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, redifferentiation 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 TGFß production by chondrocytes and whether the effect of FK506 on chondrogenic marker expression was due to TGFß. In addition, we investigated whether chondrocyte redifferentiation by TGFß 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 TGFß 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-b1, porcine TGF-b1.2, porcine TGFβ2 and recombinant, amphibian TGF-b5). To check for unwanted effects of this anti-TGFß antibody on chondrogenic marker expression or cross-reactivity to human TGFβ2, we cultured cells with anti-TGFß1 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 TGFß

Our next goal was to investigate whether redifferentiation by TGFß 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 TGFß 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). 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. The experiments were performed in triplicate samples (n=3).

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 (Eurogentec, 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. 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 TGFß2 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).

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 improve 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).

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.

Cn activity after addition of TGFß

Next we investigated whether redifferentiation by TGFß 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).

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 collagen type II, aggrecan 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 mechanism 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 rhTGFß2 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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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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FIGURE LEGENDS

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 ($p<0.05$).

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 ($p<0.05$).

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 for 10 days. **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).

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).

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.

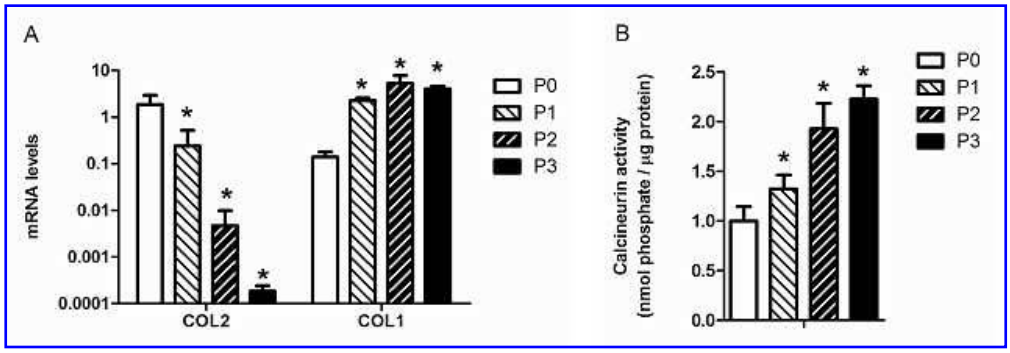

Figure 1
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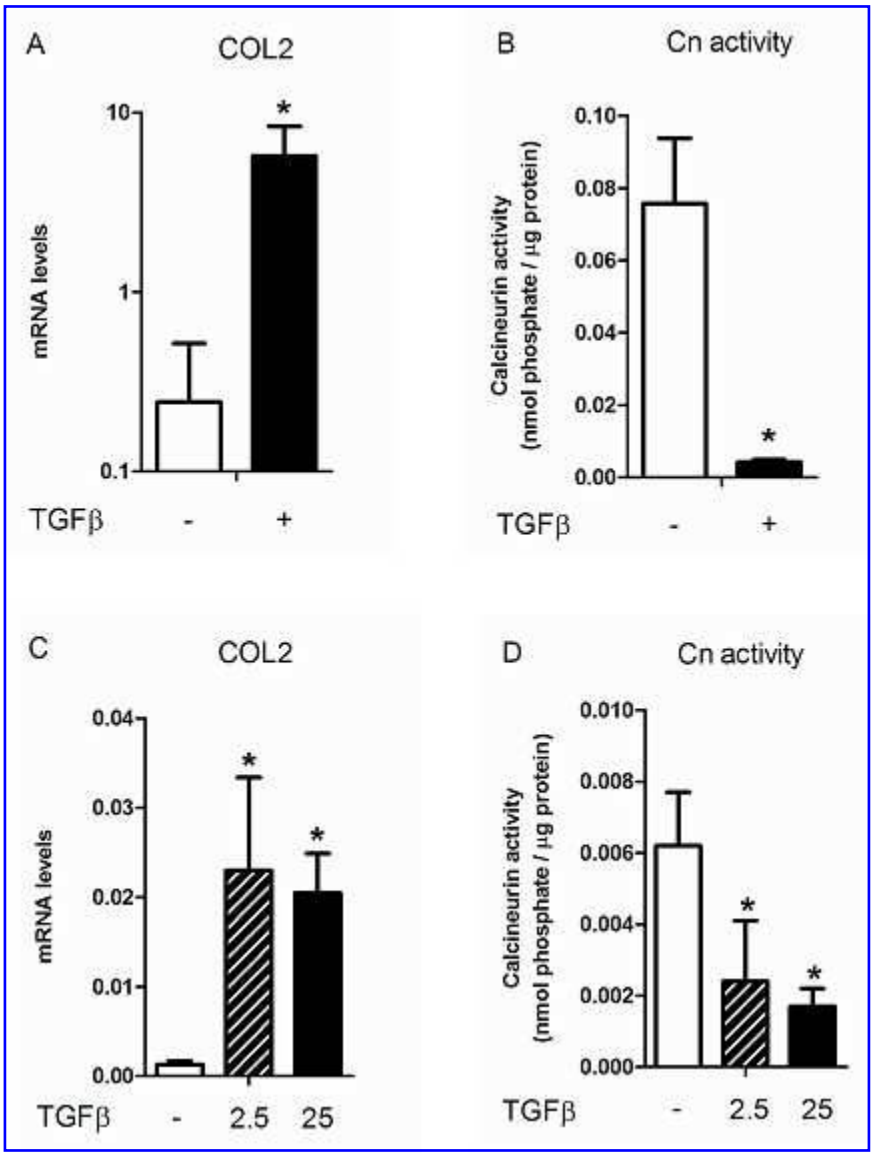

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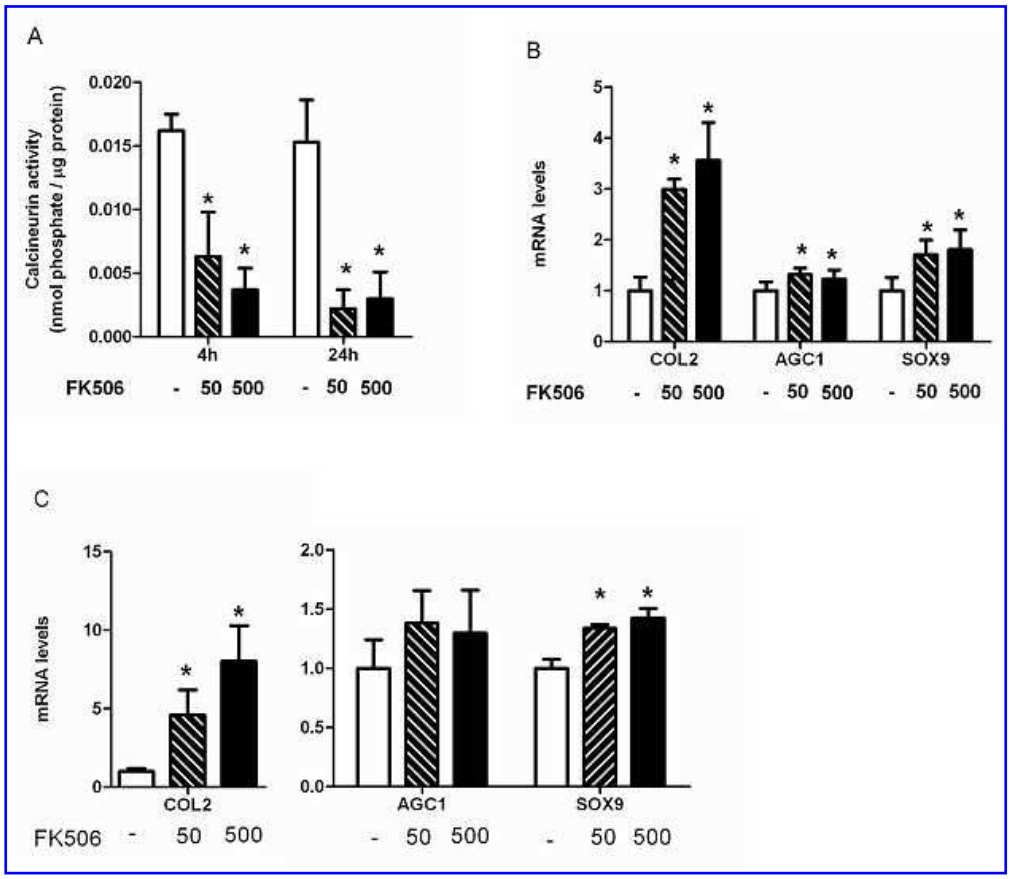

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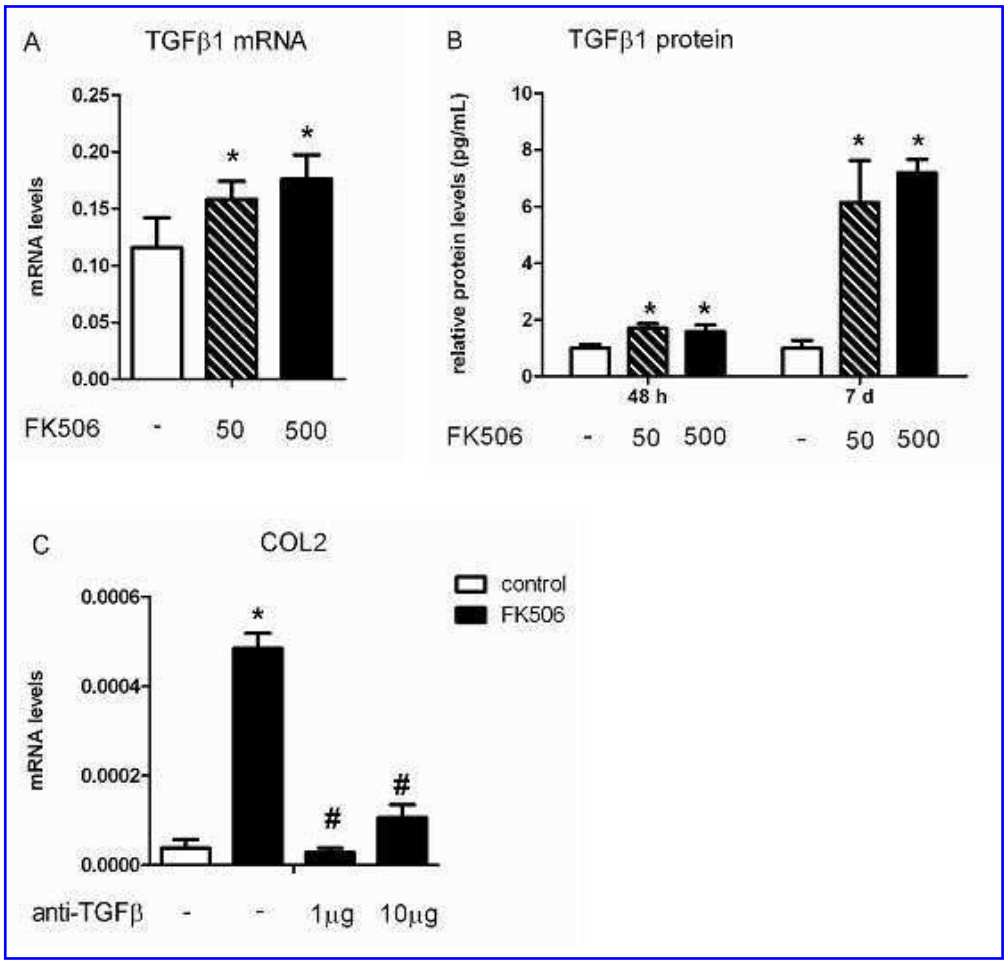

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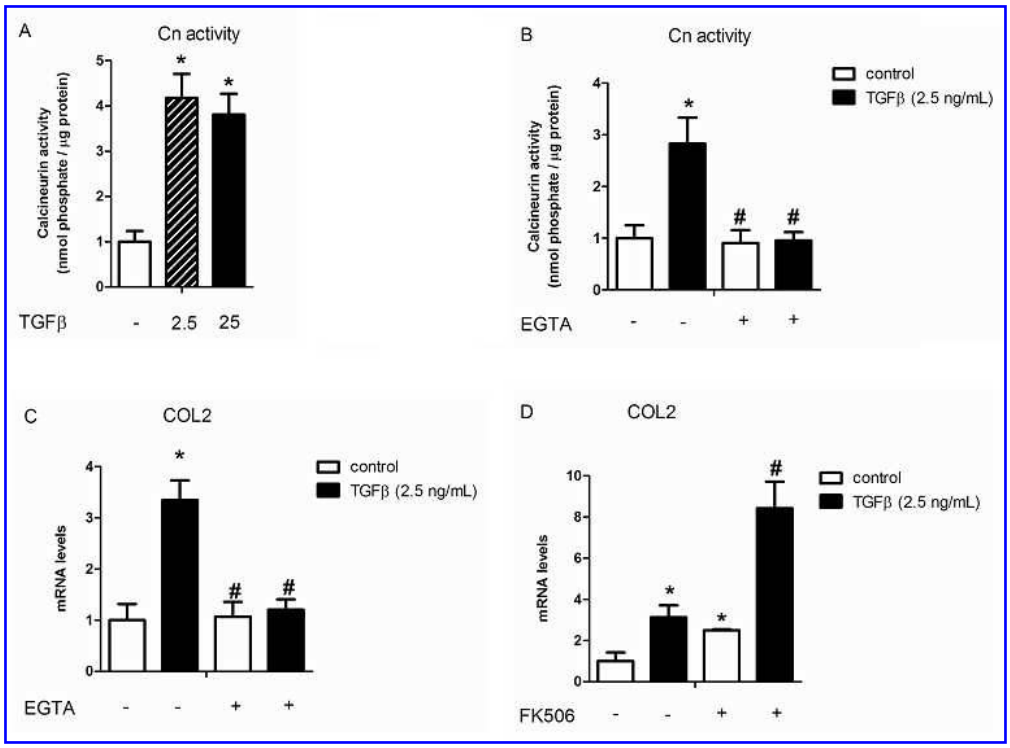

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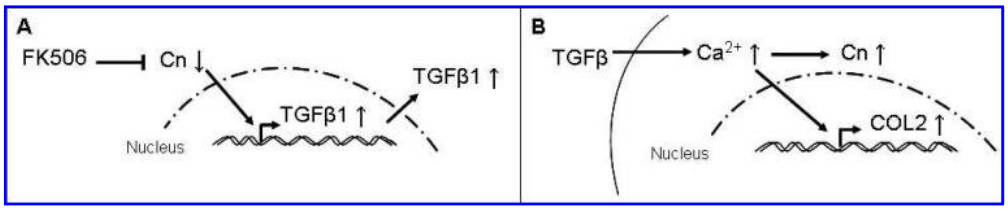

Figure 6
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