Mechanically induced osteogenic differentiation – the role of RhoA, ROCKII and cytoskeletal dynamics

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Accepted 23 October 2008
Journal of Cell Science 122, 546-553 Published by The Company of Biologists 2009
doi:10.1242/jcs.036293

Summary

Many biochemical factors regulating progenitor cell differentiation have been examined in detail; however, the role of the local mechanical environment on stem cell fate has only recently been investigated. In this study, we examined whether oscillatory fluid flow, an exogenous mechanical signal within bone, regulates osteogenic, adipogenic or chondrogenic differentiation of C3H10T1/2 murine mesenchymal stem cells by measuring Runx2, PPARγ and SOX9 gene expression, respectively. Furthermore, we hypothesized that the small GTPase RhoA and isometric tension within the actin cytoskeleton are essential in flow-induced differentiation. We found that oscillatory fluid flow induces the upregulation of Runx2, Sox9 and PPARγ, indicating that it has the potential to regulate transcription factors involved in multiple unique lineage pathways. Furthermore, we demonstrate that the small GTPase RhoA and its effector protein ROCKII regulate fluid-flow-induced osteogenic differentiation. Additionally, activated RhoA and fluid flow have an additive effect on Runx2 expression. Finally, we show RhoA activation and actin tension are negative regulators of both adipogenic and chondrogenic differentiation. However, an intact, dynamic actin cytoskeleton under tension is necessary for flow-induced gene expression.

Key words: Osteogenic differentiation, Mechanotransduction, Fluid flow, Mesenchymal stem cell

Introduction

Tissues in the musculoskeletal system differ greatly in function and phenotype, yet they are derived from common mesenchymal stem cells. In addition to local biochemical factors regulating progenitor cell differentiation, the mechanical microenvironment within a tissue may influence cell fate. Such local mechanical stimuli result in mechanotransduction, the conversion of a physical signal into an intracellular biochemical cascade with the potential to alter gene expression, protein activity and ultimately cell function (Jacobs et al., 1998; Silver and Bradica, 2002; Pavalko et al., 2003; Batra et al., 2005; Iqbal and Zaidi, 2005; Epari et al., 2006; Isaksson et al., 2006; Leclerc et al., 2006; Liedert et al., 2006; Meikle, 2006). Loading induced dynamic fluid flow is a key regulatory factor in promoting healthy bone homeostasis (Frost, 1982; Weinbaum et al., 1994; Jacobs et al., 1998; McAllister et al., 2000; Bakker et al., 2001; Batra et al., 2005; Kim et al., 2006; Leclerc et al., 2006; Mehrotra et al., 2006; Tan et al., 2006; Tan et al., 2007). Given that oscillatory fluid flow promotes osteogenic activity in mature cells, it is our hypothesis that it is also a potent regulator of mesenchymal stem-cell differentiation down the osteogenic pathway.

Previous studies have indicated that mechanical cues mediated by cell shape regulate stem-cell differentiation by eliciting alterations in the activity of RhoA, a member of the family of Rho GTPases (Hill et al., 1995; McBeath et al., 2004; Sarasa-Renedo et al., 2006). Rho GTPases play a significant role in regulating cytoskeletal dynamics and have been shown to be crucial for cell proliferation and differentiation (Hill et al., 1995; Welsh et al., 2001; Sordella et al., 2003; McBeath et al., 2004). The kinase ROCKII (also known as ROCK2) is an important effector of RhoA. Among other functions in the cell, ROCKII mediates actin cytoskeletal tension and stress fiber formation by activating myosin light chain kinase, which in turn, activates the dimerized motor protein myosin II (Rao et al., 2003; Riddick et al., 2008). Upon ATP hydrolysis, myosin II moves along neighboring actin fibers generating tension, or a state of pre-stress, within the actin network. The activation of RhoA, ROCKII and the cytoskeletal isometric tension that may accompany their activation are important factors in mesenchymal stem cell fate (McBeath et al., 2004; Woods et al., 2005; Sarasa-Renedo et al., 2006; Woods and Beier, 2006). Specifically, progenitor cells with constitutively active RhoA or ROCKII are able to undergo osteogenic differentiation downstream of soluble factors; however, inhibition of this pathway results in adipogenic differentiation (McBeath et al., 2004). Furthermore, inhibition of the RhoA/ROCKII pathway has also been shown to promote chondrogenic differentiation via increased SOX9 expression (Woods et al., 2005; Woods and Beier, 2006). Together, the activation of RhoA and the resultant isometric tension within the actin cytoskeleton may be necessary for osteogenic differentiation and inhibitory of differentiation of other lineage types; however these earlier studies were limited to intrinsic forces and did not examine the potential role of extrinsic forces that may be present within the local environment.

In this study, C3H10T1/2 progenitor cells served as a model for primary bone marrow derived mesenchymal stem cells in order to provide a homogeneous and phenotypically stable population of cells with which a large number of treatments and comparisons can be made. Such comparisons may have been difficult to conduct with primary marrow-derived stem cells, given their heterogeneous nature and phenotypic drift. C3H10T1/2 cells were utilized to examine whether oscillatory fluid flow, an exogenous mechanical

Journal of Cell Science

signal within bone, regulates the osteogenic, adipogenic and chondrogenic differentiation of murine mesenchymal progenitor cells by measuring Runx2, PPARγ and SOX9 upregulation, respectively. Additionally, we determined if oscillatory fluid flow activates RhoA GTPase and its direct effector protein ROCKII, the upstream regulators of isometric tension and actin dynamics. Finally, we examined how cytoskeletal mechanics and isometric tension within the actin cytoskeleton alters oscillatory fluid-flow-induced differentiation by the activation of RhoA, inhibition of ROCKII protein, inhibition of myosin II ATP hydrolysis, disruption of actin polymerization, and actin stabilization. Our findings suggest that loading induced oscillatory fluid flow has the potential to upregulate multiple transcription factors involved in distinct lineage pathways. Additionally, fluid flow initiates the activation of RhoA and ROCKII, both of which have essential roles in flow induced osteogenic differentiation. Furthermore RhoA has the potential to act downstream of flow to upregulate Runx2 expression and this potential is synergistically enhanced with oscillatory fluid flow exposure. Finally, although RhoA activation and isometric tension inhibit adipogenic and chondrogenic differentiation, an intact, dynamic actin cytoskeleton under tension is necessary for flow-induced PPARγ and Sox9 expression.

Results

Activity of mesenchymal progenitor cells with oscillatory fluid flow exposure

After exposing C3H10T1/2 mesenchymal progenitor cells to 1 hour of oscillatory fluid flow, we used real-time RT-PCR to determine if oscillatory fluid flow has the potential to regulate the expression of multiple transcription factors involved in osteogenic, adipogenic or chondrogenic cell fate decisions. Runx2, a transcription factor essential for osteogenic differentiation, was significantly upregulated 2.8±0.3-fold (P≤0.01). Furthermore, both PPARγ and SOX9, transcription factors involved in adipogenic and chondrogenic differentiation, respectively, were also upregulated with oscillatory fluid flow exposure 1.9±0.05-fold (P≤0.01) and 1.7±0.15-fold (P≤0.01). These results suggest that dynamic flow has the potential to regulate multiple transcription factors involved in unique musculoskeletal lineage pathways.

Based on previous studies illustrating the significance of RhoA and ROCKII in the regulation of differentiation, we chose to investigate whether oscillatory fluid flow altered their activation (McBeath et al., 2004; Woods et al., 2005; Woods and Beier, 2006). Using rhotekin-binding beads to pull down GTP-bound RhoA, we found that a 1 hour exposure of oscillatory fluid flow induced a significant twofold (P≤0.01) increase in active RhoA. Furthermore, using immunoprecipitation to isolate ROCKII followed by a kinase assay we found the activity of ROCKII was increased 3.9±0.4-fold (P≤0.01) with a 1 hour exposure to flow (Fig. 1). Thus, mechanical stimulation via fluid flow activates RhoA and ROCKII, which may ultimately enhance the tension within the actin cytoskeleton. Given that RhoA and ROCKII were activated with flow and that their activation influenced lineage commitment in stem cells, we next investigated the roles of RhoA, ROCKII and cytoskeletal tension in fluid-flow-induced gene expression.

Effects of actin cytoskeletal alterations

We used several pharmacological agents to test the importance of RhoA, ROCKII and actin cytoskeletal tension on flow-induced gene expression. Specifically, cells were exposed to (1) lysophosphatidic acid sodium salt (LPA), which activates RhoA; (2) Y-27632 dihydrochloride, which inhibits p160 ROCKII activity; (3) blebbistatin, which selectively inhibits the ATPase function of non-muscle myosin II; (4) cytochalasin D, which inhibits actin polymerization by capping the barbed end of F-actin polymers; and (5) jasplakinolide, which has been shown to stabilize actin filaments by inhibiting depolymerization.

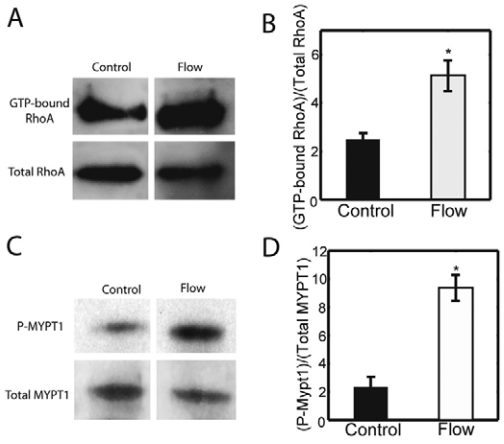

Fig. 1. Oscillatory fluid flow has the potential to regulate the activation of RhoA and its effector protein, ROCKII. (A) Using agarose beads with GST-tagged fusion protein corresponding to residues of rhotekin RhoA-binding domain, GTP-bound RhoA was isolated using a pull-down assay. (B) Western blot analysis of GTP-bound and total RhoA indicated that a 1 hour exposure to oscillatory fluid flow induces a 2.0±0.2-fold increase (P≤0.01) in active RhoA. (C) Furthermore, ROCKII was isolated by immunoprecipitation and incubated with the ROCKII substrate protein, MYPT1, and kinase activity analyzed by western blot analysis of phosphorylated MYPT1. (D) Here we show that a 1 hour exposure to dynamic fluid flow induced a significant 3.9±0.8-fold increase in kinase activity (P≤0.01). Error bars: s.e.m. (n≥4).

To characterize biochemically induced alterations in morphology and cytoskeletal microstructure, actin staining was conducted after 1 hour incubation with all five pharmacological agents with and without flow exposure (Fig. 2). Flow appeared to alter cytoskeletal density or organization under various treatments. Specifically, with 1 hour of oscillatory fluid flow exposure, actin fibril density in untreated cells appeared to increase; however, there was no specific alignment of the fibrils with respect to the direction of flow. Furthermore, LPA incubation for 1 hour without fluid flow resulted in an increase in actin fibril density similar to that of flowed untreated cells, and this was further enhanced with LPA treatment and flow. Treatment with Y27632 and blebbistatin resulted in stellate cells lacking actin fibrils. However, exposure to mechanical stimulation did not induce further alterations in cell morphology or actin organization for either treatment. Cytochalasin treatment resulted in disrupted actin fibril organization that became punctuate with flow exposure. The effects of jasplakinolide on actin cytoskeletal dynamics may be difficult to interpret based on previous reports describing contradictory outcomes. Specifically, although a number of studies use jasplakinolide to stabilize actin based on its affinity to bind to actin filaments and inhibit their depolarization by cofilin (Albinsson and Hellstrand, 2007; Woods and Beier, 2006; Woods et al., 2005), many reports also indicate that jasplakinolide enhances the rate of actin filament nucleation, causing a decrease in monomeric G-actin and the appearance of disordered polymeric actin after prolonged exposure or exposure to high concentrations (Albinsson and Hellstrand, 2007; Bubb et al., 2000; Lazaro-Dieguez, 2008).

Under our conditions, jasplakinolide treatment resulted in stabilized, dense actin fibril organization that was not altered by flow, suggesting that the concentration and exposure time in our experiments were sufficient to induce actin stabilization without the actin aggregation that ultimately occurs.

Although there were alterations in actin microstructure and cell morphology with incubation of several of the biochemical agents, for all treatments there were no gross alterations upon flow exposure that indicate impaired cell-cell contacts or cell adhesion. This is an important consideration, as earlier studies indicate that mechanotransduction can occur via cell-cell contacts as well as cell-matrix contacts (Duncan, 1995; Ralphs et al., 2002; Silver and Siperko, 2003; Tong et al., 2003; Ponik and Pavalko, 2004; Iqbal and Zaidi, 2005; Liedert et al., 2006; Liu et al., 2007; Wang et al., 2007; Woods et al., 2007).

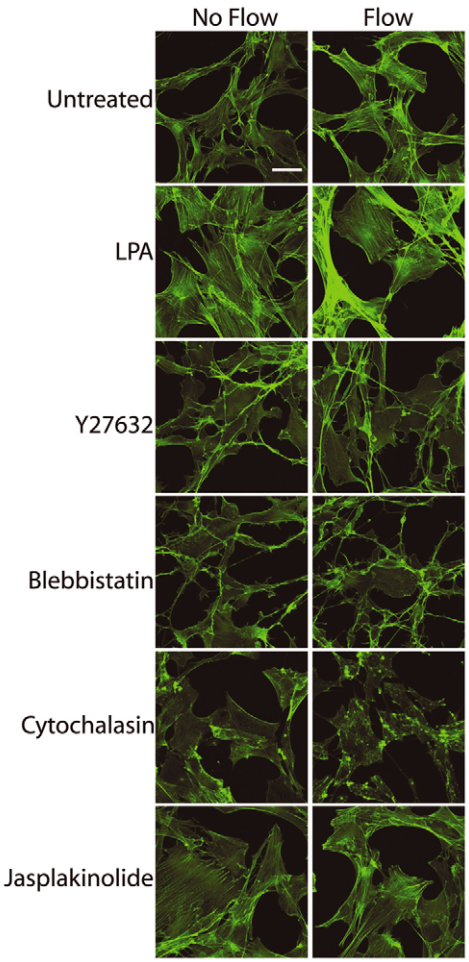

Fig. 2. Micrographs of each biochemical treatment, with or without flow, were examined to characterize alterations in actin microstructure and cell morphology. Untreated cells in the presence of flow appeared to have a denser actin misconstrue. Similar increases in actin microstructure were observed with a 1 hour incubation with LPA, and this was further enhanced with LPA treatment and dynamic flow. Inhibiting actin tension with Y27632, which inhibits ROCKII, and blebbistatin, which inhibits myosin II function, resulted in a lack of actin fibril organization and ruffled cell edges; however, there were no other gross alterations to cytoskeletal organization or cell morphology with flow. Disruption of actin polymerization with cytochalasin D incubation resulted in punctuate actin fibrils that became more pronounced with flow exposure. Treatment with jasplakinolide, which stabilizes existing filaments and inhibits actin reorganization or dynamics, appeared to have increased actin density that was not affected by flow exposure. In all cases, treatment with each biochemical in the presence of flow did not appear to induce any gross morphological alterations. Scale bar: 50 µm.

Oscillatory fluid-flow-induced Runx2 expression and effects of cytoskeletal alterations

To determine the role of RhoA, ROCKII and cytoskeletal tension in oscillatory fluid-flow-induced Runx2 upregulation, progenitor cells were incubated for 1 hour before flow exposure in one of five pharmacological agents targeting: RhoA activation, ROCKII inhibition, myosin II inhibition, actin polymerization inhibition or actin stabilization. Incubation with LPA elicited a significant twofold ($P \leq 0.01$) increase in Runx2 expression, suggesting that activated RhoA is sufficient for Runx2 upregulation and acts downstream of oscillatory fluid flow (Fig. 3A). Upon flow exposure for 1 hour, cells treated with LPA elicited a 2.4±0.02-fold ($P \leq 0.01$) increase in Runx2. In comparison with non-flowed, untreated progenitor cells, cells with activated RhoA and exposed to flow had an increased Runx2 expression of more than sixfold, suggesting that flow and RhoA have the potential to act synergistically in enhancing Runx2 expression, similar to the synergistic effect noted above on the actin cytoskeleton. By contrast, inhibiting the direct effector protein of RhoA, ROCKII, by treating the cells with Y27632 significantly decreased Runx2 expression levels threefold ($P \leq 0.01$) in non-flowed progenitor cells. Furthermore, inhibiting ROCKII abrogated flow-induced Runx2 upregulation, indicating that active ROCKII is necessary for flow-induced Runx2 expression.

Having determined that the RhoA and ROCKII signaling pathway is necessary for flow-induced Runx2 upregulation, we next investigated the role of an intact, dynamic actin cytoskeleton under isometric tension in flow-induced differentiation (Fig. 3B). Inhibiting actin polymerization with cytochalasin resulted in a significant threefold decrease ($P < 0.01$) in basal Runx2 expression

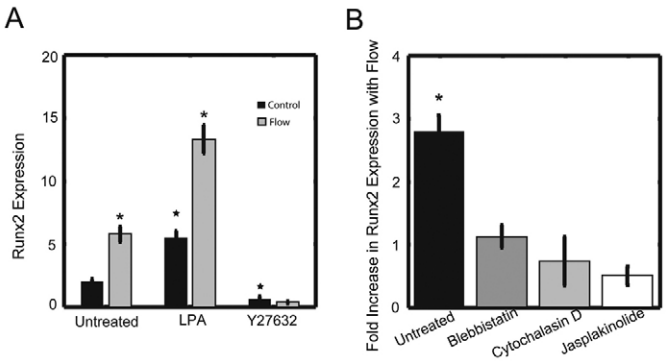

Fig. 3. The RhoA/ROCKII pathway ultimately leading to an intact, dynamic actin cytoskeleton under tension has an important role in mechanically induced Runx2. (A) One hour incubation with LPA leading to increased amounts of active RhoA elicits a 2.6±0.8-fold increase in Runx2 expression ($P \leq 0.01$). Additionally, dynamic flow further induced Runx2 expression by 2.4±0.2-fold ($P \leq 0.01$) in LPA-treated cells, suggesting that active RhoA and oscillatory fluid flow act synergistically to enhance Runx2 upregulation. On the other hand, inhibiting ROCKII function significantly decreases Runx2 expression by 3.2±0.3-fold ($P \leq 0.01$) and abrogates flow induced Runx2 expression. (B) Multiple characteristics of the actin cytoskeleton downstream of RhoA and ROCKII activity influence flow induced Runx2 expression. Specifically, if we remove cytoskeletal tension by inhibiting myosin II activity, the cells lose the ability to upregulate Runx2 with flow. Furthermore, altering cytoskeletal polymerization with cytochalasin D or inhibiting cytoskeletal dynamics with jasplakinolide abrogates flow induced Runx2 expression. Error bars: s.e.m. ($n \geq 6$).

levels. The disruption of the actin network induced by cytochalasin treatment resulted in minimal actin tension and ultimately led to a more rounded morphology, suggesting that cell shape and an intact cytoskeleton are key regulators in differentiation. These results are consistent with previous studies demonstrating that cell shape plays significant roles in stem-cell differentiation (McBeath et al., 2004). Additionally, cytochalasin D incubation attenuated flow-induced Runx2 expression. Furthermore, inhibiting actin tension by inhibiting myosin II ATPase and inhibiting reorganization with flow by jasplakinolide treatment attenuated the flow-induced Runx2 upregulation. These results indicate that an intact, dynamic cytoskeleton under tension is necessary for flow-induced increases in Runx2 expression.

Oscillatory fluid-flow-induced SOX9 expression and effects of cytoskeletal alterations

Based on previous studies demonstrating that RhoA and ROCKII inhibition increases chondrogenic differentiation, we investigated whether RhoA activation, ROCKII inhibition, myosin II inhibition, actin polymerization inhibition or polymerization stabilization altered SOX9 expression with or without the presence of mechanical stimulation (Fig. 4A,B). Consistent with earlier studies, we found that incubation with Y27623, blebbistatin, or disrupting actin polymerization with cytochalasin D elicited significant increases in SOX9 expression by twofold (P<0.01), threefold (P<0.01) and fivefold (P<0.01), respectively (Fig. 4A) (Woods et al., 2005; Woods and Beier, 2006). Furthermore, ROCKII inhibition, myosin II inhibition, actin polymerization inhibition and actin stabilization abrogated flow-induced SOX9 expression. LPA treatment did not alter Sox9 basal expression levels and treated cells maintained their ability to upregulate SOX9 1.4±0.09-fold (P≤0.05) with oscillatory fluid flow, indicating that RhoA may not be a direct inhibitor of chondrogenic differentiation (Fig. 4B). These results suggest that inhibiting tension within the actin cytoskeleton promotes chondrogenic differentiation; however, an intact cytoskeleton is necessary for flow-induced alterations in Sox9 expression.

Oscillatory fluid-flow-induced PPARγ expression and effects of cytoskeletal alterations

Recent reports suggest that cell shape plays an important role in mesenchymal progenitor cell commitment between the adipogenic and osteogenic pathways (McBeath et al., 2004). Furthermore, constitutively active RhoA and ROCKII stimulate osteogenic differentiation while preventing adipogenic differentiation (McBeath et al., 2004). In the absence of flow, incubation with cytochalasin D significantly increased expression of PPARγ 2.3-fold (P<0.01). These results indicate that a lack of actin fibril organization, which may result in a more rounded morphology, is a strong signal in initiating adipogenic differentiation. On the contrary, increasing actin cytoskeletal tension via LPA treatment significantly decreased PPARγ expression by 2.2-fold (P<0.05) (Fig. 4A). In addition to alterations in PPARγ basal expression levels, RhoA activation abrogated flow-induced upregulation of PPARγ, suggesting that activated RhoA is an antagonist of adipogenic differentiation. Moreover, ROCKII inhibition, myosin II inhibition, actin polymerization inhibition and actin stabilization, attenuated the flow-induced upregulation (Fig. 4C). Taken together, these results indicate that an intact dynamic cytoskeleton under tension is necessary for the transduction of dynamic flow into the altered gene expression of PPARγ; however additional signaling mechanisms must be initiated to antagonize the effects of flow-induced RhoA activation.

Discussion

Similar to soluble factors, oscillatory fluid flow is a potent signal capable of regulating stem-cell differentiation. For the purpose of developing regenerative medicine therapeutics, it is not only necessary to determine if specific mechanical cues elicit progenitor differentiation, but also the mechanism by which a cell transduces the physical signal into a cell fate commitment. Our study demonstrates that exposure to oscillatory fluid flow, a predicted mechanical signal in mature bone, regulates progenitor cell differentiation via the activation of the RhoA/ROCKII pathway.

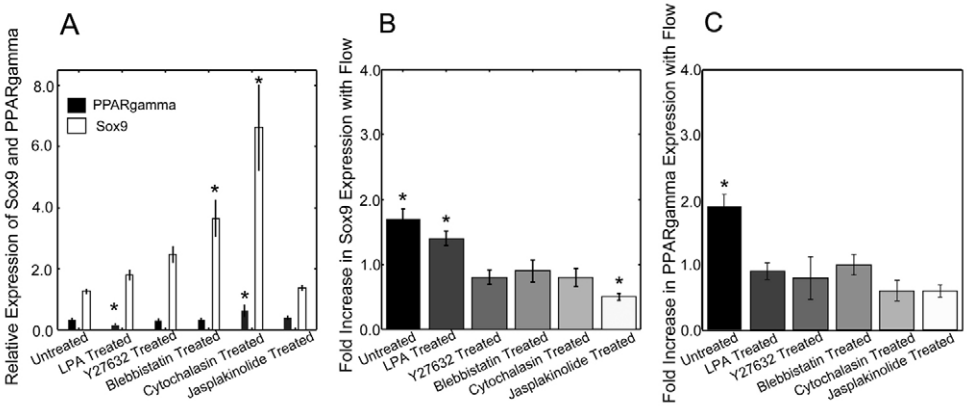

Fig. 4. A dynamic actin cytoskeleton under tension is necessary for flow-induced Sox9 and PPARγ expression. (A) Altering the cytoskeletal dynamics had significant effects on both PPARγ and Sox9 basal expression. RhoA activation, and thus increased actin organization, significantly decreased PPARγ by 2.2-fold (P<0.05), while disrupting the actin cytoskeletal organization with cytochalasin D had an opposing effect and increased expression by 2.3-fold (P<0.01). Cytochalasin D had a similar effect on basal Sox9 expression, resulting in a fivefold upregulation (P<0.01). Furthermore, inhibiting actin cytoskeletal tension using Y27632 and blebbistatin also significantly increased Sox9 basal expression by threefold (P<0.01) and twofold (P<0.01), respectively. (B) Oscillatory flow-induced Sox9 expression is attenuated in the presence of pharmacological agents that inhibit ROCKII activation, myosin II ATPase function and actin polymerization. Inhibition of actin dynamics by jasplakinolide treatment resulted in a 1.85±0.17-fold decrease in Sox9 expression (P≤0.01). LPA incubation resulting in increased levels of active RhoA did not have a significant effect on flow-induced Sox9 expression. (C) Flow-induced PPARγ expression was abrogated under all conditions, indicating that an intact, dynamic actin cytoskeleton under tension is necessary. LPA treatment also abrogated flow-induced PPARγ expression, suggesting that active RhoA has a negative effect on adipogenic differentiation. Error bars: s.e.m. (n≥6).

Additionally, we show that tension within the actin cytoskeleton is required for the activation of mechanosensors or signaling mechanisms involved in the transduction of dynamic fluid flow into cell fate decisions (Fig. 5A).

Oscillatory fluid flow induces increased Runx2 expression. Runx2 is the earliest regulator of osteoblast differentiation and targeted ablation of Runx2 results in skeletal patterning that is completely cartilaginous (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997; Huaung et al., 2006). Furthermore, Runx2 activates the expression of multiple late stage genes, including osteopontin and osteocalcin, with a distinct binding site in the promoter region of osteocalcin (Norvell et al., 2004; Sierra et al., 2004; Baksh et al., 2007). Our finding that oscillatory fluid flow initiates osteogenic differentiation via Runx2 upregulation is consistent with Li and colleagues who demonstrated that oscillatory fluid flow also induces the upregulation of the late-stage osteogenic genes, osteopontin and osteocalcin, in human marrow stromal cells (Li et al., 2004). Thus, dynamic fluid flow has the potential to regulate the expression of multiple osteogenic genes, suggesting that such mechanical stimulation may, in fact, be a potent regulator of osteogenic differentiation. However, PPARγ and Sox9 expression were also upregulated with flow. Given that PPARγ is an adipocyte-specific nuclear hormone receptor that stimulates the conversion of multiple cell types, including stem cells, fibroblasts and myoblasts into committed preadipocytes (Hu et al., 1995; Wu et al., 1995) and Sox9 is a homeodomain transcription factor expressed during chondrogenic differentiation (Ikeda et al., 2005), oscillatory fluid flow may have the potential to regulate multiple transcription factors involved in unique lineage pathways (Fig. 5A). Nonetheless, the upregulation of Sox9 with flow may also be important for osteogenic differentiation. For example, Sox9 expression has been shown to be important in endochondral ossification (Akiyama et al., 2005; Isaksson et al., 2006; Mackie et al., 2008), and previous studies also suggest that mechanical loading results in endochondral ossification during development (Carter et al., 1987; Carter and Wong, 1988; Carter and Orr, 1992; Carter et al., 1998; Stokes, 2002; Sundaramurthy and Mao, 2006; Gomez et al., 2007; Trepczik et al., 2007).

A crucial component of mechanical integrity of progenitor cells is the actin cytoskeleton. Accordingly, in the presence of oscillatory fluid flow, the cell experiences increased force transmission and tension through its cytoskeleton (Pavalko et al., 1998; Malone et al., 2007). It is well established that RhoA has the potential to regulate stem cell fate via intrinsic mechanisms; however, this is the first study to show that RhoA activation, which may result in enhanced actin cytoskeletal tension, is also initiated by extrinsic factors and has a significant role in mechanically regulated osteogenic differentiation. Our results suggest that flow-induced RhoA activation has the potential to regulate Runx2 expression. Additionally, we show that the activation of ROCKII and an intact, dynamic actin cytoskeleton under tension are necessary for flow-induced gene expression. This suggests that osteogenic differentiation requires an organized actin network under isometric tension and that this state of pre-stress can be achieved by oscillatory fluid flow, or by biochemically inducing RhoA activation.

Interestingly, we also show that an intact, dynamic actin cytoskeleton under tension is necessary for flow-induced molecular signaling, resulting in PPARγ and Sox9 expression; however, chemically induced actin tension is not sufficient. In fact, we illustrate that increased RhoA activation inhibits adipogenic differentiation, consistent with previous studies (McBeath et al., 2004). However, increased RhoA activity did not have an effect on

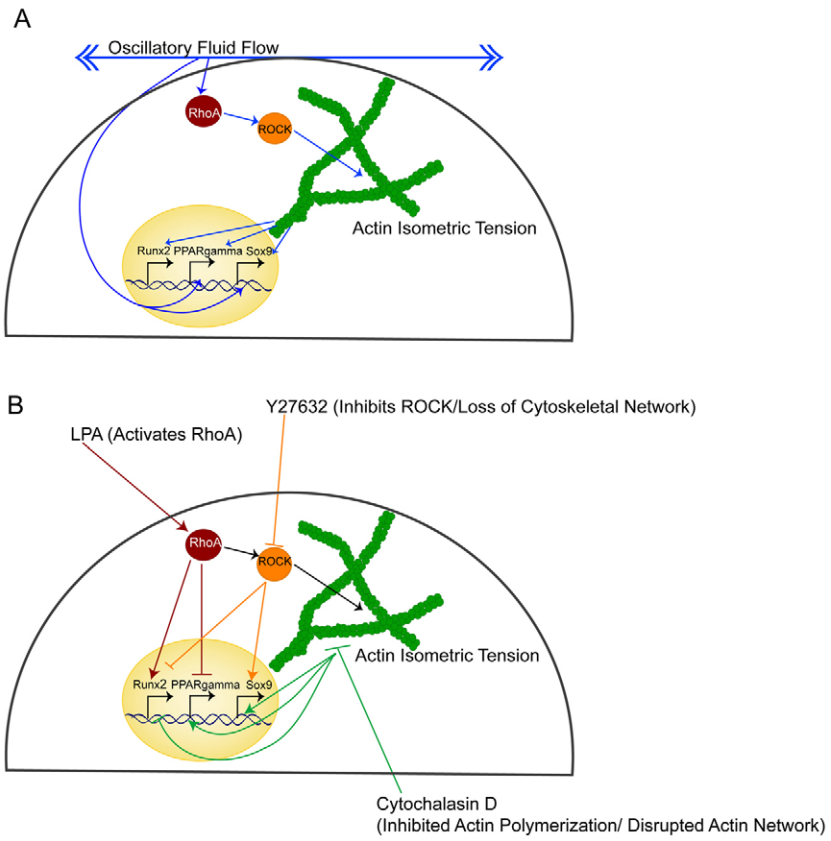

Fig. 5. Mechanical and biochemical regulation of gene expression. (A) Oscillatory fluid flow has the potential to regulate multiple transcription factors involved in unique lineage pathways; however, they are not regulated by the same mechanism, and the specific molecular pathways remain unknown. (B) Runx2 expression requires ROCKII activity and a dynamic actin cytoskeleton under tension. Furthermore, RhoA activation is sufficient to induce Runx2 upregulation and has an additive effect on flow-induced osteogenic differentiation. By contrast, RhoA activation downregulates PPARγ and attenuates its flow induced expression. RhoA does not alter Sox9 in either basal or flow-exposed conditions, suggesting that it does not have a significant role in chondrogenic differentiation. Interestingly, any biochemical that disrupted the actin network resulted in a decrease in Runx2 expression, and an increase in PPARγ and/or Sox9 indicating actin microstructure and ultimately, cell shape, may regulate commitment between these three fates; however, in all cases an intact actin network was necessary for flow-induced gene expression. Based on this, the presence of an actin cytoskeleton under 'pre-stress', controlled by RhoA and ROCKII, is necessary for the transduction of the physical signal into alterations in gene expression. Nonetheless, other signaling pathways must also be activated by with flow to induce PPARγ and Sox9 upregulation.

Sox9 expression. Interestingly, Woods and colleagues found that LPA significantly reduced Sox9 expression in the chondrogenic cell line ATDC5. This suggests that the role of RhoA in chondrogenic differentiation may be dependent on the state of differentiation. In contrast to RhoA activation, disruption of the actin cytoskeleton elicits large increases in PPARγ and Sox9 expression, while suppressing Runx2 (Fig. 5B). These results suggest that a state of minimal tension within the cytoskeleton, which may result in a more rounded cell shape similar to adipocyte and chondrocyte morphology observed in vivo, may have a role in initiating both adipogenic and chondrogenic cell fate commitment. This is consistent with previous studies showing that inhibiting actin tension and/or cell rounding promotes adipogenic and chondrogenic differentiation (McBeath et al., 2004; Woods et al., 2005; Woods and Beier, 2006). Here we demonstrate that actin cytoskeletal tension induced by oscillatory fluid flow is necessary for mechanotransduction to occur. However, in addition to the increase in cytoskeletal tension, exposure to fluid flow must initiate other signaling cascades involved in adipogenic and chondrogenic differentiation (Fig. 5A). This suggests that there are multiple mechanisms involved in the transduction of fluid flow into altered gene expression (Ralphs et al., 2002; Silver and Siperko, 2003; Tong et al., 2003; Alenghat et al., 2004; Ponik and Pavalko, 2004; Iqbal and Zaidi, 2005; Ingber, 2006; Wang et al., 2007).

In summary, we found that loading-induced oscillatory fluid flow regulates osteogenic differentiation via the activation of RhoA, ROCKII and ultimately isometric tension in the actin cytoskeleton. Furthermore, chemically activating RhoA is sufficient to induce osteogenic differentiation and acts synergistically with oscillatory fluid flow in Runx2 upregulation. There are multiple potential mechanisms whereby actin fibril tension may play a role in intracellular signal transduction, including connections to caveolae (Stahlhut and van Deurs, 2000; Hjalm et al., 2001; Swaney et al., 2006) and focal adhesions (Petit and Thiery, 2000; Rajfur et al., 2002; Humphries et al., 2007; Wehrle-Haller, 2007), as well as adherens junctions (Wheelock and Knudsen, 1991; Aberle et al., 1996; Ganz et al., 2006; Liedert et al., 2006) and primary cilia (Alenghat et al., 2004; Oishi et al., 2006; Montalbetti et al., 2007). Distinguishing between these mechanisms as well as the family of signaling proteins responsible for RhoA activation will provide novel insight into future therapeutics for tissue engineering.

Materials and Methods

Cell culture

C3H10T1/2 mesenchymal progenitor cells (ATCC, Clone 8) were cultured in Eagle's basal medium (BME) with 2 mM L-glutamine (Sigma) adjusted to contain 2.2 g/l sodium bicarbonate and supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin and streptomycin (Invitrogen). Cells were maintained at 37°C and 5% CO_2.

Oscillatory fluid flow

Cells were subcultured on fibronectin-coated glass slides (76 × 35 × 1 mm) at 140,000 cells/slide. Fluid flow was applied 48 hours following subculture, such that cells were 80-90% confluent at the time of experimentation. A previously described fluid flow device was used to deliver oscillatory fluid flow to C3H10T1/2 progenitor cells (Jacobs et al., 1998). In brief, a Hamilton glass syringe in series with rigid walled tubing and a parallel plate flow chamber drove oscillatory flow. The syringe was mounted in and driven by a mechanical loading device. The flow rate was monitored with an ultrasonic flow meter (Transonic Systems) and was selected to yield peak shear stresses of 1.0 Pa (10 dynes/cm^2). The dynamic flow profile was sinusoidal at a frequency of 1 Hz. All flow experiments were conducted for 1 hour. Following the cessation of flow, cells were incubated in fresh BME with 10% FBS and 1% penicillin/streptomycin for 30 minutes until RNA isolation.

Pharmacological agents

The following biochemical agents were employed: 1 μM cytochalasin D (Sigma), 10 μM Y-27632 dihydrochloride (Tocris Bioscience), 50 μM blebbistatin (Tocris Bioscience), 50 nM jasplakinolide (Invitrogen), 4 μM lysophosphatidic acid sodium salt (LPA) (Sigma). Cells were exposed to each pharmacological agent for 1 hour before flow.

Actin staining

Cells were fixed in 3.7% formaldehyde and membranes were removed with 0.1% Triton-X in PBS. Cells were pre-incubated in 100 μl primary blocking solution (PBS, 1% BSA, 0.1% NP-40) for 20 minutes and then incubated with 0.5 μM Alexa Fluor 488 conjugated phalloidin (Molecular Probes) in primary blocking solution for 20 minutes. Vectashield mounting medium (35 μl) (Vector Laboratories) was dispensed on the cells. A glass coverslip was placed on the slide and sealed with nail polish before observation. Actin structure was visualized with an epifluorescent microscope (Nikon Eclipse TE-300, Nikon Inc.) at 60×.

RNA isolation and real-time RT-PCR

Cells were lysed after flow exposure and total RNA isolated using Tri-Reagent (Sigma). The 260/280 absorbance ratio was measured for verification of the purity and concentration of the RNA. Reverse transcription was completed using GeneAMP RNA PCR Core kit with 0.75 μg RNA. Analysis by quantitative real-time RT-PCR (Perkin Elmer Prism 7900, Applied Biosystems) was conducted using Taqman PCR Master Mix and a 20× 18S primer and probe (Taqman Gene Expression Assays, Applied Biosystems), or by using SYBER green PCR master mix with primers and probes developed by Operon Technologies for Runx2, SOX9 and PPARγ. The primer sequences were: Runx2 forward 5′-AGAAGGCACAGACAGAAGCTTGA-3′; reverse 5′-AGGAATGCGCCCTAAATCACT-3′; SOX9 forward 5′-ATCTGA-AGAAGGAGAGCGAG-3′; reverse 5′-TCAGAAGTCTCCAGAGCTTG-3′; PPARγ forward 5′-TATGGAGTTCATGCTTGTGA-3′; reverse 5′-CGGGAAGGACTTT-ATGTATG-3′. Each sample was analyzed in triplicate.

RhoA-GTP assay

GTP-bound RhoA was assessed by a pull-down assay adapted from (Ren and Schwartz, 2000; McBeath et al., 2004). Immediately following exposure to 1 hour of oscillatory fluid flow, cells were rinsed with ice-cold PBS, and lysed in 4°C with 200 μl of lysis buffer (50 mM Tris pH 7.2) (Quality Biologicals), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml aprotinin/leupeptin and 1 mM PMSF (Sigma). Samples were centrifuged for 3 minutes at 3000 ***g*** at 4°C. Supernatant (30 μl) was removed and used to determine total RhoA; the remaining volume of supernatant was incubated with 32 μl rhotekin-binding beads (Upstate) for 45 minutes at 4°C. After incubation, samples were centrifuged for 3 minutes at 3000 ***g***, washed three times with IP buffer [10 mM Tris-HCl at pH 7.5, 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 2 mM $CaCl_2$, 0.1 mM sodium orthovanadate, 10 μg/ml aprotinin/leupeptin and 1 mM PMSF (Sigma)], and suspended in SDS-PAGE buffer. RhoA was detected by western blot.

ROCKII kinase assay

ROCKII protein was isolated using an immunoprecipitation adapted from (Sahai and Marshall, 2002; McBeath et al., 2004). Immediately following 1 hour of flow, cells were washed in ice cold PBS, lysed with 100 μl lysis buffer, sonicated for 5 seconds and centrifuged for 4 minutes at 14,000 ***g***. The supernatant was incubated with 25 μl protein G sepharose beads (Amersham Pharmacia) for 15 minutes and centrifuged for 2 minutes at 14,000 ***g***. Then the supernatant was incubated with 5 μl anti-ROCK-II antibody (Santa Cruz Biotechnology, cat no. SC-1851) for 30 minutes; 50 μl protein G sepharose beads was then added and incubated for 30 minutes. The beads were washed four times with IP buffer and resuspended in 50 μl kinase assay buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM NaF, 1 mM sodium orthovanadate, 5% glycerol, 1% NP-40, 1 mM dithiothreitol, and 1 mM PMSF (Sigma)], 100 μM of ATP (Sigma, St Louis, MO) and 500 ng recombinant MYPT1 substrate (Upstate) were incubated for 30 minutes at 37°C. SDS-PAGE buffer was added and the samples were boiled for 10 minutes at 95°C to stop the reaction. Kinase activity was detected by western blot for phosphorylated MYPT1.

Western blot analysis

Protein concentration in the supernatant was determined using a Bradford Protein Assay Kit (Bio-Rad Laboratories). Suspended samples were electrophoresed through NuPAGE 4 12% Bis-Tris polyacrylamide gels (Invitrogen) and were transferred electrophoretically onto nitrocellulose membranes in blocking buffer (Pierce). After washing, membranes were incubated with primary antibody overnight at 4°C under gentle rocking. To determine expression levels of activated RhoA or phosphorylated-MYPT1, monoclonal anti-RhoA (1:2000) antibodies (Santa Cruz Biotechnology, Cat #sc-418) and polyclonal anti-P-MYPT1 (1: 2000) antibodies (Millipore, Cat # 36-003) were used. P-MYPT1 was normalized by anti-MYPT1 antibodies (Millipore, Cat #07-672). Incubation with an HRP-conjugated anti-goat IgG (1:2000 dilution with Blotto) was carried out for 1 hour at room temperature under gentle rocking. Signals were visualized using a chemiluminescent ECL substrate (GE Healthcare, Piscataway, NJ). Densitometric analysis was performed using ImageQuant software. Protein levels were normalized using total RhoA and total MYPT1 protein levels.

Data analysis

Data are expressed as means ± s.e.m. Runx2, SOX9 and PPARγ gene expression levels were normalized against 18S rRNA assayed in the same sample tube. Statistical analysis was conducted using multiple Student *t*-tests with a Bonferroni adjustment to ensure that type I error was below 0.05 when comparing control and flow-exposed cells treated with different biochemicals. A *P*-value <0.05 was considered significant.

NIH Grant AR45989, VA Palo Alto Heath Care System Pre-doctoral Associated Health Rehabilitation Research Fellowship and the NSF Graduate Research Fellowship Program. The authors would also like to thank Dennis Carter and Diane Wagner for their involvement. Deposited in PMC for release after 12 months.

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