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N-Cadherin Interacts with Axin and LRP5 To Negatively Regulate Wnt/β-Catenin Signaling, Osteoblast Function, and Bone Formation▽

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Wnt signaling plays an important role in the regulation of bone formation and bone mass. The mechanisms that regulate canonical Wnt signaling in osteoblasts are not fully understood. We show here a novel mechanism by which the adhesion molecule N-cadherin interacts with the Wnt coreceptor LRP5 and regulates canonical Wnt/β-catenin signaling in osteoblasts. We demonstrate that N-cadherin, besides associating with β-catenin at the membrane, forms a molecular complex with axin and LRP5 involving the LRP5 cytoplasmic tail domain. N-cadherin overexpression in osteoblasts increases N-cadherin-LRP5 interaction, causing increased β-catenin degradation and altered TCF/LEF transcription in response to Wnt3a. This mechanism results in decreased osteoblast gene expression and osteogenesis in basal conditions and in response to Wnt3a. Consistent with a functional mechanism, silencing N-cadherin expression in control cells increases TCF/LEF transcription and enhances the response to Wnt3a. Using N-cadherin transgenic mice, we show that increased N-cadherin-LRP5 interaction resulting from targeted overexpression of N-cadherin in osteoblasts causes increased β-catenin ubiquitination and results in cell-autonomous defective osteoblast function, reduced bone formation, and delayed bone mass acquisition. These data indicate that a previously unrecognized N-cadherin-axin-LRP5 interaction negatively regulates Wnt/β-catenin signaling and is critical in the regulation of osteoblast function, bone formation, and bone mass.

Bone mass in adults is tightly regulated by the balance between bone resorption and formation (18). Osteoblasts are the bone-forming cells that play an essential role in bone mass acquisition (12, 27, 39). Elucidation of the molecular mechanisms that control osteoblastogenesis is therefore of major importance for a better understanding of the regulation of bone formation and bone mass (27). Recent pharmacological and genetic studies have highlighted the importance of Wnt signaling in the regulation of osteoblastogenesis, postnatal bone formation, and bone accrual (3, 4, 6, 14, 26, 31). This is exemplified by the finding that loss-of-function mutations in low-density lipoprotein receptor (LDLr)-related protein 5 (LRP5), a coreceptor for Wnt ligands, cause low bone mass, whereas LRP5 gain-of-function mutations induce a high-bone-mass phenotype (7, 16, 36). Genetic manipulations of LRP5 in mice have demonstrated that Wnt signaling mediated by this coreceptor is of major importance in the regulation of bone mass (1, 15, 28). The Wnt canonical signaling pathway mediated by LRP5 was found to promote osteoblastogenesis by stimulating preosteoblast proliferation and differentiation and reducing osteoblast apoptosis (52). The current model of the Wnt canonical pathway implies that Wnt binding to Wnt coreceptors LRP5 and Frizzled leads to the recruitment of the axin/Frat1/adenomatous polyposis coli protein (APC)/glycogen synthase kinase 3β (GSK3β)/β-catenin complex to the cytoplasmic tail of LRP5. This leads to displacement of GSK3β from the axin and β-catenin complex, inhibition of GSK3β, and decreased phosphorylation of β-catenin, which allows its stabilization and subsequent translocation into the nucleus and activation of target genes (37) (http://www.stanford.edu/~rnusse/wntwindow.html). Current evidence indicates that the Wnt canonical signaling pathway is regulated by extracellular natural antagonists, such as soluble Frizzled-related proteins (SFRPs), Cerberus, Wnt-inhibitory factor 1 (WIF1), Dickkopf (Dkk), connective tissue growth factor (CTGF), Wise, and sclerostin, which interact with Wnt proteins or Wnt signaling partners and antagonize Wnt signaling (29, 47). Cadherins, a family of transmembrane proteins that mediate cell-cell adhesion, can also modulate intracellular signaling (8, 13, 44, 53). N-cadherin is known to interact directly with β-catenin and thereby may decrease Wnt signaling (35, 54). E-cadherin was also found to negatively control Wnt signaling by sequestering β-catenin at the plasma membrane, thereby reducing the β-catenin cytosolic pool (37, 44). In bone, N-cadherin is strongly expressed by osteoblasts and is believed to play a significant role in osteogenesis (40, 42). Previous loss-of-function studies using overexpression of dominant-negative N-cadherin or heterozygous N-cadherin null mice have indicated that N-cadherin regulates osteoblast differentiation and bone formation (9, 10, 32). However, the molecular mechanisms by which N-cadherin controls intracellular signals to regulate osteoblastogenesis are not fully understood.

We hypothesized that N-cadherin may directly interact with Wnt partner molecules other than β-catenin to regulate Wnt signaling and cell function. In this study, we demonstrate that N-cadherin interacts with LRP5 and axin and that N-cadherin-axin-LRP5 interaction negatively regulates Wnt signaling

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through β-catenin degradation, resulting in decreased osteoblast differentiation. We also explored the functional importance of N-cadherin-LRP5 interaction in vivo with N-cadherin transgenic mice. Our data show that the increased β-catenin degradation induced by N-cadherin-LRP5 interaction in osteoblasts results in decreased osteoblast function and bone formation and delayed bone mass acquisition in vivo. These data identify the interaction between the cell-cell adhesion molecule N-cadherin and the Wnt coreceptor LRP5 as a novel critical mechanism that negatively regulates Wnt/β-catenin signaling, osteoblast function, bone formation, and bone mass.

MATERIALS AND METHODS

Cell cultures and transfections. MC3T3-E1 cells (ATCC) were transiently transfected with the indicated construct (1 μg total DNA) as previously described (20) using Trans-Fast transfection reagent (Promega, Charbonnières les Bains, France). MC3T3-E1 cells were stably transfected with Flag-tagged N-cadherin cloned in pcDNA 3.1 and selected using G418 (Calbiochem, San Diego, CA), and overexpression was verified by Western blot analysis. Transfection with LRP5 mutants was performed as previously described (20). Primary osteoblastic cells obtained by sequential collagenase digestion (Sigma-Aldrich, St. Louis, MO) of calvarias from 2- to 5-day-old mice. For in vitro mineralization, cells were cultured in with the presence of 50 μmol/liter ascorbic acid and 3 mmol/liter inorganic phosphate (Sigma). Mineralized extracellular matrix was identified by alizarin red staining (Sigma).

Wnt3a-CM preparation. Wnt3a-conditioned medium (Wnt3a-CM) was prepared as previously described (20).

In vitro assays. For luciferase reporter assays, 20 ng of pCMV-β-galactosidase (β-Gal) was added to the transfection mix (90 ng of TCF and 30 ng of TopFlash or FopFlash). In some experiments, luciferase activity was determined in the presence of N-cadherin small interfering RNA (siRNA) or control siRNA (2 μg/30,000 cells; Santa Cruz Biotechnology, Santa Cruz, CA). Axin siRNA was also from Santa Cruz. Luciferase activity was determined by using a luciferase assay kit (Promega) and a β-Gal gene reporter assay kit (Roche, Meylan, France). Alkaline phosphatase (ALP) activity was assayed using an ALP kit (Bio-Rad, Hercules, CA), and ALP staining was performed using a Sigma Fast 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium kit (Sigma-Aldrich). The aggregation assay was performed as previously described (19).

Cell fractionation, Western blot, and immunoprecipitation assays. For subcellular fractionation, cell layers were washed with cold A buffer (50 mM Tris/HCl [pH 7.4], 150 mM NaCl, 2 mM Na_3VO_4), scrapped, and centrifuged at 1,200 rpm for 5 min at 4°C. Cell pellets were sonicated (10 s, three times) on ice in B buffer (50 mM Tris [pH 7.4], 250 mM sucrose, 2 mM Na_3VO_4) and then ultracentrifuged at 100,000 × *g* for 1 h at 4°C. The cytosolic fractions (supernatants) were concentrated 20-fold by ultrafiltration (Centricon 10K filters, Millipore Corporation) and supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Pellets, corresponding to the membrane fraction, were resuspended in Western blot lysis buffer. The protein content was determined (BCA protein assay; Pierce Biotechnology, Inc., Rockford, IL). For nuclear extract, cells were scraped in cold phosphate-buffered saline, spinned down (12,000 × *g*, 30 s), resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl) with 25 μl of 10% NP-40 and then spinned down. Nuclei in the pellet were resuspended in buffer B (20 mM HEPES, pH 7.9; 25% glycerol; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.46 M NaCl), and protein concentration was determined.

For Western blot analysis, 15 μg of proteins were loaded on GeBa gel (4 to 12%) (Gene Bio-Application, Ltd., Kfar Hanagid, Israel). After electrophoresis, the transferred proteins were revealed with anti-Flag (Sigma-Aldrich), anti-c-Myc (AbCam, Cambridge, United Kingdom), antiaxin (Zymed), anti-N-cadherin, anti-β-catenin, anti-phospho-β-catenin, anti-GSK3, anticaveolin, anti-histone H1, anti-LDLr (Santa Cruz), or anti-LRP5 (Cell Signaling, Denver, CO) and detected using a secondary horseradish peroxidase antibody (Beckman Coulter, Fullerton, CA). Immunoprecipitation analysis was performed using microMACS protein A/G microbeads magnetic separation (Miltenyi Biotech Auburn, CA) according to the manufacturer's recommendations. Briefly, 100 μg of total protein was incubated 30 min on ice with 2 μg of the indicated antibody or immunoglobulin fraction negative control (Dako, Glostrup, Denmark), and 20 μl of protein A/G was magnetically labeled. The magnetically labeled immune complex was passed over a microcolumn placed in a magnetic field. The bound complex was washed with lysis buffer, and the immunoprecipitated protein was eluted from the column with sodium dodecyl sulfate gel loading buffer to ready it for the Western blot assay.

Immunofluorescence microscopy. Forty-eight hours after transfection or treatment, MC3T3E1 cells were fixed, permeabilized, treated with 3% bovine serum albumin, incubated with a mouse monoclonal anti-β-catenin, a rabbit polyclonal or a mouse monoclonal anti-N-cadherin (1/200), or a mouse monoclonal anti-c-Myc (5 μg/ml), and then incubated with donkey anti-rabbit antibody conjugated to Cy2 (Jackson Immunoresearch, West Grove, PA), or a donkey anti-mouse antibody conjugated to Cy3 (Jackson Immunoresearch). In experiments in which cells were treated with Wnt3a, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA). Total (1,860 cells) and nuclear β-catenin-positive cells were counted within 20 fields, and the percentage of positive nuclear β-catenin cells was recorded.

Quantitative reverse transcriptase PCR analysis. Quantitative real-time PCR analysis of total RNA was performed using a Roche light cycler and Absolute SYBR Green capillary mix (Abgene, Epson, United Kingdom). The sets of primers were 18S (forward, 5′-CGGCTACCACATCCAAGGAA-3′; reverse, 5′-GCTGGAATTACCGCGGCT-3′), collagen type 1 α1 chain (forward, 5′-CTTGGTGGTTTTGTATTCGATGAC-3′; reverse, 5′-GCGAAGGCAACAGTCGCT-3′), ALP (forward, 5′-AAGGCTTCTTCTTGCTGGTG-3′; reverse, 5′-GCCTTACCCTCATGATGTCC-3′), osteocalcin (forward, 5′-CTCACAGATGCCAAGCCCA-3′; reverse, 5′-CCAAGGTAGCGCCGGAGTCT-3′), Runx2 (forward, 5′-TTGACCTTTGTCCCAATGC-3′; reverse, 5′-AGGTTGGAGGCACACATAGG-3′) and Osterix (forward, 5′-TTTAAACAAACACGATGAGATA-3′; reverse, 5′-ATTGGACTTCCCCCTTCTTG-3′). The expression of 18S RNA used as control did not change due to overexpression of N-cadherin or addition of Wnt3a.

Generation of N-cadherin transgenic mice. A HindIII-XbaI 2.3-kb fragment of the rat collagen alpha 1 type I promoter was cloned in a pBluescript plasmid containing the human N-cadherin Flag-tagged cDNA. At the 3′ end of the N-cadherin cDNA, a XbaI-SfiI blunted fragment was introduced which contained the 3′-untranslated region of the rabbit γ-globin gene, including intronic sequences and polyadenylation signal. An XbaI-XhoI 3-kb fragment of the matrix attachment region from the chicken lysozyme locus which was blunted at the ends was cloned upstream of the promoter in the SalI site of the plasmid. The whole construct was removed using the two BssHII sites of the vector. Transgenic mice were generated by injection of the DNA construct into pronuclei from C57BL/6-B6D2 mice embryos. Founder males were mated to C57BL/6-B6D2 females. The genotype of the F1 mice was determined by PCR amplification of genomic tail DNA. F1 mice from two lines were investigated for their bone phenotypes. Heterozygotic and homozygotic mice were compared to their wild-type littermates. To determine the mineralization apposition rates and measure the active bone forming surfaces, calcein (10 mg/kg; Sigma) and tetracycline (20 mg/kg; Sigma) were injected intraperitoneally at 3 days and 1 day before sacrifice, respectively.

Northern blot analysis. Total RNA was extracted from tissues using Extract-All solution (EuroBio, Les Ulis, France). Twenty micrograms of RNA was separated and hybridized with a cDNA probe encoding the N-cadherin Flag-tagged gene. Inserts were labeled with [α-^{33}P]dCTP by using a nick translation kit (Promega). 18S and 28S subunits were revealed by ethidium bromide (EuroBio) staining and used as loading controls.

DEXA. Dual-energy X-ray absorptiometry (DEXA) measurement was performed using a PIXImus instrument (Lunar) at the ultrahigh resolution mode. The coefficient of variation was <2% for all the parameters evaluated. Total body, femoral, tibial, and caudal vertebral bone mineral content (mg) and bone mineral density (mg/cm^2) were measured in vivo.

Microtomographic and histomorphometric analyses. The microtomographic imaging system (μCT 40; Scanco Medical AG, Bassersdorf, Switzerland) is equipped with a 5-μm focal spot X-ray tube as a source. The long axis of the femur was oriented orthogonally to the rotation axis of the scanner. Scans were performed at an isotropic, nominal resolution of 20 μm (medium resolution mode). Image segmentation and morphometric characterization of the acquired three-dimensional image data included calculation of bone volume density (BV/TV) and trabecular thickness (Tb.Th), separation (Tb.Sp), and number (Tb.N) in the trabecular bone compartment in the distal metaphysis as well as cortical bone volume density (%BV) and cortical thickness (Ct.Th) in the middiaphysis (30).

For histomorphometric analysis, caudal vertebrae were fixed in 70% ethanol and embedded in methyl methacrylate. Sections obtained with a Leica microtome (SM2500S) (Wetzlar, Germany) were stained with naphthol ASTR (3-hydroxy-2-naphthoic acid 4-chloro-2-methylanilide) phosphate for tartrate-resistant acid phosphatase (TRAP) detection or with toluidine blue or were left unstained for fluorochrome evaluation (calcein/tetracycline double labeling). The trabecular bone volume, trabecular number, trabecular separation, and trabecular thickness were measured using a software package

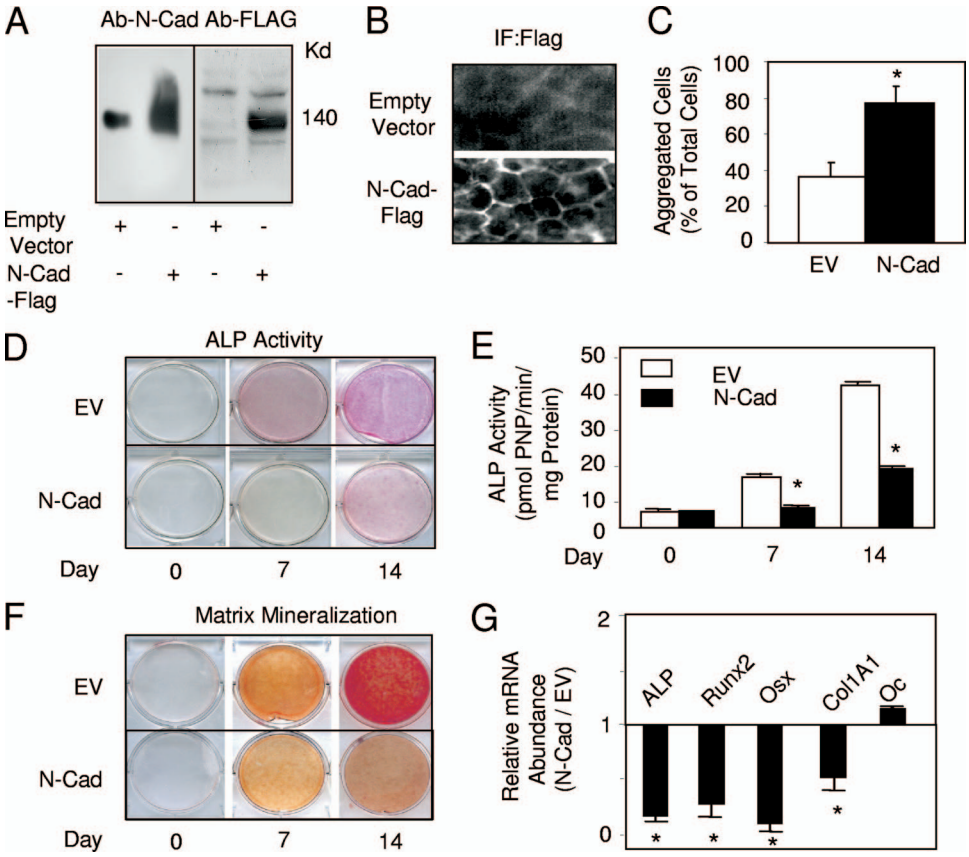

FIG. 1. Enforced expression of N-cadherin downregulates osteoblast differentiation. (A) Basal expression of N-cadherin and N-cadherin transgene expression were determined by Western blotting in stably expressed in MC3T3-E1 osteoblasts. (B) Immunofluorescence analysis of the N-cadherin transgene in transfected MC3T3-E1 osteoblasts as revealed by a Flag antibody. (C) Increased functional activity of N-cadherin in MC3T3-E1 osteoblasts stably transfected with N-cadherin or the empty vector as determined by a cell aggregation assay. (D and E) Decreased ALP staining (D) and activity (E) in MC3T3-E1 osteoblasts stably transfected with N-cadherin compared to results for control cells at 48 h. (F) Decreased extracellular matrix mineralization by MC3T3-E1 osteoblasts stably transfected with N-cadherin compared to results for control cells. (G) Decreased expression of ALP, Runx2, Osx, and Col1A1, but not osteocalcin, in MC3T3-E1 osteoblasts overexpressing N-cadherin compared to results for control cells at 24 h. Means ± SD are shown. Values that are significantly different are indicated (*, $P < 0.05$ versus empty vector cells). N-Cad, N-cadherin; WB, Western blotting; Kd, kilodaltons; IF, immunofluorescence; EV, empty vector control; Oc, osteocalcin.

(Les Ulis, France). Osteoclast surface and number were determined on TRAP-stained sections using a Leitz integrateplatte II eyepiece at ×128 magnification. The mineral apposition rate (MAR) was measured using an image analyzer (Biocom) on calcein/tetracycline double-labeled surfaces. The mineralizing surfaces were measured in the same area using the objective eyepiece Leitz integrateplatte II. The bone formation rate (BFR) was calculated according to the published nomenclature (44).

Statistical analysis. Data were expressed as means ± standard deviations (SD) and were analyzed using the statistical package Super-ANOVA (for the Macintosh; Abacus Concepts, Inc., Berkeley, CA). Differences between the mean values were evaluated with a minimal significance of P values of <0.05.

RESULTS

Osteoblasts are bone-forming cells that express large amounts of N-cadherin, which plays a role in cell adherence and differentiation (40, 42). Western blot analyses showed that MC3T3-E1 osteoblasts express N-cadherin in basal conditions (Fig. 1A). To investigate the functional role of N-cadherin in osteoblasts and to determine its role in intracellular signaling, we stably transfected murine MC3T3-E1 osteoblasts with a Flag-N-cadherin expression vector. Stable overexpression of N-cadherin increased N-cadherin expression, as revealed by Western blot (Fig. 1A) and immunofluorescence (Fig. 1B) analyses. N-cadherin overexpression resulted in a twofold increase in cell aggregation, indicating that exogenous N-cadherin was functional (Fig. 1C). We found that stable overexpression of functional N-cadherin altered osteoblastogenesis in basal conditions. This was documented by decreased ALP activity, an early marker of osteoblast differentiation, in N-cadherin-expressing cells compared to levels in control cells (Fig. 1D and E). Defective osteoblastogenesis was supported by the delayed matrix mineralization in N-cadherin-overexpressing osteoblasts compared to that in control cells (Fig. 1F). Further analysis showed that mRNA expression levels of osteoblast phenotypic markers, such as ALP, Runx2, Osterix (Osx), and type I collagen (Col1A1), were markedly downregulated in osteoblasts overexpressing N-cadherin, whereas osteocalcin, a late osteoblast marker, was not significantly changed (Fig. 1G). These results indicate that mild N-cadherin overexpression negatively regulates osteoblast differentiation and function in basal culture conditions.

The cadherin-catenin complex is known to modulate Wnt signaling (35, 54). To investigate whether N-cadherin over-

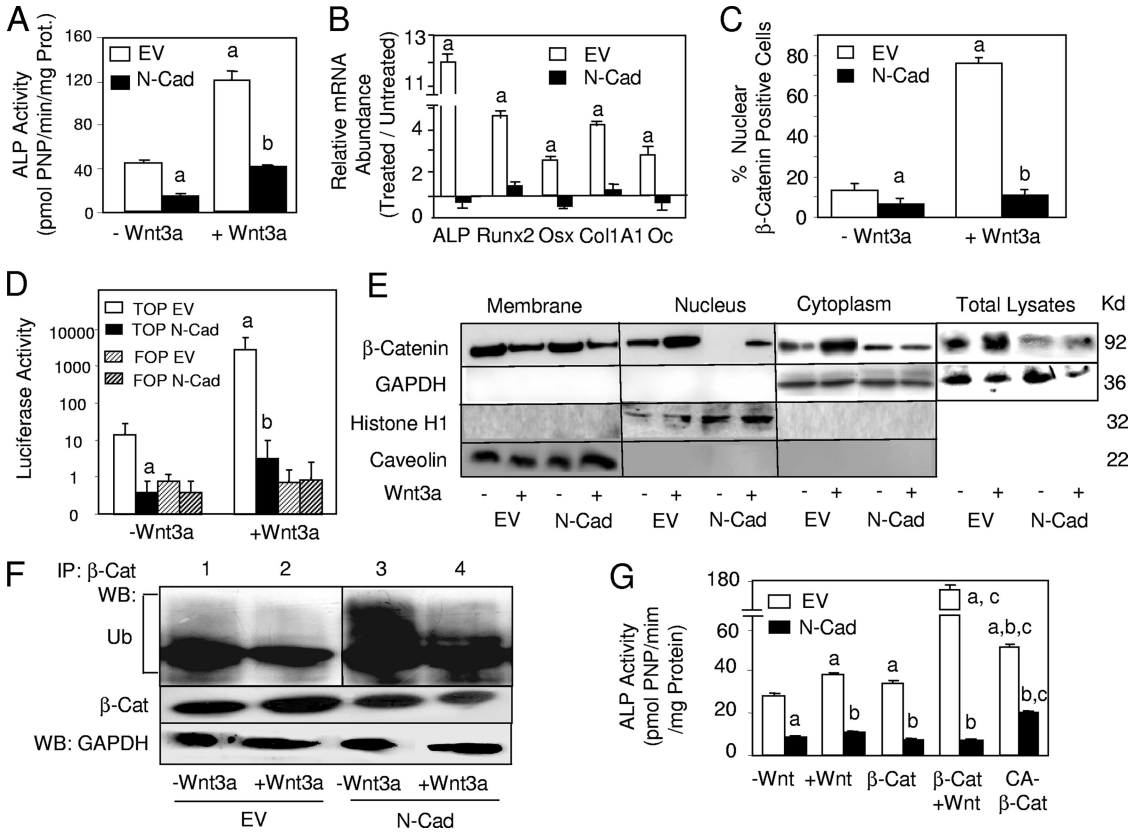

FIG. 2. Enforced expression of N-cadherin decreases Wnt/β-catenin signaling through increased β-catenin ubiquitination. (A and B) Altered ALP activity (A) and expression of phenotypic osteoblast markers (B) in response to Wnt3a in MC3T3-E1 osteoblasts stably transfected with N-cadherin compared to control cells transfected with the empty vector. Cells were treated with Wnt3a-CM (+Wnt3a; 15%) or control CM (−Wnt3a) and collected for ALP activity (48 h) or osteoblast marker expression (24 h) determined by quantitative PCR analysis. Means ± SD are shown. a, $P < 0.05$ versus empty vector untreated cells; b, $P < 0.05$ versus untreated N-cadherin cells. (C) Enforced expression of N-cadherin reduces β-catenin translocation to the nucleus. N-cadherin-transfected MC3T3-E1 cells or control cells were treated with 15% Wnt3a-CM (+Wnt3a) or control CM (−Wnt3a) for 24 h and immunostained for β-catenin and DAPI, and the number of positive nuclear β-catenin was recorded and expressed as a percentage of total cells. a, $P < 0.05$ versus EV untreated cells; b, $P < 0.05$ versus untreated N-Cad cells. (D) Enforced expression of N-cadherin in MC3T3-E1 reduces TCF/LEF transcriptional activity in basal conditions and in response to 15% Wnt3a-CM (+Wnt3a). FOP vectors were used as negative controls. a, $P < 0.05$ versus empty vector untreated cells; b, $P < 0.05$ versus untreated N-cadherin cells. (E) Enforced expression of N-cadherin MC3T3-E1 reduces total and nuclear β-catenin levels in response to 15% Wnt3a-CM (Wnt3a). N-cadherin-transfected cells or control cells were treated with 15% Wnt3a-CM (Wnt3a) or the normal CM for 24 h, and total cell lysates and membrane or nuclear fractions were analyzed by Western blotting using anti-β-catenin. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), histone H1, and caveolin were used as quality controls for cytoplasmic/total, nuclear, and membrane fractions, respectively. (F) Enforced expression of N-cadherin increases β-catenin ubiquitination for MC3T3-E1. N-cadherin-transfected cells or control cells were treated with 15% Wnt3a-CM (+Wnt3a) or the control CM (−Wnt3a) for 24 h, cells lysates were immunoprecipitated with anti-β-catenin, and immunoprecipitates were analyzed using ubiquitin or β-catenin antibodies. GAPDH immunoblotting was used as a loading control. (G) Lack of response to forced expression of β-catenin in N-cadherin-overexpressing MC3T3-E1 cells. N-cadherin-overexpressing or control cells were transiently transfected with β-catenin or constitutively active β-catenin vectors and treated with 15% Wnt3a-CM (+Wnt) or the control CM (−Wnt) for 48 h, and ALP activity was determined. a, $P < 0.05$ versus untreated EV cells; b, $P < 0.05$ versus corresponding EV cells; c, $P < 0.05$ versus β-catenin EV cells. N-Cad, N-cadherin; EV, empty vector; Prot., protein; WB, Western blotting; Kd, kilodaltons; IP, immunoprecipitation; Ub, ubiquitin; β-Cat, β-catenin; CA, constitutively active.

expression may negatively impact Wnt/β-catenin signaling in osteoblasts, we examined the response to Wnt3a in N-cadherin-overexpressing osteoblasts. Wnt3a increased ALP activity threefold in control cells and only twofold in N-cadherin-overexpressing cells (Fig. 2A). Moreover, Wnt3a markedly increased the expression of osteoblast marker genes in control cells but had no significant effect in N-cadherin-overexpressing cells (Fig. 2B). These results indicate that forced expression of N-cadherin decreases the response to Wnt signaling in osteoblasts. We then investigated the mechanism by which N-cadherin may antagonize Wnt signaling in osteoblasts. Consistent with the current model of the canonical Wnt signaling pathway (18), we found that Wnt3a increased β-catenin translocation to the nucleus in control cells, as revealed by the marked increase in the percentage of total cells with positive nuclear β-catenin staining (Fig. 2C). Consistently, Wnt3a increased TCF/LEF transcriptional activity in control cells (Fig. 2D). In contrast, Wnt3a induced little β-catenin nuclear translocation (Fig. 2C) and low TCF/LEF transcriptional activity in N-cadherin-overexpressing cells (Fig. 2D), indicating that the canonical Wnt signaling pathway is altered by N-cadherin overexpression. One reported mechanism by which cadherins may interact with

Wnt signaling is by sequestering β-catenin at the plasma membrane (35, 54). We analyzed whether N-cadherin overexpression may act by promoting β-catenin sequestration at the plasma membrane. Cell membrane-associated β-catenin levels did not differ in control and N-cadherin-overexpressing cells (Fig. 2E). Additionally, Wnt3a decreased cell membrane-associated β-catenin in both cell types, indicating that β-catenin can be released from the membrane under Wnt3a stimulation (Fig. 2E). We found that Wnt3a increased total β-catenin levels in control cells but not in N-cadherin-overexpressing osteoblasts (Fig. 2E). We also found lower nuclear β-catenin levels in basal condition and in response to Wnt3a in N-cadherin-overexpressing osteoblasts than in control cells (Fig. 2E). These results suggest that the defective Wnt/β-catenin signaling induced by N-cadherin overexpression results in part from altered β-catenin accumulation in the cytoplasm. We therefore raised the possibility that N-cadherin overexpression may induce a molecular mechanism controlling β-catenin stability. The level of β-catenin is tightly controlled by its degradation, which is dependent on GSK3β activity (37). Upon Wnt stimulation, GSK3β is normally released from its complex with APC and axin, which leads to decreased phosphorylation of β-catenin and the subsequent inhibition of ubiquitin-mediated proteasomal degradation. Consistent with this mechanism, we found that Wnt3a decreased β-catenin ubiquitinylation in control cells, resulting in increased β-catenin level (Fig. 2F, lane 2 versus lane 1). In contrast, β-catenin ubiquitinylation was higher than normal in N-cadherin-overexpressing cells (Fig. 2F, lane 3 versus lane 1). Moreover, the protective effect of Wnt3a on β-catenin ubiquitinylation was limited in N-cadherin-overexpressing cells (Fig. 2F, lane 4 versus lane 3), reflecting the increased β-catenin ubiquitinylation in these cells. These data suggest that the decreased response to Wnt3a in N-cadherin-overexpressing cells is related to increased ubiquitin-mediated proteasomal degradation of β-catenin, resulting in decreased nuclear β-catenin translocation and subsequent alteration of TCF/LEF-dependent osteoblast gene transcription. To further investigate the functional role of the increased β-catenin ubiquitinylation induced by N-cadherin overexpression, we examined the changes in ALP activity in response to various modulators of Wnt signaling. As expected from our previous results, treatment with Wnt3a increased ALP activity in control cells but had little effect in N-cadherin-overexpressing cells (Fig. 2G). Forced expression of β-catenin did not restore the response to Wnt3a in N-cadherin-overexpressing cells. Moreover, forced expression of β-catenin and Wnt3a treatment markedly increased ALP activity in control cells, but not in N-cadherin-overexpressing cells. In contrast, forced expression of a constitutively active β-catenin, which cannot be phosphorylated and degraded, did increase ALP activity in control and N-cadherin cells (Fig. 2G). These results further indicate that the increased β-catenin degradation in N-cadherin-overexpressing osteoblasts is implicated in the defective functional response to Wnt3a.

We then determined the molecular mechanism by which forced expression of N-cadherin may result in increased β-catenin ubiquitinylation and defective osteoblast differentiation. Because LRP5 is an essential transmembrane Wnt coreceptor controlling osteoblast differentiation (1, 7, 16, 28, 36), we postulated that N-cadherin, another transmembrane molecule, may interact with LRP5 to regulate β-catenin ubiquitinylation. To test this hypothesis, we examined whether exogenous LRP5 may rescue the defective osteoblast differentiation induced by N-cadherin overexpression. As shown in Fig. 3A, forced expression of LRP5 increased ALP activity in N-cadherin-overexpressing cells. The addition of Wnt3a to LRP5-overexpressing cells further increased ALP activity in N-cadherin cells, although the response remained lower than in the corresponding control cells (Fig. 3A). The finding that the defective response to Wnt3a induced by N-cadherin overexpression can be partially rescued by increasing LRP5 expression suggests that N-cadherin may interact with LRP5 to functionally control osteoblast differentiation. To test whether such interaction may control Wnt signaling in normal osteoblasts, we used a N-cadherin knockdown approach using siRNA. A Western blot analysis showed that silencing N-cadherin using siRNA efficiently decreased N-cadherin protein levels in control cells (data not shown). Silencing endogenous N-cadherin using siRNA increased TCF/TOP activity and the response to Wnt3a in control cells (Fig. 3B). This result indicates that endogenous N-cadherin downregulates Wnt signaling in normal osteoblastic cells. To further analyze N-cadherin-LRP5 interactions, we performed immunofluorescence and immunoprecipitation analyses of LRP5 and N-cadherin. Figure 3C is a representative image of 70 cells examined in 20 fields. The immunofluorescence study of LRP5 and N-cadherin shows that a subset of N-cadherin colocalized with LRP5 at the membrane level in N-cadherin-overexpressing cells. We also found that N-cadherin coimmunoprecipitates with LRP5 in control cells, suggesting that the two molecules are present in common molecular complexes (Fig. 3D). Additionally, the molecular association between N-cadherin and LRP5 was increased in N-cadherin-overexpressing cells, supporting a physical interaction between the two molecules (Fig. 3D). In contrast, we found no detectable interaction between N-cadherin and the coreceptor Frizzled, another essential Wnt component (data not shown). We also found that LRP6, a close homolog of LRP5, coimmunoprecipitates with N-cadherin in MC3T3-E1 osteoblasts (Fig. 3E). Furthermore, forced expression of LRP6 increased ALP activity in N-cadherin-overexpressing cells, and the addition of Wnt3a to LRP6 overexpression further increased ALP activity in N-cadherin-overexpressing cells (Fig. 3F). These results indicate that N-cadherin can interact directly or indirectly with LRP5 or LRP6 and that this interaction functionally interacts with Wnt signaling in osteoblasts. To determine whether the LRP5/LRP6 interaction with N-cadherin is specific, we performed immunoprecipitation experiments with LDLr, another LRP family member. As shown in Fig. 3G, LDLr was detected in osteoblasts (total lysates). However, LDLr was not found to coimmunoprecipitate with N-cadherin in normal cells or N-cadherin-overexpressing cells (Fig. 3G), suggesting that, in this model, N-cadherin interaction with LRP5 or LRP6 is specific.

To determine how N-cadherin can interact with LRP5, we investigated N-cadherin binding to LRP5 intracellular domains that are known to interact with other downstream effector proteins (31, 34) by using a number of LRP5 mutants (Fig. 4A). Immunoprecipitation analyses showed that deletion of the extracellular domain in LRP5 (LRP5C) did not alter LRP5 association with N-cadherin (Fig. 4B). In contrast, deletion of

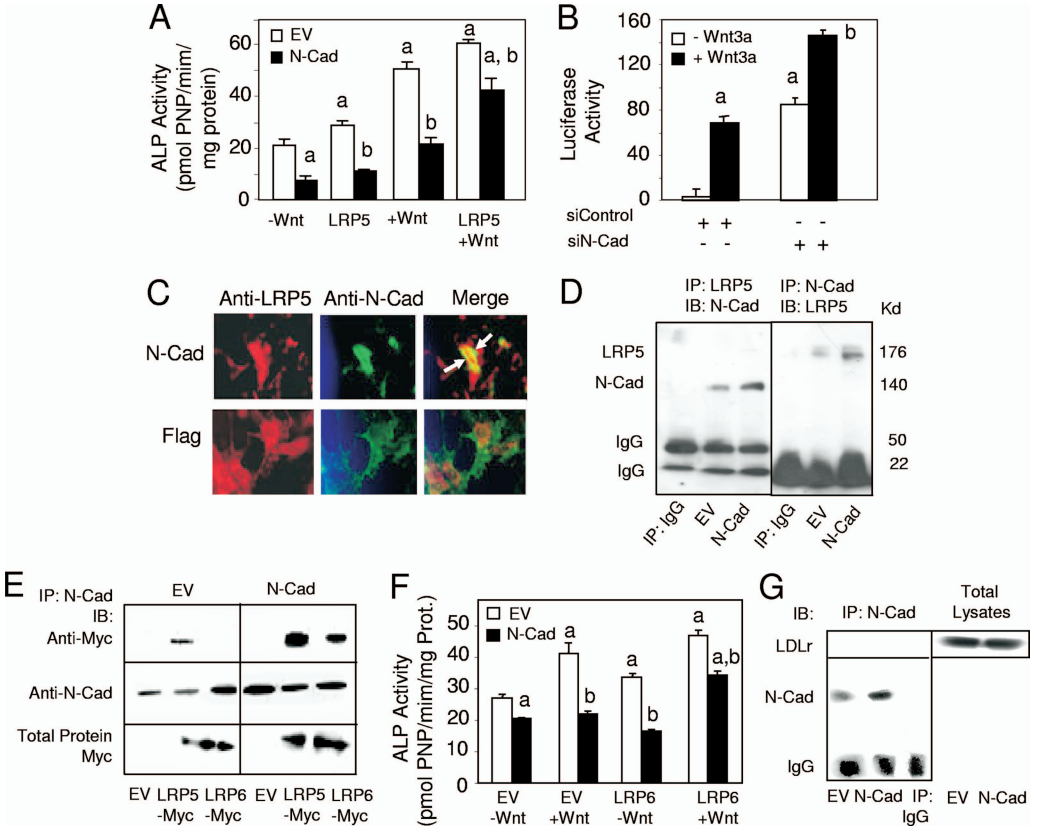

FIG. 3. N-cadherin interacts with LRP5 in osteoblasts. (A) Forced expression of LRP5 partly rescues the defective osteoblast differentiation induced by N-cadherin overexpression. MC3T3-E1 cells overexpressing N-cadherin or control cells were transiently transfected with LRP5 expression vector and treated with 15% Wnt3a-CM (+Wnt3a) or the control CM (−Wnt3a) for 48 h, and ALP activity was determined. Means ± SD are shown. a, $P < 0.05$ versus untreated EV cells; b, $P < 0.05$ versus corresponding EV cells. (B) Knocking down N-cadherin using siRNA increases TCF/LEF transcriptional activity, an effect that is enhanced by Wnt3a-CM (+Wnt3a). a, $P < 0.05$ versus untreated controls (−Wnt3a); b, $P < 0.05$ versus control siRNA-treated cells. (C) LRP5 and N-cadherin localization in N-cadherin-overexpressing cells and control (Flag) cells was determined using anti-LRP5 conjugated to Cy3 (red) and anti-N-cadherin conjugated to Cy2 (green). Arrows indicate colocalization (yellow) of a subset pool of N-cadherin with LRP5. (D) N-cadherin interacts with LRP5. Cell lysates from control or N-cadherin-overexpressing cells were immunoprecipitated with anti-N-cadherin or LRP5 antibodies, and immunoprecipitates were analyzed by immunoblotting with LRP5 or N-cadherin antibodies. Immunoglobulin G immunoprecipitates were used as negative controls. (E) N-cadherin interacts with LRP6. N-cadherin-overexpressing MC3T3-E1 cells were transfected with LRP5-Myc, LRP6-Myc, or the empty vector. Immunoprecipitation analysis was performed using anti-N-cadherin antibody, and immunoprecipitates were analyzed using Myc or N-cadherin antibodies. Western blot analysis on total proteins was performed using the Myc antibody for an analysis of LRP5/6 overexpression. (F) N-cadherin-overexpressing MC3T3-E1 cells were transfected with LRP6 or the empty vector, and ALP activity was determined at 48 h. a, $P < 0.05$ versus untreated empty vector cells; b, $P < 0.05$ versus corresponding empty vector cells). (G) N-cadherin does not coimmunoprecipitate with LDLr. Cell lysates from control or N-cadherin-overexpressing cells were immunoprecipitated with N-cadherin antibody, and immunoprecipitates were analyzed by immunoblotting with LDLr or N-cadherin antibodies. Western blot analysis of LDLr in total lysates was used as positive control of LDLr expression. Immunoglobulin G immunoprecipitates were used as negative controls. N-Cad, N-cadherin; EV, empty vector control; IP, immunoprecipitation; IB, immunoblotting; IgG, immunoglobulin G; Prot., protein.

the amino acids up to position 28 at the cytoplasmic tail domain of LRP5 (LRP5Δ28) abolished LRP5-N-cadherin binding (Fig. 4B). This result indicates that N-cadherin binding is restricted to the LRP5 cytoplasmic tail domain (last 1 to 28 amino acids). To confirm these results, a cell fractionation Western blot analysis was performed with control and N-cadherin cells transfected with LRP5C or LRP5Δ28. As shown in Fig. 4C, LRP5C, but not LRP5Δ28, was found in the membrane fraction, further suggesting that LRP5 binding to N-cadherin requires the last 28 amino acids in the LRP5 cytoplasmic tail domain.

The scaffolding protein axin is a component of the axin/GSK3/APC/Frat1 complex. Binding of axin to LRP5 is an important part of the Wnt signal transduction pathway (34). Moreover, Wnt signaling induces LRP5-axin interaction, and LRP5 transduces Wnt signals in part by binding and recruiting axin to the membrane (17, 38, 51, 55). The cytoplasmic tail of LRP5 contains four axin-like repeats which contribute to the recruitment of axin (38). We hypothesized that the cytoplasmic tail of LRP5 that contains two axin sites may link N-cadherin and LRP5 intracellular domains. To test this hypothesis, we investigated possible N-cadherin-axin-LRP5 interactions. Immunoprecipitation analysis confirmed that LRP5 was associated with N-cadherin in the absence or presence of Wnt3a (Fig. 4D). Under these conditions, axin was found associated with LRP5 in control cells, and the axin-LRP5 association was

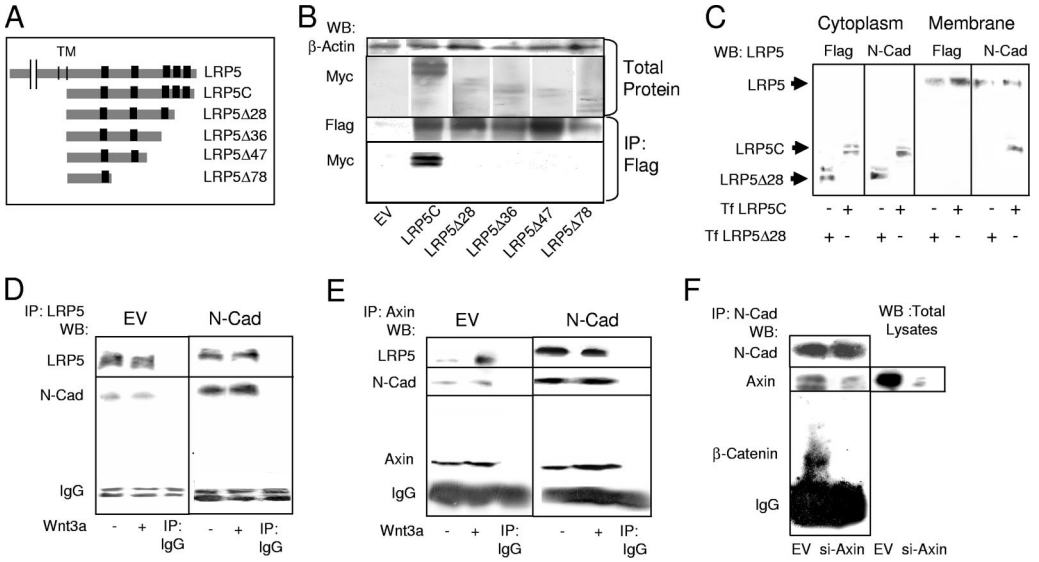

FIG. 4. N-cadherin-LRP5-axin interactions in osteoblasts. (A) Schematic representation of the LRP5 constructs used. Black bars indicate the positions of axin-binding sites in the cytoplasmic tail. (B) N-cadherin interacts with LRP5 at the cytoplasmic tail domain (last 1 to 28 amino acids). N-cadherin-overexpressing MC3T3-E1 cells were transfected with cytoplasmic LRP5 (LRP5C) or the truncated LRP5 tail Myc-Tag constructs with 28, 36, 47, or 78 C-terminal residues deleted (LRP5Δ28, LRP5D36, LRP5D47, or LRP5D78, respectively) or the empty vector. Immunoprecipitation analysis was performed using anti-Flag antibody. Immunoprecipitates were then analyzed using Flag or Myc antibodies. β-Actin antibody was used as the loading control. (C) Control and N-cadherin MC3T3-E1 cells were transfected with LRP5C or LRP5Δ28 vectors, and the localization of N-cadherin-Flag and LRP5 mutants in the cytoplasm and membrane was visualized by Western blot analysis. (D) LRP5 is associated with N-cadherin in the absence or presence of Wnt3a, and (E) Axin interacts with LRP5 and N-cadherin. Cell lysates from control or N-cadherin-overexpressing cells treated or not treated with Wnt3a-CM (15%) were immunoprecipitated with LRP5 (D) or axin antibodies (E), and immunoprecipitates were analyzed by immunoblotting with LRP5, N-cadherin, or axin antibodies. (F) Knocking down axin with siRNA decreases β-catenin interaction with N-cadherin. Cell lysates from control cells treated with axin siRNA were immunoprecipitated with anti-N-cadherin, and immunoprecipitates were analyzed by immunoblotting with N-cadherin, axin, or β-catenin antibodies. TM, transmembrane domain; N-Cad, N-cadherin; EV, empty vector control; IP, immunoprecipitation; IB, immunoblotting.

increased by Wnt3a (Fig. 4E). Moreover, we found that axin associated with LRP5 was markedly increased in N-cadherin-overexpressing cells. Additionally, we found that axin coimmunoprecipitated with N-cadherin (Fig. 4E). These data indicate that a multiprotein complex can form between N-cadherin and LRP5 and between N-cadherin and axin. In order to better determine the relative contribution of N-cadherin-β-catenin interaction versus N-cadherin-axin-β-catenin interaction, we used an effective axin siRNA. We found that knocking down axin with siRNA led to decreased β-catenin interaction with N-cadherin in control cells, as revealed by immunoprecipitation analysis (Fig. 4F). These results suggest that axin is essential for N-cadherin interaction with β-catenin in osteoblasts in basal conditions. We therefore propose that the functional interaction between N-cadherin and LRP5 in osteoblasts may be mediated at least in part by β-catenin-axin, and perhaps other proteins, linked in a multiprotein complex.

To explore the hypothesis that the negative impact of N-cadherin-LRP5 interaction on β-catenin degradation and osteoblast differentiation may be relevant to the control of bone formation and bone mass acquisition in vivo, we generated N-cadherin transgenic mice using a Col12.3-N-cadherin-construct (47) (Fig. 5A). Among the three lines generated, one was infertile and the two others showed the same phenotype; we show here one of the two phenotypes. Northern blot analyses in transgenic mice showed that the transgene was specifically expressed in bone (Fig. 5B). N-cadherin overexpression had no detectable effect on skeletal development (Fig. 5C). In

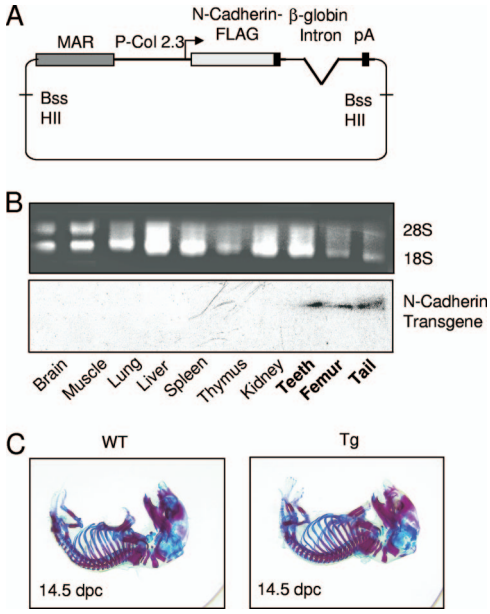

FIG. 5. Generation of N-cadherin transgenic mice. (A) Structure of the N-cadherin construct used to generate transgenic animals. (B) Northern blot analysis showing the specific expression of the N-cadherin transgene in mineralized tissues obtained from transgenic mice. 18S and 28S were used as loading controls. (C) Lack of effect of the N-cadherin transgene on skeletal development in transgenic compared to wild-type mice. Tg, transgenic; WT, wild type; dpc, days postcoitum.

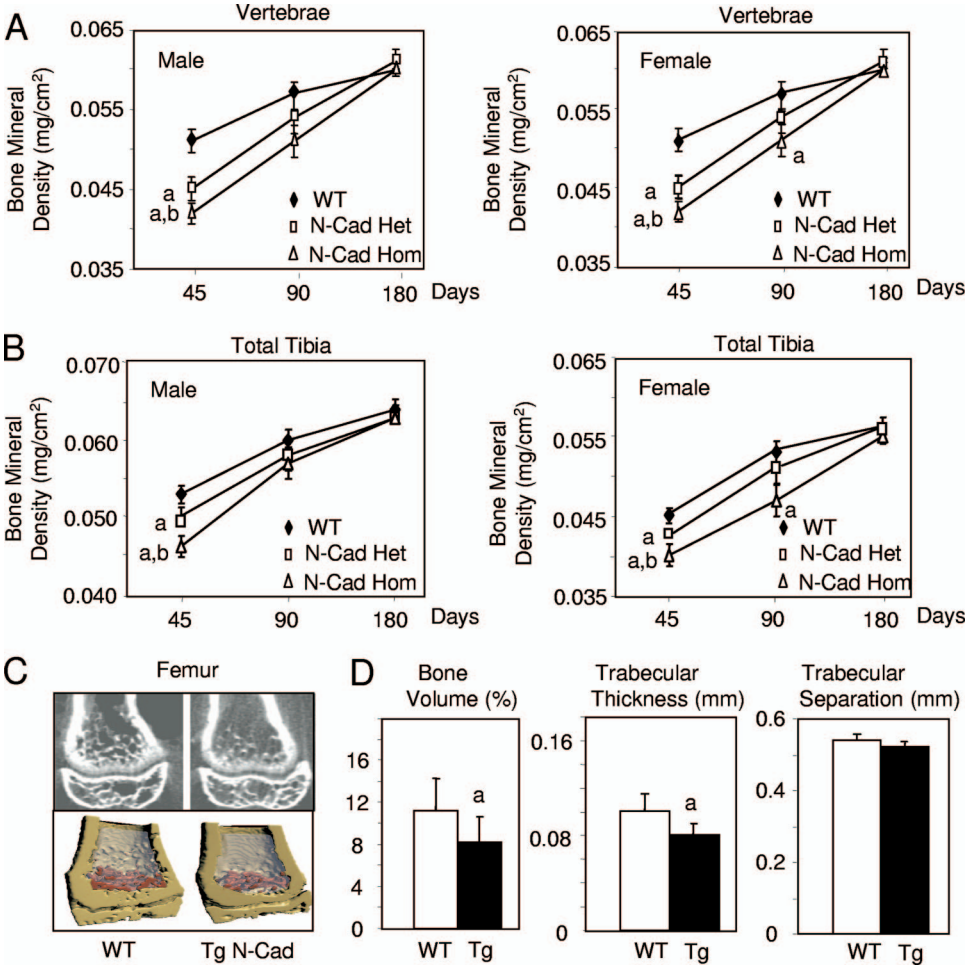

FIG. 6. Decreased postnatal bone mass acquisition in N-cadherin transgenic mice. (A and B) Decreased bone mineral density in N-cadherin heterozygotic and homozygotic transgenic mice compared to wild-type male and female mice in vertebrae (A) and tibias (B). (C) Representative μCT scan pictures showing reduced trabecular bone mass in the distal femur of 45-day-old heterozygotic N-cadherin transgenic compared to wild-type female mice. (D) μCT quantitative determination showing decreased femur bone volume and trabecular thickness and normal trabecular separation in 45-day-old female heterozygotic N-cadherin transgenic mice compared to wild-type age-matched female mice. Means ± SD from 6 to 10 animals/group are shown. a, $P < 0.05$ versus the wild type; b, $P < 0.05$ versus N-cadherin heterozygotic transgenic mice. N-Cad, N-cadherin; Het, heterozygotic; Hom, homozygotic; WT, wild type; Tg, transgenic.

contrast, we showed that N-cadherin overexpression induced a marked phenotype characterized by delayed bone mass acquisition during postnatal life (Fig. 6). The skeletal phenotype was more severe in homozygotic mice and more prominent in female than in male mice. Bone mineral density at 45 days of age was lower in heterozygotic transgenic male and female mice in the axial skeleton (vertebrae) and long bones (tibia) than in wild-type mice (Fig. 6A and B). Microcomputed tomography (μCT) analysis confirmed the decreased trabecular bone mass in N-cadherin transgenic mice at 45 days of age, whereas cortical bone was not affected (Fig. 6C). Quantitative analyses showed that the decreased trabecular bone volume in transgenic mice was associated with decreased trabecular thickness and normal trabecular separation, suggesting an alteration of bone formation (Fig. 6D). To test this hypothesis, we measured the osteoid surface, osteoblast surface, and mineralizing surface by histomorphometric analysis. We found that these parameters of bone formation were decreased at the vertebral level in 45-day-old female or male transgenic mice compared to those of wild-type mice (Fig. 7A). Furthermore, the MAR and BFR were decreased at the tissue level in transgenic mice (Fig. 7B and C). In contrast, the osteoclast surface in males (wild type versus transgenic, 12.0% ± 1.9% versus 9.9% ± 1.9%; not significant) and females (wild type versus transgenic, 11.8% ± 2.4% versus 10.1% ± 1.8%; not significant), and the osteoclast numbers (Fig. 7D and E) did not differ in transgenic and wild-type mice, further suggesting that bone resorption was not affected. These data indicate that targeted N-cadherin overexpression in osteoblasts in mice decreases trabecular bone formation as a result of decreased bone-forming surfaces and reduced osteoblast function.

To investigate whether N-cadherin overexpression reduces osteoblast activity in a cell-autonomous manner, functional analyses were performed in calvarial osteoblasts isolated from N-cadherin transgenic and wild-type neonatal mice. We found that ALP staining and activity were lower in primary osteo-

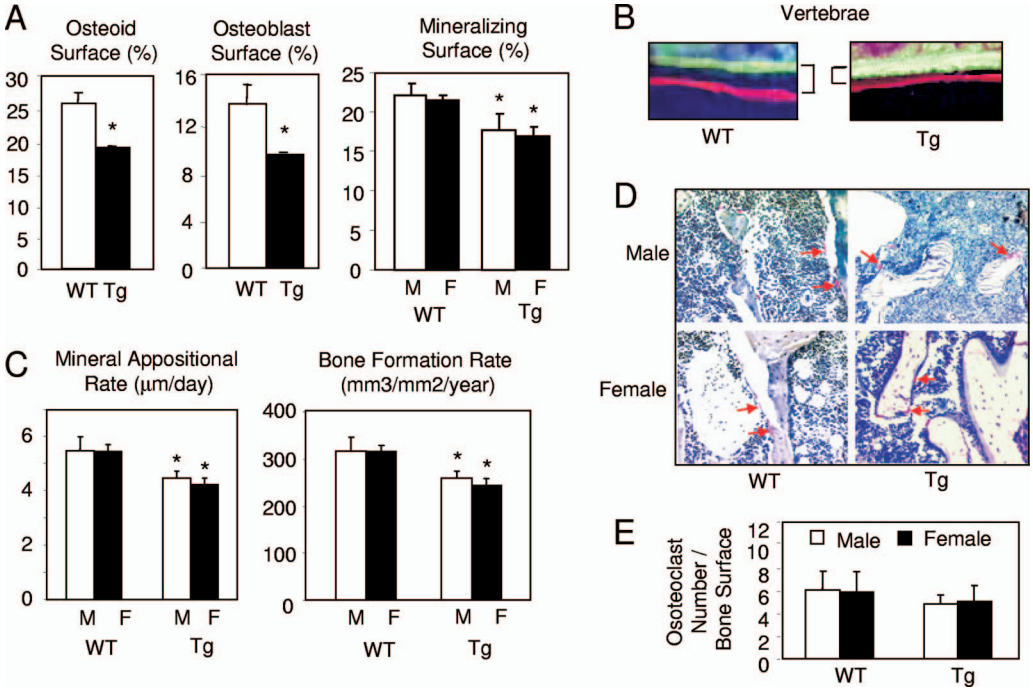

FIG. 7. Decreased postnatal bone formation in N-cadherin transgenic mice. (A) Decreased osteoid surface, osteoblast surface, and double-labeled (tetracycline/calcein) mineralizing surface in vertebral bone in male or female 45-day-old heterozygotic N-cadherin transgenic mice compared to wild-type age-matched mice. (B) Double calcein tetracycline labeling showing a decreased mineral appositional rate in 45-day-old heterozygotic N-cadherin transgenic mice compared to that for wild-type mice. (C) Decreased bone mineral appositional rate and BFR in transgenic mice compared to those for wild-type mice. (D) N-cadherin overexpression does not alter bone resorption. Histological section showing TRAP-stained osteoclasts (red, arrows) in vertebral trabecular bone from transgenic compared to wild-type mice. (E) Quantitative analysis showing that the number of TRAP-positive osteoclasts in vertebral trabecular bone does not differ in transgenic and wild-type mice. Means ± SD from 6 to 10 animals/group are shown. *, $P < 0.05$ versus the wild type. N-Cad, N-cadherin; M, male; F, female; Tg, transgenic; WT, wild type.

blasts expressing the N-cadherin transgene than in wild-type osteoblasts (Fig. 8A and B). Moreover, the basal expression of osteoblast genes, namely the Runx2, Osx, ALP, Col1A1, and osteocalcin genes, was markedly decreased in N-cadherin transgenic osteoblasts compared to wild-type osteoblasts (Fig. 8C). This indicates that N-cadherin overexpression in osteoblasts causes a cell-autonomous decrease in osteoblast differentiation. To determine the mechanism underlying the alteration in bone formation and bone mass, we performed immunoprecipitation analyses of calvarial osteoblasts isolated from N-cadherin and wild-type neonatal mice. We found that N-cadherin coimmunoprecipitates with LRP5 and axin in wild-type osteoblasts and that this association is increased in N-cadherin transgenic osteoblasts (Fig. 8D). We then determined whether this interaction had a functional implication on β-catenin degradation. We found that β-catenin levels were decreased in N-cadherin transgenic osteoblasts compared to levels in wild-type cells (Fig. 8E). Moreover, we found increased β-catenin levels associated with ubiquitin in transgenic cells (Fig. 8F), supporting a mechanism involving increased ubiquitin-mediated β-catenin degradation. Overall, these in vitro and in vivo data indicate that N-cadherin, in addition to binding β-catenin at the cell membrane, can associate with LRP5 and axin, possibly via other unknown proteins, and that the increased N-cadherin-axin-LRP5 complex results in increased β-catenin ubiquitination, resulting in reductions in osteoblast differentiation, bone formation, and bone mass acquisition (Fig. 8G).

DISCUSSION

Wnt signaling plays a major role in the regulation of bone formation and bone mass accrual (15, 52). A number of extracellular and intracellular proteins were found to antagonize Wnt signaling by acting on Wnt proteins or Wnt signaling partners (29, 47). Here, we show another mechanism by which N-cadherin interacts with LRP5/6 to trigger β-catenin degradation and the subsequent downregulation of Wnt/β-catenin signaling in osteoblasts. This reveals a novel link between LRP5, a major coreceptor of Wnt proteins, and the cell-cell adhesion molecule N-cadherin. This interaction is functional, since silencing N-cadherin using siRNA increased canonical Wnt signaling, whereas increasing N-cadherin-LRP5 interaction resulted in a decreased response to Wnt3a and reduced osteoblast marker gene expression. Our finding that the altered expression of osteoblast markers can be partly rescued by exogenous LRP5 supports the idea that N-cadherin-LRP5 interaction has a negative impact on the osteoblast phenotype. We therefore propose that N-cadherin is a novel LRP5 antagonist that negatively regulates Wnt/β-catenin signaling in osteoblasts. Importantly, the negative impact of N-cadherin mediated by LRP5 on Wnt signaling reported here differs from

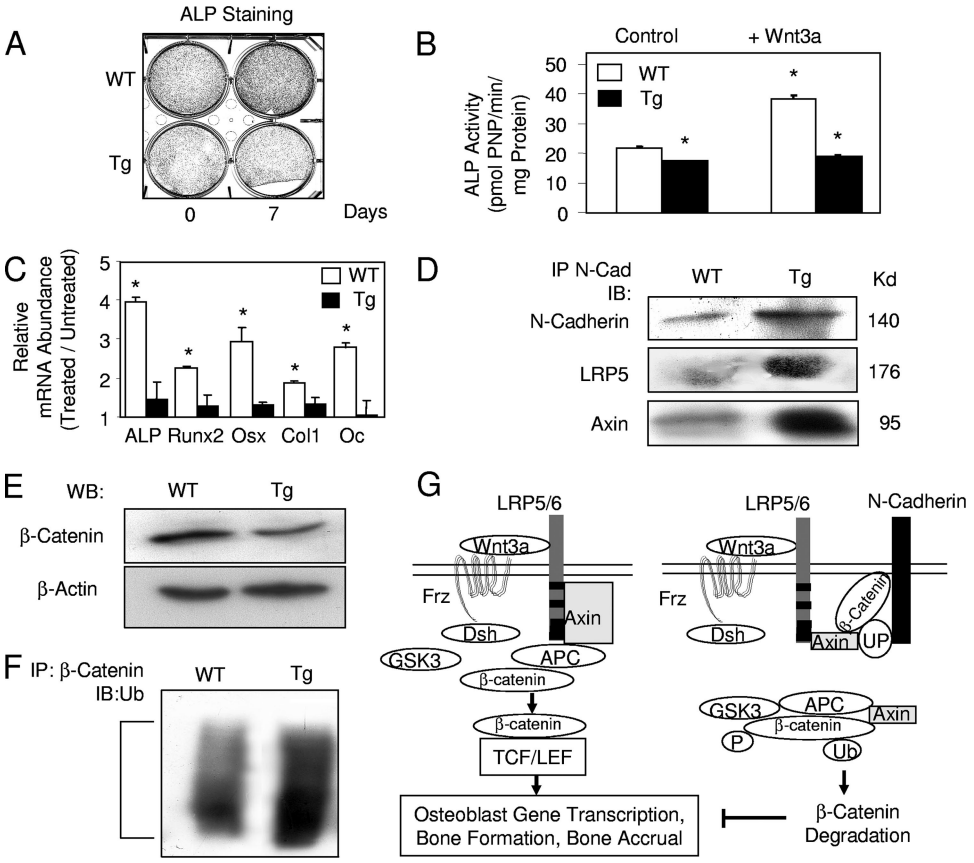

FIG. 8. Cell-autonomous defective response to Wnt signaling in N-cadherin transgenic osteoblasts. (A and B) Decreased ALP staining and activity in primary N-cadherin transgenic osteoblasts compared to wild-type osteoblasts in basal conditions and in response to Wnt3a-CM (+Wnt3a; 15%). (C) Altered expression of osteoblast marker genes in response to Wnt3a-CM (15%, 24 h) in N-cadherin transgenic osteoblasts compared to wild-type osteoblasts, as determined by quantitative PCR analysis. Means ± SD are shown. *, $P < 0.05$ versus wild-type cells. (D) Immunoprecipitation analysis showing increased axin and LRP5 coimmunoprecipitated with N-cadherin in transgenic osteoblasts compared to wild-type osteoblasts. (E) Western blot analysis showing decreased total β-catenin levels in N-cadherin transgenic osteoblasts compared to wild-type osteoblasts. (F) Immunoprecipitation analysis showing increased β-catenin ubiquitination in N-cadherin transgenic osteoblasts compared to wild-type osteoblasts. (G) Proposed model by which N-cadherin may negatively regulate Wnt/β-catenin signaling in osteoblasts. Wnt binding to LRP5 and Frizzled leads to the recruitment of the axin/APC/GSK3β/β-catenin complex to LRP5, resulting in the inhibition of GSK3β, β-catenin stabilization, TCF/LEF transactivation, and osteoblast gene expression. Our results suggest that N-cadherin, in addition to binding β-catenin at the cell membrane, interacts with LRP5 and axin, possibly via an unknown protein, in a multiprotein complex. This interaction results in increased β-catenin degradation associated with decreased TCF/LEF transcription and altered osteoblast gene expression, bone formation, and bone mass acquisition. Tg, transgenic; WT, wild type; IP, immunoprecipitation; Kd, kilodaltons; WB, Western blotting; Ub, ubiquitination; IB, immunoblotting; UP, unknown protein.

the reported action of the Wnt antagonists SFRPs, Cerberus, and WIF1, which antagonize Wnt signaling by binding with Wnt ligands (11, 22, 46). The mechanism is also distinct from the action of Dkk proteins that bind to the extracellular domain of LRP5/6 and the transmembrane protein Kremen2, which leads to degradation of the Dkk-Kremen-LRP5/6 complex (2). Other extracellular Wnt antagonists, such as connective tissue growth factor, Wise, and sclerostin, also bind to the extracellular domain of LRP5 (24, 43, 49). The present data therefore highlight a novel antagonistic mechanism by which N-cadherin increases β-catenin degradation through binding to the intracellular domain of LRP5. The finding that N-cadherin-LRP5 interaction negatively regulates Wnt signaling in part through increased β-catenin degradation, resulting in the alteration of expression of target genes, is highly relevant to the regulation of osteoblastogenesis, since both N-cadherin and LRP5 are strongly expressed in osteoblasts (15, 52, 40, 42) and Wnt signaling is a major modulator of osteoblast function (4, 14).

The N-cadherin-LRP5 interaction may negatively regulate Wnt/β-catenin signaling at different levels. One recognized effect is a direct interaction of N-cadherin with β-catenin (35, 54), resulting in β-catenin sequestration and modulation of the β-catenin cytosolic pool (44). Thus, sequestration is one mechanism that can be involved in the observed phenotype. However, we found that N-cadherin overexpression did not increase β-catenin membrane sequestration and did not prevent the release of β-catenin induced by Wnt3a stimulation, suggesting that β-catenin sequestration is not primarily responsible for the defective Wnt signaling in N-cadherin-overexpressing osteoblasts. Our data suggest that a mechanism involved in the decreased Wnt/β-signaling induced by N-cadherin-LRP5

interaction is increased β-catenin ubiquitination, resulting in decreased β-catenin cytosol levels and nuclear translocation in response to Wnt3a. Because we found increased GSK3β levels in N-cadherin-overexpressing cells (data not shown), we suggest that N-cadherin-LRP5 interaction may affect GSK3β stability and thereby β-catenin stabilization (37). Other mechanisms may be involved in the observed altered Wnt/β-catenin signaling in N-cadherin-overexpressing osteoblasts. For example, we found that N-cadherin overexpression was associated with decreased β-catenin mRNA transcripts (data not shown), suggesting that N-cadherin may modulate osteoblast gene expression by regulating β-catenin expression and availability. Furthermore, N-cadherin overexpression may alter other mechanisms, such as protein kinase C and bone morphogenetic protein signaling (33, 41), which may be dysregulated by N-cadherin overexpression.

Consistent with our finding that N-cadherin overexpression affects β-catenin degradation and osteoblast differentiation in vitro, we found that N-cadherin transgenic mice showed reduced bone formation. The inability of N-cadherin transgenic mice to reach a normal bone mass was due, at least in part, to the reduced expression of osteoblast activity, indicating that N-cadherin overexpression negatively controls bone mass accrual by suppressing bone formation. We found that the osteopenic phenotype was transient, which may be related to the age-related decreased N-cadherin and increased LRP5 mRNA levels in bone (data not shown), thus limiting the negative interaction of N-cadherin-LRP5 on bone accrual. Interestingly, both N-cadherin loss of function (9) and N-cadherin overexpression in mice (this study) resulted in osteopenia, although the mechanisms may be distinct. We found here that the alteration of bone formation induced by N-cadherin overexpression in vivo is a cell-autonomous phenotype and that N-cadherin interacts with LRP5 in osteoblasts to induce β-catenin degradation and defective Wnt/β-catenin signaling, which is consistent with our in vitro data. Overall, these data support a previously unrecognized functional mechanism by which N-cadherin-LRP5 interaction resulting in β-catenin ubiquitination critically regulates the Wnt receptor complex, osteoblast function, and bone mass.

A molecular mechanism that may explain how N-cadherin can functionally interact with LRP5 is the intracellular recruitment of axin, a key regulator of Wnt signaling. Axin binds APC in a complex with β-catenin and GSK3, leading to increased β-catenin phosphorylation and degradation as well as downregulation of Wnt signaling (21, 23, 25). Axin also interacts with Dishevelled and Frat1, which modulate β-catenin phosphorylation and degradation (34). Furthermore, axin interacts with the intracellular domain of LRP5, which is an important part of the Wnt signal transduction pathway (25, 38). Axin-LRP5 interaction induced by Wnt results in axin destabilization that contributes to β-catenin stabilization and LEF-1 transcription (38, 50). Recent data indicate that Wnt stimulation also triggers LRP6 protein aggregation on the membrane, allowing axin recruitment and β-catenin stabilization (5). Our data suggest that the interaction between LRP5 and N-cadherin is mediated by the formation of the axin-LRP5 complex, implicating axin-binding sites in the cytoplasmic tail (last 1 to 28 amino acids) of LRP5. We thus propose that part of the mechanism by which N-cadherin reduces Wnt/β-catenin signaling in osteoblasts involves the recruitment of axin, thus preventing destabilization of axin, which participates in β-catenin degradation. Since there is no reported axin-binding site on N-cadherin, the functional interaction between N-cadherin-axin and LRP5 in osteoblasts may be mediated in part by β-catenin-axin, and perhaps other proteins, linked in a multiprotein complex (Fig. 8G). This mechanism may not be restricted to LRP5, since we found that N-cadherin functionally interacts with LRP6 and axin in osteoblasts. Overall, our data indicate that a previously unrecognized interaction between N-cadherin, axin, and Wnt coreceptor LRP5/6 negatively regulates Wnt/β-catenin signaling and is critical in the regulation of osteoblast function, bone formation, and bone mass.

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