TGF-beta and BMP Signaling Pathways Regulate Chromatin Condensation in Mesenchymal Stem Cells in Response to Dynamic Loading

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Introduction

Mesenchymal stem cells (MSCs) are a promising cell source for regenerative therapies given their multipotent nature.[1] Along with soluble cues, exogenous mechanical perturbations play important roles in modulating MSC lineage-specification.[2] MSC differentiation is accompanied by chromatin remodeling and changes in gene expression.[3] In previous work, we showed that dynamic tensile loading (DL) evoked chromatin remodeling and ultimately condensation. Further, we demonstrated that ATP release and subsequent calcium signaling played a role in early signaling events mediating this DL-induced chromatin condensation. In addition to these pathways, it is well established that transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP), members of the TGF-β superfamily, regulate cellular processes including growth and differentiation.[4] Binding of TGF-β and/or BMP to cell surface receptors initiates phosphorylation of Smad proteins and transcriptional activation of target genes.[4] Recent work has shown that Smad2/3 (pSmad2/3) signaling is activated by cyclic strain.[5] However, the role of signaling through this pathway in mechanically induced chromatin condensation in MSCs has not yet been explored. In this study, we queried whether different loading conditions (i.e, frequency and duration of applied load) regulated chromatin condensation, and ascertained whether these changes were in part mediated through the TGF-β/BMP signaling pathways.

Methods

Aligned poly(ε-caprolactone) (PCL) nanofibrous scaffolds were fabricated via electrospinning and incubated in 20 μg mL^{1} fibronectin in PBS overnight to enhance cell attachment.[2] MSCs isolated from juvenile bone marrow were seeded at 200,000 cells per scaffold. Constructs were cultured in a chemically defined media (CM) without exogenous growth factors. Dynamic tensile loading (DL, 3%) for varying durations (30, 150, or 600 sec) and frequencies (0.2 ~ 2 Hz) was applied after 2 days of pre-culture, using a custom bioreactor.[2] To assess chromatin condensation, nuclei were stained with DAPI and scanned across their mid-sections using a confocal microscope (Zeiss, LSM 510). The chromatin condensation parameter (CCP)[6] was quantified using a gradient-based sobel edge detection algorithm implemented in MATLAB to measure edge density in nuclei. To determine the role of TGF-β superfamily signaling on DL induced condensation, pharmacologic inhibitors SB431542 (SB, 10μM, Sigma) to inhibit Smad 2/3 (TGFβ signaling) or LDN193189 (LDN, 100nM, Reagents Direct) to inhibit Smad 1/5/8 (BMP signaling) were applied for 2 hours and washed off prior to application of DL (3%, 1Hz, 600 sec). After DL, constructs were fixed and CCP quantified.

Given that our previous studies had shown that soluble ATP released to the media was a key mediator of condensation, we also collected conditioned media after each episode of DL, with and without TGF-β/BMP inhibition. This supernatant was then added to naïve (unloaded) MSC-seeded constructs. After 30 min, these constructs were fixed and CCP was determined (Figure 2 A). To measure the concentration of ATP in the media, a luciferin/luciferase assay was performed (ATP Assay Kit, KA1661, Abnova). To determine intracellular Ca^{2+} levels with static stretch, MSCs on scaffolds were loaded with the fluorescent calcium indicator, Cal-520™ AM (15 μM, AAT Bioquest) for 1 h at 37 °C. Constructs were placed into a micro-tensile device[7] mounted on a confocal microscope. Time-series images of baseline $[Ca^{2+}]_i$ signal were recorded (every 4s for 10 min). Grip-to-grip strain of 3% was then applied (at 0.05%/s), and $[Ca^{2+}]_i$ response was measured for an additional 10 min. A custom MATLAB program was used to analyze $[Ca^{2+}]_i$ oscillations (Figure 3,A and B).

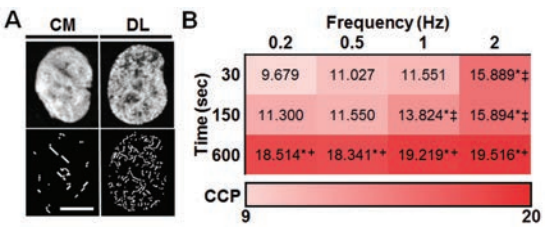

Figure 1. A) DAPI stained nuclei (Top Row) and corresponding edge detection (Bottom Row) (DL: 3%, 1hz, 600s, Bar = 3 μm), B) heat map of CCP values with changes in duration and frequency of DL (n = ~ 20, *: $p < 0.05$ vs. CM control, +: $p < 0.05$ vs. 0.2 hz, ‡: $p < 0.05$ vs. 0.5 hz).

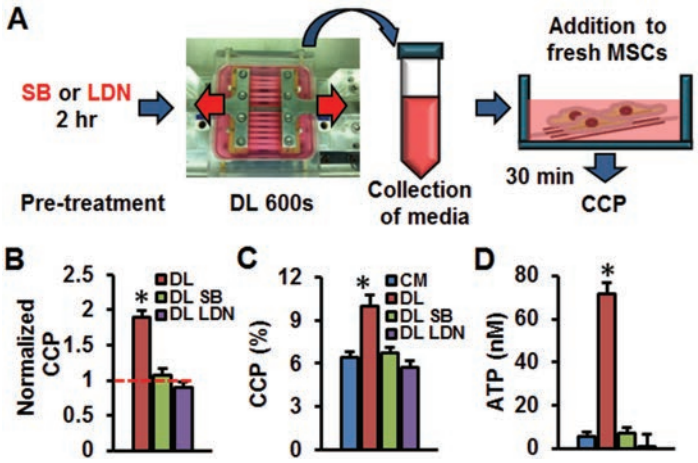

Figure 2. A) Schematic illustration of conditioned media studies with inhibition of TGFβ/BMP signaling, B) CCP with 600s of DL (3%, n = ~20, Red Line: CM control), C) CCP in unloaded scaffolds treated with DL-conditioned media (n = ~20), D) ATP in media (n = 3), *: $p < 0.05$ vs. CM

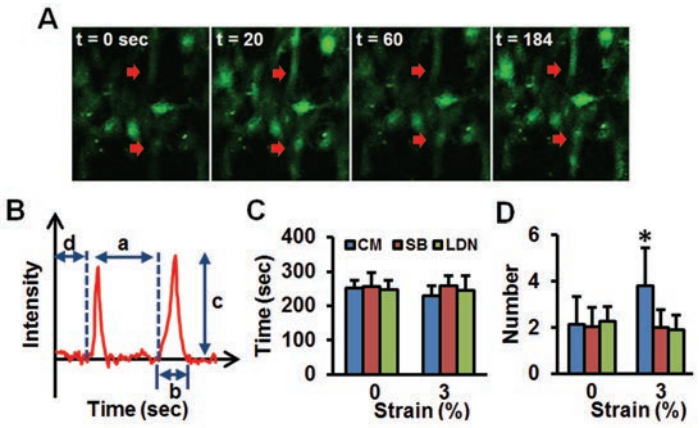

Figure 3. (A) Representative $[Ca^{2+}]i$ oscillations (Red Arrows) in MSCS as a function of time, (B) representative $[Ca^{2+}]i$ oscillation curve (a: time between peaks, b: peak duration, c: peak amplitude, d: time to peak), (C) time to peak, and (D) number of peaks in 10 nin (n = ~ 15, *: $p < 0.05$ vs. 0%).

Results

Dynamic loading led to chromatin condensation in MSC nuclei, increasing the number of visible edges (Figure 1A). No change in CCP was observed with 30 sec of DL, except in the case of DL at 2 Hz (Figure 1B). DL at 1 and 2 Hz for 150 sec increased CCP, but not at 0.2 or 0.5Hz (Figure 1B). Interestingly, with 600 sec of DL, CCP increased in all conditions, independent of loading frequency (Figure 1B).

When DL (600 sec, 3%, 1 Hz) was applied in the context of blockade of either TGF-β or BMP signaling (SB or LDN), DL induced increases in CCP were blocked (Figure 2B). Addition of media conditioned by DL (3%, 1Hz, 600s) increased CCP values in unloaded MSC-seeded constructs (Figure 2C). Conversely, pretreatment of DL-constructs with SB or LDN blocked this condensation (Figure 2C). Consistent with our previous findings, DL (3%, 1Hz, 600s) triggered ATP release into the media (Figure 2D); both SB and LDN pretreatment blocked this ATP accumulation (Figure 2C). This finding suggests that blockade of Smad signaling attenuates release of ATP upon DL, short-circuiting the load induced chromatin condensation events. To probe additional downstream responses, we also monitored $[Ca^{2+}]_i$ oscillations in MSCs (Figure 3A and B). With treatment with SB or LDN, there were no changes in average signal intensity, peak duration (not shown), or time to peak in any group (Figure 3C) when constructs were exposed to grip-to-grip strain of 3%. However, changes in the number of peaks in a 10 min observation window (Figure 3D), the time between peaks (not shown), and % of responding cells (not shown) with static stretch were all altered by inhibition of TGF or BMP signaling.

Discussion

In this study, we showed that dynamic loading leads to rapid chromatin condensation in MSCs, and that the degree of condensation depends on both the frequency and the duration of loading. In addition, we found that blockade of either Smad 2/3 or Smad 1/5/8 signaling attenuated ATP release and subsequent alterations in Ca^{2+} signaling, and thereby limited chromatin condensation. Consistent with this finding, a recent study showed that fluid shear stress results in rapid increases in cellular contractility and ATP release.[8] Likewise, addition of either BMP or TGF-β can increase contractility, and is dependent on Smad signaling. It may be that inhibition of these pathways via pre-treatment with SB or LDN decreases cellular contractility to the point where insufficient stress is generated in the cytoskeleton and so mechanically induced ATP release with applied stretch is no longer possible. Ongoing studies are now exploring changes in cell contractility with inhibition of Smad pathways, and further delineating the manner by which these pathways interact with ATP/calcium signaling. Overall, these new data suggest that dynamic tensile loading triggers ATP release by MSCs, and that this release is modulated by both TGFβ and BMP signaling pathways.

Acknowledgements

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