No evidence for a bone phenotype in GPRC6A knockout mice under normal physiological conditions

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Abstract

GPRC6A is a seven-transmembrane receptor mediating signaling by a wide range of L- α -amino acids, a signaling augmented by the divalent cations Ca^{2+} and Mg^{2+} . GPRC6A transcripts are detected in numerous mammalian tissues, but the physiological role of the receptor is thus far elusive. Analogously to the closely related calcium-sensing receptor, GPRC6A has been proposed to function as a metabolic sensor of Ca^{2+} and amino acids in bone and other tissues. In the present study, we have generated the first GPRC6A knockout mice and studied their phenotype with particular focus on bone homeostasis. The generated GPRC6A knockout mice are viable and fertile, develop normally, and exhibit no significant differences in body weight compared with wild-type littermates. Assessment of bone mineral density, histomorphometry, and bone metabolism demonstrated no significant differences between 13-week-old knockout and wild-type mice. In conclusion, our data do not support a role for GPRC6A in normal bone physiology.

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Introduction

The 'G protein-coupled receptor family C subtype 6A', GPRC6A, is the most recently identified member of family C in the superfamily of seven-transmembrane (7TM) receptors, also termed G protein-coupled receptors. Family C mammalian receptors also include the metabotropic glutamate (mGlu), γ -aminobutyric acid_B (GABA_B), T1R taste and calcium-sensing receptors. Aside from the 7TM domain governing G protein activation, family C receptors also possess a large amino-terminal domain containing the orthosteric binding site (Pin *et al.* 2003, 2004).

To date, the *in vivo* functions of GPRC6A have not been investigated, and the physiological importance of the receptor is thus unknown. Cloning and pharmacological characterization of human, mouse, and rat GPRC6A orthologues have found the receptor to be stereoselectively activated by natural L- α -amino acids, preferentially basic amino acids L-arginine, L-lysine, and L-ornithine, but also small and polar amino acids (Wellendorph & Bräuner-Osborne 2004, Kuang *et al.* 2005, Wellendorph *et al.* 2005, 2007, Christiansen *et al.* 2006a,b). Sensitivity to such a broad range of amino acids could possibly reflect the physiological role of the

receptor (Conigrave & Hampson 2006, Bräuner-Osborne *et al.* 2007, Conigrave *et al.* 2008). Using heterologous expression systems, we and others have additionally found that the L- α -amino acid response of GPRC6A is augmented by the presence of divalent cations Ca^{2+} and Mg^{2+} in physiologically relevant concentrations (Kuang *et al.* 2005, Christiansen *et al.* 2007, Wellendorph *et al.* 2007). Quarles and co-workers have reported the mouse GPRC6A (mGPRC6A) to be directly activated by high concentrations of Ca^{2+} in the absence of L- α -amino acids (Pi *et al.* 2005). Regardless of whether Ca^{2+} is a direct agonist or a potentiator of GPRC6A signaling, the concomitant binding of amino acids and Ca^{2+} appears to be a general characteristic of family C receptors. The orthosteric binding sites of family C receptors are highly conserved (Silve *et al.* 2005, Kuang *et al.* 2006) and also mGlu and GABA_B receptor signaling has been shown to be allosterically modulated by Ca^{2+} (Kubo *et al.* 1998, Saunders *et al.* 1998, Wise *et al.* 1999, Conigrave *et al.* 2000, Galvez *et al.* 2000, Francesconi & Duvoisin 2004).

GPRC6A exhibits a remarkable similarity to the calcium-sensing receptor (CaR) with regard to phylogeny, genomic organization, wide expression in tissues and organs, and ligand preferences. As inferred from

phylogenetic analyses of human family C receptors, GPRC6A has the highest overall amino acid sequence identity with CaR (45% overall amino acid identity) and vice versa (Kuang *et al.* 2005). Moreover, GPRC6A, CaR, and taste receptors (T1R), all of which respond to amino acids in a broad sense, appear to form a distinct subgroup of family C receptors (Bjarnadóttir *et al.* 2005, Kuang *et al.* 2006). The organization of the genes for GPRC6A and CaR is strikingly similar, each being composed of six protein-coding exons with intervening introns. In *Casr*, exon 1A/1B codes alternative 5'-untranslated regions (Chikatsu *et al.* 2000). The last exon in each receptor (exon VI in *Gprc6a* and exon VII in *Casr*) encodes the entire 7TM domain and C-terminus (Kuang *et al.* 2005). The widespread tissue expression of GPRC6A is also reminiscent of CaR (Mitsuma *et al.* 1999). Whereas CaR is activated by Ca²⁺ and Mg²⁺ and positively modulated by L- α -amino acids (Conigrave *et al.* 2000, Zhang *et al.* 2002), GPRC6A appears to function in a reciprocal fashion (Christiansen *et al.* 2007).

The ability of GPRC6A to respond to numerous amino acids and divalent cations, the close relationship with CaR, and the expression in major organs and tissues including heart, lung, kidney, liver, pancreas, skeletal muscle, and brain (Wellendorph & Bräuner-Osborne 2004, Kuang *et al.* 2005) has spurred several suggestions for the physiological context of GPRC6A. The receptor has been proposed to be involved in the regulation of the urea cycle, in sensing of Ca²⁺ and amino acids in the blood, in neurotransmission (Wellendorph *et al.* 2005, Bräuner-Osborne *et al.* 2007), and in the monitoring of cell death (Civelli 2005). The findings of more recent studies suggest additional putative physiological roles of the receptor, as GPRC6A transcripts have been demonstrated in specialized tissues such as bone and cultured osteoblasts (Pi *et al.* 2005), pancreatic islets (Regard *et al.* 2007), taste tissue (Wellendorph *et al.* 2007), and on the protein level in mesenteric arteries (Harno *et al.* 2008).

The functions maintained by GPRC6A are obviously of particular interest in tissues where L- α -amino acids and/or Ca²⁺ are known to play important physiological roles. Based on the expression of GPRC6A in long bone, calvarias, and cultured osteoblasts, G α_i /G α_q -mediated signaling in response to divalent and trivalent cations, and modulation of the signaling by the calcimimetic NPS R-568 and osteocalcin, Quarles and co-workers have proposed mGPRC6A to be an important receptor in bone (Pi *et al.* 2005). Also, considering the role of Ca²⁺ and L-amino acids in bone homeostasis and formation, this is a relevant hypothesis to address (Teitelbaum 2000, Dvorak & Riccardi 2004, Breitwieser 2008, Conigrave *et al.* 2008). Investigations into the *in vivo* functions of GPRC6A are, however, complicated by the absence of high-affinity ligands selectively targeting the receptor.

To overcome this problem and investigate the function of this receptor, we have generated a GPRC6A knockout mouse, and here for the first time report studies on phenotypic parameters with particular focus on normal bone physiology.

Materials and methods

Design and cloning of targeting vector

For the targeted disruption of *Gprc6a*, exon VI, containing the entire 7TM domain and C-terminal tail of the gene, was deleted using the cyclization recombination at a locus of α -over of the bacteriophage P1 (CRE-*Loxp*) and the yeast FLPase recombinase target (FLP-*Frt*) systems by a 2-*Loxp*-2-*Frt* strategy (Kwan 2002, Tronche *et al.* 2002) as outlined in Fig. 1 and detailed in the Supplementary material, see Supplementary data in the online version of the Journal of Molecular Endocrinology at <http://jme.endocrinology-journals.org/content/vol42/issue3/>.

Homologous recombination and generation of GPRC6A^{-/-} mice

GPRC6A^{-/-} mice were generated by homologous gene targeting in E14.1 embryonic stem (ES) cells (129P2/OlaHsd) as detailed in Supplementary material. Southern blot analysis using 5' and 3'-located hybridization probes derived from genomic sequences located outside the regions of homology was used to assess whether homologous recombination (HR) had occurred and a correctly targeted *Gprc6a* allele had been generated (Fig. 1A and C).

High-percentage chimeric females were mated with C57BL/6 males looking for germline transmission. The presence of a *Loxp*-flanked (floxed) allele was confirmed by PCR analysis for *Neo* using the specific primers (Fneo: 5'-aacaagatggattgcacgc-3' and Rneo: 5'-aagaactcgtcaagaagg-3'). To create a full knockout, males heterozygous for the *Neo*-targeted, floxed allele were mated to ubiquitously expressing C57BL/6 CRE-deleter females to obtain GPRC6A^{+/-Cre+} offspring (Schwenk *et al.* 1995). These were backcrossed into the C57BL/6 background to remove *Cre* and to produce a genetically pure offspring. For the current studies, the *Cre*-lacking heterozygotes were intercrossed to create littermates of all three genotypes (+/+, +/-, and -/-).

Genotyping

Genotyping of mice after *Cre* deletion was analyzed by PCR using gDNA extracted from tails of the mice. For the PCR, a primer located in exon VI (P1:

Figure 1 Targeted disruption of the mouse *Gprc6a* gene and its molecular analysis. (A) Targeting strategy used for the deletion of the *Gprc6a* exon VI. Partial scheme of the *Gprc6a* wild-type allele, at the top depicting exons V and VI, the intervening intron V, and inherent restriction enzyme recognition sites used for Southern analysis. Fragments used for Southern analysis are denoted as 5' and 3' probes. The targeting vector is shown below the wild-type allele showing the 2-*Loxp*-2-*Frt* design, the homology arms, and the site-directed engineered restriction enzyme sites marked by asterisks. (B) The correctly targeted *Gprc6a*^{neo} allele resulting from HR in ES cells. Indicated is the Southern analysis strategy and expected band sizes. (C) Southern analysis. Shown is a representative blot of screening for HR events in ES cells. To the left, DNA was digested with *SacI* and the 5' probe used. Wild-type (+) and *Neo*-targeted, floxed alleles (*neo*) are represented by DNA fragments of 7.0 and 5.1 kb respectively. To the right, DNA was digested with *SphI* and the 3' probe used. Wild-type (+) and targeted (*neo*) alleles are represented by DNA fragments of 12.7 and 5.8 kb respectively. (D) PCR genotyping. Example of a typical PCR of *GPRC6A*^{+/+}, ^{-/-}, and ^{+/-} mice. Primers P1 and P2 yield a 396 bp product ((+) allele) and primers P2 and P3 yield a 620 bp product ((-) allele). (E) RT-PCR products of expected sizes (698 bp; primers P4 and P5) were detected readily with *GPRC6A*^{+/+} but not with *GPRC6A*^{-/-} bone, lung, and 15-day embryo cDNA as template (bone tissue was analyzed in a separate PCR run). G3PDH gave consistent (982 bp) bands in all tissues examined. Only the shown bands were detected.

5'-CAGGTAGTGCTCCTTACTG-3') was used together with a primer located in intron V upstream of the first *Loxp* site (P2: 5'-GGAGATGGCCTTGAGCTATGTG-3'), a combination that amplified a 396 bp band from the wild-type allele and a 474 bp band from the floxed allele, whereas no band was amplified from the deleted allele. A primer located in the 3' homology arm (P3 5'-CAGTGATCACTCAGCCAGACAC-3') was used together with P2 to amplify a 620 bp band from the deleted allele. For routine genotyping, a PCR protocol

employing a mixture of P1, P2, and P3 was used (94 °C 2 min initial, then 94 °C 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min for 35 cycles) to generate fragments of 396 bp for the (+) allele and 620 bp for the (-) allele (Fig. 1D).

RT-PCR analysis

Lung and femur tissue from 17- to 19-week-old *GPRC6A*^{+/+} and ^{-/-} male mice and 15-day-old

GPRC6A^{+/+} and ^{-/-} embryos were isolated, preserved in pulverized dry ice and stored at -80 °C until further processing. The femur was pulverized in liquid nitrogen using a mortar and pestle and total RNA was extracted with RNeasy Lipid Tissue Mini Kit (Qiagen), whereas total RNA from lung and embryos was extracted using RNeasy Mini Kit (Qiagen). Total RNA was used for first-strand cDNA synthesis (QuantiTect Reverse Transcription Kit, Qiagen), according to the protocols of the manufacturer. Intron V-spanning primers specific for exons V (P4: 5'-gccctggctcaatgaagaaa-3') and VI (P5: 5'-tgatgtatgcccgcatgtga-3') were used for the RT-PCR using an optimized PCR protocol (94 °C 5 min pre-denaturation, then 94 °C 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min 20 s for 45 cycles). RT-PCR products were separated on 1% w/v agarose gels and detected with SYBR Safe (Invitrogen).

Animal care

Animals were maintained and bred in a commercial pathogen-free facility (Taconic, Silkeborg, Denmark). In general, mice were housed in a climate-controlled animal facility (25 °C, 55–60% humidity, 12h light:12h darkness cycle) and had free access to food and water. For analysis of body weight, eight *GPRC6A* wild-type and knockout mice of each gender were weighed every week from week 6 to 16, and every 2 weeks from week 18 to 38. Animal experiments for bone phenotyping were carried out in accordance with the European Communities Council Resolves of 24 November 1986 (86/609/ECC) and approved by the Danish Animal Experiments Inspectorate (J. No. 2006/561-1232).

Bone structural histology

The tibias were recovered from 13-week-old mice following killing and analyzed by tomodensitometry, histomorphometry, and densitometry. For tomodensitometry analysis, the right tibias were fixed overnight in 4% w/v formaldehyde in PBS, washed with PBS, and then stored in 70% v/v ethanol. Micro-computerized tomography (μCT) scans of the metaphyseal region were performed at an isotropic resolution of 9 μm, to obtain trabecular bone structural parameters. Using a two- and three-dimensional (3D) model and a semi-automatic contouring algorithm, we determined 3D bone volume, bone surface, and the trabecular thickness. 3D images were obtained on a Scanco Medical μCT scanner (μCT 20; Scanco Medical AG, Bassersdorf, Switzerland). A total of 450 images were obtained from each bone sample using a 512×512 matrix, resulting in an isotropic voxel resolution of 18×18×18 μm³. Measurements were stored in 3D image arrays with an isotropic voxel size of 9 μm.

A constrained 3D Gaussian filter was used to partly suppress the noise in the volumes. The bone tissue was segmented from bone marrow using a global thresholding procedure. In addition to the visual assessment of structural images, morphometric indices were determined from the microtomographic data sets. Cortical and trabecular bone were separated using a semi-automated contour-tracking algorithm to detect the outer and inner boundaries of the cortex. In trabecular bone, basic structural metrics were measured using direct 3D morphometry (Rüeggsegger *et al.* 1996, Kapadia *et al.* 1998). The images were also rendered for 3D display and visualization.

Bone densitometry

The left tibia was used for densitometric analysis with the Stratec peripheral quantitative computerized tomography (pQCT) XCT Research SA+ (version 5.4B; Norland Medical Systems, White Plains, NY, USA) at 70 μm resolution. Trabecular volumetric bone mineral density (BMD) was measured by metaphyseal pQCT scans positioned 1 mm from the distal growth plate and corresponding to 6% of the total length of the tibia. The trabecular region was defined as an inner area of 60% of the total cross-sectional area (Sims *et al.* 2003). Diaphyseal tibia cortical BMD was measured by pQCT scans positioned at 7 mm from the distal growth plate and corresponding to 42% of the total length of the tibia. Specific mode of analysis (peel 2) was used to calculate cortical density. The default algorithm removes voxels within the trabecular part that have an attenuation coefficient below the threshold of 710 mg/cm³. The inter-assay coefficients of variation (CV) for the pQCT measurements were <2%.

Bone biomarkers

For osteocalcin level measurements, blood was collected in tubes containing coagulation activator on the day of killing. Plasma and serum samples were separated by centrifugation at 3000 r.p.m. (750 g) for 20 min at room temperature and serum was stored at -20 °C until the analysis was performed. All assays were performed in duplicate. Serum osteocalcin was assayed with kits and reagents from Biomedical Technologies Inc. (Stoughton, MA, USA), as described previously (Clément-Lacroix *et al.* 2000). The intra- and inter-assay CVs were 2.0–2.3% and 4.0–5.0% respectively. At 11 weeks of age, urine was collected overnight in single-housing metabolic cages and stored at -20 °C. Urinary deoxypyridinoline cross-link levels (D-Pyr) were measured using a colorimetric assay from Pacific Biometrics Inc. (Tampa, FL, USA), and normalized to

creatinine concentration (measured by Metra creatinine assay kit (QUIDEL, San Diego, CA, USA)) to correct for water excretion as described by Sims *et al.* (2003). The intra- and inter-assay CVs were 3.1–4.8% and 4.3–8.4%, respectively.

Statistical analyses

Data were expressed as mean $\pm$ S.E.M. Statistical differences were calculated using Student's *t*-test or ANOVA for multiple comparisons. *P* < 0.05 were considered statistically significant.

Results

Generation of GPRC6A-deficient C57BL/6 mice

By analogy to the human *GPRC6A* gene, the mouse orthologue is encoded by six exons, which span approximately 16 kb of genomic DNA (Kuang *et al.* 2005). For the purpose of generating GPRC6A-deficient (*GPRC6A*^{-/-}) mice by HR in ES cells, we targeted exon VI using the site-specific CRE-*LoxP* recombinase technology (Kilby *et al.* 1993, Tronche *et al.* 2002). Exon VI encodes 1113 nuc and encompasses the entire 7TM domain and C-terminal of the receptor, as well as 42 nuc of the 3' UTR. The inserts were placed in regions that would not interfere with normal expression of the gene. The upstream *LoxP* was placed 300 nuc upstream of exon VI and the other inserts 300 nuc downstream of the predicted polyadenylation site. As the targeting strategy will result in the deletion of the regions of mGPRC6A involved in G protein-coupling and other putative intracellular signaling mechanisms elicited by the receptor, presumably the expression and/or folding of the amino-terminal domain of mGPRC6A will also be disrupted. A similar strategy of floxing the corresponding exon VII in *Casr* has proven efficient for the generation of conditional *Casr* knockout mice (Chang *et al.* 2008). To enable the future generation of a conditionally mutated allele, the *Neo*^r gene was additionally flanked by *Frt* sites (Fig. 1A). However, in the present study only full knockouts were generated, using CRE-*LoxP* recombination.

Correct targeting and incorporation of the targeted allele into ES cells (Fig. 1B) was confirmed by Southern blot analysis (Fig. 1C). Following restriction digestion using *SacI*, the 5' probe (a 0.82 kb *SacI*/*SpeI* fragment) detected a 7.0 kb fragment for the wild-type allele and an extra fragment of 5.1 kb corresponding to the mutant allele (Fig. 1C, left). Correspondingly, following restriction digestion using *SphI*, the 3' probe (a 0.54 kb *SacII*-fragment) detected a 12.7 kb fragment for the wild-type allele and an extra fragment of 5.8 kb for the mutant allele (Fig. 1C, right). Experiments using an

internal probe (a 0.76 kb *SacI*/*BamHI* fragment) verified that multiple integrations into the *Gprc6a* locus had not occurred (data not shown).

Targeted ES cells were used to generate germline-transmitting chimeras, which were mated to C57BL/6 mice to generate heterozygote offspring containing the *Neo*^r gene (*Gprc6a*^{neo}). *GPRC6A*^{+/-} mice were generated by crossing *Gprc6a*^{neo} with a ubiquitously expressing CRE-deleter mouse (Schwenk *et al.* 1995) and backcrossing into the C57BL/6 background. Genotyping was performed by PCR (Fig. 1D).

Confirmation of generation of GPRC6A knockout mice at the mRNA level

To confirm that the knockout strategy had led to deletion of the GPRC6A gene product, RT-PCR was performed (Fig. 1E). To this end, we designed intron-spanning primers located at exons V and VI in order to avoid contamination from genomic DNA. As expected, bands were detected in wild-type tissues reported to express GPRC6A mRNA (bone, lung and 15-day embryo; Kuang *et al.* 2005, Pi *et al.* 2005, Wellendorph *et al.* 2005) and GPRC6A protein (15-day embryo; Kuang *et al.* 2005). By contrast, no expression of mGPRC6A was observed in the same tissues from *GPRC6A*^{-/-} littermates, thus confirming the absence of GPRC6A mRNA in these mice.

Initial phenotypic characterization

Mice lacking either one or both *Gprc6a* alleles were viable and born at expected Mendelian frequencies. The mice developed normally, were fertile, and exhibited no obvious behavioral or physical phenotype. Furthermore, neither female nor male *GPRC6A*^{+/-} and ^{-/-} mice littermates displayed any statistically significant differences in body weight up to an age of 38 weeks when fed on a regular chow diet (see Supplementary material in the online version of the Journal of Molecular Endocrinology at <http://jme.endocrinology-journals.org/content/vol42/issue3/>).

Lack of a bone phenotype in GPRC6A knockout mice

To evaluate the bone structural histology, tibias from wild-type and GPRC6A knockout male and female mice were compared by 3D analysis using μ CT. No differences in bone architecture were observed between genotypes when comparing groups of either ten male or ten female wild-type and knockout mice, i.e. no statistically significant differences in trabecular bone volume (BV/TV), trabecular number, separation or thickness (Table 1). A representative 3D construction of a scan is shown in Fig. 2. In keeping with these negative

Table 1 Bone measurements of 13-week-old GPRC6A^{+/+}, ^{+/-}, and ^{-/-} mice

	Males			Females		
	+/+	+/-	-/-	+/+	+/-	-/-
Bone parameters^a						
Bone structure (tibia)						
BV/TV (%)	8.4±1.2	8.9±0.9	9.3±0.9	4.7±0.3	5.1±0.7	5.1±0.3
Trabecular number (1/mm)	3.1±0.3	3.3±0.2	3.3±0.3	2.0±0.1	2.0±0.2	2.1±0.11
Trabecular separation (µm)	418±46	388±56	375±54	717±59	883±167	626±43
Trabecular thickness (µm)	25.4±1.4	25.7±1.1	27.3±1.4	22.2±0.6	23.1±1.1	22.9±0.8
Bone biomarkers						
OCN (ng/ml)	41.2±3.2	46.0±2.8	40.6±4.0	66.3±6.5	57.2±5.4	57.3±4.0
o-Pyr/creatinine ((nmol/l)/(mmol/l))	16.0±3.3	12.7±1.8	15.9±2.0	21.1±0.9	19.4±1.1	20.3±2.1

No statistical difference between genotypes was found. BV, bone volume; TV, total volume; OCN, osteocalcin and o-Pyr, urinary deoxypyridinoline cross-link level.
^aAll values are mean±s.e.m. (n=10 per group).

structural data, osteoclast, osteoblast, and dynamic parameters were consequently not investigated. Analysis of BMD, by means of pQCT, revealed no changes in trabecular, cortical or total metaphyseal BMD, or cortical diaphysis BMD, in knockout mice compared with wild-type littermates (Fig. 3). Thus, the BMD data are in concordance with the 3D µCT dataset, since no cellular activity has been observed in regards to resorptive and formative biomarkers. Finally, we

searched for possible alterations in cellular activity, by measuring the bone biomarkers osteocalcin in serum and deoxypyridoniline (o-Pyr) in urine, relevant in relation to bone formation and resorption respectively. Again, we observed no influence of the elimination of GPRC6A gene products on the measured values (Table 1). Data obtained from our wild-type littermates are in the same range as C57BL/6 wild-type (Clément-Lacroix *et al.* 2005, Morvan *et al.* 2006).

Figure 2 Three-dimensional reconstructed trabecular bone images from µCT scans. Illustrative 3D images of the proximal tibias from 13-week-old GPRC6A wild-type and knockout mice at 9 µM resolution. Left, surface view and right, open view.

Discussion

From the present study, we have obtained no data to support a role for GPRC6A in bone homeostasis under normal physiological conditions in mice. Furthermore, we have been unable to demonstrate any changes relating to bone structure or bone turnover between 13-week-old GPRC6A wild-type and knockout mice. These findings correlate with the lack of differences in body weight between male and female wild-type and knockout mice up to 38 weeks of age. Based on the expression of mGPRC6A in long bone and its concerted Ca²⁺/amino acid signaling, as shown by several groups (Pi *et al.* 2005, Breitwieser 2008, Conigrave *et al.* 2008), the apparent absence of a phenotype related to bone physiology in the knockout mice might be ascribed to several factors. As always with knockout animals, there is an inherent possibility that compensatory mechanisms have offset the impact of the elimination of the gene (Zambrowicz & Sands 2003). Similarly, in the current scenario, it could be imagined that a protein or mechanism implicated in bone calcium sensing could substitute for the lack of GPRC6A. Given the common gene ontology and ligand preferences of CaR and GPRC6A, CaR is an obvious candidate in this respect, notwithstanding current controversies in the field. Whereas it is well established that CaR plays an essential role in calcium homeostasis of the organism through

Figure 3 Bone mineral density (BMD) measurements in *GPRC6A*^{+/+}, *+/+*, and *-/-* mice. Trabecular, cortical, and total BMD values in the metaphyseal tibia and cortical BMD values in the diaphyseal tibia by pQCT. Values are means $\pm$ S.E.M. ($n=5-11$ per group). No statistical significance was found between either wild-type and knockout males (open bars) or females (gray bars).

sensing and maintaining the extracellular calcium concentration, and by regulating hormone secretion in the parathyroid gland and kidneys, its role in bone is debated (Tfelt-Hansen & Brown 2005). Despite reports showing a lack of expression and function of CaR in bone (Pi *et al.* 1999, Kos *et al.* 2003, Tu *et al.* 2003, Pi & Quarles 2004, 2005), several groups have provided substantial evidence for the expression and function of the native receptor and a functional splice variant both at mRNA and protein level in bone, osteoblasts, osteoclasts, and calvaria (Chang *et al.* 1999, Chattopadhyay *et al.* 2004, 2007, Dvorak *et al.* 2004, Rodriguez *et al.* 2005, Mentaverri *et al.* 2006). Furthermore, a conditional osteoblast-specific CaR knockout mouse has recently been found to exhibit abnormalities in bone development (Chang *et al.* 2008). Accordingly, it would be interesting to generate a bone-specific conditional *GPRC6A* or a *GPRC6A*/CaR double knockout mouse, something which is of special pertinence to us given the already engineered *Gprc6a* conditional allele in ES cells.

When interpreting the current data, it is important to keep in mind that the role of *GPRC6A* in bone physiology was evaluated for just one age of mice (13-week-old) and in a normal physiological setting.

Although any clear effects on bone development or homeostasis would likely appear at this age, it cannot be ruled out that involvement of *GPRC6A* on bone development and/or homeostasis is age or strain dependent. For example, a transitory bone phenotype has been reported in young mice during growth (i.e. GH receptor knockout mice; Sims *et al.* 2000) and appearance of a bone phenotype has been reported in mice of old age (i.e. Δ fosB knockout mice; Sims *et al.* 2002). Furthermore, it could be speculated that the emergence of a bone phenotype in the *GPRC6A* knockout mice requires a pathophysiological condition, such as a disease-induced state or a calcium depleted/overloaded state. Scenarios including receptor overstimulation (achieved by prolonged amino acid/ Ca^{2+} ingestion), or ovariectomy leading to bone loss, may be imagined to completely determine the role of *GPRC6A* in bone physiology.

In conclusion, we have generated specific *GPRC6A* knockout mice and here for the first time report that the mice are viable, display no body weight differences compared with wild-type littermates, and lack a phenotype in relation to normal bone physiology. As mentioned previously, the broad ligand recognition and expression of *GPRC6A* has led to several

hypotheses on the physiological function of the receptor such as a metabolic sensor, a role in taste or neurotransmission or an implication in cell death (Civelli 2005, Wellendorph *et al.* 2005, Conigrave & Hampson 2006, Bräuner-Osborne *et al.* 2007). Owing to the present lack of selective agonists and antagonists, our generation of a GPRC6A knockout mouse represents an important new tool, which we are currently employing to investigate the other suggested physiological function(s) of the receptor.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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