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Supplemental

Human Developmental Chondrogenesis

as a Basis for Engineering Chondrocytes

from Pluripotent Stem Cells

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Inventory of supplemental information

1) Supplemental data

Supplemental Table 1, related to Figure 1
Supplemental Table 2, related to Table 1
Supplemental Figure 1, related to figure 2
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2) Supplemental procedures
3) Supplemental references

SUPPLEMENTAL DATA

Supplemental Table 1 (related to Figure 1).
Genetic signatures of pre-chondrocyte vs. total limb cells at 6 weeks of development.

Supplemental Table 2 (related to Table 1).
Genetic signatures of pre-chondrocyte vs resting definitive periarticular chondrocytes.

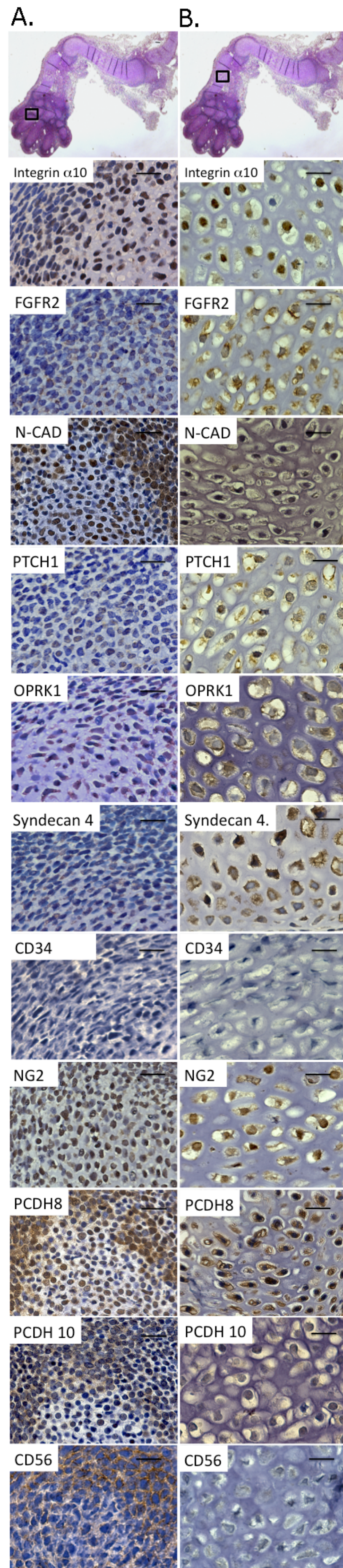


Figure S1. Related to Figure 2.

Pre-chondrocytes demonstrate a distinct surface antigen profile from differentiated chondrocytes. Immunohistochemistry for a panel of mesenchymal and chondrogenic antigens demonstrated that pre-chondrocytes at the periphery of the condensation **(A)** can be distinguished from differentiated cartilage cells in the central, more mature areas of the condensations and hypertrophic chondrocytes **(B)** via the expression of surface antigens. Positive signal is shown in brown color, nuclei counterstained with hematoxylin. Scale bar = 20 μm.

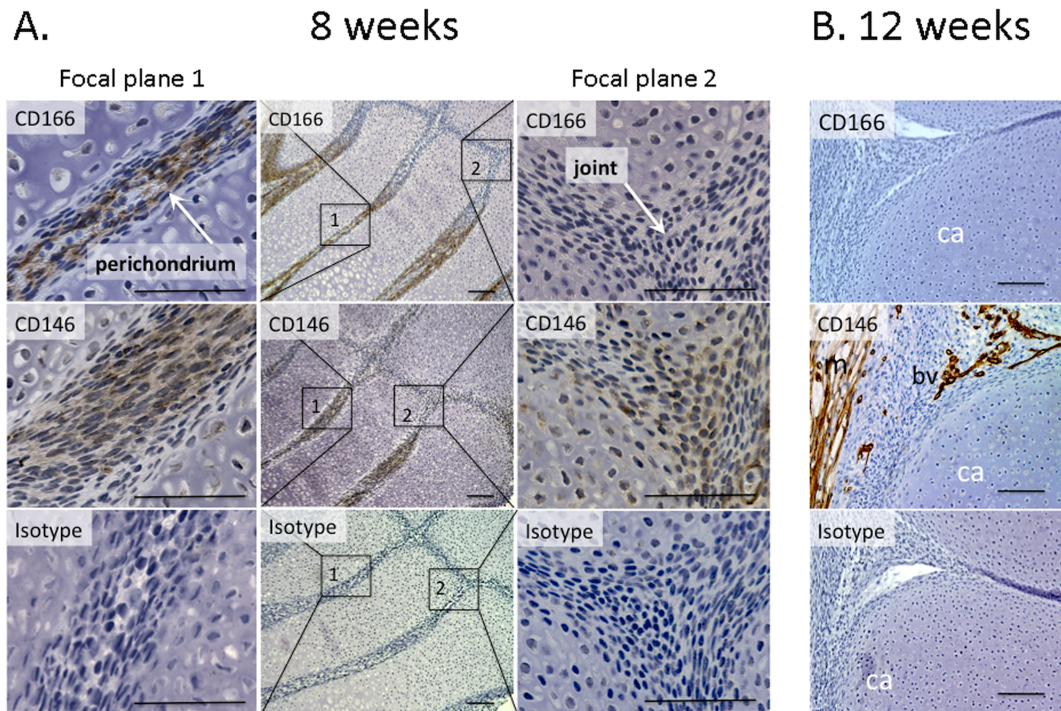


Figure S2. Related to Figure 3.

Developmental dynamics of CD166 and CD146 expression in human limbs. **(A)** At 8 weeks of development, CD146 positive pre-chondrocytes are observed in the joint-forming regions and in the perichondrium. Expression of CD166 is absent in the joint regions at 8 weeks of development, but is clearly present in the perichondrium. At 12 weeks of development (B), no CD146 and CD166 expression is observed on chondrocytes. Ca – cartilage, m – muscle, bv – blood vessels. Scale bar = 50 µm.

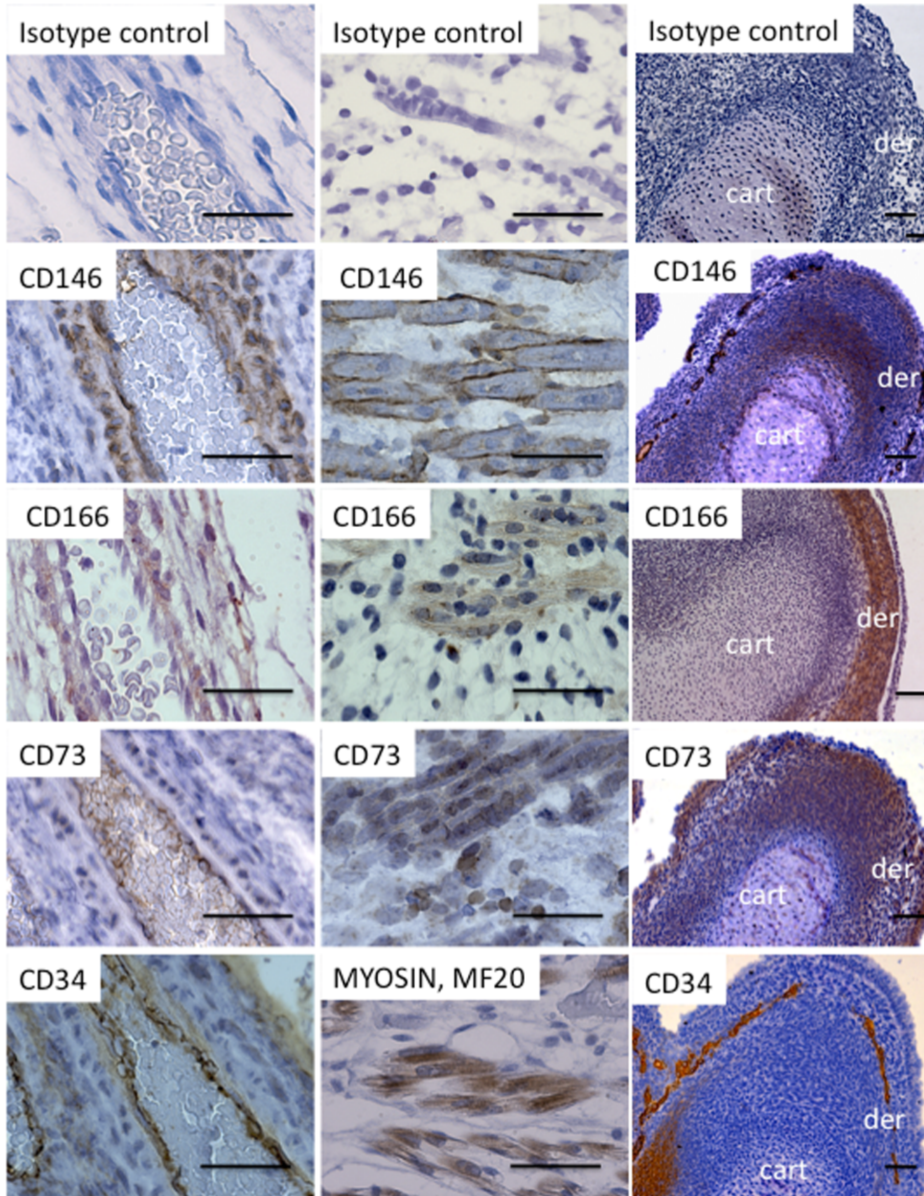


Figure S3. Related to Figure 4.

Expression of mesenchymal markers on non-chondrogenic lineages in developing human limbs. Perivascular cells **(A)** and developing skeletal myoblasts **(B)** and dermal fibroblasts (C) at 6-7 weeks of development are CD166 positive and also express other mesenchymal markers, necessitating the development of a specific sorting strategy based on multiple surface antigens to isolate pre-chondrocytes. Positive signal is shown in brown color (3,3-diaminobenzidine), nuclei counterstained with hematoxylin (blue). Control sections were stained with isotype controls. Cart – cartilage, der – dermal fibroblasts. Scale bar = 50 μm.

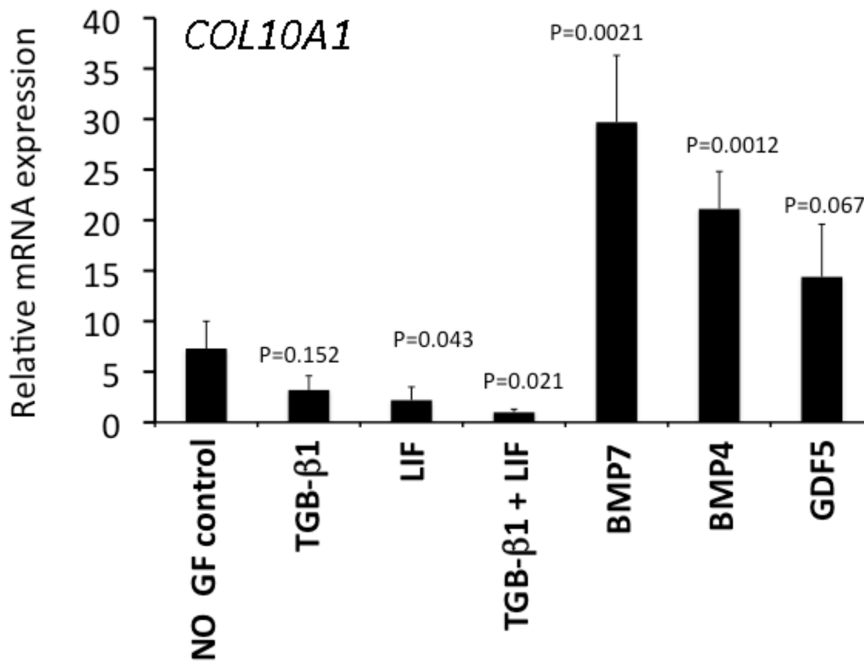


Figure S4. Related to Figure 5.
Regulation of the COL10A1 gene expression in primary chondrocytes by growth factors and morphogens. Primary human fetal chondrocytes were aggregated in serum free medium in the presence of BMP4 (50ng/ml), BMP7 (50 ng/ml), GDF5 (50 ng/ml), LIF (50 ng/ml) or TGF-β1 (10 ng/ml) and cultured for 14 days. Relative expression of *COL10A1* is shown. Error bars represent mean ± SD; 4 independent experiments. One way ANOVA was used followed by Newman Keul's test.

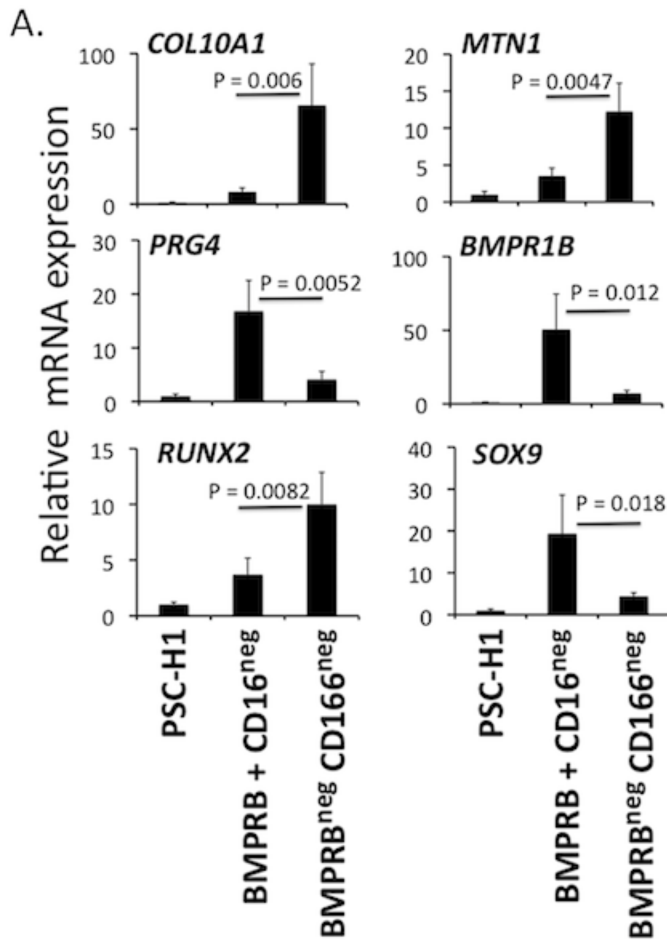
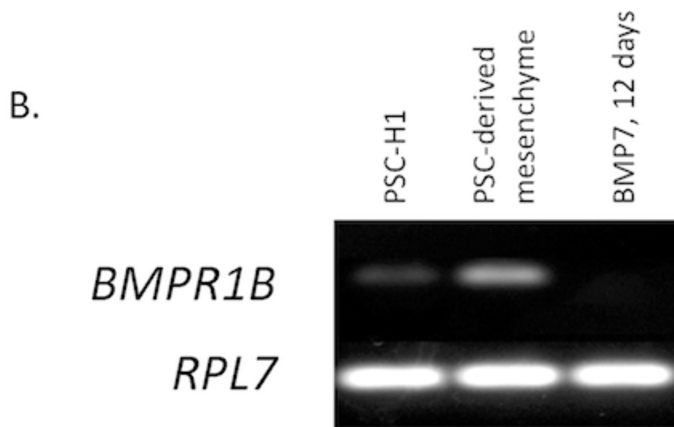
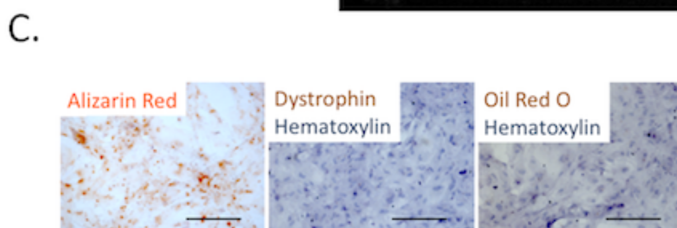


Figure S5. Related to Figure 6. Differentiation of PSC cells to chondrocytes. (A) Aggregated PSC-derived mesenchymal cells cultured in the presence of BMP7 expressed higher levels of hypertrophic genes than cells cultured in the presence of LIF and TGF-β1, but showed significantly lower expression levels of *BMPR1B*, *PRG4* and *SOX9*. (Mean ± SD; 3 independent experiments); **(B)** BMPR1B is expressed in PSC-derived mesenchymal cells 3 days after aggregation and induction of chondrogenesis, but undergoes downregulation after subsequent culture in the presence of BMP7. BMP7 was added to aggregated mesenchymal cultures for 12 days at 50 ng/ml. RPL7 is a housekeeping gene. **(C)** PSC-derived $CD146^{neg}CD166^{neg}BMPR1B^{+}$ cells show no/minimal osteogenic, myogenic and adipogenic potential as evidenced by *in vitro* analysis of mesenchymal lineage potential. Scale bar = 100 μm.





SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Laser capture microdissection of human embryonic pre-chondrocytes.

Embryonic tissues were snap frozen using OCT reagent and 10 uM sections cut using a Leica Cryostat CM1850 and placed on NF 1.0 PEN Membrane Slides (Carl Zeiss, Germany). Prior to sectioning membrane slides were irradiated with UV light for 30 minutes. Sections were either processed immediately or stored at -80° C. Sections were first fixed and dehydrated in ice-cold 70% ethanol for 2-3 min, OCT removed by dipping the slide 5-6 ties in ice-cold RNase-free water, dehydrated in ethanol again and then stained for ~30 seconds in 1% cresyl-violet acetate solution. Next, slides were placed in 70% ethanol, 100% ethanol for 1-2 min and finally, air-dried. Following dehydration, cell capture was initiated immediately. Laser capture microdissection was carried out using the Zeiss PALM microlaser system (Carl Zeiss, Germany) Following capture, RNA was extracted from cells using the RNeasy micro extraction kit (Qiagen, Germantown, MD). No amplification procedures were carried out prior to microarray. The resulting RNA was assessed on an Agilent Bioanalyser Nanochip to determine length of RNA transcripts in the samples.

Microarray hybridization and data analysis.

Human U133plus2.0 array hybridizations were performed by the UCLA Clinical Microarray Core. All specimens were analyzed separately and data processed following standard Affymetrix GeneChip Expression Analysis protocols. The acquisition of each array image was undertaken by using Affymetrix GeneChip Command Console 1.1 (AGCC). Subsequent raw data were analyzed using DNA-Chip Analyzer (D-Chip) with the .CEL files obtained from AGCC. A PM/MM difference model for estimating gene expression levels and a quantile approach for data normalization were used. Thresholds for selecting significant genes were set at a relative difference ≥ 1.5-fold for the experiment involving 5-6 week specimens and ≥ 3.0 fold for experiment involving 17 week specimens, with an absolute difference > 100 signal intensity units. Gene expression that met all three criteria simultaneously was considered as significantly changed. Global functional analyses, network analyses and canonical pathway analyses were performed using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com).

Digestion of embryonic and fetal tissues.

Tissues were first minced with scissors and then exposed to the following dissociation solution: collagenase (1 mg/mL final concentration, obtained from Sigma-Aldrich, Saint Louis, MO) or collagenase 2 (1 mg/ml, Worthington, Lakewood, NJ), dispase (0.25 mg/mL final concentration, from Invitrogen, Carlsbad, CA), DNase I (final concentration 1 mg/mL, from Sigma-Aldrich) in DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich). Digestion was carried out for 2 hours at 37 ^{o}C. 17 week cartilage was digested for 3-4 hours. The resulting cell suspension was centrifuged and washed with fresh medium before analysis.

Differentiation of human pluripotent stem cells to mesenchymal tissue.

To induce mesoderm differentiation, colonies of H1 cell line (Madison, WI) or UCLA derived and fully validated lines UCLA3 or HIPS23 were cut into uniform sized pieces using the StemProEZPassage tool (Invitrogen), transferred into 6-well plates pre-coated for 1 hour with Matrigel (growth factor reduced, no phenol red; BD Biosciences, Bedford, MA), and cultured initially in TESR media (Stem Cell Technologies, Vancouver, Canada) until 50-60% confluent (typically 2 days). To induce differentiation, TESR medium was replaced with complete serum free basal induction "X Vivo 15" medium (Lonza, Walkersville, MD). Immunophenotypic signature of PSC-derived skeletogenic mesenchymal cells has been described previously (Evseenko et al., 2010). To increase the output of skeletogenic mesenchyme and reduce the induction of hematoendothelial and cardiovascular mesoderm BMP4 and VEGF in the universal mesoderm-inducing medium permissive for all mesodermal lineages (Evseenko et al., 2010), were substituted for Noggin (50 ng/mL) and Wnt3a (10 ng/mL), following the best conditions identified in the pilot tests. After 7 days of culture, $CD166^{+}CD146^{+}PDGFR\text{-}\alpha^{+}KDR^{neg}$ PSC-derived mesenchymal cells were isolated by FACS or, in the large-scale experiments, by 2 step magnetic activated cells sorting (MACS) using magnetic columns (Miltenyi Biotec, Germany). First, cells marked with CD326, CD34, KDR and CD45 – APC labeled primary antibodies and then anti-APC magnetic bead conjugated secondary antibodies were removed using LD columns (Miltenyi Biotec, Germany). During the second step, further enrichment for $CD146^{+}CD166^{+}$ cells was carried out using an LS column after the PE-magnetic bead labeling. Quality control of the isolation performed by flow cytometry showed ≥ 95% purity of the isolated cells. After the

isolation cells were subjected to chondrogenic analysis or multilineage differentiation analysis. In the Results section data from H1 cell line is shown as representative of all tested lines.

Chondrogenic in vitro pellet and explant assays.

FACS or MACS isolated PSC-derived mesenchyme or fetal primary cells were re-plated as large (1-2 x 10^5 cells) re-aggregated colonies in low attachment culture plates or 96 well-Transwell plates (Corning) in base differentiation medium StemPro 34 (Invitrogen) or X Vivo (Lonza) supplemented BMP4 (10 ng/mL), sonic hedgehog (SHH, 25 ng/mL), FGF2 (3 ng/mL) and insulin like growth factor 1 (IGF1, 10 ng/mL) and cultured for 3 days at 37°C to induce chondrogenesis. After 3 days pellets were transferred in either hypertrophy promoting conditions (BMP4 or 7, 50 ng/mL) or conditions permissive for the generation and maintenance of resting chondrocytes (LIF 50 ng/mL, TGF beta 1, 10 ng/mL) and cultured at 5% O2 and 5% CO2 for up to 3 weeks. All cytokines were from R&D Systems.

For FACS isolation of PSC-derived chondrogenic aggregates were dissociated at 5, 10 or 15 days after aggregation using Collagenase 2 (1 mg/mL, Worthington, Lakewood, NJ) and Tryple (Invitrogen) and labeled with fluorescent antibodies on ice for 30 minutes. Following incubation, some aggregates were ether fixed for morphological evaluation. Chondrogenic aggregates were embedded in paraffin and 5 μm paraffin sections stained with Alcian Blue for cartilage associated acid mycopolisaccarides and glycosaminoglycans (all reagents obtained from Sigma-Aldrich). Immunohistochemical detection of Collagen I, II and X expression was carried out as described below.

For implantation assay, FACS isolated PSC-derived $CD166^{low/neg}$BMPR1B+ cells were pre-labeled with PKH67 (green) dye (Sigma-Aldrich) and implanted into "defects" generated in the proximal epiphysis (3-mm-thick fragment) of a femoral bone dissected from 17-week fetal specimens. 1 x 10^6 PSC derived cells were used for implantation, delivered in 2 μL of Growth Factor reduced Matigel (BD Bioscience). Explants were cultured for up to 3 weeks in X Vivo medium supplemented with 0.2% of fetal bovine serum, penicillin/streptomycin (Invitorgen) and no growth factors.

Myogenic, adipogenic and osteogenic differentiation assays.

Sorted primary pre-chondrocytes or PSC-derived chondrocytes were seeded into chamber sides (BD Biosciences, San Diego, CA). StemPro Osteogenic or StemPro Adipogenic medium (Invitrogen) was used for osteogenic and adipogenic differentiation, respectively. For myogenic differentiation, cells were cultured in myoblast differentiation medium (PromoCell, Heidelberg, Germany) for up to 3 weeks. Following incubation, cell cultures were fixed in 4% paraformaldehyde. Staining with Oil Red O was carried out for detection of neutral fat; Alizarin Red reagent was used for detection of calcium deposits.

Antibody staining and flow cytometry.

For FACS analysis, primary or PSC-derived cells were dissociated into a single cell suspension with Collagenase 2 (Worthington) and Tryple Select (Invitrogen) and then incubated with monoclonal antibodies against CD166 and BMPR1B, PE or APC conjugated; CD44, CD73, CD326, PerCP-Cy5.5 conjugated; CD31, CD45, CD325 (E-CAD), CD34 FITC conjugated; CD73 and CD146, PE-Cy7 conjugated (all from BD Biosciences or BioLegend, San-Diego, CA except BMPR1B and VEGFR2 (KDR) which was obtained from R&D Systems. Isotype control antibodies conjugated with PE, APC, FITC, Percp-Cy5.5, PE-Cy7 were purchased from BD Biosciences. After incubation, cells were washed in PBS containing 1% bovine serum albumin and analyzed using a BD FACSAria or LSRII cytometer (BD Bioscience). FACS files were exported and analyzed using FACSDiva software (BD Biosciences).

Immunohistochemistry.

For immunohistochemical analysis, fetal tissues were fixed in 4% paraformaldehyde (Sigma-Aldrich) and embedded in paraffin, 5 μm sections were incubated with hydrogen peroxide for 10 minutes, antigen retrieval was performed using citrate buffer pH 6 at 100 ^{o}C for 30 min in a steam cooker, washed with Tris buffered saline (TBS) and blocked in 2% normal horse serum for 20 minutes. Primary antibodies used were: CD146, CD140a, collagen type I, II, and X, Ki67, CD34, CD56, CD73, alpha 5-integrin/CD49e (Abcam Inc., Cambridge, MA); sarcomeric myosin (MF-20, Developmental Studies Hybridoma Bank (DSHB), Iowa City, Iowa); PCDH 8, 10, CD44, FGFR2, PTCH1 (Abgent, San Diego, CA); OPRK1 (Novus Biologicals, Littleton, CO); CD56, BMPR1B and dystrophin (Sigma-Aldrich); CD166

(Epitomics, Burlingame, CA); and Syndecan 4 from R&D systems. Primary isotype control antibodies were purchased from Abcam.

For counterstaining of cell nuclei, 4'-6-diamidino-2-phenylindole (DAPI) was added to the final PBS washing. Staining without primary antibodies served as controls. For bright field chromogen-based detection following primary antibody incubation HRP-conjugated secondary antibodies against rabbit IgG (Vector Laboratories, Burlingame, CA) were used. Antibodies were then visualized with Peroxidase Substrate Kit DAB (Vector Laboratories, Burlingame, CA). Nuclei were counterstained with hematoxylin. Overview image of cultured explants was acquired using dissecting Leica Microscope (Leica, Germany). Images were acquired using the Zeiss Axiovision software version 4.8 Carl Zeiss Microscope (Carl Zeiss, Germany) equipped with ApoTome.2: Modules for Axio Imager.2 and Axio Observer with 10, 20 and 40x (1.3 numerical aperture (NA)) and 63x (1.4 NA) oil-immersion objectives.

Quantitative real-time PCR.

SYBR Green RT-PCR amplification and detection was performed using an ABI Prism 7900 HT (Applied Biosystems) as previously described (Evseenko et al., 2006). The comparative C_t method for relative quantification ($2^{-\Delta\Delta Ct}$) was used to quantitate gene expression according to Applied Biosystems' recommendations [*7900 HT Real-Time fast and SDS enterprise and database user guide*]. A geometric average of *B2M* (β2 microglobulin), *TBP* (TATA-box binding protein) and *RPL7* (ribosomal protein L7) was used for gene normalization and expressed relative to a calibrator (sample in each set with lowest expression). Primer sequences used for qPCR are available on request.

Statistics

Descriptive statistics were performed for each data set and the data combined for collective analysis. Data was converted to graphs with Microsoft Excel 2003 (San Diego, CA). Statistical analysis was performed with SigmaStat software (Systat Software Inc., Richmond, CA). Descriptive statistics, Student T test and one-way ANOVA were applied followed by Student-Newman-Keul's test. $P<0.05$ was considered to be significant.

SUPPLEMENTAL REFERENCES

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