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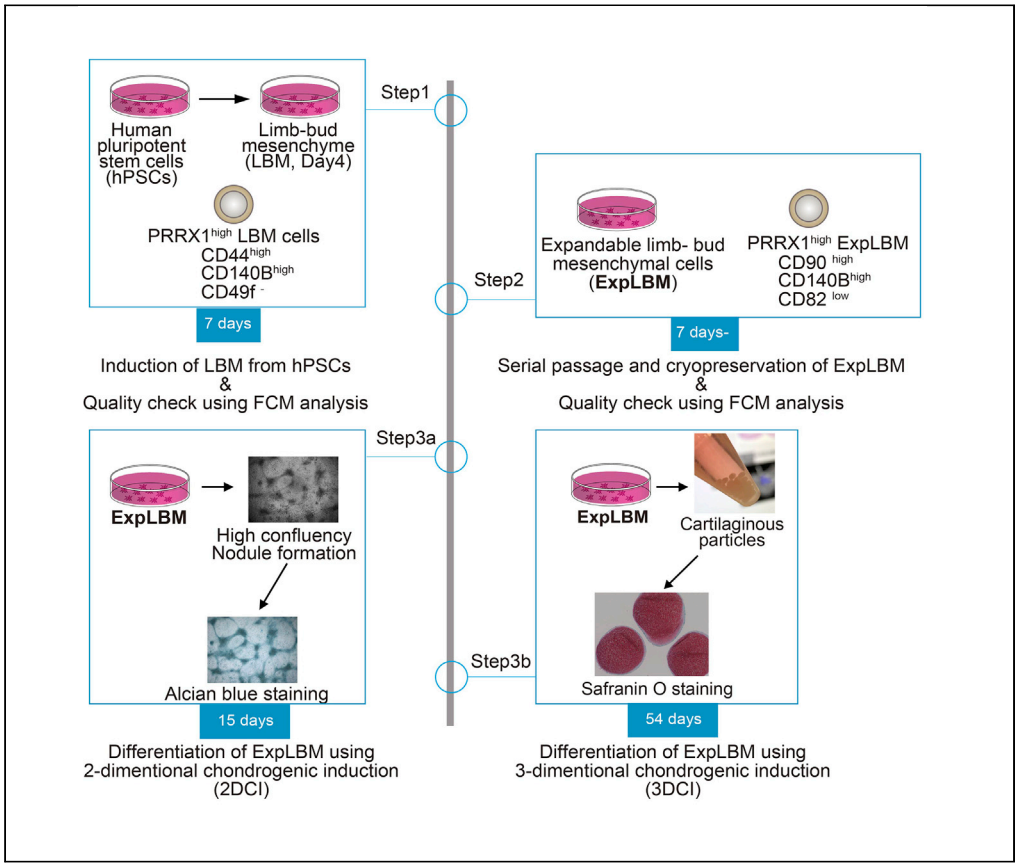
A protocol to induce expandable limb-bud mesenchymal cells from human pluripotent stem cells



Here, we present a protocol for the selective differentiation of human pluripotent stem cells mimicking human developmental processes into expandable PRRX1^{+} limb-bud mesenchymal (ExpLBM) cells. This approach enables expansion through serial passage while maintaining capacity for chondrogenic differentiation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Mimics human developmental processes for inducing hPSCs-derived limb bud mesenchyme (LBM)

Stable expansion and cryopreservation of chondroprogenitor-like ExpLBM cells

Prospective assessment of chondrogenic ExpLBM via CD90^{high} CD140B^{high}CD82^{low} population

Chondrogenic differentiation of ExpLBM using 2DCI or 3DCI protocol

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A protocol to induce expandable limb-bud mesenchymal cells from human pluripotent stem cells

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SUMMARY

Here, we present a protocol for the selective differentiation of human pluripotent stem cells mimicking human developmental processes into expandable PRRX1$^+$ limb-bud mesenchymal (ExpLBM) cells. This approach enables expansion through serial passage while maintaining capacity for chondrogenic differentiation.
For complete details on the use and execution of this protocol, please refer to Yamada et al. (2021, 2022).

BEFORE YOU BEGIN

Human pluripotent stem cells (hPSCs) can be acquired from academic organizations, such as the Center for hPSCs Cell Research and Application (CiRA) and CiRA Foundation (CiRA_F), or commercially purchased. All experiments using hPSCs must follow institutional guidelines and applicable domestic laws and regulations. All procedures were performed under sterile conditions, unless otherwise specified.

Institutional permissions

The Ethics Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, approved the experimental protocols for studies of human subjects (1707-013, 1808-017).

Prepare reagents

1. At least 1 day before the experiment.
   a. Reconstitute cytokines and chemical reagents according to manufacturers' recommendations and store aliquots at −20°C.
2. On the day of the experiments.
   a. Prepare each buffers and media, as described in the "materials and equipment" section.
   b. Pre-warm each media at room temperature.
   c. Set centrifuges to 4°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC anti-human CD44 (clone: IM7) (1:200)	eBioscience	Cat#17-0441-81; RRID: AB_469389
BB700 anti-human CD140b (clone: 28D4) (1:200)	BD Biosciences	Cat#745822; RRID: AB_2743271

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
FITC anti-human CD49f (clone: GoH3) (1:200)	BD Biosciences	Cat#561893; RRID: AB_10894397
FITC anti-human CD90 (clone: 5E10) (1:200)	BioLegend	Cat#328107; RRID: AB_893438
APC anti-human CD82 (clone: ASL-24) (1:200)	BioLegend	Cat#342113; RRID: AB_2800906
Anti-PRRX1 (1:200)	MilliporeSigma	Cat#ZRB2165
Anti-SOX9 (1:200)	Sigma-Aldrich	Cat#AB5535; RRID: AB_2239761
Anti-collagen II (COL2) (1:200)	Thermo Fisher Scientific	Cat#MA1-37493; RRID: AB_2082337
Anti-aggrecan (ACAN) (1:200)	Proteintech	Cat#13880-1-AP; RRID: AB_2722780
Anti- Type I collagen (COL1) (1:200)	SouthernBiotech	Cat#1440-01; RRID: AB_2794757
Chemicals, peptides, and recombinant proteins		
CHIR99021	Axon Medchem	Axon1386
PIK90	Sigma-Aldrich	528117
A-83-01	Tocris Bioscience	2939
Wnt-C59	Cellagen Technology	C7641-2s
LDN-193189	REPROCELL	04-0074
GDC-0449 (Vismodegib)	Cellagen Technology	C4044-5
Y-27632	MedChemExpress	MCE HY-10583
L(+)-Ascorbic acid	FUJIFILM Wako	014-04801
Chemically defined lipid concentrate (CD lipid concentrate)	Thermo Fisher Scientific	11905031
Activin A human recombinant	R&D Systems	338-AC-050
Recombinant human BMP-4 (hBMP-4)	R&D Systems	314-BP
Human FGF-2 (hFGF-2)	Katayama Chemical Industries	161-0010
Recombinant human TGF-beta1 (hTGF-β1)	PeproTech	100-21C
Recombinant human GDF-5 (hGDF-5)	PeproTech	120-01
Human recombinant EGF (hEGF)	STEMCELL Technologies	78006.1
Human insulin	Roche	11376497001
Insulin-transferrin-selenium (ITS-G) (100 x)	Thermo Fisher Scientific	41400045
Transferrin	Roche	10652202001
Fetal bovine serum (FBS)	Gibco	10270106 (Lot:42F5094K42F5094K)
0.5 M EDTA (pH8.0) solution	Nippon Gene	311-90075
Polyvinyl alcohol	Sigma-Aldrich	P8136-250G
Monothioglycerol	Sigma-Aldrich	M6145
Penicillin-streptomycin (10,000 U/mL)	Thermo Fisher Scientific	15140122
iMatrix-511 silk (iMatrix)	MATRIXOME	892021
Human plasma fibronectin (1 mg/mL)	Sigma-Aldrich	FC010
Experimental models: Cell lines		
414C2 iPSCs	CiRA	N/A
Ff-WJs513 iPSCs	CiRA-F	N/A
Ff-CLs14 iPSCs	CiRA-F	N/A
Ff-KVs09 iPSCs	CiRA-F	N/A
SEES4 hESCs	RIKEN BRC	N/A
SEES5 hESCs	RIKEN BRC	N/A
SEES6 hESCs	RIKEN BRC	N/A
SEES7 hESCs	RIKEN BRC	N/A

MATERIALS AND EQUIPMENT

We recommend that cell culture is performed in a sterile environment and sterile cell culture materials such as sterile conical tubes, microcentrifuge tubes, DNase/RNase free barrier pipet tips, and serological pipettes be purchased. If unsterile, these reagents can be sterilized by autoclaving prior to use. We also suggest that all items added to the biosafety cabinet are sprayed with 70% (v/v) ethanol in deionized water. Routine spraying of gloved hands with 70% (v/v) ethanol will also reduce the risk of contamination. Additionally, we recommend never passing items or hands over open containers of media, pipettes, or cells. Media should be prepared under sterile and endotoxin-free

conditions or can be sterilized using a 0.22-μm filter before use to reduce the activity of protein components in the media, which are crucial for cell maintenance and viability.

0.5 mM EDTA/PBS(-)		
Reagent	Final concentration	Amount
0.5 M EDTA (pH 8.0) solution	0.5 mM	250 μL
PBS(-) (Nacalai tesque, 14249-95)	N/A	250 mL
Total		250 mL
Sterilized using a 0.22 μm filter.		

0.5×TrypLE Select/EDTA		
Reagent	Final concentration	Amount
TrypLE Select (Thermo Fisher Scientific, 12563029)	N/A	25 mL
0.5 mM EDTA/PBS(-)	0.25 mM	25 mL
Total		50 mL
Store at 4°C.		

StemFit medium		
Reagent	Final concentration	Amount
StemFit AK02N (A+B+C solution) (REPROCELL, AK02N)	N/A	500 mL
Penicillin-streptomycin (10,000 U/mL)	N/A	5 mL
Total		505 mL
Store at 4°C.		

CDM2 medium		
Reagent	Final concentration	Amount
IMDM, GlutaMAX™ supplement (Thermo Fisher Scientific, 31980097)	N/A	250 mL
Ham's F-12 Nutrient Mix, GlutaMAX™ supplement (Thermo Fisher Scientific, 31765092)	N/A	250 mL
Polyvinyl alcohol (100 mg/mL)	1 mg/mL	5 mL
CD lipid concentrate	1% v/v	5 mL
Monothioglycerol	450 μM	19.4 μL
Insulin (10 mg/mL)	7 μg/mL	350 μL
Transferrin (30 mg/ml)	15 μg/mL	250 μL
Penicillin-streptomycin (10,000 U/mL)	1% v/v	5 mL
Total		515 mL
*100 mg/mL polyvinyl alcohol solution should be dissolved by heating in a 600 W microwave oven. (for 1–3 min). Store at 4°C.		

Mid-primitive streak medium		
Reagent	Final concentration	Amount
CDM2 medium	N/A	10 mL
Activin A (20 μg/mL)	30 ng/mL	15 μL
hBMP-4 (20 μg/mL)	40 ng/mL	20 μL
CHIR99021 (10 mM in DMSO (FUJIFILM Wako, 048-21985))	6 μM	6 μL
hFGF-2 (20 μg/mL)	20 ng/mL	10 μL
PIK90 (10 mM in DMSO)	100 nM	0.1 μL
Y-27632 (10 mM in DMSO)	10 μM	10 μL
Total		10 mL
Medium can be stored at 4°C for up to 2 weeks.		

Lateral plate mesoderm medium		
Reagent	Final concentration	Amount
CDM2 medium	N/A	10 mL
A-83-01 (10 mM in DMSO)	1 μM	1 μL
hBMP-4 (20 μg/mL)	30 ng/mL	15 μL
Wnt-C59 (10 mM in DMSO)	1 μM	1 μL
Y-27632 (10 mM in DMSO)	10 μM	10 μL
Total		10 mL
Medium can be stored at 4°C for up to 2 weeks.		

LBM medium		
Reagent	Final concentration	Amount
CDM2 medium	N/A	10 mL
A-83-01 (10 mM in DMSO)	1 μM	1 μL
LDN193189 (10 mM in DMSO)	500 nM	0.5 μL
CHIR99021 (10 mM in DMSO)	3 μM	3 μL
Vismodegib (1 mM in DMSO)	150 nM	1.5 μL
Y-27632 (10 mM in DMSO)	10 μM	10 μL
Total		10 mL
Medium can be stored at 4°C for up to 2 weeks.		

ExpLBM medium		
Reagent	Final concentration	Amount
CDM2 medium	N/A	100 mL
A-83-01 (10 mM in DMSO)	1 μM	10 μL
CHIR99021 (10 mM in DMSO)	3 μM	30 μL
hFGF-2 (20 μg/mL)	20 ng/mL	100 μL
hEGF (20 μg/mL)	20 ng/mL	100 μL
Y-27632 (10 mM in DMSO)	10 μM	100 μL
Total		100 mL
Medium can be stored at 4°C for up to a month.		

STEP 1 medium		
Reagent	Final concentration	Amount
CDM2 (for 2DCI) or D-MEM(FUJIFILM Wako, 044-29765) with 1% penicillin-streptomycin (for 3DCI)	N/A	10 mL
Ascorbic acid (100 mg/mL)	50 μg/mL	5 μL
ITS-G (100×)	1×	100 μL
CHIR99021 (10 mM in DMSO)	3 μM	3 μL
hFGF-2 (20 μg/mL)	10 ng/mL	5 μL
Total		10 mL
Medium can be stored at 4°C for up to 2 weeks.		

STEP 2 medium		
Reagent	Final concentration	Amount
D-MEM (for 3DCI)	N/A	10 mL
Penicillin-streptomycin (10,000 U/mL)	1%	100 μL
Ascorbic acid (100 mg/mL)	50 μg/mL	5 μL
ITS-G (100×)	1×	100 μL

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Continued		
Reagent	Final concentration	Amount
hBMP-4 (20 μg/mL)	30 ng/mL	15 μL
hTGF-β1 (20 μg/mL)	10 ng/mL	5 μL
hGDF-5 (20 μg/mL)	10 ng/mL	5 μL
hFGF-2 (20 μg/mL)	10 ng/mL	5 μL
Total		10 mL
Medium can be stored at 4°C for up to 2 weeks.		

STEP 3 medium		
Reagent	Final concentration	Amount
CDM2 (for 2DCI) or D-MEM with 1% penicillin-streptomycin (for 3DCI)	N/A	10 mL
Ascorbic acid (100 mg/mL)	50 μg/mL	5 μL
ITS-G (100×)	1×	100 μL
hBMP-4(20 μg/mL)	30 ng/mL	15 μL
hTGF-β1 (20 μg/mL)	10 ng/mL	5 μL
hGDF-5 (20 μg/mL)	10 ng/mL	5 μL
FBS (for 3DCI)	10%	1 mL
Total		10 mL
Medium can be stored at 4°C for up to 2 weeks.		

STEP 4 medium		
Reagent	Final concentration	Amount
D-MEM	N/A	500 mL
Penicillin-streptomycin (10,000 U/mL)	1%	5 mL
FBS	10%	50 mL
Total		555 mL
Medium can be stored at 4°C for up to 2 weeks.		

⚠ CRITICAL: Solutions must be prepared in a sterile environment.

Alternatives: StemFit medium is designed to maintain undifferentiated hPSCs. CDM2 medium is used for induction and maintenance of ExpLBM cells.

STEP-BY-STEP METHOD DETAILS

Thawing hPSCs

Timing: 60 min

1. Place a 60-mm culture dish on the bench.
   a. Pre-warm StemFit medium at room temperature.
2. Add 5 mL of StemFit medium supplemented with 10 μM of Y-27632 to a 15-mL conical tube.
3. Remove the vial containing hPSCs from the liquid nitrogen, and immediately thaw the vial in 37°C water bath within 1 min or until only small ice particles remain.
4. Disinfect the vial with 70% EtOH and transfer the cell suspension to the conical tube prepared in step 2 by gentle pipetting (1–2 times).
5. Centrifuge the cells at 1,500 rpm for 5 min.
6. Aspirate the supernatant and resuspend the cells in 1 mL of StemFit medium supplemented with 10 μM Y-27632.

7. Stain the cells with Trypan blue (Gibco, 15250-061) and then count the cells using a cell counter.
   a. To reduce human error in cell counting, we recommend the use of an automated cell counter (TC20 automated cell counter; size, 8–16 μm [Bio-Rad]).
8. Plate 1 × 10^4 cells/2 mL StemFit medium supplemented with 10 μM Y-27632 to one 60-mm culture dish.
9. Add 8 μL of iMatrix to a 60-mm culture dish with hPSCs.
10. Culture the dish at 37°C in 5% CO_2 incubator.
11. On the next day, change to 3 mL of fresh StemFit medium without Y-27632 and iMatrix.

Stable culture of hPSCs under feeder-free condition

Timing: 1–8 weeks

12. Replace the culture medium with StemFit without Y-27632 and iMatrix every 24–48 h.
    a. The cells should be observed every day.
    b. StemFit medium is warmed at room temperature.
    c. Until next passage, culture medium must be changed every 2 days. However, the medium color should change to orange or yellow even in 1 day, and the culture medium should be changed every day.
13. After 5–7 days culture, the cells should be passaged.
    a. When the cells become 70%–80% confluent, passage is possible.
14. Aspirate the culture medium in the 60-mm culture dish and rinse the cells with 3 mL of PBS(-).
    a. The amount of PBS(-) required may vary depending on the surface area of the culture apparatus.
15. Apply and spread 500 μL of 0.5 × TrypLE Select/EDTA, and then aspirate immediately.
16. Incubate the cells at 37°C for 5 min.
    a. The cell surface does not dry out in approximately 5 min, and thus, cell viability is not a problem. However, if left longer than 5 min, the cells will dry out. Therefore, the cells should be incubated only for 5 min.
17. Strip cells by adding 500 μL of StemFit medium containing 10 μM of Y-27632 (See troubleshooting problem 1).
18. Dissociate cell clumps into single cells by gentle pipetting (approximately 10 times).
    a. Too much pipetting increases the number of dead cells.
19. Transfer the cell suspension using a 1.5-mL microtube.
20. Determine the cell number and viability using any laboratory method. We count living cells by Trypan blue stain using cell counter.
21. Seed 1 × 10^4 cells/2 mL StemFit medium containing 10 μM of Y-27632, and then add 8 μL of iMatrix onto a 60-mm culture dish.
22. Gently agitate the culture dish to allow the cells to disperse uniformly.
23. Place the culture dish in a 5% CO_2 incubator at 37°C.
24. Go back to step 12 for maintenance of the cells.

⚠ CRITICAL: If hPSCs become overly confluent or bind each cell island colony, their pluripotent status and viability may be affected. Observe the cells periodically to ensure that they are undifferentiated and not overly confluent. If any differentiated cells or masses are observed, it is best to discard them and start all over again.

Differentiation of hPSCs into limb-bud mesenchyme (LBM)

Timing: 7 days

This section describes the production of limb-bud mesenchyme (LBM) from hPSCs based on previous reports (Yamada et al., 2021). For selective differentiation from hPSCs to LPM, refer to Loh et al.

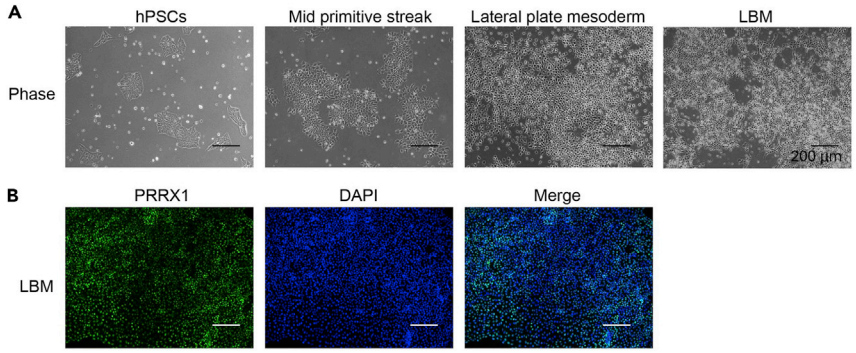

Figure 1. Induction of LBM from hPSCs

(A) Cellular morphology should be observed during stepwise induction procedure.

(B) PRRX1 staining in LBM.

(2016). Our protocol is developed based on previous researches and mimics the limb development process. The procedure of this section consists of 1) culture of hPSCs, 2) mid-primitive streak differentiation, 3) lateral plate mesoderm differentiation, and 4) LBM differentiation. This procedure should be performed on a 35-mm culture dish.

25. Seed an hPSC suspension (30,000 cells/1 mL) in a 35-mm culture dish. Cells should be suspended in StemFit medium containing 10 μM Y-27632 and 4 μL of iMatrix.
26. Culture for 24 h at 37°C in a 5% CO_2 incubator.
27. Change to 1.5 mL of StemFit medium without 10 μM Y-27632 and iMatrix.
28. After culture for 48 h, aspirate the culture medium and wash the cells with PBS(-).
29. Apply 1.5 mL of the first differentiation medium (mid-primitive streak medium) into the 35-mm culture dish.
30. After culture for 24 h, aspirate the culture medium and wash the cells with PBS(-).
31. Apply 1.5 mL of the second differentiation medium (lateral plate mesoderm medium) into the 35-mm culture dish.
32. After culture for 24 h, aspirate the culture medium and wash the cells with PBS(-).
    a. PBS(-) should be added slowly in consideration of the tendency to peel cells off.
33. Apply 1.5 mL of the third differentiation medium (LBM medium) into the 35-mm culture dish, and then culture for 48 h (See troubleshooting problem 2).
    a. The medium should be added slowly because the cells might peel off.

⚠ CRITICAL: During the LBM differentiation procedure, observe the cell morphology at each point under the microscope to validate the cellular differentiation (Figure 1). PBS(-) washing procedure prior to medium changes is necessary to achieve high efficiency of LBM induction. The quality of LBM can be assessed by the next FCM procedure instead of PRRX1 immunostaining.

Assess the quality of LBM

Timing: 60 min

We have identified the cell surface markers that define the human PRRX1$^+$ LBM cells (Yamada et al., 2022). This section describes a method enabling the assessment of LBM induction efficiency from hPSCs by checking the cell surface marker expression including CD44, CD140B, and CD49f.

34. After 48 h from changing to LBM medium, aspirate the culture medium and rinse the cells carefully with PBS(-).

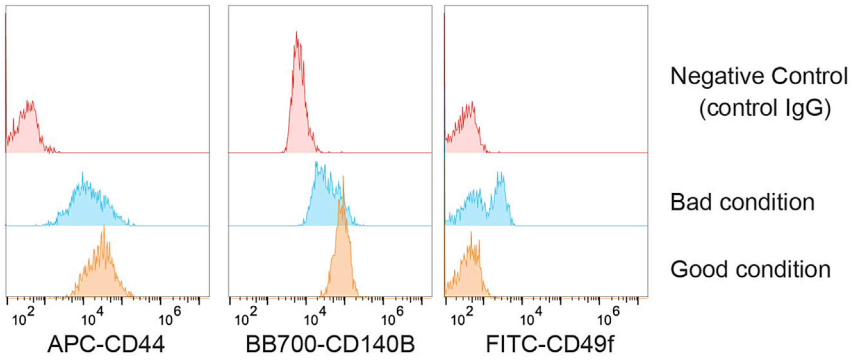

Figure 2. Flow cytometry analysis of LBM cells

Note: Cells are easily detached.

35. Apply 300 μL of Accutase (Nacalai Tesque, 12679-54), and then aspirate immediately.
36. Incubate the cells at 37°C for 5 min.
37. Strip the cells by adding 300 μL of ExpLBM medium.
38. Transfer the cell suspension to a 1.5-mL microtube.
39. Count the living cells by staining them with Trypan blue and using a cell counter.
40. Transfer the 1 × 10^5 LBM cells into a fresh 1.5-mL microtube, and then centrifuge the cells at 7,000 rpm for 1 min and then suspend the cell pellet with 100 μL of 2% FBS/PBS(-).
41. Add 0.5 μL of each antibody including APC-CD44 (good marker), BB700-CD140B (good marker), and FITC-CD49f (bad marker).
    a. "Good condition" indicates that CD44 and CD140B are highly expressed with negative CD49f compared with the negative control as shown in Figure 2. On the other hand, "Bad condition" indicates that CD140B is not expressed or less expressed with partially positive CD49f.
42. Incubate the cell suspension for 1 h on ice.
43. Add 500 μL of 2% FBS/PBS(-) to the cell suspension.
44. Centrifuge the cells at 7000 rpm for 1 min.
45. Aspirate the supernatant and suspend the cell pellets with 2% FBS/ PBS(-) containing 50 ng/mL DAPI (Thermo Fisher Scientific, D3571).
46. Analyze the cells by flow cytometry. LBM cells are defined as $CD44^{high}$ $CD140B^{high}$ CD49f (Figure 2) (See troubleshooting problem 3–4).

Establishment and serial passage of ExpLBM cells

Timing: 5–7 days

This section describes the protocol that enables the stable proliferation of LBM cells (expandable LBM cells, ExpLBM cells) using the specific culture medium named ExpLBM medium. Our LBM expansion protocols which activated WNT and FGF signaling while inhibiting TGFβ signaling, recapitulated the developmental processes for limb-bud outgrowth following chondrogenesis.

47. Coat a fresh 60-mm culture dish with 2 mL of 4 μg/mL fibronectin diluted in PBS(-) for at least 30 min at 37°C in a 5% CO_2 incubator.
48. Aspirate the fibronectin/PBS(-) and then add the ExpLBM medium to the coated dish.
49. Seed the 2 × 10^5 LBM cells suspended in a 3-mL ExpLBM medium onto fibronectin-coated culture dish.
    a. Next day after plating, check the cell adhesion. If the coating is not in place, the cells will not adhere, and such cells should not be used for further experiments because we have never characterized cells that do not adhere.

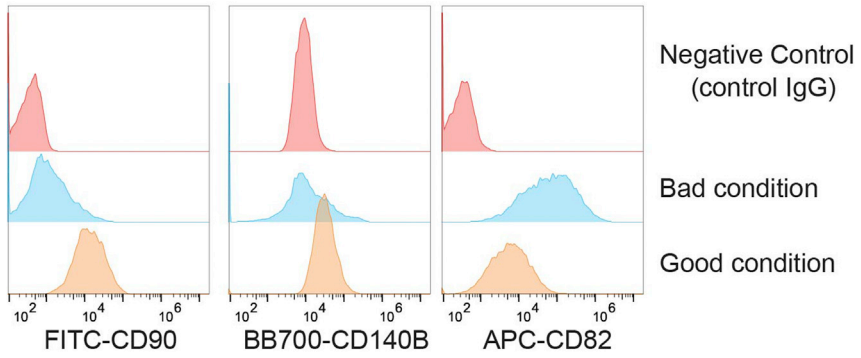

Figure 3. Flow cytometric analysis of ExpLBM cells

50. Until next passage, culture medium should be changed every 2 days.
    a. After 5–7 days culture, if the cells reached 70%–80% cell confluency, the cells should be passaged.

Assess the quality of ExpLBM cells

Timing: 60 min

This section describes a method that enables the prospective chondrogenic assessment of ExpLBM cells by checking its cell surface marker expression including CD90, CD140B, and CD82.

51. Aspirate the culture medium and rinse the cells with PBS(-).
52. Apply 400 μL of Accutase to a 60-mm culture dish, and then aspirate immediately.
53. Incubate the cells at 37°C for 5 min.
54. Strip the cells by adding 1,000 μL of ExpLBM medium.
55. Transfer the cell suspension to a 1.5-mL microtube.
56. Count the living cells by Trypan blue stain using cell counter.
57. Transfer the 1×10^5 ExpLBM cells into a new 1.5-mL microtube, and then centrifuge the cells at 7,000 rpm for 1 min and then suspend the cell pellet in 100 μL of 2% FBS/PBS(-).
58. Add 0.5 μL of each antibody including FITC-CD90 (good marker), BB700-CD140B (good marker), and APC-CD82 (bad marker).
59. Incubate the cell suspension for 1 h on ice.
60. Add 500 μL of 2% FBS/PBS(-) to the cell suspension.
61. Centrifuge the cells at 7,000 rpm for 1 min.
62. Aspirate the supernatant and suspend the cell pellets with 2% FBS/ PBS(-) containing 50 ng/mL DAPI.
63. Analyze the cells via flow cytometry. Chondrogenic ExpLBM cells are defined as CD90 ^{high} CD140B ^{high} CD82 ^{low} (Figure 3) (See troubleshooting problem 5).
    a. This assessment should be performed before 2DCI or 3DCI.

⚠ CRITICAL: It is recommended that ExpLBM cells passaged more than three times be used for the following chondrogenic induction protocols (2DCI or 3DCI).

Cryopreservation of ExpLBM cells

Timing: 30 min

64. Resuspend 1×10^6 ExpLBM cell pellets with 300 μL of STEM-CELLBANKER (ZENOGEN PHARMA, CB045).

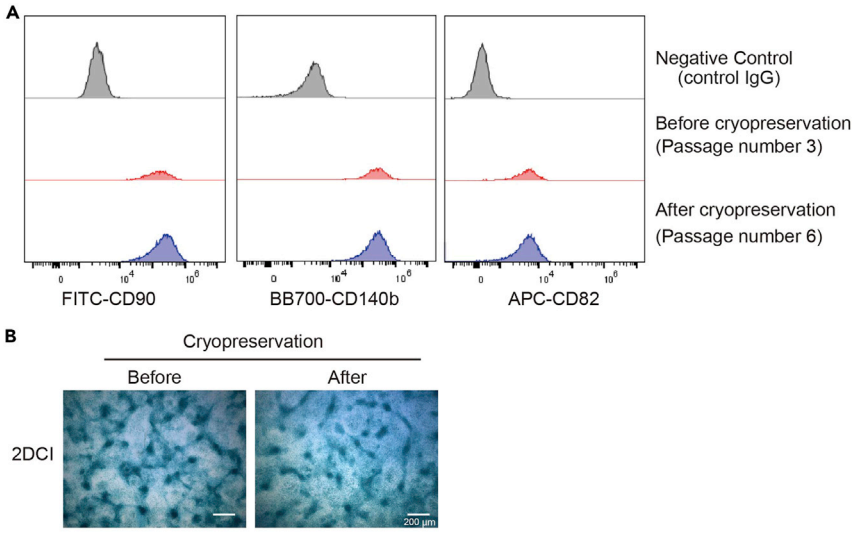

Figure 4. Effect of freeze-thaw on ExpLBM cells

(A) Flow cytometric analysis of ExpLBM cells.

(B) Alcian blue staining after the 2DCI-based differentiation.

65. Dispense 300 μL of cell suspension to cryotubes, and then put them into BICELL (Nihon Freezer, BICELL).
66. Freeze cryotubes in BICELL at a −80°C freezer for 24 h, and then transfer them in a −80°C freezer or liquid nitrogen.

⚠ CRITICAL: It is recommended that ExpLBM cells passaged more than four times be cryopreserved. The ExpLBM cells used in the paper were cryopreserved and used for chondrogenesis assay after freeze-thaw (Figure 4).

Two-dimensional chondrogenic induction (2DCI)

Timing: 15 days

This section describes a method to perform the 2-dimensional (adhesive culture) chondrogenic induction protocol of ExpLBM cells. The description below is slightly modified from the original paper by Yamada et al. (2021).

67. Prepare the ExpLBM cell suspension (70,000 cells/mL) using an ExpLBM medium containing 10% FBS.
68. Seed 200 μL of cell suspension (1.4×10^4 cells) into each well of a 96-well flat culture plate.
69. Culture for 3 days.
70. Wash the cells with PBS(-) and then add the fresh STEP 1 medium.
71. Culture for 6 days.
    a. Change the medium 3 days after changing to STEP 1 medium.
72. Wash the cells with PBS(-) and then add the fresh STEP 3 medium.
73. Culture for 6 days.
    a. Change the medium 3 days after changing to STEP 3 medium.

⚠ CRITICAL: PBS(-) washing procedure prior to medium changes is necessary to achieve high efficiency of chondrogenic induction. The chondrocyte differentiation can be assessed using Alcian blue staining (Figure 5).

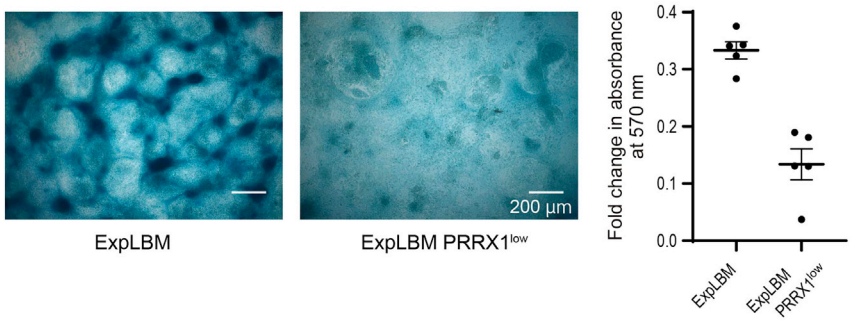

Figure 5. Alcian blue staining after the 2DCI-based differentiation of ExpLBM cells

After 2DCI procedure, the cells are fixed and then stained with Alcian blue. PRRX1 ^{low} ExpLBM (Yamada et al., 2021) shows lower intensities of Alcian blue staining.

Three-dimensional chondrogenic induction (3DCI)

Timing: >54 days

This section describes a method to perform the 3-dimensional (floating culture) chondrogenic induction protocol of ExpLBM cells. The description below is slightly modified from the original paper by Yamada et al. (2021).

74. Prepare an ExpLBM cell suspension (5 × 10^5 cells/mL) using STEP 1 medium.
75. Seed 200 μL of cell suspension (1 × 10^5 cells) into each well of a 96-well cell culture plate (CORNING, 7007, clear round bottom, ultralow attachment).
76. Centrifuge the plates at 2,000 rpm for 5 min (See troubleshooting problem 6).
77. Culture for 6 days.
    a. Change the medium 3 days after changing to STEP 1 medium.
78. Replace the culture media with fresh STEP 2 medium.
79. Culture for 6 days.
    a. Change the medium 3 days after changing to STEP 2 medium.
80. Replace the culture media with fresh STEP 3 medium.
81. Culture for 42 days (Figure 6).
    a. Change the medium every 3 days.
82. (Option) Replace the culture media with fresh STEP 4 medium, and then culture for several weeks.
    a. STEP 4 treatment induces histologically more matured cartilaginous particles.

EXPECTED OUTCOMES

This is the first protocol to guide the generation of ontogenetically defined expandable LBM cells from hPSCs that can be used in prospective assessment of chondrogenic capacity. Since the epigenetic modifications occur during development or differentiation, using our cell source resulted in new insights into signaling pathways and chromatin dynamics during human limb development and illuminates the pathogenesis of congenital skeletal diseases. In disease modeling using patient-derived iPSCs, development of a protocol to produce the desired patient-derived cell types with both reproducibility and mass productivity is critical toward achieving high-throughput drug screening in drug discovery. In fact, we showed that ExpLBM cells can be applied to disease modeling of patients with type II collagenopathy (COL2pathy), a genetic disease characterized by skeletal dysplasia caused by a *COL2A1* mutation, and COL2pathy patient-derived ExpLBM cells are useful in identifying therapeutic candidates when combined with 2DCI protocol as described in our original paper (Yamada et al., 2021). To address the regenerative medicine using human

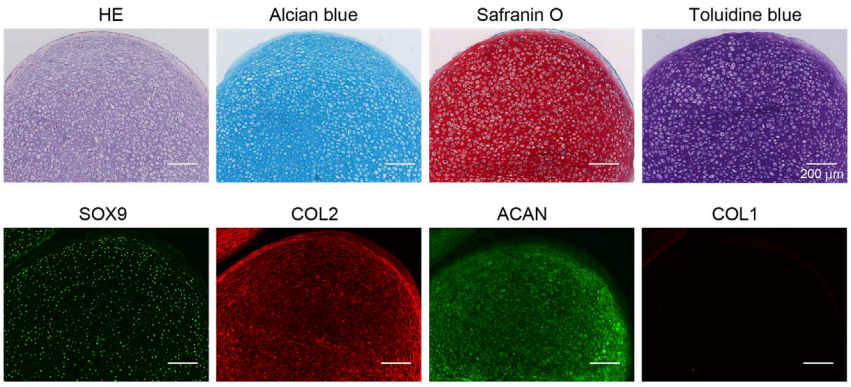

Figure 6. Histology of cartilaginous particles generated after 3DCI-based differentiation of ExpLBM cells
Cartilaginous particles were generated from ExpLBM cells, fixed and stained with hematoxylin-eosin (HE), Alcian blue, Safranin O or Toluidine blue. Immnofluorecence staining; SOX9, COL2, ACAN or COL1.

PSCs, a protocol that achieves mass productivity, high processability, and high reproducibility is critically important. In this regard, the ExpLBM cells used in our protocol allows expansion through serial passage while maintaining its capacity for chondrogenic differentiation through the analysis of cell surface markers. Especially, handling of ExpLBM cells in a single cell suspension has a high affinity to biomedical tissue engineering such as 3D bioprinting technology and cell-sheet technology. We predict that our protocol will provide new insights into human skeletal development and hereditary skeletal disease and will be applied to regenerative medicine and discovery of drugs that target cartilage-related diseases.

LIMITATIONS

Although this protocol has been validated using multiple hPSC lines described in key resources table in our laboratory, some hPSC lines that we did not use may produce less homogenous populations of LBM or ExpLBM cells. Especially, our assessment methods might not be repeatable using specific CD antigens for the several hPSC lines. We highly recommend checking the cellular characteristics by PRRX1 immunostaining.

TROUBLESHOOTING

Problem 1

hPSCs are difficult to detach from the culture dish. Can I use a cell scraper?

Potential solution

Using a cell scraper is not recommended because it significantly decreases cell viability. Many cells may not be detached, but our method can maintain high cell viability to collect a sufficient number of hPSCs to allow serial passage or LBM induction.

Problem 2

The color of the culture media becomes yellow during differentiation from LPM to LBM (day 3). Should I change them with fresh media?

Potential solution

As a 2-day culture without changing the culture media does not affect induction efficiency, the color of the culture media is negligible.

Problem 3

Induction efficiency of LBM from hPSCs is low.

Potential solution

This could be attributed to the contamination of differentiated cells in hPSC culture. Remove all differentiated cells before passaging cells, or pick and expand an undifferentiated single hPSC colony.

Problem 4

Although the differentiated cells in the hPSC culture are not apparent, CD49f positive cells are detected after inducing LBMs.

Potential solution

As the cell density of hPSCs prior to LBM induction could be high, ensuring their appropriate cell density before differentiation is a prerequisite before inducing the $CD44^{high}$ $CD140B^{high}$ $CD49f^{-}$ $PRRX1^{+}$ LBM cells. Cells must be washed with 1 × PBS(-) at each differentiation step. Without this PBS washing procedure, induction efficiency will decrease.

Problem 5

ExpLBM showed two peaks in FCM analysis of CD140B. Can $CD140B^{high}$ ExpLBM be sorted to maintain chondrogenic ExpLBM cells?

Potential solution

No. Once the expression level of PRRX1 or CD140B in ExpLBM begin to decrease, ExpLBM cannot maintain their high chondrogenic capacity. Thus, such a heterogeneous ExpLBM cells must be discarded.

Problem 6

When performing 3DCI, no single aggregate but several small spheroids were formed after centrifuging ExpLBM suspended in STEP 1 medium.

Potential solution

The centrifugation speed is not sufficient and therefore must be determined.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Takeshi Takarada (takarada@okayama-u.ac.jp).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

This study did not generate/analyze any datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

T. Takao and D.Y. performed the experiments. T. Takao wrote the original draft. T. Takao, D.Y., and T. Takarada reviewed and edited the manuscript. T. Takarada supervised the study and acquired funding.

DECLARATION OF INTERESTS

T. Takao, D.Y., and T. Takarada have a patent pending related to this work (PCT/JP2020/03551).

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