

**MECHANICAL STRAIN INHIBITS ADIPOGENESIS IN MESENCHYMAL STEM CELLS BY  
STIMULATING A DURABLE  $\beta$ -CATENIN SIGNAL**

Abbreviated title: Mechanical strain inhibits adipogenesis

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**Key Words:** obesity, exercise, osteoblast, GSK3 $\beta$

**Support:** This work was supported by NIH grants AR42360 (JR), AR52014 (JR), AR43498 (CR).

**Disclosures:** BS, ZX, NC, MM and JR have nothing to declare. C.R. has equity interests in Juvent Medical.

## **Abstract**

The ability of exercise to decrease fat mass and increase bone mass may arise through mechanical biasing of mesenchymal stem cells (MSCs) away from adipogenesis and towards osteoblastogenesis. C3H10T1/2 mesenchymal stem cells cultured in highly adipogenic medium express PPAR $\gamma$  and adiponectin mRNA and protein and accumulate intracellular lipid. Mechanical strain applied for 6h daily inhibited expression of PPAR $\gamma$  and adiponectin mRNA by up to 35 and 50% respectively after 5 d. A decrease in active and total  $\beta$ -catenin levels during adipogenic differentiation was entirely prevented by daily application of mechanical strain; further, strain induced  $\beta$ -catenin nuclear translocation. Inhibition of GSK3 $\beta$  by LiCl or SB415286 also prevented adipogenesis, suggesting preservation of  $\beta$ -catenin levels was important to strain inhibition of adipogenesis. Indeed, mechanical strain inactivated GSK3 $\beta$ , which was preceded by Akt activation, indicating that strain transmits anti-adipogenic signals through this pathway. Cells grown under adipogenic conditions showed no increase in osteogenic markers Runx2 and osterix; subsequent addition of BMP2 for 2 d increased Runx2 but not osterix expression in unstrained cultures. When cultures were strained for 5 days before BMP2 addition, Runx2 mRNA increased more than in unstrained cultures and osterix expression more than doubled. As such, mechanical strain enhanced MSC potential to enter the osteoblast lineage despite exposure to adipogenic conditions. Our results indicate that MSC commitment to adipogenesis can be suppressed by mechanical signals, allowing other signals to promote osteoblastogenesis. This data suggests positive effects of exercise on both fat and bone may occur during mesenchymal lineage selection.

## Introduction

Obesity, a disease of excess adipose tissue, and osteoporosis, indicated by decreased bone mass, are each suppressed by exercise. Linking these diseases further, adipocytes and osteoblasts arise from a common progenitor, the mesenchymal stem cell (1-3) and signals that promote bone marrow stem cell differentiation towards one lineage may preclude the formation of the other. For example, there is an inverse relation between bone marrow adiposity and the amount of bone in the axial and appendicular skeleton of young adults (4) while in aging individuals, trabecular bone is essentially replaced by fat tissue (5). Conversely when the Wnt co-receptor LRP5 is constitutively activated, causing an increase in bone mass, there is also decreased fat in the bone marrow (6). Evidence suggests that mechanical factors might have similar effects on fat and bone.

Exercise effectively combats obesity while promoting the formation of bone and muscle (7, 8). This reciprocal effect raises the possibility that exercise might influence mesenchymal stem cell (MSC) lineage allocation. Indeed, immobilization leads to a near doubling of marrow fat within 15 weeks of bed rest (9), and microgravity simulation decreases osteogenesis while increasing adipogenesis (10). Similarly, running decreases marrow fat expression (11) while exposure to extremely low magnitude mechanical signals can alter cell fate of MSCs in growing mice by inhibiting adipogenesis (12). In vitro, mechanical loading of osteoprogenitor cells promotes the proliferation (13, 14) as well as the function of osteoblasts (15-17). Mechanical strain induction of osteoblastogenesis is further associated with downregulation of Peroxisome Proliferator-Activated Receptor (PPAR $\gamma$ ) (11), a transcription factor critical for adipogenesis.

Haploinsufficiency of PPAR $\gamma$  is associated with suppressed adipogenesis and augmented osteoblastogenesis (18). Increased PPAR $\gamma$  enhances the proteasomal degradation of  $\beta$ -catenin, a critical regulator of osteoblastogenesis, with the sum effect being retarded osteogenesis (19). This suggests that preventing degradation of  $\beta$ -catenin could bias stem cell differentiation away from formation of adipocytes, leaving MSCs to respond to

environmental cues which engage other lineages. Recent work indicates that mechanical signals can activate canonical  $\beta$ -catenin signals in osteoblast cells (20) and promote expression of Wnt/ $\beta$ -catenin target genes (21). Alteration of the canonical  $\beta$ -catenin action, either by a gain of function mutation in LRP5 or by the presence of a GSK3 $\beta$  inhibitor, enhances the anabolic response of osteoblasts to mechanical loading (22). Taken together these data suggest that  $\beta$ -catenin should be a key factor influencing exercise's reciprocal control of fat and bone.

The work presented here demonstrates that mechanical signals can participate in regulating lineage selection of mesenchymal precursors. Our approach involved attempting to mechanically counteract a strong adipogenic stimulus by subjecting MSCs to a daily regimen of mechanical strain. Daily loading increased the  $\beta$ -catenin signal duration and suppressed PPAR $\gamma$  induction of adiponectin and lipid droplet accumulation. At the same time, mechanical strain activated the  $\beta$ -catenin pathway through alteration of GSK3 $\beta$  phosphorylation via Akt. Finally, mechanical strain preserved the ability of MSCs to respond to an osteogenic stimulus in spite of prior exposure to an adipogenic environment.

## Materials and Methods

**Experimental overview.** On day one of all experiments, C3H10T1/2 cells were switched to either an Adipogenic or Multipotential medium. Mechanical signals were delivered to cultures beginning on the first experimental day, continuing daily until the end of the experiment (except where noted), and compared to unstrained controls.

**Reagents.** Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA). Culture media, glutamine, trypsin-EDTA reagent, antibiotics, oligofectamine, reverse transcriptase and Taq polymerase were purchased from Invitrogen (Carlsbad, CA). Insulin, all trans-retinoic acid, 4',6-diamidino-2-phenylindole, oil red-O, L-ascorbic acid 2-phosphate, clostridium histolyticum neutral collagenase, p-nitrophenyl phosphate (PNP-p), SB415286 and lithium chloride were obtained from Sigma-Aldrich (St. Louis, MO). The RNA isolation

kit and DNase I were from Qiagen (Valencia, CA) and random primers were from Ambion (Austin, TX).

**Culture conditions.** C3H10T1/2 cells were maintained in growth medium consisting of  $\alpha$ -MEM with 10% FBS, 1.25 mM glutamine and 100  $\mu$ g/ml penicillin/streptomycin up until passage 24. For experiments, cells were plated at a density of 6,000 - 10,000 cells/cm<sup>2</sup> in BioFlex plates (FlexCell Co, Hillsborough NC) and cultured for 2 days before change to adipogenic or multipotential medium on day 1 of an experiment. For adipogenic "A" medium, 0.1  $\mu$ M dexamethasone, 5  $\mu$ g/ml insulin, and 50  $\mu$ M indomethacin were added to the growth medium. For the multipotential "M" medium, 10 nM dexamethasone, 50  $\mu$ g/ml ascorbic acid, 1  $\mu$ M  $\beta$ -glycerol phosphate, 10 nM all trans-retinoic acid, 5  $\mu$ g/ml insulin and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) were added.

**Mechanical strain.** Uniform biaxial strain was applied to C3H10T1/2 cells plated on 6-well Bioflex Collagen-I coated plates using the Flexcell FX-4000 system (Flexcell International, Hillsborough, NC). A daily regimen of 2% strain was delivered at 10 cycles/min for 3600 total cycles. Strain regimens were initiated at the beginning of each experiment except as noted.

**Real-time RT-PCR.** Total RNA was isolated by using the RNeasy mini kit (Qiagen) and treated with DNase I to remove contaminating genomic DNA. Reverse transcription was performed with 1  $\mu$ g of RNA in a total volume of 20  $\mu$ l per reaction. Real-time PCR was performed on a BioRad iCycler (Hercules, CA). 25  $\mu$ l amplification reactions contained primers at 0.5  $\mu$ M, dNTPs (0.2 mM each) in PCR buffer and 0.03 U Taq polymerase along with 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. WISP1, cyclin D1, PPAR $\gamma$ , adiponectin, Runx2, osterix (Osx) and 18S primers are shown in **Table I**. Standards and samples were run in triplicate. PCR products from all species were normalized for the amount of 18S amplicons in the RT sample, which was standardized on a dilution curve (23).

**Western blotting.** Whole cell lysates were prepared with lysis buffer (150 mM NaCl, 50mM Tris HCl, 1 mM EGTA, 0.24% sodium deoxycholate, 1%

IGEPAL, pH 7.5) containing 25 mM NaF and 2 mM Na<sub>3</sub>VO<sub>4</sub>. Aprotinin, leupeptin, pepstatin and PMSF were added prior to each lysis. 5-20  $\mu$ g of protein was loaded onto a 7 - 10% polyacrylamide gel for chromatography and transferred to PVDF membrane. After blocking, the membrane was incubated with primary antibodies overnight at 4°C. Antibodies directed against active  $\beta$ -catenin (clone 8E7, Upstate, Temecula, CA), total  $\beta$ -catenin (BD, Bedford, MA), phospho-GSK3 $\beta$  (ser9, clone 2D3, Upstate, Lake Placid, NY), total-GSK3 $\beta$  (Chemicon, Billerica, MA), phospho-Akt (ser 473, Cell Signaling, Danvers, MA), total Akt (11E7, Cell Signaling, Danvers, MA), PPAR $\gamma$ , adiponectin and actin (Santa Cruz, CA) were used. The antibody for active  $\beta$ -catenin was specific for the hypophosphorylated form of  $\beta$ -catenin (24). Secondary antibody conjugated with horseradish peroxidase was used to allow detection by ECL plus chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Images were acquired with a HP scanjet and densitometry determined using NIH ImageJ, 1.37v. To analyze differences in protein phosphorylation, arbitrary scanning units obtained for phosphoprotein band intensities were divided by arbitrary scanning units obtained for total protein band intensities for each sample analyzed. The average values obtained from at least three independent experiments for each variable were pooled.

**Immunofluorescence.** Following strain application, strained and control samples were fixed in 4% paraformaldehyde and permeabilized in 0.1% triton/PBS. An antibody to active  $\beta$ -catenin was applied overnight at 4°C. An anti-mouse FITC-conjugated antibody (Jackson ImmunoResearch, West Grove, PA) was applied for 30 minutes. Samples were mounted in anti-fade reagent (Molecular Probes, Eugene, OR) and viewed with a Leica SP2-AOBS confocal microscope.

**Histochemical staining.** After fixation in 2% formaldehyde, the cultures were then rinsed three times for 5 min in deionized water and cytoplasmic triglyceride droplets were stained with oil-red-O (25).

**Statistical analysis.** Results are expressed as the mean  $\pm$  SEM. Statistical significance was evaluated by one-way ANOVA or t-Test (GraphPad Prism). All experiments involving chromatography or

confocal imaging were replicated at least once to assure reproducibility. Densitometry data, where given, were compiled from at least 3 separate experiments.

## Results

### Adipogenic medium induces rapid acquisition of adipocyte phenotype

Lipid was appreciable in C3H10T1/2 cells cultured in adipogenic medium at 3 days, and was present in a majority of cells by 5 days (**Fig. 1A**); only cells grown in adipogenic medium stained for oil-red-O (shown as color plate in **Fig 7B**). mRNA analysis by semi-quantitative RT-PCR showed that PPAR $\gamma$  and adiponectin expression rose at 3 days in cells cultured in adipogenic medium but not in those grown in multipotential medium (**Fig. 1B**) and continued to rise. As measured by Western blot, PPAR $\gamma$  protein was barely detectable after 5 days in cells exposed to multipotential medium, but was strongly evident by 3 days in cells exposed to adipogenic medium, and continued to increase (**Fig. 1C**). Adiponectin protein was not measurable in cells cultured in multipotential medium, while in adipogenic medium adiponectin levels also continued to increase at 5 days.

### Mechanical strain inhibits adipogenesis while preventing a fall in $\beta$ -catenin

To determine whether MSC adipogenesis could be counteracted by mechanical signals, cultures were subjected to a daily regimen of mechanical strain. As  $\beta$ -catenin is associated with inhibition of adipogenesis, and mechanical strain can transiently increase  $\beta$ -catenin activity in osteoblasts (20), we hypothesized that mechanical suppression of adipogenesis might involve  $\beta$ -catenin. C3H10T1/2 cells were exposed to adipogenic medium and daily application of the strain regimen was initiated immediately. Shown in **Figure 2A**, whereas lipid droplets were evident in adipogenic conditions at 3 days, few if any lipid droplets were present in cultures subjected to strain. At 5 days, a majority of unstrained cells accumulated lipid, while less than half of strained cells exhibited lipid droplets.

Mechanical strain of C3H10T1/2 cells also modulated the expression of PPAR $\gamma$  and adiponectin (**Fig. 2B**). Compared to cells grown in the multipotential medium (control mRNA = 100%),

PPAR $\gamma$  mRNA expression in A medium increased by nearly 6-fold after 5d. Daily application of strain decreased PPAR $\gamma$  expression in adipogenic cultures by up to 35% ( $p < 0.001$ ). Adiponectin mRNA was significantly induced under adipogenic conditions at both days as expected, and was reduced by half by application of the daily strain regimen.

The  $\beta$ -catenin regulated genes cyclin D1 and WISP1 were assayed (**Fig. 2B**). Cyclin D1 mRNA decreased by half after 5 days under adipogenic conditions. Mechanical challenge increased cyclin D1 mRNA expression by nearly 3-fold at 5 days. WISP1 mRNA responses paralleled those seen for cyclin D1, dropping significantly at 5 days in A medium; in accord, mechanical strain increased WISP1 expression by nearly 6-fold over unstrained cultures at 5 days.

Both active and total  $\beta$ -catenin levels were reproducibly lower in cells in adipogenic compared to those in multipotential medium (**Fig. 2C**). This reduction in  $\beta$ -catenin was prevented by the strain regimen, such that active and total  $\beta$ -catenin levels were equivalent to those found in cells grown in the multipotential medium. Densitometry of active  $\beta$ -catenin bands was measured after 5 days showed that the decrease of  $\beta$ -catenin in adipogenic medium represented a significant change (data compiled from 3 separate experiments).

To confirm the increase in  $\beta$ -catenin due to strain, cultures were immunostained for active  $\beta$ -catenin after 3 days of daily loading. As shown in **Fig. 3**, active  $\beta$ -catenin staining increased in cell nuclei of strained cultures, shown by the overlap of DAPI and  $\beta$ -catenin antibodies on confocal microscopy.

### Inhibiting GSK3 $\beta$ mimics strain effects

To explore whether strain-mediated preservation of  $\beta$ -catenin levels was involved in the mechanism by which loading inhibits adipogenesis, we utilized pharmacological means of maintaining  $\beta$ -catenin. LiCl is a commonly used  $\beta$ -catenin activator with demonstrated effectiveness both in vitro and in vivo (26, 27). LiCl (10mM) was added to C3H10T1/2 cells grown under adipogenic conditions: lipid accumulation was reduced in the presence of LiCl at 3 days (**Fig. 4A**). The pattern of adipogenic gene expression in the presence of LiCl, as well as the GSK3 inhibitor SB415286 (20  $\mu$ M), replicated the strain effect to prevent both the rise in PPAR $\gamma$  and

adiponectin mRNA (**Fig. 4B**). As well, both LiCl and SB415286 induced expression of cyclin D1 and WISP1, as did daily application of strain (see **fig 2B**). In accordance with mRNA results, both agents also suppressed the accumulation of PPAR $\gamma$  and adiponectin proteins, with the effect on the latter nearly complete (**Fig. 4C**). As predicted,  $\beta$ -catenin levels under adipogenic conditions were preserved in the presence of both GSK3 inhibitors.

### **Strain inactivates GSK3 $\beta$**

Our results suggested that strain might maintain  $\beta$ -catenin levels by targeting GSK3 $\beta$ . Indeed, application of strain caused phosphorylation of GSK3 $\beta$  on its Ser9 site (causing inactivation) at both 3 and 5 days, as shown in **Fig. 5A**. Cultures were assayed for changes in protein at the end of the 6 h strain period on the day specified. Inactivation of GSK3 $\beta$  by strain was accompanied by increased levels of active  $\beta$ -catenin, as well as decreased expression of both PPAR $\gamma$  and adiponectin. Since Akt is known to directly phosphorylate and thereby inactivate GSK3 isoforms (28), we asked whether this mechanism was involved in the durability of  $\beta$ -catenin signaling in strained cultures subject to adipogenic conditions. Mechanical input has been shown to activate Akt in many cell types (29, 30). Strain caused significant increases in Akt phosphorylation without changing total Akt levels at 3 days, an effect also present at 5 days, as shown in the figure.

In contrast, when cultured in multipotential medium, strain had no effect on Akt or GSK3 $\beta$  phosphorylation at either 3 or 5 d after 6 h strain (**Fig. 5A**). We considered that strain-induced signaling might be more transient in non-adipogenic medium, as it has been reported in other cells. Indeed, cells cultured in multipotential medium showed a rapid response to strain with increased phosphorylation of Akt and GSK3 $\beta$  by 15 minutes, decreasing by 6 h to levels not significantly different from baseline (**Fig. 5B**). These changes were significant, as shown in averaged densitometries of phospho-proteins from 3 experiments. This rapid phosphorylation pattern was not seen in cells grown in adipogenic conditions.

### **Strain actions must occur early and be sustained**

The ability of mechanical signals to prevent adipogenesis was further explored by altering the number of loading days or by altering the day of

loading initiation. To determine if a single day of a mechanical signal was sufficient to counter the strong adipogenic bias to MSC differentiation, the strain regimen was delivered the first day only, followed by two days without loading, and was compared to daily loading. As shown in **Fig. 6A**, mRNA for PPAR $\gamma$  and adiponectin were equivalent in cultures that received a single day of loading and control cultures, in contrast to the reduction in PPAR $\gamma$  and adiponectin expression with daily loading. This was further confirmed by Western evidence that neither the rise in PPAR $\gamma$  nor adiponectin could be prevented with a single application of loading (**Fig. 6B**).

Whether mechanical signals must be applied during the early phase of adipogenesis was then tested by applying strain either immediately upon the switch to adipogenic medium, as before, or by delaying load for 2 days. In the delayed strain condition, neither PPAR $\gamma$  nor adiponectin protein levels were influenced by the later application of the mechanical regimen, and failed to prevent entry of MSCs into the adipogenic state.  $\beta$ -catenin was also not significantly activated when strain was delayed for 2 days. Furthermore, while GSK3 $\beta$  was activated by strain in both regimens compared to the no-strain condition, the effect was greatest when strain was delivered for all 5 days (**Fig. 6C**). This data suggested that once cells had committed to adipogenic differentiation, mechanical signals could not restrain this process.

Finally, cells were cultured in adipogenic media in the presence or absence of strain for 5 d and then cultured without strain for 3 further days. In **Figure 6D**, cultures strained during the initial 5 days showed higher active and total  $\beta$ -catenin as expected. Three days after stopping the daily application of the strain regimen,  $\beta$ -catenin levels were indistinguishable between the two conditions ( $\pm$ pre-strain) and a rise in adiponectin was seen in the previously strained cultures. Thus, removal of the mechanical stimulus allowed  $\beta$ -catenin to fall and adipogenesis to proceed apace.

### **Strain preserves the multipotential state of MSCs**

The expression of cyclin D1 is directly regulated by  $\beta$ -catenin (31), and cyclin D1 is associated with progression into the proliferative stage of the cell cycle (32). Since strain upregulated the expression of cyclin D1 in C3H10T1/2 cells, it was important to

determine whether proliferation was increased by the mechanical regimen. Three days after switching to adipogenic conditions, cell numbers were not different in unstrained, strained or cells treated with LiCl (**Fig. 7A**), showing that in these cells, preserving  $\beta$ -catenin did not induce proliferation.

We next asked whether MSCs exposed to adipogenic conditions would respond to an osteogenic stimulus and whether strain might enhance this response. C3H10T1/2 cells have the ability to differentiate into osteoblasts when grown in multipotential medium, as shown by alkaline phosphatase staining at 14 days, but this is a lengthy process (**Fig. 7B**). Shown in comparison are cells cultured in the adipogenic medium for 5 days and stained for oil-red O reflecting intracellular lipid. Application of strain during 5 days of adipogenic medium failed to increase expression of osteogenic transcription factors, Runx2 and osterix, as evaluated by RT-PCR, and shown in **Fig. 7C**. This was not surprising in view of our data showing that C3H10T1/2 cells require longer culture periods to induce osteogenesis as well as that of others (11, 33, 34). We thus elected, after the initial 5 days in culture, to add a strong osteogenic stimulus, BMP-2 (300 ng/ml) for 2 days. Shown in **Figure 7C**, BMP-2 was able to cause a significant increase in Runx2 expression in both unstrained and strained cultures. The increase in Runx2 was significantly more in cultures which had been strained before the BMP-2 stimulation compared to unstrained cultures, effecting a 2.5 fold increase compared to 1.8 fold in cells which had not been pre-strained ( $p < 0.05$  for a difference).

The effects of pre-straining cultures had an even greater influence on the ability of BMP-2 to induce osterix. Indeed, while BMP-2 failed to increase osterix expression in unstrained cells, cells subjected to strain showed a nearly 2-fold increase in this osteogenic transcription factor. Similar effects on these osteogenic genes were seen when BMP-2 was added at 100 ng/ml, i.e., BMP-2 induced a small increase in Runx2 but no change in osterix in unstrained cells, but caused significant increases in both mRNA species when added to cultures that were strained during exposure to adipogenic conditions. These data indicate that mechanical signals preserve C3H10T1/2 cells in a state where they can respond robustly to an osteogenic challenge despite an adipogenic environment.

## Discussion

The increase in adiposity and the progressive decline of bone mass are the key phenotypic features in obesity and osteoporosis, respectively. While the majority of treatment strategies have targeted the resident cell population, work presented here indicates that the mechanical biasing of pluripotent mesenchymal stem cells (MSCs) may represent a unique means of mitigating the pathogenesis of these distinct, but related, diseases. Given the accumulating evidence for a reciprocal relationship between emergence of adipocytic and osteoblastic lineages (3, 5, 35), it would appear that factors which drive differentiation toward one cell fate will suppress the other. Here we show that the conversion of MSCs into adipocytes driven by strongly adipogenic conditions can be inhibited by mechanical signals which also allow osteoblast lineage selection. This provides unique insight into a potential mechanism by which exercise may control fat production while conversely improving bone formation.

It is now well accepted that MSC selection of the adipocyte lineage is influenced by the presence of the nuclear receptor PPAR $\gamma$  (36). Culture of MSCs in an adipogenic medium here resulted in the rapid upregulation of PPAR $\gamma$  followed by an increase in the adipokine, adiponectin. Importantly, adipogenesis was markedly suppressed by daily exposure to mechanical signals. The efficacy of the mechanical influence relies on repeated exposure as a single day of loading was insufficient to restrict emergence of the adipocytic phenotype.

Accompanying the adipogenic transformation in MSCs, we measured a progressive decrease in both active and total  $\beta$ -catenin. Mechanical signals completely prevented decreases in  $\beta$ -catenin, while simultaneously limiting expression of both PPAR $\gamma$  and adiponectin. If the physical input was delayed by only 2 days it failed to prevent both the decline in  $\beta$ -catenin levels and the commitment towards fat, suggesting a relationship between these effects.

Mechanical preservation of the cellular  $\beta$ -catenin levels depends at least partially on the ability of strain to inactivate GSK3 $\beta$ . Strain caused sustained phosphorylation of GSK3 $\beta$  at serine 9, which prevents its association with  $\beta$ -catenin. Presumably, in the “disuse” – or unstrained -

condition, active GSK3 $\beta$  would be permissive to adipogenesis by targeting  $\beta$ -catenin for degradation. As a corollary, inhibitors of GSK3 $\beta$  maintained  $\beta$ -catenin levels in our cultures, and furthermore effectively prevented adipogenic conversion in the MSCs. Phosphorylation and deactivation of GSK3 $\beta$  can occur via the action of Akt (37). As mechanical stimulation increased the phosphorylation of both Akt and GSK3 $\beta$ , mechanical promotion of  $\beta$ -catenin level and activity likely involves this regulatory pathway. Interestingly, mechanical strain caused only a transient but rapid increase in this signaling cascade in multipotential medium; the reasons for this significant alteration in signal response are likely to be consequent to other cellular changes that accompany differentiation.

Activation of Wnt/ $\beta$ -catenin signaling suppresses PPAR $\gamma$  expression, shifting mesenchymal cell differentiation toward the osteoblast lineage (38). Importantly, there is increasing evidence that Wnt/ $\beta$ -catenin signaling can be activated by mechanical input (14, 20, 21), an event likely involved in promoting osteoblastogenesis. Indeed, mechanical signals do increase the number of osteoblasts that emerge from multipotential MSC cultures while simultaneously decreasing expression of PPAR $\gamma$  (11). The experiments we report here show that mechanical strain can suppress PPAR $\gamma$  expression even in a strongly adipogenic environment, and simultaneously prevent induction of adiponectin by PPAR $\gamma$ .

Further confirmation of a significant downstream response to mechanical activation of  $\beta$ -catenin was measured by increases in WISP-1 and cyclin D1, both known to be directly regulated by  $\beta$ -catenin (31). Although cyclin D1 can increase cell proliferation (32) and inhibit transactivating factors including PPAR $\gamma$  (39), the mechanical regimen used in our studies did not alter cell number. It is possible that the cyclin D1 response had an indirect effect to counteract PPAR $\gamma$  transactivation of adiponectin.

Given that MSCs are common precursors to both adipocytes and osteoblasts, and that lack of exercise is associated with both increased fat mass and decreased bone mass, it is possible that mechanical signals generated by exercise might bias the precursor pool against fat formation and towards bone formation. We were unable to show that mechanical stimulation caused osteogenic

differentiation of MSCs under these strong adipogenic conditions after only a week of culture, as osteogenic differentiation is known to be a more gradual process (33, 34). To overcome this time constraint, we applied a strong osteogenic differentiation factor, BMP-2. MSCs subject to mechanical loading during culture in adipogenic medium were able to respond briskly to BMP with increases in the osteogenic markers Runx2 and osterix. These data suggest that if the fall in  $\beta$ -catenin level that accompanies adipocytic differentiation can be prevented by mechanical input, not only can adipogenesis be inhibited, but MSCs will remain available for alternate cell lineage allocation. In this vein, one critical contribution of a daily input of exercise may be to preserve MSCs in a multipotential state, suppressing the emergence of adipocytes from the MSC pool by stimulating a durable  $\beta$ -catenin signal.

These in vitro experiments can not model the entirety of the complex mechanical environment in which cells contributing to skeletal architecture and remodeling exist. Osteoprogenitor cells, as well as osteoblasts in varying stages of differentiation, exist in the marrow along trabeculae, as well as on the periosteum, both sites which experience tissue strain along with oscillatory shear, pressure waves and even electrical fields (40). The levels of strain which these cells experience is controversial: while the hard tissue strain ranges from very low up to ~2000 microstrain, cell membrane strain can be at least 2 orders greater (41). Furthermore, the distortion of the bone matrix during loading will cause deformation of the marrow with changes in intramedullary pressure that are also experienced as strain by cells in the marrow (42). In sum, while in vitro experiments can not replicate the multidimensional skeletal environment, they can provide explicit mechanistic data informing our understanding of the environmental milieu.

We believe our data represent further evidence that the functional loading environment of the skeleton is central to defining bone mass and morphology, and includes mechanical influences on the bone marrow stem cell population that simultaneously influence the fat phenotype (12). That is not to say, however, that the bone marrow stem cell is the only pathway by which the skeleton recognizes and responds to functional challenges. It is accepted that osteocytes serve an essential role in

defining the mechanosensitivity of the skeleton and the three-dimensional aspect of this syncytium is ideally configured to perceive and amplify biophysical stimuli (43). The ability to respond to mechanical factors, however, long precedes the terminal placement of bone cells in this network, and the behavior of both stromal cells and osteoblasts are also known to be directly regulated by biophysical factors (40). Indeed, a direct means of promoting progenitors to skeletal lineage may represent a particularly efficient and proximal means of assuring an ever-ready population of bone forming cells. As such, mechanical factors not only regulate bone mass through osteocyte appreciation of skeletal stress and strain, but also appear to prevent osteopenia through influencing fundamental stage development of bone cells, increasing their supply.

It is well accepted that exercise in general, and mechanical signals in particular, prevents obesity. It has been presumed, however, that exercise limits fat mass expansion through metabolism and calorie expenditure. The work presented here alternatively suggests a “developmental” influence on adipogenesis, showing that the genesis of fat cells from stem cell pools is suppressed by mechanical signals. The mechanism by which the mechanical signal is transduced appears to involve activation of Akt, inactivation of GSK3 $\beta$  and preservation of  $\beta$ -catenin. Ultimately, our data suggest that particular mechanical signals may be able to inhibit the genesis of fat by promoting alternate pathway decisions for the mesenchymal stem cell pool. Indeed, given the role of mechanical signals in preventing both obesity and osteoporosis, it is conceivable that controlling precursor lineage decisions might represent a strategy to curb both diseases simultaneously.

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## Figure Legends

**Fig. 1. Adipogenic medium induces rapid adipogenesis of C3H10T1/2 cells.** C3H10T1/2 cells were seeded in 6-well Bioflex Collagen-I coated plates (100,000 cells/well) in MEM $\alpha$  and cultured in multipotential (M) or adipogenic (A) medium for 3 or 5 days. (A) Cytoplasmic triglyceride droplets appeared at 3 days under adipogenic conditions, shown at 40X. (B) Real-time RT-PCR was performed for PPAR $\gamma$  and adiponectin. mRNA is represented as a percent mRNA level measured in MEM $\alpha$  at day 0 (100%). Asterisk =  $p < 0.001$  difference between M and A media on the same day. (C) Immunoblot for PPAR $\gamma$  and adiponectin and actin: lanes refer to cells grown in M or A conditions for 3 or 5 days.

**Fig. 2. Mechanical loading inhibits adipogenesis and prevents a fall in  $\beta$ -catenin.** Strain was applied to cells in M or A medium for 3 or 5 days. (A) Cytoplasmic triglyceride droplets appeared by 3 d in unstrained cells and continued to increase; strain inhibited this accumulation; 40X. (B) Real-time RT-PCRs performed for PPAR $\gamma$ , adiponectin, cyclin D1 and WISP1 on cells  $\pm$  strain as indicated. ANOVA; a = significant difference from M3 unstrained control (100%),  $p < 0.001$ , b = significant difference between unstrained and strained condition,  $p < 0.05$ . (C) Cellular proteins extracted from cells  $\pm$  strain for the indicated times and analyzed by Western blotting for active and total  $\beta$ -catenin, and actin (loading control). M3 = M medium for 3 day culture ; A = A medium with +S referring to the strained condition at either 3 or 5 days. Densitometry compiled from 3 separate 5 d experiments shows that effects on active  $\beta$ -catenin were significant.

**Fig. 3. Active  $\beta$ -catenin is increased in strained cultures.** Cells  $\pm$  strain applied daily for 3 days in A medium were immunostained for DAPI and active  $\beta$ -catenin. DAPI nuclear images were merged with active  $\beta$ -catenin in the right of the figure and showed that strain causes elevation of active  $\beta$ -catenin and its appearance in the nucleus .

**Fig. 4. Inhibition of GSK3 $\beta$  mimics effects of strain.** Inhibitors of GSK3 were added to cells cultured in adipogenic medium for 3 days. (A) Cytoplasmic triglyceride droplets are shown with bright field microscopy and compared to effect of strain, 40X. (B) Real time RT-PCR for mRNA species as noted showed effects of 3 d treatment with LiCl (10 mM) or the GSK3 inhibitor SB415286 (SB41, 20  $\mu$ M). (C) Western blotting of total cellular proteins showed that inhibition of GSK3 prevented events associated with adipogenesis.

**Fig. 5. Strain inactivates GSK3 $\beta$ .** Cultures in M or A medium  $\pm$  mechanical strain are shown after 3 (left) or 5 (right) days. (A) Total cellular proteins were blotted with antibodies for phospho-Akt (Ser473, pAkt), phospho-GSK3 $\beta$  (Ser9, pGSK3 $\beta$ ), active  $\beta$ -catenin, PPAR $\gamma$ , adiponectin (APN) and actin (loading control). M = M medium without strain; M+S = +strain; likewise A and A+S. (B) On day 3, cells in either M or A medium were strained for 1, 15, 30 min or 6 h, and protein extracted immediately for Western analysis. Below, graphs show average densitometry from 3 experiments: both pAkt and pGSK3 $\beta$  increased with strain at 15 and 30 min in M medium but not until 6 h in A medium,  $p < 0.05$ .

**Fig. 6. Mechanical inhibition of adipogenesis requires application early and repetitively.** (A) Strain was begun on the first day of adipogenic stimulus, and either dosed for 1 (S1) or 3 (S3) days; mRNA for PPAR $\gamma$  and adiponectin was analyzed after 3 days in the adipogenic medium. Only the S3 condition inhibited adipocytic mRNA,  $p < 0.05$ . (B) PPAR $\gamma$  and adiponectin protein from 3 d cultures in A medium: S1 = cells strained first day only, S3 = strain each day of culture. (C) Strain application was delayed for 2 days (A2+S3) or applied daily (S5) and protein analyzed at 5 days. (D) Western blotting of total cellular protein of cells cultured in adipogenic medium  $\pm$  strain for 5 d shows expected strain effects (left side of blot); culture for a further 3 days without strain shows equilibration between proteins studied in the two culture conditions (right side of blot).

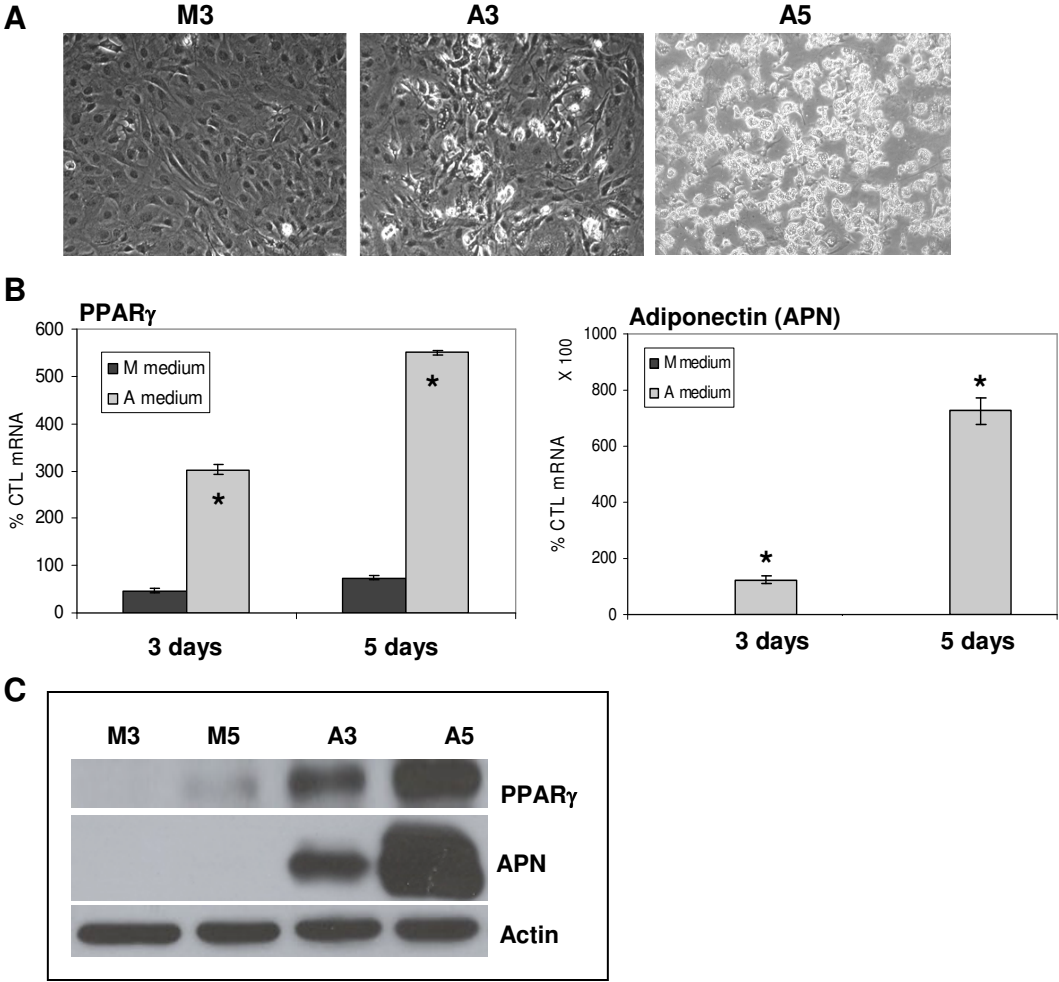
**Fig. 7. Strain preserves MSCs in a multipotential state.** (A) Cell number compiled from 3 separate experiments showed no differences between culture conditions. (B) MSCs in multipotential medium stained for alkaline phosphatase by 14 d. Shown in the right panel are cells cultured in the adipogenic medium at 5 days stained for oil-red-O as expected for cells containing lipid. Both are shown at 40X. (C) Real-time RT-

PCR was performed on cells cultured in A medium x 5 d  $\pm$  strain (strained groups are shown in gray) and then switched to M  $\pm$  BMP-2 (300ng/ml) x 2 days. ANOVA: a = difference from control unstrained (100%),  $p < 0.001$ ; b = difference from all other conditions,  $p < 0.05$ .

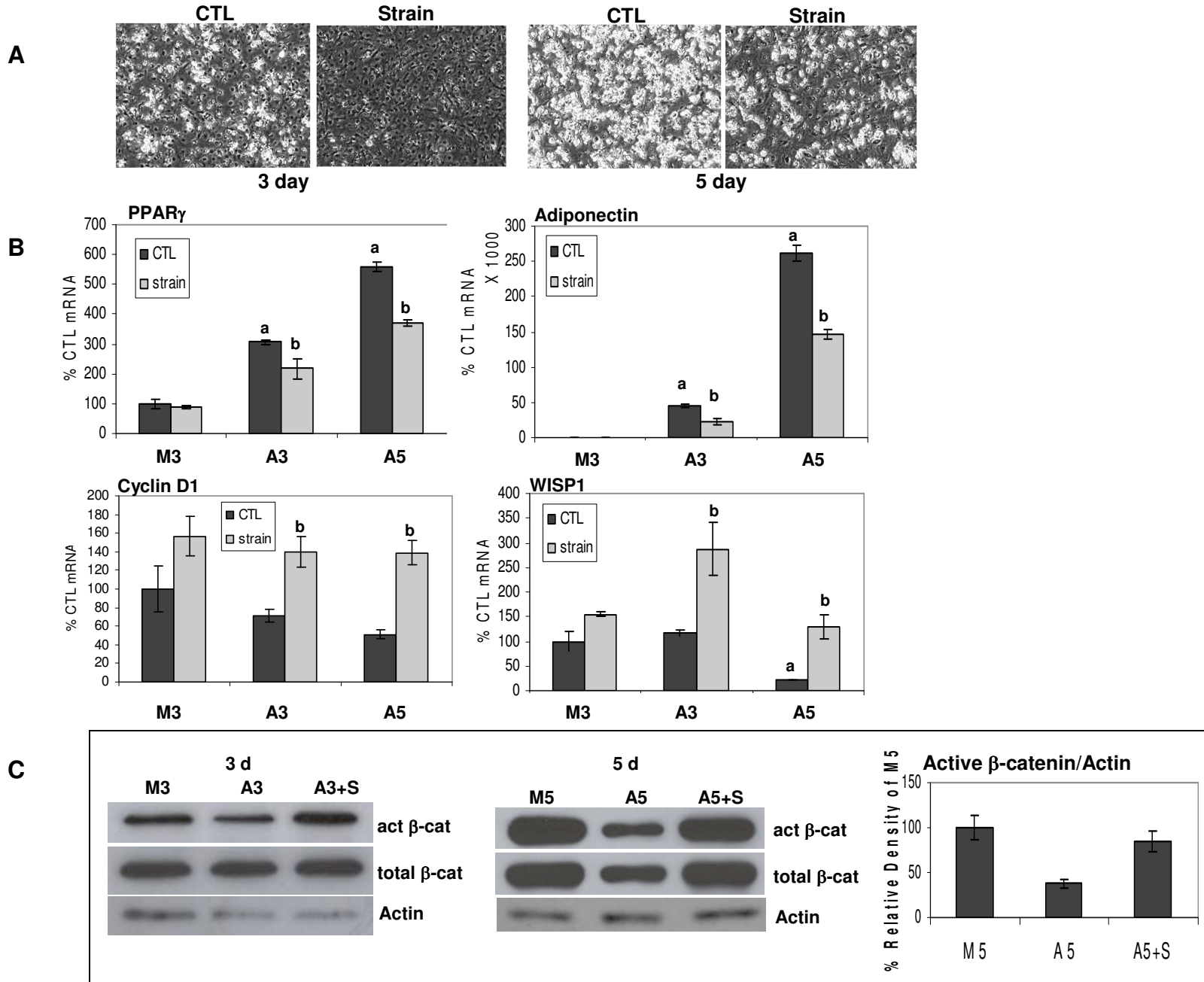
**TABLE 1. Primers for real-time PCR**

<b>Target gene (mouse)</b>		
PPAR $\gamma$	Forward	5'-GCTTATTTATGATAGGTGTGATC-3'
	Reverse	5'-GCATTGTGAGACATCCCCAC-3'
Adiponectin	Forward	5'-GCA GAG ATG GCA CTC CTG GA-3'
	Reverse	5'-CCC TTC AGC TCC TGT CAT TCC-3'
Cyclin D1	Forward	5'-GAGAAATGTACTCTGCTTTGCTGAA-3'
	Reverse	5'-GGGCTGTAGGCACTGAGCAA-3'
WISP1	Forward	5'-CTGGACAGAAAAGGGCATGT-3'
	Reverse	5'-AGGAAGGAGGGGAAATCTCA-3'
Runx2	Forward	5'-GAATGGCAGCACGCTATTAAATCC-3'
	Reverse	5'-GCCGCTAGAATTCAAAACAGTTGG-3'
OSX	Forward	5'-CCTCTCGACCCGACTGCAGATC-3'
	Reverse	5'-AGCTGCAAGCTCTCTGTAACCATGAC-3'
18S	Forward	5'-GAACGTCTGCCCTATCAACT-3'
	Reverse	5'-CCAAGATCCAACACTACGAGCT-3'

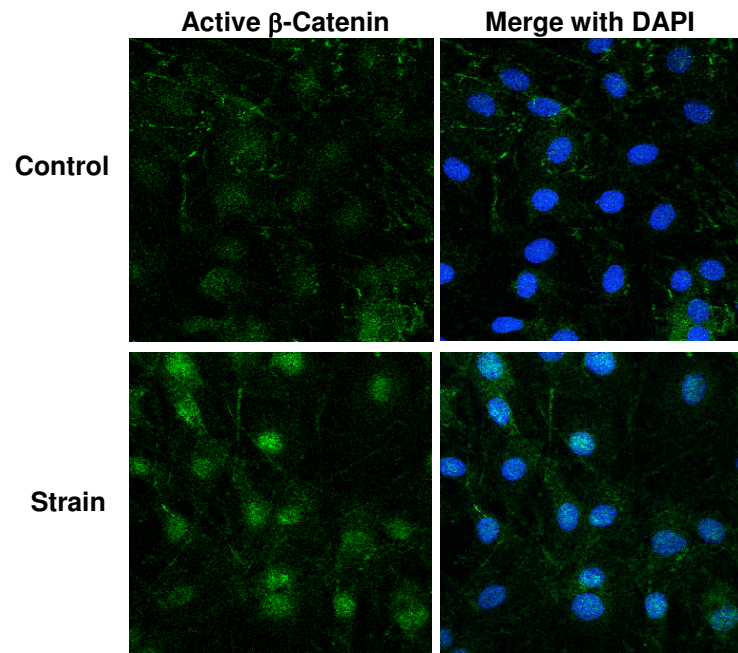
Figure 1



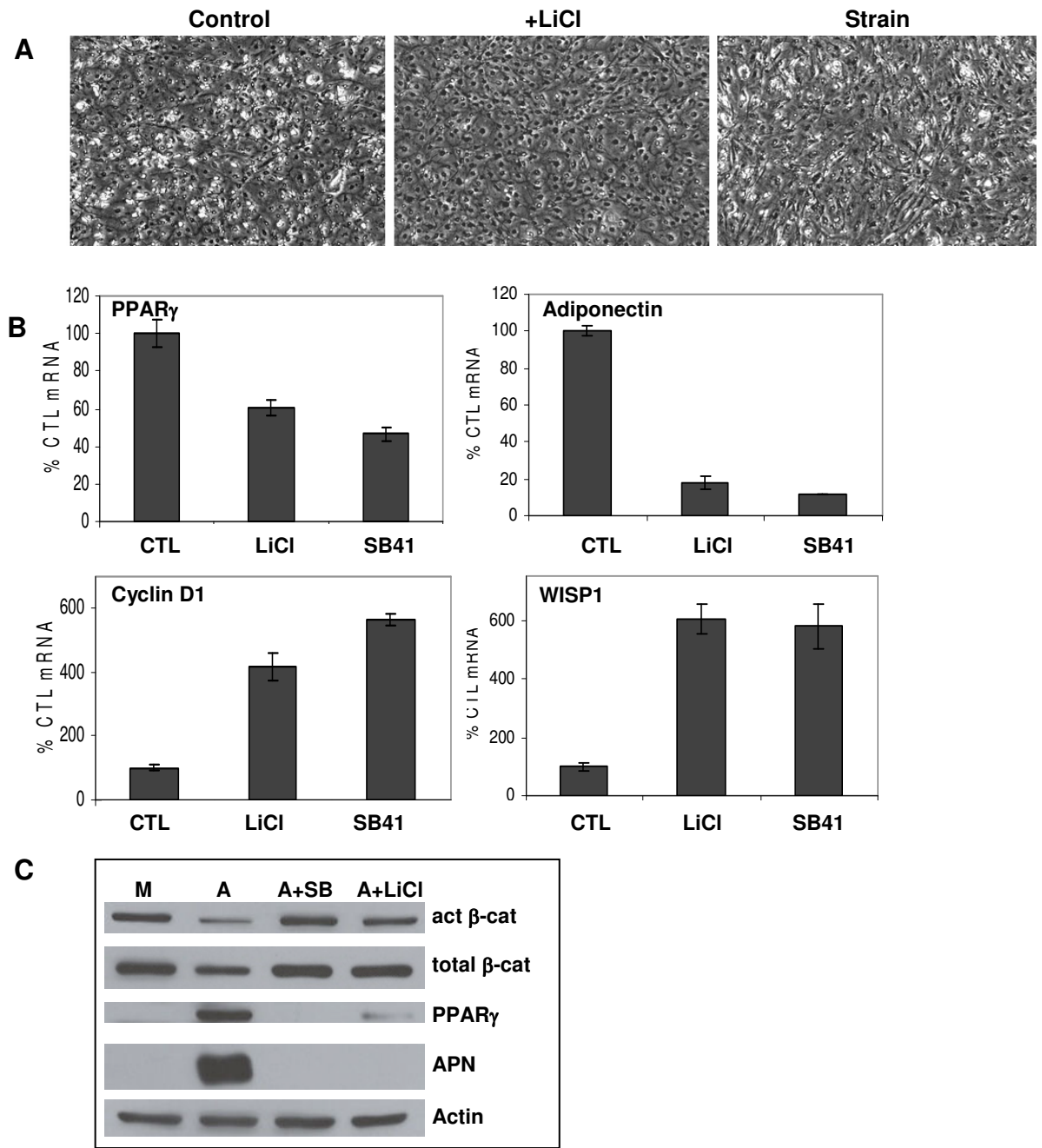
**Figure 2**



**Figure 3**



**Figure 4**



**Figure 5**

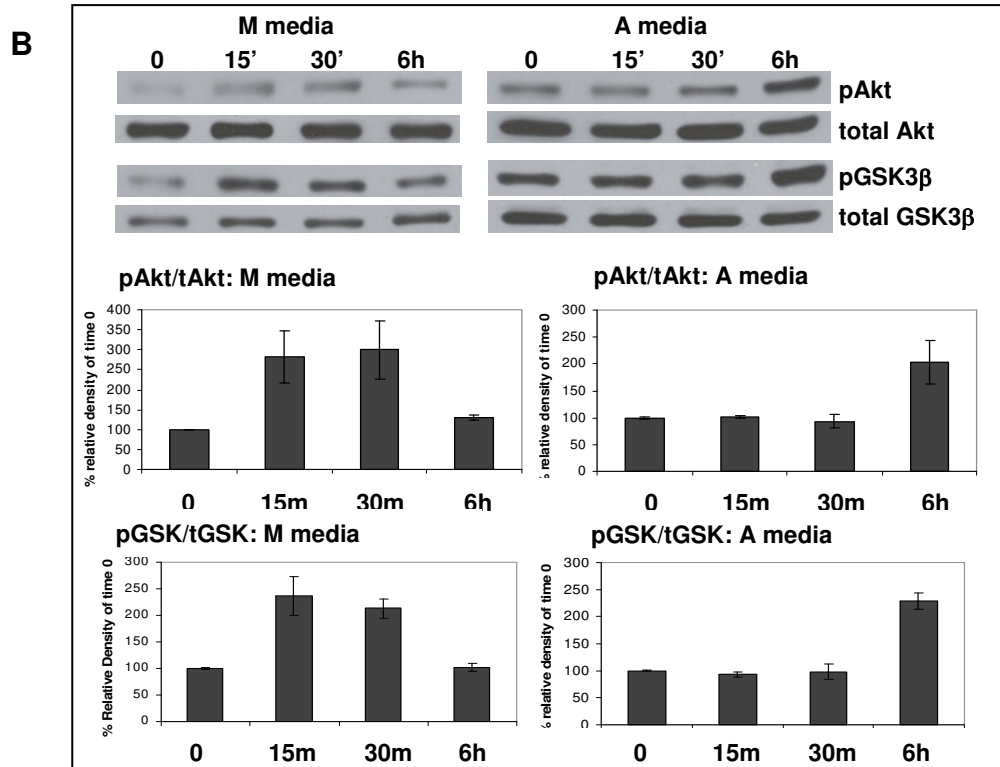
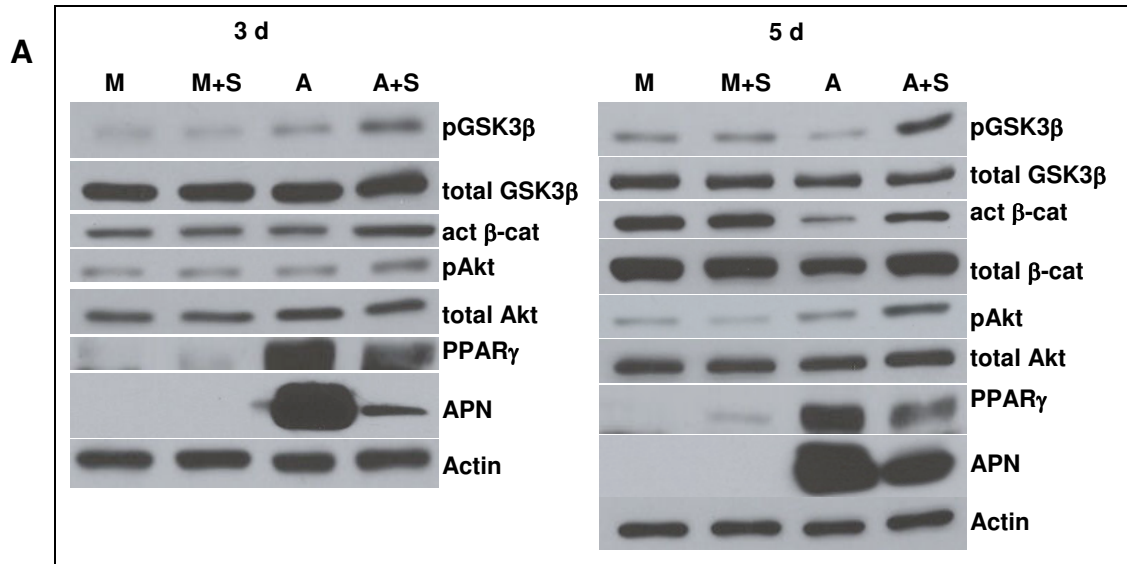


Figure 6

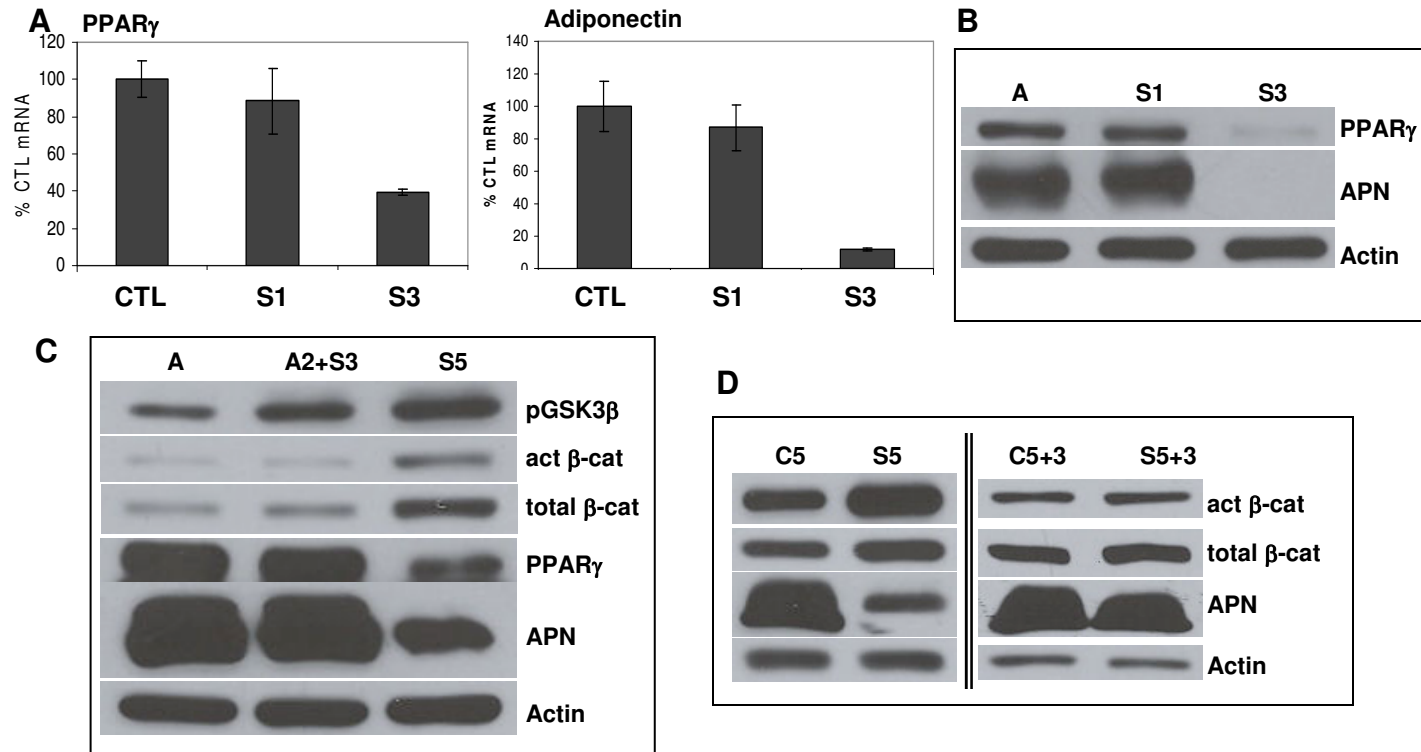


Figure 7

