



## REVIEW ARTICLE

# Tumor microenvironment of cancer stem cells: Perspectives on cancer stem cell targeting

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**Abstract** There are few tumor cell subpopulations with stem cell characteristics in tumor tissue, defined as cancer stem cells (CSCs) or cancer stem-like cells (CSLCs), which can reconstruct neoplasms with malignant biological behaviors such as invasiveness via self-renewal and unlimited generation. The microenvironment that CSCs depend on consists of various cellular components and corresponding medium components. Among these factors existing at a variety of levels and forms, cytokine networks and numerous signal pathways play an important role in signaling transduction. These factors promote or maintain cancer cell stemness, and participate in cancer recurrence, metastasis, and resistance. This review aims to summarize the recent molecular data concerning the multilayered relationship between CSCs and CSC-favorable microenvironments. We also discuss the therapeutic implications of targeting this synergistic interplay, hoping to give an insight into targeting cancer cell stemness for tumor therapy and prognosis.

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## Introduction

The concept that tumor initiation and progression are driven by a subset of cells endowed with stem-like properties was first described by Rudolf Virchow in 1855. 'Cancer stem cells (CSCs)', as they were termed, represent a subset of tumor cells that can self-renew, unlimitedly proliferate, and have the potential for tumor initiation.<sup>1</sup> These CSCs or cancer stem-like cells (CSLCs) have raised great expectations in cancer research and therapy as their eradication is expected to completely cure cancer. Alternatively, CSC-targeted therapy can also be proposed as adjuvant therapy, along with conventional therapies. However, there are still no specific markers that can mark all kinds of CSCs, for example, CD133 is the marker in ovarian CSCs and lung CSCs<sup>2,3</sup>; Meanwhile, the side population can also be used as a marker of CSCs<sup>4</sup>; ALDH can be used as a marker of solid CSCs, like head and neck squamous cell carcinoma (HNSCC), *etc.*<sup>5</sup>; CD44<sup>+</sup>/CD24<sup>-</sup> subpopulation is regarded as breast cancer stem cells (BCSCs).<sup>6</sup> Interestingly, Virchow, in suggesting a correlation between cancer and the inflammatory microenvironment, also paved the way for the 'Seed and Soil' theory proposed by Paget a few years later.<sup>7</sup> Despite these two important concepts that have been proposed for some time, the relationship between Virchow's "stem cell-like cells" and Paget's "soil" is far from being fully understood. One emerging topic is the importance of a CSC-like niche in modulating the biological traits of CSCs or CSLCs, and thus in affecting drug response. In the tumor microenvironment (TME) where CSCs are located,<sup>8</sup> there are many favorable factors for CSCs, which can be divided into two categories: cellular components and corresponding medium components. Cellular components include immune cells and non-immune cells. Medium components include cytokines, chemokines, growth factors, *etc.* These factors that can maintain or promote the functions of CSCs, interweave into the three-dimensional network in the TME, contributing to cancer recurrence, metastasis, and drug resistance (Fig. 1).

## Cellular components in the tumor microenvironment

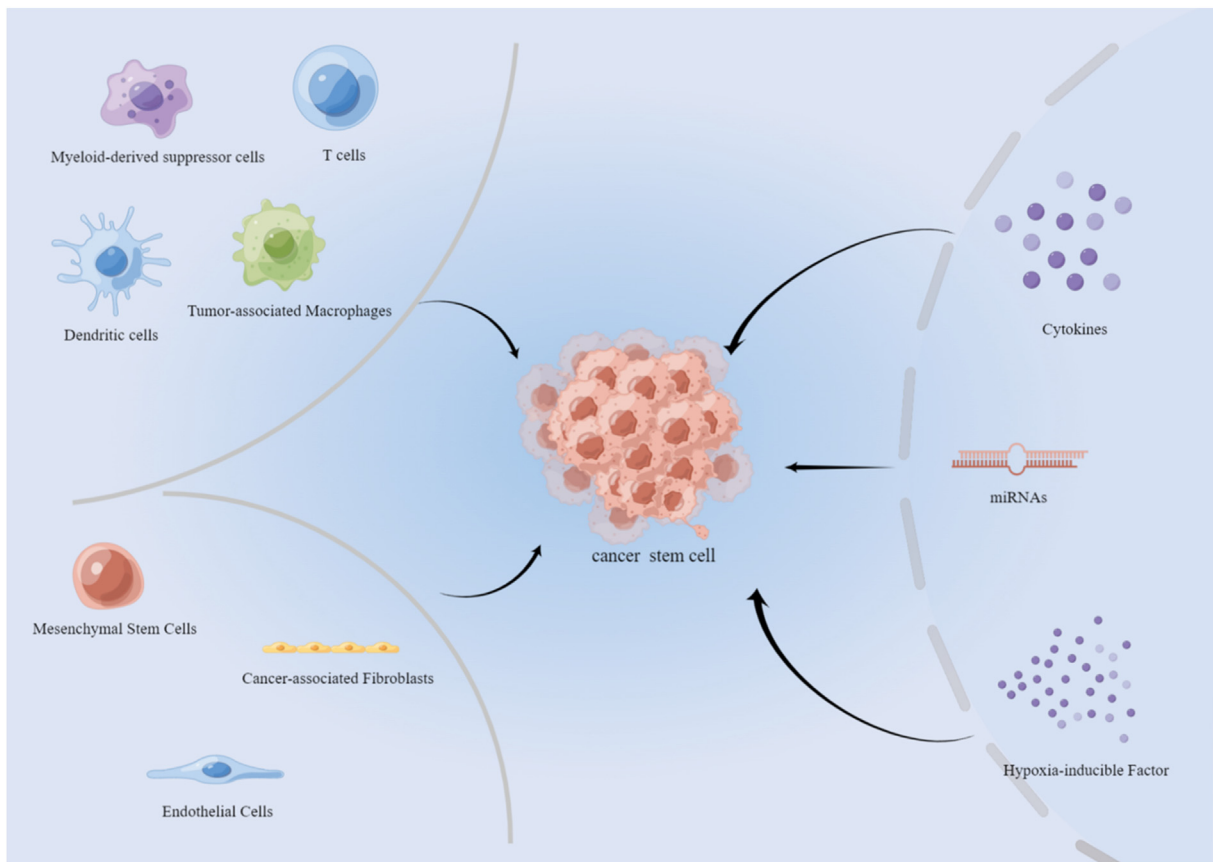
### Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stromal stem cells within most cancers and affect the function and formation of TME. The pro- or anti-tumorigenic potential of MSCs on tumor progression has been paradoxically reported. On the one hand, MSCs can function as a tumor-supporter through various mechanisms, including (i) promoting angiogenesis, (ii) suppression of the immune response, (iii) differentiation into other pro-tumorigenic stromal components, (iv) inducement of epithelial–mesenchymal transition (EMT), (v) increasing cell survival and metastasis, (vi) and enrichment of CSCs. On the other hand, MSCs have also been demonstrated to hold anti-tumorigenic functions including enhancing the immune response, suppressing angiogenesis, and promoting cell apoptosis.<sup>9</sup> Although arguments still exist, most studies point to MSCs' roles in facilitating tumor

progression within TME. Notably, a specific population of CSCs is constituted by MSCs that differentiate into mesoderm-specific cells and particular emphasis is put on the pivotal role of MSCs in supporting CSC development by various interactions and cell fusion to form hybrid tumor cells.

Numerous studies have demonstrated the interactions between MSCs and cancer cells or CSCs with the support of CSC maintenance.<sup>10</sup> MSCs can facilitate TME to maintain the activity of CSCs in direct or indirect ways. The indirect communication involves both the release of soluble molecules such as metabolites and hormones, and the exchange of microvesicles and exosomes.<sup>11</sup> Ours and other studies have shown that programmed cell death ligand 1 (PD-L1) expression is related to tumor stemness.<sup>12,13</sup> MSCs can enhance the CSC-like properties and tumorigenesis in gastric cancer via the PD-L1-CTCF (CCCTC-binding factor) association by secreting interleukin-8 (IL-8).<sup>14</sup> In addition, a recent study indicated that IL-8 secreted by MSCs bound to the membrane receptor CXCR2 of colon CSCs promotes the homing of MSCs to CSCs, which was rescued by IL-8 inhibition.<sup>15</sup> Li et al have found that tumor cell-derived interleukin-1 (IL-1) could induce MSCs to secrete prostaglandin E2 (PGE2), and PGE2 and IL-1 signaling could induce MSCs to express cytokines, such as IL-6 and IL-8, which subsequently acted on tumor cells in a paracrine manner to induce the activation of the  $\beta$ -catenin signaling and formation of CSCs.<sup>16</sup> Therefore, inhibition of the IL-8 signaling in colon tumors might have therapeutic potential to modulate disease progression, which was further confirmed by other studies.<sup>17</sup> Similarly, Luo et al found that the infiltrating bone marrow MSCs suppressed the androgen receptor signaling via secreting cytokines, to increase the population and metastatic ability of prostate CSCs.<sup>18</sup> A recent study also showed that IL-6 and hepatocyte growth factor (HGF) secreted by MSCs could facilitate CSCs' growth and maintain the stem-like characteristics of CSCs.<sup>19</sup> Besides, extracellular vesicles (EVs) are cell-derived membrane vesicles, representing an endogenous mechanism for intercellular communication.<sup>20</sup> MSCs-secreted EVs instruct stepwise dedifferentiation of breast cancer cells into dormancy at the bone marrow perivascular region for decades as CSCs, resurging as tertiary metastasis.<sup>21</sup> Moreover, MSCs can deliver nutrients, such as ketone bodies, PGE2, and glutamine, to modulate immune cells and tumor cells from cell death.<sup>16</sup> Among these nutrients, hormones as soluble agents have been indicated to hold an influence on CSCs as progesterone could induce the expansion of BCSCs.<sup>22–24</sup>

MSCs may represent a major cellular component of the TME of CSCs since numerous studies reported the mutual acquisition of properties between both interaction partners altering the original cell fate.<sup>25,26</sup> Gap junctions, also known as gap junctional intercellular communication, enable the direct interaction between two neighboring cells.<sup>27</sup> Thereby, each cell contributes equally to gap junction formation. The previous study has shown that breast cancer cell acquires CD90 expression as an MSC surface marker when it is cocultured with MSCs.<sup>25</sup> Additionally, Notch signaling starts with ligand binding from the signal-sending cell to the notch receptor of the signal-receiving cell and has been confirmed to be a reliable CSC



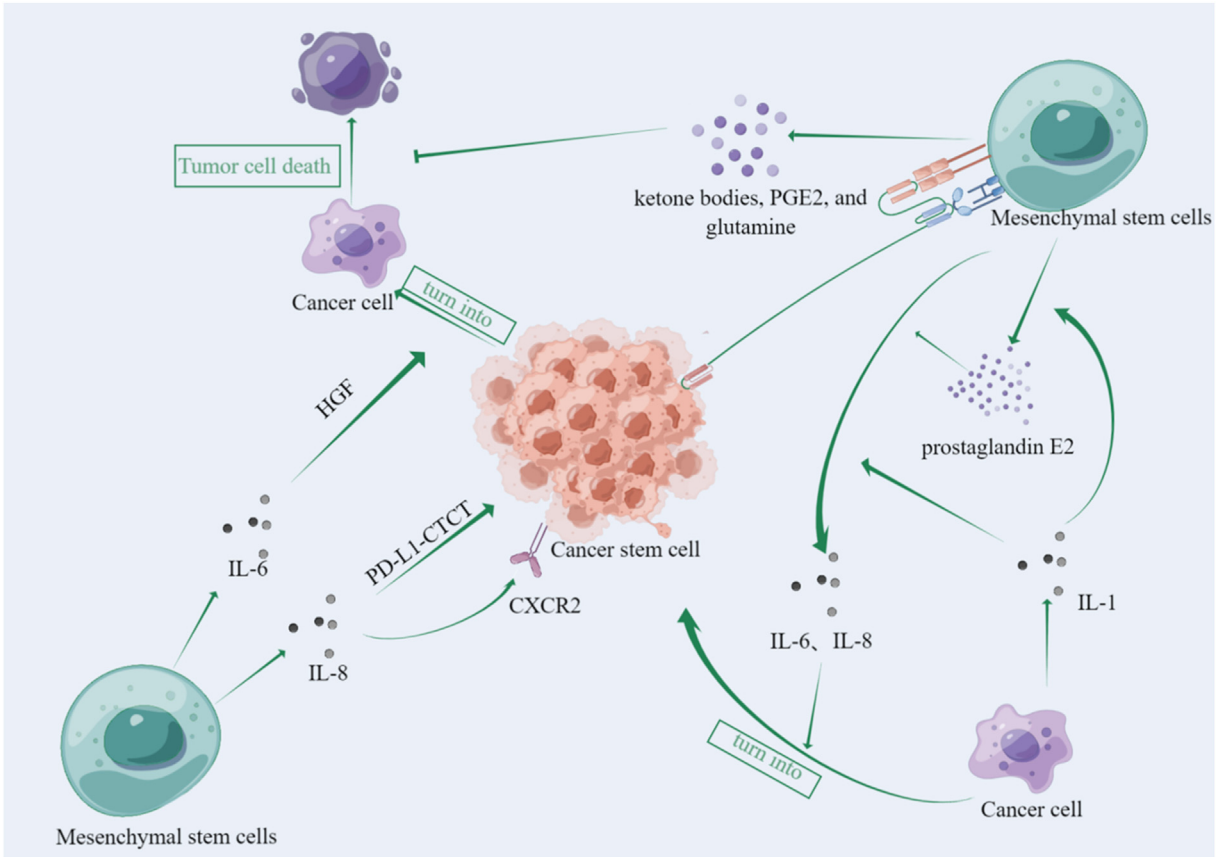
**Figure 1** CSCs' interaction with the three-dimensional network in TME. Schematic demonstration of the mechanisms of CSC interactions with TME. CSCs are capable of driving tumor growth by facilitating the formation of cancer-promoting TME around them. There are many favorable factors for CSCs, including cellular components and corresponding medium components. Cellular components include immune cells and non-immune cells. Medium components include cytokines, miRNAs, hypoxia-inducible factors, *etc.*

target.<sup>28</sup> Consistently, MSCs have been identified as signal-sending cells of the Notch signaling whereas breast cancer cells received signals, and the obtained signals, such as CD90 expression, were attenuated by blocking the Notch signaling.<sup>25</sup> Notably, CD90 has been reported as a marker for liver CSCs.<sup>29</sup> Moreover, some other evidence reveals a direct interaction between MSCs and CSCs, constituting a favorable TME for CSC existence. The previous study has shown that B cell precursor-acute lymphoblastic leukemia cells residing in the BM-MSCs TME may acquire chemoresistance by altering their phenotype to resemble that of CSCs via cell adhesion,<sup>30</sup> and BM-MSCs can promote the stemness of hypopharyngeal cancer cells.<sup>31</sup> After being co-cultured with human MSCs, the stemness of hypopharyngeal cancer cells is significantly promoted.<sup>31</sup> Interestingly, spontaneously-formed tumorigenic hybrids between bone marrow-derived MSCs and different non-small cell lung cancer (NSCLC) cell lines contribute to the highly malignant subpopulations with both EMT and CSC-like properties.<sup>32</sup> Similarly, cell fusion of MSCs and breast cancer cells leads to the formation of hybrid cells exhibiting stem cell characteristics.<sup>33</sup> Therefore, particular emphasis should be put on the pivotal role of multipotent MSCs in supporting CSC

development through various interactions and cell fusion to form hybrid tumor cells (Fig. 2).

### Endothelial cells/endothelial progenitor cells

Robert et al proposed the theory of "vasculogenic mimicry (VM) and tumor progression" in 2000.<sup>34</sup> After that, Kaur and Bajwa further recommended using the VM model to explain the phenomenon of interaction between CSCs and bone marrow-derived endothelial progenitor cells (EPCs) in 2003,<sup>35</sup> namely, vascular endothelial growth factor (VEGF) could promote the migration and tumor angiogenesis of bone marrow-derived EPCs. Migrated EPCs secrete more VEGF, thus strengthening VEGF's promotion of tumor angiogenesis and CSC multiplication. The promoting roles of endothelial cells (ECs) in CSC activity were confirmed by Tian's group which established a 3D coculture system that could study the interactions between stromal cells and CSCs, and they found that the self-renewal ability and stemness of CSCs were significantly enhanced through coculture with human umbilical vein endothelial cells (HUVECs) over an extended period.<sup>36</sup> Although this was only a recommended model based on theory, the authors still



**Figure 2** The crosstalk between MSCs and CSCs. MSCs secrete a wide variety of soluble factors to promote CSC activity or the transformation of other cells into CSCs, such as IL-6, IL-8, PGE2, and glutamine. In addition, cell fusion of MSCs and cancer cells can also form CSLCs.

pointed out the direction for future research. The interaction relationship between ECs/EPCs and CSCs can be used as a research direction in the future, as CSC-associated tumor neovascularization partially contributes to the failure of cancer treatment,<sup>37</sup> and EC-triggered signaling can enhance the survival and self-renewal of CSCs.<sup>38</sup>

Research on the interaction between CSCs and ECs/EPCs is not limited to VEGF and VM, as Ellis et al showed that conditioned medium from ECs of all organs increased the number of CSCs in colorectal cancer (CRC) cells in a paracrine/angiocrine fashion.<sup>39</sup> This study provides a theoretical basis for the antiangiogenic therapy of ovarian CSCs. A previous study suggested that ECs of tumor microvessels can secrete basic fibroblast growth factor (bFGF), which can induce well-differentiated glioma cells to acquire CSC features.<sup>40</sup> Other studies have shown that epidermal growth factor (EGF) secreted by vascular ECs can induce EMT and endow head and neck epithelial cancer cells with a stem-like phenotype,<sup>41</sup> and IL-6 secreted by ECs can increase the tumorigenic potential of CSCs in HNSCC.<sup>42</sup> Indeed, another study indicated that the blockade of IL-6 attenuated cancer tissue integration by CSCs.<sup>43</sup> Similarly, IL-8 secreted by ECs induced the CSC-like traits of glioma cells and thus promoted the 3D invasive ability.<sup>44</sup> Moreover, previous studies have indicated that the Notch pathway plays a critical role in linking angiogenesis and glioma CSC self-renewal ability.<sup>45,46</sup> Other studies also confirmed that

activating the Notch signaling pathway by hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) or interferon regulatory factor 7 contributed to glioma stem cell (GSC) maintenance.<sup>47,48</sup> This effect was further elucidated in other cancers; for example, ECs actively promoted the Notch signaling and the CSC phenotype by secreting soluble Jagged-1 in CRC.<sup>49</sup> The Jagged1-Notch1-deployed tumor perivascular niche promotes the BCSC phenotype through the transcription factor Zeb1 thus enhancing antiangiogenic therapy's efficacy.<sup>50</sup> The Notch signaling-mediated interaction between cancer cells and ECs promotes CSC-like traits, which were also revealed in other tumors, such as melanoma<sup>51</sup> and advanced colon cancer.<sup>52</sup> Thus, the stimulation mediated by Notch signaling is a potential therapeutic target for CSC-EC interactions and thus suppressing CSCs.

Additionally, CSCs can differentiate into ECs and thus facilitate their stemness. Direct observational evidence about the differentiation of CSCs into ECs was provided by Chen et al, who indicated that GSCs could differentiate into endothelial cells and promote angiogenesis in glioblastoma (GBM) using live-cell imaging.<sup>53</sup> *In vivo* cell lineage tracing with constitutive and lineage-specific fluorescent reporters also revealed that GSCs were recruited toward ECs via the SDF-1/CXCR4 axis and were induced to become pericytes predominantly by transforming growth factor-beta (TGF- $\beta$ ).<sup>54</sup> Consistently, human colorectal CSCs can give rise to vascular ECs and compose the vasculature in cancer



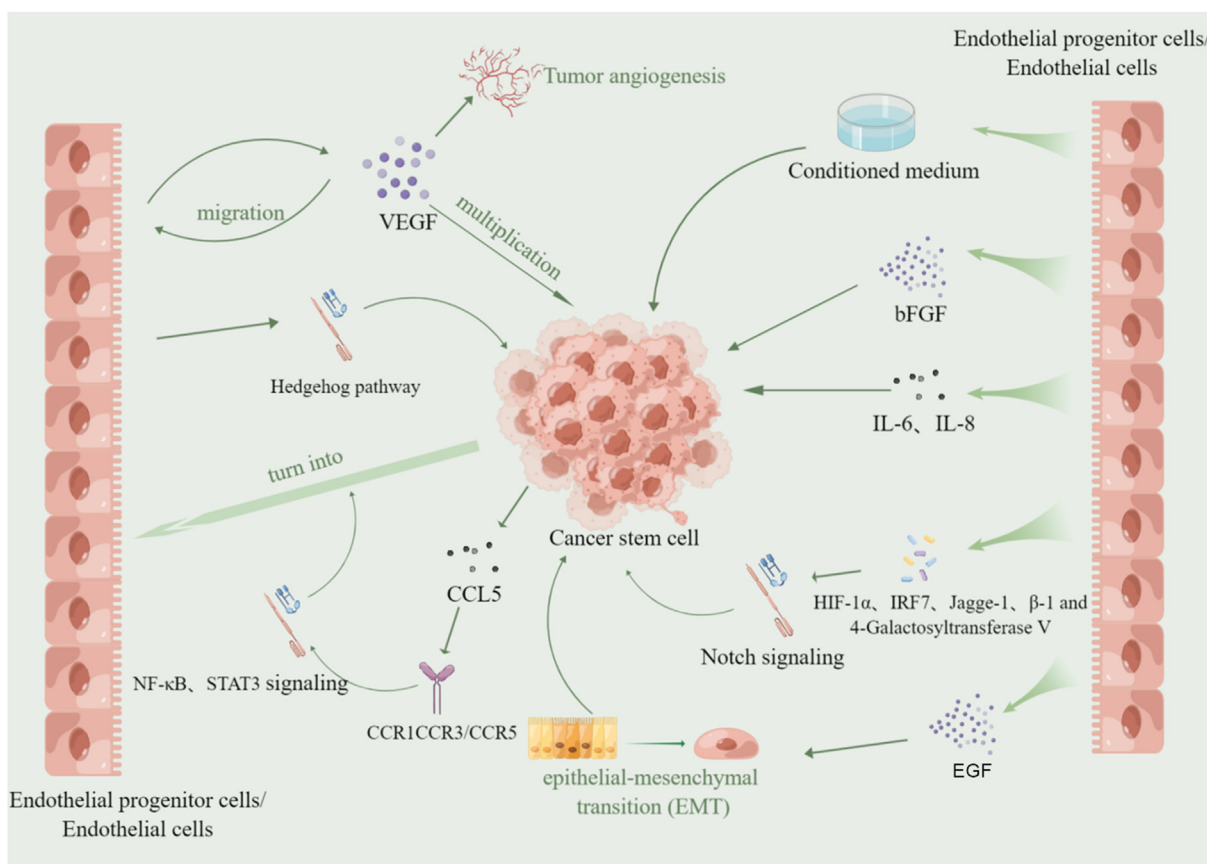
tissues.<sup>55</sup>  $\beta$ -1 and 4-galactosyltransferase V can stimulate GSCs to transdifferentiate into ECs by activating Notch1 signaling.<sup>56</sup> A recent study indicated that CSCs in ovarian cancer can secrete the chemokine CCL5, and the combination of CCL5 and its receptor CCR1/CCR3/CCR5 can activate the signal transduction pathway of NF- $\kappa$ B and STAT3 and then mediate ovarian CSCs to differentiate into ECs.<sup>57</sup> Another article suggests that persistent oxidative stress may be a critical factor in the differentiation of CSCs into tumor ECs by relying on the relationship among reactive oxygen species (ROS), the pentose phosphate pathway, and autophagy.<sup>58</sup> In addition, ECs can also promote the CSC-like traits of glioma cells by activating the Hedgehog pathway.<sup>59</sup> Notably, suppressing the endothelial differentiation of CSCs has been shown to efficiently inhibit tumor progression.<sup>60</sup> Several studies have explored potential drugs disrupting the CSC-EC interactions or the endothelial differentiation of CSCs; for example, shWNT5A@cRGD-DDD liposomes targeting WNT5A exert antiangiogenic effects *in vivo* by modulating the endothelial differentiation of GSCs.<sup>61</sup> Inhibition of adenosine A<sub>3</sub> receptor (A<sub>3</sub>AR) using MRS1220 (A<sub>3</sub>AR antagonist) reduced tumor size and blood vessel formation by suppressing the differentiation of GSCs to ECs under hypoxia.<sup>62</sup> Estradiol could change the migration, juxtacrine, and paracrine activities of CSCs by suppressing the CSC-EC interactions and affinity through a Surface Plasmon Resonance assay.<sup>63</sup> Interestingly, Xu et al explored a transformable dual-inhibition system based on a self-assembling peptide, which could provide the possibility to modulate both ECs and CSCs for cancer therapy.<sup>64</sup>

Furthermore, EPCs are considered to originate from a common hemangioblast precursor in the bone marrow with the expression of several hematopoietic and endothelial lineage markers.<sup>65</sup> When tumors grow rapidly and the mounting metabolic demands cannot be covered, distant cells from systemic recruitment, such as bone marrow-derived stem cells and progenitor cells, emerge as critical players. ECs are terminally differentiated cells and their ability to support CSC proliferation is hence limited. In this case, bone-marrow circulating EPCs provide an unlimited reservoir of cells that drives tumor angiogenesis and thus CSC activity. The migration of EPCs to the tumor vasculature largely depends on the chemokine signals emanating from the tumor tissue. A study by Folkins et al in glioma demonstrated that GSCs contributed to tumor angiogenesis by promoting local EC activity and increased mobilization and tumor recruitment of bone marrow-derived EPCs in a VEGF-dependent and SDF-1-dependent manner.<sup>66</sup> CSCs make an important contribution toward releasing proangiogenic factors,<sup>37</sup> and the number of CSCs in tumor tissue may largely affect the migration of EPCs toward the tumor. Thus, greater numbers of EPCs will be recruited by tumors with a larger CSC fraction, which raises the possibility that EPC recruitment may play a more significant role in the vascular development of tumors with a large CSC fraction. The migration of CSCs and EPCs to the tumor site ensures the formation of this premetastatic niche and then promotes angiogenesis and the formation of vascularized niches, facilitating tumor metastasis. A recent study by Hetta et al also stated a close interaction between circulating EPC and CSC levels and hepatocellular carcinoma (HCC) outcome diagnosis and prediction.<sup>67</sup> A previous study

also showed that EPCs could enhance the tumorigenic capacity of CSCs through angiogenesis and coaction of CSCs and EPCs promoted the development of colon cancer.<sup>68</sup> CSC-derived VEGF may promote the migration of EPCs and tumor angiogenesis, and EPC-derived VEGF may in turn enhance the proliferative abilities of CSCs. Thus, EPCs seem to promote tumor growth and metastasis not only by angiogenesis but also by stimulating the proliferative potential of CSCs. Since hypoxia resulting from tumor growth can also mobilize EPCs from the bone marrow, EPCs may also facilitate the formation of VM from CSCs. In contrast to the widely accepted role of EPCs in tumor angiogenesis, it must be noted that a study by Florence et al reported that EPC populations could also prevent tumor metastasis due to their phagocytic capacity similar to macrophages in breast carcinoma.<sup>69</sup> This phagocytic role of EPCs may be attributable again to the type of tumor environment. A highly metastatic environment may contribute to a reduction in the macrophagic potential of progenitor cells and an associated increase in metastatic burden, while in a less angiogenic tumor niche, EPCs may behave as macrophages and are involved in the clearance of tumor cells. Therefore, therapeutic interventions focusing only on the inherent properties of CSCs or EPCs may not be sufficient, and well-designed strategies to target the signals between EPCs and CSCs in the tumor niche as a whole are favorable. However, we must admit that the direct interaction between EPCs and CSCs is still confusing, and it is still unclear how these two cell types interact with other cells in the CSC TME. In this direction, well-designed studies focusing on *in vitro* and *in vivo* interactions between these two cell types are needed (Fig. 3).

### Cancer-associated fibroblasts/fibroblasts

Cancer-associated fibroblasts (CAFs) are a kind of fibroblast existing in solid tumors with diverse functions, including matrix deposition and remodeling, extensive reciprocal signaling interactions with cancer cells, and crosstalk with infiltrating leukocytes.<sup>70</sup> Recently, Zheng et al performed an integrated analysis of CAFs from melanoma, HNSCC, and lung cancer, and identified the molecular characteristics that were distinctly active in each CAF subtype. They found that CAF subtypes were associated with different clinical outcomes and key molecular pathways that could regulate cancer progression or were involved in immunotherapy resistance.<sup>71</sup> In 2019, Euisik et al developed a platform to reliably culture single-cell-derived spheres for functional enrichment of CSCs and discovered novel genes associated with the cancer-CAF interaction and critical to patient survival. This platform was reliable for CSC enrichment and studies of CSC-CAF interactions.<sup>72</sup> Subsequently, numerous studies have shown that CAFs could facilitate CSC activity or tumor stemness. William Matsui et al cocultured CAFs from patient tumors with tumor cells and found that both the frequency and self-renewal ability of CSCs were significantly increased in pancreatic ductal adenocarcinoma (PDAC) cells.<sup>73</sup> Wang et al showed that CAFs promoted CSC seeding and expansion in the lung during squamous cell carcinoma (SCC) metastasis, which was attenuated by a clinically relevant TGF- $\beta$  receptor



**Figure 3** The crosstalk between ECs/EPCs and CSCs. The CSC pool in the tumor sends signals including VEGF and chemokines to the EPCs to migrate to the tumor tissue. The migrated EPCs in turn secrete more angiogenic factors such as VEGF, HIF-1 $\alpha$ , and bFGF that enhance tumor growth by affecting the proliferation and expansion of the resident CSCs. EPCs may also promote the differentiation of CSCs into VM channels. Some of the migrated EPCs may also form a part of the tumor vasculature along with host ECs leading to increased tumor angiogenesis and metastasis. Notably, CSCs can be differentiated into ECs under the activation of some signaling pathways, such as the NF- $\kappa$ B and STAT3 signaling.

inhibitor, suggesting that CAFs facilitated SCC stem cell seeding and expansion in a TGF- $\beta$ -dependent manner.<sup>74</sup> Thrombospondins (THBSs or TSPs) comprise a family of five adhesive glycoproteins that are overexpressed in many types of cancers.<sup>75</sup> Recently, it was found that CAF-derived TSP-4 induced the stemness of gallbladder cancer cells through a complex TSP-4/integrin  $\alpha$ 2/HSF1/TGF- $\beta$  cascade.<sup>76</sup> Similarly, the roles of TGF- $\beta$  signaling in CAF-mediated enhancement of CSC stemness were also elucidated in gastric cancer.<sup>77</sup> Periostin (encoded by POSTN) is a matricellular protein secreted by CAFs that may promote cancer stemness, initiation, and progression and is overexpressed in several cancers.<sup>78–80</sup> POSTN secreted by CAFs promotes the CSC-like phenotype via PTK7–Wnt/ $\beta$ -catenin signaling in human HNSCC.<sup>81</sup> Autophagy is considered to be a critical process for CSC maintenance, and it has also been demonstrated that autophagic CAFs play a critical role in promoting BCSC progression.<sup>82</sup> Furthermore, CAFs can promote the stemness of cancer through other cells; for example, CAFs shape myeloid-derived suppressor cells (MDSCs) to promote the stemness of intrahepatic cholangiocarcinoma through 5-lipoxygenase.<sup>83</sup> Moreover, CAFs can promote CSC activity through exosomes; for example,

CAF-derived conditioned medium can promote the percentage, clonogenicity, and tumor growth of CSCs, which is attenuated by inhibiting exosome secretion<sup>84</sup>; CAF-derived exosomes could enhance CRC stemness by activating the TGF- $\beta$  signaling pathway, and a TGF- $\beta$ 1-neutralizing antibody inhibited this effect<sup>85</sup>; CAFs promote the stemness and chemoresistance of CRC by transferring exosomal long noncoding RNA (lncRNA) H19.<sup>86</sup> In addition to lncRNAs, exonic circRNAs have also been shown to exist in CAF-derived exosomes and modulate breast cancer stemness.<sup>87</sup> Conversely, exosomes derived from Pwll2-induced CSCs transform fibroblasts into CAFs.<sup>88</sup> Similarly, EV signaling between CAFs and CSCs may contribute to the progression, therapy resistance, and recurrence of malignant tumors.<sup>89</sup> A recent study also found that EVs from CAFs promoted BCSC properties and glycolysis via the miR-7641/HIF-1 $\alpha$  axis.<sup>90</sup> Moreover, lipid desaturation is required for CSCs, and the CD10 transmembrane hydrolase expressed on a subset of CAFs supports tumor stemness and induces chemoresistance by degenerating an antitumoral peptide termed osteogenic growth peptide, which subsequently restrains the expression of the rate-limiting desaturase SCD1 and inhibits lipid desaturation.<sup>91</sup>

Additionally, the activation of the Notch and Hedgehog signaling has been confirmed to contribute to CSC generation and sustainment, and their inhibitors have been explored to target CSCs for cancer treatment.<sup>28</sup> These two typical CSC-promoting signaling pathways were shown to be involved in CAF-mediated effects on CSC progression. CAFs can promote and maintain the stem cell-like properties of HCC cells via the IL-6/STAT3/Notch signaling pathway.<sup>92</sup> Chromatin modifying factor LSD1 has a high expression in liver CSCs and can maintain the ability of self-renewal and tumor-formation of CSCs; CAFs can activate the Notch3 signaling, induce the deacetylation of LSD1, and keep the stability of LSD1, thus promoting and maintaining the stemness of liver cancer.<sup>93</sup> The intracellular Notch1 signaling in CAFs is a molecular switch dictating the plasticity and stemness of melanoma stem cells, thereby regulating melanoma aggressiveness.<sup>94</sup> In addition, CSCs can secrete the Hedgehog ligand SHH, which regulates CAFs via paracrine activation of the Hedgehog signaling; CAFs subsequently secrete factors that promote the expansion and self-renewal of CSCs.<sup>95</sup> These results suggest that targeting the intracellular Notch and Hedgehog signaling pathway in CAFs may present a new therapeutic strategy for tumor progression by disrupting the interaction between CSCs and CAFs.

Notably, CAFs exhibit heterogeneity in tumor tissues and this heterogeneity is associated with organ-specific metastasis in PDAC.<sup>96</sup> Other studies also indicated that different types of CAFs can facilitate cancer stemness or CSC formation, for example, Song's team identified that a CD10<sup>+</sup>GPR77<sup>+</sup> CAF subset was driven by the persistent NF- $\kappa$ B activation, which was maintained by complement signaling via GPR77, a C5a receptor.<sup>97</sup> Importantly, they found that targeting these CAFs with a neutralizing anti-GPR77 antibody indeed abolished CSC-driven tumors. MRC-5 CAFs also have been found to extensively affect the production of CSC markers and inflammation-associated cell surface molecules in liver cancer<sup>98</sup> and breast cancer.<sup>99</sup> A previous study indicated that the expression of PDPN in CAFs could predict poor prognosis independently in lung SCC regardless of the expression of Podoplanin (PDPN) in tumor cells.<sup>100</sup> Furthermore, PDPN<sup>+</sup> CAFs were found to enhance tumor formation and metastasis, both of which were positively correlated with tumor stemness.<sup>101</sup> Further studies can be constructed to deeply reveal the effects and underlying mechanisms of podoplanin<sup>+</sup> CAFs on CSCs. What's more, CD90<sup>+</sup> CAFs constitute a supporting niche for cancer stemness through the paracrine insulin-like growth factor-II (IGF-II)/IGF1 receptor/Nanog pathway signaling.<sup>102</sup> CXCL12 expression is strongly enhanced in IL-7-producing CAFs, which enhance breast cancer stemness through the CXCL12/CXCR4 axis.<sup>103</sup> A previous study found that  $\alpha$ -SMA<sup>+</sup> CAFs are correlated with a poor clinical outcome in HCC and CAF-derived HGF-regulated liver CSCs via the activation of FRA1 in an Erk1/2-dependent manner using the STAM NASH-HCC mouse model.<sup>104</sup> However, some other studies demonstrated that CAFs possessed CSC-restraining functions. Recent work indicated that depletion of  $\alpha$ -SMA<sup>+</sup> CAFs increased the CSC population and the generation of an immunosuppressive TME with increased frequency of Foxp3<sup>+</sup> regulatory T cells (Tregs) and suppression of CD8<sup>+</sup> T cells, indicating that this subset of CAFs exerted CSC-

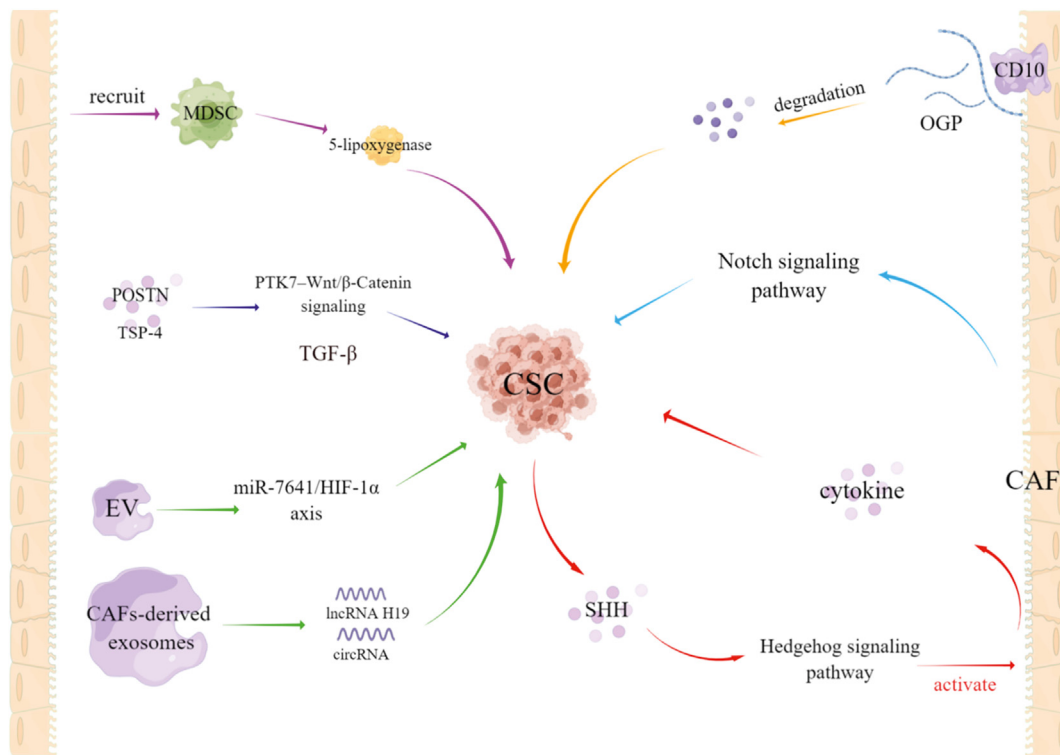
restraining functions.<sup>105</sup> Consistently, Singh et al also identified a subset of CAFs with a lower- $\alpha$ SMA expression that suppressed the stemness of oral-stem-like cancer cells.<sup>106</sup>

In addition to CAFs, a study showed that normal fibroblasts could also induce CSC formation, for example, breast fibroblasts were found to promote CSC generation in breast cancer.<sup>107</sup> When fibroblasts exist, PDPN<sup>+</sup> CSCs display a stronger invasive ability in SCC.<sup>108</sup> Similarly, human fibrocytes, differentiated from bone marrow-derived CD14<sup>+</sup> monocytes, enhance the CSC-like properties of lung cancer cells through secreted factors.<sup>109</sup> Although limited evidence has been provided, we believe that normal fibroblasts also act as a critical factor for CSC maintenance. Notably, peritumor tissue-derived fibroblasts or CAFs may exert a stronger effect on recruiting CSCs and maintaining their stemness characteristics than normal fibroblasts.<sup>110–113</sup>

These results suggest that CAFs are a potential target for optimizing therapeutic strategies against cancer. However, there are many challenges in ongoing attempts to modulate CAFs for therapeutic benefit, including a limited understanding of CAF origin and heterogeneity. CSCs could be the source of CAFs that support tumor maintenance and survival.<sup>114</sup> Additionally, adipose-derived MSCs can differentiate into pancreatic cancer-associated fibroblasts *in vitro*.<sup>115</sup> Recently, Madsen et al developed a multicolor flow cytometry strategy based on the exclusion of non-CAF and successfully employed this strategy to explore the temporal heterogeneity of freshly-isolated CAFs in triple-negative breast cancer (TNBC).<sup>116</sup> Notably, Chen et al recently established a TME-based drug screening platform to identify and repurpose compounds specifically targeting CSCs and CAFs.<sup>117</sup> This TME-based drug screening platform might comprehensively evaluate the response of cancer cells, CSCs, and CAFs to different treatments (Fig. 4).

### Tumor-associated macrophages

Tumor-associated macrophages (TAMs) are macrophages that infiltrate the stroma of tumor tissue and promote tumor angiogenesis, invasion, metastasis, immunosuppression, and chemotherapy resistance.<sup>118</sup> EMT is a process in which epithelial cells lose their epithelial phenotype and convert to mesenchymal cells.<sup>119</sup> Recent studies have shown that cancer cells undergoing EMT can become stem-like cells<sup>120</sup> and that TAMs can promote the stemness of CSCs or cancer cells by inducing the EMT process through individual factors.<sup>121</sup> A previous study showed that TAMs secreted more TGF- $\beta$ 1 than other macrophage phenotypes, by which TAMs promoted the CSC-like properties via TGF- $\beta$ 1-induced EMT in HCC.<sup>122</sup> Similarly, a recent study indicated that TAMs were associated with unfavorable prognosis in TNBC patients and promoted EMT and CSC properties by activating the chemokine (C-C motif) ligand 2 (CCL2)/AKT/ $\beta$ -catenin signaling,<sup>123</sup> which may offer new strategies for the diagnosis and treatment of TNBC. Notably, TAMs can act through the juxtacrine signaling to enhance tumor stemness, as evidence that LSECTin, a transmembrane protein highly expressed on TAMs, enhances the stemness of breast cancer cells by interacting with its receptor BTN3A3 located on breast cancer cells.<sup>124</sup>



**Figure 4** The crosstalk between CAFs/fibroblasts and CSCs. The crosstalk between CSCs and CAFs/fibroblasts is achieved by the secretion of a variety of cytokines, EV, exosomes, or other factors as indicated. Preclinical studies with mice have identified critical pathways that regulate CSC stemness and CAF/fibroblast biology (including recruitment, survival, and activation) during tumor progression. Targeting these key pathways can inhibit the crosstalk and CAFs/fibroblasts and impair tumor progression.

In canine mammary carcinomas, it was found that TAMs could facilitate CSC-induced tumor angiogenesis<sup>125</sup>; however, this effect was not elucidated in human cancer models, which could be explored in the future. TAMs can also increase the proportion of CSCs in lymphoma by secreting pleiotrophin (PTN) and thus activating the PTN/ $\beta$ -catenin pathway.<sup>126</sup> Although the critical roles of CSCs in tumorigenicity and drug resistance have been established, it is still fragmentary how TAMs regulate these two functional roles of CSCs. As a previous study demonstrated that milk-fat globule-epidermal growth factor-VIII (MFG-E8) from TAMs could confer CSCs with the ability to promote tumorigenicity and anticancer drug resistance by activating the signal transducer and activator of transcription-3 (STAT3) and sonic hedgehog pathways,<sup>127</sup> pharmacological targeting of key factors derived from TAMs might provide a unique strategy to eradicate therapy-resistant tumors by manipulating CSC activities. For example, emodin was found to block the lung metastasis of breast cancer by suppressing TAM-induced EMT and CSC aggregation<sup>128</sup>; the XIAOPI formula inhibits BCSCs by suppressing the TAM/C-X-C motif chemokine ligand 1 pathway.<sup>129</sup> Interestingly, another recent study showed that TAMs can be recruited by periostin (POSTN) in intrahepatic cholangiocarcinoma CSLCs,<sup>130</sup> indicating that there is a positive feedback mechanism in TAMs' promoting effects on CSLCs.

Notably, although the existence of CSCs has been confirmed in numerous tumor types and cell lines, and the corresponding sorting methods have been established,

there are different views on their origin, as CSCs may originate from stem cell mutation, cell fusion, embryonic stem cell residue, *etc.* A recent study demonstrated that cell-cell fusion could also contribute to the origin of different CSCs that may expand the overall CSC pool in a primary tumor.<sup>131</sup> TAMs were also confirmed to facilitate the CSC-like traits of breast cancer cells in a cell-cell fusion manner, thus TAM  $\times$  cancer cell hybrids.<sup>132</sup> Furthermore, the TAMs exposed to apoptotic cells also induced an increase in the proportion of CSCs.<sup>133</sup> Similarly, another study indicated that phagocytosis of apoptotic cancer cells by TAMs resulted in their transformation into tumor stem (initiating)-like cells.<sup>134</sup> Notably, the autoschizis-like products from CSCs had a higher activity for TAM development than those from non-CSCs.<sup>135</sup> Although limited results were presented, these results demonstrate that TAM-cancer cell fusion can facilitate cancer cell stemness or can be transformed into CSCs. Interestingly, Osman et al showed that CSCs had conversely been found to generate nontumorigenic cell types including TAMs, and this was the first study suggesting the potential differentiation of CSCs into TAMs.<sup>136,137</sup>

Mechanistically, TAMs can promote CSC progression through various endogenous factors within TAMs, including transcription factors and ligands.<sup>138</sup> Among the transcription factors, STAT3 was shown to be a critical regulator of TAM-mediated effects on CSCs; for example, TAMs can produce IL-6 and signal via the transcription factor STAT3 to promote the expansion of human hepatocellular CSCs, and



importantly, blockade of IL-6 signaling with FDA-approved tocilizumab indeed inhibits TAM-stimulated activity of CD44<sup>+</sup> hepatocellular CSCs.<sup>139</sup> Although the high infiltration of TAMs in both the tumor and stroma was found to be strongly and significantly correlated with the absence of NANOG expression in oral squamous cell carcinoma (OSCC),<sup>140</sup> TAMs were found to induce a CSC-like phenotype via IL-6 secretion and thus activate the STAT3 signaling, which subsequently and directly promoted the transcription of THBS1 in OSCC cells; conversely, exosome-transferred THBS1 activated TAMs, forming a feedback loop.<sup>141,142</sup> CSC-like phenotypes were characterized by the increased expression of stemness master regulators (Sox2, Oct4, Nanog, ABCG2, and SCA-1), in addition to the increased drug-efflux capacity, resistance to chemotherapy, and increased tumorigenicity *in vivo*. TAMs can induce murine BCSC-like traits through a novel epidermal growth factor receptor (EGFR)/STAT3/Sox2 signaling pathway, and targeting this regulatory axis using small molecule inhibitors against EGFR and STAT3, can block this crosstalk and the ability of TAM-induced CSC-like traits.<sup>143</sup> Additionally, transforming growth factor beta-induced (TGFB1), which is specifically expressed by TAMs, was found to be extremely low in GBM and GSC cells and could promote CSC-driven tumor growth through integrin  $\alpha v\beta 5$ -Src-STAT3 signaling,<sup>144</sup> indicating that high serum TGFB1 may serve as a potential diagnostic and prognostic bio-index for GBMs and the critical roles of STAT3 in TAM-mediated effects on CSCs. In addition, other transcription factors were shown to be involved in TAM-mediated CSC activity, such as Smad1/2<sup>145</sup> and arsenite-resistance protein 2 (ARS2).<sup>146</sup>

In addition to regulating the expression of stemness master regulators, TAMs can regulate CSC progression by modulating CSC markers, such as CD44, ALDH1, and CD133. The soluble glycoprotein NMB (GPNMB) produced by TAMs promotes cancer cell survival, the expansion of CSCs, and the acquisition of a metastatic phenotype through the CD44 receptor to activate tumor cells to express the cytokine IL-33 and its receptor IL-1R1.<sup>147</sup> Similarly, CD44 intracellular signaling in response to TAM signals can enhance the interaction between TAMs and CSCs at the tumor–stroma interface, which can serve as a vital area of focus for target and drug discovery.<sup>148</sup> Furthermore, TAM infiltration could up-regulate CD44 expression in a TNF- $\alpha$ - and NF- $\kappa$ B-dependent manner.<sup>149</sup> Importantly, the coexpression of CD44-positive/CD133-positive CSCs and CD204-positive TAMs was shown to be a useful predictor of PDAC survival.<sup>150</sup>

In contrast, CSCs or CSC-like cells can recruit themselves or TAMs to facilitate a pro-TME. Tao et al showed that Wnt-induced signaling protein 1 (WISP1) was preferentially expressed and secreted by GSCs, by which GSCs promote the survival of both GSCs and TAMs in an autocrine mechanism and a paracrine manner, respectively.<sup>151</sup> Cholangiocarcinoma CSCs could activate TAMs to shape a tumor-initiating niche by secreting cytokines, such as IL13 and IL34.<sup>152</sup> Consistently, CSCs remodeled their specific niche by recruiting monocytes/macrophages toward TAMs, and the CSC-recruited TAMs reciprocally promoted the stem-like properties of CSCs, progression, and androgen deprivation therapy (ADT) resistance of prostate cancer via IL6/STAT3 signaling.<sup>153</sup> Interestingly, CSC-induced macrophages

can be separated into two distinct subsets of cells, CD11c (low) and CD11c (high) cells, and only the CD11c (high) subset of cells has protumoral activity.<sup>154</sup> Lactate dehydrogenase A, a crucial glycolytic enzyme, was found to be higher in breast cancer tissues than in adjacent tissues and mediate a vicious cycle of mutual promotion between BCSC plasticity and TAM infiltration via CCL2.<sup>155</sup> Similarly, HCC CSC-like cells can recruit more TAMs to support their CSC-like traits by up-regulating secreted S100 calcium-binding protein A9 (S100A9), an inflammatory microenvironment-related secreted protein.<sup>156</sup>

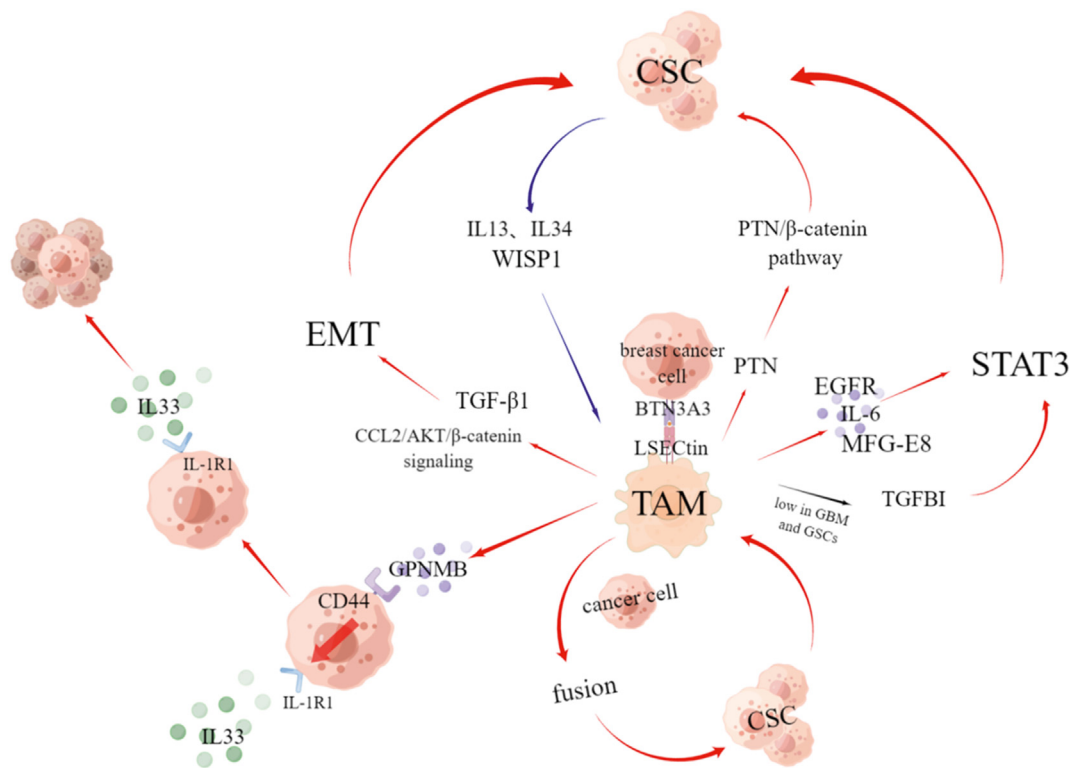
As we previously reviewed, targeting CSCs has been considered to hold the potential to treat and even cure tumors,<sup>28</sup> and the CSC-TAM interaction suggests that targeting these CSCs-TAMs may be a more powerful strategy than targeting CSCs alone. Liposomal triCurin, a synergistic combination of curcumin, epicatechin gallate, and resveratrol, was shown to repolarize TAMs and eliminate GBM and GSCs.<sup>157</sup> Rab13 sustained breast cancer stemness by supporting tumor cells to interact with TAMs, and targeting the Rab13-mediated BCSC niche with bardoxolone-methyl (C-28 methyl ester of 2-cyano-3, 12-dioxoolen-1, 9-dien-28-oic acid; CDDO-Me) prevented BCSC stemness.<sup>158</sup> Blocking the interplay between GSCs and TAMs by targeting ARS2/MAGL signaling using the MAGL-specific inhibitor, JZL184 is a potentially novel therapeutic option for GBM patients.<sup>146</sup> Additionally, 8-bromo-7-methoxychrysin (BrMC) reverses M2 polarization of TAMs induced by liver CSCs,<sup>159</sup> indicating that BrMC may be a potentially novel flavonoid agent that can be applied to disrupt the CSC-TAM interaction. Furthermore, phytosomal curcumin causes natural killer cell-dependent repolarization of GBM TAMs and stabilization of TAMs in the M1-like state to eliminate GBM and GSCs.<sup>160</sup> Furthermore, the combinatory targeting of CSCs and their interaction with TAMs effectively ameliorated ADT resistance in an orthotopic prostate cancer model.<sup>153</sup> Taken together, given the existence of CSC-TAM crosstalk during tumor progression, targeting CSC-TAM codependency is another promising strategy for cancer therapy (Fig. 5).

## Other immune cells

Except for TAMs, emerging experimental evidence has substantiated the interaction between CSCs and other immune cells, including MDSCs, T cells, and dendritic cells (DCs).

## T cells

Cytotoxic T lymphocytes (CTLs) are a subset of leukocytes that secrete various cytokines specifically for immune function. These cells build an important defense line of the body's anti-virus and antitumor immunity with natural killer (NK) cells by exerting killing effects on certain viruses, tumor cells, and other antigenic substances. During tumor progression, different types of CTLs are recruited to tumor lesions, including CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, CD3<sup>+</sup> CD56<sup>+</sup> T cells, Th17 cells, NK cells, and Tregs. Efficient antitumor immune responses include the direct killing of tumor cells by CD8<sup>+</sup> CTLs, which recognize tumor-associated antigen (TAA) in complex with MHC class I molecules. Spontaneous



**Figure 5** The crosstalk between TAMs and CSCs. At the junction of two phenotypes: TAM–CSC interactions. Both CSCs and TAMs release cytokines that mediate the interaction with each other. TAMs were also confirmed to facilitate the CSC-like traits of breast cancer cells through the cell–cell fusion manner, thus TAM  $\times$  cancer cell hybrids. Note that the different pathways shown have been studied in different cancer models (see text).

anti-CSC CTLs have been identified in various malignancies, including melanoma, adenocarcinoma, and leukemia.<sup>161–164</sup> Cioffi et al also developed a bispecific T-cell binding antibody, MT110, that efficiently targeted cytotoxic T cells to primary human pancreatic cancer cells, including a subset of highly tumorigenic CSCs.<sup>165</sup> Deleo et al proposed that ALDH1A1-specific CD8<sup>+</sup> T cells could recognize cells with CSC-like characteristics, such as ALDH (BREAT) cells in human tumor cell lines, xenograft tumors, and skin lesions, thereby killing tumors; these results support the potential of ALDH1A1-based immunotherapy to target CSCs.<sup>166</sup> Additionally, Di et al found naturally occurring multifunctional CD4<sup>+</sup> and CD8<sup>+</sup> T cells specific for the stem cell marker OCT4 among the peripheral blood mononuclear cells of both healthy individuals and ovarian cancer patients; functionally, Oct4-induced CD8<sup>+</sup> T cells could express interferon  $\gamma$  (IFN $\gamma$ )-inducible protein 10 (IP-10) and sufficient amounts of IFN $\gamma$  to induce IP-10 production, while OCT4-reactive CD4<sup>+</sup> T cells did not produce IFN $\gamma$  and IP-10 but were capable of proliferating upon stimulation with DCs loaded with an OCT4-derived peptide or OCT4 mRNA.<sup>161</sup> Furthermore, both CD8<sup>+</sup> T cells and IFN $\gamma$  could decrease CSC numbers in a 4T1 mouse model of breast cancer.<sup>167</sup> This effect suggests the presence of different types of anti-CSC-specific T cells in ovarian cancer patients. Notably, different types of tumors have different CSC markers; for example, the AC133 epitope of CD133 is an important CSC marker for GBM. Prasad et al reported a recombinant AC133  $\times$  CD3 bispecific antibody that

redirected human polyclonal T cells to AC133<sup>+</sup> GBM stem cells (GBM-SC), inducing their targeted lysis.<sup>162</sup>

Immune surveillance is an important mechanism to prevent the occurrence and development of tumors, and checkpoint inhibitors have shown remarkable success in cancer treatment.<sup>168</sup> These checkpoint inhibitors have also been shown to eliminate CSCs; for example, Wang et al found that CSCs could also evade the host immune response by up-regulating the immune checkpoint molecule CD276 (B7–H3), the antibody against which we can eliminate CSCs in a CD8<sup>+</sup> T-cell dependent manner and inhibit tumor growth and lymphatic metastasis in a mouse model of HNSCC.<sup>169</sup> In addition, Chen et al proposed that gamma delta T cells induced the up-regulation of MHC class I molecules and CD54 expression on the surface of CSC-like cells, thereby increasing the sensitivity of CD8<sup>+</sup> T cells to antigen-specific killing; and they further identified a powerful synergism between MHC-restricted and non-MHC-restricted T cells in the eradication of BCSCs.<sup>164</sup>

Tregs, a subset of tumor-infiltrating T lymphocytes expressing the transcription factor Foxp3, are one of the important factors to maintain the immune tolerance of the body.<sup>170</sup> They are produced by the thymus and exported to the periphery, and inhibit the activation and proliferation of potential self-reactive T cells in the normal body through active regulation, to regulate the immunity of the body. Recent studies have revealed that Tregs are tightly related to tumor progression, recurrence, and metastasis. Early breast cancer has an inflammatory milieu characterized by

myeloid DCs, Tregs, and CSC infiltration, and it is shown that the frequency of Tregs is associated with disease progression.<sup>171</sup> Using the murine spontaneous mammary tumor virus polyoma middle T (MMTV-PyMT) model, it has been found that Treg cell ablation increases the percentage of CSCs in the mammary compartment with a concomitant reduction in classically activated TAMs.<sup>172</sup> Furthermore, many studies indicate there is a direct and indirect interaction between Tregs and CSCs.<sup>173</sup> Intense infiltration of Tregs facilitates the qualities of GSCs through TGF- $\beta$  secretion that helps coordinately tumor growth; further mechanistic studies reveal that TGF- $\beta$  acts on cancer cells to induce the core CSC-related genes expression and sphere formation via the NF- $\kappa$ B-IL6-STAT3 signaling pathway, resulting in the increased cancer stemness and tumorigenic potential.<sup>174</sup> Besides, You et al indicated that among the various immune-related molecules, CCL5 was mostly elevated in ovarian CSCs relative to that in the non-CSCs; the expression of its receptor, CCR5, was also increased on the surface of Tregs in ovarian cancer patients.<sup>175</sup> They further revealed that ovarian CSCs recruited Tregs via this CCL5-CCR5 interaction, demonstrating that these two cell populations co-operated to promote tumor progression. In HNSCC, CD44<sup>+</sup> CSCs have been found to produce significantly higher levels of IL8, TGF- $\beta$ , and granulocyte colony-stimulating factor (G-CSF) than the CD44<sup>-</sup> cell subpopulation.<sup>176</sup> Moreover, CD44<sup>+</sup> HNSCC CSCs were shown to efficiently enhance Tregs response.<sup>176</sup> Similarly, Tregs were found to promote the stemness of acute myeloid leukemia (AML) via releasing anti-inflammatory cytokine IL-10 and subsequently activating the PI3K/AKT signal pathway.<sup>177</sup> Further transgenic mice model validated that blocking the interaction between Tregs and AML cells might be a new approach to target leukemia stem cells (LSCs) in AML treatment because disrupting the IL10/IL10R/PI3K/AKT signal prolonged mice survival and reduced the stemness of A/Ec leukemia cells.<sup>177</sup> Furthermore, Tregs are capable of inducing CRC-associated cell markers and driving the cells to be CSCs.<sup>178</sup>

## MDSCs

MDSCs are a heterogeneous group of myeloid cells that have been blocked in multiple differentiation stages, and they are a class of immature immunosuppressive cells. These cell accumulations have been observed in the blood, lymph nodes, bone marrow, and tumor sites of tumor patients, as well as in experimental animal models of cancer. These cells are characterized by their ability to suppress both innate and adaptive immune responses by secreting cytokines and thus hurt antitumor immunity<sup>179–181</sup>; for example, Cui et al found that MDSCs inhibited the corepressor gene C-terminal binding protein-2 (CtBP2) by activating the expression of microRNAs (miRNAs)-101 (miR-101) in cancer cells, and CtBP2 directly targeted stem cell core genes, thereby increasing the stemness, metastatic and tumorigenic capacity of cancer cells.<sup>182</sup> Consistently, Tang et al proposed that MDSCs induced IL6-dependent phosphorylation of STAT3 and activated Notch signaling via nitric oxide, thereby prolonging STAT3 activation and enhancing the stem cell-like properties of breast cancer

cells.<sup>183</sup> In addition, MDSC-derived exosomes are proposed to act as intercellular messengers to promote the stemness and growth of colorectal cancer cells via exosomal S100A9.<sup>184</sup> Furthermore, MDSC-generated PGE2 increases the stem cell-like properties and tumor PD-L1 expression in epithelial ovarian cancer.<sup>185</sup> Therefore, depleting MDSCs may be therapeutically effective against cancer by reducing the number of CSCs.

## DCs

DCs are another type of lymphoid and myeloid stem cell derived from the bone marrow and are present in all lymphoid organs including the thymus, spleen, and lymph nodes, and in almost all nonlymphoid tissues and organs.<sup>186</sup> DCs are the most powerful antigen-presenting cells, capable of stimulating naive cells, memory cells, and effector T cells, and play a key role in initiating immune responses. In recent years, DC-based vaccines have become promising antitumor immunotherapy.<sup>187–189</sup> One active immunotherapy strategy uses DC-based vaccination to initiate T-cell-mediated antitumor immunity, and there is much evidence showing that using human CSC antigens may improve the antitumor effect of DC vaccination against human cancer. For example, Xu et al demonstrated that DCs inoculated with GBM CSCs could induce anti-CSC CTLs and prolong the survival time of GBM CSC tumor animals.<sup>190</sup> Xu et al used A2B5 mAb to sort GSCs derived from the mouse GL261 glioma cell line and treated mouse brain gliomas with DCs induced by A2B5<sup>+</sup> GL261 cell lysates. They found that these DCs had a stronger immune response to A2B5<sup>+</sup> GL261 cells than that induced by A2B5-GL261 cell lysate.<sup>191</sup> Lu et al confirmed that CSCs enriched by the CSC marker ALDH could induce the generation of DCs, thereby significantly inhibiting tumor growth, reducing the development of lung metastases, and prolonging survival time.<sup>192</sup> In addition, CSC lysate could be used to induce DC generation in C57BL/6 mice, and injecting these CSC lysate-pulsed cells significantly reduced ALDH<sup>high</sup> CSC frequency in primary tumors and tumor growth, reduced the development of pulmonary metastases, and prolonged survival.<sup>193</sup> Similarly, Zhang et al found that DCs pulsed with antigen derived from CD105<sup>+</sup> human renal cell carcinoma (RCC) cells could induce mice to produce functionally specific activated T cells and specific antibodies against CSCs and significantly inhibited tumor growth in mice.<sup>194</sup> Yang et al found that DCs and autologous cytokine-induced killer (CIK) cells significantly increased the tumor cell apoptosis rate by increasing caspase-3 protein expression and decreasing proliferating cell nuclear antigen (PCNA) protein expression, and this DC-CIK combinatory treatment had a significant inhibitory effect on tumor growth in nude mice induced by liver CSCs.<sup>195</sup> Notably, mRNA is a naturally occurring molecule with the "blueprint" of human cells, which can produce target proteins or immunogens and activate immune responses *in vivo* to fight against various pathogens. The mRNA vaccine uses the gene sequence of the virus rather than the virus itself. Therefore, the mRNA vaccine has no viral components and no risk of infection.<sup>196</sup> Recently, many preclinical experiments have indicated that mRNA vaccines could be used in cancer immunotherapy.<sup>197</sup>

Importantly, an example of a clinical study (NCT00846456) amplified CSC-mRNA from CSCs and introduced it to monocyte-derived autologous DCs, which were vaccinated in seven patients; an immune response induced by vaccination and prolonged progression-free survival were identified in all seven patients and no patients developed adverse autoimmune events or other side effects<sup>198</sup> (Fig. 6).

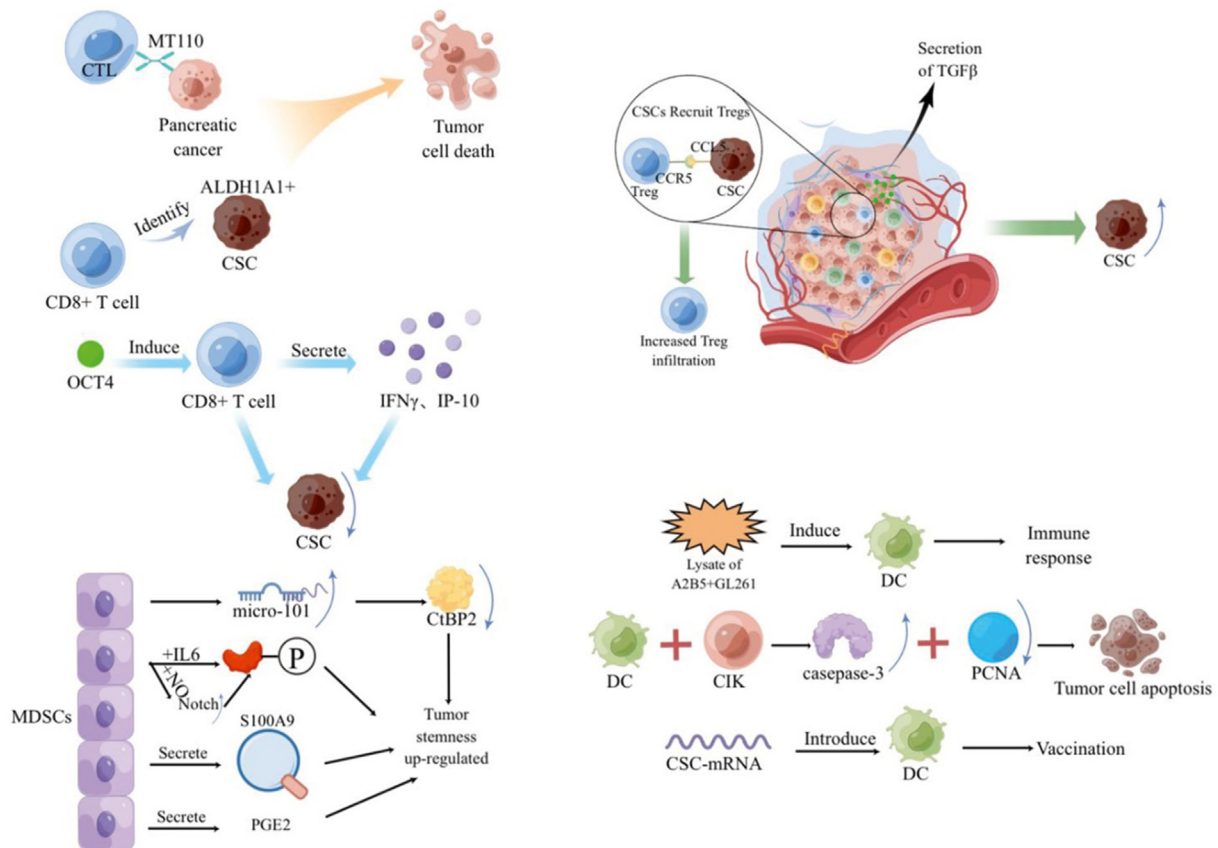
### Three medium components in the tumor microenvironment

#### Cytokines

CSCs regulate multiple cancer hallmarks through interactions with cells and the ECM in their environment by secreting EVs, including exosomes, and soluble factors, such as interleukins, cytokines, growth factors, and other metabolites, into the TME.<sup>199</sup> Cytokines (CKs) include interferon, chemokine, growth factor, tumor necrosis factor superfamily, colony-stimulating factor, interleukin, etc., and many of them participate in the formation and stemness maintenance of CSCs. CKs in the TME can adjust the self-renewal and survival of CSCs in many ways.<sup>200</sup>

The maintenance of CSCs is essentially attributed to the synergistic effect between interleukins, such as IL-6, IL-8,

IL-17, and IL-30. In breast cancer, IL-6 secreted by non-stem cells can activate the JAK1-STAT3 signaling, stimulate the expression of the Oct4 gene, and promote the conversion of non-stem cells of breast cancer into BCSCs, resulting in drug resistance in breast cancer.<sup>201</sup> Similarly, IL-6 signaling is amplified by breast cancer cell-intrinsic C/EBP $\delta$  to promote the CSC-like phenotypes.<sup>202</sup> IL-6 secreted by myofibroblasts activates HES1 to expand the CSC population in early colorectal tumors<sup>203</sup> and ovarian cancer.<sup>204</sup> Consistently, this promoting role of IL-6 in CSC activity is repeated in other tumors, such as HNSCC<sup>205,206</sup> and lung cancer.<sup>207</sup> Therefore, targeting IL-6 and the related signaling pathways has been shown to be a promising therapeutic target for CSC. Syndecan-1 ablation significantly suppresses the stemness of BCSCs by down-regulating IL-6, IL-8, gp130, Hey-1, EGFR, and p-Akt via Notch signaling.<sup>208</sup> Another study also indicated that the oncogene multiple copies in T-cell malignancy 1 (MCT-1/MCTS1) stimulated IL-6 secretion, which subsequently promoted monocytic THP-1 polarization into M2-like macrophages to increase the features of BCSCs, and this effect was further advanced by IL-6 but prevented by tocilizumab, a humanized IL-6R antibody.<sup>209</sup> Consistently, Aoi et al found that IL-6, which was expressed in lung-induced CSCs, facilitated the formation of lung cancer organoids via the conversion of MSCs into  $\alpha$ SMA<sup>+</sup> cells. Interestingly, the combination of anti-IL-6 antibody and cisplatin could destroy lung cancer



**Figure 6** CSC-immune cell crosstalk and interactions among immune cells in cancer. Different types of CSC-immune cell crosstalk (including CSC-T cells, CSC-T-reg cells, CSC-MDSCs, and CSC-DCs) in cancer. The crosstalk between two cell types is achieved by the secretion of a variety of chemokines, cytokines, exosomes, or other factors as indicated.



organoids, while cisplatin alone could not.<sup>43</sup> Tobacco smoke critically contributes to the development of lung cancer by inducing CSC-like traits in human bronchial epithelial cells, which can be attenuated by sulforaphane by targeting IL-mediated signaling.<sup>210</sup> A similar result was obtained in gastric cancer patients.<sup>211</sup> Some natural compounds were also confirmed to inhibit CSCs by modulating IL-6 signaling; for example, catechol derived from aronia juice through lactic acid bacteria fermentation inhibits BCSC formation by modulating the Stat3/IL-6 signaling pathway<sup>212</sup>; 5-desmethylsinensetin isolated from *Artemisia princeps* and Tanshinone IIA suppresses the stemness of breast cancer cells via the Stat3/IL-6 signaling<sup>213,214</sup>; and esculetoside A suppresses BCSC growth by blocking the IL-6/STAT3 signaling pathway.<sup>215</sup> In HNSCC, therapeutic inhibition of IL-6 with low-dose MEDI5117 decreases the fraction of CSCs, and adjuvant MEDI5117 inhibits the recurrence in preclinical models.<sup>216</sup> Notably, Nazari et al established a mathematical model for IL-6-mediated, stem cell-driven tumor growth and targeted treatment. This model can quantify the temporal changes in fractional occupancies of bound receptors and their influence on tumor volume.<sup>217</sup>

In addition, IL-8 produced by breast cancer cells after chemotherapy withdrawal can enhance the activity of BCSCs and generate tumor resistance through its receptor CXCR1/2, and up-regulation of IL-8/CXCR1/2 predicts a poor prognosis in breast cancer patients.<sup>218</sup> Using a mouse model of squamous cell carcinoma, Oshimori et al found that IL-33-responding Fc $\epsilon$ RI $\alpha$ <sup>+</sup> macrophages sent paracrine TGF- $\beta$  signals to CSCs, inducing invasive and drug-resistant properties and further up-regulating IL-33 expression, forming an IL-33-TGF- $\beta$  feedforward loop.<sup>219</sup> Prostate CSC-derived IL30 supported its viability, self-renewal, and tumorigenicity, and these effects were remarkably hindered by IL30 knockdown or knockout.<sup>220</sup> A similar result was acquired in breast cancer demonstrating that constitutive expression of membrane-bound IL30 enhanced BCSC activity by juxtacrine signals and via second-level mediators, mainly CXCL10 and IL23, and targeting IL30 could indeed restrain the BCSC compartment and counteract BC progression.<sup>221</sup> Moreover, numerous studies have revealed the critical roles of interleukin-17A (IL-17A) during CSC maintenance. Fiorenza Lotti et al previously indicated that chemotherapy-treated human CAFs could promote the self-renewal and tumorigenic ability of colorectal CSCs by secreting IL-17A.<sup>222</sup> Subsequently, IL-17 derived from other cell sources has also been shown to exert the same effect. For example, IL-17A secreted by lymphatic ECs promotes the tumorigenesis of hepatoma stem cells<sup>223</sup>; IL-17 from TAMs contributes to ovarian cancer malignancy by promoting the self-renewal of CSCs<sup>224</sup>; immune cell production of IL-17 induces stem cell features of pancreatic intraepithelial neoplasia cells.<sup>225</sup> Moreover, long-term stimulation of normal progenitor cells with IL-17 leads to their transformation into CSCs,<sup>226</sup> and recombinant IL-17B (rIL-17B) can also promote the sphere-formation ability and tumor growth of CSCs.<sup>227</sup> However, a recent study indicated that IL-17A did not promote stem cell expansion on PDAC cell lines and that blocking the IL-17A/IL-17RA signaling did not interfere with pancreatic cancer development and progression.<sup>228</sup> Thus, the effects of the IL-17A/IL-17RA signaling in PDAC progression need to be

further explored and currently targeting IL-17A/IL-17RA signaling may not be considered as a promising monotherapy for PDAC. These results suggest that IL-17 and its signaling pathway might serve as therapeutic targets for tumor treatment by targeting CSCs. Moreover, some other interleukins in the TME have also been shown to be involved in CSC progression, such as IL-33,<sup>229</sup> IL-23,<sup>230</sup> IL-32 $\gamma$ ,<sup>231</sup> IL-4,<sup>232</sup> and IL-12.<sup>233</sup>

Growth factor EGFR-mediated interleukin enhancer-binding Factor 3 (ILF3) contributes to the formation and survival of cancer stem-like tumorspheres in NSCLC cells, which can be suppressed by YM155, an inhibitor of ILF3.<sup>234</sup> Although numerous studies have indicated the promoting roles of TGF- $\beta$  in CSC activity,<sup>235</sup> a previous study demonstrated that growth differentiation factor 15 (GDF15), a member of the TGF- $\beta$  family, but not TGF- $\beta$ , might maintain CSC-like cells in breast cancer tissues by inducing its expression in an autocrine/paracrine manner.<sup>236</sup> In addition, cytokines, which result in tumor resistance by facilitating and maintaining CSCs, also include bFGF and the chemokine CCL2.<sup>200</sup> However, in gastric cancer, miR-106b modulates CSC characteristics through the TGF- $\beta$ /Smad signaling in CD44<sup>+</sup> gastric cancer cells.<sup>237</sup> In summary, in the TME, the cytokine network can not only be a bond between CSCs and many motivators, but cytokines themselves can also be promoting and maintenance factors of CSCs<sup>238</sup> (Table 1).

## MiRNAs

MiRNAs are a class of naturally noncoding short RNAs that negatively regulate the expression of target genes by repressing translation or cleaving mRNA.<sup>239</sup> As important regulatory factors of gene expression, miRNAs have critical roles in the self-renewal, pluripotency, differentiation, and tumorigenicity of CSCs. Recent studies indicate that some aberrantly-expressed miRNAs up-regulate the distribution of the CSC proportion in tumor tissue, and thus facilitate cancer recurrence, metastasis, and resistance.<sup>240</sup> For example, miR-1301-3p can promote the expansion of prostatic CSCs by inhibiting GSK3 $\beta$  and SFRP1 and thus activate the Wnt- $\beta$ -catenin pathway.<sup>241</sup> Similarly, miR-19b/20a/92a target E2F1 and HIPK1, activate the  $\beta$ -catenin signal transduction pathway, and thus facilitate the self-renewal ability of CSCs.<sup>242</sup> For glioma CSCs, a miR-33a-centered signaling network has been shown to promote glioma CSC maintenance by targeting phosphodiesterase 8A (PDE8A) and UV radiation resistance-associated gene (UVRAG).<sup>243</sup> We previously showed that miR-9 levels were positively correlated with the stage of breast cancer,<sup>244</sup> and another study further indicated that miR-9 and miR-221 could promote the formation of BCSCs.<sup>245</sup> In other tumors, miRNAs have also been reported to increase the stemness of CSCs; for example, miR-3120-5p can promote the stemness and invasiveness of colon CSCs by targeting Axin2.<sup>246</sup> Malignant transformation of tissue stem cells may be the root of most cancer. Accordingly, Vignesh et al identified miRNA expression patterns in the normal human colonic stem cell niche to understand how CSCs arose and found that miR-23b enhanced CSC phenotypes globally by targeting LGR5.<sup>247</sup> DDX17 is a cofactor of the Drosha-DGCR8 complex in miRNA

**Table 1** Cytokines involved in the TME of CSCs.

Cytokines	Stem cell	Potential target	Reference
IL-6	Breast CSCs	JAK1-STAT3 signaling	201
	Colorectal tumor and ovarian Cancer	HES1 gene	203,204
	Breast CSCs	MCT-1/MCTS1 gene	209
	Lung-induced CSCs	MSCs cell	43
Syndecan-1	Breast CSCs	Notch signaling	208
Sulforaphane	Lung cancer and gastric CSCs	IL-mediated signaling	210,211
Catechol	Breast CSCs	Stat3/IL-6	212
5-Desmethylsinensetin	Breast CSCs	Stat3/IL-6 signaling	213,214
Tanshinone IIA	Breast CSCs	Stat3/IL-6 signaling	213,214
Esculentoside A	Breast CSCs	IL-6/STAT3 signaling	215
MEDI5117	HNSCC	IL-6	216
IL-8	Breast CSCs	CXCR1/2	218
IL-33	Mouse model of squamous cell	TGF- $\beta$ signals	219
IL30	Breast CSCs	CXCL10 and IL23	221
IL-17A	Colorectal CSCs	Colorectal CSCs	222
	Hepatoma stem cells	Hepatoma stem cells	223
	Ovarian cancer	Ovarian cancer	224
	Intraepithelial neoplasia cells	Intraepithelial neoplasia cells	225
	Normal progenitor cell	Normal progenitor cell	226
EGFR	NSCLC cells	ILF3	234
miR-106b	Gastric cancer	TGF- $\beta$ /Smad signaling	237

biogenesis and transcriptional coactivator<sup>248</sup> and has been associated with CSC-like properties. Kao et al found that hypoxia-induced polyubiquitination of DDX17 could control its dissociation from the DROSHA-DCGR8 complex to reduce the biogenesis of anti-stemness miRNAs and thus enhance the transcription of stemness-related genes, suggesting that miRNA biogenesis was critical for maintaining cancer stemness.<sup>248</sup> Interestingly, Claudio et al also found that the miRNA profile of Ewing sarcoma family tumor (ESFT) CSCs was shared by embryonic stem cells and CSCs from divergent tumor types; mechanistically, they found that the miRNA profiles of ESFT CSCs were the result of reversible disruption of TARBP2-dependent miRNA maturation by restoring TARBP2 activity or systematically delivering synthetic forms of its two targets, miRNA-143 or miRNA-145.<sup>249</sup> Notably, chemotherapy has been shown to confer stemness to cancer cells,<sup>250</sup> and miRNAs have also been shown to play critical roles during chemotherapy-induced stemness. Xue et al revealed that chemotherapy significantly increased cell stemness by up-regulating GM-CSF expression and secretion, which subsequently increased miR-877-3p expression in gastric cancer.<sup>251</sup>

However, some miRNAs can also down-regulate the distribution of the CSC proportion in tumor tissue; for example, miR-34a inhibits the self-renewal ability of BCSCs by targeting IGF-II mRNA binding protein (IMP3), which has been shown to be highly expressed in BCSCs.<sup>252</sup> miR-34c-5p, another miR-34 family miRNA, can also inhibit the growth of ovarian CSCs by down-regulating the AREG-EGFR-ERK pathway.<sup>253</sup> Similarly, miR-628-5p can induce apoptosis of epithelial cells of ovarian cancer to decrease the percentage of ovarian CSCs.<sup>254</sup> miR-128 can directly target the stem cell regulator BMI-1 to inhibit tumor regeneration in

multiple prostate cancer xenograft models.<sup>255</sup> The miR-200 family of microRNAs has been shown to be involved in inhibiting EMT in various cancers.<sup>256</sup> They play a significant role in inhibiting mammary tumor growth and progression, and their members are being investigated as therapeutic targets.<sup>257</sup> It was also confirmed that miR-200 could suppress the self-renewal and differentiation of CSCs, regulate cell division and apoptosis, and reverse chemotherapy resistance.<sup>258</sup> Additionally, Let7d has been shown to be down-regulated in osteosarcoma CSCs and decrease their sphere- and colony-forming ability.<sup>259</sup> Our previous studies also indicated that miR-375 was expressed at low levels in tumor tissues and could suppress the stemness of breast cancer and gastric cancer cells.<sup>260,261</sup> CSCs belong to the tumor cell subpopulation at the G0 phase.<sup>262</sup> MJA-miR-35-3P can inhibit the proliferation of CSCs but has no effect on cancer non-stem cells by arresting the cell cycle in the G1 phase and inducing apoptosis by targeting the human peptidylprolyl cis/trans isomerase, never in mitosis gene a-interacting 1 (PIN1) gene, which is up-regulated in CSCs.<sup>263</sup>

Furthermore, miRNAs are engaged in the transformation of other cells into CSCs or CSCs-mediated malignant transformation, just as Fouad et al showed that long-term stimulation of liver progenitor cell (LPC) with IL-17 led to their transformation into CSCs, in which miR-122 expression was led to a 90% decrease and restored-expression of miR-122 could attenuate this transformation.<sup>226</sup> Acute or chronic exposure to arsenite could confer the stem cell-like properties of human liver epithelial L-02 cells, and during this process, miR-191 level was up-regulated and inhibition of miR-191 suppressed the acquired stem cell-like phenotype.<sup>264</sup> Dai et al also indicated that down-regulation of miR-146a-5p led to overexpression of its target gene,

heterogeneous nuclear ribonucleoprotein D, thereby promoting the malignant transformation of MSCs during interactions with glioma CSCs.<sup>265</sup>

Additionally, miRNAs are essential players in the cross-talk between CSCs and the TME. Exosomes or EVs carry biologically active miRNAs to shuttle between CSCs and the TME, thereby affecting CSC activity. On the one hand, exosome- or EV-carrying miRNAs can be used by the TME to support CSC progression. Recent work demonstrated that CRC-derived exosomes enhanced the stemness properties of CRC cells via the delivery of miR-19b *in vitro* and *in vivo*.<sup>266</sup> In AML cell-derived EVs, miR-1246 was highly-expressed and directly targeted LRIG1 to activate the STAT3 pathway, promoting the survival of leukemia stem cells.<sup>267</sup> Exosomal miR-126 derived from hepatoblastoma cells promoted the tumorigenesis of liver cancer by inducing the differentiation of BMSCs into cancer stem cells.<sup>268</sup> Furthermore, chemotherapy could also elicit exosomal miRNAs to facilitate cancer stemness or CSC activity; for example, chemotherapy-elicited exosomal miR-378a-3p and miR-378d led to activation of the Wnt and Notch stem cell pathways by targeting DKK3 and NUMB, thus increasing breast cancer stemness.<sup>269</sup> Similarly, chemotherapy-induced breast cancer cells secreted multiple EV miRNAs, including miR-9-5p, miR-195-5p, and miR-203a-3p, which simultaneously targeted the transcription factor One Cut Homeobox 2 (ONECUT2), leading to the induction of CSC traits.<sup>270</sup> Interestingly, exosomal miRNAs from one cancer type can be internalized and promote other cancer stemness, exactly as Wang et al showed that exosomal release of miR-454 by breast cancer cells sustained the biological properties of CSCs in ovarian cancer,<sup>271</sup> suggesting that exosomal miRNAs could be used as a common tool to treat cancer. Except for tumor cells, other cell types in the TME can also utilize exosome- or EV-included miRNAs to support CSC activity. TNBC cells after being internalized with EVs derived from Wharton's Jelly MSCs show impaired stem properties under hypoxic conditions. These inhibitory effects may be involved in the transfer of miRNA-125b from MSC-EVs to TNBC cells.<sup>272</sup> M2 macrophage-derived exosomal miR-27a-3p promotes the cancer stemness of HCC by down-regulating thioredoxin-interacting protein (TXNIP).<sup>273</sup> miR-21a-5p is also responsible for M2 macrophage-derived exosome-induced promoting effects on the activity of pancreatic cancer stem cells by targeting KLF3.<sup>274</sup>

On the other hand, exosome- or EV-carrying miRNAs can be used by CSCs to establish a favorable environment. A recent study has shown that miR-1246 is remarkably enriched in cisplatin-resistant lung cancer cells/exosomes and spheres, and these exosomes enhance the stemness of parental lung cancer cells; mechanistically, this promoting effect is attenuated by miR-1246 inhibition.<sup>275</sup> Exosomal miR-500a-3p is also found to have the same effect in gastric cancer.<sup>276</sup> Similarly, the expression of miR-9 was found to be significantly higher in glioma CSCs and glioma CSC-derived EVs than in GBM cells. Functional experiments showed that glioma CSC-derived EVs promoted GBM growth and migration, which could be suppressed by miR-9 inhibition.<sup>277</sup> CSC-derived exosomal miR-210-3p can promote the migration, invasion, or drug resistance of cancer cells in lung cancer and pancreatic cancer.<sup>278,279</sup> Additionally, CSC exosomes can transport miR-19b-3p into clear cell renal cell

carcinoma cells and initiate EMT to promote metastasis.<sup>280</sup> Glioma CSC-derived exosomes overexpressing miR-26a or miR-21 contribute to the enhanced proliferation and angiogenesis of human brain microvascular endothelial cells, highlighting an angiogenic role of CSC-derived exosomal miRNAs.<sup>281,282</sup> Interestingly, hypoxic glioma CSC-derived EVs exert a greater effect on GBM chemoresistance than those from normoxic glioma CSCs, which is contributed by EV-packaged miR-30b-3p.<sup>283</sup> Notably, a recent study indicated that glioma CSC-derived exosomal miR-944 could reduce glioma growth and angiogenesis by inhibiting the AKT/ERK signaling,<sup>284</sup> and overexpression of exogenous miR-504 also resulted in its delivery to cocultured microglia by glioma CSC-secreted EVs and subsequently harbored glioma CSC-derived xenografts,<sup>285</sup> suggesting that not all miRNAs derived from CSC exosomes played carcinogenic roles. Besides, the antitumor activity of EVs derived from different CSC sources has been investigated between human liver CSCs and renal CSCs<sup>286</sup> (Table 2).

### Hypoxic environment/hypoxia-inducible factor

Hypoxia is considered to be a major feature of the TME and is a potential contributor to the CSC phenotype and enhanced tumorigenicity. Through a flow cytometry assay, a previous study showed that hypoxia can enrich the side population of thyroid CSCs, proving that hypoxia can increase cancer cell stemness.<sup>287</sup> Consistently, a recent study freshly isolated hypoxic tumor cells from xenografts and found that these cells contain increased subpopulations of tumor cells with CSC-like characteristics.<sup>288</sup> Wu et al proposed that the hypoxic microenvironment might enhance the stem cell-like biological properties of laryngeal cancer cell lines by expanding the proportion of CD133<sup>+</sup> stem cells.<sup>289</sup> In addition, hypoxia induces EMT, metastasis, and drug resistance through complex machinery, which is also accompanied by changes in the expression level of stemness-related genes or stemness up-regulation.<sup>290,291</sup> The effects induced by low oxygen levels are orchestrated by hypoxia-inducible factors (HIFs), which regulate the expression of numerous genes involved in cancer progression by mediating various pathways. Hypoxia induces up-regulation of HIF-1 $\alpha$  expression, promotes the self-renewal capacity of CD133-positive human glioma-derived CSCs, and inhibits the induced differentiation of CSCs.<sup>292</sup> Hypoxia-inducible effects are primarily regulated by HIF,<sup>293</sup> which up-regulates many hypoxia-inducible pathways (such as Akt, mTOR, Notch, TGF- $\beta$ , and ER- $\alpha$ ), and promotes and maintains CSC features.<sup>290,293</sup> Under hypoxic conditions, HIF-1 $\alpha$  levels are greatly increased in glioma CSC cells, and increased HIF-1 $\alpha$  activates the JAK1/2-STAT3 axis and enhances the self-renewal ability of glioma CSCs.<sup>294</sup> Lan et al<sup>295</sup> also demonstrated that hypoxia increased the expression of adenosine receptor 2B (A2BR) in human breast cancer cells through the transcriptional activity of HIF-1. Then the binding of adenosine to A2BR promoted BCSC enrichment by activating protein kinase C- $\delta$  (PKC- $\delta$ ), which phosphorylated and activated the transcription factor STAT3, leading to increased expression of IL-6 and NANOG, two key mediators of the BCSC phenotype. Cui et al found that hypoxia could promote the deoxyribosylation

**Table 2** miRNAs involved in the TME of CSCs.

Stem cell	miRNAs	Potential target	Reference
Prostatic CSCs	miR-1301-3p	GSK3 $\beta$ , SFRP1	241
	miR-19b/20a/92a	E2F1, HIPK1	242
	miR-128	BMI-1	255
	miR-21a-5p	KLF3	274
Glioma CSC	miR-33a	PDE8A	243
	miR-146a-5p	HNRD	265
	miR-944	AKT/ERK signaling	284
Colon CSCs	miR-3120-5p	Axin2	246
CSCs	miR-23b	LGR5	247
ESFT CSCs	miRNA-143, miRNA-145	TARBP2	249
BCSCs	miR-34a	IMP3	252
	miR-378a-3p, miR-378d	DKK3, NUMB	269
	miR-9-5p, miR-195-5p, miR-203a-3p	ONECUT2	270
Ovarian CSCs	miR-34c-5p	AREG-EGFR-ERK	253
Leukemia stem cells	miR-1246	LRIG1	267
Liver cancer	miR-126	BMSCs	268
	miR-27a-3p	TXNIP	273

of HIF-1 $\alpha$  by SENP1 and increase the stability and transcriptional activity of HIF-1 $\alpha$ , thereby enhancing the stemness of hepatoma cells and the occurrence of hepatocarcinogenesis.<sup>296</sup> Yeung et al indicated that HIF-1 $\alpha$  was an important mediator of the effects of hypoxia on the clonogenicity and differentiation of CSCs differentiation.<sup>297</sup> Importantly, HIF-1 $\alpha$  also controls the expression of alarmin receptors in tumor cells that can bind to and be activated by alarmins. Hypoxic CSCs can express alarmin receptors that can bind alarmins released during necrosis, an event favoring CSC migration.<sup>298</sup> In addition to HIF-1 $\alpha$ , HIF-2 $\alpha$  also plays critical roles in the hypoxia-mediated TME facilitating CSC progression. Hypoxia induces up-regulation of HIF-2 $\alpha$ , which makes ovarian CSCs resistant to doxorubicin by promoting the expression of breast cancer resistance protein and the transport of doxorubicin<sup>299</sup>. Hypoxia-related factors switch the hypoxia response of cancer cells from HIF-1 $\alpha$ -dependent to HIF-2 $\alpha$ -dependent transcription and activate genes associated with invasion, such as MMP9, PAI-1, and stem cell factor Oct-3/4, promoting tumorigenesis progression and resistance to therapy.<sup>300</sup> Furthermore, metabolic adaptations caused by hypoxia could induce the CSC-like traits of cancer cells. Hypoxia induces the HIF-2 $\alpha$ -SOD2-mtROS-PDI/GRP78-UPR<sup>ER</sup> axis, but not HIF-1 $\alpha$ , which links mitochondrial metabolic status to endoplasmic reticulum responses through mitochondrial ROS (mtROS) levels, and induces stemness in breast cancer.<sup>301</sup> In addition, hypoxia can induce the CSC phenotype in a HIF-1 $\alpha$ - and HIF-2 $\alpha$ -dependent manner in breast cancer and endometrial cancer.<sup>302,303</sup> Therefore, the shared but distinct roles of hypoxia-inducible factors HIF-1 $\alpha$  and HIF-2 $\alpha$  exist in the TME of CSCs.

Additionally, hypoxia induces cancer stemness or CSC amplification by regulating other stemness master regulators. For example, Xiao et al found that hypoxia might increase the number of hepatocellular CSCs by altering the AR/miR-520F-3p/SOX9 signaling pathway.<sup>304</sup> Hypoxia could induce the down-regulation of dual-specificity phosphatase 2 (DUSP2), leading to the overproduction of COX-2-derived

prostaglandin E2, which promoted tumor stemness through the EP2/EP4 signaling pathway in CRC.<sup>305</sup> In neuronal tumor cells, hypoxia promotes the CSC-like function and tumorigenicity by increasing the expression of the stem cell gene DLK1.<sup>306</sup> Although hypoxia-induced chemoresistance and stemness is a well-recognized phenomenon, in which HIF-1 $\alpha$  is believed to be a key player, Soleymani Abyaneh et al showed that STAT3 rather than HIF-1 $\alpha$  was important in mediating HICR to cisplatin in MDA-MB-231 cells.<sup>307</sup> Under hypoxic conditions, glioma CSCs can rapidly release myosin-like protein 1 (MBNL1) from the nucleus, resulting in significant inhibition of MBNL1 activity, thereby impairing the ability of MBNL1 isoforms to inhibit the self-renewal and tumor-initiation ability of GCSCs.<sup>308</sup> Hypoxia-induced myeloid-derived growth factor (MYDGF) can directly affect the self-renewal of liver CSCs, indirectly aggravate the inflammatory microenvironment, and accelerate the progression of hepatocellular carcinoma.<sup>309</sup> Overexpression of epithelial cell adhesion molecule (EpcAM) can increase the expression of breast cancer stemness markers (NANOG, SOX2, and OCT4) and EMT markers (N-cadherin and vimentin) under hypoxic conditions.<sup>310</sup> Moreover, hypoxia can also drive tumor progression by altering epigenetic controls, as Wu et al found that hypoxia deregulated TET1 and TET3, both catalyzing the conversion of 5-methylcytosine (5 mC) to 5-hydroxymethylcytosine (5hmC), leading to BCSC properties.<sup>311</sup> Another study showed that TET1 and TET3 could be actively demethylated under hypoxic conditions, promoting the up-regulation of Oct4 and Nanog expression, thereby contributing to the formation of CSCs in glioma<sup>312</sup> (Table 3).

## Prospects and conclusions

At present, the 5-year survival rate of malignant tumor patients has been greatly improved, which is closely related to the emergence of early screening, diagnosis, treatment, and new treatment methods. However, there are still some



**Table 3** HIFs and hypoxic environment involved in the TME of CSCs.

Hypoxia-inducible factor/hypoxic environment	Stem cell	Potential target	Reference
Hypoxic environment	Laryngeal cancer	CD133 <sup>+</sup> stem cells	289
	Hepatoma cells	SENP1	296
	Breast cancer	HIF-2 $\alpha$ -SOD2-mtROS-PDI/GRP78-UPR(ER) axis	301
	Hepatocellular CSCs	AR/miR-520F-3p/SOX9 signaling pathway	304
	CRC	DUSP2	305
	Neuronal tumor cells	Stem cell gene DLK1	306
	Breast cancer	STAT3	307
	GCSCs	MBNL1	308
	Liver CSCs	MYDGF	309
	Breast cancer	EpCAM	310
	BCSCs	TET1 and TET3	311
HIF-1 $\alpha$	Glioma-derived CSCs	Glioma-derived CSCs	292
	Glioma CSCs	JAK1/2-STA T3 axis	294
	BCSCs	A2BR	295
HIF-2 $\alpha$	Hypoxic CSCs	Alarmin receptors	298
	Ovarian CSCs	Resistance protein	299
	Cancer cells	MMP9, PAI-1, Oct-3/4	300

patients with tumor recurrence or metastasis, and even treatment resistance after multiple treatments, ultimately leading to treatment failure. Previous studies have confirmed that these phenomena are closely related to the existence of CSCs. The complex local TME is an important factor for the formation and maintenance of CSCs, including MSCs, ECs, CAFs, and immune cells (including TAMs, Tregs, MDSCs, and Th17). On the one hand, CSCs can remodel the TME through the interaction of these stromal cells, which is conducive to the formation and maintenance of CSCs. On the other hand, these stromal cells can secrete different cytokines, growth factors, exosomes, and EVs, and stimulate the self-renewal, proliferation, and differentiation of CSCs, thus promoting tumorigenesis, progression, and immunosuppression.

It has been confirmed that several methods can be used to kill CSCs. The first strategy is to directly attach to CSC themselves. As we previously reviewed,<sup>28</sup> many FDA-approved drugs that have been confirmed to kill CSCs, such as Wnt inhibitors (niclosamide, TFP, DTX, and SFN, PP, AD, and Ts), Notch inhibitors (DAPT), Hh inhibitors (glasdegib, sonidegib, vismodegib, ciclesonide), Hippo inhibitors (verteporfin, fluvastatin, atorvastatin, CPZ), autophagy regulators (CQ, HCQ, pantoprazole), and ferroptosis inducers (TMZ and CQ, artesunate, ferumoxitol, sulfasalazine, salinomycin). Although these approved drugs are not defined as CSC-targeting drugs, these drug targets play a key role in the activation of the signaling pathways in CSCs, which also indicates that they have a potential inhibitory function on CSCs. In addition, we recently screened and found a kind of phenazine derivative that could specifically attenuate the CSC-like traits of breast cancer cells by triggering ferroptosis,<sup>313</sup> which could be used for future research. However, it must be noted that many drugs targeting CSCs have failed in clinical trials, and one of the most important reasons is the failure to correctly identify CSCs in different states, which is controlled by highly dynamic processes that are dependent on cues from the tumor stroma. Therefore, a

better result may be achieved by using combined therapies targeting multiple CSC-related pathways and CSC-targeted drugs in different states. Within targeted oncotherapy, nanomedical derivate-nanocarriers have especially presented notable prospects in enhancing targeting specificity. However, one major issue in the application of nanocarriers is that TMEs are too broad in a spectrum of targeting possibilities for these carriers to be effectively employed.<sup>314</sup> However, CSCs might portend a solution: aside from being quite heavily invested in tumorigenesis and therapeutic resistance, CSCs also show self-renewal and fluid clonogenic properties that often define specific TME niches. Therefore, new anti-CSC therapies focus on targeting these communication networks to eradicate the tumor and prevent metastasis, relapse, and drug resistance.

Although addressing cancer by suppressing CSCs and their supporting niche is still far from being a validated general strategy, there are reports available from clinical trials or preclinical experiments. First, targeting the hypoxic niche has been shown to deplete CSCs; for example, Lock et al showed that inhibition of carbonic anhydrase IX expression or activity with novel small-molecule inhibitors resulted in the inhibition of BCSC expansion in hypoxia.<sup>315</sup> Kim et al recently developed a small molecule construct, AzCDF, that allowed the therapeutic targeting of BCSCs and was effective in normally refractory hypoxic tumor environments.<sup>316</sup> Notably, a previous study indicated that hypoxia promoted the transition of mesenchymal-like (M)-BCSCs to an epithelial-like (E) state, and targeting this state equilibrium through co-inhibition of glycolysis and thioredoxin and glutathione pathways significantly suppressed tumor progression by eliminating both M- and E-BCSCs.<sup>317</sup> A study on HNSCC has shown that in the hypoxic state, after inhibiting HIF-1 $\alpha$ , CSCs are sensitive to carbon ions or photon radiation.<sup>318</sup> Second, there is growing evidence that supports the role of the secretory cytokine TGF- $\beta$  as a crucial mediator of CSC-stroma crosstalk in various tumor types, such as breast cancer,<sup>317</sup> CRC,<sup>318</sup>

**Table 4** Clinical trials targeting the TGF- $\beta$  and stromal crosstalk.

Drug name	Target	Application	Study NCT registry number
Bintrafusp alfa	TGF- $\beta$ R11 and PD-L1	Pretreated esophageal; adenocarcinoma	NCT02517398
Galunisertib	TGF- $\beta$ RI	Advanced solid tumors	NCT01682187
Fresolimumab	TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3	Renal cell carcinoma; advanced melanoma	NCT00356460
PF-03446962	TGF- $\beta$ RI	Pretreated colorectal	NCT02116894
Trabedersen versus	TGF- $\beta$ 2 RNA	Recurrent or refractory	NCT00431561

**Table 5** FDA-approved angiogenesis inhibitors.

Drug category	Drug name (targets)
Monoclonal antibodies	Bevacizumab (VEGF), Ramirumab (VEGFR2)
Small-molecule inhibitors	Sorafenib (Raf-1), SUNITINIB (VEGFR1/2/3), CaboTINIB (MET, VEGFR1/2/3, ROS1, RET, AXL, NTRK, KIT), VANDERINIB (EGFR, VEGFR, RET), APATINIB (C-KIT, VEGFR1/2/3, PDGFR- $\beta$ , FGFR1, FLT3), Savolitinib (c-Met), LENvATiniB (EGFR1, VEGFR2, VEGFR3, RTKs), REGorAFENIB (VEGFR1/2/3, TIE-1, TIE-2, RAF-1, BRAF, BRAFV600, KIT, RET, PDGFR, FGFR), furquiTiniB (VEGFR1/2/3), PazopanIB (VEGFR1/2/3, PDGFR $\alpha/\beta$ , FGFR13, Kit, ItK, Lck), AXITiniB (VEGFR1/2/3, c-KIT), NILANIB (HER1/HER2/HER4), ANlotINIB (VEGFR, PDGFR, FGFR, c-Kit)

glioma,<sup>319,320</sup> and ovarian cancer.<sup>321</sup> Some clinical trials targeting TGF- $\beta$  and stromal crosstalk are in progress (Table 4), which might be proven clinical targets for depleting CSCs by cutting the CSC-stroma connection. Third, some new antitumor therapies simultaneously target different routes to target the TME and starve CSCs. One of the most common options is to target tumor vasculature, with several FDA-approved angiogenesis inhibitors available (Table 5), such as bevacizumab (antibody directed against VEGF) or sorafenib and sunitinib, inhibitors of tyrosine kinase receptors (TKRs) that target multiple TKRs, including VEGF receptors and PDGF receptors. Nevertheless, CSCs can also exert resistance to antiangiogenic therapy, which leads to intratumor hypoxia states resulting in increased HIF expression and, therefore, an increased risk of tumor propagation, CSC self-renewal, drug resistance, and even angiogenesis activation.<sup>322,323</sup> This effect provokes the idea of antiangiogenic drugs in conjunction with other therapies, such as targeting hypoxia.<sup>324</sup> Fourth, another widely used approach is to try to prevent the function or recruitment of stromal cells because CSCs can promote their tumor niche. For example, targeting TAMs and inflammatory monocytes by inhibiting either the myeloid cell receptor colony-stimulating factor-1 receptor or CCR2 decreases the number of CSCs in pancreatic tumors.<sup>325</sup> CSCs secrete POSTN to recruit TAMs, and silencing POSTN in CCs markedly reduces TAM density, inhibits tumor growth, and increases the survival of mice bearing CSC-derived xenografts.<sup>326</sup> Fifth, targeting CSCs by immunotherapy has been largely reported as CSCs contribute to tumor development indirectly by attenuating immune surveillance within the TME. To date, immune cells such as NK cells and CD8<sup>+</sup> T cells, DC-based vaccines, oncolytic virotherapy (OVT), and chimeric antigen receptor T-cell therapy have been used to target CSCs.<sup>327,328</sup> Moreover, combination therapies composed of OVT, vaccines, and immune checkpoint blockades were also used

for targeting CSCs.<sup>329</sup> Nevertheless, different immunotherapy strategies have been presented for various cancers (Table 6).

**Table 6** Immunotherapy strategies presented in cancers.

Types of tumor immunotherapy	Drug name	Adaptation disease
Active nonspecific immunotherapy	IFN $\alpha$ -2a, IFN $\alpha$ -2b	Lymphoma
	IL-2	Melanoma, kidney cancer
Tumor vaccine	Provenge	mCRPC
Oncolytic viruses	Rigvir	Melanoma
	Oncori	Head and neck cancer
Cellular immunotherapy	T-VEC	Metastatic melanoma
	DELYTACT	Spongioblastoma
	CVT- TCR-01	Tumor cell
	Akilensai injection	Non-Hodgkin lymphoma
	Kymriah	Acute lymphoblastic leukemia
	Yescarta	B lymphoblastoma
	Tecartus	Mantle cell lymphoma
Immune checkpoint inhibitors	Abecma	Multiple myeloma
	Rikirensen injection	Diffuse large B cell lymphoma
	CTLA-4 antibody	Tumor cell
	PD1 antibody	Tumor cell
	PDL1 antibody	Tumor cell

All in all, it is becoming increasingly apparent that a single therapeutic target is unlikely to lead to effective treatment for further improvements in cancer treatment. In the interest of treating both the CSCs and cancer cell populations, we must come to understand the similarities and differences in the basic biology of CSCs and overall immune evasion. In addition, patient outcomes will emerge from a deeper understanding of the mechanisms that underlie the plasticity and dormancy of CSCs as well as the complex interactions among the CSCs and their niches and systemic factors. To do so, the preclinical models used to study anti-CSC therapies must recapitulate the genomic complexity, plasticity, and niche biology of advanced human cancers, rather than relying exclusively on simpler systems that may fail to capture these critical features. We expect, however, that higher benefit will be obtained with combined therapies that address simultaneously the CSC phenotype and the niche, and probably in the presence of another drug capable of eliminating differentiated tumor cells. With the deepening of our understanding of tumor biology and technological progress, we anticipate that immunotherapy will be at the forefront of modern clinical oncology.

## Author contributions

L.Z., X.L., and W.Z. conceived, designed, and supervised the study. Q.G. and Y.Z. drafted the manuscript. Q.G., Y.Z., T.X., W.S., H.L., and Y.Y. searched and reviewed the studies. Q.G., Y.Z., and T.X. made the figures. Q.G., Y.Z., T.X., and Y.Y. made the tables. L.Z., X.L., and W.Z. critically reviewed and revised the manuscript. All the authors approved the submission of the manuscript.

## Conflict of Interests

The authors declare that they have no competing interests.

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