

## DOWN-REGULATION OF THE GTPASE RHOB MIGHT BE INVOLVED IN THE PRE-APOPTOTIC PHENOTYPE OF OSTEOARTHRITIC CHONDROCYTES

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### 1. ABSTRACT

Anabolic activity, phenotypic alterations, and in particular survival of the chondrocytes are essential for the maintenance of proper articular cartilage and appears to fail during osteoarthritic cartilage degeneration. In this study, we investigated the presence and expression of RhoB in adult human articular cartilage and its regulation in osteoarthritic cartilage as well as in chondrocytes *in vitro*. RhoB belongs to the family of small GTPases, which are thought to be involved in a large range of activities important for eukaryotic cells.

Conventional and quantitative PCR analysis showed significant levels of RhoB expression in normal articular cartilage. Immunolocalization and confocal laser scanning microscopy showed strong cytoplasmic signals for RhoB in normal chondrocytes. In osteoarthritic cartilage, a significantly lower expression of RhoB was detectable. *In vitro* experiments showed a quick (and

transient) up-regulation of RhoB after stimulation with interleukin-1beta and serum.

Our study suggests that RhoB is constitutively expressed and essential for adult articular chondrocytes, but significantly down-regulated in osteoarthritic chondrocytes. One intriguing speculation might be that the down-regulation of RhoB in osteoarthritic chondrocytes is at least partly a prerequisite for the sustained pre- or para-apoptotic phenotype of osteoarthritic chondrocytes, because RhoB is known to be one important molecule in the induction of apoptotic cell death in response to DNA damage and osteoarthritic chondrocytes are known to have significant DNA damage. Alternatively, RhoB could be involved in the activation or deactivation and the destabilization of the functional phenotype of chondrocytes in osteoarthritic joint degeneration. Thirdly, RhoB is associated with the cell cycle, which is re-initiated in osteoarthritis.

## 2. INTRODUCTION

Articular cartilage is a highly specialized tissue that forms the smooth, gliding surface of the diarthrodial joints. It consists of an extracellular matrix which is synthesized by the sparsely distributed resident cells - the chondrocytes. Osteoarthritic cartilage degeneration is largely a process of destruction and failure of this extracellular matrix. However, matrix turnover including anabolic regeneration is solely dependent on the chondrocytes, which, in contrast to the "dead" matrix, are the active players within the tissue. Thus, anabolic activity, phenotypic alterations, and finally survival of the chondrocytes are essential for the maintenance of proper articular cartilage. Cartilage matrix failure always complicates a failure of the involved cells, in particular because there is no external cell supply to compensate for cell loss due to necrosis, apoptosis or other cellular mechanisms. Lacunar emptying in osteoarthritic cartilage (1, 2) has lead to the long standing assumption that cell degeneration is a central feature in osteoarthritic cartilage degeneration (3). More recently, apoptotic cell death has become a focus of interest (4, 5), but the extent remained controversial (6)(for review see Aigner *et al.*(7)).

Recently, gene screen experiments suggested RhoB to be expressed in articular cartilage and down-regulated in osteoarthritic cartilage (8). RhoB belongs to the family of small GTPases which are highly evolutionary conserved (9) thought to be involved in a large range of activities important for eukaryotic cells. Members of the Rho family are thought to be key regulators of the actin cytoskeleton (for review see Hall 1998(10)). Also, through their interaction with multiple target proteins, they are involved in the control of other cellular activities such as gene transcription and adhesion (for review see Fuka and others 2001 (11, 12)). Interestingly, RhoB is stabilized by TGF beta and inhibits itself TGF beta signaling within cells (13, 14), which is thought to be a main anabolic factor of articular cartilage (15). Most importantly, RhoB appears to be required for apoptotic response at least in some cell types (for review see Prendergast 2001(16)).

In this study, we tried to confirm the expression and presence of RhoB in adult human articular cartilage on the mRNA and protein level and to analyze its regulation in osteoarthritic cartilage. Furthermore, we were interested whether RhoB is also expressed in cultured chondrocytes *in vitro* and inducible by external chondrocyte-relevant growth factors and cytokines such as IL-1 and FCS as described previously for other cell systems (17, 18).

## 3. MATERIALS AND METHODS

### 3.1. Tissue sampling for mRNA analysis

For the study, cartilage from human femoral condyles of the knee joints were used. Normal articular cartilage (n = 7, 32 to 83 years, mean age 56.6 years) and early degenerated cartilage (n = 8, 43 to 91 years, mean age 69.4 years) were obtained from donors at autopsy, within 48 hours of death. Osteoarthritic cartilage samples from late stage osteoarthritic joint disease were obtained

from patients undergoing total knee replacement surgery (n = 8, 63 to 79 years, mean age 71.6 years). The cartilage was frozen in liquid nitrogen immediately after removal and stored at -80°C until required for RNA isolation. Cartilage was considered to be normal if it showed no significant softening or surface fibrillation. Early degenerated cartilage was defined as cartilage which showed moderate fibrillation and softening, but no advanced erosion of the articular cartilage. Only this cartilage was taken for the study and not (peripheral) areas showing no obvious signs of degeneration. Late stage osteoarthritic cartilage was always derived from patients undergoing knee arthroplasty due to complete destruction of the articular cartilage in major portions of the joints. Cases of rheumatoid arthritis were excluded from the study. Only primary degenerated and not regenerative cartilage (osteophytic tissue) was used for this study.

### 3.2. RNA isolation from articular cartilage

Total RNA from cartilage tissue was isolated as described previously (19). Isolated RNA was controlled for quality by electrophoresis and by spectrophotometry.

### 3.3. Cell isolation

For cDNA array analysis, normal human knee articular cartilage was obtained from a 48 yrs old male donors at autopsy without any sign of degenerative or inflammatory joint pathology.

Cartilage pieces were finely chopped and chondrocytes were enzymatically isolated from associated matrix: cells were first digested with 1 mg/ml pronase (Roche, Switzerland) in DMEM/F12 (Gibco BRL, Germany) with 10% FCS (Biochrom, FRG) for 30 minutes and subsequently with 1 mg/ml collagenase P (Boehringer Mannheim, FRG) in Hams-F12 (Gibco BRL, Germany) with 10% FCS. Finally, cells were washed several times in Hams-F12 and counted and checked for viability using the trypan blue staining.

### 3.4. High density monolayer cultures - stimulation with IL-1beta

After isolation, chondrocytes were seeded at  $2 \times 10^6$  cells/well in 6 well tissue culture plates and maintained for 48 hours in DMEM/F12 medium (Gibco BRL, Germany) supplemented with 10% fetal calf serum and 50 µg/ml penicillin/streptomycin solution (Gibco BRL, Germany) and 50 µg/ml ascorbate (Sigma, Germany).

Thereafter, chondrocytes were stimulated with 1 ng/ml rhIL-1beta (Biomol, Germany), with and without 10% fetal calf serum (Biochrom, FRG) or cultivated in medium alone for 6 hrs, 24 hrs, 48 hrs or 96 hrs. The medium was changed every day. At the end of the stimulation-period the cells were washed in sterile PBS, lysed in 70 µl lysis RLT buffer/ $10^6$  cells (Qiagen GmbH, Germany) and stored at -80°C.

### 3.5. RNA isolation

RNA was isolated from cultured cells using the RNeasy mini kit (Qiagen, Germany) (with an on-column DNase digestion step, according to the manufacturers

instructions). Briefly, cells were passed through a Qias shredder (Qiagen, Germany) and the eluted lysate was mixed 1 : 1 with 70% ethanol. The lysate was applied to a mini column and after washing and DNase digestion, the RNA was eluted in 30-50 µl of RNase-free water. The quantity and quality of RNA was assessed by ethidium bromide staining of RNA separated on 1.2% agarose gels.

### 3.6. cDNA synthesis

First strand cDNA was synthesized using 1 µg of total RNA, 400U M-MLV Reverse Transcriptase, RNase H Minus (Promega), 2 mM dNTPs (Roth) and 200 ng random primers (Promega) in a total volume of 40 µl.

### 3.7. Conventional PCR

cDNA-Aliquots equivalent to 50 ng total RNA serving as templates were amplified in 50 µl reactions containing 0.2 mM forward primer (5'-CGGACACCGACGTCATTCT-3'), 0.2 mM reverse primer (5'-CCAGGATGATGGGCACATT-3'), 10 mM dNTPs, 50 mM MgCl<sub>2</sub>, and 5 U Taq Polymerase Silver Star (Eurogentec, Belgium). PCR reaction settings were as described in the following: An initial 3 min denaturing step at 94 °C, 35 cycles with 30 sec denaturing at 94 °C, 30 sec primer annealing at 58°C and 40 sec elongation time at 72°C. After the last cycle a final 5 min extension step at 72°C was performed.

PCR products were visualized at 300 nm on 1.2 % agarose gels containing 0.5 µg/ml ethidium bromide using MBI Fermentas (Heidelberg, Germany) 100-bp DNA ladder molecular weight marker as a standard.

### 3.8. TAQMAN PCR

TAQMAN PCR was used to detect human RhoB in human articular cartilage RNA samples. The primers (MWG Biotech, Germany) and TAQMAN probes (Eurogentec, Belgium) were designed using PRIMER EXPRESS TM software (Perkin Elmer). (forward primer (50 nM): 5'-CACCGACGTCATTCT-3', reverse primer (900 nM): 5'-ACCAGGATGATGGGCACATT-3', probe (100 nM): 5'-TGGACAGCCCGGACTCGCT-3'). A separate master-mix was made up for each of the primer pairs and contained a final concentration of 200µM NTPs, 450 nM Roxbuffer and 100 nM TAQMAN probe. For all genes the final reaction mix contained besides cDNA and 0.5U polymerase (Eurogentec, Belgium) forward and reverse primers, the corresponding probes, and 5,5 nM MgCl<sub>2</sub>. All experiments were performed in triplicates.

In order to be able to obtain quantifiable results for all genes specific standard curves using sequence specific control probes were performed in parallel to the analyses. For the standard curves concentrations of 10, 100, 1000, 10000, 100.000, as well as 1.000.000 molecules per assay were used (all in triplicates). All experiments were performed in triplicates. The assay for GAPDH was described previously (20).

### 3.9. cDNA array hybridization

The Human Cancer 1.2 cDNA array (Clontech GmbH, Germany) was probed with <sup>32</sup>P-dATP labeled

cDNA probes, which were prepared from 5 µg of isolated total RNA according to the manufacturer's protocol. Membranes were hybridized for 18 hours at 68°C in Expresshyb (Clontech). The membranes were stringently washed 4x in (2x SSC, 1% SDS), 1x in (0.1x SSC, 0.5% SDS) at 68°C and 1x in 2x SSC at room temperature. Washed membranes were sealed in plastic and exposed to a phosphor plate for 72 hours. Images were captured on a Molecular Dynamics Storm phosphor imager, using Image Quant software (Molecular Dynamics).

### 3.10. Histomorphology and histochemistry

From all slices HE and Safranin O stainings were performed in order to evaluate matrix abundance, cellularity, and the content of glycosaminoglycans (GAGs).

### 3.11. Immunohistochemistry

Conventional immunohistochemical studies were performed on paraffin embedded specimens of normal (n=6) and late stage osteoarthritic (n=6) articular cartilage using a streptavidin-biotin-complex technique (Biogenex, Mainz, Germany) with alkaline phosphatase as detection enzyme as described previously (21). The specimens were fixed with 4% paraformaldehyde (in PBS pH 7.4) immediately after removal.

In order to obtain optimal staining results various enzymatic pretreatments including hyaluronidase (Boehringer, Mannheim, FRG, 2 mg/ml in phosphate buffered saline (PBS), pH 5, for 60 minutes at 37°C), pronase (Sigma, Deisenhofen, FRG, 2 mg/ml in PBS, pH 7.3, for 60 minutes at 37°C), chondroitinase ABC (sigma, Deisenhofen, FRG, 0,25 U/ml in 0,1M tris-HCl pH8, for 60 minutes at 37°C) or bacterial protease XXIV (Sigma, 0.02 mg/ml, PBS, pH 7.3, for 60 minutes at 37°C) were tested. The final protocol included a pretreatment with protease XXIV as described. The primary antibody was a mouse monoclonal antibody to Rho B (Santa-Cruz sc-8048).

Negative controls (using non-specific goat antiserum (Biogenex) or replacing the primary antibodies with PBS) never revealed any positive stainings.

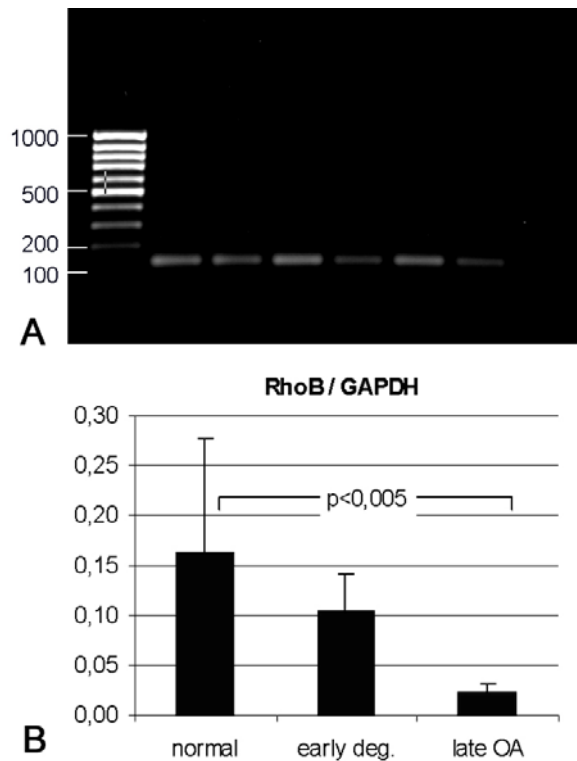
### 3.12. Confocal scanning microscopy

Confocal scanning microscopy was performed using a Leica TCS SP-II microscope (Leica, Wetzlar, FRG). For detection, Cy-5 was used as fluorochrom (Dianova, Hamburg, FRG) as cartilage tissue did not show any autofluorescence at the Cy-5 excitation wave length.

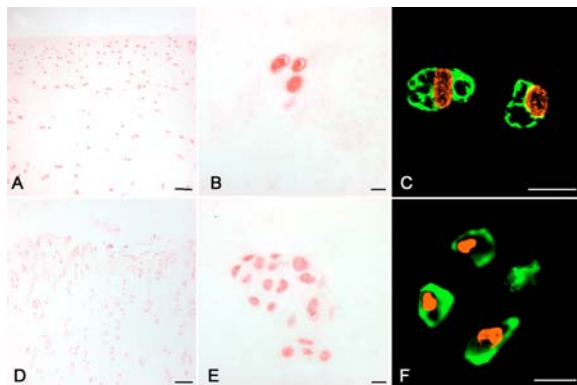
For immunofluorescence, slides were pretreated and the antibodies applied as outlined above, but the detection was performed with the tyramide amplification kit (TSA Biotin system, NEN Life Science Products, Leiden, The Netherlands) according to the manufacturer's protocol.

### 3.13. Control experiments

In control experiments, primary or secondary antibodies were replaced by PBS and the samples processed as described above. Additionally, in some test specimens similar dilutions of non-immune serum replaced the primary antibodies. All control samples were negative.



**Figure 1.** A: Demonstration of RhoB mRNA expression in normal (lanes 2-4) and osteoarthritic (lanes 5-7) articular cartilage by conventional PCR (amplificate: 121 bp). (lanes 1: 100 bp-DNA-ladder, MBI Fermentas, FRG, lane 8: water control). B: Quantitative TAQMAN-analysis for mRNA expression levels of RhoB in normal ( $n=7$ ), early degenerative ("early deg.",  $n=8$ ), and late stage osteoarthritic ("late OA",  $n=8$ ) cartilage. Given are the ratios per GAPDH.



**Figure 2.** Immunohistochemical demonstration of RhoB in normal (A-C) and osteoarthritic (D-F) cartilage. Cellular staining pattern spared the nuclei (C,F: laser scanning confocal microscopy: blue shows immunostaining for RhoB, red: nuclear counterstain). Chondrocytes in cell clusters (E) showed also positive immunostaining. (magnification bars: A,D: 100 $\mu$ m, B,C,E,F: 10 $\mu$ m).

### 3.14. Statistical analysis

Statistical evaluation of significant differences in expression levels was done by the non-parametric Wilcoxon-Mann-Whitney test for the *in vivo* investigations. For the *in vitro* probes the t-test for pairwise comparison was used. P-values below 0.05 were considered as significant.

## 4. RESULTS

### 4.1. Expression analysis of RhoB in normal adult articular chondrocytes *in vivo*

Conventional PCR analysis showed easily detectable amplification products of RhoB in all samples of normal articular cartilage investigated ( $n=3$ , Figure 1A). For exact quantification of mRNA expression levels of RhoB, a TAQMAN-assay was developed as described in materials and methods. For standardization of the gene expression levels mRNA ratios relative to GAPDH were calculated. Online quantitative PCR analysis confirmed significant levels of RhoB expression in all samples of normal adult articular cartilage (average: 0, 16/GAPDH, figure 1B).

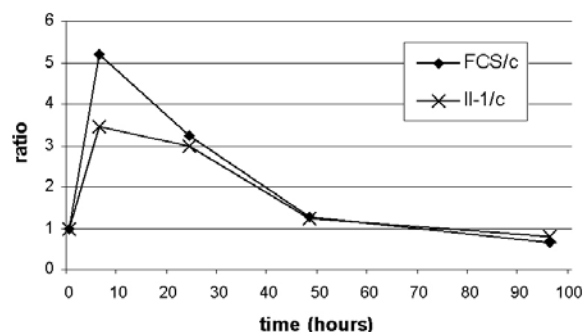
### 4.2. *In situ* localization of RhoB in adult articular cartilage - Confocal laser scanning microscopy

Next we were interested in the presence and tissue distribution of RhoB in adult articular cartilage: Immunolocalization using a RhoB specific monoclonal antibody showed strong cellular signals for RhoB in all chondrocytes of all zones of normal articular cartilage except some cells in the calcified zones which are known to be partially necrotic (6). Thus, overall no zonal distribution was detectable. By conventional immunostaining the signal for RhoB appeared to be selectively cellular with a mostly cytoplasmic distribution (Figure 2 A,B). For exact subcellular localization, immunofluorescence with subsequent confocal laser scanning microscopy was performed. These analyses showed a solely cytoplasmic staining pattern (Figure 2C). No signals were found in the nuclei and outside the cells as expected by previous localization studies (22).

### 4.3. Expression analysis of RhoB in degenerated osteoarthritic adult articular chondrocytes *in vivo*

Again, conventional PCR could show transcripts of RhoB in all samples of osteoarthritic cartilage investigated ( $n=3$ ). Quantitative expression analysis by online PCR confirmed the presence of RhoB mRNA expression also in late stage osteoarthritic chondrocytes, but at a significantly lower level (Figure 1B, 0,22x,  $p<0,005$ ). Of note, early degenerated chondrocytes did not show a significant decrease in RhoB mRNA levels compared to normal ones ( $p=0,24$ ).

Immunolocalization showed again cellular signals for RhoB in all chondrocytes of all zones, but at a reduced level (Figure 2 D,E). Confocal laser scanning microscopy showed again a solely cytoplasmic staining pattern (Figure 2 F). No signals were found in the nuclei and outside the cells as expected by previous localization studies (22, 23).



**Figure 3.** Relative ratios of RhoB mRNA expression in serum and IL-1beta-stimulated time course experiments (given are ratios compared to un-stimulated chondrocytes).

## 4.4. Expression analysis of RhoB *in vitro* - with and without anabolic (serum) or catabolic (IL-1beta) stimulation

Next, we were interested whether articular chondrocytes keep *in vitro* (after isolation from their surrounding matrix) their ability to express RhoB. RhoB was expressed by the cells, but at a very reduced level compared to chondrocytes *in situ* (6,7x,  $p < 0.02$ ). After stimulation with IL-1beta for 48 hrs we did not find a significant regulation of RhoB in the cells with and without the addition of serum ( $n=3+3$ ). Therefore, we performed a time course experiment using the cDNA array technology using IL-1beta and serum as known stimulants of RhoB. This showed a transient stimulation of RhoB by both, IL-1beta and serum at 6 and 24 hrs with largely normal expression levels after 48 hrs (Figure 3).

## 5. DISCUSSION

This study demonstrates the expression and presence of the small GTPase RhoB in adult human articular chondrocytes *in situ* as well as *in vivo*. No zonal distribution was detectable by immunostaining, suggesting that RhoB is constitutively expressed and essential for adult articular chondrocytes. The expression levels in normal cartilage were about 0.2 molecules /GAPDH similar to the expression levels of other low expressed genes in articular cartilage such as MMP-14 and collagen type II (24, 25), but considerably less than the levels of MMP-3 (11,3/GAPDH) (24). This is in line with our previously published cDNA array data which also showed stronger signals for MMP-3 than for RhoB (8). Compared to the *in vivo* situation, the levels of RhoB expression in cultured chondrocytes are low, though transiently inducible with serum (9) or interleukin-1. These experiments confirm the rather quick (and transient) up-regulation of RhoB as one type of cellular reaction to external stress stimuli (17, 18).

In this study, we were able to establish the down-regulation of RhoB in late stage osteoarthritic cartilage. The rather minor difference in staining intensity in between normal and osteoarthritic chondrocytes reflects the fact that immunostaining can not be considered to be (fully) quantitative, in particular if comparing rather abundant molecules, because good experimental evidence exists that

protein levels very closely follow mRNA expression (17, 18, 26).

From our data, one has to be careful to deduce too much for the functional role of RhoB in articular cartilage. Many of the targets of RhoB such as NFkB (27) and JNK (28) are known to be important intracellular mediators of stress signaling (i.e. MAPkinases etc.) also in articular chondrocytes (for review see Denhardt 1996 and Kracht 2002 (29, 30)), but also their role is not clearly understood within the cells. One intriguing speculation might be that the down-regulation of RhoB in osteoarthritic chondrocytes is at least partly a prerequisite for the sustained pre- or para-apoptotic phenotype of osteoarthritic chondrocytes (for review see Aigner *et al.* 2002 (7)): RhoB is known to be one important molecule in the induction of apoptotic cell death in response to DNA damage (16, 31). Osteoarthritic chondrocytes are known to have significant DNA damage (including strand breaks (4-6, 32)) among other cellular degenerative alterations. This would in normal circumstances induce RhoB expression (33, 34) and lead to apoptotic cell death. However, apoptosis is rather rare in osteoarthritic cartilage (6): instead, the chondrocytes stay in a pre- or para-apoptotic phenotype showing a picture of dis-coordinated cellular reaction pattern known from many studies of chondrocyte behavior *in vivo* and *in vitro*. Another alternative could be an involvement of RhoB in the activation or deactivation of the chondrocytes. Also, the down-regulation of RhoB could support the destabilization of the functional phenotype of chondrocytes in osteoarthritic joint degeneration (21, 35). This would fit to the fact that chondrocytes also significantly down-regulate RhoB expression *in vitro*, which similarly implicates loss of cellular phenotype and cellular activation. A third line of potential involvement might be the linkage of RhoB expression to the cell cycle: adult articular chondrocytes are thought to be largely post-mitotic (G0-cells) and are known to re-initiate proliferative activity in osteoarthritis (6) (36, 37). Similarly, adult chondrocytes regain proliferative activity after isolation from the surrounding matrix and culturing *in vitro*. This, however, suggests a different regulation pattern of RhoB in adult articular chondrocytes than in HeLa cells. In these cells, RhoB is first detectable at the G1/S phase transition with maximal expression levels during the S phase (18). At the S/G2-M transition it declines again. In contrast, adult chondrocytes show a clear signal also in the G0 phase. Still, RhoB might be involved in the delaying or stopping cell cycle progression (38) though not in the G1/S-phase transition as described for HeLa cells, because chondrocytes do not show a tetraploid DNA content (39).

So far, the cellular reaction pattern of the osteoarthritic disease process was basically divided into three categories (for review see (40)): (1) anabolic or catabolic activation, (2) phenotypic modulation, and (3) proliferation and (apoptotic) cell death. Our data as well as evidence from others (41) suggest a new important way to classify osteoarthritic chondrocytes, namely as (4) (pre-)senescent cells. This suggests that besides being related to an individual genetic background (i.e. related to gene defects) and continuous (over)load, osteoarthritis can at

least in part be considered as premature senescence of the articular cartilage and its cells. This fits very well to the well-known fact that age is the most relevant risk factor for developing osteoarthritic joint disease.

## 6. ACKNOWLEDGEMENT

We thank Drs. G. Zeiler and W. Eger (Orthopedic Hospital Rummelsberg) for providing osteoarthritic cartilage specimens and Mrs. S. Fickenscher and Mrs. O. Reimer for excellent technical assistance. Supported by the Federal Ministry of Education and Research (BMBF) and the Interdisciplinary Center of Clinical Research (IZKF) of the University Hospital of the University of Erlangen-Nürnberg.

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**Key Words:** Chondrocyte, Cell Senescence, Osteoarthritis, Cartilage, Apoptosis

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