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MiR-221 Attenuates the Osteogenic Differentiation of Human Annulus Fibrosus Cells

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Abstract

Background—In the moderate and end stages of intervertebral disc (IVD) degeneration, endochondral ossifications are found in the IVD.

Purpose—To investigate whether endochondral ossification in the late stages of disc degeneration is due to the differentiation of resident progenitor cell in the annulus fibrosus (AF) and the potential signaling pathways in vitro.

Study Design—An in vitro study of AF cell osteogenic differentiation and possible mechanisms

Methods—Normal AF (NAF) and degenerated AF (DAF) cells were isolated from tissue removed surgically from juvenile patients with idiopathic scoliosis and adult patients with degenerative scoliosis. Osteogenic differentiation was investigated using quantitative RT-PCR and histology. Effects of miR-221 on osteogenesis were measured by overexpression of miR-221 with lentivirus. BMP2 and phospho-Smad protein were detected by Western blotting.

Results—Both NAF and DAF cells underwent osteogenic differentiation, which, was confirmed by detecting mineralization of the cell cultures and by an increase in the expression mRNAs for BMP2, Runx2, ALP, and Osteocalcin. DAF cells exhibited increased osteogenic differentiation potential over the NAF cells. By contrast to the elevated phospho-Smads, the basal level of miR-221 significantly decreased in DAF cells compared with NAF cells. Cultures of both cell types in osteogenic medium showed a decrease in miR-221 expression while overexpression of miR-221 markedly decreased the level of BMP2, phospho-Smads and the expression of osteogenic genes in DAF cells. The osteogenic potential of DAF cells diminished by the overexpression of miR-221.

Conclusion—Compared to NAF cells, AF cells from degenerated discs have a greater tendency for osteogenic differentiation, which involves the BMP-Smad pathways and can be regulated by miR-221. These observations may be developed into a therapeutic to prevent the endochondral ossification.

Keywords

intervertebral disc; miR-221; annulus fibrosus; BMP; osteogenesis

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Introduction

The cause of chronic low back pain has yet to be completely discerned. Intervertebral disc (IVD) degeneration appears to be the leading reason as agreed, by consensus, among the experts in the field. Disc degeneration is a multifactorial process that involves environmental, mechanical, and genetic factors¹. Degeneration of the IVD is typically defined by loss of disc height, narrowing of disc space, fissure formation, and loss of water content, all of which result from a number of changes including biochemistry and cellular micro-environment that lead to structural and functional breakdown. However, the underlying mechanisms of the etiology and pathology are poorly defined.

During the moderate and late stages of disc degeneration, fibrocartilage-like tissue, bone formation, as well as nerve and blood vessels are found in the IVD^{2,3}. For a long time, these tissues were considered to be formed by cells that migrate into the degenerating IVD from adjacent tissues⁴. A few studies have reported the presence of progenitor/stem cells in normal IVDs⁵⁻⁷. We recently demonstrated that the normal AF cells are able to differentiate into cartilage and/or fibrocartilage cells, osteoblasts, neurons, and endothelial in response to different stimuli^{5,8}. We hypothesize that the abnormal endochondral ossification in the moderate/late stage of disc degeneration can be rationalized by demonstrating changes in environmental cues that determine the ultimate fate of the cell niche. Testing this hypothesis would lead to a mechanistic understanding of the differentiation pathways that the resident progenitor cells undergo in the pathophysiology of disc disease.

Small non-coding microRNAs have been reported to play important roles in the physiological and pathological processes by regulating gene expression via mRNA degradation or translational suppression^{9,10}. Numerous microRNAs have been found to modulate stem cell differentiation and phenotype switching. miR-134, 296, 470, 21, 145, 221 and -222 were upregulated in several types of cancer and neointimal lesion of the vascular wall after arterial angioplasty, in which they promote cell proliferation¹⁰⁻¹³. In a rat carotid artery balloon injury model, one group demonstrated that down-regulation of miR-221 suppressed neointima formation¹⁴. miR-221 and miR-222 promote cell cycle progression and proliferation by targeting cyclin-dependent kinase inhibitors p27 and p57¹⁵. Knockdown of miR-221 in human unrestricted somatic stem cells and mesenchymal stem cells increased the expression of the osteogenic genes osteopontin and osteocalcin¹⁶.

Considering the role of miR-221 in the expression of osteogenic genes, it is presumed to play a part in disc degeneration, but this has not yet been explored. In this study, we aim to answer several questions that are directed at the occurrence of endochondral ossification in the degenerated disc: 1) Are there any differences in osteogenic differentiation between normal and degenerated annulus fibrosus (AF) cells? 2) Does miR-221 promote, or suppress, osteogenic differentiation? If so, what is the signaling pathway involved? 3) Can miR-221 expression manipulation be a possible treatment option? Along these lines, we hypothesized that AF cells from degenerated disc may have increased osteogenic potential, which is modified by miR-221 via the BMP-Smad pathways, and, that overexpression of miR-221 could suppress osteogenic differentiation of degenerated AF cells.

Material and Methods

Cell Isolation and Culture

Normal and degenerated human AF cells were isolated from tissue removed surgically from patients with idiopathic scoliosis (three juveniles from 13–16 years old) or patients with disc degeneration (three adults from 48–65 years old). The study was approved by the institutional review board. The discs were exposed with an anterior approach, and an annulotomy was performed with a number-11 blade. The discs were separated from the osseous endplate with a sharp Cobb elevator. The outermost layer of AF and the inner AF adjacent to the nucleus pulposus (NP) were removed to avoid outside AF and NP contamination. The remaining transition zone containing solely AF tissue was then cut into small pieces and digested with 0.01% collagenase (Crescent Chemical, NY) at 37°C for 2–4 hours. The supernatant was carefully removed and centrifuged at 500 g for 10 minutes. The resulting cell pellet was suspended in Dulbecco's Modified Eagle Medium (DMEM) with erythrocyte lysis buffer (160 mM of NH₄Cl) and incubated at room temperature for 10 minutes with gentle agitation. The cell pellet along with undigested AF tissue were plated into a 100-mm tissue culture dish with DMEM/F12 containing 10% fetal bovine serum (Life Technology, CA) and 1% penicillin/streptomycin. The cells were cultured at 37°C in a humidified incubator with 5% CO₂. Culture medium was changed every two days. Isolated AF cells at passage 2–4 were used for later experiments.

Osteogenic Differentiation

To induce osteogenic differentiation, confluent normal AF (NAF) and degenerated AF (DAF) cells were cultured in a 24-well plate with osteogenic induction media for 1, 2, 3, and 4 weeks. Osteogenic induction media consists of DMEM/F12 supplemented with 0.01mM 1,25-dihydroxyvitamin D₃ (R & D Systems, MN), 50mM ascorbate-2-phosphate, and 10 mM β -glycerophosphate, as previously reported^{5, 17}. Control culture medium is growth medium (DMEM/F12+10% fetal bovine serum).

Alizarin Red-S Staining

After osteogenic induction, cells were fixed with 5% formalin for 15 minutes, stained with 0.2% of Alizarin Red S Solution (Chemicon International, MA) for thirty minutes, and viewed under a light microscope. The deposits were washed extensively with distilled water and then inoculated with 250 μ l of 10% cetylpyridiniumchloride (CPC) solution for 15 minutes at room temperature. The optical density was measured at 550 nm with a plate reader (Molecular Devices, CA). Alizarin Red-S in CPC was used as a standard.

RNA Isolation and Real Time RT-PCR

Total RNA was isolated from cultured cells by Trizol reagent (Life Technology, CA) and reverse transcribed to cDNA by an iScript cDNA Synthesis Kit (Bio-Rad, CA) according to the manufacturer's instructions. The quantitative polymerase chain reaction was performed using SYBR green and gene specific primer sets (Suppl table 1) on an iCycler (Bio-Rad, CA). Target gene expression was normalized to 18s using the Ct method¹⁸. To measure the expression of miR-221, total RNA was reverse transcribed with TaqMan MicroRNA

reverse transcription kit (Applied Biosystems, CA). An aliquot of the reaction was used for real time PCR using TaqMan universal master mix (Applied Biosystems, CA). The expression of miR-221 was normalized to GAPDH and 18s using Ct method. Since there is no difference in the expression of either GAPDH or 18s between NAF and DAF, in the later experiments we used 18s to normalize miR-221 expression.

miRNA Infection of DAF Cells

The miR-221 lentivirus carrying miR-221 was purchased from GeneCopoeia. Human DAF cells in a 24-well plate at 80% confluent were infected with lentiviral particles carrying either miR-221 (LP-hmiR0369-MR03) or scramble genes (LP-CmiR0001-MR03, GeneCopoeia, MD) in the presence of 8µg/ml of polybrene following manufacturer's protocol. Cells stably expressing GFP-miR-221 or GFP-scramble were selected with Puromycin. The infection efficiency was measured under a fluorescence microscope (Zeiss, Germany).

Cell Proliferation Assay

Cell proliferation assay was carried out with the WST-1 cell proliferation assay kit (Roche Applied Science, Germany) ¹⁷. Briefly, 100 µl of cells (~5,000 cells) were seeded in 96-well plates in triplicate. Cell proliferation was measured at 1, 3, 5, and 7 days. Ten microliters of the WST-1 reagent was added to the culture medium and cells were incubated for another 3 hours at 37°C. Absorbance of each well was read at an optical density of 450 nm using a plate reader. Wells without cells were assayed as the blank controls that were then subtracted from the corresponding samples.

Western Blotting

Cells were lysed with RIPA buffer (20 mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1mM Na3VO4, 1 µg/ml leupeptin) and cleared by centrifugation. Total protein concentration was determined by Bradford Dc protein assay method. Equal amounts of protein (12 µg) were loaded on reducing SDS-PAGE gels and immunoblotted with specific antibodies for Smad1, p-Smad1, Smad 5 and p-Smad 1/5/8 (Abcam, MA, 1:2000 dilution), and β-actin (loading control, Sigma, MO, 1:2000 dilution). Proteins were detected by ECL (Amersham, PA).

Statistical Analysis

Based on the pilot study, with a standard deviation of 15 and difference of 25, a power of 0.85 to detect a significant difference was achieved with a sample size of 3 for each group. Statistical difference was assessed by one-way analysis of-variance (ANOVA) using Graphpad prism 5 software. The differences between NAF and DAF cells were compared with the Student's t-test (unpaired, two tailed). A p-value <0.05 was considered significant. The data were expressed as the mean± standard deviation. Each experiment was performed in triplicate.

Results

Osteogenic and Chondrogenic Gene Profile

The expression of osteogenic and chondrogenic genes in human NAF and DAF cells were measured. As shown in Fig. 1A, the mRNA expression of *runx2* (Runt-related transcription factor 2), a key transcription factor for osteoblasts, increased 14 % ($p=0.04$); ALP (Alkaline phosphatase), an early marker for mineralization increased 55 % ($p=0.02$), and osteocalcin (OCN, a late marker for osteogenesis) elevated 172 % ($p=0.006$) in DAF cells when compared with NAF cells. No significant difference in *bmp2* mRNA was observed in these two groups ($p=0.07$). In contrast to the elevated expression of osteogenic genes, DAF cells exhibited significantly lower expression of chondrogenic genes when compared with NAF (Fig. 1B), such as type II collagen ($p=0.0002$) and aggrecan ($p=0.0006$).

DAF Cells Exhibit A Stronger Osteogenic Potential Than NAF Cells

We previously demonstrated that NAF cells have the potential for differentiation into multiple lineages, i.e adipocytes, chondrocytes, osteoblasts, neurons⁵. In this study, we compared osteogenesis between AF cells from normal and degenerated human intervertebral discs. Both NAF and DAF cells demonstrated the ability to undergo osteogenic differentiation upon induction of the cells with osteogenic medium as confirmed by Alizarin Red-S staining (Fig. 2A). Under the same induction condition in culture, DAF cells showed higher calcium deposition than the NAF cell group ($p<0.0001$) at 3 and 4 weeks of osteogenic induction. The bar graph shows quantification of Alizarin Red-S (Fig. 2B).

After 1 week of osteogenic induction, the expression of *bmp2*, *runx2*, and *alp* genes was measured by real time RT-PCR and observed to be elevated in both NAF and DAF cells when compared to the control growth medium. The expression level of osteogenic genes was the highest after the 4-weeks induction. The expression level of osteogenic genes was higher in DAF cells than in NAF cells (Fig. 3 shows 3 weeks culture, other data not shown). The order of osteogenic potential was observed to be: DAF+OIM>NAF+OIM>DAF>NAF.

miR-221 Expression Decreased in DAF Cells

Recent studies demonstrated that microRNAs play important roles in the development and pathophysiology processes, and especially miR-221 has been involved in regulating cell differentiation^{19, 20}. We discovered that miR-221 expression decreased in the DAF cells (Fig. 4, $p<0.001$). Osteogenic induction decreased the miR-221 expression in NAF cells ($p=0.007$). Osteogenic conditions further inhibited the expression of miR-221 in DAF cells ($p=0.04$) (Fig. 4).

miR-221 Promotes AF Cell Proliferation

To further test the hypothesis that miR-221 regulates AF cell phenotype, we used the gain of function approach. Human miR-221 was cloned into pEZX-MR03 vector with an EGFP tag. The DAF cells were infected with scramble or miR-221 viral particles. Cells stably expressing miR-221 were selected with puromycin on day 2. There was no difference in cell morphology between scramble microRNA and miR-221 infected groups as shown in Fig. 5A

by phase contrast and EGFP fluorescence images. Real-time RT-PCR confirmed the expression of miR-221 in these cells (Fig. 5B).

As miR-221 plays a major role in cell proliferation, we also tested cell proliferation with the WST-1 proliferation assay¹⁷. As demonstrated in Fig. 6, cell proliferation was significantly increased in DAF cells expressing miR-221 compared to the cells expressing scramble microRNA at day 3 and day 5 ($p < 0.05$ miR-221 vs scramble group).

miR-221 Regulates AF Cell Phenotype

As demonstrated in Fig. 4, the expression of miR-221 was significantly decreased in DAF cells, while the expression of osteogenic genes was elevated in these cells. We further tested the possibility of correlation by overexpressing miR-221 in DAF cells. In contrast to the increase in chondrogenic gene expression, such as collagen II and aggrecan, miR-221 expression indeed decreased the osteogenic gene expression. As shown in Fig. 7A, the expression of *bmp2*, *runx-2*, *alp*, and osteocalcin was significantly decreased in concordance with overexpression of miR-221.

In the presence of osteogenic medium, DAF cells infected with miR-221 exhibited lower osteogenic differentiation potential. After 3 weeks DAF cells infected with the control scramble microRNA showed significant calcium deposition by Alizarin Red-S staining (Fig. 7B). In contrast, miR-221 infected cells showed diminished mineralization. After 4 weeks of incubation in osteogenic medium, the miR-221 group exhibited a small amount of calcium deposition, however, compared with control cells, the amount of mineralization was much lower.

miR-221 Regulates Osteogenesis via Phosphorylation of Smads

All the above results suggested that osteogenic potential was increased in DAF cells. Smad proteins are reported to regulate the canonical signaling cascade of TGF- β super family proteins, and, the receptor-regulated Smads (Smad1, 2, 3, 5, 8) interact with membrane bound serine/threonine receptors to mediate BMP signaling pathways by stimulating the BMP receptors. Therefore, we measured the phosphorylation of Smad1, 5, 8 in NAF and DAF cells. The phosphor-Smad1 and Smad1/5/8 were increased remarkably in the DAF cells as compared to NAF cells (Fig. 8A, $p < 0.001$). Smad phosphorylation was suppressed by miR-221 overexpression (Fig. 8B). The phosphorylation level of Smad1 and Smad1/5/8 was significantly decreased in DAF cells expressing miR-221 compared to cells treated with scramble microRNA. BMP2 protein increased in DAF cells, and, overexpression of miR-221 reversed the BMP2 protein level.

Discussion

Disc degeneration is a complicated process involving both molecular and structural changes, which, results vertebral motion instability as well as compression of the dorsal root ganglia and the nerve root. The IVD is composed of a gel-like NP core surrounded by fibrous lamellae of the AF, and two thin layers of cartilaginous endplates; together, they play an important role in spine motion. AF and NP differ significantly in structure, matrix and cellular composition. NP cells are the remnant of notochord cells that have assumed a

chondrocyte phenotype and are embedded in a proteoglycan-enriched matrix. AF cells are derived from mesenchymal cells with a fibroblast like phenotype and are scattered among well-organized collagen fibers. The mesenchymal stem cell markers have been detected in the NP and AF of degenerated discs ^{21–23}. We and others have shown that progenitor cells exist in human, rat and rabbit healthy discs ^{5, 8, 24} and are able to differentiate into osteogenic, adipogenic, and chondrogenic lineages *in vitro*. In a Rhesus monkey study, another group identified the progenitor cells in NP and demonstrated that the cells are sensitive to low oxygen via hypoxia-inducible factors ²⁵. In a pig annular injury model, Mizrahi et al demonstrated that both healthy and degenerated disc cells are able to differentiate into mesenchymal lineages ²⁶. Numerous studies demonstrated that stem cells maintain homeostasis within the resident tissue and play a critical role in tissue regeneration after injury ^{27, 28}. In certain pathologic conditions, such as osteoarthritis, atherosclerosis, and end stages of the myocardial infarction, resident cells undergo a phenotypic switching that may eventually lead to alteration in cellular function and regeneration ^{29–32}. In the late stage of disc degeneration, blood vessels and neurons are observed in the disc and presumed to be involved in disease progression. Consequently, any perturbation of the cellular environment will promote disc degeneration that ultimately leads to a diminished ability to resist compressive force and low shock absorbing capability. One possible alteration is the switch in disc cell phenotype upon changes in environmental cues.

In this study, we first demonstrated that under a degenerated condition, there is a tendency for osteogenic differentiation of the inner AF cells to increase. Upon stimulation with osteogenic medium, DAF cells demonstrated higher osteogenic activity as demonstrated by real time RT-PCR and Alizarin Red-S staining. Both the early (runx2) and late (osteocalcin) osteogenic marker genes increased in the DAF cells. Then we investigated possible mechanisms that underlie the upregulated osteogenic potential. It was discovered that significantly different levels of miR-221 were present in NAF and DAF. This finding is consistent with the previous study in human unrestricted somatic stem cells and mesenchymal stem cells ¹⁶. In addition, we confirmed that overexpression of miR-221 with lentivirus suppressed the osteogenic potential and that miR-221 played its role via the BMP2-Smad pathways. The potential for osteogenic differentiation may be modulated by post-translational regulation of Smads. Moreover, the elevation of p-Smads was attenuated by overexpressing miR-221 in the DAF cells.

MicroRNAs are known to regulate developmental progression and embryonic stem cell differentiation in vertebrates ^{12, 33}. The primary microRNA transcript is processed by Drosha to form pre-miR hairpin loops. The pre-microRNAs transport from the nucleus into the cytoplasm, where a microRNA complex is formed with Dicer and then incorporated into the RNA-induced silencing complex (RISC) ^{34, 35}. Knockout of *Dicer* prevents the differentiation of embryonic stem cells ^{36, 37}. Thus, microRNAs are able to regulate the expression of target genes and are implicated in many human diseases. microRNA expression and function are cell type and tissue specific, and they also have multiple mRNA targets. miR-221 is demonstrated to play an important role in cancer and vascular diseases, and shown to decrease endothelial cell proliferation and migration by suppressing anti-angiogenic homeobox proteins and MEK/ERK pathways ³⁸. In this study, we have demonstrated that miR-221 is expressed in AF cells and its expression decreases in the

degenerated condition. An osteogenic induction condition further suppresses the expression of miR-221. These results suggest that miR-221 plays an important role in regulating osteogenesis of disc cells. A few other microRNAs also control osteoblastic differentiation, e.g. miR23a, miR-141, miR-200a, and miR-135.

A critical question is the mechanism whereby miR-221 modulates the osteogenic differentiation of disc cells. BMP and TGF- β signaling pathways play vital roles in osteogenesis via regulating runx2, which in turn interacts with receptor mediated Smads to determine cell fate³⁹. In the DAF cells, we showed increased runx2 mRNA expression and phosphorylation of Smad1, 1/5/8 compared to the NAF cells. We propose that up-regulation of osteogenic genes might contribute to the decrease in miR-221 expression, and, this may occur via Smad phosphorylation by up-regulating BMP-2 signaling. As shown in figure 8, overexpression of miR-221 in DAF cells reduced the phosphorylation of Smad, BMP2 protein and osteogenic gene expression. The discrepancy between BMP2 mRNA and protein expression may be due to post-transcriptional and post-translational regulation, which need further investigation. miR-221 suppressed osteogenesis when cells were induced with osteogenic medium up to 4 weeks. Then, it would seem consequential that overexpression of miR-221 promoted AF cell proliferation and elevated the expression of collagen II and aggrecan.

There are some limitations in the current study. The first limitation is in regard to normal and degenerated human AF cell isolation. To obtain the normal cells, we used AF tissue from patients aged 13–16 with idiopathic scoliosis, while the degenerative cells were from ones aged 48–65 with disc degeneration. This was a confounding factor because of age differences. In the future, we could use age-matched disc samples at different stages of degeneration as defined by MRI. Additionally, “normal” disc samples from patients in different age groups could be used to define the effect of aging on AF differentiation and miR-221 expression. The second limitation is that we described the relationship between miR-221 with disc degeneration, but the cause and effect are still unknown. The loss of function with miR-221 inhibitor can further prove its role in osteogenic pathology. Third, the AF cells were cultured in monolayer, which might cause de-differentiation of AF cells. Three-D culture could better maintain the AF phenotype of the cells. Also, it will be of interest to characterize miR-221 expression of AF cells in monolayer vs. 3-D culture.

In summary, AF cells from degenerated discs showed a stronger potential for osteogenic differentiation than AF cells from normal discs. The osteogenic ability may be modulated by post-transcriptional regulation of Smads (1, 5, 8), which, are crucial regulators of signal transduction by BMPs during the process of osteogenesis. Moreover, the elevation of p-Smads is attenuated by overexpression of miR-221 in DAF cells, which, has an important role in cell proliferation and migration, and may serve as a regulator of the mechanotransduction pathway. Our results demonstrate that the osteogenic potential of DAF cells is regulated by BMP-Smad pathways that are potentially modified by the expression of miR-221.

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Figure 1. Osteogenic genes were upregulated while chondrogenic genes were downregulated in DAF compared to NAF cells

Cells were cultured with growth medium and harvested for total RNA isolation. The mRNA expression of osteogenic genes (A) and chondrogenic genes (B) was measured by quantitative RT-PCR with specific primers. The target mRNA was normalized with 18S rRNA. * $p < 0.05$, # $p < 0.01$.

Figure 2. DAF cells exhibited stronger osteogenic potential than NAF cells

After the cultivation of confluent human degenerated and normal AF cells in control or osteogenic medium (OIM) for 3–4 weeks, cells were fixed with 5% formalin and stained with 0.2% Alizarin Red. Micrographs show mineralization (A). The mineralized deposits were washed extensively with water and dissolved with cetylpyridinium chloride for quantitation at O.D. 550 nm with Alizarin Red-S as a standard (B). * $p < 0.05$ con vs OIM, # $p < 0.05$ DAF vs NAF.

Figure 3. Osteogenic medium induced the expression of osteogenic-related marker genes in both DAF and NAF cells and DAF cells showed higher expression

After induction with OIM for 3 weeks, AF cells were harvested for RNA isolation and quantitative RT-PCR. Both NAF and DAF cells expressed early and late bone related marker genes. The target mRNA was normalized with 18s rRNA. * $p < 0.05$ control vs OIM, # $p < 0.05$ DAF vs NAF

Figure 4. The expression of miR-221 was significantly decreased in DAF cells

A. For quantitation of miR-221, total RNA from DAF and NAF cells were transcribed with the microRNA reverse transcription kit and amplified with master mix using human miR-221 specific primers. B. AF cells were cultured with OIM for 1 week, and then miRNA levels were measured by real-time RT-PCR.

Figure 5. Overexpression of miR-221 in DAF cells

DAF cells were infected with either GFP-miR-221 or GFP-miR-scramble lentiviral particles and cells were selected with puromycin. A. Fluorescence imaging showing the expression of GFP-scramble or GFP-miR-221 in degenerated AF cells. B. The expression of miR-221 was confirmed by real-time RT-PCR. Target gene expression was normalized to 18s.

Figure 6. miR-221 increased the proliferation of DAF cells

Cells expressing GFP-miR-221 or GFP-miR-scramble were plated on 96-well plates. The viable cells were measured with WST1 assay at day 1, 3, and 5 days. Optical density was measured at 450 nm for quantitation. *, $p < 0.05$ miR-221 vs scramble.

Figure 7. miR-221 diminished the expression of osteo-related marker/target genes and increased collagen II and aggrecan mRNA

Total RNA was isolated from DAF cells that were over-expressing miR-221. The osteogenic and chondrogenic genes were measured by real-time RT-PCR. Target gene expression was normalized to 18s.

Figure 8. miR-221 regulated the phosphorylation of Smad in DAF cells

A. Phosphor-Smad1/5/8 was markedly increased in DAF cells. p-Smad1 and p-Smad1/5/8 were detected with phosphor antibodies and normalized to total Smad1 and Smad1/5/8. Bar graphs showed the quantification of relative pixels. * $p < 0.05$ NAF vs DAF. B. Overexpressing miR-221 reduced the elevated phosphor-Smad in degenerated AF cells. Bar graphs show the relative pixels. C. BMP2 was increased in DAF cells compared to NAF. Overexpression of miR-221 had a reverse effect. * $p < 0.05$