REVIEW ARTICLE

Osteosarcoma: A review with emphasis on pathogenesis and chemoresistance

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Abstract

Osteosarcoma is the most common malignant primary bone tumor among children and adolescents. Patterns of presentation and clinical progression have been well-characterized, and cytogenetic and molecular analyses have demonstrated genomic complexity with a substantial degree of structural variation. Nevertheless, extensive research has facilitated only limited understanding of the molecular events that govern oncogenic transformation of a mesenchymal progenitor or that drive clinical phenotypes such as metastasis and chemoresponsiveness. Initial clinical management of patients is well-standardized, and the majority of patients whose tumors are localized at the time of presentation, are amenable to effective surgical resection, and exhibit extensive tumoricidal response to chemotherapy can enjoy long term survival. Outcomes for the significant proportion of patients differing with respect to any one of these clinical characteristics are much less favorable, however, and therapeutic strategies to address clinically advanced disease and chemoresistance to date have been disappointing. This review will discuss the current understanding of OS oncogenesis, clinical presentation, and the status of OS clinical management. The discussion will focus on genetic and epigenetic events associated with chemoresistance in OS and the insights such a mechanistic understanding may offer toward circumventing this major clinical barrier.

Epidimiology of Osteosarcoma

Osteosarcoma (OS) is a comparatively rare tumor that arises from malignant mesenchymal progenitor cells, but it is the most common primary bone tumor diagnosed in the pediatric and young adult population. The incidence of this malignancy is approximately 3.1 cases/million in the US, and it represents less than 1% of diagnoses in the adult population.[1] Primary bone tumors comprise the sixth most common neoplasm in children and adolescents, and the annual incidence of OS peaks at approximately 8-11 cases/million/year in the age group.[2] Among 10-19 year-olds, OS represents 15% of all extracranial malignancy diagnoses.[3] The incidence in males is increased 1.4 times compared to females.[4] An increase incidence of OS has been well-documented in patients with hereditary retinoblastoma and Li Fraumeni syndrome.[5] OS has also been reported in association with Rothmund Thompson Syndrome, Hereditary Multiple Exostoces, Werner syndrome, Bloom syndrome, RAPADALINO syndrome, Diamond Blackfan Anemia, and other disorders.(Ripperger et al and references therein)[5] Finally, a second incidence peak occurs in adults greater than 65 years of age, in whom it often presents in association with Paget's Disease or as a second cancer.[6]

Pathology and Molecular Pathogenesis of Osteosarcoma

Osteosarcoma is defined histologically based upon the presence of malignant cells producing osteoid matrix. OS variants are classified according to tumor location (central versus surface), tumor grade, histologic features, and, in some cases, radiographic features. High-grade OS is characterized by malignant cells that exhibit pleomorphic nuclei, atypical mitotic figures, and anaplasia.[7] Conventional OS, the most common high-grade variant, includes osteoblastic, chondroblastic, and fibroblastic subtypes as defined by predominant histologic features of tumor cell differentiation and characteristics of the tumor matrix.[2] (Figure 1) Rarer variants include giant-cell rich, osteoblastoma-like, clear cell type, epithelioid, and chondroblastoma-like OS.[8] Other high-grade variants include telangiectatic OS, which exhibits a histomorphology of blood-filled cysts, and small cell OS, characterized by small cells with scant cytoplasm producing lacy osteoid.[8] Low or intermediate-grade OS variants include parosteal and periosteal types, both of which arise on the surface of the bone. [8]

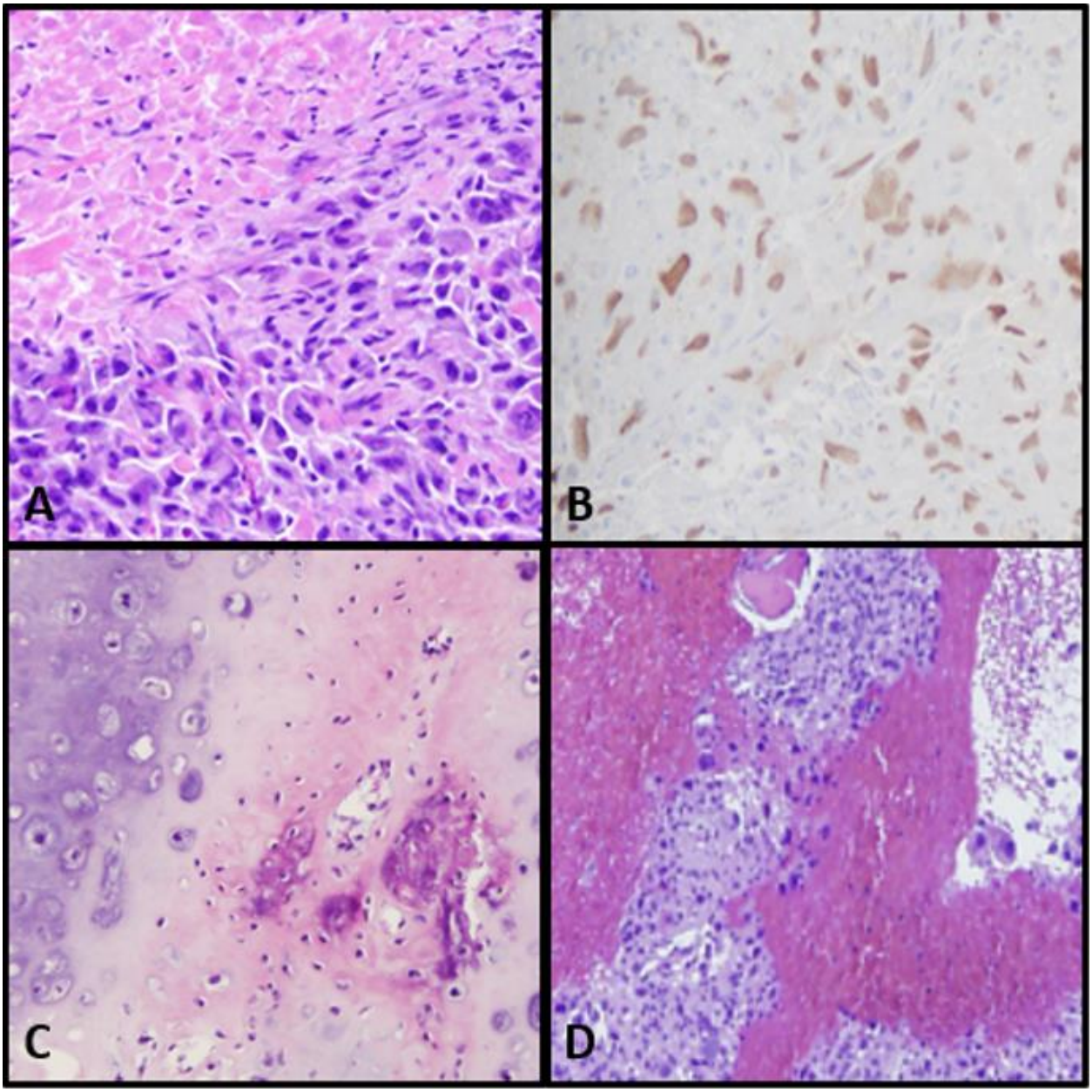

Figure 1. Conventional osteoblastic osteosarcoma (**A**) is notable for its pleomorphic cells with nuclear hyperchromasia, abundant lacy osteoid deposition, and (**B**) immunohistochemical staining of osteoid for SATB2. (**C**) The histologic variant chondroblastic osteosarcoma is characterized by malignant cells in lacunae with rare regions of osteoid production. (**D**) The telangiectatic osteosarcoma variant is notable for blood filled spaces separated by septa of malignant cells.

Mesenchymal Progenitors, Cancer Stem Cells, and the Bone Microenvironment. The interaction of mesenchymal progenitors (MP) or "committed" osteogenic progenitors with the bone microenvironment likely underlies the initiation and progression of OS. Mesenchymal stem cells (MSC) have been studied intensively over the past 25 years. These cells, which can be harvested from bone marrow, adipose, and other tissues, can undergo induced differentiation into diverse cell lineages including bone, cartilage, adipose, muscle, and others.[9,10] Nevertheless, the "stemness" of these cells

has been questioned[11,12] as has the role of this cellular population in physiologic bone development,[13] and it is by no means clear that these cells represent the cell-of-origin of osteosarcoma. This review will therefore utilize the term MSC in reference to experiments in which these cells specifically were used, but will substitute the necessarily vague term "multipotent mesenchymal progenitor" (MMP) to represent the undifferentiated mesenchymal progenitor cell that gives rise to bone. Bones develop through the processes of endochondral or intramembranous bone formation. MMPs differentiate directly to osteoblasts in membranous bone formation. In the endochondral process, by which most bones develop, MMPs differentiate into chondrocytes forming the cartilage anlagen followed by invasion of osteoblast progenitors and osteoclasts as well as angiogenic and hematopoietic elements leading to the development of primary and secondary ossification centers and deposition of cortical bone around the anlagen. As bones grow, cartilaginous structures develop at the epiphyses, between expanding ossification centers, and are referred to as the growth plate.[14] It is at these sites close to the growth plates of long bones that OS is most likely to develop in the pediatric population.[15]

Results of experiments in which oncogenic mutations have been targeted to murine MMPs or committed osteogenic progenitors support a role for early bone progenitors in the development of OS. A number of investigators have used recombinant conditional gene knock-out techniques with transgenic mice bearing floxed Trp53 or Rb1 alleles. Mice with Trp53 or Rb1 deletions targeted to MMPs or osteoblastic cells were generated by engineering the cre recombinase under the control of undifferentiated mesenchymal progenitor-restricted or osteoblast-restricted gene regulatory elements.[16–18] These investigators observed OS development in Trp53 -/+ and Trp53 -/- mice, whether deletions were targeted to MMPs or osteoblast-committed cells. Interestingly, while Rb1 deletions alone resulted in few, if any, tumors, deletion of Rb1 decreased the latency of tumor formation in mice also bearing Trp53 deletions. In contrast, Rubio and colleagues found that the stage of osteogenic differentiation of bone marrow MSCs dictated the phenotype of the sarcomas that developed compared to undifferentiated MSCs.[19] In these experiments deletion of Trp53 and Rb1 in isolated, bone marrow-derived, undifferentiated MSCs resulted in leiomyosarcoma formation, while deletion in MSCs undergoing induced osteogenic differentiation yielded tumors compatible with osteosarcoma. It is important to note, however, that these experiments utilized isolated, cultured MSCs, either undifferentiated or induced toward osteogenic differentiation *in vitro*, in which Trp53 and/or Rb1 deletion was accomplished *in vitro* by transduction of cre recombinase, and where tumorigenesis was assayed by subcutaneous injection into immune-deficient mice. In another study it was noted that overexpression of c-MYC overexpression in murine bone marrow stromal cells isolated from Ink4a/Arf-/- mice loss could induce malignant transformation to OS.[20]

Human cell models of OS tumorigenesis have likewise targeted mesenchymal or osteogenic progenitor cells. Wang and colleagues demonstrated transformation of human MSCs (hMSC) via Rb knockdown and c-Myc overexpression.[21] Cultured hMSCs and induced pre-osteoblasts were transformed with the oncogenes hTERT, SV40 large T antigen, and H-Ras and then evaluated for the OS tumorigenic potential. It was observed that cell lines derived from pre-osteoblasts developed tumors in mice with histologic features

characteristic with OS, but with restricted (osteoblastic/chondrocytic) differentiation potential.[22] In that they target arguably different target progenitor cell populations with different mechanisms of cell differentiation, and include or exclude a role for the bone/bone marrow microenvironment, these *in vivo* and *in vitro* knockout model systems cannot be regarded as exactly comparable. Given the considerable biological and clinical heterogeneity apparent in osteosarcoma, it seems reasonable to conclude that potential cells-of-origin may lie along a differentiation continuum from undifferentiated or minimally differentiated MMP to preosteoblast.[23]

The relationship between these mesenchymal progenitor-derived cells-of-origin and the tumor-maintaining cancer stem cell (CSC) is coming into focus. Cancer stem cells are self-renewing and can maintain and re-establish the full phenotypic spectrum of tumor cells.[24] Cells with these properties can be enriched and isolated from cultured OS tumors or OS cell lines based on growth properties (e.g., anchorage-independent cell spheroids) and expression of markers such as CD133, STRO1 CD117+, and CD271+ or ALDH1 activity. These cells may express MSC markers including STRO1, CD44, and CD105 and may be induced to multilineage differentiation, but exhibit robust expression of pluripotency genes such OCT-3/4, NANOG, and SOX2.[23,25,26]

The situation of mesenchymal progenitor cells, OS cells-of-origin, and OS CSCs within the bone microenvironment (BME) is key to the development and maintenance both of normal bone and OS. Cytokines, acting via paracrine or autocrine mechanisms, that regulate progenitor function and bone development may promote tumor cell survival and proliferation. Growth hormone (GH) and insulin-like growth factor 1 (IGF-1) promote cell proliferation and are prevalent in the BME during periods of skeletal growth, which correlate temporally with the interval of peak OS incidence.[15] The MMP pool is maintained in part by NOTCH signaling in the BME which suppresses osteoblastic differentiation.[27] Balancing NOTCH signaling and promoting bone formation is WNT pathway signaling, which regulates early osteoblastic differentiation through upregulation of the transcription factor osterix and by downregulation of bone resorption via induction of osteoprotegerin, an inhibitor of osteoclast development. The homeostatic control of bone resorption (osteoclast) and bone deposition (osteoblast), critical to maintenance of bone and bone marrow integrity and mediated in part by tightly-regulated interaction of receptor activator of nuclear factor kappa B protein ligand and receptor (RANKL/RANK), may be hijacked by developing OS to upregulate RANK/RANKL expression leading to increased expression of proliferation-inducing agents such as transforming growth factor β (TGF-β), fibroblastic growth factor (FGF), and bone morphogenic protein (BMP). The pro-inflammatory cytokine IL-6 increases proliferation of MSCs and OS-derived cells by activating the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway and feeds back into the RANK-RANKL axis, while inhibition of IL-6 or STAT3 activation has been shown to reduce tumor growth.[9,28]

It has been suggested that localization of OS-CSCs within discrete BME "niches" may facilitate CSC function and account for OS phenotypes.[29] MSCs are localized within the perivascular space where they interact with hematopoietic cells, fibroblasts, other mesenchymal cells, and immune cells that collectively regulate proliferation and support "stemness."[30] From this space migration to sites of injury, for example, may readily occur. OS-CSCs may similarly

"commandeer" this niche which may then support tumor development and facilitate metastasis.[29] Other hypothetical BME niches include the endosteal niche, characterized by osteoblast/osteoclast interaction, which may promote tumor proliferation, as discussed above, and the hypoxic niche, the milieu of which may promote metastatic and drug resistance phenotypes in CSCs (discussed below). Finally, the BME in the CSC niche may engender molecular crosstalk between OS-CSCs and MSCs that promotes tumor progression. Tumor secreted factors such as stromal derived factor 1 (SDF-1), macrophage migration inhibitory factor (MIF), and IL-6 can recruit MSCs to the tumor site and, in return, MSC-produced IL-6, vascular endothelial growth factor (VEGF), and transforming growth factor β (TGF-β), as well as environmental conditions such as hypoxia, may promote tumor cell proliferation, migration, and metastasis, and facilitate immune escape.[9,31] Thus, a complex network of local cell signaling pathways not only plays a critical role in the development of OS, but may impact tumor aggressiveness and efficacy of therapy.

Osteosarcoma Genetics and Epigenetics

Over the past few decades, much effort has been devoted to characterization of the complex and heterogeneous OS genome. While a pathognomonic genetic variation or mutation has not been identified for OS, a high level of chromosomal variation has consistently been observed by karyotype and molecular cytogenetic analysis with somatic copy number alterations that include both gain of chromosomes or chromosome segments and loss of chromosome or chromosome segments. One third of OS tumors may exhibit chromosomal clusters of hyper-rearrangement thought to result from a catastrophic cellular event followed by repair - a mutational process called chromothripsis.[32,33] This chromosomal instability (CIN) manifests as gain of chromosome 1; loss of chromosomes 9, 10, 13, and 17; deletions of part of chromosomes 3, 6, 9, 10, 13, 17, and 18; and duplication or amplifications of chromosomes 1, 6, 8, and 17.[33,34] CIN in OS likely reflects mutation and deregulation of cell cycle and mitotic checkpoints such as Rb and p53.[35–37] Additionally, telomerase activation and, more commonly, the Alternative Lengthening of Telomeres' mechanism appear to contribute to the CIN in OS. The latter mechanism is associated with complex chromosomal rearrangements in tumors that, like OS, lack pathognomonic genomic translocations and is associated with poor outcomes in OS.[35]

Tumor Suppressor Genes in OS Loss of the functional p53 tumor suppressor pathway has long been recognized as a central event in the development of OS. TP53 deletion or mutation has been documented in three-fourths of OS cases, occurring via allelic loss (75-80%), rearrangement (10-20%), and point mutation (20-30%).[34] TP53 encodes a transcription factor that regulates the cell cycle and apoptosis, and p53 mutations promote uncontrolled cell cycles and inhibition of senescence and cell death, thereby increasing the risk of malignant transformation.[9] Deficiency of p53 has been shown to increase expression of the transcription factors RUNX2, DLX5, and OSX in bone progenitors resulting in deregulation of normal osteoblastic differentiation.[9,38] Underscoring the importance of loss of p53 function in OS is role of aberrant p53 inhibition in OS tumorigenesis. The MDM2 and COPS3 oncoproteins inhibit p53 activity by targeting the protein for proteasomal degradation.[39,40] Amplification of *MDM2* (12q15) has been identified in roughly 3-25% of OS cases,[34,35] while amplification of *COPS3* has been observed in 30% of OS and may be associated with an adverse prognosis.[35,41]

Inactivation of the Rb tumor suppressor pathway has likewise been extensively documented in OS dating back to the initial observation of OS predisposition in individuals with hereditary retinoblastoma.[42] Rb is a regulator of the G1/S cell cycle transition, and during normal mitosis RB phosphorylation by CDK4 promotes cell cycle progression. Approximately 70% of OS cases exhibit loss of Rb function, most commonly via deletion of the RB1 locus (13q14.2).[34,35] The absence of the cell cycle arrest by RB1 silencing precludes DNA damage repair and contributes to genomic instability.[9] Inactivating deletions or rearrangements in the CDKN2A locus, which encodes an inhibitor of CDK4, occur commonly in OS. These mutations likewise negate Rb function by derepressing phosphorylation-mediated inactivation of Rb by CDK4. Functional inhibition of Rb may also result from amplification/overexpression of *CDK4*, observed in perhaps 10% of OS.[35,43] Importantly, the *CDKN2A* locus also encodes p14ARF via alternative splicing. p14ARF inhibits ubiquitin-mediated degradation of p53. Thus inactivation via deletion, mutation, or epigenetic silencing of the *CDKN2* tumor suppressor gene could accomplish functional downregulation of both p53 and Rb tumor suppressor pathways and contribute to genomic instability in OS.[9]

Deletion of the *WWOX* tumor suppressor gene (16q23.1-q23.2) has been observed in OS, and reduced expression may be a frequent event.[44] Moreover, targeted deletion of *WWOX* has been shown to result in OS formation in mice.[45] *WWOX* encodes an oxidoreductase that binds to and suppresses the RUNX2 transcription factor, which is essential for osteoblast differentiation and bone formation.[46] Gain of *RUNX2* (6p12-p21) copy number associated with overexpression has been observed in OS and may correlate with a poor chemotherapy response.[35] WWOX also interacts with p53,[47] and enforced expression of WWOX in OS cells has been shown to inhibit neoplastic phenotypes including proliferation, migration, and invasion.[48] Thus oncogenic RUNX2 overactivity driving OS tumor progression may result from overexpression of *RUNX2* or reduced expression of WWOX in the same way that suppressive p53 activity may result from inactivating mutations of *TP53* or overexpression of *COPS3* or *MDM2*.

Functional loss of the *PTEN* tumor suppressor gene has been identified in primary tumors and bone metastases of many cancers. Allelic loss and copy number loss of 10q23, to which PTEN has been mapped, occurs frequently in OS.[49] PTEN functions as a dual-specific protein phosphatase and inositol phospholipid phosphatase, and is a negative regulator of the phosphoinositol-3-kinase/AKT/MTOR pathway.[50] Loss of PTEN expression, then, deregulates this pathway, while restoration of PTEN was shown to inhibit OS cell proliferation, migration, invasion, and enhance apoptosis and may abrogate the tumor/osteoclast crosstalk discussed above.[51] Likewise, loss of expression of *TSSC3*, an imprinted tumor suppressor gene at 11p15, may facilitate OS tumorigenesis via deregulation of the PI3K/AKT/MTOR pathway.[52] Loss of function of these and other tumor suppressor genes may occur via genetic mechanisms such as loss of copy number, inactivating mutation, or gene rearrangement or via by epigenetic mechanisms, as will be discussed below.

Oncogenes in OS As is the case with many human cancers, the *MYC* oncogene has been implicated in OS tumorigenesis. The role of *MYC* in OS oncogenesis has been supported by cellular models of tumorigenesis, as discussed above. Amplification of 8q24.21, to which *MYC* is localized, has been observed with variable frequency in analyses of human

OS,[35] MYC overexpression may occur in approximately 10% of OS cases,[9] and such overexpression may be prognostically important.[53,54] MYC overexpression was shown to increase invasiveness in OS cell lines. That this phenotype could be blocked by inhibition of MEK-ERK pathway implicates this signaling pathway in the mechanism of MYC oncogenesis in human OS.[55] The potential role of copy number/amplification of oncogenes *RUNX2*, *MDM2*, and *COPS3* has been discussed above. Other oncogenes, identified as amplification targets and implicated in the pathogenesis of OS, include *CDC5L*, *MAPK7*, *PIM1*, *PMP22*, *PRIM1*, and *VEGFA*.[35] Some of these genes co-localize with the amplification targets RUNX2, CDK4, and COPS3.[35]

WNT signaling through the canonical and non-canonical pathways plays an important role in bone development, and dysregulation of WNT pathway signaling is oncogenic in OS. (reviewed in Cai et al. [56]) As has been observed with the oncogene/tumor suppressor networks discussed above, aberrant WNT pathway signaling can result from gain-of-function receptor/activator mutations and/or loss-of-function mutations of inhibitory proteins. Like WNT pathway signaling, the NOTCH receptor signaling pathway plays a central role in mesenchymal progenitor/osteoblast homeostasis, and dysregulated NOTCH signaling has been implicated in OS.[57] Upregulated NOTCH pathway signaling may support the "stemness" of OS CSCs, likely occurring in the context of the OS BME cell networks as discussed above,[58] and has been implicated in OS tumor angiogenesis and metastasis.[59]

Epigenetic Dysregulation in Osteosarcoma

It has become widely accepted over the past 25 years that cancer phenotypes reflect a disrupted epigenome as well as a disrupted genome. Epigenetic processes are biological processes that regulate or alter gene expression regulation at the transcriptional or post-transcriptional level without altering the sequence of the DNA template.[9,34] Myriad differences in epigenetic structure and function have been identified between normal stem cells, somatic cells, senescent cells, immortalized cells, and cancer cells.[60] Epigenetic processes relevant to gene expression in cancer include DNA methylation, histone post-translational modification, nucleosome remodeling, and RNA-mediated events.[34] These processes, considered in the context of the already highly complex OS genome, introduce yet more complexity into conceptual models of OS oncogenesis. While the added complexity is daunting, the molecular reversibility that is characteristic of some epigenetic processes may allow for a better conceptual framework for understanding, for example, cellular plasticity in CSCs, and presents opportunities for development of novel therapies.

DNA Methylation DNA is modified post-synthetically through methylation. In the most common DNA methylation format, a methyl group is added to the 5-carbon of cytosine, and this typically occurs at the so-called CpG dinucleotide (5'C-p-G 3') found throughout the genome.[9] Methylation is established and maintained by the DNA methyltransferases (DNMTs), some of which may also catalyze demethylation.[61] CpG dinucleotides are not distributed uniformly throughout the genome but are enriched in gene-encoding DNA and in promoter regions in particular. Approximately 70% of the gene promoters contain sequences of densely clustered CpGs that are referred to as CpG islands. CpG islands characteristically are devoid of cytosine methylation in normal somatic cells; where CpG island methylation does occur (e.g., the inactive X-chromosome), associated gene promoters generally are transcriptionally repressed.

Methylation of the CpGs distributed more sparsely in non-island sequences is much more prevalent in normal cells. Overall loss of DNA methylation was the epigenetic abnormality first described in human cancer.[62] Subsequent investigation showed, conversely, that aberrant hypermethylation of CpG islands was also common in cancer. As is the case in normal cells, aberrant CpG island methylation in cancer cells is typically associated with transcriptional repression of associated gene promoters. Aberrant CpG island hypermethylation, therefore, presents an alternative mechanism of gene silencing in cancer cells of great relevance to tumor suppressor genes.[60,63,64] As has been observed in most human cancers, osteosarcoma exhibits aberrant methylation including foci of hypermethylation and regions of hypomethylation compared to normal bone cells.[65,66]

While functional loss of the Rb and p53 tumor suppressor pathways is central to OS pathogenesis and has been documented extensively, hypermethylation-associated gene silencing of *RB1* and *TP53* specifically has not been widely observed.[9] Nevertheless, DNA methylation-associated dysregulation of pathways that regulate Rb and p53 function has been shown in OS. As noted above, *CDKN2A* encodes the p16INK4a and p14ARF proteins, which block inhibition or degradation of RB and p53, respectively. Analyses of OS samples have documented methylation-associated silencing of both transcripts expressed from this locus.[67] CpG island methylation-associated silencing of *WWOX* has been identified in a variety of cancers.[68,69] Kurek and colleagues noted reduction or absence of WWOX expression in OS, and showed that restoration of WWOX expression in OS cell lines inhibited proliferation, migration, and tumorigenicity.[48] A recent analysis confirmed CpG island methylation in OS tumors exhibiting reduced WWOX expression and found that *WWOX* silencing correlated with an poorer response to chemotherapy and adverse disease-free survival. These investigators found that WWOX regulated apoptosis and further suggested that *WWOX* silencing may facilitate tumor angiogenesis.[70] Deregulation of WNT/β-catenin signaling resulting from promoter hypermethylation of WNT pathway inhibitors has been noted by several investigators. Kansara and colleagues noted epigenetic silencing of WNT Inhibitory Factor 1 (*WNT1*) in OS cells,[71] while hypermethylation-associated silencing of *APCDD1* (APC down-regulated 1) was shown by Han and colleagues to enhance invasion and metastasis of OS cells.[72]

A number of investigators have employed multigene or whole-genome DNA methylation analyses to identify loci exhibiting differential methylation in OS samples compared to controls which then could be tested for potential relevance to OS development and clinical outcomes.[65,66,73–77] Such analyses have shown sets of differentially hypermethylated genes to be significantly enriched for pathways related to neuroactive ligand-receptor signaling, the Peroxisome Proliferator Activated Receptor (PPAR) signaling, and ion transport, while differentially hypomethylated gene groups were associated with metal ion transporter activity or Toll-like receptor signaling.[65] To focus differential methylation analyses specifically on events related to gene expression, a number of groups have integrated DNA methylation profiling with gene expression datasets. Not surprisingly, one such analysis identified *CDK4* as a gene target of hypomethylation associated with increased expression.[78] Finally, Tian and colleagues employed integrated DNA methylation and gene expression analyses in OS and then tested candidate differentially-methylated/differentially-expressed genes for prognostic significance using an

independent clinically annotated OS gene expression dataset. They found that reduced expression of the differentially methylated genes *BHMT2*, *DOCK2*, *DNALI1*, and *RIPK3* correlated with inferior survival.[74]

Comparatively few studies have associated hypomethylation of specific genes to OS tumorigenesis. Lu and colleagues, however, found that hypomethylation of the Iroquois homeobox protein 1 (*IRX1*) gene promoter was associated with overexpression in OS cell lines and primary tumors. *IRX1* overexpression was correlated with migration and invasion *in vitro* and with metastasis in a tumor xenograft model, and *IRX1* promoter hypomethylation was associated with a poorer prognosis.[79] Overall genomic hypomethylation has been associated with genomic instability in many studies, and hypomethylation of repetitive DNA elements throughout the genome has been of particular interest in that regard.[80–84] While instability is a hallmark of the OS genome, analyses to address the role of hypomethylation specifically in OS are lacking.

Histone Modification DNA is packaged with core histone proteins in the chromatin complex. Covalent posttranslational modification by addition or removal of single or multiple acetyl or methyl groups at specific amino acid residues of the tails of core histone proteins determines chromatin-protein interactions and thus specifies functions of the associated DNA including transcription.[34] These histone "marks" are maintained, modified, and recognized by a complex (and expanding) array of modification-specific "writers," "erasers," and interact with "reader" molecules and associated proteins resulting in transcriptional activation or repression (reviewed in Audia and Campbell, 2016).[85] Gain or loss of activity of these epigenetic effector proteins then, is associated with changes in the transcriptional profile of cancer cells. While a comprehensive discussion of histone marks and associated effector proteins in beyond the scope of the present review, correlation of OS phenotypes with specific histone marks and mediators has been documented in recent studies. Zhang and colleagues found that activation of the ERK1/2 signaling pathway by activated Ras reduced acetylation of histone core protein H4 at lysine 12 (H4K12ac) via accelerated degradation of histone acetyltransferase 1 (HAT1), associated with upregulated expression of target genes and increased colony formation and migration in an OS cell line. Piao and colleagues found that overexpression of the histone methyltransferase SUV39H2, which trimethylates histone 3 at lysine 9, could itself be oncogenic. Knockdown of SUV39H2 expression attenuated cell growth and promoted G1 phase cell cycle arrest, while overexpression of SUV39H2 promoted cell growth in vitro.[86]

Histone modification may be especially relevant to the biology of stem cells. A mechanism by which pluripotent differentiation potential is maintained in stem cells is related to the simultaneous presence of "activating" and "repressing" histone marks on chromatin associated with promoters of select genes – a status that has been termed "bivalence."[87] Stem cells are "poised" to express or repress genes marked in this way depending on differentiation signals. Such bivalency has been observed at gene promoters in some cancer cell lines and may facilitate phenotypic plasticity – a characteristic of "stemness."[88] La Noce and colleagues showed recently that treatment with the epigenetic modifier valproic acid, an inhibitor of histone deacetylase or HDAC), and the demethylating agent 5'-azacytidine promoted a CSC phenotype, including increased expression of gene markers of stemness, colony forming efficiency, and

tumorigenesis in OS cells.[89] Stemness phenotypes could also be promoted in OS cells via treatment with recombinant leukemia inhibitory protein (LIF) in a manner dependent upon NOTCH pathway signaling. LIF cellular expression was activated by expression of the histone 3 lysine 27 trimethyl (H3K27me3) demethylase UTX, encoded by the *KDM6A* gene, and stemness phenotypes could be attenuated by via inhibition of UTX or NOTCH in these cells.[90] These studies suggest that a more complete understanding of the epigenetic effectors that support stemness in cancer cells may lead to therapeutic targeting of these proteins

Noncoding RNA DNA encoding mRNA comprises only a small fraction of the genome. Among the protein-noncoding RNA (ncRNA) species transcribed from much of the remainder of the genome, ribosomal RNAs and transfer RNAs have long been recognized. More recently, ncRNA species corresponding to an ever-growing list of additional classes increasingly are recognized as active players in the regulation of cellular function in normal physiology and cellular dysfunction in cancer.[91] Class designations for these RNA species may refer to length (e.g., long, micro); function (small interfering); or cellular localization (e.g., small nuclear).[91] For the present review, discussion of a few of these classes is warranted. Small interfering RNAs (siRNA) and micro-RNAs (miRNA) are 21-24 nucleotides in length and are processed by Dicer proteins from precursor double-stranded molecules, complexed with Argonaute (AGO) class proteins and unwound to single-stranded molecules to form RNA-induced silencing complexes (RISC) which bind target mRNAs based on full or partial complementarity and induce mRNA cleavage and exonuclease degradation or translational inhibition.[92,93] Piwi-interacting RNAs (24-31 nucleotides) interact with a subclass of AGO proteins (piwi-family). The RISC then binds DNA based on piRNA complementarity and effects epigenetic transcriptional inhibition by removing activating histone marks, adding repressive histone marks, and inducing CpG methylation.[94] Long noncoding RNAs (lncRNA) are molecules of 200 or more base pairs. These molecules participate in a diverse array of processes based on their capacity for molecular interaction through base pairing (nucleic acids) and 3-D structure (proteins).[95] Accordingly, lncRNAs can mediate DNA-protein, chromatin-protein, chromatin-chromatin, or protein-protein interaction; they can bind and sequester proteins or RNA molecules, and they can regulate aspects of mRNA function.[95] Finally, circular RNAs (circRNA), as the name implies, form a closed loop structure through back-splicing. These molecules often act as "sponges," sequestering miRNA species or RNA-binding proteins via base complementarity. They also may enhance transcription, or mediate protein-substrate interactions.[96] The application of next generation sequencing technologies to define the noncoding RNA expression profiles of cancers, including OS, compared to normal cells, is a very active focus of research effort at the present time.

Underexpression or loss of regulatory microRNAs promotes OS tumorigenesis by deregulating some of the oncogenic pathways discussed above, including WNT/β-catenin, NOTCH2, and AKT pathways.[97–103] Upregulation or downregulation of miRNA expression could result from gain or loss of copy number,[97] but recent studies suggest that loss, especially, of tumor suppressive miRNA expression often occurs via epigenetic mechanisms. Li and colleagues described CpG island hypermethylation-associated silencing of miR-449c resulting in *MYC* overexpression.[104] Similarly, Chen and colleagues found that CpG island hypermethylation silenced miR-300 in OS

cells thereby deregulating the ubiquitin ligase $CRL4B^{DCAF13}$ E3 Ligase leading to degradation of PTEN.[105] Tumor suppressor miRNAs may also be sequestered, or "sponged" by lncRNAs or circRNAs so that overexpression of these latter RNA species, by gain of copy number, for example, results in functional downregulation of the regulatory miRNA. In this way, high-level expression of the lncRNA HULC sponged miR-122 resulting in deregulated PI3K/AKT activity and lncRNA SNHG12 sponged miR-195-5p, thus deregulating NOTCH2 signaling.[99,106] Finally, it is important to note that noncoding RNAs often target multiple molecules. The targeting "seed" regions of miRNAs and siRNAs share complementarity with multiple mRNAs, and lncRNAs may likewise sponge multiple miRNAs. For example, the lncRNA HOX transcript antisense intergenic RNA (*HOTAIR*!) is overexpressed in OS and other cancers.[107] Studies by Li and colleagues suggested that *HOTAIR* increases DNA methyltransferase 1 (DNMT1) expression by sponging its regulator miR126, thereby facilitating methylation-associated silencing of *CDKN2A*.[107] Other investigators have shown that *HOTAIR* sponges miR-217, an inhibitory regulator of the oncogenic transcription factor ZEB1.[108]

Noncoding RNAs may also mediate protein-protein interactions relevant to oncogenesis. High level expression of lncRNA LIN01116 was associated with inferior survival in a recent analysis.[109] These investigators found that LIN01116 mediated interaction between the histone lysine methyltransferase EZH2 and target genes *TP53* and *PTEN*. Knockdown of LIN01116 resulted in loss of repressive H3K4me2 histone methylation resulting in derepressed *p53* and *PTEN* expression.[109] Exemplifying yet another mechanism of noncoding RNA molecular interaction, the lncRNA *THOR* was shown to support stemness in OS cells by binding and stabilizing the mRNA encoding SOX9, a marker of stemness.[110] The foregoing discussion of epigenetic dysregulation in OS should suggest that the OS epigenome is not markedly less complex than the OS genome and that these complexities are at least additive. Recognizing this complexity, it is perhaps not surprising, as therapy of OS is next addressed, that management of OS can present such a formidable clinical challenge, as this multilayer complexity likely facilitates redundancy of oncogenic pathways, as has been discussed, and tumor survival and cellular escape pathways.

Clinical Presentation, Diagnosis, and Therapy of OS

Most OS patients present with complaint of pain (90%) and many with swelling or a palpable bony mass.[7] A delay of months from the onset of symptoms to the time of diagnosis is common, and this may be attributable to the rarity of the disease and the reassurance of initially normal findings on radiographs.[111] While history of or concern for a trauma event (e.g., running injury in a cross country athlete) may prompt medical evaluation, a pathologic fracture is noted on radiographic evaluation only in about 10% of patients.[2,7,112] While OS can occur in any bone, it arises most commonly the metaphysis of long bones, most frequently the distal femur, proximal tibia, and proximal humerus.[2] This localization may reflect conditions favorable for OS oncogenesis in regions of and during periods of accelerated bone growth.[7]

Radiographically evident metastatic disease, usually defined as three or more lesions <5mm in maximal dimension or one lesion of 1cm or greater, is present at diagnosis in approximately 20% of patients, the great majority of whom have pulmonary

metastases.[113–115] Distant bone metastasis may be seen, and likely occurs via hematogenous dissemination. Noncontiguous areas of tumor in the bone of the primary tumor or across a joint from the primary tumor, has been termed "skip" metastasis.[113] While hematogenous metastasis and regional "skip" lesions likely occur through distinct processes, both are associated with a poor prognosis.[116]

On x-ray, conventional OS often exhibit aggressive radiographic features with bony destruction, and a "sunburst" or "hair on end" periosteal reaction (Figure 2A). Tumors are described as having an ill-defined, mixed lytic-sclerotic radiographic appearance, and often a soft tissue component is evident.[2] Magnetic resonance imaging (MRI), usually of the entire bone and the adjacent joint, is typically obtained to evaluate the extent of bone marrow invasion, identify skip lesions, assess joint involvement, and identify potential compromise of surrounding structures. On MRI, tumors appear T1 hypointense, hyperintense on T2, and exhibit avid enhancement with the contrast.[7] (Figure 2B) Technetium[99] bone scintigraphy can also identify distant bony metastases, and a CT of the chest is necessary to evaluate for pulmonary metastases.

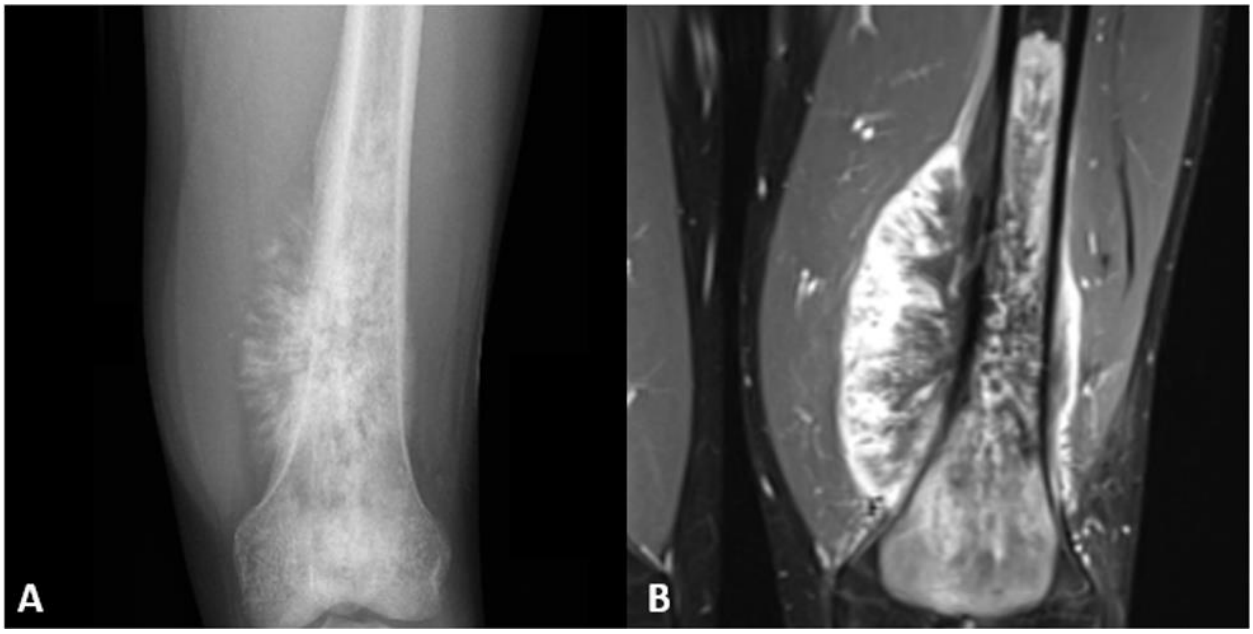

Figure 2. (**A**) X-ray an osteosarcoma of the distal femur shows the classic "sunburst" appearance and "Codman Triangle" or periosteal lifting. (**B**) Magnetic resonance imaging shows notable extension beyond the femoral cortex and avid contrast enhancement.

Diagnostic biopsy rather than definitive resection at presentation has been the norm over the past decades, and the pathology of OS has been discussed above. Two widely used surgical staging systems include the Enneking system and the staging developed by the American Joint Commission on Cancer (AJCC). While both systems take into account the histologic grade and the status of metastases, the Enneking specifically accounts presence or absence of an extra-compartmental component.[2,115]

Prior to the introduction of adjuvant chemotherapy, OS was treated with surgical resection/amputation and/or local

radiotherapy, and because most patients have microscopic distant metastases at the time of presentation, death due to progressive metastatic disease was the norm.[117] Chemotherapy trials of regimens including doxorubicin (DOXO) and methotrexate (MTX) in the 1970s showed preliminary promise.[117,118] Cisplatin (CDDP) was added to regimens in the 1980s,[119] and the regimen of CDDP/DOXO/MTX (MAP) remains the most widely-used regimen. Rosen and colleagues pioneered the administration of neoadjuvant chemotherapy, or chemotherapy given prior to definitive surgical resection. This approach permitted the assessment of and histologic response to chemotherapy, as determined by extent of tumor necrosis, and this response has become an important predictor of treatment outcomes.[120]

With current surgical approaches, approximately 90% of OS patients may be treated with limb-salvage surgery without compromise of therapeutic efficacy.[121–123] Although it has been suggested that narrower margins may be acceptable in cases of chemosensitive OS, resection with negative surgical margins remains the goal, not least because the chemotherapy response may not be assessable until the resection specimen is examined histologically.[124,125] The importance of surgical resection is underscored by results of an analysis by Isakoff and colleagues who retrospectively reviewed data from patients treated for OS on 4 cooperative group clinical trials from 1993 – 2005. Of 1054 patients, 26 (2.5%) had primary tumors localized to the pelvis. Five-year estimates of event-free (EFS) and overall survival (OS) for this group of patients were 23% and 38%, respectively, while EFS and OS estimates were 57% and 69%, respectively, for patients with non-pelvic tumors. Moreover, survival for patients with the pelvic tumors was poor whether they presented with metastatic disease or not.[126] Notably, of 5 evaluable patients who were able to undergo complete resection, 3 were alive at the time of last contact. This favorable result for patients with resectable axial tumors was confirmed in the recent EURAMOS-1 trial. While EFS was inferior among all patients with axial tumors as well as tumors of the proximal humerus or proximal femur compared to extremity, EFS was not found to be significantly different between patients with axial tumors that were completely resected and patients with tumors of the non-proximal humerus/proximal femur extremities.[114] Thus, the poor prognosis among patients with localized tumors of the pelvis and other axial bones likely reflects adequacy of local control rather than drug sensitivity of the tumor.

The current, widely-utilized chemotherapeutic regimen for treatment of localized OD includes MAP given as courses of high-dose MTX (HD MTX) and courses of DOXO/CDDP for about 10 weeks as neoadjuvant therapy followed by post-resection MAP for an additional 29 weeks.[116] While intraarterial infusion of CDDP was theorized to maximize drug delivery to tumor and improve necrosis, this mode of administration did not result in improvement in histologic or clinical responses, and given the increased complexity, is not widely utilized in children (Reviewed in Bielack et al 1993).[127] The utilization of dexrazoxane for prevention of DOXO-associated cardiac toxicity and leucovorin to mitigate the toxicity of HD MTX have permitted maximization of therapeutic dosing for these agents.[128,129] Thus dose intensity for these 3 chemotherapy agents is likely at the limit for maximal therapeutic efficacy with acceptable treatment-related and late term toxicity.[2]

Recent large cooperative group clinical trials testing this neoadjuvant (chemo – resection - postsurgical chemo) MAP regimen and modifications in children,

adolescents, and young adults include the INT0133 trial (1993 – 1997) and the EUROAM-1 trial (2005 – 2011). The former trial enrolled 662 OS patients with no clinically detectable metastatic disease and in whom complete surgical resection was deemed feasible. All patients received MAP and were then randomized to receive ifosfamide and/or the immune response modifier muramyl tripeptide phosphatidyl-ethanolamine (MTP-PE) in a 2X2 factorial design. Event-free survival at 6 years was 64% for the group overall and did not vary significantly by treatment group. An overall survival advantage, however, was observed among patients who received MTP-PE compared to those who received chemotherapy alone (78% versus 70%).[130] The EURAMOS-1 trial reported on outcomes of more than 2000 patients with localized or metastatic OS. Eligibility, for this trial also was restricted to patients with disease that was deemed surgically resectable. Event-free and overall survival at 5 years was 54% and 71%, respectively, for the entire group. Inferior EFS was associated with the presence of metastatic disease, axial primary tumors, older age, and a poor histologic response to neoadjuvant therapy (defined as <90% tumor necrosis).[114] Patients with a poor histologic response were randomized to receive post-resection MAP plus ifosfamide and etoposide (MAPIE) or MAP. Unfortunately, no benefit to incorporation of IE was observed.[131] Importantly, of EURAMOS-1 patients who presented with no clinically evident metastatic disease and in whom complete surgical remission was achieved, 48% exhibited poor histologic response to neoadjuvant chemotherapy.[114] Considering, then the poor outcomes observed among patients with metastatic disease at presentation (17% on EURAMOS-1) and patients with poor histologic response to neoadjuvant therapy, fewer than one half of OS patients have an optimal prognosis for outcome of treatment with the only regimen in common use. For the majority of patients, efficacy of treatment is compromised by chemoresistance. An understanding of the mechanisms by which OS cells acquire chemoresistance (Figure 3) will be necessary, then, if EFS above the 50%-60% range, the outcome of clinical trials since the 1980s, is to be achieved.

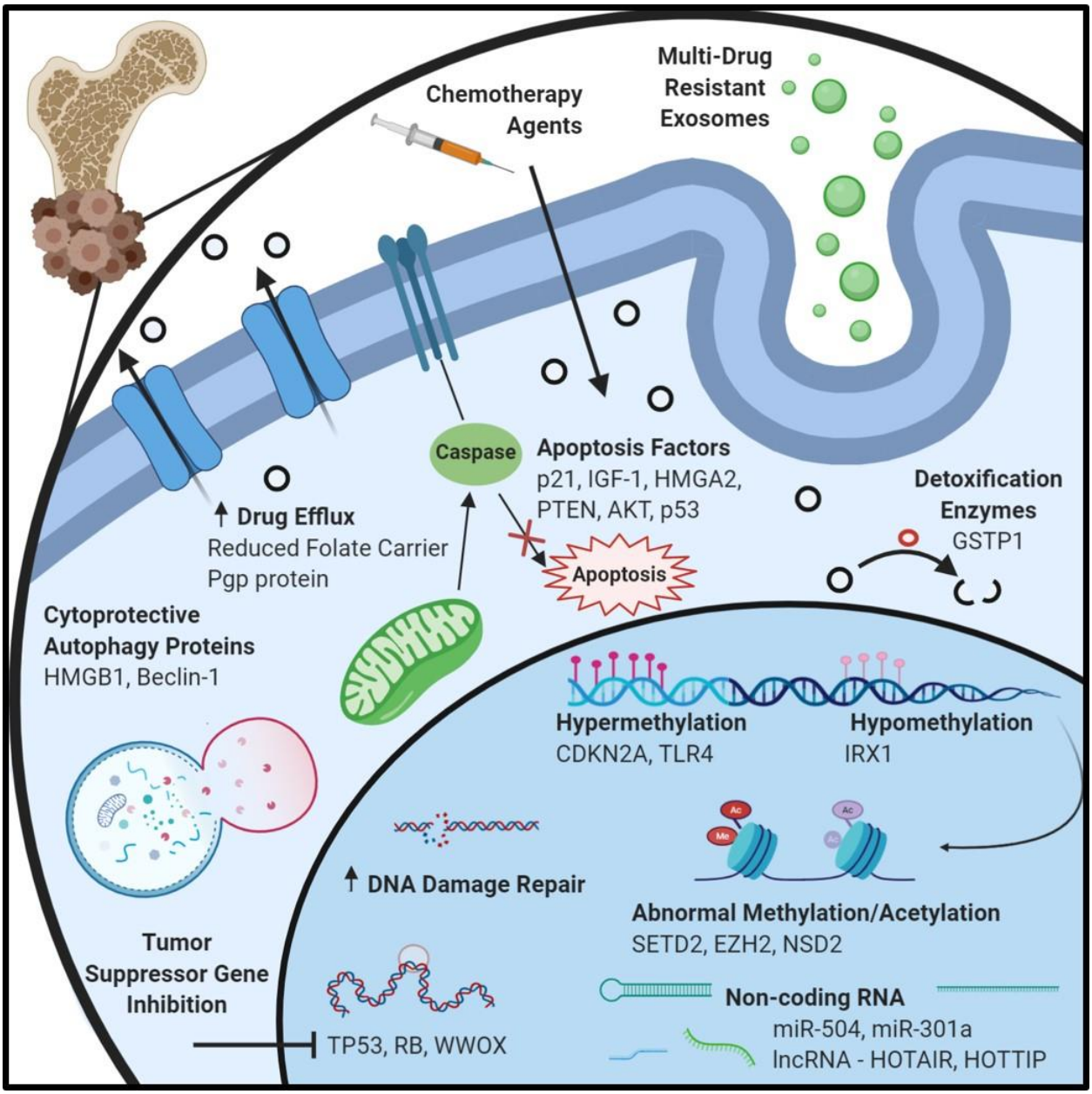

Figure. 3 – Mechanisms of chemoresistance in osteosarcoma. Key molecular processes, mediators, and targets are depicted. (Created with BioRender.com)

Mechanisms of Chemoresistance in OS

The BME, hypoxia, and stem cells As is the case with OS phenotypes generally, chemoresistance develops in the context of the BME. Han and colleagues found that tumor expression of the protein tissue inhibitor of metalloproteinase 3 (TIMP3), which blocks metalloproteinase-mediated degradation of the extracellular matrix, correlated with CDDP sensitivity in OS patients. They showed that IL-6 inhibited TIMP3 expression via STAT3 pathway signaling and promoted CDDP resistance.[132] The chromatin protein high mobility group box 1 (HMGB1) has immunomodulatory properties when secreted by hematopoietic cells in the BME and, as a chemotactic

molecule for osteoblasts and osteoclasts, participates in bone remodeling.[133] Huang and colleagues observed that upregulation of HMGB1 in OS cells induced resistance to DOXO, CDDP, or MTX. HMGB1 expression mediated a cytoprotective autophagic response (discussed below) that was reversible with HMGB1 knockdown.[134] Dysregulation of WNT and NOTCH pathway signaling may also play a role in chemoresistance. Ma et al. found that knockdown of β-catenin expression of the canonical WNT pathway sensitized Saos2 OS cells to MTX cytotoxicity and that combined inhibition of WNT/β-catenin and NOTCH pathways resulted in synergistic cytotoxicity.[135]

The relative hypoxia of the BME may condition chemoresistance in OS.[15] Increased expression of the alpha subunit of the transcription factor hypoxia inducible factor-1 (HIF1α), upregulated in response to hypoxia, may confer a poor prognosis in OS.[136] HIF1 upregulates expression of the multidrug resistance transporter, ABCB1 (discussed below)[137], and Roncuzzi and colleagues identified ABCB1 upregulation in OS cell lines that were selected for DOXO resistance.[138] Li and colleagues noted hypoxia-induced upregulation of MRP1 expression associated with HIF1α expression and activated NOTCH1 signaling.[139] Like ABCB1, MRP1 is a membrane transport protein, encoded by an ATP binding cassette subfamily gene (*ABCC1*), and has been implicated in multidrug resistance in cancer (reviewed in Lu et al, 2015).[140] Hypoxia/ HIF1α-dependent upregulation of Mxd1, a MYC family protein, was also described recently. Mxd1 expression was shown to suppress transcription of *PTEN*, thereby mediating CDDP resistance through the PI3/AKT pathway.[141] Ma and associates identified HIF1α-associated downregulation of the spindle and kinetochore complex 1 gene *SKA1* in OS cells cultured in hypoxic conditions. They found further that *SKA1* overexpression was associated with downregulation of a panel of chemoresistance effectors *in vitro*, including the multidrug transporters ABCB1 and ABCB2 as well as glutathione S-transferase P1 (GSTP1, discussed below). Enforced SKA1 expression conferred sensitization to ifosfamide and epirubicin cytotoxicity in this model.[142] Finally, hypoxia may also promote chemoresistance through mechanisms independent of HIFα. Adamski and colleagues described a hypoxia-induced pathway conferring resistance to CDDP, DOXO, and etoposide in OS cell lines. The pathway, which attenuated drug-associated TP53 activation, was not inhibitable via knockdown of HIF1α or by inhibition of PI3/AKT signaling.[143]

Hypoxia-associated factors and the milieu of the BME may uniquely condition CSCs for evolution of chemoresistance and other cancer phenotypes. Hypoxia associated activation of NOTCH1 signaling, as noted above, may facilitate both preservation of "stemness" and activation of drug efflux mechanisms. Kolenda and colleagues observed upregulation both of stem cell markers and proteins related to drug resistance in glioblastoma cell spheroids cultured under hypoxic conditions.[144] In a murine breast cancer model, Lock and coworkers demonstrated that *in vitro* inhibition of the hypoxia-response metalloenzyme carbonic anhydrase IX (CAIX) downregulated mammalian target of rapamycin (mTOR) signaling and impaired expansion of breast cancer stem cells under hypoxic conditions. CAIX inhibition in tumors *in vivo* resulted in enhanced cytotoxic response to paclitaxel.[145,146] Easwaran has suggested that the organization of the CSC epigenome in the tumor microenvironment, characterized by bivalence of promoter histone marks as discussed above, yields a highly poised configuration in which

expression or repression of multiple genes may be activated resulting in phenotypic plasticity that can confer selective advantage and facilitate tumor survival and evolution under diverse conditions.[147]

Oncogenes, tumor suppressor genes, and epigenetic dysregulation in OS chemoresistance MYC overexpression may confer MTX resistance in OS cells. Scionti and colleagues identified MYC overexpression in MTX-resistant OS cell lines compared to OS-sensitive congeners and demonstrated, further, that knockdown of *MYC* expression in resistant cell lines restored MTX sensitivity.[148] Downregulation of tumor suppressor pathways or other loss of gene function events may likewise influence tumor sensitivity in OS. Because wild type p53 mediates cell cycle arrest in response to DNA damage,[149] it is reasonable to suggest that tumor *TP53* status may be a determinant of chemotherapy response. In actuality, the utility of ascertaining tumor *TP53* status for predicting chemotherapy response has been variable in cancer.[150] Nevertheless, a few analyses have identified disruption of the p53 pathway in chemoresistant OS. A potential role for p53 in mediating DOXO resistance was demonstrated by Sun and colleagues, who showed that restoration of p53 in *TP53*-null MG-63 activated TGF-β pathway signaling leading to apoptosis following DOXO exposure.[151] Proof of principle was provided by Chen associates, albeit with respect to CDDP response. They observed overexpression of miR-504 in OS tumors compared to normal controls and found that miR-504 directly targeted *TP53* for downregulation, thereby suppressing CDDP-induced apoptosis in OS cells.[152] Yuan and colleagues assessed the role of p14ARF, which inhibits MDM2-mediated p53 degradation, in CDDP-induced cytotoxicity in OS cells. They found that p14ARF expression sensitized cells to CDDP-induced apoptotic cell death, although, interestingly, this effect appeared to be p53-independent.[153]

Upregulation of WWOX expression following MTX exposure has been shown to suppress the autophagy cellular catabolic response through the mTOR signaling pathway in OS cell lines.[47] Loss of *WWOX* function, whether by deletion or promoter hypermethylation may, therefore, compromise MTX sensitivity in OS, but this has not yet been demonstrated. Relatively little is known about promoter hypermethylation as it pertains specifically to chemoresistance, but correlation of methylation events with OS prognosis has been demonstrated in a number of studies. Rosenblum et al. undertook genome-wide DNA methylation profiling in diagnostic samples of OS and found increased methylation at more loci in samples obtained from patients who ultimately relapsed compared to samples from patients who did not relapse. They found, moreover, a strong associated between and 5-year event-free survival and promoter methylation at the *TLR4* locus, which encodes toll-like receptor 4.[154] Conversely, promoter methylation-associated silencing of the methylguanine methyltransferase gene (*MGMT*) was correlated with higher post-chemotherapy tumor necrosis in an analysis by Cui and coworkers.[155] MGMT mediates excision repair removal of O6-guanine in response to alkylating agent-induced DNA damage, and methylation-associated downregulation of MGMT activity is prognostically significant in glioblastoma and other cancers.[156] Whether *MGMT* methylation was prognostically significant in OS, however, was not determined in the Cui study. Tian and colleagues employed integrated DNA methylation and gene expression analyses in OS and then tested candidate differentially-methylated/differentially-expressed genes for prognostic significance using an independent clinically annotated OS gene

expression dataset. They found that reduced expression of the differentially methylated genes *BHMT2*, *DOCK2*, *DNALI1*, and *RIPK3* was correlated with inferior survival.[73,74] Whether these genes products mediate chemoresistance is not clear. Finally, Sonaglio and colleagues employed a panel of 18 genes to identify prognostically significant methylation markers in OS. They found that an association of estrogen receptor 1 (*ESR1*) CpG island hypermethylation with poor overall survival approached significance.[157] In support of this observation, Osuna and coworkers recently demonstrated that loss of ESR1 expression conferred a more aggressive phenotype in OS cells.[158]

In studying histone methylation in relation to cisplatin sensitivity in OS, He and colleagues found that histone demethylases KDM6A and KDM6B were upregulated in OS following CDDP treatment and that CDDP-sensitive tumors exhibited higher levels of the repressive H3K27me3 histone mark compared to CDDP-resistant tumors. They showed, furthermore, that knockdown of KDM6A or KDN6B expression conferred sensitivity to CDDP cytotoxicity while inhibition of the histone methyltransferase EZH2 rendered OS cells resistant to CDDP and upregulated expression of CSC markers.[159] Interestingly, Zhu and colleagues noted reduced expression of miR-138 in OS tumors and found that expression of this microRNA in OS cells attenuated neoplastic phenotypes and enhanced CDDP sensitivity. They found furthermore that miR-138 targeted EZH2 and that enforced EZH2 could reverse miR-138-mediated CDDP sensitivity.[160] Whether this apparent contradiction of the findings of He, et al. is related, for example, to gene modulatory effects of miR-138 independent of *EZH2* remains unresolved. Reduced expression of another histone lysine methyltransferase, SETD2, has been noted in OS tumors. SETD2 downregulates WNT/β-catenin pathway signaling through H3K36 trimethylation. Thus, overexpression of SETD2 in OS cells inhibited growth and increased cisplatin-induced apoptosis associated with repression of WNT/β-catenin pathway signaling.[161] Conversely, the histone methyltransferase NSD2, which imparts HeK36 dimethylation (H3K36me2), was shown to be upregulated in CDDP-resistant OS tumors. He and colleagues showed that *NSD2* knockdown inhibited OS cell tumor formation *in vivo* and enhanced CDDP sensitivity.[162] Thus histone modifiers can either promote or suppress chemosensitivity depending on addition or removal of specific histone marks. Although a fully consistent picture has yet to emerge, overexpressed proteins such as KDM6A, KDM6B, or NSD2 that are associated with chemoresistance may represent therapeutic targets in OS.

The list of noncoding RNAs implicated in the evolution of chemoresistance in OS is now substantial and growing rapidly. Increased expression of lncRNA *ODRUL* (OS Doxo-resistance related upregulated lncRNA) was identified in specimens of OS patients with poor chemotherapy response and in DOXO-resistant cell lines. Knockdown of *ODRUL* attenuated neoplastic phenotypes (proliferation and migration) and enhanced DOXO sensitivity associated with downregulation of *ABCB1* (MDR1) expression.[98] The relevance of WNT expression to chemoresistance in OS has been noted. Overexpression of the lncRNA *HOTTIP* has been shown to upregulate WNT/β-catenin pathway signaling associated with increased CDDP resistance in OS cells, which was reversible with WNT/β-catenin pathway inhibition.[163] MicroRNAs, whether overexpressed or underexpressed, may mediate chemoresistance through multiple pathways, and indeed miRNA profiling and correlation of expression with

chemosensitivity or chemoresistance of tumors provides a powerful tool for identification of clinically relevant chemoresistance pathways. Thus, miRNA-301a was found to be upregulated in OS specimens from patients with poor histologic response. Expression of miRNA-301a reduced DOXO-associated apoptosis in OS, while knockdown rendered cells DOXO-sensitive, phenotypes likely mediated by direct miRNA-301a targeting of AMP-activated protein kinase alpha1 (*AMPKα1*) expression.[164] A comprehensive discussion of noncoding RNAs potentially mediating drug resistance in OS is beyond the scope of the present review. The interested reader is referred to several excellent reviews specific to this topic.[165–167]

Chemotherapy intracellular efficacy Activity of transport molecules is critical to chemotherapy intracellular delivery and so may determine chemosensitivity or resistance. The reduced folate carrier (RFC) transports MTX from the extracellular to the intracellular environment. Reduced RFC activity resulting from genetic polymorphism or promoter methylation has been implicated in MTX resistance and poor chemotherapy response in a several reports.[168–170] The P glycoprotein transporter (P-GP, MDR1) encoded by the gene *ABCB1* mediates multidrug resistance in multiple tumor types (Reviewed in Robey et al and references therein).[171] P-GP upregulation is associated with chemoresistance and poor chemotherapy response in osteosarcoma.[172–174] The role of hypoxia, HIFα expression and activated NOTCH pathway signaling in upregulation of the MRP1 transporter (*ABCC1*) was noted above. Activities of these and other molecular transporters may result in efflux of chemotherapeutic drugs from the OS cell and so facilitate cell survival. Importantly, overexpression of multiple transporter molecules may be a property of OS CSCs. Sun and colleagues identified a population of cells from OS samples expressing CSC markers that exhibited overexpression of multiple members of the ATP binding cassette family of molecular transporters including ABCB1, ABCB2, ABCA1, and ABCG2. These cells were shown to be resistant to DOXO, CDDP, and MTX.[175] Metabolic chemotherapy detoxification or rescue may likewise mediate chemoresistance in OS. Expression of the detoxifying enzyme glutathione S-transferase P1 has been implicated in resistance to,[176–178] and Guo and coworkers noted increased dihydrofolate reductase expression in OS, especially in metastatic or relapsed tumor specimens.[179] Torregiani et al. reported transfer of a multidrug resistance phenotype between human OS cells. They demonstrated the intracellular transfer of MDR1 mRNA via exosomes to chemosensitive OS cells and the subsequent acquisition of resistance to doxorubicin.[180] Better understanding of the prevalence of this "one bad apple" mechanism of chemoresistance in OS is necessary.

Cell death or survival The role of the programmed cell death in chemotherapy-induced cytotoxicity is now universally recognized, and apoptosis regulatory molecules and pathways relevant to OS discussed above include p53, p21, IGF-1, HMGA2, PTEN, and AKT.[181,182] Necroptosis is a distinct cell death pathway, initially noted to be triggered by tumor necrosis factor (TNF) binding, and characterized, as the name implies, by morphologic evidence of necrosis such as cell swelling.[183] While investigation in other cancer types including hepatocellular carcinoma, breast carcinoma, glioblastoma and melanoma has suggested a role for necroptosis mediators and effectors, especially receptor interacting protein kinase 3 (RIPK3), in cancer chemosensitivity,[184] investigation of this pathway relative to OS therapy is preliminary.[181] Autophagy is a catabolic process regulated by the mTOR and

the AMP-activated protein kinase (AMPK) by which cells create energy through elimination and recycling of endogenous proteins and organelles. In reference to chemoresistance, there is a focus on the subcategory macroautophagy or the degradation of cytoplasmic material by direct engulfment by lysosomes.[185] The role of autophagy with regard to chemotherapy efficacy is binary. The pathway may mediate chemoresistance (cytoprotection) by mitigating chemotherapy-associated cell stress. Kim and coworkers noted CDDP chemoresistance associated with upregulation of glial derived neurotropic factor family receptor alpha (GFRA1) in OS cells. GFRA1 induced AMPK-dependent autophagy[186]. Likewise, knockdown of the autophagy mediator Beclin-1 conferred CDDP sensitivity in OS cells.[187,188] The upregulation of cytoprotective autophagy by HMGB1 expression, conferring OS cell resistance to DOXO, CDDP, and MTX was discussed above.[134] Alternatively, excessive autophagy can trigger cell death.[185] Thus, the ongoing dissection of these pathways to identify trigger points for cell survival versus cell death will lead to the identification of therapeutic targets. mTOR, for which multiple inhibitors with well-defined clinical profiles exist, may represent such a molecule.[189,190]

A way forward As MAP dose intensity has likely been maximized and additive or alternative "traditional" cytotoxic chemotherapeutics have not yielded clinic improvement to date, inhibitory agents targeted to tyrosine kinase signaling pathways, growth pathways such as insulin-like 1 growth factor receptor (IGF-1R), and mTOR have been or are being tested.[2,191,192] There is convincing preclinical evidence rationale for testing differentiation therapies including retinoic acid receptor α (RARα) and peroxisome proliferation-activated receptor γ (PPARγ) agonists in OS.[193] The survival benefit for patients of treatment with L-MTP-PE points to a potential role for immunomodulatory therapy, which perhaps could be augmented with epigenetic modulator therapy to reverse TLR4 silencing.[194] The properties of the OS immune cell infiltrate and the OS microenvironment have been characterized and numerous trials of immunotherapeutic agents including checkpoint inhibitors are underway.[195] Likewise, preliminary studies of CAR-T approaches show some promise in OS.[196] Further characterization of OS CSCs will identify targetable determinants of stemness. Finally, the use of existing and new demethylating agents, inhibitors of histone deacetylase, and targeted RNAs is underway. Such studies will need to be guided by analyses establishing efficacy of specific modulators for specific targets in order to achieve maximally beneficial epigenetic modulation in OS. Similarly, while a move away from one-MAP-fits-all therapy is not imminent, characterization of tumors for relevance of specific genetic and epigenetic targets will be necessary to maximize efficacy of these novel therapies.

Because chemoresistance compromises efficacy of therapy for approximately one half of patients treated for osteosarcoma, modification of the present chemotherapy approach is necessary and inevitable. Over the past 30 years, while survival rates for OS therapy have remained static, modest but meaningful improvement in outcome has been achieved for children with high-risk neuroblastoma.[197] This progress reflects, in large part, the application of intensive consolidation with high-dose chemotherapy and autologous stem cell transplantation but also the development of effective non-chemotherapeutic modalities utilizing differentiation agents and immunotherapy. Work to develop such modalities applicable to OS is underway, as discussed above. Nevertheless, given the

genetic and epigenetic complexity characteristic of OS, it is unlikely that a one-size-fits all "MAP plus X plus Y" approach will prove to be optimally efficacious. As the evolving mechanistic understanding of chemoresistance (and resistance to other therapeutic modalities) matures, however, implementation of real-time molecular diagnostics will permit optimal tailoring of treatment with chemotherapy, stem cell-directed therapy, differentiation and immunotherapies, and epigenetic therapies to reverse or circumvent resistance. Treatment outcome statistics may then become untracked and reflect benefit to these high-risk patients.

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