MECHANOBIOLOGICAL REGULATION OF HUMAN BONE MARROW STROMAL CELL DIFFERENTIATION

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INTRODUCTION

Mechanical force is a known epigenetic modulator of morphogenesis. From embryonic development to adult wound healing, mechanical forces modulate pluripotential cell activity and tissue growth and function. A significant number of studies have investigated force effects on tissue growth *in vivo* and on differentiated cells *in vitro*. However, force effects on mesenchymal stem cells, the cells that provide a progenitor cell pool for tissue repair and those that give rise to connective tissue, bone, cartilage, fat and muscle during development, are less defined. Bone marrow stromal cells (MSC) are composed of a heterogeneous cell population containing mesenchymal stem cells. Because of their multi-lineage potential and relative abundance, MSC may be valuable for clinical and regenerative therapies. Treatments under investigation manipulate cell and tissue growth via culture conditions, including biofactors delivery, scaffold matrix properties, and the mechanical environment. The purpose of this work was to elucidate how controlled perturbations in cell substrate strain alter human MSC (HMSC) differentiation down chondrogenic vs. osteogenic pathways *in vitro* and to investigate the interaction of mechanical strain with biologic factors, particularly cell substrate coating and ascorbate supplement. We hypothesize that:

- The magnitude of cell culture substrate strain has a differential effect on HMSC differentiation, where high (10%) cell stain will lead to a more fibroblastic phenotype while low (1%) strain will increase osteogenic differentiation in vitro.
- The cell substrate ligand alters the differentiation response of HMSCs to substrate deformation in vitro. Considering that fibronectin is a major ECM component in mesenchymal condensations and expression correlated with prechondrocyte patterning, a postulate is that HMSC will favor chondrogenic differentiation if cultured on fibronectin rather than a heterogeneous protein coated substrate.
- Ascorbic acid alters the differentiation response of HMSC to substrate ligand and substrate deformation in vitro. A postulate is that L-ascorbate supplement will drive HMSC to osteoblastic differentiation independent of substrate ligand via collagen type I (COL1) synthesis, presumably through COL1-integrin signaling.

METHODS

Cell Culture: HMSC were isolated from human iliac crest biopsies with IRB approval. Tissue culture polystyrene (PS) adherent passage 2 cells were seeded at 20,000 cells/cm^2 on Bioflex (Flexcell International) culture dishes (wells with flexible silicone bottoms). Serum containing media was used throughout passages and loading.

Cell Substrate Modification: FN: Fibronectin was absorbed overnight using 2 ml per well of a 10 ug/ml bovine plasma fibronectin - 0.1 M bicarbonate buffer. UV: Dishes were placed for 10 hrs under a tissue culture hood ultraviolet lamp. UV irradiation approximates the plasma treatment of commercial PS. Null: No modification control.

Cell Loading: Cells were cyclically stretched at 1 Hz for 60 min. a day at 1% or 10% strain (max strain at dish center). Experiments were performed in triplicate using cells from 3 different patients. A custom experimental system was developed to deform Bioflex dishes and uniformly coat them with cell ligands. The system provides smooth substrate deformation from low (<1%) to high (10%) strains. Loading was applied with and without ascorbate supplement (L-ascorbic acid phosphate magnesium salt at 100 uM).

RT-PCR Analysis: Cells were loaded (stretched) for 2, 5, or 9 days and total RNA extracted with Invitrogen TRIZOL. RNA was DNase treated with Qiagen RNeasy kit, RT with Applied Biosystems (ABI) N808-0234 kit, and real time PCR performed on an ABI PRISM 7700 using SYBR Green. Early and late markers of osteogenic and chondrogenic differentiation were normalized to 18S rRNA, including Osf2/Cbfa1/Runx2 (Runx2), Sox9, Collagen Type I alpha 1 (Col1), Alkaline Phosphatase (AP), Osteonectin (ON), and Aggrecan (AG). Fold changes in gene expression were calculated relative to the FN / 0% strain / no ascorbate supplement specimen (FN- 0% strain) and significant differences were determined using a generalized linear model and appropriate post-hoc tests.

AP and Mineralization Assays: Cells were cultured 1 week without load and then loaded for 3 weeks in osteogenic supplemented media (ascorbate, 0.01 uM dexamethosone, and 5 mM beta-glycerol phosphate). AP staining was performed with napthol + Fast Red Violet in DMF. Mineralization was identified with the Von Kossa method.

RESULTS

Multiple interaction effects were observed between loading, ascorbate, and substrate coating on gene expression profiles across all time points. In the specimen legend, Figure 1, + indicates with ascorbate supplement, - without. Only data for Runx2, osteonectin (ON), and aggrecan (AG) are discussed. Coating has the strongest effect on Runx2 levels (p=0.16) compared with load and ascorbate. At early time points, Runx2 increases in response to load with ascorbate supplement. But Runx2 does not appear to be significantly regulated by load as no patterns emerge across coatings and time points. Ascorbate increases Runx2 in all coats at 2 and 5 days. Coating has the most significant effect on ON levels (p=0.016, Figure 2). Loading also has a significant effect on expression across different coatings

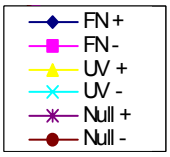

Figure 1

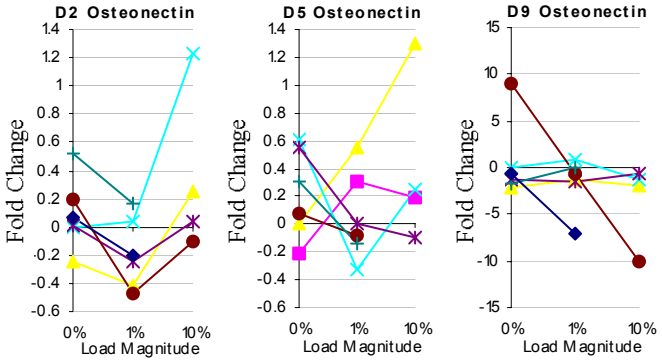

Figure 2, Osteonectin Expression Patten

(p=0.02). At 9 days, it is apparent that load increases ON earlier in the absence of ascorbate. Despite the fact that ascorbate is a supplement of osteogenic media, it lead to decreased ON transcript in the FN group. Though ascorbate is required for appropriate matrix synthesis, it does not appear to drive osteogenesis as evidenced by ON transcript (ON is expressed prior to osteocalcin in unmineralized osteoid) (Hypothesis 3). FN increased AG expression relative to other coats only at 2 days (Figure 3). Similar AG levels were observed in UV and FN coats by 5 and 9 days. However, the Null coat displayed higher AG expression at 5 and 9 days, coincidental with the cells grouping into multilayered islands, or nodules (Figure 4). This supports a role of for cell shape

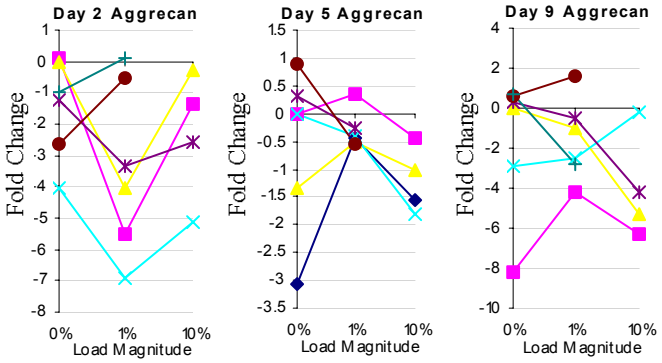

Figure 3, Aggrecan Expression Pattern

and/or oxygen tension in chondrogenic differentiation. Without supplement, loading decreases AG expression in a magnitude dependent manner at 5 and 9 days. However with ascorbate, the effect of loading was bimodal with respect to magnitude. 1 % strain increased AG relative to 0% and 10% at 5 and 9 days. Interestingly at 2 days, 1% strain caused the greatest decrease in AG expression in UV and FN dishes. FN does not appear to drive chondrogenesis in this 2D culture system (Hypothesis 2).

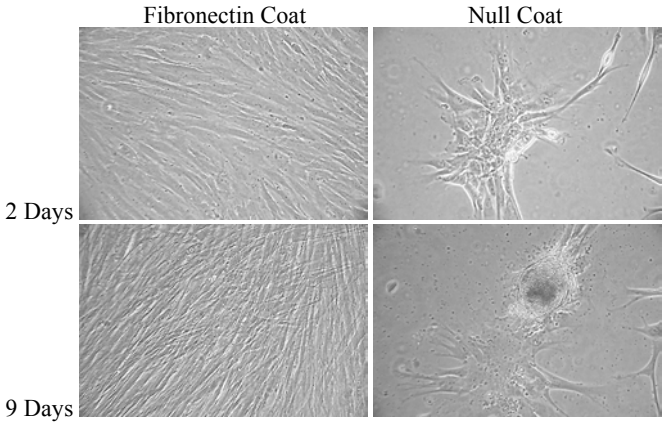

Figure 4. Cell Morphology under 1% strain

The osteogenic media inhibited cell layer detachment throughout 4 weeks of culture (Figure 5). Loaded dishes displayed stronger Von Kossa staining. The mineralization pattern depended on the dish coating. Organization of the cells into dense swirl patterns is evident in the UV groups. Null dishes also displayed staining (not shown).

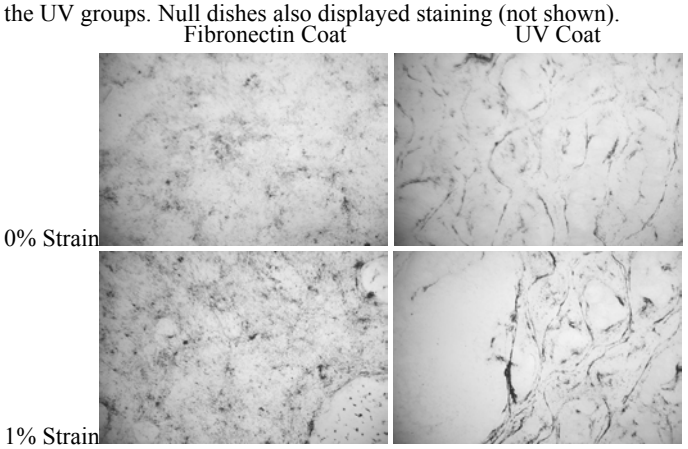

Figure 5, Mineralized Matrix at 4 weeks

DISCUSSION

10% strain appeared more osteogenic than 1% strain in FN groups, and FN more so than UV irradiation, as evidenced by Runx2 and ON expression. Under ascorbate supplement, 1% strain was more permissive for chondrogenic gene expression compared with 0% and 10% strain (Hypothesis 1). Loading and ascorbate, but not substrate, decreased AG transcript, a marker of chondrogenesis. Ascorbate exhibited a significant interaction with both substrate coating and load magnitude (Hypothesis 3). In summary, this 2D system is permissive for osteogenic and not chondrogenic differentiation. Though strain levels can modulate MSC differentiation, the ability to do so strongly depends on biologic factors like matrix ligands and ascorbate.

ACKNOWLEDGEMENTS

Research supported by NIH through The University of Michigan Center for Organogenesis Fellowship to JMT (HD07505), AR48414 and DE13835 to PHK, and DE13416 and DE13608 to SJH. Thanks to Michael Ominsky, Taocong Jin, Dennis Kayner, and Charles Roehm.