Histol Histopathol (2018) 33: 1271-1286

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Treatment of osteoarthritis with collagen-based scaffold: A porcine animal model with xenograft mesenchymal stem cells

Wo-Jan Tseng[1]*, Shu-Wei Huang[2]*, Chih-Hsiang Fang[2], Lih-Tao Hsu[3], Chih-Yu Chen[4], Hsin-Hsin Shen[3], Jenny Zwei-Chieng Chang[5], Jui-Sheng Sun[6] and Feng-Huei Lin[2]

[1]National Taiwan University Hospital Hsin-Chu Branch, Hsin-Chu City, [2]Institute of Biomedical Engineering, National Taiwan University, Taipei, [3]Center for Combination Product, Biomedical Technology and Device Research Laboratories, Industrial Technology Research Institute, Chu-Tung, Hsin-Chu County, [4]Department of Orthopedics, Shuang Ho Hospital of Taipei Medical University, New Taipei City, [5]School of Dentistry, College of Medicine, National Taiwan University and [6]Department of Orthopedic Surgery, National Taiwan University Hospital, Taipei, Taiwan.

*The first 2 authors (WR Tzeng & SW Huang) contribute equally in this work

Summary. Objective. With the goal to explore a new approach to treat the early degenerative lesions of hyaline cartilage, we implanted in a porcine OA model a collagen-based scaffold containing chondroprogenitor cells derived from human bone marrow mesenchymal stem cells (hBM-MSCs). Experimental design. Porcine knee joints were subjected to anterior cruciate ligament (ACL) transection to surgically induce OA. After 4 months, the time necessary for the development of cartilage surface damage, animals were treated either with trephination bone plug wrapped with the chondroprogenic hBM-MSCs-embedded collagen scaffold or microfractures alone. Histological and histomorphometric evaluations were performed at 5 months after surgery. Results. All animals subjected to ACL transection showed osteoarthritic changes including mild lateral femoral condyle or moderate medial femoral condyle ulcerations. After 14 days' chondrogenic induction, hBM-MSCs seeded onto the scaffold showed expression of chondroprogenitor markers such as SOX9 and COMP. At 5 months after the implantation, significant differences in the quality of the regenerated tissue were found between the hBM-MSCs-embedded scaffold group and the control group. Newly generated tissue was only observed at the site of implantation with the hBM-MSCs-embedded scaffolds. Furthermore, histological examination of the generated tissue revealed evidence of cartilage-like tissue with lacuna formation. In contrast, fibrous layers or fissures were formed on the surface of the control knee joint. Conclusions. This study shows that xenogenic hBM-MSC derived chondroprogenitor scaffolds can generate new cartilage tissue in porcine articular cartilage and have the potential as a useful treatment option for osteoarthritis.

Key words: Osteoarthritis (OA), Porcine animal model, xenograft, Bone marrow-mesenchymal stem cell (BM-MSC), Chondroprogenitor, Scaffold.

Offprint requests to: Jui-Sheng Sun, MD, PhD., Department of Orthopedic Surgery, College of Medicine, National Taiwan University, Taipei, Taiwan, No. 1, Sec. 1, Ren-Ai Rd., Taipei 10051, Taiwan, R.O.C. and Department of Orthopedic Surgery, National Taiwan University Hospital. No. 7, Chung-Shan South Rd., Taipei 10002, Taiwan, R.O.C. e-mail: drjssun@gmail.com or Feng-Huei Lin, PhD., Institute of Biomedical Engineering, National Taiwan University, Taipei, Taiwan No. 1, Sec. 1, Ren-Ai Rd., Taipei 10051, Taiwan, R.O.C. e-mail: double@ntu.edu.tw

DOI: 10.14670/HH-18-013

Introduction

Osteoarthritis (OA) is the most common form of arthritis and a leading cause of disability worldwide and is thought to be the most prevalent chronic joint disease. The incidence of osteoarthritis is rising because of the ageing population and the epidemic of obesity. Osteoarthritis (OA) is a complex degenerative disorder

involving the articular cartilage that limits the mobility of patients and is of considerable economic importance (Bijlsma et al., 2011; Neogi 2013; Neogi and Zhang, 2013). It is the leading cause of disability due to pain. The consequences of pain related to OA contribute to a substantial socioeconomic burden (Lawrence et al., 2008; Neogi, 2013; Neogi and Zhang, 2013; Christiansen et al., 2015; Kuyinu et al., 2016). Significant research is currently ongoing to understand its pathophysiology and develop successful treatment regimens. However, the direct study of OA in humans presents major difficulties, including slow progression of the disease, and asymptomatic at earlier stages of the disease (Lampropoulou-Adamidou et al., 2014). Animal models have played a key role in achieving this goal. The animal models are divided into induced and spontaneous models. Induced models are further subdivided into surgical and chemical models, according to the procedure used to induce OA. The ideal animal model for OA research should have joints that resemble human joints anatomically, physiologically, and pathologically. In addition, the animal model should have an accelerated disease progression and reproducibility to the corresponding human disease. Surgical induction of OA is the most commonly used procedure, which alters the exerted strain on the joint and/or alters load bearing leading to instability of the joint and induction of OA. Anterior cruciate ligament (ACL) transection is the most common induced OA model used nowadays (Kamekura et al., 2005; Proffen et al., 2012; Lampropoulou-Adamidou et al., 2014; Christiansen et al., 2015; Kuyinu et al., 2016). Here we present our experience by using miniature pigs as an OA model through ACL transection procedures and to explore the possibility as an animal model for treatment of osteoarthritis.

The technology of tissue engineering has improved significantly in the past decade, but total joint arthroplasty remained the gold standard treatment for end-stage OA (Bijlsma et al., 2011). For earlier stage OA patients, in addition to the symptomatic control, cartilage damage can be treated by abrasion, drilling, microfracture and mosaicplasty, autografts, allografts, and cartilage cell transplantation (Oberlander et al., 1998; Steadman et al., 2001; McAlindon et al., 2014; Fransen et al., 2015; Singh et al., 2015). With the advances in tissue engineering, scientists tried to explore a new approach to treat OA patients suffering from degenerative lesions of hyaline cartilage.

Tissue engineering involves the use of cells, scaffolds, and bioactive factors to promote cell migration, attachment, proliferation, and differentiation to the desired cell type and enhance tissue mechanical properties to eventually be suitable for clinical use (Temenoff and Mikos 2000; Daher et al., 2009; Di Luca et al., 2016). Several materials and techniques have been proposed in the literature to produce scaffolds. The satisfactory scaffold for cartilage tissue engineering provides an adequate mechanical and physio-chemical properties, appropriate porosity, pore size and interconnectivity for cell migration, cell attachment, cell proliferation, differentiated function, and eventually integrating with the host tissue (Temenoff and Mikos 2000; Garg et al., 2012; Bornes et al., 2015; Di Luca et al., 2016). In our study, we used an innovative technique to develop a new collagen-based scaffold with good integration with the host tissue, which will be used to deliver stem cells to engineered site.

Donor shortage is a real problem in clinical transplantation, xenotransplantation is an effective way to solve this problem. However, clinical use of xenotransplantation is limited due to immunological challenges such as acute vascular rejection and cell-mediated rejection (Li et al., 2017). To finally surpass this immunological barrier, more preclinical research is needed into the molecular mechanisms of rejection and the possible effects of new immunosuppressants. Bone marrow-mesenchymal stem cells (BM-MSCs) have generated a great perspective in the field of regenerative medicine, and also in the treatment of inflammatory and autoimmune diseases in the past decade due to their immunomodulatory and anti-inflammatory properties. Bone marrow-derived mesenchymal stem cells (BM-MSCs) remained the most studied stem cell source used in cartilage tissue engineering (Bornes et al., 2014, 2015; Reppel et al., 2015; DiLuca et al., 2016). BM-MSCs had the advantages of low immunogenicity and, particularly, chondrogenic differentiation potential (Stubbendorff et al., 2013; Klontzas et al., 2015; Reppel et al., 2015). Previous studies had shown favorable outcomes following BM-MSCs transplantation for treatment of focal chondral and osteochondral defects (Bornes et al., 2014, 2015; Reppel et al., 2015). Recently, in an investigation about the effect of xenogeneic BM-MSCs and pancreatic islets co-transplantation in preventing rejection or inducing tolerance to islet transplantation, the results suggest that an immunomodulatory action of BM-MSC in islet xenotransplantation has the potential to mitigate the early losses of grafts, due to the regulation of the inflammatory process of transplantation (Corradi-Perini et al., 2017). The first step toward the goal of growing human organs in animals for transplantation was marked by the Salk Institute as they highlighted the successful integration of stem cells from humans in pig embryos (Wu et al., 2017). However, some inconsistency existed between the tone of the titles and the articles that followed and it is still too early to speculate what impact this particular research will have. The benefits and concerns raised by this study will play in the public perception (Hagan-Brown et al., 2017).

In this study, an ACL transection induced porcine OA knee model was created and the collagen-based scaffold containing xenografted human BM-MSCs was implanted to evaluate the scaffold host tissue integration. The aim of this study was to investigate the effect of the immunomodulatory capacity of human mesenchymal stem cell (MSC)/extracellular matrix (ECM) collagen complex (C-MSC) *in vivo* and to test the cartilage

regenerative activity of C-MSC xenografts in a porcine model. We demonstrated that xenografted human BM-MSCs collagen based chondroprogenitor scaffold had the potential for the treatment of osteochondral defect or early osteoarthritis.

Materials and methods

Set up osteoarthritic (OA) porcine animal model

This animal study was pre-approved by Affidavit of Approval of Animal Use Protocol, National Taiwan University College of Medicine and College of Public Health; Institutional Animal Care and Use Committee (IACUC) (IACUC No.: 20120384) and was performed at Porcine model Animal Technology Co., Ltd. (Miaoli County, Taiwan). In the pilot study, ACL transection was performed and the pigs were randomized to either 1, 2.5 or 4.5 months endpoints. Briefly, following endotracheal general anesthesia, a midline incision at knee joint was made, followed by medial parapatellar arthrotomy and lateral patella subluxation; a complete mid-substance transection of the ACL was performed and care was taken to avoid damage on contacting articular cartilage. Complete ACL rupture and whole PCL integrity were verified by anterior and posterior drawer testing, respectively. The knee was lavaged with sterile saline and the arthrotomy was closed using non-resorbable sutures in an interrupted pattern. Skin was closed with resorbable suture. We selected 4 months after ACL transection as the time point for the further cartilage repair experiment.

Culture and isolation of human mesenchymal stem cells (hMSCs)

Culture of Human bone marrow mesenchymal stem cell (hMSC) specimens were harvested from bone marrow canal aspirates during total hip/knee joint replacement surgery (IRB No. 201308088RINA). MSCs were isolated from 3 donors (#1 female, age 74; #2 male, age 68; #3 female, age 73) using red blood cell lysis method. Briefly, mononuclear cell (MNC) fraction was isolated according to standard techniques by using a sterile density gradient media, Ficoll-Paque PLUS (an aqueous solution of density 1.077±0.001 g/ml, GE Healthcare, UK), and centrifuging around 300g at 20°C for 40 min. BM mononuclear cells samples were mixed with erythrocyte lysis buffer (Qiagen, Germany) and centrifuged for 5 min at 480g. After removal of the supernatant, the pellet was re-suspended with 5 ml of RPMI 1640 medium (Invitrogen, UK) and washed twice through centrifugation. The isolated cells were washed with PBS 3 times and re-suspended in low glucose Dulbecco's Modified Eagle's medium (LG-DMEM) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Israel). These cells were cultured at 37°C in 5% CO_2 atmosphere for 3 days. After 72h incubation, the non-adherent cells were removed by washing with PBS gently and leaving behind the adherent cell population to grow. When reaching 70 - 80% confluence, cells were trypsinized and subcultured for expanding. In this study, the human mesenchymal stem cells (hMSCs) were used at passage 2-3 throughout the following experiments.

Determination of surface markers for hMSCs and differentiation assay of hMSCs

In order to identify hMSCs derived, flow cytometry was carried out to identify specific cell surface markers of this cell. After reaching confluence (80% of total flask surface), cells from 20 flasks were trypsinized (MilliporeSigma, St. Louis, MO, USA) and sub-cultured for 2 days. Cells were first washed with PBS, again trypsinized, fixed with 2% paraformaldehyde (PFA) for 10 min at 4°C and permeabilized with 1×Triton (MilliporeSigma, St. Louis, MO, USA) for 5 min at 4°C. Subsequently, cells were washed with PBS/BSA 1% and incubated with primary antibodies (MilliporeSigma, St. Louis, MO, USA) for 60 min at room temperature. Antibodies used in the different samples were anti-CD29 (1:100), anti-CD34 (1:200), anti-CD44 (1:200), anti-CD45 (1:200), anti-CD73 (1:200) or anti-CD90 (1:100). Cells were then washed with PBS/1% BSA and incubated for 60 min at room temperature in the dark with secondary antibodies (goat anti-mouse or goat anti-rabbit) conjugated with fluorescein (FITC; 1:200; MilliporeSigma, St. Louis, MO, USA). The negative control was a sample of cells incubated with PBS instead of primary antibody. Samples were analyzed using a flow cytometer (BD FACSVerse, BD Biosciences, San Jose, CA, USA). A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at γ=488 nm and fluorescence was monitored at γ=527 nm. Fluorescence was detected using logarithmic amplification. Mean fluorescence intensity (MFI) values were calculated and recorded automatically. After the flow cytometry procedure the cells from the other 20 flasks were grown for 2 weeks and the medium was replaced every 2/3 days.

The differential ability of cultures BM-MSCs into different mesenchymal tissues were checked at the period around after 14-21 days' culture. The differentiation into osteo-like cells in 14 days by staining the biological apatite with Xylenol orange, the differentiation into chondro-like cells in 21 days via pellet culture and the glycosaminoglccan stained with Safranin O and the differentiation into adipo-like cells in 14 days, in which the lipid droplets stained with Nile red.

Generation of chondroprogenitor cells-embeded scaffold

Synthesis of tri-copolymer scaffolds

The fabrication of gelatin-chondroitin-hyaluronan tri-copolymer scaffolds was according to Chang et al.

(2003). Briefly, 0.5 gm gelatin powder (Sigma-Alderich, St. Louis, MO, USA), 5 mg sodium hyaluronate (HA) powder (Sigma-Alderich, USA), and 0.1 gm chondroitin-6-sulfate (C6S) powder (Sigma-Alderich, USA) were mixed with 7.5 mL double-distilled water and cross-linked for 2-3 min at room temperature by using 1% 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC, Sigma-Alderich, USA) under pH 5-6. The complex was injected into 48-well culture plate, frozen at -20°C for 1 h, transferred to -80°C for 1 h, and then lyophilized for 72h by freeze-drying technique. The dried scaffold was re-crosslinked for 48 h at room temperature by using 0.2% EDC, sterilized with 75% alcohol, then lyophilized for 72h. A tri-copolymer scaffold about 10x20 mm in length and 2 mm in height was produced for the following experiments.

Scanning electron microscopy

The morphology of collagen scaffolds was observed by field emission gun scanning electron microscopy (FEG-SEM) (JEOL, JSM-6330F, Japan). Briefly, the scaffolds were fixed with 4% para-formaldehyde (PFA) for 2h and 2% osmium tetroxide (OsO_4) solution for 1h. All the samples were dehydrated in a graded series of ethanol, dried by critical-point drying (CPD) method, and sputter-coated with gold before observation.

Quantitative real-time PCR

At confluence, the hMSC cells were trypsinized and re-suspended at a concentration of 5.0×10^7 cells/ml DMEM, then about 100 µl of cell suspension was injected into each scaffold (5.0×10^6 cells per scaffold). The scaffolds with hMSCs were placed in a 5-well culture plate for 24 h for cell adhesion, and then cultured in chondrogenic medium, consisting of serum-free DMEM with 1 ng/mL recombinant human transforming growth factor-β1 (rh-TGF-β1; R&D Systems, Minneapolis, MN, USA), 10^{-7} M dexamethasone (Sigma), 1% penicillin/gentamicin (Sigma Co., St. Louis, USA), 50 µg/ml of l-ascorbic acid 2-phosphate (Sigma Co., St. Louis, USA), 1mM sodium pyruvate (Sigma), 40 mg/mL L-proline (Sigma), and ITS premix (BD Science, Franklin Lakes, NJ) for 2 weeks.

Total RNA was extracted from scaffold using Total RNA Miniprep Purification Kit (GeneMark, Taichung, Taiwan) after 14 days' culture. The total RNA was reverse-transcribed into complementary DNA (cDNA) by using Thermo Scientific First Strand cDNA Synthesis kit (Thermo, Hudson, NH, USA) in accordance with the manufacturer's protocol. 5 µl of 5x OmicsGreen qPCR Master Mix (Omics, Taipei, Taiwan), 10 µl of primers, and 10 µl of cDNA were mixed in a final volume of 25 µl for single reaction. Induced genes expression including SOX-9, aggrecan, cartilage oligometric matrix protein (COMP), type II collagen and type X collagen were examined; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous housekeeping gene (Table 1). Reaction was performed by ABI PRISM 7500 Sequence Detection System (Life Technologies, Inc., USA) and the PCR conditions were denaturation at 95°C for 10 sec, annealing at 60°C for 20s, and extension at 72°C for 34s for up to 40 cycles. The data of relative quantitation value of gene expression was calculated using the expression of $2^{-\Delta\Delta}$Ct.

Alcian blue staining

The specimens were fixed with formalin and hydrated with distilled water, then stained in alcian blue solution (pH 2.5 in 3% acetic acid solution) for 30 minutes. After washing in running tap water for 2 minutes, then rinsing in distilled water, the specimens were counterstained in 0.1% nuclear fast red solution for 5 minutes. After washing in running tap water for 1 minute, the specimens were ready for observation. The acidic sulfated mucosubstances were stained to blue.

Hematoxylin and eosin (H&E) staining

First, we bring sections to distilled water, stain nuclei with the alum haematoxylin solution, rinse in running tap water, and then differentiate with 0.3% acid alcohol. Again rinse in running tap water 3 times, then stain with alcoholic acetified eosin Y (CI 45380)/ aq phloxine (CI 45405) for 2 minutes, dehydrate, clear and mount.

Statistics

Statistical analysis was conducted at least in triplicate, and all the results were presented as the mean ± standard deviation (SD). The differences among group means were analyzed by one-way ANOVA and the statistical significance was determined by Dunnett's t

Table 1. Oligonucleotide primer sequences used for RT-qPCR.

Gene	Sequence	Ann. T (°C)	
SOX-9	Forward	5'- AGCGAACGCACATCAAGAC-3'	65
(NM_000346)	Reverse	5'- GCTGTAGTGTGGGAGGTTGAA-3'	
ACAN	Forward	5'-ATCCCGCTACGACGCCATCTG-3'	60
(NM_13227.3)	Reverse	5'-GCTCCATGTCAGGCCAGGTCACT-3'	
COMP	Forward	5'-GCGCCAGTGCCGTAAGGACA -3'	60
(NM_000095.2)	Reverse	5'-CGTCCGTGTTGCGCTGGTCT -3'	
Col II	Forward	5'-GACAATCTGGCTCCCAAC -3'	60
(NM_001844.4)	Reverse	5'- ACAGTCTTGCCCCACTTAC-3'	
COL X	Forward	5'- CAGTCATGCCTGAGGGTTTT-3'	65
(NM_000493)	Reverse	5'- GGGTCATAATGCTGTTGCCT-3'	
GAPDH	Forward	5'- GTCAGTGGTGGACCTGACCT-3'	60
(NM_002046)	Reverse	5'- CACCACCCTGTTGCTGTAGC-3'	

SOX9, Sex determining region Y-box 9; ACAN, aggrecan; COMP, cartilage oligometric matrix protein; Col II, type II collagen; Col X, type X collagen; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

test. Statistical analysis was performed for all the quantitative results using Student's t-test for comparing means from two independent sample groups. A difference of p values less than 0.05 was considered statistically significant.

In vivo porcine implantation test

ACL transection was performed 4 months earlier before index implantation of the scaffold complex in order to establish OA model. Eight 40 kg miniature pigs were randomly assigned to a control group (microfracture only) and experimental group (trephination bone plug plus hMSC-scaffold complex). Each group contained 8 knees. The scaffold for chondrogenic induction of BM-MSC is a 2 mm thick collagen-based porous membrane with high pore interconnectivity and pore sizes varying between 200~300 μm. As described above, the expanded BM-MSC cells were seeded onto a collagen-based scaffold and then underwent chondrogenic induction for 14 days prior to implantation. In the pilot test, we observed that cells were distributed throughout the scaffold after 14 days' chondrogenic induction.

For the *in vivo* porcine implantation test, the skin incision was performed along previous surgical scar. For experimental group, a 7mm diameter, 10mm depth osteochondral defect was created with a trephine bur on

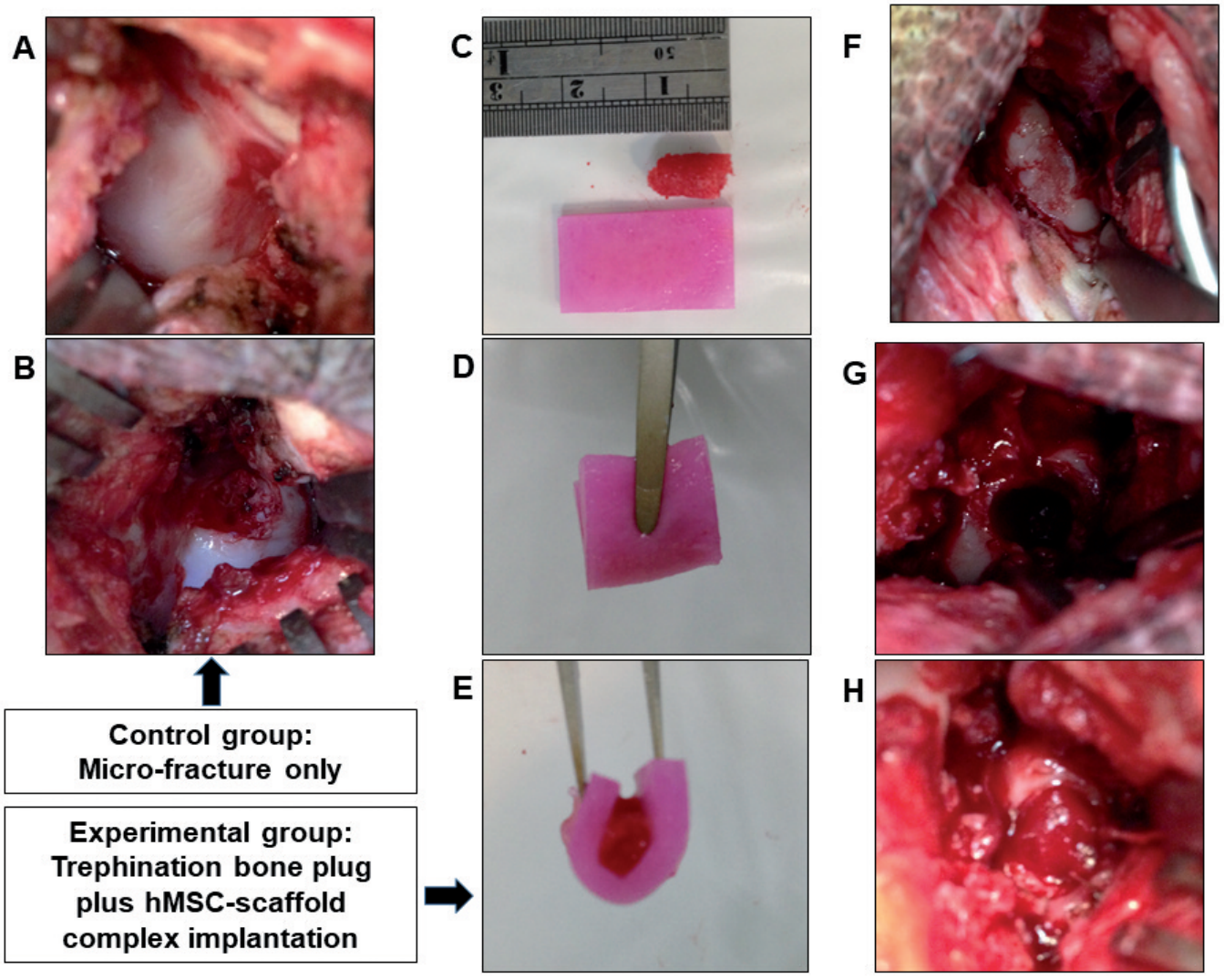


Fig. 1. *In vivo* porcine implantation. Left: Control or micro-fracture only group: The defect site **(A)** was explored and the microfracture was performed **(B)**. Middle: The bone plug plus hMSC-scaffold complex: **C:** bone plug and scaffold; **D:** side view and **E:** front view of wrapped bone plug plus hMSC-scaffold complex. Right: The experimental or trephination bone plug plus hMSC-scaffold complex implantation group: The defect site **(F)** was explored and the bone plug was removed by trephinarion **(G)**, then the wrapped around bone plug and hMSC-scaffold complex was implanted **(H)**.

the center of induced cartilage defect area of the medial femoral condyle. The defect was then filled with trephinated bone plug + chondroprogenitor cell scaffold and then fixed with round suture; for the control group, the cartilage defect area was treated with multiple drlling and then left in situ (Fig. 1). After thorough irrigation and hemostasis, the wound was then closed as routine procedure. The animals were then sacrificed 5 months after the 2nd graft implantation surgery to retrieve the knee joints for further evaluation. The study flow chart is illustrated in Fig. 2.

Evaluation of the animal study

We chose to use the Pineda score for histological evaluation with some modifications. Since the parameter of integration into host cartilage is also important and is not addressed in the scoring system, we modified the Pineda score by including this parameter to evaluate the results of our animal study. The total maximum score is 16, with five parameters as subscores (Filling of defect: 4; Reconstruction of osteochondral junction: 2; Matrix staining: 4; Cell morphology: 4; Integration of donor with host adjacent cartilage: 2; Total maximum 16) (Chang et al., 2006). The score was evaluated by the first author and corresponding author.

Results

Through the flow cytometric data, we demonstrated the cells we harvested preserved stemness and the differential ability into different mesenchymal tissue

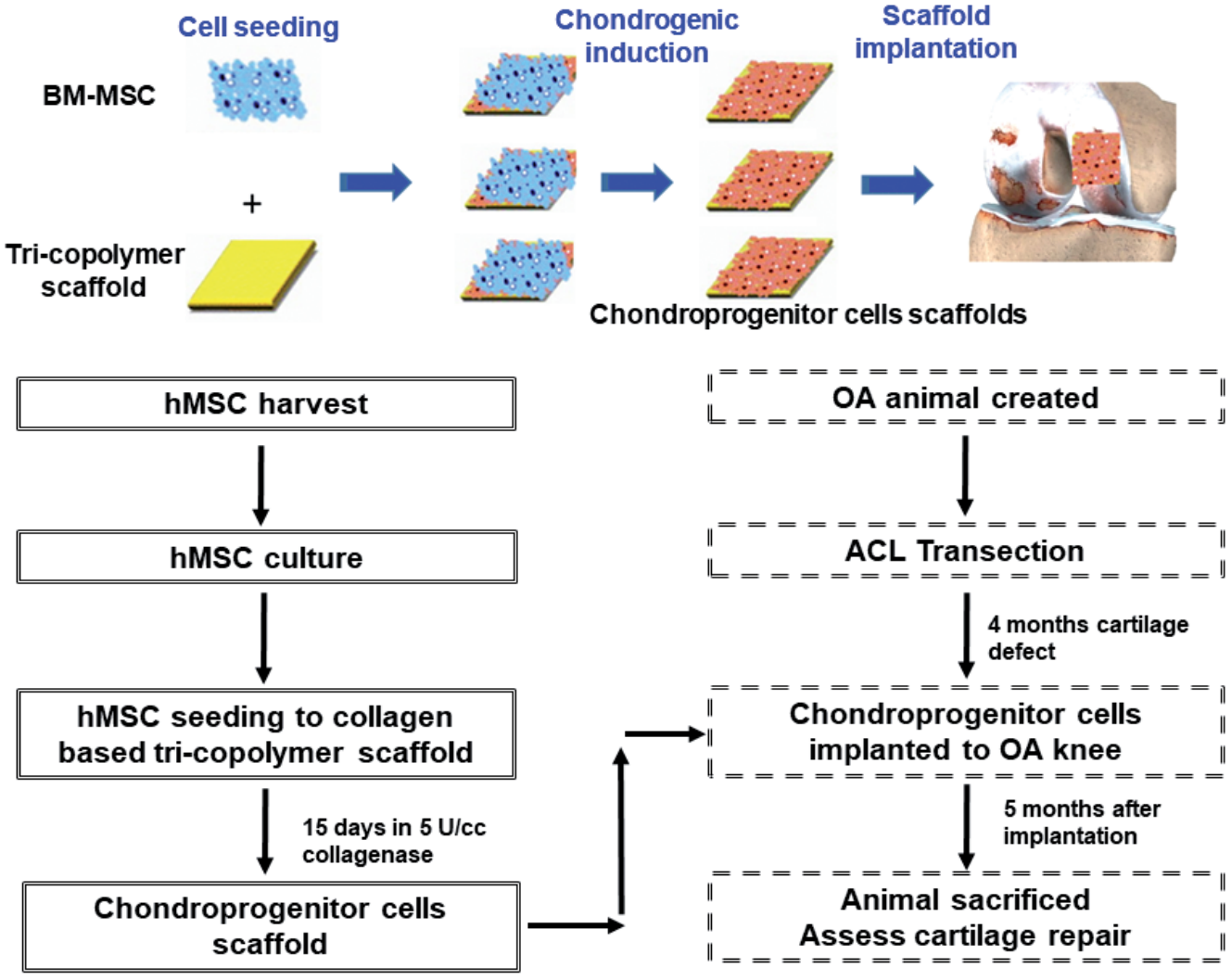


Fig. 2. The study protocol flowchart.

(Fig. 3). The scaffold for chondrogenic induction is a collagen-based porous membrane with high pore interconnectivity. The scanning electron microscopy showed a high pore interconnectivity with uniform pore size at 341±60 μm and a porosity of 75%. During the culture period, the constructs retained their initial diameter and shape; their thickness increased substantially during time, and no shrinkage was observed. The implantation of hBM-MSCs will induce the chondrogenic differentiation and maturation of scaffold. At day 7, a lower Bern score was observed, but the score gradually increased and attained higher Bern score at Day 28 (Fig. 4). When the BM-MSCs were under chondrogenic induction for 7 days, all chondrogenic genes expression was up-regulated; for the SOX-9, aggrecan, and comp they attained their plateau at the 14th days' induction; while for the Col II and Col X, they attained their plateau at the 21st days of induction (Fig. 5). We selected 14-day induction for the test in the *in vivo* chondrogenic scaffolds.

When the scaffold was implanted with hMSCs and cultured in chondrogenic induction medium for 2 weeks, the histological sections of constructs showed cartilaginous extracellular matrix (ECM) deposition and positive alcian blue staining (Fig. 6). After 2 weeks chondroprogenic induction, the hMSC cells also exhibited increased expression of chondroprogenitor cell markers such as SOX-9, aggrecan, cartilage oligometric matrix protein (COMP), type II collagen and type X collagen genes (Fig. 6).

In this study, we set up a porcine animal model of osteoarthritis (OA). At 1.0 month after anterior cruciate ligament (ACL) transection, there were minimal or no pathological changes observed; at 2.5 months after ACL transection, thinning and superficial fibrillation of cartilage (early OA) were observed; while at 4.5 months after ACL transection, vertical fissures, cartilage erosion (moderate OA), larger lesion and even cartilage matrix

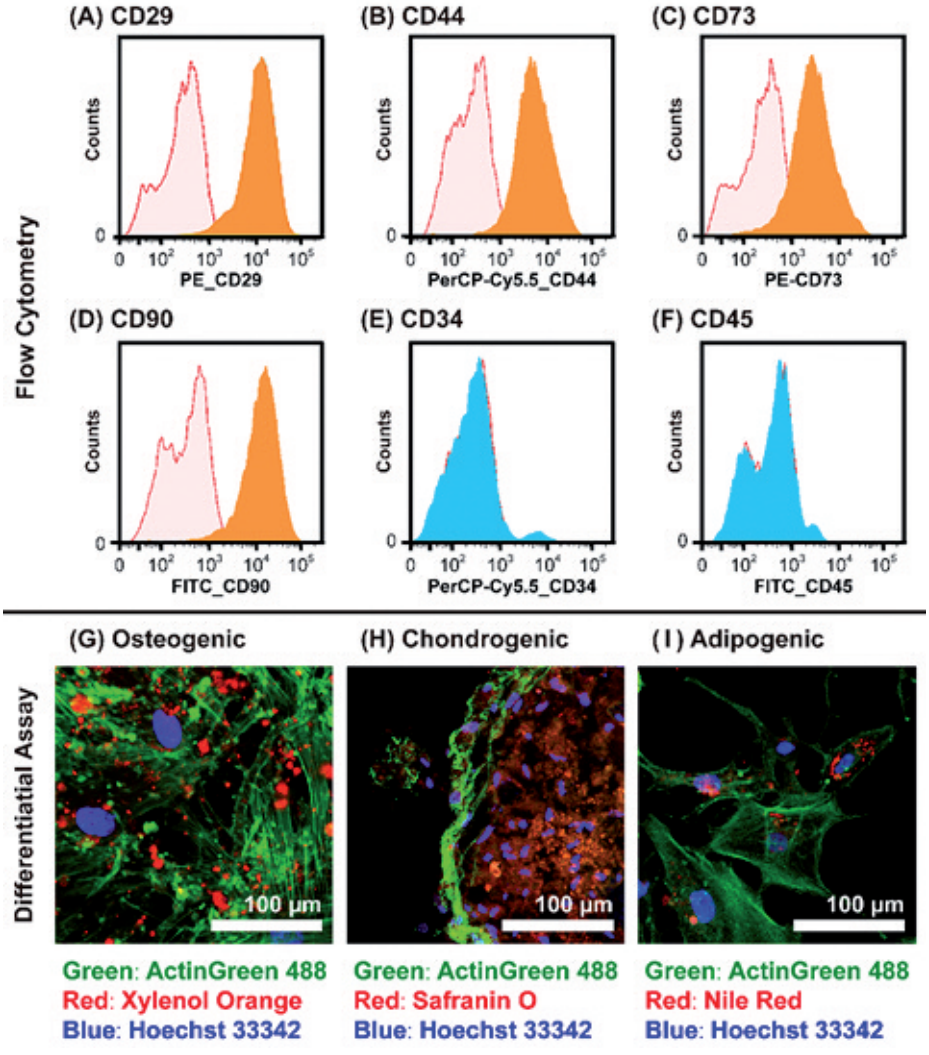


Fig. 3. The cell surface markers of human mesenchymal stem cells. Through the flow cytometric data, we could see CD29 **(A)**, CD44 **(B)**, CD73 **(C)** and CD90 **(D)** were positive, on the contrary, CD34 **(E)** and CD45 **(F)** were negative. We demonstrated the cells we harvested preserved stemness. In the differential ability into mesenchymal tissue: **G** shows the cells could be differentiated into osteo-like cells in 14 days, and the biological apatite stained in red. **H** reveals the cells could be differentiated into chondro-like cells in 21 days via pellet culture, and the glycosaminoglccan stained in red. **I** presents the cells could be differentiated into adipo-like cells in 14 days, and the lipid droplets stained in red.

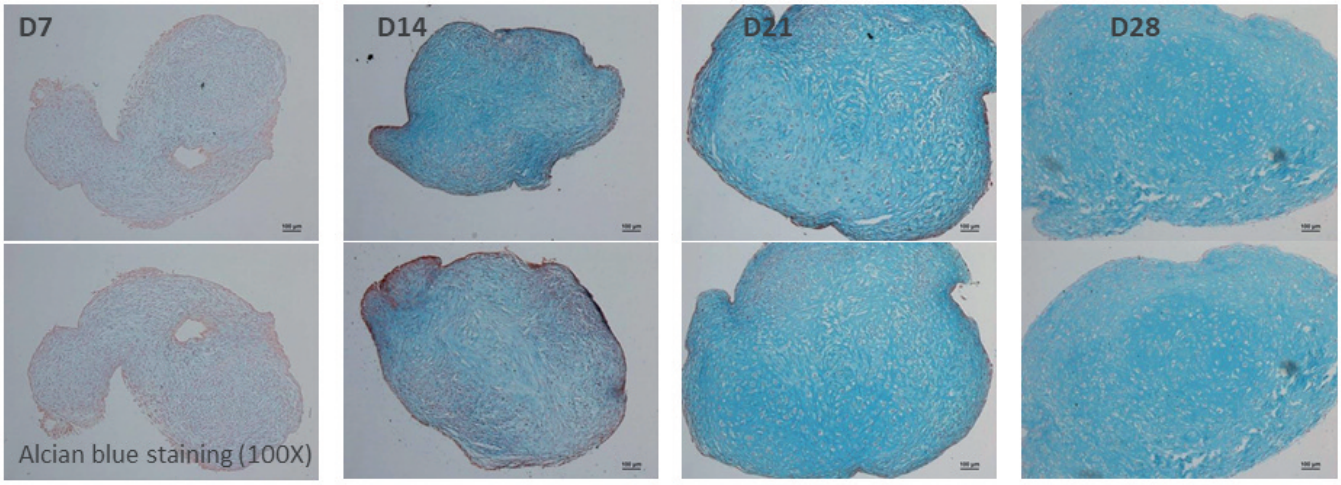
loss (severe OA) were observed (Fig. 7). We selected 4 months after ACL transection as the time point for the further cartilage repair experiment.

For the test of effects of *in vivo* implantation of hMSCs-embedded collagen-based scaffold, we designed a control or micro-fracture only group and trephination bone plug plus hMSC-scaffold complex group for comparison (Fig. 1). The animals were sacrificed 5 months after scaffold implantation (Fig. 2). A total of 8 ACL transection-induced OA knees were implanted with chondroprogenitor cells-embedded collagen scaffolds. After 5 months implantation, the experimental group (trephination bone plug plus hMSC-scaffold complex implantation) showed good gross appearance, spontaneous repair of a full thickness defect showing fibrocartilage-like tissue without collapse and was fully resurfaced; while in the control group (microfractures only), defects were filled and covered with scarring fibrous tissue and showing collapse of the articular surface and growing of adjacent cartilage into the defect, which looks like a fold (Figs. 8, 9). Histological examination of the generated tissue revealed evidence of cartilage-like tissue with lacuna formation. In contrast, no tissue regeneration was detected in the control group; only fibrous layers or fissures were formed on the surface of the control knee joint. Cartilage regeneration was observed in 6 out of 8 OA knees at 5 months following the chondrogenic hMSCs-embedded scaffold implantation.

Pineda et al. developed a semiquantitative scale for grading the natural healing process of defects drilled into articular cartilage. The scale is composed of four parameters: percent filling of the defect, reconstitution of the osteochondral junction, matrix staining and cell morphology; it has a score range from 0 (best) to 14 (worst). The scale was used to evaluate the healing of defects in rabbit knee articular cartilage (Pineda et al., 1992). In the modified Pineda score, all parameters (including filling of defect, reconstruction of osteochondral junction, matrix staining, cell morphology, and integration of donor with host cartilage) and total score showed significant differences between two groups (Fig. 10).

Discussion

Osteoarthritis (OA) is a common musculoskeletal disorder characterized by slow progression and joint tissue degeneration. Half of the world's population, aged 65 and older, suffers from OA (Musumeci et al., 2015b).



Bern score	D7	D14	D21	D28
Uniformity and darkness of Alcian blue	1	2	3	3
Distance between cells/amount of matrix accumulated	1	1	2	3
Cell morphologies represented	0	1	2	3
Total score	2	4	7	9

Fig. 4. Bern Score of scaffold at different stage after MSC implantation. Alcian blue stained histological sections of tissue-engineered (TE) cartilage scaffold (hBM-MSC cultured for 28 days in medium containing transforming growth factor β1, 1ng/mL). Sections are scored using the Bern score. At day 7, a lower Bern score was observed, but the score gradually increased and attained higher Bern score at Day 28.

Aging is one of the most prominent risk factors for the development and progression of OA (Musumeci et al., 2015a). Osteoarthritis (OA) is unquestionably one of the most important chronic health issues in humans which results in significant patient morbidity. Articular cartilage (AC) injury following joint trauma is one of the major risk factors for the development of osteoarthritis (OA). Despite widespread awareness of this disease and its devastating impact, the pathogenesis of early OA is not completely understood, hampering the development of effective tools for early diagnosis and disease-modifying therapeutics (McCoy, 2015). At the present time, end-stage OA is irreversible and eventually requires joint replacement for alleviation of pain and restoration of function as it progresses. Due to the limited capacity of AC to repair, early intervention is required to prevent progression to end-stage OA. Effective management options are limited at the present time; recently, tissue engineered scaffolds incorporating specific cell sources and bioactive molecules have been the focus in this new exciting field. The emerging field of tissue engineering, involving the use of a biocompatible, structurally and mechanically stable scaffold, has shown promising early results in cartilage tissue repair (Daher et al., 2009). Transplantation of mesenchymal stem cells (MSCs) is a promising strategy given the high proliferative capacity of MSCs and their potential to differentiate into cartilage-producing cells - chondrocytes. Biomaterial matrices are commonly used in conjunction with MSCs to aid cell delivery and support chondrogenic differentiation, functional extracellular matrix formation and three-dimensional tissue development (Bornes et al., 2014).

Surgically induced OA model had been reported in previous literature (Kamekura et al., 2005; Kuyinu et al., 2016; Lampropoulou-Adamidou et al., 2014; Proffen et al., 2012). The most well-known and common surgical procedure to induce OA model was ACL transection (Lampropoulou-Adamidou et al., 2014; McCoy 2015). ACL transection was implanted most commonly in sheep/goat. To our best knowledge, however, ACL transection procedure was rarely used to establish an OA model with miniature pigs (Wei et al., 2015). In contrast to other areas, pigs were much more easily acquired and much cheaper than sheep/goat in Asian countries. And compared with other animals, miniature pigs had the following advantages: (1) similar weight and size to adult; (2) simple and direct ACL transection by minimally invasive technique; (3) easy to perform intra-articular treatment; (4) possible regional tissue analysis; (5) cheap on housing and availability. Here we demonstrated our successful experience to establish an OA model in 4 months by using ACL transection in porcine animal model. This can be a useful guide for further study.

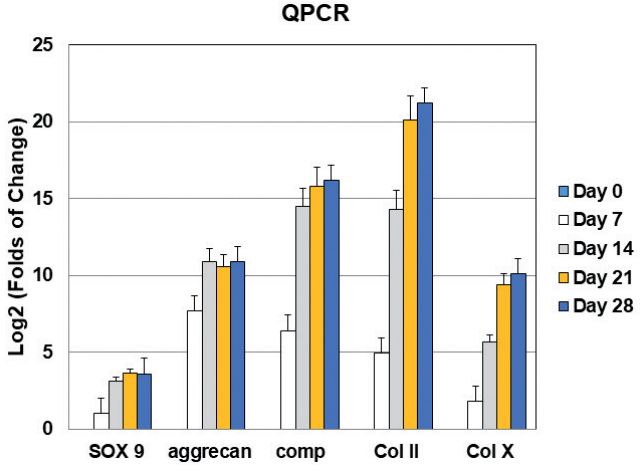


Fig. 5. Chondrocytes gene expression of BM-MSCs after chondrogenic induction. After 7 days of induction, all chondrogenic gene expression was up-regulated; for the sox9, aggrecan, and comp they attained their plateau at the 14th days of induction; while for the Col II and Col X, they attained their plateau at the 21st days of induction. We selected 14-day induction for the test in the *in vivo* chondrogenic scaffolds.

In the pilot study, X rays and MRI examination were performed to evaluate the progression of post-operation osteoarthritis; but similar to that reported by Reisig et al., there was no correlation existed the histological and MRI scores (Reisig et al., 2016). In the first part of this study, we verified the porcine OA model by ACL transection procedures as an appropriate animal model of osteoarthritis via histological evaluation. While for the treatment of osteoarthritis, we used the porcine OA model with and without specific treatment modalities for comparison. The gelatin/chondroitin sulfate/hyaluronan tri-copolymer has potential for use as a cartilage tissue engineering scaffold. In the previous study of Chang et al., they produced a gelatin/chondoitin-6-sulfate/hyaluronan tri-copolymer to mimic natural cartilage matrix for use as a scaffold for cartilage tissue engineering. They showed that cartilage tissue can be engineered *in vitro* with porcine chondrocytes and gelatin/chondoitin-6-sulfate/hyaluronan tri-copolymer which mimic natural cartilage matrix for use as a scaffold. In this study, porcine chondrocytes were seeded onto this scaffold and can be uniformly distributed in the scaffold in the spinner flask cultures (Chang et al., 2003). Later, in *in vivo* cartilage tissue engineering for the treatment of FT articular defects; Chang et al., demonstrated that gelatin/chondoitin-6-sulfate/hyaluronan tri-copolymer can be used as an excellent scaffold for treatment of full thickness (FT) articular defects by tissue engineering-treated allogenic porcine chondrocytes (Chang et al., 2006).

The poor self-repair capacity of cartilage tissue has prompted the development of a variety of therapeutic approaches, such as cellular therapies and tissue engineering based on the use of mesenchymal stem cells (MSCs). A new therapeutic strategy for articular cartilage restoration was through the use of Collagen Cell Carrier (CCC) scaffolds, to reduce the morbidity from acute cartilage injuries and degenerative joint

diseases (Szychlinska et al., 2017a). In this study, the scaffold for chondrogenic induction is a collagen-based porous membrane with high pore interconnectivity (Fig. 4); after 2 weeks chondroprogenic induction, the chondrocytes differentiated from hBM-MSCs (Fig. 4). We selected the 14-day induction before the chondrogenic scaffolds were used in the *in vivo* test as the sox9, aggrecan, and comp they attained their plateau at this 14 days' induction (Fig. 3).

Once injured cartilage needs to be reestablished, this might be approached through resorting to cell-based therapies and/or tissue engineering. Due to their chondrogenic differentiation potential, human mesenchymal stem cells (MSCs) represent a promising approach. Presently, *in vitro* chondrogenic differentiation of MSCs is limited by two main reasons as follows: aging of MSCs, which determines the loss of cell proliferative and differentiation capacity and MSC-derived chondrocyte hypertrophic differentiation, which limits the use of these cells in cartilage tissue regeneration approach (Szychlinska et al., 2017b). Cartilage aging (or "chondrosenescence") undermines

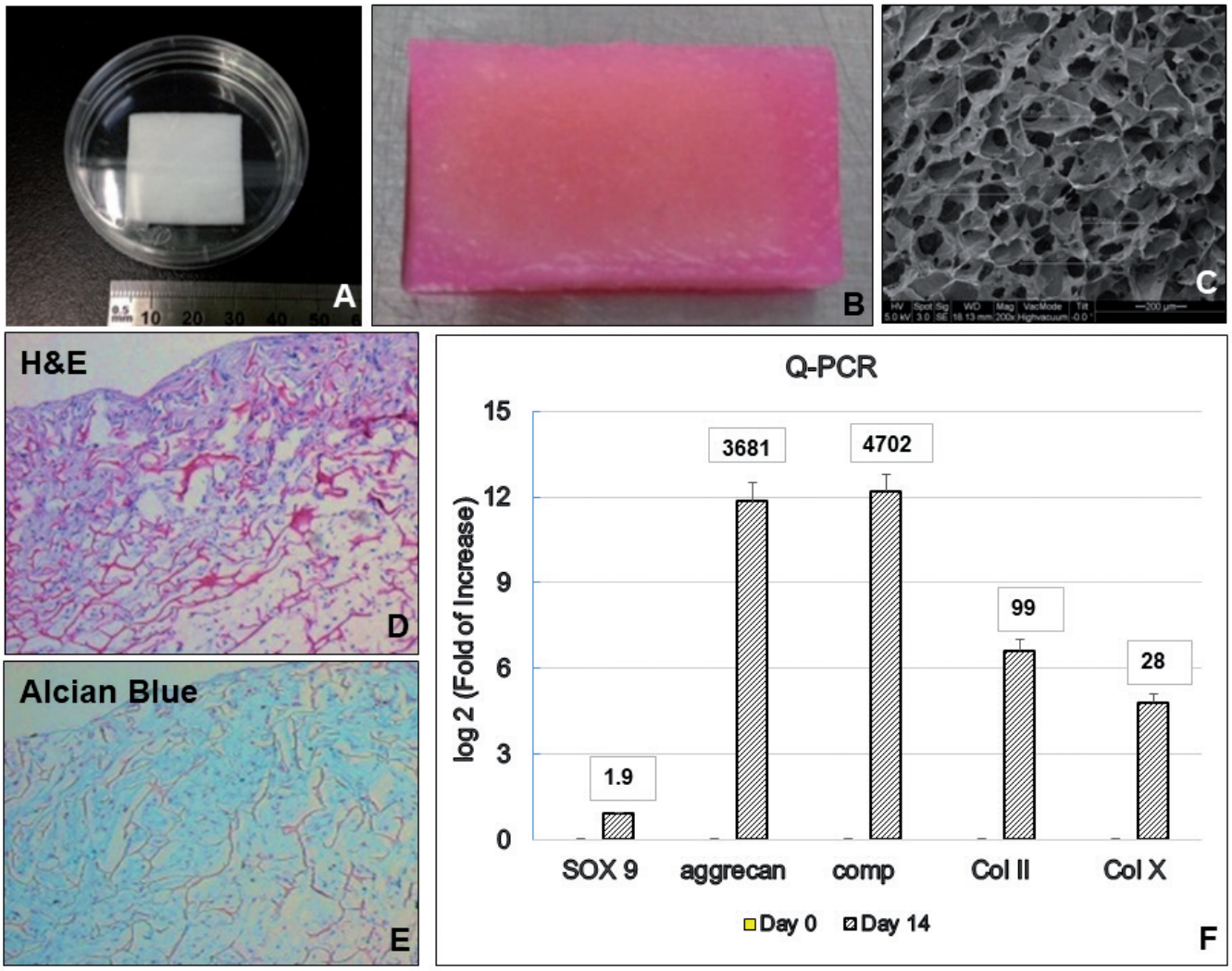


Fig. 6. Gross morphology of scaffold before implantation and histological study after MSC implantation. Upper: Gross morphology. The scaffold for chondrogenic induction is a 2mm thick collagen-based porous membrane **(A)**. Constructs retained their initial diameter and shape over the culture period. Their thickness increased substantially during time, and no shrinkage was observed **(B)**. The morphology of collagen scaffolds was revealed by SEM. The scanning electron microscopy showed a high pore interconnectivity with a uniform pore size at 341±60 µm and a porosity of 75% **(C)**. Lower (Left): histological study. When stained with H&E stain **(D)** and alcian blue stain **(E)**, histological sections of constructs at 2 weeks showed cartilaginous extracellular matrix deposition and the presence of proteoglycan around and within the cells. Lower (Right): Q-PCR analysis. After 2 weeks chondroprogenic induction, the hMSC cells exhibited increased expression of chondroprogenitor cell markers such as SOX-9, aggrecan, cartilage oligometric matrix protein (COMP), type II collagen and type X collagen at day 14 **(F)**.

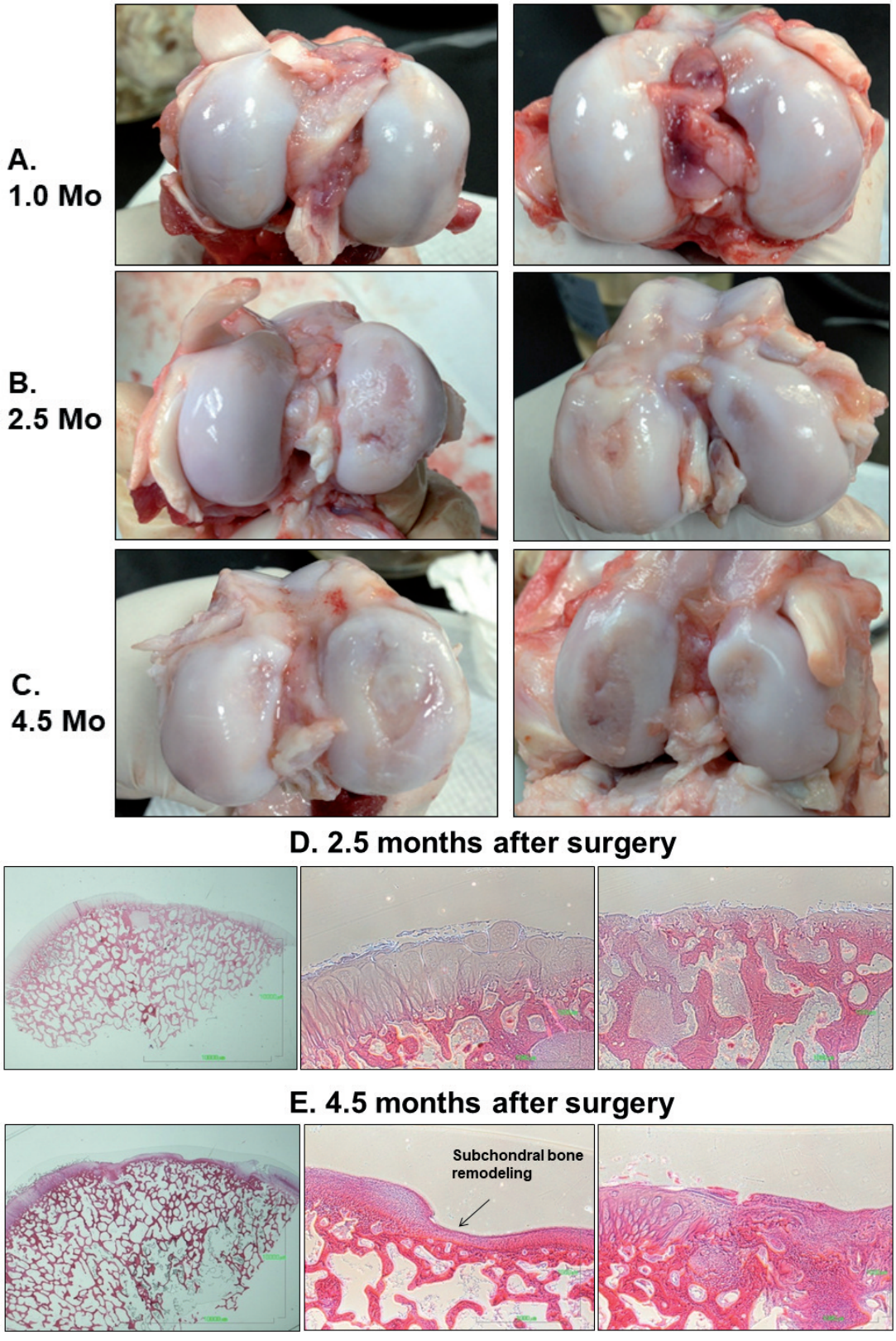


Fig. 7. Gross morphology and histomorphology of porcine osteoarthritis animal model. Gross morphology of porcine osteoarthritis animal model. At 1.0 month after anterior cruciate ligament (ACL) transection, minimal or no pathological changes were observed **(A)**. At 2.5 months after ACL transection, thinning and superficial fibrillation of cartilage (early OA) were observed **(B)**. At 4.5 months after ACL transection, vertical fissures, cartilage erosion (moderate OA), larger lesion and even cartilage matrix loss (severe OA) were observed **(C)**. Histomorphology of porcine osteoarthritis animal model. At 2.5 months after ACL transection, thinning and superficial fibrillation of cartilage were observed **(D)**. At 4.5 months after ACL transection, vertical fissures, cartilage erosion, cartilage matrix loss with subchondral bone remodeling were observed **(E)**.

cartilage function in osteoarthritis. The inflammation induced by a small number of senescent chondrocytes may be able to take advantage of the inflammatory microenvironment and then the inflammation and immunosenescence further contributes to the age-related degradation of articular cartilage, subchondral bone, synovium and other tissues (Mobasheri et al., 2015). Perturbed homeostasis caused by inflammation, oxidative stress, mitochondrial dysfunction and proapoptotic/antiapoptotic dysregulation is known to impair chondrocyte survival in joint microenvironments and contribute to OA pathogenesis (Musumeci et al., 2011). The effect of aging on MSCs is fundamental for stem cell-based therapy development, especially in older subjects. Further investigations in the potential of allogenic or even xenogenic chondrocytes as the possible cell source in cartilage tissue engineering might provide new insights into innovative strategies for cartilage regeneration and potentially inspire novel therapeutic approaches for OA treatment. In this study, we shift to xenogenic human mesenchymal stem cells as the cell source. We successfully demonstrated that this scaffold is also biocompatible with xenogenic hMSCs and when cultured in chondrogenic induction medium, cartilaginous extracellular matrix deposition and cartilage specific extracellular matrix (ECM) glycosaminoglycan within the scaffold and within the cells (Fig. 5) accompanied with increased expression of chondroprogenitor cell markers such as SOX-9, aggrecan, cartilage oligometric matrix protein (COMP), type II collagen and type X collagen genes (Fig. 5). We also demonstrated a good integration between host-tissue and hBM-MSCs scaffold complex. Smooth cartilage layer was completely restored 5 months after implantation. On the other hand, those without BM-MSCs scaffold implantation showed fibrocartilage repair on the chondral defect area (Fig. 6, Fig. Supplementary 3). We demonstrated that the experimental group (trephination bone plug plus hMSC-scaffold complex implantation) showed good full resurfacing after 5

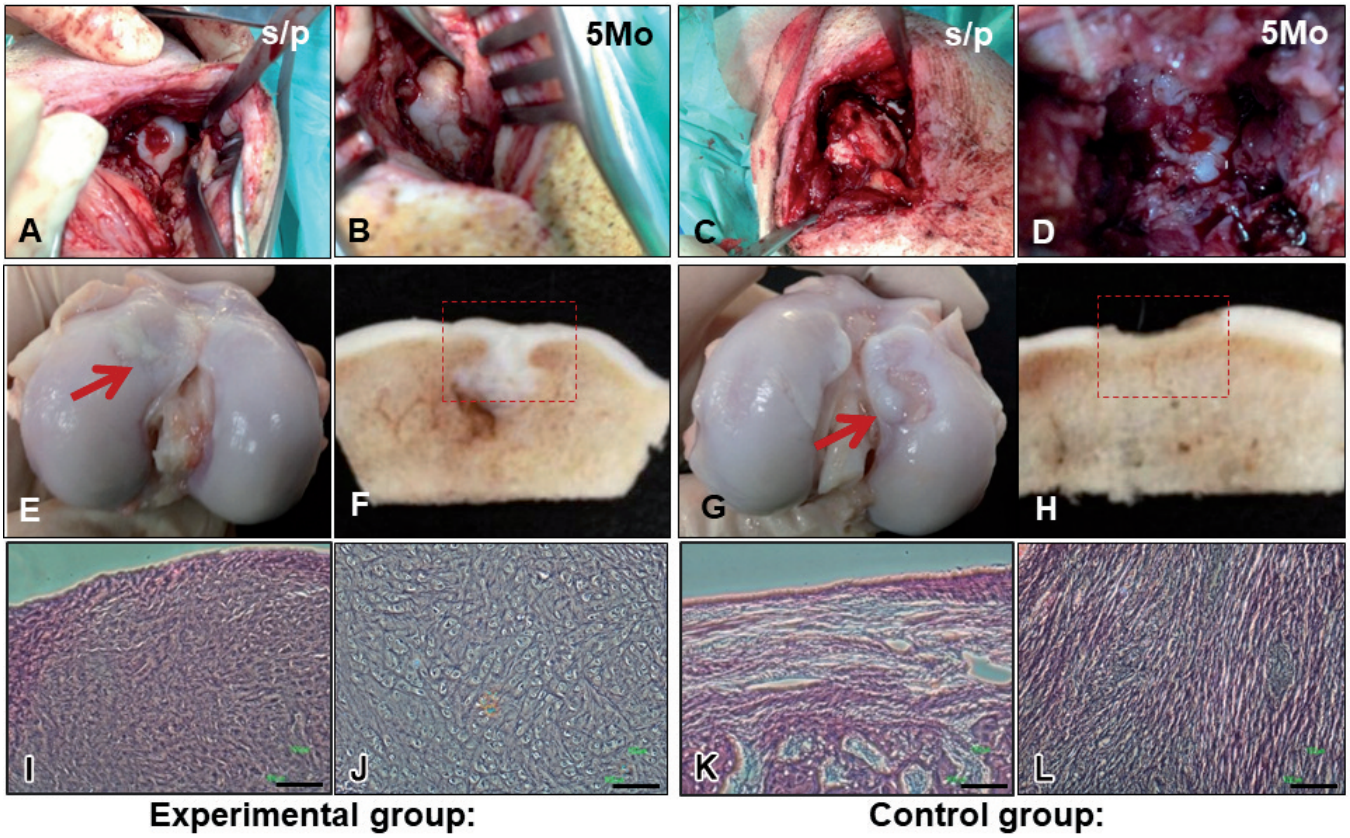


Fig. 8. Gross morphological and histomorphological comparison: the different treatment modalities. Upper row: Gross morphology at immediate postoperative day **(A, C)** and 5 months after surgery **(B, D).** Middle row: (After 5 months' healing). In the experimental group (trephination bone plug plus hMSC-scaffold complex implantation), the results showed good gross appearance, spontaneous repair of a full thickness defect showing fibrocartilage-like tissue without collapse and fully resurfaced **(E, F)**. In the control group (microfractures only), defects filled and covered with scarring fibrous tissue and secondary osteoarthritis showing collapse of the articular surface and growing of adjacent cartilage into the defect, which looks like a fold **(G, H)**. Lower: (After 5 months' healing). In the histomorphological study, the defect was filled up with chondrocytes in the experimental group **(I, J)**; while in the control (microfracture group), most of the defect was filled with fibrous tissue which was more likely fibrocartilage **(K, L)**. Scale bars: 50 µm.

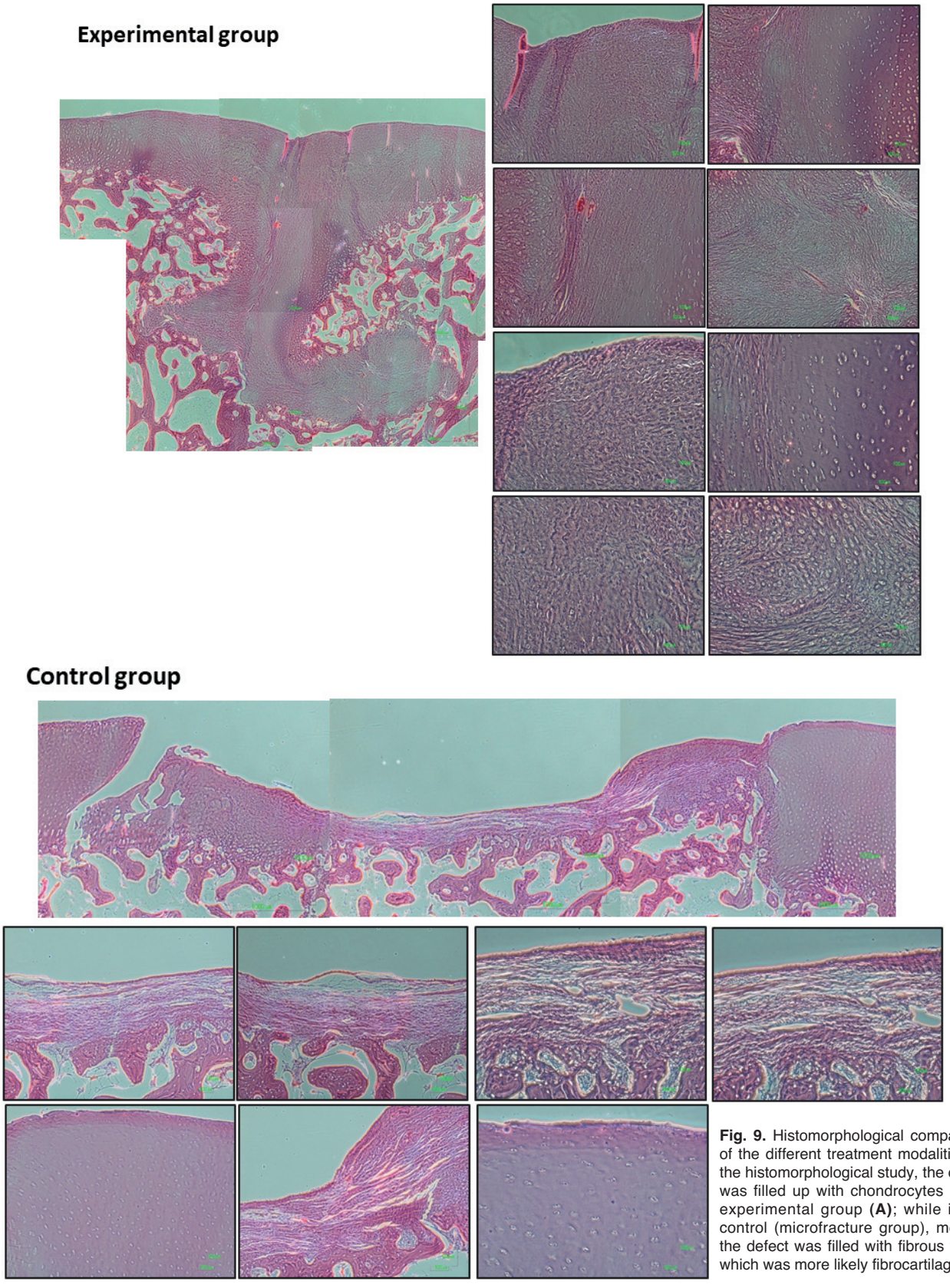


Fig. 9. Histomorphological comparison of the different treatment modalities. In the histomorphological study, the defect was filled up with chondrocytes in the experimental group **(A)**; while in the control (microfracture group), most of the defect was filled with fibrous tissue which was more likely fibrocartilage **(B)**.

months implantation. In the experimental group, the full thickness defect was grossly repaired with fibrocartilage-like tissue and without collapse; which also had a better modified Pineda score (Fig. 7). The results showed that those treated with hBM-MSC collagen-based scaffold demonstrated better cartilage integration than control group. The BM-MSCs derived collagen based chondroprogenitor scaffold had the potential for further development and to be applied to the treatment of osteochondral defect for early osteoarthritis in humans.

Regeneration of hyaline cartilage has been the focus of an increasing number of research groups around the world. One of the most important outcome measures in evaluation of its success is the histological quality of cartilaginous tissue. A variety of histological scoring systems is used to describe the quality of osteoarthritic, *in vivo* repaired or *in vitro* engineered tissue. In the study of Rutgers et al., the Histological-Histochemical Grading System (HHGS) or a HHGS-related score is most often used for evaluation of osteoarthritic cartilage; and the Osteoarthritis Research Society International (OARSI) Osteoarthritis Cartilage Histopathology Assessment System seems a valid alternative. The O'Driscoll score and the International Cartilage Repair Society (ICRS) II score may be used for *in vivo* repaired cartilage. The 'Bern score' seems most adequate for evaluation of *in vitro* engineered cartilage, while a simple alternative for O'Driscoll score system is the modified Pineda score (Rutgers et al., 2010). In this study, we selected the modified Pineda score system and Bern score for the *in vivo* and intro scoring system.

Although our study showed promising results, there were some limitations. (1) Numerous efforts have been made to implement the knowledge in the study of cartilage in the last years, and histochemistry proved to be an especially powerful tool to this aim. Although through routine staining procedure it is possible to obtain information on pathological articular cartilage, with more specialized immunohistochemistry it is possible to obtain specific information on both physiological and pathological articular cartilage. Further specific immunohistochemical techniques are crucial to know more about the physiopathology in the course and especially in early stages of OA (Musumeci et al., 2013, 2014; Szychlinska et al., 2016). (2) The study was performed with miniature pigs. The knee biomechanical behaviors between porcine and human are different, although we had promising results in porcine model; further studies are necessary to clarify if these results could be repeated in humans. Besides, although no adverse effects were observed throughout the whole course of the study, it is still compulsory to perform several kinds of examinations before the criteria are fulfilled to be able to perform further clinical trials. (3) The scaffold had only a single layer, without a stiff backbone bony layer. Thus it could not provide sufficient bony support to overcome the osteochondral defect. The bony layer on the osteochondral defect was also filled with the autogenous trephination bone graft. Newer bi-layer scaffold design with a combination of both bone and cartilage layers would be required to induce better regeneration of the bony defect. (4) The study periods were lasted only 5 months, it is not known if the BM-MSCs collagen-based scaffolds could withstand long-term integration consistency the same as native cartilage. (5) In this study, hBM-MSCs are currently being investigated as candidate cells for the repair of damaged articular cartilage. For these cells to be used clinically, it is important to understand how they will react to the complex loading environment of a joint *in vivo*. Previous *in vitro* study demonstrated that asymmetrically seeding the scaffold led to markedly improved tissue development based on histologically detectable matrix deposition (Gardner et al., 2017). Consideration of cell location, therefore, is an important aspect in the development of regenerative medicine approaches for cartilage repair. This is particularly relevant when considering the natural biomechanical

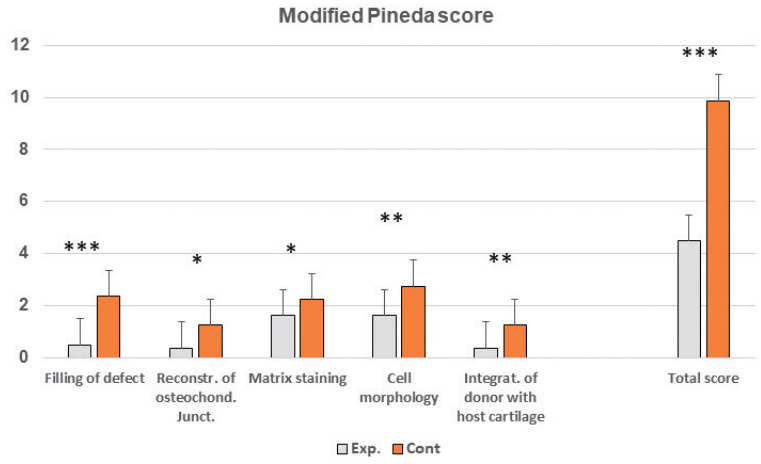


Fig. 10. Comparison of the modified Pineda scores in the study and control groups (n=8 pigs). In the modified Pineda score, all parameters (including filling of defect, reconstruction of osteochondral junction, matrix staining, cell morphology, and integration of donor with host cartilage) and total score showed significant differences between two groups. *: P<0.05; **: P<0.005; ***: P<0.0005;

environment of the joint *in vivo* and patient rehabilitation protocols. Further studies are necessary to identify and verify these questions.

In conclusion, we demonstrated a consistent, easily performed OA knee animal model by using ACL transection procedure. In addition, those treated with xenogenic hBM-MSCs-embedded collagen scaffold demonstrated better cartilage integration than control group. This study showed that hBM-MSCs derived chondroprogenitor cell scaffolds can generate new cartilage tissue *in vivo* and have the potential as a useful treatment option for osteoarthritis.

Acknowledgements: We would like to thank the National Taiwan University Hospital (Taipei, Taiwan) and Industrial Technology Research Institute (Chutung, Hsinchu, Taiwan) for their financial support. The authors declare no potential conflict of interest with respect to the authorship and/or publication of this article.

References

Bijlsma J.W., Berenbaum F., Lafeber F.P. (2011). Osteoarthritis: An update with relevance for clinical practice. Lancet. 377, 2115-2126.

Bornes T.D., Adesida A.B. and Jomha N.M. (2014). Mesenchymal stem cells in the treatment of traumatic articular cartilage defects: A comprehensive review. Arthritis Res. Ther. 16, 432.

Bornes T.D., Jomha N.M., Mulet-Sierra A. and Adesida A.B. (2015). Hypoxic culture of bone marrow-derived mesenchymal stromal stem cells differentially enhances *in vitro* chondrogenesis within cell-seeded collagen and hyaluronic acid porous scaffolds. Stem cell Res. Ther. 6, 84.

Chang C.H., Liu H.C., Lin C.C., Chou C.H. and Lin F.H. (2003). Gelatin-chondroitin-hyaluronan tri-copolymer scaffold for cartilage tissue engineering. Biomaterials. 24, 4853-4858.

Chang C.H., Kuo T.F., Lin C.C., Chou C.H., Chen K.H., Lin F.H. and Liu H.C. (2006). Tissue engineering-based cartilage repair with allogenous chondrocytes and gelatin-chondroitin-hyaluronan tri-copolymer scaffold: A porcine model assessed at 18, 24, and 36 weeks. Biomaterials 27, 1876-1888.

Christiansen B.A., Guilak F., Lockwood K.A., Olson S.A., Pitsillides A.A., Sandell L.J., Silva M.J., van der Meulen M.C. and Haudenschild D.R. (2015). Non-invasive mouse models of post-traumatic osteoarthritis. Osteoarthritis Cartilage 23, 1627-1638.

Corradi-Perini C., Santos T.M., Camara N.O.S., Riella M.C. and Aita C.A.M. (2017). Co-transplantation of xenogeneic bone marrow-derived mesenchymal stem cells alleviates rejection of pancreatic islets in non-obese diabetic mice. Transplant. Proc. 49, 902-905.

Daher R.J., Chahine N.O., Greenberg A.S., Sgaglione N.A. and Grande D.A. (2009). New methods to diagnose and treat cartilage degeneration. Nat. Rev. Rheumatol. 5, 599-607.

Di Luca A., Szlazak K., Lorenzo-Moldero I., Ghebes C.A., Lepedda A., Swieszkowski W., Van Blitterswijk C. and Moroni L. (2016). Influencing chondrogenic differentiation of human mesenchymal stromal cells in scaffolds displaying a structural gradient in pore size. Acta Biomater. 36, 210-219.

Fransen M., Agaliotis M., Nairn L., Votrubec M., Bridgett L., Su S., Jan S., March L., Edmonds J., Norton R., Woodward M., Day R. and LEGS study collaborative group. (2015). Glucosamine and chondroitin for knee osteoarthritis: A double-blind randomised placebo-controlled clinical trial evaluating single and combination regimens. Ann. Rheum. Dis. 74, 851-858.

Gardner O.F.W., Musumeci G., Neumann A.J., Eglin D., Archer C.W., Alini M. and Stoddart M.J. (2017). Asymmetrical seeding of MSCs into fibrin-poly(ester-urethane) scaffolds and its effect on mechanically induced chondrogenesis. J. Tissue Eng. Regen. Med. 11, 2912-2921.

Garg T., Singh O., Arora S. and Murthy R. (2012). Scaffold: A novel carrier for cell and drug delivery. Crit. Rev. Ther. Drug Carrier Syst. 29, 1-63.

Hagan-Brown A,, Favaretto M. and Borry P. (2017). Newspaper coverage of human-pig chimera research: A qualitative study on select media coverage of scientific breakthrough. Xenotransplantation 24, e12317.

Kamekura S., Hoshi K., Shimoaka T., Chung U., Chikuda H., Yamada T., Uchida M., Ogata N., Seichi A., Nakamura K. and Kawaguchi H. (2005). Osteoarthritis development in novel experimental mouse models induced by knee joint instability. Osteoarthritis Cartilage 13, 632-641.

Klontzas M.E., Kenanidis E.I., Heliotis M., Tsiridis E. and Mantalaris A. (2015). Bone and cartilage regeneration with the use of umbilical cord mesenchymal stem cells. Expert Opin. Biol. Ther. 15, 1541-1552.

Kuyinu E.L., Narayanan G., Nair L.S. and Laurencin C.T. (2016). Animal models of osteoarthritis: Classification, update, and measurement of outcomes. J. Orthop. Surg. Res.. 11, 19.

Lampropoulou-Adamidou K., Lelovas P., Karadimas E.V., Liakou C., Triantafillopoulos I.K., Dontas I. and Papaioannou N.A. (2014). Useful animal models for the research of osteoarthritis. Eur. J. Orthop. Surg. Traumatol. 24, 263-271.

Lawrence R.C., Felson D.T., Helmick C.G., Arnold L.M., Choi H., Deyo R.A., Gabriel S., Hirsch R., Hochberg M.C., Hunder G.G., Jordan J.M., Katz J.N., Kremers H.M., Wolfe F. and National Arthritis Data Workgroup. (2008). Estimates of the prevalence of arthritis and other rheumatic conditions in the united states. Part ii. Arthritis and Rheum. 58, 26-35.

Li Y., Xie B., Zhu M., Li X., Du F., Li Y., Li M., Yuan Q., Hua J., Xue M. Wang Z., Xia J. and Qi Z. (2017). A highly reproducible cervical cuff technique for rat-to-mouse heterotopic heart xenotransplantation. Xenotransplantation 24.

McAlindon T.E., Bannuru R.R., Sullivan M.C., Arden N.K., Berenbaum F., Bierma-Zeinstra S.M., Hawker G.A., Henrotin Y., Hunter D.J., Kawaguchi H. Kwoh K., Lohmander S., Rannou F., Roos E.M. and Underwood M. (2014). Oarsi guidelines for the non-surgical management of knee osteoarthritis. Osteoarthritis Cartilage 22, 363-388.

McCoy A.M. (2015). Animal models of osteoarthritis: Comparisons and key considerations. Vet. Pathol. 52, 803-818.

Mobasheri A., Matta C., Zakany R. and Musumeci G. (2015). Chondrosenescence: Definition, hallmarks and potential role in the pathogenesis of osteoarthritis. Maturitas 80, 237-244.

Musumeci G., Loreto C., Carnazza M.L., Strehin I. and Elisseeff J. (2011). OA cartilage derived chondrocytes encapsulated in poly(ethylene glycol) diacrylate (PEGDA) for the evaluation of cartilage restoration and apoptosis in an *in vitro* model. Histol. Histopathol. 26, 1265-1278.

Musumeci G., Trovato F.M., Pichler K., Weinberg A.M., Loreto C. and Castrogiovanni P. (2013). Extra-virgin olive oil diet and mild physical

activity prevent cartilage degeneration in an osteoarthritis model: An *in vivo* and *in vitro* study on lubricin expression. J. Nutri. Biochem. 24, 2064-2075.

Musumeci G., Castrogiovanni P., Mazzone V., Szychlinska M.A., Castorina S. and Loreto C. (2014). Histochemistry as a unique approach for investigating normal and osteoarthritic cartilage. Eur. J. Histochem. EJH. 58, 2371.

Musumeci G., Castrogiovanni P., Trovato F.M., Imbesi R., Giunta S., Szychlinska M.A., Loreto C., Castorina S. and Mobasheri A. (2015a). Physical activity ameliorates cartilage degeneration in a rat model of aging: A study on lubricin expression. Scand. J. Med. Sci. Sports 25, e222-230.

Musumeci G., Szychlinska M.A. and Mobasheri A. (2015b). Age-related degeneration of articular cartilage in the pathogenesis of osteoarthritis: Molecular markers of senescent chondrocytes. Histol. Histopathol. 30, 1-12.

Neogi T. (2013). The epidemiology and impact of pain in osteoarthritis. Osteoarthritis Cartilage 21, 1145-1153.

Neogi T. and Zhang Y. (2013). Epidemiology of osteoarthritis. Rheum. Dis. Clin. North Am. 39, 1-19.

Oberlander M.A., Baker C.L. and Morgan B.E. (1998). Patellofemoral arthrosis: The treatment options. Am. J. Orthoped. 27, 263-270.

Pineda S., Pollack A., Stevenson S., Goldberg V. and Caplan A. (1992). A semiquantitative scale for histologic grading of articular cartilage repair. Acta Anat. 143, 335-340.

Proffen B.L., McElfresh M., Fleming B.C. and Murray M.M. (2012). A comparative anatomical study of the human knee and six animal species. The Knee 19, 493-499.

Reisig G., Kreinest M., Richter W., Wagner-Ecker M., Dinter D., Attenberger U., Schneider-Wald B., Fickert S. and Schwarz M.L. (2016). Osteoarthritis in the knee joints of gottingen minipigs after resection of the anterior cruciate ligament? Missing correlation of mri, gene and protein expression with histological scoring. PLoS One 11, e0165897.

Reppel L., Schiavi J., Charif N., Leger L., Yu H., Pinzano A., Henrionnet C., Stoltz J.F., Bensoussan D. and Huselstein C. (2015). Chondrogenic induction of mesenchymal stromal/stem cells from wharton's jelly embedded in alginate hydrogel and without added growth factor: An alternative stem cell source for cartilage tissue engineering. Stem Cell Res. Ther. 6, 260.

Rutgers M., van Pelt M.J., Dhert W.J., Creemers L.B. and Saris D.B. (2010). Evaluation of histological scoring systems for tissue-engineered, repaired and osteoarthritic cartilage. Osteoarthritis Cartilage 18, 12-23.

Singh J.A., Noorbaloochi S., MacDonald R. and Maxwell L.J. (2015). Chondroitin for osteoarthritis. Cochrane Database Syst. Rev. 1:Cd005614.

Steadman J.R., Rodkey W.G. and Rodrigo J.J. (2001). Microfracture: Surgical technique and rehabilitation to treat chondral defects. Clin. Orthop. Relat. Res. 391 (Suppl), S362-369.

Stubbendorff M., Deuse T., Hua X., Phan T.T., Bieback K., Atkinson K., Eiermann T.H., Velden J., Schroder C., Reichenspurner H., Robbins R.C., Volk H.D. and Schrepfer S. (2013). Immunological properties of extraembryonic human mesenchymal stromal cells derived from gestational tissue. Stem Cells Dev. 22, 2619-2629.

Szychlinska M.A., Trovato F.M., Di Rosa M., Malaguarnera L., Puzzo L., Leonardi R., Castrogiovanni P. and Musumeci G. (2016). Co-expression and co-localization of cartilage glycoproteins chi3l1 and lubricin in osteoarthritic cartilage: Morphological, immunohisto-chemical and gene expression profiles. Int. J. Mol. Sci. 17, 359.

Szychlinska M.A., Castrogiovanni P., Nsir H., Di Rosa M., Guglielmino C., Parenti R., Calabrese G., Pricoco E., Salvatorelli L., Magro G,. Imbesi R., Mobasheri A. and Musumeci G. (2017a). Engineered cartilage regeneration from adipose tissue derived-mesenchymal stem cells: A morphomolecular study on osteoblast, chondrocyte and apoptosis evaluation. Exp. Cell Res. 357, 222-235.

Szychlinska M.A., Stoddart M.J., D'Amora U., Ambrosio L., Alini M. and Musumeci G. (2017b). Mesenchymal stem cell-based cartilage regeneration approach and cell senescence: Can we manipulate cell aging and function? Tissue Eng. Part B, Reviews. 23, 529-539.

Temenoff J.S. and Mikos A.G. (2000). Review: Tissue engineering for regeneration of articular cartilage. Biomaterials 21, 431-440.

Wei B., Zong M., Yan C., Mao F., Guo Y., Yao Q., Xu Y. and Wang L. (2015). Use of quantitative mri for the detection of progressive cartilage degeneration in a mini-pig model of osteoarthritis caused by anterior cruciate ligament transection. J. Magn. Reson. Imaging. 42, 1032-1038.

Wu J., Platero-Luengo A., Sakurai M., Sugawara A., Gil M.A., Yamauchi T., Suzuki K., Bogliotti Y.S., Cuello C., Morales Valencia M. Okumura D., Luo J., Vilariño M., Parrilla I., Soto D.A., Martinez C.A., Hishida T., Sánchez-Bautista S., Martinez-Martinez M.L., Wang H., Nohalez A., Aizawa E., Martinez-Redondo P., Ocampo A., Reddy P., Roca J., Maga E.A., Esteban C.R., Berggren W.T., Nuñez Delicado E., Lajara J., Guillen I., Guillen P., Campistol J.M., Martinez E.A., Ross P.J. and Izpisua Belmonte J.C. (2017). Interspecies chimerism with mammalian pluripotent stem cells. Cell 168, 473-486.

Accepted June 15, 2018