Large-scale production of megakaryocytes from human pluripotent stem cells by chemically defined forward programming

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The production of megakaryocytes (MKs)—the precursors of blood platelets—from human pluripotent stem cells (hPSCs) offers exciting clinical opportunities for transfusion medicine. Here we describe an original approach for the large-scale generation of MKs in chemically defined conditions using a forward programming strategy relying on the concurrent exogenous expression of three transcription factors: GATA1, FLI1 and TAL1. The forward programmed MKs proliferate and differentiate in culture for several months with MK purity over 90% reaching up to $2 \times 10^5$ mature MKs per input hPSC. Functional platelets are generated throughout the culture allowing the prospective collection of several transfusion units from as few as 1 million starting hPSCs. The high cell purity and yield achieved by MK forward programming, combined with efficient cryopreservation and good manufacturing practice (GMP)-compatible culture, make this approach eminently suitable to both in vitro production of platelets for transfusion and basic research in MK and platelet biology.

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**Megakaryocytes (MKs)** generate blood platelets whose primary role is to stop haemorrhages via localized clot formation at the site of vessel injury. MKs are polyplid cells derived from haematopoietic stem cells residing in the bone marrow where they represent only 0.01% of the total nucleated blood cells. By extension of cytoplasmic protrusions through bone marrow sinusoids, they release daily $\sim 1 \times 10^{11}$ platelets into the blood stream to sustain the count of short-lived (7–10 days) circulating platelets between 150–450 $\times 10^4$ per litre of blood. A decrease in platelet number, or thrombocytopenia, may occur following bone marrow failure (inherited or acquired, such as post-cancer treatment) or severe peripheral bleeding after trauma or surgery, and potentially leads to life-threatening haemorrhages. Currently, prophylactic and therapeutic treatment essentially relies on transfusion of ABO and RhD-matched platelet concentrates—at $> 2.4 \times 10^{11}$ platelets per unit—from voluntary donations. Recently, the increase in high-dose cancer therapy, advanced surgical procedures and the ageing population has led to a rising demand for platelets with over 4.5 million platelet units transfused per year in Europe and the United States. In addition, platelet transfusion refractoriness in platelet units transfused per year in Europe and the United States has led to a rising demand for platelets with over 4.5 million platelet units transfused per year in Europe and the United States. Moreover, platelets are a controllable source of platelets. Human pluripotent stem cells (hPSCs)—including embryonic stem cells (hESCs) derived from embryos and induced PSCs (hiPSCs) generated from post-natal somatic cells—can be maintained in vitro for prolonged periods while retaining the capacity to differentiate towards virtually any cell type upon adequate stimulation. Therefore, they offer huge opportunities for basic research and clinical applications. The production of platelets in vitro from genetically defined hPSC lines could revolutionize transfusion medicine by providing a controllable source of platelets. Moreover, platelets are anucleate and do not proliferate which means they can be irradiated before transfusion. This provides a marked safety advantage over other hPSC-derived therapeutic cells which can potentially retain oncogenic cell fractions. However, in vitro systems for the production of large amounts of MKs and subsequent platelet release to match the needs for making transfusion units still require considerable optimization.

Our work describes a novel approach for generating large quantities of functional MKs from hPSCs with unique advantages for clinical development. Existing protocols have so far relied on external signals provided by cytokines or stromal cells to mimic embryonic development in vitro and thus direct sequential differentiation of hPSCs into MKs, a process designated as ‘directed differentiation’. While mature MKs showing functional platelet release are produced, this strategy has been limited by the relatively low number of MKs generated or by the complex genetic modifications and clonal selection required to immortalize MKs post differentiation. Urged by the recent discoveries on the plasticity of cell identities controlled by limited sets of transcription factors (TFs), we adopted a radically different approach for the generation of MKs by exploring the potential of exogenous TFs to drive the differentiation process from hPSCs, a strategy called ‘forward programming’ (FOP). Proceeding from a methodically curated list of candidate genes, we discovered that the combination of GATA1–FLII–TAL1 uniquely promoted highly efficient MK-FOP from an array of hPSC lines in chemically defined conditions. Critically, the forward programmed MKs (fopMKs) matured into platelet-producing cells that could be cryopreserved, maintained and amplified in vitro for over 90 days showing an average yield of 200,000 MKs per input hPSC. This unprecedented efficiency combined with minimal cell manipulation and low cytokine requirements makes MK-FOP a promising platform for basic research as well as future clinical applications in the field of transfusion medicine.

**Results**

**GATA1–FLII–TAL1 induces hPSC to MK commitment.** Based on the knowledge that cooperative binding of TFs to target sites can activate gene expression in repressed chromatin, we hypothesized that MK-specific TFs forming complexes able to interact with chromatin remodelers would be able to rewire the hPSC gene regulatory network to induce MK-FOP. We compared TF expression in the H9 hESC line and cord blood-derived megakaryocytes (cbMKs) and narrowed down to a list of 46 TF candidates subsequently ranked based on differential gene expression level and reported protein interactions between candidates and epigenome modifiers using ViAnT (see Supplementary Fig. 1 and Methods for details). Nine TFs from the top 20 candidates were individually cloned into a lentiviral vector backbone under the control of an EF1α promoter to assess their MK-FOP potential (Fig. 1a).

H9 cells were transduced concurrently with equal amounts of each lentivector and subsequently maintained in hPSC medium (FGF2 + Activin-A) for 2 days followed by MK medium (thrombopoietin (TPO) + SCF (stem cell factor)) for a further 5 days. The transduction efficiency measured with a green fluorescent protein (GFP) reporter vector was 60.3 ± 5.6% in these conditions (n = 4). Transduction with nine TFs generated a well-defined population of cells expressing CD41a (integrin alpha-IIb: ITGA2B) on day 7 (1.1 ± 0.6%; Fig. 1b). In an attempt to identify the critical TFs responsible for the generation of this population expressing the marker typically associated with MK lineage commitment, CD41a+ cells were sorted and transgene expression measured by RT-qPCR (quantitative PCR with reverse transcription). Intriguingly, we detected a striking bias for GATA1, FLII and TAL1 (hereafter ‘3-TFs’) transgene expression in the CD41a+ population compared with its negative counterpart which suggested their instrumental role in the acquisition of the CD41a phenotype (Fig. 1b). Accordingly, the 3-TF combination tested in two hiPSC lines generated more CD41a+ cells compared with all nine TFs together demonstrating its superior efficiency (2.5 ± 0.04% versus 0.2 ± 0.04% respectively; Fig. 1c). We assessed all permutations of the 3-TFs and showed that the maximum CD41a+ cell yield was achieved upon concurrent transduction of the 3-TFs which also correlated with a higher clonogenic potential (Fig. 1d; Supplementary Fig. 2a). Importantly, no CD41a+ cells were detected after transduction with a GFP control vector demonstrating that culture conditions were not inductive per se (Fig. 1d). Finally, we combined the 3-TFs using a single lentivector carrying a polycistronic expression cassette encoding for the 3-TFs and a GFP reporter. Virtually all GFP-positive cells differentiated into CD41a+ cells by day 7, confirming the efficacy of the 3-TFs in inducing MK-FOP (Supplementary Fig. 2b).

Additional analyses confirmed that the emerging CD41a+ population at day 7 truly represented early MK lineage commitment. We first showed that the expression of key MK genes including MPL (coding for the TPO receptor), the TFs ZFPM1, RUNXI, NFE2 as well as the endogenous expression of GATA1, FLII and TAL1 were specifically induced in the CD41a+ cell population (Fig. 1e). Moreover, the MK clonogenic potential was exclusively retained in the CD41a+ cell population which further developed to mature MK colonies expressing...
CD42b (glycoprotein-llb: GPIBA) functionally demonstrating MK commitment (Fig. 1f,g). Altogether, we thereby identified GATA1, FLI1 and TAL1 as a minimal and sufficient combination of TFs to induce the formation of MK precursors from hPSCs.

Chemically defined MK-FOP generates high purity MK cultures. To facilitate transfer to the clinic, we developed a chemically defined xeno-free 3-TF MK-FOP protocol. Induction of mesoderm commitment from hPSCs by a 2-day exposure to FGF2, BMP4 and LY-294002 (ref. 25) significantly increased the percentage of CD41a+ cells at day 7 compared with pluripotency maintenance conditions (5.4±1.4% vs. 0.5±0.6%). Next, we assessed the benefit of using forced aggregation embryoid body formation instead of the base two-dimensional (2D) culture. When coupled with mesoderm-inducing conditions, embryoid body culture further improved the mesoderm commitment from hPSCs by a 2-day exposure to FGF2, BMP4 and LY-294002 (ref. 25) significantly increased the percentage of CD41a+ cells at day 7 compared with pluripotency maintenance conditions (5.4±1.6-fold increase; Supplementary Fig. 3a). Next, we assessed the benefit of using forced aggregation embryoid body formation instead of the base two-dimensional (2D) culture. When coupled with mesoderm-inducing conditions, embryoid body culture further improved the
yield of CD41a+ cells at day 7 (5.2 ± 0.9-fold increase; Supplementary Fig. 3b). The embryoid body culture had the additional advantage of providing a standardized quantity of input cells thereby conferring reproducibility. The final protocol combined lentiviral transduction with embryoid body formation over the first 24 h with mesoderm induction for the first 2 days. This protocol allowed consistent transduction efficiencies amongst hiPSC lines (68.2 ± 4.1% using a single GFP control vector, n = 11 using three hiPSC lines), resulting in 22% co-transduction efficiency as estimated using reporter vectors (Supplementary Fig. 3c). As MK maturation was not optimally sustained in the low-adherence embryoid body culture setting, we included a single-cell dissociation step which gave an optimal MK yield when performed at day 10 post transduction (Supplementary Fig. 3d) followed by a further 10 day culture in medium containing TPO and IL1β routinely used for cbMK differentiation. The optimized MK-FOP protocol using xeno-free GMP-grade basal medium and recombiant cytokines is depicted in Fig. 2a.

We observed a gradual increase in CD41a+ cells from day 4 post transduction following by the acquisition of the mature MK marker CD42a (glycoprotein IX,GP9, component of the Von Willebrand platelet receptor complex) from day 6 onwards mimicking cbMK differentiation (Fig. 2b; Supplementary Fig. 3e).

Critically, using two different hiPSC lines, MK-FOP consistently achieved MK lineage purity (>95% CD41a+ cells) by day 15 post transduction with >50% CD42a+ mature MKs by day 20 (Fig. 2b). A robust cell expansion during the single-cell culture step led to the generation of large quantities of MKs with up to 28.4 ± 7.8-fold increase relative to the hiPSC input at day 20 (iPSC#1, n = 14; Fig. 2c). Interestingly, MK-FOP achieved significantly higher cell yields compared with a standard MK-directed differentiation approach8, producing in our hands on average 26.3 and 11.7 times more mature MKs from the hiPSC lines #1 and #2, respectively (Fig. 2c). In addition, the MK purity obtained by MK-FOP was significantly higher than MK-directed differentiation (97.7 ± 0.8% versus 21.3 ± 2.9%, respectively; Supplementary Fig. 3f).

To further assess the MK identity of FOP cells, day 20 fopMKs were compared with day 10 cbMKs as a benchmark. At this stage, both cultures show similar MK maturity (50–60% CD42a+ cells; Fig. 2b; Supplementary Fig. 3e). The key platelet surface receptors for fibrinogen (αIb-β3), Von Willebrand factor (gp Ib-V-IX) and collagen (gpVI) were readily detected in both cultures (Fig. 2d). Morphologically, fopMK cultures displayed a typical mix of megakaryoblasts with large peripheral nuclei and mature MKs with increased cytoplasmic volume and frequent polyploid cells similarly to cbMK cultures (Fig. 2e,f). In accordance with published results for in vitro-derived neonate and hiPSC MKs17,28, the cell ploidy remained low on average and was comparable for both fopMKs and cbMKs (<2% 8N cells; Supplementary Fig. 3g). Analysis of the fopMK ultrastructure by electron microscopy showed lobulated nuclei, developing demarcation membrane system (cytoplasmic cell membrane supply for platelet release), cytoplasmic multi-vesicular bodies (precursors to platelet granules) and mature granules (Fig. 2g).

We confirmed by confocal microscopy that major alpha-granule proteins (P-selectin, thrombospondin, fibrinogen and vWF) were indeed correctly expressed and patterned in fopMKs (Fig. 2h). Importantly, the total expression level of the 3-TFs in fopMKs—as sum of transgenic and endogenous expression—was not significantly different from cbMKs (Supplementary Fig. 3h). Collectively, these data demonstrate the efficient production and maturation of MKs derived by forward programming in xeno-free chemically defined conditions.

MK-FOP produces expandable and cryobankable mature MKs.

We found that fopMKs could be maintained in culture and kept expanding for an extended period of up to 60 days in the TPO + IL1β condition described above (Supplementary Fig. 4a–c). The expression of KIT (the receptor for SCF, an haematopoietic progenitor pleiotropic cytokine) on a fraction of the cells suggested the persistence of a progenitor population in MK-FOP cultures (Supplementary Fig. 4d). This led us to test the continuous supplementation with SCF (50 ng ml⁻¹) instead of IL1β originally used through the second step of culture—in an attempt to improve further long-term maintenance. In addition, we reasoned that the high TPO level (100 ng ml⁻¹) may not be required in the MK-FOP context where differentiation was sustained internally by expression of the 3-TFs, and indeed high TPO may be responsible for precocious exhaustion of MK-FOP cultures by over stimulation of differentiation. Consequently, we tested a lower TPO concentration (20 ng ml⁻¹) in combination with SCF through the second step of MK-FOP (Fig. 3a). In these conditions, we were able to maintain fopMKs in culture with steady proliferation for at least 90 days, achieving close to an average 200,000 MK yield per input hiPSC (1.94 ± 1.59 × 10⁵, n = 7 for hiPSC#1 and #3 cumulatively; Fig. 3b). This was in striking contrast with the maximum 1,300 MK fold increase, earlier loss of CD42a expression and cell viability in long-term culture using the original high TPO and IL1β condition (Supplementary Fig. 4a–c). The MKs harvested from optimized long-term cultures (>30 days, defined thereafter as LT-FOP MKs) maintained a purity of over 90% CD41a+ cells with levels of CD42a expression >60% (Fig. 3c–e) and an increase in late MK-FOP production by forward programming using chemically defined conditions. (a) Schematic representation of the optimized MK-FOP protocol. Viral transduction at day 0 concurrent with embryoid body generation and mesoderm induction for 2 days was followed by a period of culture in an MK induction medium (TPO + SCF) for 8 days. Embryoid bodies showing cystic structures and actively growing cell aggregates were dissociated to single cells at day 10 and further differentiated to mature MKs (TPO + IL1β) until day 20 post transduction. (b) Time course of fopMK differentiation showing MK lineage commitment (%CD41a+ cells) and MK maturation (%CD42a+ cells) from whole culture (mean ± s.e.m. from hiPSC lines #1 and #2; n = 2 (day0–10); n = 7 (day14–22)). Representative flow cytometry dot plots for CD41a and CD42a expression are shown below. (c) The MK fold increase at day 20 relative to the day 0 hiPSC input is shown on a logarithmic scale for the hiPSC lines #1–4 differentiated by forward programming (fopMK: mean ± s.e.m.; n = 14, 7, 3, 5 respectively) or directed differentiation (hiPSCs#1 and #2; ddMK: mean ± s.e.m.; n = 3,2 respectively). **P < 0.01 and *P < 0.05 by two-tail t-test. (d) Representative histograms of the expression of major platelet receptors detected by flow cytometry are shown for day 20 fopMKs (red line) and day 10 cbMKs (blue line) against isotype control (grey shade). (e) The morphology of day 20 fopMKs and day 10 cbMKs was analysed by modified Romanowsky staining on fixed cells. Arrowheads point to multinucleated cells. Scale bars, 25 μm. (f) Cell size distribution from fopMK and cbMK cultures is shown as box plots: centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots (n = 50, 74 respectively; *P < 0.01 by two-tail t-test). (g) Cell ultrastructure of fopMKs and cbMKs was visualized by transmission electron microscopy. dms, demarcation membrane system; gr, granules; mvb, multi-vesicular body; mit, mitochondria. Scale bars, 2 μm. (h) Representative confocal pictures of fopMKs immunostained for major alpha-granule proteins (thrombospondin, fibrinogen, P-selectin and Von Willebrand Factor; iPSC#3 fopMKs, day 40). Scale bars, 25 μm.
maturation markers like GPVI (Supplementary Fig. 4e). The LT-fopMK cultures contained a mixed population of small megakaryoblasts growing in loose clusters representing the actively proliferating cell fraction together with larger polyploid MKs (Fig. 3f–h). Critically, LT-fopMKs were successfully frozen over a day 21–70 timeframe and subsequently recovered for further culture and expansion allowing cryobanking of fopMK batches (Supplementary Fig. 4f).

We found that LT-fopMK cultures were not immortalized but finite with the longest iPSC#1 and iPSC#3 cultures kept for 120 days.
and 132 days, culminating respectively in 17 million and 800,000 MK fold increases (n = 4/5 biological replicate respectively; Supplementary Fig. 4g). Beyond day 90, most LT-fopMK cultures showed a drastic loss of cell viability (Supplementary Fig. 4h) associated with a steady decrease of clonogenic potential and of CD41a+ haematopoietic progenitor content (Supplementary Fig. 4i,j). At the population level, the average expression of the 3-TFs (sum of endogenous and transgenic expression) in cbMKs (Fig. 3i). Together, we showed the 3-TF-driven MK-FOP generated a highly proliferative albeit exhaustible progenitor pool sustaining the expansion of mature MKs in vitro for over 3 months.

**Genome-wide analysis confirms long-term MK identity.** We analysed the whole-genome microarray expression data of MKs derived in vitro by different protocols and from different stem cell sources: by FOP or directed differentiation from hiPSCs (fopMKs, LT-fopMKs and ddMKs) and from cbMKs, all samples with purity greater than 95% CD41a+ and 80% CD42a+ cells.

First, enrichment analysis using hyperG test (false discovery rate (FDR) ≤ 5%) of gene ontology terms from upregulated genes versus undifferentiated hiPSC confirmed that MK-FOP efficiently induced the MK phenotype with top biological processes in fopMKs and LT-fopMKs related to haemostasis/platelet gene ontology terms as was the case for ddMKs and cbMKs (Fig. 4a).
This was mirrored by a depletion in differentiation/morphogenesis gene ontology term-associated genes indicating appropriate down-regulation of pluripotency features (Fig. 4a). Moreover, the gene set enrichment analysis against a panel of blood cells (cbMKs versus other blood cells from Haematlas; n = 4 and 46, respectively) showed specific enrichment of MK-specific genes in the list of genes upregulated in fopMKs further demonstrating the acquisition of a genuine MK phenotype (normalized enrichment score (NES) = 1.25, FDR = 21%; Fig. 4b). The highly similar expression profiles obtained from two distinct hiPSC lines demonstrates the inter-line qualitative reproducibility of MK-FOP (r² = 0.998 for iPSC#1 and #2; Fig. 4c).
Differential expression analysis comparing hiPSC-derived MKs to cbMKs revealed a number of differential expression genes that were (in accordance with the MK phenotype acquired by fopMKs) not related to haemostasis/platelet-related biological processes (503 common, 2,667 total; ≥2-fold-change and FDR ≤5% cutoff; Fig. 4d). Hierarchical clustering distinguished the four MK groups while showing a separate cluster encompassing the hiPSC-derived MKs (i.e., fopMKs, LT-fopMKs and ddMKs) distinct from cbMKs indicative of an intrinsic difference resulting from their hPSC provenance, as previously described. Altogether, the whole-genome expression analysis validated the acquisition by MK-FOP of a genuine MK phenotype that was effectively maintained throughout long-term culture.

**In vitro production of functional platelets by fopMKs.** Mature MKs produce platelets by a process of proplatelet formation whereby MKs extend cytoplasmic protrusions into the bone marrow blood stream. These protrusions contain multiple branching points and bulbous ends with active accumulation of granules into the end processes that represent the nascent platelets, which then mature further in the circulation. In culture,
fopMKS formed protoplasts containing P-selectin-positive α-granules (Fig. 5a,b; Supplementary Movies 1 and 2). For further analyses, in vitro platelet production was maximized using a static co-culture system with the murine C3H10T1/2 feeder cell line as previously described. Electron microscopy showed that platelets produced in vitro from fopMKS and cbMKS, while heterogeneous in quality reflecting the current limitations of 2D static cultures, showed the typical platelet ultrastructure notably including high alpha-granule content (Fig. 5c). We further used flow cytometry for the quantitative measurement of in vitro platelet production from fopMKS, strictly defining platelets as CD41a+/CD42a+ particles of human platelet size (Fig. 5d). A significant increase in platelet production from LT-fopMKS versus day 20 fopMKS was observed matching platelet release from cbMKS (5 ± 0.2 versus 0.8 ± 0.2 platelets per MK at day 90 and day 20, respectively; Fig. 5e). The platelet production rate was similar amongst fopMKS derived from different hiPSC lines (hiPSC#1–4; Supplementary Fig. 5a). In vitro platelets showed surface expression of the main thrombocyte receptors including the GPIIb/IIIa complex (fibrinogen receptor), GPIb and GPIX (Von Willebrand factor receptor), GPIIa and GPV1 (collagen receptors), some of them with a decreased intensity compared with donor platelets which has been previously described and likely originated from the static 37 °C in vitro culture conditions used for production (Supplementary Fig. 5b). The fopMK platelets showed a normal mean volume on a clinical Sysmex blood analyzer (8.6 ± 0.7 fl, n = 2, iPSC#1 and #5; Fig. 5f), and interestingly an increased immature platelet fraction compared with normal circulating blood (11.6 ± 3% versus 4 ± 1%, respectively; Fig. 5g). Eventually, we assessed fopMK platelet survival in vivo in immunodeficient NOD scid gamma (NSG) mice with further splenic macrophage depletion to allow human platelet maintenance in the circulation. The fopMK platelets were readily detected in the circulation for several hours while showing a shorter half-life than primary donor platelets (7.1 ± 0.8 h versus 19.7 ± 2.2 h, respectively; Fig. 5h), a result which was very similar to previously published data (7.5 and 18.3 h, respectively) and probably biased by the limitations of the current static in vitro production systems for the generation of homogenous populations of genuine resting platelets endowed with longer circulation half-life. To further distinguish functional platelets from the heterogeneous in vitro-produced pool, we used Calcein-AM as a marker of platelet viability and membrane integrity. The proportion of Calcein-AM-positive platelets within fopMK- and cbMK-derived platelet harvest was 32.4 ± 0.8% (n = 3; iPSC#1, #5) and 40.1 ± 3.6%, respectively (n = 3) and defined a phenotypically more homogenous CD41a+/CD42a+ platelet population similar to control blood (Supplementary Fig. 5c). Hereafter, in vitro generated platelets were identified using Calcein-AM staining to compare their function with donor-derived platelets.

To fulfil their haemostatic role, platelets must be able to sequentially adhere to damaged vessels (using collagen and other extra-cellular matrix receptors), increase their surface by spreading (by active remodelling of their cytoskeleton), build-up the thrombus by aggregation of other platelets (through fibrinogen binding) and eventually amplify the haemostatic response through degranulation (granule content release to the surface). We first compared event upon contact with fibrinogen presenting typical tubulin cytoskeletal reorganization (Fig. 6b). We then used a flow cytometry approach to quantitatively measure agonist-induced platelet aggregation as a broad marker of activated platelets.

Discussion

We describe here forward programming, a novel approach for the chemically defined large-scale production of MKs from hiPSCs. We drive MK development by the combined ectopic expression of three TFs: GATA1, FLI1 and TAL1. These three TFs have well-documented roles in haematopoiesis, especially in the maintenance of early haematopoietic progenitors, red blood cell and megakaryocyte differentiation. Forced expression of GATA1 or TAL1 alone in haematopoietic progenitors has been shown to bias differentiation towards MK and erythroid fates, while FLI1 cooperates with GATA1 to enable MK maturation. In addition, TAL1 and FLI1 play an earlier role in the specification of the haematopoietic programme during vertebrate embryonic development. Exogenous expression of TAL1 in hESCs has been reported to promote haematopoiesis and megakaryocyte differentiation. More recently, the combinatorial expression of TAL1 with GATA2 was found to induce an hemogenic endothelial phenotype biased towards erythro-megakaryocytic differentiation from hPSCs. Nevertheless, the MK-FOP approach described here involving co-expression of the 3-TFs GATA1, FLI1 and TAL1 is unprecedented in regard to its efficiency in rapidly imposing MK progenitor identity to hPSCs in very stringent MK-specific culture conditions. The high
hierarchical status of the 3-TFs within the MK gene regulatory network, already supported by previously published gene interaction network and ChIP-sequencing data in primary human MKs\cite{26,53} (Supplementary Fig. 2c), is now functionally confirmed in human cells by the efficiency of the MK-FOP approach.

It remains to be demonstrated how closely the 3-TF programming recapitulates normal haematopoietic development from hPSCs. Current data indicate that mesoderm commitment is strongly beneficial to MK-FOP, consistent with the normal ontogeny of blood cells in the embryo. Intriguingly, we observed an early expression of hemogenic endothelium markers (FLK1,
Calcein-AM live platelets spiked into human blood (at 1 mean two-tail)

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investigations are needed to understand the full nature and the

3-TF-driven molecular mechanisms underpinning the generation

of this long-term expanding MK progenitor.

Recently, another approach has been published that allows the expansion of MK progenitors derived from PSCs55. In contrast with MK-FOP, that system is based on the artificially controlled downregulation of GATA1 in haematopoietic progenitors allowing MK progenitor expansion and its restoration to enable subsequent MK maturation. The approach, which was developed with mouse ESCs, has yet to be demonstrated to work in human cells. In addition, the authors did not show efficient platelet production from these MKs in vitro, instead choosing an adoptive transfer of MKs into recipient mice. The injection of nucleated cells derived from human pluripotent stem cells would raise crucial issues of potential tumorigenicity that could constrain future clinical use.

The quantitative functional platelet data collected here proved that platelets derived from fopMKs in vitro are endowed with major platelet functions allowing efficient thrombus formation as shown in previous studies19,20. Although abnormalities in key TFs for the MK lineage can lead to abnormal platelet function43, the preservation of fopMK platelet function is consistent with the fact that the overall expression level of the 3-TFs is controlled throughout the programming progression and similar to cbMKs. The remaining bottleneck for application to transfusion medicine is the optimization of platelet generation in vitro: this presently remains on average 1,000-fold lower than the in vivo platelet yield per MK and is accompanied by issues regarding the purity of the final product where functional platelets represent only a fraction of the platelet size particles in the whole harvest19,20,56. In this study, which focuses on the production of the MKs themselves, we have used a previously published platelet production system based on co-culture with a mouse stromal cell line27,57. Our findings clearly confer an impetus to achieve further progress using newly developed three-dimensional laminar flow systems and bone marrow mimicking scaffolds58–60 by providing ample quantities of functional MKs from hPSCs using a simplified chemically defined protocol amenable to the generation of a clinical product.

In conclusion, our study demonstrates the feasibility of a forward programming approach to generate mature functional MKs from human PSCs that significantly transcends available directed differentiation protocols through a unique combination of key achievements. First, the methodology results in a very high cell yield and MK purity using fully chemically defined xeno-free culture conditions. To put this in the clinical context, the long-term culture expansion allows a cumulative production of 2 × 10¹¹ MKs releasing 1 × 10¹² platelets—the equivalent of 3 transfusion units—starting from only one million hiPSCs.

Figure 6 | Functional assessment of fopMK in vitro platelets. (a) Adhesion to fibroinogen of fopMK and cbMK in vitro platelets upon combined TRAP + ADP stimulation compared to blood platelets using a flow cytometry bead based assay. Percentages of adhesion on BSA or fibroinogen-coated beads are shown (mean ± s.e.m., n = 4, 4, 2 for blood, fopMK and cbMK respectively. **P < 0.01 versus blood by two-tail t-test). (b) Representative pictures from in vitro spreading assay. Washed platelets were sown on fibroinogen-coated slides, incubated for 45 min at 37 °C and immunostained for alpha-tubulin (TUBA) and F-actin (scale bars, 10 μm). (c) Aggregation of fopMK platelets upon agonists stimulation was tested both with and compared with blood platelets using a flow cytometry-based assay: representative dot plots are shown. (d) Percentages of aggregation from Calcein-AM live platelets Calcein-AM in vitro under arterial shear stress. The participation of participation of Calcein-AM live platelets spiked into human blood (at 1 × 10⁷ per ml) is shown per 100 μm² thrombus area. Normal or thrombocytopenic blood (mean ± s.e.m., n = 7; P values by two-tail t-test versus blood indicated). (f) Thrombus formation in vitro under arterial shear stress. The participation of Calcein-AM live platelets spiked into human blood (at 1 × 10⁷ per ml) is shown per 100 μm² thrombus area. Normal or thrombocytopenic blood (> 150 × 10⁹ and < 50 × 10⁹ respectively) was used as recipient. Spiked platelets were sourced from day-8 concentrate unit (blood) or from fopMK platelets (iPSCK#1 and #5; n = 30 analysed thrombi per group; P values by two-tail t-test versus blood indicated). (g) Representative pictures from in vitro thrombus formation assays. Thrombi identified using bright field images are delineated and Calcein-AM platelets fluorescing in green; in vitro platelets Calcein-AM thrombosis is intrinsically dimmer than donor-derived platelets (Supplementary Fig. 5b). Scale bar, 50 μm. (h) Thrombus formation in vivo by laser injury of an arteriole in the cremaster muscle of NSG mice and intravital confocal microscopy. The incorporation of human Calcein-AM-labelled platelets (50 million transfused per mouse) to mouse thrombi is shown per 100 μm² thrombus area at T_max (thrombus maximum size). Mean values ± s.e.m. and P values by two-tail t-test versus donor platelets are shown (n = 16/4/8 thrombi analysed for blood, fopMK#1 and #5 platelets respectively). (i) Representative snapshots of Calcein-AM + human platelets incorporated to mouse thrombus (scale bar 10 μm).
Moreover, the minimal cell handling and cytokine requirements, the cryopreservation of fopMKs enabling cell banking and future stock management and the successful differentiation of an array of hPSC lines with qualitative reproducibility are additional critical strengths of the MK-FOP approach for future manufacturing of platelets for human therapeutic applications. The effectiveness and versatility of MK-FOP opens new avenues for future basic research and functional studies on novel MK and platelet genes as well as disease modelling using hiPSCs.

**Methods**

**Human pluripotent stem cell culture.** The H9 hESC line (WiCell; passages 75–95) and hiPSC lines (iPSC1–5; A1ATD1, BBHXX8, A1ATD1-c, S4-SF3 and FF01, respectively, p30–50) were cultivated as clumps in a chemically defined medium (CDM) containing recombinant human FG2 and Activin-A (15 ng ml⁻¹ each, internal) on feeder-free gelatin or vitronectin coated wells as previously described. All hiPSC lines were obtained from the Cambridge Biomedical Research Centre iPSC Core Facility and have been derived from adult dermal fibroblasts using integrative murine retroviral vectors (iPSCP1–2), cytoplasmic Sendai viral vectors (iPSCP3–4) or episomal vectors (ipSOP5) expressing the human OCT4, SOX2, KLF4 and MYC reprogramming factors.

**Selection of transcription factor candidates.** We performed a differential gene expression analysis focused on DNA binding protein coding genes (PANTHER Classification System) from whole-genome expression data generated using the H9 hESC line (internal data, Illumina HumanWG-6 v3) and human cord blood-derived MKs. The list of 116 MK-specific genes generated was further refined by removal of 21 histone-coding genes and addition of 6 candidates based on previous knowledge of their role in megakaryopoiesis (Supplementary Fig. 1a). Using the VisANT web-based software, the resulting 101 candidate genes were subsequently ranked based on number of (1) internal protein interactions, (2) interaction with epigenetic modifiers (HAC, HDAC, DNMT, list in Supplementary Table 1) and (3) interaction with FTTC, PE and APC-conjugated antibodies (Supplementary Table 1). Background fluorescence was set against matched isotype control antibodies and compensation matrix defined using single-colour-stained cells.

**Recombinant lentiviral vectors.** Transcription factor cloning. The human coding sequences of the nine candidate genes (variants 1 from NCBI Reference Sequence Database) including the S′ Kozak consensus sequence were generated by PCR using chMK cDNA, individually cloned into the pWPT lentiviral vector backbone (Dr Trono, Addgene #12255) downstream of the human EF1-alpha ubiquitous promoter and checked for sequence integrity.

**Virral particle production.** Replication deficient lentiviral vector particles (LVPs) were produced by transient co-transfection of HEK 293T/17 cells (ATCC CRL-11268) with pWPT constructs along with the pSPAX2 and pMD2-G helper plasmids (Addgene #12260, #12259) using TransIT-LT1 transfection reagent (MirusBIO). Crude supernatants containing LVPs were concentrated by PEG-based precipitation (LentiX-concentrator, Clontech) and functional titres determined by qPCR measurement of provirus copy number in genomic DNA of transduced CD16 cells (ATCC CCL-16).

**Human pluripotent stem cell transduction.** hiPSC lines were routinely transduced by 18–24 h single exposure to LVPs using multiplicity of infection of 20 in presence of 10 μg ml⁻¹ Proamine Sulfate (Sigma) in routine culture medium.

**Megakaryocyte forward programming.** Optimized embryoid body based protocol. On transduction day (day 0), sub-confluent (50–80%) hiPSC cultures were dissociated to single cells using TrypLE (Life Technologies) Fig. 2a. Embryoid body formation was initiated with 6–12E cells in IMDM supplemented with 10% fetal bovine serum (Gibco), 1% non-essential amino acids, 1% antibiotic-antimycotic (100 ng ml⁻¹ each) and frozen at 0.5–2E + 6 cells per ml in IMDM 20% fetal bovine serum (Gibco) and 5% DMSO (Sigma).

**Adherent cell protocol.** Small cell clumps were generated from sub-confluent hiPSC cultures using a Collagenase-IV and Dispase-II mix and sonicated human fibronectin coated (50 μg ml⁻¹, Millipore) wells in CDM containing FG2 and Activin-A (15 ng ml⁻¹ each) at an approximate density of 2–5E + 5 cells per 10 cm². Cells were transduced with LVPs the next day and kept for 2 days in FG2 + Activin-A (pluripotency) or FG2 + LY-294002 + BMP4 (ref. 25) (nilosom) depending on experiment settings. For day 2, cells were maintained in CellgroSCGM with TPO and SCF as described above.

**MK-directed differentiation.** hiPSC lines were differentiated as described using batch tested serum andstromal cells from Prof Koji Etoro Laboratory.

**Cord blood-derived megakaryocytes.** Cord blood was obtained after informed consent under a protocol approved by the Cambridge 4 Research Ethics Committee (07/MRE05/44). CD34-positive cells (≥98%) isolated by magnetic cell sorting (Mylynten Biocell) were seeded at 1E + 5 cells per ml in CellgroSCGM containing TPO (100 ng ml⁻¹) and IL-3 (10 ng ml⁻¹) and cultivated for 10 days. We routinely obtained 70–90% CD41a + and 20–60% CD42a + cells by the end of the culture.

**Flow cytometry analysis.** Flow cytometry experiments were performed on a CyAn ADP (Beckman Coulter). Single-cell suspensions were generated using a Collagenase-IV/Dispase-II mix and/or enzyme free dissociation buffer. Cells were stained for 20 min at room temperature (RT) in PBS 0.5%BSA 2 mM EDTA using APC-conjugated antibodies (Supplementary Table 1). Background fluorescence was set against matched isotype control antibodies and compensation matrix defined using single-colour-stained cells. Flow cytometry analysis was performed using FACSVerse (Beckman Coulter) and DAPI (1 μg ml⁻¹) were used to determine viable cell count in samples.

**Cell morphology and phenotype analysis.** Cell morphology analysis was performed on a glass slide using cytometers at 400x for 5 min, methanol fixed and stained with the Romanowsky method.

**Megakaryocyte colony forming assay.** Around 5,000 cells per chamber were used in MethoCult methylcellulose assays (#4230, StemCell Technologies) containing screened fetal bovine serum and supplemented with TPO and SCF (100 and 50 ng ml⁻¹ respectively); Megacult collagen cultures (StemCell Technologies) were dehydrated following manufacturer’s instructions for colony immunostaining. Megakaryocyte colony forming assays. Megakaryocytes were cultivated on human fibrinogen (50 μg ml⁻¹, Millipore) coated glass cover slips for 48 h to foster adhesion and platelet formation. Cells were fixed in 2% formaldehyde, permeabilized with 0.1% Saponin/0.2% Gelatin and incubated 2 h at RT with selected primary antibodies (Supplementary Table 1) then with fluorochrome conjugated secondary antibodies for 45 min atRT, Cell nuclei were stained with DAPI. Images were acquired on a fluorescent microscope Axiovert 40 (Zeiss) or a SP5 confocal microscope for granule imaging (Leica).

**Plaoycy analysis.** Cells were fixed using 4% formaldehyde for 10 min at RT, immunostained for CD41a and CD42a expression and subsequently incubated in PBS 0.1% Tween with DAPI at 1 μg ml⁻¹ for 15 min at RT before flow cytometry analysis.

**Transmission electron microscopy.** Megakaryocytes were fixed in 2% glutaraldehyde/formaldehyde followed by post fixation, rinse embedding and staining as previously described, and eventually analysed on a FEI Tecnai G2 microscope.

**Gene expression analysis by RT-qPCR.** Total RNA was extracted using RNaseasy kit following the manufacturer’s instructions including DNase treatment. CDNA was prepared from 250–500 ng total RNA using Maxima First Strand CDNA Synthesis Kit (random hexamers and oligo(dT)₁₇₃ mix for priming; Fermentas). Two-step qPCR reactions were performed in duplicates using SYBR green chemistry on ABI 7500HT or Mx3000P instruments (Applied Biosystems; Agilent Technologies). Relative gene expression was calculated by the 2⁻ΔCt method using HMBR as housekeeping gene for normalization. qPCR primer pairs (Supplementary Table 2) designed to amplify only cDNA, to detect all known isoforms, and to have no reported off-target matches searching the human NCBI RefSeq database were tested within 80–120% PCR efficiencies with single endogen gene expression while transgene specific primer pairs used a common reverse primer specific to the viral vector RNA.

**Whole-genome expression microarray analysis.** DNA-free total RNA was extracted as above from sorted CD42b⁻ cell fractions using the Easyprep system (Stemcell Technologies; >95% purity, for chMK, dmMK and fopMK samples) or...
unsorted > 80% CD42b+ cells (LT-tol/MK samples) and 500 ng hybridized to Illumina Human HT-12 v4 BeadArrays.

Microarray data analysis. Microarray signal intensities from Genome Studio version 1.9 were variance stabilization transformed and robust spline normalized (RSN). For differential analysis, we removed low signal probes with detection P value > 0.01 in all samples, leaving only informative probes. These procedures were carried out using R package Lumi. For each pairwise differential analysis, we applied a variable selection analysis (SVA) to correct for un-modelled factors that may bring about batch effects. We then tested for differential expression using limma where statistical significance was set to 2-fold change and 5% false discovery rate or Benjamini-Hochberg adjusted P value.

Hierarchical clustering. Hierarchical clustering was performed using R package pvclust. For dissimilarity or distance measure, we used 1-correlation and average as agglomerative method.

Gene ontology enrichment analysis. We performed enrichment analysis using GOstat 2.24.0 over-represented gene ontology terms in the set of differentially expressed genes. We used standard hypergeometric test using only informative probes in the gene set as the universe.

Gene set enrichment analysis. We used geoseq-2.0.13 with datasets for megakaryocytes (n = 4) and other blood cells (n = 46) from the Haematlas study.22 (E–TABM-633; Illumina Human-v2 array).

Principal component analysis. Classification of samples in multiple dimensional factor spaces was applied by calling the function cmdscale.

Gene expression heatmaps. Heatmap builder v1.1 (Dr Ashley lab, Stanford) was used for dataset normalized representations.

In vitro platelet analysis. Co-culture on feeder cells. To promote platelet production, day 10 eDMks and day 20–90 fopMks were further cultivated for 48 h in CellGroSGM plus Heparin (25 U ml−1) without further cytokine addition at 1E+ 5 cells per cm² on gamma-irradiated 3CH10T1/2 feeder cells (Riken Institute; 1E+ 4 cells per cm²).

Platelet flow analysis. Crude supernatant containing the platelets was analysed by flow cytometry after addition of 1.9 volume of acid citrate dextrose (ACD, Sigma) and cell removal by centrifugation 150g at 10 min. Antibodies against human platelet receptors were added directly to the media (1:50 dilution, see hits method relative to the 30 min equilibrium time point66,67). All mice were kept 120 min and 24 h after transfusion using antibodies specific for human and murine platelet receptors.

Platelet size analysis. Platelet size analysis was performed using small numbers of platelets (2E+ 6 per reaction). Duplicate platelet samples were stained with Calcein-AM and anti-CD31 (clone WM59) conjugated with APC or V450 in HEPES buffer supplemented with 20 μM PPAC dhidrochloride (Calbiochem), washed then mixed in the presence or absence of the agonists thrombin receptor-activating peptide (TRAP) and ADP (10 μM each). Percentage aggregation was determined by flow cytometry for Calcein-AM-positive platelets.

Platelet-aggregation flow cytometry assay. Modified from original protocol56,57, it quantifies adhesion of platelets to single 20μm polystyrene beads (Sigma) coated with either BSA or Fibrinogen. 3 × 105 platelets stained with 100nM Calcein-AM were mixed in basal culture media with either 20 μl BSA or 20 μl fibrinogen-coated beads. Platelets (TRAP and ADP as above) incubated for 10s/37°C and subsequently stained with anti-CD41a-APC antibody. DAPI negative. Calcein-AM and CD41a positive single beads bound by platelets are quantified by flow cytometry.

Platelet survival in vivo. Platelet survival in vivo was performed using 129Sv/J mice through the tail vein as single dose of 2E+ 107 platelets. Thrombocytopenic blood was artificially prepared by collecting in 3.2% Citrate. Thrombocytopenic mice were anaesthetized with ketamine/xylazine and bled through the tail vein as single dose of 5E+ 107 platelets. Platelet survival was monitored by flow cytometry from whole-blood samples at 1, 30, 60, 120 min and 24 h after transfusion using antibodies specific for human and murine platelet receptors.

Platelet transfusions. Platelet transfusions were performed in haematology patients: are we using them appropriately? Vox Sang, 103, 284–293 (2012).

Statistical analysis. Results are presented as mean ± standard error of the mean (s.e.m.) with n representing the number of biological replicates unless otherwise stated. Statistical analyses were performed using GraphPad Prism 8 (La Jolla, CA).

References

1. Kaushansky, K. Historical review: megakaryopoiesis and thrombopoiesis. Blood 111, 981–986 (2008).
2. Michelson, A. D. Platelets 3rd Edn. (Academic Press, 2013).
3. Machlus, K. R. & Italiano, Jr, J. E. The incredible journey: from megakaryocyte development to platelet formation. J. Cell Biol. 201, 785–796 (2013).
4. Junt, T. et al. Dynamic visualization of thrombopoiesis within bone marrow. Science 317, 1767–1770 (2007).
5. Escourt, L. J. et al. Platelet transfusions in haematology patients: are we using them appropriately? Vox Sang, 103, 284–293 (2012).
6. Williamson, L. M. & Devine, D. V. Challenges in the management of the blood supply. Lancet 381, 1866–1875 (2013).
7. Stroncek, D. F. & Rebull, P. Platelet transfusions. Lancet 370, 427–438 (2007).
8. Brown, C. J. & Navarrete, C. V. Clinical relevance of the HLA system in blood transfusion. Vox Sang 101, 93–105 (2011).
9. Thomson, J. A. et al. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147 (1998).
10. Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872 (2007).
11. Murry, C. E. & Keller, G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell 152, 661–680 (2008).
12. Wu, S. M. & Hochedlinger, K. Harnessing the potential of induced pluripotent stem cells for regenerative medicine. Nat. Cell Biol. 13, 497–505 (2011).
13. Takayama, N. & Eto, K. Pluripotent stem cells reveal the developmental biology of human megakaryocytes and provide a source of platelets for clinical application. Cell. Mol. Life. Sci. 69, 3419–3428 (2012).
14. Corash, L. & Lin, L. Novel processes for inactivation of leukocytes to prevent transfusion-associated graft-versus-host disease. Bone Marrow Transplant. 33, 1–7 (2004).
15. Gaur, M. et al. Megakaryocytes derived from human embryonic stem cells: a genetically tractable system to study megakaryocytopoiesis and integrin function. J. Thromb. Haemost. 4, 436–442 (2006).
16. Klimchenko, O. et al. A common bipotent progenitor generates the erythroid and megakaryocyte lineages in embryonic stem cell-derived primitive hematopoiesis. Blood 114, 1506–1517 (2009).
17. Takayama, N. et al. Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells. J. Exp. Med. 207, 2817–2830 (2010).
18. Pick, M., Azzola, L., Osborne, E., Stanley, E. G. & Elefanty, A. G. Generation of megakaryocytic progenitors from human embryonic stem cells in a feeder- and serum-free medium. PLoS ONE 8, e55530 (2013).
19. Feng, Q. et al. Scalable generation of universal platelets from human induced pluripotent stem cells. Stem Cell Rep. 3, 817–831 (2014).
20. Nakamura, S. et al. Expandable megakaryocyte cell lines enable clinically applicable generation of platelets from human induced pluripotent stem cells. *Cell Stem Cell* **14**, 529–540 (2014).

21. Cherry, A. R. & Daley, G. Q. Reprogramming cellular identity for regenerative medicine. *Cell* **148**, 1110–1122 (2012).

22. Zaret, K. S. & Carroll, J. S. Pioneer transcription factors: establishing competence for gene expression. *Genes Dev.* **25**, 2227–2241 (2011).

23. Watkins, N. A. et al. A HaMaAtlas: characterizing gene expression in differentiated human blood cells. *Blood* **113**, e1–e9 (2009).

24. Hu, Z. et al. VisNNT-4.0: integrative network platform to connect genes, drugs and therapies. *Nucleic Acids Res.* **41**, W225–W231 (2013).

25. Bernardo, A. S. et al. BRACHYURY and CDX2 mediate BMP-induced differentiation of human and mouse pluripotent stem cells into embryonic and extraembryonic lineages. *Cell Stem Cell* **9**, 144–155 (2011).

26. Tijssen, M. R. et al. Genome-wide analysis of simultaneous GATA1/2, RUNX1, FLI1, and SCF in megakaryocytes identifies hematopoietic regulators. *Dev. Cell* **20**, 597–609 (2011).

27. Takayama, N. et al. Generation of functional platelets from human embryonic stem cell lines in vitro ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors. *Blood* **111**, 5298–5306 (2008).

28. Liu, Z. J. & Sola-Visner, M. Neonatal and adult megakaryopoiesis. *Curr. Opin. Hematol.* **18**, 330–337 (2011).

29. Mootha, V. K. et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat. Genet.* **34**, 267–273 (2003).

30. Blüteau, O. et al. Developmental changes in human megakaryopoiesis. *J. Thromb. Haemost.* **11**, 1730–1741 (2013).

31. Thon, J. N. et al. Cytoskeletal mechanics of proplatelet maturation and platelet release. *J. Cell Biol.* **191**, 861–874 (2010).

32. Gardiner, E. E. & Andrews, R. K. Platelet receptor expression and shedding: glycoprotein Ib-IX-V and glycoprotein VI. *Transfus. Med. Rev.* **28**, 56–60 (2014).

33. Hsu, Z. & Yang, Y. G. Full reconstitution of human platelets in humanized mice after macrophage depletion. *Blood* **120**, 1713–1716 (2012).

34. Hartley, P. S., Savill, J. & Brown, S. B. The death of human platelets during incubation in citrated plasma involves shedding of CD42b and aggregation of dead platelets. *Thromb. Haemost.* **95**, 100–106 (2006).

35. Versteege, H. H., Heemskerk, J. W., Levi, M. & Reitsma, P. H. New fundamentals in hemostasis. *Physiol. Rev.* **93**, 327–358 (2013).

36. Tyynjärvi, N., Wallstedt, M., Sodergren, A. L., Fazaly, L. & Ramstrom, S. Platelet adhesion changes during storage studied with a novel method using flow cytometry and protein-coated beads. *Platelets* **26**, 177–185 (2015).

37. Strauss, T., Sidilk-Muskatel, R. & Kenet, G. Developmental hemostasis: primary hemostasis and evaluation of platelet function in neonates. *Semin. Fetal Neonatal Med.* **16**, 301–304 (2011).

38. De Cuypere, I. M. et al. A novel flow cytometry-based platelet aggregation assay. *Blood* **121**, e70–e80 (2013).

39. Darbousset, R. et al. P2X1 expressed on polymorphonuclear neutrophils and platelets is required for thrombosis in mice. *Blood* **124**, 2557–2585 (2014).

40. Dore, L. C. & Crispino, J. D. Transcription factor networks in erythroid cell and megakaryocyte development. *Blood* **118**, 231–239 (2011).

41. Pimanda, J. et al. Gata2, FLI1, and SCFl form a recursively wired gene-regulatory circuit during early hematopoietic development. *Proc. Natl. Acad. Sci. USA* **104**, 17692–17697 (2007).

42. Fujiwara, Y., Chang, A. N., Williams, A. M. & Orkin, S. H. Functional overlap of GATA-1 and GATA-2 in primitive hematopoietic development. *Blood* **103**, 583–585 (2004).

43. Tijssen, M. R. & Ghevaert, C. Transcription factors in late megakaryopoiesis and related platelet disorders. *J. Thromb. Haemost.* **11**, 593–604 (2013).

44. Helfner, G. C., Clutter, M. R., Nolan, G. P. & Weissman, I. L. Novel hematopoietic progenitor populations revealed by direct assessment of GATA1 protein expression and cMPL signaling events. *Stem Cells* **29**, 1774–1782 (2011).

45. Iwasaki, H. et al. GATA-1 converts lymphoid and myelomonocytic progenitors but blocks the granulopoietic differentiation of myeloid progenitors. *Blood* **118**, 5697–5704 (2011).

46. Vallier, L. & Pedersen, R. Differentiation of human embryonic stem cells in adherent and in chemically defined culture conditions. *Curr. Protoc. Stem Cell Biol.*, Chapter 1, Unit 1D 4 1–4D 7 (2008).

47. Skepper, J. N. Immunocytochemical strategies for electron microscopy: choice or compromise. *J. Microsc.* **199**, 1–36 (2000).

48. Cazenave, J. P. et al. Preparation of washed platelet suspensions from human and rodent blood. *Methods Mol. Biol.* **272**, 13–28 (2004).

49. Nowak, P. J., Aster, R. & Boylan, B. Human platelets circulating in mice: applications for interrogating platelet function and survival, the efficacy of antiplatelet therapeutics, and the molecular basis of platelet immunological disorders. *J. Thromb. Haemost.* **5», 305–309 (2007).

50. Ghevaert, C. et al. Recombinant HPA-1a antibody therapy for treatment of fetomaternal alloimmune thrombocytopenia: proof of principle in human volunteers. *Blood* **122**, 313–320 (2013).

51. de Witt, S. M. et al. Identification of platelet function defects by multi-parameter assessment of thrombus formation. *Nat. Commun.* **5**, 4257 (2014).

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Author contributions
T.M. designed, analysed, performed and interpreted most experiments and wrote the paper. A.L.E. performed and analysed experiments. L.V. and Y.Y. performed and interpreted bioinformatics analyses pertaining to the genomic characterization of the MKs. M.R.T. contributed to megakaryocyte characterization and wrote the paper. M.W.T. performed and interpreted bioinformatics analyses pertaining to the forward programming concept. M.C. and M.A. contributed to megakaryocyte characterization. D.H., W.H.W., C.M.H., A.D., R.L., G.B. and D.C.P. performed experiments. H.P., T.P. and A.B. performed intravital microscopy experiments. N.S. interpreted bioinformatics analyses pertaining to the genomic characterization of the MKs. W.H.O. contributed platelet expert input to the forward programming concept. R.A.P. conceived the forward programming approach, designed, analysed and interpreted experiments and wrote the paper. C.G. drove the platelet biology, designed, analysed and interpreted experiments and wrote the paper.

Additional information
Accession codes: Gene expression microarray data are available from the GEO repository under the accession number GSE54822.

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Corrigendum: Large-scale production of megakaryocytes from human pluripotent stem cells by chemically defined forward programming

Thomas Moreau, Amanda L. Evans, Louella Vasquez, Marloes R. Tijssen, Ying Yan, Matthew W. Trotter, Daniel Howard, Maria Colzani, Meera Arumugam, Wing Han Wu, Amanda Dalby, Riina Lampela, Guenaelle Bouet, Catherine M. Hobbs, Dean C. Pask, Holly Payne, Tatyana Ponomaryov, Alexander Brill, Nicole Soranzo, Willem H. Ouweland, Roger A. Pedersen & Cedric Ghevaert

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This Article contains an error in the author contributions section that has resulted in incorrect credit for preparation and critical analysis of the manuscript. The correct author contributions section is as follows:

"T.M. designed, analysed, performed and interpreted most experiments and wrote the paper. A.L.E. performed and analysed experiments. L.V. and Y.Y. performed and interpreted bioinformatics analyses pertaining to the genomic characterization of the MKs. M.R.T. contributed to megakaryocyte characterization and wrote the paper. M.W.T. performed and interpreted bioinformatics analyses pertaining to the forward programming concept. M.C. and M.A. contributed to megakaryocyte characterization. D.H., W.H.W., C.M.H., A.D., R.L., G.B. and D.C.P. performed experiments. H.P., T.P. and A.B. designed and performed intravital experiments and analysed data, A.B. also participated in preparation and critical analysis of the manuscript. N.S. interpreted bioinformatics analyses pertaining to the genomic characterization of the MKs. W.H.O. contributed platelet expert input to the forward programming concept. R.A.P. conceived the forward programming approach, designed, analysed and interpreted experiments and wrote the paper. C.G. drove the platelet biology, designed, analysed and interpreted experiments and wrote the paper."