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Lipid Nanoparticle Mediated Silencing of Osteogenic Suppressor *GNAS* Leads to Osteogenic Differentiation of Mesenchymal Stem Cells *In Vivo*

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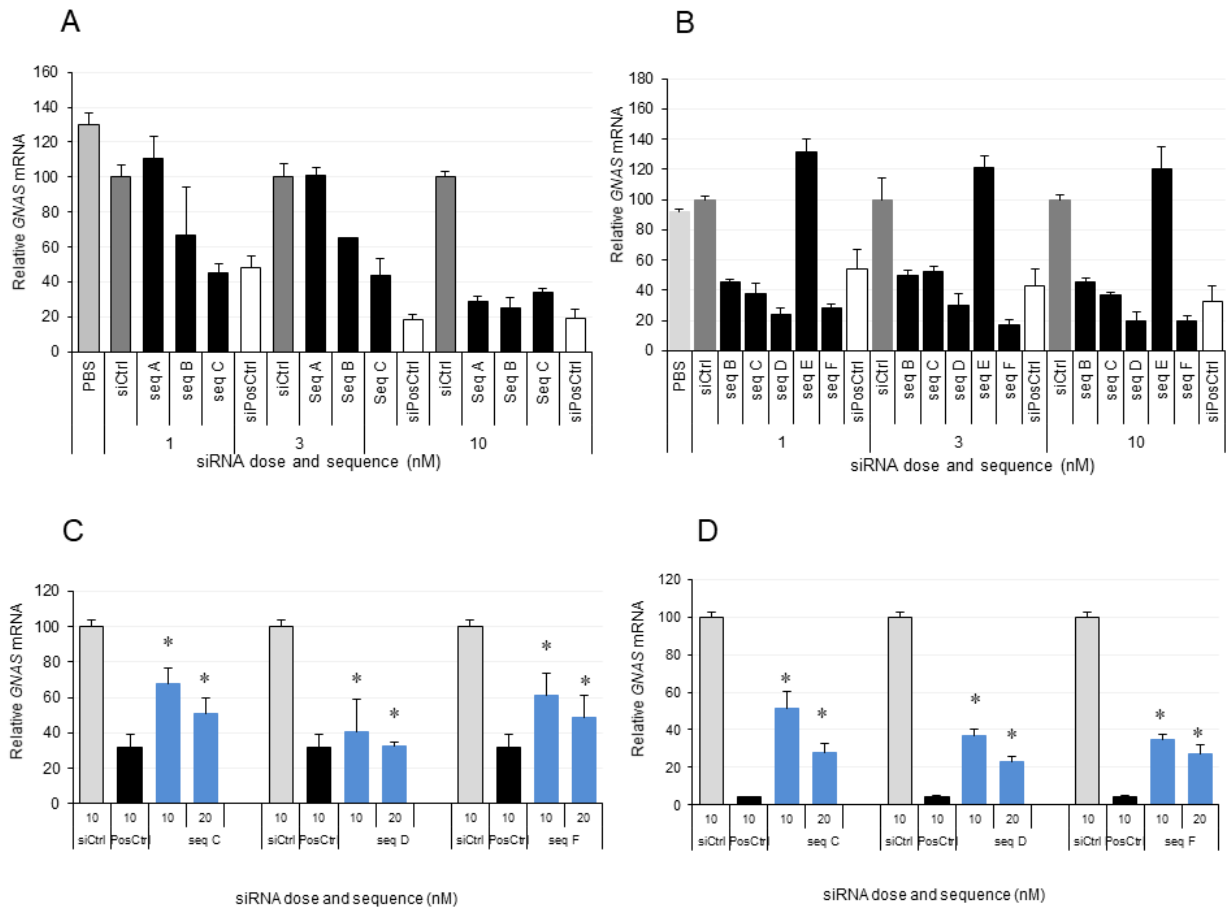


Figure S1. Identification of the siRNA sequence targeting *GNAS*. MSC-like cells were seeded in 12-well plates and incubated with DMEM followed by Osteogenic Media for 48 hours. Cells were then transfected with 6 predesigned duplexes (A, B, C D, E and F) in triplicate wells at indicated doses including a negative control siRNA (siCtrl) and a positive control (posCtrl). SiRNA targeting *GNAS* was introduced using RNAiMAX (**A**) Bar graphs depict relative *GNAS* mRNA transcript levels 48 hours post transfection (sequence A to C) and (**B**) sequence B to F, relative to *TBP* or *HPRT* housekeeping gene determined using qRT-PCR. (**C**) Bar graphs depict relative *GNAS* mRNA transcript levels 48 hours post transfection with selected siRNA sequences (seq C, D and F) using qPCR or (**D**) droplet digital PCR. Each data point represents arithmetic mean ± SEM of three technical replicates. Data are shown as mean ± SD of triplicate wells and are representative of at least 3 experiments. Data from one representative experiment of three independent experiments is shown. Bar graphs represent expression of *GNAS* mRNA transripts relative to the *HPRT* or *TBP* (not shown). *GNAS* mRNA values following siCtrl treatment are considered 100% (*: P < 00.5).

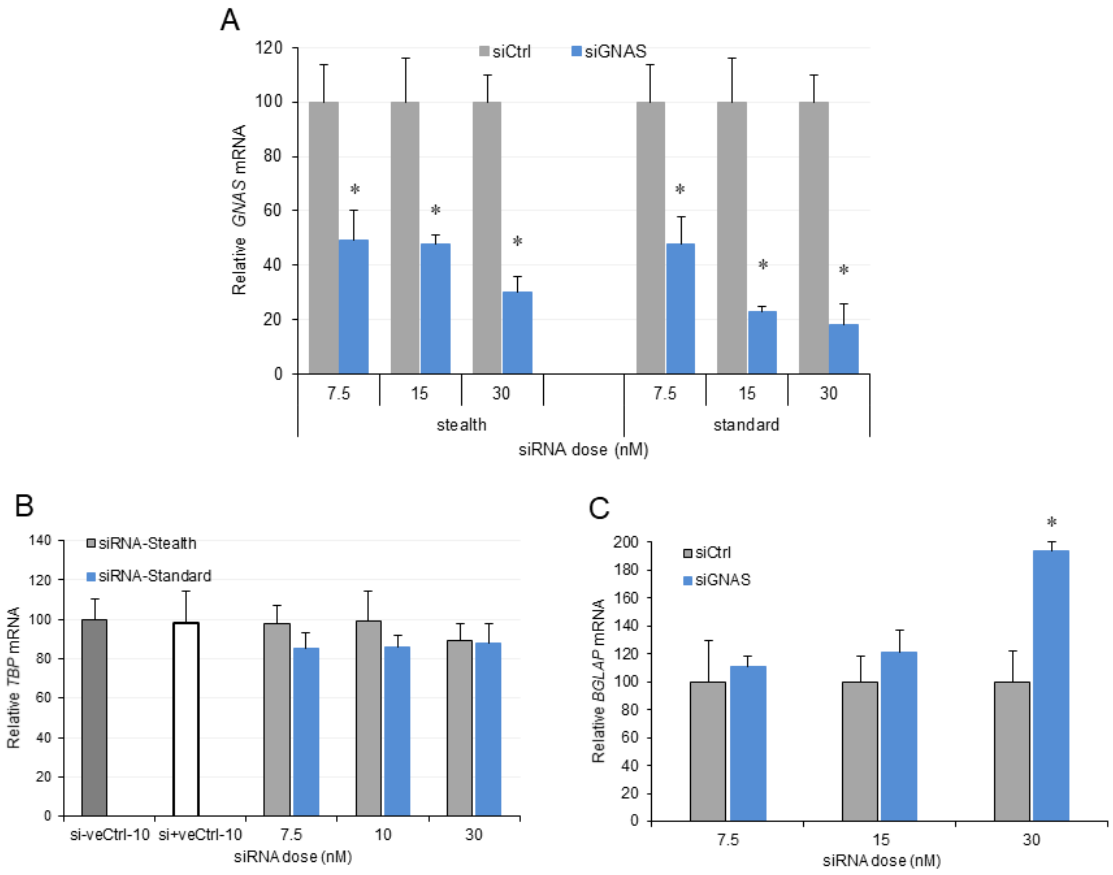


Figure S2. The 2'OMe modifications do not affect the siRNA-mediated *GNAS* knockdown. MSC-like cells were seeded in 12-well plates and incubated with DMEM followed by Osteogenic Media for 48 hours. Cells were then transfected in triplicate with unmodified (standard) or modified (stealth) siRNA at 10nM final concentration using RNAiMAX. Forty eight hours post transfection, mRNA of *GNAS*, *TBP* and *HPRT* housekeeping gene were determined using qRT-PCR. (**A**) Bar graphs represent expression of *GNAS* and (**B**) *TBP* mRNA transripts relative to *HPRT* at indicated doses. (**C**) Bar graphs depict the expression of *BGLAP* osteogenic marker following treatment with si*GNAS* at indicated doses, relative to si*Ctrl* considered 100%. Data points represent arithmetic mean ± SD of three technical replicates. Data from one representative experiment of three independent experiments is shown (*: P <0.05).

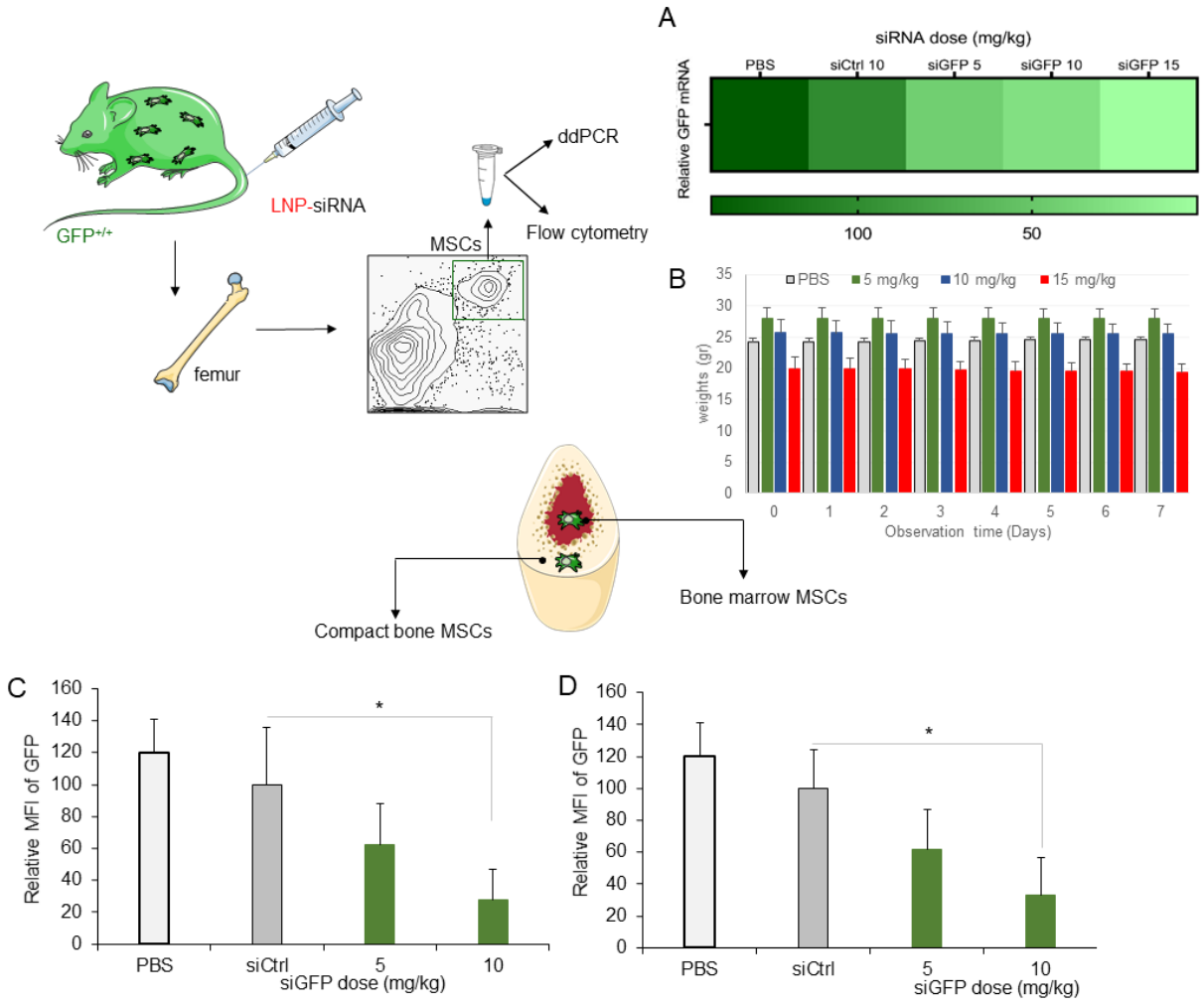


Figure S3. Silencing of GFP *in vivo* by LNP-siRNA. LNP-siRNA targeting *GFP*, LNP-si*Ctrl* (comprising PEG-DSG) or PBS, were administered i.v. into *GFP* expressing transgenic mice. Bones of 3 mice per group were harvested, pooled and approximately 5,000 MSCs isolates identified as PDGFRα^{+}/Sca-1^{+} cells were sorted. ddPCR was used to determine residual *GFP* mRNA transcripts following RNA extraction and cDNA transcription. Residual *GFP* protein in MSC isolates was also determined by FACS (**A**) Heat map depicts expression of *GFP* mRNA transcripts at indicated doses relative to si*Ctrl* treatments. Each square represents the signal obtained from bone MSCs of 3 pooled mice. Data from one representative experiment of three independent experiments is shown (**B**) Bar graphs represent the arithmetic mean ± SD weight (gr) of 3 animals administered with indicated doses of LNP-siRNA. (**C**) Bar graphs represent the arithmetic mean ± SD of relative MFI of PDGFRα^{+}/Sca-1^{+} MSCs derived from *compact* bone or (**D**) bone *marrow* of three mice, following treatment with indicated doses of LNP-si*GFP*,

normalized to si*Ctrl* which was considered to be 100%. *P < 0.05. Data from one representative experiment of three independent experiments is shown.

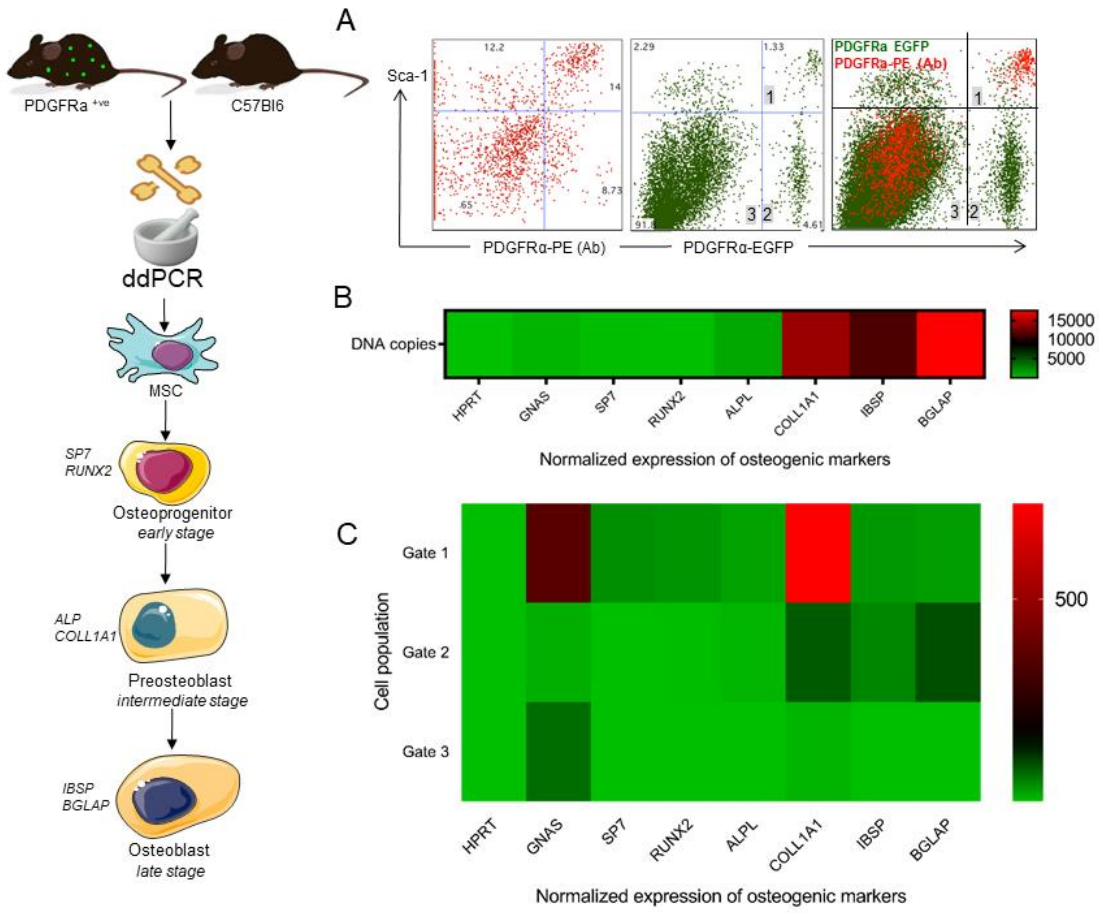


Figure S4. Molecular analysis using droplet digital PCR (ddPCR) reveals the osteogenic potential of MSCs in C57Bl6 and PDGFRα-GFP expressing transgenic mice. mRNA expression of MSCs was analyzed by ddPCR using specific primers (supplementary Table S2). (**A**) Dot plots show the MSCs identified as PDGFRα^{+}/Sca-1^{+} cells (left) as determined by antibody staining. Dot plot (middle) represents the subpopulations of cells released from the bones of PDGFRα-GFP expressing transgenic mice revealing a PDGFRα^{+}/Sca-1^{+} double positive (1), PDGFRα^{+}/Sca-1^{-} single positive (2) and PDGFRα^{-}/Sca-1^{-} double negative population (3). The dot plot (right) demonstrates the overlap of PDGFRα^{+}/Sca-1^{+} cells in PDGFRα-GFP expressing transgenic mice (green) and the same cellular phenotype (red) following staining with appropriate antibodies. The image is obtained by overlaying the respective dot plots. (**B**) The heat map exhibits transcript levels of bone osteogenic markers in

sorted PDGFRα^{+}/Sca-1^{+} MSCs of C57Bl6 mice identified by antibody staining, assessed by the color intensity. (**C**) The heat map exhibits the expression of osteogenic markers in double positive (PDGFRα^{+}/Sca-1^{+} Gate 1), single positive (PDGFRα^{+}/Sca-1^{-} Gate 2) and double negative (PDGFRα^{-}/Sca-1^{-} Gate 3) cell populations sorted from PDGFRα-GFP expressing transgenic mice, as determined by ddPCR. Each square represents the color signal obtained by pooling of bones of 4 mice.

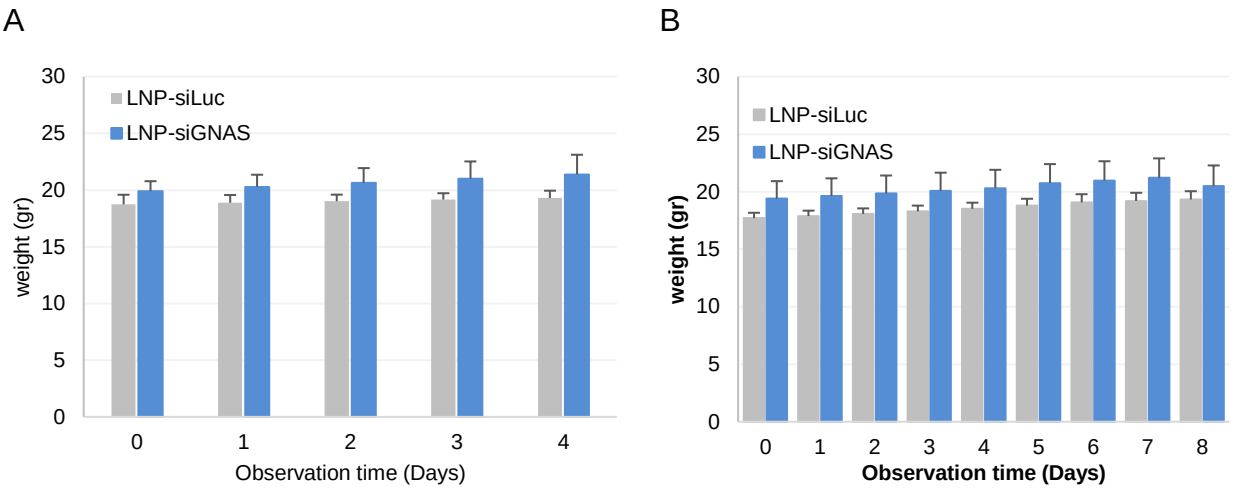
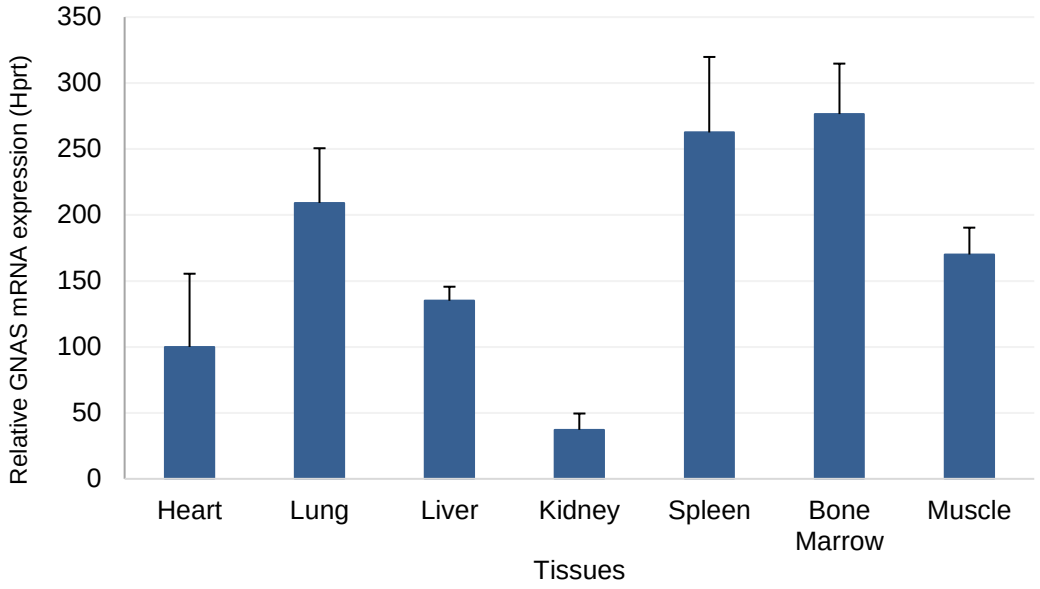


Figure S5. Animals treated with LNP-siRNAs did not show weight loss. Mice were weighted prior and post systemic administration of PBS and 10mg/kg of si*Luc* (si*Ctrl*) or si*GNAS* encapsulated in LNPs. (**A**) Each data point represents arithmetic mean ± SD of animal weight (gr) of the group observed for 4 days **(B)** and the group observed for 8 days. Data from one representative experiment of two independent experiments is shown.

siLuc: siRNA targeting Luciferase. si*GNAS*: siRNA targeting *GNAS* gene.
PBS: Phosphate Buffered Saline

A



B

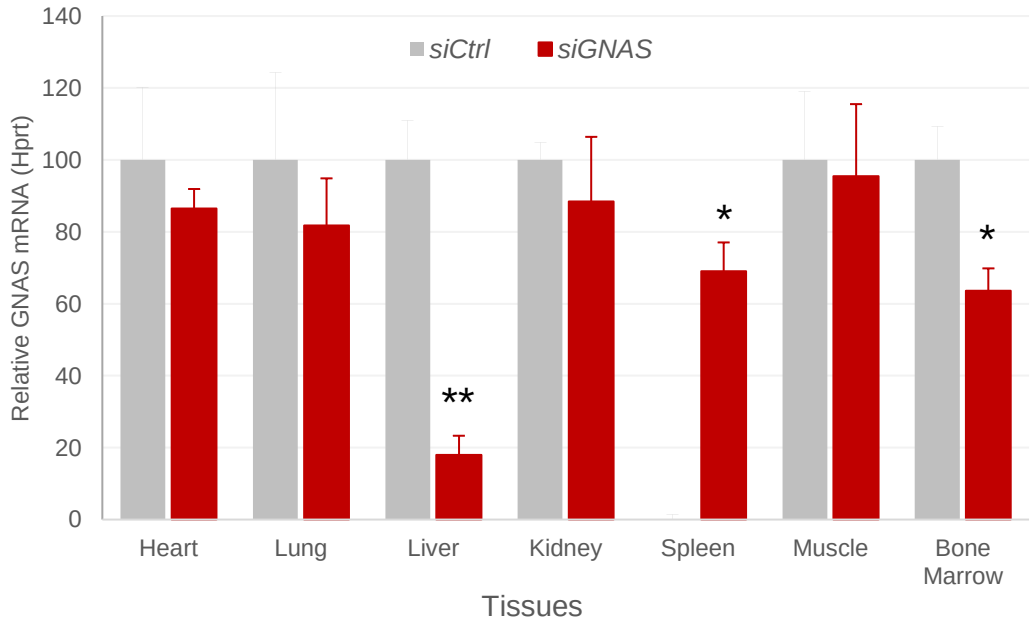


Figure S6. Systemic administration of LNP-si*GNAS* alters *GNAS* expression in tissues. *GNAS* expression was tested in various tissues of a 3 C57Bl6 mice. Next, 6 mice were divided in 2 groups of 3 and received intravenously LNP-si*Ctrl* (n=3) or LNP-si*GNAS* (n=3). After 6 days, organs as indicated were harvested and subjected to RNA extraction. Residual *GNAS* mRNA was determined using RT-qPCR and normalized to the respective LNP-si*Ctrl* which was considered 100%. Bar graphs represent the arithmetic mean ± SD (n=3) of relative *GNAS* mRNA derived from indicated tissues. Data from one experiment is shown (* $p < 0.05$, * $p < 0.01$).

siRNA sequence	Sense	Antisense
A	5'- GCAUGUUAAUGGGUUUAACGGAGAG -3'	3'- GACGUACAAUUACCCAAAUUGCCUCUC -5'
B	5'-CGCUGGCAAAUCGAAGAUUGAGGAC -3'	3'-GAGCGACCGUUUAGCUUCUAACUCCUG -5'
C	5' - GGGAGGACAACCAGACUAACCGCCT-3'	3' - AGCCCUCCUGUUGGUCUGAUUGGCGGA-5'
D	5'-GCACCAUUGUGAAGCAGAUGAGGAT -3'	3'- UUCGUGGUAACACUUCGUCUACUCCUA-5'
E	5'-GGAACACCCAAAUUUAAUUCAGCCT -3'	3'- UCCCUUGUGGGUUUAAAUUAAGUCGGA -5'
F	5'-AGAGUGAAACGUAAUUGUACAAGCA -3'	3'-AUUCUCACUUUGCAUUAACAUGUUCGU -5'
TYE 563 DS Transfection control	/5TYE563/T*CrC rUrUrC rCrUrC rUrCrU rUrUrC rUrCrU rCrCrC rUrUrG rUG*A	/5TYE563/T*CrA rCrArA rGrGrG rArGrA rGrArA rArGrA rGrArG rGrArA rGG'A
HPRT-S1 positive control	/5Phos/rGrCrC rArGrA rCrUrU rUrGrU rUrGrG rArUrU rUrGrA rArAT T	rArArUrUrUrCrArArArUrCrCrArArCrArArArGrUrCrUrGrGrCrUrU
NC1 Negative Control	rCrGrU rUrArA rUrCrG rCrGrU rArUrA rArUrA rCrGrC rGrUA T	rArUrA rCrGrC rGrUrA rUrUrA rUrArC rGrCrG rArUrU rArArC rGrArC

The asterisk symbol (*) in the duplex indicates a phosphorothioate bond to help protect against exonuclease degradation

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Table S1. siRNA sequences screened in the study.

Gene	Forward Primer	Reverse Primer
ALPL	5'-GCCTTCTCATCCAGTTCGTAT-3'	5'-CAAGGACATCGCATATCAGCTA-3'
BGLAP	5'-AGCAGAGTGAGCAGAAAGATG-3'	5'-GAACAGACAAGTCCCACACAG-3'
IBSP	5'-GAGAGTGTGGAAAGTGTGGAG-3'	5'-AGAAAATGGAGACGGCGATAG-3'
HPRT	5`-AACAAAGTCTGGCCTGTATCC-3'	5t-CCCCAAAATGGTTAAGGTTGC-3'
TBP	5'CCAGAACTGAAAATCAACG-3'	5'4GTATCTACCGTGAATCTIGGC-3'
SP7	St-CTTCTTTGTGCCTCCTTTCC-3'	5'-GCGTCCTCTCTGCTTGA-3'
COLL1A1	5'-CATTGTGTATGCAGCTGACTTC-3'	5'-CGCAAAGAGTCTACATGTCTAGG-3'
RUNX2	5'-TCCCCGGGAACCAAGAATCCCCGGGAACCAAGAA-3'	5'-GCGATCAGAGAACAAACTAGGTTTAGA-3'
GNAS	5'-GTAGGACATAGCGAAGATGGAG-3'	5'-CCATTGTGAAGCAGATGAGGA-3'

Gene	Accession number	Probe
ALPL	*NC_000070.7*	5' -/ 56-F AM/CGCCACCCA/ZEN/TGA TCACGTCGAT/31ABkFQ/-3'
BGLAP	*NC_000069.7*	5'-/56-FAM/CCCAGACCT/ZEN/AGCAGACACCATGAG/31ABkFQ/-3'
IBSP	*NC_000071.7*	5'-/56-FAM/ ATGAAGACC/ZEN/ AGGAGGCGGAGG/31ABkFQ/-3'
HPRT	*NC_000086.8*	5'-/55-FAM/CTTGCTGGT/ZEN/GAAAAGGACCICTCGAA/31ABkFQ/-3'
TBP	*NC_000083.7*	5'-/56-FAMJACTTGACCT/ZEN/AAAGACCATTGCACITCGT/31ABkFQ/-3'
SP7	*NC_000081.7*	5'-/56-FANVAAGCTCACT/ZEN/ATGGCTCCAGICCC/31ABkFCV-3'
Col1A1	*NC_000077.7*	5'456-FAM/CCGGAGGTC/ZEN/CACAAAGCTGAACA/31ABkF0/-3'
RUNX2	*NC_000083.7*	6FAM/CACAGACAGAAGCTTGATGA/MGBNFQ
GNAS	*NC_000068.8*	5'-/56-FAM/CAAGGAGCA/ZEN/ACAGCGATGGAGTCTAT/3IABkFQ/-3

Table S2. Primer sequences of the genes determined in the study.

Test performed	PBS	LNP-si*Ctrl*
ALP (U/L)	107 ± 10.2	119 ± 8.9
AST (U/L)	837 ± 30.8	715 ± 25.9
ALT (U/L)	63 ± 15	83 ± 17.5
BUN (mg/dL)	36 ± 7.01	30 ± 8.3
Creatinine (mg/dL)	1.5 ± 0.22	1.1 ± 0.29
Calcium (mg/dL)	9.0 ± 0.8	9.4 ± 0.69
Chloride (mmol/L)	107 ± 5.9	108 ± 10.1
Potassium (mmol/L)	8.91 ± 0.9	7.61 ± 0.49
Sodium (mmol/L)	149 ± 8.9	149 ± 9.8
Lipemia Index	Normal	Normal

Table S3. Clinical chemistry of representative mice treated with PBS and LNP-si*Ctrl*