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:

1) 1.1: Mention culture medium and conditions for growth.   
2) 1.4: Mention Centrifuge duration and temperature.   
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.

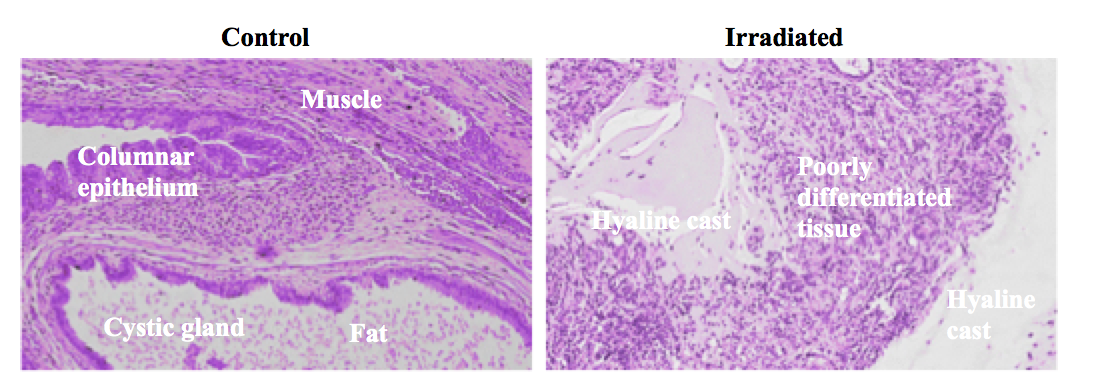
1) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.  
2) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.  
3) Notes cannot be 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 1x105 and 1x104 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*

1. 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).]

*Done*  
  
• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Matrigel, Essential 8, TrypleE, Falcon, E8, X-Rad SmART, (Precision X-ray Inc., etc.

1) Please use MS Word’s find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

*All commercial language was substituted*

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as cells, reagents, virus, instruments etc.

*Done*

• Please define all abbreviations at first use.

*Done*

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

*Done*  
   
• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Done

**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.*

We next aimed to determine whether the selective effects

of radiation on undifferentiated hPSCs contributed to growth

arrest following teratoma irradiation. We ﬁrst dissociated tera-

tomas at day 30 post-injection and used ﬂow cytometry to

quantitatively assess for presence of cell populations expressing

cell surface markers associated with pluripotency. Dissociated

teratomas were observed to contain a small population of cells

co-expressing both SSEA-4 and Tra-1–81. Single cell polymerase

chain reaction of teratoma digests conﬁrmed that teratoma

cells co-expressing SSEA-4 and Tra-1–81 expressed higher levels

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*