Article:
Odabas, S, Feichtinger, GA, Korkusuz, P et al. (2013) Auricular cartilage repair using cryogel scaffolds loaded with BMP-7-expressing primary chondrocytes. Journal of Tissue Engineering and Regenerative Medicine, 7 (10). pp. 831-840. ISSN: 1932-6254

https://doi.org/10.1002/term.1634

AURICULAR CARTILAGE REPAIR USING CRYOGEL SCAFFOLDS LOADED WITH BMP-7 EXPRESSING PRIMARY CHONDROCYTES

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Running title:

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ABSTRACT

The loss of cartilage tissue due to trauma, tumor surgery or congenital defects like 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 regeneration of cartilage tissue. In this study, primary chondrocytes were genetically modified with plasmid encoding Bone Morphogenetic Protein-7 (BMP-7) via commercially available non-viral Turbofect vector with the aim of bringing *ex-vivo* transfected chondrocytes to re-synthesize BMP-7 *n-vitro* as they would *in-vivo*. Genetically modified cells were implanted into gelatin/oxide dextran scaffolds and cartilage tissue formation was investigated in 15x15mm auricular cartilage defects *in-vivo* in 48 New Zealand (NZ) White Rabbits for 4 months. Results were evaluated via histology and early gene expression. Early gene expression results indicated a strong effect of exogenous BMP7 on matrix synthesis and chondrocyte growth. In addition, histological analysis results exhibited significantly better cartilage healing with BMP-7 modified (transfected) cells in comparison to the non-modified (non-transfected) group and as well as the control.

Key Words: 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, tumor resection, and congenital defects like 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 *et al.*, 1995; Pan *et al.*, 2007). Some other complications such as autologous scarcity, pain, donor site-directed morbidity, iatrogenic pneumothorax atelectasis and graft incompatibility are also noted (Koch *et al.,* 2002).

Polymeric biomaterials such as silicone or porous polyethylene (PE) prostheses are also a widely used for re-shaping and the support of the defect area (Romo *et al.*, 2006; Breugem *et al.*, 2011). However, there are still some important limitations also to these approaches including requirements for intensive care, infection risk, wear and tear in time and frequent replacement. There is still the possibility that these materials are seen as foreign materials by the body (Koch *et al.* 2002 , Bauer *et al.*, 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.* have summarized the initial tissue engineering efforts of *in-vivo* and *in-vitro* cartilage development for microtia treatment (Rotter *et al.*, 2008). Yamaoka and his colleagues compared various types of hydrogel matrices for auricular cartilage tissue development

(Yamaoka *et al.*, 2006). Yoo and co-workers have evaluated cell behavior and cartilage formation on hyaluronic acid modified macroporous poly(D,L-lactic acid-co-glycolic acid) scaffolds (Yoo *et al.*, 2005).

Recent approaches also include, using growth factors or stimulants for engineered therapy. Bone Morphogenetic Proteins (BMP's) are considered as good candidates for these treatments. (Reddi *et al.,* 1998; Boyne *et al.,* 2001). BMPs are growth factors that act as enhancers for extracellular matrix synthesis and are inducers of mitotic activity (Bessa *et al.*, 2008). There have been several studies reported about using BMPs or other cytokines for articular cartilage repair. Gelse and co-workers demonstrated articular cartilage repair by gene therapy, using BMP-2 and growth factor producing mesenchymal stem cells (Gelse *et. al.,* 2003). Cook and colleagues reported an articular cartilage defect repair by using BMP-7 in a canine model (Cook *et. al.,* 2003). Madry and Cucchiarini summarized the clinical potential and the challenges of using genetically modified cells for articular cartilage repair. All these studies reflect persevering attempts for 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 have 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's auricula. We have also followed early phase gene expressions. We try to describe the effects of BMP-7 expressed from genetically modified chondrocytes and whether it could support healing. We here hypothesized 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/ox-dextran scaffolds were prepared according to a previously reported procedure (Inci et al., 2011): Briefly; the cross-linker, oxidized dextran (oxDex), was synthesized by oxidation of dextran with sodium periodate in an aqueous media in the dark at room temperature for 1h (Maia et al., 2005). The oxidized dextran was separated by dialysis and freeze dried. For scaffold preparation, 2g 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 1h. The disks were then stored in a freezer at -18°C for 24hrs. 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 (JSM-5600LV, Jeol, Japan) at 8 kV. Compression tests were performed on wet scaffolds using a universal test machine (LR-5K, Lloyd Instruments, UK). Freeze-dried cryogels (Mo: dry weight) were allowed to swell until equilibrium (24 hrs) and were then weighted (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 Ethic Committee (No: G.Ü.ET-10.059). Auricular cartilage was obtained from a 2 month old male New Zealand white rabbit under sterile conditions. Cartilage was cut into small pieces and digested in collagenase (3mg/ml in PBS) solution for 24 hours. The solution was then washed twice with DMEM/F12 culture medium. Isolated cells were then seeded into 25cm^2 flasks and a condition medium (DMEM/F12, %10 FBS, %1 L-Glutamine, %0,25 Penicillin-Streptomycin, %0,25 Gentamycin, % 0,1 Insulin). Chondrocytes were cultured until passage 4 at 5% CO_2, 37^oC.

2.3 BMP-7 transfection and release studies

The hBMP-7 cDNA containing constitutive expression plasmid pVAX1-hBMP7s (Feichtinger *et al.* 2011 in submission) was provided by the Ludwig Boltzman Institute, Vienna, Austria. Plasmid transfection was performed using a Turbofect *in-vitro* transfection kit (Fermentas, USA) according to manufacturer's instructions. Briefly, 2 μl transfection agents were incubated with 5μg plasmids for about 15 minutes at room temperature. The solution was then added to the culture media/24 well plates. A day later, cells were trypsinized and seeded onto gelatin/ox-dextran scaffolds ($1x10^5$ cells/scaffold). BMP7 release into the culture media was analyzed for up to 14 days using BMP-7 Elisa Kits (RayBiotech , USA). Non-transfected cells were used as a control. In addition, transfection efficiencies of primary chondrocytes were performed using Green Florescence Protein encoding plasmid and transfection efficiencies were calculated. A student t-test was used to determine the effect of hBMP-7 to overall BMP-7 release in normal and transfected group.

2.4 Cartilage tissue regeneration in vivo

48 New Zealand (NZ) white rabbits (adult male, 12 weeks olds, 2300 ± 300 gr with open epiphysis, healthy skeletal and physiological condition) which are equivalent to 96 auricle samples were used in the following four main groups: (i) The "defect-only" group which was the "control" group called as "Group **C**"; (ii) the "scaffold only" group ("Group **S**"); (iii) scaffold seeded with primary chondrocytes ("Group **N**"); and (iv) scaffold seeded with genetically modified (transfected) primary chondrocytes group ("Group **T**"). In each group, 6 auricles were operated per month.

In a typical surgical procedure, the rabbit was generally anesthetized intramuscularly using Ketamine (3ml) and Alfasime (1ml). In order to create a critical size defect 15mmx15mm auricular cartilage tissue was removed 15 mm distal to the radix of each auricle. Critical size dimensions were selected by considering similar models applied in the literature (Cheqielski *et al.*, 2007; Haberal *et al.*, 2008). As seen in Figure 1, a special cutting device was designed and used to remove the standard dimensional cartilage tissue sample. Perichondrium and vascular territory was protected in each case. Note that the disk shaped scaffolds (diameter: 15 mm and 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 ($1x10^6$ cells per scaffold). In the last group (Group **T**), genetically modified primary cells (carrying the BMP-7 expressing plasmid) ($1x10^6$ cells

per 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 including 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 male, 12 weeks olds) were used. 3 animals/auricles were used in each time-point for each treatment group. Others were kept as a control. Gene expression analysis was performed on normal and genetically modified cells 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.

Total RNA was isolated from the samples (approximately 50-100 mg) using the TriReagent system (peqGOLD TriFastTM, Peqlab, Erlangen, Germany), according to manufacturer's instructions. The RNA-containing pellets were treated with approximately 1–5U RNase-free D Nase (DNaseI, Roche Diagnostics, Germany) per µg RNA and incubated at 37°C for 30minutes before being washed with 70% ethanol to prevent DNA contamination. Ethanol was removed by air-drying and RNA pellets were dissolved in 10-30µl of RNase and DNase free water after which it was 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 260nm and 280nm using a Nanodrop spectrophotometer (NanoDrop ND-1000, USA). Equal amounts of RNA were used for reverse transcriptions. First strand complementary DNA (cDNA) was reverse transcribed from

1μg of total RNA with the 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 qRT-PCR method with the LightCycler 480 instrument (Roche Diagnostics, GmbH, Mannheim, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene in order to normalize Aggrecan, BMP-7, Col Type I and Col Type 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 GmbH, Mannheim, Germany). Negative control experiments of each reaction were performed without the addition of template cDNA. Samples were analyzed in triplicate. In order to determine 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 Type I and Col Type II) and GAPDH amplification efficiencies were approximately equal. The data were analyzed using LightCycler Software version 3.5 (Roche Diagnostics GmbH, Mannheim, Germany). Gene expression analysis of

Aggrecan, BMP-7, Col Type I and Col Type II was performed using the Relative Expression Software Tool 2005 (REST©) (Pfaffl *et al*., 2002).

2.4.2 Histological evaluations

Histological evaluation was performed at Hacettepe University, Faculty of Medicine, Department of Histology and Embryology. 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, distilled water, Merck, Germany) and all samples were then embedded in paraffin by using an automated tissue processor (Leica Westlar, Germany) with vacuum. Five to six micrometer thick serial sections (along the entire defect) were stained with hematoxylin & eosin (HE), Masson's trichrome (MT) and Weigerts elastic stain to assess the general morphology, collagen and elastic fibril amount respectively. "Photomicrographs of each sample were generated by a light microscope (Leica, DMR, Germany) attached to a computerized digital camera (Model DFC 480, Leica Westlar Germany). Both the Pinedas (the system has been modified by assessing the presence of the elastic cartilage but not the hyaline cartilage) and the Lohans cartilage histopathology scoring systems were used to evaluate the auricular cartilage critical sized defect healing. (Lohan *et al.*, 2011; Pineda *et al.*, 1992).

Statistical analysis

A prospective randomized-controlled double-blinded in vivo study was designed. Independent variables were groups (n=8) and time (n=2) Dependent variables were

histology scores. For statistical analysis, the independent variable was groups and the dependent variables were the histology. The normality of distribution and the homogeneity of variances of the sample were established using the Shapiro-Wilk test. All parameters were analyzed by the non-parametric Kruskal-Wallis test that was used for multiple comparisons and the Dunn test for post-hoc analysis. Descriptive statistics were expressed as the median, the minimum and the maximum. Statistical significance was determined using the SPSS software (version 15.0). The differences were considered significant when $p < 0.05$.

For the gene expression results, statistical significance of differences in mRNA expression were analyzed 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 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 micrograph of the cryogel scaffolds prepared in this study. As seen here, cryogels are highly porous, with large pores (the average is over 100 μm) and inter-connective 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 behavior. They do swell in aqueous media very rapidly, within a few minutes, and reach their final size in 20-25 mins. The average swelling ratio and standard deviation (n:5) is

986.2% ± 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)

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

3.2 BMP release studies

Figure 3 depicts the 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 media as described in the previous sections. It has been reported that cells on monolayer cultures do 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, however 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 is a significant difference between normal and transfected cells in all timepoints. ($p < 0.01$ to $p < 0.0001$) Schüller and his colleagues demonstrated similar relevant findings with BMP-7 release from primary chondrocytes, transfected with pCMV-BMP7/lipofectamine system in monolayer culture (Schüller *et al.*, 2008).

Here we analyze the total BMP7 (both endogenous and in transfected cells also exogenous) release to the culture media. BMP release in this study is also related to transfection efficiency. In our study, we checked our transfection efficiencies in primary chondrocytes, which were between 15-20%, with a model plasmid (EGFP). Logically, higher transfection efficiency causes higher amounts of produced BMP. There are several issues that strongly affect the transfection efficiency. In brief; primary cells are very sensitive to the transfection and isolation procedures from the tissue can also affect the efficiency (Dinser *et al.,* 2001) Also although the 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 *et al.,* 2009)

3.3 Cartilage tissue regeneration in vivo

A detailed histological evaluation was performed in all groups. Figure 4 and Figure 5 show the average Pineda and Lohan scores of each group within 4 months.

These scoring systems are widely used and well established scoring systems for cartilage repair assessment used in the recent literature and studies. Although they are described as semi-quantitative systems, they cover and combine all of the morphologic criteria for qualitative and quantitative cartilage repair. In Lohan's scoring system the cartilage cell morphology, extracellular matrix production and the inflammation criteria were separately scored. 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 fibers), the amount of chondrocyte and non-chondrocyte cells and the amount of the fibrous and elastic cartilage were separately scored. The final total scores that included all this data were statistically analyzed.

In addition to these semi-quantitative scoring of newly formed elastic cartilage; all groups were also analyzed 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 in time but did not degrade entirely at the end of 4 months in any of the groups. All of the groups exhibited the formation of various amounts of cartilage islands and several of them revealed a varying quantity of calcified foci within the defect. Both cell seeded scaffold groups had more Weigert positive elastic cartilage islands compared to the other groups. Cartilage formation was of a more fibrous type in the control and scaffold only groups (Group C and Group 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 it was sometimes continuous with the fibrous connective tissue throughout the defect in all of the groups (Figure 6).

The normal and the genetically modified (transfected) cells with scaffold groups (Group N and Group 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 one month (Group N $p=0.023$ and $p=0.001$; Groups T $p=0.001$ and $p=0.001$) . The transfected and normal cells with scaffold groups (Group N and Group T) were significantly better than the scaffold only group (Group S) after one month (Figure 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) (Figure 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 (Figure 4 and 5).

The scaffold only group (Group S) did not significantly improve the cartilage defect healing process in the months 1, 2, 3 and 4 compared to control (Group C) (Figure 4 and 5).

3.4 Early phase gene expressions

In order to observe the effects of BMP-7 on early phase healing process, a separate group of experiments was designed, in which scaffolds carrying primary chondrocytes (normal cells) or their genetically modified forms (transfected) were implanted to the animal ear models (3 animals for each) as discussed in the previous section. The samples were taken at 3^{rd}, 7^{th}, 14^{th} and 30^{th} days and expression of four target genes, i.e., COL Type I and II, BMP-7 and Aggrecan was analyzed by quantitative Real-Time PCR.

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

Figure 7 shows the fold differences in expression with respect to the control in designated timepoints. As is seen in figure 7, 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 an activity of BMP-7, matrix protein synthesis should consequently increase (Bessa *et al.*, 2008). In this study the expression of endogenous BMP7 was on mRNA level, however, several reports show 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 Collagen type II, which is the dominant matrix protein for cartilage, were also increased, both in transfected and normal cells with scaffold groups (Group N and Group T). However, in transfected cells with scaffold group (Group T) these differences were significant and higher in all timepoints. The effect of the extra BMP-7 can be observed easily after 14 days post-operative as the matrix proteins synthesis increases. Even in the late phase (14 and 30 days), there is still a higher expression of Collagen Type II and Aggrecan in Group T. Lietman and colleagues reported similar findings on the stimulation of proteoglycan synthesis by Bone Morphogenetic Protein – 7 (Lietman *et al.,* 1997). On the other hand, Collagen Type I differences in normal cells with scaffold group (Group N) is not significant at later phases

(after 14 days) in normalized overall results. However, in Group T, a higher expression on Collagen Type 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, existence of extra BMP-7 in the microenvironment in transfected cells with scaffold group (Group T) could have also increased the level of Collagen Type I significantly. Briefly; the fold differences of the selected factors in group T were higher than normal cells (untransfected) cells with scaffold group (Group N) for every timepoint.

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 forming is 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 treatments groups and the control group presented similar inflammation scores revealing no obvious immunologic response (supplementary Table 1). Despite these facts, using a syngenic model may still refer as a limitation of our study when considering to translate into clinic.

As our histology and gene expression results indicated a significantly better cartilage healing and matrix formation in genetically modified cells individually with exogenous BMP-7. 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 an other limitation. Moreover, the expression and the release of

the plasmid encoded factors mainly depend on the transfection and expression efficiency, which is relatively low in primary cells with non-viral systems. We believe, higher expression efficiency could have an additional effect on healing. Although the histological staining and the results of the semi-quantitative scoring system exhibit a well-organized almost elastic tissue formation on transfected cells applied group (Group T), we believe that further studies are needed to elucidate the exact mechanism of the elastic formation and long term regeneration.

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 field of reconstruction surgery for head and neck surgery.

In this study, we aimed to perform a cartilage defect healing model on auricular side, which would allow the regeneration of cartilage tissue as well as forming a 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 over expressed human BMP7. Significantly improved healing and regeneration was observed which was attributed to the effect of over-expressed BMP7.

ACKNOWLEDGEMENTS

This study was supported by EU: FP6-NoE: EXPERTISSUES Project. Sedat Odabas is supported by TUBITAK (The Scientific and Technological Research Council of Turkey) as full PhD scholarship. Prof. Dr. Erhan Piskin is supported as a full member of Turkish Academy of Sciences.

Authors thank Prof. Heinz Redl for his momentous support.

Authors wish to thank Dr. James Ferguson, DVM and Dr. Sushmita Saha for language correction of this article.

REFERENCES

Al-Dosari MS, Gao X. 2009, Nonviral gene delivery: Principle, limitations, and recent progress, *The AAPS Journal,* **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, *The Journal of Bone & Joint Surgery,* **83Suppl1**: 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

Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson P, Peterson L. 1994, Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation, *N Engl J Med,* **331**: 889-895

Brodland DG. 2005, Auricular reconstruction, *Dermatologic Clinics*, **23:** 23

Cegielski M, Dziewiszek W, Zabel M, Dziegiel P, Izycki D, Zatoński M, Bochnia M. 2008, Experimental application of xenogenous antlerogenic cells in replacement of auricular cartilage in rabbits, *Xenotransplantation,* **6**: 374-83

Chen D, Harris MA, Rossini G, Dunstan CR, Dallas SL, Feng JQ, Mundy GR, Harris SE. 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. Orthopedics* **6**: 773-781

Chung C, Mesa J, Randolph MA, Yaremchuk M, Burdick JA. 2006, Influence of gel properties on neocartilage formation by auricular chondrocytes photoencapsulated in hyaluronic acid networks, *J Biomed Res Part A*, **77**: 518-525

Cook SD, Patron LP, Salkeld LS, Rueger DC. 2003, Repair of Articular Cartilage Defects with Osteogenic Protein-1 (BMP-7) in Dogs, *J Bone & Joint Surg*, **85**:116-123.

Dinser R, *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, *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, Journal of *Orthopaedic Res,* **15**: 371-380

Freed LE, Marquis JC, Nohria A, Emmanual J, Mikos AG, Langer R. 1993, Neocartilage Formation *in-vitro* and *in-vivo* using cells culture on synthetic biodegradable polymers, *J Biomed Mater Res,* **1**: 11-23

Gelse K, Mark von der K, Aigner T, Park J, Schneider H. 2003, Articular cartilage repair by gene therapy using growth factor–producing mesenchymal cells, *Arthritis & Rheumatism*, **48:** 430-441.

Grande DA, Mason J, Light E, Dines D. 2003, Stem cells as platforms for delivery of genes to enhance cartilage repair, *J Bone Joint Surg Am,* **85-A Suppl2**: 111-116

Haberal Can I, Atilla P, Cakar AN, Onerci M. 2008, An animal study on cartilage healing using auricular cartilage as a model, *Eur Arch Otorhinolaryngol,* **3**: 307-11

Haisch A, Klaring S, Groger A, Gebert C, Sittinger M. 2002, A tissue engineering model for the manufacture of auricular-shaped cartilage implants, *Eur Arch Otorhinolaryngol,* **259:** 316–21

Hidaka C, Quitoriano M, Warren RF, Crystal RG. 2001, Enhanced matrix synthesis and *in-vitro* formation of cartilage-like tissue by genetically modified chondrocytes expressing BMP-7, *J Orthopedic Res,* **19**: 751-758

Hiraki Y, Yutani Y, Fukuya M, Takigawa M, Suzuki F. 1985, Differentiation and de-differentiation 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, Mattiasson B and Piskin E. JUN 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 Suppl1** : 173-179

Kawai M, Bessho K, Maruyama H, Miyazaki J, Yamamoto T. 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, *Musculoskeletal Disorders,* **7**:62-72

Koch JR, Gorti K. 2002, Tissue Engineering with Chondrocytes, *Facial Plastic Surgery* **18**: 59-68

Lietman AS, Yanagishita M,Sampath TK, Reddi AH. 1997, Stimulation of Proteoglycan Synthesis in Explants of Porcine Articular Cartilage by Recombinant Osteogenic Protein-1 (Bone Morphogenetic Protein-7), *The J Bone and Joint Surg,* **79A**: 1132-1137

Lohan A, 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

Mandl EW, Jahr H, Koevoet JL, van Leeuwen JP, Weinans H, Verhaar JA, van Osch GJ. 2004, Fibroblast growth factor-2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear chondrocytes in monolayer culture, *Matrix Biol,* **24**: 231-241

Madry H, Cucchiarini M. 2011, Clinical potential and challenges of using genetically modified cells for articular cartilage repair. *Croat Med J,* **52:** 245-61.

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, Koevoet W, Nolst-Trenite G, Verhaar JA. 2004, Considerations on the use of ear chondrocytes as donor chondrocytes for cartilage tissue engineering, *Biorheology,* **41**: 411-421

Park C, Yoo YS, Hong ST. 2010, An update on auricular reconstruction: three major auricular malformations of microtia, prominent ear and cryptotia, *Curr Opin Otolaryngol Head Neck Surg,* **6**: 544-549

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 3rd, Presti PM, Yalamanchili HR. 2006, Medpor alternative for microtia repair. *Facial Plast Surg Clin North Am,* **14**: 129-136

Rotter N, Haisch, A, Bücheler M. 2005, Cartilage and bone tissue engineering for reconstructive head and neck surgery, *Eur Arch Otorhinolaryngol,* **262**: 539-545

Rotter N, Steiner A, Scheithauer M. 2008, Reconstruction of auricular cartilage using tissue-engineering techniques, *Operative Tech in Otolaryngology*, **19:** 278-284

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

Saha S, Kirkham J, Wood D, Curran S, Yang X. 2010, Comparative study of the chondrogenic potential of human bone marrow stromal cells, neonatal chondrocytes and adult chondrocytes, *Biochem Biophys Res Commun*, **22:** 333-8

Saraf A, Mikos AG. 2006, Gene delivery strategies for cartilage tissue engineering, *Adv Drug Delivery Rev,* **58**: 592-603

Schüler G. 2008, Overexpression of BMP-7 in monolayer chondrocytes, Diplomarbeit, Uni. of Wien.

Shieh SJ, Terada S, Vacanti JP. 2004, Tissue engineering auricular reconstruction: *in-vitro* and *in-vivo* studies, *Biomaterials*, **9**: 1545-1957

Steinert AF, Nöth U, Tuan RS. 2008, Concepts in gene therapy for cartilage repair, *Injury*, **39-S1**: 97-113

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

Tcacencu I, Carlsöö B, Stierna P, Hultenby K. 2006, Local treatment of cricoid cartilage defects with rhBMP-2 induced growth plate-like morphology of chondrogenesis, *Otolaryngology-Head and Neck Surg,* **135**: 427-433

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

Yan LP, *et al.* 2010, Genipin-cross-linked collagen/chitosan biomimetic scaffolds for articular cartilage tissue engineering applications, *J Biomed Mater Res A,* **95**: 465-475

Yoo HS, Lee EA, Yoon JJ, Park TG. 2005, Hyaluronic acid modified biodegradable scaffolds for cartilage tissue engineering, *Biomaterials,* **14:** 1925-1933

Table 1. The 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 Type I	5′-AGAACCCAGCTCGCACCT-3′	5′-CATCCTTGGTTGGGATCG-3′,	83	CAGCCACC
Col Type II	5′-GACCTGCGTCTACCCCAAC-3′	5′-GCTGCTTCTGGCTCTTGC-3′	22	TGGTGGAG

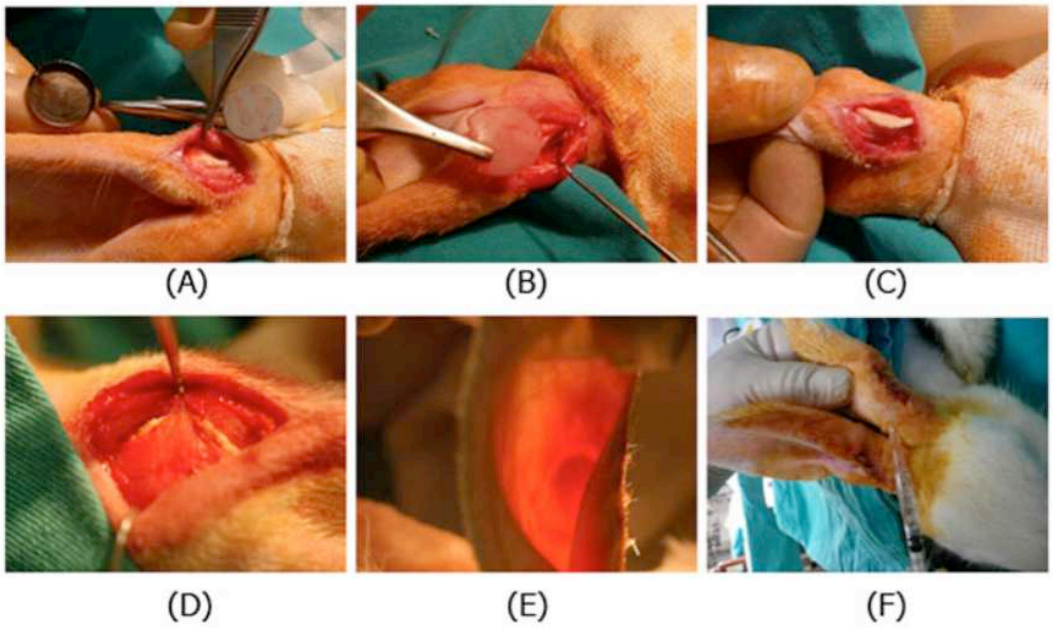

Figure 1. Rabbit ear critical size defect model. (A) Removal of auricular cartilage with a special cutting device; (B) intact auricular cartilage removed from the defect area; (C) cryogel scaffolds implanted in the defect area; (D) Replacing the perichondrium in the original site/position; (E) imaging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

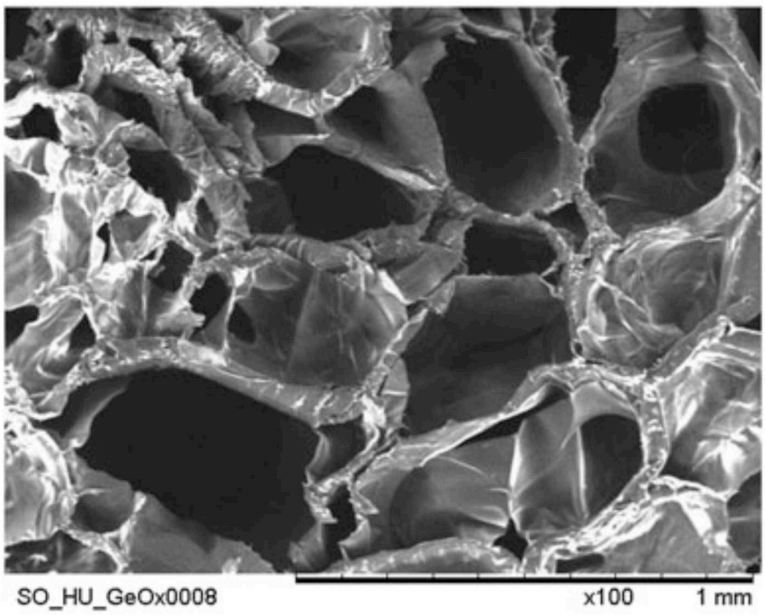

Figure 2. SEM micrograph of the cryogel scaffold used in this study.

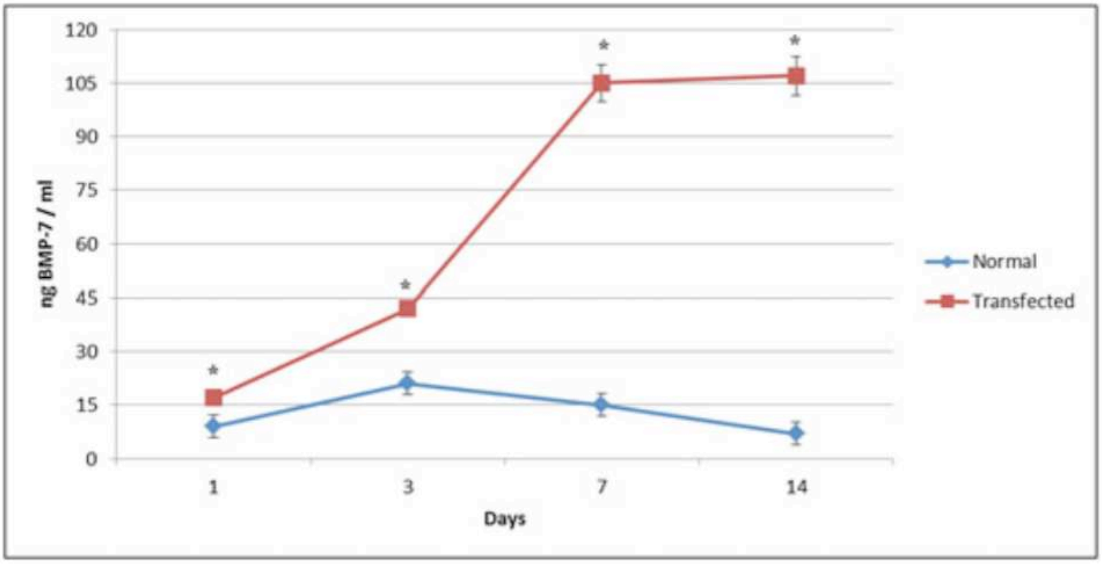

Figure 3. BMP-7 release (both endogenous and exogenous) from cryogels scaffold carrying either genetically modified (transfected) primary chondrocytes or non-transfected chondrocytes. Production per day. (N=3)

*; $p<0.05$ when comparison with normal and transfected cells.

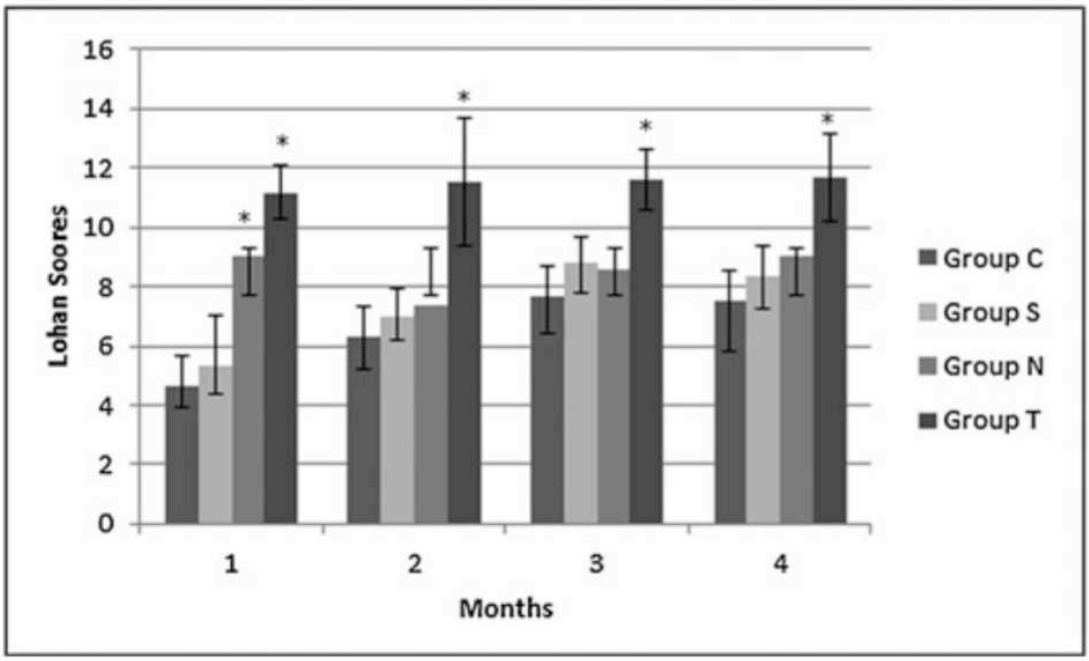

Figure 4. Lohan Histologic Scoring of each groups (N:6) (**C**: Control; **S**:Scaffolds; **N**: Scaffolds with chondrocytes; and **T**:Scaffolds with transfected cells) *; p<0.05

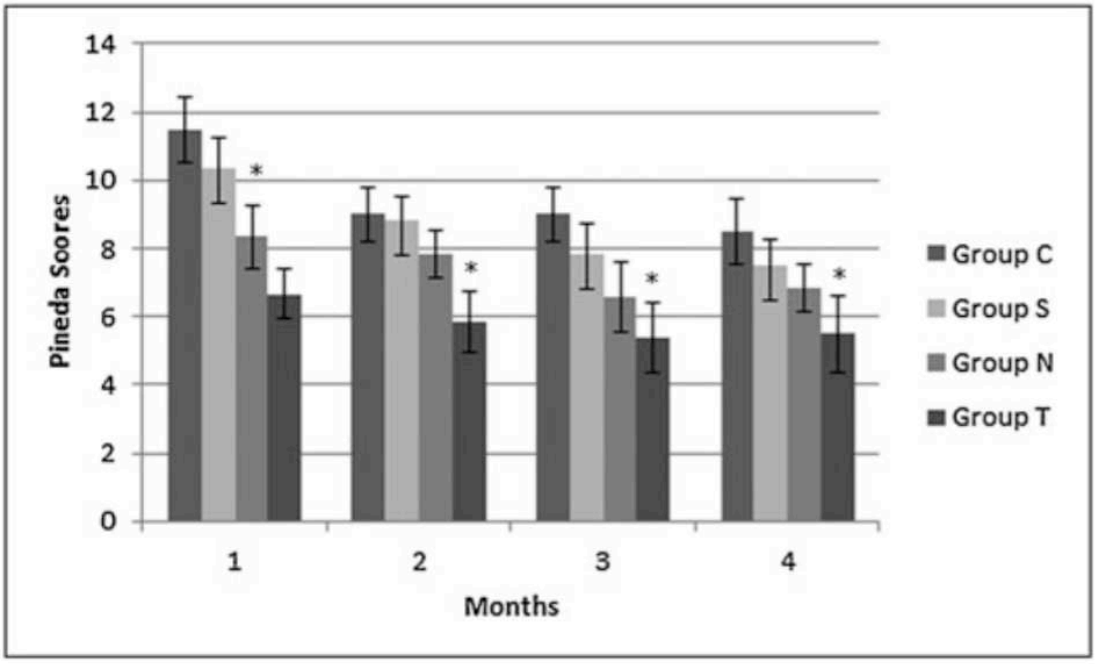

Figure 5. Pineda Histologic Scoring of each groups (N:6) (**C**: Control; **S**:Scaffolds; **N**: Scaffolds with chondrocytes; and **T**:Scaffolds with transfected cells) *; p<0.05

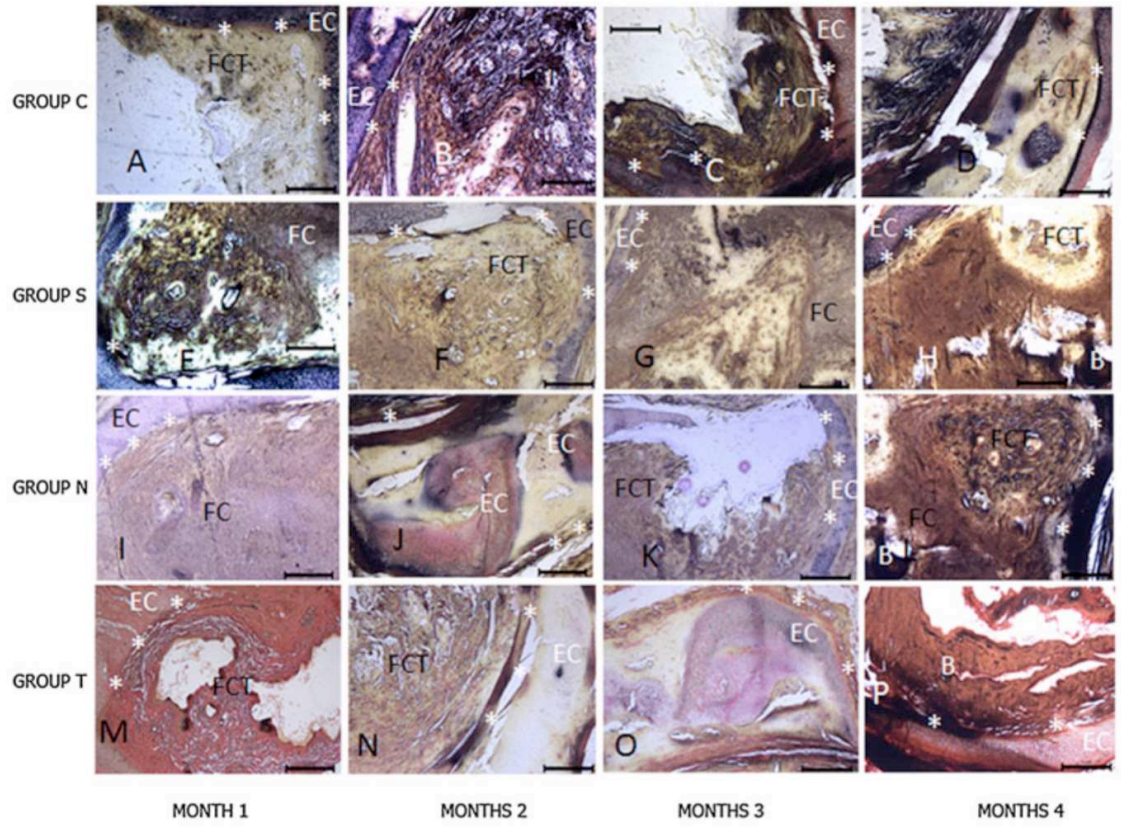

Figure 6. These are the panoramic views of the cartilage defects at lowest (25x) magnification. Elastic fibers stain in black with this dye. Bone, fibrous cartilage and the connective tissue stain in purple to yellow. The pink to purple color of the extracellular matrix exhibit an intermediate stage of the repairing or remodeling cartilage before the accumulation of the elastic fibers (in J, M, P).The asterisks show the edge between the defect and the adjacent elastic cartilage which is normal or under the remodeling process. In J and O new elastıc cartılage; ın H, L and P bone formation is observed inside the defect. **EC**: Elastic cartilage, **FC**: Fibrous cartilage, **FCT**: Fibrous connective tissue, **B**: Bone. Note that the elastic cartilage development is at the highest level in cells with scaffold applied groups at the bottom lines. Weigert elastic stain. Scales in each figure represent 1mm.

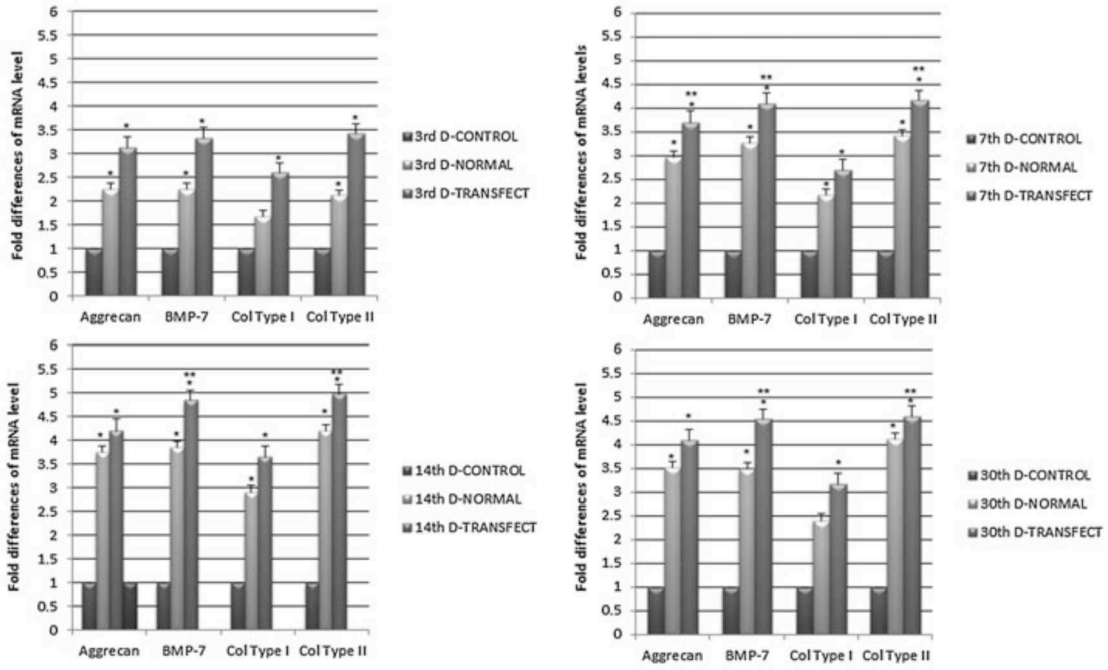

Figure 7. The fold differences in mRNA level of BMP-7, Col Type I, Col Type II, Aggrecan in cell applied groups with respect to the control up to 30 days. Non-operated tissue was used as a control. Results were normalized to GAPDH as housekeeping gene.

*; $p<0.05$ when comparison with control and cell applied groups

**; $p<0.05$ when comparison between Normal and Transfected Cells applied groups.