

3161 Liver Specific Delivery of siRNA Targeting EGLN Prolyl Hydroxylases Activates Hepatic EPO Production and Stimulates Erythropoiesis

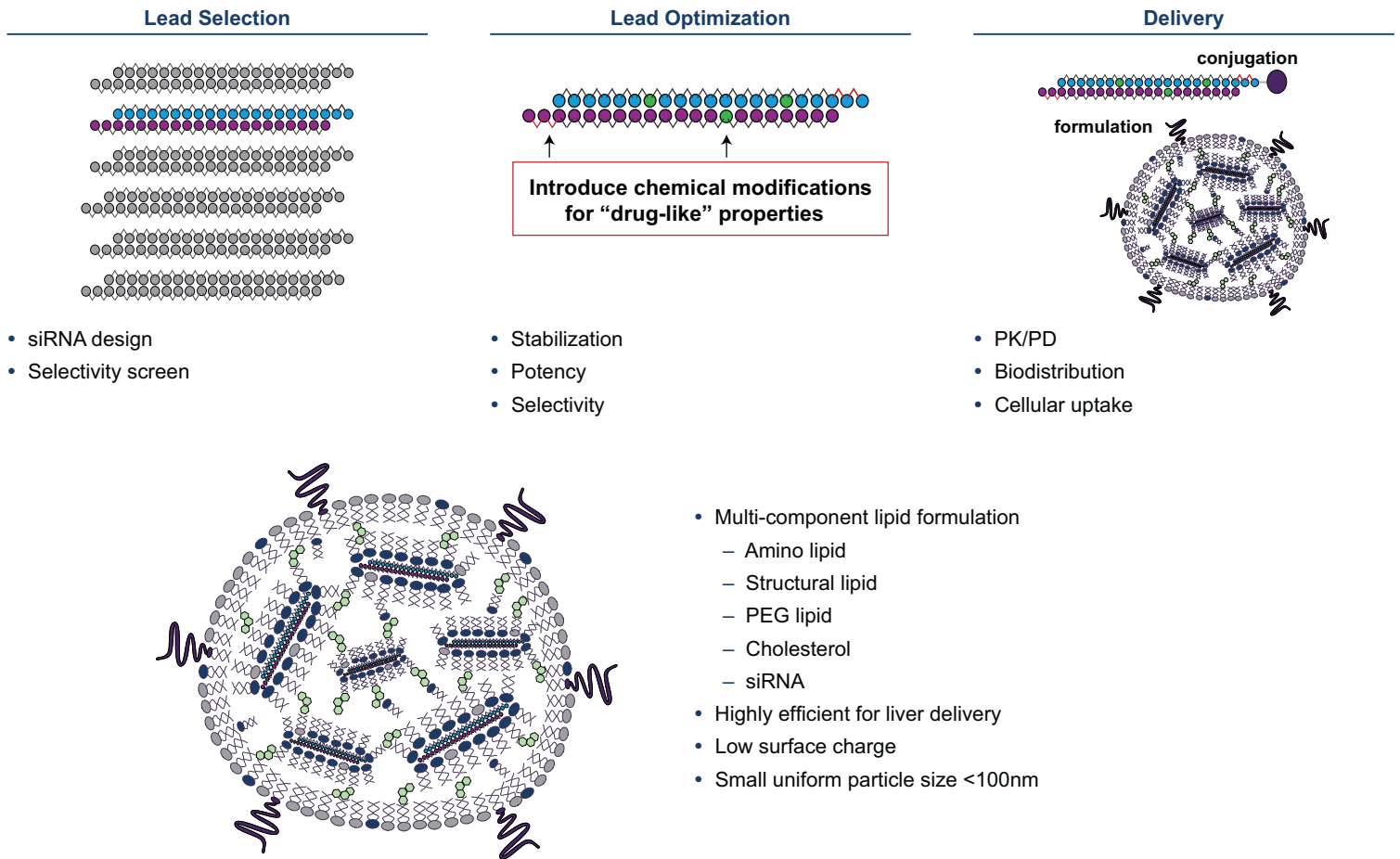
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Abstract

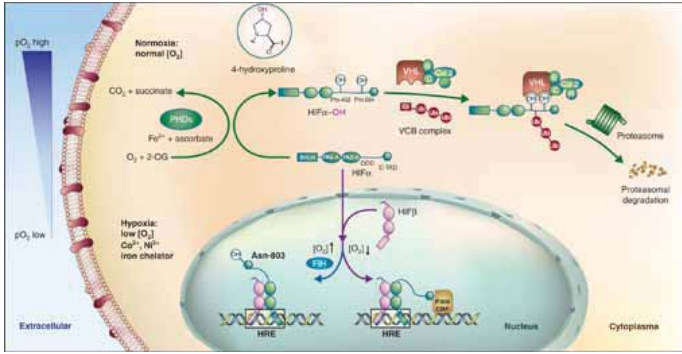
Anemia in chronic kidney disease patients due to impaired renal production of erythropoietin (EPO) and insufficient erythropoiesis is a significant public health problem. One approach to compensate for the low EPO levels in patients with impaired kidney function would be to stimulate the levels of EPO production from non-renal sources. It is well known that during fetal development the liver serves as the primary producer of EPO until the kidney becomes the dominant source after birth. Negative regulation of EPO production is mediated by the EGLN family of prolyl hydroxylases (PHDs1-3) that play an important role in oxygen sensing by targeting the transcription factor hypoxia inducible factor (HIF) for degradation via the proteasome. HIF stabilization and activity is required to mediate EPO gene transcription. Previously it has been shown that liver specific conditional knockout of all three EGLN genes is required to activate HIF and induce EPO production in liver (Minamishima et al. *Science* 2010). Here we show that simultaneous siRNA mediated knockdown of all 3 EGLN genes in mouse liver using lipid nanoparticles (LNPs) can induce hepatic EPO mRNA activation, elevation of serum EPO levels and stimulation of erythropoiesis. We extend these data by examining siRNA knockdown of different combinations of the 3 EGLN genes and demonstrate that EGLN1 is the dominant EGLN gene regulating hepatic EPO production. In addition, due to the specificity of the LNP delivery system we show that the HIF activation and increase in EPO mRNA occurs specifically in liver. Increases in serum EPO and hematocrit were durable for two weeks and one month after a single intravenous dose of LNP siRNA. Furthermore, EGLN siRNA silencing in a 5/6 nephrectomy model successfully elevates hemoglobin levels and corrects anemia. In conclusion, targeting of EGLN prolyl hydroxylase genes with siRNA therapeutics has potential in the treatment of anemia associated with chronic kidney disease and provides liver-specific target gene regulation.

Figure 1. Turning siRNAs into drugs and LNP overview

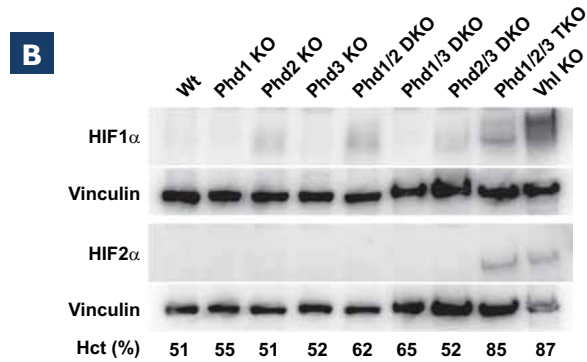
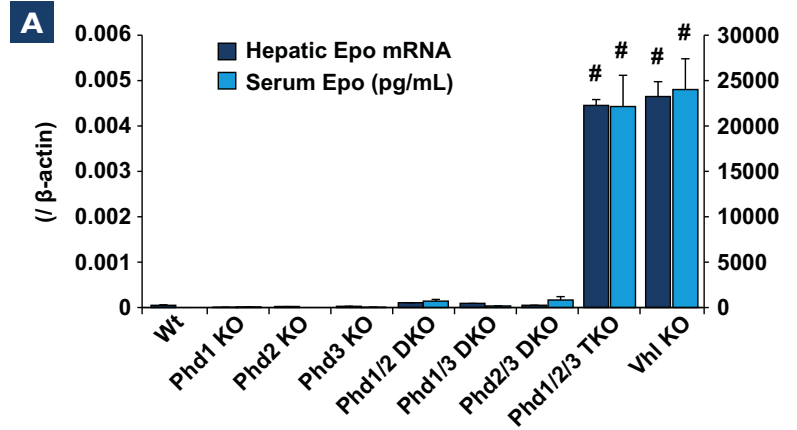


Turning siRNAs into drugs and overview of lipid nanoparticle (LNP) technology used in these studies

Figure 2. EGLN regulation of erythropoietin production

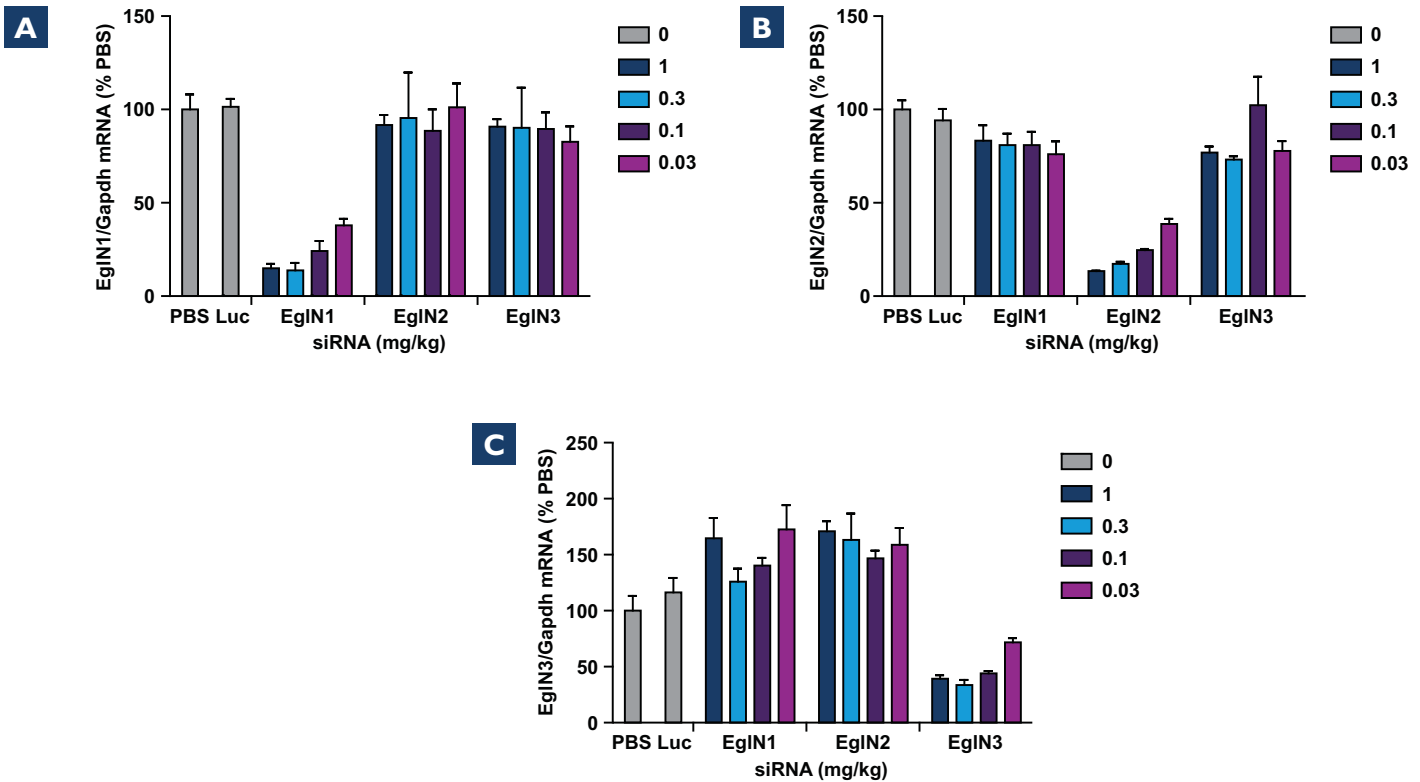


The EGLN family of prolyl hydroxylases regulate oxygen homeostasis and HIF mediated stimulation of erythropoietin. Under normal oxygen conditions EGLNs (PHDs) prolyl hydroxylate HIF- α subunits targeting them for proteasomal degradation. During hypoxia EGLNs (PHDs) are inactive allowing stabilization of HIF- α subunits which translocate to the nucleus to bind HIF beta subunits and activate hypoxia inducible gene transcription. During embryonic development, the liver is the major site of erythropoietin production which after birth is switched off and the kidney takes over as the major producer. In chronic kidney disease (CKD) patients, in many cases, the malfunctioning kidney does not produce adequate levels of erythropoietin resulting in anemia and they may benefit from increased EPO production from non renal sources to compensate. (A, B) Conditional knockout of all 3 EGLN prolyl hydroxylases (PHD1,2,3) in the liver induces hepatic EPO production, HIF activation and increase in hematocrit. We sought to try and activate hepatic EPO using siRNAs targeting EGLN prolyl hydroxylases in combination.



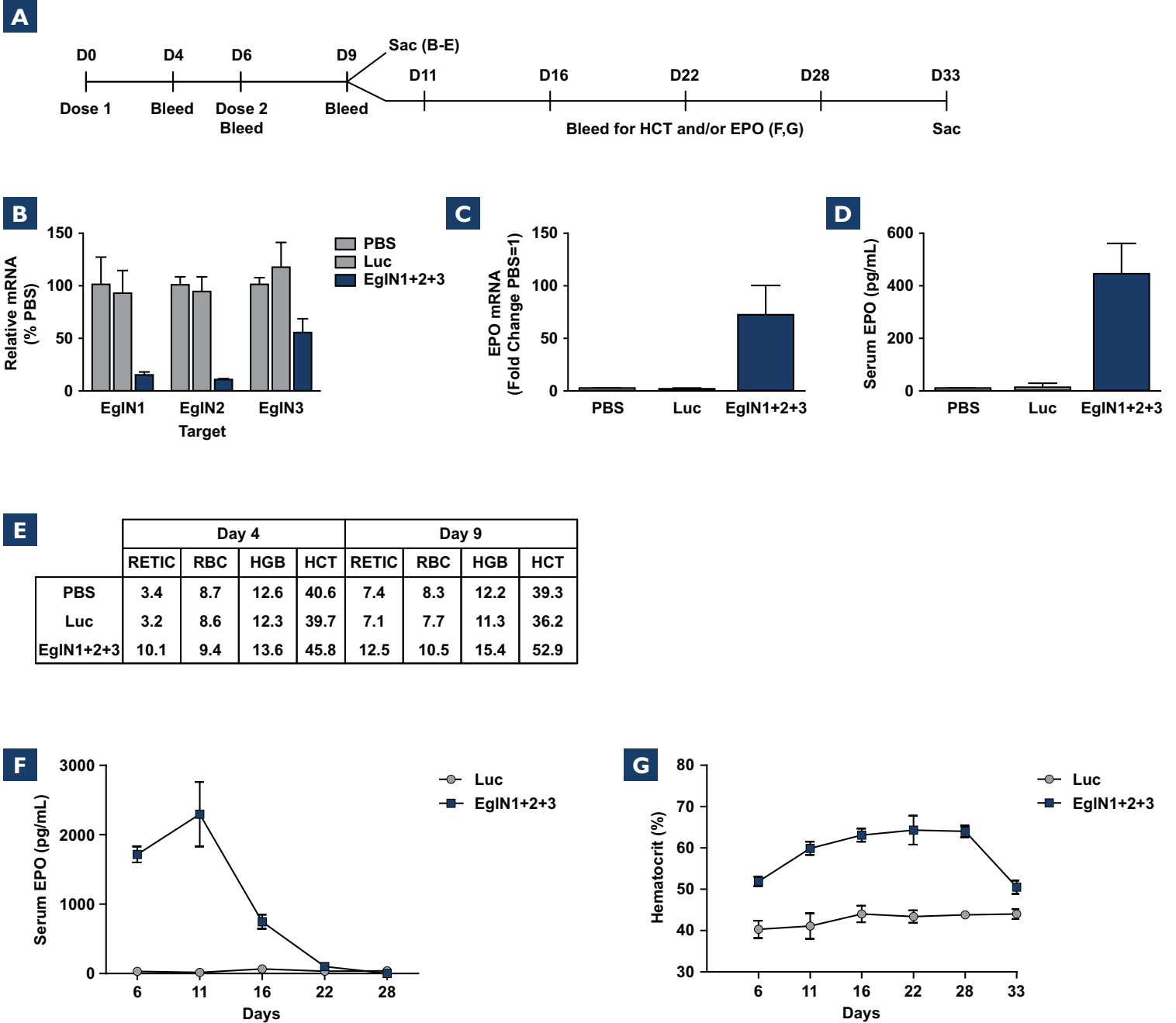
Yan et al. *Expert Opin. Ther. Patents*, 2010, Minamishima and Kaelin. *Science*, 2010

Figure 3. Validation of EgIN siRNAs *in vivo*



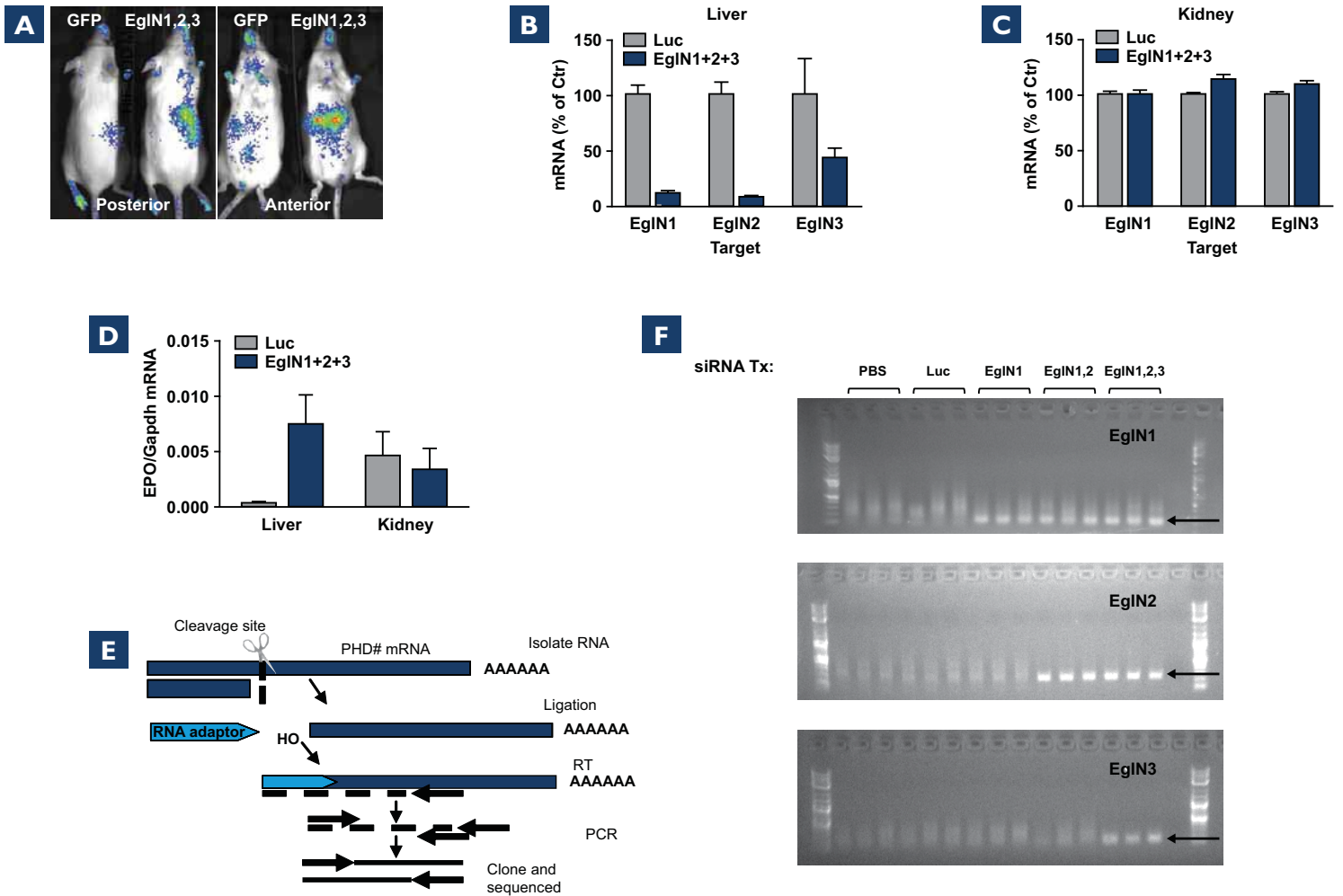
Validation of LNPs with EgIN siRNAs *in vivo*. *EgIN1* (*PHD2*), *EgIN2* (*PHD1*), and *EgIN3* (*PHD3*) hepatic mRNA levels in mice 72 hours after receiving increasing amounts of LNPs containing the indicated siRNAs by tail vein injection (0.03, 0.1, 0.3, or 1 mg/kg). LUC = firefly luciferase. mRNA levels were normalized to GAPDH and then to the corresponding value for PBS-treated mice.

Figure 4. EglN siRNA activates hepatic EPO production



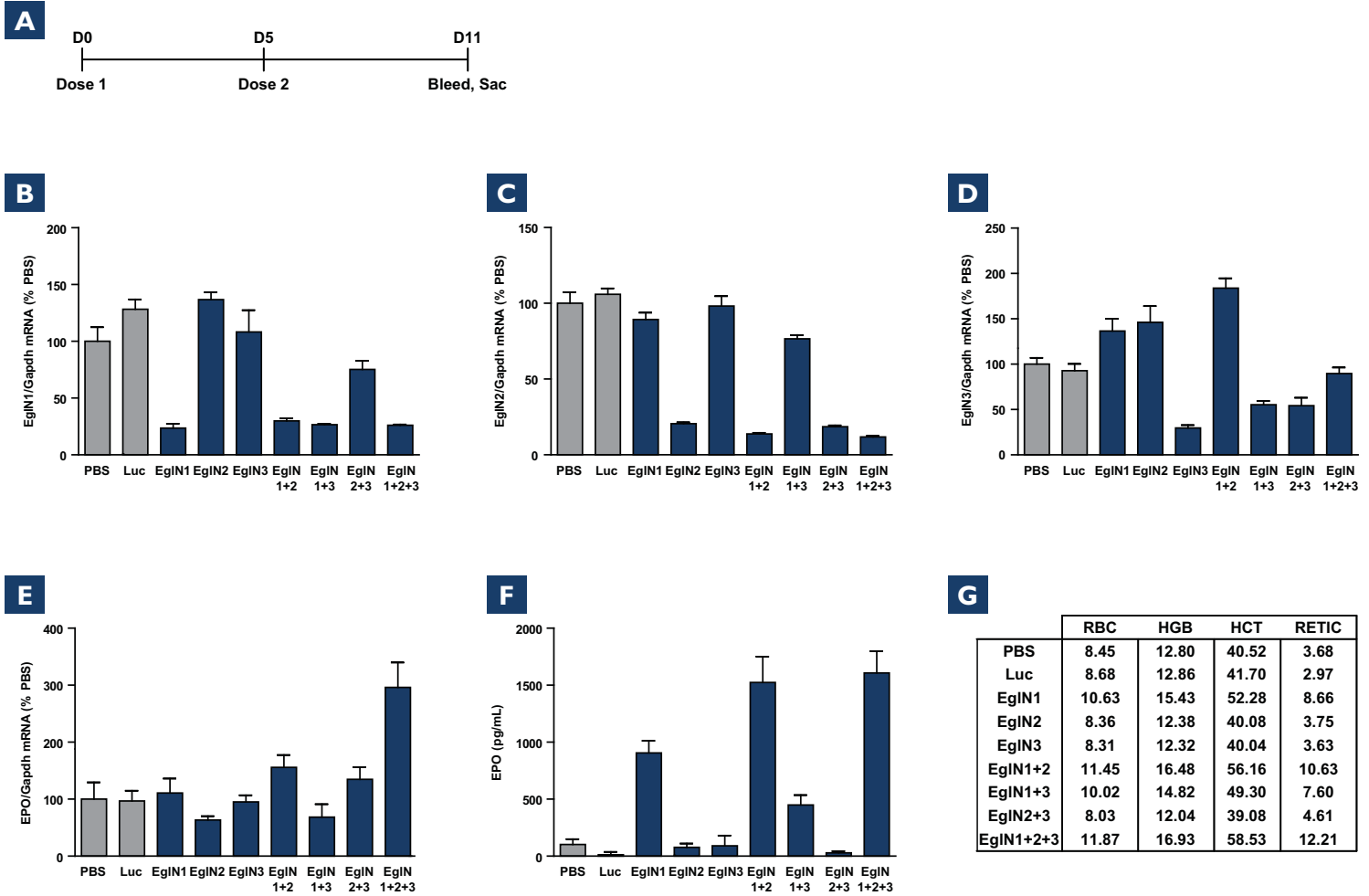
EglN siRNA activates hepatic EPO production and stimulates erythropoiesis. (A) Overview of dosing schedule and bleeds. Mice were dosed intravenously with LNPs that contained an equal mix of three siRNAs targeting EglN1, EglN2, and EglN3, respectively (total dose = 1 mg/kg). Mice treated with PBS or a similarly prepared LNP containing a single siRNA against firefly luciferase served as controls. (B-D) Hepatic *EglN* mRNA (B), Hepatic *EPO* mRNA (C) and serum EPO (D) levels at day 9. mRNA levels were normalized to *GAPDH* mRNA levels and then to the corresponding values in PBS treated mice. (E) Hematology measurements at day 4 after first dose or day 9 after 2 doses. (F and G) Serum EPO (F) and Hematocrit (G) levels in mice treated as in (A) with two doses of siRNA against the EglN family members or against firefly luciferase (LUC).

Figure 5. EgIn siRNA delivery is liver specific



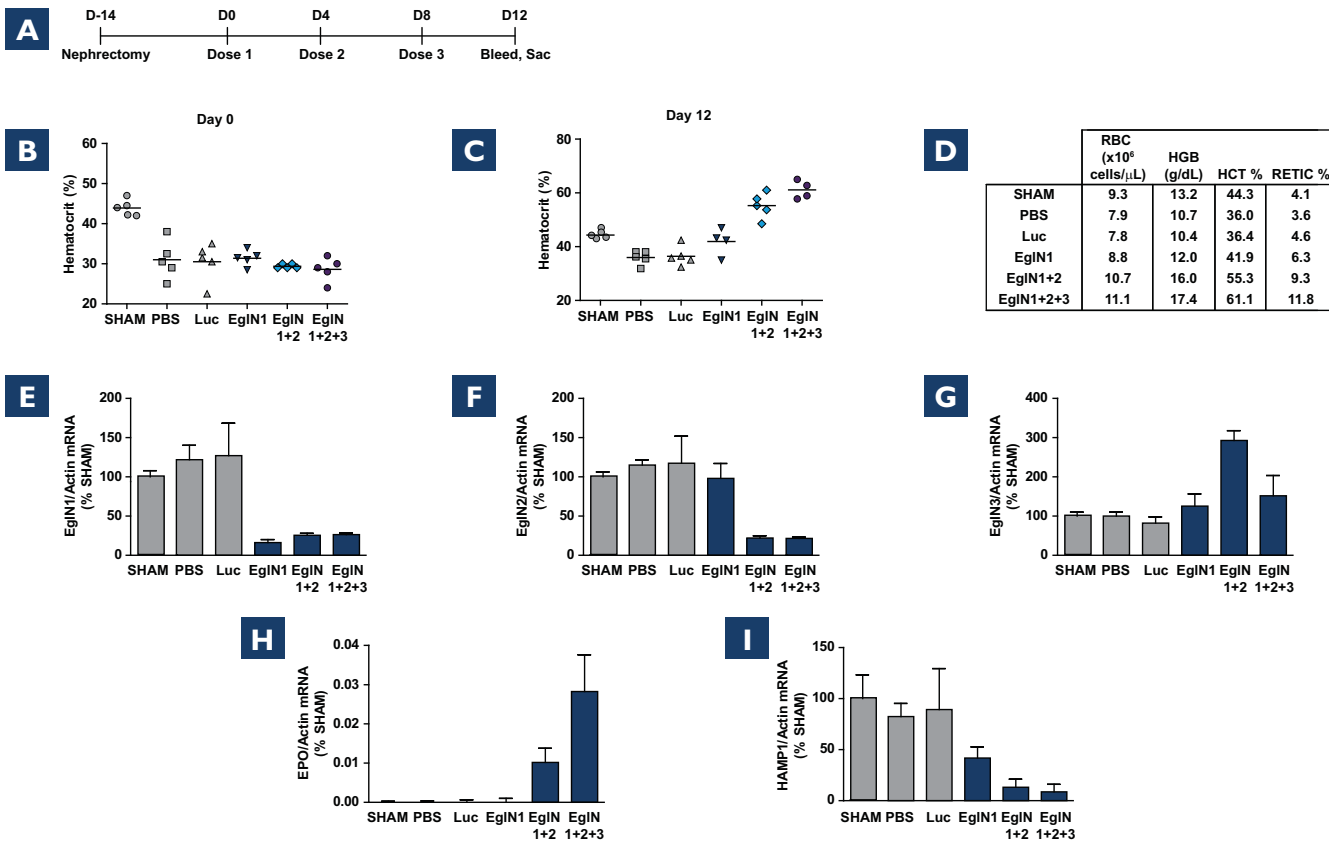
LNP-mediated EgIn siRNA delivery is liver specific and downregulates EgIn mRNA via an RNAi mechanism. (A) Bioluminescent images of HIF1a-Luc mice 72 hours after a single intravenous dose of LNPs targeting all three EgIn family members or, as a negative control, green fluorescent protein (GFP). Total dose = 1 mg/kg (0.33 mg/kg per family member). (B and C) Quantification of *EgIn* mRNA levels in livers (B) and kidneys (C) of mice 1 week after treatment with LNPs targeting all three EgIn family members or firefly luciferase. mRNA levels were normalized to *GAPDH* mRNA and then to corresponding values in mice treated with luciferase siRNA. (D) Quantification of *EPO* mRNA levels in liver and kidney in mice treated as in (B and C). (E) Overview of 5'RACE assay to monitor cleavage site of target mRNA. (F) 5'RACE of mouse liver total RNA isolates from livers treated with the indicated siRNAs. Specific cleavage sites were confirmed by sequencing of excised bands from gel.

Figure 6. Combinatorial effects of EgIn siRNAs



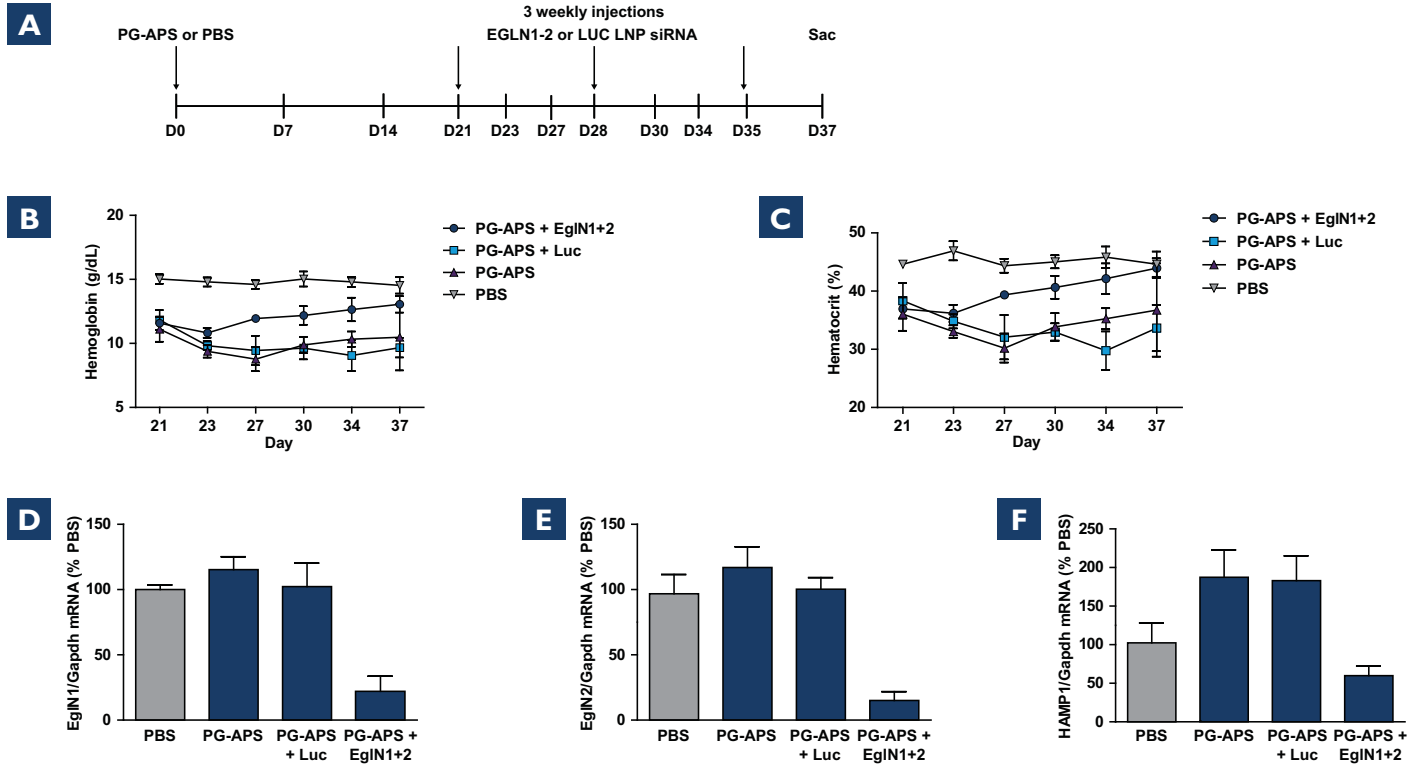
Combinatorial effects of EgIn siRNAs on hepatic EPO production. (A) Dosing Schema. (B-E). Hepatic *EgIn* (B-D) and *EPO* (E) mRNA levels for mice treated with combinations of siRNA nanoparticles targeting the indicated EgIn family members (0.5 mg/kg per EgIn family member). mRNA levels were normalized to GAPDH and then to the corresponding value for PBS-treated mice. (F and G). Serum EPO (F) and hematologic (G) values for mice treated as above.

Figure 7. EGLN knockdown in mouse anemia model



Targeting of *Egln* genes rescues anemia caused by renal failure. (A) Overview of 5/6 nephrectomy procedure and dosing schedule. (B and C) Baseline (day 0) (B) and day 12 (C) hematocrit levels in mice treated with the indicated siRNAs as depicted in (A). Sham mice underwent sham surgery rather than 5/6 nephrectomy. (D and E) Hematology (D) and mRNA (E-I) values at day 12 in mice treated with the indicated siRNAs as depicted in (A). HAMP1 = hepcidin antimicrobial peptide 1. mRNA levels were normalized to *actin* mRNA and then to corresponding sham mRNA level.

Figure 8. EGLN knockdown in rat anemia of inflammation model



Targeting of *Egln* genes corrects anemia related to inflammation in rat model. (A) Overview of rat anemia of inflammation model and dosing schedule. Rats were dosed with PG-APS polymer (or PBS) on day 0. Rats treated with PG-APS developed anemia by day 21 and were then randomized to receive LNPs targeting both *Egln1* and *Egln2*, LNPs targeting firefly luciferase (LUC), or not treated (PG-APS only). (B and C) Hemoglobin (B) and hematocrit (C) values for rats treated with the indicated siRNAs as in (A). (D-F) Hepatic mRNAs levels at termination of study on day 37. mRNA levels were normalized to GAPDH levels and then to the corresponding value for mice that received PBS instead of PG-APS.

Conclusions/Summary

- Using siRNA in LNPs we can robustly knockdown all three EGLN1,2,3 mRNAs in mouse liver
- Targeting EGLN1 alone or in combination with EGLN2,3 increases serum EPO levels
- Knockdown of EGLN1 and/or EGLN2 induces feedback loop up-regulation of EGLN3 mimicking hypoxic response
- A single dose of EGLN1,2,3 siRNA maintained elevated EPO for ~2 weeks and hematocrit for ~1 month
- Induction of serum EPO with EGLN1 knockdown (alone or combo) induces erythropoiesis:
 - increase in reticulocytes, RBCs, hemoglobin, hematocrit
- Stimulating erythropoiesis with EGLN KD negatively regulates Heparin
- The increase in serum EPO appears to be coming from liver and not kidney
- EGLN knockdown improves anemia in mouse 5/6 nephrectomy and rat anemia of inflammation models
- RNAi mediated knockdown of EGLN prolyl hydroxylases and stimulation of hepatic EPO production has potential in the treatment of anemias

Conflict of Interests

WQ, AA, JW, CZ, JQ, JH, SK, KC, DS, KF, VK are employees and/or stockowners of Alnylam Pharmaceuticals

JM received honoraria from Alnylam

WGK consults for and owns equity in Fibrogen

RB has no relevant conflict of interest to disclose