Type II TGF β receptor modulates chondrocyte phenotype

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Abstract Aging is one of the major risk factors of osteoarthritis. This pathology during which chondrocytes undergo modifications of their phenotype may result from alteration of transforming growth factor β (TGF β) signaling. This study investigates the role of TGF β response in the process of chondrocyte dedifferentiation/redifferentiation. Dedifferentiation was induced by successive passages of human articular chondrocytes, whereas their redifferentiation was performed by three-dimensional culture in alginate. Human mesenchymal stem cells were obtained from bone marrow and differentiated into chondrocyte-like phenotype by three-dimensional culture, embedded in the same scaffold. Protein and mRNA levels were analyzed by Western blot and real-time reverse transcription PCR. Regulatory mechanism was investigated using specific inhibitors (mithramycin), mRNA silencing or decoy oligonucleotides, and expression vectors. Chondrocyte

dedifferentiation interfered with TGF β signaling by decreasing T β RII mRNA and protein levels and subsequent TGF β response. T β RII ectopic expression in passaged chondrocytes permitted to increase the expression of several matrix genes, such as aggrecan or type II collagen. Redifferentiation of passaged chondrocytes permitted to restore, at least in part, T β RII expression and was related to differentiation of human bone marrow mesenchymal stem cells toward chondrocytes, where both specific protein 1 (Sp1) and T β RII mRNA levels were increased. Moreover, Sp1 manipulation by silencing or ectopic expression and pharmacologic inhibition revealed a link between expression levels of this transcriptional factor, which is crucial for constitutive expression of T β RII in cartilage, and TGF β response. Therefore, these data permit us to suggest an important role of T β RII expression in the maintenance of chondrocyte phenotype, which is altered with age, and bring new insights in our understanding of chondrogenesis process.

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Introduction

Articular cartilage consists of an abundant extracellular matrix, which is produced by chondrocytes. These highly differentiated cells are responsible for the maintenance of the cartilage as a functional entity. Aging cause changes

in the properties of this tissue, notably by modifying the matrix composition or the growth factor responsiveness (Kempson 1982; Thonar et al. 1986; Dozin et al. 2002; Loeser et al. 2000; Rosen et al. 1997; Guerne et al. 1995). During embryonic development, cartilage formation is initiated with condensation of mesenchymal cells, followed by progression of chondrocyte differentiation toward proliferation, prehypertrophy, and hypertrophy. During osteoarthritis (OA), an articular disease strongly correlated with aging (Buckwalter and Mankin 1998; van der Kraan and van den berg 2008); chondrocytes undergo morphogenic alterations accompanied by profound biochemical changes including loss of production of cartilage-specific macromolecules. For instance, type II collagen expression decreased whereas type I collagen synthesis increases. Some of alterations of chondrocyte phenotype can be reproduced in vitro. Passages induce morphogenic alterations of cells, reduction of type II collagen and induction of type I collagen synthesis (Benya et al. 1978; Archer et al. 1990; Pei et al. 2000; McDougall et al. 1996; Homicz et al. 2002). However, this model is not able to mimic other modifications undergone by OA chondrocytes and notably the induction of type X collagen, specific of hypertrophy stage. On the other hand, chondrocyte-specific phenotype can be recovered when cells are cultured in a three-dimensional culture system, such as alginate scaffold (Reginato et al. 1994; Bonaventure et al. 1994).

Several studies suggest that modifications of chondrocyte phenotype during OA result from alteration of the transforming growth factor β (TGF β) signaling. Thus, expression of TGF β and its signaling molecules is altered with age, and subsequent lack of TGF β responsiveness might be at the root of OA development (Blaney-Davidson et al. 2005). Besides, TGF β signaling is important for chondrogenesis. It promotes the differentiation of embryonic chick limb cartilage (Schonherr and Hausser 2000) and upregulates a number of molecules associated with prechondrogenic condensation (Serra et al. 1997; Chimal-Monroy and Diaz de Leon 1999; Grimaud et al. 2002). In addition, primary chondrogenesis derived from mesenchymal stem cells (MSCs) needs TGF β signals (Reddi 1994; Andriamanalijaona et al. 2008).

Members of the TGF β family transduce their signal via two serine/threonine receptors, type I (T β RI) and type II (T β RII) receptors (Wrana et al. 1994). Upon ligand binding, T β RI is phosphorylated by T β RII, resulting in activation of intracellular proteins, named

Smads. Receptor-regulated Smads (R-Smads: Smad2 and Smad3) are phosphorylated by T β RI. Phosphorylated R-Smads form heterodimers with common-partner Smad (Co-Smads: Smad4) and translocate into the nucleus where they bind to specific DNA sequences for activation of the TGF β target gene transcription (Shi and Massague 2003). Inhibitory Smads (I-Smads: Smad7) inhibit phosphorylation of R-Smads by competing with their binding to activated T β RI (Nakao et al. 1997). Recently, it was shown that besides signaling via T β RI (activin-like kinase 5, ALK5), TGF β can also signal via ALK1, thereby activating Smad1/5/8 instead of Smad2/3 (Blaney-Davidson et al. 2009; Finnson et al. 2008).

We hypothesized that the maintenance of chondrocyte phenotype, which is altered with age, required a functional TGF β signaling. We examined the influence of the members of TGF β signaling on chondrocyte phenotype. We showed that T β RII is implicated in the loss of TGF β responsiveness observed during chondrocytes passages. T β RII ectopic expression permits to restore TGF β responsiveness as well as to increase the expression of some matrix components. We also found that T β RII expression level is intimately linked to differentiation status of chondrocytes. Finally, we established a relationship between TGF β -induced effects on matrix genes and expression level of specific protein 1 (Sp1) transcription factor, which expression is decreased in the course of a dedifferentiation process (Duval et al. 2011) and is required to ensure a constitutive expression of T β RII in chondrocytes (Baugé et al. 2008).

Material and methods

Reagents

Reagents were supplied by Invitrogen (Fisher Bioblock Scientific, Illkirch, France) unless otherwise noted. Mithramycin (Sigma-Aldrich, St. Quentin Fallavier, France) and TGF β 1 (R&D Systems, Lille, France) were resuspended in phosphate buffered saline (PBS) or PBS-HCl, respectively. Alginate was provided by Sigma-Aldrich, and oligonucleotides were supplied by Eurogentec (Angers, France).

Chondrocyte culture

Human articular chondrocytes (HAC) were prepared from femoral head of postmortem individuals with no

known joint diseases or after femoral head fractures (ages between 45 and 80 years with median age of 68). The causes of donor's death were multiple, and all of them signed agreement forms in the context of a body donation program according to local legislations. Similarly, donors from arthroscopy signed the agreement for this study according to a local ethical committee (CPP). Chondrocytes were released by enzymatic digestion as previously described (Baugé et al. 2007). Cells were seeded at 4×10^4 cells/cm² and cultured in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/L D-glucose) supplemented with 10 % heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml Fungizone, in a 5 % CO₂ atmosphere. For dedifferentiation, HAC were passaged up to five times (P5) (about seven to eight population doublings) with trypsin–EDTA after reaching confluency.

For redifferentiation studies, chondrocytes, which had underwent three passages (about four to five population doublings), were encapsulated in alginate beads. Cells were suspended in alginate at the density of 2.5×10^6 cells/ml, then slowly dropped into a 100 mM CaCl₂ solution. After instantaneous gelation, the beads were allowed to polymerize further for a period of 10 min in the CaCl₂ solution. After three washes in PBS, they were finally placed in DMEM supplemented with 10 % fetal calf serum. After 3 weeks, the beads were rinsed with PBS and dissolved in a solution composed of 55 mM sodium citrate and 25 mM EDTA. The suspension was incubated at 37 °C until the beads were completely dissolved (approximately 10 min.). Centrifugation of the suspension at $800 \times g$ for 10 min allows separating the cells from their alginate matrix.

Culture of human bone marrow-derived stem cells

Human bone marrow-derived stem cells were collected from the same patients as donors used for HAC isolation. The harvested cells were washed twice with α -MEM and isolated by adherence over 24 h to tissue culture plastic and were expanded in a monolayer culture with α -MEM supplemented with glutamine, FGF (1 ng/ml), 10 % FCS and antibiotics. After amplification by successive passages (until P5), the absence of hematopoietic markers (such as CD45) was checked by reverse transcription PCR (RT-PCR) (data not shown). Thereafter, bone marrow-derived stem cells were directed toward chondrogenesis by culture in a three-dimensional scaffold (alginate bead).

For chondrogenic differentiation, isolated human bone marrow-derived stem cells were encapsulated in alginate beads, according to the same protocol to that of HAC, and cultured with a chondrogenic medium consisting of DMEM containing 100 nM dexamethasone, 50 µg/ml ascorbic acid-2-phosphate, 40 µg/ml proline, 1 mM sodium pyruvate and insulin transferin selenium supplement (Sigma), for 21 days and the media were changed twice a week.

Plasmids and transfection experiments

pCMV5-T β R2 and pEVR2-Sp1 expression vectors were described earlier (Baugé et al. 2008). Chondrocytes were transiently transfected by the calcium phosphate precipitation method as previously described (Baugé et al. 2007). After an overnight transfection, the culture medium was replaced by DMEM containing 10 % FCS and maintained in culture for 24 h.

Gene silencing experiments

Provided from Santa Cruz Biotechnology (through Tebu, Le Perray en Yvelines, France), 1.5 µg of double strand siRNA targeting Sp1 was nucleofected in HAC, according to the manufacturer's protocol with a human chondrocyte nucleofector solution (Amaxa, Köln, Germany). Negative control siRNA known to not lead to specific degradation of any cellular message was also used. After 6 h of transfection, the culture medium was replaced with DMEM containing 10 % FCS and the chondrocytes cultures were incubated for 24 h.

RNA extraction and real-time RT-PCR

Total RNA from primary HAC cultures were extracted using TRIzol. Following extraction, 1 µg of DNase-I treated RNA was reverse transcribed into cDNA in the presence of random hexamers (Applied Biosystems, Courtaboeuf, France) and Moloney murine leukemia virus reverse transcriptase. The reaction was carried out at 37 °C for 1 h followed by a further 10-min step at 95 °C. Amplification of the generated cDNA was performed by real-time PCR in an Applied Biosystems SDS7000 apparatus with appropriate primers designed with the Primer Express software. The relative mRNA level was calculated with $2^{-\Delta\Delta CT}$ method.

Protein extraction and Western blot

Cells were rinsed and scrapped in RIPA lysis buffer supplemented with phosphatase and protease inhibitors. The extracts (30 µg protein) were subjected to fractionation in a 8 or 10 % SDS-PAGE, transferred to PVDF membranes (Amersham Biosciences, Orsay, France) and reacted with rabbit aggrecan, TβRII, or Sp1 polyclonal antibodies. Subsequently, membranes were incubated with anti-rabbit secondary peroxidase-conjugated antibody. The signals were revealed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Perbio Science, Brébières, France) and exposed to X-ray film. The membranes were also reacted with anti β-actin to verify equal loading.

Synthesis and transfection of decoy oligonucleotides

Sp1 decoy oligonucleotides sequences are forward 5'-ATTTCGATCGGGGCGGGGCGAGC-3' and reverse 5'-CGACGCCCGCCCCGATCGAAT-3' (consensus sequences are underlined) and was previously shown to bind Sp1 transcriptional factor (Baugé et al. 2008). These oligonucleotides were annealed overnight and transfected into cells using the Oligofectamine reagent according to the manufacturer's instructions.

Nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared as described previously (Dignam et al. 1983). For electrophoretic mobility shift assay (EMSA), five femtomoles of [γ -³²P]ATP-labeled probes (Sp1 binding sequence) and 4 µg of nuclear extracts were incubated at room temperature for 15 min in a binding buffer (20 mM Tris-HCl pH 7.5, 1 mM DTT, 100 mM NaCl, 5 % bovine serum albumin, 0.1 % Nonidet P-40, 10 % glycerol, 25 µM ZnCl₂) and 2 µg of poly(dI-dC)·(dI-dC). Samples were resolved on a native polyacrylamide gel and exposed for autoradiography.

Statistical analysis

All experiments were repeated at least with three different donors, and similar results were obtained. Representative experiments are shown in the figures. Data are presented as the mean $\pm$ SD. Statistical significance was determined by Student's *t* test. *P* values less

than 0.05 were considered significant (****P*<0.001, ***P*<0.01, **P*<0.05 versus controls).

Results

Chondrocyte passages reduce the TGFβ responsiveness and decrease TβRII expression

In order to ascertain that subculturing chondrocytes led to the loss of cartilaginous phenotype in our hands, we monitored the decrease of type II collagen and aggrecan mRNA expression after successive passages. Their expressions were strongly decreased as soon as the first passage (about two population doublings). The maximal reduction was obtained at P2 (approximately three population doublings) and was maintained at least until P5. At opposite, collagen type I and III mRNA levels were higher at P2 (Supplementary Fig. 1A). Therefore, we limited the following experiments to two or three passages (three to five population doublings).

To investigate chondrocyte responsiveness to TGFβ during dedifferentiation process, we focused on expression of plasminogen activator inhibitor-1 (PAI-1), which is rapidly induced by TGFβ in chondrocytes (Baugé et al. 2007). Human articular chondrocytes which underwent three passages were incubated for 4 h with TGFβ1 (2 ng/ml). Figure 1a shows that passages induced a significant decrease of PAI-1 induction by TGFβ.

TGFβ initiates its cellular action by binding to TβRII, which in turn activates TβRI. Thus, we investigated the effect of passages on mRNA expression of both receptors. Whereas TβRI transcripts were increased in comparison to primary chondrocytes (P0) (Fig. 1b), TβRII is dramatically decreased following dedifferentiation (Fig. 1c). This reduction of TβRII expression was also observed at protein level (Fig. 1d).

Passages increase the expression of the R-Smad, Smad3, and decrease that of the I-Smads, Smad7

Since signaling by TGFβ is mediated by Smads, we explored the potential alteration of Smads expression during chondrocyte dedifferentiation. Our result (Fig. 2) showed that serial passages of chondrocytes in serum-supplemented medium did not modulate significantly Smad2 and Smad4 mRNA levels. Moreover, they increased Smad3 mRNA levels whereas they decreased the expression of the I-Smad, Smad7. These data suggest

Fig. 1 Chondrocyte passages reduce the TGFβ responsiveness and decrease TβRII expression. **a** Primary (P0) or passaged P1 (approximately two to three population doublings), P2 (approximately three to four population doublings), and P3 (approximately four to five population doublings). HAC were cultured for 5–6 days before incubation with TGFβ1 (2 ng/ml) for 4 h. Total RNA was isolated and analyzed by real-time RT-PCR, using PAI-1 and GAPDH primer. **b, c** Total RNA and proteins from freshly isolated chondrocytes or from chondrocytes dedifferentiated by repeated passages were extracted. TβRI and TβRII mRNA levels were defined by real-time RT-PCR. **d** TβRII protein level was analyzed by Western blot

that the loss of TGFβ responsiveness observed in dedifferentiated chondrocytes is independent of Smads expression level, since an upregulation of Smad3 and downregulation of Smad7 are usually associated with the activation of TGFβ signaling.

Ectopic expression of TβRII restores TGFβ responsiveness in passaged chondrocytes and increases expression of matrix genes

To confirm the crucial role of TβRII expression alteration in the reduction of TGFβ sensitiveness of passaged chondrocytes, we examined the effect of forced TβRII expression on the induction of PAI-1 by TGFβ (Fig. 3a). HAC were transfected with a TβRII expression vector (pCMV5-TβRII) or its corresponding insertless vector (pCMV5) for 24 h and further incubated with TGFβ. Dedifferentiation clearly causes reduced TGFβ-induced PAI-1 expression, which was restored by TβRII ectopic expression. In addition, transfection of TβRII expression vector in passaged chondrocytes induces the expression of aggrecan and collagen type II (x2-3) (Fig. 3b, c).

TβRII expression increases during chondrocyte differentiation

Since TβRII levels decreased upon dedifferentiation, we hypothesized that its expression could also be modulated during chondrocyte differentiation process. Therefore, we pursued by the examination of TβRII expression in redifferentiating chondrocytes. For that, TβRII mRNA level was evaluated prior dedifferentiation (P0), after dedifferentiation (P3), and after redifferentiation in alginate. TβRII mRNA level was increased after a 3-week culture in alginate (Fig. 4a), the time which permits to restore (at least partially) a chondrocyte phenotype in this system (Duval et al. 2011).

Similar experiments were performed using human bone marrow-derived mesenchymal stem cells, which underwent chondrogenesis by culture in alginate microbeads (Duval et al. 2012; Supplementary data 2B). This system is well known to induce chondrogenic differentiation from mesenchymal stem cells from different origins (Andriamanalijaona et al. 2008). As shown in Fig. 4b, TβRII mRNA levels strongly increased with the culture time and subsequently increased during differentiation of human bone marrow (hBM)-derived MSC into chondrocyte-like phenotype.

Fig. 2 Passages upregulates the R-Smad, Smad3, and downregulates the I-Smads, Smad7. **a–d** HAC were cultured as described in Fig. 1, and Smad2, Smad3, Smad4, and Smad7 mRNA levels were analyzed by real-time RT-PCR. Results are shown as histograms of relative expression versus GAPDH signal

Sp1 is important for T β RII expression and the maintenance of chondrocyte phenotype

Given that Sp1 expression decreases during chondrocyte dedifferentiation and is restored by alginate culture (Duval et al. 2011), we proposed that Sp1 expression and activity could increase during differentiation of stem cells. Sp1 mRNA level was raised during human bone

marrow-derived stem cell differentiation into chondrocytes (Fig. 5a). To define whether DNA-binding activity of Sp1 is also increased in these conditions, EMSAs were performed using the consensus DNA-binding site as a probe (Fig. 5b). One delayed protein–DNA complex was strongly enhanced by hBM-derived MSC culture in alginate beads after 72 or 96 h. The complex-binding activity to the probe was decreased in the

Fig. 3 Ectopic expression of T β RII restores TGF β responsiveness in passaged chondrocytes and increases expression of matrix genes. **a** Primary HAC (P0) or passaged twice (P2) were transfected with T β RII expression vector (*pCMV5-T β RII*) or with the insertless plasmid (5 μ g). After 15 h, the medium was changed for DMEM +2 % FCS for 24 h and cells were

incubated with TGF β 1 addition (2 ng/ml) for 4 h. PAI-1 expression was analyzed by real-time RT-PCR. **b**, **c** HAC were cultured and transfected as above; 24 h later, col2A1 and aggrecan mRNA expression was analyzed and normalized with GAPDH level (**b**). Aggrecan and T β RII protein levels were also analyzed by Western blot (**c**)

Fig. 4 TβRII expression increases during chondrocyte differentiation. **a** Primary chondrocytes (P0) or HAC dedifferentiated by three passages (P3) were cultured in monolayer or transferred into alginate beads for 21 days. Thereafter, TβRII mRNA level was analyzed by real-time RT-PCR. **b** Human bone marrow-

derived stem cells were cultured in alginate beads for different incubation periods, and TβRII mRNA levels were assayed. Results are shown as relative gene expression versus GAPDH signal

presence of Sp1 antibody (and not in the presence of irrelevant antibodies, namely against cKrox or Sp3), indicating that this complex contains Sp1 factor.

Then, we investigated the effect of inhibition of Sp1 on TβRII expression. First, we blocked the binding activity of Sp1 family members using decoy oligonucleotides or mithramycin (Jia et al. 2007) and showed that the loss of Sp1 binding to DNA inhibited TβRII mRNA level (Fig. 6a, b). Furthermore, we established that the specific reduction of Sp1 expression by RNA silencing, checked by Western blot, reduced TβRII expression (Fig. 6c). Mithramycin also reduced TβRII protein expression. Interestingly, aggrecan expression was decreased by mithramycin (Fig. 6d). Finally, in Fig. 7, we showed that Sp1 ectopic expression is able to restore TβRII expression in dedifferentiated chondrocytes and counteracts inhibition of aggrecan expression induced by passages.

Discussion

Modifications of chondrocyte phenotype occur with aging and during osteoarthritis. They can be mimicked, in part, in vitro by subculturing monolayer cultures of chondrocytes (Homicz et al. 2002; Pei et al. 2000; McDougall et al. 1996; Duval et al. 2011). This culture model provides a way to understand some of the molecular mechanisms responsible for chondrocyte senescence and dedifferentiation and to elucidate the transition from young/healthy to old/osteoarthritic cartilage. Conversely, studies of mechanism of redifferentiation of chondrocyte

or differentiation of mesenchymal cells into chondrocytes can permit to bring new insights in order to develop therapeutic use of MSC in cartilage disorders. In this study, we showed that chondrocyte dedifferentiation is accompanied by TβRII expression alteration, whereas redifferentiation of passaged chondrocytes or differentiation of MSC into chondrocytes is accompanied by TβRII production. Moreover, we demonstrated that forced TβRII expression is able to restore totally or partially the expression of matrix genes, suggesting a key role of this receptor and TGFβ signal in the maintenance of chondrocyte phenotype.

Our in vitro experiments show the key role of TβRII expression in chondrocyte phenotype maintenance. This result completes in vivo data which showed that TβRII-deficient mice have a reduced proliferation of chondrocytes and an accelerated early hypertrophic differentiation relative to control (Seo and Serra 2007). In addition, conditional transgenic mice in which a dominant negative form of TβRII is expressed, develop a hypoplastic cartilage (Hiramatsu et al. 2011), confirming that TβRII is required for normal cartilage development and subsequently chondrocyte phenotype.

Chondrocyte dedifferentiation, which occurs with aging, is likely associated to alteration of cell responsiveness to TGFβ by reduction of the TβRII expression. Of interest, we have previously demonstrated that interleukin-1β, a proinflammatory cytokine which is increased in OA process, rendered cells insensitive to TGFβ by reducing TβRII expression (Baugé et al. 2007). Together, these results corroborate in vivo data, which indicated that TβRII expression is dramatically

Fig. 5 Sp1 expression increases during chondrocyte differentiation. **a** Human bone marrow-derived stem cells were cultured in alginate beads for different incubation periods, and Sp1 mRNA levels were assayed. Results are shown as histograms of relative gene expression versus GAPDH signal. **b** Electrophoretic mobility shift assay (EMSA) reactions were performed using probe corresponding to Sp1 consensus DNA-binding site, incubated with 4 μ g nuclear extract from hBM-derived MSC cultured in alginate for 0, 72, or 96 h. EMSA reactions were also performed after incubation of nuclear extracts (from hBM cultured 72 h in alginate) with Sp1, Sp3, and cKrox antibodies

reduced in old mice (Blaney Davidson et al. 2005) and during OA (Boumediene et al. 1998; Verdier et al. 2005), and confirmed the relationship between TGF β expression and the reduction of cell responsiveness to TGF β observed in OA and old cartilage. Concerning TGF β expression, it increases with passages, suggesting that this receptor is not involved in the reduction of cell responsiveness to TGF β in passaged chondrocytes. This result was unexpected since TGF β

expression is reduced during experimental OA induced in mice by destabilization of the medial meniscus model (Blaney-Davidson et al. 2009).

In addition, we examined the expression levels of TGF β -signaling intracellular mediators, namely Smads. We showed that Smad3 expression is increased during chondrocyte dedifferentiation whereas that of Smad2 is not modified. Similar effects were obtained in vivo with aging (Blaney-Davidson et al. 2005). Smad3 acts as one important TGF β signaling pathway member to develop and/or maintain the phenotype of chondrocytes. Smad3, but not Smad2, stimulates primary chondrogenesis (Furumatsu et al. 2005). In addition, Smad3 deficiency accelerates chondrocyte maturation and leads to osteoarthritis (Yang et al. 2001; Van de Laar et al. 2011). Therefore, we speculate that the increased Smad3 expression observed during dedifferentiation and aging could be a compensatory mechanism to promote the chondrocyte phenotype. Moreover, these results obtained on Smad expression could be due to the loss of TGF β response since TGF β reduces Smad3 expression (Mori et al. 2000; Baugé et al. 2011).

We also observed the TGF β RII expression during in vitro chondrogenesis. Three-dimensional scaffold was used to redifferentiate dedifferentiated chondrocytes or to differentiate MSC toward chondrogenic phenotype. This culture system was described earlier to permit the induction of chondrogenic markers (Reginato et al. 1994; Bonaventure et al. 1994; Andriamanalijaona et al. 2008; Lemare et al. 1998; Cai et al. 2007; Duval et al. 2011). We showed that TGF β RII expression is induced during redifferentiation of subcultured chondrocytes and during chondrogenic process of stromal stem cells. In this regard, Mizuta and colleagues found that expression of both TGF β RI and TGF β RII receptors was upregulated during periosteal chondrogenesis induced by exogenous TGF β 1 treatment (Mizuta et al. 2002). Our data corroborate another in vitro study which proposes that TGF β signal transduction may be involved in the early stage of bone marrow MSC chondrogenesis (Huang et al. 2005) and are consistent with recent studies, which demonstrated that TGF β RII downregulation induced by retinoid acid treatment inhibits chondrogenesis of mouse embryonic palate mesenchymal cells (Yu and Xing 2006) and that TGF β signaling is needed in the chondrogenic differentiation of MSC (Longobardi et al. 2006).

Finally, we investigated the role of Sp1 in the regulation of TGF β RII expression and matrix production. This ubiquitous zinc-finger protein has earlier been proposed

Fig. 6 Sp1 regulates TβRII expression. **a** Primary HAC, transfected with a Sp1 consensus binding sequence (Sp1 decoy), were incubated in DMEM+2 % FCS for 24 h. Thereafter, TβRII mRNA versus GAPDH level was determined by RT-PCR. **b** Subconfluent cultures of chondrocytes were treated for 48 h in the presence or absence of mithramycin (100 and 150 nM). TβRII expression was analyzed at mRNA level by

real-time RT-PCR. **c** HAC were nucleofected with siRNA oligonucleotides targeted Sp1 or with control siRNA. Thereafter, the medium was replaced with DMEM+10 % FCS for 24 h. Sp1 and TβRII protein levels were defined by Western blot. **d** Chondrocytes were treated for 48 h in the presence or absence of mithramycin (100 and 150 nM). TβRII and aggrecan expression was analyzed at the protein level by Western blot

to regulate matrix production by adult articular chondrocytes maintained in primary and expansion culture (Duval et al. 2011; Dharmavaram et al. 1997; Oh et al. 2007). Furthermore, we have demonstrated the loss of Sp1 gene expression as a function of chondrocyte passage number and that Sp1 protein synthesis is restored in part by redifferentiation in three-dimensional alginate

culture (Duval et al. 2011). Here, we showed that the decrease of Sp1 induced by serial passaging of chondrocytes is responsible for TβRII reduction. However, we cannot exclude that Sp1 acts indirectly. Sp1 might permit the reexpression of TβRII in dedifferentiated chondrocytes through Sox9, which can be induced by Sp1 (Piera-Velazquez et al. 2007). Sox9 was first

Fig. 7 Sp1 regulates chondrocyte phenotype. **a, b** HAC passaged twice (P2) were transfected with Sp1 expression vector (*pEVR2-Sp1*) or with the insertless plasmid (5 μg). After 15 h, the medium was changed for DMEM+2 % FCS for 24 h. Total RNA was isolated and TβRII and aggrecan mRNA levels were analyzed and normalized with GAPDH level, in comparison to HAC with no passage (P0)

identified as a transcription factor which specifies the chondrocyte lineage and is absolutely required for complete cartilage differentiation (Bi et al. 1999). In our study, Sp1 was not able to counteract the decrease of type II collagen (data not shown) whereas it increases aggrecan mRNA level in passaged chondrocytes. Since it was previously established that T β RII gene expression occurred earlier than that of type II collagen gene expression during differentiation of bone marrow MSC under dynamic compressive loading (Huang et al. 2005), we hypothesize that longer time of Sp1 overexpression could restore the aggrecan or type II collagen expression in dedifferentiated chondrocytes. In order to validate this speculation, it will be necessary to reproduce this experiment using a stable transfection system, which could permit to maintain a high level of Sp1 during a long period.

In conclusion, we demonstrated that T β RII expression level is essential to maintain and/or promote chondrocyte phenotype and established a key role of Sp1 in this process. These findings may help in understanding the behavior of aged chondrocytes. In addition, it brings original insights in the process of MSC differentiation into chondrocytes and subsequently permits to envisage innovative strategies to optimize the therapeutic use of chondrocytes or MSC in cartilage disorders.

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