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Using Human Induced Pluripotent Stem Cells to Model Skeletal Diseases

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Abstract

Musculoskeletal disorders affecting the bones and joints are major health problems among children and adults. Major challenges such as the genetic origins or poor diagnostics of severe skeletal disease hinder our understanding of human skeletal diseases. The recent advent of human induced pluripotent stem cells (human iPS cells) provides an unparalleled opportunity to create human-specific models of human skeletal diseases. iPS cells have the ability to self-renew, allowing us to obtain large amounts of starting material, and have the potential to differentiate into any cell types in the body. In addition, they can carry one or more mutations responsible for the disease of interest or be genetically corrected to create isogenic controls. Our work has focused on modeling rare musculoskeletal disorders including fibrodysplasia ossificans progressive (FOP), a congenital disease of increased heterotopic ossification. In this review, we will discuss our experiences and protocols differentiating human iPS cells toward the osteogenic lineage and their application to model skeletal diseases. A number of critical challenges and exciting new approaches are also discussed, which will allow the skeletal biology field to harness the potential of human iPS cells as a critical model system for understanding diseases of abnormal skeletal formation and bone regeneration.

Keywords: Human iPS cells, Mineralization, Fibrodysplasia ossificans progressiva, Ossification, Skeletal diseases, Directed differentiation

1 Introduction

Musculoskeletal conditions such as osteoporosis, fractures, and skeletal malformations are among the most frequently reported medical conditions in the USA and are the second-greatest cause of disability worldwide (1). Inherited skeletal disorders are among the most common genetic diseases (2) and affect 2.4 in 10,000 births with 23 % of the affected presenting as stillbirths and 32 % mortality in the first week of life (3). Adult osteoporosis alone affects over ten million people in the United States and results in over two million fractures each year (4). Being able to model these conditions in a human model system is one critical tool for developing therapies for these medically important diseases.

1.1 Major Challenges Hinder Our Understanding of Human Skeletal Diseases

Achieving a better understanding of human skeletal development has several major challenges:

First, the genetic factors underlying skeletal diseases are complex. Many of the traits and diseases we associate with the skeleton (e.g., height; osteoporosis) are multigenic in origin (5–7). In addition, some genes have distinct functions in humans that vary significantly from what occurs in model organisms such as rodents (8–10). Although model organisms provide valuable insights into biology, these genetic complexities indicate that having a continuous source of human tissues would be extremely valuable for understanding disease pathophysiology and translating our knowledge into new treatment strategies. Until recently, this has been a major hurdle since obtaining large quantities of primary tissues from humans can be very difficult or impossible.

Second, a surprisingly large number of severe skeletal and nonskeletal medical conditions remain "undiagnosed" with only rudimentary molecular understanding of the disease pathogenesis. Patients with these rare or orphan conditions often face diagnostic and treatment delays, which can be improved when the disease process is discovered. Importantly, research into some of these rare presentations has identified key pathways leading to breakthrough discoveries and medications that benefit the wider population (e.g., the role of *SOST* in regulating bone mass (11, 12)). This demonstrates that rare disease models can highlight important pathways and help address the unmet medical needs of more complex polygenic diseases such as osteoporosis.

Third, during the past several decades, bone researchers have focused on autologous cells such as mesenchymal stem cells (MSCs) or adult stem cells (e.g., adipose-derived stem cells) (13–16). These multipotent cell types are finding applications in regenerative therapies. However, isolating large numbers of primary cells remains difficult: one report showed that 30 ml of human bone marrow yielded only 7–22 $\times$ 10^6 phenotypic MSCs after 4 weeks of culture, with some samples requiring extended culture (17). In addition, multiple donors are needed as sources for different cell types (i.e., MSCs, endothelial cells, muscle stem cells), introducing different genetic backgrounds as a new confounder. This also decreases the likelihood that a composite allograft could be created from a single donor and increases the risk of allograft rejection if a multidonor allograft was used. Finally, other cell types abundant in bone, such as neurons or hematopoietic cells, cannot be easily generated from MSCs and thus their contributions are difficult to explore. Human iPS cells help address this challenge by allowing us to potentially generate any cell type of interest.

1.2 Pluripotent Cells Are Useful for Skeletal Research

Stem cells are defined as having two basic properties: the ability to self-renew and the potential to differentiate into one or more specialized cell types. Stem cells are critical for maintaining tissues that normally have high turnover such as skin and blood. However,

it is increasingly recognized that many organs, even ones with low proliferative capacity as can be found in the skeleton, contain tissue-specific stem cells that contribute to their growth and maintenance (18). These tissue-specific cells are typically multipotent and have limited differentiation potential to create only a subset of cell types. In contrast, cells in the mammalian early embryo are pluripotent and can contribute to any tissue in the body (19, 20).

Pluripotent cells such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are well suited for modeling human physiology, pathophysiology, and development since they can create any cell types that are needed, if the appropriate differentiation protocols are available. Although multipotent stem cells like MSCs or adult stem cells are valuable for studying skeletal diseases, pluripotent cells would allow us to generate lineages that may be critical for bone formation, but outside of the normal repertoire for lineage-restricted multipotent cells (i.e., neural crest cells, neurons, immune cells). Since many of the pathways that regulate skeletal development also have critical roles in other tissue types, human pluripotent cells can be used to study these functions in nonskeletal tissues. Furthermore, starting from a pluripotent cell potentially allows us to create a continuous supply of isogenic cell types, thus minimizing the effects of variations in genetic background that may occur with primary cells.

Human ES cells are derived from human embryos created from eggs fertilized in vitro (21, 22). Briefly, these cell lines are derived from blastocysts that have been plated on a tissue culture surface to allow the inner cell mass to expand. The surviving cells grow to create a renewable cell population. Cells that maintain a normal genetic background, and remain in a pluripotent state (i.e., do not differentiate into a terminal cell type), become an embryonic stem cell line. A number of human ES cell lines are currently available. NIH supports research using a select number of lines that have met specific quality control and ethical standards (http://escr.nih.gov).

1.3 Induced Pluripotent Stem Cells Are Useful for Modeling Human Diseases

The discovery of mouse (23) and human iPS cells by Shinya Yamanaka's laboratory in 2007 (24) revolutionized the stem cell field by providing a relatively straightforward method to create pluripotent cells from a differentiated cell source. iPS cells allow us to create unlimited numbers of isogenic cell types, providing a single, renewable source of human cells with a known genetic background. These purified cell populations allow new detailed genetic, biochemical, and functional studies not previously possible while providing a high level of long-term consistency for robust experiments and allowing us to link in vitro results to a patient phenotype. The recent finding of putative pluripotent cells in somatic tissues and the creation of stem cell banks from "superdonors" that are immune-compatible with multiple recipients (25) increase the possibility that our iPS cell studies will find rapid applications in tissue engineering as immune tolerance improves.

1.4 The Method of Reprogramming May Affect the Formation of iPS Cells

All of the current methods for creating iPS cells activate a pluripotency transcriptional network to convert a more differentiated cell into a pluripotent-like cell. Many iPS cell induction methods are now widely used and demonstrate that there are many roads to pluripotency. Methods include retroviral transduction (24); DNA constructs (26); nonintegrating episomes (27); nonintegrating Sendai viruses (28); nonintegrating modified mRNA transduction (29, 30), transposons (31), and small molecules (32). The field of reprogramming continues to innovate and many new methods are constantly being made available. Many of these techniques have been used to reprogram multiple types of terminally differentiated cells.

In our own studies using iPS cells created from patients with fibrodysplasia ossificans progressiva (FOP), we were concerned that activated BMP signaling by the FOP R206H ACVR1 mutation could adversely affect our ability to create FOP iPS cells since BMPs can induce human ES cell differentiation (33). In addition, prior reports using Sendai virus indicated that Sendai-derived FOP iPS cells were not able to maintain their pluripotent state (34). In our hands, we found that retroviral and episomal methods could create FOP iPS cells (35); however, the FOP iPS cells tended to lose their iPS cell-like morphology more often when cultured for long durations in feeder-free conditions. These results indicated that there are method-specific effects on iPS cell generation that are yet to be elucidated. It also indicates that if one method of reprogramming doesn't work, a different method may be more successful to compensate for factors (genetic or otherwise) that may influence iPS cell generation.

1.5 Directed Differentiation of Pluripotent Stem Cells

A tremendous library of protocols, too large to list here, is now available describing many ways to create differentiated cell lines from pluripotent stem cells. Over the past several years, new methods have been developed specifically for human iPS cells. These methods use different approaches, including robust small molecule-directed differentiation protocols (i.e., for cardiomyocytes (36), neurons (37), and endothelial cells (38)); expression of master transcription factors (i.e., for skeletal muscle (39)); and culture in less well-defined conditions that are known to favor the formation of specific lineages (i.e., for chondrocytes and mineralizing cells).

Directed differentiation methods continue to improve, particularly with the use of newer scaffolds and culture matrices. However, several factors need to be kept in mind: the specific protocols used in directed differentiation methods may be cell type specific; many commercial differentiation mediums are proprietary (i.e., osteogenic medium often contains BMPs, which may confound an experiment if the disease already affects the BMP pathway); and a detailed optimization process may be necessary when

applying the method to different cell lines. In addition, the use of specific medium conditions can make cocultures particularly challenging since the individual cell types may not survive together if the culture conditions are not compatible. Finally, human iPS cells appear to differentiate easily into immature cell types in a dish (40); however, more mature cell types may require advanced 3D or in vivo environments (41). Despite these limitations, the ability to make specific cell types from iPS cells carrying a specific disease mutation is exciting for disease modeling since in many cases, the specific cell types that are affected by the mutation are not easily identified or obtained from primary samples.

1.6 Osteogenic Differentiation and Mineralization

One of the major challenges when differentiating human iPS cells is to obtain a large "pure" population of skeletal-lineage cells that are functional in vitro and in vivo. Traditionally, cells are assayed for their potential osteogenic capacity by detecting mineralization, a relatively late step in the bone formation process. These types of protocols were mostly adapted from methods developed for MSCs, often used monolayers of cells cultured for 12–28 days, and were dependent on fetal bovine serum (35, 42). Most of these mineralizing or osteogenic medium contain β-glycerol phosphate, ascorbic acid, and dexamethasone (35, 43). There are also a number of commercial medium available for osteoblast differentiation or mineralization, mainly tested on MSCs. However, in each of these cases, it remains important to distinguish whether the end mineral deposition was associated with other indicators of osteogenesis such as collagen fibril deposition and increased expression of osteogenic genes (44).

Several protocols have recently emerged to differentiate human iPS cells and human ES cells into osteoblasts (Fig. 1). Most protocols utilize BMPs supplemented in the culture medium and directly added to the human iPS cells (45) since BMPs are powerful promoters of osteogenesis and regulate differentiation of pluripotent cells (33, 46). Although BMPs in the medium may be useful for directed differentiation, they may confound disease modeling depending on whether BMPs are part of the disease pathogenesis or phenotype. For example, our experience with the FOP iPS cells requires using mineralization medium without BMPs to study how the increased signaling activity of the mutated BMP receptor ACVR1 affects the function of our FOP iPS cell lines (35). Similar concerns may be important for conditions using small molecules or substrates. These compounds may interact with the genetic mutations found in the cell lines.

In vitro osteogenic differentiation of human iPS cells can also be performed using embryoid body (EB) methods. A number of these protocols were initially established in murine ES or iPS cells (47, 48). Several authors have reported that treating human iPS cell-derived EBs with all-trans retinoic acid, and subsequently

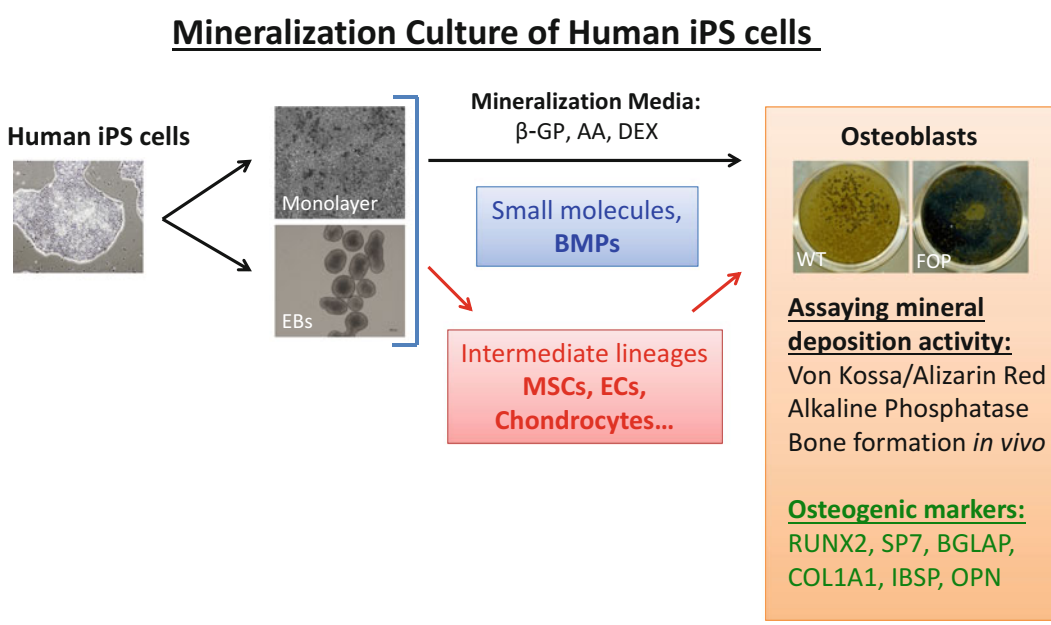

Fig. 1 Human iPS cells can be differentiated into osteoblasts via different protocols

culturing single-cell dissociated EBs in conditions that favor osteogenesis, can also lead to osteoblast differentiation in vitro and in in vivo implants (43).

A recent protocol described the use of small molecules under serum-free and feeder-free conditions to differentiate iPS cells into osteoblasts in a stepwise manner without the formation of EBs (49). These small molecules include GSK3 inhibitors (CHIR999021) to activate canonical WNT signaling cues to specify the differentiation toward mesodermal layers (50); a smoothened agonist (SAG), a hedgehog (HH) signaling activator which promotes early osteoblast differentiation in perichondrial cells consisting of osteochondroprogenitors (51); and TH (4-(4-methoxyphenyl)pyrido (4′,3′:4,5)thieno(2,3-b)pyridine-2carboxamide) for inducing osteoblast maturation (52). These defined conditions are extremely valuable as they will eventually provide more consistency, improve our ability to delineate osteoblast development and function, and facilitate more robust applications in drug screening and skeletal regeneration.

1.7 Assaying the Osteogenic Properties of Cell Lines

Determining if osteogenesis is increased in human iPS cell cultures can be challenging. As for mesenchymal stem cells, there are several surrogate markers that are commonly used including histology assays (staining for alcian blue to indicate cartilage; alkaline phosphatase to detect early mineralization activity; and calcium/phosphorous staining with von Kossa or alizarin red to detect mineral deposition). The direct assessment of osteoblast number also

remains challenging mostly because of the dearth of cell surface markers that clearly identify the specific lineages and stages of skeletogenesis. Recently, several groups have created genetically marked reporters for use in human ES and iPS cells (53, 54). These types of constructs will be extremely valuable for assaying osteoblast and chondrocyte formation directly as well as identifying additional markers for these skeletal cell lineages.

One emerging direction is the expanded use of 3D cell culture methods for studying the osteogenic micro-niche as well as combining human iPS cells with in vivo bone regeneration models in rodents. For example, critical-sized cranial defects that do not spontaneously heal can be implanted with human iPS cells seeded onto scaffolds constructed from hydroxyapatite-coated poly-L-lactic acid engineered to release BMP2. The implanted cells can facilitate the repair of these bone lesions, suggesting that human iPS cells directly contribute to osteogenesis in vivo when implanted in the right setting (45). This type of model can be very useful to acquire terminal cell fate by pluripotent cells in an in vivo setting as a complement to in vitro functional analysis.

1.8 Directed Differentiation into Other Cell Lineages Found in Bone

One significant benefit for using human iPS cells to study the skeleton is the potential ability to create all of the cell types present in bone, including endothelial cells (38), osteoclasts (55), macrophages (56), and skeletal muscle cells (39). A promising strategy is to use pluripotent cells as a source of intermediate cell types or osteoprogenitors, such as MSCs (57). Several papers have described generation of MSCs from human iPS cells using a variety of different methods, including EB formation (58, 59), small molecules such as TGFβ pathway inhibitors (60), synthetic polymer substrates (61), or coculture with murine cells (62, 63). These methods are promising and will benefit from new methods that mark tissue-specific MSCs and a better understanding of the different subsets of tissue-specific MSC-like cells. In addition, the finding that endothelial cells expressing the FOP ACVR1 R206H mutation can undergo endothelial-to-mesenchymal transition (EndoMT) to form MSC-like cells and mineralize (64) suggests that there are likely multiple routes to create osteogenic precursors.

1.9 Future Directions

Human iPS cells are a promising way to generate human cell types from patients with genetic conditions, for disease modeling, for drug screening, and for tissue engineering. iPS cells provide an important complement to adult stem cells and mesenchymal stem cells by allowing the creation of a broader array of cell types.

More widespread application of iPS cells to musculoskeletal diseases is on the forefront and will be facilitated by the development of better directed differentiation protocols that exhibit high yield, cellular uniformity, and ease of use, particularly for lineages directly relevant to musculoskeletal tissues. New approaches using

directed nucleases such as TALENs (65) and CRISPRs (66) also show great promise as a way to induce point mutations and reporter constructs into human genomic DNA. These methods will help speed the process of genomic targeting for both pluripotent and differentiated cells on a uniform background or to create corrected isogenic cell lines as research controls. Finally, new marker and reporter lines for identifying skeletal gene expression, cell surface markers for purifying mesenchymal lineages, and libraries of diseased and genetically corrected human iPS cells will be extremely valuable tools for advancing the application of pluripotent stem cells for musculoskeletal diseases.

2 Materials

2.1 Feeder Cell Preparation

1. SNL Feeder cells: Mouse fibroblast STO cell line (67), which carries the neomycin resistance and murine leukemia inhibitory factor (LIF) genes—S, STO; N, neomycin resistance; L, LIF gene.
2. Gelatin 0.1 % (Sigma #G1393). Store at 4 °C.
3. Feeder Cell Maintenance Medium:

DMEM with Glutamax	LifeTechnologies, #10566
10 % FBS	Hyclone, characterized grade, #SH30910.03 Lot#AYK176955
Pen/Strep 10 U/ml	LifeTechnologies, #15140
Sodium Pyruvate 1 mM	LifeTechnologies, #11360

4. Feeder Cell Freezing Medium:

DMEM with Glutamax	LifeTechnologies, #10566
10 % FBS	Hyclone, characterized grade, #SH30910.03 Lot#AYK176955
Pen/Strep 10 U/ml	LifeTechnologies, #15140
Sodium Pyruvate 1 mM	LifeTechnologies, #11360
10 % DMSO	Sigma, #D2650

2.2 Human iPS Cells Maintenance on Feeders and Transfer onto Matrigel

1. mTesr Medium (StemCell Technologies cat #05850). Supplement is stored at −20 °C. Thaw Supplement at 4 °C overnight. Store reconstituted medium at 4 °C for up to 2 weeks maximum.
2. BD Matrigel Basement Membrane Matrix—GFR, High concentration (BD 354263). Keep Matrigel frozen at −80 °C in aliquots of 500 μl in 50 mL conical tubes. Thaw vial overnight

at 4 °C and dilute with KO DMEM (LifeTechnologies, #19829) to a concentration of 300 μg/ml. Note: Matrigel should always be kept cold as it will polymerize at room temperature and then will not dissolve in KO DMEM.

3. Y-27632 ROCK Inhibitor (EMD cat #688000). Resuspend in DMSO and store in 10 mM aliquots at −20 °C.
4. Human iPS Cell Freezing medium.

90 % mTeSR, reconstituted with supplement	
10 % DMSO	Sigma, #D2650
10 μM Y-27632 ROCK Inhibitor (EMD cat #688000) in DMSO	

2.3 Osteoblast Differentiation from Human iPS Cells

1. mTeSR reconstituted with supplement (Stem cell Technology #05850), stable for 2 weeks at 4 °C, warm up only aliquots to room temperature prior to use.
2. Y-27632 (ROCK Inhibitor, (EMD cat #688000)).
3. Accutase (Millipore #SCR005).
4. Gelatin 0.1 % (Sigma # G1393).
5. Ascorbic Acid 2 Phosphate (Sigma # A8960), 50 mg/ml in sterile water, stable for 1 week at 4 °C.
6. Dexamethasone water soluble (Sigma #D2915), 4 mM stock solution in sterile water. Store stock solution at −20 °C, stable for 6 month. Store 4 μM working solution at 4 °C.
7. Glycerol-2-Phosphate (Sigma #G9422), 1 M stock solution in sterile water. Store at 4 °C.
8. β-mercaptoethanol (Sigma #M6250) 0.143 M in sterile water, stable for 2 weeks at 4 °C.
9. Osteoblastic base medium (OB):

KO DMEM	LifeTechnologies, #19829
20 % FBS Characterized	Hyclone, characterized grade, #SH30396.03 Lot#AVC66310, Filter sterilized
2 mM Glutamax	LifeTechnologies, #35050
1 % NEAA	Nonessential Amino-Acids, LifeTechnologies, #11140
0.1 mM β-mercaptoethanol	Sigma #6250
10 mM Dexamethasone	Sigma #D2915
10 mM glycerol-2-phosphate	Sigma #G9422

2.4 Von Kossa/ Alcian Blue Staining

1. Silver Nitrate solution (Fisher #S181-25), 0.05 g/ml in distilled water. Must be prepared and stored in the dark at room temperature. Filter the solution with funnel and filter paper before use.
2. Sodium Carbonate-Formaldehyde solution, 0.05 g/ml sodium carbonate (anhydrous, Fisher#S263-500), 0.37 % formaldehyde solution (Fisher #F79-500), prepared in distilled water. Store at room temperature.
3. 1 % Alcian Blue solution (Sigma, #A3157) pH 2.5 in 3 % acetic acid. Filter through a 0.4 μm syringe filter just prior to use. This solution is stable for up to 6 months. The final dye concentration is approximately 0.65 %. Store at room temperature.

2.5 Alkaline Phosphatase Staining

1. 95 % EtOH.
2. BCIP/NBT substrate solution (Sigma, #B5655). Keep the substrate solution protected from light. Can be at RT for 1 h max just prior to use.

3 Methods

3.1 Feeder Cell Preparation

1. Thaw one vial of 1.5×10^6 of nonirradiated STO ECACC SNL6/7 cells and plate onto one T225 with 50 ml Feeder Medium. Culture 4–5 days, no medium change needed.
2. Once the cells are 90 % confluent (small cells, just touching together), split 1:10 into ten new T225s (or use the multilayer flasks, such as BD353144). Culture 4–5 days, until confluent.
3. Harvest cells by dissociating with 4 ml trypsin/T225 for 2–5 min. Quench with 4 ml Feeder Medium. Pool five flasks together (40 ml total). Rinse the five flasks with 12 ml feeder medium, recovering >10 ml. Repeat for other set of five flasks
4. Count cells. Expected yield is $240–300 \times 10^6$ cells total for ten T225 flasks. Freeze down vials of nonirradiated cells here, if needed. Irradiate remaining cells for 60 Gy total dose. Note that many commercial sources use/suggest 40 Gy. We have had a few cases of breakthrough growth at low doses. However, do not overdose, as at >80 Gy the cells have low viability and will not support iPS cell cultures.
5. Spin down cells at $200 \times g$ for 10 min. Aspirate medium and resuspend cells in Freezing Medium. Freeze in 1 ml aliquots of 3×10^6 cells slowly (1 °C/h, using cell freezing cooler) and store long term in liquid nitrogen tank.
6. If needed, there are enough leftover cells in the TC flasks that new medium can be added on and SNLs re-expanded once (i.e., don't throw the flasks away if you want to do a second

expansion. More than two expansions reusing the same flasks are not recommended).

3.2 Human iPS Cells Maintenance on Feeders and Transfer onto Matrigel

1. *SNL Feeder Cell Thawing*
   (a) Add 1 mL of 0.1 % gelatin to each well of a six-well plate and incubate the six-well plate with gelatin at 37 °C for 15 min.
   (b) Thaw a vial of irradiated SNLs (3×10^6 cells per vial) and resuspend in MEF medium.
   (c) Remove the gelatin from the six-well plate. Add 2 mL of the cell suspension to each well of the plate and incubate at 37 °C.
   (d) Wait at least 24 h before seeding iPS cells (**Note 1**).
2. *Thawing human iPS cells.*
   (a) Prepare an aliquot of complete mTeSR + 10 μM ROCK inhibitor.
   (b) Thaw human iPS cells in a 37 °C water bath and resuspend KO DMEM.
   (c) Centrifuge for 3 min at 100 × *g*, aspirate the supernatant, and resuspend the iPS cells in 2 ml complete mTeSR +10 μM ROCK inhibitor (final concentration).
   (d) Remove the medium of the SNLs and plate cells into one to three wells of a six-well SNLs plate.
3. *Passaging iPS cells (generally 4–5 days after thawing or seeding).*
   (a) Prewarm complete mTeSR supplemented with ROCK inhibitor final concentration 10 μM.
   (b) Remove the medium from iPS cells and rinse with DPBS. Add 0.5 mL of accutase to each well and incubate at 37 °C for 3 min.
   (c) While the cells are incubating, take a six-well plate with SNLs from the 37 °C incubator and remove the medium.
   (d) Transfer the iPS cells to a 15 mL conical tube and centrifuge for 3 min at 100 × *g*.
   (e) Remove supernatant and resuspend the iPS cells in 2 mL complete mTeSR supplemented with ROCK inhibitor. Split the cells (typically 1:10, but high splits are generally not tolerated well) to a new plate of SNLs with mTesr and ROCKI; adjust the split ratio for each cell line.
   (f) Change medium every 24 h with 2 mL of complete mTeSR (no ROCKI).
   (g) Human iPS cells should be split once they are 80 % confluent, generally 4–5 days after thawing or splitting. It is important to check the phenotype and make sure that iPS cells are not differentiated. (Human iPS cells may be plated

on SNLs for maintenance or on Matrigel for further differentiation experiments, if desired. *See* below.)

4. *Matrigel-coated plates preparation.*
   (a) Prepare Matrigel plate. Frozen aliquots of Matrigel need to be thawed overnight at 4 °C.
   (b) Dilute Matrigel with *cold* KO DMEM (final concentration approx. 300 μg/ml). (Check the concentration based on the batch). Diluted Matrigel can be kept in the conical at 4 °C for 1–2 weeks.
   (c) Add one 1 ml of diluted Matrigel per well of six-well plate or 3 ml per 10 cm plate. Place in the incubator at 37 °C for 40 min.

       Note: if the plates are left in the incubator for longer, it is important to make sure that the wells don't dry out. The coated plates should be used the same day they were coated.

   (d) Before use, aspirate Matrigel, then immediately seed the iPS cells.
5. *Freezing Human iPS cell*
   (a) Centrifuge cells at 200 × *g* for 10 min. Aspirate medium and resuspend cells in Human iPS cell Freezing Medium. Freeze in 1 ml aliquots cooling cells slowly (1 °C/h, using cell freezing cooler) and store long term in liquid nitrogen tank.

3.3 Osteoblast Differentiation from Human iPS Cells

1. Culture human iPS cells in 10 cm plates to 85 % confluence. One 10 cm plate should allow preparing two 24-well plates.
2. Day 0: Gelatin coat plates based on expected number of cells. For 24-well plates, use 400,000 cells/well. Wait at least 30 min for gelatin to coat the surface at 37 °C.
3. iPS cells are washed with DPBS and then incubated with 2 ml of prewarmed Accutase for 3 min at 37 °C. To completely detach the cells you may tap the side of the plate or scrap them.
4. Add 5 volumes of KO DMEM (approx. 10 ml), gently pipette up and down to detach all the iPS cells, transfer in a 15 or 50 ml conical tube, and centrifuge at 100 × *g* for 3 min.
5. Resuspend the iPS cells in 15 ml 37 °C prewarmed mixed medium 90 % OB Medium (OB Base medium supplemented with 50 μg/ml of Ascorbic acid) / 10 % mTeSR, supplemented with 10 μM ROCK Inhibitor) for 1 × 10 cm^2 dish (increase if more dishes have been pooled).
6. Count cells. Remove the gelatin from the receiving plate.
7. Based on the amount of live cells, plate 400,000 cells/well in 1 ml of mixed medium and place the plates at 37 °C, 5 % CO_2.

8. Day1: Change medium to Osteoblastic base medium extemporaneously supplemented with 50 μg/ml of Ascorbic Acid.
9. Medium is changed every other day until day 24. The medium should be changed very carefully as the cells may start to peel off around day 10.

3.4 Von Kossa/ Alcian Blue Staining for Tissue Culture (Note 2)

1. All steps of this protocol should be performed in a chemical hood.
2. Remove medium from wells and gently rinse with DPBS.
3. Fix the cells with 4 % PFA for 15 min and rinse three times with water. Rinse wells carefully, as any residual PFA may cause the subsequent staining steps to be spuriously positive.
4. Stain in Silver Nitrate solution for 15 min in dark (wrap with aluminum foil) and wash three times with distilled water, 1 min each.
5. Develop in Sodium Carbonate-Formaldehyde solution for 2 min (time is critical) and wash two times with distilled water, 1 min each. At this time plates may be air-dried and photographed or continue with the Alcian Blue staining.
6. Optional alcian blue staining: Add 1 % Alcian Blue solution (pH 2.5) for 1 h (shorter is likely OK) and wash two times with water, 1 min each.
7. Air-dry plate and photograph or scan.

3.5 Alkaline Phosphatase Staining

1. Aspirate medium from the cell culture plates to fix and wash once with PBS 1×.
2. Fix the cell by adding 95 % EtOH and incubate at RT for 10 min minimum (1 h max).
3. Rinse three times with DPBS and add NBT/BCIP substrate solution to each well.
4. Incubate the cell culture plate for 5–10 min at 37 °C, 5 % CO_2.
5. Rinse three times with water and dry in open air.

4 Notes

1. *Feeder cell preparation.*
   Note: It is not required to change the medium every day since the cells are irradiated and will not grow. However, if they are going to stay on the plate for an extended period of time, it is recommended to change the medium every 3–4 days.
2. *Alcian blue staining.*

Generally, the two stains should be done separately at first to make sure the process is working and robust.

Alcian blue pH can be adjusted to stain different mucins:

pH 2.5 = most acid mucins (except strongly sulfated group) (blue).

pH 1.0 = only weekly and strongly sulfated acid mucins.

pH 0.2 = strongly sulfated acid mucins only.

Mineral = black

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