Abstract  The etiology of osteosarcoma (OS) remains enigmatic. Particular clinical and molecular patterns, observed with high frequency in OS, suggest that it results from some yet-to-be-discovered central driver. How else can biology generate such an aggressive, metastatic, genetically and chromosomally unstable malignancy with virtually no apparent precursor neoplasms that are partway along a disease path toward OS? With this conundrum as a backdrop, the discovery of every new native molecule with power to impact a cell’s biology is usually quickly followed by a search to see if this type of molecule contains the key to unlock OS biology.

Keywords  MircoRNA  •  miRNA  •  Prognosis  •  Apoptosis  •  Chemoresponsiveness

This pattern was followed closely as the appreciation of microRNAs (miRs) dawned on the biology of cancer and development over the last decade. MiRs are short non-coding RNAs, typically 20–22 nucleotides in length, with profound impact on the posttranscriptional control of gene expression [1]. Typically, miRs bind to the 3’ untranslated regions of target genes, limiting the level of translation. MiRs destabilize some gene transcripts, limiting the duration for which the messenger RNAs are available for translation. Other transcripts are not degraded any faster because of the bound miR, but are blocked from the ribosomal translational machinery. A single miR can impact a variety of genes. Families of miRs, sometimes grouped by sequence similarities and sometimes by co-expression from a single genomic locus, have proven to be master regulators of broad transcriptional profiles in development and
The modulating role of miRs in oncogenesis has been demonstrated in numerous cancers. In a few malignancies, dominant, cancer-initiating roles have been elucidated for specific miRs. In most cancers, they have been found to play a role.

Many investigators have sought a potential role for miRs in OS. Most have given up the initially tantalizing thought that a miR or miR family might be the driving force behind OS. However, even without some core discovery explaining OS biology, patterns of miR expression in OS and individual miRs function in OS cell lines has led to an understanding that miRs comprise an important part of the molecular landscape of OS.

A role for miRs in the management of OS has been sought by the profiling of snap frozen specimens, paraffin embedded specimens [2], and even patient serum samples [3–5]. Most of these have sought prognostic markers of survival, metastasis, or chemotherapeutic response. A variety of methods have been utilized to profile miRs, including locked nucleic acid (LNA) microarray, beads array, and TaqMan quantitative real-time PCR low density array (TLDA). Each array type has demonstrated high intra-platform reliability, but poor inter-platform reliability [6]. The wide variations in miR collection and profiling likely contribute to the difficulty of parsing the different profiles reported, but some themes are discernible.

Additional work has attempted to characterize the expression levels of individual miRs, for prognostication, deciphering of biological pathways, identification of therapeutic targets, or identification of potentially therapeutic miRs.

Profiles and Patterns of MicroRNAs in Osteosarcoma

In an early miR profiling paper, the miR-181 family (miR-181a, b, c) were overexpressed in OS tumor samples and higher expression of miR-181c was associated with development of metastasis. Multiple members of the miR-16 family were decreased in OS tumor samples, and lower levels of one family member associated with chemoresistance [7]. These two groups of oncogenic and tumor suppressive miRs have been identified by other groups as well [8]. For example, the well-known MG-63 OS cell line was found to overexpress miR-181a [9]. Others have also shown miR-181 overexpression [10]. MG-63 cells also overexpress miR-195, a miR-16 family (generally tumor suppressing) member.

A possibly explanation for the prominent role of miR-16 family members in various profiles is their participation in osteoblast differentiation [11]. Other osteoblast differentiation associated miRs, such as miR-29a/miR-29b, the let-7 family, and the miR-34 family also figure prominently in a variety of reported profiles of usually downregulated miRs [11–18]. Levels of the master osteoblast regulator, RUNX2, are related directly with miR-34 [17].

Another generally oncogenic miR group identified in OS cells is the miR-17-92 cluster. The miR-17-92 cluster and its two paralogous clusters miR-106a-92 and miR-106b-25, all associated with stemness and poor outcome in a variety of cancers, were found to be upregulated in multiple OS cell lines [19, 20].
MiR-126/126*, a well-established tumor suppressing miR in colon cancer and other carcinomas, was also found to be downregulated in osteosarcoma by a few different investigative teams [12, 20, 21]. This may be due to its recently established role antagonizing the SDF-1α cytokine, which recruits inflammatory monocytes and mesenchymal stem cells to tumors, prompting metastasis [22], or to its suppression of Sirt1 [21].

The remainder of the data available for miRs in OS either focuses on individual miRs or profiling that has not been reproduced in multiple series.

**Pronostic MicroRNAs in Osteosarcoma**

### General Aggressiveness

Some miRs have been identified as individually prognostic of survival in OS. Others have been shown in cell lines to increase invasiveness or aggressiveness. For example, silencing of the 14q32 locus has an important role in OS progression. A group of miRs expressed from this locus, including miR-382, miR-134, and miR-544, have prognostic value in OS [2, 3]. In contrast, decreased levels of miR-206 [23] and miR-145 [24] are associated with more advanced clinical stages of OS and histologic de-differentiation. Other miRs have been shown to be dysregulated in OS, but when manipulated in OS cell lines have impacted their aggressiveness. These would include miR-16, as noted above, but also miR-210 [25] and miR-21 [26], which are expressed more in OS cells than osteoblasts and modulate tumor aggressiveness in cell lines. Invasiveness due to pathologic angiogenesis is related to a loss of miR-132 expression [27].

### Metastasis

A number of individual miRs have been associated with metastasis by different study groups in OS patient cohorts. These include miR-27a and miR-181c [12], miR-206 [23], miR-145 [24], and miR-93 [28]. The modulation of expression of other miRs has been demonstrated to impact metastatic phenotypes of cell lines. This second, partly overlapping group includes miR-27a [12], miR-340 [29], miR-183 [30, 31], miR-424 [32], miR-195 [33], and miR-20a [34]. Specific gene transcript targets of some of these have also been validated. MiRs-424, -195, and -20a all target fatty acid synthase (FASN), which has previously established function in OS metastasis. The last of these, miR-20a, is part of the miR-17-92 cluster that figures prominently in more than one general profile of OS miRs. MiR-340 targets Rho-associated protein kinase 1 (ROCK1), awareness of which as a general driver of metastasis in cancer is growing rapidly [29]. MiR-183 targets ezrin, a gene central to the metastatic program for OS [30, 31].
**Chemoresistance**

In addition to miR-15b, as noted above, dysregulation of other miRs in OS tumor samples have correlated with resistance to chemotherapy. Chemoresistance was noted in OS cells that had increased levels of miR-21 [4]. Decreased responsiveness to the specific chemotherapeutic agent cisplatin was found in OS cells that had increased expression of miR-221 [35]. Five miRs were identified as being prognostic for ifosfamide response, miR-92a, miR-99b, miR-132, miR-193a-5p and miR-422a, impacting the TGF-β, Wnt, and MAP kinase signaling pathways [36]. Resistance to both methotrexate and raltitrexed (Tomudex®) was found to correlate with increased levels of miR-215 [37]. Overexpression of miR-140 in OS cells caused resistance to methotrexate and 5-fluorouracil [38].

**Pathways of Influence for MicroRNAs**

Although the appreciation of miRs has elevated to our awareness the critical impact of noncoding RNA molecules, we still interpret most of the biological influence of miRs through the language of the genes whose translation they ultimately impact. Naturally, most investigators have looked for target genes in the major developmental and oncogenic pathways. As most of these pathways have been implicated one at a time and by single miRs, it is difficult to summarize these data without apparent lists.

**Proliferation**

The group of miRs expressed from the 14q32 locus, typically lost in OS, impact a number of important pathways in proliferation, including Notch, RAS/p21, MAPK, Wnt, and the Jun/FOS [39]. Other miRs also impact Notch signaling, including miR34c, a tumor suppressor miR downregulated in OS [7]. In contradistinction, Notch signaling activity is increased by miR-199b-5p, which is often overexpressed in OS and has proven to be a potential therapeutic target. Transfection with a miR-199b-5p inhibitor decreased Notch signaling and proliferation. A downstream effect was a reduction in HES1 expression, which diminished cell invasiveness [40]. Other members of the miR-199 family, such as miR-199a-3p, reduce cell proliferation by affecting the G1/S cell cycle checkpoint of the cell cycle [41].

Signal transducer and activator of transcription 3 (STAT3), another central driver of proliferation, is a downstream target of miR-125b, which is often decreased in OS [42]. Transforming growth factor alpha (TGF-α), a ligand for the epidermal growth factor receptor, is often overexpressed in OS, functioning in autocrine fashion. MiR-376c decreases levels of TGF-α and is noted to be downregulated itself in OS [43].
Insulin-like growth factor 1 receptor (IGF-R1) is involved in the proliferation of many cancers. In osteosarcoma, it is a target of miR-16, which represses cell proliferation. When miR-16 is underexpressed, then cell proliferation increases via IGF-R1 and the Raf1-MEK1/2-ERK1/2 pathway [8]. MiR-15a and miR-16-1 also impact proliferation partly by targeting cyclin D1 [44].

Another regulator of increased OS cell proliferation is lysophosphatidic acid acyltransferase β (LPAATβ), a target of miR24. In many OS cell lines, miR-24 is downregulated, leading to increased LPAATβ activity and OS cell proliferation [45].

MiR-34a levels are low in OS cells leading to upregulation of ether à go-go 1 (Eag1) pathway activity and dependent proliferation [15].

**Apoptosis**

MiR-133a is downregulated in OS, resulting in increased cell proliferation. When levels of miR-133a are reestablished, it functions as a tumor suppressor via inhibition of Bcl-xL and Mcl-1 expression [46]. Similarly, pro-differentiation, tumor-suppressing miR-29a silenced Bcl-2 and Mcl-1 and is typically downregulated in OS [14]. Bcl-2 alone is targeted by miR-143, which is low in OS specimens [47].

Transcript levels of the regulatory gene, c-MYC, can be affected by a group of miRs at the chromosome 14q32 locus, including miR-382, miR-369-3p, miR 544, and miR-134, downregulated in OS cells. Reinstating functional levels of these miRs causes decreased c-MYC activity, triggering induction of apoptosis [48].

In contrast, suppression of the oncogenic miR-181a leads to increased apoptosis in OS [10]. Heat shock protein 90 (Hsp90) is a target of miR-223, which produces increased apoptosis in OS cells as well as G0/G1 arrest when the miR is antagonized [49].

**DNA Damage Repair**

DNA repair is aided by a phosphorylated histone H2AX. MicroRNA-138 inhibits formation of this important histone complex, thereby improving the responsiveness to both radiation therapy and chemotherapy [50].

**Invasion**

Matrix metalloproteinases (MMPs), integral in cell migration contribute to the metastatic phenotype. OS cells with decreased levels of miR-143 demonstrate resultant upregulation of its target MMP-13 and therefore metastasis [51].
**Angiogenesis**

Angiogenesis in previously dormant osteosarcoma cells was associated with decreased expression of miR-190 [52]. Vascular endothelial growth factor is down-regulated when miR-145 is over-expressed, therefore also limiting invasion of OS cells [53].

**Driving MicroRNA Dysregulation**

While so much research has focused on how miRs manage the expression levels of a variety of coding genes, much less is known about what factors directly influence the expression levels of miRs. A few associations have been identified.

For example, miR34a, a member of the illustrious miR 34 family, with its varied and sundry effects in OS, is a target of the key tumor suppressor gene p53 [13, 16]. p53 also targets miR-211, indirectly. Noncoding RNA loc 285194, a p53 regulated tumor suppressor, leads to decreased levels of miR211 as well as decreased proliferation [54].

Changes in the expression of 13 different miRs were identified by microarray and qRT-PCR when cells were transfected with apurinic/apyrimidinic endonuclease1 (APE1). This enzyme functions in both cellular DNA repair and redox regulation. Downstream pathways that were affected include p53 signaling, Wnt, TGF-β, and MAPK. Therefore, multiple cellular processes including differentiation and signaling can be regulated by APE1 through alteration of gene expression by miRs [55].

Some therapeutic chemicals applied to OS also have been shown to impact miR expression profiles. Exposure of OS cells to epirubicin increased levels of miR-302b, which inhibits OS cell proliferation via promotion of apoptosis [56].

Diallyl trisulfide (DATS) decreases angiogenesis, cell survival, and invasion of OS cells. Mechanistically, it causes a drop in the expression of Notch-1 signaling pathway and its downstream genes, such as matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF). However, expression of a group of miRs, normally decreased in OS, is increased. This group of miRs acts as tumor suppressors and include miR-34a, miR-143, miR-145, and miR200b/c [18].

**Future Directions**

Doubtless, we have only begun to understand the breadth and depth of impact miRs have on osteosarcomagenesis, progression, metastasis, and chemoresistance. As technology and our understanding of miRs continue to improve, additional utilization of miRs in diagnostic, prognostic, and hopefully therapeutic purposes will be made.
References


