Targeting loop3 of sclerostin preserves its cardiovascular protective action and promotes bone formation

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Sclerostin negatively regulates bone formation by antagonizing Wnt signalling. An antibody targeting sclerostin for the treatment of postmenopausal osteoporosis was approved by the U.S. Food and Drug Administration, with a boxed warning for cardiovascular risk. Here we demonstrate that sclerostin participates in protecting cardiovascular system and inhibiting bone formation via different loops. Loop3 deficiency by genetic truncation could maintain sclerostin’s protective effect on the cardiovascular system while attenuating its inhibitory effect on bone formation. We identify an aptamer, named aptsc56, which specifically targets sclerostin loop3 and use a modified aptsc56 version, called Apc001PE, as specific in vivo pharmacologic tool to validate the above effect of loop3. Apc001PE has no effect on aortic aneurysm and atherosclerotic development in ApoE−/− mice and hSOSTK1.ApoE−/− mice with angiotensin II infusion. Apc001PE can promote bone formation in hSOSTK1 mice and ovariectomy-induced osteoporotic rats. In summary, sclerostin loop3 cannot participate in protecting the cardiovascular system, but participates in inhibiting bone formation.

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Sclerostin can bind to low-density lipoprotein receptor-related protein 5/6 (LRP5/6) and inhibit bone anabolic Wnt signalling. Loss of function mutations in the sclerostin-encoding gene SOST resulted in increased bone mass and bone strength. Therefore, sclerostin is a target for bone anabolic therapy to reverse established osteoporosis. Humanized antibody against sclerostin for postmenopausal osteoporosis therapy was approved by the US Food and Drug Administration (FDA) in April 2019, with a boxed warning on its labelling stating that this treatment may increase the risk of heart attack, stroke and cardiovascular death and should not be used in patients who have had a heart attack or stroke within one year. Therefore, it is desirable to develop next-generation sclerostin inhibitors without cardiovascular risks. Meta-analysis of 25 cardiac ischaemic events in 4298 individuals from phase III clinical trials showed that therapeutic antibody (210 mg monthly) led to a higher risk of cardiovascular events. Estimates from a meta-analysis of ‘serious cardiovascular events’ were directionally concordant with increased vascular risk arising from therapeutic antibody treatment. To date, the mechanism for the cardiovascular risk of the therapeutic antibody remains unclear.

Sclerostin demonstrated a protective role in the cardiovascular system and could inhibit inflammatory cytokines and chemokines to prevent both aortic aneurysm (AA) and atherosclerotic development induced by angiotensin II (AngII) in Apolipoprotein E deficient (ApoE−/−) mice with transgenic introduction of sclerostin or administration of recombinant sclerostin. Therefore, the potential cardiovascular risk should be carefully examined to inhibit sclerostin in bone anabolic therapy. Sclerostin has long N- and C-terminal regions that are highly flexible and completely disordered. The remaining central residues form three loops, loop1, loop2 and loop3 (Fig. 1a). It was reported that therapeutic sclerostin antibody, which showed cardiovascular risks, could target both sclerostin loop2 and loop3. It is desirable to understand the roles of different sclerostin loops in regulating inflammatory cytokines for cardiovascular diseases and bone formation for osteoporosis and to strategically identify the functional loops in sclerostin, which cannot participate in protecting the cardiovascular system but is involved in inhibiting bone formation.

In this work, we investigated the roles of sclerostin loops using both genetic and pharmacologic approaches in vitro. For osteogenic potential, we found that loop2 and/or loop3 played important roles in the inhibitory effect of sclerostin on Wnt signalling and osteogenic potential in vitro. For cardiovascular system, we found that either loop2&3 deficiency by genetic truncation or loop 2&3 inhibition by sclerostin antibody could attenuate the suppressive effects of sclerostin on Wnt expression and chemokines, whereas loop3 deficiency by genetic truncation maintained the above suppressive effects of sclerostin on the expression of inflammatory cytokines and chemokines, whereas loop3 deficiency by genetic truncation maintained the above suppressive effects of sclerostin on macrophages and VSMCs with AngII treatment in vitro.

The role of sclerostin loop2 and loop3 in suppressing the expression of inflammatory cytokines and chemokines in vitro. RAW 264.7 macrophages were treated with AngII or vehicle for 24 h, followed by examination of the mRNA levels of inflammatory cytokines (IL-6, TNFα) and chemokines (MCP-1) by qRT-PCR. All mRNA levels were significantly higher after AngII treatment than after vehicle treatment, indicating that AngII treatment could induce the elevations of inflammatory cytokines and chemokines in macrophages. RAW 264.7 macrophages were then treated with conditioned media from the MC3T3-E1 cells transfected with Wnt and either FL hSOST or SOST truncations and treated with AngII for 24 h. All mRNA levels were significantly lower after treatment with FL hSOST, indicating that FL hSOST could suppress the expression of inflammatory cytokines and chemokines. Furthermore, compared to those with FL hSOST, all mRNA levels were significantly higher with Δloop2&3-hSOST(1-85) but were not altered with Δloop3-hSOST(1-110) treatment. Therefore, loop2&3 deficiency by genetic truncation was well maintained the above suppressive effects of sclerostin on the expression of inflammatory cytokines and chemokines, whereas loop3 deficiency by genetic truncation maintained the above suppressive effects of sclerostin in macrophages and VSMCs with AngII treatment in vitro.

The effect of sclerostin loop3 deficiency by genetic truncation on the cardiovascular system in vivo. Full-length sclerostin knock-in (hSOST) and loop3-deficient sclerostin knock-in (Δloop3-hSOST) mice were generated and crossed with ApoE−/− mice to construct hSOST, ApoE−/− and Δloop3-hSOST mice, respectively (Supplementary Fig. 3a–h). To investigate whether loop3 deficiency by genetic truncation could maintain the protective effect of sclerostin on the cardiovascular system in vivo, we determined parameters for AA and atherosclerotic development after saline or AngII infusion for four weeks. Compared to those in the ApoE−/−+AngII mice, the AA incidence for AA development (Fig. 2a and Supplementary Fig. 4a), the maximum ex vivo diameters of aortic arches and suprarenal aortas (Fig. 2b), and the atherosclerotic plaque area/total aortic root cross area ratios for atherosclerotic development (Fig. 2c & Supplementary Fig. 4b), as well as the serum levels of the inflammatory cytokines and chemokines, were significantly reduced in both the hSOST/ApoE−/−+AngII and Δloop3-SOST/ApoE−/−+AngII groups (Fig. 2d). There were no significant differences in the above parameters among the other groups. These data consistently demonstrated that loop3 deficiency by genetic truncation maintained the protective effect of sclerostin on the cardiovascular system in vivo.
Fig. 1 Effects of sclerostin loops on regulating Wnt signalling and osteogenic potential in MC3T3-E1 cells, and on regulating inflammatory cytokines/chemokines in macrophages and vascular smooth muscle cells (VSMCs). a Schematic diagram of the primary structures of full-length sclerostin and sclerostin truncations. Full-length human sclerostin (FL hSOST), sclerostin with loop2&3 deficiency by genetic truncation (Δloop2&3-hSOST(1-85)) and sclerostin with loop3 deficiency by genetic truncation (Δ loop3-hSOST(1-110)). b The effect of sclerostin on regulating Wnt signalling and osteogenic potential in MC3T3-E1 cells. *p < 0.05; **p < 0.01 and ***p < 0.005 for a comparison vs. Wnt+FL hSOST group by one-way ANOVA with Tukey’s post-hoc test. TOP-Wnt induced signal: p = 0.0001 (Wnt), p = 0.0034 (Wnt + Δloop3-hSOST), p = 0.0001 (Wnt + Δloop2&3-hSOST), p = 0.0002 (Wnt + FL hSOST + antibody); ALP: p = 0.0002 (Wnt), p = 0.0026 (Wnt + Δloop3-hSOST), p = 0.0003 (Wnt + Δloop2&3-hSOST), p = 0.0002 (Wnt + FL hSOST + antibody); OCN: p = 0.0066 (Wnt), p = 0.0404 (Wnt + Δloop3-hSOST), p = 0.0037 (Wnt + Δloop2&3-hSOST), p = 0.0045 (Wnt + FL hSOST + antibody). c The effect of sclerostin on regulating the mRNA levels of cytokines/chemokines in RAW264.7 macrophages. *p < 0.05 and **p < 0.1 for a comparison between the PBS and AngII groups by a two-tailed unpaired t test. p = 0.0022 (IL-6), p = 0.0164 (TNF-α), p = 0.0103 (MCP-1). ***p < 0.005 and ****p < 0.0001 for a comparison vs. the FL hSOST + AngII group by one-way ANOVA with Tukey’s post-hoc test. IL-6: p < 0.0001 (AngII, Wnt+AngII, Wnt + Δloop2&3-hSOST + AngII, Wnt + FL hSOST + antibody+AngII); TNF-α: p = 0.0001 (AngII, Wnt+AngII, Wnt + Δloop2&3-hSOST+AngII), p = 0.0001 (Wnt + FL hSOST+antibody+AngII); MCP-1: p = 0.0001 (AngII, Wnt+AngII, Wnt + Δloop2&3-hSOST+AngII, Wnt + FL hSOST+antibody+AngII). d The effect of sclerostin on regulating the mRNA level of chemokines in VSMCs. **p < 0.01 for a comparison between the PBS and AngII groups by an unpaired t test (two-tailed). p = 0.0016. IL-6: p < 0.01 for a comparison of with FL hSOST + AngII group by one-way ANOVA with Tukey’s post-hoc test. p = 0.0028 (AngII), p = 0.0030 (Wnt + AngII), p = 0.0012 (Wnt + Δloop2&3-hSOST + AngII), p = 0.0017 (Wnt + FL hSOST+antibody+AngII). For (b, c and d), n = 2 per group. All data were expressed as the mean ± standard deviation. Note: ALP: alkaline phosphatase; OCN: osteocalcin; AngII: angiotensin II; IL-6: interleukin 6; MCP-1: monocyte chemoattractant protein-1; TNF-α: tumour necrosis factor alpha. Source data are provided as a Source Data file.

**The effect of sclerostin loop3 deficiency by genetic truncation on bone formation in vivo.** To investigate whether loop3 deficiency by genetic truncation could attenuate the inhibitory effect of sclerostin on bone formation in vivo, we evaluated bone mass, bone microstructure and bone formation in the WT, hSOST

Δloop3-hSOST<sup>Ki</sup> mice. Microcomputed tomography (micro-CT) was used for the measurement of trabecular bone (below the growth plate) at the metaphysis of the fourth vertebrae (Fig. 3a and Supplementary Fig. 5a). Compared to those in the WT mice, the trabecular bone volume ratio (Tb.BV/TV), trabecular bone...
mineral density (Tb.vBMD), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular connectivity density (Tb.Conn.D) were significantly lower, but the trabecular spacing (Tb.Sp) was significantly higher in the hSOSTki mice. The average absolute value of the trabecular structure model index (Tb.SMI) was close to 0 in the WT mice and close to 3 in the hSOSTki mice, indicating the plate-like shape of the trabecular bone in the WT mice and the rod-like shape of the trabecular bone in the hSOSTki mice at the fourth vertebrae. There were no significant differences between the Δloop3-hSOSTki mice and WT groups in any of the above parameters.

The bone histomorphometric analysis was used for measurement of the trabecular bone (below the growth plate) at the metaphysis of the fourth vertebrae. Compared to those in the WT mice, the trabecular bone formation rate (Tb.BFR/BS) and the trabecular bone mineral apposition rate (Tb.MAR) were notably lower in the hSOSTki mice (Fig. 3b and Supplementary Fig. 5b). There were no significant differences between the Δloop3-hSOSTki and WT groups in any of the above parameters. Both micro-CT and bone histomorphometric data consistently indicated that loop3 deficiency by genetic truncation could attenuate the inhibitory effect of sclerostin on bone formation in vivo.

Selection and characterization of aptamer candidates against sclerostin loop3. The above in vivo data demonstrated that loop3 deficiency by genetic truncation could maintain the protective effect of sclerostin on the cardiovascular system while attenuating the inhibitory effect of sclerostin on bone formation. Therefore, whether specifically targeting sclerostin loop3 by pharmacologic approaches could promote bone formation and maintain sclerostin loop3-independent cardiovascular protective effects should be examined.

Oligonucleotide aptamers are single-stranded DNA/RNAs that bind to their targets by 3D complementarity. Aptamers can be tailored selected against specific domains of the protein target by...
introducing positive and negative selections through a process called Systematic Evolution of Ligands by Exponential enrichment (SELEX)\textsuperscript{12–14}. To identify aptamer candidates that target loop3 as a specific in vitro pharmacologic tool, we used full-length sclerostin as the positive target, and loop3-decient sclerostin as the negative target for the identification of aptamers from a random ssDNA library composed of ~10\textsuperscript{15} different sequences. With increasing rounds of selection, the enrichment of high-affinity aptamers through SELEX was monitored (Fig. 4a). The representative candidate sequences from the final round were identified, synthesized and characterized (Supplementary Table 1). A promising aptamer candidate, aptscl56, was finally identified (K\textsubscript{d} = 43.1 nM, IC\textsubscript{50} = 19.7 µg/ml) as a specific in vitro pharmacologic tool (Fig. 4b). Aptscl56 showed a high binding ability to sclerostin loop3 but no binding to sclerostin loop1 and loop2 (Fig. 4c).

The effect of targeting sclerostin loop3 on the expression of inflammatory cytokines and chemokines in vitro. MC3T3-E1 cells were transfected with Wnt and FL hSOST, followed by treatment with vehicle, aptscl56 and antibody. It was demonstrated that both Wnt signalling and osteogenic potential were significantly higher in the cells treated with aptscl56 or antibody.

Fig. 3 Determination of whether loop3 deficiency by genetic truncation could attenuate the inhibitory effect of sclerostin on bone formation in loop3-deficient sclerostin (Δloop3-hSOST\textsuperscript{(Δ1-110)}) knock-in mice compared to full-length sclerostin (hSOST\textsuperscript{WT}) knock-in and WT mice. Mice were injected intraperitoneally with calcein green (20 mg/kg) at 10 and 2 days before euthanasia. After euthanasia, the fourth vertebrae of all mice were collected for analysis. a Bar charts of the structural parameters of Tb.BV/TV, Tb.vBMD, Tb.Th, Tb.N, Tb.Sp, Tb.conn.D and Tb.SMI from ex vivo micro-CT examination at the fourth vertebrae. Tb.BV/TV: p < 0.0001 (hSOST\textsuperscript{WT}), p = 0.0076 (Δloop3-hSOST\textsuperscript{WT}); Tb.vBMD: p < 0.0001 (hSOST\textsuperscript{WT}), p = 0.0067 (Δloop3-hSOST\textsuperscript{WT}); Tb.Th: p < 0.0001 (hSOST\textsuperscript{WT}), p = 0.0134 (Δloop3-hSOST\textsuperscript{WT}); Tb.N: p < 0.0001 (hSOST\textsuperscript{WT}), p < 0.0001 (Δloop3-hSOST\textsuperscript{WT}); Tb.Sp: p < 0.0001 (hSOST\textsuperscript{WT}), p = 0.0001 (Δloop3-hSOST\textsuperscript{WT}); Tb.conn.D: p < 0.0001 (hSOST\textsuperscript{WT}), p < 0.0001 (Δloop3-hSOST\textsuperscript{WT}); Tb.SMI: p < 0.0001 (hSOST\textsuperscript{WT}), p = 0.0276 (Δloop3-hSOST\textsuperscript{WT}). b Analysis of dynamic bone histomorphometric parameters of Tb.BFR/BS and Tb.MAR at the fourth vertebrae. Tb.BFR/BS: p < 0.0001 (hSOST\textsuperscript{WT}), p = 0.0227 (Δloop3-hSOST\textsuperscript{WT}); Tb.MAR: p < 0.0001 (hSOST\textsuperscript{WT}), p = 0.0094 (Δloop3-hSOST\textsuperscript{WT}). For (a) and (b), data were expressed as the mean ± standard deviation. n = 10 per group. One-way ANOVA with Tukey’s post-hoc test vs. the WT group was used to determine the intergroup differences. *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.0001. Note: micro-CT: microcomputed tomography; Tb.BV/TV: trabecular relative bone volume; Tb.vBMD: trabecular volumetric mineral density; Tb.Th: trabecular thickness; Tb.N: trabecular number; Tb.Sp: trabecular spacing; Tb.conn.D: trabecular connection density; Tb.SMI: trabecular structure model index; Tb.BFR/BS: trabecular bone formation rate; Tb.MAR: trabecular mineral apposition rate. Source data are provided as a Source Data file.
than in cells treated with vehicle (Fig. 4d). These data demonstrated that targeting sclerostin loop3 with aptsc56 could attenuate the antagonistic effect of sclerostin on Wnt signalling to promote osteogenic potential in MC3T3-E1 cells.

RAW264.7 macrophages and VSMCs were treated with conditioned media from the MC3T3-E1 cells transfected with Wnt and FL hSOST, followed by treatment with vehicle, aptsc56 and antibody and then cultured in medium with AngII for 24 h. Compared to those in the macrophages treated with vehicle, the mRNA levels of IL-6, TNFα and MCP-1 were significantly higher in the macrophages treated with antibody but were not altered in the macrophages treated with aptsc56 (Fig. 4e). Consistent
results were shown in VSMCs (Fig. 4f). These data consistently suggested that sclerostin loop3 inhibition by the specific in vitro pharmacologic tool did not affect the suppressive effect of sclerostin on the expression of inflammatory cytokines and chemokines in macrophages or the expression of inflammatory chemokines in the VSMCs treated with AngII.

**Functional sites on sclerostin loop2 and loop3.** The functional sites on sclerostin loop2 and loop3 were determined by using mutational methods. Three mutants were identified for the following studies: the sclerostin loop3 mutant (loop3m: IPDAAAAAAQQLLCPGAAAAPRAAALVGS), which could bind to aptsc56 but not antagonize Wnt signalling (Supplementary Fig. 6); the aptsc56 mutant (aptsc56m: CGGGGT GTGGGTTAAATCGTTAGAAAGATTAAACAGCTGCC), which could not bind to sclerostin (Supplementary Fig. 7); and the loop2 mutant (loop2m: GPARRLPNAIGRAMWRPSGPDPR), which could bind to the sclerostin antibody but not suppress the expression of proinflammatory cytokines (Supplementary Fig. 8).

**Chemical modifications on aptsc56.** Chemical modifications were performed to improve the serum stability of aptsc56. Degradations of aptsc56 were both improved (Supplementary Fig. 9c, d). The unmodified aptsc56 were both improved (Supplementary Fig. 9c, d). The unmodified aptsc56 were both improved (Supplementary Fig. 9c, d).

**Pharmacokinetics and distribution of Apc001PE in vivo.** For a specific in vivo pharmacologic tool, the modified aptsc56 was conjugated to polyethylene glycol (PEG40k-aptsc56, named Apc001PE) to protect against renal filtration in vivo. The tissue distribution of Apc001PE was determined in mice (Supplementary Fig. 10). Apc001PE mainly accumulated in the liver and kidney rather than other tissues, indicating that Apc001PE was metabolized through the liver and kidney. Furthermore, the pharmacokinetic parameters of aptsc56 and Apc001PE were analysed by determining the plasma concentrations in Sprague-Dawley (SD) rats (Supplementary Fig. 11). It was demonstrated that nonconjugated aptsc56 had a short half-life (Elimination $T_{1/2}=1.8$ h) and a rapid clearance rate in the circulation (apparent clearance, CL/F = 0.004 L/h/kg; area under the curve, AUC = 1205 (µg h)/ml). In comparison, Apc001PE showed a 37-fold longer half-life (Elimination $T_{1/2}=66.9$ h) and a much lower clearance rate (CL/F = 0.001 L/h/kg; AUC = 11802 (µg h)/ml) in vivo. Therefore, PEG40k conjugation dramatically increased the half-life and decreased the clearance rate of aptsc56 in vivo. Apc001PE could be used as a specific in vivo pharmacologic tool.

**Immunogenicity and toxicity of Apc001PE in rats.** To evaluate the immunogenicity of the specific in vivo pharmacologic tool Apc001PE in rats, we determined the serum cytokine levels in healthy SD rats with Apc001PE administration (12 mg/kg, twice/week for six weeks). There were no significant differences in the serum levels of cytokines among the groups (Supplementary Fig. 12).

To evaluate the toxicity of Apc001PE in healthy SD rats, we determined the liver and kidney function indices and haematologic parameters by biochemical and haematological assays in rats with a single (3, 6, 12, and 24 mg/kg, respectively) and multiple administration(s) (12 mg/kg, twice/week for six weeks) of Apc001PE. There were no significant differences in the serum levels of the liver/kidney function indices or haematologic parameters among the groups (Supplementary Fig. 13).

**The effect of targeting sclerostin loop3 on cardiovascular events in ApoE−/− mice with AngII infusion.** To evaluate whether targeting sclerostin loop3 by Apc001PE could influence the cardiovascular system, we determined the parameters for both AA and atherosclerotic development in the ApoE−/− mice with AngII infusion after administration of vehicle (veh), Apc001PE (25 mg/kg, twice/week), or sclerostin antibody (25 mg/kg, twice/week) for four weeks. Compared to the veh group, the AA incidences, the maximum ex vivo diameters of thoracic aortas and suprarenal aortas, the maximum ex vivo diameters of aortic arches, the atherosclerotic plaque area/total aortic arch area, and the atherosclerotic lesion area/total cross cryosection area of the aortic root were not altered in the AngII+Apc001PE group but were higher in the AngII+antibody group (Fig. 5a–e, Supplementary Fig. 14a–c).

Immune cell infiltration, contractile phenotype loss of aortic VSMCs and cell apoptosis were involved in AA and atherosclerotic development. Compared to those in the AngII +veh group, the numbers of CD68-positive macrophages, α-SMA-positive contractile VSMCs, and cleaved caspase-3-positive apoptotic cells in the suprarenal aortas and aortic roots were not altered in the AngII+Apc001PE group but were significantly higher in the AngII+antibody group (Fig. 5f, g, Supplementary Fig. 14d–e). Furthermore, compared to those in the AngII+veh group, the serum levels of IL-6, TNF-α and MCP-1 were not altered in the AngII+Apc001PE group but were significantly higher in the AngII+antibody group (Fig. 5h).

Mechanistically, to further explore how therapeutic antibody targeting both sclerostin loop2 and loop3 increased cardiovascular risk, we determined all of the above parameters in the ApoE−/− mice with AngII infusion after administration of therapeutic antibody with and without pretreatment with loop2m (6 mg/kg, once weekly). It was found that the increased cardiovascular risk of the therapeutic antibody could be abolished by supplementation with exogenous loop2m (Fig. 5i, Supplementary Fig. 14). These data further indicated that sclerostin loop2, rather than loop3, could participate in sclerostin’s protective effect on the cardiovascular system. Thus, targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE had no effect on cardiovascular events in the ApoE−/− mice with AngII infusion.

**The effect of targeting sclerostin loop3 by Apc001PE on the cardiovascular system in the hSOSTki.ApoE−/− mice with AngII infusion.** Transgenic introduction of sclerostin protected against AngII-induced AA and atherosclerotic progression. The ApoE−/− or hSOSTki. ApoE−/− mice were treated with vehicle (veh), Apc001PE (25 mg/kg, twice/week) or sclerostin antibody (25 mg/kg, twice/week) for four weeks during AngII infusion. It was demonstrated that the AA incidence, the maximum ex vivo diameters of aortic arches, thoracic aortas and suprarenal aortas, atherosclerotic plaque area, total en face aortic arch area, atherosclerotic lesion area, total cross cryosection area of the aortic root, and serum levels of inflammatory cytokines and chemokines were significantly reduced in the hSOSTki.ApoE−/−-AngII+veh group compared to the ApoE−/−-AngII+veh group, validating the protective effect of sclerostin on the cardiovascular system (Fig. 6, Supplementary Fig. 15). Compared to those in the hSOSTki.ApoE−/−-AngII+veh group, the above parameters were not altered in the hSOSTki.ApoE−/−-AngII+veh group and were significantly elevated in the hSOSTki.ApoE−/−-AngII+anti-bodiy group. These data demonstrated that targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE could...
Fig. 5 Evaluation of whether targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE had an effect on cardiovascular events in ApoE−/− mice with Angll infusion. ApoE−/− mice with Angll infusion were subcutaneously administered vehicle, Apc001PE (12 mg/kg), humanized therapeutic sclerostin antibody (25 mg/kg), fatty acid-loop2m (6 mg/kg), or humanized therapeutic sclerostin antibody and fatty acid-loop2m (6 mg/kg) twice weekly for four weeks. a The incidence of AA formation. A two-sided chi-square test was performed to determine the difference between groups. ***p < 0.005, p = 0.0002 for all comparisons. b Ex vivo measurement of the maximum diameters of thoracic aortas and suprarenal aortas. Thoracic aorta: p < 0.0001 (Angll + antibody); suprarenal aorta: p < 0.0001 (saline), p < 0.0001 (Angll + antibody). c Ex vivo measurement of the maximum diameters of the aortic arches. p = 0.0007 (saline), p < 0.0001 (Angll + antibody). d Oil Red O staining of the aortic arch to quantify atherosclerosis. p < 0.0001 (saline), p < 0.0001 (Angll + antibody). e Quantification of positive staining per cryosection of aortic roots stained with Oil Red O. p < 0.0001 (saline), p < 0.0001 (Angll + antibody). f, g Quantification of the expression of CD68, α-SMA and cleaved caspase-3 in suprarenal aortas (f) and aortic roots (g) by immunohistochemical analysis. For (f): CD68: p = 0.0001 (saline), p < 0.0001 (Angll + antibody); α-SMA: p = 0.0001 (saline), p < 0.0001 (Angll + antibody); cleaved caspase-3: p < 0.0001 (saline), p < 0.0001 (Angll + antibody). For (g): CD68: p < 0.0001 (saline), p < 0.0001 (Angll + antibody); α-SMA: p < 0.0001 (saline), p < 0.0001 (Angll + antibody); cleaved caspase-3: p < 0.0001 (saline), p < 0.0001 (Angll + antibody). h Serum levels of inflammatory cytokines and chemokines. IL-6: p < 0.0001 (saline), p < 0.0001 (Angll + antibody); TNF-α: p < 0.0001 (saline), p < 0.0001 (Angll + antibody); MCP-1: p < 0.0001 (saline), p < 0.0001 (Angll + antibody). For (b-h), data were expressed as the mean ± standard deviation. n = 9 per group. *p < 0.05, **p < 0.01, ***p < 0.005, and ****p < 0.0001 for a comparison vs. Angll + veh by one-way ANOVA with Tukey’s post-hoc test. Note: Angll: angiotensin II; IL-6: interleukin 6; MCP-1: monocyte chemoattractant protein-1; TNF-α: tumour necrosis factor alpha; CD68: macrophage biomarker; α-SMA: contractile cell biomarker; cleaved caspase-3: apoptotic cell biomarker; loop2m: the loop2 mutant (GPARLLPNAIGR AAAWRPSGPDFR). Source data are provided as a Source Data file.
The effect of targeting sclerostin loop3 with Apc001PE on bone formation in the hSOST<sup>Ki</sup> mice. To examine whether targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE could attenuate the inhibitory effect of sclerostin on bone formation in hSOST<sup>Ki</sup> mice, mice were grouped and treated accordingly (Supplementary Fig. 16a).

After treatment, the micro-CT data showed that the hSOST<sup>Ki</sup> + Apc001PE group had significantly higher Tb.BV/TV, Tb.vBMD, Tb.Th, Tb.N, and Tb.conn.D values and lower Tb.Sp values than those in the hSOST<sup>Ki</sup>-B/L group. The Tb.SMI value was close to 0 in the hSOST<sup>Tm</sup> + Apc001PE group but close to 3 in the hSOST<sup>Ki</sup>-B/L group, indicating the plate-like shape of the trabecular bone in the hSOST<sup>Tm</sup> + Apc001PE group and the rod-like shape of the trabecular bone in the hSOST<sup>Ki</sup>-B/L group at the proximal tibia. There were no significant differences among the hSOST<sup>Ki</sup>-B/L, hSOST<sup>Ki</sup> + Veh and hSOST<sup>Ki</sup> + RANDseq groups in any of the parameters (Fig. 7a, Supplementary Fig. 16b).

In bone histomorphometric analysis of the trabecular bone at the proximal tibia, the BFR/BS and MAR/BS were notably higher in mice with AngII infusion than in mice without AngII. There were no significant differences among the hSOST<sup>Ki</sup>-B/L, hSOST<sup>Ki</sup> + Veh and hSOST<sup>Ki</sup> + RANDseq groups in any of the parameters (Fig. 7b and Supplementary Fig. 16c). Therefore, targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE could attenuate the inhibitory effect of sclerostin on bone formation in hSOST<sup>Ki</sup> mice.

Mechanistically, to further test how the specific in vivo pharmacologic tool Apc001PE attenuated the inhibitory effect of sclerostin on bone formation, we determined the bone anabolic potential of Apc001PE in the hSOST<sup>Ki</sup> mice with and without AngII infusion.
pretreatment with loop3m. The Apc001PE-mediated attenuation of sclerostin’s inhibition on bone formation could be abolished by supplementation with exogenous loop3m (Fig. 7, Supplementary Fig. 16). In contrast, the Apc001PE mutant (Apc001PEm: PEG40k-aptscl56m) was administered to hSOSTki mice. There were no significant differences between the hSOSTki + Apc001-PEm group and the hSOSTki + veh group in any of the above parameters for both micro-CT and bone histomorphometric analysis. Thus, targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE attenuated the inhibitory effect of sclerostin on bone formation by neutralizing circulating sclerostin loop3 in hSOSTki mice in vivo.

The effect of targeting sclerostin loop3 by Apc001PE on bone formation in osteoporotic rats induced by ovariectomy (OVX). To examine whether targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE could promote bone

![Fig. 7 Evaluation of whether targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE could attenuate the inhibitory effect of sclerostin on bone formation in hSOSTki mice.](image-url)
formation, increase bone mass, and improve bone micro-architecture integrity in rats with established osteoporosis induced by OVX. SD rats were grouped and treated accordingly (Supplementary Figs. 17a and 18).

Micro-CT analysis was performed for the trabecular bone (below the growth plate) at the metaphysis of the distal femur, the proximal tibia and the fifth lumbar vertebrae, as well as the cortical bone at the femoral mid-shaft. After treatment, the OVX + Apc001PE group had significantly higher Tb.BFR/BS, Tb.vBMD, Tb.Th, Tb.N and Tb.Conn.D values but lower Tb.Sp values than the OVX-B/L group, indicating a notably increased trabecular bone mass in the OVX rats treated with Apc001PE. The Tb.SMI was close to 0 in the OVX + Apc001PE group but close to 3 in the OVX-B/L group, indicating the plate-like shape of trabecular bone in the OVX + Apc001PE group and the rod-like shape of trabecular bone in the OVX-B/L group at the fifth lumbar vertebrae (Fig. 8a and Supplementary Fig. 17b). Micro-CT data for the distal femur (Supplementary Fig. 19a) and the proximal tibia (Supplementary Fig. 19b) showed consistent results. Furthermore, micro-CT of the trabecular region of the above three sites showed no significant differences among the OVX + veh, OVX-B/L and OVX + RANDseq groups in any of the parameters. For the femoral mid-shaft, the OVX + Apc001PE group had higher cortical thickness (Ct.Th.) than the OVX-B/L group. There were no significant differences among the OVX + veh, OVX-B/L and OVX + RANDseq groups for the above parameters (Supplementary Fig. 19c).

Bone histomorphometric analysis was performed on the trabecular bone at the above three sites to further examine whether targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE could promote bone formation in OVX rats. For the fifth lumbar vertebrae, the bone histomorphometric analysis showed that Tb.BFR/BS and Tb.MAR were notably higher in the OVX + Apc001PE group than in the OVX-B/L group (Fig. 8b and Supplementary Fig. 17c). The bone histomorphometric data of the distal femur (Supplementary Fig. 20a) and the proximal tibia (Supplementary Fig. 20b) showed consistent results. The bone histomorphometric analysis of the trabecular region at the above three sites showed no significant differences among the OVX + veh, OVX-B/L and OVX + RANDseq groups in any of the parameters. For the femoral mid-shaft, the bone histomorphometric analysis showed that Ct.BFR/BS and Ct.MAR were notably higher in the OVX + Apc001PE group than in the OVX-B/L group (Supplementary Fig. 20c).

To further examine whether targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE could enhance the bone mechanical properties in rats with established osteoporosis induced by OVX, we used the compression test for measurement of the fourth lumbar vertebrae. The failure force and ultimate strength were significantly higher in the OVX + Apc001PE group than in the OVX-B/L group. There were no significant differences among the OVX + veh, OVX-B/L and OVX + RANDseq groups in any of the above parameters (Supplementary Fig. 21a). Furthermore, the three-point bending test was used for measurement of the femoral midshaft. The stiffness, failure force and fracture energy were significantly higher in the OVX + Apc001PE group than in the OVX-B/L group. There were no significant differences among the OVX + veh, OVX-B/L and OVX + RANDseq groups in any of the above parameters (Supplementary Fig. 21b).

The serum levels of the bone formation biomarker procollagen type 1 N-terminal propeptide (P1NP) were determined. The serum level of P1NP was significantly higher in the OVX + Apc001PE group than in the OVX-B/L group. There were no significant differences among the OVX + veh, OVX-B/L and OVX + RANDseq groups (Supplementary Fig. 21c). Furthermore, there were no significant differences in the serum levels of sclerostin among all groups after treatments (Supplementary Fig. 21d).

For molecular analysis, OVX-induced osteoporotic rats were treated with Apc001PE with and without pretreatment with loop3m. The above bone anabolic effects of Apc001PE could be abolished by supplementation with exogenous loop3m (Fig. 8c and Supplementary Fig. 17–21). Thus, targeting sclerostin loop3 by the in vivo pharmacologic tool Apc001PE promoted bone formation, increased bone mass, improved bone microarchitecture integrity and enhanced bone mechanical properties by neutralizing circulating sclerostin loop3 in osteoporotic rats induced by OVX.

**Discussion**

This study found that sclerostin loop3 cannot participate in protecting the cardiovascular system but is involved in inhibiting bone formation by genetic and pharmacologic approaches both in vitro and in vivo.

In our in vitro studies, either loop3 deficiency by genetic truncation or targeting sclerostin loop3 by the specific in vitro pharmacologic tool aptacl56 maintained the suppressive effect of sclerostin on the expression of inflammatory cytokines and chemokines in AngII-treated macrophages and VSMCs. In our in vivo studies, either loop3 deficiency by genetic truncation or targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE maintained the protective effect of sclerostin on the cardiovascular system in ApoE−/− mice with AngII infusion. The above data consistently indicated that loop3 deficiency by genetic truncation or targeting sclerostin loop3 by the specific pharmacologic tool had no influence on cardiovascular events both in vitro and in vivo.

In our in vitro studies, either loop2&3 deficiency by genetic truncation or loop2&3 inhibition by sclerostin antibody attenuated the suppressive effect of sclerostin on the expression of inflammatory cytokines and chemokines in AngII-treated macrophages and VSMCs. In our in vivo studies, loop2&3 inhibition by sclerostin antibody significantly accelerated the development of cardiovascular events in ApoE−/− mice with AngII infusion. In a phase III clinical trial (BRIDGE), the total incidence of treatment-emergent cardiovascular serious adverse events was reported to be significantly higher in the antibody group than in the placebo group during the first 12-month period. Furthermore, in a phase III clinical trial (ARCH), the incidence of treatment-emergent cardiac ischaemic events was significantly higher in the antibody group than in the Alendronate (ALN) group during the 12-month double-blind period. Meta-analysis showed that ALN did not have a protective effect on the cardiovascular system, suggesting that the cardiovascular risk of therapeutic antibody against sclerostin still needs to be addressed. Moreover, a meta-analysis for phase III clinical trials further confirmed the higher risk of cardiac ischaemic events in patients randomized to antibody treatment. It was also found that two independent BMD-increasing SOST variants (rs7209826 and rs188810925), whose SOST expression levels were lower than those of the wild-type groups, were associated with a higher cardiovascular risk. Therefore, both genetically and therapeutically lowered sclerostin led to a higher risk of cardiovascular events, which are also reported previously.

In one nonclinical evaluation of cardiovascular events, ApoE−/− mice were fed a high-fat diet for 2 weeks, followed by OVX. Two
weeks after OVX, mice were subcutaneously administered a sclerostin antibody (r13C7 [Amgen, Inc.]) once weekly at a dose of 10 mg/kg (Multi-Discipline Review, Amgen Study No. 124609). After treatment, inflammatory cytokines and chemokines, including IL-6 and MCP-1, were locally upregulated in the aorta. In addition, sclerostin antibody enhanced the incidence of atherosclerotic plaques of types 2–5 with necrosis, indicating a potential concern of increasing plaque instability. Consistently, in our studies in ApoE−/− mice with AngII infusion, the serum levels of inflammatory cytokines and chemokines were significantly higher in the sclerostin antibody group (25 mg/kg, twice/week) than in the vehicle group. Similarly, the ratio of atherosclerotic plaques in the aortic arches and aortic roots was significantly higher in the sclerostin antibody group than in the vehicle group. Nevertheless, another group reported that sclerostin antibody (10 mg/kg, once weekly) did not influence the total atherosclerotic plaque volume or the circulating concentrations of IL-6, TNF-α and MCP-1 in ApoE−/− mice with AngII infusion21. The lack of effect on cardiovascular events could be explained by the lower dose employed in the

**Fig. 8 Determination of whether targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE could exert bone anabolic potential by neutralizing circulating sclerostin loop3 in ovariectomy-induced (OVX) osteoporotic rats.**

**a** Bar charts of the structural parameters from ex vivo micro-CT at the fifth vertebrae. Tb.BV/TV: p = 0.0086 (SHAM-B/L), p = 0.0138 (SHAM + veh), p < 0.0001 (SHAM + Apc001PE), p = 0.0277 (OVX + Apc001PE); Tb.vBMD: p = 0.0033 (SHAM-B/L), p = 0.0066 (SHAM + veh), p < 0.0001 (SHAM + Apc001PE), p = 0.0028 (OVX + Apc001PE); Tb.Th: p = 0.0169 (SHAM + veh), p < 0.0001 (SHAM + Apc001PE), p = 0.0116 (OVX + Apc001PE); Tb.N: p = 0.0084 (SHAM-B/L), p = 0.0003 (SHAM + Apc001PE), p = 0.0269 (OVX + Apc001PE); Tb.Sp: p = 0.0002 (SHAM-B/L), p = 0.0161 (SHAM + veh), p < 0.0001 (SHAM + Apc001PE), p = 0.0017 (OVX + Apc001PE); Tb.ConnD: p = 0.0050 (SHAM-B/L), p = 0.0225 (SHAM + veh), p < 0.0001 (SHAM + Apc001PE), p = 0.0328 (OVX + Apc001PE); Tb.SMI: p = 0.0006 (SHAM-B/L), p = 0.0028 (SHAM + veh), p < 0.0001 (SHAM + Apc001PE), p = 0.0012 (OVX + Apc001PE).

**b** Analysis of dynamic bone histomorphometric parameters at the fifth vertebrae. Tb.BFR/BS: p = 0.0056 (SHAM-B/L), p < 0.0001 (SHAM + Apc001PE), p < 0.0001 (OVX + Apc001PE); Tb.MAR: p = 0.0024 (SHAM-B/L), p < 0.0001 (SHAM + Apc001PE), p < 0.0001 (OVX + Apc001PE). For (a and b), data were expressed as the mean ± standard deviation followed by one-way ANOVA with Tukey’s post-hoc test vs. OVX-B/L, n = 10 per group. *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.0001. Note: Loop3m: the loop3 mutant (IPDAAAAAQLCGAAAAARRLVAAD); Tb.BV/TV: trabecular relative bone volume; Tb.vBMD: trabecular volumetric mineral density; Tb.Th: trabecular thickness; Tb.N: trabecular number; Tb.Sp: trabecular spacing; Tb.ConnD: trabecular connection density; Tb.SMI: trabecular structure model index; Tb.BFR/BS: trabecular bone formation rate; Tb.MAR: trabecular mineral apposition rate. Source data are provided as a Source Data file.
literature (10 mg/kg per week) than the higher dose employed in our study (50 mg/kg per week).

In our genetic truncation studies, loop3 deficiency by genetic truncation attenuated the inhibitory effect of sclerostin on Wnt signalling and osteogenic potential in MC3T3-E1 cells in vitro. In studies of knock-in mice, loop3 deficiency by genetic truncation attenuated the inhibitory effect of human sclerostin on bone formation in vivo. Targeting sclerostin loop3 by the specific in vitro pharmacologic tool aptsc56 inhibited sclerostin’s antagonistic effect on Wnt signalling and osteogenic potential in MC3T3-E1 cells in vitro. Moreover, targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE attenuated the inhibitory effect of sclerostin on bone formation in the hSOST°/° mice and promoted bone formation in the O VX-induced osteoratic rats in vivo. The above in vitro and in vivo data consistently demonstrated the promising bone anabolic potential of targeting sclerostin loop 3 with a specific pharmacologic tool.

In our in vitro mutation studies, sclerostin loop3m failed to inhibit Wnt signalling but remained bound to aptsc56. The aptsc56m failed to bind to sclerostin. In our in vivo studies, the attenuation of the specific in vitro pharmacologic tool Apc001PE on sclerostin’s bone formation inhibition could be completely abolished by supplementation with exogenous loop3m in hSOST°/° mice. Moreover, the bone anabolic potential of the specific in vivo pharmacologic Apc001PE could be completely abolished by supplementation with exogenous loop3m in the O VX-induced osteoratic rats. Furthermore, Apc001PE did not attenuate sclerostin’s bone formation inhibition in hSOST°/° mice or the bone anabolic potential in O VX-induced osteoratic rats. These data consistently demonstrated that targeting sclerostin loop3 by the specific in vivo pharmacologic tool Apc001PE attenuated the inhibitory effect of sclerostin on bone formation in the hSOST°/° mice and promoted bone formation in the O VX-induced osteoratic rats by neutralizing the circulating sclerostin loop3.

It was reported that sclerostin loop2, especially Asn92 and Ile94, is important for antagonizing the Wnt signalling pathway, which was mainly focused on the residues of sclerostin loop2 in regulating Wnt signalling. In our studies, we found that sclerostin loop3 could also participate in inhibiting Wnt signalling but not in protecting the cardiovascular system, which mainly focuses on the residues of loop3 in regulating Wnt signalling. Furthermore, this study is an attempt to study the key sites of loop3 in regulating Wnt signalling and in interacting with aptamer aptsc56. Extensive studies will be necessary to further confirm and validate the roles of these sites.

Taken together, this study validated that targeting sclerostin loop3 by genetic and pharmacologic approaches could maintain the protective effect of sclerostin on the cardiovascular system and promote bone formation. This study could provide a target for the development of next-generation sclerostin inhibitors that specifically target sclerostin loop3 for bone anabolic therapy, with a low cardiovascular risk.

**Methods**

**Study approval.** All procedures in the present study were approved by the Research Ethics Committee of the Hong Kong Baptist University.

**Cell culture.** MC3T3-E1 subclone 4 (mouse preosteoblast, ATCC CRL-2593) was cultured in α-MEM with 10% FBS and 100 units/ml penicillin-streptomycin. RAW 264.7 cells (macrophages, ATCC TIB-71) were cultured in DME/M with 10% FBS and 100 units/ml penicillin-streptomycin. Human VSMCs (aorta/smooth muscle, ATCC CRL-1999) were cultured in DME/M with 10% FBS, 0.2 mg/ml G418 and 100 units/ml penicillin-streptomycin. No mycoplasma infection was found for any of the cell cultures.

**TOP-Wnt-induced luciferase reporter assay.** To assess the inhibitory potency of aptamers against sclerostin’s antagonistic effect on Wnt signalling, we performed a TOP-Wnt-induced luciferase reporter assay in preosteoblast MC3T3-E1 cells. Cells were seeded in 24-well plates and transfected with the corresponding reporter plasmids, pCMV-β-gal as an internal transfection control and TOP-Wnt plasmid as necessary to facilitate transfection. Six hours after transfection, the culture medium was changed to fresh medium, and the cells were treated with aptamers or the corresponding reagents. Twenty-four hours after treatment, the cells were lysed with 100 μl/well passive lysis buffer, and 20 μl was taken for analysis. Luciferase assays were performed using the Dual-Luciferase Reporter system with parameters settings according to the manufacturer’s protocol.

**Quantitative PCR.** Total RNA from cultured cells was isolated by homogenizing using TRIzol (Invitrogen) and reverse transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCRs were performed using TaqMan Universal PCR Master Mix on the 7900 HT Sequence Detection System (Applied Biosystems). Primer sequences were designed from the following genes (GAPDH: Hs99999990_m1, Mm99999915_g1), interleukin 6 (IL-6: Hs01017143_m1), CCL-2 (MCP-1: Hs00232414_m1), TNFa (TNFa: Hs00174128_m1), ALPL (ALP: Mm00475834_m1) and Bglap (OCN: Mm03413826_mH). Relative RNA expression of genes was determined using the 2-ΔΔCt method with GAPDH as the endogenous normalizer.

**Mice and genotyping.** The procedures of all animal studies have gained ethics approval by the Research Ethics Committee of the Hong Kong Baptist University. The animals were grouped randomly and blindly to researchers. All animal experiments were carried on in compliance to the relevant ethical regulations of Hong Kong Baptist University. The animals in poor body condition were excluded. Transgenic C57BL/6 mice expressing human sclerostin (B6. hSOST°/°), 3-month, male and female; loop3-deficient human sclerostin (B6. Δloop3-hSOST°/°), 3-month, male and female; and wide-type C57BL/6 mice, 3-month, male and female were used to evaluate the bone anabolic potential (n = 10 per group). Sprague Dawley rats, 4-month, female, were used to evaluate the bone anabolic potential, serum levels immune factors and toxicities (n = 10 per group), and to perform the pharmacokinetic analysis (n = 6 per group). Apolipoprotein E null (B6. ApoE−/−) mice (C57BL/6J background), 3-month, male and female; loop3-hSOST°/° mice crossed to B6. ApoE−/− mice (B6. Δloop3-hSOST°/° mice) were purchased from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. Full-length human sclerostin knock-in (B6. hSOST°/°) and loop3-deficient human sclerostin knock-in (B6. Δloop3-hSOST°/°) mice were generated by crossing B6. Δloop3-hSOST°/° mice to generate 1465 bp (5′-RP 5′TCACAGAGACAAATGGGACATCAGT-3′ arm) and 1229 bp (3′-RP 5′TCACAGACAAATGGGACATCAGT-3′ arm) amplicons. The 5′-RP 5′TCACAGAGACAAATGGGACATCAGT-3′ arm forward primer (FP) 5′-ATGCCCACCAAAGTCATCAGTGTAG-3′ and 3′-RP 5′-CTCTCTCTCTCTCTCTCTCACACTCCTCGACGT-3′ arm reverse primer (RP) 5′-AGGGGCCGTTTTACCATGTAAGTT-3′, 3′ arm FP 5′-CTCTCTCTCTCTCTCTCTCAGACTCTCCGACGT-3′ and 3′ arm RP 5′-TCAACAAAACATATGGCCTTC-3′ to generate 1465 bp (3′ arm) and 1229 bp (5′ arm) amplicons. The Δloop3-hSOST°/° gene was genotyped using DNA extracted from mouse tail clippings and amplified using the 5′ arm FP 5′-ATGCCACAAAGTCTCAGTCTTAG-3′, the 5′ arm RP 5′-AGGGGCCGTTTTACCATGTAAGTT-3′, 3′ arm FP 5′-CTCTCTCTCTCTCTCTCTCAGACTCTCCGACGT-3′ and the 3′ arm RP 5′-TCAACAAAACATATGGCCTTC-3′ to generate 1465 bp (3′ arm) and 1229 bp (5′ arm) amplicons. The hSOST°/° mice were generated by crossing B6. Δloop3-hSOST°/° mice with B6. ApoE−/− mice. The ApoE−/− mice were generated by crossing B6. ApoE−/− mice with B6. ApoE−/− mice. The ApoE−/− allele was genotyped using DNA extracted from mouse tail clippings and amplified using FP 5′-GCTACGGGACAGGAGCAGCGC-3′ and RP 5′-TGTGAC TGGGAGACCTGGACG & GGGCCGGCAGCTCCATCTC-3′ to generate 155 bp (wild type) or 245 bp ( homozygous) amplicons. Western blot and IHC were performed to determine the expression of sclerostin in tissues using anti-sclerostin antibody (Abcam, ab85799/ab63097, 1:1000).

**AA and atherosclerosis evaluation.** For infusion, osmotic minipumps (Model 1004, ALZET, Durect Corporation, USA) were implanted into the subcutaneous space along the dorsal midline on the right flank via an incision in the scapular region under anaesthesia (4% isoflurane) to deliver AngII (500 mg/kg/min, Sigma-Aldrich) or vehicle for four weeks. Two days a week, the cages were changed. The animals were randomly divided into 3 groups: sham surgery, AngII-infused rats and AngII-infused rats after minipump implantation. Minipump implantation, mice were subcutaneously administered saline, therapeutic antibody against sclerostin (Homing-Inf USED: Creative Biolabs, 25 mg/kg).
twice/week) or Apc001PE (25 mg/kg, twice/week). The sequence of the humanized therapeutic sclerostin antibody employed in this study was the same as the sequence of a murine antibody (EVENTY™) (fomosozumab-abagq in the US). The administration dose of Apc001PE referred to the mass of apatc56. The body weight of each mouse was recorded. For AA evaluation, the aortas were perfused via the left ventricle with ice-cold saline immediately after sacrifice. The aortas were then isolated from the fat and connective tissues under a Zeiss Stemi 305 stereomicroscope with or without aneurysm formation was de

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Regions of interest (ROIs) were de

The maximum outer diameters of the aortic arch, thoracic aorta and suprarenal aorta were determined by Zeiss software (Carl Zeiss Far East Co., Ltd, Germany). For athersclerosis assessment, the atherosclerotic plaque was quantified by measuring the surface area of the Oil Red O-positive lesions on en face preparations of aortic arches. The saline-perfused upper half of the heart, including the aortic root, was directly embedded in an optimal cutting temperature compound (O.C.T., Sakura Finetek, Co. Ltd., Tokyo, Japan), frozen in liquid nitrogen, and cryosectioned (10 µm). The ratio of atherosclerotic plaque area to total cross sectional area of the aortic root was examined by Oil Red O staining (10 µm) and quantified by colorimetric analysis using ImageJ33,34.

Serum levels determination of inflammatory cytokines, chemokines, bone formation markers, immune factors, biochemical and haematological parameters, and sclerostin. The serum of mice/rats was collected after treatment. ELSA (ThermoFisher, PWN, St. Louis, MO, USA) and a 96-well plate (ThermoFisher, PWN, St. Louis, MO, USA) were used. The ssDNA library used in SELEX contained a 40-nucleotide (nt) randomized region and an 18 nt conserved region at each end for primer annealing: 5ʹ-CCTCAGTGTCAGCGTACG (N40)-CAGCTGGAGCTGCTGATCC-3ʹ, FP: 5ʹ-CAGTACCGTGGAGCTGCTGATCC-3ʹ and bio-

Enzyme-Linked OligoNucleotide Assay (ELONA). Target protein (160 ng) was coated into a 96-well microtiter plate in 100 µl of PBS and incubated at 4°C overnight. The following day, 100 µl of blocking buffer was added and incubated for 1 h. The plate was washed three times with washing buffer (PBS, 0.1% Tween 20 and 1% BSA) for 1 h at RT and washed with washing buffer (PBS, 1 µg in the 1st round and 0.1 µg in the following rounds) to remove nonspecific binding. Unbound sequences were removed with washing buffer. After washing, the bound sequence-protein beads were collected and subjected to PCR amplification with FP and Bio-TP (step 1: 95°C for 1 min for initial denaturation; step 2: 12 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s; step 3: 72°C for 2 min). PCR products were used for streptavidin magnetic beads through biotin-streptavidin binding according to the manufacturer’s instructions. Single-stranded sequences were regenerated with 0.1 Mortho-ATP. Negative selection was performed against recombinant loop3-deficient sclerostin, blank NTA beads and other His-tagged proteins. After 20 rounds of selection, the identified sequences were sent for next-generation sequencing by Illumina MiSeq (PCT No: PCT/2019/074764, PCT Pub No.: WO2019/15440).

Protein SELEX. Recombinant His6-human sclerostin was immobilized on NTA magnetic beads at 4°C for 1 h. The ssDNA library was denatured at 95°C for 10 min and rapidly cooled to 0°C for 10 min followed by incubation with immobilized sclerostin at room temperature (RT) for 0.5–1 h. The total incubation volume was 1 ml. BSA (10 µg in the 1st round and 1 µg in the following rounds) was included to avoid nonspecific binding. Unbound sequences were removed with washing buffer. After washing, the bound sequence-protein beads were collected and subjected to PCR amplification with FP and Bio-TP (step 1: 95°C for 1 min for initial denaturation; step 2: 12 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s; step 3: 72°C for 2 min). PCR products were used for streptavidin magnetic beads through biotin-streptavidin binding according to the manufacturer’s instructions. Single-stranded sequences were regenerated with 0.1 M ortho-ATP. Negative selection was performed against recombinant loop3-deficient sclerostin, blank NTA beads and other His-tagged proteins. After 20 rounds of selection, the identified sequences were sent for next-generation sequencing by Illumina MiSeq (PCT No: PCT/2019/074764, PCT Pub No.: WO2019/15440).

Micro-CT analysis. Micro-CT (version 6.5, vivaCT40, SCANCO Medical AG, Bassersdorf, Switzerland) was employed for analysis of the bone mass and bone architecture. For mice, the trabecular bone at the right proximal tibia metaphysis and the fourth lumbar vertebrae (LIV) were analysed. For rats, the trabecular bone at the right femoral mid-shaft, the right proximal tibia metaphysis, the middle third of the femur, and the fifth lumbar vertebrae, as well as the cortical bone at the right femoral mid-shaft, were analysed. Briefly, a total of 424 slices with a voxel size of 10 µm (for mice)/20 µm (for rats) were scanned at the region of the proximal tibia beginning at the growth plate and extending distally along the tibial diaphysis, the entire region of secondaries between proximal and distal aspects from the fourth (for mice)/fifth (for rats) tibial growth plate, and the region of femoral mid-shaft (for rats). Regions of interest (ROIs) were defined for trabecular (for both mice and rats) and cortical parameters (for rats) using Scanco evaluation software. Images of femurs, tibias and vertebrae were reconstructed and segmented (200 ms integration time, 0.8 sigma, 2000 thresholds). All the measurements used the same filtering and segmentation values. For the proximal tibia and distal femur of mice, 100 sequential slices beginning at 0.1 mm from the most proximal aspect of the growth plate in which both condyles were no longer visible were selected for analysis. The trabeculae were analysed by manual contouring excluding the cortical bone. For distal femoral metaphysis and proximal tibia metaphysis of rats, the entire femora or tibia were reoriented with the mid-diaphysis parallel to the z-axis, and bone length was measured as the distance between the most proximal and distal transverse plans containing the femur. Beginning from the most proximal aspect of the growth plate, the trabeculae region on 100 consecutive slices at a distance of 5 mm away from the growth plate with blocking buffer (PBS, 0.1% Tween 20 and 1% BSA) for 1 h at RT and washed with washing buffer (PBS, 1 mM MgCl2, 0.1% Tween 20 and 0.1% BSA) 4 times. The aptamer candidates were denatured at 95°C for 10 min and rapidly cooled on ice for 10 min before use. Appropriate concentrations of biotinylated aptamers were added to each well (100 µl) and incubated for 15 min. Biotinylated aptamers were directly blocked with 5% BSA, anti
cleaved caspase-3 antibody (Abcam, ab2302, 10 µg/ml), followed by incubation with goat anti-rabbit IgG (Abcam, ab20571, 1:1000). The colour reaction was then developed by adding 3,3′,5,5′-diaminobenzidine (DAB). Positive staining areas were quantified by colorimetric analysis using ImageJ36.

Bone mechanical test. The fourth lumbar vertebra (LIV) and the left femurs of the rats were directly stored at −80°C after sacrifice. The compression test and three-point test were performed by a universal testing machine (H25KS Series, Hounsfield Test Equipment Ltd, Redhill, UK) with a 2.5 kN load cell45. For the compression test, LIVs were isolated from vertebral columns and constructed into a cylinder with two parallel planes (5–7 cm) before the test. The LIVs were positioned horizontally to the base. Load was applied constantly with a displacement rate of 1 mm/min. After failure, the load vs. displacement curves were recorded, and the failure force (N) and ultimate strength (MPa) were calculated for statistical analysis. For the three-point test, femurs were loaded in the anterior-posterior direction with the span set as 17 mm. Load was applied with a constant displacement rate of 1 mm/min at the femur mid-shaft. After failure, the load vs. displacement curves were recorded, and the failure force (N), stiffness and fracture energy (J) were calculated for statistical analysis.
into PBST + 0.1% BSA) was added to each well and incubated for 30 min and washed with PBST + 0.1% BSA 4 times. Then, 50 µl of TMB was added to each well and incubated for 20 min. The reaction was stopped by adding 50 µl of 2 M H2SO4. The absorbance at 450 nm was measured with a microplate reader. Data were analysed with Origin (OriginLab, Northampton, MA, USA).

A nonlinear curve fitting model Hyperb was used to plot the binding curve. The equation of the Hyperb model is \( y = \frac{P_1 x}{P_2 + x} \), and P2 is the KD value.

Pharmacokinetic analysis of the modified aptamer after administration in vivo. After a single subcutaneous (S.C.) administration of aptsc56 or Apc001PE in rats, blood samples (~200 µl) were collected at different time points (aptsc56: 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h; Apc001PE: 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 30 h, 36 h, 48 h, 54 h, 62 h, 70 h, 76 h, 84 h, 96 h, 107 h, n = 6 in each group) via the orbital vein.26,27 The plasma was isolated within 1 h and stored at −80 °C.26,27 Prior to analysis, plasma samples were incubated with proteinase K solution (1 mg/ml proteinase K in 10 mM Tris HCl, pH 7.5, 20 mM CaCl2, 10% glycerol v/v) in digestion buffer (60 mM Tris-HCl, pH 8.0, 100 mM EDTA and 0.5% SDS) at 55 °C overnight with shaking. The samples were then centrifuged (16,900 g, 4 °C, 15 min), and the supernatant was taken for analysis.

The HPLC system was equipped with a C4 column to quantify Apc001PE in plasma samples, while a C18 column was utilized for the quantification of aptsc56. Both methods used a mobile phase elution gradient made from phase A (TEAA, pH 7.0) and phase B (acetonitrile). The column oven temperature was 50 °C. The HPLC system was equipped with a C4 column to quantify Apc001PE in plasma samples, while a C18 column was utilized for the quantification of aptsc56. Both methods used a mobile phase elution gradient made from phase A (TEAA, pH 7.0) and phase B (acetonitrile). The column oven temperature was 50 °C.

The dosing interval for Apc001PE administration could be defined based on the elimination half-life (T1/2) and the dosing ratio of the loading dosage to the maintenance dosage of Apc001PE.26,27 In the following in vivo studies, as the loading dosage and maintenance dosage of Apc001PE were set to the same dose (dosing ratio = 2), the dosing interval was twice weekly, which was slightly longer than T1/2 (66.9 h).

Statistical analysis. All variables are expressed as the mean ± standard deviation. All statistical analysis were performed by GraphPad Prism (version 8; GraphPad Software, Inc., San Diego, CA, USA), and P < 0.05 was considered statistically significant. For the in vivo experiments, the sample size was predetermined by a power calculation and our previously published paper.44 The animals were grouped randomly and blindly to the researchers. Animals in poor body condition were excluded.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. Source data are provided with this paper.

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Author contributions

G.Z., Y.Y., B.T.Z. and A.L. supervised the whole project. Y.Y., L.W. and S.N. performed the major research and wrote the manuscript in equal contributions. D.L., J.L., H.Y.C., N.L., M.S., N.L., Q.R., Z. Zhuo, C.Z., D.X., Y.L., Z.K.Z. and H.Z. provided technical support. M.L., Z. Zhang, L.C., X.P., W.X. and S.Z. provided their professional expertise.

Competing interests

The authors declare no competing interests.

Additional information

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