Mechanically Induced Purinergic and Calcium Signaling Directs Chromatin Condensation in Mesenchymal Stem Cells

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Introduction

Mechanical cues play important roles in directing lineage-specification of mesenchymal stem cells (MSCs). However, the mechanisms by which physical cues regulate chromatin condensation and gene expression remain unclear. Within the nucleus, histone methylation, mediated by the histone methytransferase EZH2, leads to chromatin condensation, silencing transcriptional events, while preserving lineage-specific gene expression. Previous work has shown that mechanical forces applied through magnetic beads induce rapid chromatin condensation in HeLa cells[1]. More recently, we have shown that dynamic tensile loading (DL) evokes heterochromatin formation in MSCs, which is dependent on acto-myosin contractility. Indeed, this mechanically induced condensation occurs more rapidly than with the addition of soluble differentiation factors. We further showed that ATP/calcium signaling played a role in early signaling events mediating this DL-induced chromatin condensation; exposure to Apyrase (an ATP diphosphohydrolase) abrogated DL-induced chromatin condensation. To further delineate the role of ATP/calcium signaling in this process, the current study interrogated key nodes in this signaling pathway via pharmacologic inhibition. Furthermore, given that mechanical inputs have been shown to imprint a 'mechanical memory' on MSCs, we queried whether multiple loading events would regulate the persistence of these changes in chromatin condensation and gene expression over the long term.

Methods

Bovine bone marrow derived MSCs (2×10^5) were seeded onto aligned poly(ε-caprolactone) nanofibrous scaffolds, and constructs were cyclically stretched (3%, 1Hz) using a custom bioreactor (short term: 600s sec, long term: 3 hour) in a chemically defined media [CM]. At each time point, an image-based edge detection algorithm was used to determine a chromatin condensation parameter (CCP) by quantifying chromatin density and organization in individual DAPI stained nuclei. To probe key signaling nodes involved in chromatin condensation, we applied selected pharmacologic inhibitors prior to loading. To investigate the role of the histone H3K27 methyltransferase EZH2, constructs were pretreated with 2.5μM of GSK343 (GSK, Sigma). Likewise, to interrupt ATP/calcium signaling, constructs were exposed to Flufenamic acid (FFA, 500 mM, a hemichannel blocker), BAPTA (50uM, an extracellular Ca^{2+} chelator), NK-62 (10uM, a Calmodulin kinase II inhibitor), and Cyclosporine A (CYPA, 5uM, a Calcineurin inhibitor). ATP in the media was also measured using an ATP assay Kit (Abnova). To monitor changes in $[Ca^{2+}]$ i within cells, constructs were placed into a micromechanical test device mounted onto confocal microscope and cells were labeled with Cal-520™ (AAT Bioquest). Constructs were treated with ATP (0.1mM or 1mM, Thermo Sci.) or were dynamically stretched (3%, 1Hz, 30sec), and $[Ca^{2+}]$i in individual cells was recorded every 4s for 10 mins. YAP (a transcriptional regulator) nuclear localization was evaluated with 1mM of ATP treatment (15 mins) or with the application of DL (30 mins). YAP staining intensity and localization was visualized by immunofluorescence (Santa Cruz Biotech.), and quantified using ImageJ (where nuclear staining was normalized cytoplasmic staining). To determine whether repeated mechanical loading introduced a mechanical memory in these cells, constructs were dynamically loaded (3%, 1Hz, 6 hour/day) for 1 day (DL×1), 3 days (DL×3) or 7 days (DL×7), after which they were returned to free swelling culture for an additional 5 days. At set time points after cessation of loading, CCP was measured and expression levels were determined by real time RT-PCR. Statistical analysis was performed by ANOVA with Fisher's post-hoc tests.

Results

Consistent with our previous findings, 600s of DL led to chromatin condensation in the nuclei of MSCs, increasing the number of visible edges (Figure 1A) and the measured CCP (Figure 1B). This increase in CCP peaked at 600s, and was reduced at 3 hours of DL. Loading also triggered ATP release (not shown). Blocking hemichannels with FFA abrogated the CCP response with 600s of DL, but did not abolish the response with 3

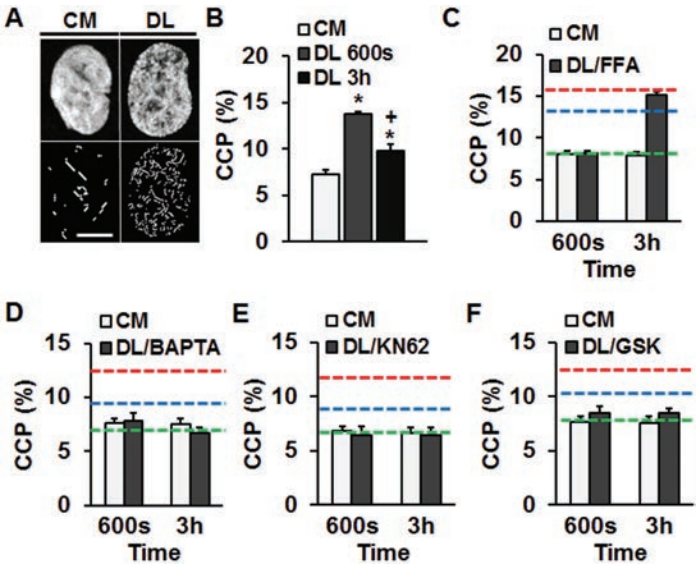

Figure 1. (A) Representative DAPI stained nuclei (top row) and corresponding edge detection (bottom row) (DL: DL for 600s, bar = 3 μm), (B) Chromatin condensation parameter (CCP) with either 600s or 3 hours of DL (n = ~20, *: $p < 0.05$ vs. CM, +: $p < 0.05$ vs. 600s, mean ± SEM), (C-F) CCP values with short and long term loading under pharmacologic inhibition of ATP/Calcium signaling (n = ~20 per group per time point; red line: DL for 600s, blue line: DL for 3hr, green line: unloaded control, mean ± SEM).

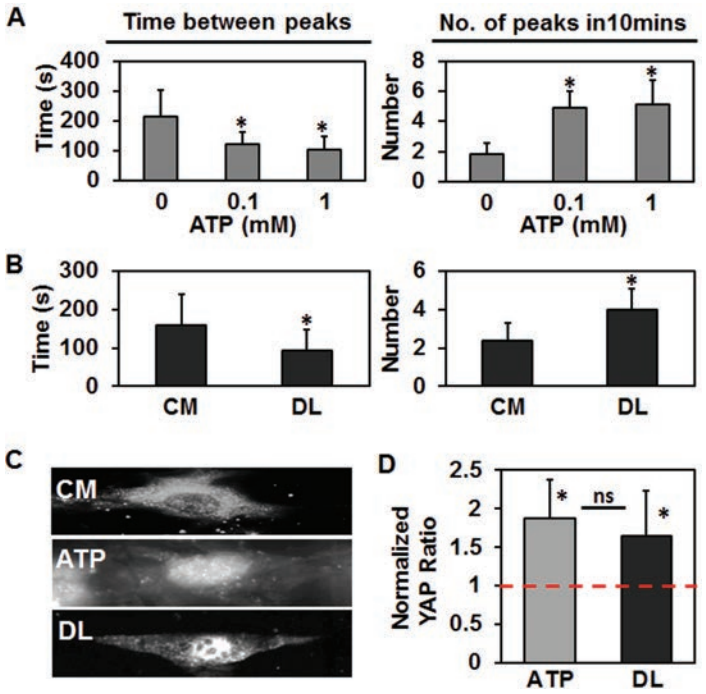

Figure 2. Time between calcium peaks (sec) and number of peaks over 10 mins following exposure to ATP (A) or application of DL (B) (*: $p < 0.05$ vs. control condition). (C) Images of nuclear localization of YAP and quantification (D) with the addition of ATP or application of DL (n = ~20, *: $p < 0.05$ vs. control (red line), mean ± SD).

hours of DL (Figure 1C). Conversely, when calcium/calmodulin/calcineurin signaling was interrupted via the inclusion of BAPTA, NK-62 and CYPA, both the short term and the longer term response was blocked (Figure 1D, 1E). Similarly, addition of a methyltransferase inhibitor (GSK), which blocks the action of EZH2, eliminated DL-induced CCP changes at both time points (Figure 1F). Monitoring internal [Ca^{2+}] showed a decrease in the time between peaks and an increase in the number of peaks when constructs were treated with ATP or exposed to 30 sec of DL (Figure 2A, 2B). With both ATP addition and DL, the transcription regulator YAP was mobilized to the nucleus (Figure 2C, 2D). In longer term studies, the number of loading cycles influenced the magnitude of chromatin condensation and the permanency of the condensation state. Increasing the number of loading events resulted in a larger increase in CCP (Figure 3A), and condensation persisted for a longer period of time after cessation of loading (Figure 3A). Additionally, aggrecan expression (AGG) increased to a greater extent with increasing number of loading events, and this expression remained elevated for prolonged periods after loading (Figure 3B).

Discussion

In this study we showed that dynamic tensile loading of MSCs seeded on aligned nanofibrous scaffolds resulted in marked chromatin condensation. Building from past findings related to ATP release with loading, we further showed that blockade of hemichannels by FFA was involved in the early signaling response, but not in the response to sustained (3 hours) dynamic loading. Conversely, when calcium in the extracellular media (BAPTA) and calcium-responsive

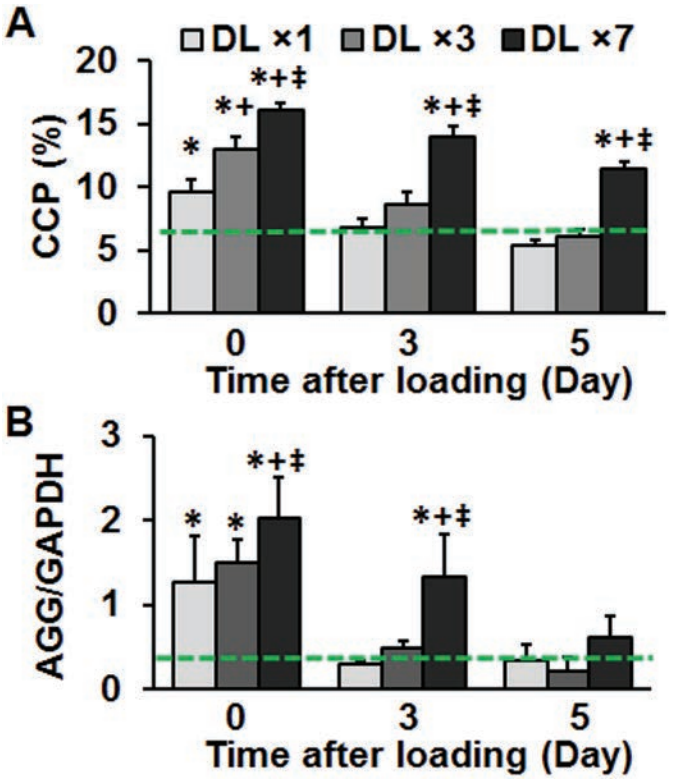

Figure 3. Changes in chromatin condensation and gene expression with multiple loading events (×1, ×3, or ×7) and with time after cessation of loading (up to five days). (A) CCP (green line: control condition, n = ~20, *: $p < 0.05$ vs. CM condition, +: $p < 0.05$ vs. DL ×1, ‡: vs. DL ×3, mean ± SEM). (B) AGG gene expression (green line: CM condition, n = 3, *: $p < 0.05$ vs. CM condition, +: $p < 0.05$ vs. DL ×1, ‡: vs. DL ×3, mean ± SD).

signaling elements (such as calmodulin and calcineurin) in the cell were blocked (using KN-62 and CYPA), both the short term and long term loading response was eliminated. This suggests that both the early and late response depend on calcium mediators, but only the early signaling events utilize hemichannels. Furthermore, when we blocked the activity of the H3K27 methyltransferase EZH2, no chromatin condensation was observed at any time point. This suggests that EZH2 serves as a common downstream integrator of the response to mechanical loading. Ongoing studies are probing the relationship between EZH2 activation and calcium signaling.When mechanical perturbation was applied multiple times, the magnitude of chromatin condensation (and ECM gene expression) increased. This suggests that there exists an extended capacity for condensation and remodeling of nuclear architecture, where repeated loading events may refine and expand locations of condensed chromatin within the genome. This advanced state of chromatin condensation also imparted a degree of permanency to the load conditioned state, where increasing the number of loading cycles sustained the condensed state for a longer period of time after cessation of loading.This implies that a mechanical memory is established in the chromatin architecture with dynamic loading. Ongoing work is now focused on identifying structural features and pathways that regulate chromatin architecture to establish this mechanical memory within the nucleus.

Significance

Mechanical cues play critical roles in directing MSC lineage specification, though the mechanisms by which they do so remain poorly understood. Here, we show that dynamic tensile loading induces chromatin remodeling through both purinergic and calcium signaling pathways, culminating in activation of an enzyme that provides epigenetic modification to histones within the nucleus. Further, we show that repeated loading imparts a mechanical memory to the MSC nucleus, suggesting that these mechanical perturbations can persistently change the trajectory of differentiation through structural remodeling of the chromatin.

Acknowledgements

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References

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