Low peak bone mass and attenuated anabolic response to parathyroid hormone in mice with an osteoblast-specific deletion of connexin43

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Summary

Connexin43 (Cx43) is involved in bone development, but its role in adult bone homeostasis remains unknown. To overcome the postnatal lethality of Cx43 null mutation, we generated mice with selective osteoblast ablation of Cx43, obtained using a *Cx43^{fl}* allele and a 2.3-kb fragment of the α_1*(I) collagen* promoter to drive *Cre* in osteoblasts (*ColCre*). Conditionally osteoblast-deleted *ColCre;Cx43$^{-/fl}$* mice show no malformations at birth, but develop low peak bone mass and remain osteopenic with age, exhibiting reduced bone formation and defective osteoblast function. By both radiodensitometry and histology, bone mineral content increased rapidly and progressively in adult *Cx43$^{+/fl}$* mice after subcutaneous injection of parathyroid hormone (PTH), an effect significantly attenuated in *ColCre;Cx43$^{-/fl}$* mice, with *Cx43$^{-/fl}$* exhibiting an intermediate response. Attenuation of PTH anabolic action was associated with failure to increase mineral apposition rate in response to PTH in *ColCre;Cx43$^{-/fl}$*, despite an increased osteoblast number, suggesting a functional defect in Cx43-deficient bone-forming cells. In conclusion, lack of Cx43 in osteoblasts leads to suboptimal acquisition of peak bone mass, and hinders the bone anabolic effect of PTH. Cx43 represents a potential target for modulation of bone anabolism.

Key words: Gap junctions, Connexin43, Teriparatide, Bone anabolic agents, Conditional gene deletion

Introduction

Bone-forming cells are highly coupled by gap junctions formed primarily by connexin43 (Cx43) and, to a lesser degree, connexin45 (Cx45) proteins (Civitelli et al., 1993; Donahue et al., 1995; Steinberg et al., 1994). Several in vitro studies have demonstrated that Cx43 is involved in modulating the differentiation and function of bone-forming cells as well as osteocytes (Cheng et al., 2001; Lecanda et al., 1998; Schiller et al., 2001a; Schiller et al., 2001c); and work from our group indicates that Cx43 controls osteoblast gene transcription via modulation of specific signaling systems required for osteoblast gene expression (Stains et al., 2003; Stains and Civitelli, 2005b).

Although this work has laid the foundation for understanding the biology of gap junction proteins in bone, only recent studies in human and mouse genetics have brought to the fore the biologic role of Cx43 in the skeleton. We had reported that targeted ablation of the *Cx43* gene in the mouse leads to a skeletal phenotype characterized by retarded intramembranous and endochondral ossification, craniofacial abnormalities and osteoblast dysfunction (Lecanda et al., 2000), providing in vivo evidence that this gap junction protein is required for normal bone development and osteoblastic differentiation (Lecanda et al., 1998; Stains et al., 2003). This notion is now further supported by findings of *Cx43* mutations in patients with oculodentodigital dysplasia (ODDD), a rare congenital disease whose phenotypic features include craniofacial malformations and syndactyly (Paznekas et al., 2003; Richardson et al., 2004). A similar, though not identical phenotype has been recently reported in a mouse with a dominant negative *Cx43* mutant allele, *Gja1^{Jrt}* (Flenniken et al., 2005). Interestingly, these animals have also generalized osteopenia, thus reinforcing the notion that functional Cx43 is important for bone mass accrual and maintenance. Such a premise could be tested in a full gene-deletion model, but unfortunately homozygous *Cx43* null mice die shortly after birth because of severe cardiovascular malformations (Reaume et al., 1995), thus precluding the use of this model to study the consequences of complete lack of *Cx43* in the adult skeleton.

In vitro studies have also shown that Cx43 is critical for bone cell response to a variety of stimuli and pharmacologic agents. For example, inhibition of gap junctional communication or

Cx43 expression hinders osteoblast responses to fluid flow (Cherian et al., 2005; Saunders et al., 2001), or to mechanically induced calcium waves (Jørgensen et al., 2000). Further, the action of parathyroid hormone (PTH), an important regulator of bone remodeling also seems to be dependent on gap junctional communication. PTH increases gap junctional communication between osteoblasts by modulating *Cx43* expression or function (Civitelli et al., 1998; Donahue et al., 1995), and interference with Cx43-mediated gap junctional communication using antisense oligonucleotides or chemical inhibitors disrupts both PTH-induced cAMP accumulation (Van der Molen et al., 1996) and osteoblast differentiation (Schiller et al., 2001b). Based on these findings, and considering the osteoblast dysfunction of *Cx43* null osteoblasts (Lecanda et al., 2000), we hypothesized that lack of *Cx43* would negatively affect skeletal responsiveness to anabolic stimuli, such as that produced by intermittent PTH administration, the only currently available modality for inducing new bone formation (bone anabolism) in patients with osteoporosis and fractures (Neer et al., 2001).

To determine the biologic importance of *Cx43* in the adult skeleton, we generated a conditional *Cx43* gene ablated mouse model based on the Cre/*lox*P system (Nagy, 2000). In this model, which overcomes the lethality of the germline *Cx43* null mutation, *Cre* expression is driven by a 2.3-kb fragment of the α_1*(I) collagen* promoter, resulting in replacement of the entire *Cx43* reading frame with the *lacZ* reporter cassette selectively in bone-forming cells (Castro et al., 2003). With this model, *Cre* is expressed just before birth and in cells that are already partially differentiated into osteoblasts, thus providing an osteoblast-specific and postnatal gene ablation model (Dacquin et al., 2002). We find that these animals are viable, but develop a low peak bone mass and remain osteopenic throughout their adult life, the result of a reduced ability of bone-forming cells to fully differentiate. They also exhibit a dramatically attenuated response to the anabolic effect of intermittent PTH administration. Thus, *Cx43* is important not only for normal skeletal development, but also for peak bone mass accrual and adult bone homeostasis. Pharmacologic stimulation of gap junctional communication may enhance the effect of osteoanabolic agents, such as PTH.

Results

Cre-mediated Cx43 gene deletion in osteoblasts

Specific osteoblast *Cx43* gene deletion was demonstrated in *ColCre;Cx43*$^{-/fl}$ mice by different approaches. PCR of genomic DNA extracted from bone revealed the expected 670-kb band corresponding to the *Cx43* deleted allele, a band that was absent from extracts of tail soft tissue (Fig. 1A). Accordingly, Cx43 immunoreactive bands were barely detectable in Western blots of bone tissue extracts from conditionally deleted mice, contrasting with strong bands in wild-type equivalent littermates and fainter bands in heterozygous equivalent mice (Fig. 1B), the latter reflecting both the loss of one *Cx43* allele and haploinsufficiency of the 'floxed' allele (Theis et al., 2001). Substantial amounts of mRNA transcripts for the *Cre* transgene were detected only in bone extracts from *ColCre;Cx43*$^{-/fl}$ mice but not in either *Cx43*$^{-/fl}$ or *Cx43*$^{+/fl}$ extracts (Fig. 1C). Whole-mount preparations of newborn animals revealed strong X-gal stain in areas corresponding to mineralized skeleton of *ColCre;Cx43*$^{-/fl}$ mice, whereas very faint stain was observed in *Cx43*$^{-/fl}$ mice, which may reflect endogenous β-galactosidase expression (Kim et al., 2004), as it is observed in animals lacking the *lacZ* reporter (Fig. 1D). In conditional *Cx43*-deleted mice, X-gal blue staining was intense in areas of more advanced ossification, such as the diaphyses of long bones, vertebral bodies, ribs, distal mandible and facial bones, whereas staining was not observed in the epiphyses of long bones, corresponding to cartilaginous growth plates, nor in the skin or internal organs (Fig. 1D). Confirming osteoblast-specific *Cx43* gene deletion, X-gal stain was selectively observed in cells lining the bone surfaces of tibial cortical endosteum and trabecular surfaces of both tibia and vertebra. As expected, most osteocytes were also X-gal positive, whereas no stain was observed in bone marrow cells (Fig. 1E).

Osteopenia and reduced osteoblast number in Cx43 conditional knockout mice

All genotypes were obtained at the expected Mendelian frequency and were viable. Whole-mount alizarin red/alcian blue staining of newborn mice did not reveal any major skeletal abnormalities in *Cx43* conditional knockout mice compared with their control littermates (Fig. 2A), consistent with post-developmental deletion of *Cx43*. However, body weight at 1 month was significantly lower in *ColCre;Cx43*$^{-/fl}$ mice relative to the other genotypes in both males and females, a difference that persisted until at least 6 months of age (Fig. 2B). Importantly, conditional *Cx43*-deficient mice exhibited significantly lower whole-body bone density by dual-energy X-ray absorptiometry (DEXA) compared with Cx43$^{+/fl}$ or *Cx43*$^{-/fl}$ littermates by two-way ANOVA (Fig. 2C). This relative osteopenia was significant as early as 1 month of age and persisted with age at least up to 6 months ($P<0.05$ and $P<0.01$, respectively). Bone mineral content (BMC) very closely resembled the bone density data, with approximately 5% lower bone mass in *ColCre;Cx43*$^{-/fl}$ mice relative to the wild-type and heterozygous equivalent mice.

Histomorphometric analysis evidenced a markedly more reduced trabecular bone mass in *ColCre;Cx43*$^{-/fl}$ mice, with approximately 40% reduction in bone volume/total volume and more than 50% reduction in osteoblast number relative to wild-type littermates (Fig. 3A-C). Trabecular thickness in the conditional Cx43 ablated mice was likewise reduced by ~30%, without differences in trabecular number (Fig. 3D,E). Mineral apposition rate was reduced by ~17%, although not statistically significantly, relative to wild-type and heterozygous equivalent littermates (Fig. 3F). By contrast, there were no statistical differences in osteoclast number among the different genotypes (Fig. 3G). The apparent discrepancy in the degree of osteopenia between DEXA and histomorphometric measurement is not uncommon (Castro et al., 2004), and reflects both a lower sensitivity of DEXA and different skeletal sites measured.

Delayed differentiation of Cx43-deficient osteoblasts

To gain further insights into the pathobiologic mechanism of this osteopenic phenotype, we studied calvaria cells isolated from genetically modified animals. Demonstrating osteoblast-specific and differentiation-dependent *Cx43* gene replacement, X-gal staining was negative in *ColCre;Cx43*$^{-/fl}$ calvaria cells upon reaching confluence, but it became progressively stronger 1 week post-confluence onward (Fig. 4A). Progressively

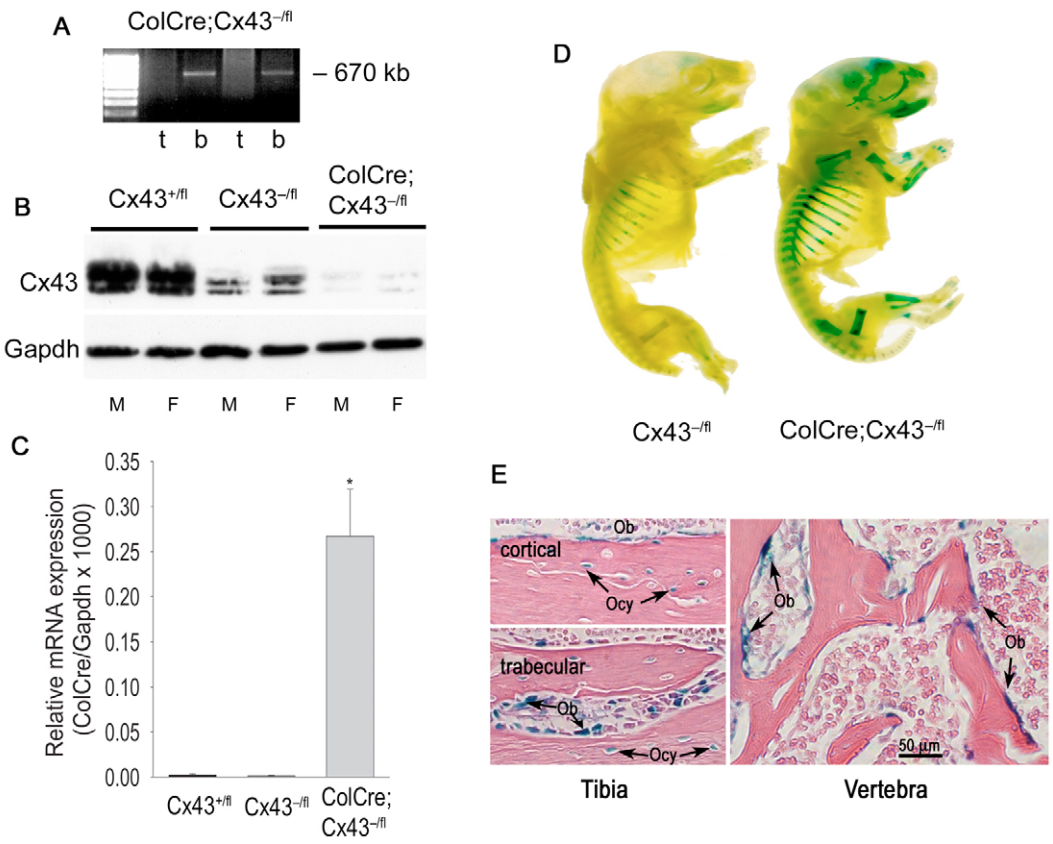

Fig. 1. Cre-mediated *Cx43* gene deletion in osteoblasts. (A) PCR of genomic DNA extracted from bone (b) or tail (t) tissue revealed the presence of a 670-kb band corresponding to the *Cx43*-deleted allele selectively in the bone tissue extract. (B) Western blot of bone tissue extracts from mice of the different genotypes showing barely detectable Cx43 immunoreactive bands in Western blots of *ColCre;Cx43*$^{-/fl}$ tissue, contrasting with strong expression of Cx43 protein in wild-type equivalent littermates, and lower but detectable expression in heterozygous equivalent mice. (C) Quantitation of mRNA for Cre by real-time PCR, showing the presence of the transgene only in extracts of *ColCre;Cx43*$^{-/fl}$ bone (one femur). (D) Whole mounts of newborn mice revealing strong X-gal stain in areas corresponding to mineralized skeleton of *ColCre;Cx43*$^{-/fl}$ mice, whereas only very faint stain was observed in *Cx43*$^{-/fl}$ mice. (E) X-gal stained sections of the mid-shaft (upper left) and metaphysis (lower left) of the tibia, and of one lumbar vertebra (right) of *ColCre;Cx43*$^{-/fl}$ mice, showing blue stain in cells lining the bone surface in both skeletal sites, but not in bone marrow cells. Non-stained surfaces correspond to areas where the cell layer was artifactually detached from bone. F, female; M, male; Ob, osteoblasts; Ocy, osteocytes.

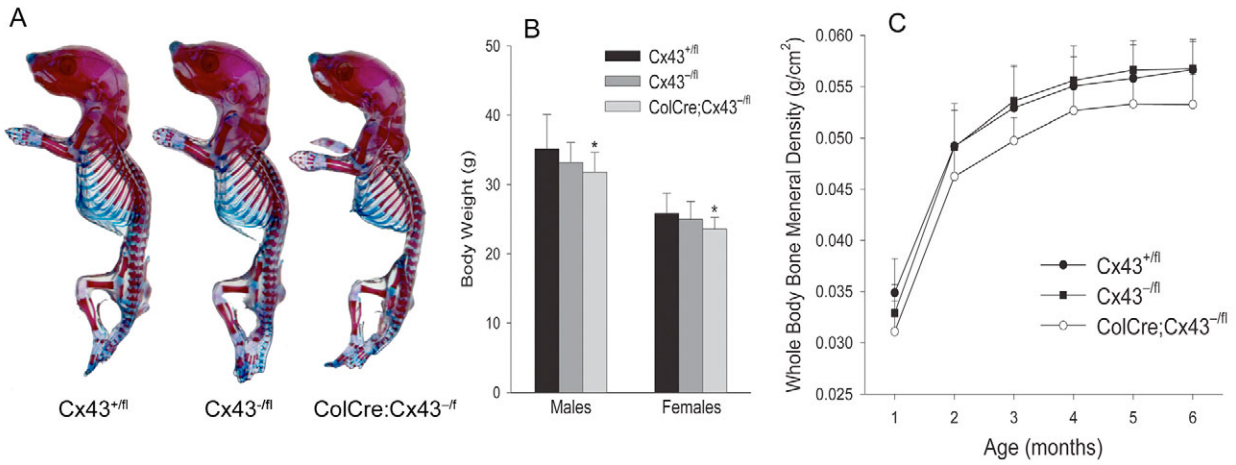

Fig. 2. Low bone mass phenotype in conditionally *Cx43* deleted mice. (A) Whole mount of alizarin red and alcian blue staining of newborn mice did not reveal any major skeletal abnormalities in *ColCre;Cx43*$^{-/fl}$ mice compared with their control littermates. (B) Lower body weight in *ColCre;Cx43*$^{-/fl}$ (n=46) relative to *Cx43*$^{+/fl}$ (n=69) and *Cx43*$^{-/fl}$ groups (n=42) at 6 months of age. (C) Whole-body BMD measured by DEXA monitored in vivo revealed significantly lower bone mass in *ColCre;Cx43*$^{-/fl}$ relative to wild-type littermates (P<0.05, for genotype effect; two-way ANOVA for repeated measures).

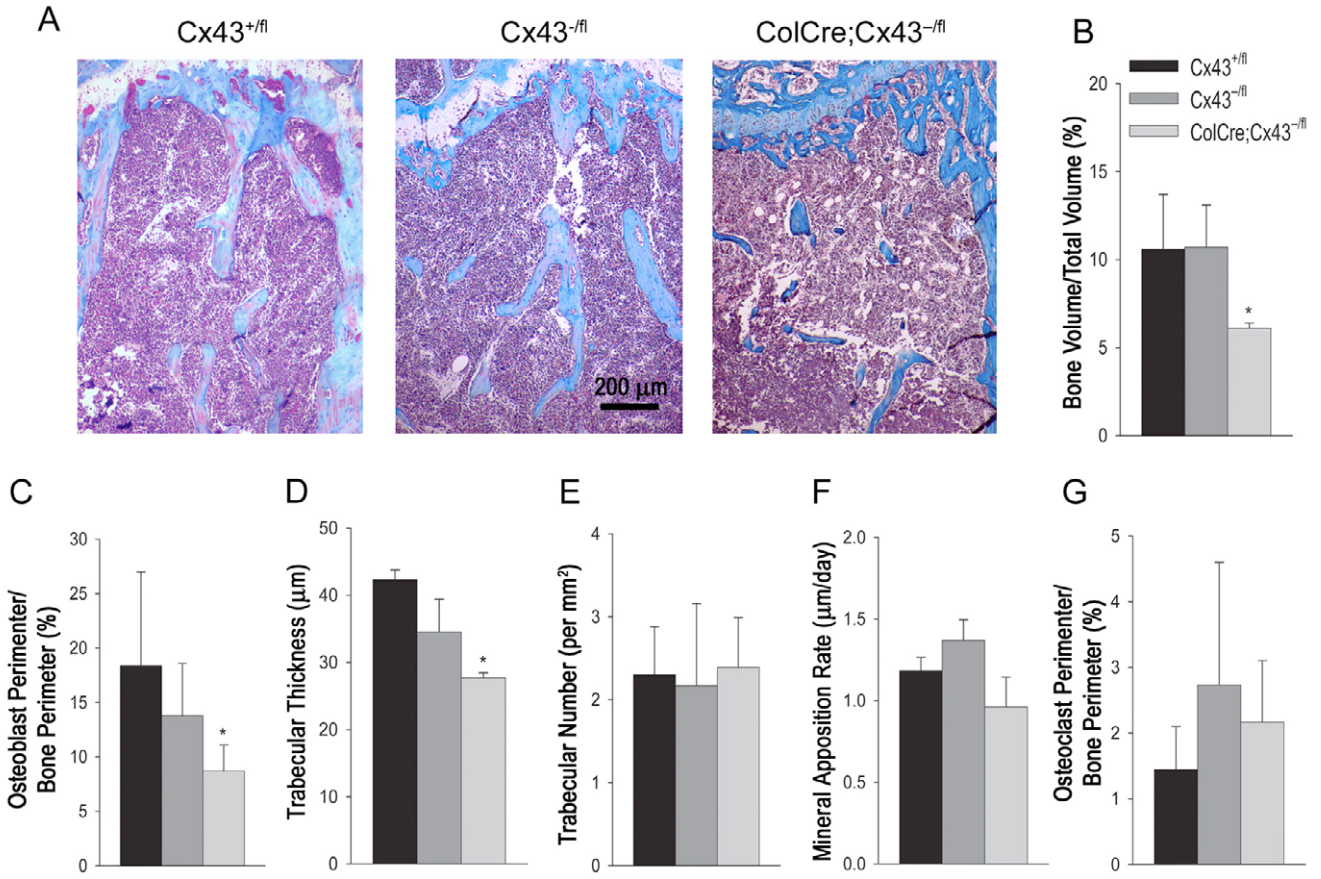

Fig. 3. Histomorphometric characterization of osteopenia in conditionally osteoblast *Cx43*-deleted mice. (A) Trichrome Masson stain of 4 μm undecalcified sections of proximal tibiae from mice of the three different genotypes at 6 months. (B-G) Quantitative histomorphometry showing significantly lower bone volume/total volume, osteoblast number and trabecular thickness in the *ColCre;Cx43*$^{-/fl}$, with non-statistically different trabecular number, mineral apposition rate and osteoclast number among the three genotype groups. *P<0.05 versus *Cx43*$^{+/fl}$ (one-way ANOVA); n=6 per genotype group.

increased X-gal stain during osteoblast differentiation is entirely consistent with the expression pattern of the promoter used to drive *Cre* (Dacquin et al., 2002). Accordingly, barely detectable Cx43 immunoreactive bands were detected in lysates of conditionally deleted cells, with reduced abundance of Cx43 in heterozygous equivalent cells (Fig. 4B). Likewise, *Cx43* mRNA abundance, assessed by real-time PCR, was reduced by ~90% and ~50% in conditionally *Cx43*-deleted and heterozygous equivalent calvaria cells after 3 weeks in culture (Fig. 4C).

Development of alkaline phosphatase activity, a marker of osteogenic differentiation, was significantly reduced in calvarial cells derived from *ColCre;Cx43*$^{-/fl}$ mice 2 weeks post-confluence, when it usually reaches a peak, as it occurred in the other two genotypes (Fig. 4D). Furthermore, after 2 weeks in culture the abundance of mRNA transcripts for other osteoblast-specific genes, namely *osteocalcin*, α_1*(I) collagen*, *osteopontin* and *Cbfa1/Runx2* was reduced by more than 50%, measured by real-time PCR, relative to wild-type equivalent cells (Fig. 4E). By contrast, neither *Cx45* nor *N-cadherin* mRNA were significantly different among the three genotypes (Fig. 4E). Importantly, *ColCre;Cx43*$^{-/fl}$ calvaria cells were not able to produce mineralized matrix until 3 weeks in culture, whereas *Cx43*$^{+/fl}$ and *Cx43*$^{-/fl}$ cells were able to start mineralization after 2 weeks (Fig. 4F,G). These in vitro data strengthen the notion that Cx43 expression is necessary for full elaboration of the osteoblast phenotype.

Attenuated bone anabolic response to intermittent PTH in osteoblast Cx43-deficient mice

We next tested the ability of conditional *Cx43*-deficient mice to respond to the anabolic stimulus provided by intermittent PTH injections. In a first study, we tested 4 doses of PTH in 5- to 6-month-old mice treated 5 days a week for 4 weeks. Because of the lower bone mass in the conditionally deleted mice relative to the other genotypes (Figs 2, 3), in these studies we monitored whole-BMC rather than bone density, to assess the absolute amount of bone gained in each group. In the wild-type equivalent *Cx43*$^{+/fl}$ group, PTH treatment induced rapid and dose-related increments in whole-body BMC, with significant increases over baseline at 4 weeks with all doses, except the lowest one. Maximal increases were 13.1% and 13.4% in *Cx43*$^{+/fl}$ and *Cx43*$^{-/fl}$ mice, respectively, with significant bone gain as early as after 2 weeks of treatment (Fig. 5A,B). However, in the conditionally Cx43-deleted *ColCre;Cx43*$^{-/fl}$ mice, only two doses of PTH resulted in statistically significant increments in bone mass, and the maximal effect obtained (9.8%) was ~30% lower than that observed in the other two genotypes (Fig. 5C).

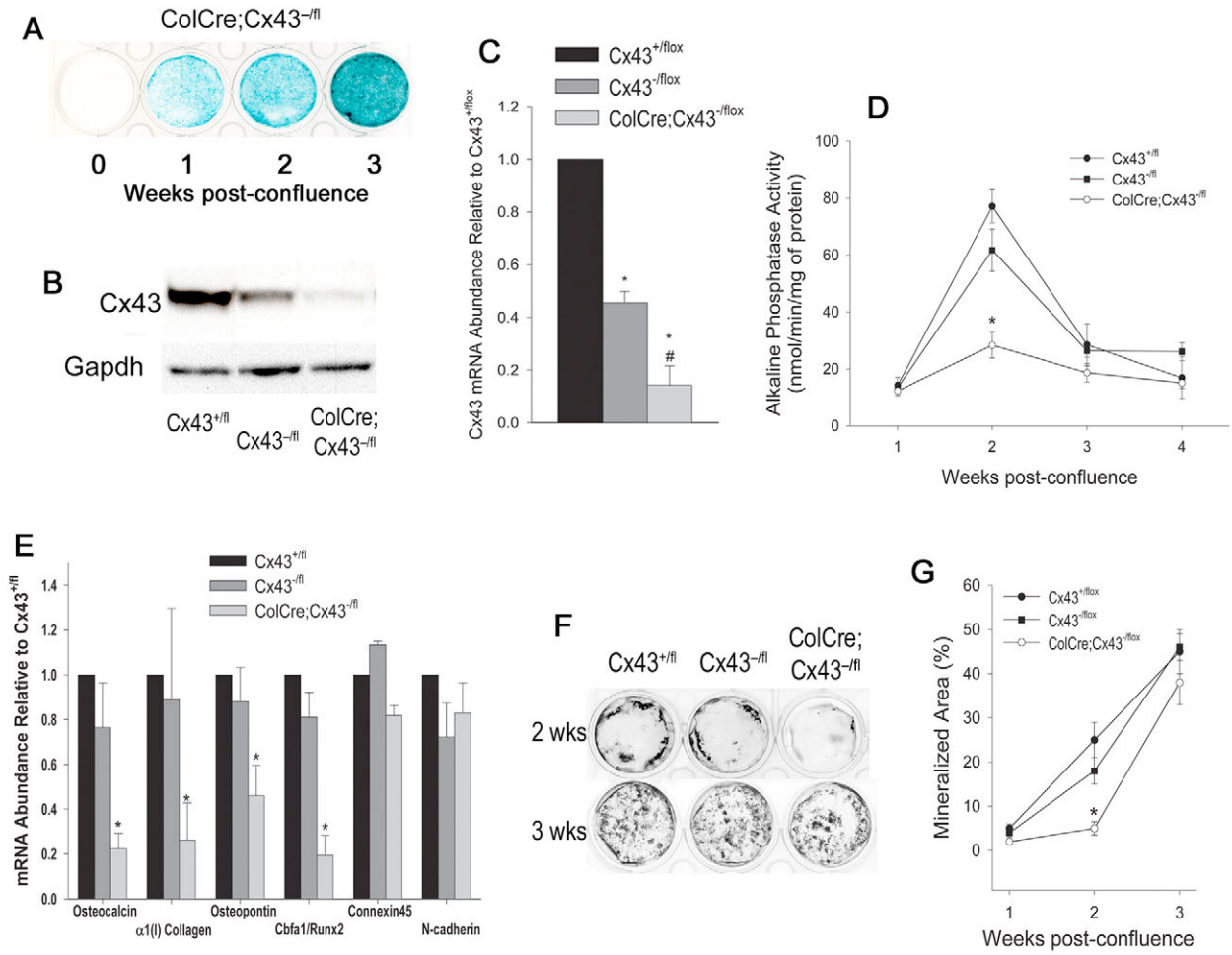

Fig. 4. Delayed differentiation of *Cx43*-deficient osteoblasts. (A) X-gal staining of post-confluent *ColCre;Cx43*$^{-/fl}$ calvaria cells grown in mineralizing medium, showing blue staining becoming stronger with time in culture. (B) Western analysis demonstrated barely detectable Cx43-specific bands in lysates of conditionally deleted cells, and reduced abundance of Cx43 in heterozygous equivalent cells. (C) Abundance of Cx43 mRNA, assessed by real-time PCR, was reduced in *ColCre;Cx43*$^{-/fl}$ calvarial cells relative to wild-type equivalent cells after 3 weeks in culture (n=3). (D) Development of alkaline phosphatase activity was significantly reduced in calvarial cells derived from *ColCre;Cx43*$^{-/fl}$ mice 2 weeks post-confluence (n=4). (E) The abundance of mRNA transcripts for other osteoblast-specific genes was reduced by more than 50%, as measured by real-time PCR, relative to wild-type equivalent cells (*P<0.05 versus *Cx43*$^{+/fl}$; one-way ANOVA; n=3). (F) Representative results of von Kossa stain of post-confluent calvaria cells and (G) quantitative data on three different preparations showing delayed in vitro matrix mineralization by *ColCre;Cx43*$^{-/fl}$ calvaria cell cultures (*P<0.05 versus *Cx43*$^{+/fl}$; Tukey test; n=3).

However, taking into account the increase occurring in untreated wild-type animals (4.8%), the difference in response amplitude would be more than 40%.

Rather surprisingly, even in vehicle-treated groups we detected a basal increase in BMC, presumably reflecting continuous bone growth in these 5-6-month-old animals. Because this may confound the extent of bone gain obtained with PTH, we repeated a similar study in older mice (7.4- to 9.6-months-old), whose bone mass should be stable. In this case, we used 40 μg/kg PTH, a dose that induced maximal effects in all genotypes in the younger animals. Again, 4-week treatment with 40 μg/kg PTH induced significant changes of whole-body bone mass in *Cx43*$^{+/fl}$ (12.5±4.7% from baseline; n=15) and *Cx43*$^{-/fl}$ (9.3±4.6%; n=11) mice, whereas the anabolic effect of PTH was reduced by 47% in the *ColCre;Cx43*$^{-/fl}$ group (6.7±5.3%; n=10). The changes in bone mass induced by PTH in the conditionally deleted animals were just slightly higher but not statistically different than the changes observed in a group of wild-type equivalent mice treated with vehicle (3.4±3.9%; n=9) (Fig. 6A). Region-specific analysis on BMC changes by DEXA also revealed that PTH significantly increased bone mass (>12%) at the lumbar spine only in the wild-type equivalent group, whereas no changes occurred at this site in the conditionally *Cx43*-deleted mice (Fig. 6B). Conversely, the anabolic effect of PTH on femur BMC was not affected by genotype, exhibiting an anabolic response of almost equal magnitude for each group (Fig. 6C).

Attenuated stimulation of bone formation after PTH treatment in osteoblast Cx43-deficient mice

Bone histomorphometric analysis was fully consistent with the DEXA results. After a 4-week treatment with 40 μg/kg PTH, bone volume (BV)/total volume (TV) was increased almost

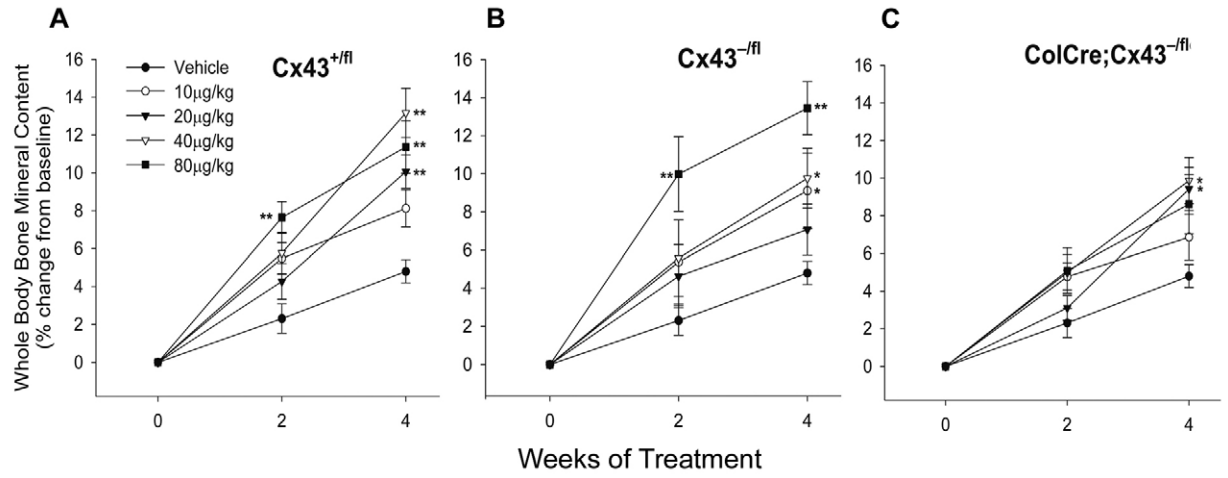

Fig. 5. Attenuated BMC response to PTH in osteoblast *Cx43*-deficient mice. Percent change from baseline of whole body BMC after a 4-week treatment with different doses of teriparatide (PTH), showing a dose-related increase of bone mass in wild-type $Cx43^{+/fl}$ mice (A). Effects of lesser magnitude were observed in heterozygous equivalent $Cx43^{-/fl}$ mice at the intermediate doses (B), whereas the effect of PTH treatment was uniformly attenuated at all doses in $ColCre;Cx43^{-/fl}$ mice (C). *P<0.05, **P<0.01 versus vehicle (one-way ANOVA); n=6-8 per each data point. Data are mean ± s.e.m.; n=12 (vehicle) and 6-8 (PTH-treated groups).

threefold in $Cx43^{+/fl}$ mice compared with mice of the same genotype treated with vehicle. A significant increase of lesser magnitude was also observed in the heterozygous equivalent group, $Cx43^{-/fl}$, whereas BV/TV was not different in the $ColCre;Cx43^{-/fl}$ group relative to the vehicle-treated group (Fig. 7A-D). Osteoblast number was increased in all genotypes with no statistical differences among groups, even though this parameter was ~30% lower in conditionally Cx43-deleted mice relative to $Cx43^{+/fl}$ littermates (Fig. 7E). Conversely, other static histomorphometric parameters of bone formation, trabecular number and thickness were significantly increased in $Cx43^{+/fl}$ (~20 and ~40%, respectively) but not in $ColCre;Cx43^{-/fl}$ or $Cx43^{-/fl}$ mice (Fig. 7F,G). Cortical thickness was also highest in $Cx43^{+/fl}$ mice, but the changes were not statistically significant (Fig. 7H). By contrast, osteoclast perimeter was higher in the $Cx43^{-/fl}$ and $ColCre;Cx43^{-/fl}$ groups, but even in this case the differences were not statistically significant (Fig. 7I).

Dynamic histomorphometric parameters of bone formation were assessed at two skeletal sites, to further investigate differences in PTH responses at the spine and femur. Abundant double-calcein labels were observed in wild-type mice after PTH treatment at both the spine and at the endosteal surface of the tibia. Double labelling was also present in the heterozygous equivalent mice, whereas in the majority of conditional knockout mice only single labels were detected

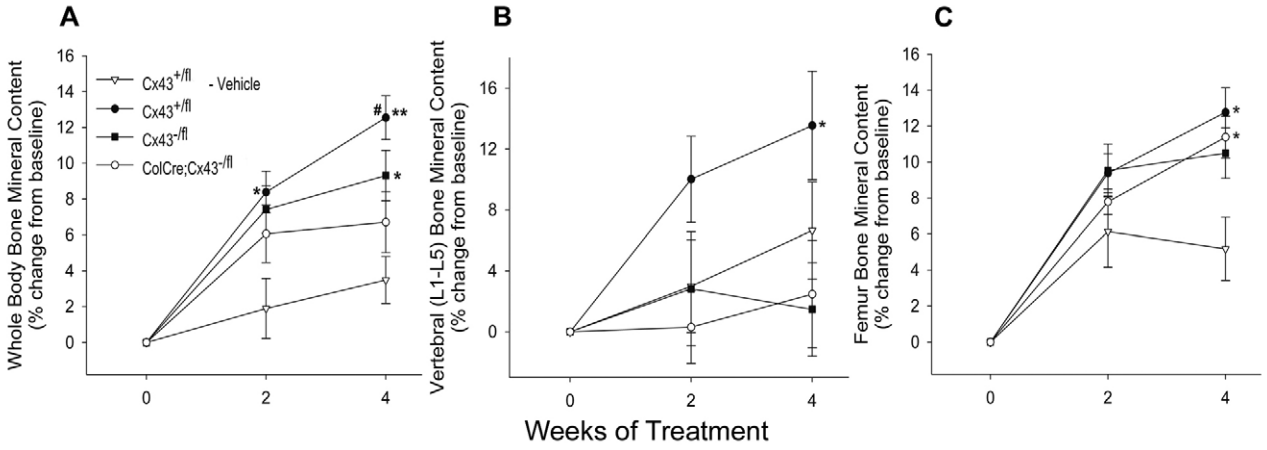

Fig. 6. Region-specific changes in BMC in osteoblast *Cx43*-deficient mice. (A) Percent change from baseline of whole body BMC after a 4-week treatment with 40 μg/kg PTH in a group of 7.4-9.6-month-old mice, showing an attenuated response in conditionally deleted $ColCre;Cx43^{-/fl}$ mice (n=10) relative to wild-type animals (n=15), whereas response in $Cx43^{-/fl}$ mice (n=11) was intermediate. (B) Response was absent at the lumbar spine in both $ColCre;Cx43^{-/fl}$ and heterozygous equivalent $Cx43^{-/fl}$. (C) Significant increases in bone mass were instead detected in all genotypes on the total femoral area. **P<0.01, *P<0.05 versus vehicle (n=9); #P<0.05 versus $ColCre;Cx43^{-/fl}$; one-way ANOVA.

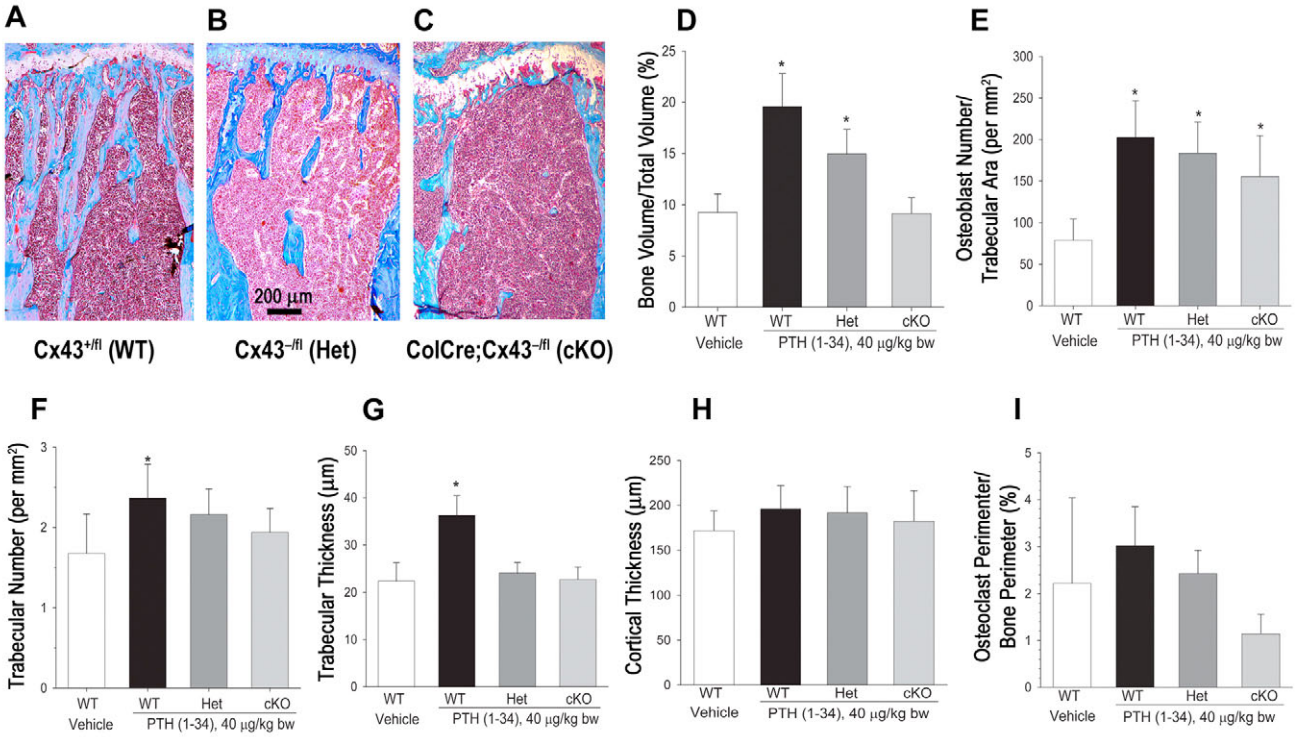

Fig. 7. Static bone histomorphometric analysis after a 4-week treatment with 40 μg/kg PTH. (A-C) Trichrome Masson stain of the proximal tibia showing less abundant trabecular bone mass in the conditional knockout (cKO) *ColCre;Cx43*$^{-/fl}$ relative to heterozygous equivalent (Het) *Cx43*$^{-/fl}$ and wild-type (WT) *Cx43*$^{+/fl}$ mice. (D) Robust anabolic response occurred in the WT group, and an attenuated response was observed in Het mice. By contrast, no significant increases in bone volume/total volume were detected in the conditionally deleted *ColCre;Cx43*$^{-/fl}$ mice relative to the other genotype groups. (E) Osteoblast number was significantly increased by PTH treatment in all groups. (F) Trabecular number was significantly increased in the wild-type groups only. (G) The same result was observed for trabecular thickness. (H) No significant changes were detected for cortical thickness. (I) Osteoclast number was not different among the different groups. **P*<0.05 versus vehicle (ANOVA); *n*=9-15.

(Fig. 8A,C). Consequently, mineral apposition rate (calculated in the trabecular and endosteal surfaces) was significantly lower in *ColCre;Cx43*$^{-/fl}$ mice than in the *Cx43*$^{+/fl}$ group in both sites (Fig. 8B,D). However, periosteal mineral apposition rate in the tibia was not significantly different among groups, even though the average was lower in *ColCre;Cx43*$^{-/fl}$ mice (0.423±0.246 μm/day) relative to *Cx43*$^{+/fl}$ (0.614±0.629 μm/day) and *Cx43*$^{-/fl}$ mice (0.710±0.497 μm/day), a result very consistent with the cortical thickness data. Finally, 5-bromo-2′-deoxy-uridine (BrdU)-positive cells were observed on the bone surface in all genotypes after PTH treatment (Fig. 8E), and the osteoblast mitotic index was not different among groups (Fig. 8F).

Discussion

The present study demonstrates that selective deletion of *Cx43* in osteoblasts leads to a marked decrease in peak bone mass and osteopenia; it also severely attenuates the bone anabolic response to intermittent administration of PTH. These abnormalities are caused by a functional defect in bone-forming cells, which fail to increase their activity in response to the hormonal stimulus. Thus, functional Cx43 is required for normal bone mass acquisition and maintenance and it is involved in the mechanism of action of PTH-induced anabolism.

An important role for Cx43 in bone homeostasis and for the function of bone-forming cells was postulated by several in vitro studies (Civitelli et al., 1993; Donahue et al., 2000; Schiller et al., 2001a), and it was established by analysis of mice with a germline null mutation of the *Cx43* gene, which exhibit delayed ossification of both endochondral and intramembranous skeleton and defective osteoblast differentiation (Lecanda et al., 2000). Craniofacial malformations are not present in *ColCre;Cx43*$^{-/fl}$ mice, most likely because in these animals *Cx43* is deleted at around birth (Dacquin et al., 2002), and thus embryonic development would be expected to be normal. However, the osteoblast defect is reproduced in *ColCre;Cx43*$^{-/fl}$ mice, a defect that leads to significant osteopenia throughout life. Interestingly, generalized osteopenia is also present in *Gja1*$^{Jrt/+}$ mice, which carry a point mutation of the *Cx43* gene (Flenniken et al., 2005), and whose phenotype resembles that of human ODDD, a rare autosomal dominant condition characterized by craniofacial (ocular, nasal and dental) malformations, limb dysmorphisms, spastic paraplegia and neurodegeneration (Loddenkemper et al., 2002; Schrander-Stumpel et al., 1993). Human ODDD has been linked to mutations of the *Cx43* gene (Kjaer et al., 2004; Paznekas et al., 2003; Richardson et al., 2004), however both *Cx43* null and *Gja1*$^{Jrt/+}$ mice exhibit impaired skull ossification (Flenniken et al., 2005; Lecanda et

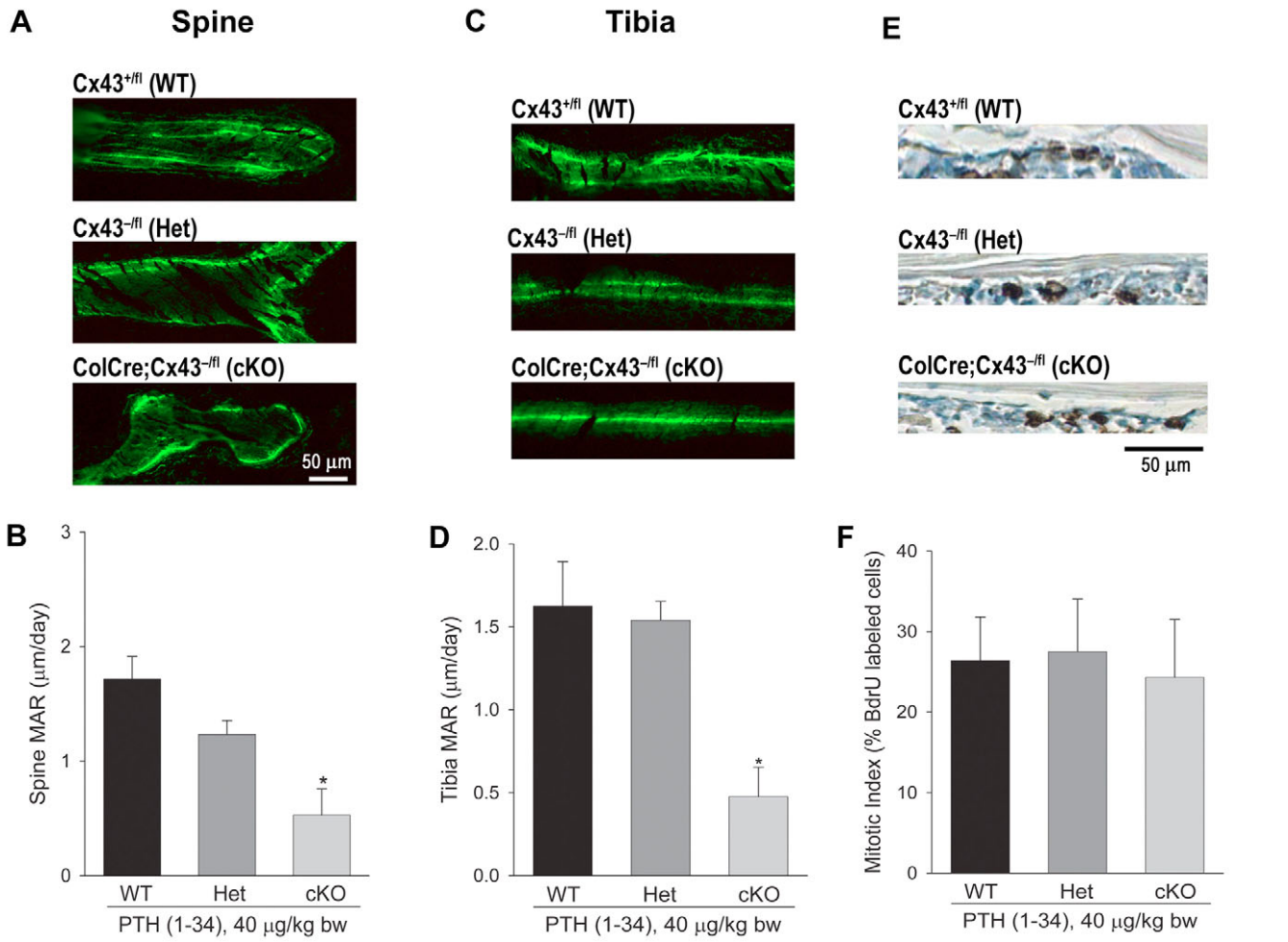

Fig. 8. Dynamic bone histomorphometric analysis and osteoblast proliferation after a 4-week treatment with 40 μg/kg PTH. (A) Fluorescent micrographs (200×) of undecalcified sections of lumbar spine trabecular bone showing double calcein labels in both wild-type *Cx43*$^{+/fl}$ (WT) and heterozygous equivalent *Cx43*$^{-/fl}$ (Het) mice, but only single labels in conditional knockout *ColCre;Cx43*$^{-/fl}$ (cKO) mice. (B) Mineral apposition rate (MAR) in the lumbar spine was significantly lower in cKO *ColCre;Cx43*$^{-/fl}$ mice than in either HT or WT *Cx43*$^{+/fl}$ group (n=5-6). (C) Calcein labeling and (D) mineral apposition rate in the endosteal surface of the tibia showing attenuated response to PTH in cKO mice (n=5-6). (E) BrdU stain of endosteal tibial surface, showing positively stained cells equally in mice of all genotypes (n=3-4). (F) Osteoblast mitotic index, expressed as percentage of BrdU-labeled cells per total number of cells on the bone surface. *P<0.05 versus *Cx43*$^{+/fl}$, one-way ANOVA.

al., 2000), whereas osteosclerotic changes are described in patients with ODDD (Paznekas et al., 2003; Schrander-Stumpel et al., 1993). Such a discrepancy may be related to species differences, or to mechanisms by which different ODDD mutations affect connexin function. Nevertheless, there is now evidence from different mouse genetic models consistently demonstrating that interference with *Cx43* in the postnatal skeleton leads to a low bone mass phenotype.

As noted, the cellular bases of the phenotype observed in conditionally *Cx43*-deleted mice suggest a defect in osteoblast differentiation and function, previously observed in the germline *Cx43* null mutants (Lecanda et al., 2000), and very likely present also in the *Gja1*$^{Jrt/+}$ mouse (Flenniken et al., 2005). Accordingly, *ColCre;Cx43*$^{-/fl}$ mice have a low osteoblast number, modestly decreased mineral apposition rate, delayed in vitro osteoblast differentiation, and profound deficit in osteoblast-specific gene expression. These results confirm that *Cx43* is required for full osteoblast differentiation and functional activity, although in vivo interference with gap junctional communication between osteoblasts and other cells on the bone microenvironment may also contribute to the phenotype. Because the 2.3-kb fragment of the α_1*(I) collagen* promoter we used to delete *Cx43* is expressed in committed osteoblasts (Dacquin et al., 2002), it is likely that the decreased osteoblast number in bone of conditionally deleted mice reflects a delayed differentiation rather than a decreased recruitment of new osteoblasts, a conclusion also supported by similar proliferation rates of bone cells in wild-type and deleted mice. Of course, this conclusion does not exclude other functions of Cx43 at earlier stages of osteoblast differentiation as postulated by studies in the *Gja1*$^{Jrt/+}$ mouse (Flenniken et al., 2005).

Although low bone mass is present in both *ColCre;Cx43*$^{-/fl}$ and *Gja1*$^{Jrt/+}$ (Flenniken et al., 2005) mutants, the molecular

mechanisms leading to osteopenia may be different. In the *Gja1*$^{Jrt/+}$ mice the mutation is germline and acts as dominant negative (Roscoe et al., 2005; Shibayama et al., 2005), whereas in our model the mutation is recessive and it occurs only in committed osteoblasts. Furthermore, osteoblasts also express *Cx45* (Civitelli et al., 1993) and although this connexin forms gap junction channels of different biophysical properties than those formed by Cx43 (Steinberg et al., 1994; Veenstra et al., 1994), Cx45 might be sufficient to support some degree of gap junctional communication in the absence of Cx43. This may provide a partial compensatory mechanism for the lack of *Cx43*, even though *Cx45* expression is not upregulated in conditionally deleted cells. By contrast, the *Gja1*$^{Jrt/+}$ variant may interfere with both connexins, or other interacting proteins, thus inhibiting the function of both Cx43 and Cx45 (Giepmans, 2004; Saez et al., 2003). These concepts are not at odds with the established notion that Cx45 overexpression reduces Cx43 function (Koval et al., 1995; Lecanda et al., 1998), because while in a mixed Cx43/Cx45 environment the biophysical properties of Cx45 prevail, in a *Cx43* null background, as it occurs in our mouse model, the presence of Cx45 would allow a certain degree of cell-cell communication that may partially compensate for lack of Cx43.

The consequences of osteoblast-specific ablation of *Cx43* are more severe under the stimulatory action of intermittent administration of PTH, reflected by the dramatic attenuation of the anabolic effect of PTH in *ColCre;Cx43*$^{-/fl}$ mice. Interestingly, although responses of lesser magnitude were also observed at intermediate doses of PTH in *Cx43*$^{-/fl}$ mice, in which the abundance of Cx43 in osteoblasts is reduced, the highest dose of PTH used (80 μg/kg) elicited a response similar to wild-type mice. By contrast, effects on bone mass that were maximal in wild-type and heterozygous equivalent animals were never achieved in the conditionally deleted mice, and no further gains were obtained with doses above 20 μg/kg. Thus, the gains in bone mass that can be induced by PTH are minimal, though not totally absent, when osteoblasts are deprived of *Cx43* in vivo, a conclusion consistent with the notion that the anabolic response to PTH requires functional Cx43. Instead, reduced Cx43 abundance in *Cx43*$^{-/fl}$ mice may be sufficient to support some osteoblast functions but not others. In particular, PTH upregulation of *Cx43* expression (Civitelli et al., 1998) is likely to be attenuated when Cx43 is decreased, and this may contribute to attenuation of PTH anabolic effect we have seen in *Cx43*$^{-/fl}$ mice. Similar observations have been made in the study of Cx43 function in astroglia, where the *Cx43*^{fl} allele shows haploinsufficiency for some phenotypical parameters but not for others (Theis et al., 2003). We also observed skeletal site-specific differences in Cx43 sensitivity to PTH anabolic effect in the conditionally deleted animals by regional DEXA analysis. It is possible that lack of *Cx43* attenuates PTH response on trabecular bone to a greater extent than it does on cortical bone, thus potentially explaining the normal response in femur observed by DEXA in conditionally *Cx43*-deleted mice. Envelope- or site-specific effects of PTH have been reported, with more pronounced bone mass increments observed in the trabecular than in the cortical component (Calvi et al., 2001; Gunness-Hey and Hock, 1984; Iida-klein et al., 2002).

The attenuated osteoanabolic response to PTH is the consequence of a failure of *Cx43*-deficient bone-forming cells to produce new bone under the hormonal stimulus, as demonstrated by ~70% lower mineral apposition rate in *ColCre;Cx43*$^{-/fl}$ than in wild-type mice after a 4-week treatment with PTH, despite a significant increase in osteoblast number. Considering that osteoblast number is decreased in untreated *ColCre;Cx43*$^{-/fl}$ mice, the results seem to indicate that the hormone is still able to stimulate osteoblast recruitment to the bone surface in conditional *Cx43*-deficient mice, although these cells are obviously impaired in their ability to synthesize new bone in response to PTH. Because *Cx43* deletion occurs in cells that are already fully committed to the osteogenic lineage, it is likely that some of PTH effects, for example recruitment or proliferation of osteoprogenitors, occur at a stage when *Cx43* deletion has not yet taken place, or are Cx43 independent. Although earlier studies indicated that intermittent PTH administration activates existing bone lining cells without affecting cell proliferation (Dobnig and Turner, 1995), an increase in bone marrow osteoprogenitor cells has been reported in response to PTH in rats (Kostenuik et al., 1999) and mice (Tanaka et al., 2004), and osteoblast number is consistently increased in mice (Iida-klein et al., 2002; Knopp et al., 2005). Furthermore, because osteoblast number was increased and osteoblast proliferation was not altered in the conditionally deleted animals, it is unlikely that our gene manipulation may have affected the anti-apoptotic action of PTH to a substantial degree (Jilka et al., 1999).

Although the molecular aspects of the interaction between Cx43- and PTH-induced bone anabolism remain to be elucidated, we had previously observed that PTH upregulates *Cx43* expression and function in osteoblasts (Civitelli et al., 1998), and more recently, we demonstrated that interference with *Cx43* alters transcriptional regulation of specific gene promoter elements, via MAP kinase- and protein kinase C-dependent pathways (Stains et al., 2003; Stains and Civitelli, 2005b). Because PTH signal transduction involves both of these pathways, it is possible that Cx43 is required to appropriately integrate PTH-activated signals and/or to equalize hormonal responses throughout the osteoblast network (Stains and Civitelli, 2005a). Consistent with this hypothesis, in preliminary results we find that interference with Cx43 function reduces the capacity of osteoblastic cells to increase osteocalcin gene transcription under stimulation by PTH (De Marzo et al., 2005). It is worth mentioning that the distribution of PTH receptors is not uniform throughout the bone tissue, and even within cell lines, certain signal responses are not homogeneous (Civitelli et al., 1992). The present results have interesting ramifications for development of therapeutic strategies for bone anabolism. The nature of the defect in response to intermittent PTH in our animal model makes it likely that similar attenuations of bone mass responses may occur for other anabolic agents or stimuli, i.e. mechanical load, because activation of bone-forming cell function is the ultimate requirement for manufacturing new bone. It is also reasonable to believe that the osteoanabolic response to PTH could be enhanced by increasing gap junctional communication using pharmacologic agents, thus allowing lesser doses or less frequent parenteral administration of PTH. Furthermore, the requirement of osteoblast/osteocyte Cx43 for the anti-apoptotic action of bisphosphonates (Plotkin et al., 2005), which are also widely used in the therapy of osteoporosis, can now be tested in vivo.

In summary, we have demonstrated that selective *Cx43* gene deletion in osteoblasts results in adult osteopenia, delayed osteoblast differentiation, and greatly attenuated osteoanabolic response to PTH, the consequence of a failure of *Cx43*-deficient bone-forming cells to mount a full response to the hormone. *Cx43*-mediated gap junctional communication represents a potential target for modulation of bone anabolic stimuli.

Materials and Methods

Transgenic mice

Development of the mouse model used in these studies has already been reported in some detail (Castro et al., 2003). Briefly, a mouse strain harboring a mutant 'floxed' Cx43 allele ($Cx43^{fl}$) (Theis et al., 2001) was mated to mice expressing *Cre* under control of a 2.3 kb $\alpha_1(I)$ *collagen* promoter fragment (abbreviated as *ColCre*) (Dacquin et al., 2002), so that Cre-mediated recombination replaces the entire *Cx43* reading frame with the *lac*Z reporter cassette. Homozygous $Cx43^{fl/fl}$ mice were generated first and crossed with *ColCre* mice also carrying a *Cx43* null allele ($ColCre;Cx43^{+/-}$). This strategy avoids potential effects of activation of *Cre* in the parental germ line. These crosses generate, in approximately equal numbers, the *Cx43* conditionally deleted mice, $ColCre;Cx43^{-/fl}$, as well as three additional genotypes, $Cx43^{+/fl}$ (wild-type equivalent), $Cx43^{-/fl}$ (heterozygous equivalent), and $ColCre;Cx43^{+/fl}$ (conditional heterozygous). All the mouse lines used in this project were developed in a mixed C57BL/6-C129/J background and littermate were used as controls. Mice were fed regular chow ad libitum and housed in a room maintained at constant temperature (25°C) on a 12 hours of light and 12 hours of dark schedule.

Genotyping was performed by PCR on genomic DNA extracted from mouse tails, after digestion with proteinase K, as described (Lecanda et al., 2000). The *Cx43* null allele was detected using primers *Cx43-5′*: 5′ GGT CAA CGT GGA GAT GCA CCT GAA GCA GAT 3′; *Cx43-3′*: 5′ AAT CGA TTG GCA GCT TGA TGT TCA AGC C 3′ and Neo^{r}*-5′*: 5′GGA TCG GCC ATT GAA CAA GAT GGA TTG CAC 3′. Primers *Cx43-5′* and *Cx43-3′* amplify a 900-bp product within the Cx43 coding region. Primer Neo^{r}*-5′* hybridizes to the neomycin resistance cassette present only in the null allele, and when used with primer *Cx43-3′*, it amplifies a 1.4-kb band, spanning the *Neo* cassette and part of the adjacent *Cx43* gene (Houghton et al., 1999). PCR was performed in a final volume of 25 μL reaction; 2 mM $MgCl_2$, 1× PCR buffer, 0.08 mM of each dATP, dCTP, dGTP, dTTP, 1 μM primers, 2.5 U *Taq* DNA polymerase, 1-5 μg genomic DNA. The DNA was denatured at 94°C for 3 minutes and amplified for 35 cycles (94°C for 30 seconds, 70°C for 45 seconds and 72°C for 120 seconds) followed by a final extension at 70°C for 20 minutes.

Primers *UMP* (5′ TCA TGC CCG GCA CAA GTG AGA C 3′) and *UMPR* (5′ TCA CCC CAA GCT GAC TCA ACC G 3′) were used for the simultaneous detection of the 'floxed' ($Cx43^{fl}$) and wild-type ($Cx43^{+}$) alleles, as described (Theis et al., 2001). These primers generate a 1 kb amplicon corresponding to the $Cx43^{fl}$ allele, and a 900 bp band, corresponding to the wild-type allele. In some experiments, the deleted *Cx43* allele was directly identified in whole bone extracts, after homogenization and phenol/chloroform extraction. This was accomplished using primers *Cx43delforw* (5′ GGC ATA CAG ACC CTT GGA CTC C 3′) and *Cx43delrev* (5′ TGC GGG CCT CTT CGC TAT TAC G 3′), which encompass the junction between the *Cx43* gene intron and the *β-galactosidase* coding region, thus generating a 670 bp amplicon, corresponding to the *Cx43*-deleted allele (Theis et al., 2001). The *ColCre* transgene was detected by using the primers *Cre 1123-1104:* 5′-AAG TGC CTT CTC TAC ACC TG-3′, *Cre 982-1002:* 5′-TGC TTA TAA CAC CCT GTT ACG-3′, *MS1*: 5′-GCT CAG CAA GCT CAC AGC AA-3′, and *LM6:* 5′-GAG CTT ACA CAT TTC GTC-3′. These primers generate 141 bp *Cre*-specific amplicon and a 448 bp *Cre*-negative amplicon.

Experimental design

A total of 157 mice were used for basal phenotypic characterization. For the PTH studies, 90 (45 males and 45 females) 5- to 6-month-old animals of three genotypes, $Cx43^{+/fl}$, $Cx43^{-/fl}$ and $ColCre;Cx43^{-/fl}$, were subcutaneously injected (5 days a week for 4 weeks) with either vehicle (0.9% saline containing 0.1% BSA and 0.001N HCl; n=12) or human recombinant PTH (1-34) (Teriparatide®, Eli-Lilly, Indianapolis, IN) at doses of 10 μg/kg (n=19), 20 μg/kg (n=18), 40 μg/kg (n=18), and 80 (n=23) μg/kg of body weight. Mice were weighed on the second week of treatment, and the amount of PTH injected was adjusted for any change in weight. A second group of older mice (7.4- to 9.6-month-old; n=45) was also treated with 40 μg/kg PTH with the same modalities as just detailed.

Whole-body mounts and X-gal staining

After sacrifice, newborn mice were skinned, eviscerated and maintained for 24 hours in ethanol 100%. After fixation in acetone for 24 hours, the carcasses were stained in a solution containing alizarin red 0.1%, alcian blue 0.3%, acetic acid and 70% ethanol (1:1:1:17). They were then transferred to a 1% KOH solution in 20% glycerol until they were cleared and then stored in glycerol for analysis of cartilage and bone, as described (Lecanda et al., 2000; McLeod, 1980). For whole-mount X-gal staining, carcasses of newborn mice were fixed for 2 hours in 2% formaldehyde, 0.02% paraformaldehyde, 5 mM EGTA, 0.1 mM $MgCl_2$ and 0.1 M $NaPO_4$, pH 7.3, then washed in a solution containing NP-40 and stained for 3 hours in X-gal substrate (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) 1 mg/ml, as described (Frendo et al., 1998). They were then transferred to a 1% KOH solution in 20% glycerol until they were cleared and then stored in glycerol. For X-gal staining of bone sections, tibiae or lumbar spine were fixed in 2% paraformaldehyde and 0.02% glutaraldehyde for 1 hour, and then decalcified in 4% EDTA for 17 days. Decalcified bones were incubated in 0.1% (v/v) X-gal substrate (see above) for 12 hours, post-fixed in 4% paraformaldehyde and embedded for paraffin sectioning (Hens et al., 2005). Sections were counterstained with eosin.

Bone mineral density (BMD) measurements

Total body BMC and BMD were monitored by DEXA using a PIXImus scanner (GE/Lunar, Madison, WI), under anesthesia with 100 mg/kg ketamine and 10 mg/kg xylazine i.p., as described (Castro et al., 2004). Heads were excluded from the analysis by masking. Region-specific BMD was also measured at the spine and femur, by identifying regions of interest corresponding to the L1-L6 area or the entire femur, respectively. In the latter case, animals were positioned with the femur at a 45° angle with the tibia, and values for both femurs averaged. Calibration was performed daily with a standard phantom as suggested by the manufacturer. The precision of whole-body BMD, assessed by the root mean square method is 1.34% (coefficient of variation) (Castro et al., 2004).

Bone histomorphometry

Mice were labeled twice by injection of calcein (15 mg/kg i.p., Sigma-Aldrich) on days 7 and 2 before euthanasia, and bone samples were prepared according to previously described methods, with some modifications (Castro et al., 2004). Briefly, dissected tibiae or lumbar spine were fixed in 70% ethanol and either decalcified in 14% EDTA for 14 days and embedded in paraffin, or left undecalcified and embedded in methyl methacrylate. Plastic sections were stained using the Masson trichrome technique, and tartrate-resistant acid phosphatase activity stain was used for paraffin sections, which were counterstained with methyl green and thionin for identification of osteoclasts and osteoblasts (Liu and Kalu, 1990). Eight μm sections were left unstained for dynamic bone histomorphometry. Quantitative histomorphometry was performed in an area 175-875 μm distal to the growth plate using the OsteoMeasure software program (Osteometrix, Atlanta, GA) in an epifluorescence microscopic system, as detailed elsewhere (Castro et al., 2004). The following parameters of bone remodeling were estimated (Parfitt et al., 1987): trabecular bone volume as a percentage of total tissue volume, trabecular thickness (in μm), trabecular number (per μm), trabecular separation (in μm), osteoblast perimeter per bone perimeter (in percent) or osteoblast number per trabecular area (in number/mm^2), osteoclast perimeter per bone perimeter (in percent), and mineral apposition rate (in μm/day), calculated as the mean distance between two fluorescent labels divided by the number of days between the labels.

Cell culture and phenotypic characterization

Osteoblast-enriched calvaria cultures were prepared from newborn mice by sequential collagenase digestion as described (Castro et al., 2004; Lecanda et al., 2000), and grown in α-modified essential medium (αMEM; Mediatech, Herndon, VA), supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) and 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma Chemicals, St Louis, MO). Approximately 3-5 calvariae were pooled to prepare the cell cultures used in each experiment. *Cx43* gene deletion was assessed in differentiating osteoblasts by β-galactosidase activity after fixation in 2% paraformaldehyde, and incubation in a solution containing 1 mg/ml X-gal substrate (see above), as described (Castro et al., 2003). Osteogenic differentiation was assessed by monitoring alkaline phosphatase activity and in vitro mineralization by von Kossa staining in the presence of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate, using standard techniques (Castro et al., 2004; Lecanda et al., 2000; Shin et al., 2000). Enzymatic activity was normalized for total protein content (Bio-Rad protein assay kit) and expressed as nmol of *p*-nitrophenol produced from *p*-nitrophenyl phosphate per minute per mg of protein. Mineralization was quantitated by calculating the surface area covered by dark stain per well, using digital image-processing software (IPLab v.3.5; Scanalytics, Rockville, MD), as previously described (Lecanda et al., 2000).

Cell proliferation

In vivo cell proliferation was assessed by BrdU incorporation, determined by immunoassay, according to the manufacturer's instructions (5-Bromo-2′-deoxy-uridine labeling and detection Kit III, Roche Molecular Biochemicals). For in vivo labeling, 100 μg BrdU (Sigma, St Louis, MO, USA) per gram of body weight in PBS was injected i.p. 2 hours before sacrifice. Longitudinal, 5 μm sections of paraffin-embedded tibiae, prepared as described above, were rehydrated and incubated for 10 minutes with 30% H_2O_2 in absolute methanol (1:9) and processed in denaturing and blocking solutions following the manufacturer's protocol. BrdU incorporated into nuclei was detected by immunostaining (Zymed Laboratories,

South San Francisco, CA). Slides were counterstained with hematoxylin and high-power field images of the cancellous bone were examined by optical microscopy. All BrdU-positive (dark-brown) nuclei in the secondary spongiosa were counted. The percentage of BrdU-positive nuclei versus total nuclei was calculated as mitotic index.

Western analysis

For whole cell lysates, calvaria cells were grown on 100 cm^2 Petri dishes and were extracted in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and protease inhibitors. For whole bone extracts, one femur was homogenized in TRIzol (Gibco) and incubated for 5 minutes at room temperature. After precipitation of DNA with ethanol, proteins were extracted from the phenol-ethanol supernatant by adding 1.5 ml of isopropanol per 1 ml of TRIzol reagent. The protein pellet was washed three times in 0.3 M guanidine hydrochloride in 95% ethanol, and dissolved in 1% SDS. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Invitrogen, Carlsbad, CA). Western blots were processed at room temperature as described (Castro et al., 2003; Lecanda et al., 2000) using an anti-Cx43 antibody (Sigma, St Louis, MO) at 1:8000 dilution or anti-GAPDH antibody, and visualized by enhanced chemiluminescence (ECL) detection.

Real-time RT-PCR

As already reported (Stains et al., 2003; Stains and Civitelli, 2005b), confluent calvaria cells or bone tissue were extracted using TRIzol (Gibco) and total RNA (1 μg) was reverse transcribed using Superscript II reverse transcriptase and oligo$(dT)_{15}$ primers. Real-time PCR analysis was performed using the SYBR green PCR method according to manufacturer's instruction (PE Biosystems, Foster City, CA). The primers used in this study have all been reported (Mbalaviele et al., 2005; Stains et al., 2003). GAPDH (PE Biosystems) was used as internal control. The cycle number at which the fluorescence exceeded the threshold of detection (CT) for GAPDH was subtracted from that of the target gene product for each well (ΔCT). Transcription levels relative to $Cx43^{+/fl}$ controls was defined as $(2^{-\Delta\Delta CT})$, where ΔΔCT equals the genotype ΔCT minus the ΔCT of $Cx43^{+/fl}$ cells. All real-time PCR experiments were performed at least three times.

Statistical analysis

Group means were analyzed by ANOVA after establishing normal distribution of data and homogeneity of variances. Where significant overall differences were observed by one-way ANOVA, the Tukey Kramer test or other post-hoc analyses were applied for multiple group comparisons. For repeated measures (PTH studies), a two-way ANOVA was applied, keeping treatment or genotype and time as independent variables. Analyses were performed using SPSS v.12.0.0 (SPSS, Chicago, IL), with the level of significance for comparison set at $P<0.05$. All data are expressed as the mean ± s.d. (unless otherwise indicated).

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References

Calvi, L. M., Sims, N. A., Hunzelman, J. L., Knight, M. C., Giovannetti, A., Saxton, J. M., Kronenberg, H. M., Baron, R. and Schipani, E. (2001). Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone. *J. Clin. Invest.* **107**, 277-286.

Castro, C. H., Stains, J. P., Sheikh, S., Szejnfeld, V. L., Willecke, K., Theis, M. and Civitelli, R. (2003). Development of mice with osteoblast-specific connexin43 gene deletion. *Cell Commun. Adhes.* **10**, 445-450.

Castro, C. H., Shin, C. S., Stains, J. P., Cheng, S. L., Sheikh, S., Mbalaviele, G., Szejnfeld, V. L. and Civitelli, R. (2004). Targeted expression of a dominant-negative N-cadherin in vivo delays peak bone mass and increases adipogenesis. *J. Cell Sci.* **117**, 2853-2864.

Cheng, B., Zhao, S., Luo, J., Sprague, E., Bonewald, L. F. and Jiang, J. X. (2001). Expression of functional gap junctions and regulation by fluid flow in osteocyte-like MLO-Y4 cells. *J. Bone Miner. Res.* **16**, 249-259.

Cherian, P. P., Siller-Jackson, A. J., Gu, S., Wang, X., Bonewald, L. F., Sprague, E. and Jiang, J. X. (2005). Mechanical strain opens connexin 43 hemichannels in osteocytes: a novel mechanism for the release of prostaglandin. *Mol. Biol. Cell* **16**, 3100-3106.

Civitelli, R., Fujimori, A., Bernier, S., Warlow, P. M., Goltzman, D., Hruska, K. A. and Avioli, L. V. (1992). Heterogeneous $[Ca^{2+}]_i$ response to parathyroid hormone correlates with morphology and receptor distribution in osteoblastic cells. *Endocrinology* **130**, 2392-2400.

Civitelli, R., Beyer, E. C., Warlow, P. M., Robertson, A. J., Geist, S. T. and Steinberg, T. H. (1993). Connexin43 mediates direct intercellular communication in human osteoblastic cell networks. *J. Clin. Invest.* **91**, 1888-1896.

Civitelli, R., Ziambaras, K., Warlow, P. M., Lecanda, F., Nelson Harley, J., Atal, N., Beyer, E. C. and Steinberg, T. H. (1998). Regulation of connexin43 expression and function by prostaglandin E2 (PGE2) and parathyroid hormone (PTH) in osteoblastic cells. *J. Cell. Biochem.* **68**, 8-21.

Dacquin, R., Starbuck, M., Schinke, T. and Karsenty, G. (2002). Mouse alpha1(I)-collagen promoter is the best known promoter to drive efficient Cre recombinase expression in osteoblast. *Dev. Dyn.* **224**, 245-251.

De Marzo, A., Stains, J. P. and Civitelli, R. (2005). Interference with connexin43 function attenuates parathyroid hormone regulation of the rat osteocalcin promoter. *J. Bone Miner. Res.* **20**, S430.

Dobnig, H. and Turner, R. T. (1995). Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. *Endocrinology* **136**, 3632-3638.

Donahue, H. J., McLeod, K. J., Rubin, C. T., Andersen, J., Grine, E. A., Hertzberg, E. L. and Brink, P. R. (1995). Cell-to-cell communication in osteoblastic networks: Cell line-dependent hormonal regulation of gap junction function. *J. Bone Miner. Res.* **10**, 881-889.

Donahue, H. J., Li, Z., Zhou, Z. and Yellowley, C. E. (2000). Differentiation of human fetal osteoblastic cells and gap junctional intercellular communication. *Am. J. Physiol. Cell Physiol.* **278**, C315-C322.

Flenniken, A. M., Osborne, L. R., Anderson, N., Ciliberti, N., Fleming, C., Gittens, J. E., Gong, X. Q., Kelsey, L. B., Lounsbury, C., Moreno, L. et al. (2005). A Gja1 missense mutation in a mouse model of oculodentodigital dysplasia. *Development* **132**, 4375-4386.

Frendo, J. L., Xiao, G., Fuchs, S., Franceschi, R. T., Karsenty, G. and Ducy, P. (1998). Functional hierarchy between two OSE2 elements in the control of osteocalcin gene expression in vivo. *J. Biol. Chem.* **273**, 30509-30516.

Giepmans, B. N. (2004). Gap junctions and connexin-interacting proteins. *Cardiovasc. Res.* **62**, 233-245.

Gunness-Hey, M. and Hock, J. M. (1984). Increased trabecular bone mass in rats treated with human synthetic parathyroid hormone. *Metab. Bone Dis. Relat. Res.* **5**, 177-182.

Hens, J. R., Wilson, K. M., Dann, P., Chen, X., Horowitz, M. C. and Wysolmerski, J. J. (2005). TOPGAL mice show that the canonical Wnt signaling pathway is active during bone development and growth and is activated by mechanical loading in vitro. *J. Bone Miner. Res.* **20**, 1103-1113.

Houghton, F. D., Thonnissen, E., Kidder, G. M., Naus, C. C., Willecke, K. and Winterhager, E. (1999). Doubly mutant mice, deficient in connexin32 and -43, show normal prenatal development of organs where the two gap junction proteins are expressed in the same cells. *Dev. Genet.* **24**, 5-12.

Iida-klein, A., Zhou, H., Lu, S. S., Levine, L. R., Ducayen-Knowles, M., Dempster, D. W., Nieves, J. and Lindsay, R. (2002). Anabolic action of parathyroid hormone is skeletal site specific at the tissue and cellular levels in mice. *J. Bone Miner. Res.* **17**, 808-816.

Jilka, R. L., Weinstein, R. S., Bellido, T., Roberson, P., Parfitt, A. M. and Manolagas, S. C. (1999). Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. *J. Clin. Invest.* **104**, 439-446.

Jørgensen, N. R., Henriksen, Z., Brot, C., Eriksen, E. F., Sorensen, O. H., Civitelli, R. and Steinberg, T. H. (2000). Human osteoblastic cells propagate intercellular calcium signals by two different mechanisms. *J. Bone Miner. Res.* **15**, 1024-1032.

Kim, J. E., Nakashima, K. and De Crombrugghe, B. (2004). Transgenic mice expressing a ligand-inducible cre recombinase in osteoblasts and odontoblasts: a new tool to examine physiology and disease of postnatal bone and tooth. *Am. J. Pathol.* **165**, 1875-1882.

Kjaer, K. W., Hansen, L., Eiberg, H., Leicht, P., Opitz, J. M. and Tommerup, N. (2004). Novel Connexin 43 (GJA1) mutation causes oculo-dento-digital dysplasia with curly hair. *Am. J. Med. Genet. A* **127**, 152-157.

Knopp, E., Troiano, N., Bouxsein, M., Sun, B. H., Lostritto, K., Gundberg, C., Dziura, J. and Insogna, K. (2005). The effect of aging on the skeletal response to intermittent treatment with parathyroid hormone. *Endocrinology* **146**, 1983-1990.

Kostenuik, P. J., Harris, J., Halloran, B. P., Turner, R. T., Morey-Holton, E. R. and Bikle, D. D. (1999). Skeletal unloading causes resistance of osteoprogenitor cells to parathyroid hormone and to insulin-like growth factor-I. *J. Bone Miner. Res.* **14**, 21-31.

Koval, M., Geist, S. T., Westphale, E. M., Kemendy, A. E., Civitelli, R., Beyer, E. C. and Steinberg, T. H. (1995). Transfected connexin45 alters gap junction permeability in cells expressing endogenous connexin43. *J. Cell Biol.* **130**, 987-995.

Lecanda, F., Towler, D. A., Ziambaras, K., Cheng, S.-L., Koval, M., Steinberg, T. H. and Civitelli, R. (1998). Gap junctional communication modulates gene expression in osteoblastic cells. *Mol. Biol. Cell* **9**, 2249-2258.

Lecanda, F., Warlow, P. M., Sheikh, S., Furlan, F., Steinberg, T. H. and Civitelli, R. (2000). Connexin43 deficiency causes delayed ossification, craniofacial abnormalities, and osteoblast dysfunction. *J. Cell Biol.* **151**, 931-944.

Liu, C. C. and Kalu, D. N. (1990). Human parathyroid hormone-(1-34) prevents bone loss and augments bone formation in sexually mature ovariectomized rats. *J. Bone Miner. Res.* **5**, 973-982.

Loddenkemper, T., Grote, K., Evers, S., Oelerich, M. and Stogbauer, F. (2002). Neurological manifestations of the oculodentodigital dysplasia syndrome. *J. Neurol.* **249**, 584-595.

Mbalaviele, G., Sheikh, S., Stains, J. P., Salazar, V. S., Cheng, S. L., Chen, D. and Civitelli, R. (2005). β-catenin and BMP-2 synergize to promote osteoblast differentiation and new bone formation. *J. Cell Biochem.* **94**, 403-418.

McLeod, M. J. (1980). Differential staining of cartilage and bone: Whole mount fetuses by alcian blue and alizarin red S. *Teratology* **22**, 299-301.

Nagy, A. (2000). Cre recombinase: the universal reagent for genome tailoring. *Genesis* **26**, 99-109.

Neer, R. M., Arnaud, C. D., Zanchetta, J. R., Prince, R., Gaich, G. A., Reginster, J. Y., Hodsman, A. B., Eriksen, E. F., Ish-Shalom, S., Genant, H. K. et al. (2001). Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N. Engl. J. Med.* **344**, 1434-1441.

Parfitt, A. M., Drezner, M. K., Glorieux, F. H., Kanis, J. A., Malluche, H., Meunier, P. J., Ott, S. M. and Recker, R. R. (1987). Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J. Bone Miner. Res.* **2**, 595-610.

Paznekas, W. A., Boyadjiev, S. A., Shapiro, R. E., Daniels, O., Wollnik, B., Keegan, C. E., Innis, J. W., Dinulos, M. B., Christian, C., Hannibal, M. C. et al. (2003). Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. *Am. J. Hum. Genet.* **72**, 408-418.

Plotkin, L. I., Aguirre, J. I., Kousteni, S., Manolagas, S. C. and Bellido, T. (2005). Bisphosphonates and estrogens inhibit osteocyte apoptosis via distinct molecular mechanisms downstream of extracellular signal-regulated kinase activation. *J. Biol. Chem.* **280**, 7317-7325.

Reaume, A. G., De Sousa, P. A., Kulkarmi, S., Langille, B. L., Zhu, D., Davies, T. C., Juneja, S. C., Kidder, G. M. and Rossant, J. (1995). Cardiac malformation in neonatal mice lacking connexin43. *Science* **267**, 1831-1834.

Richardson, R., Donnai, D., Meire, F. and Dixon, M. J. (2004). Expression of Gja1 correlates with the phenotype observed in oculodentodigital syndrome/type III syndactyly. *J. Med. Genet.* **41**, 60-67.

Roscoe, W., Veitch, G. I., Gong, X. Q., Pellegrino, E., Bai, D., McLachlan, E., Shao, Q., Kidder, G. M. and Laird, D. W. (2005). Oculodentodigital dysplasia-causing connexin43 mutants are non-functional and exhibit dominant effects on wild-type connexin43. *J. Biol. Chem.* **280**, 11458-11466.

Saez, J. C., Berthoud, V. M., Branes, M. C., Martinez, A. D. and Beyer, E. C. (2003). Plasma membrane channels formed by connexins: their regulation and functions. *Physiol. Rev.* **83**, 1359-1400.

Saunders, M. M., You, J., Trosko, J. E., Yamasaki, H., Li, Z., Donahue, H. J. and Jacobs, C. R. (2001). Gap junctions and fluid flow response in MC3T3-E1 cells. *Am. J. Physiol. Cell Physiol.* **281**, C1917-C1925.

Schiller, P. C., D'Ippolito, G., Balkan, W., Roos, B. A. and Howard, G. A. (2001a). Gap-junctional communication is required for the maturation process of osteoblastic cells in culture. *Bone* **28**, 362-369.

Schiller, P. C., D'Ippolito, G., Balkan, W., Roos, B. A. and Howard, G. A. (2001b). Gap-junctional communication mediates parathyroid hormone stimulation of mineralization in osteoblastic cultures. *Bone* **28**, 38-44.

Schiller, P. C., D'Ippolito, G., Brambilla, R., Roos, B. A. and Howard, G. A. (2001c). Inhibition of gap-junctional communication induces the trans-differentiation of osteoblasts to an adipocytic phenotype in vitro. *J. Biol. Chem.* **276**, 14133-14138.

Schrander-Stumpel, C. T., Groot-Wijnands, J. B., Die-Smulders, C. and Fryns, J. P. (1993). Type III syndactyly and oculodentodigital dysplasia: a clinical spectrum. *Genet. Couns.* **4**, 271-276.

Shibayama, J., Paznekas, W., Seki, A., Taffet, S., Jabs, E. W., Delmar, M. and Musa, H. (2005). Functional characterization of connexin43 mutations found in patients with oculodentodigital dysplasia. *Circ. Res.* **96**, e83-e91.

Shin, C. S., Lecanda, F., Sheikh, S., Weitzmann, L., Cheng, S. L. and Civitelli, R. (2000). Relative abundance of different cadherins defines differentiation of mesenchymal precursors into osteogenic, myogenic, or adipogenic pathways. *J. Cell. Biochem.* **78**, 566-577.

Stains, J. P. and Civitelli, R. (2005a). Cell-cell interactions in regulating osteogenesis and osteoblast function. *Birth Defects Res. C Embryo Today* **75**, 72-80.

Stains, J. P. and Civitelli, R. (2005b). Gap junctions regulate extracellular signal-regulated kinase signaling to affect gene transcription. *Mol. Biol. Cell* **16**, 64-72.

Stains, J. P., Lecanda, F., Screen, J., Towler, D. A. and Civitelli, R. (2003). Gap junctional communication modulates gene transcription by altering the recruitment of Sp1 and Sp3 to connexin-response elements in osteoblast promoters. *J. Biol. Chem.* **278**, 24377-24387.

Steinberg, T. H., Civitelli, R., Geist, S. T., Robertson, A. J., Hick, E., Veenstra, R. D., Wang, H.-Z., Warlow, P. M., Westphale, E. M., Laing, J. G. et al. (1994). Connexin43 and connexin45 form gap junctions with different molecular permeabilities in osteoblastic cells. *EMBO J.* **13**, 744-750.

Tanaka, S., Sakai, A., Tanaka, M., Otomo, H., Okimoto, N., Sakata, T. and Nakamura, T. (2004). Skeletal unloading alleviates the anabolic action of intermittent PTH(1-34) in mouse tibia in association with inhibition of PTH-induced increase in c-fos mRNA in bone marrow cells. *J. Bone Miner. Res.* **19**, 1813-1820.

Theis, M., de Wit, C., Schlaeger, T. M., Eckardt, D., Kruger, O., Doring, B., Risau, W., Deutsch, U., Pohl, U. and Willecke, K. (2001). Endothelium-specific replacement of the connexin43 coding region by a lacZ reporter gene. *Genesis* **29**, 1-13.

Theis, M., Jauch, R., Zhuo, L., Speidel, D., Wallraff, A., Doring, B., Frisch, C., Sohl, G., Teubner, B., Euwens, C. et al. (2003). Accelerated hippocampal spreading depression and enhanced locomotory activity in mice with astrocyte-directed inactivation of connexin43. *J. Neurosci.* **23**, 766-776.

Van der Molen, M. A., Rubin, C. T., McLeod, K. J., McCauley, L. K. and Donahue, H. J. (1996). Gap junctional intercellular communication contributes to hormonal responsiveness in osteoblastic networks. *J. Biol. Chem.* **271**, 12165-12171.

Veenstra, R. D., Wang, H.-Z., Beyer, E. C. and Brink, P. R. (1994). Selective dye and ionic permeability of gap junction channels formed by connexin45. *Circ. Res.* **75**, 483-490.