HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2023 January 10.

Published in final edited form as:

Methods Mol Biol. 2023 ; 2598: 87–114. doi:10.1007/978-1-0716-2839-3_8.

Chondrogenic Differentiation of Human-Induced Pluripotent Stem Cells

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Abstract

The generation of large quantities of genetically defined human chondrocytes remains a critical step for the development of tissue engineering strategies for cartilage regeneration and high-throughput drug screening. This protocol describes chondrogenic differentiation of human-induced pluripotent stem cells (hiPSCs), which can undergo genetic modification and the capacity for extensive cell expansion. The hiPSCs are differentiated in a stepwise manner in monolayer through the mesodermal lineage for 12 days using defined growth factors and small molecules. This is followed by 28 days of chondrogenic differentiation in a 3D pellet culture system using transforming growth factor beta 3 and specific compounds to inhibit off-target differentiation. The 6-week protocol results in hiPSC-derived cartilaginous tissue that can be characterized

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Author Contribution Statement ARD, NS, CLW, and FG were involved in the development, testing, and troubleshooting of these protocols and the writing of this paper.

2.If there is hiPSC death, cells were plated at too low of a density, media was changed too soon, Y-27632 was not added, cells were washed and fed too aggressively, or the cell line is not compatible with vitronectin. Reduce number of wells and/or flasks you are plating, make sure media is changed 24 h after plating (or try extending the time slightly longer than 24 h), make sure to add Y-27632 to media for the first 24 h, pipette media slowly and onto the side of the dish (not directly on the cells), or try other matrix substrates, such as Matrigel.

5.If cells are not differentiating or nodules form in the center of colonies, cells were induced at too high of a density. Passage cells at a lower density and induce cells 48 h after passaging.

6.If cells lift off the culture surface, cells were induced at too high of a density or were washed and fed too aggressively. Passage cells at a lower density, induce 48 h after passaging, and pipette media slowly on the side of the dish (not directly on cells).

Conflicts of Interest FG is an employee of Cytex Therapeutics, Inc. The other authors declare that they have no competing interests.

by histology, immunohistochemistry, and gene expression or enzymatically digested to isolate chondrocyte-like cells. Investigators can use this protocol for experiments including genetic engineering, in vitro disease modeling, or tissue engineering.

Keywords

Human iPSCs; Chondrogenesis; Stem cells; Tissue-engineered cartilage

1 Introduction

Articular cartilage is the tissue lining the ends of long bones in synovial joints, providing a nearly frictionless surface for joint motion while withstanding millions of cycles of loading per year [1, 2]. The unique mechanical properties of cartilage [3] are due to the composition and structure of the cartilage matrix – predominantly a variety of proteoglycans and collagens as well as hyaluronate and fibronectin [4]. Glycosaminoglycans (GAGs), largely comprising the large aggregating proteoglycan aggrecan (ACAN), make up 4–7% of the tissue [4–6]. Due to their negative charge, GAGs retain water, which composes 65–80% of the tissue weight, contributing to the compressive properties of cartilage [4–6]. Type II collagen, making up approximately 10–20% of cartilage matrix, primarily contributes to the shear and tensile properties of the tissue [4–6].

Chondrocytes, the sole cell type in articular cartilage, are responsible for maintaining the homeostasis of cartilage matrix proteins in response to genetic and environmental signals, such as growth factors or physiologic loading [7, 8]. However, under pathologic, injurious loading, the chondrocytes shift to a degradative and inflammatory phenotype [9]. Since the cartilage is avascular and aneural, it is susceptible to degeneration in an inflammatory environment, while lacking the ability to regenerate, leading to diseases such as arthritis [4, 10].

Arthritis is a family of joint diseases characterized by degradation of the cartilage matrix, which leads to joint space narrowing, along with osteophyte formation, bone remodeling, and synovitis. These progressive changes are associated with pain, swelling, and loss of motion [11, 12]. There is a variety of types of arthritis, affecting approximately 54 million adults in the United States [13], with osteoarthritis (OA) and rheumatoid arthritis (RA) as the most common. OA affects the largest number of people with increasing age, female sex, genetics, joint injury, and obesity as the primary risk factors [11, 14]. RA is an autoimmune disease [12], where the immune system leads to the inflammatory environment and onset of degeneration and symptoms [12]. Unfortunately, current treatment for OA is limited to nonsteroidal, anti-inflammatory drugs for mild-to-moderate cases and joint replacements for severe cases [11]. Disease-modifying antirheumatic drugs have been developed to treat RA; however, they are effective in only a fraction of individuals and have been associated with significant side effects [12]. Therefore, there remains a need for improved therapies for cartilage injury or arthritis. In this regard, cartilage tissue engineering not only provides a potential regenerative therapy for joint diseases but can also provide in vitro model systems for disease modeling and drug development [15–17]. Furthermore, such in vitro models

can be used to elucidate mechanisms of and develop treatments for a variety of other cartilage-associated diseases [18–25]. In this regard, the availability of large numbers of genetically defined cells that can be chondrogenically differentiated could serve as a critical component in the development of new cell-based therapies or in vitro disease models for drug screening.

1.1 Development of the Protocol

This protocol was developed based on a series of previous studies that elucidated the sequence of signaling cues required for cartilage development in vivo coupled with various reports of in vitro chondrogenic differentiation of mouse and human pluripotent stem cells [26–29]. In previous studies, we developed a method to derive chondrocytes from hiPSCs in a stepwise manner via the paraxial mesodermal lineage [30] based on the mesodermal roadmap developed by Loh et al. using embryonic stems cells and iPSCs [26]. The next steps toward directed chondrogenic differentiation were induced using bone morphogenic protein 4 (BMP4) and transforming growth factor beta 3 (TGF-β3), given their known roles in precartilaginous mesenchymal condensation and chondrogenic differentiation [29, 31–36]. In this process, hiPSC-derived sclerotome cells were treated with BMP4. The resulting chondroprogenitor cells were then cultured in a conventional 3D pellet system with TGF-β3 to further specify mesodermal cells into the chondrogenic fate. However, despite the robust generation of chondrocytes from hiPSCs using BMP4 and TGF-β3, we and others have shown significant and unpredictable cellular heterogeneity within newly formed cartilaginous tissues [30, 37–40].

To determine the identity of such nonchondrocytic cells, bulk and single-cell RNA sequencing were applied at multiple time points throughout the course of hiPSC chondrogenesis. These studies revealed that the primary off-target cells were of neural and melanocytic lineages [37, 38]. With the analysis of the gene regulatory networks, we identified that *WNTs* and melanocyte inducing transcription factor (*MITF*) are the primary hub genes responsible for off-target neurogenesis and melanogenesis during hiPSC chondrogenic differentiation, respectively. Thus, in the improved protocol, we use small molecules to inhibit Wnt and MITF signaling during chondrogenic pellet culture, significantly enhancing the efficiency and homogeneity of hiPSC chondrogenesis. We further validated our optimized protocol using multiple hiPSC lines, histological and quantitative biochemical analysis of cartilage matrix production, and real-time quantitative polymerase chain reaction (RT-qPCR) and single-cell RNA sequencing techniques to evaluate gene expression (Fig. 1).

1.2 Applications of the Protocol

The hiPSC-derived chondrocytes and tissue-engineered cartilage from this protocol can facilitate the development of patient-specific regenerative approaches for a variety of cartilaginous disorders including, but not limited to, arthritis, osteochondritis dissecans, relapsing polychondritis, chondrocalcinosis, cartilaginous tumors, and arthropathies [41]. Particularly, our protocol can allow for in vitro disease modeling and high-throughput drug screening [15]. Furthermore, in vitro modeling of specific genetic conditions can be established through targeted genome engineering of the cells (e.g., CRISPR-Cas9 genome

editing) with isogenic controls or patient-derived iPSCs, in conjunction with simulation of a diseased environment (e.g., inflammatory cytokines) [16, 42, 43]. Furthermore, since the protocol follows the developmental lineage, such models allow investigation of the mechanisms underlying developmental disorders, such as skeletal dysplasias, chondrodysplasias, collagenopathies, and aggrecanopathies [41].

Additionally, the protocol can be applied to cartilage tissue engineering and other topics in the musculoskeletal field. Studies can be developed to investigate mechanisms that promote or repress chondrogenic differentiation to improve tissue engineering and regenerative strategies. For example, various forms of loading such as osmotic, compressive, and shear forces can be applied at different magnitudes, time points, and regimens to optimize matrix production and mechanical properties [44]. The similarity between hiPSC-derived chondrocytes and primary chondrocytes allows for the study of fundamental questions regarding cell physiology, such as mechanisms of chondrocyte mechanobiology (e.g., role of ion channels, integrins, and other mechanotransduction pathways), the development and physiology of the chondrocyte circadian rhythm, and the cells' responses to metabolic syndrome and inflammation. Furthermore, the rapid expansion of genome engineering in the cartilage field provides the opportunity to apply principals of synthetic biology for the development of "smart," self-regulating cells or mechanogenetic gene circuits in human cells [45–48]. Therefore, the ability to generate a large number of chondrogenically differentiated cells from hiPSCs provides the opportunity for numerous advances in cartilage research, including other types of cartilaginous tissues [49].

1.3 Comparison with Other Methods

To tissue-engineer cartilage, a culture system is needed to differentiate and culture chondrocytes that can synthesize and accumulate cartilaginous matrix, with or without a biomaterial scaffold [6]. A variety of cell types have also been used for this purpose including primary chondrocytes, adult multipotent stem cells, and pluripotent stem cells [6]. While primary chondrocytes can synthesize a cartilaginous matrix in 3D, they are limited in their expansion potential and undergo dedifferentiation with passage in monolayer [50]. Additionally, harvesting of human chondrocytes from a patient results in donor site morbidity, while other sources (e.g., cadaveric, surgical waste) are difficult to obtain [6]. Therefore, adult stem cells, such as bone marrow-derived mesenchymal stem cells [51] and adipose-derived stem cells [52], have been used for cartilage tissue engineering. While these methods have been optimized to successfully produce cartilage-like tissue, adult stem cells represent a heterogeneous population of cells and show significant donor-to-donor variability. Furthermore, they have limited in vitro expansion capacity, making it difficult to perform gene editing and clonal isolation [53–55]. Induced pluripotent stems cells have high proliferation and differentiation capacities, allowing for the study of genetic perturbations. In addition, they can be derived in a patient-specific manner with low or no donor morbidity, and they do not have the ethical concerns associated with embryonic stem cells [42,43, 56].

Previously published chondrogenesis protocols of stem cells, including ours, have greatly extended our knowledge in chondrocyte biology and cartilage tissue engineering; however, many of these approaches rely on the application of fetal bovine serum (FBS) in culture

medium. While it may enhance cell viability, FBS can also lead to off-target differentiation because of its undefined chemical composition. Lot-to-lot variability of FBS may also make the protocols difficult to reproduce in different laboratories [34, 35, 57–61]. Here, we have established a chondrogenic differentiation protocol for hiPSCs, using serum-free, chemically defined medium, that we have validated with 8 hiPSC lines with and without genetic mutations.

1.4 Experimental Design

1.4.1 Cell Source—hiPSC lines derived from foreskin or skin fibroblasts with either retroviral or Sendai viral induction of the Yamanaka factors (i.e., *OCT3/4, SOX2, KLF4*, and *c-MYC*) [56] have been tested with this protocol. Sendai viral hiPSCs are free from genomic integration of the Yamanaka factors, unlike retrovirally transduced hiPSCs, which have the genes integrated in their genome [62]. The cells are maintained in hiPSC-maintenance medium, avoiding over-crowding and spontaneous differentiation. In some cases, cleaning, either colony picking or scraping of differentiated cells, can be carried out. hiPSC maintenance, expansion, and mesodermal differentiation are performed on vitronectin-coated plates.

1.4.2 Mesodermal Differentiation—Approximately 48 h after passaging, when the hiPSCs have reached 30–40% confluency, the cells are induced for mesodermal differentiation in monolayer. The cells are fed every 24 h with a defined growth factor and small molecule cocktail to guide differentiation. First, basic fibroblast growth factor (FGF) alongside activation of the TGF and Wnt-signaling pathways drive the cells into the anterior primitive streak. Wnt activation and FGF are continued while the TGF/BMP pathway is inhibited on the second day to derive paraxial mesodermal cells. Next, all these pathways (i.e., FGF, Wnt, and TGF/BMP) are inhibited for differentiation into the early somite. Wnt inhibition is continued in combination with sonic hedgehog (SHH) activation for the next three days achieving sclerotome cells. The following 6 days drive the cells into chondroprogenitors by adding BMP4 each day (Fig. 1). Notably, mesodermal differentiation begins along the perimeter of the hiPSC colonies, and cell morphology will change from the small, round, colonized hiPSCs into longer, more fibrotic cells that spread throughout the culture plate.

1.4.3 Chondrogenic Differentiation—After the 12-day mesodermal differentiation in monolayer, chondroprogenitor cells are disassociated with TrypLE into single cells. Cells are resuspended at a concentration of 5×10^5 cells/mL using complete chondrogenic medium containing TGF-β3. These cells are transferred to a 15 mL conical tube and centrifuged to form the 3D chondrogenic pellet culture (Fig. 1). The media is replaced every 3–4 days with complete chondrogenic medium until the time point of interest, typically 28 days after chondrogenic induction.

1.4.4 Validation of Chondrogenic Differentiation—We use five different methods to validate the outcome of the chondrogenic differentiation: isolated single cells (chondrocytes), histology, immunohistochemistry (IHC), biochemical analysis, and RT-qPCR (Fig. 1).

The chondrogenic pellets are digested to obtain hiPSC-derived chondrocytes at the single cell level (Fig. 1). Pellets are washed with $DPBS^{-/-}$ and transferred into a 0.4% (wt/vol) type II collagenase solution in a conical tube. The tubes are vortexed and placed on an orbital shaker in a 37 °C incubator. The tubes are vortexed every 20 min until the pellets are digested. After pellet digestion, the collagenase solution with single cell suspension is neutralized with medium containing 10% FBS. Cells are then resuspended in the appropriate serum-free culture medium for follow-up experiments.

For histological and IHC validation, pellets are fixed in 10% neutral buffered formalin overnight, dehydrated in 70% ethanol at 4 °C, processed following standard histological protocol (including multiple dehydration steps), and embedded in paraffin wax. Wax blocks containing the pellets are cut into 8 μm sections, and sections are placed on microscope slides. Histological slides are stained for the nuclei and proteoglycans with hematoxylin and Safranin-O (Saf-O), respectively. For IHC, slides are rehydrated, prepared for staining with a primary antibody of interest; visualized with a secondary antibody, enzyme conjugation, and a chromogen substrate, and then counterstained for the nucleus. Slides are mounted with paramount and cover slipped for imaging and storage. IHC can be semiquantified using the previously published ImageJ protocol [63].

For a more quantitative analyses and validation of our protocol, we recommend two additional methods. The first is the biochemical assay using dimethylmethylene blue (DMMB) and PicoGreen for quantitative measures of sulfated GAG (sGAG) and DNA contents, respectively [52]. Chondrogenic pellets are washed in $DPBS^{-/-}$, transferred into a 1.7 mL tube containing 200 μL of 125 μg/mL papain, vortexed, and digested at 65 °C overnight to release DNA and sGAG content. The following day, the digested samples are vortexed again and can be frozen until needed. This protocol measures the sGAG content, which can then be normalized to DNA. The second method is to quantify gene expression using RT-qPCR [52, 64]. Each pellet is washed with $PBS^{-/-}$ and transferred into a 2 mL tube to be snap frozen in liquid nitrogen and stored at −80 °C until the RNA isolation.

2 Materials

2.1 hiPSC Culture

1. hiPSC lines: This protocol has been validated with hiPSCs derived from fibroblasts reprogrammed using Sendai virus or retrovirus. It has also been successful with CRISPR-Cas9-edited hiPSCs.
2. Culture dishes: 6-well cell culture plate or T75 or T225 cm^2 cell culture flask.
3. Matrix substrate: 1 μg/mL vitronectin recombinant human protein, truncated (Invitrogen) in PBS. Coat plate at 0.5 $\mu g/cm^2$, distribute by rocking plate to ensure surface is coated, and incubate at room temperature 1 h. Plates can be used immediately or stored at 2–8 °C wrapped in plastic film. If stored, warm for 1 h at room temperature before using. Aspirate vitronectin solution and discard before culturing cells.

4. hiPSC culture medium: Essential 8 flex media (E8; Gibco). Prepare according to manufacturer instruction and store at 4 °C for up to 2 weeks. Add 10 μM Y-27632 (STEMCELL Technologies) to E8 medium for the first 24 h after thawing or passaging.
5. Disassociation solution: ReLeSR (STEMCELL Technologies).
6. Cryomedium: PSC cryopreservation kit (Gibco).
7. 2 ml cryotubes.

2.2 Mesodermal Differentiation

1. Differentiation medium (500 mL): 242.5 mL Iscove's Modified Dulbecco's Medium, glutaMAX (IMDM; Gibco), 242.5 mL Ham's F-12 nutrient mix, glutaMAX (F12; Gibco), 5 mL penicillin-streptomycin (P/S; Gibco), 5 mL insulin-transferrin-selenium (ITS+; Gibco), 19.5 μL 1-thioglycerol (Millipore Sigma), 5 mL chemically defined concentrated lipids (Thermo Fisher Scientific). Store at 4 °C for up to 2 weeks.
2. Wash medium (500 mL): 248.5 mL IMDM, 248.5 mL F12, 5 mL P/S. Store at. 4 °C for up to 2 weeks.
3. Anterior primitive streak differentiation medium (day 1): 30 ng/mL activin A protein (R&D Systems), 20 ng/mL recombinant human fibroblast growth factor basic protein (FGF2; R&D Systems), 4 μM CHIR99021 (Reprocell) in warmed differentiation medium (37 °C water bath).
4. Paraxial mesoderm differentiation medium (day 2): 20 ng/mL FGF2, 3 μM CHIR99021, 2 μM SB505124 (Tocris Bioscience), 4 μM dorsomorphin (Reprocell) in warmed differentiation medium.
5. Early somite differentiation medium (day 3): 2 μM SB505124, 4 μM dorsomorphin, 500 nM PD173074 (Tocris Bioscience), 1 μM Wnt-C59 (Cellagen Technologies) in warmed differentiation medium.
6. Sclerotome differentiation medium (days 4–6):1 μM Wnt-C59,2 μM purmorphamine (Reprocell) in warmed differentiation medium.
7. Chondroprogenitor differentiation medium (days 7–12): 20 ng/mL recombinant human bone morphogenic protein 4 (BMP4, R&D Systems) in warmed differentiation medium.
8. Disassociation reagent: TrypLE Select enzyme (Gibco).
9. Neutralization medium: 494 mL Dulbecco's Modified Eagle Medium/F12, glutaMAX (DMEM/F12; Gibco), 5 mL fetal bovine serum (FBS; Atlanta Biologicals).
10. Trypan blue stain (Invitrogen).
11. Cell counter.
12. 50 mL conical tubes.

13. Cryomedium (20 mL): 16 mL FBS, 2 mL DMEM, 2 mL dimethylsulfoxide (DMSO).
14. 2 ml cryotubes.

2.3 Chondrogenic Differentiation

1. Dexamethasone (100 μM): Add 19.62 mg dexamethasone powder (Millipore Sigma) to 1 mL absolute ethanol. Transfer 0.8 mL of the ethanol solution to 39.2 mL DMEM/F12 for a 1 mM solution. Transfer 4 mL of the 1 mM solution to 36 mL DMEM/F12 to make a 100 μM solution. Store aliquots at −80 °C up to 1 year.
2. Ascorbic acid solution: 50 mg/mL ascorbic acid (Millipore Sigma) in DMEM/F12. Store aliquots at −80 °C for up to 3 months.
3. Proline: 40 mg/mL proline (Millapore Sigma) in DMEM/F12. Store aliquots at −80 °C for up to 3 months.
4. Chondrogenic medium (500 mL): 483 mL DMEM/F12, 5 mL P/S, 5 mL ITS+, 5 mL Modified Eagle Medium (MEM) with nonessential amino acids (Gibco), 0.5 mL dexamethasone, 0.5 mL 2-Mercaptoethanol (Gibco). Store at 4 °C for up to 2 weeks.
5. Complete chondrogenic medium: 10 ng/mL recombinant human transforming growth factor beta 3 protein (TGF-γ3; R&D Systems), 1 μM Wnt-C59, 1 μM ML329 (Cayman Chemical), 0.1% ascorbic acid, 0.1% proline in warmed chondrogenic medium.
6. 15 mL conical tubes.

2.4 Chondrogenic Validation

2.4.1 Digestion of Chondrogenic Pellets

1. $DPBS^{-/-}$.
2. Neutralization medium (500 mL): 494 mL Dulbecco's Modified Eagle Medium/F12, glutaMAX (DMEM/F12; Gibco), 5 mL fetal bovine serum (FBS; Atlanta Biologicals).
3. Digestion medium: 0.4% wt/vol type II collagenase (Worthington Biochemical, activity 225 units/mL) in warmed neutralization medium. Sterile filter and use immediately.
4. 15 mL or 50 mL conical tubes.
5. Orbital shaker.

2.4.2 Histology Preparation

1. $DPBS^{-/-}$.
2. 20 mL glass scintillation vials.

3. 10% neutral buffered formalin.
4. Paraffin.
5. Ethanol, 200 proof.
6. Xylenes.
7. Microscope slides.
8. Microtome.

2.4.3 Safranin-O Staining

1. Safranin-O solution (Millipore Sigma).
2. Harris hematoxylin with glacial acetic acid (Poly Scientific).
3. Ethanol, 200 proof.
4. Xylenes.
5. Differentiation solution (Millipore Sigma).
6. Permount mounting media.
7. Glass cover slips.

2.4.4 Immunohistochemistry

1. Ethanol, 200 proof.
2. Xylenes.
3. COL1A1 antibody (Abcam, cat. no. ab90395).
4. COL2A1 antibody (Iowa Hybridoma Bank, cat. no. II-II6B3-s).
5. COL6A1 antibody (Fitzgerald Industries, cat. no. 70F-CR009X).
6. COL10A1 antibody (Millipore Sigma, cat. no. C7974).
7. Goat anti-mouse antibody (Abcam, cat. no. ab97021).
8. Goat anti-rabbit antibody (Abcam, cat. no. ab6720).
9. Hydrogen peroxide.
10. Methanol.
11. 10% goat serum (Thermo Fisher Scientific).
12. Pepsin (Thermo Fisher Scientific).
13. Proteinase K (Millipore Sigma): 0.5% wt/vol in TE buffer. Prepare fresh.
14. Quenching solution: 3% hydrogen peroxide in methanol.
15. Histostain Plus Kit (Thermo Fisher Scientific).
16. AEC substrate solution (Abcam).

17. Vector hematoxylin QS counterstain (Vector Laboratories).
18. VectaMount AQ aqueous mounting medium (Vector Laboratories).
19. Glass cover slips.
20. Aluminum foil.

2.4.5 Biochemical Analysis Preparation

1. $DPBS^{-/-}$
2. Papain solution (125 mg/L papain, pH 6.5): Weigh 125 mg of papain (Millipore Sigma), 13.8 g of sodium phosphate, 1.46 g ethylenediaminetetraacetic acid (EDTA), and 0.79 g cysteine hydrochloric acid (HCl). Mix reagents using a stir bar and plate at room temperature with 1 L ultrapure distilled water. Reagents may take 1.5–2 h to dissolve. Adjust pH to 6.5 using approximately 38 mL of 1 N NaOH. Aliquot and store at −20 °C for up to 3 months.
3. 1.7 mL tubes.

2.4.6 RT-qPCR Preparation

1. $DPBS^{-/-}$
2. 2 ml screw-top tubes.
3. Liquid nitrogen.
4. Primers. See Table 1 for suggested chondrogenic primers.

3 Methods

3.1 hiPSC Culture

≥ 1 h for coating plate(s), 30 min for plating/passaging, 30 min for feeding, 2–7 days for culture

1. Coat a 6-well plate with vitronectin.
2. Thaw a vial of hiPSCs in a 37 °C water bath.
3. Transfer the cells using sterile serological plastic pipettes into 10 mL of room-temperature hiPSC maintenance medium in a 15 mL conical tube.
4. Centrifuge cells at 200 g for 5 min at 23 °C.
5. Aspirate the supernatant.
6. Add 13 mL hiPSC maintenance medium containing 10 μM of Y-27632 to the cell pellet. Do not pipette up and down; instead gently rock the tube back and forth twice. This will prevent breaking up the colonies into single cells.
7. Aspirate the vitronectin-$PBS^{-/-}$ solution from plate.
8. Add 2 mL of cell solution to each well. Gently tilt the plate in circular and back-and-forth motions to distribute the cells evenly throughout the wells. Failure to

do so will cause the cells to cluster in one area, preventing proper proliferation, which may lead to spontaneous differentiation.

9. Incubate the plate at 37 °C.
10. After 24 h, aspirate the medium from the plate and feed with hiPSC maintenance medium without Y-27632.
11. Continue cell culture, feeding every day until cells reach approximately 80% confluency. Cells can be frozen in cryopreservation medium. We typically freeze cells sufficient for one 6-well plate in 1 mL of cryopreservation medium for future passaging. The split ratio may vary depending on hiPSC lines.
12. Before passaging, coat the appropriate number of 6-well plates, T75, and/or T225 flasks with vitronectin.
13. Aspirate medium from cell plate.
14. Wash each well with 2 mL of $PBS^{-/-}$.
15. Add 1.5 mL of ReLeSR for 1 min at RT.
16. Aspirate ReLeSR.
17. Incubate plate for 2 min at 37 °C. If the cells are ≥85% confluent or if there is spontaneous differentiation, the time can be shortened to 1 min.
18. Tape the plate several times and pipette 2 mL of hiPSC maintenance medium onto the bottom ofeach well, you should see the cells lift off with the medium, and transfer into a conical tube using sterile serological plastic pipettes. Do not pipette up and down.
19. Centrifuge cells at 200 g for 5 min at 23 °C.
20. Aspirate the supernatant.
21. Resuspend cells in hiPSC maintenance medium containing 10 μM of Y-27632. Do not pipette up and down, instead gently rock the tube back and forth twice. This will prevent breaking up the colonies into single cells.
22. Aspirate the vitronectin-$PBS^{-/-}$ solution from plate(s)/flask(s).
23. Add cell solution to plate(s)/flask(s). Gently tilt the plate(s)/flask(s) in a circular and back-and-forth motions to distribute the cells throughout the wells. Failure to do so will cause the cells to cluster, preventing proper proliferation and leading to spontaneous differentiation
24. Check cell density in microscope. If too confluent, remove some of the cell solution and add up volume with maintenance medium containing Y-27632.
25. Culture for at least 48 h until 30–40% confluent. Too high cell density inhibits mesodermal differentiation. Too low cell density prevents adhesion and increases cell death (*see* Notes 1–3).

3.2 Mesodermal Differentiation

30 min, 1 h for feeding, 12 days for culture

1. Warm appropriate volume of wash and differentiation medium in 37 °C water bath. For days 2–12: if medium changes from orange to yellow in color after 24 h, suggesting high metabolic activity of the cells, increase differentiation medium volume by 1 mL per well and/or 5 mL per flask.
2. Make the appropriate differentiation medium for the corresponding day by adding the correct growth factors. Day 1: anterior primitive streak; day 2: paraxial mesoderm; day 3: early somite; days 4–6: sclerotome; days 7–12: chondroprogenitor.
3. Aspirate maintenance media from plate(s)/flask(s).
4. Rinse plate(s)/flask(s) with wash medium.
5. Add complete differentiation medium.
6. Incubate plate at 37 °C.
7. Feed the cells every 24 h with mesodermal differentiation medium supplemented with the appropriate growth factor and small molecule cocktails. **Note that more-than-usual cell death may be observed after day 3 due to the inhibition of several major signaling pathways.** When switching to a new set of the growth factors and small molecules (i.e., days 1, 2, 3, 4, and 7), it is critical to feed at the 24 h time points to ensure proper lineage specification. Cells can be harvested after days 1, 2, 3, 6, and 12 if the specific lineage stages are of interest to the experiment (e.g., RT-qPCR) (*see* Notes 4–7).
8. On day 13, aspirate medium from cell plate(s)/flask(s).
9. Wash with $DPBS^{-/-}$.
10. Pipette TrypLE disassociation reagent onto plate(s)/flask(s). 2 mL per well of 6-well plate and 20–25 mL per T225 flask.
11. Incubate for 3 min at 37 °C.
12. Gently tap plate(s)/flask(s) several times to disassociate cells. You should see cells floating in medium (*see* Note 8).
13. Add slightly more than an equal volume of neutralization media.

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1. If hiPSCs are confluent in 2–3 days, the cell concentration of the frozen stock is too high. Plate one vial to more than one 6-well plate (e.g., 1.5 or 2 × 6-well plates).
3. If there is spontaneous differentiation, colonies were not maintained but broken into single cells or cells were plated at an incorrect density. Do not pipette up and down when plating and passaging hiPSCs or change the number of wells you plate per cell vial/plate. You can scrape differentiated cells to clean the wells.
4. If there is cell death, cells were induced at too low of a density or there was spontaneous differentiation. Wait longer after passaging to increase cell density before induction or clean wells before passaging by scraping differentiated cells. Note that more-than-usual cell death may be observed after day 3 due to the inhibition of several major signaling pathways.
7. If media is yellow, cells were metabolic and fed too low of a media volume. Increase volume by 1 mL per well (6-well plate) or 5 mL per T225 flask.
8. If cells are still stuck to the dish, they did not fully lift off the plate during disassociation. Place dishes with fresh TrypLE back in the incubator for 2 min, and increase the force of slap without causing media to splash on top of dish.

14. Pipette half of the medium into a conical tube.
15. Pipette up and down the other half in the wells/flask(s) twice to lift any remaining cells. Transfer to a conical tube.
16. Rinse the wells/flask(s) twice with neutralization medium and transfer to tubes each time.
17. Centrifuge cell tubes at 300 g for 5 min at 23 °C.
18. Aspirate supernatant.
19. Chondroprogenitor cells can be immediately used for chondrogenesis or be frozen in cryomedium for future chondrogenesis.

3.3 Chondrogenic Differentiation

1–3 h for pelleting, 28 days for differentiation

1. Warm neutralization and chondrogenic medium in 37 °C water bath.
2. Add TGFβ-3, ascorbate, proline, Wnt-C59, and ML329 to make complete chondrogenic medium.
3. Resuspend chondroprogenitor cells in complete chondrogenic medium. Combine cells if you have multiple tubes.
4. Stain 10 μL of the cell solution with 10 μL of trypan blue to count cell number on an automated cell counter or hemocytometer.
5. Using the cell count, calculate the volume needed for a concentration of 5×10^5 cells per 1 mL of complete chondrogenic medium (*see* Note 9).
6. Pipette the cell solution to make pellet cultures—1 mL per 15 mL conical tube.
7. Centrifuge tubes at 300 g for 5 min at 23 °C.
8. Loosen the caps of the tubes in the biosafety cabinet. The tubes must be loosened to provide oxygen supply to the cells. The cap should however still be screwed on so that it cannot be lifted off to prevent contamination.
9. Incubate the tubes at 37 °C.
10. Check tubes to confirm pellet formation after 24 h (*see* Note 10).
11. Feed pellets every 3–4 days.
12. Warm incomplete chondrogenic medium in 37 °C water bath.
13. Add TGF-β3, ascorbate, proline, Wnt-C59, and ML329 to make complete chondrogenic medium.

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9. If you do not have enough cells to create the desired number of chondrogenic pellets, reduce the number of cells per chondrogenic pellet (e.g., 2.5×10^5 or 3×10^5).
10. If pellets have not formed, wait an additional 24 h to allow them to form. Otherwise, dissociation reagent (TrypLE) was too aggressive, cells did not differentiate properly, or the cell line is incompatible with vitronectin. Use a milder disassociation reagent (e.g., 0.05 mM EDTA), decrease cell density at time of mesodermal induction, or try a different matrix substrate, such as Matrigel.

14. Aspirate medium from the conical tube with a 9” Pasteur pipette.

15. Use at 12 mL stereological pipette to feed 6 tubes at a time with 2 mL per pellet. Ensure lids remain loose (*see* Notes 11–12).

16. Chondrogenic pellets can be harvested at various time points as desired. Proceed to Subheading 3.4. We recommend weekly/bi-weekly harvests on days 7, 14, 28, and 42. In general, cells start to deposit matrix 14 days postchondrogenic induction (i.e., apparent pellet enlargement). Additionally, chondrocytes usually can be observed approximately 14–21 days postchondrogenic induction.

17. 28 days after chondrogenic induction, most of the cells should have differentiated into chondrocytes and formed cartilaginous matrix. We recommend digesting the chondrogenic pellet to retrieve the chondrocytes at a single cell level (Subheading 3.4.1) for further experimentation or using histology/IHC (Subheadings 3.4.2, 3.4.3, and 3.4.4), biochemical assays (Subheading 3.4.5), and/or RT-qPCR (Subheading 3.4.6) to confirm and study chondrogenic differentiation.

3.4 Chondrogenic Validation

3.4.1 Digestion of Chondrogenic Pellets—3 h

1. Warm neutralization and desired medium for the experiment. If plating the cells, we recommend using the neutralization medium.

2. Prepare digestion medium.

3. Aspirate medium from pellets.

4. Wash each pellet with 2–3 mL of $DPBS^{-/-}$.

5. Transfer pellets into a tube with digestion medium. Use 1 mL of digestion medium per pellet being digested (± 1 mL). A 15 mL or 50 mL tube should not exceed 8 mL or 25 mL of digestion medium, respectively.

6. Vortex the tube(s) and shake manually then place on an orbital shaker (80 RPMs) in a 37 °C incubator.

7. Every 20 min remove the tube(s) from the incubator to vortex and check digestion progress.

8. After approximately 2 h, the matrix of chondrogenic pellets should be mostly digested. The length of digestion time needed depends on harvest time point and size of the tube. For example, pellets harvested prior to 28 days postchondrogenic induction may only require 1 h to achieve full digestion. Additionally, we have observed faster digestion using 50 mL conical tubes (*see* Note 13).

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11. If media is yellow, pellets have grown significantly and are metabolically active or extra matrix is being produced around walls of the conical tube in addition to the pellet. Increase chondrogenic medium volume by 1 mL per tube or aspirate away excess matrix with Pasteur pipette during aspiration.

12. If black spots appear on pellet, melanin is being produced. Increase concentration of ML329.

9. Add neutralization medium to the tube(s). 4 mL for a 15 mL conical tube or 20 mL for a 50 mL conical tube.
10. Centrifuge tube(s) at 300 g for 5 min at 23 °C.
11. Aspirate supernatant.
12. Resuspend in neutralization medium.
13. Stain 10 μL of the cell solution with 10 μL of trypan blue to count on an automated cell counter or hemocytometer.
14. Centrifuge at 300 g for 5 min at 23 °C.
15. Resuspend the cells at the desired concentration. The number of cells retrieved per pellet may depend on hiPSC lines. On average, we get approximately 6.5 × 10^5 cells per pellet. The cell recovery rate may be increased if multiple pellets from the same group are pooled and digested together. If plating the chondrocytes, we recommend adding the cells to desired dish(es) and incubating for 6–8 h. This provides sufficient time for the cells to adhere without dedifferentiating and losing their phenotype.

3.4.2 **Histology Preparation—**At least 3 days

1. Transfer pellet(s) into a scintillation vial with 10% (vol/vol) neutral buffered formalin.
2. Store at 4 °C overnight.
3. Remove formalin and add 70% (vol/vol) ethanol. Pellets can be stored in 70% (vol/vol) ethanol at 4 °C long term.
4. Transfer pellet into a plastic cassette for processing. Biopsy foam pads can be used to sandwich the chondrogenic pellet within the cassette to prevent it from falling out. Processing can be done by hand or using a tissue processor.
5. Dehydrate the chondrogenic pellet for 30 min in 80% (vol/vol) ethanol followed by 30 min in 100% (vol/vol) ethanol. Exchange the 100% (vol/vol) ethanol for another 30 min. Pellets can be stored in 100% (vol/vol) ethanol overnight.
6. Clear the pellet for 30 min with a 1:1 solution of ethanol and xylene followed by 30 min of 100% (vol/vol) xylene. Exchange the 100% (vol/vol) xylene for another 30 min.
7. Begin embedding the pellet for 1 h with a 1:1 solution of xylene and paraffin wax at 60 °C followed by 1 h with 100% (vol/vol) paraffin at 60 °C. Exchange the 100% (vol/vol) paraffin for another 1 h at 60 °C.

[13] If chondrogenic pellets are not fully digested, the collagenase solution is not strong enough or the matrix is too dense. Ensure proper ratio of collagenase to medium was used and test different lots of collagenase. Remove pellets from tube and place in an empty well of 6-well plate. Use a closed, sterile 1.7 mL Eppendorf to gently “smash” pellets. Use collagenase solution to rinse the well and transfer back to tube. Digest an additional 20 min.

8. Transfer pellet into an embedding tray, place cassette on top, and fill with paraffin wax.
9. Store for a few hours or overnight at 4 °C to harden wax. Pellets can be stored in wax long term at RT.
10. Cut the wax blocks in 8 μm-thick sections and place a short ribbon of sections in a 42 °C water bath.
11. Remove a ribbon of sections from the water bath by allowing it to attach to a microscope slide. In general, there should be 3–5 sections per ribbon on each slide. Slides can be stored long term at RT.
12. Dry slides in a warmer at 37 °C for staining or in a drying rack at RT for storage overnight.
13. Perform desired histology, such as staining for sGAGs with Safranin-O (Subheading 3.4.3) or labeling of collagenous proteins with immunohistochemistry (Subheading 3.4.4).

3.4.3 Safranin-O and Hematoxylin—1–2 h

1. Remove paraffin wax with 3 rounds of soaking slide(s) in xylene for 5 min.
2. Rehydrate tissue with 100% (vol/vol) ethanol for 2 min followed by 50% (vol/vol) ethanol for 2 min.
3. Rinse slide(s) in tap water for 2 min.
4. Remove all excess water.
5. Stain slide(s) with Harris hematoxylin for 3 min (nuclei will be stained purple). Filter Harris hematoxylin staining solution before each use to remove potential precipitations and avoid deposition of particulates on slide. Do not reuse stain more than ten times.
6. Rinse slide(s) in tap water for 3 min.
7. Differentiate slide(s) in differentiation solution (acid alcohol) for 15 s.
8. Rinse slide(s) in tap water for 3 min.
9. Stain slide(s) with Safranin-O for 3–5 min (sGAGs will be stained pink/red). Do not reuse stain more than 10 times.
10. Rinse slide(s) multiple times with 100% (vol/vol) ethanol until no excess stain remains.
11. Let slide(s) partially dry before rinsing with xylene for 30 s.
12. Mount slide(s) with Permount, coverslip, let dry, and image (*see* Note 14).

[14] If there is cell and matrix heterogeneity in chondrogenic pellet, Wnt and MITF signaling is occurring and causing off-target differentiation. Increase concentrations of Wnt-C59 and ML329.

3.4.4 Immunohistochemistry—4–5 h

1. Remove paraffin wax with 3 rounds of soaking slide(s) in xylene for 5 min.
2. Rehydrate tissue by washing slide(s) with 100% (vol/vol) ethanol for 5 min twice, 95% (vol/vol) ethanol for 5 min twice, 70% (vol/vol) ethanol for 5 min, and 50% (vol/vol) ethanol for 5 min, and then tap water for 5 min. Do not let the slides dry out. Place slide(s) in a container with a hydrated paper towel.
3. Perform antigen retrieval by adding 100 μL of proteinase K or pepsin on the slide and incubating (*see* Table 2 for recommended reagent, timing, and temperature).
4. Wash the slide(s) with $DPBS^{-/-}$ for 5 min twice.
5. Quench the slide(s) for 1 h in 3% (vol/vol) hydrogen peroxide in methanol.
6. Wash the slide(s) with $DPBS^{-/-}$ for 5 min three times.
7. Use a PAP-pen to circle each piece of tissue on the slide(s) to prevent solution spreading.
8. Perform blocking by adding a couple drops (enough to cover the tissue) of 2.5% (vol/vol) goat serum to each piece of tissue for 1 h at RT.
9. Blot excess serum from bottom of inclined slide. Do not rinse.
10. Stain tissue with primary antibody for 1 h at RT in hydrated container. *See* Table 2 for antibodies and dilutions. Do not add primary antibody to one piece of tissue. Instead add more goat serum from step viii to serve as negative control.
11. Wash the slide(s) with $DPBS^{-/-}$ for 5 min three times.
12. Add a couple drops (enough to cover the tissue) of the proper secondary antibody at a 1:500 dilution for 30 min at RT (Table 2).
13. Wash the slide(s) with PBS for 5 min three times.
14. Combine 5 mL of ImmPACT AEC diluent, 2 drops of AEC reagent 1, 3 drops of AEC reagent 2, and 2 drops of AEC reagent 3. Mix well.
15. Add AEC solution to the slide(s) and incubate at RT. See Table 2 for timing (*see* Note 15).
16. Wash the slide(s) with distilled water for 5 min.
17. Rinse the slide(s) in tap water.
18. Cover tissue sections with Vector Hematoxylin QS counter-stain for 45 s.
19. Rinse the slide(s) in tap water for 10 s. Do not dehydrate slides.
20. Mount the slide(s) with VectaMount AQ aqueous mounting medium, coverslip, let dry, and image.

[15] If the positive control is not showing stain, development time is too short. Increase time of development with AEC.

3.4.5 Biochemical Assay Preparation—1 day for sample preparation, 2–3 h for assay

1. Wash chondrogenic pellets with 2–3 mL of $DPBS^{-/-}$.
2. Transfer each pellet into a 1.7 mL tube with 200 μL of papain and vortex.
3. Digest tissue at 65 °C overnight and vortex. Use of caplock on the tubes may help securely seal the tube at high temperatures. Samples can be stored at −20 °C until analysis.
4. Follow previously published protocol to determine sGAG/DNA ratio using DMMB and PicoGreen Assays [52].

3.4.6 RT-qPCR Preparation—1–2 days

1. Clean work area with RNaseZap solution.
2. Wash chondrogenic pellets with 2–3 mL $DPBS^{-/-}$.
3. Transfer pellet into a tube and snap freeze in liquid nitrogen. Samples can be frozen at −80 °C until analysis (*see* Note 16).
4. Follow previously published protocol to determine gene expression using RT-qPCR [64, 52].

3.5 Anticipated Results

During regular culture, the hiPSCs have a small, round phenotype, and they grow in dense, round colonies (Fig. 2a). When passaging and plating, caution must be taken to prevent from breaking up the colonies too much as the hiPSCs tend to trigger cell death or spontaneously differentiate if cultured as single cells. In our laboratory, most hiPSC lines had optimal survival and growth on vitronectin-coated 6-well plates while there was lower viability when cultured on 10 cm dishes. Some hiPSC lines that were originally maintained on other substrates (e.g., Matrigel) were able to be switched onto vitronectin substrate by culturing cells on vitronectin-coated plates for several passages before mesodermal differentiation. After mesodermal induction, hiPSCs begin to differentiate at the edges of the colonies. As the cells become more elongated and spread out, the center of the colony begins to differentiate as well (Fig. 2b–e). Some cell death may be observed after the third day of mesodermal induction due to the inhibition of multiple essential pathways; however, the remaining cells should recover and differentiate as expected. Excess cell death may occur if hiPSCs are induced at too low of a cell density. In our previous study, we have demonstrated a decrease in pluripotent genes *OCT4* and *NANOG* throughout the mesodermal differentiation with an upregulation of *MIXL1* indicating anterior primitive streak cells after day 1, *MSGN1* indicating the paraxial mesoderm after day 2, and *PARAXIS* indicating the early somite state after day 3 (30). After day 6, the cells are committed into the sclerotome lineage with an upregulation of *SOX9*, *PDGFRA*, and *PDGFRB*. The upregulation of *PDGFRB* continues through the chondroprogenitor stage

16. We generally use screw-top microcentrifuge tubes with O-ring cap.

alongside *COL2A1* expression after day 12 of mesodermal induction (30). We have also demonstrated that the chondroprogenitors express the surface markers CD166, CD146, and PDGFRβ, but not CD45, using flow cytometric analysis (37). Cells in the chondroprogenitor stage should be fully differentiated and spread out throughout the dish with an elongated phenotype (Fig. 2f). If the mesodermal differentiation is induced at too high of a cell density; however, one might observe formation of nodules in the center of cell colonies (Fig. 2g) and/or formation of a cell "sheet" that often spontaneously lifts off the culture plates, resulting into failed mesodermal lineage commitment.

Chondroprogenitor cells in monolayer culture (i.e., day 12 of mesodermal differentiation) should be disassociated into single cells to form 3D, chondrogenic pellets for chondrogenesis. Cells should lift off the bottom of the culture dish in the TrypLE solution after a 3 min incubation and gentle tapping. If cells do not lift off, increase the time of incubation. We have tried using cell scrapers in instances where cells do not lift after 8 min of incubation; however, the cells had poor viability and failed to form chondrogenic pellets. We average a yield of 6×10^7 chondroprogenitor cells per T225 flask; however, this value may vary due to different hiPSC lines and plating density used. Approximately 24 h after chondrogenic induction, we observe the formation of a spherical pellet in the bottom of the conical tube. Occasionally, the pellet may need additional time before the proper shape is visible. Pellets that do not form after 48 h should be discarded. This may be caused by improper mesodermal differentiation or incompatible disassociation reagent (cell line specific). In some cell lines, we have found disassociating chondroprogenitor cells in 0.05 mM EDTA and/or differentiating cells on Matrigel has improved pelleting and chondrogenesis. As pellet cultures approach 28 days, the medium may begin to turn yellow due to increased cellular metabolic activity, which can be prevented by increasing the volume of chondrogenic medium during feeding. In other cases, some excess matrix may have formed apart from the pellet in the tube; removing this during feeding will also reduce the rate the culture medium is metabolized. If black dots appear on the pellets, suggestive of off-target differentiation and presence of melanocytes, increasing the concentration of ML329 will inhibit MITF and potential melanin production. Throughout chondrogenic culture, the pellets should grow due to accumulation of cartilaginous matrix but maintain a relatively spherical shape. We have reported a significant increase in chondrogenic genes *SOX9, COL2A1*, and *ACAN* from the chondroprogenitor stage through day 42 of culture, while fibrocartilage and hypertrophic cartilage markers *COL1A1* and *COL10A1*, respectively, remain relatively low until later time points (Fig. 3a–e) [30, 37].

To obtain hiPSC-derived chondrocytes, day 28 chondrogenic pellets can be digested with type 2 collagenase. Vortexing the tubes every 20 min will facilitate the breakdown of the tissue matrix, thus increasing digestion efficiency. However, if the chunks of pellets remain intact after a lengthy period of digestion, "smashing" the pellets using a tool with a flat surface (e.g., spatula with shovel head, or lid of an Eppendorf tube) in a sterile Petri dish may help break up the tissue matrix. We do not recommend digesting for longer than 2.5 h as it may decrease the cell viability. Occasionally, small pieces of tissue may still be visible after the digestion process; however, most of the cells will be dissociated from the matrix. We average a cell viability of 85% and approximately 6.5×10^5 cells per digested pellet. We

found an increase in viability and efficiency when more pellets are digested at a time and 50 mL conical tubes are used.

To validate and visualize the cartilage matrix produced by hiPSC-derived chondrocytes, we use Safranin-O staining to reveal the presence of sGAGs (Fig. 4a–c), IHC to label various types of collagens (Fig. 4d–f), biochemical DMMB assay to quantify sGAG production (Fig. 3f), and RT-qPCR to analyze gene expression (Fig. 3a–e). We normally observe round chondrocyte-like cells within cartilaginous matrix with robust, homogenous Safranin-O staining (Fig. 4a–c). If cellular heterogeneity is observed, concentration of Wnt-C59 and ML329 can be increased to further prevent off-target differentiation. If there is little or no sGAG staining, differentiation was unsuccessful, which can be attributed to several issues addressed in Notes. If hiPSCs have successfully differentiated into chondrocytes, there should also be significant labeling for COL2A1 (Fig. 4d), with minimal labeling of COL1A1 (Fig. 4e) and COL10A1 (Fig. 4f). Biochemical analysis and RT-qPCR for these genes can further support successful chondrogenesis. Chondrogenic pellets typically have an sGAG/DNA ratio between 20 and 30 ng/ng in day-28 pellet samples, which can continue to increase with more time in culture (Fig. 3f). A significant upregulation of *SOX9, COL2A1*, and *ACAN* gene expression should be detected in the pellets at day 28 versus day-0 pellet samples (Fig. 3a–c), while the expression levels of *COL1A1* and *COL10A1* remain low until later time points (Fig. 3d–e). The hiPSC-derived chondrocytes and cartilage matrix generated using this protocol can be used for a variety of experiments including genetic engineering, in vitro disease modeling, and tissue engineering.

Acknowledgements

This work was supported by the Shriners Hospitals for Children, Nancy Taylor Foundation, Arthritis Foundation, NIH (AG46927, AG15768, AR075899, AR072999, AR074992, AR073752, AR080902, T32 DK108742, T32 EB018266), and Taiwan GSSA Scholarship.

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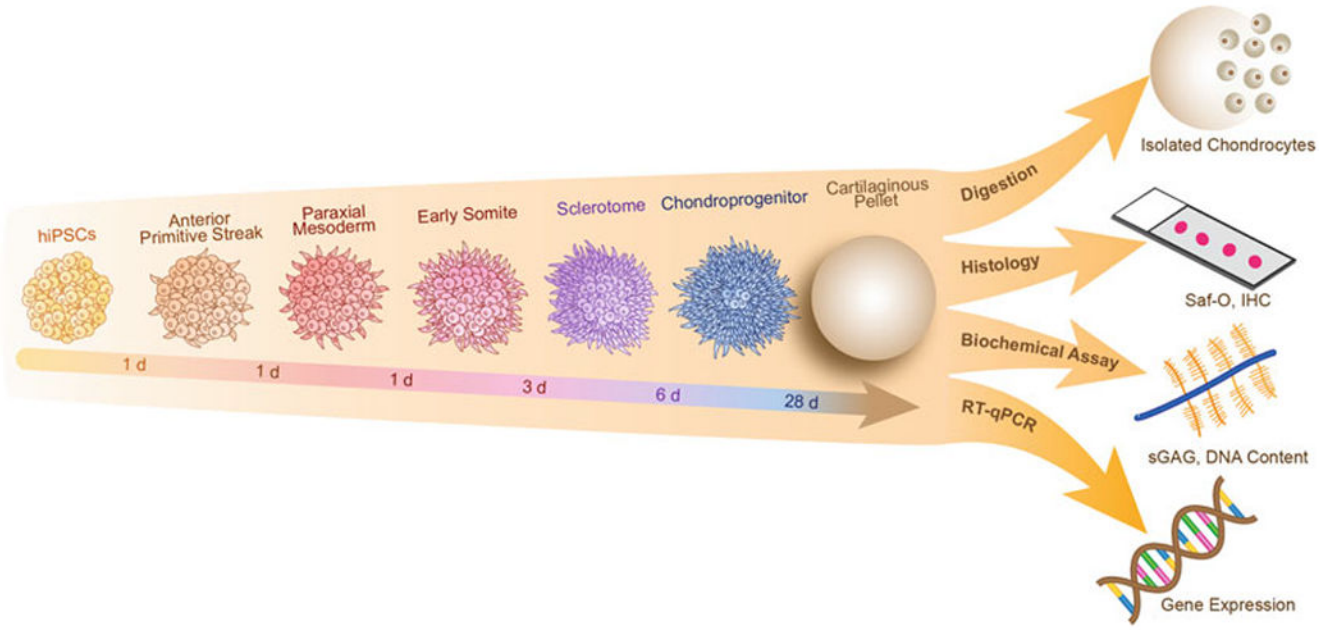

Fig. 1.

Overview schematic of the protocol. hiPSCs undergo mesodermal differentiation in monolayer for 12 days. The cells go through the anterior primitive streak, paraxial mesoderm, early somite, sclerotome, and finally chondroprogenitor stage. Cells are then cultured in a 3D pellet culture to become chondrocytes and synthesize cartilaginous matrix. The protocol then has four options to either digest the tissue to isolate single cells or validate chondrogenesis with histology (Saf-O and IHC), biochemical assays (DMMB for sGAG and PicoGreen for dsDNA), and/or RT-qPCR

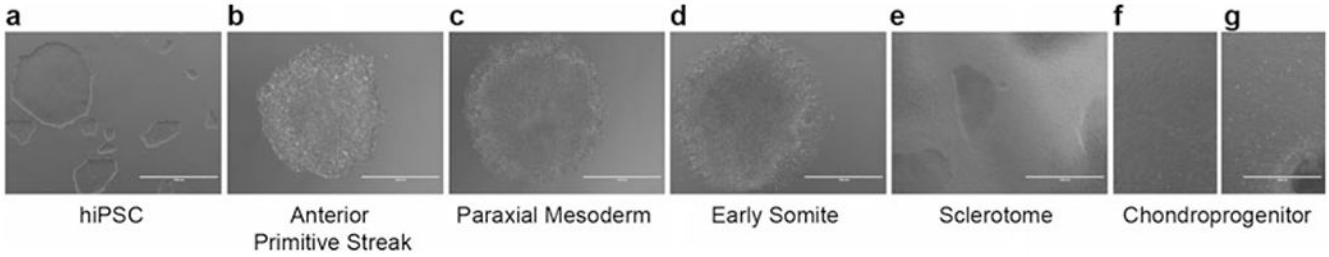

Fig. 2.

Phase contrast images of cells throughout mesodermal differentiation. (**a**) Induce cells when they are 30–40% confluent. (**b**–**f**) As cells differentiate, from (**b**) anterior primitive streak, (**c**) paraxial mesoderm, (**d**) early somite, (**e**) sclerotome, to (**f**) chondroprogenitor, they spread and become more spindled. (**g**) If cells are induced at too high of a density, they may not fully differentiate and form nodules in the center of the colonies. Scale bar = 1 mm

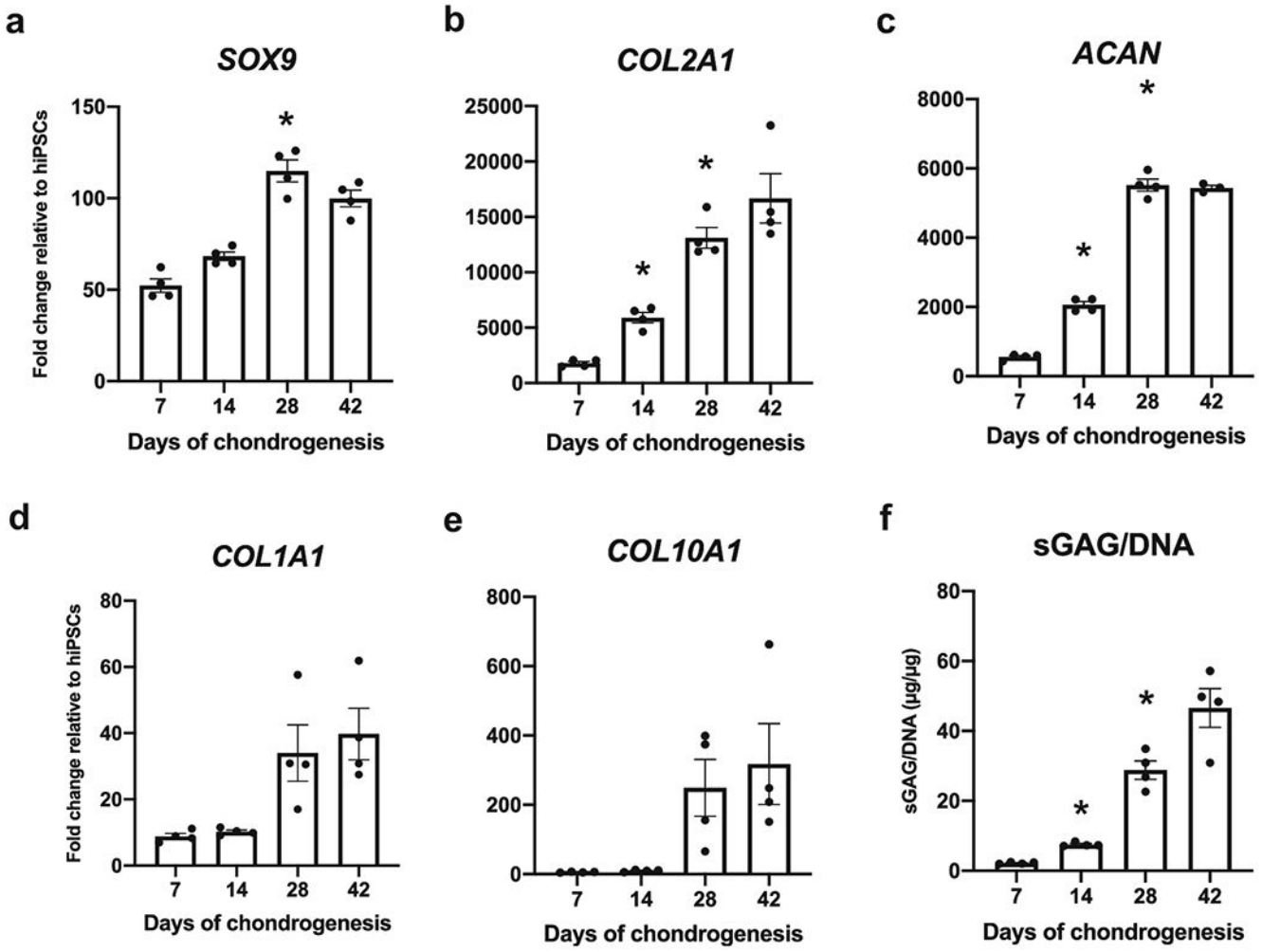

Fig. 3.

Anticipated results – gene expression and matrix quantification. (**a–c**) Chondrogenic transcription factor *SOX9* gene expression should increase early, followed by matrix genes *COL2A1* and *ACAN*. (**d–e**) Relative to the expression of *COL2A1*, lower gene expression of fibrocartilage and hypertrophic cartilage markers *COL1A1* and *COL10A1*, respectively, was observed. (**f**) sGAG/DNA ratio should increase throughout chondrogenesis, reaching 20–30 ng/ng at day 28 and over 40 ng/ng at day 42. Mean ± SEM, $n = 4$. *$p < 0.05$ compared to previous time point

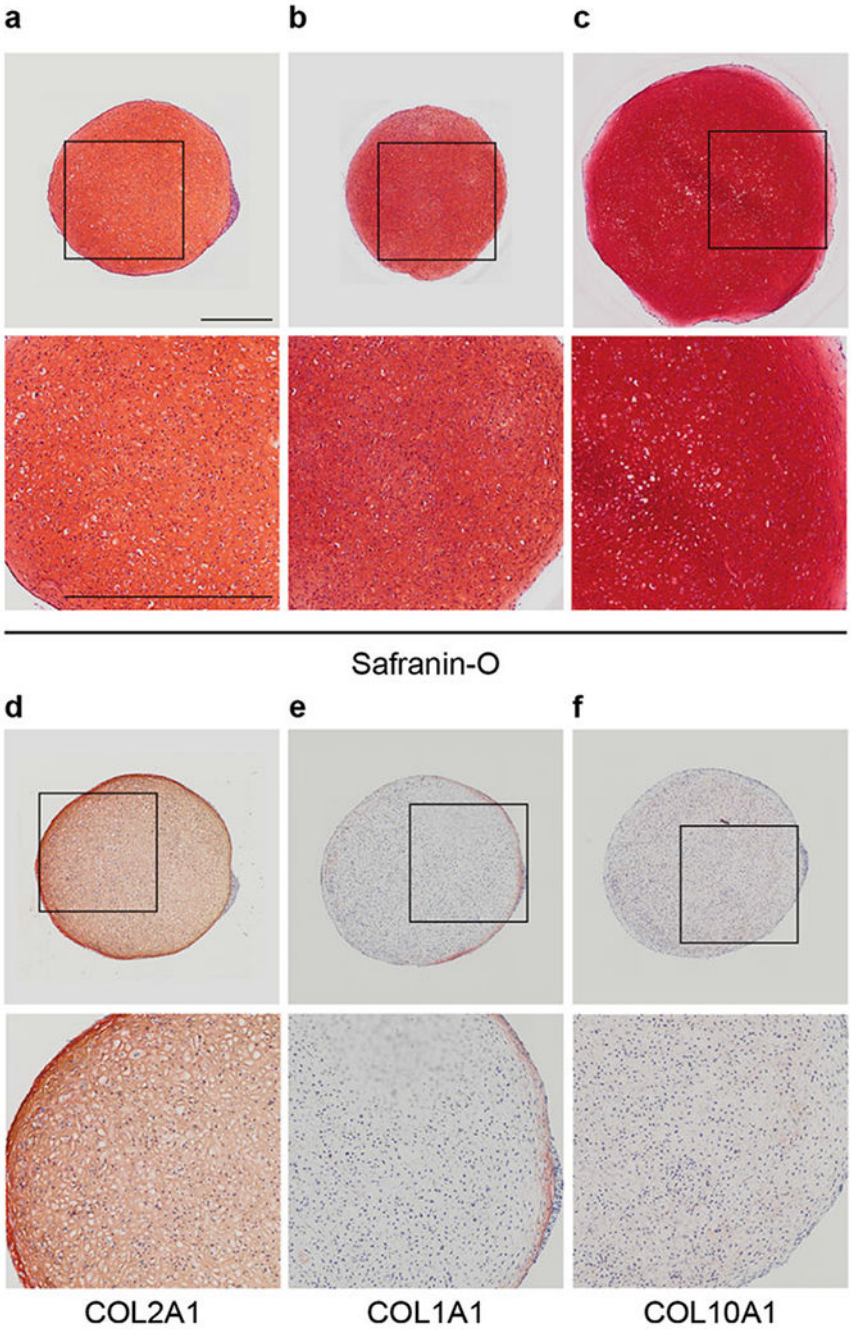

Fig. 4.

Anticipated results – histology. (**a–c**) Robust, homogenous safranin-O staining for sGAGs in three different cell lines. (**d–f**) Pellet in panel a with IHC labeling of COL2A1 (**d**), COL1A1 (**e**), and COL10A1 (**f**). Scale bar = 500 μm

Table 1

RT-qPCR primers

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
ACAN	CACTTCTGAGTTCGTGGAGG	ACTGGACTCAAAAAGCTGGG
COL1A1	TGTTCAGCTTTGTGGACCTC	TTCTGTACGCAGGTGATTGG
COL2A1	GGCAATAGCAGGTTCACGTA	CTCGATAACAGTCTTGCCCC
COL10A1	CATAAAAGGCCCACTACCCAAC	ACCTTGCTCTCCTCTTACTGC
SOX9	CGTCAACGGCTCCAGCAAGAACAA	GCCGCTTCTCGCTCTCGTTCAGAAGT
TBP	AACCACGGCACTGATTTTCA	ACAGCTCCCCACCATATTCT

Table 2

IHC antibodies

Antibody	Host	Manufacturer	Cat. No.	Retrieval (time, temp)	Dilution	AEC Time
COL1A1	Mouse	Abcam	ab90395	Pepsin (5 min, RT)	1:800	2 min
COL2A1	Mouse	Iowa Hybridoma Bank	II-II6B3-s	Proteinase K (3 min, 37 °C)	1:10	2.5 min
COL6A1	Rabbit	Fitzgerald Industries	70F-CR009X	Proteinase K (3 min, 37 °C)	1:1000	2.5 min
COL10A1	Mouse	Millipore Sigma	C7974	Pepsin (5 min, RT)	1:200	2 min
Goat anti-mouse IgG, biotin	Goat	Abcam	ab97021		1:500	
Goat anti-rabbit IgG, biotin	Goat	Abcam	ab6720		1:500	