SYMPOSIUM: MOLECULAR GENETICS IN SARCOMA

Osteosarcoma Development and Stem Cell Differentiation

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Abstract Osteosarcoma is the most common nonhematologic malignancy of bone in children and adults. The peak incidence occurs in the second decade of life, with a smaller peak after age 50. Osteosarcoma typically arises around the growth plate of long bones. Most osteosarcoma tumors are of high grade and tend to develop pulmonary metastases. Despite clinical improvements, patients with metastatic or recurrent diseases have a poor prognosis. Here, we reviewed the current understanding of human osteosarcoma, with an emphasis on potential links between defective osteogenic differentiation and bone tumorigenesis. Existing data indicate osteosarcoma tumors display a broad range of genetic and molecular alterations, including the gains, losses, or arrangements of chromosomal regions, inactivation of tumor suppressor genes, and the deregulation of major signaling pathways. However, except for p53 and/or RB mutations, most alterations are not constantly detected in the majority of osteosarcoma tumors. With a rapid expansion of our knowledge about stem cell biology, emerging evidence suggests osteosarcoma should be regarded as a differentiation disease caused by genetic and epigenetic changes that interrupt osteoblast differentiation from mesenchymal stem cells. Understanding the molecular pathogenesis of human osteosarcoma could ultimately lead to the development of diagnostic and prognostic markers, as well as targeted therapeutics for osteosarcoma patients.

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Introduction

Osteosarcoma (OS) is the most frequent primary bone sarcoma, comprising approximately 20% of all bone tumors and about 5% of pediatric tumors overall [34, 67, 73, 75, 77, 94, 127, 136, 146, 210, 252, 259]. In fact, OS is the fifth most common malignancy among individuals aged 15 to 19 years, and the second most common in adolescence after lymphoma. OS has a bimodal age distribution, with the first peak in the second decade of life and a second peak in elderly adults [146, 210]. Higher incidences in boys and in African-American children have been reported [146, 210]. The most common locations in young adults are areas with rapid bone growth, including distal femur, proximal tibia, and proximal humerus. Nevertheless, OS is relatively rare, and less than 1000 new cases are diagnosed each year in the United States, accounting for less than 2% of all new cancer cases in the U.S. [146, 210].

Although OS development is associated with several genetic predisposition conditions, most OS tumors are sporadic without familial patterns [67, 73, 77, 94, 146, 210, 252, 259]. Our current understanding of OS etiology is rather limited. Exposure to chemical beryllium oxide [51], orthopaedic prostheses [101], and the FBJ virus [51] causes OS in animal models, but their role in human OS is unknown. SV40 viral DNA has been detected in up to 50% of OS tumors [24, 125, 161], while it is unclear whether

SV40 plays any role in OS tumorigenesis [51, 62]. Radiation exposure is a well-documented risk factor for OS [51, 88, 147, 239, 256], but the interval between radiation exposure and tumor appearance is long, and hence it is likely irrelevant to the development of most conventional OS tumors. Nevertheless, radiation could be responsible for the development of secondary OS postradiation therapy of certain primary tumors.

Despite the relative low incidence of OS, more than 20,000 articles describing the pathogenic and clinical aspects of OS have been published thus far. As summarized in this survey, OS displays a broad range of genetic and epigenetic alterations, and yet no consensus changes have been identified in all OS tumors [34, 67, 73, 75, 77, 94, 127, 136, 146, 210, 252, 259]. With the rapid expansion of our knowledge about stem cell biology and cancer stem cells [204, 217, 260, 278], increasing evidence suggests OS may be considered a differentiation disease [75, 231]. Terminal differentiation of osteoblasts, which are derived from multipotent mesenchymal stem cells, is a well-orchestrated process and controlled by a cascade of regulatory signaling [36, 38, 39, 75, 82, 96, 114, 129, 136, 171, 173, 187, 270]. Pathologic and molecular features of most if not all OS tumors strongly suggest OS may be caused by genetic and epigenetic disruptions of osteoblast differentiation pathway [75, 231]. Promoting differentiation and/or circumventing differentiation defects may be exploited as an efficacious adjuvant therapy for OS since current chemotherapies mostly target the proliferative aspects of OS tumors [75, 76, 188].

In this survey, we briefly review currently identified genetic alterations that may be associated with osteosarcoma pathogenesis, and then focus on recent findings that suggest potential links between defective osteogenic differentiation of mesenchymal stem cells and osteosarcoma development. We believe this line of investigation will provide insight into the pathogenesis of osteosarcoma.

Search Strategies and Criteria

We performed PubMed searches of the literature relevant to the subject with the following keywords utilized individually or in combination: osteosarcoma (20,168 references), osteosarcoma pathogenesis (8232 references), osteosarcoma genetics (3587 references), osteosarcoma mutation (1232 references), osteosarcoma biology (238 references), osteosarcoma stem cell (669 references), osteosarcoma stem cell differentiation (99 references), and osteosarcoma lung metastasis (894 references), as of March 2008. Publications in languages other than English, pertinent review articles, and book chapters were not excluded in our searches. However, we mostly reviewed the English abstracts of the articles published in other languages and only utilized the articles that added any information to this review. Whenever information overlapped, we referenced the most recent articles built on conclusions or reports of previous articles. Our searches of articles published in the English language revealed considerable overlap in articles identified under the different search terms, and we carefully reviewed the articles for pertinence to our review article. In this review, we primarily focused on relevant publications within the past 10 years, while not excluding older but commonly referenced and highly regarded prior publications. We suspect many of the osteosarcoma cases described in the literature represent high-grade osteosarcomas.

Clinical Aspects of Human OS

Most OS patients present with pain and swelling in the affected regions after trauma or vigorous physical activities [146]. The diagnosis of OS is usually made by radiographic appearance and location of tumor lesions and a biopsy for pathologic confirmation [146]. OS can present radiographically as a lytic, sclerotic, or mixed lytic-sclerotic lesion [146]. Up to 20% of OS patients present with radiographically detectable lung metastases, whereas 80% of patients with localized OS develop metastases after surgical resection alone [146]. Death from OS is usually the result of progressive pulmonary metastasis with respiratory failure [146].

OS has a broad spectrum of histologic appearances with common characteristics containing highly proliferative malignant mesenchymal stem cells and the production of osteoid and/or bone by tumor cells [62, 146]. Histologically, OS can be divided into several subtypes. Conventional osteoblastic OS makes up about 70%, whereas chondroblastic and fibroblastic OS tumors are the next most common at 10% each [62, 73, 102, 231]. Other OS types include anaplastic, telangiectatic, giant cell-rich, and small cell OS [102]. Conventional OS is a primary intramedullary high-grade sarcoma. Current clinical management of OS includes pre- and postoperative chemotherapy and surgical resection [62]. Only about 20% of OS patients can be cured without chemotherapy [62, 102, 146]. Chemotherapy agents include doxorubicin, cisplatin, ifosfamide, and methotrexate [62, 102, 127, 146]. Surgical removal of the primary tumor requires a wide-margin resection, followed by limb salvage reconstruction [62, 102, 127, 146].

OS prognostic indicators include extent of disease at diagnosis, size and location of the tumor, response to chemotherapy, and surgical remission [62, 102, 127, 146]. For those OS patients who present without detectable metastases, approximately 70% of them can achieve long-term survival [62, 102, 127, 146]. The remaining 30% will

relapse, mostly within 5 years [62, 102, 127, 146]. Pulmonary metastasis is the most common form of distant spread. The average survival after a recurrence is less than 1 year [62, 102, 127, 146]. Removal of a surgically resectable recurrence or pulmonary metastasis improves survival [62, 102, 127, 146]. Thus, a major challenge in clinical management of OS is to identify poor responders to chemotherapy and/or to detect early metastatic lesions.

Chromosomal Abnormalities in Human OS

Unlike other sarcomas, such as synovial sarcoma, alveolar rhabdomyosarcoma, and Ewing's sarcoma, no specific translocations or genetic abnormalities have been identified in OS [34, 67, 73, 75, 77, 94, 127, 136, 146, 210, 252, 259]. Nevertheless, nearly 70% of OS tumors display a multitude of cytogenetic abnormalities [146, 210]. The ploidy number in OS has ranged from haploidy to near-hexaploidy. Chromosomal regions of 1p11–p13, 1q11–q12, 1q21–q22, 11p14–p15, 14p11–p13, 15p11–p13, 17p, and 19q13 are most commonly involved in structural abnormalities [146, 210]. Gain of chromosome 1 and loss of chromosomes 9, 10, 13, and 17 are most common overall. Less frequently involved chromosomal regions were 13q14 (locus of RB1), 12p12–pter (locus of KRAS), 6q11–q4, and 8p23 [146, 210].

A combination of several detection modalities has provided a more accurate assessment of the complex cytogenetic aberrations in OS [146, 210]. The most frequently detected amplifications include chromosomal regions 6p12–p21 (28%), 17p11.2 (32%), and 12q13–q14 (8%) [210]. Several other recurrent chromosomal losses (2q, 3p, 9, 10p, 12q, 13q, 14q, 15q, 16, 17p, and 18q) and chromosomal gains (Xp, Xq, 5q, 6p, 8q, 17p, and 20q) were also identified, as well as several recurrent breakpoint clusters and nonrecurrent reciprocal translocations [210]. These findings further highlight the complexity and the instability of the genetic makeup of OS tumors.

Genetic Alterations of Tumor Suppression Genes in Human OS

Retinoblastoma Tumor Suppressor

Individuals affected by hereditary retinoblastoma (RB) heterozygous for a germline inactivation of RB1 have an approximately 1000 times higher incidence of OS. RB1 maps to 13q14 [1, 111]. Genetic alterations of RB1 have been found in up to 70% of sporadic OS cases [2, 4, 12, 13, 164, 215, 235, 248, 267]. Loss of heterozygosity (LOH) of RB1 locus is present in 60% to 70% of OS tumors [12, 48,

271], whereas structural rearrangements and point mutations occur less commonly (30% and 10%, respectively) [2, 4, 12, 13, 164, 215, 235, 248, 267]. Furthermore, LOH at the RB1 locus has been proposed as a poor prognostic factor in OS [146, 210].

RB is an important regulator of G1/S cell cycle progression [178]. During G1/S transition, RB becomes phosphorylated, resulting in the activation of E2F factors that bind to the dephosphorylated RB protein and promote DNA synthesis and G1 to S transition [178]. CDK4 in complex with cyclin D1 phosphorylates RB. Thus, amplification or overexpression of these genes results in functional inactivation of the RB pathway. The CDKs are regulated by a series of inhibitory proteins, including p16^{INK4a}, as a negative regulator of cell cycle progression (see below). Loss of p16^{INK4a} expression occurs in osteogenic sarcomas lacking RB1 alterations [179].

p53 Tumor Suppressor

The tumor suppressor gene TP53 is located at 17p13, a region frequently identified as abnormal in OS [29, 210]. TP53 encodes a transcription factor and regulates genes involved in cell cycle, DNA damage response, and apoptosis [40, 69, 87, 126]. Alterations in TP53 observed in OS tumors consisted of point mutations (20%–30%, mostly missense mutations), gross gene rearrangements (10%–20%), and allelic loss (75%–80%) [3, 20, 28, 119, 152, 162, 163, 169, 170, 184, 186, 199, 206, 214, 221, 227, 236, 237, 241, 249, 271]. The association of TP53 with OS is further supported by the high risk of OS in patients with the Li-Fraumeni syndrome, an autosomal dominant disorder characterized by a germline mutation of TP53 [128, 144, 145, 194, 223]. Germline mutations of TP53 have been identified in a small percentage (3%) of sporadic OS cases [112, 156, 236, 237]. However, TP53 mutation status is seemingly not associated with the stages of OS tumor and/or metastasis [60]. Nevertheless, the mutation status of TP53, and to a lesser extent of RB1, could serve as a valuable indicator for predicting chemoresistance of OS [64].

p16^{INK4a} and p14^{ARF} CDK Inhibitors

INK4A (also known as CDKN2A), localized to 9p21, encodes p16^{INK4a}, a tumor suppressor that functions in part through the inhibition of CDK4 (see below) [198]. The p16^{INK4a} protein can impose a sustained G1 arrest [123, 198]. In 87 OS specimens from 79 patients, INK4A changes were observed in five of 55 cases examined (four deletions and one rearrangement), whereas no INK4A exon 2-point mutations or methylation were detected [94, 140,

162, 210, 257]. CDK4 gene amplification occurred in six of 67 tumors, but none of those with INK4A alterations [94, 210]. The absence of expression of p16[INK4a] correlated with decreased survival in pediatric OS patients [94, 210].

INK4A also encodes p14[ARF] through bicistronic transcription involving the use of an alternative reading frame [198]. The p14 protein is structurally and functionally unrelated to p16[INK4a] [66, 123]. Whereas p16[INK4a] indirectly regulates RB1 function, p14 regulates TP53 function by binding MDM2 (see below) and sequestering it in the nucleolus, thereby preventing it from shuttling p53 to the cytoplasm for degradation [192, 279]. INK4A is deleted in approximately 10% of OS, and almost all deletions would be expected to knock out expression of p14[ARF] as well [133]. Because loss of p14[ARF] should release MDM2 from this negative regulatory mechanism, deletion of INK4A represents another mechanism of functional TP53 inactivation. As the alternative products of INK4A, p14[ARF] and p16[INK4a], interact negatively with MDM2 and CDK4, deletions of the INK4A gene would be functionally equivalent to 12q13 amplification of both MDM2 and CDK4 [94, 146, 210, 252]. As a result, either of two singular genetic events (ie, INK4A deletion or 12q13 amplification) can inactivate two separate critical pathways of cell cycle control [94, 146, 210, 252].

Activation of Oncogenes in Human OS

The c-MYC product is involved in regulating cell growth and DNA replication [32, 177]. Seven to 12 percent of OS tumors have MYC amplification [9, 10, 120, 193]. This genetic alteration may be more common in Pagetic OS (see below) [210]. At the expression level, MYC expression in OS was elevated in nine of 21 (42%) patients who relapsed and in four of 17 (23%) patients who remained disease-free [53].

FOS forms heterodimeric transcription complexes with specific JUN proteins that regulate target genes involved in cell growth, differentiation, transformation, and bone metabolism [208, 255]. When the viral homolog v-FOS is injected into rodents, OS formation is induced [208, 255]. Transgenic mice overexpressing FOS in bone develop OS [208, 255]. In one report [266], 61% of OS tumors expressed high levels of FOS. The highest levels of FOS (and of JUN) expression have been reported in conventional OS [50]. FOS was expressed in nine of 21 (42%) patients who subsequently developed metastases [50, 53, 193]. Further, FOS was more frequently expressed in high-grade than in low-grade lesions [210].

MDM2, located at 12q13, encodes a protein that negatively modulates TP53 function by binding the p53 protein and physically blocking the region of p53 responsible for transcriptional activation of specific genes and targets p53 for degradation [29, 69, 87, 126, 247]. Amplification leading to MDM2 overexpression functionally suppresses p53 even in the presence of wild-type p53 protein [30, 124, 184, 185]. The 12q13 region, containing MDM2 and CDK4, is amplified in 5% to 10% of OS [132, 167, 184, 185]. However, some amplicons in this region (12q13–q14) do not include MDM2 [49]. Although MDM2 amplification has been related to progression and metastases in OS [119, 174], MDM2 amplification and TP53 mutations have not correlated with response to chemotherapy or survival [274].

Although CDK4 gene amplification has been detected in a low percentage of OS cases [93, 141], CDK4 proteins are highly expressed in 65% of low-grade OS [200]. CDK4 forms a complex with cyclin D1 and phosphorylates RB, thus releasing the E2F transcription factor from its interaction with RB [33, 179]. It has been suggested higher CDK4 levels secondary to amplification may stoichiometrically favor RB phosphorylation, thereby impairing cell cycle control [43, 107, 140, 213]. High levels of CDK4 may also drive 12q13–q15 amplification independently of MDM2 because discontinuity of the 12q13 amplicons has been identified [15, 43, 93, 257].

High levels of cyclin D1 (CCND1) have been detected in 22% of OS, and CCND1 amplification has been reported in 4% of OS [140, 257]. Furthermore, the absence of cyclin D1 expression is a powerful prognostic factor because it is associated with a metastatic phenotype [166].

ERBB2 (also known as HER2/neu and c-erbB-2) encodes a protein structurally homologous to the EGF receptor without a known ligand. At the time of initial biopsy, 20 of 47 OS (42.6%) displayed high levels of ERBB2 expression, relative to adjacent normal tissues [63]. However, the actual role of ERBB2 expression in OS development remains unclear. One study [281] found, in patients with high-grade OS without metastatic disease at presentation, increased expression of ERBB2 in tumor cells was associated with an increased probability of event-free and overall survival [281], while other studies demonstrated cytoplasmic staining of ERBB2 in pretreatment OS correlated with an increased risk of pulmonary metastases and OS cells positive for ERBB2 may represent a chemoresistant aggressive subpopulation of OS [281].

Deregulation of Major Signaling Pathways in Human OS

Wnts are a family of highly conserved, secreted proteins that play an important role in development and tumorigenesis [8, 22, 56, 58, 61, 110, 131, 134, 176, 182, 183, 197, 203, 246, 262, 264]. Many Wnts and their receptors are expressed in early bone progenitors [58, 61, 110, 134,

183]. Aberrant activation of Wnt signaling is associated with many common human cancers [8, 56, 134, 137, 203, 246]. Elevated cytoplasmic and/or nuclear localization of β-catenin, a critical mediator of the canonical Wnt pathway, has been detected in the majority of OS tumors and may correlate with OS metastasis [74, 90]. Sporadic mutations of β-catenin have also been identified [89]. OS expressing high levels of Wnt coreceptor LRP5 is less differentiated and is associated with decreased patient survival [80]. In addition, ectopic expression of the Wnt agonist DKK3 suppressed invasion and motility of OS line SAOS2 [81].

TGFβ/BMP family members play important roles in regulating cell growth and development [135, 149–151, 282]. In OS tumors, expression of TGFβ1 and TGFβ3 is higher than that of TGFβ2 [113]. TGFβ3 expression strongly related to disease progression [113]. Also, although increased expression of TGFβ2 and β3 and VEGF was correlated with OS grade, only VEGF expression was correlated with survival [92]. BMPs and their receptors (BMPRs) regulate bone and skeletal development [135, 282]. Mutations in BMPs or BMPRs lead to skeletal defects, familial primary pulmonary hypertension, and neoplasias [280]. Numerous BMPs and/or BMPRs are highly expressed in OS tumors [91, 143, 258]. Overexpression of the BMPR-II may be related to poor prognosis in malignant and metastatic OS tumors [59, 275].

MET encodes the receptor for hepatocyte growth factor (HGF/scatter factor), a cytokine that stimulates cell proliferation and motility [46, 172, 207, 216]. MET/HGF is therefore believed to play a role in stromal-epithelial interaction. Approximately 60% of OS tumors expressed MET receptor at high levels [46, 216], while some OS samples demonstrated both HGF and MET expression [46]. Thus, the activation of MET/HGF pathway may contribute to the aggressive behavior of OS tumors [14, 21, 46, 207].

GLI, originally identified as an oncogene amplified in malignant glioma, plays a role in transducing the sonic hedgehog (Shh) signal [168]. Shh is involved in anterior-posterior patterning of the limbs, and alterations in GLI1 expression may play a role in OS development [94]. GLI1 is located at 12q13.3–q14.1 and is a zinc finger transcription factor. GLI was coamplified with CDK4 in two of six OS samples [257]. An increased expression of GLI was detected in many sarcomas including seven of eight OS tumors, especially in undifferentiated tumors [205, 224].

FGFR2 plays an important role in bone and skeletal development, and inherited mutations of FGFR2 underlie skeletal dysplasias [261]. LOH of FGFR2 at 10q26 has been detected in high-grade OS, while mutations were not found in FGFR2 [160]. IGFs are produced by osteoblasts and act through their receptors to activate proliferation and differentiation. OS cells overexpress IGF1R. Additional

investigations are needed to determine whether these pathways contribute to the malignant phenotype of OS [139].

Other Genetic and/or Molecular Changes in Human OS

Paget Disease of Bone

Paget disease of bone is a heritable bone disorder characterized by rapid bone remodeling leading to abnormal bone formation. Approximately 1% of patients with Paget disease of bone develop OS [157]. Patients with Paget disease of bone account for a substantial fraction of OS occurring after the age of 60 years. Genetic linkage of Paget disease has been demonstrated to involve 18q21.1–q22 [86, 269]. Of interest is the demonstration of a possible role of the FOS gene in the pathogenesis of Paget disease [11], as well as RANK (also known as TNFRSF11A) and OPG (also known as TNFRSF11B) [68, 157, 222], although the bona fide Paget disease gene(s) remain to be identified.

Mutations of RECQ Helicases

RECQ helicases are conserved proteins that share a highly homologous DNA helicase domain, and mutations in three of the five RECQ helicases are associated with cancer predisposition syndromes, namely Rothmund-Thomson syndrome, Bloom syndrome, and Werner syndrome [252]. Rothmund-Thomson syndrome is an autosomal recessive disorder with an increased risk for OS. In one cohort of 41 patients with Rothmund-Thomson syndrome, 13 (32%) developed OS, tending to develop at a younger age (median age, 9 years) [254]. The presence of RECQL4 mutations is correlated with the risk for developing OS [253].

Bloom syndrome and Werner syndrome have some overlapping clinical features, and both exhibit predispositions to developing cancers [78]. Patients with Bloom syndrome have mutations in the BLM gene and are predisposed to all the types of cancers at a much younger age and at a higher frequency [54]. Patients with Werner syndrome have mutations in the WRN gene and are predisposed to developing OS and other tumors [65]. Of the three OS predisposition syndromes, Rothmund-Thomson syndrome appears to have the highest and most specific risk for OS tumor [252]. Thus, inactivation of the helicase pathways may contribute to OS development.

Telomerase and Telomeres in OS

Telomerase (TERT) activity is undetectable in normal cells, benign lesions, and low-grade sarcomas [272] and is

present in only a portion of OS [5, 212, 242]. TERT activity in OS tumors exhibited an inverse correlation with occurrence of pulmonary metastases in patients treated with chemotherapy [211]. Alternative lengthening of telomeres in OS may be equivalent to TERT activity [5]. Of 62 OS patients, a subset of cases lacked both TERT activity and evidence of alternative lengthening of telomeres, which was associated with a favorable prognosis [242].

Activation of Matrix Metalloproteinases (MMPs)

MMPs are zinc-dependent endopeptidases that degrade extracellular matrix proteins. MMPs are controlled by both proenzymes and inhibition of tissue inhibitor of MMPs (TIMPs). MMP2 and MMP9 were overexpressed in OS cells and associated with the ability of the cells to metastasize [18]. Increased expression of membrane-type MMP1 has been correlated with poor prognosis in OS patients [240]. Upregulation of TIMP1 is associated with poor clinical outcome for OS. Binding of TIMP-1 to an unknown receptor system reportedly triggers Ras/Raf1/FAK signaling in OS. Thus, TIMP1 may have a dual effect on tumor progression [47, 84].

Neurofibromatosis-2 (NF2)/Merlin

NF2 encodes Merlin, an ezrin-radixin-moesin (ERM)-related protein that functions as a tumor suppressor [153, 154]. NF2 null mice die in early embryogenesis, whereas NF2 heterozygous mice are viable but develop a variety of highly metastatic tumors, including OS and hepatocellular carcinoma, with long latencies [155]. Merlin mediates contact inhibition of growth through signals from the extracellular matrix. At high cell density, Merlin becomes hypophosphorylated and inhibits cell growth in response to hyaluronate through specific interaction with the cytoplasmic tail of CD44 [55, 109, 118, 218]. Well-dedifferentiated OS tumors have a higher level of CD44 [94]. Merlin may control the stability of the adherens junction by its interaction with the actin cytoskeleton. Loss of this function may lead to tumorigenesis and metastasis [106, 121]. The N-terminal region of Merlin increases p53 stability by inhibiting the MDM2-mediated p53 degradation. Thus, loss of Merlin may also destabilize p53 [108].

Additional Genetic Changes

The budding uninhibited by benzimidazole 3 (BUB3) was identified in a region of LOH at 10q26 in 20 high-grade OS tumors, although no mutations of BUB3 were observed in OS. BUB3 plays a role in chromosome homeostasis and is a component of the spindle assembly checkpoint complex [23], alterations of which could underlie the aneuploidy that is characteristic of OS [160]. Interestingly, BUB3 is a target of E2F [6]. Primase polypeptide 1 (PRIM1), located at 12q13, is amplified in nine of 22 OS tumors [276].

MDM2 and CDK4 are considered the most important amplification targets in 12q13–q15 [202], but the region also contains numerous genes, including CHOP (ie, DDIT3), SAS (sarcoma amplified sequence) [159], OS-4 [226], OS-9 [225], PRIM1 [276], and other as yet poorly characterized genes [44]. Amplification of SAS was reported in 36% of OS [180] and was linked with increased CDK4 expression [268]. Other suspected oncogenes include MAPK7 and peripheral myelin protein (PMP22/GAS3), both located at 17p11.2. MAPK7 was amplified in 10 of 19 OS samples [243]. Frequent amplification of PMP22 is observed in high-grade OS [244]. HMGIC gene (also known as HMGA2, localized to 12q14–q15) was rearranged by fusing with the keratin sulfate proteoglycan lumican gene LUM in an OS line [115]. HMGIC gene was also amplified and rearranged in two primary OS [16].

Allelic loss at 4q32–q34 was identified in 63% of OS [220]. High frequencies of allelic loss have been detected at 3q26 [79, 117], 13q, 17p, and 18q, suggesting other tumor suppressor genes may exist at 3q and 18q [271]. Expression of DCC (deleted in colon cancer), located on 18q21, decreases in OS [85]. Two potential OS suppressor gene loci were demonstrated at 6q14 (imbalance in 77% of cases) and 15q21 (58% of cases) [175].

Genome-wide Approaches to Identifications of OS-associated Genes

Microarray-based expression profiling analysis has become an increasingly common practice to identify genes associated with OS pathogenesis [103]. One such study has shown, among the 100 most up- and downregulated genes, 35 are affected in all three OS lines, with eight genes showing an increase and 27 genes a reduction in the expression level compared with normal human osteoblasts [265]. These findings have provided a proof-of-principle of genome-wide approaches to unraveling the pathogenesis of OS.

OS Metastasis-associated Genes

Ezrin, a member of the ERM proteins, has been identified as a metastasis-related gene that is differentially expressed in murine OS lines with differential metastatic potential [104–106]. Ezrin is involved in intracellular signal transduction regulating cell migration and metastasis [251] and

is expressed in a variety of cancers, some of which are associated with poor outcome [142, 148]. In a study of 19 patients with OS, the disease-free interval of OS patients with high ezrin expression was substantially shorter than that in patients with low ezrin expression, and the risk of metastatic relapse was 80% greater in the former group [106].

Expression of S100A6 was reported in 84% of analyzed OS specimens [138]. There is a trend toward decreased clinically evident metastasis with increased S100A6 staining. Overexpression of S100A6 in OS cells decreases cell motility and anchorage-independent growth [138]. These findings suggest, while S100A6 is commonly overexpressed in OS, loss of its expression may correlate with a metastatic phenotype. A cluster of 16 types of S100 genes is located on 1q21, which is frequently amplified or rearranged [25, 35, 37, 71, 158, 190, 209]. S100 proteins constitute a group of nearly 20 proteins that contain well-conserved EF-hand calcium-binding domains [209]. Several S100 proteins have been associated with human cancers [25, 35, 37, 71, 158, 190, 209].

Annexin 2 (AnxA2) was downregulated in metastatic samples [57]. AnxA2 belongs to a large family of diverse proteins characterized by conserved annexin repeat domains and the ability to bind negatively charged phospholipids in a calcium-dependent manner [7]. AnxA2 was downregulated in a subset of human OS metastases and metastatic lines [45, 165]. The actual role of AnxA2 in suppressing OS metastasis remains to be elucidated.

Chemokine stromal cell-derived factor 1 (SDF-1) belongs to cytokinelike proteins that, through binding to their CXCR receptors, play a role in cytoskeleton rearrangement, adhesion to endothelial cells, and directional migration [19]. CXCR4/SDF-1 is important in tumor progression [130, 228]. Migration and adhesion of OS cells were promoted by SDF-1 treatment, whereas the development of pulmonary metastasis after injection of OS cells in a mouse model could be prevented by the administration of T134 peptide, an inhibitor of CXCR4 [191].

## Possible Links Between Defective Osteogenic Differentiation and Bone Tumorigenesis

Human OS tumors exhibit osteoblast-like features, although the differentiation status of OS tumors can be observed within a broad range, from highly differentiated to poorly differentiated or undifferentiated phenotypes [75]. However, potential cancer stem cells responsible for OS development have yet to be identified. Understanding the molecular mechanism underlying osteogenic differentiation would help to unravel the molecular pathogenesis of human OS. Osteogenesis results from a well-coordinated

sequence of events involving epithelial mesenchymal interaction, condensation, and differentiation (Fig. 1A). Several major signaling pathways, such Wnt, BMP, FGF, and hedgehog signaling, play an important role in regulating osteogenic differentiation [58, 134–136, 203, 204]. At the transcription level, several transcriptional factors have been identified as important regulators of osteogenic lineage commitment and terminal differentiation. These transcriptional factors include Runx2, Osterix, ATF4, and TAZ [17, 31, 36, 41, 95, 97–100, 189, 229, 230, 250, 263, 273]. Among these factors, Runx2 plays an important role and serves as a hub to direct progenitors to osteogenic lineage [38, 39, 82, 96, 114, 129, 171, 173, 187, 270].

Runx2 is a member of the Runx class of transcription factors that contain a highly conserved 128 amino acid motif conferring DNA binding, protein-protein interactions, and ATP binding activities [38, 39, 82, 96, 114, 129, 171, 173, 187, 270]. $Runx2^{-/-}$ die shortly after birth and demonstrated a cartilaginous skeleton with complete absence of ossification. Despite the cartilaginous phenotype in the Runx2-null mice, histologic analysis demonstrated delayed chondrocyte maturation suggesting the importance of Runx2 in chondrogenesis and osteogenesis. Additionally, when Runx2 is overexpressed in chondrocytes via the chondrocyte-specific type II collagen promoter, it results in ectopic chondrocyte hypertrophy and endochondral ossification, thereby demonstrating the importance of Runx2 in controlling differentiation of both chondrocytes and osteoblasts [38, 39, 82, 96, 114, 129, 171, 173, 187, 270]. Runx2 transcriptional activity is regulated by numerous transcriptional co-activators and corepressors, including its interaction with Rb protein (see below).

OS can be regarded as a differentiation disease that is caused by genetic and epigenetic disruptions of osteoblast terminal differentiation (Fig. 1B). This model is supported by the following facts: First, OS tumors exhibit the characteristics of undifferentiated osteoblasts [26, 75, 83, 181, 195, 196, 219, 233, 277]. Second, differentiation-promoting agents (eg, PPARγ agonists and 9-cis-retinoic acid) induce osteoblast differentiation [75, 76]. Third, RB coactivates Runx2 through direct physical interactions at sites of active transcription, and loss of function of RB attenuates terminal osteoblast differentiation in vitro [232]. RB plays essential roles in many cellular processes including mesenchymal differentiation [70, 72, 116, 245]. Fourth, Runx2 coordinates terminal cell cycle exit through induction of $p27^{KIP}$ [1], which in turn is required for normal bone development and is lost in dedifferentiated human OS [233]. Lastly, osteogenic stimuli, such as osteogenic BMPs, failed to promote the terminal differentiation of most OS cells and rather enhanced OS tumor growth, further highlighting the existence of possible differentiation defects in OS cells [75]. Furthermore, in Ewing's sarcoma (ESW), EWS/ETS

Fig. 1A–B (**A**) Osteogenic differentiation is a well-coordinated process. Mesenchymal stem cells (MSCs) can give rise to several lineages, such as myocytes, adipocytes, chondrocytes, and osteocytes, with appropriate stimuli, presumably by activating proper lineage-specific regulators, eg, MyoD, PPARγ, Sox9, or Runx2/Osterix. Osteogenic differentiation is a tightly controlled process, which can be monitored by using alkaline phosphatase as an early marker and osteocalcin and osteopontin as late markers. (**B**) Disruption of osteogenic differentiation may lead to OS development. The defects caused by genetic (eg, activation of oncogenes or inactivation of p53 and RB tumor suppressor genes) and epigenetic alterations may occur at different stages of osteogenic differentiation. It is conceivable defects at the early stages may lead to the development of more aggressive and undifferentiated OS, or vice versa. The cells filled with black color indicate cancer-initiating cells.
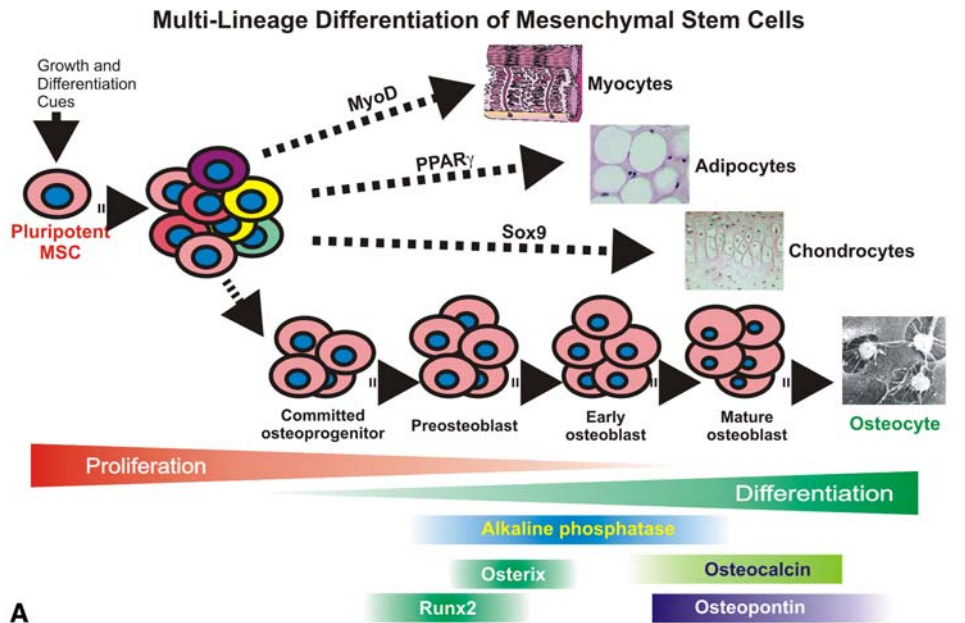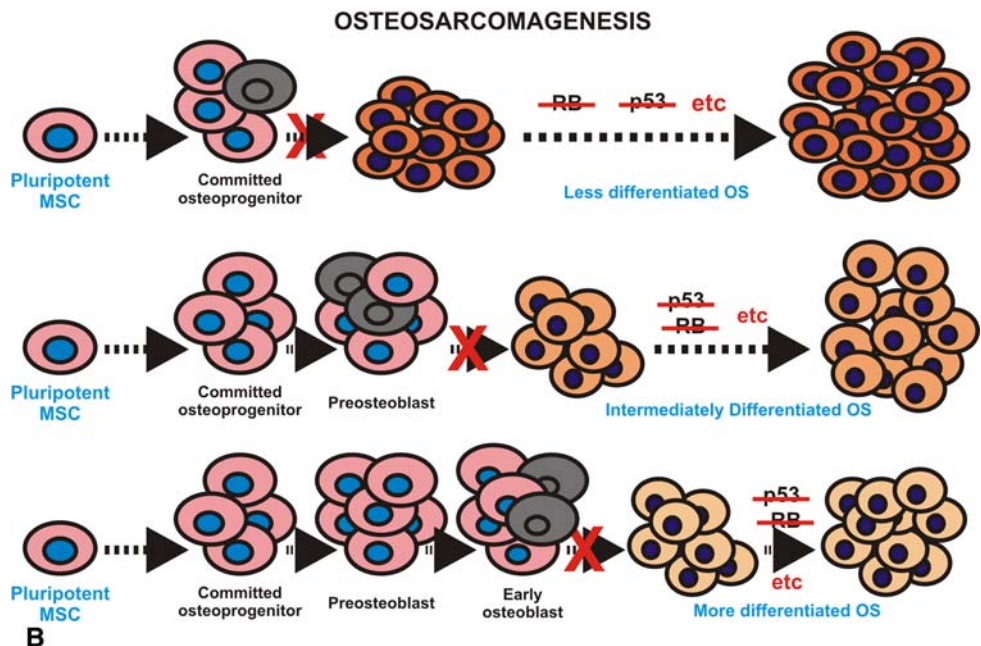
fusion proteins block differentiation along osteogenic and adipogenic lineages of marrow stromal cells [238]. In fact, expression of the EWS/FLI-1 oncogene in murine primary bone-derived cells results in EWS/FLI-1-dependent, Ewing's sarcoma-like tumors [27]. Conversely, upon EWS-FLI1 silencing, some of the ESW cell lines can differentiate along the adipogenic or osteogenic lineages when stimulated with appropriate differentiation cocktails [234]. Taken together, these emerging data strongly suggest osteosarcomogenesis may be resulted from defects in osteoblast differentiation pathway.

Discussion

Osteosarcomas are a clinically and molecularly heterogeneous group of malignancies characterized by varying degrees of mesenchymal differentiation. The genetic and epigenetic alterations described above may represent a cross-sectional endpoint view of OS. However, defining their roles in OS development has been hampered by the complexity of the genetic changes and the rarity of OS samples. Although osteosarcoma tumors display a broad range of genetic and molecular alterations, most alterations

are not frequently detected in the majority of osteosarcoma tumors. With a rapid expansion of our knowledge about stem cell biology, emerging evidence suggests osteosarcoma may be regarded as a differentiation disease caused by genetic and epigenetic changes that interrupt osteoblast differentiation from mesenchymal stem cells.

In this survey, we first reviewed the current genetic alterations and molecular biology of OS, and then focused on the possible relationship between osteogenic differentiation and bone tumorigenesis. By searching PubMed with various keywords, we found over 20,000 publications relevant to the topic. Although we only conducted the search using a single database, PubMed represents one of the most extensive databases for biomedical sciences. We believe most of the relevant and important findings related to OS have been included in this single database but cannot exclude other information that might be found in other databases (e.g., EMBASE).

It is conceivable that, at least for a subset of osteosarcomas, cancer-initiating cells may share features of a committed osteoprogenitor. Tumorigenesis may involve disruption of mechanisms, appropriately constrain the initiation of proliferation by tumor stem cells, or allow persistent expression of stem cell-like features in apparently partially committed cells. The similarities between stem cell properties and those of transformed cells are striking as both cell types possess unlimited self-renewal, express telomerase, and are undifferentiated as defined by the absence of lineage-restricted markers. In fact, a small subpopulation of self-renewing OS cells are capable of forming suspended spherical cells and colonies [55]. These OS cells as well as tissue specimens express activated STAT3 and the marker genes of pluripotent embryonic stem (ES) cells, Oct 3/4 and Nanog [55]. In support of this notion, OS is frequently observed in adolescence, a stage of intensive skeletal growth entailing increased osteoblast activity. Stem cells are more resistant to mutagenic events than somatic cells, in part due to enhanced apoptotic responses to genotoxic stress and DNA damage. The efficiency of such processes appears inversely related to the degree of terminal differentiation [231]. Thus, future investigations should be devoted to identifying the key defects in the osteoblast differentiation pathway, which is also responsible for the development of primary bone tumors.

As one of the most important factors regulate osteoblast lineage commitment and expansion, Runx2 may be deregulated and plays an important role in OS development. Runx2 levels and function are biologically linked to a cell growth-related G(1) transition in osteoblastic cells [52]. Runx2 and histone deacetylase 3-mediated repression is believed to allow high expression of bone sialoprotein-a bone matrix glycoprotein whose expression coincides with terminal osteoblastic differentiation and the onset of mineralization-in differentiating human osteoblast cells [122]. Runx2 is hyperphosphorylated by CDK1/cyclin B during mitosis, and dynamically converted into a hypophosphorylated form by PP1/PP2A-dependent dephosphorylation after mitosis to support the postmitotic regulation of Runx2 target genes [201]. A more recent study indicates Runx2-mediated activation of the Bax gene increases osteosarcoma cell sensitivity to apoptosis and Bax as a direct target of Runx2, suggesting Runx2 may act as a proapoptotic factor in osteosarcoma cells [42].

Another important factor that may play an important role in osteogenic differentiation and bone tumorigenesis is pRb. The cell cycle regulatory pathway regulated by pRb is inactivated in almost all human cancers, but individual tumor types seem to target specific components to achieve this effect. As described in Results, pRb itself is frequently inactivated in OS, and inherited heterozygous loss of the RB gene confers approximately a 1000-fold greater incidence of OS than the general population. Several lines of evidence implicate pRb in osteogenesis as pRb coactivates Runx2 through direct physical interactions at sites of active transcription, and loss of function of pRb attenuates terminal osteoblast differentiation in vitro [231]. Runx2 coordinates terminal cell cycle exit through induction of the CDK2 inhibitor p27KIP1, which in turn is required for normal bone development in vitro and in vivo, and is lost in dedifferentiated human osteosarcomas [231]. It is also possible pRb influences osteoblast differentiation through other mechanisms involving chromatin structure. Thus, it would be important to investigate how the deregulation of pRb and/or Runx2 functions may lead to the development of bone sarcomas.

Understanding the molecular pathogenesis of human osteosarcoma could ultimately lead to the development of diagnostic and prognostic markers, as well as targeted therapeutics for osteosarcoma patients. Dissecting the molecular mechanisms that control osteoblast differentiation is important not only to understand normal skeletogenesis and to pinpoint potential defects responsible for OS development but also to improve the clinical management of human OS. Although pre- and postoperative chemotherapies have improved the 5-year survival rate of OS patients, recurrent and/or metastatic OS tumors are more aggressive and usually resistant to conventional cancer therapies. In a broader sense, most current chemotherapies and/or radiation therapies target the rapidly proliferative tumor cells, with little consideration of promoting tumor cell differentiation. It is conceivable a combined therapeutic approach targeting both proliferation and differentiation phases of tumor cells would be more efficacious and less prone to inducing chemoresistance [75, 76, 217, 278]. Thus, identification of the critical differentiation defects in OS tumors may lead to a rational

design of therapeutic strategies that induce terminal differentiation of OS cells through alternative differentiation pathways and/or bypassing the differentiation defects.

The potentially important role of genetic and epigenetic events in both osteogenesis and bone tumorigenesis is now recognized. Our current knowledge of transcriptional regulation of osteoblast differentiation will provide important insights into the potential defects in osteogenic differentiation of OS cells. Future research should be directed towards identifying these differentiation defects in OS cells. This knowledge may help us develop efficacious differentiation therapies for OS by exploiting noncell autonomous signals to promote differentiation state.

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