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Mechanisms of platelet release: *in vivo* studies and *in vitro* modeling

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Abstract

Mechanisms related to platelet release in the context of the bone marrow niche are not completely known. In this review we discuss what has been discovered about four critical aspects of this process: 1) the bone marrow niche organization, 2) the role of the extracellular matrix components, 3) the mechanisms by which megakaryocytes release platelets and 4) the novel approaches to mimic the bone marrow environment and produce platelets *ex vivo*.

Introduction

Circulating platelets are highly specialized cells produced by megakaryocytes (Mks) that participate in haemostatic and inflammatory functions. Millions of platelet transfusions are conducted each year but the supply of this blood component is limited. Despite their critical role in many physiological functions, little is known about the molecular mechanisms involved in platelet production from Mks, or about the pathogenesis of platelet disorders. With such widespread impact of these diseases, and the lack of good options for clinical treatments, new insight into the formation of platelets from Mks would have a major impact on patients and healthcare. The characteristics of the environment or niche surrounding Mks play a fundamental role in the regulation of megakaryopoiesis. Despite its fundamental role in regulating haematopoiesis, analysis of the bone marrow (BM) microenvironment *in vivo* has been hampered because of the diffuse three-dimensional nature of its structure and complexity within the bone cavity, especially in humans where invasive approaches are not possible. One emerging strategy to gain such insight is through the use of relevant three-

dimensional (3D) models of human tissues via tissue engineering. These systems have also been exploited to address the clinical need for transfusable platelets by proposing novel procedures to produce fully functional platelets *in vitro.*

Here we reviewed the current state of the art on the BM niche regulation of platelet formation, the role of pro-platelet extension, and new emerging strategies to mimic platelet production in a 3D environment.

Organization of the BM niche

The current model of megakaryopoiesis consists of the ability of Mk precursors to migrate during their maturation from an endosteal niche to the sinusoids guided by chemokines and extracellular matrix (ECM) components [1, 2]. This concept is supported by the results of the co-injection of SDF1 and FGF4 into c-MPL-deficient mice that transiently increases the number of peripheral blood platelet released by directing Mks towards the BM sinusoids [3, 4]. However, observation by intravital two-photon microscopy showed that Mks barely migrate and are mostly localized in close contiguity to blood vessels [5]. Recently, a study, combining *in vivo* two-photon microscopy and *in situ* light-sheet fluorescence microscopy, confirmed that the restricted space between vessels *in vivo* allows growing Mks to reach sinusoids with minimal movements reducing the need to migrate [6].

The BM vasculature is a complex network that provides support, nutrient, and a bloodstream access point for haematopoietic cells. The BM sinusoids are the most abundant vessels of this 3D vascular mesh. During many years, the observation of Mks in the BM was restricted to a static observation leaving the door open to various hypotheses [7-9]. It is now clearly established that in mice Mks are preferentially distributed and observed close to BM sinusoids to extend proplatelets through the endothelial barrier [2, 4, 10, 11]. It has also been described that murine Mks are in the spleen, and platelet release has been observed within lung capillaries [12-14].

The extracellular matrix components of the niche

The non-cellular BM niche microenvironment contains different soluble factors secreted *in situ* by resident cells or delivered by systemic circulation. Moreover, ECM components are functioning not only as an inherent supportive scaffold but also providing direct and indirect instructions to cells regulating their behavior. The different ECM components bind to each

other to form a structural and functional meshwork guiding cell function and localization and defining the access of mature blood cells to circulation. Indeed, a precise spatio-temporal regulation guarantees the adequate blood cell generation supply by the BM niche. A basement membrane, composed by ECMs, also supports the network of different vessels that fulfill the BM. Immunofluorescence microscopy analysis revealed that the BM ECM is mainly composed of the basement membrane collagen type IV, of fibrillar collagens (type I and III), of micro-fibrillar collagen type VI, fibronectin and laminins [15-17]. While fibrillar and micro-fibrillar collagens are mostly located near the bone cortex and around the vessel of large diameters (large arterioles and arteries), type IV collagen, fibronectin, and laminins are more represented close to sinusoids where trans-endothelial migration takes place.

Mks express different ECM components and ECM-modifying enzymes that are modulated during BM reconstitution, in response to chemotherapy, thrombocytopenia, or during BM fibrosis [15, 18-20]. Recently, we demonstrated a fundamental role of fibronectin with a forced inclusion of the EDA exon domain in sustaining BM fibrosis through a TLR4 signaling axis [21]. In turn, these proteins regulate Mk maturation, proplatelet elongation, and platelet formation [17, 22, 23]. Among the studied ECM components, fibronectin and type IV collagen supported more the proliferation and maturation of haematopoietic progenitors versus the Mk lineage, while all the tested ECM components except type I collagen supported proplatelet formation [15, 17, 22-25].

Further, Semeniak *et al*. demonstrated that type I and type IV collagens bind to the same receptors on Mks competing for the final cellular behavior [17]. In line with these data, we showed a different β1-integrin internalization dynamics and consequent downstream signaling activation in Mks plated on type I and type IV collagen due to the different stiffness of the matrices [23]. Studies on ECM biomechanical properties proved that a soft microenvironment better support Mk maturation and platelet production involving membrane receptors, myosin IIA-dependent cytoskeletal rearrangements and mechano-sensitive ion channels [23, 26-28].

Hyaluronan is the most abundant glycosaminoglycan (GAG) component of the BM ECM. It has been described as an inhibitor of haematopoietic stem cell proliferation and myeloid differentiation by spatially regulating haematopoietic stem cell (HSC) lodgment [29, 30]. Although human Mks express hyaluronan receptors, *in vitro* differentiation, maturation and platelet formation are not affected by adding exogenous hyaluronan in culture [31]. On the contrary, deficiency of the hyaluronan depolymerization enzyme, hyaluronidase 2, results in

thrombocytopenia *in vivo* due to an accumulation of hyaluronan within Mks causing an imbalance in their maturation [32]. Keratan sulfate, dermatan sulfate, and chondroitin sulfate are other GAGs which role on BM homeostasis and Mk regulation has been studied. In general, it has been documented an inhibitory role of GAGs on haematopoietic progenitor differentiation toward the Mk lineage and a reduction of Mk function through direct binding to cell membrane receptors and/or binding of inhibitory cytokines [33-35].

Cytokines, growth factors, and non-protein metabolites, such as lipids or ions, are other fundamental extracellular components of the BM microenvironment [36]. Their localization follows anatomical gradients, dictated by their source of origin, finally taking part in the precise spatio-temporal regulation of BM homeostasis. Their binding to ECM components controls the biological activity of these soluble factors through biochemical and biomechanical mechanisms [37, 38].

Mechanisms of platelet shedding

Two alternative models of platelet biogenesis have been proposed so far. One model, based on electron microscopy observations, invokes preformation of “platelets territories” in the cytoplasm of mature Mks, and subsequent release of platelets through massive cytoplasm fragmentation [39]. In particular, the identification of the Demarcation Membrane System (DMS) by Yamada in 1957 led to the assumption that these membranes were responsible for defining fields of pre-formed platelets and fracture lines for further cytoplasm fragmentation [40]. A few years later, Sharnoff and colleagues observed that Mks travel through the circulation to the lungs, where they are fragmented into platelets in pulmonary capillaries [41]. This explosive-fragmentation theory retained validity for some time because of inconsistent observations such as the lack in these platelet fields of the marginal microtubule coils, a hallmark of circulating peripheral blood platelets.

The second model conceives platelet assembly through the extension of long (up to millimeters), thin cytoplasmic pseudopodia-like structures (named "proplatelets") from mature Mks [7]. These protrusions consist of multiple-platelet-size beads linked together by thin cytoplasmic bridges. The “detachment of platelet-like fragments or segments from pseudopods" of Mks was first described by Wright in 1906 [42], but proplatelets were not characterized until much later [9, 39, 43, 44]. In the “proplatelets” or “flow” model, the DMS was proposed to act primarily as a reservoir of surface membranes that evaginates to provide plasma membranes for the formation of proplatelets. In 1994, discovery and

purification of the main thrombopoiesis factor, Thrombopoietin (TPO), allowed *in vitro* Mk expansion and renewed interest in the definition of mechanisms of platelet release [45, 46]. Mk cultures provided a system to visualize these cells in the act of releasing platelets. Time-lapse video microscopy of living Mks clarified the essential nature of this complex process and supported the “proplatelets model” for platelet biogenesis [47].

Interestingly, *in vitro* released platelets, by proplatelet maturation, were structurally and functionally similar to peripheral blood platelets [48-50]. During proplatelet formation, the Mk cytoplasm is exhausted, and naked nuclei are released in the surrounding space. However, besides single platelets release, also proplatelets in bulk can detach from Mks and fragment further into platelets suggesting a potential step of platelet maturation in the blood circulation. In this regard, intermediate products named pre-platelet, able to shift back and forth between round cells and multi bodied proplatelets, have been reported [51]. The definition of proplatelets architecture and morphology provided the most suitable mechanism to explain how newly formed platelets traverse the BM vessel walls and reach the bloodstream. To prove this, in 2007, a pioneer work by Junt *et al*. used multiphoton intravital microscopy in intact BM to visualize platelet generation in mice and observed Mks that extended dynamic proplatelet-like protrusions into micro-vessels [5]. Imaging also showed Mks exhibiting fragmented protrusions and cellular processes extending into micro-vessels and releasing heterogeneous fragments resembling immature proplatelets into the BM microvasculature.

Moreover, under these *in vivo* settings, the hydrodynamic force of flowing blood was involved in the release of large proplatelet masses as well as in separating intravascular cell fragments [5]. Altogether these observations validated earlier studies that showed pseudopodia and proplatelet formation in cultured Mks and supported the idea that platelets derive from the intravascular release of fragments protruding from mature Mks. Accordingly, a few years later, Zhang *et al*., using the same technique in mice, showed that once Mks have successfully extended their proplatelets into the blood, they release fragments from the tips of their intravascular projections [1]. However, the limited resolution of intravital light microscopy in these studies could not reveal fine structural details or cytoskeletal organization during this process.

Calculations of platelet consumption and estimated platelet number released from each Mk in humans and mice suggested that platelet production via proplatelet formation is sufficient to maintain the physiological platelet turnover [1, 5]. While, rapid platelet turnover, especially

under conditions of increased demand (20-fold or more), such as during inflammatory reactions, may require different mechanisms of Mk fragmentation. Kowata *et al*., using intravital microscopy in thrombocytopenic mice, identified immature thick Mk protrusions, characterized by a peripheral zone, abundant endoplasmic reticulum, and demarcation membrane system, and random microtubule arrays, as an alternative Mk intravascular product in addition to proplatelets [52]. Consistently, Nishimura and co-workers detected an alternative Mk rupture in mice under inflammatory conditions, which entails rapid cytoplasmic fragmentation and release of much larger numbers of platelets, primarily into blood vessels [53]. Using high-speed two-photon microscopy with multicolor high-sensitive gallium arsenide (GaAs) detectors, authors demonstrated that the inflammatory cytokine IL-1α primarily regulated this novel Mk rupture-dependent pathway and this process was morphologically and mechanically distinct from proplatelet formation and typical Fas Ligand-induced apoptosis [53].

Interestingly, platelets produced with this Mk rupture displayed insufficient microtubule organization, in agreement with the nature of platelet fields described in the "fragmentation" theory. The last finding on thrombopoiesis challenges our current view of platelet generation. Lefrançais *et al*., using intravital microscopy observed, for the first time, Mks dynamically releasing platelets through proplatelet extensions in the lung microcirculation in mice [12]. The contribution of the lungs to platelet biogenesis was substantial, accounting for approximately 50% of total platelet production [12]. Brown *et al*., combining light microscopy and electron microscopy to visualize BM in living mice, found that Mks predominantly extend large protrusions into the blood vessel space by a mechanism that involves mass fusion between the internal and external membranes rather than extruding proplatelet extensions through microtubule sliding [54].

Despite recent advances, the following questions prevail: (i) Which factor/s induces platelet release through the proplatelet model? (ii) How fragments from the Mk explosion go into circulation? (iii) Does the endothelium barrier of the BM sinusoid play a role in guiding proplatelet formation or Mk fragmentation? (iv) How do fragments or intermediate products of terminal platelet formation continue their maturation in the bloodstream? Answering these questions could fill a significant gap in our knowledge of platelet production. In this context, 3D *in vitro* cultures can provide a new valuable contribution to resolve conflicting models of platelet production as they can more realistically reproduce human thrombopoiesis than 2D models, overcoming the limitations of using animals and reducing the current over-reliance on *in vivo* tests.

The ultimate *in vivo* studies and *ex vivo* three-dimensional models of BM

Bioengineering BM is emerging as a promising approach with two primary aims: (i) to develop substitutes for producing platelets for transfusion medicine to replace donor-dependent donations, (ii) to build-up a living organ to replace animal models for drug screening and modeling thrombopoiesis in health and disease. While technical limitations are still preventing the scaling up of these models for application in transfusion medicine, they are powerful tools for studying Mk physiology and pathology in a controlled environment.

The architecture and composition of the native BM is particularly challenging to replicate in light of the biochemical and mechanical properties described above, and of the hierarchical structure of its 3D microenvironment, which consists of bone lining cells, maturing blood cells always in motion, endothelial cells, adventitial reticular cells, macrophages, and adipocytes [55].

Importantly, the BM has an extensive blood supply served by numerous blood vessels of various sizes that, after entering the tissue, branch and give rise to a multitude of small arterioles and capillaries, which finally anastomose with venous sinuses [56, 57].

Besides their Mk guiding role, sinusoids have been mainly studied *in vivo* for dynamic visualization of proplatelet formation and platelet release in the bloodstream [5, 6]. The sinusoid's blood flow also appeared as a regulator of platelet biogenesis by variation of haemodynamic profile. Recently, the group of Koji Eto employed two-photon microscopy and particle image velocimetry to analyze *in vivo* BM flow dynamics. The analysis showed that resting Mks are exposed to continuous laminar flow, while, high turbulence is present around proplatelet shafts elongated by active Mks. [58]. Although we do not know the exact shear stress and turbulence that human Mks experience *in vivo*, these date were instrumental to generate high numbers of platelets from human induced pluripotent cell-derived Mks cultured *ex vivo*. In the bioreactor, mixing blades were effective in generating a dynamic flow with an optimal level of shear stress and turbulent energy for promoting the release of functional platelets [58].

By putting together all these pieces of information, tissue engineering approaches have been implemented to develop tools having different levels of complexity to advance our knowledge of the physiology and pathology of BM and thrombopoiesis [59, 60].

A BM-on-a-chip microfluidic device, consisting of a cell-inert silicon-based organic polymer filled with matrices and growth factors, enabled the culture of a whole living haematopoietic BM after subcutaneous implantation in mice [61]. After surgical removal, this system could produce blood cells continuously and release them into a microfluidic circulation while maintaining the haematopoietic staminal pool, as demonstrated by the presence of a significant proportion of long-term HSCs having self-renewal and differentiation capabilities [62]. Some challenges, including the use of animals, limited the adaptability of this strategy to the needs of producing safe human blood cells for clinical purposes. The most recent findings include a chip filled with a fibrin gel in which human HSCs and BM-derived stromal cells are co-cultured to support the differentiation and maturation of multiple blood cell lineages over 1 month while improving HSCs maintenance [63]. Similarly, macroporous hydrogel resembling the 3D architecture of trabecular bones, equipped with RGD-peptides to promote cell adhesion through integrins, demonstrated to be suitable for haematopoietic stem and progenitor cell culture and expansion into a perfusion system in co-culture with mesenchymal stem cells [64]. Further, ceramic hollow fiber constructs and bioreactors demonstrated to be a useful tool to support the culture and harvesting of human haematopoietic cells by promoting improved nutrient and metabolite diffusion and secretion of ECM components and growth factors [65, 66].

Focusing on BM models developed explicitly for studying thrombopoiesis, we can divide them into two major categories: microfluidic chips and 3D bioreactors.

First approaches included the use of microfluidics, surface patterning, coating with ECM components, such as fibronectin and von Willebrand Factor, and eventually co-cultures with endothelial cells [67-71]. Such systems remained intentionally simplistic, with the primary goal to allow high-resolution live-cell visualization rather than reproducing the 3D environment that Mks experience *in vivo*. A significant advantage of these models was the possibility to mimic haemodynamic vascular shear stress. The combined effects of anchoring to ECMs and shear were used to induce platelet release within the perfused medium. The miniaturized screening demonstrated that proplatelet formation is the result of an interplay between cytoskeleton dynamics and flow-induced deformation, this latter is fundamental to maximize the number of Mks forming platelets and speed up the process of proplatelet elongation [69-71].

Despite this knowledge, from an anatomical and physiological point of view, 3D cultures have been demonstrated to mimic more closely *in vivo* megakaryopoiesis. Photo-

crosslinked hyaluronan-based hydrogels with entrapped ECMs demonstrated the ability to promote enhanced proplatelet formation by human Mks when functionalized with fibronectin or type IV collagen [31]. Consistently, mouse Mks grown in 3D methylcellulose hydrogels, having physiological BM stiffness, resembled the phenotype of *in vivo* cells in terms of morphological aspect of the demarcation membrane system and ploidy, ultimately resulting in increased proplatelet production [72].

The ability of Mks to form platelets depends on the activation of proper autocrine regulation, mechano-transduction pathways, membrane ion channels, and consequent cytoskeleton dynamics [23, 73-76]. In this regard, researchers focused on the development of scaffolds that could provide the possibility of flexibility, altering their physical, mechanical and biochemical characteristics, to support platelet production without affecting the functionality of final platelet products. A significant breakthrough has been made by the use of silk fibroin from *Bombyx mori* silkworm cocoons [77]. Silk fibroin is a naturally-derived, biocompatible and tunable biomaterial which demonstrated fundamental features for studying thrombopoiesis, such as non-thrombogenicity, non-toxicity, and possibility to entrap ECM components (e.g., collagens, fibronectin), growth factors (e.g., VEGF) and chemokines (e.g., SDF-1α) while retaining their bioactivity on Mks [78-80]. A combination of modular flow chambers and vascular silk tubes and sponges have been used to record platelet generation, by primary human Mks, in response to variations in surface stiffness, functionalization with ECM components and co-culture with endothelial cells [78, 81, 82]. These systems were able to support efficient platelet formation and, upon perfusion, recovery of functional platelets, as assessed through multiple activation tests, including participation in clot formation and thrombus formation underflow. Importantly, increasing the size or number of the silk sponges resulted in a proportional increase in the numbers of platelets recovered, suggesting applicability to scale-up for platelet production [78, 82]. While, treatment with molecules and/or drugs that have been shown to regulate activation of biochemical pathways that are crucial for platelet formation, significantly impacted on the number of *ex vivo* produced platelets, suggesting the applicability of these models for evaluation of the effects of BM exposure to compounds that may cure Mk-related diseases [81, 83].

Benefits from a 3D spongy and porous microenvironment on *ex vivo* platelet production have been confirmed by the use of a collagen scaffold within a flow bioreactor system seeded with programmed induced pluripotent stem cell-derived Mks [84]. The graded scaffold influenced cell location while maintaining the ability to release metabolically active and

functional platelets continuously. While a 3D human marrow vascular microenvironment embedded in a matrix of type I collagen demonstrated the ability to support platelet formation by promoting interaction with endothelial cells [85]. Interestingly, Mks, once in contact with the endothelium, were able to induce pore formation to allow the release of platelets into the lumen of the artificial vessels.

The advantages of all these systems include the possibility to control cellular and ECM composition, 3D architecture, and stiffness of the biomimetic scaffolds, flow rate, and shear stress, while preserving the possibility to perform high-quality imaging. Nevertheless, there are still improvements that they must address, including the use of physiologic flow and shear rates to mimic the human bloodstream within BM sinusoids and implementation with BM endothelial cells. BM endothelial cells have a unique phenotype that is still under-characterized in humans. The understanding of these elements is fundamental to direct *ex vivo* studies of BM thrombopoiesis.

Conclusions

Insight into mechanisms is instrumental in novel approaches to understand and control Mks related to platelet functions, both in normal and disease scenarios.

Platelets derived from Mks play a central role in many physiological functions. However, there are many platelet-related diseases for which there are no current treatments other than palliative therapies, resulting in severe complications and side effects. Unravelling mechanisms of functional platelet formation from Mks would provide new options for therapeutic interventions. Further, the *in vitro* 3D models offer a broad scientific approach to design and control the local environments within which cell functions and disease states originate. The use of these systems is instrumental in gaining information on how to trigger platelet production more physiologically for applications in transfusions.

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Figure Legends

Figure 1. *In vivo* models of thrombopoiesis. Bone marrow is located within the trabecular bones, near the endosteum, where the haematopoietic stem cells are committed towards the megakaryocytic lineage under the control of Thrombopoietin. Maturing megakaryocyte (Mk) migrates towards the sinusoids prior to release platelets. In physiologic conditions, Mks elongate proplatelets through the vascular endothelium, into the lumen of sinusoidal vessels where platelets, stemming from their terminal ends, are released into the bloodstream by blood shear forces. Under acute inflammatory or cytopenic conditions acute platelet release is induced through rupture of the mature Mk membrane.

Figure 2. *Ex vivo* models of thrombopoiesis. Different human and mouse cell sources can be used to generate megakaryocytes *ex vivo* (HSC: haematopoietic stem cell; iPS: induced pluripotent stem cell). Once obtained, megakaryocytes can be cultured within bioengineered tissue-models mimicking the different features of the bone marrow environment. The most recent models are made of biocompatible materials supporting megakaryocyte function. Once in contact with the biomaterial, megakaryocytes extend proplatelets into the perfused culture medium mimicking blood shear stress.