes inflammatory cytokines and reactive oxygen intermediates and presents antigen to tumor suppressive T cells. However, the M2-subtypes, which are tumor promoting, induce T cell anergy, produce extracellular matrix components, repair damaged tissues, and induce angiogenesis [43–45]. Although the origins of macrophages in many cancers remain uncertain, most of the macrophages recruited to the tumor microenvironment, known as the TAMs, become the tumor supportive M2 subtype [46]. In glioblastoma, glioma CSCs activate the STAT3 pathway to produce cytokines, which recruit and polarize macrophages to become M2-like [47]. After recruitment, TAMs, in turn, serve as a CSC niche to support CSC growth. For example, in breast cancer, the physical interaction between TAMs and CSCs activates the EphA4 receptor on CSCs and the downstream Src and NF-κB pathways, which promote self-renewal [48]. In a murine model of breast cancer, TAMs are also demonstrated to promote CSC phenotypes in breast cancer cells through the EGF-mediated STAT3/SOX-2 cascade, and this cross talk could be abrogated by small molecule inhibitors against EGFR or STAT3 [49]. TGF-β1 and IL-6 are predominantly produced by TAMs in hepatocellular carcinoma (HCC), which induce EMT and activate the STAT3 pathway, respectively, to promote liver CSC properties [50, 51]. Milk-fat globule EGF-8 (MFG-E8), a growth factor identified to involve in phagocytosis and immune suppression [52, 53], is secreted by TAMs to activate STAT3 and Hedgehog pathways that trigger tumorigenicity and drug resistance in CSCs from various cancers [54]. It is clear that the interplay between CSCs and TAMs coordinately regulates tumor progression.

2.6. Myeloid-Derived Suppressor Cells. MDSCs are a heterogeneous population of myeloid-originated progenitor cells. In mice, these cells are characterized as $CD11b^+Gr1^+$, whereas in humans, their phenotype is $Lin^-HLA^-DR^-CD33^+$ or $CD11b^+CD14^-CD33^+$ [55–58]. As the name indicates, the main feature of MDSCs is their function on immunosuppression. MDSCs suppress immune function primarily through multiple mechanisms, including the production of arginase, inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), cyclooxygenase-2 (COX-2), and TGF-β, which together inhibit the proliferation and function of T cells [59, 60]. Recent studies have demonstrated that MDSCs are actively recruited into tumors and these tumor-associated MDSCs play an important role in tumor progression. The recruitment of MDSCs into tumor sites is primarily mediated by various cancer cells that produce chemokines, including CCL2, CCL15, CXCL5, and CXCL12 [61–64]. MDSCs are implicated in multiple stages of tumor progression, particularly the regulation of CSCs. In ovarian cancer, coculture with MDSCs stimulates the expression of miR-101 in cancer cells, which regulates CtBP2 to control the expression of stemness genes, such as NANOG, OCT-4, and SOX-2 [65]. In syngeneic mammary tumor models, CSCs displayed the elevated production of granulocyte colony-stimulating factor (G-CSF), which stimulates the recruitment of MDSCs into the tumor microenvironment. MDSCs reciprocally enhance CSC properties through the activation of Notch signaling [66]. Furthermore, tumor-infiltrated MDSCs, which showed the activation of STAT3 signaling, can enhance the stemness of pancreatic cancer cells through the induction of EMT, with a concomitant increase in the expression of stemness genes, including Snail, Slug, ZEB1, NANOG, and OCT-4 [67].

2.7. T-Regulatory Cells. The fine cross talk between CSCs and immunosuppressive cells also involves Treg cells. Treg cells are defined by the $CD4^+CD25^+FOXP3^+$ T cell subpopulation, with FOXP3 as an important transcriptional regulator of Treg cell development and function [68]. Treg cell-mediated immunosuppression primarily occurs through the production of various cytokines, such as IL-10, IL-35, and TGF-β, direct cell-cell contact via gap junctions, or metabolic disruption in which CD39 and CD73, expressed on Treg cells, facilitate the conversion of ATP to adenosine, which suppresses cytotoxic T cell and/or NK cell activity [69–71]. In tumors, Treg cells are accumulated by various mechanisms, primarily involving chemokine attractions. For example, the chemokines CCL22 and CCL28 are produced by tumor cells to attract CCR4- and CCR10-expressing Treg cells, respectively, leading to the accumulation of Treg cells in various human cancers [72–74]. Indeed, the number of Treg cells inside the tumor microenvironment is associated with clinical outcome. The higher number of Treg cells within the tumor is correlated with poor prognosis in a wide array of cancers, including gastric, esophageal, pancreatic, liver, and breast cancers [75–78]. In addition to its immune-suppressive role, the functional importance of tumor-infiltrating Treg cells in regulating CSCs is starting to emerge. A recent report demonstrated that, under hypoxia, $FOXP3^+$ Treg cells are induced to express IL-17, which drives the expansion of CSCs through the activation of Akt and MAPK signaling pathways in colorectal cancer, evidenced by the increase in the expression of colorectal CSC markers, including CD133, CD44s, and EpCAM [79]. Furthermore, Treg cells produce and secrete prostaglandin (PGE2) for immunosuppression, and PGE2 has been implicated in the regulation of CSC properties in colorectal cancer through NF-κB [80, 81].

2.8. Natural Killer Cells. The ability of natural killer (NK) cells to kill or spare depends on their expression of activating (mostly stress-induced proteins) and inhibitory (in particular MHC class I molecules) ligands on the surface of target cells. Approximately 95% of peripheral blood NK cells are $CD56^{dim}CD16^+$ which exerts strong cytotoxic activity. The remaining 5% of peripheral blood NK cells are $CD56^{bright}CD16^-$ and show cytotoxicity through strong cytokine production. $CD133^+$ glioblastoma stem cells that are able to express high levels of the activating DNAM-1 ligands PVR and Nectin-2 and low levels of MHC class I molecules have been reported to be poorly recognized and lysed by NK cells [82]. Their cytotoxic activity was revamped following IL-2

or IL-15 activation [82]. Breast cancer CSCs have also been reported to fail to express detectable levels of NK ligands, which is consistent with metastatic spread [83]. In melanoma and GBM, CSCs are highly resistant to NK cells and become susceptible to NK cytotoxicity only following stimulation with IL-2 [82]. However, the preferential resistance of CSC to NK cells is not the rule, as colon CSCs express lower MHC class I and higher levels of NK-activating ligands, including NKp30L and Nkp44L as compared to differentiated cells, which are responsible for the CSC susceptibility to NK cell killing [84]. Another mechanism by which cancer cells may evade from the cytotoxic effect of NK cells is the induction of apoptosis in microenvironmental immune cells through the interaction of CD95 (Apo1/Fas) with its ligand (CD95L). Interestingly, CD95R/L regulates CSC plasticity and its blockade reduces CSC in different tumor cell models, while activation of CD95R/L increases CSC number and is responsible for CSC reduced sensitivity to CD95-mediated apoptosis [85]. Collectively, CSCs are more refractory to the cytotoxic effect of NK cells in a variety of cancer types.

2.9. Other Stromal Cells. There is increasing evidence that mast cells (MCs) and their mediators are involved in the remodeling of the tumor microenvironment. Recent evidence has showed that MC regulates stemness of thyroid cancer through IL-8-Akt-Slug pathway [86]. In prostate cancer, MC increased stem/progenitor cell population via altering LncRNA-HOTAIR/PRC2-androgen receptor- (AR-) MMP9 signals [87]. In addition, neutrophils were found to play a crucial role in regulation of CSC populations. Wculek and Malanchi reported that neutrophils induced expansion of breast CSC population marked by $CD24^+CD90^+$, leading to induction of tumor initiation and lung metastasis [88].

3. Hypoxia

Hypoxic microenvironments in tumors result from the rapid growth of cancer cells, which exceeds the limit of blood supply [89]. In response to the hypoxia, the hypoxia-related gene expression is driven through the activated hypoxia-inducible factor (HIF) and transcription factors HIF-1α and HIF-2α that bind to the hypoxia-regulated element (HRE) gene promoters [90–92]. The capacity of HIFs to promote cancer cell stemness has been well documented. Studies have shown that HIFs can increase the expression of stem cell markers in breast cancer [93]. Bae et al. demonstrated that hypoxia can elevate the expression of the stem cell marker SOX2 in prostate cancer cell lines [94]. In addition, the overexpression of HIF-1α has been associated with stem cell marker CD44 in bladder cancer [95]. In addition to HIFs, the hypoxia-mediated overexpression of extracellular carbonic anhydrases, CAIV and CAXII, facilitates cancer cell survival and the maintenance of CSC function [96].

Given that CSC is related to metastasis and cancer cell invasion, the contribution of hypoxia to the enhanced CSC migration has been reported in several studies. The upregulation of EMT-related gene expression under hypoxic stress can enhance the invasiveness and the stem-like properties of cancer [89]. Maeda et al. showed that HIF-1α is correlated with the EMT and cell migration in $CD133^+$ pancreatic CSCs [97]. In addition to cancer cell invasion, hypoxia contributes to drug resistance by maintaining CSCs in a quiescent state to confer resistance to chemotherapeutics that commonly target actively dividing cancer cells [91]. Studies have reported that hypoxia promotes SOX-2-mediated drug resistance in ovarian CSCs via Notch signaling [98]. The downregulation of HIF-1α using a lentivirus-mediated approach can increase the chemosensitivity in triple negative breast cancer [99]. These data demonstrated that hypoxia plays an important role in the CSC niche and is substantially involved in the regulation of cancer cell stemness.

4. Extracellular Matrix

The extracellular matrix (ECM) is a collection of biochemical molecules, including proteins, glycoproteins, proteoglycans, and polysaccharides, which compose the basement membrane and interstitial matrix. In normal tissue, ECM is tightly regulated during development and primarily accomplished by controlling the expression or activities of ECM enzymes at the transcriptional and translational posttranslational levels [100]. Abnormal ECM dynamics are a hallmark of cancer. For instance, various collagens, including collagen I, collagen II, collagen III, collagen V, and collagen IX, show increased deposition in the process of tumor formation [101]. In addition, many other ECM components and their receptors such as heparan sulfate proteoglycans and CD44 are frequently overexpressed in cancer [102, 103]. As one of the major parts of the CSC niche, ECM provides both structural and biochemical support to the CSC and plays a critical role in cancer progression. ECM receptors enable the CSC to anchor in the niche where the stem cell properties could be maintained [104]. In addition, the ECM binds to various growth factors that interact with CSCs to maintain stem cells in a proliferative state. For example, in glioblastoma, the growth of glioblastoma stem cells can be enhanced by ECM protein laminin-α2 [105]. Versican G3, which is overexpressed in breast carcinoma, can inhibit cell differentiation and promote self-renewal, thereby increasing CSC properties [106, 107]. Matrix metalloproteinases that degrade and modify the ECM are upregulated in breast cancer, facilitating the EMT process [108]. Hyaluronan interacts with the cell surface protein CD44, enhancing CSC properties by activating the stem cell marker NANOG [109]. In addition to the stem cell properties, HA-CD44 interactions can also stimulate the overexpression of proteins for multidrug resistance in cancer and CSC [110]. The changes in ECM dynamics may contribute to the disruption of asymmetric stem cell division, leading to CSC overexpansion [111]. When compared with normal tissues, malignant tumors typically are characterized as stiffer due to contraction of collagen in the extracellular matrix by malignant and stromal cells [112]. On single tumor cell level, tumor stiffness was measured by atomic force microscopy mechanical measurement [113]. For in vivo measurement of tumor stiffness, compression and indentation tests were performed on fresh tumor tissues and orthotopic tumors and subcutaneous tumors derived from multiple HCC cell lines [114]. Matrix stiffness in ECM also played crucial role

Table 1: Strategies of targeting the CSC niches for cancer treatment and their respective development status.

Inhibitors/antibodies	Molecular targets	Phases of development	References
	CAFs		
PT630 (FAP inhibitor)	FAP-α	Preclinical	[5]
NK4 (anti-HGF monoclonal antibody)	HGF/MET	Preclinical	[6]
AMG337 (MET kinase inhibitor)	MET	Preclinical	[7]
Rebimastat (MMP inhibitor)	MMPs	Phase II clinical trial	NCT00040755
AMD3100 (CXCR4 antagonist)	SDF-1/CXCR4	Preclinical	[8]
GC1008 (anti-TGF-β monoclonal antibody)	TGF-β	Phase II clinical trial	NCT01401062
	Tumor vasculatures		
Sorafenib (tyrosine kinase inhibitor)	VEGFRs, PDGFRs, KIT	FDA-approved	NDA021923
Sunitinib (tyrosine kinase inhibitor)	VEGFRs, PDGFRs, KIT	FDA-approved	NDA021938
MK0752 (γ-secretase inhibitor)	γ-secretase	Phase I clinical trial	NCT00106145
OMP21M18 (anti-DLL4 monoclonal antibody)	DLL4	Phase I clinical trial	NCT01189968
OMP52M51 (anti-Notch1 monoclonal antibody)	Notch1	Phase I clinical trial	NCT01778439
	TAMs		
PLX3397 (CSF-1R inhibitor)	CSF-1R	Phase I/II clinical trial	NCT01596751
AMG820 (anti-CSF-1R monoclonal antibody)	CSF-1R	Phase I/II clinical trial	NCT02713529
Zoledronate, clodronate, ibandronate	Deplete macrophages	Phase III clinical trial	NCT00127205 NCT00009945
852A (TLR7 agonist)	TLR7	Phase II clinical trial	NCT00319748
Imiquimod (TLR7 agonist)	TLR7	Phase II clinical trial	NCT00899574 NCT00821964
	MDSCs		
Tadalafil (PDE-5 inhibitors)	PDE-5	Pilot study Phase II clinical trial	NCT00843635 NCT00752115
NCX4016 (Nitric oxide-releasing aspirin derivative)	iNOS and arginase	Phase I clinical trial (Prevention purpose)	NCT00331786
L-NAME (arginase inhibitor)	Arginase	Preclinical	[9]
All-trans retinoic acid	Inducing MDSC differentiation	Phase II clinical trial	NCT00617409
	Treg cells		
MEDI6383 (OX40 agonist)	OX40	Phase I clinical trial	NCT02221960
Ipilimumab (anti-CTLA4 monoclonal antibody)	CTLA4	FDA-approved	BLA125377
	Hypoxia		
TH-302 (hypoxia-activated prodrug)	Hypoxia	Phase III clinical trial	NCT01746979
AQ4N (hypoxia-activated prodrug)	Hypoxia	Phase I/II clinical trial	NCT00394628
	ECM		
PEGPH20 (recombinant hyaluronidase)	Hyaluronan	Phase II clinical trial Phase III clinical trial	NCT01839487 NCT02715804

in regulation of CSC plasticity. Tan et al. demonstrated that melanoma CSCs exhibited plasticity in mechanical stiffening, histone 3 lysine residue 9 (H3K9) methylation, *Sox2* expression, and self-renewal. Three-dimensional (3D) soft fibrin matrices promote H3K9 demethylation and increase *Sox2* expression and self-renewal, whereas stiff ones exert opposite effects [115]. More recently, it was found that breast CSC markers are activated synergistically in response to stiff, hypoxic conditions and that ILK is an essential regulator of breast CSCs [116]. The effect of matrix stiffness on CSC marker expression depends on cancer cell's tissue origin [117].

5. Conclusions

Mounting evidence suggests that CSCs are the root of cancers and are responsible for metastasis, resistance to conventional therapies, and tumor relapse. The state and survival of CSCs are controlled by various extrinsic factors derived from the microenvironment where the cells reside. As CSCs have to be

eradicated to prevent disease relapse or metastasis, targeting the niche factors that regulate CSCs represents an attractive therapeutic strategy for cancer treatment. Considering the encouraging results of several preclinical studies for such therapeutic approaches, targeting the CSC niche is clinically feasible [118] (Table 1). A better understanding of CSC biology and the cross talk with its niche might enable the identification of potential therapeutic targets for the development of more effective anticancer treatments.

Abbreviations

CAFs: Cancer-associated fibroblasts
CSCs: Cancer stem cells
CTSB: Cathepsin B
COX-2: Cyclooxygenase-2
EGF: Epidermal growth factor
EMT: Epithelial-to-mesenchymal transition
ECM: Extracellular matrix
bFGF: fibroblast growth factor
G-CSF: Granulocyte colony-stimulating factor
HGF: Hepatocyte growth factor
HIF: Hypoxia-inducible factor
HRE: Hypoxia-regulated element
iNOS: Inducible nitric oxide synthase
IGF: Insulin-like growth factor
IL: Interleukin
MCs: Mast cells
MDSC: Myeloid-derived suppressor cell
NK: Natural killer
PDGF: Platelet-derived growth factor
PD-L1: Programmed death-ligand 1
PGE2: Prostaglandin
ROS: Reactive oxygen species
SDF-1: Stromal-derived factor 1
TGF-β: Transforming growth factor beta
VEGF: Vascular endothelial growth factor.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

The study was supported by the Health and Medical Research Fund (03142736) and Hong Kong Research Grants Council General Research Fund (PolyU 17111614 and 17120215).

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Hindawi Publishing Corporation
Stem Cells International
Volume 2016, Article ID 2059584, 5 pages
http://dx.doi.org/10.1155/2016/2059584

Research Article

Identification of Stem-Like Cells in Atrial Myxoma by Markers CD44, CD19, and CD45

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Received 6 November 2016; Accepted 8 December 2016

Academic Editor: Jijun Hao

Atrial myxoma is the most frequent tumor arising mainly in atrial septum and its origin remains uncertain. It has been reported that a subpopulation of stem-like cells are present in benign tumors and responsible for tumor initiation and maintenance. In this study, we investigated whether stem-like cells could contribute to the atrial cardiac myxoma. Immunohistology data confirmed that a population of cells bearing the surface markers CD19, CD45, and CD44 resided in a mucopolysaccharide-rich matrix of myxoma. Moreover, we isolated myxoma cells with phase-bright culture method and confirmed that myxoma derived cells express robust level of CD19, CD45, and CD44. Furthermore, the pluripotency of this population of cells also was validated by cardiomyocytes and smooth muscle cells differentiation in vitro. Our results indicate that primary cardiac myxoma may arise from mesenchymal stem cells with the ability to generate tumors with multilineage differentiation. In conclusion, this study for the first time verified that stem-like cells are present in atrial myxoma and this population of cells may have the capacity for myxoma initiation and progression.

1. Introduction

Cardiac myxoma is the most frequent tumor of the heart [1]. It is characterized by myxoid appearance of a mucopolysaccharide-rich extracellular matrix and appears to be qualitatively distinct from different cases of histopathology. Previous studies indicated that myxoma cells arise from remnants of subendocardial vasoformative reserve cells or multipotential primitive mesenchymal cells in the fossa ovalis and surrounding endocardium, which can differentiate into a variety of cell lineages including endothelial, fibroblastic, hematopoietic, glandular, neurogenic, and smooth muscle cells [1–3]. Histopathology analysis data revealed that myxoma is mainly composed of stellate, fusiform, or elongated cell incorporated into myxoid matrix [2] and phenotypic characterization studies indicate that the origin of myxoma is from pluripotential cells [1].

The histogenesis of cardiac myxoma remains unclear and different cell phenotypes, including epithelial, endothelial, myogenic, myofibroblastic, and neural cells, were observed in myxoma by immunohistology [4]. Recent studies have indicated that cancer stem cells, a type of cancer cell that can self-renew and differentiate into multiple cell types, are responsible for tumor initiation, recurrence, and metastasis [5, 6]. Though the malignant potential of cardiac myxoma remains doubtful, a possible explanation for histogenesis of myxoma is that a population of cells with pluripotency is the origin.

It is believed that the myxoma cells are from primitive multipotent mesenchymal cells [7]. To date, a number of putative markers for cancer stem cell have been reported and CD44 is the most common cancer stem cell surface marker [5]. Adult cardiac stem cells in the myocardium have been identified using a variety of approaches, including physiological properties such as the ability to form multicellular spheroids [8]. The pattern of protein and gene expression indicates that myxoma cells may be phenotypically similar to a more primitive cardiac progenitor or primordial cardiac stem cells [1]. In this study, we isolated myxoma cells by sphere-forming methods. Moreover, the expression of cancer stem cell marker CD44 and the differentiation of myxoma derived cells were investigated.

2. Materials and Methods

2.1. Tissue Collection. Atrial myxomas were obtained from surgical specimens from the Department of Cardiothoracic Surgery, TEDA International Cardiovascular Hospital, Tianjin, China. Three patients, all female, were 52, 55, and 56 years old, respectively. And all participants gave written informed consent.

2.2. Histological Analysis. To investigate the histology of myxoma, samples were fixed in 4% paraformaldehyde, cut transversely, embedded in paraffin, and stained with hematoxylin and eosin (H&E). To carry out immunohistology, atrial myxomas were embedded into OCT compound (Miles Scientific) and cut into transverse sections at 5 μm thickness. To explore if myxomas are CD19, CD45, and CD44 positive, anti-CD19, anti-CD45, and anti-CD44 antibodies (all from BD Pharmingen, Mountain View, CA) were used. Alexa Fluor 488 and Alexa Fluor 594-conjugated secondary antibodies were applied appropriately (Invitrogen, Carlsbad, CA). DAPI was used for nuclear counterstaining.

2.3. Cell Cultures. To investigate if myxoma cells can be cultured, tissues were cut into 1 to 2 mm piece, washed with Hanks' balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA), and incubated with 0.1% collagenase II for 30 minutes at 37°C with frequent shaking [8]. Cells were then filtered through 100 μm mesh. The obtained cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 0.1 mM nonessential amino acids, 100 U/mL Penicillin G, 100 μg/mL streptomycin, 2 mmol/L glutamine, and 0.1 mmol/L β-mercaptoethanol [8]. After 2 to 3 weeks, a population of phase-bright cells appeared over the adhered fibroblast-like cells. These phase-bright cells were collected by two washes with PBS and one wash with cell dissolution buffer (Gibco, Grand Island, NY) at room temperature under microscope monitoring and subcultured with the same medium [8]. For cardiac and smooth muscle differentiation, myxoma derived cells were cultured under conditions as previously described [8, 9].

2.4. Flow Cytometry Analysis. Flow cytometry analysis of the myxoma derived cells and the subcultured cells was carried out. Antibodies used in this study were phycoerythrin (PE) conjugated anti-CD44 and allophycocyanin (APC) conjugated anti-CD45 and anti-CD19 (all from BD Pharmingen). The stained cells were analyzed using FACS LSR (Becton-Dickinson, MA). Dead cells stained by propidium-iodide (PI) were excluded from the analysis. Isotype-identical antibodies served as controls (BD Pharmingen). FlowJo software (Tree Star Inc., Ashland, OR) was used for followed data analysis.

3. Results and Discussion

3.1. Pathology of Atrial Myxoma. Histological analysis of the atrial myxoma was performed by both hematoxylin and eosin (H&E) staining and via immunofluorescent microscopic examination. H&E staining revealed that myxoma cells dispersed in myxoid matrix within myxomatous areas (Figure 1(a), Supplemental Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/2059584). Previous studies have revealed that the myxoma arises from a multipotential endocardial or subendocardial reserve mesenchymal cell which can differentiate into fibrocytes, myocytes, or endothelium [10]. To date, a number of putative markers for mesenchymal stem cells have been reported, including CD44 [11]. Our immunohistology data confirmed the expression of CD44 in myxoma. As a widely used cancer stem cell marker, CD44 expression indicates the malignant potential of cardiac myxoma [12]. It has been reported that myxomas are mainly composed of cellular elements including a variable number of blood cells [2] and amorphous myxoid matrix structures [13]. Moreover, cells in myxoid matrix rich lymphoma were more likely to express pan-B cell marker CD19 and the common leukocyte antigen CD45 [14]. And our further histology results confirmed the robust expression of CD45 and CD19 in myxoma (Figure 1(b), Supplemental Figure 1).

3.2. Isolation and Culturing of Myxoma Cells. Previous results revealed that myxoma cells express series transcription factors specific to phenotype of primitive cardiomyocytes and suggested that the development of cardiac myxoma originates from multipotential mesenchymal progenitors with a cardiomyogenic lineage [2]. We have described the isolation of cardiac stem cells that grow as phase-bright cells from murine hearts and similar methods were applied to isolate myxoma cells [9]. Explanted atrial myxoma was subjected to enzymatic digestion, and cultured phase-bright spherical cells that spontaneously separated from the myxoma samples were identified after 2 to 3 weeks (Figure 2(a)). These cells demonstrated a high nucleus-to-cytoplasm ratio (Figure 2(a), III). Flow cytometry analysis was performed to further characterize their cellular phenotypes and results revealed that this population of cells had higher expression of mesenchymal stem cell marker CD44 and pan-B cell marker CD19 and hematopoietic cell marker CD45. Cell proliferation analysis demonstrated linear growth with population doubling time

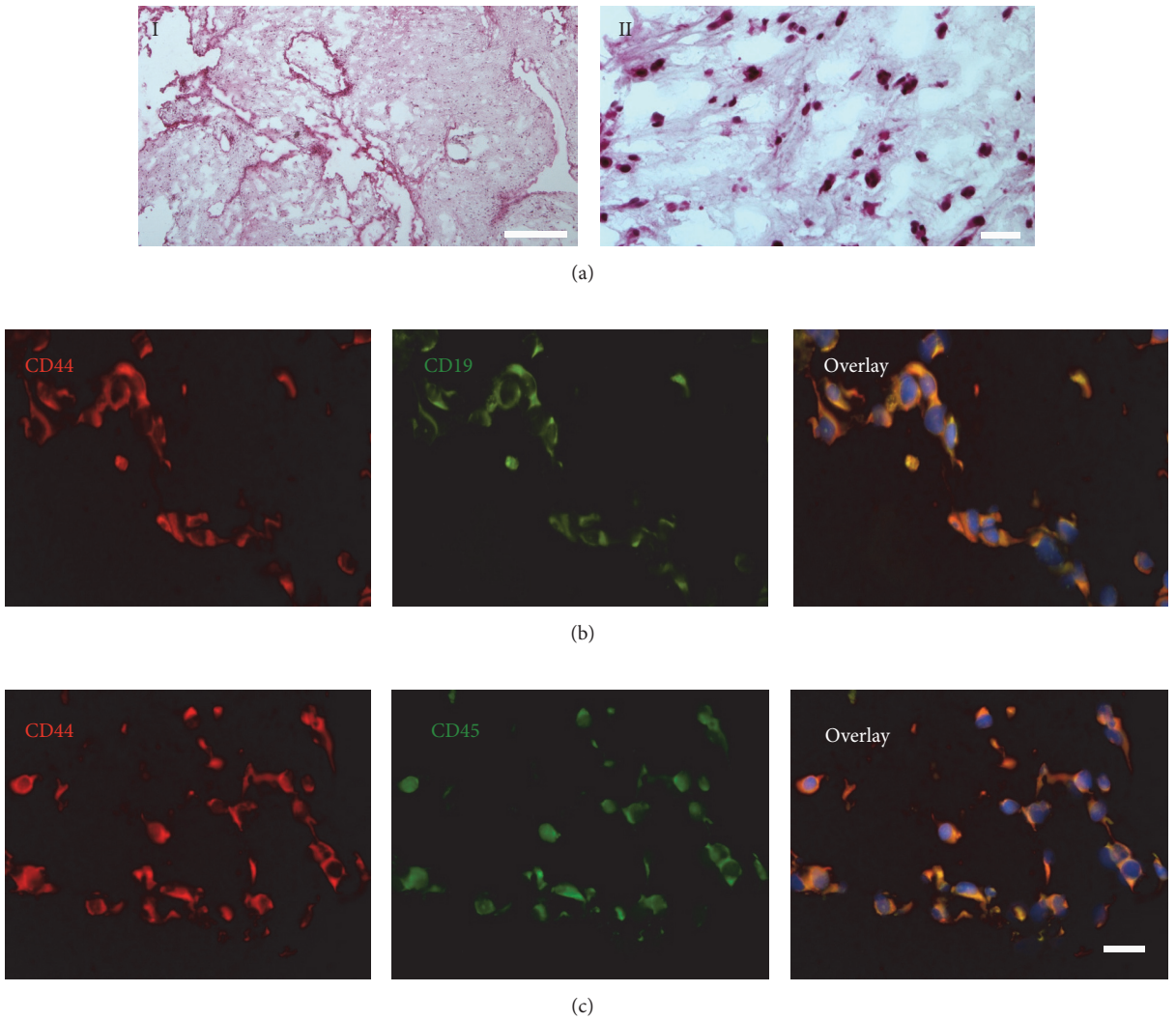

Figure 1: Phenotypic characterization of atrial myxoma. (a) Typical morphological features of myxoma by hematoxylin and eosin (HE) staining. Myxoma cells were dispersed within abundant myxoid matrix. Scale bar = 100 μm (I), =20 μm (II). (b) Myxoma cells were CD44 and CD19 double positive as confirmed by immunostaining. (c) Immunohistochemical characterization of myxomas revealed that myxoma cells were CD44 and CD45 double positive. Scale bar = 20 μm (b & c).

of approximately 7 days (data not shown). Similar results were obtained from all three samples.

3.3. In Vitro Differentiation of Myxoma Derived Cells. To investigate the multipotent differentiation of cardiac myxoma stem cells, we examined the differentiation of myxoma cells into various cell types. Similar to cardiac resident stem cells, these cells can differentiate into cardiomyocytes and smooth muscle cells, as documented by positive staining for cardiac troponin T (cTnT), connexin 43, myocyte enhancer factor 2C (MEF-2C), and α-smooth muscle actin (α-SMA) (Figure 3), similar to previous studies [1].

In this study, cardiac myxoma cells residing in a mucopolysaccharide-rich matrix were characterized in myxoma as a population of cells bearing the surface markers CD19, CD45, and CD44, which indicates these cells originating from hematopoietic cells with mesenchymal stem cell characterization. Moreover, we isolated myxoma cells with phase-bright culture method and confirmed that myxoma derived cells express robust level of CD44, CD19, and CD45. Furthermore, the pluripotency of this population cells also was verified by cardiomyocytes and smooth muscle cells differentiation in vitro.

Cancer stem cells are cancer cells that possess characteristics associated with normal stem cells, specifically the ability to give rise to heterogeneous cell populations in tumor [5]. It has been reported that tumor stem-like cells exist in benign tumors [15]. Important insights into cardiac myxoma tumorigenesis may come from studies of myxoma development and cell differentiation. Our results on identification of stem-like cells from myxoma suggested

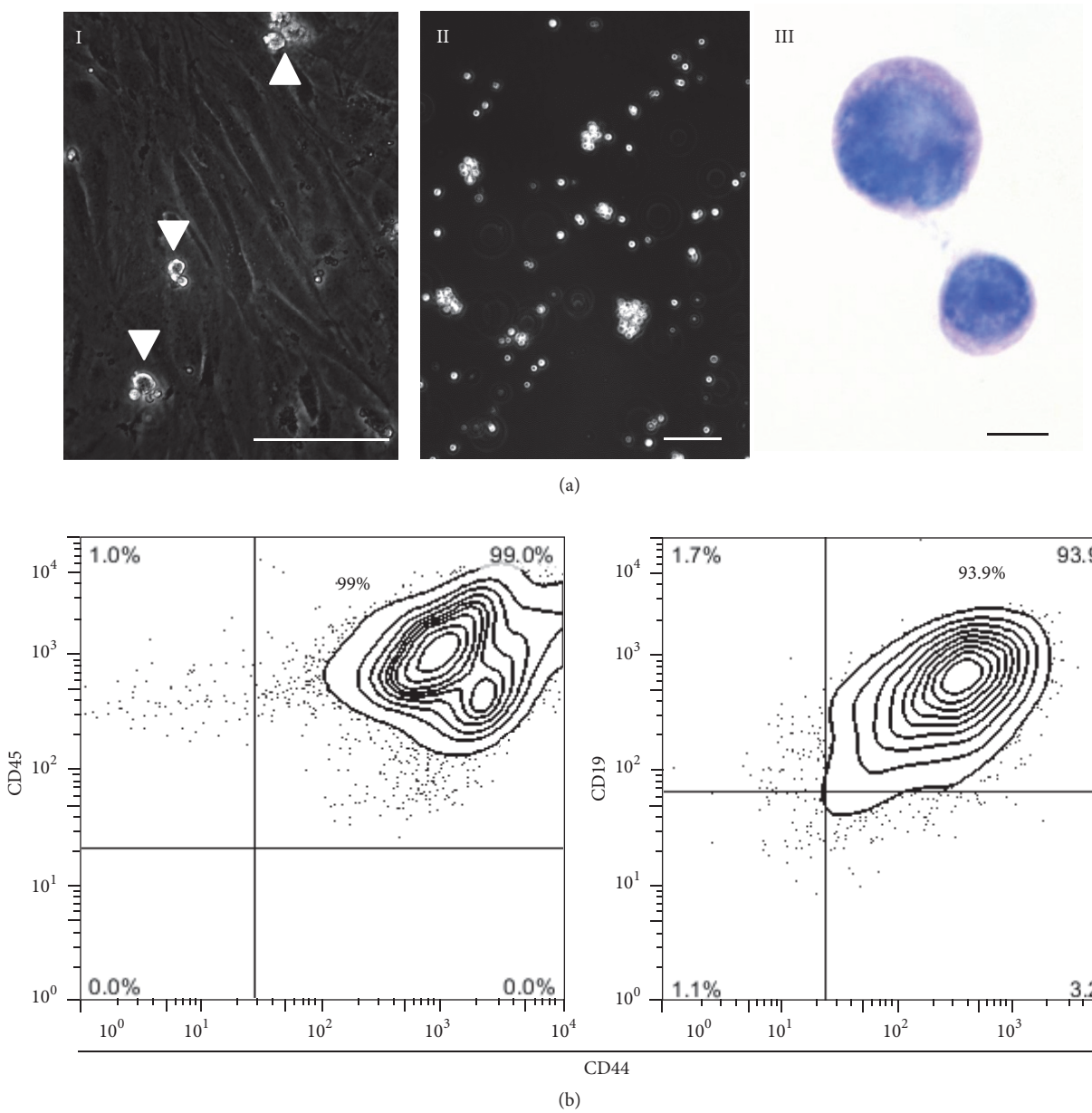

Figure 2: Characterization of subcultured myxoma cells from atrial myxoma. (a) After a period ranging from 1 to 3 weeks, phase-bright (arrow head) cells migrated over a layer of fibroblast-like cells (I, II). The phase-bright cells were collected, and Giemsa stain showed the cell with a large nucleus (III). Scale bar = 100 μm (I & II), =4 μm (III). (b) Quantification by FACS analysis of myxoma derived phase-bright cells. This cell population expresses robust CD44, CD19, and CD45.

that stem-like cells as tumor-initiating cells may be a general theme in these benign tumors.

In conclusion, our study is the first to isolate and phenotypically and functionally characterize cardiac myxoma stem-like cells by markers CD44, CD19, and CD45. In the meantime, we also verified their multipotent differentiation capabilities. This study may contribute to the elucidation of cardiac myxoma carcinogenetic mechanism and provides new insights for myxoma researches.

Competing Interests

The authors declare no competing financial interests.

Authors' Contributions

Xianghe Song and Danni Liu contributed equally to this work.

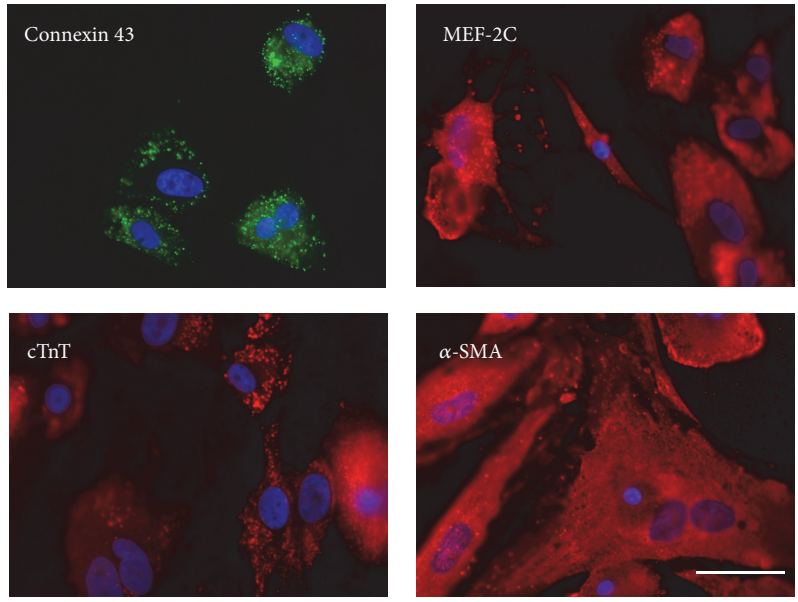

Figure 3: Multipotent capacity of myxoma derived cells. Immunostaining of subcultured myxoma derived phase-bright cells with connexin 43, myocyte enhancer factor 2C (MEF-2C), cardiac troponin T (cTnT), and α-smooth muscle actin (α-SMA). Scale bar = 10 μm.

Acknowledgments

This work was partially supported by grants from the National Natural Science Foundation of China (81371620, 81573089, and 81671734) and Program for Changjiang Scholars and Innovative Research Team in University (IRT13023).

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