In vitro and *in vivo* evidence for orphan nuclear receptor RORα function in bone metabolism

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Bone is a major target site for steroid hormone action. Steroid hormones like cortisol, vitamin D, and estradiol are responsible for principal events associated with bone formation and resorption. Over the past decade, new members of the nuclear hormone gene family have been identified that lack known ligands. These orphan receptors can be used to uncover signaling molecules that regulate yet unidentified physiological networks. In the present study the function of retinoic acid receptor-related orphan receptor (ROR) α in bone metabolism has been examined. We showed that RORα and RORγ, but not RORβ, are expressed in mesenchymal stem cells derived from bone marrow. Interestingly, for RORα we observed an increased messenger signal expression between control cells and cells undergoing osteogenic differentiation. Furthermore, the direct activation of mouse bone sialoprotein by RORα, typically 7-fold, has been shown. In contrast, transient overexpression of RORα overrides the activation of the osteocalcin promoter by 1α,25-dihydroxyvitamin D_3. In addition, we have investigated bone mass parameters and bone geometry in the mouse mutant *staggerer* (*sg*/*sg*), a mouse strain that carries a deletion within the RORα gene. Homozygote mutants have thin long bones compared with the heterozygote animals and wild-type littermates. More interestingly, the bones of the *sg*/*sg* animals are osteopenic as indicated by the comparison of bone mineral contents of *sg*/*sg* animals to the heterozygote and wild-type animals. We conclude that these *in vitro* and *in vivo* results suggest a function for RORα in bone biology. RORα most likely acts by direct modulation of a bone matrix component.

Bone is a metabolically highly active and organized tissue. The formation of bone by osteoblasts, and its remodeling by the bone multicellular unit, is a closely integrated homeostatic system. The osteoblast secretes the organic matrix, which is later mineralized. The bone extracellular matrix is composed mainly of layered type I collagen fibrils, other noncollagenous proteins such as the bone sialoprotein (BSP), a modulator of mineralization, osteopontin, which has been implicated in adhesion, and the bone-specific osteocalcin (OC), which plays an important role in bone formation (1).

Mesenchymal stem cells (MSCs) are considered to be multipotent cells that are present in the adult marrow, can replicate as undifferentiated cells, and have the potential to differentiate into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma. Recently it has been demonstrated that individual adult stem cells could indeed be induced to differentiate into adipocytic, chondrocytic, or osteocytic lineages (2). Even though the molecular basis for directing human MSCs toward the different lineages has been extensively studied, the interrelationship between each of the lineages and the control mechanism governing the differentiation of each of the lineages remains poorly understood. A key regulatory transcription factor in adipogenic differentiation belongs to the nuclear receptor peroxisome proliferator-activated receptor (PPAR) subfamily (PPARγ) whereas a runt domain protein, Cbfa1, has been identified as a crucial transcriptional activator of osteoblast differentiation (3, 4).

Steroid hormones, such as vitamin D, glucocorticoids, or estrogens, are responsible for principal events associated with bone formation and resorption. Vitamin D is a key regulator of mineral homeostasis in mammals. This hormone stimulates bone resorption and has complex effects on bone formation and bone cell differentiation by modulating the synthesis of several osteoblastic markers, including type I collagen, alkaline phosphatase, osteopontin, and OC (5, 6). Sex hormones like estrogens are essential for maintenance of normal bone mass (7). Estrogen withdrawal leads to bone loss. Estrogen effects are mediated through two estrogen receptor (ER) subtypes, ERα and the more recently described ERβ (8, 9). The finding that a mutation in the ERα allele in a male patient induces osteoporosis suggests ERα as an important regulator in bone metabolism (10). Interestingly, ERRα, an ER-related orphan receptor, also has been implicated in bone metabolism (11, 12). Recently, Vanacker *et al.* (13) showed the existence of a receptor cross talk between ERRs and ERs. Taken together, the above summarized results present evidence for an important regulatory function of nuclear receptors in bone physiology. Furthermore, these results indicate that orphan nuclear receptors may function as potent regulators of bone cell differentiation and bone metabolism in analogy to their well-characterized family members.

Therefore, we decided to investigate a possible physiological role of nuclear orphan receptors on bone function by analyzing the expression of mammalian nuclear orphan receptors during osteoblast differentiation. To this end, the availability of human MSCs (hMSCs) provided a powerful tool to monitor a possible differential expression of orphan receptor molecules during the course of osteogenic differentiation (14).

We screened for the presence of several orphan receptors (15) and decided to focus on the function of retinoic acid receptor-related orphan receptor (ROR) α in bone development. The ROR subfamily of receptors is encoded by three different genes, α, β, and γ (16). The distribution of RORα mRNA suggests that this receptor is widely expressed and functions in several organs including brain, heart, liver, testis, and skin (17). RORα exists in four splicing isoforms: RORα1–3 and RORα4 (also termed RZRα). These isoforms mainly differ by their N-terminal domains causing different DNA binding preferences, and they display differential expression profiles. RORα can bind to response elements in the promoter of target genes in a monomeric fashion and appears to act as a constitutive transcriptional activator in the absence of exogenously added ligand (16, 17).

Abbreviations: BSP, bone sialoprotein; ER, estrogen receptor; MSC, mesenchymal stem cell; hMSC, human MSC; OC, osteocalcin; ROR, retinoic acid receptor-related orphan receptor; RT-PCR, reverse transcription–PCR; *sg*, *staggerer*; $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3.

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Although RORα has been extensively studied, its true ligand remains unknown. Furthermore, it has been shown that disruption of the RORα gene results in significant cerebellar abnormalities in mice (18–20). In addition to its role in brain disorders, RORα has been implicated in the development of atherosclerosis and hypolipoproteinemia (21).

In this report, we show that RORα and RORγ are expressed in hMSCs. Interestingly, for RORα we observed a differential messenger signal expression pattern between control cells and cells undergoing osteogenic differentiation. Furthermore, we characterized the direct action of RORα on mouse BSP and the activated human OC gene promoter activity. We also provide evidence that the long bones of the mouse mutant *staggerer* (*sg*), which carries a deletion within the nuclear receptor RORα gene, are osteopenic. We conclude that these *in vitro* and *in vivo* results clearly demonstrate a function for RORα in bone biology.

Experimental Procedures

hMSC Culture. hMSCs prepared from fresh bone marrow obtained by routine iliac crest aspiration from normal human donors were obtained from Osiris Therapeutics, (Baltimore) and prepared according to Jaiswal *et al.* (14). hMSCs, derived from four different healthy donors, were cultivated according to standard protocol using DMEM low-glucose medium (Seromed, Berlin) supplemented with 10% FCS (HyClone). Osteogenic differentiation was induced by incubating the cells in a defined osteogenic supplement consisting of MEM/Ham's F-12 medium containing 0.1 μM dexamethasone, 50 μM ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate.

RORα, RORβ, and RORγ Expression During Osteogenesis. The cells were harvested after 0, 4, 8, and 15 days in culture with normal medium or with the defined osteogenic medium. The cells were processed according to the manufacturer's protocol for total RNA preparation by using the RNeasy midi RNA kit (Qiagen, Basel, Switzerland). One hundred nanograms of total RNA was added to PCRs containing the appropriate primers and the reaction mix Superscript one-step reverse transcription–PCR (RT-PCR) system (GIBCO/BRL). PCR conditions were: cDNA synthesis at 50°C, 30 min; 94°C, 2 min; 95°C, 1 min; 56°C, 30 sec; 72°C, 1 min. All experiments were performed twice by using RNA preparations from MSCs derived from different donors. The PCR fragments were visualized on a 1.5% agarose gel. Primers and annealing temperatures used were: RORα, 5′-3′forward primer GTAGAAACCGCTGCCAACA and reverse primer ATCACCTCCCGCTGCTT, 56°C; RORβ, forward primer GAACAGCGGCAGGAGCAGA and reverse primer GGTTGAAGGCACGGCACAT, 57°C; RORγ, forward primer CCCCTGACCGATGTGGACT and reverse primer CAGGATGCTTTGGCGATGA, 60°C; and β-actin, forward primer ATCTGGCACCACACCT and reverse primer CGTCATACTCCTGCTT, 60°C.

Quantitative Real-Time PCR (Taqman Assay). This technique was used to quantitatively monitor mRNA expression and has been described in detail (22). In brief, total RNA was extracted from hMSCs as described in other sections of *Experimental Procedures* and mRNA was prepared by using the oligotexmRNA kit from Qiagen. A gene-specific PCR oligonucleotide primer pair and an oligonucleotide probe labeled with a reporter fluorescent dye at the 5′ end and a quencher dye a the 3′ end were designed by using PRIMER EXPRESS 1.0 software. The primer and probes used were as follows: hRORα gene (5′-3′), forward primer GTGCGACTTCATTTTCCTCCAT, reverse primer GCTTAGGTGATAACATTTACCCATCA, and the probe CACTTCAGAATTTGAGCCAGCAATGCAA; human glyceraldehyde-3-phosphate dehydrogenase gene (5′-3′), forward primer GAAGGTGAAGGTCGGAGTC, reverse primer GAAGATGGTGATGGGATTTC, and the probe CAAGCTTCCCGTTCTCAGCC.

In general, mRNA (10 ng) was added to a 50 μl RT-PCR core reaction mix (Perkin–Elmer). The thermal cycle conditions included 1 cycle at 50°C for 30 min, 1 cycle at 95°C for 10 min, alternating 40 cycles at 90°C for 15 sec, and 40 cycles at 60°C for 1 min by using a Gene Amp 5700 Sequence Detection System (Perkin–Elmer). The relative expression of RORα was normalized to glyceraldehyde-3-phosphate dehydrogenase levels measured in the same RNA preparation.

Cell Extract Preparation and Western Blot Analysis. Cells were washed with ice-cold PBS and scraped off into ice-cold extraction buffer (20 mM Tris·HCL, pH 7.5./0.5 mM EGTA/2 mM EDTA/2 mM PMSF/1 mM DTT). They were sonicated twice on ice for 20 s each at 40 kHz, and the homogenate was centrifuged for 10 min at 600 × *g* to precipitate nuclei. The nuclei were resuspended in extraction buffer and stored at −70°C. For Western blot analysis the proteins were separated by SDS/PAGE and transferred to membranes by using standard conditions. For immunodetection we used the instruction provided for ECL detection kits (Amersham Pharmacia). The specific antibody against RORα (RORα1, sc-6062) was purchased from Santa Cruz Biotechnology.

Preparation of Promoter Constructs and Plasmids. The plasmid containing the promoter region of the BSP gene (*pBSP 2.5BSP*), a kind gift of J. Aubin (University of Toronto, Canada) was cut with *Xho*I and *Xba*I to obtain a 2.5-kb fragment of the mouse BSP promoter. The fragment was ligated into the *Xho*I and *Nhe*I sites of pGl2-basic (Promega) vector to drive the firefly luciferase gene (*BSP-luc*). The RORα1 expression construct and the DR8tk luc were obtained from M. Becker-Andre (Serrono Pharmaceutical, Geneva, Switzerland) and have been described (16, 17). The OC promoter constructs OC-344 and OC-890 have been described (23).

Cell Culture, Transient Transfections, and Luciferase Assay. ROS 17/2.8 cells were obtained from J. Fischer (University of Zurich) and cultured at 37°C in a humidified atmosphere with 5% CO_2 in DMEM/F12 nutrition mixture buffered with bicarbonate and supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Cells were seeded in 6-well plates 24 h before a transfection and transfected at 50–60% confluence by using Fugene6 transfection reagent (Boehringer Mannheim). A typical reaction mixture contained 2 μg reporter plasmid and 1 μg expression plasmid. After 4 h exposure to the transfection mix, medium was refreshed and cells were treated for 24 h with 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] when indicated. Transfected cells subsequently were harvested for luciferase assay by scraping the cells into 0.25 ml lysis buffer (Promega) after washing them in PBS. Luciferase activity was monitored according to the Promega luciferase assay kit using an automatic luminometer LB96P (Berthold, Regensburg, Germany). Results are expressed in relative light units per mg protein. All experiments were performed in triplicate on three separate occasions.

Animals. The *sg* mutant mice used in this study were maintained on a C57BL/6 genetic background in our colony at the Institut Gustave Roussy (Villejuif, France) (24). The animals received a standard diet (A04, UAR, Epinay-sur-Orge, France) and water ad libitum. They were maintained at 25°C with a 12-h light-dark cycle.

Genotype Analysis. The animals were genotyped by PCR. Genomic DNA was extracted from tail biopsies and amplified in two sets of reaction, one for each allele.

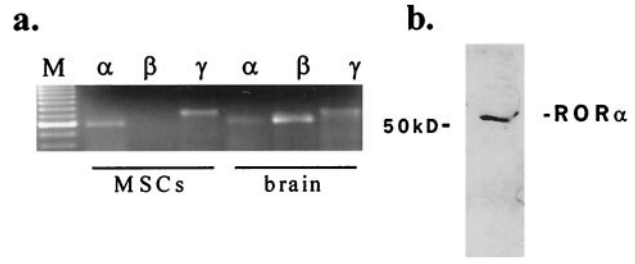

Fig. 1. Expression of RORα in hMSCs. (*a*) RT-PCR analysis using specific primers for RORα, RORβ, or RORγ as described in *Experimental Procedures*. RNA prepared from cells of different origin was used as a positive control (brain). M, size markers. (*b*) Immunodetection of RORα in hMSC nuclear extracts. Cell nuclei were prepared, and 40 μg aliquots of nuclei were separated on 10% SDS-polyacrylamide gel, transferred to plastic membranes, and probed with a polyclonal antibody raised against RORα.

The *staggerer* allele primers were: 5′-CGTTTGGCAAACTCCACC-3′ and 5′-GTATTGAAAGCTGACTCGTTCC-3′.

The + allele primers were: 5′-TCTCCCTTCTCAGTCCTGACA-3′ and 5′-TATATTCCACCACACGGCAA-3′. The amplified fragments (318 bp + and 450 bp sg) were detected by electrophoresis on agarose gel.

Bone Sample Collection. The left tibia was collected from 16-week-old homozygote (*sg*/*sg*), heterozygote (*sg*/+), and wild-type (+/+) male mice (n = 10/group). The animals were derived from seven litters, each containing *sg*/*sg*, *sg*/+, and +/+ animals.

Dual-Energy X-Ray Absorptiometry. Tibial bone mineral content (mg) and bone mineral density (mg/cm^2) were measured by using a Hologic (Waltham, MA) 6QDR-1000 instrument adapted for measurements of small animals. A collimator with 0.9 cm diameter and an ultrahigh-resolution mode (line spacing 0.0254 cm, resolution 0.0127 cm) were used. The bones were placed into a plastic container filled with 70% ethanol. The stability of the measurement was controlled daily by scanning a phantom.

Peripheral Quantitative Computed Tomography. Cortical and cancellous bone mass and geometry were monitored in a cross section of the proximal tibia metaphysis 3 mm distal to the medial and lateral intercondylar tubercle by using a Stratec-Norland XCT-2000 (Pforzheim, Germany). Cross-sectional bone mineral content (mg/mm), bone mineral density (mg/cm^3), and bone area (mm^2), cortical thickness (mm), and the cancellous bone mineral density (mg/cm^3) were determined. The following setup was chosen for the measurements: voxel size, 0.1 mm × 0.1 mm × 0.5mm (slice thickness); scan speed, scout view 10 mm/s, computer tomograph measurement 2 mm/s, 1 block, contour mode 1, peelmode 2; cortical threshold, 400 mg/cm^3. The bones were placed into a plastic container filled with 70% ethanol. The stability of the measurement was controlled daily by scanning a phantom.

Statistical Analysis. The results are expressed as mean ± standard error (SEM) or +/− SD. All statistical analysis for the *in vivo* study was carried out by using BMDP (version 1990 for VAX/VMS, BMDP Statistical Software, Cork, Ireland). The data were subjected to one-way ANOVA. Levene F test was used to test for equality of variances, and differences between groups were tested by using Dunnett test (significance level: $P < 0.05$). All statistical tests were two-tailed. Differences between all groups were tested for statistical significance.

Results

We were interested to study the expression and possible function of nuclear orphan receptors in bone biology by using hMSCs as a powerful tool in an initial characterization step. Because alkaline phosphatase is a well-defined marker during osteogenic development, we monitored the level of its activity and regarded it as a validation for the system. As expected and in line with many other similar observations, alkaline phosphatase was strongly up-regulated during the course of hMSCs differentiation. A 4-fold increase in alkaline phosphatase activity was observed when untreated control cells and cells treated with osteogenic supplement were compared at day 18. We analyzed the expression of 23 known mammalian orphan receptors (15) in hMSCs during the course of their differentiation toward osteoblasts. Fourteen of these 23 receptors were found to be expressed in hMSCs, and among them only three were found to be regulated during osteogenesis. RORα was selected among these three regulated orphan receptors. For the ROR family of orphan nuclear receptors exclusively, RORα and RORγ, but not RORβ, are expressed in hMSCs (Fig. 1*a*). During Western blot analysis, the presence of RORα has been confirmed at the protein level in hMSCs (Fig. 1*b*). Only in the case of RORα, a differential expression of messenger signals was observed between control cells and cells undergoing osteogenic differentiation. A qualitative analysis of RORα expression was performed by using RNA obtained from cells at different days of osteogenic treatment in RT-PCR assays (Fig. 2*b*). The osteogenic treatment

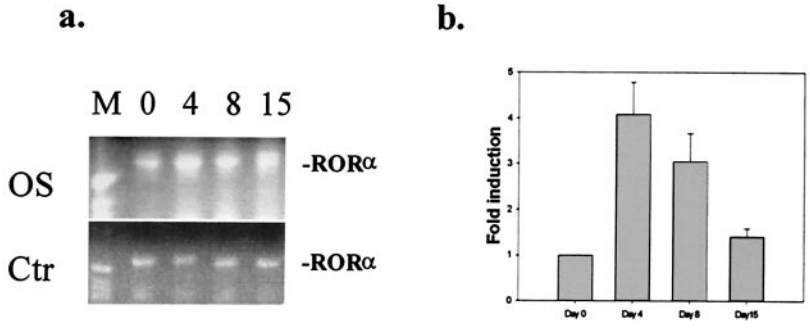

Fig. 2. Expression analysis of RORα mRNA in hMSCs during the course of osteogenesis. (a) Total RNA was prepared at the indicated days (days 0, 4, 8, and 15) and subsequently used in RT-PCR assays. The qualitative increase of RORα mRNA under osteogenic treatment (OS) is compared with untreated control cells (day 0 and Ctr). M, size markers. (*b*) Quantitative analysis of mRNA expression of RORα during hMSCs differentiation by real-time PCR. mRNA was prepared from cells undergoing osteogenic differentiation and related to the expression level of untreated cells at a similar day of culture. The relative expression of RORα was normalized to glyceraldehyde-3-phosphate dehydrogenase levels measured in the same RNA preparation. Shown is one representative experiment of three different experiments performed with different donors.

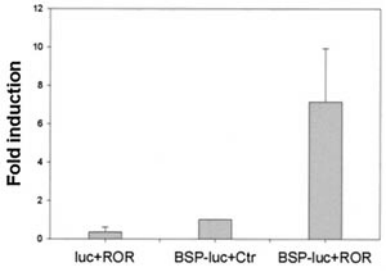

Fig. 3. Induction of mouse BSP promoter activity by RORα. Ros17/2.8 cells were transfected with parent expression vector (Ctr) or expression vector for RORα (ROR) together with a luciferase reporter gene. The reporter gene was driven by a 2,500-bp promoter fragment of the mouse BSP gene (*BSP-luc*). Luciferase activity was assayed in cells from 6-well plates and related to the activity in cells transfected with an empty expression plasmid. The results were normalized to the protein content. Shown are the mean ± SD of three experiments, each carried out with three independent triplicate analyses.

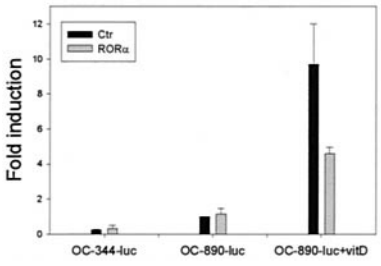

Fig. 4. RORα repression of the OC promoter is dominant over VitD activation. ROS 17/2.8 cells were transfected with a luciferase reporter gene driven by either −344/+34 (OC-344-luc) or −890/+34 (OC-890-luc) of the human OC promoter together with an expression vector for RORα (ratio 2:1 μg) (gray bars) or without (black bars). When indicated, the cells were incubated with 10 nM vitamin D. Luciferase activity was assayed in cells from 6-well plates and related to the activity in cells transfected with an empty expression plasmid. The results were normalized to the protein content. Shown are the mean ± SD of three experiments, each carried out with triplicate analyses.

resulted in a clear increase in the RORα mRNA level compared with untreated control cells. The increase in RORα messenger expression was quantitatively determined by using real-time PCR. After 4 days of treatment we obtained an approximately 4.5-fold increase in RORα expression compared with cells that remained in the untreated stage. This increase was maintained at day 8 and declined at day 15, which already corresponded to fully mineralized matrix (Fig. 2*b*). Thus, the up-regulation of RORα during osteogenic differentiation created a starting point to further investigate the function of RORα and its influence on bone biology.

The functional importance of RORα was examined in cellular transfection assays using the rat osteosarcoma cell line 17/2.8 (ROS 17/2.8) as host cells. Given the background that secreted components of the bone organic matrix like BSP or OC are important modulators of mineralization and bone formation, a conditionally active transcription factor like RORα, which is believed to be involved in regulation of bone metabolism, should regulate the promoters of these bone-specific genes. Through computer-aided sequence analysis RORα consensus binding motifs RGGTCA (R = A or G) already have been identified within the human and rat BSP promoter sequence (25). These responses elements fused to a tk minimal promoter driving a reporter were found functional. A similar consensus element GGGTCA was located within the mouse BSP promoter between positions −2007 and −2001 in respect to the transcription start site. The nucleotide in position −4 (T) was conserved between rat, human, and mouse sequence. To study a possible regulation of the mouse BSP promoter by RORα in its physiological environment, a 2,500-bp spanning fragment of the mouse BSP promoter was used to drive the firefly luciferase gene. This reporter was cotransfected with an RORα1 expression vector. RORα1 cDNA was used because the α2 or α3 version were not detectable by RT-PCR in hMSCs (data not shown) and furthermore, because it has been described that RORα1 has the strongest transcriptional activity of the three subtypes (16, 26). Coexpression of RORα resulted in a 7-fold increase in luciferase activity of the BSP luc construct compared with basal level as shown in Fig. 3. This increase was similar to the one obtained under the same conditions with a reporter construct containing two RORα response elements (DR8) (data not shown). Further *in vitro* evidence for RORα action in bone cells was collected by studying the effect of RORα on OC gene activity. As shown in Fig. 4, overexpression of RORα had no significant influence on the basal activity of OC promoter activity. Neither a reporter construct driven by the first 344 bp of the human OC promoter (OC-344-luc) nor a longer promoter sequence (OC-890-luc) was regulated by RORα coexpression (Fig. 4). As expected, only the OC-890 promoter construct was up-regulated by $1,25(OH)_2D_3$, because the only palindromic DNA sequence shown to bind the vitamin D receptor/retinoid X receptor heterodimer is located between base pairs −513 and −493 upstream of the transcription start site of the human OC promoter. As shown in Fig. 4, the addition of $1,25(OH)_2D_3$ resulted in an 8-fold reporter gene activation and the cotransfection of an RORα expression plasmid together with an OC-driven reporter-construct (OC-890) resulted in a partial suppression of the $1,25(OH)_2D_3$-activated level, usually 40%, of the up-regulated OC-890 promoter reporter gene activity.

Based on these *in vitro* observations we wanted to evaluate whether RORα also plays a role in bone metabolism *in vivo.* Therefore we examined bone mass and geometry in the long bones of the mouse mutant *sg/sg*, which has a deletion within the RORα gene. We found that the total bone mineral content of the tibia was significantly reduced in homozygote *sg/sg* mice compared with heterozygote *sg/+* and wild-type +/+ mice (Fig. 5*a*) as determined by double energy x-ray absorptiometry. This change was mainly caused by a decreased total bone mineral density (Fig. 5*b*) and not by reduced total bone area and length compared with wild-type or heterozygote animals (Fig. 5 *c* and *d*). Detailed peripheral quantitative computed tomography studies in the proximal tibia metaphysis demonstrated that the cross-sectional bone mineral content was significantly reduced in the *sg/sg* animals compared with *sg/+* and +/+ animals in this

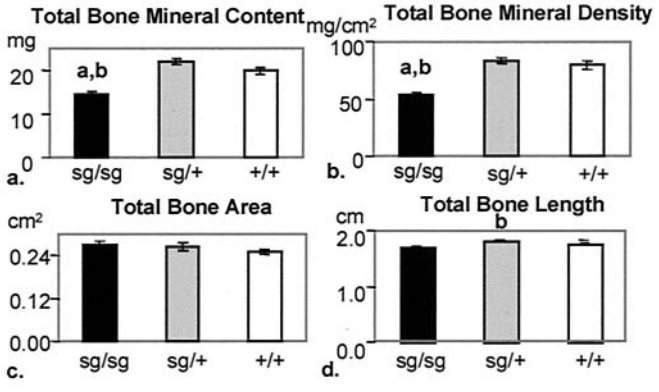

Fig. 5. Total bone mineral content (*a*), density (*b*), area (*c*), and length (*d*) of the tibia evaluated by dual energy x-ray absorptiometry. Mean ± SEM; ANOVA, Dunnett, $P < 0.05$, a = *sg/sg* ⇔ +/+, b = *sg/sg* ⇔ sg/+.

Table 1. Cross-sectional bone mineral content, density, area, cortical thickness, and cancellous bone mineral density in the proximal tibia metaphysis evaluated by peripheral quantitative computed tomography

	Bone mineral content	Bone mineral density	Bone mineral area	Cortical thickness	Cancellous bone mineral density
sg/sg	0.93 ± 0.08 a,b	423.94 ± 16.17 a,b: <.1	2.18 ± 0.11 a,b	0.298 ± 0.017 a: <.1, b	124.17 ± 3.02 a,b
sg/+	1.59 ± 0.05	469.49 ± 8.24	3.39 ± 0.11	0.371 ± 0.005	182.17 ± 10.57
+/+	1.41 ± 0.08	467.23 ± 12.64	3.10 ± 0.11	0.335 ± 0.010	169.41 ± 6.75

Mean ± SEM; ANOVA, Dunnett, $P < 0.05$, a = *sg/sg* ⇔ +/+, b = *sg/sg* ⇔ *sg/+*.

metabolically active bone site (Table 1). This reduction is the result of the decreased volumetric mineral density, indicating osteopenia, and a reduced cross-sectional bone area, indicating a thinner tibia metaphysis in those animals. Both cortical thickness and cancellous bone mineral density were reduced in the homozygote *sg/sg* animals. In contrast, the heterozygotes (*sg/+*) did not display this bone phenotype. They showed similar bone geometry and mass as their wild-type littermates (+/+) (Fig. 5; Table 1). In summary, the homozygote mutants had thin long bones compared with heterozygotes and the wild type. More interestingly, the bones of the homozygote animals were osteopenic as indicated by all quantitative x-ray-based bone mineral measurements, suggesting that RORα may be a positive regulator in bone metabolism.

Discussion

Imbalance between bone formation and bone resorption causes pathological conditions such as osteoporosis. However, cell biology of osteoblasts, their precursor cells, and factors regulating the controlled bone formation process is still not fully understood. To unravel these unidentified important physiological regulators we studied the expression and potential function of nuclear orphan receptors during osteogenic lineage progression using hMSCs.

The influence of steroid hormones like vitamin D and estrogens on regulatory events during osteoblast differentiation are striking and reflect specific stages of phenotype development. As illustrated in several reports, exposure of early progenitor cells to vitamin D resulted in inhibition of collagen type I deposition and subsequently inhibition of matrix mineralization, whereas exposure of mature osteoblasts to the hormone resulted in an increased expression of genes associated with the mineralization process such as OC (5, 14). For the ER, a correlation between ERα mRNA expression and progressive osteoblast differentiation has been described by Bodine *et al.* (27). This clearly demonstrates a functional relationship between the level of ER expression and activity on osteoblastic differentiation. Similarly the membrane receptor for parathyroid hormone is up-regulated during the osteoblastic differentiation and its expression associated with active matrix synthesis in differentiating osteoblasts (28). In the present study, we show that RORα, like other bone-active hormones, is strongly up-regulated during the differentiation of MSCs into osteoblasts. This suggests that nuclear orphan receptors like RORα may have a similar importance for an intact bone environment as classical steroid hormone receptors. Taken into consideration that RORα expression and function also has been implicated for other cells of mesenchymal origin, our findings are in line with the postulation that RORα may act as regulator in developing systems. In differentiating adipocytes RORα was up-regulated during late adipogenesis (29). Furthermore, the exogenous expression of a dominant negative RORα vector in myogenic cells impairs differentiation through direct interaction with the muscle-specific helix–loop–helix transcription factor MyoD and the general transcriptional coactivator p300 (30).

RORα binds selectively as a monomer to the consensus response element A/GGGTCA found in several genes, including the 5-lipoxygenase, the cellular retinoic acid-binding protein I, the inhibitor of cyclin-dependent kinases, and the rat BSP gene (25, 31, 32). These data primarily were collected by performing computer-assisted homology searches. The sequences found were analyzed for direct RORα regulation outside of their physiological promoter environment with the noticeable exception of Apo-A1 promoter, which has been shown to be transactivated by RORα (25). In this study we provided experimental evidence that RORα is able to regulate the natural mouse BSP promoter in bone-derived cells. In our test system, the increase in transcription of the BSP gene was comparable in magnitude to the one obtained with a consensus RORα binding site, demonstrating the potency of RORα action on BSP expression. As one of the major secretory proteins of osteoblasts, BSP functions to regulate mineralization possibly by its direct interaction with cell surface integrin receptors and/or by initiating nucleation of the bone mineral hydroxyapatite (33, 34). It is worth noticing that the analysis of BSP mRNA during the course of osteogenic differentiation with real-time PCR showed an expression pattern very close to RORα (data not shown). This observation and the strong transactivation activity of RORα on the BSP gene suggests a physiological relevance of RORα in bone metabolism. Furthermore, we showed that RORα overexpression impairs the vitamin D-dependent activation of the major noncollagenous bone matrix component OC (35). Even if the level of repression was comparable to the well-established trans-repression of OC activity by corticosteroids, the underlying mechanism seems to be different. In contrast to the cortisol-dependent repression, RORα overexpression did not change the basal reporter gene expression level (23, 36). This difference might suggest that the transrepressive mechanism depends on specific impairment of the vitamin D signal transduction pathway by RORα action. Study of the OC minus mice provided evidence that OC is a determinant of bone formation and functions as a negative regulator of bone formation (37). Therefore, the bone-forming process could benefit from a decrease in OC expression through a conditionally active transcription factor like RORα, and the repression of the vitamin-D dependent activation of the OC gene by RORα may account in part for the mechanism of action of RORα in bone. In addition, the cross talk of RORα with the major calciotrophic hormone vitamin D also suggests a significant role of RORα in bone metabolism and bone hemostasis.

Evidence for a function of RORα in bone metabolism *in vivo* was obtained by studying tibial bone mass and geometry of the *sg* homozygous mutant mouse. This mutant, which occurred spontaneously in a stock of obese mice in 1955, contains a deletion within the RORα gene (38). The cerebellar cortex is grossly underdeveloped with a deficiency of granule cells and Purkinje cells (19), underlining the importance of RORα for normal cerebellar development. Female *sg/sg* mice have a late sexual maturation, irregular estrous cycling, and a shortened postpubertal period of reproduction compared with wild-type animals (39). As estrogen greatly influences bone metabolism, we avoided complications by choosing male animals for the initial investigation. We found that the homozygote negative males had a reduced bone diameter compared with heterozy-

gotes and the wild-type male littermates. However, the tibia was not significantly shortened although the homozygote negative animals were of reduced size (40). The difference in tibial bone geometry may be related to the staggering gait, mild tremor, and hypotonia of the homozygotes (41). These factors are likely to influence muscle mass and strength and thus could indirectly also affect bone geometry.

More interestingly, the bones of the *sg/sg* animals are osteopenic. This was indicated by the significantly reduced bone mineral density as shown by dual energy x-ray absorptiometry measurements, i.e., area-based measurements, in the entire tibia and more importantly by computed tomography, which gives a true volumetric density measurement in a cross-section through the metabolically most active proximal metaphyseal region. The heterozygotes do not display this bone phenotype. These observations strongly support the notion that RORα has a regulatory function in bone metabolism. However, a few confounding factors, complicating the interpretation of the origin of the osteopenic phenotype, have to be kept in mind. For example, peripheral macrophages of *sg* mice show increased production of IL-1 and IL-6 under lipopolysaccharide treatment, demonstrating a general condition of hyperexcitability of these cells (40, 41). High cytokine levels stimulate bone resorption and thus lead to osteopenia. At present, we can conclude that functional RORα is required for a normal bone phenotype.

The discovery of the ligand could greatly contribute to the further elucidation of the physiological relevance of RORα on bone tissue. As in case of the peroxisome proliferator-activated receptor or liver X receptor families, a major insight in the biology of these receptors has been gleaned from the discovery that these receptors are molecular targets for fibrates or cholesterol derivates (42–45). The knowledge of RORα signaling pathways activated by a hormone-bound receptor molecule will be another important step of understanding its detailed physiological function.

Taken together, the results obtained in *in vitro* and *in vivo* studies implicate a physiological role for RORα during bone development. Further studies are needed to assess whether RORα has a role in maintenance of bone mass in adulthood.

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