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MicroRNA-181b regulates articular chondrocytes differentiation and cartilage integrity

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ABSTRACT

MicroRNAs are endogenous gene regulators that have been implicated in various developmental and pathological processes. However, the precise identities and functions of the miRNAs involved in cartilage development are not yet well understood. Here, we report that miR-181b regulates chondrocyte differentiation and maintains cartilage integrity, and is thus a potent therapeutic target. MiR-181b was significantly down-regulated during chondrogenic differentiation of TGF-β3-stimulated limb mesenchymal cells, but it was significantly up-regulated in osteoarthritic chondrocytes isolated from the cartilage of osteoarthritis patients. The use of a mimic or an inhibitor to alter miR-181b levels in chondroblasts and articular chondrocytes showed that attenuation of miR-181b reduced MMP-13 expression while inducing type II collagen expression. Furthermore, over-expression of anti-miR-181b significantly reduced the cartilage destruction caused by DMM surgery in mice. In sum, our data suggest that miR-181b is a negative regulator of cartilage development, and that inhibition of miR-181b could be an effective therapeutic strategy for cartilage-related disease.

1. Introduction

Chondrocytes, which are derived from mesenchymal stem cell progenitors and differentiate through chondrogenesis, synthesize a cartilaginous matrix that includes proteoglycan and macromolecules with complex carbohydrates, such as glycosaminoglycans [1,2]. Chondrogenesis is a prerequisite event for cartilage formation in the developing limb, and proceeds through the steps of mesenchymal cell recruitment/migration, condensation of progenitors, and chondrocyte differentiation and maturation [3,4]. Since mesenchymal condensation governs the pre-patterning of the limb skeleton [5], the regulation of condensation (in terms of size, shape, and timing) is important to ensuring normal skeletal pattern development. The degradation of extracellular matrix macromolecules and decreased expression of chondrocyte proteins is involved in osteoarthritis (OA), a complex degenerative joint disease characterized by recurrent arthralgia and progressive dysfunction. The current treatments for this disease only address symptoms without addressing the underlying causes [6]. Thus, further investigations into the pathology of OA are needed to facilitate the development of effective therapeutic strategies for controlling OA.

MicroRNAs (miRNAs) are single-stranded RNA molecules of 18–24 nucleotides that are generated by the sequential processing of long RNA transcripts by two key RNase III proteins, Drosha and Dicer [7]. Certain miRNAs have been shown to play key roles in diverse regulatory pathways, including development [8,9], cell proliferation/differentiation [10,11], and many other physiological and pathological processes [12,13]. Thus, they are attractive targets for the generation of novel therapeutic agents to treat various diseases [14,15].

Despite considerable evidence regarding the involvement of miRNAs in many biological responses [16,17], the identities and functions of the miRNAs that are involved in cartilage development/degeneration are poorly understood. Studies on Dicer-knockout (KO) mice showed that miRNAs play a critical role in chondrocyte proliferation [18], and a few studies have identified certain miRNAs as being involved in cartilage development. For example, miR-675 regulates type II collagen in articular chondrocytes [19], miR-18 regulates the chondrocytic phenotype by targeting Ccn2/Ctgf [20], and miR-145 can suppress TGF-β3-induced chondrogenic differentiation of mesenchymal stem cells by directly targeting Sox9, the key chondrogenic transcription factor [21]. Furthermore, studies on the expression patterns and regulatory mechanisms of miRNAs have suggested that they have tissue-, developmental stage- and disease-specific roles [22,23].

In this study, we demonstrate that miR-181b is involved in the site-specific chondrogenic differentiation induced by TGF-β3 in

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chondrogenic progenitor cells of the limb bud, and appears to be involved in maintaining cartilage integrity. These findings suggest that miR-18b is involved in the control of cartilage generation/degeneration, and thus may be a useful therapeutic target.

2. Materials and methods

2.1. Cell culture and treatments

Mesenchymal cells derived from the distal tips of Hamburger-Hamilton (HH) stage 22/23 embryo leg buds of fertilized White Leghorn chicken eggs were micromass cultured as previously described [24]. The cells were maintained in 1 ml of culture medium in the absence or presence of 5 ng/ml of TGF-β3 (Calbiochem).

2.2. Primary cultures of human chondrocytes

Human chondrocytes were prepared from macroscopically severely damaged zones of osteoarthritic knee joints obtained undergoing total knee replacement or biopsy of normal cartilages. The study was carried out in full accordance with Wonkwang University ethics guidelines and cartilage samples were collected after obtaining written informed consent of the donors. Cartilage small slices were sequentially digested with 0.06% collagenase (Sigma) then seeded at a density of 1.5×10^4 cells/cm^2 in culture medium consisting of DMEM (Gibco-Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco Invitrogen).

2.3. Analysis of cell condensation and differentiation

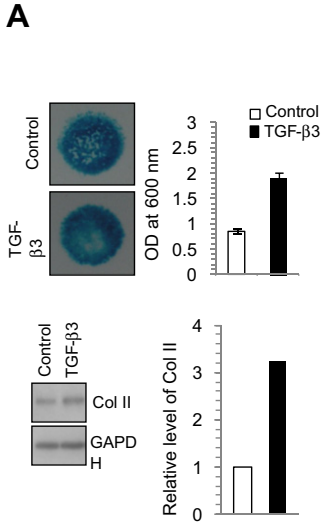
To demonstrate the deposition of cartilage matrix proteoglycans, representative cultures were stained with 0.5% Alcian blue 8GX, pH 1.0. Alcian blue bound to sulfated GAG was extracted with 6 M guanidine-HCl, and quantified by measuring the absorbance of the extracts at 600 nm. To analyze the degree of precartilage condensation, cultures were incubated with 100 μg/ml biotinylated Peanut agglutinin (PNA) (Sigma) for 1 h and bound PNA was visualized using the VECTASTAIN ABC and DAB substrate solution kit (Vector laboratories Inc., Burlingame, CA).

2.4. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

The PCR program consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 10 s at 95 °C, 15 s at 60 °C and 17 s at 72 °C using to amplify chicken type II collagen (5′-agaaaggaatccagcccaat-3′ and 5′-acacctgccagattgattcc-3′), human MMP-2 (5′-acaccaagaacttcgtctg-3′ and 5′-tgcagatctcaggagtgaca-3′), human MMP-9 (5′-atttctgccaggaccgcttctact-3′ and 5′-atgtcataggtcacgtagcccact-3′), human MMP-12 (5′-gaaccaacgcttgccaaatcctga-3′ and 5′-ttcccacggtagtgacagcatcaa-3′), or human MMP-13 (5′-ttgcagagcgctacctgagatcat-3′ and 5′-tttgccagtcacctctaagccgaa-3′) using monitoring SYBR Green I. To normalize the output, the expression of each gene of interest was divided by GAPDH gene (chicken: *gapdh* 5′-agtcatccctgagctgaatg-3′ and 5′-tactttggctggtttctccag-3′, human: *gapdh* 5′-gatcatcagcaatgcctcct-3′ and 5′-tgtggtcatgagt ccttcca-3′) expression, a commonly used housekeeping gene.

2.5. RNA preparation and miRNA real-time PCR

Total RNA was isolated using the mirVana miRNA isolation kit (Ambion). For miRNA microarray analysis, the RNA samples were processed and hybridized to a miRNA microarray (Panagene, Korea), containing 200 miRNA probes. Data were analyzed using



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miRs	Mature Sequence	miRs	Mature Sequence
gga-miR-7	UGGAAGACUAGUGAUUUUGUUG	gga-miR-130b	CAGUGCAAUAAUGAAAGGGCGU
gga-miR-9*	UAAAGCUAGAGAACCGAAUGU	gga-miR-138	AGCUGGUGUUGUGAAUC
gga-miR-15a	UAGCAGCACAUAAUGGUUUGU	gga-miR-155	UUAAUGCUAAUCGUGAUAGGGG
gga-miR-16-2	UAGCAGCACGUAAAUAUUGGUG	gga-miR-181b	AACAUUCAUUGCUGUCGGUGGG
gga-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG U	gga-miR-183	UAUGGCACUGGUAGAAUUCACUG
gga-miR-17-3p	ACUGCAGUGAAGGCACUUGU	gga-miR-193b	AACUGGCCCACAAAGUCCCGCUU U
gga-miR-18b	UAAGGUGCAUCUAGUGCAGUUA	gga-miR-194	UGUAACAGCAACUCCAUGUGGA
gga-miR-20b	CAAAGUGCUCAUAGUGCAGGUAG	gga-miR-199-1	CCCAGUGUUCAGACUACCUGUUC
gga-miR-21	UAGCUUAUCAGACUGAUGUUGA	gga-miR-200b	UAAUACUGCCUGGUAAUGAUGAU
gga-miR-22	AAGCUGCCAGUUGAAGAACUGU	gga-miR-202	AGAGGCAUAGAGCAUGGGAAAA
gga-miR-23b	AUCACAUUGCCAGGGAUUACC	gga-miR-203	GUGAAAUGUUUAGGACCACUUG
gga-miR-24	UGGCUCAGUUCAGCAGGAACAG	gga-miR-204-1	UUCCCUUUGUCAUCCUAUGCCU
gga-miR-26a	UUCAAGUAAUCCAGGAUAGGC	gga-miR-205a	UCCUUCAUUCCACCGGAGUCUG
gga-miR-30a-5p	UGUAAACAUCCUCGACUGGAAG	gga-miR-206	UGGAAUGUAAGGAAGUGUGUGG
gga-miR-30a-3p	CUUUCAGUCGGAUGUUUGCAGC	gga-miR-214	ACAGCGGGAACAGACAGGCAG
gga-miR-30b	UGUAAACAUCCUACACUCAGCU	gga-miR-215	AUGACCUAUGAAUUGACAGAC
gga-miR-30c	UGUAAACAUCCUACACUCUCAGC U	gga-miR-216	UAAUCUCAGCUGGCAACUGUG
gga-miR-30d	UGUAAACAUCCCCGACUGGAAG	gga-miR-219	UGAUUGUCCAAACGCAAUUCU
gga-miR-34a	UGGCAGUGUCUUAGCUGGUUGU U	gga-miR-222-1	AGCUACAUCUGGCUACUGGGUC UC
gga-miR-92	UAUUGCACUUGUCCCGGCCUG	gga-miR-302a	AAGUGCUUCCAUGUUUUAGUGA
gga-miR-130a	CAGUGCAAUAUUAAAAGGGCAU	gga-miR-367	AAUUGCACUUUAGCAAUGGUG

Fig. 1. TGF-β3 stimulates chondrogenic differentiation of wing limb bud mesenchymal cells. (A) Wing mesenchymal cells were treated with or without 5 ng/ml of TGF-β3 and stained with Alcian blue at day 5 of culture. Chondrogenesis was quantified by measuring the absorbance of bound Alcian blue at 600 nm (upper right panel). Changes in protein levels of type II collagen protein (lower left panel) and RNA (lower right panel) were determined by Western blotting and RT-PCR, respectively. (B) Sequences of chick miRNAs. ⁎, statistically different from control cells ($P < 0.005$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

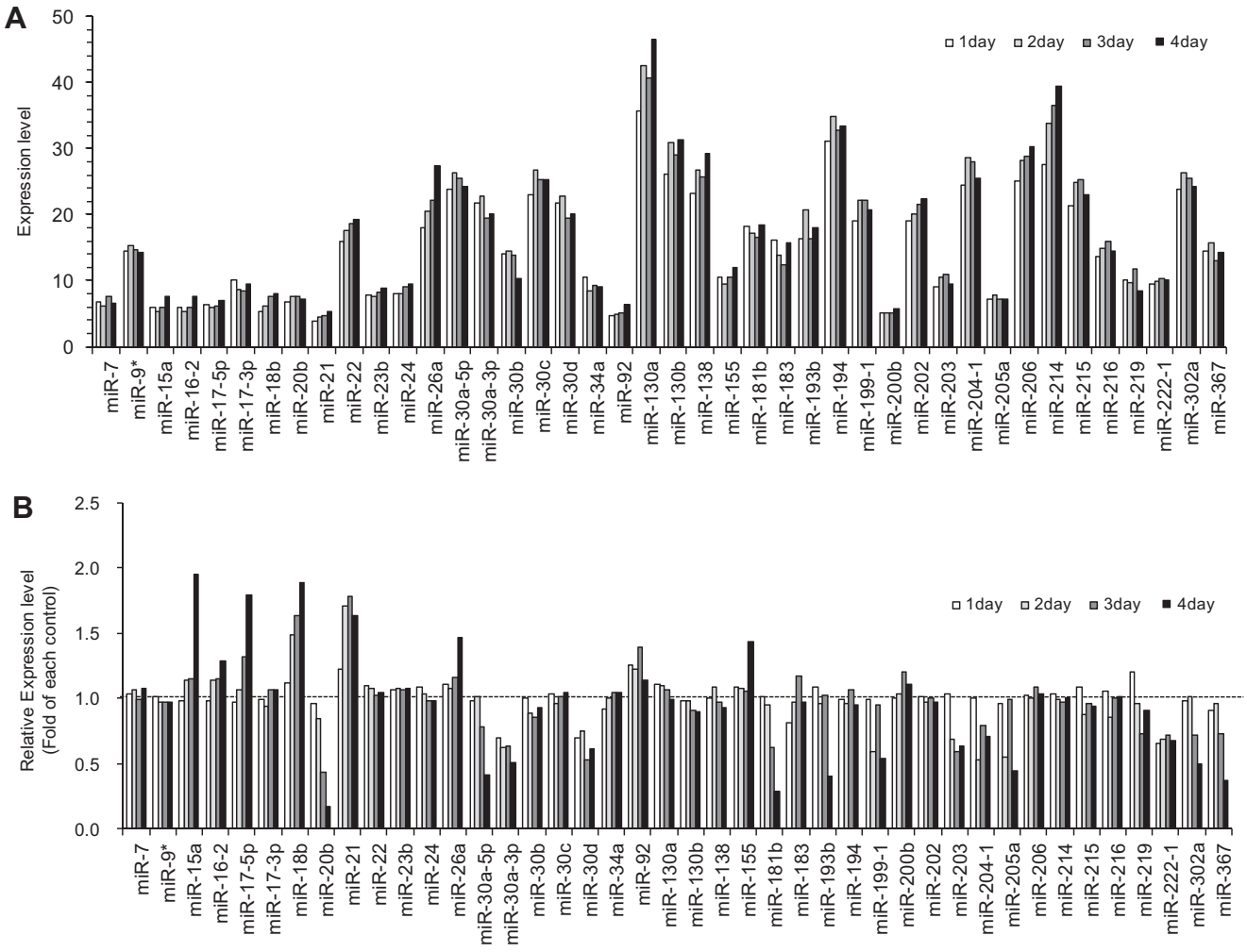

Fig. 2. miR-181b was significantly reduced during chondrogenic differentiation of limb mesenchymal cells. (A) miRNA expression profiles. Wing mesenchymal cells were cultured and performed miRNA array at culture day 1, 2, 3 and 4. (B) miRNA expression profile. Wing mesenchymal cells were cultured in the presence of 5 ng/ml of TGF-β3 and performed miRNA array at culture day 1, 2, 3, and 4 day.

analysis of variance with a multiple comparison corrected *P*-values less than 0.05 and a false discovery rate set to 0.05.

MiRNA-181 expression were independently quantified using the TaqMan MicroRNA and TaqMan gene expression assays Applied Biosystems), respectively, according to the manufacturer's protocols.

2.6. Pre-miR-181b-mediated up-regulation of miR-181b

50 nM of pre-miR-181b (Ambion) were electroporated into isolated mesenchymal cells using a square wave generator (BTX-830; Gentronics, San Diego, CA) with 20 ms, 200 square pulses.

2.7. Experimental OA, and histology of OA cartilage

Experimental OA was induced by destabilization of the medial meniscus (DMM) surgery 8-week-old male mice. Sham-operated animals injected with empty lentiviruses (mock transduction) were used as controls for DMM. Mice were killed 8 weeks after DMM surgery or 2 weeks after intraarticular injection (1×10^9 plaque-forming units (PFU)) of si-miR-181b-expressing lentiviruses for histological and biochemical analyses. Cartilage destruction in mice was examined using Safranin O staining. Briefly, knee joints were fixed in 4% paraformaldehyde, decalcified in 0.5 M EDTA (pH 7.4) for 14 days at 4 °C, and embedded in paraffin. The paraffin blocks were sectioned at 6 μm thickness. The sections were deparaffinized in xylene, hydrated with graded ethanol, and stained with Safranin O.

2.8. Immunohistochemistry

After removing paraffin, sections were incubated with the primary antibody against MMP-13 (Biovision, Milpitas, CA) or pAKT (Cell Signaling, Danvers, MA) overnight at 4 °C, followed by incubation with fluorescent-conjugated secondary antibody at room temperature for 1 h.

3. Results and discussion

Investigations into the precise regulatory mechanisms involved in cartilage development will ultimately contribute to our understanding of cartilage-degenerating diseases and facilitate the development of effective therapeutic targets. Chondrocytes, which are the only cell type in articular cartilage, are differentiated from limb mesenchymal cells via a process (called chondrogenesis) that is regulated by numerous transcription factors and growth factors. TGF-β3 is known to enhance early chondrogenesis and assist in maintaining the chondrogenic phenotype [25].

To verify the effect of TGF-β3 during chondrogenesis, chick wing bud mesenchymal cells were treated with 5 ng/ml of TGF-β3, and

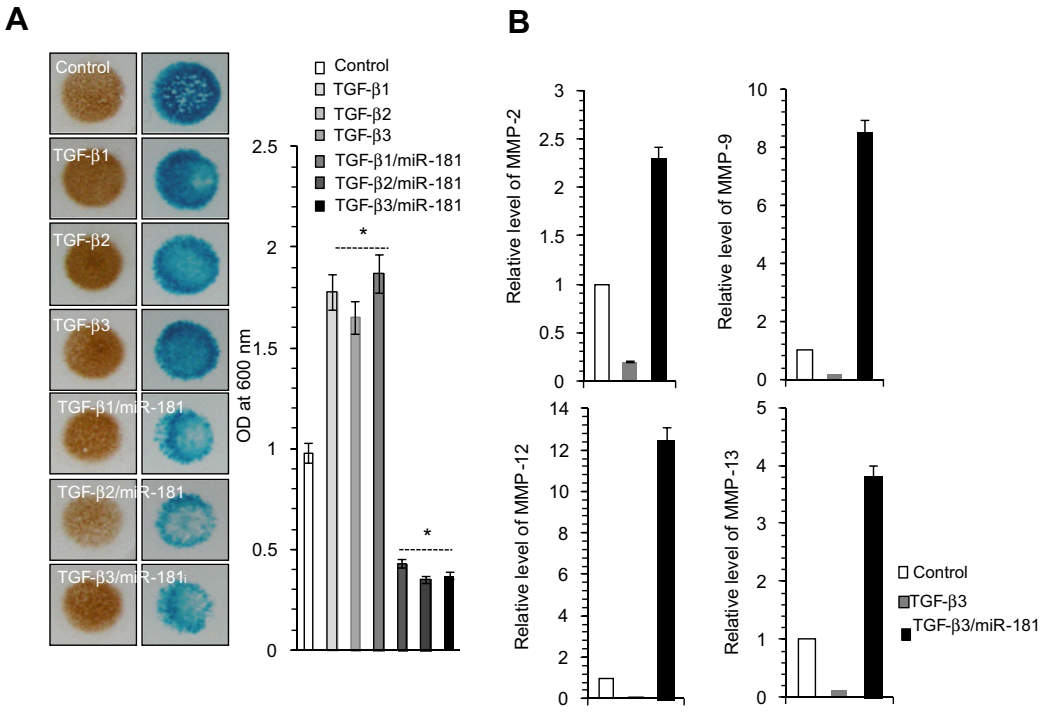

Fig. 3. Alteration of miR-181b levels regulates precartilage condensation and chondrogenic differentiation of limb mesenchymal cells. (A) Wing mesenchymal cells were treated with 5 ng/ml of TGF-β1, β2, or β1 in the absence or presence of miR-181 and stained with Alcian blue at day 5 of culture (left panel). Chondrogenesis was quantified by measuring the absorbance of bound Alcian blue at 600 nm (right panel). (B) RNA levels of MMP-2, -9, -12, or -13 were determined by RT-PCR. ∗, statistically different from control cells ($P < 0.005$).

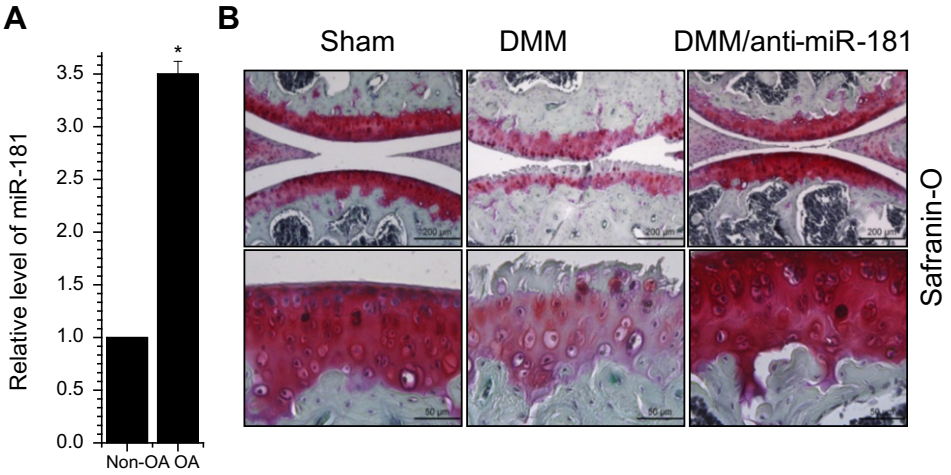

Fig. 4. miR-181 is upregulated in OA chondrocytes and suppression of miR-181 protect DMM-induced cartilage degradation. (A) Images of human articular chondrocytes isolated form damaged zone of OA cartilages (OA) or biopsy of normal cartilage (non-OA) and the expression level of miR-181 was examined by real-time PCR. (B) Mouse cartilages with OA induced by destabilization of the medial meniscus (DMM) were stained with Safranin O. sham-operated (Sham) cartilage was used as control. ∗, statistically different from control cells ($P < 0.005$).

precartilage condensation and chondrogenic differentiation were analyzed by PNA and Alcian blue staining, respectively. Our results revealed that the PNA and Alcian blue staining intensities increased following TGF-β3 treatment (Fig. 1A, upper panel). Freshly dissociated chicken chondrogenic progenitor cells produce type I collagen; as they differentiate into chondrocytes, however, these cells readily aggregate, stop synthesizing type I collagen, and increase their secretion of type II collagen (a typical marker for chondrogenic differentiation). Consistent with this, we found that the protein level of type II collagen increased when cells were exposed to TGF-β3 (Fig. 1A, lower panel). These data suggest that TGF-β3 triggers positive signaling for the chondrogenic differentiation of chick limb bud mesenchymal cells.

In an effort to identify miRNAs that are up- or down-regulated during chondrogenic differentiation, our lab performed a miRNA array screen. Total RNA was extracted and used to determine the expression levels of 42 chicken-specific miRNAs (Fig. 1B) in their mature forms at culture days 1, 2, 3, and 4. Among the miRNAs in the array, we found that miR-9, -22, -26a, -30a, -30b, -30c, -30d, -130a, -130b, -181, -194, -199, -202, -204-1, -206, -214, -215, -302a, and -367 had high expression levels in wing mesenchymal cells during chondrogenesis. Among the miRNAs whose expression was highly sustained during TGF-β3-induced chondrogenic differentiation, miR-181b was significantly down-regulated during the period of precartilage condensation (days 3 and 4 of culture) (Fig. 2). MiR-15, -17-5p, and -26 were

unaltered on days 1–3 but up-regulated on day 4, suggesting that they play roles in chondrocyte differentiation rather than the proliferation, migration, and condensation of chondrogenic progenitors.

To corroborate the involvement of miR-181b in chondrogenic differentiation, we examined the effect of miR-181b in TGF-β3-stimulated cells. We observed decreased PNA and Alcian blue staining of TGF-β3-stimulated cells treated with miR-181b (Fig. 3A), suggesting that miR-181b may negatively regulate cellular condensation in this system. With this decrease in chondrogenic differentiation, the expression levels of MMP-2, -9, -12 and -13 were significantly increased in miR-181b-treated cultures (Fig. 3B), suggesting that miR-181b inhibits precartilage condensation by up-regulating MMPs and perhaps increasing the breakdown of the extracellular matrix.

Members of the miR-181 family, particularly miR-181a and miR-181b, are known to be enriched in brain, and their aberrant expression has been associated with brain diseases [26]. MiR-181 is known to target a number of genes (*e.g.*, the phosphatases, SHP-2, PTPN22, DUSP5 and DUSP6, and the Bcl-2 family member, Mcl-1) in the settings of immune cell differentiation and leukemia [27]. A recent report demonstrated that TGF-β treatment of cancer cells induced miR-181 via smad-4 and increased the invasive ability of the cells [28]. Proteolytic degradation of cartilage is a hallmark of osteoarthritis (OA), and activated chondrocytes are known to produce matrix-degrading enzymes such as MMP-13 in OA joints [29,30]. Recent studies using experimental mouse models with surgical instability in the knee joints (DMM mice) showed that OA is initiated by the production of proteases, including MMP-13 and a disintegrin and metalloproteinase with thrombospondin motif-5 (ADAMTS-5) [31,32]. Furthermore, mice over-expressing MMP-13 showed pathological changes similar to those observed in human OA [33].

To investigate the potential involvement of miR-181b in this pathology, chondrocytes were isolated from 12 OA cartilage samples obtained from patients diagnosed with OA and five normal cartilage samples obtained from biopsies of normal patients. These samples were cultured, and the expression level of miR-181b was found to be significantly higher in OA chondrocytes compared to normal chondrocytes (Fig. 4A). To examine whether inhibition of miR-181b could protect against OA pathogenesis, we suppressed miR-181b in cartilage tissue by injecting anti-miR-9-expressing lentiviruses into DMM mouse knee joints. The cartilage destruction caused by DMM surgery was significantly reduced by expression of anti-miR-181b (Fig. 4B).

In sum, our data collectively suggest that miR-181b is a negative regulator of chondrocyte differentiation/cartilage development, and could therefore be a candidate target for the treatment of cartilage-related diseases.

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