Up-regulation of the Wnt, Estrogen Receptor, Insulin-like Growth Factor-I, and Bone Morphogenetic Protein Pathways in C57BL/6J Osteoblasts as Opposed to C3H/HeJ Osteoblasts in Part Contributes to the Differential Anabolic Response to Fluid Shear* 

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C57BL/6J (B6), but not C3H/HeJ (C3H), mice responded to mechanical loading with an increase in bone formation. A 30-min steady fluid shear of 20 dynes/cm² increased [3H]thymidine incorporation and alkaline phosphatase activity and up-regulated the expression of early mechanoresponsive genes (integrin β1 (Igtb1) and cyclooxygenase-2 (Cox-2)) in B6 but not C3H osteoblasts, indicating that the differential mechanosensitivity was intrinsic to osteoblasts. In-house microarray analysis with 5,500 gene fragments revealed that the expression of 669 genes in B6 osteoblasts and 474 genes in C3H osteoblasts was altered 4 h after the fluid shear. Several genes associated with the insulin-like growth factor (IGF)-I, the estrogen receptor (ER), the bone morphogenetic protein (BMP)/transforming growth factor-β, and Wnt pathways were differentially up-regulated in B6 osteoblasts. In vitro mechanical loading also led to up-regulation of these genes in the bones of B6 but not C3H mice. Pretreatment of B6 osteoblasts with inhibitors of the Wnt pathway (endostatin), the BMP pathway (Noggin), or the ER pathway (ICI182780) blocked the fluid shear-induced proliferation. Inhibition of integrin and Cox-2 activation by echistatin and indomethacin, respectively, each blocked the fluid shear-induced up-regulation of genes associated with these four pathways. In summary, up-regulation of the IGF-I, ER, BMP, and Wnt pathways is involved in mechanotransduction. These four pathways are downstream to the early mechanoresponsive genes, i.e. Igtb1 and Cox-2. In conclusion, differential up-regulation of these anabolic pathways may in part contribute to the good and poor response, respectively, in the B6 and C3H mice to mechanical loading.

Mechanical loading is essential for maintenance of skeletal architectural integrity. Loading stimulates bone formation and suppresses bone resorption, leading to an overall increase in bone mass (1), whereas unloading results in an overall decrease in bone mass, because of an inhibition of formation along with an increase in resorption (2). Loading produces strains in the mineralized matrix of bone, which generates interstitial fluid flow through lacunar/canalicular spaces (3). This fluid flow exerts a shear stress at surfaces of osteoblasts and osteocytes lining these spaces, which generates biochemical signals to produce biological effects. Multiple interacting signaling pathways are involved in translating the fluid shear signals into biological effects in bone cells (4), and these pathways are collectively referred to as the mechanotransduction mechanism. Mechanical loading is a key regulatory process for bone mass and strength (5). Knowledge of the mechanotransduction mechanism would not only yield information about the mechanical stimulation of bone formation but would also provide insights into the pathophysiology of osteoporosis and other bone-wasting diseases.

There is increasing evidence that genetics play a major part in determining the bone response to mechanical loading. Studies from our group (6, 7) and others (8, 9) demonstrate that C57BL/6J (B6) inbred mice responded to in vivo mechanical loading with an increased bone formation, but C3H/HeJ (C3H) mice showed no such response. We postulate that the differential osteogenic response to mechanical stress in B6 and C3H inbred strains of mice is intrinsic to bone cells and that comparative global gene expression profiling studies in osteoblasts derived from this pair of inbred mouse strains in response to fluid shear could provide information concerning potential signaling pathways involved in the mechanical stimulation of bone formation. This would also yield important information about the identity of mechanosensitive genes that determine the good and poor mechanical response in bone formation, respectively, in B6 and C3H mice.

The objectives of this study were 4-fold and are as follows: 1) to confirm that the differential anabolic response to mechanical loading in B6 and C3H strains of mice is intrinsic to osteoblasts, using an in vitro fluid flow shear stress model as a surrogate of mechanical loading (4); 2) to perform in-house microarray analyses in isolated B6 and C3H osteoblasts to identify potential signaling pathways that in part contribute to the differential osteogenic response; 3) to confirm that the pathways-of-interest are essential for fluid shear-induced cell proliferation; and 4) to determine the relationship between the pathways-of-interest and the early mechanoresponsive gene products, such as integrins and cyclooxygenase-2 (Cox-2).

The abbreviations used are: B6, C57BL/6J inbred mice; ALP, alkaline phosphatase; BMP, bone morphogenetic protein; C3H, C3H/HeJ inbred mice; Cox-2, cyclooxygenase-2; ER, estrogen receptor; Erk1/2, extracellular signal-regulated kinases 1/2; pErk1/2, phosphorylated Erk1/2; EST, expressed sequence tag; IGF-I, insulin-like growth factor-I; TGF-β, transforming growth factor-β; Wnt, wingless- and int-related protein.

* This work was supported in part by a special appropriation to the Jerry L. Pettis Memorial Veterans Affairs Medical Center, Musculoskeletal Disease Center, and by a Merit Review provided by the Office of Research and Development, Medical Research Service, Department of Veteran Affairs, and in part by Assistance Award DAMD17-01-1-0744 provided by The United States Army Medical Research Acquisition Activity (Fort Detrick MD 21702-5014). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–3.

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EXPERIMENTAL PROCEDURES

Materials—Tissue culture plasticware was obtained from Falcon (Oxnard, CA). Dulbecco’s modified Eagle’s medium was from Mediatech, Inc. (Herndon, VA). Bovine calf serum was from HyClone (Logan, UT). Trypsin and EDTA were products of Irvine Scientific (Santa Ana, CA). [3H]Thymidine (48 Ci/mmol) was obtained from Research Products International (Mount Prospect, IL). Anti-pErk1/2, anti-pan-Erk1/2, anti-β-catenin, anti-integrin β1, anti-Cox-2, and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), Upstate Biotechnology, Inc. (Lake Placid, NY), or BD Transduction Laboratories. Echistatin, endostatin, and indomethacin were products of Sigma. IC1182780 was purchased from Tocris (Ellisville, MO), and Noggin was obtained from R&D Systems (Minneapolis, MN). Other chemicals were of molecular biology grade and were from Fisher or Sigma.

Cell Culture and Fluid Shear Stress Experiments—Osteoblasts, isolated from calvaria or long bones of 8-week-old B6 and C3H mice by collagenase digestion as described previously for neonatal calvarial osteoblasts (10), were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum. Pilot studies indicated that cell passage, up to passage 7, had no significant effects on the responsiveness of primary B6 mouse osteoblasts to fluid shear stress with respect to [3H]thymidine incorporation, alkaline phosphatase (ALP) specific activity, and Erk1/2 phosphorylation. Accordingly, cells of passages 3–6 were used in this study.

50,000 cells were plated on each glass slide. At ~80% confluence, the cells were serum-deprived for 24 h and subjected to a steady fluid shear stress of 20 dynes/cm² for 30 min in the Cytodyne flow chamber as described previously (4). This dosage of fluid shear stress is believed to be within the physiologically relevant range of laminar shear stress produced by the circulation (11). Replicate glass slides of cells were placed in a parallel flow chamber but without the fluid shear stress as static controls in each experiment.

To test the potential involvement of a given signaling pathway-of-interest, cells were pretreated with a specific inhibitor of the pathway-of-interest (i.e. IC1182780 for the estrogen receptor (ER) pathway, endostatin for the canonical wingless- and int-related protein (Wnt) pathway, and Noggin for the bone morphogenetic protein (BMP) pathway), for 24 h prior to the fluid shear stress. To assess the role of integrin activation and Cox-2 on the up-regulation of these pathways, cells were pretreated with echistatin or indomethacin, respectively, for 2 h prior to the fluid shear stress.

[3H]Thymidine Incorporation Assay—Cell proliferation was assessed by [3H]thymidine incorporation during the final 6 h of the 24-h post-exposure to fluid shear as described previously (4, 12).

Cellular ALP Specific Activity Assay—Osteoblast differentiation was measured by the increase in the specific activity of ALP 24 h post-exposure to the shear stress as described previously (4, 13). The ALP-specific activity (i.e. normalized against cellular protein content) was reported to adjust for the difference in the cell number because of the increase in cell proliferation in response to fluid shear.

Western Immunoblot Assays—Cellular integrin β1, Cox-2, and β-catenin were determined by Western immunoblot assays were performed as described previously (4) using respective commercial polyclonal antibodies and normalized against each corresponding cellular actin level. The relative cellular phosphorylated Erk1/2 (pErk1/2) level (an index of Erk1/2 activation) was determined with the phospho-specific antibody against pErk1/2 and normalized against corresponding total Erk1/2 level, determined with anti-pan-Erk1/2 polyclonal antibody.

RNA Purification—Total RNA of cells on each slide was extracted with Qiagen mini RNA kit (Qiagen, Valencia, CA). The purity and integrality of each RNA sample was confirmed with Bio-analyzer (Agilent, Palo Alto, CA). Only undegraded RNA samples were used in this study.

In-house Microarray Hybridization and Data Analysis—For the preparation of our in-house microarray chips, cDNA inserts of 5,500 cDNA clones of mouse, rat, human, or monkey genes or ESTs (largely mouse and human genes) were isolated, purified, and evaluated with agarose gel electrophoresis. The microarrays were printed on aminosilane-coated microscope slides (Corning, NY) with a GMS 417 Arrayer (Genetic MicroSystems, Santa Clara, CA). Six replicates of each clone were printed on each slide. DNA was fixed to the slides by baking at 80 °C for 2 h.

The experimental strategy and analyses of the microarray experiment are described briefly as follows. Primary osteoblasts isolated from B6 or C3H inbred strain of mice were plated on glass slides and subjected to a 30-min steady shear stress as described above. Replicate plates of B6 or C3H osteoblasts were placed in the flow chamber without the fluid shear as the static control. Four hours after the fluid shear, total RNA was isolated. cDNA synthesized from 1 μg of total RNA of cells received the fluid shear, and corresponding static control cells were each fluorescently labeled with Cy5 and Cy3, respectively, as previously described (14). The microarray hybridization was performed as described previously (14). The slide was scanned using a ScanArray 4000 scanner (GSI Lumonics, San Jose, CA). The fluorescent images were acquired using ScanArray software (version 2.1; GSI Lumonics), and data were analyzed using GeneSpring Image Analysis program (Silicon Genetics, San Jose, CA). Each array spot was individually inspected using the GeneSpring Image Analysis program. The microarray analysis was repeated in osteoblasts of four pairs of B6/C3H mice. Statistically significant differences in gene expression between each pair of stressed and corresponding static control samples was analyzed using Lowess Normalization and paired t test. Differences of p < 0.05 were considered significant. Only known mouse genes were analyzed further. Because the gene annotation or accession numbers of many of the known mouse genes on our array were missing, the computer-based gene ontology and pathway analyses were not performed. Tentative classification of gene functions was determined manually based on information available on the PubMed data base.

Real Time PCR Analyses—Real time PCR was carried out with the SYBR Green method on the MJ Research DNA Engine Opticon® 2 System (Waltham, MA). The purified total RNA was used to synthesize cDNA by reverse transcription using random hexamer primers and Superscript II reverse transcriptase (Invitrogen). The cDNA was then subjected to real time PCR amplification using the gene-specific primers listed in Table 1. The primers were designed with the IDT Vector NTI software (Coralville, IA). An aliquot (25 μl) of reaction volume (consisted of 2× (12.5 μl) QuantiTect SYBR Green PCR master mix, which contained the Hot Start Taq polymerase (Qiagen), 0.5 μM of primers, and 1–5 μl of cDNA template) was used in each assay. The PCR conditions consisted of an initial 10-min hot start at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing, and extension at appropriate temperature (50–72 °C) (see Table 1) for 30 s, and a final step of melting curve analysis from 60 to 95 °C. Each reaction was performed in triplicate. The data were analyzed using Opticon® Monitor Software 2.0. Data normalization was performed against β-actin, and the normalized values were used to calculate the relative fold change between the control and the experiment groups by the threshold cycle method.

In Vivo Mechanical Loading Model—We adapted the four-point bending exercise regimen, originally developed by Akhter et al. (15) on rat tibia, as the in vivo mechanical loading model for mouse tibia as
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TABLE 1

| Gene          | Primer sequences            | Annealing temperature | Extension temperature |
|---------------|-----------------------------|-----------------------|-----------------------|
| β-Actin       | Forward primer, 5′-CAC GCA GAG CAC AGA GGA TG-3′  | 56                    | 72                    |
| Tgfβ1         | Forward primer, 5′-CGG CAG CAC ATG CAG GAC AGA CA-3′ | 53                    | 72                    |
| Ctubh1        | Forward primer, 5′-GCA TCA CGG ACT AGA GAA GGA TG-3′ | 50                    | 72                    |
| Bmpr1         | Forward primer, 5′-GCT GTA AGA GGT TCA CTA GA-3′  | 53                    | 72                    |
| Igf1r         | Forward primer, 5′-GGT CCT CAC AGA CAC AG-3′       | 54                    | 72                    |
| Wnt1          | Forward primer, 5′-TCC GGA GAC AGA AGA AG-3′       | 54                    | 72                    |
| Wnt5a         | Forward primer, 5′-TGG TGA CCA CGG CAA CA-3′       | 54                    | 72                    |
| Axin          | Forward primer, 5′-TCT GGA TAC CTC CCG ACT TA-3′   | 54                    | 72                    |
| Lef1          | Forward primer, 5′-AGC GAC AGG CAG CTA ATG CA-3′  | 54                    | 72                    |
| Lrp5          | Forward primer, 5′-GCA TCA TAT CCG CGG ACT CT-3′  | 53                    | 72                    |
| Egr1          | Forward primer, 5′-ATG TGC AGG AGG CAG CAC TG-3′  | 56                    | 72                    |
| Ncoa1         | Forward primer, 5′-TGG ACC CTC CAG CAC ACT TA-3′  | 56                    | 72                    |
| Dlx1          | Forward primer, 5′-GGC CCG CAC CAG ACT CTC AT-3′  | 56                    | 72                    |
| c-Fos         | Forward primer, 5′-ACT CTG GTC TTT CCA CAT GTG GAA-3′ | 56                    | 72                    |

Bone Histomorphometry—Both the loaded and unloaded (control) tibiae of B6 and C3H mice were removed, after euthanasia, and fixed with 10% cold neutral buffered formalin on ice. The fixed bones were then rinsed free of formalin, defleshed, and embedded in methyl methacrylate (17). Thick (0.5 mm) cross-sections were cut from the mid-diaphysis of the tibia with a wire saw (Delaware Diamond Knives), lightly ground, and stained with Goldner’s trichrome stain for mineralized bone. The stained bone slices were mounted in Fluoromount-G (Fisher) and examined under an Olympus BH-2 fluorescence/bright field microscope.

Results

Effects of Fluid Shear Stress on the Proliferation, Differentiation, and Expression of Early Mechanoresponsive Genes in Primary Osteoblasts of B6 and C3H Inbred Strains of Mice—The 30-min steady fluid shear stress of 20 dynes/cm² significantly increased (p < 0.05) the [³H]thymidine incorporation (Fig. 1A) (an index of cell proliferation) and ALP-specific activity (Fig. 1B) (a marker of osteoblast differentiation) of B6 osteoblasts. No such response was seen in C3H osteoblasts. Mechanical stimulation, including fluid shear stress, has been shown to up-regulate several early mechanoresponsive genes, such as integrins and Cox-2, within minutes in bone cells (18). Thus, we evaluated whether there was also a differential response to fluid shear in the expression of integrin β1 (Igfb1) and Cox-2 in C3H and B6 osteoblasts. Fig. 1C shows that while the 30-min steady fluid shear stress significantly increased the cellular integrin β1 protein level in B6 osteoblasts.
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by >2-fold 10 min after the stress, the same stress had no effect in C3H osteoblasts. Similarly, the fluid shear significantly increased Cox-2 protein expression by >2-fold in B6 osteoblasts but not in C3H osteoblasts (Fig. 1D). Fig. 1, C and D, also shows that the basal cellular integrin β1 and Cox-2 protein levels in C3H osteoblasts were severalfold higher than those in B6 osteoblasts. The significance of the higher basal expression of these two early mechanoresponsive genes in C3H osteoblasts is unclear. Nevertheless, these results clearly indicate that the osteogenic response to mechanical loading in this pair of inbred strains of mice is intrinsic to osteoblasts.

In-house Microarray Analysis of Shear Stress-mediated Changes in Gene Expression in Primary Osteoblasts of B6 and C3H Mice—Microarray analysis was performed with RNAs isolated from primary B6 or C3H osteoblasts 4 h after the 30-min fluid shear, using the in-house chips, whose expression was affected differentially in B6 and C3H osteoblasts (Fig. 2). Because these genes were up-regulated in both C3H and B6 osteoblasts, these mechanoresponsive genes were likely to be upstream to the mechanosensitivity genes responsible for the differential anabolic response to fluid shear between B6 and C3H osteoblasts.

The expression of 88 known mouse genes (50 up-regulated and 33 down-regulated) was altered only in C3H osteoblasts (supplemental Table 1). The up-regulated genes included a number of key regulator genes of cell proliferation and differentiation in both B6 and C3H osteoblasts, including several growth factor genes (i.e. Tgfβ1, Vegf, Igf2, Pdgfa, Fgf1, and Op2/ Bmp9β), receptor genes (Thr, Bmpr1a, Pthr, Esr2, Rarg, Forb, Osxr, Ifngr, and Tnfr), vitamin D metabolism genes (i.e. Cyp27b1), small G-protein genes (i.e. Ran and Era1), and several inhibitory transcription factor genes of osteoblast differentiation (i.e. M-twist, Id-2, and Derm-1). Because these genes were up-regulated in both C3H and B6 osteoblasts, these mechanoresponsive genes were likely to be upstream to the mechanosensitivity genes responsible for the differential anabolic response to fluid shear between B6 and C3H osteoblasts.

The expression of 129 known mouse genes was up-regulated in B6 osteoblasts but not in C3H osteoblasts (supplemental Table 2). Some of the up-regulated genes were growth factor and receptor genes (Csf1, Tgfbr1, Igfhp2, and Fgfr), transcription factor genes (Hox8.1/Msx2, and c-Myc), signal transduction genes (Pld, Hic5, Igkbαp, and Emn2), and intracellular transport and trafficking genes (Gsi5, Cacnb3, Snx3, and Aptd6). The shear stress also up-regulated Pges and Bel genes in C3H osteoblasts but not in B6 osteoblasts. Because C3H osteoblasts did not respond anabolically to fluid shear, these genes were not analyzed further.

The expression of 129 known mouse genes was up-regulated in B6 osteoblasts only (supplemental Table 3). Consistent with an anabolic response to the fluid shear in B6 osteoblasts and not C3H osteoblasts, the fluid shear differentially up-regulated in B6 osteoblasts a number of genes associated with osteoblast proliferation and differentiation. These genes include, but are not limited to, bone growth factor, receptor, and associated genes (i.e. Tgb2, Bmp4, Fgf6, Kgf/Fgf7, Pdgf, Igf1r, Ghr, and 1 protein level (Cox-2), normalized against each cellular actin protein level, was determined by Western immunoblot assay. Results are shown as mean ± S.D. (n = 6).***, p < 0.001. N.S., not significant.

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Bmpr2, Igfbp5, and Wnt5a), cytokines, and receptor genes (i.e. Osm, Il4, Il6, Il8, and Il6r), Esr1, genes involved in protein and RNA synthesis, DNA synthesis, as well as cell proliferation. A number of energy and cell metabolism genes, intracellular transport and trafficking genes, as well as oxidative stress-responsive genes, such as Hsc70, Osp94, and p47phox, were also up-regulated in B6 but not C3H osteoblasts. Similarly, a large number of transcription factors and signal transduction molecules were differentially up-regulated in B6 osteoblasts.

**Up-regulation of the Expression of Genes of Four Anabolic Signal Transduction Pathways in B6 Osteoblasts in Response to the Fluid Shear**—A manual pathway analysis of the known mouse genes whose expression was up-regulated in both C3H and B6 osteoblasts revealed that a number of genes associated with four anabolic signaling pathways were differentially up-regulated in response to the fluid shear (Fig. 3). These pathways are as follows: the Wnt pathway, the BMP/transforming growth factor(TGF)-β pathway, the ER pathway, and the growth hormone/insulin-like growth factor(IGF)-1 pathway. However, some of the upstream effector genes of these signaling pathways (with the exception of the Wnt pathway) and several downstream negative regulatory transcription factor genes of osteoblast differentiation of the BMP/TGF-β pathway (i.e. Id-2, Dermo-1, and Hox-7.1/Msx1) were also up-regulated in C3H osteoblasts, suggesting that these genes are unrelated to the mechanosensitivity genes responsible for the differential osteogenic response to mechanical stimulation in this pair of inbred strains of mice.

To confirm the microarray data, real time PCR analyses were performed on the expression levels of several genes of each of these four signaling pathways in B6 and C3H osteoblasts 4 h after the 30-min steady fluid shear. Because the in-house microarray chips contained only a limited number of known mouse genes, particularly those of the canonical Wnt signaling pathway, additional genes of the canonical Wnt pathway (i.e. Wnt1, Wnt3a, and Lrp5) were included in the real time PCR analysis. Table 2, which summarizes and compares the real time PCR and microarray results, confirms that the expression of several key genes of the Wnt signaling pathway (i.e. Wnt1, Wnt3a, Wnt5a, Lrp5, Ctnnb1, Lef1, and Axin), IGF-I pathway (i.e. Igf1 and c-Fos), ER pathway (i.e. Esr1 and Ncoa1), BMP/TGF-β pathway (i.e. Tgfb1 and Bmpr1) were differentially up-regulated by the fluid shear in B6 osteoblasts and not in C3H osteoblasts.

**Evidence That in Vivo Mechanical Loading Up-regulated Genes Associated with the Wnt, IGF-I, ER, and BMP/TGF-β Signaling Pathways**—To determine whether up-regulation of genes of these four anabolic signaling pathways in response to loading also occurred in bone *in vivo*, we determined the effects of this 2-week loading regimen in the form of a four-point bending exercise on the expression of genes associated
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TABLE 2
Microarray and real time PCR analyses of gene expression of the Wnt, BMP/TGF-β, IGF-I, and ER signaling pathways in response to the fluid shear in B6 osteoblasts as opposed to that in C3H osteoblasts

| Gene                  | B6 osteoblasts | C3H osteoblasts |
|-----------------------|----------------|-----------------|
|                       | Microarray, fold changes, mean ± S.D. (n = 4) | Real time PCR, fold changes, mean ± S.D. (n = 6) | Microarray, fold changes, mean ± S.D. (n = 4) | Real time PCR, fold changes, mean ± S.D. (n = 6) |
| The Wnt signaling pathway genes |                 |                 |                 |                 |
| Wnt1                  | 1.99 ± 0.54b   | 2.44 ± 0.71b   | ND              | 1.99 ± 0.86b   | 0.95 ± 0.10 |
| Wnt5a                 | 1.71 ± 0.52b   | 1.88 ± 0.85   | ND              | 2.19 ± 0.61b   | ND          |
| Wnt5b                 | 1.67 ± 0.49b   | 1.58 ± 0.61   | ND              | 2.64 ± 1.12b   | ND          |
| Lrp5                  | ND             | 1.90 ± 0.76   | ND              | 1.76 ± 0.83   | ND          |
| Axin                  | 1.22 ± 0.12b   | 1.18 ± 0.29   | ND              | 0.98 ± 0.13   | ND          |
| The BMP/TGF-β signaling pathway genes |                 |                 |                 |                 |
| Tgfb1                 | 1.97 ± 0.21b   | 1.74 ± 0.61a  | 1.70 ± 0.38a   | 1.29 ± 0.52a   | 1.05 ± 0.36 |
| Bmp5b                 | 2.05 ± 0.23a   | 1.90 ± 0.53a  | ND              | 1.61 ± 0.41a   | ND          |
| Bmpr2                 | 1.56 ± 0.36a   | 1.70 ± 0.38a  | ND              | 1.75 ± 0.81a   | ND          |
| The IGF-I signaling pathway genes |                 |                 |                 |                 |
| Igf2                  | 1.64 ± 0.27b   | ND             | 1.69 ± 0.53b   | ND              |
| Htra1                 | 1.93 ± 0.63a   | ND             | 2.25 ± 0.94a   | ND              |
| Igbp5                 | 2.25 ± 0.59b   | ND             | 1.28 ± 1.37    | ND              |
| Igf1r                 | 1.77 ± 0.35b   | 2.24 ± 1.42    | 1.15 ± 0.30    | 1.15 ± 0.30    |
| The ER signaling pathway genes |                 |                 |                 |                 |
| Esr1                  | 1.93 ± 0.49b   | ND             | 1.64 ± 0.33b   | ND              |
| Est1                  | 2.04 ± 0.60b   | 2.24 ± 0.57a   | 2.10 ± 0.57a   | 1.09 ± 0.57    | 1.25 ± 0.38 |
| Ncoa1                 | 2.15 ± 0.95a   | ND             | 1.75 ± 0.16a   | ND              |
| Rarg                  | 1.76 ± 0.35a   | ND             | 1.87 ± 0.83    | ND              |

a ND indicates not determined.

b p < 0.05.

c NP indicates not present in the array chip.

with these four pathways in the loaded tibia of B6 and C3H mice by real time PCR. We have shown previously with peripheral quantitative computed tomography, serum biochemical markers, and gene expression evidence that the 2-week four-point bending loading regimen significantly increased bone formation and bone mass in B6 mice but not in C3H mice in vivo (18). In this study, we showed that this 2-week loading regimen also caused massive increase in cancellous bone formation (stained in blue by the Goldner’s stain) at the periosteum of the loaded tibia of B6 mice, but not in the loaded tibia of C3H mice (Fig. 4), confirming that in vivo mechanical loading increased periosteal bone formation in B6 but not C3H mice (8, 9).

Table 3 shows that the 2-week four-point bending exercise regimen significantly enhanced the expression of genes associated with the four anabolic signaling pathways-of-interest in the loaded tibia of B6 mice, but not in the loaded tibia of C3H mice, confirming that in vivo mechanical loading also led to differential up-regulation of genes associated with these four anabolic pathways in bones of B6 and not of C3H mice.

Involvement of the ER Signaling Pathway in the Fluid Shear-induced Bone Cell Proliferation—To confirm the involvement of the ER signaling pathway in the fluid shear-induced osteoblast proliferation, B6 osteoblasts were pretreated with 200 nM of ICI182780, a pure antagonist of ER (19), for 24 h prior to the 30-min fluid shear, and the effect of this inhibitor on the fluid shear-induced proliferation in B6 osteoblasts was then determined. Because Erk1/2 activation is required for the fluid shear-induced bone cell proliferation (20), the effect of the inhibitor on Erk1/2 activation (assessed by Erk1/2 phosphorylation) was also evaluated. The ICI182780 pretreatment slightly, but not significantly, enhanced basal [3H]thymidine incorporation (Fig. 5A) and Erk1/2 phos-
phorylation (Fig. 5B). However, this pretreatment completely abolished the fluid shear-induced cell proliferation and Erk1/2 activation, confirming that the ER pathway is essential for the bone cell mitogenic action of fluid shear.

**Involvement of the Canonical Wnt Signaling Pathway in the Fluid Shear-induced Bone Cell Proliferation**—There are two major Wnt signaling pathways as follows: the canonical pathway that involves β-catenin stabilization and activation, and the noncanonical pathway that involves G-protein-dependent intracellular Ca²⁺ release (21). We focused on the canonical pathway because this pathway plays an important role in osteoblast functions and bone formation (22). Because activation of the canonical Wnt pathway blocks the glycogen synthase kinase-3β-mediated β-catenin phosphorylation, which then prevents the subsequent ubiquitination and degradation of hyperphosphorylated β-catenin, the increase in cellular β-catenin protein can be an index of activation of the canonical Wnt pathway. In this regard, the 30-min steady fluid shear yielded a significant 2-fold increase in total β-catenin protein levels in B6 osteoblasts, although it had no significant effect in C3H osteoblasts (Fig. 6), supporting the premise that fluid shear up-regulated the canonical Wnt pathway in B6 osteoblasts but not in C3H osteoblasts. Because the degradation (or "destabilization") of β-catenin is mediated by phosphorylation, we had also attempted to measure the cellular levels of phosphorylated β-catenin in this study. Unfortunately, we were unable to detect any significant levels of phosphorylated β-catenin in the stressed or control osteoblasts with any of the currently commercial phospho-specific polyclonal antibodies against β-catenin (Santa Cruz Biotechnologies).

To determine the role of the canonical Wnt pathway in the fluid shear-induced osteoblast proliferation, B6 osteoblasts were pretreated with 10 μg/ml of endostatin, a potent inhibitor of the canonical Wnt pathway (23), for 24 h prior to the 30-min fluid shear, and the effect of this inhibitor on fluid shear-induced cell proliferation and Erk1/2 activation was then determined. Although the endostatin pretreatment had no effect on basal [³H]thymidine incorporation and Erk1/2 phosphorylation, this pretreatment partially, but significantly, reduced the fluid shear-induced cell proliferation (Fig. 7A) and Erk1/2 activation (Fig. 7B). Although the primary inhibitory action of endostatin on the canonical Wnt pathway is mediated through an increase in β-catenin degradation, there is also evidence that endostatin suppressed β-catenin (Ctnnb1) gene expression (23). Thus, measurements of β-catenin mRNA transcript level may be used to assess the effect of endostatin on β-catenin activation. Accordingly, our findings that this dose of endostatin completely blocked the fluid shear-induced up-regulation of β-catenin mRNA (Fig. 7C) and protein levels (Fig. 7D) in B6 osteoblasts suggest that the partial inhibition of fluid shear-mediated cell proliferation was not due to an insufficient amount of endostatin.

**Involvement of the BMP Signaling Pathway in the Fluid Shear-induced Bone Cell Proliferation**—To assess whether the BMP signaling pathway has an essential role in the fluid shear-induced osteoblast proliferation, B6 osteoblasts were pretreated with 300 ng/ml of Noggin, an inhibitor of the BMP signaling pathway (24), for 24 h prior to the fluid shear. The Noggin pretreatment did not affect the basal cell proliferation and Erk1/2 activation but completely abolished the fluid shear-induced cell proliferation (Fig. 8A) and Erk1/2 activation (Fig. 8B) in B6 osteoblasts.

The role of the IGF-I signaling pathway in fluid shear-induced osteoblast proliferation was not evaluated in this study, because we and others have previously provided compelling evidence for the involvement.
Mechanotransduction Signaling Pathways in Mouse Osteoblasts

Global gene expression profiling by microarray analysis is a useful and complementary approach to genetic studies in the identification of genes and corresponding pathways contributing to a given phenotype (31). In this study, we have performed a microarray analysis with our in-house microarray chips on B6 osteoblasts that show good response to mechanical stimulation and C3H osteoblasts that show poor response. However, the relatively limited number of mouse gene fragments on our microarray chips precluded an extensive genome-wide assessment of the gene expression profile, and the incomplete annotation number of the gene fragments also did not permit computer-based analyses of the data. On the other hand, in contrast to previous microarray studies in chondrosarcoma cells (32) and in early osteochondroprogenitor cells (33), our study is unique in that we determined and compared a gene expression profile of B6 osteoblasts with that of C3H osteoblasts after a 30-min steady fluid shear stress. Because B6 osteoblasts, and not C3H osteoblasts, responded to fluid shear with an increase in cell proliferation and differentiation, an analysis of the genes whose expression is differentially regulated in B6 osteoblasts in response to the fluid shear could yield information about potential signal transduction pathways in the mechanotransduction mechanism, and this may help to identify potential candidate mechanosensitivity genes. Accordingly, the objective of this microarray study was not to obtain global gene expression profiling information but, rather, was to evaluate differential gene expression in B6 osteoblasts as opposed to C3H osteoblasts in response to the fluid shear to identify potential signaling pathways involved in the fluid shear-induced osteoblast proliferation. We hope to use this information to gain insights into the identity of mechanosensitivity genes that are responsible for the different bone formation response to loading in B6 and C3H mice.

Three observations of our microarray data were noteworthy. First, our study reveals that the expression of ~12% (i.e. 669 of 5,500) of the gene fragments on our in-house microarray chip was significantly affected (with more than half of them up-regulated) 4 h after the 30-min fluid shear in B6 osteoblasts. This relatively large number of mechanoresponsive genes in the good responding B6 osteoblasts underscores the complexity of mechanical stimulation in osteoblasts (4, 34). Of the 669 gene fragments whose expression was significantly altered by fluid shear, 514 (or 77%) were regulated differentially in B6 osteoblasts. This led us to postulate that the mechanosensitivity genes contributing to the different bone formation response in B6 and C3H mice may act on an upstream event of the mechanotransduction mechanism, leading to the subsequent changes in expression of up to 77% of the mechanoresponsive genes in B6 osteoblasts. Because there was also a differential up-regulation of at least two early mechanoresponsive genes, i.e. Igfb1 and Cox-2, in B6 osteoblasts as opposed to C3H osteoblasts, the mechanosensitivity genes in this pair of inbred mice are also upstream to these two early mechanoresponsive genes.

The second noteworthy observation is that a large number of the known mouse genes that were up-regulated differentially in B6 osteoblasts are associated with various anabolic cellular processes, such as cell proliferation, protein and RNA syntheses, energy metabolism, and intracellular transport and trafficking mechanisms. These findings support an anabolic action of the fluid shear in B6 osteoblasts but not in C3H osteoblasts. Also consistent with the previous findings that mechanical loading (or fluid shear) stimulated the local production of bone growth factors, such as IGF-I (35), IGF-II (36), and TGF-β1 (37, 38), we found that the fluid shear significantly up-regulated the expression of these growth factor genes as well as several other bone growth factor genes, such as Pdgf, Fgf, and Bmp, in mouse osteoblasts. The fact...
that fluid shear stress also up-regulated several bone growth factor receptor genes (i.e. Igfr1, Pthr1, and Bmpr1) suggests that the molecular mechanism whereby mechanical loading (or fluid shear) stimulates osteoblast proliferation may in part involve paracrine/autocrine actions of bone growth factors. On the other hand, because several of these growth factor (Igf2, Pdgfa, Vegfa, Fgf1, Tgfb1, and Bmp8b/Op2) and receptor (Bmpr1 and Pthr1) genes were also up-regulated in C3H osteoblasts in response to fluid shear, these growth factor and receptor genes are either unrelated to or upstream to the mechanosensitivity genes in the overall mechanotransduction mechanism in osteoblasts. We favor the latter possibility because there is strong circumstantial evidence that at least IGF-II (36) and TGF-β1 (39–41) are involved in the mechanical stimulation of bone formation.

The third and the more important observation of our microarray analysis is the finding that a number of genes associated with the four anabolic signaling pathways, namely the IGF-I, the ER, the canonical Wnt, and the BMP/TGF-β signaling pathways, were up-regulated in B6 osteoblasts and not in C3H osteoblasts in response to the fluid shear stress. The differential up-regulation of gene expression of these four signaling pathways in B6 osteoblasts was confirmed by real time PCR analyses. These findings raise the following interesting possibilities: (a) up-regulation of these four anabolic pathways is involved in the fluid shear-induced osteoblast proliferation; (b) these four signaling pathways are downstream to the mechanosensitivity genes in the overall mechanotransduction mechanism in osteoblasts. The latter possibility is consistent with our observation that the mechanosensitivity genes contributing to the different bone formation response in B6 and C3H mice may act on an upstream event of the mechanotransduction mechanism, leading to the subsequent changes of a large number of downstream mechanoresponsive genes, including genes associated with these four anabolic signaling pathways. Although our supporting data for the conclusion that each of these four pathways plays an essential role in the fluid shear-induced osteoblast proliferation were based on RNA expression data and not protein production data (with the exception of β-catenin), this conclusion was supported by the findings that a specific inhibitor of the
ER signaling pathway (IC182780), the canonical Wnt signaling pathway (endostatin), and the BMP signaling pathway (Noggin) each either completely abolished or markedly suppressed the fluid shear-induced cell proliferation in B6 osteoblasts.

We should emphasize that the in vivo application of mechanical loading through the 2-week four-point bending exercise regimen also led to up-regulation of expression of genes associated with the IGF-I, the ER, the canonical Wnt, and the BMP/TGF-β signaling pathways in the loaded tibia of B6 mice. Accordingly, inasmuch as there is a general belief that osteocytes, and not osteoblasts, are most likely the primary sensors of mechanical loading in bone (42), we conclude that our in vitro findings with the primary mouse osteoblasts are probably physiologically relevant, even though the issue as to whether osteoblasts indeed have a functional role in translating the mechanical signal into biochemical signals in bone remains controversial.

There is compelling evidence for the involvement of the IGF-I, ER, and canonical Wnt signaling pathways in mechanotransduction in bone. With respect to the IGF-I signaling pathway, it has been reported that the bone cell mitogenic response to mechanical strain is mediated through activation of the IGF-I receptor (25). We recently showed that fluid shear stress synergizes with IGF-I to stimulate Erk1/2 activation and osteoblast proliferation through integrin-dependent activation of the IGF-I signaling pathway (28), confirming a functional role of the IGF-I signaling pathway in the mechanical stimulation of osteoblast proliferation. Bikle and co-workers (26, 27) have also demonstrated that skeletal unloading induces resistance to IGF-I to induce bone formation, which is caused by inhibition of the IGF-I signaling pathway through down-regulation of the integrin pathway. Regarding the ER signaling pathway, Lanyon and co-workers (25, 43–46) have provided compelling evidence that ER, especially ERα, is essential for mechanical stimulation of bone formation. Specifically, they showed that knocking out the ERα (Esr1) expression in mice completely abolished the osteogenic response to mechanical loading in vivo (45) and in vitro (46). Their recent data suggested that ERB may also have a certain role in mediating the mechanical signal to stimulate bone formation, because knocking out ERB (Esr2) expression in female mice also blocked the mechanical stimulation of bone formation (47). In support of a role of the canonical Wnt pathway in mechanotransduction, it has been reported recently that the canonical Wnt, and the BMP/TGF-

![Mechanotransduction Signaling Pathways in Mouse Osteoblasts](image)

**FIGURE 8. Effects of Noggin pretreatment on the fluid shear-induced proliferation and Erk1/2 activation in B6 osteoblasts.** B6 osteoblasts were pretreated with 300 ng/ml Noggin for 24 h prior to the fluid shear stress. Cell proliferation was measured by [3H]thymidine incorporation, and Erk1/2 activation was assessed by the pErk1/2 level. A shows the summarized results of three separate experiments on the relative levels of pErk1/2 normalized against the total Erk1/2 (pan-Erk) protein level. The results are shown as percentage of Noggin for 24 h prior to the fluid shear stress. Cell proliferation was measured by [3H]thymidine incorporation, and Erk1/2 activation was assessed by the pErk1/2 level.

**TABLE 4**

Effects of inhibition of integrin activation and/or Cox-2 activity on the fluid shear-mediated up-regulation of gene expression of genes associated with the Wnt, BMP/TGF-β, IGF-I, and ER signaling pathways in B6 osteoblasts (mean ± S.D., n = 6)

| Gene | Inhibition of integrin activation (fold changes) | Inhibition of Cox-2 activity (fold changes) |
|------|-----------------------------------------------|-----------------------------------------------|
|      | No echistatin +100 nM echistatin               | No indomethacin +1 μM indomethacin             |
| The Wnt signaling pathway genes |                                            |                                             |
| Wnt1 | 2.51 ± 0.87*                                  | 2.59 ± 0.87*                                  |
| Wnt5a| 3.18 ± 0.68*                                  | 2.97 ± 1.00*                                  |
| Ctnnb1 | 2.26 ± 0.53*                               | 3.68 ± 1.13*                                  |
| The BMP/TGF-β signaling pathway genes |                                            |                                             |
| Tgfβ1 | 2.99 ± 1.31a                                  | 2.47 ± 0.50b                                  |
| Bmpr1 | 3.68 ± 1.49a                                  | 3.01 ± 0.17a                                  |
| Dlx1  | 2.41 ± 0.63a                                  | 2.77 ± 0.95a                                  |
| The IGF-I signaling pathway genes |                                            |                                             |
| Igf1r | 4.33 ± 1.98a                                  | 3.38 ± 1.16a                                  |
| C-Fos | ND                                           | 2.83 ± 0.89a                                  |
| The ER signaling pathway genes |                                            |                                             |
| Esr1 | 3.74 ± 0.64a                                  | 3.69 ± 0.97b                                  |
| Ncoa1 | 4.25 ± 2.03a                                  | 3.01 ± 0.39b                                  |

* p < 0.05.  
* p < 0.01.  
ND indicates not determined.
that activation of the canonical Wnt signaling pathway by the G171V mutation of the low density LRP5 led to an enhanced response in the mechanical stimulation of bone formation (48) and that mechanical loading activated the canonical Wnt signaling pathway in TOPGAL mice, which are transgenic mice expressing a β-galactosidase reporter gene driven by a T cell factor β-catenin-responsive promoter (49). There is also evidence for a role of the TGF-β signaling pathway in mechanotransduction in bone, although the evidence is less compelling. Accordingly, mechanical loading or fluid shear significantly up-regulated the expression and secretion of TGF-β1 in bone cells, whereas mechanical unloading markedly suppressed Tgfb1 expression in bone (37–41). Consequently, our findings that the IGF-I, the ER, the canonical Wnt, and even the TGF-β signaling pathways are involved in the mechanotransduction mechanism in B6 osteoblasts are not entirely surprising.

What was surprising to us is the finding that blocking the BMP signaling pathway in B6 osteoblasts by Noggin led to the complete abolition of the fluid shear-induced osteoblast proliferation. In this regard, although it is well known that BMPs are potent osteoblast differentiation agents, which stimulated bone formation primarily through its ability to promote osteoblast differentiation (50), there has been little evidence that BMPs can directly stimulate osteoblast proliferation. Consequently, the mechanism whereby inhibition of the BMP signaling pathway by Noggin blocked the fluid shear-induced cell proliferation and Erk1/2 activation in B6 osteoblasts is unclear. However, it has been demonstrated that there are significant cross-talks among the various signaling pathways involved in mechanotransduction (4, 34). Specifically, there is evidence in other cell types that the BMP signaling pathway can cross-talk with other signaling pathways, including the canonical and noncanonical Wnt pathways (51), and the ER pathway (52).

Because we have recent preliminary data that inhibition of the BMP pathway with Noggin could completely block the fluid shear-induced up-regulation of the expression of genes associated with the canonical Wnt pathway in B6 osteoblasts (53), we tentatively conclude that the canonical Wnt pathway is downstream to the BMP signaling pathway in fluid shear stress-induced osteoblast proliferation. Accordingly, we suggest that the mechanism whereby the BMP signaling pathway mediates the fluid shear-induced osteoblast proliferation involves the subsequent activation of the canonical Wnt signaling pathway.

It is also foreseeable that there would be cross-talks among these four anabolic pathways, as there is evidence that the BMP signaling pathway can cross-talk with the ER pathway in other cell types (52) and that the IGF-I signaling pathway interacts with the ER pathway in the proliferative response of osteoblasts to mechanical strain (25). Consequently, it may be speculated that these potential cross-talks may explain why inhibition of a single signaling pathway (e.g. the ER or the BMP pathway) could lead to complete abrogation of the fluid shear-induced cell proliferation and Erk1/2 activation in B6 osteoblasts.

It is also intriguing to note that endostatin was only able to partially block the fluid shear-induced Erk1/2 activation and cell proliferation, suggesting that the canonical Wnt signaling pathway is only partially involved in mechanotransduction in osteoblasts. On the other hand, fluid shear stress appeared to also up-regulate the noncanonical Wnt signaling pathway, as the expression of Wnt5a was also up-regulated in response to the fluid shear stress in B6 osteoblasts. Thus, it is possible that the mechanical stimulation of osteoblast proliferation may involve up-regulation of both canonical and noncanonical Wnt pathways. Thus, blocking the canonical Wnt pathway alone is insufficient to block completely the fluid shear-induced osteoblast proliferation.

The exact molecular mechanism as to how each of these four anabolic signaling pathways mediate the fluid shear-induced osteoblast prolifer-
C3H inbred strain of mice are upstream to the IGF-I, the ER, the Wnt, and the BMP/TGF-β signaling pathways. In this regard, we believe that the mechanosensitivity may act on an upstream event between the putative mechanosensors and the activation of integrin signaling pathway (Fig. 9). This information should be very useful in our continuing efforts to search for the identity and underlying mechanism of the mechanosensitivity genes that are responsible for the different bone formation response in this pair of inbred mice. Accordingly, we believe that the ability of a candidate gene to up-regulate the expression of genes associated with all of these four signaling pathways in response to mechanical stimulation can be a useful functional screen assay for candidate genes of mechanosensitivity. Moreover, because the mechanosensitivity genes appear to be upstream regulators, this information may allow us to narrow our search of candidate genes to upstream regulators. In this regard, the quantitative trait locus for mechanosensitivity in mouse chromosome 4 identified by Robling et al. (29) is a large 40–80-centimorgan region and contains several hundreds of genes, including a number of upstream signal transduction regulator genes. These include receptor genes (such as Lepr, Il22ra1, Ptprf, Oprd1, Htr1D, and Htr6, Epha1, and Ephb2, and Tnfr), several receptor tyrosine kinases, Ptpru, ion channel genes (Gjb3), and several other candidate genes. In order for any of these or other candidate genes to be the mechanosensitivity gene, the candidate gene must be able to up-regulate these four aforementioned anabolic signaling pathways in response to mechanical stimulation. Two genes (Bmp8/Op2 and Jun) whose expression was up-regulated by the fluid shear in osteoblasts were located within this quantitative trait locus. However, because the expression of Bmp8/Op2 was up-regulated by fluid shear in both B6 and C3H osteoblasts, and because Jun is a downstream effector of the signaling pathways-of-interest, neither of these two genes would likely be the mechanosensitivity gene(s) that contributes to the different bone formation response to mechanical loading in B6 and C3H inbred strain of mice.

In conclusion, we have demonstrated that fluid shear induced an anabolic response in B6 osteoblasts but not in C3H osteoblasts, indicating that the differential osteogenic response to mechanical stimulation in this pair of inbred strain of mice is intrinsic to osteoblasts. More importantly, we showed for the first time that fluid shear differentially up-regulated the expression of genes associated with four anabolic signaling pathways (i.e. the IGF-I, the ER, the Wnt, and the BMP/TGF-β pathways) in the good responder, B6 osteoblasts, and not in the poor responder, C3H osteoblasts. An important implication of these findings is that the mechanosensitivity genes contributing to the different bone formation response in B6 and C3H inbred strain of mice are upstream to these four anabolic signaling pathways. This information should be very helpful in our search of the identity and/or the underlying mechanism of mechanosensitivity genes.

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