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Microenvironment Signals and Mechanisms in the Regulation of Osteosarcoma

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<http://dx.doi.org/10.5772/67068>

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- β 1 (TGF- β 1) 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 haematogenous 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- β 1 (TGF- β 1) 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].

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 neuronal antigen 2/chondroitin sulfate proteoglycan 4 (Ng2/Cspg)-expressing pericytes. They also determined the role of β -catenin dysregulation in the neoplastic phenotype.

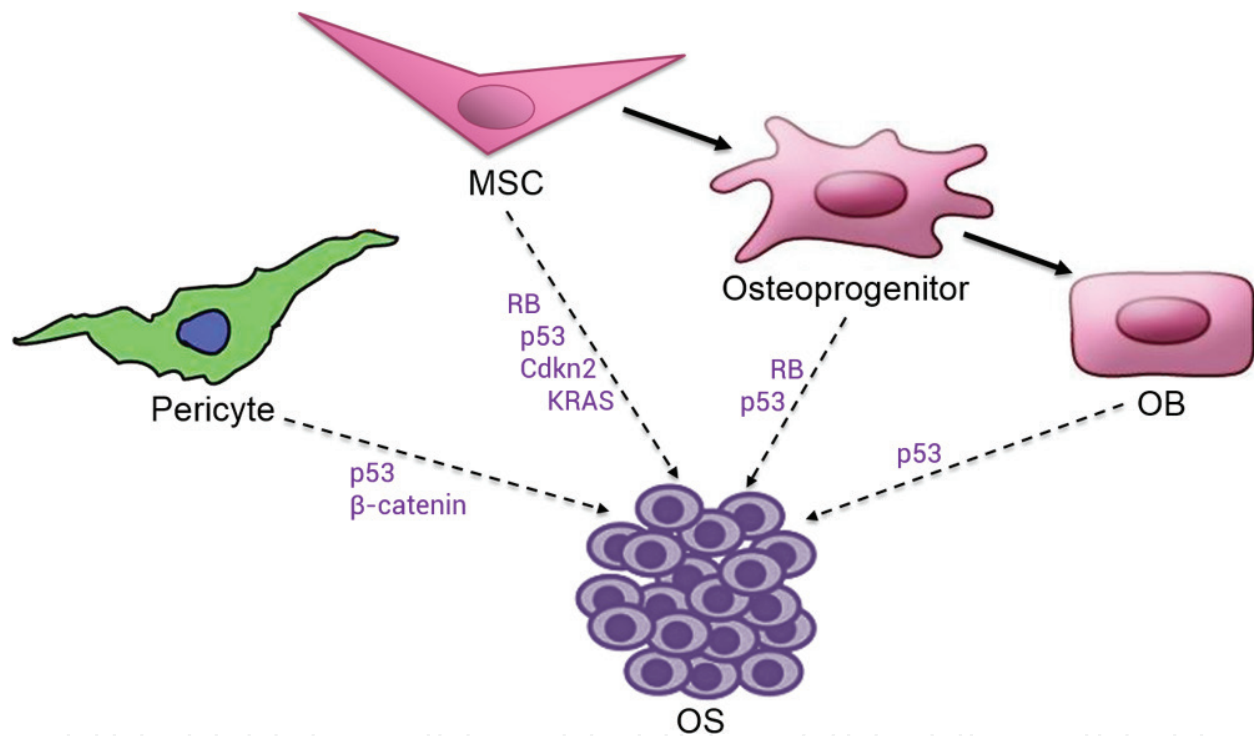


Figure 1. Cells of origin in OS. OS initiation is promoted by multiple genetic alterations (e.g., activation of oncogenes or inactivation of tumor suppressor genes).

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- β 1 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.) coinjection 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 antiapoptotic genes Birc2 (cIAP1) and Birc3 (cIAP2) are potential oncogenic drivers in the chromosome 9A1 amplicon [18].

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].

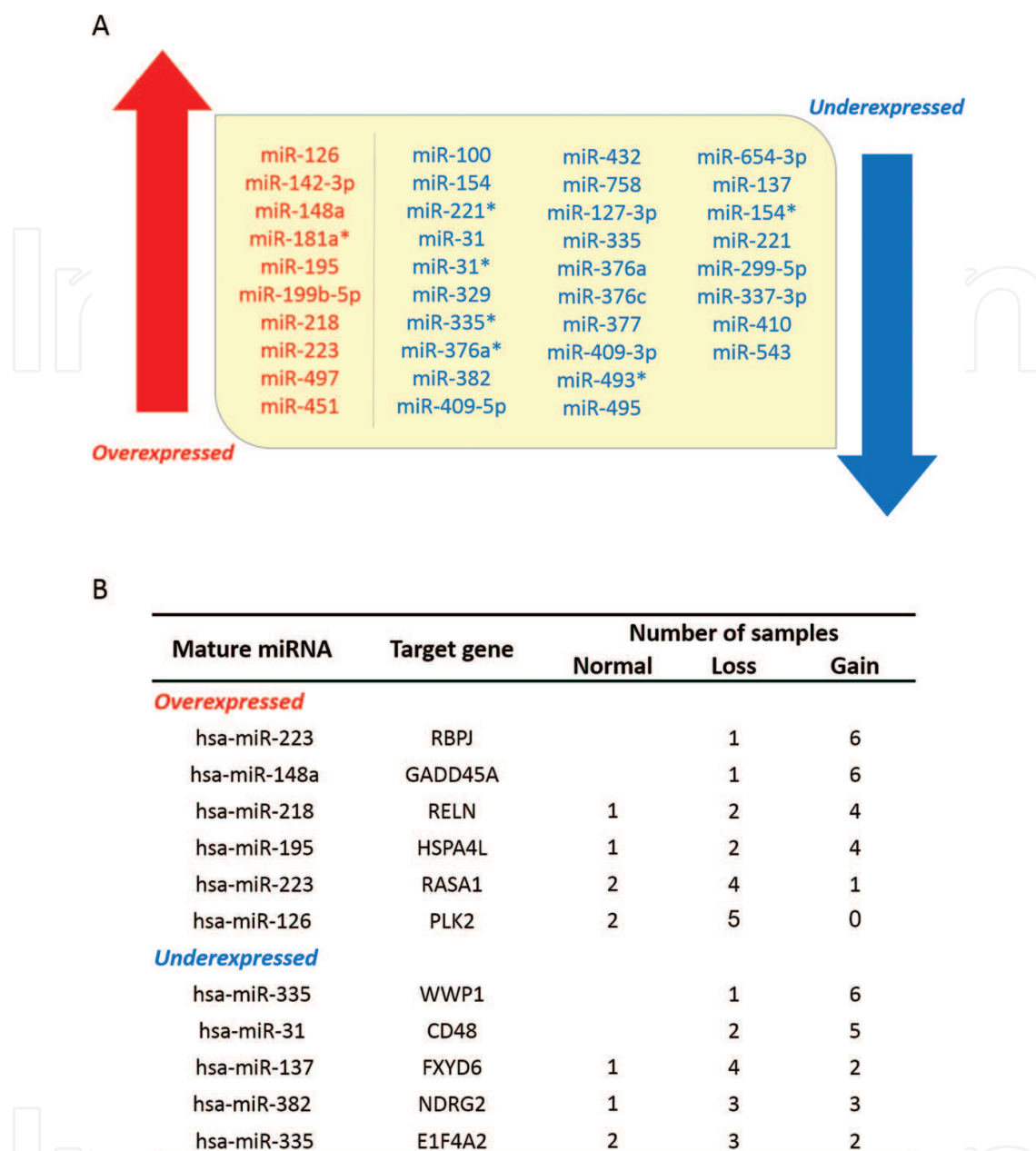


Figure 2. miRNA signature and relevant target genes in OS. (A) Differentially expressed miRNAs more than 10-fold in OSs relative to OBs in at least four tumor samples are listed. (B) Genomic status and relative expression of relevant target genes.

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 spheroids 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].

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 spheroids to doxorubicin [42]. The nuclear factor κ B (NF- κ B) 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- β 1 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- β 1 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- β 1

The expression level of TGF- β 1 is related to the metastatic potential of OS patients [45]. TGF- β 1 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- β 1 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- β 1 expression/secretion via an ERK1/2-dependent pathway [48].

OS cells can secrete factors that initiate osteoclast-mediated bone destruction, which coincides with TGF- β 1 release from the bone matrix. It was suggested that OS cells might secrete TGF- β 1 to maintain the stemness of MSCs and promote the production of pro-tumorigenic cytokines [49]. Elevated secretion of TGF- β 1 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- β 1 signaling and hypoxia.

High TGF- β 1 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- β 1 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- β 1 is responsible for promoting stemness [5, 53]. The TGF- β 1 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- β 1 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-1 α and HIF-2 α .

It has been reported that in OS, HIF-1 α 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-1 α suppresses OS cell growth by inducing apoptosis [59], and the HIF-1 α /CXCR4 pathways contribute to metastasis in human OS cells [60]. A recent meta-analysis has suggested that overexpression of HIF-1 α 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-2 α 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-2 α 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-2 α 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-1 α or HIF-2 α 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

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

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**.

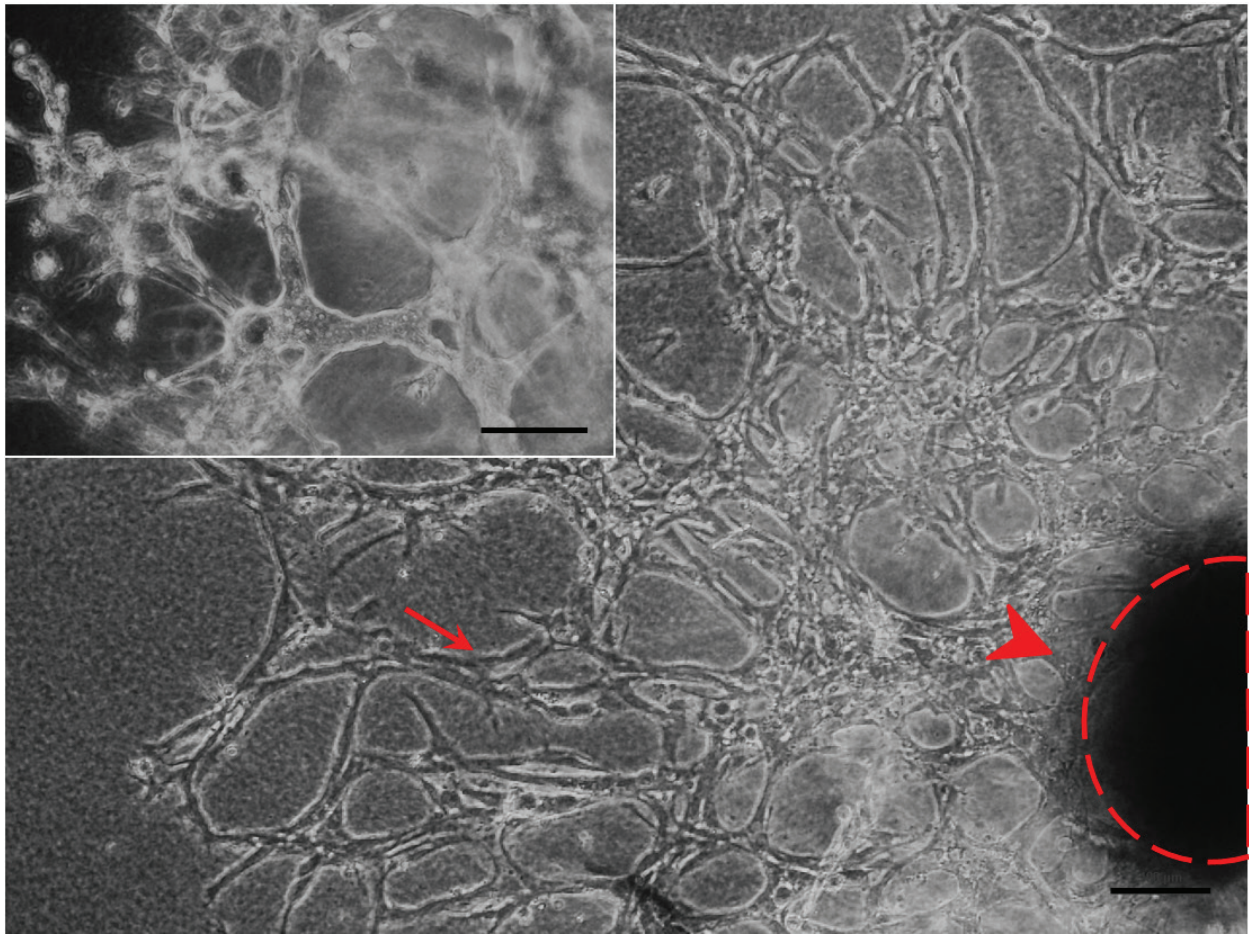


Figure 3. Differentiation potential of OSCs into vascular endothelial-like cells and formed vasculature-like network. During the transdifferentiation, vessel-like sprouts appeared around the outermost region of the OSCs (*arrowhead*), followed by the appearance of numerous branches (*arrow*). These branches 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.

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.

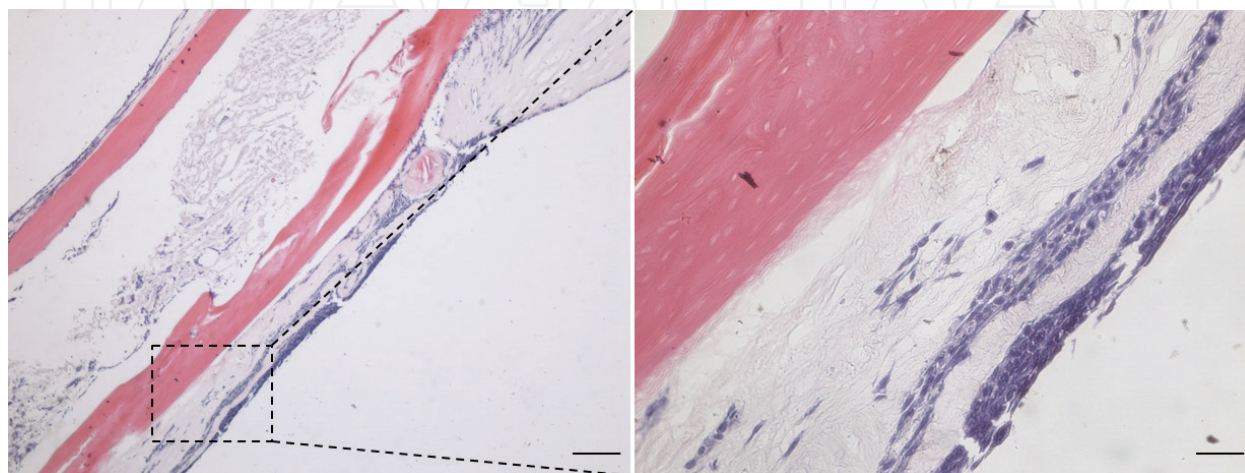


Figure 4. HE staining of mouse bone ECM after injecting MNNG/HOS (unpublished data). Scale bar = 100 μ m.

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.

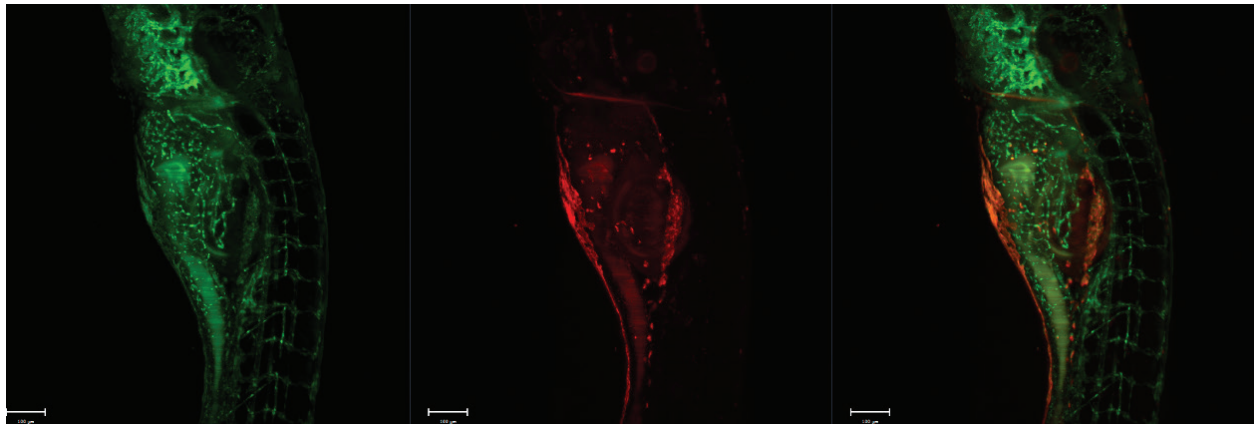


Figure 5. The FLK¹-GFP zebrafish showed a green vasculature system photographed by light sheet microscopy (unpublished data). Scale bar = 100 µm.

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, micro-environmental 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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References

- [1] Berman SD, Calo E, Landman AS, et al. Metastatic osteosarcoma induced by inactivation of Rb and p53 in the osteoblast lineage. *Proc Natl Acad Sci U S A*. 2008 Aug 19;105(33):11851–11856. DOI: 10.1073/pnas.0805462105.
- [2] Bielack SS, Kempf-Bielack B, Delling G, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *J Clin Oncol*. 20:776–790. DOI: 10.1200/JCO.20.3.776.
- [3] Overholtzer M, Rao PH, Favis R, et al. The presence of p53 mutations in human osteosarcomas correlates with high levels of genomic instability. *Proc Natl Acad Sci U S A*. 2003 Nov 25;100(24):14511. DOI: 10.1073/pnas.1934852100.
- [4] Selvarajah S, Yoshimoto M, Maire G, et al. Identification of cryptic microaberrations in osteosarcoma by high-definition oligonucleotide array comparative genomic hybridization. *Cancer Genet Cytogenet*. 2007 Nov;179(1):52–61. DOI: 10.1016/j.cancergencyto.2007.08.003.
- [5] Zhang H, Wu H, Zheng J, et al. Transforming growth factor β 1 signal is crucial for dedifferentiation of cancer cells to cancer stem cells in osteosarcoma. *Stem Cells*. 2013 Mar;31(3):433–446. DOI: 10.1002/stem.1298.
- [6] Basu-Roy U, Basilico C, Mansukhani A. Perspectives on cancer stem cells in osteosarcoma. *Cancer Lett*. 2013 Sep 10;338(1):158–167. DOI: 10.1016/j.canlet.2012.05.028.
- [7] Rubio R, Abarrategi A, Garcia-Castro J, et al. Bone environment is essential for osteosarcoma development from transformed mesenchymal stem cells. *Stem Cells*. 2014 May;32(5):1136–1148. DOI: 10.1002/stem.1647.
- [8] Mohseny AB, Szuhai K, Romeo S, et al. Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. *J Pathol*. 2009 Nov;219(3):294–305. DOI: 10.1002/path.2603.
- [9] Dung-Fang Lee, Jie Su, Huen Suk, et al. Modeling familial cancer with induced pluripotent stem cells. *Cell*. 2015 Apr 9;161(2):240–254. DOI: 10.1016/j.cell.2015.02.045.

- [10] Sato S, Tang YJ, Wei Q, et al. Mesenchymal tumors can derive from Ng2/Cspg4-expressing pericytes with β -catenin modulating the neoplastic phenotype. *Cell Rep*. 2016 Jul 13. DOI: 10.1016/j.celrep.2016.06.058.
- [11] Nowell PC. The clonal evolution of tumor cell populations. *Science*. 1976 Oct 1;194(4260):23–28. DOI: 10.1126/science.959840.
- [12] Varley JM. Germline TP53 mutations and Li-Fraumeni syndrome. *Hum Mutat*. 2003 Mar;21(3):313–320. DOI:10.1002/humu.10185.
- [13] Velletri T, Xie N, Wang Y, et al. P53 functional abnormality in mesenchymal stem cells promotes osteosarcoma development. *Cell Death Dis*. 2016 Jan 21;7:e2015. DOI: 10.1038/cddis.2015.367.
- [14] Chen X, Bahrami A, Pappo A, et al. Recurrent somatic structural variations contribute to tumorigenesis in pediatric osteosarcoma. *Cell Rep*. 2014 Apr 10;7(1):104–112. DOI: 10.1016/j.celrep.2014.03.003.
- [15] Saalfrank A, Janssen KP, Ravon M, et al. A porcine model of osteosarcoma. *Oncogenesis*. 2016 Mar;5(3):e210. DOI: 10.1038/oncsis.2016.19.
- [16] Maitra A, Roberts H, Weinberg AG, Geradts J. Loss of p16INK4a expression correlates with decreased survival in pediatric osteosarcomas. *Int J Cancer*. 95:34–38. DOI: 10.1002/1097-0215(20010120)95:1<34::AID-IJC1006>3.0.CO;2-V.
- [17] Xiong Y, Wu S, Du Q, et al. Integrated analysis of gene expression and genomic aberration data in osteosarcoma (OS). *Cancer Gene Ther*. 2015 Nov;22(11):524–529. DOI: 10.1038/cgt.2015.48.
- [18] Ma O, Cai WW, Zender L, et al. MMP13, Birc2 (cIAP1), and Birc3 (cIAP2), amplified on chromosome 9, collaborate with p53 deficiency in mouse osteosarcoma progression. *Cancer Res*. 2009 Mar 15;69(6):2559–2567. DOI: 10.1158/0008-5472.CAN-08-2929.
- [19] Ambros V. The functions of animal microRNAs. *Nature*. 2004 Sep 16;431(7006):350–355. DOI: 10.1038/nature02871.
- [20] Bartel DP. microRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004 Jan 23;116(2):281–297. DOI:10.1016/S0092-8674(04)00045-5.
- [21] Subramanian S, Lui WO, Lee CH, et al. MicroRNA expression signature of human sarcomas. *Oncogene*. 2008;27:2015–2026. DOI: 10.1038/sj.onc.1210836.
- [22] Israel A, Sharan R, Ruppin E, Galun E. Increased microRNA activity in human cancers. *PLoS One*. 2009;4:e6045. DOI: 10.1371/journal.pone.0006045.
- [23] Ambros S, Prueitt RL, Yi M, et al. Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. *Cancer Res*. 2008; 68:6162–6170. DOI: 10.1158/0008-5472.CAN-08-0144.
- [24] Maire G, Martin JW, Yoshimoto M, et al. Analysis of miRNA-gene expression-genomic profiles reveals complex mechanisms of microRNA deregulation in osteosarcoma. *Cancer Genet*. 2011 Mar;204(3):138–146. DOI: 10.1016/j.cancergen.2010.12.012.

- [25] Yang C, Yang S, Wood KB, et al. Multidrug resistant osteosarcoma cell lines exhibit deficiency of GADD45 α expression. *Apoptosis*. 2009 Jan;14(1):124–133. DOI: 10.1007/s10495-008-0282-x.
- [26] Pellegrino R, Calvisi DF, Ladu S, et al. Oncogenic and tumor suppressive roles of polo-like kinases in human hepatocellular carcinoma. *Hepatology*. 2010 Mar;51(3):857–868. DOI: 10.1002/hep.23467.
- [27] Wang S, Aurora AB, Johnson BA, et al. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Dev Cell*. 2008 Aug;15(2):261–271. DOI: 10.1016/j.devcel.2008.07.002.
- [28] He C, Xiong J, Xu X, et al. Functional elucidation of miR-34 in osteosarcoma cells and primary tumor samples. *Biochem Biophys Res Commun*. 2009 Oct 9;388(1):35–40. DOI: 10.1016/j.bbrc.2009.07.101.
- [29] Lodygin D, Tarasov V, Epanchintsev A, et al. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. *Cell Cycle*. 2008 Aug 15;7(16):2591–2600. DOI:10.4161/cc.7.16.6533.
- [30] Pu Y, Zhao F, Wang H, et al. MiR-34a-5p promotes the multi-drug resistance of osteosarcoma by targeting the CD117 gene. *Oncotarget*. 2016 Apr 1. DOI: 10.18632/oncotarget.8546.
- [31] Yan K, Gao J, Yang T, et al. MicroRNA-34a inhibits the proliferation and metastasis of osteosarcoma cells both *in vitro* and *in vivo*. *PLoS One*. 2012;7(3):e33778. DOI: 10.1371/journal.pone.0033778.
- [32] Rong S, Donehower LA, Hansen MF, et al. Met proto-oncogene product is overexpressed in tumors of p53-deficient mice and tumors of Li-Fraumeni patients. *Cancer Res*. 1995 May 1;55(9):1963–1970.
- [33] Maroun CR, Rowlands T. The Met receptor tyrosine kinase: a key player in oncogenesis and drug resistance. *Pharmacol Ther*. 2014 Jun;142(3):316–338. doi: 10.1016/j.pharmthera.2013.12.014.
- [34] Patanè S, Avnet S, Coltella N, et al. MET overexpression turns human primary osteoblasts into osteosarcomas. *Cancer Res*. 2006, 1;66(9):4750–4757. DOI: 10.1158/0008-5472.CAN-05-4422.
- [35] Wang K, Zhuang Y, Liu C, Li Y. Inhibition of c-Met activation sensitizes osteosarcoma cells to cisplatin via suppression of the PI3K-Akt signaling. *Arch Biochem Biophys*. 2012 Oct 1;526(1):38–43. doi: 10.1016/j.abb.2012.07.003.
- [36] Baldanzi G, Pietronave S, Locarno D, et al. Diacylglycerol kinases are essential for hepatocyte growth factor-dependent proliferation and motility of Kaposi's sarcoma cells. *Cancer Sci*. 2011 Jul;102(7):1329–1336. DOI: 10.1111/j.1349-7006.2011.01953.
- [37] Gibbs CP, Kukekov VG, Reith JD, et al. Stem-like cells in bone sarcomas: implications for tumorigenesis. *Neoplasia*. 2005;7:967–976.

- [38] Yan GN, Lv YF, Guo QN. Advances in osteosarcoma stem cell research and opportunities for novel therapeutic targets. *Cancer Lett.* 2016 Jan 28;370(2):268–274. DOI:10.1016/j.canlet.2015.11.003.
- [39] Adhikari AS, Agarwal N, Wood BM, et al. CD117 and Stro-1 identify osteosarcoma tumor-initiating cells associated with metastasis and drug resistance. *Cancer Res.* 2010 Jun 1;70(11):4602–4612. doi: 10.1158/0008-5472.CAN-09-3463].
- [40] Fujii H, Honoki K, Tsujiuchi T, et al. Sphere-forming stem-like cell populations with drug resistance in human sarcoma cell lines. *Int J Oncol.* 2009 May;34(5):1381–1386. DOI: 10.3892/ijoc_00000265.
- [41] Martins-Neves SR, Lopes ÁO, do Carmo A, et al. Therapeutic implications of an enriched cancer stem-like cell population in a human osteosarcoma cell line. *BMC Cancer.* 2012 Apr 4;12:139. DOI: 10.1186/1471-2407-12-139.
- [42] Gonçalves C, Martins-Neves SR, Paiva-Oliveira D, et al. Sensitizing osteosarcoma stem cells to doxorubicin-induced apoptosis through retention of doxorubicin and modulation of apoptotic-related proteins. *Life Sci.* 2015 Jun 1;130:47–56. DOI: 10.1016/j.lfs.2015.03.009.
- [43] Mongre RK, Sodhi SS, Ghosh M, et al. The novel inhibitor BRM270 downregulates tumorigenesis by suppression of NF- κ B signaling cascade in MDR-induced stem like cancer-initiating cells. *Int J Oncol.* 2015;46(6):2573–2585. DOI: 10.3892/ijoc.2015.2961.
- [44] Menendez JA, Alarcón T, Corominas-Faja B, et al. Reprogramming the epigenetic landscapes of patient-derived cancer genomes. *Cell Cycle.* 2014 Feb 1;13(3):358–370. DOI: 10.4161/cc.27770.
- [45] Yang RS, Wu CT, Lin KH, et al. Relation between histological intensity of transforming growth factor-beta isoforms in human osteosarcoma and the rate of lung metastasis. *Tohoku J Exp Med.* 1998;184(2):133–142.
- [46] Li F, Li S, Cheng T. TGF- β 1 promotes osteosarcoma cell migration and invasion through the miR-143-versican pathway. *Cell Physiol Biochem.* 2014;34(6):2169–2179. DOI: 10.1159/000369660.
- [47] Liu Y, Zheng QX, Du JY, et al. Effects of TGF beta1 autocrine blockage on osteosarcoma cells. *Chin Med Sci J.* 2004 Jun;19(2):155–156.
- [48] Silva G, Aboussekhra A. p16(INK4A) inhibits the pro-metastatic potentials of osteosarcoma cells through targeting the ERK pathway and TGF- β 1. *Mol Carcinog.* 2016 May;55(5):525–536. DOI: 10.1002/mc.22299.
- [49] Tu B, Peng ZX, Fan QM et al. Osteosarcoma cells promote the production of pro-tumor cytokines in mesenchymal stem cells by inhibiting their osteogenic differentiation through the TGF- β /Smad2/3 pathway. *Exp Cell Res.* 2014 Jan 1;320(1):164–173. DOI: 10.1016/j.yexcr.2013.10.013.

- [50] Hung SP, Yang MH, Tseng KF, Lee OK. Hypoxia-induced secretion of TGF- β 1 in mesenchymal stem cell promotes breast cancer cell progression. *Cell Transplant*. 2013;22(10):1869–1882. DOI: 10.3727/096368912X657954.
- [51] Massagué J. TGFbeta in cancer. *Cell*. 2008 Jul 25;134(2):215–230. DOI: 10.1016/j.cell.2008.07.001.
- [52] Copson ER, White HE, Blaydes JP, et al. Influence of the MDM2 single nucleotide polymorphism SNP309 on tumor development in BRCA1 mutation carriers. *BMC Cancer*. 2006;6(1):80–86. DOI: 10.1186/1471-2407-6-80.
- [53] Liu F, Kong X, Lv L, Gao J. TGF- β 1 acts through miR-155 to down-regulate TP53INP1 in promoting epithelial-mesenchymal transition and cancer stem cell phenotypes. *Cancer Lett*. 2015 Apr 10;359(2):288–298. DOI: 10.1016/j.canlet.2015.01.030.
- [54] Yue D, Zhang Z, Li J, et al. Transforming growth factor-beta1 promotes the migration and invasion of sphere-forming stem-like cell subpopulations in esophageal cancer. *Exp Cell Res*. 2015 Aug 1;336(1):141–149. DOI: 10.1016/j.yexcr.2015.06.007.
- [55] Lamora A, Talbot J, Bougras G, et al. Overexpression of smad7 blocks primary tumor growth and lung metastasis development in osteosarcoma. *Clin Cancer Res*. 2014 Oct 1;20(19):5097–5112. DOI: 10.1158/1078-0432.CCR-13-3191.
- [56] Lamora A, Mullard M, Amiaud J, et al. Anticancer activity of halofuginone in a preclinical model of osteosarcoma: inhibition of tumor growth and lung metastases. *Oncotarget*. 2015 Jun 10;6(16):14413–14427. DOI: 10.18632/oncotarget.3891.
- [57] Kumar V, Gabrilovich DI. Hypoxia-inducible factors in regulation of immune responses in tumor microenvironment. *Immunology*. 2014 Dec;143(4):512–519. DOI: 10.1111/imm.12380.
- [58] Feng H, Wang J, Chen W, et al. Hypoxia-induced autophagy as an additional mechanism in human osteosarcoma radioresistance. *J Bone Oncol*. 2016 Mar 9;5(2):67–73. DOI: 10.1016/j.jbo.2016.03.001.
- [59] Lv F, Du R, Shang W, et al. HIF-1 α silencing inhibits the growth of osteosarcoma cells by inducing apoptosis. *Ann Clin Lab Sci*. 2016 Mar;46(2):140–146.
- [60] Guan G, Zhang Y, Lu Y, et al. The HIF-1 α /CXCR4 pathway supports hypoxia-induced metastasis of human osteosarcoma cells. *Cancer Lett*. 2015 Feb 1;357(1):254–264. DOI: 10.1016/j.canlet.2014.11.034.
- [61] Ren HY, Zhang YH, Li HY, et al. Prognostic role of hypoxia-inducible factor-1 alpha expression in osteosarcoma: a meta-analysis. *Onco Targets Ther*. 2016 Mar 14;9:1477–1487. DOI: 10.2147/OTT.S95490.
- [62] Ouyang Y, Li H, Bu J, et al. Hypoxia-inducible factor-1 expression predicts osteosarcoma patients' survival: a meta-analysis. *Int J Biol Markers*. 2016 Jun 11. DOI: 10.5301/jbm.5000216.

- [63] Covello KL, Kehler J, Yu H, et al. HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes Dev.* 2006;20:557–570. DOI: 10.1101/gad.1399906.
- [64] Gordan JD, Bertout JA, Hu CJ, et al. HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. *Cancer Cell.* 2007;11:335–347. DOI: 10.1016/j.ccr.2007.02.006.
- [65] Wang Y, Yao J, Meng H, et al. A novel long non-coding RNA, hypoxia-inducible factor-2 α promoter upstream transcript, functions as an inhibitor of osteosarcoma stem cells *in vitro*. *Mol Med Rep.* 2015 Apr;11(4):2534–2540. DOI: 10.3892/mmr.2014.3024.
- [66] Zeng W, Wan R, Zheng Y, et al. Hypoxia, stem cells and bone tumor. *Cancer Lett.* 2011 Dec 27;313(2):129–136. DOI: 10.1016/j.canlet.2011.09.023.
- [67] Kawasoe Y, Yokouchi M, Ueno Y, et al. Hyperbaric oxygen as a chemotherapy adjuvant in the treatment of osteosarcoma. *Oncol Rep.* 2009 Nov;22(5):1045–1050. DOI: 10.3892/or_00000534.
- [68] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011 Mar 4;144(5):646–674. DOI: 10.1016/j.cell.2011.02.013.
- [69] Martinez-Outschoorn UE, Peiris-Pagés M, Pestell RG, et al. Cancer metabolism: a therapeutic perspective. *Nat Rev Clin Oncol.* 2016 May 4. DOI: 10.1038/nrclinonc.2016.60.
- [70] Mitsuishi Y, Taguchi K, Kawatani Y, et al. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell.* 2012 Jul 10;22(1):66–79. DOI: 10.1016/j.ccr.2012.05.016.
- [71] Bensaad K, Vousden KH. p53: new roles in metabolism. *Trends Cell Biol.* 2007 Jun;17(6):286–291. DOI: 10.1016/j.tcb.2007.04.004.
- [72] Giang AH, Raymond T, Brookes P, et al. Mitochondrial dysfunction and permeability transition in osteosarcoma cells showing the Warburg effect. *J Biol Chem.* 2013 Nov 15;288(46):33303–33311. DOI: 10.1074/jbc.M113.507129.
- [73] Wang DW, Yu SY, Cao Y, et al. A novel mechanism of mTORC1-mediated serine/glycine metabolism in osteosarcoma development. *Cell Signal.* 2016 Jun 10. DOI: 10.1016/j.cellsig.2016.06.008.
- [74] Gupte A, Baker EK, Wan SS, et al. Systematic screening identifies dual PI3K and mTOR inhibition as a conserved therapeutic vulnerability in osteosarcoma. *Clin Cancer Res.* 2015 Jul 15;21(14):3216–3229. DOI: 10.1158/1078-0432.CCR-14-3026.
- [75] Im HJ, Kim TS, Park SY, et al. Prediction of tumor necrosis fractions using metabolic and volumetric 18F-FDG PET/CT indices, after one course and at the completion of neoadjuvant chemotherapy, in children and young adults with osteosarcoma. *Eur J Nucl Med Mol Imaging.* 2012 Jan;39(1):39–49. DOI: 10.1007/s00259-011-1936-4.

- [76] Byun BH, Kong CB, Park J, et al. Initial metabolic tumor volume measured by 18F-FDG PET/CT can predict the outcome of osteosarcoma of the extremities. *J Nucl Med*. 2013 Oct;54(10):1725–1732. DOI: 10.2967/jnumed.112.117697.
- [77] Gao S, Tu DN, Li H, et al. Pharmacological or genetic inhibition of LDHA reverses tumor progression of pediatric osteosarcoma. *Biomed Pharmacother*. 2016 Jul;81:388–393. DOI: 10.1016/j.biopha.2016.04.029.
- [78] Sottnik JL, Lori JC, Rose BJ, Thamm DH. Glycolysis inhibition by 2-deoxy-d-glucose reverts the metastatic phenotype *in vitro* and *in vivo*. *Clin Exp Metastasis*. 2011 Dec;28(8):865–875. DOI: 10.1007/s10585-011-9417-5.
- [79] Maschek G, Savaraj N, Priebe W, et al. 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers *in vivo*. *Cancer Res*. 2004 Jan 1;64(1):31–34. DOI: 10.1158/0008-5472.CAN-03-3294.
- [80] Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*. 2012 Mar 20;21(3):309–322. DOI: 10.1016/j.ccr.2012.02.022.
- [81] Mueller MM, Fusenig NE. Friends or foes—bipolar effects of the tumor stroma in cancer. *Nature Nat Rev Cancer*. 2004 Nov;4(11):839–849. DOI: 10.1038/nrc1477.
- [82] Martinez-Outschoorn UE, Lisanti MP, Sotgia F. Catabolic cancer-associated fibroblasts transfer energy and biomass to anabolic cancer cells, fueling tumor growth. *Semin Cancer Biol*. 2014 Apr;25:47–60. DOI: 10.1016/j.semcancer.2014.01.005.
- [83] Chiavarina B, Whitaker-Menezes D, Migneco G, et al. HIF1-alpha functions as a tumor promoter in cancer-associated fibroblasts, and as a tumor suppressor in breast cancer cells. *Cell Cycle*. 2010 Sep 1;9(17):3534–3551. DOI: 10.4161/cc.9.17.12908.
- [84] Rattigan YI, Patel BB, Ackerstaff E, et al. Lactate is a mediator of metabolic cooperation between stromal carcinoma associated fibroblasts and glycolytic tumor cells in the tumor microenvironment. *Exp Cell Res*. 2012 Feb 15;318(4):326–335. DOI: 10.1016/j.yexcr.2011.11.014.
- [85] Nieman KM, Kenny HA, Penicka CV, et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat Med*. 2011 Oct 30;17(11):1498–1503. DOI: 10.1038/nm.2492.
- [86] Garris CS, Pittet MJ. ER stress in dendritic cells promotes cancer. *Cell*. 2015 Jun 18;161(7):1492–1493. DOI: 10.1016/j.cell.2015.06.006.
- [87] Cubillos-Ruiz JR, Silberman PC, Rutkowski MR, et al. ER stress sensor XBP1 controls anti-tumor immunity by disrupting dendritic cell homeostasis. *Cell*. 2015 Jun 18;161(7):1527–1538. DOI: 10.1016/j.cell.2015.05.025.
- [88] Chang CH, Qiu J, O'Sullivan D, et al. Metabolic competition in the tumor microenvironment is a driver of cancer progression. *Cell*. 2015 Sep 10;162(6):1229–1241. DOI: 10.1016/j.cell.2015.08.016.

- [89] Ho PC, Bihuniak JD, Macintyre AN, et al. Phosphoenolpyruvate is a metabolic checkpoint of anti-tumor T cell responses. *Cell*. 2015 Sep 10;162(6):1217–1228. DOI: 10.1016/j.cell.2015.08.012.
- [90] Chang CH, Curtis JD, Maggi LB Jr, et al. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell*. 2013 Jun 6;153(6):1239–1251. DOI: 10.1016/j.cell.2013.05.016.
- [91] Bonuccelli G, Avnet S, Grisendi G. Role of mesenchymal stem cells in osteosarcoma and metabolic reprogramming of tumor cells. *Oncotarget*. 2014 Sep 15;5(17):7575–7588. DOI: 10.18632/oncotarget.2243.
- [92] Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971 Nov 18;285(21):1182–1186. DOI: 10.1056/NEJM197111182852108.
- [93] Handa A, Tokunaga T, Tsuchida T, et al. Neuropilin-2 expression affects the increased vascularization and is a prognostic factor in osteosarcoma. *Int J Oncol*. 2000 Aug;17(2):291–295. DOI: 10.3892/ijo.17.2.291.
- [94] Habel N, Vilalta M, Bawa O, et al. Cyr61 silencing reduces vascularization and dissemination of osteosarcoma tumors. *Oncogene*. 2015 Jun 11;34(24):3207–3213. DOI: 10.1038/onc.2014.232.
- [95] Clark ER, Clark EL. Observations on living preformed blood vessels as seen in a transparent chamber inserted into the rabbit's ear. *Am J Anat*. 1932;49(3):441–477. DOI: 10.1002/aja.1000490306.
- [96] Gerhardt H, Golding M, Fruttiger M, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol*. 2003 Jun 23;161(6):1163–1177. DOI: 10.1083/jcb.200302047.
- [97] Algire GH, Chalkley HW, Legallais FY, Park HD. Vasculae reactions of normal and malignant tissues *in vivo*. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. *J Natl Cancer Inst*. 1945;6(1):73–85. DOI: 10.1093/jnci/6.1.73.
- [98] Mantadakis E, Kim G, Reisch J, et al. Lack of prognostic significance of intratumoral angiogenesis in nonmetastatic osteosarcoma. *J Pediatr Hematol Oncol*. 2001 Jun-Jul;23(5):286–289. DOI: 10.1097/00043426-200106000-00010.
- [99] Mikulić D, Ilić I, Cepulić M, et al. Tumor angiogenesis and outcome in osteosarcoma. *Pediatr Hematol Oncol*. 2004 Oct-Nov;21(7):611–619. DOI: 10.1080/08880010490501015.
- [100] Kunz P, Fellenberg J, Moskovszky L, et al. Improved survival in osteosarcoma patients with atypical low vascularization. *Ann Surg Oncol*. 2015 Feb;22(2):489–496. doi: 10.1245/s10434-014-4001-2.
- [101] Mori S, Ueda T, Kuratsu S, et al. Suppression of pulmonary metastasis by angiogenesis inhibitor TNP-470 in murine osteosarcoma. *Int J Cancer*. 1995 Mar 29;61(1):148–152. DOI: 10.1002/ijc.2910610125.

- [102] Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med.* 2003 Jun;9(6):669–676. DOI: 10.1038/nm0603-669.
- [103] Kaya M, Wada T, Nagoya S, et al. The level of vascular endothelial growth factor as a predictor of a poor prognosis in osteosarcoma. *J Bone Joint Surg Br.* 2009 Jun;91(6):784–788. DOI:10.1302/0301-620X.91B6.
- [104] Kaya M, Wada T, Kawaguchi S, et al. Vascular endothelial growth factor expression in untreated osteosarcoma is predictive of pulmonary metastasis and poor prognosis. *Clin Cancer Res.* 2000 Feb;6(2):572–577.
- [105] Kaya M, Wada T, Kawaguchi S, et al. Increased pre-therapeutic serum vascular endothelial growth factor in patients with early clinical relapse of osteosarcoma. *Br J Cancer.* 2002 Mar 18;86(6):864–869. DOI: 10.1038/sj/bjc/6600201.
- [106] Lee YH, Tokunaga T, Oshika Y, et al. Cell-retained isoforms of vascular endothelial growth factor (VEGF) are correlated with poor prognosis in osteosarcoma. *Eur J Cancer.* 1999 Jul;35(7):1089–1093. DOI: 10.1016/S0959-8049(99)00073-8.
- [107] Yang SY, Yu H, Krygier JE, et al. High VEGF with rapid growth and early metastasis in a mouse osteosarcoma model. *Sarcoma.* 2007;2007:95628. DOI:10.1155/2007/95628.
- [108] Yin D, Jia T, Gong W, et al. VEGF blockade decelerates the growth of a murine experimental osteosarcoma. *Int J Oncol.* 2008 Aug;33(2):253–259. DOI: 10.3892/ijo_00000004.
- [109] Wu X, Chen Z, Zeng W, et al. Silencing of *eag1* gene inhibits osteosarcoma proliferation and migration by targeting STAT3-VEGF pathway. *Biomed Res Int.* 2015;2015:617316. DOI: 10.1155/2015/617316.
- [110] Zhao J, Zhang ZR, Zhao N, et al. VEGF silencing inhibits human osteosarcoma angiogenesis and promotes cell apoptosis via PI3K/AKT signaling pathway. *Cell Biochem Biophys.* 2015 Nov;73(2):519–525. DOI: 10.1007/s12013-015-0692-7.
- [111] Tzeng HE, Tsai CH, Chang ZL, et al. Interleukin-6 induces vascular endothelial growth factor expression and promotes angiogenesis through apoptosis signal-regulating kinase 1 in human osteosarcoma. *Biochem Pharmacol.* 2013 Feb 15;85(4):531–540. DOI: 10.1016/j.bcp.2012.11.021.
- [112] Wang M, Wang L, Ren T, et al. IL-17A/IL-17RA interaction promoted metastasis of osteosarcoma cells. *Cancer Biol Ther.* 2013 Feb;14(2):155–163. DOI: 10.4161/cbt.22955.
- [113] de Nigris F, Schiano C, Infante T, Napoli C. CXCR4 inhibitors: tumor vasculature and therapeutic challenges. *Recent Pat Anticancer Drug Discov.* 2012 Sep;7(3):251–264. DOI: 10.2174/157489212801820039.
- [114] Fan L, Wu Q, Xing X, et al. MicroRNA-145 targets vascular endothelial growth factor and inhibits invasion and metastasis of osteosarcoma cells. *Acta Biochim Biophys Sin (Shanghai).* 2012 May;44(5):407–414. DOI: 10.1093/abbs/gms019.

- [115] Abdollahi A, Hahnfeldt P, Maercker C, et al. Endostatin's antiangiogenic signaling network. *Mol Cell*. 2004 Mar 12;13(5):649–663.
- [116] Folkman J. Antiangiogenesis in cancer therapy—endostatin and its mechanisms of action. *Exp Cell Res*. 2006 Mar 10;312(5):594–607. DOI: 10.1016/j.yexcr.2005.11.015.
- [117] Kaya M, Wada T, Nagoya S, Yamashita T. Prevention of postoperative progression of pulmonary metastases in osteosarcoma by antiangiogenic therapy using endostatin. *J Orthop Sci*. 2007 Nov;12(6):562–567. DOI 10.1007/s00776-007-1179-1.
- [118] Maniotis AJ, Folberg R, Hess A, et al. Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry. *Am J Pathol*. 1999 Sep;155(3):739–752. DOI: 10.1016/S0002-9440(10)65173-5.
- [119] Ren K, Yao N, Wang G, et al. Vasculogenic mimicry: a new prognostic sign of human osteosarcoma. *Hum Pathol*. 2014 Oct;45(10):2120–2129. DOI: 10.1016/j.humpath.2014.06.013.
- [120] Zhang LZ, Mei J, Qian ZK, et al. The role of VE-cadherin in osteosarcoma cells. *Pathol Oncol Res*. 2010 Mar;16(1):111–117. DOI: 10.1007/s12253-009-9198-1.
- [121] Alfranca A, Martinez-Cruzado L, Tornin J, et al. Bone microenvironment signals in osteosarcoma development. *Cell Mol Life Sci*. 2015 Aug;72(16):3097–3113. DOI: 10.1007/s00018-015-1918-y.
- [122] Xu WT, Bian ZY, Fan QM, et al. Human mesenchymal stem cells (hMSCs) target osteosarcoma and promote its growth and pulmonary metastasis. *Cancer Lett*. 2009 Aug 18;281(1):32–41. DOI:10.1016/j.canlet.2009.02.022.
- [123] Tsukamoto S, Honoki K, Fujii H, et al. Mesenchymal stem cells promote tumor engraftment and metastatic colonization in rat osteosarcoma model. *Int J Oncol*. 2012;40(1):163–169. DOI: 10.3892/ijo.2011.1220.
- [124] Tu B, Du L, Fan QM, et al. STAT3 activation by IL-6 from mesenchymal stem cells promotes the proliferation and metastasis of osteosarcoma. *Cancer Lett*. 2012 Dec 1;325(1):80–88. DOI: 10.1016/j.canlet.2012.06.006.
- [125] Williams SA, Maecker HL, French DM, et al. USP1 deubiquitinates ID proteins to preserve a mesenchymal stem cell program in osteosarcoma. *Cell*. 2011 Sep 16;146(6):918–930. DOI: 10.1016/j.cell.2011.07.040.
- [126] Moschoi R, Imbert V, Nebout M, et al. Protective mitochondrial transfer from bone marrow stromal cells to acute myeloid leukemic cells during chemotherapy. *Blood*. 2016 Jul 14;128(2):253–264. DOI: 10.1182/blood-2015-07-655860.
- [127] Denbo JW, Zhu L, Srivastava D, et al. Long-term pulmonary function after metastasectomy for childhood osteosarcoma: a report from the St Jude lifetime cohort study. *J Am Coll Surg*. 2014 Aug;219(2):265–271. DOI: 10.1016/j.jamcollsurg.2013.12.064.
- [128] Mirabello L, Troisi RJ, Savage S. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the surveillance, epidemiology, and end results program. *Cancer*. 2009 Apr 1;115(7):1531–1543. DOI: 10.1002/cncr.24121.

- [129] Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol*. 2014;14(3):195–208. DOI: 10.1038/nri3622.
- [130] van der Pol E, Böing AN, Harrison P, et al. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev*. 2012;64(3):676–705. DOI: 10.1124/pr.112.005983.
- [131] Lai FW, Lichty BD, Bowdish DM. Microvesicles: ubiquitous contributors to infection and immunity. *J Leukoc Biol*. 2015;97(2):237–245. DOI: 10.1189/jlb.3RU0513-292RR.
- [132] Li XB, Zhang ZR, Schluesener HJ, Xu SQ. Role of exosomes in immune regulation. *J Cell Mol Med*. 2006;10(2):364–375. DOI: 10.1038/nri3622.
- [133] Anderson MR, Kashanchi F, Jacobson S. Exosomes in viral disease. *Neurotherapeutics*. 2016 Jun 20. DOI: 10.1007/s13311-016-0450-6.
- [134] Chen WX, Cai YQ, Lv MM, et al. Exosomes from docetaxel-resistant breast cancer cells alter chemosensitivity by delivering microRNAs. *Tumor Biol*. 2014;35(10):9649–9659. DOI: 10.1007/s13277-014-2242-0.
- [135] Torreggiani E, Roncuzzi L, Perut F, et al. Multimodal transfer of MDR by exosomes in human osteosarcoma. *Int J Oncol*. 2016 Jul;49(1):189–196. DOI: 10.3892/ijo.2016.3509.
- [136] Jerez S, Araya H, Thaler R, et al. Proteomic analysis of exosomes and exosome-free conditioned media derived from human osteosarcoma cell lines reveal differential secretion of proteins related to biological functions and tumor progression. *J Cell Biochem*. 2016 Jun 30. DOI: 10.1002/jcb.25642
- [137] Mu X, Agarwal R, March D, et al. Notch signaling mediates skeletal muscle atrophy in cancer cachexia caused by osteosarcoma. *Sarcoma*. 2016;2016:3758162. DOI:10.1155/2016/3758162.
- [138] Hildenbrand R, Allgayer H, Marx A, Stroebel P. Modulators of the urokinase-type plasminogen activation system for cancer. *Expert Opin Investig Drugs*. 2010;19(5):641–652. DOI: 10.1517/13543781003767400.
- [139] Endo-Munoz L, Cai N, Cumming A, et al. Progression of osteosarcoma from a non-metastatic to a metastatic phenotype is causally associated with activation of an autocrine and paracrine uPA Axis. *PLoS One*. 2015 Aug 28;10(8):e0133592. DOI: 10.1371/journal.pone.0133592.
- [140] Vallabhaneni KC, Penfornis P, Dhule S, et al. Extracellular vesicles from bone marrow mesenchymal stem/stromal cells transport tumor regulatory microRNA, proteins, and metabolites. *Oncotarget*. 2015;6(7):4953–4967. DOI: 10.18632/oncotarget.3211.
- [141] Shimbo K, Miyaki S, Ishitobi H, et al. Exosome-formed synthetic microRNA-143 is transferred to osteosarcoma cells and inhibits their migration. *Biochem Biophys Res Commun*. 2014 Mar 7;445(2):381–387. DOI: 10.1016/j.bbrc.2014.02.007.
- [142] Lässer C. Identification and analysis of circulating exosomal microRNA in human body fluids. *Methods Mol Biol*. 2013;1024:109–128. DOI: 10.1007/978-1-62703-453-1_9.

- [143] Lässer C, Seyed Alikhani V, Ekström K, et al. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. *J Transl Med.* 2011 Jan 14;9:9. DOI: 10.1186/1479-5876-9-9.
- [144] Miranda KC, Bond DT, McKee M, et al. Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease. *Kidney Int.* 2010 Jul;78(2):191–199. DOI:10.1038/ki.2010.106.
- [145] Wang ZQ, Liang J, Schellander K, et al. c-fos-induced osteosarcoma formation in transgenic mice: cooperativity with c-jun and the role of endogenous c-fos. *Cancer Res.* 1995 Dec 15;55(24):6244–6251.
- [146] Entz-Werlé N, Choquet P, Neuville A, et al. Targeted *apc*/*twist* double-mutant mice: a new model of spontaneous osteosarcoma that mimics the human disease. *Transl Oncol.* 2010 Dec 1;3(6):344–353.
- [147] Elliott NT, Yuan F. A review of three-dimensional *in vitro* tissue models for drug discovery and transport studies. *J Pharm Sci.* 2011 Jan;100(1):59–74. DOI: 10.1002/jps.22257.
- [148] Merlino G, Khanna C. Fishing for the origins of cancer. *Genes Dev.* 21(11):1275–1279. DOI: 10.1101/gad.1563707.
- [149] Langenau DM, Keefe MD, Storer NY, et al. Effects of RAS on the genesis of embryonal rhabdomyosarcoma. *Genes Dev.* 21(11):1382–1395. DOI: 10.1101/gad.1545007.
- [150] Feitsma H, Kuiper RV, Korving J, et al. Zebrafish with mutations in mismatch repair genes develop neurofibromas and other tumors. *Cancer Res.* 68(13):5059–5066. DOI: 10.1158/0008-5472.CAN-08-0019.
- [151] Etchin J, Kanki JP, Look AT. Zebrafish as a model for the study of human cancer. *Methods Cell Biol.* 105:309–337. DOI: 10.1016/B978-0-12-381320-6.00013-8.
- [152] He S, Krens SG, Zhan H, et al. A DeltaRaf1-ER-inducible oncogenic zebrafish liver cell model identifies hepatocellular carcinoma signatures. *J Pathol.* 225(1):19–28. DOI: 10.1002/path.2936.
- [153] Alford AI, Kozloff KM, Hankenson KD. Extracellular matrix networks in bone remodeling. *Int J Biochem Cell Biol.* 2015 Aug;65:20–31. DOI: 10.1016/j.biocel.2015.05.008.
- [154] Alemany-Ribes M, Semino CE. Bioengineering 3D environments for cancer models. *Adv Drug Deliv Rev.* 2014 Dec 15;79–80:40–49. DOI: 10.1016/j.addr.2014.06.004.
- [155] Zhang W, Zhu Y, Li J, et al. Cell-derived extracellular matrix: basic characteristics and current applications in orthopedic tissue engineering. *Tissue Eng Part B Rev.* 2016 Jun;22(3):193–207. DOI: 10.1089/ten.TEB.2015.0290.
- [156] Beck EC, Barragan M, Tadros MH, et al. Approaching the compressive modulus of articular cartilage with a decellularized cartilage-based hydrogel. *Acta Biomater.* 2016 Apr;38:pp. 94–105. DOI: 10.1016/j.actbio.2016.04.019.

- [157] Datta N, Holtorf HL, Sikavitsas VI, et al. Effect of bone extracellular matrix synthesized *in vitro* on the osteoblastic differentiation of marrow stromal cells. *Biomaterials*. 2005 Mar;26(9):971–977. DOI: 10.1016/j.biomaterials.2004.04.001.
- [158] Benders KE, van Weeren PR, Badylak SF, et al. Extracellular matrix scaffolds for cartilage and bone regeneration. *Trends Biotechnol*. 2013 Mar;31(3):169–176. DOI: 10.1016/j.tibtech.2012.12.004.
- [159] Mohseny AB, Xiao W, Carvalho R, et al. An osteosarcoma zebrafish model implicates Mmp-19 and Ets-1 as well as reduced host immune response in angiogenesis and migration. *J Pathol*. 2012 Jun;227(2):245–253. DOI: 10.1002/path.3998.
- [160] Bulut G, Hong SH, Chen K, et al. Small molecule inhibitors of ezrin inhibit the invasive phenotype of osteosarcoma cells. *Oncogene*. 2012 Jan 19;31(3):269–281. DOI: 10.1038/onc.2011.245.
- [161] Rodriguez CO Jr. Using canine osteosarcoma as a model to assess efficacy of novel therapies: can old dogs teach us new tricks?. *Adv Exp Med Biol*. 2014;804:237–256. DOI: 10.1007/978-3-319-04843-7_13.
- [162] Gordon N, Koshkina NV, Jia SF, et al. Corruption of the Fas pathway delays the pulmonary clearance of murine osteosarcoma cells, enhances their metastatic potential, and reduces the effect of aerosol gemcitabine. *Clin Cancer Res*. 2007 Aug 1;13(15 Pt 1):4503–4510. DOI: 10.1158/1078-0432.CCR-07-0313.
- [163] Koshkina NV, Kleinerman ES. Aerosol gemcitabine inhibits the growth of primary osteosarcoma and osteosarcoma lung metastases. *Int J Cancer*. 2005 Sep 1;116(3):458–463. DOI: 10.1002/ijc.21011.
- [164] Rodriguez CO Jr, Crabbs TA, Wilson DW, et al. Aerosol gemcitabine: preclinical safety and *in vivo* antitumor activity in osteosarcoma-bearing dogs. *J Aerosol Med Pulm Drug Deliv*. 2010 Aug;23(4):197–206. DOI: 10.1089/jamp.2009.0773.

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