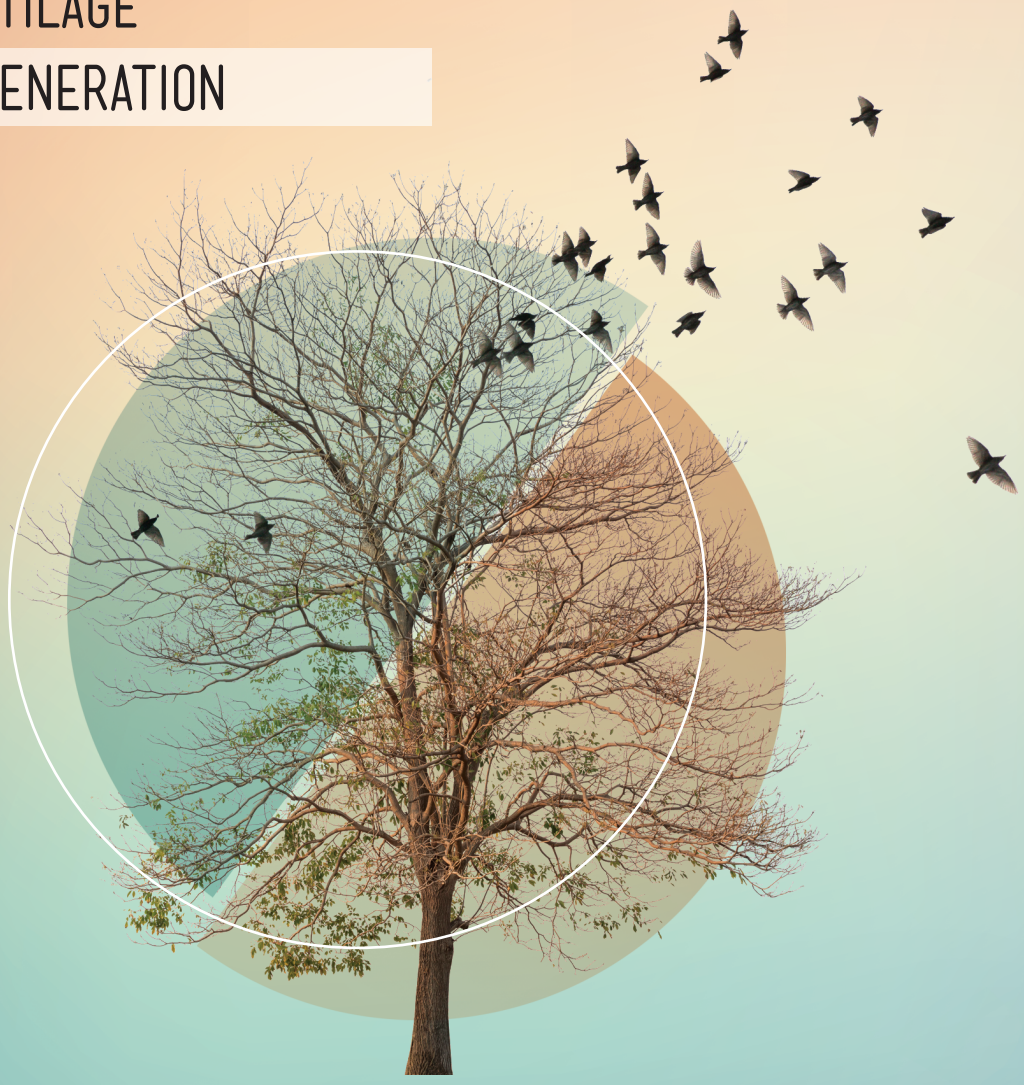
CALCINEURIN INHIBITION AT PHYSIOLOGICAL OSMOLARITY: TOWARD IMPROVING CARTILAGE REGENERATION



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Calcineurin Inhibition at Physiological Osmolarity: Toward improving cartilage regeneration

Anna E. van der Windt

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Calcineurin Inhibition at Physiological Osmolarity:

Toward improving cartilage regeneration

Remming van calcineurine bij fysiologische osmolariteit: de weg naar beter herstel van kraakbeen

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Voor mijn ouders

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General introduction and aims

Articular cartilage

Articular hyaline cartilage is a white, smooth structure covering the ends of bones in synovial joints, like in the hip and knee. Because of its unique stiff yet flexible properties, it distributes the loads, as a consequence of weight bearing and locomotion, over the surface of the joint and enables bones to smoothly glide over each other during movement. Besides in synovial joints, hyaline cartilage is also found in non-synovial joints where bones are connected via one cartilaginous structure providing load distribution and resilience, like between the vertebral bodies.

Thus, hyaline cartilage enables us to walk, dance and run a marathon with low friction between our articulating bones, providing us quality of life. However, when damaged, it mostly fails to repair itself properly. Traumatic or degenerative articular cartilage defects may be chondral or osteochondral when the subchondral bone is involved too. Chondral lesions are painless and repair poorly due to the absence of a nervous and vasculature system within the cartilage (1, 2). When untreated, small cartilage lesions will slowly progress into full-thickness lesions of the cartilage layer and involving the underlying bone, resulting in pain, reduced mobility and decreased quality of life. Osteochondral lesions may heal by an initial inflammatory response followed by the formation of so-called fibrocartilage, which is of inferior mechanical quality (2, 3), resulting in early degradation and fragmentation and eventually the onset of osteoarthritis (OA).

Osteoarthritis

Osteoarthritis is a common chronic joint disease with progressive cartilage degradation as one of its major characteristics. Other aspects of OA involve changes in the subchondral bone, ligaments and synovium. It most frequently affects the knee, hip and hands. OA is related to age and has a high prevalence in the population older than 60 years. Worldwide estimates are that 9.6% of men and 18.0% of women aged over 60 years have symptomatic osteoarthritis (data from World Health Organization, (4)). Moreover, the prevalence among younger age groups is increasing, partially due to the increase in overweight. Osteoarthritis is already one of the ten most disabling diseases in developed countries, 80% of patients with osteoarthritis will have limitations in movement and 25% cannot perform their major daily activities of life (WHO). The recently published Global Burden of Disease Study in The Lancet (5) estimated that the global years lived with disability because of OA increased by 64% over the period 1990-2010. Therefore, the economic burden of OA is high.

Current approaches in treatment of cartilage defects

Orthopedic surgeons currently use different nonoperative and operative techniques to treat cartilage defects and OA. Nonoperative treatments include weight loss guidence, analgesics and physiotherapy. Operative approaches can be classified in roughly three groups, based on their principle:

1. *Relieve of the damaged surface:* osteotomy and knee joint distraction. An osteotomy is used to realign the axis of the limb to transfer the load from the damaged surface to a healthier part of the cartilage surface. With joint distraction, an external fixation frame is bridging the knee joint, which in stages distracts the joint for 5 mm for several weeks inducing clinical improvement and decreasing the area of denuded bone (6).

2. *Replacement of the damaged surface:* mosaicplasty and osteochondral plugs, total hip arthroplasty and total/unicompartmental knee arthroplasty. Osteochondral plugs are harvested from a non-weight-bearing surface of the ipsilateral joint and placed in a prepared cylindrical hole in the cartilage defect. In mosaicplasty multiple small osteochondral plugs are harvested and placed in a larger defect. However, both techniques induce morbidity to the donor site and only limited size defects can be treated (7, 8). Total or unicompartmental joint arthroplasty is used for severe joint degeneration. The joint is replaced by a prosthetic joint, resulting in relief of pain en (partially) regain of function. However, the lifespan of these prosthetic joints is limited and revision surgery is unfavorable.

Previous mentioned nonoperative and operative treatments are mostly focused on relief of symptoms, particularly pain, and not focused on inducing cartilage repair or limiting progression of the disease. For the last decades, a lot of research worldwide has been focused on developing treatment techniques to regenerate the damaged cartilage into healthy hyaline cartilage with its unique biomechanical properties.

3. *Regeneration of the damaged cartilage:* microfracture, drilling and cell transplantation techniques. Microfracture and drilling are techniques to penetrate the subchondral bone and establish access of blood flow and bone marrow. As a result, bone marrow stem cells and inflammatory factors enter the cartilage defect, initiating a repair response. However, often fibrocartilage is formed with inferior biomechanical properties than healthy hyaline cartilage (9). Cell transplantation, like Autologous Chondrocyte Implantation (ACI), is a cell-based

repair strategy and was first described in 1994 by Brittberg (10). ACI involves harvesting cartilage from a healthy non-weight-bearing surface of the ipsilateral joint, isolating the cartilage specific cells, chondrocytes, in a laboratory, expanding the chondrocytes *in vitro* and subsequently inserting them in the cartilage defect during a second surgery. To keep the cells in place, they can be covered with a membrane or can be pre-seeded in a matrix. The main purpose of ACI is to implant chondrocytes so that hyaline cartilage is formed which resembles normal hyaline articular cartilage. Good to excellent results have been obtained with this technique on the short-to-medium term of follow-up (11-13), but the use is limited to small traumatic cartilage defects in the younger patient and not suitable for the treatment of OA. The major complications are transplant failure, delaminiation of the transplant or graft hypertrophy (11, 14).

The search for the ideal cartilage regeneration technique continues. Increasing interest is raised on improving the quality of the cartilage formed after cell transplantation for example by using different cell sources (like stem cells) or optimizing the *in vitro* culture conditions during the expansion phase. However, the development of a drug inducing hyaline cartilage repair *in vivo* in patients with OA is of course preferable and still desirable. For both goals, it is necessary to know what healthy articular cartilage is composed of and how it is formed.

Formation and composition of articular cartilage

In vivo, cartilage is only formed during embryogenesis. Most skeletal tissues arise from mesenchymal (stem) cells, which have the ability to differentiate into fibrous tissue cells, fat cells, muscle cells, chondrocytes and bone cells. Specific transcription factors regulate the differentiation of mesenchymal cells into these different lineages. Formation of the skeleton can first be seen as a condensation of these mesenchymal cells, which differentiate into precartilagenous cells and finally a cartilage template is formed with chondrocytes expressing collagen type II and Sox 9. The mesenchymal cells give rise to two different kinds of cartilage: transient and permanent. In the limb, bone is formed by terminal differentiation of the transient chondrocytes into hypertrophic chondrocytes and mineralization of the tissue. However, when a joint needs to be formed, the precartilagenous tissue splits transversely and on either side of the joint permanent articular cartilage is formed, which does not terminally differentiate into bone. Additional condensation of mesenchymal cells forms the joint capsule, ligaments and tendons (15-17).

Articular cartilage has its unique properties related to the structure and composition of its extracellular matrix (ECM), which is mainly composed of proteoglycans

(PGs, like aggrecan) entangled in a network of collagen fibers (mainly collagen type II) and large amounts of water (18, 19). Water and inorganic salts represent the bulk mass portion (65-80%), while collagens, PGs and other (glyco-)proteins constitute only 20% of the tissue's wet weight. Collagens represent 50-60% of cartilage's dry weight, while proteoglycans, as second largest solid phase portion, account for 5-10%. Only 1-5% of the cartilage volume consists of chondrocytes, the main cell type found in cartilage, embedded in the matrix (2, 18, 19)(Figure 1). The chondrocytes are responsible for maintenance of its surrounding matrix by a balance between synthesis and degradation of matrix-components.

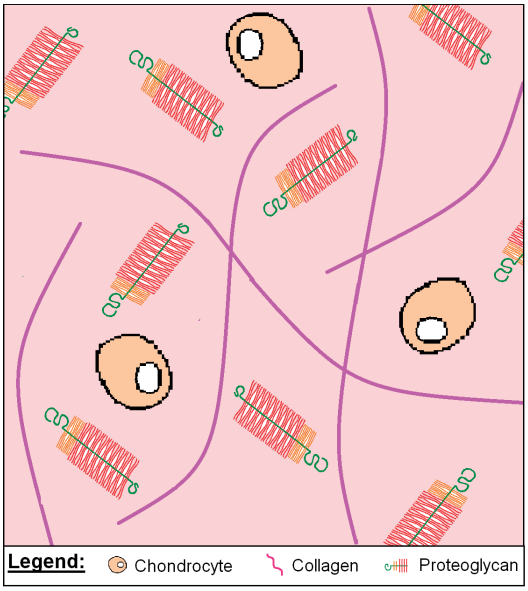

Figure 1. Schematic representation of extracellular matrix components in cartilage. Proteoglycans consist of a core protein (aggrecan) to which numerous glycosaminoglycans (GAGs) are attached. Up to 100 proteoglycans are normally attached to a hyaluronic acid filament (not shown in the figure).

Osmotic pressure in healthy and damaged articular cartilage

The extracellular matrix is crucial for the unique biomechanical properties of cartilage. Cartilage requires a high osmotic pressure to maintain its hydrostatic pressure and keep its visco-elastic properties. Proteoglycans (PGs) with negatively charged sulphated glycosaminoglycan (GAGs) side chains (20) attract mobile cations (mainly sodium) and water. An intact collagen network restricts swelling and, in combination with GAGs, determines the osmotic pressure (i.e. tonicity) of the extracellular fluid around chondrocytes, which ranges between 350 and 480 mOsm in healthy cartilage (21-23). The combination of intertwined collagens and PGs creates the tissue's rigidity by entrapping solutes and water, giving cartilage its mechanical properties, enabling it to deform and undergo large compressive and shear forces without failing (19).

In healthy cartilage (Figure 2, Panel **A**), the osmotic pressure is lowest (±350 mOsm) in the superficial (S) zone and increases to ±480 mOsm in the deep (D) zone of the cartilage, where the collagen fibers are stiffest (more stretched) and PG concentration is highest (24). The osmotic pressure is dynamic and changes under (patho)physiological conditions. In loaded cartilage (Figure 2, Panel **B**), the matrix deforms, squeezing water out of the tissue, increasing cation concentration and thus the osmotic pressure. The deformation and increase in osmotic pressure are more prominent in the superficial (S) and middle (M) layers than in the stiffer deep (D) layers of the cartilage. In osteoarthritic cartilage (Figure 2, Panel **C**), collagen fibers are degraded. The initial effect of collagen degradation is more swelling of the cartilage matrix, since there is less resistence of the collagen fibers (25). If there is also proteoglycan and GAG degradation the osmotic pressure drops (24, 26) as well as cartilage swelling and thus leading to a less stiff cartilage layer that becomes more susceptible for further degradation.

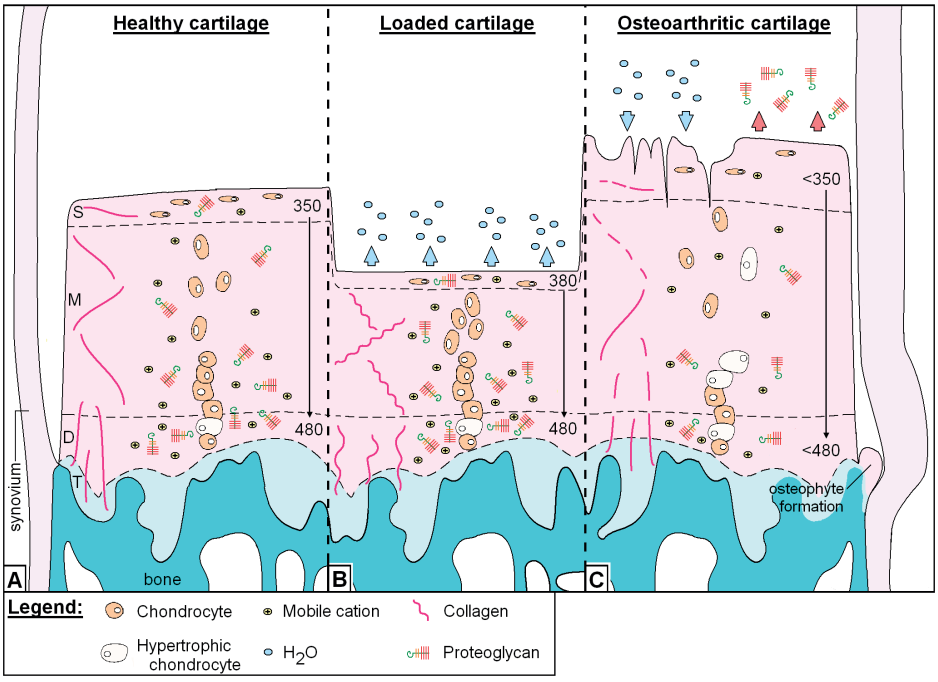

Figure 2. Illustration of the zonal regulation of osmotic pressure in healthy **(A)**, loaded **(B)** and early stage of osteoarthritic **(C)** cartilage.

However the osmolarity in the extracellular matrix can also be affected by the uptake or release of ions by the cells. Cells have an active control system using complex channels that can transport ions to keep their osmolarity within specific

boundaries. These transporters interact with various systems within and outside the cell.

Molecular mechanisms in response to changes in extracellular osmotic pressure

Molecular mechanisms involved in the response of human articular chondrocytes to changes in osmotic pressure are poorly understood. Extracellular hyperosmolarity perturbs mammalian cells by causing osmotic efflux of water, resulting in cell shrinkage (27, 28). Cells react by a rapid uptake of ions, which increase cellular ionic strength (29) with potentially detrimental effects (30-32). The initial, rapid response is the activation of transporters that exchange these ions for compatible osmolytes (32, 33). This process is controlled by Nuclear factor of activated T-cells 5 (NFAT5, also called Tonicity-responsive Enhancer Binding Protein, TonEBP), which mediates transcriptional activation of these transporters (32). NFAT5 is a member of the Nuclear factor of activated T-cells (NFAT) transcription factors (34) and was first discovered for its function in kidney cells. It targets sodium/myo-inositol cotransporter (SMIT) (35, 36), sodium/chloride-coupled acid transporters (BGT1/SLC6A12)(35), aquaporin channels (AQP1 and AQP2) (37), and calcium-binding proteins (S100A4) (38-40). Upon hyperosmotic stress, transcription of NFAT5 itself is upregulated in several cell types (41-43), but the tonicity threshold and cell signaling pathways required to activate NFAT5 may be cell type specific (44). Until recently, nothing was known about the expression or function of NFAT5 in human articular chondrocytes.

Signaling pathways influencing chondrogenesis

In the search for targets to improve the quality of the cartilage formed after cell transplantation or to induce cartilage repair in vivo, signaling pathways involved in chondrogenesis during embryonic development are frequently studied. Pathways known to be involved in this process are multiple and complex and involve for example fibroblast growth factors, transforming growth factors and Wnt/β-catenin (16). Another signaling pathway involved in chondrogenesis is the Calcineurin (Cn) – NFAT pathway (Figure 3). Where NFAT5 is osmolarity-dependent, the other members of the NFAT-family (NFAT1-4) are dependent on activation by calcineurin, which can be regulated by FK506 (also known as Tacrolimus or Prograf).

FK506 is an immunosuppressive drug discovered by Kino et al. in the 1980s (45, 46). FK506 revolutionized transplant therapy because of its ability to suppress T-cell activation, without markedly affecting bone marrow cell differentiation and proliferation (47). Through binding to FK506-binding proteins (FKBPs), FK506 inhibits

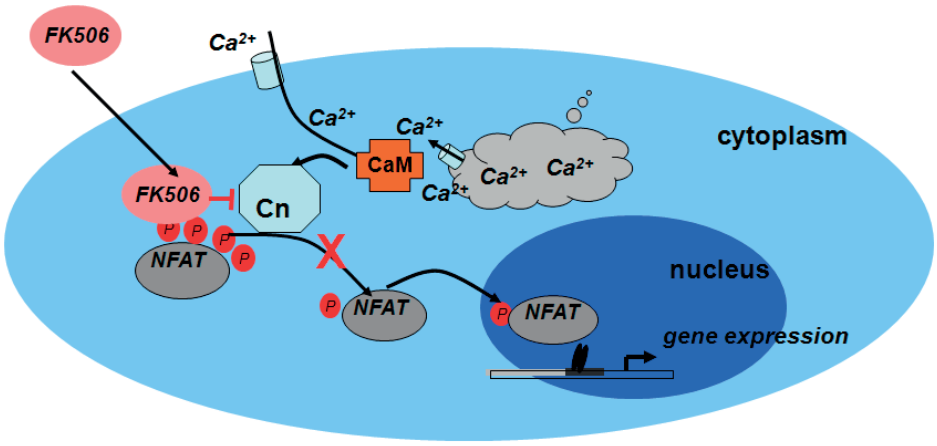

Figure 3. Schematic representation of the Cn-NFAT signaling and the inhibition by FK506. Calcineurin is activated by calmodulin and calcium from external and internal stores. Activated Calcineurin dephosphorylates the NFAT 1-4 transcription factors, which in turn translocate into the nucleus and induce gene transcription. FK506 inhibits the Calcineurin activity.

the activity of ubiquitously expressed calcium/calmodulin dependent Cn. As a consequence, the Cn-mediated dephosphorylation of transcription factors of the NFAT family (1-4) is inhibited (Figure 3).

Besides their role in T-cell activation, Cn and NFATs are now also known to play a role in physiological processes in many other cell and tissue types as well as in pathological conditions like cancer, degenerative brain diseases and cardiac hypertrophy (48). FK506 has proven to be useful in reducing inflammation and alleviating symptoms in patients with inflammatory (rheumatoid) arthritis (49-52). Interestingly, the Cn-NFAT signaling cascade is also reported to play a role in bone remodeling (53) and chondrogenesis (54, 55). FK506 has been shown to induce chondrogenic differentiation of murine chondroprogenitor cells (56, 57). This suggests that patients with non-inflammatory joint diseases, like osteoarthritis (OA), also might benefit from a treatment with Cn inhibitors.

Aims

The research that is reported in this thesis was aimed at finding new potential targets or tools for improving cartilage matrix synthesis by chondrocytes, either *in vitro* for cell transplantation techniques or *in vivo* toward a cure for osteoarthritis. The studies focus on influencing the calcineurin-dependent NFAT1-4 pathway and the osmolarity-dependent NFAT5 pathway.

The first aim was to provide further understanding in the processes that control chondrocyte differentiation. During *in vitro* expansion, required for cell-based therapies, loss of chondrocyte phenotype takes place (dedifferentiation). Chondrocytes convert into a flattened, fibroblast-like state, accompanied by a shift from collagen type II to collagen type I expression, reducing the quality of the extracellular matrix (ECM). **Chapter 2** focuses on Calcineurin (Cn) as potential target to improve chondrogenic phenotype of culture-expanded osteoarthritic chondrocytes. First, Cn activity was measured during dedifferentiation by serial passaging in monolayer and after redifferentiation with transforming growth factor β (TGFβ), a commonly used chondrogenic redifferentiation factor (58, 59). The second step was to investigate whether inhibition of Cn activity by FK506 would improve chondrogenic marker expression. Since Cn inhibitors are known to induce TGFβ in renal cells (60, 61), we investigated whether FK506 exerts its effect on chondrocyte redifferentiation through endogenous TGFβ production.

The second aim of this thesis was to investigate effects of physiological osmolarity on expression of chondrogenic markers by chondrocytes *in vitro*. As mentioned before, during cartilage degeneration as in OA, the extracellular osmolarity decreases as a result of matrix breakdown. Several studies have shown that articular chondrocytes are osmolariy-responsive (62, 63) and react with changes in aggrecan and GAG synthesis when the osmolarity is below or above the physiological range. Interestingly, chondrocyte *in vitro* expansion is mostly performed in medium of non-physiological tonicity (270 +/- 10-20 mOsm), which corresponds to physiological osmolarity of blood. However, for cartilage this level is non-physiological, since it normally has a much higher osmolarity. **Chapter 3** describes the effects of chondrocyte isolation and expansion at physiological osmolarity of 380 mOsm, by adding sterile sodiumchloride to the medium. The effects on chondrocytic markers (aggrecan, Sox9, collagen type II and type I) were studied in osteoarthritic and healthy chondrocytes. In addition, the role of the NFAT5 transcription factor in osmolarity-induced chondrocytic marker expression was elucidated.

We also wanted to elucidate whether combining both treatments would give synergistic effects. In **chapter 4** the results are described, and special attention in these studies was paid to increasing anabolic, but suppressing unwanted catabolic and hypertrophic markers.

In **chapter 5** the aim was to investigate the effects of physiological osmolarity on chondrogenic differentiation of chondroprogenitor cells and mesenchymal stem cells. Chondrogenic differentiation of progenitor cells plays an essential role during endochondral ossification for skeletal growth and bone fracture healing. Moreover, mesenchymal progenitor cells are also used as alternative cell source for cell-based cartilage regeneration. Therefore, new methodologies improving chondrogenic differentiation of mesenchymal progenitor and stem cells into chondrocytes are of interest for cartilage and bone regenerative medicine approaches. In these studies, the ATDC5 chondroprogenitor cell line and human bone marrow stem cells were used as *in vitro* models for chondrogenic differentiation. Again, the role of NFAT5 in regulating osmolarity-induced chondrogenic differentiation was elucidated.

Terminal differentiation of mesenchymal progenitor cells into hypertrophic chondrocytes and mineralization of the tissue is favorable for bone regenerative medicine, but unfavorable for cartilage purposes. Based on the results of chapter 4 and 5, we wondered whether FK506 addition to physiological osmolarity medium would be able to inhibit hypertrophic differentiation of progenitor cells. The results are described in **chapter 6**.

The last and most exciting aim of this thesis was to test the potential therapeutic effects of FK506 in an *in vivo* animal model for OA. In **Chapter 7** a recently established novel method (64) was used to induce OA in rat knee joints, showing severe degradation of articular cartilage and prominent involvement of subchondral bone and synovial macrophages. This chapter compares *in vitro* effects of FK506 on collagen and GAG synthesis on osteoarthritic cells to *in vivo* effects of FK506 on OA articular cartilage. In addition, effects of FK506 on subchondral bone and synovial macrophages were studied in this animal model for OA.

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Calcineurin inhibitors promote chondrogenic marker expression of dedifferentiated human adult chondrocytes via stimulation of endogenous TGFβ1 production

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Abstract

In-vitro chondrocyte expansion is required for several cell-based approaches for the repair of chondral lesions. During expansion, loss of chondrogenic phenotype takes place (dedifferentiation). The objective of this study was to investigate calcineurin as a potential target to improve chondrocyte phenotype for cartilage repair purposes.

Calcineurin activity in human articular chondrocytes was significantly increased during dedifferentiation and decreased during redifferentiation in vitro. Inhibition of calcineurin activity by FK506 increased the expression of chondrogenic markers collagen type 2, aggrecan and SOX9 in culture expanded cells. Addition of FK506 increased endogenous Transforming Growth Factor (TGF) β1 expression on both mRNA and protein level. The effect of FK506 on chondrogenic markers was abolished by addition of anti-TGFβ1 antibody, indicating that the endogenous TGFβ1 was necessary to increase chondrogenic marker expression. We also showed that chondrocyte redifferentiation by TGFβ requires calcium influx and does not depend on changes in calcineurin activity.

In conclusion, inhibition of calcineurin activity by FK506 increases the expression of chondrogenic markers via endogenous TGFβ1 production in human articular chondrocytes. Calcineurin inhibitors might be an alternative for the application of (recombinant) TGFβ, to promote chondrocyte phenotype for cell-based cartilage repair procedures.

Introduction

In-vitro chondrocyte expansion is required for several cell-based approaches for the repair of chondral lesions. During expansion, loss of chondrogenic phenotype takes place (dedifferentiation). Chondrocytes convert into a flattened, fibroblast-like state (1, 2), accompanied by a shift from collagen type II expression to collagen type I expression (2-4), which reduces the quality of the extra-cellular matrix. To improve the quality of cartilage generated by culture-expanded chondrocytes, re-differentiation of chondrocytes towards their chondrogenic phenotype is required. The mechanisms that regulate and control chondrocyte phenotypes are still largely unknown. Insight into these processes may provide new targets to improve cell-based cartilage repair procedures.

In this study, we focused on calcineurin (Cn), a calcium-dependent serine/threonine phosphatase, as potential target to improve chondrocyte phenotype. It has been demonstrated that osteoarthritic chondrocytes express mRNA of the α- and β-isoforms of the catalytic subunit of calcineurin (5). Furthermore, targeted inhibition of calcineurin activity with the immunosuppressive drug cyclosporin A (CsA), dose-dependently increased the production of collagen type II (5). In an in-vivo mouse model for osteoarthritis (OA), inhibition of calcineurin activity increased collagen type II expression and improved the regeneration of cartilage defects (5). In addition, inhibition of calcineurin activity by FK506 (Tacrolimus, Prograf®), another widely used immunosuppressive agent, has been reported to induce chondrogenesis in clonal mouse embryogenic carcinoma cells and in human synovial stromal cells (6, 7). Besides these stimulating effects on chondrogenesis by inhibiting calcineurin activity, an increase in calcineurin activity appeared to induce chondrogenesis in the rat calvaria chondrogenic cell line RCJ3.1C5.18 and in chicken mesenchymal cells (8, 9). Summarizing, the data suggest that calcineurin plays a role in embryonic chondrogenesis and in the progression of OA, and the effects seem to be cell type dependent. It seems likely that calcineurin is also involved in the phenotypical changes of adult human articular chondrocytes during expansion for cartilage tissue engineering procedures.

The aim of this study is to investigate the possibility to improve the chondrogenic phenotype of culture-expanded articular chondrocytes by modulating calcineurin activity. We therefore conducted the following steps: First, the relation of human adult articular chondrocyte phenotype and calcineurin activity was studied. We measured calcineurin activity during dedifferentiation by serial passaging in monolayer and after redifferentiation in vitro with Transforming Growth Factor (TGF) β, a

commonly used redifferentiation factor (10-12). The second step was to investigate whether inhibition of calcineurin activity using FK506 would improve chondrogenic marker expression of culture-expanded chondrocytes. Finally, we gained more insight in the mechanism of chondrocyte redifferentiation by calcineurin inhibition. Since calcineurin inhibitors are known to induce TGFβ signaling in renal cells (13, 14), vascular smooth-muscle cells (15) and lymphocytic B cells (16), we investigated whether addition of FK506 would stimulate endogenous TGFβ production by chondrocytes and whether the effect of FK506 on chondrogenic marker expression was due to TGFβ. In addition, we investigated whether chondrocyte redifferentiation by TGFβ is directly due to modulation of calcineurin activity, or whether calcineurin activity changes as a result of the redifferentiation.

These studies will provide further insight in the processes that control chondrocyte phenotype and may identify calcineurin as potential target to improve cell-based cartilage repair procedures.

Materials and Methods

Isolation of human adult articular chondrocytes

Human cartilage was explanted from macroscopically normal areas of the femoral condyles and tibial plateau of 12 patients (mean age 66 ± 6.2 years) undergoing total knee replacement surgery for osteoarthritis (with medical ethical approval MEC2004-322). Cartilage explants were washed with sterile physiological saline and incubated with 2 mg/mL protease XIV (Sigma-Aldrich, St. Louis, Missouri, USA) for 2 hours, followed by overnight incubation with 1.5 mg/mL collagenase B (Roche Diagnostics, Mannheim, Germany) in medium (Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 10% fetal calf serum (FCS), 50 µg/mL gentamycine and 1.5 µg/mL fungizone (all Invitrogen, Paisley, Scotland, UK)). Both enzymatic digestions were done at 37°C. After incubation, the undigested cartilage fragments were removed using a 100 µm filter, and the isolated primary chondrocytes were used for cultures.

Chondrocyte dedifferentiation

We studied calcineurin activity during chondrocyte dedifferentiation by serial passaging. To dedifferentiate chondrocytes, isolated primary chondrocytes were precultured in monolayer at a seeding density of 7,500 cells/cm^2. For the experiments, we seeded differentiated cells (without preculture: P0) and dedifferentiated cells after 1, 2 and 3 passages of monolayer culture (P1, P2 and P3 respectively)

in high-density monolayers (20,000 cells/cm^2) and cultured for four days before harvesting for analysis of mRNA expression levels (quantitative PCR; QPCR) and calcineurin activity. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in medium. Experiments were performed in triplicate samples from two donors (n=6).

Chondrocyte redifferentiation

We studied calcineurin activity during chondrocyte redifferentiation in alginate culture with added rhTGFβ2 (R&D Systems, Abingdon, U.K.). Expanded P2 chondrocytes from two donors were suspended in 1.2% alginate in physiological saline (Keltone LV, Kelco, Chicago, USA), at a density of 4x10^6 cells/mL. Beads were prepared and cultured in six-well plates (Corning, New York, USA) with 18 to 20 beads in 2 mL medium per well. Redifferentiation medium consisted of DMEM supplemented with 1:100 ITS (Becton Dickinson, Bedford, MA), 10 ng/mL IGF1 (recombinant human, Boehringer Mannheim), 25 µg/mL L-ascorbic acid 2-phosphate (Becton Dickinson), 50 µg/mL gentamycin (Invitrogen), 1.5 µg/mL fungizone (Invitrogen) and 10 ng/mL rhTGFβ2 (R&D Systems, Abingdon, Oxfordshire, UK)(10-12). After 21 days of culture, alginate beads were harvested for analyses of mRNA expression levels (QPCR) and calcineurin activity.

For greater convenience and higher throughput, we also investigated whether monolayer cultures could be used to induce these effects on chondrogenic marker expression. Passage 2 chondrocytes were seeded at a density of 20,000 cells/cm^2 and cultured for 10 days in redifferentiation medium. Culture of more than 10 days in monolayer increased the risk of contraction and loosening of the cell layer (own experience). Two concentrations of rhTGFβ2 were used: 2.5 and 25 ng/mL. Both experiments were performed in triplicate samples taken from two donors (n=6).

Inhibition of calcineurin activity with FK506

To investigate the effects of the calcineurin inhibitor FK506 on chondrocyte marker expression, we cultured chondrocytes with FK506 up to 10 days. Second-passage chondrocytes from three donors were plated at a density of 20,000 cells/cm^2 and maintained in medium with 10% FCS and with or without 50 or 500 ng/mL FK506 (60 and 600 nM respectively; Astella Pharma, Meppel, NL). In previous experiments we had ascertained that FK506 did not affect cell proliferation and cell viability up to 1000 ng/mL (data not shown). Cells were harvested after 4 and 24 hours to analyze calcineurin activity and after 24 hours and 10 days to analyze mRNA expression levels (QPCR). Experiments were performed in triplicate for three donors (n=9).

FK506 and induction of endogenous TGFβ

To evaluate the hypothesis that the effects of FK506 on chondrogenic markers are caused by endogenous TGFβ production, we measured TGFβ1 (NM_000660), TGFβ2 (NM_003238) and TGFβ3 (NM_003239) mRNA expression levels of P2 chondrocytes cultured with 50 or 500 ng/mL FK506. In addition, we measured TGFβ1 protein levels in these experiments. To investigate whether the endogenous TGFβ1 production is responsible for the FK506 effects on dedifferentiated chondrocytes, P2 chondrocytes were cultured for 10 days in medium with 50 ng/mL FK506 with or without 1 or 10 μg/mL anti-TGFβ1 antibody (Sigma-Aldrich, raised against a mixture of human TGF-β1, porcine TGF-β1.2, porcine TGFβ2 and recombinant, amphibian TGF-β5). To check for unwanted effects of this anti-TGFβ antibody on chondrogenic marker expression or cross-reactivity to human TGFβ2, we cultured cells with anti-TGFβ1 without FK506, and redifferentiated cells by adding rhTGFβ2 in the presence of 10 μg/mL anti-TGFβ1. After 24 hours and 10 days, cells were harvested to analyze mRNA expression levels (QPCR). The experiments were performed in triplicate for two donors (n=6).

Calcineurin activity after addition of TGFβ

Our next goal was to investigate whether redifferentiation by TGFβ addition is due to modulation of calcineurin activity or whether the calcineurin activity is modulated as result of redifferentiation. We therefore cultured P2 chondrocytes in redifferentiation medium supplemented with 0, 2.5 or 25 ng/mL rhTGFβ2. We used rhTGFβ2 in these experiments because this is the routine procedure in our laboratory for all studies to induce chondrocyte redifferentiation (10, 11, 17, 18). We analyzed mRNA expression and calcineurin activity after 4 and 24 hours (to analyze early modulation of Cn activity) and after 3 and 10 days (to analyze Cn modulation in time and to achieve redifferentiation). The experiments were performed in triplicate samples from two donors (n=6)

It is known that TGFβ can induce an influx of intra-cellular calcium (19-23), which can subsequently increase calcineurin activity. To investigate this mechanism, P2 chondrocytes were pretreated either with 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) tetrasodium salt (Sigma), or with 10 μM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM; Sigma) for 15 min prior to the addition TGFβ2 (2.5 ng/mL) (n=3). EGTA is a chelator of extracellular calcium, and thereby inhibits the calcium influx. BAPTA-AM is a selective Ca^{2+} chelator from intracellular stores, inhibiting an increase in intracellular calcium concentration from these intracellular stores.

Calcineurin activity

Cells from monolayer cultures were washed twice in physiological saline, before starting the cytoplasmatic extraction procedure (NE-PER Nuclear and Cytoplasmic Extraction Kit, PIERCE, Bonn, Germany). Concentrations of cytoplasmic proteins, isolated according to the supplier's instructions, were quantified using the BCA Protein Assay Kit (PIERCE, Bonn, Germany) in a microplate reader (VersaMax, Molecular Devices Ltd, NL). All samples were stored at -80°C until further use.

Calcineurin activity was measured using the Calcineurin Cellular Assay Kit Plus (BioMol, Tebu-Bio, Heerhugowaard, NL). First, extracts were purified on a Micro Bio-Spin P-6 chromatography column (Bio-Rad Laboratories B.V., Veenendaal, NL). Calcineurin activity was measured colorimetrically as relative units of free phosphate in 5 µL purified extract (1.0-1.5 µg of total cytosolic protein per assay). OD_{620nm} data were converted using a standard curve, and expressed as the amount (nmol) phosphate released per µg of total cytosolic protein.

TGFβ production

TGFβ1 secretion in the culture medium was measured in triplicate using a commercially available sandwich ezyme-linked immunosorbent assay (ELISA) kit for TGFβ1 (R&D Systems, Abingdon, Oxfordshire, UK). According to the manufacturers' protocol samples were first activated with 1 N HCl and 1.2 N NaOH/0.5 M HEPES.

mRNA expression analysis

RNA was extracted as described earlier (24) and further purified using the RNeasy Micro Kit (Qiagen, Venlo, Netherlands) with on-column DNA-digestion. Total RNA was quantified accurately using NanoDrop ND-1000 UV-Vis (Isogen Life Science, IJsselstein, Netherlands) spectrophotometer, prior to cDNA synthesis using RevertAid™ First Strand cDNA Synthesis Kit (#1622, MBI Fermentas, Germany). Expression levels of collagen type II (*COL2*) and type I (*COL1*), *SOX9* and aggrecan (AGC1) were studied as markers for chondrogenic differentiation. In the experiments with FK506, expression levels of *TGFβ1, TGFβ2, TGFβ3* and collagen type X (*COL10*; marker for hyperthrophy) were studied. The TGFβ1 (NM_000660) specific primers HsTGFb1_F GTGACAGCAGGGATAACACACTG, HsTGFb1_R CATGAATGGTGGCCAGGTC, and the FAM-labeled hydrolysis probe HsTGFb1_FAM ACATCAACGGGTTCACTACCGGC were used at 200 nM (each) and 600 nM, respectively. The other QPCR assays (*COL2, COL1, SOX9, AGC1, COL10* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)) were used as reported earlier(24-26). Reactions were performed as 20µL reactions using TaqMan® Universal PCR MasterMix (ABI, Branchburg, NJ, USA) or qPCR™ Mastermix Plus for SYBR® Green I (Euro-

gentec, Maastricht, Netherlands) according to the manufacturer's guidelines on an ABI PRISM® 7000 with SDS software version 1.7. Data were normalized to *GAPDH*, which was stably expressed across samples (data not shown), and relative expression was calculated according to $2^{-\Delta CT}$ method (27).

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Data were compared between groups by Kruskall-Wallis H test and post-hoc Mann–Whitney U test. Results represent mean ± standard deviation and $p<0.05$ was considered to indicate statistically significant differences.

Results

Chondrocyte phenotype and calcineurin activity

To investigate the relation between calcineurin activity and the phenotype of adult human chondrocytes, we first studied calcineurin activity during chondrocyte dedifferentiation by serial passaging. To confirm changes in chondrocyte phenotype resulting from serial passaging, we determined mRNA expression levels of collagen type II (*COL2*) and type I (*COL1*). During chondrocyte dedifferentiation, expression levels of *COL2* significantly decreased, while that of *COL1* increased (Figure 1A). The collagen type II / type I (*COL2/COL1*) ratio strongly decreased from 13 in primary (Passage 0; P0) chondrocytes to 0.00005 in passage 3 (P3) chondrocytes.

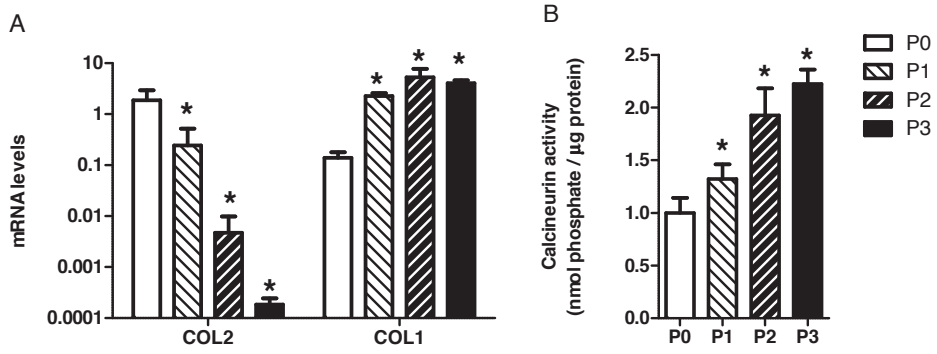

Figure 1. Relative expression of chondrogenic markers and calcineurin activity during chondrocyte dedifferentiation. **A.** Expression of collagens type II (COL2) and type I (COL1) in primary (P0) chondrocytes compared to dedifferentiated (P1-P3) chondrocytes. **B.** Relative calcineurin activity per µg of total protein in primary (P0) chondrocytes compared to dedifferentiated (P1-P3) chondrocytes. All data are means ± SD. * indicates significant difference with P0 cells ($p<0.05$).

During chondrocyte dedifferentiation, calcineurin activity significantly increased with increasing passage number (Figure 1B).

In addition, we studied calcineurin activity after chondrocyte redifferentiation in alginate culture, with addition of TGFβ2 for 21 days. *COL2* mRNA expression levels strongly increased (Figure 2A), with an increased *COL2/COL1* ratio from 0.0009 to 3, indicating redifferentiation of the cells. Redifferentiation was accompanied by a strong decrease in calcineurin activity (Figure 2B).

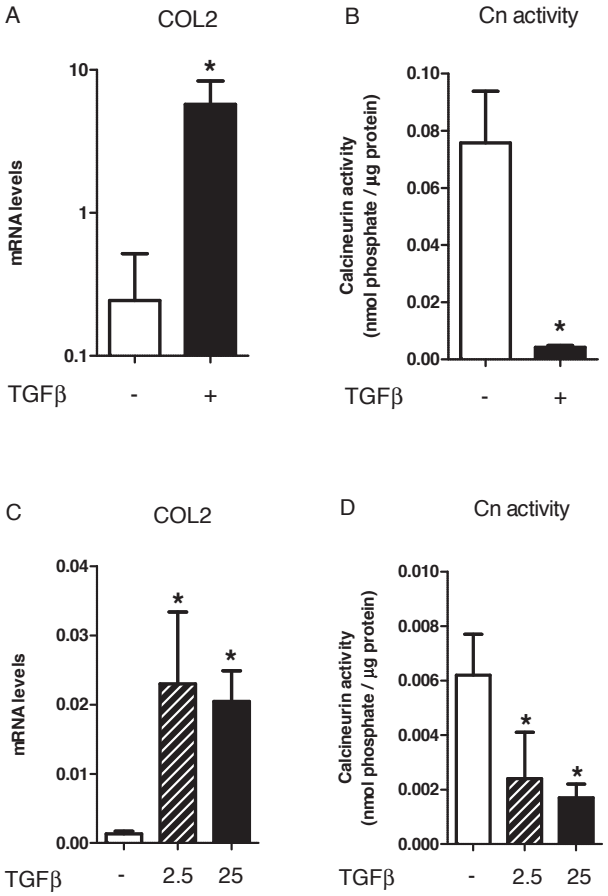

Figure 2. Collagen type II mRNA levels and calcineurin activity of chondrocytes cultured in alginate or monolayer with TGFβ2. **A.** Expression of collagen type II (COL2) mRNA in chondrocytes in alginate culture with or without TGFβ2 for 21 days. **B.** Calcineurin activity per µg of protein in chondrocytes in alginate culture with or without TGFβ2 for 21 days. **C.** Collagen type II (COL2) mRNA in chondrocytes treated with or without TGFβ2 for 10 days in monolayer culture. **D.** Calcineurin activity per µg of protein after 10 days of monolayer culture with TGFβ2. All data are means ± SD. * indicates significant difference with untreated cells ($p<0.05$).

For greater convenience and higher throughput, we also investigated whether monolayer cultures (2D-cultures) could be used to induce the effects on chondrogenic marker expression. High (25 ng/mL) and low (2.5 ng/mL) concentrations of TGFβ2 both significantly increased collagen type II mRNA expression levels (Figure 2C), leading to a 3-fold increase of the *COL2/COL1* ratio. Calcineurin activity significantly decreased by addition of TGFβ2 in monolayer (Figure 2D). Although, mRNA expressions of chondrogenic markers were lower than in alginate (Figure 2A), a clear improvement in chondrocyte marker expression was visible in the monolayer cultures, indicating that monolayer cultures can be used for the follow-up experiments using inhibitors of calcineurin activity and experiments to investigate underlying mechanisms.

Inhibition of calcineurin activity increases the expression of chondrogenic markers

As calcineurin activity increased during dedifferentiation and decreased during redifferentiation of chondrocytes, we investigated the effects of the calcineurin activity inhibitor FK506 on chondrogenic marker expression.

We first confirmed that FK506 inhibited calcineurin activity in dedifferentiated chondrocytes (P2). After 4 and 24 hours of incubation with 50 or 500 ng/mL FK506 the calcineurin activity significantly dropped (Figure 3A). Both 50 and 500 ng/mL FK506 significantly increased *COL2, AGC1* and *SOX9* expression levels after just 24 hours (Figure 3B). After 10 days of culture, the mRNA levels of *COL2* were still significantly higher in the FK506-conditions than in the control condition (Figure 3C) and similar to the effects of TGFβ2 in monolayer culture (Figure 2C). Although the expression of *COL1* was slightly upregulated by FK506, the *COL2/COL1* ratio was increased by 2.5-fold after addition of 500 ng/mL FK506 after 10 days (data not shown). In addition, the mRNA levels of *SOX9* were also still significantly higher in the FK506-conditions than in the control condition (Figure 3C). *COL10* expression was undetectable in the control and in all FK506 conditions (data not shown). We concluded that inhibition of calcineurin activity by FK506 induced expression of chondrogenic markers.

FK506-induced expression of collagen type II via endogenous TGFβ1

To explain the effects of FK506 on chondrogenic markers, we investigated the effect of FK506 on *TGFβ1, TGFβ2* and *TGFβ3* mRNA levels. After 24 hours of culture, both 50 and 500 ng/mL FK506 significantly increased *TGFβ1* mRNA levels (Figure 4A), while the levels of *TGFβ2* and *TGFβ3* were not significantly altered (data not shown). In addition, TGFβ1 protein level was significantly increased after 48 hours and even more increased at day 7 (Figure 4B).

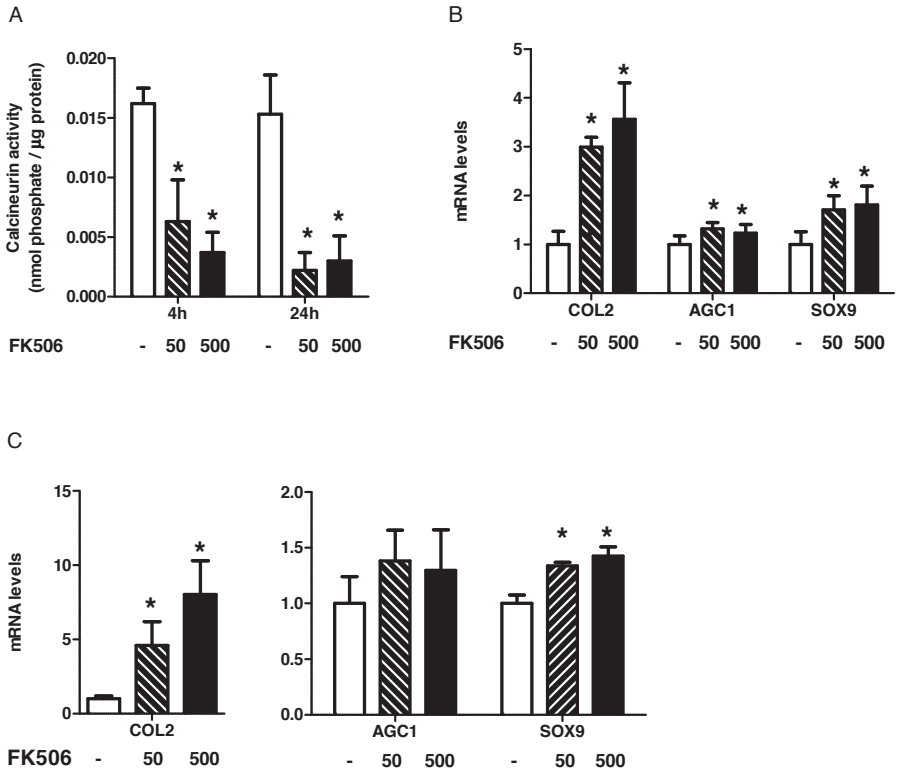

Figure 3. Effects of FK506 on calcineurin activity and on the mRNA levels of chondrogenic markers. Chondrocytes were expanded in monolayer for two passages and subsequently cultured in monolayer with 0, 50 or 500 ng/mL FK506. **A.** Calcineurin activity per µg of protein in P2 cells treated with 50 or 500 ng/mL FK506 for 4 and 24 hours, compared to untreated cells. **B** Relative mRNA levels of collagen type II (COL2), aggrecan (AGC1) and SOX9 after 24 hours of culture with FK506. **C.** Shown are mRNA levels of collagen type II (COL2), aggrecan (AGC1) and SOX9 after 10 days of culture. All data are means ± SD. * indicates significant difference with untreated cells ($p<0.05$).

Therefore, we hypothesized that this endogenous TGFβ1 production might be responsible for the chondrogenic effects of FK506 on dedifferentiated chondrocytes. Neutralizing the activity of endogenous TGFβ1 with an anti-TGFβ1 antibody significantly decreased the FK506-mediated increase in *COL2* expression after 10 days (Figure 4C). While anti-TGFβ1 also inhibited the slight increase in *AGC1* by FK506, it had no effects on *SOX9* expression (data not shown). The anti-TGFβ1 antibody had no effects on *COL2* expression levels in cells treated with rhTGFβ2 in redifferentiation medium, indicating that there was no cross-reactivity to TGFβ2 and no inhibitory effect of the antibody on chondrocyte redifferentiation otherwise.

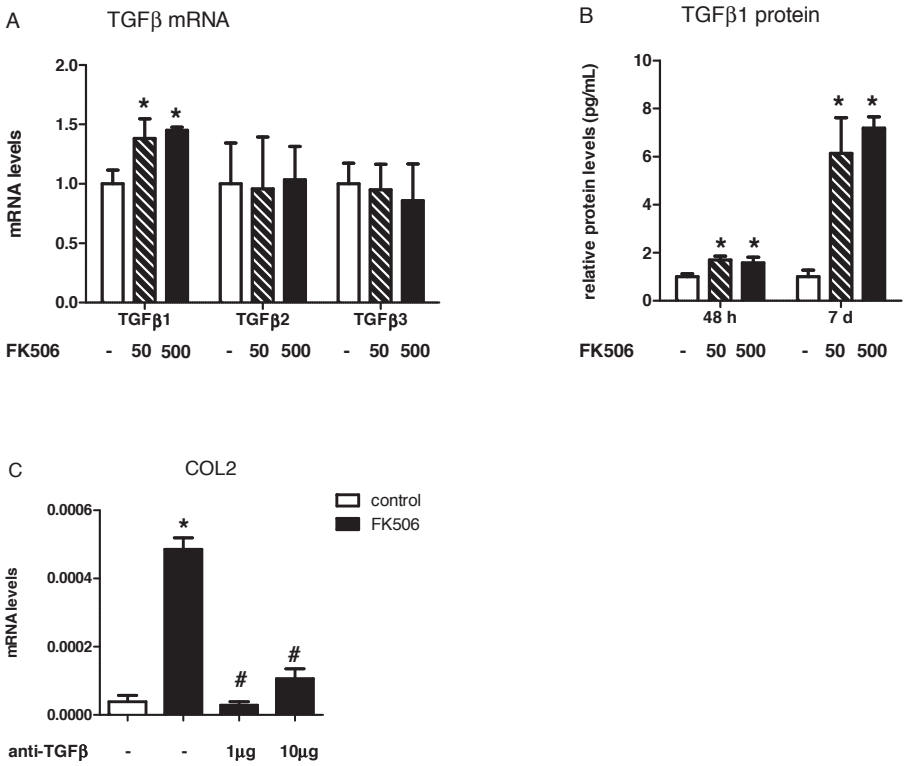

Figure 4. Effect of anti-TGFβ1 antibody on FK506-induced collagen type II expression.
A. TGFβ1 mRNA levels in cells treated with 50 or 500 ng/mL FK506 for 24 hours. **B.** TGFβ1 protein levels measured by ELISA in the culture medium after 48 hours and 7 days of culture with FK506. **C.** Collagen type II mRNA levels after 10 days of culture with FK506 with or without anti-TGFβ1 antibody. All data are means ± SD. * indicates significant difference with untreated cells ($p<0.05$). # indicates significant difference with the FK506 condition without anti-TGFβ1 ($p<0.05$).

Cn activity after addition of TGFβ

Next we investigated whether redifferentiation by TGFβ is directly due to modulation of calcineurin activity. Culturing chondrocytes in redifferentiation medium with rhTGFβ2 (serum free), significantly decreased Cn activity after 3 days (data not shown) and 10 days (Figure 2D). Surprisingly, calcineurin activity increased after 24 hours of treatment with TGFβ (Figure 5A).

As calcineurin is a calcium-dependent phosphatase, we hypothesized that the short-term peak in Cn activity by TGFβ is mediated by an increase in calcium influx. TGFβ-mediated alterations in Cn activity were completely blocked by chelation of extracellular calcium with EGTA for 15 min and 24 hours. Figure 5B confirms that Cn activation by TGFβ is altered by influx of extracellular calcium. In addition,

EGTA completely blocked the TGFβ-mediated increase in collagen type II expression after 3 days (Figure 5C). Pre-incubation with the intracellular calcium chelator BAPTA-AM did not affect TGFβ-mediated Cn activity (data not shown), indicating that TGFβ-mediated Cn activity is not dependent on intracellular calcium stores. To determine whether the short-term peak in Cn activity is necessary for TGFβ-induced redifferentiation, we cultured chondrocytes with TGFβ in redifferentiation medium supplemented with FK506. FK506 inhibits the short-term, TGFβ-induced peak in Cn activity. We found that the TGFβ-induced increase in *COL2* expression increased by FK506 (Figure 5D).

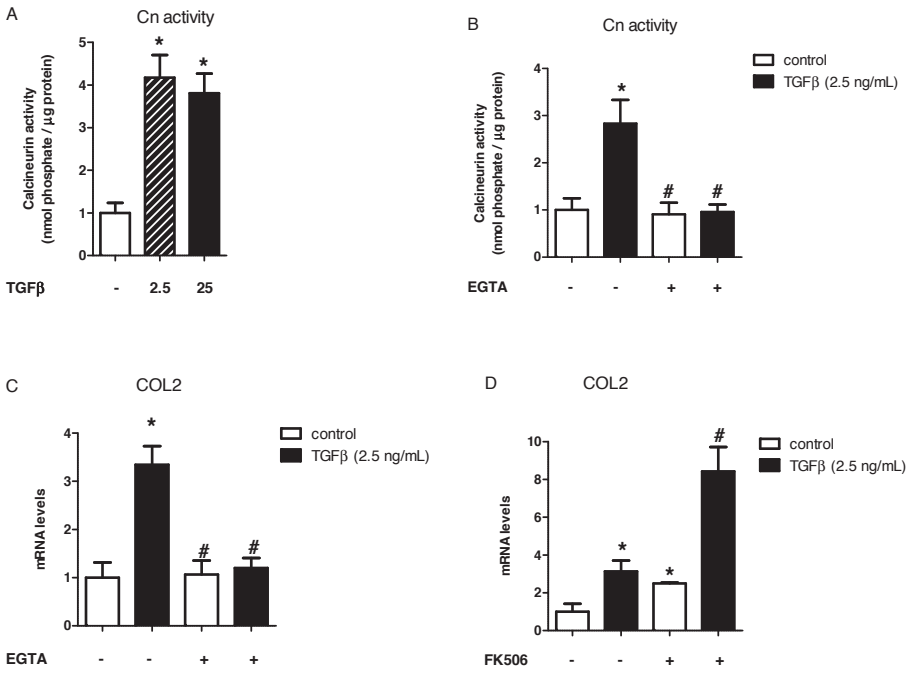

Figure 5. Effects of extracellular calcium chelation or calcineurin inhibition on TGFβ-induced collagen type II expression**. A.** Calcineurin activity per µg of protein after 24 hours of culture with 2.5 or 25 ng/mL TGFβ. Data are mean expressions relative to control (without TGFβ) ± SD means ± SD. * indicates significant difference with untreated cells ($p<0.05$). **B.** Calcineurin activity per µg of protein after chelation of extracellular calcium with 5 mM EGTA for 24 hours. Data are means ± SD. * indicates significant difference with control (without TGFβ and EGTA). # indicates significant difference with the TGFβ/ without EGTA condition. **C.** Effect of EGTA on TGFβ-induced Collagen type II (COL2) mRNA expression after 3 days. Data are mean expressions relative to control (without TGFβ and EGTA) ± SD of values normalized for GAPDH. * indicates significant difference with control. # indicates significant difference with TGFβ/ without EGTA condition. **D.** Effect of FK506 on TGFβ-induced Collagen type II (COL2) mRNA expression after 3 days. Data are mean expressions relative to control (without TGFβ and FK506) ± SD of values normalized for GAPDH. * indicates significant difference with control. # indicates significant difference with all other conditions.

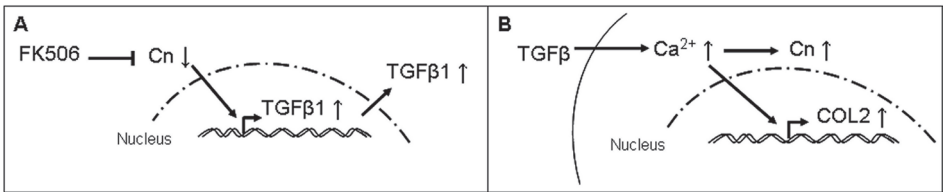

Figure 6. Schematic of FK506 signaling and TGFβ signaling in human articular chondrocytes. **A.** FK506 induces expression of chondrogenic markers via inhibition of calcineurin activity and TGFβ1 gene and protein expression. **B.** The redifferentiation effects of rhTGFβ are mediated via an increased calcium influx, leading to an increase in calcineurin activity. However, the effects of TGFβ on chondrocyte redifferentiation are not dependent on calcineurin activity modulation, suggesting that other calcium-sensitive enzymes might mediate TGFβ action. We hypothesize that improved chondrogenic marker expression by FK506 involves effect **A**, leading to effect **B** via paracrine or autocrine signaling.

In conclusion, chondrocyte redifferentiation by TGFβ is due to elevation of intracellular calcium concentration upon influx of extracellular calcium. Redifferentiation by TGFβ is not dependent on modulations in calcineurin activity.

Discussion

The present study provides evidence that FK506-induced inhibition of calcineurin activity in human adult articular chondrocytes increases the expression of chondrogenic markers via TGFβ1 signaling. Our results showed that calcineurin activity is strongly regulated during chondrocyte dedifferentiation and redifferentiation in vitro. Inhibition of calcineurin activity by FK506 increased the expression of chondrogenic markers *COL2* , *AGC1* and *SOX9*, indicating that the chondrocytes might shift towards a more chondrogenic phenotype without becoming hypertrophic. In addition to the results by Yoo et al. that inhibition of calcineurin activity protects from cartilage damage in experimental osteoarthritis (OA), we demonstrate that calcineurin inhibition can also be useful for Tissue Engineering purposes because of the anabolic effects on early differentiation of adult human articular chondrocytes in-vitro by increasing chondrogenic marker expression. Furthermore, we demonstrated that increased endogenous TGFβ1 production was responsible for the effects of calcineurin inhibition on collagen type II expression, by blocking endogenously produced TGFβ1.

The FK506-induced expression of chondrogenic markers is consistent with earlier reported effects of FK506 in other cell types (6, 7). To exclude that the effects of FK506 on the expression of chondrogenic markers were caused by unspecific

effects of FK506 and not due to calcineurin inhibition, we also performed experiments with a second calcineurin inhibitor: cyclosporine A (CsA). The effects of 1 and 10 μM CsA on calcineurin activity, on the expression of chondrogenic markers and TGFβ isoforms were comparable with the effects of FK506 (data not shown).

So far, we can conclude that both culturing with TGFβ and culturing with a calcineurin inhibitor induce chondrogenic marker expression. For many years, TGFβ has been the most commonly used chondrogenic factor to induce redifferentiation (10-12). A relation between calcineurin activity and TGFβ signaling could, at least partially, explain the effects of FK506. Our data strongly suggest that endogenous TGFβ1 is the most important TGFβ subtype responsible for the FK506-effects (Figure 6A), because the TGFβ1-antibody was specific for this subtype and blocked the effect of FK506 on collagen type II mRNA expression entirely. The relation between calcineurin inhibition and induction of TGFβ signaling has been described earlier in several other cell types, for example in renal cells (13, 14), vascular smooth muscle cells (15) and lymphocytic B cells (16) and is now also demonstrated to be present in adult human articular chondrocytes.

There are different mechanisms reported by which FK506 and/or CsA induce endogenous TGFβ. FKBP12, the 12-kDa FK506-binding protein, is a receptor for the immunosuppressant drug FK506 (28). FKBP12 occurs in high concentrations in all cells and is known to regulate fundamental aspects of cell biology (29, 30). One of its multiple biological functions, is the inhibition of TGFβ type I receptors (31, 32). FKBP12 binds to the glycine-serine region of a ligand-free TGFβ type I receptor, thereby blocking access to activators. FK506 blocks the FKBP12/TGFβ type I receptor interaction and enhances the ligand activity of the TGFβ type I receptor (31). This mechanism could explain the effects of FK506 on TGFβ signaling in our cultures, but does not explain the effects of CsA on endogenous TGFβ signaling. CsA, but not FK506, is reported to promote the release of preformed TGFβ by inducing apoptosis in human T cells (33). Next to this, there are indications that calcineurin and the calcineurin-dependent nuclear factor of activated T-cells (NFATc) transcription factors can regulate the promoter activity of TGFβ1 (33, 34), which could be the explanation for the effects of FK506 and CsA in our experiments. It would be of interest to investigate whether this mechanism plays a role in human articular chondrocytes.

Since we found this relation between calcineurin activity and endogenous TGFβ1, we also wanted to investigate whether the redifferentiation by addition of rhTGFβ2 depends on modulation of calcineurin activity. Culturing chondrocytes with TGFβ

in redifferentiation medium (serum-free), significantly decreased calcineurin activity at the long term (from 3 days of culture up to 21 days of culture). Interestingly, we found a short-term peak in calcineurin activity between 24 hours and 3 days. As calcineurin is a calcium-dependent phosphatase, we hypothesized that the short-term peak in Cn activity by TGFβ is mediated by an increase in intracellular calcium concentration. It is known that TGFβ can induce an increase of intra-cellular calcium in a variety of cell types (19-23). In rat mesengial cells, TGFβ mediated accumulation of extracellular matrix (ECM) proteins via calcium influx and thereby activation of calcineurin (19-23). In our study, we also found that the short term increase in calcineurin activity by TGFβ2, was mediated via an increase in calcium influx. It is unlikely that the Ca^{2+} influx is specific for TGFβ2, as the role of TGFβ1 in inducing a Ca^{2+} influx has also been described (20, 21, 23). Addition of EGTA to block the calcium influx abolished TGFβ-mediated increase in *COL2* expression, indicating that redifferentiation by TGFβ is due to entry of extracellular calcium (Figure 6B). Inhibition of the early increase in calcineurin activity by FK506 did not inhibit the TGFβ-induced chondrogenic marker expression. This suggests that calcium-sensitive proteins other than calcineurin might mediate TGFβ action. The short-term increase in calcineurin activity by TGFβ is likely a side-effect of the increased calcium influx (Figure 6B). Linking figure 6A and 6B would indicate that when EGTA would be added together with FK506, the endogenously induced TGFβ effect would be inhibited and thereby FK506 would have no effect. This however was not experimentally tested because this would require long term culture (at least 7 days) with EGTA which would lead to too many negative side effects on cell behavior. We conclude that the effects of TGFβ on chondrocyte redifferentiation do not depend on modulations in calcineurin activity. The TGFβ-mediated decrease in calcineurin activity after 3 days is probably a consequence of chondrocyte redifferentiation.

The aim of our study was to investigate calcineurin as potential target to improve chondrocyte phenotype for cartilage tissue engineering purposes. This study provides the first data on the relation between calcineurin activity and chondrocyte dedifferentiation and redifferentiation using gene expression as well accepted measures for chondrocyte phenotype (2, 4). In this study we used monolayer cultures of passaged adult human chondrocytes to show effects of calcineurin inhibitors on chondrogenic marker expression. Although monolayer cultures are a convenient system for studies on mechanisms of action, it is well accepted that chondrocyte phenotype is better supported in 3-dimensional systems. For future application the effects of calcineurin inhibition in 3D systems, analyses of collagen type II expression on protein levels as well as more advanced analyses on matrix assembly and effects on functional properties, would be required. Furthermore the

use of calcineurin inhibitors to control chondrocyte phenotype in-vivo deserves further investigation. As reported earlier, at the moment TGFβ is the most commonly used chondrogenic factor to induce redifferentiation in vitro (10-12). However, TGFβ has been implicated in fibrosis in many organs like eye, lung, heart, liver, kidney, skin and the synovial tissue in articular joints (35, 36). Multiple injections of high amounts (≥ 20 ng) of TGFβ in the knee induced synovial fibrosis and chondro-osteophyte formation (36-39). The use of TGFβ as therapeutic agent for cartilage repair, and even the use of TGFβ in culture before implantation of a construct in vivo, is limited due to the risk of these side-effects. Calcineurin inhibition by FK506 or CsA leads to a relatively low, sustained release of endogenous TGFβ1, which might limit the risks of fibrosis in vivo. Cyclosporine A (CsA) and FK506 are systemically used as primary immunosuppressants in hepatic and cardiac transplantation. Although systemic application of these calcineurin inhibitors is also reported to dose-dependently induce fibrosis of mainly the kidney (40-43), the dosage to be used for local application of FK506 or CsA in the joint is much lower. CsA has been reported as an effective treatment strategy for OA in a mouse model, without any reported side-effects such as fibrosis (5). Next to this, CsA has been reported to promote TGFβ transcription by synovial cells in-vitro, without displaying a profibrogenic effect in an inflamed environment (44).

Local administration of FK506 or CsA in the joint might have another advantage because of the immunosuppressive effects of these agents. Both CsA and FK506 are already used as disease-modifying antirheumatic drugs (45). They inhibit the secretion of cytokines such as interleukin (IL) -2, IL-3, IL-4, tumor necrosis factor (TNF)-α and interferon-γ from T-lymphocytes (46, 47). In tissue engineering procedures the use of cells and scaffolds might evoke inflammatory and immunological reactions that will negatively affect the performance of tissue engineered constructs (see April 2008 issue of Semin Immunol). Furthermore, in at least 50% of the patients with OA inflammation in the synovial membrane is documented (48). This inflammatory response exhibits features of a T cell immune response and several studies support the idea that T cells may play an important role in the pathogenesis and progression of OA (48). Therefore, the immunosuppressive effect of local administration of calcineurin inhibitors might be beneficial for the treatment of OA. Further animal studies are needed to investigate the beneficial and possible disadvantageous effects of calcineurin inhibitors applied during the in-vitro culture period to prepare constructs or when applied directly in the joint.

In conclusion, calcineurin inhibitors stimulate endogenous TGFβ1 production and might be a good alternative for the application of recombinant TGFβ both *in-vitro* and *in-vivo*, to promote cartilage tissue engineering and other cell-based therapies.

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Physiological osmolarity improves human chondrogenic marker expression through nuclear factor of activated T-cells 5 *in vitro*

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Abstract

Chondrocytes experience a hyperosmotic environment compared to plasma (280 mOsm) due to the high fixed negative charge density of cartilage. Standard isolation of chondrocytes removes their hyperosmotic matrix, exposing them to non-physiological conditions. During *in-vitro* expansion, chondrocytes quickly lose their specialized phenotype, making them inappropriate for cell-based regenerative strategies. We aimed to elucidate the effects of osmolarity during isolation and *in-vitro* expansion on chondrocyte phenotype.

Human articular chondrocytes were isolated and subsequently expanded at control osmolarity (280 mOsm) or at moderately elevated, physiological osmolarity (380 mOsm). The effects of physiological osmolarity on chondrocyte proliferation and chondrogenic marker expression were evaluated. The role of Tonicity-responsive Enhancer Binding Protein (TonEBP/NFAT5) in response to physiological osmolarity was investigated using NFAT5 RNA interference.

Moderately elevated, physiological osmolarity (380 mOsm) did not affect chondrocyte proliferation, while higher osmolarities inhibited proliferation and diminished cell viability. Physiological osmolarity improved expression of chondrogenic markers and NFAT5 and its target genes, while suppressing dedifferentiation marker collagen type I and improving type II/type I expression ratios >100-fold. Effects of physiological osmolarity were similar in osteoarthritic and 'normal' (non-osteoarthritic) chondrocytes, indicating a disease-independent mechanism. NFAT5 RNA interference abolished osmolarity-mediated effects and revealed that NFAT5 positively regulates collagen type II expression, while suppressing type I.

Physiological osmolarity provides a simple, yet effective, means to improve phenotypical characteristics during cytokine-free isolation and *in-vitro* expansion of human articular chondrocytes. Our findings will lead to the development of improved cell-based repair strategies for chondral lesions and provides important insights into mechanisms underlying osteoarthritic progression.

Introduction

Hyaline articular cartilage is a connective tissue covering the ends of bones in joints and is composed of specialized cells, chondrocytes, that produce a large amount of extracellular matrix. This matrix is crucial for the unique biomechanical properties of this tissue and is composed of a collagen fiber network, providing tensile strength and flexibility, and abundant ground matrix rich in proteoglycans (1). The glycosaminoglycan (GAG) side chains of the proteoglycans are sulfated and responsible for a characteristic high fixed negative charge density (2) which binds mobile cations (mainly sodium). This determines the physiological osmolarity (tonicity) of the extracellular fluid around chondrocytes *in vivo*, but the osmolarity indirectly also largely depends on the quality of the collagen network. Extracellular osmolarity in healthy cartilage ranges between 350 and 480 mOsm (3, 4). *In vivo*, osmolarity of the extracellular fluid is dynamic and changes due to alterations in matrix hydration (5). During cartilage degeneration, i.e. in osteoarthritis (OA), collagen matrix degrades and GAG concentration diminishes, resulting in a severity-depending decreased osmolarity of between 280 and 350 mOsm (3, 6). Currently, chondrocyte isolation and *in-vitro* expansion culture are performed in medium of non-physiological osmolarity (270±20 mOsm). Several studies already showed that chondrocytes are osmolarity responsive (7-9) and react with changes in matrix synthesis (4, 8, 10, 11), but focused on aggrecan (AGC1) core protein mRNA levels, AGC1 promoter activity and GAG production.

Molecular mechanisms involved in the hyperosmotic response of human articular chondrocytes (HACs) are poorly understood. Hyperosmolarity perturbs cells by causing osmotic efflux of water, resulting in cell shrinkage (12, 13). Cells react by a rapid uptake of ions, which increase cellular ionic strength (14) with potentially detrimental effects (15-17). The initial, rapid response is the activation of transporters that exchange these ions for compatible osmolytes (16, 18). This process is controlled by Tonicity-responsive Enhancer Binding Protein (TonEBP/*NFAT5*) which mediates transcriptional activation of these transporters (16). NFAT5 is a member of the Rel family of transcription factors (19) and targets sodium/myo-inositol cotransporter (SMIT) (20, 21), sodium/chloride coupled acid transporters (BGT1/SLC6A12) (20), aquaporin channels (AQP1 and -2) (22), and calcium-binding proteins (S100A4) (23-25). Upon hyperosmotic stress, transcription of NFAT5 itself is up-regulated several cell types (26-28), but the osmolarity threshold and cell signaling pathways required to activate NFAT5 may be cell type specific (29). Currently, nothing is known about the expression or function of NFAT5 in HACs.

Chondral lesions by e.g. trauma or overuse, can cause joint pain, immobility and eventually osteoarthritis (OA). The associated high prevalence (60% of all patients undergoing knee arthroscopy is diagnosed with a chondral lesion (30)) and loss of quality of life makes cartilage damage a major personal and economical burden. Treatment options of chondral lesions are limited and autologous chondrocyte implantation (ACI) is the currently most developed hyaline repair technique for the knee (31). Lately, characterized chondrocyte implantation (CCI), employing a phenotypical pre-screening prior to implantation, improved structural repair (32). Chondrocyte dedifferentiation during *in-vitro* expansion for ACI is detrimental, but almost inevitably in standard monolayer culture: spherical chondrocytes will gradually convert into fibroblast-like cells (33, 34). This morphological change is accompanied by a shift in collagen expression towards less collagen type II (COL2) and more collagen type I (COL1) (34, 35). Consequently, dedifferentiated chondrocytes produce fibrocartilage *in vivo*, with an extracellular matrix of inferior biomechanical properties due to higher collagen (especially type I) content and less proteoglycans compared to hyaline cartilage (36). Three-dimensional culture systems can partially prevent dedifferentiation, but are labor intensive and essentially impair propagation. Chondrocyte dedifferentiation might also play a role in the pathogenesis of osteoarthritis (OA), as the ability of aging chondrocytes to produce and repair the extracellular matrix is compromised (37) and collagen type I is shown to be present in clusters of fibrillated areas in late stage OA, while it is absent in healthy cartilage (38).

Here we report that physiological osmolarity (380 mOsm) during isolation and monolayer expansion can suppress chondrocyte dedifferentiation and that expression of the extracelluar matrix components collagen types I and II as well as aggrecan is NFAT5-dependent. We further show that NFAT5 contributes to the differential regulation of both collagen types. This study provides a simple, yet novel and effective, means to improve cell-based repair strategies for chondral lesions and contribute to our understanding of osteoarthritic progression.

Materials and Methods

Cartilage and chondrocyte isolation

After informed consent was obtained, human articular cartilage was explanted from macroscopically normal areas of the femoral condyles and tibial plateau of nine patients undergoing total knee replacement surgery for OA (medical ethical approval MEC2004-322). In addition to preparation of cartilage explants and isola-

tion of human articular chondrocytes (HACs) under standard conditions (DMEM, 280 mOsm) as described by Das et al. (39), medium osmolarity was also adjusted to 380, 480 or 580 mOsm, respectively by addition of sterile NaCl. Enzymatic digestion, removal of undigested fragments and subsequent chondrocyte culture are reported earlier (39). The 280 and 380 mOsm isolations were also performed with cartilage obtained from the femoral condyles and tibial plateau of two non-OA donors (further referred to as 'normal' donors), undergoing above-knee amputation surgery after trauma.

Chondrocyte proliferation and DNA measurements

Primary (passage 0; P0), P1, P2 and P3 HACs were monolayer expanded in medium corresponding to their isolation osmolarity (280, 380, 480 or 580 mOsm), with an initial seeding density of 6,000 cells/cm^2. Cells were harvested daily for cell counts and DNA assay between day two and day six. Experiments were performed in duplicate from three OA donors (n=6). At each passage, growth curves were established by cell counts using Trypan Blue (Sigma, cat. T8154) and DNA quantification. DNA measurements were performed according to Karsten et al. (40) with slight modifications (41). Doubling times within each passage were calculated from the trend line of the exponential growth phase using equation $y = x(0) \exp(kx(t))$ with $k = \ln 2 / T$ (k = growth constant, T = doubling time).

Chondrocyte expansion

Primary HACs were cultured for expansion in monolayer at a seeding density 7,500 cells/cm^2 in medium corresponding to their isolation osmolarity (280, 380, 480 or 580 mOsm). Primary through P3 cells were seeded in high-density monolayers (20,000 cells/cm^2) and cultured for additional five and seven days before analysis of mRNA (quantitative RT-PCR) and protein expression (Western Blotting) respectively. Experiments were performed in triplicate from four OA donors (n=12). Additionally, experiments were performed in triplicate from two healthy donors (n=6), to investigate whether the hyperosmotic stress response is specific for pathologically altered cells. To exclude sodium or chloride specific effects, we performed experiments using NMDG-Cl or sucrose to adjust medium tonicity to 380 mOsm.

Lentiviral NFAT5 gene knock-down

We used lentiviral vectors for non-transient shRNA-mediated gene silencing in primary chondrocytes (42). *Bam*HI/*Mun*I restriction fragments of the parental pLKO.1-puro vector, each containing the U6 promotor and one out of five different, sequence-verified anti-human NFAT5 shDNAs (MISSION shRNA library; www.

sigmaaldrich.com) were subcloned into corresponding restriction sites of recipient vector pRRL.PPT.PGK.GFPpre. This was kindly provided by L. Naldini (San Raffaele Telethon Institute for Gene Therapy, Milano, Italy) (43, 44) and optimized by A. Schambach (Department of Experimental Hematology, Hannover Medical School, Hannover, Germany) (45) to express eGFP from the phosphoglycerate kinase promoter. Lentiviral particles were produced in HEK293T cells by transient transfection using a calcium phosphate protocol (46). Cells transduced with a lentiviral vector lacking the NFAT5-specific shRNA expression cassette served as controls. All cells were grown in monolayer. TRCN0000020020 was identified as best performing anti-NFAT5 shRNA clone by QPCR-based knockdown efficiency determination and used in subsequent experiments.

Passage 1 osteoarthritic HACs from two donors were seeded (15,000 cells/cm^2) and cultured for four days in control medium (280 mOsm). Three hours prior to transduction, cells were deprived of antibiotics, transduced for ± 18 hours, refreshed with control medium with antibiotics and cultured for additional four days before harvesting for FACS analyses. Cells were resuspended in PBS with 10% FCS and antibiotics and washed. Cells were collected and stained with Hoechst 33258 (1 mg/mL, Molecular Probes) to discriminate between dead and live cells. FACS was performed on FACSAria (Becton Dickinson) and eGFP expressing cells were collected (>50%, MOI~1) and re-analyzed for purity (> 95%) using Cell Quest Pro Software.

EGFP expressing populations were seeded (10,000 cells/cm^2) and cultured in control medium up to 80% confluency. Cells were then switched to medium of 380 mOsm or kept on control medium for 24 hours prior to RNA analysis.

RNA expression analysis

RNA isolation, purification, quantification and cDNA synthesis are described elsewhere (47). Expression levels of aggrecan (*AGC1*), *SOX9* and collagen type II (*COL2*) were studied as chondrogenic markers, while collagen type I (*COL1*) was studied as dedifferentiation marker (34, 35, 48, 49). QPCR assays for *COL2, SOX9, AGC1 and COL1* were reported earlier (50). To quantify expression of *NFAT5* and its target genes, the following primers were tested for similar amplification efficiency and specificity according to Das et al. (39) and used as 20μL SYBR® Green I reactions: HsNFAT5_Fw, GGGTCAAACGACGAGATTGTG, HsNFAT5_Rv, TTGTCCGTGGTAAGCTGAGAA; HsS100A4_Fw, GTCCACCTTCCACAAGTACTCG, HsS100A4_Rv, TCATCTGTCCTTTTCCCCAAG; HsSLC6A12_Fw, ACACAGAGCATTGCACGGACT, and HsSLC6A12_Rv, CCAGAACTCGTCTCTCCCAGAA). Data were normalized to

an index of three reference genes (*GAPDH, UBC, HPRT1*) which were pre-evaluated to be stably expressed across samples (39). Relative expression was calculated according to $2^{-\Delta CT}$ method (51).

Western Blot analysis

Cells seeded at high densities were washed twice with PBS and lysed in RIPA buffer (52) with addition of protease inhibitors. Total protein concentration was quantified by BCA assay according to the manufacturer's protocol (Pierce, #23225). Ten µg aliquots were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) prior to electro-blotting onto nitrocellulose membranes (Protran BA83, Schleicher & Schuell). Blots were blocked in 5% low-fat dry milk in 1X PBS, 0.05% v/v NP-40, incubated with primary antibodies (anti-type II collagen and anti-type I collagen (both 1:100, SouthernBiotech, or 1:10,000 anti-α-Tubulin (Sigma)), washed, incubated with secondary antibodies (both 1:1,000, Dako Cytomation) and chemiluminescently detected. Signals were quantified using ImageJ 1.42 software.

Statistics

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Data were compared between groups by Kruskall-Wallis H test and post-hoc Mann–Whitney U test. Results represent mean ± standard deviation and $p<0.05$, $p<0.01$ and $p<0.001$ were considered to indicate levels of statistically significant difference.

Results

Osmolarity influences proliferation and survival of chondrocytes

We first determined the influence of osmolarity on proliferation: osteoarthritic HACs (OA-HACs) isolated at 580 mOsm hardly attached nor proliferated (Figure 1; photo D) and two days after seeding no viable cells were recovered. At 280, 380 and 480 mOsm, respectively, cells did adhere but increasing osmolarity induced marked morphological changes: at 280 mOsm, cells appeared fibroblast-like stretched-out and flattened with long filopodia (Figure 1; photo A), while at 380 mOsm cells were more sphere-shaped and had shorter filopodia (Figure 1; photo B). At 480 mOsm, cells showed few filopodia and appeared spherical (Figure1; photo C). The differences in appearance remained throughout dedifferentiation period (P0 to P3), but were most apparent at earlier passages.

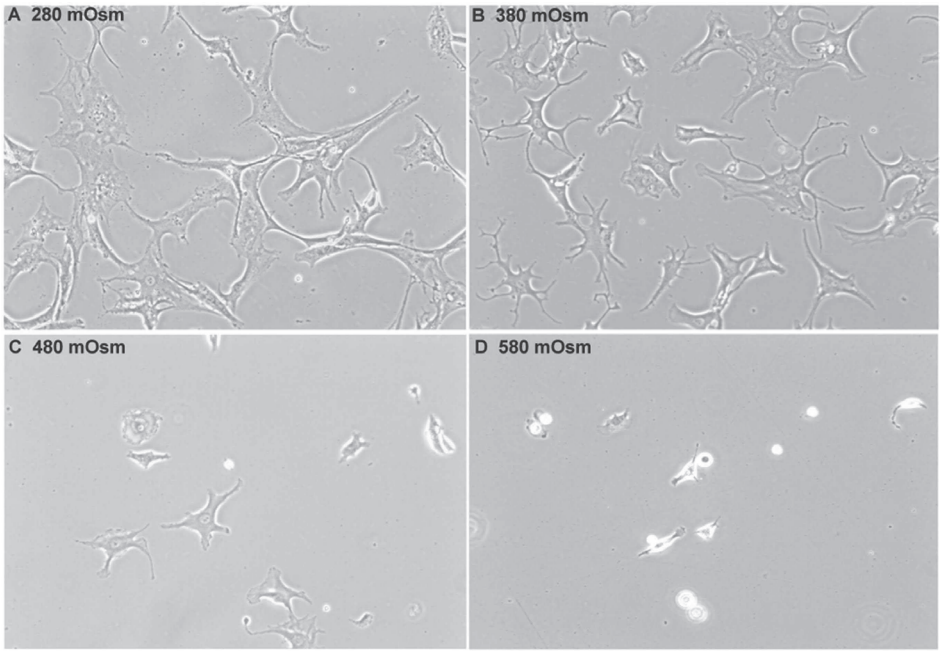

Figure 1. Hyperosmotic isolation and expansion of chondrocytes changes chondrocyte morphology. Representative images (200X) of chondrocytes cultured for 2 days at 280 **(A)**, 380 **(B)**, 480 **(C)** and 580 **(D)** mOsm.

Using cell counts and DNA assays, doubling times were calculated from growth curves established from each passage at three different osmolarities (280, 380 and 480 mOsm). Throughout dedifferentiation, OA-HACs isolated at 480 mOsm showed severely inhibited proliferation compared to cells at 280 and 380 mOsm (Table 1). In contrast, doubling times of OA-HACs at 280 and 380 mOsm never significantly differed (Table 1). Therefore, all further experiments were performed at 380 mOsm (as high osmolarity condition) and compared to 280 mOsm (control condition).

Table 1. Proliferation of chondrocytes isolated and cultured at 280, 380 and 480 mOsm. Displayed are relative doubling times in percentage (%) of cells cultured at 280 mOsm. All data are means ± SD. The absolute doubling time ± SD in hours is displayed in brackets. n=6 *$p<0.05$

Culture condition	P0	P1	P2	P3
280 mOsm	100 % (68 ± 28 hrs)	100 % (89 ± 54 hrs)	100 % (67 ± 48 hrs)	100 % (57 ± 11 hrs)
380 mOsm	113 ± 18 %	89 ± 25 %	99 ± 9 %	154 ± 41 %
480 mOsm	675 ± 405 % *	180 ± 24 % *	168 ± 28 % *	165 ± 81 % *

Isolation and expansion of chondrocytes under hyperosmotic conditions improves their phenotype

Next, we set out to determine whether expansion culture in physiological osmolarity improves the chondrocytic phenotype. Physiological osmolarity (380 mOsm) during isolation and subsequent passaging of OA-HACs significantly increased mRNA levels of both *AGC1* (Figure 2A) and *SOX9* (Figure 2B) at all passages. In expanded P3 chondrocytes in physiological culture, *AGC1* levels were still higher than in unpassaged P0 chondrocytes cultured under the standard culture conditions (280 mOsm). Physiological osmolarity also significantly up-regulated *COL2* levels from 8.5-fold in P0 to 11.6-fold in expanded P3 chondrocytes (Figure 2C) compared to controls. In contrast, *COL1* expression was significantly suppressed in physiological conditions throughout culture. Consequently, we found a significantly improved *COL2/COL1* ratio during chondrocyte expansion (Figure 2D), from 7-fold in P0 cells to 100-fold in expanded P3 cells. Physiological osmolarity also up-regulated COL2 protein expression (Figure 2E): levels significantly increased (between 1.5- and 2.2-fold) in P0, P1 and P2 chondrocytes. In contrast, physiological osmolarity significantly decreased COL1 protein expression (Figure 2F), from 2-fold in P0 cells to 13-fold in P1 cells. Physiological osmolarity also significantly increased *AGC1* (Figure 3A) and *SOX9* (Figure 3B) mRNA levels in non-osteoarthritic HACs (NHACs). Furthermore, *COL2* mRNA levels were significantly upregulated; from 5.8-fold in P0 cells to 270-fold in expanded P3 NHACs (Figure 3C). Like in OA-HACs, hyperosmolarity also down-regulated *COL1* expression with increasing passage number in NHACs: the *COL2/COL1* ratios increased during expansion (Figure 3D); from 6.8-fold in P0 cells to 355-fold in expanded P3 cells. Correspondingly, COL2 protein levels increased under these conditions (4.8-fold in P1 cells and 2.9-fold in P2 cells), while the amount of COL1 diminished (by 4.7-fold in P1 cells and 5-fold in P2 cells; Figure 3E, F).

Osmolarity activates NFAT5 in human articular chondrocytes

Compared to 280 mOsm controls, *NFAT5* mRNA levels were significantly increased in 380 mOsm OA-HACs cultures (Figure 4A), as was the expression of established NFAT5 target genes *S100A4* (in all passages; Figure 4B) and *SLC6A12* (until P2; Figure 4C). Similar effects were found in NHACs (data not shown).

NFAT5 knockdown inhibits osmolarity-induced chondrogenic marker expression

Upon transduction, sorted eGFP co-expressing OA-HACs were switched to 380 mOsm for 24 hours. In controls, not expressing *NFAT5*-specific shRNAs, a ~2-fold increase in *NFAT5* mRNA levels was observed (Figure 4A; P1) upon osmotic stimula-

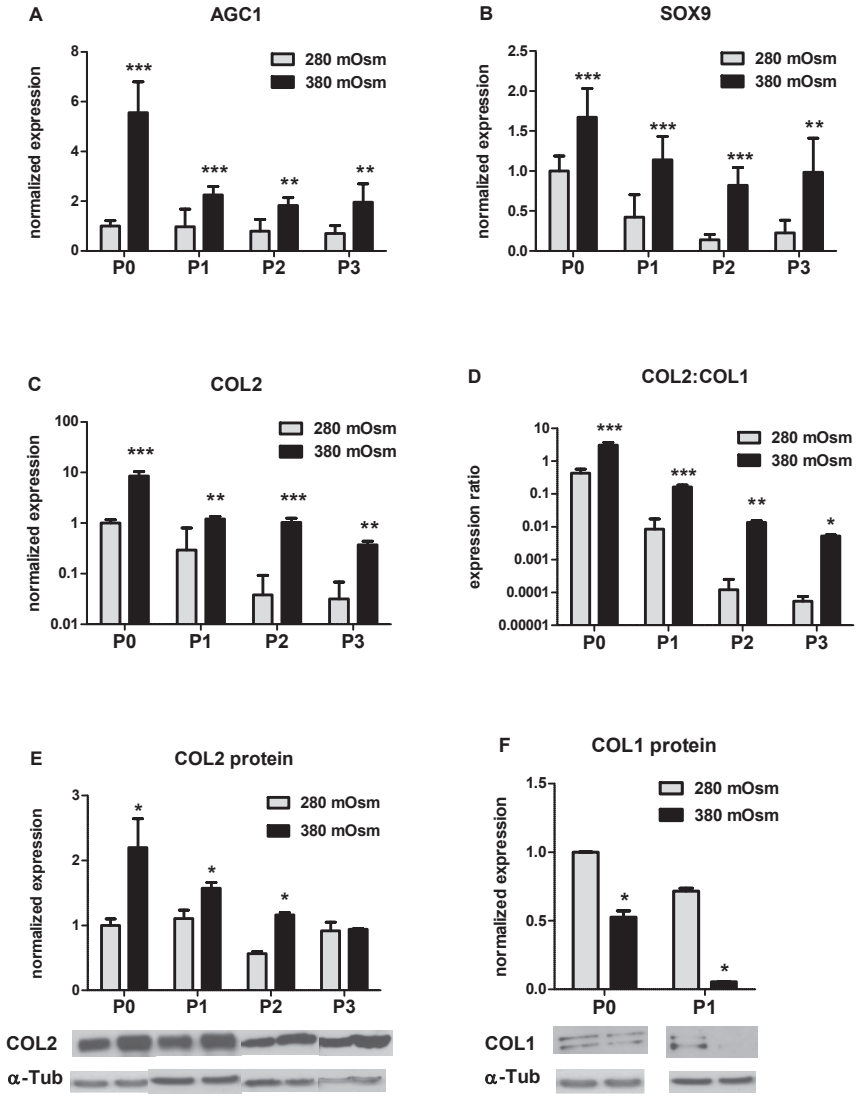

Figure 2. Hyperosmotic isolation and expansion increased marker gene expression in OA-HACs. Relative expression of *AGC1* **(A)**, *SOX9* **(B)**, *COL2* **(C)** and *COL2:COL1* ratio **(D)** in primary (P0) and passaged (P1-P3) chondrocytes cultured at 380 mOsm compared to 280 mOsm. COL2 protein expression **(E)** and COL1 protein expression **(F)** in P0 and P1 OA-HACs are shown. Protein levels are normalized to α-Tubulin. All data are means ± SD, n = 12. Differences with cells cultured at 280 mOsm are indicated with * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

tion. In contrast, likewise challenged cells expressing anti-*NFAT5* shRNAs showed a ~75% reduction in *NFAT5* levels (Figure 5A). Following *NFAT5* knockdown, also the *NFAT5* targets, *S100A4* and *SLC6A12*, were no longer osmotically inducible:

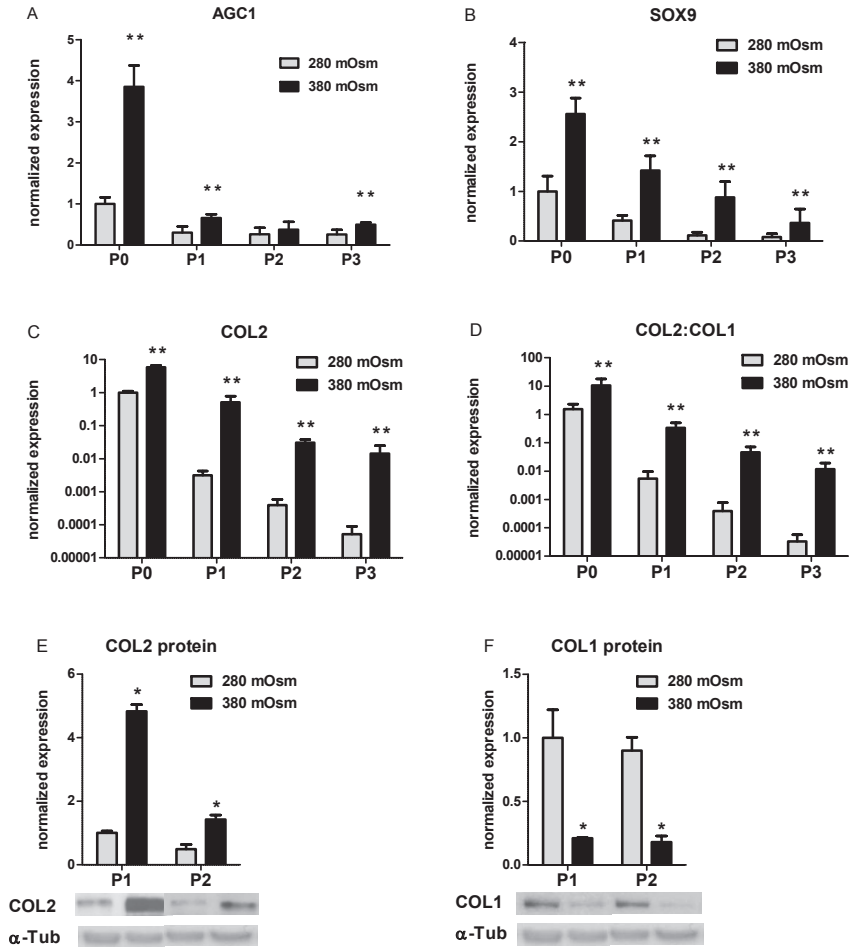

Figure 3. Hyperosmotic isolation and expansion increased chondrogenic marker expression in NHACs. Relative expression of *AGC1* **(A)**, *SOX9* **(B)**, *COL2* **(C)** and *COL2:COL1* ratio **(D)** in primary (P0) and passaged (P1-P3) NHACs cultured at 380 mOsm compared to cells cultured at 280 mOsm. COL2 **(E)** and COL1 **(F)** protein expression in P1 and P2 NHACs are shown and normalized to α-Tubulin. Data are means ± SD; n = 6. Differences to 280 mOsm controls are indicated with * (p<0.05) and ** (p<0.01).

S100A4 expression decreased 2-fold and *SLC6A12* was virtually undetectable upon *NFAT5* RNAi (Figure 5A), confirming a functional NFAT5 knockdown. At 380 mOsm, *NFAT5* RNAi also down-regulated chondrogenic markers: *AGC1* by 80%, *SOX9* by 32% and *COL2* by 84% as compared to non-RNAi controls (Figure 5B). Interestingly, expression of *COL1* increased after *NFAT5* RNAi in OA-HACs to ~300% of control levels (Figure 5B).

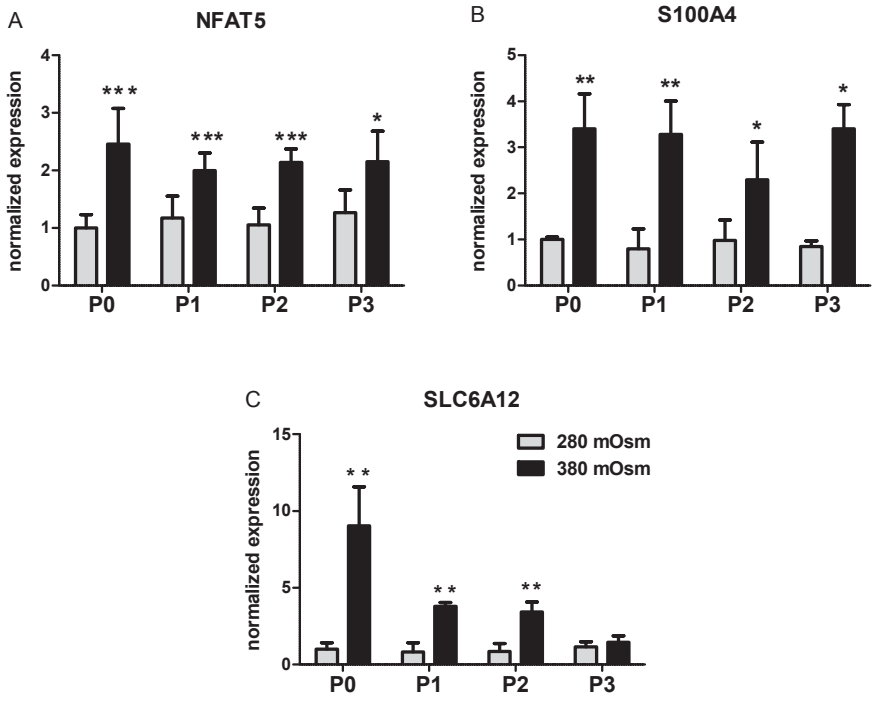

Figure 4. Hyperosmotic conditions activate NFAT5 in OA-HACs. Relative expression of *NFAT5* **(A)** and its target genes *S100A4* **(B)** and SLC6A12 **(C)** in primary (P0) and passaged (P1 to P3) chondrocytes cultured at 380 mOsm compared to 280 mOsm. All data are means ± SD, n = 12. Differences are indicated with * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Discussion

Isolation and expansion of adult human articular chondrocytes under physiological osmolarity (380 mOsm) improves expression of chondrogenic markers on mRNA and protein level. While other studies partially confirm that non-human chondrocytes respond to osmolarity with altered aggrecan and SOX9 expression (4, 8, 10), we are reporting beneficial effects of isolating and expanding human normal and osteoarthritic articular chondrocytes at physiological levels (380 mOsm). In addition, we also studied collagen type II expression, generally acknowledged to be the most important chondrogenic marker. As fibrocartilaginous collagen type I and hyaline collagen type II expression are differentially regulated in chondrocytes (34), analyzing the collagen type II/type I expression ratios is informative of chondrogenic potential (50). Interestingly, NFAT5 seems to be crucially involved in this differential regulation upon osmotic challenge: it positively regulates collagen type II, while suppressing collagen type I (Figure 5B). Fibrocartilage, occurring in areas subject

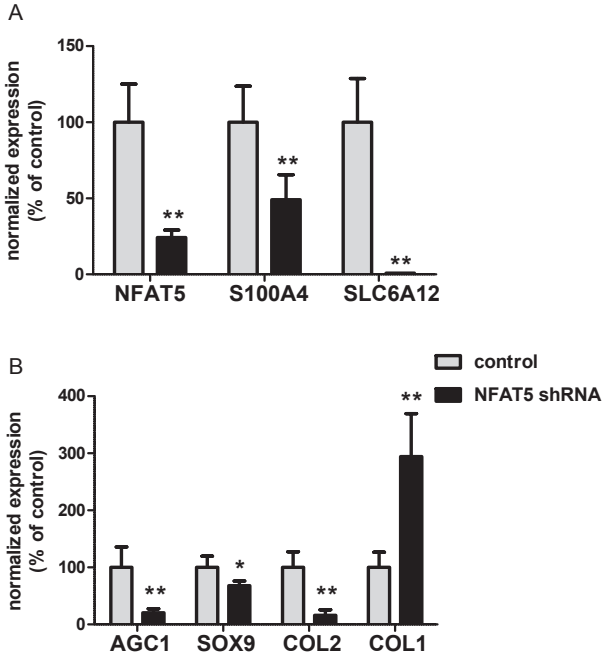

Figure 5. NFAT5 knockdown inhibits osmolarity-induced chondrogenic marker expression.
(A) Relative expression of *NFAT5* and its target genes *S100A4* and SLC6A12 in transduced chondrocytes either expressing (NFAT5 shRNA) or not expressing (control) *NFAT5*-specific shRNAs, 24 hours after increasing osmolarity to 380 mOsm. **(B)** Effects of *NFAT5* knockdown on chondrogenic markers *AGC1, SOX9, COL2* and *COL1*. All data are means ± SD, n = 6. Differences to cells transduced with control virus are indicated with * ($p<0.05$) and ** ($p<0.01$).

to frequent stress like intervertebral discs and tendon attachment sites, is more rich in collagen type I than hyaline cartilage (53). Osmolarity may thus provide a simple means to manipulate expression of these two collagens for broader applications than regenerative chondrocyte implantations (ACI or CCI) alone (54).

Under our conditions, *COL2* mRNA abundances measured by QPCR nicely correlated with protein synthesis as determined by Western Blots (Figure 2 and 3). The same holds for *COL1* expression in the early passages, but not for *COL1* expression in the later passages.

Hyperosmolarity-induced increase in NFAT5 abundance and protein synthesis rate were found to be proportional to the increase in mRNA in MDCK (28) and mIMCD3 cells (27). *NFAT5* mRNA is expressed abundantly in chondrocytes throughout passages and is further induced by osmolarity. However, we failed to show NFAT5 protein expression by Western blotting. Whether this is due to low protein abun-

dance in our cells or technical issues like poor extraction efficiency of this very large transcription factor has to be elucidated in future experiments.

Hyperomolarity induces cell shrinkage which may activate Na^+, K^+, or $2Cl^-$ co-transport allowing cellular accumulation of NaCl and KCl. The beneficial effects on chondrogenic marker gene expression therefore could have been caused by accumulation of specific inorganic ions or specific channel activity rather than primarily osmolarity-mediated effects. We used NMDG-Cl, a bulky substitute for small cations that is impermeable to almost all known channels (55), and sucrose to exclude sodium or chloride specific effects. We were not able to detect any significant differences in gene expression patterns between the NaCl, NMDG-Cl or sucrose means of osmolarity alteration (data not shown).

As our initial studies concerned adult human articular chondrocytes obtained from osteoarthritic knee joints, we aimed at eliminating interpretation bias due to the pathological state of these cells. Using identically challenged non-osteoarthritic cells (NHACs), we showed that these chondrocytes react similarly to the same order of osmolarity with respect to our marker genes: 380 mOsm significantly delayed the phenotypical deterioration of NHACs as observed in control medium. This may imply that physiological osmolarity, postulated to be around 380 mOsm for chondrocytes, is sensed by osteoarthritic and 'normal' cells in a similar fashion. We observed a slightly faster decrease in *AGC1* and *COL2* mRNA levels in P2 and P3 NHACs as compared to OA-HACs. Late stage osteoarthritic chondrocytes from fibrillated areas are dedifferentiated, flattened cells. The loss of a proper spherical shape as an integral part of the chondrocytes phenotype (56, 57) involves cytoskeletal changes (58). Exposing these cells to physiological osmolarity as re-differentiation stimulus likely induces a more enduring response as compared to spherical, normal' chondrocytes. Cell based therapies using the latter are usually restricted to younger individuals after traumatic insults. Autologous chondrocyte implantation employing osteoarthritic cells may benefit relatively more from a hyperosmotic treatment protocol.

The precise molecular mechanism by which osmolarity is sensed by cells is still poorly understood. Hyperosmolarity-increased *NFAT5* mRNA abundances have been shown for other cell types (26-28). NFAT5 is thus accepted as key transcription factor participating in the mammalian hyperosmotic stress response. Our study is the first showing the functional expression of NFAT5 in human articular chondrocytes. In both, osteoarthritic and 'normal' chondrocytes, cellular *NFAT5* mRNA levels are increased by 380 mOsm. In addition, mRNA levels of generally accepted

NFAT5 target genes, *S100A4* and *SLC6A12* (20, 59), were induced accordingly after osmotic challenge, underscoring an involvement of NFAT5. It has recently been suggested that guanine nucleotide exchange factors near the plasma membrane may be activated through cytoskeleton changes or by changes in interactions with putative osmosensors at the cell membrane in other cells (60). Sensation of such basic responses might not be different in chondrocytes than in other cells. Rho-type small G proteins (61) and p38 kinases (62, 63) might act upstream of NFAT5 in chondrocytes, too. In IMCD cells, p38 MAPK signaling was recently also shown to be involved in the NFAT5-mediated hyperosmotic induction of the osmosensitive (64, 65) serine-threonine protein kinase Sgk-1 (66, 67). As p38 MAPK plays important roles in chondrocytes and seems to be necessary for NFAT5 expression (20), further experiments employing pharmacological inhibition or knockdown experiments in HACs will hopefully shed more light into this signaling cascade in chondrocytes.

Increase in *NFAT5* mRNA is usually transient with a cell type depending time course and a 2- to 4-fold upregulation (26, 28) which fits with our data. *NFAT5* mRNA abundance might rapidly increase upon hypertonic stress by a transient increase in its mRNA stability, mediated by its 5'-UTR (27). Whether 380 mOsm is a sufficiently high osmolarity to explain our increase in mRNA by this phenomenon or whether active transcription is involved, has to be addressed in other studies. Interestingly, recently Tew et al. (68) showed that the mRNA of *SOX9*, an important regulator of COL2 expression, is stabilized by supra-physiological osmolarity. Therefore, 380 mOsm might also directly contribute to *SOX9* mRNA stability and abundance in our experiment, rather than elevating promoter activity. COL2 regulation could thus be an indirect effect of osmolarity.

Interestingly, *AGC1* seems to be more stably expressed in cultures maintained at 280 mOsm compared to 380 mOsm, with a lower overall expression in the former condition. Effects of osmolarity on promoter activity and mRNA stability of *AGC1* are incompletely understood. Other groups described the complexity of osmotic stress on gene expression (69, 70). It is tempting to speculate that gene expression may be influenced by morphological changes between our conditions: while cells cultured at 380 mOsm are rather round, cells cultured in monolayer at 280 mOsm are rather flat and more fibroblast-like (see Figure 1). Although we did not investigate actin stress fiber formation in this study, they are usually more pronounced in fibroblastic cells and have been shown to suppress *SOX9* mRNA levels in chondrocytes (49). However, aggrecan expression has been reported to be influenced by both hyper- and hypo- osmolarity (4, 8). The promoter regions of both collagen type II and aggrecan contain a plethora of potential other binding

sites for transcriptional enhancers and suppressors, such as SOX5/6 (71, 72), Barx2 (73), β-catenin (74), c-Maf (75), PIAS (76), TRAP230 (77), Bapx1 (78), and C/EBP and NF-kB (79). Chondrogenic differentiation and SOX9-dependency of aggrecan and collagen expression may also be differentially modulated by these transcriptional co-factors under different osmolarities. Interestingly, while SOX9 dependency of COL2A1 expression has been unequivocally shown, it may not actually be a key regulator of COL2A1 promoter activity in human adult articular chondrocytes (80). Of note, the human aggrecan promoter sequence has been shown to contain a conserved NFAT5 binding site (81). In nucleus pulposus cells, SOX9 mediated aggrecan expression has recently been shown to critically depend on PI3K/AKT signalling (82). Moreover, while high NaCl rapidly activates p38 MAPK, its action can be isoform specific and may exert opposing effects on NFAT5 (83), which in turn may influence COL2A1 and AGC1 transcription differently in a osmolarity-dependent manner. We are therefore currently looking into the underlying molecular mechanisms regulating aggrecan and collagen type II expression in both conditions.

With respect to regenerative medical applications, the high-end hyperosmotic conditions used by Tew et al. can be considered a limitation of that study. In our hands, these osmolarity levels (≥480 mOsm) induced chondrocyte death within 48 hours (Figure 1D) and are not likely applicable for chondrocyte expansion culture. To ensure sufficient cell numbers for cell-based repair techniques, proliferation capacity of the isolated chondrocytes should not be compromised. Cell numbers generally need to be increased during two passages (>4-10 times) for clinical application (84, 85). We found that supra-physiological conditions (480 and 580 mOsm) clearly compromised survival rates, which is in agreement with data by Racz et al. (17). From our data, we conclude that about 380 mOsm is optimal for both isolation and *in vitro* expansion culture of HACs. NFAT5 knockdown down-regulates its own transcription by 75% and compromises target gene induction (Figure 5), being in line with a functionally active NFAT5 in chondrocytes. Constitutive homodimeric NFAT5 molecules encircle DNA rather independently of osmolarity in solution (86), enabling NFAT5 to exert its biological activity over a wide osmolarity range (87, 88). It is thus reasonable to assume that NFAT5 activity is not generally compromised at 380 mOsm. However, other aspects are involved in the regulation of NFAT5 as well as its target genes. Like other proteins larger than 50kDa (89), NFAT5 dependents on nuclear localization and export sequences for its nuclear translocation (26, 86, 89). In most cells, NFAT5 is equally distributed between cytoplasm and nucleus at physiological osmolarity (±300 mOsm), whereas at 500 mOsm most of it localizes to the nucleus (19, 26, 87). To demonstrate that the hyperosmolarity-induced

chondrogenic marker expression was indeed mediated by NFAT5, we used RNAi to confirm that knockdown of NFAT5 significantly i) inhibited hyperosmotic induction of its own transcription as discussed before, (ii) suppressed the osmolarity-mediated induction of known NFAT5 targets, and (iii) most importantly, eliminated the hyperosmolarity-mediated mRNA expression of chondrogenic marker genes (*COL2, AGC1, SOX9 and COL1*).

Conclusions

We have shown that isolation and expansion of adult human articular chondrocytes in culture medium of physiological osmolarity (380 mOsm) improves chondrogenic marker expression and extracellular matrix production through NFAT5. We identified NFAT5 as a novel molecular target preserving chondrocytic marker expression. Our data provide valuable insights for the development of strategies for cell-based repair of chondral lesions and contributes to the understanding of mechanisms involving osteoarthritis.

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Inhibiting calcineurin activity under physiological osmolarity elevates anabolic but suppresses catabolic chondrocyte markers

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Abstract

The physiological interstitial osmolarity (tonicity) of healthy articular cartilage (350-480 mOsm) is lowered to 280-350 mOsm in osteoarthritis. This results in loss of tissue pre-stress, altered compressive behavior and thus inferior tissue properties. We aimed at determining potential synergistic effects of physiological osmolarity in combination with inhibiting calcineurin activity by FK506 on human articular chondrocytes and explants *in vitro*.

Osteoarthritic chondrocytes and explants and non-osteoarthritic chondrocytes were cultured in cytokine-free medium of 280 and 380 mOsm with or without calcineurin inhibition by FK506. Chondrogenic, hypertrophic and catabolic marker expression was evaluated on mRNA, protein and activity level.

Culturing osteoarthritic chondrocytes at 380 mOsm increased mRNA expression of chondrogenic markers (e.g. COL2, ~13-fold, $p<0.001$) compared to 280 mOsm, while suppressing *COL1* (~0.5-fold, $p<0.01$). Inhibiting calcineurin activity under physiological osmolarity further enhanced expression of anabolic markers on mRNA (*COL2*, ~50-fold, $p<0.001$; *AGC1*, ~2-fold, $p<0.001$; *SOX9*, ~3.5-fold, $p<0.001$) and protein (*COL2*, ~6-fold, $p<0.001$) level. Calcineurin inhibition suppressed relevant collagenases as well as hypertrophy and mineralization markers on mRNA and activity level. Aggrecanase-1 and -2 expression was not influenced by osmolarity or FK506 alone, but the combination suppressed both, by ~50% ($p<0.05$) and ~40% ($p<0.001$) respectively. Generally, similar anabolic and anti-hypertrophic effects were observed in *ex-vivo* cartilage explant cultures and non-osteoarthritic chondrocytes.

Calcineurin inhibition at physiological osmolarity exerts a superior effect than physiological osmolarity or FK506 alone: increasing anabolic, while suppressing hypertrophic and catabolic markers. Our data may aid in developing improved cell-based chondral repair and osteoarthritis treatment strategies.

Introduction

Articular cartilage has unique properties related to the structure and composition of its extracellular matrix (ECM), which is mainly composed of proteoglycans (PGs, e.g. aggrecan, *AGC1*) entangled in a network of collagen fibres (i.e. collagen type II, *COL2*) and large amounts of water (1). Water and inorganic salts represent the bulk mass portion, while collagens, PGs and other (glyco-)proteins constitute only 20% of the tissue's wet weight. Collagens represent 50-60% of cartilage's dry weight, while proteoglycans, as second largest solid phase portion, account for 5-10%.

In normal cartilage there is a balance between matrix synthesis and degradation. Depletion of aggrecan from articular cartilage by metalloproteinase (MMP) and aggrecanase (ADAMTS) action, is an essential early pathophysiological event in osteoarthritis (OA) (2-4). This decrease in PG content can result in reduced visco-elastic properties and increased deformation of cartilage under load, causing in-directly increased protease, but decreased tissue inhibitor (TIMP) production (5-7).

Cartilage requires a high osmotic pressure to maintain its hydrostatic pressure and keep its visco-elastic properties. The glycosaminoglycan (GAG) side chains of PGs are sulfated and responsible for the characteristic high fixed negative charge density (8) which binds mobile cations (sodium, calcium and potassium). The combination of intertwined collagens and PGs creates the tissue's rigidity by entrapping solutes and water, giving cartilage its unique mechanical properties, enabling it to deform and undergo large compressive and shear forces without failing (9). An intact collagen network restricts swelling and, in combination with GAGs, determines the tonicity (i.e. osmotic pressure) of the extracellular fluid around chondrocytes, which ranges between 350 and 480 mOsm in healthy cartilage (10-11). Catabolic events in the course of OA deplete PGs and degrade the collagen matrix, resulting in a severity-depending decreased osmolarity of between 280 and 350 mOsm (10, 12) and biomechanical inferiority.

Several studies have shown the presence of 'abnormal' collagens in OA cartilage: COL1 appears to be present in clusters of fibrillated areas in late stage OA, while COL10 (a hypertrophic marker) is expressed in moderately to severely affected osteoarthritic cartilage (5). COL1 expression is also increased during *in-vitro* chondrocyte expansion for several cell-based approaches, like Autologous Chondrocyte Implantation (ACI), to repair chondral lesions. During expansion, loss of chondrogenic phenotype occurs (dedifferentiation): chondrocytes convert into a flattened, fibroblast-like state, accompanied by a shift from COL2 expression

to COL1 expression (13), which reduces extracellular matrix quality. Chondrocyte dedifferentiation during *in-vitro* expansion for ACI is detrimental, but almost inevitably in standard monolayer culture.

Previously, we identified calcineurin (Cn), a calcium-dependent serine/threonine phosphatase, as a potential target to improve chondrocyte phenotype, by showing that Cn activity is strongly regulated during chondrocyte differentiation. Inhibition of Cn activity by FK506 increased the expression of the chondrogenic markers *AGC1, COL2 and SOX9*, indicating that chondrocytes might shift towards a more chondrogenic phenotype (13). We further showed that isolation and expansion of adult human articular chondrocytes (HAC) in culture medium of physiological osmolarity (380 mOsm) improves chondrogenic marker expression and extracellular matrix production through NFAT5 (14).

The aim of the current study was to elucidate whether combining physiological osmolarity with inhibiting Cn activity has a synergistic effect and thereby exceed the anabolic effects of each treatment alone. In the present study, we paid special attention to suppressing unwanted hypertrophic and catabolic markers. Finally, we also performed experiments with non-osteoarthritic chondrocytes to investigate whether our culture method holds the potential to develop strategies for cell-based repair of chondral lesions.

Materials and Methods

Cartilage and chondrocyte isolation

After informed consent (MEC2004-322), human articular cartilage was explanted from macroscopically normal areas of femoral condyles and tibial plateaus of thirteen patients undergoing total knee replacement surgery for OA and of five patients undergoing (non-osteoarthritic) trauma surgery. Isolation of primary osteoarthritic human articular chondrocytes (OAHAC) and non-osteoarthritic human articular chondrocytes (NHAC) from cartilage tissue under standard conditions (cytokine-free, 280 mOsm DMEM) was described earlier (13, 15)**.** In contrast, osmolarity of the isolation and culture media was adjusted to 380 mOsm by addition of sterile NaCl (14).

Chondrocyte expansion

Briefly, 7,500 cells/cm^2 were expanded in cytokine-free 280 mOsm or 380 mOsm DMEM, corresponding to their isolation osmolarity. Passage 1 (P1) and P2 cells were

seeded at 20,000 cells/cm^2 prior to adding 0, 50 or 500 ng/ml FK506 to the medium after 24 hours. Cells were then cultured for six days prior to mRNA (quantitative RT-PCR) and protein (Western Blotting) analyses. Experiments were performed at least in technical duplicates from five OA donors for mRNA analysis and from three OA donors for protein analysis (OAHAC).

In addition, experiments were performed with three non-osteoarthritic donors for mRNA analysis (NHAC).

Chondrocyte pellet culture (3D)

In addition to 2D culture, P2 OAHAC from three donors were isolated at 280 mOsm and cultured as triplicate pellets (2x10^5 cells/pellet) according to Hellingman et al.(16), but for 21 days in DMEM medium of 280 mOsm or 380 mOsm with addition of FK506 (50 ng/mL). The same experiments were performed with P2 NHAC from three donors. COL2 and GAG staining intensities of NHAC pellets were quantified using ImageJ 1.42 software (http://rsb.info.nih.gov/ij/download.html).

Cartilage explants

Six mm diameter full-thickness explants from femoral condyles and tibial plateaus of three OA donors were pooled prior to culturing at least two randomly taken explants per group for three days in 24-well plates with 330 µl medium per explant. Explants were cultured in DMEM at 280 mOsm or 380 mOsm, with or without addition of 50 ng/mL of FK506 for 24 hours and 7 days. RNA was extracted as described earlier (15).

RNA expression analysis

RNA purification, quantification and cDNA synthesis are described elsewhere (15). Expression levels of *AGC1*, *SOX9* and *COL2* were studied as chondrogenic markers, while *COL1* was studied as dedifferentiation marker as previously described (14). To investigate terminal chondrocyte differentiation, *COL10* and *MMP13* were used as hyperthrophic markers and alkaline phosphatase (*ALPL*) as mineralization marker. *MMP-1 ,-3, -8, -13, ADAMTS-4* and *-5* and tissue inhibitors (*TIMP-1* and *-2*) of the aforementioned were studied. While most QPCR assays were reported earlier (14-15, 17-18), ALPL expression was detected using the following primers (5' - 3'): ALPL-Fw GACCCTTGACCCCCACAAT, ALPL-Rv GCTCGTACTGCATGTCCCCT (200nM each) and ALPL hydrolysis probe FAM-TGGACTACCTATTGGGTCTCTTC-GAGCCA-TAMRA (600nM), respectively. Data were normalized to an index of at least three reference genes (*GAPDH, UBC, HPRT1*), which were pre-evaluated to be stably expressed across samples, and relative expression was then calculated

according to $2^{-\Delta CT}$ method (19). Averaged replicates per patient and condition were used to calculate the relative expression, normalized to the 280 mOsm P1 control.

Western Blotting and (immuno)histochemistry

Protein extraction, quantification, loading of SDS-polyacrylamide gels, electroblotting, detection and quantification is described in detail by van der Windt et al (14). The anti-GAPDH antibody (1:1000) was purchased from Cell Signaling Technology (Boston, MA, USA). Replicate data per patient and passage were averaged and normalized to the 280 mOsm condition.

(Immuno)histochemical staining for COL2 and GAG content of triplicate 3D pellet cultures from OAHAC and NHAC (three donors each) was performed as described earlier by our group (16).

Alkaline phosphatase activity and DNA assay

After washing human OAHAC in 1X PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per liter distilled H_2O; pH 7.4), alkaline phosphatase (ALPL) activity was determined by the colorimetric method of Lowry et al. (20) with modifications described by Janssen et al. (21). Briefly, cell lysates in 1X PBS/0.1% (v/v) Triton X-100 were heparin and RNase A (50 mg/ml in PBS) treated for 10 minutes at 37°C with gentle agitation and stored at -80°C until further use. Duplicates of 50 µl of each sample or lysis buffer (blank) were mixed with equal volumes of Substrate Solution (#P4744 Sigma-Aldrich; 20 mM *p*-nitrophenyl phosphate, 0.1M glycine, 1mM $ZnCl_2$, 1mM $MgCl_2$, pH 9.6), incubated for 10 min at 37°C in the dark and stopped by adding 125 µl of 0.1M NaOH. Samples were measured at 405nm in a standard microplate reader against a standard curve of *p*-nitrophenol (*p*NP, #1048, Sigma-Aldrich, Zwijndrecht, The Netherlands). ALPL activity was calculated from *p*NP standard curve and corrected for the amount of DNA per culture well (21). Measurements of triplicate samples from three OA donors per condition were analyzed.

MMP13 activity

MMP13 activity in OAHAC was determined using the SensoLyte® Plus 520 MMP13 Assay Kit (AnaSpec #71156, San Jose, CA) according to the manufacturer's instructions. Cell-free culture media were collected and stored at -80°C until activity of immobilized MMP13 was determined by its ability to cleave a FRET peptide substrate. Proteolysis separates the fluorophore from the quencher, with the resulting fluorescence (Ex/Em=490nm/520nm) being proportional to the MMP13 activity in the sample. Fluorescence was measured in a SpectraMAX Gemini plate reader using black, µ-clear flat-bottom 96-well plates (Greiner Bio-one, Alphen a/d Rijn,

Netherlands; #655090) and expressed as relative fluorescence units (RFU). Hence, samples were corrected for DNA content and displayed as n-fold RFU of the control condition. Measurements of replicate samples per condition were analyzed from three OA donors.

Calcineurin (Cn) activity

Cn activity was determined colorimetrically in triplicate using the Calcineurin Cellular Assay Kit Plus (BioMol, Tebu-Bio, Heerhugowaard, NL) as described earlier (13).

Statistics

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). We used the replicate raw expression data of multiple donors and tested for the effect of our experimental conditions using Linear Mixed Model (LMM) regression. We therefore accounted for the correlation in the data that exists within each donor. We further incorporated 'donor' as a random effect to correct for basal differences in expression between patients; p-values are reported as $p< 0.05$, 0.01 and 0.001.

Results

FK506 inhibits Cn activity at 280 and 380 mOsm to a similar extent

Dose-dependent inhibition of Cn activity in OAHAC cultured at 380 mOsm (data not shown) was not significantly different from that in 280 mOsm (13).

Physiological osmolarity and FK506 synergistically increase expression of chondrogenic markers of OAHAC *in vitro*

Culturing P1 and P2 osteoarthritic chondrocytes (OAHAC) in medium of physiological osmolarity (380 mOsm) significantly increased mRNA expression of chondrogenic markers *COL2, AGC1* and *SOX9* when compared to control osmolarity (280 mOsm), but significantly decreased *COL1* expression (Fig. 1), thereby confirming our earlier results (14).

Calcineurin inhibition enhanced anabolic effects of physiological osmolarity: FK506 dose-dependently increased chondrogenic marker expression to a much greater extend than in the 280 mOsm controls (Fig. 1B and D). While physiological osmolarity has a general, passage-dependent suppressive effect on *COL1* expression, addition of FK506 did not alter *COL1* gene expression and had no effect at 280

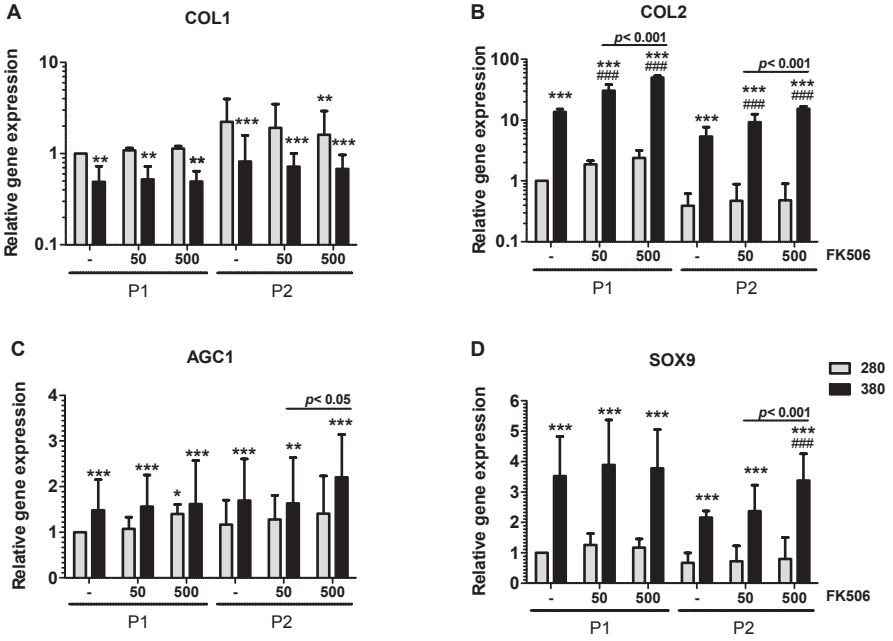

Figure 1. FK506 mediated effects on chondrogenic marker expression during isolation and expansion of OAHAC. Relative mRNA expression of **(A)** *COL1* (log scale), **(B**) *COL2* (log scale), (**C)** *AGC1*, **(D)** *SOX*9 in human osteoarthritic P1 and P2 monolayer chondrocytes from five donors, cultured at physiological (380 mOsm, black bars) or at control (280 mOsm, grey bars) omolarity with or without 50 or 500 ng/mL FK506. Data are means ± standard deviation. Differences per passage are indicated: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (compared to 280 control), #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (compared to 380 control).

mOsm either; except for P2 cells in which the highest concentration of FK506 (500 ng/mL) slightly suppressed *COL1* mRNA levels (Fig. 1A, on log scale). In contrast, *COL2* mRNA levels increased up to 13-fold in P1 chondrocytes at physiological osmolarity and increased further up to 49-fold after co-stimulation with FK506 (Fig. 1B, on log scale). At 380 mOsm, *AGC1* mRNA levels increased up to 2.1-fold and *SOX9* expression up to 3.4-fold in P2 chondrocytes after co-stimulation with FK506 (Fig. 1 C and D, respectively).

Physiological osmolarity increased COL2 protein levels at least 3-fold in P1 and P2 (Fig. 2A) chondrocytes. Addition of FK506 increased COL2 protein levels up to 6.0-fold in P1 and up to 5.2-fold in P2 chondrocytes (Fig. 2A). Physiological osmolarity did not affect COL1 protein levels in P1 chondrocytes, but addition of FK506 to the 380 mOsm medium significantly decreased COL1 protein levels (Fig. 2B). In P2 chondrocytes physiological osmolarity decreased COL1 protein levels by 35% and up to 40% when FK506 was added.

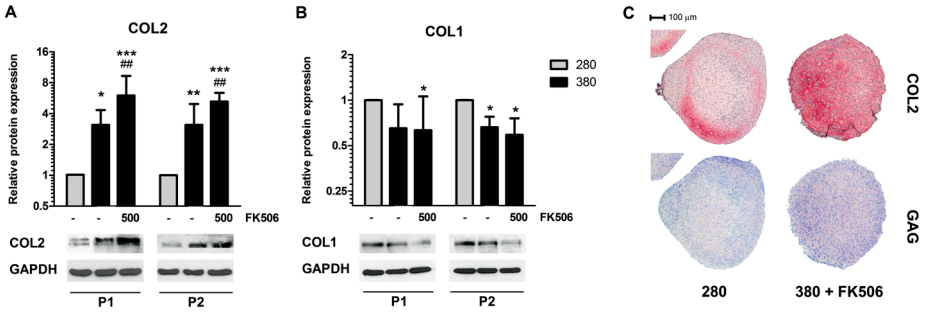

Figure 2. Improved collagen and GAG expression in expanded OAHAC. Western blot analyses of **(A)** COL2 and (**B)** COL1 levels of P1 and P2 OAHAC. Cells from three donors were cultured at physiological tonicity (380 mOsm, black bars) with or without 500 ng/mL FK506 or at control medium omolarity (280 mOsm, grey bars). Representative blots are shown (on bottom) next to densitometry data; data are means ± standard deviation. Significant differences per passage are indicated: *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$ (compared to 280 control), ##$p < 0.01$ (compared to 380 control). **(C)** Representative images of 21-day 3D cultures showing COL2 immunostaining (on top, in red) and GAG thionin staining (on bottom, in blue) of pellets from three donors cultured at 280 mOsm or 380 mOsm + FK506 (50 ng/mL). (Original magnification x 40).

As inhibition of Cn activity under physiological osmolarity decreased the de-differentiation rate of chondrocytes in 2D monolayer culture, we next evaluated this treatment in 3D micro-tissue culture (i.e. pellets) *in vitro*: COL2 protein and GAG abundance were again increased in the 380 mOsm condition with addition of FK506 as compared to the 280 mOsm control (Fig. 2C).

FK506 inhibits osmolarity-induced expression of hypertrophic and catabolic markers of OAHAC *in vitro*.

Next, we investigated the effects of FK506 at physiological osmolarity on the synthesis of terminal differentiation markers (*COL10, ALPL*), ECM-degrading enzymes (*MMPs*, *ADAMTSs*) and their inhibitors (*TIMPs*).

Physiological osmolarity also slightly increased mRNA levels of hypertrophy markers *COL10* and *MMP13* and mineralization marker *ALPL* (Fig. 3A) in P1 chondrocytes. Addition of FK506, however, inhibited these unwanted tonicity-induced effects. Moreover, FK506 exerted similar effects on the expression of collagenases *MMP1* and *-8* (Fig. 3B). Interestingly, aggrecanases *ADAMTS4* and *-5* were not significantly influenced by 380 mOsm or FK506 alone, while the combination significantly decreased their expression: *ADAMTS4* by 50% and *ADAMTS5* by 40% (Fig. 3C). The expression of tissue inhibitors of metalloproteinases, *TIMP1* and *-2*, was increased by 380 mOsm (2.5-fold and 1.5-fold, respectively). Although FK506 had limited effects on TIMP expression at 280 mOsm, it increased their expression

at 380 mOsm almost 4-fold (*TIMP1*) and 2.5-fold (*TIMP2*) (data not shown). Overall, the combination of FK506 and physiological osmolarity showed similar trends in expression levels of hypertrophic and catabolic markers in P2 chondrocytes (data not shown).

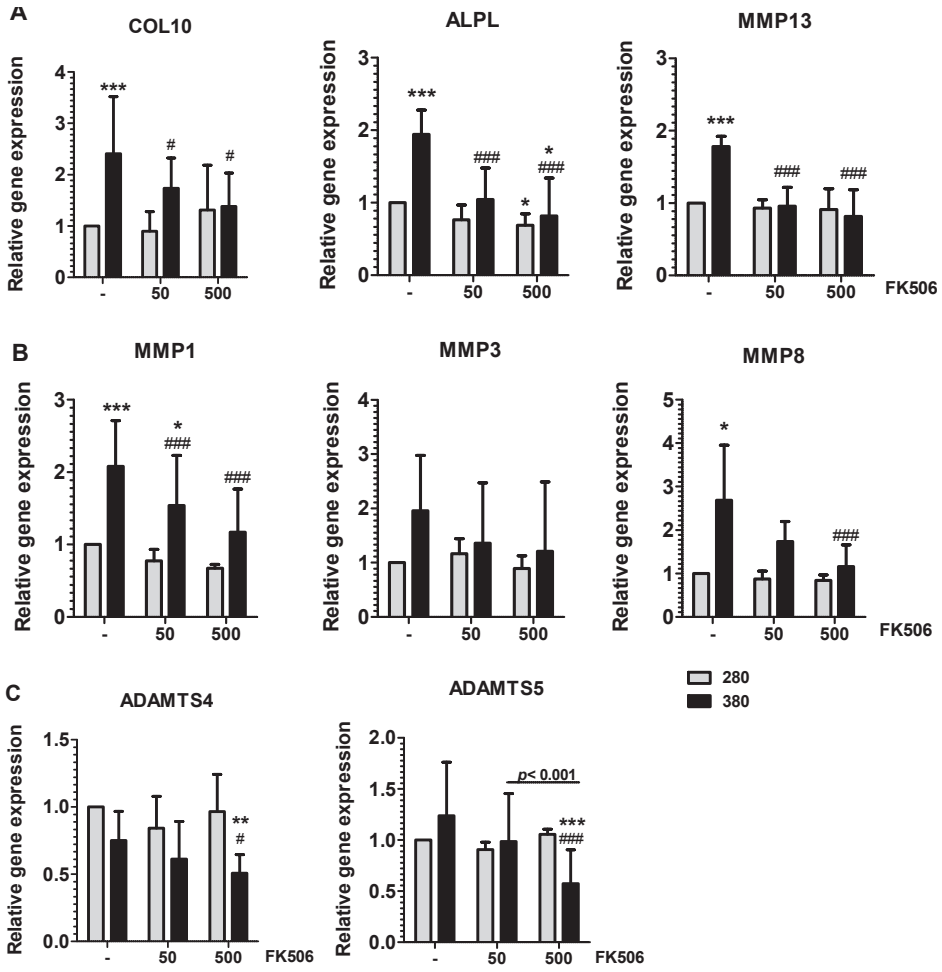

Figure 3. Treatment effects on hypertrophic and catabolic markers in OAHAC. Relative mRNA expression of **(A)** hypertrophic markers *COL10*, *ALPL*, *MMP13* and **(B,C)** catabolic markers *MMP1*, *MMP3*, *MMP8* and *ADAMTS4* and *-5*. P1 OAHAC from five donors were cultured in monolayer at physiological (380 mOsm, black bars) or control (280 mOsm, grey bars) medium omolarity with or without 50 or 500 ng/mL FK506. Data are means ± standard deviation. Differences are indicated: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (compared to 280 control), #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (compared to 380 control).

Physiological osmolarity also significantly increased ALPL activity in all three donors, while addition of FK506 to 380 mOsm medium significantly reduced osmolarity-induced ALPL activity (Fig. 4A). Although elevated osmolarity increased MMP13 activity in one patient (Fig. 4B), addition of even small doses of FK506 always suppressed its activity. Moreover, higher FK506 concentrations (500 ng/mL) suppressed MMP13 activity to, or below, control (280 mOsm) levels.

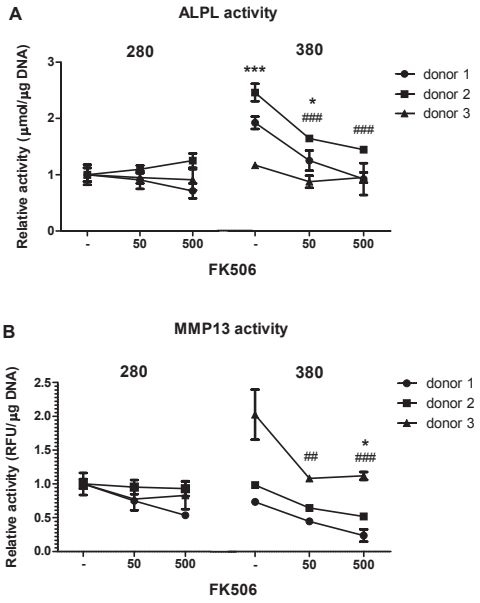

Figure 4. FK506 mediated effects on hypertrophic marker activity of OAHAC. Relative activity of **(A)** ALPL and **(B)** MMP13 in P1 monolayer cultures of OAHAC from three donors at physiological (380 mOsm, on right) or control (280 mOsm, on left) omolarity with or without 50 or 500 ng/mL FK506. Data are means ± standard deviation, normalized to 280 control. Differences are indicated: *$p < 0.05$, ***$p < 0.001$ (compared to 280 control), ##$p < 0.01$, ###$p < 0.001$ (compared to 380 control).

Synergistic effects of FK506 and physiological osmolarity in OA cartilage *ex vivo*.

Chondrocytes *in vivo* are embedded in an extensive ECM. For potential clinical applications it is essential to investigate whether chondrocytes embedded in this ECM are reacting similarly to osmolarity and FK506 as those in monolayer culture. To address whether the ECM of cartilage interferes with osmolarity-mediated responses of chondrocytes, we next performed experiments with cartilage explants.

Stimulation of cartilage explants with physiological osmolarity for 24 hours increased expression of chondrogenic markers *COL2*, *AGC1* and *SOX9*, while the expression

of *COL1* decreased (data not shown). Physiological osmolarity or FK506 had no apparent effect on hypertrophic marker expression (*COL10*, *ALPL* and *MMP13*) on the short term (data not shown). After seven days of culture, the sole osmolarity-induced upregulation of the chondrogenic markers was reduced to baseline levels (Fig. 5A), while addition of 50 ng/mL of FK506 to this medium ensured a prolonged transcriptional abundance of all tested chondrogenic markers (Fig. 5A). Osmolarity, however, also induced expression of *COL10* in explants, while no significant effects were found on *ALPL* and *MMP13* levels (Fig. 5B). Strikingly, FK506 was able to suppress *COL10* to baseline levels not only in monolayer cultures but also in cartilage tissues *in vitro*. Although physiological osmolarity did not increase *ALPL* expression in explants within 7 days, addition of FK506 under these conditions suppressed *ALPL* mRNA levels (Fig. 5B).

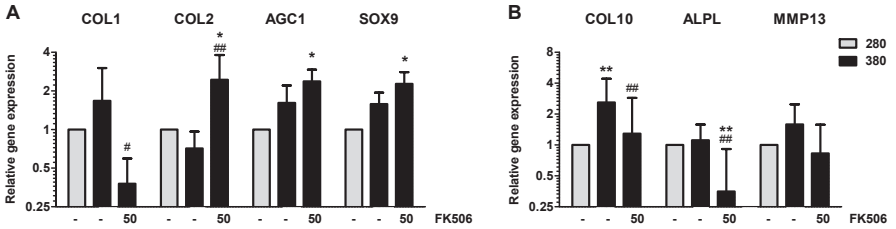

Figure 5. Treatment effects on chondrogenic and hypertrophic markers in OA cartilage explants. Relative expression of **(A)** *COL1*, *COL2*, *AGC1* and *SOX9* and **(B)** hypertrophic markers *COL10*, *ALPL* and *MMP13* in human osteoarthritic articular cartilage explants from three donors. Explants were cultured in physiological (380 mOsm, black bars) or control (280 mOsm, grey bars) medium with or without 50 ng/mL FK506. Data are means ± standard deviation. Significance is indicated by $*p < 0.05$, $**p < 0.01$ (compared to 280 control) and $\#p < 0.05$, $\#\#p < 0.01$ (compared to 380 control).

Synergistic effects of FK506 at physiological osmolarity in NHAC *in vitro*.

Finally, we evaluated our approach on NHAC from a limited number of non-osteoarthritic donors. In monolayer culture, NHAC reacted similarly to physiological osmolarity as did OAHAC: expression trends of chondrogenic and hypertrophic markers were congruent (Fig. 6), but the magnitude of the FK506 mediated expressional change was different. In both cell types, standard expansion increased *COL1* expression (Fig. 6A vs. 1A) while 380 mOsm generally suppressed *COL1* levels, although in NHAC only significantly in P2. Trends in *COL2* expression in response to osmolarity were the same in NHAC and OAHAC (Fig. 6A vs. Fig. 1B, both log scale). P2 NHAC showed a relatively larger increase in *COL2* levels by elevated osmolarity than P1 NHAC or OAHAC did (Fig. 6A vs. 1B), mainly due to a more profound suppression of *COL2* expression at 280 mOsm in P2 NHAC. In NHAC,

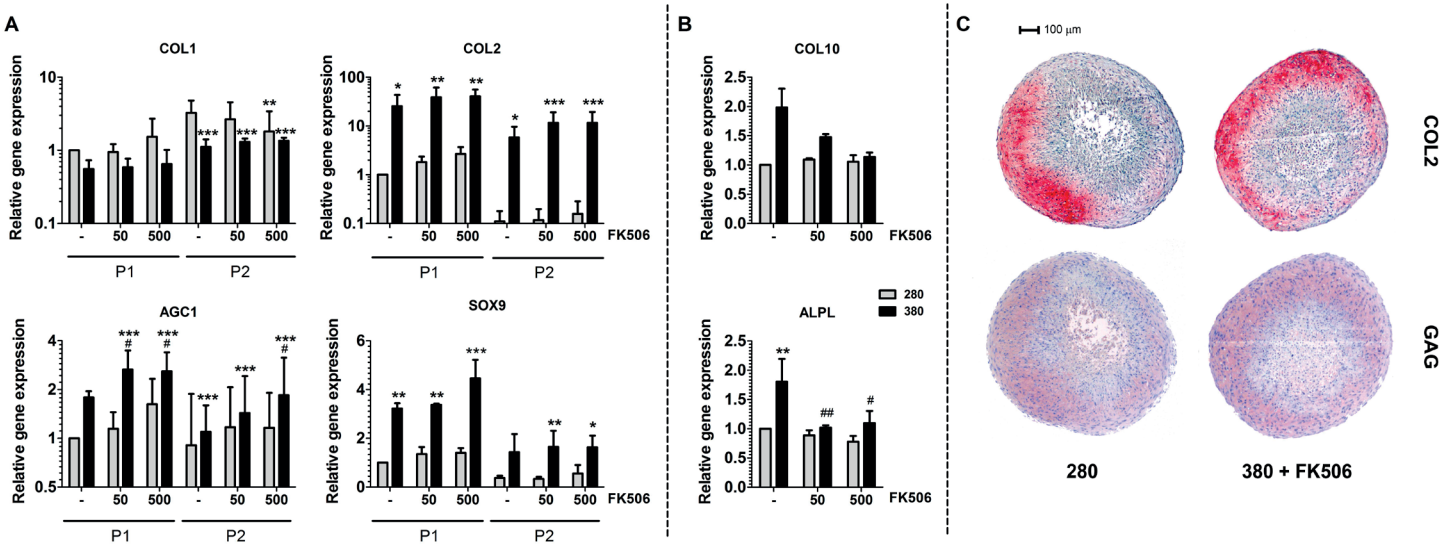

Figure 6. Effects of omolarity on NHAC. Relative mRNA levels of chondrogenic and hypertrophic marker genes in NHAC from three donors in monolayer culture: **(A)** *COL1* (log scale), *COL2* (log scale), *AGC1* and *SOX9* and **(B)** *COL10* (n=2) and *ALPL* in P1 cells. NHAC were cultured at physiological (380 mOsm, black bars) or control (280 mOsm, grey bars) medium omolarity with or without 50 or 500 ng/mL FK506. Data are means ± standard deviation. Differences are indicated: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (compared to 280 control), #$p < 0.05$, ##$p < 0.01$ (compared to 380 control). **(C)** Representative images of 21-day 3D cultures showing COL2 immunostaining (on top, in red) and GAG thionin staining (on bottom, in blue) of pellets from three donors cultured at 280 mOsm or 380 mOsm + FK506 (50 ng/mL). (Original magnification x 40).

the trends towards synergy between elevated osmolarity and FK506 were less prominent on *COL2* expression and had a more profound effect on *AGC1* mRNA levels, when compared to OAHAC (Fig. 6A). Interestingly, in NHAC, the overall expression of *SOX9* dropped in a rather passage- than stimulus-dependent manner at both osmolarities, while elevated osmolarity had a similar stimulating effect in both passages (Fig. 6A).

As OAHAC and NHAC showed similar trends in response to basal osmolarity on chondrogenic marker expression, we next analyzed their hypertrophic response. *COL10* transcripts were low abundant in NHAC and only considerably expressed by two out of three donors, but in those showed the same trend in response to osmolarity and FK506 as in OAHAC (Fig. 6B). *MMP13* transcripts were virtually absent in NHAC (data not shown). In P1, OAHAC and NHAC, revealed almost identical responses in *ALPL* expression (Fig. 3A, Fig. 6B), with FK506 suppressing the up-regulation of *ALPL* expression in response to 380 mOsm.

NHAC pellet cultures revealed a consistent trend of improved COL2 protein and GAG content (65% and 44% respectively, data not shown) upon inhibition of Cn activity under physiological osmolarity after 21 days (Fig. 6C).

Discussion

In OA, the ECM of cartilage degrades and the extracellular osmolarity drops considerably compared to healthy cartilage (10-11), resulting in a biomechanically inferior tissue. Physiological osmolarity is chondroprotective *in vitro* and *ex vivo* (14, 22-23), while Cn inhibition exerts pro-chondrogenic effects (13, 24). We now showed that Cn inhibition at physiological osmolarity exerts a superior effect: improving anabolic, while suppressing catabolic and hypertrophic markers.

Adding FK506 to medium of physiological osmolarity dose-dependently enhanced chondrogenic markers to a much greater extend than FK506 at 280 mOsm or than physiological osmolarity alone (Fig.1B-D). The already ~13-fold solely osmolarity-dependent increase of *COL2* mRNA levels in P1 cells was tripled to ~50-fold upon co-stimulation with FK506 (500 ng/mL, Fig.1B). Furthermore, physiological osmolarity suppressed unwanted *COL1* expression more efficiently than FK506 did (Fig.1A). Tew *et al*. found that osmolarity regulates *SOX9* and *COL2* expression post-transcriptionally, but stabilizing *SOX9* mRNAs while destabilizing *COL2* transcripts (25). In our study, *SOX9* up-regulation by osmolarity revealed a sub-

stantial FK506-dependency. We did not aim to elucidate the underlying molecular mechanism in this study, but rather confirmed the beneficial treatment effect on protein level (Fig.2). In both 2D and 3D cultures, COL2 mRNA and protein expression profited relatively more from osmolarity and FK506 than *AGC1* expression and GAG content did. As the maximum gain in COL2 expression diminishes in P2 compared to P1 in both OAHAC and NHAC (Fig.1B, 6A and Fig.2), our data indicate that synergism between osmolarity and Cn inhibition cannot ultimately prevent, but only delay, chondrocyte de-differentiation in monolayer culture. It is important to realize that normal adult articular chondrocytes *in vivo* do not divide, while in our study they did. Maintaining proliferation capacity and chondrogenic marker expression is excellent for clinical applications of cell-based therapies (14).

Aggrecanases *ADAMTS4* and *ADAMTS5* were not significantly influenced by 380 mOsm or FK506 alone, but the combination decreased their expression (Fig. 3). Expression of *TIMP1* and *TIMP2* was increased at 380 mOsm and further up-regulated upon co-stimulation with FK506 (data not shown), adding to a PG-protective effect of FK506. *TIMP3* transcripts were very low abundant (data not shown). Hiyama et al. (26) suggested that AP1, Sp1, and NFAT5 transcription factors control GAG synthesis in nucleus pulposus cells and we recently showed that NFAT5 is a key mediator of osmolarity responses in HAC, too (14). Although being controversial, NFAT5 expression seems to be modulated in a Cn-dependent manner in some cells (27). FK506-induced Cn inhibition likely involves several MAP kinases (28) which may contribute to an AP1/NFAT-mediated promoter activation of certain proteases. Promoter–reporter assays recently identified the promoter region of human *ADAMTS4* as a potential downstream target of NFAT and Runx2 (29) and similar transcription factor binding sites were identified in the promoter regions of MMP13 (30) and other MMPs. The cellular context is, however, important: FK506 induces chondrogenesis in murine ATDC5 cells and human synovial stromal cells, but Cn activity is necessary for chondrogenesis of mesenchymal cells (24, 31). We found that physiological osmolarity also slightly increased mRNA levels of unwanted hypertrophy markers like *COL10*, *MMP13* and *ALPL* (Fig. 3). Animal models of OA revealed crucial roles of *MMP3*, *MMP13* and *ADAMTS5* in cartilage degradation. At 380 mOsm, FK506 had a suppressing effect on these hypertrophic markers and proteases while increasing their inhibitors, *TIMP1* and *TIMP2*, respectively. Other Cn inhibitors have been shown to suppress MMP13 and ADAMTS5 expression in cytokine-stimulated cartilage explants, although possibly not through blocking NFAT signaling (32). Our data are in agreement with cyclosporine A (CsA) inhibiting *MMP1* and *MMP3* expression in chondrocytes, while increasing *COL2*, *TIMP1* and TGFβ as shown in a murine OA collagenase model (33). The cellular specificity of

FK506 action may, however, depend on selective interactions with specific isoforms of calcineurin or the existence of cell-type specific substrates. A possible role of TGFβ in calcineurin-mediated chondrogenic effects (13) may be mediated by FK506-binding immunophilins (34). MMP13 suppression in our study may involve p38γ mediated hypertonicity-induced NFAT5 activation (35-37). Hydrostatic pressure may also directly act on chondrocytes membrane transporters to activate AGC1 and COL2, although this was yet only shown for higher tonicities (38-39).

For a better prediction of the clinical applicability of our treatment, we evaluated its effect on cartilage explants. After seven days of explant culture, the sole osmolarity-induced up-regulation of the chondrogenic markers was reduced to baseline levels (Fig. 5A), while addition of 50 ng/mL of FK506 to this medium ensured a prolonged transcriptional abundance of all tested chondrogenic markers. Like in monolayer cultures, FK506 was able to suppress *COL10* and *ALPL* mRNA levels. The ionic environment of chondrocytes is unusually rich in cations, while anion concentration is low (40). It is tempting to speculate that differences in ion gradients between cells and explants *in vitro* may also contribute to differences in gene regulation at both osmolarities. GAG synthesis may involve MEK-ERK or integrin signaling (41), which might explain the slightly different responses between explants and isolated cells. Overall, both responded remarkably similar to our treatment.

Finally, we evaluated the potential of our approach for improving cell-based strategies for chondral lesion repair using 'normal', non-osteoarthritic (NHAC) rather than osteoarthritic cells. Generally, expression trends of chondrogenic and hypertrophic markers in OAHAC and NHAC monolayer cultures were similar in response to physiological osmolarity. However, the synergistic effects of 380 mOsm and FK506 on *COL2* and *SOX9* mRNA levels were less prominent in NHAC, while the effects on *AGC1* expression were more prominent. OAHAC and NHAC revealed almost identical responses to the treatment with respect to *ALPL* expression, while *MMP13* transcripts were virtually absent in NHAC. *COL10* transcripts were less abundant in NHAC than in OAHAC and virtually absent in one donor, but showed the same trends in response to tonicity and FK506 in the remaining samples (Fig. 6B). However, we were not able to perform statistics on the isolated data and therefore merged NHAC and OAHAC data for LMM expression analysis of this gene to determine whether the pathologic state of the cells is a significant factor affecting its mRNA levels in chondrocytes in response to the treatment. Osmolarity still significantly increased *COL10* mRNA levels ($p<0.01$) in the merged data, with FK506 also still inhibiting the osmolarity-induced upregulation ($p<0.05$). After the relatively short three week culture period, COL2 staining in OAHAC pellets seems

to profit relatively more from our treatment than in NHAC pellets. During OA, chondrocytes are activated and undergo phenotypic modulation, including altered COL2 regulation (42-43), possibly sensitizing OAHAC to osmolarity-induced COL2 synthesis. The combined data from NHAC monolayer and pellet cultures together indicate that FK506 supplementation under physiological osmolarity holds potential to improve the quality of non-osteoarthritic chondrocytes, although to a lesser extent when compared to OAHAC. To this end, the small number of NHAC donors may compromise the predictability of the LMM model, which is a limitation of the present study. As other statistical tests, LMM becomes less appropriate with decreasing numbers of donors even with a large number of observations on each donor.

During *in vitro* expansion, chondrocytes rapidly become more fibroblast like and this likely alters their response to osmolarity, which may explain absolute differences between cells and explants. A similar modulation of the chondroyte phenotype is commonly observed in OA chondrocytes (44), including altered gene expression regulation and subsequent changes in ECM protein synthesis and morphology (45). Chondrocytes regulate their volume upon hyperosmolarity by activating regulatory volume increase, which is altered during cell isolation involving collagenases treatment (46). Cell shape is an integral part of the chondrocyte's phenotype (47-48) and influenced, among others, by cytoskeletal structures (49). This may also account for some passage-dependent effects and differences between isolated cells and explants as observed in our study, but likely also contributes to different responses in NHAC as compared to OAHAC.

Conclusion

Using physiological osmolarity improves chondrogenic marker expression in osteoarthritic chondrocytes and cartilage explants, but to a lesser extent in non-osteoarthritic chondrocytes *in-vitro*. Adding Cn inhibitor FK506 to the culture medium improves anabolic, while suppressing catabolic and hypertrophic markers. Our current data aid in the development of novel cell-based therapies for chondral lesion repair and might alter future strategies to treat osteoarthritis.

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Osmolarity determines the *in vitro* chondrogenic differentiation capacity of progenitor cells via nuclear factor of activated T-cells 5

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Abstract

Previous studies have shown that human articular chondrocytes *in vitro* are osmolarity-responsive and increase matrix synthesis under cartilage-specific physiological osmolarity. The effects of increased osmolarity on chondrogenesis of progenitor cells *in vitro* are largely unknown. We therefore aimed to elucidate whether hyperosmolarity facilitates their chondrogenic differentiation and whether Nfat5 is involved.

ATDC5 cells and human bone marrow stem cells (hBMSCs) were differentiated in the chondrogenic lineage in control and increased osmolarity conditions. Chondrogenic outcome was measured by gene and protein expression analysis. RNAi was used to determine the role of Nfat5 in chondrogenic differentiation under normal and increased osmolarity.

Increasing the osmolarity of differentiation medium with 100 mOsm resulted in significantly increased chondrogenic marker expression (Col2a1, Col10a1, Acan, Sox9, Runx2 and GAGs) during chondrogenic differentiation of the two chondroprogenitors, ATDC5 and hBMSCs. Nfat5 knockdown under both control and increased osmolarity affected chondrogenic differentiation and suppressed the osmolarity-induced chondrogenic induction. Knockdown of Nfat5 in early differentiation significantly decreased early Sox9 expression, whereas knockdown of Sox9 in early differentiation did not affect early Nfat5 expression.

Increasing the osmolarity of chondrogenic culture media by 100 mOsm significantly increased chondrogenic gene expression during the course of chondrogenic differentiation of progenitor cells. Nfat5 may be involved in regulating chondrogenic differentiation of these cells under both normal and increased osmolarities and might regulate chondrogenic differentiation through influencing early Sox9 expression.

Introduction

Chondrogenic differentiation of progenitor cells plays an essential role during endochondral ossification for skeletal growth and bone fracture healing (1, 2). Moreover, progenitor cell-based cartilage regeneration is a rapidly evolving field and methodologies promoting chondrogenic differentiation of mesenchymal progenitor and stem cells to chondrocytes are of interest to improve cartilage and endochondral bone regenerative medicine approaches.

Chondrogenic differentiation during endochondral ossification is a multi-step developmental process during which mesenchymal progenitor cells condensate, differentiate into extracellular matrix (ECM) producing chondrocytes and ultimately terminally differentiate into hypertrophic chondrocytes (1, 3). This process is accompanied by a stage-dependent expression of chondrogenic makers: while for example Sox9 (SRY (sex determining region Y)-box 9) is a primary determinant of differentiation from early stages onwards (4, 5), its transcriptional targets collagen type 2 (Col2a1) and aggrecan (Acan) are prominently expressed by more mature, ECM producing chondrocytes. Collagen type 10 (Col10a1) and its main transcription factor Runx2 (Runt-related transcription factor 2) are specifically expressed by hypertrophic differentiating chondrocytes (4, 5).

The large amount of ECM produced by chondrocytes is mainly composed of proteoglycans (PG; like aggrecan) which are entangled in a network of collagens (mainly Col2a1) and further contains a lot of matrix-retained water (6). The glycosaminoglycan (GAG) side chains of the PGs are sulphated and responsible for a high fixed negative charge density, which attracts mobile cations and water from the ECM-environment (7, 8). This, together with the quality of the collagen network, determines the osmolarity of the extracellular fluid and provides strength and flexibility to the tissue. The extracellular osmolarity of healthy articular cartilage ranges between 350 and 480 mOsm and is thus markedly higher than that of standard culture medium (9, 10), which ranges around plasma levels (280 mOsm) (11, 12).

Several studies have shown that articular chondrocytes *in vitro* are osmolarity-responsive and increase their ECM synthesis under chondrocyte-physiological osmolarity (10, 13-15). The nuclear factor of activated T-cells 5 (Nfat5/TonEBP) plays an important role in this response (14). Nfat5 is a member of the Rel family of transcription factors (16) and mediates transcriptional activation of ion transporters like the sodium/myo-inositol transporter encoded by Slc5a3 (17, 18) and calcium binding proteins like S100a4 (19, 20) upon hypertonic stress in several cell types,

including human articular chondrocytes (14). Collectively, the Nfat5-activated transporters exchange intracellular ions for compatible osmolytes and thereby regulate intracellular ionic strength and cell volume upon hypertonic stress (21). However, a growing body of evidence indicates that Nfat5 can also be regulated independently of osmolarity (22) and has diverse osmolarity-independent transcriptional functions in cell proliferation/survival (23) , carcinogenesis (24) and many others. To the best of our knowledge, no literature is available describing the effects of increased osmolarity on chondrogenic differentiation of progenitor cells *in vitro*. In addition, the expression and a potential (osmolarity-independent) function of Nfat5 during chondrogenic differentiation remains elusive. We therefore hypothesized that Nfat5 is involved in *in vitro* chondrogenic differentiation of progenitor cells during endochondral ossification and that the differentiation process can be enhanced by increasing the osmolarity of the culture medium possibly via an Nfat5 dependent mechanism.

In the present study we used ATDC5 cells and human bone marrow stem cells (hBMSCs) as *in vitro* models for chondrogenic differentiation and provide evidence that Nfat5 expression is indeed regulated during chondrogenic differentiation independently of osmolarity status, but also responds to increased osmolarity during chondrogenic differentiation. Furthermore, we show that increased osmolarity improves chondrogenic differentiation of progenitor cells *in vitro* and that, at least in part, this is regulated via Nfat5-mediated transcriptional regulation of Sox9.

Material and methods

ATDC5 cell culture

ATDC5 cells were cultured in proliferation medium under a humidified atmosphere at 37°C, 5% CO_2. Proliferation medium consisted of DMEM/F12 (Invitrogen, Carlsbad, CA, USA), 5% FCS (PAA, Pasching, Austria), 1% antibiotic/antimycotic (mixture of 10,000 U/ml penicillin, 10,000 µg/ml streptomycin and 25 µg/ml amphotericin B; Invitrogen) and 1% NEAA (non-essential amino acids; Invitrogen) and was changed every two days. Differentiation medium comprised proliferation medium supplemented with 10 µg/ml insulin (Sigma, St. Louis, MO, USA), 10 µg/ml transferrin (Roche Applied Science, Indianapolis, IN, USA) and 30 *nM* sodium selenite (Sigma, St. Louis, MO, USA). Cells were plated at approximately 6400 cells/cm^2 in cell culture dishes, allowed to adhere overnight and the following day chondrogenesis was initiated by changing the proliferation medium to differentiation medium. Differentiation medium was changed every two days (day 0-10) and daily (from day 10

on). The osmolarity of the ATDC5 differentiation medium was determined to 310±5 mOsm using an Osmomat 030 (Gonotec GmbH, Germany). The osmolarity of this medium was increased with 100 or 200 mOsm, respectively, by adding sterile NaCl from day 0 in differentiation. For RNAi-experiments an Nfat5 siRNA-duplex (sense: 5'-CCAGUUCCUACAAUGAUAACACU-3', antisense: 5'-AGUGUUAUCAUUGUAG-GAACUGG-3'), a Sox9 siRNA duplex (sense: 5'- GACUCACAUCUCUCCUAAUTT-3', anti-sense: 5'- AUUAGGAGAGAUGUGAGUCTT-3') and a scrambled siRNA-duplex (indicated by "Mock") were used (Eurogentec). ATDC5 cells were seeded at approximately 25,000 cells/cm^2 and transfection with siRNAs (30 *nM*) was performed using ICAfectin 442 (Eurogentec) according to manufacturers' protocol. Knockdown was allowed for 1 day prior to the initiation of chondrogenic differentiation and knockdown for longer timeframes was accomplished by retransfection of the respective siRNA duplex.

hBMSC isolation and culture

Human bone marrow mesenchymal stem cells were obtained from iliac crest bone marrow aspirate from young, genetically healthy individuals (Medical ethical committee MUMC approval 08-4-056). BMSCs were isolated using Ficoll-Paque PLUS (Amersham Pharmacia, Diegem, Belgium) and plated on culture plates. Unbound cells were removed after 48 hours, and bound fraction was expanded in culture medium to five passages under a humidified atmosphere at 37°C, 5% CO_2. Culture medium consisted of DMEM high glucose (Invitrogen), 10% FCS (PAA), 1% antibiotic/antimycotic and 1% NEAA. Chondrogenesis was performed in monolayer by plating the cells at approximately 30,000 cells/cm^2 one day prior to initiating chondrogenesis with differentiation medium (proliferation medium supplemented with 1% ITS (Invitrogen), 50 μg/ml L-ascorbic acid-2-phosphate (Sigma) and 1 ng/ml TGFβ3 (R&D)) (25). The osmolarity of the differentiation medium was determined using an Osmomat 030 (Gonotec GmbH, Germany) and was measured to be 285±5 mOsm. When indicated, osmolarity of the differentiation medium was increased with 100 mOsm by adding sterile NaCl from day 0 in differentiation. Throughout the hBMSC differentiation, medium was changed every two days.

RT-qPCR

For RNA isolation, cells were washed 3 times with 0.9% NaCl w/v and disrupted with 500 μl Trizol (Invitrogen). RNA isolation, RNA quantification by UV-spectrometry (Nanodrop, Thermo Scientific), and cDNA synthesis were performed as described before (26, 27). Real time quantitative PCR (RT-qPCR) was performed using Mesagreen qPCR mastermix plus for SYBR® Green (Eurogentec, Seraing, Belgium). An Applied Biosystems ABI PRISM 7700 Sequence Detection System was used

for amplification using the following protocol: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of DNA amplification (denaturing for 15 seconds at 95°C and annealing for 1 minute at 60°C) followed by a dissociation curve. Data were analysed using the standard curve method, mRNA expression was normalized to a reference gene (β-*actin* for ATDC5 and *28S rRNA* for hBMSCs) and gene expression was calculated as fold change as compared to day 0 (t=0 in graphs). Primer sequences are depicted in Table 1.

Table 1. Oligonucleotide sequences for RT-qPCR. The 5' to 3' forward and reverse oligonucleotide sequences used for RT-qPCR are listed in the Table.

Oligo sets mouse	Forward	Reverse
Col2a1	'5-TGGGTGTTCTATTTATTTATTGTCTTCCT-3'	'5-GCGTTGGACTCACACCAGTTAGT-3'
Col10a1	'5-CATGCCTGATGGCTTCATAAA-3'	'5-AAGCAGACACGGGCATACCT-3'
Col1a1	'5-CTGACTGGAAGAGCGGAGAGTAC-3'	'5-CCATGTCGCAGAAGACCTTGA-3'
Sox9	'5-AGTACCCGCACCTGCACAAC-3'	'5-TACTTGTAGTCCGGGTGGTCTTTC-3'
Runx2	'5-GACGAGGCAAGAGTTTCACC-3'	'5-GGACCGTCCACTGTCACTTT-3'
Acan	'5-CATGAGAGAGGCGAATGGAA-3'	'5-TGATCTCGTAGCGATCTTTCTTCT-3'
Nfat5	'5-GGGTCAAACGACGAGATTGTG-3'	'5-TTGTCCGTGGTAAGCTGAGAA-3'
Slc5a3	'5-CACTTCTGTCATTGGAGCGCT-3'	'5-ATGGCAATGTCTGCTGTGTCC-3'
S100a4	'5-GTCCACCTTCCACAAGTACTCG-3'	'5-TCATCTGTCCTTTTCCCCAAG-3'
B-Actin	'5-GACAGGATGCAGAAGGAGATTACTG-3'	'5-CCACCGATCCACACAGAGTACTT-3'

Oligo sets human	Forward	Reverse
COL2A1	'5-TGGGTGTTCTATTTATTTATTGTCTTCCT-3'	'5-GCGTTGGACTCACACCAGTTAGT-3'
COL10A1	'5-ATGATGAATACACCAAAGGCTACCT-3'	'5-ACGCACACCTGGTCATTTTCTG-3'
COL1A1	'5-TGTGCCACTCTGACTGGAAGA-3'	'5-AGACTTTGATGGCATCCAGGTT-3'
SOX9	'5-AGTACCCGCACCTGCACAAC-3'	'5-CGCTTCTCGCTCTCGTTCAG-3'
RUNX2	'5-TGATGACACTGCCACCTCTGA-3'	'5-GCACCTGCCTGGCTCTTCT-3'
ACAN	'5-GCAGCTGGGCGTTGTCA-3'	'5-TGAGTACAGGAGGCTTGAGGACT-3'
NFAT5	'5-GGGTCAAACGACGAGATTGTG-3'	'5-TTGTCCGTGGTAAGCTGAGAA-3'
28SRNA	'5-GCCATGGTAATCCTGCTCAGTAC-3'	'5-GCTCCTCAGCCAAGCACATAC-3'

Immunoblotting

Cells were washed three times with 0.9% NaCl and lysed in RIPA buffer (150 *mM* NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 50 *mM* Tris pH 8.0, 5.0 *mM* EDTA pH 8.0, 0.5 *mM* dithiothreitol and 1 *mM* phenylmethylsulfonylfluoride). Extracts were sonicated on ice using the Soniprep 150 (MSE, London, UK) at amplitude 10 for 14 cycles (1 second sonication and 1 second pause). Insoluble material was

removed by centrifugation (13.000 x g, 4°C). Protein concentration was determined using the BCA protein assay (Sigma). Polypeptides were separated by SDS-PAGE (samples were equally loaded) and subsequently transferred to nitrocellulose membranes by electroblotting. Primary antibodies for immunodetection were polyclonal goat anti-Col2a1 (Southern Biotech, Birmingham, AL, USA), polyclonal goat anti-Col1a1 (Southern Biotech, Birmingham, AL, USA), polyclonal rabbit anti-Col10a1 (Calbiochem, Darmstadt, Germany), polyclonal rabbit anti-Sox9 (Abcam), mouse monoclonal anti-α-Tubulin (Sigma, Saint Louis, MO, USA), polyclonal rabbit anti-Gapdh (Cell Signaling) and polyclonal rabbit anti-Nfat5 [43]. Bound primary antibodies were detected with rabbit anti-goat, swine anti-rabbit or rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase (DakoCytomation, Glostrup, Denmark) and visualized by enhanced chemiluminescence (ECL). ECL signals were quantified using ImageJ 1.46f software, and relative differences, corrected for background and housekeeper, were determined as compared to control conditions.

Cell proliferation

Cell proliferation was assessed by crystal-violet (Sigma, Saint Louis, MO, USA) staining. Cells were washed two times with 0.9% NaCl and subsequently fixed with 4% paraformaldehyde in phosphate buffered saline for 10 minutes at room temperature. Fixed cells were washed 6 times with water and air dried for storage. Cells were incubated with 0.1% crystal-violet for 30 minutes at room temperature. Cells were washed six times with water to remove excess crystal-violet and allowed to air-dry in the dark overnight. crystal-violet was extracted from the cells by incubation with 10% acetic acid for 15 minutes under continuous agitation. Extracted crystal-violet was determined spectrophotometrically at 590 nm using a plate reader (Biorad, Hemel Hempstead, UK).

Alcian blue staining

GAG deposition was detected by Alcian blue staining. Cells were washed two times with 0.9% NaCl and subsequently fixed with 4% paraformaldehyde in phosphate buffered saline for 10 minutes at room temperature. Fixed cells were washed 6 times with water and air dried for storage. Fixed cells were incubated overnight with 1% Alcian blue (Acros Organics, Geel, Belgium) in 0.1 M HCl at room temperature. Cells were washed six times with water to remove excess Alcian blue and allowed to air-dry in the dark overnight. Alcian blue was extracted from the cells by incubation with Guanidine-HCl (6 *M*) for 2 hours under continuous agitation. Extracted Alcian blue was determined spectrophotometrically at 645 nm using a plate reader (Biorad, Hemel Hempstead, UK).

Statistics

Statistical significance was determined by two-way ANOVA (with Bonferroni *post hoc* test) for ATDC5 experiments and two-tailed student t-tests for hBMSC experiments using Graphpad PRISM 5.0 (La Jolla, CA, USA). To test for normal distribution of the input data, D'Agostino-Pearson omnibus normality tests were performed. All quantitative data sets presented passed the normality tests.

Results

Increased osmolarity improves chondrogenic marker expression in differentiating progenitor cells

To determine whether increased osmolarity improves chondrogenic marker expression in differentiating progenitor cells (14), the chondroprogenitor cell line ATDC5 (28, 29) was differentiated under control conditions and increased osmolarity (+100 mOsm and +200 mOsm). Under control conditions ATDC5 cells acquired a chondrogenic phenotype from day 7 in differentiation as determined by increased expression of collagen type II (*Col2a1*), aggrecan (*Acan*) and *Sox9* (Figure 1A). From day 14 in differentiation these cells also express increased levels of the hypertrophic markers collagen type X (*Col10a1*) and *Runx2*. Collagen type I (*Col1a1*) is only slightly induced during ATDC5 differentiation. Protein expression of the above collagens at day 14 in differentiation was confirmed by immunoblotting (Figure 1B). Increased osmolarity (+100 mOsm) significantly increased mRNA expression of *Sox9* (1.9 fold, p=0.0005), *Col2a1* (2 fold, p=0.0001), *Acan* (1.4 fold, p=0.0018), *Runx2* (1.5 fold, p=0.0061) and *Col10a1* (2.5 fold, p=0.0001) during the course of differentiation when compared to the control condition (Figure 1A). Differentiation in + 100 mOsm conditions further clearly increased COL2A1 protein expression. Expression of *Col10a1* did not increase as much at protein level as at mRNA level (Figure 1B). The decreased *Col1a1* mRNA (p=0.0035 at t=14 days) and protein expression (Figure 1B) is in line with our previous findings (14). Chondrogenic differentiation was inhibited when osmolarity of the differentiation medium was further increased to +200 mOsm, as the expression of all markers was significantly lower when compared to the control condition (at day 14 *Sox9*: p=0.0004, *Col2a1*: p=0.0184, *Acan*: p=0.0002), *Runx2*: p=0.0014 and *Col10a1*: p=0.0004) (Figure 1A/B).

Besides mRNA and protein expression of important differentiation markers, glycosaminoglycan (GAG) content is another hallmark of chondrogenic differentiation. At 14 days in differentiation, ATDC5 cells increased total GAG content by 3.2 fold (Figure 1C). Similar to mRNA and protein expression of chondrogenic

marker genes, +100 mOsm significantly increased GAG content by another 2.7 fold (control *versus* +100 mOsm, p=0.0018), whereas +200 mOsm did not increase GAG content compared to t=0. As cell proliferation is another important element in chondrogenic differentiation of ATDC5 (28) and e.g. in cartilage fracture callus development, differences in cell numbers between the conditions were determined. Increasing the osmolarity with 100 mOsm did not influence the increasing cell numbers during differentiation (Figure 1D), while culturing under +200 mOsm conditions lead to significantly lower cell numbers (reduction by ± 50%, p=0.0001)

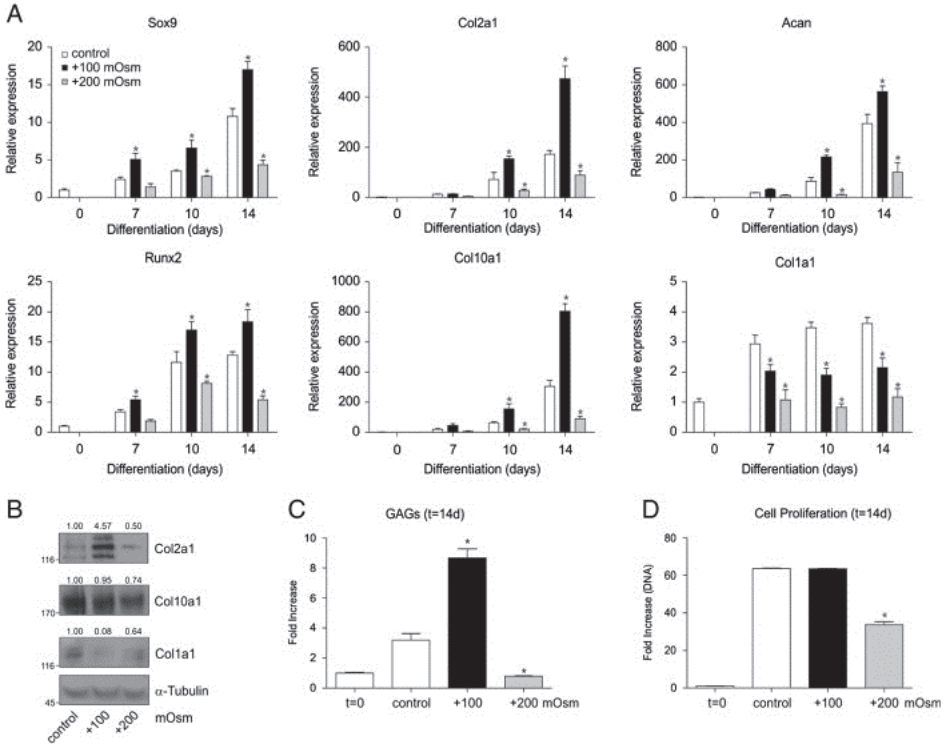


Figure 1. Increased osmolarity improves chondrogenic differentiation of ATDC5 cells. ADTC5 cells were differentiated under control osmolarity conditions (control, white bars), +100 mOsm (black bars) and +200 mOsm (grey bars). **A.** Induction of Sox9, Col2a1, Aggrecan, Runx2, Col10a1 and Col1a1 mRNA expression was determined by RT-qPCR at day 0, 7, 10 and 14 in differentiation. **B.** Protein expression of Col2a1, Col10a1 and Col1a1 in day 14 samples, α-tubulin was used as loading control. Molecular weight markers (kDa) are depicted on the left of immunoblots. Quantifications of ECL signals (corrected for the complementary α-tubulin signals and set relatively to control condition) are depicted on top of all immunoblots. **C.** Glycosaminoglycans (GAGs) were stained by Alcian Blue and fold change of t=14 samples was calculated as compared to t=0 samples. **D.** Differences in proliferation speed between conditions were determined by crystal-violet staining. Fold change (DNA) from samples from t=14 was calculated relatively to day 0. In graphs, error bars represent mean ±s.e.m.. * indicates $p < 0.05$, and is determined for control compared to +100 or +200 mOsm.

compared to the control condition. Overall, increasing osmolarity with 100 mOsm resulted in an enhanced chondrogenic marker expression during differentiation of ATDC5 cells, while further increasing the osmolarity with another 100 mOsm inhibited overall ATDC5 differentiation.

To further substantiate the pro-chondrogenic effect of increased osmolarity during chondrogenic differentiation of progenitor cells, we differentiated human bone marrow derived mesenchymal stem cells (hBMSCs) from three individuals towards the chondrogenic lineage in control medium and +100 mOsm conditions. As described earlier (26), chondrogenic/hypertrophic marker expression in these cells is detected around day 21 in differentiation. Increased osmolarity (+100 mOsm) resulted in increased *COL2A1* (p=0.0005), *ACAN* (p=0.0012) and *COL10A1* (p=0.0001) mRNA and protein expression when compared to control conditions (Figure 2A/B). Gene expression of their respective transcription factors *SOX9* and *RUNX2* was also increased in +100 mOsm conditions: *SOX9* 1.5 fold (p=0.0449) and *RUNX2* 2.1 fold higher (p=0.0010) (Figure 2A). *COL1A1* mRNA expression at day 21 in differentiation was only marginally induced in the control condition, but was found to be suppressed under increased osmolarity (p=0.0021) (Figure 2A). Decreased COL1A1 protein expression was also observed under increased osmolarity conditions (Figure 2B). Therefore, hBMSC responded similarly to increased osmolarity as ATDC5 cells.

Taken together, we found that the magnitude of chondrogenic differentiation of chondroprogenitor cells is sensitive for the osmolarity of the culture environment, providing the overall indication that chondrogenic marker expression by these cells benefits from osmolarity values in the range of that of articular cartilage.

Nfat5 is involved in chondrogenic gene expression under normal and increased osmolarity

Nfat5/TonEBP is a key regulator of the cellular response to hypertonic conditions and its expression increases during human articular chondrocyte expansion culture in 380 mOsm conditions (14). We determined the expression of *Nfat5* during chondrogenic differentiation of ATDC5 cells under control and +100 mOsm conditions. *Nfat5* mRNA expression was found to be upregulated during normal differentiation under control conditions (3.4 fold at day 7 and 5 fold at day 10 and day 14, respectively) (Figure 3A). When differentiated in +100 mOsm conditions, the expression of *Nfat5* was significantly higher as compared to the control condition (p=0.0445 for t=7, p=0.0474 for t=10 and p=0.0195 for t=14 days) (Figure 3A), indicating the osmolarity-responsiveness of *Nfat5* also during chondrogenic differentiation.

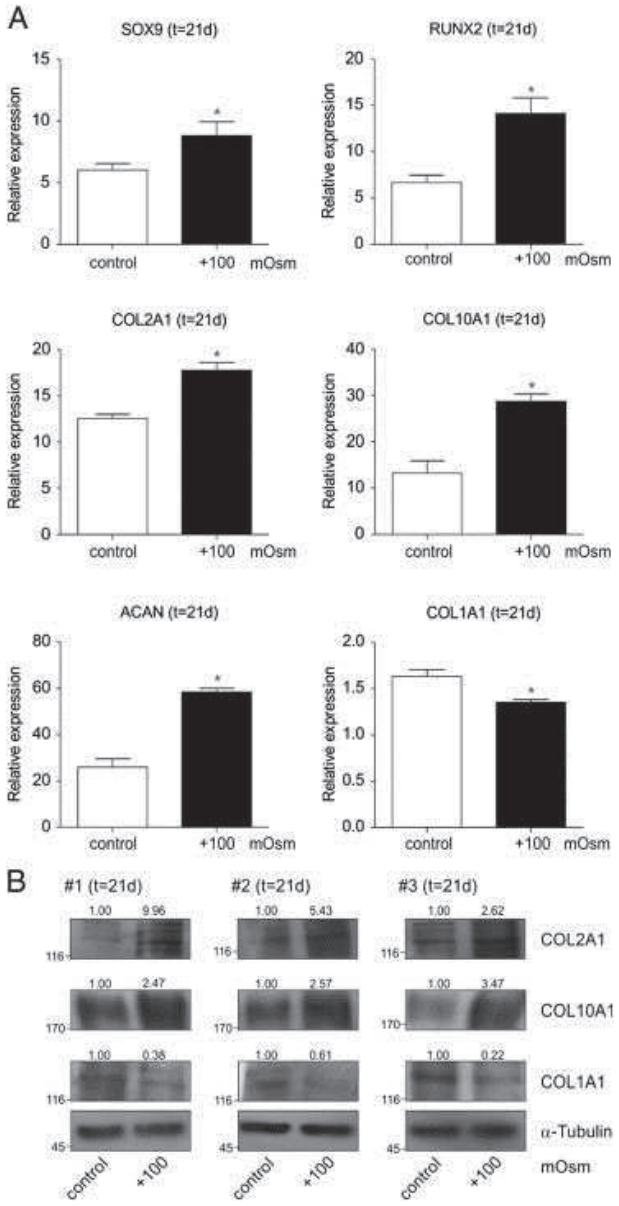


Figure 2. Increased osmolarity improves chondrogenic differentiation of hBMSCs. hBMSCs from 3 individual donors were differentiated in the chondrogenic lineage under increased osmolarity. **A.** Induction of SOX9, RUNX2, COL2A1, COL10A1, ACAN and COL1A1 mRNA expression was determined by RT-qPCR at day 0 and 21 in differentiation (control, white bars and +100 mOsm black bars) **B.** Protein expression of COL2A1, COL10A1 and COL1A1 in day 21 samples. α-Tubulin was used as loading control. Molecular weight markers (kDa) are depicted on the left of the immunoblots. Quantifications of ECL signals (corrected for the complementary α-tubulin signals and set relatively to control condition) are depicted on top of all immunoblots. In graphs, bars represent the average value of 3 individually averaged values of 3 individual samples per hBMSC isolate and error bars represent mean ±s.e.m.. * indicates $p < 0.05$, and is determined for control *versus* +100 mOsm.

Similarly, *NFAT5* mRNA expression in hBMSCs was significantly increased after 21 days of chondrogenic differentiation and also further enhanced under +100 mOsm conditions at day 21 in differentiation (p=0.0100) as compared to control conditions (Figure 3B). Confirming *Nfat5* activation upon hyperosmolarity in ATDC5, the expression of the established Nfat5 target genes *Slc5a3* and *S100a4* specifically increased in +100 mOsm cultures as compared to control cultures (p=0.0360 for *Slc5a3* at t=14 days and p=0.0175 for *S100a4* at day 14) (Figure 3C) (14).

To functionally study the involvement of Nfat5 in chondrogenic differentiation under control and +100 mOsm conditions, *Nfat5* expression was targeted using RNAi. Transfection of a target-specific siRNA duplex (30 *nM*) resulted in a 50% knockdown of *Nfat5* mRNA expression in both osmolarity conditions at day 0

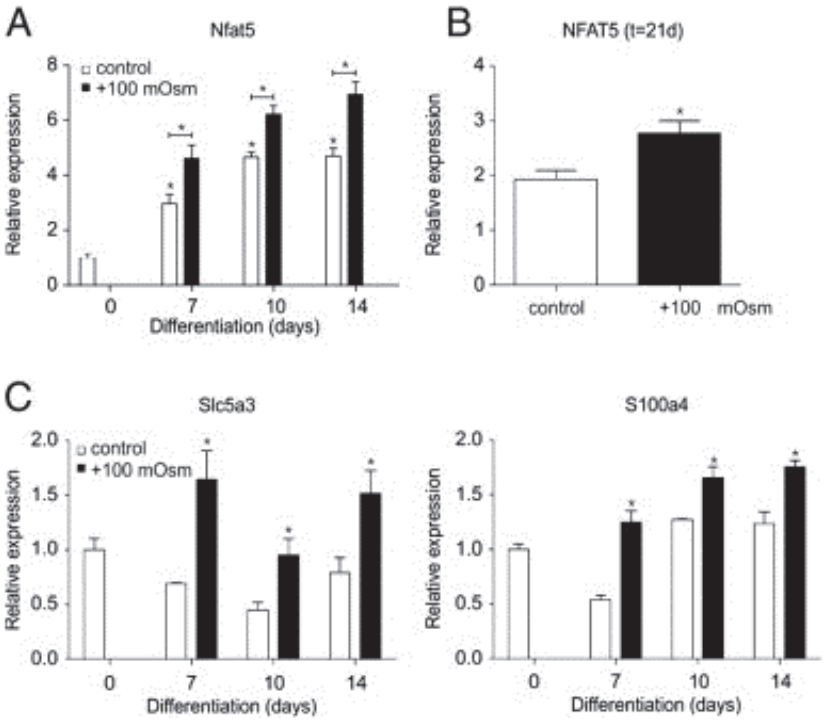
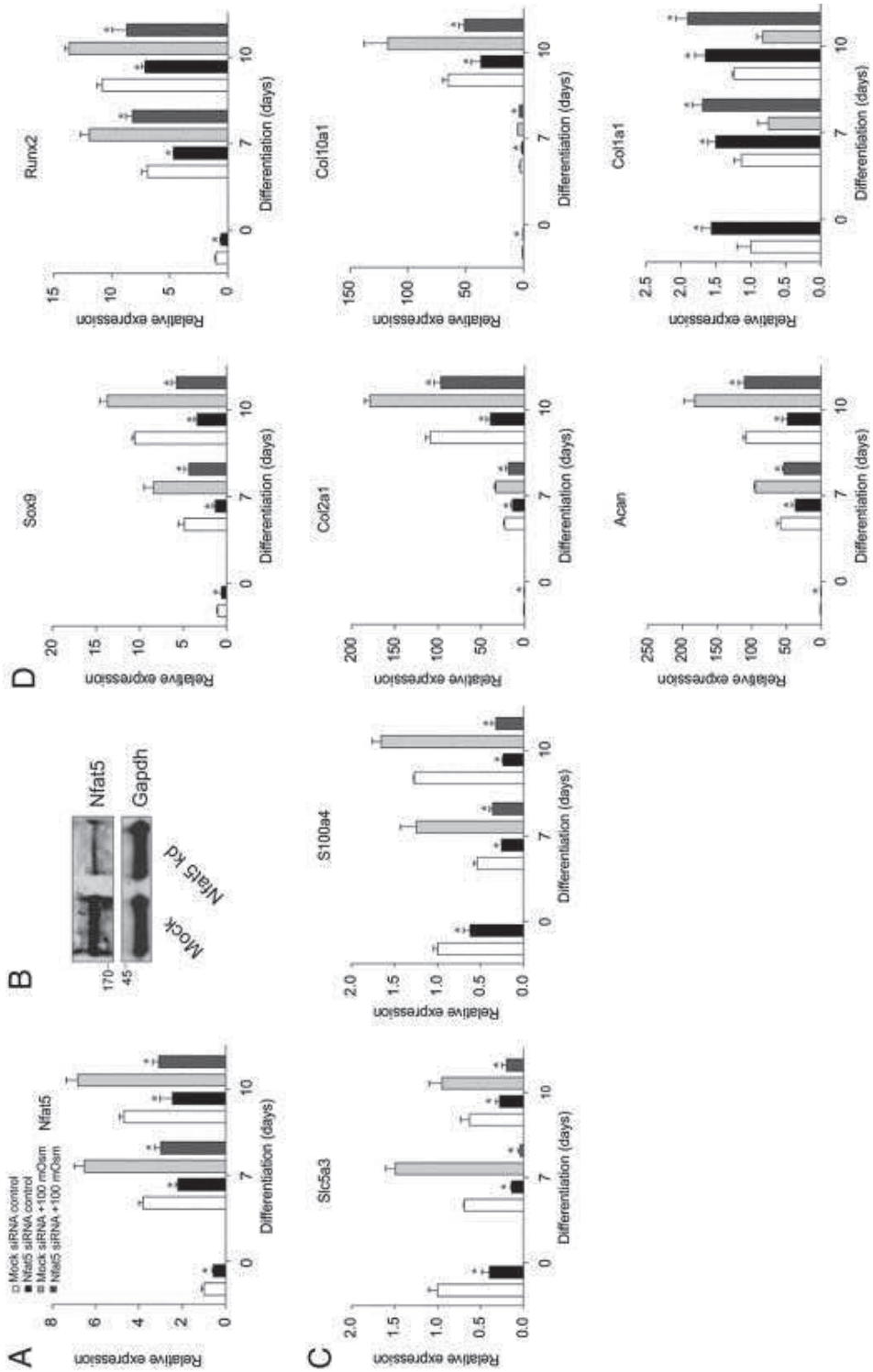


Figure 3. Nfat5 expression during chondrogenic differentiation. **A.** In similar ATDC5 samples from Figure 1A, Nfat5 mRNA expression was determined under control (white bars) and +100 mOsm (black bars) conditions. **B.** In similar hBMSC samples from Figure 2A, NFAT5 mRNA expression was determined under control (white bars) and +100 mOsm (black bars) conditions. In graphs, bars represent the average value of 3 individually averaged values of 3 individual samples per hBMSC isolate and error bars represent mean ±s.e.m.. **C.** In samples from (A) expression of established Nfat5 target genes (*Slc5a3* and *S100a4*) was determined. In graphs, error bars represent mean ±s.e.m.* indicates $p < 0.05$, and is determined for control *versus* +100 mOsm.

(p=0.0287), 7 (p=0.0415 for control and p=0.0252 for +100 mOsm) and 10 (p=0.0275 for control and p=0.0225 for +100 mOsm) in differentiation (Figure 4A; black *versus* white bars and dark grey *versus* light grey bars). Functional *Nfat5* knockdown under +100 mOsm conditions was confirmed by immunoblotting (Figure 4B), as well as prevented the upregulation of *Nfat5* target genes *Slc5a3* (p=0.0334 at day 14) and *S100a4* (p=0.0091 at day 14) under increased osmolarity (Figure 4C; dark grey *versus* light grey bars). Knockdown of *Nfat5* under control conditions resulted in a significantly suppressed upregulation of chondrogenic markers genes *Sox9* (63%, p=0.0047), *Col2a1* (59%, p=0.0038), *Acan* (51%, p=0.0033), *Runx2* (36%, p=0.0132) and *Col10a1* (53%, p=0.0350) as compared to Mock conditions (scrambled siRNA) during chondrogenic differentiation (Figure 4D; black *versus* white bars and Figure 4E). As increased osmolarity not only induced *Nfat5* expression (Figure 3A/B), but also correlated with increased chondrogenic marker expression (Figure 1), we further tested the possibility whether this osmolarity-induced chondrogenic induction may be mediated by *Nfat5*. Knocking down the expression of *Nfat5* under +100 mOsm conditions indeed suppressed the osmolarity effect on chondrogenic gene expression back to normal osmolarity conditions for most of the measured chondrogenic genes (at day 10 *Sox9*: p=0.0134, *Col2a1*: p=0.0056, *Acan*: p=0.0065, *Runx2*: p=0.0324 and *Col10a1*: p=0.0328) (Figure 4D; dark grey *versus* light grey bars and Figure 4F). The osmolarity-induced suppression of *Col1a1* mRNA and protein expression was also abrogated by *Nfat5* knockdown, where it was expressed even higher than in normal osmolarity conditions (Figure 4D; dark grey *versus* white bars and Figure 4E/F). Together, these findings indicate that *Nfat5* is involved in chondrogenic differentiation of ATDC5 cells and that the increased expression of chondrogenic markers during chondrogenic differentiation under +100 mOsm conditions is, at least in part, dependent on Nfat5.

Nfat5 is involved in Sox9 function during chondrogenic differentiation

To further explore the possibility whether osmolarity induces the increased chondrogenic marker expression via the key chondrogenic transcription factor Sox9 and to explore potential cross-talk between Nfat5 and Sox9, we determined whether osmolarity-driven chondrogenic marker expression can be abolished by interfering with Sox9 expression. Expression of *Sox9* was targeted by siRNA transfection (30 *nM*) in ATDC5 and resulted in a knockdown efficiency of 58% at mRNA level at day 0, 7 and 10 in differentiation under both control and +100 mOsm conditions (p=0.0398 at t=0, p=0.0025 at t=10 control and p=0.0123 at +100 mOsm) (Figure 5A/C; black *versus* white bars and dark grey *versus* light grey bars). Upon *Sox9* knockdown under control conditions, expression of the important Sox9 targets,


A
Mock siRNA control
Nfat5 siRNA control
Mock siRNA +100 mOsm
Nfat5 siRNA +100 mOsm
Nfat5
Relative expression
Differentiation (days)
B
Nfat5
Gapdh
170
45
Mock
Nfat5 kd
C
Slc5a3
S100a4
D
Sox9
Runx2
Col2a1
Col10a1
Acan
Col1a1

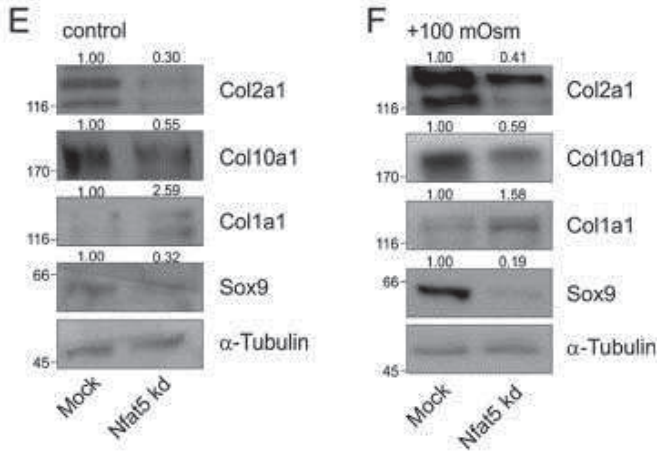


Figure 4. Nfat5 may regulate chondrogenic differentiation under normal and increased osmolarity. To determine how Nfat5 is involved in chondrogenic differentiation of ATDC5 cells, expression of Nfat5 was targeted during the course of differentiation by retransfection of an Nfat5 specific siRNA duplex. **A.** Knockdown of Nfat5 expression was confirmed at mRNA level at day 0, 7 and 10 in differentiation. White bars represent normal osmolarity conditions (control) transfected with a scrambled siRNA ("Mock") and black bars normal osmolarity conditions transfected with the Nfat5 siRNA. Light grey bars represent increased osmolarity conditions (+100 mOsm) transfected with a scrambled siRNA ("Mock") and black bars increased osmolarity conditions transfected with the Nfat5 siRNA. **B.** Knockdown of Nfat5 expression was confirmed by immunoblotting of protein samples from +100 mOsm conditions at day 10. **C.** Knockdown of Nfat5 expression was functionally confirmed by decreased Nfat5 target gene expression of Slc5a3 and S100a4. **D.** In similar samples from (A) Sox9, RunX2, Col2a1, Col10a1, Aggrecan and Col1a1 mRNA expression was determined. **E.** Protein expression of Col2a1, Col10a1, Col1a1 and Sox9 from day 10 samples in Mock and Nfat5 knockdown cells under control conditions. **F.** Protein expression of Col2a1, Col10a1, Col1a1 and Sox9 from day 10 samples in Mock and Nfat5 knockdown cells under +100 mOsm conditions. In graphs, error bars represent mean ±s.e.m.. * indicates $p < 0.05$, and is determined for Mock *versus* Nfat5 siRNA under control osmolarity and under +100 mOsm conditions, respectively.

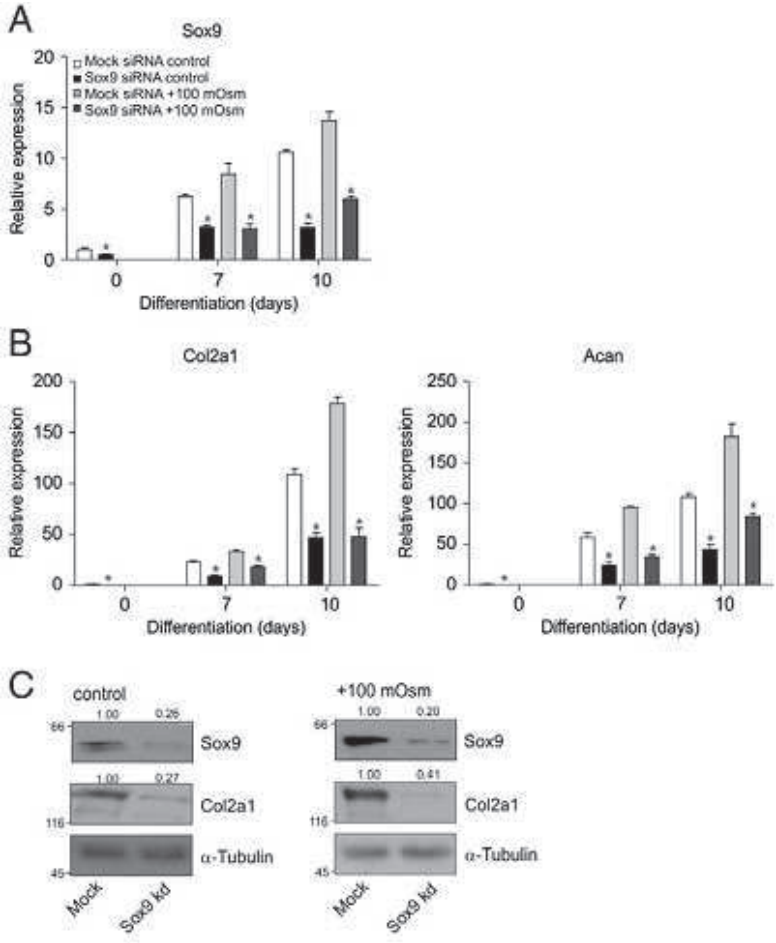


Figure 5. Osmolarity-enhanced chondrogenic differentiation is Sox9 dependent. Sox9 siRNA duplexes were retransfected under control and +100 mOsm conditions to investigate whether the osmolarity effect on chondrogenic differentiation is depending on Sox9. **A.** Knockdown of Sox9 was confirmed at mRNA level at day 0, 7 and 10 in differentiation. White bars represent normal osmolarity conditions (control) transfected with a scrambled siRNA ("Mock") and black bars normal osmolarity conditions transfected with the Sox9 siRNA. Light grey bars represent increased osmolarity conditions (+100 mOsm) transfected with a scrambled siRNA ("Mock") and black bars increased osmolarity conditions transfected with the Sox9 siRNA. **B.** The effect of the Sox9 knockdown under control and +100 mOsm conditions on chondrogenic differentiation was established by measuring *Col2a1* and *aggrecan* mRNA expression. **C.** Protein expression of Sox9 and Col2a1 from day 10 samples in Mock and Sox9 knockdown cells under control and +100 mOsm. In graphs, error bars represent mean ±s.e.m.. * indicates $p < 0.05$, and is determined for Mock *versus* Sox9 siRNA under control osmolarity conditions and under +100 mOsm conditions, respectively.

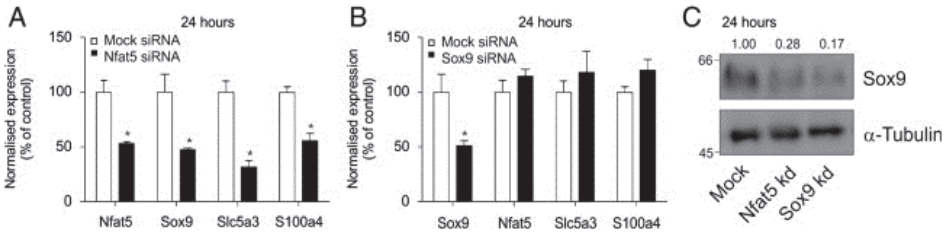


Figure 6. Nfat5 influences Sox9 expression. Signalling hierarchy between Nfat5 and Sox9 was determined early (24 hours) in ATDC5 differentiation by siRNA transfections. **A.** Expression of *Nfat5, Sox9, Slc5a3* and *S100a4* mRNAs in differentiating ATDC5 transfected with Mock or Nfat5 siRNAs in control osmolarity conditions. **B.** Same as (A) but with Mock or Sox9 siRNAs. Data is presented as percentage to control condition. **C.** Sox9 protein expression at 24 hours in ATDC5 differentiation after Mock, Nfat5 or Sox9 siRNA transfection. In graphs, error bars represent mean ±s.e.m.. * indicates $p < 0.05$, and is determined for Mock *versus* Nfat5 or Sox9 siRNA.

Col2a1 and *aggrecan*, was significantly decreased (p=0.0046 for *Col2a1* and p=0.0076 for *Acan* at t=10 days) (Figure 5B; black *versus* white bars and Figure 5C; left panel), showing that expression of these genes under control culture conditions is regulated through *Sox9*. The osmolarity-induced chondrogenic marker expression under +100 mOsm was also significantly affected by the *Sox9* knockdown (p=0.0023 for *Co2a1* and p=0.0092 for *Acan* at t=10 days) (Figure 5B; dark grey *versus* light grey bars and Figure 5C; right panel), showing that the osmolarity-induced chondrogenic marker expression functionally depends on *Sox9*. We next aimed at elucidating potential cross-talk and pathway hierarchy between Nfat5 and Sox9, which might explain how Nfat5 influences chondrogenic differentiation. We analyzed *Sox9* expression early in ATDC5 differentiation (26) at 24 hours after *Nfat5* knockdown and *Nfat5* expression upon *Sox9* knockdown at the same moment in ATDC5 differentiation as well. Functional interference with *Nfat5* expression was confirmed by decreased expression of the *Nfat5* targets *Slc5a3* and *S100a4* (Figure 6A; 68% (p=0.0202) and 44% (p=0.0293), respectively). In addition, knockdown of *Nfat*5 efficiently decreased *Sox9* mRNA (p=0.0404) and protein expression (Figure 6A/C). In contrast, knockdown of *Sox9* solely decreased *Sox9* mRNA (p=0.0339) and protein expression, without further affecting *Nfat5* mRNA levels or those of its target genes (Figure 6B/C). These data suggest that *Nfat5* may determine the outcome of ATDC5 chondrogenic differentiation by transcriptionally influencing early expression of *Sox9*.

In conclusion, increasing culture medium osmolarity from control levels (i.e. 285-310 mOsm) with 100 mOsm significantly increased chondrogenic marker expression during the course of chondrogenic differentiation of progenitor cells (ATDC5 and hBMSCs). We further found evidence that Nfat5 may play a regulatory role during

(osmolarity-induced) chondrogenic differentiation of these cells, at least in part, through influencing Sox9 expression.

Discussion

Several studies have shown that human articular chondrocytes *in vitro* are osmolarity-responsive and increase matrix synthesis under cartilage-physiological osmolarity with Nfat5 being involved in this response. The potentially beneficial effects of increased osmolarity on chondrogenic differentiation of progenitor cells are largely unknown. In addition, the expression and function of Nfat5 during this process have never been described. We here report that chondrogenic marker expression and chondrogenic extracellular matrix synthesis during differentiation of the chondroprogenitor cell line ATDC5 and in human bone marrow stem cells (both sequentially expressing a chondrocyte and hypertrophic chondrocyte phenotype, respectively) positively respond to increased osmolarity. Expression of Nfat5 is upregulated during chondrogenic differentiation and its expression is further upregulated under increased osmolarity. Nfat5 is involved in regulating chondrogenic marker expression under normal and increased osmolarity and may regulate the osmolarity-induced beneficial effects on Col2a1 and Acan through key-chondrogenic transcription factor Sox9.

In human articular cartilage the extracellular osmolarity ranges between 350-480 mOsm (9, 10), due to the high fixed negative charge density of the sulfated GAG side chains in the proteoglycan network. It may therefore not be surprising that chondrocyte phenotypic preservation during isolation and culturing of articular chondrocytes *in vitro* benefits from increased osmolarity in the culture medium (14). However, the osmolarity levels in the direct environment of chondroprogenitor cells *in vivo* are poorly investigated. Amongst others, mesenchymal stem cells are found in the bone marrow, adipose tissue, muscle, blood and periosteum (30) and most of these tissues have a normal plasma level osmolarity of around 280 mOsm. It is therefore likely that progenitor cells residing in these tissues experience an environment that is close to plasma osmolarity *in vivo*. Yet, progenitor cells express similar chondrogenic markers (e.g. Sox9, Col2a1, Acan etc.) during chondrogenic differentiation as found in mature articular chondrocytes and may create a local self-sustaining hypertonic microenvironment by excretion of ECM components during their differentiation process. It seems therefore likely that the response of mesenchymal progenitor cells to increased osmolarity may be regulated through

similar molecular mechanisms that are also responsible for increased chondrogenic marker expression in mature chondrocytes.

We used established culture protocols for ATDC5 and hBMSC differentiation and thus their corresponding differentiation media had slightly different baseline osmolarities (310 and 285 mOsm, respectively), within the range of plasma level osmolarity [11, 12]. The relative cell type-specific quantity of the osmotic challenge was, however, identical for both cell types. Noteworthy, for ATDC5 we found that, as opposed to +100 mOsm, an osmotic challenge of +200 mOsm had deleterious consequences for the chondrogenic differentiation process. This suggests the existence of an optimal osmolarity-window for the outcome of ATDC5 differentiation. The +100 mOsm condition (i.e. ~410 mOsm) is within the range of physiological articular cartilage osmolarity [9, 10]. Higher osmotic pressures, as in the +200 mOsm condition, are rather high end physiological and may exceed membrane transport capacities.

The molecular mechanism by which osmolarity is sensed by mammalian cells is only partially understood. Nfat5 is accepted as key transcription factor participating in the response to the deleterious effects of increased osmolarity (21, 31). The hypertonic stress-induced increase in intracellular ionic strength is potentially genotoxic, inducing double-strand DNA breaks, growth arrest and apoptosis (32, 33). Nfat5 is activated by hypertonic stress to compensate for these deleterious effects through activation of compensatory mechanisms like e.g. activating osmolyte transporter gene transcription (e.g. Slc5a3 and S100a4) in order to reduce the intracellular ionic strengths (21, 34). However, little is known about the osmolarity threshold required to activate Nfat5 in specific cell types and several observations suggest that Nfat5 is active already at plasma level osmolarity in tissues that normally are not exposed to a hypertonic environment (16). In addition, in our experiments knockdown of *Nfat5* under plasma level osmolarity resulted in downregulation of Nfat5 target gene expression (Figure 4C), indicating that Nfat5 is functionally active under these conditions in the tested progenitor cells. Also, it is becoming increasingly evident that Nfat5 is involved in cellular processes unrelated to hypertonic stress, such as cell proliferation, differentiation or integrin-mediated cell migration (31, 35, 36). Our present study shows that Nfat5 expression follows the course of normal chondrogenic differentiation and may play a regulating role during chondrogenic differentiation of progenitor cells. While osmolarity may enhance chondrogenic differentiation through Nfat5, its knockdown during chondrogenic differentiation under both plasma (control) osmolarity and increased osmolarity (+100 mOsm) resulted in suppressed expression of chondrogenic markers *Col2a1*, *Acan*, *Sox9*,

Col10a1 and *Runx2* (Figure 4D-F), suggesting a regulatory function of Nfat5 during chondrogenic differentiation under both control culture conditions and increased osmolarity conditions.

The mode of action by which Nfat5 may regulate chondrogenic differentiation of progenitor cells remains to be elucidated. Our data suggest that Nfat5 might act in this context through influencing the expression of key chondrogenic transcription factor Sox9. Regulation of Sox9 expression by osmolarity has been shown before. Stimulation of human chondrocytes with supraphysiological osmolarity not only induced *SOX9* mRNA levels, but also increased *SOX9* mRNA half-life (37). Post-transcriptional stabilization of *SOX9* mRNA is activated by p38 MAPK signalling (38), which is also known to be involved in the hypertonic activation and nuclear translocation of NFAT5 (39-41). Alternatively, we previously found that Sox9 expression during chondrogenic differentiation of progenitor cells *in vitro* is bi-phasic, with a first induction during the first hours of differentiation and a second peak expression later on in differentiation. Next to the well documented crucial role of Sox9 during ECM synthesis (late expression peak), we showed that the early and transient induction of Sox9 crucially determines the chondrogenic outcome of mesenchymal progenitor cell differentiation (26). Knockdown of *Nfat5* resulted in decreased *Sox9* expression (Figure 4D) on the long term, which could explain the decreased *Col2a1* and *Acan* expression seen in these cultures. In addition, and relevant to the early Sox9 induction, *Nfat5* knockdown during early chondrogenic differentiation (24 hours) affected early *Sox9* expression, as well as known Nfat5 target genes. Reciprocally, knockdown of early *Sox9* expression did not influence the expression of *Nfat5* or its target genes (Figure 6B/C), suggesting that Nfat5 may act upstream of Sox9, through yet unknown (transcriptional) mechanisms.

Conclusions

We have here shown that the magnitude of chondrogenic gene expression of differentiating chondroprogenitor cells *in vitro* can be enhanced by increasing the osmolarity levels of the culture medium. The osmolarity responsive gene Nfat5 seems to be part of the mechanism that underlies this effect and might influence chondrogenic differentiation via controlling the expression of key chondrogenic transcription factor Sox9. Future studies will focus on further elucidating the relation between Nfat5 and chondrogenic regulators. Increasing the osmolarity may be used as a relatively simple tool to modulate the formation of cartilaginous tissues

for progenitor cell-based cartilage and endochondral bone regenerative medicine approaches (42).

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FK506 promotes osmolarity-induced chondrogenic differentiation of murine ATDC5 cells

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Submitted

Abstract

Chondrogenic marker expression can be induced with osmotic stress in the range of 100 mOsm above plasma level. However, osmotic stress also induces hypertophic differentiation of chondrocytes, an unwanted event in cartilage regenerative medicine. This study investigated the potential effects of FK506 (tacrolimus) on osmolarity-induced chondrogenic and terminal differentiation of chondroprogenitor cells.

ATDC5 cells, a murine chondroprogenitor cell line, were chondrogenically differentiated under control plasma osmolarity of 312 mOsm and under a more cartilage specific physiological osmolarity (+100 mOsm). In addition, cells were costimulated with 0, 62 or 620 nM FK506 from day 7 in differentiation. Chondrogenic, hypertrophic and terminal differentiation was measured by gene expression analysis at day 14.

Cartilage physiological osmolarity (± 412 mOsm) increased chondrogenic (*Sox9, Col2, Col9, Col11*) and hypertrophic (*Runx2, Tgm2, Col10, Vegf, Bmp2*) marker expression of pre-chondrocytes, without affecting mineralization markers (*Alpl, Spp1*). FK506 further increased chondrogenic markers (*Col9,* Col11) but could not prevent hypertrophic and terminal differentiation at physiological osmolarity in this cell line.

We showed that FK506 at physiological osmolarity promotes chondrogenic differentiation of progenitor cells cultured at cartilage specific osmolarity, without altering or inhibiting differentiation to hypertrophic and calcified cells, as was found in earlier studies with osteoarthritic chondrocytes. These findings indicate that there might be a cell type dependent effect op FK506 or a timing effect during chondrogenic differentiation of ATDC5 cells.

Introduction

New methodologies promoting chondrogenic differentiation of mesenchymal progenitor cells are of interest to improve (cell based) repair of cartilage lesions in joint diseases such as osteoarthritis (OA). A clonal mouse embryonic cell line, ATDC5, has been identified to synchronously display the multistep differentiation process of mesenchymal progenitor cells during endochondral bone formation. The chondrogenic differentiation process starts with a mesenchymal condensation stage of progenitor cells, followed by differentiation into proliferating chondrocytes producing extracellular matrix (ECM), nodule formation and hypertrophic differentiation, and finally ends with matrix mineralization and apoptosis of the cells. These steps are resembled *in vitro* by ATDC5 cells as an established model to investigate regulation of chondrogenic differentiation (1, 2).

FK506 (Tacrolimus, Prograf®) is an immunosuppressive drug discovered by Kino et al. (3, 4) in the 1980s, which is clinically used for a wide range of immunological disorders. The immunosuppressive agents FK506 and Cyclosporin A (CsA) revolutionized transplant therapy because of their ability to suppress of T-cell activation without markedly affecting bone marrow cell differentiation and proliferation (5). FK506 and CsA bind to two different intracellular proteins, FKBP and Cyclophilin, but both thereby inhibiting the activity of the ubiquitously expressed calcium/calmodulin dependent Calcineurin (Cn). As a consequence, the calcineurin-mediated dephosphorylation of a specific group of transcription factors (NFATs, nuclear factor of activated T-cells) is inhibited (6, 7).

Over the last 20 years, Calcineurin and NFATs are found to play a role in many other physiological processes in other cell types and the clinical application for FK506 is still increasing. Two studies reported stimulating effects of FK506 on chondrogenic differentiation of ATDC5 cells. Nishigaki et al. (8) showed that FK506, but not CsA, stimulates production of proteoglycan and type II collagen by ATDC5 cells. Nakamura et al. (9) suggested that FK506 predominantly promotes differentiation of ATDC5 cells into proliferating chondrocytes, without terminal differentiation to hypertrophic chondrocytes leading to calcified tissue.

Recently, we used osmotic stress to induce chondrogenic marker expression in human OA chondrocytes, ATDC5 cells and human bone marrow stem cells and elucidated a role for NFAT5 (a Calcineurin independent NFAT also known as TonEBP) in this osmotic stress response (10, 11). However, in both OA chondrocytes and progenitor cells, osmotic stress also increased expression of hypertrophy and

terminal differentiation markers (11, 12). The latter being a favourable event in improving endochondral regenerative medicine approaches, but unfavourable in improving cartilage regenerative medicine treatments.

In human OA chondrocytes, FK506 was able to further enhance the osmotic stress induced anabolic marker expression (collagen type 2 (Col2) and aggrecan), while supressing unwanted catabolic and hypertrophic markers (collagen type 10 and matrix metalloproteases) (12). In the present study, we further explored these effects and investigated the potential effects of FK506 on osmolarity induced chondrogenic and terminal differentiation of the chondroprogenitor cells ATDC5.

Materials and Methods

ATDC5 cell culture

ATDC5 cells were cultured in a monolayer using proliferation medium (DMEM/F12 (Invitrogen, Carlsbad, CA, USA), 5% FCS, 0.1% (v/v) gentamycin, 0.6% (v/v) fungizione and 1% NEAA (nonessential amino acids; all Invitrogen)). Cells were plated at 6400 cells/cm^2 in cell culture dishes, allowed to adhere overnight and the following day chondrogenesis was initiated by changing the proliferation medium to differentiation medium. Differentiation medium comprised proliferation medium supplemented with 1% ITS (B&D Bioscience). Differentiation medium was changed every two days (day 0 to 10) and daily (from day 10 on). It was shown before that at standard osmolarity conditions, ATDC5 cells acquire a chondrogenic phenotype from day 7 in differentiation with increased expressions of sex determining region Y box 9 (Sox9) and Col2 (11). The osmolarity of the standard ATDC5 differentiation medium was determined using an Osmomat 030 (Gonotec GmbH, Germany) and was 312±2 mOsm. To reach a cartilage specific physiological level, the osmolarity of this medium was increased with 100 mOsm by adding sterile NaCl (10) from day 7 in differentiation. In addition, cells were co stimulated with 0, 62 or 620 nM FK506 from day 7. Experiments were performed in replicates (n=7). Cells were harvested at day 0 and 14 in differentiation for analysis of mRNA and protein expressions.

To analyze direct effects of increased osmolarity on Nfat5 protein expression, cells were cultured on 8 well culture slides (BD Falcon) and stimulated with +100 or +200 mOsm medium for 30 minutes.

RNA expression analysis

RNA purification, quantification, cDNA synthesis and RT-qPCR are described elsewhere (13, 14). Data were normalized to an index of three reference genes (*Gapdh, Hprt1,* β*-actin*), which were pre-evaluated to be stably expressed across samples, and relative expression was then calculated according to $2^{-\Delta CT}$ method (15). Replicate values (n=7) were normalized to day 0. Collagen type 1, 2, 9 and 11 and *Sox 9* were studied as markers for chondrogenic differentiation (16). *Col10* and its main transcription factor runt-related transcription factor 2 (*RunX2*) as well as vascular endothelial growth factor (*Vegf*), transglutaminase 2 (*Tgm2*), and *Mmp13* were used to evaluate induction of hypertrophic and terminal differentiation of the cells (16, 17). Bone morphogenetic protein 2 (*Bmp2*) was evaluated as general inductor of chondrogenic and subsequent hypertrophic differentiation and endochondral ossification (18). The calcium binding protein A4 (*S100a4*) was evaluated as a negative regulator of mineralization (19) and Nfat5 target gene (10). In addition, expression levels of mineralization markers alkaline phosphatase (*Alpl*) and osteopontin (*Spp1*) (20) were studied. Primer sequences for *Col2, Col1, Sox9, RunX2, Col10, Nfat5*, *s100A4* and β*-actin (11), Col9 and Col11 (21), Mmp13* (14) and *Vegf* (22) are adopted from previous studies. Primer sequences for the other tested genes are listed in table 1.

Table 1. Oligonucleotide sequences for RT-qPCR.

Oligo sets	Forward	Reverse
Hprt1	5′-TCAGTCAACGGGGGACATAAA-3′	5′-GGGGCTGTACTGCTTAACCAG-3
Gapdh	5′-AGGTCGGTGTGAACGGATTTG-3′	5′-TGTAGACCATGTAGTTGAGGTCA-3′
Tgm2	5′-GACAATGTGGAGGAGGGATCT-3′	5′-CTCTAGGCTGAGACGGTACAG-3′
Bmp2	5′-GGGACCCGCTGTCTTCTAGT-3′	5′- TCAACTCAAATTCGCTGAGGAC-3′
Alpl	5′-CCAACTCTTTTGTGCCAGAGA-3′	5′-GGCTACATTGGTGTTGAGCTTTT-3′
Spp1	5′-GCAGCAGGAGGAGGCAGAGC-3′	5′-AGTGAGTTTTCCTTGGTCGGCGT-3′

Calcineurin activity assay

FK506 is known to exert its immunosuppressive effects by inhibiting the calcineurin activity. Cells from monolayer cultures were washed twice with physiologic saline and lysed with Mammalian Protein Extraction Reagent buffer (Pierce, Bonn, Germany) according to the supplier's instructions. Samples were stored at minus 80°C until further use. Extracts were purified on a Micro Bio-Spin P-6 chromatography column (Bio-Rad Laboratories B.V., Veenendaal, NL) and protein concentrations were quantified using the BCA Protein Assay Kit (Pierce) in a microplate reader (VersaMax, Molecular Devices Ltd, NL). Calcineurin activity was measured using

the Calcineurin Cellular Assay Kit Plus (BioMol, Tebu-Bio, Heerhugowaard, NL) as described before (10).

Immunofluorescence

Next, we evaluated osmolarity-induced expression of Nfat5 protein in our experiments. Immunofluorescence was essentially performed as described earlier (23), with the conditions and the anti-Nfat5 antibody reported elsewhere (24) and the normalization procedure using a histone H3-specific antibody reported earlier by us (25).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20 software. The replicate raw expression data was tested for the effect of osmolarity, FK506 dosis and the interaction between both treatments using a general linear model analysis. The effect of FK506 significantly differed between the two osmolarity groups for some of the tested genes. Therefore, the effect of FK506 was tested separately for the 280 and 380 conditions, using univariate general linear model analysis followed by posthoc Bonferroni test. Differences were considered statistical significant when $p< 0.05$ and $p<0.01$.

Results

Cartilage-specific physiological levels of osmolarity improve chondrogenic differentiation of pre-chondrocytes

When the medium osmolarity was increased with 100 mOsm (to reach cartilage specific osmolarity of 412 mOsm) from day 7 during differentiation, expression of the chondrogenic markers *Sox9* and Collagen type 2 (*Col2*) was significantly increased at day 14 compared to control conditions (plasma level osmolarity of 312 mOsm): *Sox9* ± 2.0-fold and *Col2* ± 1.2-fold (figure 1A,B). The +100 mOsm condition also increased the *Col2:Col1* ratio by ± 1.5-fold (figure 1C). In addition, we analyzed the regulation of two other cartilage-specific collagens, collagen type 9 (*Col9*) and type 11 (*Col11*). After 14 days of differentiation at control osmolarity, the expression of *Col9* mRNA markedly increased by ± 290-fold and expression of *Col11* by ± 55-fold (figure 1D,E). Their expression was further increased when the cells were stimulated with +100 mOsm: *Col9* by 2.3-fold and *Col11* by 1.8-fold.

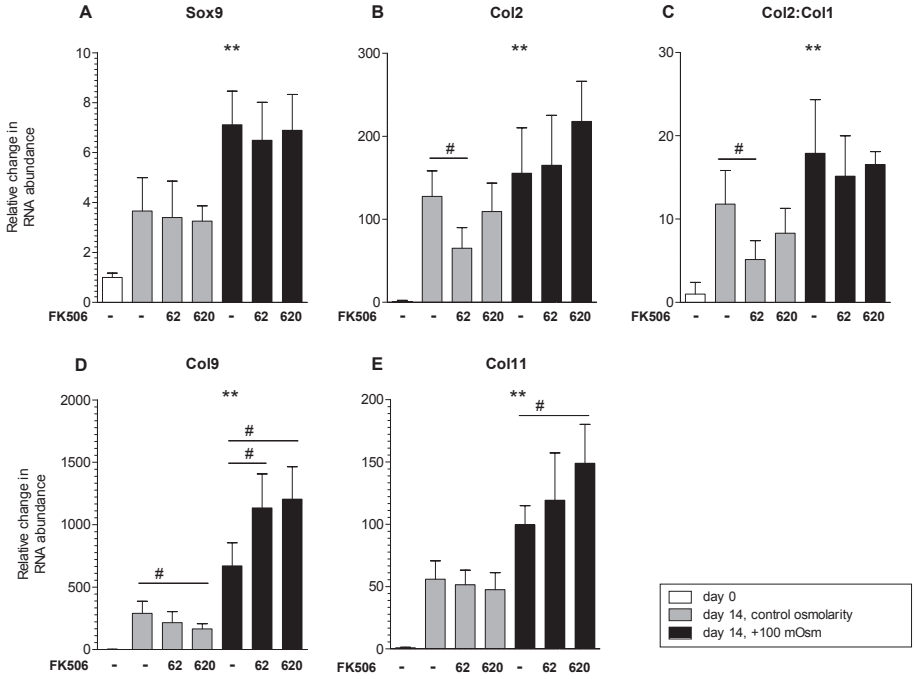

Figure 1. Increased osmolarity improves chondrogenic differentiation of ATDC5 cells and addition of FK506 further increased Col9 and Col10 expression**.** ATDC5 cells were differentiated under control osmolarity conditions for 14 days (grey bars) or at +100 mOsm from day 7 till day 14 (black bars). In addition, cells were co stimulated with 0, 62 or 620 nM FK506 from day 7. All experiments were performed in replicates (n=7). Induction of Sox9, Col2, Col2:Col1, Col9 and Col11 mRNA was determined by RT-qPCR at day 0 and day 14. Data are means ± standard deviation. Significant differences are indicated: ** $p<0.01$ between control and +100 mOsm conditions; # $p<0.05$ FK506 effect compared to the iso-osmotic condition without FK506.

Cartilage physiological osmolarity improves hypertrophic differentiation of ATDC5 cells

Besides its effects on chondrogenic marker expression, increasing the medium osmolarity with 100 mOsm to cartilage physiological levels also significantly affected subsequent expression of hypertrophic and terminal differentiation markers. We studied the effects of our treatment on expression of runt-related transcription factor 2 (*Runx2)* and transglutaminase 2 (*Tgm2*), both regulators of terminal differentiation. Both *Runx2* and *Tgm2* increased during the course of differentiation at control osmolarity and increased even further when the osmolarity was raised by 100 mOsm (figure 2A,B). The hypertrophic marker *Col10* increased by ± 4.6-fold upon osmotic stimulation, while *Mmp13* decreased ± 2.0-fold (figure 2C,D). The terminal differentiation marker *Vegf* and *Bmp2* both significantly increased by +100 mOsm (figure 2E,F).

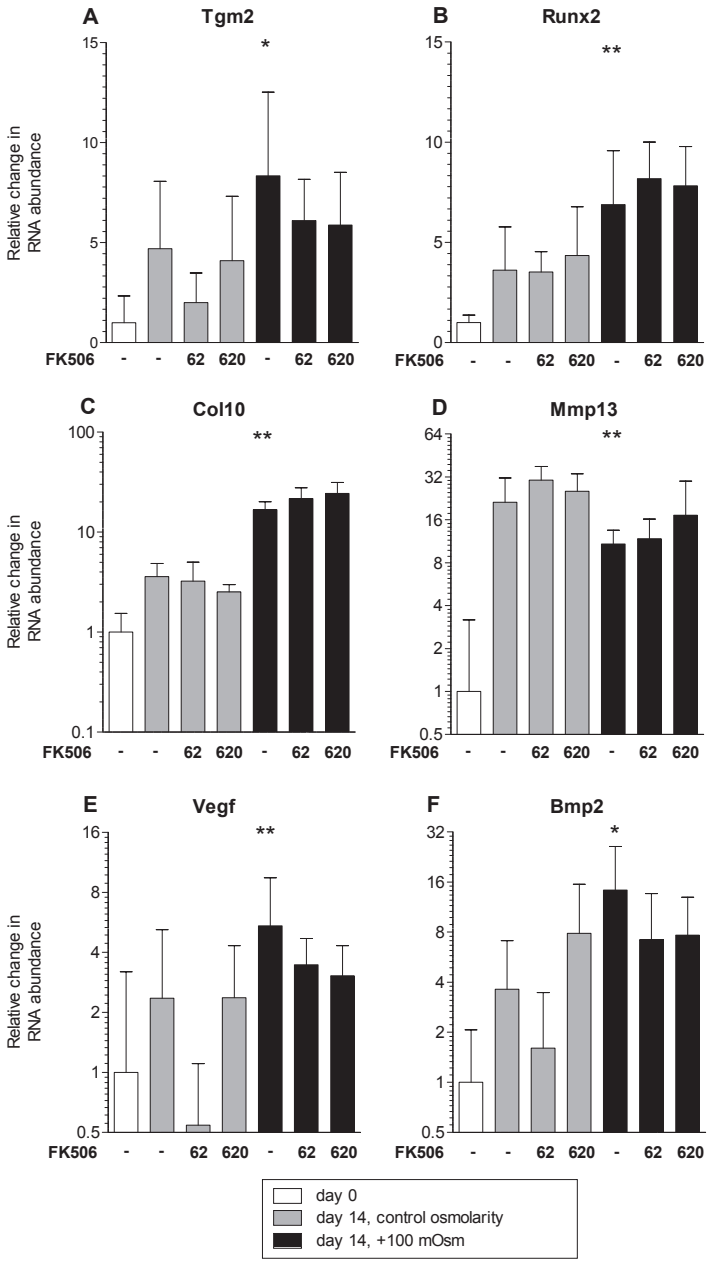

Figure 2. Effects of cartilage physiological osmolarity and FK506 on hypertrophic differentiation markers. ATDC5 cells were differentiated under control osmolarity conditions for 14 days (grey bars) or at +100 mOsm from day 7 till day 14 (black bars). In addition, cells were co stimulated with 0, 62 or 620 nM FK506 from day 7. All experiments were performed in replicates (n=7). Induction of mRNA was determined by RT-qPCR at day 0 and day 14. Data are means ± standard deviation. Significant differences are indicated: * $p<0.05$ and ** $p<0.01$ between control (grey bar) and +100 mOsm (black bar) conditions.

Cartilage specific osmolarity (+100 mOsm) slightly increased the expression S100a4, a negative regulator of mineralization (figure 3A). Expression levels of mineralization markers Alpl and Spp1 were increased during differentiation at control osmolarity, but were not further altered by +100 mOsm (figure 3B,C). These data suggest that increasing the medium osmolarity to cartilage physiological levels promotes chondrogenic and hypertrophic differentiation of pre-chondrocytes (figure 1 and 2), without promoting mineralization (figure 3).

FK506 exerts dual effects on chondrogenic differentiation between different osmolarities

Supplementation of FK506 to ATDC5 cells in differentiation medium with control osmolarity from day 7 to 14 did not improve mRNA expression of the chondrogenic markers (figure 1). On the contrary, FK506 showed a decreasing trend in chondrogenic marker expression at control osmolarity, although only significant for the *Col2:Col1* ratio and for *Col9* in one of the two FK506 conditions. At +100 mOsm, FK506 showed clear effects. While there was no effect of FK506 on *Sox9* and the *Col2:Col1* ratio, a significant increased expression of *Col9* (± 1.7-fold) by 62 nM FK506 and of *Col9* (± 1.8-fold) and *Col11* (± 1.5-fold) upon addition of 620 nM FK506 was observed (figure 1D,E). Although not significant, expression levels of *Col2* showed the same increasing trend when the cells were stimulated with FK506 in the +100 mOsm condition (figure 1B).

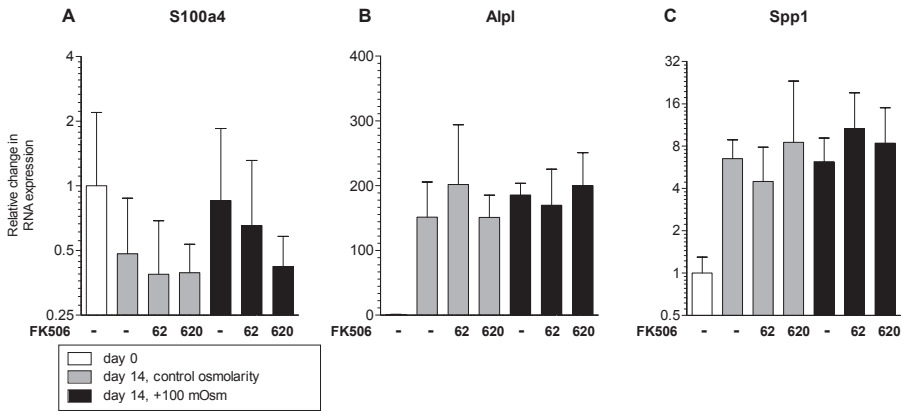

Figure 3. Mineralization markers were not altered by either increased osmolarity or FK506. ATDC5 cells were differentiated under control osmolarity conditions for 14 days (grey bars) or at +100 mOsm from day 7 till day 14 (black bars). In addition, cells were co stimulated with 0, 62 or 620 nM FK506 from day 7. All experiments were performed in replicates (n=7). Induction of mRNA was determined by RT-qPCR at day 0 and day 14. Data are means ± standard deviation.

FK506 has no effect on hypertrophic and terminal differentiation markers at both osmolarities

FK506 addition to medium of control or cartilage osmolarity (+100 mOsm) did not significantly alter any of the tested hypertrophic and mineralization markers (figure 2 and 3). There was a slightly increasing trend in *Runx2, Col10* and *Mmp13* expression and a slightly decreasing trend in *Tgm2, Vegf* and *Bmp2* expression by FK506. Currently, there is no evidence that FK506 significantly alters the osmolarity induced hypertrophic differentiation of ATDC5 cells.

Effect of FK506 on chondrogenic differentiation might be calcineurin independent

Increased osmolarity is known to target Nfat5, which was shown earlier for mature chondrocytes (10). In the current experiments this was confirmed by a 1.5 fold

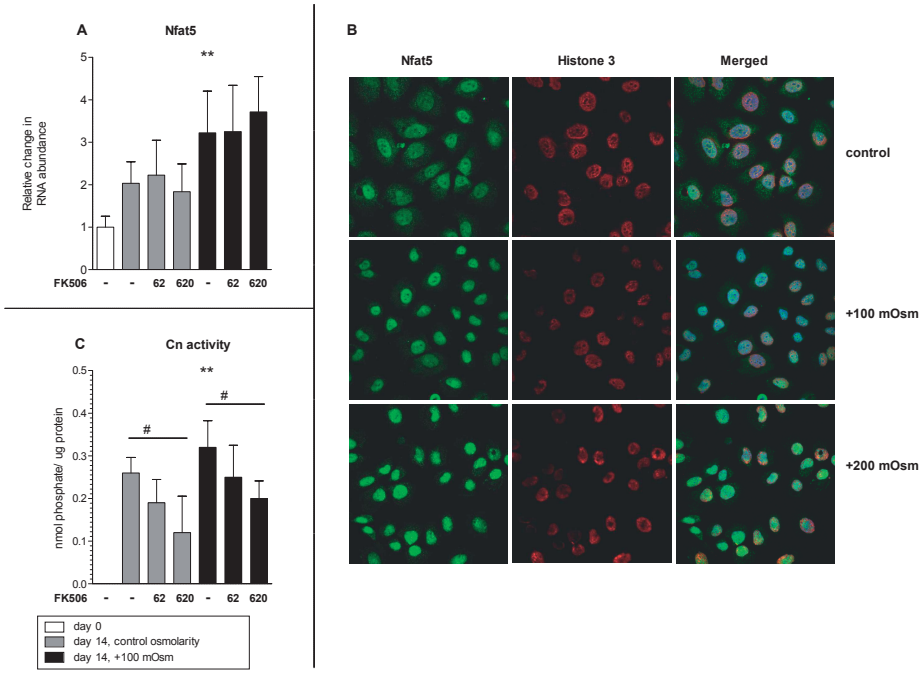

Figure 4. Correlation of Nfat5 expression and calcineurin activity. ATDC5 cells were differentiated under control osmolarity conditions for 14 days (grey bars) or at +100 mOsm from day 7 till day 14 (black bars). In addition, cells were co stimulated with 0, 62 or 620 nM FK506 from day 7. Induction of Nfat5 mRNA was determined by RT-qPCR at day 0 and day 14 (**A**) and calcineurin activity was measured (**C**). Data are means ± standard deviation. ** $p<0.01$ between control and +100 mOsm conditions; # $p<0.05$ FK506 effect compared to the condition without FK506 in the same osmolarity group. To analyse Nfat5 protein expression (**B**) cells were cultured on 8-well culture slides and stimulated with +100 or +200 mOsm medium for 30 minutes.

increase of Nfat5 mRNA expression (figure 4A). From the pattern of green colour in figure 4B it can be appreciated that, under plasma-level osmolarity (control), NFAT5 protein is more or less evenly distributed between cytoplasm and nucleus. At cartilage specific osmolarity (+100 mOsm, figure 4B), the intensity of green coloration accumulates in the nucleus. Further increasing the osmotic stress (+200 mOsm, figure 4B) also increases the NFAT5-specific label intensity in the nucleus. NFAT5 mRNA expression appears to be independent of Cn activity in ATDC5 cells (4C), because it's expression was not altered by FK506 (Figure 4A). At both osmolarity levels, FK506 significantly decreased the calcineurin activity (figure 4C), while it only affected some chondrogenic expression markers at increased osmolarity (Figure 1D,E).

Discussion

FK506 promotes osmolarity-induced terminal differentiation of the murine chondroprogenitor cell line ATDC5, an accepted model for terminal chondrogenic differentiation. The stimulating effect of cartilage physiological osmolarity on the expression of hypertrophy and terminal differentiation markers is a favourable event when aiming at improving endochondral regeneration medicine approaches. In contrast, it is unfavourable for cell-based cartilage regenerative medicine treatments. In this study we investigated the potential effect of FK506 on osmolarity induced chondrogenic and terminal differentiation of ATDC5 cells. We hypothesized that, like in human osteoarthritic chondrocytes (12), FK506 would be able to suppress the osmolarity/NFAT5 induced expression of the unwanted hypertrophic and terminal differentiation markers.

We found that cartilage physiological osmolarity (+100 mOsm; ± 412 mOsm) promoted chondrogenic and hypertrophic differentiation of pre-chondrocytes, without altering differentiation from hypertrophic chondrocytes to chondrocytes expressing mineralization markers. Expression levels of *Sox9* and collagens type 2, 9 and 11 all increased by +100 mOsm (figure 1) as well as the hypertrophic markers *Tgm2, Runx2, Col10, Mmp13, Vegf* and *Bmp2* (figure 2). Mineralization markers *Alpl* and *Spp1* (figure 3) were not further increased after stimulation by osmolarity.

In previous studies, we have shown that the osmolarity induced expression of chondrogenic markers is, at least in part, regulated via Nfat5-mediated transcriptional regulation of Sox9 (10, 11). Upon osmotic stress, transcription of Nfat5 is upregulated in several cell types (26, 27). The increased expression of Nfat5 on

mRNA level (figure 4A) by increased osmolarity support this thought. Like all transcription (co-)factors, NFAT5 needs to be translocated to the nucleus to be active. In medulla cells, upon osmotic shock, NFAT5 rapidly translocates into the nucleus to induce its own transcription (27, 28). The newly synthesised NFAT5 proteins can then amplify the induction of cytoprotective proteins (28). We found that under plasma-level osmolarity conditions, NFAT5 protein is more or less evenly distributed between cytoplasm and nucleus (figure 4B). The additional osmotic trigger of 100 mOsm stimulates transfer of NFAT5 to the nucleus (figure 4B, +100 and +200 mOsm conditions), where it appears to be functionally active as derived from the subsequent induction of its own transcription (figure 4A). NFAT5 mRNA expression appears to be independent of Cn activity in ATDC5 cells (4A).

FK506 seems to exert its effects on chondrogenic marker expression trough another mechanism than increased osmolarity, as neither *Nfat5* nor *Sox9* are significantly regulated by FK506 (figure 4A and 1A). FK506 inhibited Cn activity at both control and cartilage physiological osmolarity (figure 4C), but only improved some chondrogenic differentiation markers at physiological osmolarity (figure 1D,E). This indicates that there is an interaction between FK506 and the osmolarity, which was statistically confirmed for the FK506 effects on *Col9* and *Col11*. These data also indicate that the effect of FK506 on differentiation of progenitor cells might be independent of Cn. This is in line with the study by Nishigaki et al. where FK506 but not CsA induced production of collagen type II and proteoglycan in differentiated ATDC5 cells (8). In that study, the effect of FK506 was antagonized by rapamycin, which competitively binds to FKBP. The authors concluded that FK506 induced chondrogenic differentiation of ATDC5 cells via a Cn independent mechanism after binding to FKBP.

In human osteoarthritic chondrocytes cultured at control osmolarity we found that FK506 promoted chondrogenic marker expression via stimulation of endogenous TGFβ1 production (13). Activated TGFβ induces gene transcription by phosphorylation of Smads (29, 30). In human synovial stromal cells FK506 induced glycosaminoglycan and Col2 production via Smad 1/5/8 and Smad 3 (31), likely by means of endogenous TGFβ induction. The FK506 binding protein, FKBP12, is known to inhibit TGFβ1 receptors (32). FK506 blocks this inhibition and enhances the ligand activity of the TGFβ1-receptor. This mechanism could explain the effects of FK506 on TGFβ signaling and chondrogenic differentiation of ATDC5 cells at cartilage physiological osmolarity. However, it does not explain why FK506 only increased some chondrogenic markers at physiological osmolarity and not at control osmolarity, like it does in human adult chondrocytes. Therefore, it would be

interesting to measure endogenous TGFβ production and Smad phosphorylation in our cultures. For now, this was beyond the scope of this paper, as our aim was to investigate whether FK506 would be able to suppress the osmolarity-induced expression of the unwanted hypertrophic markers.

Unfortunately, at both tonicities, we found that FK506 could not prevent or stimulate hypertrophic and terminal differentiation of progenitor cells at cartilage physiological osmolarity (figure 2 and 3). Nakamura and colleagues (9) observed earlier that FK506 could promote the rate of cellular differentiation from pre-chondrocytes to proliferating and hypertrophic chondrocytes, without altering differentiation from hypertrophic to calcified chondrocytes. In metatarsals cultured with FK506 for 5 days, FK506 predominantly promoted differentiation into proliferating chondrocytes without altering that from proliferating into hypertrophic and calcified chondrocytes. The authors also discovered that this regulation is not related to transactivation of Sox9 or RunX2.

The results of this and of other studies indicate that FK506 promotes early chondrogenic differentiation of progenitor cells cultured at cartilage specific osmolarity, without altering or inhibiting differentiation to hypertrophic and terminally differentiated cells. The latter is in contrast to the inhibiting effect of FK506 on osmolarity-induced hypertrophy in human osteoarthritic chondrocytes (12). We previously found evidence that FK506 may be therapeutically useful in repairing articular cartilage lesions or in joints with osteoarthritis (12, 13), where it can act on human adult chondrocytes. Future studies should investigate whether the effects of FK506 are cell type specific or whether changes in FK506 concentration and/or timing during chondrogenic differentiation of progenitor cells could achieve the same inhibiting effect on cell hypertrophy. This would make FK506 more useful for cartilage tissue engineering approaches using progenitor cells.

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FK506 protects against articular cartilage collagenous extra-cellular matrix degradation

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Abstract

Osteoarthritis (OA) is a non-rheumatologic joint disease characterized by progressive degeneration of the cartilage extra-cellular matrix (ECM), enhanced subchondral bone remodeling, activation of synovial macrophages and osteophyte growth. Inhibition of calcineurin activity through tacrolimus (FK506) in *in vitro* monolayer chondrocytes exerts positive effects on ECM marker expression. This study therefore investigated the effects of FK506 on anabolic and catabolic markers of osteoarthritic chondrocytes in 2D and 3D in vitro cultures, and its therapeutic effects in an in vivo rat model of osteoarthritis.

Effects of high and low doses of FK506 on anabolic (QPCR/histochemistry) and catabolic (QPCR) markers were evaluated in vitro on isolated (2D) and ECM-embedded chondrocytes (explants, 3D pellets). Severe cartilage damage was induced unilaterally in rat knees using papain injections in combination with a moderate running protocol. Twenty rats were treated with FK506 orally and compared to twenty untreated controls. Subchondral cortical and trabecular bone changes (longitudinal microCT) and macrophage activation (SPECT/CT) were measured. Articular cartilage was analyzed ex vivo using contrast enhanced microCT and histology.

FK506 treatment of osteoarthritic chondrocytes in vitro induced anabolic (mainly collagens) and reduced catabolic ECM marker expression. In line with this, FK506 treatment clearly protected ECM integrity in vivo by markedly decreasing subchondral sclerosis, less development of subchondral pores, depletion of synovial macrophage activation and lower osteophyte growth.

FK506 protected cartilage matrix integrity in vitro and in vivo. Additionally, FK506 treatment in vivo reduced osteoarthritis-like responses in different articular joint tissues and thereby makes calcineurin an interesting target for therapeutic intervention of osteoarthritis.

Introduction

FK506 (Tacrolimus, Prograf) is an immunosuppressive drug discovered by Kino et al. in the 1980s (1). Since then, it has been used clinically for an increasing number of immunological disorders. FK506 exerts its therapeutic effects by suppression of T-cell activation, without markedly affecting bone marrow cell differentiation and proliferation (2). Through binding to FK506-binding proteins (FKBPs), FK506 inhibits the activity of ubiquitously expressed calcium/calmodulin dependent calcineurin (Cn). As a consequence, the calcineurin mediated dephosphorylation of transcription factors of the nuclear factor of activated T-cells (NFATs) family (NFAT1-4) is inhibited.

Besides their role in T-cell activation, Cn and NFATs are now also known to play a role in physiological processes in many other cell and tissue types and pathological conditions like cancer, degenerative brain diseases and cardiac hypertrophy (3). FK506 has proven to be useful in reducing inflammation and alleviating symptoms in patients with inflammatory (rheumatoid) arthritis (4, 5). Interestingly, the Cn/NFAT signalling cascade is also reported to play a role in bone remodelling (6) and chondrogenesis (7). FK506 has been shown to induce chondrogenic differentiation of murine chondroprogenitor cells (8). This suggests that patients with non-inflammatory joint diseases, like osteoarthritis (OA), also might benefit from a treatment with Cn inhibitors.

OA is a complex progressive disease and a disturbed balance between anabolic and catabolic activity of chondrocytes is an early pathophysiological event leading to matrix degradation. Progression of OA finally results in severe deterioration of articular cartilage and involves pathological changes throughout the joint, like extensive subchondral bone remodelling (9) and activation of synovial macrophages (10). We reported earlier that FK506 treatment of monolayer cultured osteoarthritic cells enhanced expression of anabolic markers like collagen type II (COL2), but suppressed relevant catabolic, hypertrophy and mineralization markers (11, 12). Another Cn inhibitor, cyclosporine A (CsA) showed similar effects on anabolic and catabolic activity of OA chondrocytes and reduced cartilage damage in a collagenase induced OA mouse model (13). However, this study only measured macroscopical and microscopical cartilage damage ex vivo and did not investigate possible effects of Cn inhibition on other tissues of the joint, like bone and synovium.

Recently, we established a novel rat OA model using a combination of papain injections with a running protocol to induce severe knee joint articular cartilage degradation together with prominent involvement of subchondral bone and synovial macrophages (14). The current study aimed to elucidate the effects of systemic FK506 treatment in this OA animal model. We first characterized whether both low and high concentrations of FK506 modulate anabolic markers in OA chondrocytes in monolayer cultures. Since chondrocytes reside in an extra-cellular matrix *in vivo,* we additionally investigated whether low dose FK506 treatment remains beneficial for extracellular matrix-embedded chondrocytes in *ex vivo* explants and pellet cultures. Finally, we tested modulating effects of FK506 in an animal model for severe cartilage degradation and analyzed articular cartilage (*ex vivo* μCT and histology), subchondral bone (in vivo μCT) and synovial macrophages activation (*in vivo* SPECT/CT) six and twelve weeks after induction of cartilage damage.

Materials and Methods

FK506 effects on osteoarthritic chondrocytes *in vitro*

Human articular cartilage was explanted from macroscopically normal areas of femoral condyles and tibial plateaus of patients (N=9, between 55-82 years old) undergoing total knee replacement surgery for OA (MEC2004-322). Isolation of primary osteoarthritic human articular chondrocytes from cartilage tissue under standard conditions (cytokine-free Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 10% fetal calf serum (FCS), 50 μg/mL gentamycine and 1.5 μg/mL fungizone; all Invitrogen, Paisley, Scotland, UK, adjusted to 380 mOsm by adding sterile sodium chloride) and monolayer experiments were performed as described earlier (11). In short, passage 1 cells were seeded in 2D monolayer, stimulated with 0, 62 or 620 nM FK506 after 24 hours and harvested for RNA analysis (quantitative RT-PCR) six days later. Experiments were performed at least in technical duplicates from four OA donors. In addition to 2D cultures, passage 2 cells from four OA donors were cultured as 3D pellets ($2x10^5$ cells/pellet) for 21 days in medium (380 mOsm) with or without addition of 62 nM FK506 (11).

To investigate the effects of FK506 on OA chondrocytes embedded in their extracellular matrix, six mm diameter full-thickness explants from femoral condyles and tibial plateaus of five OA donors were cultured as described before (11) and cultured in medium (380 mOsm) with or without 62 nM FK506 for 7 days.

RNA and protein analysis by RT-QPCR and histology

RNA from monolayer and explant cultures was extracted, purified and quantified, and cDNA was synthesized and quantified by RT-QPCR reactions as described earlier (12, 15). RNA abundance was normalized to an index of the three most stable reference genes (*GAPDH, HPRT1, 18sRNA or UBC)* replicate values were averaged per condition per patient and gene expression was calculated as fold change of control condition (0 nM FK506) (11). Primer sequences for *COL2, ACAN, MMP1, MMP13, MMP13, ADAMTS4 and ADAMTS5* were adopted from Uitterlinden et al (15), for *COL1* from Das et al (16), and for *VCAN/CSPG2* from Martin et al (17). To quantify expression of *COL9 and COL11*, the following primers were tested for similar amplification efficiency and specificity (16) , and were used as respectively 20 µl Taqman and SYBR® Green I reactions: HsCOL9A1_F GCAGCTCATGGCAAGTTTCTCT, COL9A1_R GCTTTGCTGTGCTGGGAAAA and COL9A1_FAM TGAAGTTCAAATGGAACAGAAACTTGAGGATTATCTG; HsCOL11a1_Fw AGGAGAGTTGAGAATTGGGAATC, COL11A1_Rv TGGTGATCAGAATCAGAAGTTCG.

The expression data are presented as 2-Ct values based on publication by Livak & Schmittgen (18). The cDNA abundances of each gene of interest were normalized to an index of three stable expressed reference genes to generate a normalized, so-called 2-Ct, value. Replicate values were averaged per condition, per patient, and finally expressed as fold change difference relative to the control condition (i.e. without FK506 treatment) and representing a 2-Ct value. The *COL2/COL1* ratio, like the *VCAN/CSPG2* ratio, relates the expression levels of both genes to one another. Relative higher *COL2* expression, as compared to *COL1*, is indicative of a relatively better preserved chondrocyte-specific gene expression. This also holds for *VCAN/CSPG2* ratios, and was used earlier as a measure of de-differentiation of chondrocytes (19, 20).

(Immuno)histochemical staining for COL2 and GAG on 3D pellet cultures was performed as described before (21). Staining intensities of pellets were quantified using ImageJ 1.42 software (http://rsb.info.nih.gov/ij/download.html).

FK506 therapeutic effects in a rat model for severe osteoarthritis

Forty 16-week-old male Wistar rats (Charles River Netherlands BV, Maastricht, the Netherlands) were housed in the animal facility of the Erasmus Medical Centre, with a 12-h light-dark regimen, at 21°C during the experimental period, and received standard food pellets and water ad libitum. OA-like articular destruction was induced in all animals, which were then divided over two groups: twenty rats served

as untreated controls and twenty rats were treated during the experiment with FK506. FK506 treated animals received FK506 suspension (1 mg/kg) (22) through oral probing, five days a week, not on weekends.

Severe cartilage damage was induced using intra-articular papain injections in the left knee joints combined with exposure to a moderate exercise protocol as described before (14). In short, all animals received three intra-articular injection that consisted of 15µl 4% (w/v) papain solution (type IV, double crystallized, 15 units/mg, Sigma-Aldrich, St. Louis, MO, USA) with 15µl 0.03M L-cystein (Sigma-Aldrich) (23). Their contralateral knee joint served as an internal healthy control. All rats were forced to run on a motorized rodent treadmill (LE-8700; Panlab Harvard Apparatus, Barcelona, Spain) for six weeks covering a total distance of 15km (14). During the study all animals were longitudinally monitored at baseline, 6 weeks and 12 weeks with µCT to measure subchondral bone changes (24). At six and twelve weeks, ten rats in both groups were selected for a full analysis sequence. This sequence consisted of SPECT/CT to quantify *in vivo* macrophage activation (25), and *ex vivo* EPIC-µCT and histology to measure cartilage quality (26). The details of these procedures were described earlier (14). The animal ethic committee of the Erasmus Medical Center, Rotterdam, the Netherlands, approved all conducted procedures. A detailed planning scheme of all groups and conducted tests is given in figure 1.

Subchondral bone measurements on µCT scans

Both knees of all animals were µCT scanned under isoflurane anaesthesia, using a Skyscan 1176 in vivo µCT scanner (Skyscan, Kontich, Belgium). Ten minutes of scan time was required per knee at an isotropic voxelsize of 18µm, at a voltage of 65kV, a current of 385mA, field of view of 35mm, using a 1.0mm aluminium filter, over 198° with a 0.5 degree rotation step, and a 270 msec exposure time. All datasets were segmented with a local threshold algorithm (27). Cortical and trabecular bone were automatically separated using in-house software (28). Using Skyscan software, both subchondral plate thickness (Sb. Pl. Th. in µm) and subchondral plate porosity (Sb. Pl. Por. in mm^3) of the medial and lateral compartment of the tibial plateau were measured (24). In the tibial epiphysis, the trabecular thickness (Tb. Th. in µm) and trabecular bone volume fraction (BV/TV), representing the ratio of trabecular bone volume (BV, in mm^3) to endocortical tissue volume (TV, in mm^3) were measured. Ectopic bone formation (mm^3) was also quantified as a measure for osteophyte growth in these longitudinal µCT scans.

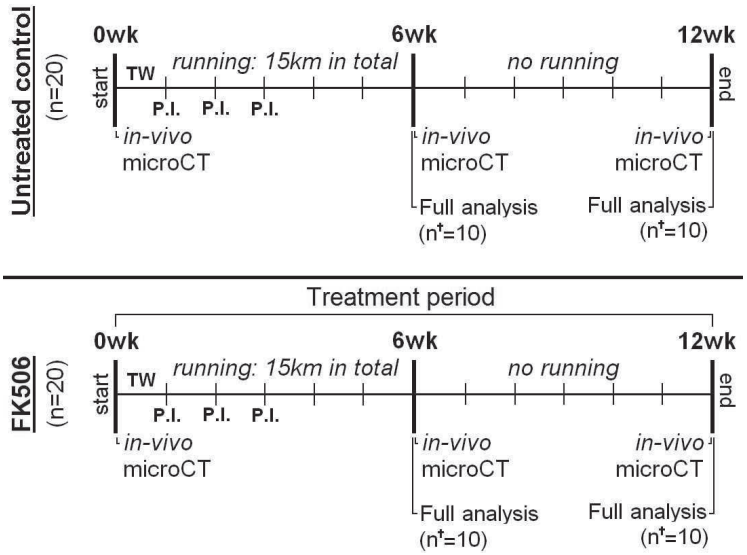

Figure 1. Experiment design indicating analytical time points and methods for each experimental group. Forty 16-week-old male Wistar rats were injected with three papain intra-articular injections (P.I.) and forced to run 15km on a motorized treadmill. Animals were divided over two different groups: an untreated osteoarthritis (OA) group (n=20) and a FK506 treated group (n=20). During the experiment three longitudinal μCT scans were made to measure subchondral bone changes (24). At six and twelve weeks a full analysis sequence was done in ten animals per group (n†=10), consisting of: determination of activated macrophages using SPECT/CT in-vivo (25); and cartilage analysis with equilibrium partitioning of an ionic contrast agent using (EPIC-)μCT (26) and histology ex-vivo.

Determination of activated macrophages by SPECT/CT using ^{111}In-EC0800

Activated macrophages express the folate receptor-β allowing monitoring macrophages *in-vivo* using folate-based radiotracers (29, 30). Phosphate buffered saline (PBS, pH 6.5) DOTA-Bz-folate (EC0800, kindly provided by Endocyte Inc., West Lafayette, USA) (31) was labelled with $^{111}InCl_3$ (Covedien, Petten, The Netherlands) as described previously (14). Quality control was performed with ITLC-SG and revealed a radiochemical yield of ~91% at a specific activity of 50 MBq/μg. ^{111}In-EC0800 (55MBq) was administered via the tail vein twenty hours prior to scanning. SPECT/CT scans were performed with a 4-head multiplex multi-pinhole small animal SPECT/CT camera (NanoSPECT/CT ™, Bioscan Inc., Washington DC, USA). All knee joints were scanned with both helical μCT (acquisition time 5min) and SPECT (acquisition time 30min). All scans were analyzed using InVivoScope processing software (Bioscan Inc.). To reduce inter-individual variation, the absolute difference in measured radioactivity (kBq/mm^3) of the OA knee joint compared to their internal control joint was calculated. This absolute difference was used when comparing means of untreated animals with FK506 treated animals.

Cartilage evaluation with contrast enhanced μCT and histology

Equilibrium partitioning of a contrast agent using μCT (EPIC-μCT) has a strong correlation with cartilage sulphated-glycosaminoglycan (sGAG) content (26). Animals were euthanized directly after the last SPECT/CT scan and both knee joints were harvested for EPIC-μCT analysis. All specimens were incubated in 40% solution of ioxaglate (Hexabrix320, Mallinckrodt, Hazelwood, MO, USA) for 24 hours at room temperature (32). EPIC-μCT was performed on the 1176 in vivo μCT scanner (Skyscan), using the following scan settings: isotropic voxel size of 18μm, a voltage of 65kV, a current of 385mA, field of view 35mm, a 0.5 mm aluminium filter, 198° with a 0.5 degree rotation step, and a 235 msec exposure time. In all EPIC-μCT datasets, X-ray attenuation (arbitrary gray values inversely related to sGAG content) and cartilage thickness (μm) was calculated for cartilage of the medial and lateral plateau of the tibia (14).

After EPIC-μCT, the separated parts of the knee joints were fixed in 3.7% phosphate buffered formaldehyde, decalcified with formic acid and embedded in paraffin. Sagittal sections were made at 300 μm intervals and stained with Safranin-O with a fast green counterstain to image the distribution of the GAGs. Sections were stained all at once, to minimize artifacts between different samples.

Statistical analysis

Statistical analysis of vitro studies was performed as described before (11). Briefly, replicate raw expression data of multiple donors was tested for the effect of FK506 using Linear Mixed Model regression and 'donor' was incorporated as a random effect to correct for basal differences in expression between donors (SPSS Inc., Chicago, USA).

For the in vivo study, differences between means of OA induced and healthy knee joints within the same animal were tested using paired t-tests at each time point for all outcome parameters (GraphPad Software, San Diego, California, USA). When comparing differences between means of untreated animals and FK506 treated animals, an unpaired t-test was used at each time point for all outcome parameters (GraphPad Software). Longitudinal data from in vivo μCT were additionally analyzed using generalized estimating equations (SPSS). For all tests, p values ≤ 0.05 were considered significant.

Results

In vitro effects of FK506 on human osteoarthritic chondrocytes

Inhibition of calcineurin activity by FK506 in monolayer cultured passage 1 osteoarthritic chondrocytes increased expressions of cartilage specific collagens. Both low and high concentrations of FK506 positively stimulated *COL2/COL1* ratio (62 nM FK506 by ± 2-fold, p = 0.067; 620 nM by ± 3-fold, p = 0.001) and *COL9* expression (62 nM FK506 by ± 1.8-fold, p= 0.037), while no effects were found on the *ACAN/VCAN* ratio or *COL11* expression (figure 2A). In cartilage explants, the osteoarthritic chondrocytes are embedded in a matrix, which might limit chondrocyte exposure to FK506. Despite the large standard deviations in the explants cultures, we found clear matrix-protective trends after FK506 treatment. A low dose of FK506 was enough to induce a similar trend of increased anabolic marker expression in ex-

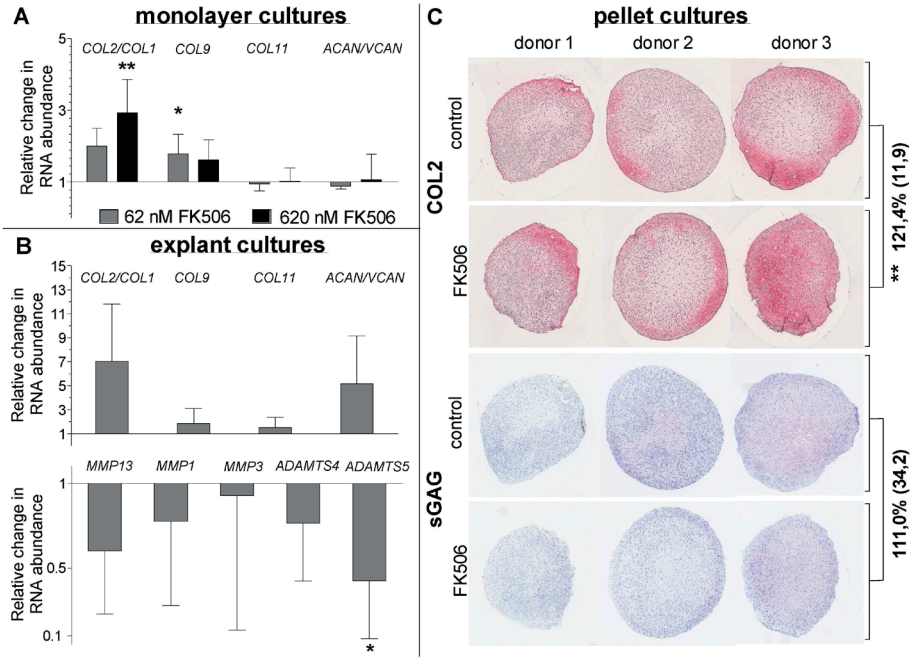

Figure 2. RNA abundance of anabolic and catabolic markers was determined in samples from human osteoarthritic monolayer **(A)** and explant **(B)** cultures. Relative changes by FK506 (grey and black bars) as compared to control (no FK506) are shown, each bar represents the fold-change compared to the control condition. Error bars indicate standard deviations. Representative images of 21-day 3D pellet cultures **(C)** show collagen type II immunostaining (on top, in red) and sGAG staining (thionin; on bottom, in blue) of pellets cultured with or without 62 nM FK506. Staining intensity in FK506 pellets is expressed as mean percentage (standard deviation) of that in control pellets *: p<0.05, **: p<0.01

plants as seen in monolayer cultured chondrocytes (figure 2B). Moreover, the FK506 induced changes in chondrogenic marker expression seem to be even higher in the explants. In line with our previous work on monolayer chondrocytes (11), FK506 also reduced the expression of the catabolic MMPs and important aggrecanases in the explant cultures (figure 2B).

The FK506 induced increase in collagen expression was confirmed by immunohistology on 3D pellets cultures of osteoarthritic chondrocytes. FK506 clearly increased COL2 protein expression (to 121.4% of control, p = 0.009) in chondrocyte pellet cultures, while no clear effect was seen on GAG staining (figure 2C).

In vivo effects of FK506 treatment

Bodyweight of all untreated rats at baseline was 416.4g (411.3 – 421.5g), during six weeks of treadmill running this decreased non-significantly to a mean weight of 408.3g (398.2 – 418.3g). During subsequent six weeks of rest, all rats increased in their mean bodyweight to 485.5g (473.0 – 498.0g). FK506 treated animals (mean weight at baseline was 413.6g; 409.4 – 417.8g) also did not increase in bodyweight during induction of OA-like articular destruction (mean weight after six weeks was 418.5g; 412.9 – 424.1g). After twelve weeks their mean bodyweight was 507.1g (498.8 – 515.4g), which was significantly higher compared to untreated controls (p = 0.004) (figure 3). During the course of the experiment, none of the animals showed signs of FK506-induced cytotoxicity, like weight or hair loss.

Osteoarthritic changes of articular cartilage

Intra-articular papain injections combined with moderate exercise in untreated controls induced severe sGAG depletion from both medial and lateral cartilage

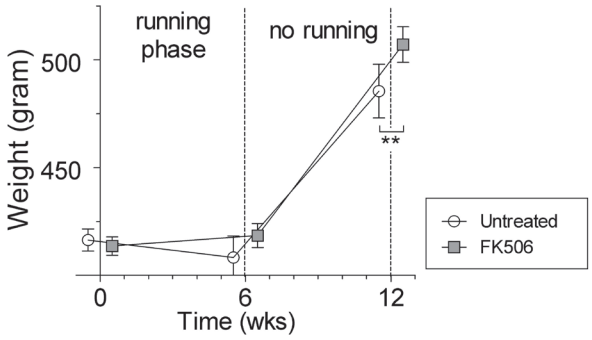

Figure 3. Increase in rat bodyweight (gram) during the experiment of OA control (white circles) and FK506 treated (gray squares) animals. **: p<0.01, error bars indicate 95% confidence intervals.

compartments of the tibia plateau. This sGAG depleted state persisted throughout the experiment (figure 4A-B). After the running protocol at six weeks, cartilage of the medial compartment was slightly reduced in thickness (figure 4C). Lateral cartilage thickness was severely degraded (figure 4D) and resulted in almost completely denuded subchondral bone (figure 4E). During subsequent six weeks of rest medial cartilage continued to degrade, in the lateral compartment an ongoing decline in cartilage thickness was absent (figure 4C). Representative medial and lateral cartilage images from safranin-O stained histology from untreated controls at six and twelve weeks are shown in figure 5.

Compared to untreated controls, FK506 treated animals had similar sGAG depleted cartilage in medial and lateral compartments of the tibia plateau (figure 4A-B). Lateral cartilage was reduced in thickness to similar extent as untreated controls (figure 4D). However, medial cartilage showed a trend towards thicker ECM compared to untreated control at six weeks, although this was not significant

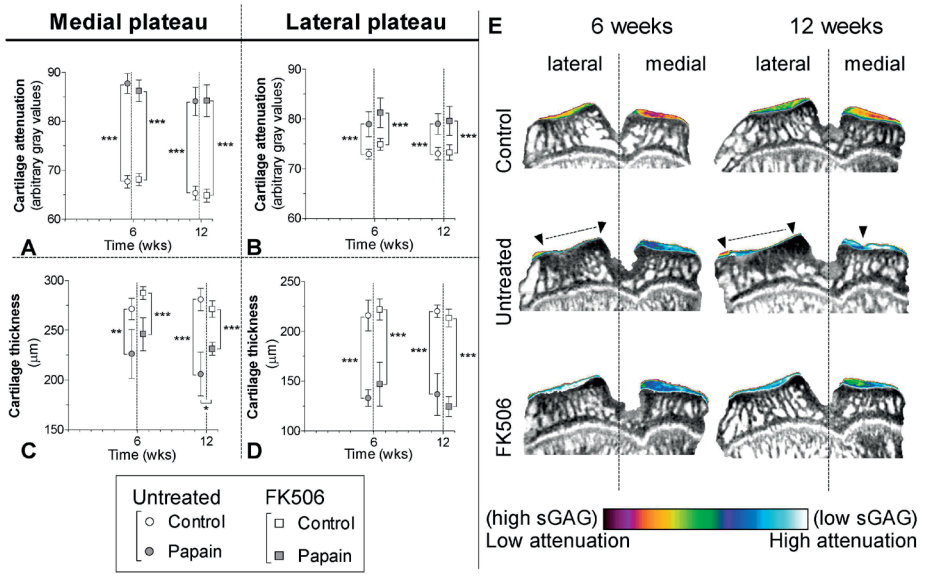

Figure 4. Cartilage quality and quantity was determined from untreated OA (circles) and FK506 treated (squares) rats with equilibrium partitioning of an ionic contrast agent using (EPIC-)µCT (**A-D**). The amount of sulphated glycosaminoglycans (sGAG) (arbitrary gray values; **A,B**) and cartilage thickness (µm; **C,D**) were measured of medial **(A,C)** and lateral (**B,D)** cartilage compartments of the tibia plateau harvested from healthy joints (blank boxes) and OA induced joints (gray boxes). Attenuation values from EPIC-µCT scans are inversely related to the sGAG content, meaning that a high attenuation corresponds to low sGAG content. Coronal images from EPIC-µCT scans of the tibia plateau show the amount of cartilage (erosions indicated with ▼ and dashed lines) and sGAG content (displayed in color). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, error bars indicate 95% confidence intervals.

(p = 0.15). In contrast to the progressive degradation of medial cartilage as seen in untreated controls at twelve weeks, medial cartilage thickness of FK506 treated animals remained constant and was significantly thicker at twelve weeks compared to untreated controls (p = 0.02) (figure 4C).

Although lateral cartilage did not differ significantly in sGAG content or thickness between untreated and FK506 treated rats, we did observe a small difference between both groups. In four FK506 treated rats, but in none of the untreated rats, there were small and focal regions of lateral tibia cartilage that showed intact but totally sGAG depleted ECM (figure 4E). A difference that was also found on safranin-O stained histology sections as shown in figure 5.

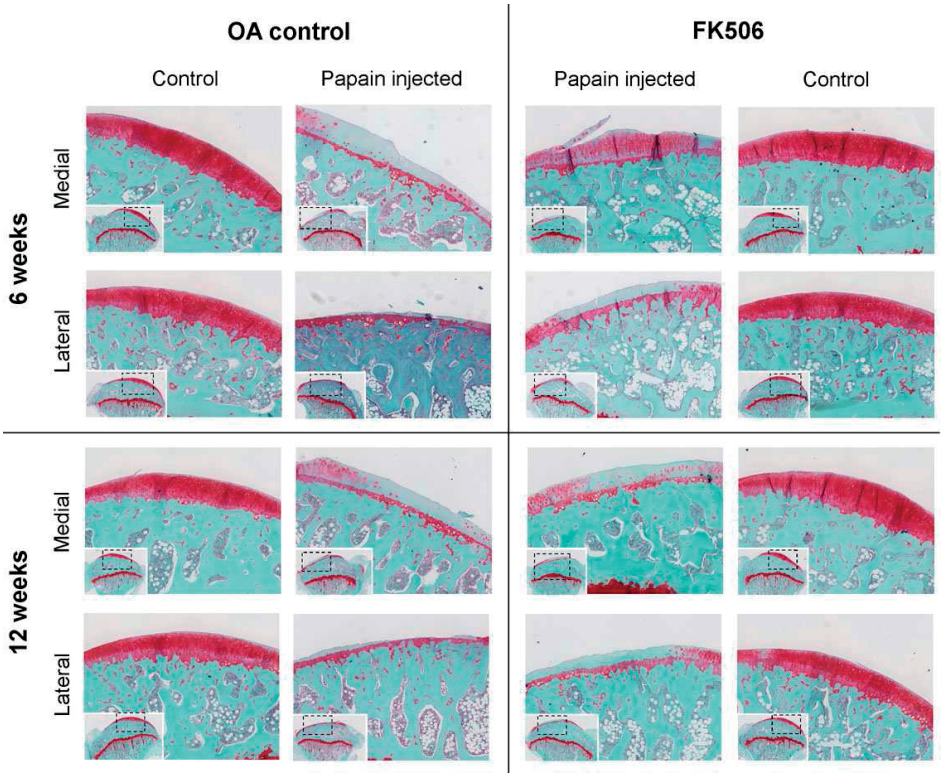

Figure 5. Safranin-O stained histology sections of medial and lateral tibial plateau cartilage after six weeks and twelve weeks of follow up. Medial cartilage of untreated OA knee joints with depleted sulphated-glycosaminoglycan at six weeks and twelve weeks, and only mildly degraded extra-cellular matrix (ECM). Lateral cartilage ECM was almost totally eroded, only the calcified cartilage layer remained present and showed ECM denudation of cartilage ECM. Much less ECM degradation occurred in FK506 treated animals. The lateral compartment cartilage was severely eroded, however 4/10 rats showed focal regions with complete sGAG depleted but partially intact ECM.

Subchondral bone changes

Subchondral bone plate thickness of medial tibia compartment in untreated controls and FK506 treated rats was slightly reduced after six and twelve weeks of follow up (figure 6A), but did not differ between both groups (p = 0.83). Medial plate porosity did not increase in both experimental groups throughout the experiment (figure 6B). Lateral compartment subchondral bone thickness of untreated OA joints was clearly increased compared to their healthy control joint at six weeks (p < 0.0001), and there was also more subchondral plate porosity (p = 0.02) (figure

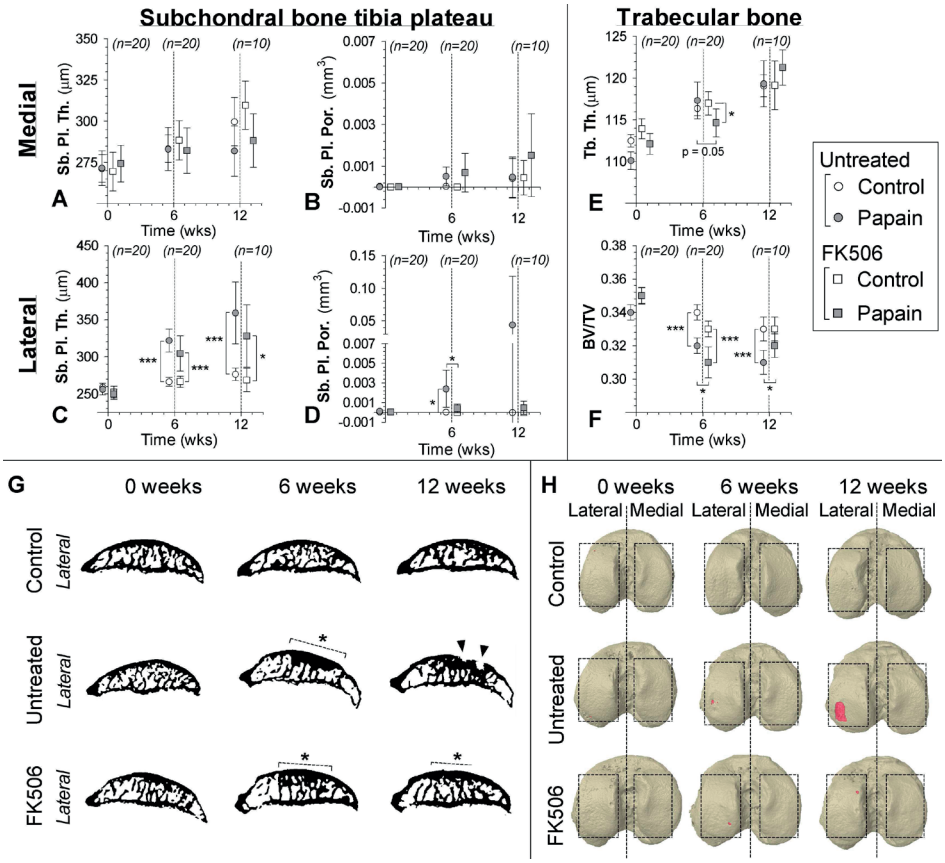

Figure 6. Subchondral bone changes analyzed with longitudinal in vivo µCT in untreated OA (circles) and FK506 treated (squares) animals. Subchondral plate thickness (Sb. Pl. Th.; **A, C)** and porosity (Sb. Pl. Por.; **B, D)** were measured in the medial (**A,B**) and lateral (**C,D**) compartment of the tibial epiphysis. Changes in trabecular thickness (Tb. Th.; **E**) and trabecular bone volume fraction (BV/TV; **F)** were measured in tibial epiphyseal bone marrow. Representative sagittal images from binary µCT scans **(G)** show pore development (indicated with q) and development of subchondral sclerosis (indicated with dashed line and *). Three-dimensional top views of the tibial plateau at different time points **(H)** show subchondral pore (red color) development. *: p<0.05, **: p<0.01, ***: p<0.001, error bars indicate 95% confidence intervals.

6C,G,H). Subchondral plate thickness further increased during subsequent six weeks of rest ($p < 0.0001$). Plate porosity also seemed to increase further, but there was no significant difference compared to internal healthy control joints. FK506 treated animals also had a thicker subchondral bone plate at six ($p < 0.0001$) and twelve ($p < 0.0001$) weeks compared to their internal healthy control joints. When longitudinal subchondral bone changes in OA joints of both groups were analyzed with generalized estimating equations, FK506 treated rats had thinner lateral subchondral bone plates compared to untreated controls ($p = 0.03$) (figure 6C,G). FK506 rats did not develop subchondral plate porosity. This was significantly lower

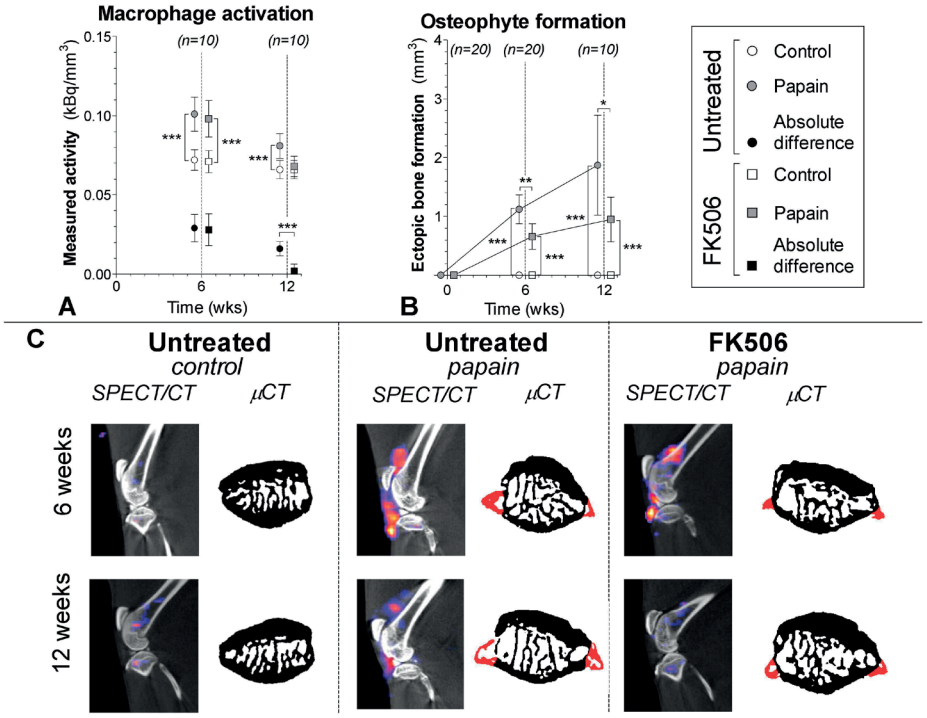

Figure 7. Macrophage activation determined in untreated OA animals (circles) and FK506 treated animals (squares) by injection of 111In-EC0800 using SPECT/CT. **A**: Quantified radioactivity in healthy joints (blank boxes) and OA joints (gray boxes) normalized to the size of the analyzed cylindrical region of interest (kBq/mm3). Absolute differences per animal were calculated (kBq/mm3) to reduce inter-individual variation (black boxes). A high radioactivity is related to more macrophage activation. **B**: Ectopic bone formation (mm3) as a measure for osteophyte development was quantified on longitudinal bone µCT scans. **C:** Sagittal SPECT/CT images of knee joints from representative animals. CT images shown in black and white were used for anatomical reference, the SPECT images are shown in color. Transaxial images from patellar bone extracted from binary µCT images show ectopic bone formation (red color). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, error bars indicate 95% confidence intervals.

at six weeks (p = 0.02), but not at twelve weeks anymore (figure 6D,H). After six weeks of treadmill exercise-mediated trabecular bone thickness (p = 0.05) and BV/TV (p = 0.03) was lower in FK506 treated animals compared to untreated controls (figure 6E,F). Reduced trabecular thickness normalized during subsequent six weeks of rest, while the BV/TV ratio increased compared to untreated controls (p = 0.02).

During six weeks of moderate running, FK506 treated animals formed less ectopic bone formation compared to untreated animals (p = 0.007) (figure 7B,C). This difference in ectopic bone formation between FK506 treated animals and untreated controls was still measured after the subsequent six weeks of rest (p = 0.04) (figure 7B,C).

Macrophage activation and osteophytes

Each animal received 54 ± 2 MBq of ^{111}In-EC0800 under isoflurane anaesthesia. There were no significant differences of injected activity between experimental groups. After completion of the running protocol, both untreated and FK506 treated rats revealed similarly increased radioactive uptake in their papain injected knee joints compared to their internal healthy control joints (figure 7A,C). After six subsequent weeks of rest, radioactive uptake in OA induced joints of FK506 treated animals dropped to control levels. The absolute difference in radioactive uptake between OA induced and healthy control joints in FK506 treated animals was lower compared to the absolute differences measured in untreated controls (figure 7A,C).

Discussion

Osteoarthritis is characterized by a loss of cartilage matrix, because chondrocytes cannot maintain tissue homeostasis due to a disturbed balance between anabolic and catabolic activities. Inhibiting calcineurin activity with immunosuppressive agents like cyclosporin A (13) or FK506 (11, 12) increases the anabolic, while suppressing the catabolic, activity of osteoarthritic chondrocytes. In this study, we found that both high and low concentrations of FK506 improved the COL2/COL1 ratio and COL9 expression in monolayer cultured human osteoarthritic chondrocytes (figure 2A). Then, in 3D chondrocyte pellet cultures, FK506 clearly increased COL2 content, while no effect was seen on sGAG staining (figure 2C). These data indicate that calcineurin inhibition through FK506 may protect the structural integrity of the ECM. Next, we studied the effects of low dose FK506 treatment in cartilage

explants, in which the chondrocytes are still embedded in their native extracellular matrix. The explants were harvested from macroscopically 'healthy' cartilage areas of the degenerated side. However, on microscopic level there might be still big differences in grade of degeneration between explants of the same donor. To limit the effects of these differences, explants were first pooled before assigning them to a certain culture condition. Despite the large standard deviations, we found a clear trend towards stimulated anabolic but reduced catabolic activities after FK506 treatment (figure 2B).

Finally, we evaluated whether FK506 also exerts similar favorable effects in a severe OA *in vivo* model with a different response in medial and lateral compartments of tibia plateau cartilage (14). Six weeks of OA-like damage induction severely erodes lateral compartment cartilage and results in complete denudation of subchondral bone. Medial cartilage becomes sGAG-depleted with a slightly degraded ECM, a process that continued progressively during the course of the experiment (figure 4 and 5). FK506 treatment *in vivo* did not increase sGAG levels nor did it protect against sGAG loss (figure 4A-B). However, it did protect against structural matrix degradation (figure 4C), which was in line with our *in vitro* results (figure 2). Loss of lateral cartilage matrix could not be prevented with FK506 treatment (figure 4D). Longitudinal μCT analysis showed reduced sclerotic bone formation in the lateral compartment of FK506 treated animals (figure 6C). Previous experiments with this severe OA model suggest that subchondral sclerosis might develop when cartilage is completely lost and subchondral bone is denuded (14). In some FK506 treated animals we found focal regions of cartilage on the lateral tibia plateau that showed totally sGAG depleted but partially intact ECM. This suggest, that FK506 might have delayed lateral cartilage matrix degradation and thus reduced formation of subchondral sclerosis. However, calcineurin inhibition is also known to modulate bone turnover (33) and therefore may have reduced sclerosis through direct modulation of osteoclast and osteoblast activity. CsA and FK506 have both been described to induce osteopenia through anti-anabolic effects on osteoblastic cells (34) and to reduce bone formation through inhibition of osteoblast differentiation (35, 36). This could be another explanation for the reduced development of subchondral sclerosis in the lateral compartment, but has to be further investigated.

Another cell type that is modulated by FK506 are macrophages (37). We determined activated macrophages using ^{111}In-EC0800 and quantitative SPECT/CT (figure 7A). During OA progression, macrophages become activated (25) and their TGFβ/BMP-2 production has previously been related to osteophyte development (38, 39). In our *in vivo* experiment, animals developed clear osteophytes at the

margins of the patella (figure 7B). FK506 treatment reduced osteophyte development while the total amount of activated macrophages was equal after six weeks of follow up. This may suggest that FK506 treatment limits cytokine production by activated synovial macrophages and previous reports on calcineurin inhibition in macrophages showed reduced cytokine production (40-42). FK506 may initially activate Toll-like receptors (TLR) pathways in activated macrophages, which can enhance NFκβ activity (42) and stimulate expression of cytokines, like TNFα, IL1α, IL1β, IL12 and iNOS (40). However, prolonged exposure to calcineurin inhibitors has been shown to also secondarily inactivate this TLR induced pro-inflammatory cytokine expression by negative feedback loops (41). As such, continuous FK506 exposure may eventually suppress NFκβ pathways, but activate caspases 3 and 9 to enhance macrophage apoptosis (42). Possibly, FK506 induced macrophage apoptosis may explain why radioactive folate uptake in our experiments was restored to levels comparable to healthy control joints (figure 7A).

Osteoclasts are large multinucleated cells of the monocyte-macrophage hematopoietic lineage and are also influenced by FK506. During osteoclastogenesis macrophage colonoy-stimulating factor (M-CSF) and receptor activator of nuclear factor-κβ ligand (RANKL) stimulate precursor cells to acquire osteoclast characteristics (43). NFATc1 is an essential terminal differentiation factor of osteoclastogenesis and can be blocked in a dose-dependent fashion using CsA or FK506 (44). CsA and FK506 treatment suppress RANKL stimulated osteoclastogenesis (45-47), and especially inhibits late stages of the osteoclast life cycle (48). By this mechanism Cn inhibition can diminish the activity of mature osteoclasts and reduce bone resorption (49-51). FK506 mediated suppression of osteoclast maturation and subsequently hindered subchondral bone resorption may therefore explain why less subchondral pores were measured in FK506 animals.

Systemic FK506 treatment is known to induce toxic side effects (52). Throughout the experiment our animals gained weight (figure 3), and FK506 animals increased more in weight from 6 to 12 weeks of follow-up. However, this result could not clearly be related to side-effect of FK506, which usually results in a loss of weight. At the end of the twelve week experiment liver function (AST, ALT, alkaline phosphatase) and kidney function (creatinin and urea) were normal (figure 8A). Liver and kidney histology gave no indication that FK506 induced liver or kidney fibrosis (figure 8B-C). Despite these promising findings, systemic FK506 treatment cannot be translated towards clinical OA care directly. FK506 induced side effects are well described in patients after long term use and is unacceptable for a therapeutic strategy in human OA patients. To reduce toxicity, local intra-articular treatment

may be used, but repetitive intra-articular injections also increase the risk of iatrogenically arthritis should be avoided. FK506-coupled biodegradable delivery systems might be able to prolong intra-articular FK506 exposure and sustain long term therapeutic action (53), hopefully without systemic adverse effects.

A

	Laboratory tests on blood samples	Untreated OA control	FK506	p value
Liver	AST	54.13 ± 10.30	78.22 ± 61.08	p = 0.22
	ALT	86.75 ± 27.32	218.11 ± 277.30	p = 0.16
	Alkaline phsophatase	91.25 ± 14.08	87.78 ± 15.21	p = 0.71
	Albumin	35.88 ± 1.46	34.56 ± 1.94	p = 0.69
Kidney	Creatinin	16.00 ± 1.60	20.33 ± 4.18	p = 0.11
	Urea	5.69 ± 0.84	7.01 ± 0.89	p = 0.11

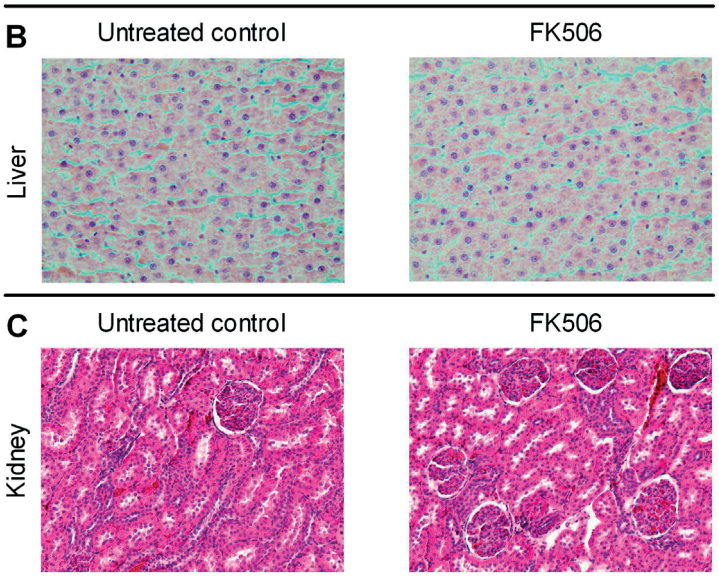

Figure 8. Toxicity analysis. Blood samples did not differ between experimental groups and gave no indication of liver or kidney toxicity **(A)**. Liver **(B)** and kidney **(C)** histology showed no indication of FK506 induced toxicity.

Conclusion

Inhibition of calcineurin activity with FK506 stimulated anabolic activity, while reducing catabolic productivity of osteoarthritic human chondrocytes. Systemic treatment with FK506 in a rat model for severe osteoarthritis also protected against

cartilage extra-cellular matrix degradation. Additionally, there was also less development of subchondral sclerosis, macrophage activation and osteophyte formation. Altogether, our data suggests that calcineurin inhibition with FK506 proves to be a promising candidate for therapeutic management of osteoarthritis.

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General discussion

Hyaline (articular) cartilage enables us to walk, dance and run a marathon without friction between our articulating bones, providing us quality of life. However, when damaged it fails to repair itself properly. Because of the high economic burden of traumatic or degenerative articular cartilage defects, the search for the ideal cartilage regeneration technique continues. Improving the quality of the cartilage formed after cell transplantation for example by optimizing the *in vitro* culture conditions during the expansion phase, is therefore of great interest. However, the development of a drug inducing hyaline cartilage repair in patients with osteoarthritis or a traumatic defect is of course preferable and still desirable.

The major aims of the research reported in this dissertation were to find new potential targets or tools for improving cartilage matrix synthesis by chondrocytes, either *in vitro* for cell transplantation techniques or *in vivo* toward a treatment for osteoarthritis. As potential targets, the calcineurin-dependent NFAT1-4 pathway and the osmolarity-dependent NFAT5 pathway were studied.

Main results and interpretation

Calcineurin as potential target to improve cartilage matrix synthesis by chondrocytes

In vitro chondrocyte expansion is required for several cell-based approaches for the repair of chondral lesions. During expansion, the chondrocytes loose their specific phenotype and dedifferentiate into more fibroblast-like cells. As a result the expression and synthesis of the cartilage specific collagen type II (COL2) decreases and that of collagen type I (COL1) increases, resulting in an inferior quality of the extracellular matrix (1-3). In **chapter 2** we identified Calcineurin (Cn) as potential target to improve chondrocytic phenotype of culture-expanded adult osteoarthritic chondrocytes. Cn activity increased during chondrocyte dedifferentiation and decreased during redifferentiation by TGFβ. The question was if Cn inhibition could stimulate the redifferentiation process. We showed that this was indeed the case. FK506-induced inhibition of Cn activity increased the gene expression of chondrocytic markers COL2, aggrecan (AGC) and SOX9, indicating that the osteoarthritic cells became more chondrogenic. This role of Cn and the effects of Cn inhibition on human adult osteoarthritic cells are consistent with other publications. Yoo et al. also described that Cn is highly expressed in chondrocytes of OA patients and that inhibition of Cn by Cyclosporin A (CsA) decreased the interleukin-1β (IL1β) stimulated production of nitric oxide (NO) and matrix metalloproteinases (MMP) 1 and 3 (4). In addition, in a mouse model for OA cyclosporin A decreased the

extent and severity of cartilage damage (4). Nakamura et al. described increased production of Col2 and Col10 in mouse costal chondrocytes after treatment with FK506 (5).

The effects of FK506 and CsA on chondrocytic marker expression has more extensively been studied in other cell types, like mesenchymal stem cells, chondroprogenitor cell lines (ATDC5, RCJ3.1C5.18) and human synovial stromal cells. FK506 induced expression of Col2, Col10 and proteoglycans in the chondroprogenitor ATDC5 cell line and in human synovial stromal cells (5-7).

However, the mechanism by which Cn inhibition leads to increased chondrocytic marker expression and the role of the NFAT1-4 transcription factors in this process remains mostly unclarified. Cn inhibitors are known to induce TGFβ signaling in renal cells, vascular smooth muscle cells and lymphocytic B cells (8-11). In **chapter 2** we demonstrated that increased endogenous TGFβ1 production was responsible for the effect of FK506 on COL2 expression in osteoarthritic chondrocytes. TGFβ is a secreted protein that controls proliferation, cellular differentiation, and other functions in many cell types. The TGFβ family is part of a superfamily, which includes, besides TGFβ, activins and bone morphogenetic proteins (BMPs). In mammalian cells there are three isotypes of TGFβ: β1, β2 and β3. Activated TGFβ induces gene transcription by phosphorylation of intracellular Smads. TGFβ has been shown to be beneficial for cartilage as it stimulates chondrocytes to elevate proteoglycan and COL2 production (12-14). In human synovial stromal cells FK506 induced glycosaminoglycan and COL2 production via Smad 1/5/8 and Smad 3 (7). There are different mechanisms reported by which FK506 and/or CsA induce endogenous TGFβ. The FK506 binding protein, FKBP12, is known to inhibit TGFβ1-receptors (15). FK506 can block this inhibition and enhances the ligand activity of the TGFβ1-receptor. This mechanism could explain the effects of FK506 on TGFβ signaling, but not the effect of CsA. An other possible mechanism is regulation of the TGFβ1 promotor activity by the Cn-dependent NFAT transcription factors (16).

The role of NFAT1-4 in regulating chondrogenic marker expression remains insufficiently clear. Tomita et al. showed that Cn and NFAT4 stimulate chondrogenesis via BMP2 in a rat mesenchymal cell line (17). However, most evidence is directed toward a role for NFAT1. In one study NFAT1 knock-out mice showed increased endochondral ossification and expression of chondrogenic markers, and the authors concluded that NFAT1 serves as a repressor of cartilage cell growth and differentiation (18). However, other studies have shown opposite roles for NFAT1. NFAT1 promoted ADAMTS-4 expression (19) and NFAT2 activates ADMTS-9 in

chondrocytes (20), which are both catabolic effects. Mice lacking NFAT1 exhibited normal skeletal development but displayed loss of specific anabolic factors (such as BMPs and TGFβ) with over-expression of specific matrix-degrading proteinases (MMPs and ADAMTSs) and proinflammatory cytokines (interleukins) in young adult articular cartilage of load-bearing joints. These initial changes were followed by articular chondrocyte proliferation/clustering, progressive articular surface destruction, periarticular chondro-osteophyte formation and exposure of thickened subchondral bone, all of which resemble human OA (21, 22). NFAT1$^{-/-}$NFAT2col2 double knockout mice displayed severe cartilage degradation with increased expression of genes encoding many matrix-degrading proteinases and increased expression of hypertrophic chondrocyte marker COL10. Expression of Sox9 and lubricin were reduced in the double knockout mice (23). These data suggest that NFAT1 supresses catabolic metabolism and hypertrophy in articular chondrocytes and thereby the development of OA. We examined the gene expression of NFAT1-4 during the dedifferentiation of osteoarthritic chondrocytes in the experiments of **chapter 2**. Consistent with most of the data above, we measured decreased expression of NFAT1 during dedifferentiation and increased expression of NFAT3, while NFAT2 and NFAT4 were not significantly regulated (data not shown). Unfortunately, we were not able to evaluate the expression and activity of NFATs on protein level, which would provide more valuable information.

Physiological omsolarity improves matrix synthesis by chondrocytes

Chondrocytes experience a hyperosmotic environment *in vivo* compared with plasma (280 mOsm) due to the high fixed negative charge density of the glycosaminoglycan side chains of proteoglycans (24). The extracellular osmolarity of healthy articular cartilage ranges between 350 and 480 mOsm (25, 26). As mentioned before, during cartilage degeneration in OA, the extracellular osmolarity decreases as a result of matrix breakdown (27). Standard isolation of chondrocytes removes the hyperosmotic matrix, exposing the cells to nonphysiological conditions in standard culture medium of 280 mOsm. In **chapter 3** we report a simple, yet effective method to improve chondrogenic marker expression by osteoarthritic and healthy human chondrocytes *in vitro*. Increasing the medium osmolarity from 280 mOsm up to physiological levels (here: 380 mOsm) by adding sterile sodium chloride during isolation and monolayer expansion increased levels of COL2, aggrecan and SOX9, while it decreased the unwanted COL1. Several studies have shown that nonhuman chondrocytes are tonicity responsive and react with changes in matrix synthesis. In 1993, incorporation of [^{35}S]sulphate and 3H-proline into glycosaminoglycans (GAGs) was reported to be maximized between 350 and 400 mOsm (within cartilage

physiological range) for both freshly isolated chondrocytes and for chondrocytes in cartilage (26). GAG production was largest in 370 mOsm and the capacity for GAG production and cell metabolism was low under hypo- and hyperosmotic conditions (28). Palmer et al. described that hyperosmotic (supraphysiological, 580-680mOsm) loading of bovine articular chondrocytes decreased aggrecan promoter activity and mRNA expression. Osmotic regulation of aggrecan gene expression was time-dependent and correlated with cell shrinkage and swelling (29). This was further investigated by Hung et al. who showed that aggrecan gene expression was elevated in monolayer chondrocytes subjected to osmotic solutions below 360 mOsm (relative cell swelling) and suppressed above 360 mOsm (cell shrinking). Interestingly, there appeared to be a direct correlation between early cell size change (shrinking) and suppression of aggrecan transcription under hyperosmotic loading, while this correlation was absent under hypotonic loading (30). These data suggest that both the osmotic environment and the applied force (cell deformation or membrane folding) regulate the transcriptional response.

Besides the effects of hyperosmotic conditions on aggrecan production, Tew et al. revealed an osmotically driven increase in post-transcriptional stability of SOX9 and activity of a COL2A1 enhancer-driven reporter (31). Cyclical osmotic loading of healthy dedifferentiated and primary OA chondrocytes but not of primary healthy chondrocytes led to an increase in SOX9 gene expression (32), indicating that the response to osmotic loading of SOX9 mRNA is dependent on the nature of the osmotic stimulation and the chondrocyte phenotype.

The molecular mechanism by which physiological osmolarity improves matrix synthesis by human articular chondrocytes has not been completely clarified. In **chapter 3** we first identified the role of NFAT5 (TonEBP) in this process. NFAT5 was abundantly expressed in both healthy and OA chondrocytes throughout passages at standard osmolarity (280 mOsm) and further increased by physiological osmolarity (380 mOsm). Unfortunately, we failed to show NFAT5 protein expression. Whether this failure is due to low protein abundance or technical issues remains to be elucidated. However, mRNA levels of well accepted NFAT5 target genes, S100A4 and SLC6A12, were induced accordingly after physiological osmotic challenge. In addition, knockdown of NFAT5 inhibited the osmolarity-induced expression of both the chondrogenic markers and S100A4/SLC6A12. So, aside from regulating intracellular osmolarity, NFAT5 seems to be a key regulator of matrix synthesis in response to osmotic changes in the extracellular environment. However, the downstream effect of NFAT5 needs to be further investigated. The human aggrecan promoter sequence has been shown to contain a conserved NFAT5 binding site

in nucleus pulposus cells and suppression of NFAT5 activity decreased aggrecan promoter activity (33). NFAT5 also regulated BMP2 and TGFβ mediated induction of β1,3-glucoronosyltransferase, an enzyme required for the synthesis of chondroitin sulfate chains of aggrecan (34, 35). On the other hand, we showed that SOX9, a known regulator of aggrecan and COL2 expression, increased by physiological osmolarity and was decreased by NFAT5 knockdown (**chapter 3 and 5**) (31). Therefore, it is also possible that NFAT5 regulates COL2 and aggecran expression in a SOX9-dependent manner rather than direct regulation of the promoter activity of COL2 and aggrecan. Details of the downstream effects of NFAT5 in regulating chondrogenic marker expression require further investigation.

Calcineurin inhibition at physiological osmolarity: effects on catabolic enzymes

In **chapter 4** we combined treatments from chapter 2 and 3 and showed that Cn inhibition by FK506 at physiological osmolarity exerts a superior effect. Adding FK506 to medium of physiological osmolarity (380 mOsm) dose-dependently enhanced chondrogenic markers to a much greater extent than FK506 or physiological osmolarity alone. In both monolayer (2D) and explant (3D) cultures, COL2 mRNA and protein expression profited relatively more from this combined treatment effect than aggrecan mRNA and GAG expression did. However, our data indicated that the combined treatment could only delay, but not prevent chondrocyte de-differentiation *in vitro*.

As OA is characterized by increased expression of catabolic enzymes, like metalloproteinases (MMPs) and aggrecanases ADAMTS4 and -5 (36-39), we also investigated the regulation of these enzymes in **chapter 4**. ADAMTS4 and -5 were not influenced by 380 mOsm or FK506 alone, but the combination decreased their expression. Interestingly, the catabolic enzymes MMP1, MMP8 and MMP13, and the hypertrophic markers COL10 and alkaline phosphatase (ALPL) were increased by physiological osmolarity in osteoarthritic human chondrocytes. Especially MMP13 is thought to be the major type II collagen-degrading collagenase in OA, that not only contributes to irreversible joint damage (progression) in OA, but importantly, also to the initiation/onset phase, wherein chondrocytes in articular cartilage leave their natural growth- and differentiation-arrested state (38). Continued overloading, stress and pro-inflammatory cytokines activate NF-κB-dependent pathways (via Toll-like receptors (TLRs) and specific protein kinase C (PKC) isoforms) and ERK/p38, which are known to activate MMP13 via other regulatory transcription factors (like ELF3 and HIF2A), facilitating the progression of articular chondrocytes to a hypertrophic-like differentiated state *in vivo*, thereby also contributing to OA onset

and/or progression (38). The role of NFAT5 in the osmolarity-induced upregulation of MMP13 and other MMPs and aggrecanases remains to be elucidated. In a murine fibroblast cell line it was discovered that NFAT5 binds to the TNF-promoter, in a pattern distinct from NFAT1 (a key factor involved in TNF gene regulation in T and B cells) and activates transcription of the pro-inflammatory cytokine TNF during hyperosmotic stress (40). In addition, NFAT5 is reported to mediate the effects of TLRs in macrophages by up-regulating IL-6, NOS2, VCAN and MMP13 (41). In inflammatory arthritis, TLR activation of NFAT5 seems to occur in a p38/MAPK-dependent manner rather than a hyperosmotic-dependent manner (42). On the other hand, hyperosmolarity-stimulated p38 activated NFAT5, which in turn directly enhances the activity of the NF-κB pathway (43). Taken together, these data suggest that besides the role of NFAT5 in protecting against cellular damage during hyperosmotic stress, it also plays a role in mediating TLR- and NF-κB activation of pro-inflammatory targets under pathological conditions (figure 1). Thereby, NFAT5 might contribute to increased expression of catabolic enzymes, like MMPs and aggrecanases, in the onset of OA.

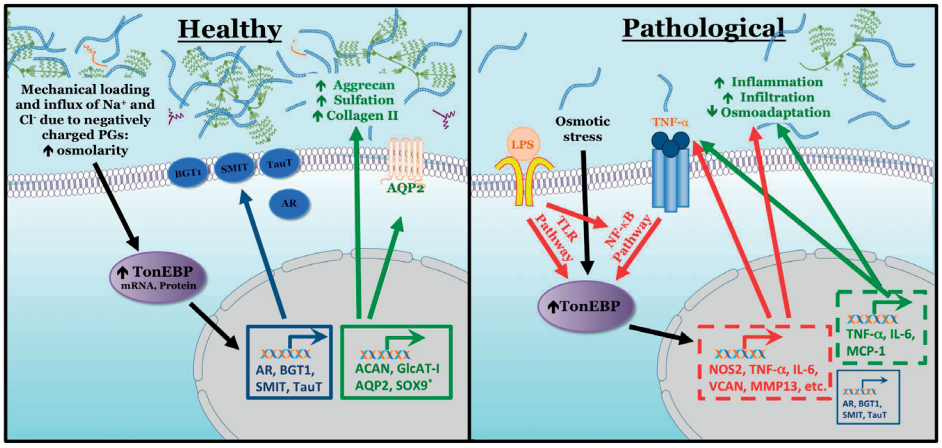

Figure 1. Adopted from Johnson et al. Matrix Biology 40 (2014) 10-16 (44).

Interestingly, in **chapter 4** we discovered that FK506 had a suppressing effect on the osmolarity/NFAT5-induced expression of the unwanted catabolic and hypertrophic markers. This data is in agreement with CsA inhibiting the IL1β-induced MMP1 and MMP3 expression in a murine model for OA (4) and with CsA inhibiting IL1α-induced MMP13, ADAMTS4 and ADAMTS5 in articular cartilage explants (45). FK506 is capable of suppressing MMP-13 synthesis via JNK pathway in rheumatoid synovium (46). Both FK506 and CsA are well known for their immunosuppressive effects. Inhibition of Cn leads to inhibition of NFAT translocation to the nucleus,

where NFATs initiate transcription of pro-inflammatory cytokines through binding to DNA in conjuction with AP-1 (47). However, some studies have linked inhibition of the Cn pathway to suppression of NF-κB signaling (48) and suppressive effects of FK506 on NF-κB signaling in peripheral human T-cells has been described (49). In addition, both CsA and FK506 are reported to impair TLR function after liver transplantation in comparison with healthy controls and there are direct correlations between serum levels and TLR function in peripheral immune cells (50). Whether FK506 suppressed the osmolarity-induced catabolic and hypertrophic markers via a Cn/NFAT-dependent or rather a TLR/NF-κB-dependent manner would be of interest in further research. Although controversial, NFAT5 might also be induced by a Cn-regulated but NFATc-independent mechanism, and NFAT5-dependent transcription can be inhibited by CsA and FK506 in T-cells (51). However, in our experiments induction of NFAT5 by a osmotic stimulus was not blocked by inhibition of Cn (51).

Treatment effects on chondroprogenitor cells

Chondrogenic differentiation of progenitor cells plays an essential role during endochondral ossification for skeletal growth and bone fracture healing. Moreover, mesenchymal progenitor cells are also used as an alternative cell source for cell-based cartilage regeneration. Therefore, new methodologies improving chondrogenic differentiation of mesenchymal progenitor and stem cells into chondrocytes are of interest for cartilage and bone regenerative medicine approaches. In **chapter 5**, the ATDC5 chondroprogenitor cell line and human bone marrow stem cells were used as *in vitro* models for chondrogenic differentiation and the effect of physiological osmolarity was studied. We found that cartilage physiological osmolarity (410 ±5 mOsm) improved chondrogenic differentiation of ATDC5 cells. Both chondrogenic (Sox9, Col2 and aggrecan, GAG content) and hypertropic (RunX2 and Col10) markers were increased. When the osmolarity was further increased up to non-physiological cartilage levels of 510 ±5 mOsm, chondrogenic differentiation was inhibited compared to control conditions (310 ±5 mOsm). In addition, we found that human bone marrow derived mesenchymal stem cells (hBMCs) responded similarly to physiological osmolarity as ATDC5 cells. Bertram et al. (52) showed that synovial fluid harvested from healthy joints ranged from 295-340 mOsm, while values from OA joints ranged from 249-277 mOsm and rheumatoid arthritis (RA) joints ranged from 273-283 mOsm. By harvesting mesenchymal progenitor cells (expressing CD90) from the synovial fluid of OA and RA joints and culturing them under different osmolarities they concluded that the cells retained increased chondrogenic potential if differentiated under the same tonicity for which they were derived within. These data confirms our results that osmolarity

regulates the chondrogenic potential of mesencymal progenitor cells. However, where healthy progenitor cells improve their chondrogenic potential at physiological (healthy) osmolarity (**chapter 5** and (52)), arthritic synovial progenitor cells did not and preferred pathological osmolarity of 264 mOsm (52). Healthy synovial fluid mesenchymal progenitor cells showed increased chondrogenic capacity compared to arthritic cells. Pathological changes in *in vivo* osmolarity of the synovial fluid may lead to a selection of a subpopulation of progenitor cells with decreased chondrogenic potential.

In **chapter 5** we also show that knockdown of NFAT5 under both control (plasma level) and cartilage physiological osmolarity decreased chondrogenic differentiation of healthy progenitor cells and suppressed the osmolarity-induced chondrogenic induction. Early expression of Sox9 in the differentiation process was decreased by NFAT5 knockdown, while knockdown of Sox9 in early differentiation did not decrease NFAT5 expression. These data suggest that NFAT5 is involved in regulating chondrogenic differentiation of chondroprogenitor cells under control and physiological osmolarity and may regulate its chondrogenic effects, at least in part, through transcription factor Sox9. Post-transcriptional stabilization of Sox9 mRNA is controlled by p38 MAPK signaling (53), which is also known to be involved in the hyperosmotic activation of NFAT5 (43, 54). The transcriptional mechanism through which NFAT5 acts upstream of Sox9 has to be addressed in future studies.

The stimulating effect of physiological osmolarity on the expression of hypertrophic and terminal differentiation markers is a favourable event in improving endochondral regeneration medicine approaches, but unfavourable in improving cartilage regenerative medicine treatments. Therefore, in **chapter 6** we investigated the effects of FK506 on osmolarity-induced chondrogenic and terminal differentiation markers in chondroprogenitor cells. We found that physiological osmolarity promoted chondrocytic and hypertrophic differentiation of pre-chondrocytes, without altering differentiation from hypertrophic to terminally differentiated chondrocytes expressing mineralization markers. FK506 inhibited Cn activity at both control and physiological osmolarity, but only improved chondrogenic differentiation markers Col9 and Col 11 at physiological osmolarity. This might indicate that the effect of FK506 on differentiation of progenitor cells is (partially) dependent on the osmolarity and not fully dependent on Cn. This is in line with the study by Nishigaki et al. (6) where FK506 but not CsA induced chondrogenic differentiation of ATDC5 cells after binding to FKBP.

However, the effects of FK506 on chondrogenic differentiation of ATDC5 cells in physiological osmolarity differ from the effects of FK506 on osteoarthritic mature chondrocytes in monolayer or explant cultures at physiological osmolarity. Where FK506 at physiological osmolarity further increased most chondrogenic markers and the COL2:COL1 ratio in osteoarthritic cells (chapter 4, figure 1+5; chapter 7, figure 2), it only increased Col9 and Col11 and did not increase the Col2:Col1 ratio in ATDC5 cells at physiological osmolarity (chapter 6, figure 1). In addition, FK506 could not decrease hypertrophic and terminal differentiation markers in ATDC5 cells at physiological osmolarity (chapter 6, figure 2+3), which is in contrast to the inhibiting effect of FK506 on hypertrophy in osteoarthritic chondrocytes (chapter 4, figure 3+5; chapter 7, figure 2). The ATDC5 cell line in these experiments is used as a model for the multistep differentiation process of chondroprogenitor cells during endochondral bone formation, which is a different process than redifferentiation of osteoarthritic mature chondrocytes. This could explain why the effects of FK506 on chondroprogenitor cells differ from the effects on osteoarthritic mature chondrocytes.

In conclusion, we found evidence that FK506 may be therapeutically useful in repairing focal articular cartilage lesions or in joints with osteoarthritis, where it can act on mature chondrocytes. The results of **chapter 6** and those of other studies (5, 7) indicate that FK506 also promotes early chondrogenic differentiation of chondroprogenitor cells, without altering differentiation to hypertrophic and terminally differentiated cells. Future studies should investigate whether changes in FK506 concentration or timing during chondrogenic differentiation of chondroprogenitor cells could achieve an inhibiting effect on cell hypertrophy, making FK506 also more useful for cartilage tissue engineering approaches using chondroprogenitor cells.

Clinical application and future directions

FK506 has proven to be useful in reducing inflammation and alleviating symptoms in patients with inflammatory (rheumatoid) joint disease (55, 56). To investigate whether patients with non-inflammatory joint diseases, like OA, also benefit from treatment with FK506, we examined in **chapter 7** the effects of systemic FK506 in a rat model for OA. FK506 treatment protected extracellular matrix integrity *in vivo* by markedly decreasing subchondral sclerosis, reducing development of subchondral pores, depletion of synovial macrophage activation and less osteophyte formation. FK506 treatment did not protect against sGAG loss in the OA model, but did protect against structural matrix degradation. OA is a complex disease and

disturbed balance between anabolic and catabolic activity of chondrocytes is an early event leading to matrix degradation. Progression of OA finally results in severe detoriation of articular cartilage and involves pathological changes throughout the joint, like extensive subchondral bone remodelling and activation of macrophages. We found indications that FK506 might not only affect chondrocytes, but may also directly modulate osteoblast and osteoclast activity and macrophage activity.

Although being a lot more potent than CsA and therefore more effective and safer at lower concentrations, even FK506 is known to cause cytotoxic effects when applied systemically. Calcineurin protein is expressed in for example the brain, spleen, thymus, heart, liver, pancreas and kidney. Both CsA and FK506 therefore induce considerable toxicity, like nephrotoxicity, neurotoxicity, glucose intolerance and diabetes, hypertension, hepatotoxicity and gastrointestinal disturbances (57). In our study the used dose of systemic FK506 did not influence liver and kidney function at the end of the 12-week experiment. Liver and kidney histology gave no indication for induced fibrosis. Several clinical trials have attempted to evaluate the efficacy and toxicity of add-on FK506 in patients with active Rheumatoid Arthritis (RA) who failed to show an adequate response to DMARDs. FK506 at dosages of mean 1.3 mg/day for 24 weeks appeared to be well tolerated and induced infection in 2.3% out of 172 patients, renal impairment in 1.2%, cardiac dysfunction in 0.6% and glucose intolerance in 0.6% (58). Minor adverse effects like gastrointestinal disorders (abdominal pain, diarrhea) occurred in 4.1% of patients. However, the systemic dosage used in these experiments is much lower than the dose used in our rat experiments (1 mg/kg/day). Therefore, despite the promising findings, systemic FK506 treatment cannot be translated toward clinical OA care directly. The possible induced side effects and toxicity are unacceptable for treating a non-life-threatening disease like OA. To reduce toxicity, local intra-articular treatment is preferable. Repetitive intra-articular injections increase the risk of iatrogenic arthritis and should be avoided. Sustained intra-articular drug delivery by loaded biodegradable hydrogels (59, 60) might be a promising solution and is of interest for future research.

In this dissertation we also showed that physiological osmolarity can be used for optimizing the *in vitro* culture conditions for cell transplantation techniques for cartilage repair. However, the *in vivo* use toward a treatment for OA or chondral lesions is again challenging and needs to be investigated. As stated before, repetitive intra-articular injections of saline upto physiological osmolarity should be avoided. The use of a hyperosmotic rinsing solution during orthopaedic surgery (61) or the use of hydrogels to deliver a sustained hyperosmotic solution in the

articular joint might be promising tools to influence the extracellular osmolarity of the damaged cartilage and hopefully leading to an increase in extracellular matrix production by the chondrocytes in situ to modulate the progression of OA. This, especially in combination with local controlled release of FK506 or more specific newer Cn- or NFAT-regulating small molecular compounds, holds potential to alter the course of OA in the future.

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Summary / Samenvatting

The research that is reported in this dissertation aimed at finding new potential targets or tools for improving cartilage matrix synthesis by chondrocytes and progenitor cells, either *in vitro* for cell transplantation techniques or *in vivo* toward a treatment for osteoarthritis (OA). The studies focus on influencing the calcineurin-dependent NFAT1-4 pathway and the osmolarity-dependent NFAT5 pathway.

In **chapter 2** we identified calcineurin (Cn) as a potential target to improve chondrogenic phenotype of culture-expanded osteoarthritic chondrocytes. During *in vitro* expansion, required for cell-based therapies, loss of chondrocyte phenotype takes place (dedifferentiation). Chondrocytes convert into a flattened, fibroblast-like state, accompanied by a shift from collagen type II to collagen type I expression, reducing the quality of the extracellular matrix (ECM). Calcineurin activity in human articular chondrocytes was significantly increased during dedifferentiation and decreased during redifferentiation *in vitro*. Inhibition of Cn activity by FK506 increased the expression of chondrogenic markers via stimulation of endogenous transforming growth factor β1 (TGFβ1). FK506 thereby became an interesting drug to promote chondrocytic phenotype for cell-based cartilage repair procedures.

Next, we focused on the effects of osmolarity on chondrocytic phenotype of culture-expanded cells. During cartilage degeneration as in OA, the extracellular osmolarity decreases as a result of matrix breakdown, in particular the loss of proteoglycans. Chondrocyte *in vitro* expansion is mostly performed in medium of non-physiological tonicity of 270 mOsm, which corresponds to physiological osmolarity of blood. Cartilage is normally characterized by a much higher interstitial osmolarity, in the range of 350 to 400 mOsm. **Chapter 3** describes the effects of chondrocyte isolation and expansion at cartilage-specific physiological osmolarity of 380 mOsm, by adding sterile sodiumchloride to the cell culture medium. Physiological osmolarity improved expression of chondrogenic markers via nuclear factor of activated T-cells 5 (NFAT5) in both osteoarthritic and normal (nonosteoartrhritic) human chondrocytes *in vitro*. These results can be used for the development of improved cell-based repair strategies for chondral lesions and provide important insights into the mechanisms underlying the progression of OA.

In **chapter 4** both treatments (FK506 and physiological osmolarity) were combined and we discovered that Cn inhibition by FK506 at physiological osmolarity exerted a superior effect than either treatment alone. The combination treatment increased anabolic, but now also suppressed unwanted catabolic and hypertrophic markers in osteoarthritic and normal (nonosteoartrhritic) human chondrocytes.

Mesenchymal progenitor cells have been introduced as alternative cell source for cell-based cartilage regeneration. Therefore, new methodologies improving chondrogenic differentiation of mesenchymal progenitor and stem cells into chondrocytes are of interest for cartilage and bone regenerative medicine approaches. In **chapter 5**, the chondroprogenitor cell line ATDC5 and human bone marrow stem cells were used as *in vitro* models for chondrogenic differentiation. Increasing the osmolarity to cartilage physiological levels also significantly increased the chondrogenic capacity of these progenitor cells. Again, we found a regulating role for NFAT5 in the chondrogenic differentiation process.

Terminal differentiation of mesenchymal progenitor cells into hypertrophic chondrocytes and mineralization of the tissue is favorable for bone regenerative medicine, but unfavorable for cartilage purposes. Based on the results of chapter 4 and 5, we wondered whether FK506 addition to physiological osmolarity medium would be able to inhibit hypertrophic differentiation of progenitor cells. In **chapter 6** we show that FK506 also improved chondrogenic differentiation of progenitor cells at physiological osmolarity. Unfortunately, FK506 could not decrease hypertrophic and terminal differentiation markers in progenitor cells. These data suggest that FK506 promotes early chondrogenic differentiation of progenitor cells, without altering differentiation to hypertrophic and terminally differentiated cells. The latter is in contrast to the inhibiting effect of FK506 on hypertrophy in human osteoarthritic chondrocytes.

Chapter 7 describes the *in vivo* effects of oral FK506 in an animal model for OA. FK506 clearly protected against articular cartilage collagenous matrix degradation in this model. In addition, it markedly decreased subchondral sclerosis and led to less development of subchondral pores as well as depletion of synovial macrophage activation and less osteophyte formation.

In conclusion, we found evidence that FK506 may be therapeutically useful in repairing articular cartilage lesions or in joints with osteoarthritis, where it can act on human chondrocytes. Future studies should investigate whether changes in FK506 concentration or timing during chondrogenic differentiation of progenitor cells could achieve an inhibiting effect on cell hypertrophy, making FK506 also more useful for cartilage tissue engineering approaches using progenitor cells.

In this dissertation we also showed that physiological osmolarity can be used for optimizing the *in vitro* culture conditions in cell transplantation techniques for cartilage repair. The use of a hyperosmotic rinsing solution during orthopaedic

surgery or the use of hydrogels to deliver a sustained hyperosmotic solution in the articular joint might be a tool to influence the extracellular osmolarity of the damaged cartilage. This, especially in combination with local controlled release of FK506, holds potential to alter the course of OA in the future.

Samenvatting

Dit proefschrift beschrijft een aantal onderzoeken gericht op het ontdekken van nieuwe technieken of aangrijpingspunten om de synthese van kraakbeenmatrix door kraakbeencellen (chondrocyten) en stamcellen te verbeteren, zowel *in vitro* tijdens cel transplantatie technieken, als *in vivo* voor een behandeling voor artrose. De onderzoeken zijn gericht op het beïnvloeden van de calcineurine afhankelijke NFAT1-4 en de calcineurine onafhankelijke NFAT5 signaal transductie cascade.

In **hoofdstuk 2** identificeerden we calcineurine (Cn) als potentieel doelwit om het kraakbeen fenotype van chondrocyten, verkregen uit een artrotisch gewricht, in een kweekcultuur te verbeteren. Tijdens het kweken en vermenigvuldigen van cellen voor cel therapieën, verliezen de chondrocyten hun specifieke fenotype (dedifferentiatie). De chondrocyten veranderen in plattere, fibroblast-achtige cellen. Dit gaat gepaard met minder collageen type 2 en meer collageen type 1 expressie, waardoor de kwaliteit van de extracellulaire matrix vermindert. De calcineurine activiteit was significant hoger tijdens de dedifferentiatie van de chondrocyten *in vitro* en was juist lager tijdens redifferentiatie. Remming van de Cn activiteit door FK506 leidde tot een hogere expressie van kraakbeen-specifieke markers doormiddel van stimulatie van de endogene groeifactor TGFβ1. FK506 lijkt hierdoor een interessant middel om het fenotype van chondrocyten bij celtherapie voor kraakbeenherstel te verbeteren.

In het volgende onderzoek bestudeerden we het effect van osmolariteit op het fenotype van chondrocyten in een kweekcultuur. Tijdens degeneratie van het kraakbeen, zoals bij artrose, daalt de extracellulaire osmolariteit door de afbraak van de matrix, met name door afbraak van proteoglycanen. Het kweken van chondrocyten gebeurt doorgaans in een kweekmedium met een niet-fysiologische osmolariteit van 270 mOsm, wat overeenkomt met de osmolariteit van bloed. Gezond kraakbeen heeft echter een veel hogere interstitiële osmolariteit van 350 tot 400 mOsm. **Hoofdstuk 3** beschrijft het effect van het kweken van chondrocyten in medium met een kraakbeen-specifieke fysiologische osmolariteit van 380 mOsm. Deze osmolariteit werd bereikt door het toevoegen van steriel natriumchloride aan het medium. Deze fysiologische osmolariteit gaf een hogere expressie van kraakbeen markers door middel van stimulatie van de transcriptie factor NFAT5, zowel in artrotische als normale (gezonde) kraakbeencellen. Deze resultaten kunnen gebruikt worden in de ontwikkeling van betere celtherapieën voor kraakbeendefecten en bieden belangrijke inzichten in de onderliggende mechanismen bij de progressie van artrose.

In **hoofdstuk 4** worden beide behandelingen (FK506 en fysiologische osmolariteit) gecombineerd en ontdekten we dat remming van Cn door FK506 in een fysiologische osmolariteit een superieur effect heeft ten opzichte van de afzonderlijke behandelingen. De combinatietherapie verhoogde de anabole, maar verlaagde de katabole en hypertrofie markers in artrotische en normale humane chondrocyten.

Mesenchymale stamcellen worden regelmatig gebruikt als alternatieve cel bron voor celtherapie voor kraakbeenherstel. Nieuwe methoden om chondrogene differentiatie van mesenchymale stamcellen richting chondrocyten te verbeteren, zijn daarom interessant voor celtherapieën voor kraakbeen- en botregeneratie. In **hoofdstuk 5** worden de chondrogene stamcellijn ATDC5 en humane beenmergstamcellen gebruikt als *in vitro* model voor chondrogene differentiatie. Het verhogen van de osmolariteit van het kweekmedium naar de kraakbeen specifieke fysiologische waarde had een significant positief effect op de chondrogene capaciteit van deze stamcellen. Ook hier vonden we een belangrijk regulerende rol voor NFAT5 in het chondrogene differentiatie proces.

Terminale chondrogene differentiatie van stamcellen naar hypertrofe chondrocyten en mineralisatie van het weefsel is gunstig voor botregeneratie therapieën, maar ongunstig voor kraakbeen behandeling. Op basis van de resultaten van hoofdstuk 4 en 5, vroegen we ons af of FK506 de hypertrofe differentiatie van de stamcellen in fysiologische osmolariteit kon remmen. In **hoofdstuk 6** werd waargenomen dat FK506 ook de chondrogene differentiatie van stamcellen in fysiologische osmolariteit verder verbeterde. FK506 had helaas geen remmend effect op de hypertrofe en terminale differentiatie markers. Deze resultaten suggereren dat FK506 de vroege chondrogene differentiatie van stamcellen stimuleert, zonder de differentiatie richting hypertrofe en terminale cellen te beïnvloeden. Dit laatste is in tegenstelling tot het remmende effect van FK506 op hypertrofie van humane artrose chondrocyten.

Hoofdstuk 7 beschrijft de *in vivo* effecten van oraal FK506 in een diermodel voor artrose. FK506 had een duidelijk beschermend effect tegen de afbraak van de collagene kraakbeenmatrix in dit model. Daarnaast verminderde de subchondrale sclerose, ontstonden er minder subchondrale poriën, werden synoviale macrofagen minder geactiveerd en werden minder osteofyten gevormd.

Samengevat vonden we bewijs dat FK506 mogelijk therapeutisch gebruikt kan worden voor het herstel van kraakbeendefecten of in gewrichten met artrose, gezien het effect op humane chondrocyten. Toekomstige onderzoeken zullen moeten

uitwijzen of aanpassingen in de dosering of het moment van toedienen van FK506 tijdens chondrogene differentiatie van stamcellen ook een remmend effect op celhypertrofie kunnen bewerkstelligen, waardoor FK506 ook beter toepasbaar wordt voor kraakbeenregeneratie therapieën met behulp van stamcellen.

Dit proefschrift toont ook aan dat fysiologische osmolariteit gebruikt kan worden voor het optimaliseren van de *in vitro* kweekcondities in celtransplantatie therapieën voor kraakbeenherstel. Het gebruik van een hyperosmotische spoelvloeistof tijdens orthopaedische operaties of het gebruik van hydrogels die langdurig een hyperosmotische vloeistof afgeven in het gewricht, kunnen methoden zijn om de extracellulaire osmolariteit van beschadigd kraakbeen te beïnvloeden. Dit, en vooral in combinatie met lokale gecontroleerde afgifte van FK506, is een potentiele manier om het beloop van artrose in de toekomst te kunnen veranderen.

Appendices

List of Abbreviations

Acan	Aggrecan
ACI	Autologous Chondrocyte Implantation
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
AGC	Aggrecan
ALPL	Alkaline Phosphatase
ATDC5	Mouse 129 teratocarcinoma AT805 derived cell line
AQ	Aquaporin Channel
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
BMP	Bone Morphogenetic Protein
BMSC	Bone marrow Mesenchymal Stem Cells
Ca^{2+}	Calcium
CaM	Calmodulin
CCI	Characterized Chondrocyte Implantation
cDNA	Complementary DNA
Cn	Calcineurin
COL/Col	Collagen
CsA	Cyclosporin A
µCT	Micro-computed Tomography
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
EPIC	Equilibrium Partitioning of an Ionic Contrast
FCS	Fetal Calf Serum
FK506	Tacrolimus
FKBP	FK506-binding Protein
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
HACs	Human Articular Chondrocytes
HPRT1	Hypoxanthine-guanine Phosphoribosyltransferase 1
IGF	Insulin-like Growth Factor
IL	Interleukin
MAPK	Mitogen-activated Protein Kinase
MMP	Matrix Metalloproteinase
mOsm	Milliosmole
mRNA	Messenger RNA
NaCl	Sodium Chloride

NFAT	Nuclear Factor of Activated T-cells
NF-κB	Nuclear Factor of Kappa-light-chain-enhancer of activated B-cells
NOS2	Nitric Oxide Synthase 2
OA	Osteoarthritis
P	Passage
PG	Proteoglycan
RUNX	Runt-related Transcription factor 2
RT-QPCR	Real time Quantitative Polymerase Chain Reaction
S100A4	S100 calcium-binding protein A4
SLC5A3	Solute Carrier Family 5 Member 3
SLC6A12	Solute Carrier Family 6 Member 12
Smad	Homolog of Drosophila Mothers Against Decapentaplegic
SMIT	Sodium/myo-inositol Cotransporter
SOX 9	SRY (sex determining region Y)-box 9
SPECT	Single-photon Emission Computed Tomography
SPP1	Osteopontin
TGFβ	Transforming Growth Factor beta
TGM	Transglutaminase
TIMP	Tissue Inhibitor of Metalloproteinases
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TonEBP	Tonicity-responsive Enhancer Binding Protein
UBC	Ubiquitin C
VCAN	Versican
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

List of Publications

Publications related to the research of this dissertation:

van der Windt AE, Jahr H, Farrell E, Verhaar JA, Weinans H, van Osch GJ. Calcineurin inhibitors promote chondrogenic marker expression of dedifferentiated human adult chondrocytes via stimulation of endogenous TGFbeta1 production. *Tissue Engineering Part A.* 2010; 16: 1-10

van der Windt AE, Haak E, Das RH, Kops N, Welting TJ, Caron MM, van Til NP, Verhaar JA, Weinans H, Jahr H. Physiological tonicity improves human chondrogenic marker expression through nuclear factor of activated T-cells 5 in vitro. *Arthritis Research & Therapy.* 2010; 12: R100

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van der Windt AE, Siebelt M, Groen HC, Sandker M, Waarsing JH, Müller C, de Jong M, Jahr H, Weinans H. FK506 protects against articular cartilage collagenous extracellular matrix degradation. *Osteoarthritis Cartilage.* 2014; 22: 591-600

van der Windt AE, Kops N, Baart EB, Verhaar JAN, Weinans H, Jahr H. FK506 promotes osmolarity-induced chondrogenic differentiation of murine ATDC5 cells. *Submitted.*

Other publications:

van der Windt AE, de Mos M, Jahr H, van Schie HT, Weinans H, Verhaar JA, van Osch GJ. Can platelet-rich plasma enhance tendon repair? A cell culture study. *The American Journal of Sports Medicine.* 2008; 36: 1171-1178

van Ginneken BT, van den Berg-Emons RJ, **van der Windt AE**, Tilanus HW, Metselaar HJ, Stam HJ, Kazemier G. Persistent fatigue in liver transplant recipients: a two-year follow-up study. *Clinical Transplantation.* 2010; 24: E10-16

PhD portfolio summary

Name PhD student:	Anna Elizabeth van der Windt
Erasmus MC Department:	Orthopaedics
Promotor(s):	Prof. Dr. ir. H. Weinans; Prof. Dr. J.A.N. Verhaar, MD.
Supervisor:	Dr. H. Jahr

1. PhD training	**Year**	**Workload**
General academic skills		
- Biomedical English writing and communication	2008	4.0
- Research Integrity (lectures only)	2008	
Research skills		
- ICRS Laboratory Skills Course	2007	1.0
- Literature meeting orthopaedic lab	2006-08, '12	4.0
(Inter)national conferences: poster presentation:		
- Calcineurin-NFAT signaling during chondrocyte differentiation in vitro. *Dutch Program for Tissue Engineering (DPTE)* Noordwijkerhout, Nederland	2007	1.0
- Calcineurin-Nuclear factor of activated T-cells pathway regulates adult human articular chondrocyte differentiation. *Orthopaedic Research Society (ORS),* San Francisco, Verenigde Staten	2008	1.0
- Inhibition of Cn activity increases chondrogenic markers via TGβ *ORS*, Las Vegas, Verenigde Staten	2009	1.0
- High tonicityimproves chondrogenic marker expression through NFAT5. *ORS*, New Orleans, Verenigde Staten	2010	1.0
- Inhibiting Cn activity under physiological tonicity, a win-win situation for cell-based chondral lesion repair. *Osteoarthritis Research Society International (OARSI)*, Brussel, België	2010	1.0

- Inhibiting Cn activity under physiological tonicity, a win-win situation for cell-based chondral lesion repair. *ORS,* Long Beach, Verenigde Staten	2011	1.0
- Inhibiting calcineurin activity under physiological conditions selectively prevents articular chondrocyte hypertrophy. *European Cells and Materials,* Davos, Zwitserland	2013	1.0

(Inter)national conferences: podium presentation:

- Platelet rich plasma enhances cell proliferation and gene expression of matrix metalloproteinase and growth factors of human tendon cells. *Nederlandse Vereniging voor Biomaterialen en Tissue Engineering,* Lunteren, Nederland. *Award voor beste presentatie*.	2006	1.0
- Calcineurin-NFAT pathway regulates adult human articular chondrocyte differentiation. *OARSI,* Fort Lauderdale, Verenigde Staten	2007	1.0
- High tonicity improves chondrogenic marker expression through NFAT5. *Nederlandse Vereniging voor Matrix Biologie,* Lunteren, Nederland. *Pauline van Wachem award voor beste presentatie*	2010	1.0
- Inhibiting calcineurin activity under physiological conditions selectively prevents articular chondrocyte hypertrophy. *European Orthopaedic Research Society,* Wenen, Oostenrijk.	2011	1.0

National local oral lectures:

- DPTE users' committee meeting	2007-09, 4x	1.0
- Research group meeting	2006-09, 13x	3.0
- Literature meeting	2006-09, 4x	1.0
- Science day, Erasmus MC, Rotterdam	2009	0.5
- Opleidingsdag ROGO Rotterdam, Delft	2014	0.5

(Inter)national conferences, attendance only		
- NOV annual meeting, Nederland	2012, '15, '16	1.5
- EORS Annual meeting, Amsterdam, Nederland	2012	0.5

2. Teaching activities	**Year**	**Workload**
Supervising practicals and excursions, tutoring		
- Tutor first year medical students	2008, '09	4.0
Supervising Master's theses		
- Supervising Angela Bastidas Coral: Heat shock proteins in osteoarthritis	2013	3.0

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Prof. dr. G.J.V.M. van Osch, beste Gerjo, mijn wetenschappelijke carrière is bij jou begonnen. Door jou en Marieke is mijn enthousiasme voor basaal wetenschappelijk onderzoek gegroeid en ik ben je erg dankbaar dat jij en Harrie mij daarna een promotietraject aanboden. Ik heb het jammer gevonden dat de onderzoeksgroepen uit elkaar gingen, ik had je er graag als begeleider bij gehouden! Gelukkig zat

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About the author

Anna Elizabeth van der Windt werd geboren op 11 oktober 1984 in Rotterdam. Zij behaalde haar VWO diploma op het Sint-Laurenscollege in Rotterdam, waarna zij in 2002 begon met de studie Geneeskunde aan de Erasmus Universiteit Rotterdam. Tijdens haar studie Geneeskunde ontstond de voorkeur voor orthopaedische chirurgie en deed zij haar afstudeer-onderzoek naar het effect van plaatjes-rijk plasma op tendinose en peesherstel op de afdeling Orthopaedie van het Erasmus MC (Prof. dr. G.J.V.M. van Osch en Prof. dr. ir. H. Weinans). Hier werd haar interesse voor wetenschappelijk onderzoek gewekt. Na het behalen van haar doctoraal diploma, werd haar studie Geneeskunde onderbroken om te kunnen starten met haar promotieonderzoek op de afdeling Orthopaedie onder begeleiding van Prof. dr. ir. H. Weinans, Dr. H. Jahr en Prof. dr. J.A.N. Verhaar. Hierna heeft Anna haar studie Geneeskunde hervat en na het behalen van haar artsexamen is zij in januari 2013 begonnen met haar vooropleiding heelkunde in het IJsselland Ziekenhuis in Capelle a/d IJssel (opleider Dr. I. Dawson). In juli 2014 begon haar opleiding tot orthopaedisch chirurg in het Erasmus MC in Rotterdam (opleiders Prof. dr. J.A.N. Verhaar en Dr. P.K. Bos) en aansluitend in Reinier de Graaf Groep in Delft (opleider Dr. R.M. Bloem).