

**Nfat1 Deficiency Promotes Progression of Post-Traumatic Osteoarthritis through Enhancement of Chondrocyte Hypertrophy and Overexpression of Proinflammatory Cytokines in Repair Cartilage**

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**Introduction:** We currently face major obstacles in our efforts to repair damaged articular cartilage due to the limited intrinsic capacity of articular cartilage to regenerate an appropriate articular surface. Human and animal studies have demonstrated that an osteochondral defect caused by joint trauma or by microfracture surgery can be repaired morphologically through the proliferation and differentiation of bone marrow mesenchymal stem cells into cartilage cells which synthesize a cartilage matrix. However, the repaired articular cartilage tissue degenerates with abnormal expression levels of cartilage markers during the late-stage of repair, and the joints with articular cartilage lesions eventually develop osteoarthritis (OA). Our previous studies revealed that transcription factor NFAT1 (Nfat1) is a key factor regulating the expression of specific proinflammatory cytokines in mouse articular cartilage, and the deletion of the Nfat1 gene may cause OA-like changes in old mice. The objective of this project is to investigate whether Nfat1 deficiency affects chondrocyte differentiation and articular cartilage regeneration during the repair of osteochondral defects in mice.

**Methods:** Nfat1 knockout (Nfat1−/−) and wild-type (WT) mice with Balb/C background, both sexes, were used for creation of osteochondral defects and various tissue analyses. All animal procedures were approved by the Institutional Animal Care and Use Committee. Animal surgery: The osteochondral defect procedure was performed on mice at the age of 2 to 3 months under general anesthesia and sterile conditions. Under a surgical microscope, the knee joint was exposed through a medial parapatellar incision. The patella was then dislocated laterally to expose the trochlear groove. A saw blade attached to a motor was used to make a 0.3-mm wide x 1.5-mm long osteochondral defect penetrating into the subchondral bone marrow cavities in the center of the trochlear groove of the lower femur. The defect was then irrigated with normal saline and the patella reduced to its normal position. The arthrotomy was closed with a size 8-0, absorbable suture and the skin closed with a size 5-0, non-absorbable suture. We also performed sham surgery in which the patellofemoral joint was visualized but an osteochondral defect was not made. Histopathology: Operated mice were euthanized at 2, 12, and 26 weeks after surgery. The knee joints were harvested and processed for histopathological analysis. At least five mice per strain group were examined at each time point. Gene expression: The healing tissue with surrounding host tissue was harvested form the articular cartilage defect sites. Total RNA was isolated from the healing tissue and cDNA was synthesized using RETROscript Kit (Ambion). Quantitative real-time PCR (qPCR) was performed using a 7500 qPCR system (Applied Biosystems) with specific primers and SYBR Green reagents. Chromatin immunoprecipitation (ChIP) assay: To determine if NFAT1 protein directly binds to the promoter of specific target genes in articular cartilage, chromatin was prepared from articular cartilage of WT mice. ChIP assays were performed according to the manual of Magna ChIP A/G Kit (Millipore) using antibodies against mouse NFAT1 protein (Santa Cruz, ChIP grade). An antibody against normal mouse IgG was used as a negative control to confirm the specificity of the ChIP assay. Statistical analyses were performed with Student’s t-test and ANOVA.

**Results:** At 2 weeks after surgery, the proliferation and migration of bone marrow stem cells were more abundant in the osteochondral defects created in Nfat1−/− mice than in WT mice. Chondrocyte differentiation was observed in the Nfat1−/− osteochondral defects but not evident in the WT osteochondral defects (Fig. 1A). qPCR analyses showed up-regulated expression levels of the mRNAs for aggrecan, type-9 collagen, type-10 collagen (a specific marker of hypertrophic chondrocytes), and specific proinflammatory cytokines and matrix metalloproteinases (Mmps) in Nfat1−/− healing tissue (Fig. 2A). At 12 weeks after surgery, osteochondral defects were filled with repair cartilage tissue in both WT and Nfat1−/− mice; however, more hypertrophic chondrocytes and endochondral ossification as well as overgrowth of repair tissues were observed in Nfat1−/− defects than in WT defects (Fig. 1B). The expression of mRNA for type-10 collagen and specific proinflammatory cytokines and Mmps was continuously up-regulated in Nfat1−/− defects (Fig. 2B). At 26 weeks after surgery, repaired subchondral bone and cartilage tissue was partially remodeled to fit the articular surface of the patella in WT mice, with mild to moderate degenerative changes. In contrast, more abundant endochondral ossification and segmentation of repair tissue were observed in the defect sites of Nfat1−/− mice, leading to severe incongruity and destruction of the articular surfaces (Fig. 1C). No significant sex differences in severity of OA were detected at any time-points. OA changes were not evident in the knee joints receiving sham surgery, although some of the Nfat1−/− knees showed mild OA changes at 26 weeks after surgery (data not shown). ChIP assays identified Adamts4, Adamts5, Acan (for aggrecan), Col2a1, Col9a1, Col10a1, Il1b, and Mmp13 as potential NFAT1 target genes in articular
chondrocytes. The expression levels of these genes were significantly altered at specific time-points during the healing of the osteochondral defects (Fig. 2A-B).

**Discussion:** The present study has established a mouse model of OA induced by surgical creation of an osteochondral defect in the patellofemoral joint. Although OA develops in the patellofemoral joints of both WT and Nfat1-/- mice at 26 weeks after the creation of osteochondral defects, destruction of the articular surface with creation of an incongruent joint is more severe in Nfat1-/- patellofemoral joints, indicating that Nfat1 deficiency promotes progression of post-traumatic OA in mice. It is noteworthy that a lack of general healing potential is not demonstrated in the Nfat1-deficient joint. On the contrary, our histological findings indicate the presence of enhanced but dysregulated cartilage proliferation. This suggests that Nfat1 deficiency-mediated development of OA may not be due to a decreased healing capacity but one that is actually more robust, although the articular cartilage is not properly regenerated and repair tissues consist of a mixture of hyaline cartilage, fibrous cartilage, endochondral bone, and fibrous tissues. The mechanisms for Nfat1 deficiency-mediated aberrant proliferation and differentiation of chondrocytes at the osteochondral defect site are not fully understood; previous studies have suggested that overexpression of proinflammatory cytokines, which is observed in the healing tissue of Nfat1-/- patellofemoral joints, may stimulate cell proliferation and differentiation. Our ChIP assays have revealed that Nfat1 is an upstream regulator of genes for specific chondrocyte markers, proinflammatory cytokines, and matrix-degrading proteinases. Thus, Nfat1 deficiency may promote progression of OA through dysregulated expression of Nfat1 target genes in joint tissue cells.

**Significance:** This study has identified that Nfat1 deficiency is a risk factor for the progression of post-traumatic OA because Nfat1 deficiency accelerates articular chondrocyte hypertrophy and articular surface destruction during the healing of articular cartilage defects. These new findings may provide new insights into the prevention and treatment of post-traumatic OA.

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**References:**
Figure 1. Photomicrographs of mouse patellofemoral joints show changes in the patella and femur with different time points: A: 2 weeks post OCD, B: 12 weeks post OCD, C: 26 weeks post OCD.

Figure 2A. qPCR analyses demonstrate temporal changes in expression levels of various genes at 2 weeks post OCD.

Figure 2B. qPCR analyses show further temporal changes in expression levels of the same genes at 12 weeks post OCD.