**TITLE:**

Targeted and Selective Treatment of Pluripotent Stem Cell-derived Teratomas Using External Beam Radiation in a Small-animal Model

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**SUMMARY:**

Research on treatment strategies for pluripotent stem cell-derived teratomas is important for the clinical translation of stem cell therapy. Here, we describe a protocol to, first, generate stem cell-derived teratomas in mice and, then, to selectively target and treat these tumors *in vivo* using a small-animal irradiator.

**ABSTRACT:**

The growing number of victims of “stem cell tourism,” the unregulated transplantation of stem cells worldwide, has raised concerns about the safety of stem cell transplantation. Although the transplantation of differentiated rather than undifferentiated cells is common practice, teratomas can still arise from the presence of residual undifferentiated stem cells at the time of transplant or from spontaneous mutations in differentiated cells. Because stem cell therapies are often delivered into anatomically sensitive sites, even small tumors can be clinically devastating, resulting in blindness, paralysis, cognitive abnormalities, and cardiovascular dysfunction. Surgical access to these sites may also be limited, leaving patients with few therapeutic options. Controlling stem cell misbehavior is, therefore, critical for the clinical translation of stem cell therapy.

External beam radiation offers an effective means of delivering targeted therapy to decrease the teratoma burden while minimizing injury to surrounding organs. Additionally, this method avoids genetic manipulation or viral transduction of stem cells—which are associated with additional clinical safety and efficacy concerns. Here, we describe a protocol to create pluripotent stem cell-derived teratomas in mice and to apply external beam radiation therapy to selectively ablate these tumors *in vivo*.

**INTRODUCTION:**

The development of stem cell therapies for tissue regeneration has encountered a number of barriers in the past several decades, hampering efforts for efficient clinical deployment. These hurdles include poor cell retention at sites of delivery, stem cell immunogenicity, and the neoplastic potential to form teratomas1. Tumorigenicity is of particular clinical concern as it can potentially harm stem cell transplant recipients2. Accounts of tumor formation due to unregulated stem cell injections have already been reported in multiple clinical settings3-5. The potential for teratoma formation is the most frequently cited clinical concern in pluripotent stem cell (PSC) development and has resulted in delays and cancellations of multiple high-profile embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) trials6-9. Thus, there is a pressing need for a translational investigation dedicated toward providing appropriate treatment, should these iatrogenic tumors arise.

To date, most strategies to control stem cell misbehavior have focused on reducing the number of PSCs with tumorigenic potential2,10. Unfortunately, only a small number of residual cells (*e.g.*, 1 x 104 to 1 x 105 cells11) is required for teratoma formation, which is far below the detection limit quoted by currently available assays12,13. Other limitations of using these preseparation methods include low efficiency and high expense, reliance on single-cell suspensions that may not be appropriate for newer tissue-engineering approaches, and the potential impairment of cell survival and engraftment.

Few studies have addressed treatment options following teratoma formation. Perhaps the most well-studied strategy is the incorporation of “suicide” genes into stem cells14,15. This method involves genetically manipulating the stem cells to incorporate an inducible apoptosis-activating gene that is inducible by pharmacological stimulation postinjection, thereby providing a rescue approach if injected cells produce teratomas. This approach, however, suffers from significant drawbacks, including off-target effects of genetic modifications of PSCs and the potential for a gradual development of drug resistance16. A similar approach utilizes small molecules to induce selective cell death of PSCs *via* the inhibition of anti-apoptotic pathways17. Other groups have targeted cell death of PSCs using antibodies against pluripotency surface markers, such as podocalyxin-like protein-1 (PODXL)18. The timing of small-molecule or antibody delivery stands to have a significant impact on the therapeutic potential of PSCs if delivered too early and may lack therapeutic efficacy if delivered too late. In addition, the systemic effects of small molecules and antibodies used in this fashion have not been studied.

An alternative approach to treating these tumors relies on using external beam radiation therapy (EBRT). EBRT is one of the primary modalities currently employed in the treatment of solid tumors19. Innovations in EBRT, including the development of the proton beam and stereotactic radiosurgery, have enabled the enhanced targeting of pathological structures while avoiding damage to normal tissue, making conformal EBRT ideal for addressing teratoma formation in anatomically sensitive structures20. Additionally, this method avoids the genetic manipulation or viral transduction of stem cells, which are both fraught with additional clinical safety and efficacy concerns15. Finally, advances in micro-irradiators have enabled the application of EBRT in rodents21.

In this article, we demonstrate how to create a small-animal model of teratoma formation by injecting human iPSCs in mice. We then show how to apply EBRT to selectively eradicate these tumors *in vivo* with minimal damage to surrounding tissue. This approach provides a targeted therapy for PSC-derived teratomas while avoiding the off-target effects of the systemic delivery of biological molecules and peptides and the genetic manipulation of the PSCs. For investigational purposes, we offer an optional step to transduce stem cells with reporter genes to track tumor response to radiation therapy *via* bioluminescence imaging (BLI).

**PROTOCOL:**

This animal experiment was approved and performed under the Institutional Review Board and the Administrative Panel on Laboratory Animal Care at Stanford University.

**1. Cell culture of iPSCs**

1.1. Grow human iPSCs derived by lentiviral reprogramming on 6-well plates coated with basement membrane matrix (*e.g.*, matrigel, referred to as matrix hereon).

1.2. Daily change the media of the iPSCs with enriched culture medium (see **Table of Materials**) incubating at 37 °C and 5% CO2.

1.3. Once the cells reach 80% - 90% confluence (approximately every 4 d), add 1 mL of recombinant cell-dissociation enzyme (see **Table of Materials**) per well and incubate at 37 °C for 5 min.

1.4. After 5 min, dissociate the cells from the well by pipetting, transfer them to a 15-mL tube, and centrifuge at 300 x *g*.

1.5. After the centrifugation, aspirate the supernatant and resuspend the cell pellet into enriched culture medium (see **Table of Materials**) enriched with Y27632 inhibitor at a 1:1,000 dilution.

1.6. Perform a cell count of the dissociated cells and replate the cells on matrix-coated 6-well plates at a density of 2 x 105 to 4 x 105.

**2. Transduction of iPSCs with a Double-fusion Reporter Gene**

2.1. Passage iPSCs in 6-well plates as per routine and add enriched culture medium (see **Table of Materials**) containing 6 ug/mL hexadimethrine bromide.

Note: The ideal colony size is 200 - 400 cells/colony to yield the highest transduction efficiency.

2.2. Concentrate self-inactivating lentivirus carrying firefly luciferase and green fluorescent protein (FLuc-eGFP) driven by human ubiquitin promotor-C by sediment centrifugation with an SW-29 rotor at 50,000 x *g* for 2 h at 4 °C.

2.3. Add the viral concentrate to the iPSCs in a 6-well plate at a multiplicity of infection (MOI) of 10 and incubate overnight at 37 °C at 5% CO2.

Note: The multiplicity of infection was determined by the expression of monomeric fluorescence protein analyzed by a fluorescence-activated cell sorting (FACS) scan.

2.4. The following day, remove the virus by centrifugation of the iPSC 6-well plates at 300 x *g* for 6 min at room temperature.

2.5. Change the media daily with enriched culture medium (see **Table of Materials**) and passage as per protocol. Utilize a fluorescence microscope to determine the approximate transduction efficiency for eGFP.

2.6. An efficiency of 30% - 40% is sufficient for FACS sorting. Proceed to the FACS of hiPSCs expressing eGFP if at least 30% - 40% of the cells express eGFP.

2.7. To confirm FLuc activity *ex vivo*, plate the cells expressing GFP sorted by FACS at a density of 5,000 cells per well.

2.8. Incubate the transduced cells and non-transduced cells (which will serve as the negative control) with the bioluminescence reporter probe D-luciferin (100 μmol/L) for 6 h. Measure the bioluminescence with a microplate spectrofluorometer.

**3. Transplantation of PSCs in the Dorsal Flank for Teratoma Formation in Immunodeficient Mice**

3.1. Add 1 mL of recombinant cell-dissociation enzyme mix per 6-well plate containing human iPSCs transduced with a double-fusion reporter gene (FLuc-GFP) in culture (see section 2) and incubate for 5 min.

3.2. After the incubation period, disperse the cells by pipette aspiration and expression. Add an equal volume of culture medium and then centrifuge at 250 x *g* for 4 min.

3.3. After centrifugation is complete, aspirate the supernatant solution, resuspend the cell pellet in 30 µl of matrix, and place it on ice to preserve its viability prior to injection. Confirm a harvest of 1 x 106 cells using a hemocytometer.

3.4. If utilizing double-fusion reporter-gene-transfected cells, suspend the double-positive FACS cells (from section 2) in 30 µL of matrix.

3.5. Induce anesthesia using 2% isoflurane 100% oxygen in 8- to 10-week-old athymic nude mice.

3.6. Using a 28.5-G syringe, inject cell/matrix mixture (see **Table of Materials**) suspension into the subcutaneous dorsal flank, aiming for an injection of in total 5 x 103 to 5 x 106 cells.

1. **Bioluminescence Imaging (BLI) of Transplanted Cells to Assess Cell Survival and Teratoma Growth** 
   1. At the desired timepoints after inoculation, perform an intraperitoneal (IP) injection of 375 mg/kg of the reporter probe D-Luciferin into the mice.
   2. 10 min after an IP injection, image the bioluminescence signal in the anesthetized animals (performed as described in step 3.5) for 30 min using 1-min acquisition windows at 5-min intervals.

Note: Weekly image acquisitions are recommended.Anesthesia is maintained during imaging by delivering inhaled isoflurane *via* a nose cone.

* 1. For data analysis, draw a region of interest (ROI) over the BLI signal and, then, normalize for the acquisition time to quantify emissions in units of maximum photons per second per square centimeter per steradian (photons/s/cm2/sr).

1. **Teratoma Irradiation Using a Preclinical Image-guided Irradiator (Figure 1)**
   1. Anesthetize a mouse in a knockdown box using 2% isoflurane in 100% oxygen at a flow rate of 1 L/min. After the mouse is fully anesthetized, transfer it to the bed of an image-guided pre-clinical irradiator (see **Table of Materials**). Maintain anesthesia by 2% isoflurane continuously *via* a nose cone.
   2. Acquire micro-CT images as a set of 400 projection images over 360° using a 40-kVp, 2-mA X-ray beam, and reconstruct those into volumetric images with an isotropic pixel size of 0.2 mm.
   3. Plan a radiation treatment using the micro-CT images using the RT\_Image software package (http://rtimage.sourceforge.net/) and perform the treatment.

Note: The treatment plan used consists of two 225-kVp X-ray beams, oriented to pass through the superficial target teratoma while skirting the surface of the rest of the mouse and sparing the underlying viscera. The exposure times for the beams are adjusted based on quarterly system calibration data so that the dose at the center of the target tumor was 6 Gy.

* 1. Repeat the treatment process on three consecutive days to deliver a total of 18 Gy to the target tumor.
  2. Maintain standard post-treatment care of the animals.

**REPRESENTATIVE RESULTS:**

Injected mice typically will demonstrate teratoma growth formation after 4 - 8 weeks as confirmed by BLI imaging (**Figure 2**). Tumors will shrink dramatically when irradiated with a cumulative dose of 18 Gy given one month after cell delivery, resulting in a significant decrease in luciferase signal (**Figure 2**). Importantly, normal tissues taken 5 mm from the irradiated site do not appear to have any significant damage (**Figure 3**).

**FIGURE LEGENDS:**

**Figure 1:** **Schematic of the protocol for the treatment of tumors with EBRT.** (**A**) The anesthetized animal is placed on the irradiator and immobilized. (**B**) A scout image is created to localize the teratoma for targeted treatment. (**C**) Using the RT\_Image software package, the X-ray beams are aligned to target the selected tumor. Prior to irradiation, the position of the collimator and the animal is confirmed. (**D**) A total of 6 Gy of radiation is delivered to the tumor target per irradiation event22.

**Figure 2: Successful seeding of cells results in sizeable tumors that can be selectively treated with radiation.** (**A**) Representative BLI of treated (right) and untreated (left) teratomas are shown. A total of 1 x 106 human PSCs constitutively expressing FLuc/eGFP were injected to both dorsal flanks of an immunodeficient mouse. While the unirradiated side continues to grow, the irradiated side shrank dramatically as shown by the decline in the luciferase signal. (**B**) This line graph demonstrates the decline of the luciferase signal in irradiated *vs.* unirradiated PSC-derived tumors. (**C**) Changes in *in vivo* caliper measurements of teratomas over time. Non-irradiated teratomas increased in size over time, whereas irradiated teratomas decreased in size. (**D**) A gross histology of the untreated (left) and treated PSC-derived tumor (right) shows a marked reduction in size after a total of 18 Gy of irradiation22.

**Figure 3:** **Targeted delivery results in minimal damage to surrounding tissue, including the liver, intestine, and muscle**. Surrounding tissues have no signs of irradiation damage, including the preservation of cellular proliferation and an absence of cellular senescence and apoptosis. Tissues were sampled 5 mm from the irradiated sites at 14 days postirradiation. (**A**)Hematoxylin & Eosin staining shows the normal architecture of the adjacent tissue. (**B**)Ki67 staining (shown in aqua) indicates that cellular proliferation is preserved in liver, intestinal, and muscle cells. Nuclei are counterstained with 4’,6-diamidino-2-phenylindole (DAPI), shown in blue. (**C**) A β-galactosidase senescence assay shows no evidence of cellular aging (*i.e.*, absence of green staining). (**D**) Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) shows no apoptosis (*i*.*e.*, absence of red staining in nuclei). Nuclei are counterstained with DAPI, shown in blue22.

**DISCUSSION:**

Preclinical data and anecdotal cases from victims of “stem cell tourism” confirm that the risk of developing teratomas is a serious drawback associated with PSC treatments23. Development of careful approaches to prevent and treat the neoplastic risk associated with stem cell therapies is, therefore, an important step in facilitating the clinical translation of regenerative stem cell therapies. In this article, we described a method of therapeutic targeting of PSC-associated teratomas using EBRT in a mouse model and showed dynamic atrophy of irradiated tumors using BLI imaging.

We utilized human iPSCs, created by a lentivirus reprogramming method and injected in a nude mice model to recapitulate the formation of teratomas *in vivo*. The use of nude mice avoids an early immunogenic rejection by a cross-species injection of cells. While the use of immune-deficient mice potentiates tumorigenic potential, the same protocol could be applied in immunocompetent mice utilizing murine PSCs. We further transduced the PSCs used in this paper with a double-fusion reporter gene that enabled serial bioluminescence imaging of the delivered cells *in vivo*. The use of reporter gene imaging enabled the serial tracking of the PSC or PSC-derivatives *in vivo* without having to rely on necropsy and histology to track the size or growth of the tumor24. Prior studies have confirmed the correlation between BLI signal intensity and tumor size25,26. Labeling the PSCs with a double-fusion reporter gene is an optional step that can be bypassed in favor of other methods of tumor burden quantification, such as necropsy.

For modifications to the radiation protocol, different dosages may be applied for preclinical tumor treatments. For the purposes of this paper, we have elected to treat the representative animals with 18 Gy administered in three doses of 6 Gy given over 3 continuous days. The advantage of not administering all 18 Gy in one setting is that lower dosages of radiation spaced apart limit adjacent tissue damage and morbidity secondary to EBRT. Patients receiving EBRT in clinical settings often receive low dosages spaced over many different treatments for these same reasons27. Other steps of the protocol should be followed as outlined.

Tumor ablation through irradiation is a promising treatment strategy for stem cell-derived teratomas, which are often delivered into surgically inaccessible areas. This study provides evidence that EBRT constitutes an effective tool for the treatment of PSC-derived teratomas. This simple approach requires the acquisition of high-resolution CT images of a subject, after which a series of radiation beams could be prescribed to irradiate a target to a desired dose while avoiding adjacent tissue20. In this study, two tangential beams were used to treat a subcutaneous PSC-derived teratoma while sparing the surrounding tissue, as well as the contralateral control teratoma. EBRT tumor treatment is both efficient and robust, and clinically feasible, in contrast to methods that rely on small molecules, antibodies, and pre-separation to prevent tumor formation.

There are significant advantages to this approach. First, external beam radiation is a clinically accepted modality of oncologic treatment that has been used in the treatment of many tumor types, including germ cell tumors19. Unlike other treatment strategies, EBRT does not modify the functional properties of the stem cells prior to or during cell delivery15. Moreover, EBRT does not interfere with the mode or number of cells delivered and, thus, has minimal impact on their potential efficacy. Targeted irradiation also reduces the off-target damage to other organs, compared to chemotherapy. Finally, regardless of pretreatment strategy, EBRT provides a “fail-safe” option which can be relied on in the event of tumor formation. Nonetheless, there are limitations to the future adoption of EBRT for treating stem cell-associated teratoma. First, the process requires repeated imaging and delivery of therapy, which, from a clinical standpoint, can be cumbersome. Also, depending on the location of the stem cell delivery, this approach may prove higher risk if radiosensitive tissue is in the beam pathway. Lastly, if stem cells disseminate systemically beyond the sites of injection and form teratomas in multiple organs, it may become difficult to apply this strategy without significant patient morbidity.

In conclusion, we provide a model of creating PSC-derived teratomas in a mouse model and demonstrate a reliable method of micro-CT irradiation that enables the targeted reduction of tumor burden. These methods can be used to compare the therapeutic efficacy of EBRT with other teratoma treatment strategies or to evaluate the value of EBRT in eradicating other types of tumors.

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**DISCLOSURES:**

The authors have nothing to disclose.

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