A Modified Method for Implantation of Pluripotent Stem Cells Under the Rodent Kidney Capsule

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Teratoma formation, the standard *in vivo* pluripotency assay, is also frequently used as a tumorigenicity assay. A common concern in therapeutic stem cell applications is the tumorigenicity potential of a small number of cell impurities in the final product. Estimation of this small number is hampered by the inaccurate methodology of the tumorigenicity assay. Hence, a protocol for tumorigenicity assay that can deliver a defined number of cells, without error introduced by leakage or migration of cells is needed. In this study, we tested our modified transplantation method that allows for transplant of small numbers of pluripotent stem cells (PSCs) under the kidney capsule with minimal cell leakage. A glass capillary with a finely shaped tip and an attached mouth pipette was used to inject PSCs into the rodent kidney capsule. H9 embryonic and induced PSCs were tagged with *Fluc* and green fluorescence protein reporter genes and divided in different cell doses for transplantation. Bioluminescence imaging (BLI) on the day of surgery showed that the cell signal was confined to the kidney and signal intensity correlated with increasing transplant cell numbers. The overall cell leakage rate was 17% and the rodent survival rate was 96%. Teratoma formation was observed in rodents transplanted with cell numbers between 1×10^5 – 2×10^6 . We conclude that this modified procedure for transplanting PSCs under the kidney capsule allows for transplantation of a defined number of PSCs with significant reduction of error associated with cell leakage from the transplant site.

Introduction

PLURIPOTENT STEM CELLS (PSCs), such as human embryonic stem cells (hESCs) and induced PSCs (iPSCs), have the potential of self-renewal and differentiation into many cell types. Hence, there is great interest in using them to treat a wide range of conditions, such as degenerative diseases, inflammatory conditions, cancer, and damaged tissues [1]. The strategy of using iPSC-derived cells is especially promising because the generation of iPSCs offers a way around the ethical issues associated with hESCs. Both PSC types possess the ability to produce an endless supply of genetically matched cells [2]. However, the generation of tissues from PSCs employs laboratory methods that may increase the risk of genetic instability, epigenetic modification, and generation of tumorigenic cells that bear the markings of cancer stem cells. Therefore, a major concern is the inclusion of small numbers of tumorigenic cells in the differentiated cell populations that are destined for clinical applications.

The teratoma assay is currently regarded as the gold standard for determining *in vivo* pluripotency of human

stem cells. It is also commonly used for evaluating the tumorigenic properties of stem cell-derived implants. However, reports from different research groups widely vary in key methodological parameters, such as preparation of cells, site of transplantation, and number of transplanted cells, and analysis of teratoma data. These discrepancies prompted Muller et al. to publish a call for the standardization of the teratoma formation assay [3].

So far, several studies indicate that teratoma formation rate by hESCs in immunodeficient mice is site dependent: subcutaneous (25%–100%), intratesticular (60%), intramuscular (12.5%), and under the kidney capsule (100%) [4]. Kidney capsule transplant offers high sensitivity, but it is difficult to estimate the number of cells implanted, especially in the low-dose range, due to the transplant technique. Generally, the cells are delivered into a small “pocket” under the kidney capsule. Syringe needles, glass capillary, or polythene tubes have been employed to deposit stem cell suspensions into this “pocket” [5–7]. These methods are complicated and are operator dependent. Another key disadvantage is the high leakage rate when you withdraw the

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syringe or capillary tube from the capsule. Others have tried to circumvent these problems by using stem cells colonies and mixing cells with feeder cells and graft [8] to form a pellet for transplant. The pellet sequesters the cells under kidney capsule. Despite these modifications, it is still difficult to determine an accurate starting cell count in the pellet. Furthermore, there is additional variability introduced by the graft material. Reduction of cell leakage after implant can be reduced by cautery of the injection site, but this in turn, can increase local inflammation and apoptosis. These issues limit our ability to determine a safe lowest number of stem cell impurities that will yield tumors in cell mixtures destined for clinical use. To increase certainty in the actual number of cells being transplanted, especially in the low-dose range, we sought to test whether it is feasible to transplant low dosage of cells into the kidney capsule using a modified technique. Here, we describe our methodology that enables low-number cell transplant into the kidney capsule with minimal cell leakage.

Materials and Methods

The Institutional Review Board of the Stanford University School of Medicine and the Stanford University Stem Cell Research Oversight Committee approved this study. Written informed consent was obtained from each subject. The Animal protocols were approved by the Stanford Animal Research Committee and were performed using appropriate aseptic techniques.

Stem cell culture

H9 hESCs and iPSCs derived from vaginal fibroblasts cell [9] were cultured using mTeSR (Stemcell Technologies, Vancouver, Canada) on Matrigel (BD Biosciences, San Jose, CA) as per manufacturer's instruction.

Stem cells transduction with double-fusion lentiviral vector

To track stem cells in vivo, H9 hESC cells and iPSC were transduced using a lentiviral vector (a gift from Dr. Joseph Wu, Stanford University, CA), which carries a human ubiquitin promoter driving firefly luciferase and enhanced green fluorescence protein (*Fluc-eGFP*) [10]. Cells were sorted for *eGFP* by a Vantage SE cell sorter (Becton Dickinson Immunocytometry Systems, San Jose, CA) after transduction. The stem cells expressing *eGFP* gene were expanded in mTeSR on Matrigel. Stable expression of the double-fusion reporter gene was confirmed with bioluminescence imaging (BLI) culture cells using Imaging System (IVIS, Alameda, CA).

Preparation of single cells for transplantation

The *Fluc-eGFP* positive stem cells were incubated with accutase [11] in the presence of 100 μ M of Rock inhibitor (Selleck Chemicals, Huston, TX) for 5 min at 37°C when they became 70%–80% confluent. The cells were then washed twice with DMEM/F12 (Invitrogen, San Diego, CA). The cells were checked under the microscope to ensure they were single cells, without clumping. If complete single cells were not observed, another accutase treatment was performed. The cell numbers and viability were determined by

trypan blue and hemocytometer. The cells then were resuspended into 40 μ L of mTeSR-1 for transplantation.

Transplantation of stem cells under the rodent kidney capsule

Female immunocompromised SCID beige mice, ages between 8–10 weeks, (Charles River Laboratories, Inc., Wilmington, MA) were used in this study. First, preparation of micro-capillary pipette tip (VWR, Radnor, PA) is essential for performing this procedure. Micro-capillary pipette was pulled on flame to make one end form a long, fine, but not very sharp pipette tip. The mice were anesthetized with ketamine (80–100 mg/kg) (Bioniche Teoranta Inverin, Co, Galway, Ireland) and xylazine (5 mg/kg), intraperitoneal injection. After sterile preparation, and 1–2 cm incision was made below the ribs and 0.5 cm off center but parallel to the spine. The kidney was gently pulled out through the incision using a glass Pasteur pipette that has been pulled into the shape of a blunt, slightly curved hook [8]. The kidney was then immobilized with a nontraumatic forceps and kept moisturized with sterile PBS. The single-cell suspension was gently sucked into the micro-capillary with a mouth pipette. The injection site was located at the upper or/and lateral side of the kidney to avoid damage to kidney blood vessels. The glass tip was slowly pushed into the capsule, as far as possible, toward the inferior pole of the kidney to avoid perforating the kidney capsule in other areas. The cells in 40 μ L of mTeSR were blown into the kidney capsule with the mouth pipette, and the fine tip was pulled out of kidney capsule slowly to avoid cell leakage. The kidney was put back into the body cavity using the blunt curved glass Pasteur pipette, without applying pressure to it. Finally, the abdominal wall was closed with suture.

Monitoring teratoma formation with BLI in vivo

The stem cells tagged with *Fluc* report gene were traced with BLI on the day of transplantation and at 1 week interval following transplantation in vivo. Mice were anesthetized by isoflurane. D-Luciferin substrate (Biosynth International, Itasca, IL) was injected into the peritoneal cavity (375 mg/kg in sterile PBS) 10 min before imaging [10]. Image shows the site of signal and light intensity. Areas of luminescence were drawn as Regions of Interest using the Igor image analysis software (Wavemetrics, Lake Oswego, OR). It was quantified for acquisition time and quantified in units of maximum photons per second per square centimeter per steradian (photons/sec/cm²/sr).

Histological analysis

Animals were sacrificed when teratoma formation was confirmed by BLI, when the signal was persistent and increasing in intensity with time. Teratoma was excised and fixed with 10% buffered formalin phosphate solution for 16 h. Paraffin-embedded specimens were cut into 5 mm sections, and hematoxylin and eosin staining was performed. The presence of all three germ layers was detected by light microscope.

Statistical analysis

The leakage and survival rates (number of affected mice/total mice) postoperation were recorded. For comparisons

FIG. 1. Characterization of human induced pluripotent stem cells (iPSC) shows stable expression of double fusion *Fluc-eGFP* reporter gene after transduction and FACS sorting. (A) Human iPSC colony show stable expression of *eGFP* report gene and strong, gray signal under fluorescence microscopy. (B) Robust expression of *luc* gene by bioluminescence imaging (BLI). (C) Strong correlation ($r^2 = 0.9965$) exists between BLI signal and human iPSC number.

FIG. 2. Procedure of low-dose stem cell suspension transplantation under kidney capsule. The trypan blue was added into the cell suspension to identify cell leakage. (A–C) Insert the fine micro-capillary pipette tip into the kidney capsule and push it toward the opposite pole, so that the capillary is traversing the length of the kidney under the capsule. (D–G) The cells were slowly blown under the kidney capsule with the mouth pipette. A blister formed under kidney capsule without leakage as the cells were transplanted under the capsule. (H–I) The micro-capillary pipette was slowly pulled out. (I) Lack of cell leakage was confirmed by absence of trypan blue spillage.

TABLE 1. RATE OF LEAKAGE ON VARIED NUMBER OF STEM CELL TRANSPLANTATION GROUPS

Single cell numbers	Leakage (n)	Recipients (n)	Leakage rate (%)
5×10^2 with trypan blue	1	10	10
5×10^2	1	11	9
1×10^3	1	11	9
1×10^4	2	14	14
1×10^5	2	12	17
1×10^6	4	19	21
2×10^6	3	6	50
Total	14	83	17

Cell leakage rate for each cell dose. Increasing doses of stem cells in 40 μ L mTeSR were transplanted under the kidney capsule. Leakage during the procedure was recorded in the different cell dose groups. The leakage rate increased with larger cell doses, but no significant difference was observed between mice transplanted with low cell doses ($\leq 1 \times 10^5$ cells) compared to the mice transplanted with high cell doses ($\geq 1 \times 10^6$ cells) ($P > 0.05$).

between two groups, chi-square test was conducted using SPSS 10.0.(IBM, New York, NY). Differences were considered significant at $P < 0.05$.

Results

Human PSC stable transduced with double fusion encoding *Fluc* and *eGFP* reporter genes

To track the human PSC in vivo, H9 hESC and iPSC were first transduced with a lentiviral vector carrying double fusion *Fluc-eGFP* gene. Stable transduction of double fusion reporter gene was confirmed by fluorescent microscopy and BLI. The colony expanded from FACS sorting GFP-positive PSC cells showed strong, green signal under fluorescent microscopy, which indicated stable expression of GFP protein (Fig. 1A). BLI showed GFP-positive stem cells that also robustly expressed luciferase protein (Fig. 1B). BLI intensity strongly correlated with increasing cell number ($r^2 = 0.9977$ for H9 hESC, $r^2 = 0.9965$ for iPSC, Fig. 1C).

Stem cell leakage rate after kidney capsule transplant

SCID mice ($n = 83$) received stem cells transplant under the kidney capsule. The mice were divided into two groups: Group A—short-term monitoring group ($n = 28$). These mice were sacrificed after operation ($n = 17$) or after recovery from operation ($n = 11$), mainly to observe cell leakage and perioperative survival. Group B—long-term monitoring group ($n = 55$). These mice were monitored for eventual teratoma formation.

Different cell doses (5×10^2 , 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , and 2×10^6) were transplanted under the mice kidney capsule. To photographically document cell leakage during the transplantation, we added trypan blue (1:4) into the cell suspensions (5×10^2) in 10 mice (Fig. 2). Lack of cell leakage was confirmed with absence of trypan blue spillage (Fig. 2I). For the rest of the mice, leakage was easily identified under the microscope by presence of leakage of the pink mTeSR-1 medium. Cell leakage rate for each group of mice trans-

planted with different cell doses is shown in Table 1. The leakage rate increased with high cell doses, but no significant difference was observed between mice transplanted with low cell doses (less than 1×10^5 cells) compared to the mice transplanted with high cell doses ($\geq 1 \times 10^6$ cells) ($P > 0.05$). The overall cell leakage rate was 17% (14/83). The cell location under kidney capsule was confirmed by BLI on the day of transplant. BLI signal was localized to the kidney area and the signal intensity correlated with increasing cell numbers. Signal was visible in both 1×10^6 and 2×10^6 hESCs and iPSCs transplantation groups (Fig. 3).

The surgical procedure was safe. The kidney bleeding rate was 14% (12/83, total operation group), two of the 12 mice died of severe bleeding during surgery. The overall survival rate was 96% (64/66, total survival surgery group). Postoperation infection was not observed in the long-term monitoring group.

Teratoma formation

Tagged stem cells were tracked using a Xenogen IVIS system at 1 week interval following transplantation. We found that signals disappeared 1 week after engraftment. Signals then reappeared from 2 to 7 weeks after transplantation in the H9 hESC transplant group. Continued persistence of the signal, with increased signal intensity over time, implied teratoma formation (Fig. 4A). Mice transplanted with 1×10^5 – 2×10^6 tagged-H9 hESC developed teratomas. Masses were not palpable, although the signal became very strong. All the tumors were removed within 16 weeks post-transplantation. Tumor sizes ranged from 0.5 cm to 1.8 cm in diameter. Smaller tumors (less than 1 cm) were solid and the larger tumors (greater than 1 cm) were cystic. Necropsy showed that the injected kidney was normal in shape and size. The tumors were located under the kidney capsule and easily separated from the kidney. The mice survived with tumors as large as 1.8 cm, which occupied most of the kidney capsule. Histological analysis revealed that all teratomas were comprised of tissues from each of the three germ layers (Fig. 4B).

Discussion

The potential for tumor formation is a major safety roadblock to clinical application of PSC technologies. The teratoma assay, in addition to being the gold standard pluripotency assay, is also being used as a tumorigenicity assay [3]. However, the current methodology for teratoma assay limits its sensitivity for detecting tumorigenicity because it does not allow reliable, defined numbers of cells to be transplanted.

Of the published protocols for stem cell transplantation under the kidney capsule, only a few articles describe the procedure in detail. It is difficult to ascertain the actual number of stem cells deposited at the specific site with these methods. This is a troublesome issue when the assay is used to detect the lower limit of cells that could be tumorigenic, rather than being used to confirm pluripotency. An accurate cell count in the low-dose range is critical for the former, while the goal of the latter is to produce teratoma regardless of the actual number of cells injected. Ritner and Bernstein [8] described a method where hESCs colonies and feeder

FIG. 3. BLI on the day of transplantation of *Fluc-eGFP*-tagged iPSC under the kidney capsule. The signal was localized to the kidney and its intensity correlated with the cell number.

cells are harvested and mixed with *Phaseolus vulgaris* lectin to form a cell pellet. This pellet is then inserted into a small pocket made by a blunted probe. While the lowest starting hESCs leading to teratoma formation could be estimated at 2.5×10^5 hESCs, confounding factors, such as *Phaseolus vulgaris* lectin and feeder cells could affect tumorigenicity analysis. Using colony pieces as engraftment, the cells number can only be estimated, rather than counted.

In our literature search, we found that other investigators have described a similar methodology as ours [4,5,12]. However, when we attempted to replicate these methods, we saw that the cell suspension leaked easily from the entry point, especially during withdrawal of the capillary or tube. Syringe needle injection methods introduced problems of kidney capsule rupture, which were associated with high rates of kidney injury [13].

We substituted the syringe needle with a capillary attached to a mouth pipette. The capillary tip was shaped into a long and fine tip. We found that these modifications gave the operator better control and ease with getting the tip under the capsule. This reduced the incidence of capsule rupture. The fine tip and blowing the cells under the capsule

with a single entry led to a decrease in the incidence of leakage, which was mainly caused by an enlarged entry point from repeat insertions during the “pocket” making process. The total leakage rate using our methodology was 17%. The leakage rate increased in high cell dose groups (1×10^6 group, 21.05%; 2×10^6 group, 50%), even though the total transplant volume was the same for all cell doses. The viscosity of the high-dose cell suspension caused difficulty with the blowing technique. This resulted in repeated draws and insertions, causing an enlarged entry point.

With respect to complications, bleeding was the most notable problem. Most mice survived after surgery, without infection. On several occasions, the fine capillary tip would scratch the surface of the kidney cortex slightly. This caused a small amount of bleeding but did not affect survival. However, severe bleeding was observed when there were deep punctures into the kidney (2 out of 83 mice). We note that the tip capillary tip caliber is critical. If the tip is made too fine, it is difficult to blow the cells out. If the tip is too wide, it is difficult to insert it into the kidney capsule, which can cause damage to the kidney tissue. Although the tip passed through the kidney tissue in some of the mice, none

FIG. 4. Teratoma formation in H9 hESC-transplanted mice. (A) Tracking of *Fluc-eGFP*-positive stem cells in vivo using a Xenogen IVIS system at 1 week interval following transplantation. BLI signal reappeared with persistence indicating teratoma formation. (B) Hematoxylin and eosin-stained teratoma sections. Scale bars = 100 μ m. Three germ layers, including neural rosettes (ectoderm), cartilage and smooth muscle (mesoderm), and secretory gland (endoderm) are shown.

FIG. 5. Immunofluorescence staining of the iPSCs line (passage 36) derived from vaginal wall fibroblasts. There is positive expression of the pluripotency markers: NANOG, OCT4, SOX2, hTERT, SSEA-3, Tra-1-60, and Tra-1-80. DAPI staining is shown on the right. Scale bars = 100 μ m.

of the teratomas infiltrated the kidney. All kidneys were normal in size and shape and the teratomas separated easily from kidney, even the larger teratoma (1.8 cm) that covered the majority of the kidney. This is not in accordance with the article from Lawrenz et al. [13] where mouse ES cells transplanted under the kidney capsule in nude mice formed tumors that infiltrated into the kidney.

Two PSC lines, H9 hESC and iPSC derived from vaginal fibroblast cell, were used to test our modified method. Different cell doses from each cell line were transplanted under the kidney capsule. Teratoma formation was observed with $1 \times 10^5 - 2 \times 10^6$ of hESCs. To our surprise, none was observed in the iPSC transplant group, even 4 months after transplantation (data not shown). The reason for this discrepancy is not clear because the iPSCs used in this study have successfully developed into teratomas in subcutaneous [9] and in skeletal muscles (data not shown). It is possible that the lack of teratoma formation in the iPSC group is related to the relatively late passage (p34–45) of cells. Another contributing cause may be the genetics of iPSCs. Narsinh, Sun, and associates noted marked heterogeneity in gene expression levels in hiPSCs compared with hESCs [14], suggesting that hiPSCs may exhibit a less stable pluripotent state. They also observed that hiPSC-derived teratomas had much slower growth kinetics than hESC-derived teratomas. However, immunofluorescence staining of our iPSC line (passage 36) was positive for expression of pluripotency markers: NANOG, OCT4, SOX2, hTERT, SSEA-3, Tra-1-60, and Tra-1-80 (Fig. 5). Furthermore, the unused iPSCs after kidney capsule transplantation survived well and formed colonies in vitro. Future studies with lower passage iPSCs are needed to examine these issues.

In summary, our modified single stem cell kidney capsule transplantation method is a relatively simple to perform and reduces cell leakage. It is particularly suitable for applications where low dosage PSC transplantation with accurate initial cell count is needed. Other transplantation methods may be more reliable for high cell dose transplantations.

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Author Disclosure Statement

The authors declare that they have no competing financial interests.

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