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The Role of Actin Cytoskeletal Tension in Oscillatory Fluid Flow Induced Osteogenesis

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1 Introduction

Loading induced oscillatory fluid flow (OFF) is a potent mechanical signal that may play an essential role not only in bone remodeling, but also in the regulation of progenitor cells. Previous studies indicate that actin cytoskeletal tension, created by the interaction between actin filaments and myosin II, drives osteogenic differentiation [1]. The RhoA/ROCK pathway regulates myosin II activity and is responsible for stress fiber formation and the generation of isometric tension within the cell [1-3]. Although OFF does not produce dramatic stress fiber reorganization [4], we hypothesize that OFF is a potent regulator of mesenchymal progenitor cell osteogenesis and that isometric tension within the actin network is necessary for such regulation.

The objectives of this study are (1) to determine the effect of OFF on C3H10T1/2 progenitor cell commitment to the osteoblast lineage via Runx2 mRNA expression; and (2) to determine if the presence of isometric tension in the actin network is necessary for such loading induced osteogenic differentiation.

2 Materials and Methods

C3H10T1/2 cells were exposed to 1 hour of OFF (1 Hz, ± 10 dynes/cm^2) and RNA was isolated at 4 time-points following the cessation of flow (0, 15, 30 or 90 min). Osteogenic differentiation was measured by Runx2 expression using real time RT-PCR. To determine if isometric tension within the actin network is necessary for flow induced osteogenic differentiation, cells were incubated for 1 hour in 1) growth media or growth media supplemented with 2) ROCK inhibitor, 30 μM Y-27632; 3) Myosin II inhibitor, 50 μM Blebbistatin; or 4) actin polymerization inhibitor, 1 μM Cytochalasin D. Cells were then exposed to 1 hour of OFF. RNA was isolated 30 minutes after the termination of flow, and Runx2 expression was determined. F-actin was stained with Phalloidin and fiber organization was observed at 60X magnification. To determine cell structural rigidity, cells were incubated in one of the 4 supplemented media discussed above, and 1 μm diameter sulfate coated fluorescent beads were allowed to attach to their surfaces. The cells were exposed to OFF for 10 seconds and time-lapse videos were used to determine peak bead displacement. Student *t*-tests were used to compare control and flowed cells. P-values were multiplied by the number of experiments being compared in agreement with the Bonferroni adjustment. A $p < 0.01$ was considered significant. Data are reported as mean ± S.E.

3 Results

Exposure to 1 hour of OFF resulted in a significant increase in Runx2 expression in cells that were lysed 30 minutes after the termination of flow ($p = 0.006$) (**Fig. 1**). In the presence of Cytochalasin D, cells had a disrupted actin fiber organization (**Fig. 2**). Both ROCK and myosin II inhibition resulted in cells lacking fibrillar actin (**Fig. 2**).

4 Conclusion

OFF regulates progenitor cell osteogenesis by inducing the upregulation of the osteogenic transcription factor, Runx2. Runx2 upregulation is attenuated by the inhibition of ROCK, an activator of myosin light chain kinase (MLCK). MLCK activates myosin II, thus ROCK inhibition attenuates actin cytoskeletal tension in the cell. To ensure that acto-myosin tension is the key cell

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function being altered by ROCK inhibition, experiments were repeated with Blebbistatin, a direct inhibitor of non-muscular myosin II ATPase. Under Blebbistatin treatment, Runx2 upregulation was similarly attenuated indicating that the presence of isometric tension in the actin network is necessary for osteogenic differentiation.

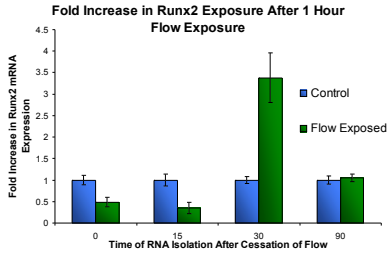

Figure 1 : After 1 hour exposure of oscillatory fluid flow, cells were lysed and RNA was isolated at 4 time points following flow termination: 0 minutes ($p = 0.028$), 15 minutes ($p = 0.032$), 30 minutes ($p = 0.006$), 90 minutes ($p = 1.0$) In all cases, $n = 8$ slides.

With the inhibition of ROCK, Myosin II and actin polymerization, flow-induced Runx2 expression was attenuated (**Fig. 3**). With both ROCK and Myosin II inhibition, there was a trend towards increased bead displacement; however, it was not significant (**Fig. 4**).

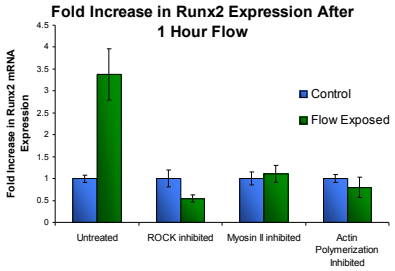

Figure 2 : Cells were incubated in 4 media types as described above and exposed to 1 hour of oscillatory fluid flow. RNA was isolated 30 minutes after the cessation of flow and Runx2 expression was determined: untreated cells ($p = 0.006$), Y-27632 treated (ROCK inhibitor) ($p = 0.112$), Blebbistatin treated (myosin II inhibitor) ($p = 1.0$), and Cytochalasin D treated (actin polymerization inhibitor) ($p = 1.0$). In all cases, $n = 7$ slides.

With both ROCK and Myosin II inhibition, compromised structural rigidity due to the loss of actin tension might lead to large cell deformations with flow, activating other mechanotransduction mechanisms. Although there was a slight decrease in rigidity as would be expected with any structural disruption, the effect appeared to be mild.

This study illustrates that OFF is a potent regulator of osteogenic differentiation in progenitor cells and that isometric tension in cytoskeletal actin is critical for such loading induced lineage commitment.

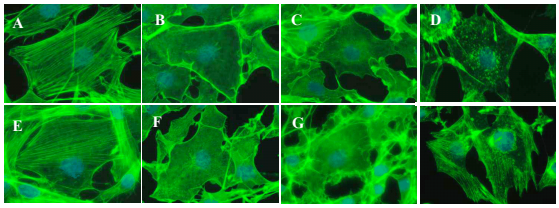

Figure 3 : Phalloidin staining of cells exposed to 1 hour fluid flow (**A**, **B**, **C**, **D**) and with no flow (E,F,G,H) in the presence of growth media (**A**, **E**), growth media with 30 µM Y-27632 (**B**, **F**), growth media with 50 µM Blebbistatin (**C**, **G**), and growth media with 1 µM Cytochalasin D (**D**, **H**). At 60X magnification.

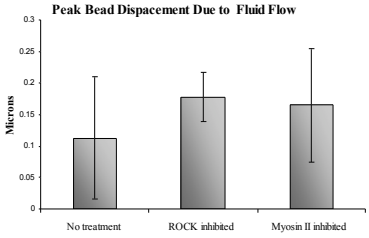

Figure 4 : Mean peak displacement of attached beads due to flow in untreated ($n = 8$ cells), Y-27632 treated (ROCK inhibitor) ($n = 12$ cells), Blebbistatin treated (Myosin II inhibitor) ($n = 11$ cells).

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