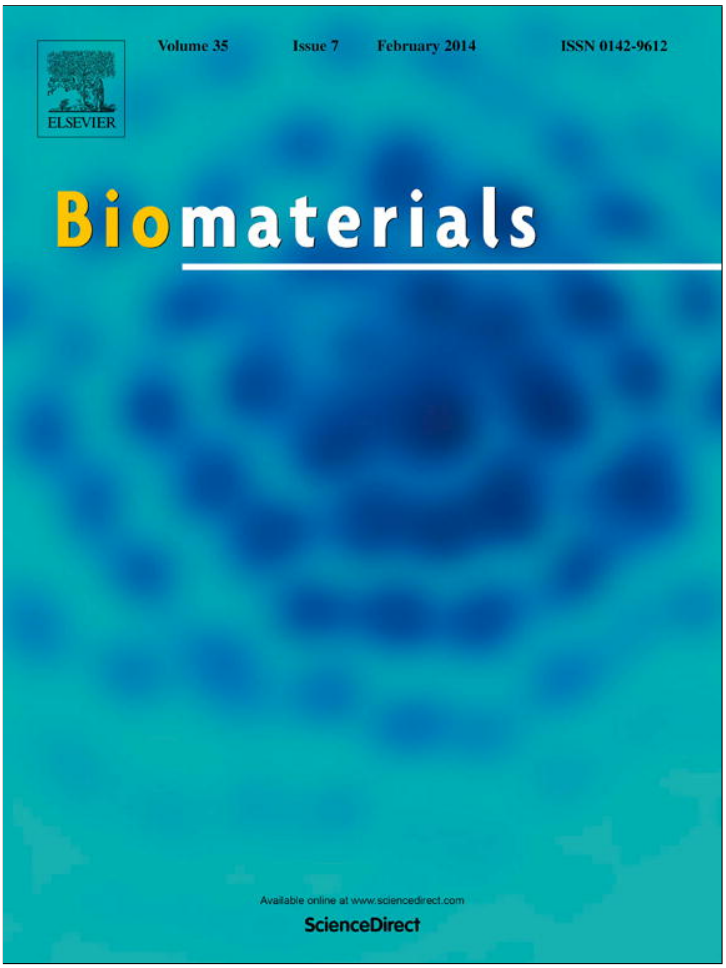

Biomaterials 35 (2014) 2199–2206

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

In vivo cell tracking by bioluminescence imaging after transplantation of bioengineered cell sheets to the knee joint

Yuko Takaku [a], Kunihiko Murai [a], Taku Ukai [b], Satoshi Ito [b], Mami Kokubo [b], Masaaki Satoh [a], Eiji Kobayashi [c], Masayuki Yamato [d], Teruo Okano [d], Mamoru Takeuchi [a], Joji Mochida [b], Masato Sato [b,*]

[a] Department of Anesthesiology and Critical Care Medicine, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
[b] Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
[c] Division of Development of Advanced Treatment, Center for Development of Advanced Medical Technology, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
[d] Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

ARTICLE INFO

Article history:
Received 9 October 2013
Accepted 22 November 2013
Available online 20 December 2013

Keywords:
Chondrocyte
Cell sheet
Tissue engineering
Transplantation
Bioluminescence imaging

ABSTRACT

In our previous studies, we have demonstrated effective regeneration of cartilage through the creation and application of layered cell sheets that combine both chondrocytes and synovial cells. In this study, we were able to demonstrate that cells derived from cell sheets can survive for long periods after transplantation into rat knee joints having osteochondral defects. We established a method for generating cell sheets from firefly luciferase-expressing chondrocytes obtained from transgenic Lewis rats, and carried out allogenic transplantation of these cell sheets into wild-type Lewis rats. We then administered luciferin and monitored the survival of the transplanted cells by using bioluminescence imaging (BLI). Our data showed that the transplanted cells survived and could be detected for more than 21 months, which was longer than expected. Furthermore, the BLI data showed that the transplanted cells remained in the knee joint and did not migrate to other parts of the body, thus confirming the safety of the cell sheets. In this study, we monitored the duration of survival of cell sheets composed of only chondrocytes, only synovial cells, or both chondrocytes and synovial cells, and found that all three types of cell sheets survived for an extended period of time.

1. Introduction

Articular cartilage tissue lacks blood vessels and has a poor ability to self-renew. Self-renewal is particularly difficult if the defect is partial and localized, and does not reach the subchondral bone. If the defect encompasses the entire articular cartilage layer, and has reached the subchondral bone, mesenchymal stem cells are introduced from the bone marrow. If the defect is small in size, regenerative repair may be possible [1]. In actual pathological instances of osteoarthritis, there is a mixture of partial- and total-thickness defects. Knee cartilage damage caused by aging or trauma easily converts to osteoarthritis and is accompanied by sharp pain.

Fortunately, the lack of blood vessels in articular cartilage results in a lower rate of rejection after grafting. Therefore, the use of allografts is more widely studied for cartilage regeneration than for the regeneration of other tissues and organs.

To date, many animal experiments and clinical studies have been carried out to evaluate strategies for improving self-renewal in articular cartilage. Recently, research involving tissue-engineered cartilage grafts has become increasingly common. In a clinical study, Ochi et al. [2] reported improvements in the response to mechanical loads and sensory innervation using tissue-engineered cartilage grafts created in atelocollagen gels. A wide variety of synthetic polymers and biomaterials are used as scaffolds to create bioengineered cartilage grafts. Consequently, biocompatibility risks associated with the application of such substances over the long term have been reported [3]. The creation of tissue-engineered cartilage tissue that does not utilize scaffolds would significantly mitigate these risks, making their application more feasible and safer for patients. Mainil-Varlet et al. [4] successfully created scaffold-free tissue-engineered cartilage using a static bioreactor system. Furthermore, Nagai et al. [5], using their rotation

* Corresponding author. Tel.: +81 463 93 1121; fax: +81 463 96 4404.
E-mail address: sato-m@is.icc.u-tokai.ac.jp (M. Sato).

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.11.071

culture method, reported that it is possible to create tissue-engineered cartilage from a lower number of chondrocytes than previously reported.

In the field of cartilage regeneration, previous research has focused on finding therapeutic methods to treat cartilage defects by using grafts made from synovial cells. Synovial cells have a critical role in the repair of cartilage defects [3]. However, in the treatment of cartilage defects using grafts derived from synovial cells, fibrous tissue is more likely to be formed on the surface of the regenerated cartilage tissue [6].

To treat osteoarthritis, our group has carried out some basic studies concerning the repair and regeneration of articular cartilage using tissue engineering. We have focused on the importance of the interaction between the cells of the recipient and the donor in the repair and regeneration of articular cartilage. If there are a minimum number of bioengineered chondrocytes at or near the surface of the cartilage layer, we have found that the recipient's cells drive the regeneration of cartilage [7–9].

Chondrocyte sheets, generated using the bioengineered cell sheet technology developed originally in Japan, can be harvested on a temperature-responsive culture dish without damaging the cells and extracellular matrix [10,11]. Layered chondrocyte sheets fabricated using temperature-responsive culture dishes have characteristics that differ from those of cultured chondrocytes using normal culture dishes. These characteristics allow them to exert regenerative effects to the same degree as other bioengineered cartilage tissue [12]. Chondrocyte sheets have already proven more effective in promoting regeneration of both partial- and full-thickness defects [12,13].

Cell sheets exhibit superior adhesion to the surface of damaged cartilage having partial- or full-thickness defects. In addition, cell sheets have superior barrier functionality, which protects the site from the catabolic factors present in the joint fluid, and also prevents leakage of the extracellular matrix of cartilage tissue into the joint fluid. Moreover, cell sheets might contribute to the repair and regeneration of cartilage by continuously providing growth factors derived from cell sheets. Layered cell sheets maintain their three-dimensional structure. In our most recent clinical studies that focused on humans, we found that multilayered sheets express a significantly larger amount of humoral factors such as transforming growth factor β, prostaglandin E2, and melanoma inhibitor activity compared with single-layered sheets and cultured chondrocyte as the same number. These data indicate that multilayered sheets will probably have higher efficacy with respect to cartilage regeneration than single-layered sheets [14].

The synovial cells existing in the knee joint secrete the synovial fluid that nourishes chondrocytes. We have developed a method of harvesting chondrocyte sheets at a faster pace and with greater certainty by co-culturing chondrocytes and synovial cells in an environment that resembles the inside of the knee joint [15]. Furthermore, in experiments on Japanese white rabbits, the transplantation of a combination of chondrocyte sheets and synovial cells led to improved repair of osteochondral tissue defects compared with the transplantation of chondrocyte sheets or synovial cells alone [16].

We sought to determine the duration for which cell sheets comprising chondrocytes and synovial cells survive and continue to secrete growth factors at defect sites within the knee joint. We hypothesized that mature chondrocytes and synovial cells, as part of cell sheets, would gradually decrease in number from the moment of transplantation, and disappear after 3–4 weeks. We also expected that grafts consisting of a combination of chondrocytes and synovial cells within an environment resembling the inside of the knee joint would survive longer than grafts of each type of cell, by enhancing the regenerative effect exerted by the recipient's own chondrocytes.

Bioluminescent reporter genes are increasingly being used to image engineered cells *in vivo*. Of these, the firefly luciferase (*luc*) gene is the most commonly used. The enzymatic reaction between luciferase and its substrate, luciferin, results in photon emission, which can be detected using a cooled charge-coupled device (CCD) camera. In recent years, transgenic animals carrying specific marker genes have been generated [17–19]. Cells derived from them can be transplanted into other animals and then used to investigate survival time, cell migration, and so forth, in those animals. These cells are also frequently used in research on the regeneration of organs and tissues [20–24]. In this study, we transplanted luciferase-expressing transgenic rat cells into nontransgenic rats and then attempted to effectively track those transplanted cells.

The aim of this study was to determine the survival time of bioengineered chondrocyte sheets and synovial cell sheets after transplantation to cartilage defects in the knee joint of rats by using *in vivo* bioluminescence imaging (BLI).

2. Materials and methods

Animal experiments were performed in accordance with the guidelines of the Institutional Regulation for Animal Experiments as well as the Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology for animal handling and care. The study was approved by the Institutional Animal Experiment Committee of Jichi Medical University.

2.1. Luciferase-expressing transgenic rats

Hakamata et al. generated transgenic rats that express the luciferase gene (Rosa/luciferase transgenic Lewis rats) [25]. This gene contains the ROSA26 promoter, which allows the stable expression of luciferase ubiquitously throughout the entire body of the rats. When the substrate luciferin is injected *in vivo*, light is emitted through oxidation catalyzed by luciferase, a reaction that uses ATP. The strength of light emission differs according to the cell type, as emission strength is affected by endogenous ATP, Mg^{2+}, the amount of expression of the luciferase enzyme, and other elements that differ across cell types.

2.2. Harvest of chondrocytes and synovial cells from luciferase-expressing transgenic rats

Luciferase transgenic (Tg) 16-week-old male Lewis rats, obtained as described previously [25], were treated with general anesthesia using 2% isoflurane in O_2. An incision was made on the inner part of the lower extremities, exteriorizing the knee joint, and severing collateral ligaments as well as anterior and posterior cruciate ligaments. Then, the synovial and cartilaginous tissue was scraped with a scalpel. The tissue cells were isolated from the tissue samples using an enzymatic procedure. Luciferase-expressing synovial cells and chondrocytes were seeded in temperature-responsive culture dishes. Co-culture of the cells on these inserts permitted a shorter proliferation time than needed with single-cell-type cultures [15].

2.3. Cell culture using temperature-responsive culture dishes

After isolating synovial cells and chondrocytes from tissues using an enzymatic procedure, the synovial cells were seeded in temperature-responsive culture dishes, while the chondrocytes were seeded in temperature-responsive inserts. Co-culture via use of the inserts reduced the proliferation time compared with that required to culture each type of cell independently.

The luciferase-expressing cartilaginous and synovial tissues harvested from Tg rats were subjected to proteolytic digestion at 37 °C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM)/F12 adjusted with 0.016% collagenase (Worthington, NJ, USA), over a period of 4 h, with continuous stirring with a strainer. Subsequently, the luciferase-expressing chondrocytes and synovial cells were separated using a cell strainer with a pore size of 100 μm (BD, NJ, USA), centrifuged, and harvested. The synovial cells were incubated in DMEM/F12 containing 10% fetal bovine serum (FBS; GIBCO, NY, USA) and 1% mycotic antibody (Wako Pure Chemical Industries, Ltd., Japan). All cells were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air.

The chondrocytes were seeded in temperature-responsive inserts (5.0 cm^2; CellSeed Inc., Tokyo, Japan). The synovial cells were evenly seeded in temperature-responsive culture dishes (9.6 cm^2; CellSeed Inc.) so that the cells were distributed at 10,000 cells/cm^2, and then co-cultured with the chondrocytes in DMEM/F12 supplemented with 20% FBS for a period of about 14 days.

After confirming that the cells were confluent after about 14 days of culture, the temperature-responsive culture dishes were transferred to a lower temperature (25 °C) incubator for an additional 30 min.

2.4. Cell sheet retrieval

A polyisopropylacrylamide (PIPAAm) membrane was fixed to the surface of the temperature-responsive culture dishes. This enabled the dish surface to reversibly change between a cell-adhesive hydrophobic surface and a cell-releasing hydrophilic surface at a temperature threshold of 32 °C. Hence, by lowering the temperature, the complete cell sheet could be harvested from the dish while maintaining the sheet structure, without requiring the use of enzymes such as trypsin, which would damage the cells.

Cell sheets harvested in this manner maintain the extracellular matrix, thus enabling a nonsuture-based transplantation procedure. Furthermore, to create a layered cell sheet, the cell sheet was covered with a polyvinylidene difluoride (PVDF) membrane, and then the cell sheet and the PVDF membrane were harvested carefully as one unit. More exactly, the PVDF membrane that covered the cell sheet was harvested by rolling the membrane up at the edge of the culture dish and then overlaying the membrane on top of another cell sheet. By repeating this procedure three times, a triple-layered chondrocyte sheet was obtained.

2.5. Transplantation of synovial cells and layered chondrocyte sheets

We used 16-week-old wild-type male Lewis rats as allograft recipients. The transplantation procedure was carried out under general gas anesthesia using 2% isoflurane in O_2. On the right side, a medial parapatellar incision was made and the patella was moved aside slightly, opening the knee cavity to expose the patellar groove of the femur. We then created osteochondral defects (φ, 3 mm) with an 18-gauge needle (it is known that natural restoration is impossible in defects of this size). We then transplanted the harvested luciferase-expressing chondrocyte sheet to the defect.

The characteristics of individual chondrocyte and synovial cell sheets and the synergistic effect achieved by combining these sheets were determined from the cell counts obtained from two-layered luciferase-expressing chondrocyte sheets (AC-AC group), two-layered luciferase-expressing synovial cell sheets (SY-SY group), and sheets comprised of both types of luciferase-expressing sheets (AC-SY group). These sheets were constructed and then transplanted to the osteochondral defects in the right knees of wild-type Lewis rats ($n = 12$, for each type of sheet).

2.6. In vivo BLI

After the bioengineered sheets were transplanted, the rats were repeatedly imaged using BLI with an IVIS system (Xenogen Corp; Hopkinton, MA, USA) to detect photons emitted by the transplanted luciferase-expressing sheet cells. For BLI, the rats were anesthetized with isoflurane and subcutaneously injected with luciferin (150 mg/kg of body weight; D-luciferin, Biosynth AG, Staad, Switzerland) near the scapula. The method of administering luciferin is described in detail below. These rats were then placed in a light-tight chamber for imaging using a CCD camera. The photon emission from the peak luciferase activity was recorded and the BLI images were aligned using the IGOR and IVIS Living Image software packages.

2.7. Comparison of several methods of luciferin injection

To determine the best method of administering luciferin, we compared three alternatives: intravenous injection (IV), subcutaneous injection (SC), and intra-articular injection (IA). We administered luciferin to the same Lewis rats to which luciferase-expressing chondrocyte cell sheets were transplanted. In all three methods, we administered luciferin at a concentration of 50 mg/ml, which is close to the saturation point. In the IV method, we administered luciferin solution at 60 mg/kg through the penile vein. In the SC method, we administered luciferin solution at 150 mg/kg near the scapula, and for the IA method, we injected luciferin at 30 mg/kg directly into the knee joint cavity under the patella. All treatments were carried out under isoflurane anesthesia. After injection, the rat was quickly placed in a light-tight chamber equipped with a CCD camera, and light emission was measured at intervals of 1 min. The change in luminescence intensity over time was monitored. Each injection method yielded differing dynamic profiles of photon emission (Fig. 1A–C).

When using the IV injection method, the amount of time required for the luminescence intensity to reach peak levels was extremely short (1–2 min after injection) and the intensity declined rapidly thereafter (Fig. 1A). Consequently, in some cases, photon emission could only be measured after the peak level had been reached. In addition, injection into the penile vein was technically challenging; if not done correctly, the vein would swell, making it difficult to repeat the injection procedure.

Following SC injection, the luminescence intensity rose gradually and peaked 20–30 min after injection, after which it declined gradually (Fig. 1B). This technique was not technically challenging; however, the method required a large amount of luciferin. Achieving the same intensity with the SC method required more than twice the dosage of luciferin used in the IV method.

Following IA injection, the luminescence intensity rose to peak levels after 4–5 min (Fig. 1C). Compared with the IV method, the IA injection was technically easier to carry out. However, in some cases, the technique failed. The technique allowed luciferin to be directly delivered to luciferase-expressing cells without having to enter the bloodstream. Of the three techniques, the IA method allowed for the maximum emission strength to be achieved using the smallest amount of luciferin. Even though this method required only 0.2 ml of drug solution, it nevertheless resulted in swelling of the articular capsule of the knee joint and increasing synovial pressure, leading to a concern about the effect that the swelling might have on the transplanted cells or the regeneration of the osteochondral defect. Of the three methods, we found that SC injection allowed us to measure the peak levels of intensity by viable luciferase-expressing cells, while at the same time being the least technically challenging method, and with minimal undesirable side effects on the knee joint.

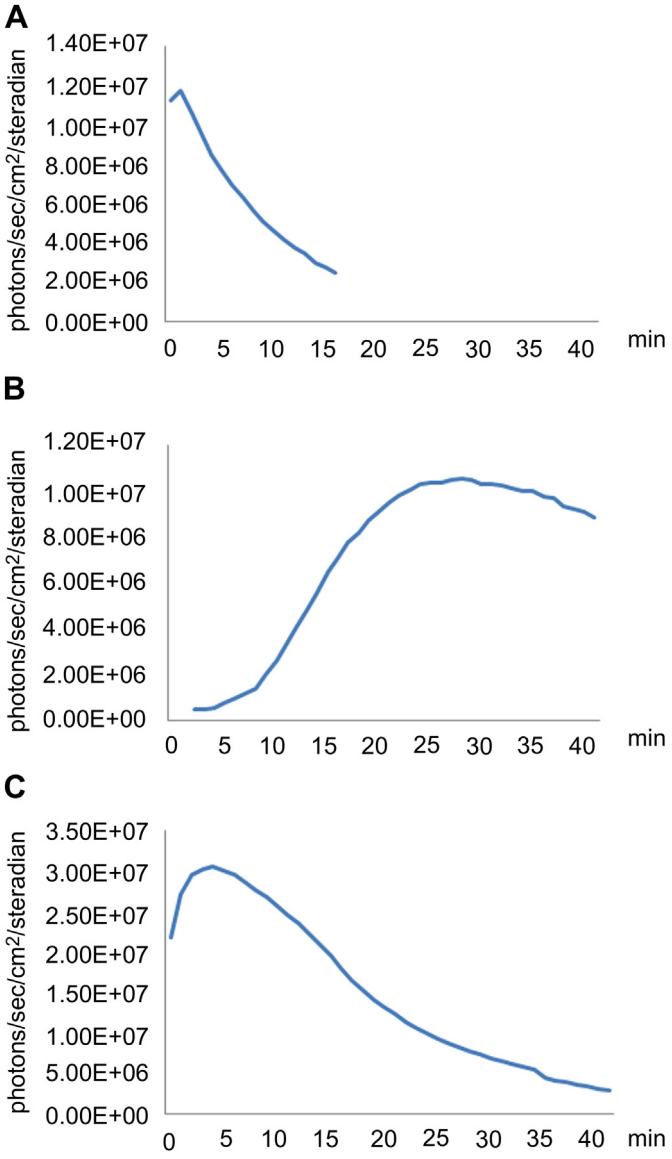

Fig. 1. Time course of BLI signals after IV (A), SC (B), or IA (C) injection of luciferin in rats. IV, intravenous injection; SC, subcutaneous injection; and IA, intra-articular injection.

2.8. Histological evaluation and scoring of the repair of osteochondral defects

We previously demonstrated in experiments with Japanese white rabbits that transplantation of cell sheets made of a combination of chondrocytes and synovial cells had a superior regenerative effect on osteochondral defects compared with those achieved with chondrocyte sheets or synovial cells alone [16]. We carried out similar experiments on Lewis rats in this study.

We used 24 of the aforementioned 36 wild-type Lewis rats that received cell sheet allografts for the following experiment ($n = 8$, per group). Two rats from each of the groups were euthanized by inhalation of CO_2 at 2, 4, 6, and 8 weeks after transplantation, and a specimen obtained from the site of the osteochondral defect on the transplanted knee was processed as follows. The tissue samples were fixed in 4% paraformaldehyde for 1 week. After decalcification for 2–3 weeks using distilled

Table 1
ICRS histological grading system.[a]

Ti
Tissue morphology
Lateral integration of implanted material

4: mostly hyaline cartilage
3: mostly fibrocartilage
2: mostly noncartilage
1: exclusively noncartilage

Matx
Matrix staining

1: none
2: slight
3: moderate
4: strong

Stru
Structural integrity

1: severe disintegration
2: cysts or disruptions
3: no organization of chondrocytes
4: beginning of columnar organization of chondrocytes
5: normal, similar to healthy mature cartilage

Clus
Chondrocyte clustering in implant

1: 25–100% of the cells clustered
2: <25% of the cells clustered
3: no clusters

Tide
Intactness of the calcified cartilage layer, formation of tidemark

1: <25% of the calcified cartilage layer intact
2: 25–49% of the calcified cartilage layer intact
3: 50–75% of the calcified cartilage layer intact
4: 76–90% of the calcified cartilage layer intact
5: complete intactness of the calcified cartilage layer

Bform
Subchondral bone formation

1: no formation
2: slight
3: strong

SurfH
Histological appraisal of surface architecture

1: severe fibrillation or disruption
2: moderate fibrillation or irregularity
3: slight fibrillation or irregularity
4: normal

FilH
Histologic appraisal of defect-filling

1:<25%
2: 26–50%
3: 51–75%
4: 76–90%
5: 91–110%

Latl
Lateral integration of implanted material

1: not bonded
2: bonded at one end/partially at both ends
3: bonded at both sides

Basl
Basal integration of implanted material

1: <50%
2: 50–70%
3: 71–90%
4: 91–100%

InfH
Inflammation

1: no inflammation
3: slight inflammation
5: strong inflammation

Hgtot
Histological grading system
Some of the histological variables:

- tissue morphology(Ti)
- matrix staining (Matx)
- structural integrity (Stru)
- cluster formation (Clus)
- tidemark opening (Tide)
- bone formation (Bform)
- histological surface architecture(SurfH)
- histological degree of defect filling (FilH)
- lateral integration of defect-filling tissue (Latl)
- basal integration of defect-filling tissue (Basl)
- histological signs of inflammation (InfH)

Maximum total: 45 points

[a] The ICRS system evaluates repair tissue based on 11 histological variables: tissue morphology (Ti), matrix staining (Matx), structural integrity (Stru), cluster formation (Clus), tidemark opening (Tide), bone formation (Bform), histological appraisal of surface architecture (SurfH), histological appraisal of the degree of defect filling (FilH), lateral integration of defect-filling tissue (Latl), basal integration of defect-filling tissue (Basl), and histological signs of inflammation (InfH). The grades for each variable were then combined to yield an overall histologic grading value (Hgtot). The total scores range from 11 to 45.

water (pH 7.4) containing 10% ethylenediaminetetraacetic acid (EDTA), the tissue was embedded in paraffin wax and sectioned perpendicularly through the center of the defect. Each section was stained with Safranin O for glycosaminoglycans for histological evaluation.

The International Cartilage Research Society (ICRS) histological grading system for evaluating tissue repair was used to assess the defect sites. This newly revised system [26–28] (Table 1), developed by O'Driscoll, Keeley and Salter, includes 11 histological variables: morphology (Ti), matrix staining (Matx), structural integrity (Stru), cluster formation (Clus), tidemark opening (Tide), bone formation (Bform), histological appraisal of surface architecture (SurfH), histological appraisal of the degree of defect filling (FilH), lateral integration of defect-filling tissue (Latl), basal integration of defect-filling tissue (Basl), and histological signs of inflammation (InfH). The assigned scores for each of these 11 histological variables range from 11 to 45 points. In our experiments, the variables were scored by two individuals.

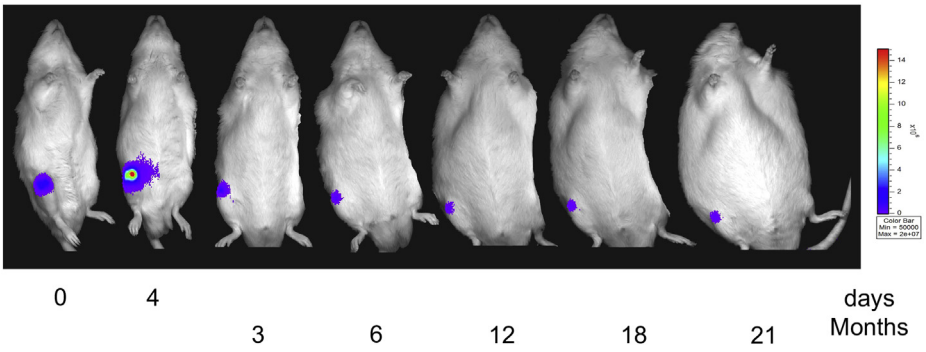

Fig. 2. CCD images from the long-term observation of luciferase activity from the right knee joint. This representative image shows the CCD image of one rat from the AC-SY group (transplanted with chondrocyte and synovial cell sheets) at the following time points (from left to right): after transplantation on day 0 and day 4, and at 3, 6, 12, 18, and 21 months. The color bar indicates the bioluminescence intensity in photons per seconds cm^2 per steradian.

2.9. Statistical analyses

The Kruskal–Wallis and Mann–Whitney *U* tests were used for the statistical analyses. *P*-values <0.05 were considered significant.

3. Results

3.1. Bioluminescence imaging

Photon emission from the right knee joints of the rats in the long-term observation group was visible by BLI for more than 21 months after graft transplantation (Fig. 2). The BLI signal was not detected anywhere other than the right knee, to which the bio-engineered chondrocyte sheets had been transplanted.

We confirmed the above observations in all but one rat in the synovial group that died 8 days after transplantation. Moreover, we also did not observe any apparent motor dysfunctions in rats in any of the groups.

3.2. Changes in luminescence intensity over time

The intensity of light emission of each group over a period of 21 months, with day 0 set as 100%, is shown in Fig. 3. The luminescence intensity of all groups peaked 3–4 days after transplantation, at which point the intensity of the AC-AC, AC-SY, and SY-SY groups was respectively 16, 5, and 7 times that on the transplantation date. However, in all groups, the intensity decreased gradually over 3–4 weeks, after which it stabilized at approximately 1/10 of the intensity on the transplantation date (Fig. 3). In other words, the intensity decreased in the three groups to values that were 1/160, 1/50, and 1/70 of the peak intensity,

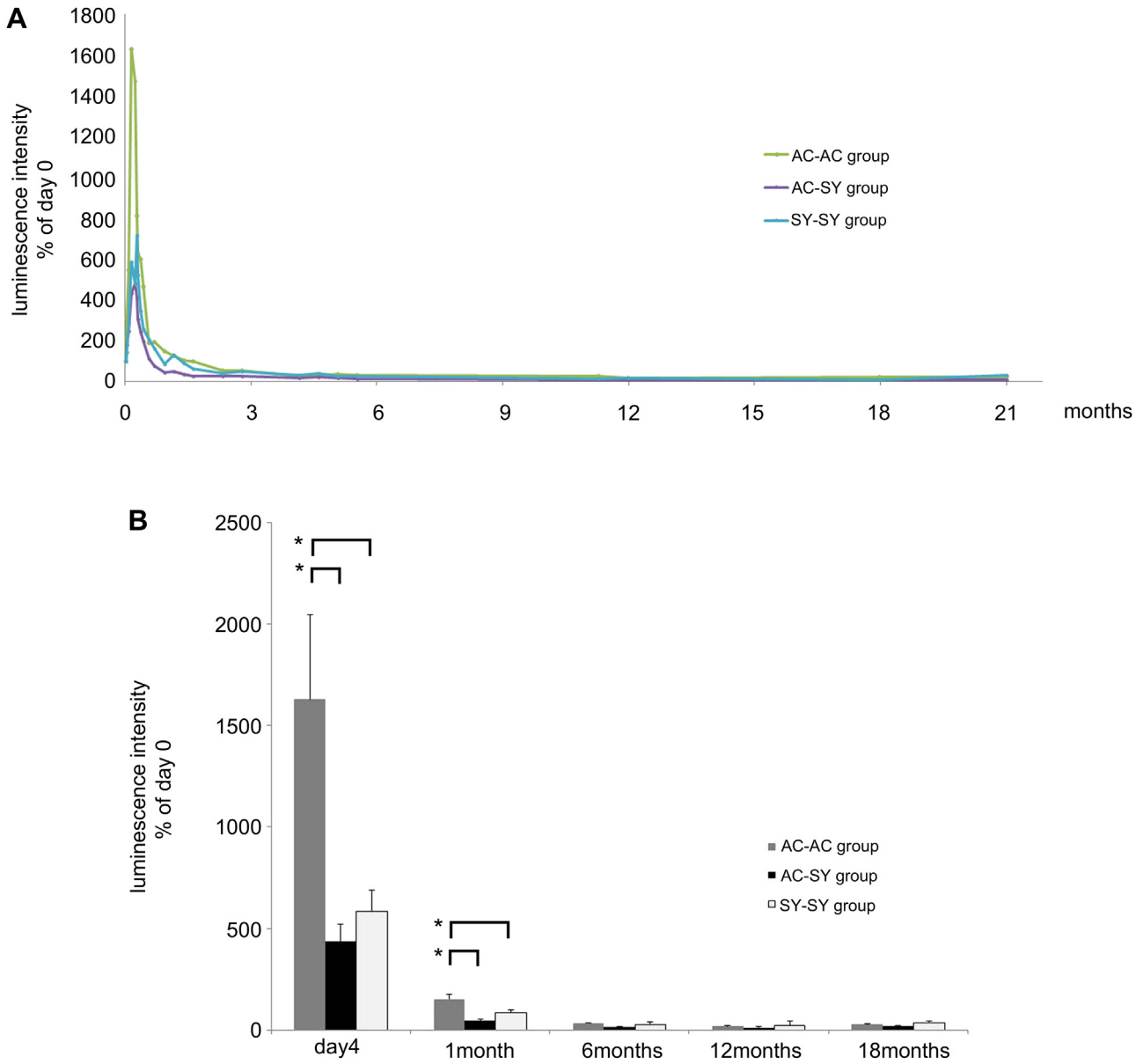

Fig. 3. Sequential quantification of luminescence intensity. Changes in the intensity of the light emission of the transplanted cells were monitored over 21 months, with the intensity on the day of transplantation being set to 100% (A). Bar graph showing the percent change in luminescence intensity in the three groups at several points after transplantation (B). Significant differences were observed between the AC-AC and AC-SY groups and the AC-AC and SY-SY groups, at 4 days and 1 month after transplantation, respectively ($p < 0.05$, *). Error bars represent standard errors. AC-AC group, transplanted with two chondrocyte sheets; SY-SY group, transplanted with two synovial cell sheets; and AC-SY group, transplanted with chondrocyte and synovial cell sheets.

respectively. However, the luminescence continued to persist at this lower value without disappearing for more than 21 months (Fig. 3A). The differences in luminescence intensity between the three groups were significant at 4 days and 1 month after transplantation. However, significant differences were not observed in the long term (Fig. 3B).

3.3. Histological evaluation and scoring for the repair of osteochondral defects

Changes in the histological features of the region encompassing the osteochondral defects were evaluated 2, 4, 6, and 8 weeks after transplantation (Fig. 4A). The ICRS grading system scores for

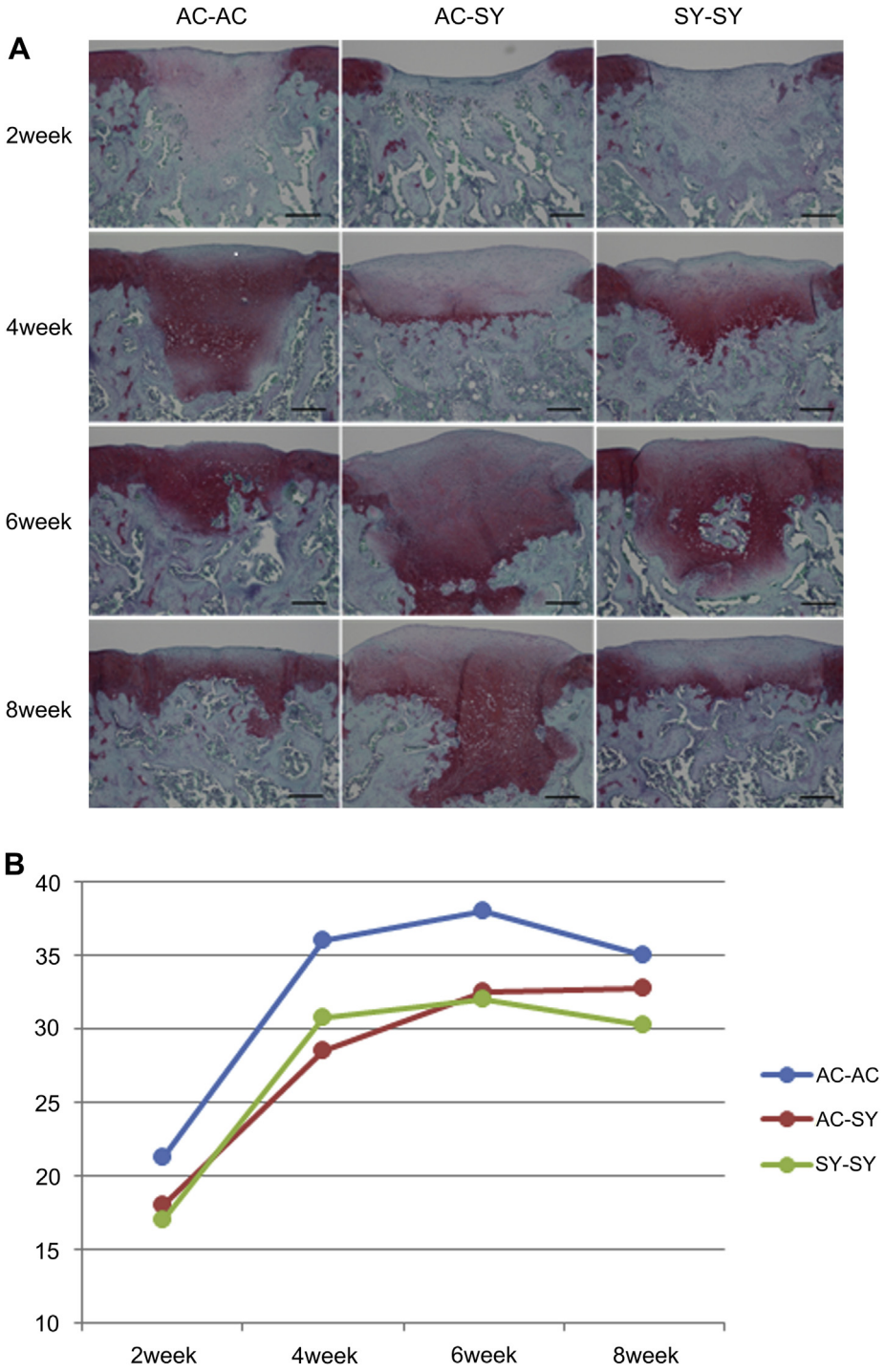

Fig. 4. Histological changes in the repair tissue 4–8 weeks after transplantation. Tissue findings (A) are stained with Safranin O. The scale bar represents 500 μm. Changes in the ICRS grading score after transplantation over time are plotted on a line chart (B). The data shown are the average ICRS grading scores for tissue repair in two rats. AC-AC group, transplanted with two chondrocyte sheets; SY-SY group, transplanted with two synovial cell sheets; and AC-SY group, transplanted with chondrocyte and synovial cell sheets ($n = 2$, per group).

regeneration of osteochondral defects at 2, 4, 6, and 8 weeks after transplantation are shown in Fig. 4B. During this period of observation, the score of each group was the highest at 6 weeks. At 8 weeks, we observed a decrease in the scores of the AC-AC and SY-SY groups. The scores for the AC-SY group remained unchanged from week 6 onward.

4. Discussion

In this study, we successfully monitored the long-term survival of bioengineered chondrocyte and synovial cell sheets by cell tracking using BLI. This method is a noninvasive, longitudinal approach for *in vivo* monitoring of transplanted cells without the need to euthanize recipient animals. Our data show that the transplanted cell sheets can survive in the knee joint for more than 21 months. Furthermore, we confirmed that the cells in the allografted sheets remained at the transplantation site and did not migrate from the knee joint to any other location.

The chondrocyte sheet is an essential initiator, providing the signals and humoral factors for cartilage formation, and tissue repair and regeneration. The initial, effective repair by host cells can be achieved after transplantation of this sheet [7–9]. We thought that transplanted chondrocyte sheets would disappear from the site of the cartilaginous defects in the knee joints within 3–4 weeks. However, our results indicate that it is possible that the defect site may function as an environmental niche appropriate for the bioengineered chondrocyte sheets, and that the surviving grafted cells acquire a dedifferentiated phenotype. The grafted stem cells may thus contribute to cartilage tissue regeneration. To confirm and extend this observation, further studies are required in other models of cartilage repair and regeneration.

We evaluated the survival duration of cell sheets composed of a combination of chondrocytes and synovial cells. In previous experiments, we demonstrated that, compared with cell sheets composed of only chondrocytes, the combination sheets were more effective in promoting the regeneration of subchondral bone and the formation of chondrocyte postregeneration [16]. Therefore, we expected that the combination of the two types of cells would result in a longer survival duration than the use of either cell type alone. However, in rats transplanted with any of the three types of cell sheets, the transplanted cells survived for more than 21 months. Therefore, it is not clear what effect, if any, using a cell sheet made of a combination of the two cell types has on survival duration.

We observed a peak in luminescence intensity in all three groups 3–4 days after transplantation. Thereafter, the intensity of the AC-AC, AC-SY, and SY-SY groups decreased to values that were 1/160, 1/50, and 1/70 of the peak intensity, respectively. One explanation for these effects is that immediately following transplantation, the bioactivity of the transplanted cells was low due to the stress associated with transplantation. In addition, the amount of luciferin that could be delivered to the transplanted cells was also low due to potent edema in the transplanted region. With the gradual recovery of bioactivity and hemodynamic stability, the intensity of the light emission increased. However, as time passed, many transplanted cells died, resulting in the detection of luminescence from only the small fraction of transplanted cells that survived. When the same amount of luciferin was added *in vitro* to a luciferase-injected cell population, the luminescence intensity was nearly directly proportional to the number of cells. Therefore, the number of cells can be measured semiquantitatively. Although a strict comparison *in vivo* is difficult, we note that, in terms of measuring cell number from luminescence intensity, transplanted cells begin to die from the moment of transplantation, so by the time of peak intensity, the number of transplanted cells has decreased. As time passes, we infer that the number of cells decreases further, meaning that the detected luminescence is being emitted from surviving cells, which are much fewer in number than those present on the transplantation date. The detection of these remaining cells and their cell forms is a topic for future study.

Our ultimate goal is to develop cell sheets that, when transplanted, do not result in the conversion of hyaline cartilage within the regenerated cartilage tissue into fibrous tissue. In previous studies using Japanese white rabbits and pigs, we reported that using a combination of chondrocytes and synovial cells for cartilage regeneration prevented the conversion from hyaline cartilage into fibrous tissue [16,29].

Cell sheets offer a distinct advantage in the therapy of knee cartilage compared with cartilage chips and other methods that utilize a hard cartilage graft. The use of such a graft requires cumbersome techniques in that the graft is sutured or fixed via filler to the area around the damaged cartilage tissue. In addition, these techniques often give rise to graft attachment problems such as the generation of intra-articular loose bodies; by contrast, cell sheets tend to have fewer occurrences of these problems.

However, currently it is difficult to generate layered cell sheets comprised of human cartilage tissue because of the differences in the proliferative capability of human chondrocytes among individuals. In particular, human chondrocytes that are commercially available have already dedifferentiated to such an extent that their proliferative capability is low. The generation of cell sheets from such chondrocytes is oftentimes unsuccessful.

To facilitate the widespread use of human chondrocyte sheets generated through the co-culture method, and for augmenting prompt clinical use of these sheets, it is necessary to consider the safety and functionality of multilayered chondrocyte sheets. Further study in animal models and subsequent clinical trials are needed to clarify the utility and safety of chondrocyte sheets. At the same time, it is also necessary to evaluate the optimal source for fresh human chondrocytes, and to establish an effective means of storing chondrocyte sheets for allografting.

We believe that the development of regenerative therapy of osteoarthritis using cell sheets will provide a ready-made therapeutic strategy that, compared with conventional therapeutic strategies, is not substantially invasive, has high effectiveness, and can be used widely.

5. Conclusions

We used the BLI method to demonstrate that allografted cell sheets in knee joints survive for unexpectedly long periods of time. Both chondrocytes and synovial cells survived for an extended period of time at the transplanted site, thereby confirming the safety and utility of cell sheets in promoting cartilage regeneration.

Acknowledgments

This work was supported by a Health and Labor Sciences Research Grant 'Research on Regenerative Medicine for Clinical Application' from the Japanese Ministry of Health, Labour and Welfare (MHLW). We are grateful for their support. We thank Takumi Teratani, Ph.D. (Division of Development of Advanced Treatment; Center for Development of Advanced Medical Technology; Jichi Medical University) for his useful comment.

References

[1] Solchaga LA, Yoo JU, Lundberg M, Dennis JE, Huibregtse BA, Goldberg VM, et al. Hyaluronan-based polymers in the treatment of osteochondral defects. J Orthop Res 2000;18:773–80.

[2] Ochi M, Uchio Y, Kawasaki K, Wakitani S, Iwasa J. Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee. J Bone Joint Surg Br 2002;84:571–8.
[3] Hunziker EB, Rosenberg LC. Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane. J Bone Joint Surg Am 1996;78:721–33.
[4] Mainil-Varlet P, Rieser F, Grogan S, Mueller W, Saager C, Jakob RP. Articular cartilage repair using a tissue-engineered cartilage-like implant: an animal study. Osteoarthritis Cartilage 2001;9(Suppl. A):S6–15.
[5] Nagai T, Furukawa KS, Sato M, Ushida T, Mochida J. Characteristics of a scaffold-free articular chondrocyte plate grown in rotational culture. Tissue Eng Part A 2008;14:1183–93.
[6] Ando W, Tateishi K, Hart DA, Katakai D, Tanaka Y, Nakata K, et al. Cartilage repair using an in vitro generated scaffold-free tissue-engineered construct derived from porcine synovial mesenchymal stem cells. Biomaterials 2007;28:5462–70.
[7] Masuoka K, Asazuma T, Ishihara M, Sato M, Hattori H, Yoshihara Y, et al. Tissue engineering of articular cartilage using an allograft of cultured chondrocytes in a membrane-sealed atelocollagen honeycomb-shaped scaffold (ACHMS scaffold). J Biomed Mater Res B Appl Biomater 2005;75:177–84.
[8] Nagai T, Sato M, Furukawa KS, Kutsuna T, Ohta N, Ushida T, et al. Optimization of allograft implantation using scaffold-free chondrocyte plates. Tissue Eng Part A 2008;14:1225–35.
[9] Sato M, Ishihara M, Furukawa K, Kaneshiro N, Nagai T, Mitani G, et al. Recent technological advancements related to articular cartilage regeneration. Med Biol Eng Comput 2008;46:735–43.
[10] Okano T, Yamada N, Okuhara M, Sakai H, Sakurai Y. Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces. Biomaterials 1995;16:297–303.
[11] Okano T, Yamada N, Sakai H, Sakurai Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide). J Biomed Mater Res 1993;27:1243–51.
[12] Kaneshiro N, Sato M, Ishihara M, Mitani G, Sakai H, Mochida J. Bioengineered chondrocyte sheets may be potentially useful for the treatment of partial thickness defects of articular cartilage. Biochem Biophys Res Commun 2006;349:723–31.
[13] Mitani G, Sato M, Lee JI, Kaneshiro N, Ishihara M, Ota N, et al. The properties of bioengineered chondrocyte sheets for cartilage regeneration. BMC Biotechnol 2009;9:17.
[14] Hamahashi K, Sato M, Yamato M, Kokubo M, Mitani G, Ito S, et al. Studies of the humoral factors produced by layered chondrocyte sheets. J Tissue Eng Regen Med. http://dx.doi.org/10.1002/term.1610. Published online in Wiley Online Library, wileyonlinelibrary.com; 2012.
[15] Kokubo M, Sato M, Yamato M, Mitani G, Kutsuna T, Ebihara G, et al. Characterization of chondrocyte sheets prepared using a co-culture method with temperature-responsive culture inserts. J Tissue Eng Regen Med. http://dx.doi.org/10.1002/term.1764. Published online in Wiley Online Library, wileyonlinelibrary.com; 2013.
[16] Ito S, Sato M, Yamato M, Mitani G, Kutsuna T, Nagai T, et al. Repair of articular cartilage defect with layered chondrocyte sheets and cultured synovial cells. Biomaterials 2012;33:5278–86.
[17] Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. FEBS Lett 1997;407:313–9.
[18] Inoue H, Ohsawa I, Murakami T, Kimura A, Hakamata Y, Sato Y, et al. Development of new inbred transgenic strains of rats with LacZ or GFP. Biochem Biophys Res Commun 2005;329:288–95.
[19] Kurome M, Ueda H, Tomii R, Naruse K, Nagashima H. Production of transgenic-clone pigs by the combination of ICSI-mediated gene transfer with somatic cell nuclear transfer. Transgenic Res 2006;15:229–40.
[20] Ono K, Takii T, Onozaki K, Ikawa M, Okabe M, Sawada M. Migration of exogenous immature hematopoietic cells into adult mouse brain parenchyma under GFP-expressing bone marrow chimera. Biochem Biophys Res Commun 1999;262:610–4.
[21] Horie M, Sekiya I, Muneta T, Ichinose S, Matsumoto K, Saito H, et al. Intra-articular injected synovial stem cells differentiate into meniscal cells directly and promote meniscal regeneration without mobilization to distant organs in rat massive meniscal defect. Stem Cells 2009;27:878–87.
[22] Hakamata Y, Tahara K, Uchida H, Sakuma Y, Nakamura M, Kume A, et al. Green fluorescent protein-transgenic rat: a tool for organ transplantation research. Biochem Biophys Res Commun 2001;286:779–85.
[23] Sekine H, Shimizu T, Dobashi I, Matsuura K, Hagiwara N, Takahashi M, et al. Cardiac cell sheet transplantation improves damaged heart function via superior cell survival in comparison with dissociated cell injection. Tissue Eng Part A 2011;17:2973–80.
[24] Sekine H, Shimizu T, Sakaguchi K, Dobashi I, Wada M, Yamato M, et al. In vitro fabrication of functional three-dimensional tissues with perfusable blood vessels. Nat Commun 2013;4:1399.
[25] Hakamata Y, Murakami T, Kobayashi E. "Firefly rats" as an organ/cellular source for long-term in vivo bioluminescent imaging. Transplantation 2006;81:1179–84.
[26] Brehm W, Aklin B, Yamashita T, Rieser F, Trüb T, Jakob RP, et al. Repair of superficial osteochondral defects with an autologous scaffold-free cartilage construct in a caprine model: implantation method and short-term results. Osteoarthritis Cartilage 2006;14:1214–26.
[27] Mainil-Varlet P, Aigner T, Brittberg M, Bullough P, Hollander A, Hunziker E, et al. Histological assessment of cartilage repair: a report by the Histology Endpoint Committee of the International Cartilage Repair Society (ICRS). J Bone Joint Surg Am 2003;85-A(Suppl. 2):45–57.
[28] O'Driscoll SW, Keeley FW, Salter RB. Durability of regenerated articular cartilage produced by free autogenous periosteal grafts in major full-thickness defects in joint surfaces under the influence of continuous passive motion. A follow-up report at one year. J Bone Joint Surg Am 1988;70:595–606.
[29] Ebihara G, Sato M, Yamato M, Mitani G, Kutsuna T, Nagai T, et al. Cartilage repair in transplanted scaffold-free chondrocyte sheets using a minipig model. Biomaterials 2012;33:3846–51.