

STAT1-mediated epigenetic regulation of LIN28A controls iPSC-derived platelet production through the let-7-RALB axis

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Abstract:

Ex vivo platelet production from induced pluripotent stem cells (iPSCs) represents a potential solution to address the limitations of donor-dependent platelet transfusion therapy. While our established immortalized megakaryocyte progenitor cell lines (imMKCLs) from iPSCs enable the large-scale production of functional iPSC-derived platelet products (iPSC-PLTs), cellular heterogeneity and senescence remain significant challenges for robust industrial-scale manufacturing. We recently identified RAS-like proto-oncogene B (RALB) as a key regulator of immune properties and platelet (PLT) productivity of imMKCLs, acting downstream of the let-7a-5p microRNA. The present study aims to identify the upstream regulators of let-7a-5p in this context. Herein, we demonstrate that the expression of Lin-28 homolog A (LIN28A), which negatively regulates let-7a-5p, is controlled in imMKCLs through DNA methylation-dependent mechanisms. Analysis of the LIN28A locus revealed distinct methylation patterns between let-7high and let-7low populations within an intronic CpG island. Overexpression of LIN28A upregulated immune-related signaling and diminished PLT production from imMKCLs. We further examined for transcriptional regulators by motif enrichment analysis and small interfering RNA (siRNA)-mediated knockdown, identifying signal transducer and activator of transcription 1 (STAT1) as an upstream regulator of LIN28A. Knockdown of STAT1 led to the suppression of immune-related gene expression resulting in increased PLT production. Inhibition of STAT1 phosphorylation with fludarabine and flavopiridol enhanced PLT generation, uncovering a novel role in platelet generation beyond their established functions in cell cycle arrest and apoptosis. In conclusion, our findings unveil the modulating roles of immune and senescence signaling in imMKCLs to optimize cell and culture conditions for large-scale PLT manufacturing.

Conflict of interest: COI declared - see note

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STAT1-mediated epigenetic regulation of LIN28A controls iPSC-derived platelet production through the let-7-RALB axis

Running title: LIN28A regulates human iPSC-platelet biogenesis

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Data Sharing Statement

The RNA-seq datasets were re-analyzed and generated using the RNA-seq data from our previous work. The raw data sets were deposited in the DNA Data Bank of Japan (DDBJ) database with association codes (DRA016355).

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Abstract

Ex vivo platelet production from induced pluripotent stem cells (iPSCs) represents a potential solution to address the limitations of donor-dependent platelet transfusion therapy. While our established immortalized megakaryocyte progenitor cell lines (imMKCLs) from iPSCs enable the large-scale production of functional iPSC-derived platelet products (iPSC-PLTs), cellular heterogeneity and senescence remain significant challenges for robust industrial-scale manufacturing. We recently identified RAS-like proto-oncogene B (RALB) as a key regulator of immune properties and platelet (PLT) productivity of imMKCLs, acting downstream of the let-7a-5p microRNA. The present study aims to identify the upstream regulators of let-7a-5p in this context. Herein, we demonstrate that the expression of Lin-28 homolog A (LIN28A), which negatively regulates let-7a-5p, is controlled in imMKCLs through DNA methylation-dependent mechanisms. Analysis of the *LIN28A* locus revealed distinct methylation patterns between let-7^{high} and let-7^{low} populations within an intronic CpG island. Overexpression of *LIN28A* upregulated immune-related signaling and diminished PLT production from imMKCLs. We further examined for transcriptional regulators by motif enrichment analysis and small interfering RNA (siRNA)-mediated knockdown, identifying signal transducer and activator of transcription 1 (STAT1) as an upstream regulator of LIN28A. Knockdown of STAT1 led to the suppression of immune-related gene expression resulting in increased PLT production. Inhibition of STAT1 phosphorylation with fludarabine and flavopiridol enhanced PLT generation, uncovering a novel role in platelet generation beyond their established functions in cell cycle arrest and apoptosis. In conclusion, our findings unveil the modulating roles of immune and senescence signaling in imMKCLs to optimize cell and culture conditions for large-scale PLT manufacturing.

Key Points:

- LIN28A regulates platelet production through the let-7 microRNA-RALB axis in human iPSC-derived megakaryocyte cell lines.
- STAT1 modulates LIN28A expression via DNA methylation, and its inhibition boosts platelet production while suppressing cellular senescence.

INTRODUCTION

Platelets, an essential cellular component in transfusion medicine, play crucial roles in hemostasis, thrombosis, and maintenance of vascular integrity. Since James Blundell's primitive success with human blood transfusion in 1818,¹ the current dependency on donor-derived platelets has substantial challenges, including limited shelf life, risk of bacterial contamination, alloimmune-mediated reactions and anticipated supply shortages due to aging societies.² *Ex vivo* blood production from induced pluripotent stem cells (iPSCs) has been proposed as a solution to these issues by offering the potential for consistent platelet supply for transfusion therapy. Several research groups, including ours, have made substantial progress in developing methods for producing platelet products *ex vivo* from human iPSCs (iPSC-PLTs).³⁻⁵ However, scaling up to achieve clinically relevant yields was difficult.

To overcome this limitation, we previously established immortalized megakaryocyte progenitor cell lines (imMKCLs) by introducing doxycycline (DOX)-inducible *c-MYC*, *BMI1* and *BCL-XL* transgenes into human iPSC-derived hematopoietic progenitor cells (HPCs).^{6,7} imMKCLs demonstrate sustained proliferative capacity over several months in the presence of DOX and produce agonist-responsive CD41⁺CD42b (GPIb α)⁺ functional iPSC-PLTs upon DOX removal. A breakthrough was achieved by implementing turbulent flow-based bioreactors, enabling the generation of clinically relevant quantities (10¹¹ level) of such functional iPSC-PLTs.⁸ These discoveries enabled not only the first-in-human clinical trial by autologous iPSC-PLTs⁹⁻¹¹ but also a robust *in vitro* system for investigating fundamental mechanisms of megakaryocyte development and thrombopoiesis.¹²⁻¹⁴

Despite these achievements, several critical issues were raised, particularly associated with the quality control of both imMKCL master cells and their derived iPSC-PLTs. In that context, we recently identified let-7 microRNA and its downstream target, RAS like proto-oncogene B (RALB), as critical regulators of immune-biased megakaryocyte subpopulations.¹⁵ These subpopulations, characterized by lower let-7 activity, exhibited immune-related signal activation, particularly interferon signaling, and compromised imMKCL quality, evidenced by arrested proliferation and impaired iPSC-PLT production. That study provided indispensable insights into the standardization of iPSC-PLT generation towards industrial-scaled manufacturing.¹⁵ However, the upstream regulators of the let-7-RALB axis and the mechanisms driving cellular heterogeneity in imMKCLs have remained elusive.

The RNA-binding protein LIN28, which exists as two paralogs (LIN28A and LIN28B), has been identified as a key regulator of stem cell maintenance and differentiation.¹⁶ The LIN28-mediated regulation of let-7 microRNA maturation has significant implications for stem cell biology, glucose metabolism, and various human diseases.^{17,18} While the role of LIN28 proteins in early development is known, their potential involvement in megakaryocyte development and thrombopoiesis remains largely unexplored. The present study focuses on elucidating the upstream regulatory mechanisms controlling iPSC-PLT production capability in imMKCLs, with an emphasis on the epigenetic and transcriptional regulations of the LIN28-let-7-RALB axis. Our findings demonstrate that LIN28A expression is regulated through DNA methylation-dependent mechanisms mediated by STAT1, ultimately influencing platelet production efficiency. This study uncovers a novel STAT1-LIN28A-let-7 regulatory network that enhances iPSC-PLT production capacity while suppressing immune-skewed properties linked to cellular senescence in imMKCLs. These findings offer valuable insights for strategizing the optimization toward an improved *ex vivo* iPSC-PLT manufacturing system.

MATERIALS AND METHODS

Cells

Human iPSC lines, TkDN-Sev2 and T-1, were established in-house.¹⁹ imMKCLs (clone 7, clone 7-3, M35-1) were induced by DOX-inducible defined factors from human iPSCs and employed in previous studies.^{7,8,15} The use of all cells was approved by the ethics committees at Kyoto University and Chiba University. imMKCLs (clone 7, clone 7-3, M35-1) were cultured as described before.⁸ Doxycycline was used to control the proliferation and differentiation stages of imMKCLs.

Overexpression of human LIN28A in imMKCLs

Viral vector use was approved by committees at Kyoto University and Chiba University. The full-length coding sequence of human *LIN28A* was cloned into the lentiviral vector LV-TRE-mOKS-Ubc-tTA-I2G.²⁰ Lentiviral production using the 293T system was described previously.⁶ imMKCLs (clone 7) were transduced with the resultant or control (MOCK) viruses.⁶ After transduction, green fluorescent protein (GFP)-positive imMKCLs were sorted using a BD FACS-Aria IIIu.

Reverse transcription and real-time PCR

Total RNA was extracted using the microRNeasy Micro Kit or microRNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed using SuperScript VILO Master Mix (Thermo Fisher Scientific) or ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Real-time PCR was performed with the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) using the StepOnePlus system (Thermo Fisher Scientific). *GAPDH* was used as the internal control. Primer sets used are listed in Supplementary Table 1. Let-7a-5p miRNA reverse transcription was performed using a TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) and miRNA-specific stem-loop RT primers according to the manufacturer's instructions.

Real-time PCR was carried out with the TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) using the StepOnePlus system. *let-7* expression was determined relative to *RNU6B* using specific TaqMan probes.

Flow cytometry

Cells were suspended in a staining medium, incubated for 30 min with appropriate antibodies on ice in the dark, and sorted or analyzed using a BD FACS Aria IIIu. Platelet counts were determined as previously described.⁸ The following antibodies were used for the analysis: allophycocyanin (APC)-conjugated anti-CD41a (integrin α IIb; HIP8 clone) (BioLegend, San Diego, CA), phycoerythrin (PE)-conjugated anti-CD42b (GPIb α) (eBioscience, San Diego, CA). Samples were analyzed using a BD FACSVerse.

DNA methylation assay

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and treated with sodium bisulfite using the MethylEasy Xceed Rapid DNA Bisulphite Modification Kit (Takara Bio). Bisulfite-treated DNA was amplified using EpiTaq HS (Takara Bio). Primers targeting CpG islands in the LIN28A region were designed by MethPrimer²¹ and listed in Supplementary Table 2. PCR amplicons were cloned into the pMD20-T vector using the Mighty TA-cloning Kit (Takara Bio) and transformed into DH5 α competent cells. The plasmid from each clone was sequenced by Macrogen Japan Corp (Kyoto, Japan). Methylation status analysis was performed using the QUantification tool for Methylation Analysis (QUMA) (RIKEN, Wako, Japan).²² Examples of sequence logos were generated based on the matrix ID. The discrepancy in the number of CpG sites may arise from differences in the sequencing read coverage and quality between *let-7*^{high} and *let-7*^{low} cells.

Expression of a dominant-negative C/EBP

The retroviral vector pGCsam of dominant-negative C/EBP with an IRES-enhanced green fluorescent protein (EGFP) was generously provided by Dr. Atsushi Iwama.²³ A dominant-negative form of C/EBP (A-C/EBP), followed by IRES enhanced green fluorescent protein (EGFP) were subcloned into a retroviral vector pGCsam. Retroviral production was performed as previously described.²³ imMKCLs (clone 7) were transduced with the resultant or control viruses. Following transduction, EGFP-positive imMKCLs were sorted using a BD FACSAria IIIu.

siRNA-mediated *STAT1* gene knockdown

The Dharmacon siGENOME siRNA SMARTpool designed against human *STAT1* (siSTAT1), comprised of four siRNAs targeting different sites, and a non-targeting control siRNA (siNT) were purchased from Horizon Discovery Ltd. Gene silencing was performed according to the Dharmacon reverse transfection protocol with minor modifications. Briefly, siRNA at 25 nM final concentration was diluted in the transfection buffer from the Stemfect RNA Transfection Kit (ReproCELL), with the transfection reagent diluted in an equal volume of buffer. The diluted siRNA and transfection reagent were combined and incubated at room temperature for 15 min. Subsequently, 12.5 μ L/well of the transfection mixture was added to the wells of a 96-well plate, followed by adding 2×10^5 imMKCLs (clone 7, DOX-ON) in 100 μ L medium to each well. After a 4-hour incubation period, cells were washed with PBS and subjected to either proliferation or differentiation culture conditions. To validate the gene knockdown efficiency, total RNA was isolated and analyzed 48 hours after the transfection.

Intracellular flow cytometry

The intracellular flow cytometry assay was optimized for imMKCLs as follows. Cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature, followed by methanol permeabilization (15 min on ice). After two washes with PBS, cells were incubated with an anti-STAT1 polyclonal antibody (Proteintech, 10144-2-AP, 0.4 μ g/100 μ L) for 1 hour on ice. Subsequently, cells were washed and incubated with a Multi-rAb CoraLite Plus 647-Goat

Anti-Rabbit Recombinant Secondary Antibody (H+L) (Proteintech, RGAR005, 1:1000) on ice for 30 mins. Stained cells and non-staining controls were then analyzed using a BD FACSVerse.

Analysis of cytokine secretion by imMKCLs

Cytokine secretion in culture supernatants was measured using the human inflammatory cytokine cytometric bead array kit (BD Biosciences) according to the manufacturer's guidelines. Briefly, bead populations with distinct fluorescence intensities coated with capture antibody proteins were mixed with PE-conjugated detection antibodies and recombinant standards or test samples and then incubated to form sandwich complexes. After acquiring sample data by flow cytometry, cytokine concentrations were calculated using the FCAP Array software (BD Biosciences).

Pharmaceutical inhibition of STAT1 phosphorylation by fludarabine and flavopiridol in imMKCLs

The effects of two STAT1 phosphorylation inhibitors, fludarabine and flavopiridol, on iPSC-PLT production from imMKCLs were evaluated. Briefly, imMKCLs (clone 7) were cultured under differentiation conditions (DOX-OFF) in the absence or presence of fludarabine (0 to 1 μ M) or flavopiridol (0 to 100 nM). Dimethyl sulfoxide (DMSO) was used as a vehicle control in all conditions.

PAC-1 binding and P-selectin expression of iPSC-PLTs

PAC-1 binding and P-selectin levels were measured according to the protocol outlined in our previous study¹⁰. Briefly, culture suspensions (iPSC-PLTs) were stimulated with or without phorbol-12-myristate-13-acetate (PMA, 0.2 μ M) or adenosine triphosphate plus thrombin receptor activator peptide 6 (ADP/TRAP6, 100 μ M and 40 μ M, respectively), or thrombin (1 U/mL). Subsequently, mixtures were incubated with BV421 mouse anti-human CD62P (BD Biosciences), APC-anti-human CD41a (BioLegend), and FITC-PAC-1 (BD Biosciences)

antibodies. Following a 30-min incubation at room temperature, samples were diluted with HEPES-Tyrode buffer and analyzed using a BD FACSVerse.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, La Jolla, CA). Data are expressed as the mean $\pm$ standard error of the mean (SEM). Values of $p < 0.05$ were considered significant. The total percentage of methylated CpGs in each sample were calculated by QUMA software²². Methylation pattern results were dichotomized. The Fisher exact test was used to evaluate the presence of any significant differences. Details of the sample size used, statistics, and statistical significance are indicated in each figure legend.

RESULTS

LIN28A regulates iPSC-PLT production through the let-7 microRNA-RALB axis in imMKCLs

Our previous study established that the let-7 microRNA-RALB axis is a major regulator in determining immune-biased imMKCL subpopulations. Specifically, reduced let-7a-5p activity leads to elevated *RALB* expression and subsequent activation of immune-related signaling, particularly interferon signals. Dysregulation of these subpopulations compromises the overall imMKCL population quality, as exemplified by reduced proliferation rate, thus leading to impaired iPSC-PLT production (Figure 1A).¹⁵ In the present study, we further investigated the regulatory mechanism upstream of the let-7-RALB axis. Our analysis focused on LIN28, a well-established developmental regulator of the let-7 family, that functions through reciprocal regulation: LIN28 proteins inhibit let-7 family maturation, while the let-7 family downregulates the expression of LIN28 proteins.^{17,18} Examination of our bulk RNA-sequencing data obtained from our previous report¹⁵ demonstrated that *LIN28A*, but not *LIN28B*, was significantly upregulated in imMKCL subpopulations with lower let-7 activity (let-7^{low}) compared to let-7^{high} cells (Figure 1B). To examine the regulatory role of LIN28A, we tested lentiviral-mediated *LIN28A* overexpression, which resulted in increased mRNA

expression of *LIN28A* and *RALB* and decreased levels of let-7a-5p (Figure 1C). These findings positioned *LIN28A* as an upstream regulator of the let-7-*RALB* axis in imMKCLs. Consistent with our hypothesis, *LIN28A* overexpression significantly increased cyclin-dependent kinase inhibitor 2A (*CDKN2A*) expression (Figure 1C), a well-known marker of cellular senescence,²⁴ impeded proliferation (Figure 1D), and subsequently impaired iPSC-PLT production under both static or turbulent flow conditions (Figure 1E-1F). These were accompanied by the activation of interferon signaling genes, which serve as markers of immune-biased imMKCL subpopulation (Figure 1G). Collectively, these results suggest that *LIN28A* functions as a key regulator of the let-7 microRNA-*RALB* axis in imMKCLs, thereby modulating iPSC-PLT production capacity.

Epigenetic regulation of *LIN28A* expression via differential DNA methylation

Previous studies have demonstrated that *LIN28A* expression is regulated by epigenetic mechanisms, including DNA methylation²⁵ and histone modification.²⁶ In this study, we analyzed predicted CpG islands in the *LIN28A* promoter region using the MethPrimer software.²¹ The criteria for prediction included an island size > 100, GC content >50.0, and an Observed/Expected number of CpG patterns (Obs/Exp) ratio > 0.6. Two CpG islands were identified: one (239 bp) located in exon 1 and the other (212 bp) within intron 1 (Figure 2A). To investigate the methylation status of these regions in imMKCLs, we performed bisulfite sequencing of both CpG islands in imMKCLs. Comparative analysis of individual CG dinucleotides revealed distinct methylation patterns exclusively in CpG island 2, but not CpG island 1, between let-7^{low} and let-7^{high} cells. The total methylation rate in let-7^{high} cells was substantially higher (51.05%) compared to let-7^{low} cells (16.42%) (Figure 2B-2C). Specifically, we identified eight specific CpG dinucleotides that exhibited hypomethylation in let-7^{low} cells but were consistently hypermethylated in let-7^{high} cells (Figure 2C). This differential methylation pattern strongly suggests that *LIN28A* expression in imMKCLs is regulated, at least in part, through epigenetic mechanisms, particularly related to let-7 activity.

Transcription factor binding sites analysis of CpG island 2 in the human *LIN28A* gene

To identify transcriptional factors (TF) that might regulate *LIN28A* expression involving epigenetic mechanisms, we conducted an in-depth analysis of potential binding sites within the methylation-sensitive CpG island 2 of *LIN28A* (212 bp, Figure 3A). Using the TFBIND algorithm,²⁷ we identified multiple candidate TF binding motifs (Figure 3B) and confirmed the expression of candidate genes using our RNA sequencing data (Figure S1). Among identified genes, the signal transducer and activator of transcription (STAT) and CCAAT/Enhancer Binding Protein (C/EBP) families were strong candidates given their known roles in hematopoietic development^{28,29} and immune system regulation^{30,31}. We hypothesized that members of the STAT or C/EBP families might manipulate the expression of *LIN28A* via DNA methylation-dependent mechanisms, where their loss of function could potentially suppress *LIN28A* expression, subsequently dampening immune signaling and improving iPSC-PLT production capacity. However, investigating C/EBP family function was particularly challenging due to the extensive functional redundancy among family members in myeloid cells, where a single gene disruption often fails to reveal their physiological roles.³² In this context, we employed a dominant-negative C/EBP approach capable of antagonizing all C/EBP family members simultaneously.²³ Contrary to our expectations, expression of the dominant-negative C/EBP led to increased expression of *LIN28A*, *IRF7*, and *ISG15* during the proliferation stage of imMKCLs. Notably, this intervention did not affect *RALB* expression, imMKCL proliferation, or subsequent platelet production (Figure S2A-C). These unexpected results may reflect the complex roles of the C/EBP family in megakaryocyte development and thrombopoiesis, but further studies are needed to clarify these functions.

***STAT1* knockdown enhances iPSC-PLT production through modulation of the *LIN28A*/let-7-*RALB* axis**

Our RNA-seq analysis revealed that *STAT1* and *STAT3* are expressed in imMKCLs (Figure S1). We first investigated the regulatory role of *STAT1* using siRNA-mediated knockdown.

A pool of siRNAs targeting *STAT1* (siSTAT1) and non-targeted control mRNA (siNT) was employed here (Figure 4A). The knockdown effectively reduced *STAT1* expression at both the mRNA (Figure 4B) and protein levels (Figure 4C), leading to concurrent suppression of *LIN28A* expression and elevated let-7a-5p levels. These results led us to conclude that *STAT1* is a potential regulator of the *LIN28A*/let-7 pathway in imMKCLs. Meanwhile, cell proliferation was slightly reduced but not significantly in *STAT1* knockdown cells (Figure S3A), consistent with a previous report on megakaryocyte proliferation.³³ The knockdown of *STAT1* notably enhanced iPSC-PLT production under both static and turbulent flow conditions (Figure 4D-4E), likely due to modulation of *RALB* and interferon signaling gene expression (Figure 4F, Figure S3B). Importantly, the functionality of iPSC-PLTs remained intact following *STAT1* knockdown, as evidenced by comparable levels of Annexin V binding, PAC-1 binding, and P-selectin expression between siSTAT1 and siNT groups (Figure S3C-S3D). Interestingly, *STAT1* knockdown also reduced the expression of *CDKN2A* and secretion of interleukin-8 (IL-8), a critical component of senescence-associated secretory phenotype (SASP) known to diminish imMKCL quality (Figure S3B, Figure 4G).¹⁵ We also validated our key findings in the M35-1 clone, the patient-derived imMKCL used in the iPLAT1 clinical trial.⁹ *STAT1* knockdown in M35-1 clone led to a significant improvement in iPSC-PLT production (Figure S3E-3F). These findings suggest a novel connection between the *STAT1* regulatory network and the senescent and aged characteristics of imMKCLs.

Pharmacological inhibition of *STAT1* enhances iPSC-PLT production under turbulent flow conditions

Given that *STAT1* activity is primarily regulated by posttranslational modifications, particularly phosphorylation of *STAT1* tyrosine and serine residues,³⁴ we hypothesized that pharmacological inhibition of *STAT1* phosphorylation could augment iPSC-PLT production. To test this, we evaluated the effects of two known *STAT1* phosphorylation inhibitors, fludarabine^{35,36} and flavopiridol³⁷ (Figure 5A). Dose optimization experiments were performed across a range of concentrations (0~5 μ M for fludarabine and 0~100 nM for

flavopiridol), with maximal efficacy on iPSC-PLT production observed at 1 μ M fludarabine and 10 nM flavopiridol (Figure 5B). While both inhibitors elicited only modest, nonsignificant increases in iPSC-PLT yield under static cultures, they significantly enhanced production under turbulent flow conditions (Figure 5C-5D). Further analysis of their effects on gene expression revealed distinct mechanism. While flavopiridol treatment upregulated let-7a-5p and downregulated *STAT1*, *RALB* and interferon signaling which aligns well with our hypothesis, fludarabine did not, suggesting an alternative mechanism (Figure 5E-5F). Nevertheless, our findings establish *STAT1* as a critical upstream regulator of the LIN28A/let-7/RALB axis, modulating iPSC-PLT production.

DISCUSSION

We initiated the first-in-human clinical trial of transfusion by autologous iPSC-PLTs.⁹ The trial involved a 52-year-old patient suffering from aplastic anemia and allo-platelet transfusion refractoriness (allo-PTR), who had been treated with standard medicine. We generated iPSCs from the patient's circulating T cells and derived autologous imMKCLs.^{9,10} Through three cohort clinical trials and further in-depth study, we recognized distinct differences between the aged donor-derived M35-1 imMKCL clone and our control clone, clone 7 (established from neonate fibroblasts-derived iPSCs), in terms of both iPSC-PLT production and gene expression profile.¹⁵ Based on such points, we identified a novel regulatory mechanism controlling iPSC-PLT production capacity, with a particular focus on the regulation of the LIN28A-let-7-RALB axis. We demonstrate that *LIN28A* expression, regulated by DNA methylation and *STAT1*, acts as a critical upstream modulator of the let-7-RALB pathway, ultimately influencing platelet production capacity from imMKCLs. Our findings provide new insights into how transcription factors and epigenetic modifications determine imMKCL differentiation, senescence, and subsequent iPSC-PLT emergence and quality, with significant implications for optimizing iPSC-PLT manufacturing.

One of the major findings of this study is the identification of *LIN28A* as a key regulator of the let-7 microRNA-*RALB* axis in imMKCLs (Figure 1). Both *LIN28A* and *LIN28B* are critical pluripotency genes highly expressed in undifferentiated ESCs and iPSCs.²⁶ Human hematopoietic development at the transition stage from fetal liver to adult bone marrow is marked by let-7 upregulation and accompanied by *LIN28B* downregulation.³⁸ In murine thrombopoiesis, *Lin28b* also exhibits high expression in fetal megakaryocytes and negatively regulates the levels of platelet P-selectin.³⁹ Moreover, the developmentally restricted expression of *Lin28b* suppresses a megakaryocyte-biased HSC pool.⁴⁰ We previously demonstrated that suppressed endogenous let-7a-5p activity leads to *RALB* upregulation, thereby accelerating the development of immune-biased imMKCL subpopulations that compromise the overall quality of imMKCLs.¹⁵ Our imMKCLs, which mimic early embryonic megakaryocyte development, represent a heterogeneous population with varying let-7 expression and activity, resulting in distinct iPSC-PLT production capacities and senescence phenotypes.¹⁵ We found difference in the expression of *LIN28A*, but not *LIN28B*, between let-7^{low} and let-7^{high} cells (Figure 1), and sequential analysis of these populations revealed the differential expression of *LIN28A* to be epigenetically regulated by DNA methylation (Figure 2). *LIN28A* overexpression experiments demonstrated its role as a key regulator controlling the senescence phenotype in imMKCLs that recapitulate embryonic development. Altogether, our findings demonstrate that epigenetic modulation of *LIN28A* expression, particularly through DNA methylation, contributes to cellular heterogeneity within imMKCLs.

Meanwhile, our investigation of transcription factor regulation revealed STAT1 as a key modulator of *LIN28A* expression, whereas the C/EBP family's involvement was more intricate than initially hypothesized (Figure 3 and S2). The STAT family has been extensively studied as central mediators for interferons and innate inflammatory response, with established roles in hematopoiesis.^{30,41} In particular, thrombopoietin (TPO), the primary megakaryocyte-potentiating factor, activates multiple STAT proteins (STAT1, STAT3, and

STAT5) through its receptor c-Mpl in megakaryocytes and other hematopoietic cells.⁴² Previous studies demonstrated that Stat3 activation promotes the expansion of megakaryocytic progenitor cells,⁴³ while Stat1 can rescue multiple defects in *Gata1*-deficient megakaryopoiesis in mice.³³ Moreover, *Stat1* deletion in mice shifts hematopoietic balance by reducing megakaryopoiesis while favoring erythropoiesis.⁴⁴ In our *ex vivo* human iPSC-PLT manufacturing system, *STAT1* knockdown yielded different results, as it suppressed *LIN28A* expression and increased let-7a-5p levels, leading to significantly enhanced iPSC-PLT production without compromising platelet functionality, as evidenced by preserved activation markers (PAC-1 and P-selectin, Figure S3). Intriguingly, *STAT1* depletion reduced the expression of cellular senescence markers, including *CDKN2A* and the key SASP factor, *IL8*. While co-upregulation of *STAT1* and *CDKN2A* has been observed in senescent cells and cancers, their regulatory relationship appears to be context dependent.⁴⁵ These findings highlight critical and evident differences between murine and human hematopoiesis. We previously demonstrated that interferon alpha (IFN- α), an upstream regulator of *STAT1*, impairs both imMKCL proliferation and iPSC-PLT generation. However, RNA sequencing analysis revealed no detectable expression of the interferon family in imMKCLs.¹⁵ Alternative *STAT1* activation pathways, including damage-associated molecular patterns (DAMPs) via toll-like receptor signaling,⁴⁶ the cGAS/STING pathway, and interleukin-6 (IL-6) signaling,⁴⁷ may be involved. We hypothesize that senescent imMKCLs may release DAMPs and interleukins, suggesting that identifying *STAT1* activators could lead to optimized culture conditions preventing imMKCL quality decline. These findings indicate that *STAT1* inhibition could benefit clinical applications by enhancing PLT production while preventing cellular senescence, a major obstacle to maintaining the long-term stability and quality of imMKCLs.

Fludarabine, a purine analog, and flavopiridol, a cyclin-dependent kinase (CDK) inhibitor, are both clinically applied agents for treating leukemias.^{48,49} In our study, pharmacological inhibition of *STAT1* phosphorylation by these compounds exhibited promotive effects on

iPSC-PLT production from imMKCLs via distinct mechanisms (Figure 5). While primarily recognized for their roles in inducing cell cycle arrest and apoptosis³⁶, our findings reveal an unexpected functional benefit of these compounds in the context of *ex vivo* platelet production. Given their pleiotropic effects and cytotoxicity, further studies are needed to clarify their mechanisms, optimize dosing, and assess safety for potential clinical translation.

In summary, our study identifies key regulatory networks involving STAT1, LIN28A, and the let-7-RALB axis that determine iPSC-PLT production capacity and quality and provides new insights into the reality of cellular heterogeneity in imMKCLs. These findings advance not only the comprehensive understanding of platelet biogenesis but also practical approaches for optimizing platelet manufacturing.

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Authorship

Contribution: K.H. and S.J.C. designed and performed the experiments, analyzed the data, interpreted the results, prepared figures, and wrote the manuscript. K.F. and A.K. assisted with the experiments. N.S. and M.E. provided intellectual contributions and edited the manuscript. N.T. contributed to the experimental design, motif analysis, data interpretation, and edited the manuscript. K.E. managed the overall project, contributed to the funding acquisition, experimental design, data interpretation, and edited the manuscript.

Conflict-of-interest disclosure: K.E. was a founder of Megakaryon Co. Ltd. but with no stock currently. The interests of K.E. were reviewed and managed by Kyoto University in accordance with its conflict-of-interest policies. The other authors declare no conflict of interest.

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Figure legends

Figure 1. LIN28A regulates the let-7 microRNA-RALB axis in imMKCLs

A. A schematic illustration depicting the let-7 miRNA-RALB axis regulating immune-related signaling, which alters the proliferation and platelet production capacity of imMKCLs. **B.** *LIN28A* but not *LIN28B* shows increased expression in let-7^{low} cells compared to let-7^{high} cells. Bar graphs show TPM values of the indicated genes from three distinct imMKCL clones. **C.** The lentiviral-mediated overexpression (O/E) of *LIN28A* significantly increased *LIN28A* and *RALB* mRNA expression while suppressing let-7a-5p expression. **D.** Growth curve and

relative fold change of the cell count on day 14 of MOCK and *LIN28A* O/E imMKCLs (clone 7). **E.** *LIN28A* overexpression led to deficient iPSC-PLT generation by imMKCLs (Clone 7). **F.** Representative flow cytometry plots of iPSC-PLTs generated from MOCK or *LIN28A* O/E imMKCLs in static or turbulent flow conditions. CD41⁺CD42b⁺ PLTs were identified from a platelet-sized gate. **G.** *LIN28A* overexpression induced mRNA expression of representative immune-related genes in imMKCLs. Data are expressed as the mean $\pm$ SEM from three to four independent experiments. Unpaired two-tailed Student's *t*-tests were used to assess statistical significance.

Figure 2. Distinct DNA methylation at human *LIN28A* CpG island 2 in let-7 low and let-7 high imMKCLs

A. A schematic representation of CpG islands in the human *LIN28A* gene. Two CpG islands sequences were identified using the MethyPrimer platform. Bisulfite PCR was performed, and the methylation status of each CpG island was evaluated. **B.** The methylation status of individual CpG dinucleotides in human *LIN28A* CpG islands was analyzed by bisulfite sequencing in let-7^{high} and let-7^{low} cells. Results are shown by methylated (closed) or unmethylated (open) circles. **C.** Individual and total methylation rates of two CpG islands are shown. Fisher's exact tests were used to assess statistical significance by QUMA software. * $p < 0.05$, ** $p < 0.01$.

Figure 3. Transcription factor binding motif analysis of CpG island 2 in the human *LIN28A* gene and functional enrichment of candidate transcription factors

A. A schematic representation of CpG island 2 in the human *LIN28A* gene. Transcription factor binding sites of CpG island 2 were identified using the TFBIND software. **B.** The top motifs identified are listed in the table.

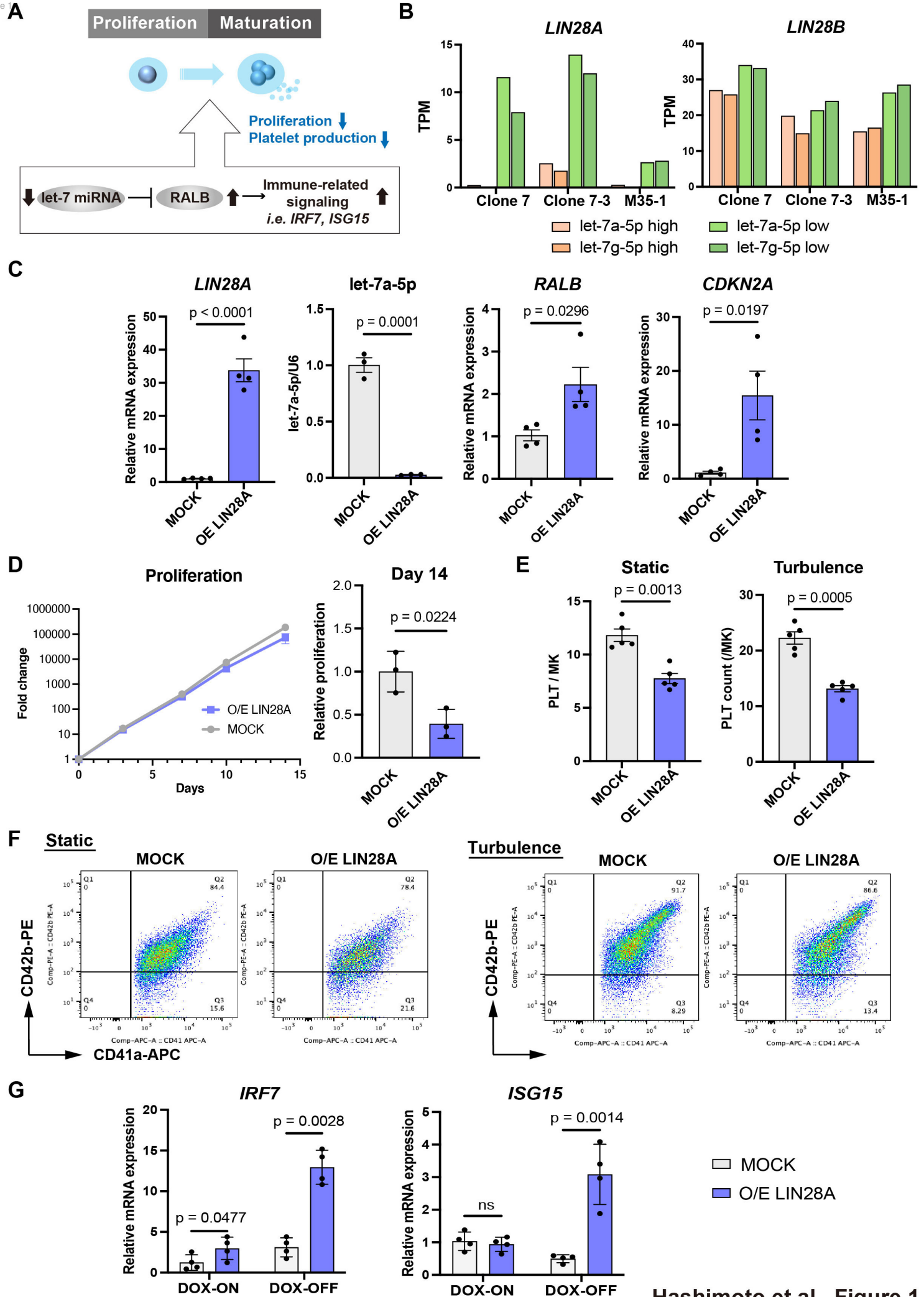
Figure 4. STAT1 is the upstream regulator of *LIN28A* in imMKCLs

The siRNA SMARTpool designed against human STAT1 (siSTAT1) and a non-targeting control siRNA (siNT) were transfected into imMKCLs. **A.** A schematic illustration of the experimental procedure of siRNA-mediated *STAT1* knockdown in imMKCLs. **B.** *STAT1* knockdown significantly decreased *STAT1* and *LIN28A* mRNA expression and increased let-7a-5p expression. **C.** Intracellular protein levels of STAT1 (mean fluorescence intensity, MFI) detected in siNT or siSTAT1 imMKCLs by intracellular flow cytometry. *STAT1* expression was compared using a histogram overlay in the left panel. **D.** *STAT1* knockdown improved iPSC-PLT generation by imMKCLs (Clone 7). **E.** Representative flow cytometry plots of iPSC-PLTs generated from siNT or siSTAT1 imMKCLs in static or turbulent flow conditions. CD41⁺CD42b⁺ PLTs were identified from a platelet-sized gate. **F.** *STAT1* knockdown decreased mRNA expression of representative immune-related genes in imMKCLs. **G.** The secretion of IL-8 from clone 7 and clone 7-3 during the maturation stage (DOX-OFF Day 6). Data are expressed as the mean $\pm$ SEM from three to four independent experiments. Unpaired two-tailed Student's *t*-tests were used to assess statistical significance.

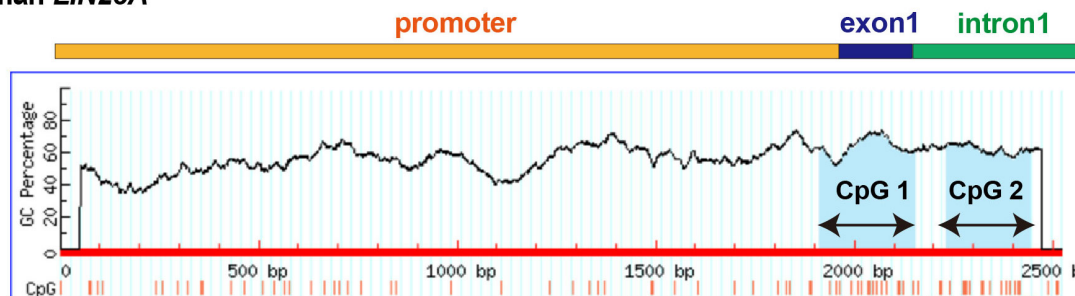
Figure 5. Pharmacological inhibition of STAT1 enhances iPSC-PLT production under turbulent flow conditions.

A. Chemical structures of fludarabine and flavopiridol, two established inhibitors for STAT1 phosphorylation. **B.** Dose optimization on iPSC-PLT production of fludarabine (0~5 μ M) and flavopiridol (0~100 nM). **C.** iPSC-PLT generation by imMKCLs (Clone 7) in the absence and presence of fludarabine (1 μ M) or flavopiridol (10 nM) under static or turbulent flow conditions. **D.** Representative flow cytometry plots of iPSC-PLTs generated in the absence and presence of fludarabine (1 μ M) or flavopiridol (10 nM) in static or turbulent flow conditions. CD41⁺CD42b⁺ PLTs were identified from a platelet-sized gate. **E.** Fludarabine (1 μ M) or flavopiridol (10 nM) treatments elevated let-7a-5p expression. **F.** Flavopiridol (10 nM) treatment reduced mRNA expression of representative immune-related genes in imMKCLs at the maturation stage. Data are expressed as the mean $\pm$ SEM from four to five

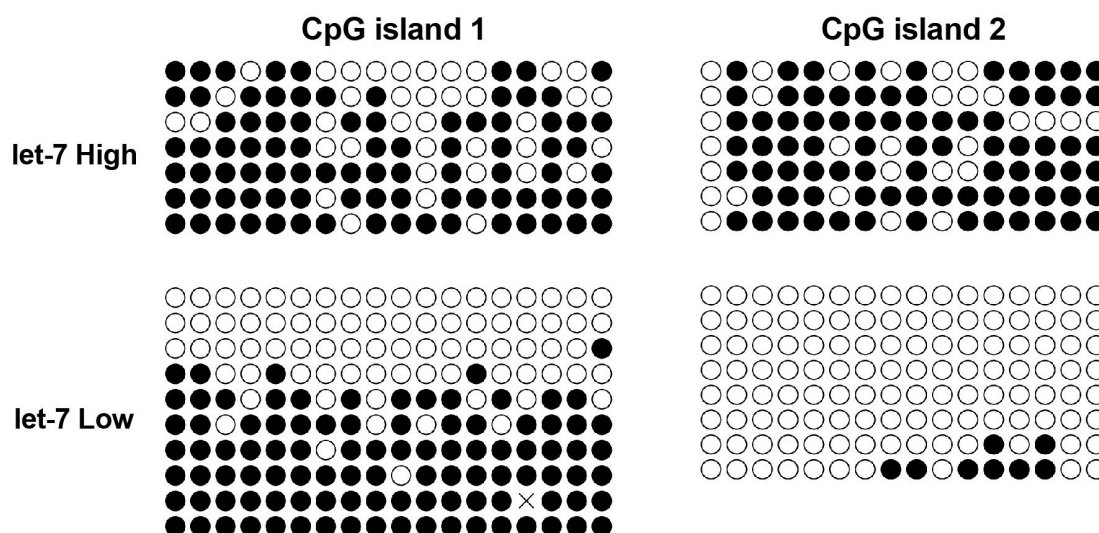
independent experiments. Statistical significance was assessed using one-way ANOVA or unpaired two-tailed Student's *t*-tests .



A

Human *LIN28A*

B



C

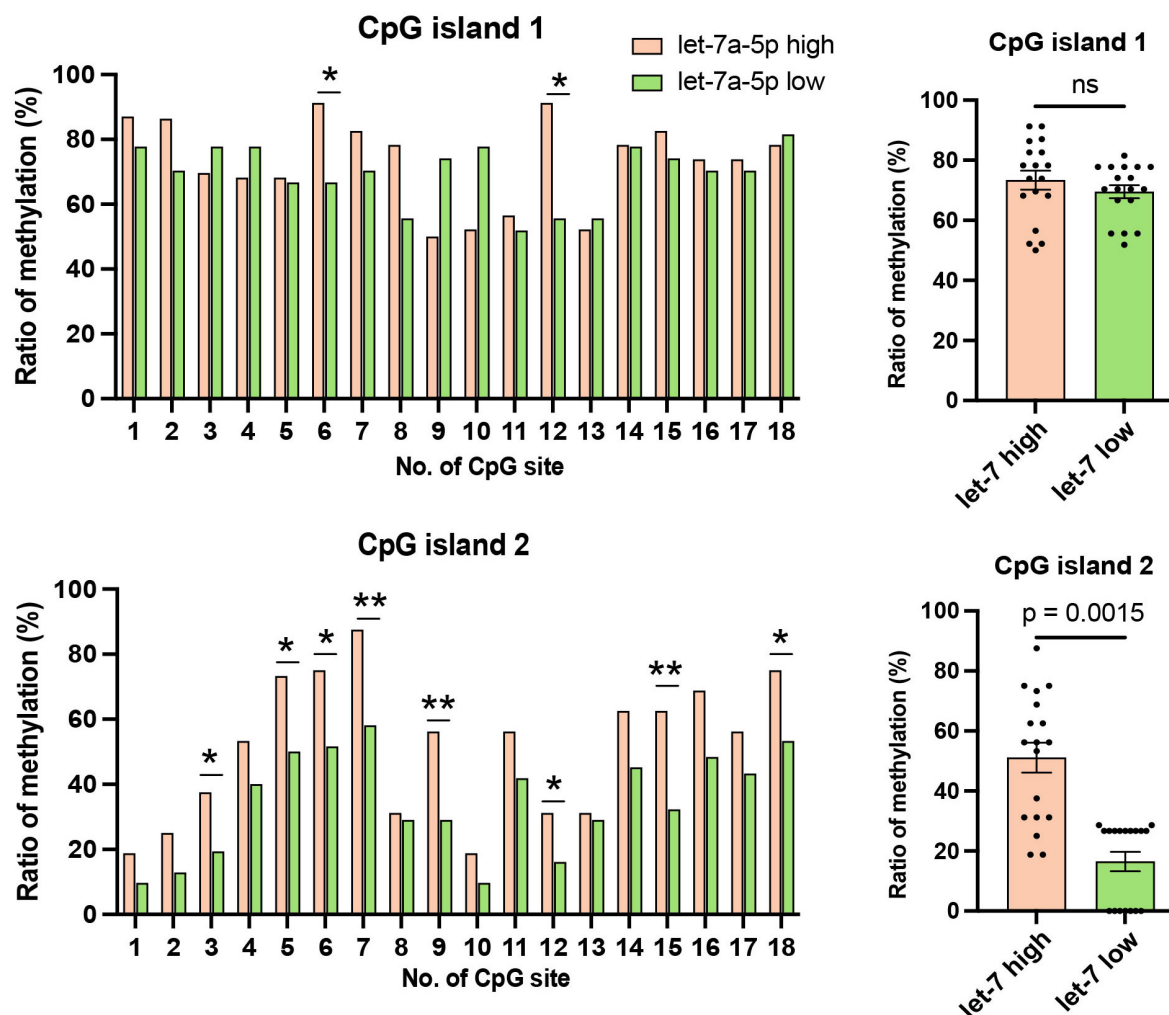
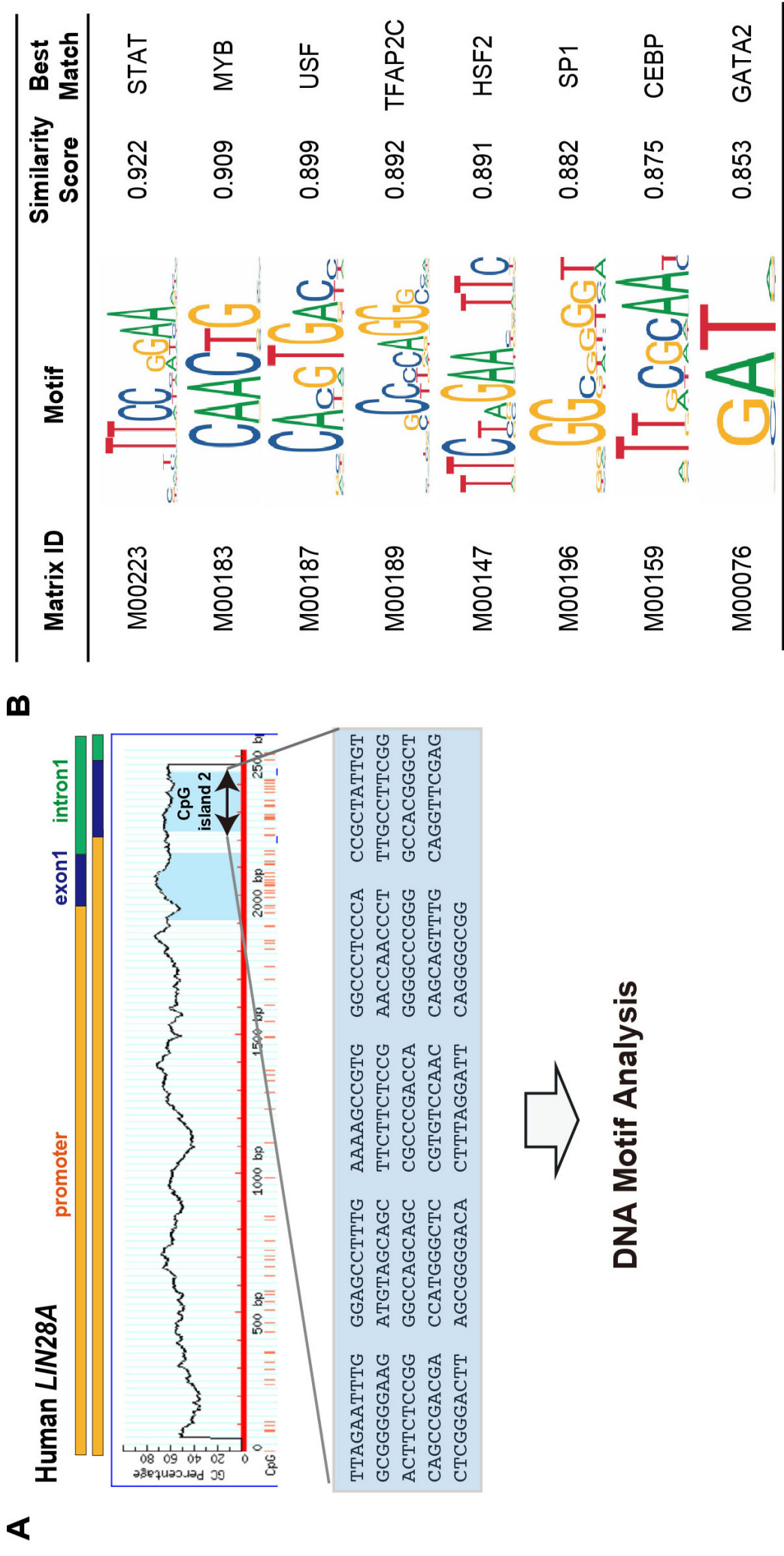
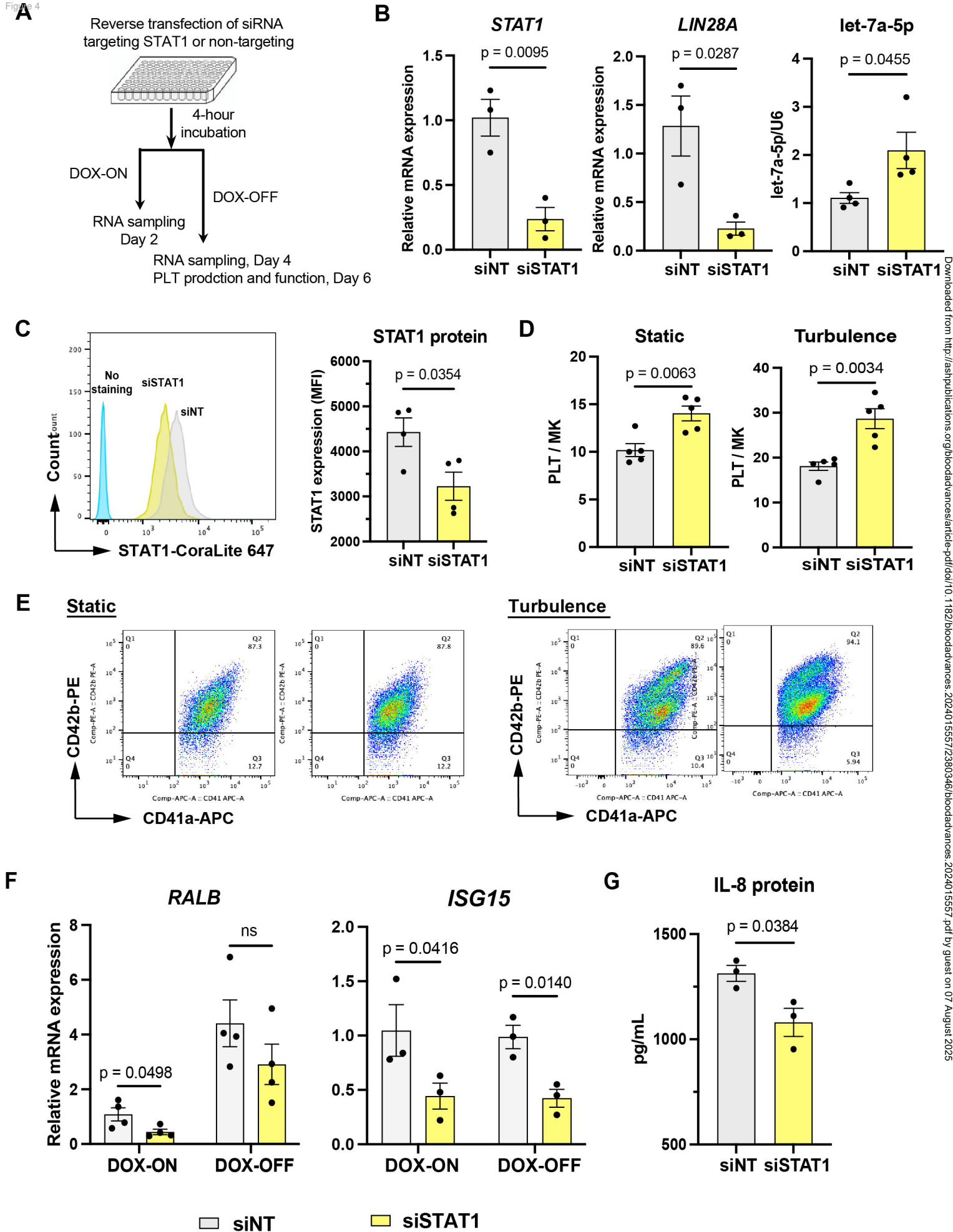
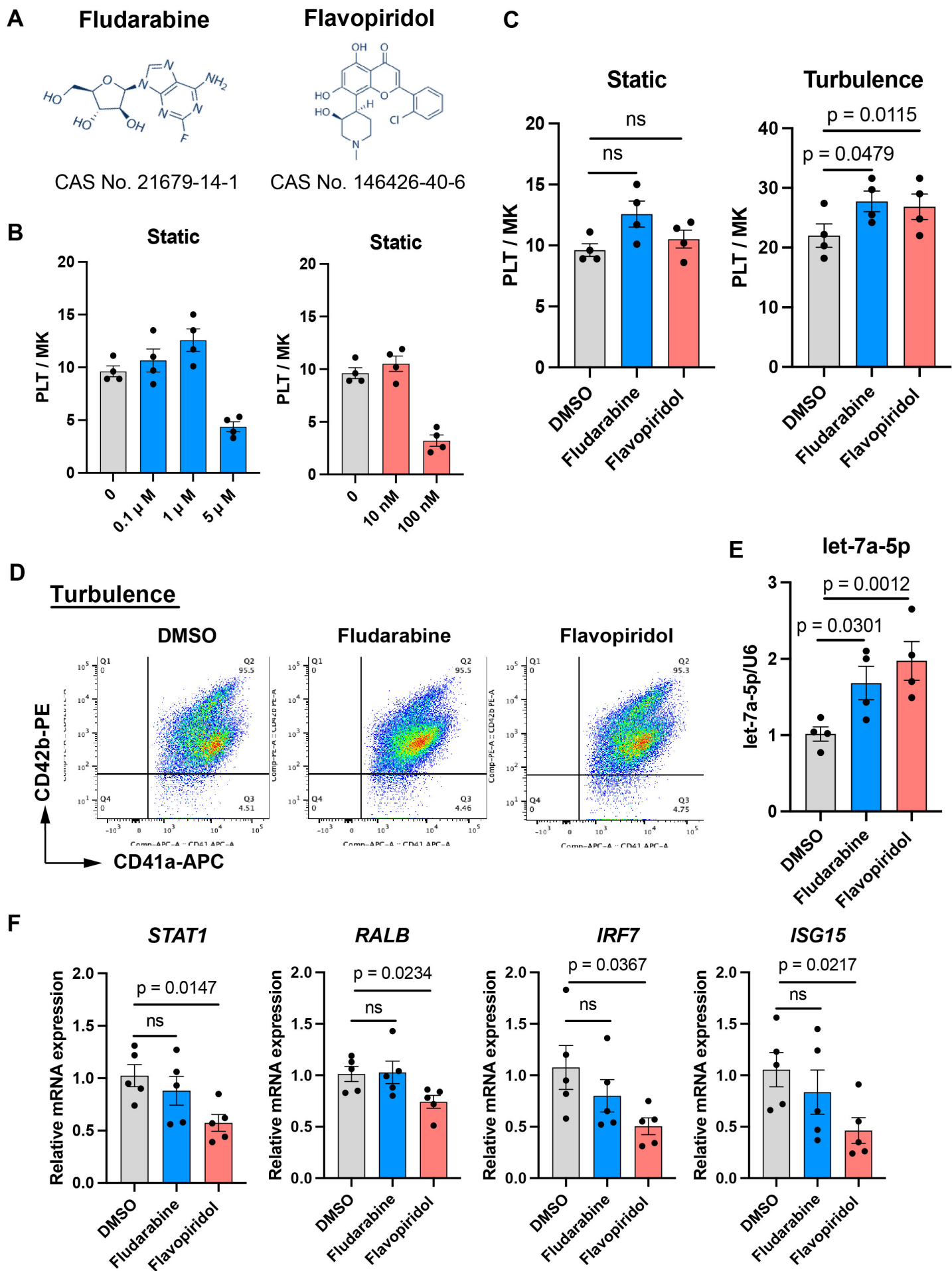


Figure 3



Hashimoto et al., Figure 3





Supplemental Materials

STAT1-mediated epigenetic regulation of LIN28A controls iPSC-derived platelet production through the let-7-RALB axis

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Supplementary Table 1. Primer pairs used in RT-qPCR

Gene symbol	Primer	Sequences (5'- 3')
<i>RALB</i>	Forward	GACTACGCAGCCATTCGAGA
	Reverse	CCGCTCCTCTAGGTCAGACT
<i>IRF7</i>	Forward	CTTCCAAGAAGAGCTGGTGGAAAT
	Reverse	TTCCAGCTTCACCAGGAC
<i>ISG15</i>	Forward	GAGAGGCAGCGAACTCATCT
	Reverse	CTTCAGCTCTGACACCGACA
<i>LIN28A</i>	Forward	CTTGAAGGAGGGTGAGGCAG
	Reverse	TGATGCTCTGGCAGAAAGTGG
<i>CDKN2A</i>	Forward	AGGCCGATCCAGGTCAT
	Reverse	ACCAGCGTGTCCAGGAA
<i>STAT1</i>	Forward	GTTAACGTTCGCACTCTGTG
	Reverse	GTACCACTGAGACATCCTGC
<i>STAT3</i>	Forward	TCTGTGTGACACCAACGAC
	Reverse	AAGGTGAGGGACTCAAACCTG
<i>MXI</i>	Forward	ACAGGACCATCGGAATCTTG
	Reverse	CGGCACTTGACAATCATG
<i>GAPDH</i>	Forward	GTTGCCATCAATGACCCCTTC
	Reverse	CATTGATGACAAGCTTCCCG

Supplementary Table 2. Primer pairs used in Bisulfite PCR

	Primer	Sequences (5'- 3')
<i>LIN28A</i> CpG island 1	Forward	TTAGGTTGTTTAAAGGATTTTAAGA
	Reverse	AAACAACCTACCAAACAACAAAAAAA
<i>LIN28A</i> CpG island 2	Forward	TTTTTAGAATTGTTGGGAGTTTTTGAAAAGT
	Reverse	AACCTAAAACCTC AATCCCCACCTC

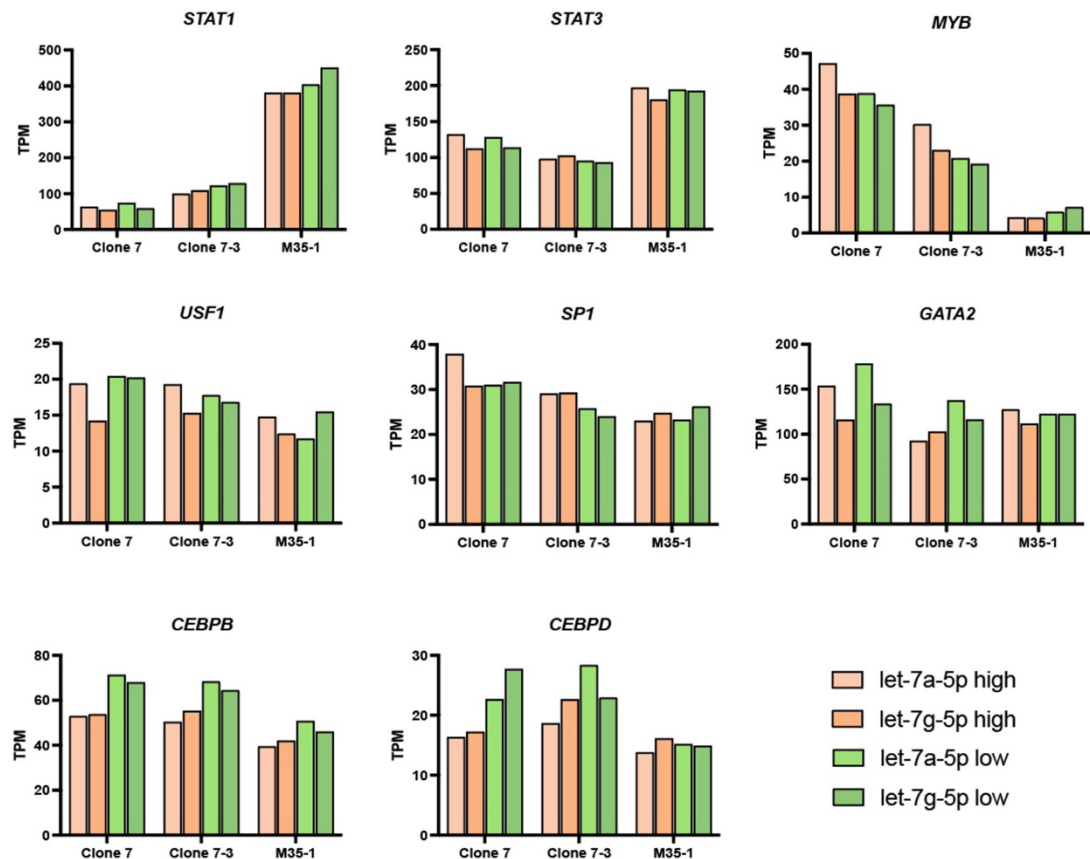


Figure S1 related to Figure 3

Figure S1, related to Figure 3 The mRNA expression of candidate genes (best match) identified in Figure 3. Bar graphs show TPM values of the indicated genes from three distinct imMKCL clones.

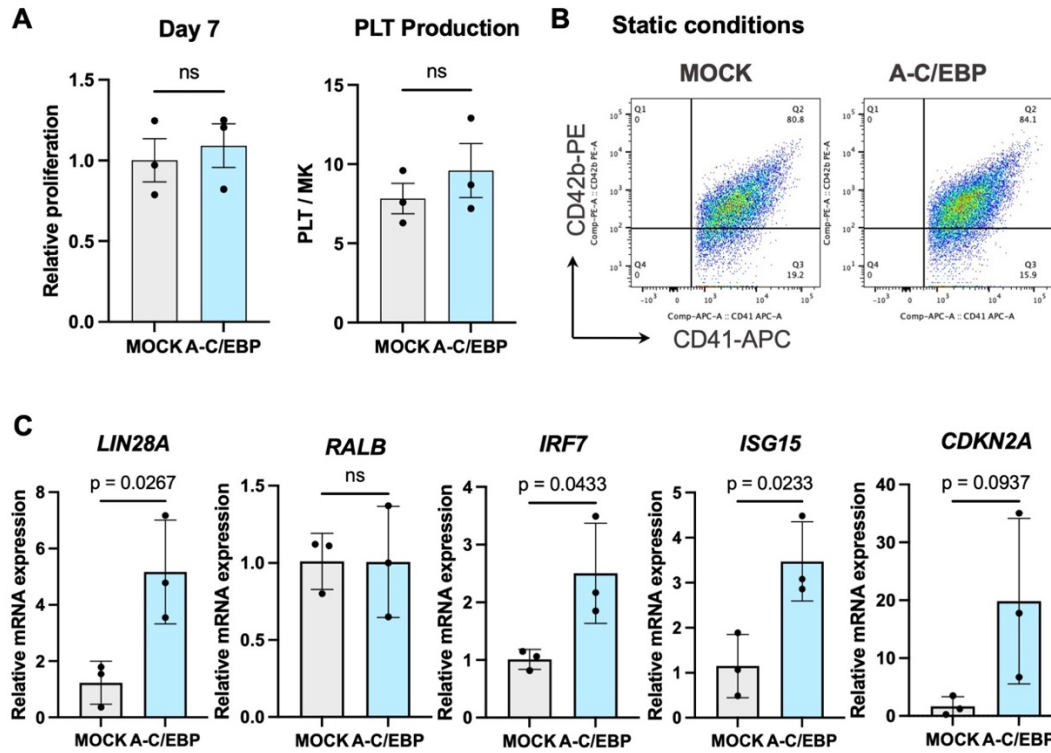


Figure S2 related to Figure 3

Figure S2, related to Figure 3. C/EBP family loss of function analysis. A retroviral vector encoding A-C/EBP, a dominant-negative C/EBP, or a control vector (MOCK) was transduced into imMKCLs. After transduction, EGFP-positive cells were sorted. **A.** MOCK and A-CEBP imMKCLs demonstrated comparable proliferative potential and iPSC-PLT production. **B.** Representative flow cytometry plots of iPSC-PLTs generated from MOCK and A-C/EBP imMKCLs in static conditions. **C.** mRNA expression of indicated genes in imMKCLs during the proliferation stage. Data are expressed as the mean $\pm$ SEM from three independent experiments. Unpaired two-tailed Student's *t*-tests were used to assess statistical significance.

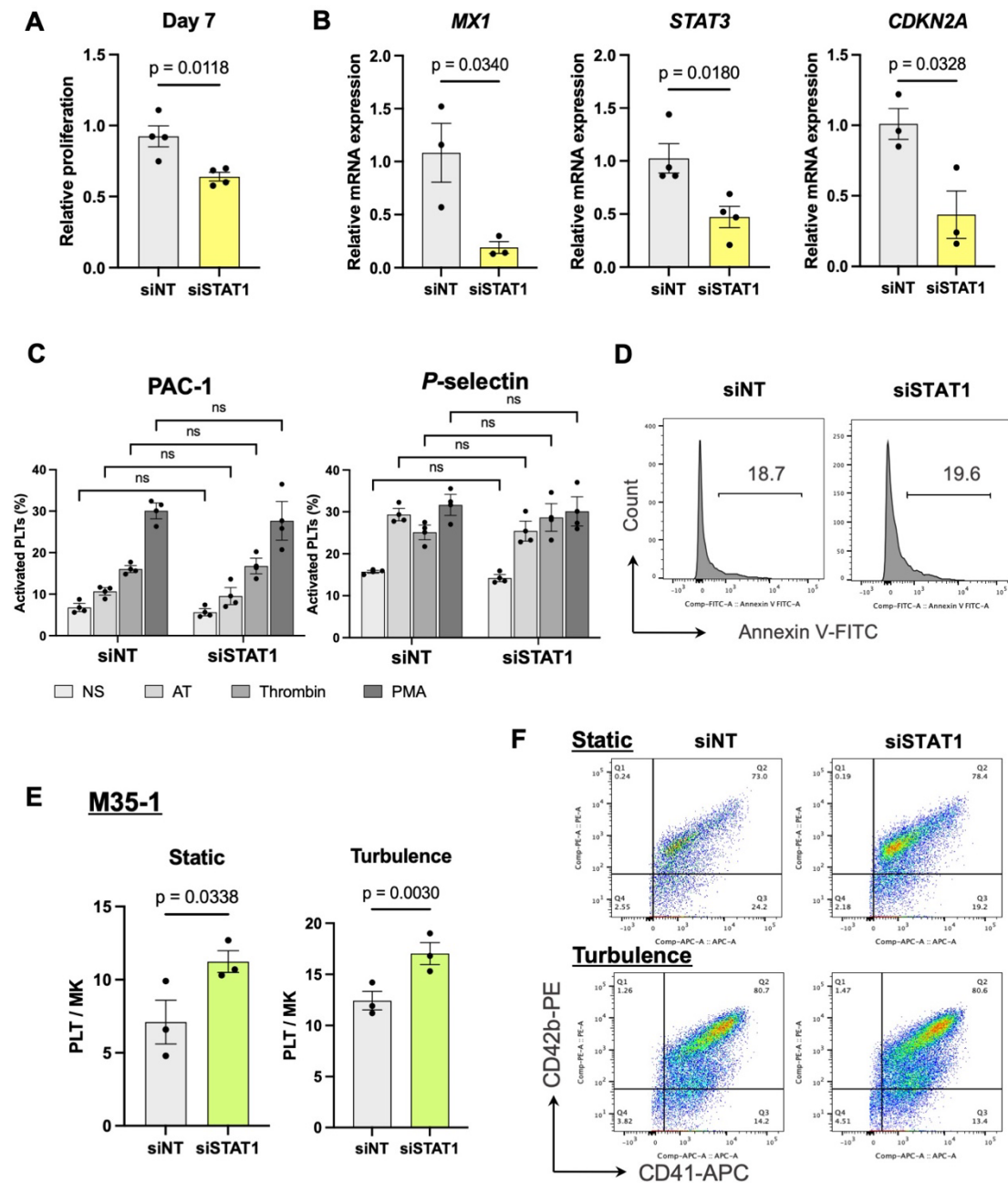


Figure S3 related to Figure 4

Figure S3, related to Figure 4. **A.** The relative fold change of siNT and siSTAT1 imMKCL (clone 7) cell counts on day 7. **B.** siRNA-mediated *STAT1* knockdown decreased mRNA expression of indicated genes in imMKCLs. Unpaired two-tailed Student's *t*-tests were used to assess statistical significance. **C.** Comparable PAC-1 binding and P-selectin expression on iPSC-PLTs generated from siNT or siSTAT1 imMKCLs with or without PMA or ADP/TRAP6

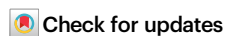
(AT) or thrombin stimulation. No stimulation (NS). **D.** Representative histograms of Annexin V bound to CD41⁺ iPSC-PLTs derived from siNT or siSTAT1 imMKCLs. **E.** STAT1 knockdown improved iPSC-PLT generation by imMKCLs (M35-1). **F.** Representative flow cytometry plots of iPSC-PLTs generated from siNT or siSTAT1 imMKCLs in static or turbulent flow conditions. CD41⁺CD42⁺ PLTs were identified from a platelet-sized gate. iPSC-PLTs were generated in turbulent flow conditions. Data are expressed as the mean $\pm$ SEM from three to four independent experiments.

A let-7 microRNA-RALB axis links the immune properties of iPSC-derived megakaryocytes with platelet producibility

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We recently achieved the first-in-human transfusion of induced pluripotent stem cell-derived platelets (iPSC-PLTs) as an alternative to standard transfusions, which are dependent on donors and therefore variable in supply. However, heterogeneity characterized by thrombopoiesis-biased or immune-biased megakaryocytes (MKs) continues to pose a bottleneck against the standardization of iPSC-PLT manufacturing. To address this problem, here we employ microRNA (miRNA) switch biotechnology to distinguish subpopulations of imMKCLs, the MK cell lines producing iPSC-PLTs. Upon miRNA switch-based screening, we find imMKCLs with lower let-7 activity exhibit an immune-skewed transcriptional signature. Notably, the low activity of let-7a-5p results in the upregulation of RAS like proto-oncogene B (*RALB*) expression, which is crucial for the lineage determination of immune-biased imMKCL subpopulations and leads to the activation of interferon-dependent signaling. The dysregulation of immune properties/subpopulations, along with the secretion of inflammatory cytokines, contributes to a decline in the quality of the whole imMKCL population.

The first successful whole blood transfusion took place in 1818 by James Blundell¹. This historic achievement was followed by Karl Landsteiner's discovery of ABO blood types in 1901² and the development of a platelet isolation device for transfusion by Emil J. Freireich in the 1950s-1960s³. Since then, donor-dependent platelet transfusion has become a standard healthcare practice despite persistent challenges of supply shortages resulting from various factors including viral contamination, alloimmune transfusion refractoriness, and the recent COVID-19 pandemic. In this context, we have adopted the strategy of ex vivo blood pharming using human induced pluripotent

stem cells (iPSCs). We developed immortalized MK progenitor cell lines (imMKCLs) as the starting cell source for ex vivo iPSC-derived platelet (iPSC-PLT) manufacturing^{4,5}. imMKCLs exhibit sustained proliferation over several months in the presence of doxycycline (DOX) and shed iPSC-PLTs after DOX removal, enabling the generation of over 10¹¹ iPSC-PLTs in a turbulent flow-based bioreactor⁶. Building upon these achievements, we initiated the first-in-human clinical trial, iPLAT1⁷⁻⁹. While iPLAT1 demonstrated promising outcomes without significant adverse events, a transient increase in D-dimer levels and leukocyte count was observed after transfusion of the maximal dose⁷.

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These observations indicate the potential involvement of recently identified immune-skewed MKs^{10,11}, raising concerns about the quality control of imMKCLs as master cells. Meanwhile, we noticed considerable variation in the quality of imMKCL clones with regards to their proliferation and iPSC-PLT production capacity¹². Specifically, certain imMKCL clones displaying cellular senescence exhibited a diminished capacity to generate iPSC-PLTs, but the capacity could be restored through the knockdown of p53 and CDKN1A¹². However, the molecular factors underlying this heterogeneity, which significantly hinder the efficiency and standardization of iPSC-PLT manufacturing, remain unknown.

MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate the stability and translation of the target mRNAs by binding with the complementary mRNA sequences. In hematopoietic cells, miRNAs have been identified as key players in cell fate decisions and functions¹³. Based on the evidence that DNA-based genetic circuits regulate protein expression depending on miRNA activities rather than miRNA expression¹⁴, we developed an innovative biotechnology method, miRNA switch, which enables the identification of specific cell types^{15,16}. MiRNA switches can detect endogenous miRNA activity to distinguish heterogeneous cell populations without the need of antibody labeling. This technology has proven successful for various cell types, including iPSC-derived hepatocytes, endothelial cells, cardiomyocytes¹⁵, mouse embryonic stem cell (ESC)-derived neurons¹⁷, and undifferentiated human ESCs and iPSCs¹⁸. We therefore hypothesized that the miRNA switch can be employed to distinguish subpopulations among imMKCLs with heterogeneous miRNA activities (i.e., a gradient variation in miRNA activity).

In this study, we screened a library containing 269 target miRNA switches, leading to the identification of let-7a-5p and let-7g-5p miRNAs with heterogeneous activity among imMKCLs. By a transcriptional analysis of imMKCL subpopulations exhibiting either high or low let-7 activity (hereafter referred as let-7 high and let-7 low, respectively), we discovered that let-7 low imMKCLs exhibited an immune-skewed transcriptional signature. Further investigation unveiled the pivotal role of let-7a-5p and its downstream target, RAS like proto-oncogene B (*RALB*), in modulating the lineage determination of 'immune' MKs within imMKCLs. Importantly, we elucidated that the dysregulation of immune properties/subpopulations within imMKCLs, along with the secretion of inflammatory cytokines, results in a decline of their quality based on their arrested proliferation and deficient iPSC-PLT generation. This study provides valuable insights into the correlation between immune-biased MKs and their role in quality control during ex vivo iPSC-PLT manufacturing.

Results

Identification of miRNAs with endogenous activity in imMKCLs

MiRNA switch technology describes synthetic mRNA-based genetic circuits that regulate transgene expressions through miRNA activity^{15,16}. We hypothesized that miRNA switches can identify imMKCL subsets with distinct miRNA activities, providing a valuable tool for exploring the heterogeneity within imMKCLs. Figure 1a depicts a schematic illustration of miRNA switch technology. We designed synthetic mRNAs that contain complementary sequences of target miRNAs in the 5' untranslated region (UTR) and encode a reporter fluorescent protein to monitor endogenous miRNA activity. A pair of mRNAs were synthesized and co-transfected into imMKCLs, including Azami-Green 1 (AG1)-coding mRNA as a transfection control and TagBFP-coding mRNA containing the target miRNA sequence (Fig. 1b). TagBFP expression levels were measured by flow cytometry in cells with endogenous activity for a specific miRNA (Fig. 1c). A miRNA switch-based screening was conducted using a library containing 269 miRNA switches (Supplementary Table 1) and resulted in the identification of 24 types of miRNAs with endogenous activity in imMKCLs (Fig. 1d).

Let-7 miRNA switches enrich immune-skewed imMKCLs

Among the active miRNAs, we focused on let-7a-5p and let-7g-5p, as they showed heterogeneous activity among imMKCLs (Fig. 2a). We sorted subpopulations with high or low let-7 activity, with the let-7 low subset representing less than 5% of the total population. We confirmed that the let-7 activity levels in these populations correlated with their expression levels in imMKCL subpopulations (Supplementary Fig. 1a). To characterize molecular differences between the let-7 high and let-7 low subpopulations, we performed a bulk RNA-seq analysis using three different imMKCL clones (M35-1, clone 7, clone 7-3) in both the proliferation and maturation phases. The properties of clone 7 imMKCLs and the functionality of the derived iPSC-PLTs were reported previously^{5,6}. Besides, clone 7 has been employed by multiple research groups for the investigation of megakaryopoiesis and thrombopoiesis^{19–21}, highlighting its importance as a research tool. Clone 7-3, which is derived from clone 7 and displays an aged phenotype characterized by reduced proliferation and diminished iPSC-PLT production following repeated cultures, was also employed (Supplementary Fig. 1b). M35-1 was established from patient iPSCs for the iPLAT1 clinical trial due to its relatively superior expandability and iPSC-PLT productivity when compared with other competent patient-derived imMKCL clones⁸. Comparable iPSC-PLT generation was observed in the three clones for the let-7 low or let-7 high subpopulations, respectively (Supplementary Fig. 1c).

The sampling procedure for the bulk RNA-seq is illustrated in Fig. 2b. A differential gene expression analysis revealed many genes with altered expression profiles between let-7 high and let-7 low imMKCLs. To identify enriched transcriptional pathways in each subpopulation, we performed a gene set enrichment analysis (GSEA). Notably, we observed a series of immune-related gene sets that were significantly enriched in let-7 low imMKCLs in both the proliferation and maturation phases (Fig. 2c, d). Specifically, in the proliferation phase, gene sets in response to tumor necrosis factor (TNF), lipopolysaccharide (LPS), and enterotoxin were enriched in let-7 low cells, consistent with the reported properties of naïve mouse and human MKs with regard to sensing invading pathogens^{22,23}. TNF-driven inflammation has also been suggested to induce platelet hyperreactivity in aging²⁴. In the maturation phase, gene sets related to interferon signaling were significantly enriched in let-7 low cells. Some immune related properties were retained from the proliferation phase to maturation phase (Fig. 2e–h, Supplementary Table 2). These findings suggest that imMKCLs with lower let-7 activity preferentially enrich an immune-skewed MK phenotype. Our bulk RNA-seq analysis also identified the upregulation of several genes encoding chemokines and cytokines, including C-X-C motif chemokine ligand 10 (*CXCL10*), C-C motif chemokine ligand 2 (*CCL2*), and *CCL3*, in the immune-skewed let-7 low subpopulations of imMKCLs (Fig. 2f, h), indicating the consistency of multiple immune responses through the secretion of these molecules in human MKs²⁵. Interestingly, elevated mRNA levels of these molecules have been reported in patients with severe acute respiratory syndrome (SARS) and middle east respiratory syndrome (MERS)²⁶. Moreover, in COVID-19 patients, increased mRNA expression levels of *CXCL10*, *CCL2*, and *TNF* were correlated with an increased number of MKs^{27,28}.

We further investigated whether immune MKs could be identified by let-7 miRNA switches in MK progenitors directly differentiated from human ESCs. We utilized CD34+ hematopoietic progenitor cells (HPCs) generated by our hPSC-sac method^{29,30} and performed a bulk RNA-seq analysis on two separate cell populations with different let-7a-5p and let-7g-5p activities (Supplementary Fig. 2a). A GSEA revealed similar enriched immune-related gene sets in let-7 low ESC-derived HPCs (Supplementary Fig. 2b, c, Supplementary Table 2), suggesting that the fate decision of immune-skewed MKs in MK development may occur as early as the hematopoietic progenitor stage.

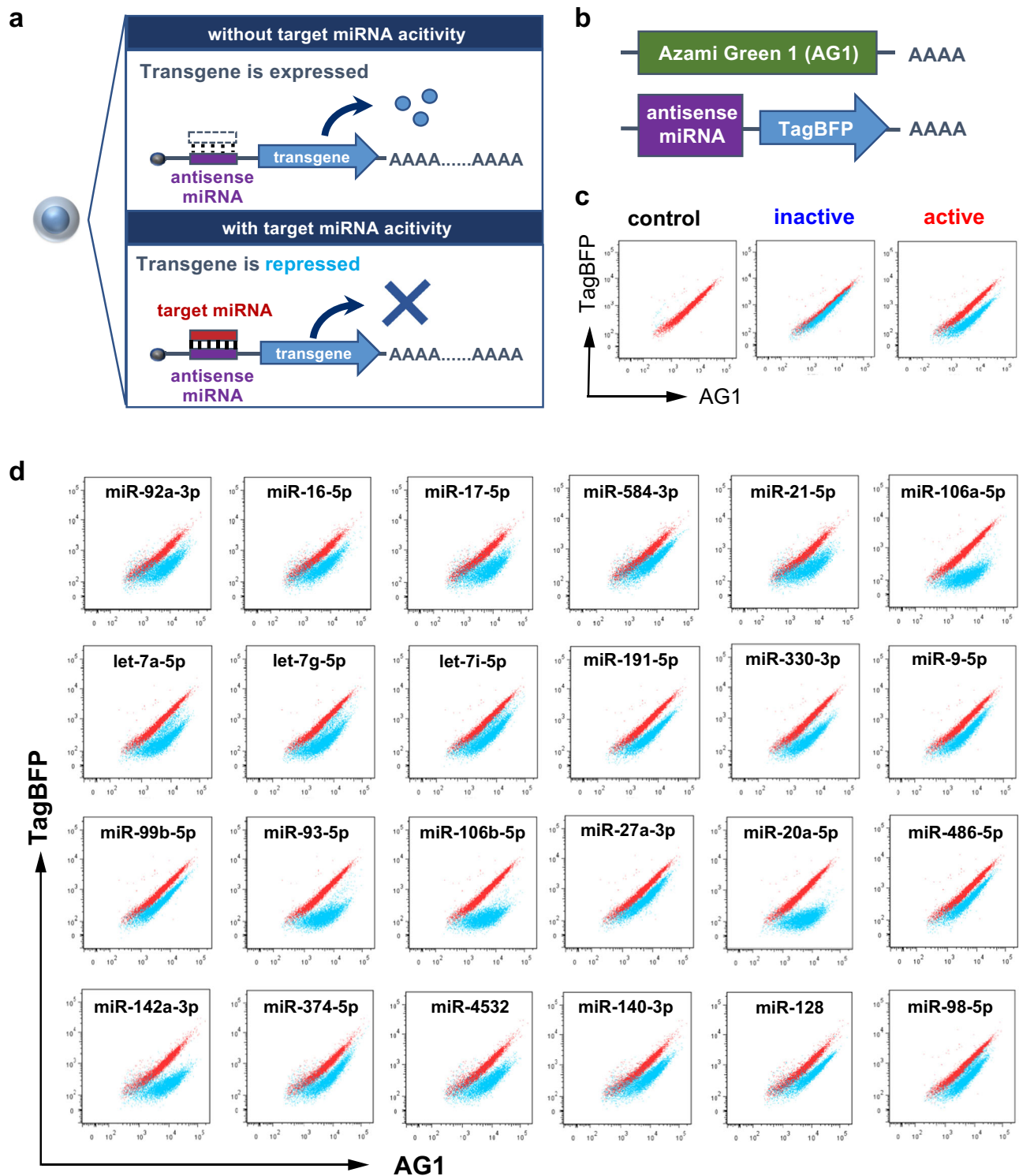


Fig. 1 | A miRNA switch-based screening identifies miRNAs with endogenous activity in imMKCLs. **a** A schematic illustration of the design of miRNA switches. The mRNA is composed of the antisense sequence of the target miRNA and a reporter transgene. In the presence of an active target miRNA, the expression of the reporter gene is repressed. **b** Design of miRNA switches for imMKCLs. A pair of mRNAs, one encoding TagBFP containing the antisense sequence of the miRNA target in the 5' UTR and the other of Azami Green protein, were synthesized in vitro. The two mRNAs were co-transfected into imMKCLs via lipofection. 24 h after the

transfection, the cells were analyzed by flow cytometry to identify the miRNA activity in imMKCLs through a screening. **c** Representative dot plots of target miRNA activity in imMKCLs. Active miRNA showed less TagBFP expression. **d** Flow cytometry analysis of the target miRNAs showing endogenous activity in imMKCLs. A miRNA switch-based screening was performed from a library containing 269 kinds of miRNA switches (Supplementary Table 1), and 24 kinds of miRNAs were identified with endogenous activity in imMKCLs.

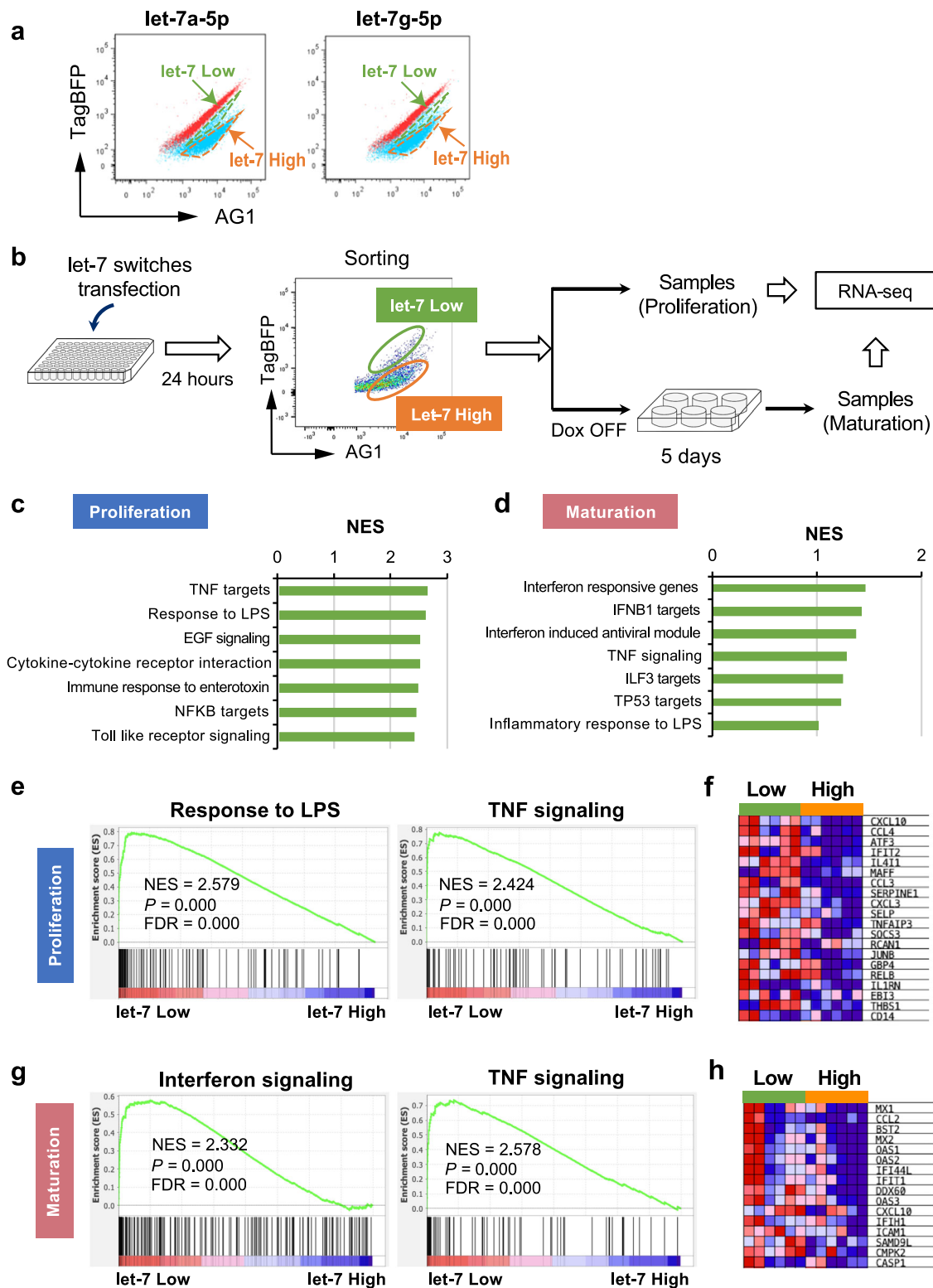


Fig. 2 | Let-7 miRNAs enable the identification of immune-skewed imMKCLs.

a Heterogenous responsive activities in imMKCLs were identified with let-7a-5p or let-7g-5p miRNA switches, resulting in let-7 low-responsive and let-7 high-responsive subpopulations. **b** A schematic illustration of the bulk RNA-seq sampling workflow. Let-7a-5p and let-7g-5p switches were transfected into imMKCLs, followed by the sorting of let-7 low- and high-responsive cells by flow cytometry. Three distinct imMKCL clones (clone 7, clone 7-3, M35-1) were employed for the bulk RNA-seq analysis. Gene set enrichment analysis (GSEA) were performed to compare let-7 low ($n = 6$ independent biological samples) and let-7 high ($n = 6$)

subpopulations. Bar graphs showing the GSEA results of the top enriched immune-related gene sets in let-7 low-responsive imMKCLs in the proliferation phase (**c**) and maturation phase (**d**). GSEA plots showing typical enriched immune-related gene sets in the proliferation phase (**e**) and maturation phase (**g**). Representative enrichment plots from each group are displayed with the normalized enrichment score (NES), the determined nominal (non-adjusted) p -value, and the false discovery rate (FDR) derived from GSEA software. Heatmaps of the top differentially expressed TNF targets of let-7 low- or high-responsive imMKCLs in the proliferation phase (**f**) and maturation phase (**h**). Source data are provided as a Source Data file.

scRNA-seq reveals the heterogeneity and functional diversity of imMKCLs

To further characterize the cellular heterogeneity of imMKCLs and explore the mechanism(s) underlying the enrichment of ‘immune’ imMKCLs by let-7 miRNA switches, we performed a single-cell (sc)RNA-seq analysis on let-7 low and high imMKCLs in the proliferation phase. Since both let-7a-5p and let-7g-5p miRNA switches demonstrated similar behaviors accordingly to the bulk RNA-seq analysis (Fig. 2), we focused our investigation on let-7a-5p. We identified five transcriptionally heterogeneous subpopulations of imMKCLs (Fig. 3a), with let-7 low imMKCLs being relatively concentrated in clusters 3 and 5 (Fig. 3b). We further determined the expression of the top differentially expressed genes (DEGs) and characterized the gene ontology (GO) terms enriched in each cluster (Fig. 3c, d).

Cluster 1 exhibited an enrichment of GO:BP (Biological Process) terms associated with oxidative phosphorylation, indicating their role in energy supply. Conversely, clusters 2 and 4 represented subsets of cycling MK progenitors with enriched GO:BP terms related to cell cycle and mitotic nuclear division. The identification of proliferating cell nuclear antigen (*PCNA*) and tropomyosin 4 (*TPM4*), two marker genes of cycling MKs, is consistent with recent studies on human bone marrow (BM) cycling MKs³¹. Cluster 3 highly expressed genes associated with “platelet activation” and “blood coagulation”, suggesting a subset of MK progenitors dedicated to platelet emergence. In particular, thrombopoiesis-associated genes, such as thrombospondin 1 (*THBS1*)¹⁰, von Willebrand factor (*VWF*)³², and platelet glycoprotein IX (*GP9*)³³, were highly expressed in cluster 3 compared to the other clusters (Fig. 3f). Moreover, cluster 3 was enriched with genes related to the immune system process, suggesting that it may represent a subset of MK progenitors with functional duality. For instance, platelet factor 4 (*PF4*, also known as *CXCL4*), one of the top DEGs enriched in cluster 3, promotes blood coagulation (Fig. 3c)³⁴ and is a crucial regulator in innate immunity through its activity on macrophages³⁵ and neutrophils³⁶. Additionally, pro-platelet basic protein (*PPBP*, also known as *CXCL7*), an activator of neutrophils against bacteria³⁷, was also significantly upregulated in cluster 3.

Both clusters 3 and 5 may represent subpopulations of MK progenitors with highly upregulated potential immune-related genes. The annotated immune genes in clusters 3 and 5 are involved in immune system process and immune response according to the GO analysis (Fig. 3e). Cluster 5 highly expressed genes sets in response to type I interferon, cytokine, and virus, whereas cluster 3 appeared to be responsive to myeloid leukocyte-mediated immunity. *CCL5*, a key proinflammatory chemokine³⁸, was one of the top DEGs enriched in cluster 5 (Fig. 3f). Notably, *CCL5* has been reported to promote platelet formation in thrombopoiesis³⁹, and its expression was upregulated in cluster 3 compared to clusters 1, 2, or 4 (Fig. 3c, f). The interferon-stimulated gene for interferon-stimulated 15 (*ISG15*), a ubiquitin-like protein that can be covalently bound to host and viral proteins⁴⁰, was identified as the top DEG in cluster 5. Given that the secretion of type I interferons from virus-infected cells is a hallmark of antiviral immunity⁴¹, our results indicated that cluster 5 represents the representative ‘immune’ MKs involved in antiviral functions. Recent in vivo models of naïve MKs have demonstrated the presence of ‘immune’ MKs, the hematopoietic stem cell (HSC) niche supporting MKs, and thrombopoiesis-biased MKs^{10,11,31}. Notably, our results suggest that imMKCLs have a thrombopoiesis-biased subset (cluster 3) and a representative immune subset (cluster 5), similar to those found in endogenous human MKs.

The inhibition of let-7a-5p activity drives the development of ‘immune’ imMKCLs

We next investigated whether let-7 functionally drives the development of ‘immune’ subsets in imMKCLs by conducting loss-of-function experiments (Fig. 4a). We inhibited let-7a-5p expression/activity using

a let-7a-5p inhibitor (Fig. 4b, c) and found enhanced gene expressions of several immune-related molecules identified in clusters 3 and 5, including *PF4*, *PPBP*, *ISG15*, and interferon induced protein with tetra-ricopeptide repeats 3 (*IFIT3*) (Fig. 4d). We also investigated whether the inhibition of let-7a-5p activity in imMKCLs alters the response to immune stimuli. The cells were stimulated with the pathogen receptor agonist LPS or control buffer. The supernatants were analyzed using a cytometric bead array kit by flow cytometry. We found that the inhibition of let-7a-5p in imMKCLs promoted the secretion of interleukin-8 (IL-8) (Fig. 4e), a critical proinflammatory chemokine⁴². IL-8 and its receptors have been shown to control MK proliferation and maturation⁴³. These results are consistent with a previous report that found human cord blood-derived MKs produce IL-8 even in the absence of stimuli⁴⁴. Collectively, we concluded that let-7a-5p is a functional player that modulates the development of ‘immune MKs’ among imMKCLs.

RALB is a functional target of let-7a-5p in the development of immune-skewed imMKCLs

The preceding experiments revealed the critical role of let-7a-5p in immune-skewed imMKCLs. To further determine the underlying factors that contribute to immune-related outcomes, we conducted an ingenuity pathway analysis (IPA) to investigate the upstream regulators of cluster 3 and cluster 5 (let-7 low imMKCLs). Figure 5a shows a flowchart depicting the overall analysis design. IPA analysis identified the MK transcriptional factor *GATA1*⁴⁵ and the myeloid development regulator *KLF2* as potential transcription activators in cluster 3 (Fig. 5b). Notably, *KLF2* was shown to regulate host innate immune responses to polymicrobial infections⁴⁶. On the other hand, known activators of virus-inducible cellular genes, such as interferon regulatory factor 7 (*IRF7*)⁴⁷ and *IRF3*⁴⁸, were identified as potential upstream regulators in cluster 5 (Fig. 5b). We also compared common upstream regulators of clusters 3 and 5 with the predicted let-7a-5p targets (Fig. 5c). Our analysis identified twenty candidate upstream regulators, of which eight candidates showed detectable expression levels in imMKCLs (Fig. 5d). Based on their increased expression levels in the enriched let-7 low clusters (clusters 3 and 5), we suspected that cut-like homeobox 1 (*CUX1*) and RAS like proto-oncogene B (*RALB*) are targets of let-7a-5p (Fig. 5e) and may contribute to the observed immune-related outcomes. Accordingly, the inhibition of let-7a-5p upregulated the mRNA expression levels of *CUX1* and *RALB* in both the proliferation and maturation phases (Fig. 5f). While the overexpression of *CUX1* did not cause significant effects (Supplementary Fig. 3), *RALB* overexpression upregulated the mRNA expression of *IRF7*, *ISG15*, and *IFIT3* (Fig. 5g, h, Supplementary Fig. 4a, b), but not the expression level of let-7a-5p (Supplementary Fig. 4c). On the other hand, despite being predicted as an upstream regulator of both clusters 3 and 5, *RALB* overexpression did not influence the expression of thrombopoiesis-related genes identified in Cluster 3 (Supplementary Fig. 4d), indicating that distinct let-7 targets may be responsible for regulating immune-related pathways and thrombopoietic pathways in imMKCLs. Combining our findings with the IPA (Fig. 5b), we propose that let-7a-5p tunes the immune properties of imMKCLs by targeting *RALB* (Fig. 5i).

The dysregulated immune properties/subsets are associated with the quality of imMKCLs

We previously reported that imMKCL clones exhibiting slower proliferation rates at the proliferation stage are less capable of generating iPSC-PLTs (Fig. 6a)¹². However, the factor(s) responsible for inducing the low quality of imMKCLs, along with the cellular senescent/aging transcriptional signature (Supplementary Fig. 5a, b), remains unclear. Therefore, we reexamined our GSEA of the different qualities of MKCLs utilized in our previous study¹². Similar to the GSEA of the let-7 low and high populations (Fig. 2), we found TNF signaling and

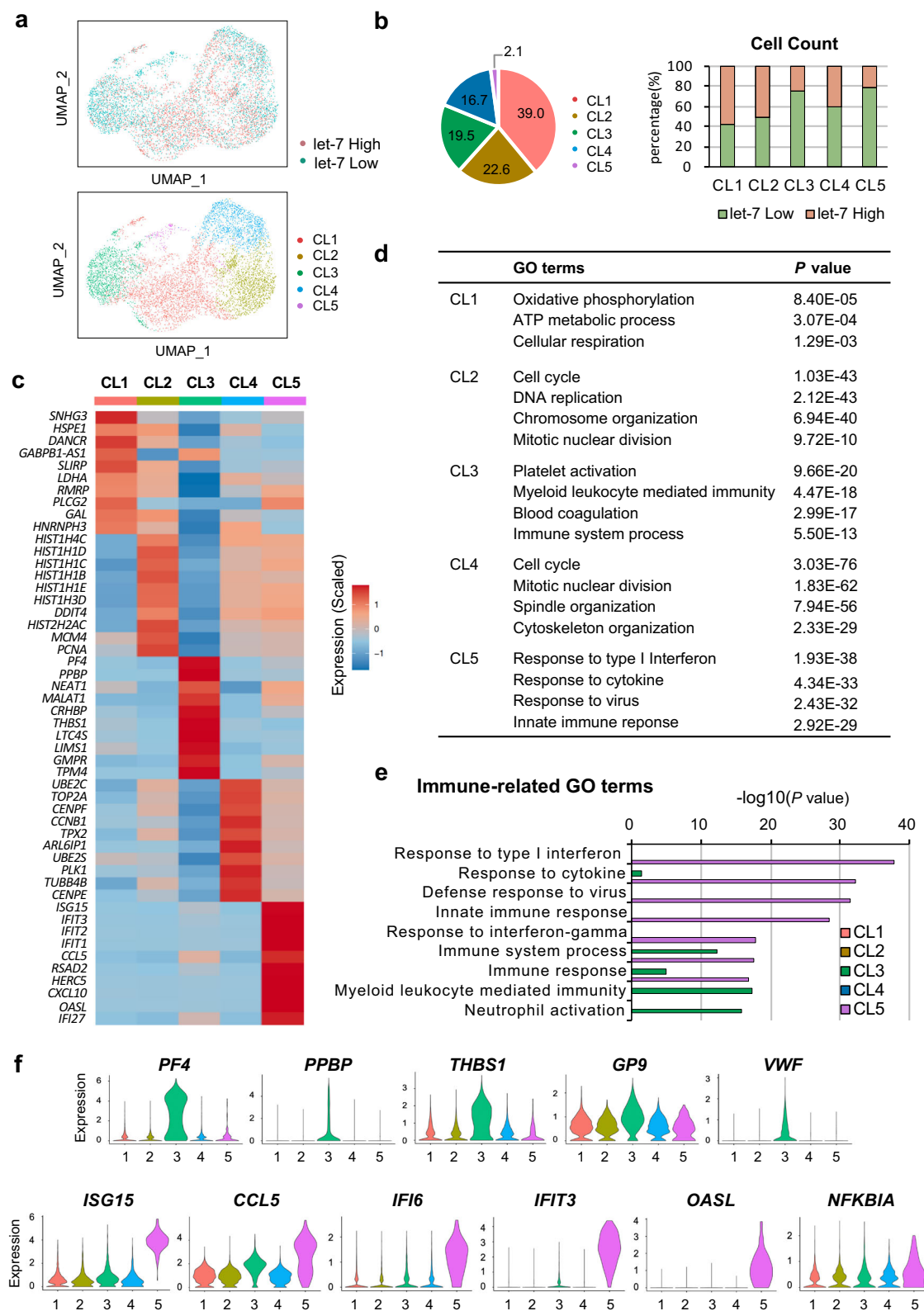


Fig. 3 | Single-cell RNA-seq reveals the presence of transcriptionally distinct immune-skewed subpopulations that are abundant in let-7a-5p low-responsive imMKCLs. a Visualization of imMKCLs (clone 7) by uniform manifold approximation and projection (UMAP) and colored according to let-7a-5p high/low-responsive imMKCLs and imMKCL sub-clusters. **b** The distribution of let-7a-5p low- and high-responsive cells in each cluster, and a bar plot showing the distribution of let-7a-5p low- and high-responsive cells in each cluster. **c** The relative expression level of the top ten DEGs in each cluster. **d** The representative terms of GO:BP enriched in each

cluster. GO terms under the biological process category for upregulated differentially expressed genes (DEGs) in each cluster were analyzed by g:Profiler, *p*-values were determined by a default hypergeometric test and correction for multiple testing has been performed by the g:SCS algorithm. **e** Typical immune-related GO:BP terms in each cluster. **f** Violin plots showing the expression level of typical thrombopoiesis-related and immune-related genes in each cluster. Source data are provided as a Source Data file.

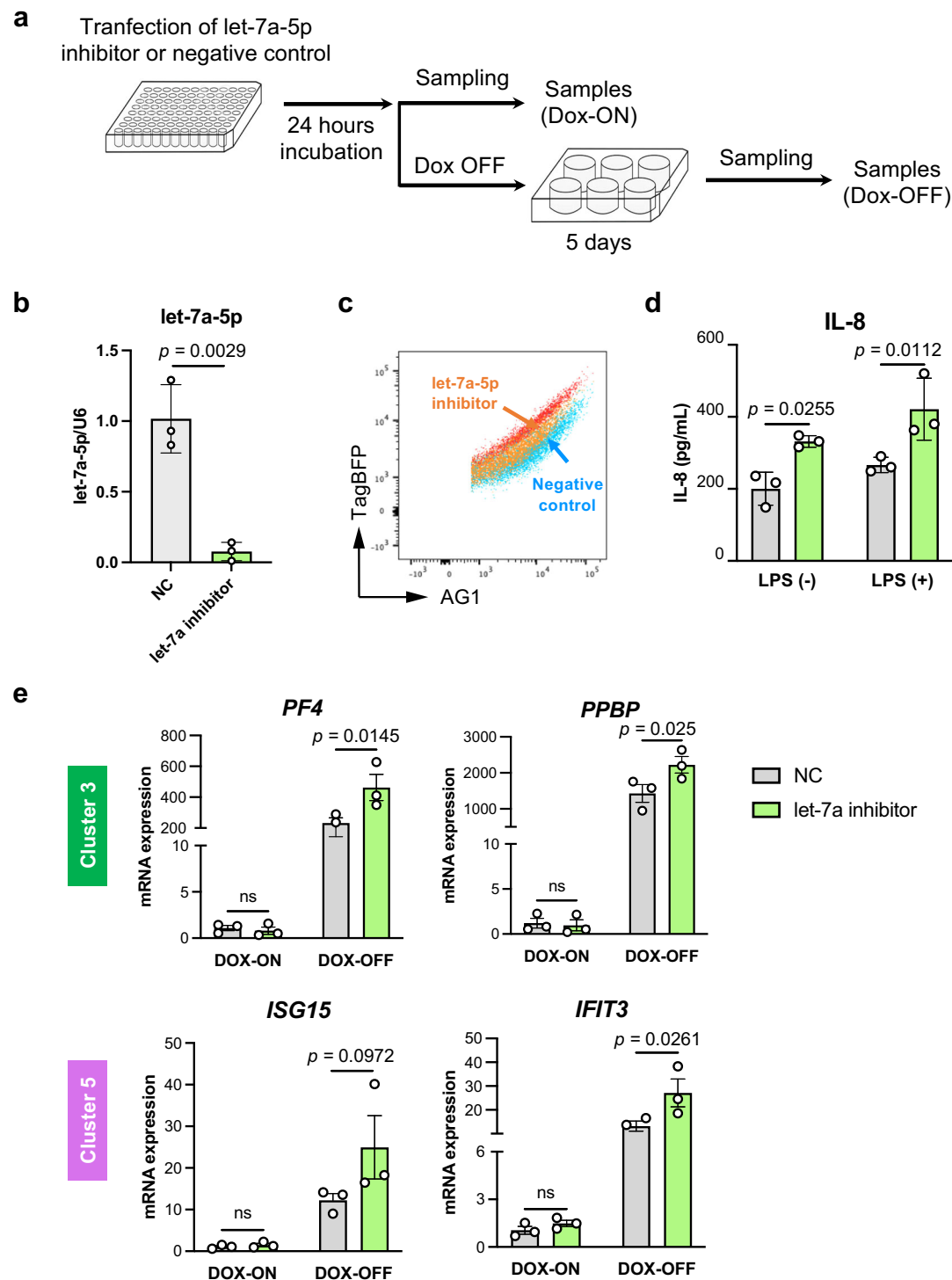


Fig. 4 | Let-7a-5p is a functional player in the development of immune-skewed subsets in imMKCLs. **a** A schematic illustration of the experimental workflow using a specific let-7a-5p inhibitor. **b** Let-7a-5p expression levels in imMKCLs (clone 7) treated with a negative control or let-7a-5p inhibitor. The expression levels were measured by qRT-PCR and normalized to an endogenous control, RNU6B (U6). **c** Let-7a-5p activity patterns were analyzed by FACS. **d** The inhibition of let-7a-5p in imMKCLs (clone 7) increased the mRNA expression levels of the marker genes identified in clusters 3 and 5 (Fig. 3). The mRNA expression levels were measured

using qRT-PCR and normalized to GAPDH. **e** Let-7a-5p inhibition induced a greater secretion of IL-8 from imMKCLs at the proliferation stage (DOX-ON). After transfection of the let-7a-5p inhibitor or negative control, the imMKCLs were incubated for 24 h in the absence or presence of LPS (50 ng/mL) in the proliferation phase. Inflammatory molecules were measured by a cytometric bead array in the supernatant. Data are expressed as the mean $\pm$ SEM from three independent experiments. Unpaired two-tailed student's *t*-tests were used to assess statistical significance. Source data are provided as a Source Data file.

interferon responsive gene sets were significantly enriched in intermediate-to-low (low-intermediate) quality MKCLs (Fig. 6b, Supplementary Fig. 5c). We also found that clone 7 and clone 7-3, despite being derived from the same iPSC clone with the same genetic

background^{5,6}, display different characteristics with regards to proliferation and iPSC-PLT production in both static and turbulent flow conditions (Fig. 6c–e) and let-7a-5p activity patterns (Supplementary Fig. 6a). Clone 7-3 showed enriched TNF signaling and interferon

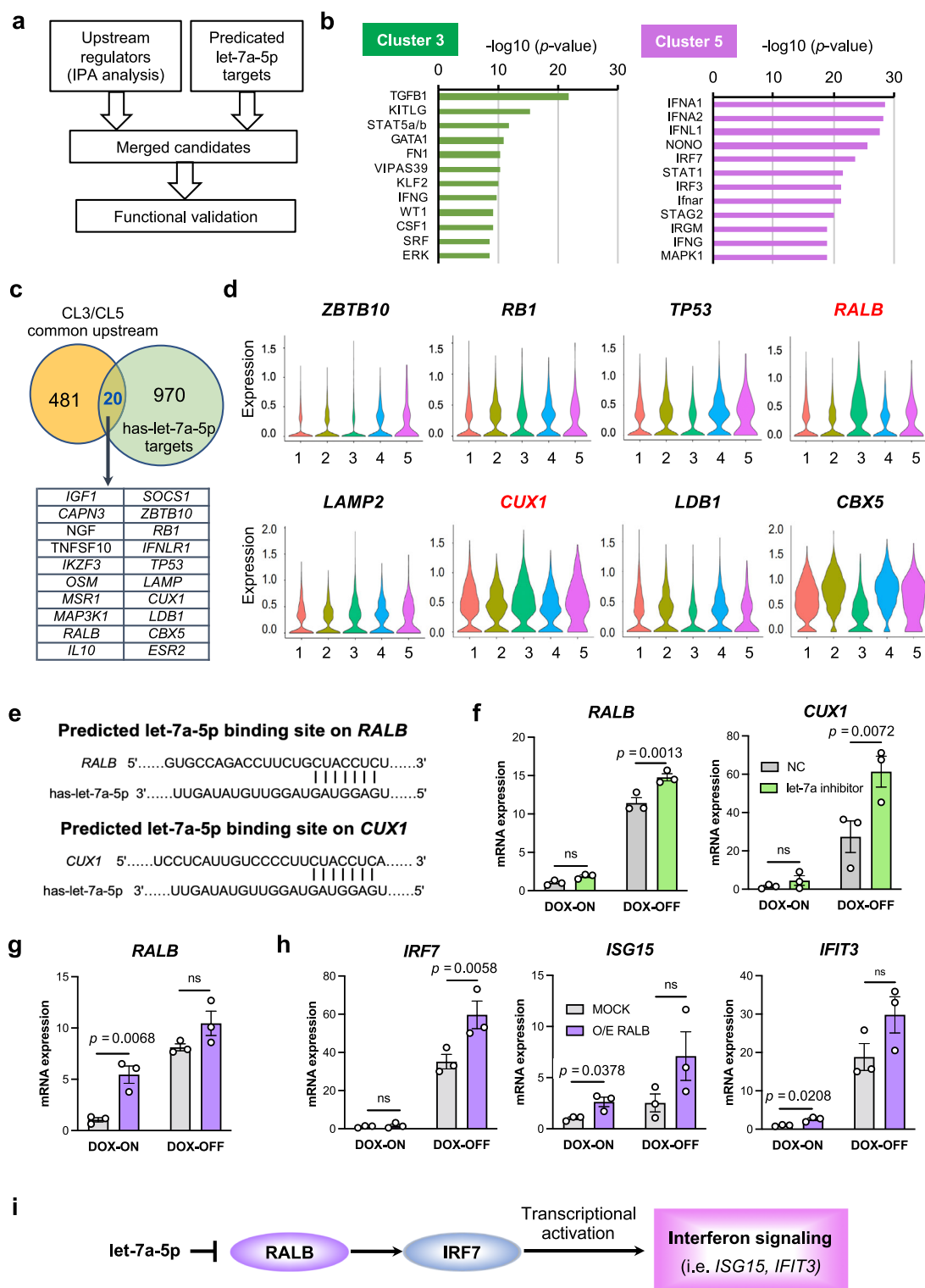


Fig. 5 | RALB is a target of let-7a-5p in the development of immune-skewed imMKCLs. a A flow chart of the upstream regulator analysis. **b** Bar plots showing the top 10 upstream regulators for cluster 3 and cluster 5 that were identified by the IPA based on scRNA-seq datasets. **c** The identification of eight potential upstream regulators by comparing the common upstream regulators of clusters 3 and 5 and predicted has-let-7a-5p targets. **d** Violin plots showing the expression levels of identified upstream regulators in each cluster. The regulators CUX1 and RALB showed increased expression levels in let-7a-5p low cells (clusters 3 and 5) and are colored red. The overlap p -values are calculated using Fisher's Exact Test, and significance is generally attributed to p -values < 0.01 . **e** The let-7a-5p binding site on

CUX1 and RALB predicted by TargetScan. **f** Let-7a-5p inhibition induced the mRNA expression of CUX1 and RALB in imMKCLs. **g** The lentiviral-mediated overexpression of RALB elevated the mRNA expression of RALB at both the proliferation (DOX-ON) and maturation stages (DOX-OFF). **h** The overexpression of RALB induced the expression of interferon signaling genes. **i** A schematic illustration showing how the let-7 miRNA-RALB axis modulates interferon signaling in imMKCLs. Data are expressed as the mean $\pm$ SEM from three independent experiments. Unpaired two-tailed student's t -tests were used to assess statistical significance. Source data are provided as a Source Data file.

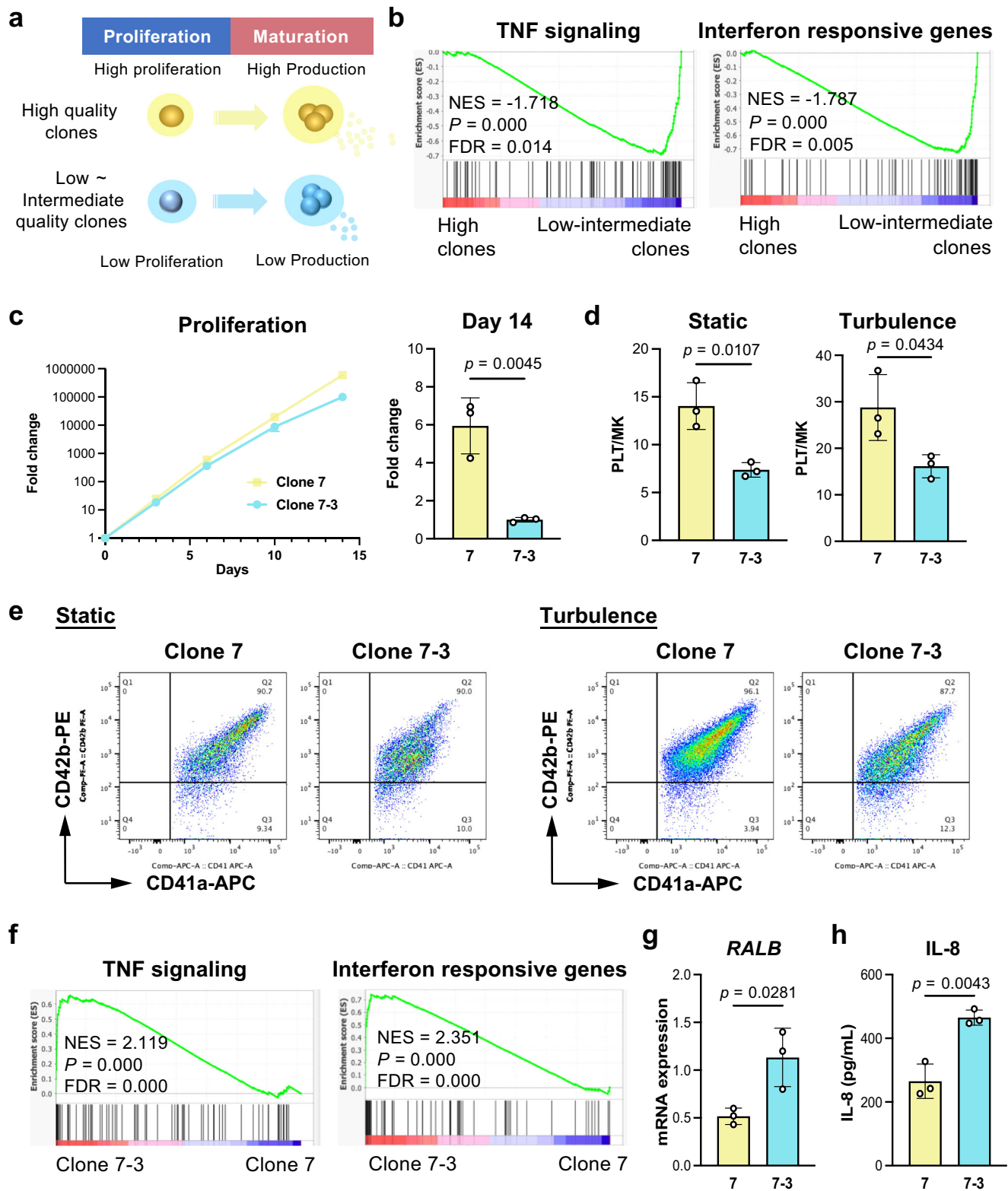


Fig. 6 | Dysregulated immune properties are associated with arrested proliferation and deficient iPSC-PLT generation of imMKCL clones. **a** A schematic illustration showing the correlation of proliferation and iPSC-PLT production by a previous study⁴². **b** GSEA were performed to compare high quality clones ($n = 5$) and Low -intermediate clones ($n = 7$). GSEA plots showing enriched TNF signaling and interferon responsive gene sets in low-intermediate quality clones compared with high quality clones. **c** The proliferation and fold change of the cell count at day 14 of clone 7 and of clone 7-3. **d** iPSC-PLT production of clone 7 and of clone 7-3 in static or turbulent flow conditions. **e** Representative flow cytometry plots of iPSC-PLTs

generated from clone 7 and clone 7-3 in static or turbulent flow conditions. **f** GSEA plots showing enriched TNF signaling and interferon responsive gene sets in clone 7-3. Representative enrichment plots from each group are displayed with the NES, non-adjusted p -value, and FDR derived from GSEA software. **g** Clone 7-3 shows elevated RALB mRNA expression compared with clone 7. **h** The secretion of IL-8 from clone 7 and clone 7-3 at the proliferation stage. Data are expressed as the mean $\pm$ SEM from three independent experiments. Unpaired two wo-tailed student's t -tests were used to assess statistical significance. Source data are provided as a Source Data file.

responsive signaling compared to clone 7 (Fig. 6f, Supplementary Fig. 6b). These results suggest that the induced immune-related pathways are associated with low-quality imMKCLs. The GSEA further demonstrated that downregulated senescent tumor protein 53 (TP53) targets are enriched in clone 7 (Supplementary Fig. 6c), indicating the cellular senescence of clone 7–3. The mRNA expression of *RALB* and IL-8 secretion of clone 7–3 was higher compared with clone 7 (Fig. 6g, h). It is known that inflammatory cytokines are secreted by senescent cells with persistent DNA damage⁴⁹. In addition to IL-8 secretion (Fig. 6h), genes encoding inflammatory cytokines and chemokines (*IFNB1*, *CXCL8*, *CXCL10*, *CXCL11*) also showed higher expression levels in clone 7–3 (aged clone) compared with clone 7 (younger clone) (Supplementary Fig. 6d). Notably, the addition of recombinant IL-8 into imMKCL culture resulted in diminished iPSC-PLT production (Supplementary Fig. 7a–c). Blocking IL-8 signaling with Reparixin⁵⁰, a specific CXCR1/2 inhibitor, improved iPSC-PLT production (Supplementary Fig. 7d–g) without affecting proliferation. Furthermore, the administration of recombinant interferon- α 2a led to lower proliferation rates (Supplementary Fig. 8a) as well as impaired iPSC-PLT production in a dose-dependent manner (Supplementary Fig. 8b, d). Interferon treatment elevated mRNA expression of the senescence marker *CDKN2A* as well as the interferon-responsive genes *ISG15* and *IFIT3* in imMKCLs (Supplementary Fig. 8c). Taken together, these findings suggest that the dysregulation of immune properties/subpopulation within imMKCLs, along with the secretion of inflammatory cytokines, leads to arrested proliferation and deficient platelet generation of the whole imMKCL population, potentially due to the upregulation of *RALB* levels. Moreover, these results emphasize the effect of immune cytokines on ex vivo iPSC-PLT manufacturing, thus bearing implications for clinical applications.

Intriguingly, our investigation of *RALB* revealed that the upregulation of *RALB* levels induced arrested proliferation, deficient iPSC-PLT production, and increased IL-8 secretion (Fig. 7a–d) as well as being accompanied by a cellular senescent/aging transcriptional signature (Supplementary Fig. 9a). We assumed that this mechanism could recapitulate the characteristics of low-quality imMKCLs. A bulk RNA-seq analysis revealed enriched TNF signaling and interferon response in *RALB*-overexpressing imMKCLs (Fig. 7e and Supplementary Fig. 9b), providing further support for our assumption. Additionally, *RALB* expression levels correlated with the expression levels of several interferon responsive genes in multiple imMKCL clones (Fig. 7f).

We employed two additional approaches to validate the effects of *RALB*. The small molecule inhibitor RBC8 (Supplementary Fig. 10a), which is described as a selective inhibitor against the GTPase Ral⁵¹, at 0.1 μ M resulted in accelerated proliferation (Supplementary Fig. 10b) and ameliorated iPSC-PLT production (Supplementary Fig. 10c–e). siRNA-mediated *RALB* knockdown also enhanced iPSC-PLT production (Supplementary Fig. 10f, g). Although no significant difference was observed in IL-8 secretion (Supplementary Fig. 10h), *RALB* knockdown induced a decline in the mRNA expression levels of *IRF7*, *ISG15*, *IFIT3* (Supplementary Fig. 10i). Finally, the iPSC-PLTs generated from low quality clone (clone 7–3) showed decreased PAC-1 binding and P-selectin expression (Supplementary Fig. 11). These results collectively suggest that dysregulated immune properties/subsets within imMKCLs are associated with diminished imMKCL proliferation and iPSC-PLT generation, a phenotype potentially attributed to the upregulation of *RALB* expression.

Lastly, we verified our findings in an in vitro differentiated MK model derived from cord blood CD34⁺ cells (Supplementary Fig. 12a). The inhibition of let-7 had no significant effect on interferon signaling or platelet production (Supplementary Fig. 12b–d). However, the overexpression of *RALB* resulted in the upregulation of interferon-responsive genes and a reduction in the production of platelet-like particles (Supplementary Fig. 12e–g). While our results suggest imMKCL and primary MKs respond differently to let-7 inhibition,

they indicate a common effect of *RALB*, highlighting the pivotal role of *RALB* in determining immune properties/subsets in MK development.

Discussion

In this study, we applied miRNA switch technology to explore the heterogeneity of imMKCLs, a promising ex vivo source of iPSC-PLTs for transfusion therapy, at the proliferating stage. By screening a library containing 269 miRNA switches, we identified heterogeneous let-7 miRNA activity among imMKCLs. By conducting bulk and scRNA-seq, we revealed that let-7 miRNA switches effectively enrich immune-skewed subpopulations. Our results demonstrate that the inability of let-7a-5p to suppress the expression of *RALB*, which lead to the activation of interferon-dependent signaling, is crucial for the lineage determination of immune-biased imMKCLs. While a senescence/aging signature is dominant in clone 7–3, we found the dysregulation of immune properties/subpopulations contributes to the secretion of inflammatory cytokines, resulting in a declined quality of the whole imMKCL population based on arrested proliferation and deficient iPSC-PLT generation. This senescence/aging related phenotype is potentially modulated by the let-7 microRNA/*RALB*/interferon signaling axis, as evidenced by *RALB* overexpression and knockdown. Consistently, we found the overexpression of *RALB* induced immune-skewed transcriptional phenotypes in a cord blood-derived MK model.

Non-coding RNAs, particularly miRNAs, play a crucial role in the post-transcriptional regulation of megakaryopoiesis⁵². For instance, miR-150⁵³, miR-155⁵⁴, and miR-146a⁵⁵ have been proposed to act in MK lineage specification, while miR-125a-5p positively promotes proplatelet formation and platelet generation by targeting the expression of actin-bundling protein L-plastin⁵⁶. Although the let-7 family is the most abundant miRNAs in megakaryocytic-erythroid progenitors⁵⁷, its role in MK development has not been elucidated. Here we employed miRNA switch technology and observed the heterogeneous activities of let-7a-5p and let-7g-5p among imMKCLs. We discovered that immune-skewed phenotypes were preferentially enriched in imMKCLs with lower let-7 activity and characterized by the enhanced gene expression of immune-related molecules (i.e., *ISG15*, *IFIT3*; Fig. 3). The enriched gene sets in let-7 low imMKCLs (Fig. 2, Supplementary Table 2) partially overlapped with the enriched gene sets found in naïve lung MKs^{22,58,59}. Interestingly, previous studies have suggested that the let-7 family is involved in the post-transcriptional control of innate immune responses to pathogenic agents⁶⁰ and cytokine production by T lymphocytes in adaptive immunity⁶¹. In the present study, we observed that internal let-7 activity did not affect iPSC-PLT production (Supplementary Fig. 1c) and that the modulation of let-7 by mimics and inhibitors showed no discernible effect on iPSC-PLT production. These observations could be attributed to the intricate interplay of let-7 in regulating both the immune subset (cluster 5) and the thrombopoietic subset (cluster 3). We speculate that the inhibition of let-7 may compensate for any negative impact on iPSC-PLT production induced from the immune subset by influencing the thrombopoietic subset.

The classical view of megakaryopoiesis and thrombopoiesis has not provided insights about immune MKs in development. However, emerging evidence shows that lung-resident MKs in mice, particularly those in the non-vasculature area of the lungs, exhibit an immune-skewed transcriptional signature compared with BM-resident MKs^{22,58}. Human embryonic MKs with immune characteristics were suggested to be generated along a distinct programmed trajectory¹⁰. Interestingly, our findings here show that heterogeneity is evident even within the immune-skewed MK population. We found imMKCLs contain ‘immune’ MKs in cluster 5, which displayed several immune programs (Fig. 3). The enriched gene sets in cluster 5 partially overlapped with those identified in human BM³¹. Cluster 3, on the other hand, exhibited

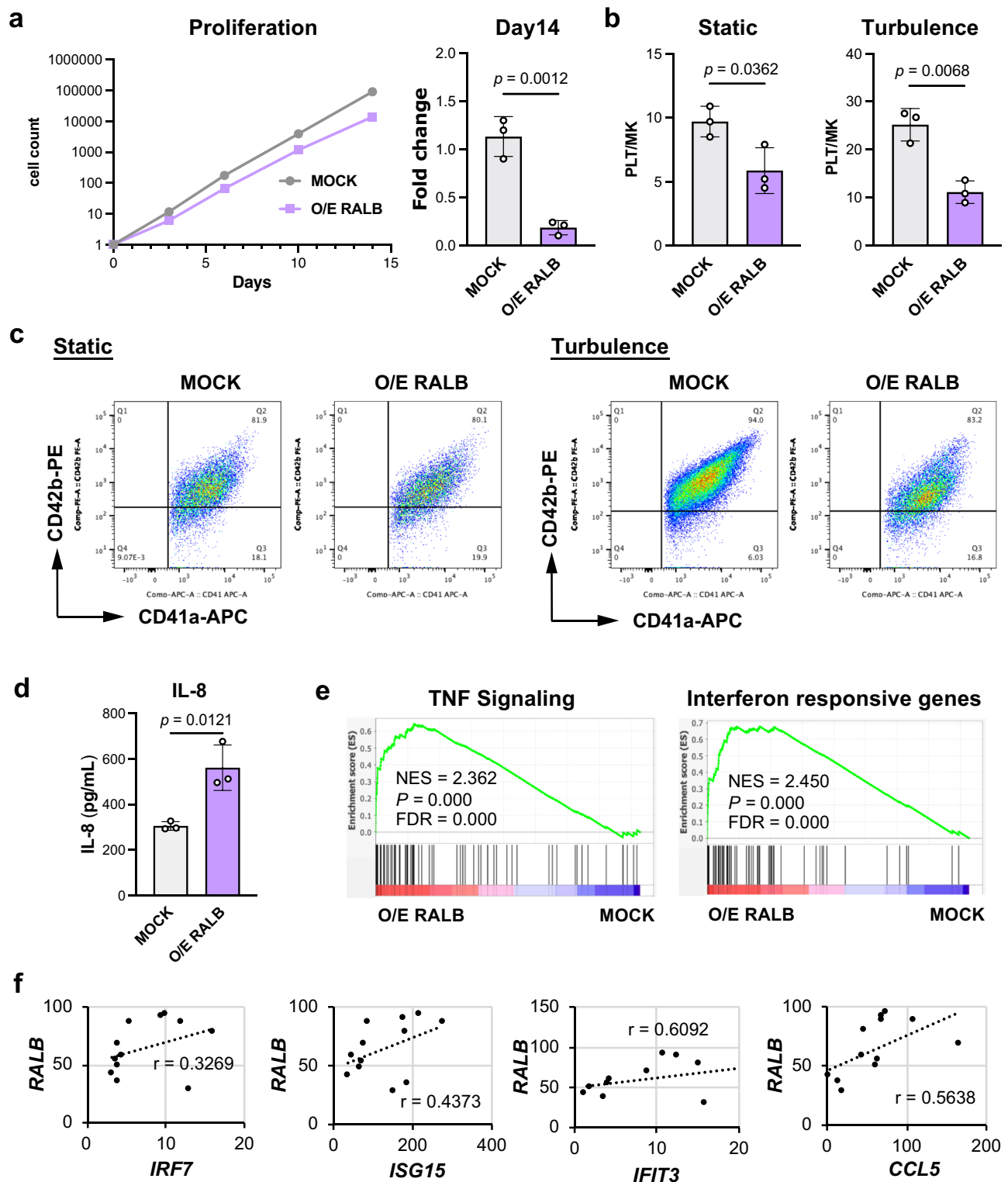


Fig. 7 | The overexpression of RALB diminishes the proliferation and iPSC-PLT generation capacity of imMKCLs, potentially by inducing the dysregulation of their immune properties. The lentiviral-mediated overexpression (O/E) of RALB leads to arrested proliferation (**a**) and deficient iPSC-PLT generation (**b**) in imMKCLs (Clone 7). **c** Representative flow cytometry plots of iPSC-PLTs generated from MOCK and O/E RALB in static or turbulent flow conditions. **d** IL-8 is secreted more by O/E RALB than by MOCK imMKCLs. **e** GSEA were performed to compare mock ($n = 3$) and RALB overexpressing cells ($n = 3$). GSEA plots showing enriched

TNF signaling and interferon responsive gene sets in O/E RALB imMKCLs at the maturation stage. Representative enrichment plots from each group are displayed with the NES, non-adjusted p -value, and FDR derived from GSEA software. **f** A gene expression correlation analysis of RALB and immune-related genes. Data are expressed as the mean $\pm$ SEM from three independent experiments. Unpaired two-tailed student's t -tests were used to assess statistical significance. Source data are provided as a Source Data file.

functional duality in both thrombopoiesis and immunity. We suspect that cluster 3 contains two kinds of iPSC-PLT-generating MKs: one generating iPSC-PLTs for hemostasis specifically, and the other generating iPSC-PLTs with preferential immune functions. Cluster 3 may help explain why platelets have immune functions, while previously identified naïve immune MKs are mostly diploid and seemingly unable to produce platelets^{11,22}.

Previous studies utilizing scRNA-seq have identified functionally distinct subsets of adult BM MKs^{11,31}, including thrombopoiesis-biased MKs, immune MKs, and HSC niche-supporting MKs. However, the key molecular event(s) governing the development of the heterogeneous ontology is poorly understood. The current study supports the functional diversity of imMKCLs, where lower *let-7* activity enriches immune-skewed subpopulations. This heterogeneity may arise at or before the HPC stage (Supplementary Fig. 2). Furthermore, our findings reveal *RALB* as a functional target of *let-7a-5p* in modulating the lineage determination of ‘immune’ MKs within imMKCLs (Figs. 5, 7). *RALB* also drives the immune-skewed transcriptional phenotypes of cord blood-derived MKs (Supplementary Fig. 12), shedding light on the regulation of human immune MKs. RAL GTPases, encoded by *RALA* and *RALB*, are known for their roles in cell growth, granule secretion, and cancer metastasis⁶². Within the context of platelet biology, the activation of the RAL family in platelets occurs through α -thrombin-induced Ca^{2+} /calmodulin binding to either RAL⁶³. Additionally, the RAL family is recognized as critical regulators of *P*-selectin expression and platelet-leukocyte interactions in mice⁶⁴. In cancer biology, evidence has highlighted the importance of the RALB/TBK1 pathway in cell autonomous survival by establishing a link between tumor formation and the innate immune response⁶⁵. Considering the protective role of platelets in shielding cancer cells from immune surveillance by cytotoxic lymphocytes⁶⁶, it is reasonable to suspect that the RAL family serves as the molecular conduit connecting MKs/platelets and cancer. While another identified upstream regulator candidate, *CUX1* (Fig. 5), is essential for modulating endothelial senescence⁶⁷, our study highlighted the involvement of *RALB* in the senescence process during hematopoiesis. For ex vivo iPSC-PLT manufacturing, our findings indicate *RALB* may serve as a hallmark/predictor of imMKCL quality. Indeed, some low-quality imMKCL clones exhibited elevated *RALB* expression levels (Fig. 6g, Supplementary Fig. 5d). In addition, a decline in imMKCL quality, along with senescent phenotypes, was observed following the overexpression of *RALB* (Fig. 7).

The success of our ex vivo manufacturing strategy for iPSC-PLTs predominantly relies on maintaining high-quality imMKCLs preserved in a master cell bank (MCB)^{8,9}. To meet the demand for platelet transfusion therapy, which typically requires $2\text{--}3 \times 10^{11}$ platelets per standard dose⁶⁸, it is mandatory to ensure a substantial quantity of imMKCLs. However, the heterogeneity of imMKCLs poses a considerable challenge to achieving efficient and standardized iPSC-PLT manufacturing and may result in the heterogeneous functionality of iPSC-PLTs⁶⁹. In the iPLAT1 study, which used patient-derived imMKCLs (originally an iPSC clone derived from T cells of the patient), a slightly elevated white blood cell count and D-dimer level, which were observed after the transfusion of 10^{11} iPSC-PLTs in cohort 3⁷, indicated the possibility of immuno-thrombosis and the existence of immune subpopulations within imMKCLs. In the present study, we discovered a significant correlation between the immune properties/subpopulations of imMKCLs and their capacity to generate iPSC-PLTs. Whilst the immune subpopulations of MKs have been indicated as a genetically programmed ontogeny¹⁰, our findings suggest that the dysregulation of immune subpopulations/properties can have a significant impact on the overall imMKCL population, resulting in a decline of imMKCL quality. This decline is potentially mediated through the *let-7* miRNA/*RALB*/interferon signaling axis, as evidenced by the immune-skewed transcriptional signatures and

deficient iPSC-PLT production upon *RALB* overexpression (Figs. 5–7). Furthermore, our study revealed that utilization of the small molecule inhibitors RBC8 (Supplementary Fig. 10) and Reparxin (Supplementary Fig. 7) against *RALB* and IL-8 signaling favors iPSC-PLT production. The consistent efficacy of these small molecules across various clones suggests their universal functionality, highlighting their potential applicability in future clinical settings. Taken together, this study highlights the significance of considering the immune/senescent attributes of donor cells and allogenic imMKCL MCB in future iPSC-PLT transfusion therapies, as they profoundly impact both the quantity and quality of the produced iPSC-PLTs. Thus, this study provides important insights into the standardization of iPSC-PLT generation towards industrial-scaled manufacturing.

Methods

Cells

The human iPSC lines TkDN-Sev2 and T-1 were established in house⁷⁰. imMKCLs (clone 7, clone 7–3, M35-1) were induced by DOX-inducible defined factors from human iPSCs and employed in previous studies^{5,6}. The human ESC line KhES-3 was obtained from the Institute for Frontier Medical Sciences, Kyoto University (Kyoto, Japan). The cord blood-derived CD34+ cells were provided by the Japanese Red Cross Society Kanto-Koshinetsu Blood Center. The use of all cells was approved by the ethics committees at Kyoto University and Chiba University.

Cell culture

The imMKCLs (clone 7, clone 7–3, M35-1) were cultured as described before⁶. Doxycycline was used to control the proliferation and differentiation stages.

MicroRNA switches

miRNA-responsive mRNAs (miRNA switches) were generated using a MegaScript T7 kit (Ambion) as described previously¹⁵. The miRNA switches were encoded on modified mRNA to post-transcriptionally regulate TagBFP, a blue fluorescent protein, in response to the activity of an arbitrary miRNA (miR-X) expressed in imMKCLs. TagBFP-coding mRNA includes the target miR-X sequence in 5' UTR such that the expression level of TagBFP is suppressed in response to increased miR-X activity. Azami Green 1 (hmAG1)-coding mRNA served as the transfection control (Fig. 1B).

miRNA switch-based screening

The miRNA switch-based screening was performed using an original constructed library (total of 269 miRNA switches). Two different mRNA coding for TagBFP and AG1, respectively, were co-transfected into the cells. The transfection of miRNA switches was performed as previously described¹⁵. Briefly, in the proliferation phase of imMKCLs, the reverse transfection of 150 ng mRNAs (75 ng each) was done with StemFect mRNA (Stemgent) in 50 μL of the cell suspension (1×10^6 cells/mL) for 30 min, followed by dilution to 200 μL using culture medium in 96-well plates. After 24 h, flow cytometry was performed using a LSRFortessa (BD Biosciences, San Jose, CA, USA). Candidate miRNA switches were selected based on the active responsive pattern of imMKCLs to the target miR-X (“active” in Fig. 1c). MiRNAs that did not show strong responsive activities in imMKCLs were defined as “inactive” (Fig. 1c). For *let-7a-5p* and *let-7g-5p*, we individually transfected the candidate switches with control hmAG1-coding mRNA into imMKCLs. Following a 24 h incubation, the cells were sorted using a BD FACSaria II and subjected to further investigation.

Cell sorting and flow cytometry

Cells were suspended in staining medium, incubated for 30 min with appropriate antibodies on ice in the dark, and sorted or analyzed using

a BD FACSAria II. Platelet count was determined as previously described⁶. The following antibodies were used for the flow cytometry: allophycocyanin (APC)-conjugated anti-CD41a (integrin α IIb β 3 complex: HIP8 clone) (Biolegend, San Diego, CA), phycoerythrin (PE)-conjugated anti-CD42b (GPIb α) (eBioscience, San Diego, CA), and PE-conjugated anti-CD41a (HIP8 clone) (Biolegend). The gating strategies are provided in the Supplementary Fig. 13.

Reverse transcription and real-time PCR

Total RNA was extracted using the microRNeasy Micro Kit or microRNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed using SuperScript VILO™ Master Mix (Thermo Fisher Scientific). qPCR was carried out with SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using a StepOnePlus system (Thermo Fisher Scientific). *GAPDH* was used as the internal control. The primer sets used are listed in Supplementary Table 3. Let-7a-5p and let-7g-5p miRNA reverse transcription was performed using a TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) and miRNA specific stem-loop RT primers according to the manufacturer's instructions. qPCR was carried out with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) using the StepOnePlus system. The let-7 expression levels were determined relative to RNU6B using specific TaqMan probes.

Analysis of cytokine secretion by imMKCLs

Cytokine secretion in culture supernatants was measured using the human inflammatory cytokine cytometric bead array kit (BD Biosciences) according to the manufacturer's guidelines. Briefly, bead populations with distinct fluorescence intensities coated with capture antibody proteins were mixed with PE-conjugated detection antibodies and recombinant standards or test samples, and then incubated to form sandwich complexes. After acquiring sample data by flow cytometry, the cytokine concentrations were calculated using FCAP Array™ software (BD Biosciences).

Transfection of let-7a-5p inhibitor

An mirVana let-7a-5p-specific inhibitor and negative control were purchased from Thermo Fisher Scientific and used according to the manufacturer's instructions. The transfection procedure was performed using a Stemfect RNA Transfection Kit (ReproCell, Yokohama, Japan).

Lentiviral production

The use of viral vectors was approved by committees at Kyoto University and Chiba University. The full-length coding sequences of human CUX1 and RALB were cloned into the lentiviral vector CS2-Ubiquitin-GFP or CS2-Ubiquitin-IB. Lentiviral production using the 293 T system was described previously⁴.

Bulk RNA-sequencing analysis

Total RNA was extracted using the microRNeasy Micro Kit. RNA-seq libraries were prepared according to the manufacturer's protocol. Briefly, ~10 ng of total RNA was used for the cDNA synthesis using a SMART-Seq v4 Ultra Low Input RNA Kit for sequencing (Takara Bio). cDNA was fragmented using an S220 Focused-ultrasonicator (Covaris, Woburn, MA, USA). The cDNA library was then generated using a NEBNext® Ultra™ DNA Library Prep Kit for Illumina (New England BioLabs, Beverly, MA, USA). Finally, the NEBNext library size was estimated using a bioanalyzer with an Agilent High Sensitivity DNA Kit. Sequencing was performed using a HiSeq2500 (Illumina) or a NextSeq 500 (Illumina) platform with a single-read sequencing length of 60 bp. TopHat (version 2.1.1) was used to map to the reference genome (UCSC/hg19) with annotation data from iGenomes (Illumina). Gene expression levels were quantified using Cuffdiff

(Cufflinks version 2.2.1) and expressed as fragments per kilobase of exon per million mapped sequence reads (FPKM).

Gene sets enrichment analysis (GSEA)

A GSEA was applied to screen pathways enriched in (1) let-7 (let-7a-5p and let-7g-5p) low or high imMKCLs (M35-1, clone 7, clone 7-3), (2) let-7 low imMKCLs of clone 7-3 or of clone 7, and (3) MOCK or RALB-overexpressing imMKCLs (clone 7) based on bulk RNA-seq datasets. The plots were generated using GSEA software (version 4.2.3) from the Broad Institute. We also re-analyzed bulk RNA-seq datasets from high-, intermediate- and low-quality MKCL clones¹². The normalized gene expression matrix was input for the GSEA, and FDR and *P*-values < 0.05 were considered statistically significant.

Chromium 10x single-cell RNA-seq library construction

imMKCLs (clone 7) with distinct let-7a-5p activity were sorted separately and resuspended at 1000 cells/μL in PBS with 0.4% BSA (Fig. 3). The cells were loaded onto a Chromium Next Gel Beads-in-Emulsion (GEMs) Chip G Single Cell Kit (10 × Genomics, USA). GEM generation and barcoding, reverse transcription, and cDNA generation and library construction were performed following the manufacturer's protocol (Chromium Next GEM Single Cell 3' Reagent Kits v3.1 Dual Index, 10 × Genomics). Dual-indexed, single-cell libraries were pooled and sequenced in paired-end reads on a Novaseq6000 (Illumina).

Bioinformatics analysis

10 × Genomics-derived datasets were collected, and quality control was performed to filter out low-quality and contaminated cells. Generally, reads were pre-processed with the Cell Ranger pipeline v3.0.2 (10 × Genomics). Downstream analysis and visualization were done using Seurat (version 4.0.5) implemented in R (version 4.1.1). After inspection of the quality control metrics, cells with > 15% of mitochondrial content or <2500 detected genes were excluded from the downstream analysis. We normalized and scaled the unique molecular identifier (UMI) counts using regularized negative binomial regression. Afterward, we performed linear dimensionality reduction (principal component analysis) and used the top 20 principal components to perform the unsupervised Uniform Manifold Approximation and Projection (UMAP) and clustering, which were computed at a range of resolutions from 1.2 to 0.05. The let-7a-5p high and low imMKCL populations were combined for all subsequent analyses. Cell clusters were identified using the FindClusters function from Seurat. Five clusters for DOX-ON samples were identified (resolution 0.2). The FindAllMarkers function implemented in Seurat was used to identify DEGs between different clusters. The Wilcoxon test was performed on each gene, and the *P*-values and adjusted *P*-values for statistical significance were computed. Genes with adjusted *P*-values < 0.01 were considered significant. The g:Profiler was used for the identification of enriched functional terms from the GO.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA). Data are expressed as the mean standard error of the mean (SEM). Values of *P* < 0.05 were considered significant. Details of the sample size used, statistics, and statistical significance are indicated in each figure legend.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The raw sequence data have been deposited in the DNA Data Bank of Japan (DDBJ) database under the association number [PRJDB15883](https://www.ncbi.nlm.nih.gov/PRJDB15883),

composing of the following subseries: DRA016355, DRA016356, DRA016432. The project is also available at the NCBI BioProject [<http://www.ncbi.nlm.nih.gov/bioproject/>] under the same association number. The processed data sets were deposited with the association codes [E-GEAD-615](#), [E-GEAD-616](#), [E-GEAD-617](#). Source data are provided with this paper. The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information file. Source data are provided with this paper.

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Author contributions

S.J.C. designed and performed the majority of the experiments, analyzed the data, interpreted the results, prepared figures, and wrote the manuscript. K. Hashimoto and K.F. designed and performed experiments regarding miRNA switch screening. K. Hayashi prepared the miRNA switches. S.K.P., A.Y., W.-Y.Q. E.N., and M.A.K. assisted with the experiments. A.K. sequenced the bulk RNA-seq libraries and provided intellectual contributions. T.Y. and M.K. contributed to the bioinformatics analysis of the scRNA-seq. S.N. provided intellectual contributions. N.S. provided intellectual contributions and edited the manuscript. H.S. provided guidance on miRNA switch-related experiments and edited the manuscript. N.T. provided guidance on the experimental design, contributed to the bioinformatics analysis, and edited the manuscript. K.E. managed the overall project, proposed ideas, contributed to the experimental design and data interpretation, and wrote the manuscript.

Competing interests

S.J.C. and K.E. have applied a patent related to this study. K.E. was a founder of Megakaryon Co. Ltd. but with no stock currently. H.S. is a founder and an outside director of aceRNA Technologies Ltd. The

interests of K.E. and H.S. were reviewed and are managed by Kyoto University in accordance with its competing interest policies. The other authors declare no competing interests.

Additional information

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Supplementary Information

A let-7 microRNA-RALB axis links the immune properties of iPSC-derived megakaryocytes with platelet producibility

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Supplementary Methods

Differentiation of CD34⁺ cells from human ESCs

The human ESC line KhES-3 was obtained from the Institute for Frontier Medical Sciences, Kyoto University (Kyoto, Japan), and maintained in AK02N medium (Ajinomoto, Tokyo, Japan) on iMatrix-511 silk (Takara Bio, Shiga, Japan)-precoated 6-well plates. CD34⁺ HPC differentiation was performed using our previously established Sac method^{1, 2}. Briefly, small clumps of equivalent cell numbers (1.5 to 1.6×10^5 cells) were transferred onto a 10-cm dish coated with mitomycin C-treated C3H10T1/2 mouse feeder cells (Riken Bio-Resource Center, Tsukuba, Ibaraki, Japan) and cultured in basal medium (IMDM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA), insulin-transferrin-selenium (Thermo Fisher Scientific), 50 $\mu\text{g/mL}$ ascorbic acid (Sigma-Aldrich), 450 μM α -monothioglycerol (Sigma-Aldrich), and 20 ng/mL recombinant human VEGF (Wako, Osaka, Japan). Then, 50 ng/mL basic FGF (Wako) and 10 μM SB431542 (Wako) were added on days 4 to 7, and 10 U/mL Heparin (AY pharmaceuticals, Tokyo, Japan) on days 4 to 10. Medium changes were performed on days 4, 7, and 11 (Supplementary Fig. 2a). On day 14, the cells were collected with a cell scraper and filtered with a cell strainer for further investigation.

Purification of human cord blood-derived CD34⁺ cells

Fresh umbilical cord blood (UCB) samples were obtained from healthy donors sourced from the Japanese Red Cross Society Kanto-Koshinetsu Blood Center. Mononuclear cells (MNC) were isolated through density centrifugation using lymphocyte separation medium (Ficoll). The cells were subsequently incubated with the immunomagnetic beads (CD34) (Miltenyi Biotech) and selected using a QuadroMACS® separator according to manufacturer's instructions. The efficiency for the purification was verified by flow cytometry. The resulting CD34⁺ fraction was aliquoted and stored in liquid nitrogen until use.

Differentiation of megakaryocytes from cord blood-derived CD34⁺ cells

The *in vitro* differentiation of cord blood-derived CD34⁺ cells was performed in accordance with a previously published protocol with minor modifications^{3, 4}. Briefly, CD34⁺ cells were expanded in X-VIVO 10 media (Lonza), supplemented with 1% bovine serum albumin (BSA, StemCell Technologies), 100 ng/mL human SCF (R&D Systems), 100 ng/mL human Flt-3 ligand (PeproTech EC Ltd.), 50 ng/mL human thrombopoietin (TPO, R&D Systems), 10 ng/mL human IL-6 (PeproTech EC Ltd.), and 10 ng/mL human IL-3 (PeproTech EC Ltd.). Following a 9-day culture period, the cells were harvested and counted. 4×10^5 cells were seeded and cultured for an additional 11 days in a total volume of 1 mL X-VIVO 10 media supplemented with 1% BSA, SCF, TPO, and IL-6. RNA isolation was isolated on day 16, and flow cytometry analysis was

conducted on day 20 to evaluate CD41a⁺CD42b⁺ platelet-like particles. The media was changed every two days across the differentiation period (Supplementary Fig. 11a).

siRNA-mediated gene knockdown

The Dharmacon™ siGENOME siRNA SMARTpool designed against human RALB (siRALB), which is comprised of four siRNAs targeting different sites, as well as a non-targeting control siRNA (siNT) were purchased from Horizon Discovery Ltd. The gene silencing procedure was performed based on the Dharmacon reverse transfection protocol with minor modification. Briefly, siRNA at 10 nM final concentration was diluted in transfection buffer from the Stemfect RNA Transfection Kit (ReproCELL), and transfection reagent was diluted in an equal volume of buffer. The diluted siRNA and transfection reagent were combined and incubated at room temperature for 15 min. Subsequently, 12.5 µL/well of transfection mixture was added to the wells of a 96-well plate, followed by the addition of 2×10⁵ cells in 100 µL medium to each well. After a 4-hour incubation period, the cells were washed with PBS and subjected to either proliferation or differentiation culture conditions. To validate the gene knockdown efficiency, total RNA was isolated and analyzed 48 hours after the transfection.

Addition of recombinant human interferon-α2a or IL-8 in imMKCL culture

To examine the potential involvement of interferons or IL-8 in the proliferation and iPSC-PLT production of imMKCLs, cells were cultured under either proliferation or differentiation culture conditions with the addition of recombinant human interferon-α2a (Miltenyi Biotec) or IL-8 (R&D systems). Concentrations ranging from 0 to 100 ng/mL were utilized as working concentrations. The proliferation and iPSC-PLT production capacity of imMKCLs were analyzed accordingly.

Addition of small molecules in imMKCL culture

The impact of two small molecules on the proliferation and iPSC-PLT production of imMKCLs was investigated: (1) Reparixin (Selleckchem), a specific inhibitor of CXCL8 receptor CXCR1/2 that is commonly used to inhibit the IL-8 signaling pathway; and (2) RBC8 (Selleckchem), a selective inhibitor of the GTPases RALA and RALB. imMKCLs were cultured under either proliferation or differentiation culture conditions in the absence or presence of Reparixin or RBC8 at working concentrations ranging from 0 to 10 µM. For these studies, dimethyl sulfoxide (DMSO) was added in control wells. Notably, the application of a high concentration (10 µM) of either molecule arrested the proliferation.

Intracellular flow cytometry

The intracellular flow cytometry assay was optimized for imMKCLs as follows. Cells were fixed in 4% paraformaldehyde (PFA) for 15 mins at room temperature, followed by methanol permeabilization (15 mins on ice). After two washes with PBS, the cells were incubated with anti-RalB antibody (Merck Millipore, 1:3000) for 1 hour on ice. Subsequently, the cells were washed and incubated with a goat anti-mouse secondary antibody, Alexa Fluor™ 647 (Invitrogen, 1:1000) on ice. The stained cells and non-staining controls were then analyzed by flow cytometry.

PAC-1 binding and *P*-selectin expression of iPSC-PLTs

The measurement of PAC-1 binding and *P*-selectin expression levels adhered to the protocol outlined in our previous study⁵. Briefly, the culture suspensions (iPSC-PLTs) were stimulated with or without phorbol-12-myristate-13-acetate (PMA, 0.2 μ M) or adenosine triphosphate plus thrombin receptor activator peptide 6 (ADP/TRAP6, 100 μ M and 40 μ M, respectively). Subsequently, the mixtures were incubated with BV421 mouse anti-human CD62P (BD Biosciences), APC-anti-human CD41a (Bio Legend), and FITC-PAC-1 (BD Biosciences) antibodies. Following a 30-min incubation at room temperature, the samples were diluted with Hepes-Tyrode buffer and analyzed by flow cytometry.

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Supplementary Table 1 The target miRNAs included in the miRNA switch screening library.

miR-1234-5p	miR-185-3p	miR-301a-5p	miR-425-3p	miR-497-5p	miR-10b-3p
miR-329	miR-144-3p	miR-211-3p	miR-485-5p	miR-450-5p	miR-379-5p
miR-629-5p	miR-362-3p	miR-589-5p	miR-671-5p	miR-506-3p	miR-125b-1-3p
miR-30b-3p	miR-296-3p	miR-506-5p	miR-361-3p	miR-582-3p	miR-422a
miR-98-3p	miR-499a-5p	miR-24-2-5p	miR-105-5p	miR-381-3p	miR-181b-3p
miR-122-5p	miR-27a-5p	miR-505-5p	miR-454-3p	miR-301a-3p	miR-510
miR-411-5p	miR-29c-5p	miR-148b-5p	miR-204-3p	miR-486-3p	miR-125b-2-3p
miR-212-5p	miR-582-5p	miR-197-5p	miR-222-5p	miR-93-3p	miR-483-5p
miR-576-5p	miR-146a-3p	miR-548-3p	miR-34a-3p	miR-190a	miR-383
miR-140-5p	miR-326	miR-576-3p	miR-219-5p	miR-342-5p	miR-590-3p
miR-133a	miR-17-3p	miR-17-5p	miR-1	miR-206	miR-21-5p
miR-367-3p	miR-373-5p	miR-92a-3p	miR-16-5p	miR-197-3p	miR-24-3p
miR-339-5p	miR-224-5p	miR-127-3p	miR-365a-3p	miR-183-5p	miR-331-3p
miR-203a	miR-214-3p	miR-137	miR-298	miR-325	miR-449a
miR-498	miR-661	miR-429	miR-1182	miR-208a-3p	miR-320b
miR-423-5p	miR-140-3p	miR-378c	miR-185-5p	miR-101-3p	miR-378b
miR-128	miR-509-5p	miR-340-5p	miR-25-5p	miR-92b-3p	miR-320c
miR-508-3p	miR-744-5p	miR-3180-3p	miR-30d-3p	miR-215	miR-4510
miR-1260a	miR-151a-5p	miR-584-5p	miR-503-5p	miR-34c-5p	miR-3180
miR-514a-3p	miR-374b-5p	miR-28-3p	miR-1307-5p	miR-21-3p	miR-193a-5p
miR-1269a	miR-365b-3p	miR-4286	miR-4454	miR-138-5p	miR-1307-3p
miR-652-3p	miR-502-3p	miR-92b-5p	miR-501-3p	miR-1285-3p	miR-126-3p

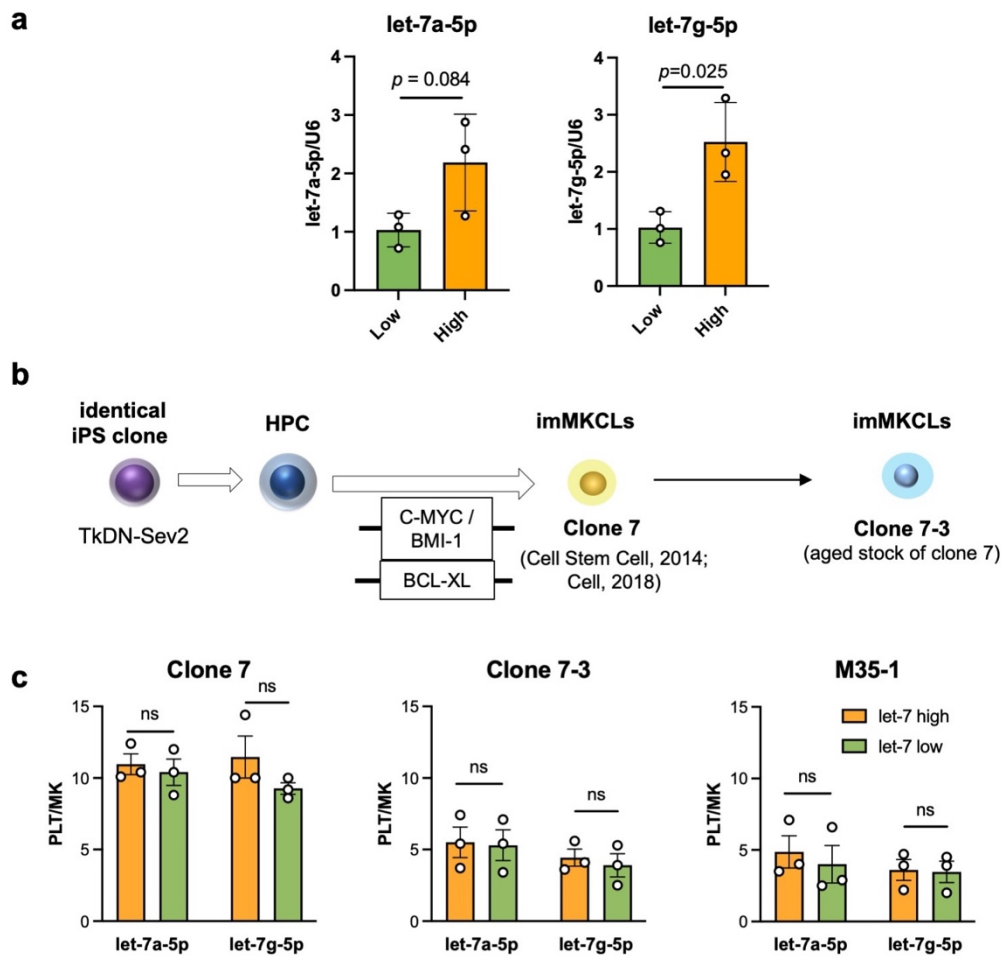
miR-4531	miR-193b-5p	miR-181a-2-3p	miR-1301	miR-877-5p	miR-4443
miR-425-5p	miR-320d	let-7a-3p	miR-92a-1-5p	miR-365b-5p	miR-142-3p
miR-320e	miR-106b-3p	miR-548	miR-1261	miR-9-3p	miR-129-1-3p
miR-424-3p	miR-760	miR-365a-5p	miR-374a-5p	miR-873-5p	miR-30c-2-3p
miR-99b-3p	miR-4532	20b-3p	miR-4448	miR-363-5p	miR-145-3p
miR-574-5p	miR-223-3p	miR-4521	miR-22-5p	miR-339-3p	miR-16-2-3p
miR-374a-3p	miR-542-3p	miR-452-5p	miR-513a-3p	miR-532-3p	miR-548a-3p
miR-27b-5p	miR-3180-5p	miR-4324	let-7d-3p	miR-184	miR-95
miR-664a-5p	let-7a-5p	let-7g-5p	let-7i-5p	miR-191-5p	miR-125b-5p
miR-100-5p	miR-342-3p	miR-210	miR-193b-3p	miR-99b-5p	miR-106b-5p
miR-27a-3p	miR-30a-3p	miR-423-3p	miR-409-3p	miR-186-5p	miR-324-3p
miR-22-3p	miR-378a-3p	miR-196b-5p	miR-296-5p	miR-99a-5p	miR-34a-5p
miR-501-5p	miR-500a-3p	miR-155-5p	miR-182-5p	miR-10a-5p	miR-345-5p
miR-335-5p	miR-362-5p	miR-193a-5p	miR-134	miR-195-5p	miR-26a-5p
miR-151a-3p	miR-200c-3p	miR-212-3p	miR-143-3p	miR-149-5p	miR-28-5p
miR-505-3p	miR-192-5p	miR-187-3p	miR-129-5p	miR-106a-5p	miR-299-3p
miR-483-3p	miR-18a-3p	miR-330-3p	miR-200b-3p	miR-154-5p	miR-150-5p
miR-145-5p	miR-199a-5p	miR-135a-5p	miR-200a-3p	miR-133b	miR-141-3p
miR-148a-3p	miR-9-5p	miR-520c-3p	miR-328	miR-361-5p	miR-375
miR-378a-5p	miR-382-5p	miR-484	miR-486-5p	miR-7-5p	miR-124-3p
miR-302a-5p	miR-370	miR-373-3p	miR-492	miR-509-3p	miR-512-5p
miR-516b-5p	miR-518b	miR-518c-3p	miR-518c-5p	miR-519d	miR-520f
miR-520g	miR-523-3p	miR-525-5p	miR-526a	miR-98-5p	

Supplementary Table 2 Significantly enriched immune-related gene sets in let-7 low imMKCLs at the proliferation or differentiation stages and in let-7 low ESC-derived CD34⁺ cells. The normalized enrichment score (NES), the determined nominal (non-adjusted) *p* value, and the false discovery rate (FDR) were derived from GSEA software.

GSEA Gene Sets	NES	<i>P</i> value	FDR
imMKCLs (Dox-ON)			
Immune response to enterotoxin	2.471	0.000	0.000
TNF signaling via NFKB	2.295	0.000	0.000
NFKB targets	2.172	0.000	0.000
IFNB1 targets	2.304	0.000	1.54E-04
Inflammatory response to LPS	2.279	0.000	2.42E-04
Interferon responsive genes	2.215	0.000	0.003
imMKCLs (Dox-OFF)			
Immune response to enterotoxin	2.665	0.000	3.41E-04
TNF signaling via NFKB	2.578	0.000	0.000
NFKB targets	2.214	0.000	0.008
IFNB1 targets	2.843	0.000	0.000
Inflammatory response to LPS	2.041	0.000	0.002
Interferon responsive genes	2.931	0.000	0.000
ESC-derived CD34⁺ HPCs			
Immune response to enterotoxin	1.926	0.000	0.005
TNF signaling via NFKB	1.870	0.000	0.009
NFKB targets	1.719	0.003	0.032
IFNB1 targets	2.317	0.000	0.000
Inflammatory response to LPS	1.665	0.000	0.045
Interferon responsive genes	2.408	0.000	0.000

Supplementary Table 3 Primer pairs used in RT-qPCR.

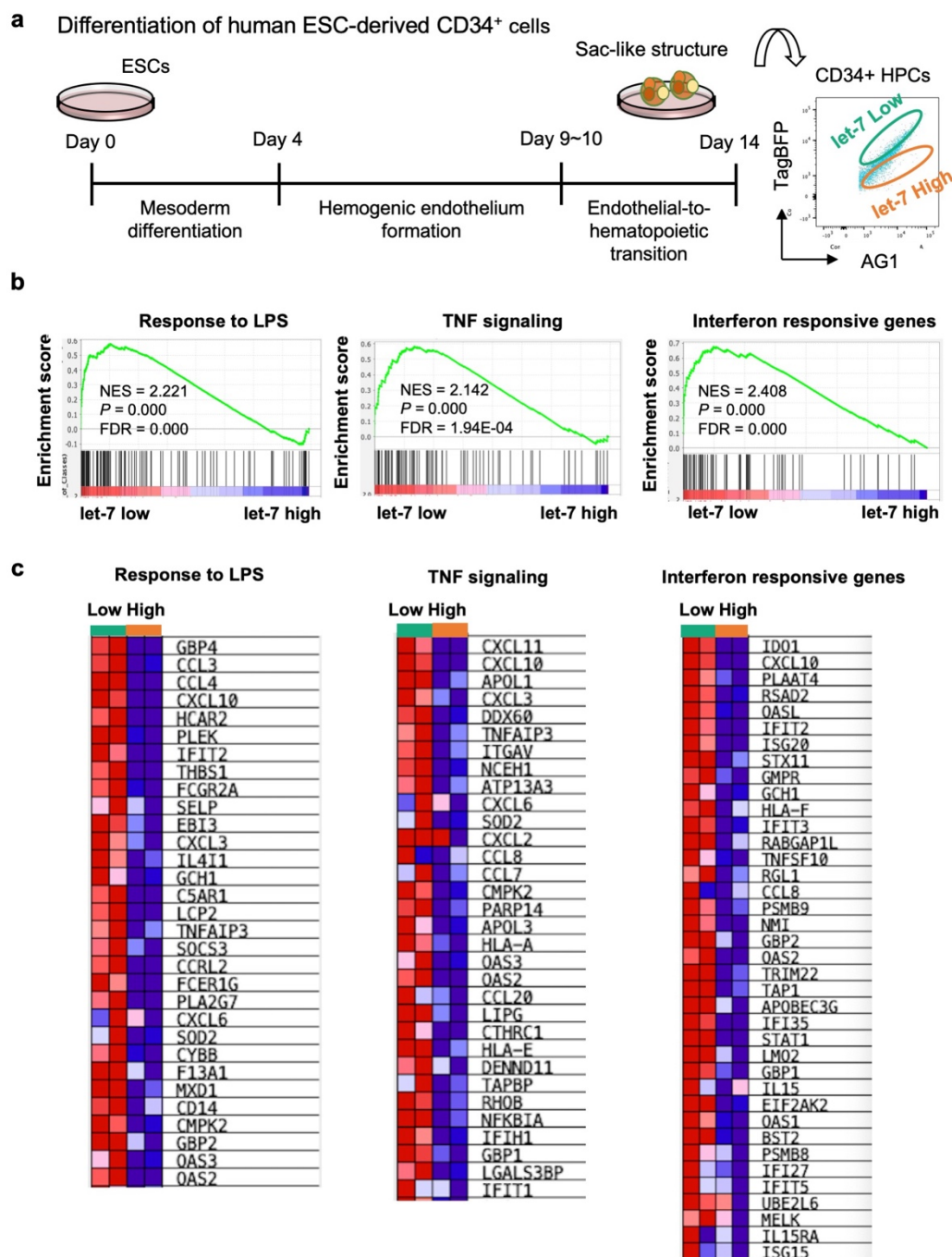
Gene symbol	Primer	Sequences (5'- 3')
ISG15	Forward	GAGAGGCAGCGAACTCATCT
	Reverse	CTTCAGCTCTGACACCGACA
CCL5	Forward	ATCCTCATTGCTACTGCCCTC
	Reverse	GCCACTGGTGTAGAAATACTCC
IFIT3	Forward	GAAGGAACTGGGCCGCCTGCTAAG
	Reverse	GCCCTGGCCCATTTCCTCACTACC
PF4	Forward	GTGTGAAGACCACCTCCCAG
	Reverse	GCTTGCAGGTCCAAGCAAAT
PPBP	Forward	TTGTAGGCAGCAACTCACCC
	Reverse	TGCAAGGCATGAAGTGGTCT
RALB	Forward	GACTACGCAGCCATTCGAGA
	Reverse	CCGCTCCTCTAGGTCAGACT
CUX1	Forward	ACAGACACTGAAGAACCAAGCCGA
	Reverse	TGTGAGGCCAGCTGGAGGGA
IRF7	Forward	CTTCCAAGAAGAGCTGGTGGAAT
	Reverse	TTCCAGCTTCACCAGGAC
CDKN2A	Forward	GCCCAACGCACCGAATAGTT
	Reverse	AATCGGGGATGTCTGAGGGA
GAPDH	Forward	GTTGCCATCAATGACCCCTTC
	Reverse	CATTGATGACAAGCTTCCCG



Supplementary Figure 1

Supplementary Fig. 1

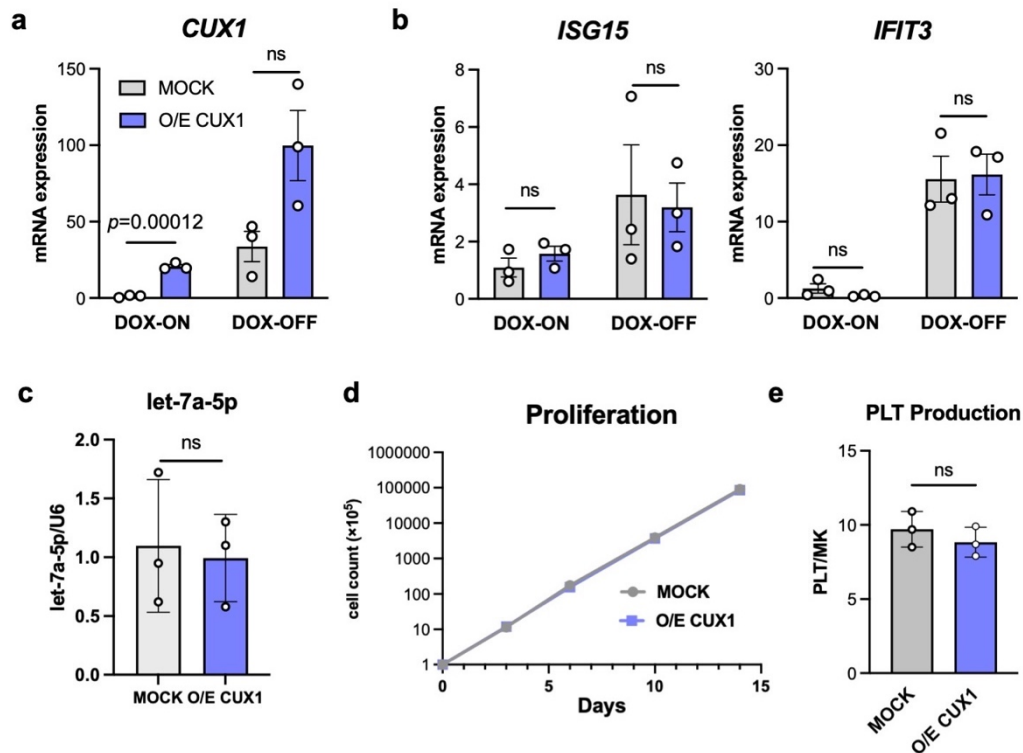
(a) let-7a-5p and let-7g-5p expression levels were determined in let-7 high/low imMKCLs (clone 7) by RT-qPCR. The expression levels were normalized to RNU6B (U6). Sampling was performed 48 hours after sorting. Data are expressed as the mean $\pm$ SEM from three independent experiments. (b) Clone 7-3 represents an aged stock of clone 7 that shares the same genetic background. (c) iPSC-PLT production from let-7 high/low imMKCLs in static conditions. The cells were proceeded to the DOX-OFF step directly after sorting. Three distinct imMKCL clones were employed. Data are expressed as the mean $\pm$ SEM from three independent experiments. Unpaired two-tailed student's *t*-tests were used to assess statistical significance. Source data are provided as a Source Data file.



Supplementary Figure 2

Supplementary Fig. 2

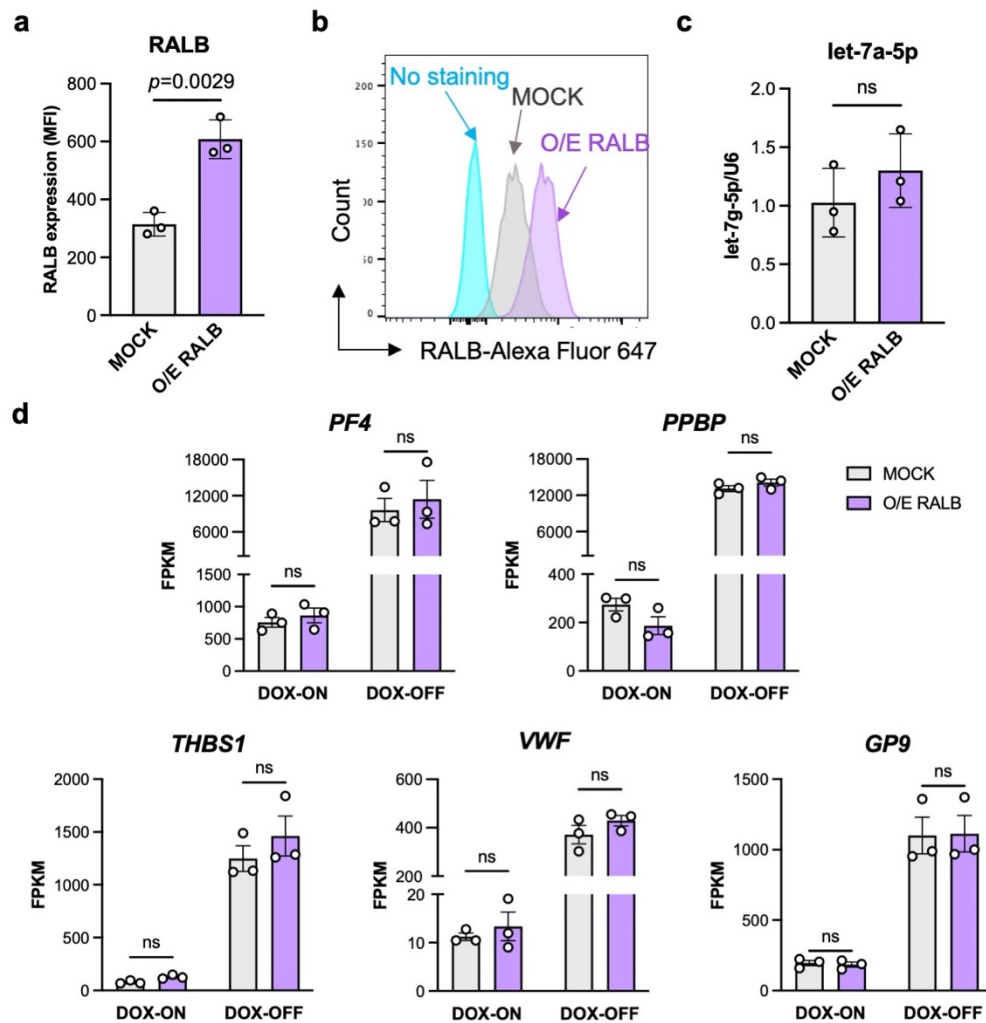
In vitro ESC-derived CD34⁺ HPCs exhibited immune-skewed transcriptional signatures in let-7 low subpopulations. **(a)** A schematic illustration of the ESC-derived HPC induction by the Sac method. **(b)** GSEA plots showing the significantly enriched immune-related gene sets in let-7 low ESC-derived HPCs. **(c)** Heatmaps of the gene sets enriched in the same cells.



Supplementary Figure 3

Supplementary Fig. 3

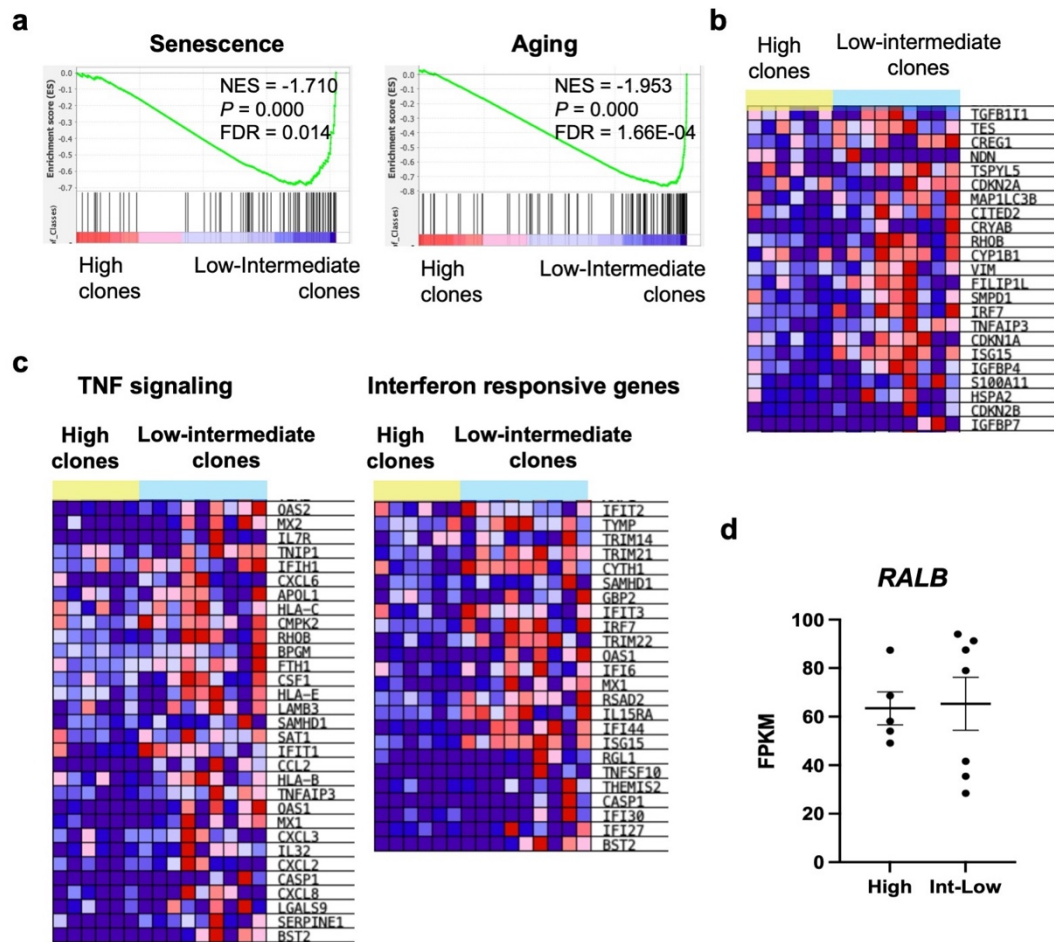
(a) The lentiviral-mediated overexpression (O/E) of *CUX1* elevated the mRNA expression of *CUX1* at both the proliferation (DOX-ON) and maturation (DOX-OFF) stages in imMKCLs (clone 7). **(b)** *CUX1* overexpression did not affect the mRNA expressions of *ISG15* or *IFIT3*. The expression levels were normalized to *GAPDH*. **(c)** The expression of *let-7a-5p* was not significantly affected by the overexpression of *CUX1*. The expression levels were normalized to *RNU6B*. Neither the proliferation rate **(d)** nor the iPSC-PLT production **(e)** in static conditions were affected by *CUX1* overexpression. Data are expressed as the mean $\pm$ SEM from three independent experiments. Unpaired two-tailed student's *t*-tests were used to assess statistical significance. Source data are provided as a Source Data file.



Supplementary Figure 4

Supplementary Fig. 4

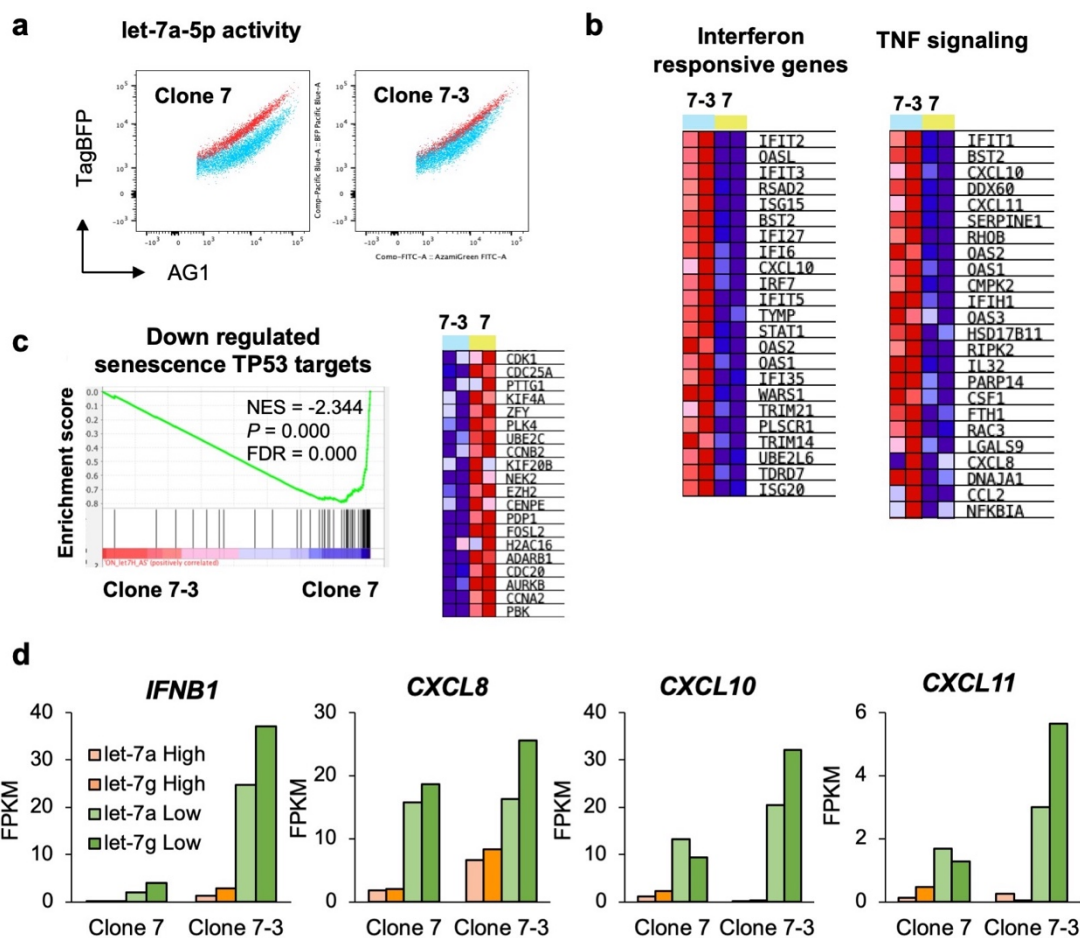
(a) The intracellular protein expression of RALB (mean fluorescence intensity, MFI) detected in MOCK or RALB-overexpressing imMKCLs (O/E RALB) by intracellular flow cytometry. (b) The RALB expression was compared using a histogram overlay. (c) Comparable let-7a-5p expression was observed in MOCK and RALB-overexpressing imMKCLs. (d) The overexpression of RALB did not significantly impact the expression of genes related to MK maturation. The bar graphs show FPKM values of the indicated genes. Data are expressed as the mean $\pm$ SEM from three independent experiments. Unpaired two-tailed student's t -tests were used to assess statistical significance. Source data are provided as a Source Data file.



Supplementary Figure 5

Supplementary Fig. 5

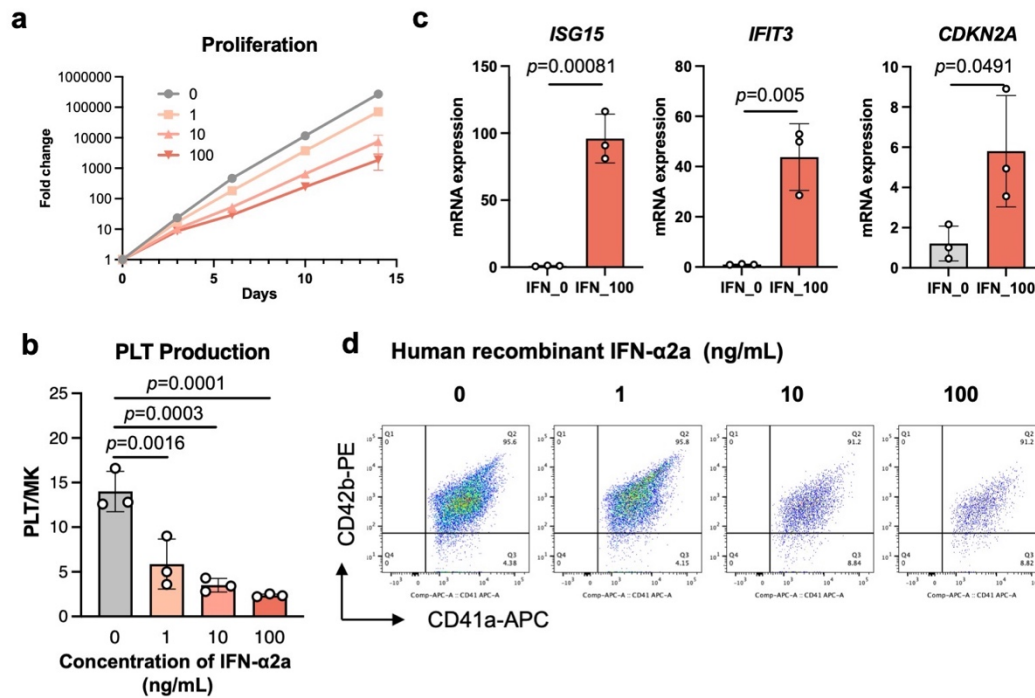
GSEA were performed to compare high quality clones (n=6) and Low ~ intermediate clones (n=9). (a) GSEA plots and (b) a heatmap showing the enrichment of senescence and aging-related gene sets in low~intermediate quality clones compared with high quality clones. Representative enrichment plots from each group are displayed with the NES, non-adjusted p value, and FDR derived from GSEA software. (c) Heatmaps of immune-related gene sets enriched in low~intermediate quality clones. (d) *RALB* expression levels in clones with distinct qualities. Source data are provided as a Source Data file.



Supplementary Figure 6

Supplementary Fig. 6

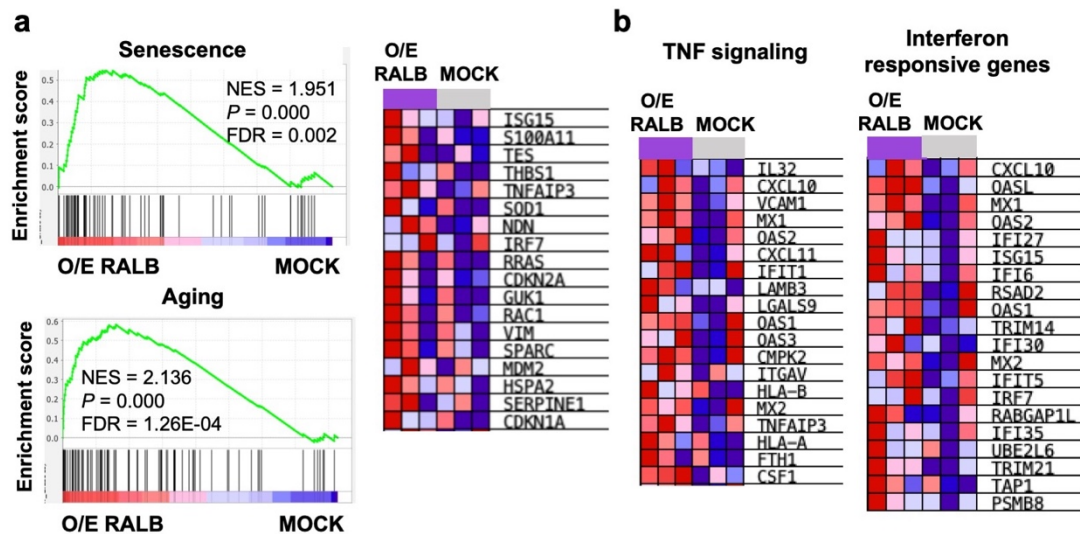
(a) A flow cytometry analysis of let-7a-5p activity patterns in clone 7 and clone 7-3 by miRNA switch technology. (b) Heatmaps of gene sets enriched in clone 7-3. The let-7 low cells from each clone were compared. (c) GSEA plots showing the enrichment of downregulated senescence TP53 targets in clone 7-3 compared with clone 7. (d) Inflammatory cytokine encoding genes exhibited increased expression levels in clone 7-3 compared with clone 7. Source data are provided as a Source Data file.



Supplementary Figure 8

Supplementary Fig. 8

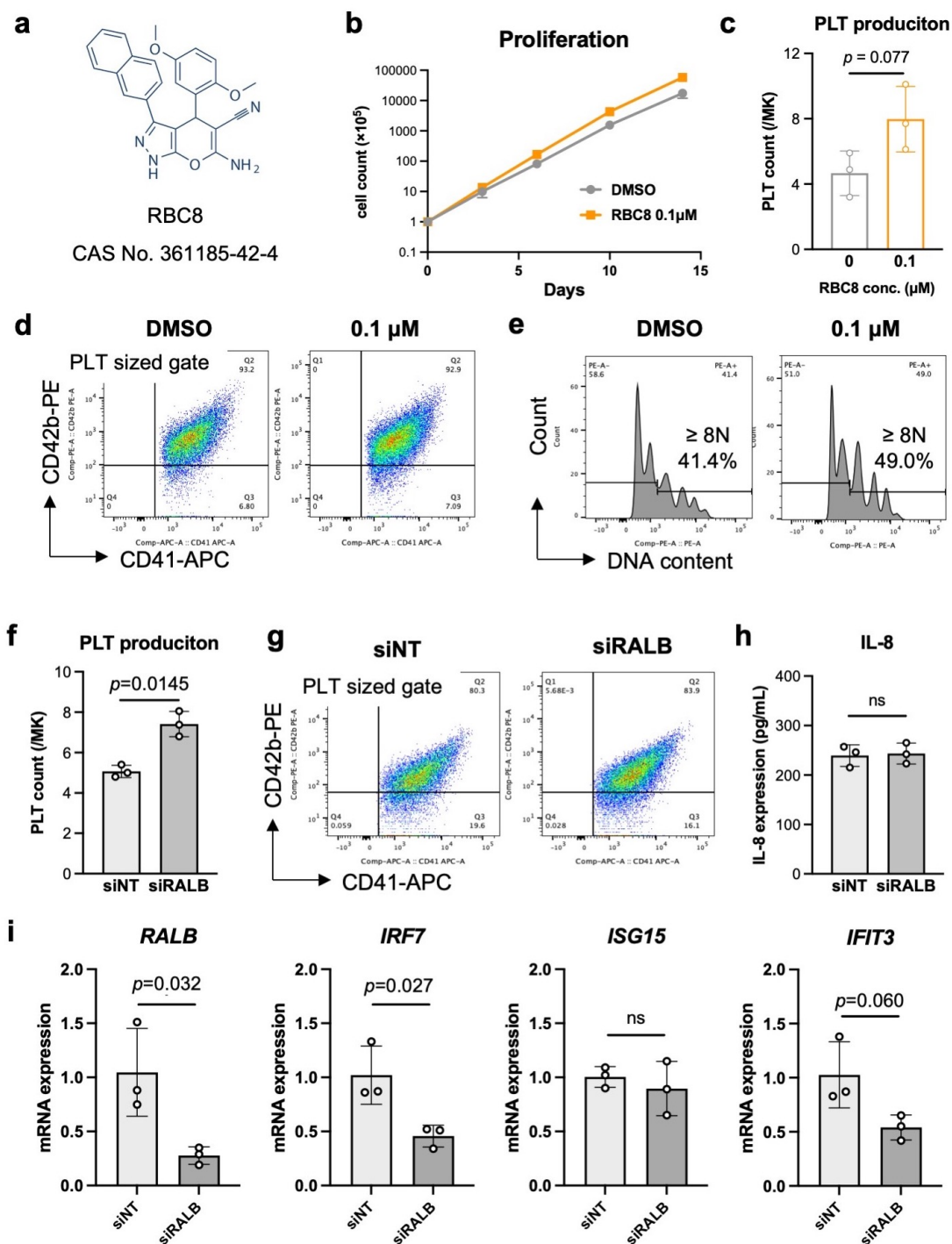
Treatment with human recombinant interferon-α2a inhibited the proliferation (**a**) and iPSC-PLT production (**b**) of imMKCLs in a dose-dependent manner. imMKCLs (clone 7) were treated with human IFN-α2a at concentrations of 0, 1, 10, or 100 ng/mL in static conditions. (**c**) The induced mRNA expression of interferon-related genes and *CDKN2A* in imMKCLs. mRNA expression levels were measured by RT-qPCR and normalized to *GAPDH*. (**d**) Representative flow cytometry plots of iPSC-PLTs generated under the indicated conditions. Data are expressed as the mean ± SD from three independent experiments. One-way ANOVA with multiple comparisons or unpaired two-tailed student t-tests were used to assess statistical significance. Source data are provided as a Source Data file.



Supplementary Figure 9

Supplementary Fig. 9

(a) GSEA plots and a heatmap showing the enrichment of senescence and aging-related gene sets in RALB-overexpressing imMKCLs (O/E RALB) compared with MOCK. (b) Heatmaps of the gene sets enriched in O/E RALB (clone 7).

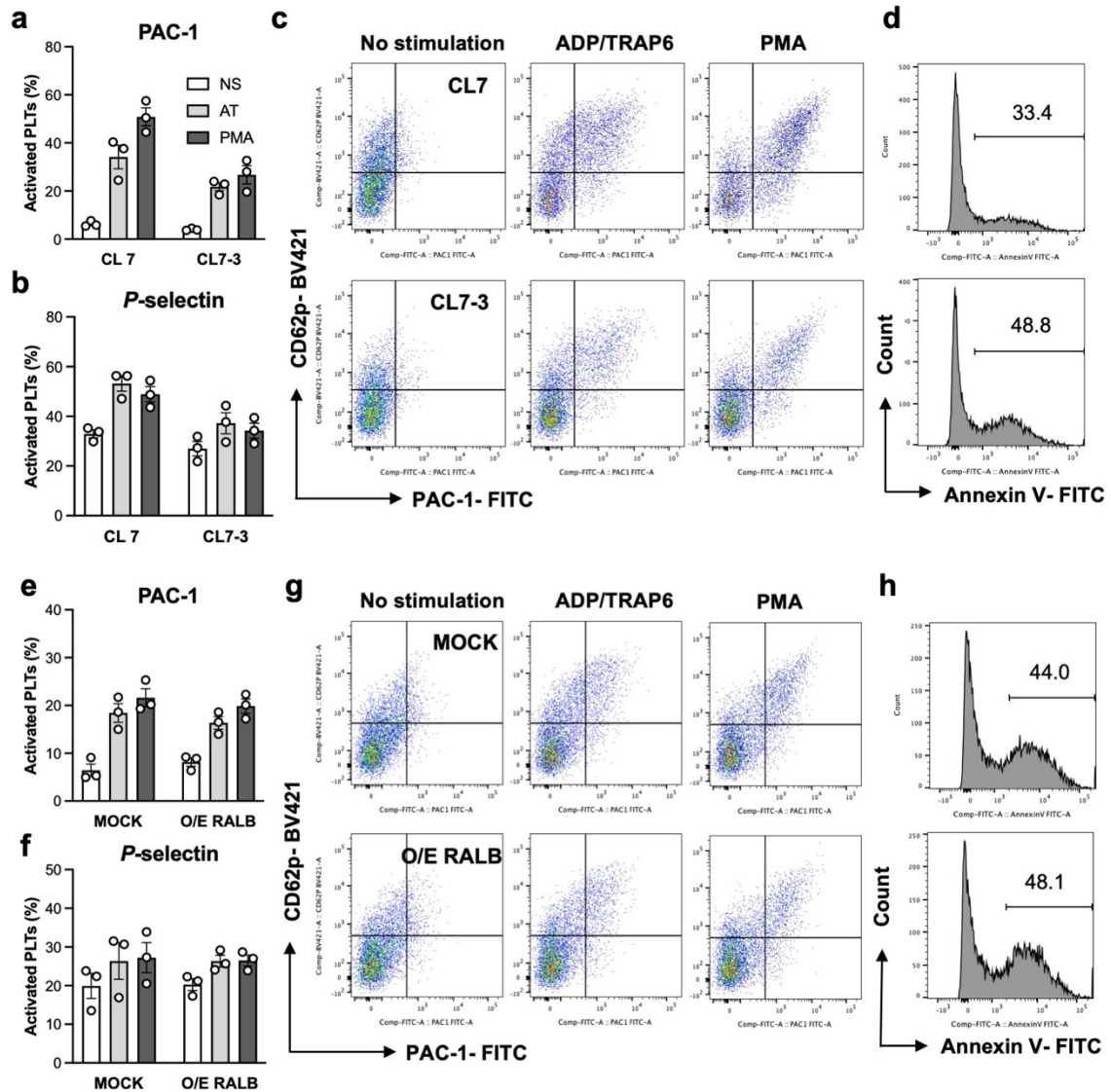


Supplementary Figure 10

Supplementary Fig. 10

(a) Molecular structure of RBC8, a specific inhibitor of RALA and RALB. (b) The administration of 0.1 μ M RBC8 inhibited the proliferation of imMKCLs (clone 7). (c) iPSC-PLT production under the indicated conditions. (d) Representative flow cytometry plots of iPSC-PLTs generated

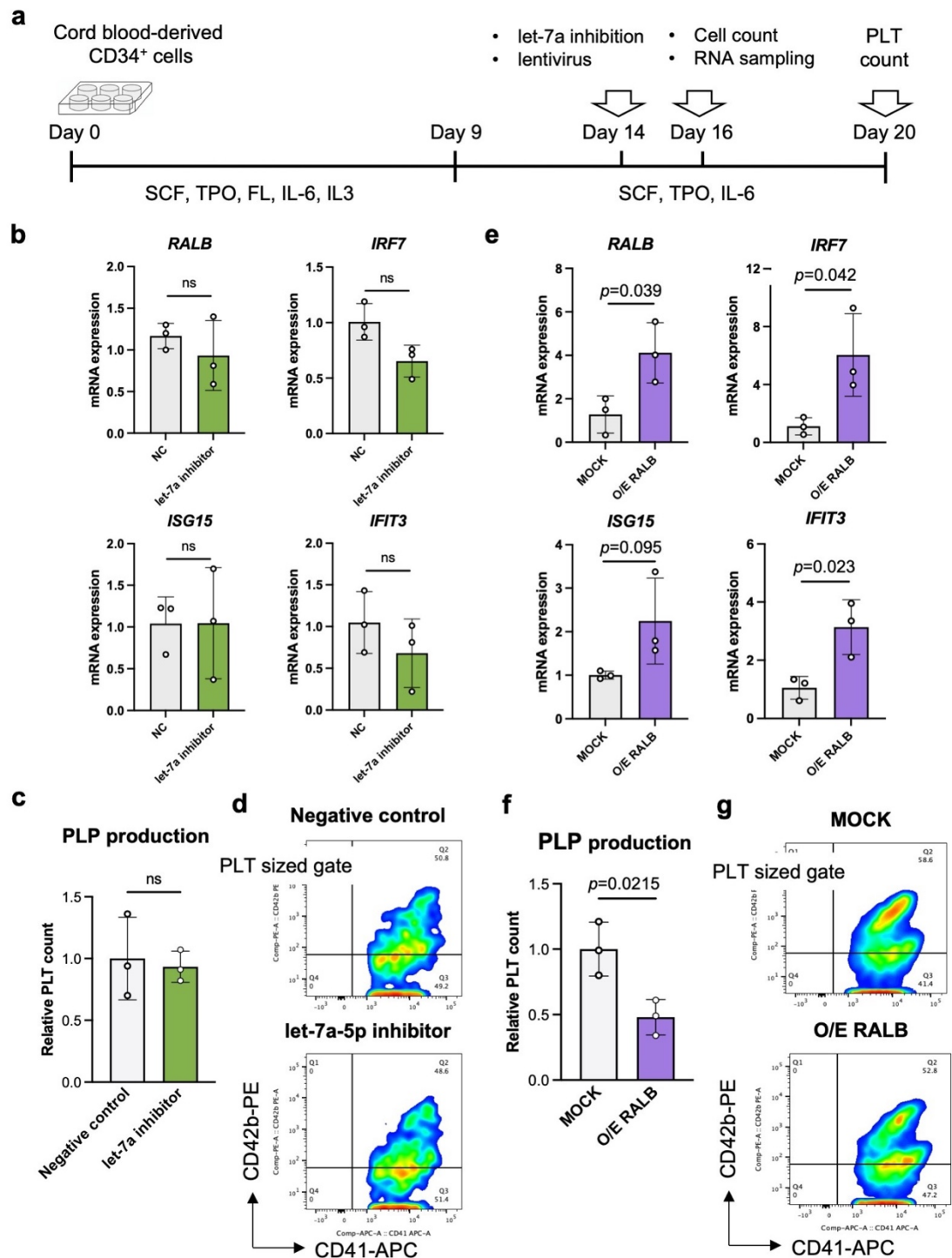
from RALB-overexpressing imMKCLs (clone 7) in the presence or absence of RBC8. **(e)** Flow cytometric analysis of ploidy. **(f)** siRNA-mediated *RALB* knockdown ameliorated the iPSC-PLT production of imMKCLs (clone 7). Cells were transfected with siRNA targeting *RALB* (siRALB) or non-targeting control (siNT). **(g)** Representative flow cytometry plots of iPSC-PLTs generated from siNT or siRALB. **(h)** RALB knockdown did not significantly impact IL-8 secretion at the maturation stage. **(i)** The relative mRNA expression of the indicated genes was assessed in imMKCLs 48 hours following the reverse transfection procedure. mRNA expression levels were measured by RT-qPCR and normalized to *GAPDH*. The iPSC-PLTs were generated in static conditions. Data are expressed as the mean $\pm$ SEM from three independent experiments. Unpaired two-tailed student's *t*-tests were used to assess statistical significance. Source data are provided as a Source Data file.



Supplementary Figure 11

Supplementary Fig. 11

(a) PAC-1 binding and (b) *P*-selectin expression on iPSC-PLTs generated from clone 7 or clone 7-3 with or without PMA or ADP/TRAP6 stimulation. (c) Representative flow cytometry plots of activated iPSC-PLTs. (d) Representative histograms of Annexin V bound to CD41⁺ iPSC-PLTs derived from clone 7 or clone 7-3. (e) PAC-1 binding and (f) *P*-selectin expression on iPSC-PLTs generated from MOCK or RALB-overexpressing imMKCLs (O/E RALB) with or without PMA or ADP/TRAP6 stimulation. (g) Representative flow cytometry plots of activated iPSC-PLTs. (h) Representative histograms of Annexin V bound to CD41⁺ iPSC-PLTs derived from MOCK or O/E RALB. The iPSC-PLTs were generated in turbulence conditions. Data are expressed as the mean $\pm$ SEM from three independent experiments. Source data are provided as a Source Data file.

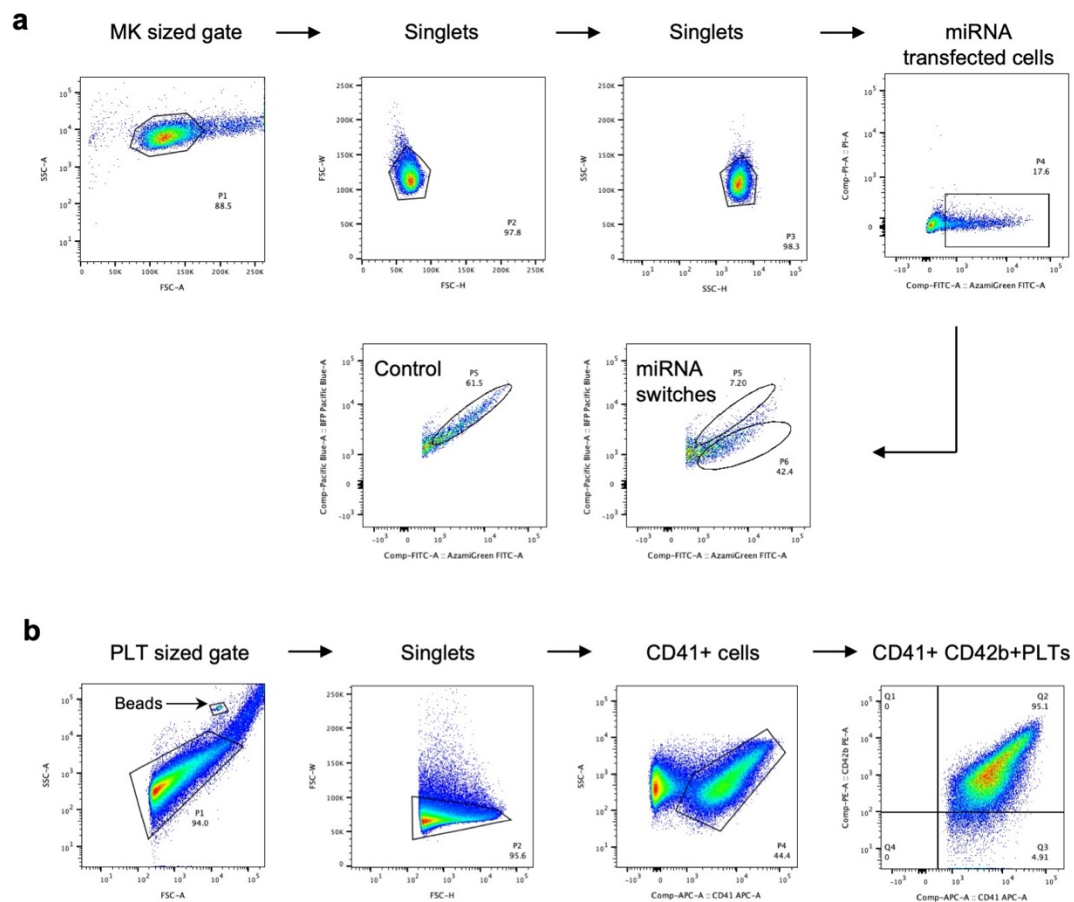


Supplementary Figure 12

Supplementary Fig. 12

(a) A schematic illustration of the *in vitro* differentiation of MKs from cord blood-derived CD34⁺ cells. The inhibition of let-7a-5p significantly impacted neither mRNA expression of the indicated

genes **(b)** nor platelet-like particles (PLP) production **(c-d)**. **(e)** Significantly elevated expression levels of *RALB*, *IRF7*, *ISG15*, and *IFIT3* were observed in RALB-overexpressing (O/E) cells. Total RNAs were isolated from day-16 cells. The mRNA expression levels were measured by RT-qPCR and normalized to *GAPDH*. **(f-g)** The overexpression of RALB induced a significant decline of CD41a⁺CD42b⁺ PLPs generated from cord blood-derived MKs at day 20. Data are expressed as the mean $\pm$ SEM from three independent experiments. Unpaired two-tailed student's *t*-tests were used to assess statistical significance. Source data are provided as a Source Data file.



Supplementary Figure 13

Supplementary Fig. 13

Flow cytometry gating strategies for imMKCL subpopulations distinguished by miRNA switches **(a)** and for iPLC-PLTs counted with the aid of absolute counting beads **(b)**.