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SPECIES-SPECIFIC EXPRESSION OF TYPE II TGF- β RECEPTOR ISOFORMS BY ARTICULAR CHONDROCYTES: EFFECT OF PROTEOGLYCAN DEPLETION AND AGING



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Recently, a new isoform of the type II transforming growth factor beta receptor (TGF- β RII) was identified. This isoform (TGF- β RII₂) contains an insertion of 25 amino acids in the extracellular domain of the receptor. Using RT-PCR the authors demonstrated that both TGF- β RII₁ and TGF- β RII₂ are expressed by chondrocytes in murine and human articular cartilage. Bovine articular chondrocytes expressed TGF- β RII₁ mRNA but did not express detectable levels of TGF- β RII₂ mRNA, suggesting that the new isoform does not play an important role in normal bovine cartilage physiology. Because TGF- β responses seem to be age related and differential TGF- β responses have been described between normal cartilage and cartilage undergoing repair the authors studied if the relative mRNA expression between these isoforms is altered during cartilage repair and aging. No differences in the relative mRNA expression of the two isoforms of the type II TGF- β receptor could be demonstrated in murine cartilage during aging or during the repair phase after mild PG depletion indicating that it is unlikely that age-related TGF- β responses and differential TGF- β responses between normal cartilage and cartilage undergoing repair are the result of differences in the relative expression of the two TGF- β RII isoforms.

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Transforming growth factor beta (TGF- β) is a potent regulator of cell metabolism.^{1,2} A role of TGF- β in cartilage physiology is suggested by the presence of high concentrations of TGF- β in normal articular cartilage³ and its regulatory role in chondrocyte proliferation, differentiation and extracellular matrix production.⁴⁻¹¹ Since TGF- β expression in the joint is enhanced in patients with rheumatoid arthritis^{12,13} and active TGF- β is present in the synovial fluid of patients with rheumatoid arthritis or osteoarthritis¹⁴ it is assumed that TGF- β also plays a role in the regulation of chondrocyte metabolism during these joint diseases.

TGF- β responses are mediated by specific cell surface receptors. Articular chondrocytes express at least four distinct types of TGF- β receptors: type I, type II, type III and type V TGF- β receptors.^{1,15} The

type I and type II TGF- β receptors seem to be the most important TGF- β receptors since they are directly involved in signal transduction.¹⁶⁻²⁰

Recently a new isoform of the type II TGF- β receptor was isolated from a mouse brain cDNA bank,²¹ a human glioblastoma cDNA bank²² and from a human endothelial cell cDNA bank.²³ This new isoform (TGF- β RII₂) contains an insertion of 25 amino acids in the extracellular domain of the receptor. It is most likely that this isoform is generated by differential splicing of mRNA that is transcribed from the original type II TGF- β receptor gene. An important role of this isoform is suggested because the insertion of the human isoform and the murine isoform are localized at the same site and the amino acid sequence of the insertion is highly conserved between these species (76% identical).²³ It has been shown that TGF- β RII₂ mRNA is expressed in different murine tissues like stomach, intestine, kidney, lung and brain.²¹ In addition, TGF- β RII₂ mRNA is expressed in several human cell-types like glioblastoma cells, vein endothelial cells, embryonal lung fibroblasts, omental microvascular endothelial cells and lung carcinoma cells.²³ However, no data are available about the expression of TGF- β RII₂ mRNA in articular cartilage. Therefore, the expression of this isoform in normal articular cartilage of different species was studied.

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Received 28 August 1996; accepted for publication 28 October 1996

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1043-4666/97/050347 + 5 \$25.00/0/ck960175

KEY WORDS: cartilage/isoforms/receptor/TGF- β

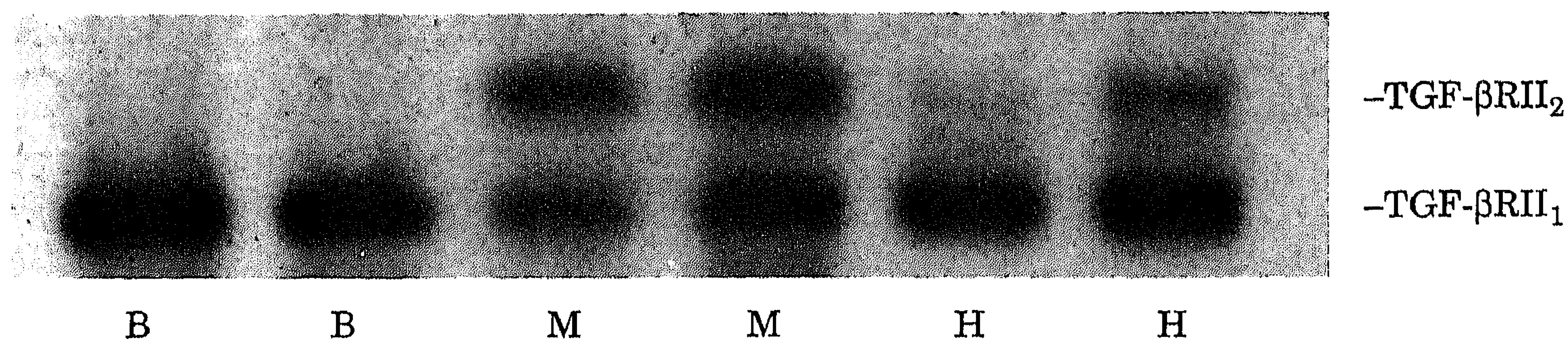


Figure 1. Expression of TGF- β RII₁ and TGF- β RII₂ mRNA in normal bovine (B), murine (M) and human (H) articular cartilage.

Total RNA was isolated from normal articular cartilage and RT-PCR was performed. PCR products are separated on a 1.6% agarose gel. Each lane shows the products of RT-PCR using RNA from different donors. RNA from human and bovine cartilage was isolated from individual donors while RNA of murine cartilage was isolated from pooled patellar cartilage of five mice.

Because TGF- β effects on articular cartilage seem to be age related²⁴⁻²⁷ and differential TGF- β responses have been described between normal cartilage and cartilage undergoing repair,²⁸⁻³⁰ the authors also studied if the relative expression of TGF- β RII₁ and TGF- β RII₂ is altered during aging or during matrix repair after PG depletion.

RESULTS

TGF- β RII₁ and TGF- β RII₂ mRNA expression in normal cartilage

RT-PCR on isolated RNA from human and murine articular cartilage using TGF- β RII specific primers resulted in two PCR products. The sizes of the PCR products corresponded to the expected size of amplified TGF- β RII₁ mRNA (525 bp) and TGF- β RII₂ mRNA (601 bp) demonstrating that both TGF- β RII₁ and TGF- β RII₂ mRNA are expressed in normal articular cartilage. However, there were striking differences in the relative expression of the two isoforms between species. Murine articular chondrocytes expressed almost equal amounts of TGF- β RII₁ and TGF- β RII₂ mRNA, while human articular chondrocytes expressed about three times more TGF- β RII₁ mRNA than TGF- β RII₂ mRNA.

When RNA from bovine articular chondrocytes was used only TGF- β RII₁ mRNA was detected demonstrating that the expression of the TGF- β RII₂ isoform is very low or absent (Fig. 1).

TGF- β RII₁ and TGF- β RII₂ mRNA expression in cartilage of different age

It has been reported that TGF- β has different effects on old cartilage compared to young cartilage.²⁴⁻²⁷ To investigate if these age-related TGF- β responses are the result of differences in the relative expression of the two isoforms of the TGF- β type II receptor the authors determined the relative mRNA expression of TGF- β RII₁ and TGF- β RII₂ in murine articular cartilage of different ages. RNA was isolated from patellar cartilage 3, 6, 12, 18 and 24 months of age, and RT-PCR was performed. As shown in Fig. 2, articular cartilage from mice of all ages express both TGF- β RII₁ and TGF- β RII₂ mRNA. No differences could be demonstrated in the relative expression of TGF- β RII₁ and TGF- β RII₂ mRNA between 3 months old (young adult) cartilage and cartilage up to 24 months of age (very old).

TGF- β RII₁ and TGF- β RII₂ mRNA expression in cartilage during a repair phase

Because differential TGF- β responses have also been described between normal cartilage and cartilage

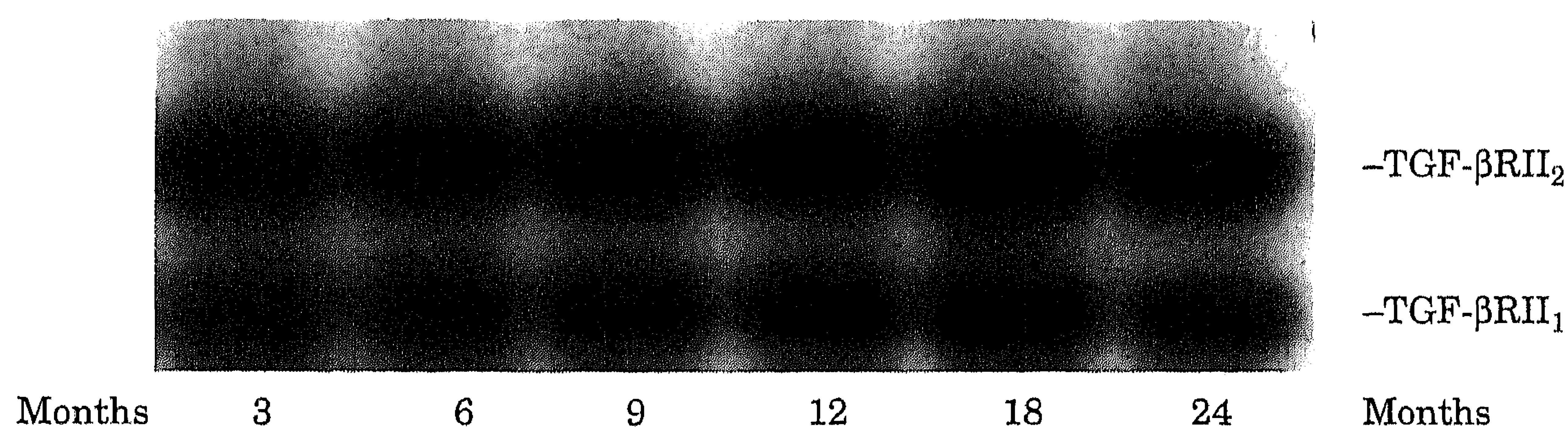


Figure 2. Expression of TGF- β RII₁ and TGF- β RII₂ mRNA in murine articular cartilage between 3 and 24 months of age.

RT-PCR was performed using RNA isolated from patellar articular cartilage of different age. RNA was isolated from pooled patellar cartilage of five mice. Shown is a representative experiment of three.

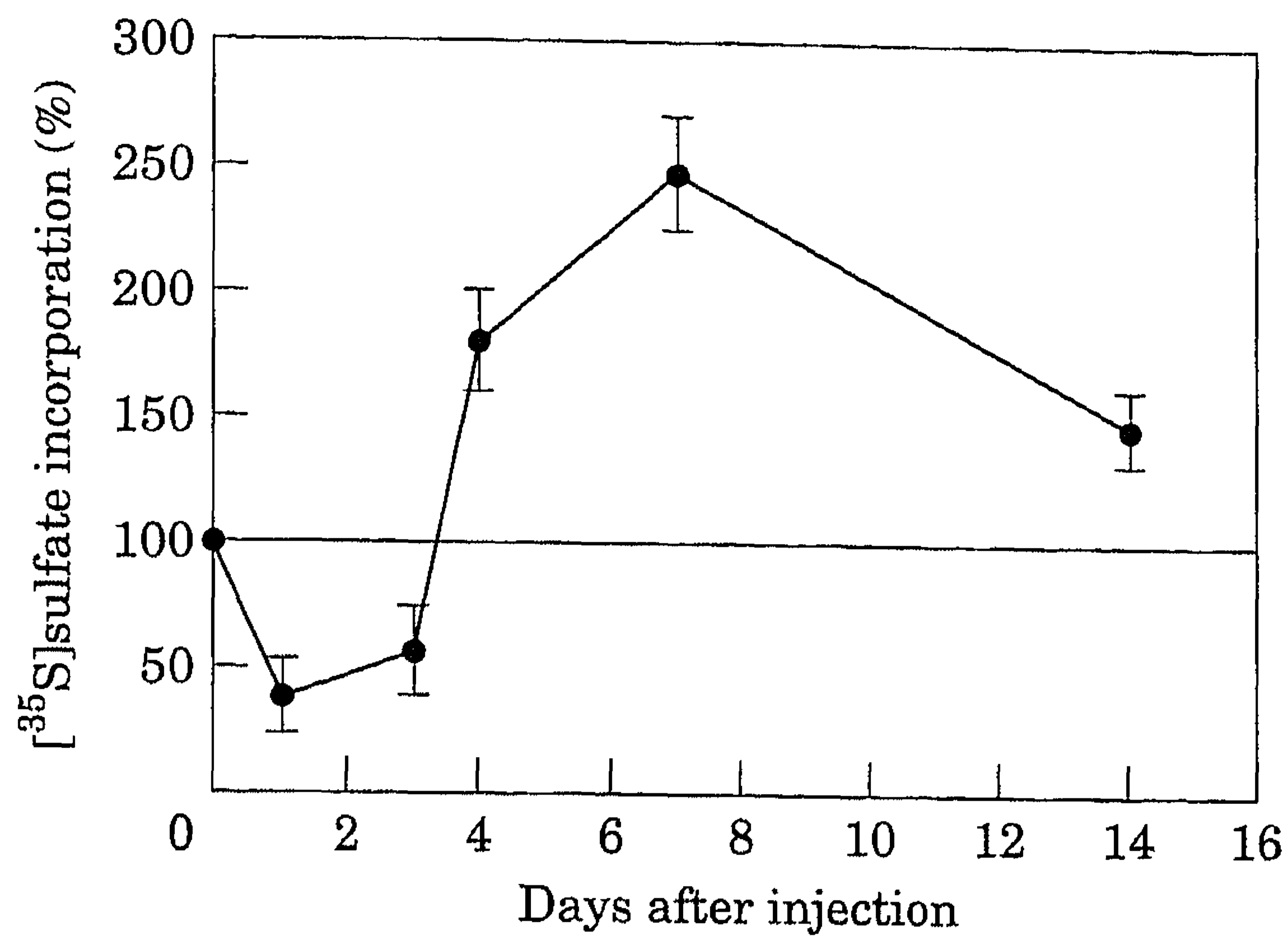


Figure 3. Effect of intra-articular injection of 0.5% papain on the PG synthesis of patellar cartilage.

Glycosaminoglycan synthesis was measured *ex vivo* by [³⁵S]sulfate incorporation using five patellae at each point of time. The results are expressed as percentage of [³⁵S]sulfate incorporation of patellar cartilage from joints which were intra-articular injected with saline. Shown is a representative experiment of three.

undergoing repair after cartilage injury^{11,28,29} whether the relative expression of TGF- β RII₁ and TGF- β RII₂ mRNA is altered during the repair phase after mild PG depletion was investigated. A murine cartilage repair model was used in which 0.5% papain was injected in the murine knee joint. Intra-articular injection of papain results in PG depletion³¹ and an inhibition of the PG synthesis up to 50% at one day after injection (Fig. 3). The PG synthesis is normalized between three and four days after injection whereafter the PG synthesis is supranormal up to 14 days with a maximal stimulation at 7 days after injection. The relative TGF- β RII₁ and TGF- β RII₂ mRNA expression in cartilage was determined by RT-PCR at different points of time after injection of papain. No differences in the relative mRNA expression of the two isoforms could be demonstrated between normal articular cartilage and cartilage after papain treatment on any point of time (Fig. 4).

DISCUSSION

Recently a new isoform of the type II TGF- β receptor was identified.²¹⁻²³ This new isoform (TGF- β RII₂) contains an insertion of 25 amino acids in the extracellular domain of the receptor. An important role of this isoform is suggested by the high conservation of this insertion between species. Since the original type II receptor (TGF- β RII₁) has a low affinity for TGF- β 2^{32,33} it was investigated whether the new isoform was a TGF- β 2-specific receptor. However, binding studies using transfected cells showed that TGF- β RII₂ also had a much higher affinity for TGF- β 1 than for TGF- β 2.^{21,23} These results indicate that the new isoform is not a TGF- β 2-specific receptor. Transfection studies using a TGF- β resistant cell line which lacks endogenous TGF- β RII demonstrated that TGF- β RII₁ and TGF- β RII₂ were indistinguishable in their biological functions.²³ However, these studies are non-physiological and limited to only a few specific TGF- β responses.

Using RT-PCR it has been demonstrated for the first time that both TGF- β RII₁ and TGF- β RII₂ mRNA are expressed in normal human and murine articular cartilage. The authors were not able to detect TGF- β RII₂ mRNA in bovine articular chondrocytes. Although murine primers were used in these studies we can exclude the possibility that the inability to detect TGF- β RII₂ mRNA in bovine articular chondrocytes is an artefact due to differences in the primer-annealing sequences. Both TGF- β RII₁ and TGF- β RII₂ mRNA were amplified in the same tube, using the same primers and under the same conditions so the isoforms are internal controls for each other. Since TGF- β RII₁ mRNA could be detected in bovine chondrocytes it can be concluded that bovine articular chondrocytes do not express TGF- β RII₂ mRNA or express this isoform at a very low level. This implicates that it is unlikely that TGF- β RII₂ plays an essential role in the metabolism of normal bovine articular cartilage.

TGF- β responses on articular chondrocytes are shown to be age related.²⁴⁻²⁷ For example, articular

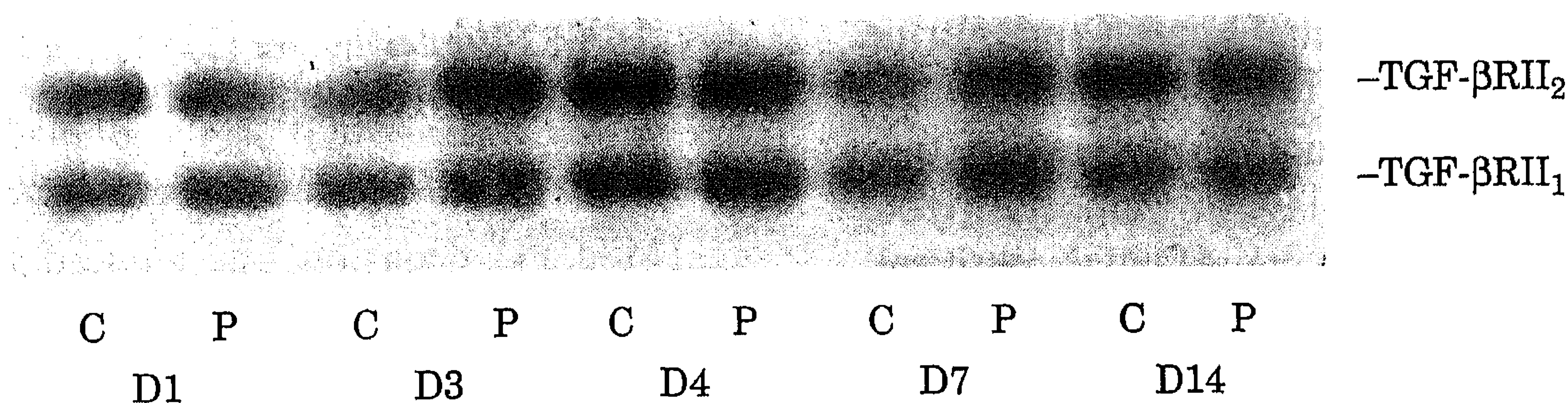


Figure 4. Expression of TGF- β RII₁ and TGF- β RII₂ mRNA in patellar cartilage after PG depletion.

Murine patellae were dissected at several points of time after intra-articular injection of 0.5% papain (P) or saline (C). RNA was isolated from pooled patellar cartilage of five mice. RT-PCR was performed and the PCR products are separated on a 1.6% agarose gel. Shown is a representative experiment of three.

cartilage from old adult pigs is more sensitive to TGF- β induced elaboration of extracellular inorganic pyrophosphate (ePPI) than cartilage from juvenile and young adult pigs.²⁶ Young murine articular cartilage appears to be more sensitive to TGF- β -induced stimulation of PG synthesis than old murine articular cartilage.²⁵ Because various TGF- β responses are age related, the authors investigated if one of the isoforms is selectively upregulated during aging. However, it was demonstrated that the ratio between TGF- β RII₁ and TGF- β RII₂ mRNA does not differ in murine articular cartilage between 3 months and 24 months of age. These results suggest that the described differential TGF- β responses between old and young articular cartilage are not mediated by differences in the relative expression of the two isoforms of TGF- β RII.

It has also been demonstrated that TGF- β has different effects on normal articular cartilage and cartilage during a repair phase. For example, TGF- β stimulated PG synthesis of human osteoarthritic cartilage while under the same conditions TGF- β did not have a significant effect on the PG synthesis of normal human cartilage.²⁹ In addition, TGF- β enhanced the PG synthesis of interleukin 1 (IL-1)-treated cartilage but the effects of TGF- β on normal cartilage PG synthesis were minimal.^{28,30} The relative expression of TGF- β RII₁ and TGF- β RII₂ mRNA in the repair phase after mild PG depletion was investigated. Mild PG depletion in murine articular cartilage was induced by injection of 0.5% papain in the murine knee joint. No differences in the relative expression of TGF- β RII₁ and TGF- β RII₂ mRNA could be demonstrated between normal cartilage and cartilage undergoing repair after mild PG depletion. This indicates that the supranormal PG synthesis after PG depletion by papain is not mediated by selective upregulation of mRNA of one of the two isoforms. This also suggests that the described differential TGF- β responses between normal cartilage and cartilage undergoing repair^{28,30} are not the result of differences in the relative expression of TGF- β RII₁ and TGF- β RII₂ mRNA.

In summary, this study demonstrates that the newly identified isoform of the type II TGF- β receptor (TGF- β RII₂) is expressed in normal human and murine articular cartilage. TGF- β RII₂ mRNA was not detectable in bovine articular cartilage indicating that it is unlikely that TGF- β RII₂ plays an important role in the physiology of normal bovine articular cartilage. No differences in the relative mRNA expression of the two isoforms of the type II TGF- β receptor could be demonstrated in murine cartilage during aging or during the repair phase after mild PG depletion indicating that it is unlikely that age related TGF- β responses and differential TGF- β responses between normal cartilage and cartilage undergoing repair are

the result of differences in the relative expression of the two TGF- β RII isoforms.

MATERIALS AND METHODS

Animals

Male C57Bl/10 mice were used to study age-related differences. Male C57Bl/6 mice were used in experiments in which cartilage depletion was induced. Mice were kept in cages with a wood chip bedding in a room kept at constant temperature. They were fed a standard diet and tap water *ad libitum*.

Isolation of cartilage

Murine articular cartilage was isolated from patellae. Patellae were decalcified in 3.5% EDTA for 4 h at 4°C, when the whole cartilage layer was stripped off. Because old cartilage is more calcified, decalcification of patellae of old mice (>3 month) was performed overnight at 4°C. In control experiments it was demonstrated that decalcification by EDTA does not affect efficiency of RNA isolation or RT-PCR (data not shown). Bovine cartilage was isolated from metacarpophalangeal joints within 8 h after death of the animals. Human cartilage was isolated from femoral knee condyles within 18 h after death of the donor. Only cartilage samples which were histologically defined as normal were used. Isolated human and bovine articular cartilage was immediately frozen in liquid nitrogen.

RNA isolation and RT-PCR

Total RNA was isolated using TRIzol Reagent (Life Technologies). Human and bovine articular cartilage was ground to powder in a freeze mill and defrosted in TRIzol. Murine articular cartilage was directly after isolation extracted with TRIzol. Before reverse transcription total RNA was treated with DNase (Life Technologies). Reverse transcription was performed with M-MLV Reverse Transcriptase (Life Technologies) using the 3' PCR primer. Taq DNA Polymerase (Life Technologies) was used in the PCR reaction. cDNA was cycled 35 times at 92°C for 1 min, 55°C for 1 min and 72°C for 1 min. The amplified products were separated on an 1.6% agarose gel and visualized by chemiluminescence using the Digoxigenin (DIG) System (Boehringer Mannheim). The PCR primers used for amplification of TGF- β RII₁ and TGF- β RII₂ mRNA are described by Suzuki *et al.*²¹ Since these primers are located on both sides of the insertion, the PCR products of TGF- β RII₁ or TGF- β RII₂ differ in size (525 bp and 601 bp, respectively) and show different mobility on an 1.6% agarose gel.

Induction of mild proteoglycan depletion

Mild PG depletion was induced in 10-week-old male C57Bl/6 mice by intra-articular injection of papain as described by van der Kraan *et al.*³¹ In short, the right knee-joints of the mice were injected once with 6 μ l 0.5% papain (Type IV, 15 U/mg, Sigma in 0.03 M L-cysteine.HCl, Sigma). The left control knees were injected with saline. At several points of time after the injection patellae were isolated and used for determination of patellar PG synthesis and RNA extraction for RT-PCR.

Determination of patellar cartilage proteoglycan synthesis

Proteoglycan synthesis was measured *ex vivo*. Whole patellae, with a standard amount of surrounding tissue, were dissected from the knee joints. Patellae were pulse-labelled (2 h, 37°C) with [³⁵S]sulfate (1.1 MBq/ml). After labelling the patellae were washed, fixed, decalcified, punched out of the surrounding tissue, dissolved and counted by liquid scintillation counting as described previously.³⁴

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