

Teratoma Formation Assay for Assessing Pluripotency and Tumorigenicity of Pluripotent Stem Cells

Shingo Miyawaki^{1, 2, 3}, Yohei Okada^{2, 4}, Hideyuki Okano² and Kyoko Miura^{1, 2, *}

¹Biomedical Animal Research Laboratory, Institute for Genetic Medicine, Hokkaido University, Hokkaido, Japan; ²Department of Physiology, Keio University School of Medicine, Tokyo, Japan; ³Division of Epigenome Dynamics, Institute for Advanced Medical Science, Tokushima University, Tokushima, Japan; ⁴Department of Neurology, Aichi Medical University School of Medicine, Aichi, Japan

*For correspondence: miura@igm.hokudai.ac.jp

[Abstract] Pluripotent stem cells such as induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) form teratomas when transplanted into immunodeficient mice. As teratomas contain all three germ layers (endoderm, mesoderm, ectoderm), teratoma formation assay is widely used as an index of pluripotency (Evans and Kaufman, 1981; Hentze *et al.*, 2009; Gropp *et al.*, 2012). On the other hand, teratoma-forming tumorigenicity also represents a major risk factor impeding potential clinical applications of pluripotent stem cells (Miura *et al.*, 2009; Okano *et al.*, 2013). Recently, we reported that iPSCs derived from naked mole-rat lack teratoma-forming tumorigenicity when engrafted into the testes of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice due to an ES cell-expressed Ras (ERAS) and Alternative reading frame (ARF)-dependent tumor-suppression mechanism specific to this species (Miyawaki *et al.*, 2016). Here, we describe a method for transplanting pluripotent stem cells into the testes of NOD/SCID mice to generate teratomas for assessing the pluripotency and tumorigenicity.

Keywords: Pluripotent stem cells, Teratoma, Tumorigenicity, NOD/SCID mice, Testis

[Background] iPSCs and ESCs are exploited for applications in cell transplantation therapy for regenerative medicine. However, these cells form tumors called teratoma containing differentiated tissues when transplanted into immune-deficient mice. Therefore, the risk of their teratoma-forming tumorigenicity limits their clinical application. Several studies have reported the methods to overcome the risk of teratoma-forming tumorigenicity (Itakura *et al.*, 2017; Vazquez-Martin *et al.*, 2012). Recently, we reported that iPSCs derived from naked mole-rats lack teratoma-forming tumorigenicity when engrafted into the testes of NOD/SCID mice due to species-specific activation of tumor-suppressor ARF and a disruption mutation of the oncogene ERAS (Miyawaki *et al.*, 2016). In this protocol, we describe a method for transplanting pluripotent stem cells into the testes of NOD/SCID mice to generate teratomas. This approach can minimize the immune rejection due to the presence of the testicular–blood barrier (Cheng and Mruk, 2012). In addition, this approach is advantageous because transplanted cells are easily identified around the injection site even when they do not form tumors. Thus, the

technique described herein is useful for assessing the pluripotency and tumorigenicity of pluripotent stem cells.

Materials and Reagents

1. Falcon 15 ml conical tube (Corning, Falcon®, catalog number: 352196)
2. BIO-BIK 1.5 ml centrifuge tube (Ina-optika, catalog number: CF-0150)
3. 26-gauge needle (Terumo Medical, catalog number: NN-2613S)
4. Laboratory wipe (Kimwipes) (KCWW, Kimberly-Clark, catalog number: 62011)
5. 0.22-µm filter unit (TPP Techno Plastic Products, catalog number: 99155)
6. 10 cm tissue culture dish (Corning, Falcon®, catalog number: 353046)
7. Pluripotent stem cells stably expressing a fluorescent marker, such as green fluorescent protein (GFP), for detection of injected cells

Note: We used naked mole-rat (NMR) iPSCs (clones 24 and 27), mouse iPSCs (clones 20D17 and 38C2: Okita et al., 2007), and human iPSCs (clone 201B7: Takahashi et al., 2007), as described in our previous study (Miyawaki et al., 2016).

8. Sterile phosphate-buffered saline (PBS) (NACALAI TESQUE, catalog number: 27575-31)
9. 0.1% trypsin-EDTA (Thermo Fisher Scientific, Gibco™, catalog number: 15090046)
10. Fetal bovine serum (Biowest, catalog number: S1820-500)
11. Dulbecco's modified Eagle's medium (Sigma-Aldrich, catalog number: D5796)
12. Penicillin/streptomycin (Wako Pure Chemical Industries, catalog number: 168-23191)
13. Non-essential amino acids (NACALAI TESQUE, catalog number: 06344-56)
14. β-Mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
15. L-Glutamine (NACALAI TESQUE, catalog number: 04260-64)
16. DMEM/F12 (Sigma-Aldrich, catalog number: D6421)
17. KnockOut™ Serum Replacement (Thermo Fisher Scientific, Gibco™, catalog number: 10828028)
18. Fibroblast growth factor 2 (PeproTech, catalog number: 100-18B)
19. Isoflurane (Wako Pure Chemical Industries, catalog number: 099-06571)
20. 70% ethanol
21. Ampicillin (Wako Pure Chemical Industries, catalog number: 012-23303)
22. Paraformaldehyde (Junsei Chemical, catalog number: 58295-1201)
23. Sodium hydroxide (NACALAI TESQUE, catalog number: 31511-05)
24. Paraffin
25. Hematoxylin and eosin (HE)
26. iPS medium for mouse iPSCs (see Recipe 1)
27. iPS medium for NMR and human iPSCs (see Recipe 2)
28. 4% paraformaldehyde (PFA) (see Recipe 3)

Equipment

1. Two humidified 5% CO₂ cell culture incubators: 32 °C for NMR iPSCs, 37 °C for mouse and human iPSCs (Thermo Fisher Scientific, Thermo Scientific™, model: Heracell™ 150i)
2. Centrifuge (TOMY DIGITAL BIOLOGY, model: AX-501)
3. Coulter counter (Beckman Coulter, catalog number: 6605698)
4. Isoflurane vaporizer, chamber, and nose cone (Shinano, catalog number: SN-487-OT)
5. Heating pad (Nissin, model: NHP-M30N)
6. Operating scissors and tweezers (see Figure 1)
7. 25 µl Hamilton syringe (Hamilton, model: 702 N, catalog number: 80400)
8. Microbalance (Shimadzu, model: ATX84)
9. Icebox



Figure 1. Surgical instruments used in this protocol

Software

1. GraphPad Prism software (GraphPad software, model: version 6.0)

Procedure

A. Preparing cells for transplantation

1. Maintain iPSCs according to the previous reports (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007; Miyawaki *et al.*, 2016). It is necessary to prepare at least 2×10^6 cells.
2. Replace with fresh culture medium 1 h before dissociation.
3. Aspirate culture medium and wash twice with PBS.
4. Detach the cells using trypsin-EDTA (1 ml/10 cm dish).
5. Stop the trypsin reaction with 15% fetal bovine serum-containing medium for mouse iPSCs (see Recipe 1) or trypsin inhibitor for NMR and human iPSCs (see Recipe 2).

6. Collect cells in a 15 ml conical tube, and re-suspend cells in 4 ml of PBS.
7. Count cells using a Coulter counter.
8. Centrifuge cells at 200 x *g* for 5 min at room temperature.
9. Aspirate supernatant and re-suspend iPSCs in an appropriate volume of PBS to yield a final concentration to 5 x 10⁷ cells/ml (equal to 1 x 10⁶ cells/20 µl).
10. Transfer cells into a 1.5 ml tube.
11. Place cells on ice until used for injection.

B. Injection into mice

1. (Optional) Administer ampicillin-containing water (1 g/L) orally to mice from 3 days before until 1 week after surgery.
2. Place NOD/SCID mice on a heating pad and anesthetize with isoflurane.
3. Wash surgical area with 70% ethanol and remove hair from the dorsal region.
4. Make a 1 cm incision above the top of the preputial gland and open the abdomen.
5. Carefully pull out the epididymal fat pad along with the testes (Figure 2A).
6. Fill a Hamilton syringe with 20 µl (1 x 10⁶ cells) of cell suspension.
7. Cut the tunica vaginalis of the testis using a 26-gauge needle (Figure 2B).
8. Use the Hamilton syringe to inject 20 µl of cell suspension (Figure 2C).
9. Slowly remove the needle to avoid backflow of cells.
10. Return the testes to their original location.
11. Suture the wound.

Note: Demonstration of the injection procedure can be viewed in Video 1.

Video 1. Injection procedure. The cell suspension is blue stained for demonstration.



C. Defining pluripotency and tumorigenicity

1. At 4, 10, 20, or 28 weeks after transplantation, anesthetize and sacrifice the mice in accordance with the Guide for the Care and Use of Laboratory Animals. Thereafter, dissect the tumors and testes (Figures 2D and 2E).

Note: Tumors could be observed 4 weeks and 10 weeks after the injection of mouse iPSCs and human iPSCs, respectively.

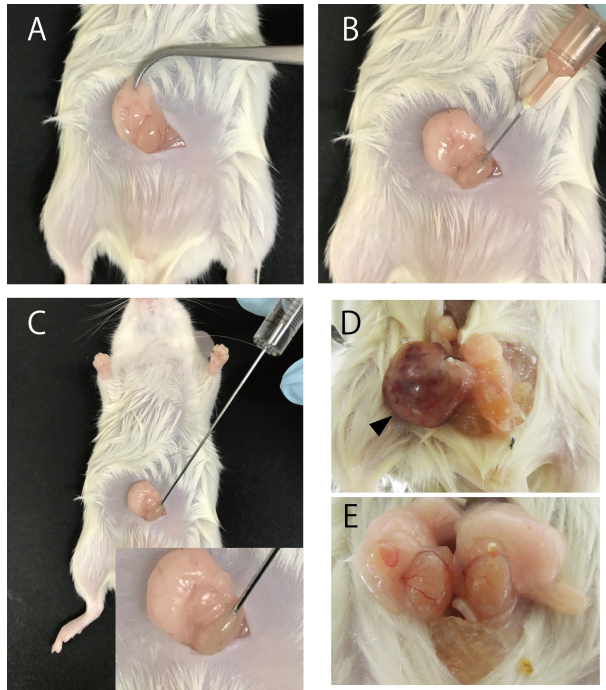


Figure 2. Injection iPSCs into testis of NOD/SCID mice. Transplant procedure (A-C). Representative photographs of teratoma (D) and testis without tumor formation (E). Arrowhead indicates teratoma formed by mouse iPSCs.

2. After removing moisture within the laboratory wipe, measure the weight of the tumor.
3. Fix the tumors or testes overnight in PBS containing 4% PFA (see Recipe 3) for embedding in paraffin.
4. Stain sections with hematoxylin and eosin (HE), or subject to immunohistochemical analysis, to detect expression of GFP and differentiation markers. The section of tumors and testes in our previous study are shown in Figure 3 (Miyawaki *et al.*, 2016).

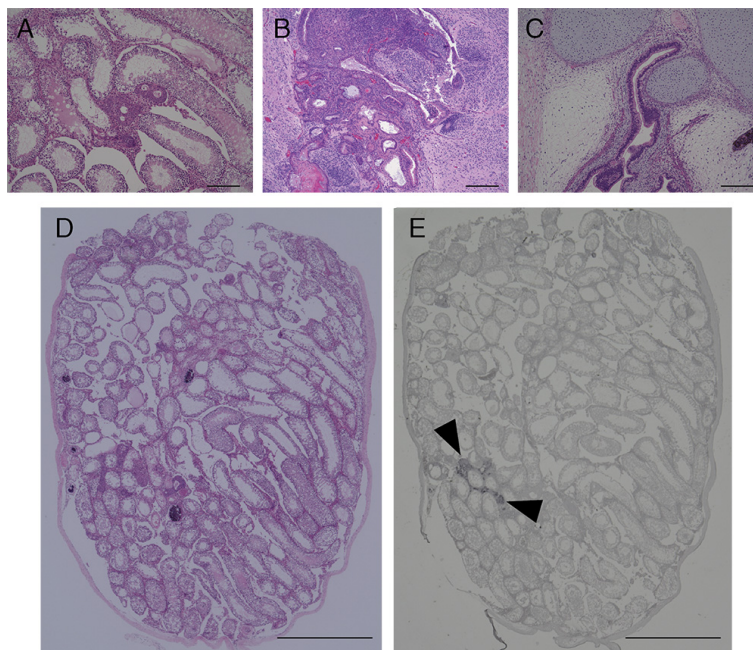


Figure 3. Image of HE and immunohistochemical stained section of tumors and testis after transplantation of iPSCs. HE staining section of tumors and testis of mice transplanted with NMR iPSCs 10 weeks after transplantation (A and D), mouse iPSCs 4 weeks after transplantation (B) or human iPSCs 10 weeks after transplantation (C). Immunohistochemical analysis of GFP (E). Arrowheads indicate the transplanted GFP-labeled NMR iPSCs. Scale bar, 200 μ m (A-C) and 1 cm (D and E).

Data analysis

The weights of tumors or testes were analyzed after logarithmic transformation (arbitrary units: $6 + \log 2$). The Bartlett test was used to verify equal variances across populations. The statistical significance of the difference between experimental groups was determined by one-way analysis of variance or the Kruskal Wallis test followed by the Dunn's method using GraphPad Prism 6 software. Statistical significance was considered when $P < 0.05$ and all data are displayed as mean $\pm$ SEM as in Miyawaki *et al.*, 2016.

Weight of tumors and testes in our previous study are shown in Table 1 for reference (Miyawaki *et al.*, 2016).

Table 1. Weight of each teratoma or testis, and mean weight

	Weight (g)									
	mean	SD	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8
Without injection	0.082	0.007	0.090	0.089	0.085	0.085	0.082	0.078	0.073	0.073
NMR iPSCs	0.073	0.018	0.099	0.087	0.085	0.074	0.073	0.064	0.060	0.042
mouse iPSCs	1.73	0.999	3.123	2.890	2.143	1.989	1.691	0.914	0.555	0.538
human iPSCs	2.902	2.057	5.665	4.994	4.825	2.591	2.583	1.663	0.711	0.187

Recipes

1. Mouse iPSCs medium

Dulbecco's modified Eagle's medium (Sigma-Aldrich)
15% fetal bovine serum (JRH or BioWest)
1% penicillin/streptomycin (Wako Pure Chemical Industries)
2 mM L-glutamine (NACALAI TESQUE)
0.1 mM non-essential amino acids (NACALAI TESQUE)
0.1 mM β -mercaptoethanol (Sigma-Aldrich)
Human recombinant leukemia inhibitory factor (NACALAI TESQUE)
Filtered by 0.22- μ m filter unit, stored at 4 °C

2. NMR and human iPSCs medium

DMEM/F12 (Sigma-Aldrich)
20% KnockOut Serum Replacement (Thermo Fisher Scientific)
1% penicillin/streptomycin (Wako Pure Chemical Industries)
2 mM L-glutamine (NACALAI TESQUE)
0.1 mM non-essential amino acids (NACALAI TESQUE)
0.1 mM β -mercaptoethanol (Sigma-Aldrich)
4 ng/ml fibroblast growth factor 2 (PeproTech)
Filtered by 0.22- μ m filter unit, stored at 4 °C

3. 4% PFA

4 g PFA
100 ml of PBS
Heat on hot plate stirrer
Add 20 μ l of 1 M sodium hydroxide to dissolve residual PFA
Aliquot and freeze at -20 °C for storage

Note: The investigator must wear a facemask, gloves, lab coat and eye protection during PFA preparation.

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