Essential Role of β-Catenin in Postnatal Bone Acquisition*

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Sheri L. Holmen‡‡, Cassandra R. Zylstra‡, Aditi Mukherjee§, Robert E. Sigler¶, Marie-Claude Faugere**, Mary L. Bouxsein‡‡, Lianfu Deng§§, Thomas L. Clemens‡§, and Bart O. Williams$$$%

From the ¥Laboratory of Cell Signaling and Carcinogenesis and ||Laboratory of Analytical, Cellular, and Molecular Microscopy, Van Andel Research Institute, Grand Rapids, Michigan 49503, ¥Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294, **Department of Medicine, University of Kentucky, Lexington, Kentucky, 40536, and §§Orthopedic Biomechanics Laboratory, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215

Mutations in the Wnt co-receptor LRP5 alter bone mass in humans, but the mechanisms responsible for Wnts actions in bone are unclear. To investigate the role of the classical Wnt signaling pathway in osteogenesis, we generated mice lacking the β-catenin or adenomatos polyposis coli (Apc) genes in osteoblasts. Loss of β-catenin produced severe osteopenia with striking increases in osteoclasts, whereas constitutive activation of β-catenin in the conditional Apc mutants resulted in dramatically increased bone deposition and a disappearance of osteoclasts. In vitro, osteoblasts lacking the β-catenin gene exhibited impaired maturation and mineralization with elevated expression of the osteoclast differentiation factor, receptor activated by nuclear factor-κB ligand (RANKL), and diminished expression of the RANKL decoy receptor, osteoprotegerin. By contrast, Apc-deficient osteoblasts matured normally but demonstrated decreased expression of RANKL and increased osteoprotegerin. These findings suggest that Wnt/β-catenin signaling in osteoblasts coordinates postnatal bone acquisition by controlling the differentiation and activity of both osteoblasts and osteoclasts.

The Wnt signaling pathway is implicated in the regulation of bone mineral density (1–5). Wnt ligands bind and activate a specific cellular receptor complex composed of a member of the frizzled family of seven transmembrane-spanning proteins and either LRP5 or LRP6 (6). LRP5 and LRP6 are members of a distinct subfamily of the low density lipoprotein receptor proteins (6). Loss of LRP5 causes osteoporosis pseudoglioma syndrome in humans, which is characterized by low bone density at an early age (7, 8), whereas point mutations in LRP5 are associated with extremely high bone density in an autosomal dominant inheritance pattern in humans and mice (2, 4, 5, 9).

Binding of cognate receptors by Wnt ligands can initiate several downstream signaling cascades (6). Activation of the “canonical” pathway involves the stabilization of cytoplasmic levels of β-catenin. The main function of the adenomatous polyposis coli (APC)2 protein appears to be the normal degradation of β-catenin. In the absence of APC, β-catenin levels are elevated, which ultimately contributes to increased proliferation (11). This pathway requires either LRP5 or LRP6 for activity (6), but LRP5 and LRP6 may have other cellular functions that are not directly related to regulation of Wnt signaling. For example, mice lacking LRP5 display defects in cholesterol and glucose metabolism (12). It remains unclear whether the bone defects seen in humans and mice lacking the Lrp5 gene are due solely to defects in Wnt signaling or if other downstream signaling cascades are involved.

In this work, we have shown that osteoblast-specific deletion of the β-catenin gene leads to early onset, severe osteoporosis and is associated with defective osteoblast differentiation in vitro. In contrast, loss of Apc leads to early onset, severe osteoporosis leading to lethality early in life. Interestingly, we show that these osteoblast-specific deletions are associated with alterations in the regulation of osteoprotegerin (OPG) and receptor activated by nuclear factor-κ B ligand (RANKL) and with altered osteoclastogenesis in vivo. Thus, this work provides the first genetic evidence that dysregulation of β-catenin in osteoblasts leads to defects in bone development and supplies the first link between Wnt/β-catenin signaling in osteoblasts and functional alterations of the osteoclast regulated by the OPG/RANKL signaling axis.

EXPERIMENTAL PROCEDURES

Mouse Crosses—OC-cre mice (20) were mated with homozygous conditional mutants carrying modified Apc (19) or β-catenin (18) alleles to generate OC-cre/Apclox−/lox and OC-cre/β-cateninlox−/lox progeny, which were used in subsequent matings. All experiments performed were in compliance with the guiding principles of the “Care and Use of Animals” available at www.nap.edu/books/0309053773/html and approved prior to use by the Van Andel Research Institute Institutional Animal Care and Use Committee.

Genotype Analysis—DNA was prepared from tail biopsies using an AutoGenprep 960 automated DNA isolation system. PCR-based strategies were then used to genotype these mice (details available upon request).

Demineralized Bone Histology—Tissue samples were fixed in formalin overnight, decalcified in ImmunoCal decalcifying agent (Decal, Baltimore, MD) overnight, and then dehydrated through a graded alcohol series in a Ventana Renaissance processor (Ventana Medical Systems, Tucson, AZ). Tissues were paraffin-embedded, and 5-μm sections were adhered to glass slides. Slides were de-paraffinized and stained with hematoxylin and eosin or left unstained for immunohistochemistry.

Mineralized Bone Histology—Femurs were fixed in ethanol at room

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‡ A Pfizer Fellow of the Life Sciences Research Foundation.

§§ To whom correspondence should be addressed. Tel.: 616-234-5308; Fax: 616-234-5309; E-mail: bart.williams@vri.org.

1 R. Nusse, personal communication.

2 The abbreviations used are: APC, adenomatous polyposis coli; OPG, osteoprotegerin; μCT, microcomputed tomography; RANKL, receptor activated by nuclear factor-κB ligand; RT, reverse transcription; OC, osteocalcin.
β-Catenin Signaling in Osteoblasts

21163

RESULTS

Mice with Osteoblast-specific Deletions of β-Catenin or Apc Die by 4 Weeks of Age—Because global inactivation of either the β-catenin gene (14, 15) or Apc (16, 17) results in early embryonic death, we crossed mice containing conditionally inactivatable alleles of β-catenin (β-catenin-flox) (18) or Apc (Apc-flox) (19) to mice expressing cre under the control of the osteocalcin (OC) promoter (20) to disrupt these genes in osteoblasts. Previous analysis of the OC-cre strain via crossing it to strains carrying reporter transgenes indicated that expression of cre recombinase was specific to the osteoblast lineage with no detectable expression or function in other cell lineages (20). As expected, deletion of the β-catenin gene led to loss of β-catenin protein (Fig. 1, a and b). Consistent with its role in mediating β-catenin degradation (11), deletion of Apc was associated with significantly elevated levels of β-catenin as assessed by immunohistochemistry (Fig. 1, b and c). By 1 week of age mice homozygous for either mutation could be identified by their reduced size (Fig. 1, d–g). OC-cre;β-catenin-flox/flox (Δβ-catenin, or Δβ-cat) mice died within 5 weeks (Fig. 1h). Growth retardation was even more severe in the OC-cre;Apc-flox/flox (ΔApc) mice (Fig. 1g), and these mice generally succumbed within 2 weeks (Fig. 1f). At weaning, only 75% of the expected number of Δβ-catenin mice and only 10% of the expected number of ΔApc mice were identified (Fig. 1, h and i). No defects in the incisors were observed either grossly or histologically, and the teeth erupted normally in both mutants (data not shown). The cause of the postnatal lethality in these mutant mice is currently unclear but is certainly not because of aberrant expression of the osteocalcin promoter in extraosseous tissues (20). Importantly, mice carrying osteoblast-specific deletions of both the Apc and β-catenin genes (ΔApc/Δβ-catenin) display growth and survival characteristics similar to those lacking only the β-catenin gene (data not shown), suggesting that the severe phenotype induced by loss of Apc is due to dysregulation of β-catenin signaling.

Δβ-Catenin and ΔApc Mice Have Dramatic Defects in Bone Development—Analysis of femurs from Δβ-catenin mice by μCT revealed striking reductions in both the trabecular and cortical bone compartments (Fig. 2, a and b). In contrast, analysis of ΔApc mice revealed a significant accumulation of bone matrix in the femur, to the point where the marrow space was almost completely filled (Fig. 2, d and e). Bone in the metaphyseal region was poorly mineralized, whereas osteoid in the diaphysis was more completely mineralized and entirely filled the marrow cavity. μCT images of femurs from ΔApc/Δβ-catenin mice were similar to those seen in Δβ-catenin mice, again suggesting that loss of Apc induced phenotypes in a β-catenin-dependent manner (Fig. 2c).

Examination of undecalcified sections from tibia of the Δβ-catenin mice disclosed a dramatic reduction in mineralized cortical and trabecular bone (Fig. 3, a–d), consistent with changes observed by μCT. By contrast, the ΔApc mice had dramatically increased bone deposition associated with disturbances in bone architecture and composition (Fig. 3, e–h). For example, the growth plate of ΔApc mice lacked a secondary ossification center and was misshapen (Fig. 3g), possibly because of the rapid rate of bone formation and the lack of osteoclasts (see below) that would normally function to shape the ends of the bone. Further analysis of decalcified bone sections from 4-week-old Δβ-catenin and 2-week-old ΔApc mice showed marked abnormalities in all bone examined, including vertebrae, long bones, and calvaria (Fig. 4).

Effects of Dysregulation of β-Catenin on Osteoblast Differentiation In Vitro—To examine the cellular mechanisms responsible for these disturbances in the mutant mice, we studied the effect of conditional deletion of the β-catenin and Apc genes in calvarial osteoblasts in vitro. Cells derived from mice carrying the floxed alleles were infected with adenovirus expressing the cre recombinase (Cre+) or a control adenovirus directing the expression of green fluorescent protein (Cre−) and then differentiated in the presence of β-glycerol phosphate and ascorbate (mineralizing medium). No obvious qualitative differences in proliferation rates or osteoblast density were observed in osteoblasts mutant for either gene (data not shown). However, consistent with the osteopenic phenotype of the Δβ-catenin mice, osteoblasts deficient in β-catenin showed delayed and diminished expression of osteocalcin as well as a marked reduction in calcified nodule formation (von Kossa staining). In-
FIG. 1. Comparison of mutant and control mice. a–c, immunohistochemical detection of β-catenin protein expression in cultured osteoblasts. a, Δβ-catenin osteoblasts, 40×; b, cre-flox/flox osteoblasts, 40×; c, ΔAPC osteoblasts, 40×. d, appearance of Δβ-catenin mice at postnatal day 10; e, ΔAPC mice at postnatal day 11. Growth curve of Δβ-catenin (f) and ΔAPC (g) mice compared with control littermates. Survival curve for Δβ-catenin (h) and ΔAPC (i) mice.
Interestingly, Runx-2 expression levels were similar in control and β-catenin osteoblasts (Fig. 5a), suggesting that early events in the osteoblast differentiation program (21) do not depend on signaling through β-catenin. In contrast, APC osteoblasts appeared to mature and mineralize normally in vitro, although osteocalcin expression levels increased somewhat prematurely as compared with controls (Fig. 5b). It appears, therefore, that excess β-catenin signaling does not severely

**FIG. 2.** μCT of femurs from Δβ-catenin, ΔAPC, and control mice. a, 31-day-old wild-type mouse; b, 31-day-old Δβ-catenin mouse; c, 31-day old ΔAPC/Δβ-catenin mouse; d, 12-day-old wild-type mouse; e, 12-day-old ΔAPC littermate. All panels show images ranging from the midshaft (left side) to the epiphysis (right side).

**FIG. 3.** Mineralized histology of bones from Δβ-catenin and ΔAPC mice. a and b, 30-day-old wild-type mouse; c and d, 30-day-old Δβ-catenin littermate. d, inset, a small portion of bone lined by a group of osteoclasts (*). e and f, 12-day-old wild-type mouse; g and h, 12-day-old ΔAPC littermate. Note the dramatic increase in mineralized bone (blue staining).
impact the ability of osteoblasts to differentiate, at least in vitro. Thus, the more dramatic effects of disruption of the β-catenin gene observed in vivo may be due to non-cell autonomous functions of APC in osteoblasts.

*Dysregulation of β-Catenin in Osteoblasts Is Associated with Abnormal Osteoclastogenesis*—Initial histological analysis (Fig. 3) suggested a disturbance in osteoclastogenesis in both mutants. Indeed, quantitation of osteoclasts in representative long bone sections showed that osteoclast numbers were dramatically increased in the Δβ-catenin mutants but entirely absent in the ΔAPC mice (Fig. 6a). In addition, osteoclasts were dramatically decreased in Δβ-catenin mutants at 4 weeks of age and were absent in ΔAPC mice at 2 weeks of age (data not shown). However, the dramatic deposition of osteoid material in ΔAPC mice suggests they were present at an earlier age. These findings suggested that dysregulated β-catenin signaling in osteoblasts not only causes cell-autonomous osteoblast defects but can also impact bone resorption by altering the numbers of osteoclasts. To explore this possibility further, we measured the expression of RANKL, the major osteoclast differentiation factor, and the osteoclast inhibitory factor, OPG (22). In Δβ-catenin osteoblasts, OPG mRNA expression was decreased compared with controls after 5 and 10 days of differentiation in mineralization medium, whereas RANKL expression was increased (Fig. 6b). The reverse pattern was observed in the ΔAPC osteoblasts (Fig. 6c). In addition, levels of serum OPG in the ΔAPC mice were 3-fold higher than controls (Fig. 6d). Serum OPG levels were similar to controls in both Δβ-catenin and ΔAPC/Δβ-catenin mice (data not shown). Taken together, these observations suggest that alterations in β-catenin signaling in osteoblasts brought about by each mutation lead to marked disturbances in osteoclast differentiation.

**DISCUSSION**

In this study we used conditional mutagenesis to disrupt selectively the genes encoding β-catenin and Apc in mouse
osteoblasts. In both models, osteoblast-specific disruption of the canonical Wnt signaling pathway led to postnatal death. In the case of the ∆APC mice, we speculate that deficiencies in hematopoiesis may be responsible. As shown, ∆APC mice develop bone in which the vast majority of the marrow component is absent. In other situations where osteopetrosis is observed in humans and mice, it is often accompanied with hepatosplenomegaly associated with extramedullary hematopoiesis. Interestingly, despite severe osteopetrosis, we have not observed any evidence of extramedullary hematopoiesis in these mice. Importantly, mice carrying osteoblast-specific deletions of both the Apc and the β-catenin genes are phenotypically similar to those lacking only the β-catenin gene (Fig. 2), suggesting that the majority of the phenotype induced by loss of Apc is because of dysregulated β-catenin signaling. In the case of ∆β-catenin mice, we occasionally observe animals with paralysis, consistent with osteopetrosis-related fractures, accounting for some portion of the lethality observed. However, we find that many of these animals die very suddenly between 26 and 30 days of age (Fig. 1) without prior evidence of paralysis. One explanation is that catastrophic fractures may occur leading to full paralysis and sudden death. Alternatively, defects in hematopoietic cell regulation or other systemic defects may play a role.

The skeletal phenotypes observed in these mice allow two important conclusions regarding the role of Wnt signaling in skeletal development. First, Wnt signaling controls postnatal bone acquisition by determining β-catenin levels in osteoblasts, and the previously identified alterations in bone mass in humans carrying mutations in LRP5 (1, 2, 4, 9) result from aberrant β-catenin signaling. However, it is important to note that the bone phenotypes resulting from inactivation of osteoblast β-catenin signaling, although consistent with the alterations seen in patients carrying mutations in LRP5, were more severe. One explanation for this difference is the potential role of LRP6 in β-catenin regulation in osteoblasts. We speculate that the continued presence of LRP6 in the context of LRP5 deficiency allows residual Wnt signaling through β-catenin. In agreement with this idea, we (23) and others (24) have shown that mice carrying mutations in both Lrp5 and Lrp6 show synergistic defects in bone development. In an alternative model, functions of β-catenin independent of Wnt signaling contribute to the observed phenotype. For example, β-catenin also plays a key role in mediating cell-cell adhesion through its interaction with E-cadherin (25). E-cadherin is a single-pass transmembrane protein that forms homotypic dimers with E-
cadherin proteins expressed on adjacent cells (25). The intracellular portion of E-cadherin binds directly to either β-catenin or the related plakoglobin protein, which then associates with α-catenin. The association of α-catenin with the actin cytoskeleton completes the coupling of cell-cell adhesion to the cytoskeleton. Although some studies have suggested that plakoglobin and β-catenin are interchangeable in mediating these cell adhesion complexes (26), it is possible that loss of β-catenin could also affect cell-cell adhesion in osteoblasts. However, the fact that ΔAPC mice exhibit the converse phenotype suggests that the functions of β-catenin in canonical Wnt signaling underlie the observed phenotypes. Also, our observations that mice carrying osteoblast-specific mutations in both Apc and β-catenin genes have phenotypes similar to those seen in mice mutant for only the β-catenin gene suggests that the osteopetrosis seen in ΔAPC animals is due to dysregulation of Wnt signaling observed in the osteoblast lineage, based on Runx2 expression (3, 21), but appears to be essential for the performance of the more mature osteoblast (e.g. osteocalcin expression and mineralization) (27, 28). However, the marked alterations in osteocalcin expression and reciprocal patterns of expression of OPG and RANKL in the context of dysregulated β-catenin signaling suggest that non-cell autonomous effects on osteoblast progenitors also impact overall bone acquisition.

Further support for this latter effect has come from a recent report indicating that the OPG gene may be a direct transcriptional target for complexes containing the β-catenin protein (10).

These combined effects of Wnt signaling through the canonical pathway in mature osteoblasts are therefore critical in the control of normal bone acquisition, and this pathway represents a plausible pharmacological target for treating osteoporosis and other bone disorders.

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