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RESEARCH LETTER

Reduced Sclerostin Expression in Human Atherosclerotic Plaques Links to Ischemic Stroke

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Vascular calcification (VC) in the form of punctuated and spotty manifestations called microcalcifications resembles ossification during bone development. It is particularly prominent in vulnerable atherosclerotic plaques, triggering plaque rupture, myocardial infarction, or stroke. Atherogenic mediators trigger vascular smooth muscle cells (VSMCs) transdifferentiation to *bone-forming* osteoblast-like (OBL) cells, which actively promotes VC. Sclerostin (*SOST* gene) inhibits Wnt signaling, osteoblast function, and bone formation but also plays a role in VC. It is noteworthy that treatment with the sclerostin monoclonal antibody romosozumab increased the risk of myocardial infarction in the ARCH (post-menopausal women with osteoporosis) clinical trial.[1] Moreover, the role of sclerostin in cardiovascular disease is surrounded by controversy.[1] The present study investigated the link between sclerostin and VSMCs transdifferentiation to OBL cells and its correlation to VC microcalcification and incidence of ischemic stroke. Our single-cell RNA sequencing (scRNAseq) analyses revealed that *SOST* is downregulated in cells derived from the athero-prone aortic root and arch where calcifications appear first, as opposed to *SOST* upregulation in the more atheroresistant descending aorta.[2] Viable aorta CD45$^-$ cells from male Apolipoprotein E$^{-/-}$C57Bl/6 mice fed a high-cholesterol diet were isolated, sorted, and loaded on a C1 Single-Cell mRNA Seq HT IFC chip for scRNAseq analysis (n=6 mice/group; as previously described for exclusion criteria, weight loss, sample randomization, control groups, animal care, and experimental procedures carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Geneva University School of Medicine).[2] After obtaining an institutional review committee approval and informed patient consent, the upstream carotid plaque specimens of symptomatic (ischemic stroke) and asymptomatic (no ischemic stroke events) patients (n=16–20 per group) undergoing endarterectomy (>70% luminal narrowing) and a part of a previously published cohort with described clinical characteristics and plaque morphology[3] were used for immunofluorescence staining. To minimize the autofluorescence, paraformaldehyde fixation for the minimum time required and TrueVIEW (Vector Laboratories, Inc. Newark, CA) treatment were applied. The cryosections were costained with

Key Words: osteoblast-like cells ■ slerostin ■ vascular calcification ■ vascular smooth muscle cells ■ Wnt signaling

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For Sources of Funding and Disclosures, see page 3.

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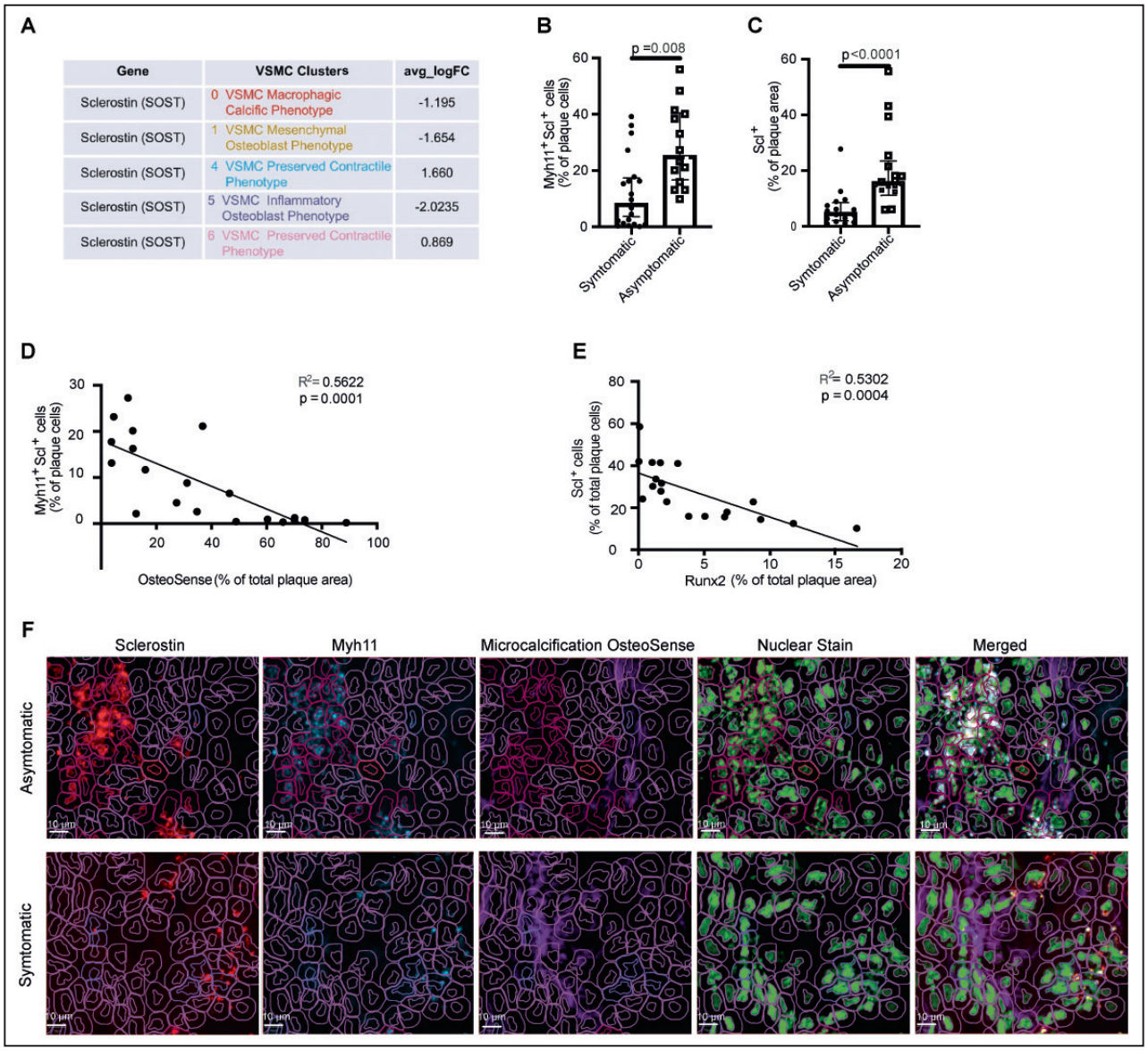

Figure 1. Sclerostin expression in atherosclerotic plaques.

A, Single-cell RNA sequencing data demonstrate log fold-change in *SOST* average expression in VSMC clusters (avg_logFC; log fold-change of the average expression adjusted *P* value <0.05). **B**, Bar graph represents the median with interquartile range of the percentage of Myh11$^+$Scl$^+$ and (**C**) Scl$^+$-positive area in symptomatic and asymptomatic carotid artery disease patients, respectively (Mann–Whitney *U* test). **D**, A negative correlation between the percentage of Myh11$^+$Scl$^+$ l cells and the extent of microcalcification (OsteoSense positive plaque area) and (**E**) between the percentage of total Scl$^+$ cells and induction of VSMCs phenotype switch to osteoblast-like cells quantified as Runx2-positive area in atherosclerotic plaques of symptomatic and asymptomatic carotid artery disease patients. Simple linear regression test with *P* value calculated from an F test. (**F**) Representative immunofluorescence staining of sclerostin (red), Myh11 (a marker of VSMCs with preserved phenotype, blue), fluorescent nucleic acid stain (SYTO13, green) and microcalcification (OsteoSense, violet), expression in atherosclerotic lesions of symptomatic and asymptomatic coronary artery disease patients, respectively. Not shown in the figure are endogenous tissue controls (no primary or secondary antibody) and primary antibody controls (only secondary antibody) to reveal the level of autofluorescence and nonspecific binding in human plaques. Myh11 indicates myosin heavy chain 11; Scl, sclerostin; *SOST*, sclerostin gene; and VSMCs, vascular smooth muscle cells.

anti–myosin heavy chain 11 (Myh11) (VSMCs preserved phenotype marker), and anti-sclerostin antibodies, cell-permeant SYTO13 green fluorescent nucleic acid stain for bright, high-affinity, high-specificity nucleic acid staining (Thermo Fisher, Waltham, MA), anti-Runx2 antibody (VSMC switch to OBL marker cells) (Novus Biologicals, Centennial, CO) and OsteoSense 680EX Fluorescent microcalcification agent (PerkinElmer, Waltham, MA), acquired with Axioscan Z1 and quantified with QuPath. Endogenous tissue controls were used (no primary or secondary antibody) and primary antibody controls (only secondary antibody). DEseq2 was applied to calculate

fold changes and *P* values of the scRNAseq. Statistical and simple linear regression analysis were performed with Prism 8 (GraphPad Software, Inc., La Jolla, CA) and Mann–Whitney *U* test with statistically significant *P* values <0.05. All the data supporting the findings of this study are available from the corresponding author upon reasonable request. The well-characterized by scRNAseq analysis phenotypically modified clusters of VSMCs presenting macrophagic calcific, mesenchymal osteoblast, and inflammatory osteoblast phenotypes[2] showed pronounced downregulation of *SOST* expression (clusters 0, 1, and 5) (Figure [A]). In contrast, VSMCs with preserved contractile phenotype[2] overexpressed *SOST* (clusters 4 and 6) (Figure [A]). To further explore the link between sclerostin expression and vascular microcalcification promoting plaque rupture, we used upstream carotid plaque specimens of symptomatic (ischemic stroke) and asymptomatic (no ischemic stroke events) patients.[3] The analysis of human carotid plaque specimens was focused on atherosclerotic plaque intima microcalcification occurring in association with the thinning of the fibrous cap, triggering plaque rupture and ischaemic stroke. Plaques of asymptomatic patients exhibited a significantly higher percentage of sclerostin-expressing VSMCs with preserved phenotype ($Myh11^{+}Scl^{+}$) and $sclerostin^{+}$ plaque area (Figure (B) and (C)) which cell percentage was negatively correlated with the extent of microcalcification (OsteoSense area) (Figure [D] and [F]) and VSMCs phenotype switch to OBL cells (Runx2-positive area) (Figure [E] and [F]).

The present study demonstrated that the upregulated sclerostin expression by VSMCs with preserved contractile phenotype in response to atherosclerosis disease progression is associated with reduced microcalcification and improved plaque stability in human atherosclerosis. In contrast, calcific VSMCs OBL-like cells showed downregulation of sclerostin expression linked to increased vascular microcalcification and plaque destabilization. The present findings are in line with studies showing that lower arterial sclerostin is associated with a higher risk of cardiovascular events,[1,4] and linked to vascular microcalcification as demonstrated by the present study. Furthermore, the high blood sclerostin concentrations associated with cardiovascular disease[1,4] may be due to excessive sclerostin produced by VSMCs with preserved phenotype in response to the disease rather than being a disease cause. In this regard, the present study supports previous investigations assuming that excessive vessels' local production of sclerostin during VC progression works as a defense mechanism preventing further progression of VC but potentially contributing to the inhibition of bone turnover.[4] Localized expression of sclerostin in atherosclerotic plaques may therefore appear to act as a defense mechanism against microcalcification and plaque destabilization. Given the accumulating data regarding the side effects of romosozumab, used to treat osteoporosis at present there is a safety warning from the Food and Drug Administration and the European Medicines Agency to avoid romosozumab treatment of patients with a history of major adverse cardiac events (MACE).[5] In this context, the present finding supports the hypothesis that sclerostin could act as a vascular local defence mechanism against microcalcification and plaque destabilization, and argue for extension of the contraindication of romosozumab treatment to patients at risk of MACE.

ARTICLE INFORMATION

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Disclosures

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