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The Epigenetic Regulation of SOX9 by miR-145 in Human Chondrosarcoma

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ABSTRACT

Chondrosarcoma is the most common primary bone malignancy in the adult population with a high rate of pulmonary metastasis. Chondrosarcoma is managed with surgical excision as the tumors do not respond well to conventional chemotherapy or radiation therapy. Thus, there exists a dire need to develop systemic treatment options to target chondrosarcoma cells for metastatic spread. We hypothesized that the expression of miR-145 is low in chondrosarcoma, leading to decreased transcriptional control of SOX9 (the master regulator of chondrogenesis), and downstream activation of the transcription factor ETV5. We have previously shown that ETV5 activates MMP-2 expression in chondrosarcoma, which in turn increases local bone matrix resorption. In this study, we confirm high expression of SOX9 in human chondrosarcoma using real-time PCR, Western blotting, and immunofluorescence. An ETV5 promoter–reporter plasmid was transfected into chondrosarcoma cells to determine if SOX9 directly regulates the expression of ETV5. Co-transfection of the ETV5 promoter-plasmid with SOX9 lentivirus significantly increased the luciferase activity derived from the ETV5 promoter, from which the regulatory relationship between SOX9 and ETV5 is established. MiR-145 was found to be down-regulated in chondrosarcoma cell lines, patient samples, and further confirmed with a public sarcoma database. After stable miR-145 lentiviral transfection, the subsequent mRNA expression levels of SOX9, ETV5, and MMP-2 were significantly decreased in chondrosarcoma cells. The results generated by this study may have important clinical significance in the treatment of patients with chondrosarcoma in that targeted miRNA may have the potential to downregulate the upstream activators of proteases such as MMP-2. J. Cell. Biochem. 116: 37–44, 2015.

KEY WORDS: CHONDROSARCOMA; SOX9; ETV5; microRNA; MiR-145

Chondrosarcoma is a malignancy of cartilage and is the most common primary bone malignancy in the adult population [Hameed and Dorfman, 2011]. Chondrosarcoma is unique among bone sarcomas in that it does not respond well to chemotherapy and traditional radiotherapy, thus surgical excision, when feasible, is the sole treatment option currently available. Due to the lack of systemic control of chondrosarcoma, patients with advanced disease die of lung metastases [Rizzo et al., 2001]. Hence, survival rates in this patient population as a whole are significantly lower than those of patients with other bone sarcomas [Eriksson et al., 1980; Dai et al., 2011]. There exists a critical need to develop systemic treatment to target chondrosarcoma cells and prevent metastatic spread.

The hallmark of metastasis is the ability of cancer cells to invade into their surrounding matrix. Indeed, chondrosarcoma is defined pathologically as malignant cartilage tissue that exhibits invasion of the surrounding bone tissue. Our lab has previously shown that the ability of chondrosarcoma cells to invade into and dissolve calcified bone matrix is due, at least in part, to upstream regulation of matrix

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metalloproteinase-2 (MMP-2) by the ETS transcript variant 5 (ETV5) [Power et al., 2013]. MMP-2 is an enzyme secreted from the cell and degrades extracellular gelatinase denatured collagen, whereas ETV5 plays diverse roles in multiple types of invasive tumors [Monge et al., 2007; Planaguma et al., 2011]. Thus, the upstream activation of ETV5 is of significant interest in the study of metastasis in chondrosarcoma. The intrinsic molecular trigger for ETV5 regulation of MMP-2 in chondrosarcoma remains unknown. In other biologic processes, a known upstream regulator of ETV5 is the SRY-related high mobility group-Box gene 9 (SOX9) as identified in kidney development [Reginensi et al., 2011]. This connection is particularly relevant to chondrosarcoma given the fact that SOX9 is the master regulator of the process of cartilage differentiation (chondrogenesis).

MicroRNAs (miRNAs) are short (21–24 nucleotides) noncoding RNAs, which are post-transcriptional modulators of gene expression which play important roles in tumorigenesis and cancer metastasis [Farazi et al., 2013]. Because miRNAs are often highly deregulated in various types of cancer, they can potentially be effective treatment targets. MiRNA profiling studies of chondrocytes have led to the identification of miR-145 as a regulator of the expression of SOX9 in normal cartilage cells [Martinez-Sanchez et al., 2012]. In particular, epigenetic regulation of SOX9 has been demonstrated by Yang et al. [2011] who reported that miR-145 inhibits SOX9 in chondrogenic differentiation in mesenchymal stem cells. It is not known if the regulation of SOX9 by miR-145 in cartilage development is recapitulated in cartilage malignancy. In this study, we hypothesized that the lack of miR-145 epigenetic suppression of SOX9 in chondrosarcoma cells results in SOX9 overexpression and downstream activation of ETV5. The objectives of this study were to determine the baseline expression of SOX9 and miR-145 in chondrosarcoma cells and clinical chondrosarcoma specimens, to determine SOX9 regulation of the ETV5 gene promoter in chondrosarcoma, and to overexpress miR-145 in chondrosarcoma cells in order to determine the subsequent effects of the expression of downstream invasive genes in chondrosarcoma cells.

METHODS

ETHICS

This study received ethics approval from the Hamilton Integrated Research Ethics Board (REB 05–302) and Montreal General Hospital Research Ethics Committee (REC#03–012). Patient informed consent was obtained individually.

CHONDROSARCOMA SAMPLE COLLECTION

Specimens were obtained at either the time of biopsy or surgical excision. A bone pathologist verified the diagnosis of chondrosarcoma. Four chondrosarcoma patient samples were collected and studied (Table I).

CELL LINES

The human chondrosarcoma cell lines JJ012 (JJ) and105KC (KC) were kindly provided by Dr. Joel Block. The chondrosarcoma cell lines were cultured in monolayer until confluent in 37°C humidified air with 5% CO_2 in media consisting of DMEM/MEM/F12 (Gibco) supplemented with 10% fetal bovine serum. Human fetal osteoblast 1.19 cells (hFOB) (American Type Culture Collection, ATCC#CRL-11372) and human chondrocyte cells CHON-002 (CHON) (ATCC#CRL-2847) were used as control cell lines. The control chondrocyte cell line CHON is a normal, non-malignant cell line that can be used for normalization expression in abnormal chondroid cell lines (chondrosarcoma cell lines). Similarly, these cell lines were maintained in supplemented DMEM under 37°C humidified air containing 5% CO_2 as previously described [Mak et al., 2009].

TABLE I. Chondrosarcoma Patient Demographics

Patient sample	Gender	Age	Tumor location	Grade
1	Female	57	Right femur	High
2	Male	19	Left humerus	High
3	Male	21	Left humerus	High
4	Female	36	Right ulna	Low

Chondrosarcoma samples were collected and studied from four patients.

RNA, miRNA PURIFICATION AND REVERSE TRANSCRIPTION (RT)

Total RNA was isolated from the chondrosarcoma cell lines and control cell lines using the illustra RNAspin Mini RNA Isolation Kit (GE). Single-stranded complementary DNA (cDNA) was synthesized from 1.0 μg of total RNA using the qScript cDNA SuperMix for RT–PCR (Quanta) as optimized in our lab [Mak et al., 2012]. Chondrosarcoma patient samples were harvested from human surgical specimens and homogenized in lysis buffer using a motorized rotor–stator homogenizer. Total RNA was isolated using the mirVana miRNA Isolation Kit (Life Technologies). The MicroRNA Reverse Transcription Kit (Applied Biosystems) was used with miRNA-specific primers provided with the TaqMan MicroRNA Assay (Applied Biosystems) to convert miRNA to cDNA.

REAL-TIME POLYMERASE CHAIN REACTION (PCR)

The expression of SOX9, ETV5, MMP-2, and miR-145 was analyzed using real-time RT–PCR. In brief, real-time PCR analysis was performed on cDNA synthesized from the total RNA of chondrosarcoma and control cells using the StepOnePlus Real-Time PCR System (Applied Biosystems) with the LuminoCt SYBR Green qPCR ReadyMix (Sigma–Aldrich Co.) according to the manufacturer's instructions. Cycling consisted of 40 cycles of 3 s at 95°C and 30 s at 60°C, operated with the StepOne Software v2.3. PCR experiments were performed in triplicate and included negative no-template controls. mRNA Primer pairs (Table II) were designed using the Real-time PCR Primer Design software (VWR GenScript Corp.), and synthesized (Integrated DNA Technologies). In addition, primer pairs for two housekeeping/reference genes, GAPDH and RPS18, were also designed and synthesized. For miRNA, the TaqMan MicroRNA Assay (Applied Biosystems) was performed with hsa-miR-145 TaqMan probes on the mature miR-145 sequence GUCCAGUUUUCCCAG-GAAUCCCU to quantitate only mature miRNAs by real-time PCR.

RELATIVE QUANTIFICATION USING REAL-TIME PCR

RPS18 was designated as the reference gene for relative quantification, through which the expression of endogenous mRNA from

TABLE II. Human mRNA Primer Sequences Special Designed for Real-Time PCR Amplification

Gene	Forward/Reverse	Primer sequence	Accession #	Size (bp)
GAPDH	F	5′ CAT GAG AAG TAT GAC AAC AGC CT 3′	NM_002046	113
	R	5′ AGT CCT TCC ACG ATA CCA AAG T 3′		
RPS18	F	5′ GAT GGG CGG CGG AAA ATA G 3′	NM_022551.2	165
	R	5′ GCG TGG ATT CTG CAT AAT GGT 3′		
SOX9	F	5′ GAG GAA GTC GGT GAA GAA CG 3′	NM_000346	136
	R	5′ GGA GTG CAC CTC GCT CAT 3′		
ETV5	F	5′ TCA GCA AGT CCC TTT TAT GGT C 3′	NM_004454.2	119
	R	5′ GCT CTT CAG AAT CGT GAG CCA 3′		
MMP-2	F	5′ ACA TCA AGG GCA TTC AGG AG 3′	NM_004530.4	131
	R	5′ CTG AGC GAT GCC ATC AAA TA 3′		

chondrosarcoma cells was normalized. Similarly, a small-nucleolar reference RNA, RNU48, was used as the endogenous control for real-time quantitation of miRNAs (control RNU48 sequence: GATGACCCCAGGTAACTCTGAGTGTGTCGCTGATGCCATCACCG-CAGCGCTCTGACC). Cycle threshold (Ct) numbers were derived from the exponential phase of PCR amplification. Relative changes in mRNA and miRNA expression were calculated using the comparative ΔΔCT (crossing point) method. All results in this study are calculated from the mean of triplicate experiments.

WESTERN BLOTTING

Chondrosarcoma cells were plated and grown to confluence. Cells were scraped and collected in Nonidet P-40 (NP40) lysis buffer containing protease inhibitor cocktail tablets (Hoffmann-La Roche). The lysate was centrifuged at 10,000*g* for 20 min. Protein concentration was determined by the BCA Protein Assay (Pierce) following the manufacturer's instructions. Lysates were electrophoresed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) at 90 V for 90 min, and then transferred to a PVDF membrane using a semi-dry transfer cell (Bio–Rad) at 20 V for 45 min. Blots were blocked overnight with 5% skim milk in 1 × TBS-T (Tris-buffered saline with Tween 20) and then incubated with polyclonal anti-human SOX9 antibody (1:1000, Millipore) or monoclonal anti-human beta actin antibody as the loading control (1:2000, Millipore) for 2 h at room temperature. Blots were subsequently incubated with appropriate secondary antibody and visualized using the Amersham ECL Western Blotting System (GE Healthcare) according to the manufacturer's instructions. The western blots were repeated in triplicate and densitometry was measured and plotted with ImageJ software version 1.48.

IMMUNOFLUORESCENCE ASSAY

Cells were grown on cover slips and were fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.2% Triton x-100 for 5 min. Subsequently, slides were incubated for 1 h at room temperature with polyclonal anti-human SOX9 antibody (1:1000, Millipore). Slides were further incubated in secondary antibody conjugated with Texas red for 1 h. Slides were washed, incubated with DAPI and phalloidin conjugated with Alexa 488, mounted with 50% glycerol, and visualized with fluorescence microscopy as optimized in our lab [Mak et al., 2011].

REPORTER LUCIFERASE ASSAY

Construction of the ETV5 reporter was performed according to the standard cloning protocols. Briefly, the sequence for the human ETV5 promoter (1333 bp) was amplified and subcloned into a pEZX-PG04 vector (GeneCopoeia Inc.). The plasmid pEZX-PG04-ETV5 encodes Gaussia Luciferase (Gluc) under the control of ETV5-promoter and secreted alkaline phosphatase (SEAP) under the control of the CMV promoter.

Cells were plated and transfected with a pEZX-PG04-ETV5 plasmid using EndoFectin Plus reagent (GeneCopoeia Inc.). Forty-eight hours after transfection, cell-free conditioned medium samples were assayed for Gluc and SEAP using a luminometer according to the manufacturer's instructions. Transfection efficiency was normalized to the level of SEAP activity. The EF1α promoter reporter positive control clone (with GLuc, Puromycin-resistance, and SEAP) and a negative non-promoter sequence control clone (with GLuc, Puromycin-resistance, and SEAP as well) were used as controls.

LENTIVIRUS PRODUCTION AND INFECTION FOR SOX9 AND miR-145

The full-length human SOX9 gene was cloned into a lentivirus vector with GFP expression (Applied Biological Materials Inc.). For miRNA, the full-length mature miR-145 in precursor sequence was cloned into a lentiviral vector with eGFP (GFP-tagged hsa-mir-145 microRNA lentivirus, GeneCopoeia Inc.) for miR-145 overexpression. *Escherichia coli* DH5-alpha competent bacteria cells were transformed with the lentiviral vector, and positive bacterial clones producing optimal plasmids were selected. Then, lentiviral particles were collected and packaged in 293 T cells by co-transfection with the third generation lentiviral packaging system including pMDG-VSVG envelope, pMDLg/pRRE and pRSV-Rev packaging vectors. The lentiviral titer was determined by qPCR (Applied Biological Materials Inc.). Chondrosarcoma cells were infected with 5 multiplicity of infection, and 5 μg/mL polybrene added to the cultures. After overnight culturing medium was changed, cells were split 48 h later, and grown thereafter in 4 μg/mL puromycin for selection of infected cells. Negative blank vectors expressing red fluorescence were included as negative controls for both SOX9 and miR-145 (miRNA scrambled control clone) and all plasmids were verified by DNA sequencing.

TRANSIENT PROTEIN TRANSFECTION

Chondrosarcoma cells were transfected with the SOX9 recombinant proteins (Abnova) and ProteoJuice protein transfection reagent (EMD Chemicals Inc.) according to the manufacturer's protocol. Transfections were performed in serum-free media. Three hours after exposure to the recombinant protein/ProteoJuice complex, media containing 10% fetal bovine serum was added. Cells were incubated

for 24 h following transfection and then medium were collected. Conditioned medium were studied with luciferase assays.

STATISTICAL ANALYSIS

GraphPad Prism software (GraphPad Software, Inc.) was used for statistical analysis. All data are presented as mean ± standard error of the mean (SEM), and are representative of measurements that were performed on four different chondrosarcoma patient samples (n = 4). To assess variations in real-time PCR gene expression, analysis of variance (ANOVA) and the post-hoc multiple comparison Tukey test (* $P < 0.05$ or $P < 0.01$) were applied. Measurements were normalized to the negative control. P values <0.05 were considered to be statistically significant. Each experiment was performed at least three times for each tumor specimen and cell line.

RESULTS

EXPRESSION LEVEL OF SOX9 IN CHONDROSARCOMA CELLS

To determine the baseline expression level of SOX9 in chondrosarcoma cells, multiple approaches including real-time PCR, Western blotting and immunofluorescence were used in this study. Both chondrosarcoma cell lines JJ and KC showed much higher mRNA expression of the SOX9 gene than the control cell line hFOB after normalization (Fig. 1A). SOX9 expression in JJ was sevenfold higher and in KC was 2.5-fold higher than control cell lines. A correspondingly high SOX9 protein expression level was observed by Western blotting, confirmed with densitometry (Fig. 1B). Given the significantly higher level of SOX9 expression, the chondrosarcoma cell line JJ was selected to be the working cell line for all subsequent experiments. Lastly, the location and distribution of the SOX9 protein in chondrosarcoma cells was detected with Texas red, and co-stained with DAPI for the nuclei and Alexa 488-conjugated phalloidin (Fig. 1C). The expression of SOX9 in human chondrosarcoma cells was strongest within the nucleus as demonstrated by in situ fluorescent immunocytochemistry.

LUCIFERASE ASSAY WITH ETV5 PROMOTER AND SOX9 LENTIVIRUS CO-TRANSFECTION

To determine if SOX9 regulates ETV5, co-transfection of an ETV5 gene promoter and a SOX9 lentiviral vector in chondrosarcoma cells

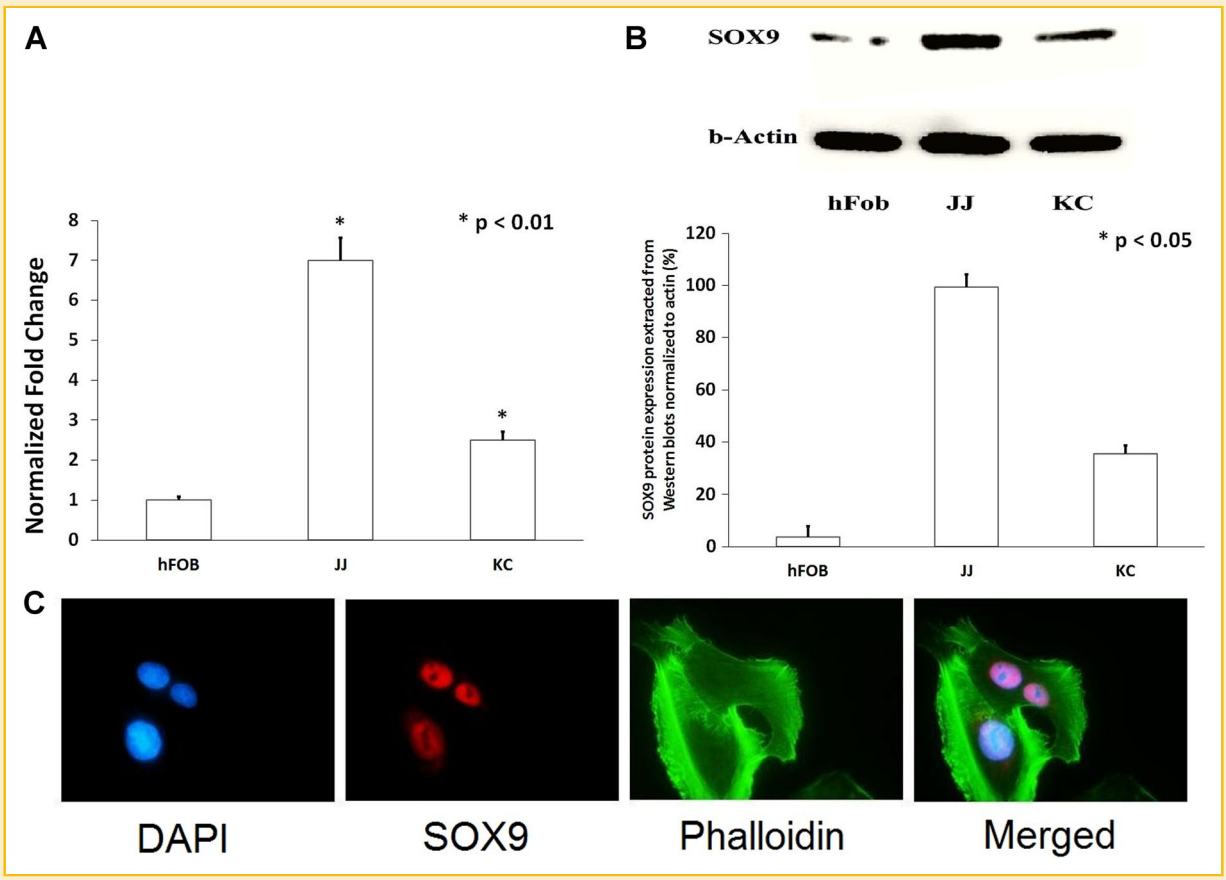

Fig. 1. Expression of SOX9 in chondrosarcoma cells. (A) Total RNA extracted from chondrosarcoma cell lines JJ, KC and the control cell line hFOB was analyzed using real-time PCR. MRNA expression of SOX9 gene exhibits higher fold changes in both chondrosarcoma cell lines than hFOB using RPS18 mRNA for normalization. Normalized fold changes of three repeats with $P < 0.01$ were plotted (*). (B) SOX9 protein level was detected using Western blot. Beta actin protein was used as a loading control. JJ chondrosarcoma cells express high protein levels of SOX9 compared to controls. Densitometry was measured from triplicate western blots and plotted in bar graph format. (C) In situ fluorescent immunocytochemistry shows strong nuclear expression of SOX9 labelled with Texas red (red), and co-stained with DAPI for the nuclei (blue) and Alexa 488-conjugated phalloidin (green) in JJ chondrosarcoma cells. Representative pictures were taken with corresponding filters using a fluorescent microscope with 100× magnification.

was performed and assessed with luciferase assays and real-time PCR. This experiment examines both endogenous and transfected ETV5 expression. Chondrosarcoma cell transfection with the ETV5 promoter-reporter plasmid resulted in significantly up-regulated luciferase activity of the ETV5 promoter compared to a negative control plasmid (Fig. 2A). This experiment confirmed that the ETV5 gene is naturally regulated in chondrosarcoma cells. Correspondingly, ETV5 mRNA expression demonstrated a 2.5-fold increment with insertion of the ETV5 promoter–reporter plasmid (Fig. 2B). After confirming that the ETV5 promoter-luciferase assay was functioning in our cell model, the chondrosarcoma cells were infected with a SOX9 stable lentiviral vector (titer of 1×10^8 TU/mL; MOI of 5). Co-transfection of the ETV5 promoter-plasmid with the SOX9 lentivirus significantly increased the luciferase activity derived from the ETV5 promoter (Fig. 2C), indicating a regulatory relationship between ETV5 and SOX9. Likewise, SOX9 mRNA levels increased by 13-fold when the SOX9 lentiviral vector was co-transfected in chondrosarcoma cells (Fig. 2D).

EXPRESSION OF miR-145 IN CHONDROSARCOMA CELLS AND HUMAN CHONDROSARCOMA TISSUE

The expression level of miR-145 was examined in chondrosarcoma cell lines, chondrosarcoma patient samples and data from a public sarcoma database [Sarver et al., 2010]. The intrinsic expression of miR-145 in chondrosarcoma cells was significantly lower than that of RNU48, the stably expressed snRNA (small nuclear RNA) used for normalization (Fig. 3A). Similarly, the expression level of miR-145 in four chondrosarcoma patient samples were all down-regulated compared to the chondrocyte control cell line, CHON (Fig. 3B). In addition, a database set up by Sarver et al. (2010) based on 22 different sarcoma types identified the expression of over 700 miRNAs using miRNA beadarrays [Sarver et al., 2010]. Extracting the sets of miR-145 data points from this public database, the expression of miR-145 is consistent (Fig. 3C) with our observation in that miR-145 is under-expressed in human chondrosarcoma, comparing to normal bone, and similar to the expression in Ewing's sarcoma and osteosarcoma.

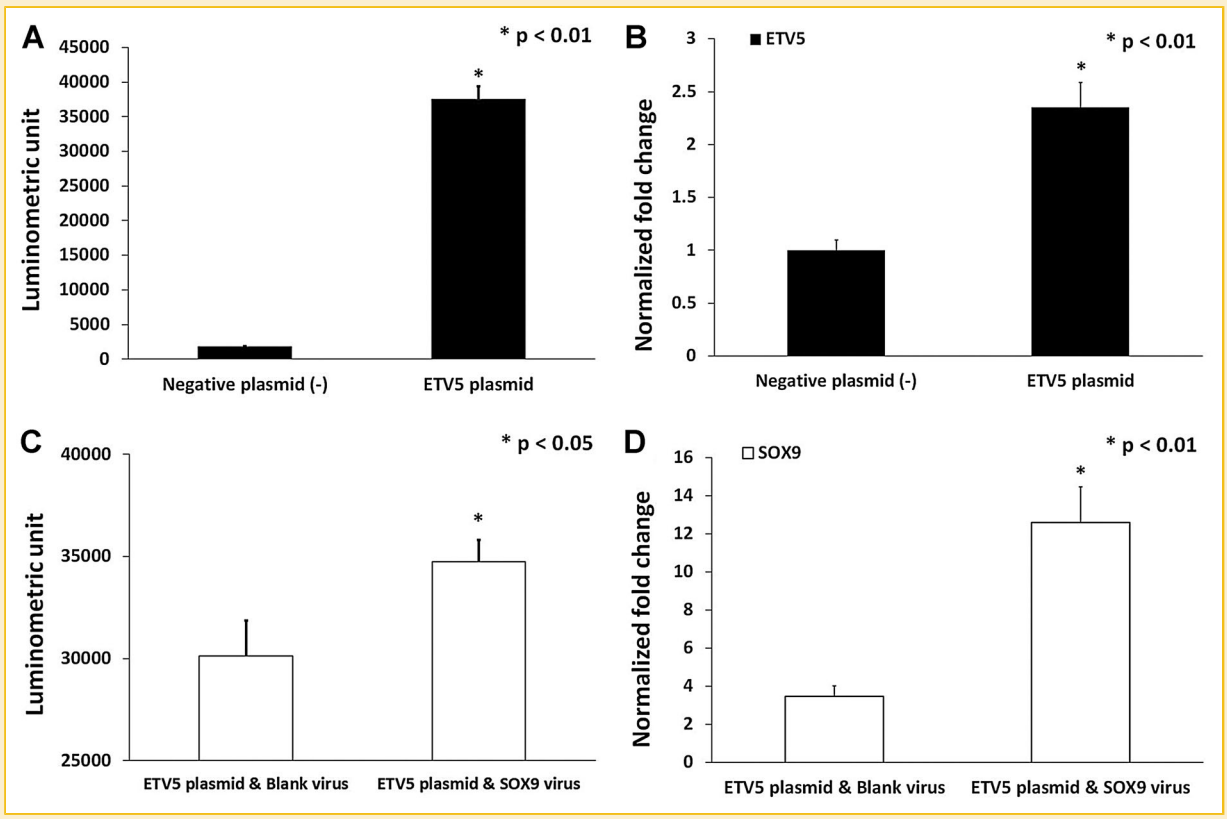

Fig. 2. Luciferase assay with ETV5 promoter reporter and co-transfection with a SOX9 lentivirus. (A) Luciferase activity of chondrosarcoma cells transfected with the ETV5 reporter plasmid and negative plasmid controls are shown. (B) Total RNA extracted from chondrosarcoma cells transfected with the ETV5 reporter plasmid was analyzed using real-time PCR. MRNA expression of ETV5 exhibits significantly higher fold changes in chondrosarcoma cells with the ETV5 promoter-reporter plasmid compared to baseline using RPS18 mRNA for normalization. (C) Luciferase activity in co-transfection of the ETV5 promoter-reporter plasmid and SOX9 lentivirus chondrosarcoma cells versus co-transfection of the ETV5 promoter-reporter plasmid and blank control lentivirus are shown. (D) Total RNA extracted from chondrosarcoma cells transfected with the ETV5 plasmid and SOX9 lentivirus was analyzed using real-time PCR. MRNA expression of SOX9 exhibits significantly higher fold changes in chondrosarcoma cells with the ETV5 promoter-reporter plasmid transfected with SOX9 lentivirus, using RPS18 mRNA for normalization. Normalized luminometric units and fold changes of three repeats with $P < 0.05$ or $P < 0.01$ were plotted (*).

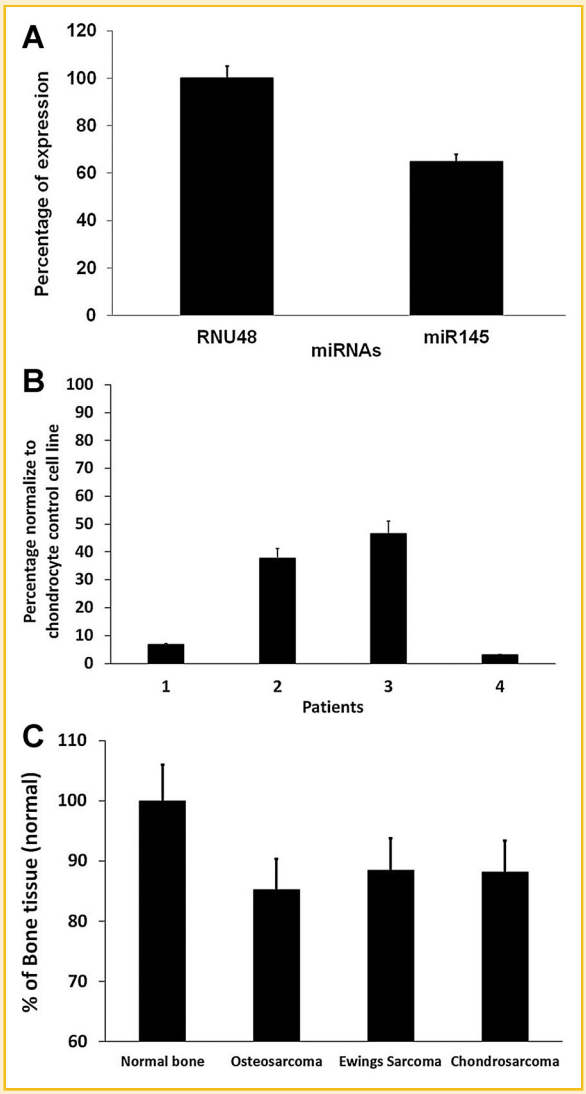

Fig. 3. Expression level of miR-145 in chondrosarcoma cells. Total RNA was isolated and miRNA was reverse transcribed with miRNA-specific primers to cDNA. Real-time PCR was performed with TaqMan probes on the mature miRNA sequence. (A) The intrinsic level of miR-145 in chondrosarcoma cells was low compared to RNU48 (reference snRNA) for normalization. (B) MiRNA was extracted from four chondrosarcoma patient samples. MiR-145 expression from all four chondrosarcoma patients was under 100% compared to the chondrocyte control cell line, CHON. (C) Data of miR-145 measurement was extracted from the S-Med public database for normal bone tissues, osteosarcoma, Ewing's sarcoma, and chondrosarcoma. MiR-145 is under-expressed in these three human bone malignancies.

OVEREXPRESSION OF miR-145 IN CHONDROSARCOMA CELLS

Using stable lentiviral transfection of miR-145, the effect of miR-145 expression on the subsequent mRNA expression of SOX9, ETV5, and MMP-2 in chondrosarcoma cells was examined. In chondrosarcoma cells, GFP expression was observed at day 1 (Fig. 4A) following infection with the miR-145 recombinant lentivirus (titer of 1×10^8 TU/mL; MOI of 5), suggesting that the lentiviral infection of the chondrosarcoma cells was effective and stable. MiR-145 expression was significantly up-regulated (approximately threefold) in the lentiviral-transfected chondrosarcoma cells (Fig. 4B). Conversely, the mRNA expression levels of SOX9, ETV5 and MMP-2 were significantly decreased compared to the negative plasmid-transfected control (Fig. 4C).

DISCUSSION

The etiology of bone cancer remains unknown as it is not a common malignant disease. Among various types of bone malignancies, osteosarcoma contributes the highest proportion, followed by chondrosarcoma and Ewing's sarcoma [Dai et al., 2011]. Chondrosarcoma is the most common primary bone malignancy in the adult population. With the advent of systemic chemotherapy in the management of osteosarcoma and Ewing's sarcoma, a dramatic increase in the long-term survival has been observed [Eriksson et al., 1980; Dai et al., 2011]. In contrast, chondrosarcoma continues to have a poor prognosis owing to not responding well to adjuvant therapy [Dai et al., 2011], regardless that anti-angiogenic agents and radiotherapy may delay progression and sometimes some rare cases of regression are noted [Versleijen-Jonkers et al., 2014]. Due to the fact that chondrosarcoma shows a predilection for pulmonary metastasis, it is important to investigate the potential molecular targets for preventing and treating chondrosarcoma metastasis.

In this study, we confirmed high intrinsic expression of SOX9 in human chondrosarcoma cell lines using real-time PCR, Western blotting and immunocytochemistry. As we have previously verified the expression of ETV5 in chondrosarcoma [Power et al., 2013], the ETV5 promoter-reporter plasmid was further transfected into chondrosarcoma cells to determine if SOX9 regulates the expression of ETV5. Co-transfection of the ETV5 promoter-plasmid with the SOX9 lentivirus significantly increased the luciferase activity derived from the ETV5 promoter, from which the relationship between SOX9 and ETV5 is confirmed. MiR-145 was found to be down-regulated in chondrosarcoma cell lines, chondrosarcoma patient samples, and further confirmed by data extracted from a public sarcoma database. After stable miR-145 lentiviral transfection, the subsequent mRNA expression levels of SOX9, ETV5, and MMP-2 were significantly decreased. Therefore, the relatively low intrinsic levels of miR-145 in chondrosarcoma may trigger downstream MMP-2 overexpression either by allowing SOX9 overexpression, or by directly affecting EVT5 and/or MMP-2 expression. This is a particularly interesting finding given that we have shown that MMP-2 is responsible, at least in part, for bone matrix destruction in chondrosarcoma [Power et al., 2013].

SOX9 is the essential transcription factor for initial chondrocyte differentiation and cartilage formation [Bobick et al., 2009]. Chondrocytes, which differentiate following the condensation of mesenchymal stem cells, are triggered by SOX9 for the secretion of cartilaginous matrix molecules, such as collagens and proteoglycans [Bobick et al., 2009]. SOX9 has been demonstrated to be highly expressed in chondrosarcoma [Soderstrom et al., 2002]. Moreover, the presence of SOX9 suggests that the tumor cells of mesenchymal chondrosarcoma undergo a normal progression

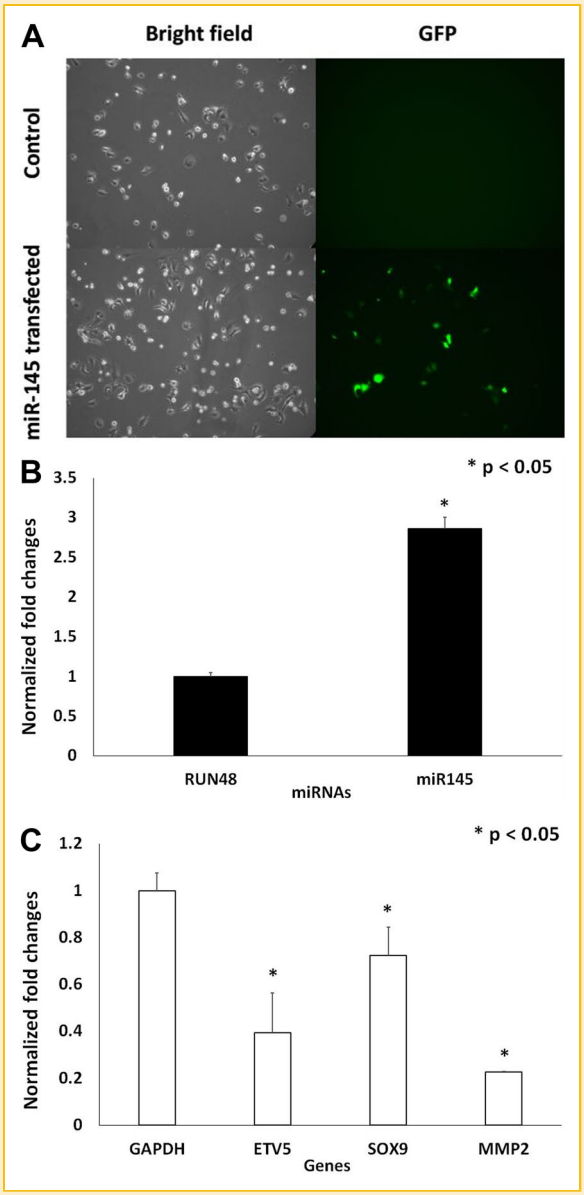

Fig. 4. Overexpression of miR-145 in chondrosarcoma cells. (A) Representative photos taken using the fluorescent microscopy showed successful miR-145 recombinant lentivirus infection to the chondrosarcoma cells comparing to the miRNA scrambled negative blank vector lentiviral-infected control in the bright field photos. (B) Total RNA was isolated and miRNA was reverse transcribed with miRNA-specific primers to cDNA. Real-time PCR was performed with TaqMan probes on the mature miRNA sequence. MiR-145 level in the miR-145-overexpressed chondrosarcoma cells was threefold higher compared to RNU48 for normalization. (C) Total RNA extracted from chondrosarcoma cells transfected with the miR-145 lentivirus was analyzed using real-time PCR. MRNA expression of SOX9, ETV5 and MMP-2 genes were depleted in miR-145-overexpressed chondrosarcoma cells using RPS18 mRNA for normalization. Normalized fold changes of three repeats with $P < 0.05$ were plotted (*).

from condensation to hypertrophy, similar to the process of chondrogenesis seen during fetal development [Fanburg-Smith et al., 2010]. Our findings of SOX9 expression in chondrosarcoma cell lines support data from previous reports and confirm its importance as a marker for cartilage phenotype. Therefore the confirmation that SOX9 is overexpressed in chondrosarcoma highlights the importance of determining SOX9 epigenetic regulation. A recent study showed that SOX9 knockdown causes apoptosis of human chondrosarcoma and chondrocyte cell lines [Ikegami et al., 2011]. Therefore, SOX9 loss-of-function studies would not be considered in this project since it would result in apoptosis in chondrosarcoma cells and chondrocytes.

To further study the downstream effect of SOX9, ETV5 was examined as one of the downstream target genes of SOX9 as it is activated by SOX9 in kidney development [Reginensi et al., 2011]. Our study verified that SOX9 expression leads to a downstream activation of ETV5 in chondrosarcoma. Although we have shown that there is a regulatory link between Sox9 and ETV5, future work would determine direct promoter region binding or protein-protein interaction. ETV5 belongs to the PEA3 subfamily of Ets transcription factors [Oh et al., 2012]. ETV5 has arisen as an inducer of epithelial to mesenchymal transition (EMT) and metastatic target in cancer. The ETV5 over-expressed endometrial carcinoma [Colas et al., 2012] and ovarian cancer cell lines [Planaguma et al., 2011] showed the main features of EMT including modulation of cell adhesion, cell–cell contact, cellular junctions, and actin cytoskeleton reorganization. Concomitant with EMT, the up-regulation of ETV5 in endometrial carcinoma also regulated the MMP-2 promoter regions [Monge et al., 2007]. Similarly, previous studies in our lab have shown that MMP-2, regulated by ETV5, is responsible for calcified bone matrix invasion by chondrosarcoma cells [Power et al., 2013].

One of the challenges in chondrosarcoma research is the identification of therapeutically approachable targets. miRNAs have been presented as a successful diagnostic and therapeutic tool in several cancers [Farazi et al., 2013]. Several studies demonstrated that the differential expression patterns of miRNAs are a promising tool for the diagnosis and treatment of bone malignancies including osteosarcoma [Miao et al., 2013]. In particular, low miR-145 expression has been identified as an independent prognostic factor for osteosarcoma patients [Tang et al., 2013] and miR-145 inhibits invasion and metastasis in osteosarcoma cells [Fan et al., 2012]. A recent study has shown that a wide variety of miRNAs were differently regulated in chondrosarcoma using miRNA arrays and real-time PCR [Yoshitaka et al., 2013]. To our knowledge, we are the first group to identify a regulatory relationship between miR-145 and SOX9 expression in bone malignancy. MiR-145 has been shown to inhibit SOX9 in chondrogenic differentiation in mesenchymal stem cells [Yang et al., 2011]. MiR-145 directly represses SOX9 expression in human articular chondrocytes through a unique binding site in its 3′-UTR [Martinez-Sanchez et al., 2012]. Correspondingly, our results with lentiviral overexpression of miR-145 leading to down-regulation of SOX9, ETV5, and MMP-2 suggest that miR-145 may be an epigenetic regulator of SOX9, affecting downstream ETV5 and MMP-2 in human chondrosarcoma.

The pathophysiology of chondrosarcoma is complex while advances in our understanding of this tumor at the molecular levels are improving. This study closes the loop from our previous work between the invasive properties of MMP-2 in chondrosarcoma and

the upstream epigenetic regulation of MMP-2 through SOX9 and ETV5. It is clear that the expression of miR-145 is aberrantly low in chondrosarcoma, hence the lack of transcriptional control of SOX9, leading to downstream activation of ETV5 and subsequent over-expression of MMP-2 and bone invasion. Several diseases are currently being approached with therapeutic miRNA [Nana-Sinkam and Croce, 2013] and therefore the information generated by this study may have important clinical significance in the treatment of patients with chondrosarcoma in that targeted miRNA may have the potential to decrease chondrosarcoma cell invasion and metastasis by targeting MMP-2 upstream. The rarity of higher-grade chondrosarcoma reinforces the need for multi-institutional studies with long-term follow-up in conjunction with scientific research. Advances in molecular pathway targeting therapeutics will likely be the future of systemic treatment of chondrosarcoma and the current research is aimed at identifying these pathways.

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