Rapamycin-Induced Hypoxia Inducible Factor 2A Is Essential for Chondrogenic Differentiation of Amniotic Fluid Stem Cells

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ABSTRACT

Amniotic fluid stem (AFS) cells represent a major source of donor cells for cartilage repair. Recently, it became clear that mammalian target of rapamycin (mTOR) inhibition has beneficial effects on cartilage homeostasis, but the effect of mTOR on chondrogenic differentiation is still elusive. Therefore, the objectives of this study were to investigate the effects of mammalian target of rapamycin complex 1 (mTORC1) modulation on the expression of SOX9 and on its downstream targets during chondrogenic differentiation of AFS cells. We performed three-dimensional pellet culturing of AFS cells and of in vitro-expanded, human-derived chondrocytes in the presence of chondrogenic factors. Inhibition of mTORC1 by rapamycin or by small interfering RNA-mediated targeting of raptor (gene name, RPTOR) led to increased AKT activation, upregulation of hypoxia inducible factor (HIF) 2A, and an increase in SOX9, COL2A1, and ACAN abundance. Here we show that HIF2A expression is essential for chondrogenic differentiation and that AKT activity regulates HIF2A amounts. Importantly, engraftment of AFS cells in cell pellets composed of human chondrocytes revealed an advantage of raptor knockdown cells compared with control cells in their ability to express SOX9. Our results demonstrate that mTORC1 inhibition leads to AKT activation and an increase in HIF2A expression. Therefore, we suggest that mTORC1 inhibition is a powerful tool for enhancing chondrogenic differentiation of AFS cells and also of in vitro-expanded adult chondrocytes before transplantation. Stem Cells Translational Medicine 2016;5:580–590

SIGNIFICANCE

Repair of cartilage defects is still an unresolved issue in regenerative medicine. Results of this study showed that inhibition of the mammalian target of rapamycin complex 1 (mTORC1) pathway, by rapamycin or by small interfering RNA-mediated targeting of raptor (gene name, RPTOR), enhanced amniotic fluid stem cell differentiation toward a chondrocytic phenotype and increased their engrafting efficiency into cartilaginous structures. Moreover, freshly isolated and in vitro passaged human chondrocytes also showed redifferentiation upon mTORC1 inhibition during culturing. Therefore, this study revealed that rapamycin could enable a more efficient clinical use of cell-based therapy approaches to treat articular cartilage defects.

INTRODUCTION

Human articular cartilage is an avascular tissue solely composed of chondrocytes, which secrete matrix components crucial for cartilage architecture and function. There is no direct supply of nutrients and oxygen to the chondrocytes; therefore, chondrogenic cells depend primarily on anaerobic metabolism [1]. Trauma and other injuries can lead to partial cartilage defects, for which cell-based therapy approaches are currently viewed as the most promising treatment options [2].

Autologous chondrocyte implantation (ACI) is a frequently used method to relieve patients from pain associated with cartilage degeneration. For this kind of treatment, chondrocytes have to be isolated from areas of the articular joint that bear less weight. Subsequently, isolated chondrocytes are expanded in vitro before transplantation into the defective area [3]. Unfortunately, cultured adult chondrocytes dedifferentiate over time and thereby lose their ability to secrete collagen type II, which negatively impacts their successful use during therapy [4].

Amniotic fluid stem (AFS) cells are considered to be a major source of donor cells for the treatment of cartilage defects because they are broadly multipotent [5, 6], can be differentiated into the
chondrocyte lineage [7–9], and are a safe alternative to potentially tumorigenic embryonic stem cells [10]. Stem cells can be biobanked and expanded to large amounts. Hence, patients only need to undergo a single surgical procedure when cells are transplanted into the joint tissue. These findings make AFCs cells very attractive for cell-based articular cartilage repair. However, practical experience with ACI has shown that the differentiation efficiency of the donor cells determines successful clinical outcome [11]. Therefore, AFCs-cell-based transplantation approaches will rely on efficient ways to differentiate cells before or during the transplantation process.

The mammalian target of rapamycin (mTOR) signaling pathway has been recognized as the master regulator of cellular turnover and metabolism [12]. The mammalian target of rapamycin complex 1 (mTORC1) signaling pathway plays a major role in the ability of cells to sense the availability of nutrients, growth factors, and oxygen [13, 14]. Several studies have already demonstrated the positive effects of the mTORC1 inhibitor rapamycin for the treatment of osteoarthritis in vivo and in vitro. One mechanism is that rapamycin leads to induction of autophagy [15, 16]. This has a protective impact on osteoarthritic-related degradation of extracellular matrix [17]. Additionally, mTORC2-controlled Akt activation has been shown to regulate chondrocyte differentiation as well as proteoglycan synthesis and SOX9 expression [18–20]. Because adult chondrocytes reside in an environment nearly devoid of nutrients and oxygen, it is tempting to speculate that inhibition of mTOR might lead to a cellular phenotype reminiscent of a physiologic chondrocyte status. Therefore, we aimed to analyze the effect of mTORC1 modulation during chondrogenic differentiation. We hypothesized that the blockage of mTORC1 elicits a beneficial effect on chondrogenic marker expression. As source cells, we used in vitro-expanded, patient-derived chondrocytes, as well as human AFS cells. We applied three-dimensional pellet culturing, which mimics the environment found in articular cartilage. Additionally, we used pharmacologic and genetic interventions to target key signaling molecules. To our knowledge, this is the first analysis of whether mTOR modulation can enhance chondrogenic marker expression in human AFS cells during chondrogenic differentiation.

### MATERIALS AND METHODS

**Human Amniotic Fluid Stem Cells and Human Chondrocyte Cell Culture**

High Oct4-expressing single-cell clones, derived from the CD117/2 population, were used in this study [21]. Cells were maintained in Chang media, as previously described [21]. Chondrocytes were provided by Danube University Krem and were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific, Waltham, MA, [https://www.thermofisher.com](https://www.thermofisher.com)) supplemented with 10% fetal calf serum, 2.5 mM L-glutamine (GE Healthcare, Pasching, Austria, [http://www3.gehealthcare.com](http://www3.gehealthcare.com)), 50 mg/l streptomycin sulfate (GE Healthcare), 30 mg/l penicillin (GE Healthcare), and 50 μg/ml ascorbic acid. All cells were cultured at 37°C in 5% CO2. The study protocol for the use of articular cartilage tissue and the isolation of chondrocytes thereof was approved by the institutional review board of the Danube University Krem (GS4-EK-4/199-2013).

**Differentiation of Human AFS Cells Into Chondrocytes**

Human monoclonal AFS cells were cultured until they were 80% to 90% confluent. AFS cells were dissociated with 0.25% trypsin/EDTA (GE Healthcare) and counted via CASY measurement (Roche Diagnostics, Risch-Rotkreuz, Switzerland, [http://www.roche.com](http://www.roche.com)). We seeded 2.5 × 105 cells into chondrogenic differentiation media containing DME (Thermo Fisher Scientific) supplemented with 0.5% fetal calf serum, 1% ITS (i.e., recombinant human insulin, human transferrin, and sodium selenite; Sigma-Aldrich, St. Louis, MO, [http://www.sigmaaldrich.com](http://www.sigmaaldrich.com)), 100 nM dexamethasone (Sigma-Aldrich), 50 μg/ml ascorbic acid (Sigma-Aldrich), 100× nonessential amino acids (GE Healthcare), 1 mM sodium pyruvate (Sigma-Aldrich), 5 ng/ml transforming growth factor β-1 (Pepro-technol, London, U.K., [https://www.peprotech.com](https://www.peprotech.com)), and 4% methyl cellulose (Sigma-Aldrich). Subsequently, cells were centrifuged at 1,500g for 10 minutes. Pellets were cultured for 14 days at 37°C in 5% CO2 and the medium was changed every 3 days. To selectively block mTOR downstream pathways, 25 nM of the mTORC1 inhibitor rapamycin (Merck Millipore, Billerica, MA, [http://www.merckmillipore.com](http://www.merckmillipore.com)), 1μM of MK2206, or 1μM of torin 1 (both from Selleckchem, Houston, TX, [http://www.selleckchem.com](http://www.selleckchem.com)) was added to the medium upon preparation and was freshly added when differentiation medium was changed.

**siRNA Preparation and Transfection**

Transfection of cells with siRNA was performed according to an established protocol [22]. Cells were incubated in a final siRNA concentration of 50 nM for 24 hours. The following siRNAs were used in our experiments: raptor (gene name, RPTOR) siRNA, HIF2A siRNA, rictor (gene name, RICTOR) siRNA, and control siRNA (all siRNAs were obtained from GE Healthcare).

**Lactate Dehydrogenase Assay**

The lactate dehydrogenase assay (Promega, Madison, WI, [http://www.promega.com](http://www.promega.com)) was performed according to the manufacturer’s protocol.

**Proteoglycan Staining**

Safranin O and 1,9-dimethylmethylene blue (DMMB) staining were performed as previously described [23, 24].

**Immunohistochemical Staining**

Human articular cartilage, chondrocyte pellets, and chondrogenic differentiated AFS cell pellets were fixed dehydrated and embedded in paraffin. Paraffin was removed using xylol, isopropanol, and an alcohol gradient in descending order (all from Carl Roth, Karlsruhe, Germany, [https://www.carlroth.com](https://www.carlroth.com)). For antigen unmasking, sections were incubated in modified citrate buffer (pH 6.1; Agilent Technologies, Glostrup, Denmark, [http://www.agilent.com](http://www.agilent.com)) at 120°C. Slides were incubated in 1% H2O2 and treated with 0.1% Tween 20 (both, Sigma-Aldrich). Sections were blocked with either 2.5% horse serum (Vector Laboratories, Burlingame, CA, [https://www.vectorlabs.com](https://www.vectorlabs.com)) or 1% bovine serum albumin (Sigma-Aldrich). The following antibodies were used for staining: pS6 (1:150); Cell Signaling Technology, Danvers, MA, [http://www.cellsignal.com](http://www.cellsignal.com); SOX9 (1:200); Santa Cruz Biotechnology, [http://www.scbt.com](http://www.scbt.com); COL2A1 (1:100); Developmental Studies Hybridoma Bank, Iowa City, IA, [http://dshb.biology.uiowa.edu](http://dshb.biology.uiowa.edu); and HIF1A (1:100), HIF2A (1:300), and ACAN (1:100), from Santa Cruz Biotechnology. Primary antibodies were incubated overnight at 4°C. For immunohistochemistry, biotinylated secondary antibodies (Vector Laboratories) were added for 45 minutes on the
next day. Sections were treated with streptavidin-horseradish peroxidase (HRP) complexes (Leica, Milton Keynes, UK, http://www.leica Microsystems.com) conjugated with peroxidase. To visualize positive staining, sections were incubated with aminothiol carbazpole (Agilent Technologies) for 20 minutes and hematoxylin (Carl Roth) was used for counterstaining.

**CMFDA Staining**

For coculture experiments, AFS cells were stained with 5-chloromethylfluorescein diacetate (CMFDA; Santa Cruz Biotechnology), whereas primary chondrocytes remained unstained. On the day of pellet formation, AFS cells were incubated in serum-free media containing 8 μM CMFDA at 37°C and in 5% CO2. After 1 hour, the medium was replaced with medium containing complete media, and cells were cultivated for another hour. CMFDA-labeled AFS cells and chondrocytes were harvested and pellets were formed containing 1.67 × 10^8 AFS cells and 8.3 × 10^5 primary chondrocytes.

**RNA Extraction and Polymerase Chain Reaction**

Total RNA was isolated from differentiated pellets on indicated days according to the manufacturer’s instructions (Peqlab, Erlangen, Germany, https://de.vwr.com). cDNA synthesis and polymerase chain reaction (PCR) were performed as described [21]. The human primers used are listed in supplemental online Table 1. Primers were designed using Primer3 (http://bioinfo.ut.ee/primer3). For normalization, β-actin (gene name, ACTB) was used and relative gene expression was calculated using the comparative Ct method (2^−ΔΔCt).

**Protein Analysis**

Proteins of AFS cells, human chondrocytes, and chondrogenic differentiated pellets were harvested as previously described [21]. The following antibodies were used: from Cell Signaling Technology: rabbit antiphosphorylated S6 ribosomal protein S240/244 (1:10,000; catalog no. 2215); rabbit antiphosphorylated Akt S473 (1:10,000; no. 4690), rabbit anti-raptor (1:1,000; no. 2280), rabbit anti-rictor (1:1,000; no. 2114), mouse anti-Akt (1:2,000; no. 2920), mouse anti-6PProtein (1:1,000; no. 2317), and rabbit anti-GAPDH (1:10,000; no. 5174); from Santa Cruz Biotechnology: rabbit anti-SOX9 (1:1,000; no. sc-20095) and mouse anti-HIF2A (1:250; no. sc-13596); from BD (Franklin Lakes, NJ, http://www.bd.com): mouse anti-HIF1A (1:250; no. 610958); and from Sigma-Aldrich: mouse anti-actin (1:3,000; no. A2228). Membranes were incubated with HRP-linked heavy and light chain antibodies (anti-mouse immunoglobulin G [IgG], 1:3,000, no. 31430; anti-rabbit IgG, 1:3,000, no. 31460; all Thermo Fisher Scientific). Enhanced chemiluminescence (Advansa, Menlo Park, CA, https://advansa.com) was used for detection.

**Statistical Analysis**

All experiments were performed in triplicate. Data were averaged and are presented as mean ± SEM. Statistical significance was tested by Student’s unpaired t test, comparing each experimental setup with the control, unless otherwise indicated. GraphPad Prism version 4.00 software (GraphPad Software, San Diego, CA, http://www.graphpad.com) was used; p < .05 was considered significant and p < .001 was considered highly significant.

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**RESULTS**

**mTORC1 Signaling in Human Cartilage and Effects of mTORC1 Inhibition on Human Chondrocyte Pellets**

Safranin O staining was performed to highlight the distribution of proteoglycans within human articular cartilage of the knee joint. As expected, in the superficial zone, which is rich in nutrients and oxygen, a low expression of proteoglycans was detected (Fig. 1A). With decreasing oxygen levels toward the deep zone of the cartilage, a strong increase of Safranin O-positive areas was observed. To visualize activity of the mTOR signaling pathway, we stained consecutive sections of the same sample for the mTORC1 substrate pS6 (Fig. 1B). In the superficial zone, a strong phosphorylation of S6 at Ser240/244 was identified. This signal decreased toward the deep zone as low oxygen levels and nutrient depletion dominated. This staining pattern was observed in a total of five healthy human cartilage samples.

Next, we determined the effect of rapamycin, a mTORC1 inhibitor, on in vitro-expanded human chondrocytes. Cells were subjected to a pellet formation protocol and were cultured in chondrogenic differentiation media supplemented either with or without rapamycin. After 21 days of differentiation, pellets were stained with DMMB, which reacts with proteoglycans to yield a violet color (Fig. 1C, 1D), whereas nuclei are stained blue. The extracellular matrix protein ACAN (Fig. 1E, 1F) and S6 at Ser240/244 were stained by immunohistochemistry. For all stainings, a similar pattern was observed: In control treated pellets, a proteoglycan- and ACAN-negative outer rim was observed and the expression of these markers was induced toward the middle of the pellet where oxygen levels declined (Fig. 1C, 1E), whereas high pS6 levels were detected in the outer area (Fig. 1G). When treated with the mTORC1 inhibitor rapamycin, proteoglycan and ACAN were expressed already at the edge and on the inside of the pellet (Fig. 1D, 1F). To verify the block of mTORC1 by rapamycin, treated pellets were stained for pS6 (Fig. 1H).

**Inhibition of mTORC1 Enhances Chondrogenic Differentiation of AFS Cells and Induces HIF2A**

The findings derived from human chondrocytes prompted us to investigate the effect of mTORC1 inhibition during chondrogenic differentiation of AFS cells. Therefore, we used established monoclonal, c-kit-positive stem cell lines and subjected them to chondrogenic differentiation [21, 25]. The treatment protocols performed in the following experiments are outlined in Figure 2A.

During AFS cell differentiation, SOX9 protein amounts constantly increased (Fig. 2B). To study the role of mTOR signaling during chondrogenic differentiation, pellets were treated with the mTORC1 inhibitor rapamycin and with the mTORC1 and mTORC2 inhibitor torin during the whole period of differentiation. Phosphorylation of p70 S6K at threonine 389 was assessed to quantify mTORC1 activity and SOX9 protein amounts were measured in the same lysates at the end of the differentiation process (Fig. 2C). SOX9 protein abundance was increased after rapamycin treatment, but not after torin treatment and quantitative reverse transcription (RT)-PCR also showed a significant increase of SOX9, COL2A1, and ACAN mRNA (Fig. 2D). Interestingly, COL1A1, a marker for immature chondrocytes, but also for hypertrophic chondrocytes, decreased upon rapamycin treatment during differentiation (Fig. 2D).

To prove that inhibition of mTORC1 is beneficial for the chondrogenic differentiation of AFS cells, the mTORC1-specific
component raptor was removed by transfection with siRNA oligonucleotides targeting RPTOR. Subsequently, pellets were generated and cultured for 14 days in chondrogenic differentiation media. Loss of raptor resulted in an induction of SOX9 protein expression (Fig. 3A). Cells were also treated with rapamycin and the result was comparable to the siRNA approach; mRNA levels of SOX9 and COL2A1 were increased when mTORC1 was blocked pharmacologically (Fig. 3B). To further verify this finding, pellets were subjected to immunohistochemistry and stained for SOX9 after 14 days of differentiation (Fig. 3C). SOX9 was expressed predominantly in the nucleus and its abundance was increased in rapamycin and siRNA raptor-treated pellets compared with control pellets.

Because we observed chondrogenic marker expression in the presence of rapamycin at the surface of chondrocyte pellets, which usually only occurs at the inside of pellets, we speculated that mTORC1 inhibition might induce a hypoxic response in cells. Hence, we examined expression of the two main α subunits, HIF1A and HIF2A, which regulate HIF activity. To selectively induce HIF protein amounts, AFS cells were treated with the chemical hypoxia inducers dimethyloxaloylglycine (DMOG), cobalt chloride (CoCl₂), and sodium sulfite. As expected, DMOG and CoCl₂ induced accumulation of HIF1A and HIF2A, whereas sodium sulfite only induced HIF2A (Fig. 3D). Importantly, rapamycin-induced HIF2A protein expression in a dose-dependent manner. This upregulation was accompanied by phosphorylation of the mTORC2 substrate AKT at Ser 473, whereas ribosomal protein S6 phosphorylation at Ser 240 and 244 was completely inhibited.

We next differentiated AFS cells in the presence of 1 mM DMOG or 150 μM CoCl₂ for 14 days. Subsequently, pellets were

**Figure 1.** Mammalian target of rapamycin signaling in chondrocytes residing in healthy articular cartilage and in cell culture pellets. (A): Safranin O staining of human articular cartilage, showing a typical proteoglycan expression pattern of the tissue. (B): A consecutive section was stained for ribosomal protein S6 phosphorylated at S240/244 (pS6), indicating active mammalian target of rapamycin complex 1 signaling. (C, D): DMMB staining of chondrocytes cultured in chondrogenic differentiation media or media supplemented with rapa for 21 days. Pellets were immunohistochemically stained for ACAN (E, F) and pS6 (G, H). Scale bars = 80 μm. Abbreviations: DMMB, 1,9-dimethylmethylene blue; rapa, rapamycin.
fixed and stained immunohistochemically for HIF1A and HIF2A (supplemental online Fig. 1A). A strong expression of HIF1A was only determined when pellets were treated with CoCl2. On the contrary, DMOG selectively induced expression of HIF2A. These data were also validated by Western blot (supplemental online Fig. 1B). Because DMOG and CoCl2 selectively induced expression of HIF2A or HIF1A, respectively, we next quantified chondrogenic marker expression in the treated pellets (supplemental online Fig. 1C). Pellets cultured in differentiation media containing DMOG revealed higher expression levels of the chondrogenic markers SOX9, ACAN, and COL2A1 compared with pellets treated with CoCl2. Although both hypoxia inducers reduced the expression of COL1A1, only CoCl2 boosted VEGFA expression. These findings indicate that the expression of chondrogenic markers is associated with the presence of HIF2A.

We determined HIF expression on day 14 of differentiation (Fig. 3E). Although we observed no change regarding HIF1A expression, a strong increase of HIF2A was monitored after 14 days of differentiation compared with undifferentiated AFS cells. This gain was further enhanced when mTORC1 was blocked either by siRNA-mediated silencing of raptor or by rapamycin treatment. These data suggest that HIF2A, but not HIF1A, is associated with chondrogenic differentiation of AFS cells and that mTORC1 is able to control HIF2A expression.

**Chondrogenic Differentiation of AFS Cells Depends on HIF2A**

Next, we evaluated the contribution of HIF2A to the chondrogenic differentiation effect. Therefore, we transfected AFS cells with siRNA targeting HIF2A and generated chondrogenic pellets from these cells. To determine, whether rapamycin is able to rescue the effect observed by the loss of HIF2A, siRNA-treated cells were also incubated in the presence of rapamycin. After 14 days of differentiation, pellets were harvested and the siRNA-mediated knockdown of HIF2A was still detectable by Western blot (Fig. 4A).
Quantification of chondrogenic marker expression by quantitative RT-PCR showed that knockdown of HIF2A led to impaired expression of SOX9, ACAN, and COL2A1 on day 7 of differentiation and could not be reversed when rapamycin was added (Fig. 4B).

AKT Activation Is Essential for HIF2A Expression

Because SOX9 and its target genes were upregulated upon inhibition of mTORC1, we next aimed to investigated the role of mTORC2 during this process. We treated cell pellets with MK2206, a specific inhibitor of the mTORC2 substrate AKT. During the first days of differentiation, massive cell death was observed, leading to disintegration of cell pellets, indicating an essential function of AKT for AFS cell survival during differentiation. Cell death was observed when pellets were treated with MK2206 (supplemental online Fig. 2A).

To be able to perform further experiments regarding the effect of AKT inhibition on chondrogenic differentiation, we used in

![Figure 3](image-url)
vitro-expanded human chondrocytes because they proved to be more enduring when subjected to inhibitor treatment. We cultured the chondrogenic pellets in differentiation media supplemented with either rapamycin, MK2206, or both. Immunohistochemical analysis and Western blotting revealed a strong induction of HIF2A expression in control pellets and pellets treated with rapamycin, whereas MK2206 led to a reduction of HIF2A expression (Fig. 5A, 5B). Also, the expression pattern of the chondrogenic markers SOX9 and COL2A1 was strongly reduced when AKT activity was inhibited by MK2206 (Fig. 5A, 5C).

Successful downregulation of the mTORC1 and mTORC2 substrates pS6 (S240/S244) and pAKT (S473) upon treatment with the respective inhibitors was visualized by Western blotting (Fig. 5B). High magnification of the pellet-liquid interface revealed co-expression of SOX9 and COL2A1 in deeper areas of the pellet located about 50 μm from the pellet surface. In contrast, rapamycin treatment increased the intensity of SOX9 staining in the outer pellet area and COL2A1 expression was detected already at the pellet surface. Analysis of mRNA harvested from chondrocyte pellets demonstrated that rapamycin was able to increase expression of SOX9 and COL2A1 in the whole tissue area when compared with control treated pellets (Fig. 5C). Furthermore, treatment with MK2206 inhibited expression of SOX9 and COL2A1, and this inhibition could not be reversed in the presence of rapamycin.

Grafting of AFS cells Into Chondrogenic Pellets From Adult Human Chondrocytes

The ability of differentiated AFS cells to integrate into chondrogenic pellets generated from healthy human chondrocytes can be used to determine the efficiency of the differentiation process. We transfected AFS cells with either control siRNA or siRNA targeting raptor and labeled the cells with a fluorescent dye. Subsequently, we mixed the treated cells with adult human chondrocytes to form achimeric, cartilaginous pellets. After 14 days of cultivation in differentiation media, labeled cells resided in clusters within the chondrocytic pellet (Fig. 6A). Furthermore, AFS-derived cells, regardless of whether they harbored raptor knockdown, showed a chondrocyte-like morphology. Moreover, quantification of SOX9-positive AFS cells showed that AFS cells depleted for raptor expressed more SOX9 (50%) than control siRNA-treated AFS cells (42%). Successful knockdown of raptor was verified by Western blot, which also showed a decrease of ribosomal...
protein S6 phosphorylation and a concomitant increase in AKT phosphorylation (Fig. 6B).

To further validate the importance of HIF2A for upregulation of chondrogenic markers during development, we analyzed expression data generated by Wu et al. that were derived from articular cartilage of fetuses of 6 and 17 weeks of age [26]. We calculated the Pearson correlation coefficient for HIF2A (gene name, EPAS1) and HIF1A with the chondrogenic markers SOX9, COL2A1, and ACAN (Fig. 6C). In contrast to HIF1A, HIF2A showed positive correlation in all cases; thus, we conclude there is also coregulation of HIF2A and chondrogenic markers during human fetus development.
DISCUSSION

AFS cells are broadly multipotent cells and can be differentiated into each embryonic germ-cell layer [5, 6]. We previously used monoclonal AFS cells for the generation of Schwann cells [21] and several studies have shown their differentiation to chondrocytes, which we recently summarized [27]. AFS cells can be obtained via immunoselection of c-kit-positive cells from amniotic fluid-derived cells [5]. They show expression of mesenchymal stem cell markers like CD29, CD44, CD73, CD90, and CD105, but also of embryonic stem cell markers like SSEA4 and Oct4. Isolated cells can be grown in large quantities, and selected AFS cells show differentiation potential exceeding that of unselected AFS cells [28]. In contrast to embryonic stem cells, AFS cells are not tumorigenic and their isolation per se does not harm the unborn child [10]. When compared with induced pluripotent stem cells, AFS cells do not require epigenetic reprogramming before differentiation and, because of their isolation during fetal development, they potentially harbor fewer somatic mutations than cells isolated from adults. The use of AFS cells as donor cells for chondrogenic differentiation has been documented in several studies [7–9]. However, the mechanisms that are effective during the differentiation process have not been fully elucidated.

Recent studies have shown that autophagy, controlled by mTORC1, is crucial for chondrocyte homeostasis in the articular cartilage and that rapamycin is able to protect cartilage from experimentally induced degeneration [15–17, 29]. Generally, mTORC1 is the main sensor in cells for the availability of oxygen and nutrient supply, and its activity regulates key metabolic processes involved in cellular growth [12]. Hence, the presence of little or no oxygen tension leads to deactivation of the mTORC1 pathway, and cells reduce their metabolic turnover. In studies that used different cellular models of chondrogenic differentiation, hypoxic conditions proved to be beneficial for the expression of chondrogenic markers like SOX9, ACAN, and COL2A1, while suppressing the hypertrophic state [30–34].

Here we focused on the impact of mTORC1 inhibition on the expression pattern of chondrogenic markers during cellular differentiation. We used ribosomal protein S6 phosphorylation as a bona fide mTOR activity readout. We identified strong S6

Figure 6. AFS cells integrate into cartilaginous pellets and differentiate into chondrocytes. (A): Adult chondrocytes were mixed with AFS cells, treated either with control siRNA or raptor siRNA targeting RPTOR, and labeled with 8 μM CMFDA. Cell pellets were formed and, after 14 days of differentiation, AFS cells were identified by green fluorescent staining. Successful differentiation of AFS cells into chondrocytes was assessed by SOX9 staining (red). *: area of differentiated AFS cells. Scale bars = 60 μm. (B): Western blot analysis of AFS cells transfected with either control siRNA or raptor siRNA, pS6, and pAKT. β-Actin is shown as the loading control. (C): Scatter plots derived from GSE 51812 comparing EPAS1 (HIF2A) and HIF1A expression with SOX9, COL2A1, and ACAN expression. Blue dots represent human fetal cartilage at age 6 weeks and green dots represent human fetal cartilage at the age 17 weeks. Pearson correlation coefficient and p values are given within the graphs. Abbreviations: AFS, amniotic fluid stem; CMFDA, 5-chloromethylfluorescein diacetate; Expr. Int., normalized expression intensity; siRNA, small interfering RNA.
phosphorylation at the superficial zone of normal articular cartilage, as well as at the outer zone of three-dimensional cultivated, adult human chondrocytes. Both are areas with high nutrient and oxygen exposure. Remarkably, in all cases, S6 activity was inversely correlated with proteoglycan expression. When we used rapamycin to block mTORC1 activity, we could induce proteoglycan expression at the outer surface of pellets derived from cultured human chondrocytes. These findings prompted us to apply mTORC1 inhibition during chondrogenic differentiation of AFS cells.

It has been shown that hypoxia, which is a main inhibitor of mTORC1, increases expression of SOX9 and extracellular matrix genes in human chondrocytes [34–36]. In the present study, we provide comprehensive evidence that mTORC1 inhibition leads to SOX9 upregulation accompanied by COL2A1 and ACAN induction. Use of rapamycin or depletion of raptor both inhibited mTORC1 activation, and led to an increase in chondrogenic marker expression. Importantly, mTORC1 inhibition was accompanied by marked HIF2A upregulation, whereas HIF1A was not induced. We noticed that rapamycin treatment or siRNA targeting of raptor always led to increased AKT activation. This phenomenon can be attributed to the suppression of AKT activation by mTORC1 [37, 38], which is not a tumor-specific process, because it can also occur in articular cartilage [39]. When we blocked AKT activation by MK2206 treatment or siRNA-mediated depletion of rictor, survival of AFS cells during differentiation was severely compromised. In vitro-expanded chondrocytes were more resistant to AKT inhibition and, in this model system, we could clearly monitor a decrease in HIF2A expression, as well as a decrease in SOX9 expression and its target genes. A connection between AKT activation and HIF1A accumulation has been shown in fibroblasts, osteoblasts, and mesenchymal stem cells [40–42] but, as far as we know, this is the first report demonstrating that AKT activation is necessary for HIF2A expression in human chondrocytes.

HIF2A plays a major role in the induction of cartilage matrix synthesis in human articular chondrocytes [43, 44]. We showed that HIF2A was specifically induced by DMOG treatment and that HIF2A was essential for the effect of rapamycin during chondrogenic differentiation of AFS cells. We observed that depletion of HIF2A inhibited expression of chondrogenic markers and additional rapamycin treatment could not rescue the observed effect.

To test whether mTORC1 inhibition is beneficial for AFS cell differentiation in an environment mimicking human articular cartilage, we grafted raptor-depleted AFS cells together with adult human chondrocytes in a three-dimensional culture system. It has been shown that adult chondrocytes are able to provide chondroinductive cues to mesenchymal stem cells and to promote their chondrogenic phenotype [45]. In our test system, we also observed an integration of AFS-derived cells into cartilaginous architecture provided by adult human chondrocytes. In this setting, raptor-knockdown AFS-derived cells had a higher frequency of SOX9-positive cells than control samples. This also indicates that cell-based transplantation approaches can benefit from protocols involving mTORC1 inhibition. Because we have shown that AFS cells and adult chondrocytes respond to rapamycin treatment, we propose adjuvant local treatment with sirolimus in future studies involving patients undergoing cartilage regeneration therapy. Our findings are also supported by data derived from human developing cartilage, in which we could clearly show a correlation of HIF2A, but not of HIF1A, with chondrogenic marker expression.

CONCLUSION
To our knowledge, we are the first to show that mTORC1 inhibition leads to upregulation of chondrogenic markers during AFS cell differentiation and during redifferentiation of in vitro-expanded human chondrocytes. The process depends on availability of HIF2A, which is controlled by AKT activation. We conclude that physiologic deactivation of mTOR can be mimicked via rapamycin treatment and that mTORC1 inhibition represents an opportunity to enhance chondrogenic differentiation of AFS cells and in vitro-expanded chondrocytes. Because the latter are frequently used for autologous implantation, our findings may help achieve better-quality chondrocytes, resulting in higher success rates when treating articular cartilage defects.

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AUTHOR CONTRIBUTIONS
A.P. and M.M.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; D.S., K.K., and B.S.: collection and/or assembly of data, final approval of manuscript; H.Z., J.G.J., and M.H.: data analysis and interpretation, final approval of manuscript; M.R. and S.N.: provision of study material or patients, data analysis and interpretation, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST
The authors indicated no potential conflicts of interest.

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