Supplemental materials

Continuous PTH in Male Mice Causes Bone Loss Because It Induces Serum Amyloid A (SAA)

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Supplemental Figure legends (Fig. 1 to Fig. 4); Supplemental Table 1 and 2; and Supplemental Figure 1 to Figure 4.

Legends for Supplemental figures

Supplemental Figure 1. Generation of SAA3 KO mice. (A) SAA3 mice were generated by CRISPR/Cas9. The *Saa3* single-guided RNA (*Saa3*gRNA), Cas9 mRNA and the targeting single-stranded oligo-directed nucleotides (ssODN) bearing two in-frame tandem termination codons (TAA and TGA) and a Sal1 restriction enzyme digestion site were microinjected into the pro-nucleus of CD-1 one-cell embryos. Through specific CRISPR/Cas9 editing and homology-directed repair, the termination codons and the Sal1 point mutation were knockin into the first coding exon of *Saa3* gene, which resulted into a founder mouse with one WT allele and one knockin allele. (B and C) The heterozygous (HET) founder mouse was first identified by PCR

followed by Sal1 digestion and then confirmed by sequencing of PCR product. (D) The HET founder mouse was bred with WT CD-1 mice to generate more HET mice, which were genotyped by PCR, using allele specific primers as listed in the Methods section. For experiments, WT and SAA3 KO mice were generated by HET x HET mating.

Supplemental Figure 2. RANKL increased SAA3 protein expression and its secretion from WT BMMs. Continuous PTH increased gene expression for *Tnfsf11* (RANKL) and *Tnfrsf11b* (OPG) similarly in WT and SAA3 KO BMSC cultures. Continuous PTH increased osteoclast-like cells similarly in WT and SAA3 KO bone marrow cultures. (A) Immunofluorescence for SAA3 protein in BMMs and ELISA of SAA3 protein in the medium of BMMs. WT and SAA3 KO BMMs were treated with M-CSF+RANKL (30 ng/ml each) for 1 day. BMMs were labeled for SAA3 (red) and DAPI (blue) proteins. Representative 200x magnification (scale bars: 50 μm) merged microscopic images. Und= Undetectable. (B) Osteoclast-like cells in PTH-stimulated WT and SAA3 KO BMSCs cultured with vehicle (VEH) or PTH (10 nM) for 7 days. Representative image of TRAP+ cells, marked by blue arrow, showing partial coverage by canopy of cells, at 40x magnification (scale bars: 500 μm). Expression of genes for *Tnfsf11* and *Tnfrsf11b* measured on day 7 by qPCR and reported as relative quantification (RQ) values. (C) Bone marrow cultures were treated with PTH (10 nM). Representative TRAP stained 100x magnification (scale bars: 200 μm) microscopic images. Data are means ± SEM for n=3 independent samples. $^{**}P < 0.01$, determined by 2-way ANOVA, post-hoc Bonferroni pairwise multiple comparisons.

Supplemental Figure 3. Continuous PTH stimulated prostaglandinE_2 (PGE_2) in hBMSCs. Human BMSCs were cultured in osteoblast differentiation medium in the presence of vehicle (VEH) or PTH (10 nM) ± NS398 (100 nM), a selective inhibitor of COX2 activity. At each medium change, the medium was collected and assessed for PGE_2 by ELISA. Nd=Not detectable. In this dataset, the data are from the same experiment as Figure 6A but expanded to show that NS398 blocked all PGE_2 production. Bars are means ± SEM for n=3 independent samples. $^{**}P < 0.01$, determined by 1-way ANOVA, post-hoc Bonferroni pairwise multiple comparisons.

Supplemental Figure 4. Addition of conditioned medium (CM) from RANKL-treated hBMMs, but not from RANKL + NS398 treated hBMMs, inhibited PTH-stimulated osteoblast differentiation in hBMSC cultures. *BGLAP* mRNA expression in hBMSC cultures at day 21. hBMSCs were cultured in the presence of OPG (100 ng/ml), vehicle (VEH) or PTH (10 nM) ± CM from hBMMs. hBMMs were treated with M-CSF+RANKL (30 ng/ml each) in the absence or presence of NS398 (100 nM) for 8 days. CM from days 4-6 of hBMM cultures were pooled and added to hBMSCs as 3 parts CM and 1 part osteoblast differentiation medium. Bars are means ± SEM for n=3 independent samples. $^{**}P < 0.01$, determined by 1-way ANOVA, post-hoc Bonferroni pairwise multiple comparisons.

Supplemental Table 1. Primers used for quantitative real-time PCR (qPCR)

Gene Name	Taqman® probe number	Gene Name	Taqman® probe number
Bmp2	Mm01340178_m1	*BMP2*	Hs00154192_m1
Dkk1	Mm00438422_m1	*DKK1*	Hs00183740_m1
Gapdh	Mm99999915_g1	*GAPDH*	Hs02758991_g1
Igf1	Mm00439560_m1	*IGF1*	Hs01547656_m1
Runx2	Mm00501584_m1	*RUNX2*	Hs01047973_m1
Saa1	Mm00656927_g1	*SAA1*	Hs00761940_s1
Saa2/*Saa1*[*]	Mm04208126_mH	*SAA2*	Hs01667582_m1
Tnfsf11 (RANKL)	Mm00441908_m1	*TNFSF11* (RANKL)	Hs00243522_m1
Wnt10b	Mm00442104_m1	*WNT10B*	Hs00559664_m1
Ctnnb1 (catenin, beta 1)	Mm00483039_m1	*ALPL* (alkaline phosphatase)	Hs01029144_m1
Ptgs2 (COX2)	Mm00478374_m1	*BGLAP* (osteocalcin)	Hs01587814_g1
Pthlh (Pthrp)	Mm00436057_m1		
Ramp3	Mm00840142_m1		
Saa3	Mm00441203_m1		
Sost	Mm00470479_m1		
Tnfrsf11b (OPG)	Mm01205928_m1		
Wnt4	Mm01194003_m1		
Wnt7b	Mm01301717_m1		

Gene Name	SYBR Green validated primer sequence
Bglap (osteocalcin)	Reverse TGG TCT GAT AGC TCG TCA CAA G Forward CTG ACC TCA CAG ATC CCA AGC
Actb (actin, beta)	Reverse CCA GTT GGT AAC AAT GCC ATG T Forward GGC TGT ATT CCC CTC CAT CG

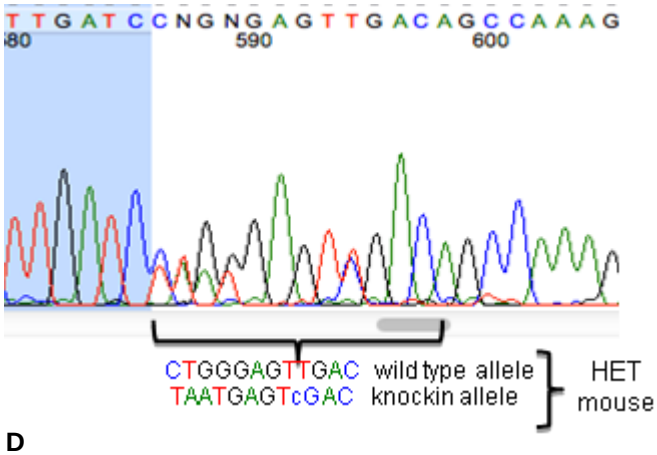
[*]The gene assay ID Mm04208126_mH for *Saa2* detected both *Saa2* (RefSeq NM_011314.2) and *Saa1* (RefSeq NM_009117.3) transcripts.

Supplemental Table 2. **Mendelian ratios of WT and SAA3 KO mice**

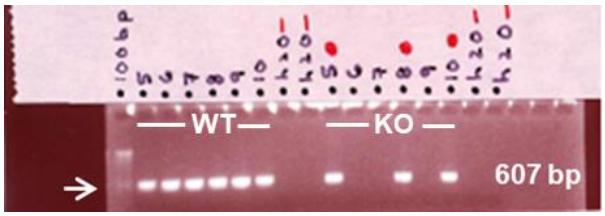
	Males (368**)**		**Females (**360**)**	
Genotype	**Number**	**Ratio**	**Number**	**Ratio**
Wild type (WT)	88	0.96	90	1.00
SAA3 knockout (KO)	90	0.98	92	1.02
SAA3 heterozygous (HET)	190	2.06	178	1.98

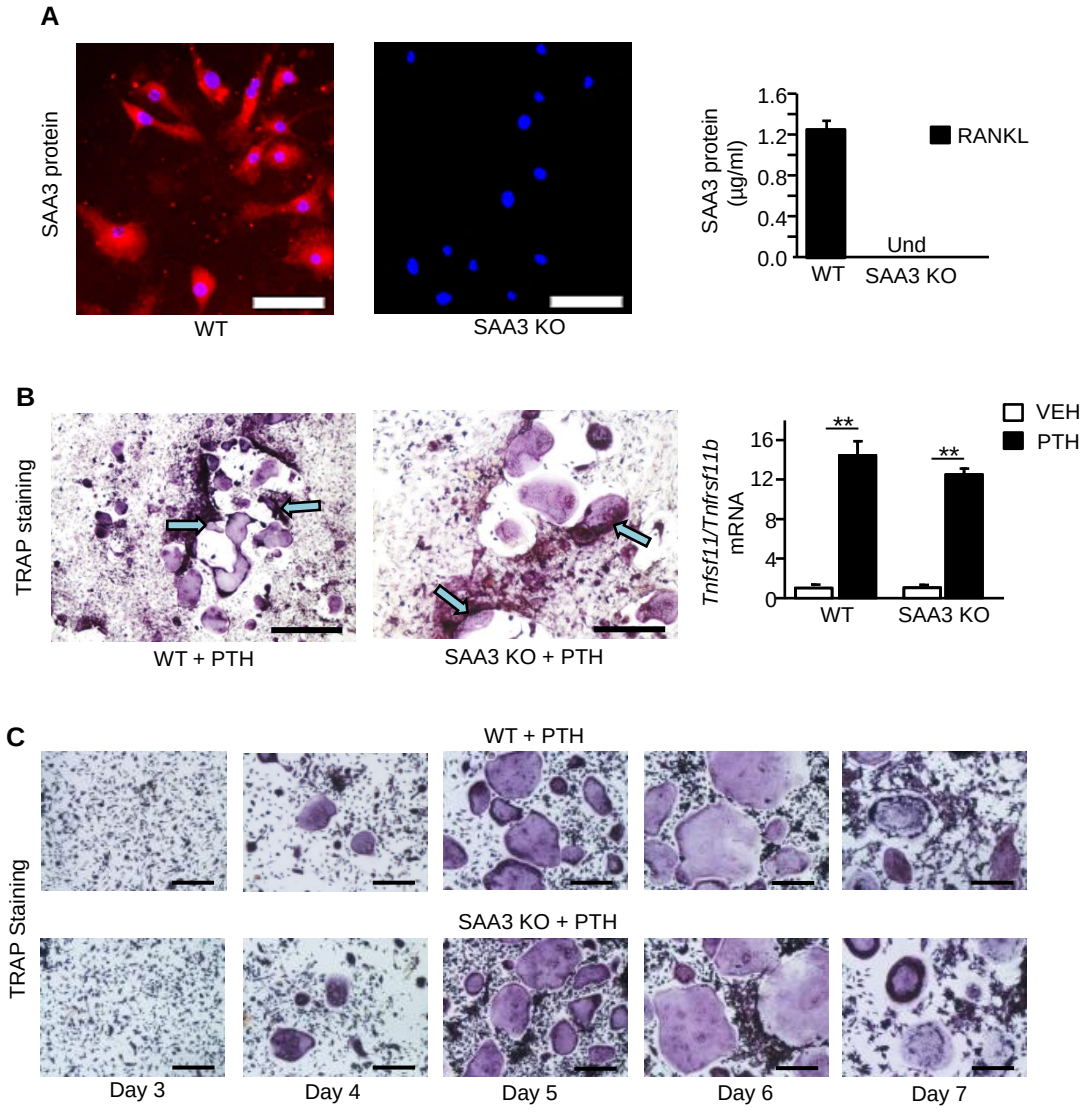
All experimental SAA3 KO and WT mice were obtained by HET x HET mating. All three genotypes were obtained in the expected Mendelian ratios.

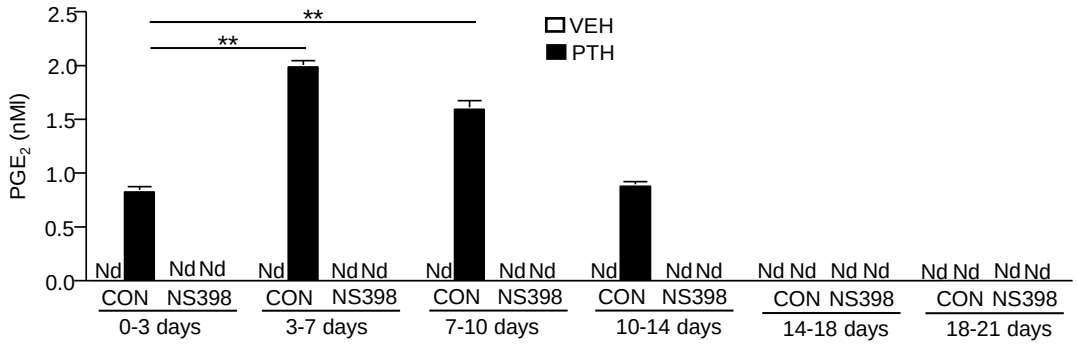

A
Wild type allele
Exon 2
TCCTCCACCCCACGAAATGGCATGCTCATCTTTGTTTTCCTGGCCCTATAGCAGGATGAAGCCTTCCAT
M K P S I
TGCCATCATTCTTTGCATCTTGATCCTGGGAGTTGACAGCCAAAGATGGGTCCAGTTCATGAAAGAAGC
A I I L C I L I L G V D S Q R W V Q F M K E A
Homology-directed repair
Saa3gRNA + Cas9mRNA + targeting ssODN
Pronuclear injection
Knockin allele
Exon 2
TCCTCCACCCCACGAAATGGCATGCTCATCTTTGTTTTCCTGGCCCTATAGCAGGATGAAGCCTTCCAT
M K P S I
SalI
TGCCATCATTCTTTGCATCTTGATCTAATGAGTcGACAGCCAAAGATGGGTCCAGTTCATGAAAGAAGC
A I I L C I L • • V D S Q R W V Q F M K E A


C
T T G A T C C N G N G A G T T G A C A G C C A A A G
580
590
600
CTGGGAGTTGAC wild type allele
TAATGAGTcGAC knockin allele
HET mouse


B
PCR followed by Sal1 digestion


D
WT
KO
607 bp


A
SAA3 protein
WT
SAA3 KO
SAA3 protein (µg/ml)
1.6
1.2
0.8
0.4
0.0
RANKL
Und
WT
SAA3 KO
B
TRAP staining
WT + PTH
SAA3 KO + PTH
Tnfsf11/Tnfrsf11b mRNA
16
12
8
4
0
**
**
VEH
PTH
WT
SAA3 KO
C
TRAP Staining
WT + PTH
SAA3 KO + PTH
Day 3
Day 4
Day 5
Day 6
Day 7


2.5
2.0
1.5
1.0
0.5
0.0
PGE2 (nMl)
**
**
VEH
PTH
Nd
Nd Nd
Nd
Nd Nd
Nd
Nd Nd
Nd
Nd Nd
Nd Nd
Nd Nd
Nd Nd
Nd Nd
CON
NS398
CON
NS398
CON
NS398
CON
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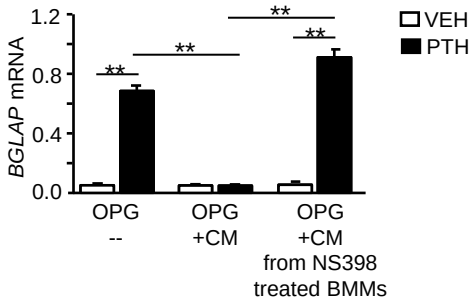
CON
NS398
CON
NS398
0-3 days
3-7 days
7-10 days
10-14 days
14-18 days
18-21 days


1.2
0.8
0.4
0.0
BGLAP mRNA
**
**
**
VEH
PTH
OPG
--
OPG
+CM
OPG
+CM
from NS398
treated BMMs