Teratoma formation: A tool for monitoring pluripotency in stem cell research

Wendy Y. Zhang[1,2], Patricia E. de Almeida[1,2] and Joseph C. Wu[1,2,3,4],
[1]Department of Medicine, Division of Cardiology; [2]Department of Radiology;
[3]Institute for Stem Cell Biology and Regenerative Medicine; [4]Cardiovascular Institute; Stanford University School of Medicine, Stanford, CA 94305, USA

Abstract

Human embryonic stem cells and human induced-pluripotent stem cells are uniquely defined by their pluripotent differentiation potential and endless self-renewing ability. This capability to become any somatic cell type within the human body has garnered significant attention and interest in the fields of cell biology and regenerative medicine. In studying these promising cells, quality-control assays that can characterize their pluripotency and determine the tumorigenicity of their therapeutic progenies become critical. The most rigorous and arguably accurate among current assays is teratoma formation *in vivo*. This chapter will provide a brief description of teratoma biology, discuss its clinical relevance, and cover methods of forming, monitoring, and analyzing teratomas. The protocols outlined in this chapter have been extensively utilized in peer-reviewed literature.

Teratoma formation, pluripotency testing, human embryonic stem cells, human induced pluripotent stem cells, molecular imaging

A. Introduction

1.1 Methods of pluripotency testing

The ability to differentiate into almost all tissue types is the hallmark of human pluripotent stem cells (hPSCs). However, as we study the biology of hPSCs for future clinical applications – whether they be under the influence of growth factors (1–4), pro-survival cocktails (5,6), genetic modification (7–10), or other manipulations (1) – pluripotency testing remains a fundamental component of every research design. Assays generally used to test such "stemness" include genomic profiling for relative quantification of pluripotency genes, immunocytochemistry to detect pluripotency markers, embryoid body formation to test 3-germ-layer differentiation capability *in vitro* or *in vivo*, and teratoma formation to test 3-germ-layer differentiation capability *in vivo* (Table 1). Notwithstanding the *in vitro* assays, teratoma formation *in vivo* is considered the most stringent of pluripotency assays because it provides

Last revised March 28, 2012. Published June 10, 2012. This chapter should be cited as: Zhang Wendy, Y., de Almeida Patricia, E., and Wu Joseph, C., Teratoma formation: A tool for monitoring pluripotency in stem cell research (June 10, 2012), StemBook, ed. The Stem Cell Research Community, StemBook, doi/10.3824/stembook.1.53.1, http://www.stembook.org.

Copyright: © 2012 Wendy Y. Zhang, Patricia E. de Almeida, and Joseph C. Wu. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Correspondence: Joseph C. Wu, MD, PhD, Lorry Lokey Stem Cell Research Building, 265 Campus Drive, Room G1120B, Stanford, CA 94305-5111. Ph: 650-736-2246 Fax: 650-736-0234, E-mail: joewu@stanford.edu

Table 1. Summary of pluripotency testing methods

Pluripotency Assay	Purpose	Length of Assay	Nature of Assay	Strength/Definitiveness of Assay
Colony morphology	Verify ESC colony-like morphology of clustered, border-definied colonies.	10 minutes	*in vitro*	Low
Immunohistochemistry	Stain for standard pluripotency markers such as Oct4, Tra-1-60, Sox2, Tra-1-81, Nanog and SSEA.	1–4 days	*in vitro*	Medium
Real-time PCR	Detect and quantify expression levels of selected pluripotency genes; limited by gene number.	4–6 hours (from RNA extraction to RT-PCR	*in vitro*	Medium-High
Embryoid body formation and analysis	Test differentiation capability of PSCs to tissues of all 3 germ layers *in vitro* or *in vivo*; should be coupled with relative quantification expression of gene-expression from the three germ-layers, which can be done by RT-PCR. Genes include: Nanog, Oct4, Sox2, and Klf4 (pluripotency markers); Ncam and NeuroD (ectodermal markers); Runx2, HNF4a, and Nkx2.5 (mesodermal markers); and Sox17, Albumin, Glut2, and Insulin (endodermal markers).	2–3 weeks	*in vitro* *or* *in vivo*	Medium-High
Microarray	A comprehensive measurement of gene expression levels.	1–2 days	*in vitro*	Medium-High
Teratoma formation	Test differentiation capability into all 3 germ layers *in vivo*.	1–2 months	*in vivo*	High

more reliable and comprehensive confirmation than does testing cells on a simplified, artificial petri dish. This *in vivo* assay, coupled with noninvasive, longitudinal imaging, have proven to be invaluable not only in the visualization of stem cell survival and migration post-delivery, but also have been crucial in studying the safety and viability of future stem cell applications (11–13).

1.2 Teratoma biology

Teratomas are benign tumors characterized by their rapid growth *in vivo* and their haphazard mixture of tissues, and thus often have semi-semblances of organs, teeth, hair, muscle, cartilage, and even bone. In fact, it is rare that a teratoma does not contain remnants of all three germ layers. These are key characteristics of robust pluripotency and explain why teratoma formation is widely viewed in stem cell research as the "gold standard" for assessing pluripotency. Following their establishment *in vivo*, teratomas typically progress by remodeling their microenvironment to support their growth and the formation of blood vessels for nutrients. Upon engraftment, teratoma formation is affected by three main factors: PSC type, cell number, and delivery route. For the first major factor, teratoma incidence has been shown to vary depending on PSC type. One study observed human induced pluripotent stem cells (hiPSCs) teratoma formation rate to be slower than that of human embryonic stem cells (hESCs) due to the heterogeneity in hiPSC gene expression levels that suggests a less stable pluripotent state in these cells (14), but another study demonstrated hiPSCs to be more efficient (15). Such variability may be attributed to the different methods of reprogramming, techniques in differentiation, and original somatic sources used by different groups. For example, Miura et al. observed a difference in teratoma-forming propensity across neurospheres derived from varying iPSCs that were reprogrammed in the presence and absence of both c-*Myc* retroviral transduction and drug selection for *Nanog* and *Fbxo15* (16). The investigators delivered secondary neurospheres (free-floating aggregates of cultured neural stem cells) generated from 36 iPSC clones derived in 11 different ways into immunodeficient mice and observed that although *c-Myc* retrovirus did not significantly affect teratoma-forming propensity, iPSC tissue of origin did have an effect. A thorough comparison of tumorigenicity between iPSCs and ESCs is outlined and reviewed by Ben-David and Benvenisty (17). As to the site of cell implantation, some researchers have observed that injection site does affect teratoma formation efficiency (18, 19), while others have found little or no effect (14). Lastly, regarding the injected cell number, the "critical threshold" that needs to be achieved for teratoma formation has been investigated. For example, Lee et al. observed that the minimum number of cells needed to form intramyocardial teratomas was approximately 1×10^5 cells, compared to an approximate cell count of 1×10^4 for teratoma formation in skeletal muscles (20), though an earlier study had

observed that as few as $0.5 \times 10^3 - 1 \times 10^3$ ESCs were capable of inducing teratoma formation in subcutaneous dorsal regions (21).

1.3 Clinical implications of teratomas

The same pluripotent characteristic that makes hPSCs a powerful tool in the future of medicine also creates major clinical hurdles, highlighting the fine line that both separates and connects pluripotency and tumorigenicity. hESCs and human embryonal carcinoma cells (hECs) are two pluripotent cell types that share many characteristics. In the clinic, teratocarcinomas derived from hECs are most commonly found in the gonads and are one of the most common forms of cancer in young adult males (22,23). Teratoma formation from hPSC delivery in humans, on the other hand, has not yet been reported due to the predominantly pre-clinical stage stem cell therapy research currently occupies. However, in 2009 there was one reported incidence of donor stem cell-derived tumors in a boy who had received fetal neural stem cell transplantation for treatment of ataxia-telangiectasia in 2001. The patient started having recurrent headaches in 2005, which MRI scans later revealed to have been caused by abnormal growths in both his brain and spinal cord. Investigators biopsied the growths and confirmed them to be multifocal tumors (24). This report highlights on the potential risk of tumor formation in stem cell therapy as well as the urgent need to assess the safety of such therapies. Within that same year, Geron Corp. received a temporary FDA hold on the first ever FDA-approved clinical trial using hESC derivatives for spinal cord injury after observing a few incidents of animals developing microscopic, nonproliferative cysts at the lesion sites (25). The FDA hold was lifted approximately 10 months later, after Geron stated that it had determined the cysts "did not lead to any adverse consequences to the animals" (26). Most recently, in the first ever report of hESC-derived cell transplantation into humans, investigators saw no signs of tumorigenicity, ectopic tissue formation, rejection, or hyperproliferation after 4 months from subretinally transplanted hESC-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy (27). These developments underline the importance of carefully assessing stem cell tumorigenicity to evaluate complex risks and benefits for patients. As clinical translation of hPSCs progresses, the utilization of "gold standard" teratoma formation to test pluripotency as well as other hPSC behavior *in vivo* will continue to be indispensable for investigators.

B. Teratoma formation

2.1 Materials

Pluripotent stem cell culture

- hESC line or hiPSC line stably expressing firefly luciferase (Fluc), monomeric red fluorescent protein (mRFP), and herpes simplex virus thymidine kinase (HSVtk).
  - We have successfully and reproducibly performed teratoma formation assays using human embryonic stem cell lines H7, H9 (National Stem CellBank, cat. no. WA07, WA09, respectively), and iPSC lines generated in our lab expressing these reporter genes.
- To culture hPSCs on feeder layers: irradiated mouse embryonic fibroblasts (MEFs)
  - MEF growth medium: DMEM high glucose containing L-glutamine (Invitrogen, Gibco, Grand Island, NY; cat. no. 11965) and supplemented with 10% FBS (Invitrogen, Gibco, cat. no. 10437) and 5% penicillin/streptomycin (Invitrogen, Gibco, cat. no. 15070).
  - Gelatin (Sigma-Aldrich, St. Louis, MO; cat. no. G1890): Dilute to 0.1% solution and autoclave before using.
- 0.1 M PBS, pH 7.4, without Ca^{2+} and Mg^{2+} (Invitrogen, Gibco, Grand Island, NY; cat. no. 10010)
- Growth factor reduced, LDEV-free ES cell qualified Matrigel (BD Biosciences, San Jose, CA; cat. no. 354277)
- mTESR-1 hES Growth Medium (STEMCELL Technology, Vancouver, BC; cat. no. 05850)
- Collagenase Type IV (Invitrogen, Grand Island, NY; cat. no. 17104-019)
- Hydrophilic 0.45 μm syringe filter (i.e. Millex-HV Filter, 0.45 μm, PVDF, 13 mm, ethylene oxide sterilized. Millipore, Billerica, MA; cat. no. SLHV013SL)

Transplantation of cells into animals

- Mice or rat models (i.e., Charles River Laboratories, Wilmington, MA)
  - A variety of animal strains can be used. Immunodeficient strains (i.e., Nu/Nu nude, BC nude, or SCID for mice; Rowett nude or Athymic for rats), however, form teratomas at a higher incidence. There is a significant chance of cell rejection in non-immunocompromised models.
- Matrigel (BD Biosciences, San Jose, CA; cat. no. 354277)

- Hemacytometer or other cytometers
- 28.5 gauge insulin syringe
- Anesthesia system
  - We generally use isoflurane vapor in oxygen.
- Preferred: 37°C heat pad

2.2 Methods

The following steps delineate the culturing and harvesting of hPSCs pre-injection followed by animal transplantation. We followed standard lentiviral tranduction methods for stable integration of our reporter genes outlined by Sun et al. (28). Previous studies have demonstrated that expression of such reporter genes does not significantly affect ESC proliferation, viability, and differentiation (21,29–31). Our reporter genes consist of a triple fusion (TF) construct containing an ubiquitin promoter (other constitutive promoters can also be used) driving GFP for flow cytometry sorting and fluorescence imaging (FLI), firefly luciferase (Fluc) for bioluminescence imaging (BLI), and herpes simplex virus thymidine kinase (HSVtk) for PET imaging. The double fusion (DF) construct is the TF construct without the distal HSVtk component. Following transplantation, viable cells with integrated genes will readily produce the luciferase, which reacts with delivered D-luciferin to emit low-intensity photons that can be captured into images by cool-charged couple device camera. Similarly, viable cells expressing HSVtk reporter gene will react with intravenously administered radioactive reporter probe 9-4-[^{18}F]fluoro-3-(hydroxylmethylbutyl)guanine ([^{18}F]-FHBG) to produce high-energy photons detectable by PET.

1. Pre-coat tissue plates with Matrigel for at least 45 minutes in 37°C. For making Matrigel-coated plates, refer to Braam et al. (32) and BD's Matrigel manufacturer's information).
   - Note: Although our hPSC culturing was done on Matrigel-coated, feeder-free tissue plates, the culture of hPSCs on feeder layers is also a commonly used method. It is our experience, however, that a higher efficiency of transduction and more straightforward culturing conditions are better achieved under feeder-free conditions. For feeder layer preparation, pre-coat tissue culture plates with 0.1% gelatin (∼2 mL for six-well plate, ∼4 mL for 10-cm plates) and incubate at 37°C for at least 30 minutes. Afterwards, plate inactivated mouse embryonic fibroblasts (MEFs) at ∼90% confluency in each plate. Incubate for 24 hours at 37°C with MEF growth medium for strong attachment before plating ESCs or iPSCs.
2. Expand the DF or TF hPSCs on the Matrigel coated tissue plates until a sufficient number of cells for injection is achieved.
   - As previously mentioned in the introduction, varying cell numbers will form teratomas. We typically inject 100,000−1,000,000 cells to maximize teratoma formation efficiency. If stronger engraftment and faster teratoma growth are desired, a higher cell number should be delivered, volume permitting.
3. When the desired cell number is achieved, prepare cells for injection. First prepare the Collagenase Type IV for cell disaggregation. Prepare the Collagenase Type IV solution at a concentration of 50 mg/mL in PBS, pH 7.4; make sure to mix thoroughly. Approximately 1 ml per well for six-well plates and 4 ml for 10 cm plates are needed. Filter the homogenized solution through a hydrophilic 0.45 μm syringe filter.
4. Aspirate the mTeSR-1 cell medium and wash the cells gently with PBS.
5. Aspirate the PBS and add 1 ml per well for six-well plates and 4 ml for 10 cm plates of filtered Collagenase Type IV. Incubate at 37°C and 5% CO_2 for 45–90 minutes, until hPSC colonies completely detach. Other methods of harvesting such as dissociation (incubation for 2–4 min) or gentle scraping may be used.
   - Note: Single-cell dissociation of hPSC colonies should not be used and will not be achieved under these conditions; efficiency of teratoma formation is typically higher when cells are injected as small clusters or aggregates.
   - In parallel, if Matrigel had previously been aliquoted and stored in –80°C (the optimal condition for long term storage), thaw 40–50 μL of Matrigel per injection site (this could take up to 1 hour). Make sure to always keep Matrigel on ice as it will solidify rapidly at room temperature.
6. Centrifuge cells down at 500 g, 4°C for 5 minutes.
   - Note: When handling the hPSC pellets and resuspending the cells, it is extremely important to pipette GENTLY to avoid cell death.
7. Aspirate the supernatant and wash the cells with 4–5 mL of PBS by gentle swirling in the tube. Then centrifuge cells down at 500 g, 4°C for 5 minutes. Resuspend cells in PBS at a concentration of [desired number of cells]/[40–50 μL of PBS].
   - Note: Try to resuspend cells in a lower volume of PBS as a higher density of cells promotes survival and engraftment post-injection.

8. Add an equal volume of chilled, liquid Matrigel to the cell/PBS mixture and keep on ice until injection.
   - Note: It is important to always keep samples on ice to maximize cell viability upon delivery.
9. Using a 28.5 gauge syringe, inject the hPSCs into the desired anatomical locations for teratoma formation. Anesthetize and handle the animals according to respective institution's animal use protocol. We generally use isoflurane vaporized in oxygen at a constant flow of 1–2% for mice and 2–3% for rats; animals are placed into a container circulating the isoflurane for inhalation. Carefully monitor animals during the duration of anesthesia as death from overdosing and hypothermia from long periods of knockdown can occur.
   - Note: When drawing the cells into the syringe, do so slowly and gently to prevent shearing.
10. To enhance cell engraftment post-injection, allow the Matrigel/PBS/cell mixture to solidify in the localized transplantation site before allowing the animal to wake up. Keep the animal(s) under anesthesia, preferably on a 37°C heat pad, for 10–20 minutes after injection.
11. If the transplantation of cells required surgery, it is best to keep animals on a 37°C heat pad for 24 hours to avoid hypothermia during post-operative recovery. Place half of the cage on the heat pad and half off so as to allow the animals to choose a more comfortable mode of recovery.
    - Note: Imaging can be performed immediately following cell delivery or at later hours/days. It is preferable to allow animals to recover from anesthesia fully before subjecting them to extensive period of anesthesia imaging often required for imaging. If imaging is to be performed directly post-transplantation, use extra caution because during this time animals are much more susceptible to infections (especially post-surgery), are undergoing an extensive period of anesthesia, and are more likely to die from other complications.

A note on selection of anatomical locations for hPSC delivery: Implantation of cells for teratoma formation has been performed in the following anatomical locations: sub-renal capsule, subcutaneous, myocardium, intra-muscular, intra-hepatic, and in the brain (20,21,33–36). However, for the sake of pluripotency testing, the simplest and most efficient sites are intra sub-renal capsule, intra-muscular, and subcutaneous as the other sites require more technical expertise and often result in significantly higher mortality rates. We most commonly perform teratoma formations subcutaneously with a very high success rate (∼85–95% depending on the cell line).

C. Longitudinal tracking of teratoma formation *in vivo*

3.1 Materials

Note: Chemicals listed below may be hazardous. It is important that the Material Safety Data Sheet for all chemicals are reviewed before use.

Bioluminescence imaging (BLI)

- Xenogen *In Vivo* Optical Imaging System (Xenogen Corporation, Alameda, CA)
- Living Image Software (Caliper Life Sciences, Hopkinton, MA) such as Living Image 4.2
- D-luciferin (Biosynth, Hertfordshire, UK; cat. no. L-8220)
  - Dissolve 1 g of D-luciferin in 22 mL sterile PBS.
  - Store prepared D-luciferin solution in 1–1.5 mL aliquots at 20°C to prevent repeated freeze-thaw cycles. Try to maintain a consistent batch within each experiment to minimize variability in BLI signal due to variations in D-luciferin lots.
  - D-luciferin is light-sensitive and should be stored in light blocking tubes or under constant cover.
- 28.5 Gauge insulin syringe
- Anesthesia system
  - We generally use isoflurane vapor in oxygen.

Positron emission tomography (PET)

- MicroPET scanner such as the eXplore Vista (GE Healthcare, Waukesha, WI) or MicroPET rodent R4 (Concorde Microsystems, Knoxville, TN)
- MicroPET analysis software such as Igor image analysis software (WaveMetrics) or ASI Pro (Concorde Microsystems, Knoxville, TN)
- [^{18}F]-FHBG (can be synthesized by a cyclotron at respective institution's radiochemistry facility)
- Anesthesia system
  - We generally use isoflurane vapor in oxygen.

3.2 Methods

BLI

12. Anesthetize experimental and control animals with 1–2% isoflurane. Control animals are used to establish the background levels of bioluminescence. Intraperitoneally inject D-luciferin (375 mg/[kg animal body weight]) using a 28.5 gauge insulin syringe and wait for 10 minutes with animals still anesthetized to allow D-luciferin absorption and luciferase/luciferin reaction.
    - Note: Always weigh animals at each time point prior to injection in order to determine correct D-luciferin dose and to ensure consistent and accurate results.
13. Place the animal into the Xenogen IVIS machine's imaging chamber. Make sure the isoflurane is readily flowing into the chamber's designated anesthesia delivery channels.
14. Acquire images at 1–2 minute intervals for 30–45 minutes to ensure the capture of bioluminescence signal peak (photons/sec/cm^2/sr). Fluc signal usually peaks at 25–30 minutes.
15. To ensure the capture of Fluc signal peak, keep track of the BLI max radiance signal throughout the imaging session. Due to the nature of luciferase/luciferin reaction kinetics, as imaging approaches the 20 minute mark, maximum radiance signals will increase at a slower rate than before, which suggests that the the signal peak is approaching. At this time, make sure to acquire images more frequently (i.e., every 30 seconds) until the max radiance signal definitively starts to decrease. For a more detailed description of BLI techniques, refer to Wu et al. (37).
    - Note: As teratomas form in the animals, the signal might saturate so adjust exposure time and binning to ensure that an accurate and representative image is captured.
16. Image the same animals at set time points over the span of the designed experiment. We generally image the animals at the following time points: day 1, 2, 4, 7, 14, 21, etc.
    - Note: It is possible that signal may temporarily decrease around days 2–4 as there will be some initial cell death, but the signal should recover by days 7–10 if cells have engrafted and are viable.
17. Analyze acquired images using the Living Images Software; draw ROIs (regions of interest) to encircle the appropriate and intended area(s) of signal. Representative ROIs and longitudinal teratoma formation are shown in Figure 1A and 1B, respectively.

MicroPET

18. Freshly prepare a sufficient amount of [^{18}F]-FHBG. Normally, one should use ~100 μCi [^{18}F]-FHBG per mouse. One may refer to Yaghoubi et al. (38) for a more detailed description of [^{18}F]-FHBG preparation. Once again, make sure to include control animals (i.e., without cell transplantation or with sham surgery) for background signal acquisition.
    - Note: It is of great importance to go over the appropriate institutional radiation safety protocols and to take extreme caution when handling [^{18}F]-FHBG as it is radioactive and hazardous.
19. Draw 100 μCi [^{18}F]-FHBG per animal (measure with the dose calibrator) into a 28.5 gauge insulin syringe. Inject the appropriate dose of [^{18}F]-FHBG through the tail veins. Wait for ~60 minutes to allow the tracer to distribute throughout the body before imaging.
    - Note: Minimize the tail vein injection-time as this tracer is radioactive. To do so, practice tail vein injections ahead of time until the procedure becomes comfortable as such injections are relatively difficult. It may also be helpful to place heat packs on the animal's tail to dilate the tail vein prior to injection.
20. Anesthetize animal and secure it onto the bed of the mircoPET scanner. Image animal per microPET manufacturer's instructions.
21. Reconstruct the acquired images using microPET software programs. We generally utilize filtered back projection algorithms for image reconstruction.
22. Analyze these reconstructed images using Amide's A Medical Imaging Data Examiner (AMIDE) software or the programs provided by the microPET scanner manufacturer. AMIDE may be downloaded for free at http://www.amide.sourceforge.net.
23. Similar to BLI acquisitions, animals can be imaged longitudinally as needed. However, because weekly images and quantifications from BLI are already being acquired and radiotracers are quite expensive, microPET imaging may be performed either weekly or even monthly.

D. Teratoma explantation and analysis ex vivo

Four to six weeks post-injection, teratomas will have formed and are often very palpable if cells were transplanted subcutaneously or intraperitoneally. This time period, as previously mentioned, depends on the number of cells

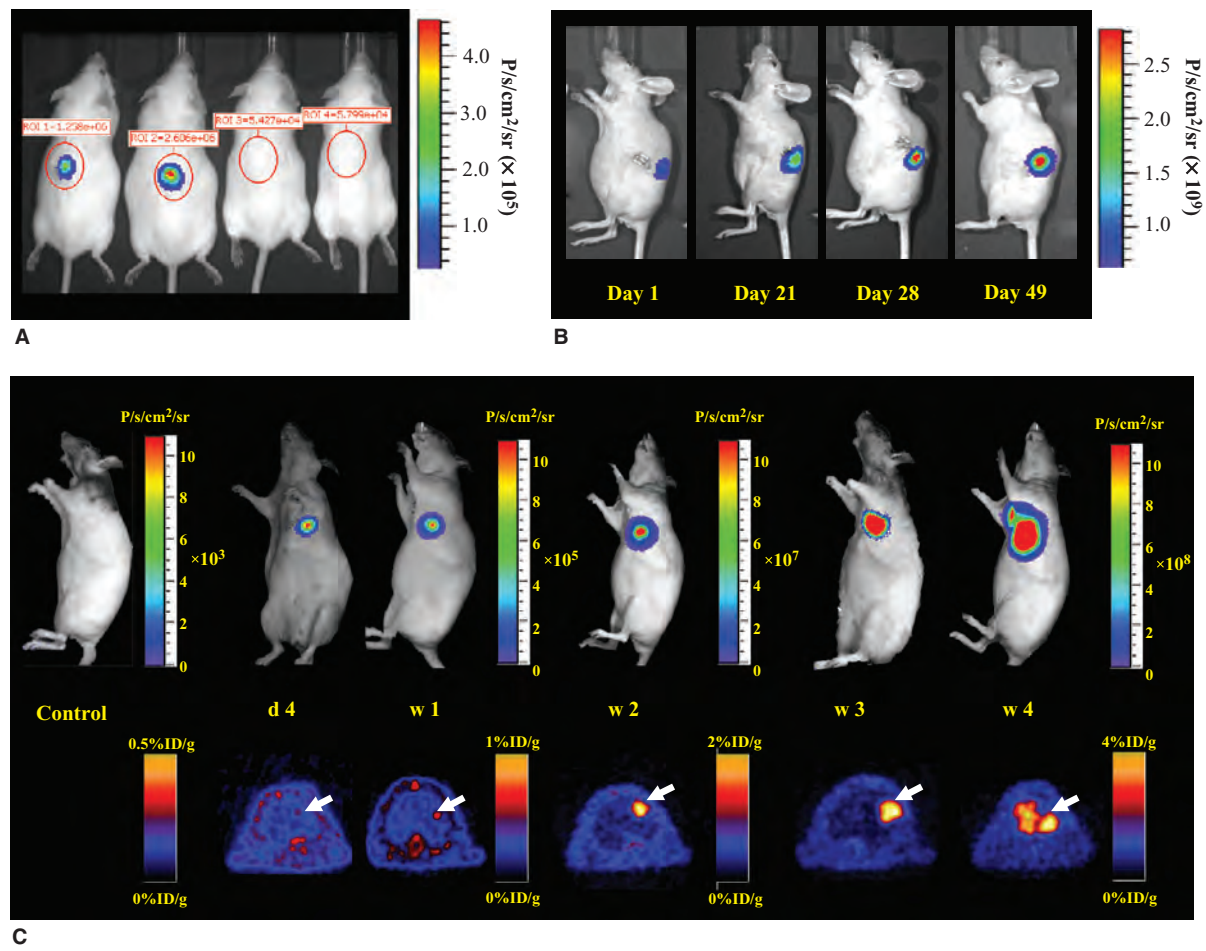

Figure 1. Noninvasive molecular imaging of transplanted hPSCs. (A) Image of an ROI drawn over localized bioluminescence signal using Living Image Software. **(B)** BLI of teratoma formation in the sub-renal capsule of immunodeficient NU/NU Nude mouse over the course of 7 weeks. **(C)** Longitudinal assessment of survival and proliferation by hESCs expressing TF reproter genes in the myocardium of adult nude rats over the course of 4 weeks; bioluminescence signal (top) and microPET signal (bottom). (Panel **C** reprinted with permission from [31]).

implanted. Teratomas should be removed once they reach a dimension when they affect the animals' behavior, motility, food and water intake (such parameters may vary between institutions). To analyze the characteristic composition of the teratomas, explant the teratomas and perform histological and immunohistochemical staining to visualize the interiors. Make sure to sacrifice and to process animals according to the institution's approved protocols.

4.1 Materials

Note: Chemicals listed below may be hazardous. It is important that the Material Safety Data Sheet for all chemicals are reviewed before use.

- Surgical scissors
- Surgical forceps
- 4% Paraformaldehyde (Electron Microscopy Science, Hatfield, PA; cat. no. 15700)
- OCT tissue embedding compound (Electron Microscopy Science, Hatfield, PA; cat. no. 62550-01)
- Ethanol
- Paraffin
- Tissue cassette (i.e., Simport biopsy cassettes, Fisher Scientific)
- Positively-charged Superfrost® microscope slides (Electron Microscopy Science, Fisher Scientific, or Menzel-Gläser)
- Cryostat sectioner
- Hematoxylin, Mayer's (Sigma-Aldrich, St. Louis, MO; ca. no. MHS1)

- Aqueous Eosin Y (EM Diagnostics, Gibbstown, NJ; cat no. 592-75)
- Xylene
- Gelatin (Sigma-Aldrich, St. Louis, MO; cat. no. G1890)
- Triton X-100 if antigens are intracellular (Sigma-Aldrich, St. Louis, MO; cat. no. 9002-93-1)
- Serum from host of primary antibodies
- Desired primary and secondary antibodies for immunohistochemistry staining.
- DAPI

4.2 Methods

Teratoma explantation

24. When teratomas are ready for explantation, anesthetize the mice and sacrifice via cervical dislocation, CO_2, or other methods approved by one's respective institution.
25. Carefully cut the area immediately surrounding the formed mass so as not to damage the teratoma but rather excise it as a whole (Figure 2A).
26. Gently grasp the teratoma and cut its connecting tissues to release the mass. Fix teratoma in 4% paraformaldehyde overnight and proceed to paraffin embedding, tissue sectioning, and immunostaining. Alternatively, embed tissue in OCT and freeze it for cryosectioning. (Explanted teratoma - Figure 2B).
    - Note: To preserve tissues for future molecular analyses, samples can be snap-frozen in liquid nitrogen following explantation and stored at −80°C until further processing. Previous studies have determined that there is no visible DNA degradation after 24 weeks of storage and only mild degradation following 1 year in freeze-dried samples preserved at room temperature (39,40). RNA, however, is more susceptible to degradation so samples must be snap-frozen imediately following tissue explantation and stored at −70°C or lower to prevent RNA degradation (41,42). Alternatively, samples can be preserved in a RNA stabilization reagent such as RNAlater® (Qiagen, Valcencia, CA) for up to 1 day at 37°C, 7 days at 18 to 25°C, or 4 weeks at 2 to 8°C. The use of such stabilization reagent is a practical alternative for when samples must be transported or shipped because it does not require liquid nitrogen or dry ice. Tissue samples should be stored at −70°C or lower. DNA and RNA can also be extracted from tissue samples immediately after explantation.

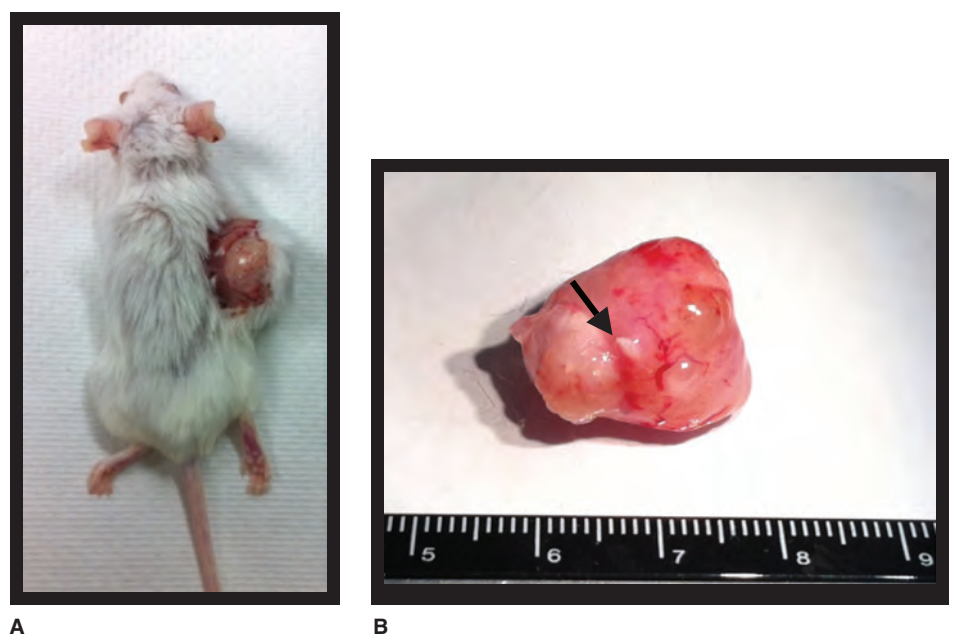

Figure 2. Gross images of teratoma growth in subcutaneous tissue of mouse. (A) The teratoma mass formed in the subcutaneous region of an immunodeficient SCID mouse pre-explantation. **(B)** Image of the explanted teratoma. Notice the presence of blood vessels and varied composition of the tumor, visible even without histological sectioning. There is a sharp, white tissue mass (black arrow) protruding from the center of the teratoma that resembles cartilage or bone.

Histological and immunohistochemical staining

27. Dilute paraformaldehyde (PFA) to a 4% solution in 0.1 M PBS, pH 7.4, and make enough for complete immersion of the teratoma.
28. After explantation, place the teratoma in the 4% PFA solution and fix the mass for 24–48 hours at room temperature.
29. After fixing, transfer mass into 70% ethanol. This will start the dehydration process. Move up the gradient of ethanol in a series over time (70% for 1 hour, 90% for another hour, 95% for 1 hour, and 100% for 1–2 hours).
    - Note: Excess ethanol must be removed prior to next steps.
30. Remove fat tissues surrounding teratoma that will make the paraffin blocks difficult to cut, and immerse tissue in xylene for 2 hours (transfer tissue to fresh xylene at 1 hour) at room temperature.
    - Note: Xylene is hazardous in case of eye contact (irritant), skin contact (irritant, permeator), ingestion, or inhalation. Use with caution.
31. Transfer tissue to a standard immunohistochemistry cassette and add liquid paraffin that has been heated to 60°C to the tissue. Allow the paraffin to harden.
    - Note: This tissue block can now be stored for a long time with little degradation.
32. Cryosection the paraffin-embedded teratomas into 4–6 μm serial sections, mount onto microscope slides, stain with hematoxylin and eosin, and analyze (Figure 3).

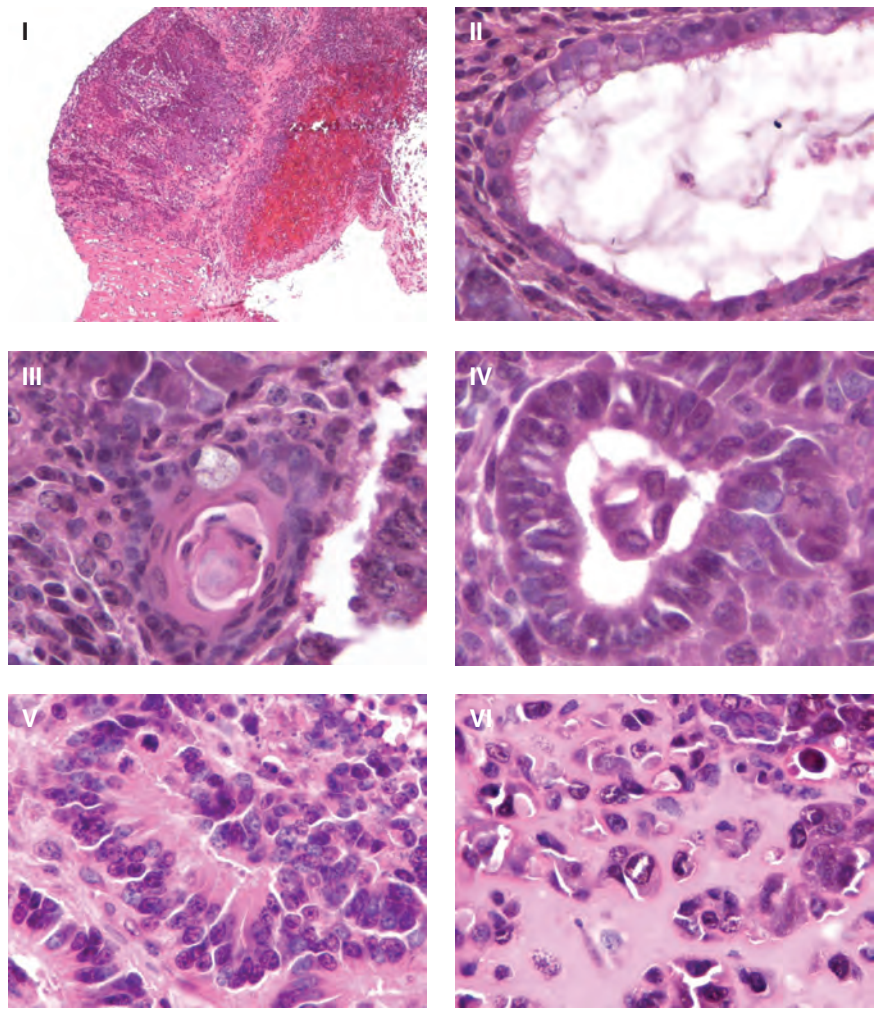

Figure 3. Histological characterization of an explanted intramyocardial teratoma formed from undifferentiated mouse ESCs. Histological analysis of a representative explanted intramyocardial teratoma section showed (I) anterior wall of the myocardium injected with cells surrounding area of hemorrhage (40×); (II) respiratory epithelium with ciliated columnar and mucin-producing goblet cells (1000×); (III) squamous cell differentiation with keratin pearl; (IV) nonciliated columnar gland (1000×); (V) rosette consistent with neuroectodermal differentiation (1000×); and (VI) osteoid (nonmineralized bone) formation (1000× magnification). Reprinted with permission from [31].

- Note: H&E stained slides should be interpreted by an expert pathologist.
- For a more detailed protocol on embedding and sectioning, refer to protocols produced by Fischer et al. (43,44).

33. If immunohistochemistry staining is needed, deparaffinize the slides using xylene and rehydrate using distilled water.
34. First block endogenous peroxidase enzyme activity with a 3% hydrogen peroxidase solution in methanol for 30–45 minutes at room temperature.
35. Wash the slides with PBS (pH 7.4) three times (5 minutes each).
36. Block the slides with 10% serum (serum type may vary depending on primary and secondary antibodies' hosts), 0.1% Triton X-100 (if antigens are intracellular), and 0.1–0.2% gelatin in PBS for 1 hour at room temperature.
    - Note: Triton X-100 is hazardous in case of eye contact, ingestion, or inhalation. Use with caution.
37. Incubate in primary antibody diluted in blocking buffer (from step 36) for 1–2 hours at room temperature or overnight at 4°C. Optimal antibody dilution should be determined by titration.
38. Wash samples gently three times with PBS (5 minutes each wash).
39. Incubate in fluorescent-conjugated secondary antibody diluted in blocking buffer for 30–60 minutes at room temperature.
40. Wash samples gently three times with PBS (5 minutes each wash).
41. Add DAPI (1:1000) for 5–10 minutes at room temperature for nuclear counter-staining.
42. Wash samples once with PBS. Carefully remove excess PBS, add mounting media and coverslip. Allow mounting media to harden prior to immunofluorescence microscopy.

E. Outsourcing

Labs from various research institutions and commercial companies offer teratoma formation and analysis services. For example, Applied StemCell, Inc. offers ESC/iPSC characterization via teratoma formation, EB formation, and publication quality analysis at a cost of around $3000 or more and a turnaround time of 12–15 weeks. These available services can be found at http://www.appliedstemcell.com/product/lists/8. If experimental resources are limited, this might be an effective means of teratoma formation, but the high cost and lengthy duration of service should be factored into the experimental design.

F. Summary and conclusion

Overview Timeline

*DF construct (eGFP-Fluc) and TF construct (mRFP-Fluc-HSVtk) lentiviral production.

4 days

*Lentiviral transduction of hPSC cells.

3–5 days

*Selection of DF or TF transduced hPSC cell clones.

3–6 weeks

(Note: * Steps are outlined in detail by Sun et al. [28])

Steps 1–2 Culture and expand hPSCs until sufficient amount achieved.

Multiple days-weeks. Exact timeframe varies depending on the number of cells needed. hPSC often can be passaged every 3 days at a 1:3 ratio.

Steps 3–8 Harvest and prepare DF or TF hPSCs in Matrigel for transplantation.

45–90 minutes

Steps 9–11 Cell implantation into animals.

30–60 minutes

Step 12–17 Molecular imaging of hPSC cell behavior *in vivo*: BLI.

1–2 hours weekly or monthly

Step 18–23 Molecular imaging of hPSC cell behavior *in vivo*: microPET.

1–2 hours weekly or monthly

Step 24–26 Explantation of teratoma.

15–30 minutes

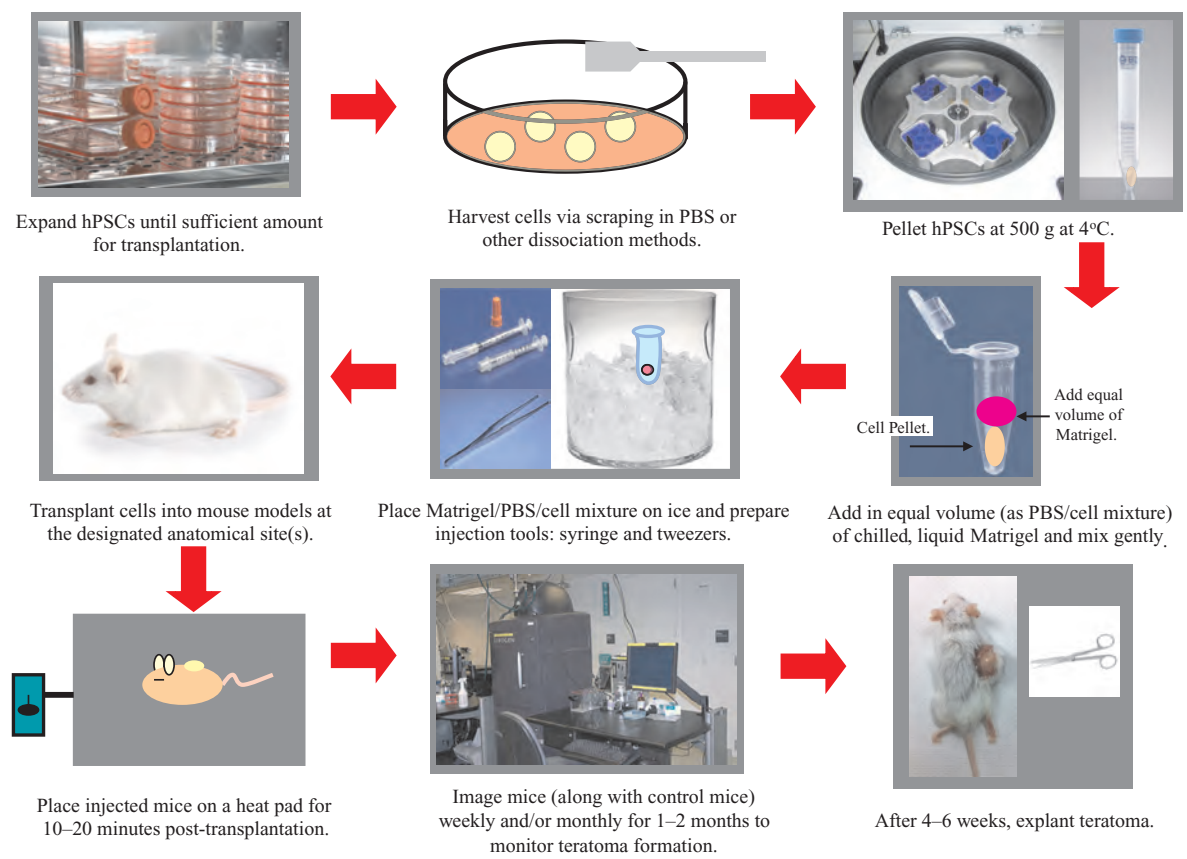

Figure 4. Schematic of teratoma formation. An overview of teratoma formation: culture hPSCs until the desired amount is achieved, harvest the cells using scrapers or Collagenase IV, pellet cells, add equal volume of liquid Matrigel to the cell mixture (make sure to keep cells on ice), transplant mixture into mouse models, allow mice to recover on heat pad, image mice regularly, and explant teratomas for analysis.

Step 27–42 Histological and immunohistochemical staining, processing, and analysis of the teratomas.

1–4 days

As human pluripotent stem cells and their derivatives approach clinical use, it is of paramount importance that we are able to accurately and comprehensively characterize their pluripotency and behavior *in vivo*. To accomplish such tasks is no small feat, but teratoma formation assay (summarized in Figure 4) has proven to be a key tool for researchers. This pluripotency testing method can study hPSC behavior not only in a more functional manner, but also in a more representative manner, because it examines the anatomical microenvironment *in vivo* rather than relying on that of a petri dish. Therefore, teratoma formation assays have demonstrated great potential to significantly help investigators understand both basic stem cell biology and tumorigenic potential of therapies prior to their clinical use.

G. Acknowledgements

This work was supported in part by Stanford VPUE (WYZ) and Burroughs Wellcome Foundation, NIH DP2OD004437, and NIH R01EB009689 (JCW). We thank Dr. Joseph Gold and Blake Wu for careful proofreading.

References

1. Discher, DE., Mooney, DJ., and Zandstra, PW. (2009). Growth factors, matrices, and forces combine and control stem cells. Science *26324*(5935), 1673–1677.

2. Sato, N., Meijer, L., and Skaltsounis, L., et al. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med *10*(1), 55–63.

3. Schuldiner, M., Yanuka, O., and Itskovitz-Eldor, J., et al. (2000). Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A *10;97*(21), 11307–11312.

4. Xu, C., Rosler, E., and Jiang, J., et al. (2005). Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells *23*(3), 315–323.

5. Herszfeld, D., Wolvetang, E., and Langton-Bunker, E., et al. (2006). CD30 is a survival factor and a biomarker for transformed human pluripotent stem cells. Nat Biotechnol *24*(3), 351–357.

6. Laflamme, MA., Chen, KY., and Naumova, AV., et al. (2007). Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol *25*(9), 1015–1024.

7. Lombardo, A., Genovese, P., and Beausejour, CM., et al. (2007). Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotechnol *25*(11), 1298–1306.

8. Lebkowski, JS., Gold, J., and Xu, C., et al. (2001). Human embryonic stem cells: culture, differentiation, and genetic modification for regenerative medicine applications. Cancer J *7*(Suppl 2), S83–S93.

9. Zou, J., Maeder, ML., and Mali, P., et al. (2009). Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. Cell Stem Cell *25*(1), 97–110.

10. Gropp, M., Itsykson, P., and Singer, O., et al. (2003). Stable genetic modification of human embryonic stem cells by lentiviral vectors. Mol Ther *7*(2), 281–287.

11. Kooreman, NG., and Wu, JC. (2010). Tumorigenicity of pluripotent stem cells: biological insights from molecular imaging. J R Soc Interface *7*(Suppl 6), S753–S763.

12. Cao, F., Drukker, M., and Lin, S., et al. (2007). Molecular imaging of embryonic stem cell misbehavior and suicide gene ablation. Cloning Stem Cells *Spring; 9*(1), 107–117.

13. Cao, F., Li, Z., and Lee, A., et al. (2009). Noninvasive de novo imaging of human embryonic stem cell-derived teratoma formation. Cancer Res *69*(7), 2709–2713.

14. Gutierrez-Aranda, I., Ramos-Mejia, V., and Bueno, C. (2010). Human induced pluripotent stem cells develop teratoma more efficiently and faster than human embryonic stem cells regardless the site of injection. Stem Cells *28*(9), 1568–1570.

15. Narsinh, KH., Sun, N., and Sanchez-Freire, V., et al. (2011). Single cell transcriptional profiling reveals heterogeneity of human induced pluripotent stem cells. J Clin Invest *121*(3), 1217–1221.

16. Miura, K., Okada, Y., and Aoi, T., et al. (2009). Variation in the safety of induced pluripotent stem cell lines. Nat Biotechnol *27*(8), 743–745.

17. Ben-David, U., and Benvenisty, N. (2011). The tumorigenicity of human embryonic and induced pluripotent stem cells. Nat Rev Cancer *11*(4), 268–277.

18. Prokhorova, TA., Harkness, LM., and Frandsen, U., et al. (2009). Teratoma formation by human embryonic stem cells is site-dependent and enhanced by the presence of Matrigel. Stem Cells Dev *18*(1), 47–54.

19. Cooke, MJ., Stojkovic, M., and Przyborski, SA. (2006). Growth of teratomas derived from human pluripotent stem cells is influenced by the graft site. Stem Cells Dev *15*(2), 254–259.

20. Lee, AS., Tang, C., and Cao, F., et al. (2009). Effects of cell number on teratoma formation by human embryonic stem cells. Cell Cycle *15;8*(16), 2608–2612.

21. Cao, F., van der Bogt, KE., and Sadrzadeh, A., et al. (2007). Spatial and temporal kinetics of teratoma formation from murine embryonic stem cell transplantation. Stem Cells Dev *16*(6), 883–91.

22. Solter, D. (2006). From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research. Nat Rev Genetics *7*(4), 319–327.

23. Andrews, PW.. (1988). Human teratocarcinomas. Biochem Biophys Acta *948*(1), 17–36.

24. Amariglio, N., Hirshberg, A., and Scheithauer, BW., et al. (2009). Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med *6*, 1000029.

25. Geron Corporation. (2010). Geron's IND for Spinal Cord Injury Placed on Hold [Press release]. Retrieved from http://ir.geron.com/phoenix.zhtml?c=67323&p=irol-newsArticle&ID=1636249&highlight=.

26. Johnson, S. (2010). FDA approves Geron's groundbreaking study of embryonic cells. *San Jose Mercury News*. Retrieved from http://www.geneticsandsociety.org/article.php?id=5305.

27. Schwartz, SD., Hubschman, JP., and Heilwell, G., et al. (2012). Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet *379*(9817), 713–720.

28. Sun, N., Lee, A., and Wu, JC. (2009). Long term non-invasive imaging of embryonic stem cells using reporter genes. Nat Protoc *4*(8), 1192–1201.

29. Wu, JC., Cao, F., and Dutta, S., et al. (2006). Proteomic analysis of reporter genes for molecular imaging of transplanted embryonic stem cells. Proteomics *6*(23), 6234–6249.

30. Wu, JC., Spin, JM., and Cao, F., et al. (2006). Transcriptional profiling of reporter genes used for molecular imaging of embryonic stem cell transplantation. Physiol Genomics *25*(1), 29–38.

31. Cao, F., Lin, S., and Xie, X., et al. (2006). In vivo visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery. Circulation *113*(7), 1005–1014.

32. Braam, SR., Denning, C., and Matsa, E., et al. (2008). Feeder-free culture of human embryonic stem cells in conditioned medium for efficient genetic modification. Nat Protoc *3*(9), 1435–1443.

33. Tang, C., Lee, AS., and Volkmer, JP., et al. (2011). An antibody against SSEA-5 glycan on human pluripotent stem cells enables removal of teratoma-forming cells. Nat Biotechnol *29*(9), 829–834.

34. Nussbaum, J., Minami, E., and Laflamme, MA., et al. (2007). Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J *21*(7), 1345–1357.

35. Lees, JG., Lim, SA., and Croll, T., et al. (2007). Transplantation of 3D scaffolds seeded with human embryonic stem cells: biological features of surrogate tissue and teratoma-forming potential. Regen Med *2*(3), 289–300.

36. Kawai, H., Yamashita, T., and Ohta, Y., et al. (2010). Tridermal tumorigenesis of induced pluripotent stem cells transplanted in ischemic brain. J Cereb Blood Metab *30*(8), 1487–1493.

37. Wu, JC., Sundaresan, G., and Iyer, M., et al. (2001). Noninvasive optical imaging of firefly luciferase reporter gene expression in skeletal muscles of living mice. Mol Ther *4*(4), 297–306.

38. Yaghoubi, SS., Jensen, MC., and Satyamurthy, N., et al. (2009). Noninvasive detection of therapeutic cytolytic T cells with 18F-FHBG PET in a patient with glioma. Nat Clin Pract Oncol *6*(1), 53–58.

39. Takahashi, R., Matsuo, S., and Okuyama, T., et al. (1995). Degradation of macromolecules during preservation of lyophilized pathological tissues. Pathol Res Pract *191*, 420–426.

40. Matsuo, S., Toyokuni, S., and Osaka, M., et al. (1995). Degradation of DNA in dried tissues by atmospheric oxygen. Biochem Biophys Res Commun *208*, 1021–1027.

41. Eikmans, M., Baelde, HJ., and De Heer, E., et al. (2000). Processing renal biopsies for diagnostic mRNA quantification: improvement of RNA extraction and storage conditions. J Am Soc Nephrol *11*(5), 868–873.

42. Roos-van Groningen, MC., Eikmans, M., and Baelde, HJ., et al. (2004). Improvement of extraction and processing of RNA from renal biopsies. Kidney Int *65*(1), 97–105.

43. Fischer, AH., Jacobson, KA., and Rose, J., et al. (2008). Paraffin embedding tissue samples for sectioning. CSH Protoc. 1:pdb.prot4989.

44. Fischer, AH., Jacobson, KA., and Rose, J., et al. (2008). Cutting sections of paraffin-embedded tissues. CSH Protoc. 1:pdb.prot4987.