Tracking the Seed Cells in Cell-based Intra-Articular Injection Therapies of Articular Cartilage Defect

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Abstract

Articular cartilage is hyaline cartilage. The physiological function of articular cartilage is to reduce friction of bones and provide lubrication to the joint. When injured, articular cartilage can be repaired autonomously, but resulted in fibrous-cartilage-like filling tissue which is inferior to original cartilage in both biological and mechanical properties. In order to repair, regenerate and restore the function of tissue, tissue engineering employs various types of seeding cells and scaffold materials of biological or non-biological sources and constructs specific cell growing system. Stem cells are capable of differentiating into various types of cells. Both animal and clinical trial proved the feasibility of employing mesenchymal stem cells to therapeutic treatment of cartilage defect. However, the mechanism underlying the cell-based therapy is not clearly elucidated. We intra-articularly inject seeding cell-scaffold complex and collect cells at different time point post injection. Less than 1% of injected cells survive within 6 hours post injection, and no cell is detected in regenerated tissue by 7 days post injection. Tracking the cells could provide insight into potential mechanism of articular cartilage regeneration.

Keywords: regenerative medicine, cartilage regeneration, stem cell-based therapy, intra-articular injection, cell tracking

Introduction

Articular cartilage is hyaline-like cartilage, composed by abundant water, extracellular matrix (mainly Type II collagen and proteoglycans), and sparsely distributed chondrocytes. There is pressure that is about 3.5 times of human body weight applied to knee joint when standing, and multiple forms of force such as squeezing, stretching, friction and cutting force are exerted to articular cartilage when walking and doing sports. The physiological function of articular cartilage is to reduce friction of bones and provide lubrication to the joint. There is no blood vessel, lymphatic system and nerve in articular cartilage. Substance exchange is performed by osmosis(Smith, 2002). When damaged, articular cartilage can be repaired by the body itself. However, this results in formation of a fibrocartilage-like filling at the lesion site, which is far inferior to the hyaline cartilage in biomechanical properties, and cell death, degradation of extracellular matrix, and tissue degeneration occur within a year(Glyn-Jones et al., 2015). Because of its simple structure and cell type composition, and its absence of blood vessels, lymph, and innervation, cartilage has long been considered the most promising part of the body to be repaired or reconstructed by tissue engineering. The current repair methods include microfracture, autologous chondrocyte transplantation, growth factor injection, and predifferentiated stem cell transplantation(Kreuz et al., 2006; Peterson et al., 2010; Behery et al., 2013).

Stem cells have pluripotency for differentiation, and thus mesoderm-derived mesenchymal stem cells are receiving attention. Mesenchymal stem cells have the potential for chondrogenic differentiation, are capable of secreting multiple growth factors, and are characterized by anti-inflammation and immunosuppression (Caplan and Dennis, 2006). Potential candidates include bone marrow stem cells, adipose derived mesenchymal stem cells, umbilical cord blood stem cells, and synovial stem cells. Animal experiments and clinical trial reports have confirmed the feasibility of mesenchymal stem cells in the treatment of cartilage defects. Intra-articular injection of adipose-derived mesenchymal stem cells can increase the thickness of cartilage in the knee and promote cartilage regeneration in clinical trials(Jo et al., 2017). In a mouse model of a cartilage defect, injection of articular synovial stem cells into the joint cavity

can promote cartilage repair, and the proteoglycan content in the regenerated tissue is similar to that of articular cartilage(Mak et al., 2016).

However, the mechanism by which mesenchymal stem cells promote repair is not yet clear. Since the injection or filling of non-differentiated stem cells can promote cartilage repair, there are two main points of view. One is that stem cells are recruited to the defect site in vivo, proliferate and differentiate into chondrocyte; another is that mesenchymal stem cells secrete growth factors and cytokines and recruit stem cells reside in the joint cavity or in the bone marrow to repair cartilage defects. To elucidate the manner in which stem cells affect cartilage regeneration, it is necessary to study the fate of filled or injected stem cells. By sectioning and staining of articular cartilage after transplantation of xenogeneic mesenchymal stem cells, it is found that xenograft cells gradually decreased with time, and after 8 weeks post-surgery no cell could be observed; but the existence of a strong inflammatory response is controversial(Yan and Yu, 2007). Tatebe et al. reported that by staining the same kind of stem cells before injection, cells with fluorescent pigment markers were distributed throughout the new cartilage region 8 weeks post-surgery, which coincided with the region of type II collagen distribution, suggesting that the transplanted stem cells differentiate into chondrocytes(Tatebe et al., 2005). Knee anterolateral cruciate ligament resection is another common osteoarthritis model. SPIO-labeled adipose-derived stem cells are injected 6 weeks after excision of the sheep's anterior cruciate ligament and the distribution of cells in the joint cavity is tracked. The injected cells can stay in the knee for up to 14 weeks(Feng et al., 2018). In the sheep cartilage defect model, bone marrow stem cells and adipose tissue-derived stem cells stained with fluorescent dye PKH26 were injected through the joints and tracked by articular cartilage sectioning and staining. There is fluorescent signal in the regenerated cartilage slice(Ude et al., 2012). However, as the dye can be metabolized, the possibility of entering other cells could not be ruled out. Therefore, the use of reliable viable cell labeling methods to track the survival and migration of cells is crucial for the assessment of the reliability of stem cell therapies, especially cell injection therapy.

To track exogenous cells in living animals, the cells must first be labeled. The

existing methods are mainly divided into two categories, the use of dye in vitro staining, and the use of reporter genes to label cells. Commonly used cell dyes include superparamagnetic iron oxide (SPIO), non-radioactive and radioactive isotope dyes. The detection of the presence and distribution of cells in tissue can be performed by tissue section staining or flow cytometry, with high resolution and quantification, but animals need to be sacrificed and the dynamics of cells in individual animals cannot be continuously tracked. Using live imaging methods can continuously track the number and differentiation of cells. The use of dyes or probes for in vitro labeling has the potential for dyes to enter the tissues from the cells due to metabolism and diffusion, resulting in reduced signal or false positives (dye is absorbed by other cells). Krawetz et al. screened synovial fluid stem cell subtypes distributed around cartilage defect with strong chondrogenic potential, and injected the specific cell types with stably expressed GFP into the knee joints of the cartilage defect mice model. Synovial fluid and defects were analyzed, and the results showed that depending on the strain of the stem cells, the fluorescence cells migrated to the cartilage defect within 2 to 4 weeks, but the fluorescence signal disappeared after 4 weeks. This confirms that some cells have migrated to the defect, but the fate of the cells after migration remains unclear(Mak et al., 2016).

We injected hyaluronic acid mixed with stem cells into the joint cavity of the cartilage defect rabbit pathological model. Through sampling at different time points, we investigated the location of injected cells at the early stage of cartilage repair, aiming to explore the possible repair mechanism of mesenchymal stem cells to promote cartilage defects repair.

Results

Lentiviruses are packaged in the 293T cell line. Strong red fluorescence could be observed 48 hours after transfection in the cells (Figure 1A) and the cells began to float. Lentiviral particles were obtained by direct collection of supernatants. In the subsequent transfection experiments, transfection efficiency is relatively high, so no enrichment of the lentivirus solution is required in this experiment.

The vector plasmid pT375-mCherry-puro^{r} containing the red fluorescent protein

gene bears a puromycin resistance gene, so that the screening of transfected cells can be performed using both flow cytometric sorting and drug screening. Lentivirus transfection efficiency can be determined by flow sorting. The transfection efficiency of adipose-derived stem cells (ADSCs) transfected with PR8.74 and VSVG packaging systems is about 58.2% (Figure 1B, 1C). Among the two screening methods, cells obtained using drug screening has stronger fluorescence (Figure 1D) and possibly due to the high concentration of puromycin used in the experiment (1.0 μg/mL). Besides, rabbit bone marrow mesenchymal stem cells (BMSCs) and adipose-derived mesenchymal stem cells stably expressing GFP were extracted from GFP transgenic New Zealand white rabbits, and Rabbit chondrocytes stably expressing GFP are kindly provided by Dr. LI Junxiang.

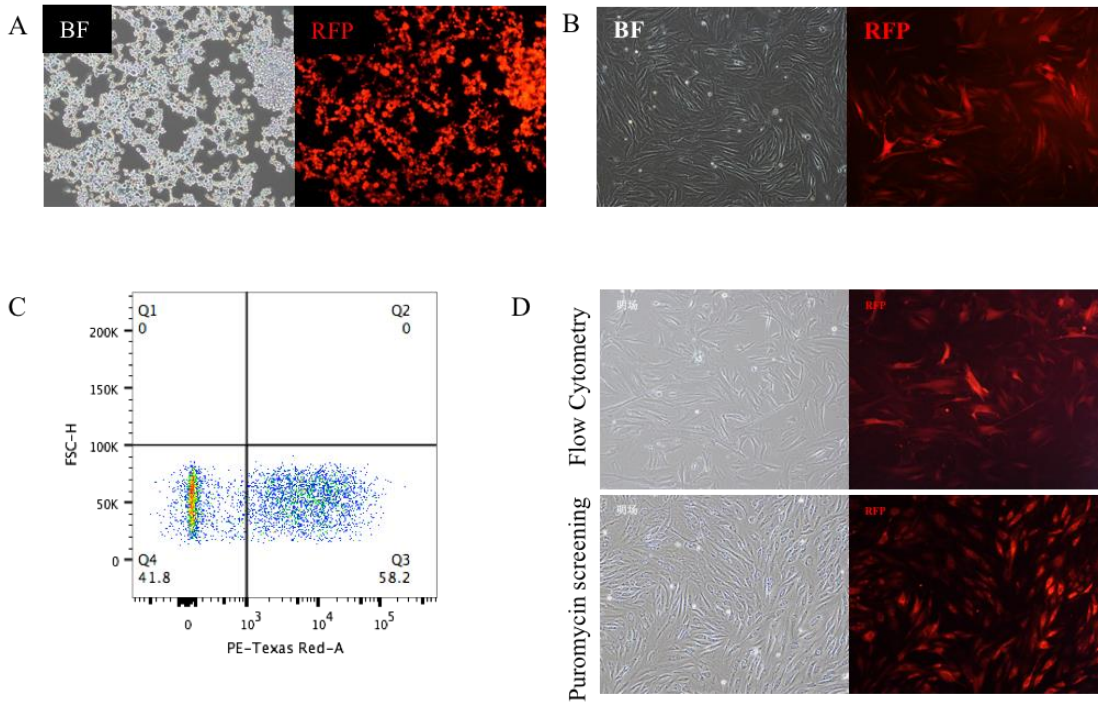

Figure 1 Lentivirus package and transfection. A, lentivirus packaging was performed in the 293T cell line. Left, bright field; right, excited by green light, under 10× field of vision. B, adipose stem cells transfected with lentivirus harboring pT375-mCherry-puro^r, under 10× field of vision. C, flow cytometry analysis of transfected cells. Transfection efficiency is approximate to 58%. D, screening for stably transfected cells, both flow cytometric sorting and drug screening are employed in the study. Upper panel, flow cytometry sorting. Lower panel, puromycin (1.0 μg/mL) screening.

In this study, a common method of generating acute cartilage injury is used to create a defect with a diameter of about 4 mm in the cartilage of the femoral condyle in rabbits. It is generally believed that defects with a diameter of more than 4 mm cannot

be repaired by the body. The incision of the joint capsule heals 7 days after surgery, and there may be a strong inflammatory reaction, possibly due to the surgical procedure. 14 days after the surgery, the wounds on the rabbit skin and joint capsules all heal well. There is no severe inflammatory reaction if no severe infection occurred. Regenerated tissue is observed at 7 days after the operation. The regenerated tissue is white in color, opaque, soft in texture, which is distinctly different from the surrounding cartilage tissue (Figure 2). Regenerated tissue is not tightly integrated to the surrounding cartilage, so it can be easily removed with surgical instruments. Stem cell are injected 7 days after surgery. The volume of regenerated tissue at the site of defect increases, and the tissue is harder than 7 days before. In the experimental group injected with hyaluronic acid and stem cells, the surface of the repaired tissue is smoother than that of the experimental group that injected only cells.

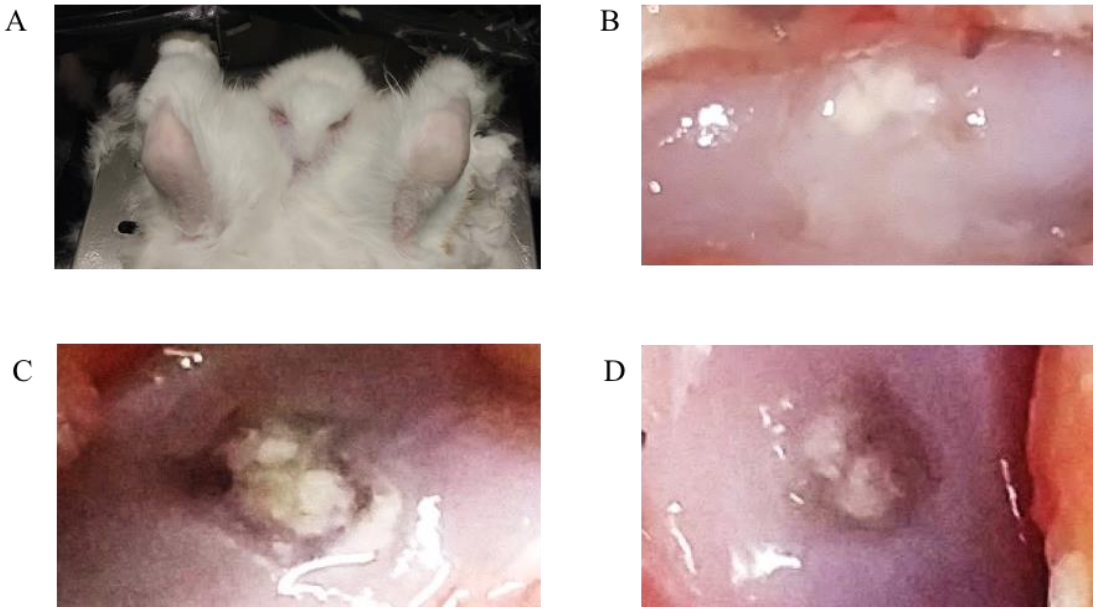

Figure 2 New Zealand white rabbits weighing about 3 kg are used as model animal and a hole of about 4 mm in diameter is drilled on the cartilage of the knee joint as a model of acute articular cartilage injury. A, the appearance of knee joints at 7 days after surgery, the wounds recover well, and there is no suppuration. B, there is a small amount of regenerative tissue in the cartilage defect, the color is white and opaque, and the texture is soft. C, stem cell injections are performed 7 days after surgery. The regenerated tissue at the site of defect increases 7 days after the injection, and the texture is harder than 7 days before. D, in the group injected with hyaluronic acid and stem cells, the surface of the regenerated tissue is smoother than that of the group that injected with only cells.

Before fluorescent proteins are widely used in biological research, fluorescent dyes are widely used to label living cells for observation of their dynamics. Fluorescent dyes

can bind to specific intracellular components. To test the reliability of the fluorescent protein label used in the study, an experimental group labeled with the fluorescent dye CFSE is set (Table 1). Cells were injected into the joint cavity 7 days after the operation. Approximately 60,000 cells injected into each joint cavity. In the experiment group injected with GFP-chondrocytes, cultured GFP-chondrocytes is used as a positive control, and cells with red fluorescence are sorted by flow cytometry. No cell is collected from neither the experimental group injected with PBS and GFP-chondrocytes or that injected with hyaluronic acid and GFP-chondrocytes (Figure 3A). In the experiment group injected with RFP-ADSC, cultured RFP-ADSC is used as a positive control, and cells with red fluorescence are sorted by flow cytometry. No cell is collected from neither the experimental group injected with PBS and adipose stem cells nor that injected with hyaluronic acid and adipose stem cells (Figure 3B), suggesting that the synovial fluid contained almost no injected cells 7 days after the injection.

In experimental groups injected with CFSE-stained cells, cultured CFSE-stained adipose stem cells and chondrocytes are used as positive controls, and cells with green fluorescence were sorted by flow cytometry (Figure 3C, 3D). 26.4% of injected cells are collected from the group injected with PBS and adipose stem cells, and 5.1% of cells are collected from the group that receives injection of hyaluronic acid and adipose stem cells. 5.1% of injected cells are collected from the group injected with PBS and chondrocytes, and 5% of injected cells are collected from the group injected with hyaluronic acid and chondrocytes. Because the disparity of retrieval rate of two labeling methods is so significant, there is likely to be problems with the reliability of fluorescent dye-labeled cells (Figure 3E). Therefore, in subsequent experiments we use cell lines stably expressing fluorescent protein.

	PBS+chondrocytes	**HA+chondrocytes**	**PBS+ADSCs**	**HA+ADSCs**
CFSE	3 060	3 000	15 180	3 063
GFP/RFP	0	0	1	3

Table 1 Cell recovery at the 7th day after injection. ~60 000 cells are injected into each joint cavity.

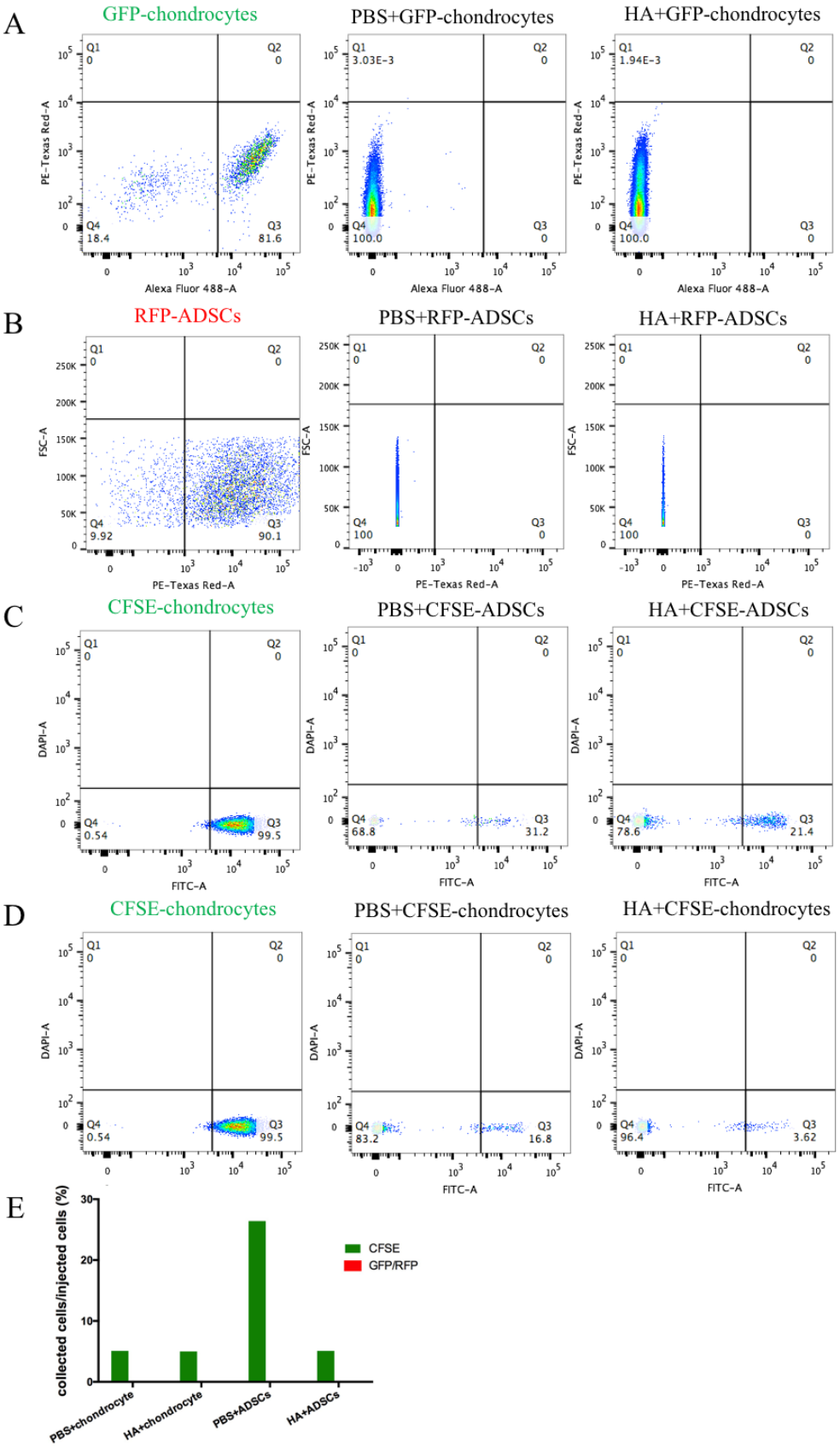

Figure 3 Results of cell recovery from synovial fluid at 7th day after injection. 60 000 cells are injected into each joint cavity. A, intra-articular injection with GFP-chondrocytes. Left panel, GFP-chondrocytes

as positive control. Middle panel, injected with GFP-chondrocytes and PBS. Right panel, injected with GFP-chondrocytes and sodium hyaluronate. No cell is collected. B, intra-articular injection with RFP-ADSCs. Left panel, RFP-ADSCs as positive control. Middle panel, injected with RFP-ADSCs and PBS. Right panel, injected with RFP-ADSCs and sodium hyaluronate. C, intra-articular injection with CFSE-stained ADSCs. Left panel, CFSE-stained chondrocytes as reference for fluorescence intensity. Middle panel, injected with CFSE-stained ADSCs and PBS. Right panel, injected with CFSE-stained ADSCs and sodium hyaluronate. D, intra-articular injection with CFSE-stained chondrocytes. Left panel, CFSE-stained chondrocytes as positive control. Middle panel, injected with CFSE-stained chondrocytes and PBS. Right panel, injected with CFSE-stained chondrocytes and sodium hyaluronate. E, retrieval ratios of two labeling methods are significantly different.

Since the number of cells collected from the synovial fluid on the seventh day after the injection is not sufficient. We shift the sampling time point earlier to 24 hours after the injection in the following experiment. P5 RFP-ADSCs and GFP- BMSCs are used in the experiments. 24 hours after the injection of cells, cultured RFP-ADSC is used as a positive control, and cells with red fluorescence were sorted using flow cytometry (Table 2, Figure 4A, 4B). The cell recovery rates are 0.01% (PBS + cells) and 0.004% (HA + cells). In the experimental group injected with GFP-BMSCs, the cultured BMSC is used as a negative control, and cells with green fluorescence were selected by flow cytometry. No cell is collected from either PBS/cell group or HA/cell group.

Since it is still difficult to collect the injected cells from the synovial fluid 24 hours after the injection, the sampling time is again advanced to 6 hours after the injection. GFP-BMSCs were used in the experiment. Cultured BMSC is used as a negative control, and cells with green fluorescence were sorted by flow cytometry (Table 2, Figure 4C, 4D). The recovery rate is approximately 0% in both group.

Within 6 hours after injection, the proportion of positive cells that could be collected is already very low. In order to rule out the possibility that insufficiency of injected cells causes poor recovery, the number of the cells injected is increased to approximately 800,000 cells per joint. 6 hours after injection, cultured GFP-ADSCs are used as positive control, and the recovered synovial fluid is subjected to flow cytometry sorting. The recovery rate is approximately 0.56% in the group injected with PBS and GFP-ADSCs, and 0.88% for the group injected with DMEM/F12 medium (Figure 5, Table 3). Within 6 hours after injection, the number of seed cells in the synovial fluid drastically decreased.

Time	PBS+RFP-ADSCs	HA+RFP-ADSCs	PBS+GFP-BMSCs	HA+GFP-BMSCs
7d	1	3	-	-
24h	6	3	1	0
6h	-	-	13	0

Table 2 Dynamics of cell count in synovial fluid within 7 days after injection. ~60 000 cells are injected into each joint cavity.

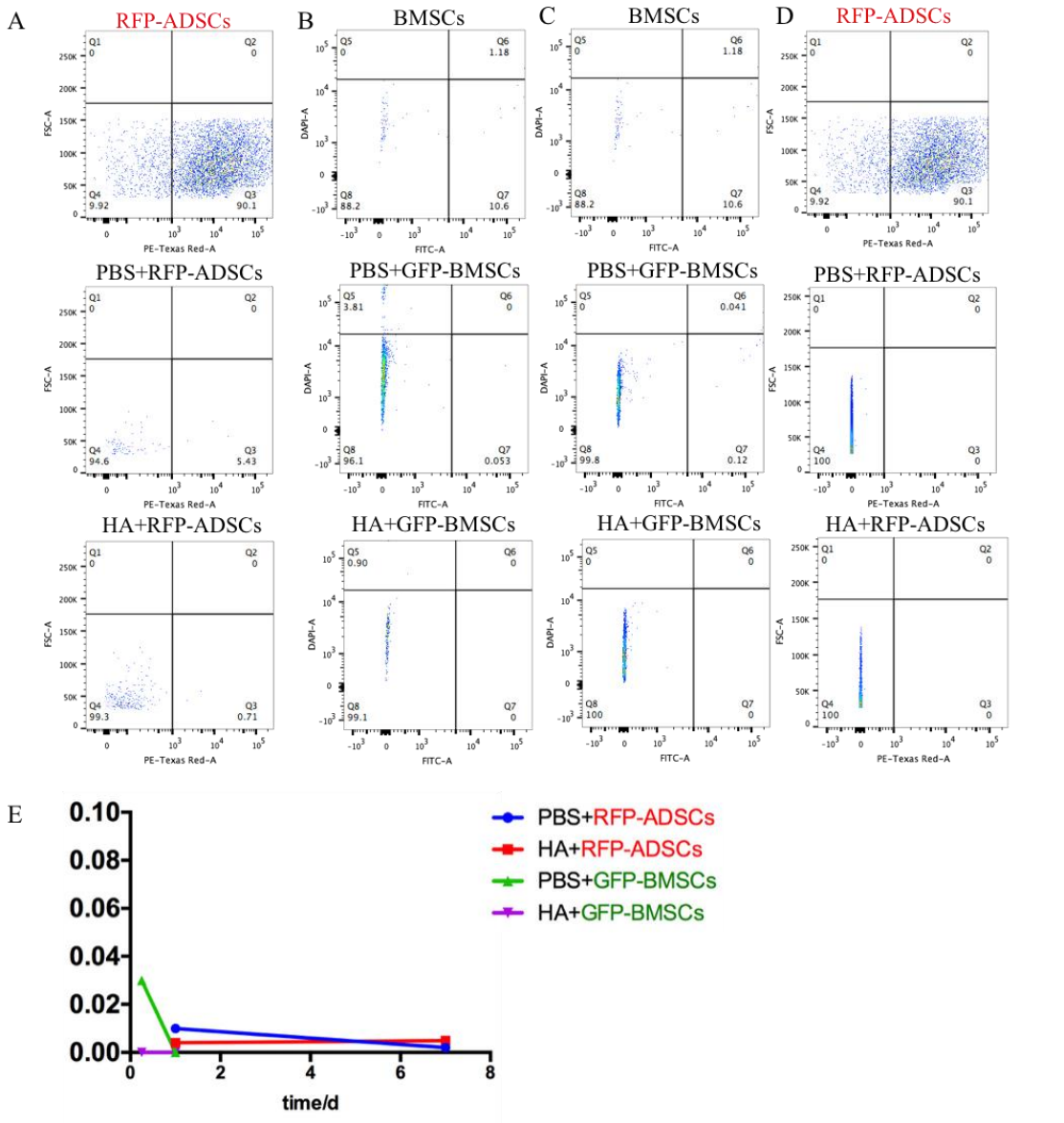

Figure 4 Results of cell recovery from synovial fluid within 24 hours after injection. ~60 000 cells are injected into each joint cavity. A, flow cytometry analysis of collected cells at 24 hours after intra-articular injection with RFP-ADSCs. Upper panel, RFP-ADSCs as positive control. Middle panel, injected with RFP-ADSCs and PBS. Lower panel, injected with RFP-ADSCs and sodium hyaluronate. B, flow cytometry analysis of collected cells at 24 hours after intra-articular injection with GFP-BMSCs. Upper panel, BMSCs as negative control. Middle panel, injected with GFP-BMSCs and PBS. Lower

panel, injected with GFP-BMSCs and sodium hyaluronate. C, flow cytometry analysis of collected cells at 6 hours after intra-articular injection with GFP-BMSCs. Upper panel, BMSCs as negative control. Middle panel, injected with GFP-BMSCs and PBS. Lower panel, injected with GFP-BMSCs and sodium hyaluronate. D, flow cytometry analysis of collected cells at 6 hours after intra-articular injection with RFP-ADSCs. Upper panel, RFP-ADSCs as positive control. Middle panel, injected with RFP-ADSCs and PBS. Lower panel, injected with RFP-ADSCs and sodium hyaluronate. E, dynamics of cell recover in synovial fluid within 7 days after injection.

Time	PBS+ GFP-ADSCs	DMEM/F12+ GFP-ADSCs
6h	4445	7045
Recovery	**0.56 %**	**0.88 %**

Table 3 Recovery of cells from synovial fluid 6 hours after intra-articular injection of GFP-ADSCs. ~800 000 cells are injected.

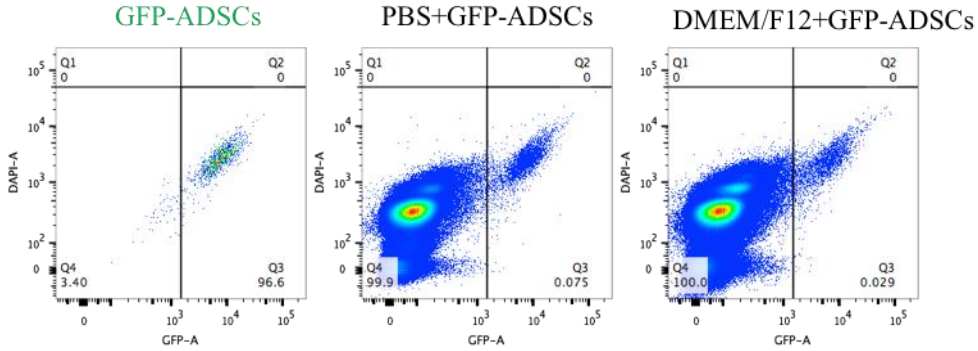

Figure 5 Flow cytometry analysis of cells collected from synovial fluid 6 hours after intra-articular injection of GFP-ADSCs. ~800 000 cells are injected. Left panel, GFP-ADSCs as positive control. Middle panel, injected with GFP-ADSCs and PBS. Right panel, injected with GFP-ADSCs and DMEM/F12 cell culture medium.

To explore whether cells have migrated to other parts of the joint cavity and colonized, especially migrate to the defect of cartilage and differentiate to chondrocytes and directly participate in the construction of regenerated cartilage like those reported previously, we extract the regenerated tissue and digest it, then employ flow cytometry analysis to perform the cell count (Table 4).

For the experimental group injected with RFP-ADSCs, the regenerated tissue is digested 24 hours after the injection and microscopic examination revealed that no cell is observed in the regenerated tissue. 7 days after the injection, the number of cells recovered in the experimental group injected with PBS and adipose stem cells is 1, and the number of cells recovered in the experimental group that injected hyaluronic acid

and adipose stem cells is 3 (Figure 6). It can be considered that almost no injected cells were found in the repaired tissue 7 days after the injection. We lyse cells from regenerated tissue and attempt to extract total RNA. The final RNA concentration is less than 0.1 ng/μL, too low for further reverse transcription and real-time quantitative PCR experiment.

For the experimental group injected with GFP-BMSCs, the regenerated tissue at the defect site is digested 6 hours after the injection, and microscopic examination revealed no cell. Twenty-four hours after the injection, the filling at the defect is digested and microscopically examined. No cell is found. The regenerated tissue at the defect is removed and observed under a fluorescence microscope. Only the autofluorescence of the tissue is observed.

Time	PBS+RFP-ADSCs	HA+RFP-ADSCs	PBS+GFP-BMSCs	HA+GFP-BMSCs
7d	1	3	-	-
24h	0	0	0	0
6h	-	-	0	0

Table 4 Dynamics of cell count in regenerated tissue within 7 days after injection. ~60 000 cells are injected into each joint cavity.

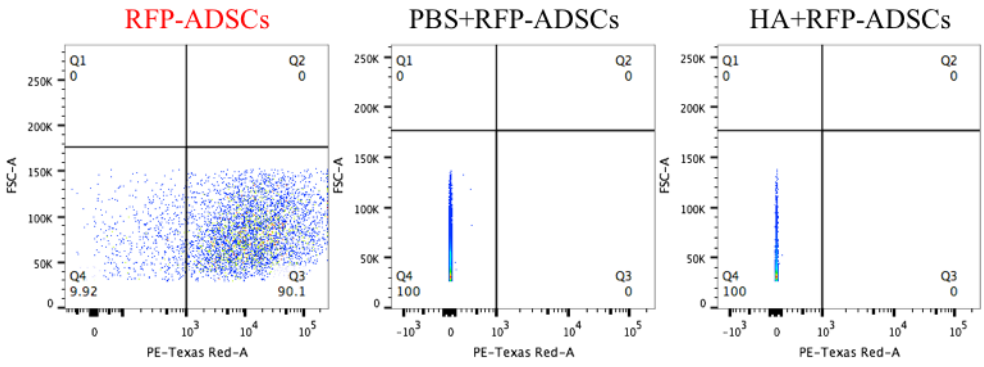

Figure 6 Flow cytometry analysis of cells collected from regenerated tissue at the 7th day after intra-articular injection of GFP-ADSCs. ~60 000 cells are injected. Left panel, RFP-ADSCs as positive control. Middle panel, injected with RFP-ADSCs and PBS. Right panel, injected with RFP-ADSCs and sodium hyaluronate. No positive cell retrieved.

Discussion

In the experiment, due to the insufficiency of retrieved cells, gene expression assays could not be performed. There are several reasons why there is few cells

recovered. First, the survival of the transplanted cells in the body depends on whether or not an effective supply of nutrients and oxygen can be obtained; before the new blood vessels have generated in the regenerated tissues, the injected cells are confronted to problems of nutrition and oxygen deficiency. Mesenchymal stem cells can secrete various growth factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), stem cell growth factor (HGF), transforming growth factor -β (TGF-β), etc. Under hypoxic conditions, the secretion of VFGF, HGF, and bFGF by adipose stem cells increased. VEGF can promote the development of new blood vessels. In mouse ischemic models, transplantation of adipose stem cells can reverse the symptoms of hindlimb ischemia. Therefore, adipose-derived stem cells are considered to be promising solution to the problems of hypoxia and malnutrition in transplantation. At the same time, there are multiple growth factor receptors on the surface of adipose stem cells, such as VEGF receptor, HGF receptor, epidermal growth factor receptor (EGFR) and bFGF receptors, which can be regulated by various signals in the environment. *In vitro* experiments show that VEGF can promote the migration of adipose stem cells and promote the differentiation of cells into chondrocytes; HGF can promote the differentiation of adipose stem cells into hepatocytes; EGF inhibits the differentiation of adipose stem cells into adipocytes; bFGF promotes the proliferation of adipose stem cells, promotes the differentiation to the chondrocytes.

Although studies have shown that adipose-derived stem cells secrete factors that promote angiogenesis and have a high survival rate in ischemic conditions, articular cartilage is a tissue that lacks nerves, lymph and blood vessels, the environment inside the joint cavity is not suitable for the survival of a large number of free cells. This may be one of the reasons why cell recovery efficiency is very low.

It was observed that as the cell being passaged, the morphology of the cells changed from the mesenchyme cell-like shape into slender fibroblast-like cells. The state of the cells changed, which may also affect the survival of the cells in the body. Senescence begins as soon as the cells are taken from the body(Wagner et al., 2008; Barretto et al., 2014). Due to the limited number of primary cells obtained, the mesenchymal stem

cells have to be passaged multiple times to obtain a sufficient number of cells; this results in a continuous decrease in the actual viability of the injected mesenchymal stem cells, which may be more likely to occur after injection.

The adipose stem/adipocyte precursor cell isolated from adipose tissue is not a homogeneous group but a group of mixed cells containing several subpopulations(Zuk et al., 2002). Different subgroups can be distinguished by surface antigens. As the cell being passaged, the expression level of the surface antigen of a single cell subpopulation will change(Mitchell et al., 2006). Therefore, the number of passages may affect the interaction between cells and the environment.

The cartilage defect surgery is invasive and creates an open wound in the skin and joint cavity, and therefore requires suturing. The gut sutures that can be absorbed by body are used during surgery to reduce the number of stitch removal steps and to speed up wound healing. However, wound healing after suturing still takes about a week, during which time the wound will produce different degrees of inflammation. After exposing the interior of the joint cavity and extracting the repair tissue 8 days after surgery, it was found that most knee joints of the rabbit healed well without any suppuration or redness, but some rabbits had moderate to strong inflammatory reactions in the knee joint. The infection is caused by the cartilage defect accidentally injuring the subchondral bone, or the wound healing condition is not good. Inflammation may cause apoptosis of mesenchymal stem cells(Luo et al., 2012). In the process of extracting repair tissue from a very small number of experimental animals, we observe that the wound on the tendon surrounding the joint cavity shows an incomplete healing state before the joint cavity is exposed again; the cause cannot be determined.

The mechanism by which stem cells promote cartilage repair is generally considered to be cell differentiation into various cell types(O'Sullivan et al., 2011), and most of these reports have attempted to demonstrate stem cell differentiation in order to repair chondrocytes in tissues. On the other hand, stem cells may create a protective environment conducive to the repair of tissue formation by secreting some biologically active molecules(Caplan and Dennis, 2006).

Considering the results of our study, the adipose-derived stem cells and bone

marrow stem cells actually stayed in the joint cavity for less than 1 day, and no fluorescent cells were found under the microscope in the repaired tissue. Controversy lies in whether the injected stem cells are directly involved in the construction of regenerated cartilage. Given the very short time it takes for the injected cells to stay in the joint cavity and the lack of convincing literature, the “differentiation hypothesis” should be well assessed further.

In addition to cell differentiation, another widely supported hypothesis is the paracrine hypothesis. Mesenchymal stem cells secrete a number of growth factors and anti-inflammatory factors, such as interleukin X and interleukin I receptor antagonists, which may have immunosuppressive and nutritional effects on sites of inflammation(Veronesi et al., 2014).

Both adipose-derived stem cells and bone marrow stem cells do not express MHC-II molecules on the cell surface, so they do not cause aggregation of allogeneic mononuclear cells. Therefore, they can be used for allogeneic cell transplantation(Niemeyer et al., 2007). Besides, adipose-derived stem cells and bone marrow stem cells can also inhibit mixed lymphocyte reactions(Puissant et al., 2005). This characteristic does not change after being induced to differentiate into osteoblasts or chondrocytes(Niemeyer et al., 2007). The immunosuppressive properties of adipose-derived stem cells and bone marrow stem cells may depend on cell-cell contact(Puissant et al., 2005). At the same time, small soluble molecules may act as more important factors to regulate immunosuppressive effects. Adipose-derived stem cells secrete prostaglandin-2 (PGE2) and leukocyte inhibitory factors. If these factors are specifically inhibited or neutralized, the immunosuppressive effects of adipose-derived stem cells disappear(Najar et al., 2010). Therefore, adipose-derived stem cells are likely to slow the destruction of articular cartilage by the inflammatory response by reducing the immune response.

The paracrine effect of mesenchymal stem cells is verified by in vitro co-culture experiments. Co-culture of bone marrow stem cells with chondrocytes enhances the secretion and formation of extracellular matrix(Tsuchiya et al., 2004). The survival rate of chondrocytes co-cultured with adipose-derived stem cells was significantly higher

than that of monocultured chondrocytes. Chondrocytes secretion of matrix metalloproteinases increases in patients with osteoarthritis(Nummenmaa et al., 2015; Zeng et al., 2015). Among them, MMP-13 is considered to be one of the major factors causing articular cartilage defects in osteoarthritis(Shirai et al., 2011). If adipose-derived stem cells are co-cultured, the concentration of MMP-13 in the medium can be significantly reduced. Intraarticular injection of adipose-derived stem cells in osteoarthritis model animals also reduced intra-articular MMP-13 levels(Kuroda et al., 2015).

According to the experimental results in this study and the evaluation of existing reports, it is speculated that the main mechanism of intra-articular injection of mesenchymal stem cells to promote cartilage repair may be through the secretion of cytokines, inhibition of local inflammation and immune response, thereby inhibiting the cartilage degradation, creating a more stable and suitable microenvironment for cartilage regeneration.

In the therapy of intra-articular injection of cells to repair cartilage defects, the actual survival time of cells in the joint cavity may be very short: the number of seed cells in the synovial fluid drops sharply within 6 hours after injection, and there is almost no surviving injected cells in the synovial fluid at 24 hours after injection; within 7 days after injection, the regenerated tissues contain no injected cells; it is presumed that the vast majority of the injected cells may die within 24 hours after the injection. The rapid decrease in cell number may be due to the lack of blood vessels in the joint cavity, insufficient supply of oxygen and nutrients, inflammation of the joint after cartilage defect, and apoptosis of injected cells. At the same time, the reliability of fluorescent dye-labeled cells is doubtful and does not apply to in vivo tracking.

Materials and Methods

Lentivirus production vector plasmid pT375-mCherry-puro^{r} is extracted from mCherry puro^{r} spec^{r} strain using TIANprep Mini Plasmid Kit II (TIANGEN®). Packaging plasmids VSVG and PR8.74 are kindly provided by Dr. YU Yingting. Lentivirus is packaged in human renal epithelial cell line 293T using Lipofectamine® 3000 Reagent Kit.

Cell transfection cultured cells with convergence of 70%-90% are used for transfection. Polybrene is added with lentivirus to cell culture and incubate at 37℃ 5% CO_2 for 6 hours. Add identical volume of cell culture medium with Polybrene. 48 hours after transfection, change culture medium and examine fluorescence under microscope. Positive cells are sorted by flow cytometry or screened by 1.0 μg/mL puromycin.

Isolation and culture of rabbit ADSCs anaesthetize rabbit by 0.1 mL/kg body weight 4% pentobarbital sodium solution. Carefully isolate subcutaneous fat pad on the back of the animal and suture. Sterilize the fat pad by 75% ethanol and wash twice by PBS solution. Chop the tissue and digest in 0.1% collagenase II at 37℃ 5% CO_2 for 4 hours. Centrifuge at 1 000 × rpm for 5 min. Discard the supernatant. Culture cells with DMEM/F12 (add 10% FBS and Penicillin/Streptomycin before use) at 37℃ 5% CO_2. Subculture when cell reach the convergence of 80%-90%. Digest by 0.25% trypsin-EDTA for 1 min at 37℃ 5% CO_2, Centrifuge at 1 000 × rpm for 5 min. Discard the supernatant. Culture cells with DMEM/F12 cell culture medium at 37℃ 5% CO_2.

Isolation and culture of rabbit SF-MSCs anaesthetize rabbit by 0.1 mL/kg body weight 4% pentobarbital sodium solution. Inject 1 mL of 0.5 mg/mL hyaluronidase into the joint cavity. Move the knee up and down for 1 min. Inject 2 mL of fresh PBS with antibiotics into the joint cavity and move the knee. Use 5 mL syringe to extract fluid in the knee joint. Add alpha MEM cell culture medium (add 10% FBS and Penicillin/Streptomycin before use) two times of the fluid extracted and culture at 37℃ 5% CO_2. Subculture when cell reach the convergence of 80%-90%. Digest by 0.25% trypsin-EDTA for 1 min at 37℃ 5% CO_2, Centrifuge at 1 000 × rpm for 5 min. Discard the supernatant. Culture cells with alpha MEM cell culture medium at 37℃ 5% CO_2.

Isolation and culture of rabbit BMSCs inject 10 mL of air into the ear vein to kill the animal. Isolate limb bones and cut off the cartilage at the end of bones. Use low glucose DMEM cell culture medium to wash out the bone marrow inside. Culture in low glucose DMEM cell culture medium (add 10% FBS and Penicillin/Streptomycin before

use) at 37℃ 5% CO_2. Subculture when cell reach the convergence of 80%-90%. Digest by 0.25% trypsin-EDTA for 1 min at 37℃ 5% CO_2, Centrifuge at 1 000 × rpm for 5 min. Discard the supernatant. Culture cells with low glucose DMEM cell culture medium at 37℃ 5% CO_2.

Animal model New Zealand rabbits with body weight approximate to 3 kg are used. anaesthetize rabbit by 0.1 mL/kg body weight 4% pentobarbital sodium solution. Use electric drill to construct a defect with diameter of ~4 mm on the femur articular cartilage and be careful not to reach the subchondral bone. Suture the wounds. Intramuscularly inject penicillin potassium and anodyne.

Cell injection and retrieval wash cultured cell twice by PBS solution, Digest by 0.25% trypsin-EDTA for 1 min at 37℃ 5% CO_2, Centrifuge at 1 000 × rpm for 5 min. Discard the supernatant. Re-suspend the cells in PBS or 2% hyaluronic sodium solution. Count cells using a hemocytometer. Inject 0.5 mL of cell into the joint cavity. Cell retrieval is performed under the same protocol of *isolation of SF-MSCs*.

RNA isolation RNA is isolated using RNAprep pure Cell / Bacteria Kit (TIANGEN®) or miRcute miRNA Isolation Kit (TIANGEN®).

Acknowledgements

Sincerely I would like to thank PhD candidate WANG Zhaoyan from Prof. WU Qiong's lab for his kind help and patience. Sincere thanks to Prof. WU Qiong and Prof. CHEN Guoqiang. Also, many thanks to all lab members from Wu Lab, and all the members in microbiology laboratory. And finally, thanks to PhD candidate XU Kun from Department of Computer Sciences for his help in processing the figures.

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