Maintenance of Human iPS Cells in a Feeder-free Culture System

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Introduction

The establishment of human induced pluripotent stem cells (iPSCs) has revealed a new path for the generation of stem cells without the use of embryos. This new approach eliminates ethical concerns and provides an invaluable contribution to the field of regenerative medicine and stem cell biology. The direct reprogramming of somatic cells to pluripotency was first accomplished in 2006, when Takahashi and Yamanaka converted adult mouse fibroblasts to iPSCs through ectopic expression of a select group of transcription factors[1]. Subsequent reports optimized this technique, demonstrating that regardless of the gene combination used iPSCs were highly similar to embryonic stem cells (ESCs) in their morphology, ability to express cell surface markers typical of pluripotent cells, form viable chimeras, and give rise to teratomas containing cell types representative of all three germ layers[2,3].

Human ES and iPS cells require a specific culture system for successful expansion. Originally, human ES cells were cultured on a feeder layer of mouse embryonic fibroblasts (MEFs). While MEFs provide a robust surface for long-term culture of hES cells, there are a number of concerns associated with the use of a cell feeder layer. Feeder cells may secrete undefined components into the media and cause batch-to-batch variability in feeder layers, or there may be xenogenic contamination of hES and hiPS cells grown in this system. BD Matrigel™ matrix is a well established growth substrate for feeder-free hES culture. Recently, BD Matrigel matrix has been used for culturing undifferentiated human iPS cells in a feeder-free environment. This has been accomplished using BD Matrigel matrix in conjunction with MEF-conditioned medium, culture additives or defined media such as mTeSR®1 (STEMCELL Technologies), and for in vivo differentiation studies eg, teratoma formation[4–6]. BD Matrigel™ matrix is a reconstituted basement membrane isolated from the Engelbreth-Holm-Swarm mouse sarcoma and contains extracellular matrix molecules such as laminin, collagen IV, entactin, and heparan sulfate proteoglycan[7]. It is highly biologically active and closely resembles basement membrane in both consistency and activity[8]. STEMCELL Technologies has developed mTeSR1 and TeSR™2 as standardized media for feeder-independent maintenance of human pluripotent stem cells (hPSCs). These media formulations have been designed to maintain and expand undifferentiated hPSCs in conjunction with BD Matrigel™ hESC qualified matrix (BD Cat. No. 354277) as a growth substrate. Furthermore, each batch of BD Matrigel hESC qualified matrix is pre-qualified by STEMCELL Technologies to ensure consistency, reproducibility, and reliability in performance.

Human iPS cells were first established using inactivated MEFs and hESC media, but subsequently shown to grow well in a feeder-free system using BD Matrigel™ matrix and mTeSR®. When using the MEF feeder layer method a higher replating efficiency is achieved when picking iPSC colony. The disadvantage of this approach is that many colonies emerge from partially reprogrammed cells, which may not be easy to distinguish from true human iPS colonies. The BD Matrigel matrix and mTeSR feeder-free system is a more stringent culture method that more clearly distinguishes true human iPS cells from partially reprogrammed cells[9].

This report details the feeder independent culture method for adult human iPS cells using BD Matrigel hESC-qualified matrix and mTeSR1 media, thus eliminating concerns regarding batch-to-batch variability associated with feeder layers. A variety of methods exist for iPS cell derivation, all reliant upon manipulation of a select group of transcription factors[10]. A human iPS cell clone derived from myoblasts (clone C5-139) was established by transduction of retroviral expressed factors including Oct4, Sox2, Klf4, in combination with lentiviral vectors expressing lgT and cMyc. In this study, we demonstrate the maintenance of undifferentiated iPS cells on BD Matrigel hESC-qualified matrix once the round(s) of infection have been completed, and several colonies have appeared on MEF layer. The iPS cells grown on BD Matrigel matrix in mTeSR1 media exhibit undifferentiated colony morphology, express markers of undifferentiated iPS cells, and maintain pluripotency after 30 passages. Cells were able to form teratomas and differentiate into the cells of all three-germ layers.

Materials and Methods

Reagents

- BD Matrigel hESC-qualified matrix (BD Cat. No. 354277) and BD™ Dispase (BD Cat. No. 354235).
- For immunocytochemistry, human embryonic stem cell marker antibody panel (R&D Systems Cat. No. SC008).
- TRA-1-60 (EMD Millipore Cat. No. MAB4360).
- Alexa Fluor® 555 donkey anti-goat IgG (Life Technologies Cat. No. A21432), and Alexa Fluor 555 goat anti-mouse IgG (Life Technologies Cat. No. A21422) secondary antibodies.
- mTeSR media (STEMCELL Technologies Cat. No. 05850).
- Accumax (EMD Millipore Cat. No. SCR006).
- Penicillin-Streptomycin (Gibco Cat. No. 15070-063).

Preparation of mTeSR Media

The components of mTeSR media: mTeSR Basal Media and 5X mTeSR Supplement were mixed according to the recommendations in the product specification sheet. Penicillin-Streptomycin was added to the mTeSR media. For optimal results, the lot numbers of both components of the mTeSR1 Medium kit (mTeSR1 Basal Medium and mTeSR1 5X Supplement) should end with the same letter (eg, D). If desired, 5X Supplement can be aseptically dispensed into working aliquots and stored at -20°C. Frozen aliquots were used within 3 months. Thawed aliquots should be used within 1 day to prepare complete mTeSR1 medium.

Coating Procedure

BD Matrigel™ hESC-qualified matrix was thawed on ice according to the recommendations provided in the product specification sheet. Repeated freeze thaw is not recommended. For coating, one aliquot of BD Matrigel hESC-qualified matrix was added to 25 mL ice-cold DMEM/F-12 (HyClone™, Thermo Fisher). Instructions for aliquot volume is lot-specific based on the protein concentration and is found on the product specification sheet, as well as on the BD Biosciences support webpage. The coating solution was kept on ice at all times. One mL of the coating solution was added to each well of a BD Falcon™ 6-well multiwell plate (kept on ice) and spread by rocking gently (if air bubbles get trapped in the wells, centrifuge the plate at 300 x *g* for 10 minutes at 4°C. The centrifuge should be pre-cooled to 4°C). Plates were incubated at room temperature for 1-2 hours. The coating solution was aspirated from the plate, and cells were plated. Coated plates with coating solution can be stored at 4°C and should be used within one week of coating. Coating solution should be aspirated just before using the plates.

Picking Colonies

Once the round(s) of infection has been completed, and several colonies appeared on the the MEF layer, the colonies were passaged using BD™ Dispase once or twice until clear, human ES-like colonies appear. Once clear colonies have appeared on MEF layer, they were picked to establish a clonal population. Colonies to be picked were selected and circled. Human iPS cells were prepared for sub-culture by aspirating the media and rinsing the plate with PBS. BD Dispase (5 mL for 10 cm dish, or 3 mL for 6 cm dish) was added and incubated for 10 minutes or until cells appear to be lifting off from the plate. BD Dispase was then diluted by adding an equal volume of PBS. The cells were scraped using a glass pipet, or each colony was picked with a p200 pipet and placed in an Eppendorf® tube. DMEM F-12 (1 mL) was added to each tube followed by 200 µl of Accumax™ (Accumax added after all colonies have been picked and tubes are ready to spin). The cells were spun immediately at 800 rpm for 5 minutes. The colony was resuspended in 0.5 mL of mTeSR®1 + Rock inhibitor (1 µl/mL) and plated on a 48-or 24-well plate (depending on size of the colony) coated with BD Matrigel hESC-qualified matrix and fed every day. Rock inhibitor was left in culture for one day after passage.

Culture of Picked Clones

The iPSC clone C139 derived from human myoblasts was then maintained on BD Matrigel hESC-qualified matrix-coated BD Falcon™ multiwell plates in mTeSR1 media. Cells were passaged using Accumax at a 1:4-1:6 split ratio. Media was changed every day during one passage cycle, except once per week when a double volume of media was added to skip changing media on one day.

Feeding Human iPS Cells

The iPS cells were monitored and fed daily until the cells required passaging. The spent medium was aspirated with a sterile Pasture pipet and 3.0 mL of fresh mTeSR® medium was added to each well of a 6-well BD Matrigel™ hESC-qualified matrix-coated plate and incubated at 37°C. To reduce potential contamination, pipets or Pasteur pipets were used only once.

Passaging iPS Cells

The cells were split when one of the following was observed:

1. iPSC colonies are becoming too large or too dense.
2. Increased differentiation.

The split ratio is variable, but generally between 1:3 and 1:9. The split ratio was adjusted depending on the growth rate of cells.

Cells grown on BD Matrigel hESC-qualified matrix and mTeSR were always passaged with Accumax™. Accumax (0.5 mL) was directly added to the cells after removing the media. The cells were monitored closely since Accumax is a very powerful disaggregating agent (usually the incubation time with Accumax is about 5 minutes). The cell suspension was transferred to a tube containing culture media at 5X the volume of Accumax reagent used (DMEM F-12 media or variants like IMDM, DMEM, etc). Immediately after diluting the Accumax, the cells were briefly agitated by gentle pipetting, and were spun at 800 rpm for 5 minutes. The cells were resuspended in mTeSR + Rock inhibitor (1 µl/mL) and replated in a plate previously coated with BD Matrigel hESC-qualified matrix. Cells grown in BD Matrigel hESC-qualified matrix and mTeSR generally need to be split every 3-5 days.

Immunocytochemical Detection of Embryonic Antigens in iPS cells

Human iPS cells cultured on BD Matrigel hESC-qualified matrix and mTeSR were analysed for the expression of undifferentiated markers on the cells. The iPS cells were fixed with 4% paraformaldehyde (prepared fresh in PBS) for 20 minutes at room temperature and washed 3 times with PBS for 5 minutes. For Specific Embryonic Antigen 4 (SSEA-4), TRA-1-60 and Alkaline Phosphatase (AP) staining, the cells were blocked with 1% BSA, and 10% normal donkey serum in PBS (blocking buffer) at room temperature for 45 minutes. For Oct-3/4 and Nanog staining, the cells were permeabilized prior to staining with antibodies for intracellular markers and blocked with 0.1% Triton™ X-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 45 minutes. After blocking, cells were probed with anti-SSEA-4, Oct-3/4, Nanog, AP, and TRA-1-60 primary antibody at a final concentration of 10 µg/mL and incubated overnight at 2-8°C. The cells were washed three times with 2 mL of PBS containing 1% BSA for 5 minutes. After washing, the cells were incubated with Alexa Fluor®-conjugated secondary antibody (Alexa Fluor 555 donkey anti-goat IgG, or goat anti-mouse IgG) diluted 1:1000 in PBS containing 1% BSA for 60 minutes at room temperature in the dark. The cells were then washed three times with PBS containing 1% BSA for 5 minutes. Nuclei were counterstained with 4,6-Diamidino-2-phenylindole (DAPI, 0.1 µg/mL). The plates were stored wrapped in aluminum foil at 4°C in 2 mL PBS until visualized under a fluorescence microscope.

Teratoma Formation

Human iPS cells were cultured for 15-20 passages on BD Matrigel™ hESC-qualified matrix-coated culture dishes in mTeSR media and subsequently tested for pluripotency by teratoma formation. Cells were injected into the hind leg muscle of immunodeficient mice. BD Matrigel matrix was added at 1/3 total volume to the cell suspension prior to injection. Tumors formed at 6 weeks post-injection were processed for hematoxylin and eosin staining and histological analysis.

Results and Discussion

Morphology of Human iPS Cells cultured on Mouse Embryonic Fibroblast Feeder Layer, and on BD Matrigel matrix in a Feeder-free Culture System

Colonies displaying hESC morphology were expanded on MEFs (after the completion of infection), and the clones were successfully maintained under feeder-free conditions using BD Matrigel hESC-qualified matrix-coated surface and mTeSR®1 media. Figure 1 (A-D) shows representative examples of healthy human iPSC colonies that are good candidates for selection from the MEF layer. The cells in these colonies exhibit ES-like morphology. Once clear colonies appeared on the MEF layer, they were picked to establish a clonal population and then maintained under feeder-free culture conditions.

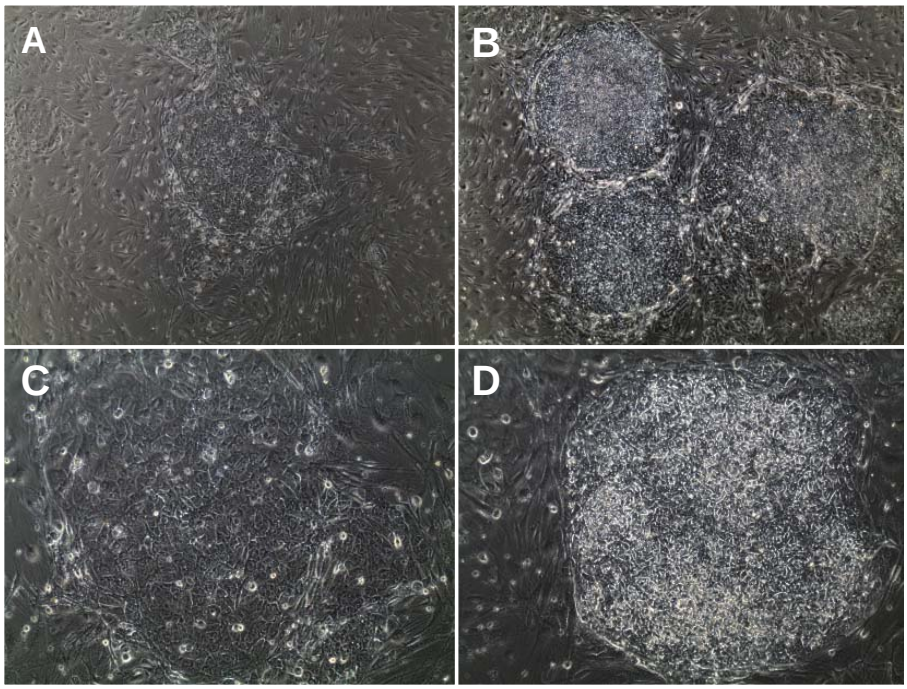

Figure 1. Phase contrast micrographs showing representative examples of human iPSC colonies on MEF feeder layer that are suitable for selection. Magnification: **A** and **B** (4X); **C** and **D** (20X).

Figure 2 (A-D) represents phase contrast micrographs showing the morphology of good human iPSC clones cultured on BD Matrigel™ hESC-qualified matrix-coated surface. The cells have formed densely packed colonies, and the individual cells had a high nuclear to cytoplasmic ratio, as well as a prominent single nucleolus that is typical of human ES cells. Furthermore, the colonies exhibit features indicative of pluripotency, such as sharply defined edges and round, three-dimensional colony morphology.

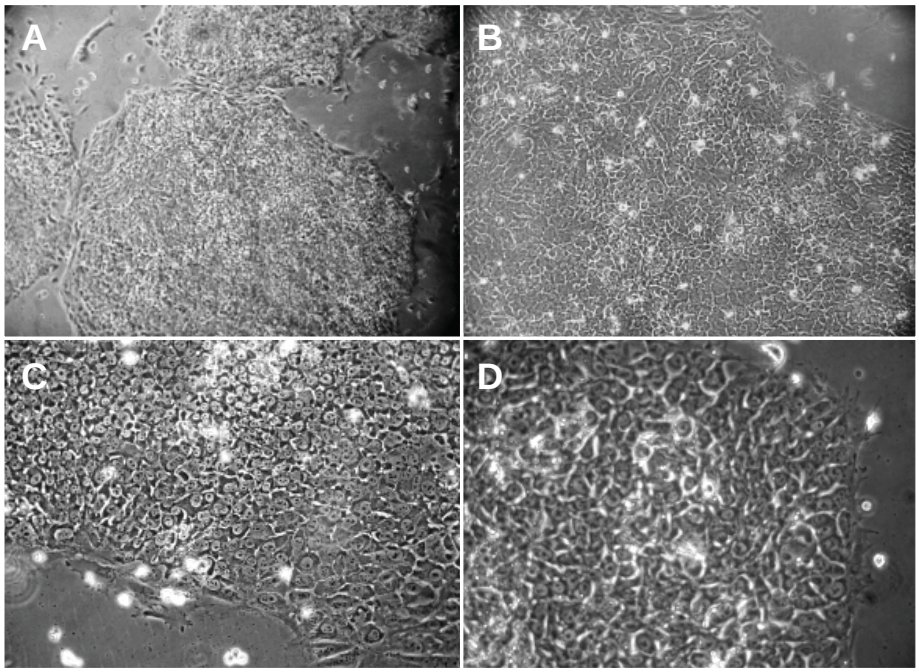

Figure 2. Representative images of human iPS cells cultured on feeder-free BD Matrigel hESC-qualified matrix-coated surface and mTeSR®1 media. Phase contrast micrographs showing the morphology of human iPSC clones. Magnification: **A** (5X); **B** (10X); **C** (20X); and **D** (35X).

Immunocytochemical Analysis of Human iPS Cells for Undifferentiated Cells

To confirm their ability to express ES cell proteins, human iPS cells were immunostained with antibodies directed against AP, SSEA-4, Oct-3/4, TRA-1-60, and Nanog. Consistent with their hES cell-like morphology, iPS cells maintained on BD Matrigel hESC-qualified matrix expressed characteristic markers for undifferentiated cells, including Nanog, Oct-3/4, TRA-1-60, SSEA-4, and AP (Figure 3).

As anticipated, expression of SSEA-1 was not detectable (data not shown). Corresponding images stained with DAPI show nuclei of individual cells in the colonies (Figure 3, Panel B). Panels C and D are merge of images in (A) and (B). These results demonstrate that human iPS cells can be readily maintained under feeder-free conditions and reduce variability associated with feeder cells.

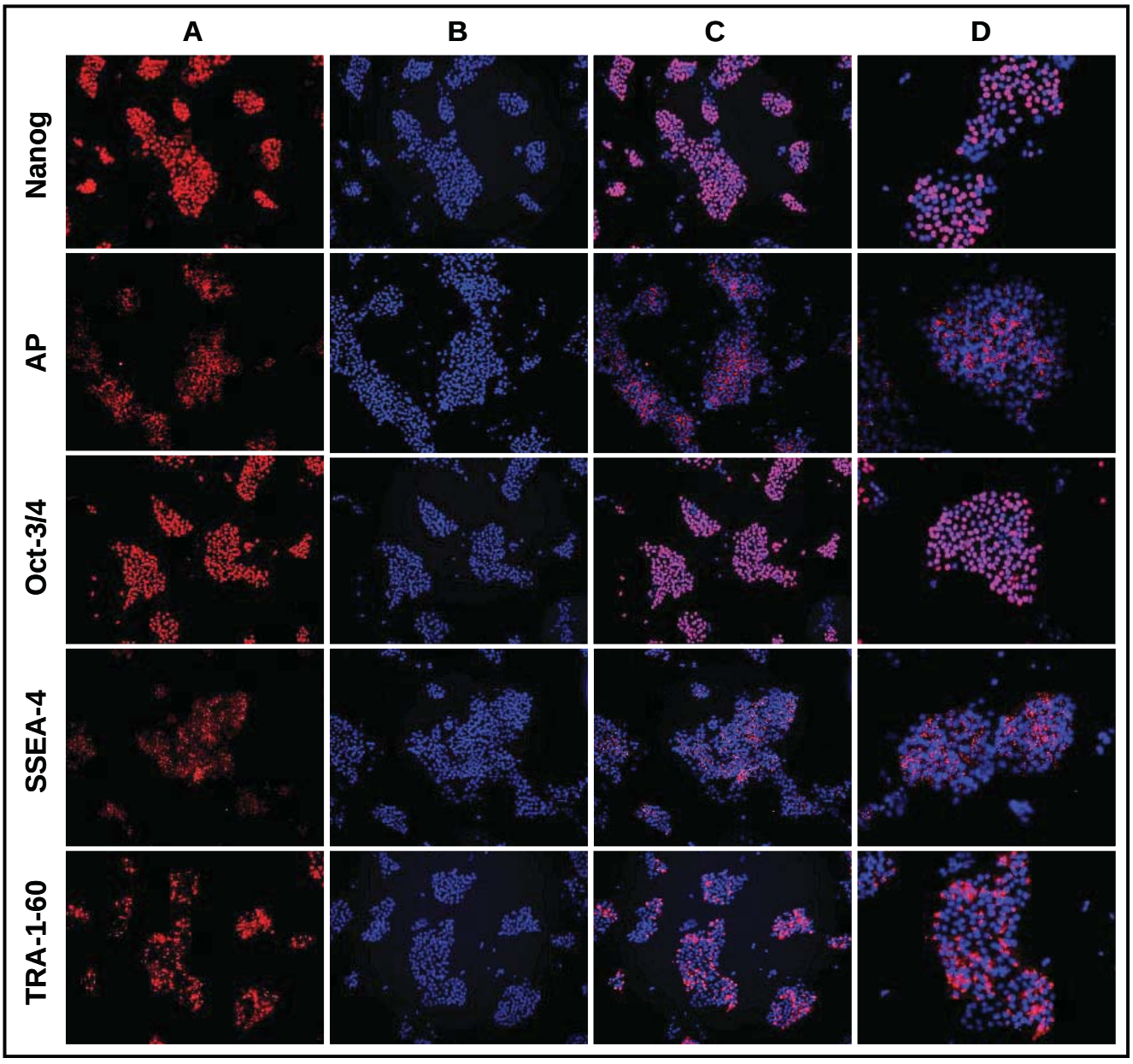

Figure 3. Immunocytochemistry of human iPSC cells expressing markers of undifferentiated cells. Human iPS cells maintained pluripotency on BD Matrigel hESC-qualified matrix-coated surface after 10 passages. The iPS cells were stained using antibodies directed against Nanog, AP, Oct 3/4, SSEA-4, and TRA-1-60 (red; Panel **A**), and the nuclei were counterstained with 4,6-Diamidino-2-phenylindole (DAPI) (blue; Panel **B**) to indicate the total cell content per field. Panels **C** and **D** are merged images of Panels **A** and **B** Magnification: Panels **A-C** (10X); Panel **D** (20X).

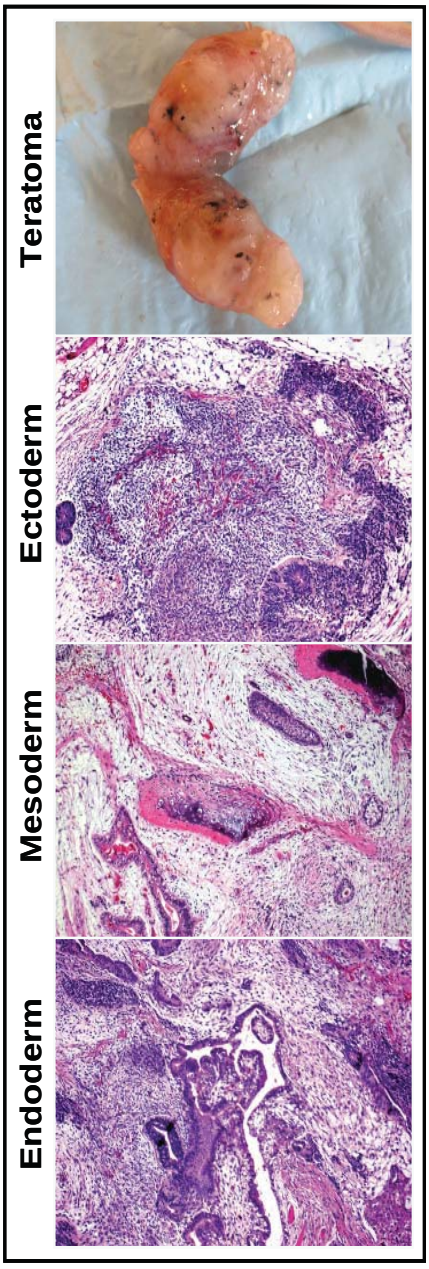

Figure 4. Representative images demonstrating iPS cells cultured on BD Matrigel hESC-qualified matrix form teratomas after 6 weeks post-injection, and differentiate into all three embryonic germ layers including ectoderm, mesoderm, and endoderm as indicated by hematoxylin and eosin staining of paraffin sections through teratomas. Magnification: 5X.

Human iPS cell Differentiation

The teratoma assay is routinely used to demonstrate the pluripotency of newly established hPSC lines, long-term cultures, and genetically modified hPSCs. To further establish pluripotency of human iPS cells on a functional level in vivo, the teratoma differentiation assay was performed. Representative results (Figure 4) demonstrate that hiPSCs maintained in BD Matrigel™ hESC-qualified matrix and mTeSR®1 for multiple passages are capable of forming teratomas 6 weeks post-injection into immunocompromised mice, and differentiated into cells derived from all three germ layers.

We have successfully cultured human iPS cells in BD Matrigel hESC-qualified matrix and mTeSR media. Furthermore, human iPS cells cultured from frozen stocks expressed pluripotency markers when maintained on BD Matrigel hESC-qualified matrix and mTeSR media. All of the ES like characteristics of the human iPSC clones were maintained after multiple freezing (data not shown).

Conclusions

BD Matrigel matrix is an effective surface that supports feeder-free expansion and maintenance of undifferentiated human iPS cells. Collectively, our analyses demonstrate the successful maintenance of human iPS cells on BD Matrigel hESC-qualified matrix under feeder-free conditions, which potentially reduces the variability associated with using mouse feeder cells. Human iPSC cultures exhibited normal morphology, expressed markers of undifferentiated human iPS cells, and differentiated into all three germ layers.

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