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

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Abstract

Introduction: Chondrocytes experience a hypertonic environment compared to plasma (280 mOsm) due to the high fixed negative charge density of cartilage. Standard isolation of chondrocytes removes their hypertonic 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 tonicity during isolation and *in-vitro* expansion on chondrocyte phenotype.

Methods: Human articular chondrocytes were isolated and subsequently expanded at control tonicity (280 mOsm) or at moderately elevated, physiological, tonicity (380 mOsm). The effects of physiological tonicity on chondrocyte proliferation and chondrogenic marker expression were evaluated. The role of Tonicity-responsive Enhancer Binding Protein (TonEBP/NFAT5) in response to physiological tonicity was investigated using nuclear factor of activated T-cells 5 (NFAT5) RNA interference.

Results: Moderately elevated, physiological, tonicity (380 mOsm) did not affect chondrocyte proliferation, while higher tonicities inhibited proliferation and diminished cell viability. Physiological tonicity 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 tonicity were similar in osteoarthritic and 'normal' (non-osteoarthritic) chondrocytes, indicating a disease-independent mechanism. NFAT5 RNA interference abolished tonicity-mediated effects and revealed that NFAT5 positively regulates collagen type II expression, while suppressing type I.

Conclusions: Physiological tonicity 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 tonicity (i.e. osmotic pressure) of the extracellular fluid around chondrocytes *in vivo*, but the tonicity indirectly also largely depends on the quality of the collagen network. Extracellular tonicity in healthy cartilage ranges between 350 and 480 mOsm [3, 4]. *In vivo*, tonicity 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 tonicity of between 280 and 350 mOsm [3, 6]. Currently, chondrocyte isolation and *in-vitro* expansion culture are performed in medium of non-physiological tonicity (270±20 mOsm). Several studies already showed that chondrocytes are tonicity 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 hypertonic response of human articular chondrocytes (HACs) are poorly understood. Hypertonicity 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 hypertonic stress, transcription of NFAT5 itself is up-regulated several cell types [26-28], but the tonicity 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 tonicity (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 isolation of human articular chondrocytes (HACs) under standard conditions (DMEM, 280 mOsm) as described by Das et al. [39], medium tonicity 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 tonicity (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 tonicity (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

hypertonic 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 [43]) 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) [44, 45] and optimized by A. Schambach (Department of Experimental Hematology, Hannover Medical School, Hannover, Germany) [46] to express eGFP from the phosphoglycerate kinase promoter. Lentiviral particles were produced in HEK293T cells by transient transfection using a calcium phosphate protocol [47]. 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 knock-down efficiency determination and used in subsequent experiments.

Passage 1 OA-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 [48]. 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, 49, 50]. QPCR assays for *COL2, SOX9, AGC1 and COL1* were reported earlier [51]. 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 [52].

Western Blot analysis.

Cells seeded at high densities were washed twice with PBS and lysed in RIPA buffer [53] 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

Hypertonicity influences proliferation and survival of chondrocytes.

We first determined the influence of tonicity 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 tonicity 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 (Figure 1; photo C). The differences in appearance remained throughout dedifferentiation period (P0 to P3), but were most apparent at earlier passages.

Using cell counts and DNA assays, doubling times were calculated from growth curves established from each passage at three different tonicities (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 tonicity condition) and compared to 280 mOsm (control condition).

Isolation and expansion of chondrocytes under hypertonic conditions improves their phenotype.

Next, we set out to determine whether expansion culture in physiological tonicity improves the chondrocytic phenotype. Physiological tonicity (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 tonicity 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 tonicity 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 tonicity significantly decreased COL1 protein expression (Figure 2F), from 2-fold in P0 cells to 13-fold in P1

cells. Physiological tonicity also significantly increased *AGC1* (Figure 3A) and *SOX9* (Figure 3B) mRNA levels in non-osteoarthritic HACs (NHACs). Furthermore, *COL2* mRNA levels were significantly up-regulated; from 5.8-fold in P0 cells to 270-fold in expanded P3 NHACs (Figure 3C). Like in OA-HACs, hypertonicity 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).

Hypertonicity 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 hypertonicity-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 hypertonic stimulation. 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 hypertonically inducible: *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).

Discussion

Isolation and expansion of adult human articular chondrocytes under physiological tonicity (380 mOsm) improves expression of chondrogenic markers on mRNA and protein level. While other studies partially confirm that non-human chondrocytes respond to tonicity 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 [51]. Interestingly, NFAT5 seems to be crucially involved in this differential regulation upon hypertonic challenge: it positively regulates collagen type II, while suppressing collagen type I (Figure 5B). Fibrocartilage, occurring in areas subject to frequent stress like intervertebral discs and tendon attachment sites, is more rich in collagen type I than hyaline cartilage [54]. Tonicity may thus provide a simple means to manipulate expression of these two collagens for broader applications than regenerative chondrocyte implantations (ACI or CCI) alone [55].

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.

Hypertonicity 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 hypertonicity. However, we failed to show NFAT5 protein expression by Western blotting. Whether this is due to low protein abundance in our cells or technical issues like poor extraction efficiency of this very large transcription factor has to be elucidated in future experiments.

Hypertonicity 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 tonicity-mediated effects. We used NMDG-Cl, a bulky substitute for small cations that is impermeable to almost all known channels [56], 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 tonicity 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 tonicity 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 tonicity, 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 [57, 58] involves cytoskeletal changes [59]. Exposing these cells to physiological tonicity 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 hypertonic treatment protocol.

The precise molecular mechanism by which tonicity is sensed by cells is still poorly understood. Hypertonicity-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 hypertonic 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, 60], were induced accordingly after hypertonic 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 [61]. Sensation of such basic responses might not be different in chondrocytes than in other cells. Rho-type small G proteins [62] and p38 kinases [63, 64] 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 hypertonic induction of the

osmo-sensitive [65, 66] serine-threonine protein kinase Sgk-1 [67, 68]. 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 tonicity to explain our increase in mRNA by this phenomenon or whether active transcription is involved, has to be addressed in other studies. Interestingly, very recently Tew et al. [69] showed that the mRNA of SOX9, an important regulator of COL2 expression, is stabilized by supra-physiological tonicity. 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 tonicity.

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 tonicity on promoter activity and mRNA stability of AGC1 are incompletely understood. Other groups described the complexity of osmotic stress on gene expression [70, 71]. 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 [50]. However, aggrecan expression has been reported to be influenced by both hyper- and hypo-tonicity [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 [72, 73], Barx2 [74], β-catenin [75], c-Maf [76], PIAS [77], TRAP230 [78], Bapx1 [79], and C/EBP and NF-kB [80]. Chondrogenic differentiation and SOX9-dependency of aggrecan and collagen expression may also be differentially modulated by these transcriptional co-factors under different tonicities. 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 [81]. Of note, the human aggrecan promoter sequence has been shown to contain a

conserved NFAT5 binding site [82]. In nucleus pulposus cells, SOX9 mediated aggrecan expression has recently been shown to critically depend on PI3K/AKT signalling [83]. Moreover, while high NaCl rapidly activates p38 MAPK, its action can be isoform specific and may exert opposing effects on NFAT5 [84], which in turn may influence COL2A1 and AGC1 transcription differently in a tonicity-dependent manner. We are therefore currently looking into the underlying molecular mechanisms regulating aggrecan (AGC1) and collagen type II (COL2) expression in both conditions.

With respect to regenerative medical applications, the high-end hypertonic conditions used by Tew et al. can be considered a limitation of that study. In our hands, these tonicity 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 [85, 86]. 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 tonicity in solution [87], enabling NFAT5 to exert its biological activity over a wide tonicity range [88, 89]. 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 [90], NFAT5 dependents on nuclear localization and export sequences for its nuclear translocation [26, 87, 90]. In most cells, NFAT5 is equally distributed between cytoplasm and nucleus at physiological tonicity (±300 mOsm), whereas at 500 mOsm most of it localizes to the nucleus [19, 26, 88].

To demonstrate that the hypertonicity-induced chondrogenic marker expression was indeed mediated by NFAT5, we used RNAi to confirm that knockdown of NFAT5 significantly i) inhibited hypertonic induction of its own transcription as discussed before, (ii) suppressed the tonicity-mediated induction of known NFAT5 targets, and (iii) most importantly, eliminated the hypertonicity-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 tonicity (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.

Abbreviations

HACs, human articular chondrocytes; mOsm, milliosmoles per kg of water; NFAT, nuclear factor of activated T-cells; NHAC, non-osteoarthritic HACs; NMDG-Cl, N-methyl-D-glucamine chloride ; OA, osteoarthritis; PG, proteoglycan; RNAi, RNA interference; TonEBP, Tonicity-responsive Enhancer Binding Protein.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HJ conceived the study. AW, HW, JV, HJ designed the study. AW, EH, RD analyzed the data. AW, EH, NK, TW, MC performed the experiments. NT, TW, MC contributed reagents/materials/analysis tools. AW, HJ wrote the paper. All authors read and approved the final manuscript.

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References

1. Mow VC RA (Ed.). **Basic Orthopaedic Biomechanics** Lippincott-Raven; 1997.
2. Lesperance LM, Gray ML, Burstein D: **Determination of fixed charge density in cartilage using nuclear magnetic resonance.** *J Orthop Res* 1992, **10:**1-13.
3. Maroudas AI: **Balance between swelling pressure and collagen tension in normal and degenerate cartilage.** *Nature* 1976, **260:**808-809.
4. Urban JP, Hall AC, Gehl KA: **Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes.** *J Cell Physiol* 1993, **154:**262-270.
5. Urban JP: **The chondrocyte: a cell under pressure.** *Br J Rheumatol* 1994, **33:**901-908.
6. Bank RA, Soudry M, Maroudas A, Mizrahi J, TeKoppele JM: **The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation.** *Arthritis Rheum* 2000, **43:**2202-2210.
7. Bush PG, Hall AC: **Passive osmotic properties of in situ human articular chondrocytes within non-degenerate and degenerate cartilage.** *J Cell Physiol* 2005, **204:**309-319.
8. Palmer GD, Chao Ph PH, Raia F, Mauck RL, Valhmu WB, Hung CT: **Time-dependent aggrecan gene expression of articular chondrocytes in response to hyperosmotic loading.** *Osteoarthritis Cartilage* 2001, **9:**761-770.
9. Bush PG, Hall AC: **Regulatory volume decrease (RVD) by isolated and in situ bovine articular chondrocytes.** *J Cell Physiol* 2001, **187:**304-314.
10. Hung CT, LeRoux MA, Palmer GD, Chao PH, Lo S, Valhmu WB: **Disparate aggrecan gene expression in chondrocytes subjected to hypotonic and hypertonic loading in 2D and 3D culture.** *Biorheology* 2003, **40:**61-72.
11. Negoro K, Kobayashi S, Takeno K, Uchida K, Baba H: **Effect of osmolarity on glycosaminoglycan production and cell metabolism of articular chondrocyte under three-dimensional culture system.** *Clin Exp Rheumatol* 2008, **26:**534-541.
12. Burg MB: **Macromolecular crowding as a cell volume sensor.** *Cell Physiol Biochem* 2000, **10:**251-256.
13. Neuhofer W, Woo SK, Na KY, Grunbein R, Park WK, Nahm O, Beck FX, Kwon HM: **Regulation of TonEBP transcriptional activator in MDCK cells following changes in ambient tonicity.** *Am J Physiol Cell Physiol* 2002, **283:**C1604-1611.
14. Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN: **Living with water stress: evolution of osmolyte systems.** *Science* 1982, **217:**1214-1222.
15. Kultz D, Chakravarty D: **Hyperosmolality in the form of elevated NaCl but not urea causes DNA damage in murine kidney cells.** *Proc Natl Acad Sci U S A* 2001, **98:**1999-2004.
16. Woo SK, Lee SD, Kwon HM: **TonEBP transcriptional activator in the cellular response to increased osmolality.** *Pflugers Arch* 2002, **444:**579-585.
17. Racz B, Reglodi D, Fodor B, Gasz B, Lubics A, Gallyas F, Jr., Roth E, Borsiczky B: **Hyperosmotic stress-induced apoptotic signaling pathways in chondrocytes.** *Bone* 2007, **40:**1536-1543.
18. Burg MB, Kwon ED, Kultz D: **Regulation of gene expression by hypertonicity.** *Annu Rev Physiol* 1997, **59:**437-455.
19. Miyakawa H, Woo SK, Dahl SC, Handler JS, Kwon HM: **Tonicity-responsive enhancer binding protein, a rel-like protein that stimulates transcription in response to hypertonicity.** *Proc Natl Acad Sci U S A* 1999, **96:**2538-2542.
20. Lopez-Rodriguez C, Antos CL, Shelton JM, Richardson JA, Lin F, Novobrantseva TI, Bronson RT, Igarashi P, Rao A, Olson EN: **Loss of NFAT5 results in renal atrophy and lack of tonicity-responsive gene expression.** *Proc Natl Acad Sci U S A* 2004, **101:**2392-2397.
21. Na KY, Woo SK, Lee SD, Kwon HM: **Silencing of TonEBP/NFAT5 transcriptional activator by RNA interference.** *J Am Soc Nephrol* 2003, **14:**283-288.
22. Kasono K, Saito T, Tamemoto H, Yanagidate C, Uchida S, Kawakami M, Sasaki S, Ishikawa SE: **Hypertonicity regulates the aquaporin-2 promoter independently of arginine vasopressin.** *Nephrol Dial Transplant* 2005, **20:**509-515.
23. Chen M, Sinha M, Luxon BA, Bresnick AR, O'Connor KL: **Integrin alpha6beta4 controls the expression of genes associated with cell motility, invasion, and metastasis, including S100A4/metastasin.** *J Biol Chem* 2009, **284:**1484-1494.
24. Ito T, Kimura Y, Uozumi Y, Takai M, Muraoka S, Matsuda T, Ueki K, Yoshiyama M, Ikawa M, Okabe M, Schaffer SW, Fujio Y, Azuma J: **Taurine depletion caused by knocking out the**

taurine transporter gene leads to cardiomyopathy with cardiac atrophy. *J Mol Cell Cardiol* 2008, **44:**927-937.
25. Nakamura N, Takenaga K: **Hypomethylation of the metastasis-associated S100A4 gene correlates with gene activation in human colon adenocarcinoma cell lines.** *Clin Exp Metastasis* 1998, **16:**471-479.
26. Ko BC, Turck CW, Lee KW, Yang Y, Chung SS: **Purification, identification, and characterization of an osmotic response element binding protein.** *Biochem Biophys Res Commun* 2000, **270:**52-61.
27. Cai Q, Ferraris JD, Burg MB: **High NaCl increases TonEBP/OREBP mRNA and protein by stabilizing its mRNA.** *Am J Physiol Renal Physiol* 2005, **289:**F803-807.
28. Woo SK, Dahl SC, Handler JS, Kwon HM: **Bidirectional regulation of tonicity-responsive enhancer binding protein in response to changes in tonicity.** *Am J Physiol Renal Physiol* 2000, **278:**F1006-1012.
29. Morancho B, Minguillon J, Molkentin JD, Lopez-Rodriguez C, Aramburu J: **Analysis of the transcriptional activity of endogenous NFAT5 in primary cells using transgenic NFAT-luciferase reporter mice.** *BMC Mol Biol* 2008, **9:**13.
30. Widuchowski W, Widuchowski J, Trzaska T: **Articular cartilage defects: study of 25,124 knee arthroscopies.** *Knee* 2007, **14:**177-182.
31. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L: **Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation.** *N Engl J Med* 1994, **331:**889-895.
32. Saris DB, Vanlauwe J, Victor J, Haspl M, Bohnsack M, Fortems Y, Vandekerckhove B, Almqvist KF, Claes T, Handelberg F, Lagae K, van der Bauwhede J, Vandenneucker H, Yang KG, Jelic M, Verdonk R, Veulemans N, Bellemans J, Luyten FP: **Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture.** *Am J Sports Med* 2008, **36:**235-246.
33. von der Mark K, Gauss V, von der Mark H, Muller P: **Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture.** *Nature* 1977, **267:**531-532.
34. Schnabel M, Marlovits S, Eckhoff G, Fichtel I, Gotzen L, Vecsei V, Schlegel J: **Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture.** *Osteoarthritis Cartilage* 2002, **10:**62-70.
35. Benya PD, Padilla SR, Nimni ME: **Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture.** *Cell* 1978, **15:**1313-1321.
36. Setton LA, Mow VC, Muller FJ, Pita JC, Howell DS: **Altered structure-function relationships for articular cartilage in human osteoarthritis and an experimental canine model.** *Agents Actions Suppl* 1993, **39:**27-48.
37. Loeser RF: **Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix.** *Osteoarthritis Cartilage* 2009, **17:**971-979.
38. Gouttenoire J, Valcourt U, Ronziere MC, Aubert-Foucher E, Mallein-Gerin F, Herbage D: **Modulation of collagen synthesis in normal and osteoarthritic cartilage.** *Biorheology* 2004, **41:**535-542.
39. Das RH, van Osch GJ, Kreukniet M, Oostra J, Weinans H, Jahr H: **Effects of individual control of pH and hypoxia in chondrocyte culture.** *J Orthop Res* 2009.
40. Karsten U, Wollenberger A: **Improvements in the ethidium bromide method for direct fluorometric estimation of DNA and RNA in cell and tissue homogenates.** *Anal Biochem* 1977, **77:**464-470.
41. de Mos M, van der Windt AE, Jahr H, van Schie HT, Weinans H, Verhaar JA, van Osch GJ: **Can platelet-rich plasma enhance tendon repair? A cell culture study.** *Am J Sports Med* 2008, **36:**1171-1178.
42. Stewart SA, Dykxhoorn DM, Palliser D, Mizuno H, Yu EY, An DS, Sabatini DM, Chen IS, Hahn WC, Sharp PA, Weinberg RA, Novina CD: **Lentivirus-delivered stable gene silencing by RNAi in primary cells.** *RNA* 2003, **9:**493-501.
43. **Sigma-Aldrich** [http://www.sigmaaldrich.com]
44. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L: **A third-generation lentivirus vector with a conditional packaging system.** *J Virol* 1998, **72:**8463-8471.
45. Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D: **Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery.** *J Virol* 1998, **72:**9873-9880.

46. Schambach A, Bohne J, Baum C, Hermann FG, Egerer L, von Laer D, Giroglou T: **Woodchuck hepatitis virus post-transcriptional regulatory element deleted from X protein and promoter sequences enhances retroviral vector titer and expression.** *Gene Ther* 2006, **13:**641-645.
47. Follenzi A, Naldini L: **HIV-based vectors. Preparation and use.** *Methods Mol Med* 2002, **69:**259-274.
48. Uitterlinden EJ, Jahr H, Koevoet JL, Jenniskens YM, Bierma-Zeinstra SM, Degroot J, Verhaar JA, Weinans H, van Osch GJ: **Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants.** *Osteoarthritis Cartilage* 2006, **14:**250-257.
49. 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 Eng Part A*, **16:**1-10.
50. Tew SR, Hardingham TE: **Regulation of SOX9 mRNA in human articular chondrocytes involving p38 MAPK activation and mRNA stabilization.** *J Biol Chem* 2006, **281:**39471-39479.
51. Mandl EW, Jahr H, Koevoet JL, van Leeuwen JP, Weinans H, Verhaar JA, van Osch GJ: **Fibroblast growth factor-2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear chondrocytes in monolayer culture.** *Matrix Biol* 2004, **23:**231-241.
52. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.** *Methods* 2001, **25:**402-408.
53. Kraveka JM, Schady D, Obeid LM, Ogretmen B: **Immunoprecipitation of human telomerase reverse transcriptase with telomerase activity.** *Anal Biochem* 2001, **291:**166-169.
54. Benjamin M, Ralphs JR: **Biology of fibrocartilage cells.** *Int Rev Cytol* 2004, **233:**1-45.
55. Wuertz K, Urban JP, Klasen J, Ignatius A, Wilke HJ, Claes L, Neidlinger-Wilke C: **Influence of extracellular osmolarity and mechanical stimulation on gene expression of intervertebral disc cells.** *J Orthop Res* 2007, **25:**1513-1522.
56. Duranton C, Huber SM, Tanneur V, Brand VB, Akkaya C, Shumilina EV, Sandu CD, Lang F: **Organic osmolyte permeabilities of the malaria-induced anion conductances in human erythrocytes.** *J Gen Physiol* 2004, **123:**417-426.
57. Darling EM, Athanasiou KA: **Retaining zonal chondrocyte phenotype by means of novel growth environments.** *Tissue Eng* 2005, **11:**395-403.
58. Murphy CL, Sambanis A: **Effect of oxygen tension and alginate encapsulation on restoration of the differentiated phenotype of passaged chondrocytes.** *Tissue Eng* 2001, **7:**791-803.
59. Eric M. Darling PEP, Benjamin A. Evans, Richard Superfine, Stefan Zauscher, Farshid Guilak: **Mechanical Properties and Gene Expression of Chondrocytes on Micropatterned Substrates Following Dedifferentiation in Monolayer** *Cellular and Molecular Bioengineering* 2009, **2:**395-404.
60. Jeon US, Kim JA, Sheen MR, Kwon HM: **How tonicity regulates genes: story of TonEBP transcriptional activator.** *Acta Physiol (Oxf)* 2006, **187:**241-247.
61. Kino T, Takatori H, Manoli I, Wang Y, Tiulpakov A, Blackman MR, Su YA, Chrousos GP, DeCherney AH, Segars JH: **Brx mediates the response of lymphocytes to osmotic stress through the activation of NFAT5.** *Sci Signal* 2009, **2:**ra5.
62. Kelkar N, Standen CL, Davis RJ: **Role of the JIP4 scaffold protein in the regulation of mitogen-activated protein kinase signaling pathways.** *Mol Cell Biol* 2005, **25:**2733-2743.
63. Lopez-Rodriguez C, Aramburu J, Rakeman AS, Rao A: **NFAT5, a constitutively nuclear NFAT protein that does not cooperate with Fos and Jun.** *Proc Natl Acad Sci U S A* 1999, **96:**7214-7219.
64. Ho SN: **Intracellular water homeostasis and the mammalian cellular osmotic stress response.** *J Cell Physiol* 2006, **206:**9-15.
65. Nishida Y, Nagata T, Takahashi Y, Sugahara-Kobayashi M, Murata A, Asai S: **Alteration of serum/glucocorticoid regulated kinase-1 (sgk-1) gene expression in rat hippocampus after transient global ischemia.** *Brain Res Mol Brain Res* 2004, **123:**121-125.
66. Rozansky DJ, Wang J, Doan N, Purdy T, Faulk T, Bhargava A, Dawson K, Pearce D: **Hypotonic induction of SGK1 and Na+ transport in A6 cells.** *Am J Physiol Renal Physiol* 2002, **283:**F105-113.

67. Chen S, Grigsby CL, Law CS, Ni X, Nekrep N, Olsen K, Humphreys MH, Gardner DG: **Tonicity-dependent induction of Sgk1 expression has a potential role in dehydration-induced natriuresis in rodents.** *J Clin Invest* 2009, **119:**1647-1658.
68. Webster MK, Goya L, Ge Y, Maiyar AC, Firestone GL: **Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum.** *Mol Cell Biol* 1993, **13:**2031-2040.
69. Tew S, Peffers M, McKay T, Lowe E, Khan W, Hardingham T, Clegg P: **Hyperosmolarity regulates SOX9 mRNA post transcriptionally in human articular chondrocytes.** *Am J Physiol Cell Physiol* 2009.
70. Chao PH, West AC, Hung CT: **Chondrocyte intracellular calcium, cytoskeletal organization, and gene expression responses to dynamic osmotic loading.** *Am J Physiol Cell Physiol* 2006, **291:**C718-725.
71. Finan JD, Guilak F: **The effects of osmotic stress on the structure and function of the cell nucleus.** *J Cell Biochem*, **109:**460-467.
72. Ikeda T, Kamekura S, Mabuchi A, Kou I, Seki S, Takato T, Nakamura K, Kawaguchi H, Ikegawa S, Chung UI: **The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage.** *Arthritis Rheum* 2004, **50:**3561-3573.
73. Lefebvre V, Behringer RR, de Crombrugghe B: **L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway.** *Osteoarthritis Cartilage* 2001, **9 Suppl A:**S69-75.
74. Meech R, Edelman DB, Jones FS, Makarenkova HP: **The homeobox transcription factor Barx2 regulates chondrogenesis during limb development.** *Development* 2005, **132:**2135-2146.
75. Akiyama H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, Zhang Z, Deng JM, Taketo MM, Nakamura T, Behringer RR, McCrea PD, de Crombrugghe B: **Interactions between Sox9 and beta-catenin control chondrocyte differentiation.** *Genes Dev* 2004, **18:**1072-1087.
76. Huang W, Lu N, Eberspaecher H, De Crombrugghe B: **A new long form of c-Maf cooperates with Sox9 to activate the type II collagen gene.** *J Biol Chem* 2002, **277:**50668-50675.
77. Hattori T, Eberspaecher H, Lu J, Zhang R, Nishida T, Kahyo T, Yasuda H, de Crombrugghe B: **Interactions between PIAS proteins and SOX9 result in an increase in the cellular concentrations of SOX9.** *J Biol Chem* 2006, **281:**14417-14428.
78. Zhou R, Bonneaud N, Yuan CX, de Santa Barbara P, Boizet B, Schomber T, Scherer G, Roeder RG, Poulat F, Berta P: **SOX9 interacts with a component of the human thyroid hormone receptor-associated protein complex.** *Nucleic Acids Res* 2002, **30:**3245-3252.
79. Yamashita S, Andoh M, Ueno-Kudoh H, Sato T, Miyaki S, Asahara H: **Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes.** *Exp Cell Res* 2009, **315:**2231-2240.
80. Ushita M, Saito T, Ikeda T, Yano F, Higashikawa A, Ogata N, Chung U, Nakamura K, Kawaguchi H: **Transcriptional induction of SOX9 by NF-kappaB family member RelA in chondrogenic cells.** *Osteoarthritis Cartilage* 2009, **17:**1065-1075.
81. Aigner T, Gebhard PM, Schmid E, Bau B, Harley V, Poschl E: **SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes.** *Matrix Biol* 2003, **22:**363-372.
82. Tsai TT, Danielson KG, Guttapalli A, Oguz E, Albert TJ, Shapiro IM, Risbud MV: **TonEBP/OREBP is a regulator of nucleus pulposus cell function and survival in the intervertebral disc.** *J Biol Chem* 2006, **281:**25416-25424.
83. Cheng CC, Uchiyama Y, Hiyama A, Gajghate S, Shapiro IM, Risbud MV: **PI3K/AKT regulates aggrecan gene expression by modulating Sox9 expression and activity in nucleus pulposus cells of the intervertebral disc.** *J Cell Physiol* 2009, **221:**668-676.
84. Zhou X, Ferraris JD, Dmitrieva NI, Liu Y, Burg MB: **MKP-1 inhibits high NaCl-induced activation of p38 but does not inhibit the activation of TonEBP/OREBP: opposite roles of p38alpha and p38delta.** *Proc Natl Acad Sci U S A* 2008, **105:**5620-5625.
85. Brittberg M: **Autologous chondrocyte implantation--technique and long-term follow-up.** *Injury* 2008, **39 Suppl 1:**S40-49.
86. Brittberg M, Peterson L, Sjogren-Jansson E, Tallheden T, Lindahl A: **Articular cartilage engineering with autologous chondrocyte transplantation. A review of recent developments.** *J Bone Joint Surg Am* 2003, **85-A Suppl 3:**109-115.

87. Lopez-Rodriguez C, Aramburu J, Rakeman AS, Copeland NG, Gilbert DJ, Thomas S, Disteche C, Jenkins NA, Rao A: **NF-AT5: the NF-AT family of transcription factors expands in a new direction.** *Cold Spring Harb Symp Quant Biol* 1999, **64:**517-526.
88. Lopez-Rodriguez C, Aramburu J, Jin L, Rakeman AS, Michino M, Rao A: **Bridging the NFAT and NF-kappaB families: NFAT5 dimerization regulates cytokine gene transcription in response to osmotic stress.** *Immunity* 2001, **15:**47-58.
89. Lee SD, Woo SK, Kwon HM: **Dimerization is required for phosphorylation and DNA binding of TonEBP/NFAT5.** *Biochem Biophys Res Commun* 2002, **294:**968-975.
90. Cyert MS: **Regulation of nuclear localization during signaling.** *J Biol Chem* 2001, **276:**20805-20808.

Figure legends

Figure 1. Hypertonic 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.

Figure 2. Hypertonic 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).

Figure 3. Hypertonic 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).

Figure 4. Hypertonic 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).

Figure 5. NFAT5 knockdown inhibits hypertonicity-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 tonicity 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).

Table 1. Proliferation of chondrocytes isolated and cultured at 280, 380 and 480 mOsm

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 % *

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 *$p<0.05$. mOsm, milliosmoles per kg of water.

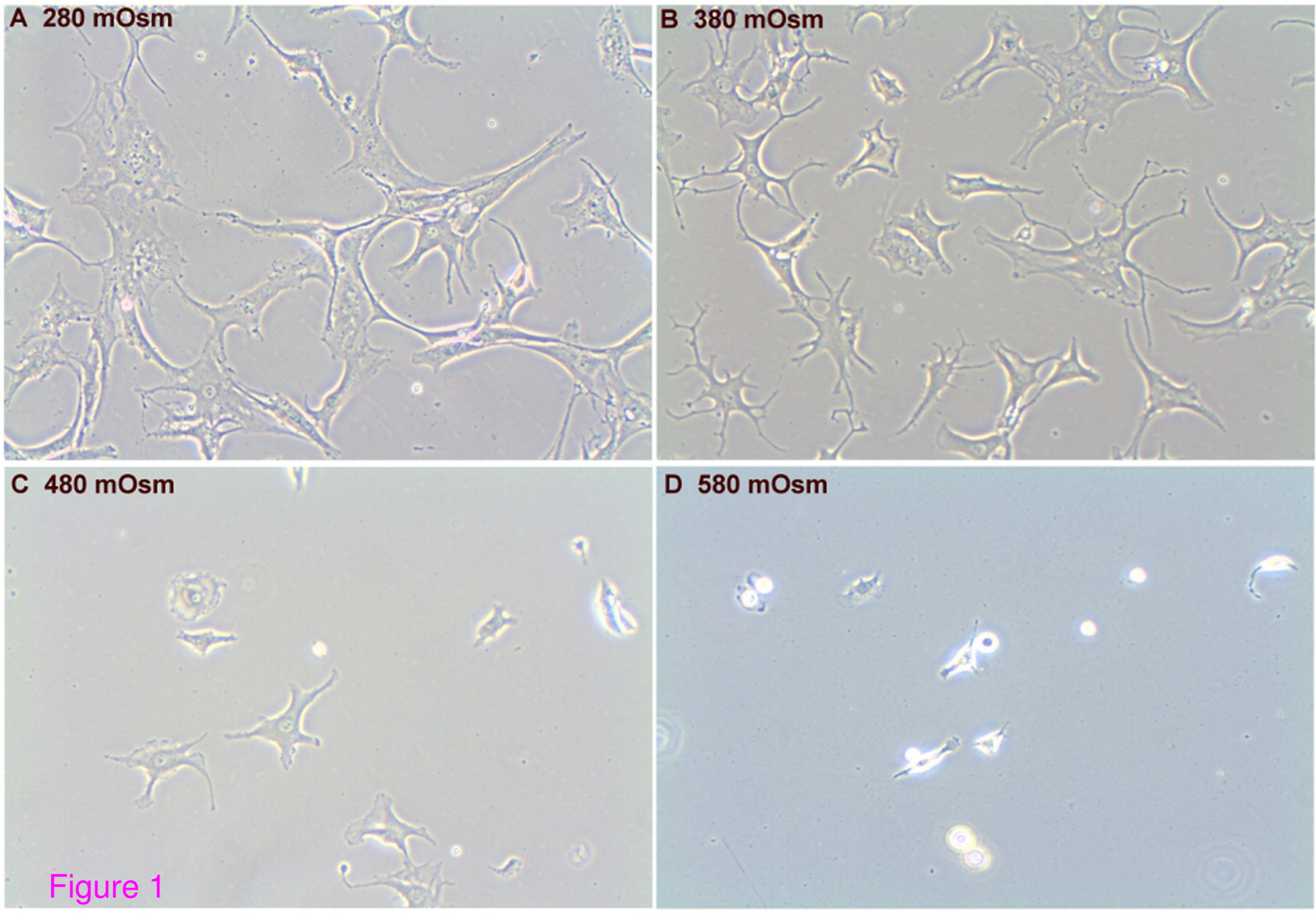


Figure 1

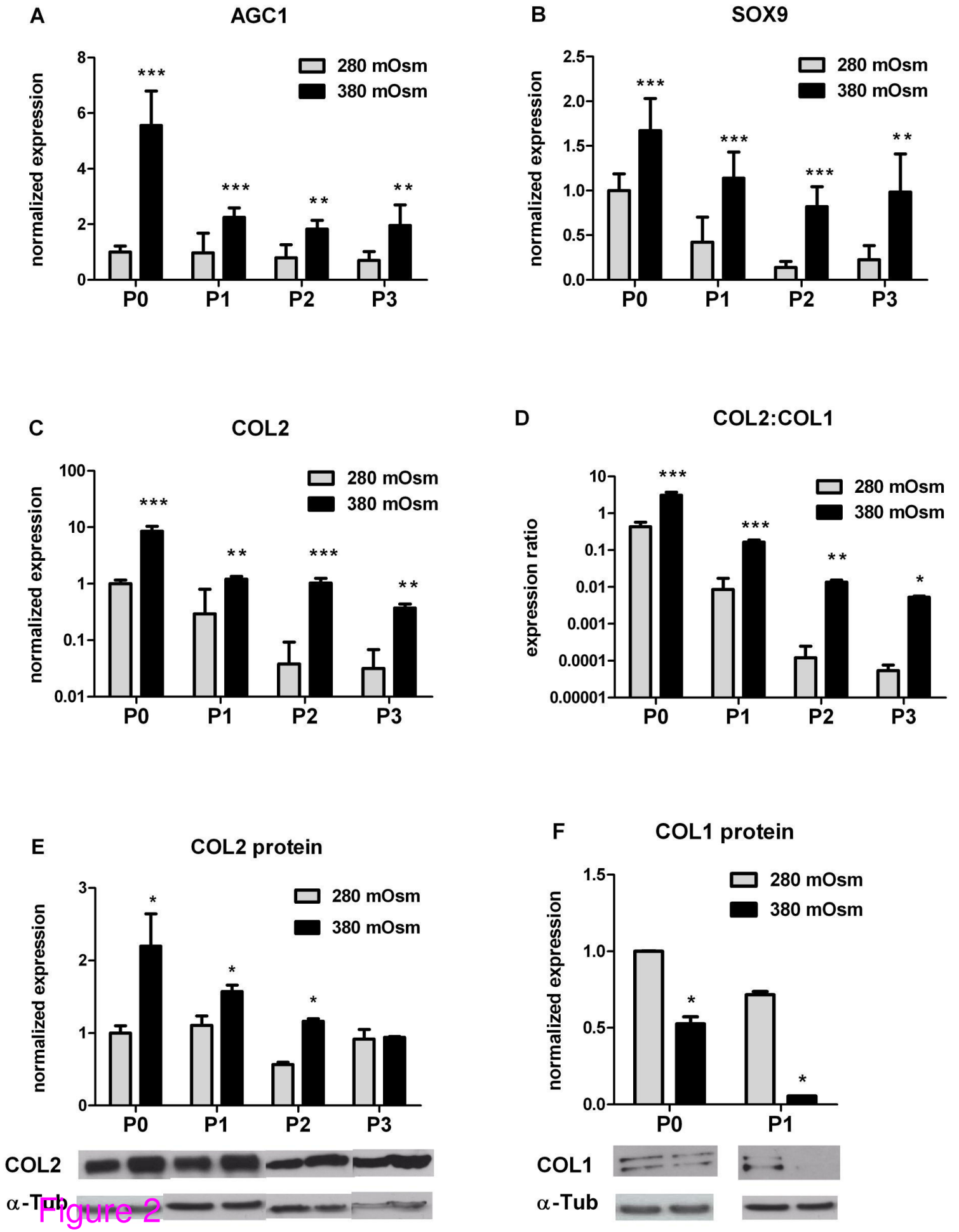
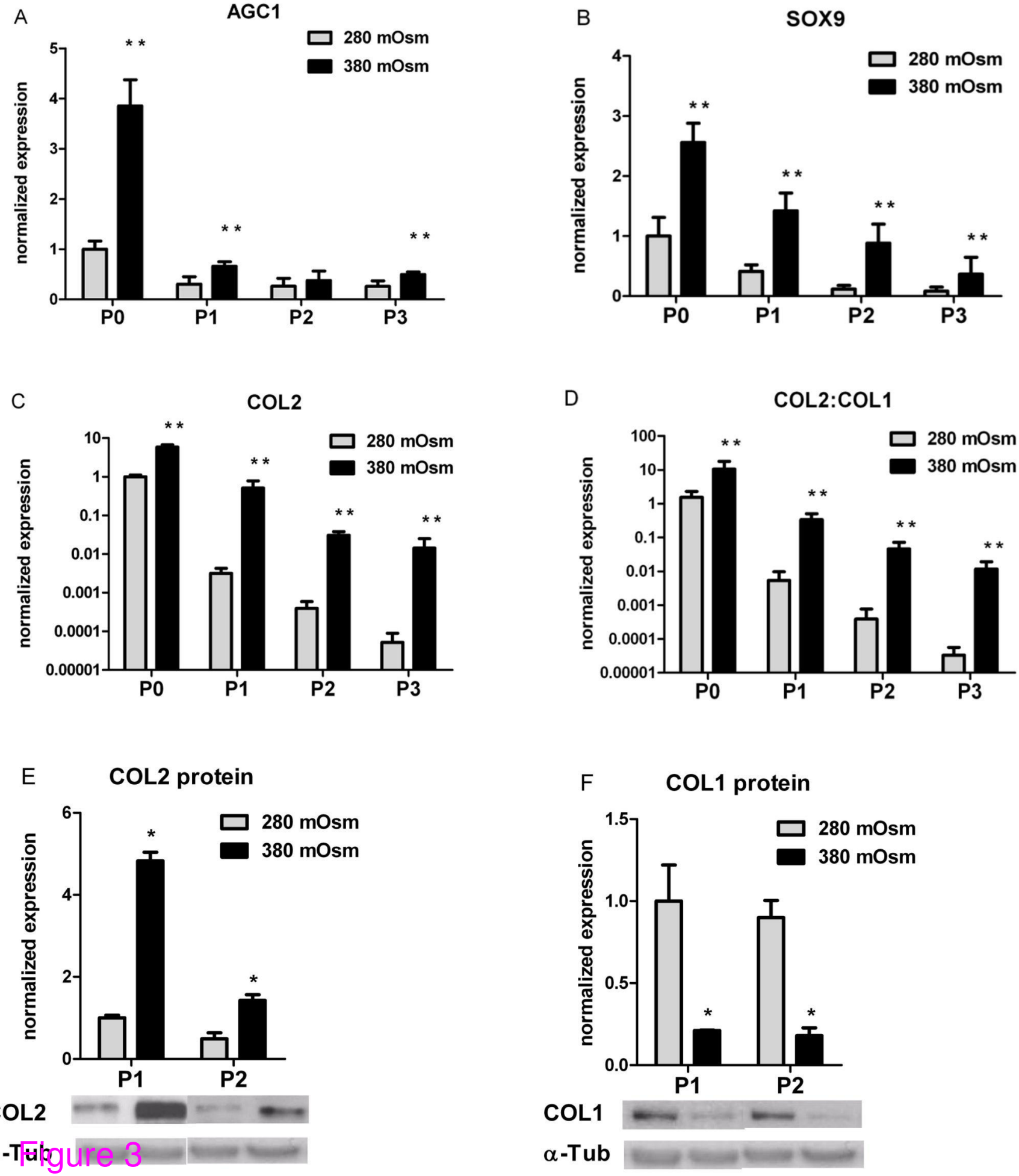

A
AGC1
normalized expression
280 mOsm
380 mOsm
P0
P1
P2
P3
B
SOX9
C
COL2
D
COL2:COL1
expression ratio
E
COL2 protein
COL2
α-Tub
F
COL1 protein
COL1
α-Tub

Figure 2


A
AGC1
280 mOsm
380 mOsm
normalized expression
P0
P1
P2
P3
B
SOX9
C
COL2
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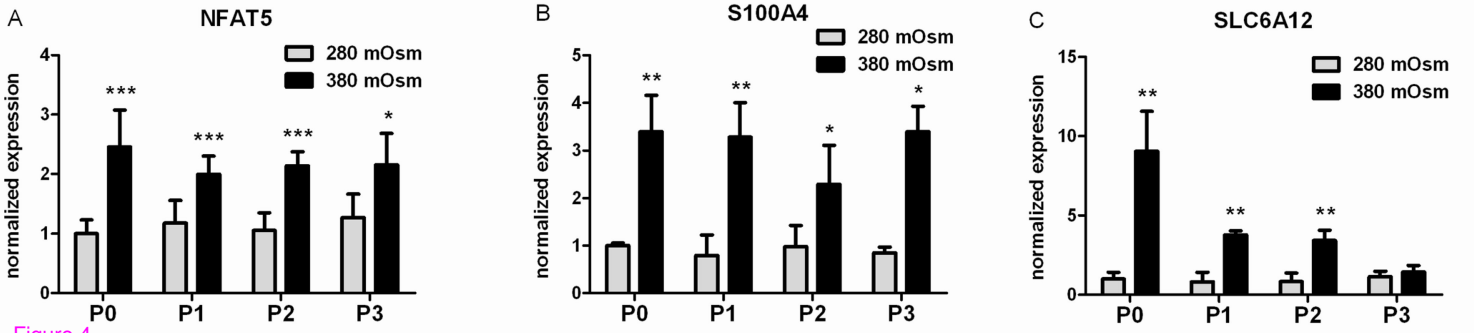
COL1 protein
COL2
α-Tub
COL1
α-Tub

Figure 3


A
NFAT5
280 mOsm
380 mOsm
normalized expression
4
3
2
1
0
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5
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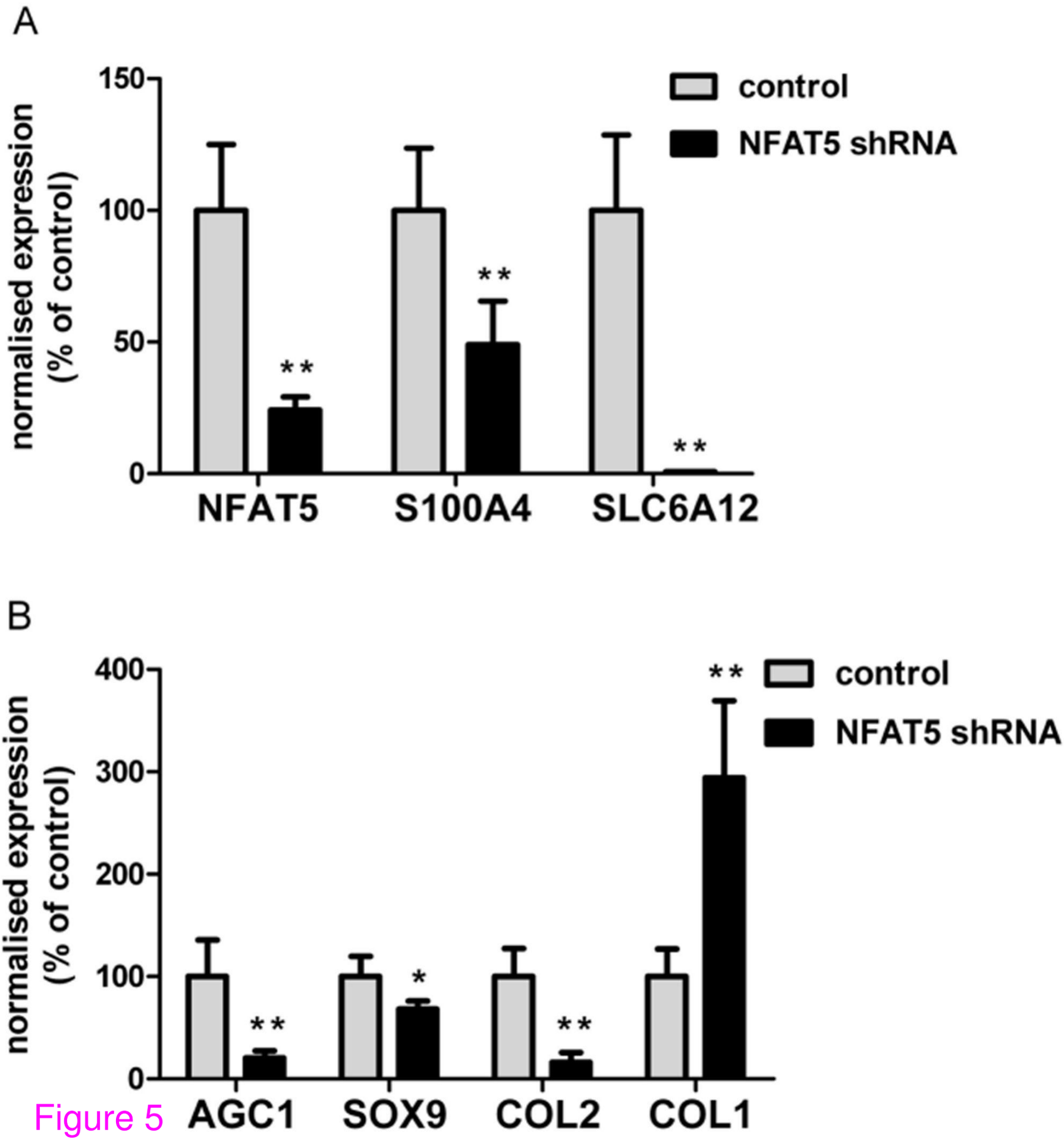
Figure 4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normalised expression
(% of control)
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NFAT5 shRNA
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SLC6A12
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normalised expression
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control
NFAT5 shRNA
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AGC1
SOX9
COL2
COL1

Figure 5