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BRIEF REPORT

Genetic engineering of megakaryocytes from blood progenitor cells using messenger RNA lipid nanoparticles

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Abstract

Background: Platelets are an essential component of hemorrhage control and management, and engineering platelets to express therapeutic proteins could expand their use as a cell therapy. Genetically engineered platelets can be achieved by modifying the platelet precursor cells, megakaryocytes (MKs). Current strategies include transfecting MK progenitors *ex vivo* with viral vectors harboring lineage-driven transgenes and inducing the production of *in vitro* modified platelets. The use of viruses, however, poses challenges in clinical implementation, and no methods currently exist to genetically modify MKs with nonviral techniques. Lipid nanoparticles (LNPs) are a nonviral delivery system that could enable a facile strategy to modify MKs with a variety of nucleic acid payloads.

Objectives: To investigate whether LNPs can transfect cultured hematopoietic stem/progenitor cell–derived MKs to express exogenous proteins and induce functional changes.

Methods: MK and MK progenitors differentiated from cord blood–derived hematopoietic stem/progenitor cells were treated with LNP formulations containing messenger RNA and resembling the clinically approved LNP formulations. Transfection efficiency was assessed through flow cytometry by expression of enhanced green fluorescent protein. Functional changes to the MKs were assessed through rotational thromboelastometry by expression of exogenous coagulation factor (F)VII, a representative physiologically relevant protein.

Results: LNPs enabled transfection efficiencies of 99% in MKs and did not impair MK maturation, viability, and morphology. MKs engineered to express exogenous FVII decreased clotting time in FVII-deficient plasma following clot initiation.

Conclusion: This approach provides an easy-to-use modular platform to genetically modify MK and MK progenitors, which can be potentially extended to producing genetically modified cultured platelets.

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Jerry Leung and Asel Primbetova contributed equally to this study.

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KEYWORDS
cell therapy, genetic engineering, megakaryocytes, mRNA, nanoparticle drug delivery system

1 | INTRODUCTION

Platelets play important roles in hemostasis [1], inflammatory diseases [2], wound healing [3], and cancer among other processes [4]. Although platelet transfusions are largely restricted to hemostatic indications, genetically modifying platelets with new or enhanced function could expand their use as a cell therapy to treat other pathological conditions [5,6]. However, limited tools are available for genetically modifying platelets. Although platelets used for transfusions are collected from donors, platelets can also be cultured *in vitro*. Indeed, improved cell culture systems can now enable large-scale production of *in vitro* transfusable platelets, which coupled with gene therapy technologies could lead to the production of engineered *in vitro* platelets designed for specific transfusion needs [7].

Platelets are produced by megakaryocytes (MKs), large polyploid cells derived from hematopoietic stem cells. Engineering platelets *in vitro* currently involves genetically modifying hematopoietic or induced pluripotent stems cells *ex vivo* via viral vectors with lineage-specific transgenes. Transduced stem cells further differentiate into MKs to ultimately produce modified, *in vitro* platelets [8,9]. While viral transduction is an important tool for engineering MKs and platelets, its clinical implementation remains challenging. Virus type can affect transfection efficiency and payload size, therefore limiting efficacy [10]. Current viral-based strategies also rely on transducing stem cells prior to differentiation, which can affect cellular viability and differentiation [11]. Alternative methods may help mitigate these challenges; however, there are currently no reported nonviral transfection agents for genetically modifying MKs.

Lipid nanoparticles (LNPs) are well-established vectors for efficient cellular transfection, demonstrated by their clinical application as SARS-CoV-2 vaccines and treatment for hereditary transthyretin amyloidosis [12–14]. Compared with viral vectors, LNPs are less immunogenic, amenable to various nucleic acid payloads, and easier to synthesize with lower manufacturing costs [15]. LNPs containing messenger RNA (mRNA; mRNA-LNPs) have also enabled expression of exogenous proteins directly in donor platelets [16], suggesting that similar LNPs transfect MKs. Here, we demonstrate that LNPs resembling clinically approved RNA-LNP formulations transfect hematopoietic stem/progenitor cell (HSPC)–derived MKs and elicit functional changes when evaluated *ex vivo*.

2 | METHODS

2.1 | MK expansion

Cord blood–derived cryopreserved $CD34^+$ HSPCs were provided by the laboratory of Dr Connie Eaves and processed as previously described [17]. Briefly, mononuclear cells were isolated by density gradient centrifugation in RosetteSep (STEMCELL Technologies). $CD34^+$ cells were then isolated using EasySep Human $CD34^+$ Positive Selection Kit (STEMCELL Technologies), cryopreserved in fetal bovine serum with dimethyl sulfoxide (9:1 volume per volume), and stored at −150 °C. When needed, cells were thawed at 37 °C, and 10× volume of 37 °C warmed Iscove's Modified Dulbecco's Medium (Gibco) was added dropwise. Cells were centrifuged for 7 minutes at 300 × *g* and cultured for 12 days at 300 000 cells/mL in StemSpan Serum-Free Expansion Medium (STEMCELL Technologies) supplemented by 25 ng/mL stem cell factor, and 50 ng/mL of thrombopoietin. Unless otherwise stated, media was replaced every 3 days. The study was approved by the University of British Columbia Research Ethics Board (H20-01839).

2.2 | Formulating mRNA-LNPs

mRNA-LNPs were formulated as previously described [18]. Briefly, an ethanolic lipid mixture containing an ionizable lipid (DLin-MC3-DMA, ALC-0315 (DC Chemicals), or SM-102 (Cayman Chemical)), distearoylphosphatidylcholine (Avanti Polar Lipids), cholesterol (Sigma-Aldrich), and a PEGylated lipid (PEG-c-DMG, ALC-0159 (Cayman Chemical), or PEG-DMG (Avanti Polar Lipids); 50:10:38.5:1.5 mol%) was mixed via a T-junction with an aqueous solution containing mRNA (enhanced green fluorescent protein [EGFP], firefly luciferase [FLuc], or murine coagulation factor [F]VII, 5-methoxyuridine or N^1-methyl-Ψ modified; TriLink Biotechnologies, NanoVation Therapeutics, RNA Technologies and Therapeutics) at a 1:3 ratio and amine-to-phosphate ratio of 6. The subsequent mixture was dialyzed 500× against 1× phosphate-buffered saline overnight, sterile-filtered with a 0.2-μm syringe filter, and concentrated in Amicon 10 000 kDa molecular weight cutoff ultracentrifugation units (MilliporeSigma). RNA encapsulation and concentration were determined using the RiboGreen Quant-it Kit (Thermo Fisher Scientific).

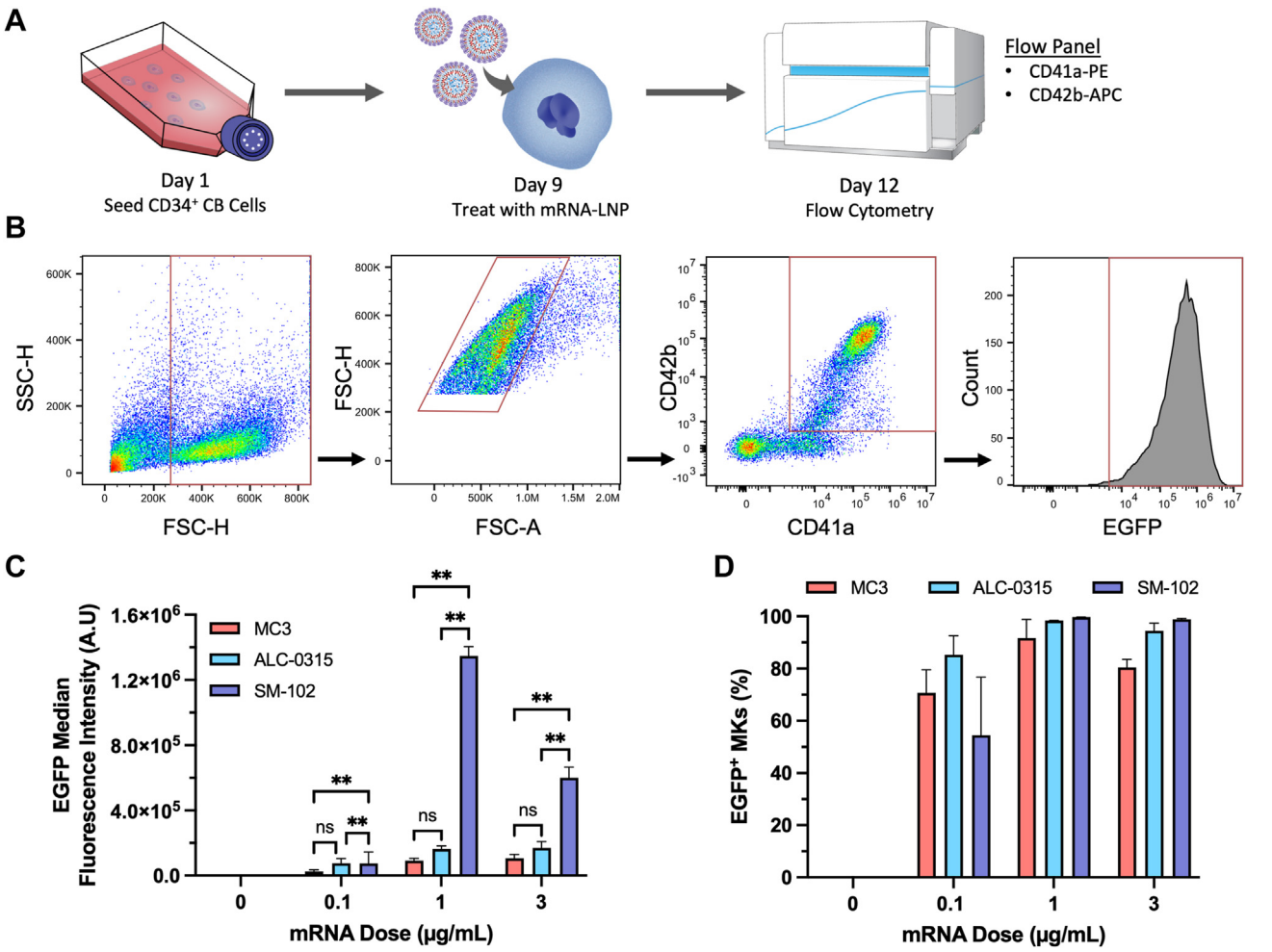

FIGURE 1 Exogenous messenger RNA (mRNA) delivered to megakaryocytes (MKs) via lipid nanoparticles (LNPs) can be translated with high transfection efficiency. (A) Schematic of the culturing and transfection of MKs. (B) Representative gating scheme for flow cytometry. Cells were first gated by the forward (FSC-H) and side scatter height (SSC-H), whereas single cells were then gated by the forward scatter area (FSC-A) and height. MKs were then identified as cells positive for both CD41a and CD42b before a final gate for enhanced green fluorescent protein (EGFP) fluorescence. Gated populations are denoted by the red box. Quantification of the (C) median fluorescence intensity and (D) percentage of MKs positive for EGFP 72 hours after treatment with mRNA-LNPs encoding for EGFP and containing the ionizable lipids MC3, ALC-0315, or SM-102. Data were analyzed by 1-way analysis of variance followed by a Bonferroni post hoc correction. Data represented by mean ± SEM (n = 3 biological replicates). APC, allophycocyanin; CB, cord blood; ns, not significant; PE, phycoerythrin. $^{**}P < .01$.

2.3 | Treating with mRNA-LNPs

On day 9 or 11 of culture, cells were treated with 0.1, 1, or 3 µg/mL mRNA-LNP at approximately 300 000 cells/mL. Media was supplemented with 1 µg/mL apolipoprotein E. Cells were then further incubated at 37 °C (5% CO_2 and 20% O_2) before assaying for EGFP or FVII expression on day 12. Untreated cells were supplemented only with apolipoprotein E.

2.4 | Flow cytometry

On days 9, 11, and 12 of culture, cells were labeled with phycoerythrin (PE)-anti-CD41a (HIP8, BD Pharmingen, diluted 1:100), allophycocyanin (APC)-anti-CD42b antibodies (HIP1, Invitrogen, diluted 1:200), and the viability marker Zombie UV (BioLegend, diluted 1:500) for 30 minutes on ice. The cells were then washed twice in Hanks' Balanced Salt Solution (Gibco) supplemented with 2% fetal bovine serum (HF), before fixing with 4% paraformaldehyde for 15 minutes at room temperature. The cells were washed twice with HF and analyzed using the CytoFLEX LX cytometer (Beckman Coulter). All data were analyzed using FlowJo v10.8.1 (Becton, Dickinson & Company).

2.5 | MK enrichment

MKs were enriched by magnetic separation using a MACS Cell Separation Kit (Miltenyi Biotec) following the manufacturer's protocol. Briefly, day 11 cultured cells were stained with PE-anti-CD41a antibodies (HIP8, BD Pharmingen, diluted 1:100) for 10 minutes in the

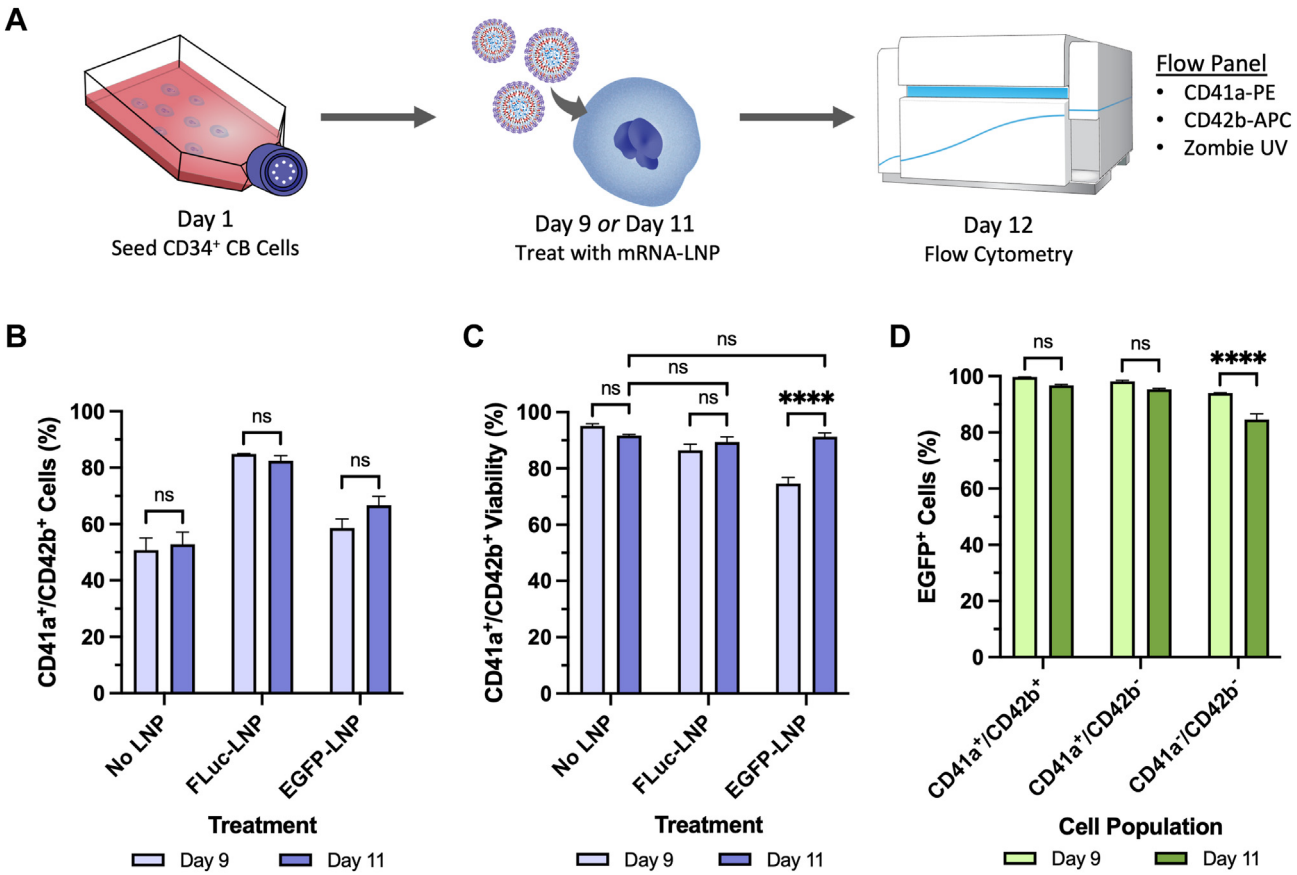

FIGURE 2 Transfecting megakaryocytes (MKs) cultured for 11 days does not impact MK maturation and viability. (A) Schematic of culturing and transfection of MKs. (B) Frequency and (C) viability of cells positive for both CD41a and CD42b without lipid nanoparticles (LNPs; No LNP), or treated with messenger RNA (mRNA)–LNP encoding firefly luciferase (FLuc; FLuc-LNP) or enhanced green fluorescent protein (EGFP; EGFP-LNP). MKs were transfected on either day 9 or day 11 and population frequencies and viability were assessed at day 12. (D) Frequency of EGFP-positive cells in each subset of cells transfected with mRNA-LNP at either day 9 or 11 and assayed by flow cytometry on day 12. Data represented by mean ± SEM (n = 3 biological replicates). APC, allophycocyanin; CB, cord blood; ns, not significant; PE, phycoerythrin. ****$P < .0001$.

dark at 4 °C before being washed twice with HF buffer. The cells were then incubated with anti-PE microbeads (130-048-801, Miltenyi Biotec) for 15 minutes on ice and washed with HF. Cells resuspended in HF were passed through a MiniMACS MS separation column (Miltenyi Biotec), eluted using HF, washed with PBS, and counted using a hemocytometer before rotational thromboelastometry (ROTEM) analysis or preparation for mass spectrometry.

2.6 | Mass spectrometry

Samples were lysed with trifluoroethanol:water (1:1 volume per volume) as described [19], and digested with trypsin (P8108S, New England Biolabs; 1:50 trypsin:protein weight per weight) overnight at 37 °C, followed by a second spike-in (1:200 trypsin:protein weight per weight) for 4 hours. Peptides were desalted using C18 STAGE-tips (made in-house) [20], then reconstituted in 0.1% formic acid and 0.5% acetonitrile. Twenty-five nanograms were loaded on a TimsTOF Pro2 (Bruker Daltonics) coupled to a nanoElute 2 ultra-high-performance liquid chromatography system (Bruker Daltonics) using Aurora Series Gen2 (CSI) analytical column. TimsTOF Pro 2 was operated with Parallel Accumulation-Serial Fragmentation scan mode for data-independent acquisition. The data were searched on DIA-NN (1.8.1) [21] using label-free quantification at a peptide and protein false discovery rate of 1% against the *Homo sapiens* UniProt database (UP000005640) modified to include EGFP and murine FVII. Missing values were imputed with random small numbers (23-29.5). Data are deposited to the ProteomeXchange Consortium via the Proteomics Identifications (PRIDE) database partner repository (dataset identifier PXD050741) [22].

2.7 | Confocal microscopy

$CD41a^+/CD42b^+$ cells were first sorted on the Cytek Aurora CS (Cytek Biosciences) and then allowed to adhere for 24 hours to a 96-well glass bottom black plate or microscope circular glass cover glasses coated with fibrinogen (0.1%) before fixing with 4% paraformaldehyde for 15 minutes at room temperature washing twice with HF. Cells were then labeled with PE-anti-CD41a (HIP8, BD Pharmingen, diluted 1:100), Vybrant DyeCycle Violet (Invitrogen, diluted 1:4000), and Alexa Fluor-647 anti-α-tubulin (EP1332Y, Abcam, diluted

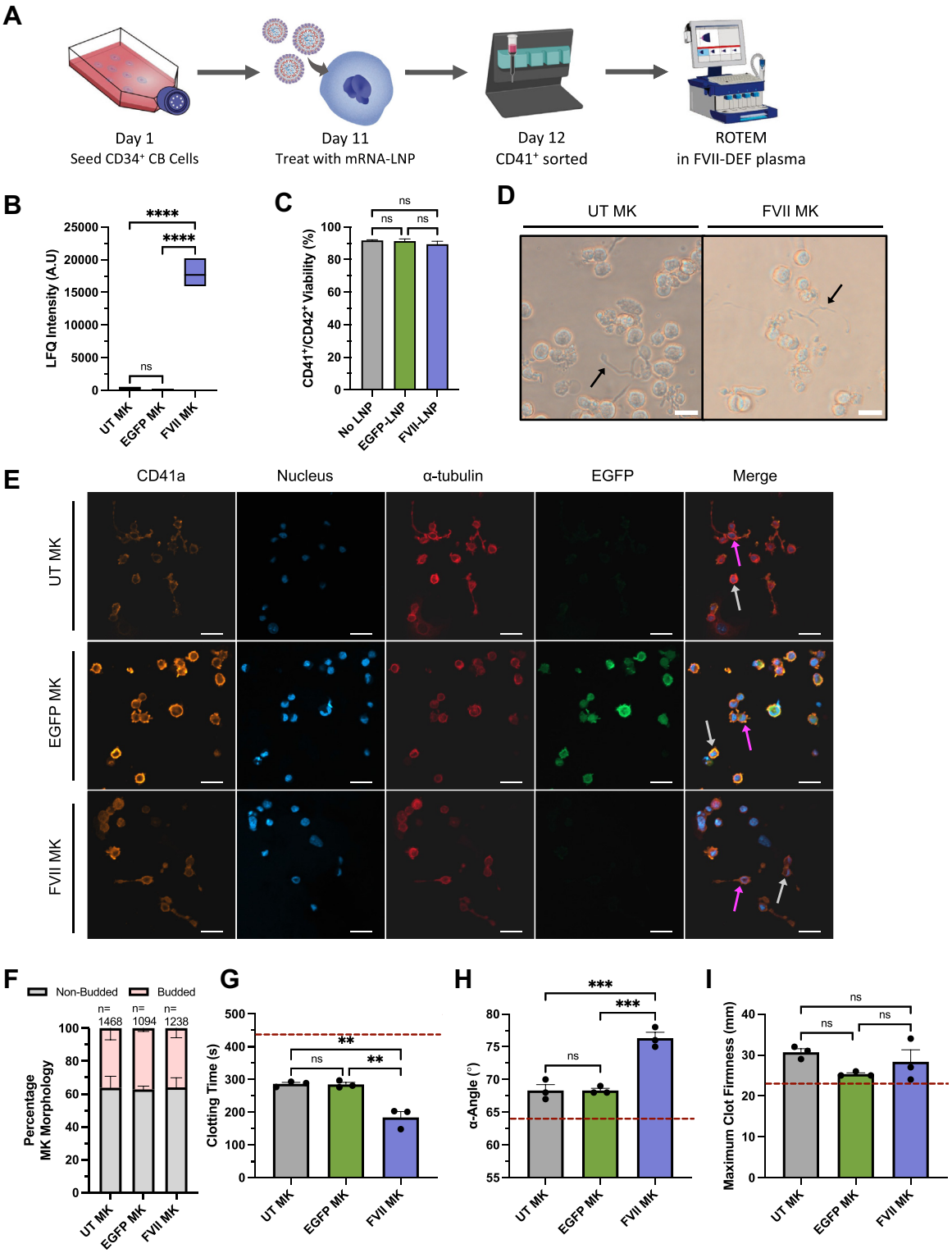

FIGURE 3 Megakaryocytes (MKs) express exogenous factor (F)VII and reduce clotting time in FVII-deficient (FVII-DEF) plasma. (A) Schematic of the transfection and analysis procedure, where MKs transfected on day 11 were enriched by magnetic separation on day 12 and assayed by rotational thromboelastometry in FVII-DEF plasma. (B) Label-free quantification (LFQ) intensity values of murine FVII detected through mass spectrometry in untreated MKs (UT MK), or MKs treated with messenger RNA (mRNA)–lipid nanoparticles (LNPs) encoding enhanced green fluorescent protein (EGFP; EGFP MK) or FVII (FVII MK). FVII expression was assessed 24 hours after treatment. (C) Viability of UT MKs or mRNA-LNP–treated MKs 24 hours after treatment assessed via flow cytometry. (D) Brightfield images of UT MKs and FVII MKs displaying proplatelet formation (black arrows). Scale bar represents 20 μm. (E) Representative confocal microscopy images along with the (F) corresponding quantification of UT MKs, EGFP MKs, or FVII MKs displaying nonbudded (gray bars and arrow) or proplatelet-like and budded (pink bars and arrow) morphologies. The number of cells counted (*n*) are indicated above each bar. MKs were stained for CD41a (orange),

1:250), washed once with HF, and imaged on the Zeiss LSM 800. Images were analyzed using ZEN Blue v2.5 (ZEISS). Approximately 200-500 $CD41a^+$ cells/well and 4 wells/sample were counted for morphological analyses.

2.8 | ROTEM analysis

MK function was measured using ROTEM by an adapted protocol described previously [23]. 150×10^3 untreated, FVII- or EGFP-expressing MKs purified by CD41a microbead enrichment were centrifuged at $405 \times g$ for 10 minutes, resuspended in 105-μL FVII-deficient plasma (Affinity Biologicals), and incubated at 37 °C for 30 minutes. The hemostatic profile of FVII-deficient plasma and MKs was then measured by ROTEM delta (Werfen) using mini cups and pins. Seven microliters of STAR-TEM (503-10-US, Tem Innovations GmbH) and EXTEM (503-05-US, Tem Innovations GmbH) reagents were added into the mini cup followed by 105-μL of FVII-deficient plasma with or without MKs.

2.9 | Statistical analysis

All data are reported as mean ± SEM unless otherwise noted, and analyzed by 1-way or 2-way analysis of variance corrected with either a post hoc Tukey or Bonferroni test as noted. Analysis and graphs were generated using GraphPad Prism v9.0 (GraphPad Software). A P value of <.05, 95% CIs, was considered significant.

3 | RESULTS AND DISCUSSION

To determine whether LNP formulations can transfect MKs, MKs were produced from umbilical cord blood-HSPCs and treated with mRNA-LNPs on the ninth day of culture (Figure 1A). The LNPs used for transfection resembled the 3 clinically approved LNP products: Onpattro (Alnylam Netherlands), Comirnaty (BioNTech), and Spikevax (Moderna Biotech Spain), designated by their ionizable lipids MC3 [24], ALC-0315 [25], and SM-102 [26], respectively. All LNPs encapsulated mRNA encoding for either EGFP or FLuc as a negative control. MKs were defined as cells expressing both CD41a and CD42b (Figure 1B). All MKs treated with any of the 3 mRNA-LNPs encoding for EGFP expressed the EGFP protein (Figure 1C, D), with up to 99% of MKs positive for EGFP. MKs treated with SM-102 mRNA-LNPs yielded the highest EGFP expression, with approximately 15-fold higher expression at a dose of 1 μg/mL than MC3, which yielded the lowest. These results suggest that mRNA-LNPs can readily transfect MKs to yield high-protein expression levels.

A concern with transfection methods that rely on DNA or viral vectors is transgene protein expression early in the differentiation cycle, which can affect cellular maturation and other downstream processes, as well as low transduction efficiencies [27]. A potential benefit of transfecting with LNPs is the ability to transfect at later stages of differentiation, mitigating impaired differentiation or cell function. To investigate whether mRNA-LNP transfection at different time points affects MK viability and maturation from HSPCs, cells were treated with SM-102 mRNA-LNPs encoding for either EGFP (EGFP-LNPs) or FLuc (FLuc-LNPs) at days 9 and 11 of culture (Figure 2A). Treatment was chosen on these 2 days as the cells exhibited the most rapid increase in the population of $CD41a^+$/$CD42b^+$ cells during this time. Population frequency, maturation, and viability were evaluated by flow cytometry at day 12. Cells transfected on days 9 and 11 with FLuc- or EGFP-LNPs yielded a similar frequency of MKs compared with HSPCs without mRNA-LNPs (Figure 2B). However, HSPCs treated with EGFP-LNPs on day 9 resulted in significantly lower viability compared with HSPCs transfected on day 11 (Figure 2C). This suggests that the type of protein expressed and the time of transfection may impact MK viability. While EGFP expression is generally considered to have negligible effects on cells, fluorescent protein expression can induce oxidative stress or apoptosis, contributing to the difference between EGFP and FLuc mRNA-LNP–treated cells [28,29]. Regardless of the day of transfection, however, cells treated with mRNA-LNPs on either day 9 or day 11 yielded similar transfection efficiencies, with over 95% of $CD41a^+$/$CD42b^+$ MKs expressing EGFP and 80% to 90% of cells not identified to be MKs ($CD41a^-$/$CD42b^-$) expressing EGFP (Figure 2D).

We next determined if MK function could be modulated with mRNA-LNPs. MKs respond to agonist stimulation and contribute to the strength of growing clots *in vitro* which can be measured by viscoelastic testing [23]. MKs were treated on the eleventh day of culture with SM-102 mRNA-LNPs encoding for FVII (FVII-LNP), as a proof-of-concept enzyme that helps initiate blood clotting, or EGFP as a control. One day after treatment, MKs were enriched via magnetic separation and assayed by ROTEM in FVII-deficient plasma to assess the contribution of MK-derived FVII toward clot formation (Figure 3A). MKs treated with FVII-LNP highly expressed FVII, with no significant impact on cell viability (Figure 3B, C). Transfected MKs also formed proplatelets (Figure 3D), and displayed similar proplatelet-like or budding morphologies—suggested to pre-empt platelet formation [30]—to untreated MKs (Figure 3E). The percentage of proplatelet-like or budded MKs was not significantly impacted after LNP transfection (Figure 3F). When assessed by ROTEM, FVII-expressing MKs decreased the clotting time by one-third compared with untreated

nuclei (blue), and α-tubulin (red), with EGFP expression displayed in green. Scale bar represents 20 μm. Quantification of (G) clotting time, (H) α-angle, and (I) maximum clot firmness of UT MKs (gray) or MKs treated with mRNA-LNP encoding EGFP (EGFP MK, green) or FVII (FVII MK, purple) in the presence of FVII-DEF plasma. The red dotted line in each graph represents the values for FVII-DEF plasma alone without any MKs (No MK). Data were analyzed by 1-way analysis of variance followed by a Bonferroni post hoc correction. Data represented by mean ± SEM (n = 3 biological replicates). CB, cord blood; ns, not significant. $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

MKs or MKs treated with EGFP-LNPs (EGFP-MKs; Figure 3G). There was a significant difference in the measured α-angle, with an increase of about 12° compared with FVII-deficient plasma (Figure 3H). Conversely, untreated MKs and EGFP-MKs had α-angles similar to FVII-deficient plasma alone. There was no significant difference in the maximum clot firmness between the FVII-MKs and untreated or EGFP-MKs (Figure 3I). These results suggest the MKs can be functionally changed using mRNA-LNP.

One limitation of this study is that it was limited to MKs, and engineered platelets produced from transfected MKs were not validated. Though producing platelets *in vitro* typically has low yields, technologies already exist to produce platelets *en masse* [31]. Transfecting MKs derived from other stem cells may also be beneficial. While cord blood–derived HSPCs are a readily available source of renewable stem cells, they also yield MKs that may not be fully mature, with lower ploidy and platelet generation potential [32–35]. The small size (<20 μm) and low ploidy of the MKs generated in this study also suggest that the isolated and transfected $CD41a^{+}/CD42b^{+}$ population consisted of mostly MK progenitors rather than mature MKs. The impact of LNP transfection on platelet production and more mature MK populations, among other biological processes, therefore warrants further investigation. Leveraging mRNA-LNP with *in vitro* platelet production can ultimately provide an easy-to-use modular platform to genetically modify MKs, potentially extending to large-scale modified platelets.

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AUTHOR CONTRIBUTIONS

J.L. and A.P. designed the study, performed the experiments, analyzed the data, generated the figures, and wrote and edited the manuscript. C.S. designed and performed the ROTEM experiments illustrated in Figure 3, analyzed the data, generated the figures, and wrote and edited the manuscript. B.N.H. designed and performed the mass spectrometry illustrated in Figure 3 and edited the manuscript. H.H.H. performed the experiments illustrated in Figure 3 and edited the manuscript. A.H. designed the study illustrated in Figure 1, performed the experiments, and edited the manuscript. L.J.F., D.D., P.R.C., P.W.Z., and C.J.K. helped design the study and analyze data and wrote and edited the manuscript.

DECLARATION OF COMPETING INTERESTS

P.R.C. and C.J.K. are cofounders of and hold equity in NanoVation Therapeutics Inc. P.R.C. and C.J.K. are cofounders of and hold equity in SeraGene Therapeutics Inc. C.J.K. is a cofounder of and holds equity in CoMotion Drug Delivery Systems Inc. P.R.C. is a cofounder of and holds equity in Acuitas Therapeutics. P.W.Z. is a cofounder of and holds equity in Notch Therapeutics. P.W.Z. is the Canada Research Chair in Stem Cell Engineering. J.L., C.S., P.R.C., and C.J.K. have submitted intellectual property on RNA-LNP. A.P., B.N.H., H.H.H., A.H., L.J.F., and D.D. have no conflicts of interest to declare.

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