

Research article

**THE ROLE OF ALK-1 AND ALK-5 IN THE MECHANOSENSING
OF CHONDROCYTES**PATRICIA SANZ-RAMOS¹, JAVIER DOTOR²
and IÑIGO IZAL-AZCÁRATE^{1,*}¹Laboratory for Orthopaedic Research, University of Navarra, School
of Medicine, Pamplona, Spain, ²DIGNA Biotech, Pamplona, Spain

Abstract: We aim to demonstrate the role of Alk receptors in the response of hydrogel expansion. Chondrocytes from rat knees were cultured onto plastic and hydrogel surfaces. Alk-1 and Alk-5 were overexpressed or silenced and the effects on cells during expansion were tested and confirmed using peptide inhibitors for TGF β . Overexpression of Alk-5 and silencing of Alk-1 led to a loss of the chondrocyte phenotype, proving that they are key regulators of chondrocyte mechanosensing. An analysis of the gene expression profile during the expansion of these modified cartilage cells in plastic showed a better maintenance of the chondrocyte phenotype, at least during the first passages. These passages were also assayed in a mouse model of intramuscular chondrogenesis. Our findings indicate that these two receptors are important mediators in the response of chondrocytes to changes in the mechanical environment, making them suitable targets for modulating chondrogenesis. Inhibition of TGF β could also be effective in improving chondrocyte activity in aged or expanded cells that overexpress Alk-1.

Keywords: Chondrocyte, Mechanosensing, Mechanobiology, Mechanotransduction, Collagen hydrogels, TGF β , Differentiation, Signalling, Cartilage, Chondrogenesis

* Author for correspondence. inizal@me.com, phone: +34 48 425600 (6403), fax: +34 48 425649

Abbreviations used: Alk-1 – activin receptor-like kinase 1; Alk-5 – activin receptor-like kinase 5; FGFR3 – fibroblast growth factor receptor 3; FA – focal adhesions; MSCs – mesenchymal stem cells; OA – osteoarthritis; TGF β – transforming growth factor β ; Col2 – type II collagen; Col1 – type I collagen; Col10 – type X collagen

INTRODUCTION

The chondrogenic capacity of cartilage cells can be modulated *in vitro*. Several parameters may affect chondrocyte behavior and these parameters have become the subject of research aiming to restore extracellular matrix gene expression that has been lost during joint pathologies or during *in vitro* monolayer expansion. Cultures of chondrocytes on commonly used polymer surfaces undergo a progressive and, beyond a certain number of passages, irreversible loss of the cartilage phenotype [1–5]. This is characterized by a cessation in the expression of collagen type II, aggrecan and the transcription factor Sox9, among others.

In our laboratory, we used collagen hydrogels that support the culture of chondrocytes on substrates with low mechanical properties (in the range of pascals) [6, 7]. We achieved an efficient expansion method that slowed down the dedifferentiation process that takes place when cells are cultured onto plastic [8]. The mechanisms responsible for this behavior of chondrocytes in the hydrogel culture are still unknown, but some clues have been found, such as the interesting pattern for transforming the growth factor β (TGF β) receptors activin receptor-like kinase 1 and 5 (Alk-1 and Alk-5) [8]. We described an increase in the Alk-1 level in the plastic culture. This was found to slow down in hydrogels. The opposite pattern was found for Alk-5. The relevance of this behavior is that these TGF β receptors have opposing effects in chondrocytes [9–16]. When TGF β signals through Alk-1, it activates Smad 1/5/8 and produces hypertrophic chondrocytes and terminal differentiation [14, 15]. When it binds to Alk-5 (also known as TGF β receptor 1), it enhances the expression of cartilage-related genes through Smad 2/3 activation [9–13]. The balance between these two receptors determines the capacity of the cell to respond to TGF β .

We hypothesized that these receptors could be implicated in the mechanosensing capacity of chondrocytes. Therefore, we aimed to effectively demonstrate the role of these two receptors in the process of chondrocyte mechanosensing. We used the model of hydrogel culture with collagen type I hydrogels, which simplify synthesis and handling and have offered good results previously. We used a plasmid system to induce cells to overexpress or silence Alk-1 and Alk-5 in order to study their effect on the chondrogenetic capacity of cartilage cells.

MATERIALS AND METHODS

Preparation of hydrogels

Hydrogels were prepared as described previously using 3 mg/ml of rat tail collagen type I (BD Biosciences). They yielded an elastic modulus of 22.42 Pa, which was determined via rheology [6].

Isolation and culture of chondrocytes

The source of tissue was skeletally mature Wistar rats (average weight of 200–250 g), obtained using a protocol approved by our institutional committee

(number 066-11, date 5/27/2011). We used cells pooled from 5 rats for the experiments and replicated each experiment three times with different cell extractions. The whole knee joint was exposed under sterile conditions and cartilage pieces were obtained from the femoral cartilage using surgical blades. The tissue was placed in culture dishes, minced, and treated overnight with 0.5 mg/ml dispase and 60 units/ml collagenase (Sigma), both of which had been dissolved in the expansion medium (see below). After digestion, the cells were pelleted, washed and finally resuspended in the expansion medium, which was DMEM-Glutamax supplemented with 10% fetal bovine serum and 1 mM L-cysteine and penicillin/streptomycin (both from Invitrogen).

The cells were seeded on 175-cm² flasks for the control expansion and on collagen hydrogel-coated 175-cm² flasks, and grown under standard hypoxic conditions (37°C, 5% CO₂ and 5% O₂) unless otherwise indicated. For normoxic conditions, we used 37°C and 5% CO₂. Both cultures (hydrogel and plastic) were two-dimensional. The plastic-expanded cells were detached using trypsin (Invitrogen) and the hydrogel-cultured cells were detached using 100 µg/ml collagenase (Sigma) for 45 min at 37°C. To inhibit TGFβ, peptides P17 and P144 (Digna Biotech) were added separately at 0.2 mg/ml. A sample for every passage was collected to detect chondrocyte markers using RT-PCR (see below). To study the chondrogenic capacity, we performed micromass culture and injected chondrocytes into the adductor muscles of nude athymic mice. A total of 200,000 expanded chondrocytes (from plastic or hydrogel-coated surfaces) were pelleted (centrifuged at 400 x g for 5 min) and placed overnight in a 15-ml conical tube with an inclination of about 45°. Pellets were suspended in expansion medium for 7 additional days and collected to be analyzed using RT-PCR and histology. For the mouse model, 4 million chondrocytes were collected and implanted in a PBS suspension in the adductor muscle of nude mice weighing 30–40 g, as previously described [17]. This protocol was approved by our institutional committee (number 065-12, date 5/18/2012). The cells were maintained for 1 week and then the complete limb was extracted, fixed in formalin, decalcified using 10% formic acid for 2 weeks, and subjected to histological analysis using Sirius red staining.

RT-PCR assays

Cells used for the RT-PCR assay were collected from hydrogels, plastic surfaces and micromass structures as described above. RNA was extracted using 1 ml of Trizol (Invitrogen), following the manufacturer's guidelines. After determining the RNA concentration using spectrophotometry with nanodrop (Thermo Fisher Scientific), we performed retrotranscription with the cDNA SuperMix qScript system (Quanta Biosciences), using 1 µg of RNA for each reaction. A total of 0.5 µl of the product of the previous reaction was subjected to a gene expression assay via real-time PCR. To study relative expression, we followed the 2^{-ΔΔCt} method as described by Livak and Schmittgen [18] using Taqman assays (Applied Biosciences) for type II collagen (Col2), Sox9, fibroblast growth factor

receptor 3 (FGFR3), Alk-5, Alk-1, aggrecan, type I collagen (Col1) and type X collagen (Col10). Cells on plastic were taken as the controls and the values obtained for hydrogel-cultured cells were subsequently referred to them.

Western blot analysis

For the detection of Alk-1, Alk-5 and SMADs, we performed western blot analyses of protein extracts from chondrocytes. Proteins were extracted using Phosphosafe extraction reagent (Novagen), and after quantification using the Bradford assay, loaded onto SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes and blocked using 5% milk in TBST. We used specific antibodies diluted 1:1000 in blocking buffer. After washing the blot extensively, secondary antibodies were used diluted 1:2000 in blocking buffer and then washed again. Finally, the blots were visualized using Lumilight Plus western blotting substrate (Roche). In order to visualize SMADs, the chondrocytes first underwent a treatment with TGF β (R&D systems) at 1 ng/ml for 1 h.

Histological analysis

The chondrogenic capacity of expanded cartilage cells was determined via histological processing of the micromass structures. Sections 4 μ m in thickness were stained using Sirius Red. We also used specific antibodies to detect aggrecan (Abcam), Col2 (Biogenesis) and Col1 (BD Biogenesis) via immunofluorescence. For this purpose, the sections were initially treated with trypsin (0.1% in PBS containing 0.1% calcium chloride) for 1 h at 37°C. They were then blocked with normal goat serum (Sigma) diluted 1:20 in PBS, treated with the primary antibody diluted 1:50 overnight at 4°C and finally washed and incubated with the secondary antibody 1:200 for 45 min at room temperature (RT). Samples were stained using Sirius red and Safranin-O. Samples from non-injected mice were used as controls.

Overexpression/silencing

Overexpression of Alk-1 and Alk-5 was induced using cDNA obtained from Origene, which was then cloned in *Escherichia coli*. Then, 2 μ g of Alk-1 or Alk-5 in solution was transfected into chondrocytes using 4 μ l Lipofectamine (Invitrogen) per well in 6-well plates to give a final concentration of 8 ng/ μ l. The silencing was performed using the HuSH silencing system for Alk-1 and Alk-5 from Origene. They were cloned and transfected using the same protocols.

Methylation analysis

We used the DNA modification procedure for the methylation analysis of matrix-related gene promoters. Genomic DNA from chondrocytes cultured on plastic or hydrogels was modified using the CpGenome DNA Modification Kit (Millipore). A total of 1 μ g of DNA was subjected to the DNA modification procedure and then to conventional PCR using primers to detect differences in modified and unmodified DNA. The primers used are listed in Table 1. The outcome of the PCR was loaded on 1% agarose gels and resolved in TAE buffer. The gels were photographed using the Gel-Doc system (Bio-Rad).

Table 1. Primers used for the RT-PCR.

Alk-1	
Left modified primer	AAATGGTTTTGTTTAGAATTTACGG
Right modified primer	TAACTTCTTCCTACCTAACCCCG
Left unmodified primer	AATGGTTTTGTTTAGAATTTATGG
Right unmodified primer	TAACTTCTTCCTACCTAACCCCACT
Alk-5	
Left modified primer	TTTTTTTTGAGTAATTTTTACGG
Right modified primer	GCACTACTAAACCTCTAACCACG
Left unmodified primer	TTTTTTTTGAGTAATTTTTATGG
Right unmodified primer	ACACTACTAAACCTCTAACCACACC

Statistical analysis

The results from gene expression experiments were compared using the nonparametric Kruskal-Wallis test, using the software SPSS 15.0 for Windows. We considered values of $p < 0.05$ to be significant.

RESULTS

Overexpression and silencing of Alk-5 and Alk-1

The roles of Alk-5 and Alk-1 in chondrocytes were tested through overexpression or silencing of these two receptors. For this purpose, chondrocytes were liposome transfected using Alk-5 and Alk-1 cDNA and silencing RNAs developed by Origene. Transfection was developed using different concentrations of Lipofectamine with cDNA corresponding to green fluorescent protein (GFP), achieving transfection efficiencies close to 100% (Fig. 1A). The overexpression or silencing method was confirmed via RT-PCR (Fig. 1B), which shows an increased expression of the two receptors when the cDNA was transfected and a downregulation when the silencing RNA was used. Finally, the effect that the overexpression or silencing has on the chondrocyte phenotype was determined through RT-PCR analysis of cartilage-specific markers (Fig. 1C). When the Alk-5 was overexpressed or Alk-1 was silenced, aggrecan and Col2 expressions increased compared to normal chondrocytes. Alternatively, when Alk-5 was silenced or Alk-1 was overexpressed, aggrecan and Col2 were both downregulated. Col1 displayed the opposite regulation. When we combined the overexpression with the silencing of the antagonistic receptor, we did not detect a potentiation of the effect that each Alk had on its own. In order to confirm these results, we placed chondrocytes in pellet cultures and showed using immunofluorescence how the expression of cartilage markers was enhanced when Alk-5 was overexpressed or Alk-1 silenced (Fig. 1D). We selected the overexpression of Alk-5 and the silencing of Alk-1 as the conditions for the expansion of chondrocytes in subsequent experiments.

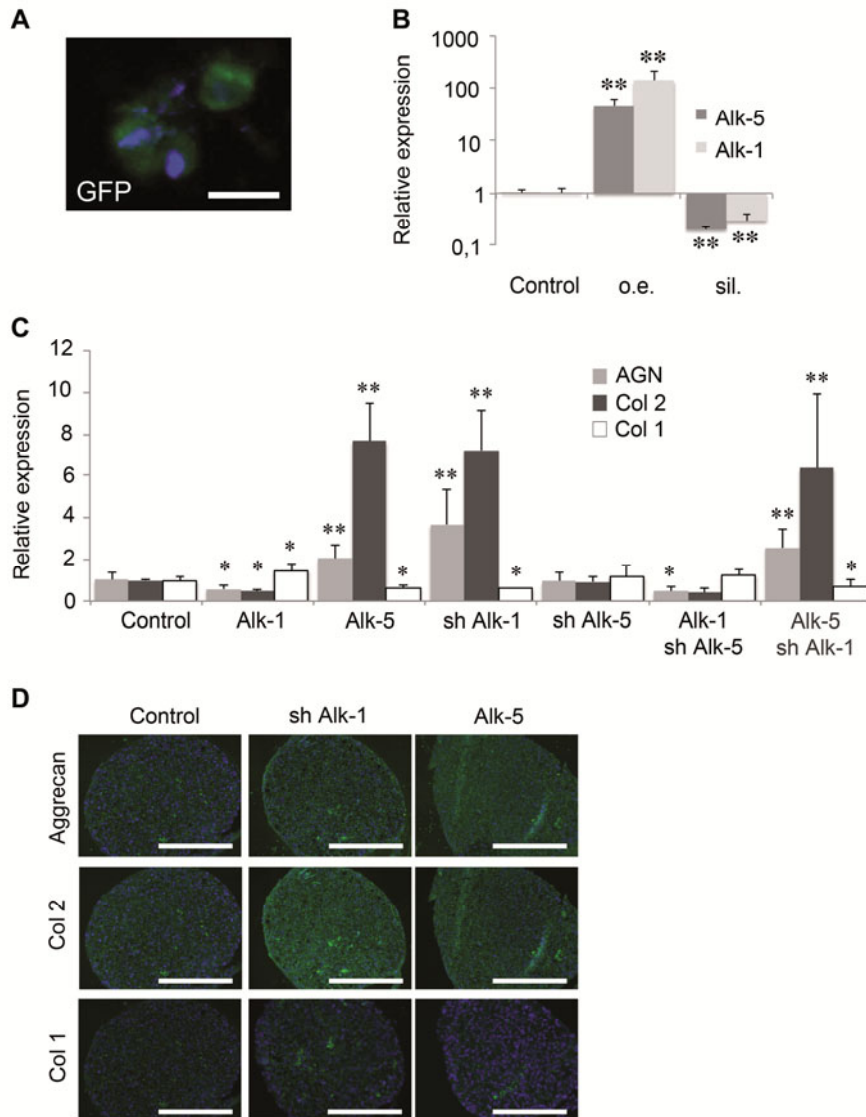


Fig. 1. Efficient transfection of chondrocytes using lipofectamine (LFP). A – Control plasmid containing the cDNA for GFP were transfected into freshly isolated chondrocytes using LFP (8 ng/ μ l) and the cells were photographed under a fluorescence microscope showing the positive staining for approx. 100% of the cells. B – Under those conditions, plasmids for the overexpression and silencing of receptors Alk-1 and Alk-5 were transfected and their expression analyzed via RT-PCR. C – The expression of cartilage markers aggrecan, Col2 and Col1 was used as a control to monitor the effect of overexpressing or silencing both receptors. D – Chondrocytes were cultured in 3D micromass systems and analyzed for the expression of cartilage genes by immunofluorescence. Scale bar: 50 μ m for (A) and 500 μ m for (D). The average \pm SD for every group (n = 6) is represented. *p < 0.05 and **p < 0.001 relative to the control cells.

Expansion of chondrocytes after Alk-5 upregulation and Alk-1 downregulation

Selected chondrocytes were expanded up to passage 8 and their RNA was extracted for RT-PCR analysis of cartilage markers (Fig. 2A). When genetically modified chondrocytes were expanded (Alk-5 for cells overexpressing Alk-5, and shAlk-1 for cells where Alk-1 was silenced), we observed a delay in the progressive loss of the chondrocytic markers aggrecan and Col2, and a delayed upregulation of Col1 with respect to unmodified control cells during the course of the culture. In addition, and confirming our previous results, hydrogel-expanded cells did show a delayed decay of the cartilage markers aggrecan and Col2 and an increased expression of Col1. We also confirmed the expression of Alk-1 and Alk-5 for the modified Alk-5 and shAlk-1 cells during the experiment and found an overexpression for Alk-5 and a downregulation of Alk-1 consistent with the modification of the cells. These changes were particularly evident in the first passages. Data concerning the expressions of Alk-1 and Alk-5 were confirmed using western blot analysis (Fig. 2B). A band corresponding to Alk-5 appears in passage 0 cells. Its size decreases when the cells reach passage 4 under either set of conditions, but this decrease is enhanced when passage 0 cells were transfected with the corresponding plasmids. Alk-1 on its own increases with the passage number and decreases strongly when silenced.

In order to test if changes in the expression of receptors lead to changes in the signaling pathways, we performed a western blot analysis of SMAD2/3 and SMAD1/5/8 (Fig. 2B). The results confirmed findings for the RT-PCR, showing the presence of SMAD2/3 only in unpassaged cells and hydrogel-expanded cells. It was potentiated when cells were transfected with plasmids to overexpress the Alk-5 receptor. SMAD1/5/8 also appeared in unpassaged cells, but it was maintained only in plastic-expanded chondrocytes, showing no band for the hydrogel-expanded cells and poor band intensity in cells where Alk-1 had been silenced.

The capacity to form a matrix during the course of the experiment was analyzed by placing expanded cells in passages 0–8 in pellet cultures. In all cases (Fig. 3A), the expression of the cartilage markers aggrecan and Col2 was significantly upregulated when Alk-5 and shAlk-1 cells were assayed and referred to unmodified cells, showing a much-delayed decay for these markers. In addition, when the expansion was performed onto hydrogels, the expressions of these two markers were much increased during the first passages, showing an interesting synergy for the genetic modification and the hydrogel culture effect, which involved an improved expression of cartilage markers and confirmed previous results. In the assay of Col1, a significant decrease was observed when Alk-5 and shAlk-1 cells were used.

The chondrogenetic capacity was also studied *in vivo* by injecting the control, Alk-5 and shAlk-1 cells into the adductor muscle of nude mice. We could not detect any positive staining for Safranin-O (data not shown). Images in Fig. 3B for Sirius red staining showed more intensely stained samples for animals transfected with shAlk-1 cells. No significant differences were found for Alk-5 and control cells.

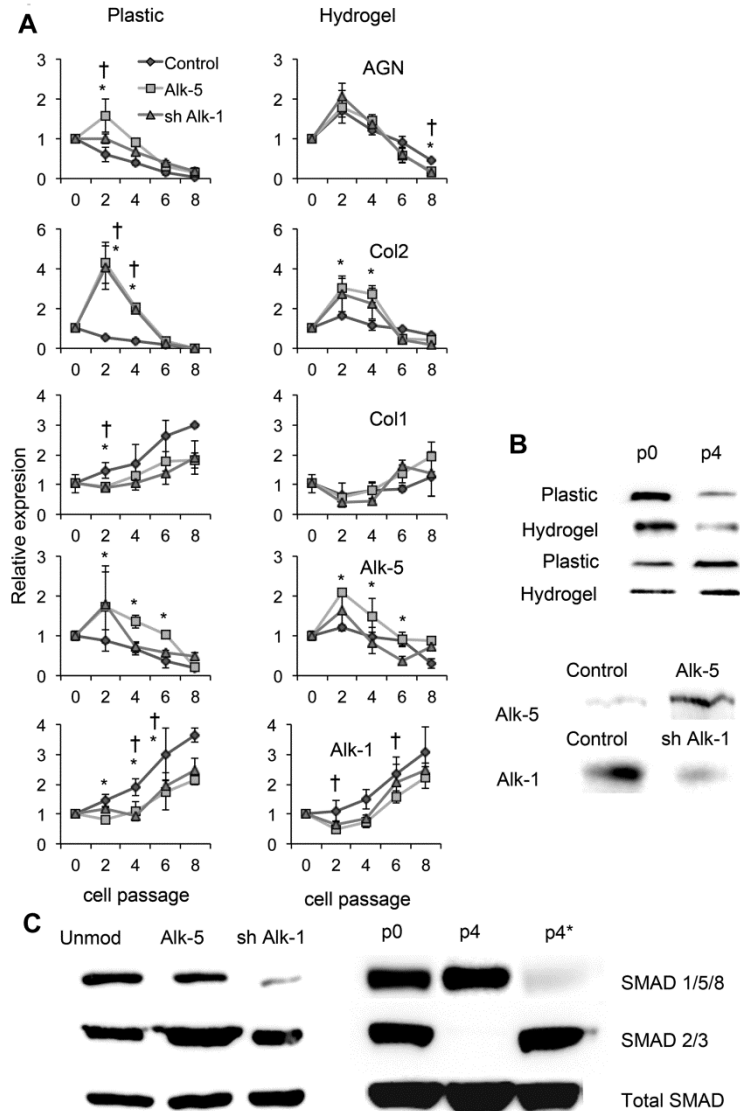


Fig. 2. The expression of cartilage markers during expansion. A – Expanded chondrocytes were analyzed for the expression of markers using RT-PCR. The average \pm SD for every group ($n = 6$) is represented for passages 0, 2, 4, 6 and 8. B – Western blot of Alk-1 and Alk-5 in passage 0 and passage 4 cells both in plastic and in hydrogel culture. The band for Alk-1 and Alk-5 also shows in cells induced to overexpress Alk-5 (marked as Alk-5) and for cells induced to silence Alk-1 (marked as shAlk-1). C – Phosphorylation of SMADs after TGF- β treatment (1 ng/ml during 1 h) in normal and passaged cells was determined by western blot analysis. P4 stands for passage 4 cells and p4* for cells in passage 4 cultured in hydrogels. * $p < 0.05$ for Alk-5 cells relative to the control cells in every passage. † $p < 0.05$ for Alk-5 cells relative to the control cells in every passage.

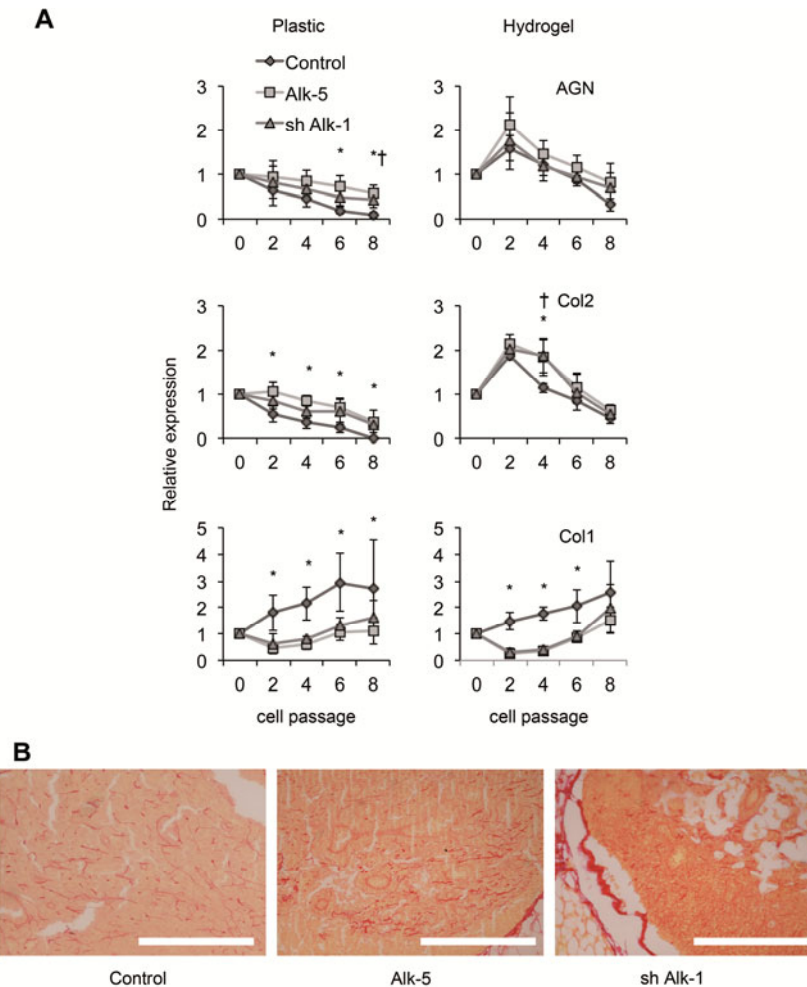


Fig. 3. The expression of cartilage markers in pellets obtained for cells in every passage. A – Pellets were dispersed and analyzed for the expression of markers using RT-PCR. The average \pm SD for every group ($n = 9$) is represented for passages 0, 2, 4, 6 and 8. B – The in vivo assay was developed by injecting chondrocytes from passage 2 into the adductor muscles of nude mice. Sections were stained using Sirius red. Scale bar: 200 μ m. * $p < 0.05$ for Alk-5 cells relative to the control cells in every passage. † $p < 0.05$ for Alk-5 cells relative to the control cells in every passage.

Inhibition of TGF β in hydrogel-cultured Alk-5 and sh-Alk-1 cells

In order to study the biological role of Alk-1 and Alk-5 receptors in chondrocytes, we used the TGF β inhibitors P17 and P144. Here, we report the data for P17, since all of the results are similar to those obtained using P144 (data not shown). Plastic-expanded cells were cultured in the presence of P17. Cartilage markers were assayed using RT-PCR (Fig. 4). In the control, unmodified cells, the effect of the inhibition was hardly noticeable, but for Alk-5

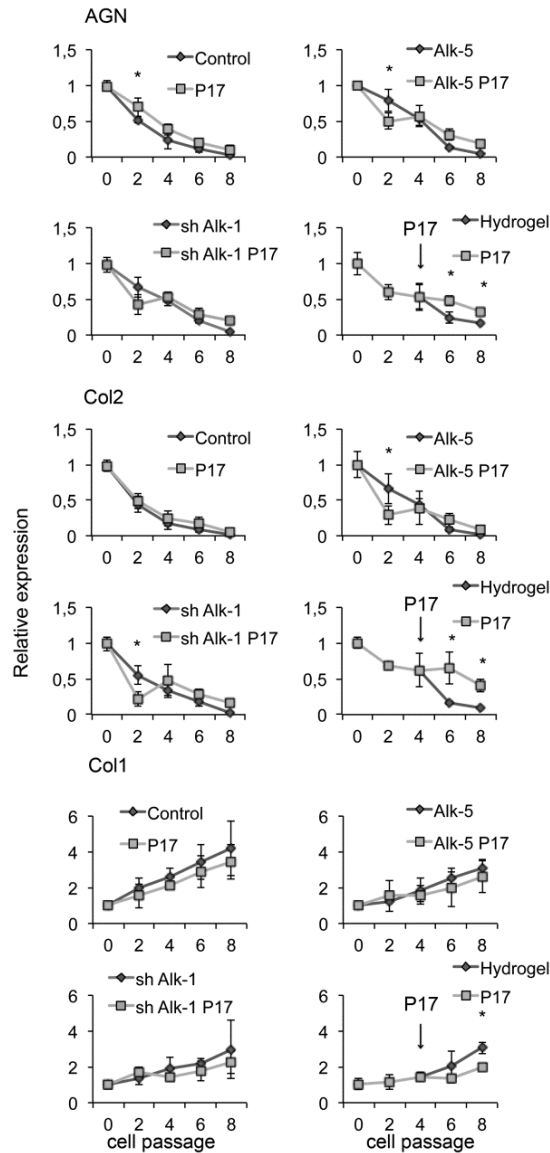


Fig. 4. Inhibition of TGF β during expansion of genetically modified chondrocytes. A – Plastic-expanded chondrocytes were treated with peptide inhibitor P17 and analyzed for the expression of cartilage markers aggrecan, Col2 and Col1 using RT-PCR. The peptide inhibitor for TGF β was also added to plastic cultures of cells overexpressing Alk-5 (Alk-5 cells, B) and chondrocytes silenced for Alk-1 (shAlk-1 cells, C). The average \pm SD for every group (n = 6) is represented for passages 0, 2, 4, 6 and 8. D – We also used wild-type chondrocytes cultured onto hydrogels to which peptide was added after passage 4, when the cells had shown to increase the expression of Alk-1 receptor [8] and it would be desirable to inhibit TGF β activity. *p < 0.05 for peptide-treated cells relative to the control cells in every passage.

and shAlk-1 cells, a clear effect was observed in passage 2 cells: an increase in aggrecan and Col2 expression and a decrease in Col1 expression, which suggests increased signaling through Alk-5, an effect that would positively influence the chondrocyte phenotype. We also used hydrogel expansion for the inhibition of TGF β , adding P17 after the 4th passage, when we had observed an increased level of Alk-1 [8]. At this point, the effect of inhibition was clear and significant, showing an increase in the expression of aggrecan and Col2, and a decrease in Col1 after the addition of the peptide.

DNA methylation analysis

We used primers specific for selected CpG islands on the promoter of Alk-1 and Alk-5, which are capable of amplifying modified or unmodified DNA, respectively. Fig. 5 shows the presence of bands in the gel for those primers specific for unmodified DNA, indicating the presence of methylation in the CpG islands studied for the promoter of Alk-1 both in plastic and in hydrogel-cultured chondrocytes. This is an indication of the lack of regulation of the gene for Alk-1 in our model of chondrocyte culture by methylation, at least in the islands studied. For Alk-5, we could detect the appearance of a band for the primers specific for modified DNA in its promoter. This indicates that, at least in our model, the cells cultured in collagen hydrogels suffer a de-methylation of the promoter for Alk-5.

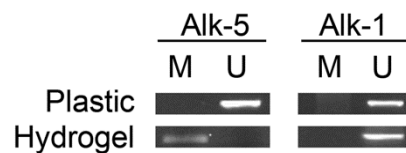


Fig. 5. Methylation analysis of CpG islands in the promoters of Alk-1 and Alk-5. Genomic DNA was transformed using the bisulfite modification and subjected to PCR using primers for the specific sequence of modified (M) or unmodified (U) DNA, respectively indicating unmethylated and methylated DNA in chondrocytes from passage 2 cultured both on plastic and on collagen hydrogels. The band pattern remains constant for Alk-1 (positive using primers specific for unmodified DNA) but changes for Alk-5 suggest a de-methylation process.

DISCUSSION

These results concur with those from our earlier research, in which we suggested that Alk-1 and Alk-5 are suitable candidates for the final effector of the response of chondrocytes to changes in substrate rigidity [8]. These two transmembrane proteins function as opposite receptors for TGF β [19]. In chondrocytes, Alk-5 has been described as the natural receptor, leading to a potentiation of the chondrogenetic capacity through the activation of SMAD 2/3 [9–11], while when Alk-1 is present, TGF signals activate SMAD 1/5/8, which leads to the hypertrophic and terminal differentiation of chondrocytes [12–14]. Our data

(Fig. 3) strongly confirm these earlier findings, showing SMAD 2/3 activation by TGF β only when cells were expanded onto hydrogels, which had been shown in a previous experiment to induce an improved expression for Alk-5 [8].

These two receptors have been described to have a role in the changes that age has on the reparative capabilities of cartilage. An alteration in the ratio of Alk-5 to Alk-1 to a higher expression of Alk-1 is observed in older chondrocytes [16, 20], in osteoarthritis (OA) [16] and during expansion in plastic [8]. We demonstrate here that both are relevant effectors of chondrocyte mechanosensing and modulators of chondrocyte phenotype since, by modifying their expression (increasing or silencing), we are able to modulate their chondrogenetic capacity. Expansion of cells with an overexpression of Alk-5 or a downregulation of Alk-1 mimics the effect that expansion onto hydrogels had on dedifferentiation of chondrocytes [8]. Indeed, a combination of the two factors (overexpression and silencing and hydrogel culture) could offer a synergistic effect that could be of interest in efforts to find optimal culture systems for cartilage cells (Figs 2 and 3). The data in Fig. 2 point to a regulation of Alk-1 by Alk-5 and vice versa. This coordinated regulation has not yet been demonstrated, but the opposing effects for the two receptors described here and in previous studies [16, 20] strongly support this hypothesis.

Interestingly, the methylation analysis performed showed how culture onto hydrogels produced a decrease in the methylation of the promoter of Alk-5. We suspect that this change is probably responsible for the increase in the expression of Alk-5 through the duration of the culture. We did not detect a similar behavior for Alk-1, which points to a relevant role of epigenetics in chondrocyte biology. In previous studies, epigenetic mechanisms have also been related to cartilage biology. The epigenetic state of the Sox9 promoter has been described to change during the process of OA [21]. In the same way, promoters for several metalloproteinases have been described as less methylated in OA-derived samples [22–24]. However, other studies have yielded results that could not demonstrate significant gene methylation in the aggrecan promoter of aged and OA chondrocytes [25].

To the best of our knowledge, there are no studies describing methylation-silencing mechanisms for Alk receptors *in vitro*, but epigenetic changes during *ex vivo* expansion (including methylation) have been described for mesenchymal stem cells (MSCs). Choi et al. showed how MSCs from late passages increased their methylation state in genes related to DNA replication, cell cycle and adipogenic differentiation [26, 27]. We can suggest that the changes occurring during expansion and prior to cell therapy applications may have special relevance for the final behavior of cells. Additionally, since one of the key factors implicated in these changes is the biomechanics of the culture system [6–8], we would like to open a new line of research in the epigenetic mechanisms that could be implicated in the sensing of matrix stiffness. In any case, our data point to a key role for the two receptors Alk-1 and Alk-5 in the physiology of chondrocytes. They are possible targets to modulate cartilage cell behavior. In order to confirm this role for Alk-1 and Alk-5, we used peptide

inhibitors against TGF β P17 and P144 (data not shown for the latter) [28–31]. Our results (Fig. 4) demonstrate an increased chondrocytic phenotype when TGF β was inhibited in Alk-1 expressing cells and, on the contrary, a lower chondrogenic capacity when we inhibited TGF β on cells expressing Alk-5. All of this is consistent with the role described for TGF β receptors. Of particular interest are the results found for P17. When added to the culture medium at the moment of the switch from Alk-5 to Alk-1 (around the 4th passage), P17 causes the cells to stop or at least slows down the dedifferentiation process. If the expansion of chondrocytes on hydrogels delays the dedifferentiation to the 4th passage, treatment with P17 at that moment could maintain the differentiated state in passages 6 through 8.

In any case, changes in stiffness as observed in our model are caused by a reduction in the collagen content of the hydrogel. This could also lead to a reduced availability of binding sites for integrins in the soft hydrogels that may cause the cells to decrease the FA sites and, subsequently, the signal transduction intensity. In fact, it has been extensively reported that decreased rigidities have precisely this effect on the turnover of FA, since the number of FA is dependent on the rigidity of the substrate [32, 33]. All of this evidence suggests that the mechanisms evaluated in this study are valid as a model for studying the effect of changing the substrate mechanics on chondrocytes. Thus, we offer relevant data that help to increase the understanding of mechanosensing mechanisms. We demonstrate that Alk-1 and Alk-5 may be important targets for modulating chondrocyte behavior in cartilage pathologies, which include the strategies that try to inhibit TGF β when expression of Alk-1 cannot be silenced.

Acknowledgments. This study is funded by PIUNA (Plan de Investigación, Universidad de Navarra, 2010-40). We would like to thank Digna Biotech for kindly providing peptides to inhibit TGF β . Patricia Sanz-Ramos received a Fellowship from the Asociación de Amigos de la Universidad de Navarra. The authors have no conflict of interests.

REFERENCES

1. Glowacki, J., Trepman, E. and Folkman, J. Cell shape and phenotypic expression in chondrocytes. **Proc. Soc. Exp. Biol. Med.** 172 (1983) 93–98.
2. Brodtkin, K.R., García, A.J. and Levenston, M.E. Chondrocyte phenotypes on different extracellular matrix monolayers. **Biomaterials** 25 (2004) 5929–5938.
3. Benya, P.D. and Shaffer, J.D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. **Cell** 30 (1982) 215–224.
4. Binette, F., McQuaid, D.P., Haudenschild, D.R., Yaeger, P.C., McPherson, J.M. and Tubo, R. Expression of a stable articular cartilage phenotype without evidence of hypertrophy by adult human articular chondrocytes in vitro. **J. Orthop. Res.** 16 (1998) 207–216.

5. Darling, E.M. and Athanasiou, K.A. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. **J. Orthop. Res.** 23 (2005) 425–432.
6. Sanz-Ramos, P., Mora, G., Ripalda-Cemboráin, P., Vicente-Pascual, M. and Izal-Azcárate, I. Identification of signalling pathways triggered by changes in the mechanical environment in rat chondrocytes. **Osteoarthr. Cartil.** 20 (2012) 931–939.
7. Sanz-Ramos, P., Mora, G., Vicente-Pascual, M., Ochoa, I., Alcaine, C., Moreno, R., Doblaré, M. and Izal-Azcárate, I. Response of sheep chondrocytes to changes in stiffness in the range from 2 to 20 Pa. Effect of cell passaging. **Connect. Tissue Res.** 54 (2013) 159–166.
8. Sanz-Ramos, P., Duart, J., Rodríguez-Goñi, M.V., Vicente-Pascual, M., Dotor, J., Mora, G. and Izal-Azcárate, I. Improved chondrogenic capacity of collagen hydrogel-expanded chondrocytes. In vitro and in vivo analysis. **J. Bone Joint Surg. Am.** 96 (2013) 1109–1117.
9. Nakao, A., Imamura, T., Souchelnitskiy, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C.H., Miyazono, K. and ten Dijke, P. TGF β receptor mediated signalling through Smad2, Smad3 and Smad4. **EMBO J.** 16 (1997) 5353–5362.
10. Lin, H.Y. and Moustakas, A. TGF β receptors: structure and function. **Cell. Mol. Biol.** 40 (1996) 337–349.
11. Moustakas, A., Souchelnitskiy, S. and Heldin, C.H. Smad regulation in TGF β signal transduction. **J. Cell. Sci.** 114 (2001) 4359–4369.
12. Inman, G.J., Nicolas, F.J. and Hill, C.S. Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF β receptor activity. **Mol. Cell.** 10 (2002) 283–294.
13. Roberts, A.B. TGF β signaling from receptors to the nucleus. **Microbes Infect.** 1 (1999) 1265–1273.
14. Goumans, M.J. and Mummery, C. Functional analysis of the TGF β receptor/Smad pathway through gene ablation in mice. **Int. J. Dev. Biol.** 44 (2000) 253–265.
15. Ito, H., Akiyama, H., Shigeno, C. and Nakamura, T. Noggin and bone morphogenetic protein-4 coordinately regulate the progression of chondrogenic differentiation in mouse clonal EC cells, ATDC5. **Biochem. Biophys. Res. Commun.** 260 (1999) 240–244.
16. Blaney Davidson, E.N., Remst, D.F., Vitters, E.L., van Beuningen, H.M., Blom, A.B., Goumans, M.J., van der Berg, W.B. and van der Kraan, P.M. Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. **J. Immunol.** 182 (2009) 7937–7945.
17. Dell’Accio, F., De Bari, C. and Luyten, F.P. Molecular markers predictive of the capacity of expanded human articular chondrocytes to form stable cartilage in vivo. **Arthritis Rheum.** 44 (2001) 1608–1619.
18. Livak, K.J. and Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. **Methods** 25 (2001) 402–408.

19. Finsson, K.W., Parker, W.L., ten Dijke, P., Thorikay, M and Philip, A. ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes. **J. Bone Mineral Res.** 23 (2008) 896–906.
20. van der Kraan, P.M., Goumans, M.J., Blaney Davidson, E. and ten Dijke, P. Age-dependent alteration of TGF- β signalling in osteoarthritis. **Cell Tissue Res.** 347 (2012) 257–265.
21. Kim, K.I., Park, Y.S. and Im, G.I. Changes in the epigenetic status of the SOX-9 promoter in human osteoarthritic cartilage. **J. Bone Mineral Res.** 28 (2013) 1050–1060.
22. Bui, C., Barter, M.J., Scott, J.L., Xu, Y., Galler, M., Reynard, L.N., Rowan, A.D. and Young, D.A. cAMP response element-binding (CREB) recruitment following a specific CpG demethylation leads to the elevated expression of the matrix metalloproteinase 13 in human articular chondrocytes and osteoarthritis. **FASEB J.** 26 (2012) 3000–3011.
23. Cheung, K.S., Hashimoto, K., Yamada, N. and Roach, H.I. Expression of ADAMTS-4 by chondrocytes in the surface zone of human osteoarthritic cartilage is regulated by epigenetic DNA de-methylation. **Rheumatol. Int.** 29 (2009) 525–534.
24. Roach, H.I., Yamada, N., Cheung, K.S., Tilley, S., Clarke, N.M., Oreffo, R.O., Kokubun, S. and Bronner, F. Association between the abnormal expression of matrix-degrading enzymes by human osteoarthritic chondrocytes and demethylation of specific CpG sites in the promoter regions. **Arthritis Rheum.** 52 (2005) 3110–3124.
25. Poschl, E., Fidler, A., Schmidt, B., Kallipolitou, A., Schmid, E. and Aigner, T. DNA methylation is not likely to be responsible for aggrecan down regulation in aged or osteoarthritic cartilage. **Ann. Rheum. Dis.** 64 (2005) 477–480.
26. Choi, M.R., In, Y.H., Park, J., Park, T., Jung, K.H., Chai, J.C., Chung, M.K., Lee, Y.S. and Chai, Y.G. Genome-scale DNA methylation pattern profiling of human bone marrow mesenchymal stem cells in long-term culture. **Exp. Mol. Med.** 44 (2012) 503–512.
27. Zhang, Z.X., Guan, L.X., Zhang, K., Wang, S., Cao, P.C., Wang, Y.H., Wang, Z. and Dai, L.J. Cytogenetic analysis of human bone marrow-derived mesenchymal stem cells passaged in vitro. **Cell Biol. Int.** 31 (2007) 645–648.
28. Dotor, J., López-Vázquez, A.B., Lasarte, J.J., Sarobe, P., García-Granero, M., Riezu-Boj, J.I., Martínez, A., Feijoó, E., López-Sagaseta, J., Hermida, J., Prieto, J. and Borrás-Cuesta, F. Identification of peptide inhibitors of transforming growth factor beta 1 using a phage-displayed peptide library. **Cytokine** 39 (2007) 106–115.
29. Ezquerro, I.J., Lasarte, J.J., Dotor, J., Castilla-Cortázar, I., Bustos, M., Peñuelas, I., Blanco, G., Rodríguez, C., Lechuga, M.C., Greenwel, P., Rojkind, M., Prieto, J. and Borrás-Cuesta, F. A synthetic peptide from

- transforming growth factor beta type III receptor inhibits liver fibrogenesis in rats with carbon tetrachloride liver injury. **Cytokine** 22 (2003) 12–20.
30. Santiago, B., Guitierrez-Cañas, I., Dotor, J., Palao, G., Lasarte, J.J., Ruiz, J., Prieto, J., Borrás-Cuesta, F. and Pablos, J.L. Topical application of a peptide inhibitor of transforming growth factor-beta1 ameliorates bleomycin-induced skin fibrosis. **J. Invest. Dermatol.** 125 (2005) 450–455.
 31. Hermida, N., López, B., González, A., Dotor, J., Lasarte, J.J., Sarobe, P., Borrás-Cuesta, F. and Díez, J. A synthetic peptide from transforming growth factor-beta1 type III receptor prevents myocardial fibrosis in spontaneously hypertensive rats. **Cardiovasc. Res.** 81 (2009) 601–609.
 32. Bershadsky, A.D., Balaban, N.Q. and Geiger B. Adhesion-dependent cell mechanosensitivity. **Annu. Rev. Cell. Dev. Biol.** 19 (2003) 677–695.
 33. Pelham, R.J. Jr. and Wang, Y. Cell locomotion and focal adhesions are regulated by substrate flexibility. **Proc. Natl. Acad. Sci. USA** 94 (1997) 13661–13665.