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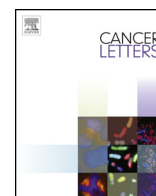
Mini-review

Advances in osteosarcoma stem cell research and opportunities for novel therapeutic targets

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ABSTRACT

Osteosarcoma is the most common type of bone cancer, especially in children and young adults. The primary treatment for osteosarcoma is a combination of surgery and chemotherapy, however prognoses remain poor due to chemoresistance and early metastases. Osteosarcoma stem cells appear to play central roles in tumor recurrence, metastases and chemoresistance *via* self-renewal and differentiation. Targeting these cells may provide a novel strategy in the treatment of osteosarcoma. This review summarizes current knowledge of this rare phenotype and recent advances in understanding the functions OSCs (osteosarcoma stem cells) in osteosarcoma, with the aim of improving therapies in the future.

Introduction

Osteosarcoma is the most common type of bone cancer and the second leading cause of cancer-related deaths in children and young adults [1]. The majority of osteosarcomas originate in the long bones, such as the distal femur and proximal tibia. It is highly aggressive with a metastatic rate of ~20%, with the most common targets being the lung and other bones [2]. Although the origins of osteosarcoma remain unclear, the most likely candidates are considered to be mesenchymal stem cells (MSCs) or osteoprogenitor cells [3,4]. Currently, the primary treatment for osteosarcoma is a combination of surgery and chemotherapy. However, osteosarcoma frequently develops resistance to conventional chemotherapies resulting in tumor recurrence. Amputation of the affected limbs is often the only option but even this usually fails to save a patient's life due to early metastases [5]. A better understanding of tumor pathology in osteosarcoma and the mechanisms of initiation and recurrence are urgently needed to improve patient prognosis.

Discovery of osteosarcoma cancer stem cells

Emerging evidence has indicated that malignant tumors contain a hierarchy of cells responsible for tumor initiation, propagation, recurrence and resistance to therapy [6]. These include a rare phenotype termed cancer stem cells (CSC), that have the ability to retain their stem cell-like properties through self-renewal and differen-tiation [7]. CSCs are generally more malignant than differentiated cancer cells and may present a precise therapeutic target that circumvents conventional treatments to the tumor bulk. Elucidating the role of CSCs in osteosarcoma may improve prognosis in the treatment of osteosarcoma [8,9].

The existence of OSCs (Osteosarcoma stem cells) was first demonstrated by Gibbs et al. who identified a subpopulation of cells in human osteosarcoma tissue samples and cell lines that were capable of growing sarcospheres, or osteospheres, in serum-free conditions [10,11]. Martins-Neves et al. identified a subpopulation of cells in MNNG/HOS osteosarcoma cell lines that showed resistance to chemotherapeutic agents and irradiation and exhibited stem-like properties [12]. Other *in vitro* studies supporting the existence of OSCs included the formation of sarcospheres in MG63 cells under anchorage-independent conditions [13] and isolation and characterization of OSCs in human and canine osteosarcoma [14].

Side population (SP) cells have also been identified in osteosarcoma. These exhibit increased sphere-forming and colony formation capabilities and higher tumorigenicity compared to non-SP cells [15]. This was confirmed by Yang et al. who demonstrated that only the SP fraction had the capacity to self-renew [16].

Research now focuses on the regulatory mechanisms that underlie OSC initiation and activities and their potential as therapeutic targets in the treatment of osteosarcoma [1,8,11].

Techniques to isolate osteosarcoma stem cells

Surface markers, such as CD133, are commonly used to identify CSCs; however their biological function in osteosarcoma is unclear and suitable markers for separating OSCs from differentiated cells

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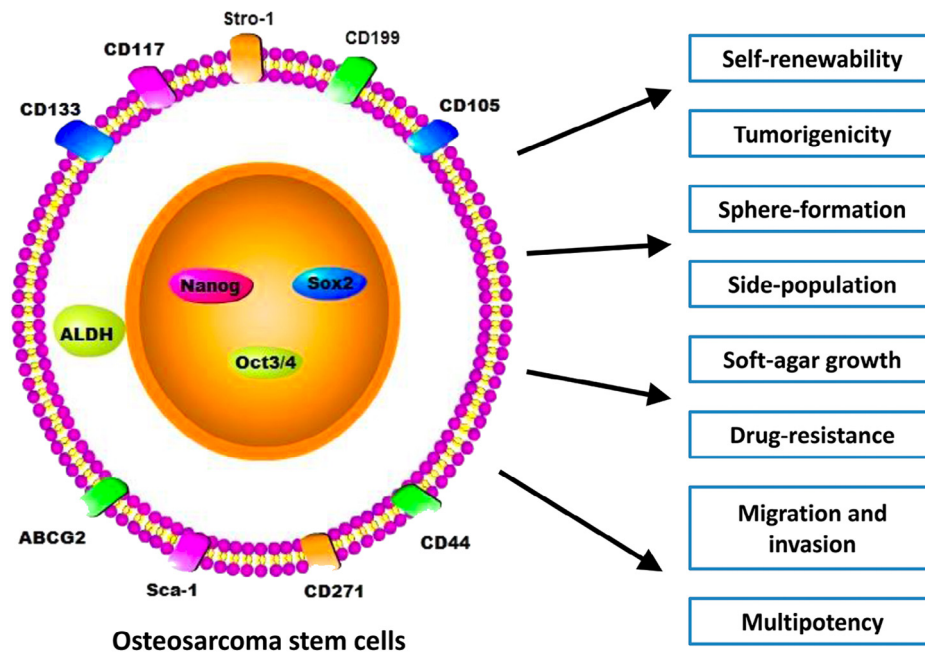
Fig. 1. Molecular markers used to isolate OSCs in tumors. The schematic diagram illustrates the functional roles of three key pluripotent molecules Oct3/4, Sox2 and Nanog and associated markers commonly used to identify OSCs.

remain to be found [17]. Gemei et al. detected 50 out of 245 membrane proteins that were differentially expressed between OSCs and differentiated cells through gene expression profiling. Their results have provided valuable data towards defining a cell-surface protein signature for OSC [18].

Fig. 1 provides a schematic illustration of these markers in OSCs; Table 1 summarizes their functions.

CD133

CD133 (AC133), a member of prominin family, is a glycoprotein with 3 isoforms that has been wildly used for isolating CSCs. Several studies have linked CD133-positive cells with a stem-cell phenotype. These include CD133-positive subpopulations exhibiting stem-like properties in SAOS2, MG63 and U2OS osteosarcoma cell lines [19–21]; elevated mRNA expression levels in stemness genes Oct4, Nanog and CXCR4 [22,23]; higher migration and invasive capabilities, particularly in lung metastasis [24]; and increased drug resistance [22,23]. In addition, cell populations with elevated levels of CD133 and CD199, along with low levels of CD44, exhibited higher levels of stem cell markers [25]; and CD49f-negative/CD133-positive cells possessed strong tumorigenicity and self-renewal capacities and were able to differentiate to CD49f-positive cells with more limited tumorigenicity [26].

CD117 and Stro-1

CD117 and Stro-1 are MSC markers. They have been found to be preferentially expressed in spheres and doxorubicin-resistant osteosarcoma cells and CD117-Stro-1 double-positive OSCs have been identified in both human and murine osteosarcoma cells and were found to exhibit high degrees of multipotency, invasiveness, drug resistance and elevated levels of self-renewal and metastatic potential [27]. Furthermore, they were enriched in metastasis-associated marker CXCR4 and drug-resistance marker ABCG2 [27]. Adhikari et al. also showed them to be highly effective in forming transplantable tumors. In contrast, CD117-Stro-1

double-negative cells lacked this ability [27]. Several studies have confirmed these findings [35,36,54], demonstrating the importance of CD117 and stro-1 as molecular markers for identifying and isolating OSCs in osteosarcoma. CD105 and CD44 have also been identified as effective MSC markers in osteosarcoma along with CD117 and stro-1 [10].

Other molecular markers

Other molecular markers that are associated with the OSC phenotype include the following: enhanced aldehyde dehydrogenase (ALDH) activity has been linked to increased levels of tumorigenicity, self-renewal and differentiation potential in osteosarcoma cell lines [28]; increased expression of ATP-binding cassette subfamily G member 2 (ABCG2) and tumor metastasis-associated marker CXCR4 have both been reported in sphere cells [16,24,27]; CD271, a marker of MSCs and human melanoma cancer stem cells, was able to distinguish a subpopulation of osteosarcoma cells that displayed stem-like features such as self-renewal, drug resistance and tumorigenicity [31]; and hematopoietic and MSC antigen Sca-1 was identified as an effective OSC marker [29,30]. Lower expression levels of CD326, CD24, CD44 and CBX3 have been identified in sarcospheres associated with the OSC phenotype, suggesting that ABCA5 may be a putative biomarker for OSCs [55].

Side population cells

Side population (SP) cells exhibit high levels of multidrug resistance and share similarities with CSCs. For example, SP cells have been linked to ABC protein transporters with high expressions of stemness genes Oct4 and Nanog [16]. The long-term label retention dye pKh26 can differentiate between rapidly dividing cells and quiescent cells, and studies using pKh26hi have identified SP cells as a subpopulation capable of self-renewal and tumor generation [32]. Hoechst dye exclusion assays are a common way to isolate drug-resistant cells and thereby establish SP cells [17].

Signaling pathways that influence osteosarcoma stem cells

The roles of signaling pathways, epigenetic regulators and the microenvironment in the regulation and maintenance of OSCs are illustrated in Fig. 2 and summarized in Table 1.

Oct3/4, Sox2 and Nanog

Transcription factors Oct3/4, Sox2 and Nanog play central roles in the development of CSCs in a variety of tumors and maintaining pluripotency and self-renewal in undifferentiated embryonic stem cells [56–61], and have been used to establish stemness in OSCs [10,16,19,21,31]. Levings et al. engineered an osteosarcoma cell line that stably expressed human Oct4 promoter-driven GFP reporter gene and were significantly more tumorigenic than GFP-depleted cells. The Oct4/GFP+ cells also expressed MSC markers CD105 and ICAM-1 and had a propensity to metastasize to the lung [33]. Sox2 was highly expressed in both human and murine osteosarcoma cell lines and tissue samples. Knockdown of Sox2 resulted in increased differentiation in osteosarcoma cells and a reduction in clone formation, migration and invasion, while activating the Wnt pathway and thereby inhibiting tumor formation. In contrast, Sox2-depleted osteosarcoma cells failed to form osteospheres and differentiated into mature osteoblasts [29]. These observations were confirmed by Tang et al. who also showed that overexpression of Oct4 and Sox2 led to an enrichment of tumor stem cells in osteosarcoma cell lines [34]. Less is known about the actions of Nanog in osteosarcoma, however studies have indicated

that it may target CSCs [62]. Our previous study indicated that suppression of Nanog, through overexpression of TSSC3 (PHLDA2), inhibited the OSC phenotype in osteosarcoma cell lines [35].

TGF-β1

Transforming growth factor β1 (TGF-β1) is a pleiotropic cytokine that promotes tumor propagation and cytokine secretion [63]. TGF-β1 signaling combined with a hypoxic environment dramatically induced self-renewal in non-stem osteosarcoma cells and increased metastatic potential, chemoresistance, tumorigenicity and neovasculogenesis in OSCs [36]. It was suggested that osteosarcoma cells may secrete TGF-β to maintain MSC stemness and promote production of pro-tumor cytokines [37]. TGF-β1 treatment has also been linked to expression of Sox2 in immature or undifferentiated cells, and to a marginal increase in expressions of Nanog and Oct4 [36].

BMP-2

BMP-2 is an active inducer of osteoblastic differentiation in immature osteoblasts and less committed cells. Reports have implicated BMP-2 in the induction of tumorigenic factors and the proliferation and aggressiveness of osteosarcoma MSCs (OSMSC) [38]; and elevation of BMP-2 has been linked to overexpression of VEGF, EMMPRIN and MMP-9 in OSMSCs [38]. In contrast, researchers reported BMP-2 downregulated Oct3/4, Sox2 and Nanog expression in osteosar-

Table 1
Summary of findings in OSC research.

	Specific	Findings	Reference
Markers	CD133	Over-expressing Sox2, Nanog, Oct4, Nestin, CXCR4	[19–26]
		Tumorigenic, differentiable, side-population, drug-resistance	
		Migrative, invasive and higher lung metastasis rate	
		CD199+, CD44–, CD49f–	
	CD117 and Stro-1	Tumorigenic and multipotent	[10,27]
		Invasive, metastatic, self-renewal, drug resistant	
		Over-expressing CXCR4, ABCG2, CD105, CD44	
	ALDH	Tumorigenic, self-renewal, differentiable	[28]
	ABCG2 and CXCR4	Sphere-forming	[16,24,27]
	Sca-1	Related to Sox2 and Wnt pathway	[29,30]
		Sphere-forming, differentiable	
	CD271	Self-renewal, drug resistant and tumorigenic	[31]
	Side population	Multidrug resistance, self-renewal, tumorigenic, sphere-forming	[15–17]
		Over-expressing Oct4 and Nanog	
	pKh26	Self-renewal, tumorigenic	[32]
Regulators	Oct3/4	Tumorigenic and metastasis, expressing CD105 and ICAM-1	[33]
	Sox2	Sphere-forming, differentiable, invasive, tumorigenic	[29,34]
		Repress Wnt pathway	
	Nanog	Repress by TSSC3	[35]
	TGF-β	Related to hypoxia, self-renewal, metastatic, chemoresistant	[36,37]
		Tumorigenic, neovasculogenesis, supporting neighboring MSCs	
		Induce Sox2, Oct4, Nanog,	
	BMP-2	Over-expressing VEGF, EMMPRIN, MMP-9, tumorigenic	[38]
		Decrease of Nanog, Oct4, Sox2	[39]
		Decrease of tumorigenicity and proliferation	
	MAPKs	Invasive and migrative, tumorigenic, undifferentiated	[18,40]
		Activate by TNFα and IL-1	
	Notch	Tumor initiation, propagation, aggressive	[41–44]
		Regulate ALDH expression	
	Hedgehog	Tumor initiation, regulate Yap1 and H19	[45–47]
Epigenetical regulators	TSSC3	Reduce self-renewal, cell vitality, induce apoptosis	[35,48]
		Decrease of Oct4, Sox2, Nanog	
	HIF2PUT	Decrease CD133, sphere-forming	[49]
	miR-133a	Invasive and aggregative	[50]
	let-7/98 and miR-29a,b,c	Microarray analysis	[51]
	miR-29b-1	Reduce growth and sphere-forming	[52]
		Repress CD133,Oct4, Nanog, Sox2	
	Telomerase	Sphere-forming, invasive, chemoresistant, tumorigenic	[53]
		Over-expressing CD117 and Stro-1	
	MLH1 and MSH2	Chemoresistant and sphere-forming	[13]
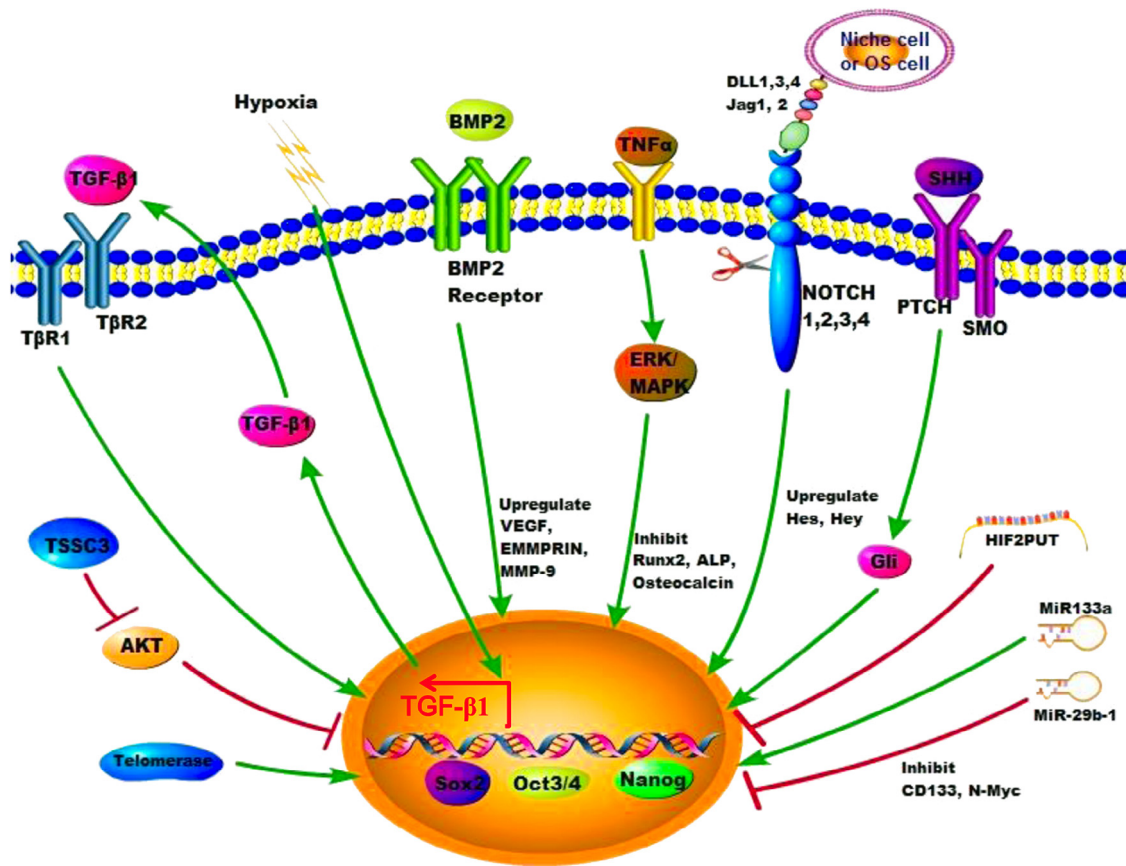
Fig. 2. The roles of signaling pathways, epigenetic regulators and the microenvironment in the maintenance of OSCs. Although the regulatory mechanisms of Oct3/4 and Sox2 in stemness have been studied extensively, the role of Nanog in OSCs remains largely unexplored. Key regulatory pathways in the initiation of OSCs include Notch and Hedgehog; MAPK-related pathways have been associated with invasiveness and motility in OSCs; additional pathways are described in the text. The epigenetic imprinting gene TSSC3 is considered to facilitate OSCs in evading apoptosis *via* inhibition of Akt; telomerase is reported to promote self-renewal; whereas, hypoxia may induce TGF-β1 expression and promote self-renewal. The stem cell niche may provide a microenvironment for OSCs to maintain stemness and generate differentiated progeny.

coma cells and upregulated transcription of osteogenic markers Runx-2 and Collagen Type I, resulting in decreased tumorigenicity [39], and BMP-2 expression has been linked to decreased expression of ki67, potentially inhibiting the proliferation of OSCs [39].

MAPK pathway

Mitogen-activated protein kinases (MAPK) play key roles in many cellular programs, including cell proliferation, differentiation, motility and survival [64], and studies have indicated they may influence the OSC phenotype: MAPK signaling has been linked to cytoskeletal rearrangement during tumoral invasion in osteosarcoma cells; and the ERK/MAPK pathway has been associated with differentially expressed proteins involved in the enhanced invasiveness and motility of OSCs [18]. ERK activation of TNFα and IL-1 was implicated in maintaining AX cells, a OSC cell line, in an undifferentiated state, thereby promoting tumor progression and reducing osteoblastic gene expression [40].

Notch and Hedgehog pathway

Notch proteins are transmembrane receptors activated by ligands on adjacent cells. Following activation, the Notch intracellular domain (NICD) is released and enters the nucleus where it regulates expressions of Hey and Hes family proteins. These are involved in the maintenance of stem cells [65]. Dysregulation of Notch signaling was shown to result in osteosarcoma, demonstrating the significance of Notch signaling in the initiation and progression of

osteosarcoma [41,42]. Furthermore, cell lines derived from Notch-induced osteosarcoma formed aggressive high-grade-type tumors in nude mice; and Notch-activated mutation acted as a second hit in a p53-loss-driven osteosarcoma model, significantly shortening tumor latency and aggressiveness [43]. Meanwhile, both the aggressive metastatic phenotype and ALDH activity were reduced in a murine osteosarcoma cell model following inhibition of Notch signaling. This finding suggests that Notch signaling may influence OSCs *via* suppression of ALDH [44]. Hedgehog pathways have been shown to participate in the regulation of CSCs in a variety of tumors, including glioblastoma [66,67] and melanoma [68]; furthermore, they have been implicated in the initiation and regulation of OSCs [45]. Hedgehog signaling regulates the expressions of downstream genes by activating GLi through membrane receptors, such as PTCH and Smoothened [69]. Recent reports have shown that Hedgehog is overexpressed in osteosarcoma cell lines; however, cell growth could be suppressed through inhibition of Smoothened [46]. Confirmation of the role of Hedgehog signaling in osteosarcoma development was demonstrated in a mouse model: aberrant Hedgehog signaling in mature osteoblasts *via* Yes-associated protein 1 (Yap1) and H19 led to the development of osteoblastic osteosarcoma [47].

Epigenetic regulators in osteosarcoma stem cells

Imprinting gene TSSC3

Genomic imprinting is the epigenetic modification of gene loci resulting in altered allelic expression. A malignant-transformed

osteosarcoma cell line (MTF) has been developed in our laboratory composed primarily of OSCs capable of forming spheres [70]. Microarray analysis identified 10 imprinting genes that were aberrantly expressed, including tumor-suppressing STF cDNA 3 (TSSC3) in this cell line. This imprinting gene is related to cell death and was significantly downregulated [70]. Overexpression of TSSC3 has also been reported to suppress cell vitality and growth, induce apoptosis and downregulate expressions of Oct4, Sox2 and Nanog in osteosarcoma cells, leading to dramatic reductions in the OSC phenotype [35,48].

MicroRNAs

MicroRNAs (miRNA) have been implicated in the epigenetic regulation of cancer cells and the following miRNAs have been identified in tumor-related functions in osteosarcoma. Hypoxia-inducible factor-2α (HIF-2α) promoter upstream transcript (HIF2PUT) is a novel long non-coding RNA (lncRNA); overexpression of HIF2PUT markedly decreased the percentage of CD133-expressing cells and impaired OSC sphere-forming capacity in MG63 osteosarcoma cells [49]. Silencing miR-133a reduced cell invasion and prolonged survival in osteosarcoma-bearing mice, suggesting that miR-133a may play a role in OSC regulation [50]. A total of 189 miRNAs were reported to be differentially expressed in 3AB-OS CSCs relative to their parental cells. These included two miRNA families, let-7/98 and miR-29a, b, c, and their anticorrelated mRNAs (MSTN, CCND2, Lin28B, MEST, HMGA2 and GHR), suggesting they may comprise a set of OSC markers [51]. MiR-29b-1 was found to negatively regulate expressions of CD133, N-Myc, Oct3/4, Sox2 and Nanog in OSCs; in contrast overexpression of miR-29b-1 consistently reduced growth and the sphere-forming ability of OSCs in this cell line [52].

Telomerase and DNA repair

Telomerase activity has been detected in most malignant cancer cells, including osteosarcoma, and is crucial for the maintenance of stem/progenitor cells. However the relationship between telomerase expression and CSCs remains unclear [71]. High telomerase activity has been linked to enhanced stem cell-like properties in osteosarcoma cells, including sphere-forming capacity, invasiveness, metastatic potential and resistance to chemotherapeutic agents [53]. In addition, sphere-driving OSCs displayed increased expressions of CD117 and Stro-1 compared to telomerase-negative cells; conversely inhibition of telomerase resulted in decreased tumorigenic potential in osteosarcoma [53]. Increased expression of DNA repair enzyme genes, MLH1 and MSH2, has also been linked to chemoresistance in osteosarcoma sphere cells [13].

Microenvironment of osteosarcoma stem cells

Stem cell niche defines the microenvironment in which stem cells reside. It is comprised of stem cells, neighboring supportive cells, inflammatory cells, microvessels, the extracellular matrix and soluble factors such as chemokines and cytokines. The niche microenvironment facilitates stem cells in entering quiescence and undergo differentiation and to maintain their stemness including self-renewal and to regulate differentiation [72,73]. This has been demonstrated in OS99-1 cells: OS99-1 cells with high ALDH activity exhibited CSC-like behavior when the cells were isolated from subcutaneous tumors *in vitro* and not from adherent cultures [28]. Three types of stem cell microenvironments have been reported in glioblastoma: perivascular niche, hypoxic niche and metastatic niche. Tumor stem cells are thought to induce vascularization and differentiate into endothelial-like cells to mimic microvessels in order to supply oxygen and nutrition to the tumor [74,75]. GSCs (Glioblastoma stem cells) have been found adjacent to capillaries and adhered to endothelial cells in brain tumors [76]. This was further demonstrated through increased tumor formation in mice following co-injection of CD133-positive human medulloblastoma cells with endothelial cells [76]. Further investigation revealed that the process was mediated by VEGF/VEGFR and SDF-1/CXCR4 pathways [77,78]. Osteosarcoma is a tumor enriched in vasculature, supporting the existence of a perivascular OSC niche, and reports have confirmed that the hypoxic niche is central in osteosarcoma propagation and CSC maintenance [79,80]. Hypoxia-inducible factor (HIF) has been reported as highly expressed in CSCs in several tumors; conversely, blocking HIF-1α or HIF-2α activity resulted in dramatic decreases in CSC proliferation and self-renewal [81]. A hypoxic environment in non-stem osteosarcoma cells has been reported to dramatically induce the self-renewal capacity of OSCs [36]. Interestingly, expression of the epigenetic gene TSSC3, which is also known as PHLDA2, reported to repress OSC self-renewal, can be regulated by hypoxia [82]. Both the Notch and Hedgehog pathways have been implicated in the stem cell niche: The Notch pathway was reported to be activated and to promote stem-like characteristics in glioblastoma and colorectal cancer. This was believed to be through Nitric oxide and Notch ligands released from tumor endothelial cells in the stem cell niche [83–85]; the Hedgehog pathway was activated in the glioblastoma stem cell (GSC) niche, probably through ligands secreted by endothelial cells, suggesting a paracrined function may support CSCs [6]. Although there is few data for understanding microenvironment of OSCs, it is without doubt that microenvironment could notably influence the biological behaviors of OSCs. Exploration of molecular interactions among OSCs, microvessels and hypoxia in future might develop new therapeutic strategy to target OSCs.

Therapeutic targeting of osteosarcoma stem cells

CSCs exploit various mechanisms to deregulate apoptotic signaling pathways, including the ability to enter quiescence and slow the rate of proliferation [86]. This can be combined with overexpression of ABC drug efflux transporters to enhance chemoresistance [87]. Inhibition of drug efflux transporters was found to enhance cellular uptake of doxorubicin and initiate apoptosis in OSCs; whereas, efflux activity of drug transporters P-glycoprotein and breast-cancer related proteins facilitated OSCs entering quiescence through activation of anti-apoptotic mediators Bcl-2 and Bcl-xL, downregulation of Bak, and caspase-3/7 inactivation [88]. The anticancer agent salinomycin has been shown to be an effective inhibitor of OSCs *in vivo* and *in vitro*. Salinomycin inhibited expressions of Oct4 and Sox2 and suppressed the sphere-forming capacity and chemoresistance in OSCs without severe side effects. Salinomycin can inhibit Wnt pathway activity suggesting the involvement of Wnt/β-catenin signaling [34]. Bufalin is the active component in the Chinese medicine Chan Su. Bufalin treatment of immunodeficient mice and OSCs derived from the MG63 cell line resulted in their inability to differentiate. Further investigation revealed the expression of stem cell markers CD133 and Oct4. Treatment with bufalin also inhibited sphere forming and proliferation in these cells [25].

Signaling pathways associated with OSC niche functions may present novel targets for the treatment of osteosarcoma: studies have shown that the Hedgehog and Notch pathways participate in the perivascular niche; and VEGF/VEGFR and SDF-1/CXCR4 contributed to angiogenesis and reprograming of endothelial cells in the GSC niche. Conversely, anti-VEGF and CXCR4 could suppress tumor angiogenesis and may break down the perivascular niche.

TSSC3 is an imprinting gene that is primarily regulated by methylation. As reported above, expression of TSSC3 was found to be significantly lower in OSCs than in differentiated osteosarcoma cells; conversely overexpression of TSSC3 could increase apoptosis and inhibit drug resistance in OSCs [35]. These findings suggest that

TSSC3 expression in osteosarcoma could be targeted by demethylation drugs. MST312 [53] and caffeine are telomerase and DNA repair inhibitors, respectively, suggesting they may also be candidates for OSC-targeted therapies [13]. Telomerase is partially responsible for immortalization in malignant tumor cells, and elevated telomerase activity has been implicated in self-renewal and chemoresistance in OSCs [89]; caffeine enhanced the efficacy of doxorubicin and cisplatin, indicating that drug resistance in sarcosphere cells was related to an efficient DNA repair capacity in OSCs. Other potential therapeutic agents for TSC (Tumor stem cells) ablation in osteosarcoma include the epigenetic regulator (5-azacytidine), anti-microtubule drug (vincristine) and telomerase inhibitor (RHPS4) [55].

Conclusion

Cancer stem cells have been implicated in malignant tumor initiation, recurrence, drug resistance, invasion and metastasis. Therefore therapeutic targeting of OSCs offers a promising strategy for the treatment of osteosarcoma. However, such treatments require accurate identification and isolation of OSCs. Currently, the most effective markers for OSCs are CD133, CD117, Stro-1 and ALDH. Although each of these have displayed partial success in isolating CSCs in other types of cancer [90–92], a definitive set of markers that can specifically isolate OSCs remains to be found. It may be possible to improve the efficiency and accuracy of OSC-isolation by combining CSC markers with sphere-forming assays and SP cell sorting. However, sphere-forming and SP subpopulations are not exclusively comprised of CSCs, resulting in an overlap of cell types [93]. Therefore, further research is required to fully understand the biological functions of CSC markers in order to accurately isolate OSCs from non-OSCs.

There has been increasing interest in the key signaling pathways involved in CSC actions with the aim of developing new therapies to target CSCs. However, relatively few studies have focused on OSCs. The roles of TGF-β1, Sox2 and Notch pathways in the initiation and regulation of OSCs have attracted the most attention and the findings have been promising. Recent research into epigenetic intervention has also been encouraging, particularly in relation to the imprinting gene TSSC3 and the effects of demethylation drugs in OSCs. The OSC niche and the influence of the microenvironment *in situ* may provide an alternative approach to elucidate the events that contribute to OSC generation and regulation.

Despite recent advances, much of the research into OCSs has focused on individual functions or mechanisms and studies have not been correlated. Further development will require systematic and in-depth understanding of the molecular mechanisms that underlie regulation and maintenance of the OSC phenotype and its influence in osteosarcoma in order to improve patients' prognoses.

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Conflict of interest

The authors declare that they have no conflict of interests.

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