Recombinant sclerostin inhibits bone formation *in vitro* and in a mouse model of sclerosteosis

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Supplementary material

Microscale thermophoresis (MST)

A Monolith NT.115 instrument (NanoTemper Technologies) was used to measure the interaction between recombinant mScl and LRP6-E1E2 (UCB) [1] in DPBS. LRP6-E1E2 was labelled with fluorescent NT647 dye using a Monolith NT RED NHS Protein Labelling Kit (NanoTemper Technologies). The MST experiments were carried out in DPBS (pH 7.4) containing 0.05% Tween 20 and 50% (w/v) PEG8000, with increasing concentrations (0 nM to 250 nM) of recombinant mScl titrated against 12.5 nM labelled LRP6-E1E2. Interactions were measured at 23.9 °C with 5s/30s/5s infrared (IR) laser off/on/off times, 100% LED power and 40% MST power. Each MST run was performed in triplicate and dose response curves were fitted to a 1:1 interaction model using the MO.Affinity Analysis v2.2.6 software (NanoTemper Technologies).

Surface plasmon resonance competition assay

The interaction of recombinant mScl with LRP6-E1E2-Fc (UCB) was measured at 25 °C on a Biacore 3000 instrument (GE Healthcare Life Sciences). HBS-EP pH 7.4 (10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.05% v/v surfactant P20; GE Healthcare Life Sciences, BR-1006-69) was used as running buffer for the immobilisation of human sclerostin to the surface. A flow cell on a CM5 carboxymethyl dextran coated sensor (GE Healthcare Life Sciences, BR100399), was activated by the addition of a mixture, in water, of N-hydroxysuccinimide and ethyl(dimethylaminopropyl)carbodiimide (GE Healthcare Life Sciences, BR-1000-50; injection volume: 60 µL; flow rate: 10 µL/min). Human sclerostin

(hScl; UCB Celltech; 20 µg/mL), in 10 mM sodium acetate pH 4, was added to immobilise ~1600 RU on the surface. Unreacted activated sites were blocked by the addition of 1 M ethanolamine (50 µL, flow rate 10 µL/min). A reference flow cell was activated and blocked with 1 M ethanolamine in a similar manner. A range of dilutions (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81 and 0 nM) of recombinant mScl in HBS-EP pH 7.4, containing 1 mg/mL CM-dextran (Fluka BioChemica, 27560), were pre-incubated with 100 nM LRP6-E1E2-Fc for 30 minutes at room temperature and passed over the hScl and reference flow cells, using 30 µL injections and a flow rate of 10 µL/min. HBS-EP containing 1 mg/mL CM-dextran was used as the running buffer. Dilution curves were fitted with XLfit version 5.5.0.5 (IDBS) and an EC50 derived for each recombinant mScl protein.

P1NP ELISA

Procollagen Type I N-terminal propeptide (P1NP) is a reference bone formation marker that increases during bone formation and decreases during bone resorption [2]. P1NP concentrations were measured using P1NP ELISA kits (Immunodiagnostic Systems Holdings PLC). The assays were performed as described in the kit manual. Individual serum samples (collected at predose, 1, 7 and 42 days; N=6 at each time point, except $SOST^{-/-}$ Vehicle N=7) were each analysed in duplicate. Two independent ELISAs were performed. Absorbance was measured at 450 nm (reference 650 nm) and P1NP concentrations in the control and serum samples were determined by interpolation from a P1NP standard curve.

Anti-drug antibody assay

The severity of immune responses against the administered mScl constructs were determined by electrochemiluminescence (ECL) anti-drug antibody (ADA) assays. Unchanged mScl and mScl hFc PD were biotinylated with EZ-Link NHS-LC-LC-Biotin (Thermo Fischer Scientific) as per manufacturer's instructions. Unchanged mScl and mScl hFc PD were labelled with MSD GOLD SULFO-TAG NHS-Ester (150 nmol) (Meso Scale Diagnostics) as per provided instructions. Excess Biotin and SULFO-TAG was

removed using 2 mL 7K molecular weight cut-off Zeba Spin Desalting Columns (Thermo Fischer Scientific). Sclerostin neutralising antibody (Anti-Scl Ab [3]) (UCB) standards (50 µL/sample; 2000, 667, 222, 74, 25, 8.2, 2.7 and 0 ng/mL) were prepared in PBS/1% BSA/10% WT serum. A 50 µL solution of 1 µg/mL biotinylated and SULFO TAG labelled mScl (for mScl treated mice) and mScl hFc PD (for mScl hFc PD treated mice) were added to the 50 µL standards, the experimental serum samples (50 µL; 1:10 dilution), and two controls (50 µL 500 ng and 20 ng anti-Scl Ab in PBS/1% BSA/10% WT serum). Sample solutions were incubated overnight at room temperature, in a shaking incubator. MSD GOLD 96-well Streptavidin SECTOR plates (Meso Scale Diagnostics) were washed (PBS/0.1% Tween wash buffer), blocked with 150 µL PBS/3% BSA blocking buffer and incubated overnight at 4 °C. Prepared samples were added (50 µL/well) the following day, after washing the blocked plates with wash buffer (as above). Plates were sealed and incubated, shaking at room temperature, followed by three washing steps (as above). Two times MSD Read Buffer (Meso Scale Diagnostics) was added (150 µL/well) and signal was read immediately at 450 nm (570 nm as reference) using an MSD SECTOR Imager 6000 system (Meso Scale Diagnostics). A standard curve was plotted, and Anti-Scl Ab serum concentrations were calculated by interpolation.

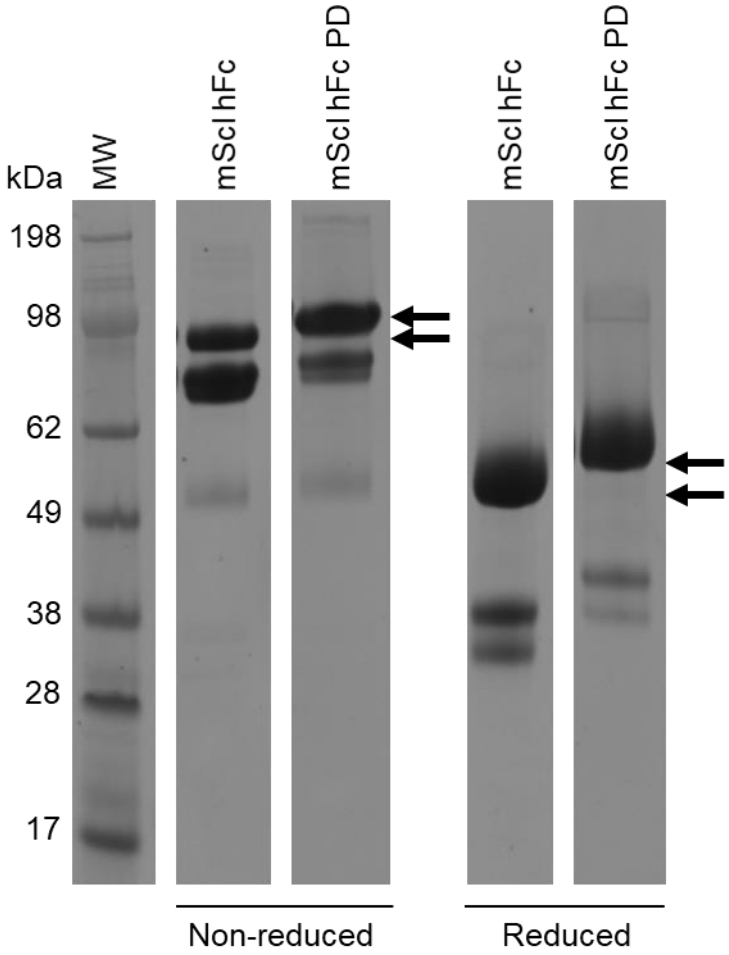
References

[1] Holdsworth G, Slocombe P, Doyle C, Sweeney B, Veverka V, Le Riche K, et al. Characterization of the Interaction of Sclerostin with the Low Density Lipoprotein Receptor-related Protein (LRP) Family of Wnt Co-receptors. J Biol Chem 2012;287:26464–77. https://doi.org/10.1074/jbc.M112.350108.

[2] Vasikaran S, Eastell R, Bruyère O, Foldes AJ, Garnero P, Griesmacher A, et al. Markers of bone turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: a need for international reference standards. Osteoporos Int J Establ Result Coop Eur Found Osteoporos Natl Osteoporos Found USA 2011;22:391–420. https://doi.org/10.1007/s00198-010-1501-1.

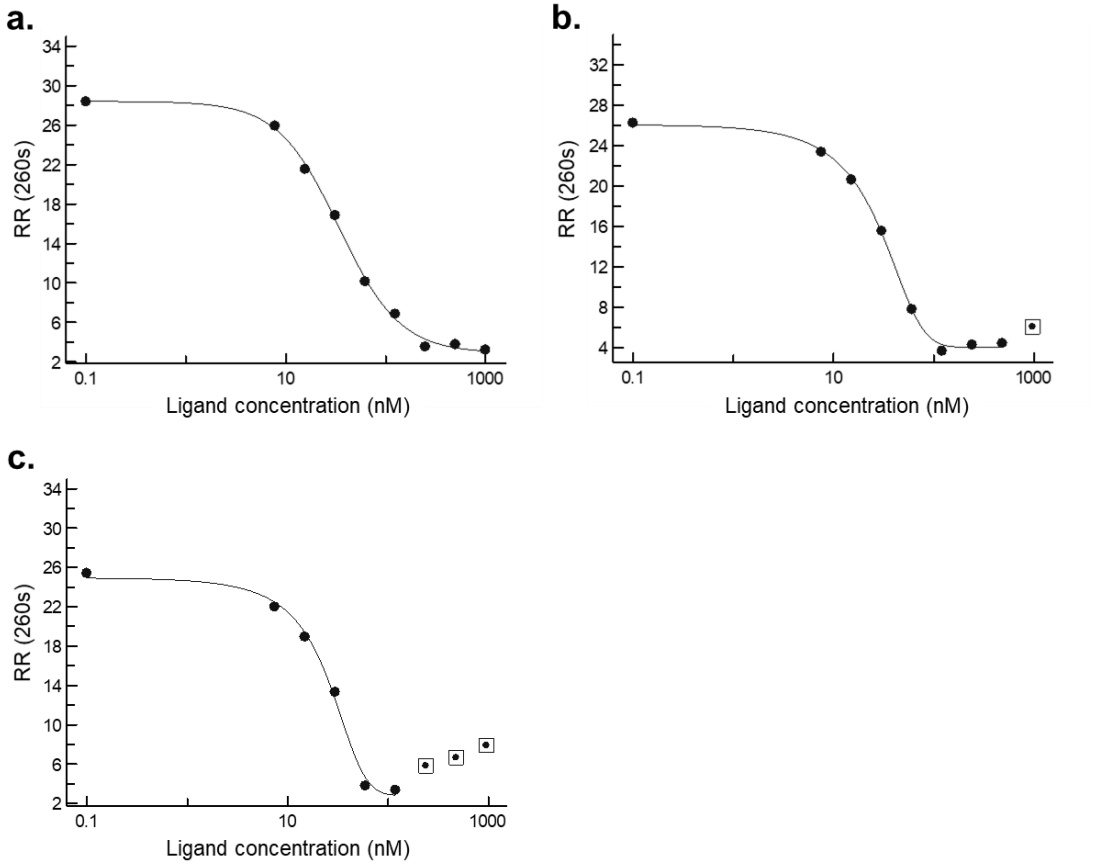
[3] Veverka V, Henry AJ, Slocombe PM, Ventom A, Mulloy B, Muskett FW, et al. Characterization of the Structural Features and Interactions of Sclerostin. J Biol Chem 2009;284:10890–900. https://doi.org/10.1074/jbc.M807994200.

Supplementary Figure S1



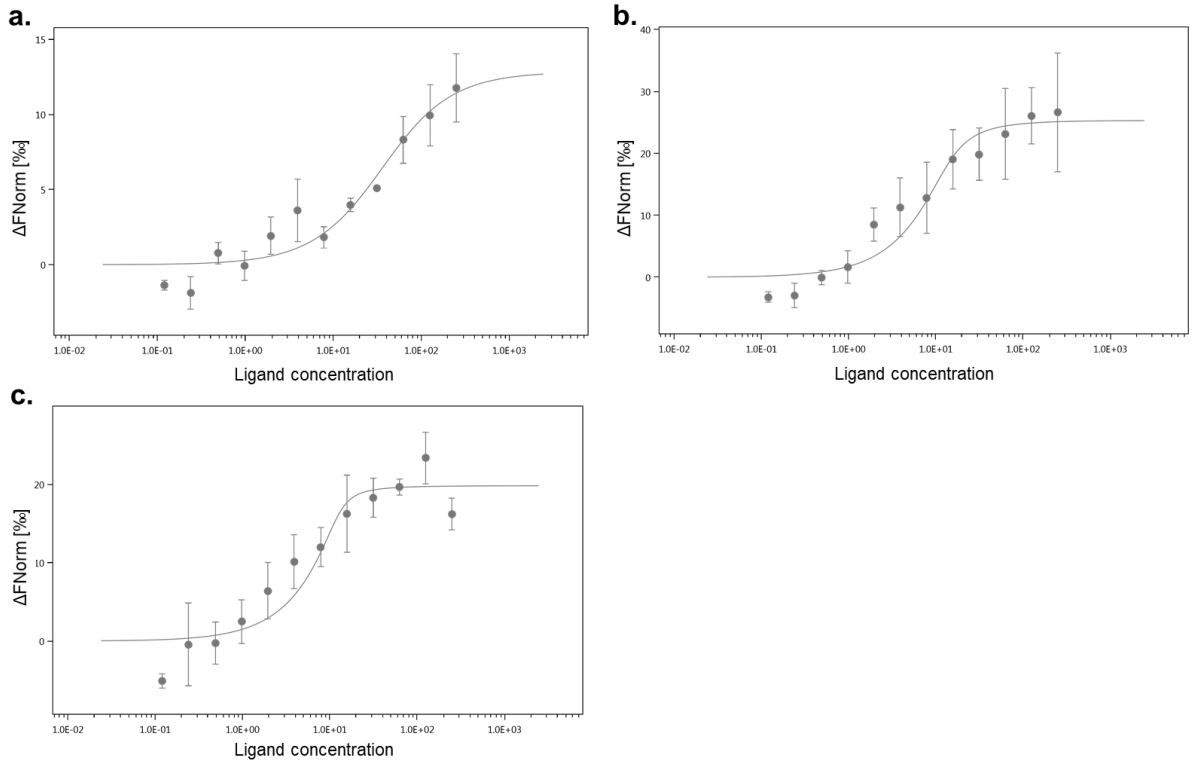
SDS PAGE of purified mScl hFc and mScl hFc PD under reduced and non-reduced conditions. Dimeric mScl fusion proteins are indicated by ~98 kDa (left arrows) bands under non-reducing conditions and ~50-55 kDa under reducing conditions (right arrows). Some additional minor abundance bands were observed for each protein construct. Cropped gels are juxtaposed, and complete gels are presented in Supplementary Fig. S9. Lane 1 is from Supplementary Fig. S9c (lane 1), lanes 2 and 5 are from Supplementary Fig. S9b (lanes 4 and 8), and lanes 3 and 6 are from Supplementary Fig. S9a (lanes 3 and 8).

Supplementary figure S2



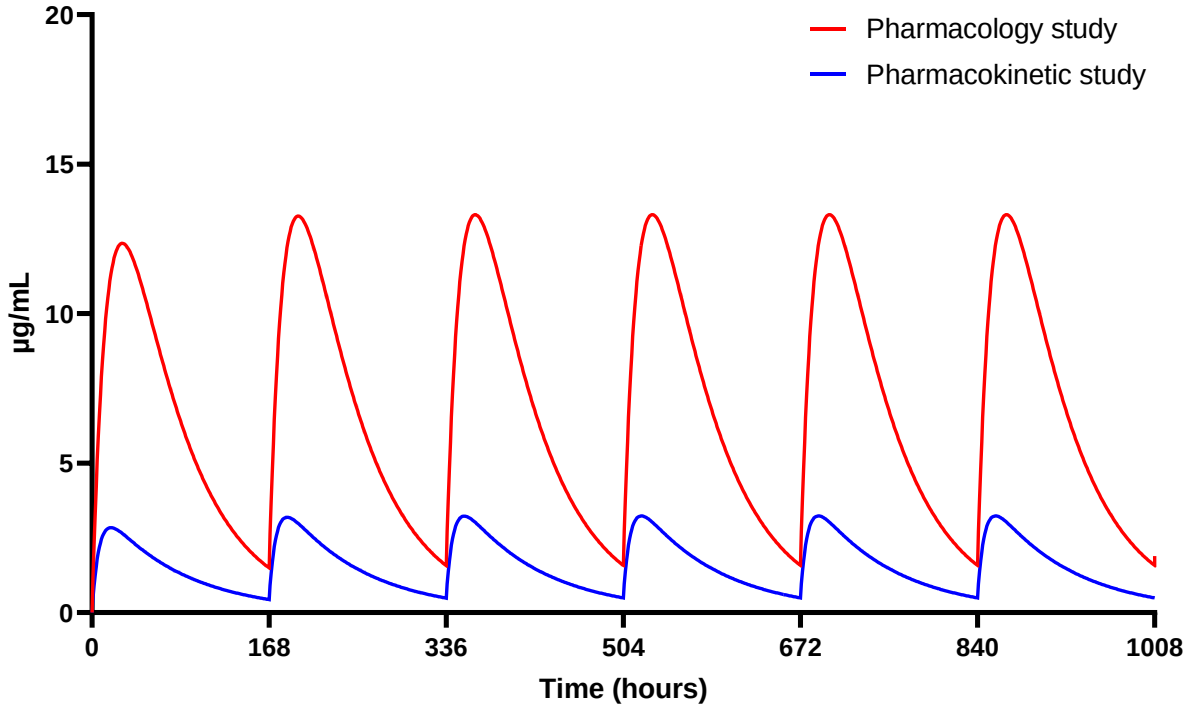
SPR dilution curves of hScl (UCB), mScl, mScl hFc and mScl hFc PD. (**a, b, c**) mScl (**a**), mScl hFc (**b**) and mScl hFc PD (**c**) interaction with LRP6-E1E2-Fc. Relative response (RR) at 260s was plotted against the concentration of recombinant sclerostin in solution. Points with squares were marked as outliers.

Supplementary Figure S3



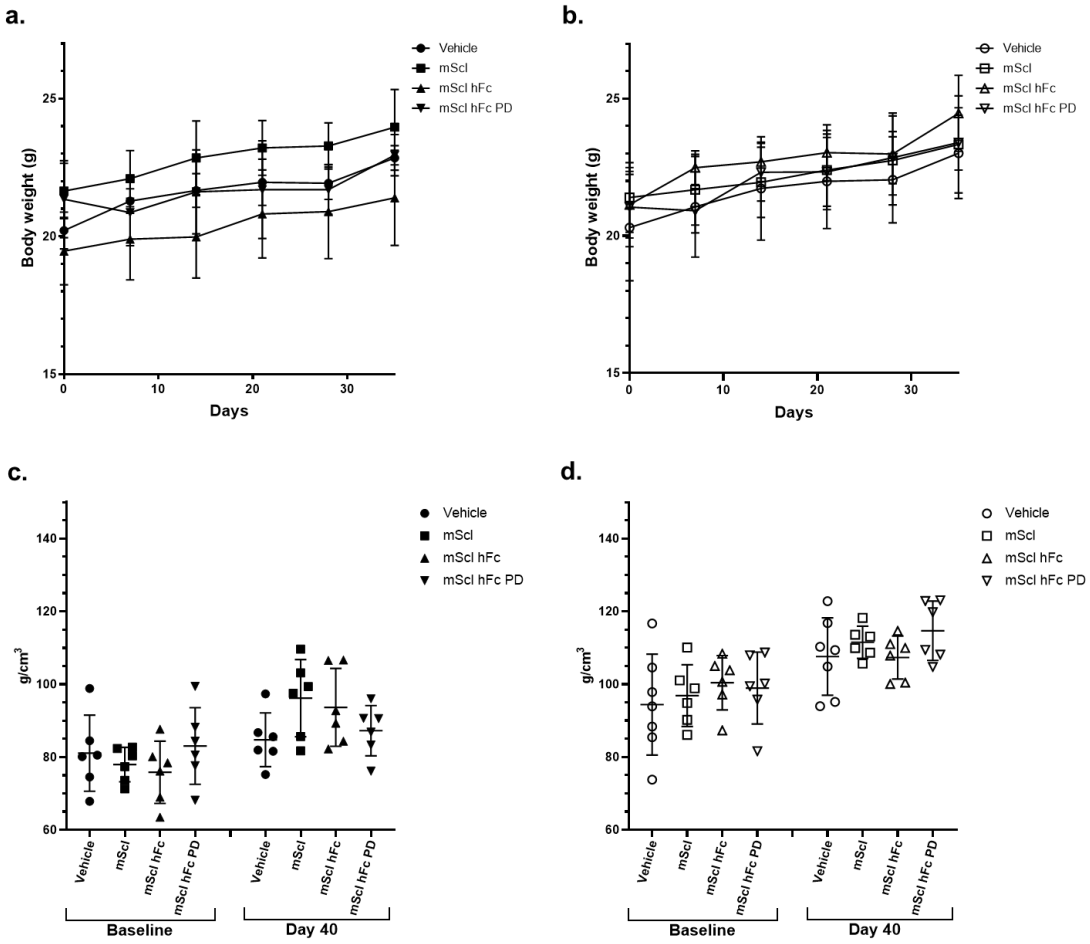
MST dose response curves for mScl, mScl hFc and mScl hFc PD. (**a, b, c**) mScl (**a**), mScl hFc (**b**) and mScl hFc PD (**c**) interaction with fluorescently labelled (NT647) LRP6-E1E2. Gradual change in thermophoresis is plotted as ΔFnorm versus ligand concentration to yield a 12-point dose response curve fitted to a one-site binding model. Mean±SD of triplicates from three independent experiments shown.

Supplementary Figure S4



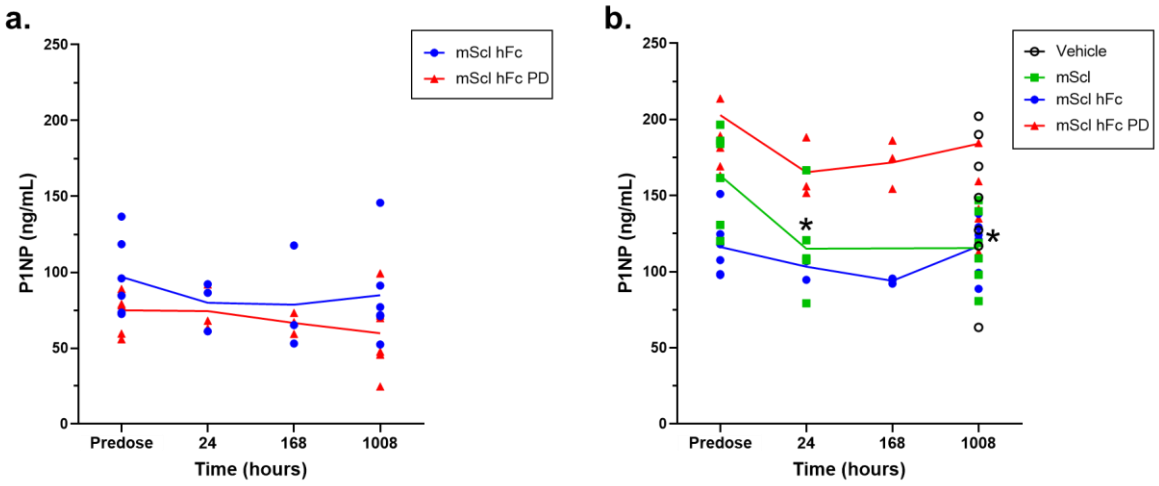
Pharmacology study exposure compared to the 42-day exposure prediction model. mScl hFc concentration results from a 6-week multidose pharmacology study (10 mg/kg mScl hFc administered subcutaneously) fitted to a compartmental pharmacokinetic model (red line). Prediction model (assuming 50% bioavailability and 0.1/hour absorption) (blue line) simulated using results from a single-dose study (10 mg/kg mScl hFc administered intravenously) that was fitted to a compartmental pharmacokinetic model. Berkeley Madonna 9.1.19 software (University of California) was used to fit models and simulate the prediction model.

Supplementary Figure S5



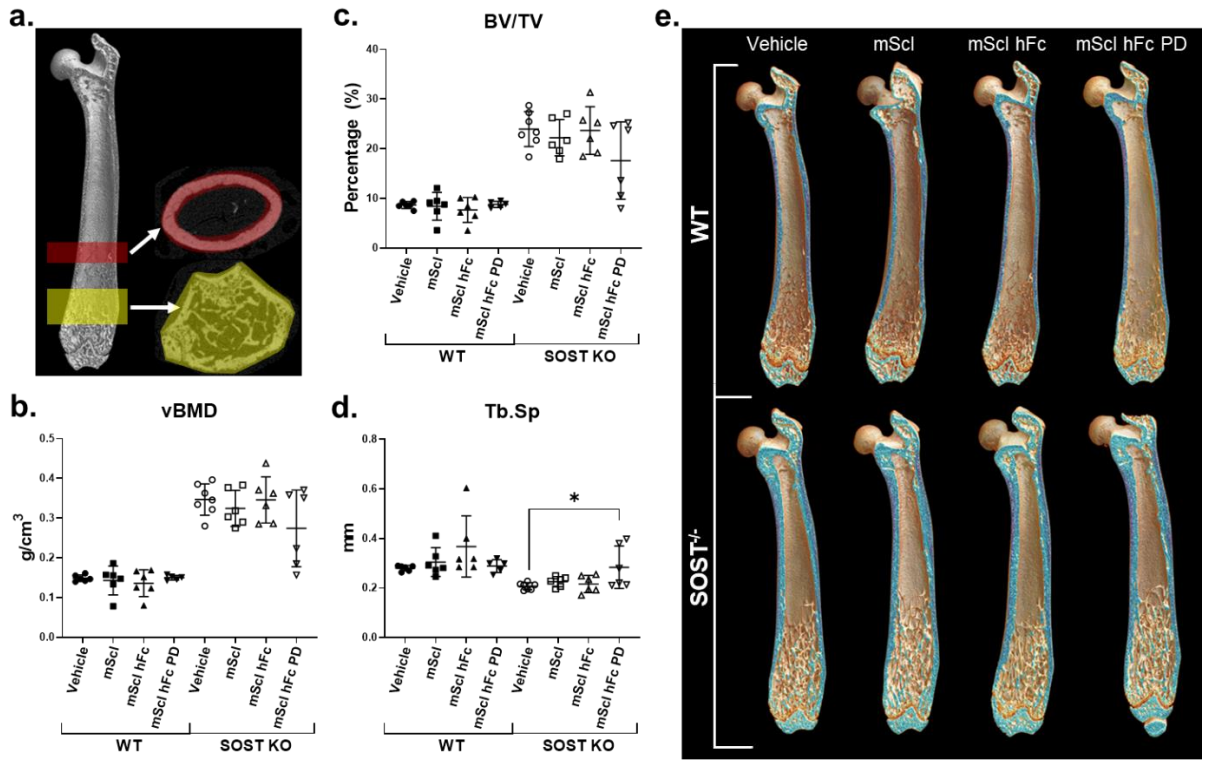
Body weights and whole body aBMD of WT and SOST$^{-/-}$ mice groups. (**a, b**) Weight of 7-10 week old female WT (**a**) and SOST$^{-/-}$ (**b**) mice ranged from 18-23 g and increased gradually over the experimental period. (**c**, **d**) Whole body areal BMD of WT (**c**) and SOST$^{-/-}$ (**d**) mice measured by DXA at Baseline and at Day 40, which was just prior to the end of the study. Mean±SD from each group shown (n=6 mice/group except for SOST$^{-/-}$ vehicle n=7).

Supplementary Figure S6



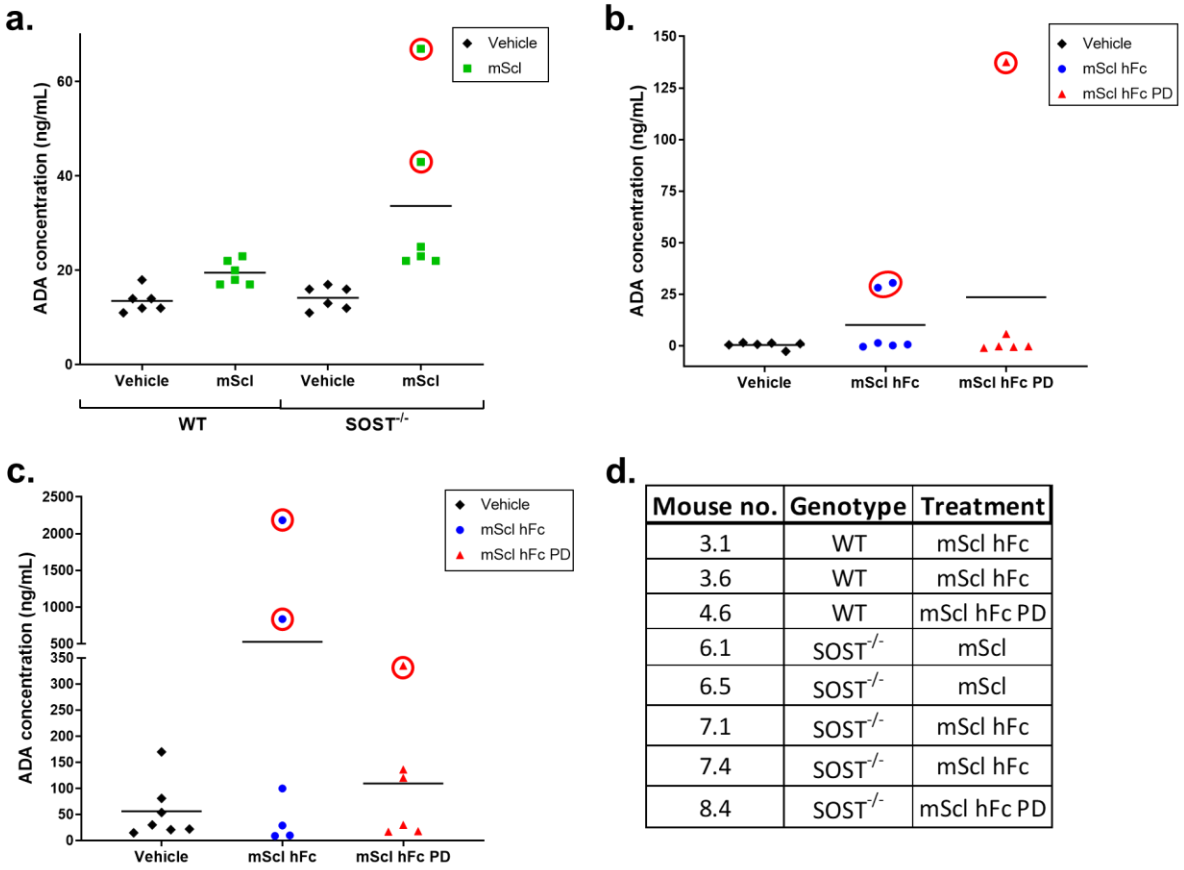
WT and SOST$^{-/-}$ P1NP levels in the six-week study. (**a**) P1NP concentrations of WT mice treated with mScl hFc and mScl hFc PD. (**b**) P1NP concentrations of SOST$^{-/-}$ mice treated with Vehicle, mScl, mScl hFc and mScl hFc PD. N=6 at each time point, except SOST$^{-/-}$ Vehicle N=7. Vehicle data was collected at Day 42 only. Unpaired t-test was performed for statistical analysis of mScl (Predose vs 24 hour), and mScl vs Vehicle (Day 42) comparison (*$p<0.05$).

Supplementary Figure S7



Femoral trabecular bone phenotype of WT and SOST$^{-/-}$ mice treated with Vehicle, mScl, mScl hFc and mScl hFc PD. Trabecular microarchitecture was assessed by µCT in WT and SOST$^{-/-}$ mice aged ~3 months. (a) Region of interest for femoral trabecular (yellow) and cortical (red) bone analysis. (**b, c, d**) vBMD (**b**), BV/TV (**c**), and Tb.Sp (**d**). (**e**) Representative µCT images of the right femurs of WT and SOST$^{-/-}$ mice. Blue indicates bone density and are for illustration purposes only. Bars represent mean±SD of n=6 mice/group (except WT mScl hFc PD group: n=5; SOST$^{-/-}$ vehicle group: n=7). Ordinary one-way ANOVA was performed for statistical analysis: *p<0.05.

Supplementary Figure S8

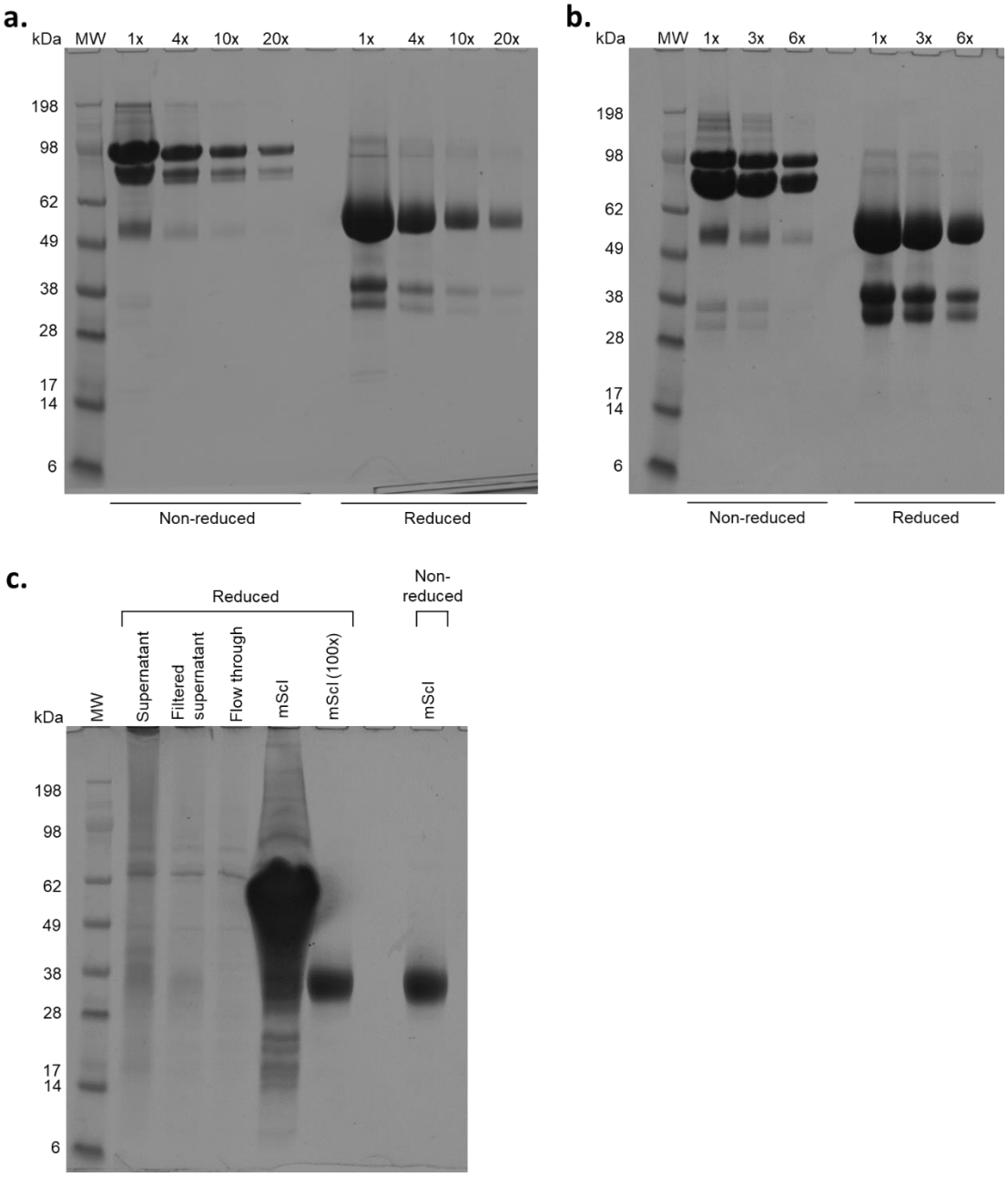


d.

Mouse no.	Genotype	Treatment
3.1	WT	mScl hFc
3.6	WT	mScl hFc
4.6	WT	mScl hFc PD
6.1	SOST$^{-/-}$	mScl
6.5	SOST$^{-/-}$	mScl
7.1	SOST$^{-/-}$	mScl hFc
7.4	SOST$^{-/-}$	mScl hFc
8.4	SOST$^{-/-}$	mScl hFc PD

Anti-drug antibodies against recombinant mScl in WT and SOST$^{-/-}$ mice. (**a**) Anti-drug antibody (ADA) assay (biotinylated and SULFO TAG labelled mScl added to samples) of WT and SOST$^{-/-}$ mice treated with wild type mScl. (**b, c**) ADA assay of WT (**b**) and SOST$^{-/-}$ (**c**) treated with mScl hFc or mScl hFc PD (biotinylated and SULFO TAG labelled mScl hFc PD added to samples). Potential ADA responses are indicated with a red circle around the data point(s). (**d**) Table indicating genotype and treatment of samples with potential ADA responses.

Supplementary Figure S9



Complete gels that were cropped for presentation in main manuscript (Fig. 1) and supplementary material (Supplementary Fig. S1). (**a**) Various concentrations of mScI hFc PD at non-reduced and reduced conditions (lanes cropped for Fig. 1: 4x reduced (lane 3); Supplementary Fig. S1: 4x reduced and non-reduced (lanes 3 and 8)). (**b**) Various concentrations of mScI hFc at non-reduced and reduced conditions (lanes cropped for Fig. 1: 6x reduced (lane 8); Supplementary Fig. S1: 6x reduced and non-reduced (lane 4 and 8). (**c**) Partial purification process of mScI (lanes cropped for Fig.1: MW (lane 1) and mScI (100x) reduced (lane 6)).

Supplementary Table 1

Pharmacokinetic summary of mScl WT, mScl hFc and mScl hFc PD.

Mouse strain	Treatment	Dose (mg/kg)	Route	Half-life	C_{max} (µg/mL)	AUC (hour*µg/mL)	Clearance (mL/day/kg)	V_{ss} (mL/kg)
WT	mScl	4.4	IV	≤5 mins	ND	ND	ND	ND
$SOST^{-/-}$	mScl	4.4	IV	≤5 mins	ND	ND	ND	ND
WT	mScl hFc	10	IV	1.5 days	12.0	273.8	529.8	1109.8
$SOST^{-/-}$	mScl hFc	10	IV	1.8 days	9.8	236.1	566.1	1390.7
WT	mScl hFc PD	10	IV	4 days	20.9	829.8	80.4	483.2
$SOST^{-/-}$	mScl hFc PD	10	IV	ND	21.9	577.1	ND	ND

Half-life was not well estimated for mScl hFc PD in $SOST^{-/-}$ mice. IV: intravenous; C_{max}: maximum concentration; AUC: area under curve; V_{ss}: steady-state volume of distribution; ND: not determined.

Supplementary Table 2

Trabecular bone histomorphometry of bone close to the femoral distal growth plate.

	WT								$SOST^{-/-}$							
	Vehicle		mScl		mScl hFc		mScl hFc PD		Vehicle		mScl		mScl hFc		mScl hFc PD	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
vBMD (g/cm^3)	0.15	0.01	0.14	0.04	0.14	0.03	0.15	0.01	0.35	0.04	0.32	0.05	0.35	0.06	0.27	0.10
BV/TV (%)	8.66	0.71	8.41	2.81	7.66	2.52	8.78	0.63	23.96	3.51	22.20	3.66	23.67	4.80	17.61	7.79
Tb.Th (mm)	0.05	0.00	0.06	0.01	0.06	0.00	0.05	0.00	0.09	0.00	0.09	0.00	0.09	0.01	0.09	0.01
Tb.Sp (mm)	0.28	0.01	0.31	0.06	0.37	0.12	0.29	0.03	0.21	0.01	0.23	0.02	0.22	0.03	0.28	0.09*
Tb.N (mm^{-1})	1.62	0.11	1.50	0.46	1.31	0.44	1.66	0.14	2.62	0.34	2.44	0.30	2.63	0.50	1.95	0.78
Tb.Pf (mm^{-1})	29.14	2.63	29.07	5.74	26.05	3.49	25.88	2.30	13.58	2.48	13.47	2.16	13.30	2.65	16.76	5.22
SMI	2.25	0.13	2.30	0.23	2.21	0.19	2.07	0.12	1.85	0.27	1.82	0.17	1.82	0.37	2.09	0.42
DA	0.38	0.03	0.34	0.03	0.36	0.06	0.39	0.03	0.49	0.03	0.47	0.02	0.45	0.04	0.46	0.04

Data are expressed as the mean±SD of n=6 mice/group (except WT mScl hFc PD group: n=5; $SOST^{-/-}$ vehicle group: n=7). Ordinary one-way ANOVA was performed for statistical analysis: *$p<0.05$ compared with $SOST^{-/-}$ vehicle group. The distal metaphyseal region of interest of the right femur was analysed. vBMD: volumetric bone mineral density; BV/TV: bone volume fraction; Tb.Th: trabecular thickness; Tb.Sp: trabecular space; Tb.N: trabecular number; Tb.Pf: trabecular pattern factors; SMI: structure model indexes; DA: degree of anisotropy.

Supplementary Table S3

Cortical bone histomorphometry near the femoral midshaft.

	WT								SOST$^{-/-}$							
	Vehicle		mScl		mScl hFc		mScl hFc PD		Vehicle		mScl		mScl hFc		mScl hFc PD	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
vBMD (g/cm^3)	1.06	0.07	1.01	0.04	1.02	0.05	1.01	0.05	1.41	0.06	1.38	0.07	1.43	0.12	1.37	0.12
BV/TV (%)	44.73	2.41	42.80	1.42	43.23	1.84	42.88	1.66	56.95	2.26	55.90	2.64	57.72	4.22	55.51	4.22
T.Ar (mm^2)	1.84	0.09	1.89	0.12	1.73	0.11	1.91	0.07	2.59	0.18	2.57	0.15	2.48	0.16	2.56	0.11
B.Ar (mm^2)	0.82	0.05	0.81	0.06	0.75	0.07	0.82	0.05	1.47	0.12	1.44	0.14	1.44	0.19	1.42	0.14
Ma.Ar (mm^2)	1.02	0.08	1.08	0.06	0.98	0.05	1.09	0.05	1.11	0.10	1.13	0.05	1.05	0.06	1.14	0.10
T.Pm (mm)	5.29	0.15	5.34	0.20	5.11	0.15	5.40	0.13	6.34	0.28	6.29	0.19	6.20	0.28	6.25	0.15
B.Pm (mm)	9.39	0.32	9.49	0.37	9.09	0.22	9.63	0.26	11.54	0.61	11.51	0.78	11.30	0.58	11.25	0.56
Es.Pm (mm)	4.10	0.19	4.16	0.17	3.98	0.09	4.22	0.14	5.21	0.36	5.22	0.61	5.11	0.34	5.00	0.45

Data are expressed as the mean±SD of n=6 mice/group (except WT mScl hFc PD group: n=5; SOST$^{-/-}$ vehicle group: n=7). The diaphyseal region of interest of the right femur was analysed. vBMD: volumetric bone mineral density; BV/TV: bone volume fraction; T.Ar: tissue area; B.Ar: bone area; Ma.Ar: medullary area; T.Pm: tissue perimeter; B.Pm: bone perimeter; Es.Pm: eccentric perimeter.