Sost Haploinsufficiency Provokes Peracute Lethal Cardiac Tamponade without Rescuing the Osteopenia in a Mouse Model of Excess Glucocorticoids

Behzad Javaheri,* Eleanor Herbert,* Mark Hopkinson,* Ahmed Al-Jazzar,† and Andrew A. Pitsillides*

From the Department of Comparative Biomedical Sciences,* The Royal Veterinary College, London, United Kingdom; Experimental Histopathology,† The Francis Crick Institute, London, United Kingdom; and the College of Veterinary Medicine,‡ King Faisal University, Al-Hofuf, Saudi Arabia

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Address correspondence to Andrew A. Pitsillides, Ph.D., Department of Comparative Biomedical Sciences, The Royal Veterinary College, Royal College St., London NW1 0TU, United Kingdom. E-mail: apitsillides@rvc.ac.uk.

Glucocorticoid-induced secondary osteoporosis is the most predictable side effect of this anti-inflammatory. One of the main mechanisms by which glucocorticoids achieve such deleterious outcome in bone is by antagonizing Wnt/β-catenin signaling. Sclerostin, encoded by Sost gene, is the main negative regulator of the proformative and antiresorptive role of the Wnt signaling pathway in the skeleton. It was hypothesized that the partial inactivation of sclerostin function by genetic manipulation will rescue the osteopenia induced by high endogenous glucocorticoid levels. Sost-deficient mice were crossed with an established mouse model of excess glucocorticoids, and the effects on bone mass and structure were evaluated. Sost haploinsufficiency did not rescue the low bone mass induced by high glucocorticoids. Intriguingly, the critical manifestation of Sost deficiency combined with glucocorticoid excess was sporadic, sudden, unprovoked, and nonconvulsive death. Detailed histopathologic analysis in a wide range of tissues identified peracute hemopericardium and cardiac tamponade to be the cause. These preclinical studies reveal outcomes with direct relevance to ongoing clinical trials that explore the use of antisclerostin antibodies as a treatment for osteoporosis. They particularly highlight a potential for increased cardiovascular risk and may inform improved stratification of patients who might otherwise benefit from antisclerostin antibody treatment. (Am J Pathol 2019, 189: 753–761; https://doi.org/10.1016/j.ajpath.2018.12.007)
essential for osteocyte viability and maintenance of the bone’s material properties. These changes may explain the impairment of the biomechanical properties in the surrounding bone and may account for the loss of bone strength that occurs before the loss of bone mineral density.

One of the mechanisms by which GCs drive bone loss is by inhibition of the Wnt/β-catenin signaling pathway, critical for the differentiation of mesenchymal cells toward mature osteoblasts, bone formation, and mechanoadaptive responses. A natural antagonist of Wnt signaling, sclerostin (Sost gene product), predominantly secreted by osteocytes, is a potent inhibitor of osteoblastic mineralization. Thus, its deficiency provokes marked increases in bone mass achieved by a range of targets, without any significant impact on osteocyte differentiation.

Several studies have reported contradictory results on the relationship between GC excess and sclerostin. Previous studies have reported that in vivo antisclerostin antibody treatment prevents the reduction in bone mass and strength induced by GC excess. Despite these established links between sclerostin levels and the prevention of GC-induced changes in bone mass, there remains controversy about how these links affect patients, in which there have been discordant reports of decreased serum sclerostin in patients after 1 week of GC treatment and increased serum sclerostin at later time points. Similar disparities in the levels of sclerostin are also seen in GC-related disease states, in which both increased and decreased levels are reported in patients with excess GCs. The reasons for these contradictory observations on the relationship between sclerostin and excess GCs are unclear, raising the question whether modulation of sclerostin function indeed counteracts the deleterious effect of endogenously raised GC levels on bone mass and strength.

We tested this possibility by determining whether genetically determined sclerostin deficiency was capable of rescuing the compromised bone mass that occurs with GC excess. To address this question, sclerostin-deficient mice were crossed with an established mouse model of GC excess because of an N-ethyl-N-nitrosourea–induced mutation in corticotropin-releasing hormone (Crh), and the effects on bone mass and structure were evaluated along with detailed histopathologic analysis in a wide range of tissues. Our preclinical findings are relevant to ongoing clinical trials in which serious fatal cardiovascular adverse events were reported in patients receiving antisclerostin antibody. They particularly highlight a potential need for patient stratification to help realize the potential benefit of such treatment.

**Materials and Methods**

**Animals**

Frozen sperm from a male Sost knockout (KO) mouse in the C57BL/6J background was purchased from the Knockout Mouse Project Repository at the University of California Davis, and used to fertilize ova from C57BL/6J wild-type (WT) mice as described previously; heter/haplozygosity was confirmed by genotyping. The mouse model for excessive circulating GC concentrations was a gift from Medical Research Council (Oxfordshire, UK) and was generated in C57BL/6J mice by an N-ethyl-N-nitrosourea–induced mutation in corticotropin-releasing hormone (Crh) at −120 bp of the promoter region, resulting in a gain-of-function mutation (Crh+/−/−) and mated with C3H/HeH as described previously. Female Sost homozygous KO (Sost+/−) mice were crossed with male Crh+/−/− to produce Sost+Crh+/−/− (Sost heterozygous/Crh heterozygous) and Sost+/− mice. Mice were housed in polycarbonate cages under 12 hours light/dark cycle at 21 ± 2°C with free access to Rat/Mouse One maintenance diet (Special Diet Services, Witham, UK) and water ad libitum. The studies used only male mice throughout. All procedures complied with UK Animals (Scientific Procedures) Act 1986, were approved by the Royal Veterinary College’s Ethics committee, and followed Animal Research: Reporting of In Vivo Experiments guidelines.

**X-Ray MicroCT**

In vivo scanning of the entire right tibia at 1 month of age under 2% isoflurane-induced anesthesia and postmortem at 2 months of age were achieved with the use of a Skyscan 1176 X-ray microcomputed tomography machine (Skyscan, Kontich, Belgium). The X-ray tube was operated at 40 kV, 600 μA, with a voxel size of 9 μm, an exposure of 2000 ms, and a rotation step of 0.800 degrees. The radiation dose from the microcomputed tomography (microCT) scanning was estimated to be approximately 500 mGy for each scan, which has been proved to cause no significant effect on bone adaptations. Slices were reconstructed with the use of NRecon 1.6; trabecular and whole bone analyses were performed as described previously. For morphometric trabecular analysis appearance of the trabecular bridge that connected the two primary spongiosa bone islands was set as a reference point for analysis of proximal tibia metaphyseal trabecular bone; 5% of the total bone length from this point (toward diaphysis) was used. For cortical analysis, after segmentation, alignment, and removal of fibula a minimum threshold was used in Slice Geometry to calculate mass: cross-sectional area (CSA), mean cross-sectional thickness, second moment of area around minor axis, second moment of area around major axis, and predicted resistance to torsion along a central 70% portion of the entire tibia length excluding regions that contained trabecular bone.

**Histologic Analysis**

Tibia, heart, lungs, liver, spleen, kidneys, and brain were fixed in 4% formaldehyde (from paraformaldehyde Alfa Aesar Inc., Ward Hill, MA) and stored in 70% ethanol before routine processing into paraffin and production of
slides sectioned at 4 μm and stained with hematoxylin and eosin. Stained slides were evaluated by a board-certified veterinary pathologist (E.H., The Francis Crick Institute, London, UK). Total numbers of animals examined were Sost+/+ WT (n = 4), Sost+/− heterozygous KO (n = 7), Sost−/− homozygous KO (n = 4), Crh+/+ WT (n = 4), Crh−/+−120 heterozygous KO (n = 4), and Sost-Crh−/+−120 heterozygous (n = 5) mice. In addition, Sost−/+−120 (n = 3) mice which spontaneously died were stored in 70% ethanol and examined histologically. Hearts from these animals were examined by using multiple step levels and extensive sectioning throughout the block.

Statistical Analysis

Statistical analyses were performed with R, version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria; http://www.r-project.org, last accessed August 29, 2018). Measurements were summarized as means ± SEM. Linear model (two-way analysis of variance) was used to determine the effects of age (1 and 2 months) and genotype (Sost heterozygous and homozygous as well as Crh heterozygous and double Sost-Crh heterozygous and their corresponding WT littermates) and their interaction on all phenotypic measurements. Bonferroni post hoc correction was performed for whole bone measurements. The statistical significance level was set at 5%.

Results

Sost Haploinsufficiency Does Not Rescue Compromised Bone Mass Induced by Excessive GCs

The designed studies tested whether genetically determined Sost deficiency rescued low bone mass induced by excessive GCs in vivo. Trabecular bone was analyzed, and the entire tibial cortex was analyzed proximodistally in a non-biased fashion at 1 and 2 months of age. Both age and genotype independently affected the cortical CSA, and significant interaction was evident between age and genotype along the entire tibial length (Figure 1). The detailed post hoc analyses at 1 month of age revealed, as expected, significantly higher CSA in Sost homozygous KO (Sost−/−) mice than in Sost+/+ WT mice (Figure 1) as well Sost−/− mice compared with Sost−/+ mice along the entire tibia. Unexpectedly, lower cortical CSA was observed in restricted regions of the tibia in Sost−/+ mice compared with Sost+/+ WT littermates (Figure 1). At 2 months of age, CSA was significantly higher in Sost−/− mice than in Sost+/+ WT mice between 15% and 85% of the length and also in Sost−/− mice compared with Sost+/+ WT and Sost+/+ mice across the entire tibial cortex.

Analysis of mice with excessive endogenous GCs at 1 month of age failed to find any significant differences in cortical CSA at any location along the tibia in WT Crh (Crh+/+ WT), heterozygous Crh (Crh+/−−120), or mice heterozygous for both Crh and Sost (Sost-Crh+/−−120) (Figure 1). In contrast, in mice aged 2 months, CSA was, as expected, significantly lower in Crh+/−−120 mice compared with Crh+/+ WT mice and also lower in Sost-Crh+/−−120 mice compared with Crh+/+ mice between approximately 25% and 85% of the tibial length. Intriguingly, no significant rescue in the reduction of CSA was observed at any location in tibia from Sost-Crh+/−−120 mice compared with Crh+/−−120 mice. Evaluation of torsion, a measure of predicted resistance to torsion, showed identical trends, indicating that Sost haploinsufficiency also failed to rescue GC-induced decreases in the tibia’s architectural strength (Supplemental Figure S1). Further interrogation of cortical bone revealed that mean cortical cross-sectional thickness (Supplemental Figure S2), second area around minor axis (Supplemental Figure S3), and around major axis (Supplemental Figure S4) also indicated that Sost haploinsufficiency failed to rescue Crh+/−−120-induced compromise in cortical bone mass and architecture. Our evaluation of age-related changes in the WT mice of both Sost−/− (C57BL/6NTac/C57BL/6J) and Crh+/−−120 (C57BL/6J/C3H/HeH) backgrounds revealed greater growth kinetics within the latter, between 1 and 2 months of age, suggesting that there was ample scope for any effects of the combined genetic manipulation to be realized (Supplemental Figure S5).

In addition to microCT analysis of cortical bone, tibial trabecular bone was analyzed. Percentage of bone volume [trabecular bone volume (BV)/trabecular total volume (TV)] was significantly greater in mice lacking both copies of Sost (Sost−/−), whereas a trend that did not reach levels of statistical significance was observed for enhanced BV/TV in mice lacking a single copy (Sost+/−) (Figure 2A). This elevation of BV/TV in Sost−/− mice was linked with enhanced BV, trabecular number, and thickness, and lower trabecular separation; neither of these variables alone reached statistical significance (Figure 2A). The analysis also showed a trend for lower trabecular BV/TV in mice with excess GCs (Crh+/−−120) and no significant modification of BV/TV in Sost-Crh+/−−120 mice (Figure 2A). In agreement with the microCT data, detailed histologic evaluation of tibia structure revealed thicker cortices in tibiae from mice lacking either one (Sost+/−) or both (Sost−/−) copies of functional Sost compared with tibia from Sost+/+ WT mice (Figure 2, B–G). Thinner cortices were also observed in Crh+/−−120 mutant mice compared with tibiae from their Crh+/+ WT littermates. In agreement with three-dimensional quantification, tibiae from mice harboring both Sost+/+ and Crh+/−−120 (Sost-Crh+/−−120), were similar to Crh+/−−120 mice, indicating a failure of Sost+/− to rescue the effects of GC excess on bone mass (Figure 2, E–G). Together, the detailed comparison of bone by both histology and microCT revealed at 2 months of age that i) both heteroand homozygous Sost-deficient mice exhibited significantly greater bone mass and predicted strength, ii) heterozygous Crh mutation led to a compromised bone mass and strength.
and, iii) Sost haploinsufficiency fails to rescue the compromised bone mass produced by excess GCs.

Combined Sost Haploinsufficiency and Crh$^{+/120}$ Related GC Excess Provoke Peracute Lethal Cardiac Tamponade

In the studies that examined potential rescue of excess GC-related low bone mass by deletion of a single Sost allele, sudden, unprovoked, nonconvulsive death of approximately 10% of total Sost-Crh$^{+/−}$ offspring was observed between 1 to 2 months of age. No such incidences occurred in either Crh$^{+/120}$, Sost$^{−/−}$ mice or their respective WT littermates. To identify the cause of this sudden death, a comprehensive histopathologic examination of multiple tissues from all mouse strains was performed hematoxylin and eosin staining, which was scored with a semiquantitative grading system. Examination of the brain, kidney, liver, lung, and

Figure 1  Sost haploinsufficiency does not rescue low bone cross-sectional area (CSA) induced by excessive glucocorticoids (GCs). A: Average CSA of Sost wild-type (WT) (Sost$^{+/+}$), Sost heterozygous knockout (KO) (Sost$^{+/−}$), Sost homozygous KO (Sost$^{−/−}$), Crh WT (Crh$^{+/+}$), Crh heterozygous (Crh$^{+/−}$), and double Sost-Crh heterozygous (Sost-Crh$^{+/−}$) mice at 1 and 2 months of age. Lines represent the means; shading, the SEM. B: Graphic heatmap representation of statistical significance of the effect of genotype, age, their interactions as well as post hoc analysis along a 15% to 85% portion of the whole tibia length, excluding proximal and distal metaphyseal bone. Line graphs represent means ± SEM (shadow). n = 8 mice per group.
spleen showed no signs of overt pathology in $Sost^{+/+}$ WT, $Sost^{+/\times}$, $Sost^{-/-}$, $Crh^{+/+}$ WT or $Crh^{-/-120}$, or $Sost-Crh^{-/-120}$ mice in which a propensity for sudden death was observed.

Intriguingly, evaluation of heart tissue in $Sost-Crh^{-/-120}$ mice with sudden death revealed all to have hemopericardium with a markedly expanded pericardial space that contained a large number of densely packed, homogenously distributed erythrocytes and compression of the right ventricular (Figure 3A) and right atrial lumen (Figure 3B) in all mice that experienced sudden death. Therefore, the cause of sudden death in these animals was considered to be due to peracute hemopericardium that led to cardiac tamponade. Hearts were examined at multiple step levels through the tissue to try to ascertain the origin of the hemorrhage; however, none could be identified. No significant findings were observed in the hearts of the remainder of the $Sost^{-/-}$ mice in which a propensity for sudden death was observed.

Figure 2  Trabecular bone phenotype of Sost wild-type (WT) ($Sost^{+/+}$), Sost heterozygous knockout (KO) ($Sost^{+/\times}$), Sost homozygous KO ($Sost^{-/-}$), $Crh$ WT ($Crh^{+/+}$), $Crh$ heterozygous ($Crh^{+/\times}$), and double $Sost-Crh$ heterozygous ($Sost-Crh^{-/-120}$) mice at 2 months of age. A: Ex vivo high-resolution analyses of the proximal metaphyseal tibia to determine trabecular bone volume (BV), trabecular total volume (TV), percentage of bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). B–G: Representative hematoxylin and eosin–stained sections of tibia at 2 months of age. B: $Sost$ WT ($Sost^{+/+}$) mice. C: $Sost$ heterozygous KO ($Sost^{+/\times}$) mice. D: $Sost$ homozygous KO ($Sost^{-/-}$) mice. E: $Crh$ WT ($Crh^{+/+}$) mice. F: $Crh$ heterozygous ($Crh^{+/\times}$) mice. G: $Crh$ heterozygous ($Crh^{+/\times}$) mice. H: Double $Sost-Crh$ heterozygous ($Sost-Crh^{-/-120}$) with corresponding higher magnification demonstrating the structural differences between groups. Data are expressed as means ± SEM, $n = 8$ mice per group. *$P < 0.05$. Original magnification: ×2 (B–G, main images); ×10 (B–G, enlarged images).

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Discussion

Osteopenia because of excess GCs mainly involves a decrease in bone formation, secondary to the effects on the activity and viability of osteoblasts and osteocytes, which ultimately leads to an impairment in bone strength. One of the main mechanisms by which GCs achieve such deleterious outcome in bone is by antagonizing Wnt/β-catenin signaling. Sclerostin, the product of the Sost gene, is the main negative regulator of the Wnt signaling pathway. Thus, sclerostin normally acts to suppress the proformative and antiresorptive role of the Wnt signaling pathway. Herein, the hypothesis that the partial inactivation of sclerostin function by genetic manipulation will be capable of rescuing the low bone mass induced by high endogenous GC levels was tested.

In contrast, it was found instead that Sost+/− mice, or in any of the Sost+/−, Sost−/−, Crh+/−, WT, Crh+/−, WT, and Crh+/−120 mice.

It is possible that these dissimilar observations arise due solely to differences in experimental design and analysis. These studies undertook a nonbiased analysis of almost the entire tibial cortex adjusting for GC-induced divergence in bone length and thus ensure comparisons are made at precisely matched anatomic locations and volumes. Although Marenzana et al reported small, significant protective effects of antisclerostin antibody treatment against an exogenous GC-induced reduction in cortical bone volume at the femur mid-shaft, they did not find similar rescue of GC-induced reduction in bone length. The methods used, however, made no apparent correction for this growth retardation; thus, measurement of equivalent bone volumes was not ensured. An alternative explanation is that our studies encompass the developmental effect of excessive GCs and Sost haploinsufficiency, and it remains possible that this may diverge from the outcome of transient antisclerostin antibody treatment of adult mice that have induced GC excess.

Sclerostin has long been considered an effective target for treating osteoporosis. The most recent phase 3 clinical trial of romosozumab (an antisclerostin antibody), to our knowledge, was however hampered by unforeseen, increased adverse cardiovascular risk, resulting in refusal to approve this treatment by the Food and Drug...
Administration. The finding that a combination of high endogenous GC levels together with Sost haploinsufficiency results in sudden death with histopathologic, marked hemopericardium, is therefore intriguing. The pericardial space in these mice was found to be markedly enlarged by densely packed erythrocytes with compression of the heart lumen, consistent with sudden death from cardiac tamponade.

To our knowledge, cardiac tamponade secondary to spontaneous hemopericardium has not been reported before in mice. In other species, causes of spontaneous hemopericardium occur because of rupture of the intra-pericardial portion of the aorta or pulmonary artery, rupture of a coronary vessel, cardiac rupture after an acute myocardial infarct or rupture of an atrium secondary to atrial thrombosis, blood dyscrasias, and neoplasia. The histologic examinations, however, did not localize the site of rupture/hemorrhage or reveal an overt underlying pathology. Microscopic examination of the heart and major blood vessels in both affected and unaffected animals also failed to disclose any possible predisposing lesions such as aneurysm, congenital blood vessel anomaly, or cardiac disease.

Several possible explanations may clarify the underpinning mechanisms whereby sclerostin exerts a regulatory role in the cardiovascular system. Although sclerostin is reported to be expressed in the aorta,51–53 neither Sost KO mice in this study or others nor human patients with osteosclerosis or van Buchem’s disease due to mutations in the SOST gene exhibit any greater risk of cardiovascular complications.54–57 Furthermore, a case study by van Lierop et al58 reported that GC treatment does not lead to adverse cardiovascular effects in a van Buchem high bone mass patient with mutation in the SOST gene. In addition, Sato et al59 reported that Sost homozygous KO mice treated with GCs maintain structural and material mechanical properties despite increased osteocyte apoptosis and that no cardiovascular complications were reported. It is possible that differences in our findings to those reported by aforementioned studies can be explained by the fact that the excess GCs present in Crh+/−-120 mice during developmental and later life-course may affect multiple organs, including the cardiovascular system.

Other studies have also reported up-regulation of sclerostin in foci of vascular and valvular calcification.60–63 Our detailed histopathologic examinations were not able to find any signs of calcification in any mice in this study. This agrees with other animal studies showing that sclerostin is unlikely to be involved in vascular mineralization.62,64 No evidence suggests that sclerostin plays a causal role in cardiovascular disease.43,44,45,46,47,48 Recent studies have reported an association between sclerostin levels and cardiovascular disorders.65–68 The precise function of sclerostin in the regulation of the cardiovascular system remains to be defined.

A limitation in our studies is the lack of any measured levels of circulatory sclerostin; thus, it remains to be verified that the Sost genetic haploinsufficiency in our studies is necessarily reflected in a change in expression levels. The middle level bone phenotype observed in the Sost haploinsufficient mice (either in mice with or without the Crh+/−-120 mutation) in which true heterozygosity was also confirmed by genotyping makes it highly likely that sclerostin levels are modified accordingly. The levels might be lower in the Sost-Crh+/−-120 mice than in Crh+/−-120 mice. Another limitation is that whether antisclerostin antibody-mediated blockade of sclerostin function is equivalent to genetic insufficiency cannot be predicted.

Nonetheless, the critical manifestation of Sost-deficiency combined with GC excess in our study is sporadic, sudden, unprovoked, and nonconvulsive death linked to peracute hemopericardium and cardiac tamponade. Tamponade has been linked to many conditions, including cancer, uremia, hypothyroidism, and pericarditis, where enlargement of pericardium arises gradually.69–72 No evidence for such conditions was found in this study. The rapid hemopericardium-related tamponade observed is more consistent with myocardial rupture, but this was not found on detailed pathologic examination using multiple step levels sectioned throughout the heart. The cause of this tamponade and death in some Sost-Crh+/−-120 mice remains unexplained; this is confounded by a lack of any abnormal preceding changes evident in their living equivalents; no predisposing lesions, such as aneurysm, focal thinning, or disruption of the myocardium or coronary vessels were observed histologically in the unaffected animals. It is tempting to speculate on the likely mechanisms. Experiments that explore whether unrestricted Wnt signaling, due to Sost deficiency, interacts with regulators of inflammatory processes, as seen in circumstances such as GC excess, may explain such serious adverse cardiovascular events. These findings that use mouse preclinical models, therefore, prompt further studies aimed at deciphering their clinical significance and the underpinning molecular mechanisms by which GC-induced osteopenia might be better-targeted without risk of cardiovascular side effects.

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Supplemental Data

Supplemental material for this article can be found at https://doi.org/10.1016/j.ajpath.2018.12.007.
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