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## **Type II TGF $\beta$ receptor modulates chondrocyte phenotype**

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1 **Abstract:**

2 Aging is one of the major risk factors of osteoarthritis. This pathology during which chondrocytes undergo  
3 modifications of their phenotype may result from alteration of TGF $\beta$  signaling. This study investigates the role of  
4 TGF $\beta$  response in process of chondrocyte dedifferentiation/redifferentiation.

5 Dedifferentiation was induced by successive passages of human articular chondrocytes, whereas their  
6 redifferentiation was performed by tridimensionnal culture in alginate. Human mesenchymal stem cells were  
7 obtained from bone marrow and differentiated into chondrocyte-like phenotype by 3D-culture, embedded in the same  
8 scaffold. Protein and mRNA levels were analyzed by Western-Blot and real time RT-PCR. Regulatory mechanism  
9 was investigated using specific inhibitors (mithramycin), mRNA silencing or decoy oligonucleotides, and expression  
10 vectors.

11 Chondrocyte dedifferentiation interfered with TGF $\beta$  signalling by decreasing T $\beta$ RII mRNA and protein levels and  
12 subsequent TGF $\beta$  response. T $\beta$ RII ectopic expression in passaged chondrocytes permitted to increase the expression  
13 of several matrix genes, such as aggrecan or type II collagen. Redifferentiation of passaged chondrocytes permitted  
14 to restore, at least in part, T $\beta$ RII expression, and was related to differentiation of human bone marrow mesenchymal  
15 stem cells toward chondrocytes, where both Sp1 and T $\beta$ RII mRNA levels were increased. Moreover, Sp1  
16 manipulation by silencing or ectopic expression and pharmacologic inhibition revealed a link between expression  
17 levels of this transcriptional factor, which is crucial for constitutive expression of T $\beta$ RII in cartilage, and TGF $\beta$   
18 response.

19 Therefore, these data permit us to suggest an important role of T $\beta$ RII expression in maintenance of chondrocyte  
20 phenotype, which is altered with age, and bring new insights in our understanding of chondrogenesis process.

21

22 **Keywords:** TGFbeta receptors, chondrocytes, dedifferentiation/differentiation, Sp1, osteoarthritis, aging

23

## 1 INTRODUCTION

2 Articular cartilage consists of an abundant extracellular matrix, which is produced by chondrocytes. These highly  
3 differentiated cells are responsible for the maintenance of the cartilage as a functional entity. Aging cause changes in  
4 the properties of this tissue, notably by modifying the matrix composition or the growth factor responsiveness  
5 (Kempson,1982 ; Thonar et al., 1986 ; Dozin et al., 2002; Loeser et al., 2000; Rosen et al., 1997; Guerne, et al.,  
6 1995). During embryonic development, cartilage formation is initiated with condensation of mesenchymal cells,  
7 followed by progression of chondrocyte differentiation toward proliferation, prehypertrophy and hypertrophy.  
8 During osteoarthritis (OA), an articular disease strongly correlated with ageing (Buckwalter and Mankin, 1998, van  
9 der Kraan et van den berg, 2008), chondrocytes undergo morphogenic alterations accompanied by profound  
10 biochemical changes including the loss of production of cartilage-specific macromolecules. For instance, type II  
11 collagen expression decreased wheareas type I collagen synthesis increases. Some of the alterations of chondrocyte  
12 phenotype can be reproduced *in vitro*. Passages induce morphogenic alterations of cells, reduction of type II  
13 collagen, and induction of type I collagen synthesis (Benya et al., 1978; Archer et al., 1990; Pei et al., 2000;  
14 McDougall et al., 1996, Homicz et al., 2002). However, this model is not able to mimic others modifications  
15 undergone by OA chondrocytes, and notably the induction of type X collagen, specific of hypertrophy stage. On the  
16 other hand, the chondrocyte-specific phenotype can be recovered when the cells are cultured in three-dimensional  
17 culture system, such as alginate matrices (Reginato et al., 1994 ; Bonaventure et al., 1994).

18 Several studies suggest that modifications of chondrocyte phenotype during OA result from alteration of  
19 Transforming Growth Factor  $\beta$  (TGF $\beta$ ) signaling. Thus, the expression of TGF $\beta$  and its signaling molecules is  
20 altered with age, and the subsequent lack of TGF $\beta$  responsiveness might be at the root of OA development (Blaney-  
21 Davidson et al., 2005). Besides, TGF $\beta$  signaling is important for chondrogenesis. It promotes the differentiation of  
22 embryonic chick limb cartilage (Schonherr et al., 2000), and upregulates a number of molecules associated with  
23 prechondrogenic condensation (Serra et al., 1997; Chimal-Monroy et al., 1999; Grimaud et al., 2002). In addition,  
24 primary chondrogenesis derived from mesenchymal stem cells (MSCs) needs TGF $\beta$  signals (Reddi, 1994;  
25 Andriamanalijaona et al., 2008).

26 Members of TGF $\beta$  family transduce their signal via two serine/threonine receptors, type I (T $\beta$ RI) and type II (T $\beta$ RII)  
27 receptors (Wrana et al., 1994). Upon ligand-binding, T $\beta$ RI is phosphorylated by T $\beta$ RII, resulting in activation of  
28 intracellular proteins, named Smads. Receptor-regulated Smads (R-Smads: Smad2 and Smad3) are phosphorylated  
29 by T $\beta$ RI. Phosphorylated R-Smads form heterodimers with common-partner Smad (Co-Smads: Smad4), and  
30 translocate into the nucleus where they bind to specific DNA sequences for activation of TGF $\beta$  target gene  
31 transcription (Shi et al., 2003). Inhibitory Smads (I-Smads: Smad7) inhibit phosphorylation of R-Smads by  
32 competing with their binding to activated T $\beta$ RI (Nakao et al., 1997). Recently, it was shown that besides signaling  
33 via T $\beta$ RI (Activin-like kinase 5, ALK5), TGF-beta can also signal via ALK1, thereby activating Smad1/5/8 instead  
34 of Smad2/3 (Blaney-Davidson et al., 2009; Finnson et al., 2008).

35 We hypothesised that the maintenance of chondrocyte phenotype, which is altered with age, required a functional  
36 TGF $\beta$  signalling. We examined the influence of members of TGF $\beta$  signalling on chondrocyte phenotype. We  
37 showed that T $\beta$ RII is implicated in the loss of TGF $\beta$  responsiveness observed during chondrocytes passages. T $\beta$ RII

1 ectopic expression permits to restore TGF $\beta$  responsiveness as well as to increase the expression of some matrix  
2 components. We also found that T $\beta$ RII expression level is intimately linked to differentiation status of chondrocytes.  
3 Finally, we established a relationship between TGF $\beta$ -induced effects on matrix genes, and expression level of  
4 Specific protein 1 (Sp1) transcription factor, which expression is decreased in the course of dedifferentiation process  
5 (Duval et al., 2011) and is require to ensure constitutive expression of T $\beta$ RII in chondrocytes (Baugé et al., 2008).

## 8 MATERIAL AND METHODS

### 9 Reagents.

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

### 15 Chondrocyte culture

16 Human articular chondrocytes (HAC) were prepared from femoral head of post-mortem individuals with no known  
17 joint diseases or after femoral head fractures (ages between 45 and 80 years with median age of 68). The causes of  
18 donor's death were multiple and all of them signed agreement forms in the context of a body donation program  
19 according to local legislations. Similarly, donors from arthroscopy signed the agreement for this study according to  
20 local ethical comitee (CPP). Chondrocytes were released by enzymatic digestion as previously described (Baugé et  
21 al., 2007). Cells were seeded at  $4 \times 10^4$  cells/cm<sup>2</sup> and cultured in Dulbecco's modified Eagle's medium (DMEM) (4.5  
22 g/L D-glucose) supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 IU/ml penicillin, 100  $\mu$ g/ml  
23 streptomycin and 0.25  $\mu$ g/ml fungizone, in a 5 % CO<sub>2</sub> atmosphere. For dedifferentiation, HAC were passaged up to  
24 five times (P5) (about 7-8 population doubling) with trypsin-EDTA after reaching confluency.

25 For re-differentiation studies, chondrocytes, which had underwent three passages (about 4-5 population doubling),  
26 were encapsulated in alginate beads. Cells were suspended in alginate at the density of  $2.5 \times 10^6$  cells/ml, then slowly  
27 dropped into a 100 mM CaCl<sub>2</sub> solution. After instantaneous gelation, the beads were allowed to polymerize further  
28 for a period of 10 min in CaCl<sub>2</sub> solution. After three washes in PBS, they were finally placed in DMEM  
29 supplemented with 10% foetal calf serum. After 3 weeks, the beads were rinsed with PBS and dissolved in a solution  
30 composed of 55 mM sodium citrate and 25 mM EDTA. The suspension was incubated at 37°C until the beads were  
31 completely dissolved (approximately 10 min.). Centrifugation of the suspension at 800 g for 10 min allows  
32 separating the cells from their alginate matrix.

### 34 Culture of human bone marrow-derived stem cells

35 Human bone marrow-derived stem cells were collected from the same patients as donors used for HAC isolation.  
36 The harvested cells were washed twice with  $\alpha$ -MEM, and isolated by adherence over 24h to tissue culture plastic and  
37 were expanded in monolayer culture with  $\alpha$ -MEM supplemented with glutamine, FGF (1 ng/ml), 10% FCS and

1 antibiotics. After amplification by successive passages (until P5), the absence of hematopoietic markers (such as  
2 CD45) was checked by RT-PCR (data not shown). Thereafter, bone marrow-derived stem cells were directed toward  
3 chondrogenesis by culture in 3-D scaffold (alginate bead).

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

#### 9 **Plasmids and transfection experiments**

10 ~~p3TPLux~~, pCMV5-TβRII, pEVR2-Sp1 expression vectors were described earlier (Baugé et al., 2008). Chondrocytes  
11 were transiently transfected by the calcium phosphate precipitation method as previously described (Baugé et al.,  
12 2007). After overnight transfection, the culture medium was replaced by DMEM containing 10% FCS, and  
13 maintained in culture for 24 h.

#### 15 ~~Luciferase assay~~

16 ~~Luciferase assay was performed with Luciferase Assay kit from Promega (Charbonnières, France), as previously~~  
17 ~~described (Baugé et al., 2007).~~

#### 19 **Gene silencing experiments**

20 1.5 µg of double strand siRNA targeting Sp1, provided from Santa-Cruz Biotechnology (through Tebu, Le Perray en  
21 Yvelines, France), were nucleofected in HAC, according to the manufacturer's protocol with human chondrocyte  
22 Nucleofector solution (Amaxa, Köln, Germany). Negative control siRNA known to not lead to specific degradation  
23 of any cellular message was also used. After 6 h of transfection, the culture medium was replaced with DMEM  
24 containing 10% FCS and the chondrocytes cultures were incubated for 24 h.

#### 26 **RNA extraction and real-time RT-PCR.**

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

#### 34 **Protein extraction and Western-blot.**

35 Cells were rinsed, scrapped in RIPA lysis buffer supplemented with phosphatase and protease inhibitors. The  
36 extracts (30 µg protein) were subjected to fractionation in a 8 or 10% SDS-PAGE, transferred to PVDF membranes  
37 (Amersham Biosciences, Orsay, France), and reacted with rabbit aggrecan, TβRII or Sp1 polyclonal antibodies.

1 Subsequently, membranes were incubated with anti-rabbit secondary peroxidase-conjugated antibody. The signals  
2 were revealed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Perbio Science, Brébières, France)  
3 and exposed to X-ray film. The membranes were also reacted with anti  $\beta$ -actin to verify equal loading.  
4

#### 5 **Synthesis and transfection of decoy oligonucleotides.**

6 Sp1 decoy oligonucleotides sequences are: Forward 5'- ATTCGATCGGGGCGGGGCGAGC -3' and Reverse 5'-  
7 CGACGCCCCGCCCGATCGAAT -3' (consensus sequences are underlined). This decoy oligonucleotide was  
8 previously shown to bind Sp1 transcriptional factor (Baugé et al., 2008). These oligonucleotides were annealed  
9 overnight and transfected into cells using the Oligofectamine reagent according to the manufacturer's instructions.  
10

#### 11 **Nuclear extracts and EMSA**

12 Nuclear extracts were prepared as described previously (Dignam et al., 1983). For EMSA, 5 femtomoles of ( $\gamma$ -  
13 <sup>32</sup>P)ATP-labelled probes (Sp1 binding sequence) and 4  $\mu$ g of nuclear extracts were incubated at room temperature  
14 for 15 min in binding buffer (20 mM Tris-HCl pH 7.5, 1 mM DTT, 100 mM NaCl, 5% bovine serum albumin, 0.1%  
15 Nonidet P-40, 10% glycerol, 25  $\mu$ M ZnCl<sub>2</sub>) and 2  $\mu$ g of poly(dI-dC).(dI-dC). Samples were resolved on native  
16 polyacrylamide gel and exposed for autoradiography.  
17

#### 18 **Statistical analysis:**

19 All experiments were repeated at least with 3 different donors, and similar results were obtained. Representative  
20 experiments are shown in the figures. Data are presented as the mean  $\pm$  SD. Statistical significance was determined  
21 by Student's *t*-test. *P* values less than 0.05 were considered significant. \*\*\*= *P* <0.001; \*\*= *P* < 0.01; \*= *P* < 0.05  
22 versus controls.  
23  
24

## 1 RESULTS

### 2 **Chondrocyte passages reduce the TGF $\beta$ responsiveness and decrease T $\beta$ RII expression.**

3 In order to ascertain that subculturing chondrocytes led to the loss of cartilaginous phenotype in our hands, we  
4 monitored the decrease of type II collagen and aggrecan mRNA expression after successive passages. Their  
5 expressions were strongly decreased as soon as the first passage (about 2 cell doubling). The maximal reduction was  
6 obtained at P2 (approximately 3 doubling) and was maintained at least until P5. At opposite, collagen type I and III  
7 mRNA levels were higher at P2 (suppl figure 1A). Therefore, we limited the following experiments to 2 or 3  
8 passages (3-5 cell doubling).

9 To investigate chondrocyte responsiveness to TGF $\beta$  during dedifferentiation process, we focused on the expression  
10 of Plasminogen Activator Inhibitor-1 (PAI-1), which is rapidly induced by TGF $\beta$  in chondrocytes (Baugé et al.,  
11 2007). Human articular chondrocytes which underwent three passages were incubated for 4 h with TGF $\beta$ 1 (2 ng/ml).  
12 Figure 1A shows that passages induced a significant decrease of PAI-1 induction by TGF $\beta$ .

13 TGF $\beta$  initiates its cellular action by binding to T $\beta$ RII, which in turn activates T $\beta$ RI. Thus, we investigated the effect  
14 of passages on mRNA expression of both receptors. Whereas T $\beta$ RI transcripts were increased in comparison to  
15 primary chondrocytes (P0) (fig. 1B), T $\beta$ RII is dramatically decreased following dedifferentiation (fig. 1C). This  
16 reduction of T $\beta$ RII expression was also observed at protein level (fig 1D).

17

### 18 **Passages increases the expression of the R-Smad, Smad3, and decreases that of the I-Smads, Smad7.**

19 Since signalling by TGF $\beta$  is mediated by Smads, we explored the potential alteration of Smads expression during  
20 chondrocyte dedifferentiation. Our result (fig 2) showed that serial passages of chondrocytes in serum supplemented  
21 medium did not modulate significantly Smad2 and 4 mRNA levels. Moreover, they increased Smad3 mRNA levels  
22 whereas they decreased the expression of the I-Smad, Smad7. These data suggest that the loss of TGF $\beta$   
23 responsiveness observed in dedifferentiated chondrocytes is independent of Smads expression level, since an up-  
24 regulation of Smad3 and down-regulation of Smad7 is usually associated with activation of TGF $\beta$  signalling.

25

### 26 **Ectopic expression of T $\beta$ RII restores TGF $\beta$ responsiveness in passaged chondrocytes and increases expression 27 of matrix genes.**

28 To confirm the crucial role of T $\beta$ RII expression alteration in the reduction of TGF $\beta$  sensitiveness of passaged  
29 chondrocytes, we examined the effect of forced T $\beta$ RII expression on the induction of PAI-1 by TGF $\beta$  (fig 3A). HAC  
30 were transfected with a T $\beta$ RII expression vector (pCMV5-T $\beta$ RII) or its corresponding insertless vector (pCMV5) for  
31 24h and further incubated with TGF $\beta$ . Dedifferentiation clearly causes reduced TGF $\beta$ -induced PAI-1 expression,  
32 which was restored by T $\beta$ RII ectopic expression. In addition, transfection of T $\beta$ RII expression vector in passaged  
33 chondrocytes induces ~~an increased mRNA level~~ the expression of aggrecan and collagen type II (x2-3) (fig 3B-C).

34

### 35 **T $\beta$ RII expression increases during chondrocyte differentiation**

36 Since T $\beta$ RII levels decreased upon dedifferentiation, we hypothesized that its expression could also be modulated  
37 during chondrocyte differentiation process. Therefore, we pursued by examination of T $\beta$ RII expression in re-  
38 differentiating chondrocytes. For that, the T $\beta$ RII mRNA level was evaluated prior dedifferentiation (P0), after



1 dedifferentiation (P3) and after redifferentiation in alginate. TβRII mRNA level was increased after a 3 week-culture  
2 in alginate (fig 4A), time which permit to restore (at least partially) a chondrocyte phenotype in this system (Duval et  
3 al., 2011).

4 Similar experiments were performed using human bone marrow-derived mesenchymal stem cells, which underwent  
5 chondrogenesis by culture in alginate microbeads (Duval E/Boumediene K, unpublished data; suppl data 2B). This  
6 system is well-known to induce chondrogenic differentiation from mesenchymal stem cells from different origins  
7 (Andriamanalijaona et al., 2008). As shown in figure 4B, TβRII mRNA levels strongly increased with the culture  
8 time, and subsequently increased during differentiation of hBM derived-MSc into chondrocyte-like phenotype.

### 10 **Sp1 is important for TβRII expression and the maintenance of chondrocyte phenotype**

11 Given that Sp1 expression decreases during chondrocyte dedifferentiation and is restored by alginate culture (Duval  
12 et al., 2011), we proposed that Sp1 expression and activity could increase during differentiation of stem cells. Sp1  
13 mRNA level was raised during human bone marrow derived stem cells differentiation into chondrocytes (Fig 5A).  
14 To define whether DNA binding activity of Sp1 is also increased in these conditions, EMSAs were performed using  
15 the consensus DNA binding site as a probe (fig 5B). One delayed protein-DNA complex was strongly enhanced by  
16 hBM-MSc culture in alginate beads after 72h or 96h. The binding activity of the complex to the probe was  
17 decreased in the presence of Sp1 antibody (and not in the presence of irrelevant antibodies, namely against cKrox or  
18 Sp3), indicating that this complex contains Sp1 factor.

19 Then, we investigated the effect of inhibition of Sp1 on TβRII expression. First, we blocked the binding activity of  
20 Sp1 family members using decoy oligonucleotides or mithramycin (Jia et al., 2007) and showed that the loss of Sp1  
21 binding to DNA inhibited TβRII mRNA level-expression (fig 6A-B). Furthermore, we established that the specific  
22 reduction of Sp1 expression by RNA silencing, checked by Western-blot, reduced TβRII expression (fig 6C).  
23 Mithramycin also reduced TβRII protein expression. Interestingly, aggrecan expression was also decreased by  
24 mithramycin (figure 6D).

25 Finally, in figure 7, we showed that Sp1 ectopic expression is able to restore TβRII expression in dedifferentiated  
26 chondrocytes and counteracts inhibition of aggrecan expression induced by passages.

## 1 DISCUSSION

2 Modifications of chondrocyte phenotype occur with aging and during osteoarthritis. They can be mimicked, in part,  
3 *in vitro* by subculturing monolayer cultures of chondrocytes (Homicz et al., 2002; Pei et al., 2000; McDougall et al.,  
4 1996; Duval et al., 2011). This culture model provides a way to understand some of the molecular mechanisms  
5 responsible for chondrocyte senescence and dedifferentiation, and to elucidate the transition from young/healthy to  
6 old/osteoarthritic cartilage. Conversely, studies of mechanism of re-differentiation of chondrocyte or differentiation  
7 of mesenchymal cells into chondrocytes can permit to bring new insights in order to develop the therapeutic use of  
8 mesenchymal stem cells (MSC) in cartilage disorders. In this study, we showed that chondrocyte dedifferentiation is  
9 accompanied by T $\beta$ R2 expression alteration, whereas redifferentiation of passaged chondrocytes or differentiation of  
10 MSC into chondrocytes are accompanied by T $\beta$ R2 production. Moreover, we demonstrated that forced T $\beta$ R2  
11 expression is able to restore totally or partially the expression of matrix genes, suggesting a key-role of this receptor  
12 and TGF $\beta$  signal in the maintenance of chondrocyte phenotype.

13 Our *in vitro* experiments show the key-role of T $\beta$ R2 expression in chondrocyte phenotype maintenance. This result  
14 completes *in vivo* data which showed T $\beta$ R2-deficient mice have a reduced proliferation of chondrocytes and an  
15 accelerated early hypertrophic differentiation relative to control (Seo et al., 2007). In addition, conditional transgenic  
16 mice in which a dominant negative form of T $\beta$ R2 is expressed, develop a hypoplastic cartilage (Hiramatsu et al.,  
17 2011), confirming that T $\beta$ R2 is required for normal cartilage development, and subsequently chondrocyte  
18 phenotype.

19 Chondrocyte dedifferentiation, which occurs with aging, is likely associated to alteration of cell responsiveness to  
20 TGF $\beta$  by reduction of T $\beta$ R2 expression. Of interest, we have recently demonstrated that interleukin-1 $\beta$ , a  
21 proinflammatory cytokine which is increased in OA process, rendered cells insensitive to TGF $\beta$  by reducing T $\beta$ R2  
22 expression (Baugé et al., 2007). Together, these results corroborate *in vivo* data, which indicated that T $\beta$ R2  
23 expression is dramatically reduced in old mice (Blaney Davidson et al., 2005) and during OA (Boumediene et al.,  
24 1998; Verdier et al., 2005), and confirmed the relationship between T $\beta$ R2 expression and the reduction of cell  
25 responsiveness to TGF $\beta$  observed in OA and old cartilage. Concerning T $\beta$ R1 expression, it increases with passages,  
26 suggesting that this receptor is not involved in the reduction of cell responsiveness to TGF $\beta$  in passaged  
27 chondrocytes. This result was unexpected since T $\beta$ R1 expression is reduced during experimental OA induced in mice  
28 by destabilization of the medial meniscus model (Blaney-Davidson et al., 2009).

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

1 We also observed the TBR2 expression during *in vitro* chondrogenesis. Three-dimensional scaffold was used to re-  
2 differentiate dedifferentiated chondrocytes or to differentiate MSC toward chondrogenic phenotype. This culture  
3 system was described earlier to permit the induction of chondrogenic markers (Reginato et al., 1994; Bonaventure et  
4 al., 1994; Andriamanalijaona et al., 2008; Lemare et al., 1998; Cai et al., 2007; Duval et al., 2011). We showed that  
5 TBR2 expression is induced during redifferentiation of subcultured chondrocytes and during chondrogenic process  
6 of stromal stem cells. In this regard, Mizuta and coll. found that expression of both TBR1 and TBR2 receptors was  
7 upregulated during periosteal chondrogenesis induced by exogenous TGF $\beta$ 1 treatment (Mizuta et al., 2002). Our data  
8 corroborate another *in vitro* study which proposes that TGF $\beta$  signal transduction may be involved in the early stage  
9 of bone marrow-MSC chondrogenesis (Huang et al., 2005), and are consistent with recent studies, which  
10 demonstrated that TBR2 down-regulation induced by retinoid acid treatment inhibits chondrogenesis of mouse  
11 embryonic palate mesenchymal cells (Yu et Xing, 2006) and that TGF $\beta$  signalling is needed in the chondrogenic  
12 differentiation of MSC (Longobardi et al., 2006).

13 Finally, we investigated the role of Sp1 role in the regulation of TBR2 expression and matrix production. This  
14 ubiquitous zinc-finger protein has earlier been proposed to regulate matrix production by adult articular chondrocytes  
15 maintained in primary and expansion culture (Duval et al., 2011; Dharmavaram et al., 1997 ; Oh et al., 2007).  
16 Furthermore, we have demonstrated loss of Sp1 gene expression as a function of chondrocyte passage number and  
17 that Sp1 protein synthesis is restored in part by re-differentiation in 3D-alginate culture (Duval et al., 2011). Here,  
18 we showed that the decrease of Sp1 induced by serial passaging of chondrocytes is responsible for TBR2 reduction.  
19 However, we cannot exclude that Sp1 acts indirectly. Sp1 might permit the re-expression of TBR2 in  
20 dedifferentiated chondrocytes through Sox9, which can be induced by Sp1 (Piera-Velazquez et al., 2007). Sox9 was  
21 first identified as a transcription factor which specifies the chondrocyte lineage and is absolutely required for  
22 complete cartilage differentiation (Bi et al., 1999). In our study, Sp1 was not able to counteract the decrease of type  
23 II collagen (data not shown) whereas it increases aggrecan mRNA level in passaged chondrocytes. Since it was  
24 previously established that TBR2 gene expression occurred earlier than that of type II collagen gene expression  
25 during differentiation of bone marrow-MSC under dynamic compressive loading (Huang et al., 2005), we  
26 hypothesize that longer time of Sp1 overexpression could restore the aggrecan or type II collagen expression in  
27 dedifferentiated chondrocytes. In order to validate this speculation, it will be necessary to reproduce this experiment  
28 using stable transfection system, which could permit to maintain a high level of Sp1 during a long period.

29 In conclusion, we demonstrated that TBR2 expression level is essential to maintain and/or promote chondrocyte  
30 phenotype, and established a key-role of Sp1 in this process. These findings may help in understanding the behaviour  
31 of aged chondrocytes. In addition, it brings original insights in the process of MSC differentiation into chondrocytes  
32 and subsequently permits to envisage innovative strategies to optimize the therapeutic use of chondrocytes or MSC  
33 in cartilage disorders.

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4    Sp1, respectively.

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9    CB conceived, carried out experiments, analyzed data and wrote paper. ED participated to data collection and  
10   analysis. DO, NG and SL participated to data collection. PG participated to data interpretation. KB conceived  
11   experiments, analyzed data and was involved in writing the paper. All authors had final approval of the submitted  
12   and published versions.

13

14    **DISCLOSURE**

15    The authors confirm that there are no conflicts of interest.

16

17

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7

8

1 **FIGURE LEGENDS**

2 **Figure 1: Chondrocyte passages reduces the TGFβ responsiveness and decreases TβRII expression.**

3 A: Primary (P0 : about 1-2 population doubling) or passaged (P1 (~2-3 population doubling), P2 (~3-4 population  
4 doubling) and P3 (~4-5 population doubling)) HAC were cultured for 5-6 days before incubation with TGFβ1 (2  
5 ng/ml) for 4 h. Total RNA was isolated and analyzed by real-time RT-PCR, using PAI-1 and GAPDH primers.

6 B-C: Total RNA and proteins from freshly isolated chondrocytes or from chondrocytes dedifferentiated by repeated  
7 passages were extracted. TβRI and TβRII mRNA levels were defined by real-time RT-PCR.

8 D: TβRII protein level was analyzed by Western-blot.

9  
10 **Figure 2: Passages up-regulates the R-Smad, Smad3, and down-regulates the I-Smads, Smad7.**

11 A-D: HAC were cultured as described in figure 1 and Smad2, 3, 4 and 7 mRNA levels were analyzed by real-time  
12 RT-PCR. Results are shown as histograms of relative expression versus GAPDH signal.

13  
14 **Figure 3: Ectopic expression of TβRII restores TGFβ responsiveness in passaged chondrocytes and increases  
15 expression of matrix genes.**

16 A: Primary HAC (P0) or passaged twice (P2) were transfected with TβRII expression vector (pCMV5-TβRII) or  
17 with the insertless plasmid (5 μg). After 15 h, the medium was changed for DMEM+2% FCS for 24 h and cells were  
18 incubated with TGFβ1 addition (2 ng/ml) for 4 h. PAI-1 expression was analyzed by real-time RT-PCR.

19 B-C: HAC were cultured and transfected as above. 24 h later, col2A1 and aggrecan mRNA expression was analyzed  
20 and normalized with GAPDH level (B). Aggrecan and TβRII protein levels were also analysed by Western-blot (C).

21  
22 **Figure 4: TβRII expression increases during chondrocyte differentiation**

23 A: Primary chondrocytes (P0) or HAC dedifferentiated by 3 passages (P3) were cultured in monolayer or transferred  
24 into alginate beads for 21 days. Thereafter, TβRII mRNA level was analyzed by real-time RT-PCR.

25 B: Human bone marrow-derived stem cells were cultured in alginate beads for different incubation-period and TβRII  
26 mRNA levels were assayed. Results are shown as relative gene expression versus GAPDH signal.

27  
28 **Figure 5: Sp1 expression increases during chondrocyte differentiation**

29 A: Human bone marrow-derived stem cells were cultured in alginate beads for different incubation-period and Sp1  
30 mRNA levels were assayed. Results are shown as histograms of relative gene expression versus GAPDH signal.

31 B: Electrophoretic mobility shift assay (EMSA) reactions were performed using probe corresponding to Sp1  
32 consensus DNA binding site, incubated with 4 μg nuclear extract from hBM-MSC cultured in alginate for 0h, 72h or  
33 96h. EMSA reactions were also performed after incubation of nuclear extracts (from hBM cultured 72h in alginate)  
34 with Sp1, Sp3 and cKrox antibodies.

35  
36 **Figure 6: Sp1 regulates TβRII expression.**

1 A: Primary HAC, transfected with a Sp1 consensus binding sequence (Sp1 decoy), were incubated in DMEM+2%  
2 FCS for 24 h. Thereafter, T $\beta$ R $\text{II}$  mRNA versus GAPDH level was determined by RT-PCR.  
3 B: Subconfluent cultures of chondrocytes were treated for 48 h in the presence or absence of mithramycin (100 and  
4 150 nM). T $\beta$ R $\text{II}$  expression was analyzed at mRNA level by real-time RT-PCR.  
5 C: HAC were nucleofected with siRNA oligonucleotides targeted Sp1 or with control siRNA. Thereafter, the  
6 medium was replaced with DMEM+10% FCS for 24 h. Sp1 and T $\beta$ R $\text{II}$  protein levels were defined by Western-Blot.  
7 D: Chondrocytes were treated for 48 h in the presence or absence of mithramycin (100 and 150 nM). T $\beta$ R $\text{II}$  and  
8 aggrecan expression was analyzed at protein level by Western-Blot.

9  
10 **Figure 7: Sp1 regulates chondrocyte phenotype**

11 A-B: HAC passaged twice (P2) were transfected with Sp1 expression vector (pEVR2-Sp1) or with the insertless  
12 plasmid (5  $\mu$ g). After 15 h, the medium was changed for DMEM+2% FCS for 24 h. Total RNA was isolated and  
13 T $\beta$ R $\text{II}$  and aggrecan mRNA levels were analyzed and normalised with GAPDH level, in comparison to HAC with no  
14 passage (P0).

15  
16 **Suppl data 1:**

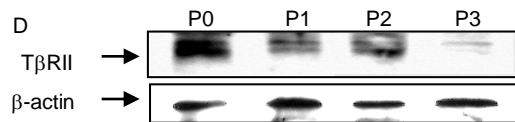
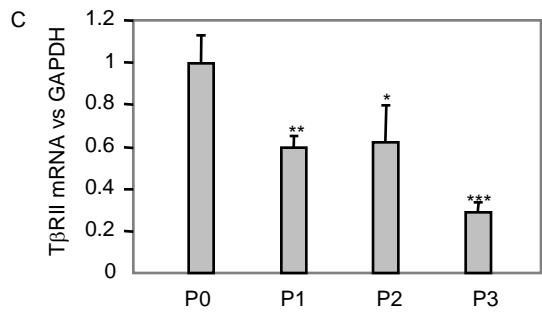
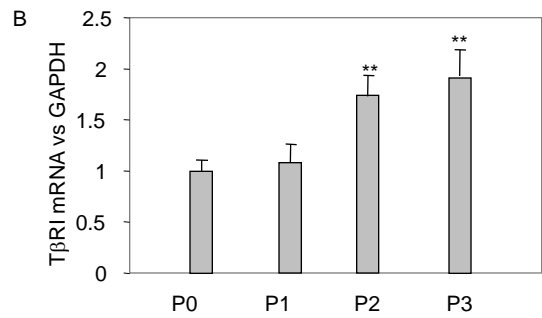
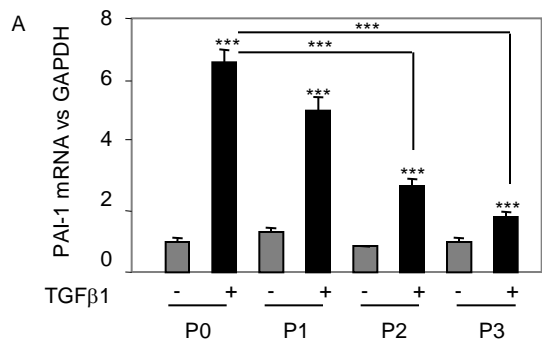
17 Primary (P0) or passaged (until P5  $\sim$  6-7 population doubling) HAC were cultured in monolayer for 5-6 days.  
18 Thereafter, COL1A1, COL2A1, COL3A1 and aggrecan mRNA levels were analyzed by real-time RT-PCR.

19  
20 **Suppl data 2:**

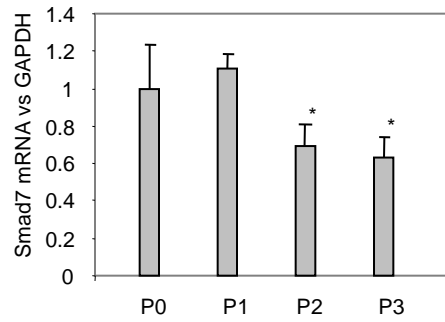
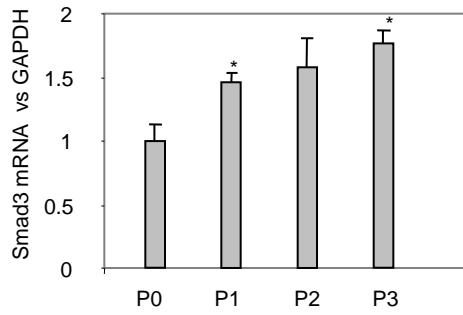
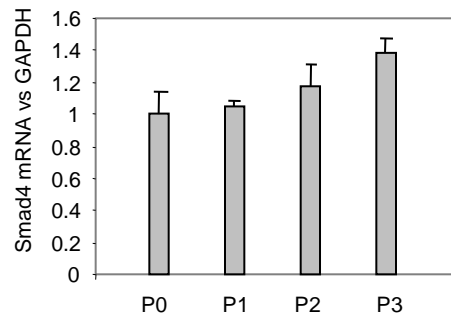
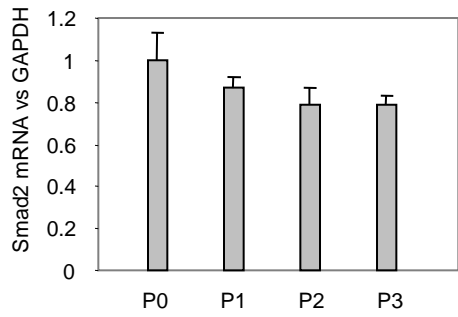
21 A: P3 ( $\sim$  4-5 population doubling) chondrocytes were cultured in monolayer or transferred into alginate beads for 21  
22 days. Thereafter, COL1A1, COL2A1, COL3A1 mRNA levels were analyzed by real-time RT-PCR.

23 B: Human bone marrow-derived stem cells were cultured in alginate beads for 7 days and COL2A1 and aggrecan  
24 mRNA levels were assayed. Results are shown as histograms as relative gene expression versus GAPDH signal.

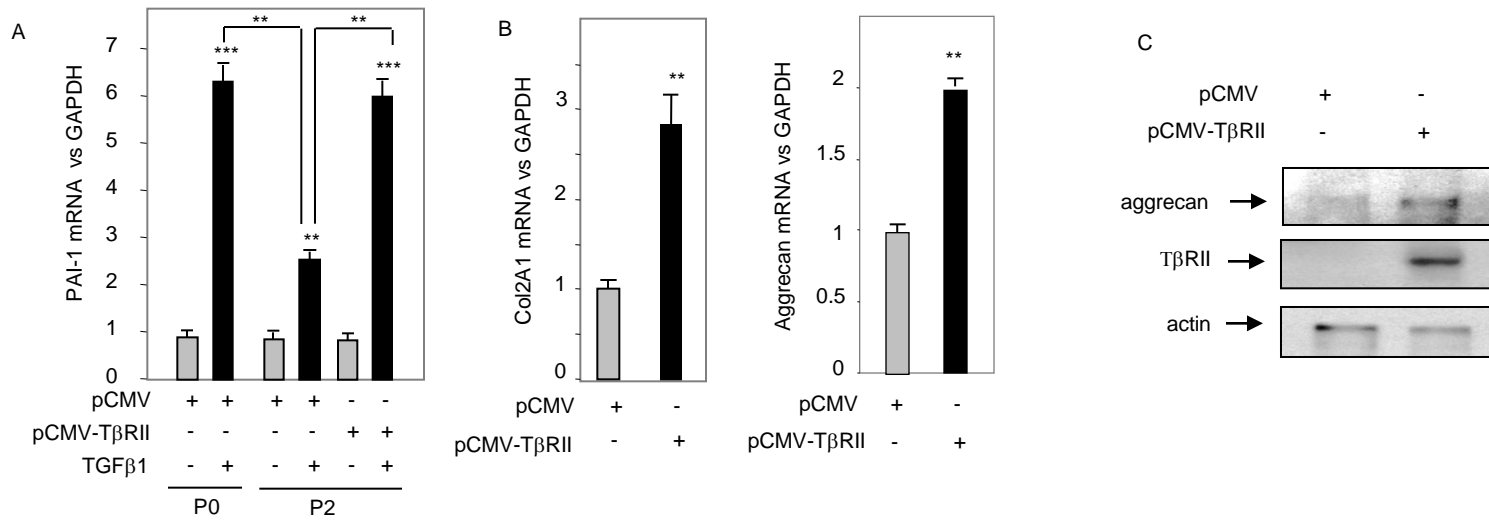
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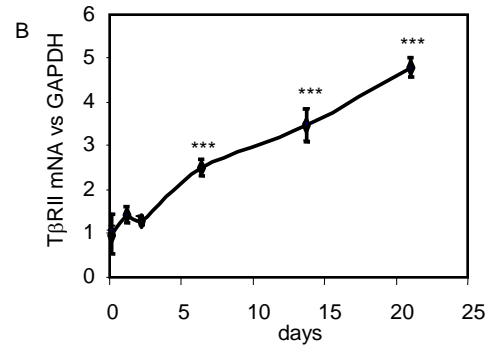
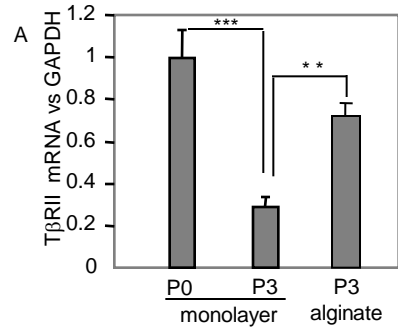
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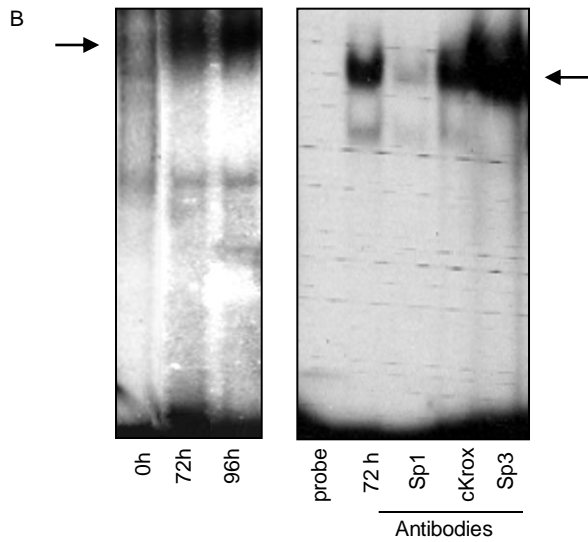
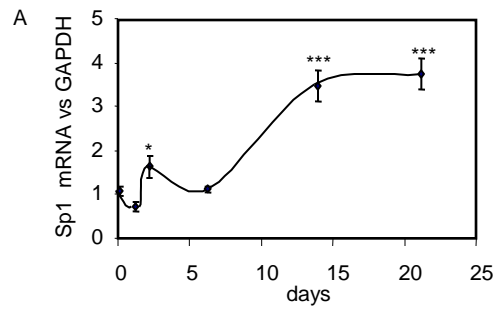
**Figure 2:**



**Figure 3:**

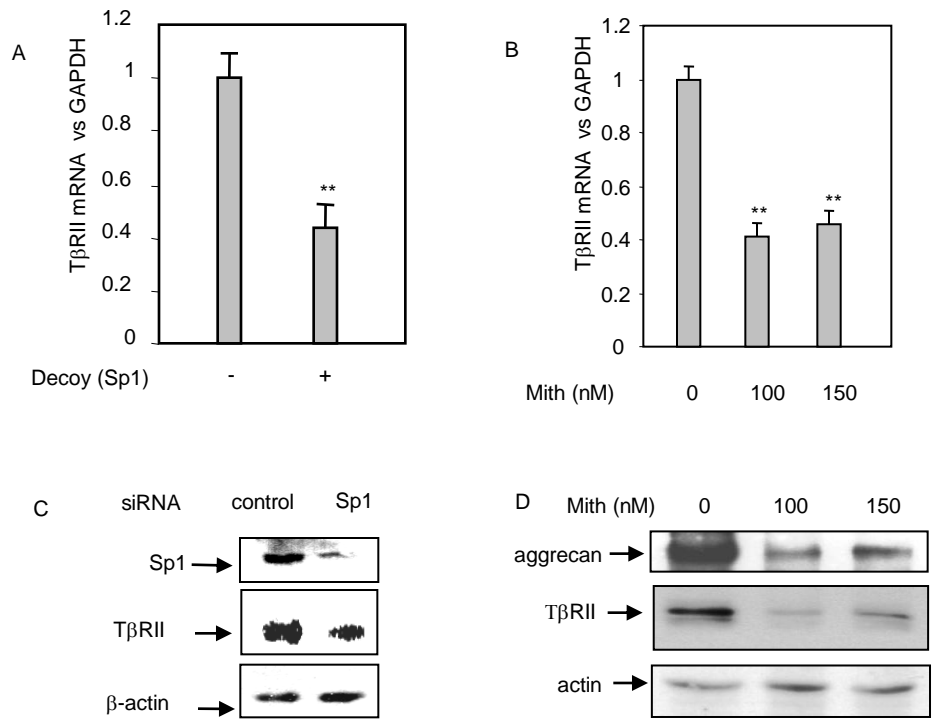


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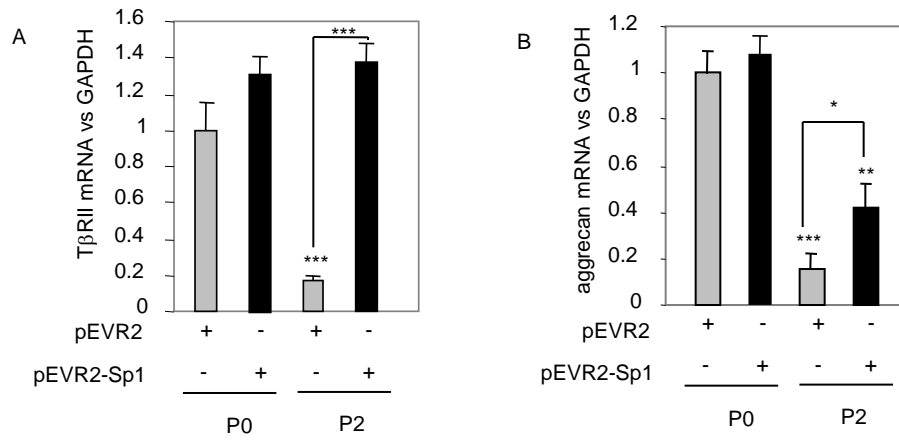


**Figure 5**

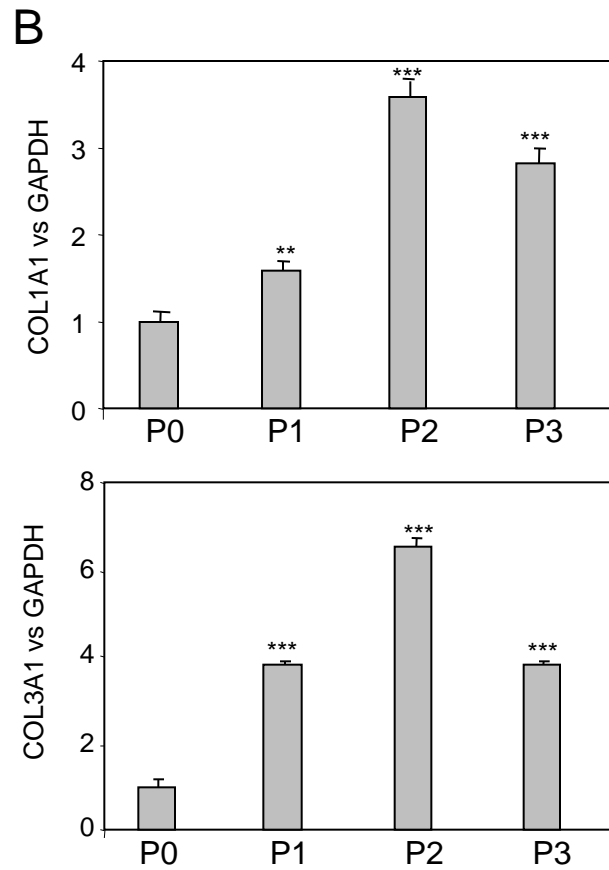
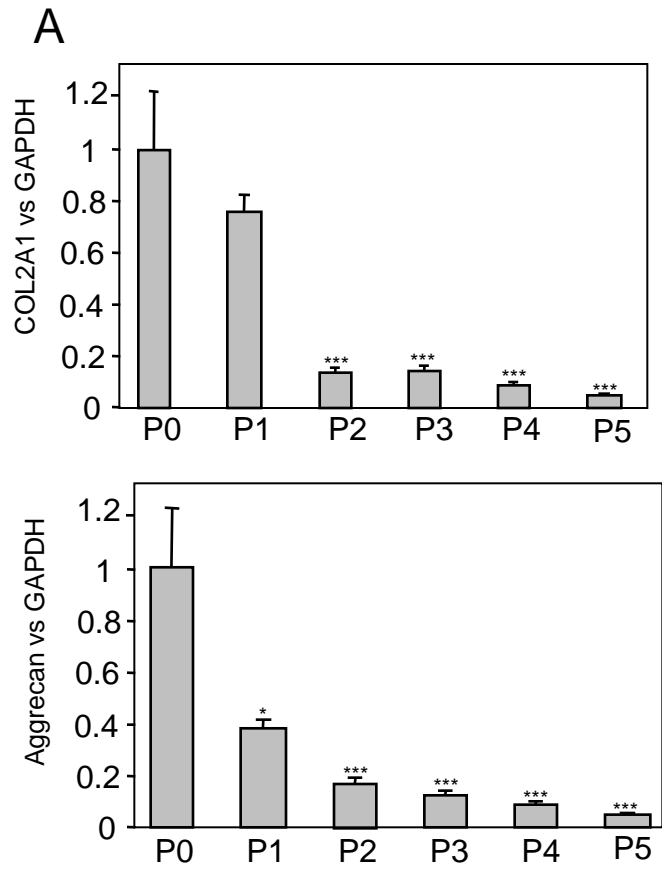




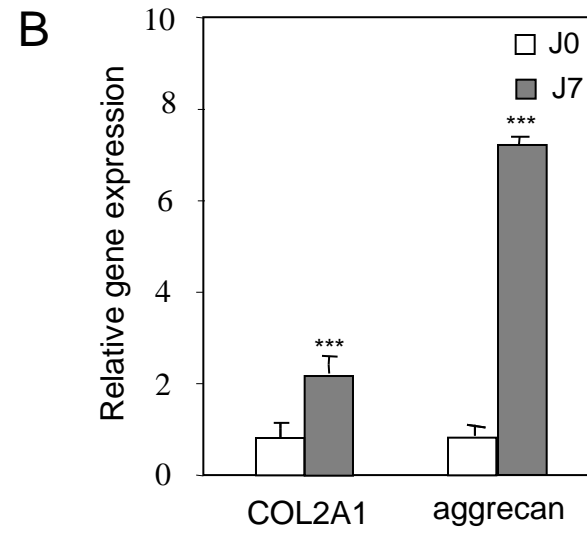
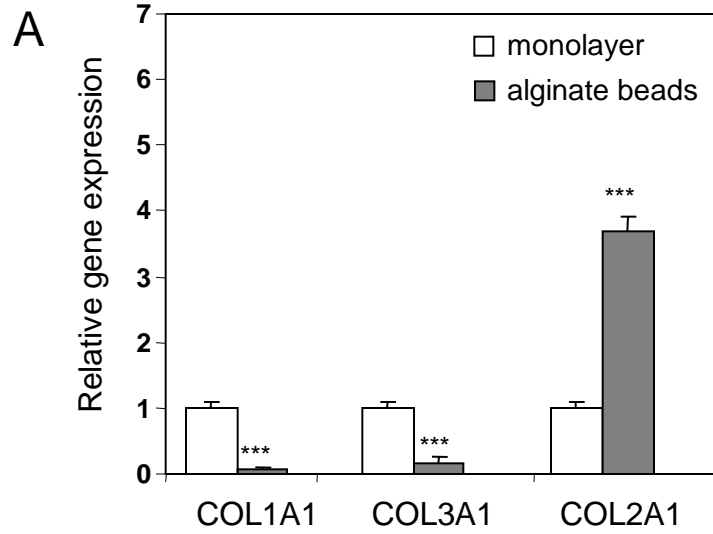
**Figure 6**



**Figure 7**



Suppl figure 1



**Suppl figure 2**