

Review Article

Role of the WWOX tumor suppressor gene in bone homeostasis and the pathogenesis of osteosarcoma

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Abstract: Osteosarcoma is the most common primary bone malignancy in children with unknown etiology and often with poor clinical outcome. In recent years, a critical role has emerged for the WW domain-containing oxidoreductase (WWOX) in osteosarcoma and bone biology. WWOX is a tumor suppressor that is deleted or attenuated in most human tumors. *Wwox*-deficient mice develop osteosarcoma and a bone metabolic disease characterized by hypocalcemia and osteopenia. Studies of human osteosarcomas have revealed that the WWOX gene is deleted in 30% of cases and WWOX protein is absent or reduced in ~60% of tumors. Further, WWOX levels are attenuated in the majority of osteosarcoma cells, in which ectopic expression is associated with reduced proliferation, migration, invasion and tumorigenicity. At the molecular level, WWOX associates with RUNX2 and suppresses its transcriptional activity in osteoblasts and in cancer cells. This review provides new insights on the current knowledge of the spectrum of WWOX activities and future directions for the role of WWOX in bone biology and osteosarcoma.

Keywords: Tumor suppressor, WWOX, osteosarcoma, WW domain, RUNX2, osteoblast

Introduction

Osteosarcoma is the most common primary malignant bone tumor in childhood and adolescence [1, 2]. This rare, highly aggressive tumor usually involves long bones and frequently metastasizes to the lungs, carrying a significant risk of mortality. Little is known of the etiology of human osteosarcoma and lesser still of the various interactions that occur between host and tumor cells that govern growth and progression of osteosarcoma *in vivo*. Tumors are believed to originate from primitive bone-forming mesenchymal cells, and although several predisposing environmental (e.g., ionizing radiation) and genetic (e.g., *TP53*, *RB*) factors have been identified, for most the etiology is unknown [3]. After tumor staging, the most important predictor of disease-free survival in osteosarcoma is responsiveness to neoadjuvant chemotherapy. Ninety percent or greater tumor necrosis at resection (a good response) is associated with a favorable prognosis. Currently, there are no molecular

markers to further stratify the poor responding patients (0-89% necrosis) for overall prognosis and for additional therapy [4-6]. Despite initial major discoveries in the chemotherapeutic regimens for osteosarcoma treatment more than 25 years ago, the survival rate has plateaued as no new therapies have been introduced for decades [7].

Osteosarcoma research has been hindered by the extensive genetic heterogeneity of tumors. Tumors contain multiple random chromosomal aberrations and only a few deletions and amplifications appear common to comparative genomic hybridization (CGH) studies [8, 9]. Nevertheless, several lines of evidence suggest that osteosarcoma may originate from a small number of basic genetic alterations. For example, two more frequently occurring deletions involve the *TP53* and *RB* tumor suppressor genes. Recently it has been demonstrated that mice with osteoblast-restricted loss of p53 and Rb and/or PRKAR1A uniformly develop osteosarcoma [10,

11]. Molecular analyses of osteosarcoma have also revealed alterations in TGF- β /BMP, Wnt/ β -catenin, RUNX2 and cell cycle related pathways [12-16]. Molecular characterization of key genes controlling osteosarcoma metastases have implicated FAS [17], EZRIN [18], and more recently NOTCH and HES1 [19]. Since controlling metastasis is the key to improving survival in osteosarcoma, a better understanding of how these genes regulate metastasis may identify new therapeutic targets for osteosarcoma patients. Despite these advances, a true molecular classification of osteosarcoma with markers predictive of tumor progression, metastasis, and response to chemotherapy is lacking. This has prevented the development of biologically tailored therapies to minimize toxicity in low-risk patients and maximize potential for cure in high-risk patients with metastatic disease.

In our lab, we have found that targeted ablation of the *Wwox* gene in mice is associated with osteosarcoma development [20]. Subsequent studies in human osteosarcomas revealed frequent alterations of the WWOX gene by genome-wide array CGH analysis [21], of WWOX mRNA by real-time PCR [22], and of WWOX protein by immunohistochemical staining [21, 23]. Further, manipulation of WWOX in osteosarcoma cells attenuates tumor cell growth and invasiveness [23]. At the molecular level, WWOX, via its first WW domain, associates with RUNX2, the master regulator of osteoblast differentiation, suppressing its transactivation function and inversely altering its expression level [23, 24].

WWOX structure-function relationship

The WWOX gene, also known as *FOR* and *WOX1*, spans a genomic locus of >1 megabase that encompasses nine exons and encodes an open reading frame of 1245 bases [25-27]. The protein sequence includes two WW domains and a short chain dehydrogenase/reductase (SDR) domain (reviewed in [28]). The gene spans the common fragile site *FRA16D*, a region involved in loss of heterozygosity (LOH) and homozygous deletions (HDs) in many cancers and cancer-derived cell lines, and containing four chromosomal translocation breakpoints in multiple myeloma [28-30]. Numerous studies have correlated loss of WWOX expression with cancer development, including some associating WWOX alteration with poor prognosis and outcome in various cancer types (reviewed in

[31]), suggesting a growth advantage for tumors with loss of WWOX. Ectopically over-expressed WWOX has been reported to promote apoptosis, suppress anchorage-independent growth and colony formation, and diminish tumor growth in immunocompromised mice, suggesting a tumor suppressor function [31-33].

The mouse ortholog, *Wwox*, at murine chromosome 8E1 is highly homologous to the human locus and shares its fragility [34]. WWOX is expressed in most organs, but is highest in hormonally regulated cells such as those of breast, ovary, testes and prostate [20, 35]. Importantly, WWOX is also expressed in chondrocytes and osteoblasts of limbs, calvarium and vertebral bone [24]. Characterization of mouse strains with targeted knockout of the *Wwox* gene has led to important clues of the roles of WWOX in metabolism and tumorigenesis [reviewed in [28]]. *Wwox*-deficient (*Wwox*^{-/-}) pups are smaller than control littermates and all *Wwox*^{-/-} mice die by 4 weeks after birth with severe metabolic defects [24, 36]. Macroscopic and histological examination of the organs confirmed atrophy of organs in *Wwox*^{-/-} mice, gonadal abnormalities and bone growth retardation [24, 36]. Importantly, analysis of tumor growth in *Wwox*^{-/-} mice revealed *bona fide* tumor suppressor function of WWOX [20].

Via its WW domains, WWOX binds proline-rich ligand PPxY containing proteins (reviewed in [37, 38]). Among these proteins are RUNX2 [24], ERBB4 [39], c-JUN [40], and EZRIN [41]. These specific interactions have been shown to regulate transcription, apoptosis and cytoskeleton organization [28, 37]. Other WWOX binding partners have been suggested to interact independently of the WW domain and PPxY motifs, and include p53 [42], JNK1 [43], TAU [44], MDM2 [45] and TGF β 1 [46]. Moreover, WWOX can regulate gene function by competing with other WW domain-containing proteins, such as co-activators and ubiquitin ligases, for binding with targets, thus affecting their transactivation and degradation rate [38, 39]. The nature of the various interacting partners with which WWOX can physically associate suggests that WWOX plays a central role in various signal transduction pathways.

Significance of WWOX in bone biology

Mouse studies demonstrate conclusively that

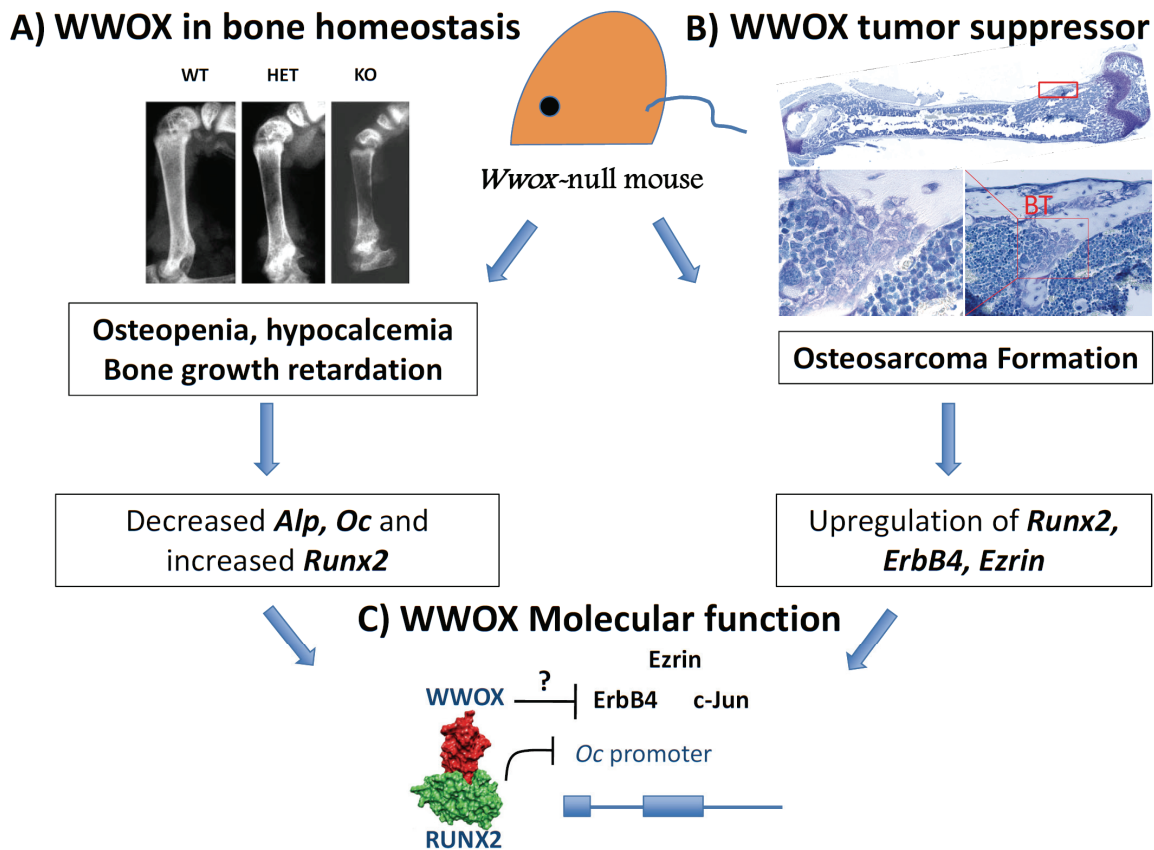


Figure 1. Bone phenotypes in *Wwox*-null mice. Targeted ablation of *Wwox* in the mouse is associated with changes in bone homeostasis and development of osteosarcoma. **A)** Upper panel with radiographs of femoral bones of 3-week old mice showing reduced mineralization and bone density in *Wwox*-null mice (KO) mice as compared with wild type (WT) and heterozygous (HET) mice. This osteopenic phenotype seems to be a result of increased activity of osteoclasts, perhaps secondary to hypocalcemia, and reduced expression of osteogenic markers such as osteocalcin (Oc) and alkaline phosphatase (Alp). **B)** Upper panel shows Toluidine blue stained paraffin embedded section of a *Wwox*-KO mouse femur (14 days old) obtained after micro-CT scanning. Lower panels show tumor tissue and cells (BT, bone tumor) found on the lateral side of the limb. Left panel shows further enlarged section of the tumor section showing malignant proliferating cells. Real-time PCR analysis of RNA isolated from these femurs revealed upregulation of *Runx2*, *ErbB4* and *Ezrin* levels. **C)** At the molecular level, WWOX associates with RUNX2 and represses its transactivation function on the *osteocalcin* promoter. Whether WWOX also suppresses expression of other interacting partners associated with osteosarcoma development is yet to be determined.

WWOX is necessary for normal bone development. Radiographic analyses in our *Wwox*^{-/-} mice reveal decreased bone density as compared with wild type littermates (**Figure 1**) [24]. Further histologic sectioning and three-dimensional microcomputed tomography (μCT) analyses confirm an osteopenic phenotype in *Wwox*^{-/-} mice characterized by thinner cortical bone and decreased bone volume and mineral density. This phenotype has been independently validated in another *Wwox*^{-/-} mouse model generated by the Aldaz group [36] and in *Ide/Ide* dwarfed rats [47]. Together, these results con-

firm reduced bone formation in *Wwox*-deficient animals as compared to control littermates. *Wwox*^{-/-} mice also exhibit increased bone resorption (osteoclast activity) as assessed by tartrate-resistant acid phosphatase (TRAP) histochemical staining [24]. Although WWOX is expressed in osteoclasts, it is not upregulated following RANKL-induced osteoclast differentiation, suggesting a limited role in osteoclast function. The increased osteoclast activity in *Wwox*^{-/-} mice is likely secondary to the observed hypocalcemic phenotype [24]. These results are in sharp contrast to the observations of Ludes-

Meyers et al. [36], who observed no differences in osteoclast/osteoblast activity in *Wwox* null mice despite osteopenia. The reason(s) for the discrepancies between these studies remain to be determined. Nonetheless, the metabolic consequences of WWOX deficiency are well known to contribute to osteopenia, which is largely considered the precursor to osteoporosis [48]. Whether WWOX or its genomic locus is involved in osteoporosis is not known. Further investigation of WWOX status shall uncover its potential role in these conditions.

Molecular analysis of osteogenesis markers in *Wwox*^{-/-} bones and osteoblast cultures reveal that WWOX deletion results in significantly decreased levels of osteoblastic genes related to bone mineralization including alkaline phosphatase (*Alp*) and osteocalcin (*Oc*) [24]. Using *ex vivo* calvarial osteoblast differentiation assays, we reported an autonomous osteoblast mineralization defect and reduced expression in osteogenesis markers [24]. These findings are consistent with the reduced bone formation and osteopenic phenotype observed in *Wwox*^{-/-} mice [24, 36]. Intriguingly, femurs of *Wwox*^{-/-} mice exhibit increased levels of *Runx2* [24] and *Osterix* (unpublished data), essential transcription factors for bone formation. This change in definitive markers of committed osteoprogenitors may reflect a stimulated population of cells recruited into the osteoblast lineage to compensate for the bone loss, and further might be related to osteosarcoma formation in *Wwox*^{-/-} mice (see below).

Importance of WWOX in osteosarcoma

Targeted ablation of the murine *Wwox* gene leads to post-natal lethality, although by 4 weeks of age mice develop focal lesions along the diaphysis of their femurs resembling early osteosarcomas (**Figure 1**) [20, 23]. In our initial studies we observed that ~30% of *Wwox*-deficient mice develop spontaneous tumors by analysis of histologic sections of paraffin-embedded bone [20]. Since the small tumors were present in only a few serial sections and could easily be missed, we developed a more rapid and sensitive screening approach using μ CT imaging of intact limbs. Irregular endosteal or periosteal cortical protrusions are identified and then confirmed by histologic analysis, using imaging as a guide for sectioning [23]. Using this protocol, osteosarcomas are detected in

100% of the post-natal mice prior to their death. Analyses by others of other *Wwox* null rodent models failed to detect osteosarcomas [36, 47], most likely due to the lack of a sensitive detection method such as μ CT to find these tiny tumors. Since *Wwox*^{-/-} mice generated using conventional techniques die very early in life, conditional *Wwox* knockout mice were generated to study the WWOX function in both normal and cancer tissues [unpublished data]. This new model will greatly facilitate the functional analysis of *Wwox* in adult mice and will allow more refined investigations of neoplastic transformation in bone tissues.

Recent studies on human osteosarcoma specimens have reported constitutively reduced or absent WWOX expression. Initially, Yang et al. reported deletion of WWOX in 3 of 10 (30%) of osteosarcoma samples as assessed by CGH [21], suggesting that the WWOX locus is targeted in the pathogenesis of osteosarcoma. Immunohistochemical staining analyses demonstrate that loss of WWOX protein is even more frequent, occurring in up to 60% of clinical samples [21, 23]. Our analysis specifically found that normal WWOX expression is detected in 42% of primary tumors, while 58% exhibited absent or reduced WWOX immunoreactivity (**Figure 2**) [23]. Using patient-matched biopsy and resection samples, we further showed that WWOX levels frequently increase in tumors resected following chemotherapy when compared with their primary biopsies. For these tumors, chemotherapy appears to induce tumor cell normalization rather than death, accompanied by restoration of WWOX expression [23]. Further exploration of these results may shed light on the cause(s) of WWOX attenuation in osteosarcoma and raise the possibility of a reversible process. In contrast with primary resections, tumor lung metastases often exhibit reduced WWOX levels relative to the primary tumors, suggesting that WWOX loss is associated with osteosarcoma progression [23]. In a more recent study, Diniz et al. found that osteosarcomas are associated with reduced WWOX mRNA expression by nested reverse transcription-PCR and real-time PCR [22]. These observations clearly indicate that loss of WWOX expression is a common event in the pathogenesis of osteosarcoma.

In vitro analyses of osteosarcoma have also confirmed significantly reduced WWOX mRNA

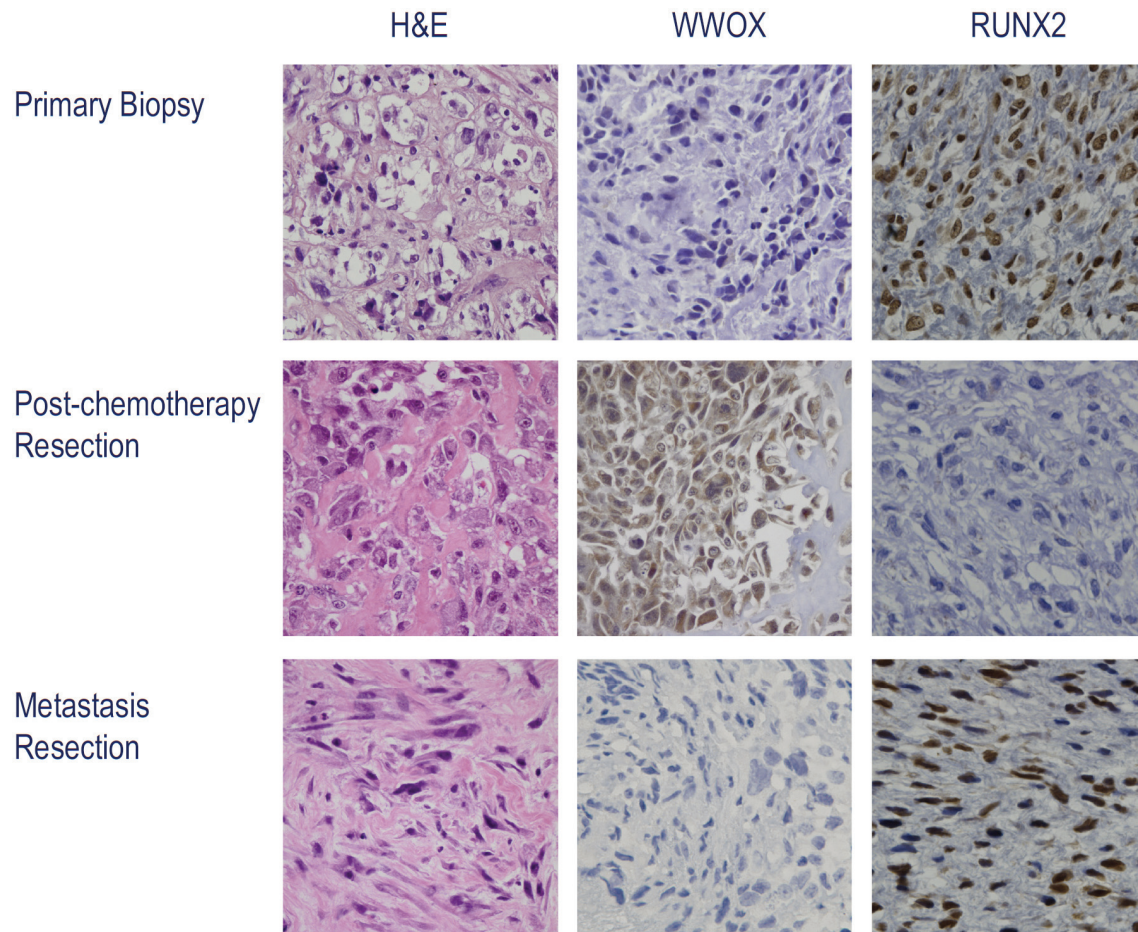


Figure 2. Changes in Immunohistochemical Expression Patterns of WWOX and RUNX2 in an Osteosarcoma Patient's Tumor. The patient's primary biopsy, obtained before chemotherapy, had no detectable WWOX expression but was positive for RUNX2 (Top Row). Following chemotherapy, the resected tumor reversed its expression pattern and was positive for WWOX and negative for RUNX2 (Middle Row). The patient subsequently developed a lung metastasis that, despite occurring after chemotherapy, retained the expression pattern found in the primary tumor biopsy, suggesting it originated from a chemotherapy-resistant clone (Bottom Row). All images were obtained at 600X magnification.

and protein levels in many osteosarcoma cell lines [23]. Importantly, overexpression of WWOX in WWOX-negative osteosarcoma cells using adenoviral or lentiviral vectors suppresses tumorigenicity *in vitro* and *in vivo*, suggesting that WWOX expression can potentially reverse the malignant properties of osteosarcoma [23]. Further, our preliminary observations suggest that restoration of WWOX expression in metastatic osteosarcoma cell lines reduces their cell migration and Matrigel invasion capabilities [23]. Collectively, these findings strengthen the hypothesis that WWOX deficiency may be a significant factor in promoting the onset and progression of human osteosarcomas.

Molecular function of WWOX in osteosarcoma

The complex functionality of WWOX in bone homeostasis suggests that deregulation of WWOX could contribute to the pathogenesis of osteosarcoma. WWOX has been shown to partner with several proteins implicated in the pathogenesis of osteosarcoma, including EZRIN [41], ERBB4 [39] and RUNX2 [24]. EZRIN is a membrane-cytoskeleton linker protein that was first characterized based on its differential expression between highly metastatic and poorly metastatic murine osteosarcoma cell lines [49]. High expression of EZRIN in primary tumors is associated with a significantly shorter disease-

free interval and higher risk of recurrence in pediatric osteosarcoma patients [50]. Whether WWOX through its physical interaction with EZRIN in osteosarcoma cells can attenuate EZRIN's pro-metastatic function is still to be determined.

WWOX also interacts with the ERBB4 receptor tyrosine kinase via its PPxY motifs, sequestering it in the cytoplasm and suppressing transcriptional coactivation of its intracellular domain (ICD), in a process mediated by YAP [39]. ERBB4 plays an important role in cellular differentiation and proliferation [51], and its ICD (p80) is highly expressed in the nuclei of osteosarcoma cells, suggesting its involvement in the pathogenesis and progression of osteosarcoma [52]. Our attempts to assess the clinical significance of the WWOX-ERBB4 association in breast cancer revealed that membranous expression of ERBB4, together with WWOX expression, is associated with favorable patient survival when compared to expression of membranous ERBB4 in the absence of WWOX [53]. This may be explained by the fact that WWOX prevents translocation of ERBB4-ICD into the nucleus and stabilizes the full-length ERBB4 at the cell membrane. It is likely that WWOX plays a similar role in osteosarcoma through regulating ERBB4-ICD cellular localization. When WWOX is reduced or absent in osteosarcoma cells, ERBB4-ICD would be expected to be mostly nuclear, contributing to ERBB4 pro-survival function. The precise role of the interaction between WWOX and ERBB4 in the pathogenesis of osteosarcoma requires further detailed investigation.

WWOX-RUNX2 functional crosstalk

The most significant contribution of WWOX to the regulation of osteoblast differentiation during bone development appears to be its association with the osteoblast-specific master transcription factor, RUNX2. Biochemical analyses of WWOX binding partners have revealed both physical and functional associations of WWOX with RUNX2 [24]. In particular, we initially reported that this association suppresses RUNX2's transactivation function, as assessed by osteocalcin transactivation [24]. Furthermore, ectopic expression of WWOX in MDA-MB231 breast cancer cells reduces *RUNX2* levels and levels of its target genes, including *VEGF* and *osteocalcin* [24]. Since we observed

an impaired osteoblast differentiation phenotype in *Wwox*^{-/-} mice, we hypothesize that osteosarcoma formation could be related to a differentiation defect in the osteoblast compartment. In fact, WWOX appears essential in regulating the proliferation and maturation of osteoprogenitor cells during bone formation [24]. Since *RUNX2* mRNA and protein levels increase in bony tissues of *Wwox*-deficient mice and show an inverse correlation in WWOX-manipulated osteosarcoma cell lines, the WWOX-RUNX2 interaction may be essential for the development of osteosarcoma [23, 24].

Recently, we reported that *RUNX2* expression is significantly deregulated in osteosarcoma, in agreement with previously published data (reviewed in [16]). Our study specifically examined *RUNX2* and WWOX expression in matched primary tumor biopsies, resected primary tumors following chemotherapy and from metastatic lesions [23]. We observed that 60% of primary tumor biopsies, 16% of post-chemotherapeutic resections and ~70% of metastatic tumors were positive for *RUNX2* staining. While these numbers suggest a loss of *RUNX2* expression with chemotherapy mirroring the gain of WWOX, an inverse relationship between WWOX and *RUNX2* was not evident in paired human tumors as in osteosarcoma cell lines [23]. This observation might suggest that the relationship between WWOX and *RUNX2* is more complex in tumors, perhaps due to the complexity of multiple additional aberrations that take place *in vivo*. Further analyses are necessary to decipher the functional crosstalk between WWOX and *RUNX2* in osteosarcomas and other malignancies. These results may also suggest that interactions with other WWOX signaling members are critical in osteosarcoma tumorigenesis.

Intriguingly, *RUNX2* is a target of several WW domain-containing proteins, including co-activators and ubiquitin ligases [54]. How the different WW domain proteins control *RUNX2* activity is not fully elucidated. Importantly, in the absence of WWOX, the balance amongst remaining WW domain containing-proteins may determine the transcriptional outcome of *RUNX2*. Since *RUNX2* is upregulated in osteosarcoma, we may speculate that WWOX loss in osteosarcoma may be, at least in part, responsible for this altered expression. Further dissection of the molecular and functional asso-

ciation between WFOX and RUNX2 in osteoblasts and in osteosarcomas is necessary to better understand this relationship.

Conclusions and future directions

Despite knowledge of many of the genetic abnormalities in osteosarcoma, its complexity precludes placing its biology into a simple conceptual framework. WFOX is consistently deleted in the majority of human osteosarcomas, and targeted ablation of *Wfox* in mice is associated with osteopenia, osteoblast maturation defects and osteosarcoma development [20, 23, 24]. It is possible that WFOX deletion resulting from loss of heterozygosity and/or deletion of chromosome 16q23.2 is a causative factor in the pathogenesis of osteosarcoma. It is worth mentioning that osteosarcomas are also seen in a number of inherited cancer predisposition disorders such as Retinoblastomas, Li-Fraumeni syndrome, Bloom's syndrome, Paget's disease and Rothmund-Thomson syndrome [55-58]. In recent years there has been cumulative evidence that these diseases are associated with chromosomal instability [59]. Following DNA replication stress during growth and development, common fragile loci including *FRA16D*, where WFOX resides, may present intrinsic replication difficulties and cause DNA breaks and deletions [60]. For example, Chan et al [61] proposed that cancer predisposition in Bloom's syndrome patients may be due to "accumulated loss of tumor suppressor function of genes residing at fragile site loci." Since the relative risk of osteosarcoma development increases greatly with ionizing radiation and chemotherapy [62], it is plausible that these treatments lead to chromosomal instabilities affecting tumor suppressor genes in fragile sites, such as WFOX, thus contributing to osteosarcoma development. Recent evidence has also shown that "chromothripsis", a new mechanism for genetic instability in cancer cells, is very common in osteosarcomas and chondromas [63] suggesting that the genome of bone cells is susceptible to chromosomal rearrangements. Whether inactivation of the DNA damage response machinery may contribute to loss of WFOX expression in these cancers, and possibly vice versa, is yet to be determined. The complexity of osteosarcoma has also hindered efforts to identify novel prognostic factors and therapeutic targets. Independent of tumor necrosis, WFOX expression might serve as a useful prognostic marker [23]. In particular, a

number of patients with a poor response by tumor necrosis demonstrate restoration of WFOX, implying normalization of tumor cells rather than cell death, potentially identifying a subset of so-called poor responders with a good prognosis based on tumor biology. Conversely, tumors that fail to undergo necrosis or normalization will likely define a population at risk for aggressive, metastatic disease. Additional WFOX binding partners critical in bone development, such as RUNX2, may be important in elucidating this biology. Large cohorts of patients are necessary to further our understanding of the role of WFOX in the pathogenesis of osteosarcoma and to define its definitive link with treatment response and overall prognosis. A better understanding of the functions of WFOX in bone development and homeostasis would also be necessary. Finally, specific ablation of *Wfox* in mouse bone without the compound effects of total *Wfox* deletion will likely be instrumental to specifically address the role of WFOX in bone homeostasis and the pathogenesis of osteosarcoma.

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