Osteoporosis in Sparc (osteonectin)-deficient mice: characterization of phenotypic determinants of femoral strength and changes in gene expression

Fiona C. Mansergh,1 Timothy Wells,1 Carole Elford,2 Samuel L. Evans,3 Mark J. Perry,4 Martin J. Evans,1 and Bronwen A. J. Evans2

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Submitted 12 July 2007; accepted in final form 11 September 2007

Mansergh FC, Wells T, Elford C, Evans SL, Perry MJ, Evans MJ, Evans BA. Osteopenia in Sparc (osteonectin)-deficient mice: characterization of phenotypic determinants of femoral strength and changes in gene expression. Physiol Genomics 32: 64–73, 2007. First published September 18, 2007; doi:10.1152/physiolgenomics.00151.2007.—Sparc null mutants have been generated independently via targeted mutations in exons 4 and 6. Previous studies have identified low-turnover osteopenia in the 129Sv/C57BL/6 exon 4 knockout. Since both Sparc null mutations result in complete absence of Sparc protein, similar phenotypic outcomes are likely. However, genetic background (strain) and/or linkage disequilibrium effects can influence phenotypic type. Different inactivating mutations should be tested in various mouse strains; similar phenotypic outcomes can then confidently be assigned to the mutated gene. We have evaluated the bone phenotype in the 129Sv/EvSparc<sup>tm1cam</sup> exon 6 knockout at 4 and 9 mo, using physical measurement, mechanical strength tests, and DXA scanning. We have also quantified bone marrow adiposity and circulating leptin levels to assess adipose tissue metabolism. 129Sv/EvSparc<sup>tm1cam</sup> null mice show decreased bone mineral density and bone mineral content and increased mechanical fragility of bone, in line with previous studies. Differences were also noted. Increased body weight and levels of bone marrow adiposity but decreased circulating leptin concentrations were identified at 4, but not 9 mo, and 129Sv/EvSparc<sup>tm1cam</sup> null mice also had shorter femurs. Molecular phenotyping was carried out using mouse HGMP NIA microarrays with cortical femur samples at various ages, using semiquantitative RT-PCR validation. We identified 429 genes highly expressed in normal bone. Six genes (Sparc, Zfp162, Bysl, E2F4, two ESTs) are differentially regulated in 129Sv/EvSparc<sup>tm1cam</sup> cortical femur vs. 129Sv/Ev controls. We confirm low-turnover osteopenia as a feature of the Sparc null phenotype, identifying the usefulness of this mouse as a model for human osteoporosis.

osteoporosis; knockout mice; microarray; adiposity

TOTAL BONE MASS VARIES throughout life and is regulated via relative rates of bone resorption and deposition. Osteoblasts deposit bone (and are responsible for its developmental formation), while osteoclasts resorb bone, resulting in the constant remodeling of skeletal homeostasis. Osteoblasts are derived from mesenchymal cell populations (20), while osteoclasts are formed via fusion of cells of the monocyte/macrophage family (37); precursors of both are present in the adult bone marrow (27). Osteoporosis is a disorder of skeletal turnover; bone is resorbed faster than it is reformed as a result of imbalances in the activity, or relative numbers of, osteoblasts and osteoclasts (37). Risk factors include sex, hormonal status, race, nutritional status, inadequate resorption/retention of calcium via the digestive system and kidney, lack of weight bearing exercise and low peak bone mass in youth.

Human and animal linkage studies have identified multiple genetic loci that influence bone mass [which is highly variable in humans and between different inbred mouse strains (1, 4, 17, 24, 32)]. Polymorphisms in the COL1A1, TGFβ1, SOST, VDR, ERα, LRP5, BMP2, and IL-6 genes, among others, have been implicated in increased osteoporosis risk (17, 21, 32). Moreover, genetically defined mouse mutants have been of particular utility in identifying underlying causes of bone thinning in humans. Gain of function p53 mutants, PAG5 hypomorphs, PolG knock-in mutants, and knockouts of BGN, SHIP, OPG, c-Abl, Irs1, klotho, Ku86, XPD, and Sparc all show reductions in bone density (15, 30, 35, 38).

Sparc (a.k.a. osteonectin or BM40) is a calcium, hydroxyapatite and collagen binding protein, implicated in cell proliferation, tissue morphogenesis, and repair and the modeling of extracellular matrix (9). It is widely expressed in development and is present at high levels in bone, possibly linking organic and mineral phases of bone tissue (27). Two independent Sparc knockout lines have been generated. One, Sparc<sup>tm1cam</sup>, was generated on both a purebred 129Sv/Ev and a mixed 129Sv/Ev/Mf1<sup>nu</sup> background, with an inactivating mutation in exon 6 of the Sparc gene (19). The other knockout (not specifically named but referred to as the Sparc exon 4 knockout) was generated on a 129Sv/C57BL/6 background with an inactivating mutation in exon 4 (2). Both Sparc null mutations have been shown to result in complete absence of Sparc protein via Western blot. Furthermore, testing of a wide variety of tissues and cell lines identified no splice variants of Sparc in mouse (29). Therefore, similar phenotypic outcomes would be likely. Initial analyses revealed subcortical posterior cataract in both lines (2, 19); differences in the timing of cataract onset is most probably genetic background dependent (28). Progressive low-turnover osteopenia has been identified in the 129Sv/C57BL/6 Sparc exon 4 knockout (5, 6, 7, 15, 16). The numbers of osteoblasts and osteoclasts have been shown to be reduced, resulting in a decrease in the rate of bone formation which exceeds the reduction in bone resorption (15). Increased mineral content, crystallinity and collagen maturity were noted, consistent with decreased bone formation and remodeling (5). These Sparc<sup>−/−</sup> mice attain a lower peak bone mass with progressive bone loss thereafter (15). Elevated serum leptin concentrations with advancing age, and excessive fat deposits have also been reported; marrow stroma from exon 4 Sparc<sup>−/−</sup> mice contained fewer osteoblasts and showed an increased tendency to form adipocytes (30, 35, 38). Interestingly, others...
have reported that Sparc seems to play a role in adipose tissue physiology (11), and Sparc expression is also altered in at least some forms of human obesity (23).

Genetic background would, however, be expected to influence this phenotype; C57BL/6 mice have comparatively low bone density, while that of 129 strains is typically high (3, 4). We have now studied the bone phenotype in purebred 129Sv/Ev Sparc<sup>tm1cam</sup> null mice. Given data suggesting increased adiposity at the expense of bone formation, we have also assessed body weight, bone marrow adipocyte size and number, and circulating leptin concentrations. Measurements of femoral size, strength, and mineral content have confirmed that loss of Sparc causes an osteopenic phenotype, regardless of the nature of the null mutation or the genetic background of the mice. Some differences have been noted between the two Sparc knockout lines, namely differences in femoral length and leptin metabolism not found in previous work with the exon 4 Sparc knockout animals.

We have also used our purebred mice with matched controls to carry out microarray analysis to assess consequent changes in femoral gene expression in ageing Sparc null mice. Six genes, including Sparc, are downregulated in 129Sv/Ev Sparc<sup>tm1cam</sup> null mice; we have also used data from controls to identify 429 expressed sequence tags (ESTs) that are highly expressed in normal bone. These may be of importance to the etiology of bone loss during ageing, and also provide a valuable resource for identifying candidate genes for association or linkage studies.

**MATERIALS AND METHODS**

**Mouse nomenclature and strain choice.** 129Sv/Ev Sparc<sup>tm1cam</sup> null mice: 129Sv/Ev refers to mice of inbred strain 129 and substrain SvEv. Sparc<sup>tm1cam</sup> refers to “Sparc, targeted mutation 1, University of Cambridge.” This targeted mutation is in exon 6. (The exon 4 knockout has not been similarly named and is referred to here as the exon 4 knockout). Mice used in this study were derived either from mutant homozygote lines or from 129Sv/Ev controls. Male mice were used to minimize the effect of hormonal variation on gene expression levels. For all measurements, experimental and control male animals were killed at 17 and 40 wk time points (4 and 9 mo). Sample sizes of at least six controls and six knockouts were used for each assay. Our inbred mice have been inbred for generations prior to the introduction of the Sparc<sup>tm1cam</sup> mutation; moreover, the ES cell lines used to generate this line were themselves of 129Sv/Ev origin. There should therefore be no genetic variation between control and experimental lines other than the Sparc<sup>tm1cam</sup> mutation. This should eliminate the possibility of error arising from linkage disequilibrium or from genetic drift between control and experimental lines derived from a mixed background. Elimination of such sources of error is particularly important when assessing results from microarray analyses.

**Animal husbandry and tissue extraction.** Mice were maintained and killed under Home Office license in accordance with British law (comparable with US Public Health Service Policy on Humane Care and Use of Laboratory Animals). Transgenic and control animals were maintained on RM3 diet (1.15% calcium, 0.82% phosphorus, 4,088.65 IU/kg vitamin D; Special Diet Services, Witham, Essex, UK) ad libitum. Most animals (except for those used in leptin assays) were killed via cervical dislocation. Whole femurs and tibias were dissected free of surrounding tissue. Bones intended for measurement and/or structural assay were wrapped in sterile PBS-soaked gauze and frozen at -20°C prior to assessment. Tibiae to be sectioned prior to adipocyte counting were placed in 4% paraformaldehyde (PFA). Trunk blood samples for the determination of circulating leptin levels were obtained from halothane-anesthetized mice via decapitation.

**Leptin assay.** Blood samples were collected in heparinized tubes and separated plasma was stored at -20°C prior to determination of plasma leptin concentrations via radioimmunoassay (Linco Research, St. Charles, MO). Intra-assay coefficient of variation (CV) was 4.3%.

**Bone marrow adipocyte counting.** Tibiae were fixed in 4% PFA for 2 days after dissection. The PFA was then replaced with a 10% EDTA solution (in 0.3 M NaOH). This solution was refreshed every second day for 3 wk. Following decalcification, the final EDTA wash was replaced with 70% ethanol. Bones were then embedded in paraffin wax, sectioned, and stained with toluidine blue. Sections were visualized using a Leica DMLB microscope and photographed with a Leica DFC300FX camera. Image analysis was carried out using Scion Image. Longitudinal sections of middiaphyseal marrow were assessed for adipocytes, following the method previously described by Gevers et al. (18). The total area counted was measured, along with adipocyte number, the area of each adipocyte, total adipocyte area and % of the field lateral and by adipocytes. Three sections were counted per animal; sections from six animals were assessed for each of the following four sample types: 4-mo male 129Sv/Ev, 4-mo male 129Sv/Ev Sparc<sup>tm1cam</sup>, 9-mo male 129Sv/Ev, 9-mo male 129Sv/Ev Sparc<sup>tm1cam</sup>. Statistical analysis of results was carried out using GraphPad Prism v. 2.0.

**Femoral length, strength, and morphology.** Previously frozen femurs were thawed at room temperature, and the lengths were measured with a hand-held micrometer. Each bone was then loaded in three-point bending between 2.5-mm-diameter rollers (6.5 mm apart), with the middle roller 3.25 mm from the outer rollers and positioned over the thinnest part of the femoral shaft, level with the distal end of the lateral ridge that runs along the proximal part of the femur toward the greater trochanter. The posterior aspect of the condyles rested on the side of the outer roller, and the bones were orientated such that they were loaded in a roughly posterior direction. Each bone was loaded at a crosshead speed of 2 mm/min until failure, with load and displacement data recorded by the computer controlled testing machine (Lloyd LRX tensile testing machine with 100N load cell; Lloyd Instruments, Segensworth, Hants, UK). Middiaphyseal cortical medio-lateral and anterior posterior diameters, and lateral, medial, anterior, and posterior wall thicknesses were measured at the fracture site using a Pye traveling microscope. Using these measurements and simple beam theory, ultimate tensile stress (UTS) was calculated using:

\[
I = \frac{\pi}{64} (b_i d_i^3 - b_o d_o^3)
\]

where \(b\) and \(d\) are the breadth and depth of the cross-section, respectively, and the subscripts \(o\) and \(i\) indicate the outside and inside dimensions, respectively.

**Femoral mineralization.** Bone mineral content (BMC) was measured by dual-energy X-ray absorptiometry using the Lunar Pixi small animal scanner. The accuracy of this technique in measuring calcium content was confirmed in preliminary studies, in which a highly significant correlation between femoral total BMC and ash weight (\(r = 0.86\); \(P < 0.0001\)) was obtained (34). The CV for femoral bone mineral density (BMD) for five repeated scans of 30 femurs, with repositioning between scans, was 2.7%. Bones were thawed at room temperature for 30 min before measurement and aligned anterior-posteriorly relative to the scanning beam. Measurements of total BMC (g) and scanned bone area (BA; cm²) were made, and areal BMD (aBMD; g/cm²) was calculated as BMC/BA. In addition, distal femur aBMD were determined by measuring BMC in a fixed area (0.03 cm²) of each region of interest.

**Statistical analysis.** All data are presented as means ± SD. The differences between two experimental groups were compared by the
unpaired Student t-test (*, **, and *** P < 0.05, 0.01 and 0.001, respectively, vs. control of the same age; +, ++, and +++ P < 0.05, 0.01, and 0.001, respectively, vs. same animal at different age).

RNA extraction for array analysis. Whole femurs were dissected free of surrounding tissue. Bone marrow was flushed via needle and syringe, using 0.5 ml PBS per femur. Cellular components of bone marrow were isolated via centrifugation for 5 min at 2000 rpm. Pellets were resuspended in TRIzol (Invitrogen, Paisley, UK) prior to RNA extraction. Flushed femurs were also retained. A 0.4-cm section of the femoral midshaft was obtained to minimize contamination by muscle and cartilage adhering to femoral epiphyses and standardize anatomical localization. Material from two to six femoral cortices was pooled in RNAlater (Ambion, Huntingdon, Camb., UK). Where tissue from more than one animal was used, material from litter mates was pooled. Femoral midshaft samples were later transferred into TRIzol (Invitrogen) and immediately homogenized using a Yellowline DH18 basic electronic homogenizer (Yellowline; IKA, Staufen, Germany) prior to RNA extraction via the manufacturer’s protocol. RNA samples were quantitated using formaldehyde gel electrophoresis and spectrophotometry (CamSpec, Sawston, Camb., UK). Different pooled RNA samples were used for labeling array repetitions. Total RNA (10 µg) was labeled with either Cy3 or Cy5 dyes using the CyScribe labeling system (GE Healthcare, Chalfont St. Giles, Bucks., UK), according to the manufacturer’s protocol. Labeled cDNA (1 µl) was combined with 2 µl 50% glycerol, run on a “John gel” (a microscope slide-sized, 1.5% agarose gel) and scanned using a GeneTac LS IV scanner (Genomic Solutions, Huntingdon, Camb., UK) to assess successful incorporation of label. Control and experimental samples were then combined and prepared for hybridization.

Arrays: hybridization, image, and data analysis. Microarray analysis was performed using the mouse National Institute on Aging (NIA) 15000 set from the Human Genome Mapping Project (HGMP), as described at the following website: http://www.ncbi.nlm.nih.gov/geo/ (platform accession no. GPL 5458). The following comparisons were carried out: 1) 17-wk 129Sv/Ev Sparc<sup>tm1cam</sup> null, male vs. 17-wk 129Sv/Ev wild-type male, bone marrow; 2) 17-wk 129Sv/Ev Sparc<sup>tm1cam</sup> null, male vs. 17-wk 129Sv/Ev wild-type male, femoral midshaft; 3) 40-wk 129Sv/Ev Sparc<sup>tm1cam</sup> null, male vs. 40-wk 129Sv/Ev wild-type, male, femoral midshaft; 4) 40-wk 129Sv/Ev Sparc<sup>tm1cam</sup> null, male vs. 40-wk 129Sv/Ev wild-type, male, bone marrow; 4) 40-wk 129Sv/Ev Sparc<sup>tm1cam</sup> null, male vs. 40-wk 129Sv/Ev wild-type male, femoral midshaft.

Hybridization and scanning were carried out as previously described (17). Arrays were repeated five times with fluorescent switching, to counteract any issues of dye bias that may have arisen from direct labeling. As the arrays contained duplicate spots, this allowed analysis of 10 spots per EST. Repetitions were derived from different RNA samples, to control for biological variation. Scanned images were stored and filtered and then analyzed using the GeneTac Analyser spot finding software (Genomic Solutions, Huntingdon, Camb., UK). Data were analyzed as previously described (28), but, in brief, ESTs that were deemed to be significantly differentially regulated were changed in expression levels by at least twofold in 8 out of 10 replicates, were above background plus 2 SD in at least one channel and showed a delta value of at least 0.5 using Significance Analysis of Microarrays software (http://www-stat.stanford.edu/~tibs/SAM). These ESTs were subjected to bioinformatic analysis and were assessed via semiquantitative RT-PCR to confirm array results. Data presented here are in full compliance with MIAME standards (8).

Data are available from the GEO database, under series number GSE8381. We have also exceeded the requirements of MIAME in PCR testing every potentially differentially regulated gene. We also analyzed the femur dataset from the control animals to identify genes highly expressed in normal bone. ESTs that were expressed above background + 2 SD in all five array repetitions, in both 4-mo and 9-mo animals were selected. These were analyzed using DAVID (http://niaid.abcc.ncifcrf.gov/). Gene details were later checked and amended where necessary, with reference to the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Gene details are presented in Supplementary Table S1.1

RT-PCR array confirmations. We tested all genes arising from the four experiments using semiquantitative RT-PCR. RNAs were quantitated as described above. Even quantities of control and experimental RNA (usually 2–4 µg) were treated with DNA free (Ambion) according to the manufacturer’s protocol. Reverse transcription (RT) reactions were carried out using the Superscript II First Strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer’s instructions. A “no RT” control corresponding to each sample was also produced; these were treated in exactly the same way as the samples except that Superscript II reverse transcriptase (Invitrogen) was not added. Standard primers were used for housekeeping genes; β-actin, β-microglobulin, and GAPDH (see Table 1 for primer sequences). We used three housekeeping genes in conjunction to ensure that biased expression of one would not adversely affect results. Moreover, the three housekeeping genes used were present on the NIA arrays and were not significantly altered in expression. Primers for ESTs were designed as previously described (28) (see also Table 1). PCRs were initially carried out at the optimum melting temperature indicated, including No RT controls. Conditions were varied in order that only single bands appeared and the number of cycles for minimum visibility on a gel was always identified. We ensured that PCR bands obtained for housekeeping controls were even at minimum visibility cycles before testing other genes. To minimize false positive results, we used at least two sets of Sparc null and two sets of control cDNAs from different animals. Variation was observed in both sets in order for a gene to be confirmed as variably expressed. PCRs were carried out in 20-µl volumes using 0.025-µmol concentrations of each dNTP (100 mM dNTP set, Invitrogen) and 1 unit Taq DNA polymerase (Sigma) per reaction. PCRs were usually carried out using PCR buffer (Sigma) containing 15 mM MgCl<sub>2</sub>; however, we varied magnesium concentrations where necessary to optimize results. Annealing temperature and the number of cycles were varied, up to a maximum of 50 cycles; finally, some primers were also redesigned. Final PCR conditions are indicated for confirmed genes in Fig. 4. PCRs changes were quantified from .tif files using Scion Image (Scion), via the Scion Image protocol for analyzing electrophoretic gels. PCR intensity changes (Fig. 4) were calculated via the following equation:

\[
\frac{\text{Spare average band intensity/wild-type average band intensity}}{\text{Spare average housekeeping gene intensity/wild-type average housekeeping gene intensity}} = X
\]

Changes <1 were converted to fold changes as follows: −1/X.

RESULTS

Body weight and adiposity. The body weight of wild-type 129Sv/Ev mice increased by 20% from 4 mo to 9 mo (P < 0.001; Fig. 1A) and this gain was accompanied by a 40% increase in circulating leptin (P < 0.05, Fig. 1E). At 4 mo 129Sv/Ev Sparc<sup>tm1cam</sup> mice were significantly (20%) heavier than their wild-type counterparts (P < 0.001, Fig. 1A), but this difference was not sustained, 129Sv/Ev Sparc<sup>tm1cam</sup> mice being similar to their wild type counterparts at 9 mo (Fig. 1A). Despite being heavier than their wild-type counterparts at 4 mo, circulating leptin was 40% lower in 129Sv/Ev Sparc<sup>tm1cam</sup> mice than in wild-type animals (P < 0.01, Fig. 1E). Plasma leptin concentration increased at a much higher rate in 129Sv/Ev Sparc<sup>tm1cam</sup> mice than control animals between 4 and 9 months.

1 The online version of this article contains supplemental material.
9 mo of age, however, such that leptin concentrations were not significantly different between the two groups at 9 mo of age.

In the tibial marrow compartment total marrow adiposity did not change between 4 and 9 mo in 129SvEv mice (Fig. 1 D). At 4 mo of age marrow adiposity was doubled in 129SvEv Sparc<sup>tm1cam</sup> mice (P < 0.05) but declined significantly by 9 mo (P < 0.05), becoming similar to that in their wild-type counterparts (Fig. 1 D). These differences in marrow adiposity were not dependent upon any changes in the number of adipocytes (Fig. 1 D) but were entirely attributable to the parallel differences in adipocyte size, adipocyte size being increased by 55% at 4 mo of age (P < 0.01) and declining by 25% at 9 mo (P < 0.05, Fig. 1 C).

**Femoral length and morphology.** In 129SvEv mice femoral length increased by 4% between 4 and 9 mo (P < 0.01, Fig. 2 A). Over the same period, there was no significant increase in femoral length in 129SvEv Sparc<sup>tm1cam</sup> mice, femoral length becoming 4% shorter by 9 mo (P < 0.001, Fig. 2 A). Measurement of middiaphyseal femoral diameters revealed that in 129SvEv mice neither medio-lateral, nor anterior-posterior diameters changed over the same period (Fig. 2 B and C). However, in 129SvEv Sparc<sup>tm1cam</sup> mice, medio-lateral diameter increased by 10% over this age range (P < 0.01, Fig. 2 B), whilst anterior-posterior diameter remained unaltered. At no point were either measures of diameter different between 129SvEv Sparc<sup>tm1cam</sup> mice and their wild-type counterparts.

**Femoral mineralization.** Total femoral mineral content (BMC) increased by 18% between 4 and 9 mo in 129SvEv mice (P < 0.05, Fig. 2 E), but when corrected for femoral area, aBMD was not significantly increased (Fig. 2 D). Total femoral BMC in 129SvEv Sparc<sup>tm1cam</sup> mice was reduced by 20% in 4-mo-old 129SvEv Sparc<sup>tm1cam</sup> mice (P < 0.01), but despite increasing significantly by 9 mo (P < 0.01), total BMC remained 14% lower than that in 129SvEv mice (P < 0.05, Fig. 2 E). When corrected for femoral area, aBMD remained significantly reduced in 129SvEv Sparc<sup>tm1cam</sup> mice (Fig. 2 D). At 4 mo aBMD was 17% lower in 129SvEv Sparc<sup>tm1cam</sup> mice (P < 0.01), but despite a 13% increase (P < 0.001), remained 11% lower than that in 129SvEv mice at 9 mo (P < 0.001, Fig. 2 D).

**Femoral strength.** Strength testing of middiaphyseal femora revealed that failure load did not increase significantly between 4 and 9 mo in 129SvEv mice (Fig. 3 A). Despite a similar femoral strength at 4 mo, failure load in 129SvEv Sparc<sup>tm1cam</sup> mice diverged from that in their wild-type counterparts, becoming 20% lower at 9 mo of age (P < 0.01, Fig. 3 A). The absence of any significant age- or strain-related differences in the second moment of area revealed that there was no significant contribution of geometric variables to the recorded differences in femoral strength (Fig. 3 B). Although in 129SvEv

### Table 1. Primers used for amplification

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**Housekeeping Genes**

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Genes detected after array analysis as significantly differentially regulated in 129SvEv Sparc<sup>tm1cam</sup> null bone. All significant genes are listed here, including those not eventually confirmed by RT-PCR. The columns list the GenBank accession numbers of the expressed sequence tag (EST) originally printed on the array, the gene to which this EST is homologous (if any), and the forward and reverse primer sequences. Our standard housekeeping gene primer sequences are listed separately at the bottom. The final date of search is given beside uncharacterized ESTs.

### References

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Sparc<sup>tm1cam</sup> mice the mean UTS at 9 mo was only 78% of that in 4-mo-old 129SvEv Sparc<sup>tm1cam</sup> mice, none of the means were significantly different (Fig. 3B).

**Array results.** As stated above, four different sets of array comparisons were carried out: 1) 17-wk 129SvEv Sparc<sup>tm1cam</sup> null, male vs. 17-wk 129Sv/Ev wild-type, male, bone marrow; 2) 17-wk 129SvEv Sparc<sup>tm1cam</sup> null, male vs. 17-wk 129Sv/Ev wild-type, male, femoral midshaft; 3) 40-wk 129SvEv Sparc<sup>tm1cam</sup> null, male vs. 40-wk 129Sv/Ev wild-type, male, bone marrow; 4) 40-wk 129SvEv Sparc<sup>tm1cam</sup> null, male vs. 40-wk 129Sv/Ev wild-type, male, femoral midshaft.

**Bone marrow.** Analysis of bone marrow samples at both 4 and 9 mo showed no statistically significant differences between 129SvEv Sparc<sup>tm1cam</sup> null mice and 129SvEv controls. When we looked at Sparc expression in controls, it was often so low that values were beneath background + 2 SD, thereby eliminating Sparc from further analysis. If Sparc is not highly expressed in a majority of marrow cells, the effect of knocking it out in this tissue may be minimal. Another possibility is that, owing to high heterogeneity of cell type within the bone marrow, significant expression differences between cell subtypes may be difficult to detect.

**Femoral midshaft.** Analysis of femoral midshaft bone arrays showed that five genes (including Sparc) were differentially regulated at 4 mo between 129SvEv Sparc<sup>tm1cam</sup> null mice and 129SvEv controls, while 15 genes (also including Sparc) were differentially regulated at the 9-mo time point (see Table 1). The presence of the Sparc gene acted as a good internal control and was reliably detected in each of the femur datasets as downregulated.

Via semiquantitative RT-PCR, we were able to confirm the downregulation of Sparc at 4 and 9 mo and of five other genes, zinc finger protein 162 (Zfp162), Bystin-like 1 (Bysl), transcription factor E2F4, and two uncharacterized ESTs, BG066811 and BG065585, at 9 mo (Fig. 4). Bioinformatic analysis of these genes is presented in Table 2. We also analyzed the femur dataset from the control animals to identify genes highly expressed in normal bone. Details of 429 highly expressed ESTs are presented in Supplementary Table S1. Some of these represent the same gene; having taken redundancies into account, we find there are a total of 403 entries. Of these, 204 genes are mapped to UniGene clusters, some of which are well characterized functionally; 16 map to the mouse mitochondrion; and 183 are completely uncharacterized as of 23rd February, 2007. We have compared the composition of our dataset with other array studies previously presented. Microarray datasets involving biglycan-deficient preosteoblasts, glucocorticoid-treated osteoblasts, and early osteoblast differentiation did not contain any of the genes we noted above (12, 14, 26). However, all of these studies involved cultured osteoblasts as opposed to live bone samples. Similarly, we have compared the chromosomal locations of our genes with a quantitative trait locus (QTL) mapping study using 129S1/SvImJ mice (the only mapping study we could find where strain 129 mice were used to map bone density) (24). None of the locations of our genes matched to QTLs identified; how-
ever, we have included mouse and human chromosomal locations in Table 2 for ease of comparison with other such studies.

**DISCUSSION**

Sparc is an important structural constituent of bone, not only binding hydroxyapatite and collagen, but also regulating cell proliferation, cell-matrix interactions, angiogenesis, and MMP production (15). We have now confirmed that the inactivation of Sparc results in osteopenia, regardless of mutation location or genetic background. This is important, as numerous phenotypes attributed solely to the lack of the ablated gene have later been explained by genetic background differences between experimental and control animals or by linkage disequilibrium (13).

Our Sparc null mutation is present in a genetic background that normally specifies high bone density (3, 4); as a result we have a milder phenotype than that previously reported (15). Differences in BMC and aBMD were noted at both 4- and 9-mo time points, but mechanical strength only differed significantly at 9 mo. This impairment of strength appears to be accounted for by a change in material properties rather than a change in the size of the bones. We also noted a small but significant reduction (3–4%) in femoral length in 129SvEv Sparc<sup>tm1cam</sup> null mice. Length is inversely related to strength,

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Fig. 2. Development of femoral geometry and mineral content in control (■) and 129SvEv Sparc<sup>tm1cam</sup> null mice (●). Parameters shown are femoral length (A), mediadiaphyseal medio-lateral (M/L) diameter (B), mediadiaphyseal anterior-posterior (A/P) diameter (C), areal bone mineral density (aBMD, D), bone mineral content (BMC, E). Values shown are means ± SD (n=10–16 for A, B, and C, and 6–13 for D and E; *, **, and ***P < 0.05, 0.01, and 0.001, respectively, vs. control of the same age; +, ++, and +++P < 0.05, 0.01, and 0.001, respectively, vs. same animal at different age).

Fig. 3. Development of femoral strength in control (■) and 129SvEv Sparc<sup>tm1cam</sup> null mice (●). Parameters shown are failure load (A), second moment of area (B), and ultimate tensile stress (C). Values shown are means ± SD (n=13–16; **P < 0.01 vs. control of the same age).
but the potential of this variable is largely negated in our study via the use of a fixed span in the three-point bending protocol. Previous reports had indicated no change in skeletal size (2, 19). However, Sparc\textsuperscript{tm1cam} null mice were studied initially on a M\textsuperscript{f1} outbred mixed background. Preliminary observations using these mice and M\textsuperscript{f1} controls showed such high degrees of individual variability in most skeletal parameters (unpublished data), which we subsequently used inbred 129SvEv mice. Given the small difference observed, it is also likely that this would be undetectable even on a “fixed” mixed background derived from two inbred lines (such as that studied in the exon 4 knockout).

Previous reports note increased adiposity in exon 4 Sparc null mice (7); conversely, Sparc expression is increased in three different mouse models of obesity and altered in human obesity cases (36). These results point to a complex role for Sparc in adipose regulation. We therefore assessed body weight and adiposity in these animals. Adipocytes, osteoblasts, and cartilage originate from the same lineage, mesenchymal stem cells (6, 7, 20), therefore absence of Sparc may perturb mesenchymal differentiation at the expense of the osteoblast lineage. Skeletal size differences are minor and only appear in later life, indicating that an effect on cartilage differentiation and skeletal patterning is unlikely, but marrow stroma from 129SvC57BL/6 Sparc null mice contains fewer osteoblasts and shows an increased tendency to form adipocytes (16), suggesting preferential adipocytic differentiation at the expense of the osteoblast lineage. To address these issues, we have assessed the size and number of bone marrow adipocytes. These studies, involving staining and counting of adipocytes in fixed tibial sections, are advantageous in that this method rules out any effect of cell culture on cell numbers and may be more indicative of any perturbations in stem cell differentiation than analysis of peripheral fat depots. The numbers of marrow adipocytes do not vary significantly (although their volume does at one time point), indicating that the lack of Sparc has more effect on lipid accumulation than on differentiation or proliferation. However, adipocytes in marrow are thought to secrete leptin; larger adipocytes secreting proportionately more. It is conceivable that marrow-derived leptin may exert a

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**Fig. 4. RT-PCR confirmation of genes differentially regulated in 4- and 9-mo 129SvEv Sparc\textsuperscript{tm1cam} null mice.** Gene name, number of PCR cycles, and annealing temperature used are given to the right. Lane labels identify the following: C = control, 129SvEv bone; Sp = 129SvEv Sparc\textsuperscript{tm1cam} null bone; NC and NSp indicate the corresponding No RT controls. Two sets of samples from different control and experimental animals were used. β-Actin, β-globulin, and Gapdh are housekeeping controls. Residual Sparc expression is often detected by PCR in knockouts, but Western blots have confirmed the absence of functional protein (19).
Table 2. Confirmed genes

<table>
<thead>
<tr>
<th>Accession</th>
<th>Sample</th>
<th>Gene Description</th>
<th>Array Fold Change</th>
<th>Unigene Cluster</th>
<th>Mn. Chromosome</th>
<th>Hs. Chromosome</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>AW538347</td>
<td>9-mo femur</td>
<td>E2F4: E2F transcription factor 4, mRNA</td>
<td>down, -3.82</td>
<td>Mm.34554</td>
<td>8 (8 D3)</td>
<td>E2F4, Hs.108371, 16q21-q22</td>
<td>role in early adipogenic differentiation; knockout mice show craniofacial and erythropoic defects; OMIM 600659; human Unigene cluster lists bone and bone marrow as tissue source</td>
</tr>
<tr>
<td>BG064802</td>
<td>4-mo + 9-mo femur</td>
<td>Sparc Secreted acidic cysteine rich glycoprotein (Osteonectin)</td>
<td>4 mo, down, -9.04 9 mo down, -11.15</td>
<td>Mm.35439</td>
<td>11</td>
<td>SPARC, Hs.111779, 5q31.3-q32</td>
<td>knocked out in Sparc mice, working control; mouse Unigene cluster lists bone and bone marrow as tissue source uncharacterized not well characterized</td>
</tr>
<tr>
<td>BG065585</td>
<td>9-mo femur</td>
<td>EST Ccde96: Coiled-coil domain containing 96</td>
<td>down, -4.83</td>
<td>no Unigene</td>
<td>Mm.42368</td>
<td>5 (5 B2)</td>
<td>cohiled-coil domain containing 96, Hs.646895, 4p16.1 highly expressed in macrophages; contains the KH module, a sequence motif indicating a major role in regulating cellular RNA metabolism (10); OMIM 601516; mouse Unigene cluster lists bone and bone marrow as tissue source uncharacterized not well characterized</td>
</tr>
<tr>
<td>BG066811</td>
<td>9-mo femur</td>
<td>EST Ccde96: Coiled-coil domain containing 96</td>
<td>down, -5.23</td>
<td>Mm.256422</td>
<td>19 (19B)</td>
<td>Splicing factor 1 (SF1), Hs.502829, 11q13</td>
<td>highly expressed in macrophages; contains the KH module, a sequence motif indicating a major role in regulating cellular RNA metabolism (10); OMIM 601516; mouse Unigene cluster lists bone and bone marrow as tissue source uncharacterized not well characterized</td>
</tr>
<tr>
<td>BG077386</td>
<td>9-mo femur</td>
<td>zinc finger protein 162, mRNA (cDNA clone MGC: 3157495 IMAGE: Zip162)</td>
<td>down, -4.99</td>
<td>Mm.256422</td>
<td>19 (19B)</td>
<td>Splicing factor 1 (SF1), Hs.502829, 11q13</td>
<td>highly expressed in macrophages; contains the KH module, a sequence motif indicating a major role in regulating cellular RNA metabolism (10); OMIM 601516; mouse Unigene cluster lists bone and bone marrow as tissue source uncharacterized not well characterized</td>
</tr>
<tr>
<td>BG079188</td>
<td>9-mo femur</td>
<td>bstand-like, mRNA (cDNA clone MGC: 27710 IMAGE:4925307) (Bysl)</td>
<td>down, -5.52</td>
<td>Mm.27291</td>
<td>17</td>
<td>bstand-like (BYSL), Hs.106880, 6p21.1</td>
<td>bstand is involved in implantation, also cell adhesion; OMIM 603871; mouse Unigene cluster lists bone and bone marrow as tissue source</td>
</tr>
</tbody>
</table>

paracrine influence on bone formation without changing levels of circulating leptin.

Exon 4 129SvC57BL/6 Sparc null mice have been shown to have larger deposits of subcutaneous and epididymal fat and to display raised serum leptin levels between 6–8 mo (7). In contrast, we failed to note a difference in circulating leptin concentration at 9 mo, but noted a significant decrease in leptin concentrations at 17 wk. We also noticed a substantial increase in body weight at 17 wk [a time point not tested in the prior study (7)], which had disappeared at 40 wk (9 mo). These differences are probably not diet related, as food fat contents varied (28, 33).

There are a number of genes that have a high homology to Sparc [hevin/Sc1, Spock 1-3 (also known as testicans 1-3), Smoc1, and Smoc2]. Our arrays have not detected differential regulation of any of these to compensate for the absence of

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Sparc. It should be noted however, that the NIA set contains only 15,000 ESTs, in which there is a degree of redundancy (28). This hypothesis has not been tested on an array dataset that is representative of the entire mouse genome.

The nature of the differentially regulated genes is interesting. The Unigene clusters representing three of these genes, E2F4, Bysl, and Zfp162, quote bone and sometimes bone marrow as a tissue source, demonstrating their relevance to bone biology. E2F4 knockout mice have been generated; erythroid abnormalities during development and craniofacial abnormalities have been noted (22). Of further relevance is the fact that this gene regulates adipocyte differentiation. Zfp162 and Bysl are less well investigated; however, high levels of expression in macrophages (which are bone marrow derived and which fuse in some cases to form osteoclasts) have been noted for Zfp162 (10). The remaining two genes are functionally uncharacterized. Notably, despite the number of ESTs present on the array, we have not seen relative changes in biomarkers for bone metabolism, in either bone or in bone marrow, which contains both osteoblast and osteoblast precursors.

Using data from control animals, we have also identified 403 genes that are highly expressed in bone. Of these, 183 are completely uncharacterized. These data may be a useful resource in identifying novel candidate genes for skeletal disease, especially as a large number of linkage and association studies have delineated wide intervals in which important genes reside, but, owing to the number of genes within the critical regions, many have not yet been identified (21).

Despite the wealth of data from various genome and EST projects (23, 25), biochemical pathways identified in bone are still incomplete, while large proportions of genes or ESTs identified by large scale sequencing projects are relatively functionally uncharacterized, implying that many vital regulators of skeletal homeostasis remain to be elucidated. The use of microarrays in combination with knockout mice explores phenotypes in a complex world of development, ageing, and environment and can also be used to elucidate currently unidentified components of essential biological processes such as skeletal metabolism. Identification of genes dysregulated during abnormal bone thinning may lead us to novel routes of therapeutic intervention for osteoporosis. This topic is also important in that it will allow us to develop our knowledge of the biology and biochemical mode of action of Sparc, which we have confirmed as an important therapeutic target for osteoporosis.

In summary, our data predicate the utility of 129SvEv Sparcnullcam pull mice as a model of low turnover osteopenia. We have also identified a number of genes that may contribute to the impairment of bone in the absence of Sparc.

ACKNOWLEDGMENTS

We thank The HGMP Centre for provision of the NIA microarray slides used in this study. We also thank Anna Hurley and Steve Turner, Vicky Workman, and Steffan Adams at the Cardiff University Array facility for assistance with array protocols, scanning, image analysis, data storage, and bioinformatics.

S. L. Evans, M. J. Evans, and B. A. J. Evans are members of CITER (Cardiff Institute of Tissue Engineering and Repair).


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GRANTS

This work was funded by the Wellcome Trust and the Biotecnology and Biological Sciences Research Council.

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