Mechanical Loading Regulates NFATc1 and β-Catenin Signaling through a GSK3β Control Node*

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Mechanical stimulation can prevent adipogenic and improve osteogenic lineage allocation of mesenchymal stem cells (MSC), an effect associated with the preservation of β-catenin levels. We asked whether mechanical up-regulation of β-catenin was critical to reduction in adipogenesis as well as other mechanical events inducing alternate MSC lineage selection. In MSC cultured under strong adipogenic conditions, mechanical load (3600 cycles/day, 2% strain) inactivated GSK3β in a Wnt-independent fashion. Small interfering RNA targeting GSK3β prevented both strain-induced induction of β-catenin and an increase in COX2, a factor associated with increased osteoprogenitor phenotype. Small interfering RNA knockdown of β-catenin blocked mechanical reduction of peroxisome proliferator-activated receptor γ and adiponectin, implicating β-catenin in strain inhibition of adipogenesis. In contrast, the effect of both mechanical and pharmacologic inhibition of GSK3β on the putative β-catenin target, COX2, was unaffected by β-catenin knockdown. GSK3β inhibition caused accumulation of nuclear NFATc1; mechanical strain increased nuclear NFATc1, independent of β-catenin. NFATc1 knockdown prevented mechanical stimulation of COX2, implicating NFATc1 signaling. Finally, inhibition of GSK3β caused association of RNA polymerase II with the COX2 gene, suggesting transcription initiation. These results demonstrate that mechanical inhibition of GSK3β induces activation of both β-catenin and NFATc1 signaling, limiting adipogenesis via the former and promoting osteoblastic differentiation via NFATc1/COX2. Our novel findings suggest that mechanical loading regulates mesenchymal stem cell differentiation through inhibition of GSK3β, which in turn regulates multiple downstream effectors.

The output of differentiating cells from the marrow mesenchymal stem cell (MSC) pool reflects a reciprocal relationship between the numbers of adipocytes and osteoblasts (1). This suggests that regulation of MSC differentiation should be an important point where control of osteoprogenitor and adipocyte output is delineated by microenvironmental factors. It is known that marrow is replaced by fat in sedentary and aged individuals (2, 3). Exercise, with subsequent physical loading of marrow MSC, increases bone mass, and generally represses fat (4, 5). Indeed, MSC are highly responsive to mechanical signals during differentiation, and mechanical load can apprehend adipogenesis even under strong adipogenic culture conditions (6).

As such, the effects of mechanical strain or exercise to promote bone formation and limit adipogenesis are conveyed, at the very least, through direct effects on MSC differentiation.

Our previous work suggests that β-catenin signaling is largely responsible for mechanical limitation of adipocyte differentiation (6). β-Catenin is known to regulate adipogenesis at multiple loci, including attenuation of PPARγ expression (7) and a negative effect on PPARγ activation of gene targets (8). The fall in β-catenin that accompanies the rising levels of PPARγ and adiponectin consequent to adipogenesis can be prevented by a regimen of daily application of mechanical strain. Furthermore, preservation of β-catenin levels in MSC cultures through pharmacological GSK3β inhibition, which limits β-catenin degradation, also prevents adipogenesis as we and others have shown (6, 9), providing further evidence that mechanical load utilizes β-catenin signaling. Mechanical regulation of β-catenin signaling is not only apparent in MSC but is also measured in cultured pre-osteoblasts (10–12). Regulation of β-catenin activity is known to be influenced by GSK3β, an enzyme that is inhibited via phosphorylation through multiple inputs such as insulin-stimulated Akt (13), as well as Wnt binding to LRP targets (14, 15).

Another target of mechanical input to bone cells is cyclooxygenase-2 (COX2), which rises sharply after mechanical strain (10) and fluid shear (16). An increase in COX2 is associated with differentiation of osteoblasts (17, 18), which designates this gene as important to understanding MSC lineage allocation. COX2 is known to be a target of β-catenin (19–22), raising the possibility that mechanical activation of β-catenin promotes COX2 gene transcription. Pharmacologic inhibition of GSK3β, which regulates β-catenin, has been shown to increase both COX2 and bone formation (12). COX2 expression is suggested to inhibit adipocyte differentiation (23, 24). Thus, much experimentation implicates mechanical regulation of GSK3β, with consequent effects on β-catenin, as an important pathway directing MSC lineage allocation.

We here investigate the contribution of mechanical inhibition of GSK3β, resulting in increased β-catenin signaling to both adipogenesis and COX2 activation. Although we will show that β-catenin is largely responsible for transducing mechani-
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cal inhibition of adipogenesis, we find that β-catenin is not involved in mechanical stimulation of COX2. Instead GSK3β regulation of NFATc1 nuclear accumulation controls COX2. Our results are shown both for C3H10T1/2 MSC and marrow-derived MSC.

EXPERIMENTAL PROCEDURES

Reagents—Fetal bovine serum was from Atlanta Biologicals (Atlanta, GA). Culture media, trypsin-EDTA reagent, antibiotics, Lipofectamine 2000, reverse transcriptase, and Taq polymerase were from Invitrogen. Insulin, SB415286, iodoniamycin, tacrolimus, and actinomycin at the concentrations specified in the legends were from Sigma-Aldrich. DKK-1 and Wnt3a were purchased from R & D Systems (Minneapolis, MN).

Cells and Culture Conditions—C3H10T1/2 cells were maintained in growth medium (10% fetal bovine serum, 100 µg/ml penicillin/streptomycin). For experiments, the cells were plated at a density of 6,000–10,000 cells/cm² in collagen-I coated silicone membrane plates and cultured for 2 days before beginning experiments. Adipogenic medium included 0.1 µM dexamethasone, 5 µg/ml insulin, and 50 µM indomethacin.

Key experiments were replicated in a marrow-derived mesenchymal stem cell line generated from C56/BL6 wild-type mice using the procedure of Peister et al. (25). These cells readily undergo differentiation into osteogenic, adipogenic, or alternative lineages using standard modifiers. The cells were plated under similar adipogenic conditions as above. We have termed these cells “marrow-derived MSC” (mdMSC) in the text.

Mechanical Strain—Uniform biaxial strain was applied to C3H10T1/2 cells or mdMSC plated on collagen-I coated silicone membrane plates using the Z-Strain cell deformation device (6, 26). A daily regimen of 2% strain was delivered at 10 cycles/min for 3600 total cycles. Strain levels experienced by cells within marrow environment are unknown. By virtue of their adherence to substrate and the architecture of the plasma membrane as well as the intracellular compartment itself, the cells will experience a complex and heterogeneous strain distribution, which could include strains of this magnitude (27).

RNA Interference—The cells were transfected with siRNA (100 nm) in serum-free OptiMEM overnight before replacing the medium.

Nuclear and Cytoplasmic Protein Fractionation—The cells were washed with 1× phosphate-buffered saline, the cell pellet was resuspended in 0.33 M sucrose, 10 mM Hepes, pH 7.4, 1 mM MgCl₂, 0.1% Triton X-100 (pellet versus buffer, 1:5) and placed on ice for 15 min. After 3,000 rpm for 5 min, the supernatant was collected (cytoplasmic fraction). The pellet was resuspended in 0.45 M NaCl and 10 mM Hepes, pH 7.4, and placed on ice for 15 min. After centrifugation at 12,000 rpm for 5 min, the nuclear fraction supernatant was collected.

Real Time RT-PCR—Total RNA was isolated with the RNeasy mini kit (Qiagen) and treated with DNase I. Reverse transcription of 1 µg of RNA in a total volume of 20 µl was performed prior to real time PCR (Bio-Rad iCycler). 25-µl amplification reactions contained primers (0.5 µM), dNTPs (0.2 mM each), 0.03 units of Taq polymerase, and SYBR-green (Molecular Probes, Eugene, OR) at 1:150,000. Aliquots of cDNA were diluted 5–5000-fold to generate relative standard curves to which sample cDNA was compared. PPARγ, adiponectin, COX2, WISP1, and 18 S primers were as in Refs. 6 and 10. For β-catenin the forward and reverse primers were 5′-CCCTGAGGCTAGATGAGG-3′ and 5′-TGTCAGCTCAGAATTTCAC-3′, respectively. Standards and samples were run in triplicate. PCR products were normalized to 18 S amplicons in the RT sample, and standardized on a dilution curve from RT sample.

Western Blotting—Whole cell lysates were prepared with lysis buffer (150 mM NaCl, 50 mM Tris HCl, 1 mM EDTA, 0.24% sodium deoxycholate, 1% Igepal, pH 7.5) containing 25 mM NaF and 2 mM Na₃VO₄, aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride were added prior to each lysis. 5–20 µg of fractionated or whole lystate proteins were loaded onto a 7–10% polyacrylamide gel for chromatography and transferred to polyvinylidene difluoride membrane. After blocking, primary antibody was applied overnight at 4 °C including antibodies against active β-catenin (clone 8E7; Upstate, Temecula, CA), total β-catenin (BD, Bedford, MA), phospho-GSK3β (ser9, clone 2D3; Upstate, Lake Placid, NY), total GSK3β (Chemicon, Billerica, MA), PPARγ, adiponectin, Cox-2, NFATc1, and tubulin (Santa Cruz, CA). Secondary antibody conjugated with horseradish peroxidase was detected with ECL plus chemiluminescence kit (Amerham Biosciences). The images were acquired with a HP Scanjet and densitometry determined using NIH ImageJ, 1.37v.

Luciferase Assay—C3H10T1/2 cells were seeded at 100,000 cells/well; 24 h after seeding, the cells were transfected with 2.5 µg/well COX2 reporter plasmid (gift from H. Herschman, UCLA) and 5 µl/well Lipofectamine, and the medium was replaced at 24 h. β-Galactosidase plasmid (1 µg/well) was co-transfected together to control for transfection efficiency. The assays were performed with luciferase assay (Promega, Madison, WI) and Galacto-StarTM (Applied Biosystems, Bedford, MA).

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation was performed as described previously (28, 29). C3H10T1/2 cells were treated with or without SB415286 for 4 h. The cells were subjected to cross-linking with 1% formaldehyde for 10 min at 37 °C, followed by a wash with phosphate-buffered saline. The cells were extracted in 5 mM Pipes, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, and then with 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1. Chromatin pellets were sonicated to an average of 300–500-bp fragments of DNA, centrifuged, and then diluted into chromatin immunoprecipitation buffer (16.7 mM Tris-HCl, pH 8.1,150 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA). Immunoprecipitations were performed overnight at 4 °C with the indicated antibodies and then collected following a 1-h incubation with salmon sperm DNA- and bovine serum albumin-pretreated Zysorbin (Zymed Laboratories Inc., San Francisco, CA). The precipitates were then washed sequentially, and the cross-links were reversed with an overnight incubation at 65 °C in 1% SDS and 0.1 M NaHCO₃. DNA fragments were purified using Qiagen QIAquick Spin Kits (Valencia, CA) and subjected to PCR techniques using primers (forward, 5′-TTGACAAGTCGCTGAAATGGG-3′; reverse, 5′-GGGACGAGAGAGCAGACG-
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FIGURE 1. **Mechanical effect on β-catenin is critical for inhibition of adipogenesis.** A, C3H10T1/2 cells in adipogenic medium were treated with or without DKK-1 (50 ng/ml), and strain was applied daily for 4 days. Total cellular proteins were immunoblotted for adiponectin (APN), PPARγ, β-catenin (β-cat) both total and active, and tubulin as designated. B, DKK-1 (50 ng/ml, added 30 min prior to Wnt3a) prevents Wnt3a (100 ng/ml) stimulation of GSK3β phosphorylation and increase in active β-catenin measured at 3 h. C, DKK-1 does not prevent GSK3β phosphorylation induced by 1 h of mechanical strain. D, β-catenin mRNA and protein shown 48 h after addition of siCat or siScr (CTL). E, 48 h after the addition of siScr or siCat, the strain regimen (left panel) or SB415286 (20 μM, right panel, noted as SB) was applied for 4 days. Adiponectin mRNA was amplified by real time RT-PCR. For mRNA experiments, significant change from control condition is shown by an asterisk, p < 0.01.

TCC-3′) designed to amplify fragments of the murine COX2 promoter region (residues −136 to +159). All of the PCR analyses for the primer set were carried out in a predetermined linear range of DNA amplification.

**Statistical Analysis**—The results are expressed as the means ± S.E. Statistical significance was evaluated by one-way analysis of variance or t test (GraphPad Prism). All of the experiments were replicated at least once to assure reproducibility. Densitometry data, where given, were compiled from at least three separate experiments.

**RESULTS**

**Mechanical Effect on β-Catenin Is Critical for Inhibition of Adipogenesis**—As MSC enter the adipogenic lineage and express PPARγ and adiponectin, the levels of both active and total β-catenin drop rapidly as we have previously shown (6). Mechanical load applied daily inhibits adipogenesis while maintaining β-catenin levels; pharmacological inhibition of GSK3β with subsequent preservation of β-catenin levels is even more potent. This work implicated GSK3β in the preservation and activation of cellular β-catenin given that phosphorylation of serine 9, causing inhibition of GSK3β, is demonstrated within 30 min of applying strain. Here, to further understand mechanical control of adipocyte differentiation, we first wished to ascertain whether the mechanical effect was separate from Wnt signaling, which has been implicated by others as essential to mechanical signaling (30). As shown in Fig. 1A, after 4 days of culture in adipogenic medium, the application of a daily regimen of mechanical strain (3600 cycles/day at 10 cpm, 2%) prevented the decrease in levels of active and total β-catenin, as well as limited expression of PPARγ and adiponectin proteins in this series of experiments, with the consequent inhibition of lipid laden adipocyte morphology. We show here that the addition of the Wnt signaling inhibitor DKK-1 (31) had no effect on the ability of strain to prevent adipogenesis. To confirm that DKK-1 blockade was effective, Fig. 1B shows that exogenous Wnt3A was unable to induce GSK3β phosphorylation or activation of β-catenin once DKK-1 was added. Importantly, DKK-1 blockade of Wnt-activated signaling over the 4 days of culture did not interfere with strain effect to inhibit GSK3β, demonstrated in Fig. 1C as an increase in phospho-GSK3β after application of the daily strain regimen for 4 days.

To investigate whether mechanical preservation of β-catenin was critical to mechanical inhibition of the MSC response to adipogenic conditions, β-catenin was knocked down through siRNA targeting. Shown in Fig. 1D, 100 nm siRNA-targeting β-catenin (siCat) reduced β-catenin mRNA levels by 80% compared with MSC treated with a control siRNA (siScr). In the right panel, shown 3 days after treatment with siRNAs, siCat treatment effectively decreased both active and total β-catenin proteins. Silencing β-catenin almost entirely prevented the ability of the daily mechanical regimen to decrease adipogenesis, demonstrated as a lack of significant effect of mechanical strain on expression of adiponectin mRNA and shown in the left panel of Fig. 1E. Similarly, inhibition of GSK3β with the specific GSK3β ATP-competitor SB415286 (32), shown in the right panel of Fig. 1E, even more strongly inhibited adiponectin mRNA, an effect almost entirely prevented in cultures where β-catenin knockdown was evident. These data indicate that mechanical prevention of adipogenesis is largely due to the
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Maintenance of active levels of β-catenin, a process likely regulated through mechanical phosphorylation of GSK3β.

Mechanical Stimulation of COX2 Does Not Require β-Catenin Activation—We next examined the effect of mechanical loading on COX2, a gene that responds to multiple types of mechanical stimulation in bone cells, as we and others (10, 12, 16) have reported. Importantly, COX2 expression is known to inhibit adipocyte differentiation (23, 24), and β-catenin has both transcriptional and post-transcriptional effects on COX2 (19–21, 33). To verify that COX2 was subject to mechanical regulation in MSC, the cells were cultured in adipogenic medium for 4 days, and the 3600 cycle strain regimen was applied daily. COX2 mRNA was expressed at a level 4-fold greater than that in unstrained MSC, as shown in Fig. 2A. Concurrently, shown in the next panel is the reduction in adiponectin mRNA, as well as an increase in the expression of WISP1, a gene we have reported is increased through mechanical activation of β-catenin (6, 10). To verify that COX2 was subject to mechanical regulation in MSC, the cells were cultured in adipogenic medium for 4 days, and the 3600 cycle strain regimen was applied daily. COX2 mRNA was expressed at a level 4-fold greater than that in unstrained MSC, as shown in Fig. 2A. Concurrently, shown in the next panel is the reduction in adiponectin mRNA, as well as an increase in the expression of WISP1, a gene we have reported is increased through mechanical activation of β-catenin (6, 10).

The mechanical regulation of COX2, however, is independent of β-catenin. As shown in Fig. 2C, MSC treated with siCat expressed not only increased basal levels of COX2 after knockdown of β-catenin but also an enhanced response to strain. In the representative Western shown, active and total β-catenin levels were almost entirely silenced for 4 days after the introduction of the siRNA targeting β-catenin compared with cultures treated with siScr. The blunting of strain inhibition of adiponectin is seen in the figure. To confirm this, densitometry data for adiponectin bands allowed quantitation of effect: adiponectin protein density from four separate knockdown experiments are shown in the bottom graph of Fig. 2C, revealing that there was a significant impairment of the ability of strain to inhibit adiponectin expression.

Similarly, pharmacological inhibition of GSK3β with SB415286, which we have previously shown prevents adipogenesis while increasing β-catenin levels in this culture system (6), both inhibits adiponectin expression and, here, stimulates COX2 protein expression (Fig. 2D). Confirming the role of β-catenin in attenuating adipogenesis, inhibition of GSK3β was less effective when β-catenin was reduced, shown by the decreased ability of mechanical strain to inhibit adiponectin shown in the Western; the graph below shows compiled densitometry data for four experiments. In contrast, COX2 continued to respond to inhibition of GSK3β with a substantial increase in protein level, despite the lack of response of active
β-catenin. These data suggest that GSK3β inhibition is involved in the mechanical regulation of both downstream genes in MSC. The differential requirement for β-catenin for mechanical inhibition of adipogenesis compared with mechanical stimulation of COX2 indicates that GSK3β regulates COX2 through an alternate effector.

GSK3β Is Involved in Regulation of Both Adiponectin and COX2—Because inhibition of GSK3β by both mechanically induced phosphorylation and pharmacologic means produces differential effects on adiponectin and COX2, we wished to confirm the involvement of GSK3β. siRNA targeting GSK3β was used to knock down this molecule in MSC cultures. In Fig. 3A, knockdown of GSK3β 4 days after siRNA treatment limits the expression of adiponectin in the presence or absence of strain. This is likely due to the increase in β-catenin level, which occurs upon removing the known tonic effect of GSK3β to induce β-catenin proteolysis (34, 35). Strain application, however, was still effective in reducing adiponectin expression further, as was the addition of SB415286, shown in Fig. 3B. That the specific pharmacological inhibition had similar effects on adiponectin as did strain in the presence of GSK3β knockdown suggests that there was enough remaining GSK3β to allow further reductions in adiponectin. Remaining actionable GSK3β is suggested by our data showing that total GSK3β falls after strain and treatment with SB415286 in the siRNA GSK3β condition; this reduction in total GSK3β may represent continuous or intermittent activation, because it has been shown that inhibition of GSK3β leads to decreases in total levels (13).

Because knockdown of GSK3β strongly increased COX2 expression in the basal state at 4 days (data not shown), an event consistent with the known effects of GSK3β inhibition on COX2 mRNA levels (36), we examined cell cultures at an earlier time point after GSK3β silencing. Two days after introducing siRNA targeting GSK3β, basal COX2 was unchanged. In this situation, both strain (Fig. 3C) and SB415286 (Fig. 3D) were ineffective in stimulating COX2. In comparison, siRNA silencing of β-catenin had no effect on the ability of either strain or pharmacologic inhibition of GSK3β to induce COX2. This indicates that strain induction of COX2 is not dependent on β-catenin but does require inactivation of GSK3β.

Mechanical Strain Promotes NFATc1 Nuclear Accumulation—Recent evidence shows that NFAT nuclear transcription factors can regulate COX2 expression through increased transcription; the promoter of COX2 is known to contain an NFAT consensus response element (37). GSK3β can modulate NFAT signaling through enhancing NFAT rephosphorylation and exit from the nucleus (38). We thus considered whether mechanical effects on GSK3β might modulate COX2 via NFATc1. In Fig. 4A, mechanical strain caused accumulation of nuclear NFATc1, which remained elevated 4 h after initiating strain; PARP was run on stripped NFATc1 blots to prove that nuclear proteins were present. NFATc1 has several splice variants that are differentially expressed in tissue. In MSC, we typically see three bands in the nuclear fraction that increase in a parallel fashion. COX2 started to rise in the cytoplasm by 30 min, consistent with an early increase in nuclear NFATc1; lactate dehydrogenase was run on stripped COX2 blots to confirm that cytoplasmic proteins were present. Inhibition of GSK3β by SB415286 revealed the same pattern of early nuclear translocation of NFATc1, an effect sustained for at least 4 h and accompanied by increased COX2 protein (Fig. 4B).

To ascertain that GSK3β was involved in the translocation of NFATc1 and its potential subsequent effect on COX2, GSK3β was silenced with siRNA. As shown in Fig. 4C, after GSK3β knockdown, NFATc1 and COX2 proteins were not affected by mechanical strain; NFATc1 did not translocate to the nucleus, and COX2 did not rise. Taken together, these data indicate that mechanical inhibition of GSK3β leads to nuclear accumulation of NFATc1 and that appearance of this transcription factor in the nucleus is involved in the mechanical regulation of COX2.

Sustained nuclear translocation of NFATc1 and increased COX2 expression at 4 h after strain application were unaffected by siRNA silencing of β-catenin, as shown in Fig. 4D. NFATc1 nuclear translocation caused by pharmacological inhibition of GSK3β was also unaffected by β-catenin knockdown (Fig. 4E).

Inhibition of Calcium/Calcineurin Signaling Does Not Block Strain-induced NFAT Nuclear Accumulation—Calcium/calcineurin signaling causes dephosphorylation of NFATc1 with subsequent COX2 expression (39). We analyzed whether this pathway might be involved in the mechanical activation of NFATc1 in MSC. MSC were treated with ionomycin to activate calcineurin. As shown in Fig. 5A, NFATc1 was rapidly translocated to the nucleus by 30 min and peaked at 60 min. Accompanying the ionomycin-induced increase in nuclear NFAT was
a substantial increase in COX2, as expected. Tacrolimus, a calcineurin inhibitor, abolished the ionomycin stimulation of NFATc1 and COX2, shown in Fig. 5B. Tacrolimus did not, however, block the effects of either mechanical loading (Fig. 5C) or pharmacologic inhibition of GSK3β to induce NFATc1 nuclear accumulation, nor did it affect COX2 induction (Fig. 5D). It was evident that tacrolimus reduced the basal levels of NFATc1, such that although strain and SB415286 increased the nuclear accumulation of NFATc1, the levels were actually lower, as was the effect on COX2 stimulation. However, that tacrolimus did not prevent stimulatory effects on either NFATc1 or COX2 indicates that mechanical regulation of NFATc1 nuclear accumulation does not require activation of calcineurin. Thus, alternation of GSK3β activity will have consequences for NFATc1 activity.

**NFATc1 Is Critical for Mechanical Control of COX2, but Not for Mechanical Inhibition of Adipogenesis**—To investigate whether NFATc1 was responsible for the mechanical stimulation of COX2, we next applied mechanical strain in the presence of siRNA targeting NFATc1. As shown in Fig. 6A, in cells 2 days after NFATc1 silencing, neither application of strain nor pharmacologic inhibition of GSK3β (Fig. 6B) were able to induce COX2. In cells treated with a scrambled siRNA, COX2 was increased by both regulatory GSK3β processes as shown in the first two lanes of each blot. The addition of siNFATc1 alone had an inconsistent effect on the “basal” level of COX2 protein.

In contrast, the effect of GSK3β inhibition by mechanical or pharmacological means to reduce adiponectin and PPARγ
expression was unaffected when NFATc1 was silenced (Fig. 6, C and D). This suggests that NFATc1 is not involved in the mechanical inhibition of adipogenesis but that COX2 expression requires activation of NFATc1.

mdMSC Respond to Mechanical Strain with Inhibition of GSK3 and Downstream Signaling—To confirm that our findings have relevance for MSC derived from bone marrow, we performed key experiments in a MSC derived directly from mouse marrow using an established protocol (25). These mdMSC readily acquire adipogenic characteristics upon exposure to adipogenic medium as shown in Fig. 7A, and strain prevents the development of Oil-Red O consistent with intracellular lipid. Analogous to results obtained with C3H10T1/2 MSC, mdMSC express high levels of adiponectin when cultured in the adipogenic medium and respond to a daily strain regimen by retaining β-catenin, as well as suppressing expression of adiponectin as shown in Fig. 7B. In mdMSC that are treated with siRNA targeting β-catenin, the strain effect to inhibit adipogenesis is attenuated, shown in the rows where siRNA β-catenin rather than siScr was added. Strain also induced COX2 protein expression in these cells, an effect that was not affected by β-catenin knockdown. When siRNA targeting GSK3β was added (Fig. 7C), strain did not cause increases in active β-catenin, as seen in the cells dosed with control siRNA. As in the C3H10T1/2 MSC, siRNA GSK3β reduced adipogenesis, likely through inhibition of early C/EBPβ phosphorylation necessary to adipogenesis. The further decrements in adipogenesis in the presence of strain are likely due to further decrease in this early step caused by GSK3β phosphorylation. Finally, in mdMSC, mechanical strain caused NFATc1 nuclear accumulation (shown in Fig. 7D, measured at 4 h). This was associated with increased COX2 protein expression. In the presence of strain GSK3β, neither NFATc1 accumulation nor COX2 protein induction resulted from the application of mechanical strain.

GSK3β Activates COX2 Gene Transcription—To ascertain the mechanism by which NFATc1 increased COX2 protein in MSC, activity of the murine 720-base pair proximal COX2 promoter was tested (40). Luciferase activity of this promoter did not change after treatment with either ionomycin or SB415286, shown in Fig. 8A. Because similar proximal promoter sequences have been shown to be weakly stimulated after treatment with phorbol myristate and...
prostaglandin E2 in osteoblasts (40), we verified that this was also true in MSC (Fig. 8B).

We next considered that inhibition of GSK3β might increase COX2 expression through post-transcriptional means, prolonging the half-life of the COX2 mRNA, which is known to be highly unstable. As shown in Fig. 8C, the half-life of COX2 mRNA was less than 1 h, as expected. The addition of complete GSK3β inhibition, although increasing COX mRNA by 4–6-fold after 24 h of treatment (Fig. 2A), had no effect on COX2 mRNA half-life. This was also true when examined at shorter time periods (data not shown). This result excluded the possibility that NFATc1 induction of COX2 in MSC was due to the stabilization of COX2 mRNA.

The rapid and robust response of COX2 mRNA to NFATc1 stimulus suggested a direct transcriptional regulatory effect. Because the presence of an NFATc1 responsive element in a more distant promoter region is unknown, we queried transcriptional regulation by asking whether RNA polymerase II had an increased association with the COX2 gene after inhibition of GSK3β. Using chromatin immunoprecipitation after treatment with SB415286, DNA-protein-DNA interactions were evaluated. Complexes were either precipitated with IgG (control) or with an antibody to RNA polymerase II. The COX2 gene (−136 to +159) was amplified by PCR. As shown in Fig. 8D, the association of RNA polymerase II with COX2 was increased in cells where GSK3β was inhibited. This evidence supports transcriptional regulation of COX2 through GSK3β-regulated NFATc1.

**DISCUSSION**

It has long been known that skeletal tissue responds to mechanical signals generated during daily loading, and more recently, it was discovered that mechanical signals are recognized by mesenchymal stem cells resulting in promotion of osteoblastic lineage and decreased allocation into the adipocyte line (5, 6). With the work presented here we show that multiple downstream effectors are conscripted toward this end through a single regulatory node, GSK3β, which is inhibited by biomechanical input.

In its basal active state, GSK3β directly phosphorylates β-catenin, targeting it for proteasomal degradation. Induction of phosphorylation at serine 9 inhibits GSK3β (41) and is a central mechanism invoked in allowing β-catenin activation during canonical Wnt signaling (42). In this manner, Wnt has effects both to repress adipogenesis (34) and to stimulate osteoblastogenesis (43–45). Mechanical loading similarly stimulates osteoblastogenesis and represses adipogenesis (4), an effect that most certainly involves inhibition of GSK3β activity (6, 11). However, despite suggestions that mechanical stimulation increases osteogenesis through Wnt signaling (30, 46), we have
previously shown that mechanical inhibition of GSK3β is Wnt/ 
LRP-independent in pre-osteoblasts (10). Inhibition of GSK3β 
by substrate strain in MSC, as well as subsequent causal effects, 
is independent of Wnt association with its receptor complex as 
we have shown here. The identity of the kinase responsible for 
strain induction of GSK3β phosphorylation is as yet unknown 
but may be AKT, which we and others have shown responds to 
strain and is known to target GSK3β (6, 10, 11).

An important regulatory role of β-catenin during adipocyte 
differentiation has been firmly established (8, 47). We 
previously showed that mechanical loading inhibits the 
expression of PPARγ and adiponectin in MSC grown under 
adipogenic conditions while maintaining β-catenin levels 
(6). To ascertain that β-catenin was critical to mechanical inhi-
bition of adipogenesis, we here demonstrated that β-catenin 
silencing almost entirely prevents the mechanical inhibitory 
effect. That strain moderately reduces adipogenesis during 
β-catenin knockdown likely results from several factors. First, 
the efficiency of siRNA silencing is not complete. More impor-
tantly, active GSK3β phosphorylates C/EBPβ, which in turn is 
important during the mitotic clonal expansion of early adipocy-
te precursors (48) as well as increasing transcription of mul-
tiple adipocyte genes (49). Thus, mechanical inhibition of 
GSK3β should be expected to limit adipogenesis in a partially 
β-catenin-independent fashion.

Although adipogenesis is limited through preservation of 
β-catenin levels, osteogenesis is enhanced. This has been 
inferred by the association of activating mutations in the Lrp5 
receptor, which result in high bone mass through multiple 
mechanisms that include stimulation of osteoprogenitor emer-
gence from the MSC and increase in osteoblast function (50– 
52). Osteoblastogenesis is also dependent on COX2, an enzyme 
that, through its generation of prostaglandin E2, is important 
for bone formation (53) and stimulates MSC maturation fur-
ther along the osteoblast phenotype (18). During adipogenesis 
COX2 is decreased (24), and PPARγ also directly down-regu-
lates COX2 (17). As a positive stimulus of bone formation, 
COX2 is a known target of mechanical stimulation in bone cells 
(10, 12) and is itself regulated by β-catenin (21). Indeed, β-cate-
nin activates COX2 transcription in gastrointestinal cells (19, 
54, 55). Thus, although we expected that in MSC that COX2 
would be regulated by mechanical strain and have demon-
strated that here (Fig. 2), we were surprised that the robust 
mechanical regulation of COX2 was independent of β-catenin.

GSK3β has been shown to regulate COX2 through post-
transcriptional processes (36). Our data indicated that although 
β-catenin was not involved, COX2 expression induced by 
mechanical input, and inhibition of GSK3β was prevented by 
RNA silencing of GSK3β. This indicated that other targets of 
GSK3β might be involved in affecting MSC lineage fate. 
NFATc1 was a strong candidate because it is not only regulated 
by calcium signaling, itself subject to mechanical regulation (56, 
57), but is also thought to have multiple roles in regulating bone 
mass (58, 59). Furthermore, COX2 is increased by NFAT acti-
vation in T-cells (37), keratinocytes (60), and colon cells (61).

Importantly, we have now shown that mechanical strain 
causes NFATc1 translocation to the nucleus in MSC. Because 
multiple types of mechanical input have been shown to increase 
intracellular calcium concentration (56, 62, 63), perhaps an 
NFAT response should not be surprising because NFAT is acti-
vated through calcineurin-stimulated dephosphorylation. A 
second regulatory control of NFAT activity is through its 
rephosphorylation and return to the cytoplasm enacted by 
kinasas such as GSK3β, (38), which can be found in the nucleus 
(64). The time course of mechanical NFATc1 activation is con-
sistent with both early NFATc1 translocation through calcium/ 
calcineurin and prolonged nuclear retention through inhibition 
of GSK3β. Because tacrolimus completely inhibited nuclear 
NFATc1 translocation caused by ionophore treatment but had 
little to no effect on the nuclear NFATc1 levels caused by 
mechanical stimulation, or on pharmacological inhibition of 
GSK3β, we contend that inhibition of GSK3β is the primary 
mechanism by which NFATc1 was retained in the nucleus at 
4 h. Silencing NFATc1 prevents mechanical stimulation of 
COX2, while having little to no effect on mechanical inhibition of 
adiponectin, an effect opposite to that occurring during 
β-catenin silencing.

Interestingly, the cytoplasmic to nuclear cycling of NFAT 
proteins is subject to cellular calcium oscillations in MSC (65). 
These oscillations are damped during adipogenic differen-
tiation, leading to decreases in both total and nuclear NFAT 
(66). This effect likely explains the decreased NFATc1 seen 
even 30 min after the addition of tacrolimus to our MSC 
cultures; inhibition of calcineurin blocks the calcium/cal-
cineurin signal initiated by ATP autocrine/paracrine signal-
ing, which causes spontaneous [Ca2+]i oscillations. Adding 
further complexity to this situation, although silencing 
NFATc1 does prevent COX2 induction by strain, it also can 
increase basal levels of this enzyme. This may arise out of the 
very early requirement for NFATc1 during adipogenesis 
(35), an effect that we have also seen, silencing NFATc1 MSC 
cultured under adipogenic conditions prevents adipocyte 
differentiation (data not shown). Thus, higher expression of 
COX2 in the presence of NFATc1 silencing may be due to 
the limitation of early adipogenesis.

NFAT is reported to regulate COX2 expression at the tran-
scriptional level (60, 61), and at least one NFAT response ele-
ment is found in the COX2 proximal promoter (37). We were 
unable to show that NFATc1 activation accompanying either 
ionophore or GSK3β inhibition had any effect on the activity of 
the murine 720-nucleotide proximal COX2 promoter, despite 
the more than 10-fold increases in COX2 mRNA measured 
after these treatments. AU-rich elements of the 3′-untranslated 
region of COX2 also contribute to mRNA stability (20, 67), a 
post-transcriptional mechanism that has been suggested to be 
as sensitive to regulation by GSK3β (36). In MSC, the very short 
half-life of COX2 mRNA was unaffected by inactivation of 
GSK3β. To support a transcriptional mechanism for effects of 
GSK3β/NFATc1, we queried whether the association of RNA 
polymerase II with the COX2 gene would be increased after 
inhibition of GSK3β using a chromatin immunoprecipitation 
strategy (68). Indeed, 4 h after treating MSC with the specific 
GSK3β inhibitor, RNA polymerase II association with the 
COX2 promoter is increased, supporting our contention that 
transcription of COX2 is increased. Subtle effects on mRNA 
pool sizes might also explain our results.
Mechanical Regulation of GSK3β as a Control Node

This evidence leads us to conclude that mechanical control of GSK3β regulates adipocyte differentiation through preservation of β-catenin, an effect that also promotes the expansion of the osteoprogenitor pool. At the same time mechanical inhibition of GSK3β regulates COX2 through NFATc1, a second effector involved in promotion of osteogenesis. GSK3β has thus emerged as a control locus regulating downstream genes through multiple signaling pathways in MSC. Mechanical control of this major control locus in MSC lineage allocation begins to explain positive effects of exercise that are not calorie-driven.

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