

# Mechanical load modulates chondrogenesis of human mesenchymal stem cells through the TGF- $\beta$ pathway

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## Abstract

This study investigated the effect of mechanical load on human mesenchymal stem cell (hMSC) differentiation under different exogenous transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) concentrations (0, 1 or 10 ng/ml). The role of the TGF- $\beta$  signalling pathway in this process was also studied. Human MSCs were seeded into fibrin-biodegradable polyurethane scaffolds at a cell density of  $5 \times 10^6$  cells per scaffold and stimulated using our bioreactor. One hour of surface motion superimposed on cyclic compression was applied once a day over seven consecutive days. Scaffolds were analysed for gene expression, DNA content and glycosaminoglycan amount. Addition of TGF- $\beta$ 1 in the culture medium was sufficient to induce chondrogenesis of hMSCs. Depending on the TGF- $\beta$ 1 concentration of the culture medium, mechanical load stimulated chondrogenesis of hMSCs compared to the unloaded scaffolds, with a much stronger effect on gene expression at lower TGF- $\beta$ 1 concentrations. With TGF- $\beta$ 1 absent in the culture medium, mechanical load stimulated gene transcripts and protein synthesis of TGF- $\beta$ 1 and TGF- $\beta$ 3. TGF- $\beta$  type I receptor inhibitor LY364947 blocked the up-regulation on TGF- $\beta$ 1 and TGF- $\beta$ 3 production stimulated by mechanical load, and also blocked the chondrogenesis of hMSCs. Taken together, these findings suggest that mechanical load promotes chondrogenesis of hMSCs through TGF- $\beta$  pathway by up-regulating TGF- $\beta$  gene expression and protein synthesis.

**Keywords:** chondrogenesis • human bone marrow mesenchymal stem cells • fibrin-polyurethane scaffold composites • mechanical load • TGF- $\beta$ 1 • TGF- $\beta$  pathway

## Introduction

Articular cartilage has a very poor ability to self repair. Tissue engineering is believed to be the future of articular cartilage healing because of the unsatisfying results of the current clinical procedures. Mesenchymal stem cells (MSCs) derived from bone marrow have demonstrated the multipotential to differentiate into several cell lineages, including chondrocytes [1].

It has been documented that cyclic hydrostatic pressure [2–4] and cyclic compressive load [5–11] can stimulate chondrogenic differentiation of MSCs. However, the precise mechanisms by which physical loads affect biological responses have yet to be resolved. Furthermore, the ideal mechanical parameters remain

unknown. Since the physiological joint movement is kinematically very complex, sole mechanical compression alone is unlikely to sufficiently stimulate the generation of functional tissue from MSCs *in vitro*. To address the combined interplay of joint-level compression, shear and articular motion, we developed a bioreactor that allows for simultaneous compression, shear and articular fluid transport of developing constructs [12, 13]. Our recent study established a fibrin-polyurethane scaffold composite culture system, which is able to provide an environment for chondrogenesis of human mesenchymal stem cells (hMSCs) [14]. Therefore in this study, cyclic compressive load and surface shear strain were applied on hMSCs fibrin-polyurethane scaffolds constructs to modulate chondrogenesis, aiming to more accurately mimic the *in vivo* situation *in vitro*. Furthermore, some scaffolds were cut into top and bottom sections to investigate the mechanotransduction effect on different parts of the stimulated constructs.

Various growth factors (*i.e.* fibroblast growth factor-2, transforming growth factor- $\beta$  [TGF- $\beta$ ], insulin-like growth factor-1 and

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osteoprogenitor factor-1) have been used to modulate chondrocyte phenotype, proliferation and biosynthesis rates. In particular, 'chondrogenic medium' containing dexamethasone and TGF- $\beta$ <sub>1</sub> has been developed to induce chondrogenic differentiation of chondroprogenitor cells [1]. Dimeric ligands of the TGF- $\beta$  superfamily signal across cell membranes by assembling heterotetrameric complexes of structurally related serine/threonine-kinase receptor pairs, designated types I and II. TGF- $\beta$  complexes assemble cooperatively through recruitment of the low-affinity (type I) receptor by the ligand-bound high-affinity (type II) pair. The type II receptor phosphorylates the type I receptor, which in turn activates type I receptor kinase activity [15]. It is known that TGF- $\beta$  and mechanical loads modulate chondrogenesis of MSCs. However, the interaction between TGF- $\beta$  and mechanical load remains unclear. Therefore we applied mechanical loads on hMSCs fibrin-polyurethane scaffold constructs, either alone or in the presence of 1 ng/ml or 10 ng/ml TGF- $\beta$ <sub>1</sub>, specifically to determine whether there is a dose response of this factor on any mechanical stimulation. In addition, the inhibition of TGF- $\beta$  signalling by LY364947, a selective, ATP-competitive inhibitor of TGF- $\beta$  type I receptor kinase (TGF- $\beta$  RI) [16–18], was used to investigate the role of TGF- $\beta$  signalling pathway in hMSC chondrogenic differentiation induced by mechanical loading.

## Materials and methods

### Biodegradable polyurethane scaffold

Cylindrical (8 × 4 mm) porous scaffolds (average pore size of 90–300  $\mu$ m) of biodegradable polyurethane were prepared as described elsewhere [19]. The polymers used for scaffold preparation were synthesized with hexamethylene diisocyanate, poly( $\epsilon$ -caprolactone) diol and isosorbide diol (1,4:3,6-dianhydro-D-sorbitol) as chain extender [20].

### Isolation and expansion of hMSCs

Bone marrow was obtained with ethical approval and the written consent of patients undergoing total hip replacement. MSCs were isolated by Ficoll cushion as described elsewhere [14].

### Fibrin-polyurethane composite culture of hMSCs

A fibrin-polyurethane hybrid system was used for 3D hMSC culture. P3 hMSCs were trypsinized, suspended in fibrinogen solution and mixed with thrombin solution immediately prior to seeding into the polyurethane scaffold at a cell density of  $5 \times 10^6$  per scaffold. The final concentrations of the fibrin gel were 17 mg/ml fibrinogen and 0.5 U/ml thrombin [21]. After 7 days in pre-culture in six-well plates (5 ml medium per scaffold, medium changed every 2 to 3 days), cell-scaffold constructs were exposed to mechanical loading in special holders for 7 days as described below (3 ml medium per scaffold, medium changed everyday). Constructs were divided into three groups. Each group had eight scaffolds in total. All groups were cultured in medium consisting of DMEM

(Gibco, Basel, Switzerland), ITS (10  $\mu$ g/ml insulin from bovine pancreas, 5.5  $\mu$ g/ml human transferrin (substantially iron-free), 5 ng/ml sodium selenite, 0.5 mg/ml bovine serum albumin and 4.7  $\mu$ g/ml linoleic acid; Sigma), 100 units/ml Penicillin (Gibco), 100  $\mu$ g/ml Streptomycin (Gibco), 1% non-essential amino acid (Gibco), 50  $\mu$ g/ml ascorbate 2 phosphate (Sigma, Buchs, Switzerland), 5  $\mu$ M  $\epsilon$ -amino-caproic acid (Sigma) [22] and  $10^{-7}$  M dexamethasone (Sigma). Either 0 ng/ml, 1 ng/ml or 10 ng/ml recombinant human TGF- $\beta$ <sub>1</sub> (Fitzgerald, Concord, MA, USA) was added into the medium of three groups respectively prior to and during mechanical loading.

For the TGF- $\beta$  RI inhibitor study, in the group without TGF- $\beta$ <sub>1</sub> in culture medium, inhibition of TGF- $\beta$  RI signalling was investigated by adding 1  $\mu$ M LY364947 (Sigma) (an inhibitor of TGF- $\beta$  RI) into the medium.

### Mechanical loading

Mechanical conditioning of cell-scaffold constructs was performed using our previously described bioreactor system [12]. Briefly, a ceramic hip ball (32 mm in diameter) was pressed onto the cell-seeded scaffold. Interface motion was generated by oscillation of the ball about an axis perpendicular to the scaffold axis.

Samples were assigned in quadruplicates to one of two groups: The loaded group was exposed to ball oscillation of  $\pm 25^\circ$  at 1 Hz. Simultaneously, dynamic compression was applied at 1 Hz with 10% sinusoidal strain, superimposed on a 10% static offset strain, resulting in an actual strain amplitude of 10–20%. Mechanical loading was performed 1 hr a day over 7 consecutive days. The group of unloaded constructs served as controls. After 7 days in pre-culture and 7 days of loading the top 10% of the construct was used for gene expression analysis.

### Biochemical analysis

Whole samples were digested with 0.5 mg/ml proteinase-K at 56°C overnight. DNA content was measured spectrofluorometrically using Hoechst 33258 (Sigma) [23]. The amount of glycosaminoglycan (GAG) in the scaffolds and medium was determined by the dimethylmethylene blue dye method [24].

### Gene expression analysis

Total RNA was extracted from homogenized constructs with TRI Reagent. Reverse transcription was performed with TaqMan reverse transcription reagents, using random hexamer primers and 1  $\mu$ g of total RNA. Table 1 shows the sequence of human primers and TaqMan probes for collagens type-I (COL1), type-II (COL2), type-X (COL10), aggrecan (AGG) and proteoglycan4 (PRG4). Primers and probe for amplification of 18S ribosomal RNA (18S), osterix transcription factor (Sp7, Hs00541729\_m1), TGF- $\beta$ <sub>1</sub> (TGFB1, Hs00171257\_m1) and TGF- $\beta$ <sub>3</sub> (TGFB3, Hs00234245\_m1) were from Applied Biosystems (Rotkreutz, Switzerland). Relative quantification of target mRNA was performed according to the comparative C<sub>T</sub> method with 18S ribosomal RNA as the endogenous control [25, 26].

### TGF- $\beta$ <sub>1</sub> and TGF- $\beta$ <sub>3</sub> ELISA

During 7 days of mechanical loading, conditioned medium of loaded and control samples were pooled for each construct and analysed for TGF- $\beta$ <sub>1</sub>

**Table 1** Oligonucleotide primers and probes (human) used for real time PCR

COL1	Primer fw (5'-3')	CCCTGGAAAGAATGGAGATGAT
	Primer rev (5'-3')	ACTGAAACCTCTGTGCCCTTCA
	Probe (5'FAM/3'TAMRA)	CGGGCAATCCTCGAGCACCCCT
COL2A1	Primer fw (5'-3')	GGCAATAGCAGGTTACGTACA
	Primer rev (5'-3')	GATAACAGTCTTGCCCCACTTACC
	Probe (5'FAM/3'TAMRA)	CCTGAAGGATGGCTGCACGAAACATAC
COL10A1	Primer fw (5'-3')	ACGCTGAACGATACCAAATG
	Primer rev (5'-3')	TGCTATACCTTTACTCTTTATGGTGTA
	Probe (5'FAM/3'TAMRA)	ACTACCCAACACCAAGACACAGTTCTTCATTCC
Aggrecan	Primer fw (5'-3')	AGTCCTCAAGCCTCCTGTACTCA
	Primer rev (5'-3')	CGGGAAGTGGCGGTAACA
	Probe (5'FAM/3'TAMRA)	CCGGAATGGAAACGTGAATCAGAATCAACT
PRG4	Primer fw (5'-3')	GAG TAC CCA ATC AAG GCA TTA TCA
	Primer rev (5'-3')	CCA TCT ACT GGC TTA CCA TTG CA
	Probe (5'FAM/3'TAMRA)	CAA TCC CAT GCT TTC CGA TGA GAC CAA T

and TGF- $\beta_3$  concentrations by ELISA (DY240 and DY243, R&D Systems, Abingdon, UK) according to manufacturers' instruction.

## Statistical analysis

SPSS 16.0 statistical software was used for statistical analysis (significance at  $P < 0.05$ ). Gene expression values were normalized to the values of cells before 3D culture (day 0), if this value was not detectable, the data were then normalized to the expression level of control samples treated with 0 ng/ml TGF- $\beta_1$  (day 14). One-sample Kolmogorov–Smirnov test was used to define if the data are normally distributed (normal distribution at  $P > 0.1$ ). For comparison of all the gene expression data, Mann-Whitney  $U$  test was used. For comparison of GAG/DNA value, TGF- $\beta_1$  and TGF- $\beta_3$  protein concentrations in medium, between control and loaded samples, an independent  $t$ -test was used; among the 3 groups with different concentrations of TGF- $\beta_1$  in medium, ANOVA LSD *post hoc* test was used.

## Results

### TGF- $\beta_1$ and mechanical loading experiments

To address the effect of mechanical loading on chondrogenesis of hMSCs and the interaction between mechanical loading and TGF- $\beta_1$ , hMSCs were cultured in presence or absence of mechanical loading and with 0, 1 or 10 ng/ml TGF- $\beta_1$  in the culture medium.

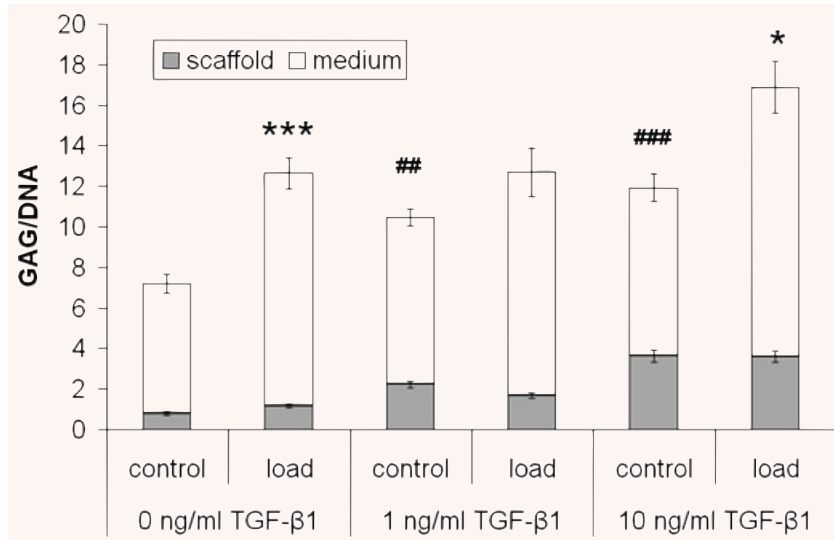
## Biochemical analysis

There was no difference in DNA content between control samples cultured in medium with different concentrations of TGF- $\beta_1$  (data not shown). Mechanical loading decreased the DNA content of constructs cultured in medium with 10 ng/ml TGF- $\beta_1$  compared to control samples ( $P < 0.05$ ), but not in the constructs cultured in medium with 0 or 1 ng/ml TGF- $\beta_1$ .

Total GAG synthesized (scaffolds plus medium) was normalized to DNA content (Fig. 1). The control samples cultured in medium with 1 ng/ml ( $P < 0.01$ ) or 10 ng/ml TGF- $\beta_1$  ( $P < 0.001$ ) had significantly higher GAG/DNA value compared to samples cultured in medium without TGF- $\beta_1$  (Fig. 1 control). In all the three groups where samples were cultured in medium with different concentrations of TGF- $\beta_1$ , the total GAG/DNA value showed a trend of up-regulation by mechanical load, this difference was significant in the groups with 0 ng/ml ( $P < 0.001$ ) or 10 ng/ml TGF- $\beta_1$  ( $P < 0.05$ ).

## Gene expression

Without mechanical stimulation, addition of TGF- $\beta_1$  in the culture medium significantly up-regulated the mRNA gene expression of COL2, AGG, COL10 and Sp7, markedly down-regulated the mRNA gene expression of PRG4, while having no effect on the mRNA gene expression of COL1, TGFB1 and TGFB3 (Fig. 2A–H). By day 14, the COL2, AGG, COL10 and Sp7 gene expression of hMSCs cultured in presence of 1 ng/ml TGF- $\beta_1$  increased 46,392 ( $P = 0.004$ ),



**Fig. 1** GAG/DNA value of scaffolds cultured in medium with 0, 1 or 10 ng/ml TGF-β<sub>1</sub> for 14 days, and their conditioned medium. Mean ± S.E.M., n = 6. ##P < 0.01, ###P < 0.001 versus control samples (0 ng/ml TGF-β<sub>1</sub>). \*P < 0.05, \*\*\*P < 0.001 versus respective control samples for a given medium condition.

687- ( $P = 0.002$ ), 60- ( $P = 0.002$ ) and 121- ( $P = 0.004$ ) fold respectively compared to cells cultured in the absence of TGF-β<sub>1</sub>. This increase was greater when 10 ng/ml TGF-β<sub>1</sub> was added to the medium. In contrast, treatment with 10 ng/ml TGF-β<sub>1</sub> decreased PRG4 gene expression to 0.13- ( $P = 0.009$ ) fold compared to cells cultured in the absence of TGF-β<sub>1</sub>.

Significant increases in chondrogenic gene expression due to mechanical load were only noted when TGF-β<sub>1</sub> was not added to the culture medium. When hMSCs were cultured in medium without TGF-β<sub>1</sub>, mechanical loading significantly stimulated gene expression by 1663- ( $P = 0.018$ ), 269- ( $P = 0.004$ ), 42- ( $P = 0.004$ ), 174- ( $P = 0.006$ ), 3- ( $P = 0.037$ ) and 6- ( $P = 0.004$ ) fold for genes COL2, AGG, COL10, Sp7, TGFB1 and TGFB3 respectively (Fig. 2A, B, E, F, G and H; 0 ng/ml TGF-β<sub>1</sub>). In the absence of TGF-β<sub>1</sub>, COL1 and PRG4 gene expression was up-regulated 2.7- and 3.4-fold by mechanical loading (Fig. 2C and D; 0 ng/ml TGF-β<sub>1</sub>). However, this difference is not significant. With 1 ng/ml TGF-β<sub>1</sub> in culture medium, AGG gene expression was stimulated 3.8-fold by mechanical loading ( $P = 0.004$ ) (Fig. 2B; 1 ng/ml TGF-β<sub>1</sub>). The remaining gene expression levels were not affected by mechanical loading.

In the group where 10 ng/ml TGF-β<sub>1</sub> was added, only Sp7 gene expression (2-fold,  $P = 0.037$ ) was significantly up-regulated by mechanical loading (Fig. 2F; 10 ng/ml TGF-β<sub>1</sub>). All the other genes in the other groups did not show significant difference between loaded and control samples.

### TGF-β<sub>1</sub> and TGF-β<sub>3</sub> ELISA

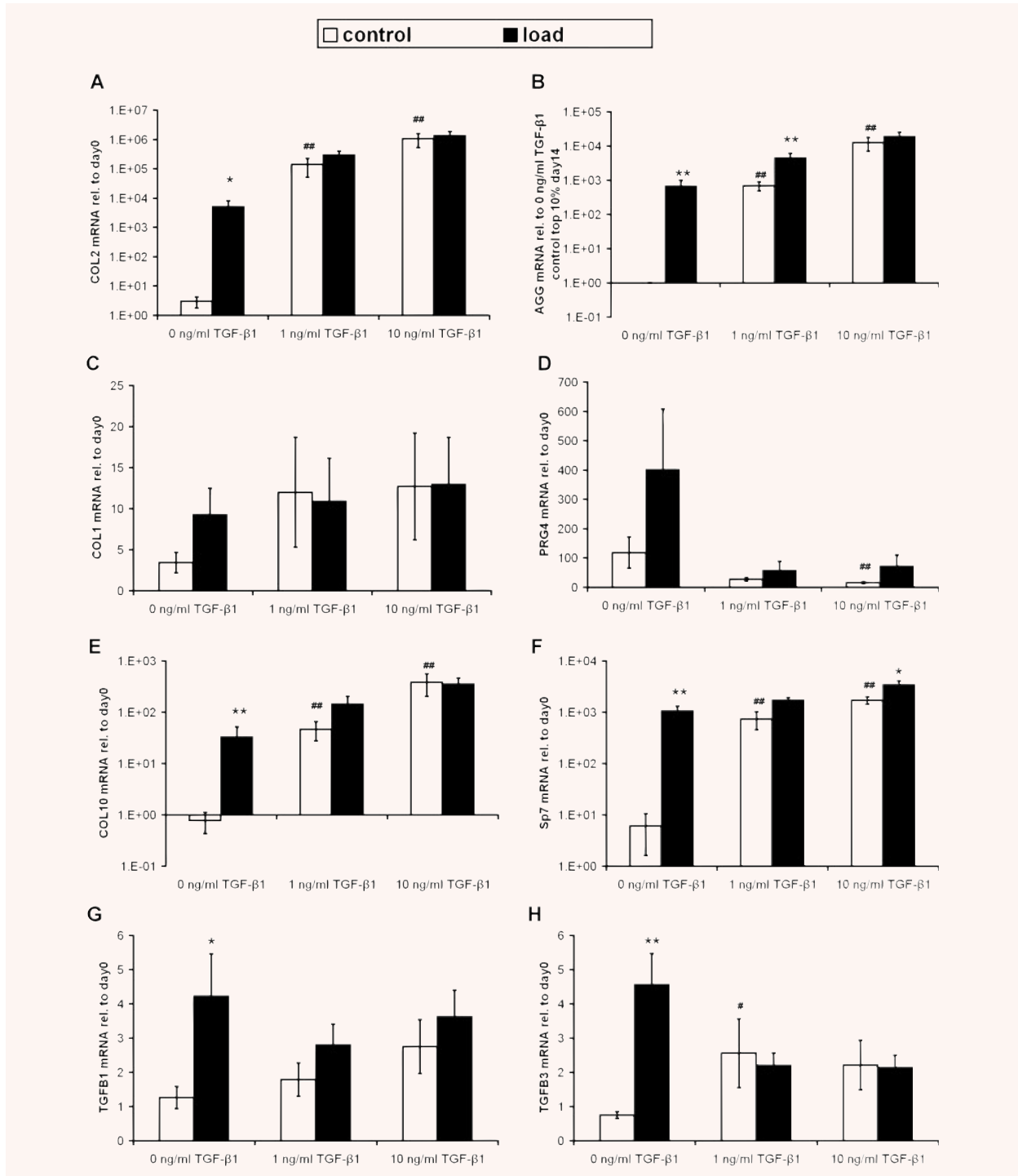
The concentrations of TGF-β<sub>1</sub> and TGF-β<sub>3</sub> protein in the conditioned medium on days 8–14 (7 days of loading) were determined by ELISA (Fig. 3).

The TGF-β<sub>1</sub> protein concentrations in control sample conditioned medium treated with 1 ng/ml ( $0.75 \pm 0.05$  ng/ml,  $P < 0.001$ ) or 10 ng/ml ( $3.42 \pm 0.13$  ng/ml,  $P < 0.001$ ) TGF-β<sub>1</sub> were both significantly higher than that of control samples cultured in the absence of TGF-β<sub>1</sub> ( $0.17 \pm 0.02$  ng/ml) (Fig. 3A control). The greatest up-regulation of TGF-β synthesis was seen in the loaded group cultured in the absence of exogenous TGF-β<sub>1</sub>. Mechanical loading significantly up-regulated TGF-β<sub>1</sub> protein synthesis in groups with 0 (2.98-fold,  $P < 0.001$ ) or 1 ng/ml (1.34-fold,  $P = 0.001$ ) TGF-β<sub>1</sub> in the culture medium (Fig. 3A). While in the group with 10 ng/ml TGF-β<sub>1</sub> in the culture medium, the amount of TGF-β<sub>1</sub> protein was down-regulated by mechanical loading by 20% ( $P < 0.001$ ) (Fig. 3A).

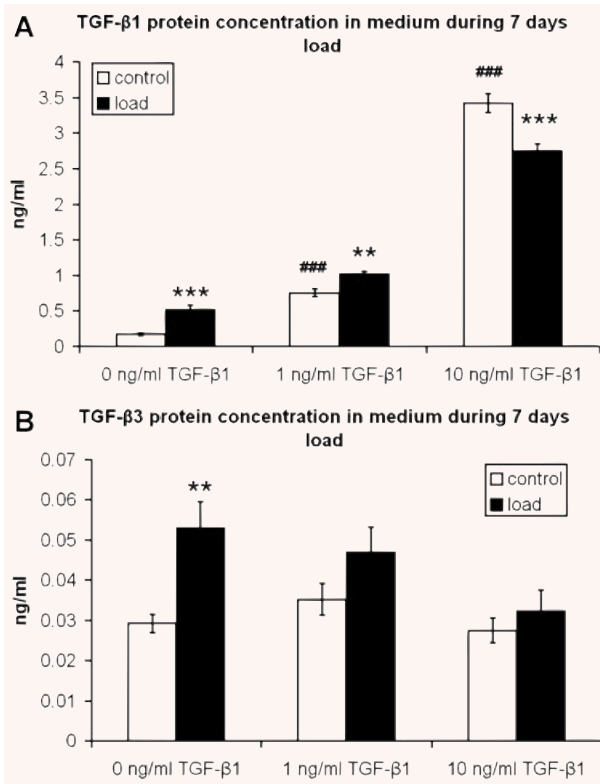
No difference in TGF-β<sub>3</sub> protein concentrations was observed in the conditioned medium of control samples from three groups treated with different exogenous levels of TGF-β<sub>1</sub> (Fig. 3B control). Mechanical loading significantly up-regulated TGF-β<sub>3</sub> protein synthesis in samples cultured without TGF-β<sub>1</sub> in the medium (1.82-fold,  $P = 0.002$ ) (Fig. 3B).

### TGF-β RI inhibitor and mechanical loading experiments

As we had determined that chondrogenesis could be induced by mechanical load in the absence of TGF-β we decided to investigate this further. To investigate the role of TGF-β signalling in chondrogenic induction of hMSCs by mechanical loading when no TGF-β<sub>1</sub> was added into the culture medium, hMSCs were cultured in the presence and absence of mechanical loading and the presence and absence of TGF-β RI inhibitor LY364947.



**Fig. 2** Relative mRNA expression of hMSCs cultured in the top 10% sections of scaffolds for 14 days with (load) or without (control) mechanical stimulation on days 8–14. AGG data were normalized to control samples cultured in medium with 0 ng/ml TGF-β<sub>1</sub> (day 14), all the other data were normalized to day 0. Mean ± S.E.M., *n* = 6. #*P* < 0.05, ##*P* < 0.01 versus control (0 ng/ml TGF-β<sub>1</sub>). \**P* < 0.05, \*\**P* < 0.01 versus respective control samples for a given medium condition.



**Fig. 3** TGF-β<sub>1</sub> (A) and TGF-β<sub>3</sub> (B) protein concentrations in the conditioned medium during 7 days of mechanical loading. Mean ± S.E.M., n = 12. ###P < 0.001 versus control samples cultured in medium with 0 ng/ml TGF-β<sub>1</sub>. \*\*P < 0.01, \*\*\*P < 0.001 versus respective control samples for a given medium condition.

## Gene expression

When LY364947 was not added into the culture medium, mechanical loading significantly up-regulated COL1, COL2, COL10, AGG, Sp7, TGFB1 and TGFB3 gene expression in the absence of additional TGF-β (Fig. 4; LY364947<sup>-</sup>). When LY364947 was added into the culture medium during 7 days of loading, mechanical load did not have any effect on the expression of any of these genes demonstrating a pivotal role in TGF-β up-regulation due to mechanical load (Fig. 4; LY364947<sup>+</sup>).

## Discussion

TGF-β<sub>1</sub> increased the gene expression of chondrogenic, hypertrophic and osteogenic markers (Fig. 2), and also stimulated the synthesis of GAG (Fig. 1). This has been shown in both pellet culture and scaffold culture of MSCs [1, 27–31], indicating the ability of TGF-β<sub>1</sub> to promote chondrogenesis and terminal ossification.

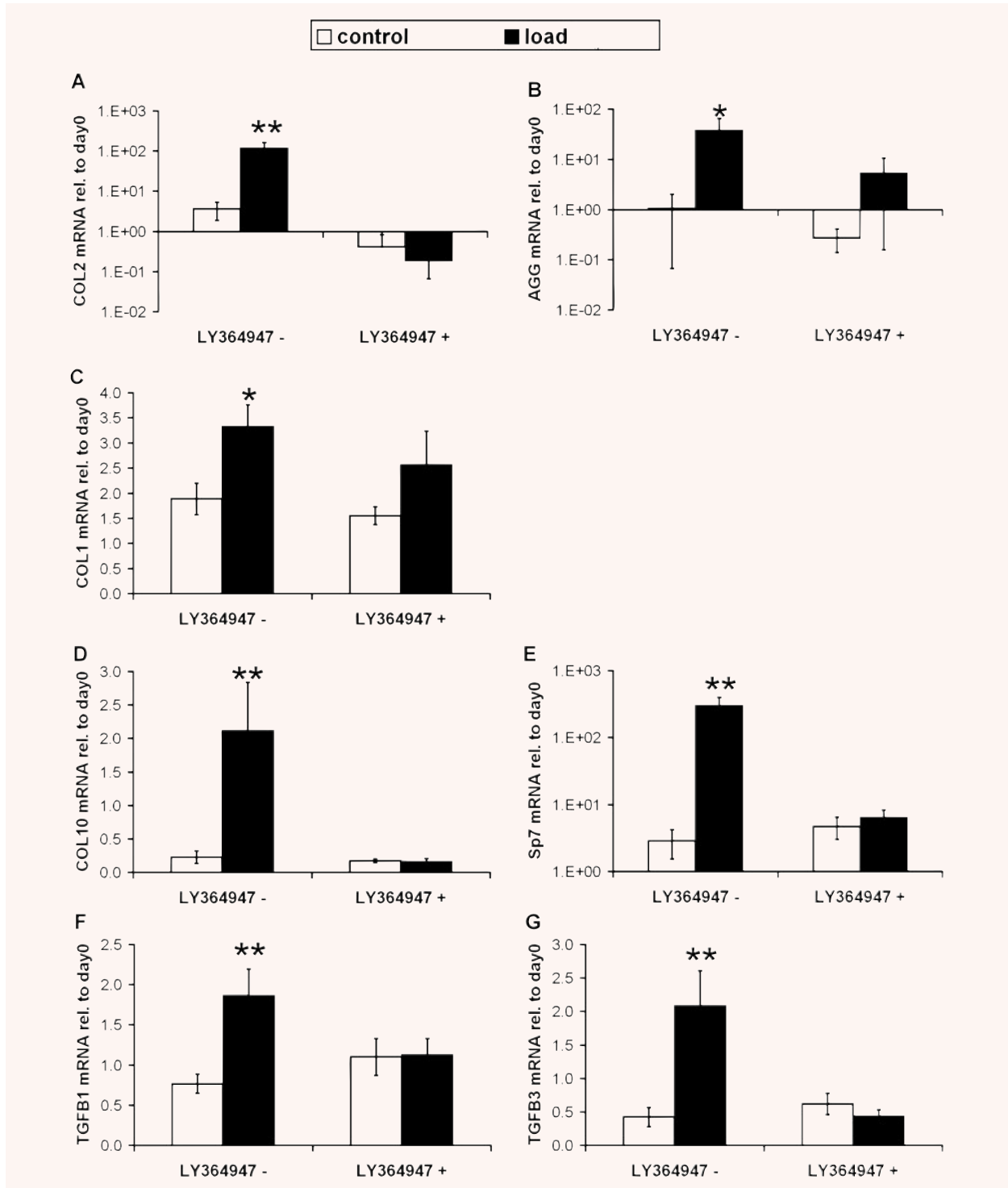
Even though it is widely believed that stem cells react to mechanical load, according to the literature chondrogenic response is only mildly enhanced by load. The data presented here demonstrated that under lower concentrations of TGF-β<sub>1</sub> in the culture medium, a large increase in chondrogenic marker expression (type II collagen and aggrecan) was induced by dynamic compression and surface shear strain. However, we found this response was greatly reduced when 10 ng/ml TGF-β<sub>1</sub> was added to the culture medium, this suggests a feedback mechanism. We propose two possible explanations. (1) The concentration of TGF-β<sub>1</sub> in culture medium influences the effect of mechanical load on the chondrogenesis of stem cells. The standard chondrogenic TGF-β<sub>1</sub> level (10 ng/ml) is non-physiological and artificially high. In this situation the effects of mechanical load on the chondrogenesis of hMSCs are masked, as seen by very small increase in the type II collagen (1.2-fold) and aggrecan (1.5-fold) gene level between control and loaded samples in our study (Fig. 2A and B).

Most of the published studies related to mechanical load and chondrogenesis of stem cells were performed using 10 ng/ml TGF-β, which may explain why chondrogenic differentiation was minimally stimulated by load [3, 6, 10, 11]. This is supported by the study of Terraciano *et al.* They showed that when goat MSC-PEGDA hydrogel constructs were cultured in the absence of TGF-β<sub>1</sub>, aggrecan gene expression was up-regulated 3.8-fold after 1 hr of cyclic compression; however, when 10 ng/ml TGF-β<sub>1</sub> was present, aggrecan gene expression only increased 1.4-fold after load of 2.5 hrs per day for 14 days [11].

Miyanishi *et al.* reported that exposing hMSCs pellets to intermittent hydrostatic pressure in the absence of TGF-β increased SOX9, type II collagen and aggrecan mRNA levels by 4.0-, 5.7- and 4.4-fold compared to the respective control. In the presence of 10 ng/ml TGF-β<sub>3</sub>, intermittent hydrostatic pressure increased SOX9, type II collagen and aggrecan mRNA levels by 1.9-, 3.3- and 1.6-fold compared to the respective control [4]. This is consistent with our present study that the trend of chondrogenic gene expression increase is lower under high exogenous TGF-β level. Under comparable culture conditions (10<sup>-7</sup> M dexamethasone, no TGF-β) and load, the up-regulation of type II collagen and aggrecan mRNA expression in Miyanishi paper [4] was 5.7- and 4.4-fold, respectively. Using the device in our study load increased type II collagen and aggrecan mRNA expression by 1663- and 666-fold, respectively. This indicates the advantage of our system, although other factors such as the frequency which the medium is changed will play a role [7, 9].

We also observed that the preparation of the medium played a role. Due to the serum-free nature of the chondrogenic medium if the TGF-β<sub>1</sub> was added prior to sterile filtration, the final concentration was 0.5 ng/ml, not the 10 ng/ml as expected. This led to differences when load was applied. We prepared a sterile filtered stock solution through a small volume filter that would adsorb little protein. The TGF-β<sub>1</sub> was then added after the main medium had been sterile filtered.

(2) Dexamethasone is necessary in the process of chondrogenic differentiation stimulated by mechanical load. We performed an experiment with neither dexamethasone nor TGF-β<sub>1</sub> in the



**Fig. 4** Relative mRNA expression of hMSCs cultured for 14 days, with (load) or without (control) mechanical stimulation, and with (LY364947<sup>+</sup>) or without (LY364947<sup>-</sup>) LY364947 in the culture medium on days 8–14. Data were normalized to day 0. Mean  $\pm$  S.E.M.,  $n = 8$ . \* $P < 0.05$ , \*\* $P < 0.01$  versus respective control samples for a given medium condition.

culture medium. Mechanical load did not affect the gene expression profile except for 7.45-fold increase of PRG4 mRNA expression (data not shown). Huang *et al.* and Mouw *et al.* have also carried out studies without dexamethasone and TGF- $\beta_1$ , where mechanical load did not lead to highly increased type II collagen or aggrecan gene expression [8, 10]. Although dexamethasone is not a specific chondrogenic differentiation factor [1], our previous study has shown that combination of dexamethasone and TGF- $\beta_1$  is crucial for chondrogenesis of hMSCs [14]. With dexamethasone present in the culture medium, addition of TGF- $\beta_1$  did not affect the DNA content of the hMSCs scaffolds constructs, which is consistent with other investigations [7, 28, 29].

When TGF- $\beta_1$  was absent in the medium, we detected an increase in type X collagen and Sp7 gene expression, although this increase was much lower than the up-regulation of type II collagen. We have previously demonstrated that the fibrin-polyurethane construct leads to a significantly lower expression of hypertrophic markers compared to pellet culture [14]. A limited degree of up-regulation of type X collagen may be unavoidable as the level found in monolayer expanded MSCs is so low. Even a very low level of absolute expression would seem like a large increase when the baseline levels are so low. We have compared the dCt of type X collagen expression to that found in normal, healthy adult human articular cartilage, using 18S as a control. We found that the expression was similar to that found within our loaded samples without exogenous TGF- $\beta_1$  treatment (data not shown). Other groups have suggested Col X may not be an optimal marker during *in vitro* chondrogenesis as an increase was detected prior to type II collagen expression during chondrogenesis of human MSCs [32, 33]. There is also the possibility that a small subset of cells undergo hypertrophy, leading to the increases seen.

It is also interesting to note that addition of TGF- $\beta_1$  into the culture medium down-regulated PRG4 expression (Fig. 2D). Samples loaded in the absence of additional TGF- $\beta_1$  led to an increase in endogenous TGF- $\beta$  synthesis (Fig. 3). The final measured concentration of TGF- $\beta_1$  in the conditioned medium of 0 ng/ml and 1 ng/ml groups was similar, and yet PRG4 was only down-regulated in the 1 ng/ml group. This suggests that there is a subtle difference in the way PRG4 expression is regulated by TGF- $\beta$ , the significance which is still unclear, as PRG4 is required for adequate lubrication at the interface.

Mechanical load up-regulated the gene expression and protein synthesis of TGF- $\beta_1$  and TGF- $\beta_3$  in hMSCs in the absence of exogenous TGF- $\beta_1$ . This finding is consistent with other studies which have demonstrated that mechanical loading can promote

TGF- $\beta$  production in cell culture [7, 8, 34] and tissues [35, 36]. Several researchers have proposed that mechanical stimulation may activate TGF- $\beta$  signalling and hence promote the chondrogenesis of MSCs [8, 10, 37]. Therefore, we applied the TGF- $\beta$  type I receptor inhibitor LY364947 in the group without TGF- $\beta_1$  in the culture medium, and stimulated hMSCs-polyurethane scaffolds constructs with dynamic compression and surface rotation. Results showed that when LY364947 was absent in the medium, mechanical load induced chondrogenesis of hMSCs and TGF $\beta$  gene expression in the absence of exogenous TGF- $\beta$  (Fig. 4; LY364947<sup>-</sup>). With LY364947 in the medium, chondrogenesis of hMSCs stimulated by load was blocked as determined by no up-regulation of chondrogenic and hypertrophic marker genes (Fig. 4A–E; LY364947<sup>+</sup>). The TGF $\beta$  gene expression was also not up-regulated (Fig. 4F and G; LY364947<sup>+</sup>). To the best of our knowledge, this is the first study which demonstrated that the induced chondrogenesis of hMSCs by mechanical load is an outcome of TGF- $\beta$  synthesis and signalling. It is a direct mechanism that mechanical loading in the absence of exogenous TGF- $\beta$  enhances the production of TGF- $\beta$  through up-regulation of mRNA gene transcripts, hence inducing chondrogenesis. The chondrogenic response is then prevented when TGF- $\beta$  signalling is inhibited *via* the blocking of the receptor. This process might also involve up-regulation of TGF- $\beta$  receptors, as Huang *et al.* have shown that cyclic compressive loading promoted gene expression and protein synthesis of both TGF- $\beta$  type I and type II receptors [8]. This finding helps to understand the rationale by which mechanical loading affects the differentiation of hMSCs, and will be useful in cartilage tissue engineering.

In conclusion, this study has demonstrated that mechanical loading can stimulate chondrogenesis of hMSCs cultured in polyurethane-fibrin composites; however, it is only observable under low concentrations of TGF- $\beta_1$  and in the presence of dexamethasone. Mechanical load induces chondrogenesis of hMSCs through the TGF- $\beta$  pathway and this can be blocked using a specific inhibitor of TGF- $\beta$  RI.

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## References

1. Johnstone B, Hering TM, Caplan AI, *et al.* *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res.* 1998; 238: 265–72.
2. Angele P, Yoo JU, Smith C, *et al.* Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated *in vitro*. *J Orthop Res.* 2003; 21: 451–7.
3. Miyazishi K, Trindade MC, Lindsey DP, *et al.* Dose- and time-dependent effects of cyclic hydrostatic pressure on transforming growth factor-beta3-induced chondrogenesis by adult human mesenchymal



- stem cells *in vitro*. *Tissue Eng.* 2006; 12: 2253–62.
4. **Miyanishi K, Trindade MC, Lindsey DP, et al.** Effects of hydrostatic pressure and transforming growth factor-beta 3 on adult human mesenchymal stem cell chondrogenesis *in vitro*. *Tissue Eng.* 2006; 12: 1419–28.
  5. **Elder SH, Goldstein SA, Kimura JH, et al.** Chondrocyte differentiation is modulated by frequency and duration of cyclic compressive loading. *Ann Biomed Eng.* 2001; 29: 476–82.
  6. **Angele P, Schumann D, Angele M, et al.** Cyclic, mechanical compression enhances chondrogenesis of mesenchymal progenitor cells in tissue engineering scaffolds. *Biorheology.* 2004; 41: 335–46.
  7. **Huang CY, Hagar KL, Frost LE, et al.** Effects of cyclic compressive loading on chondrogenesis of rabbit bone-marrow derived mesenchymal stem cells. *Stem Cells.* 2004; 22: 313–23.
  8. **Huang CY, Reuben PM, Cheung HS.** Temporal expression patterns and corresponding protein inductions of early responsive genes in rabbit bone marrow-derived mesenchymal stem cells under cyclic compressive loading. *Stem Cells.* 2005; 23: 1113–21.
  9. **Campbell JJ, Lee DA, Bader DL.** Dynamic compressive strain influences chondrogenic gene expression in human mesenchymal stem cells. *Biorheology.* 2006; 43: 455–70.
  10. **Mouw JK, Connelly JT, Wilson CG, et al.** Dynamic compression regulates the expression and synthesis of chondrocyte-specific matrix molecules in bone marrow stromal cells. *Stem Cells.* 2007; 25: 655–63.
  11. **Terraciano V, Hwang N, Moroni L, et al.** Differential response of adult and embryonic mesenchymal progenitor cells to mechanical compression in hydrogels. *Stem Cells.* 2007; 25: 2730–8.
  12. **Wimmer MA, Grad S, Kaup T, et al.** Tribology approach to the engineering and study of articular cartilage. *Tissue Eng.* 2004; 10: 1436–45.
  13. **Li Z, Yao S, Alini M, et al.** Different response of articular chondrocyte subpopulations to surface motion. *Osteoarthritis Cartilage.* 2007; 15: 1034–41.
  14. **Li Z, Kupcsik L, Yao SJ, et al.** Chondrogenesis of human bone marrow mesenchymal stem cells in fibrin-polyurethane composites. *Tissue Eng Part A.* 2008; DOI: 10.1089/ten.tea.2008.0247.
  15. **Groppe J, Hinck CS, Samavarchi-Tehrani P, et al.** Cooperative assembly of TGF-beta superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding. *Mol Cell.* 2008; 29: 157–68.
  16. **Peng SB, Yan L, Xia X, et al.** Kinetic characterization of novel pyrazole TGF-beta receptor I kinase inhibitors and their blockade of the epithelial-mesenchymal transition. *Biochemistry.* 2005; 44: 2293–304.
  17. **Li HY, Wang Y, Heap CR, et al.** Dihydropyrrrolopyrazole transforming growth factor-beta type I receptor kinase domain inhibitors: a novel benzimidazole series with selectivity versus transforming growth factor-beta type II receptor kinase and mixed lineage kinase-7. *J Med Chem.* 2006; 49: 2138–42.
  18. **Shiou SR, Datta PK, Dhawan P, et al.** Smad4-dependent regulation of urokinase plasminogen activator secretion and RNA stability associated with invasiveness by autocrine and paracrine transforming growth factor-beta. *J Biol Chem.* 2006; 281: 33971–81.
  19. **Gorna K, Gogolewski S.** *In vitro* degradation of novel medical biodegradable aliphatic polyurethanes based on epsilon-caprolactone and pluronics with various hydrophilicities. *Polymer Degradation and Stability.* 2002; 75: 113–22.
  20. **Gorna K, Gogolewski S.** Biodegradable polyurethanes for implants. II. *In vitro* degradation and calcification of materials from poly(epsilon-caprolactone)-poly(ethylene oxide) diols and various chain extenders. *J Biomed Mater Res.* 2002; 60: 592–606.
  21. **Lee CR, Grad S, Gorna K, et al.** Fibrin-polyurethane composites for articular cartilage tissue engineering: a preliminary analysis. *Tissue Eng.* 2005; 11: 1562–73.
  22. **Kupcsik L, Alini M, Stoddart MJ.** Epsilon-aminocaproic acid is a useful fibrin degradation inhibitor for cartilage tissue engineering. *Tissue Eng Part A.* 2008; DOI: 10.1089/ten.tea.2008.400.
  23. **Labarca C, Paigen K.** A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem.* 1980; 102: 344–52.
  24. **Farndale RW, Buttle DJ, Barrett AJ.** Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta.* 1986; 883: 173–7.
  25. **ABI PRISM 7700 Sequence Detection System User Bulletin.** 1997; 2: 11–5.
  26. **Lee CR, Grad S, Maclean JJ, et al.** Effect of mechanical loading on mRNA levels of common endogenous controls in articular chondrocytes and intervertebral disk. *Anal Biochem.* 2005; 341: 372–5.
  27. **Lisignoli G, Cristino S, Piacentini A, et al.** Cellular and molecular events during chondrogenesis of human mesenchymal stromal cells grown in a three-dimensional hyaluronan based scaffold. *Biomaterials.* 2005; 26: 5677–86.
  28. **Mauck RL, Yuan X, Tuan RS.** Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. *Osteoarthritis Cartilage.* 2006; 14: 179–89.
  29. **Li WJ, Tuli R, Okafor C, et al.** A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. *Biomaterials.* 2005; 26: 599–609.
  30. **Kisiday JD, Kopesky PW, Evans CH, et al.** Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures. *J Orthop Res.* 2008; 26: 322–31.
  31. **Huang CY, Reuben PM, D'Ippolito G, et al.** Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture. *Anat Rec A Discov Mol Cell Evol Biol.* 2004; 278: 428–36.
  32. **Mwale F, Stachura D, Roughley P, et al.** Limitations of using aggrecan and type X collagen as markers of chondrogenesis in mesenchymal stem cell differentiation. *J Orthop Res.* 2006; 24: 1791–8.
  33. **Peltari K, Winter A, Steck E, et al.** Premature induction of hypertrophy during *in vitro* chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. *Arthritis Rheum.* 2006; 54: 3254–66.
  34. **Klein-Nulend J, Roelofs J, Sterck JG, et al.** Mechanical loading stimulates the release of transforming growth factor-beta activity by cultured mouse calvariae and periosteal cells. *J Cell Physiol.* 1995; 163: 115–9.
  35. **Raab-Cullen DM, Thiede MA, Petersen DN, et al.** Mechanical loading stimulates rapid changes in periosteal gene expression. *Calcif Tissue Int.* 1994; 55: 473–8.
  36. **Robbins JR, Evanko SP, Vogel KG.** Mechanical loading and TGF-beta regulate proteoglycan synthesis in tendon. *Arch Biochem Biophys.* 1997; 342: 203–11.
  37. **Schumann D, Kujat R, Nerlich M, et al.** Mechanobiological conditioning of stem cells for cartilage tissue engineering. *Biomed Mater Eng.* 2006; 16: S37–52.