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Large-Scale Cultures and Bioreactors for the Production of Megakaryocytes and Platelets

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Abstract

A constant rising demand on platelets has been observed which is associated with population ageing and rapid progresses in the development of advanced therapeutic strategies. Therefore, innovative attempts towards the *in vitro* production of platelets and their precursors are inevitable. Extensive research has been performed on the exploration of reliable cell sources and the establishment of efficient protocols for the differentiation of functional platelets. In particular, the design of bioreactors mimicking the bone marrow microenvironment has gained plenty of attention to achieve clinically relevant platelet yields. In this review, we summarize major advances and perspectives on the manufacture and application of *in vitro* generated megakaryocytes and platelets.

Keywords: Bioreactors; Large-scale production; Megakaryocytes; Platelets

Current Demand and Challenges for the *In Vitro* Production of Megakaryocytes and Platelets

Platelet (PLT) [1,2] transfusions serve as the standard clinical therapy for patients suffering from thrombocytopenia or PLT dysfunctions. Moreover, advanced experimental approaches exploit the regenerative characteristics of PLTs for tissue remodeling [3] or anti-cancer therapies [4]. Demographic changes as well as rising incidence and prevalence of hematological diseases constantly increase the clinical demand for donated PLTs. However, the availability of donors is limited [5]. Hence, in the recent decade, specialized *in vitro* techniques were developed to generate PLTs and their precursors, the megakaryocytes (MKs), for therapeutic needs. These meet the challenge to provide MKs and PLTs in adequate practicability, safety and clinical grade to satisfy the quantitative and qualitative demands for a Good Manufacturing Practice (GMP)-compliant translational perspective [6].

First, the availability and expandability of the cell source for differentiation is essential. This endorses the use of induced pluripotent stem cells (iPSCs) in contrast to $CD34^+$ hematopoetic progenitors or embryonic stem cells. For an optimal effort-benefit ratio, the differentiation protocol for MK or PLT production would allow the cryopreservation of an intermediate or mature stage and the technically simple harvest of high yields of cell products from a scalable culture setup. Several protocols starting from iPSCs, described in the following paragraph, yet fulfill these expectations. However, often iPSCs are generated by lentiviral mediated reprogramming. Here, optimally non-integrative vectors should be applied to reduce a potential tumorigenic risk of the derived stem cell-derived products [6].

Finally, the quality of *in vitro* generated MKs or PLTs is of tremendous relevance. *In vitro* MKs or PLTs have to match the phenotype, morphology and ultrastructure of their natural paragons [7]. Importantly, appropriate *in vivo* models and lately clinical studies need to demonstrate the functional capacity of MKs to produce PLTs in the recipient's circulation. Also, the shedded PLTs should be able to respond to stimuli and aggregate [8]. In the following paragraphs, distinct approaches to produce MKs and PLTs in large-scale for clinical yields including latest trends in bioreactor design will be described – Highlighting practicability, safety and quality aspects.

Towards the Large-Scale *In Vitro* Production of Megakaryocytes

While there are currently no protocols available describing the *in vitro* production of MKs or PLTs in a clinical relevant scale, several groups have presented substantial work on the establishment of such processes with good prospects.

The group of Jiang has established a promising approach differentiating human cord blood (CB)-derived $CD34^+$ hemaptopoetic progenitors to MKs to substitute PLT transfusion. After initially establishing a static, two stage culture systems [9], the group recently presented an augmented, up-scaled device represented by a 2 L turning bottle device [10]. This design allowed the production of ~2×10^{10} MKs from 1×10^6 $CD34^+$ cells after 13 days. The authors hypothesize that starting with one CB unit only they can produce sufficient MKs for the treatment of 30 patients. However, as previously discussed, $CD34^+$ cells do not represent a highly accessible cell source to satisfy the intense demand for PLTs.

Other groups suggest the differentiation from human pluripotent stem cells (hPSC), with a recent focus on the production from iPSCs. To induce MK differentiation from iPSCs one approach combined the formation of embryonic bodies (EBs) with the parallel transduction of three transcription factors (GATA1, FLI1 and TAL1), a process called forward programming [11]. After 10 days the EBs were dissociated and the suspension cells were further expanded for in total 90 days, resulting in 2×10^5 MKs per input hPSC which were able to produce functional PLTs in co-culture with murine feeder cells. Another approach focuses on the establishment of immortalized megakaryocyte

progenitor cell lines (imMKCL) from iPSC-derived hematopoietic progenitor cells by lentiviral transduction with BMI1 and c-MYC and subsequent transduction with BCL-XL. This strategy allows an expansion phase of 5 months [12]. While BMI1 and BCL-XL suppress senescence and apoptosis, c-MYC promotes the proliferation. When the transgene expression was turned off using a doxycyclin-regulated system, functional PLTs were produced. By the use of this system, theoretically, one PLT transfusion unit of 1×10^{11} PLTs could be produced in 25-50 ml medium. The authors highlight the option to cryopreserve imMKCLs which would allow a subsequent production of MKs within 14 days post-thawing. Moreover, they suggest an implementation of master cryo banks to enable a matching of HLA and HPA types between the *in vitro* generated cells and their recipient. However, the establishment of such master cell banks comprises high costs and efforts, as the HLA system is very polymorphic. A high number of cell lines would be necessary to match different patients [13] and possible clones would have to be tested for their quality.

To circumvent an immunological reaction against *in vitro* generated MK or PLTs, alternatively a genetic knock out [14,15] or knockdown [16] of HLA class I in the source cells is possible, targeting the conserved domain beta2-microglobulin by TALEN, CRISPR/Cas9 or RNA interference, respectively. As the complete absence of HLA class I is activating the recognition by natural killer (NK) cells, an HLA knock out is useful for the production of PLTs only, since PLTs are not recognized by NK cells [17], in contrast to MKs.

Although the genetic modification of the iPSCs may involve the use of lentiviral vectors, this is associated with low safety concerns as MKs and PLTs might be irradiated before transfusion and thereby reducing the risk for tumorigenesis.

Specialized Bioreactors for the High Quality production of Platelets *In Vitro*

While there are several approaches available aiming at a technically simple, large-scale production of MKs, challenges to produce high quality PLTs *in vitro* have driven the development of complex bioreactors mimicking the natural bone marrow (BM) microenvironment. For the generation of PLTs in such setups, often MKs are produced ahead in a static system, and subsequently introduced to the fluidic bioreactor systems, some of which are not larger than chip-sized [18].

Several approaches considered the optimization of technical bioreactor parameters, such as a continuous media perfusion [1,2,18-22] and gas exchange, the control of flow rate or shear stress [18-23] or a real-time monitoring [18,20,21] of the production process. To stimulate a most efficient MK trapping within the intended bioreactor niche some setups aimed to create an appropriate scaffold composition [1,2,18,21] mimicking the natural BM architecture. Several designs also manufactured a contact of MKs to endothelial cells [2,18,20,21], components of the extracellular matrix [1,2,18,21] or particular chemo-attractants [1,2,21]. Other reactors yet enable a downstream processing such as the separation and concentration of PLTs [22,24].

Currently, the highest yield of PLTs derived from an *in vitro* bioreactor corresponds to a ratio of 100 PLTs per MK [18,22] which clearly does not match the natural output of 1000s of PLT per MK. As yet discussed by Thon et al. 2017, in order to produce an equivalent to one PLT apheresis unit (3×10^{11} PLTs), the implementation of 3×10^{9} MKs into a bioreactor of thousand-fold scale would be required [24].

Further improvements on the modeling of physical parameters the cell is exposed within the fluidic system and understanding of their effects, could pave the way to increase the capacity of bioreactors to produce appropriate clinical yields. Moreover, the development of scaled designs that support a commercial application could represent an important driving force for translational perspective. To address economic aspects a reduction of the media volume and product concentration would further be advantageous.

Advantages and Disadvantages of *In Vitro* Produced Megakaryocytes and Platelets for Transfusion

Besides the distinct technical challenges to produce MKs or PLTs *in vitro*, the two cell types differ in their applicability for a potential future transfusion. In several aspects of practicability, safety and quality, either MKs or PLTs hold advantages over the other.

While PLT transfusion is yet the clinical standard for the treatment of thrombocytopenia, MK transfusion was not conceivable before the upcoming of *in vitro* production strategies. Hence, besides the challenge to generate clinical grade MKs *in vitro*, still MK transfusion has to be proven as a safe and beneficial therapeutic concept. However, first clinical studies on the transfusion of MKs generated *ex vivo* from $CD34^{+}$ cells, showed promising results in terms of safety and a reduced necessity for additional PLT transfusions [25-27]. Due to the current difficulties to generate sufficient amounts of functional PLTs *in vitro*, there are no clinical studies available demonstrating a transfusion of *in vitro* manufactured PLTs. However, *in vitro* PLTs could serve as a direct functional support for the recipient's hemostasis.

In contrast, the onset of PLT production upon infusion *in vitro* MKs demands time, and does not allow to estimate or control the amount of released PLTs, since this varies significantly between *in vivo* and *in vitro* conditions. The number of *in vitro* PLTs instead can be determined ahead of transfusion. Therefore, a combination of both MK and PLT transfusions could be therapeutically desirable - PLT transfusion for urgent needs, such as for arrest of severe acute bleeding and MK transfusion in non-emergency cases [27], such as adjuvant therapy after scheduled high dose chemotherapy.

Still, *in vivo* produced PLTs might possess a longer half live within circulation than PLTs collected *in vitro* prior to transfusion. Here, *in vitro* systems addressing a harmonization of MK differentiation and a synchronized PLT release could bear the potential for a better control of PLT age at the time point of transfusion.

Further obstacles in PLT transfusion are contingent on the short half-life of PTLs [28] as well as their problematic collection and storage which often leads to a loss of PLT quality [18], particularly related to the preservation of PLT quiescence and ability to activate. Interestingly, *in vitro* generated MKs or MK progenitors (MKPs) can be stored frozen and thereafter further cultured [11,12,15]. In case of the cryopreservation of MKPs, however, a further maturation is required upon thawing. Moreover, the classical cryoprotectant DMSO is cytotoxic and therefore another obstacle for the instant application of thawed transfusion products.

Importantly, the safety of cellular transfusion products is a crucial aspect to consider, especially when discussing SC-derived products. PLTs as enucleated cells may be exposed to irradiation in order to inhibit a potential co-transfusion of undifferentiated or transformed cells with tumorigenic potential originating from the SC culture [29]. For MKPs irradiation is not applicable, however, for mature MKs

which no longer undergo cellular division but produce proPLTs instead irradiation may be an option to improve the safety of their application [30]. Another safety concern for transfusion products is the maintenance of sterility. Despite of the standard test are subjected to, donated PLT concentrates contain a residual risk for undetected contamination. In contrast, the *in vitro* production of cell therapeutics under GMP compliant conditions allows a further reduction of contamination risks by the implementation of closed and/or automated systems in clean room environments [31].

Lately, a follow-up of MK distribution and homing upon transfusion is indispensable. In a mouse model a short time engraftment of MK progenitors (MKPs) in BM for several weeks was demonstrated which lead to a prolonged release of PLTs into the circulation [9]. However, a certain risk of MKs homing to secondary loci of thrombopoiesis such as the lung has likewise to be addressed. Available studies from mouse models reveal divergent results [16,32] which might be explained by a potentially abnormal behavior of human MKs within the murine circulation system.

To date the amount of PLTs released per MK *in vitro* in all technical setups is far below the natural production yields, resulting probably from the challenge to ideally reconstruct the physiological niche of PLT production with its numerous microenvironmental factors [18]. Presumably, transferring the final differentiation step from MKs to PLTs to physiological conditions *in vivo*, would allow a reduction of differentiation costs while optimizing outcome and PLT quality.

Conclusion

Multifaceted innovative work was done to develop alternative strategies for the prevention and treatment of thrombocytopenia. In summary, *in vitro* MKs can be generated in very promising although not in clinical scale yet. Still, initial clinical trials indicate MK therapeutic suitability for the treatment of thrombocytopenia. In contrast, the *in vitro* production of functional PLTs remains technically very complex. However, given these hurdles are overcome, *in vitro* PLTs could be used as a direct alternative to the standard therapies using donated PLTs. Hence, in the future, *in vitro* generated cells could represent an alternative to complement or even replace the transfusion of donor PLTs. Remarkably, the feasibility to genetically modify *in vitro* produced MKs and PLTs may allow their manufacture as personalized cell products to match the specific needs of each patient. Conceivably, one or a combination of the reviewed methods holds the key to significantly improve the management of thrombocytopenic patients.

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