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Leveraging platelet for plasmid DNA delivery

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Leveraging platelet for plasmid DNA delivery

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ABSTRACT OF THE THESIS

Leveraging platelet for plasmid DNA delivery

by

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Master of Science in Bioengineering

University of California, Los Angeles, 2020

Professor Zhen Gu, Chair

Techniques for targeted delivery of nucleic acids are important for biomedical applications of gene therapy. Here, we leverage platelet to specifically deliver plasmid DNA to the target cells. It is demonstrated *in vitro* that the platelets transfected with GFP-encoding plasmids can deliver plasmids to HepG2 cells. Furthermore, platelets tend to interact more with HepG2 cells during co-culture compared with HeLa cells. Platelets deliver plasmids to HepG2 cells by actin-dependent endocytosis, whereas both asialoglycoprotein receptors of HepG2 cells and GPIbα of platelets are not involved in this process. This study provides evidences that platelet can be potentially engineered for liver targeted plasmid DNA delivery.

The thesis of Xuan Xu is approved.

Jun Chen

Song Li

Zhen Gu, Committee Chair

University of California, Los Angeles

2020

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1. Introduction

Gene therapies, including RNA interfering and genome editing, hold great promise for treating inherited diseases. However, several inherent features of the nucleic acid, the mediator of gene therapy, hinders its clinical translation. Specifically, nucleic acids have limited ability to penetrate the cell membrane, and they could not reach target tissues efficiently to exert effective treatment[1,2]. Therefore, techniques for *in vivo* delivery of therapeutic nucleic acids, such as small interfering RNA (siRNA) and DNA is important for the clinical translation of gene therapies[3–5]. Up to now, various types of materials, including cationic lipids[6,7], polymers[8,9] and inorganic nanoparticles[10], have been developed and used for nucleic acids delivery. These materials can physically interact with nucleic acids by electrostatic interactions or chemically conjugate to nucleic acids *via* covalent bonds[11,12]. With the aid of delivery tools, nucleic acids can be selectively delivered to target tissues and organs *via* tuning the size and composition of the delivery systems or *via* introducing targeting ligands[13,14]. In the meantime, various biological delivery systems based on viruses, cells and cell-derived components have also been developed, leveraging their inherent abilities of cargo delivery and tissue targeting[4,15–17]. For example, adeno-associated virus (AAV) vectors have been most commonly used for delivering nucleic acids *in vivo* due to their high transduction efficiency[4]. However, challenges associated with immunogenicity and low DNA package capacity remain to be solved[18,19]. In addition, cells and cell-derived particles, including autologous hematopoietic stem cell (HSC) therapy[20], chimeric antigen receptors (CARs) T-Cell therapy[21], exosomes[22], red blood cells[23] and platelets[24,25], have also been developed to deliver *ex vivo* gene. These biological delivery tools have inherent abilities to interact with specific cells and tissues, including participating in biological activities and transporting to target tissues and organs[15–17,20].

Platelets hold unique characteristics that have been leveraged to design drug delivery systems[17]. First, they play important roles in hemostasis and thrombosis; they are first responders being recruited to the site of vasculature damage for triggering hemostasis while they are the culprit in thrombosis[26,27]. Therefore, they have been engineered to deliver therapeutic drugs in the vascular system by leveraging their roles in hemostasis and thrombosis[28–31]. Second, platelets have been reported to interact with various cell types, including cancer cells, T cells, dendritic cells and hepatocytes[32–36]. The ability of platelets to interact with different cell types offers advantages for developing platelet-based drug delivery systems to target cells or tissues. For example, the platelet membrane has been utilized to coat nanoparticles to generate platelet mimetics for targeted drug delivery[37,38].

Recently, efforts have been made to utilize platelets for delivering synthetic nucleic acids. RNA was introduced into platelets by liposomes, making platelets a bioreactor for translating exogenous mRNA. Besides, plasmid DNA was introduced into megakaryocytes by electroporation, and vesicles shed by megakaryocytes were detected to contain plasmids. Moreover, nanoparticles coated with platelet membrane could deliver siRNA to treat breast cancer by RNA interference[24,25,39,40].

Platelets, anucleate cells with a diameter of 2-4 μm, are mainly derived from megakaryocytes residing in bone marrow and lung[41]. There are three major steps in platelet biogenesis. First, megakaryocytes need to undergo maturation before platelet biogenesis. Cells grow to a diameter of 100 μm after multiple rounds of DNA amplification, resulting in polyploid nucleus with 4N to

128N DNA. Meanwhile, interconnected membrane network structures are formed and platelet-associated proteins are synthesized in the cells. Second, the tubular pseudopodia structures branch out from megakaryocytes membrane with swelling tips in the end. And these swelling tips later become the major sites for platelet generation and release. Through these tubular structures, platelet-associated proteins and organelles are transported from megakaryocytes to distant tips for platelets generation. Third, the tips extend into bone marrow vascular sinusoids and nascent platelets are released from tips into the bloodstream[42]. Chemical and physical stimuli are required during this process. For example, Thrombopoietin (TPO) is one of the cytokines regulating platelet biogenesis from megakaryocytes, while blood shear forces help release platelets into the blood stream[43–45].

After entering the blood stream, the platelets can circulate for 3-5 days in mice and 7-10 days in human before being cleared[46]. Desialylation is one of the mechanisms regulating platelet clearance in liver[47]. Platelet surface proteins are heavily modified by polysaccharides, including *N*-linked and *O*-linked glycans and sialic acids are usually linked to the terminal of glycans[48–50]. Desialylation of *N*-linked glycans is triggered in some scenarios such as sepsis, senescence, and thrombocytopenia, which causes platelet accumulation and clearance in liver[36,51,52]. Studies reported that asialoglycoprotein receptor in the hepatic cells, including hepatocytes and kupffer cells, mediate accumulation and clearance of platelets in liver by recognizing desialylated terminal GalNAc or Gal of glycans[36,47].

Based on the properties of platelets, we propose that the platelet could act as a vector to deliver therapeutic plasmid DNA to hepatocytes in the liver after we transfected platelets with plasmids,

treated them with sialidase *ex vitro* and transfused them back into the body. Here, platelets were transfected with plasmid DNA by chemical reagents. In particular, transfected platelets could specifically deliver plasmid DNA to HepG2 cells through actin-dependent endocytosis. Therefore, the results provided preliminary evidences that platelets could potentially be engineered for delivering therapeutic plasmid DNA to the liver.

2. Materials and methods

2.1. Chemicals and reagents

Lipofectamine™ 3000 transfection reagent and CellTracker™ Green CMFDA Dye were purchased from Thermo Scientific. Neuraminidase from *Clostridium perfringens* (*C. welchii*), prostaglandin E_1 (PGE1), asialofetuin and 12-*O*-Tetradecanoylphorbol-13-acetate (PMA) were purchased from Sigma-Aldrich. Label IT® Nucleic Acid Labeling Reagent, Cy®5 for plasmid DNA labeling was purchased from Mirus Bio. Murine CD41, CD42d, CD62p (P-selectin) for fluorescence-activated cell sorting (FACS) analysis and human CD42b (GPIbα) for receptor inhibition were purchased from BioLegend. Buffer A (5 mM HEPES, 0.137 M NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.5 mM glucose, pH 7.4) and buffer B (140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, and 12.5 mM sucrose pH 6.0) were prepared and stored in 4 ℃ and warmed to room temperature before use.

2.2. Cell culture

The human hepatocellular carcinoma cell line, HepG2 cells, and HeLa cells were purchased from ATCC® and cultured in RPMI 1640 with 10% Fetal Bovine Serum (FBS). Murine megakaryocyte cell line L8057 was kindly provided by professor Alan Cantor (Boston Children's Hospital, Dana-

Farber Cancer Institute) and cultured in RPMI 1640 with 20% FBS with or without PMA.

2.3. Preparation of platelets

The platelets were extracted from megakaryocyte cell line L8057 cells. The cells were stimulated by 0.4 μM PMA for three days before the extraction. To isolate platelets from megakaryocytes, the cells culture medium was centrifuged at 200 g for 10 minutes (brake off). Then, the supernatant was carefully pipetted without disturbing the cell pellet and went through a 5 μm cell strainer (pluriStrainer, USA). Next, the filtered medium was centrifuged again at 200 g for 5 minutes (brake off), and then the supernatant was isolated to be centrifuged at 10,000 g for 20 minutes (brake off). After centrifugation, the supernatant was carefully removed. The white pellet was gently resuspended by buffer A with 1 μM PGE1 added, and the suspension was centrifuged at 100 g for 5 minutes (brake off) to remove the cell debris. The purified platelets in the supernatant was collected for downstream studies.

2.4. Transfection and purification of platelets

The plasmid pCAG-GFP (Addgene plasmid # 11150; http://n2t.net/addgene:11150; RRID:Addgene_11150) was a gift from Connie Cepko and Lipofectamine™ 3000 that was used for the plasmid transfection (amount of plasmid DNA : P3000™ reagent : Lipofectamine™ 3000 reagent = 1:2:2). After adding the transfection mixture to platelet suspension, it was incubated at 37 ℃ for 1 hour. Next, the suspension was centrifuged at 1200 g for 10 minutes and the white sediment is resuspended by buffer A with PGE1 added (note that in this step resuspended platelets must be pipetted to a new Eppendorf tube to avoid contamination of residue transfection reagent). The platelets were then washed twice by buffer A with PGE1 added.

2.5. *In vitro* platelet-mediated plasmid DNA delivery assay

HepG2 cells and HeLa cells were seeded in a 24-well plate (5×10^4 cells/well) one day before the experiment. During the experiment, the purified platelets were resuspended by buffer B with 5 mU μL^{-1} neuraminidase added and incubated for 30 minutes at 37℃. After centrifuged at 1200 g for 5 minutes, enzymatically treated platelets were resuspended with RPMI 1640 with 10% FBS and pipetted into each well. The platelets were incubated with cells for 30 minutes at 37℃ and then washed by Phosphate-Buffered Saline (PBS, pH 7.4). Next, cells were incubated in RPMI 1640 with 10% FBS for 48 hours. The GFP expression of cells was detected by a confocal microscope (Zeiss LSM 880, 10 ×). The number of GFP-positive cells was analyzed by a BD FACSCalibur flow cytometer and flow cytometric data were processed by FlowJo (version 10.0.7; TreeStar, USA).

2.6. *In vitro* platelet-cell interactions study

The plasmid was labeled by Cy5 following the protocol provided by the manufacturer. And the platelets were transfected with labeled plasmid DNA using Lipofectamine™ 3000 transfection reagent. Next, the transfected platelets were labeled with 5 μM CMFDA for 30 minutes at 37 ℃. After treated with 5 mU μl^{-1} neuraminidase, the labeled platelets were incubated with cells for 30 minutes at 37 ℃ and washed by PBS (pH 7.4). The cells were then fixed in 4% paraformaldehyde for 15 minutes at room temperature. The interactions between cells and platelets were observed by a confocal microscope (Zeiss LSM 880, 20 ×).

2.7. *In vitro* endocytosis and receptors inhibition study

To study the mechanism of the interactions between platelets and cells, cells or platelets were

treated with inhibitors and the effect of inhibition on the GFP expression was observed in the cells. we treated HepG2 cells with 10 μM cytochalasin D, an inhibitor of actin polymerization, to investigate whether or not the actin-dependent endocytosis was involved in the interactions of platelets with cells. Furthermore, we either treated HepG2 cells with 100 μg mL^{-1} asialofetuin, an inhibitor of asialoglycoprotein receptor, or blocked GPIbα on platelet surface with 10 μg mL^{-1} CD42b antibody to investigate whether these two potential receptors could mediate the interaction between platelets and cells. GFP expression in the cells was observed after 48 hours by confocal microscope (Zeiss LSM 880, 10 ×) or fluorescence microscope (Nikon, 10 ×).

2.8. Statistical analysis

All results are expressed as the mean ± SD. Biological replicates were used in all experiments unless otherwise stated. Two-tailed Student's t-test was used to analyze 2 experimental groups. All statistical analysis was performed by using the Prism (version 6.01; GraphPad software, USA). The threshold for statistical significance was $p < 0.05$.

3. Results

3.1. Platelets can be transfected with plasmids

The megakaryocyte cell line L8057 was used to extract platelets, and extracted platelets were confirmed by three platelet markers, CD41, CD42d, and CD62p (Fig. 1A). Platelets were transfected with 1 μg plasmids by Lipofectamine™ 3000 transfection reagent, resulting in ~28% platelets associated with plasmids (Fig. 1B-C). The results have show that megakaryocytes could be used to generate platelets *in vitro* and platelets could be transfected with plasmids.

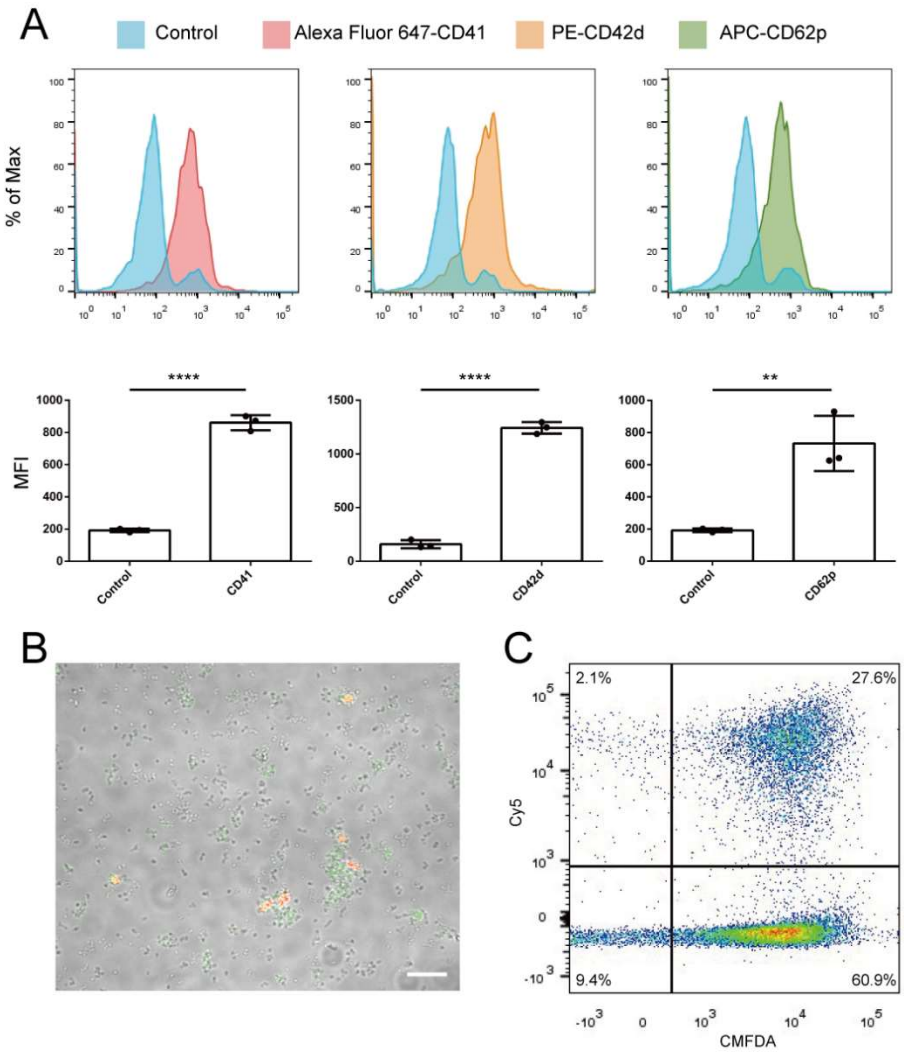

Fig. 1. Platelets transfection with plasmids. (A) Flow-cytometric analysis of platelets extracted from L8057 cells. The histogram graphs are representatives of three experiments. Group of extraction without staining was set as control. Data of the bar graphs are represented as mean ± SD (n=3). **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (B) Representative confocal image of platelets (CMFDA, green) after transfected with plasmids (Cy5, red). Scale bar, 10 μm. (C) Flow-cytometric analysis of platelets (CMFDA, green) after transfected with 1 μg plasmids (Cy5, red). The platelets associated with plasmids were identified as CMFDA and Cy5 double positive. The dot plot is representative of three experiments.

3.2. Platelets can specifically deliver plasmids to HepG2 cells

To investigate whether platelets can specifically deliver plasmids to hepatocytes, HepG2 cells were used as an *in vitro* cell model for hepatocytes, and HeLa cells were used as a negative control. Platelets were transfected with 1 μg plasmids encoding the green fluorescent protein (GFP) and then treated with 5 mU μL^{-1} neuraminidase for 30 minutes. Then, the treated platelets were incubated with HepG2 cells or HeLa cells for 30 minutes followed by washing with PBS (pH 7.4). After 48 hours, the GFP expression rate is higher in HepG2 cells than that of HeLa cells (Fig. 2A-B). To further observe the platelet interactions with HepG2 cells during co-culture, CMFDA (green) and Cy5 dye (red) were used to label platelets and plasmids, respectively. After a 30-minute co-culture, more platelets transfected with plasmids were found to be colocalized with HepG2 cells than with HeLa cells (Fig. 2C). These results suggested that GFP expression in HepG2 cells was attributed to platelets rather than non-specific transfection such as remaining Lipofectamine reagent.

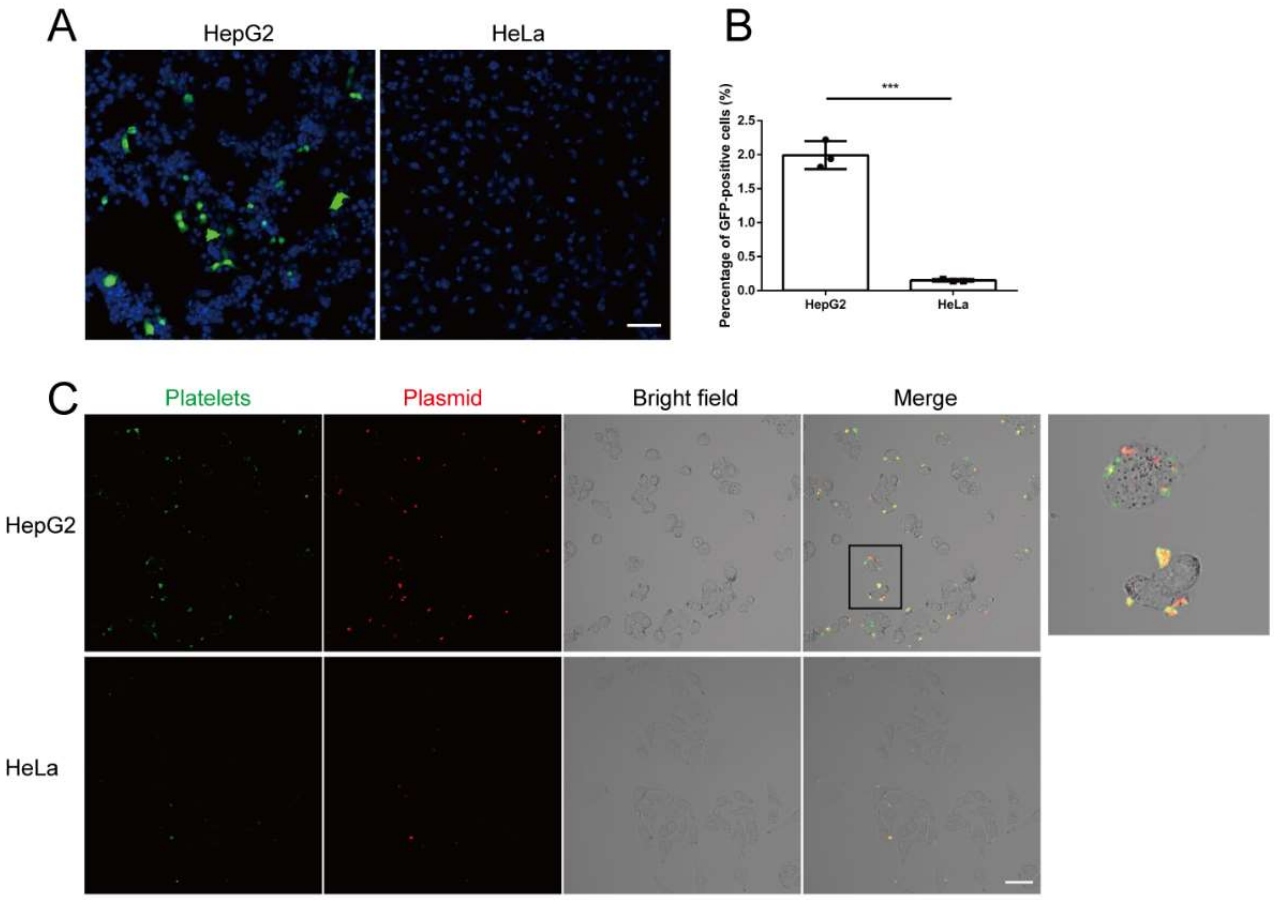

Fig. 2. Platelets can specifically deliver plasmids to HepG2 cells *in vitro*. (A) Representative confocal images of GFP-expressing HepG2 cells and Hela cells after 48 hours. HepG2 cells and HeLa cells were cocultured with platelets which were transfected with 1 μg plasmids and treated with 5 mU μL^{-1} neuraminidase. Both cells were stained by Hoechst 33342 (blue). Scale bar, 100 μm. (B) Quantification of GFP-positive HepG2 cells and HeLa cells after 48 hours. Data are represented as mean ± SD (n=3). ***$p < 0.001$. (C) Representative confocal images of platelet interaction with HepG2 cells and HeLa cells after a 30-minute co-culture. Platelets (CMFDA, green) were transfected with 1μg plasmids (Cy5, red). Scale bar, 50 μm.

3.3. Platelets deliver plasmids to HepG2 cells through actin-dependent endocytosis

To study the interaction mechanisms between HepG2 cells and platelets, HepG2 cells were treated with 10 μM cytochalasin D to inhibit actin polymerization and coculture with platelets. GFP expression of treated cells could not be detected by confocal microscope (Fig. 3). To investigate whether receptor-ligand interactions were involved in cell-platelet interactions, HepG2 cells were treated with asialofetuin and platelets were treated with CD42b antibody, respectively. However, both treatments did not inhibit GFP expression in HepG2 (Fig. S1). The results suggested that the plasmids were specifically delivered into HepG2 cells by actin-dependent endocytosis while neither asialoglycoprotein receptors of HepG2 cells or GPIbα of platelets were involved in this process.

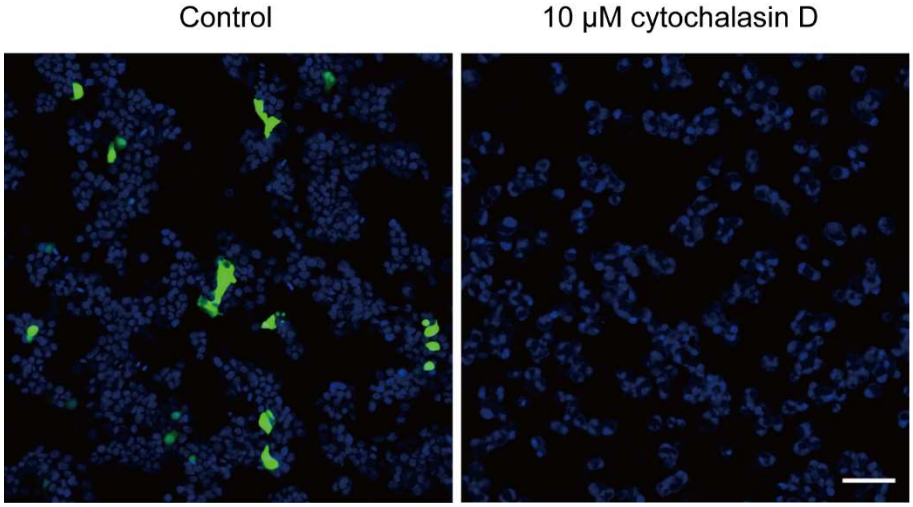

Fig. 3. Actin-dependent endocytosis is involved in platelet-mediated plasmid delivery. Representative images of GFP-expressing HepG2 cells with or without cytochalasin D treatment after 48 hours. Scale bar, 100 μm.

4. Discussion

The potential usage of plasmid DNA delivery is genome editing for gene therapy. Since

desialylated platelets can accumulate in the liver, platelets could potentially serve as a drug delivery vehicle targeting the liver tissues[36]. There are several metabolism diseases associated with hepatic genes[53,54]. For example, knocking down proprotein convertase subtilisin/kexin type 9 (Pcsk9) in the liver can decrease the risk of cardiovascular diseases[55]. Here, we shown that platelet could specifically deliver plasmids into HepG2 cells, which indicates that platelets can be potentially engineered to deliver therapeutic plasmid DNA to liver. For example, CRISPR-Cas9 system can be packed in plasmids and delivered to the liver by platelet for therapeutic genome editing (Fig. 4).

Furthermore, in addition to gene knockdown, CRISPR-Cas systems have been engineered for different functions, including gene expression regulation, DNA base editing, RNA editing and detection[56–58]. Plasmid DNA can potentially bring these functions to *in vivo* application and expand versatility of systems. The large size of plasmid constructs of CRISPR-Cas systems remain challenge to biological delivery tools. For example, ~8 kb Cas9 can not packed into AAVs which have maximum capacity of ~4.5 kb. Given the size of plasmid used for studies is ~4.8 kb, our studies suggest that platelet can become biological delivery tool for delivering plasmid DNA which might not be packed into AAVs.

The blood donation is the major source for platelet production. However, the platelet production at scale and the risk of contamination remain to be challenging to this approach[59]. In the meantime, techniques to produce platelets by megakaryocytes *in vitro* have been reported[60,61]. In our studies, the murine megakaryoctyes cell line has been used to produce platelets, and platelet associated markers were identified on the surface of them. Furthermore, either platelets extracted from blood

or they produced from cells can specifically deliver plasmids to HepG2 cells (Fig. S2), which might indicate two types of platelets have similar characteristics that can be recognized by HepG2 cells during the interactions such as receptors, secretory proteins and cytokines. Our studies serves as proof of concept for cell-based platelet production techniques. However, whether they have native physiological characteristics need to be further examined because platelets formation needs chemical and physical stimuli which were absent in *in vitro* cell culture[43–45,62].

Cellular hitchhiking is the method by which cells deliver cargo adsorbing onto the membrane and it is observed in nature. For example, *Listeria monocytogenes* can attach to platelet surface, and be shuttled and presented to the splenic dendritic cells[35]. This natural process has been leveraged as the drug delivery strategy. For example, circulation time for liposomes is prolonged by adsorbing to the red blood cell surface[63–65]. In the studies, there was the colocalization between plasmids and aggregation of platelets after transfection (Fig. 1B), indicating that platelet surface was charged by plasmid DNA-lipid complex adsorbing on the surface. Thus, it is possible that plasmid DNA was delivered to HepG2 cells by cellular hitchhiking, whererin plasmid DNA was first adsorbed onto the surface of platelets facilitated by the cationic lipids in the chemical reagents and then enters the cells after the platelet was ingested.

Although plasmid DNA can be specifically delivered to HepG2 cells by platelets, the efficiency is low, given that only ~2 % of cells measured express GFP after 48 hours incubation. The transfection reagent might be one of the major factors determining delivery efficiency by affecting the transfection efficiency of platelets[25]. To increase the efficiency of platelet transfection, optimization of the transfection reagents could be performed based on previous studies. Of note,

two factors have been reported to affect the uptake efficiency of synthetic lipids by platelets. First, cationic lipids can be more efficiently internalized by platelets than neutral ones. Second, platelets can take up particles with small size (< 200 nm in diameter) more efficiently[25,66–68]. In addition, surface modification of the nanoparticles can also facilitate platelet uptake. For example, fibrinogen can enhance platelet uptake of liposomes by being conjugated onto the liposome surface[66].

Asialoglycoprotein receptors of HepG2 cells and GPIbα of platelets have been reported to be involved in platelet clearance in liver by interacting with each other[36]. In addition, there are other receptors have been reported to be involved in recruiting platelets to liver. For example, hyaluronan-CD44 binding regulates platelets to be recruited to the liver to accelerate non-alcoholic steatohepatitis[69]. Our results shown that blocking either asialoglycoprotein receptors or GPIbα did not cause significant decrease of GFP expression in the cells, which might indicate that platelet interactions with liver are synergistically regulated by other receptors in addition to asialoglycoprotein receptors and GPIbα.

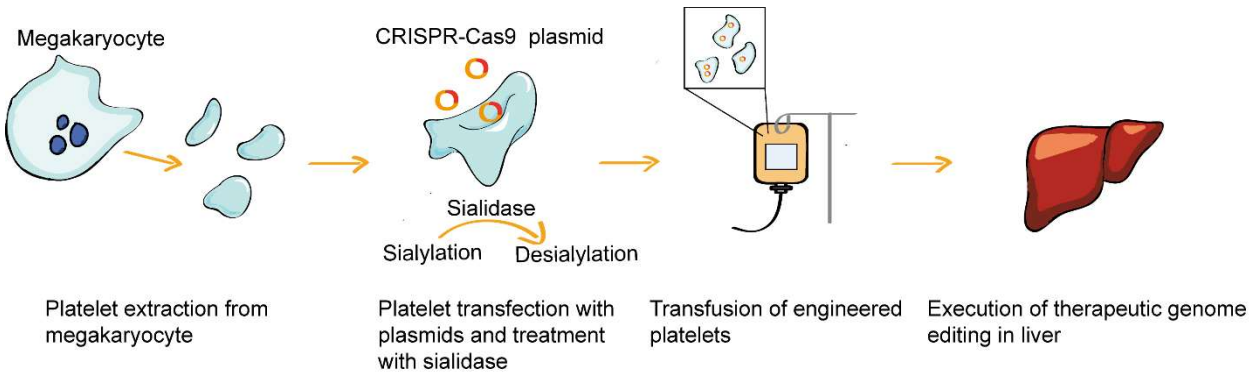

Fig. 4. Schematics of the workflow to engineer platelet to deliver CRISPR-Cas9 plasmid for therapeutic genome editing in liver.

5. Appendices

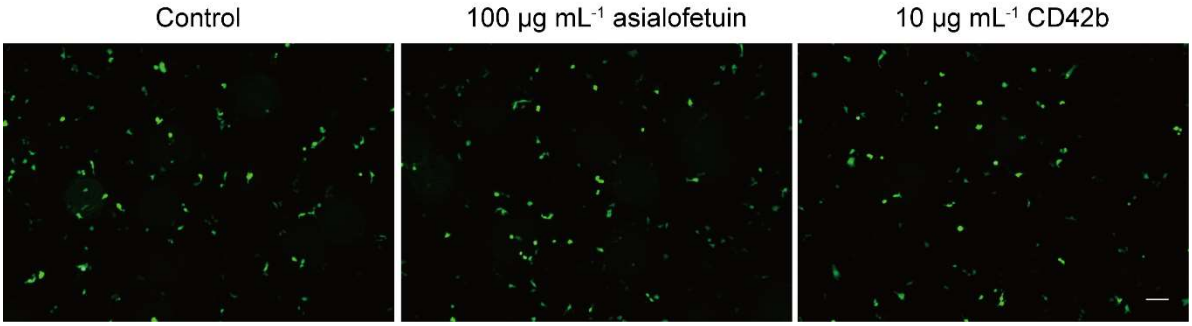

Fig. S1. **Asialoglycoprotein receptors and GPIb*α* are not involved in platelet-mediated plasmid delivery.** Representative images of GFP-expressing HepG2 cells after treated with asialofetuin or CD42b treatment for 48 hours. HepG2 cells without treatment were used as a control. Scale bar, 100 μm.

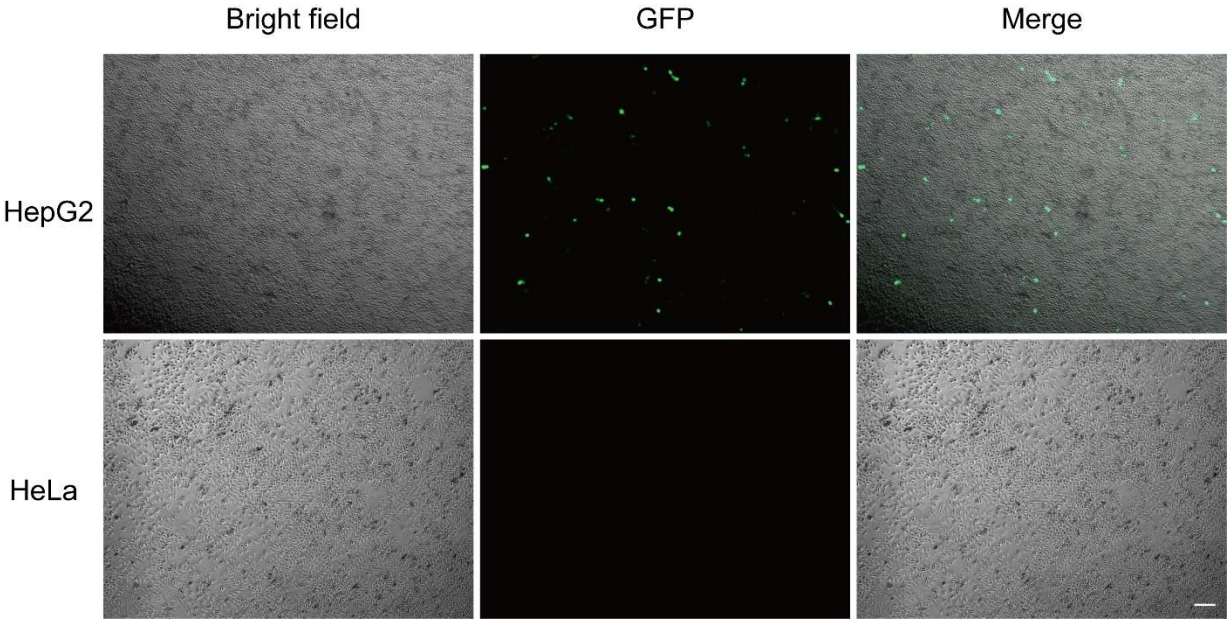

Fig. S2. Blood-derived platelets can specifically deliver plasmids to HepG2 cells *in vitro*. Representative images of GFP-expressing HepG2 cells and HeLa cells after 48 hours. HepG2 cells and HeLa cells were cocultured with blood-derived platelets that were transfected with 1 μg plasmids and treated with 5 mU μL^{-1} neuraminidase. Scale bar, 200 μm.

References

1. Han, S. O., Mahato, R. I., Sung, Y. K. & Kim, S. W. Development of biomaterials for gene therapy. *Molecular Therapy* **2**, 302–317 (2000).
2. Whitehead, K. A., Langer, R. & Anderson, D. G. Knocking down barriers: Advances in siRNA delivery. *Nature Reviews Drug Discovery* **8**, 129–138 (2009).
3. Setten, R. L., Rossi, J. J. & Han, S. ping. The current state and future directions of RNAi-based therapeutics. *Nature Reviews Drug Discovery* **18**, 421–446 (2019).
4. Dunbar, C. E. *et al.* Gene therapy comes of age. *Science* **359**, eaan4672 (2018).
5. Doudna, J. A. The promise and challenge of therapeutic genome editing. *Nature* **578**, 229–236 (2020).
6. Felgner, P. L. *et al.* Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proceedings of the National Academy of Sciences* **84**, 7413-7417 (1987).
7. Malone, R. W., Felgner, P. L. & Verma, I. M. Cationic liposome-mediated RNA transfection. *Proc. Proceedings of the National Academy of Sciences* **86**, 6077–6081 (1989).
8. Boussif, O. *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proceedings of the National Academy of Sciences* **92**, 7297–7301 (1995).
9. Park, T. G., Jeong, J. H. & Kim, S. W. Current status of polymeric gene delivery systems. *Advanced Drug Delivery Reviews* **58**, 467–486 (2006).
10. Sokolova, V. & Epple, M. Inorganic Nanoparticles as Carriers of Nucleic Acids into Cells. *Angewandte Chemie International Edition* **47**, 1382–1395 (2008).
11. Laemmli, U. K. Characterization of DNA condensates induced by poly(ethylene oxide) and polylysine. *Proceedings of the National Academy of Sciences* **72**, 4288–4292 (1975).

12. Jeong, J. H., Mok, H., Oh, Y. K. & Park, T. G. siRNA conjugate delivery systems. *Bioconjugate Chemistry* **20**, 5–14 (2009).

13. Love, K. T., Mahon, K. P. *et al.* Lipid-like materials for low-dose, *in vivo* gene silencing. *Proceedings of the National Academy of Sciences* **107**, 1864–1869 (2010).

14. Rozema, D. B., Lewis, D. L. *et al.* Dynamic PolyConjugates for targeted *in vivo* delivery of siRNA to hepatocytes. *Proceedings of the National Academy of Sciences* **104**, 12982–12987 (2007).

15. Kalluri, R. & LeBleu, V. S. The biology, function, and biomedical applications of exosomes. *Science* **367**, eaau6977 (2020).

16. Villa, C. H., Anselmo, A. C., Mitragotri, S. & Muzykantov, V. Red blood cells: Supercarriers for drugs, biologicals, and nanoparticles and inspiration for advanced delivery systems. *Advanced Drug Delivery Reviews* **106**, 88–103 (2016).

17. Lu, Y., Hu, Q., Jiang, C. & Gu, Z. Platelet for drug delivery. *Current opinion in biotechnology* **58**, 81–91 (2019).

18. Mingozzi, F., Maus, M. V., Hui, D. J. *et al.* $CD18^{+}$ T-cell responses to adeno-associated virus capsid in humans. *Nature medicine* **13**, 419–422 (2007).

19. Rogers, G. L., Martino, A. T., Zolotukhin, I., Ertl, H. C. J. & Herzog, R. W. Role of the vector genome and underlying factor IX mutation in immune responses to AAV gene therapy for hemophilia B. *Journal of translational medicine* **12**, 25 (2014).

20. Morgan, R. A., Gray, D., Lomova, A. & Kohn, D. B. Hematopoietic Stem Cell Gene Therapy: Progress and Lessons Learned. *Cell Stem Cell* **21**, 574–590 (2017).

21. Sadelain, M., Rivière, I. & Riddell, S. Therapeutic T cell engineering. *Nature* **545**, 423–431 (2017).

22. Alvarez-Erviti, L., Seow, Y. *et al.* Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nature biotechnology* **29**, 341–345 (2011).

23. Byun, H. M. *et al.* Erythrocyte ghost-mediated gene delivery for prolonged and blood-targeted expression. *Gene therapy* **11**, 492–496 (2004).

24. Chan, V., Novakowski, S. K., Law, S., Klein-Bosgoed, C. & Kastrup, C. J. Controlled Transcription of Exogenous mRNA in Platelets Using Protocells. *Angewandte Chemie International Edition* **54**, 13590–13593 (2015).

25. Novakowski, S., Jiang, K., Prakash, G. & Kastrup, C. Delivery of mRNA to platelets using lipid nanoparticles. *Scientific reports* **9**, 552 (2019).

26. Lefrançais, E., Ortiz-Muñoz, G. *et al.* The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature* **544**, 105–109 (2017).

27. Sun, H. *et al.* Platelet kainate receptor signaling promotes thrombosis by stimulating cyclooxygenase activation. *Circulation research* **105**, 595–603 (2009).

28. Korin, N. *et al.* Shear-activated nanotherapeutics for drug targeting to obstructed blood vessels. *Science* **337**, 738–742 (2012).

29. Hansen, C. E. *et al.* Platelet-Microcapsule Hybrids Leverage Contractile Force for Targeted Delivery of Hemostatic Agents. *ACS Nano* **11**, 5579–5589 (2017).

30. Wang, C. *et al. In situ* activation of platelets with checkpoint inhibitors for post-surgical cancer immunotherapy. *Nature Biomedical Engineering* **1**, 1-10 (2017).

31. Zhang, X. *et al.* Engineering PD-1-Presenting Platelets for Cancer Immunotherapy. *Nano letters* **18**, 5716–5725 (2018).

32. Karpatkin, S. & Pearlstein, E. Role of platelets in tumor cell metastases. *Annals of Internal Medicine* **95**, 636–641 (1981).

33. Camerer, E. *et al.* Platelets, protease-activated receptors, and fibrinogen in hematogenous metastasis. *Blood* **104**, 397–401 (2004).

34. Guidotti, L. G., Inverso, D. *et al.* Immunosurveillance of the Liver by Intravascular Effector $CD8^+$ T Cells. *Cell* **161**, 486–500 (2015).

35. Verschoor, A. *et al.* A platelet-mediated system for shuttling blood-borne bacteria to $CD8\alpha^+$ dendritic cells depends on glycoprotein GPIb and complement C3. *Nature immunology* **12**, 1194–1201 (2011).

36. Rumjantseva, V. *et al.* Dual roles for hepatic lectin receptors in the clearance of chilled platelets. *Nature medicine* **15**, 1273–1280 (2009).

37. Hu, Q. *et al.* Anticancer Platelet-Mimicking Nanovehicles. *Advanced Materials* **27**, 7043–7050 (2015).

38. Hu, C. M. J., Fang, R. H., Wang, K. C. *et al.* Nanoparticle biointerfacing by platelet membrane cloaking. *Nature* **526**, 118–121 (2015).

39. Kao, C. Y. & Papoutsakis, E. T. Engineering human megakaryocytic microparticles for targeted delivery of nucleic acids to hematopoietic stem and progenitor cells. *Science advances* **4**, eaau6762 (2018).

40. Zhuang, J. *et al.* Targeted gene silencing in vivo by platelet membrane-coated metal-organic framework nanoparticles. *Science advances* **6**, eaaz6108 (2020).

41. Lefrançais, E., Ortiz-Muñoz, G. *et al.* The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature* **544**, 105–109 (2017).

42. Patel, S. R., Hartwig, J. H. & Jr, J. E. I. The biogenesis of platelets from megakaryocyte proplatelets. *The Journal of Clinical Investigation* **115**, 3348–3354 (2005).

43. Kaushansky, K. & Drachman, J. G. The molecular and cellular biology of thrombopoietin:

The primary regulator of platelet production. *Oncogene* **21**, 3359–3367 (2002).

44. Kaushansky, K. The molecular mechanisms that control thrombopoiesis. *The Journal of Clinical Investigation* **115**, 3339–3347 (2005).

45. Jiang, J., Woulfe, D. S. & Papoutsakis, E. T. Shear enhances thrombopoiesis and formation of microparticles that induce megakaryocytic differentiation of stem cells. *Blood* **124**, 2094–2103 (2014).

46. Harker, L. A. The kinetics of platelet production and destruction in man. *Clinics in haematology* **6**, 671–693 (1977).

47. Sørensen, A. L. *et al.* Role of sialic acid for platelet life span: exposure of β-galactose results in the rapid clearance of platelets from the circulation by asialoglycoprotein receptor-expressing liver macrophages and hepatocytes. *Blood* **114**, 1645–1654 (2009).

48. Lewandrowski, U., Moebius, J., Walter, U. & Sickmann, A. Elucidation of *N*-glycosylation sites on human platelet proteins: A glycoproteomic approach. *Molecular & Cellular Proteomics* **5**, 226–233 (2006).

49. Lewandrowski, U., Lohrig, K., Zahedi, R. P., Wolters, D. & Sickmann, A. Glycosylation site analysis of human platelets by electrostatic repulsion hydrophilic interaction chromatography. *Clinical Proteomics* **4**, 25–36 (2008).

50. Crocker, P. R. & Varki, A. Siglecs, sialic acids and innate immunity. *Trends in Immunology* **22**, 337–342 (2001).

51. Grewal, P. K. *et al.* The Ashwell receptor mitigates the lethal coagulopathy of sepsis. *Nature medicine* **14**, 648–655 (2008).

52. Li, J., Van Der Wal, D. E., Zhu, G., *et al.* Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia. *Nature*

communications **6**, 7737 (2015).

53. Aponte, J. L. *et al.* Point mutations in the murine fumarylacetoacetate hydrolase gene: Animal models for the human genetic disorder hereditary tyrosinemia type 1. *Proceedings of the National Academy of Sciences* **98**, 641–645 (2001).

54. Angulo, P. Nonalcoholic Fatty Liver Disease. *New England Journal of Medicine* **346**, 1221–1231 (2002).

55. Cohen, J. C., Boerwinkle, E., Mosley, T. H. & Hobbs, H. H. Sequence Variations in *PCSK9,* Low LDL, and Protection against Coronary Heart Disease. *New England Journal of Medicine* **354**, 1264–1272 (2006).

56. Tanenbaum, M. E., Gilbert, L. A., Qi, L. S., Weissman, J. S. & Vale, R. D. A protein-tagging system for signal amplification in gene expression and fluorescence imaging. *Cell* **159**, 635–646 (2014).

57. Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A. & Liu, D. R. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **533**, 420–424 (2016).

58. Cox, D. B. T., Gootenberg, J. S., Abudayyeh, O. O. *et al.* RNA editing with CRISPR-Cas13. *Science* **358**, 1019–1027 (2017).

59. Strassel, C., Gachet, C. & Lanza, F. On the Way to *in vitro* platelet production. *Frontiers in medicine* **5**, 1–8 (2018).

60. Thon, J. N., Dykstra, B. J. & Beaulieu, L. M. Platelet bioreactor: accelerated evolution of design and manufacture. *Platelets* **28**, 472–477 (2017).

61. Heazlewood, S. Y. *et al.* Progress in bio-manufacture of platelets for transfusion. *Platelets* **28**, 649–656 (2017).

62. Leiva, O. *et al.* The role of extracellular matrix stiffness in megakaryocyte and platelet development and function. *American Journal of Hematology* **93**, 430–441 (2018).

63. Anselmo, A. C. & Mitragotri, S. Cell-mediated delivery of nanoparticles: Taking advantage of circulatory cells to target nanoparticles. *Journal of controlled release* **190**, 531–541 (2014).

64. Chambers, E. & Mitragotri, S. Prolonged circulation of large polymeric nanoparticles by non-covalent adsorption on erythrocytes. *Journal of controlled release* **100**, 111–119 (2004).

65. Chambers, E. & Mitragotri, S. Long circulating nanoparticles via adhesion on red blood cells: Mechanism and extended circulation. *Experimental biology and medicine* **232**, 958–966 (2007).

66. Nishiya, T. & Toma, C. Interaction of platelets with liposomes containing dodecapeptide sequence from fibrinogen. *Thrombosis and haemostasis* **91**, 1158–1167 (2004).

67. Fröhlich, E. Action of Nanoparticles on Platelet Activation and Plasmatic Coagulation. *Current medicinal chemistry* **23**, 408–430 (2016).

68. Gupalo, E., Kuk, C., Qadura, M., Buriachkovskaia, L. & Othman, M. Platelet–adenovirus vs. inert particles interaction: Effect on aggregation and the role of platelet membrane receptors. *Platelets* **24**, 383–391 (2013).

69. Malehmir, M., Pfister, D., Gallage, S., Szydlowska, M., Inverso, D. *et al.* Platelet GPIb*α* is a mediator and potential interventional target for NASH and subsequent liver cancer. *Nature medicine* **25**, 641-655 (2019).