Supplementary Material

Materials and Methods

***Luciferase expression in vivo*.** Mice were injected intradermally with 10 µg of RNA in 100 µl of Ringer's lactate solution. One day post injection, mice were sacrificed, skin from the injection site isolated, and frozen in liquid nitrogen. After addition of 800 µl of lysis buffer (25 mM Tris pH 7.5, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM DTT, 1 mM PMSF), tissue was homogenized in a ball mill. Luciferase activity in the supernatant after centrifugation was measured in a LUMAT LB 9507 luminometer at 15 s measuring time using 50 µl of lysate.

***Measurement of cytokine secretion upon administration of LNP-encapsulated engineered mRNA*.** Macaques received a single injection of LNP-encapsulated M. fascicularis Epo mRNA. Before and 6 hours after treatment, blood was drawn and samples were analyzed for cytokines using the MILLIPLEX Map Non-human primate cytokine magnetic bead panel kit (Merck Millipore, Schwalbach, Germany) at Huntingdon.

Sequence information

Optimized mRNA sequences harboring a HSD17B4 5'-UTR, a GC-enriched ORF, an ALB 3'-UTR, and a polyA plus histone stem loop are given as encoded by the respective production vectors. For luciferase (PpLuc), the full sequence is depicted. For mouse (MmEpo), pig (SsEpo), and macaque (MfEpo) erythropoietin, only sequences of the ORF cassettes (including the flanking cloning sites which are highlighted) are shown.

PpLuc

GGGTCCCGCAGTCGGCGTCCAGCGGCTCTGCTTGTTCGTGTGTGTGTCGTTGCAG
GCCTTATTCAAGCTTGAGGATGGAGGACGCCAAGAACATCAAGAAGGGCCCGGC
GCCCTTCTACCCGCTGGAGGACGGGACCGCCGGCGAGCAGCTCCACAAGGCCAT
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GTCGACATCACCTACGCGGAGTACTTCGAGATGAGCGTGCGCCTGGCCGAGGCCA
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GCCTGCAGTTCTTCATGCCGGTGCTGGGCGCCCTCTTCATCGGCGTGGCCGTCGCC
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MmEpo

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SsEpo

…AAGCTTACCATGGGCGCCCGCGAGTGCCCCGCCCGGCTGCTCCTGCTGAGCCTC
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GT…

MfEpo

…AAGCTTACCATGGGCGTGCACGAGTGCCCGGCCTGGCTCTGGCTGCTGCTCAGC
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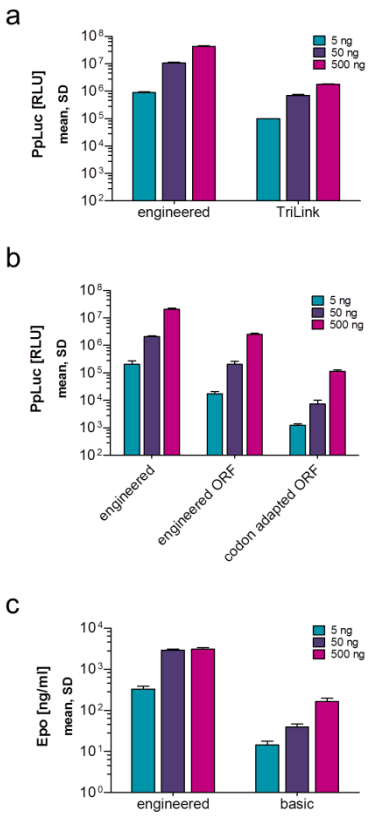

Figure S1 mRNA sequence-engineering improves protein expression in vitro. (**a**) Engineered, unmodified luciferase mRNA outperforms a doubly modified luciferase mRNA from a commercial supplier. Human fibroblasts were lipofected in triplicate with the indicated amounts of two different mRNAs. Luciferase expression was quantified 24 h after transfection. engineered, mRNA without chemical nucleoside modifications comprising a cap, an optimized open reading frame, a 5'-UTR from HSD17B4 (hydroxysteroid (17-beta) dehydrogenase 4), a 3'-UTR from ALB (albumin), a polyA, and a histone stem loop; TriLink, commercially available mRNA harboring pseudouridine and 5-methylcytidine of which further details are not disclosed. (**b**) Optimization of the open reading frame sequence as well as the combination with specific regulatory flanking sequences contributes to improved luciferase expression in vitro. HeLa cells were lipofected in triplicate with the indicated amounts of three different luciferase-encoding mRNAs and protein expression was determined 24 h later. engineered, mRNA harboring a cap, an optimized open reading frame, a 5'-UTR from HSD17B4, a 3'-UTR from ALB, a polyA, and a histone stem loop; engineered ORF, mRNA harboring a cap, a sequence-engineered open reading frame, a 3'-UTR from HBA (hemoglobin alpha), and a polyA; codon adapted ORF, mRNA harboring a cap, a codon-adapted open reading frame, a 3'-UTR from HBA, and a polyA. (**c**) Sequence-engineering enhances erythropoietin expression in vitro. HeLa cells were lipofected in triplicate with the indicated amounts of either of two mouse EPO-encoding mRNAs. EPO levels in cell supernatants were determined 24 h after transfection. engineered, mRNA harboring a cap, an optimized open reading frame, a 5'-UTR from HSD17B4, a 3'-UTR from ALB, a polyA, and a histone stem loop; basic, mRNA harboring a cap, the wildtype open reading frame, the 5'- and 3'-UTR sequences from HBB (hemoglobin beta), and a polyA.

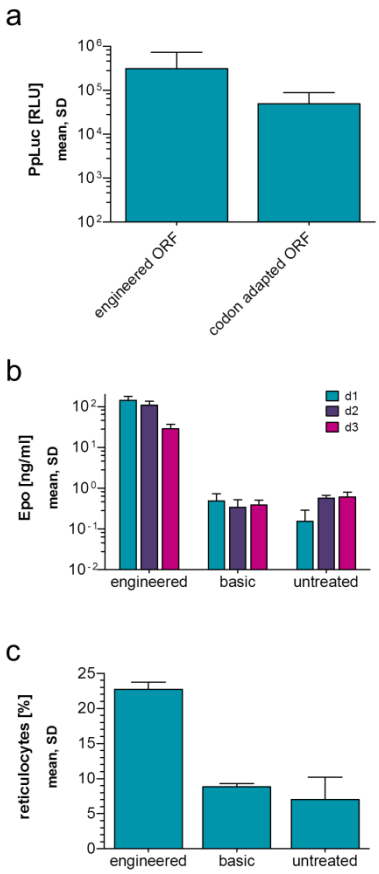

Figure S2 mRNA sequence-engineering improves protein expression in vivo. (**a**) Optimization of the open reading frame sequence augments protein expression in vivo. Mice received 10 µg of uncomplexed luciferase mRNA harboring either a sequence-engineered or a codon adapted open reading frame via intradermal injection. Protein expression was determined 24 hours after administration. The mRNAs consisted of a cap, the respective open reading frame, a 3'-UTR from HBA, and a polyA. (**b, c**) Sequence-engineering enhances erythropoietin expression and physiological effects in vivo. Mice were intraperitoneally injected with 1 µg of TransIT-complexed mRNA. Plasma EPO levels (**b**) were analyzed at the indicated times after administration and the level of reticulocytes (**c**) was determined 4 days after treatment. Mice received either an engineered (fully optimized) or a standard (basic) mRNA. Untreated animals were used as control. engineered, mRNA harboring a cap, an optimized open reading frame, a 5'-UTR from HSD17B4, a 3'-UTR from ALB, a polyA, and a histone stem loop; basic, mRNA harboring a cap, the wildtype open reading frame, the 5'- and 3'-UTR sequences from HBB, and a polyA.

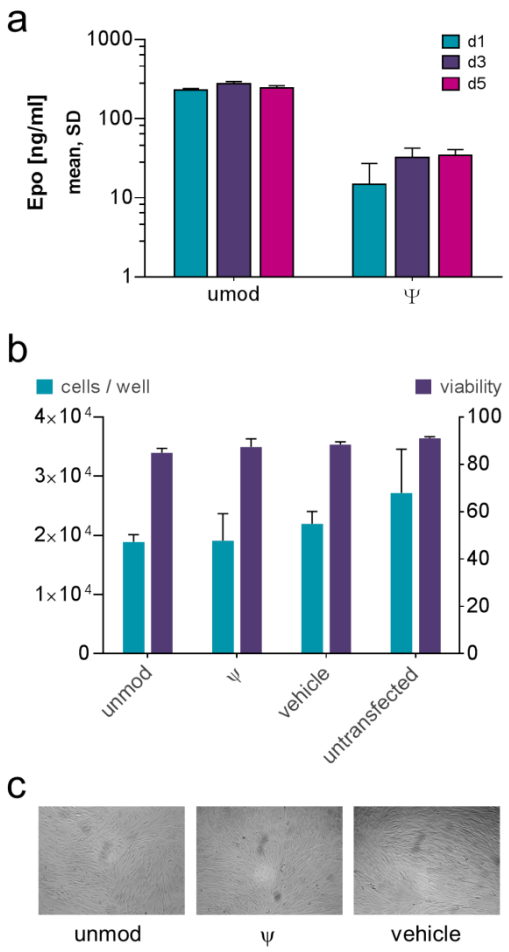

Figure S3 Repeated transfections of primary cells with mRNA do not require chemical modifications. Ten thousand human dermal fibroblasts were seeded per well onto a 96-well plate and transfected in triplicate with 40 ng of either unmodified or pseudouridine-harboring mEpo mRNA on five consecutive days using RNAiMAX transfection reagent according to the manufacturer's instructions. Cells treated with vehicle only or left untreated served as controls. (**a**) mRNA without chemical modifications did not lose expression efficacy upon repeated transfections. Expression of erythropoietin was quantified 24 hours after the first, third and last transfection with unmodified and the corresponding pseudouridine-modified mRNA, respectively. (**b**) Repeated transfections with unmodified mRNA did not negatively impact cell number or viability. Parameters were determined 24 hours after the last transfection and did not differ among transfection groups. (**c**) Repeated transfections with unmodified mRNA did not alter cell morphology. Cell morphology was investigated 24 hours after the last transfection and did not differ among treatment groups. Representative microscopic pictures are shown.

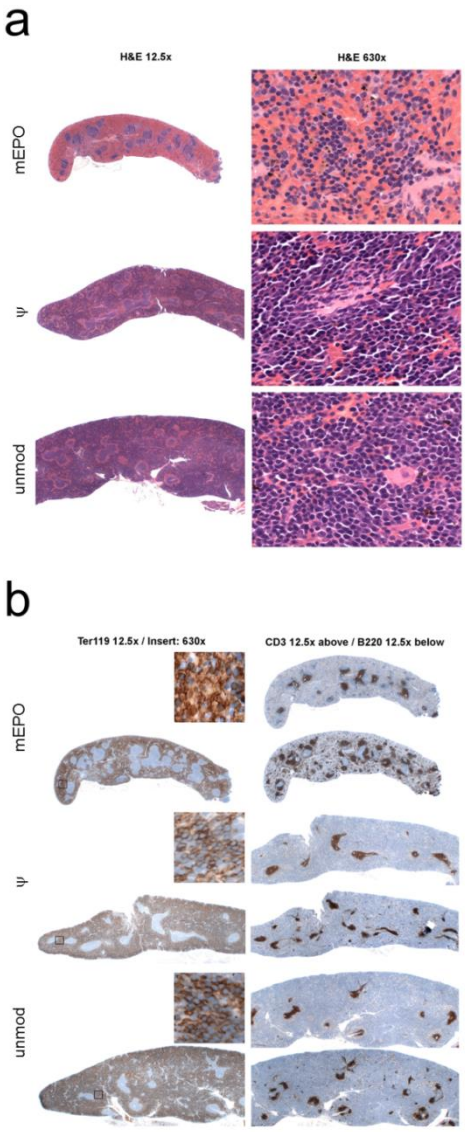

Figure S4 TransIT-complexed Epo-mRNA induces massive erythropoiesis in the spleen without causing histological or immunological disturbances. Mice received in total 5 intraperitoneal doses of either 0.8 µg of recombinant murine erythropoietin or 1 µg of TransIT-complexed Epo-mRNA at an interval of 2 weeks. mRNA was either unmodified (unmod) or harbored pseudouridine (Ψ). (**a**) Hematoxylin and eosin (H+E) staining of spleen sections reveal massive erythropoiesis in mRNA-treated animals as well as the absence of histological abnormalities. Except a weaker erythropoiesis, probably due to lower protein expression, the spleens of mice injected with pseudouridine-modified mRNA do not differ from those of animals that received unmodified mRNA. (**b**) Immunohistochemical staining for Ter119 (pro-erythroblasts to mature erythrocytes), CD3 (T cells) and B220 (B cells) reveals a normal spleen architecture in mRNA-treated animals. While the relative intensities of Ter119 staining are in line with an excess of erythrocyte progenitors in the spleen of mRNA-injected animals, CD3 and B220 stainings provide no evidence for an abnormal immunological status of the organ. Beyond a stronger effect of unmodified mRNA on erythropoiesis, pseudouridine-modified and unmodified mRNAs are indistinguishable with respect to their physiological effects. Figures show representative examples of an experiment with n = 4 for all groups.

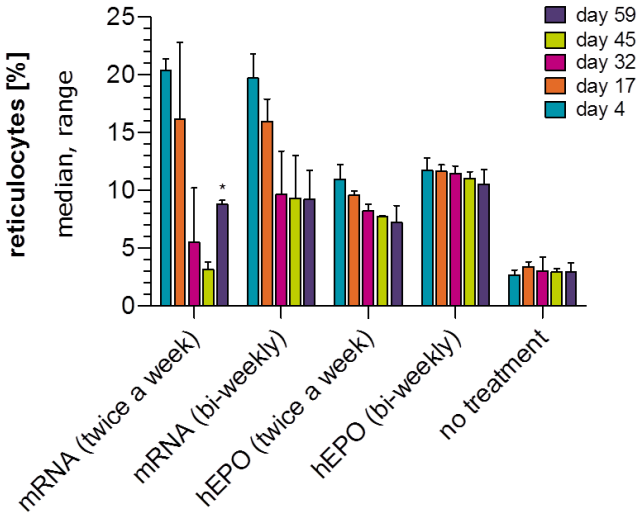

Figure S5 Desensitization by high and/or frequent EPO expression transiently reduces the physiological response to the hormone. Mice were injected with either 1 µg of TransIT-complexed Epo-mRNA or 100 U of recombinant human EPO protein either twice a week or every other week, with one exception where the interval to the previous treatment was 2 weeks instead of half a week (marked by an asterisk). The levels of reticulocytes were analyzed 4 days each after distinct treatments. The declining reticulocyte response upon repeated EPO treatments appears to exacerbate with increasing serum EPO levels, which are much higher from mRNA compared to recombinant protein (Fig. 2B), and frequency of injections. Whereas frequent treatments with a very effective mRNA dose finally lead to almost undetectable reticulocyte responses, i.e., the number of reticulocytes in response to treatments is barely above background levels, pausing of treatment can restore substantial physiological responses to mRNA treatment (see measurement marked by an asterisk). n = 4 for all groups.

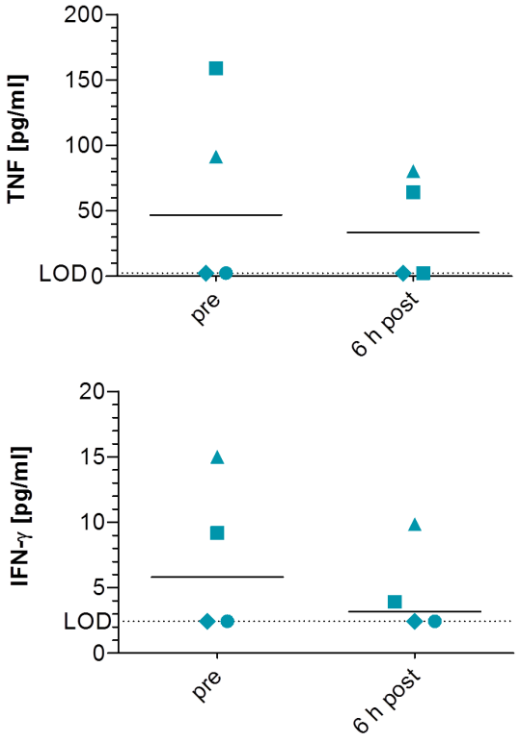

Figure S6 LNP-encapsulated engineered, unmodified Epo mRNA does not show inappropriate stimulation of the innate immune system. A single intravenous injection of engineered, unmodified M. fascicularis Epo mRNA into cynomolgus monkeys did not lead to any substantial cytokine secretion. Macaques received 100 µg of LNP-encapsulated Epo mRNA (0.037 mg/kg). Blood was collected before (pre) and 6 hours after (6 h post) treatment and analyzed for the levels of different cytokines. Distinct symbols are assigned to individual animals. LOD, limit of detection.

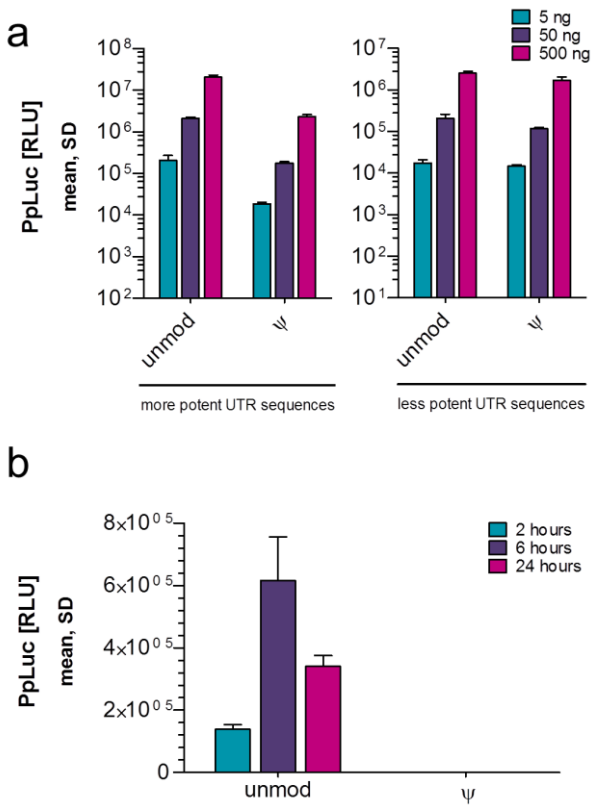

Figure S7 Incorporation of pseudouridine shows negative interference with functional RNA elements. (**a**) Comparison of the effects of pseudouridine incorporation on the expression of luciferase-encoding mRNAs that differ in the sequence and potency of their UTR elements. HeLa cells were lipofected with either of two mRNA species that were either unmodified of harbored pseudouridine instead of uridine. Luciferase expression was determined 24 h after transfection. With less potent UTR sequences (right), the incorporation of pseudouridine had no longer a negative effect. Untranslated regions comprised either a 5'-UTR from HSD17B4, a 3'-UTR from ALB, a polyA, and a histone stem loop (left) or a 3'-UTR from HBA and a polyA (right). (**b**) Incorporation of pseudouridine impairs the internal ribosome entry site (IRES) of the encephalomyocarditis virus. HeLa cells were transfected with bicistronic mRNAs that were either unmodified or harbored pseudouridine. Expression of the second cistron encoding firefly luciferase was quantified at different times after transfection. unmod, mRNA harboring the nucleotides A, U, G, and C; ψ, mRNA in which pseudouridine replaces U.

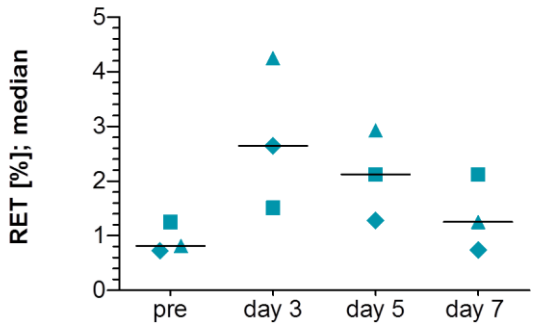

Figure S8 TransIT-complexed engineered Epo mRNA can elicit systemic physiological responses in swine. Intraperitoneal mRNA administration into pigs induces a transient increase of reticulocytes. Pigs received two injections of TransIT-complexed porcine Epo mRNA (360 µg each, corresponding to 0.018 mg/kg per dose) on two consecutive days (days -1 and 0; total dose per animal: 0.036 mg/kg) and were analyzed for the number of reticulocytes in the blood on various days. Each pig represented by an individual symbol revealed a distinct physiological response and, although the temporal pattern of the response slightly differed among animals, the increase of reticulocyte numbers on day 3 was statistically significant compared to the pre-treatment level ($p < 0.05$, student's t-test). pre = prevalue before treatment, n = 3 for treatment group.