Wnt/β-Catenin Signaling Is a Component of Osteoblastic Bone Cell Early Responses to Load-bearing and Requires Estrogen Receptor α*

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The Wnt/β-catenin pathway has been implicated in bone cell response to their mechanical environment. This response is the origin of the mechanism by which bone cells adjust bone architecture to maintain bone strength. Osteoporosis is the most widespread failure of this mechanism. The degree of osteoporotic bone loss in men and women is related to bio-available estrogen. Here we report that in osteoblastic ROS 17/2.8 cells, osteoblastic ROS 17/2.8 cells rotic bone loss in men and women is related to bio-available estrogen. We examined the relationship between responsiveness to loading and LRP5 function to be examined directly. When the bones of mice with the LRP5 gain of function mutation are exposed to short periods of mechanical loading they show a more osteogenic response than their wild-type (WT) controls (10). Those lacking LRP5 function because of absence of the gene locus show a lower osteogenic response (11, 12). This supports the hypothesis that LRP5 status influences the osteoregulatory outcome of strain-related stimulation of bone cells.

LRP5 is the membrane-associated co-receptor of the transmembrane protein Frizzled (Fzd) (13). When this receptor is inactive β-catenin within the cell is phosphorylated by glycogen synthase kinase-3β (GSK-3β) enabling its ubiquitination and proteasomal degradation. The Fzd/LRP5 receptor is active, GSK-3β activity is inhibited. The activated (hypophosphorylated) β-catenin, which then accumulates is free to move to the nucleus in conjunction with T cell factor (TCF) and lymphoid enhancer factor (LEF) where they displace inhibitory transcription factors localized to TCF/LEF consensus binding sites to activate TCF/LEF responsive target genes (14–17).

Recent studies have shown that mechanical loading in vivo up-regulates “stress”-responsive and Wnt/β-catenin genes (18, 19) and that this effect is enhanced in LRP5 gain of function mice (19). Furthermore, the strain and flow-related up-regulation of a number of gene products is enhanced by concurrent stimulation of the Wnt pathway (19). The available evidence therefore suggests that the Wnt/β-catenin signaling pathway provides an important contribution to bone cell adaptive responses to mechanical stimulation. However, there are at present no data, of which we are aware, that link function of Wnt/β-catenin signaling with the most widespread failure of...
the mechanically related pathway; postmenopausal osteoporosis in women, and age-related osteoporosis in men.

The rapid loss of bone in women at the menopause and the later, more gradual, age-related decline in bone mass in men and women, both represent an acquired failure to match bone mass and architecture to prevailing loads. In both instances this failure is linked to levels of bio-available estrogen (20–22) (reviewed in Ref. 23). Despite this association in vivo the level of estrogen has not been linked to bone cell ability to respond to mechanical strain, or one of its immediate consequences, fluid flow. An explanation for this could be the finding that, in vitro and in vivo, it is ERα, rather than estrogen itself, that is necessary for full expression of bone cells adaptive response to mechanical stimulation. In vitro the proliferative response of bone cells to applied strain or fluid shear is absent when ERα is absent or blocked by receptor modulators (18, 24), and enhanced when ERα numbers are increased by transfection (25). In the absence of ERα in vivo bone adaptive response to loading is substantially less than when it is present (26). Because the level of expression of ERα is normally regulated by circulating estrogen, and unaffected by local strain (27), it may be that when estrogen levels are low the amount of functional ERα available to effectively process the cell strain-related response falls below critical levels. In this situation even high strains are no longer converted into adequately osteogenic responses. In the absence of these responses, bone mass declines. Whereas this hypothesis (28) provides a conceptual explanation for estrogen-related bone loss, the mechanisms have yet to be established. The studies reported here were designed to assess the extent to which the functioning of the Wnt/β-catenin pathway in bone cell early responses to mechanical strain are modified by the presence or activity of estrogen and ERα.

EXPERIMENTAL PROCEDURES

Reagents—Dulbecco’s minimal essential medium (DMEM) without phenol-red, the l-glutamine, penicillin/streptomycin, and trypsin/EDTA were purchased from Invitrogen (Invitrogen Ltd, Paisley, Scotland, UK). Heat-inactivated fetal calf serum was purchased from LabTech International (East Sussex, UK). Tissue culture plasticware was obtained from Falcon (BD Biosciences, Cowley, Oxford, UK) or Nunc (Nalg, Nunc International) unless stated otherwise. For in vitro straining, cells were cultured on custom-made tissue culture-treated plastic slides purchased from Nunc (Dossel, Germany) which were placed in 4-well plates purchased from quadriPERM, (Greiner bio-one, Stonehouse). For immunocytochemistry, in non-strained experiments, cells were cultured onto 13-mm diameter tissue culture-treated plastic slides (for reporter assays) and 8-well plates (for Western blotting) or plastic slides (when subjected to mechanical strain) and incubated at 37 °C with 5% CO2 in a humidified atmosphere.

Constructs—The super8XTOPFLASH (superTOP) reporter construct containing 8 TCF/LEF binding sites within a pTA-Luc vector of Clontech driving the expression of firefly luciferase under the control of a minimal TA viral promoter and the super8XFOPFLASH (superFOP) reporter construct containing mutated TCF/LEF binding sites were designed in the Randall Moon Laboratory and were kindly donated by Prof. Moon (29). The osteopontin (OPN) luciferase reporter construct OPN-pGL3 containing a 2.3-kbp fragment of the 6-kbp rat OPN promoter containing two TCF/LEF binding sites in both the wild-type and mutant forms (−1960/−1463) (30) were a gift from Prof. Lukas A. Huber. A control plasmid containing Renilla luciferase gene under the control of a cytomegalovirus (CMV) promoter from Promega (Southampton, UK) was used to normalize for transfection efficiency. Cells were transfected using TransFectin purchased from Bio-Rad or Effectene purchased from Qiagen (Crawley, West Sussex, UK).

Antibodies—The antibody recognizing active β-catenin (8E7) was purchased from Upstate Signaling, Dundee, UK. The mouse monoclonal β-catenin (E-5), rabbit polyclonal ERα (MC-20), goat polyclonal β-actin (I-19), and goat polyclonal Lamin B (C-20) were purchased from Santa Cruz Biotechnology (La Jolla, CA). The rabbit polyclonal phospho-GSK-3β (Ser9) antibody (rabbit monoclonal GSK-3β (27C10) and rabbit polyclonal IGFIR primary antibodies were obtained from Cell Signaling Technologies (Hitchin, UK). For Western blotting, the primary antibodies were detected using horseradish peroxidase-linked anti-mouse, -goat, or -rabbit conjugates as appropriate (Dako, Ely, UK). For immunocytochemistry fluorescein isothiocyanate-conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibodies (Alexa Fluor 488) were used as appropriate (Molecular Probes). The real-time RT-PCR primers for CREBBP and β-actin were purchased from MWG-Biotech-AG (85560 Ebersberg, Germany).

Animals—Female C57/BL/6 mice were purchased from Charles River Laboratories (Margate, Kent, UK). ERα-null mutant mice (ERα−/−) (31) were a gift from Professor Pierre Chambon.

Cell Culture—Primary osteoblast-like cells were prepared from the long bones of 17-week-old female ERα−/− mice and their WT littermates as previously described (32).

The ROS 17/2.8 cells or primary osteoblast-like cells were seeded in complete media (Dulbecco’s modified Eagle’s medium without phenol-red supplemented with 10% (v/v) fetal calf serum, 2 mM l-glutamine, and 100 units/ml penicillin and 100 μg/ml streptomycin) at a density of 2 × 10^4 cells onto thermomax coverslips (for immunocytochemistry) or 24-well plates (for reporter assays) and 8 × 10^5 cells/cm^2 onto 6-well plates (for Western blotting) or plastic slides (when subjected to mechanical strain) and incubated at 37 °C with 5% CO2 in a humidified atmosphere.

Treatments—To test the potential involvement of the ER in LiCl or strain-induced β-catenin signaling ROS 17/2.8 cells, WT osteoblasts or ERα−/−-derived osteoblasts were pretreated with 1 μM or 100 nM with selective estrogen receptor modulators (SERMs) ICI 182,780 or tamoxifen (33, 34) for 3 h prior to stimulation with 10 mM NaCl, 10 mM LiCl, or mechanical strain and then cultured (in the presence of the SERM or vehicle) over a period of 1–48 h dependent on the experimental endpoint. These concentrations of ICI 182,780 and tamoxifen are those (between 1 nM and 100 nM) that produced maximal reductions in LiCl-induced TCF/LEF reporter activity without any effect in controls. Cells were incubated with ethanol-based 17β-estradiol (E2) at a dose of 10–100 nM (as previously described (25)) over a period of 1–48 h. Control cells received an equal volume
of vehicle (ethanol), the volume of vehicle in both control and treated cells did not exceed 0.1% (v/v). The dose of LiCl used in these studies has been reported to inhibit GSK-3β (35).

Mechanical Straining in Vitro—Cells cultured on plastic slides were subjected to a single period of 600 cycles of four-point bending at a frequency of 1 Hz. The waveform of each strain cycle consisted of a ramped square wave with strain rates on and off of 23,000 μe/s, dwell times on and off of 0.4 and 0.75 s, respectively and a peak strain of 3400 μe (32). Following strain treatment, the cells were maintained in the media exposed to the strain and cultured over a time course as appropriate. Controls and treated cells on slides and in wells or dishes were maintained under similar conditions for specified times after treatment.

Transient Transfections and Luciferase Assay—ROS 17/2.8 cells were transiently transfected in 24-well plates or on the plastic slides with 0.1 μg or 0.25 μg, respectively, of a control plasmid (RL-pCMV) and 0.5 μg or 2.5 μg (well or slide respectively) with the superTOP, or superFOP reporter constructs. Slides were also transfected with 2.5 μg of the OPNpGL3 reporter construct and the RL-pCMV reporter using Transfection or Effectene according to the manufacturer’s protocol. After treatment, the cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed with 1× passive lysis buffer (Promega). 20 μl of lysate was used to determine the relative luciferase activity of firefly and Renilla luciferase using the Dual-Luciferase assay system (Promega).

Western Blotting—Cells were briefly washed in ice cold PBS and lysed in denaturing lysis buffer (2% SDS, 2 M urea, 8% sucrose, 20 mM sodium β-glycerophosphate, 1 mM NaF, and 5 mM Na<sub>2</sub>VO<sub>4</sub>) using 100 μl/l/slide. Genomic DNA was sheared by passage through a Qiashredder column (Qiagen), and proteins and DNA denatured by boiling for 5 min. Nuclear and cytoplasmic fractionation was performed by a modification of the method described by Sunters et al. (36). Briefly, cells were trypsinized and washed twice in ice-cold PBS and lysed on ice for 15 min in 100 μl of cytoplasmic lysis buffer (10 mM HEPES pH 7.4, 10 mM KCl, 0.01 mM EDTA, 1 mM dithiothreitol, 5 mM Na<sub>2</sub>VO<sub>4</sub>, 20 mM sodium β-glycerophosphate, 0.1% Nonidet P-40, and Halt Protease inhibitor mixture (Perbio, Chester, UK)). Nuclei were sedimented by centrifugation and the supernatant containing the cytoplasmic fraction removed. The nuclei were then washed in 1 ml of cytoplasmic lysis buffer to remove any contaminating cytoplasm and resedimented. The nuclei were then lysed in 100 μl of denaturing lysis buffer as before. Protein concentrations were determined by the BCA assay (Perbio, Chester, UK). 20 μg of protein were size-fractionated using SDS-PAGE and electrotransferred onto Protran nitrocellulose membranes (Schleicher and Schuell Dassel, Germany). Membranes were blocked for 1 h in 0.2% (w/v) 1-block (Topix, Bedford, MA) before being incubated with primary and secondary antibodies. Proteins were visualized using the enhanced chemiluminescence detection system (ECL) (GE Healthcare, Amersham Biosciences).

Immunocytochemistry—After treatment, slides or coverslips were washed in PBS, and the cells were fixed with ice-cold methanol on ice for 10 min followed by two PBS washes. The cells were then permeabilized in buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.05% sodium azide, 0.5% Triton X-100 (Surface- Amps×-100, Pierce), pH 7.0) for 10 min on ice. Non-specific antigen binding sites were blocked by incubating the slides in wash buffer (10% fetal calf serum, 0.05% sodium azide in PBS) for 1 h at room temperature. Slides were incubated with primary antibodies recognizing either active β-catenin (1:100 dilution) or ERα (1:100 dilution) overnight at 4 °C in a humidified chamber after which they were washed for 30 min at room temperature before incubation with the secondary antibody. Fluorescin isothiocyanate-conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibodies were used in a 1:100 dilution for 45 min in the dark at room temperature. Cells were then washed twice in wash buffer. Slides were mounted in PBS before visualization by confocal microscopy. Confocal micrographs shown are merged through-focus images for stack of xy images. For clarity, micrographs for ERα staining are shown with red fluorescence, images were altered using Adobe Photoshop software (Version 5.0).

Quantification of Immunocytochemical Images—200 osteoblasts were examined on the coverslips or slides and the intensity of staining evaluated in arbitrary units (pixel intensity per unit area under standardized conditions as in Horton et al. (37) using Leica Sp5 software (Leica Microsystems LAF AF-TCS SP5, Heidelberg, Germany).

Mechanical Loading of the Mouse Tibia in Vivo—The right tibia of 32 female C57BL/6 mice (15 weeks old) and 24 female ERα<sup>-/-</sup> mice were subjected to single period of dynamic axial load using a hydraulic actuator under feedback control (38) (Dartec HC10, Zwick/Roell UK). The load was applied with a sine waveform, at a frequency of 2 Hz for 30 s (60 cycles). The peak load magnitude was 12 N producing a compressive strain measured at the medial midshaft of 1300 με. In separate experiments in similar mice this transient exposure to dynamic strain change has been shown to produce an osteogenic response. The left tibia was not loaded and served as an internal control. To restrain the mice during loading they were anesthetized with halothane for ~3 min and allowed to recover immediately after loading. The animals were sacrificed at 3, 8, 12, and 24 (C57BL/6) and 3, 8 and 24 h (ERα<sup>-/-</sup>) after loading (8 mice at each time point).

RNA Isolation from Mouse Bones and Analysis by Microarray—The right and left tibiae were carefully dissected and all their surrounding muscle removed leaving the periosteum intact. The cartilaginous ends of the bones were removed and the remaining shaft spun at 5000 rpm for 2 min (Eppendorf centrifuge) to remove marrow before being snap-frozen in liquid nitrogen. Frozen tissues were pulverized under liquid nitrogen using a mortar and pestle and lysed in Qiazol lysis reagent (Qiagen). Total RNA for microarray experiments was purified and DNase-treated using RNase Mini kit (Qiagen). The quantity and the integrity of the purified RNA were assessed using the Agilent RNA Bioanalyzer (Agilent Technologies UK Limited, Stockport, UK).

cDNA was synthesized from total RNA using a SMART protocol (39) and labeled using the Bioprime labeling kits (Invitrogen Ltd) according to the manufacturer’s recommendation. The labeled cDNA was hybridized onto an RNG-MRC mouse set 25K microarray printed on the GE Codelink slide. The
arrays were scanned using a ProScanArray HT (PerkinElmer, Beaconsfield, UK) at 7 different PMT gain settings from 40 to 70. The images were then processed using ImaGene 6.0.1 (Bio Discovery, El Segundo, CA), where all 14 images were overlaid and gridded and the feature data extracted.

These data were then processed using Mavi 2.6.0 (MWG Biotech AG, Ebersberg, Germany), which increases the dynamic range while avoiding saturation problems. The data were then loaded into R Project for Statistical Computing for further analysis. A two dimensional loess normalization, from the YASMA5 (Yet Another Statistical Microarray Analysis) library, was performed on each array to correct for any spatial variation within the slide. The LIMMA library (Linear Models for Microarray Analysis (40) from the BioConductor software project was used to further normalize the data and to select differentially expressed genes. In brief, a linear model is fitted to the data for each gene to fully model the systematic part of the data and provide estimates for each coefficient (samples in this case). These coefficients can then be compared and differentially expressed genes were selected using an empirical bayes moderated t-statistic. Differential genes were selected for the comparisons of interest based on their moderated t-statistic (41) after using a false discovery rate control of 5% (42). The fitted values for each sample were then converted back to red and green intensities and loaded into GeneSpring GX (Agilent technologies, Stockport, UK) to allow for easy comparison of lists of differential genes. Lists of differential genes were loaded into Ingenuity Pathways Analysis software.

**Quantitative Real Time RT-PCR**—Quantitative real time RT-PCR (qRT-PCR) was used to confirm changes in transcription suggested by microarray analysis. Total RNA from loaded and control tibiae was reverse-transcribed with Superscript II Reverse Transcriptase (Invitrogen). RT-PCR was carried out using QuanTect Syber Green PCR kit (Qiagen) and Opticon 2 lightcycler (MJ Research, Waltham, MA). Sequences of the primers were: CREBBP (forward 5’-CAATCCCAACCTCCATTTTC-3’, reverse 5’-CTCTTGTGGGGGTATTCAG-3’) and β-actin (forward 5’-CTATGAGCTGCTAGCGTTC, reverse 5’-AGTTTCTAGGTATCCAGCAGGT-3’). The PCR conditions used a 15 min initial enzyme activation step followed by 40 cycles of 15 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C. The final elongation step was 7 min at 72 °C.

**Statistical Analysis**—Statistical significance was determined by a 2-tailed unpaired Student’s t test or one way analysis of variance (ANOVA) followed by the post hoc (Bonferroni) multiple comparisons between treatment groups using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). p < 0.05 was considered statistically significant. Although for clarity some data are presented as mean percentage compared with control (±S.E.), all comparisons are made with raw data.

**RESULTS**

**Mechanical Strain in Osteoblastic Cells Increases the Levels of Active (Hypo-phosphorylated) β-Catenin, Stimulates Its Accumulation in the Nucleus and Increases TCF/LEF Transcriptional Activity of the Osteopontin Promoter**—The deformation of bone tissue that occurs when bones are loaded changes the shape of resident bone cells and causes movement of fluid within the interstices of the bone that these cells occupy. Many studies have shown that osteoblasts and osteocytes are responsive to both strain and fluid flow by increasing their production of prostanooids (43, 44), nitric oxide (45), and growth factors (46) and by proliferation (18, 47–50). Hens et al. (51) and Norvell et al. (52) have also shown mechanically related activation of the Wnt pathway.

In the experiments reported here we sought to determine in cells of the ROS 17/2.8 osteoblastic cell line, whether a single short period of four-point bending of their substrate, sufficient to stimulate their proliferation, would result in changes in the localization, amount, or activity of β-catenin similar to the changes caused by LiCl, which activates the β-catenin signaling pathway by inhibiting GSK-3β.

Immunocytochemistry and confocal microscopy reveal that in unstrained static (control) cells there was only weak punctate nuclear staining for activated β-catenin evident at all time points investigated (a representative image after 3 h is shown in Fig. 1A, panel a). Thirty minutes after exposure to strain the staining intensity for activated β-catenin in the nucleus had increased (Fig. 1A, panel b) compared with the control, remaining high at 1 and 3 h after straining (Fig. 1A, panels c and d). By 6 h (Fig. 1A, panel e) and 24 h (data not shown) the pattern and intensity of staining was similar to that in static, non-strained control cells.

As a positive control for nuclear accumulation of activated β-catenin, ROS 17/2.8 cells were treated with 10 mM LiCl (in a parallel experiment). In time-matched control cells treated with 10 mM NaCl, faint punctate staining for activated β-catenin was observed in the nucleus (shown by a representative image after 3 h Fig. 1B, panel a). At the earlier time points (30 min to 3 h) the intensity and pattern of staining for activated β-catenin was similar to that after mechanical strain (Fig. 1B, panels b–d). After 6 h, the nuclear staining declined to levels similar to those after 30 min and 1 h of LiCl stimulation (Fig. 1B, panel e). By 24 h levels were the same as in the controls (data not shown).

To confirm our immunocytochemical data, we performed Western blot analysis on whole cell lysates at various time points after the cells had been subjected to strain. Fig. 1C shows that 10 min after strain there was an increase in the level of activated β-catenin, which reached a maximum at 30 min, remained high at 1 and 3 h, and had declined by 24 h. In comparison, levels of total cellular β-catenin (both phosphorylated and hypophosphorylated forms) remained constant throughout. Western blotting of the nuclear and cytoplasmic fractions confirmed that both strain and LiCl treatment were associated with increased levels of active β-catenin in the nucleus (Fig. 1D). Lack of cross contamination of the fractions was validated by the absence of the membrane receptor IGFIR in the nuclear fraction, and lamin B (a component of the nuclear membrane) in the cytoplasm. Although there was a slight strain-related increase in activated β-catenin in the cytoplasm, this was not as marked as the increase in the nucleus (Fig. 1D). These Western blots also showed that the changes in the levels of activated β-catenin were paralleled by inhibitory phosphorylation of GSK-3β (Fig. 1C). This is consistent with strain-related activa-
ERα Mediates β-Catenin Response to Strain in Osteoblasts

To confirm the effect of E2 treatment on the transcriptional activation of β-catenin in the nucleus of ROS 17/2.8 cells we performed a reporter assay using the superTOP construct containing multiple TCF/LEF binding sites. This showed that 10–100 nM of E2 treatment over 24 h (Fig. 2C) or 48 h (data not shown) had no influence on TCF/LEF transcriptional activity. In contrast a significant increase was observed following LiCl stimulation (396 ± 31%, p < 0.001) over the same time period.

mean ± S.E. of data pooled from three separate experiments, each normalized to its mean static control. ***, p < 0.001 versus unstrained static control (Student’s t test).
These data suggest that in osteoblastic ROS 17/2.8 cells E2 alone had no influence on β-catenin activation or localization within the cell.

The Estrogen Receptor Modulators ICI 182,780 and Tamoxifen Inhibit LiCl-stimulated Nuclear Localization and Activation of β-Catenin in ROS 17/2.8 Cells—Although movement of β-catenin from the cytoplasm to the nucleus is a prerequisite for its ability to regulate transcription, β-catenin has no nuclear recognition sequence of its own and requires association with other molecules to pass through the nuclear membrane (57). It has been documented that nuclear steroid receptors play a role in the nuclear translocation of activated β-catenin (58–62).

However, while the ER has been shown to physically interact with β-catenin (63), the only published data of which we are aware suggests that in the cell types studied ER does not play a role in the nuclear accumulation of β-catenin (62). We have previously reported that ERα rapidly translocates to the nucleus following mechanical strain (27) and have hypothesized that the effect of estrogen on the ability of bone cells to adjust bone architecture adequately in response to mechanical strain is indirect via estrogen regulation of ERα.

To investigate whether ER plays a role in the regulation and translocation of β-catenin within osteoblastic cells we cultured ROS 17/2.8 cells on coverslips with vehicle, ICI 182,780 or tamoxifen for 3 h prior to treatment with LiCl for a further 3 or 6 h in the presence of the SERM, thereby exposing the cells to ICI 182,780 or tamoxifen for 6 or 9 h, respectively. In the vehicle-treated control cells immunocytochemical staining for ERα was mostly distributed in the cytoplasm (Fig. 3A, panel a). After 3 h of treatment with LiCl the intensity of cytoplasmic staining for ERα decreased and punctate staining in the nucleus could be seen (Fig. 3A, panel b). As expected, LiCl treatment also stimulated nuclear translocation of activated β-catenin (Fig. 3A, panel d) compared with the weak punctate nuclear staining evident in the NaCl-treated control (Fig. 3A, panel c). Following 6 h of ICI 182,780 treatment cytoplasmic staining for ERα was reduced (Fig. 3A, panel e) while the nuclear staining for ERα induced by LiCl also appeared to decline (Fig. 3A, panel f). The ICI 182,780 treatment alone had no apparent effect on either the level or location of activated β-catenin (Fig. 3A, panel g), yet the LiCl-induced nuclear accumulation of activated β-catenin was abrogated in the presence of ICI 182,780 (Fig. 3A, panel h). This finding was also observed after 6 h of LiCl treatment in which the cells were exposed to a longer period of ICI 182,780 (data not shown). Treatment with tamoxifen for 6 h had no effect on cytoplasmic staining for ERα and LiCl-induced nuclear accumulation of activated β-catenin was not modulated in the presence of tamoxifen at 6 h (data not shown). In contrast, when the cells were exposed to tamoxifen for 9 h cytoplasmic staining for ERα was substantially lower (Fig. 3A, panel i). The LiCl-induced nuclear accumulation of ERα was also reduced (Fig. 3A, panel j). The longer period of tamoxifen treatment had no apparent effect on the localization of activated β-catenin compared with control (Fig. 3A, panel k), while the LiCl-stimulated nuclear accumulation of activated β-catenin was inhibited (Fig. 3A, panel l).

To determine whether the modulation of LiCl-induced nuclear translocation of active β-catenin was reflected in changes in transcriptional activity, ROS 17/2.8 cells were transfected with superTOP and pretreated with vehicle, ICI 182,780 or Tamoxifen for 3 h prior to LiCl stimulation for 24 h. LiCl caused a statistically significant increase in TCF/LEF activation (489 ± 51%, p < 0.001). ICI 182,780 had no effect on the basal activity of the superTOP reporter compared with that in vehicle controls, however it significantly decreased (91%, p < 0.01) the TCF/LEF activation in response to LiCl (Fig. 3B). The data in Fig. 3B clearly show that tamoxifen had no effect on basal activity of the reporter and did not significantly modulate LiCl-mediated TCF/LEF activation, this may reflect the delayed action of tamoxifen on ERα expression.
**ERα Mediates β-Catenin Response to Strain in Osteoblasts**

17/2.8 cells, we sought to determine whether ERα was involved in mediating strain-related β-catenin signaling.

ROS 17/2.8 cells were exposed to strain in the presence of ICI 182,780 (as described for the LiCl stimulation) and the cellular localization of ERα and active β-catenin determined. In the vehicle-treated static control cells immunocytochemical staining for ERα was mostly distributed in the cytoplasm (Fig. 4A, panel a). Three hours after treatment with strain the intensity of nuclear staining for ERα appeared to increase while cytoplasmic staining was still evident (Fig. 4A, panel b). As shown in Fig. 1A, panel a weak punctate nuclear staining for activated β-catenin was evident in the static control (Fig. 4A, panel c). As expected, 3 h following strain accumulation of active β-catenin could be seen in the nucleus (Fig. 4A, panel d). Once again ICI 182,780 substantially reduced the intensity of staining for ERα in the cytoplasm and the nucleus in the absence and presence of strain (Fig. 4A, panels e and f). There was no discernible effect of ICI 182,780 treatment alone on the intensity or distribution of activated β-catenin in the nucleus (Fig. 4A, panel g); however, it inhibited nuclear accumulation of activated β-catenin in response to strain (Fig. 4A, panel h).

Western blot analysis on subcellular fractions confirmed that ICI 182,780 had no effect on the levels of either nuclear or cytoplasmic β-catenin in control cells (Fig. 4B) but had an inhibitory effect on the strain-related increase in nuclear accumulation of activated β-catenin. As previously described (Fig. 1D) we confirmed the absence of cross contamination between nuclear and cytoplasmic fractions (Fig. 4B).

To assess whether the strain-induced increase in TCF/LEF activity was modulated by the ER, ROS 17/2.8 cells transfected with the OPN reporter construct were strained in the presence of ICI 182,780 and lysed 48 h later. Fig. 4C demonstrates that ICI 182,780 inhibited the statistically significant increase in TCF/LEF activity following strain (21 ± 3.8%, p < 0.01) compared with static controls. No significant change in the level of luciferase activity between treatment groups was detected in cells transfected with the double mutant construct (Fig. 4C).

Taken together, these data suggest the involvement of ERα in strain and LiCl-related nuclear accumulation, activation and transcriptional regulation of β-catenin/TCF/LEF responsive genes. That movement of β-catenin and ERα to the nucleus was reduced by ER modulators suggests that these two molecules co-translocate.

**Absence of ERα Abrogates Nuclear Accumulation of Active β-Catenin in Response to LiCl or Strain in Primary Cultures of Mouse Osteoblast-like Cells**—To further investigate the role of ERα in mediating bone cell response to LiCl-induced, and strain-related activation of the Wnt/β-catenin pathway, we established primary cultures of osteoblast-like cells isolated from long bones of female ERα−/− and their WT littermates.

Cells were stimulated by LiCl or strain in the presence of ICI 182,780 as described previously. As observed in ROS 17/2.8 cells, weak immunostaining for activated β-catenin was detected in WT control cells (Fig. 5A, panel a) and was not modulated by the presence of ICI 182,780 (Fig. 5A, panel b). LiCl stimulated nuclear accumulation of activated β-catenin over a period of 30 min to 6 h, was maximal at 3 h (as shown by a representative image in Fig. 5A, panel c) and was reduced in

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**FIGURE 3.** The effect of the SERMs ICI 182,780 and tamoxifen on LiCl-induced nuclear localization of activated β-catenin and transcriptional capability of the TCF/LEF consensus binding sequence containing reporter plasmid. A, ROS 17/2.8 cells were seeded onto thermax cover slips and treated with vehicle, 100 nm ICI 182,780 or 100 nm tamoxifen for 3 h prior to 10 mM LiCl stimulation for 3 h and 6 h in the presence of the SERM. Cells were fixed in ice-cold methanol, and the subcellular localization of ERα (red staining) and active β-catenin (green staining) were determined by immunocytochemistry and confocal microscopy. Top panel shows ERα staining after: panel a, 6 h of vehicle control; panel b, 3 h of vehicle pretreatment prior to 3 h of 10 mM LiCl treatment; activated β-catenin staining after: panel c, 6 h of vehicle; panel d, 3 h of vehicle pretreatment prior to 3 h of 10 mM LiCl. Middle panel shows ERα staining after: panel e, 6 h of ICI 182,780; panel f, 3 h of ICI 182,780 pretreatment prior to 3 h of LiCl stimulation; activated β-catenin staining after: panel g, 6 h of ICI 182,780; panel h, 3 h of ICI 182,780 pretreatment prior to 3 h of LiCl stimulation. Bottom panel shows ERα staining after: panel i, 9 h of tamoxifen; panel j, 3 h of tamoxifen pretreatment to 6 h LiCl stimulation; activated β-catenin staining after: panel k, 9 h of tamoxifen; panel l, 3 h of tamoxifen pretreatment prior to 6 h of LiCl stimulation. Images captured are representative of two separate experiments. Scale bar, 50 μm. B, ROS 17/2.8 cells were transiently transfected with the superTOP luciferase reporter and a control plasmid that constitutively expresses Renilla luciferase. Cells were either treated with vehicle, 1 μM ICI 182,780 or 1 μM tamoxifen prior to 10 mM LiCl stimulation and harvested 24 h later. Firefly luciferase activity was determined and normalized to that of Renilla. Data represent corrected luciferase activity, mean ± S.E. Data were pooled from three separate experiments, each normalized to the mean vehicle control. ***, p < 0.001 versus vehicle, ICI 182,780 and tamoxifen alone. **, p < 0.01 versus LiCl and Veh.

Taken together, these data suggest that within osteoblastic cells ERα is involved in the regulation of β-catenin activity, translocation of activated β-catenin to the nucleus, and its transcriptional activity.

The Estrogen Receptor Modulator ICI 182,780 Inhibits Mechanical Strain-induced Nuclear Localization and Activation of β-Catenin in ROS 17/2.8 Cells—Because ICI 182,780 reduced the activation, nuclear accumulation and transcriptional transactivation of β-catenin stimulated by LiCl in ROS
FIGURE 4. The effect of ICI 182,780 on strain-induced nuclear localization of activated β-catenin, and transcriptional activation of the OPN promoter containing two TCF/LEF consensus binding sequences. A, ROS 17/2.8 cells were seeded onto plastic slides, pretreated for 3 h with vehicle or 100 nM ICI 182,780, and then subjected to mechanical strain in the presence of vehicle or ICI 182,780 and cultured for a further 3 h. Cells were fixed and the subcellular localization of ERα (red staining) and activated β-catenin (green staining) was determined by immunocytochemistry and confocal microscopy. Top panel shows staining for ERα following: panel a, static control (in the presence of vehicle for 6 h); panel b, 3 h after strain; panel c, static control; panel d, 3 h after strain (in presence of vehicle). Bottom panel shows staining for ERα after: panel e, 6 h of 100 nM ICI 182,780; panel f, 3 h of pretreatment with ICI 182,780 prior to strain and incubated for a further 3 h (in the presence of the SERM); localization of activated β-catenin: panel g, 6 h of 100 nM ICI 182,780; panel h, 3 h of pretreatment with ICI 182,780 prior to strain and incubated for a further 3 h (in presence of the SERM). Scale bar, 50 μm. B, Western blot of nuclear and cytoplasmic fractions of ROS 17/2.8 cells pretreated for 3 h with either vehicle or 100 nM ICI 182,780, and then subjected to mechanical strain. Data are representative of three separate experiments. C, ROS 17/2.8 cells were seeded onto collagen-coated plastic slides, transiently transfected with either the OPNpGL3 reporter construct (black bars) or the double mutant (−1660/−1463) reporter (white bars) construct and the control plasmid (RL-pCMV), which constitutively expresses Renilla luciferase. Cells were pretreated for 3 h with vehicle or 1 μM ICI 182,780 then subjected to mechanical strain and harvested 48 h later. Firefly luciferase activity was determined and normalized to that of Renilla. Mean ± S.E. of data pooled from three separate experiments, each normalized relative to its mean static control is shown. For the fully functional construct, **, p < 0.01 versus Veh and Static; ICI 182,780 and Static, ICI 182,780 and Strain (one way analysis of variance).

FIGURE 5. The effect of LiCl on the nuclear accumulation of activated β-catenin in ERα-deficient osteoblast cultures. Osteoblast-like cells were derived from the long bones of 17-week-old female ERα−/− mice or their WT littermates and cultured onto thermox coverslips. A, cells were pretreated with vehicle or 100 nM ICI 182,780 for 3 h prior to 10 mM LiCl treatment for 3 h. Cells were fixed, and the subcellular localization of activated β-catenin (green staining) was determined by immunocytochemistry and confocal microscopy. Top panel shows WT-treated cells: panel a, 6 h of vehicle; panel b, 6 h of ICI 182,780; panel c, 3 h of LiCl treatment in the presence of vehicle; panel d, 3 h of LiCl in the presence of ICI 182,780; Bottom panel shows ERα−/−-treated cells: panel e, 6 h of vehicle; panel f, 6 h of ICI 182,780; panel g, 3 h of LiCl treatment in the presence of vehicle; panel h, 3 h of LiCl in the presence of ICI 182,780. Scale bar, 50 μm. B, intensity of nuclear staining for activated β-catenin was evaluated using Leica Sp5 software and is represented as pixel intensity per 100 μm². Data are representative of three separate experiments. *** p < 0.001 versus vehicle, ICI 182,780, LiCl and ICI 182,780 for WT cells (one way analysis of variance). There were no significant differences detected between the treatment groups in the ERα−/− cells.

the presence of ICI 182,780 (Fig. 5A, panel d). In ERα−/− cells treated with vehicle (Fig. 5A, panel e) weak immunostaining for activated β-catenin was observed and this appeared unchanged after ICI 182,780 treatment (Fig. 5A, panel f). However, in contrast to the findings in ROS 17/2.8 and WT cells, LiCl in the absence and presence of ICI 182,780 had no stimulatory effect on nuclear accumulation of active β-catenin (Fig. 5A, panels g and h). Changes in expression of activated β-catenin were analyzed by quantitative confocal microscopy after immunostaining. As shown in Fig. 5B a 1.7-fold increase (p < 0.001) in nuclear staining for activated β-catenin was observed in WT cells exposed to LiCl for 3 h. This effect was inhibited in the presence of ICI 182,780 (p < 0.001). In ERα-deficient cells, 3 h of LiCl treatment had no influence on the level of the fluorescence intensity of activated β-catenin in the presence or absence of ICI 182,780 compared with controls (Fig. 5B).

As shown in Fig. 6A, panel a, the weak punctate staining for activated β-catenin was detected in the nucleus of non-strained vehicle-treated WT cells, and this was unchanged following ICI 182,780 treatment (data not shown). However, 3 h after exposure to strain, nuclear staining for active β-catenin was strong (shown by a representative image, Fig. 6A, panel b) and was similar to that observed in ROS 17/2.8 cells (Fig. 1A). Pretreatment with ICI 182,780 substantially reduced the strain-related increase in nuclear staining for active β-catenin (Fig. 6A, panel...
ERα Mediates β-Catenin Response to Strain in Osteoblasts

**FIGURE 7.** Microarray analysis of the genes from the Wnt/β-catenin signaling pathway altered significantly in response to mechanical loading in mouse tibia. Total RNA was extracted from the loaded and the contralateral control tibiae of WT and ERα−/− mice (at 3, 8, and 24 h after treatment, 8 animals in each group). Gene profiles were run on a GE Codelink slide spotted with a 25,000 mouse oligo set. Differential genes were selected for the comparisons of interest based on their moderated t-statistic after using a false discovery rate control of 5%. Lists of differential genes were loaded into the Ingenuity Pathways Analysis software (Ingenuity Systems), and the analysis focused on genes previously highlighted as contributory to the Wnt/β-catenin signaling pathway. Schematic shows gene products associated with the Wnt/β-catenin signaling canonical pathway. Genes that were up or down-regulated in response to mechanical strain at the early time points (3 and 8 h after treatment) are highlighted on a black background. Abbreviations, those in italics, also relate to Table 1: CBP, CAMP-response element-binding protein-binding protein; Frizzled, Frizzled-2; GNAQ, guanine nucleotide-binding protein; P2, protein phosphatase 2; WNT, Wnt family members; RAR, retinoic acid receptor, DKK, dickkopf; SFRP, secreted frizzled-related protein; GBP, grouscho-binding protein; Dsh, dishevelled; APC, adenomatous polyposis coli; p, phosphate.

gene expression profile of 25,000 genes in seven different RNA pools from *in vivo* loaded and contralateral control tibiae from both WT C57BL/6 and ERα−/− mice.

Fig. 7 shows that 6 genes previously reported to be involved in the Wnt/β-catenin pathway (*WNT2B, Fzd2, GNAQ, PPP2R3A, CREBBP, RARγ*) were differentially expressed in loaded versus control bones in WT mice 3 h after treatment (expression of ten genes changing over a period of 24 h). This differential expression was less evident at 8 and 12 h after loading and returned to the levels observed in the contralateral control limb 24 h after treatment (Table 1). In contrast, in ERα−/− mice, only one gene (*RARγ*) showed a change in its expression between loaded and control bones at 3 h and none at 24 h.

From the list of differentially expressed Wnt pathway-related genes, CREBBP was chosen to verify microarray expression pattern by qRT-PCR. This gene was chosen because its expression changed at a number of time points in the loaded tibia in both WT and ERα−/− mice. Table 1 shows that the qRT-PCR
expression pattern of *CREBBP* matches the microarray expression pattern in the wild type and ERα−/− control and loaded samples.

These microarray data indicate differential changes in the regulation of ten genes, previously reported to be involved in the Wnt/β-catenin signaling pathway, in loaded versus control bones in the 24 h after loading *in vivo* in normal C57BL/6 WT mice. Only two genes were differentially regulated by load over the same period in mice lacking ERα; one (*RARγ*) at 3 h after loading and one (*CREBBP*) at 8 h. This suggests that the presence of ERα in osteoblastic cells facilitates a more extensive response in β-catenin signaling to mechanical strain than occurs when ERα is absent.

**DISCUSSION**

The data presented here clearly show changes in the regulation, phosphorylation status, and intracellular localization of β-catenin in osteoblastic bone cells *in vitro* within 3 h of a single period of dynamic strain of similar magnitude to that which these cells would experience *in vivo*. These changes are consistent with exposure to strain-initiating changes within the cell cytoplasm that involve reduction in the level of GSK-3 with exposure to strain-initiating changes within the cell and ER. The microarray showed no change in the expression between control and loaded tibia.

**TABLE 1**  
Complete set of genes from the Wnt/β-catenin pathway that changed significantly in response to mechanical strain in the tibiae of the WT and ERα−/− mice at the time points studied

Microarray results were verified through Syber Green quantitative real-time reverse transcriptase (qRT-PCR) analysis for *CREBBP*. The qRT-PCR expression pattern of *CREBBP* matches the microarray expression pattern in the wild type and ERα−/− control and loaded samples. Italics font for *CREBBP* is used for the qRT-PCR data where the microarray showed no change in the expression between control and loaded tibia.

| Time point after treatment | Wild type | ERα−/− | Fold change | Fold change |
|----------------------------|-----------|--------|-------------|-------------|
|                             | GenBankTM Name | GenBankTM Name | qRT-PCR | qRT-PCR |
| **3 h**                    |            |        | 2.2        | 1.0 |
| AK051071                   | CREBBPa   | M32070 |           |     |
| NM_020510                  | FZD2a     |        | ▲1.0       | 1.7 |
| NM_008139                  | GNAQa     |        | ▲2.1       | 0.9 |
| BG031532                   | PPP2R3Aa  |        | ▲2.3       |    |
| M32070                     | RARGa     |        | ▲1.9       |    |
| NM_009520                  | WNT2Ba    |        | ▲2.4       |    |
| AK031007                   | CREBBPa   |        | ▲2.0       |    |
| NM_144880                  | PPP2R5Aa  |        | ▲2.5       |    |
| X56567                     | RARAg     |        | ▲1.5       |    |
| AK017548                  | TLE4a     |        | ▲0.5       |    |
| AK051071                  | CREBBPa   |        |           |     |
| **8 h**                    |            |        | 0.6        | 0.5 |
| AK051071                  | CREBBPa   |        | ▲1.0       | 1.7 |
| NM_020510                  | FZD2a     |        | ▲2.5       | 0.8 |
| AK051071                  | CREBBPa   |        | 0.5        | 0.6 |
| **12 h**                   |            |        | 0.6        | 0.5 |
| AK051071                  | CREBBPa   |        | ▲0.5       | 0.9 |
| NM_020510                  | FZD2a     |        | ▲0.5       | 1.2 |
| **24 h**                   |            |        | NDb        | 0.9 |
| No change detected in Wnt/β-catenin pathway genes expression using microarray | AK051071 CREBBPa | No change detected in Wnt/β-catenin pathway genes expression using microarray |

a CREBBP, cAMP response element-binding protein-binding protein.
b FZD2, frizzled-2.
c GNAQ, guanine nucleotide-binding protein.
d PPP2R3A, protein phosphatase 2 (formerly 2A), regulatory subunit Bα.
e RARG, retinoic acid receptor.
f WNT2B, Wnt2b.
g PPP2R5A, protein phosphatase 2 regulatory subunit B (B56α).
h RARA, retinoic acid receptor.
i TLE4, transducin-like enhancer of split 4.
j ND, not determined.

When these cells were seeded onto collagen strain also produced activation of the OPN reporter containing TCF/LEF binding sites. This is consistent with findings in primary calvarial osteoblasts cultured from TOPGAL mice in which TCF/LEF activation was increased (12.5%) following exposure to stretching using a Flexercell model, when on a collagen substrate but not when seeded onto a plastic substrate (51).

This is also consistent with reports from mesenchymal cells (64) that GSK-3β inhibitors only partially re-capitulate the transcriptional events triggered by Wnt. Thus β-catenin-related control of transcription involves factors in addition to the physical presence of β-catenin in the nucleus. Nevertheless β-catenin cannot mediate transcription unless it is activated and transported to the nucleus. Whether initiated by strain or LiCl, the effectiveness of this process appears to be modified substantially by the ER modulators ICI 182,780 and tamoxifen and is much reduced in cells constitutively lacking ERα. This suggests that activation of β-catenin and its transport to the nucleus is facilitated, if not mediated completely, by ERα. Consistent with this inference is the finding from microarray analysis of RNA extracted from resident cells in loaded and non-loaded bones in WT and ERα−/− mice that early loading-related up-regulation of Wnt-responsive genes is practically abrogated in mice lacking ERα.

The implication of these data relate directly to our understanding of the etiology of post-menopausal osteoporosis in women and age-related osteoporosis in men. In both men and women, for most of their lives, bone cell adaptive responses to physical load bearing are adequate to ensure that their skle-
tons are sufficiently robust to withstand the loads of day to day physical activity without damage. This situation deteriorates with age so that the lifetime risk of a fragility fracture for a 50-year-old Caucasian woman is 45% and for a man 13% (65). This increase in fracture risk is associated directly with loss of bone tissue, the severity of which, in both sexes, is related primarily to levels of bio-available estrogen. Almost by definition this bone loss, and the consequent diminished ability to withstand the loads of everyday activity without damage, represents an acquired failure of the loading-related mechanism by which bone cells previously maintained an adequately robust structure. This raises the question “what is the mechanism of that failure?” We have previously hypothesized that the estrogen-associated deterioration in adaptation to mechanical events characteristic of osteoporosis is not due to a direct effect of estrogen on the activity or recruitment of bone cells but an indirect effect because of the decline in ERα that estrogen withdrawal entails. We suggested that this decline in ERα availability may be sufficient to be a limiting factor for the effectiveness of the mechanically adaptive response (28). This interpretation is supported by the finding that the adaptive response to loading in mice lacking ERα is substantially less than in their WT controls (26). This in turn is consistent with the data presented here, that in the absence of ERα the Wnt pathway contribution to bone cells adaptive response to strain is severely limited.

The microarray data from our present study identified ten genes previously associated with the Wnt pathway that were differentially regulated within 24 h of bone loading in vivo. This number was reduced to only two genes in the absence of ERα. The loading-related increase in Wnt co-receptor Fzd10 (FZD2) levels is consistent with increased activation of the Wnt pathway. The G-protein Gαq (GNAQ) has been shown to enhance canonical Wnt signaling via Fzd (66), but also by disrupting GSK-3β activity in a casein kinase 1 (CK1)-dependent fashion (67). Protein phosphatase 2a (PPP2R3A gene, PP2a protein) regulates β-catenin stability by dephosphorylating both it and APC (68). The CREB-binding protein CBP (CREBBP gene) is an acetyl transferase, which interacts with over 40 different transcription factors including ERα and β-catenin to transactivate the RNA polymerase II holoenzyme (reviewed in Ref. 69). Transcription of Fzd10 is dependent on ERα and estrogen (70). Little is known about the transcriptional regulation of either Gαq or CBP. However, it is attractive to speculate that enhanced expression of Gαq would potentiate Fzd-mediated signaling. That the expression of most of these targets approaches those in controls after 8 h is similar to the timing of activation and nuclear localization of β-catenin observed in ROS 17/2.8 and WT osteoblast-like cells. It is remarkable that the microarray study we report here did not indicate loading-related regulation of any of the usual bone-related candidates. This may be primarily a matter of timing.

The precise role of Wnt/β-catenin signaling in bone cell responses to strain has yet to be defined. One way in which strain in bone tissue could influence regulation of the Wnt pathway is through the agency of sclerostin, a protein produced by osteocytes (71, 72) in amounts that are reduced by their exposure to mechanical strain (73). Binding of sclerostin to the LRP5 receptor prevents LRP5/Fzd complex formation (74) and inhibits down stream signaling initiated by Wnt ligand binding. To the extent that activity of the Wnt pathway leads to higher levels of osteogenesis, by reducing sclerostin levels, this could provide a mechanism for strain-related control of bone mass. In the LRP5 mutant situation the low strains associated with high bone mass permit maximum sclerostin production by osteocytes but, because it is incapable of binding to the mutated LRP5 receptor (75), no regulation of the Wnt pathway is achieved.

This scenario is almost certainly only a partial explanation of one of the mechanisms by which mechanical strain in bone tissue influences osteoblast activity to adjust bone mass and architecture. Many reports have identified a number of factors associated with strain-related or flow-related stimulation of cells of the osteoblast lineage notably prostanoïds and nitric oxide. The interaction of these strain-related pathways with Wnt/β-catenin signaling is unclear, as is the identity of the ligands that normally occupy the LRP5 co-receptor and so regulate the activity of the Wnt pathway. However, regardless of the origin or identity of these ligand(s), if they are to influence transcription through the canonical Wnt pathway β-catenin must be transported to the nucleus where it can regulate transcription of genes associated with one or more of the functions of osteoblasts including their control of osteoclasts. Our data clearly show that the accumulation of active (hypophosphorylated) β-catenin in the nucleus is limited when ERα activity is absent or reduced by ER modulators. It is attractive to speculate that in post-menopausal osteoporosis in women, and age-related osteoporosis in men, the diminished effectiveness of osteoblast responsiveness to loading is associated with the lack of sufficient functional ER to facilitate translocation of β-catenin, and thus transcription of genes associated with this aspect of osteoblast function. Unfortunately data on the ER status of osteoblasts in the relevant human population is sparse. What little there is supports the concept that ER function influences bone adaptive responses to loading. In young females ER polymorphisms are associated with different levels of responsiveness to loading (76) and after the menopause ER numbers in osteoblasts appear to be reduced (77–79).

The association between steroid receptors and β-catenin has also been reported in other situations. For example ligand bound androgen and retinoid receptors, but not ERα, have been reported to be responsible for nuclear translocation of β-catenin in the prostate (59). Ectopic expression of ERα in Drosophila increases the retinal disc apoptosis caused by mutant Armadillo (the Drosophila homologue of β-catenin), and the addition of estrogen increases apoptosis still further (63). Similarly, immunoprecipitation/chromatin immunoprecipitation (ChIP) analysis demonstrates physical association between ERα and β-catenin on ERE and TCF/LEF target promoters (63). ERα expression correlates with nuclear localization of β-catenin in endometrial cancers (80), and β-catenin signaling is essential for the effects of estrogen in the uterus (81). ERα has been shown to be an activator or a repressor of transcription through its effects on β-catenin-mediated TCF/LEF transcription of the OPN gene in breast cancer (82) (reviewed in Ref. 83). Taken together, the biochemical and physiological evidence suggests that the interaction of ERα and β-catenin is of a more wide-
spread importance than the response of osteoblasts to mechanical load.

In conclusion, the study reported here clearly indicates involvement of the Wnt/β-catenin signaling pathway in the early responses of osteoblastic cells to levels of strain similar to those that they are subjected to in vivo. Strained cells show increased levels of activated β-catenin in the nucleus and, when cultured on collagen, increased activity of a TCF/LEF reporter on the OPN promoter. Microarray analysis of RNA extracted from loaded and non-loaded mouse tibiae in vivo show differential regulation of a small number of genes previously reported to be involved in the Wnt pathway. Inhibition of the activity of ERα by the ER modulators ICI 182,780 and tamoxifen, or the absence of ERα in primary cultures of osteoblasts extracted from ERα−/− mice, reduce the activity of the Wnt/β-catenin pathway stimulated either by strain or LiCl-mediated inhibition by GSK-3β. The number of Wnt pathway-related genes differentially regulated by bone loading in vivo is substantially reduced in osteoblastic cells from mice lacking ERα. These data suggest, to our knowledge for the first time, that the contribution of Wnt/β-catenin signaling in bone cell early responses to mechanical strain requires ERα.

Acknowledgments—We thank Rosemary Suswillo for expert technical assistance. We are grateful to Professor Chambon for the gift of ERα knockout mice. We acknowledge Professor Moon for kindly donating the superTOPFLASH/FOPFLASH reporter constructs and Professor Huber for donating the OPNpGL3 and mutant reporter constructs. Preliminary studies were performed on a confocal microscope with kind permission from Professor M. A. Horton.

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