

CARTILAGE CONSTRUCTS ENGINEERED FROM CHONDROCYTES OVEREXPRESSING IGF-I IMPROVE THE REPAIR OF OSTEOCHONDRAL DEFECTS IN A RABBIT MODEL

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Abstract

Tissue engineering combined with gene therapy is a promising approach for promoting articular cartilage repair. Here, we tested the hypothesis that engineered cartilage with chondrocytes overexpressing a human insulin-like growth factor I (IGF-I) gene can enhance the repair of osteochondral defects, in a manner dependent on the duration of cultivation. Genetically modified chondrocytes were cultured on biodegradable polyglycolic acid scaffolds in dynamic flow rotating bioreactors for either 10 or 28 d. The resulting cartilaginous constructs were implanted into osteochondral defects in rabbit knee joints. After 28 weeks of *in vivo* implantation, immunoreactivity to β -gal was detectable in the repair tissue of defects that received *lacZ* constructs. Engineered cartilaginous constructs based on IGF-I-overexpressing chondrocytes markedly improved osteochondral repair compared with control (*lacZ*) constructs. Moreover, IGF-I constructs cultivated for 28 d *in vitro* significantly promoted osteochondral repair *vis-à-vis* similar constructs cultivated for 10 d, leading to significantly decreased osteoarthritic changes in the cartilage adjacent to the defects. Hence, the combination of spatially defined overexpression of human IGF-I within a tissue-engineered construct and prolonged bioreactor cultivation resulted in most enhanced articular cartilage repair and reduction of osteoarthritic changes in the cartilage adjacent to the defect. Such genetically enhanced tissue engineering provides a versatile tool to evaluate potential therapeutic genes *in vivo* and to improve our comprehension of the development of the repair tissue within articular cartilage defects. Insights gained with additional exploration using this model may lead to more effective treatment options for acute cartilage defects.

Keywords: Tissue engineering; gene therapy; articular chondrocytes; bioreactor; insulin-like growth factor I; transplantation; scaffold; osteochondral defects; cartilage repair; osteoarthritis.

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Introduction

Articular cartilage has only limited ability to repair following an acute osteochondral injury (Hunziker, 2009). Mesenchymal cells from the bone marrow repopulate the defect and form a fibrocartilaginous tissue of lesser quality than the original cartilage (Shapiro *et al.*, 1993). Although different reconstructive surgical interventions are available, none of them allows for complete and durable articular cartilage regeneration (Hunziker, 2002; Redman *et al.*, 2005; Safran and Seiber, 2010; van Osch *et al.*, 2009). It is because of this inability to restore the original cartilage structure that there is great clinical interest in developing novel strategies for cartilage repair (Dell'Accio and Vincent, 2010).

Tissue engineering (Langer and Vacanti, 1993) and gene therapy (Cucchiari and Madry, 2005; Evans *et al.*, 2000) are two promising alternate approaches to cartilage regeneration. Functional substitutes for native cartilage can be generated from articular chondrocytes (Wolf *et al.*, 2008) and transplanted into cartilage defects *in vivo*, to enhance cartilage repair compared to defects left empty or treated with cell-free scaffolds (Schaefer *et al.*, 2002). Gene delivery of regulatory signals to the chondrocytes within these constructs could further enhance chondrogenesis (Saltzman, 1999).

Insulin-like growth factor I (IGF-I) is a particularly compelling candidate for gene transfer to chondrocytes (Mauck *et al.*, 2003; Nixon *et al.*, 2000; Nixon *et al.*, 2005; Pei *et al.*, 2002), as it simultaneously increases chondrocyte proliferation (Trippel *et al.*, 1989) and the synthesis of type-II collagen (van der Kraan *et al.*, 2002) and proteoglycans (Luyten *et al.*, 1988; Morales and Hascall, 1989). Isolated IGF-I overexpressing articular chondrocytes led to enhanced cartilage repair *in vivo* when implanted in a lapine osteochondral defect model (Madry *et al.*, 2005). *In vitro*, tissue engineering is improved when such chondrocytes overexpressing IGF-I are used to engineer cartilage, as shown by the enhanced morphological, biochemical and biomechanical parameters of these constructs (Madry *et al.*, 2002). When applied to the problem of cartilage repair, this approach would allow immediate filling of the defect with a structure that actively supports chondrogenesis (Evans, 2011; Ivkovic *et al.*, 2011); in contrast to the less targetable intra-articular delivery of a therapeutic gene vector (Madry *et al.*, 2011a). It remains unknown, however, whether such spatially defined overexpression of an IGF-I sequence in tissue-engineered cartilaginous constructs enhances the repair of osteochondral defects *in vivo*.

Here, we utilised enhanced chondrogenesis within tissue-engineered cartilage *via* genetically modified chondrocytes *in vitro* to repair an acute cartilage defect model *in vivo*. We specifically tested the hypothesis that tissue-engineered cartilaginous constructs created from chondrocytes overexpressing a human IGF-I cDNA can enhance the repair of osteochondral defects *in vivo*. We also tested the hypothesis that a longer cultivation period of the constructs prior to implantation (28 d *in vitro*) results in improved repair *in vivo*, *vis-à-vis* a shorter cultivation period (cultured for 10 d *in vitro*). Moreover, since osteoarthritis (Goldring and Goldring, 2010) is a clinically significant potential long-term consequence of a focal cartilage defect (Schinhan *et al.*, 2012), osteoarthritic changes in the adjacent articular cartilage were assessed.

Materials and Methods

Experimental design

Cartilaginous constructs were engineered using allogeneic lapine articular chondrocytes transfected with expression plasmid vectors carrying either the *Escherichia coli* (*E. coli*) β -galactosidase (*lacZ*) gene (referred to as *lacZ* constructs) or a human IGF-I cDNA (IGF-I constructs) (Fig. 1). Previously, we have documented the histological, biochemical, and biomechanical *in vitro* properties of these constructs (including transgene expression), and demonstrated an enhancement of structural and functional

features of engineered cartilage *in vitro* by spatially defined overexpression of human IGF-I (Madry *et al.*, 2002).

One day after transfection, modified chondrocytes were dynamically seeded onto polyglycolic acid scaffolds that allow for sustained transgene expression and controlled chondrogenesis *in vitro* and *in vivo* (Freed *et al.*, 1994; Freed *et al.*, 1998; Madry *et al.*, 2002; Schaefer *et al.*, 2002). Two days after seeding, both types of constructs were transferred into rotating bioreactors and cultivated for either 10 or 28 d. The constructs were then removed from the bioreactors at these two time points and transplanted into osteochondral defects in the patellar groove of rabbits.

Four experimental groups were formed: (1) *lacZ* and (2) IGF-I constructs cultivated for 10 d *in vitro* and implanted in the left and right knees ($n = 7$ animals); (3) *lacZ* and (4) IGF-I constructs cultivated for 28 d *in vitro* and implanted in the left and right knees ($n = 7$ animals). Twenty-eight weeks post implantation, animals were euthanised and the distal femurs were analysed.

Materials

Reagents were obtained from Invitrogen/GIBCO (Karlsruhe, Germany) unless otherwise indicated. The polyglycolic acid scaffold was from Cellon (Strassen, Luxembourg), collagenase type I (activity: 232 U/mg) from Biochrom (Berlin, Germany), and bovine testicular hyaluronidase from Sigma (Munich, Germany). Plastic ware was from Falcon (Becton Dickinson, Pont de Claix, France).

Table 1. Inverse semi-quantitative score.

| Category | Evaluation | Points |
|-------------------------------------|----------------------------------|-----------|
| Contracture | No | 0 |
| | Yes | 1 |
| Effusion | No | 0 |
| | Yes | 1 |
| Intra-articular adhesions | No | 0 |
| | Yes | 1 |
| Synovialitis | No | 0 |
| | Yes | 1 |
| Osteophytes | No | 0 |
| | Yes | 1 |
| Colour of the defect | White | 0 |
| | Translucent | 1 |
| | Dark | 2 |
| Surface of the defect | Smooth | 0 |
| | Fibrillated | 1 |
| | Total degeneration | 2 |
| Integration with adjacent cartilage | Complete integration | 0 |
| | Demarcating border | 1 |
| Filling of the defect | Protruding | 1 |
| | In level with adjacent cartilage | 0 |
| | 50 % repair of defect depth | 1 |
| | 0 % repair of defect depth | 2 |
| Maximal points | | 12 |

Values of all individual categories were added. A macroscopic normal joint and defect appearance would receive 0 points.

Cell culture, transfection, and seeding onto scaffolds

All animal procedures were approved by the local governmental animal care committee and were conducted in accordance with the German legislation on protection of animals and the *NIH Guidelines for the Care and Use of Laboratory Animals* (NIH Publication #85-23 Rev. 1985). Animals were housed one per cage in air-conditioned rooms with constant temperature and a regular light/dark scheme. They had free access to tap water and were fed a standard diet.

Articular chondrocytes were isolated from articular cartilage obtained from the knee and hip joints of two male skeletally immature Chinchilla bastard rabbits to allow for a high rate of chondrogenesis (Wei and Messner, 1999) (mean weight: 2.1 ± 0.5 kg; Charles River, Sulzfeld, Germany) as previously described (Madry *et al.*, 2003) and expanded in DMEM with 50 $\mu\text{g}/\text{mL}$ ascorbic acid, 100 U/mL penicillin G and 100 $\mu\text{L}/\text{mL}$ streptomycin (basal medium). The cell number was determined by haemocytometry. Viability was determined by trypan blue exclusion and always exceeded 90 %.

Chondrocytes were transfected using the nonliposomal lipid formulation FuGENE 6 (Roche, Mannheim, Germany) with endotoxin-free expression plasmid vectors carrying either *lacZ* (pCMV*lacZ*) (Madry and Trippel, 2000) or a human IGF-I cDNA (pCMVhIGF-I) (Madry *et al.*, 2002; Madry *et al.*, 2001) under the control of the human cytomegalovirus (CMV) immediate-early promoter/enhancer as previously described (Madry *et al.*, 2003) using chondrocytes at passage 2 on days 10-14 after cell isolation, seeded at a density of 1.2×10^6 cells/flask in basal medium containing 10 % foetal bovine serum (growth medium) in T-75 flasks. Aliquots of chondrocytes used for seeding in the scaffolds were also processed for X-Gal staining.

One day after transfection, chondrocytes were dissociated from the culture plates, re-suspended in growth medium and dynamically seeded onto polyglycolic acid (PGA) scaffolds (3.5 mm diameter x 1.5 mm fibrous discs, 2.5×10^6 cells/scaffold) as previously described (Madry *et al.*, 2002). Aliquots (10 μL) of the seeding medium were removed after 1, 24, 36 and 48 h and cell numbers were determined to estimate the number of cells per scaffold and time point. Two days after initiating the seeding,

cell-polymer constructs were transferred into rotating bioreactors containing basal medium (RCCV-110, Cellon, Strassen, Luxembourg) (Freed *et al.*, 1998; Madry *et al.*, 2002). The vessel rotation rate was adjusted to allow for a free suspension of each construct in the rotational flow. The constructs were assessed for wet weight at the time of transfer to the bioreactor and implantation. The medium was replaced every 5 d (50 %). Gas exchange was continuous.

Implantation of genetically modified cartilaginous constructs

The implantation of the *lacZ* or IGF-I constructs was performed either after 10 or 28 d of bioreactor cultivation *in vitro* (Fig. 1). Male Chinchilla bastard rabbits ($n = 14$; mean weight: 2.8 ± 0.3 kg; Charles River) were anaesthetised by intramuscular injection of Ketavet (0.75 mg/kg of body weight; Pharmacia & Upjohn, Erlangen, Germany) and Rompun (0.2 mL/kg of body weight; Bayer, Leverkusen, Germany). The knee joint was entered through a medial parapatellar approach, the patella was dislocated laterally and the knee was flexed to 90° . A cylindrical osteochondral cartilage defect of standardised depth (4.0 mm) was created in each patellar groove ($n = 28$ defects) with a manual cannulated burr (3.2 mm in diameter; Synthes, Umkirch, Germany). Each defect was washed with saline and blotted dry. Engineered cartilaginous constructs were press-fit into the defects. The right and left knees alternately received *lacZ* or IGF-I constructs. Constructs from one single preparation were employed in all defects. The patella was reduced, the knee was put through a range of motion to assure the stability of the constructs and the incisions were closed in layers. Immediately postoperatively, the animals were allowed unrestricted activity and full weight bearing without any immobilisation, then to climb and jump 6 weeks after the implantation.

Detection of transgene expression

Detection of β -galactosidase activity in transfected chondrocytes was performed by X-Gal staining using a standard protocol (Madry *et al.*, 2003) and in tissue sections by incubation with a 1:100 dilution of a mouse anti- β -gal antibody (GAL-13) (Sigma). The production of IGF-I was determined from the cell culture supernatants

Table 2. Effects of tissue-engineered constructs based on chondrocytes overexpressing *lacZ* and IGF-I on the on the histological grading of the synovial membrane after 28 weeks *in vivo*.

| Category | 10 days | | 28 days | |
|------------------------------|------------------------|------------------|------------------------|------------------|
| | <i>lacZ</i> constructs | IGF-I constructs | <i>lacZ</i> constructs | IGF-I constructs |
| Villus thickening | 1.3 (0.5) | 1.0 (0.6) | 1.2 (0.4) | 1.0 (0) |
| Villus architecture | 1.0 (0.9) | 0.5 (0.5) | 0.8 (0.4) | 0.3 (0.5) |
| Inflammatory cell infiltrate | 0.8 (1.0) | 0.2 (0.4) | 0.3 (0.5) | 0 (0) |
| Average total score | 3.2 | 1.7 | 2.3 | 1.3 |
| SD | 2.3 | 1.4 | 1.4 | 0.5 |

Each category and total score is calculated as the average score performed by two blinded evaluators. Data are expressed as mean \pm S.D.

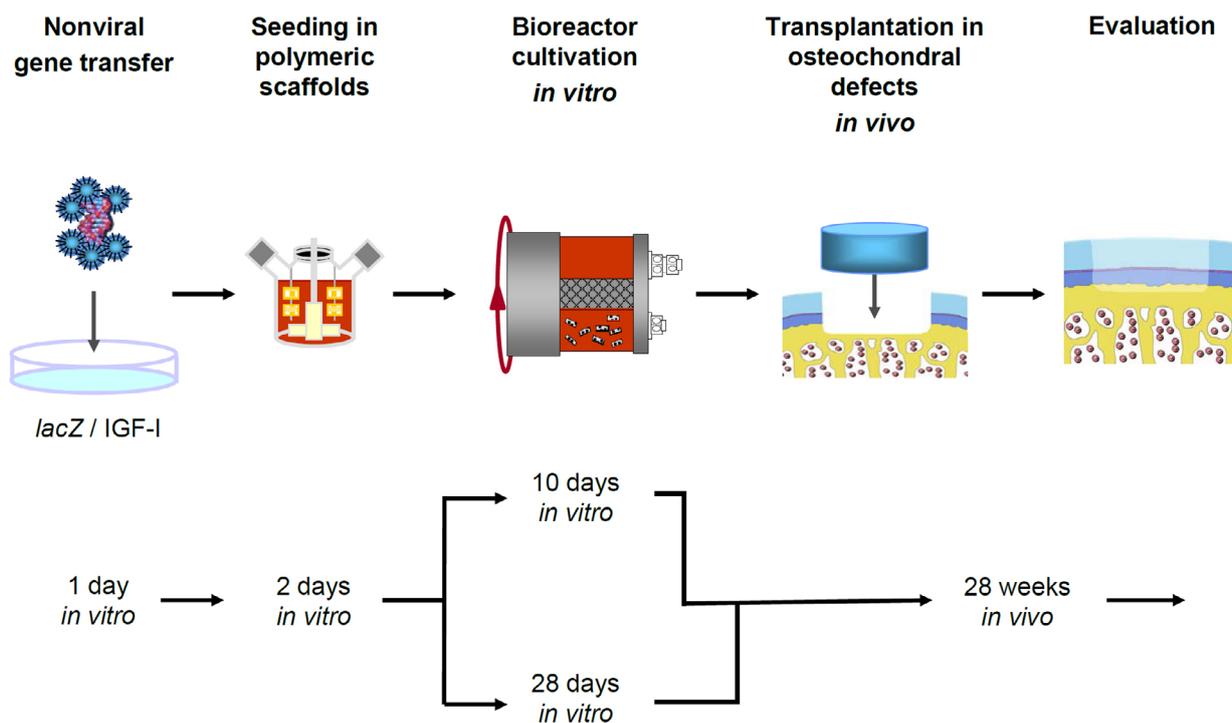


Fig. 1. Experimental design. Primary rabbit chondrocytes were isolated, placed in monolayer culture and transfected with either *lacZ* or IGF-I expression plasmid vectors using FuGENE 6. At day 1 post transfection, the chondrocytes were released and dynamically seeded into polymeric scaffolds in spinner flasks. At day 3 post transfection (day 2 post seeding), the resulting *lacZ* or IGF-I constructs were transferred into rotating bioreactors and cultivated for either 10 or 28 d *in vitro*. At these two time points, the *lacZ* and IGF-I constructs were removed from the bioreactors and transplanted into osteochondral defects in the patellar groove of rabbits. Twenty-eight weeks post implantation, animals were euthanised and the distal femora were retrieved and analysed.

of transfected chondrocytes cultivated in monolayer and of engineered cartilaginous constructs. To determine IGF-I secretion from the constructs, 2 samples from each group were removed from the bioreactors at days 3, 5, 10, 13, 18, 24 and 31 after transfection and individually cultivated in 24-well plates in 500 μ L basal medium. After 12 h, the conditioned medium was collected and the constructs were returned to the bioreactors.

To monitor the IGF-I amount in the synovial fluid, a lavage (1 mL phosphate-buffered saline, PBS) of the knees was performed at the time of sacrifice. The samples were analysed after extraction of binding proteins using an IGF-I ELISA (R&D Systems, Wiesbaden, Germany) with a detection limit of 26 pg/mL. To examine the expression of IGF-I in the defects, immunohistochemical analysis of tissue sections was performed using a 1:25 dilution of a polyclonal goat anti-IGF-I antibody (AF-291-NA) (R&D Systems). Immunoreactivity to IGF-I was compared to articular chondrocytes of a normal lapine osteochondral unit.

Macroscopic evaluation of the specimen

Two rabbits were removed from the protocol because of death caused by gastrointestinal infections unrelated to the surgical treatment at weeks 2 and 24 post operation. At 28 weeks post-operation, the animals were euthanised. The knee joints were exposed and macroscopically examined using an inverse semi-quantitative score (Table 1) (Orth *et*

al., 2011). Values of all individual categories were added. A macroscopic normal joint and defect appearance would receive 0 points.

Histological and immunohistochemical analyses

The distal femurs were retrieved, fixed in 4 % phosphate-buffered formalin, trimmed to 2.0 x 1.5 x 1.0 cm, and decalcified. Following decalcification, distal femurs were trimmed close to the proximal border of that interface and embedded, to assure that the subsequent cutting was moving from the periphery of the repair tissue in the defect towards the centre. Paraffin-embedded frontal sections (5 μ m) of the distal femora starting from the proximal border of the defect were taken at 200 μ m intervals. Peripheral sections as identified by image analysis of their width (less than 2.0 mm) were excluded. All sections were taken within approximately 1.2 mm from the centre of the defects ($n = 5-10$ per defect).

Sections were stained with Safranin O and haematoxylin and eosin (HE) (Kiernan, 1999). For type-I and type-II collagen immunostaining, deparaffinised sections were submerged for 30 min in 0.3 % hydrogen peroxide. After washing with PBS, sections were incubated for 30 min in 0.1 % trypsin, washed with PBS and blocked with 3 % bovine serum albumin in PBS (blocking buffer) for 30 min. The sections then were incubated with a 1:50 dilution of a monoclonal mouse anti-human type-I collagen IgG or a 1:10 dilution of a monoclonal mouse anti-human

type-II collagen IgG (Medicorp AF-5610 and AF-5710, respectively, Montréal, Canada) in blocking buffer for 24 h at 4 °C, washed and exposed to a 1:100 dilution of a biotinylated anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The sections were washed, incubated for 30 min with avidin-biotin-peroxidase reagent (Vectastain Elite ABC kit; Vector Laboratories), washed and exposed to diaminobenzidine (Vector Laboratories). Control tissues for the primary antibodies included lapine skin and lapine articular cartilage. To examine the potential infiltration of immune cells in the defects, immunohistochemical analysis of tissue sections was performed using a mouse monoclonal anti-CD11b antibody (Ly-40; Acris, Hiddenhausen, Germany) to detect activated macrophages, a mouse monoclonal anti-CD3 antibody (PC3/188A; DakoCytomation, Hamburg, Germany) to detect CD3 (T-lymphocytes) and a mouse monoclonal anti-HLA-DR α antibody (TAL.1B5; DakoCytomation) to detect class II MHC antigens. These antibodies have been shown to cross-react with a large panel of species, including the rabbit. To control for all secondary immunoglobulins, sections were processed as described above with omission of the primary antibody.

The synovial membrane adjacent to the articular cartilage was evaluated on HE-stained sections using a previously published scoring system (Fortier *et al.*, 2002; Nixon *et al.*, 1999) (Table 2). Visualisation of the collagens within the repair tissue was performed on HE-stained histological sections using polarised light microscopy (BX-45, Olympus, Hamburg, Germany).

Immunoreactivity to type-I collagen in the repair tissue was compared to articular cartilage (negative control), and to that of the subchondral bone (positive control), both of a normal lapine osteochondral unit. Immunoreactivity to type-II collagen in the repair tissue was compared to articular cartilage (positive control), and to that of the subchondral bone (negative control), both of a normal lapine osteochondral unit. A 0-4 point score was given to each knee: 0, no immunoreactivity; 1, significantly weaker immunoreactivity; 2, moderately weaker immunoreactivity; 3, similar immunoreactivity; 4, stronger immunoreactivity (Kaul *et al.*, 2006; Madry *et al.*, 2005).

The adjacent articular cartilage was evaluated for early osteoarthritic changes based on the part of the histological score described by Solchaga *et al.* (2000), which determines osteoarthritic changes using Safranin O-fast green-stained sections. A 2 mm area of articular cartilage adjacent to both defect edges (excluding the area of integration that is independently scored in the score described by Sellers) was evaluated. Score points were given as follows: normal cellularity, no clusters, and normal staining: 0 points; normal cellularity, mild clusters, and moderate staining: 1 point; mild or moderate hypocellularity, and slight staining: 2 points; severe hypocellularity, and poor or no staining: 3 points.

For a quantitative assessment of the repair tissue, Safranin O-fast green stained sections ($n = 9-10$ per defect) were analysed using the complex cartilage repair score described by Sellers *et al.* (Orth *et al.*, 2012b; Sellers *et al.*, 1997; Sellers *et al.*, 2000). Values ranged from 31 points

(i.e. empty defect without repair tissue) to 0 points (normal articular cartilage, complete regeneration).

All images were acquired by a solid-state CCD camera (Olympus) mounted on a microscope (BX 45; Olympus) and analysed with the analySIS program (Soft Imaging System Corp., Munster, Germany) using standardised parameters, including light intensity. A total of 173 sections were scored independently and in a blinded fashion by three individuals.

Statistical analysis

Based on previous experiments (Kaul *et al.*, 2006; Madry *et al.*, 2005; Madry *et al.*, 2011b; Orth *et al.*, 2012b), we estimated standard deviation of 25 % for the mean total score to determine sample size. For a power of 80 % and a two-tailed alpha level of 0.05, a sample size of 6 defects per group would be needed to detect a mean difference of 5 points between groups (assuming a pooled standard deviation of 2.5 points; effect size = $5/2.5 = 2.0$, using the two-sample Student's *t*-test (version 5.0, nQuery Advisor, Statistical Solutions, Saugus, MA, USA). To accommodate possible postoperative complications, each test condition was performed with 7 defects per group and time point for *in vivo* experiments.

Statistical analysis of the data was performed using the SPSS software package (version 12.0, SPSS Inc., Chicago, IL, USA). To evaluate the *in vivo* experiments, points for each category and total score were compared between the two groups using a mixed general linear model with repeated-measures (knees nested within the same animals). All continuous variables, including IGF-I production and histological scoring for parameters such as cell morphology, architecture and tidemark, were tested for normality using the Kolmogorov-Smirnov goodness-of-fit method and no significant skewness or kurtosis was detected. Therefore, continuous data are expressed in terms of the mean \pm SD or mean \pm 95 % confidence interval. Categorical data including type-I and type-II collagen immunoreactivity did not conform to a Gaussian distribution and were thus compared nonparametrically between the defects receiving *lacZ* versus IGF-I constructs using the Wilcoxon signed-ranks test. A two-tailed $P < 0.05$ was considered statistically significant, shown in both figures and tables by superscripts of a.

Results

Cellular characterisation of the *lacZ* and IGF-I constructs

Transfection efficiency determined at day 3 after transfection was 37.3 ± 8.2 % ($n = 4$). Cell viability at the time of dynamic seeding (one day after transfection) was 97.6 ± 0.6 % and 97.7 ± 0.3 % for *lacZ*- and IGF-I-transfected chondrocytes, respectively ($n = 4$, $P > 0.05$). Seeding of *lacZ*- and IGF-I-transfected chondrocytes into PGA scaffolds was efficient, as indicated by the steady decline in the cell numbers of the seeding medium over the 2 days of seeding. At the time of transfer to the bioreactors, the *lacZ* and IGF-I constructs contained 2.5 ± 0.1 and $2.6 \pm 0.1 \times 10^6$ viable chondrocytes, respectively

($n = 14$, $P > 0.05$) (Fig. 2). There was no difference in cell viability ($97.9 \pm 4.2\%$ and $97.7 \pm 4.5\%$, respectively, $n = 2$, $P > 0.05$) and in the wet weights between groups at this time (32.8 ± 4.5 and 34.6 ± 4.5 mg, respectively, $n = 14$, $P > 0.05$), suggesting that transfection, expression of the transgenes, and scaffold material had no adverse effect upon chondrocyte viability and attachment. After 10 d *in vitro*, the IGF-I constructs had a 1.5-fold higher wet weight than the *lacZ* constructs (18.9 ± 2.5 and 12.4 ± 2.1 mg, respectively; $n = 14$, $P = 0.003$). At 28 d of cultivation, the wet weight of the IGF-I constructs was 1.8-fold higher than that of the *lacZ* constructs (14.3 ± 2.7 mg and 7.8 ± 1.8 , respectively; $n = 7$, $P < 0.001$).

Expression of the transgenes *in vitro*

Mean transfection efficiency was $37.3 \pm 8.2\%$. Mean IGF-I production by chondrocytes transfected with pCMVhIGF-I cultivated in monolayer was 186 ± 37 ng/L $\times 10^7$ cells/24 h ($n = 2$) on day 2 after transfection and 289 ± 41 ng/L $\times 10^7$ cells/24 h ($n = 2$) on day 4. IGF-I constructs produced 2.21 ± 0.31 ng IGF-I per construct/24 h ($n = 3$) on day 2 and 0.36 ± 0.08 ng IGF-I per construct/24 h ($n = 3$) on day 13 (day 10 of bioreactor cultivation) after transfection, and were significantly elevated until day 18 (0.19 ± 0.05 ng IGF-I per construct/24 h; $n = 3$, $P < 0.05$) compared to *lacZ* constructs (day 28 of bioreactor cultivation: 0.08 ± 0.01 ng IGF-I per construct/24 h; $n = 3$). At all time points tested, the conditioned medium of the *lacZ* constructs contained less than 0.08 ± 0.02 ng IGF-I per construct/24 h ($n = 3$).

Implantation of genetically modified engineered constructs

Macroscopic evaluation of treated knees after 28 weeks *in vivo*

Both types of constructs (*lacZ* and IGF-I constructs, each cultivated for 10 or 28 d *in vitro*) were next implanted into osteochondral defects. There was no effect of the differences in wet weight of the constructs on the press-fit implantation. No joint effusion, macroscopic synovialitis, heterotopic ossifications, or osteophytes were evident in

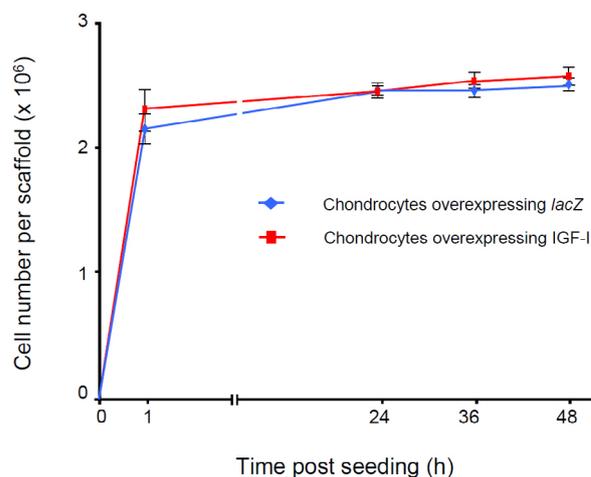


Fig. 2. Number of transfected articular chondrocytes overexpressing *lacZ* and IGF-I attached to the polymeric scaffolds in spinner flasks over time *in vitro*. Shown are the number of cells attached per polyglycolic acid (PGA) scaffold at the designated times during the 48 h course of dynamic seeding. Two days after initiating of the seeding, cell-polymer constructs were transferred into the rotating bioreactors.

any of the specimen at 28 weeks after transplantation. Semi-quantitative macroscopic evaluation of the repair tissue revealed a trend towards better scores for defects that received IGF-I constructs than those where *lacZ* constructs were implanted, regardless of the duration of cultivation in the bioreactor, without reaching statistical significance ($P > 0.05$) (Fig. 3).

Histological analysis of the synovial membrane after 28 weeks *in vivo*

Semi-quantitative evaluation of the constructs cultivated for 10 d in the bioreactor revealed a 1.9-fold lower (better) score for the synovial membrane of knee joints that received the IGF-I constructs compared with those where *lacZ* constructs were applied ($P > 0.05$) (Table 2). When constructs were cultivated for 28 d in the bioreactor,

Table 3. Effect of tissue-engineered constructs based on chondrocytes overexpressing IGF-I on the histological grading of the repair tissue – 10 d of bioreactor cultivation.

| Category | <i>lacZ</i> constructs | IGF-I constructs |
|----------------------------|---------------------------|---------------------------------------|
| | Mean (95 % CI) | Mean (95 % CI) |
| Filling of defect | 1.63 (1.23 - 2.02) | 0.81 (0.41 - 1.20) ^a |
| Integration | 1.27 (1.12 - 1.43) | 1.08 (0.93 - 1.23) |
| Matrix staining | 3.26 (3.09 - 3.44) | 2.81 (2.64 - 2.98) ^a |
| Cellular morphology | 3.30 (2.96 - 3.65) | 2.25 (1.91 - 2.58) ^a |
| Architecture of defect | 2.36 (1.84 - 2.88) | 2.16 (1.64 - 2.68) |
| Architecture of surface | 2.53 (2.14 - 2.91) | 2.57 (2.18 - 2.95) |
| Subchondral bone | 2.33 (2.01 - 2.65) | 1.52 (1.20 - 1.84) ^a |
| Tidemark | 3.01 (2.59 - 3.42) | 2.28 (1.86 - 2.70) ^a |
| Average total score | 19.7 (17.7 - 21.7) | 15.4 (13.5 - 17.5)^a |

Histological scoring is based on the complex system described by Sellers *et al.* (Sellers *et al.*, 1997; Orth *et al.*, 2012b). The score for a normal osteochondral unit is 0 points; an empty defect receives 31 points. ^a Statistically significant treatment effect. CI = confidence interval.

the score for the synovial membrane of knee joints that received IGF-I constructs was 1.8-fold lower (better) compared with those where *lacZ* constructs were applied ($n = 6$, $P > 0.05$).

Immunological analysis of immune cell infiltration

No immune cells infiltrating the defects of knees receiving either *lacZ* or IGF-I constructs were identified by immunohistochemical analysis of tissue sections using specific antibodies to detect CD3- (T-lymphocytes), CD11b- (activated macrophages), and HLA-DR α (class II MHC antigens) (data not shown).

Expression of the transgenes *in vivo*

Cytoplasmic immunoreactivity to β -gal was present in about 5-10 % of cells randomly distributed within the repair tissue of defects that received the *lacZ* constructs, with no preference for any cartilage layer and regardless of the duration of cultivation in the bioreactor (Fig. 4). IGF-I levels in the lavage fluids were not significantly different at 28 weeks between knee joints that received *lacZ* or IGF-I constructs (11.6 ± 2.2 and 9.3 ± 2.0 ng/mL, respectively, $n = 12$, $P > 0.05$). Immunoreactivity to IGF-I was always stronger in the repair tissue of defects that received IGF-I constructs than the *lacZ* constructs, regardless of the duration of cultivation in the bioreactor (Fig. 4).

Articular cartilage repair and chondrogenesis in osteochondral defects by implantation of genetically modified tissue-engineered constructs *in vivo*

Histological analysis of the repair tissue *in vivo* after transplantation of *lacZ* and IGF-I constructs cultivated for 10 d in bioreactors

Filling of the defect, matrix staining with Safranin O, cellular morphology, and restoration of the subchondral bone and of the tidemark were significantly improved in defects treated with IGF-I constructs compared with defects that received *lacZ* constructs (Table 3, Fig. 5). When the individual scores were combined, the average

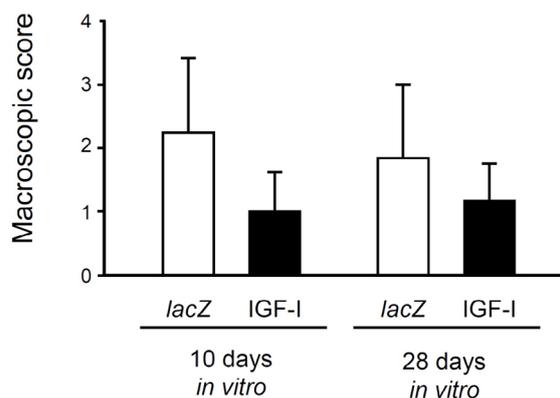


Fig. 3. Macroscopic articular cartilage repair by tissue-engineered constructs, based on chondrocytes overexpressing *lacZ* and IGF-I after 28 weeks of implantation. Data were obtained applying the inverse semi-quantitative score described in Table 1 by two blinded evaluators. A macroscopic normal joint and defect appearance would receive 0 points. Data are expressed as mean \pm S.D ($P > 0.05$ for all comparisons).

total score was significantly better for defects treated with the IGF-I constructs compared with those receiving the *lacZ* constructs (15.4 and 19.7 points, respectively, $P < 0.01$, Table 3).

Histological analysis of the repair tissue *in vivo* after transplantation of *lacZ* and IGF-I constructs that were cultivated for 28 d in bioreactors

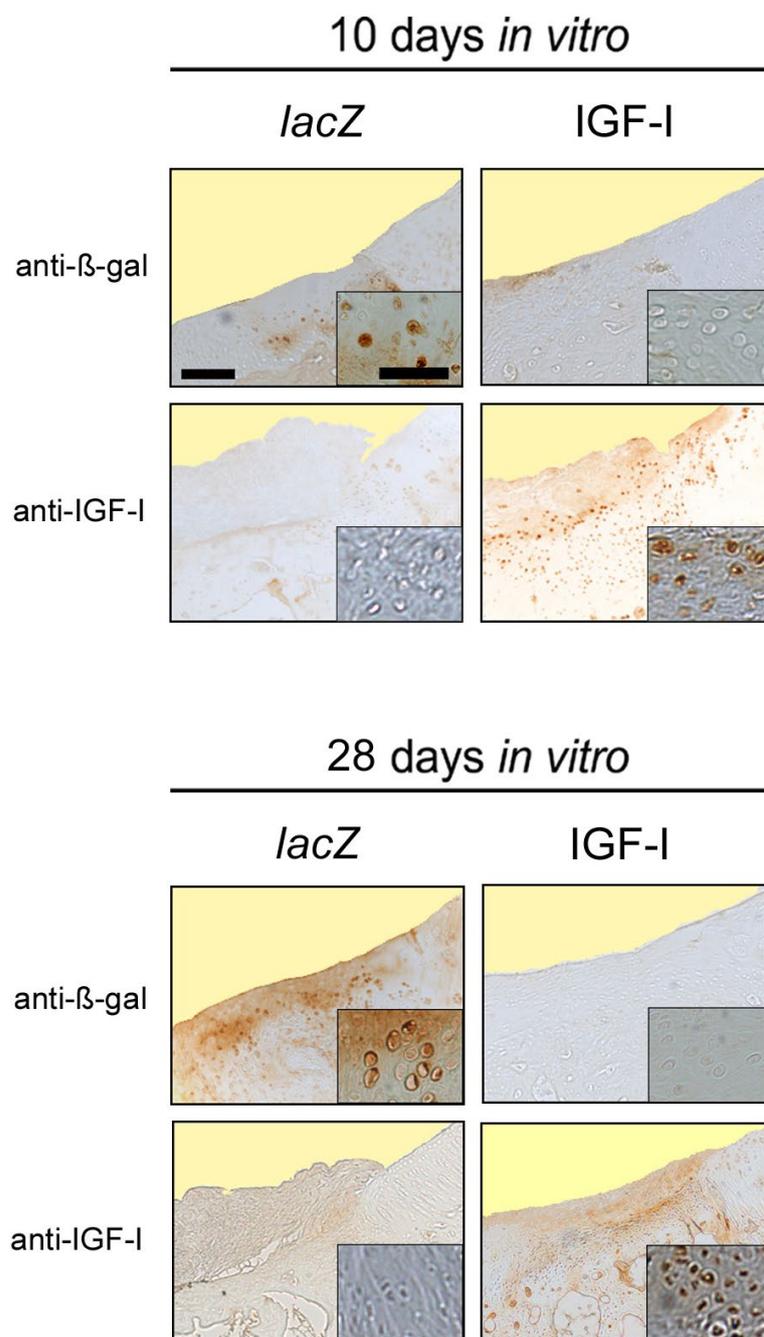
A repair tissue filled the cartilage defects treated with IGF-I constructs with a similar level to that of the surrounding normal articular cartilage, corresponding to the lowest (best) average individual score value (0.53) of all categories evaluated at 28 weeks *in vivo* (Table 4), were significantly different when compared with defects receiving the *lacZ* constructs. Similarly, individual score values for integration of the new tissue with the adjacent

Table 4. Effect of tissue-engineered constructs based on chondrocytes overexpressing IGF-I on the histological grading of the repair tissue – 28 d of bioreactor cultivation.

| Category | <i>lacZ</i> constructs | IGF-I constructs |
|----------------------------|---------------------------|---------------------------------------|
| | Mean (95 % CI) | Mean (95 % CI) |
| Filling of defect | 0.95 (0.65 - 1.24) | 0.53 (0.23 - 0.83) ^a |
| Integration | 1.15 (1.08 - 1.21) | 1.03 (0.97 - 1.09) ^a |
| Matrix staining | 2.44 (2.04 - 2.83) | 2.47 (2.07 - 2.86) |
| Cellular morphology | 2.90 (2.46 - 3.33) | 2.15 (1.72 - 2.58) ^a |
| Architecture of defect | 2.48 (2.19 - 2.77) | 1.30 (1.01 - 1.59) ^a |
| Architecture of surface | 2.07 (1.71 - 2.43) | 1.83 (1.48 - 2.19) |
| Subchondral bone | 2.11 (1.79 - 2.44) | 1.93 (1.61 - 2.26) |
| Tidemark | 3.01 (2.75 - 3.27) | 2.75 (2.49 - 3.01) |
| Average total score | 17.1 (15.4 - 18.8) | 13.9 (12.2 - 17.1)^a |

Histological scoring is based on the complex system described by Sellers *et al.* (Sellers *et al.*, 1997; Orth *et al.*, 2012b). The score for a normal osteochondral unit is 0 points; an empty defect receives 31 points. ^a Statistically significant treatment effect. CI = confidence interval.

Fig. 4. Transgene expression *in vivo*. Representative histologic sections of the area of integration between the repair tissue (left side of each picture) with the adjacent articular cartilage (right side of each picture) of the osteochondral defects 28 weeks after the implantation of tissue-engineered constructs based on chondrocytes overexpressing *lacZ* (left columns) and IGF-I (right columns) after 10 (top 4 images) and 28 d (bottom 4 images) of bioreactor cultivation stained with an anti- β -gal and anti-IGF-I antibody. No immunoreactivity to β -gal was present in the cytoplasm of cells in the repair tissue of defects that received an IGF-I constructs at both time points. Immunoreactivity to β -gal was present in the cytoplasm of about 5-10 % of cells randomly distributed within in the repair tissue of defects that received the *lacZ* constructs at both time points. Immunoreactivity to IGF-I in the repair tissue is mainly located in the superficial and middle parts of the cartilaginous repair tissue of the defect receiving an IGF-I construct at 10 and 28 days. Photomicrographs were obtained using standardised photographic parameters, including light intensity. Insets are taken from the middle parts of the cartilaginous repair tissue. Scale bars = 300 μ m, and 30 μ m (insets).



articular cartilage, cellular morphology and architecture were significantly improved for defects receiving IGF-I constructs compared with those receiving *lacZ* constructs (Fig. 6). The average total score was also significantly better in the IGF-I constructs that were cultivated for 28 d in bioreactors *in vitro* compared with the *lacZ* constructs (13.9 and 17.1 points, respectively; $P = 0.01$).

Effect of IGF-I overexpression and duration of bioreactor cultivation on type-I and type-II collagen synthesis

Polarised light microscopy revealed collagen fibrils within the repair tissue of defects receiving both types of constructs, mainly oriented parallel to the joint surface without reproducing a hyaline articular cartilage organisation. Birefringence was weaker within the repair

tissue than in the surrounding normal articular cartilage of both groups (Fig. 5 and 6). Birefringence in the subchondral bone was similar between the defect areas and the adjacent tissue.

Immunoreactivity to type-I collagen was stronger in the repair tissue of defects that received cartilaginous constructs composed of *lacZ*-modified chondrocytes than in those where the IGF-I constructs were applied, regardless of the duration of cultivation in the bioreactor (Fig. 7). No difference in immunoreactivity to type-I collagen was seen between both groups when cultivated for 10 d in the bioreactor *in vitro* ($P > 0.05$). Transplantation of *lacZ* constructs that were cultivated for 28 d *in vitro* resulted in a 1.4-fold stronger immunoreactivity to type-I collagen compared with IGF-I constructs ($P > 0.05$).

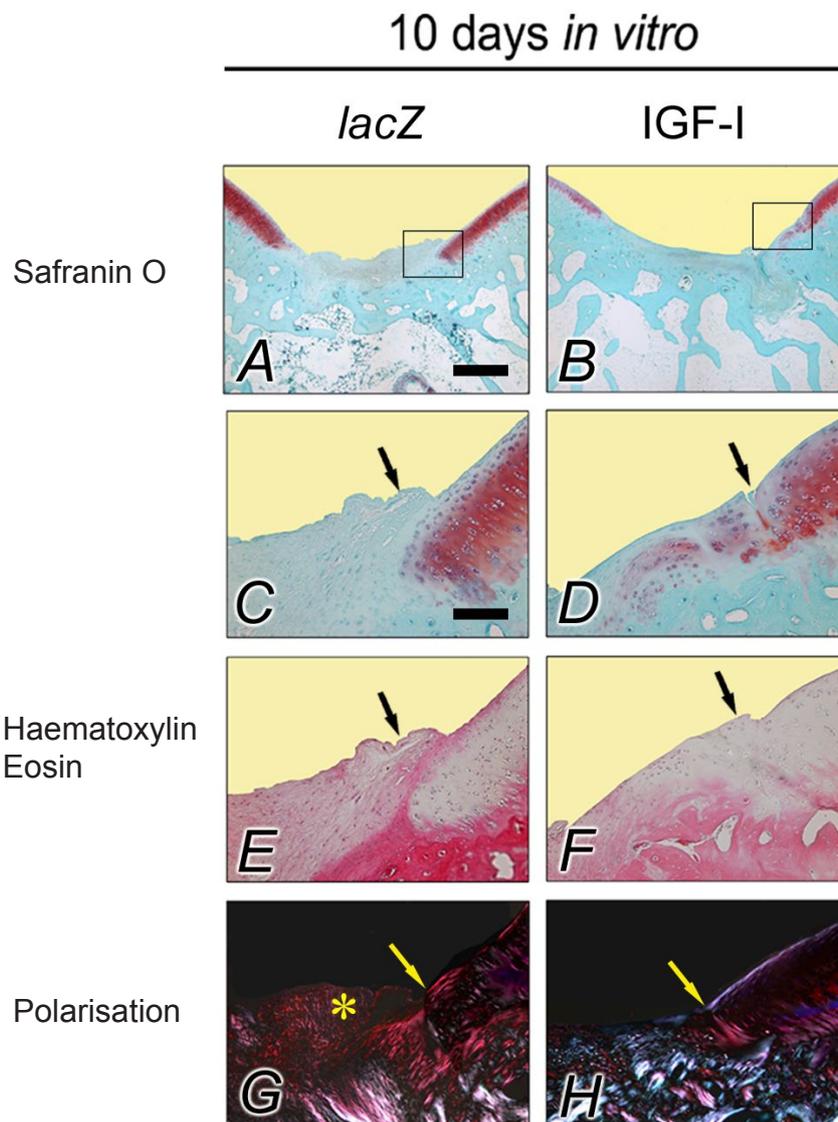


Fig. 5. Osteochondral repair following 10 d of *in vitro* bioreactor cultivation. Representative histologic sections of osteochondral defects 28 weeks after the implantation of tissue-engineered constructs based on chondrocytes overexpressing *lacZ* (left columns; *A*, *C*, *E*, *G*) and IGF-I (right columns; *B*, *D*, *F*, *H*) after 10 d of bioreactor cultivation. The top two images give an overview of the entire defect treated with a *lacZ* (left; *A*) and IGF-I construct (right; *B*) (Safranin O-fast green). Images below (*C-H*) are magnified views of the insets in the top two images, illustrating the area of integration (arrows) between the repair tissue (left side of each picture) with the adjacent articular cartilage (right side of each picture) (Safranin O-fast green; *A-D*). Note the difference in cellular morphology of the repair tissue between the groups, with less than 50 % of round cells with the morphology of chondrocytes following transplantation of a *lacZ* construct (left; *E*), compared with the repair tissue composed mostly of round cells with the morphology of chondrocytes following transplantation of an IGF-I construct (right; *F*) (haematoxylin and eosin). The repair of the subchondral bone in the defect receiving an IGF-I construct is significantly improved (polarised light microscopy, right; *H*), in contrast to the defect receiving a *lacZ* construct (left; *G*). Here, a part of the repair tissue extends into the joint space; asterisk). All sections illustrated were taken from defects having a histological rating equal to the mean score for its respective treatment group. Photomicrographs were obtained using standardised photographic parameters, including light intensity. Scale bars = 1 mm (*A*, *B*); and 300 μ m (*C-H*).

Immunoreactivity to type-II collagen was always stronger in the repair tissue of defects that received IGF-I constructs than in those where the *lacZ* constructs were applied, regardless of the duration of cultivation in the bioreactor (Fig. 8). Transplantation of IGF-I constructs that were cultivated for 10 days in the bioreactor *in vitro*

resulted in a 1.9-fold stronger immunoreactivity to type-II collagen compared with *lacZ* constructs ($P > 0.05$). Transplantation of IGF-I constructs that were cultivated for 28 d in the bioreactor *in vitro* led to a 1.6-fold stronger immunoreactivity to type-II collagen compared with *lacZ* constructs ($P > 0.05$).

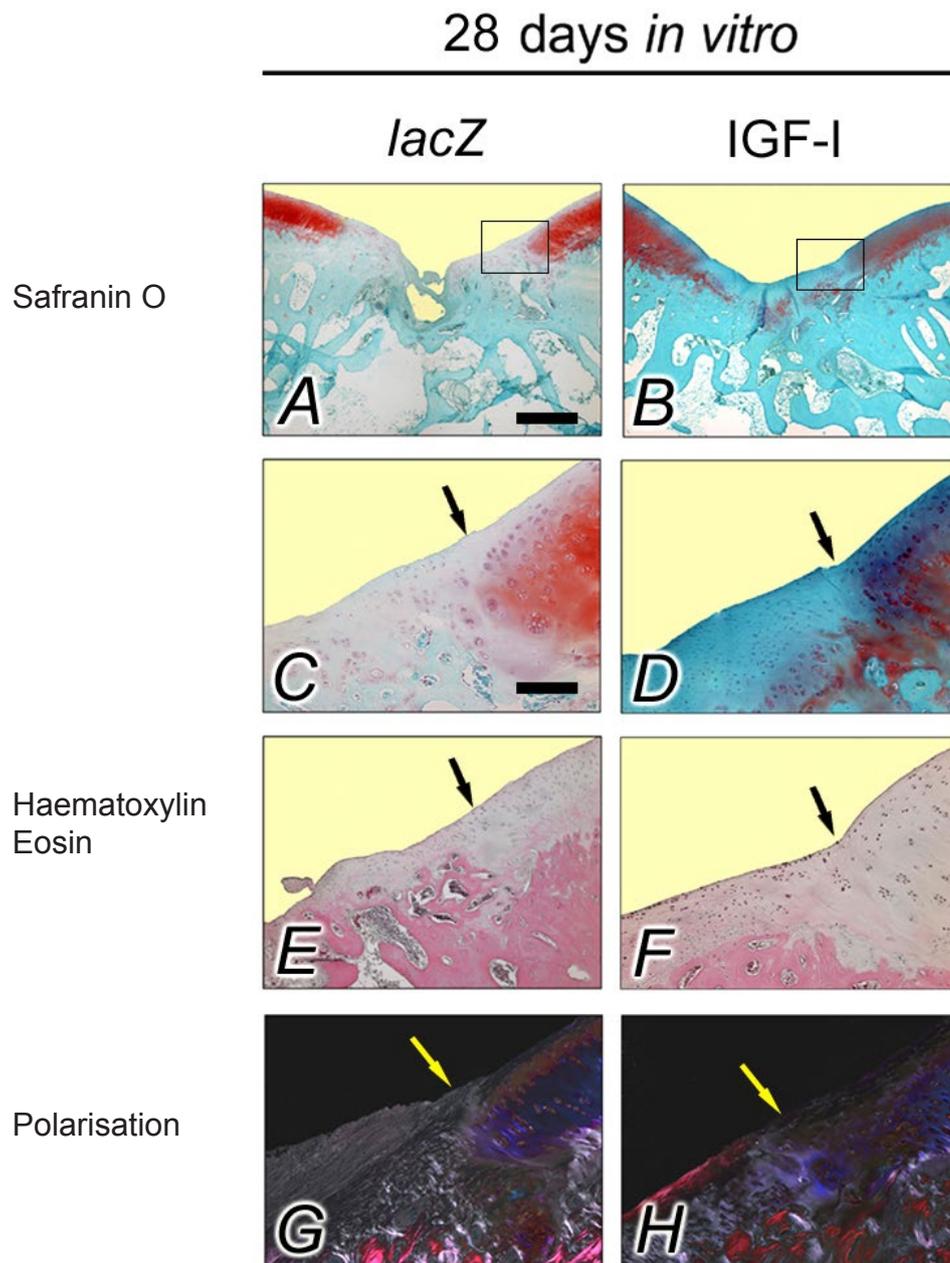


Fig. 6. Osteochondral repair following 28 d of *in vitro* bioreactor cultivation. Representative histologic sections of osteochondral defects 28 weeks after the implantation of tissue-engineered constructs based on chondrocytes overexpressing *lacZ* (left columns; *A*, *C*, *E*, *G*) and IGF-I (right columns; *B*, *D*, *F*, *H*) after 28 d of bioreactor cultivation *in vitro*. The top two images give an overview of the entire defect treated with a *lacZ* (left; *A*) and IGF-I construct (right; *B*) (Safranin O-fast green). Images below (*C-H*) are magnified views of the insets in the top two images, illustrating the area of integration (arrows) between the repair tissue (left side of each picture) with the adjacent articular cartilage (right side of each picture) (Safranin O-fast green; *A-D*). Note the significantly lesser degree in filling of the defect and overall increased degeneration of the repair tissue following transplantation of a *lacZ* construct (left; *A*) (Safranin O-fast green), the lesser developed cellular morphology following transplantation of a *lacZ* construct (left; *E*) compared with the cellular morphology following transplantation of an IGF-I construct (right; *F*) (haematoxylin and eosin), together with the significantly improved restoration of the subchondral bone (polarised light microscopy, right; *H*). Photomicrographs were obtained using standardised photographic parameters, including light intensity. Scale bars = 1 mm (*A*, *B*); and 300 μ m (*C-H*).

Osteoarthritic changes in the articular cartilage adjacent to the osteochondral defects

Osteoarthritic changes in the cartilage adjacent to the defects were worse following transplantation of *lacZ* constructs cultivated for 10 d *in vitro* compared with IGF-I

constructs (1.8-fold; $P > 0.05$) (Fig. 9). Transplantation of *lacZ* constructs that were cultivated for 28 d *in vitro* also resulted in a 2.5-fold higher (worse) score for osteoarthritis in the adjacent articular cartilage, significantly different compared to IGF-I constructs ($P = 0.007$).

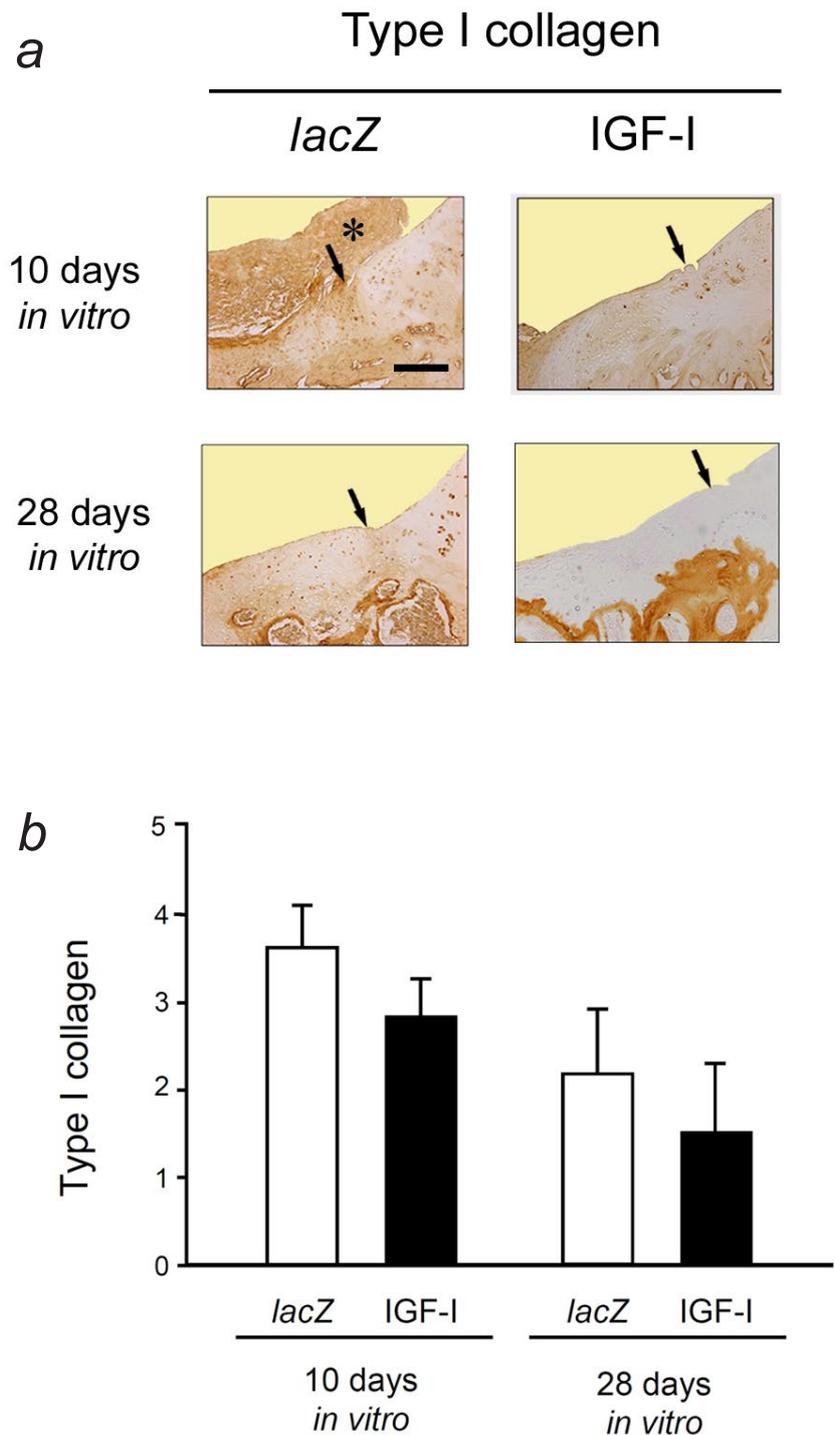


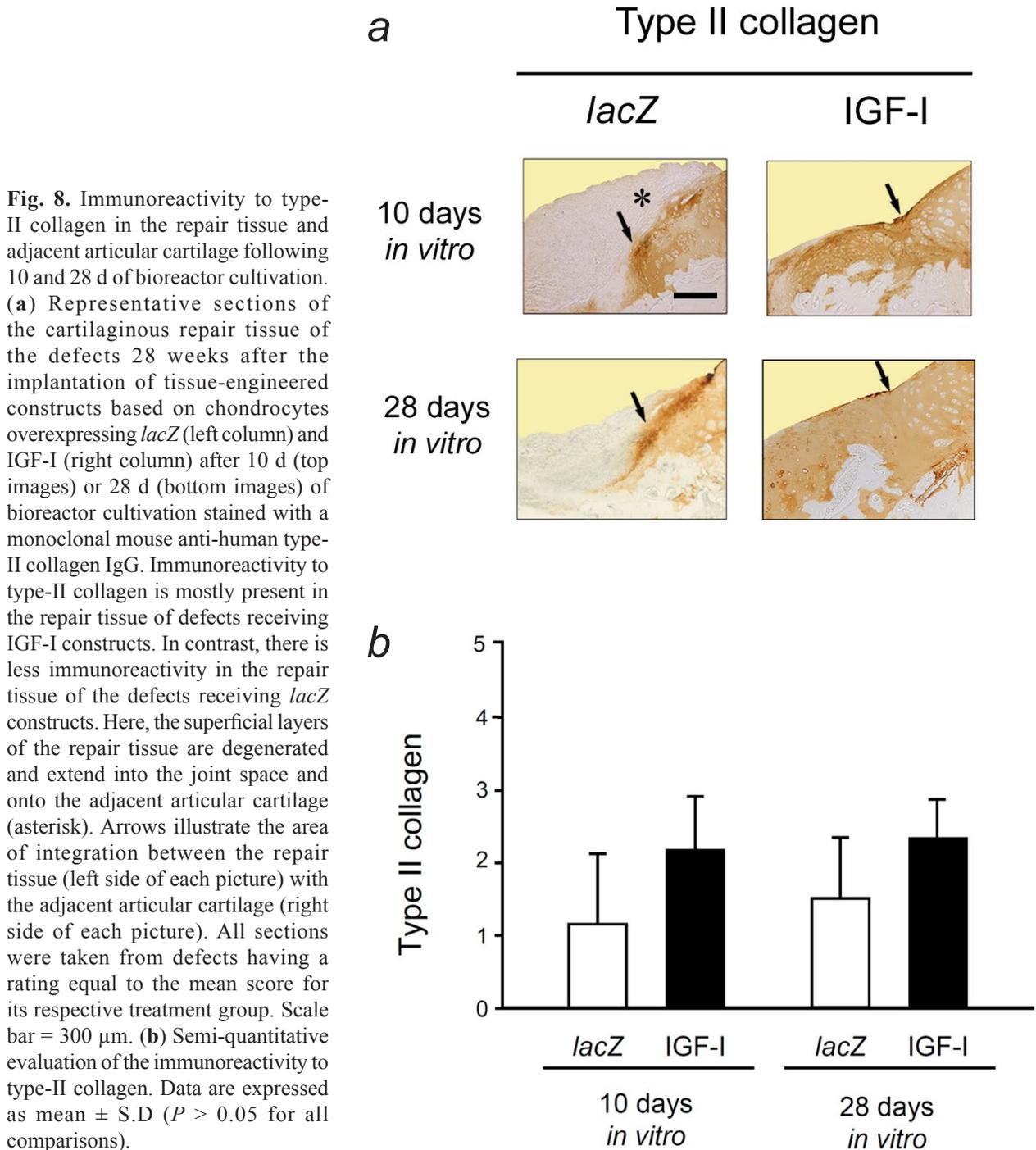
Fig. 7. Immunoreactivity to type-I collagen in the repair tissue and adjacent articular cartilage following 10 and 28 d of bioreactor cultivation. **(a)** Representative sections of the cartilaginous repair tissue of the defects 28 weeks after the implantation of tissue-engineered constructs based on chondrocytes overexpressing *lacZ* (left column) and IGF-I (right column) after 10 d (top images) or 28 d (bottom images) of bioreactor cultivation stained with a monoclonal mouse anti-human type-I collagen IgG. A large part of the superficial repair tissue in the defect receiving a *lacZ* construct (following 10 d of bioreactor cultivation) is degenerated and extends both into the joint space and onto the adjacent articular cartilage; asterisk). The cartilaginous repair tissue in defects receiving *lacZ* is characterised by a higher degree of immunoreactivity to type-I collagen, compared with defects receiving IGF-I constructs. Arrows illustrate the area of integration between the repair tissues (left side of each picture) with the adjacent articular cartilage (right side of each picture). All sections were taken from defects having a rating equal to the mean score for its respective treatment group. Scale bar = 300 μ m. **(b)** Semi-quantitative evaluation of the immunoreactivity to type-I collagen. Data are expressed as mean \pm S.D. ($P > 0.05$ for all comparisons).

Effect of the duration of bioreactor cultivation *in vitro* on the quality of the repair tissue *in vivo*

A statistical comparison between the two time points (10 *versus* 28 d) within each single treatment group (*lacZ* and IGF-I) was performed. Among the *lacZ* constructs, filling of the defect ($P = 0.04$) and matrix staining ($P = 0.01$) were improved when the constructs were cultivated for 28 d compared with 10 d. For all other individual parameters, the duration of bioreactor cultivation did not influence overall repair, as reflected in the similar total score ($P > 0.05$).

For the IGF-I constructs, a comparison for each single parameter between the 10 and 28 d of cultivation groups

revealed significantly better repair at 28 d with respect to filling ($P = 0.03$), architecture of the defect ($P < 0.01$), and architecture of the surface ($P < 0.01$). Other individual categories were not significantly different between 10 and 28 d. The average total score of the IGF-I constructs was significantly improved after 28 d of bioreactor cultivation compared with 10 d ($P = 0.02$), in contrast to the *lacZ* constructs. This was also reflected in the lowest (best) total score value (13.9) of all 4 groups.



Discussion

Combining tissue engineering and gene therapy may be beneficial compared with cell transplantation or gene transfer alone. We investigated if overexpression of a human insulin-like growth factor I (IGF-I) gene in chondrocytes used to engineer cartilage can enhance the repair of osteochondral defects in a rabbit model.

The present study has three major new findings. First, overexpression of a human IGF-I cDNA in tissue-engineered cartilaginous constructs enhances the repair of osteochondral defects in a rabbit model after 28 weeks *in vivo*, regardless whether the constructs were cultivated

for 10 or 28 d *in vitro*. Second, maximal promotion of cartilage repair was initiated following implantation of IGF-I constructs subjected to a longer cultivation period (cultivated for 28 d *in vitro*). Third, lesser osteoarthritic changes in the cartilage adjacent to the defects resulted when IGF-I constructs cultivated for 28 d *in vitro* were transplanted.

This *in vivo* study is a direct translation of previous work *in vitro* which showed that gene transfer of a human IGF-I cDNA enhanced both structural and functional parameters of tissue-engineered cartilage (Madry *et al.*, 2002). To the best of our knowledge, the present study is the first that moved the concept of IGF-I overexpression to

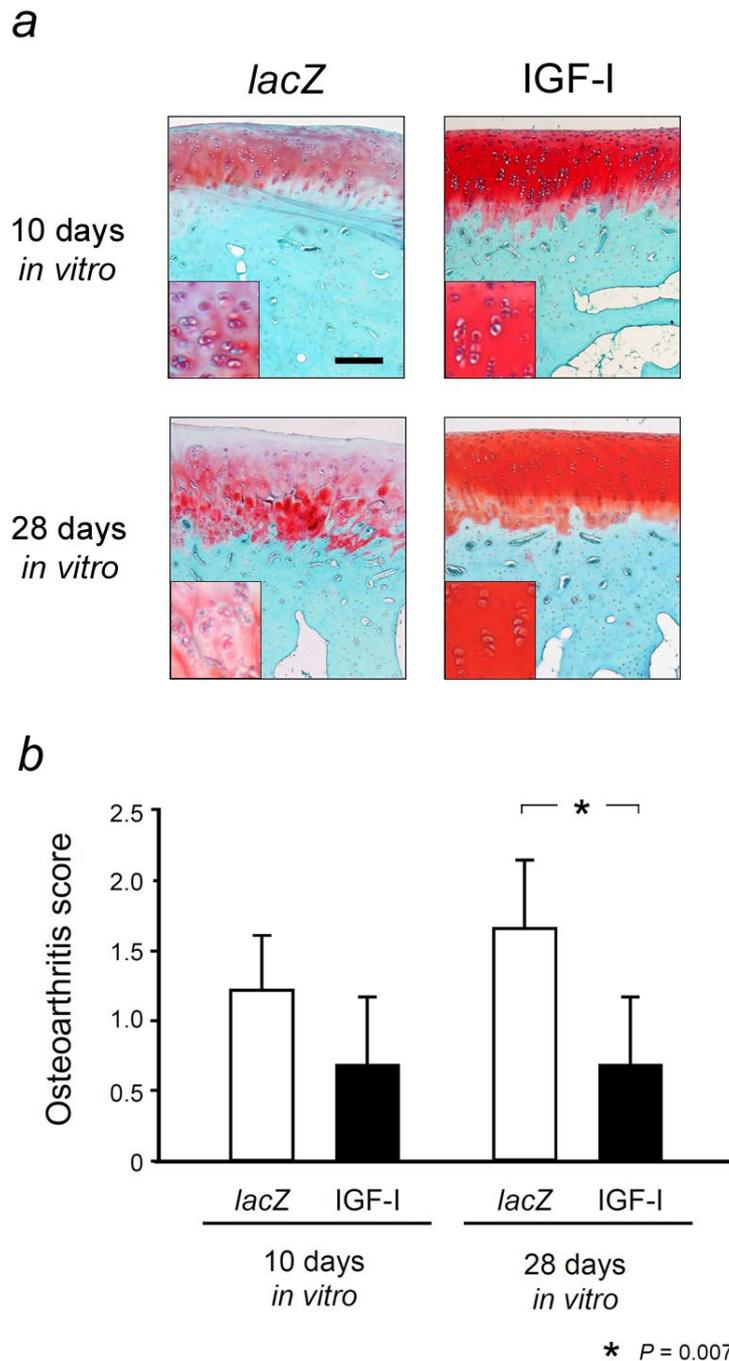


Fig. 9. Osteoarthritic changes in the articular cartilage adjacent to the defects in the trochlea after 28 weeks *in vivo*. **(a)** Representative histologic sections of the articular cartilage adjacent to the osteochondral defects 28 weeks after the implantation of tissue-engineered constructs based on chondrocytes overexpressing *lacZ* (left column) and IGF-I (right column) after 10 d (top images) or 28 d (bottom images) of bioreactor cultivation *in vitro*. All sections represent a 2 mm² area of articular cartilage adjacent to the defect (excluding the area of integration that is independently scored) and have a histological rating equal to the mean score for its respective treatment group. Insets are highly magnified views of representative regions of the middle zone of the articular cartilage. Photomicrographs were obtained using standardised photographic parameters, including light intensity. Safranin O-fast green. Scale bar = 250 μ m. **(b)** Semi-quantitative microscopic evaluation of osteoarthritis changes in the articular cartilage adjacent to the defect in the trochlea after 28 weeks *in vivo* were evaluated using a grading system according to Solchaga *et al.* (2000), which includes single parameters of cellularity, staining intensity and cluster formation. Data are expressed as mean \pm S.D.

improve tissue engineering of cartilage to an animal model and investigated the effects of two different periods of *in vitro* construct cultivation, both on cartilage repair and osteoarthritis development in the adjacent cartilage.

The most important finding was the enhanced cartilage repair following overexpression of IGF-I tissue-engineered constructs *in vivo*. As the animals were allowed unrestricted activity postoperatively under full-load bearing to maximise exposure of the defects to loading, these data suggest that even under physiological loads, resilience was better for the IGF-I-treated defects than for the controls. This approach may be advantageous compared to the implantation of isolated native (Brittberg *et al.*, 1994) or genetically modified (Madry *et al.*, 2011a; Madry *et al.*, 2011b) cells or to direct gene transfer approaches (Evans

et al., 2011). So far, only few studies have been performed to test the concept of tissue-engineered cartilage based on genetically modified cells for articular cartilage repair. Chondrocytes transfected with a bone morphogenetic protein 7 (BMP-7) gene and encapsulated in collagen-fibrin gels that were statically cultured for 2 weeks led to improved repair of cartilage defects after 12 weeks in rabbits *in vivo* (Che *et al.*, 2010). When de-differentiated rabbit chondrocytes transduced *ex vivo* with a recombinant baculoviral BMP-2 vector were cultured in scaffolds for 3 weeks, cartilage repair was improved at 8 weeks following transplantation in rabbit osteochondral defects (Chen *et al.*, 2009).

Previous *in vivo* studies have demonstrated the potential of IGF-I delivered as protein to enhance the repair of

experimental chondral (Hunziker and Rosenberg, 1996) and osteochondral defects (Fortier *et al.*, 2002; Nixon *et al.*, 1999). Similarly, improved repair also resulted following transplantation of IGF-I-overexpressing isolated cells such as adenovirally-transduced perichondrial cells (Gelse *et al.*, 2003) and transfected (Madry *et al.*, 2005; Orth *et al.*, 2011) or adenovirally-transduced articular chondrocytes (Goodrich *et al.*, 2007). Interestingly, the positive immunoreactivity to type-I and -II collagen supports (fibro-) cartilaginous repair of the defect, whereas Safranin O staining of the same tissues was lower compared with previous studies without or with transplantation of IGF-I-overexpressing cells (Madry *et al.*, 2005; Madry *et al.*, 2011b; Orth *et al.*, 2012a; Orth *et al.*, 2011). When related to the results following implantation of IGF-I-overexpressing articular chondrocytes in alginate spheres, average total score values for both control and treatment groups were better after 14 weeks *in vivo* compared to the results after 28 weeks of the present study (Madry *et al.*, 2005; Orth *et al.*, 2012a; Orth *et al.*, 2011). Whether these discrepancies are due to differences in the structure of the repair tissue at the different observation times *in vivo* or reflect a possible effect of the scaffold biomaterial on proteoglycan deposition remains to be determined.

Providing an IGF-I gene sequence *via* a tissue-engineered construct allows for a spatially defined delivery of a therapeutic factor to articular cartilage defects while simultaneously supplying a template filling the defect (Evans, 2011; Ivkovic *et al.*, 2011). For example, when porous collagen-glycosaminoglycan scaffolds were loaded with IGF-I, a large part of the protein was released as a burst within the first day (Mullen *et al.*, 2010). Here, secretion of IGF-I was significantly elevated until day 18 compared to *lacZ* constructs. Of note, levels of IGF-I in the conditioned medium may not reflect the total extent of transgene expression, as some of the IGF-I may have been associated with the newly deposited extracellular matrix of the constructs, possibly potentiating its effects (Jones *et al.*, 1993). The positive immunoreactivities to β -gal and IGF-I in the respective repair tissues of defects indicates prolonged expression of the transgene and presence of the growth factor following orthotopic implantation, as the half-life of free IGF-I is very short (Frystyk *et al.*, 1999). This IGF-I produced by the chondrocytes within the tissue-engineered construct may have not only autocrine, but also paracrine effects on the mesenchymal cells from the subchondral bone marrow, as IGF-I can induce chondrogenesis in mesenchymal stem cells (Im *et al.*, 2006; Longobardi *et al.*, 2006; Uebersax *et al.*, 2008). In contrast, IGF-I levels in the synovial fluid were normal – a desirable outcome for a possible clinical translation – where intra- and extra-articular side-effects of the therapeutic factor need to be avoided. Also, IGF-I overexpression did not result in adverse effects such as synovitis or infiltration of immune cells. These data are consistent with preceding reports (Brower-Toland *et al.*, 2001; Madry *et al.*, 2005; Madry *et al.*, 2002; Smith *et al.*, 2000) and correspond well with the normal IGF-I levels already after day 4 post-operation when 25,000 ng recombinant IGF-I (a \sim 1,000-fold higher dose than the \sim 2.2 ng secreted by the constructs on day 2) were applied to osteochondral defects

in a horse model (Fortier *et al.*, 2002). Such normal IGF-I levels may likely result from a decreased clearance of the IGF-I caused by its retention within the newly formed cartilaginous matrix of the repair tissue, for example by interaction with IGF-I binding proteins (Garcia *et al.*, 2003; Sah *et al.*, 1994; Weimer *et al.*, 2012; Yoon and Fisher, 2008), increased binding to IGF-I receptors and extracellular matrix components, and/or elution of the protein. Altogether, this suggests that at least some of the transfected, transplanted allogeneic chondrocytes continue to produce the growth factor within their new matrix (besides the endogenous IGF-I). Transgene expression in cartilage defects has been previously shown to last for at least 36 weeks *in vivo*, following implantation of allogeneic chondrocytes modified by adenoviral-mediated gene transfer in fibrin glue into osteochondral defects in an equine model (Hidaka *et al.*, 2003). With the potential use of alternative strategies (e.g., use of viral vectors), transfection efficiency and expression of the transgene *in vivo* may be further increased (Cucchiari *et al.*, 2005; Kupcsik *et al.*, 2009).

Another key finding is the influence of the *in vitro* cultivation period (Sekiya *et al.*, 2002) of the engineered cartilage on defect repair *in vivo*. Jin *et al.* (2010) reported better repair when using mature constructs. Histological scores were also improved when engineered cartilage, based on BMP-2 overexpressing chondrocytes, was cultured for 3 weeks compared with 1-week cultivation (Chen *et al.*, 2009). Interestingly, immature constructs from non-modified chondrocytes with lesser mechanical properties allowed for a better integration (Khan *et al.*, 2008) than mature constructs (Obradovic *et al.*, 2001). We have previously shown that engineered cartilage composed of genetically modified chondrocytes overexpressing IGF-I contains 10-fold more glycosaminoglycans, 2-fold more collagens, and 1.5-fold more DNA and had a 3.6-fold higher equilibrium modulus than constructs based on *lacZ*-overexpressing chondrocytes after 28 days *in vitro* (Madry *et al.*, 2002). In general, the morphological, biochemical, and biomechanical characteristics of engineered cartilage composed of naïve (Freed *et al.*, 1998) and genetically modified chondrocytes (Madry *et al.*, 2002) continue to improve for at least the first 4-6 weeks of bioreactor culture *in vitro*, (Freed *et al.*, 1998; Madry *et al.*, 2002; Miot *et al.*, 2012; Miot *et al.*, 2006). Thus, more mature constructs contain more cells and extracellular matrix, reflected by an increase in Safranin O staining intensity (Madry *et al.*, 2002), which may protect the chondrocytes from mechanical stresses *in vivo* (Ball *et al.*, 2004), together with persisting transgene expression (Madry *et al.*, 2002). These improved structural characteristics, as well as a possibly higher concentration of paracrine factors secreted by the higher number of cells within the constructs may be involved in the enhanced repair process. Of note, construct maturity – as determined by the wet weights of the IGF-I constructs – was significantly higher than in *lacZ* constructs at both time points *in vitro*, highlighting the positive effect of IGF-I on structural parameters of tissue-engineered cartilage *in vitro* (Madry *et al.*, 2002). The decreasing wet weights of constructs of both groups over time most likely result from a degradation and resorption of the scaffold

that exceeded the deposition of new matrix, as also seen in other cell-scaffold systems (Meinel *et al.*, 2004). These findings underscore the importance of the duration of the *in vitro* cultivation period for articular cartilage repair based on tissue-engineered constructs *in vivo* (Deponti *et al.*, 2012; Miot *et al.*, 2012).

A clinically very critical consequence of a cartilage defect is the osteoarthritic degeneration (Martel-Pelletier *et al.*, 2008) of the adjacent cartilage (Lefkoe *et al.*, 1993; Schinhan *et al.*, 2012). Importantly, implantation of IGF-I constructs cultivated for 28 d *in vitro* led to a significantly reduced degree of osteoarthritis in the adjacent articular cartilage. It is possible that the improved defect repair translated into a better resilience to withstand the loads applied to the defects. However, it may also represent a protective paracrine effect of the secreted IGF-I on the adjacent articular cartilage (Clemmons *et al.*, 2002; Martel-Pelletier *et al.*, 1998; Rogachefsky *et al.*, 1993). Although upregulation of IGF-I gene expression has been demonstrated in human osteoarthritic articular cartilage (Middleton and Tyler, 1992), the response of osteoarthritic chondrocytes to IGF-I is reduced (Loeser *et al.*, 2000). In addition, the duration of transgene expression *in vivo* could play a role. Osteoarthritic changes in human cartilage decreased following targeted overexpression of IGF-I *in situ* after 90 d (Weimer *et al.*, 2012). In the present study, IGF-I expression was prolonged in IGF-I constructs of both cultivation periods *in vivo*. Whether the maturity of the cartilaginous construct affects the duration of transgene expression *in vivo* may be the subject of further studies.

Limitations of this study include: the lack of a detailed *in vitro* evaluation of the constructs implanted in the present study; the use of a small animal model as opposed to a larger animal; the lack of a time-course of transgene expression *in vivo*; the absence of a biomechanical evaluation of the constructs and the repair tissue, as well as animal gait analysis.

As the experimental foundation to generate cartilage by means of tissue engineering is well established (Angele *et al.*, 1999; Freed *et al.*, 1999; Grad *et al.*, 2011; Langer and Vacanti, 1993; Martin *et al.*, 2007; Mikos *et al.*, 2006; Stoddart *et al.*, 2009), its principle has already found entry into the surgical treatment of large symptomatic articular cartilage defects – as evidenced by the widespread use of matrix-induced autologous chondrocyte implantation (ACI), where the cells are attached to a biodegradable scaffold (Brittberg, 2009). Such supportive biomaterials may be similarly used to culture genetically modified human articular chondrocytes in bioreactors, with the aim of a spatially controlled application of the modified chondrocytes *in vivo*. When delivered in a clinical setting, a construct that actively supports chondrogenesis would then fill the focal cartilage defect (Madry and Cucchiari, 2011). In view of this consideration, further steps are necessary to move this approach towards a clinical translation. These include proofs of its safety in preclinical and clinical trials, the selection of the optimal therapeutic gene, the establishment of the optimal cell number per defect surface area needed for implantation and the choice of an appropriate gene vector system that allows for efficient gene transfer and controlled gene expression

(Madry and Cucchiari, 2011). Here, low-level, long-term transgene expression may be more appropriate when compared to high-level, short-term gene expression as the repair tissue develops over a long period of time (Safran and Seiber, 2010; Madry *et al.*, 2011a). *Ex vivo* nonviral gene transfer provides such characteristics and therefore holds clinical potential (Al-Dosari and Gao, 2009). During the bioreactor cultivation, the cartilaginous constructs may additionally be subjected to direct mechanical forces (Salzmann *et al.*, 2009), allowing for an even more defined stimulation based on the interaction between growth- or transcription-factors and dynamic loading (Kupcsik *et al.*, 2009; Mauck *et al.*, 2003).

In summary, application of gene transfer to cartilage tissue engineering opens doors that would remain closed to either technology working alone. This concept of genetically enhanced tissue engineering of cartilage may be used to evaluate the effect on articular cartilage repair of genes involved in chondrogenesis. It may also provide improved future clinical treatment options for acute cartilage defects.

Conclusions

Spatially defined delivery of a gene sequence within a tissue-engineered cartilaginous construct that actively supports chondrogenesis enhances the repair of osteochondral defects *in vivo*. Genetically enhanced tissue engineering provides a versatile tool to evaluate potential therapeutic genes and to improve our comprehension of the process of new tissue formation within articular cartilage defects. Insights gained with additional explorations using this model may lead to more effective treatment options for acute cartilage defects.

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Discussion with Reviewers

Reviewer I: There are still transfected cells detectable after 24 weeks. When do you expect that no transfected cells will be detectable in the tissue and is there a need to modify the lifetime of the transfected cells?

Authors: Longer studies are required to answer this important question. As the recombinant protein was only detected within the repair tissue, without intra-articular effects, we believe that there is no need to further expand the lifetime of the transfected and implanted chondrocytes.

Reviewer I: The authors correctly highlight that the described engineered cartilage model could not only help understanding the process of articular cartilage repair, but also possibly lead to a new treatment option for acute cartilage defects. In view of the latter consideration, which further steps would the authors envision to be necessary to move the approach towards a clinical translation?

Authors: The experimental foundation to generate cartilage by means of tissue engineering is well established, and its principle has already found entry into the surgical treatment of large symptomatic articular cartilage defects, as evidenced by the widespread use of matrix-induced autologous chondrocyte implantation (ACI), where the cells are attached to a biodegradable scaffold. Such supportive biomaterials may be similarly used to culture genetically modified human articular chondrocytes in bioreactors with the aim of a spatially controlled application of the modified chondrocytes *in vivo*. When delivered in a clinical setting, a construct that actively supports chondrogenesis would then fill the focal cartilage defect. In view of this consideration, further steps necessary to move this approach towards clinical translation include proofs of its safety in preclinical and clinical trials, the selection of the optimal therapeutic gene(s), the establishment of the optimal cell number per defect surface area needed for implantation and the choice of an appropriate gene vector system that allows for efficient gene transfer and controlled gene expression. Here, low-level, long-term transgene expression may be more appropriate compared to high-level, short-term gene expression as the repair tissue develops over a long period. *Ex vivo* nonviral gene transfer provides such characteristics and therefore holds clinical potential. During the bioreactor cultivation, the cartilaginous constructs may additionally be subjected to direct mechanical forces, allowing for an even more defined stimulation based on the interaction between growth- or transcription factors and dynamic loading.

Reviewer II: Preventive effects of the constructs on subsequent arthritis in adjacent cartilage are of great interest. The authors suggested a protective paracrine effect of the secreted IGF-1 on adjacent cartilage as a potential mechanism, but there are two conflicting results in the present study. In Figs. 3 and 4, immunohistochemistry demonstrated the prolonged expression of IGF-1 in the reparative tissues. However, the other data revealed that there was no significant difference in IGF-1 levels in the

lavage fluids of the knees between IGF-1 and the *lacZ* group. The reviewer would like the authors to explain such discrepancy in IGF-1 expression *in vivo*.

Authors: Providing an IGF-I gene sequence *via* a tissue engineered construct allows for a spatially defined delivery of a therapeutic factor to articular cartilage defects, while simultaneously supplying a template filling the defect (Evans, 2011; Ivkovic *et al.*, 2011). For example, when porous collagen-glycosaminoglycan scaffolds were loaded with IGF-I, a large part of the protein was released as a burst within the first day (Mullen *et al.*, 2010). The positive immunoreactivity to IGF-I in the repair tissue of defects treated with both immature and mature IGF-I constructs, together with the positive immunoreactivity to β -gal in the repair tissue of defects treated with both immature and mature *lacZ* constructs, indicates prolonged expression of the transgene. This IGF-I produced by the chondrocytes within the tissue-engineered construct may have not only autocrine, but also paracrine effects on the mesenchymal cells (MSCs) from the subchondral bone marrow, as IGF-I can induce chondrogenesis in MSCs (Im *et al.*, 2006; Longobardi *et al.*, 2006; Uebersax *et al.*, 2008). In contrast, IGF-I levels in the synovial fluid were normal, a desirable outcome for a clinical translation, where side-effects of the therapeutic factor need to be avoided. Also, IGF-I overexpression did not result in adverse effects such as synovitis or infiltration of immune cells. These data are consistent with preceding reports (Brower-Toland *et al.*, 2001; Madry *et al.*, 2005; Madry *et al.*, 2002; Smith *et al.*, 2000) and correspond well to the normal IGF-I levels present already after day 4 post operation when 25,000 ng recombinant IGF-I (a \sim 1,000-fold higher dose than the \sim 2.2 ng secreted by the constructs on day 2) were applied to osteochondral defects in a horse model (Fortier *et al.*, 2002). Such normal IGF-I levels may result from the retention of the IGF-I within the repair tissue, for example by interaction with its binding proteins (Garcia *et al.*, 2003; Sah *et al.*, 1994; Weimer *et al.*, 2012; Yoon and Fisher, 2008), increased binding to IGF-I receptors and extracellular matrix components, and/or elution of the protein.

Reviewer III: The defects were analysed after 24 weeks; do you expect that the transfected cells still produce IGF-I at this time point (if they are still alive)?

Authors: There was positive immunoreactivity to IGF-I in the repair tissue of defects treated with immature and mature IGF-I constructs, together with the positive immunoreactivity to β -gal in the repair tissue of defects that received *lacZ* constructs. Although IGF-I levels in the synovial fluid were not elevated, this suggests that at least some of the transfected, transplanted allogeneic chondrocytes continue to produce the growth factor within their new matrix. Transgene expression in cartilage defects has been previously shown to last for at least 36 weeks *in vivo* following implantation of allogeneic chondrocytes modified by adenoviral-mediated gene transfer in autogenous fibrin glue into 15 mm diameter osteochondral defects in an equine model (Hidaka *et al.*, 2003).