Comparative evaluation of isogenic mesodermal and ectomesodermal chondrocytes from human iPSCs for cartilage regeneration

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Generating phenotypic chondrocytes from pluripotent stem cells is of great interest in the field of cartilage regeneration. In this study, we differentiated human induced pluripotent stem cells into the mesodermal and ectomesodermal lineages to prepare isogenic mesodermal cell–derived chondrocytes (MC-Chs) and neural crest cell–derived chondrocytes (NCC-Chs), respectively, for comparative evaluation. Our results showed that both MC-Chs and NCC-Chs expressed hyaline cartilage–associated markers and were capable of generating hyaline cartilage–like tissue ectopically and at joint defects. Moreover, NCC-Chs revealed closer morphological and transcriptional similarities to native articular chondrocytes than MC-Chs. NCC-Ch implants induced by our growth factor mixture demonstrated increased matrix production and stiffness compared to MC-Ch implants. Our findings address how chondrocytes derived from pluripotent stem cells through mesodermal and ectomesodermal differentiation are different in activities and functions, providing the crucial information that helps make appropriate cell choices for effective regeneration of articular cartilage.

INTRODUCTION

Hyaline cartilage, originating from either mesoderm or ectoderm, is a connective tissue on an articular surface that transmits loads or in the nasal septum that provides structural support (1). During development, the mesoderm layer develops into somite and lateral plates, and cells of these mesodermal structures further generate hyaline cartilage in limbs, while the ectoderm layer gives rise to external ectoderm, neural tube, and neural crest (2). Specifically, the neural crest, occasionally called “the fourth germ layer” due to its astonishing multipotency, is able to generate a variety of ectodermal and mesodermal cell types. During craniofacial development, neural crest–derived cells (NCCs), as an outgrowth of epithelium cells near the neural tube (3), convert into ectomesodermal cells and then become cranial NCCs. This allows the formation of head mesenchyme for further generation of craniofacial hyaline cartilage (4). Because mesoderm-derived cells (MCs) and NCCs share the capacity to generate hyaline cartilage, their progeny is considered appropriate cell sources for hyaline cartilage regeneration.

Mesenchymal stem/stromal cells (MSCs) derived from adult tissues, such as bone marrow and fat, have multilineage differentiation ability and have been extensively studied as a promising cell source for tissue repair such as cartilage reconstruction. To date, there are more than a thousand ongoing clinical trials using MSCs for therapies (5). While many of the trials focus on cartilage repair, increasing similarities to native articular chondrocytes through differentiation potential (6) and are considered alternative stem cell sources for cartilage regeneration. Studies have demonstrated the induction of PSCs into chondrocytes along the mesodermal or ectomesodermal lineage. For example, several groups have shown that through stepwise induction by growth factors, mesoderm–derived chondrocytes can be generated from hESCs (9–11) or hiPSCs (12–15), and these MCs are capable of repairing cartilage lesions in rodent joints (13, 14, 16). Besides mesodermal derivation, other studies have shown that chondrocytes can be obtained through differentiation of NCCs derived from hESCs (17–19) or hiPSCs (20) along the ectomesodermal lineage. Recently, our group has also reported that chondrocytes differentiated from hiPSC–derived NCCs express chondrocyte markers and matrix (21). These findings suggest that cartilage formation during development can be emulated to some degree in culture by inducing chondrogenic differentiation of PSCs along distinct developmental lineages.

While previous studies have demonstrated chondrogenic differentiation of PSCs through stepwise induction by chemically defined medium, it remains unclear whether isogenic mesodermal and ectomesodermal lineage–derived chondrocytes from hiPSCs are different in phenotypes and properties and how similar the derived cells are to native chondrocytes (NCs). In light of the emerging evidence suggesting the potential of nasal chondrocytes for articular cartilage regeneration (22), ectomesodermal lineage–derived chondrocytes are promising as potential alternative cells to mesodermal lineage–derived chondrocytes for the repair of joint defects. Therefore, this study to determine biological and functional differences between...
chondrocytes derived from these two developmental lineages is expected to provide the crucial information to strengthen our current knowledge of cell choices for articular cartilage regeneration. In this study, blood-derived hiPSCs were induced for mesodermal and ectomesodermal differentiation to prepare isogenic MCs and NCCs, respectively, following modified published protocols (9, 18), and then induced to differentiate into chondrocytes. Differences in phenotype and capability of cartilage regeneration between the distinct lineage-derived chondrocytes were characterized by both in vitro and in vivo assays. Genome-wide transcriptome analysis was also performed to compare hiPSC-derived chondrocytes to NCCs to detect key differences and identify potential molecular candidates for priming chondrogenic induction of MCs and NCCs and enhancing hyaline cartilage formation.

RESULTS

Blood-derived hiPSCs differentiate into chondrocyte-like cells along mesodermal or ectomesodermal lineages

The pluripotency of hiPSCs was examined by the analysis of cell morphology, characterization of pluripotency markers, and teratoma formation. The results showed that compact colonies similar to those of hESCs were formed in culture (fig. S1A) and expressed alkaline phosphatase (ALP) (fig. S1B). Immunofluorescence and flow cytometry results demonstrated that the colonies exhibited pluripotency-related markers OCT4, NANOG, SOX2, PODPCALYXIN, SSEA4, and CD9 (fig. S1, C and D). Results of teratoma formation in severe combined immunodeficient (SCID) mice showed that the hiPSC was capable of giving rise to ectodermal, endodermal, and mesodermal lineage-derived cells (fig. S2). All these together confirm the pluripotency of the blood-derived hiPSCs.

To determine the phenotypic differences between chondrocytes derived from the two different developmental lineages, blood-derived hiPSCs were induced into MCs and NCCs and further into isogenic MC-derived chondrocytes (MC-Chs) and NCC-derived chondrocytes (NCC-Chs), respectively (Fig. 1A), following previously published protocols with modifications (9, 21). The morphology of mesendodermal cells showed similar compactness to that of hiPSCs at day 4, while the morphology of differentiated mesoderm revealed cell cluster formations at day 8 (Fig. 1B). On the other hand, during induction, NCCs exhibited compact morphology at day 5 and later became stellate-shaped at day 15 (Fig. 1B). Results of immunofluorescence assessment indicated that MCs expressed mesoderm markers, CD34 and alpha–smooth muscle actin, after 8 days of MC induction (Fig. 1C), and NCCs were stained positive for neural crest markers, P75 and human natural killer 1, at day 15 of induction (Fig. 1C). These results demonstrate that the derivation of MCs and NCCs from hiPSCs was achieved with the use of the induction protocols.

Specific lineage-derived chondrocytes were generated through chondrogenic induction of cell pellets, and the results showed that pellets of NCC-Chs were significantly larger than those of MC-Chs (Fig. 1D). NCC-Ch pellets demonstrated stronger staining of Alcian Blue (Fig. 1E) and higher glycosaminoglycan (GAG) content (Fig. 1F) than MC-Ch ones. The flow cytometry result showed that chondrocyte-related surface markers, CD44 and CD151, were expressed in 94.1 and 95.6% of MC-Chs and in 73.5 and 95.7% of NCC-Chs, respectively (Fig. 1G). Immunofluorescence staining indicated that the expression of collagen type 1 alpha 1 chain (COL1A1) and COL2A1 in MC-Ch pellets was comparable to that in NCC-Ch pellets, while the COL10A1 content of MC-Ch pellets was greater than that of NCC-Ch pellets (Fig. 1H). Since COL10A1 is a marker of hypertrophic chondrocytes (23), the results suggest that the cells of MC-Ch pellets undergo hypertrophy.

To identify cell derivatives at different stages of differentiation from hiPSCs to NCC-Chs or MC-Chs, quantitative polymerase chain reaction (qPCR) was performed to detect the mRNA expression of cell specific markers (Fig. 1I). The expression of pluripotency markers—OCT4, NANOG, and SOX2—highly present in hiPSCs gradually decreased during differentiation and was undetectable or low in MC-Chs and NCC-Chs. The mesoderm-related markers—GATA4, FOXA2, CXCR4, KDR, MIXL1, and CDH1—and the neural crest-related markers—SOX1, PAX6, ZIC1, and P75—were highly expressed in MCs and NCCs, respectively. The progressive increase in the expression of cartilage-associated markers, SOX9 and ACAN, during chondrogenesis of both lineage cells indicated successful generation of MC-Chs and NCC-Chs from hiPSCs. Other lineage-associated markers, COL1A1 and COL2A1, significantly increased, whereas COL10A1 markedly decreased in both lineage-derived chondrocytes compared to those in MCs and NCCs. Together, these results indicate that MC-Chs and NCC-Chs derived from the blood-derived hiPSCs are chondrocyte-like cells expressing hyaline cartilage–associated extracellular matrix (ECM) and markers.

MC-Ch and NCC-Ch implants form hyaline cartilage–like tissue ectopically in mice

After 21 days of chondrogenic induction, pellets of MC-Chs and NCC-Chs were subcutaneously implanted in mice for 30 days to ectopically generate hyaline cartilage. Semitransparent and smooth MC-Ch and NCC-Ch pellets significantly increased from 0.89 ± 0.29 mm3 to 5.44 ± 2.33 mm3 and 7.63 ± 2.72 mm3 to 35.73 ± 8.31 mm3 in volume, respectively, during implantation (Fig. 2A). The increase in pellet size is likely resulted from the accumulation of cartilage-associated ECM. The morphology of MC-Chs stained by hematoxylin and eosin (H&E) appeared to be spindle-shaped, while that of NCC-Chs was round-shaped (Fig. 2B), suggesting that, compared to MC-Chs, NCC-Chs in lacunae more closely resemble spherical chondrocytes in native hyaline cartilage. Safranin O/Fast Green staining revealed that GAG-rich matrix had accumulated in MC-Ch and NCC-Ch pellets (Fig. 2B). The immunofluorescence analysis detecting human vimentin showed that the harvested pellets comprised mainly human cells (Fig. 2C) and that detecting mouse VEGFR1 indicated no occurrence of vascular invasion in the pellets (fig. S3A). Moreover, while both MC-Ch and NCC-Ch pellets contained abundant COL2A1 and a small amount of COL1A1, there was significantly more COL2A1 and less COL1A1 produced by NCC-Chs than that by MC-Chs (Fig. 2D). COL1A1 was sparsely present in MC-Ch pellets, whereas the molecule was absent in NCC-Ch pellets. Together, these results suggest that both chondrocyte lines form cartilage-like tissue subcutaneously in mice, but the tissue derived from NCC-Ch pellet is more similar to hyaline cartilage than that from MC-Ch pellet.

MC-Ch and NCC-Ch pellets repair joint defects of rats

To further examine the capacity of cartilage generation, pellets of MC-Chs and NCC-Chs were implanted in osteochondral defects and sealed with fibrin glue at the femoral trochlea groove of athymic nude rats (Fig. 3A). Fibrin glue used to secure cell pellets was chosen as an acellular control, and intact cartilage without surgical intervention as a reference control of desired repair. Macroscopic evaluation
Fig. 1. Differentiation of blood-derived hiPSCs toward isogenic MC-Chs and NCC-Chs. (A) Schematic of procedures inducing differentiation from hiPSCs to chondrocytes and corresponding timelines. hiPSCs were induced to differentiate into MCs and NCCs and then into MC-Chs and NCC-Chs, respectively. D, day. (B) Morphology of mesodermal and ectomesodermal lineage cells determined by microscopic imaging. (C) Immunofluorescence staining of hiPSC-derived MCs for detection of alpha-smooth muscle actin (α-SMA) and CD34 at day 8 and that of hiPSC-derived NCCs for detection of P75 and human natural killer 1 (HNK1) at day 15. Nuclear DNA was labeled by 4′,6-diamidino-2-phenylindole (DAPI). (D) Representative macrographs and volume quantification of MC-Ch and NCC-Ch pellets. (E) MC-Ch and NCC-Ch pellets stained by Alcian Blue. (F) Quantification of glycosaminoglycan (GAG) in MC-Ch and NCC-Ch pellets analyzed by dimethylmethylene blue. (G) Flow cytometry analysis of cells for detection of chondrocyte-related markers, CD44 and CD151. (H) Immunofluorescence staining of MC-Ch and NCC-Ch pellets for detection of COL1A1, COL2A2, and COL10A1. Nuclear DNA was labeled with DAPI. (I) Dynamics of the mRNA expression of pluripotency markers (OCT4, NANOG, and SOX2), mesoderm-associated markers (GATA4, FOXA2, CXCR4, KDR, MIXL1, and CDH1), neural crest–associated markers (SOX1, PAX6, ZIC1, and P75), and cartilage-associated markers (SOX9, ACAN, COL1A1, COL2A1, and COL10A1) during differentiation of hiPSCs into chondrocytes. Bars are color-coded to represent different cell types. N.D., not detected. n = 3. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars, 200 μm.
showed that defects of both the MC-Ch and NCC-Ch groups were filled with regenerated tissue after 4 weeks, whereas those of the fibrin glue group were not (Fig. 3B). At week 16, increased joint repair was found in all groups with the NCC-Ch group exhibiting more complete repair than the other groups. Further determined by the ICRS-I scoring system, the NCC-Ch group scored significantly higher in the visual assessment of articular cartilage repair (Fig. 3C and fig. S4A). Human vimentin localization indicated that the repaired tissue at rat joint defects implanted with MC-Ch and NCC-Ch pellets comprised mainly human cells (Fig. 3D), and rat vascular endothelial growth factor receptor 1 (VEGFR1) localization showed no vascular invasion in the regenerated cartilage (fig. S3B). The H&E staining results showed that, in the fibrin glue group, fibrous tissue resulting from host cells was formed after 4 weeks and later replaced by bone-like tissue after 16 weeks (Fig. 3E). On the other hand, the groups implanted with cell pellets showed that the defects were filled with highly cellular tissue after 4 and 16 weeks. Particularly, the regenerated tissue derived from NCC-Ch pellets integrated well with the surrounding native cartilage at week 16 (Fig. 3E). Safranin O/Fast Green staining further revealed that the regenerated tissue by MC-Chs or NCC-Chs was rich in GAG content and contained lacunae with spindle-shaped or round-shaped cells, respectively, but both groups fell short of fully restoring the osteochondral structure of cartilage as that shown in the control (Fig. 3F). The corresponding histological assessment by ICRS-II scoring showed that the 16-week NCC-Ch group scored significantly higher in superficial assessment, matrix staining, cell morphology, surface architecture, abnormal calcification, and overall assessment than the 16-week MC-Ch one (Fig. 3G and fig. S4B). Together, these results demonstrate the capacity of MC-Chs and NCC-Chs for cartilage regeneration of joint defects and a more desired outcome of cartilage repair by the NCC-Ch group.

**Global transcriptome of MC-Chs and NCC-Chs largely resembles that of NCs**

To further investigate cell differences at the molecular level, the global transcriptome of MC-Chs and NCC-Chs, compared to that of NCs, was analyzed by RNA sequencing (RNA-seq). Overall differences in the transcriptome between MC-Chs or NCC-Chs and
NCs were revealed by the Euclidean distance in multidimensional scaling plots (Fig. 4A) and also demonstrated by hierarchical clustering heatmaps (Fig. 4B). About 8 or 6% of the total transcript was differentially expressed between MC-Chs or NCC-Chs and NCs, respectively, and 5% of the total transcript between MC-Chs and NCC-Chs (Fig. 4C). The differentially expressed transcripts with the information of fold change, $P$ value, and false discovery rate (FDR) are listed in tables S1 to S3. The Gene Ontology (GO) enrichment result showed that transcripts associated with lineage specifications, including epithelium development and skeletal system development, were differentially regulated in MC-Chs, whereas those, including neurogenesis and nervous system development, were up-regulated in NCC-Chs compared to NCs (Fig. 4D). A number of transcripts highly expressed in NCC-Chs compared to those in MC-Chs were neuron development related (Fig. 4D). Further analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, the result showed that the transcripts up-regulated in both MC-Chs and NCC-Chs, compared to NCs, were related to complement and coagulation cascades and glutathione metabolism (Fig. 4E), which were previously found involved in the regulation of chondrogenesis.

Fig. 3. Implantation of human MC-Ch and NCC-Ch pellets in rat joints. (A) Schematic of chondrocyte pellet implantation. The pellet placed in the joint defect was covered with fibrin glue for secure integration with host tissue. (B) Repair of cartilage defects 4 and 16 weeks after pellet implantation. The control shown is contralateral joints of animals receiving implants. Black arrows point to the site of implantation. (C) ICRS-I scoring for visual assessment of repaired rat joints. (D) Immunofluorescence staining of the regenerated cartilage for detection of human vimentin. Nuclear DNA was labeled with DAPI. (E) Rat femur joints analyzed by H&E staining. (F) Rat joints analyzed by Safranin O/Fast Green staining. Solid boxed (superficial) and dashed boxed (subchondral bone) areas in the left column are shown at a higher magnification in the central and right column, respectively. (G) ICRS-II scoring for histological assessment of regenerated cartilage. $n = 3$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Scale bars, 200 µm.
The transcripts of molecules in the pathways mediating tight junction and focal adhesion in MC-Chs and cell adhesion molecules and ECM receptor interaction in NCC-Chs, critical to cell condensation of chondrogenesis, were up-regulated compared to those in NCs. In addition, the results showed that transcripts of molecules in the pathways controlling cholesterol metabolism, peroxisome proliferator–activated receptor signaling, and fat digestion and absorption in MC-Chs were increasingly expressed compared to those in NCs. In addition, the results showed that transcripts of molecules in the pathways controlling cholesterol metabolism, peroxisome proliferator–activated receptor signaling, and fat digestion and absorption in MC-Chs were increasingly expressed compared to those in NCs.
NCC-Chs (Fig. 4E), which were previously found related to the phenotype of growth plate chondrocytes (26–28). Together, the bioinformatic results indicate that both MC-Chs and NCC-Chs are phenotypically similar but not identical to NCs.

Transcripts of cartilage development–associated molecules are differentially expressed between MC-Chs or NCC-Chs and NCs

Cartilage development–associated genes (GO: 0051216) of the GO database were selected to further characterize the differentially expressed transcripts between MC-Chs, NCC-Chs, and NCs. The analysis showed that 34 and 29% of the cartilage development–associated transcripts were significantly regulated in MC-Chs and NCC-Chs, respectively, compared to those in NCs (Fig. 5, A and B), while 11% of the transcripts between MC-Chs and NCC-Chs were differentially expressed (Fig. 5C). Heatmaps of differential expression of the transcripts between MC-Chs, NCC-Chs, and NCs showed that many of the listed molecules were associated with chondrogenesis and ECM organization (Fig. 5, D to F). Furthermore, the analysis of functional protein association networks determining interactions of the differentially expressed molecules between MC-Chs, NCC-Chs, and NCs revealed that transcription factors and growth factors were the most interconnected molecules in the networks (Fig. 6). In particular, bone morphogenetic protein 2 (BMP2), BMP6, and fibroblast growth factor 2 (FGF2) were highly interactive in the networks of 54 transcripts down-regulated in MC-Chs, and BMP2, BMP6, FGF2, and growth and differentiation factor 5 (GDF5) in the networks of 45 transcripts down-regulated in NCC-Chs compared to those in NCs (Fig. 6, A and B). None of these growth factors was found in the association network of differentially expressed molecules between MC-Chs and NCC-Chs (Fig. 6C). These results indicate that chondrocytes derived from hiPSCs using the induction protocols described in Fig. 1A are close but not identical to phenotypic articular chondrocytes, suggesting that the protocols require further improvement to reduce the phenotypic differences between MC-Chs or NCC-Chs and NCs.

Chondrogenic induction with selected growth factors enhances differentiation of hyaline cartilage chondrocytes from MCs and NCCs

To test the hypothesis that the growth factors, down-regulated in MC-Chs or NCC-Chs compared to NCs (Fig. 6), are essential to the induction of phenotypic articular chondrocytes, we induced chondrogenic differentiation of MCs and NCCs using transforming growth factor beta 1 (TGFβ1)–based medium with or without a combination of the previously identified growth factors (Fig. 7A). After 21 days of chondrogenic differentiation, MC-Ch and NCC-Ch pellets of the treated groups gained a significant increase in volume (Fig. 7B), GAG content (Fig. 7C), and intensity of Alcian Blue staining (Fig. 7D) compared to those of the control groups. The result of mRNA expression revealed that the COL2A1 transcript in treated MC-Ch and NCC-Ch pellets was greater than that of treated MC-Ch ones (Fig. 7F). Results of immunofluorescence staining revealed that the COL2A1 content in treated MC-Ch and NCC-Ch pellets was comparable to that in control ones (Fig. 7G). Consistent with the mRNA expression results, COL10A1 and COL1A1 were significantly reduced in treated MC-Ch and NCC-Ch pellets, respectively, compared to that of control ones. It is worth noting that the increase in the expression of COL10A1 transcripts between treated and control NCC-Chs was not shown in the protein expression. Together, these results demonstrate that our modified protocols using the growth factors identified in this study promote chondrogenesis of MCs and NCCs toward the generation of hyaline cartilage chondrocytes.

Growth factor–treated NCC-Ch implants promote regeneration of articular cartilage at rat joints

MC-Chs and NCC-Chs induced by the selected growth factors were further evaluated for the capability of articular cartilage repair in rats. NCs serving as a relevant cell control were included here for comparison. Macroscopic examination showed that joint defects of all groups were filled with regenerated cartilage after 6 weeks (Fig. 8A), and comparable ICRS-I scores indicated no significant differences in visual assessment of the repair either between NCC-Chs without (NCC-Ch_C) and with (NCC-Ch_T) or between MC-Chs without (MC-Ch_C) and with (MC-Ch_T) the treatment of selected growth factors (Fig. 8B and fig. S5A). Human vimentin localization showed that the repaired cartilage of all groups consisted mainly of human cells (Fig. 8C). The H&E staining results demonstrated that the regenerated cartilage of both MC-Ch_T and NCC-Ch_T were highly integrated with the surrounding native cartilage and presented similar histological structures as that of the NC control (Fig. 8D). Furthermore, the regenerative cartilage of NC, MC-Ch_T, and NCC-Ch_T showed comparable intensities of Safranin O/Fast Green staining, which were noticeably greater than those of the MC-Ch_C and NCC-Ch_C ones (Fig. 8E). Following the ICRS-II scoring system to quantify the outcome of cartilage repair for comparison among all the groups, the results of histological assessment showed that the treated MC-Ch and NCC-Ch groups scored significantly higher in the categories of matrix staining, cell morphology, mid/deep assessment, and overall assessment than the control ones (Fig. 8F), indicating an enhancement in cartilage repair by cells of the treated groups. Notably, the NCC-Ch_T group showed significantly higher scores in several of the categories of repair assessment than the MC-Ch_T one (Fig. 8F and fig. S5B) and comparable scores to the NC control one. The regenerated cartilage of MC-Ch_T and NCC-Ch_T had greater surface and bulk stiffness, determined by nanoindentation and microindentation testing, respectively, than that of MC-Ch_C and NCC-Ch_C (Fig. 8G). Implanted pellets of NCC-Ch_T resulted in the generation of stiffer cartilage compared to those of MC-Ch_T. The immunofluorescence staining results showed that COL2A1 was a marker of fibrocartilage, was significantly reduced in NCC-Chs in response to the treatment whereas that in MC-Chs remained comparable between the control and treated groups. Markers of hypertrophic chondrocytes, COL10A1 and ALPL, were down-regulated in treated MC-Chs compared to those in control cells; in contrast, expression levels of these two markers in treated NCC-Chs were higher than those in the control. Mechanical testing by nanoindentation and atomic force microscopy (AFM) revealed that, induced by a combination of the growth factors, both MC-Ch and NCC-Ch pellets gained a significant increase in surface stiffness, and the stiffness of treated NCC-Ch pellets was greater than that of treated MC-Ch ones (Fig. 7F).
Fig. 5. Analysis of differentially expressed cartilage development–associated transcripts between different chondrocytes. (A to C) Pie charts depicting percentages of the up-regulated (Up), down-regulated (Down), and nonsignificant (NS) transcripts comparison between MC-Chs, NCC-Chs, and NCs. (D to F) Heatmaps of differentially expressed cartilage development–associated transcripts comparison between MC-Chs, NCC-Chs, and NCs. The color intensity of each grid denotes the extent of change in transcript expression, where up-regulated transcripts are shown in red and down-regulated ones are shown in turquoise. n = 3.
Fig. 6. Interactions of cartilage development–associated molecules differentially expressed in hiPSC-derived and NCs. Functional protein association networks established using the STRING database revealed known and predicted interactions of cartilage development–associated molecules differentially expressed between (A) MC-Chs and NCs, (B) NCC-Chs and NCs, and (C) MC-Chs and NCC-Chs.
Fig. 7. Chondrogenic induction of MCs and NCCs by identified growth factors.  

(A) Schematic of procedures and corresponding timelines for inducing differentiation from hiPSCs to chondrocytes.  

(B) Macrophages and size quantification of representative MC-Ch and NCC-Ch pellets of control and treated groups.  

(C) GAG quantification of MC-Ch and NCC-Ch pellets of control and treated groups.  

(D) Alcian Blue staining of MC-Ch and NCC-Ch pellets of control and treated groups.  

(E) Levels of mRNA expression of general cartilage–associated markers (SOX9, ACAN, COL1A1, and COL2A1), hyaline cartilage–associated markers (SIX1, THBS4, and ABI3BP), and hypertrophic–associated markers (COL10A1, CBFA1, and ALPL) in MC-Ch and NCC-Ch pellets.  

(F) Stiffness of control and treated MC-Ch and NCC-Ch pellets measured by atomic force microscopy (AFM).  

(G) Immunofluorescence staining of MC-Ch and NCC-Ch pellets of control and treated groups for detection of COL1A1, COL2A1, and COL10A1. Nuclear DNA was labeled with DAPI.  

n = 3. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars, 200 μm.
Fig. 8. Implantation of human MC-Ch and NCC-Ch pellets treated with identified growth factors in rat joints. (A) Repair of rat cartilage defect 6 weeks after pellet implantation. Black arrows point to the site of implantation. (B) ICRS-I scoring for visual assessment of repaired rat joints. (C) Immunofluorescence staining of implanted chondrocyte pellets for detection of human vimentin. Nuclear DNA was labeled with DAPI. (D) Regenerated cartilage analyzed by H&E staining. (E) Rat joint analyzed by Safranin O/Fast Green staining. The central and bottom rows are magnifications of the solid boxed (superficial) and dashed boxed (subchondral bone) regions in the top row. (F) ICRS-II scoring for histological assessment of regenerated cartilage. (G) Surface and bulk stiffness of regenerated cartilage measured by AFM and Mark-10 indentation testing, respectively. (H) Immunofluorescence staining of regenerated cartilage for detection of COL1A1, COL2A1, and COL10A1. Nuclear DNA was labeled with DAPI. n = 3. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars, 200 μm.
highly expressed in the regenerated cartilage of all nonsham groups (Fig. 8H). Both COL1A1 and COL10A1 were notably reduced in the treated groups compared to those in the control ones. The regenerated cartilage of NCC-Ch_T containing only COL2A1 without COL1A1 and COL10A1 represents the characteristic of hyaline cartilage. Together, these results suggest that NCC-derived chondrocytes induced by the growth factors identified in this study are capable of enhancing articular cartilage repair.

**DISCUSSION**

Research focusing on derivation of chondrocytes from iPSCs for cartilage regeneration has gained increasing attention in recent years, though it still remains challenging to obtain phenotypic articular chondrocytes (30, 31). Furthermore, to the best of our knowledge, the direct comparison in the phenotype and capability of cartilage repair between isogenic chondrocytes derived from the mesodermal and neural crest lineages has not been reported. In this study, the phenotype, chondrogenic capability, and transcriptome of isogenic MC-Chs and NCC-Chs differentiated from blood-derived hiPSCs along the mesodermal and ectomesodermal lineage, respectively, were characterized. We have found that while capable of producing cartilaginous matrix to generate cartilage-like tissue ectopically and at joint defects, both MC-Chs and NCC-Chs are transcriptionally similar but not identical to NCs. Our major finding is that NCC-Chs induced by a combination of growth factors identified in this study express greater levels of hyaline cartilage–associated markers and produce stiffer matrix with more GAG content than MC-Chs. Notably, NCC-Chs lack hypertrophic chondrocyte–associated markers. In addition, results of in vivo studies have further shown that a cartilage layer with proteoglycan-rich ECM and hyaline cartilage chondrocyte–like cells expressing COL2A1 without COL1A1 and COL10A1 is regenerated from implanted NCC-Chs at a joint defect. The outcome of reconstruction of articular cartilage by NCC-Ch implants is comparable to that by control NC implants, determined based on the results of similar ICRS assessment scores. This study demonstrates that hiPSC-derived NCC-Chs express phenotypic characteristics of hyaline cartilage chondrocytes and are capable of regenerating articular cartilage at a joint defect.

We derive MC-Chs and NCC-Chs from hiPSCs using previously published protocols with our modifications. While our findings are largely in agreement with those reported by other groups, there exist differences between their and our methods and results. To derive chondrocytes along the mesodermal lineage, we follow the protocol published by Kimber’s group (9) to induce hiPSCs into MCs. Instead of culturing MCs on a gelatin-coated two-dimensional (2D) surface with induction of FGF2 and GDF5, we pack the cells in a 3D cell pellet to increase cell-cell interaction and induce chondrogenesis with TGFβ1. The modified protocol is adopted to address concerns associated with the use of a 2D surface for chondrogenesis. A previous study has shown that chondrocytes generated from hiPSCs in 2D culture contain undifferentiated hiPSCs and implantation of those chondrocytes at a joint defect results in teratoma formation (13). The findings indicate that complete chondrogenesis is not achieved in 2D culture due to lack of cell condensation, critical for early chondrogenic induction and cartilage formation (32). In the current study, MC-Chs synthesize noticeable COL1A1 and COL10A1 in pellets (Figs. 1H and 2D), whereas the cell generated by Kimber’s group expresses negligible mRNA levels of the molecules (16). The discrepancy in the COL10A1 content of differentiated chondrocytes between these studies likely results from the effect of FGF2. As a previous study has shown that FGF2 is able to ameliorate chondrocyte hypertrophy (33), the induction of FGF2 during chondrogenesis of MC pellets seems critical to the prevention of COL10 production. In our experiment using a growth factor mixture containing FGF2 to increase the derivation of hyaline cartilage chondrocytes, the result showing a significant reduction of COL10 in treated MC-Chs confirms the speculation (Fig. 7, E and G). For derivation of NCC-Chs, we follow our previously reported protocol (21) to induce differentiation of hiPSCs along the ectomesodermal lineage. Chondrocytes derived from NCCs express abundant COL2 and GAG but little COL1 and no COL10 (Figs. 1H and 2D). The current finding is discrepant with previously reported results that hESC-derived NCs induced by platelet-derived growth factor and BMP4 increasingly express COL10 during chondrogenesis and that subcutaneous implantation of the cells leads to bone formation (34). Together, these results suggest that the culture environment is critical to the production of hyaline cartilage chondrocytes from PSCs, and we are able to generate mesoderm- and ectomesoderm-derived chondrocytes using the induction protocols performed in this study.

One of the key findings in this study is that 92 and 94% of the mRNA transcript of MC-Chs and NCC-Chs, respectively, show expression levels comparable to that of NCs, suggesting that the hiPSC-derived cells are similar but not identical to phenotypic articular chondrocytes. Specifically, our results indicate that the differentially expressed genes between MC-Chs and NCs are associated with epithelium development and blood vessel formation, whereas those between NCC-Chs and NCs are associated with neuron generation and nervous system development. Similar findings that stem cell–derived chondrocyte culture contains multiple cell populations have been reported by other groups. For example, using the approach of single-cell analysis, Dicks et al. (35) have found that at least nine different cell clusters—including chondrogenic, neurogenic, and other nonbone/noncartilage mesenchymal cells—are identified in hiPSC-derived mesodermal chondrocyte culture. Similarly, Umeda et al. (19) have demonstrated that the culture of ectomesodermal chondrocytes derived from hESCs and hiPSCs contains cell populations capable of differentiating into neural cells, melanocytes, and endothelial cells. These results indicate that it has remained challenging to derive a homogenous population of target cells from PSCs (36, 37). The approaches that have been applied to address the challenge include inhibiting the formation of undesired cell populations (38) and improving the efficiency of chondrogenesis (15, 39). In this study, we identify the growth factors down-regulated in hiPSC-derived chondrocytes compared to those in reference NCs and then add the identified molecules in culture during chondrogenic differentiation of MCs and NCCs to increase the generation of the chondrocyte population. The strategy leading to the use of other growth factors in addition to TGFβs to enhance chondrogenesis has previously been demonstrated in several studies (40–43), including one of which identifying the growth factor signaling critical to chondrogenesis through comparing the transcriptome of bone marrow MSCs with that of chondrocytes derived from these MSCs (43). Our study demonstrates a potential solution to the challenge of heterogeneity of PSC-derived cells.

The analysis of differential expression of cartilage development–associated genes reveals differences between hiPSC-derived and NCs, indicating that TGFβ alone is inadequate to induce complete chondrogenesis of hiPSC to generate hyaline cartilage. While TGFβ1 and
TGFβ3 are used as key growth factors for chondrogenic induction of stem cells, they have been shown to increase the production of hypertrophic chondrocytes with up-regulated COL10 (44, 45). It has also been demonstrated that TGFβs are involved in the induction of fibrocartilage chondrocytes (46, 47). We have found the similar response of hiPSC-derived chondrocytes to TGFβ1 as shown in Figs. 1H and 2D. However, with the supplementation of BMP2, BMP6, and FGF2 with or without GDF5 in TGFβ1-based induction medium, the phenotypes of MC-Chs and NCC-Chs are increasingly driven toward that of hyaline cartilage chondrocytes. Specifically, the increase in pellet size during chondrogenesis is likely resulted from the effect of FGF2. We and others have shown that FGF2 promotes cell proliferation and increases COL2 expression in chondrogenic cells (48, 49). BMPs are also critical to the regulation of chondrogenesis and the development of hyaline cartilage. For example, BMP2 enhances COL2 and aggrecan production in chondrocytes (20), BMP6 promotes chondrogenic induction and hyaline cartilage regeneration (50), and GDF5, also known as BMP14, increases the expression of SOX9, COL2, and ACAN and significantly reduces the expression of hypertrophic chondrocyte–associated markers (51). In addition to these effects, BMPs are capable of reducing fibrous tissue formation. For instance, BMP2 has been shown to ameliorate TGFβ-induced fibrosis in liver (52), and BMP6 has been shown to down-regulate COL1 expression to reduce fibrocartilage formation (50). These findings provide a plausible explanation to why the addition of BMPs reduces COL10 production by MC-Chs and COL1 production by NCC-Chs. Nevertheless, our results indicate that further optimization of the induction medium may be required for enhancing the phenotypes of hiPSC-derived chondrocytes. As shown in Fig. 7, E and G, there is discrepancy between the mRNA and protein results of hypertrophic chondrocyte–associated markers of NCC-Chs treated with and without a combination of the growth factors. Although the transcript result alone is inadequate to confirm hypertrophy of NCC-Chs, it still indicates the cell may be overinduced by suboptimal doses of the growth factors such that it undergoes the early stages of hypertrophy. To overcome this issue, our future studies will focus on optimizing induction doses and timing of the growth factors during chondrogenesis of NCCs and MCs to generate phenotypic hyaline cartilage chondrocytes.

Our current study uses articular chondrocytes as the reference cell to determine differences in the expression of transcriptome between hiPSC-derived and desired chondrocytes. We are aware that our approach to compare ectomesodermal NCC-Chs to mesodermal articular chondrocytes may be less than ideal for finding developmentally relevant molecules to enhance NCC chondrogenesis. However, considering that our purpose is to generate articular cartilage for the comparative evaluation of ectomesodermal lineage–derived cells for cartilage repair. The use of isogenic cells allows an accurate comparison without the variable of genetic differences. In addition, it is a fresh approach to analyze differences in the expression of global transcriptome between hiPSC-derived chondrocytes and reference NCs to identify growth factors for improving hyaline cartilage generation. We believe that our findings revealing the advantageous capability of NCC-Chs for articular cartilage repair are significant for filling the knowledge gap as to some degree they can provide an explanation of the biology behind the previous findings demonstrating articular cartilage repair by nasal chondrocytes (22, 53). With the robust evidence supporting the use of neural crest cells for hyaline cartilage regeneration, our findings provide the crucial information that may affect the choice of appropriate cell types for the repair of joint cartilage in future clinical applications.

MATERIALS AND METHODS

Culture of blood-derived hiPSCs

Three independent blood-derived hiPSC lines—JHU-62i, JHU-106i, and JHU-160i—derived from different donors were obtained from the WiCell Laboratory (Madison, WI, USA). These cell lines were cultured with Essential 8 (E8) medium in fibronectin-coated six-well plates and maintained in an incubator at 37°C in a humidified 5% CO2 atmosphere. Cells were stained with Fast blue RR salt (Sigma-Aldrich, St. Louis, MO, USA) to detect the localization of ALP. When reached about 80% confluence, cells were collected using Versene solution (Thermo Fisher Scientific, MA, USA) and replated at a split ratio of 1:6. The hiPSC lines at passage 12 were used in this study.

Generation of hiPSC-derived MCs and NCCs

To obtain distinct lineage-derived chondrocytes, individual hiPSC lines were induced into mesodermal and ectomesodermal lineages following previously published protocols by Ordershaw et al. (9) and our collaborative team (21), respectively. For MC induction, hiPSCs were seeded in fibronectin-coated (Thermo Fisher Scientific) six-well plates and induced by mesodermal differentiation basal medium [Dulbecco’s modified Eagle’s medium (DMEM)/F12, 2 mM l-glutamine, 1% nonessential amino acid, 1% Insulin–Transferrin–Selenium (ITS), 2% B27, and 90 μM beta-mercaptoethanol] composed
of a combination of chemicals with different dosages, as listed in table S4, dependent on induction stages. Briefly, in the initial 4-day period, hiPSCs were induced into mesendodermal cells by basal medium containing activin-A (10 to 50 ng/ml), wingless-type MMTV integration site family member 3A (WNT3A) (20 ng/ml), BMP2 (20 ng/ml), and BMP4 (40 ng/ml). In the next 4 days, mesendodermal cells were then induced into MCs with basal medium containing FGF2 (20 ng/ml), BMP4 (40 ng/ml), follistatin (100 ng/ml), and NT4 (2 ng/ml). For NCC induction, ectomesodermal NCCs were differentiated from hiPSCs by simultaneously inhibiting SMAD and activating WNT signaling (21). Briefly, hiPSCs seeded at a density of 67,500 cells/cm² were induced by differentiation medium containing Essential 6 (E6) medium, 5 mM SB431542, 10 mM dorsomorphin, heparin (30 mg/ml), 10 mM CHIR99021, and FGF2 (100 µg/ml) for 12 days. Cells were passaged using Accutase (STEMCELL Technologies, Vancouver, BC, Canada) when reaching about 90% confluence. To prepare NCCs, passaged cells were washed three times with ice-cold Dulbecco’s phosphate-buffered saline (D-PBS) washing buffer, treated with Neural Crest Stem Cell MicroBeads for 30 min at 4°C, and sorted by magnetic-activated cell sorting. The sorted NCCs were then seeded and expanded in growth factor–reduced Matrigel-coated (WisCell Laboratory) six-well plates for subsequent chondrogenic induction.

In vitro chondrogenic induction and assessment
To prepare mesoderm- and ectomesoderm–derived chondrocytes, MCs and NCCs were further induced for chondrogenesis following our previously published protocol (55). Briefly, 500,000 MCs or NCCs were collected and centrifuged at 600g for 5 min to form a high-cell density pellet before induced by chondrogenic medium containing high-glucose DMEM, 1% antibiotics, 1% ITS + Premix (Corning, Corning, NY, USA), 0.9 mM sodium pyruvate, 1-ascorbic acid-2-phosphate (50 mg/ml), l-proline, 0.1 µM dexamethasone, and TGFβ1 (10 ng/ml) (PeproTech, Rocky Hill, NJ, USA). Medium was changed every 2 days during the period of chondrogenic induction. To further enhance the derivation of phenotypic chondrocytes, MC and NCC pellets were induced by TGFβ1-based differentiation medium with BMP2 (10 ng/ml), BMP6, and FGF2 each. For chondrogenesis of NCC pellets, GDF5 (10 ng/ml) was additionally added. The chondrogenesis of cells by TGFβ1 alone was used as a control.

To analyze differences in the size of the generated cell pellets, the length (L), width (W), and height (H) of a 3D pellet were measured by a digital caliper and its volume (V) was calculated on the basis of the equation \( V = \pi/6 \times (L \times W \times H) \) commonly used to determine the size of an irregular-shaped biological specimen (56). For analysis of chondrogenic differentiation, chondrocyte pellets were fixed in 4% formaldehyde, dehydrated by a series of gradient ethanol, infiltrated with xylene, and then embedded in paraffin. Embedded cell pellets were cut into 8-µm sections using a microtome, deparaffinized, and stained with Alcian Blue (Polysciences, Warrington, PA, USA) for GAG detection. In addition, the total GAG content of cell pellets was quantified by the dimethylmethylene blue assay (Sigma-Aldrich) and normalized with the DNA content determined separately by the PicoGreen assay (Thermo Fisher Scientific) following the manufacturer’s instructions.

NCs harvested from human articular cartilage
Human articular cartilage as part of surgical waste obtained from three donors without osteoarthritis was provided by the University of Wisconsin Hospital and Clinics with approval from the Institutional Review Board. To isolate chondrocytes, cartilage was individually minced, washed twice with D-PBS, and incubated with pronase solution (1 mg/ml; Roche, Basel, Switzerland) for 30 min at 37°C and additionally with collagenase solution (1 mg/ml; Roche) for 12 to 18 hours at 37°C with agitation. The digestion solution was collected and centrifuged at 600g for 5 min to prepare uncultured NCs for RNA-seq analysis.

Flow cytometry analysis
Cells were suspended and washed three times with washing buffer composed of ice-cold D-PBS, 1% bovine serum albumin (BSA), 5 mM EDTA, and 25 mM Heps before antibody incubation. hiPSCs were incubated with the primary antibody goat anti-human OCT3/4 (R&D Systems, Minneapolis, MN, USA), mouse anti-human SOX2 (R&D Systems), goat anti-human NANOG (R&D Systems), mouse anti-human PODPCALYXIN (R&D Systems), mouse anti-human SSEA4 (R&D Systems), or mouse anti-human CD9 (R&D Systems) for 30 min at 4°C. After three subsequent washes with the buffer, cells were incubated with secondary antibody Alexa Fluor 546 donkey anti-mouse (Thermo Fisher Scientific) or fluorescein isothiocyanate (FITC) donkey anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA) for another 30 min at 4°C. For analysis of MC-Chs and NCC-Chs, cells were incubated with phycoerythrin–conjugated mouse anti-human CD44 or CD151 (BD) for 30 min at 4°C. Fluorescent cells were then detected by the Attune NxT Flow Cytometer (Life Technologies, Carlsbad, CA), and the resulting flow cytometry data were analyzed by FlowJo (Tree Star, Ashland, OR), following the manufacturer’s instructions.

Immunofluorescence analysis
hiPSCs were fixed with 4% formaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100 in D-PBS for 5 min at 4°C before incubation with the primary and secondary antibodies previously described in the method of flow cytometry analysis. Cell pellets harvested from chondrogenic culture or implants from animals were cut into 8-µm sections, deparaffinized, and unmasked with 0.1% (w/v) pepsin in 0.01 N HCl for 15 min, permeabilized with 0.1% Triton X-100 in D-PBS for 30 min, and blocked with 0.1% (w/v) BSA in D-PBS for 30 min. Slides with sectioned specimens were incubated with primary antibody goat anti-human COL1A1 (Santa Cruz Biotechnology), COL2A1 (Santa Cruz Biotechnology), COL10A1 (Santa Cruz Biotechnology), rabbit anti-human vimentin (Abcam, Cambridge, UK), or rabbit anti-mouse/rat VEGFR1 (Abcam) for an hour at 4°C. The slides were then washed three times with ice-cold D-PBS washing buffer, incubated with secondary antibody FITC donkey anti-goat (Santa Cruz Biotechnology) or Alexa Fluor 550 donkey anti-rabbit (Thermo Fisher Scientific) for 45 min at 4°C, and mounted with coverslips using mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector, Burlingame, CA). The dilution of antibodies for optimal concentration was set following individual manufacturer’s instructions. For fluorescence detection, specimens were observed under the Nikon A1R-s Confocal Microscope (Nikon, Tokyo, Japan).

Total RNA extraction and real-time qPCR
Total RNA was extracted from cells using the NucleoSpin RNA II Kit (Clontech Laboratories, Mountain View, CA, USA). The quality and quantity of total RNA were measured by Nanodrop 1000 (Thermo Fisher Scientific), followed by reverse transcription with the High-Capacity cDNA Reverse Transcription Kit for real-time qPCR. To
quantify the mRNA expression of markers of interest, cDNA was amplified by qPCR with IQSYBR Green Premix (Bio-Red, Hercules, CA, USA) and specific primers are listed in table S5. The level of mRNA expression was determined using the $2^{ΔΔCT}$ method with ubiquitin C as a housekeeping gene.

**Mechanical assessment of chondrocyte pellets and regenerated cartilage**

The surface stiffness of chondrocyte pellets was determined by nanoindentation testing using the Bruker Catalyst BioAFM Atomic Force Microscope. Following a published method (57), freshly harvested chondrocyte pellets were immobilized by 2% agarose gel (Benchmark, NY, USA) was used to perform microindentation testing to evaluate with a M5-012 Force Gauge (CSC Force Measurement, Copiague, NY, USA) attached. For the mechanical assessment of regenerated cartilage, rat distal femurs were harvested and the posterior portions of condyles were removed to create tissue of uniform thickness. The remaining tissue section consisting of the trochlear groove with implant was secured by a silicon nitride cantilever (spring constant $k = 0.06 \text{ N/m}$) with a 5-μm-diameter borosilicate glass bead (Novascan Technologies, Boone, IA, USA) attached. For the mechanical assessment of regenerated cartilage, rat distal femurs were harvested and the posterior portions of condyles were removed to create tissue of uniform thickness. The remaining tissue section consisting of the trochlear groove with implant was secured by a silicon nitride cantilever (spring constant $k = 1.00 \text{ N/m}$) with a 12-μm-diameter borosilicate glass bead (Novascan Technologies) attached. Five different regions of each testing specimen were probed at the indentation velocity of 5 μm/s and a trigger force of 10 to 50 nN to get an average value of the surface stiffness. In addition, the Mark-10 ESM030 Motorized Test Stand with a M5-012 Force Gauge (CSC Force Measurement, Copiague, NY, USA) was used to perform microindentation testing to evaluate the bulk stiffness of regenerated cartilage. Briefly, three regions of each specimen were measured using a 0.34-mm cylindrical steel probe at the indentation velocity of 0.5 mm/min. The Young’s modulus of a detected region with the Poisson ratio of 0.04 was calculated following a previously published method (58).

**In vivo evaluation of hiPSCs and derivatives**

In vivo studies were performed following the animal protocols approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. For the teratoma formation assay, 1 million hiPSCs in 0.1 ml of Matrigel were mixed with DMEM/F12 at the ratio of 1:1 and subcutaneously injected into the groin of SCID mice (NOD.Cg-Ptkdc<sup>−/−</sup>Prkdc<sup>scid</sup>Il2rg<sup>−/−</sup>Mm<sup>−/−</sup>IL2rg<sup>−/−</sup>/SzJ) (The Jackson Laboratory, Bar Harbor, ME, USA) between the ages of 11 and 12 weeks. The tissue was harvested 8 weeks after injection and prepared for histological analysis by H&E staining. The same strain of mice was also used for ectopic implantation of MC-Ch and NCC-Ch pellets. Briefly, a 10-mm incision was created for insertion of a single pellet into the mouse. After 30 days of subcutaneous implantation, pellets were collected and processed for immunohistochemical, H&E, and Safranin O/Fast Green staining.

To evaluate the capacity of hiPSC-derived chondrocytes for articular cartilage repair, 18 athymic nude rats (Hsd:RH-Foxn1<sup>−/−</sup>) (Envigo, Indianapolis, IN, USA) between the ages of 7 and 8 weeks were equally divided into 4- and 16-week treatment groups, with each further divided into the acellular fibrin glue control (n = 3), MC-Ch pellet (n = 3), and NCC-Ch pellet (n = 3) subgroups. A cylindrical defect of 1 mm in diameter and 2 mm in depth was created by a biopsy punch at the right trochlear groove of a rat for implantation, and the intact left joint of the animal was used as a contralateral control. Cell pellets were implanted and secured by fibrin glue at joints defects. In a separate in vivo study to evaluate the capability of hiPSC-derived chondrocytes enhanced by a combination of growth factors for joint repair, 24 athymic nude rats (Hsd:RH-Foxn1<sup>−/−</sup>) between the ages of 7 and 8 weeks were randomly divided into the sham (n = 4), MC-Ch pellet-control (n = 4), MC-Ch pellet-treated (n = 4), NCC-Ch pellet-control (n = 4), and NCC-Ch pellet-treated (n = 4) groups. Cylindrical defects on joint cartilage were created as described and then implanted with cell pellets, secured by fibrin glue. The animals were kept for 6 weeks before euthanasia for analysis.

Tissue specimens were harvested after animals were euthanized, fixed with 4% formaldehyde, decalcified in 22% neutral-buffered formic acid with end point as determined by radiography (Faxitron UltraFocus DXA, Tucson, AZ, USA) before paraffin embedding and sectioning for immunohistochemical and histological staining and microscopic analysis (Nikon, Japan). The visual and histological assessments of specimen images were performed to evaluate the repair of cartilage defects. Briefly, macrographs and micrographs of reconstructed cartilage were blindly scored by four independent reviewers following the ICRS-I (59) and ICRS-II (60) scoring system, respectively. For visual assessment, macrographs of femoral condyles were scored on the categories of integration to border zone, macroscopic appearance, and degree of cartilage repair. For histological assessment, micrographs of regenerated cartilage defects were scored on 12 different evaluation categories. Each score presented in the results was calculated as the mean of the four independent reviewers’ scores.

**RNA-seq assessment and data analysis**

NCs, MC-Chs, and NCC-Chs were collected, lysed in TRIzol (Life technologies), and stored at −80°C until extraction of total RNA using the Direct-zol RNA Kit (Zymo Research). The optical density value of total RNA was measured using the NanoDrop spectrophotometer (Thermo Fisher Scientific) to confirm the ratio of A<sub>260</sub> to A<sub>280</sub>. The RNA integration number was measured using the BioAnalyzer (Agilent Technologies) RNA 6000 Nano Kit. cDNA libraries were prepared using the SMART-Seq v4 Ultra Low Input RNA Kit for the Sequencing (TAKARA Bio) and Nextera XT DNA Library Prep Kit (Illumina). The size distribution and concentration of the final products were then evaluated using the BioAnalyzer High Sensitivity DNA Kit (Agilent Technologies). The libraries were pooled and diluted to 3 nM using 10 mM tris-HCl, (pH 8.5) and then denatured using the Illumina protocol. The denatured libraries were loaded onto an S1 flow cell on Illumina NovaSeq 6000 (Illumina) and ran for 2 × 50 cycles according to the manufacturer’s instructions. Demultiplexed sequencing reads were generated using Illumina bcl2fastq (released version 2.18.0.12), allowing no mismatches in the index read. Files containing RNA-seq raw sequences have been deposited in National Center for Biotechnology Information Gene Expression Omnibus under the accession number GSE152080.

BBDuk was used to trim/filter low quality sequences using the “-trimql=10” option. Next, alignment of the filtered reads to the human reference genome (GRCh38) was done using HISAT2 (version 2.1.0) applying “--no-mixed and --no-discard” options. Read counts were calculated using HTSeq by supplementing Ensembl gene annotation (GRCh38.78). Gene expression values were calculated as counts per million (CPM). Genes with no detected CPM in all samples were filtered out.
To identify the transcripts and associated expression levels, the genomic origin of sequenced cDNA fragments must be determined. Raw fastq files were preprocessed with the trimming software Skewer (61), and the trimmed single-end reads were aligned to the reference human genome using STAR (Spliced Transcripts Alignment to a Reference) (62). In addition, samples were normalized by the method of trimmed mean of M values (TMM) (63), and the analysis of differentially expressed genes was performed with a generalized linear model using the edgeR package (64). All genes with $P < 0.05$ and FDR $< 0.25$ were selected to conduct the test of KEGG analysis (www.kegg.jp), which is a collection of manually curated pathway maps representing hypotheses of the molecular interaction and action networks for many biological processes. A subset of differentially expressed genes with $P < 0.05$, FDR $< 0.25$, and $\log_2$ fold change greater or less than ±2 was selected and uploaded to STRING and TargetMine for further functional protein association network and gene set enrichment analysis, respectively. Both samples and genes are clustered using Euclidean distances. For genes, an additional elbow function is applied to estimate the number of gene clusters present. Calculated relationships are depicted by dendrograms drawn at the top (samples) and to the left (genes) of the heatmap. The graduation of color is determined by a $z$ score that is computed and scaled across rows of genes normalized by TMM.

**Statistical analysis**

Each assay was performed with samples of three biological replicates ($n = 3$). All quantitative data were calculated on the basis of results of biological replicates and presented as the means ± SD. Statistical comparison between groups was analyzed using Student’s $t$ test or one-way analysis of variance (ANOVA) with a post hoc Tukey’s test. A $P$ value of 0.05 was considered statistically significant.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/21/eabf0907/DC1

View/request a protocol for this paper from Bio-protocol.

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suppression of Aisenbrey, G.
Vemuri, M.
regenerating cartilages by engineered ASCs: Prolonged TGF-
and B.
Hu, Y.-H.
induction of growth factors influence chondrogenesis of 

Acknowledgments: We thank C. Ma, R. Weishar, T. Wang, B. Li, and M. Leiferman for assistance with the experimental preparations, and all researchers including E.-L. Hsieh and C. Ma.
Funding: This work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the NIH under award numbers R01 AR064803 (to W.-J.L.) and 5103844 (to E.V.S. and S.P.P.). M.J.S. was supported by NIH Biotechnology Training grant T32 GM008349. B.E.W. was supported by NIH National Institute on Aging (T32 AG00213-26), University of Wisconsin-Madison Institute of Clinical and Translational Research (CTS UL1TR000429 and CTS TL1TR000429). We also thank the University of Wisconsin-Madison Translational Research Initiatives in Pathology Laboratory, supported by the UW Department of Pathology and Laboratory Medicine and UWCCC (P30 CA14520); the Small Animal Imaging and Radiology Facility and Flow Cytometry Laboratory, supported by UWCCC (P30 CA14520) and NIH Equipment grant for Faxitron (1S10OD023676-01); the UW Materials Research Science and Engineering Center, supported by the National Science Foundation (DMR-1720415) for use of Equipment grant for Faxitron (1S10OD023676-01); the UW Materials Research Science and Engineering Center, supported by UWCCC (P30 CA14520) and NIH Equipment grant for Faxitron (1S10OD023676-01); the UW Materials Research Science and Engineering Center, supported by the National Science Foundation (DMR-1720415) for use of the facilities and services; and the Biotechnology Center's Bioinformatics Resource Center for the assistance with data analysis. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
Author contributions: M.-S.L. and W.-J.L. designed the experiments. M.-S.L., E.M.L., H.J., H.-C.H., and W.-J.L. contributed data. M.J.S. was supported by NIH Biotechnology Training grant T32 GM008349. B.E.W. was supported by NIH National Institute on Aging (T32 AG00213-26), University of Wisconsin-Madison Institute of Clinical and Translational Research (CTS UL1TR000429 and CTS TL1TR000429). We also thank the University of Wisconsin-Madison Translational Research Initiatives in Pathology Laboratory, supported by the UW Department of Pathology and Laboratory Medicine and UWCCC (P30 CA14520); the Small Animal Imaging and Radiology Facility and Flow Cytometry Laboratory, supported by UWCCC (P30 CA14520) and NIH Equipment grant for Faxitron (1S10OD023676-01); the UW Materials Research Science and Engineering Center, supported by the National Science Foundation (DMR-1720415) for use of the facilities and services; and the Biotechnology Center's Bioinformatics Resource Center for the assistance with data analysis. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Citation: M.-S. Lee, M. J. Stebbins, H. Jiao, H.-C. Huang, E. M. Leiferman, B. E. Walzack, S. P. Palecek, E. V. Shusta, W.-J. Li, Comparative evaluation of isogenic mesodermal and ectomesodermal chondrocytes from human iPSCs for cartilage regeneration. Sci. Adv. 7, eabf0907 (2021).
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Sci Adv 7 (21), eabf0907. DOI: 10.1126/sciadv.abf0907