Interaction between ERK and Retinoic Acid Signaling in the Regulation of *COL10A1*-expressing Cartilage-forming Potential of hPSC-derived Chondroprogenitors

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INTRODUCTION: Chondrogenesis from human pluripotent stem cell (hPSC)-derived mesoderm provides a valuable human model for growth plate as well as articular cartilage development. The mesodermal cells can be expanded using exogenous growth factors such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), along with pharmacological agents including SB43154, which inhibits transforming growth factor-beta (TGF-β) signaling, and CHIR99021 that mimics canonical WNT signaling [1-3]. However, changes in medium composition give rise to two distinct types of chondrogenic mesenchymal cells: SOX9$^+$ cells and GDF5$^+$ cells. In the presence of TGF-β3 and BMP4, SOX9$^+$ cells preferentially differentiate into *COL10A1*$^+$*PRG4*$^-$ chondrocytes, whereas GDF5$^+$ cells form *COL10A1*^{lo}*PRG4*$^+$ chondrocytes [3]. Notably, cartilage pellets derived from SOX9$^+$ cells readily mineralize *in vivo*, consistent with endochondral ossification, whereas GDF5$^+$cell-derived pellets remain unmineralized for up to 8 weeks, indicative of stable cartilage [3]. We previously reported at ORS (#1896) that endogenous BMP signaling enhances the ability of SOX9$^+$ cells to form *COL10A1*$^+$ chondrocytes, whereas inhibition of aldehyde dehydrogenase 1A (ALDH1A), the enzyme responsible for retinoic acid (RA) synthesis, increases their potential to form *COL10A1*^{lo} chondrocytes. A defining characteristic of GDF5$^+$ cells is their slow growth. To explore the underlying mechanisms, we examined the potential role of the extracellular signal–regulated kinase (ERK) pathway, which is commonly stimulated by growth factors such as FGF. ERK regulates diverse cellular processes, including cell-cycle progression, and recent studies indicate that it can also be activated by non-genomic RA signaling. Here, we focused on elucidating whether and how ERK activation, potentially induced by FGF and coordinated with RA signaling, influences the developmental potential of SOX9$^+$ cells to generate *COL10A1*$^+$ cartilage.

METHODS: SOX9$^+$ cells derived from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) were treated with the ERK inhibitor GDC0994, all-trans RA (atRA), and the pan-retinoic acid receptor (RAR) reverse agonist BMS493—which inhibits transcriptional [i.e., genomic] activities of RARs—to assess effects on chondrogenic properties of SOX9$^+$ 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]. Mesodermal cells were maintained in CDM containing FGF2, PDGF, SB431542 and CHIR99021 (FPSbC). The mesodermal progeny was then cultured under FSbC (FPSbC minus PDGF) conditions to generate SOX9$^+$ cells [3] and treated with 1 μM GDC or 30-100 nM atRA for 8 days. Treated cells were subjected to cartilage pellet culture. Pellet chondrogenesis was induced using PDGF, TGF-β3 and BMP4 as described previously [1-3], and expressions of *COL2A1* and *COL10A1* was assessed by RT-PCR (Fig. 1).
RESULTS: Treatment of SOX9$^+$ cells with either 1 μM GDC or 30-100 nM atRA reduced *COL10A1* expression in the developed cartilage pellets compared with untreated controls. GDC also reduced the growth rate of SOX9$^+$ cells, as expected. However, when GDC and atRA were applied simultaneously, the suppressive effect of GDC on *COL10A1*$^+$ chondrocyte formation was attenuated, and *COL10A1* expression was instead elevated in the cartilage pellets (Figs. 2, 3).
DISCUSSION: Our results indicate that ERK and RA signaling interact to regulate the chondrogenic fate of SOX9$^+$ cells. Treatment with either an ERK inhibitor or atRA alone suppressed *COL10A1* expression in subsequent cartilage pellets (Figs. 2,3); however, their combined application unexpectedly increased *COL10A1* expression, and these effects were maintained even in the presence of 100 ng/mL Noggin, a BMP inhibitor (data not shown), while we have previously demonstrated that BMP preconditioning of SOX9$^+$ cells promote their ability to generate *COL10A1*$^+$ chondrocytes (ORS #1896). This suggests that the opposing actions of atRA and ERK inhibition on the chondrogenic fate determination occur independently of BMP signaling. Interestingly, the effect of atRA contrasts with our previous finding that inhibition of ALDH1A by 673A (which reduces intracellular RA levels) suppressed the *COL10A1*$^+$ cartilage-forming potential in SOX9$^+$ cells. At present, the precise contributions of ERK and RA pathways to the acquisition of *COL10A1*$^+$ cartilage-forming potential remain unclear. Ongoing studies aim to further elucidate the mechanistic basis of *COL10A1*$^+$ versus *COL10A1*^{lo} chondrogenic fate determination in both SOX9$^+$ and GDF5$^+$ cells.

SIGNIFICANCE/CLINICAL RELEVANCE: Controlling chondrocyte hypertrophy within regenerated cartilage is critical for the clinical success of cell-based cartilage therapies. Therefore, elucidating the molecular mechanisms governing chondrogenic fate determination in hPSC-derived SOX9$^+$ and GDF5$^+$ chondroprogenitors may inform strategies to regulate the differentiation potential of therapeutically relevant adult chondrogenic stem/progenitor cells, such as mesenchymal stromal cells (MSCs). Our results support the hypothesis that modulation of ERK and RA signaling pathways in hPSC-derived SOX9$^+$ cells influence their propensity to generate either *COL10A1*$^+$ or *COL10A1*^{lo} chondrocytes in the presence of TGF-β3 and BMP4. Further mechanistic investigations will be essential not only to enhance the therapeutic utility of hPSC-derived mesenchymal chondroprogenitors in cartilage regeneration, but also to improve the effectiveness of MSC-based cartilage repair.

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Fig. 1. *Our experimental strategy and future direction*

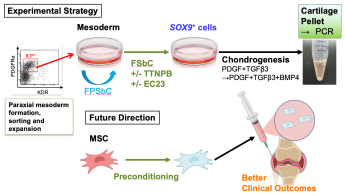

Fig. 2. *COL10A1 expression in cartilage pellets generated from hESC-derived mesodermal cells with and without factors.*

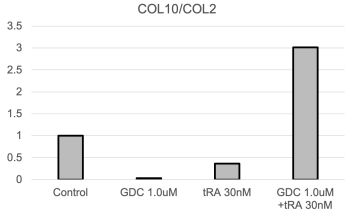

Fig. 3. *COL10A1 expression in cartilage pellets generated from hiPSC-derived mesodermal cells with and without factors.*