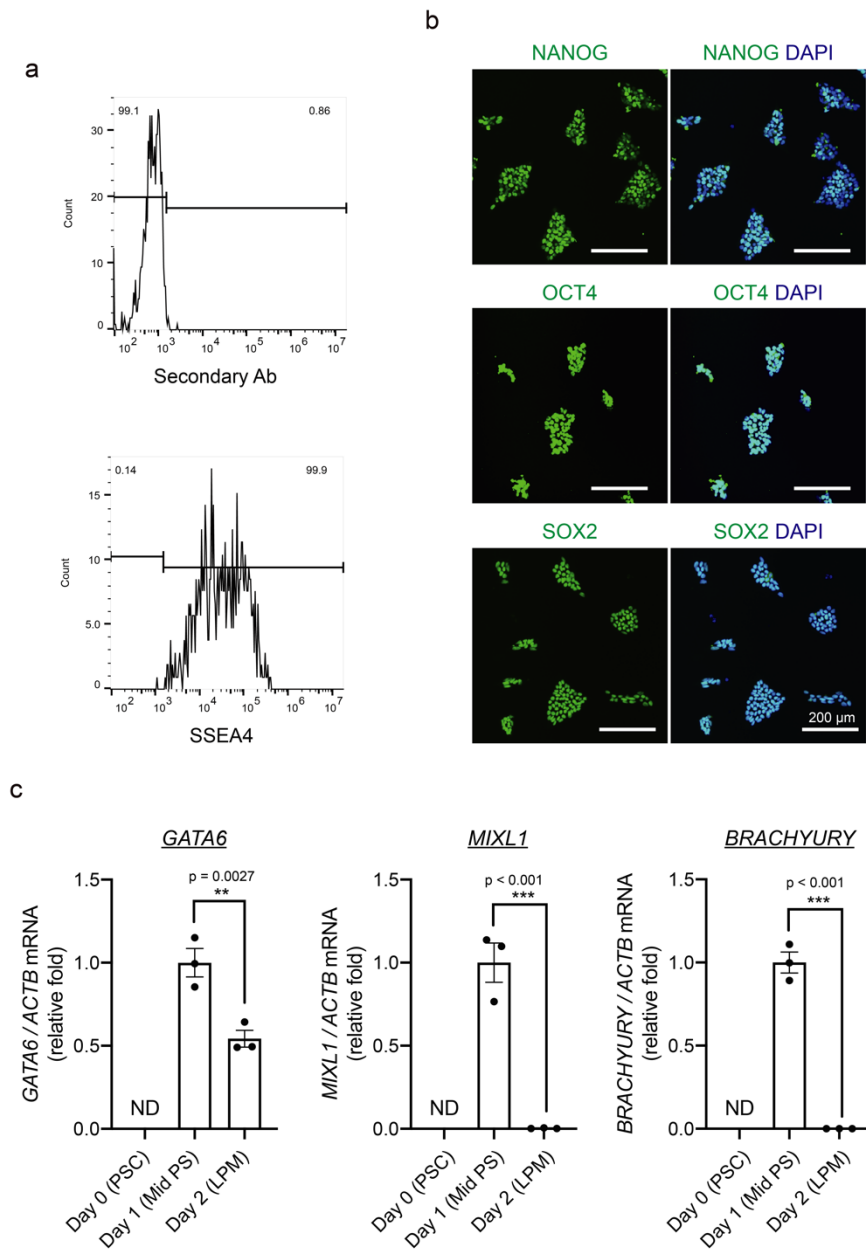
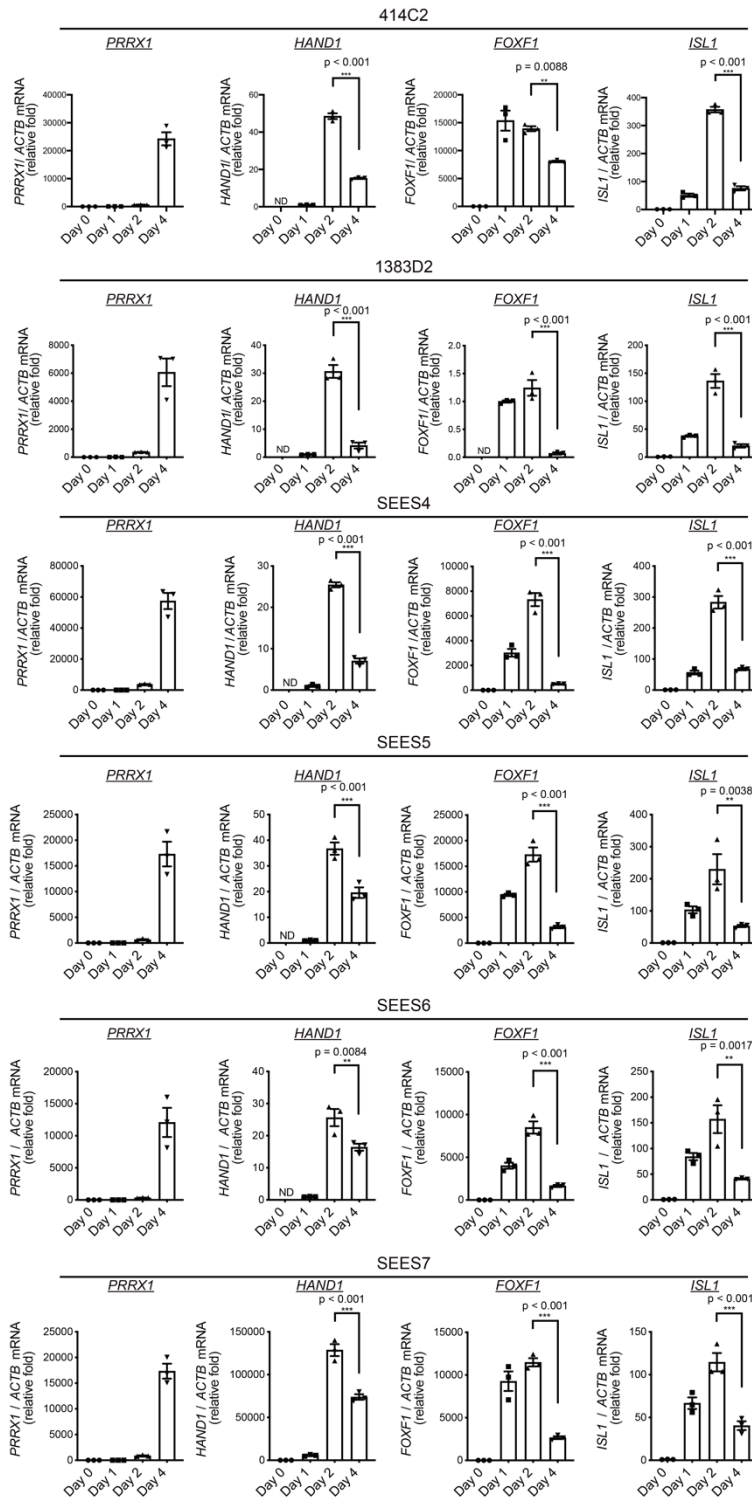

Supplementary information

Induction and expansion of human PRRX1⁺ limb-bud-like mesenchymal cells from pluripotent stem cells

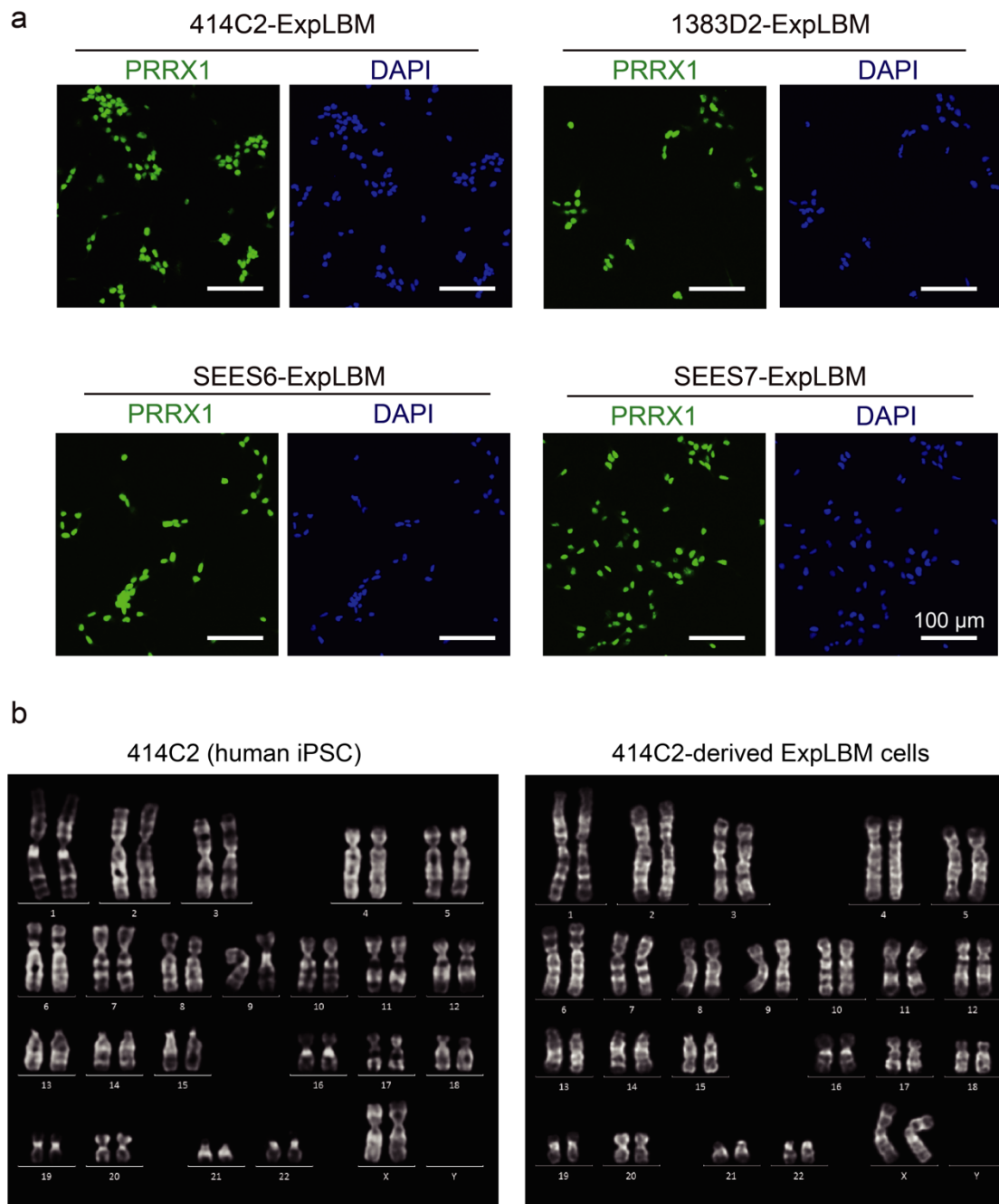
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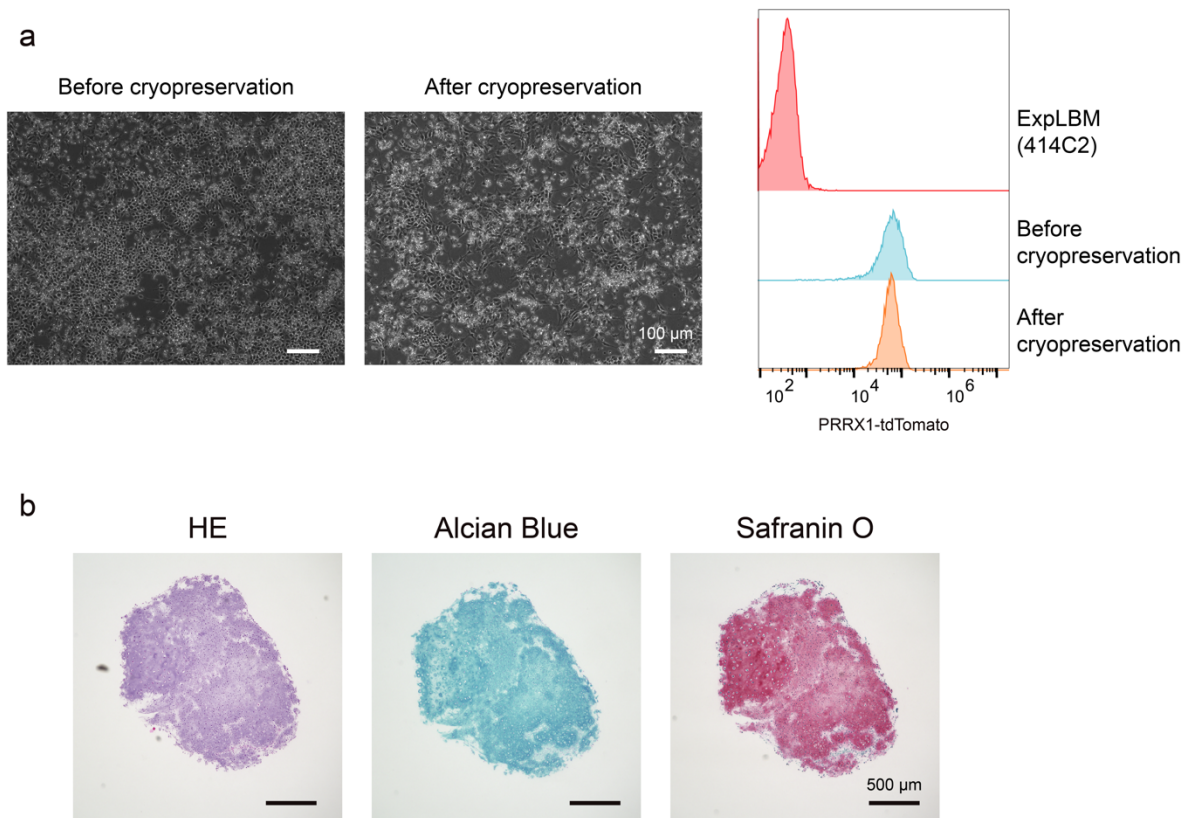
Supplementary Figure 1 | Expression of pluripotent marker and mid-primitive streak marker in PRRX1^{3' tdTomato}-reporter-containing iPSCs. (a) Representative flow cytometric analysis data of SSEA4 in undifferentiated PRRX1^{3' tdTomato}-reporter iPSCs. (b) Immunocytochemical analysis of NANOG, OCT4, or SOX2 expression in PRRX1^{3' tdTomato}-reporter iPSCs. The nuclei were counterstained with DAPI. Representative images are shown. (c) Comparison of mid-primitive streak (Mid-PS) marker gene expression (*GATA6*, *MIXL1*, *BRACHYURY*) during differentiation from PSC to LPM. Total RNA was extracted at days 0, 1 and 2, and the expression levels of marker genes were compared using qRT-PCR. All levels were normalised to the *ACTB* mRNA level (n = 3 from three biologically independent experiments). ***P* < 0.01, ****P* < 0.001. Statistical significance was determined by one-way ANOVA with correction for multiple testing using the Bonferroni method (c).



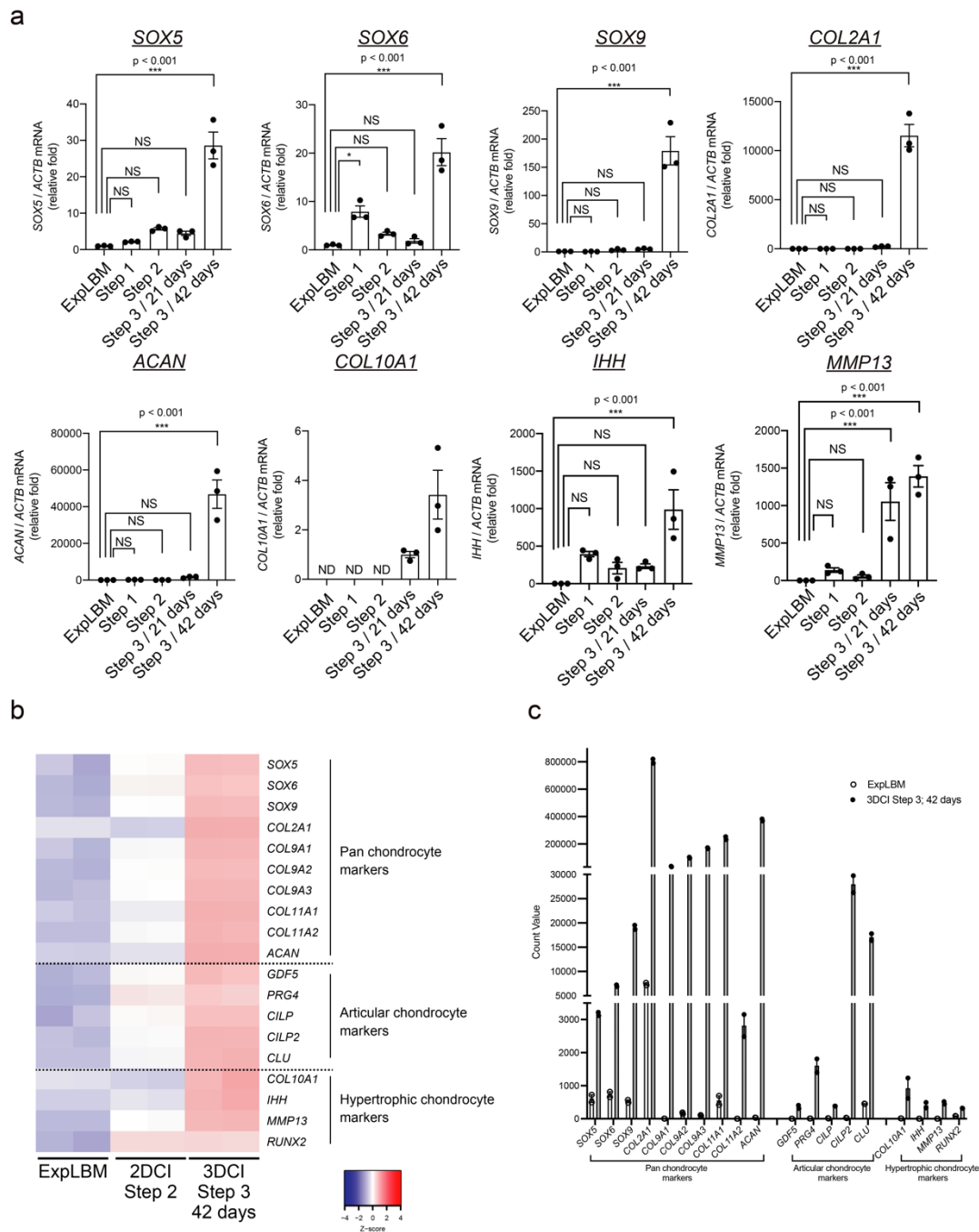
Supplementary Figure 2 | Induction of LBM-like cells from hiPSC lines (414C2 and 1383D2) or hESC lines (SEES4, SEES5, SEES6 and SEES7). Comparison of marker gene expression in the LBM (*PRRX1*) or LPM (*HAND1*, *FOXF1*, *ISL1*) after treating LPM cells with LBM medium (combination 8, Supplementary Table 1). Total RNA was extracted at day 0, 1, 2 and 4 and the levels of marker genes were compared using qRT-PCR. All the values were normalised to *ACTB* mRNA level (n = 3, three biologically independent experiments). ** $P < 0.01$, *** $P < 0.001$. Statistical significance was determined by one-way ANOVA with correction for multiple testing using the Bonferroni method.



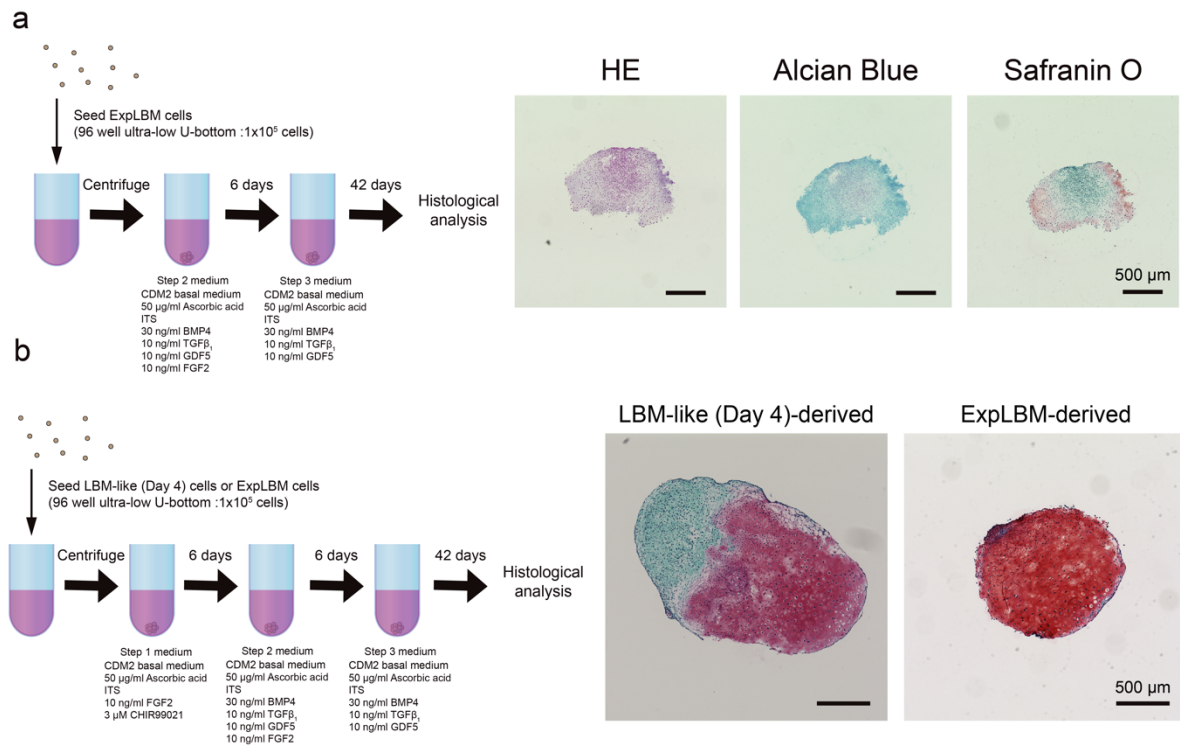
Supplementary Figure 3 | PRRX1 expression in each hPSC-derived ExpLBM line and karyotype analysis of 414C2-derived ExpLBM cells. (a) Immunostaining of PRRX1 in ExpLBM cells derived from hiPSC lines (414C2 and 1383D2) or hESC lines (SEES6 and SEES7). (b) Comparison of karyotype between 414C2 (hiPSCs) and 414C2-derived ExpLBM cells (passage number = 3). 414C2-derived ExpLBM cells had the same karyotype as that of 414C2-derived cells.



Supplementary Figure 4 | Cryopreservation of ExpLBM cells. (a) Flow cytometric analysis of tdTomato expression after cryopreservation. After storage in liquid nitrogen, ExpLBM cells were cultured and analysed to detect the expression of tdTomato. (b) Chondrogenic differentiation capacity of ExpLBM after cryopreservation. ExpLBM cells were added to 96-well ultra-low U-bottom plates and then centrifuged to pellet the cells. Culture media were changed at each indicated time point (steps 1–3) to generate the hyaline cartilaginous-like particles.

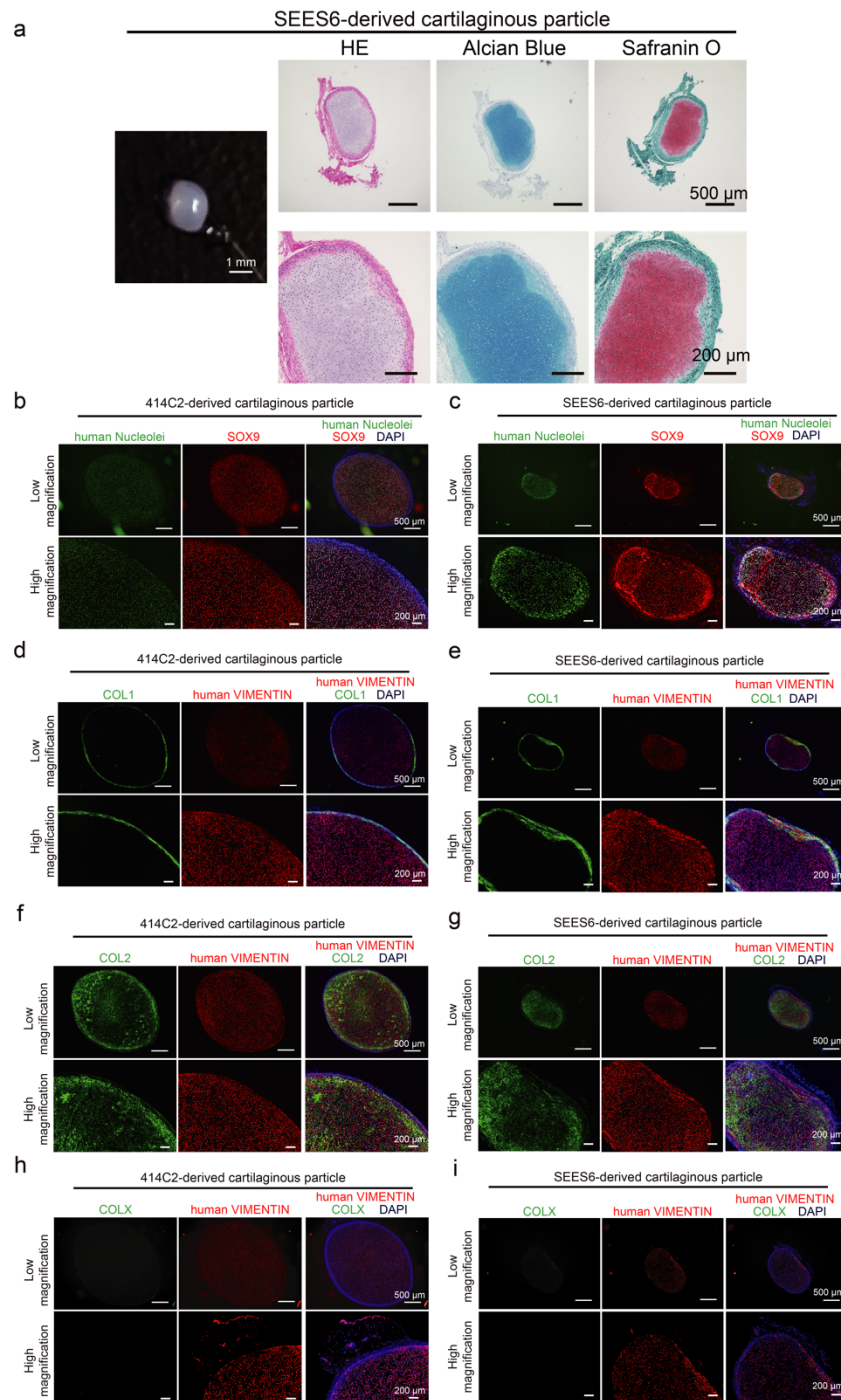


Supplementary Figure 5 | qRT-PCR and RNA-seq analyses of chondrocyte marker gene expression. (a) During the 3DCI protocol, total RNA was extracted at the ExplBM cell state, step 1 (6 days), step 2 (6 days), and step 3 (21 days) or step 3 (42 days), and the levels of marker genes were compared using qRT-PCR. All levels were normalised to the *ACTB* mRNA level ($n = 3$, three biologically independent experiments). (b) Heatmap of the expression levels of each marker gene in the RNA-seq analysis of ExplBM (PRRX1^{high}, PN = 4 or 5), PRRX1^{low} ExplBM, 2DCI-treated ExplBM, and 3DCI-treated ExplBM (step 3, 42 days) (PRRX1^{3'utTomato}-reporter-derived samples). (c) Count value of each chondrocyte marker in ExplBM or 3DCI-treated ExplBM (step 3: 42 days), related to Supplementary Figure 5b. N.D., not detected. * $P < 0.05$, *** $P < 0.001$. Statistical significance was determined by one-way ANOVA with correction for multiple testing using the Bonferroni method (a).



Supplementary Figure 6 | Effects of 3DCI on ExpLBM and LBM cells.

(a) Chondrogenic induction of ExpLBM cells using 2 step-3D culture conditions without step 1. ExpLBM cells were added to 96-well ultra-low U-bottom plates and then centrifuged to pellet the cells, which were cultured from step 2 and subjected to histological analysis, including staining using HE, Alcian Blue or Safranin O stain, which was performed after step 3. (b) Chondrogenic induction of LBM-like (Day 4) or ExpLBM cells under 3DCI. LBM-like (Day 4) or ExpLBM cells were added to 96-well ultra-low U-bottom plates and then centrifuged to pellet the cells. Culture media were changed at each indicated time point (steps 1–3) to generate hyaline cartilaginous-like particles.

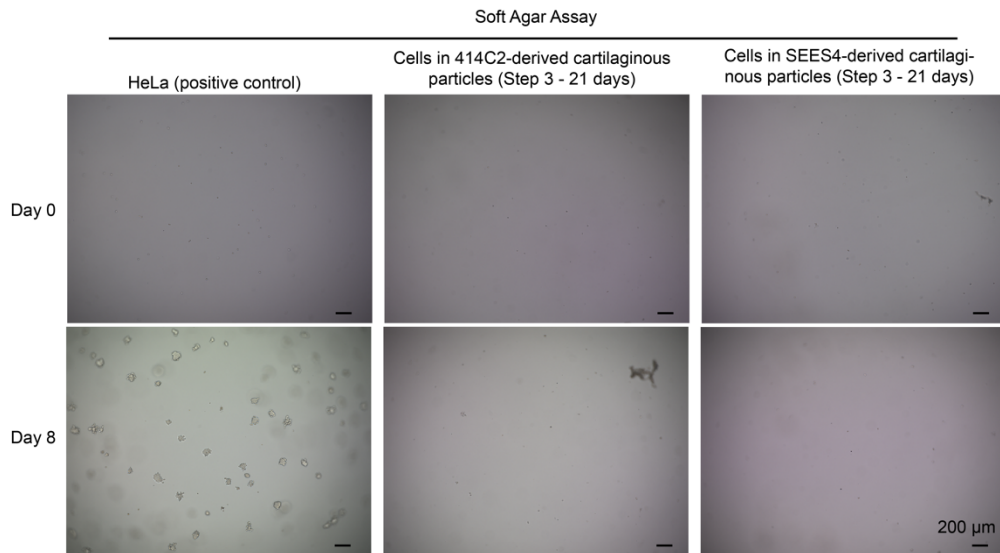


Supplementary Figure 7 | Subcutaneous transplantation of the cartilaginous particles in NOD-SCID mice. (a) Subcutaneous inoculation of the cartilaginous particles in NOD-SCID mice. SEES6-derived cartilaginous particles (step 3 treatment; 21 days) were subcutaneously inoculated into NOD-SCID mice, and their engraftment was assessed using HE, Alcian blue or Safranin O staining at 8 weeks after the transplantation. Representative images are shown. (b–i) Immunostaining of the subcutaneously engrafted 414C2 or SEES6-derived cartilaginous tissues in NOD-SCID mice with human nucleoli/SOX9 (b, c), human VIMENTIN/COL1 (d, e), human VIMENTIN/COL2 (f, g) or human VIMENTIN/COLX (h, i). Tissue sections were stained with each indicated antibody, and the nuclei were counterstained with DAPI. Representative images are shown.

a

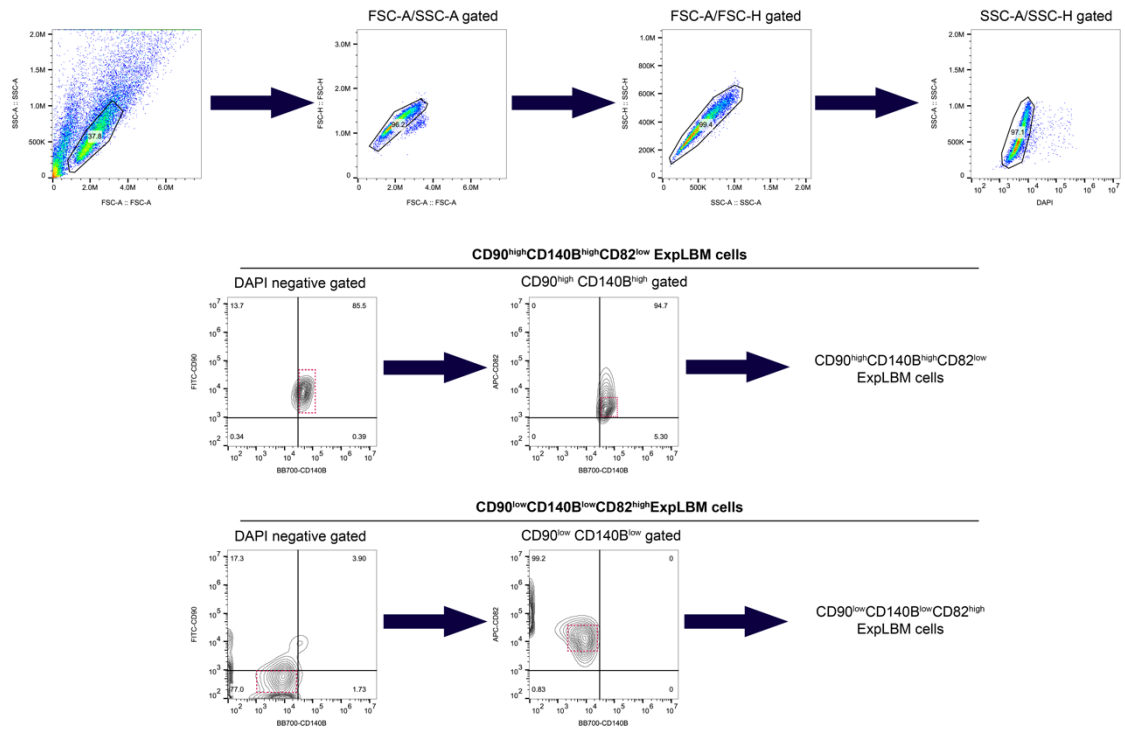
Host (Implantation site)	Successful engraftment / Number of transplantation	Teratoma incidence / Number of transplantation
NOD-SCID mice (Subcutaneous)	4 / 4	0 / 4
SCID rat (Drill hole in articular cartilage)	8 / 8	0 / 8

b

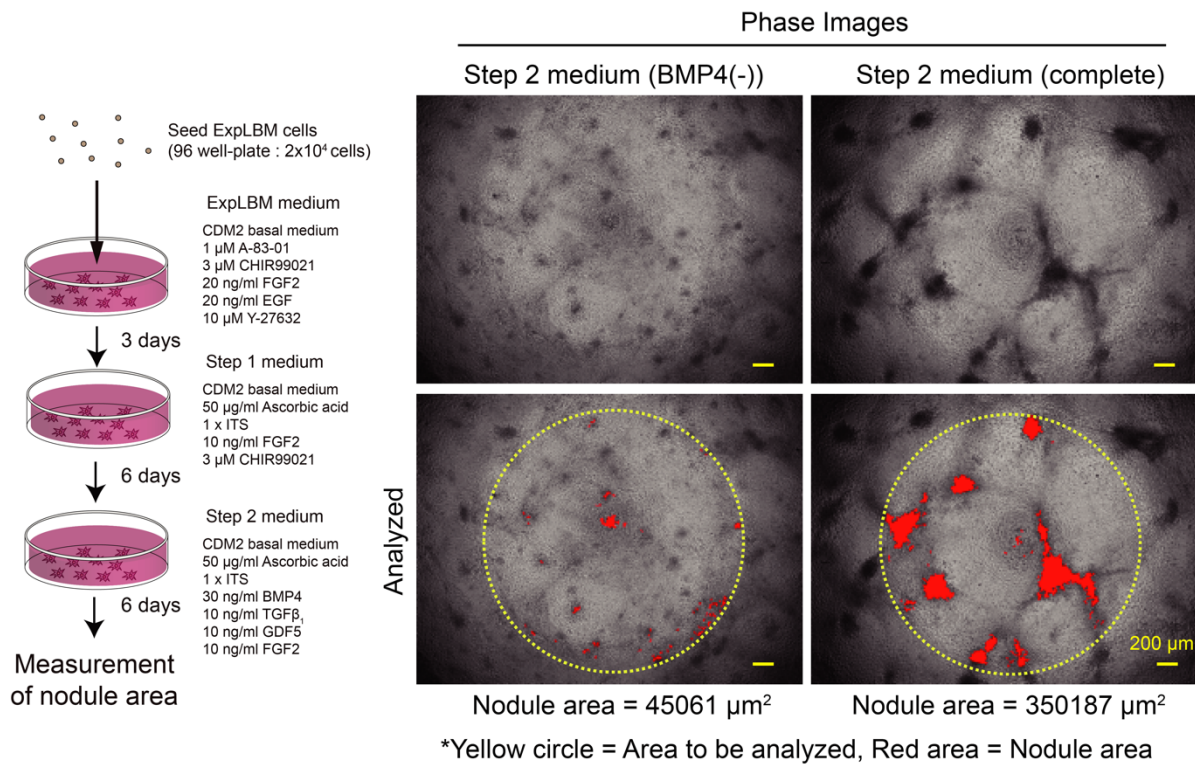


Supplementary Figure 8 | Tumorigenic assay of ExpLBM cells.

(a) Ratio of successful engraftment and teratoma incidence among various animals that received transplantation of ExpLBM-derived cartilaginous particles. (b) Assessment of the tumorigenic potential of ExpLBM-derived cartilaginous particles by soft-agar assay. Cells in cartilaginous particles were dissociated into single cells and their colony-forming capacity was compared with that of HeLa cells (n = 3, three biologically independent experiments).



Supplementary Figure 9 | Gating strategy used in Figure 4h and 4i. The populations shown as red dotted area in each panel were isolated as CD90^{high}CD140B^{high}CD82^{low} and CD90^{low}CD140B^{low}CD82^{high} ExpLBM cells, respectively.



Supplementary Figure 10 | Establishment of chemical screening system based on 2DCI method. Experimental procedure on the evaluation of chondrogenic differentiation. The 414C2-derived ExpLBM cells were seeded into 96-well plates and differentiated by following the indicated procedure. After taking phase images of each well, the nodule area (red region) in the yellow circle was measured to compare the chondrogenic effect between step 2 media (BMP4 (-)) and complete step 2 media. Representative images are shown.