Targeted expression of a dominant-negative N-cadherin in vivo delays peak bone mass and increases adipogenesis

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Summary
We studied the function of osteoblast cadherins in vivo by transgenic expression of a truncated N-cadherin with dominant-negative action, driven by an osteoblast-specific promoter (OG2-NcadΔC). During the first 3 months of life, bone mineral density was reduced, whereas percent body fat was increased in transgenic animals compared with wild-type littersmates, with associated decreased bone formation rate and osteoblast number, but normal osteoclast number. Osteoblast differentiation was delayed in calvaria cells isolated from transgenic mice. Likewise, the number of osteoblast precursors in bone marrow stromal cells from OG2-NcadΔC mice was decreased compared with wild-type cultures, whereas the number of adipogenic precursors was increased. In vitro, a transcriptionally active β-catenin mutant reversed the delay in osteoblast differentiation and the exuberant adipogenesis. Thus, in vivo disruption of cadherin function hinders osteoblast differentiation and favors, indirectly, bone marrow progenitor cell commitment to the alternative adipogenic lineage via interference with β-catenin signaling. This results in decreased bone formation, delayed acquisition of peak bone mass and increased body fat.

Key words: Cell-cell adhesion, Mesenchymal differentiation, Cadherins, Adipogenesis, Transgenic mice

Introduction
Cadherins are a family of integral transmembrane proteins that mediate calcium-dependent cell-cell adhesion, a process necessary for cell aggregation and sorting during tissue morphogenesis, and for development and maintenance of cell polarity, cell migration, proliferation and survival (Gumbiner, 1996; Takeichi, 1995). In addition to their role as a structural component of adherens junctions, cadherins can also modulate intracellular signaling. The cytoplasmic domain of cadherins binds to β-catenin and plakoglobin, two vertebrate homologs of Drosophila armadillo, which link cadherins to the actin cytoskeleton via α-catenin (Nagafuchi, 2001). Through their association with β-catenin, cadherins can also interfere with the Wnt signaling system, of which β-catenin is an integral component. In this function, β-catenin participates in the formation of a transcriptionally active complex which also includes members of the T-cell factor/lymphoid enhancer factor (Tcf/Lef) family of DNA-binding proteins (Cadigan and Nusse, 1997; Peifer and Polakís, 2000). Cadherin modulation of β-catenin signaling is complex. On the one hand, β-catenin bound to cadherins may represent a readily available pool for nuclear translocation, thus favoring Wnt signaling. On the other hand, excess binding to cadherin on the cell surface may decrease β-catenin nuclear pools, thus antagonizing Tcf/Lef transactivation (Fagotto et al., 1996; Sadot et al., 1998).

Osteoblasts, bone forming cells, express multiple cadherins, primarily N-cadherin, cadherin-11 and cadherin-4/R-cadherin (Okazaki et al., 1994; Cheng et al., 1998; Ferrari et al., 2000). We and others have previously shown that upregulation of N-cadherin and cadherin-11 defines osteogenic cell differentiation from mesenchymal precursors (Shin et al., 2000; Kawaguchi et al., 2001b), and disruption of cadherin-mediated cell-cell adhesion hinders osteoblast differentiation and bone matrix production (Cheng et al., 1998; Cheng et al., 2000; Ferrari et al., 2000). Importantly, targeted disruption of the cadherin-11 gene results in mild skeletal defects with reduced bone density (Kawaguchi et al., 2001a). This mild skeletal phenotype and the overlapping distribution patterns of N-cadherin and cadherin-11 in the skeletal tissue suggest that N-cadherin may compensate for lack of cadherin-11, and raise the possibility that the two osteoblast cadherins may serve common functions in controlling bone formation (Civitelli et al., 2002). Unfortunately, homozygous loss of the N-cadherin gene results in early embryonic lethality, thus precluding the use of this model for any studies of adult bone (Radice et al., 1997).
We have generated a transgenic mouse line expressing a truncated N-cadherin with dominant-negative action in osteoblasts. This approach allows for non-isotype specific interference with cadherin function, thus overcoming a potential redundancy. The truncated cadherin we used lacks most of the extracellular domain but its cytoplasmic C terminal tail is intact, including the β-catenin binding site, and it has been proven to inhibit cadherin function in vivo (Kintner, 1992; Hermiston and Gordon, 1995) and in osteoblast cultures (Cheng et al., 2000; Ferrari et al., 2000). We found that targeted expression of this dominant-negative cadherin in osteoblasts in vivo reduced bone formation and osteoblast number, leading to a delay in achievement of peak bone mass, while increasing body adiposity. This phenotype is associated with altered lineage allocation of mesenchymal bone marrow precursors, resulting in reduced osteogenesis and increased adipogenesis – an abnormality that in vitro can be reversed by exogenous expression of transcriptionally active β-catenin.

Materials and Methods
Generation of transgenic mice
A 1.3 kb DNA fragment containing the OG2 promoter was excised from pH11.3-Luc, a kind gift of Dr Gerard Karsenty, Baylor College of Medicine, Houston, TX (Ducy and Karsenty, 1995), and ligated into pcDNA3 after digestion with NraI and HindIII (Invitrogen). This resulted in replacement of the CMV promoter with the OG2 promoter (OG2-pcDNA3). To clone NcadAC into this vector, two PCR primers were designed to amplify a 850 bp product from pSP72NcadAC, courtesy of Dr Chris Kintner, Salk Institute, San Diego, CA (Kintner, 1992). The PCR product contains the entire NcadAC reading frame, an EcoRI linker at the 5′ end and a FLAG epitope tag, followed by a stop codon and a HindIII site. The product was ligated in OG2-pcDNA3, yielding OG2-NcadAC (Fig. 1A). A 2.9 kb fragment containing the OG2 promoter, NcadAC, and BGH polyA was excised from OG2-NcadAC for microinjection into the male pronucleus of fertilized eggs (E0.5), harvested from superovulated, 4-week-old B6C3F1 females. The eggs were implanted into the reproductive tract of 3-5-month-old Swiss Webster pseudopregnant female mice. Twenty-five founder mice from three litters were screened for the expression of the transgene using both PCR primers designed to amplify a sequence encompassing the 3′ end of the OG2 promoter and the 5′ end of NcadAC; and a cDNA probe hybridizing to the transition region and a small sequence of NcadAC (Fig. 1A). Hybridization was performed on genomic DNA after digestion with PsI, and transgene-positive founders were identified by a 1.1 kb band in Southern analysis, or a 256 bp PCR product (Fig. 1B). Mice were fed regular chow ad libitum and housed in a room maintained at constant temperature (25°C) on a schedule of 12 hours of light and 12 hours of dark.

Bone mineral density measurements and body composition analysis
Total body bone mineral density and percent body fat were monitored by dual-energy X-ray absorptiometry using a PIXImus scanner (GE/Lunar, Madison, WI). Calibration of the instrument was conducted daily as suggested by the manufacturer. After induction of anesthesia with 100 mg/kg ketamine and 10 mg/kg xylazine i.p., mice were placed on the imaging-positioning tray in a prostrate position. One investigator (CHMC) performed and analyzed all the scans using the software provided by the manufacturer. Heads were excluded from the analysis by masking. The precision of this technique, assessed by the root mean square method after scanning 17 mice three times with repositioning, was 1.34% for bone mineral density and 3.31% for percent body fat.

Bone histology and histomorphometry
Histomorphometric analysis was performed on tibial metaphyses of 2-month-old mice. Bone samples were dissected out and fixed in 70% ethanol. Samples were left undecalcified, dehydrated, infiltrated and embedded in methyl methacrylate. Longitudinal sections were cut frontally in the proximal tibial metaphysis using a Leica RM 2165 microtome. Four-micrometer sections were stained with Goldner’s trichrome technique or for tartrate resistant acid phosphatase activity and counterstained with methyl green and thionin for identification of osteoclasts and osteoblasts (Liu and Kalu, 1990), while 8 μm sections were left unstained for dynamic bone histomorphometry. Quantitative histomorphometry was performed in an area 175-875 μm distal to the growth plate at 200× magnification (dynamic parameters) or 400× magnification (cellular and structural parameters), using the OsteoMeasure software program (Osteometrix, Atlanta, GA), a Nikon Eclipse E400 light/epifluorescent microscope, a CalComp Drawing Board III digitizing tablet and a video subsystem. The width of growth plate was measured by tracing the upper and lower margins of the growth plate at 200× magnification. Following an established nomenclature (Parfitt et al., 1987), the following static parameters of bone remodeling were estimated: trabecular bone volume as a percentage of total tissue volume, trabecular thickness (μm), trabecular number (per mm), trabecular separation (μm), osteoblast perimeter per bone perimeter (percentage), osteoblast number per bone perimeter (per mm), osteoclast perimeter per bone perimeter (percentage), osteoclast number per bone perimeter (per mm) and growth plate width (μm). For assessment of dynamic histomorphometric parameters, 2-month-old mice were labeled twice by injection of calcine (20 mg/kg i.p., Sigma-Aldrich) on days 8 and 3 before killing. After methylmethacrylate embedding, 8 μm thick sections were mounted unstained in Fluoromount (Electron Microscopy Sciences, Fort Washington, PA) for examination by fluorescent microscopy and calculation of the following dynamic parameters on two nonconsecutive sections per animal: mineral apposition rate (μm/day; mean distance between two fluorescent labels divided by the number of days between the labels), and bone formation rate/tissue volume (%/year; annual fractional volume of trabecular bone formed/ tissue volume). Results represent the average±s.d. of at least three sections per mice from four transgenic and four wild-type mice.

Cell culture and phenotypic characterization
Calvarial cells (CLVC)
Osteoblast-enriched calvaria cultures were prepared from newborn mice by sequential collagenase digestion as previously described (Lecanda et al., 2000), and grown in alpha 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 three to five calvariae were pooled to prepare the cell cultures used in each experiment.

Bone marrow stromal cells (BMSC)
After dissection, the epiphyseal ends of femora and tibiae of 2-3-month-old mice were removed and the marrow cavity of the shafts was flushed with 5 ml of αMEM with penicillin and streptomycin using a 25-gauge needle. The material was then filtered through a 70 μm cell strainer (Falcon). Red blood cells in marrow cells were hemolyzed in 0.017 M Tris-HCl buffer, pH 7.5, containing 0.8% NH₄Cl, and the resulting BMSC suspension was rinsed twice in PBS and plated at 1·2.5×10⁶ cells/cm².

Alkaline phosphatase activity
As a marker of osteogenic differentiation, alkaline phosphatase activity was measured in cell extracts according to previously
published and established methods (Lecanda 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.

In vitro mineralization
To assess the capacity of osteoblastic cells to mineralize in vitro, CLVC were cultured in the presence of 50 µg/ml ascorbic acid (AA) and 10 mM β-glycerophosphate (β-GP) for 2-3 weeks, and subjected to Von Kossa staining, as previously described (Lecanda et al., 2000).

Adipogenesis assay
For adipogenesis BMSC were seeded at 10^5/cm^2 in αMEM containing 15% FBS and cultured for 14 days. One-half of the medium was replaced with fresh medium on day 5 and adipogenesis was stimulated by adding 0.5 mM IBMX, 60 µM indomethacin and 0.5 µM hydrocortisone (Sigma-Aldrich, St Louis, MO) on day 11. On day 14, cultures were fixed and stained as described above (adipogenesis was observed as red, lipid vacuole-containing cells). They were fixed and stained as described above (adipogenesis assay), and adipocytes were identified as red, lipid vacuole-containing cells. Wells containing more than 20 Giemsa positive fibroblastoid cells were considered positive for CFU-F. For the CFU-Adipocytes (CFU-A) assay, cells were cultured for 4 weeks in the presence of an adipogenic cocktail (0.5 mM IBMX, 60 µM indomethacin, and 0.5 µM hydrocortisone) during the second week. They were fixed and stained as described above (adipogenesis assay), and adipocytes were identified as red, lipid vacuole-containing cells. Wells containing more than 20 red-stained cells were considered positive for CFU-A. To determine the number of CFU-Osteoblasts (CFU-O), cells were cultured for 4 weeks in the presence of a mineralizing medium (50 µg/ml AA and 10 mM β-GP), with weekly medium replacement. After 4 weeks, the wells were stained by the Von Kossa method, as described above, and the number of wells with more than 20 cells having a dark brown precipitate with more than 20 Giemsa positive cells were considered positive for CFU-O. The number of wells with more than 20 cells having a dark brown precipitate with more than 20 Giemsa positive cells were stained by the Von Kossa method, as described above, and the number of wells with more than 20 cells having a dark brown precipitate with more than 20 Giemsa positive cells were considered positive for CFU-O.

Detection and quantification of bone marrow mesenchymal progenitor cells
Limiting dilution analysis (Bellows and Aubin, 1989) was applied to determine the number of bone marrow mesenchymal progenitor cells that were able to produce colony forming units (CFU). BMSC harvested from the femur and tibia of 2-3-month-old mice were diluted in growth medium (MEM + 15% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2% fetal bovine serum) and plated at densities ranging between 10^5 and 10^3 cells/well in 96-well plates (corresponding to 3.2×10^4 to 3.2×10^2 cell/cm^2). After 1 week in culture, the medium was replaced with fresh growth medium with or without additives, as noted below. To determine the number of CFU-Fibroblasts (CFU-F), the plates were fixed in 100% ethanol after 2 weeks in culture and stained with Giemsa. Wells containing more than 20 Giemsa positive fibroblastoid cells were considered positive for CFU-F. For the CFU-Adipocytes (CFU-A) assay, cells were cultured in the presence of an adipogenic cocktail (0.5 mM IBMX, 60 µM indomethacin, and 0.5 µM hydrocortisone) during the second week. They were fixed and stained as described above (adipogenesis assay), and adipocytes were identified as red, lipid vacuole-containing cells. Wells containing more than 20 red-stained cells were considered positive for CFU-A. To determine the number of CFU-Osteoblasts (CFU-O), cells were cultured for 4 weeks in the presence of a mineralizing medium (50 µg/ml AA and 10 mM β-GP), with weekly medium replacement. After 4 weeks, the wells were stained by the Von Kossa method, as described above, and the number of wells with more than 20 cells having a dark brown extracellular matrix were considered positive for CFU-O. The number of CFUs in each assay and at each dilution was estimated according to the formula, F0 = e^-kn; where F0 is the fraction of colony-negative wells relative to the total number of wells seeded; e=2.71 (base of natural logarithms); and k is the number of CFUs per well (Bellows and Aubin, 1989).

Immunoprecipitation, western analysis and subcellular fractionation
For tissue extracts, calvaria from wild-type or transgenic mice were dissected, rinsed in phosphate-buffered saline, cut into small pieces, and incubated in RIPA buffer (10 mM Tris, pH 8.0; 150 mM NaCl; 1% NP-40; 0.1% sodium deoxycholate and 1% sodium deoxycholate containing a protease inhibitor cocktail (Sigma)) for 30 minutes at 4°C. Samples were subsequently sonicated on ice, and the insoluble material removed by centrifugation. The protein content of the supernatants was determined using the BCA protein assay (Pierce). The samples (250 µg) were then diluted in 1 ml immunoprecipitation buffer (10 mM Tris, pH 8.0; 150 mM NaCl; 1% NP-40 and 1% BSA). Following pre-clearing of the supernatants with Protein A/G Plus beads (Santa Cruz), the polyclonal antibody PEP1, which recognizes the C-terminal intracellular tail of Xenopus N-cadherin (generous gift of Dr Barry Gumbiner, University of Virginia, Charlottesville, VA), was added and the samples incubated overnight at 4°C with gentle shaking. Protein A/G Plus beads were washed and incubated with the sample for 1 hour, then pelleted by centrifugation. The beads were washed three times in immunoprecipitation buffer, then twice in PBS. Bound material was eluted from the beads by boiling in SDS-PAGE sample buffer. Samples were then electrophoresed on SDS-PAGE gels and examined by immuno blotting with anti-FLAG M2 antibodies (Sigma).

For whole-cell lysates, CLVC grown on 100 cm^2 Petri dishes 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. Proteins were separated by 10% SDS-PAGE, and electroblotted to a nitrocellulose membrane. For subcellular fractionation of β-catenin, cells were rinsed with cold PBS, scraped from the plate and centrifuged at 10,000 g for 5 minutes at 4°C. The pellets were gently resuspended in a hypotonic buffer (10 mM HEPES-KOH, pH 7.9; 10 mM KCl; 1.5 mM MgCl; 1.5% NP-40 and 1 mM PMSF) and centrifuged at 10,000 g for 5 minutes at 4°C to generate supernatants containing the cytosolic and membrane proteins. The pellets were used for nuclear extraction using a high-salt lysis buffer (20 mM HEPES-KOH pH 7.9; 1.22 mM MgCl; 420 mM NaCl; 0.2 mM EDTA; 25% glycerol; 1 mM NaF; 1 mM Na3VO3; 0.5 mM DTI; and 1 mM PMSF), and spun at 10,000 g for 5 minutes at 4°C to generate cytosolic containing nuclear extracts. Protein concentration was measured using Bio-Rad protein assay kit. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Invitrogen, Carlsbad, CA). Western blots were processed at room temperature as described (Lecanda et al., 2000) using either the PEP1 antibody (1:500) or N-cadherin (N19) antibody (1:1000; Santa Cruz), or an anti-β-catenin antibody (1:5000; Upstate, Charlottesville, VA), and visualized by ECL detection. In some cases, the membranes were washed an re-probed with an anti-GAPDH antibody to control for loading.

Retroviral transduction of active β-catenin
Retroviral particles were generated as described previously (Lai et al., 2001) by transfecting the SFG viral vector containing the mutant β-catenin construct ΔN151 into the packaging cells 293PGP, which express MuLV gag-pol and vesicular stomatitis virus G glycoprotein under tetracycline regulation, using LipofectAMINE (Gibco, Grand Island, NY). To clone ΔN151 into the SFG, the pUHD10-3/ΔN151 vector (kind gift of Dr James Nelson, University of California San Francisco) was digested with NcoI, blunt-ended, and then released with BamHI. The insert was then blunt-end ligated into the BamHI site of SFG. In order to generate the highest titer of viral particles (>5×10^6 CFU/ml), conditioned medium was harvested daily from day 3 to day 7 after the withdrawal of tetracycline. The media were combined, filtered through a 0.45 µm membrane and used for transduction in the presence of 8 µg/ml polybrene. As a negative control, a virus carrying the SFG-LacZ cDNA was generated in the same fashion. Retroviral transduction was performed either the day after seeding (CLVC), or after 5 days in culture (BMSC), by adding conditioned medium containing either ΔN151 or LacZ retroviral particles, and incubating for 24 hours. These conditioned media were not cytotoxic based on pilot titration experiments. At the end of the incubation period, the virus-containing medium was removed,
and cells were grown according to the procedures described for the CFU assays. Reverse transcription and PCR (RT-PCR) 
Femurs were dissected out, cleaned of soft tissue and snap frozen in liquid nitrogen then homogenized using a mortar and pestle. Total RNA was extracted using TRizol (Gibco) according to manufacturer’s directions. BMSC and CLVC were cultured in MEM medium containing 10% FCS for 3-4 weeks and total RNA isolated as just described. For RT-PCR, 1 µg of total RNA was reverse transcribed using oligo-dT primers and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) in a 20 µl reaction. Reverse transcription was carried out for 60 minutes at 42°C. The temperature was then raised to 95°C for 15 minutes to inactivate the transcriptase and activate the polymerase, and first strand cDNA was amplified by 35 cycles (94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and 72°C for 10 minutes). The presence of NcadΔC mRNA was detected using primers that amplify a fragment of the NcadΔC coding region (bases 52 to 246), yielding a 195 bp product. GAPDH was used as an internal control for cDNA quality and PCR efficiency (Table 1). The reaction products were separated on a 2% agarose gel electrophoresis, and visualized by ethidium bromide staining.

Real time-RT-PCR
Total RNA (1 µg) isolated from CLVC and BMSC was reverse transcribed using Superscript II reverse transcriptase and oligo(dT)15 primers. One fiftieth of this reaction was used for real-time PCR analysis of mRNA using the SYBR green PCR method according to

Table 1. List of primers used in this work

| Gene product | Forward primer | Reverse primer | Product size (bp) |
|--------------|----------------|----------------|------------------|
| NcadΔC       | AAACAGCCCTTTCTGCTACC | TCATGCTCAGAGAGGAGACC | 195              |
| GAPDH        | ACTTTGCTGAAGCTTTTCA   | TGCAGCGAACTTTATTGATG | 265              |
| PPARγ2       | ACCACTCGCATTTCCGCAATCA | TGGGTCAGCTTGGTGAATG |                 |
| Adipsin      | TGCCATGTGCTGCTGTCAGTAGC | TGAGCTGCCAGACGAGATG |                 |
| Col1a1       | GATGAGGCAATGGGATTCAAATGA | TGATAGTATTCCTCGCCGGAGAGA |                 |
| Osteopontin  | GTATTGCCTTTGCGCTTGG | TGAGCTGCCAGACGAGATG |                 |
| Osteoprotein | TCCCCGAGGACCACCTGAGAC | TGAGCTGCCAGACGAGATG |                 |
| RANK-L       | CTTGAGGCCGCCAGCATT | GGTACCAAGAGGACAGAGTGACTTAA |                 |

and cells were grown according to the procedures described for the CFU assays.

Reverse transcription and PCR (RT-PCR)
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Disruption of osteoblast cadherins in vivo

manufacturer’s instruction (PE Biosystems, Foster City, CA). In brief, a mixture of 1× SYBR green master mix, 50 nM forward primer and 50 nM reverse primer with the cDNA template (2 μl) was heated at 95°C for 10 minutes. DNA was then amplified for 40 cycles (95°C for 15 seconds, 60°C for 1 minute) on the GeneAmp 5700 sequence detector. The primers used are listed in Table 1. 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 level of expression, relative to wild-type controls, was defined as (2–ΔΔCT), where ΔΔCT equals the OG2-NcadΔC ΔCT minus the ΔCT of wild-type cells. All real-time PCR experiments were performed at least three times.

Results

Selective expression of the NcadΔC transgene in cells of the osteoblastic lineage

We used the OG2 promoter (1.3 kb) to express the truncated cadherin mutant NcadΔC selectively in osteoblasts, because it had previously been shown to drive the in vivo expression of other genes in bone forming cells only postnatally (Ducy et al., 1999). To establish an OG2-NcadΔC homozygous transgenic line, we identified five founder transgenic mice among 25 pups produced after reimplantation of the microinjected embryos. We selected two founder male mice for high copy number of the transgene, and mated them with wild-type B3CF1 female mice. Hemizygous F1 transgenic animals were then cross-bred to produce homozygous F2 animals. The presence of NcadΔC transgene transcript was confirmed as a 195 bp band detected by RT-PCR in extracts of femur and calvaria of OG2-NcadΔC mice, but not in extracts from other tissues (Fig. 1C), thus confirming osteoblast-specific expression of the transgene. Expression of NcadΔC protein in bone was detected by immunoprecipitation using an antibody that recognizes the intracellular tail of Xenopus N-cadherin (PEP.1) and subsequent western analysis using an anti-FLAG M2 antibody that recognizes the epitope tag fused to the NcadΔC construct. A band of approximately 45 kDa, corresponding to the truncated NcadΔC, was detected in the input fraction from transgenic calvarium and was markedly enriched in the immunoprecipitated fraction (Fig. 1D, lanes 2 and 4). The 45kDa protein was not detected in protein extracts from wild-type littermate calvarium (Fig. 1D, lanes 1 and 3).

Low bone mass and high body fat phenotype in OG2-NcadΔC mice

As shown in Fig. 2A, during the first 3 months of life – when bone mass accumulates – total body mineral density was significantly reduced in OG2-NcadΔC mice compared with wild-type littermates, with maximal difference of 10% at age of 3 months (51.54±2.01 mg/cm², n=26; vs. 57.3±2.45 mg/cm², n=13; P<0.001; respectively). Thereafter, no statistically significant difference in bone density was detected between wild-type and transgenic mice. By contrast, body composition analysis showed reciprocal changes at the earliest time-points, with consistently higher percent body fat in OG2-NcadΔC relative to wild-type mice (Fig. 2B), and maximal difference of 27% at 3 months of age (29.8±5.7% vs. 22.8±2.3%; P=0.011). During the same time period, body weight (Fig. 2C) and food intake were also significantly higher in OG2-NcadΔC mice than in wild-type controls (4.59±0.67 g/day vs. 3.03±0.33 g/day; P<0.01). At age 4 months and higher, percent body fat and body weights were no longer different between mutant and wild-type animals.

Fig. 2. Low bone mass and high body fat in OG2-NcadΔC mice. Bone density and body composition were analyzed by dual energy X-ray absorptiometry in live animals at different times up to 4 months. (A) Total body bone mineral density (BMD) was significantly reduced in OG2-NcadΔC mice during the first 3 months of age – when BMD accumulates – compared with wild-type littermates, whereas percent body fat (B) and body weight (C) were significantly higher in OG2-NcadΔC mice compared with wild-type littermates during the first 3 months of age. *P<0.05, two-way ANOVA. Error bars represent standard deviation.
Histomorphometric analysis of long bones revealed an even more pronounced low bone mass phenotype in OG2-NcadΔC transgenic mice than was detected by radio-absorptiometric measurements. Trabecular bone volume in the proximal tibial metaphysis was reduced 73% in 2-month-old transgenic mice relative to wild-type littermates (Fig. 3A,B). This decreased bone volume was associated with a decrease in trabecular number and thickness, and a significant increase in the mean...
Fig. 3. Reduced bone formation in OG2-NcadΔC transgenic mice: histologic and histomorphometric analysis. (A) Goldner’s trichrome stained, 4 μm undecalciﬁed sections of proximal tibiae from OG2-NcadΔC (Tg) or wild-type (WT). Micrographs on the right are enlargements (400×) of the rectangular areas shown on the left micrographs (200×). (B) Bone volume, expressed as a percentage of total tissue volume (BV/TV) in the entire area of the bone marrow cavity. 175–875 μm from the growth plate was signiﬁcantly reduced in transgenic mice compared with those of wild-type controls (n=8). (C–E) OG2-NcadΔC mice also exhibited a signiﬁcantly lower trabecular number and thickness and higher trabecular separation compared with wild-type mice. (F) Fluorescence micrograph of calcein labeling in 8 μm unstained sections of cancellous bone, showing no double labels in sections of transgenic mice bone. Accordingly (G,H), dynamic parameters of bone formation, bone formation rate/tissue volume and mineral apposition rate were signiﬁcantly reduced in transgenic mice compared with wild-type littermates. (I) Four-micrometer sections of proximal tibiae were stained for tartrate-resistant acid phosphatase activity and counterstained with methyl green and thionin for identiﬁcation of osteoclasts (Oc) and osteoblasts (Ob). Micrographs on the right are enlargements (400×) of the rectangular areas shown on the left micrographs (200×). (J,K) The number of osteoblasts expressed as osteoblast perimeter per bone perimeter (%) was reduced in OG2-NcadΔC mice compared with wild-type mice. There was no signiﬁcant difference in the number of osteoclasts between transgenic and wild-type animals. In all panels, asterisks denote *P<0.05, t-test.

distance between individual trabeculae (Fig. 3C–E). Dynamic indices of bone formation, reﬂected as an osteoblast defect in transgenic animals, reﬂected by a few double labeled seams and reduced distance between the two calcine labels visualized by ﬂuorescence microscopy (Fig. 3F). Bone formation rate was reduced 74% in OG2-NcadΔC mice (Fig. 3G), commensurate with the decreased bone volume, and mineral apposition rate was also signiﬁcantly reduced (Fig. 3H). The number of osteoblasts was lower in transgenic than in wild-type mice (Fig. 3I,J), although the difference was not as large as that seen for bone formation rate. However, there was no signiﬁcant difference in osteoclast number between transgenic and wild-type animals in sections stained for the osteoclast-speciﬁc tartrate-resistant acid phosphatase activity (Fig. 3K). There were also no differences in osteoid labeling or thickness, or in the width of the growth plate between wild-type and transgenic mice (89.57±1.46 vs. 81.40±6.69 μm, respectively).

NcadΔC expression in vivo retards osteoblast differentiation

We next examined osteoblast differentiation and function in cells isolated from newborn mouse calvaria. As shown in Fig. 4A, alkaline phosphatase activity, an index of osteogenic differentiation, was reduced after 1 and 2 weeks post-conﬂuence in CLVC derived from OG2-NcadΔC mice compared with wild-type controls, but it was not different after 3 weeks in culture. Matrix mineralization was modestly but not substantially reduced in mutant relative to wild-type cells cultured for 2 and 3 weeks after conﬂuence in the presence of AA and β-GP, despite expression of the transgene at these time-points (Fig. 4B). However, using the highly sensitive real time RT-PCR we did ﬁnd that expression of many osteoblast products was indeed altered in OG2-NcadΔC CLVC after 2 weeks of culture. The abundance of mRNA transcripts for Cbfa1, osteopontin, osteoprotegerin and RANK-L were all reduced in OG2-NcadΔC relative to wild-type cells, whereas type 1 collagen expression was slightly higher 2 weeks post-conﬂuence (Fig. 4B). Detection of osteocalcin mRNA yielded inconsistent results, but because we used the OG2 promoter to drive expression of NcadΔC, which in turn alters osteoblast differentiation, the interpretation of osteocalcin expression data is problematic. Using the PEP.1 antibody in immunoblots or an antibody against the N-terminus of mouse N-cadherin (N19) in immunoprecipitations, we did not detect any differences in endogenous N-cadherin abundance between transgenic and wild-type cells (Fig. 4D), consistent with our previous ﬁndings in osteoblastic cells showing that NcadΔC expression alters the function but not the expression of osteoblast cadherins (Cheng et al., 2000).

NcadΔC expression in vivo alters bone marrow mesenchymal cell differentiation

To test whether the ability to generate osteogenic precursors was defective in OG2-NcadΔC mice, we analyzed lineage allocation by bone marrow mesenchymal precursors using a limiting dilution method. As shown in Fig. 5A, unfractonated BMSC from OG2-NcadΔC mice seeded at low density and then cultured in mineralizing medium for 3 weeks yielded a signiﬁcantly lower number of Von Kossa positive wells relative to wild-type cultures, resulting in 50% fewer CFU-O than wild-type controls (Fig. 5C). Conversely, OG2-NcadΔC BMSC cultures incubated with an adipogenic cocktail (IBMX, indomethacin and hydrocortisone) generated a higher number of cells positive for Oil Red O lipid droplets relative to wild-type cultures (Fig. 5B), resulting in ﬁvefold higher CFU-A relative to wild-type controls (Fig. 5C). But the number of CFU-F, an index of mesenchymal cell precursor number, did not differ between the two genotypes (Fig. 5C).

The evidence of an osteoblastogastic to adipogenic shift was corroborated by quantitative real-time PCR assessment of molecular markers of mesenchymal cell differentiation. The abundance of mRNA for PPARY2, one of the earliest transcription factors that are expressed in the adipogenic pathway, and for adipin, a marker of terminally differentiated adipocytes, were 11-fold and 3-fold higher, respectively, in OG2-NcadΔC BMSC cultures than in wild-type cells (Fig. 5D). By contrast, the abundance of Cbfa1 mRNA, one of the earliest transcriptional factors expressed during the osteoblast differentiation program, was decreased 40% in OG2-NcadΔC marrow cells compared to wild-type cultures. Conﬁrming expression of the transgene in these conditions, a PCR product of the correct size was ampliﬁed from cDNA obtained from cultures of BMSC isolated from transgenic mice only after culture in mineralizing medium to allow for osteoblast differentiation (Fig. 5E). The PCR product was not present in freshly isolated, undifferentiated BMSC (not shown).

Viral transduction of constitutively active β-catenin rescues the osteoblast defect and reduced adipogenic commitment in NcadΔC cells

Expression of the truncated NcadΔC mutant can theoretically not only interfere with cell-cell adhesion, as we previously
showed (Cheng et al., 2000), but it can also sequester β-catenin in the plasma membrane, reducing its cytoplasmic pool and thus inhibiting its transcriptional activity (Fujimori and Takeichi, 1993). To explore this possibility, we first determined the cellular distribution of β-catenin in NcadΔC-expressing cells. Cell fractionation of β-catenin showed that in calvaria cells isolated from newborn OG2-NcadΔC mice compared with wild-type (WT) cells at 1 and 2 weeks post-confluence, *P<0.001, t-test (means ± s.e.m.). (B) Von Kossa staining of CLVC cultures isolated from newborn OG2-NcadΔC transgenic (Tg) or wild-type (WT) mice, after 2 or 3 weeks in culture in the presence of AA (50 µg/ml) and β-GP (10 mM). There is no apparent difference in the mineralized (dark stain) area between the transgenic and wild-type cells at either time-point. Expression of the transgene NcadΔC was detected at the corresponding time-points by RT-PCR of total RNA extracted from parallel cultures, with GAPDH as control for RNA integrity and abundance (lower panels). (C) Total RNA was extracted from CLVC grown for 2 weeks after confluence. The amount of mRNA for osteoblast products was determined by real-time PCR and expressed as mRNA abundance in OG2-NcadΔC relative to wild-type (WT) cells. Type I collagen expression levels were increased 1.8 times in transgenic animals compared with wild-type controls, whereas osteoprotegerin, osteopontin, Cbfα1 and RANK-L mRNA abundance was significantly reduced. (D) Whole-cell lysates obtained from transgenic (Tg) and wild-type (WT) CLVC grown for 3 weeks post-confluence were separated by SDS-PAGE and blotted using antibodies that recognize the intracellular tail of Xenopus N-cadherin (PEP.1) or the N-terminus of N-cadherin (N19; Santa Cruz). Two bands of ~100 and 120 kDa were detected in both cases, corresponding to endogenous N-cadherin and possibly other cross-reacting cadherins. A band of ~45 kDa, corresponding to the truncated NcadΔC, was detected by the PEP.1 antibody only in CLVC from transgenic animals, but not from wild-type littermates (left panel). This membrane was stripped and blotted again with an anti-GAPDH antibody to control for loading.
Disruption of osteoblast cadherins in vivo

Discussion

We have shown that in vivo expression of a dominant-negative cadherin, NcadΔC, in osteoblasts leads to transient osteopenia and increased adiposity in mice. Expression of this truncated cadherin in osteoblasts in vivo interferes with the osteoblast differentiation program and shifts bone marrow mesenchymal cell commitment from osteogenesis to adipogenesis. In vitro, the osteoblast abnormality can be reversed by expression of a stabilized, active β-catenin mutant. Thus, the integrity of cadherin function in osteoblasts is necessary for the differentiation and function of bone forming cells.

The osteopenia of young transgenic animals was global, as determined by whole body bone mineral density, but it was particularly evident on the trabecular component of long bones, where the decrease of trabecular bone volume was >50% at 2 months. Histologically, the osteopenic phenotype of young NcadΔC transgenic mice is the consequence of decreased bone formation rate and perhaps reduced osteoblast number, but not increased bone resorption. The inherent high variability in the histomorphometric measurements of osteoblast number and the less distinctive morphologic features of mouse osteoblasts compared with rat or human osteoblasts (Jilka et al., 1996) may explain the only modestly reduced osteoblast number compared with the dramatically decreased dynamic indices of bone formation in transgenic mice. Very consistent with our findings is a similar, though milder, osteopenic phenotype observed in 3-month-old mice genetically deficient in cadherin-11 (Kawaguchi et al., 2001a). Although the authors did not report whether the low bone mass corrected with time in older animals, they did observe decreased bone formation rates in cadherin-11 deficient mice. The relatively more severe osteopenia in our mice, a model of global, non-isotype specific interference with cadherin function may indicate that other cadherins are involved in control of bone formation. The most
likely candidate is N-cadherin, which is abundantly expressed in osteogenic cells and known to be involved in chondro osteogenesis (Haas and Tuan, 1999; Marie, 2002; Tuan, 2003). As NcadΔC did not affect expression of endogenous osteoblast cadherins, a finding consistent with our previous results in MC3T3-E1 cells (Cheng et al., 2000), it is likely that the phenotype we observed is related to an interference of the dominant-negative transgene with cadherin-regulated signaling, as discussed further below. The mechanism by which peripheral fat is increased in OG2-NcadΔC mice is under investigation, but it may be related to the exuberant adipogenesis induced by the dominant-negative cadherin (see below). It is interesting to note that the reciprocal changes in body fat and bone mass simultaneously correct at 4 months of age, pointing to a common cause. We were able to detect the presence of NcadΔC mRNA in osteoblasts up to 6 months of age (data not shown), thus ruling out inactivation of the OG2 promoter as an explanation for the transient phenotype. Although the reasons for the recovery of bone mass at 4 months remain unexplained, normalization of β-catenin cellular distribution in BMSC suggests that cadherin-independent compensatory mechanisms might reverse the abnormality of β-catenin signaling induced by the dominant-negative cadherin by 4 months of age.

At the cellular level, the reduced development of alkaline phosphatase activity and the lower abundance of most osteoblast-specific products, osteopontin, osteoprotegerin, RANK-L and, in particular, Cbfa1, in post-confluent CLVC cultures (which consist of fully committed osteogenic cells), suggest a disruption of the osteoblast differentiation program by NcadΔC. The modest increase of type I collagen mRNA in NcadΔC in CLVC, a finding also previously noted in the MC3T3-E1 cell line (Cheng et al., 2000), may simply reflect a slow progression to full differentiation in the genetically altered cell cultures, as production of type I collagen decreases with full differentiation. However, in vitro CLVC mineralization was only modestly affected by NcadΔC expression. It should be noted that CLVC cultures are tested when fully confluent, and in such conditions the number of osteoblasts that are able to fully differentiate may be sufficient to produce substantial mineralized matrix, thus masking subtle differences. However, the osteogenic to adipogenic shift in bone marrow precursor cell differentiation was unexpected, since the transgene is driven by the mouse OG2 promoter, which is expressed in mature, differentiated osteoblasts (Ducy et al., 1999), and thus should not be present in undifferentiated cells. In fact, we could detect the transgene in BMSC only when these cells were pushed towards osteoblast differentiation by incubation with AA and β-GP. Yet, the reciprocal down- and upregulation of early, lineage-specific...
transcription factors, Cbfa1 and PPARγ2, implies an alteration of early commitment. Perhaps the lower number of cells that can go through the osteogenic pathway in the presence of the dominant-negative cadherin may simply increase the number of uncommitted cells at that will default to the adipogenic pathway. Another more intriguing possibility may be that differentiated osteoblasts release signals that feedback onto uncommitted precursors and regulate their fate. Disruption of such signals by dominant-negative cadherin may negatively affect mesenchymal cell commitment towards osteogenesis. Whichever the mechanism, the present in vivo results strengthen previous in vitro work from our and other groups, showing that cadherins are critically involved in chondro-osteogenic differentiation. In uncommitted C3H10T1/2 and C2C12 mesenchymal cells, stimulation of osteoblast differentiation is associated with increased expression of N-cadherin and cadherin-11, whereas the abundance of these two cadherins decreases with adipogenic differentiation, to completely disappear in fully differentiated adipocytes (Shin et al., 2000; Kawaguchi et al., 2001b). Likewise, N-cadherin is critically involved in cell-cell interactions during TGF-β-induced cartilage condensation, and it is sequentially up- and downregulated in chondrogenesis (Tuli et al., 2003).

The increased membrane/cytoplasmic and decreased nuclear localization of β-catenin in CLVC of young transgenic mice is consistent with others’ reports indicating that NcadAC can retain β-catenin on the cell surface (Kintner, 1992; Fujimori and Takeichi, 1993). A decreased nuclear β-catenin pool may reduce β-catenin available for binding to Tcf/Lef, thus inhibiting its transcriptional activity (Orsulic et al., 1999; Sadot et al., 1998; Fagotto et al., 1996). This mechanism of action is not specific for any cadherins and provides a global inhibition of cadherin-mediated cell-cell adhesion – via interference with lateral clustering and dimerization of endogenous cadherins – and β-catenin signaling (Nienman et al., 1999). The reversal of both the osteogenic defect in CLVC and the exuberant adipogenesis in BMSC by retroviral transduction of a stable, constitutively active β-catenin mutant strengthens the contention that the dominant-negative effect of NcadAC is probably related to an interference with the canonical β-catenin signaling pathway. Importantly, the β-catenin mutant we used, ΔN151, lacks the α-catenin binding site (Barth et al., 1997). Although α-catenin seems to have a role in signaling, rescue from the shift in lineage allocation seems to be independent of α-catenin binding, as ΔN151 was fully effective in our system. An emerging body of evidence indicates that activation of the canonical β-catenin pathway provides key regulatory cues in mesenchymal cell fate decisions, favoring chondro-osteogenic differentiation (Rawadi et al., 2003; Fischer et al., 2002; Bain et al., 2003), while inhibiting adipogenesis (Ross et al., 2000; Bennett et al., 2002). The decreased osteogenic and increased adipogenic precursors in OG2-NcadAC mice is perfectly consistent with these reports, showing that, in addition to Wnt signaling, cadherins are critically involved in regulating β-catenin activity.

In conclusion, our data show that in vivo expression of a truncated cadherin mutant in osteoblasts interferes with their differentiation and function and increases adipogenesis, resulting in decreased bone formation, delayed acquisition of peak bone mass and increased peripheral adiposity in young mice. This phenotype is probably determined by interference of the dominant-negative cadherin with β-catenin signaling. Therefore, cadherins are integral modulator of osteoblast function in vitro and in vivo.

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