Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice

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One of the well characterized cell biologic actions of lithium is the inhibition of glycogen synthase kinase-3β and the consequent activation of canonical Wnt signaling. Because deficient Wnt signaling has been implicated in disorders of reduced bone mass, we tested whether lithium could improve bone mass in mice. We gavage-fed lithium chloride to 8-week-old mice from three different strains (*Lrp5*$^{-/-}$, SAMP6, and C57BL/6) and assessed the effect on bone metabolism after 4 weeks of therapy. *Lrp5*$^{-/-}$ mice lack the Wnt coreceptor low-density lipoprotein receptor-related protein 5 and have markedly reduced bone mass. Lithium, which is predicted to act downstream of this receptor, restored bone metabolism and bone mass to near wild-type levels in these mice. SAMP6 mice have accelerated osteoporosis due to inadequate osteoblast renewal. Lithium significantly improved bone mass in these mice and in wild-type C57BL/6 mice. We found that lithium activated canonical Wnt signaling in cultured calvarial osteoblasts from *Lrp5*$^{-/-}$ mice *ex vivo* and that lithium-treated mice had increased expression of Wnt-responsive genes in their bone marrow cells *in vivo*. These data lead us to conclude that lithium enhances bone formation and improves bone mass in mice and that it may do so via activation of the canonical Wnt pathway. Lithium has been used safely and effectively for over half a century in the treatment of bipolar illness. Prospective studies in patients receiving lithium should determine whether it also improves bone mass in humans.

anabolic | osteoporosis | therapy

The skeleton is in a dynamic state, being continually degraded and renewed in a tightly regulated remodeling process that involves a complex network of systemic hormones and local factors (1, 2). Among the local signaling factors implicated in this process are Wnt ligands. Their role was inferred after the identification of mutations in the Wnt coreceptor low-density lipoprotein receptor-related protein 5 (*LRP5*) in patients with heritable skeletal diseases. "Loss-of-function" mutations in *LRP5* were found to cause the Osteoporosis–Pseudoglioma syndrome (OPPG), an autosomal recessive disorder characterized by extremely low bone mineral density (BMD) and skeletal fragility (3). Missense mutations in *LRP5* that are thought to create a "gain of function" cause autosomal dominant high bone mass phenotypes in which BMDs are well above the population mean (4, 5).

LRP5 transduces Wnt signal via the canonical pathway, in which the interaction of Wnt ligand with LRP5 and a member of the Frizzled family of coreceptors at the cell surface ultimately leads to the nuclear accumulation of β-catenin (6). Nuclear β-catenin interacts with members of the Tcf/Lef transcription factor family to regulate gene transcription. The importance of the canonical Wnt signaling cascade in bone biology is supported by studies in mice. *Lrp5*-deficient mice and transgenic mice expressing one of the described gain-of-function mutations have similar phenotypes to human OPPG and high bone mass, respectively (7, 8). Mice in which the β-catenin gene has been inactivated in a tissue/cell-specific manner fail to form osteoblasts (9–11), whereas mice in which β-catenin levels have been increased in osteoblasts form excessive bone and too few osteoclasts (9, 12). Skeletal homeostasis is also perturbed by the genetic manipulation of other genes involved in canonical Wnt signaling in mice (13, 14).

Genetic variation, or manipulation, of the canonical Wnt pathway has clearly indicated the pathway's importance to skeletal growth and homeostasis. Pharmacologic modulation of this pathway should therefore be able to affect bone mass. Activation of the canonical Wnt signaling pathway *ex vitro* and *in vivo* can be achieved with lithium chloride (LiCl), which has been shown to inhibit glycogen synthase kinase-3β (GSK-3β), an enzyme that phosphorylates β-catenin in the cytoplasm, targeting it for ubiquitination and degradation (15–17).

In this study, we tested whether LiCl could increase bone mass *in vivo* and whether this effect would be independent of LRP5. We treated Lrp5-deficient mice, osteopenic senescence accelerated SAMP6 mice, and wild-type C57BL/6 mice, and found that LiCl significantly increased bone formation in each strain. The serum level of lithium in the treated animals appeared sufficient to inhibit GSK-3β and to alter the expression of several Wnt-responsive genes, implying that lithium or other GSK-3β inhibitors may be efficacious in treating disorders of low bone mass such as OPPG and senile osteoporosis.

Methods

Supporting Information. For further details, see *Supporting Text*, Figs. 4–9, and Tables 2 and 3, which are published as supporting information on the PNAS web site.

Generation of Lrp5 Mutant Mice. The *Lrp5* locus was targeted by homologous recombination in the 129/Sv hybrid R1 ES cell line (18). *Lrp5* knockout mice generation and Southern and Northern blot hybridization were performed as described in *Supporting Text* (Fig. 5*a*).

Calvaria cells were recovered from *Lrp5*$^{-/-}$ or their corresponding wild-type littermate mice 1–2 days after birth by sequential collagenase digestion at 37°C as described by Garcia *et al.* (19). Calvaria cell treatment, transfection, osteogenic differention assays, and real-time PCR are detailed in *Supporting Text*.

Embryonic murine mesenchymal stem cells were isolated from murine embryo limb buds at day 11.5. mMCS were prepared as

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Abbreviations: OPPG, Osteoporosis–Pseudoglioma syndrome; GSK-3β, glycogen synthase kinase-3β; BMD, bone mineral density; PTH, parathyroid hormone; hPTH, human PTH.

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described (20). These cells were used to test for ALP activity and *Collagen1α1* (*ColIα1*) expression in response to either recombinant Wnt3a or LiCl (20 mM) as described in *Supporting Text*.

Lithium and Parathyroid Hormone (PTH) Treatment Regimens and Assessment of the Biochemical and Skeletal Responses. Three mouse strains ($Lrp5^{-/-}$, SAMP6, and C57BL/6) were used to assess the affect of orally administered LiCl on bone metabolism. Detailed treatment regimens and protocols are provided in *Supporting Text* and Fig. 4.

MicroCT scans, quantitative computerized tomography and histomorphometry of mouse tibia were performed as described (21, 22) (for details see *Supporting Text*).

Statistical Analyses. Data were expressed as mean ± SEM. Statistical differences were calculated by using Student's *t* test or ANOVA for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

***Lrp5*$^{-/-}$ Mice Have Reduced Bone Mass Because of a Deficiency in Osteoblast Number and Function.** Correct targeting of *Lrp5* (Fig. 5*a*) was confirmed by Southern blot hybridization (Fig. 5*b*). Heterozygous crosses produced litters with expected Mendelian ratios of wild-type ($Lrp5^{+/+}$), heterozygous ($Lrp5^{+/-}$), and homozygous mutant ($Lrp5^{-/-}$) pups. Mice did not differ in size or weight from their same-sex littermates at birth or at death, and sexually mature female and male $Lrp5^{-/-}$ mice were fertile. Although the targeting construct was intended to generate a Lrp5–LacZ fusion protein, no Lrp5-containing transcripts were detectable by Northern blot (Fig. 5*c*), and no Lrp5 or Lrp5–LacZ fusion polypeptides were detected by Western blot or histochemical staining by using tissues (liver and bone) known to normally express Lrp5 (data not shown). RT-PCR analyses of transcript from these mice indicated that the targeted allele misspliced the LacZ-containing exon, leading to nonsense-mediated mRNA decay (data not shown). Consequently, these mice are functionally null for Lrp5. Importantly, the inactivation of *Lrp5* did not result in significant changes in the expression of its closest homologue and functionally related receptor *Lrp6* in calvaria cells (Fig. 5*d*).

Analysis of tibiae from 12-week-old mice revealed significantly decreased BMD in $Lrp5^{-/-}$ mice compared with $Lrp5^{+/+}$ littermates (Table 2), as has been reported in mice with other targeted mutations in *Lrp5* (7, 23). Static and dynamic histomorphometry was performed in 12-week-old mice and significant differences were observed between $Lrp5^{-/-}$ and $Lrp5^{+/+}$ mice (Fig. 1 *a* and *b* and Table 2). Total BMD, trabecular BMD, trabecular bone volume to total bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), mineralizing surface (MS), bone formation rate (BFR/BS), mineral apposition rate (MAR), osteoblast number (N.Ob/T.AR) and osteoclast number (N.Oc/T.AR) in secondary spongiosa were lower in $Lrp5^{-/-}$ mice (Fig. 1 and Table 2). These results demonstrate that osteoblast number and function are diminished in $Lrp5^{-/-}$ mice. The urine levels of deoxypyridinoline crosslinks (Dpyr/Creat), a biochemical marker of bone resorption, were similar in $Lrp5^{+/+}$ and $Lrp5^{-/-}$ mice, suggesting that bone resorption was not overtly affected by *Lrp5* deficiency (Table 2). The reduction in bone anabolism by osteoblasts, coupled with normal catabolism by osteoclasts, accounts for the marked decrease in bone mass.

LiCl Improves Bone Formation in *Lrp5*$^{-/-}$ Mice. The Lrp5 receptor has a role in the transduction of Wnt signaling. To determine whether LiCl, which is expected to act downstream of Lrp5 and activate Wnt target genes through inhibition of GSK-3β, could improve bone anabolism, we analyzed the effect of LiCl in $Lrp5^{-/-}$ cells *ex vivo* and in $Lrp5^{-/-}$ mice *in vivo*. We recovered primary osteoblasts from $Lrp5^{+/+}$ and $Lrp5^{-/-}$ mice, transfected them with the canonical

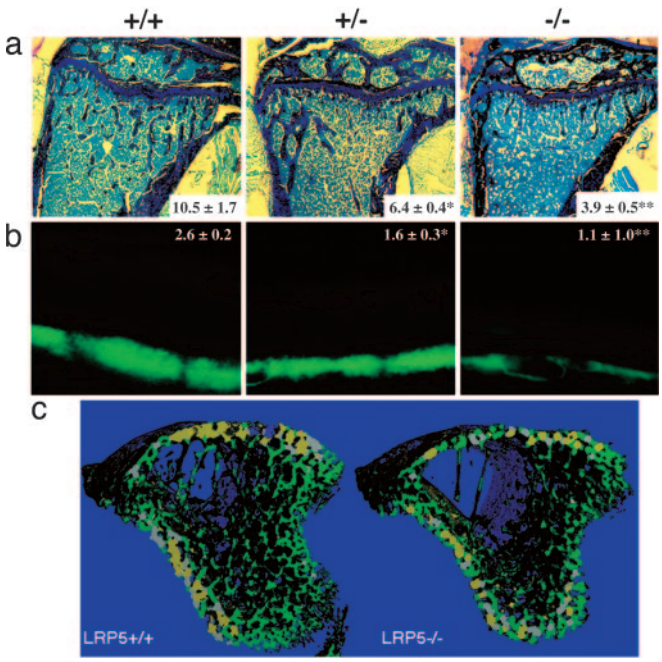

Fig. 1. Bone phenotype of $Lrp5^{-/-}$ (−/−) mice. (*a*) Photomicrographs of coronal sections through proximal tibias of 12-week-old $Lrp5^{+/+}$ (+/+), $Lrp5^{+/-}$ (+/−), and $Lrp5^{-/-}$ (−/−) mice stained with Toluidine blue. Note the decreased numbers of trabeculae in heterozygous and homozygous mutant mice. The inset numbers represent trabecular BV/TV based on analysis of 6–12 mice of each genotype. (*b*) Fluorescent photomicrograph of bone trabecula from 12-week-old $Lrp5^{+/+}$ (+/+), $Lrp5^{+/-}$ (+/−), and $Lrp5^{-/-}$ (−/−) mice after calcein double labeling. Inset numbers represent mineral MAR based on analysis of nine $Lrp5^{+/+}$ (+/+) mice and six $Lrp5^{-/-}$ (−/−) mice. (*c*) Three-dimensional microCT reconstruction of proximal tibias from 4-week-old $Lrp5^{+/+}$ and $Lrp5^{-/-}$, showing reduced trabecular bone volume in the mutant mouse. *, Significant difference from wild type at $P < 0.05$; **, significant difference from wild type at $P < 0.01$.

Wnt signaling reporter construct Topflash, and then treated the cells with either Wnt3a or LiCl. Wild-type osteoblasts were able to respond to Wnt3a by increasing luciferase activity, whereas $Lrp5^{-/-}$ osteoblasts had a minimal increase in activity (Fig. 2*a*), possibly because of the unaffected expression of *Lrp6*. Significantly, LiCl treatment increased luciferase activity in both $Lrp5^{-/-}$ and in $Lrp5^{+/+}$ cells (Fig. 2*a*), demonstrating that the Wnt signaling pathway is functional in $Lrp5^{-/-}$ cells and that LiCl activity is independent of Lrp5 expression. As predicted, the extracellular Wnt antagonist Dkk1 did not inhibit the lithium effect, but it did reduce Wnt3a-induced activity (Fig. 2*a*). We have also further confirmed that, in calvaria cells, LiCl is able to increase β-catenin stabilization and induce β-catenin nuclear translocation (Fig. 7). Interestingly, LiCl treatment significantly decreased the percentage of apoptotic calvarial cells derived from $Lrp5^{-/-}$ mice, whereas it did not affect apoptosis of wild-type cells (Fig. 2*b*). It also reduced the number of cells derived from $Lrp5^{+/+}$ that spontaneously differentiated toward the adipocyte lineage (Fig. 2*c*). These *in vitro* data indicate that *Lrp5* deficient marrow-derived stromal cells and osteoblasts fail to respond to Wnt3a but can respond to a GSK-3β inhibitor.

We further tested whether LiCl is able to increase osteoblast differentiation in either progenitor cells such as mesenchymal stem cells or mature osteoblast. As shown in Fig. 2 *e* and *f*, in murine mesenchymal stem cells isolated from $Lrp5^{-/-}$ and $Lrp5^{+/+}$ embryos, both LiCl and Wnt3a were able to stimulate ALP activity and *ColIα1* expression, two early markers of osteoblast differentiation. On the other hand, LiCl or Wnt3a failed to affect the expression of these same markers in both $Lrp5^{-/-}$ and $Lrp5^{+/+}$ calvaria cells (data

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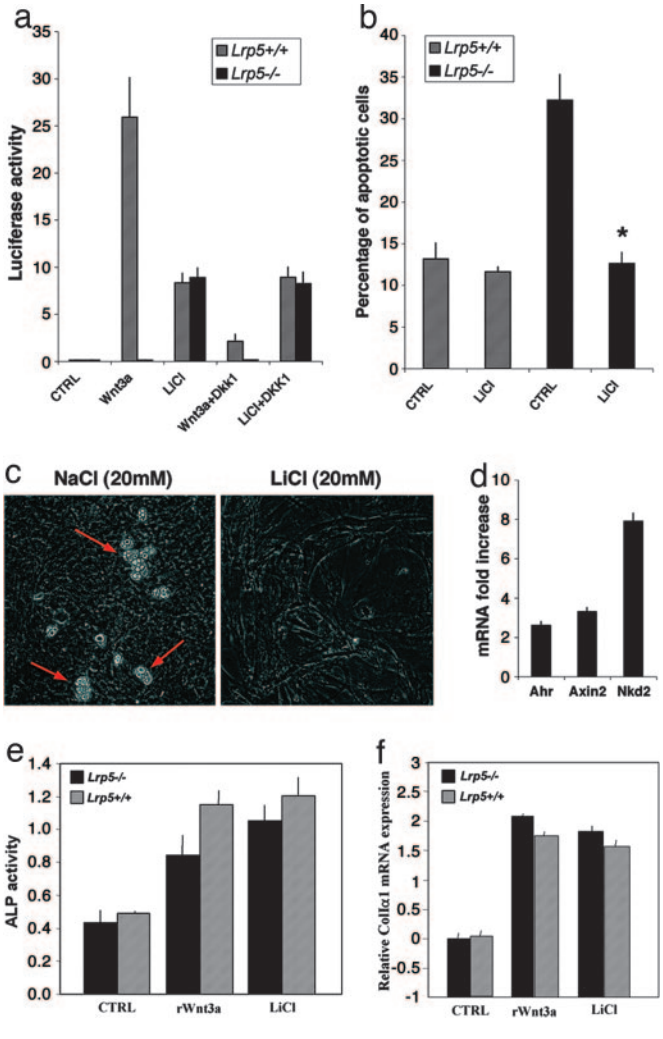

Fig. 2. LiCl effects in cells derived from $Lrp5^{+/+}$ and $Lrp5^{-/-}$ mice. (*a*) Fold-induction of firefly luciferase normalized to renilla luciferase in osteoblasts harvested from $Lrp5^{+/+}$ and $Lrp5^{-/-}$ mice. Cells were transiently transfected with Topflash and *Renilla* reporters and then cultured in the presence of combinations of 20 mM LiCl, 50 ng/ml Wnt3a, and 50 ng/ml Dkk1. Wnt3a induces luciferase expression in $Lrp5^{+/+}$, but not in $Lrp5^{-/-}$ cells. LiCl induces expression in both genotypes and is not inhibited by Dkk1. (*b*) Percent apoptotic calvarial osteoblasts harvested from $Lrp5^{+/+}$ and $Lrp5^{-/-}$ mice and cultured with or without 20 mM LiCl. LiCl reduces apoptosis in $Lrp5^{-/-}$ osteoblasts. (*c*) Phase contrast micrographs of calvaria cells harvested from $Lrp5^{+/+}$ mice and cultured in medium containing either 20 mM NaCl or 20 mM LiCl. In contrast to cells cultured in NaCl that can differentiate along the adipocyte lineage (arrows), cells cultured in LiCl do not become adipocytes. (*d*) Fold increase in mRNA for the Wnt responsive genes *Ahr*, *Nkd2*, and *Axin2*. Total RNA were prepared from bone marrow cells recovered from LiCl-treated and from vehicle-treated mice. The expression level of selected genes was determined by real-time PCR and normalized on the basis of *Gapdh* expression. Data are presented as fold increase in mRNA expression level in LiCl treated to control vehicle treated cells. (*e* and *f*) ALP activity and *Col*α*1* gene expression in embryonic murine mesenchymal stem cells derived from from $Lrp5^{+/+}$ and $Lrp5^{-/-}$ mice and cultured in the presence of either Wnt3a (50 ng/ml) or LiCl (20 mM). After 4-day stimulation, ALP activity was assessed in cell lysates. Total RNA was also extracted and *Col*α*1* expression was analyzed by real-time PCR and normalized on the basis of *Gapdh* expression. Both Wnt3a and LiCl were able to increase the activity of ALP and *Col*α*1* gene expression.

not shown and Fig. 6). These data correlate with previous reports showing that Wnt3a induces osteoblast commitment in pluripotent mesenchymal cells with no effect on mature osteoblast (24).

We then tested the effect of LiCl on bone formation *in vivo* by treating 8-week-old $Lrp5^{-/-}$ mice with LiCl for 1 month. LiCl therapy did not affect weight gain, activity levels, and grooming behavior in the mice (Table 1). Oral dosing of 200 mg/kg per day (a dose giving plasma levels comparable with levels used to treat humans with bipolar illness) increased bone volume in $Lrp5^{-/-}$ mice compared with untreated $Lrp5^{-/-}$ mice (Fig. 3 *a–h* and Table 1) and restored bone volume close to that of untreated $Lrp5^{+/+}$ mice. Histomorphometric analyses confirmed that $Lrp5^{-/-}$ mice treated with LiCl had significant increases in bone volume (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th) (Table 1), and that LiCl affected bone formation and not bone resorption (Table 1). We observed increases in osteoid thickness (O.Th), and osteoblast number (N.Ob/T.Ar), but no changes in osteoclast number (N.Oc/T.Ar). We also found significant increases in bone formation rates (BFR/TV) and percent mineralizing surface (MS) in LiCl-treated mice not only relative to $Lrp5^{-/-}$ mice but also compared with control animals (Tables 1 and 2), demonstrating a true anabolic action of this compound.

We measured serum lithium levels in the treated animals and found that the concentration was in the range reported to inhibit GSK-3β *in vivo* (17, 25). We then isolated bone marrow cells from 9-week-old $Lrp5^{-/-}$ animals that had been treated with LiCl for 1 week, quantified the expression levels of three Wnt target genes, Ahr, Nkd2, and Axin2 (26), and compared them with bone marrow cells from $Lrp5^{-/-}$ mice treated only with vehicle. Quantitative RT-PCR revealed increased expression for each of these genes in the lithium-treated mice (Fig. 2*d*). These results indicate that LiCl doses that increased bone volume also stimulated Wnt-responsive genes in target tissues *in vivo*.

In humans, chronic lithium treatment can affect calcium metabolism by causing hypersecretion of PTH (27, 28). Therefore, we measured serum PTH levels in LiCl-treated mice to determine whether the bone anabolic effect was caused by an increase in PTH. No change in PTH concentration was observed (Table 1). Thus, LiCl activates bone formation downstream and independent of Lrp5, likely through the intracellular activation of canonical Wnt signaling and not through a sustained increase in PTH secretion.

LiCl Improves Bone Formation in C57BL/6 and SAMP6 Mice. $Lrp5^{-/-}$ mice are deficient in their ability to transduce Wnt signal. To determine whether LiCl could improve bone mass in mice with functioning Lrp5 receptors, we treated 8-week-old C57BL/6 mice with LiCl for 4 weeks. These mice also had significant increases in bone mass compared with controls (Table 1). However, because these mice had normal starting levels of bone mass and bone formation, their response to LiCl was not as great as that observed in the $Lrp5^{-/-}$ mice.

We next tested the effect of LiCl in SAMP6 mice that display an accelerated osteoporosis phenotype due to reduced osteoblastogenesis (29–31). Eight-week-old SAMP6 mice were treated for 4 weeks, killed, and evaluated by histomorphometry. LiCl treatment resulted in a significant increase in bone volume, osteoblast number, and mineral surface, whereas osteoclast numbers remained unchanged (Fig. 3 *i* and *j* and Table 1).

PTH is a well known bone anabolic agent (32). Therefore, we compared the effect of human PTH 1–34 (hPTH) and LiCl on bone parameters in SAMP6 animals. As expected, hPTH treatment increased formation in trabecular bone, and LiCl activity was comparable with that of hPTH (Fig. 3 *i* and *k* and Table 1). Human PTH also increased cortical BMD in treated animals, a known activity of PTH in rodents (32, 33), whereas the effect of LiCl on cortical BMD was not significantly different (Table 1). Interestingly, D-Pyr crosslinks were reduced in SAMP6 mice that were treated with either LiCl or hPTH, suggesting that each agent could decrease bone resorption activity in this strain (Table 1). LiCl did not affect bone resorption activity in the $Lrp5^{-/-}$ or the C57BL/6 mice.

We have presented strong data suggesting that LiCl both *ex vivo* and *in vivo* triggers Wnt/β-catenin signaling, most likely through

Table 1. Effects of LiCl therapy

	Lrp5$^{-/-}$		SAMP6			C57BL/6	
12 weeks of age	Vehicle (n = 8)	LiCl (n = 6)	Vehicle (n = 10)	LiCl (n = 10)	hPTH (n = 8)	Vehicle (n = 9)	LiCl (n = 6)
BV/TV, %	1.81 ± 0.3	10.04 ± 3.87**	4.86 ± 0.98	7.72 ± 0.94*	9.07 ± 0.23*	8.28 ± 0.98	11.21 ± 1.13*
Tb.Th, μm	31.98 ± 6.46	34.27 ± 5.73	32.77 ± 1.56	51.66 ± 6.36*	42.44 ± 2.20*	38.80 ± 2.75	46.36 ± 4.11*
Tb.N	0.62 ± 0.16	2.67 ± 0.81**	1.37 ± 0.10	1.63 ± 0.13*	1.86 ± 0.77*	1.78 ± 0.24	2.50 ± 0.19*
O.Th, μm	2.89 ± 1.44	7.89 ± 2.08*	6.47 ± 0.51	8.74 ± 1.69	9.02 ± 1.48*	4.78 ± 1.91	18.21 ± 4.37*
N.Ob/T.Ar	14.03 ± 2.90	69.79 ± 7.93*	12.64 ± 2.70	16.58 ± 5.32*	14.72 ± 4.35*	27.07 ± 7.73	43.02 ± 5.70*
N.Oc/T.Ar	2.50 ± 0.87	3.12 ± 1.12	2.59 ± 0.72	4.30 ± 2.47	2.33 ± 0.77	2.21 ± 0.81	1.45 ± 0.72
N.Ad/T.Ar	33.3 ± 0.9	5.09 ± 2.95*	97.4 ± 22.2	43.0 ± 12.1*	19.0 ± 4.8**	6.0 ± 3.1	ND
MS, %	23.74 ± 14.1	38.4 ± 11.3	22.2 ± 12.4	28.2 ± 7.2	30.4 ± 7.4	34.2 ± 12.2	44.5 ± 10.2*
MAR, μm/day	1.2 ± 0.5	2.1 ± 0.7*	0.9 ± 0.3	1.4 ± 0.2*	1.7 ± 0.4*	1.0 ± 0.7	2.3 ± 0.5*
BFR/TV	53.4 ± 38.0	122.2 ± 12.4*	52.4 ± 34.0	124.2 ± 12.6*	164.2 ± 16.4*	52.2 ± 22.8	68.7 ± 20.5
OCN, ng/ml	137 ± 18	136 ± 9	ND	ND	ND	125 ± 14	197 ± 24*
Dpyr/Creat, nM/mM	12.56 ± 2.51	11.88 ± 3.02	10.77 ± 1.03	8.22 ± 0.45*	6.81 ± 0.57**	17.5 ± 2.9	17.9 ± 2.9
PTH, ng/ml	53.99 ± 2.75	54.35 ± 4.97	ND	ND	ND	47.2 ± 4.3	52.7 ± 6.0
LiCL, μmol/ml	0.05 ± 0.01	0.413 ± 0.05**	ND	ND	ND	ND	ND
Body weight, g	27.70 ± 0.94	26.42 ± 1.03	29.11 ± 1.09	27.49 ± 0.71	28.31 ± 1.03	22.43 ± 1.24	23.02 ± 0.77
Cortical BMD, mg/cm^2	984.2 ± 9.2	992.3 ± 14.0	1,072.2 ± 5.3	1,091.5 ± 2.6	1,121.3 ± 12.8*	907.9 ± 4.7	912.1 ± 7.0

BC/TV, trabecular bone volume to total bone volume; Tb.Th, trabecular thickness; O.Th, osteoid thickness; N.Ob/T.AR, osteoblast number; N.Oc/T.AR, osteoclast number; N.Ad/T.AR, adipocytes per bone marrow area; MS, mineralizing surface; MAR, mineral apposition rate; BFR/TV, bone formation rate; OCN, osteocalcin; Dpyr/Creat, deoxypyridinoline crosslinks; ND, not determined; *, $P < 0.05$; **, $P < 0.01$.

inhibition of GSK-3β. To further investigate the role of GSK-3β, we have synthesized and treated C57BL/6 mice with a recently reported specific and bioavailable GSK-3β inhibitor (Fig. 8; ref. 34). Similar to LiCl, treatment of C57BL/6 animals with this specific GSK-3β inhibitor (at 3 mg/kg per day) for 4 weeks results in a significant increase in BV/TV as well as trabeculae number and thickness (Fig. 9 and Table 2).

LiCl-Treated *Lrp5*$^{-/-}$ and SAMP6 Mice Have Reduced Numbers of Bone Marrow Adipocytes. Adipocytes and osteoblasts are thought to arise from common marrow stromal cell progenitors, and their differentiation pathways are governed by Wnt signaling. Therefore, we analyzed the number of adipocytes in the tibial bone marrows of *Lrp5*$^{-/-}$ and *Lrp5*$^{+/+}$ mice. *Lrp5*$^{-/-}$ mice have significantly more adipocytes per bone marrow area (N.Ad/T.Ar) compared with

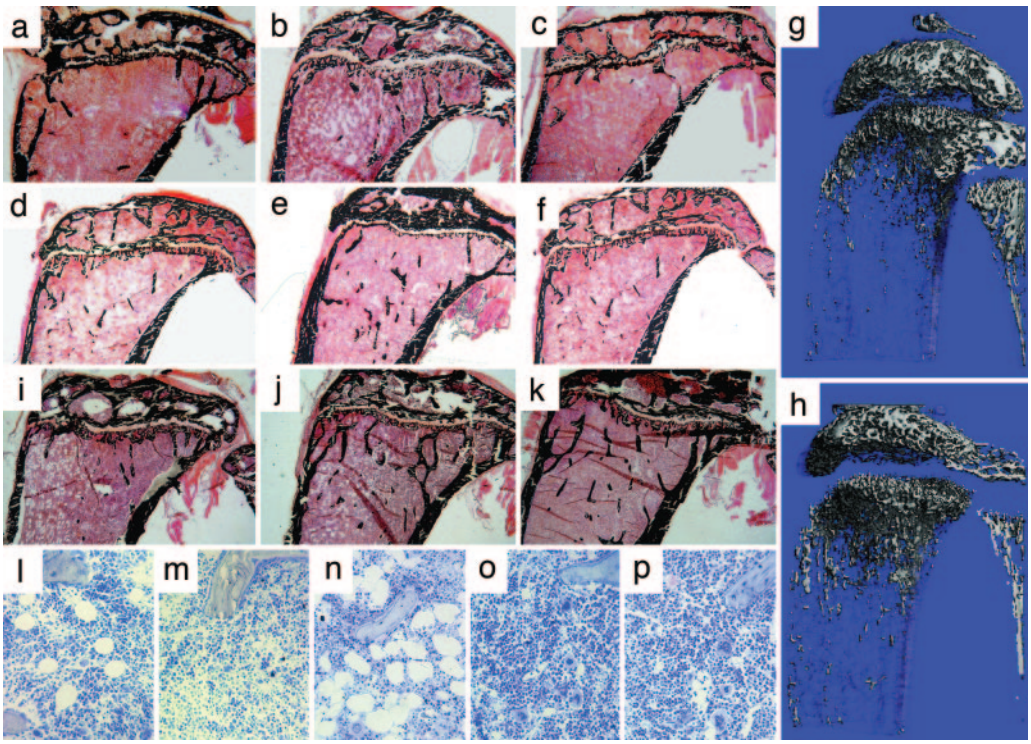

Fig. 3. Lithium therapy increases bone mass and decreases bone marrow adiposity. (*a–f* and *i–k*) Von Kossa-stained coronal sections through proximal tibias of individual 12-week-old mice. Note the general increase in number of bone trabeculae (arrows) in LiCl mice. hPTH-treated SAMP6 mouse serves as a positive control. (*a–c*) *Lrp5*$^{-/-}$ mice that were gavage fed vehicle. (*d–f*) *Lrp5*$^{-/-}$ mice that were gavage fed LiCl. (*g* and *h*) Three-dimensional microCT reconstruction of proximal tibias from *Lrp5*$^{-/-}$ treated with vehicle (*g*) and treated with LiCl (*h*), showing increased trabecular bone volume in response to lithium. (*i*) SAMP6 mouse gavage fed vehicle. (*j*) SAMP6 mouse gavage fed LiCl. (*k*) SAMP6 mouse treated with hPTH. (*l–p*) H&E-stained trabecular bone from the proximal tibias of individual 12-week-old mice looking at marrow adiposity. (*l*) *Lrp5*$^{-/-}$ mouse that was gavage fed vehicle. (*m*) *Lrp5*$^{-/-}$ mouse that was gavage fed LiCl. (*n*) SAMP6 mouse that was gavage fed vehicle. (*o*) SAMP6 mouse that was gavage fed LiCl. (*p*) SAMP6 mouse that received s.c. hPTH.

Lrp5$^{-/-}$mice (Fig. 3 *l–p* and Table 1). Treatment with LiCl decreased N.Ad/T.Ar in *Lrp5*$^{-/-}$ mice to a level equivalent to that of *Lrp5*$^{+/+}$ littermates (Table 1). SAMP6 mice also have a higher bone marrow fat content than wild-type mice (30). LiCl therapy also significantly reduced N.Ad/T.Ar in these mice. Interestingly, treatment with PTH had a similar effect. Therefore, the effect of LiCl upon adipogenesis *in vivo* mirrors the effect that was observed *ex vivo* when calvaria cells were treated with LiCl (Fig. 2*c*).

Discussion

We have clearly shown that lithium therapy enhances bone anabolism and increases BMD in mice. Our data suggest that the enhancement of bone anabolism results, at least in part, from the activation of the canonical Wnt signaling pathway downstream of Lrp5. Several lines of evidence support this interpretation. First, we saw the greatest improvement in bone mass in the *Lrp5* mutant mice (Table 1). These mice have reduced canonical Wnt signaling because of the absence of the Wnt coreceptor Lrp5. Lithium-induced increases in canonical Wnt signaling are more likely to have substantial effects in these mice than in mice with functioning signaling pathways. Second, we found reduction in marrow adiposity in the *Lrp5*$^{-/-}$ and SAMP6 mice that received lithium (Table 1 and Fig. 3). We observed this same reduction in adipocyte number in calvaria cells that had been cultured in the presence of LiCl (Fig. 2*c*) and Wnt3a (data not shown). Other investigators have also shown that Wnt ligands are able to divert marrow stromal cells from the adipocyte lineage into the osteoblast lineage *in vivo* (14). Third, we found that at the time of death serum lithium levels were at a level that can have therapeutic activity in humans. Fourth, we found no evidence to indicate that the activity of the known bone anabolic agent, PTH, was being increased by lithium; serum PTH levels were unchanged in the lithium-treated mice. Fifth, quantitative RT-PCR revealed increases in Wnt-responsive gene mRNA transcripts in lithium-treated mice compared with vehicle-treated controls. Lastly, LiCl treatment *ex vivo* was able to increase Topflash activity in *Lrp5* mutant cells, whereas exogenous Wnt ligand did not. We think the aforementioned data make a strong case for lithium affecting canonical Wnt signaling *in vivo*, but we cannot exclude the possibility that lithium improves bone mass by affecting other cell biologic pathways as well.

Prior studies have implicated canonical Wnt signaling in the regulation of bone metabolism at several levels, for example, in modulating the commitment of undifferentiated cells to the osteoblast lineage (11, 14), the proliferation of preosteoblasts (23), the terminal differentiation of osteoblasts (3), and the production by osteoblasts of OPG (12, 26), an inhibitor of osteoclast differentiation. Therefore, the action of lithium in bone could be occurring at more than one level. Our present data are compatible with three likely sites of lithium action. One is in the commitment of undifferentiated cells to the osteoblast lineage, as indicated by the reduction in marrow adipocyte number and the increase in gene expression of osteoblast-specific markers in murine embryonic mesenchymal stem cells. A second is in the proliferation of preosteoblasts, as suggested by the increased number of osteoblasts per total area. A third is at the level of osteoblast apoptosis, which we found to be reduced by lithium therapy in cultures of calvaria cells *ex vivo*. Despite published data that clearly show a role for canonical Wnt signaling in regulating osteoclast differentiation (12, 26), we found no evidence that lithium therapy had this effect in the mice. Osteoclast numbers were not reduced in lithium-treated mice. We also detected no change in the mRNA expression level of OPG in recovered bone marrow cells from lithium-treated mice compared with vehicle-treated controls.

The effect of lithium we observed in mice is not at odds with prior studies in which β-catenin levels have been genetically altered. Mice with cell-specific disruption or stabilization of β-catenin would be expected to have more profound changes in canonical signaling than would mice that have been treated with lithium, because the serum lithium concentration in treated mice was 0.4–0.5 mM, which is below lithium's *in vitro* IC_{50} of 2 mM for GSK-3β inhibition (16). We suspect that the modulation of β-catenin levels by lithium is more reflective of the normal physiologic alteration in levels that result from Wnt signaling than the alteration in levels that occurs in genetically manipulated mice. Other *in vivo* studies of LiCl also support this interpretation (17). Furthermore, we have tested the activity of a specific and bio-available GSK-3β inhibitor (34) on bone formation. Treatment with this specific inhibitor showed similar increase in bone formation than LiCl.

Oral lithium has been used to treat humans with bipolar disease for over a half-century, with substantial benefit (35). Patients maintained on chronic lithium therapy experience few long-term side effects, although toxicity can occur when serum levels become high due to overdose or poor renal excretion. There have been concerns about the potential for deleterious effects of lithium therapy on the skeleton, because lithium does increase serum PTH levels (36). The increase in PTH that occurs in patients receiving lithium has been attributed to the ion's ability to reset the calcium sensor in the parathyroid gland (37). Another complication of lithium therapy is the development of parathyroid adenomas that require surgical resection because of hypercalcemia (38). Few human studies have specifically addressed the effect of lithium therapy on bone metabolism (39). Despite concerns that chronic elevations of PTH would promote bone catabolism, most studies found no detrimental effect of lithium on bone mass. In fact, data from two studies suggest that lithium could have bone anabolic activity in humans, similar to what we observed in mice. One, a cross-sectional study in a cohort of 26 patients who had been receiving lithium for >10 years, found that the mean BMD of lithium-treated patients was >1 standard deviation higher than the mean BMD of healthy controls, who the investigators had matched for sex, age, and body mass index (40). The second study, a 2-year prospective longitudinal study of 53 patients beginning chronic lithium therapy, looked at calcium balance and found that, despite increases in serum PTH, urine calcium excretion actually decreased (41). Although measures of bone density and metabolic labeling of bone were not performed in this study, it is intriguing to speculate that calcium was depositing into bone. Our study, using mice in which we were able to assess bone metabolism *in vivo* by calcein labeling, provides the most compelling evidence for anabolic effects of lithium on bone, in large part because we were able to control for polygenic and environmental factors that often confound human studies.

We have not yet determined whether the dosing regimen of lithium is important for its therapeutic activity. In humans, lithium has a half-life of 24 h and is taken two or three times daily to maintain stable lithium concentrations. We gavage-fed LiCl to mice as a single daily dose and measured plasma lithium levels 2 h after dosing. Therefore, we do not know whether this dosing schedule caused therapeutic peaks in lithium levels, with troughs falling outside the therapeutic range, or maintained therapeutic lithium concentrations. The large volume of blood required to measure plasma lithium precludes obtaining serial measurements in mice. There have been conflicting reports on the effect of LiCl on bone metabolism in larger animals such as the rat (42–44). However, these disparities likely reflect methodologic differences in study design and interpretation. In the one study that identified deleterious effects, LiCl was injected i.p. into skeletally immature rats, and serum lithium levels were 1.6 mM (43), which is at the high end of the therapeutic range and associated with an increased incidence of side effects in humans (35). Furthermore, the investigators looked only at cortical bone growth rather than trabecular bone growth. In our study, mice had lower LiCl doses and serum lithium levels (0.4–0.5 mM). Differences in neurologic responses to different serum levels of lithium have been demonstrated in mice (17), so a dose-response effect on bone metabolism might be expected. Rats that were given lower doses of LiCl in their drinking water for 40

days achieved serum levels of 0.4 mM and had no observed alterations in their bone metabolism (44), and growing rats given s.c. low-dose injections of LiCl had a trend toward increased trabecular bone formation (42). Lack of statistically significant differences, beneficial or deleterious, between treated and control animals in these latter two studies could be due to the small cohort sizes and the relative imprecision of the assays that were available when the studies were performed. We had the ability to quantify bone mass using high-resolution microCT coupled with standardized histomorphometry. Because it is well recognized that responsiveness to the bone anabolic agent PTH depends on whether the elevation is transient or sustained (45–47), it will be important to determine whether the same will hold true for LiCl.

Several other issues must be considered before contemplating the use of lithium as a bone anabolic agent in humans. For example, it is essential to determine whether alterations associated with lithium therapy might be cancer predisposing. Transcriptional activation of Wnt1 expression in mice causes mammary tumors (48), and loss-of-function mutations in the β-catenin stabilizer APC predispose to malignant colon cancer in humans (49). Short-term lithium therapy (60 days) in mice with a cancer predisposing *Apc* mutations did not cause an increased rate of tumor formation, but did cause a modest increase in tumor size (25). Importantly, increased rates of cancer have not been reported in patients receiving chronic lithium therapy (50). A second theoretical consideration is whether deposition of lithium ions in bone matrix could have detrimental effects on the material properties of bone. Our results indicate that lithium may be useful in increasing bone mass in humans. Although its greatest efficacy may be in patients with mutant LRP5 receptors, it may also be effective for other forms of osteopenia and osteoporosis. It was also recently demonstrated that inhibition of Wnt signaling was associated with lytic bone lesions in patients with multiple myeloma (51, 52), and it is reasonable to anticipate that lithium therapy might also be beneficial for myeloma related bone disease. Well powered prospective studies of bone metabolism in humans receiving lithium therapy for bipolar illness should help address this possibility.

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