Equine induced Pluripotent Stem Cell culture

Chapter x

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Running head: culture of equine iPSCs

i. Abstract

Ground-breaking work by Takahashi and Yamanaka in 2006 demonstrated that non-embryonic cells can be reprogrammed into pluripotent stem cells (PSCs) by forcing the expression of a defined set of transcription factors in culture, thus overcoming ethical concerns linked to embryonic stem cells. Induced PSCs have since revolutionized biomedical research, holding tremendous potential also in other areas such as livestock production and species conservation. iPSCs exhibit broad accessibility, having been derived from a multitude of cell types and species. Apart from humans, iPSCs hold particular medical promise in the horse. The potential of iPSCs has been shown in a variety of biomedical contexts in the horse. However, progress in generating therapeutically useful equine iPSCs has lagged behind that reported in humans, with the generation of footprint-free iPSCs using non-integrative reprogramming approaches having proved particularly challenging. A greater understanding of the underlying molecular pathways and essential factors required for the generation, and maintenance of equine iPSCs and their differentiation into relevant lineages will be critical for realising their significant potential in veterinary regenerative medicine. This article outlines up-to-date protocols for the successful culture of equine iPSC, including colony selection, expansion, and adaptation to feeder-free conditions.

Key Words:

Equine, iPSCs, cell reprogramming, feeder-free culture

1. Introduction

Induced pluripotent stem cells (iPSCs) have significantly transformed biomedicine, owing to their immense potential in disease modelling, drug screening and cell-based therapies, in addition to highly exciting non-medical applications such as species conservation.[1,2] Back in 2006, ground breaking work by Takahashi and Yamanaka demonstrated that by introducing a combination of four transcription factors (OCT4, SOX2, c-MYC, and Klf4, referred to as OSKM), non-embryonic cells could be reprogrammed to a developmental state akin to embryonic stem cells (ESCs). As such, iPSC are devoid of the ethical concerns associated with the use of embryos.[3] Remarkably, iPSCs have been derived from most cell types, including for example urine-derived cells[4], and from a multitude of species, making them easily accessible for research purposes.[1] Moreover, through their large developmental plasticity and ability to differentiate into a variety of cell lineages, iPSCs provide powerful tools for a wide array of applications in biomedical research and therapy.[1]

As well as human medicine, iPSC technology holds significant promise in veterinary species, specifically in horses, in which stem cell therapies are already well established and could considerably benefit, in terms of improved efficacy, from the introduction of iPSCs.[5]

Non-integrative or excisable reprogramming methods including Sendai virus, episomal vectors, RNA-based technologies or piggyBac expression systems provide a feasible route to the therapeutic use of iPSCs, and are now routinely employed to reprogram human cells [6–8]. However, the use of these approaches with horse cells has met with little success [9,10], leading to the predominant use of integrating viral systems to generate equine iPSC lines.[5]

Moreover, advancements in iPSC technology have led to the elimination of xeno- and animal-dependent cultures in favour of feeder-free conditions. This involves substituting feeders with a suitable coating matrix and using serum replacement or small-molecule cocktails in the place of fetal bovine serum (FBS).[11] Thus, numerous clinical-grade products are available to support growth of iPSCs from humans and rodents, but the culture of iPSCs from domestic species still relies heavily on the use of feeders[5,12]. Yet, transitioning to feeder-free iPSCs will be imperative for any future therapeutic applications for horses.

Considering how slow the field of equine iPSCs has advanced compared to human and mouse, gaining a better understanding of the molecular pathways and critical factors involved in the generation and maintenance of equine iPSCs will be crucial.

The protocols in this article have been successfully used over the years in our laboratory to grow equine iPSCs of different origins and generated using different approaches. Hopefully their use will aid future advancements in realising the significant potential of these cells in veterinary regenerative medicine.

2. Materials

2.1 Cell culture equipment

1. 70 % Ethanol for surface sterilisation
2. Class 2 biological safety hoods
3. Laminar flow hood
4. Water bath set at 37°C
5. Inverted Microscope
6. Neubauer Hemocytometer (or other cell counting device)

7. Incubator, suitable for cell culture (set to 37 ºC, 21% O2, 5% CO2, 95% relative humidity)
8. Centrifuges (suitable for small tubes, and 15-50mL tubes)
9. Sterile cell culture plastic pipettes and pipette tips
10. Sterile centrifuge tubes in various sizes
11. Different size multi well tissue culture plates and flasks
12. GammaCell 1000 irradiator
13. Syringe with 18G x 1 1/2" (1.2 x 40 mm) needle

2.2 Cell culture reagents

1. Phosphate-buffered saline (PBS)
2. **Mouse embryonic fibroblast (MEF) media:** DMEM, high glucose (41965039, Thermo Fisher), 10%FBS, 0.1mM MEM Non-Essential Amino Acids (NEAA), 2mM L-glutamine and Penicillin/Streptomycin (stable for up to 4 weeks at 4 ºC).
3. **Equine iPSC media:** DMEM, high glucose (41965039, Thermo Fisher) containing 20% FBS or KnockOut™ Serum Replacement (10828028, Thermo Fisher), 1x GlutaMAX™ (35050061, Thermo Fisher) 0.1mM β-mercaptoethanol, 0.1 mM MEM non-essential amino acids and 1% penicillin-streptomycin supplemented with 10 ng/ml human bFGF and 1000 U/mL human LIF (should be prepared before use and stable for up to 7 days at 4 ºC).
4. **Equine embryonic fibroblast (EEF) media** used to prepare conditioned media (CM): KnockOut™ DMEM (10829018, Thermo Fisher) containing 20% KnockOut™ Serum Replacement (10828028, Thermo Fisher), 1x GlutaMAX™ (35050061, Thermo Fisher) 0.1mM β-mercaptoethanol, 0.1 mM MEM non-essential amino acids and 1% penicillin-streptomycin (stable for up to 4 weeks at 4 ºC).
5. **Trypsin-EDTA** (0.05%), phenol red (25300054, Thermo Fisher)
6. **StemPro™ Accutase™ Cell Dissociation Reagent** (A1110501, Thermo Fisher)
7. **Corning® Matrigel® Matrix** Before use, thaw stock solution at 4ºC to avoid gel formation and dilute as appropriate in cold DMEM. Then coat tissue culture plates and

flasks overnight at 4ºC or at 37 ºC for 1h and use immediately or stored sealed at 4ºC for up to a week.

8. **Gelatin:** Before use, dilute 2% gelatin solution (G1393, Sigma-Aldrich) in PBS to 0.1% working solution. Cover tissue culture plastic surfaces with working solution and incubate for 15 min at 37ºC, use immediately or stored sealed at 4ºC for up to a week.
9. **Freezing media:** 10 % (v/v) DMSO (cell culture grade), 90 % (v/v) FBS

2.3 Cell stocks

1. Equine iPSCs
2. MEFs
3. EEFs

3 Methods

3.1 Preparation of MEF feeder cells

This protocol describes the preparation of irradiated MEF feeder cells for culture of equine iPSCs (see **Note 1**).

1. Use low passage MEFs grown in suitable culture vessel with MEF media.
2. When ready for irradiation, detach cells by incubating for 2-8 min with trypsin at 37 ºC, then spin at 300 x *g* for 5min, and re-suspend in fresh pre-warmed media. Count cells on hemacytometer and re-plate in suitable vessel at $5.7x10^3$ cells/cm^2 to expand further or proceed to irradiation as described below.
3. For irradiation add desired cell number in 50 mL falcon in culture media and expose to 12.5 Gray in Gamma Irradiator.
4. Plate cells immediately (see step 7) or freeze down in freezing media for later use (see **Note 2**).
5. One day before plating iPSC culture, coat wells in a culture vessel of desired size with 0.1% gelatin solution and incubate for 15min at 37 ºC. Use immediately or store sealed at 4 ºC for up to 7 days.
6. Quickly thaw stock of irradiated MEFs by placing in 37 ºC water bath and then dilute in MEF culture media. Centrifuge cells 300 x *g* for 5 min at RT. Discard supernatant and resuspend in fresh MEF media.
7. Seed MEFs in gelatin coated surfaces ($2x10^6$ cells per 6-well plate; see **Note 3**) and culture overnight to allow cell attachment before use for iPSC culture.

3.2 Picking, expansion and culture of equine iPSC colonies on feeder cells

Equine iPSCs can be generated using different reprogramming approaches, and relevant protocols have been published [9,13,14]. The present is a protocol for establishing and maintaining iPSC lines in feeder culture once bona fide colonies have been generated using one of the approaches above, and describes the selection of individual iPSC colonies and their subsequent expansion in culture.

1. Individual colonies should be selected for clonal expansion based on assessment by daily microscope inspection. Colonies selected for picking should show clear growth and appear compact with well-defined edges. Colonies observed to increase in size for 2-4 days and devoid of a dark area in the center should be picked (Figure 1).
2. One day before picking, prepare MEF feeders in 96- or, alternatively, 24-well plates (see 3.1).
3. It is easiest to pick the colonies under a microscope or stereomicroscope, which is integrated into a laminar flow hood. Alternatively, a microscope can be brought into the hood while leaving the glass shield fully open and taking care to work as much as possible under aseptic conditions (e.g. avoid external airflow from people walking past, and work as quick as possible once culture dish is open).
4. Position the colony directly under the microscope and use a 20uL pipette to carefully pick the colony by gentle aspiration. Then transfer onto well containing feeders in equine iPSC media and visualize under microscope to confirm the transfer has been successful by identifying the colony. If picking proves difficult, try using a syringe to cut the colony out before aspirating.
5. Repeat as above for other colonies as required, and place each in a separate well.
6. Culture picked colonies overnight and then confirm each has attached to feeder layer. Colonies should start proliferating 2-4 days after picking. If colony shows no sign of growth after 7 days of picking, they can be discarded. Once it has been proliferating for 3-5 days, and as long as the center is not turning dark or there are no signs of differentiation, a colony should be split for expansion into a larger vessel (24 or 12-well size) with fresh MEF feeders. Splitting can be done by mechanical disruption (see 3.2.7) or using Accutase (see 3.2.8).

7. To disrupt a colony mechanically use a microscope set up as described in Step 3.2.3. Put the colony in focus under the microscope and use a syringe to carefully cut it into 4-8 pieces. Carefully aspirate the pieces with a 20- or 100uL pipette and transfer into a well with fresh feeders. If multiple colonies in a well are disrupted, then media in the well should be aspirated in its entirety and spun for 4 min 200 x *g*, before carefully resuspending in fresh culture media.
8. To dissociate colonies with Accutase, carefully aspirate the media and wash with PBS. Add enough Accutase to cover the surface of the well. Place in incubator for 2-8 min and observe cells under microscope. When the outside of the colony starts to lift (cells will seem brighter) and colony becomes less compact, add equine iPSC media to stop reaction. Use 1mL pipette to carefully scrape and disrupt iPSCs, before aspirating. Do not pipette cells up and down more than twice, to avoid disruption into single cells. Spin down cells for 4 min 200 *x* g, then carefully resuspend in fresh culture media and plate on new feeders.
9. After splitting cells into larger vessel as described above, check daily to ensure colonies keep their compact morphology with clear edges. If cells become confluent, use Accutase as above to split into larger vessel (6-well plate).
10. iPSC cells can be maintained from then on in 6-wells plates using equine iPSC media ~~e~~(or other suitable media that has been previously optimized). Typically cells need to be split every 3-5 days at a ratio of 1:4-1:10 depending on the culture (See **Note 4**).

3.4 Growth of equine iPSCs in feeder free culture

Feeder-free culture greatly facilitates work with iPSCs and it is a requirement for many downstream applications, whether involving their use as in vitro models or in prospective therapeutic applications. As reprogramming of equine cells is simpler using feeder layers, feeder-free cultures usually result from adaptation of iPSC lines generated on feeders[13]. However, adaptation to feeder free conditions can be challenging. Choosing the right coating substrate and culture media is crucial, and different combinations may need to be tested in each particular instance. The following protocol uses matrigel and EEF CM. It was originally used to adapt iPSCs generated through reprogramming of equine keratinocyte with retroviral vectors, and has later been successfully used with other equine iPSC lines in our laboratory.[13]

1. Harvest CM from confluent layers of mitotically inactivated EEFs (see **Note 5**) in T175 flasks at 37 ºC and 5% CO2. Do this by collecting and replacing EEF media daily from each flask for a total of 5 days. Stock store collected media at -20C until use. Discard cells after the 5 days. Before use, supplement the conditioned media with 10 ng/ml human bFGF and 1000 U/mL human LIF (keep at 4 ºC for up to 7 days).
2. Start transition to feeder-free culture by gradually reducing feeder density during iPSC passaging. When preparing feeders before iPSC passaging, plate 75% of the usual feeder number (e.g. if normally using $2x10^6$ per well of a 6 Well plate, reduce to $1.5x10^6$ per well). Split iPSC culture as usual using prepared feeders.
3. Monitor morphology of passaged iPSCs and use same feeder density for at least one more passage. After transition to reduced feeders some iPSC colonies may undergo morphology changes indicative of loss of pluripotency (Figure 2). In that case, try to remove abnormal colonies mechanically and keep the rest of the culture growing. If on the contrary only a few colonies maintain typical morphology, then try to pick those and plate them in smaller vessel (see 3.2.1), and discard the rest. Once colonies are growing well, move on to next step.
4. In the next step reduce feeder density to 50% of the original following the same principle as in 3.4.2. In addition, when plating cells on reduced feeder density, replace half of equine iPSC media with CM collected in Step 3.4.1. Follow same procedures as in Step 3.4.3.
5. Once colonies are growing well in 50% feeder density, then prepare a Matrigel coated culture dish and seed feeders at 25% of the original density. Passage iPSCs onto reduced feeders and culture in 100% CM.
6. Follow same procedures as in Step 3.4.3 (See **Note 6**).
7. Once colonies are growing well on a minimal amount of feeders, then passage onto Matrigel only and 100% CM.
8. Follow same procedures as in Step 3.4.3.
9. Continue to expand cells in feeder free conditions as in 3.4.7 and confirm cells maintain normal karyotype once adapted.

4. Notes

1. In addition to MEFs, equine embryonic fibroblasts or SNLs can be used succesfully as feeder layers to generate and maintain equine iPSCs. These cells can be inactivated either by the use of γ-irradiation or mitomycin C treatment.[5,15]
2. It is useful to freeze irradiated MEFs at a "working concentration" e.g. in vials each containing the required amount for seeding a 6-well plate($2x10^6$ MEF per 6-well plate).
3. Feeder cell density may need to be optimised based on the performance of iPSC cultures.
4. Once colonies have been expanded into multiple wells, further optimization of dissociation method and/or culture media can be performed if needed, by determining in the first instance growth rate and morphology of colonies under different conditions. Different media may be suitable for culture of equine iPSC, including chemically defined media.[5,10]
5. Our laboratory has always used fibroblasts derived from the same single equine fetus[13], therefore the effects of fetal age (and other potential variables) on the suitability of EEFs for iPSC culture are yet to be determined.
6. If at this point colonies stop growing or loose typical iPSC morphology, then changing culture conditions should be attempted. Different coating matrix and culture media combinations can be tried to establish feeder-free culture. Matrigel can be replaced with Vitronectin, Laminin-521[16,17] or other commercially available matrices, in combination with media such as Essential 8 (Gibco) or mTeSR plus (Stemcell technologies).[18,19]

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Figure Captions

Figure 1: Representative bright field image of *bona-fide* equine iPSCs grown on MEF feeders, and showing compact colonies with defined edges. Colonies indicated by arrows displayed sustained growth over 2-5 days and were ready for picking.

Figure 2: Bright field images showing (A) equine iPSC colonies (indicated by arrows) which failed to adapt to feeder free conditions as shown by distinct

morphological changes and halted growth, and (B) iPSC population which adapted to feeder free conditions successfully, as evidenced by highly proliferative, compact colonies with well-defined edges.