CellR4 2015; 3 (1): e1394

TGF-β3-Releasing Pharmacologically Active Microcarriers Combined with Human Cartilage Microparticles Drive MIAMI Cells Toward a Hyaline Cartilage Phenotype

G.J.-R. Delcroix[1,2,3], G. D'Ippolito[1,2,3,4], T. Reiner[2], T. Malinin[1], H. T. Temple[1], C.N. Montero-Menei[5,6], P.C. Schiller[1,2,3,7]

[1]Department of Orthopaedics, University of Miami Miller School of Medicine, Miami, FL, USA
[2]GRECC & Research Service, Bruce W. Carter Veterans Affairs Medical Center, Miami, FL, USA
[3]Interdisciplinary Stem Cell Institute, University of Miami Miller School of Medicine, Miami, FL, USA
[4]Department of Biomedical Engineering, University of Miami College of Engineering, Miami, FL, USA
[5]Inserm U1066, MINT, Angers, France
[6]LUNAM, Angers University, France
[7]Department of Biochemistry & Molecular Biology, University of Miami Miller School of Medicine, Miami, FL, USA

Corresponding Author: Gaëtan Jean-Robert Delcroix, Ph.D/Engineer, Gdelcroix@med.miami.edu
Paul C. Schiller, Ph.D, P.Schiller@miami.edu

Keywords: Cartilage, tissue engineering, MIAMI cells, stem cells, scaffolds, microcarriers, drug delivery.

Abstract

Introduction: **Articular cartilage defects are common, particularly in the young and active population.**

Background: **Most treatments available for articular cartilage defects are not completely satisfactory as they tend to result in the production of fibrocartilage instead of hyaline cartilage.**

Aim: **We evaluated the chondrogenic differentiation of marrow-isolated adult multilineage inducible (MIAMI) cells in contact with pharmacologically active microcarriers (PAMs) releasing TGF-β3, microcartilage, or a combination thereof. We hypothesize that combination of TGF-β3-releasing PAMs and microcartilage will enhance and guide the chondrogenic differentiation of MIAMI cells toward a hyaline phenotype.**

Materials and Methods: **MIAMI cells were isolated from swine bone marrow. PAMs are biocompatible and biodegradable 60 *μ*m PLGA microspheres coated with a biomimetic surface and delivering TGF-β3 in a controlled fashion. Microcartilage is made of micronized and dried cadaveric human articular cartilage. The attachment, survival and chondrogenic differentiation of swine MIAMI (swMIAMI) cells in contact with PAMs and/or microcartilage was assessed by microscopy, histological staining, RT-qPCR and immunohistochemistry of the cartilage pellets formed after 21 days of chondrogenic differentiation *in vitro*.**

Results: **SwMIAMI completely attached to PAMs and microcartilage in 24 hours *in vitro*, and were then pelleted to undergo *in vitro* chondrogenesis. Microcartilage and PAMs TGF-β3 contributed to the overall size of the neo-cartilage, with PAMs having a strong effect on swMIAMI cell survival and glycosaminoglycan production. Using RT-qPCR, we observed the strongest effect in terms of hyaline-specific cartilage gene expression, i.e. high *aggrecan* and low *type X collagen*, with the combination of both PAMs TGF-β3 and microcartilage. We confirmed by immunohistochemistry the overexpression of aggrecan and down-regulation of type X collagen in swMIAMI cells exposed to both microcartilage and PAMs delivering TGF-β3.**

Discussion: **Our primary finding was that the exclusive combination of the natural microcartilage and synthetic PAMs delivering TGF-β3 provide a unique environment with adequate support and signaling molecules to direct the differentiation of swMIAMI cells *in vitro* towards a phenotype closely resembling that of hyaline cartilage instead of a more fibrocartilaginous phenotype.**

Conclusion: **In the future, we hope this study will help designing a minimally invasive combinatorial strategy to provide a faster healing of the damaged articular cartilage with improved microarchitecture and mechanical properties.**

Introduction

Articular cartilage defects are common, particularly in the young and active population. Joint trauma affects all joint tissues to some degree but the damage to articular cartilage, almost always accompanied with subchondral bone defect, appears most significant as it is largely irreversible. The acute symptoms following joint injury include joint pain and swelling due to intra-articular bleeding, synovial effusion and inflammatory cell infiltration[1], which may ultimately lead to post-traumatic osteoarthritis[2] with the resulting reduced physical activity and deconditioning of the musculoskeletal system[3]. Joint replacement in this young patient group is complicated by the limited lifespan of the implants[3] and alternatives are therefore needed. Repair strategies can be categorized in three main groups: those involving the direct transplantation of whole tissue grafts or progenitor/differentiated cells with chondrogenic potential onto the cartilage defect, those involving the use of scaffolds applied onto the lesion, or a combination thereof[4, 5].

Microfractures, allogeneic or autologous osteochondral grafts harvested from a non-load bearing area of the joint are techniques still widely used, despite major drawbacks such as the formation of fibrocartilage while the viability of chondrocytes within grafted tissue and the capacity of the graft to withstand the stress of a load-bearing area are uncertain[4, 6]. In addition, graft-based strategies frequently suffer graft integration problems, delamination, tissue hypertrophy, as well as harvest site morbidity[4]. Autologous chondrocyte implantation (ACI) is currently a well-established clinical technique[7] but faces problems such as the low expansion potential of chondrocytes, dedifferentiation in culture[4, 8]. Current strategies for joint repair using graft, cellular therapy, and tissue engineering strategies in autologous or allogeneic settings as well as tissue banking are well-described in the book of Malinin and Temple[6].

Although chondrocytes have been primarily used for cartilage repair, researchers are now investigating the potential of many other cell types to better repair articular cartilage[9, 10]. Mesenchymal stromal cells (MSCs) are a very attractive source of cells for cartilage repair due to their ease of expansion and potential to differentiate toward the chondrogenic lineage[11-15]. MSCs represent a heterogeneous population of mixed cells including stem, progenitor, and committed cells with different molecular and functional properties[16-18]. MSCs are easily obtained from the iliac crest bone marrow[19, 20], are available for autologous transplantation, can be rapidly expanded *ex vivo*, have immune-modulatory properties[21], and can migrate to areas of inflammation. MSCs immunomodulatory properties are also considered essential to avoid the onset of post-traumatic osteoarthritis[22]. Several preclinical and a few clinical studies have described the therapeutic potential of MSCs for cartilage repair[23-25] but have also revealed the main limitation of this strategy, which is a poor cell engraftment[20]. In addition, most MSC-based strategies suffer from undesired mineralized cartilage production with high type X collagen expression[26], characteristic of fibrocartilage found in intervertebral discs[27] compared to the more flexible hyaline articular cartilage[28]. To overcome these cell survival and differentiation limitations, the use of matrix scaffolds in tissue engineering has become increasingly popular[29] to help improve the survival of the grafted cells and therefore their physiological contribution to an improved outcome of the therapeutic strategy.

Hyaluronan/hyaluronic acid[30-35] and collagen-based matrices[36-38] are among the most popular natural scaffolds as they offer a substrate that would normally be found in the structure of native articular cartilage. Many other types of scaffolds have been investigated for cartilage repair, alone or in combination with MSCs, such as fibrin glue[39], agarose gels[40], modified marine-based polysaccharides[41], alginate[42], synthetic materials[43-46], nanoscaffolds[47-49] as well as more complex strategies based on composite scaffolds made of several materials[37, 50-52].

Interestingly, human allogeneic cartilage-derived scaffolds have also recently been investigated to repair articular cartilage defects, the rationale for these strategies being to take advantage of the natural cartilage tissue properties, presumably extracellular matrix molecules and growth factor content, to facilitate cartilage repair[53-58]. Two such products currently distributed by the University of Miami Tissue Bank, namely cartilage microparticulate (microcartilage) and cartilage fluff[59, 60], have shown very promising results for the filling of artificially created osteochondral lesions within 16 weeks in a baboon animal model[61] and are now routinely used for the repair of isolated cartilage defects in humans.

In addition to the use of stem cells and scaffolds, growth factors have demonstrated an important effect in guiding the survival and differentiation of the stem cells towards a chondrocytic phenotype[62, 63]. Consequently, combinatorial strategies relying on the use of cells, scaffolds and growth factors aiming at guiding the fate of the transplanted cells are being

investigated. Various growth factors are currently known to be critical in the chondrogenic differentiation of progenitor cells resulting in research studies investigating the use of scaffolds containing or releasing FGF-2[64], PTH[65], BMPs[66,67], TGF-β[68,69] or more complexes ones combining various molecules and growth factors[70-73].

Nevertheless, most recent strategies lead at best to a mixture of fibrocartilage and hyaline cartilage that, on the long term, usually fails to restore articular cartilage molecular, biochemical, biomechanical and functional properties. When hyaline cartilage is produced, it is often of an immature nature and lack a true articular surface. Functionally, the repaired tissue may fail to withstand the mechanical demands of articular cartilage and a lack of successful lateral integration between the host and repaired tissue, causing tissue degeneration, is often observed[4]. This could probably be due to the choice of the cytokine/growth factor, the pharmacokinetic bioavailability of the factor during the repair process, the inconsistent behavior/response of the cells, the cytokine milieu in the injury site during the repair process, and the biomechanical forces influencing tissue organization during the repair process, among other factors. Thus, novel tissue engineering strategies are needed in order to fully recover the biomechanical, tissue organization, and functional properties properties of lesioned articular cartilage in a rapid and minimally invasive fashion.

A tissue engineered product for cartilage repair should provide a potent chondro-inducing support for the articular hyaline tissue, and should be preferably injectable into the joint, thereby avoiding an open-knee surgery and reducing the patient's recovery time. Ideally, this product should be readily available as an off-the-shelf manufactured product. The strategy we describe in the present paper combines the use of 1) marrow-isolated adult multilineage inducible (MIAMI) cells, a subpopulation of MSCs delivered in a hybrid scaffold made of 2) biocompatible/biodegradable microcarriers, termed pharmacologically active microcarriers (PAMs), that release a proven chondrogenic growth factor (TGF-β3) in a sustained and physiological fashion with an ideal pharmacokinetic release profile and 3) human cartilage microparticles (microcartilage). See Figure1 for an overview of this combinatorial strategy. Compared to MSCs, MIAMI cells are significantly more homogeneous, developmentally more immature, and have a distinct proteome and secretome profile[74-76] of potential benefit for cell therapy strategies. PAMs are biocompatible and biodegradable PLGA microspheres engineered to continuously release an active protein such as TGF-β3 and present a surface of extracellular matrix molecules supplying a three-dimensional (3D) scaffold for the transported cells[77-79]. These combined pa-

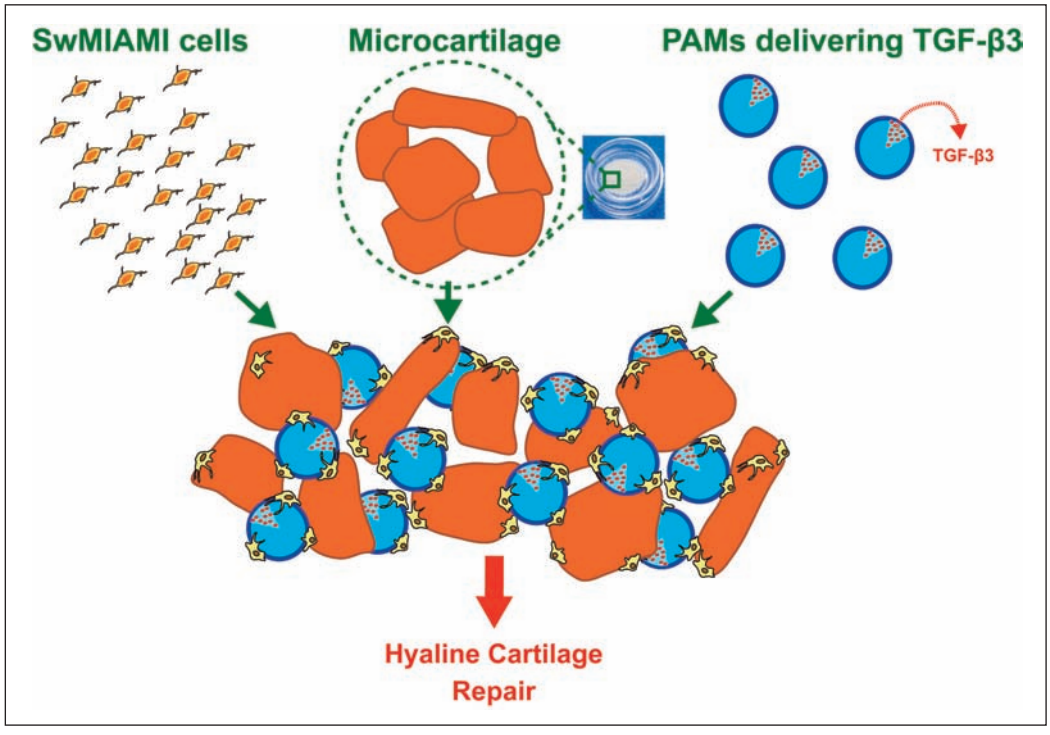

Figure 1. Combinatorial tissue engineering strategy for hyaline cartilage repair.

rameters act both on the transported cells and the surrounding tissue, enhancing cell engraftment in different transplantation paradigms[80-82] as well as MSC chondrogenic differentiation[83, 84]. The microcartilage is made of micronized articular cartilage dried under conditions minimizing thermal denaturation of the proteins found in the hyaline matrix [59, 60]. Thus, we hypothesize that both PAMs delivering TGF-β3 and microcartilage should help guiding the cells toward the adequate hyaline cartilage phenotype, while the microcartilage should also provide the biomechanical and chondro-regenerative support necessary to the healing process. To test this hypothesis, we isolated MIAMI cells from swine bone marrow[85] and performed micropellet culture of MIAMI cells in presence of PAMs, microcartilage, or a combination thereof and performed RT-qPCR and histology/immunohistochemistry analysis on the neo-cartilage pellets formed *in vitro*. The rationale for using swine MIAMI cells lies in the perspective of future *in vivo* work on a swine animal model of cartilage repair.

Materials and methods

Bone marrow harvesting, selection & expansion of swine MIAMI cells

Whole bone marrow (BM) was obtained through iliac crest aspiration from a 3 months old female Yorkshire swine. The animal was placed under general anesthesia and a bone marrow aspiration needle with stylet inserted into the dorsal aspect of the tuber coxae. The stylet was removed and 20 ml of BM was collected with a syringe containing 200 U/ml heparin. SwMIAMI cells were isolated from the BM as previously described[85]. Briefly, whole BM cells, including adherent and non-adherent cells, were plated at a density of $1.0x10^5$ cells/cm^2 in 10 ng/ml fibronectin-coated T-75 flasks (Nunc, Thermo scientific, Waltham, MA) in the presence of DMEM-LG with 5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (P/S) (Sigma-Aldrich, St-Louis, MO), 100 μM ascorbic acid 2-phosphate (Sigma-Aldrich), and a mixed solution of essential fatty acids (expansion medium). Bone marrow nucleated cells were counted with a hemocytometer using 2% acetic acid (to lyse red blood cells) and 0.4% trypan blue (to exclude dead cells). Cells were incubated in a tri-gas incubator in a 100% humidified atmosphere of 3% O_2, 5% CO_2, and 92% N_2 (low oxygen tension). Whole medium was changed after one week. Thereafter, half medium was replaced twice a week. SwMIAMI cells were cultured up to 40% confluence before splitting. For expansion, swMIAMI cells were plated at a density of 100-300 cells/cm^2 in expansion medium at low oxygen tension and one half medium changed twice a week. Only cells from passages 2 to 4 were used.

Formulation of PAMs releasing TGF-β3

The three main steps necessary for PAM formulation (TGF-β3 nanoprecipitation, microsphere formulation and surface functionalization with fibronectin and Poly-D-Lysine) as well as all the characterization work were performed as previously described in Morille et al. 2013[84]. The PAMs releasing TGF-β3 used in the present study were prepared as the one referred as the optimized "PLGA-P188-PLGA PAMs/TGF-β3" in Morille et al. 2013[84].

Preparation of microcartilage

All the procedures related to microcartilage processing were performed as previously described[59, 60]. Each donor was tested for a panel of communicable diseases (including HIV, HCV, HBV, HAV, Syphilis, and HTLV.3) and the tissues were processed aseptically, with microbiologic cultures taken at each step of the process. Slices of articular cartilage were removed from cadaveric human bones with a scalpel, washed in cold Lactated Ringer's solution and blotted dry before hypothermic dehydration. Hypothermic dehydration was performed by placing the samples for 3-4 days in a vacuumed desiccator containing silica gel beads at 4ºC. Dehydrated cartilage slices were then micronized at liquid nitrogen temperature using a CryoMill apparatus (Retsch, Newtown, PA). The resulting cartilage preparation was then sieved to select microparticles within the 100-300 μm range before packaging.

Preparation of the swMIAMI cells/PAMs/ microcartilage aggregates

SwMIAMI cells were washed with DMEM-LG, detached with a 3:1 mixture of trypsin-EDTA 0.05% (GIBCO, Life Technologies, Carlsbad, CA) and Versene (GIBCO), and pelleted at 295 g for 5 min before resuspension in expansion medium. Lyophilized PAMs or microcartilage were resuspended in expansion medium for at least 15 min before use, and then sonicated and briefly vortexed

prior to addition of the cell suspension. Samples of up to $2.0x10^6$ cells attached onto 1.5 mg of materials (either PAMs, microcartilage or a combination thereof) were prepared in a final volume of 1.5 mL. The mixture was then plated in 1.9 cm^2 Costar ultra-low cluster plate (#3473, Corning, Corning, NY) and incubated at 37ºC for up to 24 hours to allow cell attachment on the surfaces of PAMs/microcartilage.

Transduction of swMIAMI Cells

SwMIAMI cells were transduced to express enhanced green fluorescent protein (eGFP) to study their attachment to PAMs and microcartilage using fluorescent microscopy. Transduction of swMIAMI cells was performed in a similar way than previously described for human MIAMI cells[86]. Briefly, subconfluent swMIAMI cells were transduced with p24 lentiviruses containing a pRRLsinPPT-EGFP construct (provided by Dr. Anthony Oliva, Viral Vector Core, The Miami Project, University of Miami Miller School of Medicine, Miami, FL) to express enhanced green fluorescent protein (eGFP). Lentiviruses were diluted in MIAMI cell expansion medium supplemented with 10 ng/mL protamine sulfate (Sigma) at a concentration of 1 transduction unit per cell. Fresh viral solution was applied another time after 24 hours and left in contact with the cells for 3 additional days.

Microscopic evaluation of cell attachment

SwMIAMI cell adhesion to the PAMs/microcartilage surface was assessed under phase microscopy as well as fluorescent microscopy to visualize eGFP expressing MIAMI cells (Nikon Eclipse Ti, Nikon Instruments, Melville, NY). Scanning electron microscopy (SEM) was also used to obtain high resolution images. For SEM, aggregates of cells and PAMs and/or microcartilage were washed in PBS (GIBCO), fixed with 1% glutaraldehyde (Sigma-Aldrich) and then dehydrated with alcohol. Afterwards, samples were soaked in hexamethyldisylasane (Sigma-Aldrich) and covered by a thick layer of carbon before observation using a FEI XL-30 Field Emission SEM (FEI, Hilsboro, OR).

Chondrogenic differentiation-micropellet culture

Micropellet cultures were performed as elsewhere described[87, 88], with modifications. Briefly, the aggregates described in the previous section were then recovered and pelleted in 15 mL Falcon tubes by centrifugation at 550 g for 5 min, in a final volume of 1.5 mL chondrogenic media composed of DMEM/HG (GIBCO), 100 U/mL penicillin, 0.1mg/mL streptomycin, 0.25 mg/mL amphotericin B (Sigma-Aldrich), 100 μM Ascorbic Acid 2-phosphate (Sigma-Aldrich), 1X ITS (Sigma), 50 μg/ml L-Proline (Sigma) and 100 nM dexamethasone (Sigma). Overall, five conditions were used to test the effect of the PAMs-TGF-β3, microcartilage and a combination thereof on the chondrogenic differentiation of the MIAMI cells: #1 cells, #2 cells in medium supplemented with 10 ng/mL TGF-β3, #3 cells with PAMs-TGF-β3, #4 cells with microcartilage, #5 cells with microcartilage and PAMs-TGF-β3. Samples were incubated in a 5% CO_2 humidified cell culture incubator at 37ºC with caps loosened. The medium was changed every 3-4 days and the experiment terminated after 21 days. Untransduced swMIAMI cells were used in the chondrogenic differentiation experiment. The neo-cartilage pellets formed after 21 days were analyzed by RT-qPCR, histology and immunohistochemistry.

mRNA extraction, reverse transcription and real-time quantitative PCR

Design of primers specific for swine genes (Table 1) was performed using the NCBI primer-blast website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). RNA extraction was performed using the RNAqueous-4PCR Kit (Ambion, Life Technologies, grand Island, NY), following the manufacturer's guidelines with modifications. Micropellet cultures were lysed with 250μL of lysis buffer followed by homogenization with 18g needles, before being applied onto Qiashredder columns (Qiagen, Valencia, CA) to achieve an optimal homogenization. Microcartilage alone (with no cells) was used as a control. The rest of the procedure was performed following the manufacturer's instructions. Retrotranscription of the isolated mRNA was performed using the High Capacity cDNA Reverse transcription Kits (Ambion) and the double-stranded product was finally purified using the Qiaquick PCR Purification kit (Qiagen) and eluted in 50 μL RNAse free water. RT-qPCR was performed as previously described[89], with modifications: 10 μL of cDNA (diluted 1:10) were mixed with 12.5 μL SYBR green (Brilliant II SYBR Green QPCR Master mix, Stratagene, Agilent technologies, Santa Clara, CA), 0.375 μL ROX (diluted 1:1000) and 2 μL of primer pairs (2 μM final concentration

of both forward and reverse primers, Eurofins MWG Operon, Huntsville, AL) in a final volume of 25 μL. Amplification was carried on a Stratagene Mx3005P thermocycler (Agilent technologies) with a first denaturation step at 95ºC for 10 min and 40 cycles of 95ºC for 30 s, 56ºC for 45 s and 72ºC for 30s. After amplification, a melting curve of the products determined the specificity of the primers for the targeted genes. A mean cycle threshold value (Ct) was obtained from 2 measurements for each cDNA. Two housekeeping genes known to be adequate for MIAMI cells[90], *elongation factor 1a* (*EEF1A1*) and *ribosomal protein L13a* (*RPL13A*), were used for normalization. The relative transcript quantity (Q) was determined by the delta cT method $Q=2^{(Ct\ min\ in\ all\ the\ samples\ tested-Ct\ of\ the\ sample)}$. Relative quantities (Q) were normalized using the geometric mean of the Q values obtained with both housekeeping genes:

$$Q\ normalized = \frac{Q}{Mean\ (Q\ EEF1A1\ and\ Q\ RPL13A)}$$

Histology, staining and immunohistochemistry

After fixation in 10% buffered formalin (Sigma-Aldrich), the cartilage pellets were dehydrated, embedded in paraffin, sectioned at 4 μm, and incubated at 57°C overnight. Knee osteochondral biopsies were used as positive control for the validation of the antibodies (data not shown). Pellet sections were stained with hematoxylin and eosin (H&E) (Sigma-Aldrich) following standard procedures as well as with Alcian Blue/Fast Red (ScienCell, Carlsbad, CA) for analysis of proteoglycan deposition[40]. For immunohistochemistry (IHC) antigen retrieval was done by incubating sections in 80-90°C 10 mM sodium citrate buffer (pH 6, Sigma-Aldrich). Sections were incubated with primary antibodies diluted 1/50 in M.O.M. diluents (Vector Laboratories, Burlingame, CA): anti-type II collagen monoclonal antibody (Clone #5B2.5, Abcam, Cambridge, England), anti-cartilage proteoglycan (aggrecan) monoclonal antibody, (Clone #MAB2015, Millipore, Billerica, MA) and anti-type X collagen monoclonal antibody (Clone #COL-10, Abcam). The slides were washed several times in PBS and were incubated with the secondary biotinylated anti-mouse IgG (Vector Labs), following the manufacturer's instructions. Specific color was developed with the Vectastain Elite ABC kit Immunoperoxidase system according to the manufacturer's recommendations (Vector Labs) using 3, 3'-diaminobenzidine tetrahydrocloride (DAB) as substrate. Sections were counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene, and mounted with synthetic resin under a glass coverslip. Sections were examined using a Microphot-FXA microscope (Nikon Instruments).

Statistical analysis

All quantitative data are expressed as mean ± SD. Statistical analysis was performed with the GraphPad Prism software using one-way ANOVA followed by Dunnet post-hoc multiple comparison test. Values of $p<0.01$ were considered statistically significant and noted*.

Results

Attachment of swMIAMI cells to PAMs and microcartilage

SwMIAMI cells were incubated *in vitro* with microcartilage alone or in the presence of microcartilage and PAMs delivering TGF-β3. SwMIAMI cells started to attach to microcartilage and PAMs after 4 hours as seen under phase contrast (Fig. 2 A, C) and fluorescent microscopy to visualize swMIAMI cells

Table I. Primer sequences used for RT-qPCR.

Gene	*Full Name*	*Accession number*		*Sequences*	*Amplicon size*
EEF1A1	*Sus scrofa eukaryotic translation elongation factor 1 alpha 1*	NM_001097418.1	F	CCACCACTACTGGCCATCTG	146
			R	ACGCTCACGTTCAGCCTTTA	
RPL13A	*Sus scrofa ribosomal protein L13a*	NM_001244068.1	F	AGGCCAAGATCCATTACCGC	104
		NM_001244069.1	R	CTTGAGGACCTCCGTGAACC	
COLL10A1	*Sus scrofa collagen, type X, alpha 1*	NM_001005153.1	F	GCCAACCAGGGAGTAACAGG	129
			R	TGGGTCATAGTGCTGTTGCC	
ACAN	*Sus scrofa aggrecan*	NM_001164652.1	F	CAGTCACACCTGAGCAGCAT	87
			R	GTTCAAGCCAATCCACTGGT	

expressing eGFP (Fig. 2 E, G). At 4 hours, many cells were still unattached to the materials and it was shown using SEM microscopy that most of the cells presented a rounded morphology characteristic of an incomplete attachment to the materials (Fig. 2 I, K). After 24 hours, almost all the living cells (expressing eGFP) were attached to the microcartilage and PAMs, and exhibited a spread morphology in close contact with the materials (Fig. 2 B, D, F, H). Interestingly, the combination of swMIAMI cells with microcartilage/PAMs formed homogeneous aggregates in which the cells appeared to contribute to maintain together the microcartilage and PAMs as macroscopically uniform clusters. These aggregates formed after 24 hours attachment (Fig. 2 J, L) were able to easily pass through a 16-gauge needle, which would allow non-invasive *in vivo* studies using an arthroscopic strategy in the future. For chondrogenic differentiation assessment, these aggregates were then pelleted and cultured for 21 days under chondrogenic differentiation conditions (Fig. 2 M, N).

Pellet morphology and histological staining

After 21 days under chondrogenic differentiation,

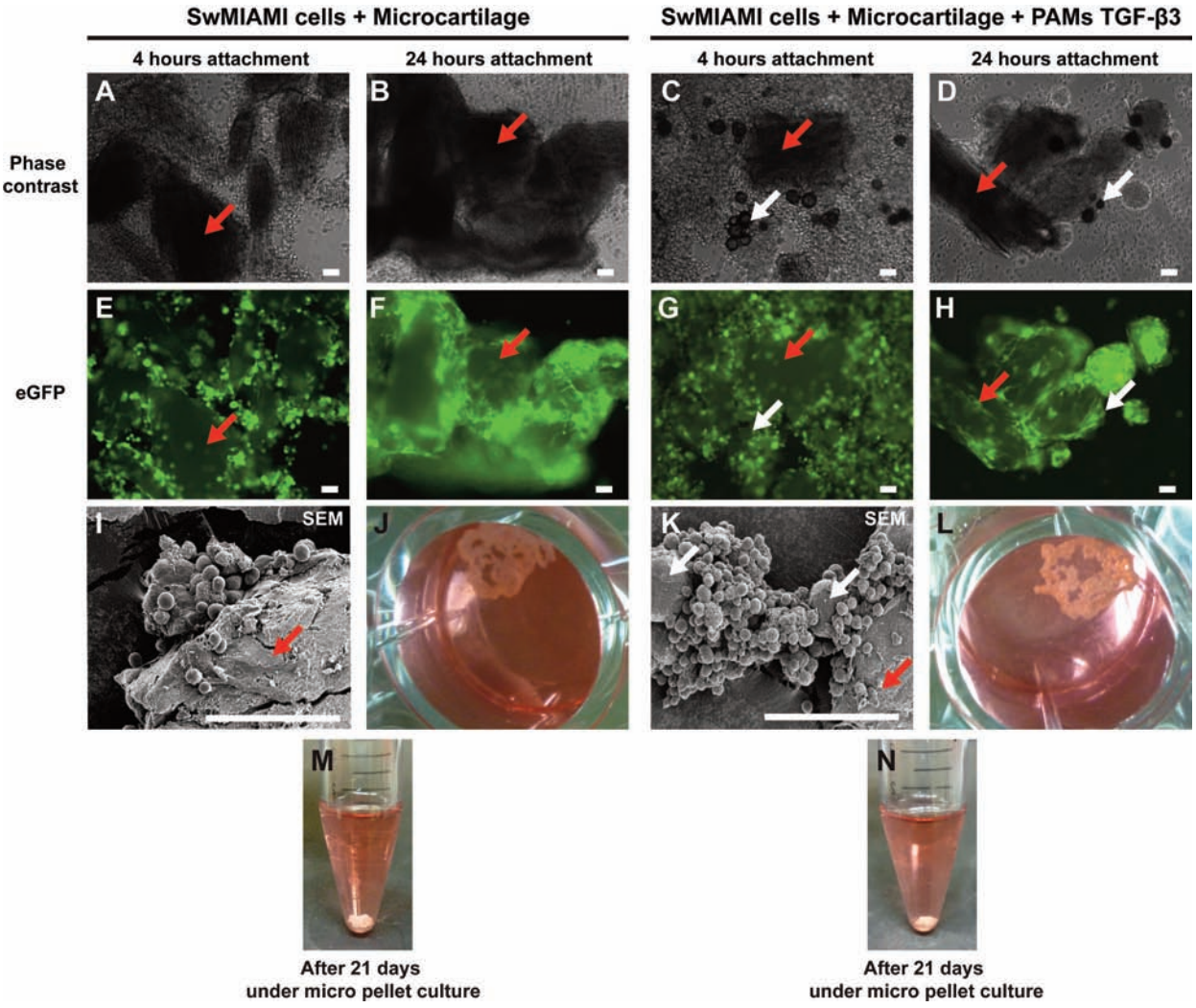

Figure 2. SwMIAMI cells fully attach to PAMs & microcartilage within 24 hours to form homogeneous, injectable, complexes *in vitro*. SwMIAMI cells were incubated with microcartilage (left panel); or a combination of microcartilage and PAMs (right panel). After 4 hours, a significant fraction of the cells started to attach to the microcartilage and PAMs, even though they do not display yet a flat/spread morphology (I, K). Almost all the living cells (expressing eGFP) were attached after 24 hours and were spread on the surface of the materials (F, H). The complexes formed (J, L) were then pelleted and cultured for 21 days under chondrogenic differentiation conditions (M, N). A-C are phase contrast images; E-H the corresponding images showing eGFP; I and K are SEM; J,L,M,N are macroscopic images. White arrows: PAMs. Red arrows: microcartilage. 1.5 mg material in total was used for each condition. Scale bar: 100μm.

we observed using H&E staining that the microcartilage and PAMs not only contributed to the overall size of the neocartilage pellets, but also affected the amount of swMIAMI cells detected. Indeed, a higher density of swMIAMI cells was observed when they were combined to microcartilage and PAMs TGF-β3 compared to microcartilage alone (Fig. 3 A, B). SwMIAMI cells pelleted without supporting scaffolds at all resulted in even smaller cartilaginous pellets (data not shown). The content of glycosaminoglycans was also increased with the presence of PAMs TGF-β3 compared to microcartilage alone (Fig. 3 C, D). Thus, adhesion onto microcartilage and PAMs TGF-β3 promoted the survival of the swMIAMI cells in the course of the chondrogenic differentiation *in vitro* as well as a stronger production of glycosaminoglycans.

RT-qPCR assessment of chondrogenic differentiation

At the end of the 21-day chondrogenic differentiation, the neocartilage pellets were lysed, total RNA extracted, and gene expression was quantified by RT-qPCR. Transcript levels were normalized to that of both *EEF1A1* and *RPL13A* mRNAs. Significant effects of the PAMs and microcartilage were observed on the a*ggrecan* and t*ype X collagen* expression. SwMIAMI cells expressed low levels of *aggrecan* under control conditions and, as expected, addition of TGF-β3 to the culture media increased *aggrecan* mRNA expression (2.57±0.16 fold) and decreased *type X collagen* expression (0.8±0.05 fold). However, a controlled delivery of TGF-β3 by the PAMs had an even stronger effect on the expression of *aggrecan* and t*ype X collagen* (5.96±0.96 and 0.26±0.01 folds, respectively), underlining the importance of the growth factor deliv-

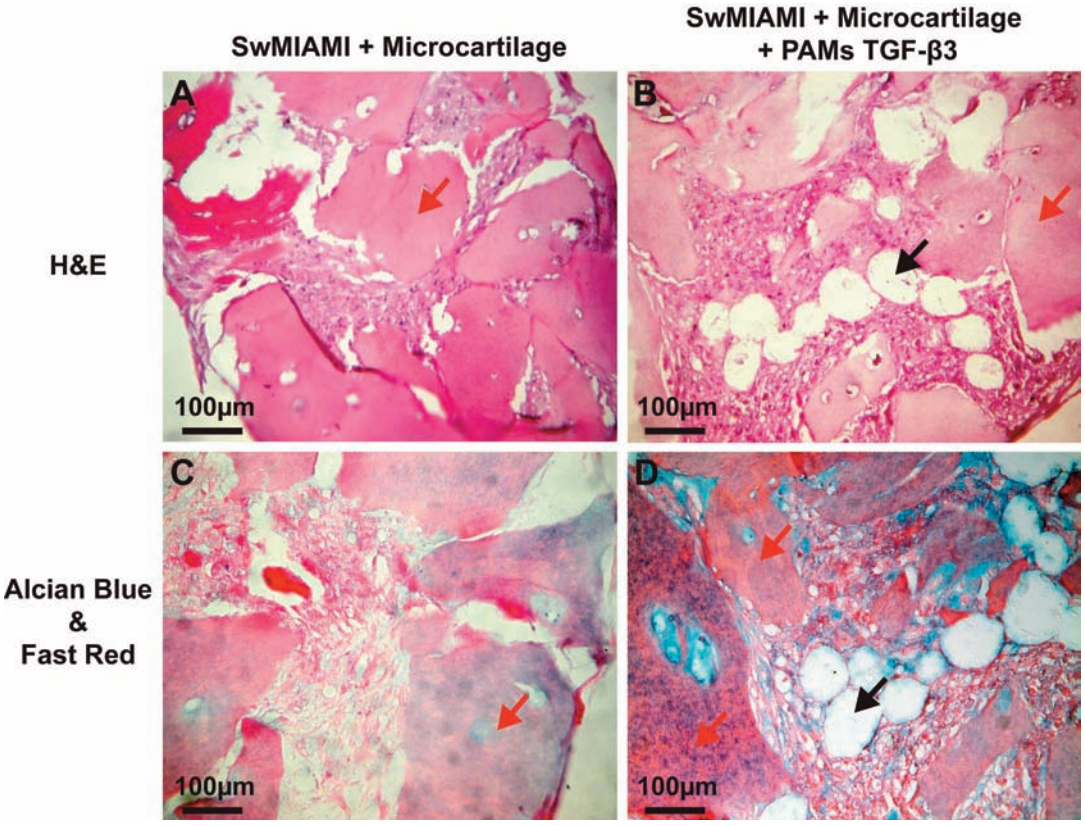

Figure 3. PAMs TGF-β3 combined to microcartilage increased swMIAMI cell survival and glycosaminoglycan production during chondrogenic differentiation. Microcartilage significantly contributed to the size of the neocartilage pellets obtained after 21 days of chondrogenic differentiation, as observed with H&E staining (A). A higher cell survival was observed after 21 days of chondrogenic differentiation in the presence of microcartilage and PAMs TGF-β3 (B) compared to microcartilage alone (A), thereby underlining the importance of TGF-β3 release on cell survival. The content of glycosaminoglycans (stained blue with Alcian Blue) was also increased with the presence of PAMs TGF-β3 (D) compared to microcartilage alone (C). Red arrows point to microcartilage, black arrows point to PAMs.

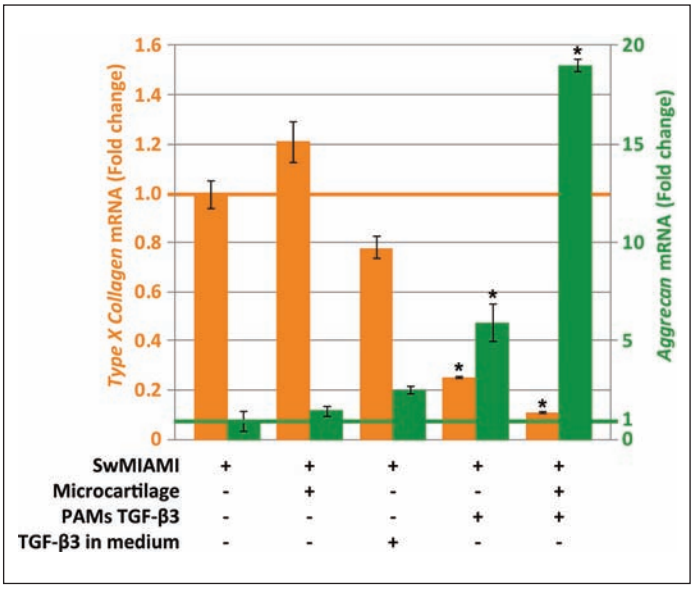

Figure 4. Specific hyaline profile induction: *aggrecan* mRNA expression is increased while *type X collagen* mRNA is drastically reduced in swMIAMI cells when cultured with both microcartilage and PAM TGF-β3 *in vitro*. SwMIAMI cells alone or combined to microcartilage and/or PAMs releasing TGF-β3 were subjected to chondrogenic differentiation conditions *in vitro* for 21 days. At the end of this period, cells were lysed and gene expression was quantified by RT-qPCR. Gene expression levels were normalized to that of the endogenous expression of *EEF1A1* and *RPL13A* transcripts. TGF-β3 supplementation in the medium had some effects in increasing *aggrecan* (2.57±0.16 fold) while slightly decreasing *type X collagen* expression (0.8±0.0 fold). TGF-β3 released in a continuous manner from the PAMs had an even stronger effect on *aggrecan* and *type X collagen* expression (5.96±0.96 and 0.12±0.01 folds, respectively). The combination of PAMs TGF-β3 and microcartilage had a strong effect on *aggrecan* expression and drastically reduced *type X collagen* expression (19.11±0.28 and 0.12±0.01 folds, respectively).

ery mode. Interestingly, culturing swMIAMI cells in the presence of both microcartilage and PAMs TGF-β3 had a significant effect on *aggrecan* and *type X collagen* expression, with 19.11±0.28 and 0.12±0.01 fold change, respectively (Fig. 4). In addition, parallel expression of *type X collagen* (known to promote cartilage calcification and possibly hypertrophy) was dramatically reduced in the presence of PAMs TGF-β3 and even more so when swMIAMI cells formed complexes with both microcartilage and PAMs TGF-β3 (Fig. 4). Importantly, no cDNAs were ever amplified from human microcartilage alone, without cells, used as a control (data not shown). Thus, combination of microcartilage and PAMs delivering TGF-β3 directed the chondrogenic differentiation of swMIAMI cells toward a hyaline phenotype while minimizing the expression of the default fibrocartilage phenotype.

IHC assessment of chondrogenic differentiation

At the end of the 21-day chondrogenic differentiation, the neocartilage pellets were fixed, embedded, and sectioned for histological examination. IHC staining confirmed the strong hyaline-like nature of the explant, characterized by high expression of aggrecan (Fig. 5 B) with concomitant low expression of type X collagen (Fig. 5 D), when swMIAMI cells were combined with both microcartilage and PAMs TGF-β3 compared to swMIAMI cells alone (Fig. 5 A, C). Importantly, we observed that the cells in vicinity of the microcartilage particles had a somewhat increased expression of aggrecan (Fig. 5 B), albeit less than those in the vicinity of the PAMs, thereby confirming the RT-qPCR findings that demonstrated the possible contribution of the microcartilage to the hyaline cartilage phenotype. Noteworthy, we also observed an increased expression of type II collagen by the cells in contact with the PAMs (data not shown), therefore confirming our previous study performed with human MSCs[84].

Discussion

We previously demonstrated the capacity of swMIAMI cells to undergo chondrogenic differentiation, thereby forming neo-cartilage pellets expressing type II collagen[85]. However, the 2 main types of cartilage found in the body, namely fibrocartilage and hyaline cartilage, are different in the way the tissue is organized and its molecular composition. Hyaline cartilage consists of an amorphous collagen matrix rich in aggrecan, while fibrocartilage contains low aggrecan, high type X collagen and a more fibrous organization[28]. Aggrecan is a crucial proteoglycan and key contributor to the generation of a hyaline cartilage phenotype because it has an increased water attraction capacity. A higher aggrecan content in hyaline cartilage therefore contributes to its higher flexibility and cushioning capacity. Type X collagen contributes to mineralization and makes fibrocartilage stronger, more rigid, and only slightly flexible and therefore well-suited for its main role in

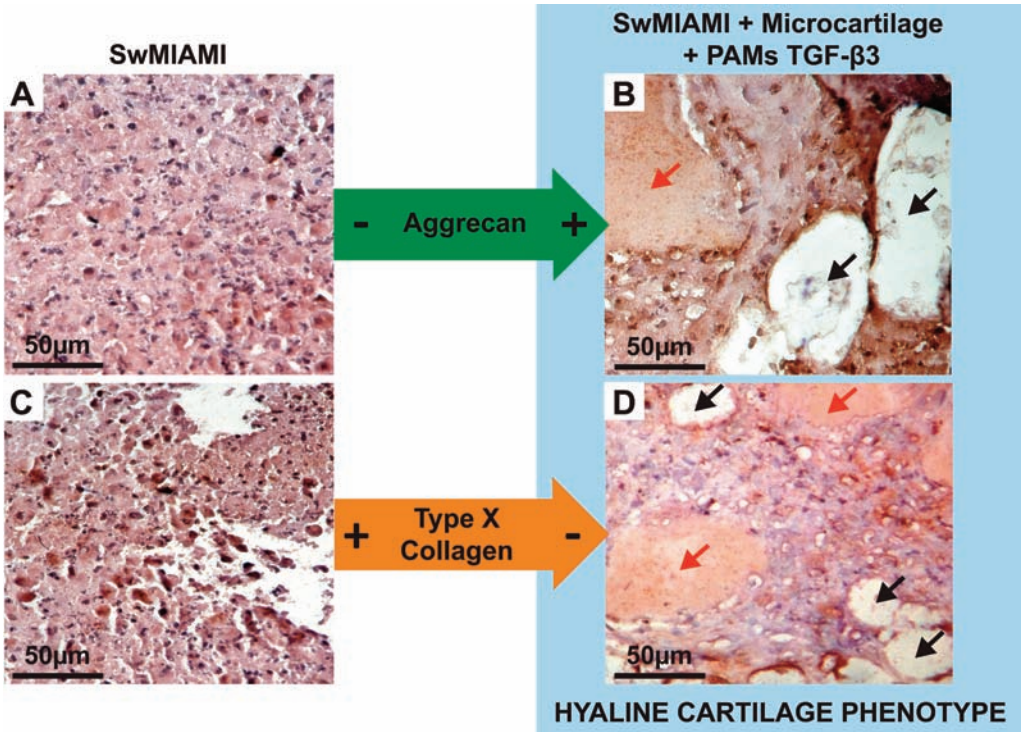

Figure 5. Microcartilage combined to PAMs TGF-β3 directs the chondrogenic differentiation of swMIAMI cells toward hyaline cartilage *in vitro*. SwMIAMI cells in presence of microcartilage and PAMs releasing TGF-β3 highly expressed aggrecan (B) while exhibiting a decreased expression of type X collagen (D) compared to swMIAMI cells alone (A, C). Noteworthy, cells in the vicinity of microcartilage expressed a somewhat increased expression of aggrecan (B), which was even stronger in the vicinity of the PAMs (B). *Red arrows* point at microcartilage. *Black arrows* point at PAMs.

the intervertebral discs in the spine. High aggrecan and low type X collagen expression are crucial molecular determinants of the unique mechanical properties and articular function of hyaline cartilage[28]. Thus, it is essential to determine the effect of the different conditions on the expression levels of these genes by the swMIAMI cells in order to assess their capacity to differentiate into the desired hyaline phenotype. Our primary result indicated that the exclusive combination of the natural microcartilage and synthetic PAMs delivering TGF-β3 provide a unique environment with adequate support and signaling molecules to direct the differentiation of swMIAMI cells with a phenotype that closely resembles that of hyaline cartilage.

We demonstrated that swMIAMI cells were able to attach very quickly not only to the fibronectin/poly-D-lysine surface of PAMs, which confirmed our previous data[83,84], but also to the microcartilage. The adhesion behavior we observed was in accordance with a recent paper that describes the adhesion kinetics of MSCs to articular cartilage surface[91] in which the authors describe that 50% of the cells attach to the cartilage in about 2 hours. Another possible advantage described for MSCs is that their resistance to shear stress after attachment to cartilage appears higher than that of chondrocytes[91]. The stronger expression of glycosaminoglycans, type II collagen and aggrecan we detected for the cells in contact with the PAMs TGF-β3 confirmed PAMs bioactivity and induction of chondrogenic differentiation we observed in other studies[83,84]. Furthermore, the cells in close contact with microcartilage also had a somewhat stronger expression of aggrecan. This finding underlines another possible benefit of microcartilage, used alone or in a combinatorial strategy, for cartilage repair as it may support and guide stem cells differentiation as well as provide mechanical stability of the neo-cartilage in a clinical setting. Moreover, use of microcartilage may facilitate the filling of larger lesion as we observed that its use resulted *in vitro* in neo-cartilage pellets of larger size, while the additional presence of PAMs also improved further the survival of the swMIAMI cells during *in vitro* chondrogenesis. This is in accordance with several recent studies that describe the bioactivity of cartilage-de-

rived scaffolds on various stem cells chondrogenesis and of PAMs in the enhancement of cell survival[80,84]. For example, our group recently demonstrated that cultivation of chondrocytes onto cartilage fluff helped in expanding the chondrocytes while avoiding their de-differentiation[92]. Other *in vitro* studies performed with an oriented cartilage extracellular matrix-derived scaffolds seeded with MSCs demonstrated that the scaffold alone, without the addition of exogenous growth factor, positively affected MSC chondrogenic differentiation[53]. Similar data supporting cartilage-based scaffolds bioactivity have been obtained *in vivo* after subcutaneous injection in nude mice of cartilage-derived scaffolds seeded with either adipose-derived stem cells[54] or MSCs[55,56]. All those studies demonstrated that cartilage-derived matrices could induce by themselves, to some extent, a chondrogenic differentiation of the stem cells used. Importantly, only two of those studies examined the expression of type X collagen, and demonstrated variable outcomes with either a decrease[54] or an increase of type X collagen expression[56]. A recent study also described the effect of human acellular cartilage matrix powder on MSC chondrogenic differentiation, with an observed reduction of hypertrophy when growth factor was supplemented to the media[57]. This is in accordance with our results, while our PCR data also suggest that delivering the growth factor in a controlled and constant manner by means of the PAMs is crucial for the adequate behavior of the cells. It has to be noted that in opposition to our strategy, most of these studies would not allow for an injectable approach, because of the larger size of the scaffolds used.

In an attempt to understand the molecular mechanisms triggering chondrogenesis of MSCs in contact of articular cartilage, a recent study[93] described that molecules contained in native cartilage such as decorin, hyaluronan (HA), osteopontin and biglycan may be responsible for aggrecan overexpression while HA may also be responsible for type X collagen down-regulation. Despite some studies demonstrating that HA may induce chondrocytic chondrolysis[94], a preliminary clinical trial using a hyaluronan-based scaffold seeded with autologous chondrocytes has also recently been conducted in the treatment of cartilage defects with positive outcomes[95]. Finally, aggrecan has also been described as playing an important role in the maintenance of chondrocyte phenotype *in vitro*[8].

Importantly, in our study, the strongest effect in terms of hyaline-specific cartilage gene expression (high aggrecan and low type X collagen) was obtained with the combination of both PAMs releasing TGF-β3 and microcartilage, and this is, to the best of our knowledge, the first study describing the possible benefit of combining natural and growth factor-releasing synthetic scaffolds for cartilage repair cell therapy. However, the scope of this study is limited in that it is relatively short term (21 days), focuses only on key molecular markers of hyaline cartilage, and was performed only *in vitro*. To address this limitation, our laboratory recently developed an *ex vivo* model of cartilage repair allowing us to biomechanically stimulate the damaged osteochondral tissue during the course of repair. This model will be extremely valuable in the future to test the best combination of materials to use, the dose and the cell/material ratio, and to assess in greater detail the molecular changes and mechanisms associated with the phenotypic determination and phenotype stability before moving forward in a larger animal model of osteochondral defect. Swine would be a candidate of choice because of its physiological, mechanical, and chemical parameters similar to those observed in humans[67,96,97]. On the long term, we expect that combination of MIAMI cells/PAMs TGF-β3/microcartilage will help to improve the microarchitecture and mechanical properties of the repaired cartilage tissue in a more rapid and minimally invasive fashion compared to the currently available strategies.

Acknowledgments

We are grateful to all our colleagues that contributed to this work, and more particularly to Ximena Vial (Biomedical Engineering Department, University of Miami) for her help with the scanning electron microscopy as well as to Laurence Sindji, Florian Fouchet (Inserm U1066, University of Angers, France) and Marie Morille (University Montpellier I, Montpellier, France) for their help with the formulation and characterization of PAMs. Finally, we thank Carine Bouffi (Cincinnati Children's Hospital Medical Center, Ohio, USA) for her kind advice on the micro-pellet histology techniques.

Funding source

This work was funded by the University of Miami Tissue Bank and the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development (Biomedical Laboratory Research and Development) Merit Review award (BX000952) of Dr. Paul C. Schiller. INSERM and the University of Angers funded the work of Dr Claudia N. Montero-Menei.

Disclaimer

The contents of this manuscript do not represent the views of neither the Department of Veterans Affairs nor the United States Government.

References

1. Lotz MK, Kraus VB. New developments in osteoarthritis. Posttraumatic osteoarthritis: pathogenesis and pharmacological treatment options. Arthritis Res Ther 2010; 12(3): 211.
2. Brown TD, Johnston RC, Saltzman CL, Marsh JL, Buckwalter JA. Posttraumatic osteoarthritis: a first estimate of incidence, prevalence, and burden of disease. J Orthop Trauma 2006; 20(10): 739-744.
3. Anderson DD, Chubinskaya S, Guilak F, Martin JA, Oegema TR, Olson SA, et al. Post-traumatic osteoarthritis: Improved understanding and opportunities for early intervention. J Orthop Res 2011; 29(6): 802-809.
4. Redman SN, Oldfield SF, Archer CW. Current strategies for articular cartilage repair. Eur Cell Mater 2005; 9: 23-32; discussion 23-32.
5. Hunziker EB. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. Osteoarthritis Cartilage 2002; 10(6): 432-463.
6. Malinin T, Temple HT. Musculoskeletal Tissue Transplantation & Tissue Banking. Jaypee Brothers Medical Publishers, 2013.
7. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994; 331(14): 889-895.
8. Hoshiba T, Yamada T, Lu H, Kawazoe N, Chen G. Effects of extracellular matrix proteins in chondrocyte-derived matrices on chondrocyte functions. Biotechnol Prog 2013; 29(5): 1331-1336.
9. Beane OS, Darling EM. Isolation, Characterization, and Differentiation of Stem Cells for Cartilage Regeneration. Ann Biomed Eng 2012; 40(10): 2079-2097.
10. Schiller PC, D'Ippolito G. Adult and Embryonic Stem Cells in Cartilage Repair. Current Rheumatology Reviews 2009; 5: 15-23.
11. Djouad F, Mrugala D, Noel D, Jorgensen C. Engineered mesenchymal stem cells for cartilage repair. Regen Med 2006; 1(4): 529-537.
12. Veronesi F, Giavaresi G, Tschon M, Borsari V, Nicoli Aldini N, Fini M. Clinical use of bone marrow, bone marrow concentrate and expanded bone marrow mesenchymal stem cells in cartilage disease. Stem Cells Dev 2012; 22(2): 181-192.
13. Dashtdar H, Rothan HA, Tay T, Ahmad RE, Ali R, Tay LX, et al. A preliminary study comparing the use of allogenic chondrogenic pre-differentiated and undifferentiated mesenchymal stem cells for the repair of full thickness articular cartilage defects in rabbits. J Orthop Res 2011; 29(9): 1336-1342.
14. Gardner OF, Archer CW, Alini M, Stoddart MJ. Chondrogenesis of mesenchymal stem cells for cartilage tissue engineering. Histol Histopathol 2013; 28(1): 23-42.
15. Stoltz JF, Huseltein C, Schiavi J, Li YY, Bensoussan D, Decot V, De Isla N. Human Stem Cells and Articular Cartilage Tissue Engineering. Curr Pharmaceutical Biotechnol 2012; 13(15): 2682-2691.
16. Phinney DG, Kopen G, Righter W, Webster S, Tremain N, Prockop DJ. Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. J Cell Biochem 1999; 75(3): 424-436.
17. Ho AD, Wagner W, Franke W. Heterogeneity of mesenchymal stromal cell preparations. Cytotherapy 2008; 10(4): 320-330.
18. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8(4): 315-317.
19. Peterbauer-Scherb A, van Griensven M, Meinl A, Gabriel C, Redl H, Wolbank S. Isolation of pig bone marrow mesenchymal stem cells suitable for one-step procedures in chondrogenic regeneration. J Tissue Eng Regen Med 2010; 4(6): 485-490.
20. Arthur A, Zannettino A, Gronthos S. The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair. J Cell Physiol 2009; 218(2): 237-245.
21. Trivedi HL, Vanikar AV. Mesenchymal stem cells and solid organ transplantation. CellR4 2013; 1(2): e377.
22. Qi Y, Feng G, Yan W. Mesenchymal stem cell-based treatment for cartilage defects in osteoarthritis. Mol Biol Rep 2012; 39(5): 5683-5689.
23. Mokbel AN, El-Tookhy OS, Shamaa AA, Rashed LA, Sabry D, El Sayed AM. Homing and reparative effect of intra-articular injection of autologus mesenchymal stem cells in osteoarthritic animal model. BMC Musculoskelet Disord 2011; 12(1): 259.
24. Lee KB, Hui JH, Song IC, Ardany L, Lee EH. Injectable mesenchymal stem cell therapy for large cartilage defects--a porcine model. Stem Cells 2007; 25(11): 2964-2971.
25. Yoshioka T, Mishima H, Ohyabu Y, Sakai S, Akaogi H, Ishii T, et al. Repair of large osteochondral defects with allogeneic cartilaginous aggregates formed from bone marrow-derived cells using RWV bioreactor. J Orthop Res 2007; 25(10): 1291-1298.
26. Hellingman CA, Koevoet W, van Osch GJ. Can one generate stable hyaline cartilage from adult mesenchymal stem cells? A developmental approach. J Tissue Eng Regen Med 2012; 6(10): e1-e11.
27. Fujioka H, Wang GJ, Mizuno K, Balian G, Hurwitz SR. Changes in the expression of type-X collagen in the fibrocartilage of rat Achilles tendon attachment during development. J Orthop Res 1997; 15(5): 675-681.
28. Valiyaveettil M, Mort JS, McDevitt CA. The concentration, gene expression, and spatial distribution of aggrecan in canine articular cartilage, meniscus, and anterior and posterior cruciate ligaments: a new molecular distinction between hyaline cartilage and fibrocartilage in the knee joint. Connect Tissue Res 2005; 46(2): 83-91.
29. Kon E, Filardo G, Roffi A, Andriolo L, Marcacci M. New trends for knee cartilage regeneration: from cell-free scaffolds to mesenchymal stem cells. Curr Rev Musculoskelet Med 2012; 5(3): 236-243.

30. Kaplan LD, Lu Y, Snitzer J, Nemke B, Hao Z, Biro S, et al. The effect of early hyaluronic acid delivery on the development of an acute articular cartilage lesion in a sheep model. Am J Sports Med 2009; 37(12): 2323-2327.
31. Erickson IE, Kestle SR, Zellars KH, Dodge GR, Burdick JA, Mauck RL. Improved cartilage repair via in vitro pre-maturation of MSC-seeded hyaluronic acid hydrogels. Biomed Mater 2012; 7(2): 024110.
32. Cavallo C, Desando G, Columbaro M, Ferrari A, Zini N, Facchini A, et al. Chondrogenic differentiation of bone marrow concentrate grown onto a hylauronan scaffold: rationale for its use in the treatment of cartilage lesions. J Biomed Mater Res A 2013; 101(6): 1559-1570.
33. Srinivasan PP, McCoy SY, Jha AK, Yang W, Jia X, Farach-Carson MC, et al. Injectable perlecan domain 1-hyaluronan microgels potentiate the cartilage repair effect of BMP2 in a murine model of early osteoarthritis. Biomed Mater 2012; 7(2): 024109.
34. Bulpitt P, Aeschlimann D. New strategy for chemical modification of hyaluronic acid: preparation of functionalized derivatives and their use in the formation of novel biocompatible hydrogels. J Biomed Mater Res 1999; 47(2): 152-169.
35. Coates EE, Riggin CN, Fisher JP. Photocrosslinked alginate with hyaluronic acid hydrogels as vehicles for mesenchymal stem cell encapsulation and chondrogenesis. J Biomed Mater Res A 2013; 101(7): 1962-1970.
36. Stark Y, Suck K, Kasper C, Wieland M, van Griensven M, Scheper T. Application of collagen matrices for cartilage tissue engineering. Exp Toxicol Pathol 2006; 57(4): 305-311.
37. Shainer R, Gaberman E, Levdansky L, Gorodetsky R. Efficient isolation and chondrogenic differentiation of adult mesenchymal stem cells with fibrin microbeads and micronized collagen sponges. Regen Med 2010; 5(2): 255-265.
38. Mimura T, Imai S, Okumura N, Li L, Nishizawa K, Araki S, et al. Spatiotemporal control of proliferation and differentiation of bone marrow-derived mesenchymal stem cells recruited using collagen hydrogel for repair of articular cartilage defects. J Biomed Mater Res B Appl Biomater 2011; 98(2): 360-368.
39. Shao XX, Hutmacher DW, Ho ST, Goh JC, Lee EH. Evaluation of a hybrid scaffold/cell construct in repair of high-load-bearing osteochondral defects in rabbits. Biomaterials 2006; 27(7): 1071-1080.
40. Huang CY, Reuben PM, D'Ippolito G, Schiller PC, Cheung HS. Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture. Anat Rec A Discov Mol Cell Evol Biol 2004; 278(1): 428-436.
41. Merceron C, Portron S, Vignes-Colombeix C, Rederstorff E, Masson M, Lesoeur J, et al. Pharmacological Modulation of Human Mesenchymal Stem Cell Chondrogenesis by a Chemically Over-Sulphated Polysaccharide of Marine Origin: Potential Application to Cartilage Regenerative Medicine. Stem Cells 2011.
42. Tay LX, Ahmad RE, Dashtdar H, Tay KW, Masjuddin T, Ab-Rahim S, et al. Treatment Outcomes of Alginate-Embedded Allogenic Mesenchymal Stem Cells Versus Autologous Chondrocytes for the Repair of Focal Articular Cartilage Defects in a Rabbit Model. Am J Sports Med 2012; 40(1): 83-90.
43. Kim SH, Jung Y. A biocompatible tissue scaffold produced by supercritical fluid processing for cartilage tissue engineering. Tissue Eng Part C Methods 2013; 19(3): 181-188.
44. Hu J, Feng K, Liu X, Ma PX. Chondrogenic and osteogenic differentiations of human bone marrow-derived mesenchymal stem cells on a nanofibrous scaffold with designed pore network. Biomaterials 2009; 30(28): 5061-5067.
45. Singh M, Sandhu B, Scurto A, Berkland C, Detamore MS. Microsphere-based scaffolds for cartilage tissue engineering: Using subcritical CO2 as a sintering agent. Acta Biomater 2010; 6(1): 137-143.
46. Grande DA, Halberstadt C, Naughton G, Schwartz R, Manji R. Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts. J Biomed Mater Res 1997; 34(2): 211-220.
47. Stenhamre H, Thorvaldsson A, Enochson L, Walkenstrom P, Lindahl A, Brittberg M, et al. Nanosized fibers' effect on adult human articular chondrocytes behavior. Materials Sci Engineering C, Materials for biological applications 2013; 33(3): 1539-1545.
48. He X, Fu W, Feng B, Wang H, Liu Z, Yin M, et al. Electrospun collagen-poly(L-lactic acid-co-epsilon-caprolactone) membranes for cartilage tissue engineering. Regen Med 2013; 8(4): 425-436.
49. Accardi MA, McCullen SD, Callanan A, Chung S, Cann PM, Stevens M, Dini D Effects of fibre orientation on the frictional properties and damage of regenerative articular cartilage surfaces. Tissue Eng Part A 2013; 19(19-20): 2300-2310.
50. Giannoni P, Lazzarini E, Ceseracciu L, Barone AC, Quarto R, Scaglione S. Design and characterization of a tissue-engineered bilayer scaffold for osteochondral tissue repair. J Tissue Eng Regen Med 2012 Nov 21. doi: 10.1002/term.1651. [Epub ahead of print].
51. Grigolo B, Fiorini M, Manferdini C, Cavallo C, Gabusi E, Zini N, et al. Chemical-physical properties and in vitro cell culturing of a novel biphasic bio-mimetic scaffold for osteochondral tissue regeneration. Journal of biological regulators and homeostatic agents 2011; 25(2 Suppl): S3-13.
52. Hwang NS, Varghese S, Li H, Elisseeff J. Regulation of osteogenic and chondrogenic differentiation of mesenchymal stem cells in PEG-ECM hydrogels. Cell Tissue Res 2011; 344(3): 499-509.
53. Jia S, Liu L, Pan W, Meng G, Duan C, Zhang L, et al. Oriented cartilage extracellular matrix-derived scaffold for cartilage tissue engineering. J Biosci Bioeng 2012; 113(5): 647-653.
54. Cheng NC, Estes BT, Awad HA, Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells by a porous scaffold derived from native articular cartilage extracellular matrix. Tissue Eng Part A 2009; 15(2): 231-241.
55. Xue JX, Gong YY, Zhou GD, Liu W, Cao Y, Zhang WJ. Chondrogenic differentiation of bone marrow-derived mesenchymal stem cells induced by acellular cartilage sheets. Biomaterials 2012; 33(24): 5832-5840.
56. Chen CC, Liao CH, Wang YH, Hsu YM, Huang SH, Chang CH, et al. Cartilage fragments from osteoarthritic knee promote chondrogenesis of mesenchymal stem cells without exogenous growth factor induction. J Orthop Res 2012; 30(3): 393-400.
57. Chang CH, Chen CC, Liao CH, Lin FH, Hsu YM, Fang HW. Human acellular cartilage matrix powders as a biological scaffold for cartilage tissue engineering with syn-

ovium-derived mesenchymal stem cells. J Biomed Mater Res A 2014; 102(7): 2248-2257.
58. Zhao YH, Yang Q, Xia Q, Peng J, Lu SB, Guo QY, et al. In vitro cartilage production using an extracellular matrix-derived scaffold and bone marrow-derived mesenchymal stem cells. Chin Med J (Engl) 2013; 126(16): 3130-3137.
59. Malinin T. Method for regenerating cartilage. US2007/0098759A1 2007.
60. Malinin T. Cartilage Material. US2011/0104242A1 2011.
61. Malinin TI, Temple HT, Carpenter EM. Induction of regeneration of articular cartilage defects by freeze dried particulate cartilage allografts. ICRS 2009 Meeting; Poster presentation.
62. Cals FL, Hellingman CA, Koevoet W, Baatenburg de Jong RJ, van Osch GJ. Effects of transforming growth factor-beta subtypes on in vitro cartilage production and mineralization of human bone marrow stromal-derived mesenchymal stem cells. J Tissue Eng Regen Med 2012; 6(1): 68-76.
63. Bosetti M, Boccafoschi F, Leigheb M, Bianchi AE, Cannas M. Chondrogenic induction of human mesenchymal stem cells using combined growth factors for cartilage tissue engineering. J Tissue Eng Regen Med 2012; 6(3): 205-213.
64. Maehara H, Sotome S, Yoshii T, Torigoe I, Kawasaki Y, Sugata Y, et al. Repair of large osteochondral defects in rabbits using porous hydroxyapatite/collagen (HAp/Col) and fibroblast growth factor-2 (FGF-2). J Orthop Res 2010; 28(5): 677-686.
65. Zhang W, Chen J, Tao J, Hu C, Chen L, Zhao H, Xu G, Heng BC, Ouyang HW. The promotion of osteochondral repair by combined intra-articular injection of parathyroid hormone-related protein and implantation of a bi-layer collagen-silk scaffold. Biomaterials 2013; 34(25): 6046-6057.
66. Tamai N, Myoui A, Hirao M, Kaito T, Ochi T, Tanaka J, et al. A new biotechnology for articular cartilage repair: subchondral implantation of a composite of interconnected porous hydroxyapatite, synthetic polymer (PLA-PEG), and bone morphogenetic protein-2 (rhBMP-2). Osteoarthritis Cartilage 2005; 13(5): 405-417.
67. Gavenis K, Heussen N, Hofman M, Andereya S, Schneider U, Schmidt-Rohlfing B. Cell-free repair of small cartilage defects in the Goettinger minipig: The effects of BMP-7 continuously released by poly(lactic-co-glycolid acid) microspheres. J Biomater Appl 2013; 28(7): 1008-1015.
68. Re'em T, Kaminer-Israeli Y, Ruvinov E, Cohen S. Chondrogenesis of hMSC in affinity-bound TGF-beta scaffolds. Biomaterials 2012; 33(3): 751-761.
69. Madry H, Rey Rico A, Venkatesan JK, Johnstone B, Cucchiarini M. TGF-beta-releasing scaffolds for cartilage tissue engineering. Tissue Eng Part B Rev 2014; 20: 106-125.
70. Lim SM, Oh SH, Lee HH, Yuk SH, Im GI, Lee JH. Dual growth factor-releasing nanoparticle/hydrogel system for cartilage tissue engineering. J Mater Sci Mater Med 2010; 21(9): 2593-2600.
71. Park JS, Yang HN, Woo DG, Jeon SY, Park KH. SOX9 gene plus heparinized TGF-beta 3 coated dexamethasone loaded PLGA microspheres for inducement of chondrogenesis of hMSCs. Biomaterials 2012; 33(29): 7151-7163.
72. Wu G, Cui Y, Ma L, Pan X, Wang X, Zhang B. Repairing cartilage defects with bone marrow mesenchymal stem cells induced by CDMP and TGF-beta. Cell Tissue Bank 2014; 15(1): 51-57.
73. Ertan AB, Yilgor P, Bayyurt B, Calikoglu AC, Kaspar C, Kok FN, Kose GT, Hasirci V. Effect of double growth factor release on cartilage tissue engineering. J Tissue Eng Regen Med 2013; 7(2): 149-160.
74. D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. J Cell Sci 2004; 117(Pt 14): 2971-2981.
75. Roche S, D'Ippolito G, Gomez LA, Bouckenooghe T, Lehmann S, Montero-Menei CN, et al. Comparative analysis of protein expression of three stem cell populations: models of cytokine delivery system in vivo. Int J Pharm 2013; 440(1): 72-82.
76. Schiller P, D'Ippolito G. Multilineage-inducible cells and uses thereof. US7,807,458B2 2010.
77. Tatard VM, Venier-Julienne MC, Saulnier P, Prechter E, Benoit JP, Menei P, et al. Pharmacologically active microcarriers: a tool for cell therapy. Biomaterials 2005;26(17):3727-37.
78. Delcroix GJR, Schiller PC, Benoit J-P, Montero-Menei CN. Adult cell therapy for brain neuronal damages and the role of tissue engineering. Biomaterials 2010; 31(8): 2105-2120.
79. Garbayo E, Delcroix GJ-R, Schiller PC, Montero-Menei CN. Advances in the Combined Use of Adult Cell Therapy and Scaffolds for Brain Tissue Engineering. "Tissue Engineering for Tissue and Organ Regeneration" book, Chapter 18, 2011.
80. Delcroix GJ, Garbayo E, Sindji L, Thomas O, Vanpouille-Box C, Schiller PC, et al. The therapeutic potential of human multipotent mesenchymal stromal cells combined with pharmacologically active microcarriers transplanted in hemi-parkinsonian rats. Biomaterials 2011; 32(6): 1560-1573.
81. Tatard VM, Sindji L, Branton JG, Aubert-Pouessel A, Colleau J, Benoit JP, et al. Pharmacologically active microcarriers releasing glial cell line-derived neurotrophic factor: Survival and differentiation of embryonic dopaminergic neurons after grafting in hemiparkinsonian rats. Biomaterials 2007; 28(11): 1978-1988.
82. Garbayo E, Raval AP, Curtis KM, Della-Morte D, Gomez LA, D'Ippolito G, et al. Neuroprotective properties of marrow-isolated adult multilineage-inducible cells in rat hippocampus following global cerebral ischemia are enhanced when complexed to biomimetic microcarriers. J Neurochem 2011; 119(5): 972-988.
83. Morille M, Van-Thanh T, Garric X, Cayon J, Coudane J, Noël D, Venier-Julienne MC, Montero-Menei CN. The role of pharmacologically active microcarriers releasing TGF-beta3 in cartilage formation in vivo by mesenchymal stem cells. Biomaterials 2010; 31(25): 6485-6493.
84. Morille M, Van-Thanh T, Garric X, Cayon J, Coudane J, Noel D, et al. New PLGA-P188-PLGA matrix enhances TGF-beta3 release from pharmacologically active microcarriers and promotes chondrogenesis of mesenchymal stem cells. J Control Release 2013; 170(1): 99-110.
85. D'Ippolito G, Gomez LA, Curtis KM, Delcroix GJ-R, Hare JM, Hatzistergos KE, Oskouei BM, Howard GA, Reiner

T, Schiller PC. Isolation and characterization of swine MIAMI cells: a valuable animal model for adult stem cell therapy. CellR4 2014; 2(5): e1215.
86. Delcroix GJ, Kaimrajh DN, Baria D, Cooper S, Reiner T, Latta L, et al. Histologic, biomechanical, and biological evaluation of fan-folded iliotibial band allografts for anterior cruciate ligament reconstruction. Arthroscopy 2013; 29(4): 756-765.
87. Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 1998; 4(4): 415-428.
88. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 1998; 238(1): 265-272.
89. Delcroix GJ, Curtis KM, Schiller PC, Montero-Menei CN. EGF and bFGF pre-treatment enhances neural specification and the response to neuronal commitment of MIAMI cells. Differentiation 2010; 80(4-5): 213-227.
90. Curtis K, Gomez L, Rios C, Garbayo E, Raval A, Perez-Pinzon M, Schiller P. EF1alpha and RPL13a represent normalization genes suitable for RT-qPCR analysis of bone marrow derived mesenchymal stem cells. BMC Mol Biol 2010; 11: 61.
91. Hung BP, Babalola OM, Bonassar LJ. Quantitative characterization of mesenchymal stem cell adhesion to the articular cartilage surface. J Biomed Mater Res A 2013; 101(12): 3592-3598.
92. Malinin T, Buck BE. Cultivation of human chondrocytes on cartilage fluff matrix without loss of phenotypic expression. Transplantation Technol Res 2014; 4(2).
93. Grogan SP, Chen X, Sovani S, Taniguchi N, Colwell Jr CW, Lotz M, et al. Influence of cartilage extracellular matrix molecules on cell phenotype and neocartilage formation. Tissue Eng Part A 2013; 20(1-2): 264-274.
94. Knudson W, Casey B, Nishida Y, Eger W, Kuettner KE, Knudson CB. Hyaluronan oligosaccharides perturb cartilage matrix homeostasis and induce chondrocytic chondrolysis. Arthritis Rheum 2000; 43(5): 1165-1174.
95. Pavesio A, Abatangelo G, Borrione A, Brocchetta D, Hollander AP, Kon E, et al. Hyaluronan-based scaffolds (Hyalograft C) in the treatment of knee cartilage defects: preliminary clinical findings. Novartis Foundation Symposium 2003; 249: 203-217; discussion 29-33, 34-8, 39-41.
96. Jung M, Kaszap B, Redohl A, Steck E, Breusch S, Richter W, et al. Enhanced early tissue regeneration after matrix-assisted autologous mesenchymal stem cell transplantation in full thickness chondral defects in a minipig model. Cell Transplant 2009; 18(8): 923-932.
97. Gotterbarm T, Breusch SJ, Schneider U, Jung M. The minipig model for experimental chondral and osteochondral defect repair in tissue engineering: retrospective analysis of 180 defects. Lab Anim 2008; 42(1): 71-82.