Reprogramming megakaryocytes to engineer platelets as delivery vehicles

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We developed a method for the efficient generation of engineered platelets that can be filled with any recombinant therapeutic protein during the differentiation process by reprogramming megakaryocytes, the progenitor cells of platelets. To demonstrate the versatility of this approach, we loaded cytoplasmic and secreted proteins that can be delivered as active enzymes to recipient cells, be released upon platelet activation, or be continuously secreted by platelets over time.

Cell therapy is a rapidly advancing field with the potential to transform medicine across disease areas with significant therapeutic need[1-4]. A goal of synthetic biologists is to build genetic tools to reprogram cells for improved performance and new functions to enhance cellular capabilities for a variety of applications[5-7]. Platelets are anucleate blood cells that circulate throughout the body with diverse roles in hemostasis, wound healing, inflammation, and clot formation. The production of platelets begins when their progenitor cells, megakaryocytes (MKs), undergo a dynamic maturation process in which rapid cytoplasmic expansion occurs that includes an accumulation of essential proteins and α-granules required for platelet development and function. During this process, the MK cytoskeleton is reorganized to promote long branched structures, called proplatelets, that traffic proteins from the MK cell body into developing platelets that are released from their ends[8]. As platelets are released from proplatelets, they are naturally filled with proteins, many of which are packaged into the secretory α-granules that are released upon platelet activation to participate in a myriad of physiological processes[9]. Therefore, platelets are well equipped for protein delivery.

We have previously shown that pluripotent stem cells can be reprogrammed to load MKs with non-native proteins during the differentiation process[10]. Similarly, previous work has shown that activated platelets shed microparticles containing organelle, RNA, and protein content from MKs that can be transferred to recipient cells[11-14]. This led us to the hypothesis that platelets can be engineered and loaded with non-native proteins to be used as novel vectors for modulating target cells by delivering their payloads. To test this hypothesis, we developed a method to genetically

reprogram MEG-01 cells, a human megakaryoblast leukemia cell line known to release platelet-like particles (PLPs) that have characteristics similar to human platelets[15-17].

To achieve the goal of engineering platelets to deliver therapeutic cargos, we first transfected MEG-01 cells with green fluorescent protein (GFP), differentiated them into megakaryocytes, and harvested GFP-filled PLPs (Fig. 1a, b). We observed robust differentiation in the presence of thrombopoietin (TPO) (Fig. 1c) and GFP expression from the PLPs (Fig. 1d). To investigate whether the engineered PLPs can transfer functional proteins to target cells, we chose to deliver Cre recombinase to the Cre reporter HEK cell line, HEK293-loxP-GFP-RFP. Cre recombinase is an enzyme that catalyzes the site-specific recombination of DNA between loxP sites. In the HEK293-loxP-GFP-RFP cell line, GFP and a stop sequence are flanked by two loxP sites followed by a red fluorescent protein (RFP) gene.

We used Cre recombinase fused to an estrogen receptor (Cre/ER) which remains cytoplasmic in the absence of the activating ER-ligand, 4-hydroxy tamoxifen (4-OHT), thereby enabling efficient Cre/ER packaging into platelets from MKs (Supplementary Fig. 1). Engineered PLPs loaded with Cre/ER were co-cultured with the HEK293-loxP-GFP-RFP cell line and 4-OHT was added to the medium to activate the Cre/ER and promote its translocation to the nucleus in the HEK293-loxP-GFP-RFP cells after the delivery of Cre from the engineered PLPs (Fig. 1e). This enables the Cre to perform the DNA excision of GFP and the stop sequence, turning the cells from green to red (Fig. 1f). For this assay, the appearance of RFP-expressing HEK293 cells demonstrates intracellular transfer of a functional recombinase. After 48 hrs of co-culture, almost 40% of the HEK293-loxP-GFP-RFP cells expressed RFP demonstrating that MKs can be engineered to package non-native active enzymatic proteins into platelets for their delivery to other cell types (Supplementary Fig. 2).

An alternative to the direct transfer of proteins is the controlled release of protein cargo from engineered PLPs upon platelet activation. To evaluate this mode of delivery, we targeted our protein of interest to the α-granules of MKs for their packaging into the α-granules in PLPs (Fig. 2a). MKs naturally store a large number of bioactive proteins in their α-granules that are packaged into platelets. Initially we targeted GFP to the α-granules by fusing it to a short peptide sorting signal derived from the human cytokine RANTES to its 5' end [18]. Normally the distribution of GFP in MKs is evenly distributed throughout the cytoplasm (Fig. 2b), however, when targeted to the α-granules, the RANTES-GFP is clustered into discrete areas of the cell, consistent with being

packaged into granules[19] (Fig. 2b). In addition to targeting GFP to the α-granules, we studied the controlled release of a secretory protein from platelet α-granules. Secreted alkaline phosphatase (SEAP) is a widely used secreted reporter used to analyze protein secretion from cells into the culture medium. Expressing SEAP in engineered PLPs without targeting it to the α-granules resulted in the constant secretion from the engineered PLPs over 10 hrs (Fig. 1c, blue squares), whereas when SEAP is targeted to the α-granules in PLPs, most of its release occurs after activation with thrombin (Fig. 2c, red triangles).

In summary, we capitalized on the innate storage, trafficking, and release capabilities of platelets to rationally reprogram MKs to build delivery vehicles that can release bioactive proteins. Our proof-of-concept studies reveal an innovative and flexible approach to deliver therapeutic protein payloads to recipient cells using engineered platelets. We show that the delivery of these proteins is diverse and can be used to genetically reprogram target cells, to continuously release proteins over time during their circulation, or for the therapeutic protein to remain localized in α-granules to be released upon platelet activation. These results highlight the utility of reprogramming MKs with therapeutic proteins to be packaged into platelets that can be engineered with a variety of release profiles depending on the therapeutic need and offer an exciting entry point to develop new therapies for delivering therapeutic proteins and reprogramming cells *in vivo*. Future efforts are underway to engineer orthogonal triggers for platelet activation, including receptor engineering methods, for the targeted delivery of payloads[20-22].

Online content

Methods, including statements of data availability and any associated accession codes and references are available at (link inserted by journal).

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Author contributions

TLD conceived of the study and designed the experiments. FI conducted the MEG-01 transfections with GFP for Fig. 1b, SJ constructed the α-granule targeted GFP and SEAP plasmids He also carried out the Cre and SEAP experiments including all of the flow cytometry in this study. TLD constructed the Cre plasmid and performed all of the microscopy in this study. TLD wrote the manuscript with contributions from FI and SJ.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at (link inserted by journal).

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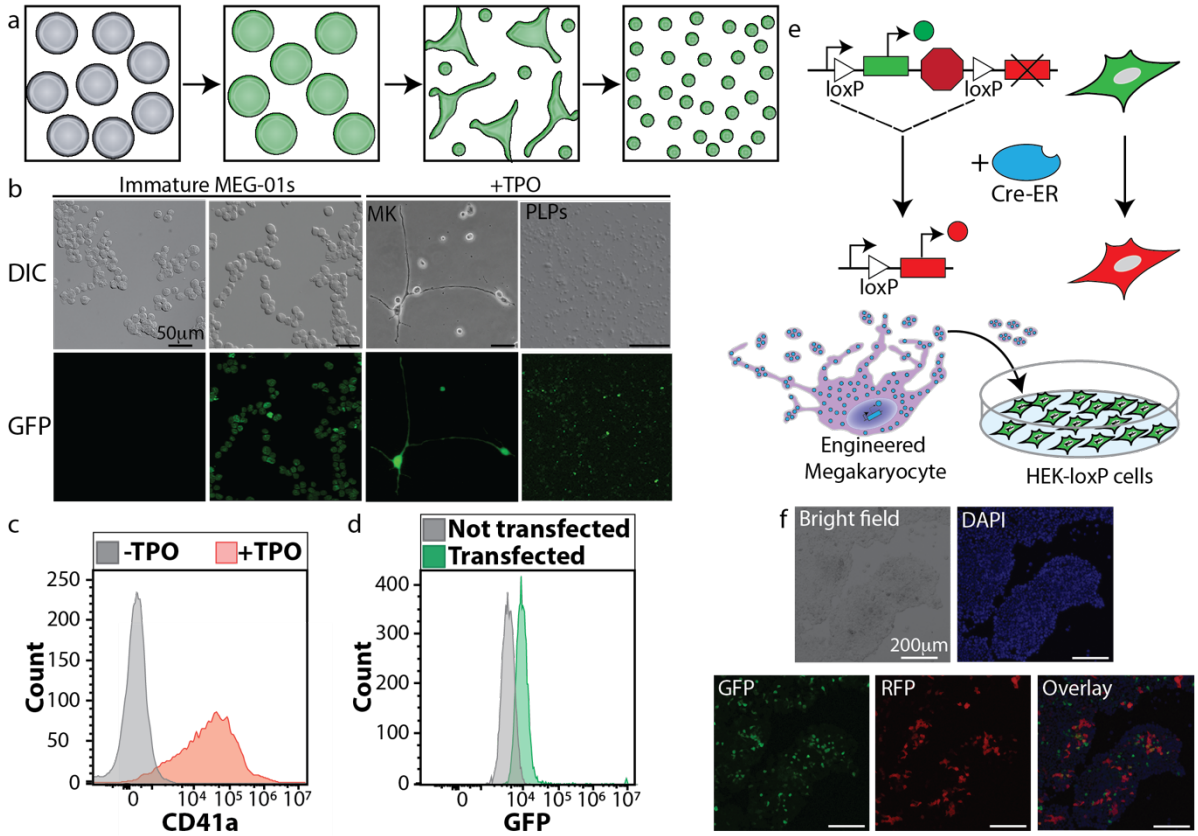

Figure 1. Reprogramming megakaryocytes to engineer platelets as delivery vehicles. a Schematic of the reprogramming process. First MEG-01s are transfected with a plasmid expressing GFP and differentiated in medium containing TPO for 48 hrs. Loaded PLPs were harvested from the medium. **b** DIC and GFP images of the cells during the reprogramming and differentiation process. **c** Flow cytometry of undifferentiated (-TPO, grey) and differentiated (+TPO, orange) MKs stained with the MK cell marker, CD41a. **d** Flow cytometry of MKs not transfected with GFP (grey) and MKs transfected with GFP (green). **e** LoxP-GFP-RFP Cre reporter HEK cell line. In the presence of Cre, the HEK cells turn from green to red with the removal of GFP and the stop sequence. Reprogrammed MKs are filled with Cre-ER and PLPs are harvested. **f** Engineered platelets filled with Cre-ER were co-cultured with the HEK-loxP cell line and supplemented with 4-OHT for 48 hrs. Images of the HEK-loxP cells after co-culture.

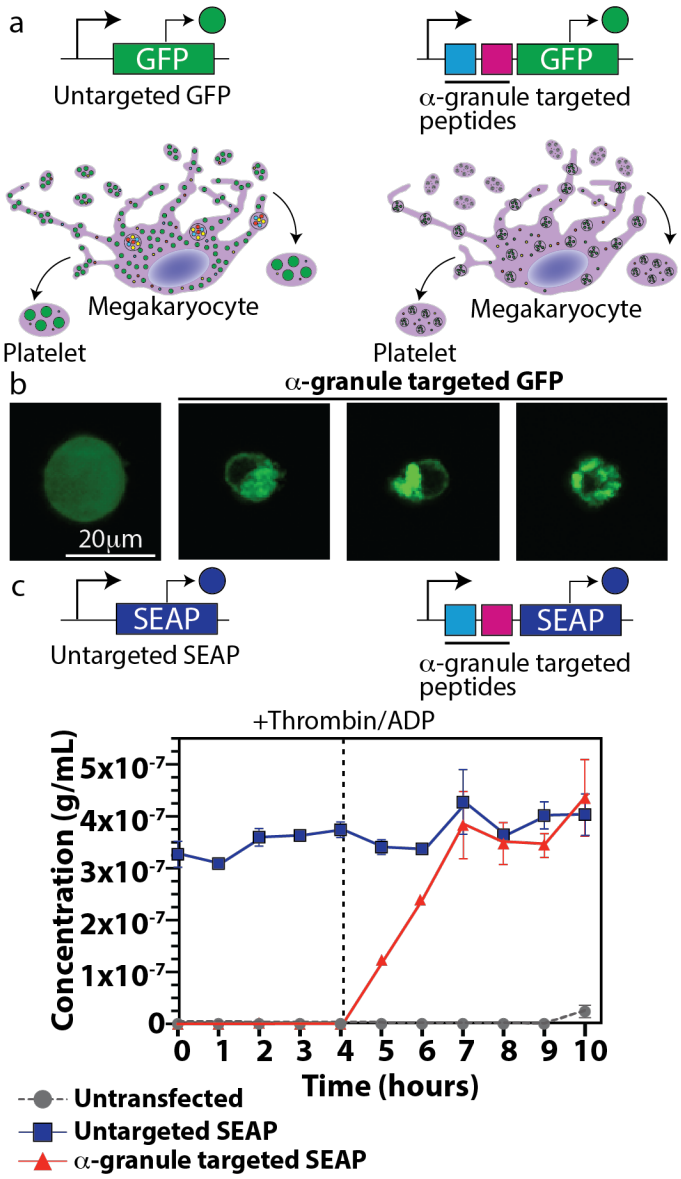

Figure 2. Targeting proteins to α-granules for their activated release. a Non-α-granule targeted GFP proteins in MEG-01s and PLPs (left) and GFP fused to peptides for targeting to α-granules (right). b Fluorescent image of non-targeted GFP in MEG-01s (left) compared to GFP that is targeted to the α-granules (right). c Non-α-granule-targeted SEAP and its release from PLPs over time (blue squares). SEAP targeted to the α-granules (red triangles) and its release upon activation with the addition of thrombin and ADP (dotted line). These experiments were repeated

independently at least three times with similar results. Error bars indicate the standard error of the mean.

Methods

DNA plasmids and molecular cloning TLD_010 (Addgene #19696) was used to constitutively express GFP in MKs. Due to complexity, the plasmid that expresses Cre-ER was constructed in parts by gBlock synthesis of DNA fragments (Integrated DNA Technologies) and assembled using standard molecular biology techniques including restriction enzyme digest (NEB) to cut the DNA fragments and T4 ligase (Thermo Fisher) to ligate the pieces together. Information on the cloning and deposition of plasmids constructed during this study can be found in Supplementary Fig. 1 and Supplementary Table 1. CMV-SEAP (Addgene #24595) was used to constitutively express SEAP in MKs. The α-granule targeted EGFP plasmid was made by gBlock synthesis of the short peptide sorting signals derived from the human cytokine RANTES and cloned into the TLD_010 plasmid to target the GFP to α-granules (Supplementary Fig. 4). The α-granule targeted SEAP plasmid was made by gBlock synthesis of the short peptide sorting signals derived from the human cytokine RANTES and cloned into the CMV-SEAP plasmid (Supplementary Fig. 6). Information on the cloning can be found in Supplementary Table 1.

Cell culture and transfection

<u>MEG-01 cells</u>

MEG-01 cells (ATCC) are semi-adherent cells and were maintained in RPMI 1640 medium (Gibco) supplemented with 10% Fetal Bovine Serum (FBS; Gibco) and 200 units/ml penicillin and streptomycin (Gibco). MEG-01s were passaged every three to four days, as needed. Briefly, the adherent cells were scraped using a cell scraper and the resulting suspended cells were counted and 1-2x10^5 cells/mL were centrifuged at 300*g* for 5 minutes and the pellet was resuspended and plated into a new flask. The standard transfection protocol was as follows, briefly: 300,000 cells were plated in a 24-well format and transfected 24 h later with 0.8μg of DNA using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For transfecting MEG-01s to load PLPs, 500,000 cells were plated in a 6-well format and transfected 24 hr later with 4μg of DNA using Lipofectamine 2000 following the manufacturer's instructions. The medium was replaced with fresh medium containing 100 ng/mL of recombinant human thrombopoietin (TPO; PeproTech) to mature the cells 8-12 h after the transfection. 48 h after transfection cells were

either examined using a Nikon TS100 epifluorescent microscope, scraped and prepared for flow cytometry analysis, and/or the PLPs were harvested.

Mature MK cell isolation

48 hr after exposure to 100 ng/mL of TPO cells, the culture flask was scraped with a cell scraper. All cells and medium were transferred to a conical tube and centrifuged at 100g for 5 minutes. The supernatant was either transferred to a fresh tube for PLP isolation or discarded. The MK pellet was resuspended in 100 μL PBs containing 3% bovine serum (BSA; Fisher Scientific) for antibody labeling.

PLP isolation

PLPs were isolated from MEG-01 cultures treated with 100 ng/mL of TPO for 48 hr by gently pipetting the medium over the bottom of the flask and transferring it to a 15 mL conical tube. Cells were centrifuged at 100*g* for 5 minutes and the medium was transferred to a fresh 15 mL conical tube and the pellet was discarded or saved for MK isolation. The cells were centrifuged again at 1,200*g* for 15 minutes and the PLP pellet was resuspended in 100 μL PBS containing 3% BSA for antibody labeling.

HEK-293-loxP-GFP-RFP cells

HEK-293-loxP-GFP-RFP cells (GeneTarget Inc) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco), and supplemented with 10% FBS and 200 units/ml penicillin and streptomycin.

Coincubation of PLPs with HEK-293-loxP-GFP-RFP cells. 10,000 HEK-293-loxP-GFP-RFP cells were plated in a 24-well format. The next day loaded PLPs were isolated from 2 wells of transfected MEG-01 cells, resuspended in HEK-293 growth medium, and coincubated with the HEK-293-loxP-GFP-RFP cells in one well. To enable the Cre recombinase to translocate to the nucleus of the HEK-293-loxP-GFP-RFP cells, a 1 mg/mL stock solution of 4-OHT (Millipore Sigma) was prepared in

ethanol and added to the medium to a final concentration of 40 ng/mL. 48 hr after coincubation, the HEK-293-loxP-GFP-RFP cells were analyzed by flow cytometry and microscopy.

All cell lines were grown in a humidified 5% CO_2, 37°C incubator.

SEAP Assay

SEAP levels were determined by adding QUANTI-Blue reagent (Invitrogen), whose change in color intensity from pink to purple/blue is proportional to the enzyme's activity. In brief, medium was replaced 8-12 hrs after transfection with fresh medium containing 100 ng/mL of TPO for ~40 hr before starting the time-course measurements. For each time point, 180 μL of Quanti-Blue working stock was added to each well of a 96-well plate and 20 μL of cell culture supernatant from the SEAP-expressing cells and incubated at 37°C, 5% CO_2 for 2 hrs. After incubation, SEAP activity was assessed by measuring the OD_{625} (Synergy HTX Reader, Biotek). Immediately after measuring the 3-hr timepoint, MKs and PLPs were activated with 1 U/mL of thrombin (Millipore Sigma) using a 50 U/mL stock solution that was prepared in PBS, 7.4 and 100 μM of adenosine 5'diphosphate (ADP; Millipore Sigma) that was from a stock solution of 25 mg/mL prepared in sterile Milli-Q water. Because FBS in the culture medium contains some basal alkaline phosphatase, all OD_{625} data were base-line corrected by subtracting the average basal OD_{625} value at each time point for untransfected MEG-01 cell culture medium. To assess the time for the reaction to reach a plateau, the CMV-SEAP plasmid was transfected in MEG-01 cells and the at OD_{625} was measured (Supplementary Fig. 3). Before base-line correction, we found that the enzymatic reaction reached a plateau at 2 hr. Baseline=corrected OD_{625} was plotted as a function of time using GraphPad Prism (GraphPad Inc). To determine the concentration of protein secreted by the cells, recombinant SEAP was serially diluted in culture medium at known concentrations and measured 2 hr later in assay media with an n=3 of independent replicates (Supplementary Fig. 5). GraphPad Prism was used to fit the data to a dose-response curve for converting OD_{625} values into concentrations of SEAP using the standard approach of:

$$Y = Bottom + (X\text{^}Hillslope) * (Top - Bottom)/(X\text{^}HillSlope + EC50\text{^}HillSlope)$$

Microscopy

In Figure 1b, cell fluorescent images were acquired using the Nikon TS100 microscope equipped with a SOLA light engine, 380-680 nm wavelength range and a Nikon EF-4 ENDOW GFP HYQ excitation/emission filter, and a ProgRes high resolution monochrome camera. All other images were acquired using a Zeiss Axio Observer 7 live cell imaging inverted fluorescence microscope.

Cell staining

For nucleated cells (MEG-01s, MKs, and HEK-293-loxP-GFP-RFP cells), cells were stained with a 1:100 dilution of a 1 mg/mL stock of Hoechst 33342 and incubated at 37°C, 5% CO_2 for 30 minutes. After incubation, the cells were washed with PBS and resuspended in 500 μL of PBS for flow cytometry analysis. The anucleate PLPs were stained with calcein red-orange AM at a dilution of 1:40 from a 1mM stock (Invitrogen) following the manufacture's protocol. For confirming mature MKs and PLPs, APC-conjugated mouse anti-human CD41a (BD Biosciences) was used at 1:100 dilution. For the cells stained with CD41a, cells were resuspended in PBS with 3% BSA and after Hoechst incubation, CD41a antibody was added and cells were incubated at 37°C, 5% CO_2 for an additional 30 minutes.

Flow cytometry

EGFP transfected cells and HEK-293-loxP-GFP-RFP cells before and after PLP coincubation were measured with a CytoFLEX S cytometer (Beckman Coulter) using 488 nm laser with a 525/50 filter for measuring EGFP expression and a 561 nm laser with a 610/20 filter for measuring RFP. Gates were constructed inside forward and side scatter plots (FSC-A vs SSC-A) to exclude debris and non-singlet events (Supplementary Fig. 2). Cytometry data were analyzed and processed with FlowJo software (FlowJo LLC).