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Targeted and selective treatment of pluripotent stem cell-derived teratomas using external beam radiation in a small animal model

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TITLE:

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

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KEYWORDS:

pluripotent stem cells, teratomas, irradiation, induced pluripotent stem cells, external beam radiation therapy, tumors

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 teratomas[1]. Tumorigenicity is of particular clinical concern as it can potentially harm stem cell transplant recipients[2]. Accounts of tumor formation due to unregulated stem cell injections have already been reported in multiple clinical settings[3-5]. 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) trials[6-9]. 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 potential[2,10]. Unfortunately, only a small number of residual cells (*e.g.*, 1×10^4 to 1×10^5 cells[11]) is required for teratoma formation, which is far below the detection limit quoted by currently available assays[12,13]. 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 cells[14,15]. 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 resistance[16]. A similar approach utilizes small molecules to induce selective cell death of PSCs *via* the inhibition of anti-apoptotic pathways[17]. 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 tumors[19]. 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 structures[20]. Additionally, this method avoids the genetic manipulation or viral transduction of stem cells, which are both fraught with additional clinical safety and efficacy concerns[15]. Finally, advances in micro-irradiators have enabled the application of EBRT in rodents[21].

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% CO_2.

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 10^5 to 4 x 10^5.

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% CO_2.

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 10^6 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 10^3 to 5 x 10^6 cells.

4. Bioluminescence Imaging (BLI) of Transplanted Cells to Assess Cell Survival and Teratoma Growth

4.1. At the desired timepoints after inoculation, perform an intraperitoneal (IP) injection of 375 mg/kg of the reporter probe D-Luciferin into the mice.

4.2. 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.

4.3. 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/cm^2/sr).

5. Teratoma Irradiation Using a Preclinical Image-guided Irradiator (Figure 1)

5.1. 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.

5.2. 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.

5.3. 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.

5.4. Repeat the treatment process on three consecutive days to deliver a total of 18 Gy to the target tumor.

5.5. 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 event[22].

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 10^6 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 irradiation[22].

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 blue[22].

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 treatments[23]. 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 tumor[24]. Prior studies have confirmed the correlation between BLI signal intensity and tumor size[25,26]. 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 reasons[27]. 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 tissue[20]. 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 tumors[19]. Unlike other treatment strategies, EBRT does not modify the functional properties of the stem cells prior to or during cell delivery[15]. 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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Figure 1

Animal placement and immobilization	CT imaging	Target identification	• Collimator positioning • Animal positioning • Verification	Radiotherapy delivery
1 minute	5 minutes	5 minutes	1 minute	10-20+ minutes

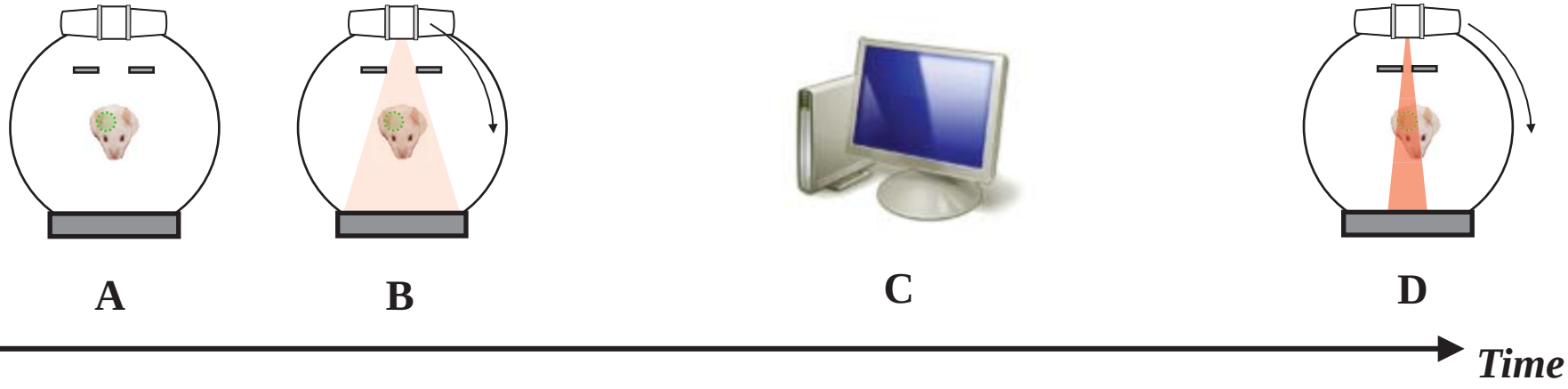

Figure 2

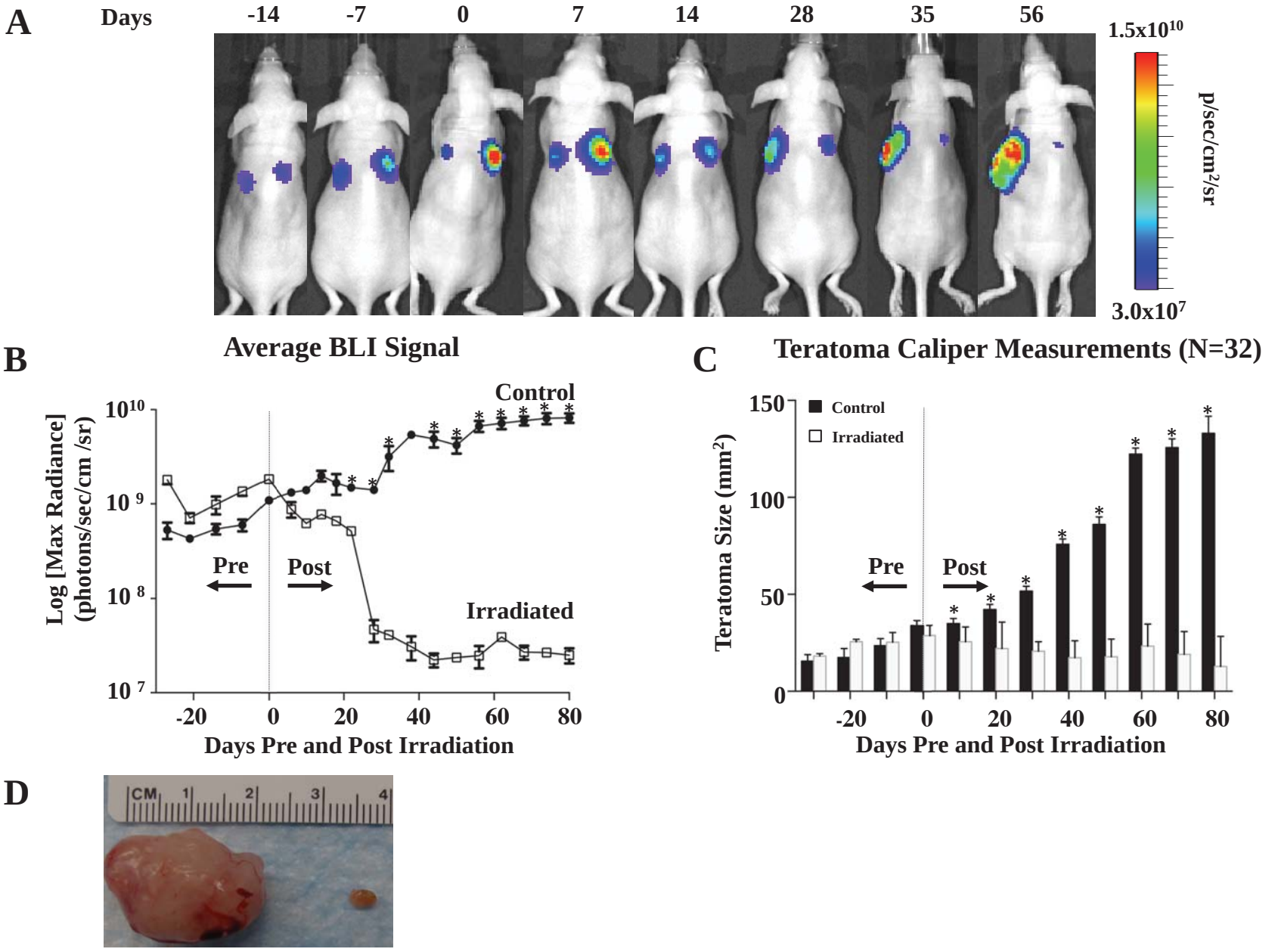

Figure 3

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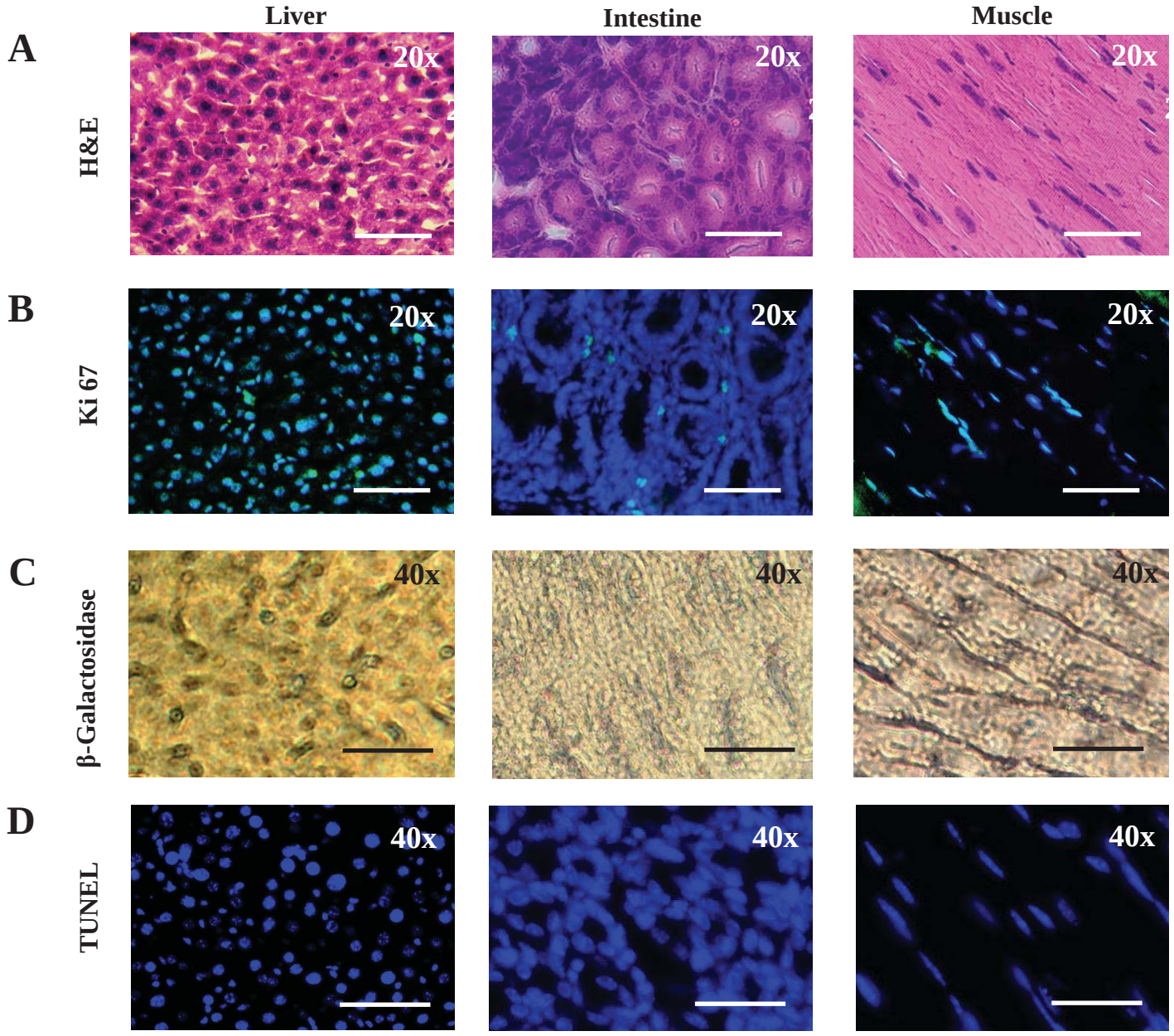

Name of Material/ Equipment	Company	Catalog Number
Induced Pluripotent Stem Cell Control Line	Stanford University	Nguyen Lab
Corning matrigel basement membrane matrix 354234	Fisher Scientific	CB-40234
Essential 8 culture medium	ATCC-The global bioresource center	30-2203
Tryple E	Gibco	12605-036
Y27632 inhibitor 2 HCL (ROCK Inhibitor)	Fisher Scientific	S104950MG
Lentivirus	Cyagen	P170721-1001cjn
Polyrbrene Infection/Transfection Reagent	Millipore Sigma	TR-1003-G
Fluc-eGFP reporter gene driven by ubiquitin promoter	Stanford University	Sam Gambhir lab
D-luciferin	Perkin Elmer	122799
Flow cytometer (BD FACSARIA III)	BD Biosciences	FACSAria
microplate spectrofluorometer (Glomax Navigator System)	Promega Bio Systems, Sunnyvale, CA	GM2000
Xenogen IVIS 200	Perkin Elmer	124262
Isoflurane	Sigma-Aldrich	CDS019936
X-Rad SmART image-guided irradiator	Precision X-ray Inc., North Branford, CT	X-Rad SmART
RT_Image software package	Stanford University (http://rtimage.sourceforge.net/)	RT_Image v0.2β

Comments/Description	
Cell culture of iPSC	
Cell culture of iPSC	
Cell culture of iPSC	
Cell culture of iPSC	
Cell culture of iPSC	
Transduction of iPSC with double fusion reporter gene	
Transduction of iPSC with double fusion reporter gene	
Transduction of iPSC with double fusion reporter gene	
Transduction of iPSC with double fusion reporter gene and BLI	
Transduction of iPSC with double fusion reporter gene	
Transduction of iPSC with double fusion reporter gene	
BLI	
irradiation	
irradiation	
Irradiation	

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Department: Medicine

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Article Title: Targeted and selective treatment of pluripotent stem cell-derived teratomas using external beam radiation in a small animal model

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We would like to sincerely thank the editors and reviewers for reading our manuscript and their critical appraisal. We reviewed the feedback closely and incorporated significant revisions in the manuscript that addressed concerns raised and clarified. We are appreciative this feedback has enhanced the quality of the manuscript.

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Introduction:** Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

We have revised the introduction to focus on explaining the method in the wider context of the body of the literature.

1) Please cite relevant references to the following lines: 51-54, 76, 77-79, 80, 87.

Line 76 we removed stem cell tourism, to the rest we have added relevant references.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples of level of detail required:
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3) 1.6: Mention counting method.
4) 2.2: Please cite a reference for the lentivirus used. Please also mention which ubiquitin promotor is used and at what concentration (relative to lentivirus concentration). Mention centrifugation temperature.
5) 2.3: How is MOI estimated?
6) 2.4: temperature?
7) 4.1: Please specify I.P injection.
8) Section 4 : Mention BLI steps include acquisition settings, durations etc.
9) 5.2: Should microCT imaging be done before irradiation?
10) Is imaging repeated after radiation treatment?
11) Please add a single-line space after each step.
All above edits were incorporated.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

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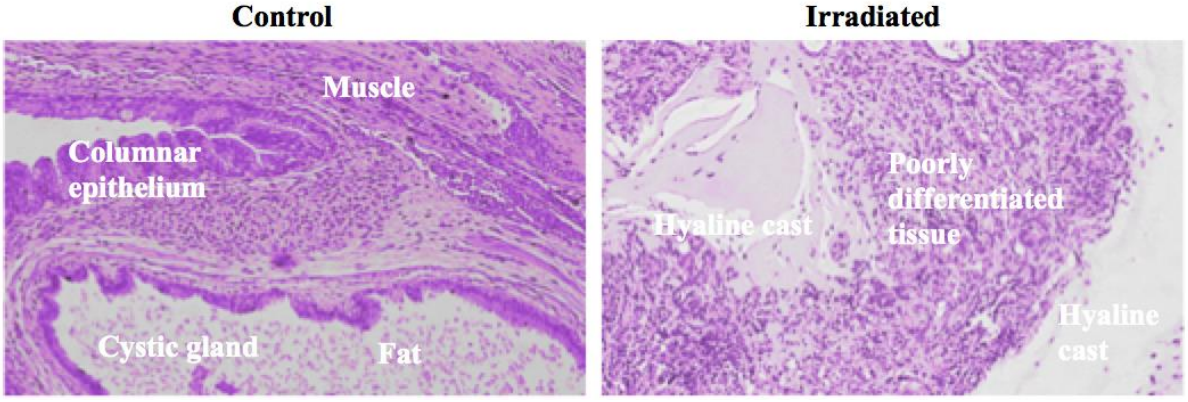
2) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
3) Notes cannot be filmed and should be excluded from highlighting.
4) Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.

Protocol highlighted.

• **Results:**
1) Unclear how the tumor model is validated. Also it is unclear where " targeted and selective" treatment is shown.

The teratoma model has been validated in a previous study (Lee, Cell Cycle, 2009). . In 2009, our group first investigated the relationship between the number of embryonic cells and the development of teratomas in immunocompromised mice. In the study, a minimum of $1x10^5$ and $1x10^4$ ES cells expressing a bioluminescence reporter gene were required to form teratomas in the myocardium and in skeletal muscle. The development of teratomas was monitored by serial bioluminescence imaging and confirmed by histology that showed the presence of three germ layers (e.g., cartilage [mesoderm], mucinous glandular epithelium [endoderm], and neural tissue [ectoderm]). Similarly, we demonstrated the presence of three germ layers by histology. As shown in the figure below (left), the control teratoma was trilaminar and containing mature derivatives from the three germ layers. The irradiated teratoma (right) was found to lack derivatives from all three germ layers, suggesting that radiation exposure causes cell death and inhibits differentiation of tumor cells.



2) What is the animal survival post-treatment? Do you have survival curves for the treated group?

All animals survived post treatment until they were sacrificed. We do not have survival curves for the treated groups because all the animals survived until they were sacrificed.

3) What is the sample size?

The sample size was 32 animals per group as indicated in figure 2.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
We have modified the discussion section accordingly

• **Figures:**

1) Fig 2A: please add a color bar (graded scale to indicate intensities represented by the pseudocolor) to each panel. Ideally all should be on the same scale.: reconfigure picture
   The figure was modified to include color scale
2) 2) Fig 2B: Define error bars. How many animals per group? Resubmit picture
   The figure has been revised with defined error bars defined. The sample size was 32 samples per group and this is now indicated in the figure.
   3) Fig 3C: how many animals did you see such size reduction in?
   All teratomas had a 75% reduction in size. About 30% of teratomas reached the size reduction depicted in the figure.

• **References:**Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]

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Reviewer #1:
Manuscript Summary:
In their paper, the authors describe a protocol for creating pluripotent stem cell-derived teratomas in a immunedeficient mice base on External-Beam-Radiation (EBRT), thereby demonstrating a method of microCT irradiation enabling targeted reduction of tumor burden. The authors suggest this approach to be useful for comparison of the therapeutic efficacy of EBRT with other treatment strategies or evaluate the value of EBRT in eradicating other types of tumors. In my opinion, the paper deals with a highly relevant topic (as the authors state: „stem cell tourism"). The protocol is well described and understandable.

Major Concerns:
none

Minor Concerns:
Statistics of Fig. 2B are not clear to me.

*We thank the reviewer for this comment. We have revised Figure 2 to display time points where there was a significant difference (*p<0.001) in bioluminescence between the control and treated group. A repeated ANOVA was used to compare the two groups at several time points.*

Reviewer #2:

Manuscript Summary:

The manuscript describes a pluripotent stem-cell derived teratoma therapy by the external beam radiation in nude mice. In the abstract, introduction and discussion authors extensivelly write about "the stem cell turism" and dangers that are possible in medical practice after transplantation of stem cells or their derivatives. They say that it is important to control stem cell "misbehavior" and propose targeted external beam radiation as therapy that spares other organs. In their protocol they describe the methods that they used but some important data are missing (see below). The result is really showing that the therapy worked and that irradiated teratomas were much smaller, while the adjacent tissue seems intact.

Major Concerns:

Maybe a bit too much was written about dangers in human therapy but I think that one cannot totally disregard safety measures such as the selection of differentiated cells because of the high cost and wait and see whether teratoma will appear or not. Maybe such a consensus will be reached among medical professionals at some point?!

We agree with the reviewer that this area remains a dynamic area in the field without clear consensus. We have shifted the focus of our introduction and discussion sections to discuss the approach of EBRT methods presented rather than the broader topic.

I suggest rather that the animal model that was used is more to roughly described and proposed for further preclinical research. What is necessary to describe:

I cannot find a description of the induced pluripotent cell line that was used for transplantation. How was it induced? Is it a mouse cell line? If so, why the nude mice were used? Please, explain.

We appreciate the reviewer allowing us to elaborate on this important point. To clarify, we use a human induced pluripotent stem cell line reprogrammed by lentivirus (hence the use of nude mice). This was clarified in the methods section. Also, we added the following statement in our discussion "The use of nude mice avoids early immunogenic rejection by cross-species infection of cells. While use of immune-deficient mice potentiates the tumorigenic potential, one could apply the same protocol in immunocompetent mice utilizing mouse pluripotent stem cells."

Explain why lentiviral vectors for reporter genes and is there a danger after their incorporation into the genome or not? Should genetics of teratoma cells be investigated at some point? Before or after therapy?

We used lentiviral vector for the double fusion reporter gene given that this approach was reliably described in the literature to label stem cells and used for in vivo tracking with no detectable impact on stem cell pluripotency or differentiation potential. Genetics of the teratoma in response to EBRT (was investigated as part of prior work Lee at al. Stem Cells 2017).

I do not see a histological analysis of the teratoma. Was it a trilaminar teratoma as required for the usual pluripotency assay? Was it immature, did it contain EC cells? What was the histology of the small tumor that remained?

This excellent point was raised by the editor and please see our detailed response above. In summary as part of prior work (Lee, Cell Cycle, 2009) we performed histological analysis of the teratoma confirming trilaminar nature and identified a population of cells expressing pluripotency markers. The residual tumor was largely made of poorly differentiated tissue and hyaline casts.

Minor Concerns:
Some of the references are not complete. The journal and the year is mentioned but the volume and pages are missing.

This was carefully revised and formatting of the references per journal style.

Reviewer #3:
Manuscript Summary:
The authors described the technical approach to ablate the already formed teratomas using external beam radiation therapy.

Major Concerns:
In the abstract, the phrase "from de-differentiation of stem cell derivatives post transplantation" should be carefully reviewed because of lack of evidence so far. I agree that it would be a possible concern. However, because there is no report that teratoma can be formed by de-differentiation of stem cells, this possibility should be discussed in the discussion if necessary.

We thank the reviewer for this insight and we have removed the phrase "de-differentiation" given the controversy surrounding this issue in the field.

The references #9 and #10 are less relevant to the notion of authors. These demonstrated that approaches to get rid of undifferentiated stem cells for inhibiting teratoma formation. Failure of isolating differentiated cells using FACS would not be relevant to the limitation of these approaches.

We appreciate the reviewer's astute point. Towards that end, we took out the FACS sorting statement and revised this portion of the introduction.

Suicide gene approach the authors criticized in the introduction, would be applied in vitro (not in vivo) to induce selective cell death of undifferentiated stem cells prior to transplantation. Thus, the 'significant drawback' that the authors described in the introduction would not be applicable. Instead, genetic modification would be issue for clinical application.

This is an excellent point the reviewer raises. We clarify that we are describing the approach of genetically manipulating pluripotent stem cells with suicide genes and then after injection using a drug. Both references cited utilize this method. This was clarified by adding the following statement to our introduction "This method involves genetically manipulating the stem cells to incorporate an inducible apoptosis activating gene that is inducible by pharmacological stimulation post-injection; providing a rescue approach if injected cells produce teratomas."

The authors should provide appropriate reference to support that "Because these tumors harbor a

small population of cells expressing pluripotent markers.". To my knowledge, presence of undifferentiated stem cells in teratomas (expressing OCT-4) is quite rare unless pluripotent stem cells are genetically unstable. Additionally, it is not clear how it can be justified that presence of 'small population of cells expressing pluripotent markers' can make this type of teratoma more radio-sensitive 'unlike other teratoma'.

We thank the reviewer for bringing up this important point. While some reports have been published suggesting there are residual cells in teratoma expressing markers of pluripotency, the impact of these cells remains unknown and controversial. Thus, we have removed this part of the introduction.

The authors achieved the regression of teratoma by 18Gy (3 X 6Gy). Unlike proton beam therapy, the normal cell damage could not be avoided. As the authors agree, due to possible normal cell damage during SBRT, radiation cancer therapy is normally applied in brain or lung, of which organ is considered to be radio-resistant. For determining radiation effect, gamma-H2AX staining should be performed in Figure 3.

We agree with the reviewer's point regarding staining for gamma-H2AX to examine the effect of cell damage. This was previously performed in a separate body of work (Lee et al. Stem Cells 2017). Our data provides a functional surrogate of drop in BLI signal of teratoma (Figure 2) and preservation of surrounding tissue (Figure 3). We feel this data is most relevant to utilizing the protocol and methods as indicated by the editors.

The authors should carefully state the risk-benefit of this approach. Thanks to development of variety of techniques to selectively ablate the undifferentiated stem cells prior to transplantation in vitro (not in vivo), the application of EBRT for teratoma formation would not be practical. I also agree that this approach would be useful once the teratoma is formed regardless of the pretreatment. Thus, 'fail-safe' approach using suicide gene system was suggested. This should be clearly noticed in the discussion.

We appreciate the reviewer's feedback. We have modified the discussion section to explicitly review the risks and benefits of this approach.

Minor Concerns:
Discussion is too long and there are too much contents, less relevant to the main notion.

We modified the discussion section significantly based on reviewer and editor's feedback to focus primarily on the protocol outlined.

The authors need to carefully review the reference #30. This work is not relevant to the statement.

Removed

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