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Assessing Constitutive Activity of Extracellular Calcium-Sensing Receptors *In Vitro* and in Bone

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Abstract

Constitutive activity of the extracellular calcium-sensing receptor (CaSR) has been studied in kindreds with the human disorder autosomal dominant hypocalcemia (ADH) and in an animal model called the *Nuf* mouse. These families generally showed reduced parathyroid hormone (PTH) secretion and excessive renal calcium (Ca^{2+}) excretion. Soft tissues calcifications in the kidney and basal ganglia are frequent (10–50% of ADH cases), and there is a single report of skeletal abnormalities in a family resulting in short stature and premature osteoarthritis. In the latter, a causative mechanism could not be determined. The phenotype of the *Nuf* mouse is one of ectopic calcifications and cataracts in addition to biochemical abnormalities (low serum Ca^{2+} and high serum phosphate concentrations). To better understand the role of CaSRs in the control of osteoblastic function, we generated a transgenic mouse model with constitutively active CaSRs in mature osteoblasts. An analysis of the skeletal phenotype of that mouse indicates that strong signaling by CaSRs in this cell lineage induces alterations in the bone homeostasis reflected in mild osteopenia in male and female mice during growth and in adulthood. These studies indicate that this approach can be readily adapted to assess CaSR actions in other cell systems.

1. Introduction

Studying the relationship between the serum $[\text{Ca}^{2+}]$ and parathyroid hormone (PTH) gave rise to the concept of a membrane mechanism for mediating sensing changes in the extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_e$). Small increases in the $[\text{Ca}^{2+}]_e$ within the physiological range inhibit PTH secretion and cell proliferation. These events are transduced via multiple G protein-mediated signaling pathways, including the stimulation of phospholipase C, A2, and D (PLC, PLA2, and PLD) activities, intracellular Ca^{2+} release, and mitogen-activated protein (MAP) kinase activity. High $[\text{Ca}^{2+}]_e$ also inhibit adenylate cyclase activity in acutely dispersed bovine parathyroid cells (PTCs; Hofer and Brown, 2003). Cloning of the extracellular calcium-sensing receptor (CaSR) cDNA from bovine parathyroid glands (PTGs) established the molecular basis for extracellular Ca^{2+} -sensing. When expressed in exogenous cell systems after cDNA transfection, CaSRs couple minute changes in $[\text{Ca}^{2+}]_e$, in the sub-millimolar range, to signaling cascades similar to those in PTCs (Brown *et al.*, 1993).

Generalized or conditional knockout in PTCs of the *Casr* gene produces hypercalcemia and hyperparathyroidism (HPT) in mice due to the inability of the cells to respond to elevated serum $[\text{Ca}^{2+}]$ (Ho *et al.*, 1995). In humans, activating and inactivating CaSR mutations induce hypocalcemia and hypercalcemia, respectively. PTH levels are low (or inappropriately normal) in patients with activating mutations and high (or inappropriately normal) in patients with inactivating mutations (Egbuna and Brown, 2008). These observations clearly support the critical functional role of the CaSR in mediating Ca^{2+} -sensing and secretion in PTCs. The phenotype of the global CaSR knockout mouse and these genetic disorders of Ca^{2+} -sensing further establish the central role of the CaSR in renal

Ca^{2+} handling. Inactivating mutations in the CaSR are associated with hypocalciuria, while activating mutations are accompanied by often profound hypercalciuria.

A mouse model of *Casr* activation has suggested potential roles for activated CaSRs *in vivo*. The *Nuf* model comprises *Nuf*⁺ (heterozygous) and *Nuf*⁺*Nuf*⁺ (homozygous) mice with one or both *Casr* alleles carrying a substitution of Leu at position 723 with Gln, respectively (Hough *et al.*, 2004). *Nuf*⁺*Nuf*⁺ mice demonstrate severe hypocalcemia, hyperphosphatemia, and low PTH levels. These animals also manifest sudden death, cataracts, and ectopic calcifications throughout its tissues. An elevated $\text{Ca} \times \text{phosphate}$ product may be responsible for these calcifications, or alternatively, excessive and uncontrolled CaSR signaling may produce these pathologic soft tissue calcifications. Families with heterozygous activating CaSR mutations also show basal ganglia and renal calcifications which may be due to the same factors. These observations shed light on the phenotype of global CaSR activation but do address the role of CaSRs in specific tissues.

CaSRs are broadly expressed in tissues outside the PTG, including bone, cartilage, brain, kidney, skin, breast, gastrointestinal tract, and smooth and cardiac muscle. Understanding the role of CaSRs in these tissues using generalized CaSR knockout mice (CaSR^{+/-} or CaSR^{-/-}) is challenging. At baseline, these mice have chronic mild to severe HPT (Ho *et al.*, 1995). Their HPT obscures the role of CaSRs in tissues like bone and cartilage because high PTH levels can affect those target organs. Assessment of CaSR functions selectively in these and other tissues is now feasible, owing to a newly developed floxed CaSR mouse model (Chang *et al.*, 2008). Using this mouse model, we demonstrated that CaSRs in osteoblasts and chondrocytes are critical regulators of skeletal development.

Based on its topology and sequence homology (Fig. 13.1), the CaSR is classified in family C of the G protein-coupled receptor (GPCR) superfamily. This receptor has a large extracellular domain (ECD, ~600 amino acids), the classic seven-transmembrane domain (7-TMD, ~250 amino acids), and a long carboxyl-terminal tail (C-tail, ~200 amino acids) (Brown *et al.*, 1993; Chang and Shoback, 2004; Hofer and Brown, 2003). The CaSR likely functions as a dimer (homo- or heterodimer) like other family C GPCRs. Both dimerization and glycosylation are critical steps for efficient cell-surface targeting and ligand binding to the receptor.

As a typical GPCR, the 7-TMD of the CaSR has three extracellular (EC1–3) and three intracellular (IC1–3) loops that are responsible for transducing the extracellular stimulus (after ligand binding) into intracellular signals by interacting with different G-protein subunits (Gαq, Gαi, Gα11, Gβγ, etc.). Mutational analysis of CaSR mutants revealed amino acid residues in IC2 and IC3 that are critical for PLC signaling and efficient cell-surface expression of the receptor (Chang *et al.*, 2000). Substitutions of specific residues in these receptor domains with alanine or nonconserved amino acids altered efficiency of the CaSR's coupling to downstream effectors. Studies of CaSRs in which the carboxyterminal tail is either truncated or mutated revealed the importance of an alpha-helical structure in the N-terminus of the tail, for both signaling and cell-surface expression of the receptor (Chang *et al.*, 2001). Others have shown that there is a filamin-A binding domain in the middle of the C-tail that is required for interaction with the cytoskeleton and for the formation of signaling complexes with other intracellular proteins (Hofer and Brown, 2003).

Family C GPCRs are thought to have evolved from the bacterial periplasmic proteins (PBPs) based on the homology of protein sequences in their ECDs. The PBPs contain a Venus Flytrap (VFT) domain that binds and transports nutrients such as Ca^{2+} , Mg^{2+} , and amino acids across the periplasm and the peptidoglycan mesh (Brown *et al.*, 1993; Chang and Shoback, 2004; Hofer and Brown, 2003; Huang *et al.*, 2009; Silve *et al.*, 2005; Wellendorph

and Brauner-Osborne, 2009). Crystallography and computer modeling reveal a VFT-like structure in the ECD of the CaSR (residues 1–540). The CaSR-VFT domain contains three Ca^{2+} -binding sites that include several amino acids in the N-terminus of the ECD (between residues #100 and #300) (Fig. 13.1). The efficiency of Ca^{2+} binding to these sites depends on the overall tertiary structure of the ECD that is maintained by intra- and intermolecular disulfide bonding, interactions of hydrophobic and hydrophilic domains, and more importantly, local ionic strength. Any substitution of an amino acid residue within or adjacent to Ca^{2+} -binding sites that alters the conformation of the ECD will likely alter the Ca^{2+} binding of the receptor. Furthermore, amino acids, aminoglycosides, polyamines, pH, and salinity, which alter local ionic strength, can also potentiate or suppress the response of receptor to Ca^{2+} , potentially by altering the conformation of ECD and its affinity to Ca^{2+} .

The biological significance of mutations in the CaSR is clearly demonstrated by three genetic disorders—familial benign hypocalciuric hypercalcemia (FBHH), neonatal severe HPT (NSHPT), and autosomal dominant hypocalcemia (ADH). Heterozygous and homozygous inactivating (loss-of-function) mutations produce FBHH and NSHPT, respectively, while activating (gain-of-function) mutations cause ADH (Egbuna and Brown, 2008; Huang *et al.*, 2009; Pidasheva *et al.*, 2004; <http://www.casrdb.mcgill.ca>). The majority of these naturally occurring mutations are found in the ECD of the CaSR (Fig. 13.1). In contrast, the *Nuf* mouse has mutations in a CaSR residue in the fourth transmembrane domain of the receptor (Hough *et al.*, 2004). These CaSR mutations, in general, cause a shift (to the right for the inactivating mutations and to the left for the activating mutations) in their Ca dose–response curves when expressed in the exogenous cell systems, further underscoring the role of ECD in Ca^{2+} -binding.

The above disorders predominantly affect parathyroid and renal function. The CaSR is, however, expressed in many other tissues, but it has been difficult to determine what role the CaSR plays in these tissues on the basis of these disease phenotypes. Growing interest has focused on exploring the functional impact of the CaSR in different systems using cultured cells. Many of these studies support a role for CaSRs in modulating distinct cell functions depending on the system. Translating the findings from *in vitro* studies to the biological actions of the CaSR *in vivo* has been challenging. To address this, we and others have begun to study gain-of-function and loss-of-function in mouse models. The tissue-specific, conditional knockout approach using Cre-lox recombination is being used to delete CaSRs from specific cell populations *in vivo*. Using another strategy, we have developed a transgenic mouse model with tissue-specific overexpression of the CaSR in mature osteoblasts to study the gain-of-function of CaSR signaling in this cell type. This report describes methods to generate mice that overexpress a constitutively active CaSR in mature osteoblasts to investigate the role of CaSR in bone remodeling.

2. Materials and Methods

2.1. Construction of activating CaSR mutant constructs

A pcDNA3.1 vector (Wt-CaSR/pcDNA3.1) containing a bovine CaSR cDNA was subjected to site-directed mutagenesis using Chameleon Mutagenesis Kit (Stratagene, La Jolla, CA) and a set of selection ($5'$ -CCGCTGGAGAAGACGACAAT- $3'$) and mutagenic ($5'$ -CCACCCTG AGTTTTGTG GCCCGATCAAGATTGACCCTTTGAAGCTTGGTGAGTTCTGC A- $3'$) primers to make the Act-CaSR/pcDNA3.1 vector which encodes five point mutations [Q118P; N119I; S123P; N125K; and D127G] in the ligand-binding region of the receptor. This mutant was previously identified by Jensen *et al.* (2000) using a random saturation mutagenesis approach and shown to exert constitutive activity in transfected NIH-3T3 and tsA (a transformed HEK-293 cell) cell lines. Wt-CaSR/pcDNA3.1 and Act-CaSR/pcDNA3.1

vectors were amplified in TOP-10 *Escherichia coli*, purified using a Qiagen Maxi-Prep Kit (Qiagen, Valencia, CA), and stored in a sterile Tris–HCl buffer (10 mM, pH 8.5) at a concentration of 1 µg/µl.

2.2. Cell transfection

HEK-293 cells were cultured in T75 flasks containing growth medium (Dulbecco's Minimal Essential Medium, containing 10% fetal calf serum and penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 °C in 5% CO₂ until confluence). Confluent cells were split (1:6) into fresh T75 flasks containing 10 ml of growth medium, cultured for 4–6 h, and then transfected with CaSR cDNA using Ca–phosphate precipitation method (Chang *et al.*, 2000, 2001). Ten micrograms of vector DNA was first mixed with 0.5 ml of 0.25 M CaCl₂. The DNA/CaCl₂ mixture was then added dropwise over 60 s to 0.5 ml of twofold concentrated HES buffer (in mM, 280 NaCl, 10 KCl, 1.5 NaH-PO₄·2H₂O, 12 dextrose, 50 HEPES (pH 7.5)) in a 15 ml test tube with gentle agitation by swirling the tube continually. The resulting mixture was incubated for 15 min at room temperature and then added to the culture flasks. After 18–24 h incubation at 37 °C in 5% CO₂, transfected cells were trypsinized and plated into wells of a 6-well Falcon culture plate (Nunc, Rochester, NY). Cells from one T75 flask were dispensed into a total of 24 wells and cultured for 24–48 h before assays.

2.3. Inositol phosphate (InsP) assays

Transfected HEK-293 cells were prelabeled with [³H]myoinositol (2 mCi/ml; Amersham, Piscataway, NJ) for 18–24 h in the growth media (Chang *et al.*, 2000, 2001). After three washes with fresh growth media, cells were pretreated with LiCl (to block Ins polyphosphate 1-phosphatase activity) in a MEM–LiCl solution [Eagle's modified essential medium with Earle's salts custom-prepared without CaCl₂, MgSO₄, and NaHCO₃ and supplemented with 0.5 mM CaCl₂, 0.5 mM MgSO₄, and 10 mM LiCl] for 15 min at 37 °C. For experimental treatments, media were replaced with fresh MEM–LiCl media supplemented with different concentrations of CaCl₂ (0.1–10 mM) and incubated for 60 min at 37 °C. Total ³H-InsPs were extracted from cells and quantitated by anion-exchange chromatography (Chang *et al.*, 1998).

2.4. Expression of Act-CaSR in osteoblasts

In vivo expression of the Act-CaSR transgene was driven by the 3.5 kb human osteocalcin (OC) promoter which is active in mature osteoblasts (Dvorak *et al.*, 2007). Act-CaSR cDNA was subcloned from the Act-CaSR/pcDNA3.1 vector into ClaI/XbaI sites between the OC promoter and a bovine growth hormone poly A (bGH-poly A) sequence in the pBluescript II vector (Fig. 13.2). The transgene containing the OC promoter, Act-CaSR, and bGH-poly A was released by digestion with Kpn I restriction enzyme, purified using Qiagen Gel Extraction Kit (Qiagen, Valencia, CA), and stored in 10 mM Tris–HCl buffer (pH 8.5). The DNA was microinjected into the pronuclei of fertilized ova harvested from the oviducts of newly plugged FVB/N mice by standard techniques at the University of California San Francisco Transgenic Core Facility. Surviving microinjected embryos were reimplanted into the oviduct of pseudopregnant female recipients. After birth, transgenic FVB/N mice were maintained under standardized environmental conditions, with access to food and water *ad libitum*. Genotyping of tail DNA was performed using two sets of transgene-specific primers (set 1: 5'-GTGCTGCCTCCGCCACTGAT-3' and 5'-GCCCCACCTGCTGCTTTGAGT-3', spans end of OC promoter and 5' end of transgene amplifying a 1379 bp fragment; set 2: 5'-GGGTATGGTGCGGAGGAAGG-3' and 5'-TTGTGCTGCCCCGAC CCTTTC-3', spans end of OC promoter and 3' end of transgene amplifying a 1539 bp fragment). All protocols were approved by the Animal Care Committee of the San Francisco Veterans Affairs Medical Center.

2.5. Assessment of Act-CaSR expression in transgenic osteoblasts: Immunoblotting

Crude membrane proteins were prepared from cultured bone marrow stromal cells from Wt and transgenic Act-CaSR mice as described previously (Dvorak *et al.*, 2007). Immunoblotting with a polyclonal anti-CaSR antiserum and peroxidase-conjugated anti-rabbit IgG secondary antibodies (GE Healthcare, Little Chalfont, UK) was performed as described previously (Chang *et al.*, 1998). Specificity of the antiserum was confirmed by the absence of signal after preincubating anti-CaSR antiserum with the peptide against which it was raised.

2.5.1. Quantitative PCR (qPCR)—Femora of 6-week-old Wt and Act-CaR littermates were isolated and separated into epiphyseal/metaphyseal and diaphyseal compartments. Bone marrow was flushed out with PBS before bones were frozen in liquid nitrogen. For RNA isolation, the bones were powdered using a multisample biopulverizer (RPI, Mt. Prospect, IL) and homogenized with a rotor-stator homogenizer (Polytron PT 3000; Brinkmann Instruments, Westbury, NY) in RNA-Stat reagent (Tel-Test, Friendswood, TX). cDNAs were synthesized with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA), and expression of Act-CaSR transgene was quantified using TaqMan qPCR kits and ABI PRISM 7900HT Sequence Detection System with SDS software (Applied Biosystems Foster City, CA). A threshold cycle (number of PCR cycles required to generate a fluorescent signal exceeding a preset threshold) was determined for the Act-CaSR transgene and normalized to the threshold cycle for a housekeeping gene (L19) in the same sample. Primers and probes for Act-CaR (forward primer: 5'-AGGCCAGCTGCTCGAGAGT-3', reverse primer: 5'-CTTGAGTCTTCAGAAGTCACATCATG-3', and probe: 5'-FAM-ACTCAGCTCAGCAGCTGGGAAGC-BHQ-3') were custom made by Integrated DNA Technologies (Skokie, IL) according to published nucleotide sequences.

2.6. Assessment of skeletal phenotypes in transgenic mice

The approach used to monitor temporal changes in bone mass and structure was *in vivo* microcomputed tomography (μ CT) because it allows quantitation of mineralized tissue and assessment of three-dimensional structural parameters in live animals over time. μ CT scans were performed on transgenic Act-CaSR versus Wt mice using a SCANCO VivaCT 40 scanner (SCANCO Medical, Bassersdorf, Switzerland). Animals were anesthetized with 2% isoflurane (Baxter Healthcare Corporation, Deerfield, IL), mixed in oxygen, and scanned every 6 weeks from 6 to 30 weeks of age. To examine trabecular bone, 100 serial cross-sectional scans (1.05 mm) of the secondary spongiosa of the left distal femoral metaphysis were obtained, from the end of the growth plate extending proximally to the shaft. The isotropic voxel (volumic pixel) size was 10.5 μ m, and X-ray energy was 55 kV. A global threshold (27.5% of the grayscale) was applied to segment mineralized from soft tissue in the μ CT images. Linear attenuation was calibrated using preset hydroxyapatite blocks. 3-D image reconstruction and analysis were performed using software provided by the manufacturer.

2.7. Statistics

Data from two groups were compared using unpaired Student's *t* test. Data from multiple groups were compared using ANOVA with Tukey's *post hoc* analysis. Significance was assigned for $p < 0.05$.

3. Results

3.1. Assessment of activity of Act-CaSR *in vitro*

In principle, the activity of mutant CaSRs can be assessed by their ability to stimulate PLC, raise the intracellular $[Ca^{2+}]_i$, enhance MAPK activity, and inhibit cAMP production. However, measurement of PLC activity and intracellular Ca^{2+} mobilization are more standard methods for assessing receptor activation because they are straightforward, economical, and adaptable to high-throughput assays. More importantly, these methods can quantify changes in the efficacy of CaSR's response to its ligand, in this case, high $[Ca^{2+}]_e$.

By measuring the accumulation of total InsPs as an index of PLC activation, we observed different signaling responses in HEK-293 cells expressing Wt-CaSR versus Act-CaSR cDNAs (Fig. 13.3). We observed a clear left-shift in the EC_{50} of the Ca dose-response curves from ≈ 3.5 mM in Wt-CaSR-expressing cells to < 0.3 mM in Act-CaSR-expressing cells. We also observed an elevation of basal activity at 0.1 mM Ca^{2+} in Act-CaSR-expressing versus Wt-CaSR-expressing cells, compatible with the notion that this was a constitutively active receptor mutant.

3.2. Transgenic expression of Act-CaSR in osteoblasts

Extensive biochemical and mutational analyses have identified CaSR mutants that demonstrate constitutive activity (Egbuna and Brown, 2008; Pidasheva *et al.*, 2004). Little is known about whether and how these mutants impact cellular functions outside the PTGs *in vivo*, especially in bone and cartilage, because the resulting alterations in parathyroid function (hypoparathyroidism) can affect those tissues directly. Our transgenic approach, using a highly cell-specific OC promoter to express the Act-CaSR in mature osteoblasts, allowed us to target the mutant receptor to bone and avoid the metabolic changes of a more generalized promoter. Indeed in two transgenic Act-CaSR mouse lines that we established, we observed no significant differences in levels of serum Ca^{2+} or PTH between Wt and Act-CaSR transgenic mice (Dvorak *et al.*, 2007).

We further confirmed targeting of the transgene to bone by qPCR analysis of RNA extracted from femurs and calvariae but not in the other tissues tested (Fig. 13.2). Both the epiphyseal/metaphyseal and diaphyseal femoral compartments, which primarily contain cancellous and cortical bone, respectively, exhibited significant transgene expression (Fig. 13.2). Immunoblotting of crude membrane fractions of cultured bone marrow stromal cells from Act-CaSR mice revealed immunoreactive bands corresponding to the bovine CaR (120, 140, and 150 kDa) (Fig. 13.2).

3.3. Skeletal phenotype of transgenic Act-CaSR mice

By *in vivo* μ CT scanning, we observed profound changes in trabecular bone of the transgenic Act-CaSR mice, from 6 to 30 weeks of age (Fig. 13.4). Act-CaSR mice consistently showed reduced trabecular bone mass (Fig. 13.4) as indicated by the decreased ratio of total bone volume (BV) over total tissue volume (TV) (BV/TV) and an osteopenic phenotype supported by the reduced bone mineral density (BMD; Fig. 13.4). Reduced bone mass in the Act-CaSR mice was mainly due to decreases in the number and connectivity of trabecular bones and increases in trabecular spacing. Similar bone phenotypes were observed in both male and female mice. These data suggest that constitutive activity of CaSRs in osteoblasts is detrimental to skeletal function and causes osteopenia likely due to increased bone breakdown. Bone histomorphometric parameters (increased osteoclast surface per eroded surface) further support that conclusion (Dvorak *et al.*, 2007).

4. Conclusions

While human activating mutations uncovered during the genetic evaluation of individuals with ADH (hypocalcemia, low PTH levels, and hypercalciuria) demonstrate the prominence of the CaSR in regulating parathyroid and renal function, it has been difficult to discern the role of CaSRs in other tissues from studying these families. Mouse models of generalized knockout of the CaSR are dominated by the mild to severe disturbances in serum $[Ca^{2+}]$ and PTH levels that have secondary effects on skeletal tissues. This report describes the analysis in transgenic mice of a constitutively activated CaSR mutant targeted to mature mineralizing osteoblasts through the use of a well-defined OC promoter. In the absence of abnormalities in serum $[Ca^{2+}]$ and PTH, this strategy demonstrated that the CaSR is likely playing a role in controlling the balance of bone remodeling—modulating levels of bone resorption most likely. Use of this and other well-defined mutants in transgenic models should give additional information relevant to understanding CaSR function outside the PTG.

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Figure 13.1.

Topology of the bovine CaSR. The legend (lower left) shows symbols representing naturally occurring activating (gain-of-function) mutations causing ADH and inactivating (loss-of-function) mutations responsible for FBHH. Modified, with permission, from Brown *et al.* (1993).

Figure 13.2.

Expression of Act-CaSR transgene in osteoblasts. (A) Act-CaSR transgenic construct containing a 3.5 kb fragment of the human OC promoter, Act-CaSR coding sequence, and bovine growth hormone poly A (bGH-poly A) signal sequence in a pBluescript II SK vector. (B) qPCR confirmed the expression of Act-CaSR RNA in bone (distal femur, epiphysis/metaphysis, femoral midshaft, and calvaria) but not in kidney and brain in transgenic Act-CaSR mice or in any tissue tested in Wt mice. RNA expression was normalized to the expression of the housekeeping gene L19. (C) Immunoblotting for the CaSR was performed on crude membrane proteins of bone marrow stromal cells (75 μ g) cultured from Wt and Act-CaSR mice, using polyclonal antibodies against the ECD of the bovine parathyroid CaR (Brown *et al.*, 1993). Reproduced with permission from Dvorak *et al.* (2007).

Figure 13.3.

Effects of raising $[Ca^{2+}]_e$ from 0.1 mM to the concentrations shown on total 3H -InsP accumulation in HEK-293 cells transiently expressing cDNAs encoding Wt-CaSR and Act-CaSR. Results are expressed as the percentage of the maximal response at 10 mM Ca^{2+} in cells expressing either Wt-CaSR or Act-CaSR as noted ($N = 4$ independent transfections). Data were reanalyzed from experiments previously reported (Dvorak *et al.*, 2007).

Figure 13.4.

In vivo μ CT scans to investigate changes in trabecular bone mass and structure in Wt versus Act-CaSR mice. μ CT analysis was performed on the distal femur of 6–30-week-old female mice. (A) Three-dimensional μ CT image reconstruction of trabecular bones from 12-week-old Wt and transgenic Act-CaSR mice. (B) Structural parameters derived from the μ CT analyses, including trabecular bone volume fraction (BV/TV), bone mineral density (BMD), trabecular number (Tb.N), trabecular spacing (Tb.Sp), trabecular thickness (Tb.Th), and connectivity density (Conn. D). Data represent mean $\pm$ SE ($n = 8$ mice per group; ANOVA; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Reproduced with permission from Dvorak *et al.* (2007).