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Microvesicles, but not platelets, bud off from mouse bone marrow megakaryocytes

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Abstract:

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TO THE EDITOR:

For the past 75 years, debate has focused on two primary mechanisms for platelet generation-cytoplasmic fragmentation and proplatelet production. The cytoplasmic fragmentation model was based on high magnification images visualizing the internal ultrastructure of the megakaryocyte.[1] These images showed that megakaryocytes contain abundant membrane that divide the cell into "platelet territories," a name indicating a role in partitioning off assembling platelets that are formed by fracture of the megakaryocyte cytoplasm. By contrast, the proplatelet model proposes that megakaryocytes generate platelets by remodeling their cytoplasm into long, beaded processes that function as the assembly lines of platelet production (Figure 1A).[2] Proplatelets are thought to function as an intermediate structure in platelet production, and platelets generated from proplatelets manifest many properties of blood platelets.[3] Intravital microscopy visualizing megakaryocytes forming extensions in the bone marrow of living mice also supports a physiological role for proplatelets. According the the proplatelet model, the demarcation system provides a reservoir of platelet membrane to cover growing proplatelet processes.[4] In 2007, Junt and colleagues observed living megakaryocytes extending proplatelets into the vascular lumen, and demonstrated how flowing blood sheared these processes off into the circulation.[5] Based on two-photon and light sheet microscopy data from Geue et al, Münzer et al, and Stegner et al approximately 1,620 MKs per mm^3 bone marrow of mice make proplatelets at steady state.[6-8]

In May 2020, Potts et al. published an article in JEM in which they offer a completely different hypothesis for platelet production proposing that membrane budding is the major mechanism of platelet production (Figure 1B).[9] Using 3D and 4D imaging methods that enable

megakaryocyte behavior to be studied in its native environment, the authors suggest that membrane budding is a common event that results in the substantial release of platelets directly into the peripheral circulation during both fetal and adult life. They show that buds derive from the surface of megakaryocytes and suggest that these buds are directly released as platelets. Using the NF-E2 knockout mice, which exhibit severe thrombocytopenia, the authors suggest that a block in megakaryocyte blebbing explains the low platelet count observed in these mice.

While the findings of Potts et al. are provocative, whether the blebbing of the megakaryocyte surface generates *bona fide* platelets remains uncertain. We have previously noted that microvesicles can be shed from maturing megakaryocytes (MKMVs).[10] This work showed that cultured megakaryocytes produced MKMVs that were smaller and less regular than platelets and lacked standard platelet organelles such as granules and mitochondria. However, these earlier studies did not include imaging of MKMV formation *in vivo*. In contrast, Potts et al. included *in vivo* studies, but not high magnification imaging of megakaryocytes in bone marrow. They were therefore unable to directly address the question of whether or not the structures they studied contained a normal allotment of organelles. To fill this gap in our knowledge and assess the nature of the blebs emanating from megakaryocytes *in vivo*, we have now compared micrographs of *bona fide* platelets to the structures observed by Potts et al. and evaluated high magnification images of megakaryocytes in bone marrow.

Assessment of the images in Potts et al. lead us to question whether the objects that they described are actually platelets. There are clear morphological and ultrastructural differences

between platelets and MKMVs (Figure 1C-E). If membrane budding was a major mechanism of platelet production, one would expect the budding megakaryocyte cytoplasm to exhibit characteristics of a resting platelet and possess an array of intracellular components. Blood platelets display several distinguishing features, including a discoid shape, microtubule coil, open canalicular system, and distinct organelles (Figure 1C). In the initial figure of Potts et al., there is very little evidence indicating that budding megakaryocytes have ultrastructural features of blood platelets as electron micrographs do not show clear evidence of microtubule coils, granules, and an open canalicular system (Figure 1D). In fact, this cell fragment looks more like a microvesicle coming from a cultured blebbing megakaryocyte (Figure 1E). In addition to functioning as the band aids of the blood, platelets also serve as a major transport and delivery system. To accomplish this, platelets contain major secretory granules, including α-granules and dense granules, which are clearly visible by electron microscopy in blood platelets (Figure 1C).

To better understand the *in vivo* significance of the blebbing phenomenon, we evaluated megakaryocytes in their native bone marrow environment at high magnification using transmission electron microscopy (Figure 2). Strikingly, these images demonstrated (1) a central area replete with vesicles and other organelles and (2) a rim of cytoplasm around the central area that is devoid of organelles and from which the blebs can be seen budding off (Figure 2A; Supplementary Fig. S1). These images suggest that this rim of cytoplasm gives rise to MKMVs (Figure 2B), which are reminescent of the budding vesicular structures observed by Potts et al. Megakaryocytes situated in the bone marrow were frequently observed extending blebs towards blood vessels (Figure 2C,D). However, these blebs

appeared to lack microtubule coils, granules, and open canalicular system, hallmark features of assembling blood platelets. To assess the nature of megakaryocyte buds more comprehensively, we performed a quantitative comparison of the α-granule content of 32 megakaryocyte buds and 26 mature platelets. In order to correct for sample error inherent in analysis of TEM images and for differences in size between megakaryocyte buds and platelets, α-granule density (i.e., α-granules/μm^2) was calculated for buds and platelets (Fig. 2E). Areas and α-granule numbers were quantified from images of bone marrow megakaryocytes and mature circulating platelets (Supplementary Fig. S2). This analysis showed that most megakaryocyte buds had no α-granules and the overall α-granule density of megakaryocyte buds was <0.09 α-granules/μm^2, while mature platelets had an α-granule density of 2.8 α-granules/μm^2. These observations support a model wherein the cytoplasmic rim surrounding the organelle-replete central area of the megakaryocyte actively contributes to the formation of blebs that are mostly devoid of α-granules or other organelles observed in mature platelets.[10]

The megakaryocyte is remarkable in its ability to produce platelets that are relatively uniform in size and content. Any theory of platelet production by megakaryocytes must provide a mechanism by which homogenous platelet populations are generated. Production of platelets via proplatelet formation involves the elongation of slender tubules of uniform diameter that enable generation of uniform platelets.[11] In contrast to the uniformity of platelets, platelet microvesicles vary significantly in size, much like the blebs on maturing megakaryocytes. A thorough model of platelet production needs to explain how each platelet receives the proper allotment of essential organelles- mitochondria, dense granules, α-granules, etc. Cytoskeletal

transport pathways provide a mechanistic explanation for loading of assembling platelets with organelles and granules.[12] An indiscriminate budding mechanism fails to address this issue. In contrast, MKMVs vary substantially in size and do not possess the full allotment of organelles that characterize a platelet.

In summary, we propose that the studies described in the Potts et al. paper do not describe a novel pathway for generating mature platelets, but rather describe the generation of an alternative megakaryocyte-derived product. It is possible that these blebbing structures are related to circulating MKMVs, which derive from megakaryocytes and do not possess a normal repertoire of organelles.[10] MKMVs have been shown to have several important functions in megakaryopoiesis, thrombosis, and cell-cell communication.[13-15] Our high resolution electron microscopy of megakaryocytes in the bone marrow environment demonstrates that MKMV-like structures form *in vivo* from a rim of cytoplasm that blebs off into vesicular structures in close apposition to the bone marrow vasculature. Whether the MKMVs that we observe in bone marrow are the same that we previously described in the circulation[10] and whether they are the same structures described by Potts et al. will require additional studies. Nonetheless, our evaluation of bone marrow megakaryocytes at high resolution raises substantial doubt about whether the structures derived from megakaryocyte budding in Potts et al. are *bona fide* platelets. The argument that megakaryocyte blebbing is not a physiological mechanism for generating genuine circulating platelets derives not from a single observation, but from a confluence of evidence. Lack of morphological platelet features, of bleb uniformity, of demonstration of bleb disconnection, and of integrated regulation in response to physiologic cues all challenge the contention that mature, circulating platelets form from megakaryocyte

blebs. These concerns underscore the importance of rigorous assessment of platelet morphology and careful consideration MKMV production in proposing alternative models of platelet biogenesis.

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Disclosures: J.E.I. is a co-founder and has financial interest in PlateletBio, a biotechnology company that aims to produce donor-independent platelet-like cells. R.F. is a founder and has a financial interest in PlateletDiagnostics. The interests of R.F. are reviewed and managed by the Beth Israel Deaconess Medical Center Office of Compliance and Business Conduct.

Figure Legends

Figure 1. Schematic of current concepts of platelet production showing mature megakaryocytes localized close to bone marrow sinusoids. (A) *Proplatelet model*. Megakaryocytes polarize their demarcation membrane system (DMS) as prerequisite for directed release of long cytoplasmic protrusions called proplatelets, which are driven by

reorganization of microtubules (*shown in green*) into the sinusoidal lumen. Platelet intermediates (preplatelets) and platelets are shed into the circulation by blood shear forces. The terminal stage of platelet production occurs in the bloodstream. (B) *Membrane budding model*. Continuous release of final platelets directly from the megakaryocyte via membrane budding. (C) Thin-section electron micrograph showing the hallmark features of blood platelets. Resting platelets have a discoid shape with clear α-granules (I), microtubules (II), and dense granules (III). (D) Electron micrograph from Potts et al. showing a megakaryocyte membrane bud. (E) Electron micrograph showing a bleb from a cultured mouse megakaryocyte that lacks the characteristic features of an assembling platelet. Scale bar, 1 μm. Cells were fixed, processed, and imaged as previously described.[10]

Figure 2. Thin-section electron micrographs showing blebbing megakaryocytes in the bone marrow. (A) Bone marrow megakaryocytes demonstrate a central organelle-rich area (*thick arrows*) surrounded by an organelle-deplete cyoplasmic rim (*thin arrows*). (B) Megakaryocyte blebs lack the characteristic contents and features of an assembling platelet. (C,D) Blebbing megakaryocytes are frequently observed in the marrow adjacent to blood vessels. EC, endothelial cell; RBC, red blood cell. Scale bars, 2 μm. (E) Comparison of α-granule content in megakaryocyte buds versus mature platelets. TEM images of megkaryocyte buds (n = 32) and mature platelets (n = 26; see Supplementary Fig. S2) were analyzed for α-granule number and total area. α-Granule density (α-granule/μm^2) is shown as mean±SD.

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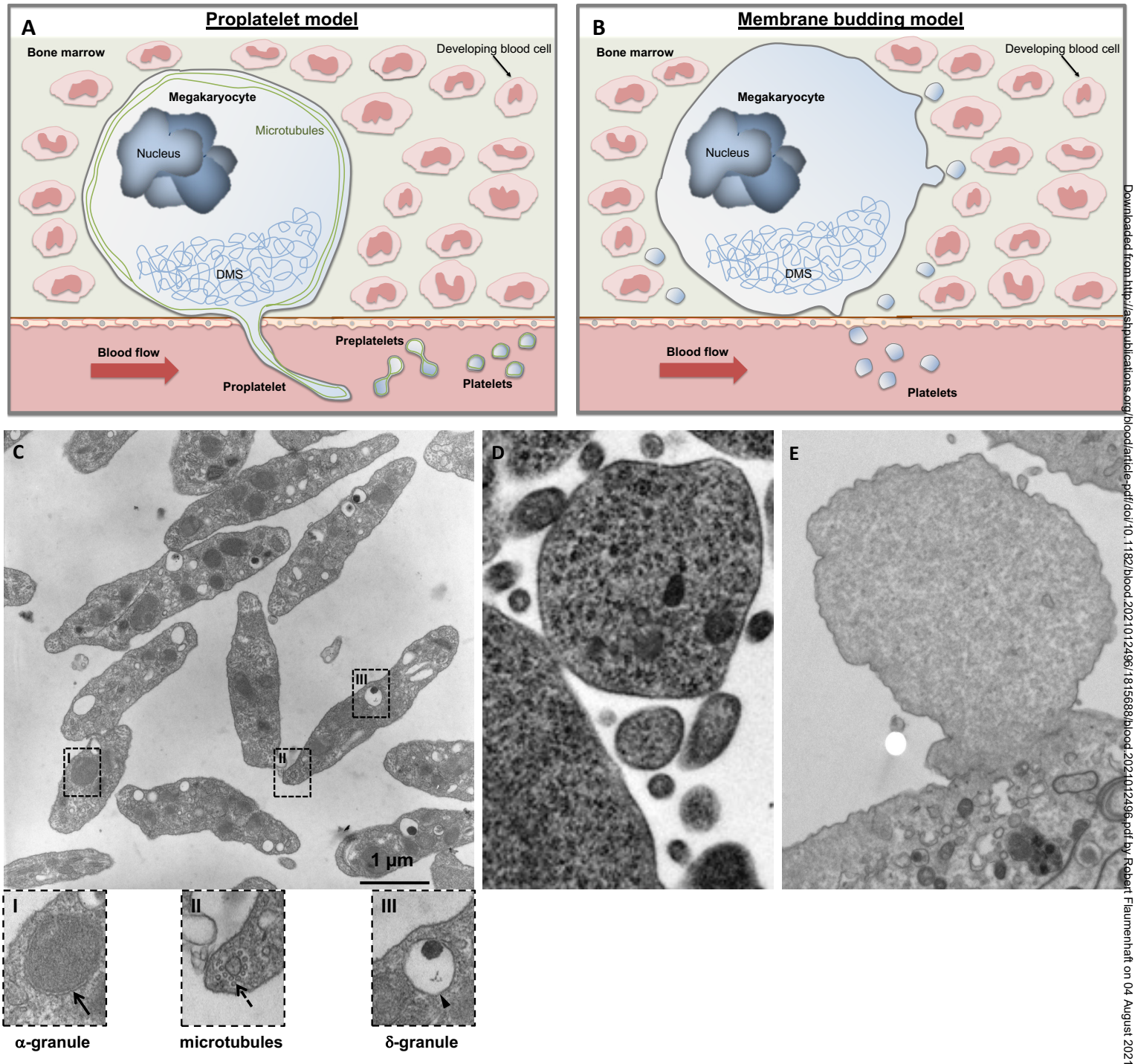
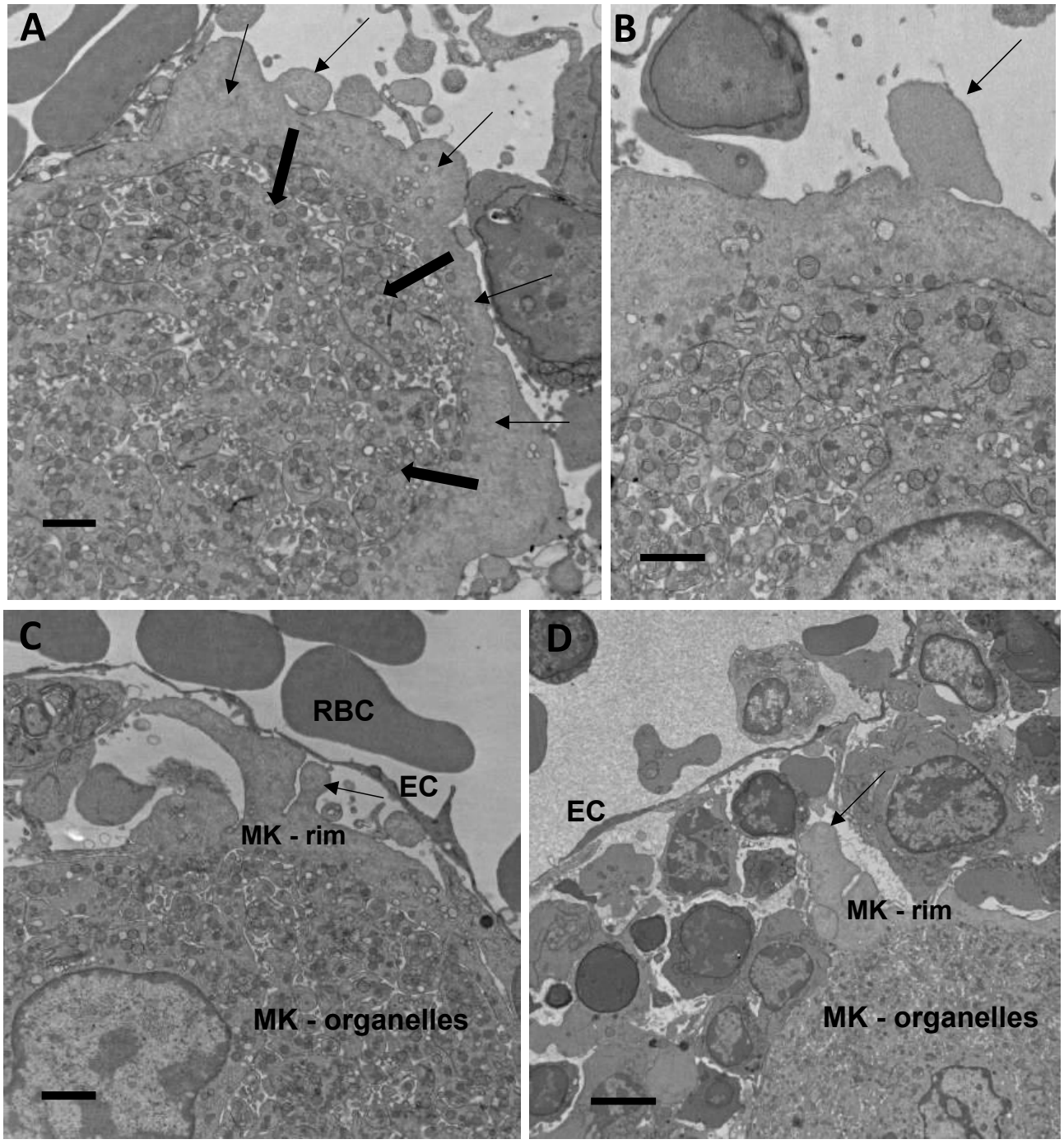


Figure 1



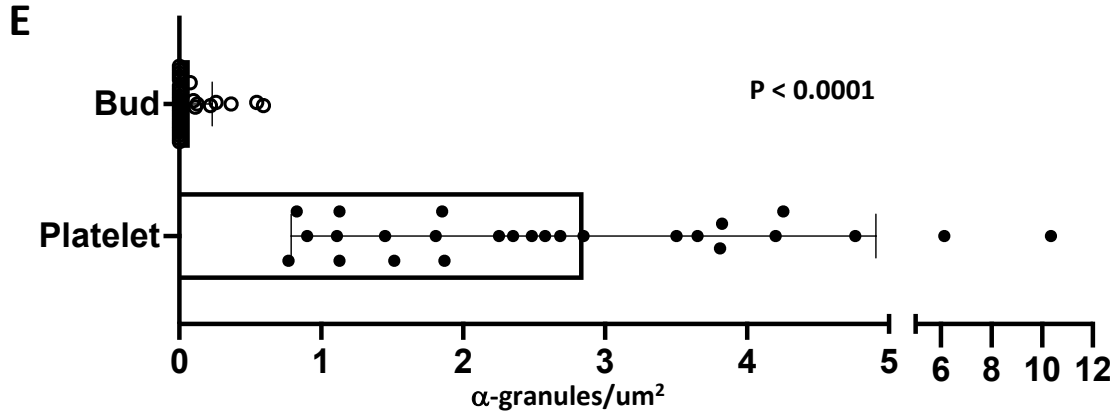


Figure 2