Cancer Metastasis and Cancer Stem Cells

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Abstract: Increasing amounts of evidence have indicated the important role of cancer stem cells (CSCs) in tumorigenesis and relapse. Metastasis is a key biological characteristic of malignant tumors. How cancer cells spread from the original tumor into the circulation and then infiltrate distant organs remains a topic of debate. Moreover, understanding the differences between metastasized and non-metastasized cancer cells is the key to develop strategies to block metastasis. In this review, we summarized the development of the CSC theory related to tumor metastasis over the last two decades.

Keywords: CSCs, cancer metastasis, immune escape, angiogenesis, drug resistance, cancer therapy.

THE MAIN STEPS OF TUMOR METASTASIS

Metastasis is a biological characteristic of malignant tumors and is an important reason for poor prognosis. As the fatal step in the progression of solid tumors, metastasis includes several fundamental biological processes: tumor initiation, epithelial-mesenchymal transition (EMT), basement break down, invasion into the stroma (local invasion), entering the blood circulation (intravasation), and dissemination to distant organs. Changes in various genes and cytokines have been identified as a foundation for tumor metastasis. MTA1 is a component of nucleosome remodeling and histone deacetylase (NuRD), and its high expression is closely related to tumor metastasis. Yi *et al*. [1] analyzed 48 ovarian carcinoma specimens and found that the frequency of MTA1 overexpression was 100% in primary ovarian carcinoma and 87.5% in lymph nodes with metastasis, but 38.5% in primary tumors and 23% in lymph nodes without metastasis. Therefore, the study of tumor metastasis has become increasingly important and may yield strategies to prevent tumor metastasis.

THEORIES ON TUMOR METASTASIS

"Seed and Soil" Hypothesis

Paget first proposed the "seed and soil" hypothesis in 1889, which suggests that the successful growth of metastatic cells depends on the interactions and properties of cancer cells (seeds) and their target organ microenvironment (soil) [2-3]. He hypothesized that cancer cells metastasize to locations that are biochemically and physiologically favorable for implantation and growth [2]. Although many findings support this hypothesis, our understanding of the mechanisms that drive this phenomenon remains incomplete. It has been known that lung, breast and melanoma cancers, among others, can metastasize to the brain, while ovarian, liver, and gastric cancers do not metastasize to the brain [4-8]. In other words, tumors only metastasize when the appropriate "seeds" and "soils" are matched. The attention has been focused on circulating tumor cells (CTCs) and found that CTCs seem to survive in the "soil" of the target organ for colonization and subsequent growth, which could constitute strong evidence to explain the occurrence of brain metastasis. Several researchers have shown that the hydrogen peroxide generated by cancer cells and cancer-associated cells may act as a "fertilizer" for cells, driving accelerated aging, inflammation, DNA damage and metabolism [9]. Conversely, the malignant tumor "seed" affects the "soil" to form a vicious cycle and promote tumor metastasis. The latest report indicates that mesenchymal-like breast cancer cells are more likely to activate macrophages to a tumor-associated macrophage (TAM)-like phenotype *via* granulocyte-macrophage colony-stimulating factor (GM-CSF) than non-mesenchymal-like breast cancer cells. Moreover, TAMs secrete Chemokine (C-C motif) ligand 8 (CCL8) to induce cancer cell EMT, which forms a positive feedback loop *in vitro* and *in vivo*. Inhibiting GM-CSF and CCL18 can break the loop and reduce tumor metastasis [10]. These findings confirm mutual influence of the tumor "seed" and growth "soil", providing an experimental basis for developing new targeting drugs against the tumor microenvironment. This hypothesis has provided a general direction for tumor therapy, suggesting that both the tumor cells themselves and the microenvironment may play important roles in future treatment strategies.

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Epithelial-Mesenchymal Transition (EMT) and Metastasis

EMT is known as a crucial event in tumor metastasis by which cells lose epithelial properties while acquiring mesenchymal-like properties, which promotes tumor cells invasiveness and migration [11]. EMT has been suggested to be a late stage event in malignant tumor development and plays an important role in promoting metastasis in epithelium-derived tumors. The role of EMT in tumor metastasis has been a topic of research. EMT is rooted in extracellular matrix (ECM) degradation and ECM proteins re-synthesis [12]. The activation of EMT is thought to allow tumor cells to lose cell-to-cell junctions and dissociate from each other for single cell migration and invasion [13]. This process is characterized by a switch from the expression of epithelial to mesenchymal markers, including three families of transcription factors, SNAI1/2, zinc finger E-box binding homeobox (ZEB)1/2, and TWIST1/2, which could increase the invasiveness of epithelial cells. Putz *et al*. [14] collected tumor cells from prostate, colon, and breast cancer bone metastases and found that all of these cells expressed EMT markers, which confirmed the occurrence of EMT in tumor metastasis. E-cadherin is an important cell adhesion molecule in the calcium mucin family. A reduction or loss of E-cadherin expression is the most notable feature of EMT and can be used as a clinical prognostic indicator of tumor metastasis. E-cadherin has also been identified as a direct target of Snail [15]. Snail, a transcription repressor during EMT, directly represses E-cadherin promoter activity and induces epithelial cells to lose their polarity [16]. Ikenouchi *et al*. [17] studied the relationship between Snail and the promoters of claudins and occludins and found that the integral membrane proteins located at tight junctions maintain epithelial cell polarity. Finally, EMT was induced with a concomitant repression of the expression of claudin and occludin when Snail was overexpressed. Transforming growth factor (TGF)-β is a type of complex functional cytokine and plays an important role in promoting tumor metastasis during tumor progression. Krueppel-like factor 8 (KLF8) is a downstream transcription factor of TGF-β1 and has an important role in EMT induction. TGF-β1 has been shown to induce EMT by down-regulating E-cadherin and up-regulating vimentin expression, while KLF8 is regulated by TGF-β1 and can block TGF-β1-induced EMT when silenced [18]. Li *et al*. [19] demonstrated that the down-regulation of B-cell-specific moloney murine leukemia virus insertion site 1 (Bmi1) restores E-cadherin expression and cell-to-cell junctions in breast cancer cells, while the up-regulation of Bmi-1 decreases E-cadherin expression while increasing N-cadherin and vimentin expression, which are mesenchymal markers that could promote EMT development. Because the EMT is a process of cell dedifferentiation, numerous findings showed that EMT may be related to CSCs generation and the emergence of the CSCs phenotype [20]. Mani *et al*. [21] first demonstrated the direct link between EMT and CSCs, which showed that the induction of EMT in epithelial cells resulted in the acquisition of mesenchymal traits and expression of stem cell markers. In recent years, Zhang *et al*. [22] demonstrated that endothelial cell-secreted epidermal growth factor (EGF) induces EMT and bestows human head and neck tumor cells with stem-like characteristics. Zhao *et al*. [23] investigated the effect of Nestin on proliferation, survival and metastasis and surprisingly found that Nestin silencing could inhibit CSCs metastasis by suppressing the EMT phenotype in breast cancer stem cells. In conclusion, EMT governs the sensitivity of oncotherapy, and EMT inhibition may be able to reduce or eliminate metastatic tumor cells.

Tumor Dormancy and Metastasis

Clinically, tumors recur in distant organs, and these tumors frequently cannot be detected until years to decades after the primary tumor diagnosis. It has inferred that some of these tumor cells may not have immediately begun to proliferate after disseminating to the metastatic target organs, living in a dormant state and forming no metastasis for many years. This process is referred to as “tumor dormancy” [24]. Breast cancer can recur after the removal of the primary tumor and treatment to eliminate remaining tumor cells, and tumor dormancy can explain this phenomenon. Disseminated estrogen receptor-positive tumor cells carrying a dormancy signature undergo a prolonged dormancy period before resuming metastatic growth [25]. Therefore, dormant tumor cells may form new metastatic foci rapidly after reactivation, leading to a poor prognosis. The mechanisms of tumor dormancy remain poorly understood. One theory is cellular dormancy, which refers to a down-regulation of uPAR (metastasis-associated urokinase receptor) and inactivation of integrin α5β1, decreases the phosphorylation of extracellular signal-related kinase (ERK) and the activation of p38 protein [26]. Another may be the micrometastasis dormancy theory, which includes angiogenic dormancy and immunologic

mechanism. Angiogenesis is an essential step in tumor metastasis. In recent years, it has been proposed that angiogenic dormancy restricts the revascularization and prevents micrometastases from developing into macrometastases. A study tested the angiogenesis factors in dormant cancer cells and found that 6 of 15 were up-regulated, such as tissue inhibitor of metalloproteinases 3 (TIMP3), Cadherin1 (CDH1), and thrombospondin 1 (TSP1). They also suggested that the addition of vascular endothelial growth factor (VEGF) could maintain tumor cells in a dormant state [27]. Romero *et al*. [28] used a non-transgenic mouse model to investigate the mechanisms involved in dormant metastasis and found that when tumor-bearing mice were immune-depleted of T lymphocytes or asialo GM1-positive cells, growth limitation of dormant disseminated metastatic cells was relieved and lung metastases progressed. Therefore, an immunologic mechanism may underlie tumor dormancy, and this model system may be valuable for more in-depth analyses of metastatic dormancy. Metastatic cells undergoing reactivation result in specialized ECM niches that support positive signals, such as Wnt and Notch, and decrease negative signals, such as BMP [29]. One study aimed to investigate the potential role of ECM in tumor dormancy, and the results showed that the number of cells in the S-phase was reduced when cells were cultured in an ECM-adherent environment, and the migration ability of cells significantly reduced after release from the ECM gel. These findings all suggest a crucial role for ECM in tumor dormancy and provide a useful *in vitro* model system to thoroughly study the molecular mechanisms of tumor dormancy [30]. Therefore, tumor dormancy may be a novel target therapy for tumor treatment in the future.

CSCs and Metastasis

In the 1970s, it was found that a fraction of cancer cells could form colonies in soft agar. However, they only studied the behavior of CSCs from metastatic sites; they thought that CSCs from the primary site would also be important and perhaps clarify the metastatic process [31]. By the late 1990s, Bonnet *et al*. [32] first identified CSCs in hematopoietic malignancies and proposed the concept of CSCs. In 2003, Al-Hajj *et al*. [33] isolated the CD24+CD44- breast cancer stem cells. These findings indicate that CSCs exist in solid tumors and suggest that CSCs may be the root cause of tumorigenesis. Like stem cells (SCs), CSCs have the capacity to self-renew and are pluripotent. In addition, CSCs also have the same biological characteristics as tumor cells. The chromosome of CSCs is polyploid, and CSCs are highly tumorigenic and metastatic after inoculation. These findings all suggest that CSCs account for tumorigenesis and metastasis (Figure **1**).

Tumor metastasis, which is considered the result of the selective cloning of tumor cells, is the result of a very small fraction of millions of tumor cells. This finding suggests that tumor cells that leave the primary tumor and form metastases have the stem cell properties. Chen *et al*. [34] injected gastric adenocarcinoma (GAC) CSCs (GCSCs) and peripheral blood from GAC patients into the immunodeficient mice and surprisingly found that the generated tumors strongly resembled the original tumors. Subsequently, the GCSCs captured from the circulating system showed that tumor metastases may be due to CSCs.

The Metastatic Microenvironment of CSCs

It was known that the stem cells microenvironment is closely related to the proliferation and differentiation of stem cells and directly or indirectly decides the fate of stem cells. Like the microenvironment of SCs, CSCs also have a unique microenvironment that maintains the characteristics of CSCs, such as their ability to self-renew and pluripotency. The CSC microenvironment contains various stromal cells, such as mesenchymal and immune cells, ECM, a vascular network, and many factors derived from microenvironmental cells. Evidence has shown that mesenchymal cells can regulate breast CSCs through cytokine loops by involving interleukin (IL)-6 and the chemokine Chemokine (C-X-C motif) Ligand (CXCL7) [35]. Regarding the ECM, Malanchi *et al*. [36] showed that periostin, a component of ECM,secreated by fibroblasts, is needed in the secondary target organ to initiate colonization, that is to say, the stromal niche signals are crucial for CSCs during the secondary expansion process. Zhu *et al*. [37] found that hypoxia-inducible factor (HIF)-1α and autophagy can induce non-stem pancreatic cancer cells to differentiate into the pancreatic stem cells. The vascular microenvironment is also crucial for CSCs survival and development. One study demonstrated that endothelial cells induce sonic hedgehog homolog (Shh) to activate the Hedgehog pathway in glioma cells, which promotes CSCs properties and glioma propagation [38]. Recently, a report suggested that cytokines secreted by tumor associated cells, such as hepatocyte growth factor (HGF) and stromal cell-derived factor-1 (SDF-1), increased CD44v6 expression in colorectal stem cells,

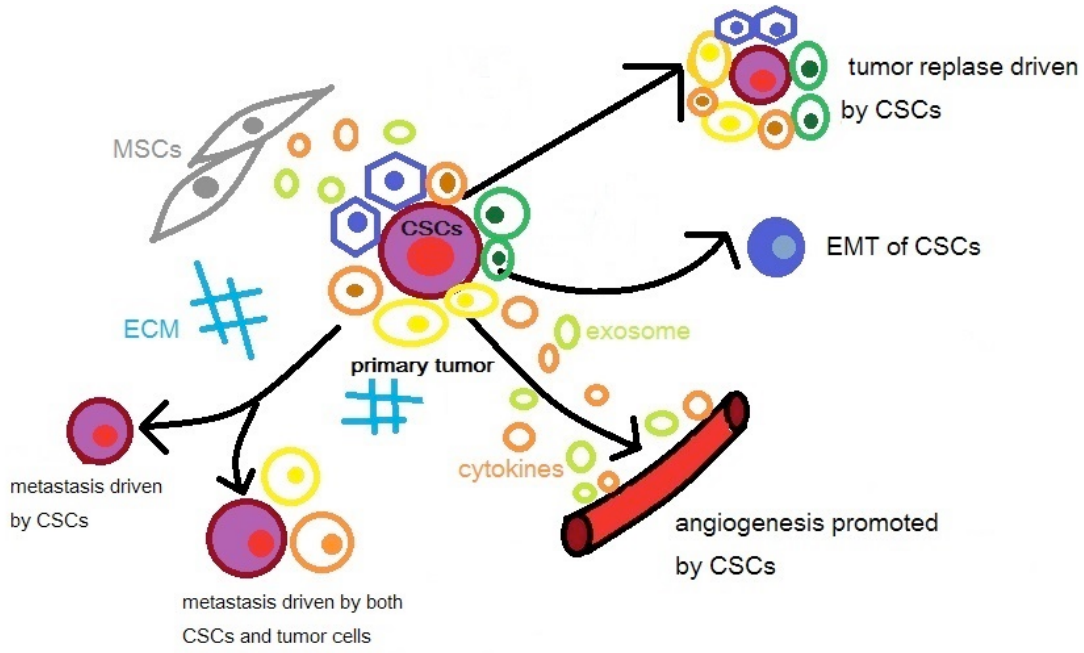

Figure 1: The potential contribution of CSCs to tumor metastasis.

CSCs in primary tumor can metastasize to distant tissues directly or contribute to cancer metastasis by mediating EMT, angiogenesis, and drug resistance, etc.

which could strongly promote migration and metastasis [39]. Thus, the microenvironment of CSCs is essential for CSCs and affects the biological characteristics of CSCs in many ways. Targeting the CSC microenvironment may be a new direction for cancer therapy in the future.

Metastasis of CSCs Characterized by Different Markers

Emerging evidences suggest that CSCs are connected to metastasis. As such, CSCs may be the origin of metastasis. Aldehyde dehydrogenase (ALDH) enzymes are considered aldehyde scavengers that eliminate toxic aldehydes [40]. Recently, ALDH has been identified as a reliable CSCs marker in various cancer types. Charafe *et al*. [41] used the ALDEFLUOR assay to isolate CSCs from human breast cancer cell lines and injected them into NOD/SCID mice, finding that ALDH+ CSCs are responsible for mediating metastasis. Brain metastases are very common in adults with lung cancer. Nolte *et al*. [42] showed that the stem-like population in brain metastases originated from the lung. Brain metastatic tumor spheres not only expressed stem cell markers but also possessed the original patient tumor heterogeneity. Niess *et al*. [43] also demonstrated that CSCs are responsible for metastasis in pancreatic cancer using a Hoechst 33342 and orthotopic xenograft. The results demonstrated that the CSCs are highly metastatic after orthotopic injection. EMT is one of the main steps in tumor metastasis. Evidence showed that CD24+ CSCs are susceptible to EMT induction because of the expression of CSCs markers, such as TGF, which is an effective EMT inducer [44]. Accordingly, specific stem cell features, such as the migration into diseased areas, open new avenues towards the development of tracking and delivering anti-cancer drugs to the disseminated metastatic lesions [45].

MicroRNAs, CSCs and Metastasis

MicroRNAs are small non-coding regulatory RNAs that can regulate gene expression [46]. Recent studies suggest that microRNAs may play important roles in CSCs metastasis. Huang *et al*. [47] found that the up-regulation of miR-888 could increase the potential migration and invasion of breast CSCs. In contrast, CSCs could metastasize to the bone and brain when expressing significantly lower levels of miR-7 [48]. Increasing amounts of miRNAs have been identified in EMT, suggesting that miRNAs may connect CSCs to metastasis by regulating EMT [49]. The down-regulation of miR-21 by antagonists reversed the EMT, decreasing the expression of HIF-1α and suppressing the invasion and migration, which indicates a potential role in regulating CSCs-associated metastasis [50]. Evidences showed that miRNAs may regulate the cancer metastasis of CD133(+) glioma stem cell (GSC) by targeting tissue inhibitor of metalloproteinases-2

(TIMP-2). Down-regulation of miR-20a/106a increased endogenous TIMP-2 protein level and inhibited GSC invasion. Thereby, miRNAs may be a potential therapeutic target in CSCs metastasis in clinical scientific research in the future.

CSCs AND ANGIOGENESIS

Tumor cells alone are not sufficient to engender distant metastasis; they need the assistance of blood and lymph vessels to transport malignant cells. A number of studies have indicated that CSCs may promote angiogenesis, while the vascular microenvironment could maintain and even induce cells to transform into Scs. Fantozzi *et al*. [52] reported that EMT up-regulated the expression of the pro-angiogenic factor VEGF-A and increased tumor angiogenesis in murine breast cancer stem cells. Other strong evidence has also been reported in human cancers. Cao *et al*. [53] sorted and analyzed the SP cells, which represent putative CSCs, surprisingly finding that SP cells overexpressed VEGF-A, VEGF-B, FGF-2, etc., which could induce the migration of human umbilical vein endothelial cells. In a CD133(+) liver tumor initiating cell (TIC) study, Tang *et al*. [54] found that CD133(+) liver TICs can promote angiogenesis by regulating nucleus tractus solitarii (NTS), IL-8, CXCL1, and Mitogen-activated protein kinase (MAPK) signaling. In addition, Hypoxia and the interaction between chemokine receptors and ligands play regulatory roles in angiogenesis for CSCs. The transcriptional activity of HIF-1α and β-catenin were enhanced in prostate CSCs and EMT had taken place, which maintained angiogenesis and selected an invasive and metastatic phenotype [55]. Ping *et al*. [56] studied CXCL12 and its receptor C-X-C chemokine receptor type 4 (CXCR4) in GSC and found that the expression levels of CXCL12 and CXCR4 were higher in CD133(+) GSC than in CD133(-) GSC. Moreover, CXCL12 and CXCR4 can induce VEGF expression, which could promote angiogenesis. Further studies should be conducted to confirm the mechanism of tumor migration and invasion and provide guidance for anti-angiogenic therapy.

CSCs AND IMMUNE ESCAPE

Immune escape is a hallmark of tumor progression and relapse. CSCs may play an important role in this process. As the origin of tumorigenesis, CSCs can evade immune surveillance *via* a variety of effective mechanism, such as changing surface antigen expression, the expression of immune escape related genes, or a low level of some cytokines that can suppress the immune system to help CSCs escape recognition and attack from the immune system [57]. Wu *et al*. [58] investigated CD133+ brain CSCs in astrocytoma and glioblastoma multiforme samples and found that CD133+ brain CSCs do not express major histocompatibility complex (MHC) I or natural killer (NK) cell-activating ligands, which allows CSCs to escape immune surveillance. CD44 is a surface marker of CSCs in the head and neck squamous cell carcinoma. Chikamatsu *et al*. [59] demonstrated that CD44+ CSCs express rare human leukocyte antigen (HLA)-A2, class II and transporter antigen processing (TAP) 2 to escape immune attack. Furthermore, CD44+ CSCs also expressed a variety of cytokines to induce immune tolerance, such as interleukin (IL)-8, granulocyte colony-stimulating factor (G-CSF), and transforming growth factor (TGF)-β. In general, the reason why tumor cells escape from the host immune system may be that CSCs could specifically tolerate current immunotherapy. Therefore, novel immunotherapeutic strategies need to be developed to overcome CSCs-derived immune suppression.

CSCs AND DRUG RESISTANCE

Since research showed that ALDH(high) CD44(+) CSCs tolerate cisplatin, It has been known that CSCs are resistant to chemotherapy and radiation [60]. This resistance could be due to the following reasons: (1) CSCs may live in a dormant state and without proliferation. (2) CSCs can effectively repair DNA damage. (3) CSCs can highly express the multi-resistant membrane transporters that are associated with drug resistance. (4) CSCs highly express apoptosis genes. (5) CSCs have high telomerase activity. Therefore, tumors could resist chemotherapy and radiation after nonfatal treatment, which means tumor cells themselves naturally select the CSCs. As such, it has been aimed to develop strategies to overcome or inhibit tumor drug resistance. ABCB5, a drug efflux transporter, is associated with therapeutic resistance and can identify CSCs in human malignancies. Wilson *et al*. [61] proved that ATP-binding cassette sub-family B member 5 (ABCB5) regulates CSCs maintenance circuit *via* IL1β secretion, which is involved in the IL1β/IL8/CXCR1 cytokine signaling circuit. Because ABCB5 can reverse the resistance to multiple chemotherapeutic agents when blocked, it was defined as a novel target direction in CSCs maintenance. ATP-binding cassette, subfamily G, member 2 (ABCG2) is highly expressed in CSCs and may play an important role in cancer malignant behaviors. Because the down-regulation of ABCG2 can

significantly decrease the doxorubicin resistance, proliferation, migration and invasion potential, scientists suggested it as a potential marker for CSCs and drug resistance in hepatocellular carcinoma [62]. Eliminating CSCs and signal transduction pathways they depend on would become an important direction to prevent drug resistance.

THERAPY TARGETING CANCER STEM CELLS AND THEIR METASTATIC MICROENVIRONMENT

In recent years, it has been continued to investigate methods to target CSCs without wounding the normal SCs in order to effectively prevent metastasis and recurrence of the tumor and ultimately permanently cure the tumor. The separation and identification of CSCs as well as their role in metastasis could improve the understanding of the occurrence, development, metastasis, and relapse of the tumor at the molecular, genomic and epigenetic levels.

A novel mechanism has been proposed that the SDF-1–CXCR4 axis may be a crucial pathway in the interaction between CSCs and their surrounding supportive cells. They used a CD44(+) CXCR4(+) cell line to monitor the influence of SDF-1α on CSCs metastasis and found that SDF-1α could enhance the cell migration, that is to say, SDF-1α is a chemoattractant for CSCs and demonstrate the role in the interaction between CSCs and their supportive cells in the CSCs niche. Therefore, using the peptide receptor agonist may be a feasible target therapy method [63-64].

The microRNAs that affect CSCs and the metastatic progress could be potential targets for tumor therapy. MicroRNA-145 is an important regulator of tumorigenesis. Hu *et al*. [65] showed that as a tumor suppressor, miR-145 can down-regulate markers of lung CSCs and EMT progress by targeting Oct4, leading to the inhibition of tumor growth and metastasis. Multiple evidence showed that microRNAs are involved in EMT, which suggests that microRNAs might link CSCs to metastasis *via* EMT. It has been known that the EMT inducer zinc finger E-box binding homeobox 1(ZEB1) could promote metastasis not only by enhancing migration and dissemination but also by maintaining the SC phenotype *via* the inhibition of miR-200 family members, which is essential for metastasis. As a tumor suppressor, miR-200c can markedly down-regulate ZEB1 expression to reduce the migration and invasion of melanoma CD44+CD133+ CSCs, which suggests a potential role of miR-200c as a therapeutic target for CSCs [66].

It has also found other novel target therapeutic methods. Silencing Grp78, which is important in cytoprotection and tumorigenesis, could improve chemo-radiosensitivity to inhibit cell invasion and reverse EMT transition in CD24-CD44+ head and neck CSCs. Thus, Chiu *et al*. [67] suggested that Grp78 may serve as a molecular target that can be developed to eradicate refractory head and neck CSCs. Deng *et al*. [68] also identified a hopeful therapeutic target called Compound2(C2), a new gamboge derivative, which could effectively inhibit the stem-like properties of CSCs in head and neck squamous cell carcinoma. Components in the CSC microenvironment may also be potential targets for future cancer therapy. Astrocytes activated by IL-1β can promote the metastatic growth of breast CSCs by activating the Notch signaling pathway in the brain. This pathway could be utilized to identify a novel therapeutic target for brain metastatic cancer [69].

CONCLUSIONS AND PROSPECTS

CSCs and their microenvironment play important roles in cancer development and progression. The in-depth study of CSCs has gradually led to the acceptance of the theory that CSCs are the best candidate for tumor metastasis. Although many questions related to CSCs remain unanswered, such as the mechanism of CSCs generation and metastasis, the isolation and molecular analysis have already supplied a large amount of opportunities to study novel tumor therapy. Therefore, it should been focused on CSCs-targeting therapy in future tumor treatment, especially the interactional mechanism between CSCs and their microenvironment, which helps us to understand the innate character of tumor metastasis.

CONTRIBUTIONS

Huan Liu wrote the paper; Haijuan Wang and Haili Qian reviewed the paper.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

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