Ivković A., Pascher A., Hudetz D., Matičić D., Jelić M., Dickinson S., Loparić M., Hašpl M., Windhager R., Pećina M. (2010) ***Articular cartilage repair by genetically modified bone marrow aspirate in sheep.*** **Gene Therapy, 17 (6). pp. 779-89. ISSN 0969-7128**

http://www.nature.com/gt

http://dx.doi.org/10.1038/gt.2010.16

Articular Cartilage Repair by Genetically Modified Bone Marrow Aspirate in Sheep

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Running title: Gene therapy in ovine articular cartilage repair

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word count: 8877

Summary

Bone marrow presents an attractive option for the treatment of articular cartilage defects as it is readily accessible, it contains mesenchymal progenitor cells which can undergo chondrogenic differentiation, and coagulated it provides a natural scaffold that contains the cells within the defect. This study was performed to test whether an abbreviated *ex vivo* protocol that utilizes vector-laden coagulated bone marrow aspirates for gene delivery to cartilage defects may be used for clinical application. Ovine autologous bone marrow was transduced with adenoviral vectors containing cDNA for GFP or TGF-β1. The marrow was allowed to clot forming a gene plug and implanted into partial-thickness defects created on the medial condyle. At 6 months the quality of articular cartilage repair was evaluated using histological, biochemical and biomechanical parameters. The results of the repair assessment showed that the groups treated with constructs transplantation contained more cartilage-like tissue than the untreated controls. Improved cartilage repair was observed in groups treated with unmodified bone marrow plugs and Ad.TGF-β1 transduced plugs, but the repaired tissue from TGF treated defects showed significantly higher amounts of collagen II ($p<0.001$). The results confirmed that the proposed method is fairly simple and a straightforward technique for the application in clinical settings. Genetically modified bone marrow clots are sufficient to facilitate articular cartilage repair of partial thickness defects *in vivo*. Further studies should focus on improvement of the combination of different genes to promote more natural healing.

Key words: *adenovirus – articular cartilage defects – transforming growth factor-β1 – bone marrow – cell transplantation – gene therapy*

Introduction

Hyaline cartilage in adults is highly specialized tissue and its unique three-dimensional structure enables it to withhold tremendous mechanical forces inflicted during joint movement, and its smooth surface lowers friction between articular surfaces of the joint. Hyaline cartilage is avascular, aneural and alymphatic tissue with low number of cells imbedded into extracellular matrix, and therefore it has very modest reparative and regenerative capabilities. Articular cartilage defects are very frequent, especially among younger and working population. Such defects do not heal, and often, with time they lead to premature osteoarthritis, and consequently a decrease in quality of life and increase in costs of health care.

Both, for scientist and treating clinicians, the restoration of damaged cartilage remains one of the biggest challenges in modern orthopaedics. There is no pharmacological treatment that promotes the repair of the cartilage, and numerous clinical and experimental procedures that have been utilized result in fibrocartilage repair tissue that is inferior to normal cartilage.[1] Current treatment modalities include microfracture, transplantation of ostechondral grafts and chondrocytes, use of biodegradable scaffolds or combination of these.[2,3] Although mentioned procedures produce good clinical result in terms of pain relief and improvement of joint function, long-term outcomes are less predictable and satisfactory.

New biological approaches to cartilage repair offer alternative to current treatment options, particularly those that are based on the use of cells and molecules that promote chondrogenesis or/and inhibit cartilage breakdown.[4] Any successful strategy that attempts to repair hyaline cartilage defects must include sufficient number of cells, appropriate signal to modulate cellular response and a scaffold that would contain the cells within the defect.

Mesenchymal stromal cells (MCSs) present very attractive option for cell-based strategies since they can be easily isolated, expanded, and under appropriate conditions, differentiated into mesenchymal tissues such as cartilage, bone or muscle.[5] Numerous gene products such as transforming growth factor- β (TGF-β)[6], bone morphogenetic protein-7 (BMP-7)[7], insulin-like growth factor-1 (IGF-1)[8] and bone morphogenetic protein-2 (BMP-2)[9], have shown promises in regulating the process of growth, repair and regeneration of cartilage in animal models, but their use is limited by delivery problems and rapid clearance from the joint.

Gene therapy has become attractive alternative system for delivering therapeutic gene products to specific designated tissues, which may overcome some of the mentioned problems.[11,12] Viral vectors have been successfully used to target graftable articular chondrocytes, periosteal cells and bone marrow-derived MCSs as a chondroprogenitors, as well as the synovial lining.[13,14] The use of scaffolds in cartilage repair is a very promising strategy since they contain, deliver and orient cells within their three-dimensional structure. Many different types have been tested in clinical and experimental settings, but the search for the optimal one is still ongoing.[10]

Ex vivo or indirect approach is usually utilized in the treatment of cartilage defects with genetically modified cells. It includes harvesting and expansion of the cells, transduction with a therapeutic gene, seeding on the scaffold and reimplantation into the defect. This approach is technically demanding, expensive and requires at least two surgical procedures. Pascher et al.[15] have recently proposed a novel gene transfer protocol for the repair of articular cartilage. It is an abbreviated *ex vivo* protocol that utilizes vector-laden coagulated bone marrow aspirates (gene plugs) for gene delivery to cartilage defects. The study showed that nucleated cells within fresh autologous bone marrow aspirates may be successfully transduced with adenoviral vectors

sufficient to secret transgene products up to 21 days. In theory, this approach provides all necessary ingredients for successful cartilage repair: transduced mononuclear cells secrete signals which stimulate mesenchymal progenitors to differentiate along the chondrogenic lineage The bone marrow clot itself provides a natural autolougus three-dimensional scaffold to be used for containment of cells and vectors within the defect and guaranties biological ingrow of the construct in the defect.

To examine whether direct implantation of genetically modified bone marrow clots (gene plugs) might be used in situations that closely mimic real-life clinical situations, a sheep model was established. Partial-thickness chondral defects were created on the weight-bearing surface of the femoral condyle in sheep. Fresh autologous bone marrow aspirates were transduced with adenoviral constructs carrying therapeutic or marker genes, and the clots were pressfit implanted in the defect. The objectives of the current study were to: 1) determine feasibility of the proposed abbreviated *ex vivo* protocol to be used as a novel treatment tool in clinical settings, 2) to determine whether transgene expression of TGF-β1 within the gene plug enhances cartilage repair, and 3) to test whether there is a presence of adenoviral genome within the cells of synovial lining, which would suggest vector leakage from the clots.

Results

Histological assessment

The mean scores of the histological assessment are shown in Table 1 and the representative histological sections are shown in Figure 1. Six months after the surgical procedure, all groups treated with bone marrow clot transplantation were superior to empty control in terms of overall score according to the ICRS Visual Histological Assessment Scale,

although statistical significance was not observed (p=0.061) (Table 1). Each histological parameter was analyzed by Kruskal-Wallis test. Statistical significant difference was observed in one category - cell distribution: TGF and BMC groups had a higher score than the CON group (Table 1).

Biochemical properties

GAG analysis did not reveal any statistical difference between the mean values for repaired cartilage in treatment groups and native cartilage from contralateral knee ($p>0.050$ for all comparisons paired samples t-test; Figure 2a). There were no statistically significant differences in GAG mean values for repaired cartilage between the treatment groups ($F_{2,19}=0.6$, $p=0.581$, *one-way ANOVA;* Figure 2a).

The collagen type I content was found to be significantly higher in all treatment groups when compared to native cartilage ($p<0.050$ for all comparisons, paired samples t-test; Figure 2b). The three treatment groups also significantly differed in collagen type I content of repaired cartilage ($F_{2,19}=13.9$, $p<0.001$, *one-way ANOVA;* Figure 2b). The collagen type I content in BMC group was significantly lower from that detected in GFP and TGF treated groups respectively ($p<0.001$ and $p=0.001$, respectively, Tukey post-hoc test), while there was no difference among GFP and TGF groups ($p=0.482$, Tukey post-hoc test).

The collagen type II content was significantly lower in BMC and GFP treatment groups when compared to native cartilage (Figure 2c). There was no difference between the GFP and BMC groups ($p=0.079$, Tukey post-hoc test). Collagen type II content in the TGF group was significantly higher ($F_{2,19}=56.2$, $p<0.001$, *one-way ANOVA*; Figure 2c) than that detected in GFP and BMC treated groups ($p<0.001$ for both, Tukey post-hoc test)

When compared to native cartilage, water content in the repaired tissue was significantly lower in the TGF and GFP groups (p<0.001 and p=0.005 respectively, paired samples t-test; Figure 2d) where as the water content of the TGF group was significantly lower than the one detected in the GFP ($F_{2,19}$=5.9, p=0.01, *one way ANOVA*; Figure 2d) and the BMC group (p=0.008, Tukey post-hoc test).

Biomechanical properties

Cartilage stiffness at micrometer scale

Dynamic elastic modulus |E^*| values for native articular cartilage and reparative cartilage obtained after the treatment with genetically modified bone marrow are shown in Figure 3. The measurements are obtained with microspherical tip, nominal radius of 7.5 μm, and they reflect structural changes at the micrometer scale. Dynamic elastic modulus $|E^*|_{micro}$ values gradually increased from native cartilage, to the TFG, BMC and GFP treated group (Figure 3). $|E^*|_{micro}$ was significantly higher in all repair groups when compared to native cartilage (BMC p<0.001, GFP p=0.003, TGF p<0.001 respectively, paired samples t-test). Treatment groups significantly differed in $|E^*|_{micro}$ ($F_{2,19}$=5.3, p=0.015; *one-way ANOVA*). $|E^*|_{micro}$ in the TGF group was significantly lower than that detected in the GFP group (p=0.014, Tukey post-hoc test). $|E^*|_{micro}$ values of the TGF group were also lower compared to values of the BMC and GFP groups but no statistically significant differences were observed.

Dynamic elastic modulus $|E^*|_{micro}$ was moderately positive associated with water and moderately negative with collagen type II, but not with GAG and collagen type I content (Table 2).

Cartilage stiffness at nanometer scale

Dynamic elastic modulus $|E^*|$ values for native articular cartilage and reparative cartilage obtained after the treatment with gene plugs are shown in Figure 4. The measurements are obtained with sharp pyramidal tip, nominal radius of 20 nm, and they reflect structural changes at the nanometer scale. Obtained dynamic elastic modulus $|E^*|_{nano}$ values showed that the BMC treated group had very similar stiffness to the native cartilage (p=0.345, paired samples t-test), but it was higher in TGF and GFP treated groups (p=0.028 and p=0.005 respectively, paired samples t-test; Figure 4). Furthermore, we found statistically significant difference in $|E^*|_{nano}$ between treatment groups (p<0.001, Kruskall-Wallis test). Stiffness was significantly higher in the GFP control group than in the TGF group (p=0.007, Mann-Whitney test). BMC had lower $|E^*|_{nano}$ when compared to GFP and TGF (p=0.001 and p=0.004 respectively, Mann-Whitney test).

$|E^*|_{nano}$ was strongly positively associated with collagen I and moderately negatively with water, but not with GAG and collagen II content (Table 2).

PCR analysis

To determine the expression levels of the respective vectors driven by cytomegalovirus promoter within the synovial membrane 6 months following surgery, PCR was performed using the CMV promoter and sheep β-actin primer sets. The analysis included 5 groups of specimens according to treatment, namely TGF-β1 vector treated, GFP vector treated as a positive control,

bone marrow treated, empty defect group and controls from contralateral knee. PCR analysis of the synovial tissue revealed no presence of CMV promoter in all of the treatment groups and the control group 180 days following implantation. Expression of the β-actin gene was detected in all of the analyzed samples.

Discussion

It is a well known fact that the healing of focal lesions in adult articular cartilage is very limited and, over time, they may progress to osteoarthritis. Articular cartilage damage is a growing health care problem and a recent study showed that approximately 2/3 of patients undergoing knee arthroscopies have been diagnosed with cartilage lesion.[16] On the other hand growing armamentarium of novel biological methods and technologies offer scientists as well as clinicians powerful tools in developing new and effective methods in treating damaged cartilage. Cell based therapies, signaling and scaffolds are key topics on which any successful tissue-engineering strategy builds.[17]

The approach to cartilage repair of local defects described in this study utilizes vector-laden coagulated bone marrow aspirates for gene delivery to cartilage defects. Aspirated autologous bone marrow contains progenitor cells, the matrix is completely natural and native to the host, and the contained fibrin fibers adhere the whole construct to the surface of the defect. Preliminary *in vitro* and *in vivo* studies on small animals showed that clotted mixtures of adenoviral suspensions with fresh aspirated bone marrow resulted in levels of transgenic expression in direct proportion to the density of nucleated cells within the clot.[15] The current study is a step forward towards a clinical application of genetically modified bone marrow (gene plugs) to treat local cartilage lesions. The whole study was conceived in a way to simulate potential clinical situation where one would have to treat isolated chondral defect situated on the load-bearing surface of the femoral condyle. Therefore a sheep model as a large animal model was chosen. The drawback of the proposed model is the fact that sheep cartilage on the medial condyle is very thin. Ahern et al. performed a detailed systematic review of preclinical animal models in single site cartilage defect testing, and according to their analysis the ovine cartilage is

variable in thickness and it measures from 0.40 to 1.68 mm.[18] Minor variability in the obtained results might be contributed to that fact, nevertheless, reproducible standardized chondral defects could be created in all animals, using a adapted punch-drill device. For implantation of the gene plugs standard operation instruments were used. The proposed method proved to be fairly simple and a straightforward technique for application in clinical settings. It is a single step operation, which can be easily done by two surgeons within 30 to 45 minutes.

The use of TGF-β1-transduced bone marrow clots for articular cartilage defects repair

Adult MSCs present a very interesting platform for the development of treatment strategies in orthopaedic tissue engineering. They can be obtained relatively easily from various tissue sources such as bone marrow, fat and muscle, and under appropriate conditions they have the capacity of differentiation into various mesenchymal lineages including bone and cartilage.[5,19] Numerous *in vitro* studies showed that primary MSCs undergo chondrogenic differentiation when cultured in the presence of specific media supplements, including dexamethasone and certain extracellular biological cues.[20,21]

TGF-β1 has been used as a key stimulator of chondrogeneseis in many in vivo and in vitro studies, as it stimulates cell proliferation and synthesis of major components of extracellular matrix (ECM) - GAG and collagen.[22,23,24] TGF-β1 was chosen because it is one of the best characterized and the most potent chondrogenic growth factors. The results of the present study showed that all groups that underwent transplantation of bone marrow clots have a high content of GAGs, but only the repair tissue of TGF-β1 gene plug treated defects had a very high content of collagen type II similar to native cartilage. The fact that only TGF treated defects scored statistically higher in terms of cellular distribution leads to the conclusion that residing

mesenchymal progenitors within the gene plug responded to the local expression of TGF-β1 in terms of chondrogenic differentiation, which in the end resulted in higher ECM turnover and better quality of the cartilage repair. Guo et al.[25] reported similar results in a rabbit model of full-thickness cartilage defects using an ex vivo approach and a chitosan scaffold. Another study by Pagnotto et al.[26] showed improved cartilage repair in osteochondral defects implanted with MSCs transduced with adeno-associated virus (AAV) carrying cDNA for TGF-β1. In their study transgene expression slowly decreased from 100% at two weeks to 17% at 12 weeks, but it proved that gene therapy enables sustained delivery of the bioactive molecules for a period of time that is sufficient to induce and govern cellular response within the defect. Owing to its safe profile, AAV is considered to be the most suitable viral vector for human application, and is currently being tested in phase I clinical trial.[27]

Although practical, the use of a single biological factor to stimulate and regulate process of chondrogenic differentiation has obvious limitations in ability to produce cartilage of optimal quality. Chondrogenesis is a finely regulated mechanism, which includes numerous growth and transcription factors, and a combination of these might be more effective. Synergistic effect on chondrogenesis has been reported for TGF-β1 when co-administered with IGF-1.[28] Steinert at al.[29] recently used aggregate culture system to study effects of co-expression of TGF-β1, IGF-1 and BMP-2 on MSCs. Their results showed larger aggregates, higher levels of GAG synthesis, and greater expression of cartilage specific marker genes by adding different combinations of growth factors to MSCs. Furthermore, it is known that TGF stimulation of MSCs promotes hypertrophy and the increased expression of collagen type I and X. However, Kafienah et al.[30] have shown that including parathyroid hormone-related protein (PTHrP), down-regulates collagen type I and X in cartilage tissue engineered from MSCs. It should be also noted that

some transcription factors such as SOX-9 (which is known to be essential for the full expression of chondrocyte phenotype) and Wnt are not chondrogenic itself, but can make cells more responsive to growth factors and other chondrogenic stimuli. Along these lines, in order to optimize the proposed method, delivery of multiple genes might be more reliable option, and further studies are needed to pinpoint the exact protocol in terms of concentration and temporal sequence of delivery of chosen genes.

Apart from the fact that in the current study only TGF-β1 was used to induce chondrogenesis, another important drawback is the fact that we were not able to control weight-loading conditions in operated animals. Inconsistencies in repair quality in between the treated groups could be attributed to the influence of the weight-loading conditions of the joint immediately after the surgical procedure. In human patients proper rehabilitation protocols are crucial to optimize the results of bone marrow-stimulating as well as cell based techniques, including postoperative continuous passive motion exercises along with crutch-assisted restrictions of weight-bearing up to 6 to 8 weeks.[31,32] Practical limitations prevented postoperative ambulation restrictions, possibly inflicting detrimental shear forces on the construct, leading to a reduced quality of produced matrix. In our study these limitations might be reflected in the fact that TGF treated groups have good concentrations of GAGs and collagen II, but very high content of collagen I and low content of water.

Determining biomechanical properties of cartilage repair tissues by indentation-type atomic force microscopy (*IT AFM)*

Biochemical and histological parameters provide information regarding the amount and spatial distribution of the major components comprising repaired cartilage. However, only biomechanical analysis can assess the load-bearing capabilities of the cartilage and therefore biomechanical parameters reflect the true nature of the repaired tissue. To determine load-bearing capabilities of examined tissue, IT AFM was used to determine *stiffness* - a mechanical parameter that describes the relation between an applied, nondestructive load and resultant viscoelastic deformation of cartilage tissue. Furthermore, biomechanical data with biochemical content was correlated.

Hyaline cartilage is highly specialized tissue with unique three-dimensional structure, which allows it to behave mechanically as viscoelastic solid.[33] It reflects unique ultrastructure of cartilage extracellular matrix composed of proteoglycans embedded into a network of different types of collagen fibrils. Furthermore, under cyclic loading, the applied stress and resulting strain are not in phase. In order to determine stiffness of the cartilage, compressive force is applied and the ratio of stress to strain – *dynamic elastic modulus* $|E^*|$ is calculated. Several studies describe use of differently shaped probes for indentation testing of cartilage where data are typically assessed at millimeter scale. However, this is insufficient to detect local mechanical property variations of the examined tissue that reflect differences in cartilage structural organization at the molecular level. [34,35]

To overcome these limitations, Stolz et al.[36] proposed a novel, AFM-based approach they termed IT AFM. Their protocol enabled absolute measurements of the dynamic elastic modulus $|E^*|$ at two different length scales of tissue organization – micrometer and nanometer scale. This is technically possible because two different probe types are used for these measurements: the microspherical tips for micrometerscale measurements and sharp pyramidal tips for nanometerscale measurements. In our study dynamic elastic modulus $|E^*|_{nano}$ of the native sheep cartilage is approximately 0.02 MPa, and $|E^*|_{micro}$ is ~1 MPa, which is in agreement with studies performed on the healthy human cartilage, where stiffness values averages around 0.015 MPa, and 2.6 MPa respectively for healthy individuals without OA.[37,38]. According to Stolz et al., this 100-fold modulus difference between micrometer and nanometer scale is a result of assessing different levels of cartilage hierarchical organization. On the micrometer scale, articular cartilage behaves as relatively amorphous material while at the nanometer scale ultrastructural differences are resolved.

Microstiffness values were lowest for native cartilage and gradually rose from TGF and BMC to GFP treated groups respectively (Figure 3). This would suggest that the repair tissue of the TGF treated group is qualitatively superior to the other two groups showing biomechanical properties close to native cartilage. However, nanoscale measurement showed that the BMC treated group has very similar nanostiffness to that of native cartilage, and the stiffness values of the TGF and GFP treated groups are much higher (Figure 4). We hypothesized that this observation could reflect different amount and spatial orientation of newly synthesized extracellular components and/or amount of water within the repair tissue in the last two groups. To test this hypothesis correlation analysis was performed which showed that the dynamic elastic modulus $|E^*|_{micro}$ correlated moderately positively with water and moderately negatively with

collagen type II, but not with GAG and collagen type I content (Table 2). At the same time $|E^*|_{nano}$ correlates strongly positive with collagen type I and moderately negative with water, but not with GAG and collagen type II content (Table 2). At micrometer level, biomechanical properties of cartilage repair tissue are only moderately correlated with the biochemical content. This observation leads us to conclusion that, at micrometer level of tissue organization, it is not possible to determine contribution of individual ECM components to biomechanical properties of repaired cartilage. However, at nanometer level dynamic elastic modulus correlates with collagen I content, which is barely present in native cartilage. A sharp AFM tip has nominal radius of 20 nm that is smaller than an individual collagen fibril, which typically measures around 50 nm.[36] While microspherical tip is too big to detect subtle differences in orientation and amount of collagen fibrils, sharp pyramidal tip can discriminate such differences, resulting in higher stiffness values.

Presence of adenoviral vector in the surrounding synovial lining

The use of viral-based gene therapy has always raised lot of controversies regarding its safety. Although very effective in terms of gene transfer and expression, viral vectors induce immune response and their presence in the surrounding tissue may result with detrimental side effects For example TGF-ß1 if administered into the joint in higher concentration leads to chondrophyte formation at the margins of the joint, which at later stages calcify and become real osteophytes.[39] One of the major goals of this study was to determine if there is any residual presence of virus within the synovium. Following sacrification of the animals, joints were inspected for any signs of osteophyte formation and/or arthrofibrosis, but none were detected.

PCR analysis of the synovial lining tissue could not detect residual presence of the vectors in any of the experimental groups, which suggests that the virus is well contained within the clot, and there is no leakage to the surrounding tissue, consistant to previous studies from Pascher et al.[15] in rabbits.

In conclusion, the present study systematically explores benefits and pitfalls of the novel technique to treat local cartilage defects by using gene plugs in clinical settings. Contrary to more complex approaches in tissue engineering we advocate the use of simpler methods that harness the intrinsic regenerative potential of endogenous tissues using biological stimuli to initiate and promote natural healing *in situ*. This concept has been termed *facilitated endogenous repair* by Evans et al.[40] and the ultimate goal is to enable clinicians to use tissue engineering that is not only successful but also cheap, safe and clinically expeditious. The proposed method is a single-step procedure that can be easily implemented in standard clinical settings, avoids usual drawbacks associated with gene therapy because administered locally, and avoids the use of expensive *in vitro* production of autologous and engineered tissues.

Materials and methods

Vector Construction

The first generation recombinant vector used in this study originated form replication-deficient type 5 adenovirus lacking E1 and E3 loci (Ad.).[41] The recombinant Ad.TGF-β1 and Ad.GFP were constructed by Cre-lox recombination using the system of Hardy et al.[42] Briefly, the adenoviral vectors were propagated in 293-CRE8 cells and purified on three successive $CsCl_2$ density gradients between 1.2 and 1.4 g/ml. Following dialysis in 10mM Tris-HCL, pH 7.8, 150mM NaCl, 10mM $MgCl_2$ and 4% sucrose, the preparations were aliquotted and stored at -80°C. Viral titers were estimated by optical density and standard plaque assay.

Animals

Twenty eight skeletally mature sheep (female, 1 to 3 years old) were used for this study. The sheep were randomly assigned to one of four groups. In the bone marrow clot group (BMC) (*n=6*), the sheep were implanted with untreated autologous bone marrow clot that was aspirated from iliac crest of respected animal. In the green fluorescent protein group (GFP) (*n*=6) autologous bone marrow clots genetically modified with Ad.GFP (GFP gene plug) to express green fluorescent protein were implanted in sheep as a positive control. In the TGF-β1 treated group (TGF) (*n*=10) autologous bone marrow clots genetically modified to over express transforming growth factor-β1 (TGF-ß1 gene plugs) were implanted in the sheep. In the negative control group defects were left empty (n=6) (CON, defect without implant). Native cartilage from the contralateral knee was harvested from each animal and compared to the repair tissue of the defect sites. The experimental protocol was approved by the local Animal Experiment Ethical Committee.

Anesthesia Protocol

The sheep were operated on under general anesthesia and aseptic conditions. The premedication was performed with 0.1 mg/kg of intramuscular xylazine (Xylapan, Vetoquinol, Bern, Switzerland) and cephalic vein was prepared for administration of drugs. Induction of anesthesia was performed intravenously with 2.5% solution of thiopentale sodium (Thiopental, Nycomed, Ismaning, Germany) in a dose 5 mg/kg and small boluses of drug were administered until the jaws were relaxed for endotracheal intubation. Cefuroxime (Ketocef, Pliva, Zagreb, Croatia) was administered perioperatively. Carprofen (Rymadil, Pfizer Animal Healthcare, Exton, PA, USA) was administered postoperatively in a dose of 2 mg/kg.

Surgical Procedure

Medial parapatellar arthrotomy was performed on the right knee of each animal, and both condyles were exposed. A standardized partial-thickness chondral defect of 6.2 mm in diameter (Fig 5a and 5b) was made on the weight-bearing surface of the medial condyle using an adapted punch-drill device of an mosaicplasty instrumentary (Smith & Nephew Inc., Andover, MA, USA). Special care was taken not to damage the subchondral bone, as well as to create sharp edges as the boder of the defects, perpendicular to the joint surface. Defects were then treated as described below.

Pressfit implantation of gene plugs and native bone marrow plugs into the defects

Under sterile surgical conditions 3 ml of bone marrow was aspirated form the right iliac crest of an anesthetized sheep using Trapsystem®Set (H-S Medical, Inc., Boca Raton, FL, USA)

or a 16g needle. Using a 1-ml micropipette, aliquots of 250 ml were rapidly mixed with 25µl suspension of 1 x 10^{10} viral particles of Ad.GFP or Ad.TGF-β1. The mixtures were pipetted into the defects being covered with a paper to build a chamber and allowed to coagulate in situ for five minutes. The paper was then removed, the implants rinsed with saline solution and checked for stability by repetitive flexion and extension of the knee. The joint was closed by suturing in two layers (Fig 5c,d).

Harvesting the samples

All sheep were euthanatized by intravenous injection of an overdose of barbiturate 6 months after surgery. The medial condyle containing the cartilage defect was removed and divided into two halves using a cooled saw, one being used for histology and one for biochemistry and biomechanical testing. Undamaged articular cartilage was taken from the medial condyle of the contralateral knee joint for control. The specimens for histology, biochemical and biomechanical analysis were prepared as described below. Synovial lining specimens were taken from each joint and stored in liquid nitrogen for PCR analysis.

Morphologic Analysis—Histology

The osteochondral samples fixed for histology were decalcified in 10% EDTA. The samples were dehydrated in alcohol, embedded in paraffin, and sectioned at 5 µm. Sections were stained with hematoxylin and eosin to evaluate morphology, and safranin-O to assess proteoglycan distribution in the pericellular matrix. Slides were examined blinded by two observers by light microscopy and graded semi-quantitatively using the ICRS Visual Histological Assessment Scale.[43] The scoring system was based on articular surface morphology,

matrix composition, cellular distribution, cell population viability, subchondral bone morphology and cartilage mineralization.

Quantitative Biochemical Characterization

Samples were frozen and stored at -80°C until ready for analysis. Wet and dry weights of the cartilage or repair tissue were determined before and after freeze-drying. The samples were then solubilized using digestion with trypsin and processed for complete biochemical analysis, as described by Dickinson et al.[44]

Each sample was milled in liquid nitrogen using a stainless steel percussion mortar and pestle, to obtain a fine particulate, and weighed after freeze-drying to obtain the dry weight. Bovine pancreatic trypsin was prepared at 2 mg/mL in Tris buffer (pH 7.5), containing 1mM iodoacetamide, 1mM ethylenediaminetetraacetic acid and 10 mg/mL pepstatin A (all from Sigma). An initial incubation for 15 h at 37°C with 250 mL trypsin was followed by further 2h incubation at 65°C after the addition of a further 250 mL of the freshly prepared proteinase. All samples were boiled for 15 min at the end of incubation, to destroy any remaining enzyme activity.

Type I collagen. The digests were assayed using inhibition enzyme-linked immunosorbent assay (ELISA) using a rabbit antipeptide antibody to type I collagen, as previously described.

Type II collagen. The digests were assayed using inhibition ELISA using a mouse immunoglobulin G monoclonal antibody to denatured type II collagen, COL2–3/4m, as previously described, but modified for use on 384-well plates to allow the use of a smaller volume of sample than is required for a 96-well plate.[19]

Glycosaminoglycans (GAG). A previously described colorimetric assay for GAG was modified for use on 384-well plates to allow the use of a smaller volume of sample than is required for a 96-well plate.[19,45]

Water content. The percentage of water was calculated by subtracting the dry weight of the sample and dividing the difference by the wet weight.

Polymerase chain reaction (PCR) analysis

To detect the presence of adenoviral genome in the synovial lining, PCR analysis was performed. Synovial membranes of the joints were digested with proteinase K for 4 hours at 37 °C. Total DNA was then extracted using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA), following the manufacturer instructions. Amplification of the CMV promoter sequence in the vector and the sheep *β*-actin gene was performed using the following primers: cytomegalovirus forward 5′-TCATATGCCAAGTACGCCCCC-3′, reverse 5′-TGGGGCGGAGTTGTTACGAC-3′; *β*-actin forward 5′CATGCCATCCTGCGTCTGGACC-3′, *β*-actin reverse 5′ TACTCCTGCTTGCTGATCCACATCTGC-3′. Amplification products were visualized on agarose gel with ethidium bromide.

Biomechanical properties

Biomechanical properties of the repair tissue compared to regular cartilage from the contralateral knee was assessed by IT AFM of 2 mm diameter samples, harvested by using a skin biopsy punch and scalpel. Care was taken to include the full thickness of the repair tissue but to exclude any subchondral bone. The specimens were then stored in the cold room at 4º Celsius in

PBS supplemented with protease inhibitor cocktail (Complete, Boehringer Mannheim, Germany).

Mechanical properties (i.e. stiffness) of articular cartilage and repair tissue were determined by measurements of $|E^*|$, the dynamic elastic modulus of articular cartilage at two different length scales of tissue organization – micrometer ($|E^*|_{micro}$) and nanometer ($|E^*|_{nano}$). Preparation of the cartilage samples, data acquisition and processing was done as described by Stolz et al.[36] Briefly, spherical tips with radius of 7.5 μm (SPI Supplies, West Chester, PA, USA) were mounted onto the end of rectangular tipples silicon nitride cantilevers having nominal spring constants of 0.35 N/m (MicroMasch, San Jose, CA, USA) and used for micrometer-scale experiments. For nanometer-scale experiments, square-based pyramidal silicon-nitride tips with a nominal tip radius of 20 nm on V-shaped 200-mm-long silicon nitride cantilevers with a nominal spring constant of 0.06 N/m (Veeco Instruments Inc., Plainview, NJ, USA) were used. The IT AFM was operated in the force-volume mode where the load-displacement curves were recorded at five different sites on the sample surface at a frequency of 3 Hz with scan areas of 0 μm x 0 μm and 10 μm x 10 μm. Data sets recorded at any given sample site consisted of 256 load-displacement curves (each curve consisting of 512 data points) which were analyzed to compute the dynamic elastic modulus $|E^*|$.

Statistics

A Kolmogorov-Smirnov test was used to test distributions of biochemical (GAG, collagen I, collagen II and water) and biomechanical data ($|E^*|_{micro}$ and $|E^*|_{nano}$) for normality. Distributions were normal for all the variables except for $|E^*|_{nano}$. Therefore parametric tests to analyze all biochemical and biomechanical data were used, except for mentioned elastic modulus

data where we used non-parametric tests. Biochemical and biomechanical properties of repaired cartilage were expressed as the mean (M) ± standard deviation (SD). As the contralateral knee in each animal served as its own control, a 2-tailed paired samples *t* test was used to compare treatment versus control groups. Data from each test subsets were compared by one-way analysis of variance (ANOVA) with Tukey post-hoc test where required. To analyze $|E^*|_{nano}$ we used Wilcoxon matched pairs test as a non-parametric equivalent of paired samples *t* test and Kruskal-Wallis and Mann-Whitney tests as non parametric equivalents of ANOVA and Tukey post hoc . Non-parametric tests were also used to analyze semi-quantitative histological scores. Association between biomechanical properties and biochemical content were determined using Spearman correlation. Statistical significance was set at $p < 0.05$. All analyses were performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL).

Acknowledgement

We thank Pierre Mainil-Varlet, MD, PhD, Davor Jezek MD, PhD, Andreja Vukasovic and Ivan Cerovecki for assistance in histological analysis; Snjezana Martinovic, MD, PhD for valuable insight in designing this study; Mario Kreszinger, DVM, PHD, Drazen Vnuk, DVM, PHD and Norbert Kastner, MD for assistance during animal surgeries; and Fran Borovecki MD, PhD for his work with PCR analysis. This study was supported by the Croatian Ministry of Science (projects No. 108-0000000-3652 and 108-1080327-0161). Marko Loparic acknowledges an NCCR ‘‘Nanoscale Science’’ grant, awarded by the Swiss National Science Foundation to Ueli Aebi and Ivan Martin.

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Titles and legends to figures

Figure 1 Representative histological sections of the repair tissue filling the ovine chondral defects stained with hematoxylin-eosin (left panel) and safranin-O (right panel). Panel bars: 100 μm. (a,b) CON group showing acellular tissue (AC) within the defect with intact subchondral bone (BS). (c,d) BMC group. The defect is predominantly filled with fibrocartilage (FC). There is clear demarcation between native hyaline cartilage (Hc) and fibrocartilage (Fc) separated by a defect gap (DG). The subchondral bone (BS) is intact. (e,f) GFP group. Irregular filling of the defect with fissures. The defect is filled with mixture of hyaline and fibrocartilage. (g,h) TGF group. Improved histological appearance of the repair tissue within the defect. Hyaline cartilage (Hc) and columnar organization of chondrocytes is detected on both sides of the defect gap (DG). The subchondral bone is intact (BS).

Figure 2 Biochemical analysis of repaired cartilage compared to native cartilage. (a) GAG. (b) Collagen I. (c) Collagen II. (d) Water.

* 2-tailed paired *t* test: comparison of repaired and native cartilage ($p<0.05$)

† 1-way ANOVA with Tukey post-hoc test: comparison of the treatment groups ($p<0.05$)

Figure 3 Micrometer measurements. Dynamic elastic modulus $|E^*|_{micro}$ of native articular cartilage and repair tissue of the BMC, GFP, and TGF group. Average microstiffness (M±SD) increased from native cartilage to GFP treated group: $|E^*|_{micro} = 1.025 \pm 0.098$ (native cartilage), $|E^*|_{micro} = 1.577 \pm 0.285$ (TGF), $|E^*|_{micro} = 1.863 \pm 0.079$ (BMC), $|E^*|_{micro} = 2.025 \pm 0.371$ (GFP).

* 2-tailed paired *t* test comparison of repaired versus native cartilage ($p<0.05$)

† one-way ANOVA with Tukey post-hoc test comparison of repaired cartilage between treatment groups ($p<0.05$)

Figure 4 Nanometer measurements. Dynamic elastic modulus $|E^*|_{nano}$ of native articular cartilage and repair tissue of the BMC, GFP, and TGF group. $|E^*|_{nano}$ =19.28 ± 3 (native cartilage), $|E^*|_{nano}$ = 21.54 ± 1.24 (BMC), $|E^*|_{nano}$ = 39.26 ± 104.56 (TGF), $|E^*|_{nano}$ = 189.21 ± 39.26 (GFP).

* 2-tailed paired *t* test comparison of repairedand native cartilage ($p<0.05$)

† one-way ANOVA with Tukey post-hoc test comparison of treatment groups ($p<0.05$)

Figure 5 Implantation of a gene plug. (a) An adapted standardized mosaciplasty instrumentary was used to create a chondral defect on the weight-bearing surface of the medial condyle in sheep. (b) Care was taken not to penetrate the subchondral plate. The defect measured 6.2 mm in diameter. (c) Pressfit Implantation of the bone marrow construct into the defect. (d) The plug is stable, well-placed within the defect, joint is rinsed with saline and ready to be closed.

Tables

Table 1 Histological grading of the repair tissue at 6 months according to ICRS Visual Histological Assessment Scale [a] (medians ± interquartile range)

ICRS Score	*Treatment group*								
	CON		*BMC*		*GFP*		*TGF*		*p**
	C	*Q*	*C*	*Q*	*C*	*Q*	*C*	*Q*	
Surface	0.00	0	0.00	0	0.00	0	0.00	0	0.343
Matrix	1.50	2	3.00	1	3.00	2	3.00	0	0.062
Cell ditribution	1.00†	0	2.00	0	2.00	2	2.00	0	0.016
Cell population viability	0.00	1	1.00	3	0.00	0	0.00	0	0.095
Subchondral bone	2.50	1	3.00	0	3.00	3	3.00	0	0.177
Cartilage mineralization	1.50	3	3.00	0	3.00	3	3.00	0	0.162
Median total score	6.50	6	11.50	4	11.00	9	11.00	0	0.061

Abbreviations: CON, control group; BMC, bone marrow clot group; GFP, green fluorescent protein group; TFG, transforming growth factor-β1 group, C, median; Q, interquartile range.

[a]The table shows the medians from each group for each subcategories, and the total medians for each group.

***** Kruskal-Wallis test (Mann-Whitney test was used as a post-hoc procedure when K-W test revealed statistically significant difference).

† Significantly lower score from TGF and BMC groups (p=0.002 and p=0.008 respectively, Mann-Whitney test).

Table 2 Associations between dynamic elastic modulus measured on micrometer and nanometer scale and biochemical parameters (GAG, collagen I, collagen II and water).

	Association [Spearman's ρ(p)]	
	$\|E^*\|_{micro}$	$\|E^*\|_{nano}$
GAG	-0.35 (0.108)	-0.24 (0.288)
Collagen I	-0.08 (0.710)	0.80 (<0.001)†
Collagen II	-0.56 (0.007)†	0.29 (0.191)
Water	0.44 (0.038)*	-0.46 (0.033)*

†. Association is significant at the 0.01 level (2-tailed).

*. Association is significant at the 0.05 level (2-tailed).