

The Nuclear Factor of Activated T Cells (NFAT) Transcription Factor NFATp (NFATc2) Is a Repressor of Chondrogenesis

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Abstract

Nuclear factor of activated T cells (NFAT) transcription factors regulate gene expression in lymphocytes and control cardiac valve formation. Here, we report that NFATp regulates chondrogenesis in the adult animal. In mice lacking NFATp, resident cells in the extraarticular connective tissues spontaneously differentiate to cartilage. These cartilage cells progressively differentiate and the tissue undergoes endochondral ossification, recapitulating the development of endochondral bone. Proliferation of already existing articular cartilage cells also occurs in some older animals. At both sites, neoplastic changes in the cartilage cells occur. Consistent with these data, NFATp expression is regulated in mesenchymal stem cells induced to differentiate along a chondrogenic pathway. Lack of NFATp in articular cartilage cells results in increased expression of cartilage markers, whereas overexpression of NFATp in cartilage cell lines extinguishes the cartilage phenotype. Thus, NFATp is a repressor of cartilage cell growth and differentiation and also has the properties of a tumor suppressor.

Key words: cartilage • NFATp • mesenchymal stem cells • chondrosarcoma • differentiation

Introduction

Nuclear factor of activated T cells (NFAT)¹ is a family of transcription factors critical in regulating early gene transcription in response to T cell receptor-mediated signals in lymphocytes (1–6). There are currently four known NFAT genes, NFATp (NFATc2, NFAT1), NFATc (NFATc1, NFAT2), NFAT3 (NFATc4), and NFAT4 (NFATc3, NFATx) (7–11), that

share homology within a region distantly related to the Rel domain. As described below, evidence is emerging that this family of transcription factors controls processes of cell differentiation, likely in response to changes in calcium flux, in progenitor cells of multiple lineages.

In resting T cells, NFAT proteins are present in the cytoplasm as phosphorylated species. Upon activation, sustained increases in calcium activate the phosphatase calcineurin, which subsequently dephosphorylates NFAT (12–14), which is then quickly translocated into the nucleus to drive gene expression in association with other factors such as c-Maf, activator protein 1 (AP-1), and NFAT-interacting protein 45 (NIP45) (15–18). Dephosphorylated NFAT is then rephosphorylated on serine residues by glycogen synthase kinase 3 (GSK3) and translocated back to the cytoplasm (19). The activation-dependent dephosphorylation and translocation of NFAT in lymphocytes and in cardiac endothelial and

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¹Abbreviations used in this paper: BMP, bone morphogenetic protein; CDMP, cartilage-derived morphogenetic protein; COMP, cartilage oligomeric protein; EA, extraarticular cartilage cell line; GDF, growth/differentiation factor; HPRT, hypoxanthine phosphoribosyltransferase; MSC, mesenchymal stem cell; NFAT, nuclear factor of activated T cells; PTHrP, parathyroid hormone-related protein; RT, reverse transcription; wt, wild-type.

muscle cells can be blocked by immunosuppressive drugs such as cyclosporin A or tacrolimus (FK506), which block calcineurin (20).

Much has been learned about the function of NFAT proteins from the generation of NFAT genetic mutant mouse strains. Mice lacking NFATc die in utero from failure to form the semilunar cardiac valves (5, 6), and a role for NFAT3 in cardiac hypertrophy has been elegantly demonstrated (21). T cells from mice lacking NFATc in the lymphoid system (as evaluated by recombination activating gene [RAG]-2 blastocyst complementation) hypoproliferate and have impaired IL-4 production (22, 23), consistent with a function of NFATc as a direct transcriptional activator of the IL-4 gene. Recently, NFATc has also been shown to regulate HIV-1 replication in T cells (24). Evidence that NFATp and NFAT4 might repress proliferative responses, Th2 cell formation, and lymphocyte activation was obtained from the characterization of NFATp and NFAT4 single and double deficient animals. Mice lacking NFAT4 have normal peripheral T cell proliferation and cytokine production, although there is an increase in the number of memory and/or activated T and B cells (25). We and others have previously described the phenotype of mice lacking NFATp (26–28). Such animals display modest splenomegaly with hyperproliferation of T and B lymphocytes and enhanced Th2 responses as measured by increased IL-4 and IgE production. Mice lacking both NFATp and NFAT4 have massive lymphoproliferation and selective activation of the Th2 compartment (29).

The widespread distribution of NFATp in the adult animal suggested that this family member might control cellular differentiation programs in organ systems unrelated to the immune system, and indeed recent evidence suggests that NFATp may participate in processes of adipogenesis and myogenesis (30, 31). Here, we demonstrate that NFATp is a potent repressor of cartilage cell growth and differentiation in the adult animal. Few molecular regulators of chondrogenesis have been identified, and the majority of these affect the embryonic formation of cartilage. To our knowledge, NFATp is the first transcription factor described to control the differentiation of adult mesenchymal stem cells (MSCs) into cartilage. Mice lacking NFATp should prove valuable for the study of cell lineage commitment decisions in chondrogenesis. Further, the potential use of NFATp inhibitors in degenerative and inflammatory joint diseases such as osteoarthritis and rheumatoid arthritis, where cartilage has been destroyed, and in the stimulation of endochondral bone formation to achieve repair of bone defects and fractures, is obvious.

Materials and Methods

Mice. NFATp^{-/-} mice were generated as described (5, 26) and have been backcrossed onto a BALB/c background for at least eight generations. Wild-type (wt) controls were +/+ or +/- littermates. Mice were housed in sterilized microisolator cages, fed autoclaved food and water, and handled in laminar airflow hoods. All animal studies were approved by the Harvard University Institutional Review Board.

Light Microscopic Analysis. For paraffin sections, bone specimens were fixed in 10% buffered formalin (0.1 M PBS, pH 7.4) for 2 wk, decalcified in 25% formic acid for 2–3 wk, processed and embedded in paraffin, cut into 6- μ m sections, and stained with hematoxylin and eosin or safranin O–fast green. For plastic-embedded sections, bones were fixed and decalcified as above and infiltrated for 2–3 wk before embedding in JB4 medium (Polysciences) and sectioned at 4.5- μ m thickness (32). They were stained with 0.5% toluidine blue or safranin O–fast green.

Preparation of Cartilage Cells and Cell Lines, and Stable Transfection of the S12 and Extraarticular Cartilage Cell Lines. Hind limbs of normal and NFATp^{-/-} and NFATp^{+/-} mutant mice were dissected from the torso leaving the femoral joint intact. The femoral head was isolated from the joint, and the articular surface was cut upwards from the neck of the femur. The articular cap was released by producing a small incision through the femoral head and applying pressure with a pair of forceps to release the secondary center of ossification containing trabecular bone. Cells within the articular cartilage that had been cleanly removed free of the underlying trabecular bone or cells from physically distinct masses in the extraarticular soft tissues (EA cells) were dissociated by digestion of the tissue with trypsin/collagenase (33) for 6 h. Cells were plated at a density of 2.5×10^5 /100-mm dish and grown in DMEM/10% fetal bovine serum until reaching confluence at 2–3 wk. Cells were trypsinized and replated at 2×10^6 /100-mm dish. All experiments depicted in these studies are from second passage primary cells.

To generate the S12 cell line, rapidly dividing cultures of NFATp^{-/-} primary cells that had reached confluence were subcloned by limiting dilution (1,000 cells/100-mm dish) and allowed to grow until separate colonies could be visualized. Colonies were picked with cloning discs (Scienceware®; Bel-Art Products) and expanded to establish multiple clonal lines. One such line, S12, was chosen for further study. Stable transfection of S12 and of a bulk population of EA cells was achieved by electroporation at 250 V, 975 μ F of 5×10^6 S12 or EA cells in 400 μ l RPMI 1640 medium without supplements containing 20 μ g of an NFATp expression plasmid in the vector pRep4, or empty vector alone. Selection with hygromycin at 25–50 μ g/ml resulted in the appearance of hygromycin-resistant colonies in \sim 2 wk.

Immunohistochemistry Analysis. Immunofluorescent staining was carried out with antibodies to type II collagen (PF4Z; Caltag) and cartilage oligomeric protein (COMP), provided by Dr. D. Heinegard (Lund University, Lund, Sweden). Immunoreactions were carried out as reported previously using culture pretreatment with monensin to enhance cellular staining of secreted proteins (34). Both phase-contrast and immunofluorescent photomicroscopy were performed using an OM-2 microscope (Olympus Co.) on T-Max® p3200 film (Eastman Kodak Co.).

Reverse Transcription PCR, and Northern and Western Blot Analysis. RNA was prepared from wt and NFATp^{-/-} cartilage cultures and from S12 and EA transfected cell lines. Reverse transcription (RT)-PCR was performed using primers specific for NFATp (upper, 5'-tctcaatcagtcgggctcctatg; lower, 5'-gctcggggcagctgtgtgtgg), NFATc (upper, 5'-tccggcgcagtcgagccgtcatcgtgtgtggatcctga; lower, 5'-ggaccgggtcaattggcaggaaggtactgtgaaacg), NFAT4 (upper, 5'-cttcccagcagcctctcatcc; lower, 5'-ccgtggtgggcaaaaggctcagtg), NFAT3 (upper, 5'-gaagctacctccggtagacag; lower, 5'-gcttcatactgctgtgtagcc), type II collagen (upper, 5'-cctgtctctctttaaacc; lower, 5'-acagaggtgtttgacacag), type X collagen (upper, 5'-cttctcaggatctcagtg; lower, 5'-gagcattgagtgatgacc), cartilage-derived morphogenetic protein (CDMP)-1 (growth/differentiation factor [GDF]5) (upper, 5'-acgggacctgtctttaaag; lower, 5'-cttataccac-

gtgttgg), β -actin (upper, 5'-ctggagaagagctatgagct; lower, 5'-gcc-atgccaatgtgtctct), and hypoxanthine phosphoribosyltransferase (HPRT) (33, 35–37). All RT-PCR was carried out using 1 μ g of total mRNA using a Titan™ One Tube kit (Boehringer Mannheim) according to the manufacturer's instructions. Individual salt and primer annealing temperatures were determined for each primer set. For semiquantitative RT-PCR, initial RT reactions were carried out followed by sequential amplification for five cycles. The resultant cDNAs were then diluted serially 3 times, and PCR was carried out for 24 cycles. Amplicons from control reactions carried out with mRNA isolated from articular cartilage were confirmed by sequence analysis. The products were resolved on a 1.4% agarose gel, stained with GelStar®, and individual band intensities were determined using an Alpha Innotech Image Analysis system. The slopes of the titration curves normalized to that of β -actin were used as a relative approximation of the individual mRNA quantities.

For Northern blot analysis, 10 μ g of RNA was fractionated by electrophoresis on 1.2% agarose/6% formaldehyde gels. Identical gels were blotted to Gene Screen and hybridized with at least 1×10^6 cpm of random primer-labeled cDNA fragments per milliliter of QuikHyb® solution according to the manufacturer's instructions (Stratagene). cDNA fragments were purified from plasmids TGF- β 1,2,3 (gift of J. Letterio, National Institutes of Health, Bethesda, MD) and GDF5,6 (gift of S. Lee, Johns Hopkins Medical School, Baltimore, MD). Nuclear and cytoplasmic extracts for Western blot analysis were prepared from S12 and EA NFATp transfectants. Nuclei were isolated as described (38). Extracted proteins were separated by 8% PAGE followed by electrotransfer to nitrocellulose membranes and probed with an mAb specific for NFATp (Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated goat anti-mouse IgG and enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Pharmacia Biotech).

Proliferation Assays. Wt cartilage cells and NFATp^{-/-} cartilage cells were plated at varying densities in triplicate in 96-well microtiter plates for 48 h and pulsed with [³H]thymidine 16 h before harvesting to measure incorporation into DNA.

Karyotypic Analysis and Generation of Cartilage Cells from MSCs In Vitro. Karyotypic analysis was performed by K. Au (Diagnostic Cytogenetics, Inc., Seattle, WA). The human and mouse MSCs were provided by Drs. A. Caplan and J. Dennis (Case Western Reserve Medical School, Cleveland, OH) and generated as described (39, 40). Chondrogenesis was induced by culturing human MSCs in micromass pellets in the presence of a defined medium that includes dexamethasone and TGF- β 3 (40–45).

Results

Skeletal Abnormalities in Mice Lacking NFATp. We have previously described the generation and immune system phenotype of mice with a targeted disruption at the NFATp locus (26). These mice express reduced amounts of a mutant NFATp protein that fails to bind DNA. Hence, we refer to these mice as NFATp^{-/-}. NFATp^{-/-} mice appeared healthy and were fertile, and the development of the skeletal system was normal. However, after ~6 mo of age, the animals developed progressive difficulty in ambulation accompanied by fixed contractures and a significantly decreased range of joint motion. The phenotype persisted upon multiple backcrosses of the disrupted NFATp locus to the

BALB/c strain. All affected animals had involvement of the hip joints, and in some female mice, similar changes were also observed in other peripheral joints such as the shoulder, knee, and ankle. The phenotype affected 100% of females and approximately one third of males, and symptoms occurred earlier and were much more severe in female animals. Radiograms (Fig. 1) revealed extensive joint destruction and the deposition of extraarticular calcified masses that proved to be cartilage and bone by light microscopy. No abnormalities were noted in the axial skeleton.

Recapitulation of Endochondral Bone Formation in NFATp^{-/-} Connective Tissue. Skeletal morphogenesis of long bones occurs when undifferentiated MSCs differentiate into chondroblasts that synthesize a cartilage matrix. The central portion of this cartilaginous matrix is resorbed to form the marrow cavity (cavitation). The more superficial cartilage cells at the ends of the long bones further differentiate and synthesize the matrix components of the surface and superficial layers of the true articular cartilage. A second group of cartilage cells distally also proliferates and undergoes a progressive differentiation and synthesis of a matrix, which calcifies and is ultimately resorbed and replaced by osteoblasts to form bone (endochondral ossification).

In NFATp^{-/-} mice, visual inspection of the hip joint revealed abnormalities of the articular cartilage and also revealed extraarticular masses of cartilage that not only were spatially distinct from one another in the extraarticular soft tissues but also were easily separable manually from the joint. The articular cartilage in NFATp^{-/-} mice was grayish in color compared with wt littermates and had a visibly roughened surface in contrast to the smooth, polished appearance of the wt control articular cartilage. Both gross analysis and a careful longitudinal histologic analysis were performed on many animals, ranging in age from 2.5–20 mo, and representative sections are shown in Fig. 2. Light microscopic analysis

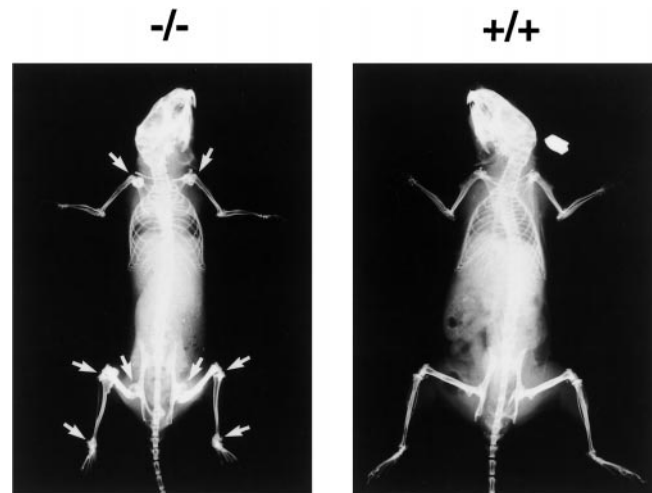


Figure 1. Cartilage masses and joint destruction in NFATp^{-/-} mice. X ray of a severely affected 20-mo-old NFATp^{-/-} female (left) and a +/+ female littermate (right). Multiple calcified masses in the shoulder, knee, hip, and ankle joints and joint destruction are present. X-OMAT Diagnostic film (Eastman Kodak, Inc.) was used, and the exposure time was set at 50 s, 20 kV.

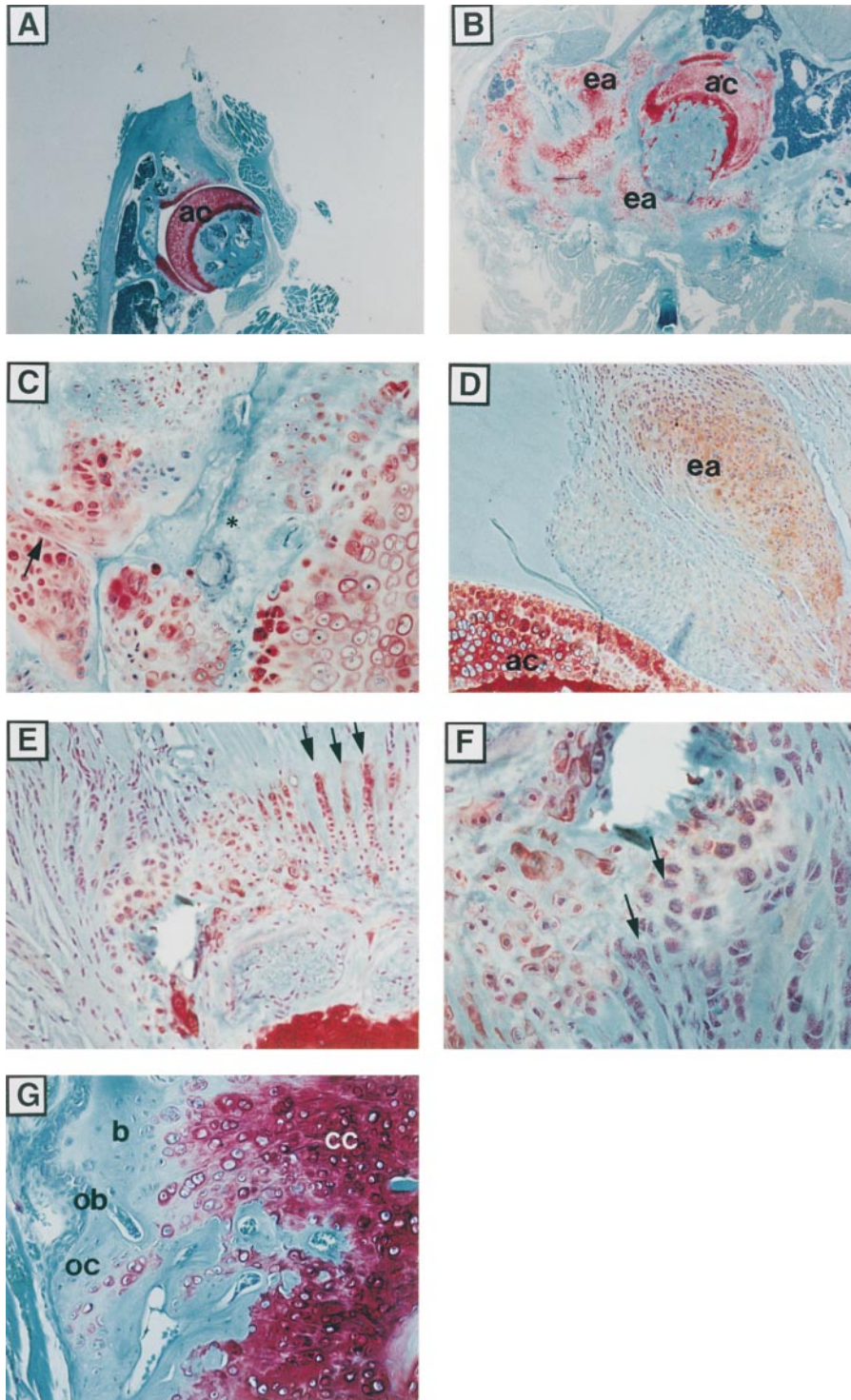


Figure 2. Cartilage cell proliferation in articular cartilage and in extraarticular connective tissue in $\text{NFATp}^{-/-}$ mice. (A and B) Low power (original magnification: $\times 20$) of the femoral heads and acetabula of 12-mo-old control $+/+$ (A) and $\text{NFATp}^{-/-}$ mutant (B) mice, showing thickening of articular cartilage and sites of extraarticular cartilage proliferation and joint destruction in the mutant animal. (C) High power (original magnification: $\times 200$) of B, showing invasion of the acetabulum by proliferating cartilage cells (black arrows) and obliteration of the joint space (white arrows) with loss of safranin-O staining (*). (D) Femoral head and extraarticular connective tissues of 3-mo-old $\text{NFATp}^{-/-}$ mouse (original magnification: $\times 100$), clearly demonstrating the physical separation of the sites of articular and extraarticular cartilage proliferation. (E) Extraarticular connective tissues of 3-mo-old $\text{NFATp}^{-/-}$ mouse demonstrating the differentiation of resident cells into ordered columnar cartilage (arrows). (F) High power (original magnification: $\times 200$) of E. (G) Extraarticular connective tissues of 6-mo-old $\text{NFATp}^{-/-}$ mouse showing calcification of cartilage and the beginnings of endochondral ossification in the extraarticular soft tissue (original magnification: $\times 200$). ac, articular cartilage; fh, femoral head; a, acetabulum; ea, extraarticular; ob, osteoblasts; oc, osteocytes; cc, calcified cartilage; b, bone.

demonstrated proliferation of abnormal-looking cartilage cells in the articular cartilage (Fig. 2, A and B), most apparent in older female animals. When this proliferation occurred, it was apparent in the layer of already existing cartilage cells above the zone of calcified cartilage (Fig. 2, A–C). In the most severe cases, as in the 1-yr-old animal shown, there was extensive degradation of the cartilage and destruction of the joint (Fig. 2, B and C).

In all $\text{NFATp}^{-/-}$ female mice, ectopic formation of cartilage occurred in the extraarticular soft tissues. In extraarticular connective tissue, resident cells rapidly differentiated to safranin-O staining cartilage cells beginning as early as 3 mo of age. Multiple, spatially distinct sites of cartilage formation were formed with time, accompanied by progressive endochondral differentiation of the cartilage cells, columnar arrangement of the tissue, invasion of the cartilage tissue by

capillaries and chondroclasts (osteoclasts), calcification of the cartilage, and replacement of cartilage by bone synthesized by osteoblasts (endochondral ossification) (Fig. 2, D–G). Thus, at 3 mo of age, induction of chondrogenesis in the extraarticular soft tissues occurred with resident cells in the extraarticular soft tissue beginning to differentiate into cartilage and align into typical columnar fashion (Fig. 2, D–F). At 6 mo of age, sequential differentiation of the chondrocytes and the onset of endochondral ossification were apparent (Fig. 2 G). From 3 to 20 mo, there was a steady and progressive increase in the volume of cartilage and bone in the individual extraarticular masses as the process of endochondral bone formation continued, as well as the initiation of new sites of cartilage induction (Fig. 2, D–G). The uncontrolled induction, proliferation, and differentiation of the resident extraarticular connective tissue cells over the 20-mo period studied also extended into and between the fibers of the overlying muscle layers (Fig. 2, B and C).

Proliferation of NFATp^{-/-} Cartilage Cells Is Cell Intrinsic. One explanation for this phenotype is induction of uncontrolled cartilage proliferation by an extrinsic factor(s). Alternatively, the dysregulation observed may be intrinsic to the cartilage cell precursor. To determine whether these characteristics were extrinsic or intrinsic to the cells, we more carefully characterized their phenotype and behavior. Cartilaginous tissue was removed from the surfaces of the femoral and humeral heads and from the extraarticular cartilage masses of affected NFATp^{-/-}, control wt, and NFATp^{+/-} heterozygous mice and placed in culture. NFATp is widely expressed in many adult tissues, but has not been described to be present in cartilage. Examination by RT-PCR of RNA prepared from cells from the cartilage cultures established above did reveal the presence of transcripts encoding NFATp in wt cartilage cells. Further, a mutant transcript corresponding in size to the gene-targeted NFATp allele was detected in the cells established from NFATp^{-/-} cartilage (Fig. 3 a). Interestingly, transcripts specific for the three other NFAT family members, NFATc, NFAT4, and NFAT3, were also present in wt and NFATp^{-/-} cartilage (Fig. 3 a). Examination of the NFATp^{-/-} cultures revealed cuboidal cells that grew in sheets (Fig. 3, b and c). Immunohistochemical analysis confirmed that a high percentage of cells expressed type II collagen and COMP, antigens that are characteristic of dif-

ferentiated cartilage cells (Fig. 3, d and e). These results demonstrated that differentiated cartilage cells expressed NFATp as well as NFATc, NFAT4, and NFAT3, and that the proliferating cells present in the articular and extraarticular cartilage of NFATp^{-/-} animals were indeed of cartilage origin.

Cartilage cells from NFATp^{-/-} mice proliferated in culture, and in some instances displayed uncontrolled growth consistent with malignant transformation (see below). A variety of growth and differentiation factors has been shown to cause induction and proliferation of cartilage cells from primitive mesenchymal progenitor cells in vitro, and in some instances, in vivo. Most notable among these are members of the TGF-β family and the related bone morphogenetic protein factors (BMPs), although the latter appear to be more critical in skeletal morphogenesis than in mature cartilage growth (46, 47). It was possible that NFATp negatively regulated the transcription of one or more of these factors. Therefore, we scanned the NFATp^{-/-} cartilage cell lines for their expression of the TGF-β family members as well as other genes most often thought to be involved in chondrogenesis, and found normal or reduced levels of TGF-β1, TGF-β2, TGF-β3, GDF5 (CDMP-1), GDF6 (CDMP-2), and Sox9 (normal) transcripts compared with wt cartilage cells (not shown). Thus, overexpression of these known regulators of chondrogenesis does not appear to account for the induction of proliferation and differentiation of connective tissue precursor cells to cartilage cells or the proliferation of articular cartilage cells observed in the absence of NFATp in the adult animal. To further address the possibility that these cellular events were driven by secreted factors, wt cartilage cells were cultured with supernatants derived from the NFATp mutant cell lines. No effect on proliferation of wt cartilage was observed (not shown). We conclude that the uncontrolled induction and proliferation of endochondral lineage cartilage cells and of the already existing articular cartilage cells in the absence of NFATp are likely to be intrinsic to the cell, although we cannot rule out a secreted factor that requires other culture conditions to be active.

NFAT Expression Is Regulated during Cartilage Cell Differentiation. NFATp is not expressed in the skeletal system during embryonic development at embryonic day (E)11 and E13.5 (not shown), and mice lacking NFATp display

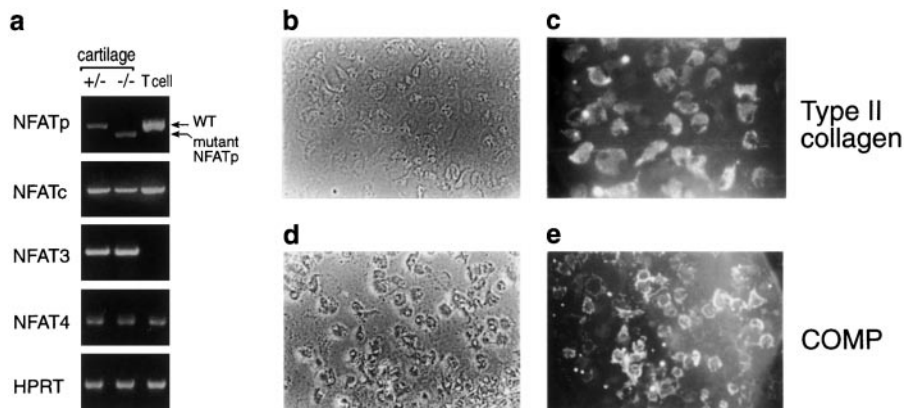


Figure 3. Characteristics of cartilage cells derived from NFATp^{-/-} cartilage. (a) RT-PCR of RNA prepared from wt and NFATp^{-/-} cartilage cells showing presence of wt and mutant NFATp transcripts as well as transcripts for NFATc, NFAT4, and NFAT3. (b) Phase-contrast micrograph of NFATp^{-/-} cartilage cells. (c) Immunohistochemical analysis of the same field stained with an antibody to type II collagen. (d) Phase-contrast micrograph of NFATp^{-/-} cartilage cells. (e) The same field stained with an antibody to COMP.

normal skeletal morphogenesis. Further, we could not detect NFATp protein in articular cartilage cells or in articular cartilage *in vivo* despite multiple attempts using Western blot analysis, immunofluorescence, immunohistochemistry, or labeling with immunogold on frozen or paraffin sections of articular cartilage. We concluded that NFATp is present at very low levels in differentiated cartilage, detectable only by RT-PCR. Therefore, we believed it was more likely that NFATp controlled cartilage cell differentiation at the level of the adult mesenchymal progenitor cell. Recently, Caplan and colleagues have described an *in vitro* pellet culture system whereby purified MSCs harvested from human or rabbit bone marrow can be differentiated along a chondrogenic or osteogenic lineage (39–41, 43). For chondrogenesis, such cultures rely on the addition of TGF- β and the morphometric constraints of a pellet culture system. The expression of NFAT family members was assessed in human MSCs cultured under chondrogenic conditions (Fig. 4). In bone marrow–derived stem cells untreated with exogenous growth factors, so-called “control,” NFATp transcripts were detectable. By day 3 of differentiation, NFATp mRNA levels had markedly increased in stem cells cultured under chondrogenic conditions, and by day 14, NFATp transcripts had further increased. In contrast, in stem cells cultured under osteogenic conditions, transcripts for NFATp were not detectable. Thus, levels of NFATp are specifically regulated during chondrogenesis. Transcripts for the three other NFAT family members, NFATc, NFAT4, and NFAT3, were also present in differentiating chondrocytes. However, in contrast to NFATp, their level of expression did not fluctuate during differentiation and was not restricted to the chondrogenic pathway, as they were also detected in cultures differentiated along an osteogenic pathway (not shown). We conclude from these data and from the *in vivo* phenotype described above that NFATp expression is regulated in undifferentiated human MSCs during chondrogenesis, and that NFATp is the family member likely to control lineage commitment along this pathway in postembryonic MSCs.

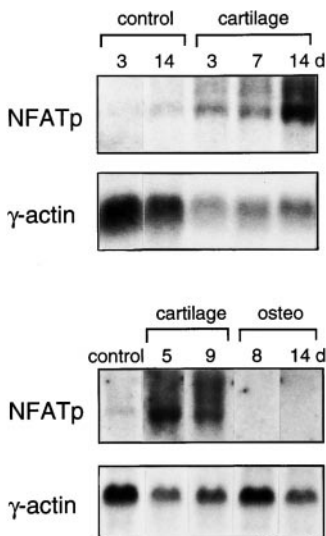


Figure 4. Expression of NFATp is regulated during chondrogenesis. Northern blot analysis of human MSCs undifferentiated or differentiated under chondrogenic (top) or osteogenic (bottom) conditions. Expression of NFATp and actin was determined.

NFATp^{-/-} Cartilage Cells Have Increased Expression of Cartilage Markers, Whereas Ectopic Expression of NFATp in Two NFATp^{-/-} Cartilage Cell Lines Derived from Both Articular and Extraarticular Tissues Extinguishes the Cartilage Phenotype. The uncontrolled proliferation of resident connective tissue cells and their differentiation to cartilage cells and the proliferation of existing articular cartilage cells, coupled with the regulated expression of NFATp in MSC cultures, suggested that NFATp represses the chondrogenic program. To further investigate this possibility, we took two approaches. First, we compared the levels of transcripts for type II and X collagen by semiquantitative RT-PCR in primary articular chondrocytes established from wt and NFATp^{-/-} femoral heads. Fig. 5 A demonstrates that articular chondrocytes lacking NFATp have much higher (at least eightfold) levels of both type II and X collagen mRNA than wt articular cartilage cells.

Second, we stably reintroduced NFATp into S12 and EA, two cartilage cell lines derived from bulk-cultured NFATp^{-/-} articular cartilage and extraarticular cartilage, respectively. Several transfected subcloned S12 lines and bulk-transfected but not subcloned EA lines were derived and analyzed by Western blot for expression of NFATp (Fig. 5 B). A panel of S12 and EA transfectants expressing different levels of NFATp were then analyzed by semiquantitative RT-PCR for expression of characteristic cartilage cell markers (Fig. 5 C). Expression of type II collagen, type X collagen, and CDMP-1 were markedly repressed in all of the S12 NFATp transfected sublines, and a correlation between levels of NFATp protein and repression of cartilage cell phenotype was observed. In two of the three bulk-transfected EA cells, overexpression of NFATp similarly extinguished expression of the type II and type X collagen genes compared with two of the three control vector (Rep) transfectants. The third line (EA.NFAT3) did not show repression of the cartilage phenotype, whereas the third control line (EA.Rep3) did. Therefore, we conclude that the level of expression of NFATp in most instances controls the extent to which a cell manifests a cartilage phenotype, as determined by expression of markers characteristic of differentiated cartilage.

NFATp Has the Features of a Murine Tumor Suppressor Gene. Several pieces of data suggest that the proliferating cartilage cells in NFATp-deficient animals may sometimes undergo transformation. First, the proliferating cartilage cells both in articular cartilage (Fig. 6 D) and in the extraarticular soft tissues (Fig. 6, E and F) varied in size but were usually very large, had increased chromatin content, noticeable mitotic figures, and were safranin O positive (Fig. 6, D–F). Cells with multiple nucleoli were observed (Fig. 6, E and F). Further, this uncontrolled formation of extraarticular cartilage and bone often invaded and extended beyond the connective tissues, progressing between the overlying muscle fibers (Fig. 6, A–C). This cytogenetic picture coupled with the invasion of the acetabulum by proliferating cartilage cells in the ligamentum teres (Fig. 6, A–C) strongly suggested a malignant process, likely accounting for the destruction of the joint in the older animals. Second, in contrast to the slow

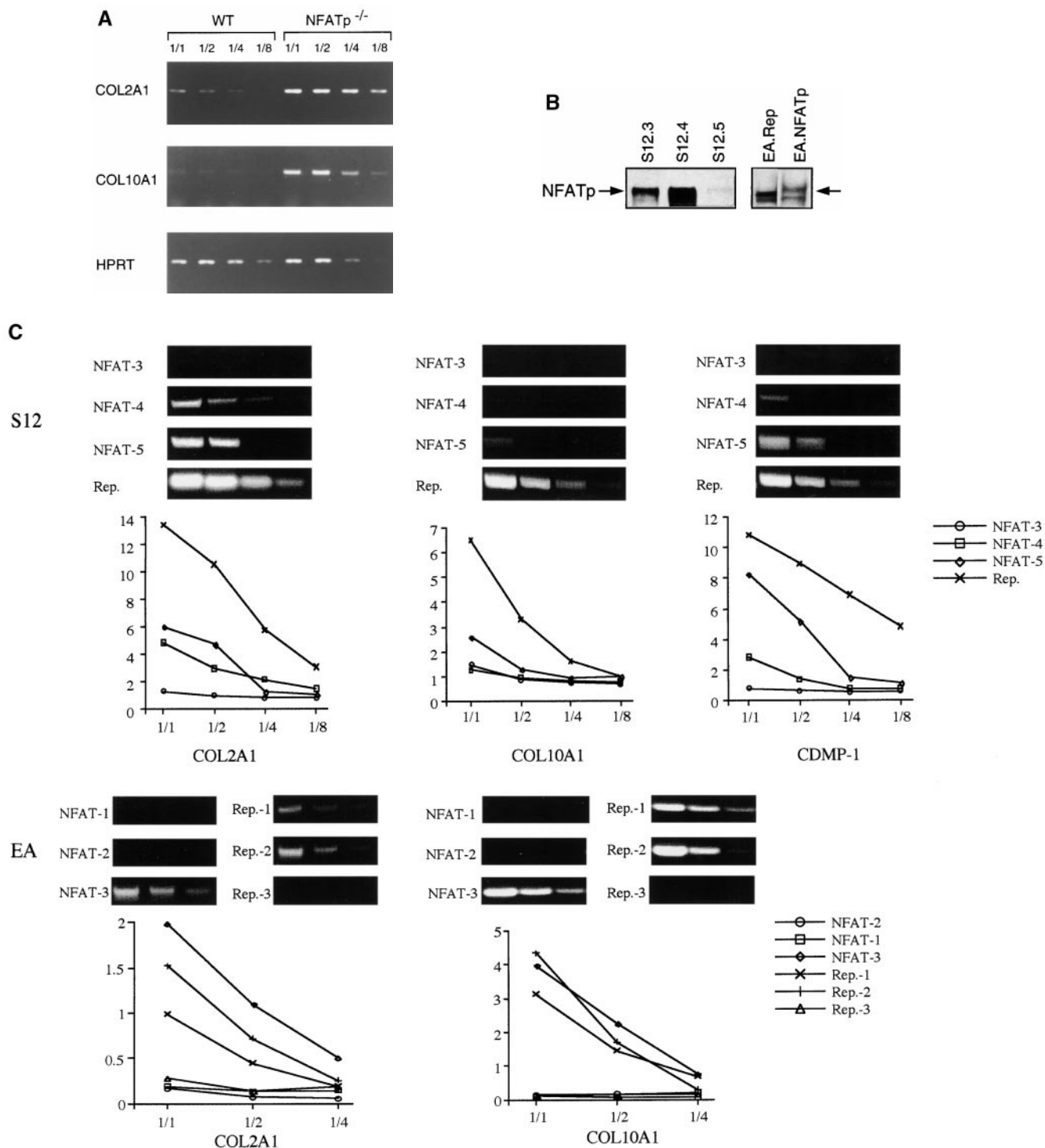


Figure 5. Absence of NFATp enhances, whereas overexpression of NFATp represses the cartilage phenotype. (A) RT-PCR analysis of mature cartilage gene expression in primary wt and NFATp^{-/-} articular chondrocytes using types II and X collagen and HPRT primers. (B) Western blot analysis of S12 and EA control (Rep) and NFATp transfectants with anti-NFATp antibody. (C) RT-PCR analysis of mature cartilage gene expression of S12 and EA control and NFATp transfectants using type II collagen, type X collagen, CDMP-1, and actin primers (references 36–38).

growth of cartilage cells observed in cultures established from wt and heterozygous mice, some of the cultures established from both the articular and extraarticular sites from NFATp^{-/-} mice expanded rapidly, necessitating frequent

passaging. Thus, in contrast to wt cartilage cells, NFATp^{-/-} cartilage cells did not display contact-induced growth inhibition since they continued to proliferate in vitro even when plated at confluency (Fig. 7). Third, karyotypic analysis of

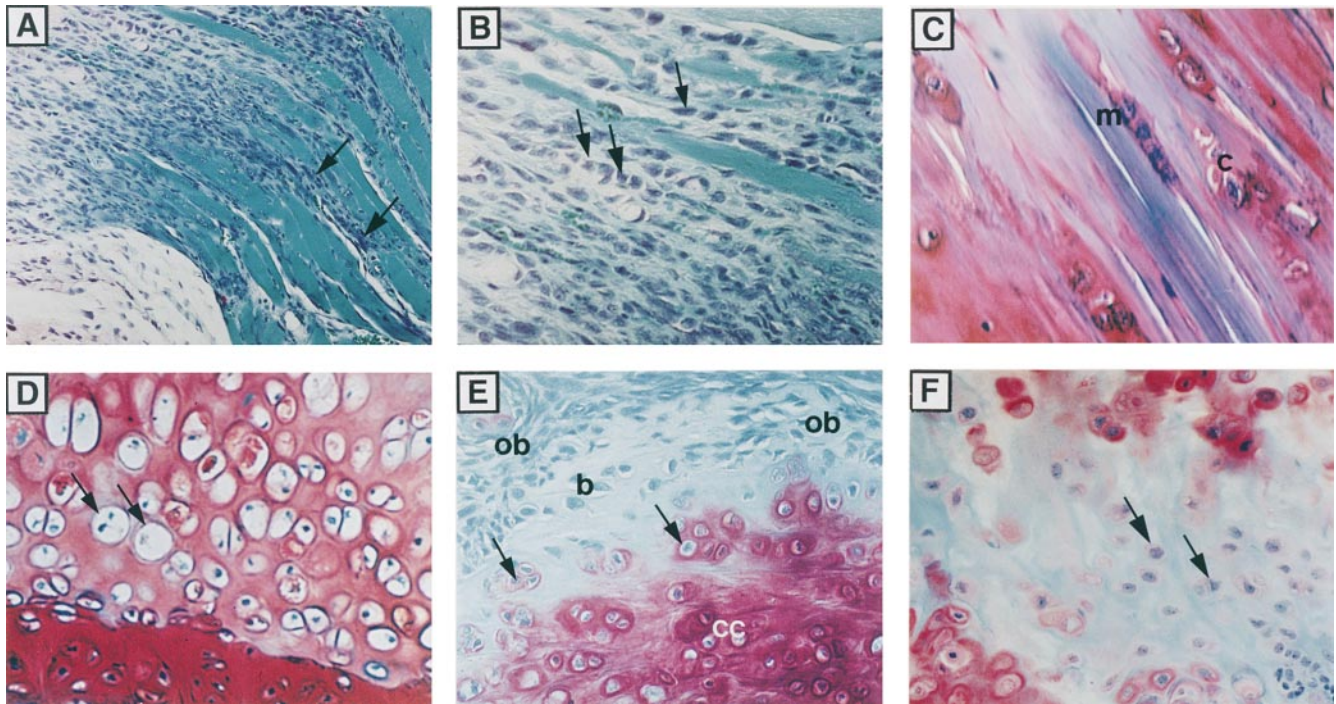


Figure 6. Transformation of and invasion by NFATp^{-/-} cartilage cells. (A) Extraarticular connective tissues of 6-mo-old NFATp^{-/-} mouse showing invasion and differentiation of cartilage cells into muscle. Primitive transformed proliferating cells show early differentiation into chondroblasts (arrows); original magnification: $\times 100$. (B) High power (original magnification: $\times 200$) of A, showing columns of primitive transformed cells differentiating into prechondroblasts. (C) High power (original magnification: $\times 200$) of 1-yr-old NFATp^{-/-} mouse showing cartilage cells invading muscle (arrows). (D) High power (original magnification: $\times 400$) of articular cartilage of 1-yr-old NFATp^{-/-} mouse showing abnormal-looking cartilage cells with multiple nuclei (arrows). Note the normal appearance of the layer of calcified cartilage. (E) High power (original magnification: $\times 400$) of extraarticular cartilage cells from 1-yr-old NFATp^{-/-} mouse showing multiple cells in clusters (arrows). (F) Same as E, showing multiple nucleoli (arrows); original magnification: $\times 400$. m, muscle; c, cartilage cells; cc, calcified cartilage; ob, osteoblasts; b, bone.

four cartilage cell lines (one male, three female) established from NFATp^{-/-} mice revealed aneuploidy in the three female lines, although there were no consistent karyotypic abnormalities observed (Table I). The histologic phenotype, coupled with the loss of contact inhibition and the presence of aneuploidy, suggests that the cartilage cells arising in NFATp-deficient mice may occasionally undergo transformation to a malignant state. Thus, some of the cartilage tumors in the NFATp^{-/-} mice may be best classified as chondrosarcomas even though a pathologic survey of lungs, brain, and intestine did not reveal metastatic lesions. However, spontaneous solid tumors in mice rarely metastasize. It should be noted also that NFATp^{-/-} cartilage cells do retain a differentiated phenotype similar to some, but not all, human chondrosarcomas (“low grade”).

Discussion

The NFATp Transcription Factor Is a Regulator of Chondrogenesis. Levels of NFATp control cartilage cell induction and proliferation, resulting in a spectrum of growth abnormalities ranging from hyperproliferation to neoplastic transformation, although the mechanism by which NFATp inhibits cartilage cell growth and differentiation is not understood. Few molecular regulators of chondrogenesis are known, and most of these operate during skeletal morphogenesis rather than in

the adult animal. Our studies demonstrate that a member of the NFAT family of transcription factors, NFATp, appears to be a repressor of cartilage cell growth and differentiation in the adult animal. Further, the uncontrolled induction and proliferation of cartilage cells that result from its absence presumably increase the likelihood of a “second-hit” that then can result in frank transformation of these cells.

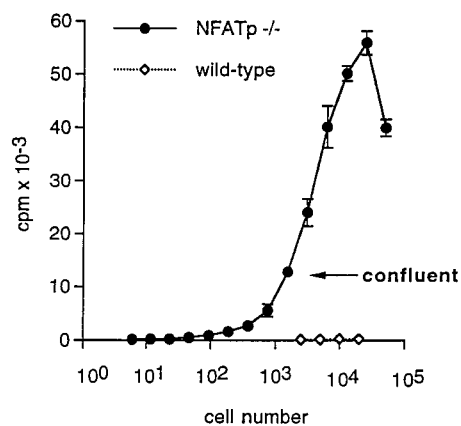


Figure 7. NFATp^{-/-} cartilage cells show loss of contact-induced growth inhibition. Varying numbers of wt or NFATp^{-/-} cartilage cells were plated, and cell division was monitored. Cell division continued even in cells that had reached confluency.

Table I. Cytogenetic Analysis of NFATp^{-/-} Cartilage Cell Lines

Tumor	Karyotype
1	40,XY
2	40-41,XX[cp7]/39-40,xx-4[6],-12[7],+17[2], +mar[5][cp7]/43,X,der(?X),add(1),der(?6),+?7, +9,+mar5[3]
3	40,X-X,+mar[3]/39,X-X,-14,+mar[2]/41, XX+mar[4]/40,XX[41]
4	71-89,XXXX-2[6],-3[10],-4[12],-4[10],-6[2], -7[8],-9[6],-10[2],-11[4],-12[3],-13[2],-14[3], -15[2],-16[2],-17[2],+18[4],+1-6mar[9][cp12]

Aneuploidy in NFATp^{-/-} tumor cells. Karyotypic analysis of four tumor cell lines from NFATp^{-/-} mice revealed aneuploidy in three of them. The first line was a normal male with random chromosome loss. The second line had a modal chromosome no. of 40 in 7 cells with monosomy of chromosomes 4 and 12, trisomy of chromosome 17, and gain of markers in most of the cells. An additional three cells displayed additional structures and numerical abnormalities. 2 clones were observed in the third tumor, a normal 40XX clone in 41 cells, and an abnormal clone that demonstrated clonal evolution. A marker chromosome was observed in all cells in the clone, and in addition, five cells demonstrated loss of an X and two of these also had loss of chromosome 14. The fourth tumor, derived from extraarticular cartilage, was pseudotetraploid with a modal chromosome no. of 71-76 (range 71-89), with a consistent finding of loss of chromosomes 4 and 16. One to six marker chromosomes were observed in most cells, and there were no normal diploid or tetraploid cells. mar, marker; der, derivative; add, additional material of unidentified origin.

To our knowledge, NFATp is the first transcription factor to be identified that controls the induction of chondrogenesis from adult connective tissue progenitor cells. There are three other known members of the NFAT family, all of which are expressed in cartilage. Although all four known NFAT family members are present in cartilage cells, only the expression of NFATp is regulated during the process of chondrogenesis. Further, only the expression of NFATp and not NFATc, NFAT4, or NFAT3 was restricted to MSCs undergoing chondrogenesis rather than osteogenesis. We conclude that NFATp is the member of this family involved in regulating the chondrogenic program, but it will be of interest to carefully examine cartilage morphology in mice lacking other members of the NFAT family.

NFATp Regulates Chondrogenesis in the Adult Animal. Consistent with the normal skeletal development of mice lacking NFATp, we did not detect NFATp expression in cartilage during embryogenesis. Rather, our data implicate NFATp as a critical regulator of chondrogenesis in the adult animal. Thus, NFATp expression is specifically regulated in human adult MSCs during in vitro chondrogenesis, whereas overexpression of NFATp represses the mature cartilage phenotype.

This is in contrast to the function of the cartilage-specific transcription factor, Sox9, which is critical in cartilage morphogenesis (48). Mutations in the Sox9 gene or in the Sox9

promoter are responsible for the human disease campomelic dysplasia (49, 50). Several additional regulators of chondrogenesis during embryonic development have been identified (51-53). Analysis of mutant mice has provided evidence for a role in cartilage development and differentiation for Indian Hedgehog (54, 55), parathyroid hormone-related protein (PTHrP [54, 56, 57]), fibroblast growth factors (58-60), insulin-like growth factors (61), Noggin (62), and BMPs, a family of secreted signaling molecules originally isolated by virtue of their ability to induce ectopic cartilage and endochondral bone formation when implanted into subcutaneous tissues of adult animals (46, 47, 55, 63-66). It is unlikely that NFATp acts through controlling these known regulators of chondrogenesis, since these appear to play a more important function in morphogenesis affecting both skeletal and nonskeletal organ systems than they do in controlling growth of adult cartilage cells. For example, the mouse *short ear* mutation is secondary to inactivation of the BMP5 gene (47), whereas mutations of the GDF5 gene account for limb alterations in *brachypodism* mice (67). Indian Hedgehog is expressed in prehypertrophic chondrocytes where it controls the rate of hypertrophic differentiation in part through inducing the expression of a second signal, PTHrP, in the perichondrium. PTHrP^{-/-} mice have defects in formation of hypertrophic cartilage (54, 57). Indeed, we found no evidence that NFATp directly regulated the transcription of several members of the BMP family or the TGF-β proteins themselves or acted through a secreted factor. Rather, the dysregulation of cartilage growth appeared to be cell intrinsic. However, it is of interest that NFATc and the type III TGF-β receptor are both required for endocardial cell transformation in the heart (5, 6, 68), raising the possibility that signal transduction pathways stemming from TGF-β receptors intersect with NFAT proteins.

We speculate that initiation of chondrogenesis results in activation of NFATp, which then sets in motion a genetic program to control cartilage differentiation and proliferation. Presumably, NFATp-regulated genes in cartilage must act in part to repress this program, since overexpression of NFATp extinguished the differentiated cartilage phenotype while loss of NFATp resulted in spontaneous activation of chondrogenesis. Future experiments will focus on identifying the upstream targets and downstream effectors of NFATp both in the MSC and in mature articular cartilage. One can speculate that some of the NFATp-regulated genes in MSCs will control proliferation, as evidenced by the uncontrolled division of these cells in its absence. Some of these may well be modifiers of NFATp activity that can account for the substantial sex difference in the development of the phenotype. In contrast to the complete penetrance (100%) of the cartilage phenotype in female mice, only approximately one third of male mice is affected, and the onset of symptoms is later. There is much precedent for gender differences in diseases: systemic lupus erythematosus, rheumatoid arthritis, and ankylosing spondylitis are well-known examples. A reasonable guess is that female or male sex hormones are involved in the phenotype described here because of direct or indirect regulation by NFATp.

NFATp Controls Differentiation of MSCs into Cartilage. NFATp acts as a repressor of cartilage cell differentiation from primitive MSCs, since in its absence, cartilage cells, many of which undergo the endochondral sequence of ossification, are induced to form and proliferate in the surrounding extraarticular connective tissues. Thus, the developmental sequence of endochondral bone formation is recapitulated in the absence of NFATp. It has been difficult to recapitulate the process of chondrogenesis from stem cells in vitro, although there has been some recent success in doing so under very stringent culture conditions (39–41, 43). It will be interesting to determine whether NFATp^{-/-} MSCs derived from mice that also express an SV40 T antigen transgene (H-2K^b-tsA58 “immortomouse” [44, 69]) will spontaneously differentiate into cartilage in vitro. In this regard, the effect of the NFAT inhibitors, CsA and FK506, on in vitro chondrogenesis from wt MSCs will be interesting to establish. Given the extremely low level of NFATp expression in mature cartilage cells, we suspect that the proliferation of articular cartilage cells observed is secondary to recruitment of MSCs or stem cells that reside in synovial lining tissue to this site. MSC recruitment, their differentiation into chondrocytes, and their mitotic expansion at different stages of commitment are all separable activities that could be affected by NFATp. Compounds that block the function of NFATp in cartilage may prove valuable in achieving sustained differentiation and growth of cartilage from MSCs in vitro or in vivo. Such inhibitors might control MSC recruitment and chondrogenesis in response to environmental injuries such as occur during normal mechanical wear and tear. The potential use of such NFATp inhibitors in degenerative joint diseases such as osteoarthritis, where cartilage has been destroyed, as well as in the stimulation of endochondral bone formation to repair bone defects and fractures in which endochondral bone formation plays an important role, is obvious.

NFATp May Act as a Tumor Suppressor in Cartilage. We show here that NFATp displays properties of a tumor suppressor gene in cartilage, as evidenced by the neoplastic changes in NFATp^{-/-} cells in both articular cartilage and extraarticular cartilage. The invasive nature of the cartilage cell proliferation in vivo, coupled with their behavior in vitro and ability to form tumors in syngeneic mice (Gerstenfeld, L.C., unpublished observations), suggests that these are chondrosarcomas. There has been controversy about what constitutes malignancy in cartilage tumors in humans (70). Chondrosarcomas are second only to osteosarcomas as the most frequent primary malignant tumors of bone (70, 71). Unlike osteosarcoma or Ewing’s sarcoma, chondrosarcomas are minimally responsive to chemotherapy and are largely treated by surgical excision. Outcome is difficult to predict in part because chondrosarcomas are difficult to grade and can be confused with benign enchondroma or osteochondrogenous exostosis (70, 71). This has recently prompted the use of other methods of assessing tumor virulence such as imaging, mean DNA content, and presence of aneuploidy to help predict outcome (72, 73). Although a variety of chromosomal abnormalities has been noted in human

chondrosarcomas, including loss of heterozygosity at the EXT1 locus (74, 75), no consistent cytogenetic phenotype has been described, similar to what was observed in the NFATp^{-/-} tumor lines. Unlike osteosarcoma and Ewing’s sarcoma, which almost always involve mutations in the Rb (76) and EWS1 (Ewing’s sarcoma) genes, no frequent association with known tumor suppressor genes has been reported for chondrosarcomas, although abnormalities in p53 expression have been reported (77, 78). In preliminary experiments, we have not detected abnormalities in p53 expression, but it will be important to analyze the NFATp^{-/-} transformed cell lines presented here for mutations in other known tumor suppressor genes. Further, since almost no transformed, differentiated, rapidly growing, and transfectable cartilage cell lines are available, these cell lines should prove very valuable for the analysis of gene regulation and function in cartilage. Finally, our data suggest that a careful examination of human chondrosarcoma tissue for the presence of NFATp gene mutations may be warranted.

We are very grateful to A. Caplan and J. Dennis for their generous provision of human differentiated MSCs. We thank D. Heinegard and G. Crabtree for providing the anti-COMP and NFATp antibodies, J. Letterio for providing TGF- β 1,2,3 cDNAs, S. Lee for providing GDF5/6 cDNAs, J. Knoll, A. Schiller, A. Hecht, and H. Mankin for helpful discussions, S. Ashkar for help with photography, and H. Cantor and M. Grusby for a thoughtful review of the manuscript.

This work was supported by National Institutes of Health grants AI/AG37833 (to L.H. Glimcher), HD22400 (to L.C. Gerstenfeld), and AG14701 (to M.J. Glimcher), a grant from The Peabody Foundation (to M.J. Glimcher), and a grant from The G. Harold and Leila Y. Mathers Charitable Foundation (to L.H. Glimcher).

Submitted: 2 September 1999

Revised: 7 October 1999

Accepted: 19 October 1999

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