



HAL
open science

Type II TGF β receptor modulates chondrocyte phenotype

Catherine Baugé, Elise Duval, David Ollitrault, Nicolas Girard, Sylvain Leclercq, Philippe Galéra, Karim Boumédiene

► **To cite this version:**

Catherine Baugé, Elise Duval, David Ollitrault, Nicolas Girard, Sylvain Leclercq, et al.. Type II TGF β receptor modulates chondrocyte phenotype. *AGE*, 2013, 35 (4), pp.1105-16. 10.1007/s11357-012-9433-7 . hal-01149519

HAL Id: hal-01149519

<https://hal.science/hal-01149519>

Submitted on 24 Sep 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Type II TGFbeta receptor modulates chondrocyte phenotype.**

2
3 **Catherine Bauge^{1¶}, Elise Duval¹, David Ollitrault¹, Nicolas Girard¹, Sylvain Leclercq^{1,2}, Philippe Galéra¹, and**
4 **Karim Boumédiene¹**

5
6 1: Université de Caen Basse-Normandie, MILPAT, Caen, France

7 2: Department of Orthopaedic Surgery, Saint-Martin Private Clinic, Caen, France

8
9 ¶: Correspondence and copy request: Catherine Bauge, catherine.bauge@unicaen.fr EA4652 Microenvironnement

10 Cellulaire et Pathologies, Université de Caen, 14032 Caen cedex, France; tel: +33 231068218; fax: +33
11 231068224

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 β signaling. This study investigates the role of
4 TGF β 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 β signalling by decreasing T β RII mRNA and protein levels and
12 subsequent TGF β response. T β 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 β RII expression, and was related to differentiation of human bone marrow mesenchymal
15 stem cells toward chondrocytes, where both Sp1 and T β 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 β RII in cartilage, and TGF β
18 response.

19 Therefore, these data permit us to suggest an important role of T β 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 β (TGF β) signaling. Thus, the expression of TGF β and its signaling molecules is
20 altered with age, and the subsequent lack of TGF β responsiveness might be at the root of OA development (Blaney-
21 Davidson et al., 2005). Besides, TGF β 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 β signals (Reddi, 1994;
25 Andriamanalijaona et al., 2008).

26 Members of TGF β family transduce their signal via two serine/threonine receptors, type I (T β RI) and type II (T β RII)
27 receptors (Wrana et al., 1994). Upon ligand-binding, T β RI is phosphorylated by T β RII, resulting in activation of
28 intracellular proteins, named Smads. Receptor-regulated Smads (R-Smads: Smad2 and Smad3) are phosphorylated
29 by T β 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 β 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 β RI (Nakao et al., 1997). Recently, it was shown that besides signaling
33 via T β 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 β signalling. We examined the influence of members of TGF β signalling on chondrocyte phenotype. We
37 showed that T β RII is implicated in the loss of TGF β responsiveness observed during chondrocytes passages. T β RII

1 ectopic expression permits to restore TGF β responsiveness as well as to increase the expression of some matrix
2 components. We also found that T β RII expression level is intimately linked to differentiation status of chondrocytes.
3 Finally, we established a relationship between TGF β -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 β 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 β 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 4×10^4 cells/cm² 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 μ g/ml
23 streptomycin and 0.25 μ g/ml fungizone, in a 5 % CO₂ 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 2.5×10^6 cells/ml, then slowly
27 dropped into a 100 mM CaCl₂ solution. After instantaneous gelation, the beads were allowed to polymerize further
28 for a period of 10 min in CaCl₂ 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 α -MEM, and isolated by adherence over 24h to tissue culture plastic and
37 were expanded in monolayer culture with α -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 β -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 CGACGCCCCGCCCCGATCGAAT -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 (γ -
13 32 P)ATP-labelled probes (Sp1 binding sequence) and 4 μ 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 μ M ZnCl₂) and 2 μ 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.

1 RESULTS

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

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

25

26 **Ectopic expression of T β RII restores TGF β responsiveness in passaged chondrocytes and increases expression 27 of matrix genes.**

28 To confirm the crucial role of T β RII expression alteration in the reduction of TGF β sensitiveness of passaged
29 chondrocytes, we examined the effect of forced T β RII expression on the induction of PAI-1 by TGF β (fig 3A). HAC
30 were transfected with a T β RII expression vector (pCMV5-T β RII) or its corresponding insertless vector (pCMV5) for
31 24h and further incubated with TGF β . Dedifferentiation clearly causes reduced TGF β -induced PAI-1 expression,
32 which was restored by T β RII ectopic expression. In addition, transfection of T β 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 β RII expression increases during chondrocyte differentiation**

36 Since T β 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 β RII expression in re-
38 differentiating chondrocytes. For that, the T β 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-MSK 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-MSK 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 β RII expression alteration, whereas redifferentiation of passaged chondrocytes or differentiation of
10 MSC into chondrocytes are accompanied by T β RII production. Moreover, we demonstrated that forced T β RII
11 expression is able to restore totally or partially the expression of matrix genes, suggesting a key-role of this receptor
12 and TGF β signal in the maintenance of chondrocyte phenotype.

13 Our *in vitro* experiments show the key-role of T β RII expression in chondrocyte phenotype maintenance. This result
14 completes *in vivo* data which showed T β RII-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 β RII is expressed, develop a hypoplastic cartilage (Hiramatsu et al.,
17 2011), confirming that T β RII 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 β by reduction of T β RII expression. Of interest, we have recently demonstrated that interleukin-1 β , a
21 proinflammatory cytokine which is increased in OA process, rendered cells insensitive to TGF β by reducing T β RII
22 expression (Baugé et al., 2007). Together, these results corroborate *in vivo* data, which indicated that T β RII
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 β RII expression and the reduction of cell
25 responsiveness to TGF β observed in OA and old cartilage. Concerning T β R1 expression, it increases with passages,
26 suggesting that this receptor is not involved in the reduction of cell responsiveness to TGF β in passaged
27 chondrocytes. This result was unexpected since T β 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 β 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 β 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 β response since TGF β 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 β 1 treatment (Mizuta et al., 2002). Our data
8 corroborate another *in vitro* study which proposes that TGF β 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 β 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.

34
35
36
37
38

1 **ACKNOWLEDGMENTS**

2 We would like to thank Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New-York, USA) and Dr Suske
3 (Institut für Molekularbiologie and tumorforschung, Marburg, Germany) for providing pCMV5-TβRII and pEVR2-
4 Sp1, respectively.

5 This work was supported by a grant from European Community in the context of Genostem integrated project (6th
6 framework program - Contract grant number: 503161) and by Lions Club of Normandy. E Duval and D. Ollitrault
7 were recipient of fellowship from “Conseil Régional de Basse-Normandie” and from “Ministère de l’Enseignement
8 Supérieur et de la Recherche”, respectively.

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

1 **REFERENCES**

- 2 Andriamanalijaona R, Duval E, Raoudi M, Lecourt S, Vilquin JT, Marolleau JP, Pujol JP, Galera P, Boumediene K
3 (2008) Differentiation potential of human muscle-derived cells towards chondrogenic phenotype in alginate beads
4 culture. *Osteoarthritis Cartilage* 16: 1509-1518.
- 5 Archer CW, McDowell J, Bayliss MT, Stephens MD, Bentley G (1990) Phenotypic modulation in sub-populations
6 of human articular chondrocytes in vitro. *J Cell Sci* 97: 361-371.
- 7 Baugé C, Beauchef G, Leclercq S, Kim SJ, Pujol JP, Galera P, Boumediene K. (2008) NFkappaB mediates IL-1beta-
8 induced down-regulation of TbetaRII through the modulation of Sp3 expression. *J Cell Mol Med* 12: 1754-66.
- 9 Baugé C, Cauvard O, Leclercq S, Galéra P, Boumédiene K (2011) Modulation of transforming growth factor beta
10 signalling pathway genes by transforming growth factor beta in human osteoarthritic chondrocytes: involvement of
11 Sp1 in both early and late response cells to transforming growth factor beta. *Arthritis Res Ther.* 13:R23
- 12 Baugé C, Legendre F, Leclercq S, Elissalde JM, Pujol JP, Galera P, Boumediene K (2007) Interleukin-1beta
13 impairment of transforming growth factor beta1 signaling by down-regulation of transforming growth factor beta
14 receptor type II and up-regulation of Smad7 in human articular chondrocytes. *Arthritis Rheum* 56: 3020-3032.
- 15 Benya PD, Padilla SR, Nimni ME (1978) Independent regulation of collagen types by chondrocytes during the loss
16 of differentiated function in culture. *Cell* 15: 1313-1321.
- 17 Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrughe B (1999) Sox9 is required for cartilage formation. *Nat*
18 *Genet* 22: 85-89.
- 19 Blaney Davidson EN, Remst DF, Vitters EL, van Beuningen HM, Blom AB, Goumans MJ, van den Berg WB, van
20 der Kraan PM. (2009) Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in
21 humans and mice. *J Immunol.* 182:7937-45.
- 22 Blaney Davidson EN, Scharstuhl A, Vitters EL, van der Kraan PM, van den Berg WB. (2005) Reduced transforming
23 growth factor-beta signaling in cartilage of old mice: role in impaired repair capacity. *Arthritis Res Ther.* 7:R1338-
24 1347.
- 25 Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Fresinger P (1994) Reexpression of
26 cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. *Exp Cell Res*
27 212: 97-104.
- 28 Boumediene K, Conrozier T, Mathieu P, Richard M, Marcelli C, Vignon E, Pujol JP (1998) Decrease of cartilage
29 transforming growth factor-beta receptor II expression in the rabbit experimental osteoarthritis--potential role in
30 cartilage breakdown. *Osteoarthritis Cartilage* 6:146-149.

- 1 Buckwalter JA, Mankin HJ (1998) Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and
2 transplantation, *Instr. Course Lect.* 47: 487–504.
- 3 Cai X, Lin Y, Ou G, Luo E, Man Y, Yuan Q, Gong P (2007) Ectopic osteogenesis and chondrogenesis of bone
4 marrow stromal stem cells in alginate system. *Cell Biol Int* 31: 776-783.
- 5 Chimal-Monroy J, Diaz de Leon L (1999) Expression of N-cadherin, N-CAM, fibronectin and tenascin is stimulated
6 by TGF-beta1, beta2, beta3 and beta5 during the formation of precartilaginous condensations. *Int J Dev Biol* 43:59-67.
- 7 Dharmavaram RM, Liu G, Mowers SD, Jimenez SA (1997) Detection and characterization of Sp1 binding activity in
8 human chondrocytes and its alterations during chondrocyte dedifferentiation. *J Biol Chem* 272: 26918-16925.
- 9 Dignam JD, Lebovitz RM, Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble
10 extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11: 1475-1489.
- 11 Dozin B, Malpeli M, Camardella L, Cancedda R, Pietrangelo A (2002) Response of young, aged and osteoarthritic
12 human articular chondrocytes to inflammatory cytokines: molecular and cellular aspects. *Matrix Biol* 21: 449–459
- 13 Duval E, Bigot N, Hervieu M, Kou I, Leclercq S, Galéra P, Boumediene K, Baugé C (2011) Asporin expression is
14 highly regulated in human chondrocytes. *Mol Med.* 17:816-23. doi: 10.2119/molmed.2011.00052.
- 15 Finnon KW, Parker WL, ten Dijke P, Thorikay M, Philip A (2008) ALK1 opposes ALK5/Smad3 signaling and
16 expression of extracellular matrix components in human chondrocytes. *J Bone Miner Res* 23:896-906.
- 17 Furumatsu T, Tsuda M, Taniguchi N, Tajima Y, Asahara H (2005) Smad3 induces chondrogenesis through the
18 activation of SOX9 via CREB-binding protein/p300 recruitment. *J Biol Chem* 280: 8343-8350.
- 19 Grimaud E, Heymann D, Redini F (2002) Recent advances in TGF-beta effects on chondrocyte metabolism.
20 Potential therapeutic roles of TGF-beta in cartilage disorders. *Cytokine Growth Factor Rev* 13: 241-57.
- 21 Guerne PA, Blanco F, Kaelin A, Desgeorges A, M. Lotz M (1995) Growth factor responsiveness of human articular
22 chondrocytes in aging and development. *Arthritis Rheum* 38: 960–968
- 23 Hiramatsu K, Iwai T, Yoshikawa H, Tsumaki N (2011) Expression of dominant negative TGF-β receptors inhibits
24 cartilage formation in conditional transgenic mice. *J Bone Miner Metab.* 29 : 493-500.
- 25 Homicz MR, Schumacher BL, Sah RL, Watson D (2002). Effects of serial expansion of septal chondrocytes on
26 tissue-engineered neocartilage composition. *Otolaryngol Head Neck Surg.* 127:398-408.

- 1 Huang CY, Reuben PM, Cheung HS (2005) Temporal expression patterns and corresponding protein inductions of
2 early responsive genes in rabbit bone marrow-derived mesenchymal stem cells under cyclic compressive loading.
3 *Stem Cells*.23:1113-21.
- 4 Jia Z, Zhang J, Wei D, Wang L, Yuan P, Le X, Li Q, Yao J, Xie K. Molecular basis of the synergistic antiangiogenic
5 activity of bevacizumab and mithramycin A. *Cancer Res*. 2007 May 15;67(10):4878-85.
- 6 Kempson GE (1982) Relationship between the tensile properties of articular cartilage from the human knee and age.
7 *Ann Rheum Dis* 41: 508–511
- 8 Lemare F, Steimberg N, Le Griel C, Demignot S, Adolphe M (1998) Dedifferentiated chondrocytes cultured in
9 alginate beads: restoration of the differentiated phenotype and of the metabolic responses to interleukin-1beta. *J Cell*
10 *Physiol* 176:303-313.
- 11 Loeser RF, Shanker G, Carlson CS, Gardin JF, Shelton BJ, Sonntag WE (2000) Reduction in the chondrocyte
12 response to insulin-like growth factor 1 in aging and osteoarthritis: studies in a non-human primate model of
13 naturally occurring disease. *Arthritis Rheum* 43:2110–2120F
- 14 Longobardi L, O'Rear L, Aakula S, Johnstone B, Shimer K, Chytil A, Horton WA, Moses HL, Spagnoli A (2006)
15 Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-
16 beta signaling. *J Bone Miner Res* 21: 626-636.
- 17 McDougall S, Fu YH, Lowe GN, Williams A, Polendo R, Benya PD, Iida-Klein A, Fang MA, Hahn TJ (1996)
18 Surface adhesion-mediated regulation of chondrocyte-specific gene expression in the nontransformed RCJ 3.1C5.18
19 rat chondrocyte cell line. *J Bone Miner Res* 11: 1130-1138.
- 20 Mizuta H, Sanyal A, Fukumoto T, Fitzsimmons JS, Matsui N, Bolander ME, Oursler MJ, O-Driscoll SW (2002) The
21 spatiotemporal expression of TGF-beta1 and its receptors during periosteal chondrogenesis in vitro. *J Orthop Res*
22 20:562-574.
- 23 Mori Y, Chen SJ, Varga J (2000) Modulation of endogenous Smad expression in normal skin fibroblasts by
24 transforming growth factor-beta. *Exp Cell Res* 258:374-383.
- 25 Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, ten Dijke P
26 (1997) Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* 389:631-635.
- 27 Oh JE, Han JA, Hwang ES (2007) Downregulation of transcription factor, Sp1, during cellular senescence. *Biochem*
28 *Biophys Res Commun*. 353:86-91
- 29 Pei M, Yu C, Qu M (2000) Expression of collagen type I, II and III in loose body of osteoarthritis *J Orthop Sci* 5:
30 288-293.

- 1 Piera-Velazquez S, Hawkins DF, Whitecavage MK, Colter DC, Stokes DG, Jimenez SA (2007) Regulation of the
2 human SOX9 promoter by Sp1 and CREB. *Exp Cell Res* 313: 1069-1079
- 3 Reddi AH (1994) Bone and cartilage differentiation. *Curr Opin Genet Dev.* 4: 737-744.
- 4 Reginato AM, Iozzo RV, Jimenez SA (1994) Formation of nodular structures resembling mature articular cartilage in
5 long-term primary cultures of human fetal epiphyseal chondrocytes on a hydrogel substrate. *Arthritis Rheum* 37:
6 1338-1349.
- 7 Rosen F, McCabe G, Quach J, Solan J, Terkeltaub R, Seegmiller JE, Lotz L (1997) Differential effects of aging on
8 human chondrocyte responses to transforming growth factor beta: increased pyrophosphate production and decreased
9 cell proliferation. *Arthritis Rheum* 40: 1275–1281
- 10 Schonherr E, Hausser HJ (2000). Extracellular matrix and cytokines: a functional unit. *Dev Immunol* 7: 89-101.
- 11 Seo HS, Serra R (2007) Deletion of Tgfb2 in Prx1-cre expressing mesenchyme results in defects in development of
12 the long bones and joints. *Dev Biol* 310: 304-316.
- 13 Serra R, Johnson M, Filvaroff EH, LaBorde J, Sheehan DM, Derynck R, Moses HL (1997) Expression of a
14 truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte
15 differentiation and osteoarthritis. *J Cell Biol* 139:541-552.
- 16 Shi Y, Massague J (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113: 685-700.
- 17 Thonar EJ, Buckwalter JA, Kuettner KE (1986) Maturation-related differences in the structure and composition of
18 proteoglycans synthesized by chondrocytes from bovine articular cartilage. *J Biol Chem* 261: 2467–2474
- 19 van de Laar IM, Oldenburg RA, Pals G, Roos-Hesselink JW, de Graaf BM, Verhagen JM, Hoedemaekers YM,
20 Willemsen R, Severijnen LA, Venselaar H, Vriend G, Pattynama PM, Collée M, Majoor-Krakauer D, Poldermans D,
21 Frohn-Mulder IM, Micha D, Timmermans J, Hilhorst-Hofstee Y, Bierma-Zeinstra SM, Willems PJ, Kros JM, Oei
22 EH, Oostra BA, Wessels MW, Bertoli-Avella AM (2011) Mutations in SMAD3 cause a syndromic form of aortic
23 aneurysms and dissections with early-onset osteoarthritis. *Nat Genet.* 43 : 121-6
- 24 van der Kraan PM, van den Berg WB (2008) Osteoarthritis in the context of ageing and evolution. Loss of
25 chondrocyte differentiation block during ageing. *Ageing Res Rev.* 7: 106-113.
- 26 Verdier MP, Seite S, Guntzer K, Pujol JP, Boumediene K (2005) Immunohistochemical analysis of transforming
27 growth factor beta isoforms and their receptors in human cartilage from normal and osteoarthritic femoral heads.
28 *Rheumatol Int* 25:118-124.

1 Wrana JL, Attisano L, Wieser R, Ventura F, Massague J (1994) Mechanism of activation of the TGF-beta receptor.
2 Nature 370:341-247.

3 Yang X, Chen L, Xu X, Li C, Huang C, Deng CX (2001) TGF-beta/Smad3 signals repress chondrocyte hypertrophic
4 differentiation and are required for maintaining articular cartilage. J Cell Biol 153:35-46.

5 Yu Z, Xing Y (2006) All-trans retinoic acid inhibited chondrogenesis of mouse embryonic palate mesenchymal cells
6 by down-regulation of TGF-beta/Smad signaling. Biochem Biophys Res Commun 340: 929-934

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 β R 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 β R 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 β R 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 β R 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 μ g). After 15 h, the medium was changed for DMEM+2% FCS for 24 h. Total RNA was isolated and
13 T β R 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.

25

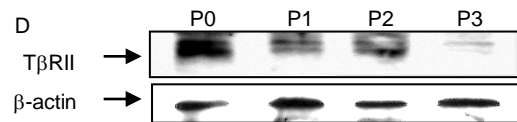
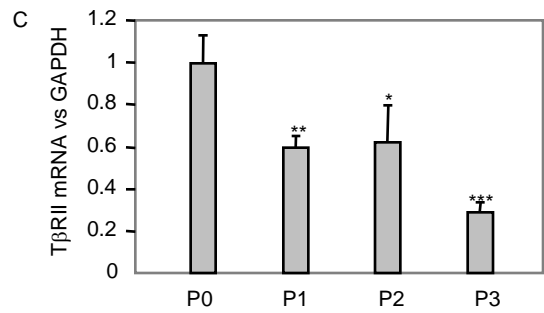
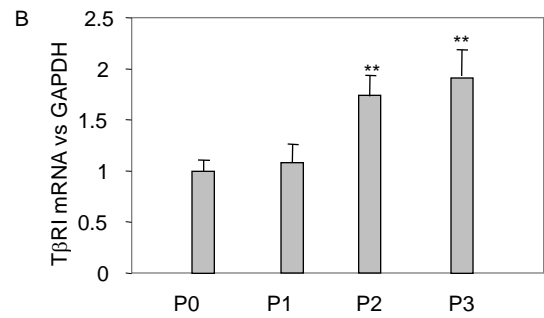
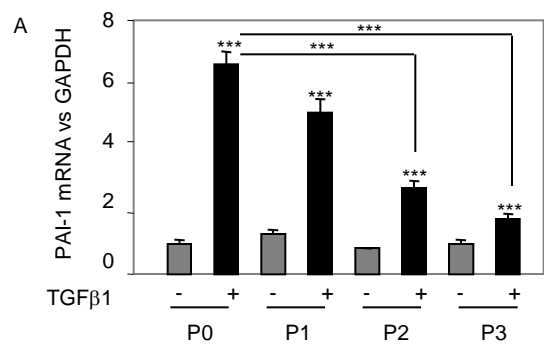


Figure 1:

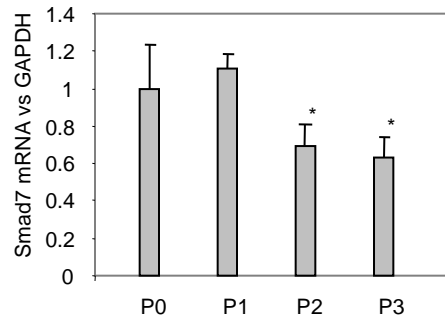
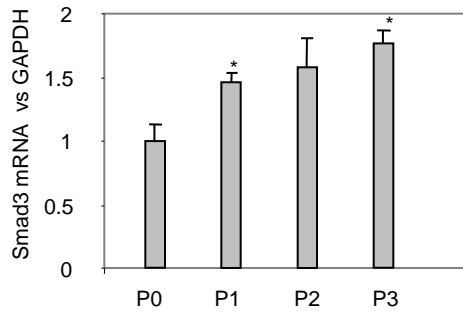
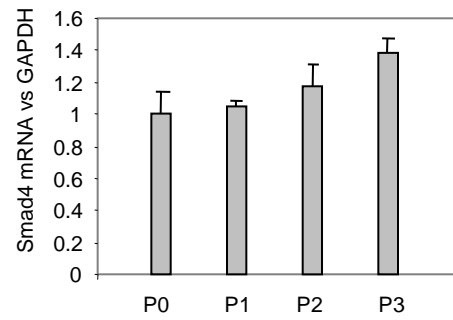
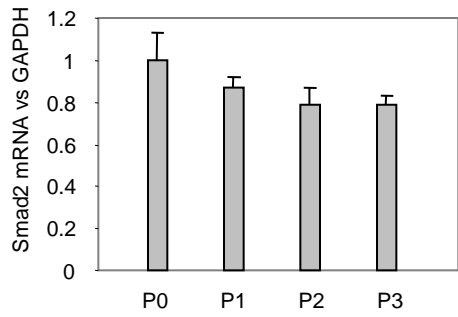


Figure 2:

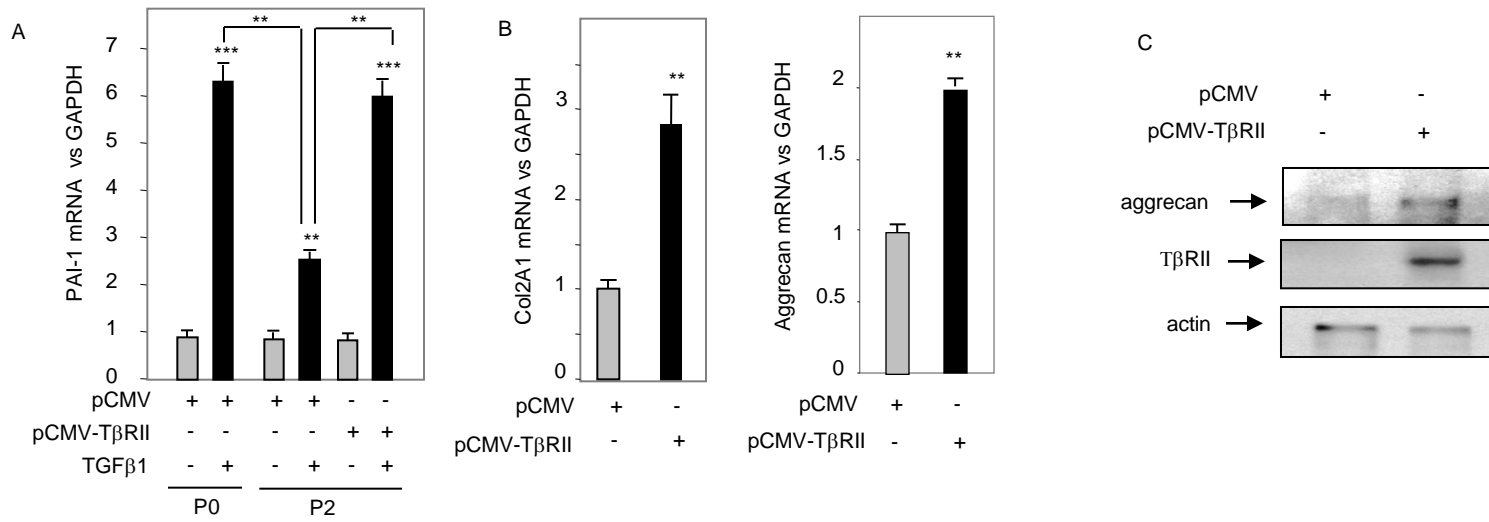


Figure 3:

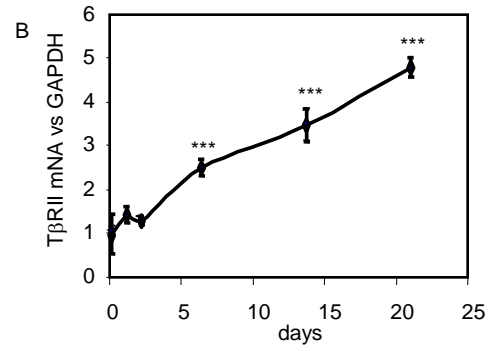
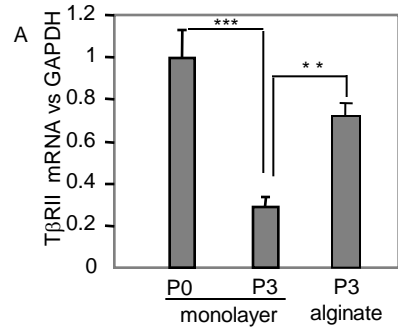


Figure 4:

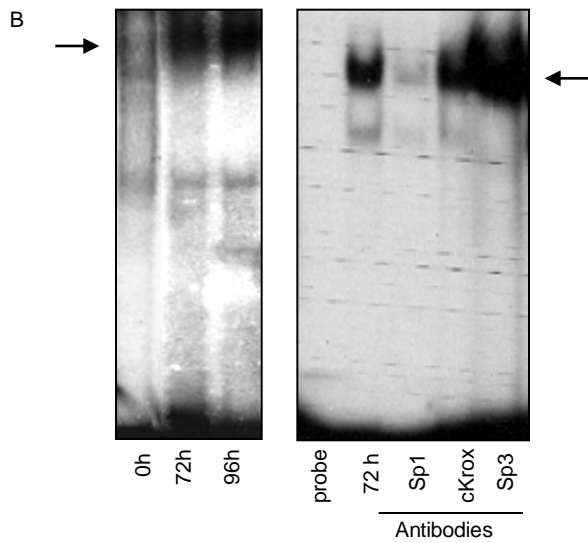
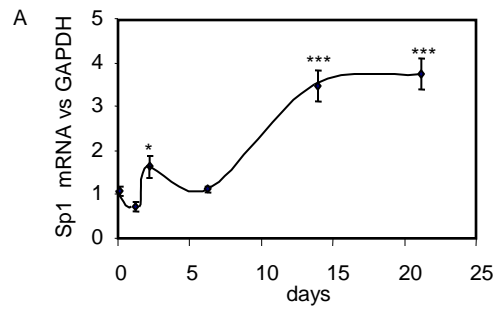


Figure 5

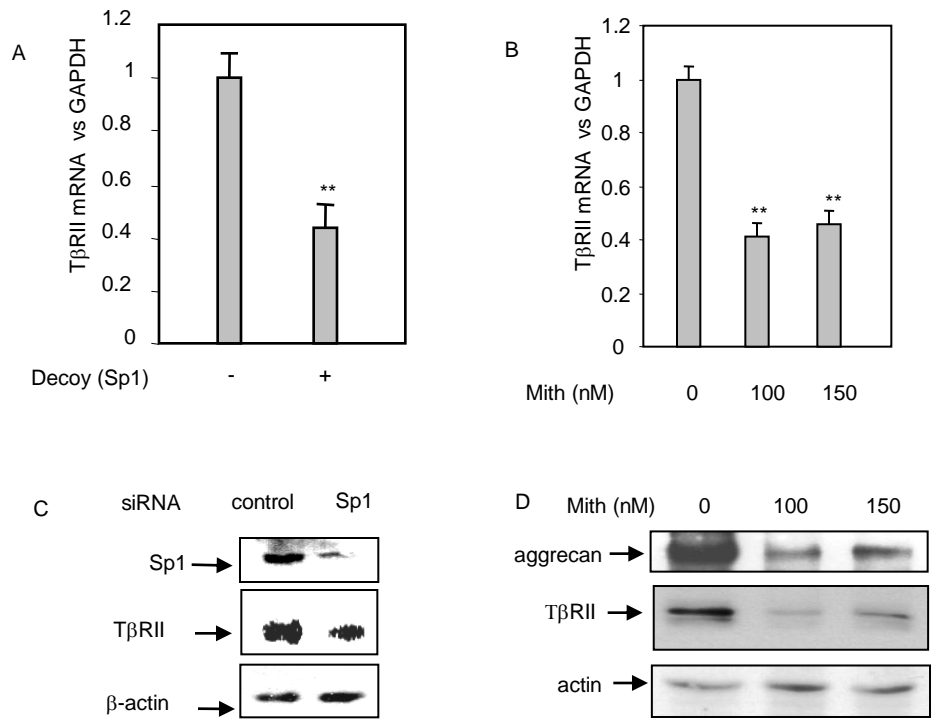


Figure 6

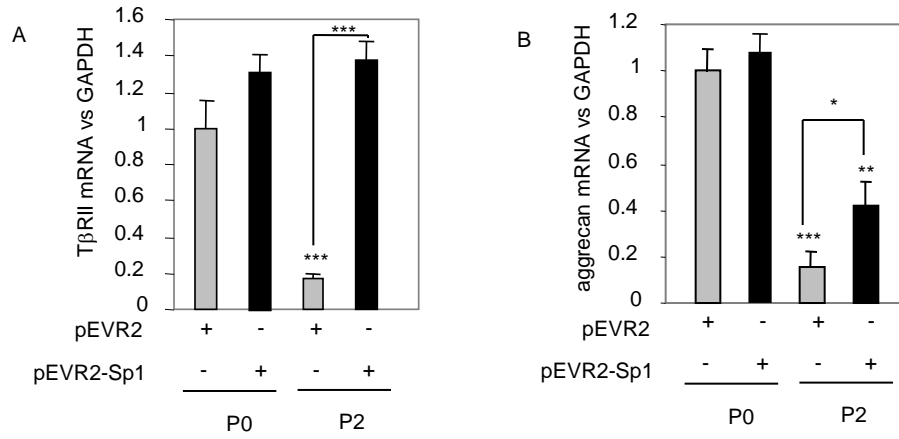
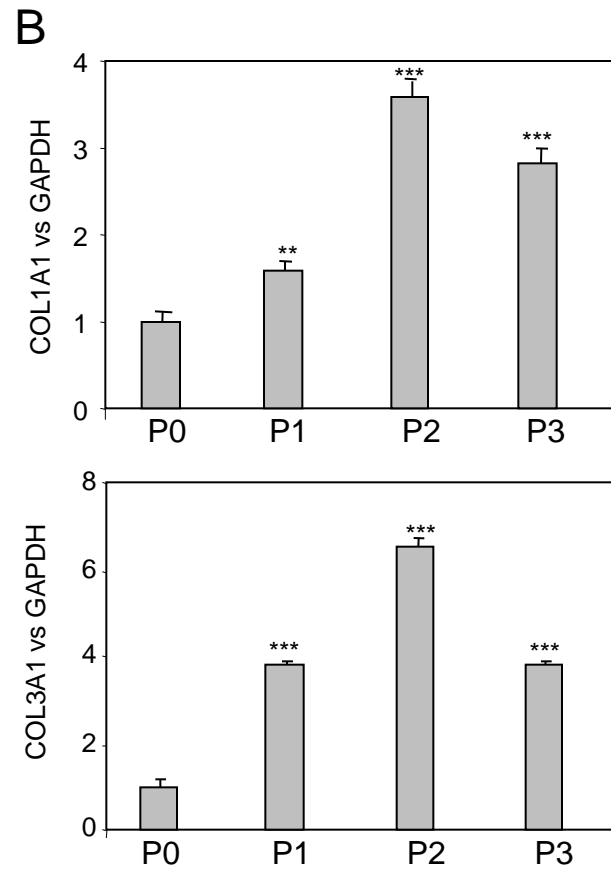
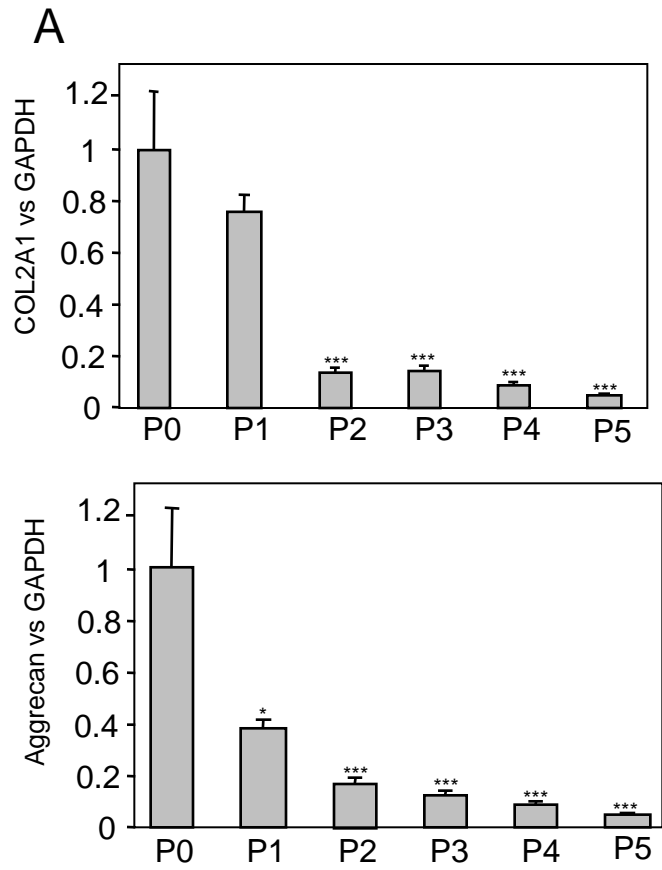
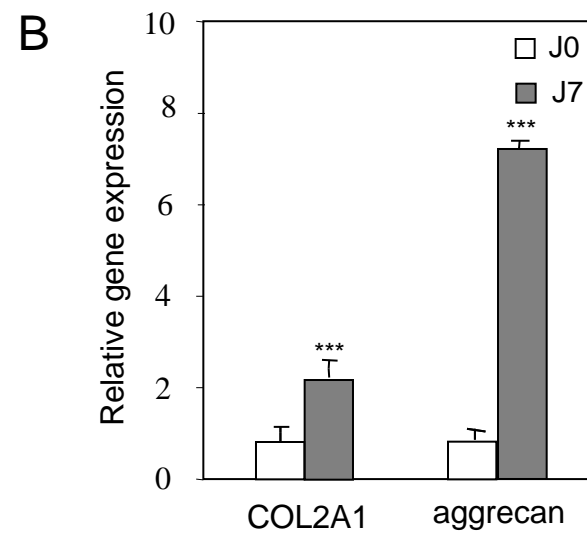
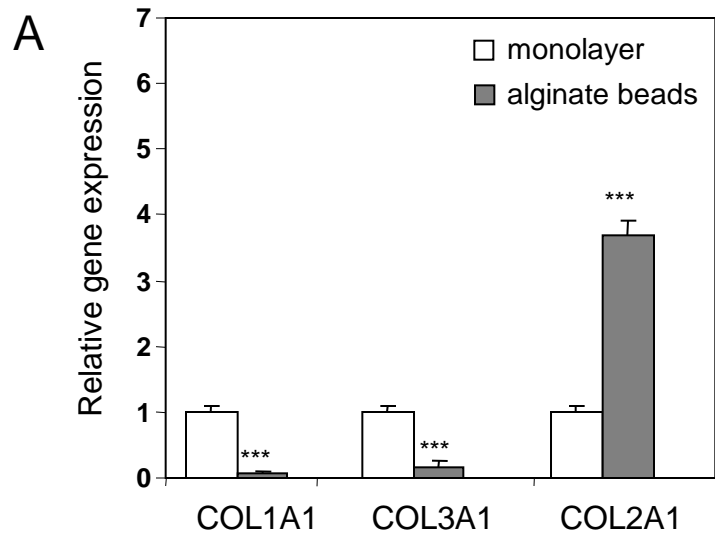


Figure 7



Suppl figure 1



Suppl figure 2