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Review

Mass production of iPSC-derived platelets toward the clinical application

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ABSTRACT

The *ex vivo* production of platelets from induced pluripotent cells (iPSCs) may offer a safer and sustainable alternative for transfusions and drug delivery systems (DDS). However, the mass production of the clinically required number of iPSC-derived platelets (iPSC-PLTs) is challenging. Here, we introduce recent technologies for mass production and the first-in-human clinical trial using *ex vivo* iPSC-PLTs. To this end, we established immortalized megakaryocyte progenitor cell lines (imMKCLs) as an expandable master cell bank (MCB) through the overexpression of c-MYC, BMI1 and BCL-XL, which modulated megakaryopoiesis and thrombopoiesis. We also optimized a culture cocktail for maturation of the imMKCLs by mixing an aryl hydrocarbon receptor (AhR) antagonist, SR1/GNF-316; a Rho-associated protein kinase (ROCK) inhibitor, Y-27632/Y-39983; and a small-molecule compound replacing recombinant thrombopoietin (TPO), TA-316. Finally, we discovered the importance of turbulence on the manufacturing of intact iPSC-PLTs, allowing us to develop a turbulence-based bioreactor, VerMES. Combination of the MCB and VerMES enabled us to produce more than 100 billion iPSC-PLTs, leading to the first-in-human clinical trial. Despite these advancements, many challenges remain before expanding the clinical implementation of this strategy.

Contents

Abbreviations: PLT, platelet; MK, megakaryocyte; allo-PTR, alloimmune platelet transfusion refractoriness; AhR, aryl hydrocarbon receptor; BM, bone marrow; B2M, beta2-mirocglobulin; CCI, corrected count increment; DMS, demarcation membrane system; GMP, good manufacturing practice; HPC, hematopoietic progenitor cell; HSC, hematopoietic stem cell; HLA-I, human leukocyte antigen class I; HPA, human platelet antigen; IGFBP2, insulin growth factor binding protein 2; imMKCLs, immortalized megakaryocyte progenitor cell lines; iPSCs, induced pluripotent stem cells; iPSC-PLTs, induced pluripotent stem cell-derived platelets; KIR, killer immunoglobulin-like receptor; MCB, master cell bank; MSC, mesenchymal stromal cell; MIF, macrophage migration inhibitory factor; MLC2, myosin light chain 2; MBS, myosin-binding subunit; NRDC, nardilysin; NK, natural killer; PIV, particle image velocimetry; PBMC, peripheral blood mononuclear cell; PMDA, Pharmaceuticals and Medical Devices Agency; rhTPO, recombinant human TPO; ROCK, Rho-associated protein kinase; STR, short tandem repeat; SCF, stem cell factor; TPO, thrombopoietin.

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1. Background

The first successful human-to-human blood transfusion was carried out in 1818 by the British obstetrician James Blundell. Following the discovery of the ABO blood group in 1901 [1] and the Rh blood group in 1940–1941 [2,3], blood banks and transfusion services were established worldwide. To date, blood transfusions and blood-derivatives save millions of lives per year. However, these methods depend completely on a consistent supply of donated blood, which is globally threatened by falling birthrates and ageing societies. The fragility of this dependence was laid bare by blood shortages experienced upon the stay-at-home orders in response to the COVID-19 pandemic. Moreover, concerns persist regarding the risk of emerging infectious agents and alloimmunization. Therefore, cell-cultured blood products may offer a safer and more sustainable alternative to donated blood in the future [4].

PLT products are extremely vulnerable to shortages, having a shelf life of only 4 days in Japan and 7 days in the US, and need to be stored in plasma at room temperature (20-24 °C) with gentle agitation to maintain their hemostatic capacity [5,6]. 2–3 x 10^{11} PLTs are commonly transfused to prevent or treat bleeding complications in thrombocytopenia [7,8]. However, 5 %–15 % of these patients are complicated with alloimmune PLT transfusion refractoriness (allo-PTR) caused by alloantibodies against human leukocyte antigen class I (HLA-I) or human PLT antigens (HPAs), which presumably manifested by random PLT transfusions or pregnancies [9]. In these cases, compatible donor PLTs are required, even for patients with rare HLA-I or HPA types.

The first-in-human clinical trial for *ex vivo* induced pluripotent stem cell-derived PLT (iPSC-PLTs), the iPLAT1 study (phase 1), was conducted from 2019 to 2021 as an autologous transfusion to a 55-year-old Japanese woman diagnosed with severe aplastic anemia and following allo-PTR due to alloimmunization to HPA-1a [10,11]. The required HPA-1b/1b PLT phenotype is exhibited by less than 0.002 % of the Japanese population [12], and no registered donor was found in the Japanese Red Cross repository. Autologous iPSC-PLTs were decided as the complete solution for her life-threatening scarce supply. The study was conducted in accordance with Japanese regulations of regenerative medicine, and the autologous iPSC-PLT transfusion products were prepared based on good manufacturing practice (GMP) with extensive preclinical assessment.

However, the *in vitro* production of PLTs suffers from low yields [13,14]. Although our group reached the clinical-scale production of more than 2–3 x 10^{11} of iPSC-PLTs, the yield of iPSC-PLTs per megakaryocyte (MK) was low compared with the native process that can generate 800–2000 PLTs per MK *in vivo* estimated from endogenous PLT production in mice [15].

In this review, we introduce technologies for the mass production of clinically relevant iPSC-PLTs used in the iPLAT study and remaining issues found in the clinical trial that need solving before iPSC-PLTs are clinically practical.

2. Establishment of immortalized megakaryocyte progenitor cell lines (imMKCLs)

Even though iPSCs may represent a potent source for *ex vivo* PLT biogenesis, the extensive expansion and differentiation into mature MKs to produce 10^{11}-scale PLTs involve laborious, time-consuming, and costly processes and are nearly impossible to achieve with consistent quality. To overcome these problems, imMKCLs were established as a master cell bank (MCB) that displays long-term expansion capability in liquid culture even after cryopreservation [16].

imMKCLs were generated through the sequential overexpression of c-MYC and BMI1 and of BCL-XL in $CD34^+$ multipotent hematopoietic progenitor cells (HPCs) and differentiated megakaryocyte progenitor cells, respectively, under the control of doxycycline. Although c-MYC overexpression drives cell cycle for proliferation, its cellular manipulation simultaneously promotes senescence by activating INK4A/ARF and apoptosis through the caspase 3/7 pathways [16]. BMI1 is a polycomb group (PcG) protein that suppresses the INK4/ARF locus encoding the $p16^{INK4A}$ and $p14^{ARF}$ proteins – key regulators of cellular senescence [17]. BCL-XL is a critical anti-apoptotic protein for the sustained maturation of MKs [18]. The overexpression of c-MYC and BMI1 only leads to a limited exponential proliferation of MK progenitors, presumably reflecting the increased caspase activities at the later stage of proliferation. The overexpression of BCL-XL (14–21 days later from the HPC stage) successfully enables the exponential proliferation of MK progenitors for more than 5 months by suppressing caspase activities, thus enabling the establishment of imMKCLs [16].

In the presence of doxycycline, imMKCLs expand through self-renewal by the overexpression of c-MYC, BMI1 and BCL-XL. Upon removal of doxycycline, imMKCLs cease to express these transgenes, causing the cells to mature with large polyploidization and yield iPSC-PLTs in 4–6 days (Fig. 1). Because the quick down-regulation of c-MYC in MKs is required for maturation and PLT production [19], the doxycycline-controlled expression system perfectly regulates the self-renewal stage and the maturation and PLT production stage of imMKCLs by modulating c-MYC expression levels.

For the iPLAT study, autologous iPSCs were generated from peripheral blood mononuclear cells (PBMCs) by electroporating episomal reprogramming vectors, then cultured on mitomycin C-treated SNL feeder cells, and finally cryopreserved. The cryopreserved iPSCs were thawed and verified for their ability to differentiate into MKs. Then, a selected iPSC clone was differentiated into HPCs by the revised "PSC-sac method." [20,21]. From the HPCs, several patient-derived imMKCL clones were established, and the best clone was selected after examinations of proliferation and PLT production. Finally, the selected clone was expanded and cryopreserved as an MCB for the GMP production. To confirm compatibility with GMP, imMKCLs were extensively tested for sterility and potential pathogens and confirmed to have DNA

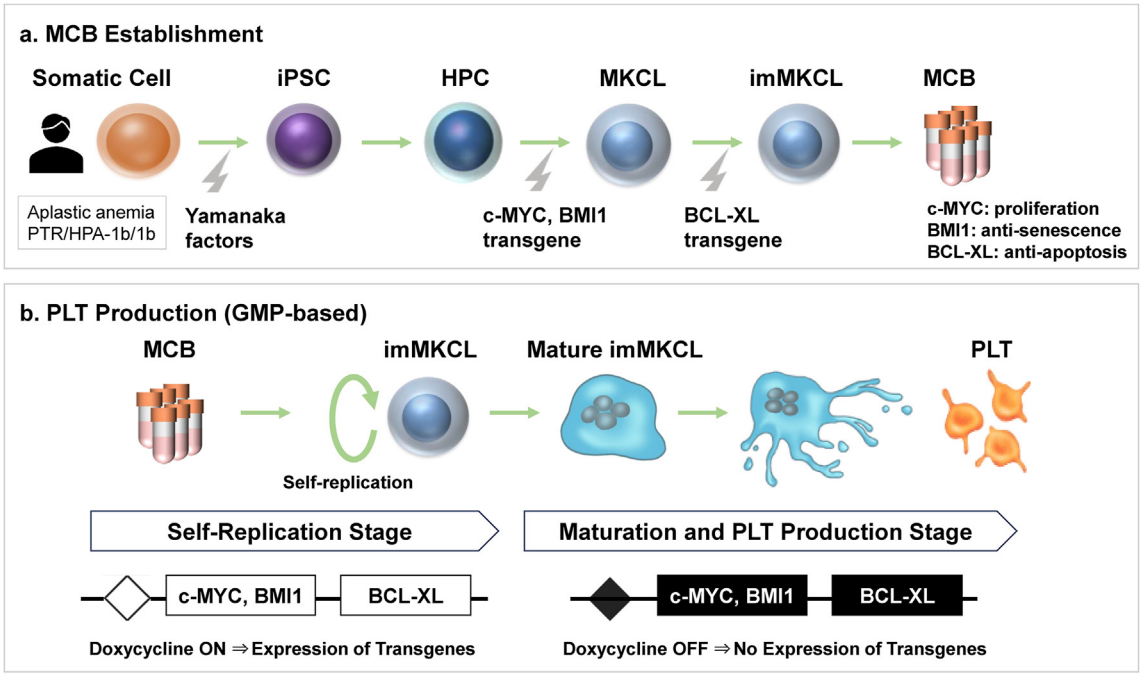

Fig. 1. Schematic overview of PLT production from the imMKCL MCB. (a) An MCB was established from iPSCs derived from patient somatic cells by introducing c-MYC and BMI1 at the HPC stage and BCL-XL at the early MK stage. (b) The addition of doxycycline induces the expression of the transgenes, forcing imMKCLs to self-replicate and expand (self-replication stage). The removal of doxycycline ceases the expression of the transgenes, allowing imMKCLs to mature and produce PLT-like particles (maturation and PLT production stage).

identical to the patient's PBMCs based on short tandem repeat (STR) analysis and HPA genotyping.

3. Clinical-scale production of PLTs from imMKCLs

3.1. Small-molecule thrombopoietin (TPO) alternative

TPO is a glycosylated cytokine primarily produced in the liver. TPO signaling through its receptor, c-MPL, is critical for hematopoietic stem cell (HSC) maintenance, MK lineage differentiation and PLT production as well as imMKCL expansion. Several non-peptidyl small-molecule c-MPL agonists have been developed, such as eltrombopag, an orally administered drug used to treat immune-thrombocytopenia and aplastic anemia. The advantages of these small-molecules over recombinant human TPO (rhTPO) include greater stability, less immunogenicity and lower manufacturing costs.

Among these TPO receptor agonists, TA-316 was selected. Notably, TA-316 promotes more megakaryopoiesis and thrombopoiesis *ex vivo* than rhTPO or eltrombopag and has a higher affinity for c-MPL [22]. It facilitates a 1.5-fold increase of imMKCL expansion during the self-renewal stage and more than doubles the relative number of iPSC-PLTs produced per single imMKCL cell in the PLT production stage compared with rhTPO [22].

3.2. MK maturation enhancers – AhR antagonist and ROCK inhibitor

One of the challenges of *ex vivo* PLT production is the low percentage of thrombopoiesis. The bone marrow (BM) microenvironment, which is constituted of cellular interactions, cytokines and extracellular matrix (ECM), is primarily maintained by mesenchymal stromal cells (MSCs). In terms of PLT production, coculturing $CD34^{+}$ cells with BM-derived MSCs was reported to significantly increase the percentage of proplatelet-producing MKs, and the number of PLTs increased from 10 to 28 PLTs per MK compared with other culture conditions [23]. This increase was caused by MSC-derived suppression of the Aryl hydrocarbon receptor (AhR) signaling pathway during MK maturation, especially the downstream effector CYP1B1 gene, which is a member of the cytochrome P450 superfamily [23]. A small-molecule AhR antagonist recapitulated this MSC effect, and a high-affinity AhR antagonist, such as SR1 or GNF-316, was found to promote MK maturation more than the MSC coculture by its stronger suppression of AhR signaling [23].

Another key signaling pathway involving MK maturation is the Rho/Rho-associated protein kinase (ROCK) pathway. ROCK is a key Rho effector that phosphorylates myosin light chain 2 (MLC2) at Ser19 and the myosin-binding subunit (MBS) of myosin phosphatase, thereby inhibiting its phosphatase activity [24–27]. MLC2 phosphorylation is necessary for actin/myosin contractile forces in a variety of cellular processes such as cytokinesis and membrane blebbing [26–28]. Inhibition of MLC2 phosphorylation leads to a significant increase in PLT release but with increased PLT size, presumably due to the lower contractile forces [29]. During endomitosis, ROCK is inactivated to prevent cytokinesis; otherwise, RhoA-activated ROCK promotes formation of the contractile ring and cell division [30]. ROCK inhibitors, such as Y-27632/Y-39983, accelerate MK polyploidization and promote the demarcation membrane system (DMS) and proplatelet formation by driving transcriptional changes [30,31].

3.3. Feeder-independent imMKCL culture system

For scalable *ex vivo* PLT production, the most straightforward culture system is a feeder-free suspension culture. The original imMKCL culture required adhesion condition for PLT production [16]. To overcome this problem, a chemical screening revealed that the AhR inhibitor in combination with Y-27632/Y-39983 effectively promotes PLT production under feeder-free conditions [32]. Although the detailed mechanism needs to be investigated, because

the number of functional PLTs increased almost 20-fold when both compounds were added compared to either compound alone, synergistic effects between the two compounds are likely involved.

The discovery of the two compounds enabled a feeder-independent imMKCL culture system, in which imMKCLs in the maturation and PLT production stage are cultured with SR1/GNF-316 and Y-27632/Y-39983 in addition to TPO/TA-316 and stem cell factor (SCF). This system allows for the mass production of iPSC-PLTs in a scalable manner in liquid tanks. Accordingly, the following bioreactor further enabled 10^{11}-order manufacturing.

3.4. Turbulence-based bioreactor, VerMES

The physical conditions of MKs play a crucial role in PLT production *in vivo*. Through the visualization and analysis of PLT release in mouse BM *in vivo* by two-photon microscopy and particle image velocimetry (PIV), it was observed that proplatelets of MKs actively releasing PLTs were exposed to high turbulence at vascular branching points, whereas resting MKs without PLT releases resided in laminar flow without turbulence [32]. Mimicking blood flow *in vitro*, it was confirmed that laminar flow with only shear stress and vorticity does not induce efficient thrombopoiesis, but turbulent flow with optimal levels of shear stress and turbulent energy dramatically improves the yield and quality of PLTs [32]. This discovery is consistent with both the lung and BM being sites of PLT biogenesis [15], since the lung has dense vascular branching with complex turbulent flow produced through respiration.

Optimized turbulent flow promotes the migration of nardilysin (NRDC) into the plasma membrane upon MK maturation. NRDC specifically binds to β1-tubulins in α4A/β1-tubulin dimers to be involved in transmembrane shedding for a higher PLT yield [32]. Tubulin also interacts with HDAC6 to regulate tubulin or cortactin remodeling through deacetylation. Turbulence promotes the release of insulin growth factor binding protein 2 (IGFBP2) and macrophage migration inhibitory factor (MIF) from imMKCLs to promote the production of several ECM proteins, including vitronectin, fibrinogen, VCAM-1, and von Willebrand factor, for higher PLT yield [32].

Based on our *in vivo* observation of turbulence, we applied mathematical simulations to a vertical reciprocal motion type liquid culture bioreactor to examine how turbulent flow facilitates PLT production. The optimal bioreactor, VerMES, has two oval-shaped mixing impellers reciprocally moving up-and-down at adjustable speeds generating turbulence (Fig. 2). The number of functional $CD41^{+}CD42b^{+}$ PLTs generated from imMKCLs in VerMES is ~100 PLTs per MK, which is ~4-fold higher than the flask-shaking condition and capable of clinical-scale (10^{11}-scale) production.

3.5. Production of iPSC-PLTs for transfusion

A cryopreserved MCB vial was thawed and cultured with TA-316, SCF and doxycycline. imMKCLs were expanded to 2–4 x 10^{10} in 23 days by moving the cells from culture plates to rotating flasks and finally to 20-L WAVE bags. The cells were transferred to 4 vessels of 8-L scale VerMES containing a medium of TA-316 and SCF, GNF-316 and Y-39983 for feeder-free culture and with KP-457 (ADAM17 inhibitor) to prevent GPIbα (CD42b) shedding and retain PLT adhesion capability (Fig. 3a). After a 6-day culture with continuous turbulent flow, iPSC-PLTs were purified, washed, concentrated and packed at approximately 1×10^{11} in 200 mL bicarbonate Ringer's solution [33] with 10 % ACD-A and 2.5 % human serum albumin. Then the iPSC-PLTs were irradiated with 25 Gy γ-rays as in the standard procedure for blood products to eliminate the potential tumorigenicity of any residual imMKCLs (Fig. 3b).

4. Nonclinical evaluation

iPSC-PLTs are functionally comparable to human donor PLTs even after 5 days of storage in terms of adhesion, aggregation, activation, circulation, and hemostasis according to several *in vitro* assays and *in vivo* models. Stimulation with ADP and TRAP-6 caused comparable P-selectin expression, a marker of α granule release, and PAC-1 binding, a marker for GPIIb/IIIa (CD41a/CD61) activation, which is required for PLT aggregation [34]. Additionally, ADP and collagen induced comparable PLT aggregation according to the light transmission method. Meanwhile, in PLT circulation study, rabbit

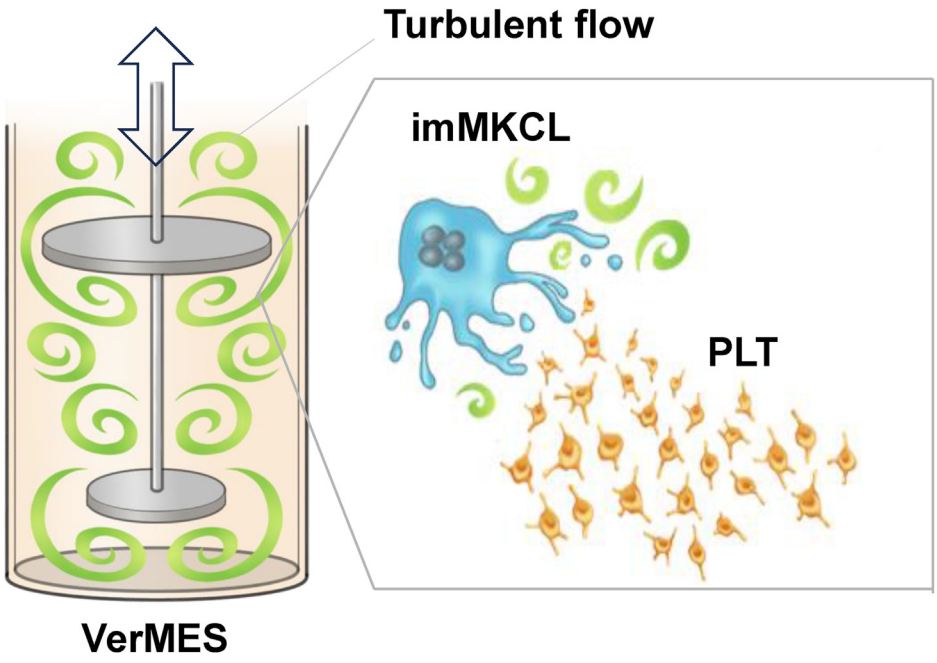

Fig. 2. Turbulence-based bioreactor, VerMES. Vertical reciprocal motion by the mixing impellers with optimized speed produces turbulent flow with optimal shear stress, which improves the yield and quality of the PLTs.

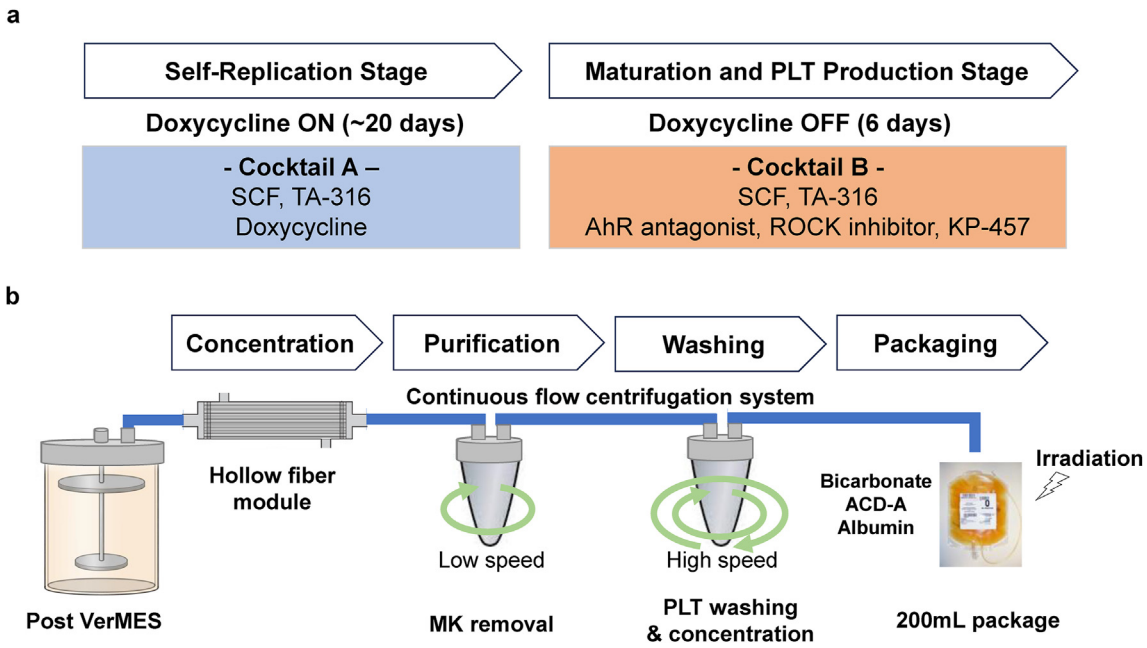

Fig. 3. (a) imMKCL media is supplemented with cocktail A (SCF, TA-316, and doxycycline) during the self-replication stage and cocktail B (SCF, TA-316, AhR antagonist, ROCK inhibitor, KP-457) during the maturation and PLT production stage. (b) Schema of the iPSC-PLT processing. The post VerMES media is concentrated from 8 L to 1–2 L through a hollow fiber filtration module. Then, remaining imMKCLs are removed by continuous centrifugation. Washed iPSC-PLTs are resuspended in PLT storage solution (bicarbonate Ringer's solution with 5 % ACD-A solution plus 2.5 % human serum albumin) and irradiated with 25 Gy γ-rays.

models showed an initial dip in the iPSC-PLT count immediately after the transfusion followed by a slight increase over 6 h and a decrease from 8 to 24 h [11]. An IVIS analysis in mice of the patient imMKCLs showed a substantial accumulation of iPSC-PLTs in the lungs, which may explain the initial dip in the rabbit models [10,11]. In addition, circulating laboratory clone iPSC-PLTs in mice showed a gradual decrease in their size over time [32], suggesting the iPSC-PLTs fragmented to an optimal size during circulation *in vivo,* which may gradually increase the circulation number.

The autologous iPSC-PLTs for the HPA-1b/1b patient were confirmed to be negative for HPA-1a antigen. The ultrastructures of the iPSC-PLTs resembled human donor PLTs but were larger, with diameters of ~3.5–4 μm compared with ~2.5 μm for donor PLTs. Furthermore, the surface expressions of CD41a, CD42b, CD61 and HLA-A/B/C were comparable to that of human donor PLTs, but those of CD36 and CD49b were lower. Also, ABO antigens were negative for the iPSC-PLTs even though the patient had type A blood. Since the expression level of ABO antigens in PLTs varies between individuals [35], this phenotype must be further investigated.

The safety of iPSC-PLTs was extensively evaluated through a preclinical assessment, from raw materials through manufacturing to packaging, following consultation with the Japanese Pharmaceuticals and Medical Devices Agency (PMDA). No general toxicity was observed in single-dose tests on NOG mice and repeated-dose tests on rats. *In vitro* proliferation tests confirmed no tumorigenicity after irradiation with 25 Gy γ-rays. Genetic toxicity tests confirmed no mutagenic potential. Drug residues were below the limit stated in the ICH's M7 guideline.

5. Clinical trial

The autologous iPSC-PLTs were transfused to the patient with three sequentially escalating doses of 1×10^{10}, 3×10^{10}, and maximally 1×10^{11}, as compared to the standard dose of $2–3 \times 10^{11}$. There were no observed clinical symptoms or signs of adverse events from the patient after each transfusion and during the 1-year observation period after cohort 3. Although slight increases in the coagulation marker D-dimer and white blood cell count were observed after 24 h in cohort 3, the levels spontaneously returned to normal and showed no apparent signs of pulmonary embolism or deep vein thrombosis.

On the other hand, the corrected count increment (CCI) at 1 h and 24 h were measured as ~0, meaning no increase in PLT count after the transfusion, which disagrees with the preclinical *in vivo* circulation test in rabbit models [36]. Because a few large PLTs were detected and gradually decreased over time according to a flow cytometry analysis (3.51 % after 1 h and 1.75 % after 14 days), the lack of CCI increase may be partly due to the resolution of the common blood count device, which was not calibrated for large PLTs. However, the primary cause may lie elsewhere. One hypothesis is that PLT accumulated in the liver, where they were cleared by Kupffer cells due to defective glycosylation or sialylation of PLT glycoproteins [37–39]. Because iPSC-PLTs are shed from iPSC-derived MKs, which are mostly of the embryonic/fetal phenotype, they may have some embryonic/fetal characteristics including an aberrant status of glycosylation or sialylation. Slight PLT activation by physical stimuli during the processing, such as filtration and irradiation, could be another cause, as this activation may induce *in vivo* micro-aggregation and clearance of the iPSC-PLTs.

6. Future perspectives

The establishment of imMKCLs as an MCB and the development of a feeder-independent culture system that includes a turbulence-based bioreactor and novel drugs has enabled the *ex vivo* manufacturing of iPSC-PLTs at clinical scale. Through nonclinical evaluations, the quality, efficacy, and safety of both the MCB and iPSC-PLTs were confirmed, paving the way for the iPLAT study. The study validated the safety of the transfusion but revealed a discrepancy in the circulation between animal models and the human patient.

For future clinical implementation to be practical, methods that reduce the manufacturing costs are necessary. Ideally, the production efficiency of iPSC-PLTs will reach >800 PLTs per MK, which

is the estimated rate *in vivo*. Many conditions reported to enhance megakaryopoiesis and thrombopoiesis have yet to be tested in our culture system. Advanced PLT processing technologies are also needed because the existing purification-washing-packaging process results in an approximately 50 % loss of iPSC-PLTs and possibly an increase of disialylated iPSC-PLTs. At the same time, raw materials and the culture system itself could be further optimized for cost effective production.

As a commercial product, allogeneic PLTs with HLA homozygous haplotype are preferred to autologous PLTs, because the latter require a long time and high costs for their production and quality testing for each patient. Alternatively, genetically modified HLA-I deficient PLTs could be developed as a single off-the-shelf PLT product. One common knockout target for HLA-I is beta-2-mirocglobulin (B2M), a component of HLA-I heterodimer and requirement for HLA-I cell surface expression [40–44]. It should be noted that HLA-I-deficient products risk activating natural killer (NK) cells because HLA-I molecules act as inhibitory ligands for killer immunoglobulin-like receptors (KIRs) and CD94/NKG2 on NK cells [45,46]. However, HLA-I deficient B2M-knockout iPSC-PLTs do not elicit an NK cell response *in vitro* [43]. The compatible result was obtained *in vivo* with humanized immune system mice, which were reconstructed with enough human NK cells to reject B2M-knockout iPSC-derived hematopoietic mononuclear cells [43]. As for HPA incompatibility, PLT products for the desired HPA type are needed because, for instance, most HPAs are derived from GPIIb/IIIa (CD41a/CD61), which is required for PLT aggregation.

Additionally, cell-cultured PLT products and their derivatives have not only in terms of safety and sustainability and meeting unfilled demands for cases with rare HLA-I or HPA phenotypes, but also in terms of applications to novel clinical treatments. Recent studies have revealed the importance of PLTs outside of hemostasis and maintenance of vascular integrity. For example, PLTs involve innate immune responses, releasing cytokines and interact with immune cells to modulate immune responses [47,48]. Conversely, PLTs involves in pathogenesis of several diseases such as cardiovascular diseases [34], cancer metastasis [35], rheumatoid arthritis [49] and even severe COVID-19 symptoms [36]. PLTs also contribute to development, homeostasis, and tissue regeneration. PLT-rich plasma (PRP) has been used for regeneration therapies of bone, cartilage, tendon, and muscle damages. Several studies are going on to leverage engineered PLTs as a drug or drug delivery system (DDS) [50,51]. Thus, iPSC-PLT products and their derivatives could have a wide variety of clinical applications beyond the treatment of bleeding complications.

Declaration of competing interest

K.E. was the founder of Megakaryon, which conducted a clinical trial using allogenic iPSC-PLTs (but K.E. has no stock of Megakaryon). Otsuka Pharmaceutical Co. Ltd., Megakaryon Co., and Satake Multimix Co. provided collaborating project fundings.

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