Retinoid Signaling Controls the Developmental Fate of hPSC-derived Chondroprogenitors: Toward Articular-like Permanent Chondrocytes or Endochondral Ossification-prone Chondrocytes.

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INTRODUCTION: Chondrogenesis from human pluripotent stem cell (hPSC)-derived mesodermal cells serves as a valuable human model for cartilage development. These mesodermal cells can be expanded through the application of exogenous growth factors, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), along with inhibitors, SB431542 to counteract transforming growth factor-beta (TGF-β), and CHIR99021 to activate canonical WNT signaling [1-3]. However, changes in the medium composition result in two distinct types of chondrogenic mesenchymal cells: those expressing SOX9 ($SOX9^+$), and those expressing GDF5 ($GDF5^+$) in addition to tendon/ligament markers such as *SCX*, *MKX*, and *TNMD*. Interestingly, these two types of cells preferentially give rise to different types of chondrocytes: i.e., $SOX9^+$ cells to endochondral ossification-prone and $GDF5^+$ cells to endochondral ossification-resistant (i.e., permanent) chondrocytes [3]. Notably, cartilage pellets derived from $SOX9^+$ cells readily mineralize, while those from $GDF5^+$ cells remain unmineralized for up to 8 weeks *in vivo* [3]. Comparative transcriptome analyses reveal distinct endogenous signaling mechanisms are at play during development for each cell type (some are shown in Fig. 1). We hypothesize that some of the predicted endogenous signaling mechanisms influence the hPSC-derived mesodermal cells to acquire different chondrogenic activities in the $SOX9^+$ and $GDF5^+$ progeny.

METHODS: To test this hypothesis, we treated the mesodermal cells during proliferation and differentiation cultures toward $SOX9^+$ cells and $GDF5^+$ cells with activators and inhibitors of the predicted signaling mechanisms to assess their effects on the chondrogenic potential of $SOX9^+$ cells and $GDF5^+$ cells. First, hPSC lines were differentiated toward paraxial mesodermal progeny in a chemically-defined medium (CDM) as previously described [1-3]. Then, we isolated the mesodermal fraction by cell sorting [3, 4]. These mesodermal cells were maintained in CDM containing FGF2, PDGF, SB431542 and CHIR99021 and $SOX9^+$ mesenchymal cells were conventionally generated by removing PDGF [3]. To produce $GDF5^+$ mesenchymal cells, we transferred the mesodermal medium to CDM supplemented with PDGF and Noggin (a BMP inhibitor) [3]. Chondrogenesis was performed under the same condition for $SOX9^+$ cells and $GDF5^+$ cells, using PDGF, TGF-β3 and BMP4 as described previously [1-3], and *in vitro* generation of endochondral ossification-prone (hypertrophic) chondrocytes (i.e., [$COL2A1^+$] $COL10A1^+PRG4^{lo}$) and articular-like permanent (non-hypertrophic) chondrocytes (i.e., [$COL2A1^+$]$COL10A1^{lo}PRG4^+$) was demonstrated by RT-PCR analyses.

RESULTS: Cartilage pellets generated from $GDF5^+$ cells express *COL10A1* at lower (1-10%) levels, and *PRG4* at higher levels than those from $SOX9^+$ cells *in vitro* [3]. Previous research showed that IL-6 family cytokine and Activin/TGF-β signaling enhanced *GDF5* and *SCX* expression, respectively, during the $GDF5^+$ cell genesis, but they did not significantly affect $COL10A1^{lo}$ permanent chondrocyte formation from the developed $GDF5^+$ cells [5], suggesting that expression levels of *GDF5* and *SCX* are not proper predictors for their biological properties. Interestingly, however, treatment of mesoderm while expanded, with inhibitors of Aldehyde dehydrogenase 1A (ALDH1A) (673A and CM10) to reduce intracellular retinoid levels led to $GDF5^+$ cells differentiated from them which showed an improved capacity to generate $COL10A1^{lo/-}$cartilage. Inhibition of retinoic acid (RA) receptor (RAR) signaling by a RAR reverse agonist (BMS493), did not show the same effects (Fig. 2). In contrast, treatment of mesoderm while expanded or during the genesis culture for $SOX9^+$ cells with retinoid analog (EC23) to enhance intracellular RA signaling resulted in cells showing improved capacity to form $COL10A1^{+/hi}$ cartilage. However, pan-RAR agonist (TTNPB) appeared to show only a weak effect (Fig. 2).

DISCUSSION: While our previous RNA-seq analyses of the $SOX9^+$ and $GDF5^+$ cells [3] predicted many potential endogenous signaling mechanisms, the most significant thus far is activation of BMP7 plus NRG1 signaling (data not shown) or retinoid signaling that enhances the $COL10A1^+PRG4^{lo/-}$ chondrocyte forming activity in mesodermal cells. In addition, inhibition/reduction of intracellular RA signaling in mesodermal cells are sufficient to improve the capacity of $GDF5^+$ cells differentiated from such cells to form $COL10A1^{lo}PRG4^+$ primitive articular-like chondrocytes (Fig. 3). Thus, a treatment of chondrogenic mesenchymal progenitor cells prior to chondrogenic differentiation seems to have a sustained effect on the biological properties of their differentiated progeny, chondrocytes. It is tempting to speculate that key difference between the limb mesenchyme that gives rise to growth plate cartilage and the joint progenitor cells responsible for articular cartilage formation may simply be the level of intracellular RA.

SIGNIFICANCE/CLINICAL RELEVANCE: These results support our hypothesis that the fate of chondrocytes originating from chondrogenic mesenchymal cells can be predetermined during the mesenchymal cell stage, which seems to depend on how the mesenchymal cells are influenced during their maintenance/expansion cultures. Further biological studies will have profound implications for enhancing the potential of hPSC-derived mesenchymal chondroprogenitors in cartilage regenerative therapy. Moreover, these insights might extend to improving cell-based cartilage therapy involving adult chondrogenic cells, such as bone marrow mesenchymal stromal cells, by applying similar treatments to improve therapeutic outcomes.

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Fig. 1. *Distribution of IL-6 family cytokine signaling genes (pink) and RA signaling genes (blue).*

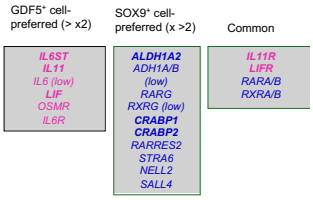

Fig. 2. *Retinoid effects on hPSC-derived mesodermal cell's potential to give rise to COL10Ai^{high} and COL10A1^{low} cartilage.*

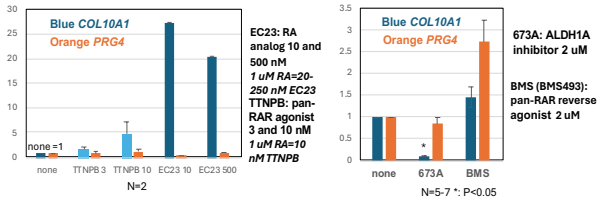

Fig. 3. *Working model for development of SOX9$^+$ and GDF5$^+$ cells.*