Potential for Tumorigenesis and Repair of Osteochondral Defects by iPS Cell Transplantation in Rat

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Abstract

Articular cartilage repair remains a challenge in the field of orthopedic medicine. Cell-based therapy for cartilage repair, such as autologous chondrocyte implantation, was established in the 1990s. However, the issue of the source from which the lesion-targeting cells are harvested remains a limitation of this approach as larger lesions require more cells for repair, and thus, more healthy tissue must be damaged to harvest the needed cells. Reprogramming of induced pluripotent stem (iPS) cells is a promising tool for cell-based regenerative therapy because of their proliferative capacity and pluripotency; however, these characteristics also create a risk of tumorigenesis. This study aimed to determine the probability of iPS cell-derived tumor occurrence as a function of injection or transplantation site, and to assess whether transplanted iPS cells can promote cartilage defect repair. Pluripotent mouse iPS cells (5x10^6 cells/ml) were subcutaneously injected or transplanted into experimentally induced lesions in the knee cartilage of immunodeficient rats. Subcutaneous teratoma formation was observed in 30% of animals (3 of 10) at 4 weeks, and 41% of animals (7 of 17) at 12 weeks after iPS cell injection. Cartilage repair as indicated by modified Wakitani’s score was similar in the cell-free group and in the iPS cell implantation group at 4 weeks [11.8 ± 1.8 (n = 8) vs. 10.3 ± 2.8 (n = 18)]. iPS cell implantation yielded a score of 7.8 ± 2.0 (n = 10) at 12 weeks, significantly better than the cell-free group [10.5 ± 0.6 (n = 4)]. There was no macro- or microscopic evidence of tumor formation at the cartilage repair site after iPS cell implantation. Although we could not use the iPS cells directly for cartilage repair, the results of our study indicate the potential for a new therapy for cartilage repair by developing iPS reprogramming technology.

Keywords: Osteochondral defect repair; iPS cells; Tumorigenesis; Subcutaneous space

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1. Introduction

Articular cartilage is an avascular tissue composed of cells dispersed within an extracellular matrix (ECM) of collagen and proteoglycans (Verwoerd-Verhoef et al., 1998). Damaged cartilage has a limited capacity for spontaneous repair (Hunter, 1995; Shapiro et al., 1993), possibly because the absence of vascularity means there is an inadequate supply of progenitor cells and/or growth factors (Urist and Adams, 1968). Many studies have used cell transplantation and/or the introduction of growth factors into defective cartilage to stimulate tissue repair (Nawata et al., 2005; Nishimoto et al., 2004; Wakitani et al., 1994; Wei et al., 2009; Zscharnack et al., 2010), and the moderate success of these approaches has led to clinical testing (Wakitani et al., 2004b; Wakitani et al., 2007; Wakitani et al., 2011). However, the mechanism of the repair process remain obscure (Dell’accio and Vincent, 2010). We previously observed that peripheral blood cell contribute to cartilage defect repair (Okano et al., 2012), and that systemic administration of granulocyte colony-stimulating factor (G-CSF) enhanced early filling of osteochondral lesions in a rat experimental model (Okano et al., 2014). Thus, the number and type of cells employed is likely to be critical to ensuring an effective outcome.

Induced pluripotent stem (iPS) cells represent a promising source of cells for the repair of many types of tissue (Yamanaka, 2007). These cells can be generated artificially by reprogramming somatic cells via introduction of specific transcription factors (Nakagawa et al., 2008; Takahashi et al., 2006), which is far less invasive than harvesting ES cells from embryos (Takahashi et al., 2007). Thus, iPS cells are potentially more useful than ES cells for autologous cell therapy, tissue regeneration, and the treatment of hereditary diseases, for which iPS cells can be derived from a patient’s own cells (Yamanaka, 2007). Some studies have reported the potential of this technology for cartilage regeneration (Outani et al., 2013; Saito et al., 2013). However, the beneficial properties of iPS cells also produce the risk of tumorigenesis.

Uto et al. reported that the risk of tumor formation in bone or cartilage increased when iPS cells were transplanted at higher concentrations of $10^7$ to $10^8$ iPS cells/mL or when cells were transplanted into a heterologous host strain (Uto et al., 2013). However, they also showed that iPS cells promote cartilage defect repair in cases of 100% homology and the same higher concentrations of iPS cells (Uto et al., 2013), consistent with previous reports on cell therapy for cartilage defect repair (Dell’accio and Vincent, 2010; Nakajima et al., 2008; Wakitani et al., 2004a). Thus, the fate of iPS cells might be altered by strain mismatch and cell number. Our previous studies showed that ES cell transplantation into the joints of immunodeficient rats resulted in the formation of teratomas that subsequently destroyed the joint, but only when the joint was fixed with a pin; without this attachment, cartilage repair was instead promoted (Nakajima et al., 2008; Wakitani et al., 2004a; Wakitani et al., 2003). ES cells transplanted into an osteochondral lesion formed cartilage and promoted tissue repair (Wakitani et al., 2004a), Although the reasons for these effects are unclear, we hypothesized that the local environment, including mechanical constraints, dictates the behavior of transplanted ES cells; moreover, the mobilization of ES cells to the lesion site is essential for the normal repair process. Thus, as in the case of ES cells, the fate of iPS cells may also be defined by the local environment. Nevertheless, previous studies have been limited by small sample sizes that preclude generalization, and an open question is whether iPS cell-derived tumors are more likely to form in cartilage than in other tissues.
The present study investigated the probability of tumorigenesis from iPS cells injected into the subcutaneous space or transplanted into the injured cartilage of immunodeficient rats, and assessed whether this approach can be beneficial for cartilage tissue repair.

2. Materials and Methods

2.1 Cell preparation

iPS-MEF-Ng-178B-5 cells generated from mouse embryonic fibroblast by the retroviral introduction of three pluripotency factors, Oct3/4, Sox2 and Klf4, were purchased from RIKEN cell bank (RIKEN BRC, Tsukuba, Japan) with frozen stock. The cells were plated onto a feeder layer of irradiated mouse embryonic fibroblast (MEF) grown in a 100-mm gelatin coated dish at $1 \times 10^6$ cells/dish. The cells were cultured in high glucose Dulbecco’s modified Eagle’s medium (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) with 15% Fetal calf serum (FCS; Biowest, Kansas city, MO, USA) containing of 0.1mM of nonessential amino acids (Gibco Life technologies Inc., Carlsbad, CA, USA), 0.1mM 2-Mercaptoethanol (Sigma-Aldrich), 1000U/mL of Leukemia Inhibitory Factor (LIF; Wako Ltd., Osaka, Japan )(Fig. 1). The medium was changed daily and cells were passaged every 2 to 3 days with 0.25% trypsin/0.02%EDTA (Gibco Life technologies), and resuspended in FCS at a concentration of $5 \times 10^6$ cells/mL determined with reference to previous studies of injection or transplantation (Mera et al., 2009; Nakajima et al., 2008; Saito et al., 2013; Uto et al., 2013; Wakitani et al., 2004a; Wakitani et al., 2003).

![Morphology of iPS cells immediately prior to harvesting for transplantation. Cells formed clusters on the mouse embryonic fibroblast feeder layer in vitro.](image)

2.2 Animals

Female immunodeficient rats (F344/Njcl-run/run, female) purchased from Clea Japan, Inc. Tokyo, Japan, were maintained on a 12:12 h light: dark cycle with access to food and water ad libitum. All animal procedures were approved by and conducted in accordance with the regulations of the Osaka City University School of Medicine Committee on Animal Research. Animals were 9-10 weeks
old, weighed about 145.9±10.3g, and were divided into two groups: 13 rats were examined for teratoma formation and cartilage defect repair at 4 weeks after the surgery, while 8 were examined at 12 weeks (Table 1).

2.3 Teratoma formation assay

Resuspended iPS cells (100 µl) were subcutaneously injected as described previously (Mera et al., 2009; Saito et al., 2013; Takayama et al., 2010) into the backs of 18 rats; the same concentration of cells was transplanted into a lesion in the knee cartilage. The characteristics of animals with or without teratoma assay are shown in Table 1.

Table 1 Summary of animal experiments

<table>
<thead>
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<th>Animal ID</th>
<th>iPS cells transplantation</th>
<th>w/wo teratoma assay</th>
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<tbody>
<tr>
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<td>left knee</td>
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<tr>
<td>#21</td>
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</table>

#1-#13 samples for 4 weeks after surgery; 11 animals 18 knees for iPS transplantation, 10 animals for teratoma assay
#14-#21 samples for 12 weeks after surgery; 8 animals 12 knees for iPS transplantation, all 8 animals for teratoma assay
2.4 Cartilage lesioning

Animals were anesthetized by subcutaneous injections of ketamine (50mg/mL; Sankyo, Co., Ltd., Tokyo, Japan) and xylazine (0.2mg/ml; Bayer Co., Ltd., Tokyo, Japan) in a ratio of 10:3 at a dose of 1 mL/kg body weight. A skin incision was made at the midline of the knee to expose the joint and the patella was dislocated laterally using medial parapatellar methods. The patellar groove of the femoral bone in both knees was subjected to osteochondral injury (diameter 1.5 mm and depth 1.0 to 2.0 mm) by means of a hand drill (Fig. 2) (Okano et al., 2014; Okano et al., 2012). Nineteen of the rats were randomly selected for the cartilage repair experiment with the iPS cells: the cells suspension (2 to 3 µL) was transplanted into the lesion site as shown in Table 1. After allowing the cells to remain at the lesion for several seconds, the dislocated patella was returned to the original position and the incision was sutured. For the sham-operated control group, the lesions were left unfilled. Animals were allowed to recover and move freely after surgery.

Fig. 2. Experimental cartilage lesion formation at the patellar groove of the femoral bone of immunodeficient rats. The lesion was subjected osteochondral injury (diameter 1.5 mm and depth 1.0 to 2.0 mm). The cell suspension (2 to 3 µL) was transplanted into the lesion site as shown in Table 1.

2.5 Histology

Rats were euthanized by CO₂ inhalation. The knees were removed and immediately fixed in 10% buffered formalin (Nacalai Tesque Inc., Kyoto, Japan) overnight at 4°C. The tissue was decalcified with 0.5M EDTA (pH 7.4) for 4 weeks, then embedded in paraffin and sectioned axially to the femur at a thickness of 5µm. Sections were stained with hematoxylin/eosin or toluidine blue for light microscopic examination of teratomas and cartilage defects, respectively.

2.6 Histology evaluation and statistical analyses

The microscopic findings were evaluated and quantified with the modified Wakitani’s score (Orth et al., 2012), and the average and standard deviation of each group was calculated. Values were compared by Mann–Whitney U test between the frequency distribution of each group and statistical tests were considered significant at the level of p < 0.05 with Prism ver 6.0e (GraphPad Software Inc., La Jolla, CA, USA). All p-values were 2-sided.
3. Results

One of 21 animals that received bilateral iPS transplantation in the knee cartilage died for unknown reasons and was excluded from analyses. Three of 10 animals (Labeque et al.) at 4 weeks and four of the remaining seven animals (57.1%) at 12 weeks had tumors in the subcutaneous space of the back; however, of 18 knee tissue samples examined at 4 weeks and the 10 examined at 12 weeks, none had developed tumors at the site of iPS cell transplantation (Table 2).

Table 2 Probability of tumorigenesis following iPS cell injection into the subcutaneous space or transplantation into a lesion site in knee cartilage

<table>
<thead>
<tr>
<th></th>
<th>teratoma formation at cartilage defect</th>
<th>teratoma formation at subcutaneous space</th>
</tr>
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<tbody>
<tr>
<td>4 weeks</td>
<td>0/18 (0%)</td>
<td>3/10 (30%)</td>
</tr>
<tr>
<td>12 weeks</td>
<td>0/10 (0%)</td>
<td>4/7 (57.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>0/28 (0%)</td>
<td>7/17 (41.2%)</td>
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</table>

Animals with subcutaneous tumors had a characteristic swelling in their backs around the injection site, which was readily distinguished at 4 weeks and still more apparent at 12 weeks after the procedure (Fig. 3A, B). A histological examination of the tumors showed a mixture of immature neuroectodermal elements with rosette formation (Fig. 3C), mesodermal cartilage elements (Fig. 3D), and endodermal epithelial-like tissue (Fig. 3E), confirming their identity as immature teratomas.

![Fig. 3. Teratoma formed by iPS cell injection into the subcutaneous space of an immunodeficient rat. Tumors were visible by macroscopic examination on the backs of animals(A, B). Hematoxylin and eosin staining of a section from a tumor revealed a mixture of immature (C) neuroectodermal elements with rosette formation; (D) mesodermal cartilage elements; and (E) endodermal epithelial-like tissue. Scale bar = 100 μm.](image-url)
The cartilage lesion site was filled with cells and tissues that had partially integrated into the surrounding cartilage. At 4 weeks after the procedure, the non-implanted lesions had been repaired by a mixture of mostly fibrous tissue and some hyaline cartilage with an irregular surface (Fig. 4A); the iPS-transplanted lesion had better hyaline cartilage with a smoother surface (Fig. 4B). The metachromasy observed in some tissue samples suggested that limited tissue repair had occurred at 12 weeks (Fig. 4C), and in comparison, the cell morphology and matrix staining in most of the samples with iPS implantation were more likely to show better repair and integration to the hyaline cartilage (Fig. 4D). The modified Wakitani’s score was similar in the cell-free group [11.8 ± 1.8 (n = 8)] and in the iPS group at 4 weeks [10.3 ± 2.8 (n = 18)]. At 12 weeks, the iPS groups had a score of 7.8 ± 2.0 (n = 10), significantly better than the cell-free group [10.5 ± 0.6 (n = 4)] (Table 3, and Fig. 5). Although there was evidence that iPS cell transplantation promoted cartilage repair, there was no evidence of tumor formation microscopically (Table 2).

Fig. 4. Cartilage repair by iPS cell transplantation. Knee tissue sections stained with toluidine blue show the cartilage defect in a sham-operated animal at (A) 4 and (C) 12 weeks after the procedure. Representative images of cartilage into which iPS cells were transplanted at (B) 4 and (D) 12 weeks after the procedure are shown. Scale bar = 100 μm.
Fig. 5. Dot-plots of modified Wakitani’s scores for each sample. The iPS cell implantation group was significantly better than the cell-free group at 12 weeks. *P < 0.05, Significant difference by Mann-Whitney U test

Table 3

<table>
<thead>
<tr>
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<th>4 weeks</th>
<th>12 weeks</th>
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<tbody>
<tr>
<td>iPS cells (-)</td>
<td>11.8 ± 1.8 (n = 8)</td>
<td>10.5 ± 0.6 (n = 4)</td>
</tr>
<tr>
<td>iPS cells (+)</td>
<td>10.3 ± 2.8 (n = 18)</td>
<td>7.8 ± 2.0 (n = 10) *</td>
</tr>
</tbody>
</table>

average ± sd (number of samples)
* P < 0.05, Significantly different by Mann–Whitney U test

4. Discussion

In the present study, mouse iPS cells were transplanted into an experimentally induced cartilage lesion or injected into the subcutaneous space of immunodeficient rats to assess the potential for tissue repair and tumor formation. Here, iPS cells formed teratomas when introduced into the subcutaneous space but not the cartilage, suggesting that iPS cells have low potential for tumor formation in cartilage despite their pluripotency, possibly because the local environment is unfavorable. Moreover, there were also beneficial effects for cartilage repair, especially at 12 weeks after the procedure.

iPS cells represent a promising source of cells for the repair of many types of tissue (Yamanaka, 2007). Some studies have reported the potential of this technology for cartilage regeneration (Outani et al., 2013; Saito et al., 2013). However, the pluripotency and proliferative capacity of iPS cells makes them a risk for tumorigenesis (Uto et al., 2013). We previously transplanted ES cells into joint spaces or osteochondral defects in immunodeficient rats (Nakajima et al., 2008; Wakitani et al., 2004a; Wakitani et al., 2003), and determined that the probability of teratoma formation was dependent on the local tissue environment, including mechanical constraints, since the mobility of ES cells is essential during tissue repair. Uto et al. showed the risk of tumorigenesis associated with
the transplantation of iPS cells into cartilage defects in Severe Combined Immunodeficiency (SCID) mice arises in part from the incompatibility in the genetic backgrounds of the donor and host, and the high iPS cell densities also required to promote effective tissue repair in same genetic background (Uto et al., 2013). They transplanted iPS cells into the SCID mice at a density that was 2 to 20 times higher when the risk of tumorigenesis was present, but 20% less without the tumorigenesis risk (Uto et al., 2013), than what was used in our present study. Saito et al. performed the teratoma formation assay with mouse iPS cells transplanted into the subcutaneous space of SCID mice at density of $10^6$ iPS cells/mL, also 20% less than in our present study (Saito et al., 2013). SCID mice are defective in T- and B-cell immune function and are thus more likely to produce tumors from iPS cells than rats with a defect in only T-cell function. Thus, we determined a cell density of $5 \times 10^6$ cells/mL carried the minimum expected risk of tumorigenesis at the cartilage defect, but may still produce tumors in the subcutaneous space (Mera et al., 2009; Nakajima et al., 2008; Wakitani et al., 2004a). In this condition, the probability of tumorigenesis from iPS cells transplanted into the subcutaneous space is at least 41.2% (7 of 17) in immunodeficient rats. Moreover, the probability at 12 weeks is 57.1% (4 of 7) and 30% (3 of 10) at 4 weeks (Table 2), suggesting that the tumor would grow and have a greater chance of existing microscopically in subcutaneous space. However, we did not even microscopically confirm the tumors at the site of iPS cell transplantation in 18 knee tissue samples at 4, and 10 at 12 weeks (Table 2). The volume of cells transplanted into the defect (2 to 3 µL of $5 \times 10^6$ iPS cells/mL) was much less than that injected into the subcutaneous space (100 µL), but consistent with Uto, who used 1 µL of $10^6$, $10^7$, or $10^8$ iPS cells/mL (Uto et al., 2013). The sample size in our study was sufficient to determine the probability of tumorigenesis in the cartilage defect (Table 2). Therefore, iPS cell-derived tumors are not likely to form in cartilage as they do in the subcutaneous space of immunodeficient rats (Table 2).

Cartilage defect repair with iPS cell implantation was significantly better than the cell-free group at 12 weeks after the procedure (Table 3, Fig. 4 and 5). The beneficial effect at this density was consistent with previous reports (Nakajima et al., 2008; Uto et al., 2013; Wakitani et al., 2004a). Actually, it is not realistic to use iPS cells directly for application in the human body, even though the possibility of the risk of tumorigenesis might be low. We look forward to the development of technology to regulate cell differentiation regardless of the implantation environment through the evolution of iPS reprogramming technology.

Induced chondrogenic (iChon) cells have been derived from dermal cells harvested from humans and mice using reprogramming technologies similar to those applied to iPS cells (Outani et al., 2013). Production of iPS cells requires the transcriptional factors c-Myc, klf-4, and Oct 3/4 or Sox2, which maintain cellular immaturity similar to that of ES cells (Takahashi and Yamanaka, 2006). iChon cells were developed by introducing the cartilage-specific transcriptional factor Sox 9, which is required for fetal cartilage formation(Akiyama et al., 2002); Oct 3/4 or Sox2 do not mediate this process (Outani et al., 2013). iChon cells are thought to be more restricted in their differentiation potential than iPS cells since they are generated directly by the introduction of the transcriptional factor, Sox9, which chondrocyte have already acquired to identify (Outani et al., 2013). When human-derived iChon cells were transplanted into the subcutaneous space or osteochondral lesions of immunodeficient mice or rats, cells formed cartilaginous tissue but not tumors in the subcutaneous space and stimulated repair of the cartilage after 4 weeks (Outani et al., 2013). Thus, iChon cells may differentiate into chondrocyte regardless of implantation environment and may
have a superior safety profile (i.e., lower tumorigenic potential) and effectiveness for osteochondral tissue repair.

In conclusion, the increased risk of tumorigenesis associated with the pluripotency of iPS cells rely on the local environment of the implantation site in this study with enough sample size, iPS cell-derived tumors are not likely to form in cartilage, and would make more fate-restricted progenitor cells attractive alternatives for the repair of specific types of tissue. Although this was examined in the context of damaged cartilage in the present study, the same principle applied to other tissues offers new possibilities for the clinical application of reprogramming technologies.

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