Chapter 5

Microenvironment Signals and Mechanisms in the Regulation of Osteosarcoma

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Additional information is available at the end of the chapter

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Abstract

Osteosarcoma (OS) is the most common malignant primary bone tumor in children and adolescents and features rapid development, strong metastatic ability, and poor prognosis. It has been well established that diverse genetic aberrations and metabolic alterations confer the tumorigenesis and development of OS. The intricate metabolism and vascularization that contributes to the nutrient and structural support for tumor progression should be thoroughly clarified to help us gain novel insights into OS and its clinical diagnoses and treatments. With regard to the complex bone extracellular matrix (ECM) and local cell populations, we intend to illustrate the interrelationship between various microenvironmental signals and the different stages of OS evolution. Solid evidence has noted two crucial factors of the OS microenvironment in the acquisition of stem cell phenotypes - transforming growth factor-β1 (TGF-β1) signaling and hypoxia. Different cell subtypes in the local environment might also serve as unique contributors that interact with each other and communicate with distant cells, thus participating in local invasion and metastasis. Proper models have been established and improved to reveal the evolutionary footsteps of how normal cells transform into a neoplastic state and progress toward malignancy.

Keywords: microenvironment, genetic aberrations, vasculogenesis, niches, models

1. Introduction

Osteosarcoma (OS) is the second highest cause of cancer-related death in children and adolescents. Unfortunately, complete surgical resection fails to eliminate OS due to the early hematogenous spread of pulmonary metastases. Despite advanced multi-agent neoadjuvant and adjuvant chemotherapies, the clinical outcome for patients with OS unfortunately remains

discouraging, and the long-term survival rate for high-grade OS remains poor [1]. It is urgent to identify innovative diagnostic and prognostic markers as well as effective therapeutic targets.

The vast majority of OS arises in the metaphyseal regions adjacent to physes with a strong capacity of proliferation, including the distal femur, proximal tibia, and proximal humerus [2]. Evidence has elucidated that the complex etiology of OS is characterized by genomic instability, highly abnormal karyotypes, and multiple genomic aberrations with copy number variations occurring in multiple chromosomes [3, 4]. The story of how OS originates and develops is mysterious and is still the subject of exploration on many fronts.

In addition to the complexity of OS cells, the microenvironment of OS is also dynamic and variable with a complex bone extracellular matrix (ECM) and diverse populations of localized cells. Regulating various microenvironmental signals and different niches in OS warrant attention. Importantly, the OS microenvironment is characterized by abundant transforming growth factor-β1 (TGF-β1) and hypoxia. These conditions induce non-stem-like OS cells to adopt cancer stem cell characteristics, which in turn promote tumorigenesis and chemoresistance [5]. In addition, identifying distinct metabolic patterns and vascularization in OS should be considered in more detail and could provide a potential framework for clinical applications.

By reviewing the literature on classical and cutting-edge studies, we will discuss the regulation of microenvironmental signals during OS development and illustrate novel models for the study of OS.

2. Cells of origin: tumorigenesis

When a normal cell acquires the first cancer-promoting mutation(s) and initiates neoplasm, it is termed as cell of origin. As more information is gathered on the characteristic features of cell of origin, it is not difficult to create a clear assessment and better understanding on tumor evolution, which may remarkably lead to clinical improvements.

OS was believed to originate from bone mesenchymal stem cells (MSCs) or osteoprogenitors [6]. The deficiency of p53 alone or in combination with pRb in undifferentiated adipose-derived MSCs (ASCs) or bone marrow-derived MSCs (BM-MSCs) promotes metastatic osteoblastic OS development upon intrabone (i.b.) or periosteal (p.) orthotopic inoculation in immunodeficient mice [7]. In addition, the protein expression of cyclin-dependent kinase inhibitor 2A (CDKN2A)/p16 was identified as a sensitive prognostic marker in OS patients. Aneuploidy, translocations, and homozygous loss of the Cdkn2 region might have caused the malignant transformation of MSCs, which eventually evolved to OS in xenografted mice [8]. These findings proved that MSCs with genetic mutations might eventually develop into OS. Moreover, excision of p53-floxed alleles, which are p53 genes flanked by loxP sites that could be edited, in the osteoblastic lineage mediated by an osterix (OSX)-Cre transgene would cause spontaneous OS in mice. This model traced the cells of origin to osteoprogenitors because the excision was driven by the osterix promoter expressed in osteoprogenitor cells [6].

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Nonetheless, there have been some other disputes as to the cell of origin for OS (**Figure 1**). Induced pluripotent stem cells (iPSCs) were generated from fibroblasts obtained from a family with Li-Fraumeni syndrome (LFS), a rare autosomal dominant syndrome characterized by the occurrence of diverse mesenchymal and epithelial neoplasms at multiple sites. LFS iPSC-derived osteoblasts (OBs) from these individuals have provided a sophisticated model system to study the early stages of OS development and elucidate the pathological mechanism of p53 mutant-associated OS development [9]. Recent research has provided evidence that pericytes, a mesenchymal cell population surrounding endothelial cells, could be a cell of origin for benign and malignant mesenchymal neoplasms [10]. Lineage-tracing studies in mice were accomplished to reveal sarcomas that are driven by the deletion of p53, and desmoid tumors that are driven by a mutation in adenoma polyposis coli (Apc) could be derived from neuron-glial antigen 2/chondroitin sulfate proteoglycan 4 (Ng2/Cspg)-expressing pericytes. They also determined the role of β-catenin dysregulation in the neoplastic phenotype.

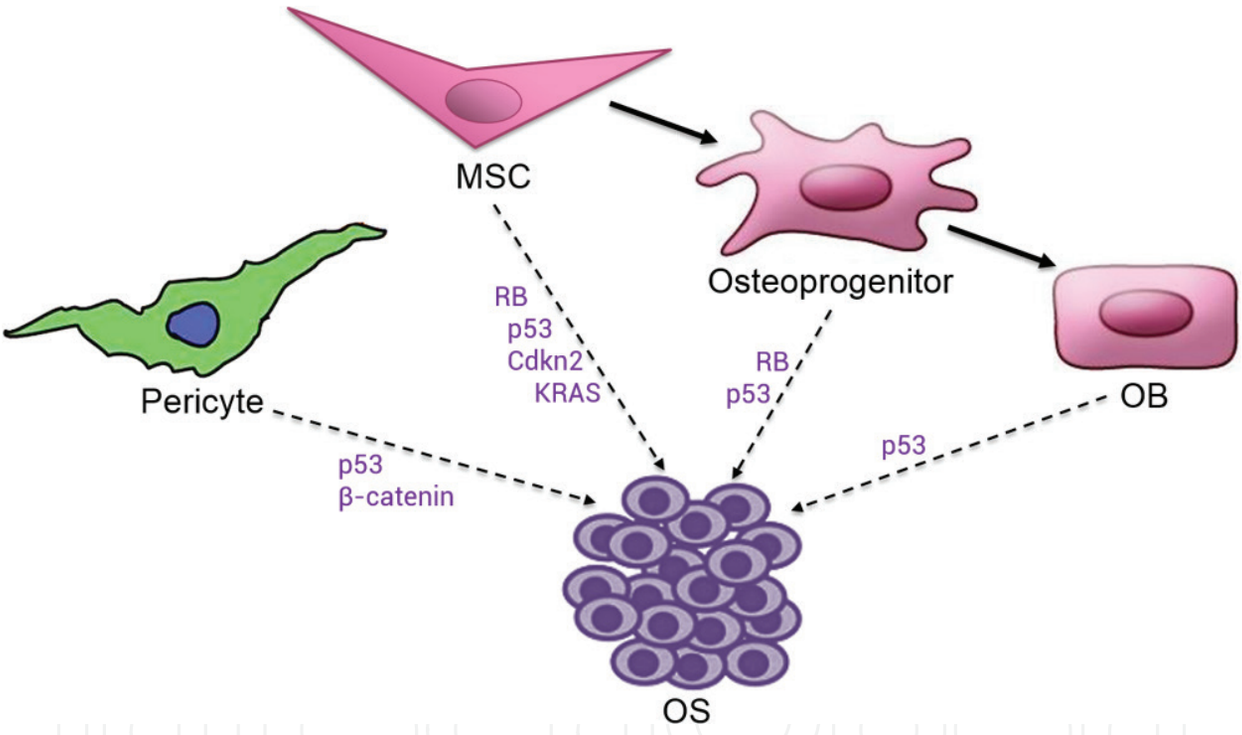

Figure 1. Cells of origin in OS. OS initiation is promoted by multiple genetic alterations (e.g., activation of oncogenes or inactivation of tumor suppressor genes).

The etiology of OS is still vague, while its pathogenesis remains mysterious. Generally, tumorigenesis is closely associated with inherited gene defects or mutations and exposure to exogenous carcinogens. These factors will affect the mutation rate and continually play a role in tumor evolution [11]. In the most likely scenario, the unique properties of OS might be related to either the genetic or epigenetic aberrations generated from either the cell of origin or components in the bone marrow microenvironment, such as the elevated levels of TGF-β1 and low oxygen tension. Uncovering the relationship between cytogenetic changes and microenvironmental signals in tumorigenesis will provide solutions for tumor eradication.

2.1. Tumor suppressor genes and oncogenes

OS results from multiple factors and gene aberrations. During the initiation and progression of OS, diverse oncogenes or tumor suppressor genes cause aberrant expression and hence dysregulate cell proliferation, apoptosis, and angiogenesis. Currently, the etiology and pathogenesis research on OS mainly focus on these oncogenes, tumor suppressor genes, and multidrug-resistant genes.

OS is a malignant bone cancer with severe chromosomal abnormalities and often has mutations of p53 and pRb. Up to 22% of OS patients carry an abnormal TP53 gene, and the allelic loss on chromosome 17p13 was confirmed in 75% of patients by a detection of mutation in the germ line [12, 13]. Strong evidence also suggested that p53 could regulate the genomic stability, proliferation, and immune properties of MSCs. p53 loss of function in MSCs compromises osteogenic differentiation and affects bone tumor microenvironment, both of which influence the development of OS [14].

A German group generated the first porcine model of OS by introducing oncogenic $TP53^{R167H}$ and $KRAS^{G12D}$ mutations as well as overexpressing Myc in porcine MSCs. These transformed porcine MSCs, with genomic instability and complex karyotypes, had the ability to develop into sarcomas upon transplantation into immunodeficient mice [15]. Other models also indicated that intrabone or periosteal inoculation of p53−/− or p53−/−RB−/− BM-MSCs or ASCs originated metastatic osteoblastic osteosarcoma (OS). Moreover, the subcutaneous (s.c.) coinfusion of p53−/− RB−/− MSCs together with BMP-2 resulted in appearance of tumoral osteoid areas [7]. pRb and p16(INK4a) are crucial G1-checkpoint proteins that maintain the balance of cellular proliferation. Deletion of p16 expression is significantly associated with decreased survival in a univariate analysis. The loss of pRb activation permits the hyper-proliferation of aberrant cells [16].

The progression of health informatics and the comprehensive study of "big data" have brought about new insights of genomic research. OS gene expression was first compared in gene expression omnibus (GEO) datasets and genomic aberrations in the International Cancer Genome Consortium (ICGC) database to identify differentially expressed genes (DEGs) and correlate these with both single-nucleotide polymorphisms (SNPs) and copy number variants (CNVs) in OS. The functional annotation of SNP- or CNV-associated DEGs was accomplished in accordance with gene ontology analysis, pathway analysis, and protein-protein interactions (PPIs). The PPI network analysis showed that chaperonin containing TCP subunit 3 (CCT3), COP9 signalosome subunit 3 (COPS3), and WW domain-containing E3 ubiquitin-protein ligase 1 (WWP1) could be candidate driver genes in OS tumorigenesis [17].

Another study performed a microarray-based comparative genomic hybridization (array-CGH) analysis on genomic DNA isolated from 41 patients with p53 +/− OS and 10 rhabdomyosarcoma samples. Results showed either gains or losses in the recurrent copy number, and the regions indicated known candidate oncogenes on mouse chromosomes 9 and 15. Furthermore, functional assays proved that the matrix metalloproteinase 13 (MMP13) gene, the antiapoptoticgenes Birc2 (cIAP1) and Birc3 (cIAP2) are potential oncogenic drivers in the chromosome 9A1 amplicon [18].

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2.2. MicroRNAs and their target genes

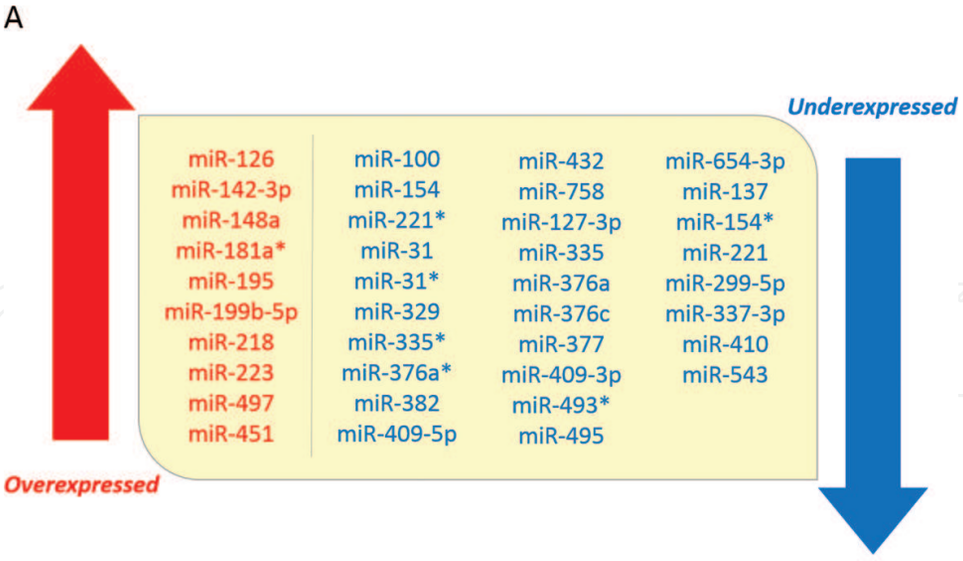
MicroRNAs (miRNAs) are a class of small, single-stranded RNA molecules ranging from 18 to 25 nucleotides in length. miRNAs play important roles in proliferation, differentiation, apoptosis, and other cellular activities through posttranscriptional regulation of genes [19, 20]. miRNA signatures are detected in diverse types of cancers such as sarcoma, breast and prostate cancer [21–23]. Emerging evidence suggests that miRNAs are involved in the pathogenesis of OS and could potentially be developed for use as diagnostic biomarkers and therapeutic strategies.

Expression profiling of 723 human miRNAs was performed in seven OS specimens. Of the miRNAs tested, 38 were differentially expressed ≥ 10-fold (28 under- and 10 overexpressed) as shown in **Figure 2A**. In this analysis, miRNA-mRNA pairings were identified along with copy number changes of their corresponding target genes (**Figure 2B**). Many of the predicted gene targets of differentially expressed miRNAs are involved in intracellular signaling pathways important for OS, which include the c-Met, Notch, RAS/p21, mitogen-activated protein kinase (MAPK), Wnt, and Jun/Fos pathways [24]. For example, GADD45A, a putative target of miR-148a, could promote DNA repair and cell cycle arrest via the p38 MAPK and c-Jun N-terminal kinase (JNK) pathways. Overexpression of miR-148a contributed to the downregulation of GADD45A in OS, which was associated with multidrug resistance [25]. In this set of OS specimens, miR-126 was overexpressed and reported to downregulate the expression of polo-like kinase 2 (PLK2). PLK2 was proven to undergo transcriptional silencing via methylation in various cancer types, thus acting as a presumptive tumor suppressor gene [26]. Furthermore, miR-126 could stimulate developmental angiogenesis via vascular endothelial growth factor (VEGF) signaling [27].

The expression and either genetic or epigenetic alterations of the miR-34 family were examined in 117 primary OS samples. The miR-34 family was found to be decreased and undergo minimal deletions and epigenetic inactivation in OS cells [28]. Mutations in the TP53 gene sequence, functional inhibition of p53 protein, and hypermethylation of the miR-34a promoter are all associated with the loss of miR-34a expression in tumors [29]. miR-34a was proven to be involved in the drug resistance, proliferation, and metastasis of OS [30, 31]. Sarcomas occur at a high frequency in p53-deficient mice and patients with Li-Fraumeni syndrome (LFS). The overexpression of c-Met in these tumors suggested that the miR-34-p53-c-Met axis could comprise a regulatory gene network that cooperatively controls tumor progression in OS [32].

As one of the common target of miR-34a, c-Met is encoded by the MET oncogene, which is the receptor for hepatocyte growth factor (HGF). This receptor is overexpressed in a variety of human malignancies and stimulates cell proliferation, local invasion, and distant migration [33]. Researchers transformed OBs into malignant cells characterized with OS properties via overexpression of MET [34]. HGF-c-Met signals can activate the downstream signals of RAS/MAPK and PI3K-Akt, which enhances the drug resistance of OS and promotes the motility and proliferation of sarcoma cells [35, 36].

A



B

Mature miRNA	Target gene	Number of samples		
		Normal	Loss	Gain
Overexpressed				
hsa-miR-223	RBPJ		1	6
hsa-miR-148a	GADD45A		1	6
hsa-miR-218	RELN	1	2	4
hsa-miR-195	HSPA4L	1	2	4
hsa-miR-223	RASA1	2	4	1
hsa-miR-126	PLK2	2	5	0
Underexpressed				
hsa-miR-335	WWP1		1	6
hsa-miR-31	CD48		2	5
hsa-miR-137	FXYD6	1	4	2
hsa-miR-382	NDRG2	1	3	3
hsa-miR-335	E1F4A2	2	3	2

Figure 2. miRNA signature and relevant target genes in OS. (A) Differentially expressed miRNAs more than 10-fold in OSs relative to OBs in at least four tumor samples are listed. (B) Genomic status and relative expression of relevant target genes.

3. Osteosarcoma stem cells and dedifferentiation

Cancer stem cells (CSCs) are characterized by self-renewal, pluripotency, and increased cell plasticity. Some OS cells expressed specific surface markers of MSCs such as Stro-1, CD105, and CD44 [37]. Other evidences suggested that single-cell suspensions were able to form sarcospheres in anchorage-independent and serum-free conditions. These spheroids showed increased expression of the pluripotency-associated genes OCT4, NANOG, and SOX2 compared with adherent cells [38].

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The currently embraced notion assumes that CSCs are critical for the recurrence and metastasis of malignancies, and common chemo- and radiotherapies are ineffective at killing CSCs. Thus, there is a need to explore the characteristics of CSCs in OS. CSCs isolated from OS are able to self-renew, sustain tumor generation, and confer metastatic potential and drug resistance [39]. The enhanced chemoresistance of the CSC subpopulation appears to be related to a more tolerant DNA repair ability [40] as well as an increased drug efflux capacity due to the high expression of ATP-binding cassette (ABC) transporters such as P-glycoprotein (MDR-1) and the breast cancer-resistant protein (BCRP/ABCG2) [41]. Developing CSC-targeted therapies could yield exciting new approaches for clinical application. The inhibition of ABC transporters is able to sensitize OS-derived sarcospheres to doxorubicin [42]. The nuclear factor κB (NF-κB) inhibitor BRM270 can specifically target the SaOS-2 stemlike cell population to undergo apoptosis [43].

Normal cells and cancer cells can acquire stem-like properties by several dedifferentiation inducers, including transcriptional networks involving key transcription factors (e.g., Oct4, Sox2, Nanog), miRNAs (e.g., let-7, miR-200 family), microenvironmental signals (e.g., hypoxia, inflammation, autocrine/paracrine oncogenic signaling pathways), epigenetic modifications (e.g., DNA demethylation, histone acetylation/methylation), and metabolic reprogramming [44].

Our group has demonstrated the role of the microenvironment and the intracellular context of OS on dedifferentiation. TGF-β1 and hypoxia are crucial factors that induce OS cells toward a CSC phenotype, which is characterized by the ability to self-renew and pluripotency. The dedifferentiated cells induced by TGF-β1 and hypoxia could differentiate into vascular endothelial-like cells (CD31 positive) in either a 3D culture system or xenografts. These cells could also form lipid droplets in an adipogenic differentiation medium. Gene set enrichment analysis (GSEA) revealed that gene alterations during the process of dedifferentiation are closely correlated with chemoresistance and metastasis in OS patients [5].

3.1. TGF-β1

The expression level of TGF-β1 is related to the metastatic potential of OS patients [45]. TGF-β1 suppressed miR-143 expression through a SMAD2/3-dependent mechanism and collaboratively upregulated the expression of versican to promote OS cell migration and invasion in vitro [46]. Blockage of the TGF-β1 autocrine loop inhibited OS cell proliferation and enhanced chemotherapy sensitivity, which might serve as a viable clinical treatment [47]. The tumor suppressor p16(INK4) inhibited the paracrine pro-migratory effect on OS stromal fibroblasts through the inhibition of TGF-β1 expression/secretion via an ERK1/2-dependent pathway [48].

OS cells can secrete factors that initiate osteoclast-mediated bone destruction, which coincides with TGF-β1 release from the bone matrix. It was suggested that OS cells might secrete TGF-β1 to maintain the stemness of MSCs and promote the production of pro-tumorigenic cytokines [49]. Elevated secretion of TGF-β1 by MSCs under hypoxic conditions could promote the growth, motility, and invasiveness of breast cancer cells [50]. This result indicated a possible link between TGF-β1 signaling and hypoxia.

High TGF-β1 expression occurs in many other types of cancer and is related to the state of ECM, angiogenesis, and immune escape [51]. The activation of TGF-β1 signaling triggers the epithelial-mesenchymal transition (EMT) and ensures that the transformed cancer cells possess a stronger capacity of self-renewal, tumorigenesis, and chemo-/radioresistance [52]. In OS or other tumor types, solid evidence suggests that TGF-β1 is responsible for promoting stemness [5, 53]. The TGF-β1 inhibitor SB525334 significantly inhibited the migration and invasion of sphere-forming stemlike cells [54]. In an OS mouse model, either overexpression of the natural TGF-β/SMAD signaling inhibitor SMAD7 in OS cells or treatment with the TGF-β receptor inhibitor SD208 affected the microarchitectural parameters of the bone and inhibited lung metastasis [55]. The natural alkaloid halofuginone, an inhibitor of the TGF-β/Smad3 cascade, specifically hindered OS progression against lung metastatic dissemination [56]. All of these studies revealed that blocking TGF-β resulted in the repression of the tumorigenic potential of OS cell lines, tumor-associated bone remodeling, and the development of metastasis, highlighting TGF-β1 as a promising therapeutic target.

3.2. Hypoxia

The hypoxic niche plays a vital role in regulating tumor cell behavior. During tumor proliferation, oxygen is unable to diffuse completely throughout the tumor. On the other hand, if newly formed blood vessels cannot reach the tumor region, these results in an imbalance between oxygen consumption and acquisition and creates a hypoxic microenvironment. Hypoxia-inducible factors (HIFs) are associated with the maintenance of cellular oxygen equilibrium and hypoxia adaptation when oxygen levels cannot meet the demand [57]. Hypoxic signaling promotes the expression and function of HIF-1α and HIF-2α.

It has been reported that in OS, HIF-1α is associated with drug resistance and/or radioresistance via either activation of Bcl-2 proapoptotic family-induced AMP-activated protein kinase (AMPK) signaling or an autophagy mechanism [58]. The downregulation of HIF-1α suppresses OS cell growth by inducing apoptosis [59], and the HIF-1α/CXCR4 pathways contribute to metastasis in human OS cells [60]. A recent meta-analysis has suggested that overexpression of HIF-1α is a predictive factor for poor outcomes in OS and could serve as a promising prognostic biomarker to predict the outcome of OS patients [61, 62].

HIF-2α plays a role in the maintenance of stem cell properties in both normal and cancer stem cells [63, 64]. It has been indicated that the long noncoding RNA (lncRNA) TCONS_00004241, also known as HIF-2α promoter upstream transcript (HIF2PUT), was associated with the sphere-forming capacity of CD133-positive OS stem cells. Overexpression of HIF2PUT markedly decreased the percentage of CD133-expressing cells in the MG-63 OS cell line and impaired their proliferation, migration, and self-renewal capacities [65]. These results suggest that HIF2PUT and the HIF-2α axis could provide a hypoxia-mediated therapeutic strategy to targeting stemlike cells in OS.

HIF is highly expressed in CSCs in various types of cancer, and blockade of either HIF-1α or HIF-2α activity would significantly attenuate the proliferation and self-renewal of CSCs [66]. Targeting the hypoxic microenvironment could be a possible therapeutic strategy to eradicate the CSC population in malignant tumors including OS. Researchers exposed highly metastatic

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mouse OS cells to hyperbaric oxygen and measured the cell viability. Cell proliferation was significantly suppressed under hyperbaric oxygen conditions, and a hyperbaric oxygen treatment in combination with carboplatin exhibited significant synergy in the suppression of cell proliferation. Concomitant hyperbaric oxygen enhanced the chemotherapeutic effects of carboplatin on both tumor growth and lung metastasis and reduced the mortality of OS-bearing mice. These findings suggested that the concomitant treatment of hyperbaric oxygen plus carboplatin could be an efficient therapeutic strategy for OS treatment [67].

4. Glycolysis in osteosarcoma

Metabolic reprogramming is considered to be a prominent hallmark in cancer [68]. In the 1920s, Otto Warburg found that cancer cells were prone to glycolysis even under aerobic conditions, while most of the surrounding normal cells underwent oxidative phosphorylation. This phenomenon, known as the "Warburg effect," has been confirmed in cancers from different tissues [69]. Although ATP productivity via glycolysis is lower than that via oxidative phosphorylation, glycolysis provides tumor cells with a stronger adaptability to a hypoxic environment caused by the lack of vasculature. Furthermore, glycolysis intermediates can provide precursors such as lipids, proteins, and nucleotides for the synthesis of macromolecules needed for proliferation [70].

The oxidative phosphorylation levels in different OS cell lines (LM7, 143B, SaOS-2, and HOS) were evaluated compared with those in noncancerous counterpart osteoblastic hFOB cells. The results showed that two of the OS cell lines (SaOS-2 and HOS) were actively respiring, whereas LM7 and 143B were highly glycolytic. Further analysis of the mitochondrion in the latter cell lines indicated mitochondrial swelling, depolarization, and membrane permeabilization, all of which could explain their reliance on glycolysis [13].

In OS, glycolysis might be caused by either gene mutation or a hyperactivated metabolic pathway. For example, the tumor suppressor p53, which is well characterized in safeguarding the body from developing OS [71], is important in the maintenance of the cytochrome C oxidase complex. The dysfunction of p53 can lead to reduced oxygen consumption from mitochondrial respiration and enhanced glycolysis [72]. The PI3K-Akt-mTOR pathway, a key oncogenic pathway in multiple human cancers that promotes glucose metabolism and cell proliferation, is frequently hyperactivated in OS and leads to glycolysis [73, 74].

Although the significance of glycolysis in OS is still under investigation, its value regarding clinical diagnosis and treatment has already been proven. 18F-Fluorodeoxyglucose (FDG)-positron emission tomography/computed tomography (PET/CT) has emerged as a promising tool for the diagnosis and prognosis for OS based on its ability to quantify glucose consumption. In several studies, patients with OS had undergone 18F-FDG PET/CT scans to measure imaging parameters such as the maximum standardized uptake value, metabolic tumor volume, and total lesion glycolysis both before and after chemotherapy. Significant differences between nonresponding tumors and responding lesions were observed and therefore could be used as predictors of the histological response to chemotherapy and patient survival [75, 76].

Lactate dehydrogenase A (LDHA) is a key enzyme involved in anaerobic glycolysis and converts pyruvate into lactate. It is upregulated in OS compared to normal OB cells (hFOB1.19). LDHA inhibition could decrease lactate production, inhibit cell proliferation and invasion in vitro, and compromise tumorigenesis in vivo [77]. 2-Deoxy-D-glucose (2DG), a glucose analogue, can be used as a glycolysis inhibitor which decreases lactate production, enhances oxidative phosphorylation, inhibits the metastatic phenotype in vitro, and delays metastasis in an orthotopic post-surgical model [78]. 2-DG is also used in combination with either adriamycin (ADR) or paclitaxel in animal models for the treatment of human OS and non-small-cell lung cancer [79].

As a heterogeneous entity with multicomponent interactions, the progression of OS depends upon reciprocal interactions between the neoplastic cells and the dynamic microenvironment. Tumor microenvironments include ECM, immune cells, endothelial cells, pericytes, fibroblasts, MSCs, adipocytes, and other components [80, 81]. Recent studies have described metabolic coupling among stromal cells such as cancer-associated fibroblasts (CAFs), adipocytes, immune cells, and neoplastic cells [82–90]. Glycolytic CAFs can provide nutrients such as lactates and ketones as fuel for tumor cells [82–84]. Adipocytes produce free fatty acids and promote fatty acid oxidation in tumor cells [85]. MSCs cocultured with OS cells can lead to metabolic reprogramming in both MSCs and neoplastic cells as described by the Warburg effect. After coculturing, MSCs underwent a metabolic shift toward aerobic glycolysis with increased lactate production and efflux due to the upregulation of monocarboxylate transporter-4 (MCT-4). In the meantime, OS cells would utilize lactate by increasing MCT-1 expression to enhance mitochondrial biogenesis and oxidative phosphorylation. Interestingly, these MSC-activated SaOS-2 and HOS cells also acquired an increased migratory capacity [91].

5. Angiogenesis and vasculogenic mimicry

Vascularization plays an important role in tumor survival and progression. Angiogenesis and vasculogenic mimicry (VM) have been demonstrated to be the two major processes in the development of tumor vascularization system, which supplies cancer cells with blood.

The growth, invasion, and metastasis of solid tumors require an adequate blood supply to transport nutrition and oxygen as well as metabolic waste and carbon dioxide [68, 92]. Tumors have their own vascular system, which is, however, highly abnormal and different from the normal vasculature with respect to organization, structure, and function.

OS is a type of malignant bone tumor with abundant blood vessels, indicating the prominent functions of the vasculature in OS progression. Increased vasculature could be a poor prognostic factor in human OS [93]. Similarly, a decrease in the number of vessels was shown to significantly reduce primary OS growth in a mouse model [94]. Here, we intend to summarize the theoretical and clinical findings in OS angiogenesis and VM.

5.1. Angiogenesis in OS

Angiogenesis is a dynamic and programmed process in which new capillaries sprout from preexisting vessels, and is induced by different triggers (e.g., hypoxia) that modulate a broad

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range of molecular mechanisms manipulating tip cells and stalk cells [95]. Angiogenesis firstly demonstrated its correlation to tumor growth by inserting a transparent chamber into mouse ears [96]. Subsequently, in vitro tumor-induced angiogenesis was established with a wound chamber [97].

Clinical studies on OS angiogenesis are highly controversial. The first clinical discussion on the relationship between angiogenesis and long-term outcomes of patients with OS was published in 2001 [98]. A retrospective immunohistochemical study was performed on biopsy specimens from non-metastatic OS patients with CD34 antibody staining and quantified the average intratumoral microvessel density (MVD) per field, but results showed no correlation with long-term outcome in patients with non-metastatic OS. Additionally, angiogenesis was correlated with the overall and disease-free survival as well as the metastasis rates because patients with a higher MVD had a shorter survival time and a higher metastatic rate [99]. However, the quantification and analysis have been hampered by heterogeneous OS vascularization and non-standardized methods in detecting microvessels and small study cohorts. Recent study applied highly standardized whole-slide imaging to overcome these limitations. Intratumoral vascularization was quantified at the time of diagnosis in whole sections from a multicenter cohort of 131 osteosarcoma patients. The results suggested that patients with low OS vascularization have a prolonged survival and good response to neoadjuvant chemotherapy [100]. Moreover, inhibition of angiogenesis in murine OS by the angiogenic inhibitor TNP470 indicated an antitumor ability with higher cancer cell death rate and an effective suppression of pulmonary metastasis in an OS mouse model [101].

Vascular endothelial growth factor (VEGF), a homo-dimeric protein also known as VEGFA, is a key trigger to induce either physiological or pathological angiogenesis including OS [102]. Elevated expression of VEGF in primary OS notably promotes angiogenesis, increases the local MVD and perimeter, and subsequently leads to a prominently higher rate ($p < 0.05$) of pulmonary metastasis. These findings correlate with a worse outcome in terms of the disease-free survival and overall survival in untreated patients [103, 104]. Furthermore, patients with serum VEGF > 1000 pg/ml had significantly worse survival than patients with levels < 1000 pg/ml ($p = 0.002$) despite the lack of a link between serum VEGF levels and the tumor volume as well as the sensitivity to preoperative chemotherapy [105]. The transcription level of VEGF isoform variants and VEGF receptors (Flt-1 and KDR) was detected in 30 OS samples. Interestingly, the cell-retained VEGF isoforms VEGF165 and VEGF189 might be critical for neovascularization in OS, while the soluble VEGF121 isoform is insufficient to stimulate neovascularization in this type of neoplasm [106]. This also indicated that only specific types of VEGF isoforms have the ability to induce OS angiogenesis. Orthotopic injection of human OS cells with either high or low VEGF expression into severe combined immunodeficient mice uncovered that high VEGF-expressing OS cells developed more malignant xenografts with earlier neoplasm formation, larger tumor size, more frequent invasion to the peritumoral tissue, and a higher rate of lung metastasis [107]. VEGF blockade by sFlt1 in a murine model partially abrogated the angiogenesis and delayed VEGF-promoted tumor growth [108]. In view of the substantial influence of VEGF in OS progression, molecular regulation of VEGF in tumorigenesis and progression of OS has been studied in recent years. STAT3 has been determined as an important upstream regulator in VEGF expression, while the

PI3K-Akt pathway has been suggested as the main signaling cascade downstream of VEGF that mediates OS angiogenesis [109, 110]. Several studies also showed that members of the interleukin (IL) family, such as IL-6 and IL-17, could induce VEGF expression and promote angiogenesis in OS [111, 112]. The CXCL12-CXCR4 axis has additionally been demonstrated to be involved in promoting VEGF expression [113]. As opposed to the factors mentioned above, miR-145 targets VEGF and inhibits angiogenesis as well as the invasion and metastasis of OS cells [114].

Endostatin, a 20 kDa fragment of collagen XVIII, is a member of a group of endogenous anti-angiogenic proteins activated by proteolytic processing. Endostatin inhibits endothelial cell proliferation, migration, and invasion by modifying 12% of the human genome to downregulate pathological angiogenesis without exerting side effects, which makes this protein a broad-spectrum angiogenesis inhibitor. Anti-angiogenic therapy by endostatin was performed in OS-burdened mice models [115, 116]. Notably, the number of pulmonary metastatic lesions was lower, and the size of the pulmonary metastatic lesions was smaller in the group treated with endostatin compared to control group. Thus, anti-angiogenic therapy might be a potential treatment for OS because it provides patients with a promising improvement to their prognosis, although anti-angiogenic therapies cannot thoroughly cure OS [117].

5.2. Vasculogenic mimicry

Apart from the important role of angiogenesis in OS vessel network formation, VM has emerged as another effective pathway in OS vascular development. VM is defined as a type of vasculature-like lumen formed by tumor cells and the extracellular matrix instead of by endothelial cells and becomes incorporated into the tumor blood microcirculation. It was first reported in melanoma and identified by CD34-negative and periodic acid-Schiff (PAS)-positive staining in which red blood cells could be detected [118].

VM also has been detected in OS in vivo and in vitro. Immunohistochemical staining for endothelial cell marker CD34, OB-related marker osteocalcin, and PAS was performed on OS clinical samples. VM channels were confirmed in OS specimens in which the channel wall was positive for osteocalcin and PAS but negative for CD34 [119]. Further investigation by using the Kaplan-Meier survival analysis found that the present rate of VM in OS patients after preoperative chemotherapy was correlated with both the overall survival ($p = 0.011$ and 0.040) and metastasis-free survival ($p = 0.002$ and 0.045). Additionally, as a strong mediating factor in vascular formation, inhibition of VEGF by siRNA in the human OS cell line MG-63 could suppress VM formation in vitro [103]. Furthermore, vascular endothelial-cadherin (VE-cadherin) seems to be critical in the formation of VM. After knocking down VE-cadherin, OS cells could not form OS-generated endothelial-like networks in vitro [120].

Notably, unlike the typical $CD31^-/CD34^-/PAS^+$ VM, our group found that osteosarcoma stem cells (OSCs) had the capability to construct a CD31-positive vascular network de novo either under hypoxia or upon VEGFA induction [5]. This neo-VM subtype was formed by a type of vascular endothelial cell-like cells that transdifferentiated from OSCs as shown in **Figure 3**.

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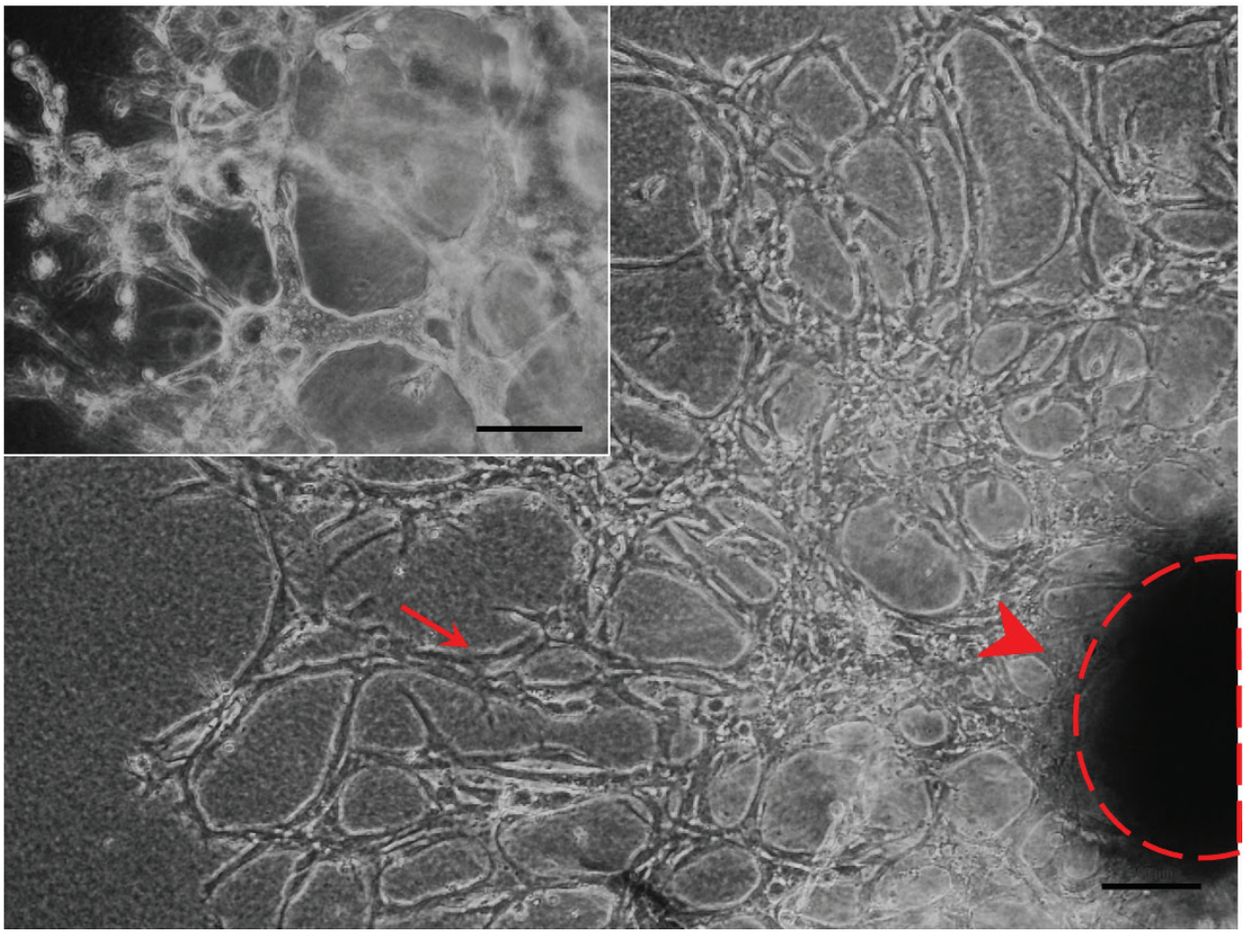

Figure 3. Differentiation potential of OSCs into vascular endothelial-like cells and formed vasculature-like network. During the transdifferentiation, vessel-like sprouts appeared around the outermost region of the OSCs (*arrowhead*), followed by the appearance of numerous branches (*arrow*). These branches extended out from the spheres and eventually formed a vasculature-like network. The *dotted line* and *arrowhead* show the region of the OSCs. The arrow indicates the vasculature-like network which is formed by vascular endothelial-like cells. High magnification image of the vasculature-like network is shown as an inset. Scale bar = 100 μm.

6. Stromal niche: bone marrow mesenchymal stem cell

OS is more often found in the distal femur and proximal tibia, which are also the major milieu of bone marrow MSCs. MSCs are a heterogeneous subpopulation of adult stem cells with immunomodulatory properties and a potential to differentiate into several tissue-specific cells such as OBs, adipocytes, and chondrocytes [121].

It is widely accepted that the tumor microenvironment is correlated with tumorigenesis and cancer progression. Since MSCs are one of the important components in the OS microenvironment, many studies have investigated the contribution of bone marrow MSCs to OS growth and progression. MSCs isolated from primary OS tissue, which show no neoplastic features, are similar to their bone marrow counterparts with regard to morphology, specific gene expression, and differentiation potential. Exogenous MSCs could target the OS site and promote OS growth and progression in a mouse xenograft model [122]. Similar results were also found in a rat model [123]. IL-6 secreted by MSCs could activate STAT3 signaling in OS

cells, which in turn augment cell proliferation, migration, invasion, and pulmonary metastasis [124]. Interestingly, IL-6/STAT3 signaling could also respond to MSCs to enhance drug resistance. MSC-conditioned medium could improve the survival of U-2 OS and SaOS-2 cells and reduce apoptosis in the presence of therapeutic concentrations of either doxorubicin or cisplatin via the IL-6/STAT3 signaling pathway by increasing the expression of multidrug-resistant protein (MRP) and MDR-1 and decreasing the expression of caspase 3/7 activity and annexin V binding. Furthermore, the proliferation and progression of neoplastic cells need to be initiated and induced by certain pro-tumor cytokines secreted by MSCs. Therefore, OS cells could inhibit MSC differentiation into OBs via the TGF-β/Smad2/3 signaling pathway to promote the secretion of cytokines from MSCs [49].

Basic helix-loop-helix (bHLH) transcription factors belong to the third largest family of recognized transcription factors in the human genome and are essential regulators of development and differentiation via DNA-binding elements known as E boxes. DNA binding of bHLH proteins is restricted by heterodimerization with inhibitors of DNA binding (IDs). ID ubiquitination by ubiquitin-specific peptidase 1 (USP1) has been demonstrated to not only be necessary for the proliferation of several OS cell lines but also sufficient to prevent normal mesenchymal cell differentiation and sustain the cells in a stemlike state [125]. Meanwhile, a recent study uncovered a phenomenon of functional mitochondrial transfer from bone marrow stromal cells to acute myeloid leukemia (AML) cells during chemotherapy, which confers survival advantages for AML cells [126]. Altogether, preventing the differentiation of MSCs into OBs might remodel the bone microenvironment and provide OS cells with a more suitable survival niche.

As a vital component of the OS environment, MSCs might play a critical role in OS malignancy and could be a potential target in cancer therapy.

7. Emerging role: exosomes

Tumor cell function not only depends on self-regulation but also requires a significant assistance from the microenvironment to support growth and help with immune escape and motility through the local area. Approximately 15–20% of patients diagnosed with OS are observed as having detectable metastasis via X-ray examination. Additionally, more than 30% of patients will develop metachronous lung metastases, which makes clinical treatment more challenging [127, 128]. There is an urgent need for more studies on the early diagnosis of distant metastasis of OS. In recent years, more researchers have focused their concentration on an emerging role of extracellular vesicles, also referred to as exosomes, in cancer metastasis.

Exosomes are extracellular vesicles that originate within microvesicular bodies and are shed from plasma membrane with sizes in the range from 30 to 100 nm [129, 130]. Exosomes are unilamellar vesicles composed of a lipid bilayer and have a homogenous cup-shaped appearance based on scanning electron microscopy [131, 132]. The contents of exosomes are varied and heavily depend on the originating cells, but these are broadly considered to include proteins, mRNAs, miRNAs, lipids, and carbohydrates [133]. Exosomes have been recognized as important to intercellular communication among tumor cells [134]. However, related papers focusing on exosomes in OS are scarce and limited.

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Exosomes isolated from the multidrug-resistant human OS cell line MG-63DXR30 by differential centrifugation of the culture media could be taken up into secondary cells and induce a doxorubicin-resistant phenotype, suggesting that exosomes play a potential critical role in transferring the multidrug-resistant phenotype [135]. A systematic comparison of the proteomes, exosomes, and exosome-free fractions was performed in MG-63, U-2 OS, and SaOS-2 cells. The results showed that OS cells can secrete different exosomes involved in angiogenesis, cell adhesion, and migration [136]. Additionally, it has been indicated that Notch-activating factors can be delivered to the murine muscle cells by exosomes from the murine OS cell line K7M2 and specifically increase Notch signaling pathway activation [137]. The urokinase plasminogen activator (uPA) is a serine protease involved in ECM degradation and plays a significant role in the progression and metastasis of various solid tumors including the breast, lung, prostate, pancreas, ovary, kidney, and colon [138]. The levels of uPA and the uPA receptor (uPAR) were exclusively elevated in metastatic OS cells. These metastatic OS cells secrete both an active soluble form and an exosome-encapsulated form of uPA to drive the migration or metastatic conversion of OS cells [139]. Other research demonstrated that exosomes secreted by human MSCs could exhibit antiapoptotic function or cell-protective function to increase OS survival under serum starvation conditions [140]. Exosomes may also be a neo-drug vector for OS treatment. For example, synthetic miR-143 can be enveloped in exosomes and transferred to OS cells exhibiting that the delivery of miR-143 via exosomes could significantly reduce the migration of OS cells [141].

In the future, research of the effects of exosome should be focused on its constituents in OS. As these microvesicles are involved in tumor progression, they might be the promising targets for cancer therapy. We could possibly identify tumor antigens to improve the diagnosis and prognosis of OS if exosome contents are associated to different levels of aggressiveness. Importantly, exosomes are easily isolated from the peripheral blood and other bodily fluids and could be used as a noninvasive diagnostic tool [142–144].

8. Mimicking the bone microenvironment

To reveal the process in detail that normal cells take to evolve to a neoplastic state and their subsequent progression to metastasis, proper research models need to be established. Establishment of an OS research model has always been challenging. Researchers initially used transgenic technology to reedit key genes in mice [145], but since then great strides have been made for the establishment and improvement of various OS animal models [6–10, 15, 146]. Despite all this, animal models and patient tissues are often limited by the availability of test subjects, feasibility of the testing procedure, and maintaining viable tissue. Furthermore, there are important ethical concerns regarding the compassion for experimental animals that may suffer pain or discomfort during the study. In vitro models have the advantage of easier availability and operability as well as reducing time and monetary costs.

Traditional two-dimensional cultures are most commonly used for the in vitro study of mammalian cells and have made remarkable contributions to scientific discovery. Even so, cultivation either on plastic dishes or in flasks rarely recapitulates the conditions of cell activities in vivo. The limitations of flat culturing regarding the cellular microenvironment have

prompted the use of three-dimensional (3D) cultures [147]. The advantages of 3D cell culture include better mimicry of the cell-cell interactions and of the intricate microenvironment. In recent years, zebrafish models have been generated as a comprehensive stand-in for malignancy research and are especially appealing for OS because of their similarities to human osteogenesis [148–152]. More high-tech models are being created with the rapid development of engineering techniques. It is promising that these novel technologies could be applied in drug testing as well as other physiological and biochemical studies with the goal of replacing animal models to reduce the use of experimental animals.

8.1. Extracellular matrix

ECM is a collection of extracellular molecules that provides structural and biochemical support to the surrounding cells and therefore plays a vital role in cell adhesion, cell communication, and maintenance of function. In the case of the bone, the organic portion of ECM primarily comprises type I collagen secreted by OB lineage cells, while calcium phosphate in the form of hydroxyapatite composes its mineralized portion. Bone ECM provides a scaffold for mineral storage and regulates OB lineage and osteoclast lineage cell function and differentiation of MSCs to OBs [153]. The usage of bone ECM in tissue engineering and biological studies has attracted attention [154, 155]. Porcine cartilage was decellularized, solubilized, and then methacrylated, and ultraviolet (UV) photocrosslinked to create methacrylated solubilized decellularized cartilage hydrogels. These hydrogels were characteristically similar to native cartilage tissue and could support ECM production. Additionally, these hydrogels supported the growth of rat bone marrow-derived MSCs that were encapsulated in the gel networks and caused significant upregulation of chondrogenic genes [156]. Bone-like ECM synthesized by OBs was used to enhance the osteoblastic differentiation of MSCs in vitro [157], and decellularized cartilage ECM was applied as a treatment for osteochondral defects [158].

Our group has generated tissue-derived bone ECM from humans, mice, and rats and established an OS model that could mimic an intact OS environment in vitro by injecting OS cells into bone ECM. Bone ECM is soaked in cell-cultured medium after decalcification and decellularization, and OS cells are injected into ECM and cultured under complete medium. As shown in **Figure 4**, bone ECM provides a scaffold for OS cell proliferation and shows amazing biocompatibility.

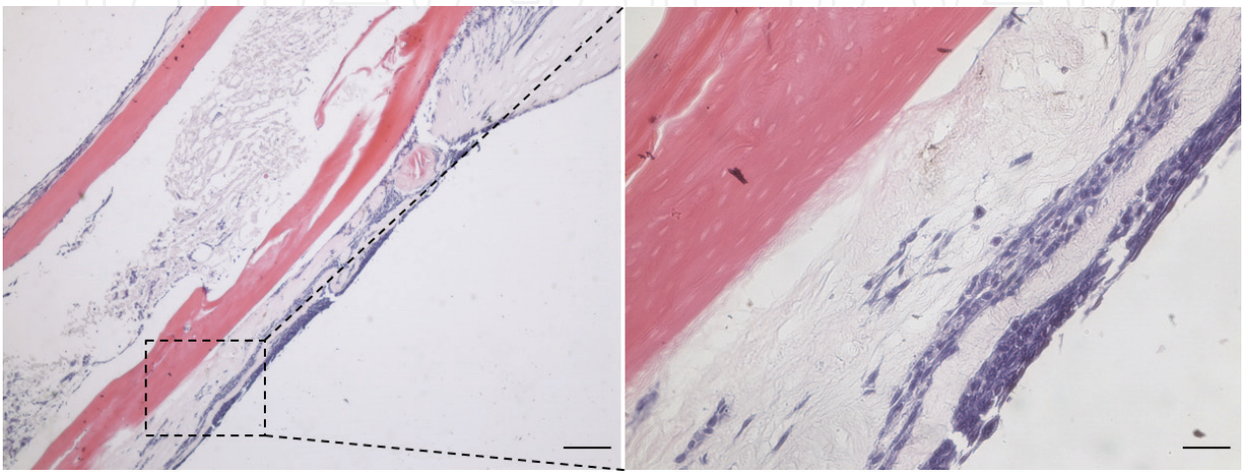

Figure 4. HE staining of mouse bone ECM after injecting MNNG/HOS (unpublished data). Scale bar = 100 μm.

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8.2. Zebrafish: an in vivo model for OS research

Zebrafish is an important and widely used vertebrate model in scientific research. In recent years, they have become a useful model for cancer and other diseases due to their straightforward genome information with abundantly conserved regions homologous with those in human beings, their small size and ease of manipulation, and their transparent bodies which make observation of organ systems easy. Compared to the 3D model, zebrafish can address the issue of maturation, which is a virtually insurmountable barrier of in vitro development.

As a multifunctional model, zebrafish with genetic modifications have been used in a large number of experiments. Transgenic zebrafish with a GFP-tagged vasculature provide an advanced approach for the study of angiogenesis and cancer metastasis and can easily be observed by either light microscopy (**Figure 5**) or laser confocal microscopy. Furthermore, leukemia, melanoma, pancreatic adenocarcinoma, intestinal hyperplasia, and other types of solid tumor have been studied in zebrafish models, which are stable and effective assay method for investigating pathogenesis.

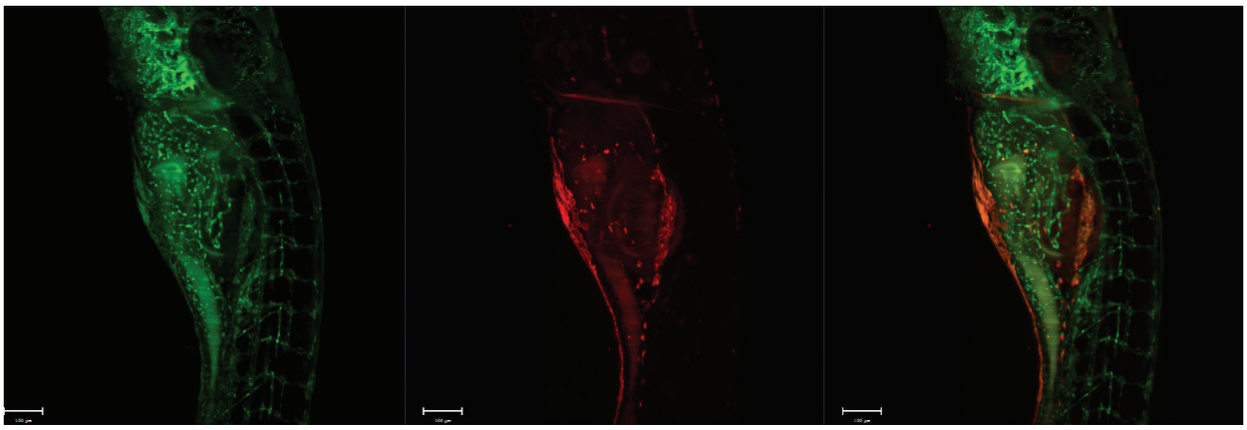

Figure 5. The FLK^{+}-GFP zebrafish showed a green vasculature system photographed by light sheet microscopy (unpublished data). Scale bar = 100 μm.

An OS xenograft zebrafish model has also been reported recently [159]. Since OS probably originates from MSCs mutated in the process of differentiation toward OBs, one group injected two MSC cell lines, after 8 months of culturing, and found that the cells gained a malignant transformation. The results found that transformed MSCs formed an OS mass, induced angiogenesis, and migrated through the bodies of the embryos of zebrafish, which was not observed in the normal MSC controls. Whole-genome analysis indicated higher expression of matrix metalloproteinase 19 (MMP-19) and erythroblastosis virus E26 oncogene homologue 1 (Ets-1) in the mutated cells compared to normal cells. Furthermore, upon investigation the host response, zebrafish embryos injected with transformed MSCs showed decreased expression of immune response-related genes, especially major histocompatibility complex class I (MHC-I), compared to embryos injected with normal MSCs. The above experiments also reproduced tumorigenesis, progression of OS, including angiogenesis, migration, and metastasis in vivo and identified potential molecular regulators by using a zebrafish model.

Zebrafish is also a useful tool for screening for OS therapeutic drugs. The development of metastases is still the major cause of death of patients with OS as well as other cancers. Ezrin, the prototypical ezrin/radixin/moesin (ERM) protein family member, is associated with the actin cytoskeleton and the plasma membrane. Ezrin has been demonstrated to be a vital protein related to cancer metastasis. Microinjection of ezrin small-molecule inhibitors, NSC305787 and NSC668394, into zebrafish embryos prominently inhibited cell mobility during embryonic development. The results supported an approach using ezrin protein as a putative target molecule in OS therapy [160].

8.3. Other novel OS models

With their advantages of in vivo vascularization and an immune system, animal models can be instrumental for executing drug screens and studying the etiology of OS. Apart from the cell-of-origin transgenic models and the zebrafish models mentioned above, there are more novel therapeutic interventions in various models that have already been reported or are in current veterinary clinical trials [161].

OS is an aggressive primary bone cancer with highly metastatic capacity, and the development of pulmonary metastases is the most common reason for treatment failure. K7M3 cells were injected into the tibia of wild-type BALB/c mice to induce a primary bone tumor or into the tail vein of wild-type BALB/c and gld mice to form pulmonary metastases [162]. To assess the importance of Fas in the process of OS lung metastasis, two animal models for lung metastases were generated through intravenous injection or subcutaneous injection in mice, and those proved the efficacy of aerosol gemcitabine (GCB) which targets Fas pathway [163].

The assessment of the safety issue of a regional aerosol GCB delivery and evaluation of the effect of GCB on Fas pathway in lung metastasis of OS-bearing dogs further confirmed clinical and pathological findings in mice [164]. The clinical and pathological findings in mice were further confirmed and extended in a canine model, which supports the notion that aerosolized gemcitabine may be useful against the pulmonary metastasis of OS and can allay patient tolerability concerns to a certain extent.

9. Conclusion

Multiple genomic aberrations together with abnormal activation of receptor kinases greatly contribute to the complex etiology of OS. There is no escaping the fact that in many respects, microenvironmental signals can either support or interrelate with tumor cells to regulate the biological behavior of OS. Although the remodeling systems established heretofore still require more precise characterization in vivo with respect to the extent of recapitulation, the utilization of physiological and biochemical studies can eventually be applied to clinical pharmacokinetic studies and evaluations of therapeutic efficiency. To gain exact and further insight on the cross talk between tumor cells and the microenvironment, both in vivo and in vitro novel models should be created and applied in research.

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