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RESEARCH ARTICLE

Auricular cartilage repair using cryogel scaffolds loaded with BMP-7-expressing primary chondrocytes

S. Odabas[1], G. A. Feichtinger[2], P. Korkusuz[3], I. Inci[1], E. Bilgic[3], A. S. Yar[4], T. Cavusoglu[5], S. Menevse[4], I. Vargel[5] and E. Piskin[1]*

[1]*Chemical Engineering Department and Bioengineering Division and Centre for Bioengineering (Biyomedtek), Hacettepe University, Beytepe, Ankara, Turkey*
[2]*Ludwig Boltzmann Institute for Experimental and Clinical Traumatology/AUVA Research Centre, Austrian Cluster for Tissue Regeneration, European Institute of Excellence on Tissue Engineering and Regenerative Medicine Research (Expertissues EEIG), Vienna, Austria*
[3]*Department of Histology and Embryology, Faculty of Medicine, Hacettepe University, Sihhiye, Ankara, Turkey*
[4]*Department of Medical Biology and Genetics, Faculty of Medicine, Gazi University, Ankara, Turkey*
[5]*Department of Plastic and Aesthetic Surgery, Faculty of Medicine, Kirikkale University, Kirikkale, Turkey*

Abstract

The loss of cartilage tissue due to trauma, tumour surgery or congenital defects, such as microtia and anotia, is one of the major concerns in head and neck surgery. Recently tissue-engineering approaches, including gene delivery, have been proposed for the regeneration of cartilage tissue. In this study, primary chondrocytes were genetically modified with plasmid-encoding bone morphogenetic protein-7 (BMP-7) via the commercially available non-viral Turbofect vector, with the aim of bringing *ex vivo* transfected chondrocytes to resynthesize BMP-7 *in vitro* as they would *in vivo*. Genetically modified cells were implanted into gelatin–oxidized dextran scaffolds and cartilage tissue formation was investigated in 15 × 15 mm auricular cartilage defects *in vivo* in 48 New Zealand (NZ) white rabbits for 4 months. The results were evaluated via histology and early gene expression. Early gene expression results indicated a strong effect of exogenous BMP-7 on matrix synthesis and chondrocyte growth. In addition, histological analysis results exhibited significantly better cartilage healing with BMP-7-modified (transfected) cells than in the non-modified (non-transfected) group and as well as the control. Copyright © 2012 John Wiley & Sons, Ltd.

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Supporting information may be found in the online version of this article.

Keywords **auricular cartilage; primary chondrocytes; plasmid DNA; non-viral; *ex vivo* transfection; bone morphogenetic proteins**

1. Introduction

In head and neck surgery, cartilage tissue reconstruction still needs state-of-the-art approaches to replace the loss of cartilage tissue after trauma, tumour resection and congenital defects, such as microtia and anotia. Using autologous costal cartilage is the traditional method of surgery; however, there may not be enough cartilage tissue available and the type of cartilage may not be suitable for this kind of application. In addition, costal cartilage is difficult to form to a proper shape, which may lead to symmetrical and aesthetic problems (Quatela and Goldman, 1995; Pan *et al*., 2008). Other complications, such as autologous scarcity, pain, donor site-directed morbidity, iatrogenic pneumothorax atelectasis and graft incompatibility are also noted (Koch and Gorti, 2002).

Polymeric biomaterials, such as silicone or porous polyethylene (PE) prostheses, are also a widely used for

*Correspondence to: E. Piskin, Chemical Engineering Department and Bioengineering Division and Centre for Bioengineering (Biyomedtek), Hacettepe University, Beytepe, 06800 Ankara, Turkey. E-mail: piskin@hacettepe.edu.tr; erhanpiskin@biyomedtek.com

reshaping and support of the defect area (Romo *et al*., 2006; Breugem *et al*., 2011). However, there are also some important limitations to these approaches, including requirements for intensive care, infection risk, wear and tear over time and frequent replacement. There is still the possibility that these materials are seen as foreign materials by the body (Koch and Gorti, 2002; Bauer, 2009). Also, all these surgical operations are technically very demanding and multi-step procedures that require well-trained surgeons. In the hand of inexperienced surgeons, results may be poor.

Several promising tissue-engineering approaches for cartilage defect healing and regeneration have been proposed (Osch *et al*., 2004; Chung *et al*., 2006). Rotter *et al*. (2008) have summarized the initial tissue-engineering efforts of *in vivo* and *in vitro* cartilage development for microtia treatment. Yamaoka and colleagues (2006) compared various types of hydrogel matrices for auricular cartilage tissue development. Yoo and co-workers (2005) have evaluated cell behaviour and cartilage formation on hyaluronic acid-modified macroporous poly(D,L-lactic acid-co-glycolic acid) scaffolds.

Recent approaches also include using growth factors or stimulants for engineered therapy. Bone morphogenetic proteins (BMPs) are considered to be good candidates for these treatments (Reddi, 1998; Boyne, 2001; Chubinskaya *et al*., 2007). BMPs are growth factors that act as enhancers for extracellular matrix (ECM) synthesis and are inducers of mitotic activity (Bessa *et al*., 2008). There have been several studies reported using BMPs or other cytokines for articular cartilage repair. Gelse and co-workers (2003) demonstrated articular cartilage repair by gene therapy, using *BMP-2* and growth factor-producing mesenchymal stem cells. Cook and colleagues (2003) reported an articular cartilage defect repair using BMP-7 in a canine model. Madry and Cucchiarini (2011) summarized the clinical potential and challenges of using genetically-modified cells for articular cartilage repair. All these studies reflect persevering attempts in the repair of articular cartilage. However, there are still no significant studies on using genetically modified cells/gene therapy for auricular cartilage repair.

In the study reported here, we aimed to use this promising approach in an auricular cartilage defect. The defects were critical sized, which means they cannot heal spontaneously. We used primary chondrocytes; genetically modified with BMP-7-encoding plasmids, and furthermore applied them together with cryogel scaffolds for healing of cartilage defect in rabbit auriculae. We also followed early phase gene expressions. We attempt here to describe the effects of BMP-7 expressed from genetically modified chondrocytes and whether it could support healing. We here hypothesize that using a tissue-engineering approach with genetically modified BMP-7-expressing cells could be utilized in auricular cartilage defects that may exhibit improved cartilage tissue formation in a defined time.

2. Materials and methods

2.1. Preparation of cryogel scaffolds

All materials were obtained from Sigma-Aldrich (Germany) unless otherwise stated. Gelatin–oxidized dextran scaffolds were prepared according to a previously reported procedure (Incı *et al*., 2012). Briefly, the cross-linker, oxidized dextran (oxDex), was synthesized by oxidation of dextran with sodium periodate in an aqueous medium in the dark at room temperature for 1 h (Maia *et al*., 2005). The oxidized dextran was separated by dialysis and freeze-dried. For scaffold preparation, 2 g gelatin was mixed with an appropriate amount of oxDex and the mixture was transferred into 10 mm diameter tubes, which were then frozen at –12 °C in an ethanol-cooled cryostat for 1 h. The disks were then stored in a freezer at –18 °C for 24 h. The frozen samples were taken from the tube, thawed at room temperature and washed with distilled water.

Pore morphologies of the cryogels were examined using a scanning electron microscope (SEM; JSM-5600LV, Jeol, Japan) at 8 kV. Compression tests were performed on wet scaffolds, using a universal test machine (LR-5 K, Lloyd Instruments, UK). Freeze-dried cryogels (*Mo*; dry weight) were allowed to swell until equilibrium (24 h) and were then weighed (*Me*; swollen weight). The swelling ratio (*Sr*) was calculated using the following equation:

$$Sr = [(Me - Mo)/Mo] \times 100 \quad (1)$$

2.2. Auricular chondrocytes isolation

All procedures were approved by Gazi University Animal Ethics Committee (No. G.Ü.ET-10.059). Auricular cartilage was obtained from a 2 month-old male New Zealand white rabbit under sterile conditions. The cartilage was cut into small pieces and digested in collagenase solution (3 mg/ml in PBS) for 24 h. The solution was then washed twice with Dulbecco's modified Eagle's medium (DMEM)/F12 culture medium. Isolated cells were then seeded into 25 cm^2 flasks and a conditioned medium [DMEM/F12, 10% fetal bovine serum (FBS), 1% L-glutamine, 0.25% penicillin–streptomycin, 0.25% gentamycin, 0.1% insulin]. The chondrocytes were cultured until passage 4 at 5% CO_2 and 37 °C.

2.3. BMP-7 transfection and release studies

The hBMP-7 cDNA containing constitutive expression plasmid pVAX1-hBMP-7 s was provided by the Ludwig Boltzman Institute (Vienna, Austria). Plasmid transfection was performed using a Turbofect *in vitro* transfection kit (Fermentas, USA) according to the manufacturer's instructions. Briefly, 2 µl transfection agents was incubated with 5 µg plasmids for about 15 min at room temperature. The solution was then added to the culture medium in 24-well

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plates. A day later, the cells were trypsinized and seeded onto gelatin/ox-Dex scaffolds (1×10^5 cells/scaffold). BMP-7 release into the culture medium was analysed for up to 14 days, using BMP-7 Elisa Kits (RayBiotech, USA). Non-transfected cells were used as control. In addition, the transfection efficiencies of primary chondrocytes were investigated using a green fluorescent protein (GFP)-encoding plasmid and the transfection efficiencies were calculated. Student's *t*-test was used to determine the effect of hBMP-7 to overall BMP-7 release in the normal and transfected groups.

2.4. Cartilage tissue regeneration *in vivo*

New Zealand (NZ) White rabbits (48 adult males, 12 weeks old, weighing 2300 ± 300 g, with open epiphyses and in healthy skeletal and physiological condition), equivalent to 96 auricle samples, were used in the following four main groups: (a) the defect-only group, which was the control group called group C; (b) the scaffold-only group (group S); (c) scaffold seeded with primary chondrocytes (group N); and (d) scaffold seeded with genetically modified (transfected) primary chondrocytes (group T). In each group, six auricles were operated per month.

In a typical surgical procedure, the rabbit was generally anaesthetized intramuscularly, using ketamine (3 ml) and Alfasime (1 ml). In order to create a critical sized defect, 15×15 mm auricular cartilage tissue was removed 15 mm distal to the radix of each auricle. Critical sized dimensions were selected by considering similar models applied in the literature (Cegielski *et al*., 2008; Haberal Can *et al*., 2008). As seen in Figure 1, a special cutting device was designed and used to remove the standard dimensional cartilage tissue sample. The perichondrium and vascular territory was protected in each case. Note that the disk-shaped scaffolds (diameter 15 mm × height 2 mm) were sterilized with 70% ethanol and dried before implantation. After implantation, the defect was closed with 3.0 Caprosyn sutures (Syneture, Ireland).

In Group C, the defect was closed without any further operation. In Group S, the cryogel scaffolds were implanted in the defect area. In Group N, the scaffold were first placed in the defect area and primary chondrocytes isolated from rabbit auricular cartilage tissues were then injected into the scaffold (1×10^6 cells/scaffold). In the last group (group T), genetically modified primary cells (carrying the BMP-7-expressing plasmid; 1×10^6 cells/scaffold) were used. Cartilage reconstruction was monitored for up to 4 months. At the end of each month, operated cartilage tissue was removed from the defect area and the surrounding tissue.

2.4.1. Early phase gene expression in vivo

In order to investigate the effects of genetic modification, early gene expression of selected genes was performed in another set of animal studies. Here, 12 NZ White rabbits (adult males, 12 weeks old) were used; three animals/auricles were used at each time-point for each treatment group. Others were kept as controls. Gene expression analysis was performed on normal and genetically modified cell groups. Non-operated tissue was used as a control. All animals were operated as described previously. Samples were collected from the implant sites on days 3, 7, 14 and 30 and all samples were immediately put into liquid nitrogen and kept at –80 until processed.

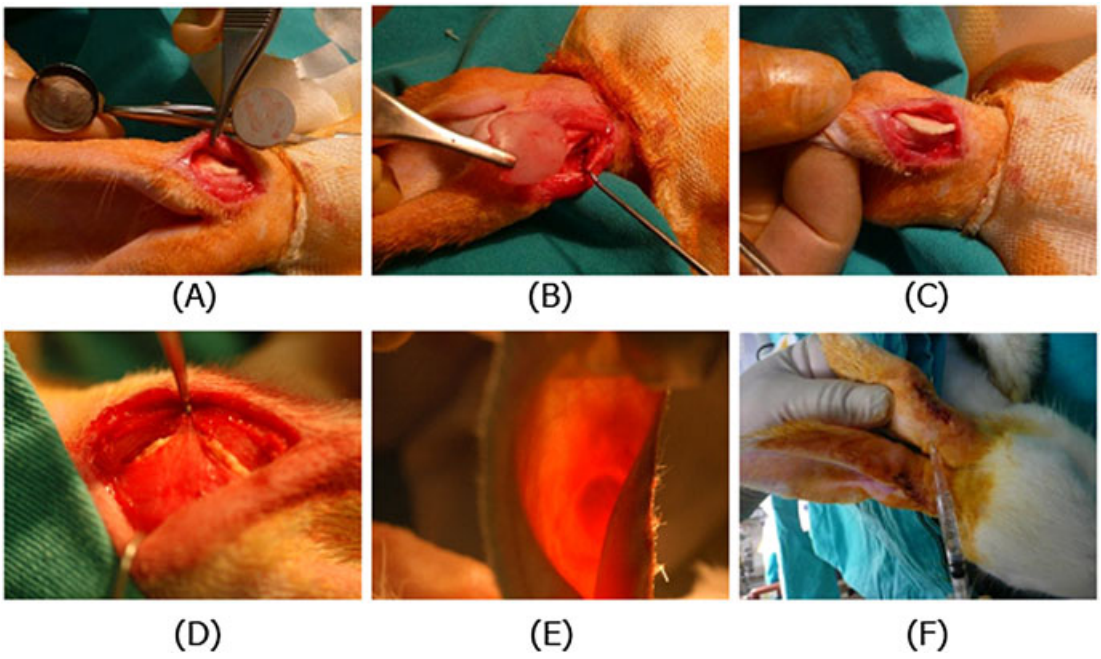

Figure 1. Rabbit ear critical size defect model: (A) removal of auricular cartilage using a special cutting device; (B) intact auricular cartilage removed from the defect area; (C) cryogel scaffolds implanted in the defect area; (D) replacing the perichondrium in the original site/position; (E) imaging of the transplanted site by light illumination; (F) injection of cells into the implanted scaffold

Total RNA (approximately 50–100 mg) was isolated from the samples using the TriReagent system (peqGOLD TriFastTM, Peqlab, Erlangen, Germany), according to the manufacturer's instructions. The RNA-containing pellets were treated with approximately 1–5 U RNase-free DNase (DNaseI; Roche Diagnostics, Germany)/μg RNA and incubated at 37 °C for 30 min before being washed with 70% ethanol to prevent DNA contamination. Ethanol was removed by air-drying and the RNA pellets were dissolved in 10–30 μl RNase- and DNase-free water, after which they were air-dried. Isolated RNA was then stored at –80 °C. The yield and quantity of the RNA of each sample was quantified by measuring absorbances at 260 and 280 nm, using a Nanodrop spectrophotometer (NanoDrop ND-1000, USA). Equal amounts of RNA were used for reverse transcription. First-strand complementary DNA (cDNA) was reverse-transcribed from 1 μg total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany), according to the manufacturer's instructions. cDNA products were stored at –20 °C.

For the expressions, *aggrecan*, *BMP-7*, collagen type I (*Col I*) and collagen type II (*Col II*) mRNA levels were measured using a quantitative real-time PCR (qRT–PCR) method with a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the housekeeping gene in order to normalize *aggrecan*, *BMP-7*, *Col I* and *Col II* mRNA expression levels. Probes and exon–exon-spanning primers for each gene assay were designed using the Universal Probe Library (UPL) Assay Design Center (Roche Applied Science, GmbH, Mannheim, Germany). The gene-specific primer sequences (exon–exon junction to allow discrimination between cDNA and genomic DNA) and UPL numbers are provided in Table 1.

The reaction mixture was prepared in a 96-well plate, using LightCycler Taq-Man Master Mix solution (Roche Diagnostics). Negative control experiments of each reaction were performed without the addition of template cDNA. The samples were analysed in triplicate. In order to determine the amplification efficiencies of the target genes, standard curves were constructed from samples used in a series of dilutions for both the gene of interest (GOI) and the housekeeping gene (*GAPDH*). GOI (*aggrecan*, *BMP-7*, *Col I* and *Col II*) and *GAPDH* amplification efficiencies were approximately equal. The data were analysed using LightCycler Software v 3.5 (Roche Diagnostics). Gene expression analysis of *aggrecan*, *BMP-7*, *Col I* and *Col II* was performed using the Relative Expression Software Tool 2005 (REST©) (Pfaffl *et al*., 2002).

2.4.2. Histological evaluation

Histological evaluation was performed at the Department of Histology and Embryology, Faculty of Medicine, Hacettepe University. The observers were two experienced histologists (among the authors) and the samples were evaluated blindly. The auricular specimens were removed and immersed in 10% neutral formalin solution, pH 7.0, at room temperature. Specimens were decalcified in De Castro solution (chloral hydrate, nitric acid and distilled water; Merck, Germany) and all samples were then embedded in paraffin using an automated tissue processor (Leica Westlar, Germany) with vacuum. Serial sections along the entire defect, 5–6 μm thick, were stained with haematoxylin and eosin (H&E), Masson's trichome (MT) and Weigert's elastic stain to assess the general morphology, collagen and elastic fibril amount, respectively. Photomicrographs of each sample were generated using a light microscope (Leica, DMR, Germany) attached to a computerized digital camera (Model DFC 480, Leica Westlar, Germany). Both the Pineda (the system was modified by assessing the presence of the elastic cartilage but not the hyaline cartilage) and the Lohan cartilage histopathology scoring systems were used to evaluate auricular cartilage critical sized defect healing (Lohan *et al*., 2011; Pineda *et al*., 1992).

2.5. Statistical analysis

A prospective randomized-controlled double-blinded *in vivo* study was designed. The independent variables were groups ($n = 8$) and time ($n = 2$) and the dependent variables were histology scores. For statistical analysis, the independent variables were groups and the dependent variables were histology. The normality of distribution and the homogeneity of variances of the sample were established using the Shapiro–Wilk test. All parameters were analysed using the non-parametric Kruskal–Wallis test for multiple comparisons and the Dunn test for *post hoc* analysis. Descriptive statistics were expressed as median, minimum and maximum. Statistical significance was determined using SPSS software v 15.0. The differences were considered significant at $p < 0.05$.

For the gene expression results, statistical significance of differences in mRNA expression were analysed by a pairwise fixed reallocation randomization test as a statistical model included in the relative expression software tool (REST©, Qiagen, USA) developed for group-wise

Table 1. Gene-specific primer and probe sequences

Gene	Forward primer	Reverse primer	UPL probe no.	UPL probe 5′-FAM-sequence-TAMRA-3′
GAPDH	5′-CACAGTTTCCATCCCAGACC-3′	5′-TGGTTTCATGACAAGGTAGGG-3′	25	TGGAGGAG
Aggrecan	5′-CAGGAGGCAGCCAGTGAG-3′	5′-GGTAGAGCTGGCCTGTGGT-3′	28	GCGGCTGG
BMP-7	5′-CCTGTTAACCAGCCAAGTCG-3′	5′-CGCTCGGTTACCTCTGGA-3′	71	CTGGCTGC
Col I	5′-AGAACCCAGCTCGCACCT-3′	5′-CATCCTTGGTTGGGATCG-3′	83	CAGCCACC
Col II	5′-GACCTGCGTCTACCCCAAC-3′	5′-GCTGCTTCTGGCTCTTGC-3′	22	TGGTGGAG

comparison and statistical analysis of relative expression results. The differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Cryogel scaffolds

Figure 2 shows a representative SEM image of the cryogel scaffolds prepared in this study. As seen here, cryogels are highly porous, with large pores (average $> 100\,\mu m$) and interconnective pore morphology, which are very suitable structural properties for porous materials in tissue engineering (Nickerson *et al.*, 2006; Jain *et al.*, 2008).

One of the important properties of these cryogel scaffolds is their swelling ability and behaviour. They swell in aqueous media very rapidly, within a few minutes, and reach their final size in 20–25 min. The average swelling ratio and standard deviation (SD; $n = 5$) is $986.2 \pm 134.0\%$, which is in the range of the data reported in the related literature for similar dextran-based cryogels (Nickerson *et al.*, 2006; Jain *et al.*, 2008).

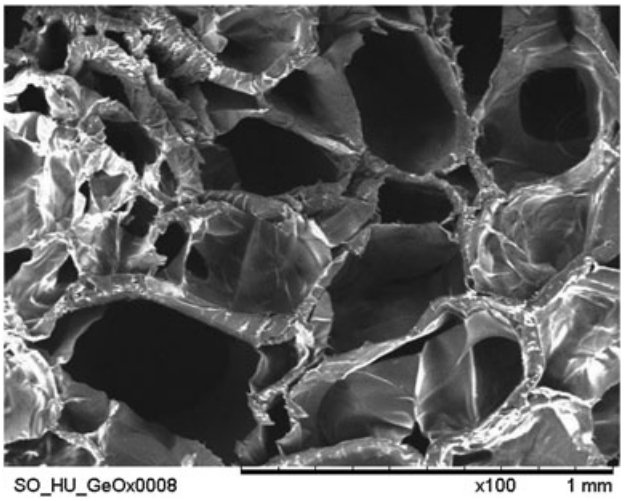

Figure 2. SEM micrograph of the cryogel scaffold used in this study

The average elastic moduli and toughnesses, with the corresponding standard deviations ($n = 5$), obtained from the compression tests were 8.4 ± 1.8 kPa and $261.0 \pm 67.5\,kJ/m^3$, respectively, which are comparable to the mechanical properties of similar cryogels/hydrogels reported in the literature (Incı *et al.*, 2012; Nickerson *et al.*, 2006; Al-Munajjed and O'Brien, 2009).

3.2. BMP release studies

Figure 3 depicts BMP-7 release from cryogels loaded with genetically modified chondrocytes (transfected, carrying BMP-7-expressing plasmids) and scaffolds seeded with non-transfected chondrocytes obtained in the *in vitro* cell culture medium, as described in the previous sections. It has been reported that cells on monolayer cultures lose their ability to encode the specific genes for ECM proteins and change their morphology from chondrocytic to fibroblastic (Hiraki *et al.*, 1985; Stokes *et al.*, 2001). As seen in Figure 3, the non-transfected cells also synthesize BMP-7, although to a much lower extent and decreasing with time. As was our aim in this study, transfected cells expressed/released BMP-7 in much higher amounts and for longer periods. There was a significant difference between normal and transfected cells at all time points ($p < 0.01$–0.0001). Schüler (2008) demonstrated similar relevant findings with BMP-7 release from primary chondrocytes, transfected with the pCMV–BMP-7–lipofectamine system in monolayer culture.

We analysed the total BMP-7 (both endogenous and in transfected cells, also exogenous) release to the culture medium. BMP release in this study was also related to transfection efficiency. In our study, we checked our transfection efficiencies in primary chondrocytes, which were 15–20%, with a model plasmid (EGFP). Logically, higher transfection efficiency causes higher amounts of BMP to be produced. There are several issues that strongly affect the transfection efficiency. In brief, primary cells are very sensitive to transfection, and isolation procedures from the tissue can also affect efficiency (Dinser *et al.*, 2001). Also, although

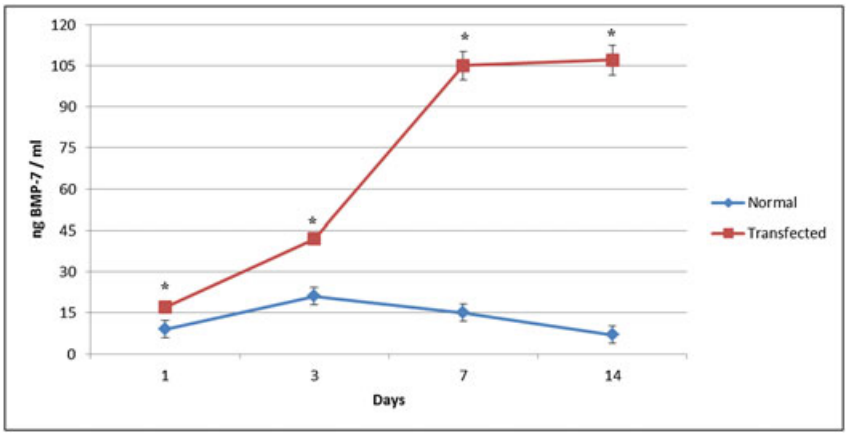

Figure 3. BMP-7 release (both endogenous and exogenous) from cryogels scaffold carrying either genetically modified (transfected) primary chondrocytes or non-transfected chondrocytes. Production per day ($n = 3$); *$p < 0.05$ compared with normal and transfected cells

transfections with non-viral vectors are much safer in primary cell culture, transfection efficiencies with non-viral vectors are lower than with viral vectors (Al-Dosari and Gao, 2009).

3.3. Cartilage tissue regeneration *in vivo*

A detailed histological evaluation was performed in all groups. Figures 4 and 5 show the average Pineda and Lohan scores of each group within 4 months. These scoring systems are widely used and well established for cartilage repair assessment in the recent literature and studies. Although they are described as semi-quantitative systems, they cover and combine all of the morphological criteria for qualitative and quantitative cartilage repair. In Lohan's scoring system, the cartilage cell morphology, extracellular matrix production and inflammation criteria are scored separately. In Pineda's scoring system the filling percentage of the defect, the reconstruction of osteochondral junction, extracellular matrix staining by Weigert elastic stain (which reveal the presence of elastic fibres), the amount of chondrocyte and non-chondrocyte cells and the amounts of fibrous and elastic cartilage are scored separately. The final total scores that included all these data were statistically analysed.

In addition to this semi-quantitative scoring of newly formed elastic cartilage, all groups were also analysed by histological staining. Figure 6 shows histological staining for all groups.

According to histological assessment, all of the defects were closed by the formation of fibrous connective tissue and cartilage (fibrous and elastic) at the end of 4 months. Repair in the control group was mainly by way of connective tissue formation. The biomaterial degraded over time, but had not degraded entirely at the end of 4 months in any of the groups. All the groups exhibited the formation of various amounts of cartilage islands and several of them

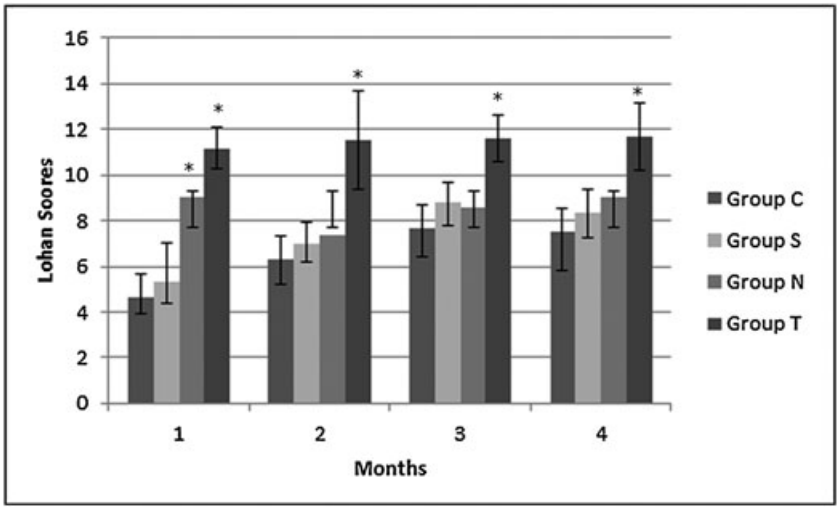

Figure 4. Lohan histological scoring of each group ($n = 6$); C, control; S, scaffold; N, scaffold with chondrocytes; T, scaffold with transfected cells; *$p < 0.05$

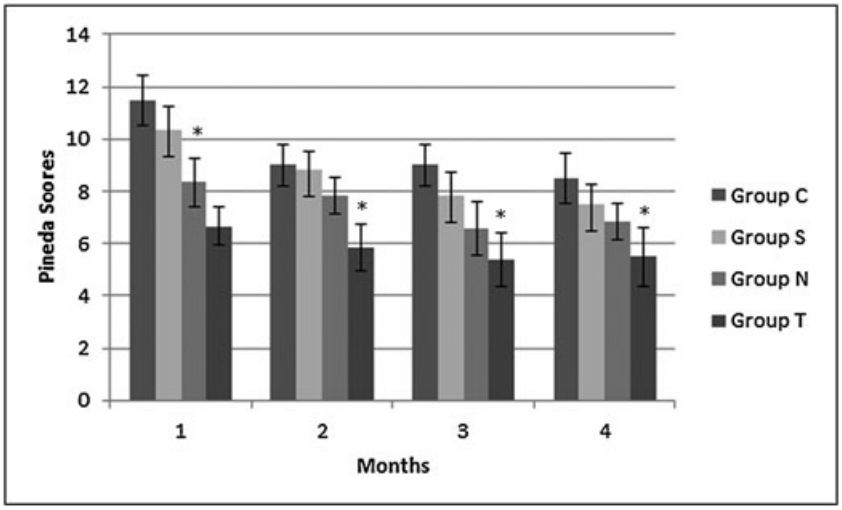

Figure 5. Pineda histological scoring of each group ($n = 6$); C, control; S, scaffold; N, scaffold with chondrocytes; T, scaffold with transfected cells; *$p < 0.05$

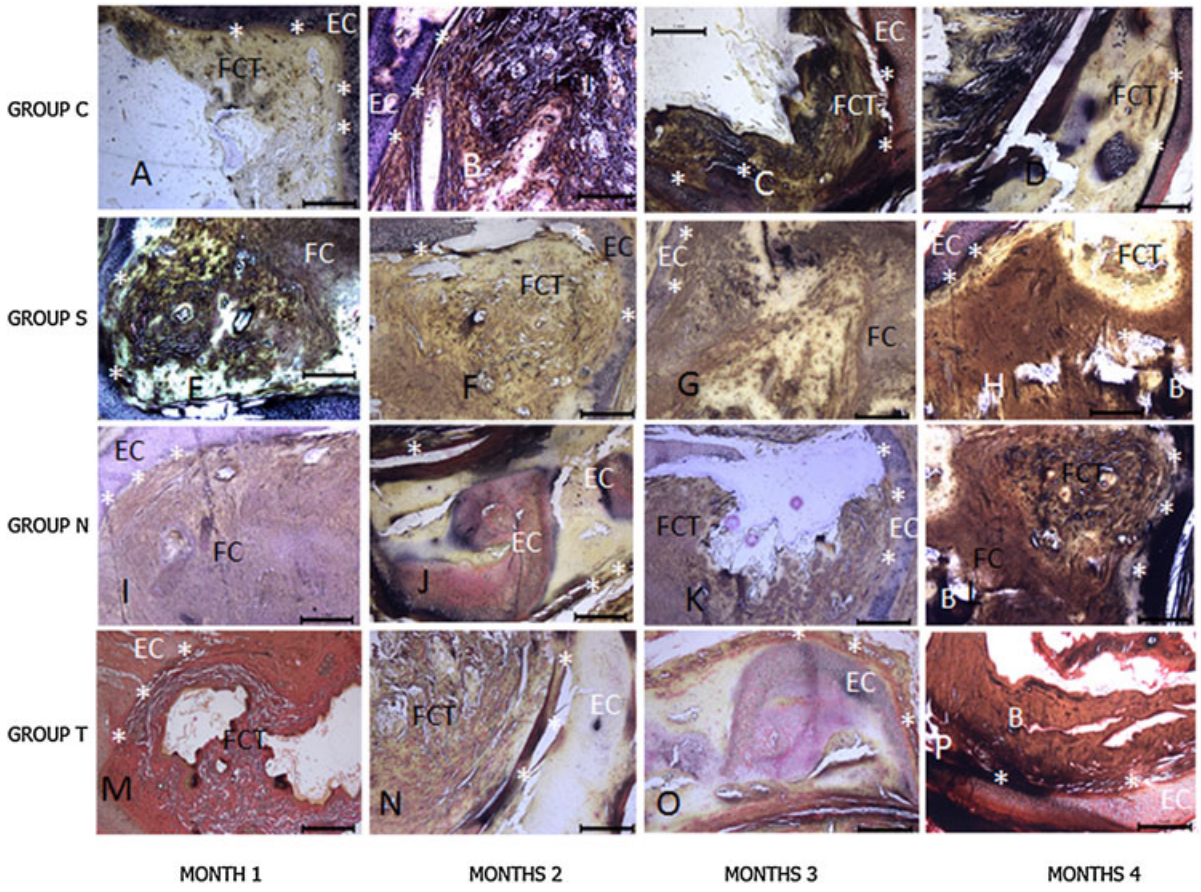

Figure 6. Panoramic views of the cartilage defects at lowest (×25) magnification. Weigert elastic stain: elastic fibres stain black with this dye; bone, fibrous cartilage and connective tissue stain purple to yellow; the pink to purple colour of the extracellular matrix exhibits an intermediate stage of the repairing or remodelling cartilage before the accumulation of elastic fibres (J, M, P). *Edge between the defect and adjacent elastic cartilage which is normal or under the remodelling process. (J, O) New elastic cartilage; (H, L, P) bone formation is observed inside the defect. EC, elastic cartilage; FC, fibrous cartilage; FCT, fibrous connective tissue; B, bone. Note that elastic cartilage development is at the highest level in cells in scaffold-applied groups. Bars = 1 mm

revealed a varying quantity of calcified foci within the defects. Both cell-seeded scaffold groups had more Weigert-positive elastic cartilage islands than the other groups. Cartilage formation was of a more fibrous type in the control and scaffold-only groups (groups C and S). Although the scaffold was biocompatible, allowing good guidance for cartilage regeneration, it was not enough to support elastic cartilage formation over a period of 4 months in the entire defect *in vivo*. The new cartilage-like tissue was not completely homogeneous and was sometimes continuous with the fibrous connective tissue throughout the defect in all groups (Figure 6).

The normal and genetically modified (transfected) cells in the scaffold groups (groups N and T) received significantly better Pineda and Lohan defect healing scores than those of the control (group C) and the scaffold-only group (group S) at 1 month (group N, $p = 0.023$ and $p = 0.001$; group T, $p = 0.001$ and $p = 0.001$). The transfected and normal cells with scaffold groups (groups N and T) were significantly better than the scaffold-only group (group S) after 1 month (Figures 4 and 5; group N, $p = 0.03$ Lohan; group T, $p = 0.001$ and $p = 0.001$).

The transfected cells with scaffold group (group T) received significantly better Pineda and Lohan defect healing scores than those of the control at 2 months (group T, $p = 0.001$ and $p = 0.001$). The transfected cells with scaffold group was better than the scaffold-only group (group S) according to Lohan scoring at 2 months ($p = 0.001$; Figure 4). At 3 months, the transfected cells with scaffold group (group T) was better than the control according to Lohan ($p = 0.003$) and the normal cells with scaffold group (group N) was better than the control according to Pineda ($p = 0.007$; Figures 4 and 5). At 4 months, the transfected cells with scaffold group (group T) was better than the control according to Pineda and Lohan ($p = 0.001$ and $p = 0.002$). The transfected cells with scaffold group (group T) was significantly better than the scaffold-only group (group S; $p = 0.012$) according to Lohan after month 4 (Figures 4 and 5). The scaffold-only group (group S) did not significantly improve the cartilage defect healing process in months 1, 2, 3 and 4 compared to control (group C; Figures 4 and 5).

3.4. Early-phase gene expressions

In order to observe the effects of BMP-7 on the early-phase healing process, a separate group of experiments was designed, in which scaffolds carrying primary chondrocytes (normal cells) or their genetically modified (transfected)

forms were implanted to animal ear models (three animals for each), as discussed in the previous section. The samples were taken at days 3, 7, 14 and 30 and the expression of four target genes, *COL I*, *COL II*, *BMP-7* and *aggrecan*, was analysed by qRT–PCR.

The selected factors/genes were related to cartilage tissue formation and ECM synthesis, as follows: BMP-7, which stimulates chondrogenesis and matrix synthesis; COL II, which is the dominant ECM protein in auricular cartilage; Aggrecan, which is a cartilage-specific proteoglycan core protein; and finally COL I, which exists naturally in bone and fibrous cartilage.

Figure 7 shows the fold differences in expression with respect to the control at designated time points; as can be seen, there was an increase in synthesis of all selected factors over time. BMP-7 plays a role in enhancing matrix synthesis in cartilage tissue formation; therefore, if there is activity of BMP-7, matrix protein synthesis should consequently increase (Bessa *et al.*, 2008). In this study the expression of endogenous *BMP-7* was at the mRNA level; however, several reports have shown that exogenous BMPs (that come from the plasmid) can elevate the levels of endogenous BMPs and have an induced effect on the synthesis of mRNAs as well as matrix synthesis and tissue development (Erickson *et al.*, 1997; Chen *et al.*, 1997; Kawai *et al.*, 2006). In addition; Aggrecan, which is a specific matrix protein for cartilage, and Coll II, which is the dominant matrix protein for cartilage, were also increased in both transfected and normal cells with scaffold groups (groups N and T). However, in the transfected cells with scaffold group (group T) these differences were significant and higher at all time points. The effect of the extra BMP-7 can be easily observed 14 days post-operatively as the matrix protein synthesis increases. Even in the late phase (14 and 30 days) there is still a higher expression of Coll II and Aggrecan in group T. Lietman *et al.* (1997) reported similar findings on the stimulation of proteoglycan synthesis by BMP-7. On the other hand, Coll I differences in the normal cells with scaffold group (group N) are not significant at later phases (after 14 days) in the normalized overall results. However, in group T, a higher expression of Coll I was observed at later phases (14 days). This observation can be explained by the role of BMP-7 in bone and cartilage regeneration (Bessa *et al.*, 2008). BMP-7 activates cartilage matrix synthesis and has a role in chondrogenesis; however, it also activates bone formation and dominates the synthesis of bone-related matrix proteins. Therefore, the existence of extra BMP-7 in the microenvironment in the transfected cells with scaffold group (group T) could also have significantly increased the level of Coll I. Briefly; the fold differences of the selected factors in group T were higher than in the normal (untransfected) cells with scaffold group (group N) for every time point.

Cartilage tissue has a limited regenerative capacity, due to its avascular structure. In order to regenerate the tissue, essential growth factors should be present in the microenvironment. The use of genetically engineered cells to enhance cartilage healing and tissue formation was the main target of the present study. In our study, we used a syngenic animal model belonging to an inbred strain. The animals were sufficiently identical and immunologically compatible. In addition, inflammation is a critical point during tissue regeneration. In our study both treatment groups and the control group presented similar inflammation scores,

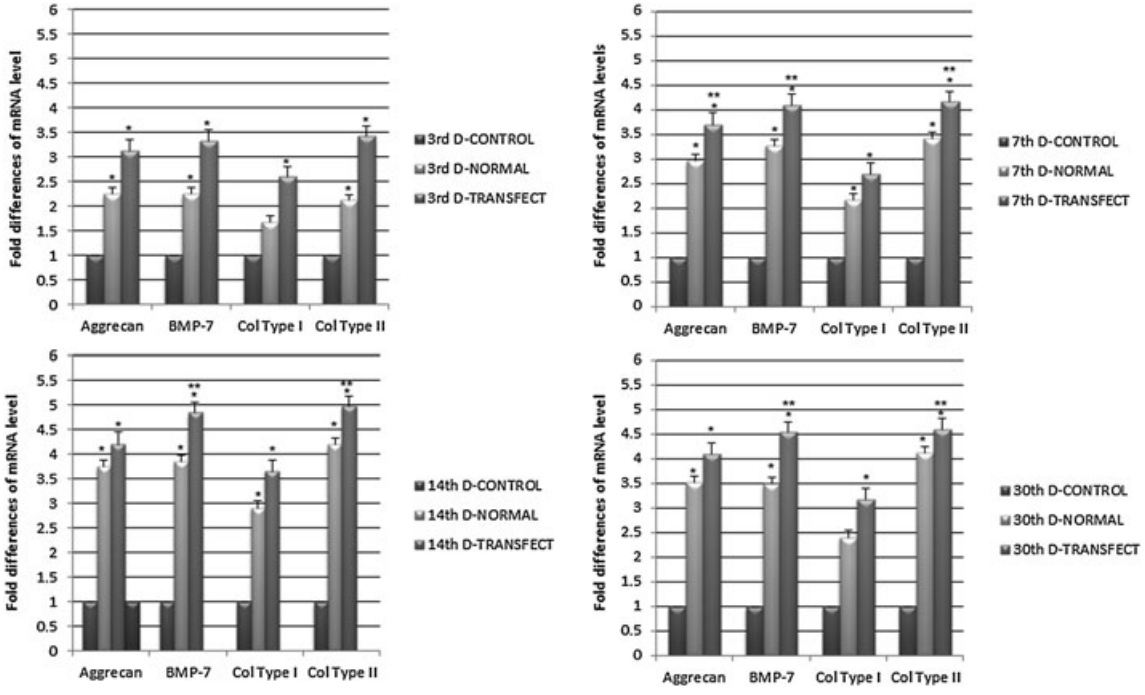

Figure 7. Fold differences in mRNA level of *BMP-7*, *Col I*, *Col II* and *Aggrecan* in cell-applied groups with respect to the control up to 30 days. Non-operated tissue was used as a control. The results were normalized to *GAPDH* as the housekeeping gene: *$p < 0.05$ compared to control and cell-applied groups; **$p < 0.05$ between the normal and transfected cell-applied groups

revealing no obvious immunological response (see Supporting information, Table S1, Table S2). Despite these factors, using a syngenic model may still be seen as a limitation of our study when considering translation into the clinic.

Our histology and gene expression results indicated significantly better cartilage healing and matrix formation in genetically modified cells individually with exogenous BMP-7 (see Supporting information Figure S1, S2 and S3). However, we could not correlate the results of histology and early gene expression together, due to the different variable numbers and incompatible time points, which might be stated as another limitation to our study. Moreover, the expression and release of the plasmid-encoded factors mainly depended on transfection and expression efficiency, which is relatively low in primary cells with non-viral systems. We believe that higher expression efficiency could have an additional effect on healing. Although the histological staining and the results of the semi-quantitative scoring system exhibited a well-organized, almost elastic, tissue formation on the transfected cell-applied group (group T), we believe that further studies are needed to elucidate the exact mechanism of the elastic formation and long-term regeneration.

4. Conclusion

There is still a need for novel therapies for the reconstruction of auricular cartilage. Although there are some surgical approaches, including autologous cartilage grafts or prostheses, they all have some disadvantages and risks. Therefore, auricular reconstruction still remains one the most difficult fields of reconstruction surgery for head and neck surgery.

In this study, we aimed to perform a defect healing model on auricular cartilage that would allow the regeneration as well as the formation of cartilage tissue very similar to native auricular cartilage. We envisage the possible treatment of cartilage defects via tissue-engineering approaches.

Our results demonstrate that proper cartilage tissue was formed at the end of 4 months using genetically modified primary chondrocytes that overexpressed human BMP-7. Significantly improved healing and regeneration was observed, which was attributed to the effect of overexpressed BMP-7.

Conflict of interest

The authors have declared that there is no conflict of interest.

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Supporting information on the internet

The following supporting information may be found in the online version of this article:

Figure S1. Haematoxylin&Eosin micrographs obtained from the edge of the defect

Figure S2. Weigert elastic fibers stain

Figure S3. The fold differences of mRNA levels normalized to the control

Table S1. Lohan sub-scoring for the regeneration of elastic cartilage

Table S2. Pineda modified sub-scoring for the early regeneration of elastic cartilage

References

Al-Dosari MS, Gao X. 2009; Nonviral gene delivery: principle, limitations, and recent progress. *AAPS J* **11**: 671–681.

Al-Munajjed AA, O'Brien FJ. 2009; Influence of a novel calcium-phosphate coating on the mechanical properties of highly porous collagen scaffolds for bone repair. *J Mech Behav Biomed* **2**: 138–146.

Bauer BS. 2009; Reconstruction of microtia. *Plast Reconstr Surg* **124**: 14–26.

Boyne PJ. 2001; Application of bone morphogenetic proteins in the treatment of clinical oral and maxillofacial osseous defects. *J Bone Joint Surg* **83**(suppl 1): 146–150.

Bessa PC, Casal M, Reis RL. 2008; Bone morphogenetic proteins in tissue engineering: the road from the laboratory to the clinic, part I (basic concepts). *J Tissue Eng Regen Med* **2**: 1–13.

Breugem CC, Stewart KJ, Kon M. 2011; International trends in the treatment of microtia. *J Craniofac Surg* **4**: 1367–1369.

Cegielski M, Dziewiszek W, Zabel M, *et al*. 2008; Experimental application of xenogenous antlerogenic cells in replacement of auricular cartilage in rabbits. *Xenotransplantation* **6**: 374–383.

Chen D, Harris MA, Rossini G, *et al*. 1997; Bone morphogenetic protein 2 (*BMP-2*) enhances *BMP-3*, *BMP-4* and bone cell differentiation marker gene expression during the induction of mineralized bone matrix formation in cultures of fetal rat calvarial osteoblasts. *Calcif Tissue Int* **60**: 283–290.

Chubinskaya S, Hurtig M, Rueger DC. 2007; OP-1/BMP-7 in cartilage repair. *Inter Orthop* **6**: 773–781.

Chung C, Mesa J, Randolph MA, *et al*. 2006; Influence of gel properties on neocartilage formation by auricular chondrocytes photoencapsulated in hyaluronic acid networks. *J Biomed Res A* **77**: 518–525.

Cook SD, Patron LP, Salkeld LS, *et al*. 2003; Repair of articular cartilage defects with osteogenic protein-1 (BMP-7) in dogs. *J Bone Joint Surg* **85**: 116–123.

Dinser R, Kreppel F, Zaucke F, *et al*. 2001; Comparison of long-term transgene expression after non-viral and adenoviral gene transfer into primary articular chondrocytes. *Histochem Cell Biol* **116**: 69–77.

Erickson DM, Harris SE, Dean DD, *et al*. 1997; Recombinant bone morphogenetic protein (BMP)-2 regulates costochondral growth plate chondrocytes and induces expression of BMP-2 and BMP-4 in a cell maturation-dependent manner. *J Orthop Res* **15**: 371–380.

Gelse K, von der Mark K, Aigner T, *et al*. 2003; Articular cartilage repair by gene therapy using growth factor-producing mesenchymal cells. *Arthrit Rheumat* **48**: 430–441.

Haberal Can I, Atilla P, Cakar AN, *et al*. 2008; An animal study on cartilage healing using auricular cartilage as a model. *Eur Arch Otorhinolaryngol* **3**: 307–311.

Hiraki Y, Yutani Y, Fukuya M, *et al*. 1985; Differentiation and dedifferentiation of cultured chondrocytes: increase in monomeric size of 'cartilage-specific' proteoglycans by dibutyryl cyclic AMP and complete inhibition of their synthesis by retinoic acid. *Biochem Int* **2**: 267–272.

Incı I, Kirsebom H, Galaev IG, *et al*. 2012; Gelatin cryogels cross-linked with oxidized dextran and containing freshly formed hydroxyapatite as potential bone tissue engineering scaffolds. *J Tissue Eng Regen Med* doi: 10.1002/term.1464.

Jain E, Srivastava A, Kumar A. 2008; Macroporous interpenetrating cryogel network of poly(acrylonitrile) and gelatin for biomedical applications. *J Mater Sci Mater Med* **20**(suppl 1): 173–179.

Kawai M, Bessho K, Maruyama H, *et al*. 2006; Simultaneous gene transfer of bone morphogenetic protein (*BMP*)*-2* and *BMP-7* by *in vivo* electroporation induces rapid bone formation and *BMP-4* expression. *Musculoskel Disord* **7**: 62–72.

Koch JR, Gorti K. 2002; Tissue engineering with chondrocytes. *Facial Plastic Surg* **18**: 59–68.

Lietman AS, Yanagishita M, Sampath TK, *et al*. 1997; Stimulation of proteoglycan synthesis in explants of porcine articular cartilage by recombinant osteogenic protein-1 (bone morphogenetic protein-7). *J Bone Joint Surg* **79A**: 1132–1137.

Lohan A, Marzahn U, El Sayed K, *et al*. 2011; *In vitro* and *in vivo* neo-cartilage formation by heterotopic chondrocytes seeded on PGA scaffolds. *Histochem Cell Biol* **136**: 57–69.

Maia J, Ferreira L, Carvalho R, *et al*. 2005; Synthesis and characterization of new injectable and degradable dextran-based hydrogels. *Polymer* **46**: 9604–9614.

Madry H, Cucchiarini M. 2011; Clinical potential and challenges of using genetically modified cells for articular cartilage repair. *Croat Med J* **52**: 245–261.

Nickerson MT, Farnworth R, Wagar E, *et al*. 2006; Some physical and microstructural properties of genipin-crosslinked gelatin–maltodextrin hydrogels. *Int J Biol Macromol* **38**: 40–44.

Osch GJVM, Mandl EW, Jahr H, *et al*. 2004; Considerations on the use of ear chondrocytes as donor chondrocytes for cartilage tissue engineering. *Biorheology* **41**: 411–421.

Pan B, Jiang H, Guo D, Huang C, Hu S, Zhuang H. 2008; Microtia: ear reconstruction using tissue expander and autogenous costal cartilage. *J Plast Reconstr Aesthet Surg* **61**(Suppl 1): S98–103.

Pfaffl MW, Horgan GW, Dempfle L. 2002; Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* **9**: 36.

Pineda S, Pollack A, Stevenson S, *et al*. 1992; A semiquantitative system for histologic grading of articular cartilage repair. *Acta Anat* **143**: 335–340.

Reddi A. 1998; Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nat Biotech* **16**: 247–252.

Romo T III, Presti PM, Yalamanchili HR. 2006; Medpor alternative for microtia repair. *Facial Plast Surg Clin North Am* **14**: 129–136.

Rotter N, Steiner A, Scheithauer M. 2008; Reconstruction of auricular cartilage using tissue-engineering techniques. *Op Techn Otolaryngol* **19**: 278–284.

Quatela VC, Goldman ND. 1995; Microtia repair. *Facial Plast Surg* **11**: 257–273.

Schüler G. 2008; Overexpression of *BMP-7* in Monolayer Chondrocytes. Diploma Study, University of Vienna.

Stokes DG, Liu G, Dharmavaram R, *et al*. 2001; Regulation of type II collagen gene expression during human chondrocyte dedifferentiation and recovery of chondrocyte-specific phenotype in culture involves Sry-type high-mobility group box (SOX) transcription factors. *Biochem J* **360**: 461–470.

Yamaoka H, Asato H, Ogasawara T, *et al*. 2006; Cartilage tissue engineering using human auricular chondrocytes embedded in different hydrogel materials. *J Biomed Res A* **1**: 1–11.

Yoo HS, Lee EA, Yoon JJ, *et al*. 2005; Hyaluronic acid modified biodegradable scaffolds for cartilage tissue engineering. *Biomaterials* **14**: 1925–1933.