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Engineered red blood cells carrying PCSK9 inhibitors persistently lower LDL and prevent obesity

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Abstract

Low plasma levels of Proprotein Convertase Subtilisin/Kexin 9 (PCSK9) are associated with decreased low-density lipoprotein (LDL) cholesterol and a reduced risk of cardiovascular disease. PCSK9 binds to the epidermal growth factor-like repeat A (EGFA) domain of LDL receptors (LDLR), very low-density lipoprotein receptors (VLDLR), apolipoprotein E receptor 2 (ApoER2), and lipoprotein receptor–related protein 1 (LRP1) and accelerates their degradation, thus acting as a key regulator of lipid metabolism. Antibody and RNAi—based PCSK9 inhibitor treatments lower cholesterol and prevent cardiovascular incidents in patients, but their high-cost hampers market penetration. We sought to develop a safe, long-term and one-time solution to treat hyperlipidemia. We created a cDNA encoding a chimeric protein in which the extracellular N- terminus of red blood cells (RBCs) specific glycophorin A was fused to the LDLR EGFA domain and introduced this gene into mouse bone marrow hematopoietic stem and progenitor cells (HSPCs). Following transplantation into irradiated mice, the animals produced RBCs with the EGFA domain (EGFA-GPA RBCs) displayed on their surface. These animals showed significantly reduced plasma PCSK9 (66.5% decrease) and reduced LDL levels (40% decrease) for as long as 12 months post-transplantation. Furthermore, the EGFA- GPA mice remained lean for life and maintained normal body weight under a high-fat diet. Hematopoietic stem cell gene therapy can generate red blood cells expressing an EGFA—glycophorin A chimeric protein as a practical and long-term strategy for treating chronic hyperlipidemia and obesity.

Introduction

Hyperlipidemia, characterized by elevated plasma LDL (low-density lipoprotein) levels, is a risk factor for cardiovascular disease, the leading cause of death worldwide. Therapeutics that reduce plasma LDL levels are the mainstay in the prevention of cardiovascular disease. Statins, small molecule inhibitors of HMG-CoA reductase, impede the generation of LDL cholesterol and are effective in reducing the incidence of cardiovascular disease and mortality in high-risk individuals. Because statins inhibit a crucial metabolic pathway, patients who receive statin medication can suffer adverse effects, ranging from minor cognitive decline and muscle pain to an increased risk of diabetes as well as liver and kidney failure [1].

The discovery of proprotein convertase subtilisin-like kexin type 9 (PCSK9) was a possible breakthrough in tackling hypercholesterolemia. PCSK9 is produced by the liver and accelerates degradation of LDLR. A gain of function mutation in PCSK9 in two French families that exhibit a dominant form of Familial Hypercholesterolemia suggested that reducing PCSK9 levels is an attractive mechanism to lower cholesterol [2]. A woman of African descent who had a loss of function mutation in both PCSK9 alleles had significantly lower plasma LDL levels while otherwise healthy. Combined, these genetic findings suggested that inhibition of PCSK9 could be a novel and safe target to treat hypercholesterolemia [3].

PCSK9 is a serine protease encoded by a gene comprising 12 exons located on chromosome 1p32. PCSK9 binds to the epidermal growth factor-like A domain (EGFA) on the stalk of the LDLR and blocks a structural transition of LDLR in the endosome. Instead of recycling LDLR to the surface [4] it is targeted for lysosomal degradation. Inhibitory peptides that mimic EGFA prevent degradation of LDLR and can lower plasma LDL levels [5]. The catalytic sub-unit of PCSK9 binds structurally similar EGFA domains on LDLR superfamily members including very low-density lipoprotein receptors (VLDLR), apolipoprotein E receptor 2 (ApoER2), and lipoprotein receptor-related protein 1 (LRP1), implicating PCSK9 as key regulator in lipid metabolism [6]. PCSK9 also regulates triglyceride metabolism by enhancing the degradation of CD36 (cluster of differentiation 36), a scavenger receptor involved in transport of long-chain fatty acids and triglyceride storage in adipocytes and liver [7]. Independent of its action on LDLR, PCSK9 modulates cholesterol transport and metabolism in the intestines by upregulating the intestinal epithelial cholesterol transporter NPC1L1 (Niemann-Pick C1-like protein 1) and increasing the expression of apolipoprotein B48 [8]. These results highlight PCSK9's diverse roles in regulating lipid homeostasis.

Multiple molecular strategies and therapeutics have been developed that inhibit LDLR degradation by PCSK9 and thus lower LDL levels (for review see reference [9]). While showing promise, these therapeutics have drawbacks, such as a transient reduction of LDL that must be counteracted by frequent injections of antibodies, peptides, or small molecule drugs. FDA-approved antibody-based drugs, while showing efficacy in patients, are expensive and limit their wide-use as a preventative strategy [10]. Disabling the PCSK9 gene using gene editing technologies may allow a one-shot injection as a preventative treatment to reduce the risk of cardiovascular disease [11], however, the safety of such treatments are still being extensively evaluated. Red blood cells are an attractive vehicle to introduce therapeutics into the body due to their systemic circulation, long half-life, and lack of DNA and RNA [12–14]. Hematopoietic stem cell (HSC) gene therapy–autologous transplantation of HSCs in which a cDNA is expressed by a lentivirus vector–has proven successful in treating X-Linked Severe Combined Immunodeficiency, Wiskott Aldrich Disease, Sickle Cell Anemia, and other conditions [15–18].

Here we generated a cDNA that encodes a chimeric protein in which the extracellular N-terminus of glycophorin A was fused to the LDLR EGFA domain. We introduced this gene

into mouse bone marrow hematopoietic stem and progenitor cells (HSPCs). Following transplantation into irradiated mice, the animals produced red blood cells (RBCs) with the EGFA domain displayed at the cell surface (EGFA-GPA RBCs). We assessed the potential of PCSK9 inhibitor-carrying RBCs as a long-term strategy to treat hyperlipidemia. We observed significant decreases in both plasma PCSK9 and LDL levels for at least 12 months. Interestingly, we also observed that the transplanted mice weighed significantly less than the control mice.

Materials and method

Animal care

All of our animal experimental procedures were approved by the Committee of Animal Care (the Institutional Animal Care and Use Committee/IACUC for Massachusetts Institute of Technology and the Whitehead Institute). Protocol #0218-012-21.

Plasmids and generation of recombinant retroviruses

cDNA sequences of mouse EGFA and human GPA, National Center for Biotechnology Information (NCBI) reference sequences NC_000075.6 and NM_002099.6, respectively, were synthesized and then cloned into XhoI and EcoRI-cut XZ201 using a Thermo Scientific Rapid DNA ligation kit following the manufacturer's protocol. The GFP-expressing, XZ201 plasmids were used for the production of murine stem cell virus (MSCV)-based retrovirus vectors to infect erythroid progenitors (Fig 1A). Therefore, positive transduction is indicated by GFP expression.

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37˚C. XZ201-based plasmids and pCLECO packaging plasmids were transformed into HEK293T cells with Fugene 6 (Promega) according to the manufacturer's protocol. The medium was replaced after 8 hours, and retrovirus—containing supernatant was collected and filtered after 24 hours for transduction.

Flow cytometric analyses and antibodies

All flow cytometric data were acquired on a Fortessa flow cytometer (BD Biosciences) and analyzed using Flowjo software (Tree Star). All stainings were carried out in FACS buffer (2mM EDTA and 1% FBS in phosphate-buffered saline/PBS) for 20 minutes on ice. Samples were washed twice with FACS buffer prior to flow analyses. The following are the antibodies used, all at 1:100 dilution: anti-myc tag-PE (Cell Signaling Technology, 3739), anti-mouse Ter119--APC (eBioscience, 17-5921-83), and anti- mouse CD71-PE (Affymetrix, 12-0711-83). Hoechst 33342 (Life Technologies, H1399) was used to visualize nuclei.

Isolation, viral infection and culture of murine erythroid progenitors

Pregnant C57BL/6J mice at embryonic day 14.5 (E14.5) were euthanized by CO_2 asphyxiation. Fetal livers were isolated from the embryos and suspended in cold PBS with 2% FBS and 100 μM EDTA. Mature RBCs in the cell suspension were lysed by incubation in sterile ammonium chloride solution (Stemcell) for 10 min at 4˚C. Lineage- positive cells were magnetically depleted using the BD Pharmingen Biotin MouseLineage Panel (559971; BD Biosciences) and BD Streptavidin Particles Plus-DM (557812; BD Biosciences), per the manufacturer's protocol. Lineage-negative fetal liver cells, enriched for erythroid progenitors, were then plated in 24-well plates at 100,000 cells per well, covered by 1 ml of virus containing supernatant, produced as described above, and 0.1% polybrene (Sigma Aldrich), and spin-infected at 500 g for 90 minutes at 25˚C. Cells were then cultured in fresh erythroid maintenance medium

A.

N' | SP | EGF-A domain of LDL-R | myc-tag | glycophorin A | C'

E14.5 fetal
liver-derived
HSPCs

2 days
in vitro
differentiation

B.

Gated on: Ter119+ GFP+

☐ empty vector
☐ EGFA-GPA

Normalized to mode

myc-tag ⟶

C.

Cell Number

●— Empty Vector
●— EGFA-GPA

n.s.

Day

D.

Empty vector

EGFA-GPA

10μm

E.

Day 0   Day 1   Day 2

Empty
vector

EGFA-
GPA

CD71

Ter119 ⟶

F.

Day 2

21.5%

Hoechst

21.4%

Ter119 ⟶

G.

recombinant
myc proteins

kDa

vector only
EGFA-GPA
10ng
50ng
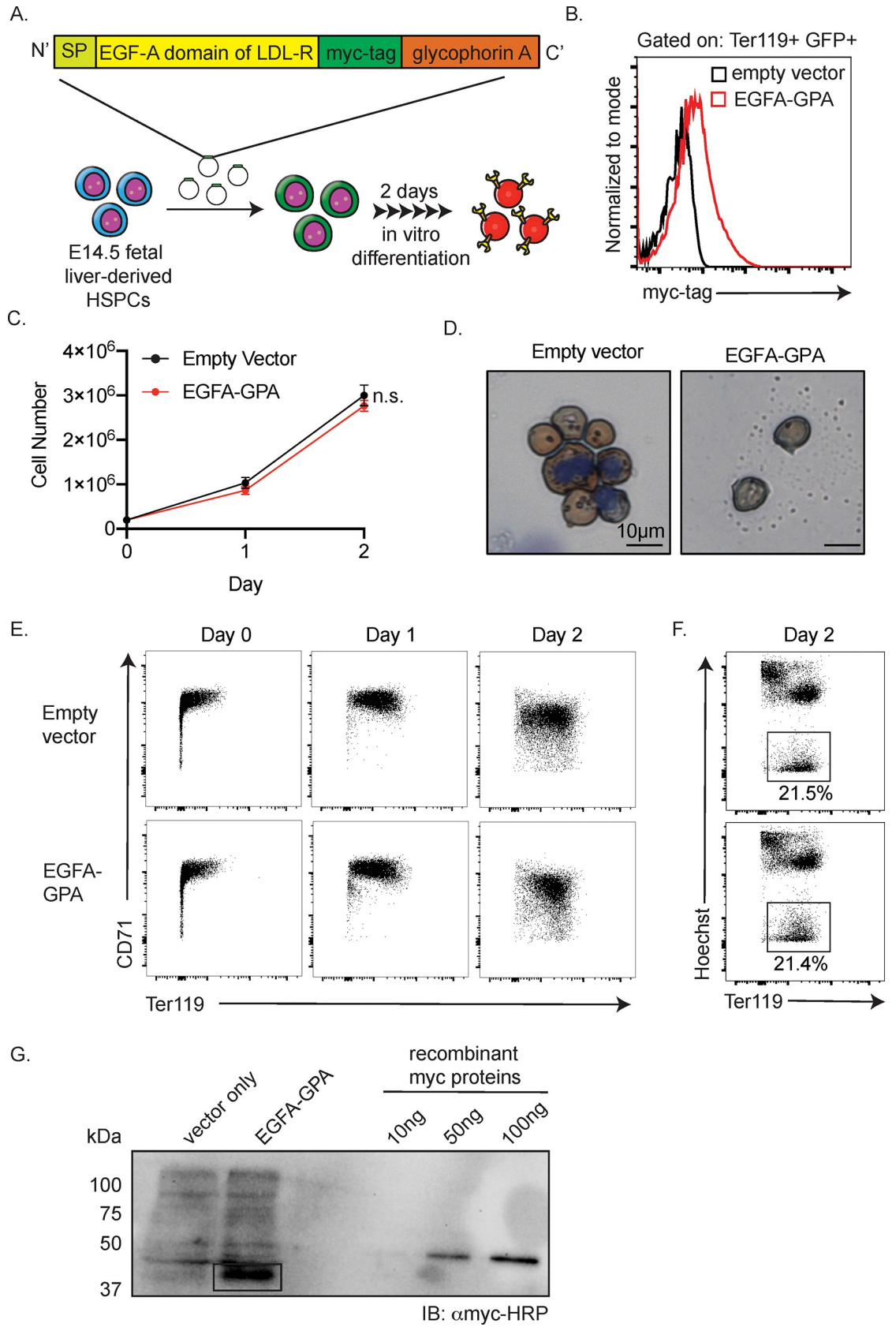100ng

100
75

50

37

IB: αmyc-HRP

Fig 1. Reticulocytes produced in cell culture can be engineered to express on their surface the EGFA domain of LDLR without compromising their normal biological properties. (A) Design of the chimeric EGFA-GPA protein. Schematic depicting the generation of engineered reticulocytes produced by *in vitro* culture of murine fetal liver- derived HPSCs (SP = signal peptide) (B) Surface myc-tag expression of murine reticulocytes generated in culture that express empty vector (expressing GFP under the control of the CMV promoter to help indicate positive transduction in cells) or EGFA-GPA together with GFP, as evaluated by flow cytometry. (C) Proliferation of *in vitro*-differentiated erythroid cells (n = 3) (D) Benzidine-Giemsa staining of the resulting reticulocytes. (E) Representative flow cytometry evaluation of erythroid differentiation progression as indicated by surface expression of CD71 (transferrin receptor) and Ter119 (marker of mature erythroid cells) and (F) enucleation rate of erythroid cells as indicated by low Hoechst and high Ter119 staining. (G) Quantification of the number of EGF-A molecules per erythroid cell by western blot. Lysates from 1 million erythroid cells collected at day 2 terminal differentiation were analyzed. As quantification standard, we analyzed 10, 50, and 100 ng of full-length recombinant human c-Myc protein (molecular weight = 55 kD). Western blots were performed with anti-myc antibody. Calculated from the band's density, each EGFA-GPA erythroid cell expresses ~9.36 million EGFA-GPA protein.

(StemSpan- SFEM; StemCell Technologies) supplemented with 100 ng/ml recombinant mouse SCF (R&D Systems), 40 ng/ml recombinant mouse IGF1 (R&D Systems), 100 nM dexamethasone (Sigma), and 2 U/ml erythropoietin (Amgen) at 37˚C for 24 hours.

To collect virally infected erythroid progenitors, after 24 hours in culture cells were sorted for GFP+ by flow cytometry and cultured in fresh erythroid differentiation medium (IMDM containing 15% (vol/vol) FBS (Stemcell), 1% detoxified bovine serum albumin (BSA; Stemcell), 500 μg/ml holo-transferrin (Sigma-Aldrich), 0.5 U/ml Erythropoietin (Epo; Amgen), 10 μg/ml recombinant human insulin (Sigma-Aldrich), and 2mM L- glutamine (Invitrogen) at 37˚C for 48 hours. Cells in differentiation medium were counted manually with a hemocytometer at 0 hour, 24 hours, and 48 hours. After 48 h, erythroid cells were fixed on slides with cold methanol and stained with May-Grünwald–Giemsa (Sigma) and diaminobenzidine hydrochloride reagents (Sigma-Aldrich GS-500 and D-9015) for morphological analyses.

Calculation of the number of chimeric proteins on murine RBCs

Western blotting on 1,000,000 in vitro—differentiated murine erythroid cells was conducted with anti-myc-Tag (9B11) mouse monoclonal antibody (Cell Signaling no. 2040, 1:1,000 dilution). The copy number of recombinant human GPA protein per erythroid cell was derived using a linear signal intensity plot generated with 10, 50, and 100 ng of recombinant human c-Myc tagged proteins (55 kDa). As determined by the myc signal intensity in the western blot with an anti-myc antibody and as quantified by ImageJ, 1,000,000 murine EGFA-GPA cells contained ~855 ng EGFA-GPA protein. The molecular weight of EGFA-GPA is ~40,000 Da. Thus, each reticulocyte expresses ~9,000,000 copies of EGFA-GPA.

Transplantation of transduced mouse fetal liver cells

A total of 1,000,000 virally infected murine hematopoietic stem/ progenitor cells cultured in erythroid maintenance medium, produced as described above, were collected and resuspended in 100μl PBS. These cells were then injected retro-orbitally into a C57BL/6J (Jackson Laboratory) mouse irradiated at 1050 rad, 1 day prior to transfer, in a Gammacell 40 irradiator chamber (Nordion International Inc.), and transplanted mice were monitored for 4 weeks before further analyses.

Transfusion and in vivo survival of EGFA-GPA RBCs

A total of 200 μl blood from transplanted mice containing RBCs expressing the EGFA- GPA chimeric protein was collected into heparinized tubes (Fisher Scientific, 365965). The RBCs were washed twice in PBS and resuspended in PBS. Labeling was then carried out with 5 μM CellTrace Violet Dye for 20 minutes at room temperature (Life Technologies). 20% FBS in

PBS was then added to RBCs for quenching the staining reaction. Violet-labeled RBCs were washed twice with PBS and resuspended in 200μl sterile PBS for intravenous injection into recipient mice. An equal volume of unmodified RBCs, similarly stained, served as a control. A drop of blood, ~20 μl, was collected into heparinized tubes by retro-orbital bleeding 1 h after transfusion at day 0 and every 3–4 days for 1 month as indicated in the text. These blood samples were washed once with FACS buffer and then subjected to staining with anti-Ter119-APC and anti-myc tag-PE for 20 minutes on ice. Samples were washed twice with FACS buffer prior to analyses on a FACS Fortessa flow cytometer for violet fluorescence and for GFP, Ter119, and myc signals.

PCSK9 and LDL measurement

Blood samples ($\sim$ 100 μL) were collected by retro-orbital bleeding 3 h after transfusion at day 0 and at time points indicated in the text for the sortagging approach, or for transplanted mice every month after bone marrow reconstitution. Plasma from these samples were isolated and stored at -80°C after quick freezing with liquid nitrogen until all samples were collected for simultaneous analysis. The plasma samples were diluted (20 μL in 200 μL PBS) then plasma PCSK9 levels quantified using a Mouse Proprotein Convertase 9/PCSK9 Quantikine ELISA Kit (R&D systems) and plasma LDL levels measured using Cholesterol Assay Kit—HDL and LDL/VLDL (Abcam #ab65390). The detailed procedures were provided with the kit.

Normalization of PCSK9 levels

PCSK9 levels are determined by the average of triplicate assay reads from each plasma samples. PCSK9 levels of the treated group is normalized to the average levels of the control group at each time point and shown as percentage (with control cohort depicted as 100%) to highlight the significance of PCSK9 reductions.

Results

Engineered mouse RBCs that express the EGFA peptide at the cell surface

Our goal was to test the feasibility of a genetically engineered erythroid stem and progenitor cell therapy as a method to reduce LDL cholesterol. We generated retroviral constructs that can infect hematopoietic stem/progenitor cells (HSPCs) and allow expression of chimeric proteins on the surface of mature RBCs. We targeted glycophorin A (GPA) as the RBC membrane protein for the creation of a fusion protein, based on its abundance and RBC-specific expression. We genetically fused the EGFA- like domain of LDLR (EGFA) to the N-terminus of mature GPA to expose EGFA on surface of the RBC plasma membrane (Fig 1A). A myc-tag was inserted between the C-terminus of EGFA and N-terminus of GPA to facilitate detection. To mark all transfected cells and their progeny, the retroviral vector also contained a green fluorescent protein (GFP) under the control of the CMV promoter. We infected E14.5 mouse fetal liver derived HSPCs with these retroviruses as well as with an empty vector that expressed GFP as a control and cultured them *in vitro* to differentiate into reticulocytes. All terminally differentiated transfected and thus GFP+ reticulocytes expressed the chimeric GPA on their surface, as indicated by myc surface expression (Fig 1B). In this *in vitro* culture system, the EGFA-expressing cells underwent enucleation at a level similar to control cells. They showed similar CD71 and Ter119 surface expression, proliferation, and morphology compared to control cells, suggesting that the presence of the fusion does not disturb normal erythroid differentiation (Fig 1C–1F). Each reticulocyte was estimated to express ~9 million copies of EGFA-GPA (Fig 1G; see Methods for calculation).

Engineered mouse RBCs expressing EGFA peptides do not perturb erythropoiesis *in vivo*

Since our engineered erythroid progenitors underwent normal erythropoiesis *in vitro*, we next produced engineered RBCs *in vivo* by transplanting sub-lethally irradiated mice with fetal liver hematopoietic stem/ progenitor cells (HSPCs) infected by the retrovirus expressing both GFP and EGFA-GPA. Complete blood counts (CBCs) and myc surface expression (indicating the expression of the EGFA-GPA chimera) from one batch of EGFA-GPA-transplanted mice were followed over time (Fig 2B). Compared to control 7-week-old female mice, the blood parameters of the transplanted mice were within the normal range reported in the Mouse Phenome Database on the Jackson Laboratory website. Moreover, all of the GFP+ cells, the progeny of the transfected HSPCs, expressed myc on their surface (Fig 2C). In a separate batch of mice transplanted with HSPCs expressing EGFA-GPA, 7.59 ± 1.34% of the RBCs were myc + 4-weeks after bone marrow reconstitution (±S.E.M.; n = 12). There was no noticeable change in morphology and apoptotic marker in the engineered RBCs compared to controls (Fig 2D and 2E). To determine whether the introduction of EGFA-GPA impacts the circulatory half-life of RBCs, we stained isolated RBCs from the transplanted mice with the CellTrace Violet Dye prior to transfusion into normal recipients to monitor the total population of transfused RBCs. Cells expressing EGFA-GPA were monitored by the green fluorescent protein (GFP) signal (from the GFP expression cassette in the retroviral vector; Fig 2F). The circulatory lifespan of the transfused control and EGFA-GPA RBCs is similar to that of normal mouse RBCs (Fig 2F).

Mice transplanted with HSPCs expressing EGFA-GPA maintained lower plasma PCSK9 and LDL levels for at least one year

Mice expressing engineered RBCs were fed a normal diet for 12 months, and their weight, plasma LDL and PCSK9 were measured every 3 months. Over the course of this period, the numbers of EGFA-GPA RBCs, as detected by GFP+ myc+ RBCs, in transplanted mice, remained around 3–6% of total RBCs (Fig 3A; S1 Fig). We observed significant decreases in plasma LDL and PCSK9 levels in EGFA-GPA transplanted mice. Engineered RBCs thus sequester circulatory PCSK9 (Fig 3B and 3C). Lower levels of plasma LDL in these mice are correlated with a significant reduction in body weight gain over the ensuing 12 months (Fig 3D).

EGFA-GPA transplanted mice resist high-fat diet-induced obesity

To better mimic the type of diet that causes obesity, we fed mice a high-fat diet (~60% fat chow) for 8 weeks, and measured their weight, plasma LDL, and PCSK9 biweekly. The high-fat diet for mice also caused elevated LDL levels that are more similar to humans. The numbers of EGFA-GPA RBCs in transplanted mice, as indicated by GFP+ myc+ RBCs, remained steady at about 9–12% (Fig 4A; S2 Fig). Circulating PCSK9 levels in mice that carry EGFA-GPA RBCs were significantly lower than in controls across all time points, although PCSK9 levels did gradually increase over these 8 weeks (Fig 4B). Unexpectedly we did not observe a significant decrease in plasma levels of LDL (Fig 4C). Nonetheless, EGFA-GPA transplanted mice were still able to resist high-fat diet induced weight gain and maintained their weight for 8 weeks. This established the efficacy of the engineered RBCs in preventing diet-induced obesity (Fig 4D). Hematoxylin and eosin staining of various organs including heart, kidney, liver and spleen showed no abnormalities. We conclude that the presence of EGFA-GPA RBCs does not have obvious side effects despite their protection against obesity (Fig 4E).

A.

E14.5 fetal
liver-derived
HSPCs

Transplant

1 month
in vivo
differentiation

B.

C.

Gated on: Ter119+ GFP+

myc-tag ⟶

% GFP+ myc+ RBCs

EGFA-GPA

D.

Empty vector   EGFA-GPA

10μm

E.

Gated on: Ter119+ GFP+

Annexin V ⟶

F.

Fraction of cell survival

Days post-transfusion

Empty Vector
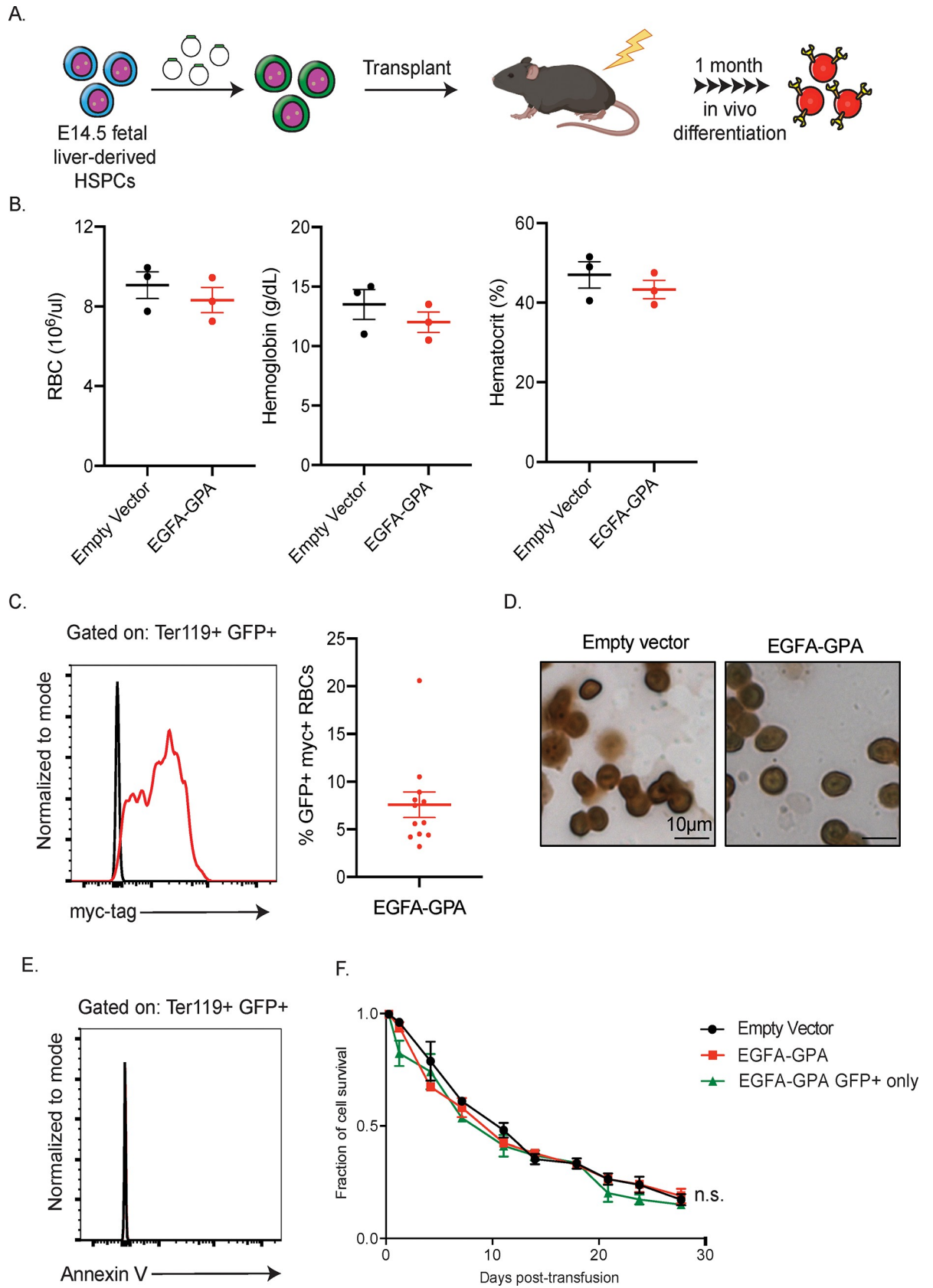EGFA-GPA
EGFA-GPA GFP+ only

n.s.

Fig 2. *In vivo* production of mature RBCs expressing EGFA-GPA. (A) Schematic depicting the generation of engineered mature RBC produced by transplanting genetically modified HSPCs. (B) Complete blood counts of 8-week-old female C57BL/6J mice subjected to bone marrow transplantation with progenitor cells expressing vector or EGFA-GPA and bled at 1-month post-transplantation. (n = 3/group, mean ± SEM) (C) At 1-month post-transplantation blood was analyzed by flow cytometry for the percentage of cells that were myc positive (right). As plasmids used to engineer the transplanted HSPCs contain GFP under the control of the CMV promoter to help indicate positive transduction, thus Ter119+ erythroid cells derived from the transplanted HSPCs were also GFP+ and were then analyzed for myc expression. (n = 12, mean ± SEM). (D) Benzidine-Giemsa and (E) Annexin V staining of the resulting RBCs. (F) Circulatory half-life of the transfused RBCs. 100 μl of blood from transplanted mice with ~8% of their RBCs expressing vector only or EGFA-GPA were stained with violet-trace dye and transfused into recipient mice. The fraction of transfused RBCs in recipients was analyzed by flow cytometry at the indicated time points. The violet-trace dye represents the total population of transfused RBCs, of which only ~8% are GFP+ and express the exogenous chimeric protein, while the GFP signal represents only the 8% of the transfused RBCs expressing the EGFA-GPA. (n = 3/group, mean ± SEM).
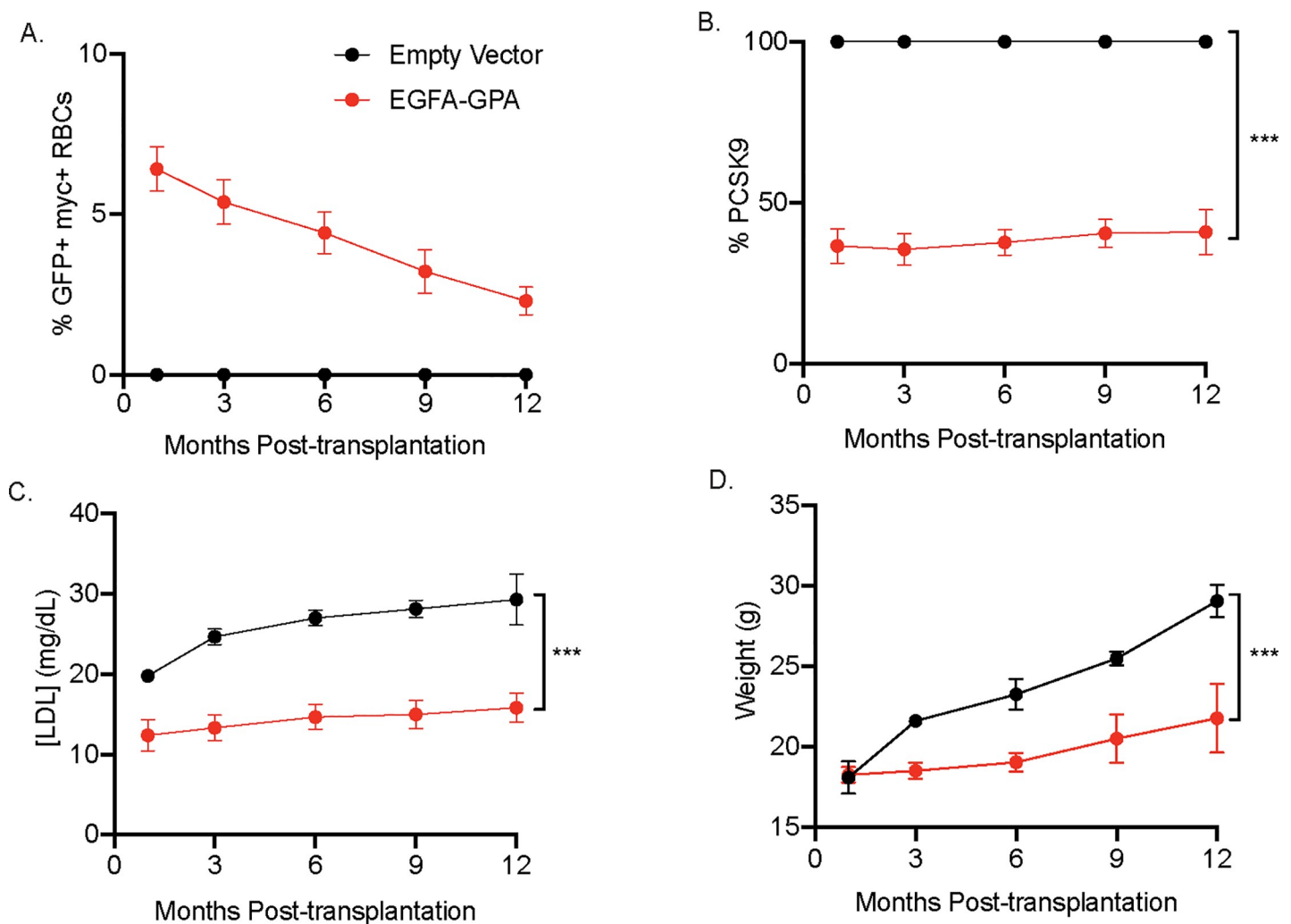
https://doi.org/10.1371/journal.pone.0259353.g002

Fig 3. EGFA-GPA—Expressing mice maintain lower plasma LDL level and weight for the remainder of their lifetime. (A) Long-term expression of myc+ cells (n = 12, mean ± SEM) in mice transplanted with HSPCs expressing the Glycophorin A- EGF-A chimeric protein. (B) Plasma PCSK9 and (C) LDL levels of transplanted mice were quantified via ELISA assay at the indicated time points. Engineered RBCs act as a sink in capturing circulating PCSK9 molecules, inhibiting LDLR degradation that resulted in lower plasma LDL concentration. (n = 8/group, mean ± SEM). (D) Mice expressing EGFA-GPA RBCs weigh significantly less than control mice throughout their observed lifespan (n = 8/group, mean ± SEM).
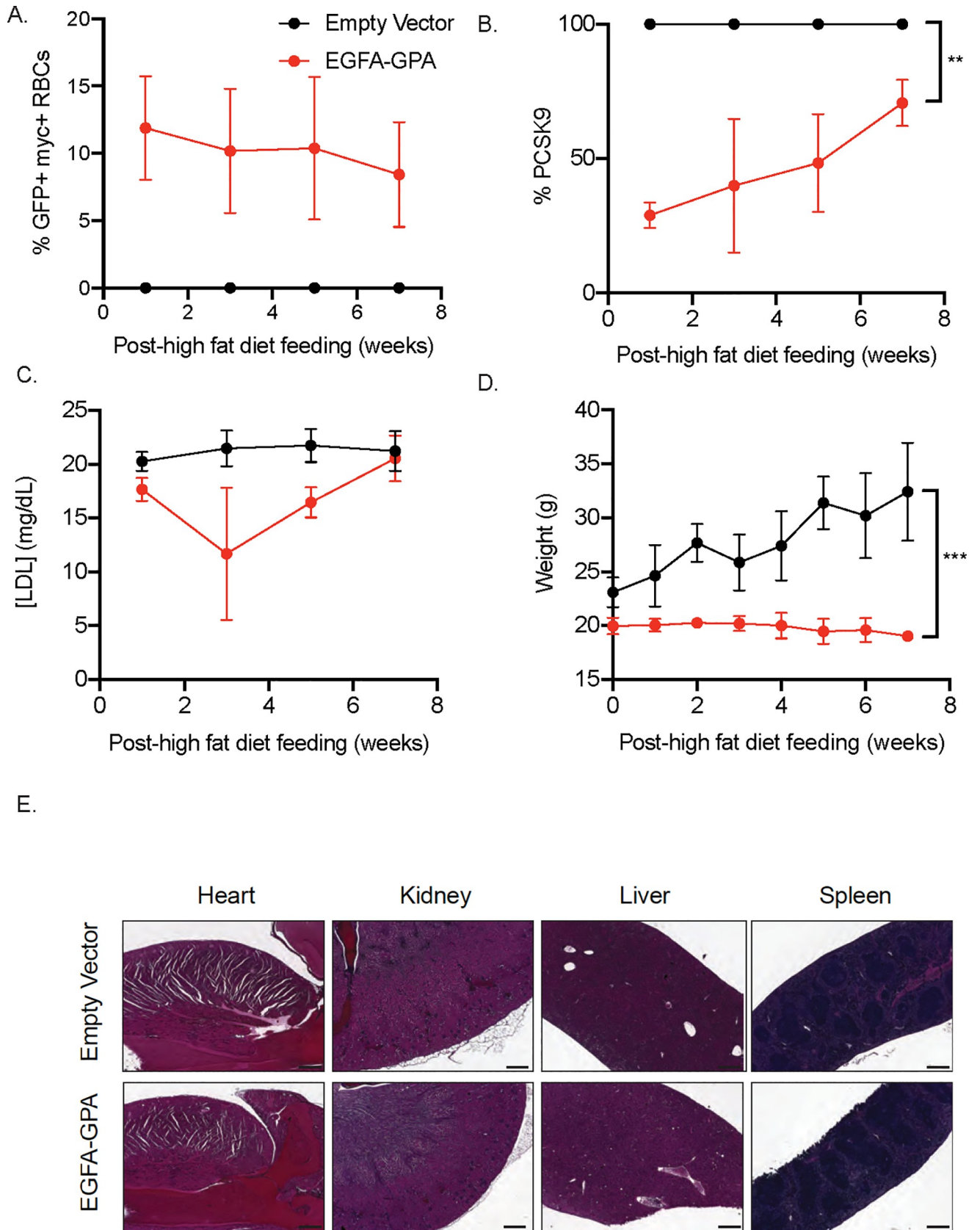
https://doi.org/10.1371/journal.pone.0259353.g003

Fig 4. Hematopoietic transplantation of engineered RBCs prevent weight gained in mice fed with a high-fat diet but unable to lower LDL levels.
Mice were placed on a high-fat diet 4 weeks after transplantation with HSPCs expressing the EGFA-GPA chimeric protein and monitored biweekly. (A)
Long-term expression of myc+ cells (n = 3, mean ± SEM). (B) Plasma PCSK9 and (C) LDL levels of transplanted mice were quantified via ELISA assay at
indicated time points. (n = 3/group, mean ± SEM). (D) Mice expressing EGFA-GPA RBCs weigh significantly less than control mice (n = 3/group,
mean ± SEM). (E) Hematoxylin and eosin staining of various organs: heart, kidney, liver and spleen.

Discussion

We showed that mouse RBCs, engineered to expressing on their surface a peptide PCSK9
inhibitor, can deplete circulatory PCSK9 by acting as a systemic sink. This depletion results in
a persistent reduction of plasma LDL levels. Animals that carry EGFA-GPA RBCs upon receiv-
ing modified hematopoietic stem cells, were resistant to diet-induced obesity. Transplantation
of autologous, genetically modified HSPCs that express an EGFA-GPA chimeric protein,
could therefore be a practical and one-shot preventative treatment for cardiovascular disease
and obesity in humans, especially those with a genetic predisposition to high cholesterol levels.

Selective bone-marrow depletion techniques that target and kill specific hematopoietic pro-
genitors, especially HSPCs, avoid complete myeloablation. This means that patients can
undergo bone-marrow transplantation without chemotherapy or radiotherapy and avoid the
toxic side effects that come with such treatments [19, 20]. We envision that this approach can
provide a lasting effect on lowering LDL and preventing obesity. We observed that the percent-
age of engineered RBC expressing the EGFA-GPA decreases overtime in the transplanted
cohort. However, the levels of PCSK9 and LDL did not correlate with the decreasing
EGFA-GPA RBC levels. This result suggested that we could potentially reach a similar decrease
of PCSK9 and LDL levels at a lower percentage of RBC reconstitution (<2%). This could also
explain the low variation in PCSK9 levels of the transplanted cohort despite the variation in
the engineered RBC expression. We generated another cohort of EGFA-GPA expressing mice
by transplanting fewer numbers of transduced hematopoietic stem cells/ progenitors (300,000
cells). The fewer number of transplanted HCS weren't enough in reconstituting the bone mar-
row after irradiation, and we were only able to generate four healthy mice. This new cohort
expressed EGFA-GPA RBCs in the range of 0.5–1.3%. In the previous cohort, the mouse that
expressed 0.8% of GPA-RBCs at month 12th, exhibited 48% of LDL reduction, which is
around the maximum levels of LDL reduction achieved throughout the study. Similarly, in the
lower GPA-EGFA expressing cohort, the mice with 0.8% GPA-EGFA had 45% reduction of
LDL. We believe 0.8% GPA-EGFA is the lowest number of RBCs to achieve clinically relevant
LDL reduction, since we notice a trend of decreasing percentage of GPA-EGFA RBCs that are
inversely correlated with LDL reduction levels (S3 Fig). We concluded the study at month 3,
since the lower GPA-EGFA expressing mice no longer expressed the modified RBCs at month
6.

We also envision that more potent inhibitors than the EGFA peptide might achieve even
greater reductions of plasma LDL levels and with fewer engineered RBCs. For example, one
can covalently attach EGFA peptide variants with enhanced affinity for PCSK9 act as a sys-
temic sink, or anti-PCSK9 camelid-derived single domain antibody fragments onto the glyco-
phorin A protein [21].

It is an intriguing finding that we observed a reduction in weight gain and resistance to diet
induced obesity in the EGFA-GPA mice. Since PCSK9 also enhances degradation of vLDLR
expressed in adipocytes, we actually expected the opposite result. Indeed, PCSK9 knock-out
mice show increased uptake of chylomicrons and vLDL accumulation in visceral fat, adipo-
cytes, heart and muscles, i.e. in tissues where vLDLR is expressed predominantly. However,
PCSK9 knock-out mice are not prone to obesity [22]. We suggest that using the EGFA-GPA

modified red blood cells, which persistently deplete only the circulatory PCSK9, may allow PCSK9 to continue to function normally in the liver in autocrine and paracrine manner. This may have affected lipid metabolism differently in comparison with complete ablation of PCSK9 in knock-out mice. Similarly, antibody treatment allows for inhibition of both circulating and liver PCSK9. Hence, we speculate that the decrease of weight gain is attributed to the inhibition of only circulating PCKS9 that is uniquely achieved in transplanted mice.

The depletion of only the circulating PCSK9 could also explain the reason why this approach is unable to lower LDL levels when mice are fed with a high-fat diet. Mice lipoprotein profile is different from humans, in which the majority of plasma cholesterol are in the form of HDL while in humans LDL accounts for the majority of total plasma cholesterol. The high-fat diet caused a significant increase of LDL in mice to levels that are more similar to humans. Our engineered RBC approach robustly decreased the PCSK9 levels in plasma, however, it may fail to significantly decrease the levels of PCSK9 in the extracellular spaces on the liver. Since liver accounts for the majority of LDL clearance, inhibition of only the circulating PCSK9 activity may not be sufficient to cause significant increase of LDL uptake, hence we observe very little to no decrease of LDL levels in transplanted mice. We did still observe weight difference in the transplanted vs. control cohort even to a greater degree, suggesting an unexplored function of circulating PCSK9 in lipid accumulation in peripheral tissues.

However, in trying to explain the unexpected findings, we acknowledged the limitation of the technique we used to determine LDL levels. The Cholesterol Assay kit by ABCAM quantifies free cholesterol level, in which the LDL/VLDL levels are determined after HDL are separated from serum samples. This approach is not the most reliable quantification method for LDL levels. For follow up studies, we are planning to perform LC/MS based quantification to profile the serum lipids in order to dissect the effect of depletion of only the circulating PCSK9 to lipid homeostasis.

In this study, we sought to develop a more practical treatment to lower LDL via a single transfusion of engineered hematopoietic stem cells to produce PCSK9 inhibitor-carrying RBCs. The depletion of circulating PCSK9 lowered LDL levels in transplanted mice, however, failed to effectively treat hypercholesterolemia under high-fat diet. Unexpectedly, we observed a significant difference in total body weight of the transplanted vs. control cohort. Since PCSK9 has a variety of functions outside the liver, not all of which are fully understood, the EGFA-GPA mice reported here could serve as a model to systematically explore the function of circulatory PCSK9 in the pathogenesis of diabetes, inflammation, sepsis, viral infections, and especially, obesity. With obesity and cardiovascular disease as main causes of death worldwide, a safe, effective and permanent therapy to inhibit PCSK9 can improve health span and quality of life. This preliminary study suggested the potential of using red blood cells genetically modified as circulating PCSK9 inhibitors to treat hyperlipidemia and obesity.

Supporting information

S1 Fig. Individual values corresponding to data depicted in Fig 3.
(TIFF)

S2 Fig. Individual values corresponding to data depicted in Fig 4.
(TIFF)

S3 Fig. Determining the minimum percentage of engineered RBCs reconstitution to sustain LDL reduction. (A) Expression of myc+ cells (n = 4, mean ± SEM) in mice transplanted with a reduced number of HSPCs expressing the Glycophorin A- EGF-A chimeric protein. (B) Plasma PCSK9 and (C) LDL levels of transplanted mice were quantified via ELISA assay at the

indicated time points. (D) weight of mice expressing EGFA-GPA RBCs. (n = 4, mean ± SEM) (E) Correlation of % GFP-EGFA expression with LDL reduction.

(PDF)

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