Protocol 7

Reprogramming Mouse Fibroblasts with *piggyBac* Transposons

This protocol describes the procedure for doxycycline-inducible reprogramming when transcription factors are introduced into mouse fibroblasts or other cells using Protocol 5 or 6.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Recipes for reagents specific to this protocol, marked <R>, are provided at the end of the protocol. See Appendix 1 for recipes for commonly used stock solutions, buffers, and reagents, marked <A>.

Reagents

Cells with reprogramming plasmids introduced by Protocol 5 or 6
Complete ES cell medium <R>
+doxESC medium

Complete ES cell medium with doxycycline at 1500 ng/mL final concentration. Prepare only sufficient +doxESC medium that will be used within 2 d.

Doxycycline (Sigma-Aldrich D9891-25G)
Phosphate-buffered saline (PBS) (Invitrogen 10010-23)
Trypsin–EDTA (GIBCO 25200-072)

Equipment

Centrifuge
Incubator, humidified 37°C, 5% CO_2
Conical tubes (15 mL), plastic, sterile
Microscope, inverted
Pipette, multichannel
Pipette (P20)
Pipettes, sterile
Plates (96 well) (Costar 3894), V-bottom
Tissue culture dishes, plastic, sterile

METHOD

1. The day after transfection (after Protocol 5 or 6), remove the medium and add +doxESC medium.
2. Culture the cells at 37°C in 5% CO_2. Initially, change the +doxESC medium every other day. Once cell proliferation is evident and/or colonies appear, change the +doxESC medium daily.
3. Induced pluripotent (iPS) cell colonies should begin to appear after 7–10 d. If the iPS cell colonies will be subcloned, do not passage the mouse embryonic fibroblasts (MEFs) after transfection because they may lead to multiple lines of the same clone.
4. Pick iPS cell colonies 8–12 d after addition of +doxESC medium.

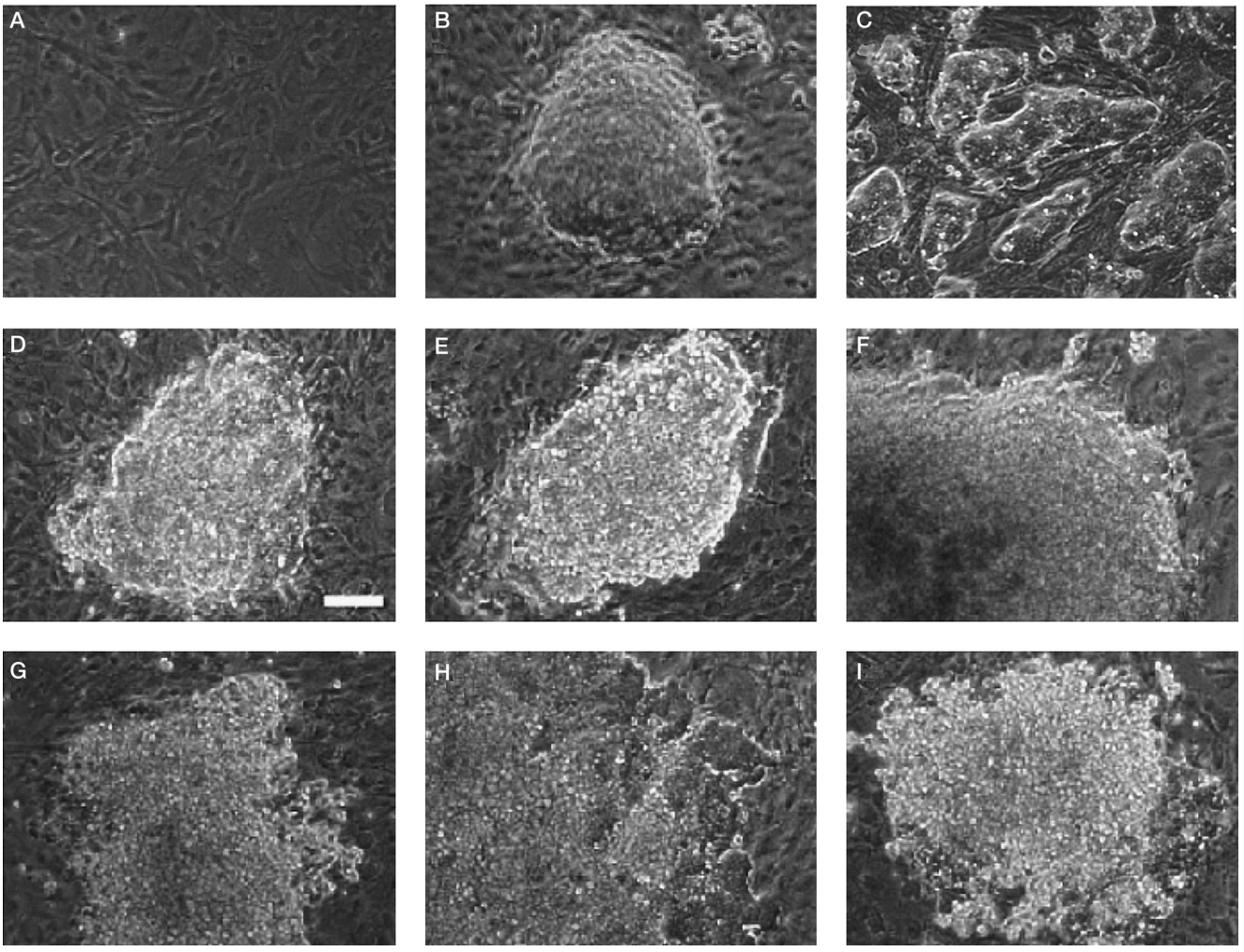

FIGURE 1. Variations in cell morphology forming colonies 10–12 d after inducing reprogramming of mouse embryonic fibroblasts (MEFs). (*A*) Initial MEFs at the time of transfection. Note the cell density. (*B*) ES cell–like primary colony before picking. (*C*) The iPS cell culture after several passagings of clone in *B*. (*D–I*) Different non-ES cell–like colonies. After doxycycline withdrawal, they may stop growing or change to more ES cell–like morphology.

5. Add 20 μL of trypsin–EDTA to each well of a V-bottomed 96-well plate.
6. Remove the medium from the plate with the doxycycline-induced colonies and carefully wash once with PBS. Remove the PBS. Gently add 10 mL of PBS. Colony morphology will vary (Fig. 1), and you may want to pick colonies with ES cell-like morphology.
7. Using a dissecting microscope and a P20 pipette, carefully dislodge the colonies one at a time by nudging the colony with the pipette tip. Pick the colony into the pipette along with not more than 5 μL of PBS. Transfer the colony into one of the trypsin-containing wells of the 96-well plate. Do not pick more iPS cell colonies at a time than can be handled within 5–10 min.
8. Once all of the desired colonies are picked into the 96-well plate, allow the colonies to incubate for 2–3 min at 37°C.
9. Add 50 μL of +doxESC medium to feeders in a flat-bottomed 96-well plate. Using a multichannel pipette, add 80 μL of +doxESC medium, and dissociate the colony by pipetting up and down. Passage the cells onto feeders in a flat-bottomed 96-well plate (Chapter 11, Protocol 3).

10. Expand the clones when the wells become subconfluent by passaging to feeders with a larger surface area.
11. Grow the clones in +doxESC medium for 7–10 d before withdrawing the doxycycline. Some clones will differentiate or not survive upon doxycycline withdrawal. If conditions were right, the majority of the clones will become doxycycline independent and the cells will self-renew, keeping their ES cell–like morphology in standard ES cell culture conditions (Chapter 8, Protocol 3).

RECIPE

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Complete ES Cell Medium (ES-DMEM)

Component	Final concentration
DMEM	
Fetal bovine serum (FBS) or serum replacement	10%–20%
L-Glutamine or L-alanyl L-glutamine[a]	2–4 mM
β-Mercaptoethanol (b-ME)[b] or monothioglycerol[c]	0.055–0.1 mM
Nonessential amino acids	0.1 mM
Sodium pyruvate (optional)	1 mM
Nucleosides[d] (optional)	
Penicillin-streptomycin (optional)	50–100 units (μg)/mL
LIF	500–2000 units/mL

[a]L-Alanyl L-glutamine is a stable dipeptide substitute of L-glutamine (e.g., GlutaMAX from Invitrogen Life Technologies).

[b]Miscalculations that increase b-ME concentration are guaranteed to cause ES cell culture failure.

A 55 mM solution is commercially available (e.g., Invitrogen Life Technologies 21985-023). Alternatively, 10 mM stock can be made from a 14.3 M solution by adding 7 μL of b-ME to 10 mL of PBS.

[c]Monothioglycerol is less volatile than b-ME and can be used as a substitute typically at a final concentration of 0.15 mM.

[d]Addition of the nucleosides was described in the original formulations for ES cell culture (Robertson 1987). It is used for culture of some ES cell lines. A 100× stock is commercially available (e.g., Millipore ES-008-D).