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Inducible nonviral gene expression in the treatment of osteochondral defects¹

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Introduction

Articular cartilage defects belong to the very challenging therapeutic problems^{1,2}. A variety of therapeutic strategies have the potential to stimulate the formation of new articular surface, including stimulation of fibrocartilaginous tissue repair^{3–5}, osteochondral graft transplantation^{6,7} or autologous chondrocyte transplantation⁸. Of importance is that no synthetic material comes close to the articular cartilage

level of performance^{9,10}. Although decreased symptoms are reported in some patients treated with these techniques, the long-term clinical value is still uncertain and the need for new treatment methods obvious^{11,12}. Tissue engineering has become a major field in biotechnology during the last years¹¹. Techniques were developed to isolate and cultivate cells of a patient to induce cartilage-like tissue. Type I collagen sponges and other matrices and gels have previously been shown to be suitable carriers for three-dimensional culture of chondrocytes^{13,14}.

The use of growth factors in combination with tissue engineering seems to be reasonable for the therapy of osteochondral lesions^{11,15}. Some specific cytokines stimulate the cell proliferation and matrix synthesis and can be used to support healing^{16–20}.

Residual problems are the transient effect and the difficulty of growth factor administration²¹. A short half-life hampers the direct injection of recombinant cytokines into the affected joints²². Among different strategies to overcome this problem gene transfer techniques were

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developed and used^{21,23–25}. Nonviral gene transfer into primary chondrocytes of various species has been demonstrated by others^{26–29}. Using constitutive expression systems, they achieved high transfection rates *in vitro* and were able to demonstrate persistent transgene expression *in vivo*. These authors imply that constitutive transgene expression for therapeutic goals ultimately will not be useful in view of the fact that regulated transgene expression has several definite advantages. Cells genetically modified *ex vivo* with growth factors prior to tissue engineering lead to an enhanced matrix synthesis^{15,30–33}.

However, a gene product involved in chondrocyte-defect repair is only required during the repair phase. Constitutive, unregulated expression is not required in most cases and may lead to an abnormal regulation of cell growth and side-effects due to chronic over-expression of the cytokine thereafter³⁴.

For these reasons, a control of timing is preferable over constitutive gene expression. Among the various inducible gene expression systems that have been described³⁵ the tetracycline-inducible system^{36,37} has the advantage that it is dependent on a drug (tetracycline or doxycycline) that is both highly specific and non-toxic.

Regulation of gene expression by tetracycline is rapid, reversible and can be modulated³⁸. Tet-controlled expression has been demonstrated in different cells³⁹, in transgenic mice^{40,41}, in the photoreceptor system⁴², in the heart⁴³, the brain^{44,45} and skeletal muscle^{46,47} but it has not been previously investigated in chondrocytes or in osteochondral defects. The recently described doxycycline-dependent reverse transactivator rTA2^s-M2⁴⁸ was already successfully used to achieve tightly regulated gene expression *in vitro* and *in vivo*^{47,49}.

The objective of this study was to establish a method for tetracycline regulated gene expression in chondrocytes transplanted into osteochondral defects of living animals. Using chondrocytes transfected with a tetracycline-inducible expression plasmid, we demonstrate that transgene expression in these cells can be induced *in vivo* after implantation.

Materials and methods

ANIMALS

The study has been approved by the appropriate institutional review board. Animals were kept according to FELASA-guidelines. Female CD1 nu/nu mice (6–8 weeks old) and 6 weeks old New Zealand white rabbits were obtained from Charles River (Sulzfeld, Germany) and kept under conventional housing conditions. Quarantine lasted 7 days. The animals were housed with appropriate bedding, provided with free access to drinking water and food. Mice were maintained in IVC-cages, rabbits in V2A-cages under conditions of controlled temperature and light.

CHEMICALS

Reagents were obtained from Biochrom (DMEM, Pen/Strep, Glutamin, FBS, PBS, Trypsin), Roche (Collagenase A, FuGENE 6[®]), Sigma–Aldrich (X-Gal, Doxycycline), Invitrogen (LipofectAmine[®]), and Resorba Clinicare[®] (Collagen Sponge).

CELL CULTURE/TISSUE ENGINEERING

NIH-3T3-cells were obtained from ATCC (CCL-1658), 293T cells⁵⁰ were obtained from R. Willemssen (Daniel den Hoed Cancer Center, Rotterdam, The Netherlands).

Articular cartilage was obtained from knee joints of New Zealand white rabbits under general anesthesia. At the time of biopsy, 6 weeks old New Zealand white rabbits (average weight 1.8 kg) were used. Sterile harvested cartilage was washed twice in PBS, cut in 1 mm³ pieces, minced and digested on a shaker with 0.25% trypsin (30 min) and 50 mg/20 ml (0.21 U/mg) Collagenase A (4–6 h). The resulting solutions were centrifuged at 900 rpm for 4 min. Cell pellets were resuspended in medium. The isolated chondrocytes were seeded in 25 cm² flasks, grown to a density of 80%, then seeded in 75 cm² flasks and splitted at a ratio of 1:3 every 5th day.

Cell viability and numbers were determined by trypan blue staining. Ten to 14 days following biopsy, the adherent cells were released from the dishes by a 3 min-exposure to 0.25% trypsin. Chondrocytes (6×10^5) were seeded on a 4 mm³ piece of a commercially available type I collagen sponge (Resorba[®], Nürnberg, Germany) by injection with a needle (27G). After seeding, the medium was changed every 2nd day.

Cells were grown in Dulbecco's modified Eagles medium containing 10% fetal bovine serum, 1% Pen/Strep and 1% glutamine and maintained at 37°C, 5% CO₂. After 14 days in culture, the collagen sponges were dissolved with Collagenase A, cells isolated and cell viability and number were determined by trypan blue staining.

PLASMIDS

All tet-regulatory elements were cloned into one construct to circumvent the need for double transfection of each cell. The plasmid used for tetracycline-controlled gene expression is based on a modified version of the retroviral construct pN2A⁵¹, comprising a neomycin resistance gene under the control of the 5'LTR promoter and a multiple cloning site (MCS) located in the U3 Region of the 3'LTR. pN2A was modified by insertion of a poly(A) signal in antisense orientation in the MCS, yielding the vector pN2AP.

In the first step pTRE2 (Clontech, Palo Alto, CA, USA) was digested with *Xho*I and *Eco*RV and treated with Klenow enzyme. The 537 bp fragment containing the Tet-responsible element fused to the minimal CMV promoter followed by an MCS was isolated and cloned in antisense orientation into the *Sna*BI site of the linearized vector pN2AP. The resulting plasmid was designated pN2ATRE.

In the second step rTA2^s-M2 under the control of the hCMV promoter was obtained by *Xho*I and *Bam*HI digestion of the plasmid pUHrt 61-1⁴⁸ (kindly provided by H. Bujard, Heidelberg). After treatment with Klenow enzyme the 1.5 kb fragment was cloned into the *Sfu*I digested pN2ATRE to obtain pN2ATetOn.

The *lacZ* gene was cloned into the Klenow enzyme treated *Not*I site of pN2ATetOn, yielding pN2ATetOnLacZ.

TRANSFECTION OF CELLS AND INDUCTION OF GENE EXPRESSION

NIH-3T3 cells were stably transfected using Lipofect-Amine[®] and selection with G 418 (1 mg/ml). Cell clones were picked and screened for tetracycline-dependent transgene expression.

Chondrocytes were cultured for 24 h before transfection. Then 2×10^5 cells were trypsinized and transferred to a 60 mm plate. To determine best transfection conditions, lipid/DNA ratios and the DNA dose were varied according to the suggestions of the manufacturer (2–6 μ g DNA per well, FuGENE:DNA = 2 or 3 v/w). We next investigated the best time course of LacZ-induction. Doxycycline at a dosage of 1 μ g/ml was added and cells were incubated for 12, 24 and 36 h before fixation and staining. The efficiency of gene transfer was measured by histochemical examination of β -galactosidase activity in the cells using the chromogenic substrate X-gal and also by flow-cytometry (FACS) with the fluorescein-containing LacZ-substrate fluorescein- β -D-galactopyranosid (FDG)⁵² with an adaptation according to Krüger *et al.*⁵³.

INJECTION OF NIH-3T3-CELLS AND *IN VIVO* INDUCTION OF β -GALACTOSIDASE EXPRESSION

Cell suspensions were injected subcutaneously in 8 nude mice in both flanks ($1.5\text{--}9 \times 10^6$ cells in 200 μ l PBS). Four weeks later, after the tumors were grown, lacZ-gene-expression was induced in 6 mice by addition of doxycycline to the animals' drinking water (1 mg/ml). Drinking water was changed daily as doxycycline is light sensitive and labile in solution. Two mice received water without the antibiotic as control.

PREPARATION OF TUMOR EXTRACTS AND HISTOLOGICAL EVALUATION

Mice were sacrificed after sedation by an overdose of pentobarbital 24, 48 resp. 72 h after induction and the tumors excised. One half of each tumor was cut in 3 mm³ pieces, fixated immediately in 0.5% glutaraldehyde for 1 h at room temperature and stained with X-gal staining solution. For histologic staining of LacZ, the other half of the tumors were cut into slices with a thickness of 7 μ m in a cryotome. The slices were fixed in glutaraldehyde and incubated for 6 h in X-gal staining solution. The tissue slices were counterstained by hematoxylin–eosin.

GENERATION OF OSTEOCHONDRAL LESIONS AND IMPLANTATION OF CHONDROCYTE-SEEDED COLLAGEN SPONGES

By the time of implantation of the cell-seeded sponges, animals had reached an average weight of 3 kg. The rabbits were anesthetized by i.m. injection of 0.25 mg/kg medetomidine and 17 mg/kg S-ketamine. After intubation 30 mg/kg metamizole i.v. and 4 mg/kg carprofen s.c. were administered. Using sterile technique a medial parapatellar arthrotomy was performed and the patella was laterally displaced. In the first surgery a small biopsy (1 $\times$ 1 mm) was taken from the trochlear groove. In the second surgery a steel drill (3.6 mm in diameter) with a stop-device was used to create a full-thickness defect (3 mm deep) in the articular cartilage of the trochlear groove of the contralateral knee. The defects were cleaned and rinsed with sterile saline. The chondrocyte-collagen implants were press-fitted into the drill-holes. In one animal of the doxycycline induced group two defects were generated to have an internal control: non-transfected cells were implanted into a proximal and pN2TetOnLacZ-transfected cells into a distal defect, as shown in Fig. 4B. The sponges were held in place within the defects by repositioning of the patella within the trochlear groove. After extubation, all rabbits were returned to their

cages and were allowed free cage activity. Postoperative analgesia consisted of 0.03 mg/kg buprenorphin on the surgery day and 4 mg/kg carprofen for 3 days.

In 10 rabbits gene expression was induced 14 days after surgery for 7 consecutive days by daily subcutaneous injections of 1 mg/kg doxycycline per day. This antibiotic is well distributed into the synovial fluid, the amount measured in the joint approaches that in plasma⁵⁴. The control group of 4 rabbits did not receive doxycycline.

PREPARATION OF FEMORAL CONDYLES AND HISTOLOGICAL EVALUATION

Rabbits were sacrificed after anesthesia with propofol by fast injection of 5 ml pentobarbital 3 weeks after implantation. The knee joints were harvested, assessed macroscopically and photographed after fixation (2% formaldehyde, 0.2% glutaraldehyde in PBS) and color-development after X-gal staining. The condyles were decalcified in EDTA (10%) for 3 weeks and embedded in paraffin. Serial sections were cut transversely, from the proximal aspect to the distal aspect of the trochlear groove. Sections (6 μ m) from the center of each defect were counterstained with hematoxylin–eosin, eosin and nuclear fast red for histological evaluation.

STATISTICAL ANALYSIS

Data are expressed as mean $\pm$ standard deviation (SD) of separate experiments. Each test condition was performed in quadruplicate for the determination of transfection conditions and time-course experiments. Student's *t*-test was used to determine the significance of differences between groups. *P* values less than 0.05 were considered significant.

Results

DOXYCYCLINE-INDUCIBILITY OF LACZ EXPRESSION OF PN2TETONLACZ IN CELL LINES *IN VIVO*

To verify that LacZ expression of our plasmid construct was doxycycline-inducible, we injected a tumorigenic subline of NIH-3T3-fibroblasts, stably transfected with N2TetOnLacZ, into 8 nu/nu mice subcutaneously in both flanks. Four weeks after injection 14 tumors grew to an average size of 341 mm³. Forty-eight hours after induction of gene expression with doxycycline, mice were sacrificed and tumors analysed histologically after X-gal staining to evaluate LacZ-activity. Sections of the tumors showed a slight blue background-staining in the non-induced group, possibly due to basal activity of the TRE promoter in the absence of doxycycline [Fig. 1(A)] and an intensive LacZ-staining in the induced group [Fig. 1(B)]. Tumors were not fully stained, because the staining solution did not penetrate the specimen within 6 h. Histologic evaluation demonstrated the slight stained cells in the control group and the intensive blue cells in the induced group (data not shown).

TRANSFECTION OF CHONDROCYTES AND INDUCTION OF GENE EXPRESSION

To test, whether these results could be reproduced with articular chondrocytes, we transfected these cells with N2TetOnLacZ. The efficiency of gene transfer was measured by histochemical examination of β -galactosidase

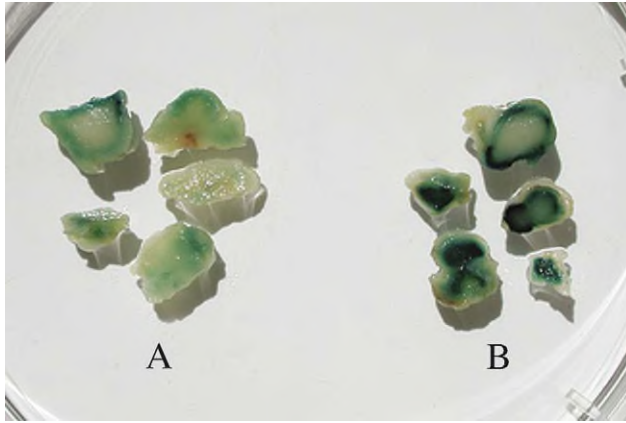


Fig. 1. Sections of NIH-3T3 cell-induced tumors in nude mice. A stably N2TetOnLacZ-transfected NIH-3T3-clone was used. Tumors were excised, cut and stained with X-gal for 6 h. (A) Non-induced controls demonstrate a background staining; (B) administration of doxycycline in drinking water induced LacZ-expression *in vivo*. Tumors were not fully stained, because the staining solution did not penetrate the specimen within 6 h.

activity in the cells using the chromogenic substrate X-gal and also by flow-cytometry (FACS). Transfection efficiency varied with the total DNA dose, but not with the lipid/DNA ratio. Maximal transfection efficiency ($13.5 \pm 1.32\%$) was seen with $12 \mu\text{l}$ FuGENE and $4 \mu\text{g}$ DNA (ratio 3:1) in a 60 mm plate in induced cells (Fig. 2), although there was no significant difference compared to lipid/DNA ratio of 2:1 ($13.2 \pm 0.74\%$). Non-induced controls demonstrated LacZ-staining in $0.55 \pm 0.17\%$ (lipid/DNA ratio 2:1) and $0.6 \pm 0.2\%$ (lipid/DNA ratio 3:1) of cells (Fig. 2). LacZ staining was much more intensive in the induced cells compared to the non-induced controls [Fig. 3(A and B)]. Due to leakiness of transgene expression caused by the basal activity of the TRE promoter in the absence of doxycycline, cells expressed background LacZ activity. No further increase in efficiency was obtained with lower or higher DNA concentrations. Maximal induction was generally achieved after 24 h treatment with doxycycline.

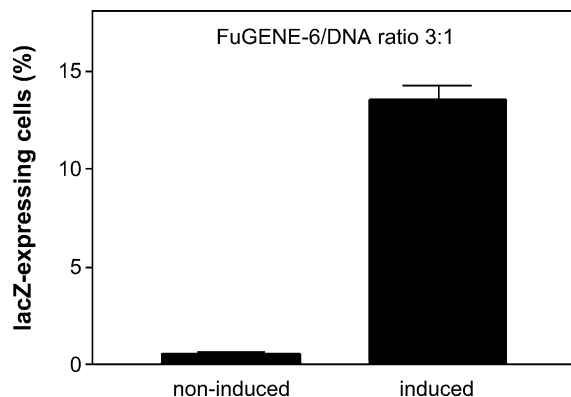


Fig. 2. LacZ-expression in N2TetOnLacZ-transfected rabbit articular chondrocytes. Chondrocytes were transfected with FuGENE 6/ DNA ratios of 2:1 and 3:1. Forty-eight hours after transfection cells were induced by doxycycline ($1 \mu\text{g/ml}$) and incubated for additional 24 h. Efficiency of gene transfer was measured by flow-cytometry (FACS). Data are expressed as mean transfection efficiency ($n = 3$) $\pm$ standard deviation.

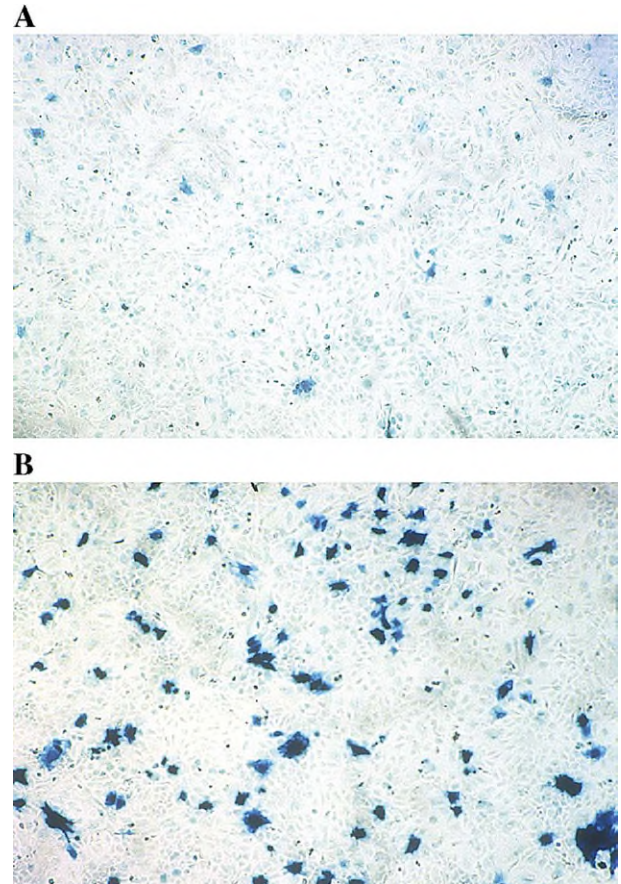


Fig. 3. Light microscopy of N2TetOnLacZ-transfected chondrocytes in cell culture. (A) Transfected chondrocytes without doxycycline-induction. Cells express a slight blue LacZ-activity likely due to leakiness referring to basal activity of the TRE promoter in the absence of doxycycline; (B) transfected chondrocytes with doxycycline-induction: LacZ-expression was detected 24 h after induction with $1 \mu\text{g/ml}$ doxycycline in 9.6% of the cells. Original magnification $\times 100$.

CHONDROCYTE-SEEDED COLLAGEN SPONGES *IN VITRO*

We next determined the growth and viability of transfected cells seeded onto collagen sponges. Seeding the chondrocytes on collagen sponges resulted in growth of cells only on the surface of the scaffold. Only after injection with a fine needle into the center of the sponges, cells were distributed throughout the entire matrix (data not shown). After 14 days in culture the mean number of cells recovered from the collagen sponges after treatment with Collagenase A was 5.2×10^5 (± 0.6) or 86% of the number of cells originally injected into the sponge prior to incubation. Cell viability amounted to 94% ($\pm 2.3\%$) of the cells recovered from the sponge. In tissue culture *in vitro*, living cells could be maintained in collagen sponges up to 6 weeks.

Because cells were harvested from superficial layers of hyaline cartilage in the trochlear groove of the patella, we considered them to be chondrocytes.

REGULATION OF LACZ-EXPRESSION IN OSTEOCHONDRAL DEFECTS *IN VIVO*

Three weeks after implantation of the chondrocyte-collagen sponges, animals were sacrificed and femoral

condyles were harvested. In macroscopic evaluation, the regenerative tissue appeared smooth and resembled the surrounding articular cartilage [Fig. 4(A)]. Some areas showed slight indentation. Small fissures could be determined in the central parts of most of the transplants. No chondral reactions occurred on the opposite patella. In treated joints a mild synovitis could be seen.

To exclude unspecific X-gal staining, one animal with two defects in the same joint was evaluated: in the proximal defect, we implanted non-transfected cells on the collagen sponge, in the distal defect N2TetOnLacZ-transfected cell-bearing sponges were used. After treatment with X-gal, only the distal defect exhibited macroscopically a blue staining, whereas the proximal defect remained unstained [Fig. 4(B)]. Notably, both the induced and the uninduced cells were stained to a lesser degree. A three-dimensional view of

a transverse cut section through the condyle revealed the depth of the stained cells in the implant [Fig. 4(C)].

Histologically, the implants appeared well integrated into the cartilage and bone [Fig. 5(A)]. Chondrocytes showed a columnar appearance and proliferation in lacunae even in the center of the osteochondral defect [Fig. 5(B)]. Collagen fibers of the cell-bearing sponge as well as newly synthesised collagen could be demonstrated. Non-induced controls showed several lightly stained cells across the entire section of the condyle but did not show LacZ-activity over the background (data not shown). In the doxycycline-treated group, transfected chondrocytes in the collagen matrices were strongly stained, demonstrating an efficient induction of LacZ-expression [Fig. 6(A–C)]. Counterstaining with eosin and nuclear fast red was used to highlight β -galactosidase positive blue cells (data not shown).

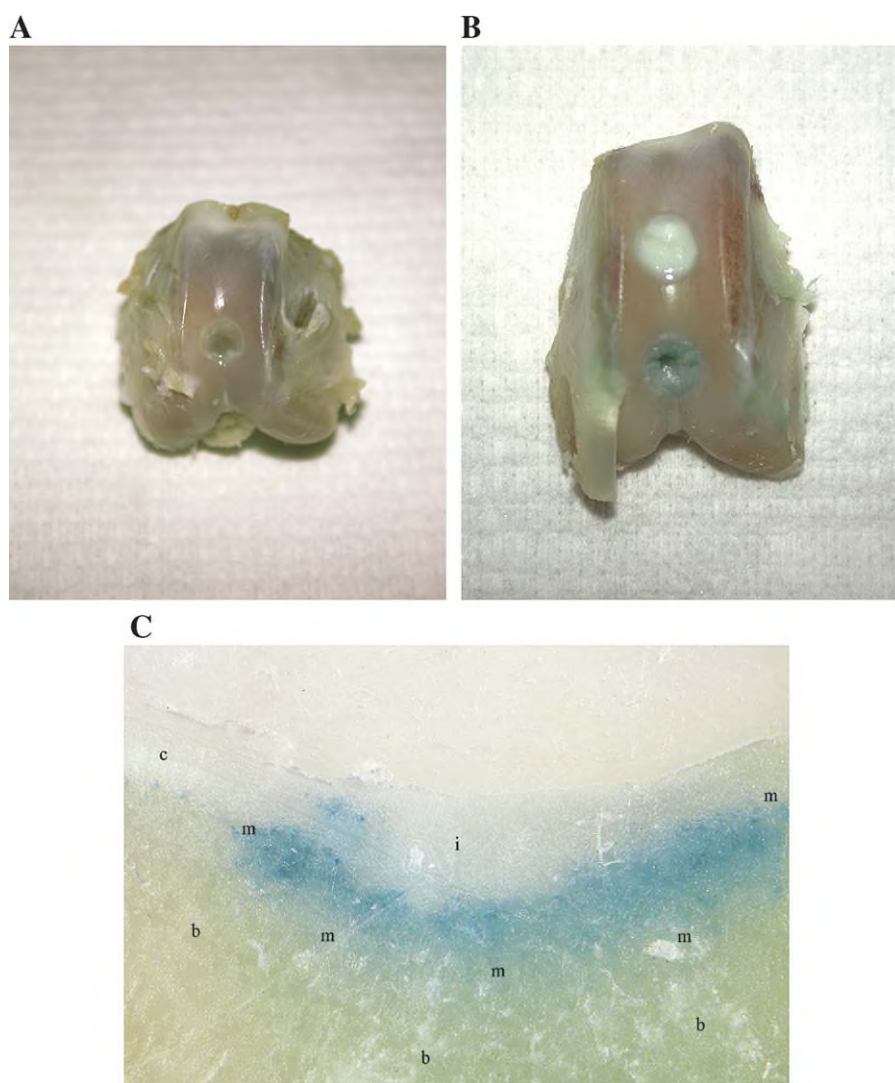


Fig. 4. Femoral condyles 3 weeks after implantation of chondrocyte-seeded collagen sponges. (A) Non-induced control specimens did not demonstrate X-gal staining macroscopically; (B) as an internal control and to detect possible unspecific X-gal staining two defects were generated in one animal: non-transfected cells were implanted into the proximal and N2TetOnLacZ-transfected cells into the distal defect. X-gal staining was detected in the defect with the transfected cells after treatment with doxycycline only; (C) transversely cut section through a femoral condyle of the induced group demonstrating the depth and the cumulative effect of X-gal stained cells in the defect; b = bone, c = cartilage, i = implant, m = margins of the defect.

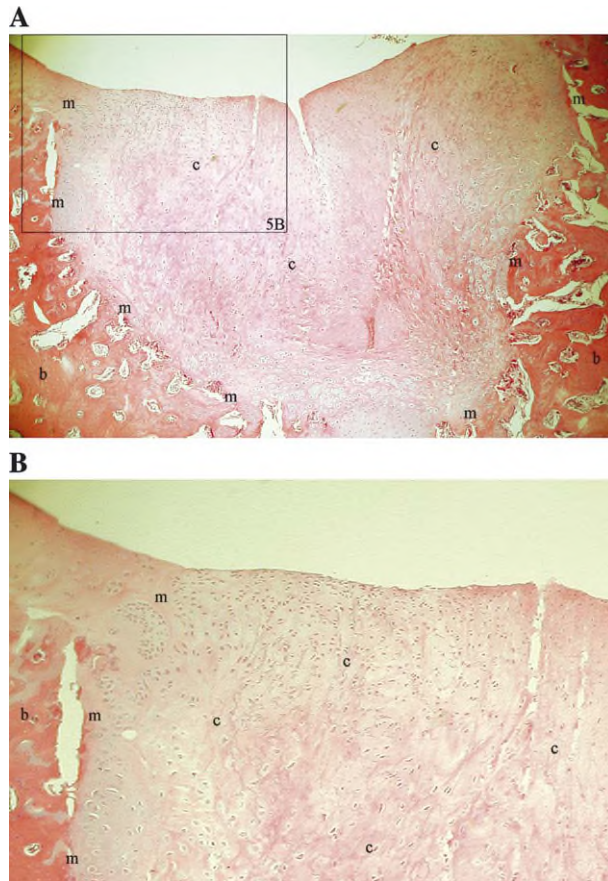


Fig. 5. Histologic analysis of an osteochondral defect 3 weeks after implantation, demonstrating repair of the lesion by the collagen-cell-sponge; b = bone, c = chondrocytes and collagen fibers, m = margins of the defect. Section from a control animal that did not receive doxycycline, the uninduced transgene cells did not reveal LacZ-staining. X-gal staining and hematoxylin/eosin, original magnification A: $\times 40$, B: $\times 100$, detail in higher magnification.

Interestingly, some of the implants of the doxycycline-induced group showed infiltrating lymphocytes on the margins of the defect, possibly an immunologic response to the β -galactosidase-protein. Immunohistochemistry with CD-3 and CD-20 was done to differentiate between T- and B-lymphocytes. Both stainings were positive, demonstrating an immunologic reaction was taking place. These lymphocyte infiltrations were not seen in the non-induced group (data not shown).

A difference between both groups regarding regeneration of hyaline cartilage was not expected and could obviously not be determined.

Discussion

The goal of our study was to develop a system of controllable transgene expression in articular chondrocytes to be used in the therapy of osteochondral defects. To achieve our aim, we chose the tetracycline-inducible gene expression system.

After *in vitro* characterization of transfected chondrocytes, inducible gene regulation was assessed *in vivo*. To transplant the manipulated cells, we seeded them onto

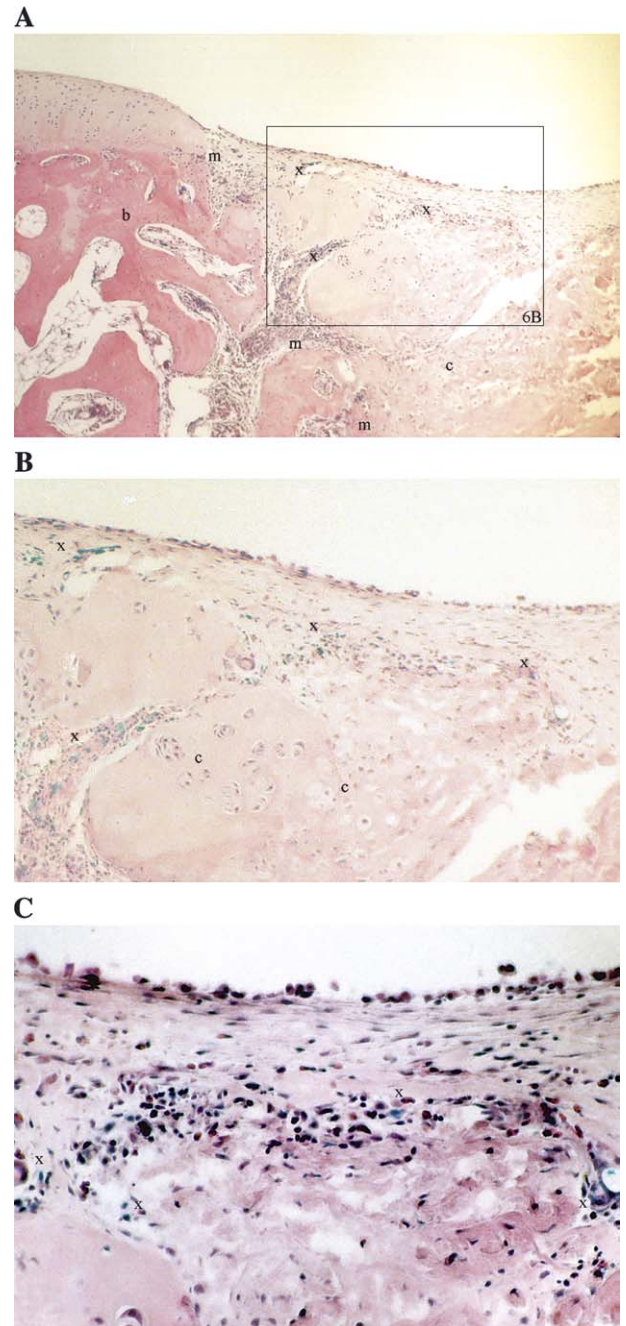


Fig. 6. Inducible LacZ-expression in chondrocytes after doxycycline treatment; b = bone, x = induced LacZ-expressing cells, m = margins of the defect. Section from an animal given doxycycline 1 mg/kg subcutaneously for 1 week. Induced transgene cells demonstrate intensive LacZ-staining. X-gal staining and hematoxylin/eosin, original magnification A: $\times 40$, B: $\times 100$, C: $\times 200$.

collagen-sponges. Previous studies have used a periosteal cap, as well as collagen I sponges⁵⁵ to retain transplanted chondrocytes in cartilage defects. We choose to transplant our genetic manipulated chondrocytes on collagen I sponges, because cells grown on these matrices are phenotypically stable, metabolically active and express extracellular proteins characteristic for articular cartilage⁵⁶. With our technique injecting the cells into the collagen sponge

with a fine needle, we achieved a cell growth even in the deeper areas of the scaffold whereas other groups observed cell growth only on the surface⁵⁵.

N2TetOnLacZ-transfected autologous chondrocytes, seeded on type I collagen sponges and implanted in artificial osteochondral defects in the trochlea femoris of NZW-rabbits, expressed the marker gene LacZ and became LacZ-positive in animals which obtained doxycycline to induce expression of the transgene. In contrast, transduced cells of animals in the non-induced group showed only slight transgene expression, which is consistent with our observation in cell culture. In a recent study, Lamartina *et al.*⁴⁷ reported tight control of transgene expression *in vitro* and *in vivo* in a nonviral vector system by the novel reverse transactivator rTA2^S-M2 and another mutant rTA2^S-S2. This is also reported by Koponen *et al.*⁴⁹ who used rTA2^S-M2 in a lentiviral vector system. Obtaining high levels of induction, both groups observed very low basal activity in the non-induced state of their systems. This is consistent with our data which show slight basal expression of our transgene in the non-induced state both *in vitro* and *in vivo*.

How many of our transfected cells survived *in vivo* could not be determined but the number was substantial as shown in Fig. 6.

Of interest was the observation of mononuclear cell infiltration in the adjacent cell layers in the transgene expressing knee joints, documenting the immunogenicity of the transfected cells, probably due to the expression of the reporter gene^{57,58}. This is not envisaged to represent a problem if the transgene is one coding for an endogenous growth factor.

Thus, our experiments document the feasibility of using a nonviral tet-inducible system to regulate transgene expression *in vivo* after implantation of genetically modified chondrocytes. In future, this approach might be used for a controlled delivery of therapeutic genes such as Bone Morphogenetic Proteins (BMP), Transforming Growth Factor- β (TGF- β), or Insulin-like Growth Factor-1 (IGF-1) in cartilage defects to study the mechanism of the growth factor cascade in the articular healing process.

The successful clinical application of nonviral vectors will rely on a better understanding of the barriers to gene transfer and on the development of vectors that can overcome such barriers.

These results may serve as a first step of combining inducible gene expression in chondrocytes with tissue engineering. More information further defining safety questions of gene delivery into primary cells is needed before this approach enters the clinic. Certainly a system that allows controlled gene expression is a step in the right direction.

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