The Microtubule Network and Cell Death Are Regulated by an miR-34a/Stathmin 1/βIII-Tubulin Axis

Nancy S. Vetter, E. A. Kolb, Christopher C. Mills, and Valerie B. Sampson

Abstract

MicroRNA-34a (miR-34a) is a master regulator of signaling networks that maintains normal physiology and disease and is currently in development as a miRNA-based therapy for cancer. Prior studies have reported low miR-34a expression in osteosarcoma; however, the molecular mechanisms underlying miR-34a activity in osteosarcoma are not well-defined. Therefore, this study evaluated the role of miR-34a in regulating signal transduction pathways that influence cell death in osteosarcoma. Levels of miR-34a were attenuated in human osteosarcoma cells and xenografts of the Pediatric Preclinical Testing Consortium (PPTC). Bioinformatics predictions identified stathmin 1 (STMN1) as a potential miR-34a target. Biotin pull-down assay and luciferase reporter analysis confirmed miR-34a target interactions within the STMN1 mRNA 3′-untranslated region. Overexpression of miR-34a in osteosarcoma cells suppressed STMN1 expression and reduced cell growth in vitro. Restoration of miR-34a led to microtubule destabilization and increased βIII-tubulin expression, with corresponding G1–G2 phase cell-cycle arrest and apoptosis. Knockdown of the Sp1 transcription factor, by siRNA silencing, also upregulated βIII-tubulin expression in osteosarcoma cells, suggesting that miR-34a indirectly affects Sp1. Validating the coordinating role of miR-34a in microtubule destabilization, when miR-34a was combined with either microtubule inhibitors or chemotherapy, STMN1 phosphorylation was suppressed and there was greater cytotoxicity in osteosarcoma cells. These results demonstrate that miR-34a directly represses STMN1 gene and protein expression and upregulates βIII-tubulin, leading to disruption of the microtubule network and cell death.

Implications: The miR-34a/STMN1/βIII-tubulin axis maintains the microtubule cytoskeleton in osteosarcoma, and combining miR-34a with microtubule inhibitors can be investigated as a novel therapeutic strategy. Mol Cancer Res; 1–12. ©2017 AACR.

Introduction

Osteosarcoma is the most common bone tumor that occurs in children (1). About 70% to 75% of patients achieve a complete clinical response with multimodal therapy of standard chemotherapy and surgery. These treatments are ineffective for metastatic and relapsed disease. For patients with metastasis at diagnosis, the overall 5-year survival rate is approximately 30% (2) and declines to 15% for those with recurrent osteosarcoma (3). Rigorous preclinical studies are identifying novel agents and treatments that may lead to the development of new and effective therapies to improve survival outcomes for these patients. Understanding the molecular events that control disease progression and metastasis and how these affect drug response is essential for identifying agents that efficiently overcome mechanisms of drug resistance.

MicroRNAs (miRNAs) are a class of short, single-stranded, noncoding RNAs that are intricately involved in the posttranscriptional regulation of genes through mRNA silencing (4). These highly conserved RNAs contain 6- to 8-nucleotide seed sequences that bind mRNA 3′-untranslated region (UTR) sequences. Full sequence complementarity of miRNA:mRNA binding is not required for miRNAs to function effectively (4) to promote transcript degradation and translational repression. A single miRNA can target and change the expression of many genes, or many miRNAs can target a single gene. Several studies show that miRNAs have central roles in normal physiologic processes as well as in pathologic conditions, including cancer development and progression. Altered expression of specific miRNAs in cancer may predict prognosis and response to therapy (5). For example, high expression of the miR-200 family has been detected in ovarian cancer and is associated with early relapse and decreased overall survival (6). Expression signatures of local or systemically circulating miRNAs that are underepressed (tumor suppressors) or highly expressed (oncogenes) can be developed as biomarkers for discriminating tumor origins, subtypes, and oncogenic mutations (7, 8). For miRNAs that function as tumor suppressors, miRNA mimics can be used to restore miRNA gene expression and inhibit target oncogenes.

MiR-34a is a master regulator of signaling networks that maintain normal physiology and disease and is in clinical development as a diagnostic, prognostic, and therapeutic agent for several
malignancies, including cancer. miR-34a belongs to an evolutionarily conserved miRNA family of genes which also contains miR-34b/c and was first characterized as a cancer-related miRNA by Welch and colleagues, in neuroblastoma (9). In their study (9), the encoding gene was mapped on chromosome 1p36.22. Gene deletions and rearrangements are frequently observed in the 1p36 region in a variety of human cancers including neuroblastoma, glioma, leukemia, and breast cancer (10). In osteosarcoma, 1p36 is a region of chromosomal gains with the amplified expression of several genes (11, 12). miR-34a was identified as an inhibitor of cancer cell growth in a wide range of solid and hematologic malignancies (13, 14), including osteosarcoma tumors (15, 16). Restoration of expression of miR-34a blocked cell migration, cell proliferation, and led to cell-cycle arrest in G1 and G2 phases and apoptosis in vitro and in vivo in preclinical osteosarcoma models (15, 17). Antitumor effects are mediated by repressing targets such as the transcription inducer of cell-cycle progression E2F3 (E2F transcription factor 3), histone deacetylase sirtuin (SIRT1), cell-cycle regulators, cyclin-dependent kinase 4/6 (CDK4, CDK6), and the anti-apoptotic protein b-catenin 2 (Bd2; reviewed in ref. 13). Moreover, miR-34a is directly regulated by p53 transcriptional activation and epigenetic modulation, and inactivating mutations of p53 often correlate with the decrease in expression of miR-34a in tumors (18). The low frequency of minimal deletions for miR-34a in primary osteosarcoma samples suggests that epigenetic alterations may play an important role in modulating the expression of miR-34a (15).

Stathmin 1 (STMN1), also known as oncprotein 18 (Op18), is a microtubule-destabilizing protein that is ubiquitously expressed in vertebrates. The STMN1 gene is located in the 1p36.11 chromosomal region and encodes a 19-kDa cytosolic phosphoprotein. STMN1 binds b-tubulin and regulates the dimerization and polymerization of tubulin (19). These are critical processes in the signaling cascade that controls the assembly and disintegration of the mitotic spindle during cell-cycle progression and cell movement. Dephosphorylation activates STMN1 for microtubule destabilization in interphase and cytokinesis, and phosphorylation deactivates STMN1 to allow microtubules to polymerize to form the mitotic spindle in prophase and for cell movement (19, 20). Kumar and colleagues (21) identified STMN1 in bone cells, and the protein was proposed to play a role in altering osteoblast growth and response to various hormonal stimuli. High STMN1 expression is frequently demonstrated in numerous types of cancer including acute leukemia, gastric, and ovarian cancer (22–24) as well as osteosarcoma (21, 25). Elevated STMN1 expression may represent one mechanism of resistance to the antitumor effects of microtubule inhibitors. STMN1 inhibition results in cell-cycle arrest in the G2/M phase and apoptosis as well as the suppression of lung metastasis (25–27). Other studies show that resistance to docetaxel in gastric cancer is mediated via upregulation of STMN1 by FOXM1 transcription factor, and STMN1 knockdown inhibits in vivo tumor growth of gastric cancer cells (28). Overexpression of miR-31 in ovarian cancer restored sensitivity to chemotherapy by directly repressing STMN1 (29), and miR-101 also inhibited STMN1 and sensitized nasopharyngeal carcinoma cells to radiation (30).

The broad activity of miR-34a across several types of cancer suggests that miR-34a shares common molecular mechanisms of tumor suppression. Successful clinical application of this miRNA requires detailed knowledge of target gene regulation and a comprehensive understanding of the biologic mechanism of action. In the current study, the mechanism of action of miR-34a in osteosarcoma was investigated using a gain-of-function approach in p53-null and wild-type p53 human osteosarcoma cell lines. We identified an association between miR-34a and STMN1 in vitro and evaluated the miR-34a signal transduction on STMN1 and pathways downstream involving the microtubule network. Our results highlight a novel regulatory pathway involving miR-34a, STMN1, and bIII-tubulin that controls microtubule stability and dynamics and mediates survival of osteosarcoma cells.

Materials and Methods

Cell culture

Human osteosarcoma cell lines SaOS (p53-null), 143B, HOS, U2OS (wild-type p53), MG-63 (mutant p53), and human osteoblasts (CRL-1132) were purchased from ATCC. SaOS and U2OS cells were cultured in McCoy 5A medium; 143B, HOS, and MG-63 cells in Modified Eagle’s Medium (MEM); and osteoblasts in Ham F12 Medium. Growth media were supplemented with 10% FBS, 2 mmol/L L-glutamine, 25 U/mL penicillin, and 25 µg/mL streptomycin. Cells were maintained at 37°C in humidified incubators, in an atmosphere of 5% CO2.

Osteosarcoma xenograft tumors

The OS1, OS2, OS9, OS17, OS31, OS33, OS46, OS51, and OS56 tumor lines were generously provided by the Pediatric Preclinical Testing Consortium (PPTC), previously called the Pediatric Preclinical Testing Program (PPTP). These lines are maintained by serial passage in severe-combined immune deficient (SCID) mice as described previously (31).

Antibodies and reagents

Primary antibodies against STMN1, phospho-STMN1 (S38), sirtuin 1 (SIRT1), b-tubulin, bIII-tubulin, specificity protein 1 (Sp1), cyclin D1, p27, PARP, microtubule-associated protein light chain 3-II (LC3-II), GAPDH, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology. Alexa 488–labeled secondary antibodies were from Jackson ImmunoResearch Laboratories. Gemcitabine was purchased from Selleckchem. Eribulin was obtained from the Pharmacy, Alfred. I duPont Hospital for Children.

RT-qPCR and PCR

Total RNA was isolated from cells and tumors using Trizol reagent (Invitrogen). First-strand cDNA was synthesized with the High Capacity cDNA Synthesis Kit (Applied Biosystems). TaqMan miRNA gene expression assays for human miR-34a and miR-361 (internal reference) were purchased from Applied Biosystems. Gene amplification was performed using the 7900HT Fast Real-Time PCR system (Applied Biosystems). The primer sets were STMN1: F - 5' TCTCGTCGCTGCTCTCCTCA 3' and R - 5' TCTCGTCGCTGCTCTCCTCA 3'; TUBB2: F - 5' AAATACTATGCCTGGGCTCATC 3' and R - 5' GTATATCCGCCGCTCCTCCT 3'; TUBB3: F - 5' GCAACTCGGTCGGGCACT 3' and R - 5' CGAGGCGACGTACTCGCTGAG 3'; E2F3: F - 5' GGACGTCTCCTACAACCAC 3' and R - 5' AGTACGTAGACCCGCTTCTT 3' and GAPDH: F - 5' TGCCACCACTGGTGCTACG 3' and R - 5' GCCATGGACTGTGGCATGAG 3'. cDNA loading was normalized to
GAPDH, and gene expression was determined using the ∆∆Ct method of relative quantification. Data are presented as mean ± SE of 3 measurements.

Transfections with Lipofectamine (for miRNA and siRNA oligonucleotides)

SaOS and 143B cells were seeded into 6-well plates (2.5 × 10^5 cells/well) in medium without antibiotics. After 24 hours, each cell line was transiently transfected with either precursor hsa-miR-34a-5p (miR-34a; Thermo Fisher Scientific) or the nontargeting miRNA control (miR-C) using RNAi max Lipofectamine (Invitrogen) according to the manufacturer’s protocol. SMARTpool siRNA oligonucleotides targeting STMN1 and Sp1 and a nontargeting control were purchased from Dharmacon. After 6 hours, the cells were grown in medium supplemented with 10% FBS and antibiotics. Cells were harvested for analysis 48 hours after transfections.

Transfections with nucleofector (for plasmid DNA)

Human STMN1 (transcript variant 1) expression plasmid was purchased from OriGene. Osteosarcoma cells were transfected with STMN1 plasmid using the Amaxa nucleofector (Lonza) according to the manufacturer’s protocol.

Cell viability assay

SaOS and 143B cells were seeded into 96-well plates (2 × 10^3 cells/well) in medium without antibiotics and grown for 24 hours. Cells were transfected with miRNAs for 72 hours, siRNAs for 48 hours or treated with eribulin or gemcitabine for 24 hours. Viable cells were measured using the Cell Titer Blue Assay (Promega) according to the manufacturer’s protocol. Absorbance values were read at 570 nm using a Victor4 plate reader (PerkinElmer). Data are represented as the mean of 6 measurements ± SE.

miRNA biotin pull-down

Biotin-labeled miR-34a (bi-miR-34a) was synthesized by Dharmaco. Bi-miR-34a or biotin-labeled cel-miR-67 (bi-miR-C) control molecules were transfected into SaOS and 143B cells using RNAi max Lipofectamine (Invitrogen). After 24 hours, cells were incubated with lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 300 mmol/L KCl, 12 mmol/L MgCl2, 1% NP-40, 1 mmol/L dithiothreitol (DTT)] supplemented with complete protease inhibitor EDTA-free (Roche), RNase inhibitor (Promega, 200 U/mL), and ribonucleoside-vanadyl complex (Sigma-Aldrich). Bi-miR-34a and mRNA complexes were captured with Streptavidin-Dynabeads–coated magnetic beads (Invitrogen) for incubation for 4 hours. Beads were washed 5 × [100 mmol/L Tris-HCl (pH 7.5), 3 mmol/L MgCl2, 14 mmol/L NaCl, 1 mmol/L DTT, and 5% glycerol], and bound mRNA was purified using TRizol (Invitrogen) and analyzed by PCR using forward and reverse primers against STMN1, TUBB3 (negative control), and E2F3 (positive control). Nonspecific binding of RNA to the magnetic beads was analyzed using bi-miR-C.

Luciferase assay

The pMIRTarget plasmid containing the STMN1 mRNA 3’UTR sequence fused to the luciferase reporter gene was constructed by Origene. SaOS and 143B cells were co-transfected with pMIRTargetSTMN1 3’TUTR and either pre-miR-34a or nontargeting control miR-C oligonucleotides with Lipofectamine 3000 (Invitrogen). After 24 hours, cell extracts were prepared, and reporter gene activity was determined using the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer’s protocol. The Victor plate reader was used to measure luciferase reporter units (PerkinElmer). Luciferase measurements were normalized to Renilla luciferase used as the internal control.

Immunoblot

Cells were treated with miR-34a, siRNA, or microtubule-targeting agents (MTA) as single agents or in combination and harvested at time points specified. Cells were lysed in RIPA buffer (Invitrogen) supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific). lysates were precleared by centrifugation, and protein concentration was determined (Pierce). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, incubated with primary antibodies and HRP-conjugated secondary antibodies, and subjected to ECL analysis (GE Healthcare).

Immunofluorescent staining and confocal microscopy

Poly-l-lysine–coated glass coverslips were placed in 12-well plates and overlaid with 2 × 10^5 SaOS and 143B cells. The cells were grown overnight and then transfected with miR-34a before processing for immunofluorescence. Cells were fixed with formaldehyde for 20 minutes at room temperature and rehydrated with PBS containing CaCl2 (0.1 mmol/mL), MgCl2 (1 mmol/L), and 0.2% BSA. Subsequently, the cells were incubated with the primary antibodies (1:200 dilution) followed by secondary antibodies (1:200). Hoechst dye (Thermo Fisher Scientific) was used for nuclear staining at 1:10,000. Fluorescent images were captured using a Leica TCS SP5 scanning confocal microscope (Leica Microsystems) with the 63 × oil objective lens. Images were processed using Photoshop (Adobe Systems Inc.).

Cell-cycle analysis

SaOS and 143B cells (2 × 10^5) were transfected with miR-34a for 72 hours and subjected to propidium iodide (PI) staining (Sigma). Briefly, cells were trypsinized, washed with PBS, and fixed with 70% ethanol for 30 minutes at −20°C. Cells were washed, pelleted, and resuspended in 500 μL of PI/RNase solution for 15 minutes at room temperature. Cell-cycle analysis was performed using the NovoCyte Flow Cytometer (ACEA Biosciences).

Statistical analysis

The relationship between the miRNA and mRNA targets was tested with Pearson correlation coefficient. Synergy of miR-34a and drug combination therapies was quantified using the CompuSyn software (CombuSyn, Inc.) to calculate the combination index (CI) based on the Chou–Talalay method (32) where CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively. All statistical analyses used the 2-tailed unpaired Student t test and were calculated using GraphPad Prism software (GraphPad Software Inc.). Data was expressed as mean value ± SE. Statistical significance was defined as P < 0.05 and is denoted by asterisks in the figures.
Results

Endogenous expression of miR-34a correlates inversely with STMN1 mRNA expression in osteosarcoma cell lines and xenograft tumors

Expression of miR-34a was evaluated by RT-qPCR in 5 human osteosarcoma cell lines (SaOS, 143B, HOS, U2OS, MG-63) and 9 tumors of the PPTC osteosarcoma xenograft panel (OS1, OS2, OS9, OS17, OS31, OS33, OS46, OS51, OS56). In agreement with earlier reports (15), all osteosarcoma specimens demonstrated attenuated levels of miR-34a (Fig. 1A), relative to human osteoblasts (CRL11372). Bioinformatics (www.microRNA.org) was used to predict candidate genes that are potentially regulated by miR-34a, and STMN1 was identified as a novel target that has not been studied. The STMN1 protein regulates microtubule dynamics and high expression correlates with tumor progression in osteosarcoma (25). The alignment predicted between the miR-34a seed sequence and the STMN1 3’UTR recognition sequence is shown in Fig. 1A. RT-qPCR analysis of STMN1 mRNA levels in the osteosarcoma cell lines and xenograft tumors showed highSTMN1 expression, in comparison with osteoblasts (Fig. 1A). Pearson correlation analysis revealed that levels of STMN1 were negatively correlated with miR-34a (r = –0.35, P < 0.05; Fig. 1B). To confirm this finding, SaOS, 143B, HOS, and U2OS cell lines were transfected with miR-34a precursors. Overexpression of miR-34a relative to control cells transfected with miR-C (Supplementary Fig. S1) significantly reduced STMN1 mRNA levels in SaOS and 143B cells (by 0.12-fold and 0.52-fold, respectively, P < 0.05; Fig. 1C). In addition, immunoblot analysis demonstrated that STMN1 protein levels were downregulated in miR-34a–expressing cells (Fig. 1D). Taken together, these results imply that miR-34a is involved in the regulation of STMN1 in osteosarcoma.

miR-34a targets the STMN1 mRNA 3’UTR in vitro

The biotin pull-down assay was used to assess target interactions between miR-34a and STMN1 mRNA. SaOS and 143B cells transfected with either bi-miR-34a or bi-miR-C controls were treated with streptavidin beads. Bound mRNA was isolated and subjected to PCR analysis. Using STMN1-specific

Figure 1.

Gene expression of endogenous miR-34a and STMN1 for osteosarcoma cell lines and osteosarcoma xenografts. A, Top, Schematic of the alignment between the miR-34a seed sequence and the target recognition sequence of STMN1 mRNA 3’UTR, predicted by microRNA.org. Bottom, Expression of miR-34a and STMN1 mRNA in osteosarcoma cell lines (n = 5) and xenografts (n = 9) was measured by RT-qPCR and expressed relative to normal osteoblasts. Values are mean of 3 measurements. B, Correlation between miR-34a expression in osteosarcoma cells and xenografts and STMN1 mRNA was analyzed by Pearson correlation coefficient. C, SaOS and 143B cells were transfected with nontargeting miR-C (control) oligonucleotides or miR-34a precursors, and STMN1 mRNA was measured by RT-qPCR. Results are represented as fold change relative to miR-C. Graph depicts mean ± SE of one representation of 3 independent experiments. *, P < 0.05. D, STMN1 protein expression in untreated, miR-C-, and miR-34a-transfected SaOS, 143B, HOS, and U2OS cells was measured by immunoblot. GAPDH was loading control. Representative results of 3 analyses are shown.
primers, amplification of STMN1 in cells expressing bi-miR-34a, but not those with bi-miR-C (Fig. 2A), was observed, validating STMN1 as a direct miR-34a target gene in vitro. There was no amplification of the nontargeted TUBB3 gene (negative control), whereas E2F3, a known miR-34a target (13), was detected (positive control; Fig. 2A). We examined the specific interaction site of miR-34a on the STMN1 3′UTR using the luciferase reporter assay. Co-transfection of SaOS and 143B cells with the pMiRTarget plasmid, containing the STMN1 3′UTR sequence inserted downstream from the firefly luciferase gene, was performed with either miR-34a or miR-C. Red fluorescence protein was used as a reporter to monitor transfection efficiency and both cell lines were more than 80% efficient. Osteosarcoma cells expressing miR-34a showed significantly lower luciferase activity in comparison to controls (Fig. 2B). These results confirmed that miR-34a binds the recognition sequence of STMN1 mRNA within the 3′UTR. In addition, cell viability measurements demonstrated that the restoration of miR-34a reduced the percentage of viable SaOS cells by 37.1%, and of 143B by 38.9% after 72 hours (P < 0.05; Fig. 2C). Collectively, our data demonstrate that miR-34a binds STMN1 mRNA at the 3′UTR and induces growth inhibition in vitro in osteosarcoma cells.

**In vitro disruption of miR-34a on tubulin and the microtubule network**

The STMN1 protein binds and sequesters β-tubulin monomers to destabilize microtubules and prevent mitotic spindle assembly (19). This suggests that microtubule stability could be affected by the observed effects of miR-34a on STMN1. Levels of class II and III β-tubulin isotypes were analyzed in osteosarcoma cells transfected with miR-34a. Overexpression of miR-34a did not affect the expression of TUBB2A (encoding βII-tubulin), data not shown. In contrast, levels of TUBB3 (encoding βIII-tubulin) were significantly higher, relative to miR-C controls (Fig. 3A). The increase in gene expression correlated with increased βIII-tubulin protein levels (Fig. 3B, top). We also determined whether siRNA-mediated STMN1 inhibition alters β-tubulin expression. Immunoblot analysis confirmed that STMN1 knockdown was >70% and >90% efficient for SaOS and 143B, respectively (Fig. 3B, bottom). Notably, direct silencing of STMN1 increased protein levels of βIII-tubulin, but there was no effect on total β-tubulin protein (Fig. 3B, bottom). Immunofluorescent staining and confocal imaging of osteosarcoma cells using antibodies against STMN1 and βIII-tubulin were analyzed. Representative images shown for the 143B cell line (Fig. 3C) demonstrate that STMN1 protein, which is distributed throughout the cytoplasm in control cells (top, i–iii), was lower in miR-34a-expressing cells (top, iv–vi). Inducible βIII-tubulin expression was observed (bottom, iii–vi), confirming immunoblot results. Furthermore, miR-34a-expressing cells demonstrated reduced polymerized tubulin, in comparison to untreated controls (Fig. 3D, iv–vi). Similar disruption of the microtubule skeleton was observed in cells subjected to siRNA-mediated STMN1 knockdown (Fig. 3D, vii–ix). Thus, miR-34a disrupts the microtubule network and acts...
miR-34a upregulates βIII-tubulin and destabilizes microtubules in vitro in osteosarcoma cells. **A**, Expression of TUBB3 in miR-34a–expressing SaOS and 143B cells was measured by RT-qPCR. Data are shown relative to miR-C controls. Expression of TUBB3 is normalized to GAPDH. Values are expressed as mean ± SE of triplicate measurements and are representative of 2 separate experiments. *P < 0.05. **B**, SaOS and 143B cells were transfected with either miR-34a (top) or with siRNA targeting STMN1 (bottom), and STMN1, β-tubulin, and βIII-tubulin protein expression were determined by immunoblot. GAPDH was loading control. Representative results of 3 analyses are shown. **C**, Confocal microscopy images for 143B cells transfected with miR-34a for 48 hours and immunostained for STMN1 (orange) and βIII-tubulin (green). **D**, Confocal microscopy images for SaOS and 143B cells transfected with miR-34a (iv–vi) and siRNA-STMN1 (vii–ix) for 72 hours and immunostained for total β-tubulin (green). Cells were counterstained with Hoechst (blue) to visualize nuclei. Scale bars are 25 μm.
through the inhibition of STMN1 and increased expression of βIII-tubulin in vitro in osteosarcoma.

miR-34a mediates cell-cycle arrest, autophagy, and apoptosis through the regulation of STMN1

Next, we investigated putative downstream effectors of miR-34a. Consensus binding sequences for the Sp1 transcription factor are contained within the βIII-tubulin promoter (33), but the molecular mechanisms regulating transcription of βIII-tubulin are not clear. Immunoblot analysis revealed that siRNA silencing of Sp1 in SaOS, 143B, HOS, and U2OS upregulated βIII-tubulin protein (Fig. 4A, lane 4), indicating that Sp1 negatively regulates βIII-tubulin expression. Comparative increases in levels of βIII-tubulin protein in miR-34a–expressing cells (Fig. 4A, lane 3) suggest that Sp1 may be an indirect downstream effector of miR-34a that is involved in the regulation of βIII-tubulin. We determined the effect of miR-34a on the cell-cycle distribution in SaOS and 143B cells transfected

---

**Figure 4.**

miR-34a represses STMN1 and induces cell-cycle arrest, apoptosis, and autophagy. **A**, SaOS, 143B, HOS, and U2OS were transfected with either miR-34a or with siRNA-targeting Sp1 (bottom), and STMN1 and βIII-tubulin protein expression were measured by immunoblot. GAPDH was loading control. Representative results of 3 analyses are shown. **B**, 143B cells were transfected with miR-34a or an STMN1 expression plasmid. Immunoblot for STMN1 protein (left) is indicated. Cell-cycle distribution analysis for untreated, miR-34a–expressing, or STMN1-overexpressing 143B cells was assessed by PI staining and flow cytometric analysis. Arrow indicates apoptotic cells. **C**, Immunoblot analysis of cell-cycle markers (cyclin D1 and p27) and apoptotic marker (cleaved PARP) in miR-34a–expressing and STMN1-overexpressing osteosarcoma cells. GAPDH was loading control. Representative results of 3 immunoblot analyses are shown. **D**, Confocal microscopy images for 143B cells transfected with miR-34a for 48 hours and immunostained for LC3 I/II (red) and βIII-tubulin (green). Cells were counterstained with Hoechst (blue) to visualize nuclei. Scale bars are 25 μm.
with miR-34a, followed by STMN1 overexpression, using an STMN1 expression plasmid (Fig. 4B). Our data revealed that treatment with miR-34a only led to cell-cycle arrest in G1 and G2–M phase and apoptosis (sub-G1; Fig. 4B, ii, and Table 1). Restoration of STMN1 reduced the number of miR-34a–expressing cells in G1 and G2–M (Fig. 4B, iii). Notably, cell-cycle markers cyclin D1 and p27 were upregulated in miR-34a–expressing cells (Fig. 4C), to confirm that both G1 and G2 phase arrest occurred in these cells. Similar upregulation in levels of cyclin D1 and p27 was observed in HOS and U2OS (Supplementary Fig. S2). In addition, decreases in both proteins were evident when STMN1 was restored. Protein expression of cleaved PARP was also higher, confirming that miR-34a induced apoptosis (Fig. 4C). Immunofluorescent images (Fig. 4D) illustrate that cells transfected with miR-34a expressed high levels of LC3 I/II protein. These cells show morphologic changes (rounded) and aberrant nuclei (enlarged and multinucleated) that are in line with cellular alterations associated with cell death response to miR-34a (Fig. 3C and D). Taken together, these data demonstrate that miR-34a triggers autophagy and cell death in vitro in osteosarcoma.

Combining miR-34a with microtubule inhibitors and chemotherapy enhances cytotoxicity in vitro in osteosarcoma cells

On the basis of the ability of miR-34a to regulate key components in the microtubule network, we analyzed the effects of miR-34a with eribulin and gemcitabine on osteosarcoma cell growth. SaOS and 143B cells were treated with multiple combinations of both agents (miR-34a/eribulin or miR-34a/gemcitabine), and cell viability was assayed to determine effect levels (fraction affected, Fa) and CI values. Drug interactions were classed as additive (CI = 1), antagonistic (CI > 1), or synergistic (CI < 1). Results presented in Fig. 5A and Table 2 show that combination therapies consisting of...
miR-34a/eribulin or miR-34a/gemcitabine were synergistic for osteosarcoma cell lines. Interestingly, immunoblot analysis of STMN1 protein revealed increases in levels of phosphorylated STMN1 by eribulin treatment, in both cell lines (Fig. 5B). STMN1 phosphorylation is associated with formation of the mitotic spindle and microtubule stabilization and is implicated in resistance mechanisms to MTAs (34). Importantly, the expression of STMN1 protein and consequently STMN1 phosphorylation were abrogated when the 2 agents were combined. Representative immunostaining and confocal imaging for 143B cells using antibodies against STMN1 confirmed that eribulin did not affect STMN1 protein levels (Fig. 5C, iv), whereas co-treatment with miR-34a and eribulin showed substantial downregulation of STMN1 by eribulin treatment, in both cell lines (Fig. 5B).

The survival of pediatric patients with metastatic and recurrent osteosarcoma has not improved over the past 20 years due, in part, to the fact that few new therapies are effective in these patients. Rigorous preclinical studies are identifying novel agents and treatment strategies that may lead to the development of new clinical trials to improve survival outcomes. miR-34a has emerged as a promising agent for the management and therapy of liver cancer and is the first miRNA-based cancer therapy that was tested in clinical trials (35). The antitumor activity of miR-34a has been demonstrated in vitro and in vivo in osteosarcoma (15), but the molecular mechanism of action is not well-characterized. Our current findings identify miR-34a as a critical molecule that controls microtubule stability and growth of osteosarcoma cells and add to the rationale for clinical investigation of miR-34a as a therapeutic agent.

In this study, human osteosarcoma cell lines and pediatric osteosarcoma xenograft tumors were used to confirm previous reports of low levels of miR-34a in osteosarcoma (15, 36). Previous studies have demonstrated the function of miR-34a as a tumor suppressor in the control of cell-cycle progression, cell proliferation, migration, and survival in cancer, including osteosarcoma (13, 15). Our findings are consistent with these functions of miR-34a and reveal that microtubule destabilization is a key event in the antitumor activity. The validation of STMN1 as a miR-34a target gene attributes a novel role to miR-34a in the regulation of microtubule stability. Relative to normal osteoblasts, mir-34a and STMN1 mRNA were inversely correlated in osteosarcoma cells and xenograft tumors, and inhibition of the STMN1 oncogene by the restoration of miR-34a supports the tumor-suppressing characteristics of miR-34a.

STMN1 is highly expressed in several types of cancer (22, 23), as well as osteosarcoma (21, 25), and this correlates with poor prognosis and resistance to chemotherapy agents. Restoration of downregulated miR-34a significantly reduced STMN1 gene and protein expression and arrested the growth of osteosarcoma cells, suggesting that the antitumor activity of miR-34a in osteosarcoma is mediated through the microtubule network. The sensitivity of osteosarcoma cells that lack endogenous p53, and that express wild-type p53, demonstrated that the p53 status is not a prevailing indicator of miR-34a activity, as the general responses to miR-34a restoration were similar for all cell lines. This is in line with emerging studies that suggest that endogenous p53 is not a prerequisite for miR-34a tumor suppressor activity and miR-34a is capable of inhibition in p53-null and p53-expressing cancer cell lines (37).
Evidence supporting the oncogenic role of STMN1 in osteosarcoma can be inferred from experiments demonstrating high levels of expression in osteosarcoma tumors (21, 25), the cytogenetic location to 1p36, a known cancer-related region in osteosarcoma (11, 12), and the role in growth regulation (34, 38). Attenuation of STMN1 suppresses growth of STMN1-expressing osteosarcoma cells (33, 36), indicating elevated levels of STMN1 play an important role in disease progression. Selective strategies to inhibit cell division through the silencing of STMN1 have been studied using ribozymes (38), siRNA targeting (34), and miRNAs (28, 29). The therapeutic inhibition of STMN1 using a bifunctional shRNA plasmid (pbi-shRNA STMN1) that cleaves STMN1 and reduces the stability of microtubule networks and decreases the availability of βIII-tubulin in vitro in osteosarcoma.

Sp1 transcription factor modulates the expression of a number of essential oncogenes and tumor suppressors (41). The promoter region for βIII-tubulin contains multiple potential binding sequences for Sp1 as well as for activating protein-2 (AP2) and a central nervous system enhancer regulatory element and an E-box (33), but the regulation of βIII-tubulin remains largely unknown. Silencing Sp1 with siRNA increased the expression of βIII-tubulin and activated apoptosis. These observations imply that the Sp1 transcription factor may be an indirect downstream effector of miR-34a signaling that is involved in the upregulation of βIII-tubulin. Furthermore, the miR-34a involvement on Sp1 activity could be mediated through posttranslational modifications or other transcriptional co-factors (41). Several reports indicate that the constitutive expression of βIII-tubulin is frequently linked to tumor aggressiveness, resistance to chemotherapy, and patient relapse (42–44), and both high STMN1 and βIII-tubulin expression are implicated with poor survival in patients with ovarian cancer (22). Mechanistically, microtubules that are enriched in heterodimers formed by βIII-tubulin are unstable polymers, and the overexpression of βIII-tubulin increases the rate of microtubule detachment from microtubule-organizing centers and reduces the stability of microtubule networks (45, 46). Our data are in agreement with these studies, as inducible expression of βIII-tubulin in response to miR-34a overexpression correlated with microtubule destabilization, cell-cycle arrest, and apoptosis. In this context, inducible expression of βIII-tubulin cannot be associated with an aggressive phenotype, nor linked to resistance and survival. Therefore, while high constitutive βIII-tubulin expression could be a marker for aggressive tumors, low constitutive expression and inducible βIII-tubulin expression are associated with destabilization of microtubules and osteosarcoma cell death.

The influence on multiple cellular pathways may also suggest that miR-34a can act synergistically with conventional, cytotoxic therapies. The elevated expression of STMN1 and βIII-tubulin may be important in regulating sensitivity to broad classes of chemotherapy drugs. Mechanisms underlying resistance to microtubule inhibitors that occur at the microtubules include mutations, alterations of tubulin isotypes, or regulatory proteins (47). In the current study, we have demonstrated that cells that were exposed to eribulin showed higher levels of phosphorylation of STMN1. The phosphorylation of STMN1 disrupts the formation of STMN1–tubulin complexes and increases the availability of free β-tubulin for microtubule assembly, which can confer resistance to MTAs. Combining miR-34a and MTAs confirmed that osteosarcoma cells expressing STMN1 protein were more sensitive to microtubule inhibition by STMN1 silencing. In line with these findings, the strategy of combining mir-34a with paclitaxel has been reported to overcome resistance to paclitaxel in hormone-refractory prostate cancer cells (48) and to treat murine melanoma metastasized to the lungs of mice (49). The implication is that in a selective molecular background involving STMN1 deletion, inducible βIII-tubulin may enhance the response to these therapies.

This is the first report demonstrating that miR-34a regulates STMN1 and has a modulatory role in microtubule stability. Birnie and colleagues (50) demonstrate a miR-223/STMN1/tubulin-regulated pathway in malignant pleural mesothelioma and suggest a possible conservation of function. Our findings are in line with the anti-tumor signal transductions for miR-34a and support further investigations into combining miR-34a with microtubule inhibitors as a therapeutic strategy. Microtubule inhibitors show limited efficacy in patients with osteosarcoma and have not been incorporated into standard neoadjuvant therapy regimens or in common retrieval regimens. Osteosarcoma cells and tumors resistant to MTAs may survive in the presence of microtubule inhibition through the cellular efflux of drug, ineffective target interaction, and deficiencies in the apoptotic pathway. Several MTAs are in active clinical development in pediatric sarcomas. A recent Children’s Oncology Group trial (NCT02097238) failed to clearly demonstrate a role for eribulin in neoadjuvant chemotherapy for recurrent and refractory osteosarcoma. As new agents move forward in clinical development, understanding mechanisms of resistance and identifying biomarkers of response will be imperative. While an association between high levels of STMN1 and βIII-tubulin in cancer is not novel (22), our data specifically demonstrate that STMN1 inhibition and inducible βIII-tubulin in osteosarcoma could alter microtubule stability. We have also identified STMN1 and βIII-tubulin expression as biomarkers of response to therapy.

In conclusion, these mechanistic studies have identified a key role of miR-34a in osteosarcoma, centered on the regulation of STMN1 and microtubule stability. Our studies demonstrate the feasibility of using miR-34a as a miRNA-based therapy for reducing STMN1 expression in vitro in osteosarcoma to promote microtubule-mediated cell death. Our data also show that mir-34a targets cell processes distinct from MTAs, through the coordinated regulation of the miR-34a/STMN1/βIII-tubulin axis. Ongoing studies aim to elucidate the effect miR-34a can exert on STMN1 and microtubules, in relevant in vivo osteosarcoma preclinical models. Of note, the recent failure of MR34X as a miR-34a replacement therapy for patients with liver cancer highlights that current miRNA delivery approaches are limited in their safety and efficacy. Comprehensive molecular studies such as this, as well as the development of reliable miRNA delivery systems, and characterization of the adaptive and innate immune response will be crucial to support clinical trials for miR-34a replacement in patients with osteosarcoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.S. Vetter, V.B. Sampson

Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): C.C. Mills, V.B. Sampson

Writing, review, and/or revision of the manuscript: E.A. Kolb, V.B. Sampson

Study supervision: E.A. Kolb, V.B. Sampson

Acknowledgments

The authors thank Dr. Richard Gorlick (The Children’s Hospital at Montefiore, Bronx, NY) for providing the PPTIC OS xenograft tumors for this study.

Grant Support

This work is supported by the Clinical and Translational Research (CTR) Pilot Award to V.B. Sampson under grant number NIH/NIH-5-GM104941, the Cell Science Core of the Center for Pediatric Research which is supported by Institutional Development Awards (IDEA) under grant numbers P20GM103464 and P30GM114736, and the Biomolecular Core Lab which is supported by grants P20GM103464 (COBRE), P30GM114736 (COBRE), and P20GM103446 (INBRE).

The costs of publication of this article were defrayed in part by the payment of page charges. This paper must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

The Microtubule Network and Cell Death Are Regulated by an miR-34a/Stathmin 1/βIII-Tubulin Axis


Mol Cancer Res  Published OnlineFirst March 8, 2017.

Updated version  Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-16-0372

Supplementary Material  Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2017/03/08/1541-7786.MCR-16-0372.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.