Investigating the Effect of Synthetic Extracellular Matrix Stiffness on Osteogenic Differentiation of iPSC-derived Mesenchymal Stem Cells

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INTRODUCTION: Induced pluripotent stem cells (iPSCs) are generated through the genetic reprogramming of somatic cells through ectopic expression of pluripotency factors. iPSCs can perpetually self-renew in culture and exhibit multi-differentiation potential. Their ability to specialize into distinct cell types holds tremendous potential for bone tissue engineering and the broader field of regenerative medicine. The differentiation process of iPS-derived mesenchymal stem cells toward specific lineages is guided by several biophysical and chemical cues present within the extracellular matrix (ECM), including stiffness, composition, and mechanical characteristics. Notably, increasing mechanical stiffness of the ECM has emerged as a significant regulatory factor in the osteogenic differentiation of MSCs, facilitating the process of bone formation [1]. In this context, our study investigates the effect of varying the synthetic ECM stiffness on guiding osteogenic differentiation of iPS-derived MSCs within a 3D *in vitro* environment.

METHODS: 3D culture: iPSCs were cultured and seeded at a density of 4.6 x 10^5 in GelMa scaffolds of 7.5, 10, or 15%, with increasing GelMa concentration corresponding to increased stiffness [2]. Scaffolds were cultured for 28 days in Advanced Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum, 100 nM dexamethasone, 10 uM B-glycerolphosphate, 50 uM ascorbic acid, 1% anti-anti (100 U penicillin, 100 ug streptomycin, 0.25 ug amphotericin), 100 ng/mL BMP2, and 100 uM vitamin D to induce osteogenic differentiation. **RT-qPCR:** Osteogenic markers of Osteopontin (SPP1) and Osteonectin (SPARC) mRNA expressions were quantified by RT-PCR, normalized against the housekeeping gene of GAPDH, and analyzed using the delta-delta CT method. **Alizarin Red Staining**: The scaffolds were fixed with 4% PFA in PBS for 6 hours and dehydrated with 15% sucrose, followed by 30% sucrose overnight. To analyze matrix mineralization, 10 uM sections of the scaffolds were stained with Alizarin red (AR), imaged on a bright field, and converted to a binary grayscale via ImageJ. A threshold was set to illuminate only the stained regions, and then the areas of calcium mineralization were measured. **Alkaline phosphatase staining**: Slide sections were incubated with the Alkaline Phosphatase (ALP) staining mixture (ThermoFisher NBT/BCIP Substrate Solution) at 4º overnight. After staining, the areas of mineralization were measured using ImageJ. **H&E Staining:** Slide sections were also stained with hematoxylin and eosin to visualize the cell morphology and distribution.

RESULTS: SPP1 and SPARC are well-established gene markers of osteogenesis due to their roles in regulating bone mineralization processes, selective expression by osteogenic cells, and functions in mediating cell-matrix interactions crucial for bone remodeling and formation. As depicted in Figure 1**,** RT-qPCR analysis revealed statistically significant increases in SPP1 expression under the 10% and 15% conditions compared to the 7.5% condition, along with a statistically significant increase in SPARC expression in the 15% condition. Upregulation of SPP1 and SPARC in the scaffold with higher GelMa concentration suggests enhanced osteogenic differentiation in stiffer extracellular matrices. AR and ALP staining assays enable quantification of osteogenesis, as AR measurement enables visualization of mineralized matrix deposition, while ALP measurements provide an indication of early osteoblastic differentiation and bone matrix formation. Similar to the RT-qPCR, AR, and ALP staining results (Figure 2) suggest greater matrix mineralization and osteogenic differentiation in the 15% GelMa condition compared to the 7.5% condition.

DISCUSSION: Previous studies have reported the role of extracellular matrix stiffness in directing osteogenic differentiation of MSC cells; however, the potential of iPSCs in this regard remains an understudied area. The multi-differentiation potential of patient-specific iPSCs provides a potential platform to develop personalized bone regeneration and treatment of bone injury and disease [3]. We constructed a 3D system that replicates a more authentic, applicative environment compared to a 2D cell culture condition to investigate the feasibility of synthetic EMCs stiffness in facilitating osteogenic differentiation of iPS-derived MSCs. Our 3D system demonstrates the concentration-dependent impact of GelMa on the osteogenic differentiation of iPSC-derived MSCs, with higher GelMa concentration, and thus stiffer ECMs favoring osteogenic lineage. This research underscores the potential for refining bone regenerative strategies using iPSC-based approaches.

SIGNIFICANCE/CLINICAL RELEVANCE: Investigating iPSC osteogenesis can provide insight into strategies to promote bone formation and bone regeneration in difficult-to-heal clinical situations, e.g., non-unions. Furthermore, 3D scaffolds encapsulating iPSCs allow for investigating osteogenesis in a physiologically relevant *in vitro* model.

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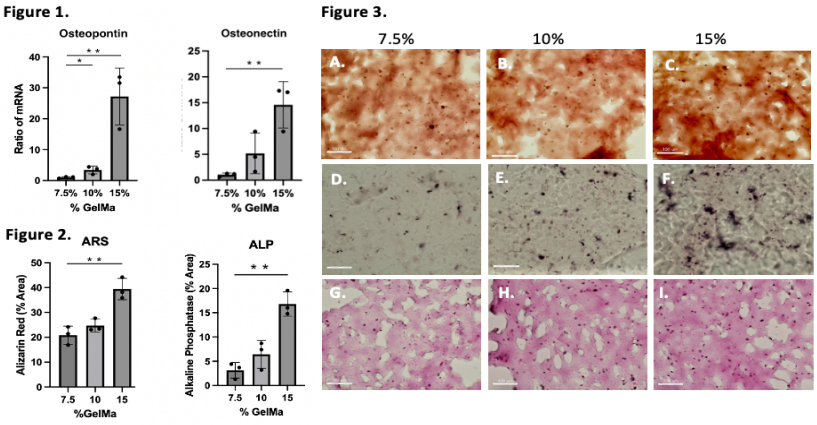

Figure 1. Real-time qPCR was performed to analyze the expression of Osteopontin (SPP1) and Osteonectin (SPARC). SPP1 and SPARC genes were upregulated with increasing matrix stiffness. **Figure 2.** Alizarin Red (ARS) and alkaline phosphatase (ALP) staining indicate greater matrix mineralization with stiffer matrices. **Figure 3.** Images of ARS (A-C), ALP (D-F), and H&E Staining (G-I). All scale bars are 100 um. P < 0.05* and P < 0.01** were considered statistically significant.