Photocrosslinkable Hydrogels with Stiffness Gradients to Interrogate MSC Chondrogenic Differentiation

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INTRODUCTION: Oxidized methacrylated alginate (OMA) can be used to form photocrosslinkable hydrogels with tunable physical and cell adhesive properties for regenerative medicine applications. Previous studies encapsulated human bone marrow-derived mesenchymal stem cells (MSCs) in OMA hydrogels to investigate cytocompatibility, cell adhesivity, spreading, and proliferation. However, there has been limited investigation on the combined influence of controlled adhesivity and stiffness for chondrogenic differentiation. Here, we aimed to 1) establish the role of RGD presentation (to provide adhesion ligands) on encapsulated MSCs in OMA gels and 2) interrogate the effects of chondrogenic differentiation on MSC mechanosensing of a continuous stiffness gradient. The role of RGD modification in chondrogenesis was determined in encapsulated MSCs in bulk OMA gels. We then formulated OMA hydrogels using a grayscale photomask to generate hydrogels possessing a continuous stiffness gradient. We further examined the role of mechanosensing in our gradient hydrogels using an actin polymerization inhibitor. Collectively, these results establish a new approach for exploring the interplay of substrate stiffness and adhesion on MSC mechanosensing and chondrogenic differentiation.

METHODS: Human bone marrow-derived MSCs (RoosterBio) were suspended in 8% w/v 2OX20MA (2% oxidation, 20% theoretical methacrylation) at 5 x 10^6 cells/mL and pipetted into 8 mm square molds. Prior to crosslinking, we placed a grayscale photomask gradient on top of the 8 mm square molds. Gels were crosslinked under UV light (20 mW/cm^2 (320–500 nm, Omnicure S2000)) for 2 min. Bulk gels were formed similarly without the photomask. To induce chondrogenic differentiation, the constructs were cultured in chondrogenic induction media for 21 days at 37ºC with 5% CO_2. The elastic moduli of photomask gels were determined across the gradient using a microindenter.

To measure chondrogenic gene expression, samples were collected in TRIzol Reagent (Invitrogen) for PCR analysis according to the manufacturer's instructions. Following RNA isolation, 800 ng of RNA was reverse transcribed with the QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA) and we ran qPCR using the QuantiFast Probe PCR Kit (QIAGEN) on a QuantStudio 6 system (Applied Biosystems). Primers and probes for housekeeping genes *GAPDH* (Hs_02786624_g1), *Col10A1* (Hs_00166657_m1) were purchased from Thermo Fisher Scientific. Amplification conditions were 95°C for 3 min, followed by 45 cycles at 95°C for 3 s and 60°C for 30 s. The qPCR results were normalized to *GAPDH* transcript levels to yield ΔC_t and then to the same starting number of D0 MSCs to yield $2^{-\Delta\Delta Ct}$.

To determine the role of stiffness on MSC mechanosensing, encapsulated MSCs in bulk and stiffness gradient gels were treated with 0.1 µM Latrunculin A (LatA), an actin polymerization inhibitor. Following 21 days in chondrogenic medium and LatA treatment, the samples were fixed in 4% paraformaldehyde for 1 hour and stored in phosphate-buffered saline until tissue processing. The samples were processed, embedded in paraffin, and sectioned at 5 µm. To qualitatively assess chondrogenesis, samples were stained using Safranin-O/Fast Green for glycosaminoglycan (GAG) using standard protocols.

All experiments were performed with a minimum of three independent replicates and compared using mean and standard deviation. Statistical analysis was performed using GraphPad Prism *via* one-way ANOVA with multiple comparisons test. A p-value of less than 0.05 was considered statistically significant, which is denoted by different letters.

RESULTS: We quantified the expression of the chondrogenic differentiation and cartilage formation marker *COL10A1* (encoding for collagen X) to determine the influence of RGD modification on chondrogenesis. Encapsulated MSCs in RGD-modified 2OX20MA hydrogels exhibited significantly higher relative expression of the gene encoding for collagen X than unmodified hydrogels, emphasizing the importance of cell adhesion to the material (n=3, p<0.05) (**Fig. 1A**). We fabricated a novel greyscale photomask to create a stiffness gradient from 3 kPa to 15 kPa, moduli previously reported to promote MSC chondrogenic differentiation. Following 7 days in culture, the elastic moduli of the acellular hydrogels decreased, suggesting degradation by hydrolysis (n=3) (**Fig. 1B**). We assessed the role of stiffness on MSC mechanosensing using Safranin-O/Fast Green for visualization of GAG. The stiffness gradient gels exhibited decreasing GAG presence in concordance with initial stiffness. Groups treated with LatA exhibited similar GAG levels to the untreated bulk control gels, suggesting that MSCs were unable to mechanically sense the stiffness gradient (n=3) **(Fig. 1C).**

DISCUSSION: These data demonstrate that chondrogenic differentiation of MSCs can be modulated by RGD modification and exposure to mechanical stiffness. This research provides insight on the underlying mechanisms of MSC behavior and differentiation, which are critical for tissue engineering.

SIGNIFICANCE/CLINICAL RELEVANCE: This study aimed to create a continuous stiffness gradient that is physiologically relevant to investigate chondrogenic differentiation of MSCs and the role of stiffness on MSC mechanosensing. The photomask platform is useful to generate biomaterials with continuous gradients that may be more indicative of native tissues.

IMAGES AND TABLES:

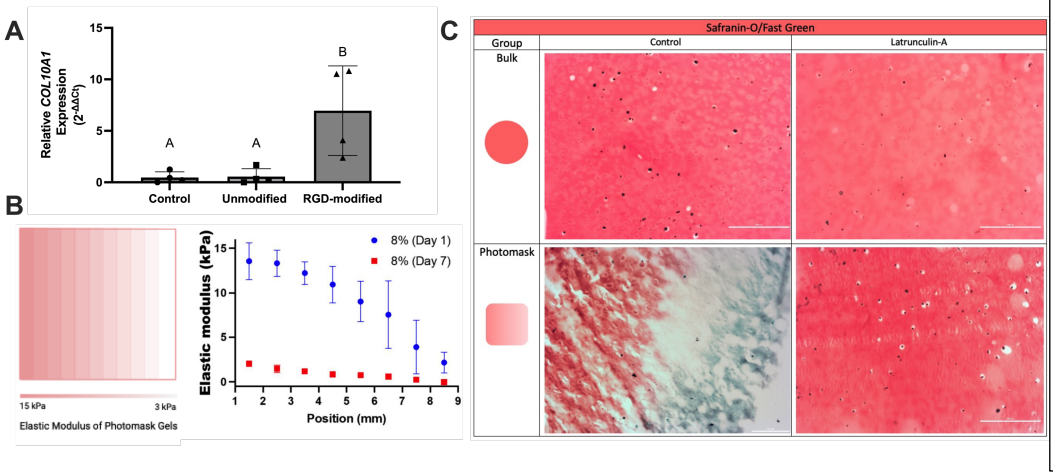

Figure 1. Effect of RGD modification and stiffness on chondrogenic differentiation of MSCs. (A) RGD-modified 2OX20MA induced significantly higher gene expression of *COL10A1* than unmodified gels (n=3). **(B)** Schematic (left) and measured values (right) of elastic moduli of gradient gels, displaying a stiffness gradient with a decrease over 7 days (n=3). **(C)** Stiffness gradient modulated GAG levels. LatA treatment inhibits MSC's ability to mechanosense stiffness gradient (n = 3). Scale bar is 500 µm.