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Amylin inhibits bone resorption while the calcitonin receptor controls bone formation in vivo

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Amylin is a member of the calcitonin family of hormones cosecreted with insulin by pancreatic β cells. Cell culture assays suggest that amylin could affect bone formation and bone resorption, this latter function after its binding to the calcitonin receptor (CALCR). Here we show that *Amylin* inactivation leads to a low bone mass due to an increase in bone resorption, whereas bone formation is unaffected. In vitro, amylin inhibits fusion of mononucleated osteoclast precursors into multinucleated osteoclasts in an ERK1/2-dependent manner. Although *Amylin* +/− mice like *Amylin*-deficient mice display a low bone mass phenotype and increased bone resorption, *Calcr* +/− mice display a high bone mass due to an increase in bone formation. Moreover, compound heterozygote mice for *Calcr* and *Amylin* inactivation displayed bone abnormalities observed in both *Calcr* +/− and *Amylin* +/− mice, thereby ruling out that amylin uses CALCR to inhibit osteoclastogenesis in vivo. Thus, amylin is a physiological regulator of bone resorption that acts through an unidentified receptor.

Introduction

Bone remodeling is the process by which bone mass is maintained constant throughout life in vertebrates. This is a dynamic two-step process that begins with bone resorption by osteoclasts followed by bone formation by osteoblasts. The dynamic nature of bone remodeling suffices to explain why our molecular understanding of this process has gained so much from in vivo studies and especially from mouse genetic studies. These latter studies have identified transcription factors, growth factors and their receptors, hormones and intracellular signaling molecules that affect one or the other arm of bone remodeling at the level of cell differentiation, proliferation, or function (Boyle et al., 2003; Teitelbaum and Ross, 2003).

Mouse genetic studies have also uncovered unexpected functions for known regulators of bone remodeling. One example of these surprising results has been the characterization of calcitonin function in vivo. Indeed, mice lacking calcitonin and calcitonin gene–related peptide (CGRP) have a high bone mass phenotype due to an increase in bone formation parameters (Hoff et al., 2002). This was surprising as the calcitonin receptor (CALCR) is expressed on osteoclasts but not on osteoblasts (Nicholson et al., 1986; Lee et al., 1995). Moreover, numerous studies have demonstrated that it is a specific marker of osteoclast differentiation and that calcitonin can inhibit bone resorption in vitro and in vivo (Quinn et al., 1999; Cornish et al., 2001). The increase in bone formation observed in *Calcitonin/Cgrp*-deficient mice suggests that the expression of *Calcr* in osteoclasts may not be pivotal for the function of calcitonin/CGRP itself in bone remodeling and/or that CALCR on osteoclasts may have other ligands.

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Abbreviations used in this paper: BFR, bone formation rate; BMM, bone marrow macrophage; CALCR, calcitonin receptor; CGRP, calcitonin gene–related peptide; ERK1/2, extracellular signal–regulated protein kinase1/2; M-CSF, macrophage colony–stimulating factor; PTH, parathyroid hormone; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase; WT, wild-type.

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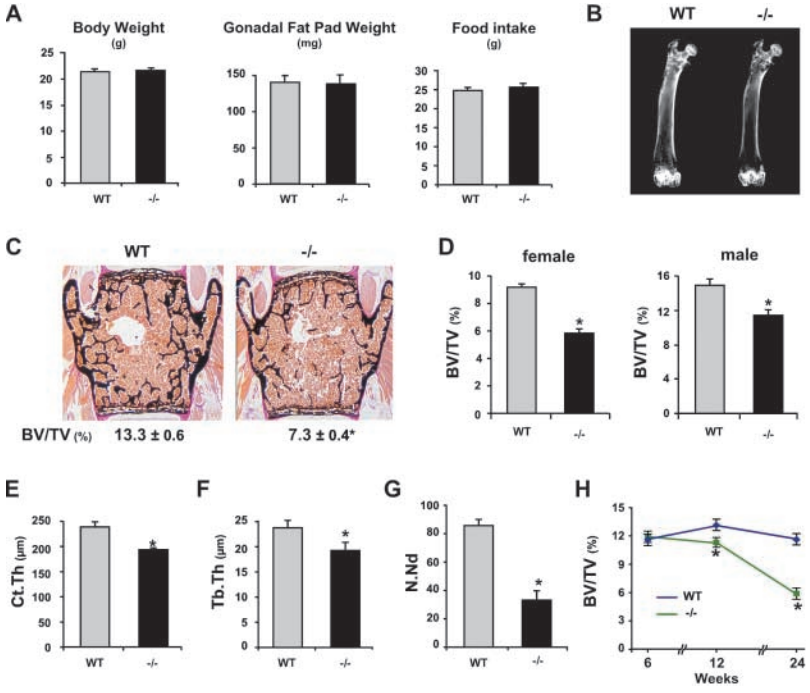

Figure 1. **Low bone mass in *Amylin*-deficient mice.** (A) Body weight, gonadal fat pad weight, and food intake are identical between *Amylin*-deficient and wild-type (WT) mice. (B) X-ray analysis showing a decrease in mineral density in 6-mo-old *Amylin*-deficient compared with WT femurs. (C) Histological analysis of 6-mo-old WT and *Amylin*-deficient vertebrae showing a decrease in bone volume over tissue volume (BV/TV). (D) Low bone mass phenotype in both male and female *Amylin*-deficient mice. (E–G) Microarchitecture parameters in *Amylin*-deficient mice. Cortical thickness (E), trabecular thickness (F), and number of nodes (G) were decreased in *Amylin*-deficient compared with WT mice. (H) Bone mass of 6-, 12-, and 24-wk-old WT and *Amylin*-deficient mice vertebrae. Asterisks indicate statistically significant differences ($t \leq 0.01$) between two groups ($n \geq 8$). Error bars represent SEM.

Calcitonin is the founding member of a small family of polypeptide hormones that comprises of CGRP, adrenomedullin, intermedin, CALCR stimulating hormone, and amylin or islet amyloid polypeptide (Wimalawansa, 1997; Katafuchi et al., 2003; Roh et al., 2003). Amylin, the focus of this paper, was identified as a protein present in pancreatic β cells that is cosecreted with insulin after food ingestion (Westermark et al., 1987; Kahn et al., 1990). Despite many efforts it has been difficult to ascribe a significant function to amylin in the control of glucose metabolism in vivo (Gebre-Medhin et al., 1998). In contrast, in the last 10 yr a growing body of cell culture–based studies have suggested that amylin may be a regulator of bone remodeling by favoring bone formation and possibly by inhibiting bone resorption (Su et al., 1992; Zaidi et al., 1993; Cornish et al., 1999, 2001); that amylin is able to bind in vitro to CALCR has raised the hypothesis that it may be a physiological ligand of CALCR on osteoclasts (Christopoulos et al., 1999).

To address the role of amylin in bone remodeling and to determine whether it is a physiological ligand of CALCR we analyzed *Amylin*-deficient as well as *Amylin* +/− and *Calcr* +/− mice. Here, we show that *Amylin*-deficient mice develop a low bone mass phenotype mimicking an osteoporosis that is due solely to an increase in bone resorption. We also provide genetic and histological evidences ruling out that amylin acts through CALCR to regulate osteoclastogenesis.

Results and discussion

Low bone mass phenotype in *Amylin*-deficient mice

Amylin-deficient mice are born at the expected mendelian ratio, have a normal life span, are fertile, and do not display any overt phenotypic abnormalities (Gebre-Medhin et al., 1998). Unlike what has been shown in gain of function experiments (Cornish et al., 1998), body and gonadal fat pad weights were normal in *Amylin*-deficient mice as were food intake, serum insulin, and glucose levels (Fig. 1 A; Table I). Thus, amylin is dispensable for regulation of food intake, body weight, and glucose metabolism in vivo.

To determine whether amylin affects bone remodeling in vivo, we analyzed wild-type (WT) and *Amylin*-deficient mice by X rays and histology. In 24-wk-old mice, X ray revealed a decrease in bone density in *Amylin*-deficient compared with WT long bones (Fig. 1 B). Histological analysis revealed a 50% decrease of bone mass as measured by bone volume over tissue volume (Fig. 1 C, BV/TV). Further analysis revealed a decrease in cortical and trabecular thickness and in connectivity between trabeculae in *Amylin*-deficient mice, this latter feature being a hallmark of osteoporosis (Fig. 1, E–G). This low bone mass phenotype observed in both sexes, starting in 12-wk-old mice (Fig. 1, D and H), was not secondary to metabolic abnormalities because all hormonal and metabolic parameters measured were normal in *Amylin*-deficient mice (Fig. 1 A; Table I).

Table I. **Hormonal and metabolic measurements in amylin-deficient mice**

	WT mice	Amylin-deficient mice
Serum calcium (mg/dl)	9.40 ± 0.29	9.45 ± 0.34
Serum phosphate (mg/dl)	7.04 ± 0.31	6.98 ± 0.45
Serum PTH (mg/ml)	20.32 ± 1.0	22.74 ± 3.9
Serum insulin (pg/ml)	218.2 ± 10.5	222.1 ± 26.0
Blood glucose (mg/ml)	150.4 ± 9.9	144.88 ± 5.4
Urine calcium/Creat. (mg/dl)	0.52 ± 0.13	0.57 ± 0.02
Urine phosphate/Creat. (mg/dl)	27.64 ± 0.9	25.46 ± 1.3

Creat, creatine.

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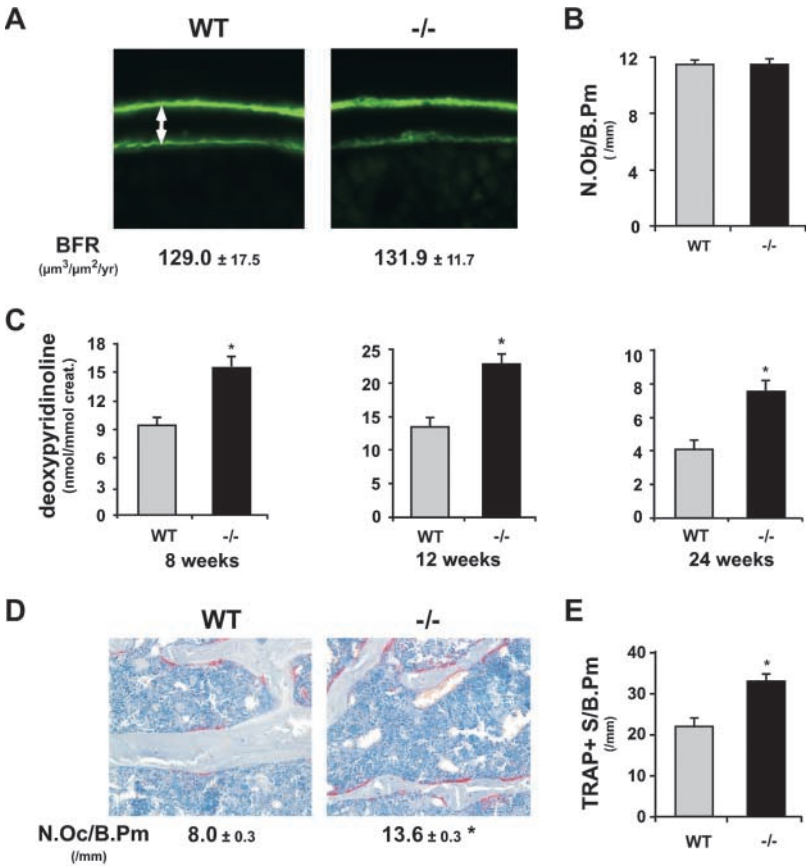

Figure 2. **Increased bone resorption in *Amylin*-deficient mice.** (A and B) Calcein double labeling in 6-mo-old WT and *Amylin*-deficient mice. The bone formation rate (BFR) is not affected in *Amylin*-deficient mice nor is the number of osteoblasts. (C) Urinary deoxypyridinoline cross-links elimination was significantly increased in *Amylin*-deficient compared with WT mice at 8, 12, and 24 wk old. (D) Increased number of TRAP-positive multinucleated osteoclasts in *Amylin*-deficient compared with WT bones. Note the thinner appearance of the trabeculae. (E) Increased TRAP-positive surface per bone perimeter in *Amylin*-deficient compared with WT osteoclasts. Asterisks indicate statistically significant differences ($t \leq 0.01$) between two groups ($n \geq 8$). Error bars represent SEM.

Increased bone resorption in *Amylin*-deficient mice

To determine whether the low bone mass phenotype of the *Amylin*-deficient mice was due to a decrease in bone formation and/or an increase in bone resorption, we performed histomorphometric, cell biology, and biochemical analyses. Bone formation assessed by measuring the bone formation rate (BFR) after double injection of calcein was comparable in 12- and 24-wk-old WT and *Amylin*-deficient mice; likewise the number of osteoblasts was similar in *Amylin*-deficient and WT mice at all ages analyzed (Fig. 2, A and B; not depicted). To exclude that any cellular phenotype in osteoblasts not revealed by histology existed, we cultured osteoblast progenitor cells and analyzed them after 10 d of culture for alkaline phosphatase activity, Type I collagen synthesis, and the presence and size of mineralization nodules. We did not observe any differences between WT and *Amylin*-deficient osteoblasts for these parameters (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200312135/DC1). These data do not support the hypothesis that amylin affects osteoblast differentiation or function in vivo and rule out that the low bone mass phenotype of *Amylin*-deficient mice is secondary to a defect in bone formation.

Next, we studied bone resorption through biochemical and histological means. Urinary elimination of deoxypyridinoline, a degradation product of collagen and an indicator of bone resorption was increased in 8-, 12-, and 24-wk-old *Amylin*-deficient mice (Fig. 2 C). There was an increase in the number of tartrate-resistant acid phosphatase (TRAP)–positive cells and surface, i.e., osteoclasts, in *Amylin*-deficient bones (Fig. 2, D and E). The increase in deoxypyridinoline excretion in 8-wk-old mutant mice establishes that the abnormalities in osteoclast biology underlying the low bone mass phenotype observed in older *Amylin*-deficient mice were present early during life. These results suggest that the phenotype of the amylin-deficient mice is related, at least in part, to bone loss as it occurs in osteoporosis.

Amylin inhibits osteoclastogenesis

The increased osteoclast number in *Amylin*-deficient mice led us to test whether amylin affects differentiation and/or function of the osteoclasts. To study osteoclast differentiation, we used ex vivo culture of bone marrow macrophages (BMMs) that were cultured in medium supplemented with serum from *Amylin*-deficient mice to achieve a better controlled amylin concentration in the extracellular medium when added.

In the presence of receptor activator of NF-κB ligand (RANKL) and macrophage colony–stimulating factor (M-CSF) and in absence of amylin, BMMs differentiate into TRAP-positive multinucleated osteoclasts (Fig. 3, A and B). This was observed whether we used WT or *Amylin*-deficient BMMs (unpublished data), thus, ruling out the existence of a cell-autonomous defect in *Amylin*-deficient osteoclasts. In contrast, when amylin was added to the culture medium, we consistently observed a decrease in the number of TRAP-positive multinucleated osteoclasts (Fig. 3 A). This decrease was dose dependent and was observed when using a physiological concentration of amylin ($0.5.10^{-10}$ M; Fig. 3 A). Moreover, multinucleated osteoclasts cultured in the presence of amylin were smaller and had consistently fewer nuclei per cell than those cultured in absence of amylin (Fig. 3

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B). Similar results were obtained when spleen cells were used as a source of osteoclast progenitors (unpublished data). That the number of TRAP-positive mononucleated cells was similar whether cells were treated with amylin or not, whereas the number of multinucleated osteoclasts was lower after amylin treatment indicates that amylin does not affect the early steps of osteoclast differentiation but only fusion of TRAP-positive mononucleated cells into TRAP-positive polynucleated osteoclasts (Fig. 3 C). Consistent with this function of amylin in vitro, the number of nuclei was significantly increased in *Amylin*-deficient osteoclasts in vivo when compared with WT osteoclasts (unpublished data).

To determine if amylin affects the function of TRAP-positive multinucleated osteoclasts, WT BMMs were differentiated into osteoclasts on dentine slices and resorption pit surface was measured. In the presence of amylin, dentine slice resorption was decreased compared with what was observed when osteoclasts were cultured in absence of amylin. However, this decrease was proportional to the decrease in the number of multinucleated osteoclasts induced by the addition of amylin (Fig. 3 D). These results demonstrate that amylin is a regulator of osteoclast differentiation and that it does not affect overtly the function of osteoclasts once differentiated.

Amylin inhibition of osteoclastogenesis requires ERK1/2 phosphorylation

Next, we tested whether signaling events occurring after binding of calcitonin to CALCR in vitro also occurred when osteoclasts were treated with amylin (Chen et al., 1998). Primary osteoclasts generated from BMMs were stimulated with amylin. Western blot analysis using an antibody specific for the phosphorylated form of extracellular signal–regulated protein kinase 1/2 (ERK1/2) showed that ERK1/2 was transiently and rapidly phosphorylated after amylin stimulation of osteoclasts (Fig. 3 E). To demonstrate that ERK1/2 activation is required for amylin inhibition of osteoclast differentiation, BMMs were infected with a retrovirus containing a dominant negative form of ERK1/2 (Robinson et al., 2002) and differentiated in the absence or presence of increasing concentrations of amylin. In osteoclast cultures expressing the dominant negative form of ERK1/2 amylin could not affect osteoclast fusion or the number of TRAP-positive multinucleated osteoclasts, regardless of the dose used (Fig. 3 F). These data indicate that ERK1/2 phosphorylation is a necessary intracellular event for amylin inhibition of osteoclast differentiation.

Calcr and *Amylin* inactivation affect bone remodeling in opposite manner

The data presented are compatible with the hypothesis that amylin inhibitory action on osteoclastogenesis occurs via CALCR. This hypothesis would predict that haploinsufficiency of either CALCR's physiological ligands or of *Calcr* itself should result in identical phenotypic and cellular abnormalities in bone. Thus, to determine whether CALCR is the main receptor for amylin in vivo, we generated and analyzed *Calcr*-deficient mice and compared their bone phenotype to the one of *Amylin*-deficient and *Calcitonin/Cgrp*-deficient mice (Fig. 4, A–C). Because homozygous *Calcr*-deficient embryos die before skeletogenesis is initiated (unpublished data), this analysis was conducted in heterozygous

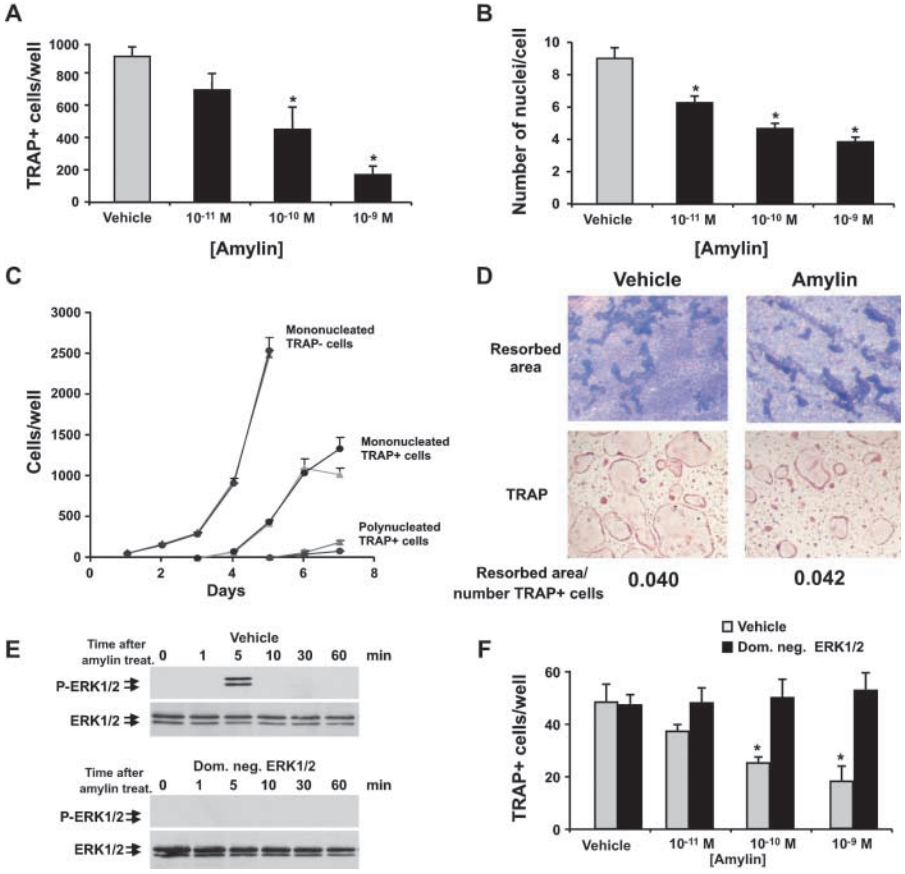

Figure 3. **Amylin inhibits osteoclastogenesis ex vivo.** (A) In vitro differentiation of osteoclasts is inhibited by amylin at a physiological concentration (10^{-10} M). (B) Multinucleated osteoclasts differentiating in the presence of amylin are smaller and have fewer of nuclei. (C) BMMs proliferation and early steps of osteoclast differentiation was not affected by amylin. (●) 10^{-10} M amylin; (▲) vehicle. (D) Dentine slice resorption assay in the presence of vehicle or physiological levels of amylin. Resorption is decreased by the presence of amylin in a manner proportional to the decrease in the number of osteoclasts. (E) In vitro differentiation of ERK1/2 dominant negative infected and noninfected osteoclasts in presence of amylin. ERK1/2 is phosphorylated 5 min after stimulation by amylin in noninfected culture, whereas this phosphorylation is abolished in ERK1/2 dominant negative infected cells. (F) Amylin inhibition of osteoclastogenesis is abolished in ERK1/2 dominant negative infected culture. Error bars represent SEM. The asterisks indicate a statistical difference ($t \leq 0.05$) between vehicle and treated culture.

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mice. That *Calcr* expression is decreased twofold in *Calcr* +/− osteoclasts allowed us to use *Calcr* +/− mice as a true model of *Calcr* haploinsufficiency (Fig. 4 D).

Calcr +/− mice displayed a high bone mass phenotype due to an increase in bone formation, whereas bone resorption was normal (Fig. 4, G and F). This observation indicates that *Calcr* is a regulator of bone formation. *Calcitonin/Cgrp* +/− mice also had an increase in bone mass (unpublished data) suggesting that calcitonin and/or CGRP are ligands of CALCR in vivo. In contrast, *Amylin* +/− mice presented a low bone mass phenotype almost as severe as that observed in homozygous *Amylin*-deficient mice. This phenotype was also due to an increase in bone resorption, whereas bone formation was unaffected (Fig. 4, F and G; Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200312135/DC1). That the bone histological abnormalities caused by *Amylin* haploinsufficiency were opposite to those observed in *Calcr* +/− mice, indicates that the CALCR is not the main receptor through which amylin affects osteoclastogenesis in vivo. This notion was further established by the fact that compound heterozygote mice for *Amylin* and *Calcr* inactivation presented an increase in osteoclast number similar to the one observed in *Amylin* +/− mice and an increase in BFR similar to the one observed in *Calcr* +/− mice (Fig. 4, F and G).

Thus, to date, amylin is the only physiological regulator of bone resorption among the members of the calcitonin family of peptide. The fact that *Amylin* +/− mice develop a low bone mass phenotype illustrates how important this hormone is for the regulation of bone resorption in vivo. The observation that amylin is a physiological inhibitor of bone resorption has medical relevance. Indeed, the predominant expression of *amylin* in pancreatic β cells support the hypothesis that the low bone mass phenotype observed in Type I diabetes patients (Levin et al., 1976) may be secondary, at least in part, to the absence of amylin secretion in these patients. This hypothesis can now be tested in animal models. In view of the important role that amylin plays in the control of bone resorption, it is now necessary to identify a specific amylin receptor in order to fully understand how this novel pathway regulates osteoclastogenesis in vivo.

Materials and methods

Biochemistry

Parathyroid hormone (PTH) was measured using a mouse intact PTH ELISA kit (Immunotopics). Insulin and glucose were measured after 6 h of fasting using the immunoassay kit purchased from Peninsula Laboratories and the Accu-Check glucometer (Roche). Deoxypyridinoline cross-links and creatine were measured in evening urines using the Pyrilinks-D immunoassay and creatine kit (Metra Biosystems).

Morphological and histological analyses

X rays were performed using a Faxitron (Philips). For histological analyses, mice were injected with 25 mg/kg calcein 10 and 2 d before sacrifice and undecalcified bones were embedded in methylmethacrylate according to standard protocols (Parfitt et al., 1987). Measurements were performed using a microscope (model DMLB; Leica), a 3CCD color video DXC-390

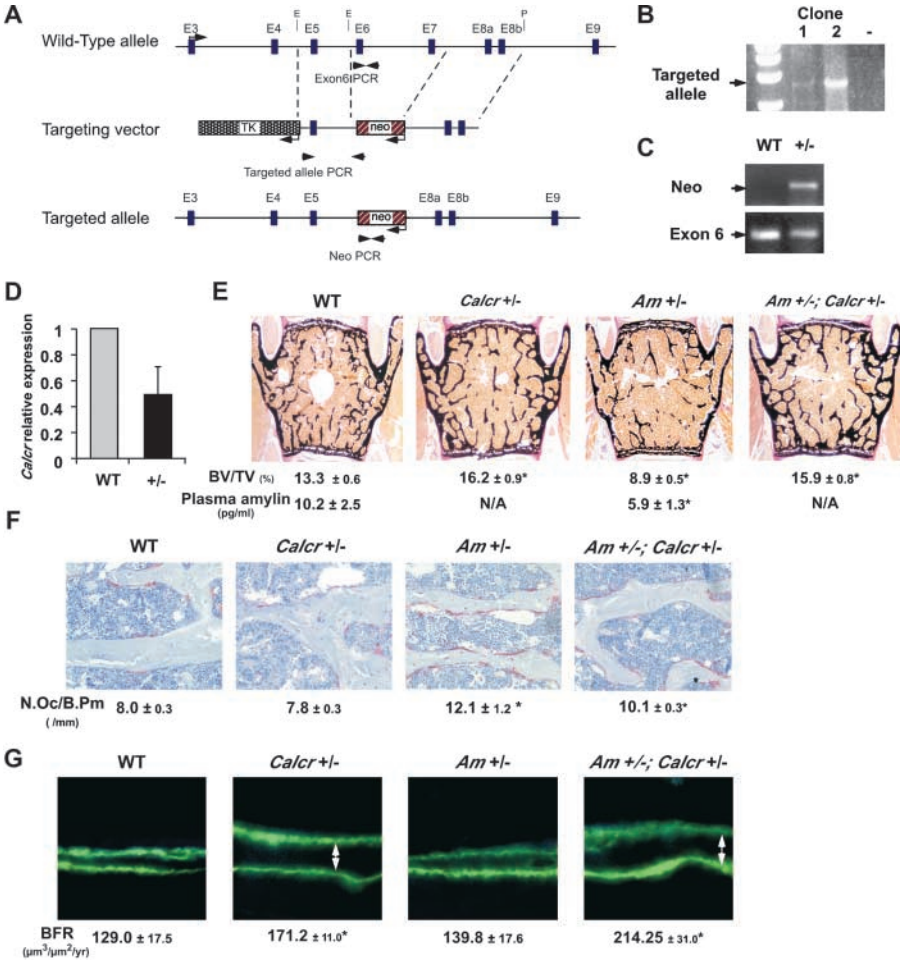

Figure 4. **Different bone phenotypes in *Amylin* +/− and *Calcr* +/− mice.** (A) Targeted disruption of *Calcr* (arrowheads represent primers). (B) PCR analysis using primers specific for the targeted construct confirming efficient recombination at the *Calcr* locus. (C) Presence of the *Calcr* targeted allele was assessed by PCR. (D) Decreased *Calcr* expression in *Calcr* +/− compared with WT osteoclasts as measured by real time PCR. (E) Bone volume over tissue volume (BV/TV) is increased in *Calcr* +/−, decreased in *Amylin* +/− (*Am* +/−) mice, and increased in *Am* +/−; *Calcr* +/− mice compared with WT littermates. (F) Osteoclasts number per bone perimeter is normal in *Calcr* +/− mice and increased in both *Amylin* +/− and *Am* +/−; *Calcr* +/− compared with WT mice. (G) BFR is increased in *Calcr* +/−, and *Am* +/−; *Calcr* +/− mice, whereas it is normal in *Amylin* +/− mice. Asterisks indicate statistically significant differences ($t \leq 0.01$) between WT and mutant mice ($n \geq 6$). Error bars represent SEM.

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camera (Sony) and the OsteoMeasure Analysis System (Osteometrics). Statistical differences between groups were assessed by *t* test.

Cell biology and protein chemistry

For in vitro osteoclast differentiation, bone marrow of 6–8-wk-old C57Bl6 mice were flushed and the monocyte fraction isolated by centrifugation on a lymphocyte separation medium gradient (ICN Biomedicals). Cells were washed and seeded at 2,500 cells/mm^2 and cultured for 7 d in differentiation medium: α-MEM containing 10% FCS (Invitrogen), 30 ng/ml M-CSF (R&D Systems), and 50 ng/ml soluble recombinant RANKL (Sigma-Aldrich). TRAP staining was performed as described previously (Suda et al., 1997). To assess bone resorption, bone marrow monocytes were plated on dentine discs (ALPCO Diagnostics) and cultured for 9 d in differentiation medium. Cells were removed by immersion in 0.5 M ammonium hydroxide and dentine slices stained with Toluidine blue. Resorption areas were analyzed using osteomeasure software.

Osteoclast infection

BMMs were isolated over a gradient of LSM and 5×10^{-6} cells were plated in α-MEM supplemented with 10% FBS and 100 ng/ml M-CSF (R&D Systems). After 2 d, cells were infected with ERK1/2 dominant retrovirus (a gift from R. Faccio and S. Teitelbaum, Washington University School of Medicine, St. Louis, MO) for 24 h in the presence of 100 ng/ml M-CSF and 4 μg/ml of polybrene (Sigma-Aldrich). Infected cells were cultured for an additional 2–3 d. For in vitro differentiation, 5,000 cells/well were plated in 96 well plates in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL (Sigma-Aldrich). After 5 d, cells were stained for TRAP activity. For Western blot analysis using P-ERK1/2 and ERK1/2 antibodies (Cell Signaling Technology), osteoclasts were lysed in RIPA buffer containing 5 mM iodoacetamide, 10 mM NaF, and 0.4 mM Na_2VO_4 with protease inhibitor cocktail (Roche).

Mice

Generation of *Amylin*-deficient mice has been described previously (Gebre-Medhin et al., 1998). For the generation of *Calcr*-deficient mice, a neomycin cassette was inserted in reverse orientation into the *Calcr* allele in place of exons 6 and 7 of mouse *Calcr* (Anusaksathien et al., 2001). After electroporation of the targeting construct into embryonic stem cells and subsequent G418/Gancylcovir selection, surviving clones were isolated and screened by PCR using *Neo* and *Calcr* exon 5–specific primers. Heterozygous mice were obtained from these embryonic stem cells by standard procedures. PCR genotyping was performed on tail DNA using *Neo* and *Calcr* exon 6–specific primers. For *Calcr* expression analysis, RNA was isolated from osteoclasts generated from WT or *Calcr* +/− BMMs precursors using TRIzol and reverse transcribed using the superscript II kit (Invitrogen). 12.5 ng total RNA equivalent was analyzed by TaqMan technology (primer and probe Mm00432271_m1) using an ABI7000 apparatus (Applied Biosystems). *Calcr* relative expression value was obtained after normalization to rodent *Gapdh* expression (primer and probe 4308329).

Online supplemental material

Fig. S1 provides evidence of the normal osteoblast biology in Amylin-deficient mice. Fig. S2 shows histomorphometric analysis of Amylin +/− mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200312135/DC1.

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