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Forming megakaryocytes from murine induced pluripotent stem cells by the inducible overexpression of supporting factors

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Abstract

Background: Platelets are small anucleate cells that circulate in the blood in a resting state but can be activated by external cues. In case of need, platelets from blood donors can be transfused. As an alternative source, platelets can be produced from induced pluripotent stem cells (iPSCs); however, recovered numbers are low.

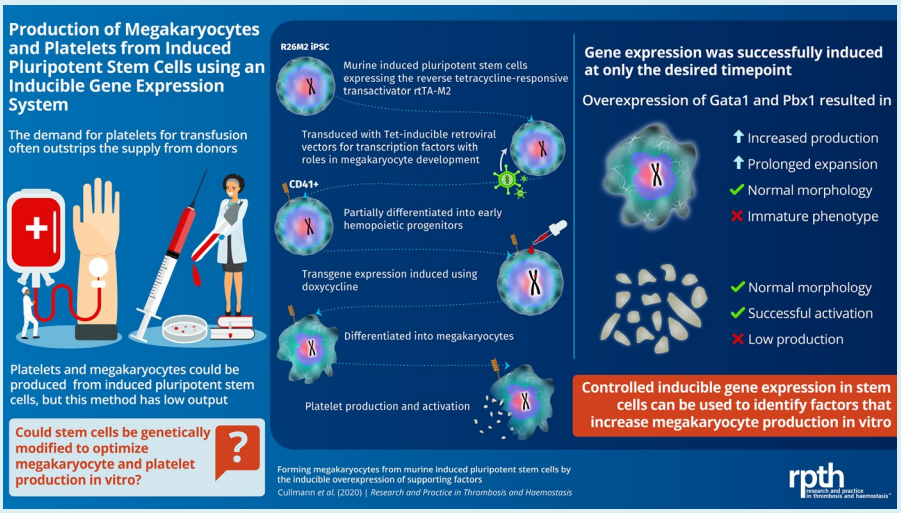
Objectives: To optimize megakaryocyte (MK) and platelet output from murine iPSCs, we investigated overexpression of the transcription factors GATA-binding factor 1 (GATA1); nuclear factor, erythroid 2; and pre–B-cell leukemia transcription factor 1 (Pbx1) and a hyperactive variant of the small guanosine triphosphatase RhoA (RhoAhc).

Methods: To avoid off-target effects, we generated iPSCs carrying the reverse tetracycline-responsive transactivator M2 (rtTA-M2) in the Rosa26 locus and expressed the factors from Tet-inducible gammaretroviral vectors. Differentiation of iPSCs was initiated by embryoid body (EB) formation. After EB dissociation, early hematopoietic progenitors were enriched and cocultivated on OP9 feeder cells with thrombopoietin and stem cell factor to induce megakaryocyte (MK) differentiation.

Results: Overexpression of GATA1 and Pbx1 increased MK output 2- to 2.5-fold and allowed prolonged collection of MK. Cytologic and ultrastructural analyses identified typical MK with enlarged cells, multilobulated nuclei, granule structures, and an internal membrane system. However, GATA1 and Pbx1 expression did not improve MK maturation or platelet release, although in vitro–generated platelets were functional in spreading on fibrinogen or collagen-related peptide.

Conclusion: We demonstrate that the use of rtTA-M2 transgenic iPSCs transduced with Tet-inducible retroviral vectors allowed for gene expression at later time points

during differentiation. With this strategy we could identify factors that increased in vitro MK production.



KEYWORDS

Genetic modification, iPS cells, megakaryocytes, retroviral vectors, Tet-inducible system

Essentials

- Megakaryocytes (MKs) and platelets differentiated from induced pluripotent stem cells (iPSCs) may represent an alternative source for transfusion.
- Tet-regulated retroviral systems allow inducible expression in MK differentiation from iPSCs.
- Expression of GATA-binding factor 1 (GATA1) and pre–B-cell leukemia transcription factor 1 (Pbx1) improve MK differentiation from murine iPSCs.
- GATA1 and Pbx1 MKs display an immature phenotype and release platelets only with low efficiency.

1 | INTRODUCTION

Platelets are small anucleate blood cells that circulate in a resting state in large amounts in the mammalian body. Their major task is to regulate blood coagulation to prevent bleeding, but they also participate in several other processes such as immune responses and inflammation.[1] Platelet transfusions are frequent therapies in patients with a lack of (functional) platelets due to, for example, trauma, surgery, or chemo-/radiotherapy or for patients with inherited platelet disorders. To date, platelets for transfusion are exclusively obtained from human donors, which might not be sufficient for the increasing need of platelet transfusion units.[2,3] This becomes especially important in societies with an aging population that will have expanding needs for blood products for transfusion.[4] Even though platelet collection, processing, and transfusion are well established, they can be associated with major complications such as immunity against transfused platelets leading to platelet refractoriness.[5] Furthermore, transfusion units must be stored at room temperature to prevent platelet activation, which in turn also increases the risk of bacterial contamination and thereby limits the shelf life of the products to about 5 days.[6]

Therefore, platelets produced in vitro may be an alternative source for transfusion. Platelets can be generated in vitro from megakaryocytes (MKs) that were differentiated from primary hematopoietic stem cells (HSCs),[7,8] embryonic stem cells,[9,10] or induced pluripotent stem cells (iPSCs).[11,12] Among those cell sources, pluripotent stem cells, in contrast to short-lived HSCs, offer the opportunity to establish a cell line with special properties that allows the most efficient MK differentiation. However, although platelet production from iPSCs is possible, the output in vitro is not as efficient as it is from MKs in vivo. Whereas one MK generates up to 1000 platelets in vivo, they produce only about 10 to a maximum of 80 platelets in vitro[13] also when using shear force systems to enhance platelet release.[14]

To generate platelets in vitro, in the first instance MKs must be produced and expanded. This can be achieved by the use of cytokines and specified culture conditions; however, supporting factors may also be expressed in the cells. In this respect, the combined overexpression of the transcription factors GATA-binding factor 1 (GATA1), stem cell leukemia 1/T-cell acute lymphocytic leukemia protein 1 (SCL/TAL1), and Friend leukemia integration 1 (FLI1)[13,15] in human iPSCs has been shown to enhance MK differentiation.

In addition, expression of MYC proto-oncogene, bHLH transcription factor in combination with BMI1 proto-oncogene, polycomb ring finger and B-cell lymphoma-extra large in a different approach generated expandable and cryopreservable MKs.[16] In the study by Nakamura and colleagues,[16] an inducible system was used that allowed for expression of factors for MK expansion and the destruction of proteins by a destabilizing domain fused to the factors for differentiation. This inducible system was transduced into hematopoietic progenitors generated from iPSCs but not into pluripotent cells. In the study by Moreau et al,[13] the transcription factors were introduced by single, constitutive active lentiviral vectors and differentiation initiated thereafter. In both expression systems, no modified iPSC lines could be established, as expression of the factors cannot be tolerated in pluripotency.

In our study, we wanted to express supporting factors to enhance MK and platelet output from murine iPSC. To allow the tight regulation of transgene expression during differentiation, we generated iPSCs from Rosa26 reverse tetracycline-responsive transactivator M2 (rtTA-M2) mice.[17] In these cells, the rtTA-M2 will be constitutively expressed from the Rosa promoter in the Rosa26 safe harbor locus. These Rosa rtTA-M2 iPSCs were then transduced with retroviral vectors expressing from a Tet-inducible promoter. With this strategy, transgene expression was expected to be well regulated and protected from potential silencing during differentiation. As proof of concept for our inducible system, we selected factors with well-known roles in megakaryopoiesis that were expected to improve MK differentiation. GATA1 is the major transcription factor controlling MK maturation.[18-21] The transcription factor nuclear factor, erythroid 2 (Nfe2) controls proplatelet formation and release.[22,23] Furthermore, we selected Pbx1, the cofactor of myeloid ecotropic viral integration site 1 homolog (MEIS1) and hematopoietic Hox transcription factors such as HoxA9. Pbx1 knockout mouse embryos display reduced numbers of HSC, common myeloid progenitors, and MK-erythrocyte progenitors.[24,25] In addition to transcription factors, also the small Rho–guanosine triphosphatase (GTPase) RhoA influences MK maturation and platelet release as indicated by the occurrence of macrothrombocytopenia in knockout mouse models.[26,27] Point mutations at F30L push their GTPase activity to enhanced intrinsic guanosine triphosphate (GTP) ↔ guanosine diphosphate (GDP) exchange rates and, therefore, GTPases become spontaneously activated.[28]

In contrast to most of the studies on in vitro platelet production that focus on human cells, in our study, we aimed to develop a protocol for murine cells. We believe that for studying the functionality of in vitro–derived MK and platelets and their interaction with the host environment, the all-murine model is indispensable. Otherwise, interspecies differences between mouse and human hampers their physiological responses. In addition, platelet function during infection can adequately be studied only in immune competent models. Murine in vitro platelets can be tested in syngeneic mouse models. The "all murine" model will therefore cover the gap between basic research and clinical translation. Using our Tet-inducible iPSC model, we identified GATA1 and Pbx1 to improve MK differentiation and to allow prolonged maintenance of MK progenitors with mostly immature phenotype. MKs were able to produce platelets in vitro that had all typical morphologic features and responded to activation by spreading on respective matrices.

2 | MATERIAL AND METHODS

2.1 | Cell lines

293T and murine fibroblast Schwann cell factor 1 (SC1) cells were cultured in Dulbecco's modified Eagle medium (DMEM, BioWest, Nuaillé, France) supplemented with 10% heat inactivated fetal calf serum (FCS; Biochrom, Holliston, MA, USA), 1 mM L-glutamine (Life Technologies, Rockville, MD, USA) and 1% penicillin/streptomycin. OP9 cells were cultivated in α-Medium (MEM; (Biochrom) with 20% FCS (Biochrom) 1 mM L-glutamine (Life Technologies) and 1 mM penicillin/streptomycin.

2.2 | Retroviral vector production

Gammaretroviral self-inactivating vectors containing the Tet-inducible T11 promoter were used[29]; 5×10^6 293T cells were seeded in 10-cm plates and transfected by the calcium-phosphate transfection method the next day with 10 μg gammaretroviral transfer vector plasmid, 15 μg gag/pol plasmid (pcDNA3.MLVg/p) and 1.5 μg plasmid encoding the vesicular stomatitis virus glycoprotein envelope (pMD.G plasmid), in medium supplemented with 25 μM chloroquine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Retroviral supernatants were collected 36 and 48 hours after transfection and concentrated by high-speed centrifugation at 11 500 *g* for 16 hours. Concentrated viral particles were resuspended in StemSpan medium (Stem Cell Technologies, Köln, Germany) and stored at −80°C. Viral vector titers were determined by transducing SC1 cells in serial dilutions in the presence of protamine sulfate (4 μg/mL) and doxycycline (1 μg/mL). SC1 cell were analyzed for enhanced green fluorescent protein (eGFP) expression by flow cytometry 5 days after transduction.

2.3 | Culture and transduction of murine iPSC

iPSCs were cultured on mitomycin C–treated mouse embryonic fibroblast–feeder cells in knockout-DMEM supplemented with 15% FCS Superior (Biochrom), 1% penicillin/streptomycin, 2 mM glutamate, 0.1 mM nonessential amino acids (Life Technologies), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and 10^3 U/mL leukemia inhibitory factor. For transduction, iPSCs were depleted from their feeders by allowing their attachment to gelatin-coated culture dishes. Transduction was performed once with a multiplicity of infection (MOI) of 10 after seeding iPSCs on gelatin-coated cell culture dishes with protamine sulfate (4 μg/mL).

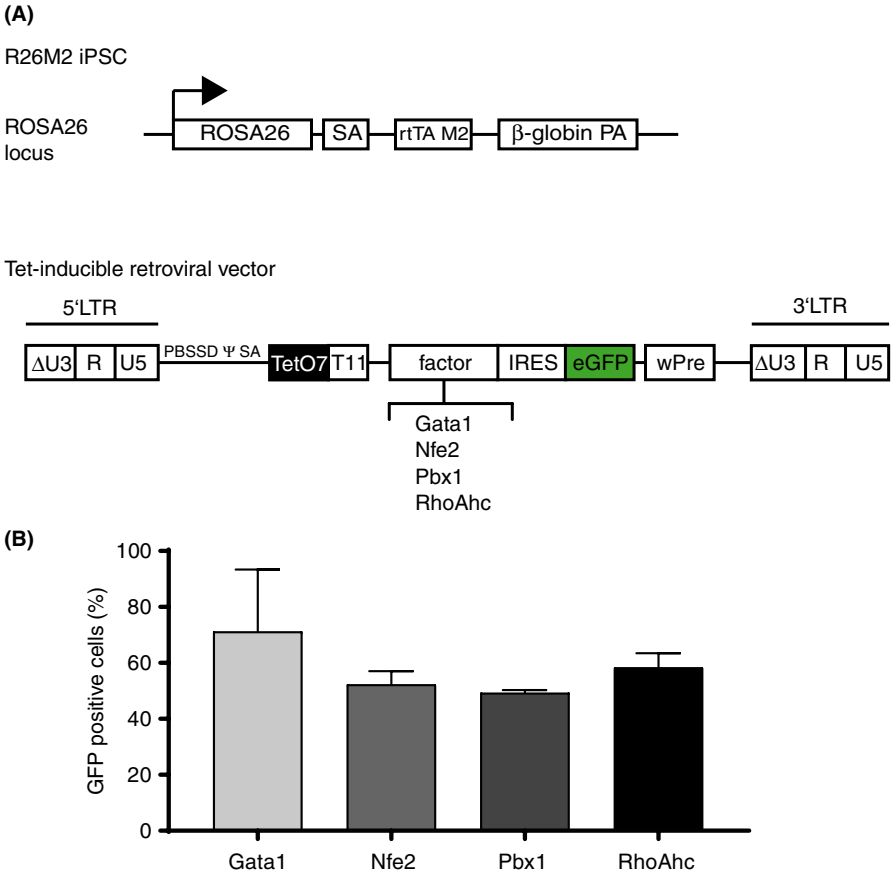


FIGURE 1 (A) R26M2K2 induced pluripotent stem cells (iPSCs) and retroviral vectors. Murine R26M2K2 iPSCs express the reverse tetracycline-responsive M2 (rtTA-M2) transactivator from the Rosa26 promoter. Schematic representation of the expression cassette inserted into the Rosa locus: The coding sequence of the rtTA-M2 is inserted downstream of the Rosa26 promoter after an additional splice acceptor site (SA). Polyadenylation of RNA is guaranteed by the β-globin polyadenylation site (PA). R26M2 iPSCs were transduced with self-inactivating (SIN) gammaretroviral vectors expressing the candidate factors GATA1, Nfe2, Pbx1, and RhoAhc from a T11 minimal promoter. (B) Transduction efficiency of R26M2K2 iPSCs. The transduction rate was determined by splitting the cultures into induced and noninduced cultures. The noninduced cultures were taken for differentiations in later experiments, the induced cultures were analyzed by flow cytometry to determine the transduction efficiencies (mean ± SD, n = 3). Ψ, packaging signal; eGFP, coding sequence for the enhancer green fluorescent protein; IRES, internal ribosomal entry site; LTR, long terminal repeat; PBS, primer binding site; R, repeat region; SD, splice donor; TetO7, repeat of seven Tet operons; T11, minimal promoter; factors, coding sequence of one of the four factors; U3, 3′ unique region, U5, 5′ unique region; wPRE, woodchuck hepatitis virus posttranscriptional regulatory element

2.4 | Differentiation of murine iPSCs into MKs and platelets

Differentiation followed previously established protocols with modifications.[30,31] iPSCs were depleted from their feeder cells on 6-well gelatin-coated cell culture dishes and cultivated without feeders for 2 days on 6-well gelatin-coated cell culture dishes or flasks in Iscove's modified Dulbecco's medium (Thermo Fisher Scientific, Waltham, MA, USA), 15% FCS (Biochrom), 1% penicillin/streptomycin, 1 mM L-glutamine, 50 ng/ml ascorbic acid (Sigma-Aldrich) and 150 mM monothioglycerol (Sigma-Aldrich). For embryoid body (EB) formation, 15 000 cells/mL were seeded into 6-well suspension cell culture dishes in 3 mL medium and grown for 7 days on an orbital shaker. At day 5 of EB formation, medium was supplemented with 10 ng/mL murine interleukin-3 and 30 ng/mL murine stem cell factor (SCF) (PeproTech, Rocky Hill, NJ, USA). At day 7 of differentiation, EBs were dissociated with collagenase IV (Life Technologies) (250 U/mL), and CD41-positive early hematopoietic progenitors were enriched via magnetic-associated cell sorting (MACS) using the biotinylated anti-CD41 antibody (1:100) (eBioscience, San Diego, CA, USA) and antibiotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions; 10^5 of the CD41 + cells were seeded onto mitomycin C-treated OP9 feeder cells in MK differentiation medium (MEM, Biochrom), 20% FCS, 1% penicillin/streptomycin, 1 mM L-glutamine, 50 ng/mL murine thrombopoietin (THPO), and 25 ng/mL murine SCF (PeproTech). After 2 weeks, MK and platelet-like particles (PLPs) were harvested and analyzed or replated onto fresh mitomycin C–treated feeder cells.

2.5 | Flow cytometry

MKs were incubated with fluorescent-labeled antibodies for 30 minutes at 4°C. For PLP analysis, antibodies were incubated for 10 minutes at 37°C followed by 10 minutes at room temperature. The antibodies are listed in Table S3. Flow cytometry was performed using the CytoFLEX (BeckmanCoulter, Krefeld, DE, USA).

2.6 | Electron microscopy

MKs and PLPs were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) in culture medium for 45 minutes at room temperature. After washing in phosphate buffered saline (PBS), cells were centrifuged and gently mixed with 2% warm liquid agarose. After cooling and gelling, small agarose blocks were cut containing the cells. These blocks were postfixed with 2% osmium tetroxide in PBS and treated with 1% tannic acid.[32] Then, cells were dehydrated in a graded series of ethanol and finally embedded in epoxy resin (Sigma-Aldrich, Steinheim, Germany). Ultrathin sections were cut and stained with 2% uranylacetate for 15 minutes followed by 2% lead citrate for 5 minutes. All preparations were examined in a JEM 1400T flash electron microscope (Jeol USA Peabody, MA, USA), and pictures were taken with a XAROSA digital camera using Radius software (EMSIS GmbH, Muenster, Germany).

2.7 | Platelet spreading

For the functional analysis of in vitro–produced PLPs, cover slips were coated overnight at 4°C either with 10 μg fibrinogen or with 1 μg collagen-related peptide (CRP) in PBS (coating area ~ 1cm^2). For resting conditions, cover slips were coated with poly-L-lysin in H_2O for at least 30 minutes at room temperature. After blocking with 1% sterile bovine serum albumin (BSA)/PBS for 1 hour, cover slips were washed with prewarmed PBS and HEPES-Tyrodes buffer. Thrombin (final concentration, 0.01 U/mL) or adenosine diphosphate (ADP; 10 μM)/thromboxane analog (U46619, 3 μM) were added to the PLP suspension and immediately transferred to the coated cover slides. After 60 minutes, the PLPs were fixed with 4% paraformaldehyde in PHEM Buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA. 2mM MgCl2, pH 7.0) + 0.1% Nonidet P40 for at least 20 minutes. Samples were washed with PBS and blocked with 5% BSA/PBS. CD41/CD61 was stained using Alexa647-conjugated anti-CD41/CD61 monoclonal antibodies (1 μg/mL, Nieswandt Laboratory, Würzburg, Germany), CD42a was stained using Alexa488-conjugated anti-CD42a monoclonal antibodies (1 μg/mL, Nieswandt Laboratory, Würzburg), β1-tubulin was stained using anti–β1-tubulin polyclonal antibodies (20 μg/mL, GeneTex: GTX107175) and Cy3-conjugated donkey anti-rabbit IgG antibodies (2.5 μg/mL, Jackson ImmunoResearch, West Grove, PA, USA). Images were acquired with a TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

3 | RESULTS

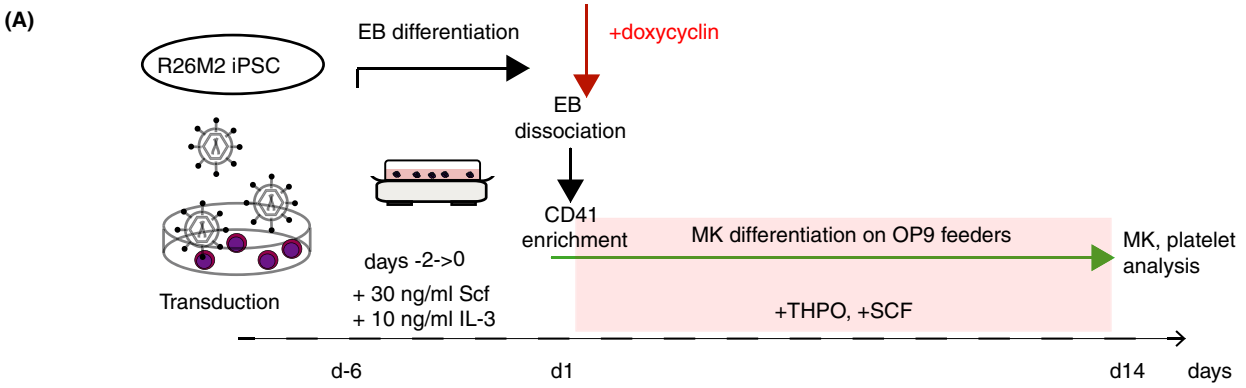
3.1 | Murine iPSCs and vectors for Tet-inducible expression

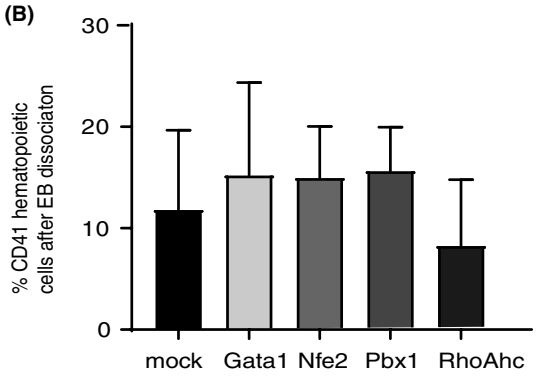
In our study, we sought to develop a Tet-inducible expression system for the differentiation of murine iPSCs to MKs by avoiding expression in the pluripotent state. To guarantee the presence of the rtTA-M2 transactivator protein in all cells, we generated iPSCs from B6.Cg-Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$/J mice, which express the rtTA-M2 from the Rosa promoter.[17] Reprogramming was performed by lentiviral expression of octamer-binding transcription factor 4 (Oct4), Kruppel-like factor 4, sex-determining region Y–box 2 (Sox2), and c-Myc.[33,34] From 56 selected iPSC clones, 31 were tested for their potential to give rise to CD41-positive early hematopoietic progenitors after 7 days of EB formation. Of those, two were selected because they produced 36% and 48% CD41 + cells, respectively, very similar to the murine embryonic stem cell line OG2 (Figure S1) and were, therefore, well suited to differentiate into hematopoietic cells. These two clones (R26M2K2, R26M2K3) were analyzed further for their pluripotency. Stage-specific embryonic antigen 1 expression was in the range of that in OG2 cells and alkaline phosphatase activity was verified (Figure S2A, B). We detected expression of Sox2, Nanog, and Oct4 on the mRNA and protein level (Figure S2C,D). R26M2K2 was selected for further studies.

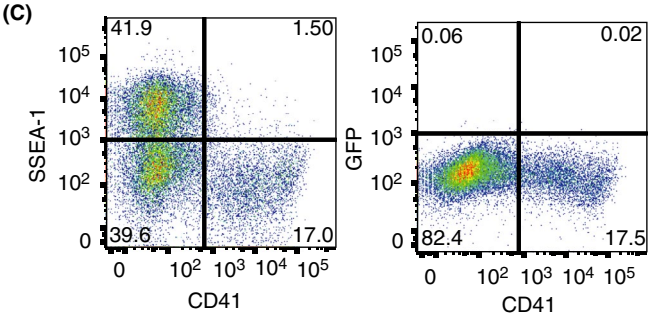
To express supporting factors in R26M2K2 iPSCs that would be inducible at later time points of differentiation but would not express in pluripotency, Tet-inducible gammaretoviral vectors[29] were generated encoding one of the selected factors (GATA1, Nfe2, Pbx1, and RhoAhc). These vectors can be induced by the application of doxycycline (Tet-on system) and also coexpressed eGFP by an internal ribosomal entry site to label the induced transgenic cells (Figure 1A). The vectors could be produced with titers of 10^8 infectious particles/mL (Figure S3) and transduced R26M2K2 iPSCs with an efficiency between 50% and 70% with a MOI of 10 (Figure 1B).

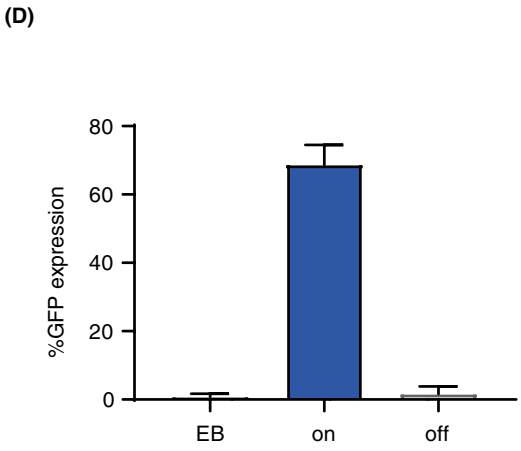
3.2 | GATA1 and Pbx1 overexpression during differentiation increased CD41-positive MKs

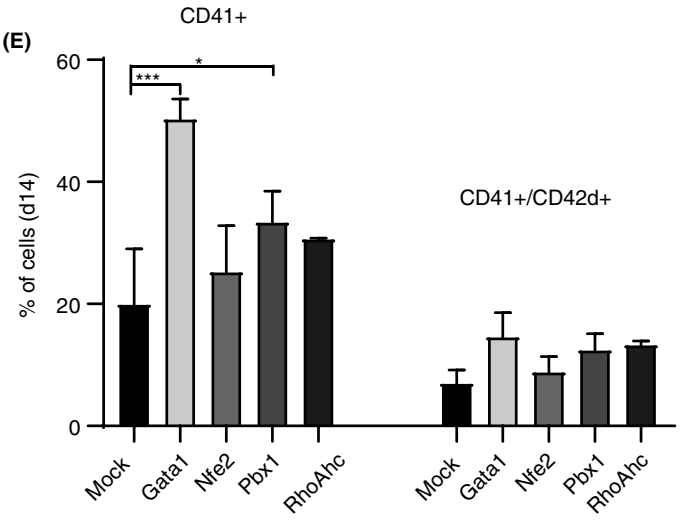
First, we established the MK differentiation protocol using untransduced R26M2K2 iPSC. Differentiation was initiated by EB formation for 6 days (Figure 2A). At day 5 of EB formation, the culture medium was supplemented with SCF and interleukin-3 to support hematopoietic commitment. After EB dissociation, hematopoietic progenitors characterized by CD41 expression were enriched by MACS and plated on OP9 feeder cells in the presence of THPO and SCF. In the mouse, CD41 marks the early embryonic hematopoietic progenitors,[35,36] and expression declines along the differentiation to mature blood cells except for MK. After 2 weeks, MKs in the cultures could be identified by flow cytometry using the MK-lineage marker CD41, but also by their typical morphology of enlarged cells with multilobed nuclei (Figure S4). In addition to CD41, cell surface expression of CD42d, the

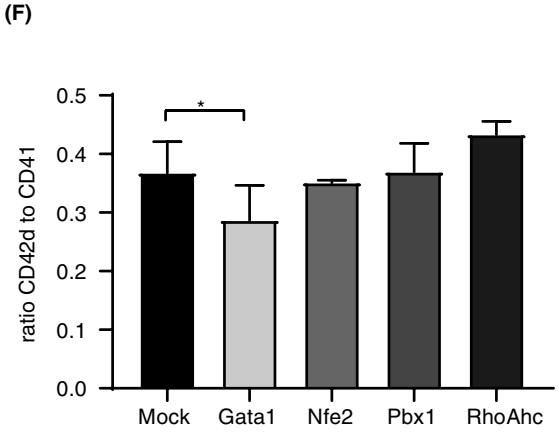

(A)
R26M2 iPSC
EB differentiation
+doxycyclin
EB dissociation
CD41 enrichment
MK differentiation on OP9 feeders
MK, platelet analysis
Transduction
days -2->0
+ 30 ng/ml Scf
+ 10 ng/ml IL-3
+THPO, +SCF
d-6
d1
d14
days


(B)
% CD41 hematopoietic cells after EB dissociaton
mock
Gata1
Nfe2
Pbx1
RhoAhc


(C)
SSEA-1
CD41
41.9
1.50
39.6
17.0
GFP
0.06
0.02
82.4
17.5


(D)
%GFP expression
EB
on
off


(E)
CD41+
CD41+/CD42d+
% of cells (d14)
Mock
Gata1
Nfe2
Pbx1
RhoAhc


(F)
ratio CD42d to CD41
Mock
Gata1
Nfe2
Pbx1
RhoAhc

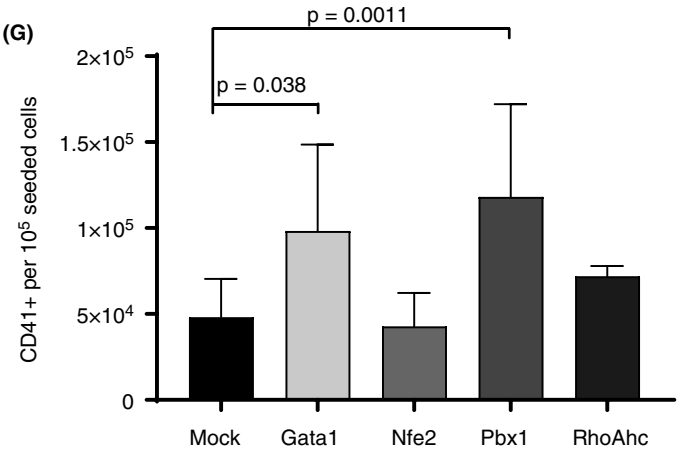

(G)
p = 0.0011
p = 0.038
CD41+ per 10^5 seeded cells
Mock
Gata1
Nfe2
Pbx1
RhoAhc

FIGURE 2 (MK) differentiation from R26M2K2 iPSCs. (A) Differentiation scheme for MK and platelet generation from murine iPSCs. After retroviral transduction, iPSCs were differentiated into EBs by orbital shaking for 6 days (day −6); at day 5, hematopoietic differentiation was enforced by addition of SCF and interleukin-3. At day 1, CD41 positive early hematopoietic progenitors were selected by magnetic-associated cell sorting and seeded onto OP9 feeder cells with thrombopoietin and stem cell factor and induced by doxycycline. After day 14, MK morphology and surface marker expression were analyzed. (B) CD41 expression on early hematopoietic progenitors after EB dissociation. (n = 3-9, mean ± SD, no differences by one-way analysis of variance ANOVA with Dunnett's multiple comparison test). (C) Representative flow cytometry plots of cells after embryoid body (EB) dissociation showing expression of CD41 that is not coexpressed with the pluripotency marker Stage-specific embryonic antigen 1 (left plot). No GFP expression could be detected at that time, (right plot). (D) Green fluorescent protein (GFP) expression in EBs measured at the day of dissociation in the uninduced state. GFP expression in MKs after 2-week induction with doxycycline. No expression after 1-week withdrawal of doxycycline during further cultivation of MKs (GATA-1 transduced MKs are shown, mean ± SD, n = 3-5) (E) Percentage of CD41 expression and CD41/CD42d double positive cells at days 14 of differentiation on hematopoietic cell differentiated on OP9 feeders, (n = 3-8, mean ± SD, one-way ANOVA with Dunnett's multiple comparison test, $^{***}P < .00$; Mann-Whitney test, $^{*}P = .006$). (F) Ratio of CD42d to CD41 expression as a measure of differentiation. (G) Quantification of CD41 + MK at day 14 ((F) and (G): n = 3-8, mean ± SD, Mann-Whitney test)

glycoprotein (GP) V subunit of the GPIb/IX/V complex, was analyzed, indicative for MK maturation. After 2 weeks of coculture, untransduced R26M2K2 iPSCs gave rise to ~20% CD41-positive MK, which coexpressed CD42d on one-third of the cells (Figure 2E).

We then differentiated the R26M2K2 iPSCs that were transduced with the Tet-inducible retroviral vectors encoding one of the four factors. During pluripotency and EB formation, expression from the vectors was not induced and the factors thus not expressed. The transduction did not influence the percentage of CD41 + early hematopoietic progenitors after EB dissociation and varied between 8% and 17% (Figure 2B, C). Unwanted expression from the vectors in the noninduced state ("leakage") could be ruled out as no green fluorescent protein (GFP) signal could be detected by flow cytometry at that time point (Figure 2C, D). The expression of GATA-1, Pbx1, Nfe2, or RhoAhc was induced at the first day of coculture on OP9 feeder cells. After 2 weeks, overexpression of GATA1 increased the contribution of CD41 + MKs 2.5-fold compared to control cultures (50% vs. 20%, Figure 2E). In the cultures transduced to express Pbx1, 32% of the cells expressed CD41 ($P = .006$; Figure 2E). In cultures from Nfe2 or RhoAhc transduced iPSCs, no significant increase of MK differentiation compared to the untransduced cultures could be detected. One-third of the CD41-positive cells coexpressed CD42d, however, none of the factors increased MK maturation (Figure 2E). We further calculated the ratio of CD42d to CD41 positive cells with higher numbers being indicative for better MK maturation (Figure 2F). GATA1 expression significantly reduced CD42d coexpression on CD41-positive MK ($P = .04$ Mann-Whitney test; Figure 2F). Next, we determined the total output of generated MK by evaluating the numbers of CD41 + MK per 10^5 initially seeded early progenitors. The highest number of CD41 + MKs was generated by overexpression of Pbx1, which was increased 2.5-fold compared to the nontransduced cultures, followed by expression of GATA1 with 2-fold increase (Figure 2G). MK cell numbers generated from Nfe2 or RhoAhc overexpressing cultures were not improved.

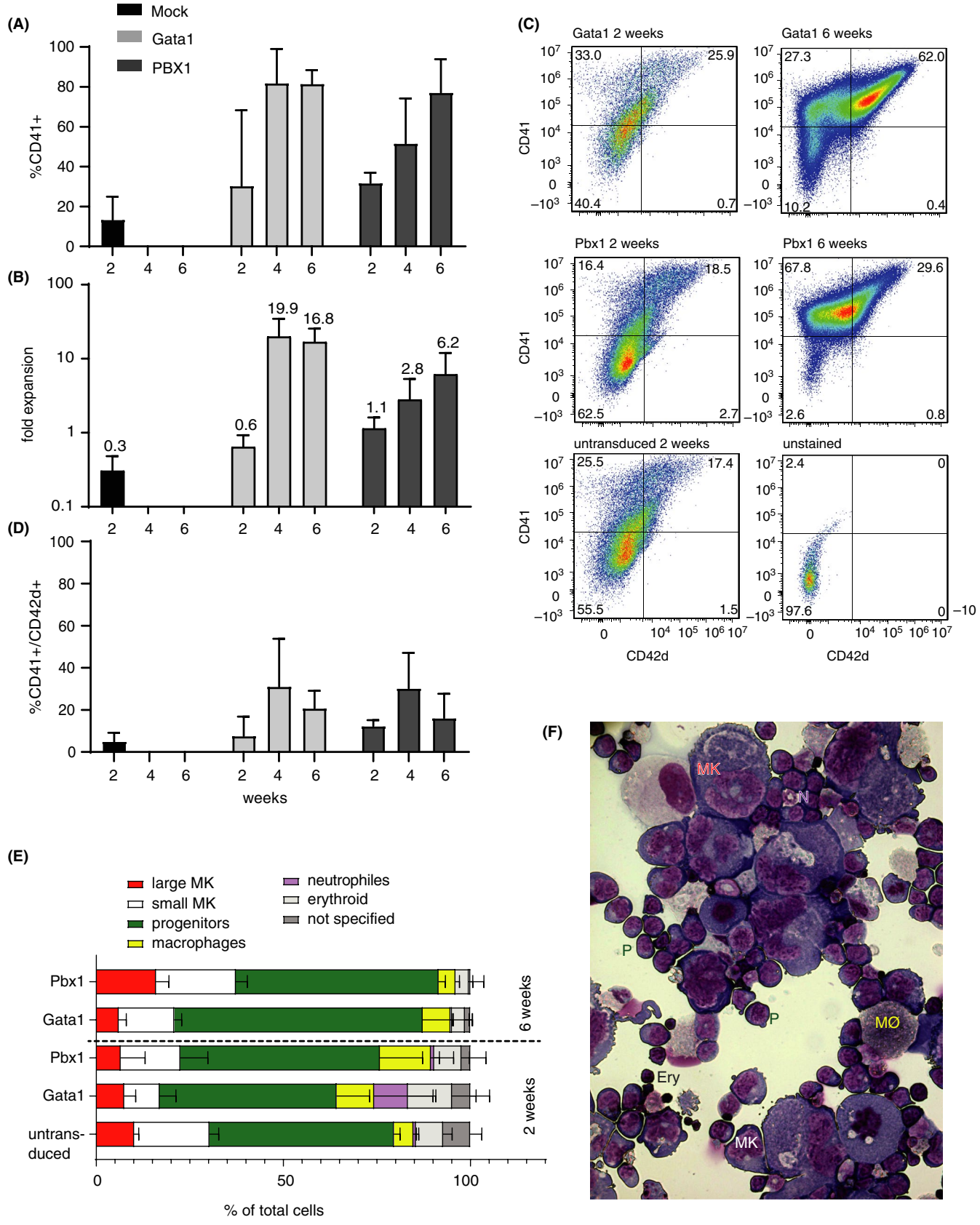
3.3 | GATA1 and Pbx1 expression supported prolonged MK production

For in vitro platelet production, it would be advantageous to expand MK to higher numbers. We therefore investigated whether MK generation could be maintained for longer periods of time. We concentrated on GATA1 and Pbx1 transduced cultures, because these two genes improved MK production in our previous experiments. After 2 weeks, MK progenitors were split and reseeded onto new feeder cells for further cultivation. Pbx1 and GATA1 expressing cultures could generate MK for at least 6 weeks after EB dissociation, while this was not possible for untransduced cultures (Figure 3A). With each passage, MK progenitors expanded 19.9- and 16.8-fold (GATA1) and 2.8- and 6.2-fold (Pbx1) at 4 and 6 weeks, respectively (Figure 3B). In addition, with prolonged culture time, the intensity of CD41 expression improved and the percentage of CD41-positive cells in the cultures increased to >90% (Figure 3A, C). However, coexpression of CD42d, indicative for MK maturation, increased only during the first 4 weeks but not thereafter (Figure 3D, C). Cytomorphological analyses of MK differentiated over this prolonged period showed an increasingly uniform picture of MK and MK progenitors and a declining contribution of blood cells of other lineages, such as macrophages, neutrophils, or erythroid cells (Figure 3E, F). Taken together, our culture condition and overexpression of GATA1 and Pbx1 allowed the enrichment of immature MK but did not support their final maturation.

MK of GATA1 expressing cultures were analyzed by transmission electron microscope (TEM, Figure 4A-C). MK had enlarged nuclei and contained granule structures reminiscent of alpha and dense granules. The intracytoplasmic membranous webs (demarcation membrane system [DMS]) was visible but less well developed compared to the DMS in MKs from the murine bone marrow (BM) (Figure 4E), with oval membranous structures close to the nucleus. No pre-DMS could be visualized in MK differentiated from murine BM cell in vitro underlining the difficulties of maturing MKs in vitro (Figure 4F). Von Willebrand factor expression in iPS-derived MK, as a marker for alpha granules, was confirmed by immunostaining (Figure 4D). TEM analysis of our untransduced cultures was not possible, due to low cell numbers.

3.4 | Transcription factor expression during early hematopoietic differentiation

GATA1 and Pbx1 are expressed during early hematopoietic commitment.[37,38] To investigate the endogenous expression levels during EB



formation, we isolated RNA from untransduced Rosa26M2K2 EBs through a course of 8 days and measured GATA1 and Pbx1 mRNA contents (Figure S5). GATA1 expression increased from day 2 on and declined after 5 days reaching background levels at day 8. In contrast, Pbx1 was expressed at moderate levels throughout the EB differentiation. In addition to these transcription factors, we investigated the

FIGURE 3 Percentages of (A) CD41-positive and (D) CD42d/CD41 double positive megakaryocytes (MKs) differentiated from R26M2K2 induced pluripotent stem cells (iPSCs) transduced to express Pbx1 or GATA1 compared to nontransduced iPSCs followed over a period of 6 weeks; at each time point, 100 000 MKs were reseeded on OP9 cells and analyzed. (n = 4-12, mean ± SD). (B) Fold expansion of cells of the MK lineage (based on CD41 + by flow cytometry) after replating of 100 000 hematopoietic progenitors from EBs (week 2) or 100 000 cells from the previous cultures (weeks 4 and 6, n = 4-12; mean ± SD). (C) Representative examples of a flow cytometry analysis of GATA1 and Pbx1 transduced cultures compared to untransduced cultures. Two different time points are shown (2 and 6 weeks of differentiation). Unstained sample as gating control. (E) Summarized cytomorphological analysis. Cells on cytospins were stained and distinguished according to their phenotype: large or small MKs, progenitors, macrophages, neutrophils, cells of the erythroid lineage, other cells that could not be assigned to a certain lineage (n = 3-5). (F) Representative cytology of a differentiated cultures of GATA1 transduced R26M2K2 iPSCs after 2 weeks (May-Grünwald staining; MK, megakaryocyte; MØ, macrophage; N, neutrophile; Ery, erythroid cells; P, progenitors)

levels of MEIS1, the cofactor of Pbx1, FLI1, and SCL/TAL1, the latter two factors reported to support human MK differentiation.[13] SCL/TAL1 had its highest expression on day 3, while MEIS1 increased during the last days of EB formation. FLI1 expression was not modulated during the EB differentiation. In our experiments, we induced the factors in hematopoietic precursors after EB dissociation, but

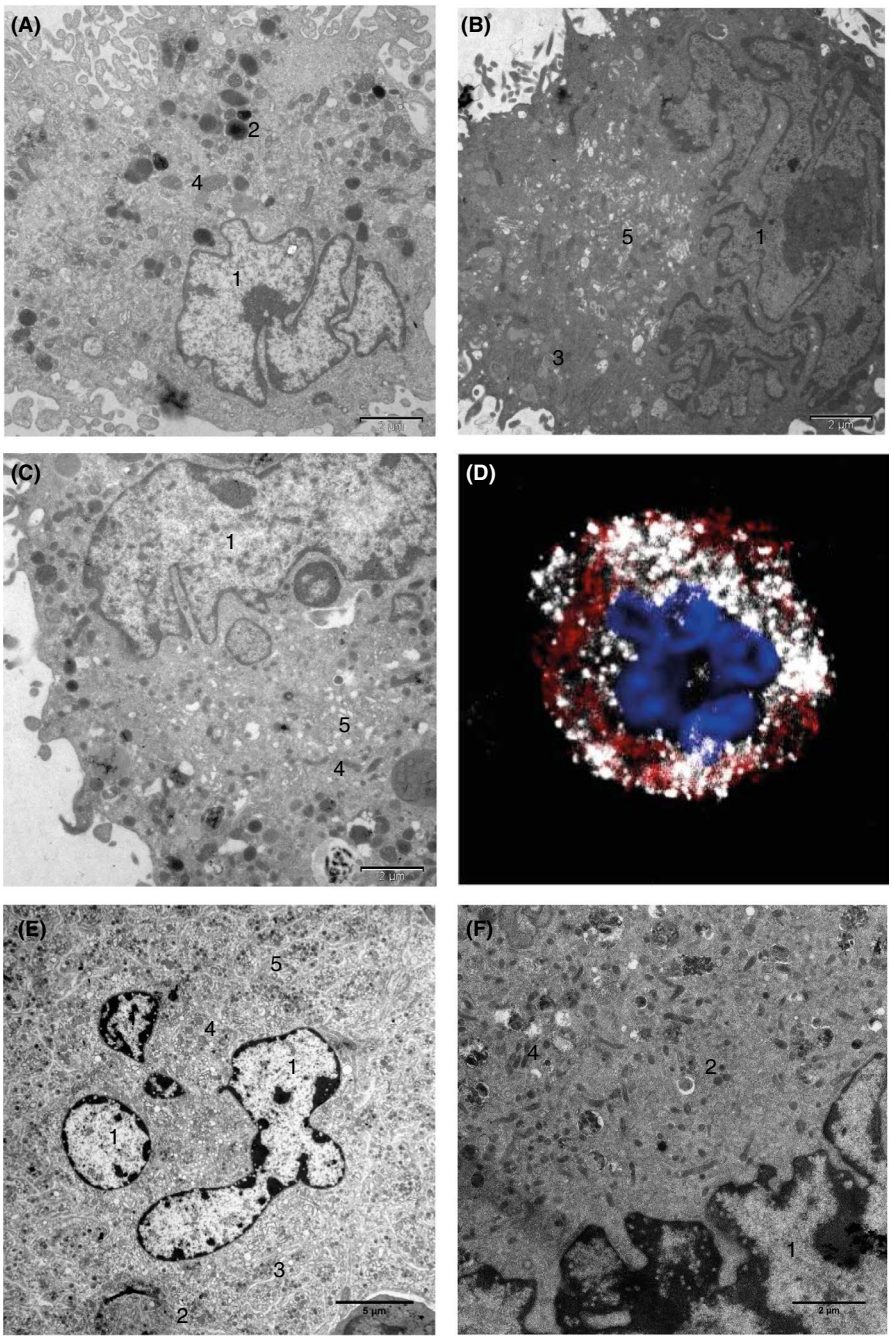


FIGURE 4 Electron microscopic analysis of megakaryocytes (MKs) differentiated from induced pluripotent stem cells (iPSCs). (A-C) (1) nucleus, (2) alpha granule-like, (3) dense granule-like (4) mitochondria, (5) Pre–demarcation membrane system. (D) Laser scanning confocal microscopy of an iPSC-derived in vitro MK detecting the alpha granules by staining of von Willebrand factor (VWF). White, VWF; red, F-actin; blue, DAPI. (E) MK from the murine bone marrow (BM) in vivo. (F) MKs differentiated from murine BM cells in vitro

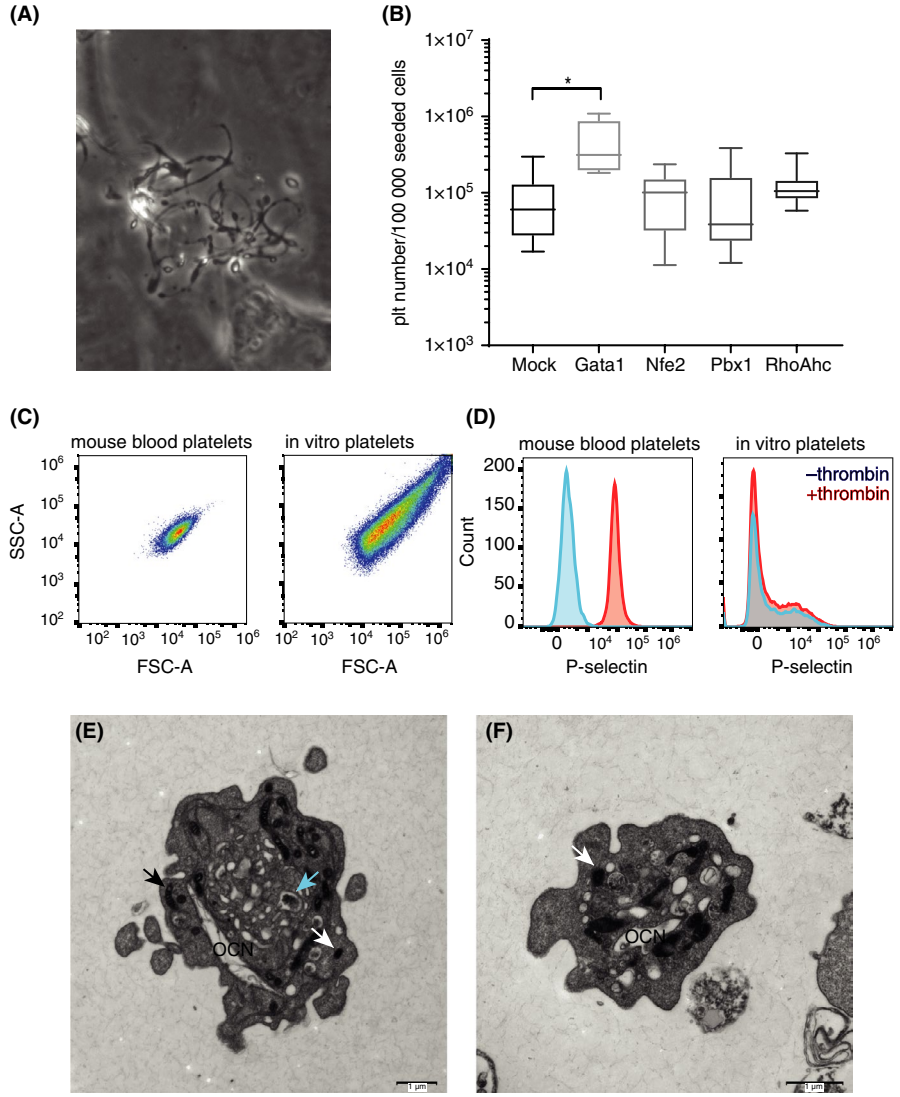


FIGURE 5 Platelets differentiated from R26M2K2 induced pluripotent stem cells (iPSCs) overexpressing the candidate factors. (A) Representative image of cellular elongations of a megakaryocyte (MK) differentiated from murine iPSCs (magnification 400-fold). (B) CD41 + platelet numbers from 10^5 seeded cells (box and whisker blot, median + range, n = 5-9, Kruskal Wallis test). (C) Representative flow cytometer plot showing the size of the in vitro–differentiated platelets compared to platelets derived from murine blood. (D) P-selectin expression on murine blood platelets and in vitro–produced platelets after activation with thrombin. (E, F) Electron microscopic analysis of platelets differentiated from GATA1-overexpressing R26M2K2 iPSCs harvested after 2 weeks of differentiation. Open canalicular network (OCN); black arrow, mitochondria, white arrow, alpha granule; blue arrow, dense granule

not during EB formation. Limited experiment in our hands inducing GATA1 at day 5 of EB formation did not increase hematopoietic differentiation, while induction at the first day of EB formation inhibited EB growth (data not shown).

Analysis of genome integrity by array– comparative genome hybridization (CGH) performed at later stages of our experiments uncovered trisomy 11 in the Rosa26M2K2 iPSCs (data not shown). As the clone was selected based on its good hematopoietic differentiation potential, the trisomy may have contributed to hematopoietic differentiation. However, the course of transcription factor expression during EB formation in our clone was similar to expression profiles reported by others,[37,38] and also not expressed in the pluripotent state at different levels compared to the expression in CD45.1 iPSC control cells (Figure S4).

3.5 | Platelet production from murine in vitro MKs

As a further step in differentiation, MKs can release platelets in vitro. Cellular protrusions and elongations from MKs were detected by light microscopy 2 weeks after coculture, indicative for beginning proplatelet formation (Figure 5A). PLPs were defined by size (relative to murine platelets in circulation) and CD41 expression and could readily be detected in the cell culture supernatant. In agreement with our observation of increased numbers of MKs differentiated from GATA1-overexpressing R26M2 iPSC, also the highest number of PLPs per plated hematopoietic progenitor could be found in these cultures (5.5-fold increase; Figure 5B). In contrast, in Pbx1-transduced cultures, we found no increase in PLP numbers.

PLPs produced from iPSCs were on average slightly larger than mouse blood–derived platelets (Figure 5C). CD42d coexpression within the CD41 + PLP population was between 15% and 25% and did not significantly improve in any of the groups (Figure S6). Moreover, we could detect only low levels of P-selectin expression on in vitro–differentiated platelets, which did not change after induction with thrombin (Figure 5D). In ultrastructural analysis, typical platelet features were identified like the absence of a nucleus, the open canalicular network, mitochondria, and the occurrence of different types of granules, presumably α-granules and δ-granules (Figure 5E, F).

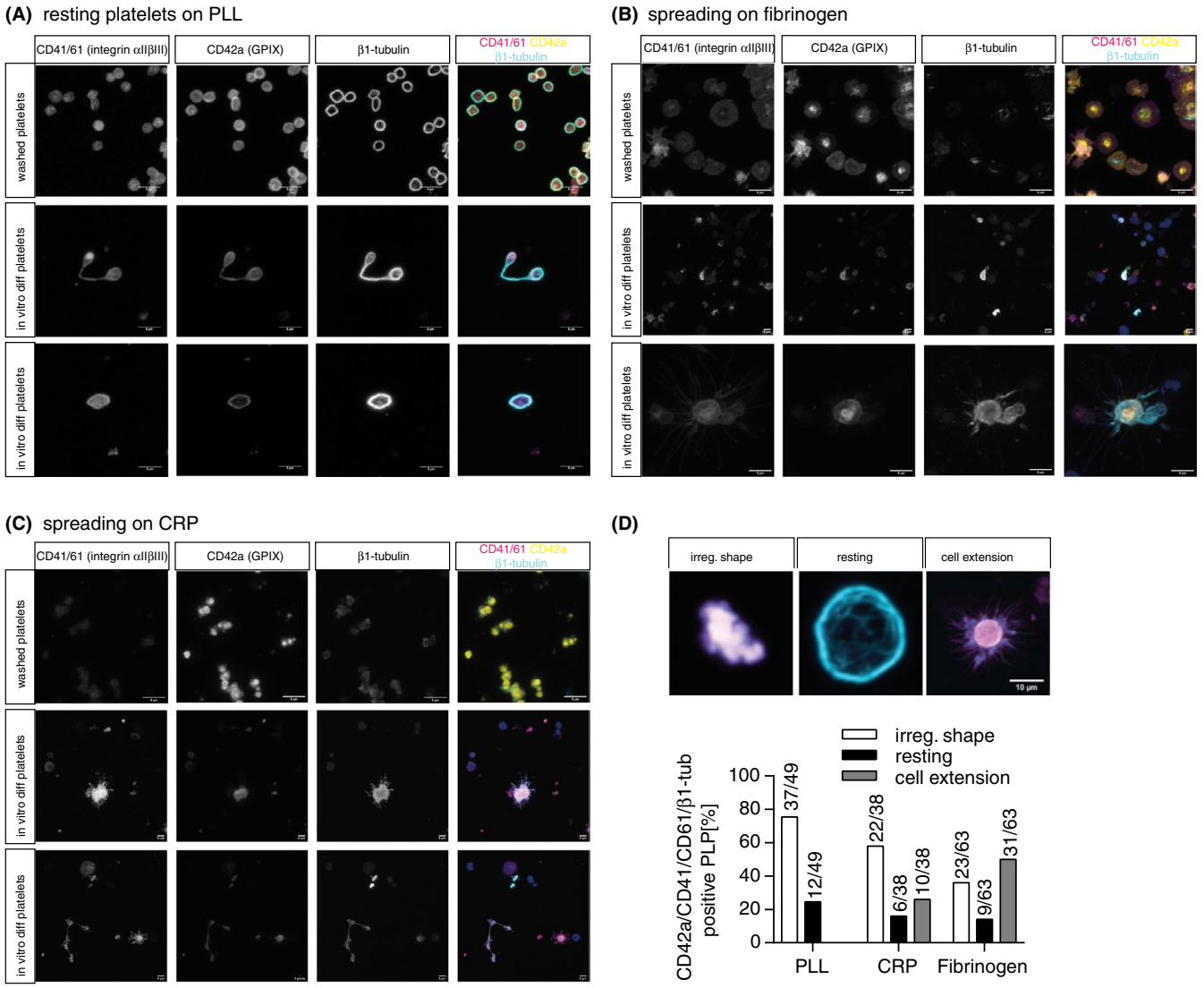


FIGURE 6 Functional analysis of in vitro platelets in platelet spreading assays. Platelets from mouse blood (washed platelets, top rows) or in vitro–differentiated platelets (platelet-like particles [PLPs], second and third rows) were analyzed for their spreading capacity on different matrices and activation. For identification, CD41/61 (red), CD42a (glycoprotein IX, yellow) and β1-tubulin (magenta) were detected by specific antibodies. Residual nucleated cells were labeled by staining with DAPI (blue). (A) Platelets were incubated on poly-L-lysine (PLL)-coated cover slips and remained nonactivated. (B) Platelets were activated with thrombin and spread on fibrinogen for 60 min. (C) Platelets were activated with ADP/U46619 and spread on collagen-related peptide (CRP)-coated cover slips. Scale bar indicates 5 µm. (D) Summary of analyzed CD42a/CD41/CD61/ β1-tubulin positive PLP contribution to the respective phenotypes: resting, cell extensions or irregular shaped. (Data shown from one representative experiment out of two. For the spreading assays, in each experiment platelets were collected and pooled from different independent differentiation cultures)

3.6 | Platelets differentiated from GATA1-overexpressing R26M2K2 can spread on matrices

Finally, we asked whether the in vitro–produced platelets can be activated and were functional. To this end, we isolated PLPs and allowed them to adhere and spread on fibrinogen or CRP-X_L coated surfaces in the presence of thrombin or ADP/thromboxane analog (U46619). Under resting conditions on poly-L-lysine coated surfaces around 24% of PLPs stained positive for CD42a/CD41/CD61/ β1-tubulin and displayed a marginal band, which is a cytoskeletal hallmark of lentiform/discoid platelets in circulation. Most of the PLPs (~76%) exhibit an irregular shape (Figure 6A). However, upon activation with thrombin or ADP/U46619 and seeding on fibrinogen or CRP-X_L, PLPs can form slender cytoplasmic projections (~50% of PLPs on fibrinogen and ~ 26% on CRP-X_L), reminiscent of filopodia from peripheral blood platelets under the same conditions (Figure 6B, C, D). These data indicate that the in vitro–produced PLPs were able to rearrange their cytoskeleton upon external stimulation and were thus functional in this aspect.

4 | DISCUSSION

In our study, we developed a strategy for inducible expression of supporting factors during differentiation of MKs from iPSCs. In

many cases, the pluripotent state cannot tolerate the expression of transcription factors because they would initiate differentiation. Using the Tet-inducible system, genetic modification can be made in iPSCs, but expression then induced at later stages. However, during differentiation from iPSCs, epigenetic mechanisms especially active during transition from pluripotent to committed cells may shut off certain genomic regions. In addition, retroviral vectors integrated into the genome can be actively silenced,[39] a mechanism especially active in iPSCs. With our strategy to generate iPSCs that carry the rtTA-M2 transactivator in the Rosa26 safe harbor locus, rtTA-M2 expression was ensured in all cells of the iPSC line and induction of the Tet promoter could not be hampered by low levels or no transactivator. To accomplish the system, Rosa26M2 iPSCs were then transduced with retroviral vectors carrying the Tet-inducible promoter. In our experiments, we could demonstrate that this system was well capable to regulate expression with no detectable expression in the uninduced state, but expression was effectively switched on after application of doxycycline. Importantly, expression could also be completely switched off at later time points during differentiation after many weeks in the "on state," which can be of great importance for factors that may negatively impair final maturation.

To test our system, we investigated the expression of three transcription factors and one small GTPase of which supportive effects on megakaryopoiesis could be expected. Among them, we identified GATA1 and Pbx1 to increase the percentage of CD41 positive MKs as well as the absolute numbers of CD41 + MKs. GATA-1 and Pbx1 MKs could be reseeded and cultured for several weeks, while this was not possible for MK from untransduced cultures. During the replating, the main MK expansion took place. When calculating the total output of MKs per iPSC, in the best setup we could generate ˜300 MK and 1500 platelets per iPSC. There was, however, no positive effect of GATA1 and Pbx1 on MK maturation or the production of platelets, indicating that these transcription factors supported only the early differentiation steps during megakaryopoiesis. Although positive influences were reported from BM-derived hematopoietic cells [40], Nfe2 did not improve MK differentiation from iPSCs.

MKs and platelets derived from iPSCs resemble more closely embryonic rather than definitive hematopoiesis. Megakaryopoiesis in the mouse embryo initiates early in the extraembryonic yolk sac, starting from a bilineage precursor that gives also rise to erythroid cells [41]. Expression of P-selectin in murine fetal platelets is low to absent [42,43]. In agreement with this, we had difficulties detecting P-selectin on activated iPSC-derived platelets. In contrast, platelet activation was possible in the spreading assay, indicating that the lack of P-selectin presentation after activation with thrombin was not due to the inability of platelets to be activated. Furthermore, in vitro production keeps platelets at 37°C, while it is recommended to store platelets at room temperature. For example, under these conditions, metalloproteinase enzyme activity can lead to shedding of platelet cell surface receptors (CD42b, GPVI).[44] In general, the functionality of in vitro–generated platelets is still under discussion[45,14,46] and needs consideration also in the context of the platelet granule cargo.[47]

The immature phenotype of our in vitro expanded MKs may have contributed to the low platelet output. We also show that the DMS of in vitro MKs was not fully matured, a phenomenon also observed in MK differentiated from murine BM in vitro.[48] It was proposed that the missing environmental mechanical forces may be responsible for insufficient DMS maturity. One can speculate that the immature DMS in our MK contributed to inefficient platelet release. In vitro platelet production under shear force conditions mimicking the in vivo situation may help MK maturation and increase platelet production.[14,49-51] So far, we have not addressed the option of placing our in vitro produced MK progenitors under shear force conditions; however, the expansion of our GATA1 and Pbx1 MK progenitors opens the possibility to transfer them into a bioreactor for large-scale production of murine platelets. The expansion and maturation of MKs and progenitors in bioreactor-based approaches will certainly be necessary to produce large numbers of platelets for transfusion, even in the context of animal models.

Finally, our study presents an ideal system for inducible gene expression during MK differentiation from iPSCs. With this system in hand, it becomes possible to screen more candidate factors in an unbiased approach. By encoding more factors on one vector is will also be feasible to investigate synergies by coexpression of different proteins.

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RELATIONSHIP DISCLOSURE

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

KC, MJ, FS, AM generated the vectors, performed research and analyzed the data. MS, GM, HS, MB performed the platelet spreading assays, analyzed the data, and wrote the manuscript; DS performed the array CGH analysis. KB investigated MK and platelets by electron microscopy. KC, MJ, TM, UM designed the study and wrote the manuscript.

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SUPPORTING INFORMATION

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