Published in final edited form as:

Biomaterials. 2017 November ; 146: 60–71. doi:10.1016/j.biomaterials.2017.08.006.

Modular flow chamber for engineering bone marrow architecture and function

Christian A. Di Buduo^{a,b}, Paolo M. Soprano^{a,b}, Lorenzo Tozzi^{a,b,c}, Stefania Marconi^d, Ferdinando Auricchio^d, David L. Kaplan^c, and Alessandra Balduini^{a,b,c,*}

^aDepartment of Molecular Medicine, University of Pavia, Pavia, Italy

^bBiotechnology Research Laboratories, IRCCS San Matteo Foundation, Pavia, Italy

^cDepartment of Biomedical Engineering, Tufts University, Medford, MA, USA

^dDepartment of Civil Engineering and Architecture, University of Pavia, Pavia, Italy

Abstract

The bone marrow is a soft, spongy, gelatinous tissue found in the hollow cavities of flat and long bones that support hematopoiesis in order to maintain the physiologic turnover of all blood cells. Silk fibroin, derived from *Bombyx mori* silkworm cocoons, is a promising biomaterial for bone marrow engineering, because of its tunable architecture and mechanical properties, the capacity of incorporating labile compounds without loss of bioactivity and demonstrated ability to support blood cell formation. In this study, we developed a bone marrow scaffold consisting of a modular flow chamber made of polydimethylsiloxane, holding a silk sponge, prepared with salt leaching methods and functionalized with extracellular matrix components. The silk sponge was able to support efficient platelet formation when megakaryocytes were seeded in the system. Perfusion of the chamber allowed the recovery of functional platelets based on multiple activation tests. Further, inhibition of AKT signaling molecule, which has been shown to be crucial in regulating physiologic platelet formation, significantly reduced the number of collected platelets, suggesting the applicability of this tissue model for evaluation of the effects of bone marrow exposure to compounds that may affect platelet formation. In conclusion, we have bioengineered a novel modular system that, along with multi-porous silk sponges, can provide a useful technology for reproducing a simplified bone marrow scaffold for blood cell production *ex vivo*.

Keywords

Silk; Bone marrow; Hematopoiesis; Megakaryocyte; Platelet

*Corresponding author. Department of Biomedical Engineering, Tufts University, 4 Colby Street, Medford, MA 02155, USA. alessandra.balduini@tufts.edu (A. Balduini).

Contributions

Alessandra Balduini: conceived the idea, supervised the project, designed the flow chamber and the experiments, analyzed the data and wrote the manuscript; Christian A. Di Buduo: conceived the idea, designed the flow chamber and the experiments, performed the experiments, analyzed the data and wrote the manuscript; Paolo M. Soprano and Lorenzo Tozzi: performed the experiments; Stefania Marconi and Ferdinando Auricchio: gave technical assistance for 3D printing; David L. Kaplan: analyzed the data and edited the manuscript.

Disclosure of conflicts of interest

The authors declare no competing financial interests.

1. Introduction

Numerous approaches have been made towards development of an “ideal” bone marrow scaffold for blood cell production for transfusions and tissue repair as well as for drug testing *ex vivo* with tissue engineering solution being one of the greatest emerging option to address these needs [1,2].

The bone marrow is a soft, spongy, gelatinous tissue found in the hollow cavities of axial and long bones, which accounts for approximately 5% of the body weight in adult humans and consists of hematopoietic tissue islands and adipose cells surrounded by vascular sinusoids interspersed within a meshwork of trabecular bone [3,4]. In long bones, one or more arteries pass through the cortical bone entering the marrow cavity obliquely, while in flat bones, the bone marrow is served by numerous blood vessels of various sizes entering the marrow via large and small canals [3]. After entry, the artery splits into multiple branches that give rise to a multitude of small thin-walled arterioles and sinusoids that let cells to enter the bloodstream [4–6]. The main bone marrow scaffolding is represented by the components of the extracellular matrix (ECM), which provide, depending on their composition, localization and stiffness, the ideal microenvironment to support hematopoietic stem cell (HSC) differentiation into committed lineages and release of mature cells into blood [7–9].

Research on the bone marrow is currently achieved by bone marrow histopathology which allow the analysis of mature stages of the blood cells differentiation, but not of dynamic processes such as cell migration and platelet production. Further, the quality of the marrow sections is governed by numerous variables related to specimen collection and processing, including fixation, decalcification, embedding, sectioning and staining, which may affect the integrity of the sample [3]. To this regard, laboratory research has intensively investigated *in vitro* culture techniques and animal models to extrapolate invaluable insights into hematopoiesis. However, *in vitro* cultures fail to reproduce the three-dimensional bone marrow architecture, while interspecies differences between humans and common animal models sometimes make it difficult to translate the achieved results.

One of the major challenges for the field is the development of bio-compatible scaffolds that can meet the needs to support platelet formation and functions *ex vivo* [9–11]. To this regard, silk fibroin is a biologically-derived protein polymer purified from domesticated silkworm (*Bombyx mori*) cocoons that has demonstrated excellent properties for biomedical applications, including biocompatibility, robust mechanical strength, and slow, controlled degradation to nontoxic products *in vivo* [12–14]. Further, silk can be processed entirely in aqueous systems using mild, ambient conditions of temperature and pressure, allowing the incorporation of labile compounds, as well as complex fluids, without loss of bioactivity [15,16]. Importantly, silk can be prepared in a range of material formats, including films, hydrogels, microspheres and sponges, already tested in a wide range of tissue engineering challenges, from bone to vessels modeling [17]. In particular, silk sponges are a promising candidate material for development of a bone marrow scaffold because of their demonstrated ability to support megakaryocyte (Mk) function and platelet production [16,18]. Importantly,

the low thrombogenicity, non-toxicity and low-immunogenicity of silk provide a unique and versatile system for reconstituting bone marrow properties for blood cell production.

In this study, we developed a modular chamber design, rapid to manufacture by 3D printing and easy to manage, to hold a silk-based 3D sponge with interconnected and controlled-size pores. The advantages of the system include the presence of inlet and outlet ports for perfusion of medium throughout the scaffold and the tunable properties of the silk biomaterials that can be chemically and mechanically tailored to entrap bioactive molecules while retaining bioavailability. We envision this system as a manageable and versatile tool for culturing cells in a 3D environment under perfusion. The validation of this technology for studying hematopoiesis was achieved by culturing human cord blood-derived MkCs within the system and by collecting released platelets through perfusion into a transfusion bag. Importantly, collected platelets displayed comparable structure, granule content, antigen expression and functionality of human peripheral blood platelets. Thus, the silk-based 3D sponge presents major advantages for studying released platelet phenotype and functionality that are not easily captured with conventional 2D static culture systems.

2. Materials and methods

2.1. Materials

B. mori silkworm cocoons were supplied by Tajima Shoji Co., Ltd. (Yokohama, Japan). Pharmed tubing was from Cole-Parmer (Vernon Hills, IL, USA). Transfer bags for platelet collection were from Fenwal (Mont Saint Guibert, Belgium). Thrombin was from Sigma Aldrich (Saint Louis, MO, USA and Milan, Italy). AKT inhibitor VIII, isozyme-Selectine, AKTI-1/2 was from Calbiochem (La Jolla, CA). Convulxin was from Enzo Life Sciences (Rome, Italy). Type I collagen was purified as described previously [19]. Immunomagnetic separation system was from Miltenyi Biotech (Bergisch Gladbach, Germany and Bologna, Italy). Recombinant human thrombopoietin (TPO) and interleukin 11 (IL-11) were from Peprotech (London, UK). TruCount tubes, human fibronectin and PAC-1 FITC were from Becton Dickinson (S. Jose, CA, USA). 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from BioLegend (London, UK). The following antibodies were used: mouse monoclonal anti-CD61, clone SZ21, from Immunotech (Marseille, France); rabbit monoclonal anti- β 1-tubulin was a kind gift of Prof. Joseph Italiano (Brigham and Women's Hospital, Boston, USA); rabbit polyclonal anti-human von Willebrand Factor (Dako, Milan, Italy); goat polyclonal anti-CD61 (clone C-20) (Santa Cruz Biotechnology, CA, USA); PE mouse monoclonal anti-human CD42b (clone HIP1), FITC mouse monoclonal anti-human CD61 (clone PM6/13) and mouse monoclonal anti-Thrombospondin (clone A4.1) (Abcam, Cambridge, UK); mouse monoclonal anti-CD42b (clone SZ2) (Beckman Coulter, Milan, Italy); rabbit polyclonal anti-fibronectin and mouse monoclonal anti-phosphotyrosine (clone 4G10) (Millopre, Milan, Italy); mouse monoclonal anti-fibronectin (clone EP5) (Santa Cruz Biotech, TX, USA); mouse monoclonal anti- β -actin (clone AC-15) and mouse monoclonal anti- α -tubulin (clone DM1A) (Sigma Aldrich, Milan, Italy); Alexa Fluor-conjugated secondary antibodies and Hoechst 33258 were from Life Technologies (Monza, Italy); anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, California, USA); anti-mouse and anti-rabbit HRP-conjugated secondary

antibody and Precision Plus protein standard (Bio-Rad, Milan, Italy). Enhanced chemiluminescence reagents (ECL) were from Millipore (Milan, Italy).

2.2. Cell culture

Human umbilical cord blood was collected following physiologic pregnancies and deliveries upon informed consent of the parents. All human samples were obtained in accordance with the ethical committee of the IRCCS Policlinico San Matteo Foundation and the principles of the Declaration of Helsinki. Mks were differentiated from human CD34⁺ hemopoietic progenitor cells using previously described methods [20,21]. Briefly, CD34⁺ cells were separated by an immunomagnetic beads technique and cultured for 13 days in Stem Span medium (STEMCELL Technologies, Vancouver, Canada) supplemented with 10 ng/mL TPO, IL-11, 1% penicillin-streptomycin and 1% L-glutamine, at 37 °C in a 5% CO₂ fully-humidified atmosphere.

2.3. Peripheral blood platelet sample preparation

Human whole blood was collected from healthy volunteers, in accordance with the ethical committee of the IRCCS Policlinico San Matteo Foundation and the principles of the Declaration of Helsinki, using citric acid-citrate-dextrose (ACD) as anticoagulant. Human peripheral blood platelets were isolated from whole blood that was centrifuged at 200 × g for 10 min to obtain platelet-rich plasma (PRP). Platelet were finally washed in Tyrode's buffer (134 mM NaCl; 0.34 mM Na₂HPO₄; 2.9 mM KCl; 12 mM NaHCO₃; 20 mM HEPES; 5 mM glucose) in presence of 0.2 U/ml apyrase and 1 μM PGE 1 (Sigma, Milan, Italy) and allowed to rest at room temperature for 1 h, before being used. Morphology and functionality of peripheral blood platelets were analyzed by microscopy, western blotting or flow cytometry, as subsequently described. For immunofluorescence microscopy, whole blood was smeared on a glass microscope slide, air dried and then stain, as subsequently described.

2.4. Modular flow chamber design and 3D printing

A modular flow chamber has been designed to re-create the characteristic features of the bone containing spongy marrow. The chamber consists of one well of 19.8 × 36.0 × 8.2 mm having a hollow cavity of 3.4 × 20 × 5 mm, enclosed in a block of 35.4 × 51.6 × 21.58 mm and connected to the outside of the chamber through two channels of 3.4 mm diameter. Stainless steel needles (10 G) were inserted into the channels, to connect the chamber to the perfusion system (Syringe Pump PHD 2000, Harvard Apparatus, Holliston, MA) at the inlet and a blood collection bag containing acid-citrate-dextrose (ACD) at the outlet. The chamber can be closed by a cover top of 35.4 × 52.0 × 12.2 mm that can be secured by means of plastic tips to be inserted into coaxial holes in the cover and in the chamber. A cover top having a hollow cavity, parallel to the one of chamber, can be used to allow gas exchange into the system during 3D cell culture.

The chamber was manufactured using 3D printing technology and molding technique. We first designed the geometry of the chamber and of the cover, then we created the two respective molds, using CAD software (Solidworks, Dassault Systèmes). Each mold is composed by two interlocking parts, namely a main structure and a base (Supplementary

Fig. 1), in order to aid the cleaning of the 3D printed pieces. The mold components were exported from the CAD software as STL (Standard Triangulation Language) files and 3D printed using a Projet460 Plus (3DSystems), based on binder jetting technology. The 3D printer works using a gypsum-based powder (VisiJet® PXL™) deployed in layers of 100 µm and a binder (VisiJet® PXL™ Clear) to harden the powder and create the final object. After the printing, the remaining powder was accurately cleaned off the molds, which were subsequently treated with an infiltrating adhesive (ColorBond), to increase their mechanical properties. The molding was performed using a polydimethylsiloxane (PDMS) (Sylgard®184, Dow Corning), mixed in a 10:1 ratio of base material and curing agent. The selected material is stable both at low and high temperatures (−45 to 200 °C) and it is resistant to UV, water and solvents. The PDMS was poured into the 3D printed molds that were positioned into a vacuum chamber to remove all the air bubbles. The curing of the PDMS took 48 h at room temperature (about 24 °C); the molds were then separated and the final models were extracted and sterilized by autoclave.

2.5. Silk solution preparation

Silk fibroin aqueous solution was obtained from *B. mori* silkworm cocoons according to previously published literature [22–24]. Briefly, dewormed cocoons were boiled for 10 min in 0.02 M Na₂CO₃ solution at a weight to volume ratio of 5 g to 2 L. The fibers were rinsed for 20 min for three times in ultrapure water and dried overnight. The dried fibers were solubilized for 4 h at 60 °C in 9.3 M LiBr at a weight to volume ratio of 3 g–12 mL. The solubilized silk solution was dialyzed against distilled water using a Slide-A-Lyzer cassette (Thermo Scientific, Waltham, MA, USA) with a 3500 MW cutoff for three days and changing the water a total of eight times. The silk solution was centrifuged at maximum speed for 10 min to remove large particulates and stored at 4 °C. The concentration of the silk solution was determined by drying a known volume of the solution and massing the remaining solids.

2.6. Silk sponge fabrication and assembly

In order to explore silk as a scaffold for reproducing the spongy architecture of bone marrow niche, silk solution (8% w/v) [24] was mixed with 25 µg/mL fibronectin and dispensed into the modular chamber. NaCl particles (approximately 500 µm in diameter) were then sifted into the solution in a ratio of 1 mL–2 g of NaCl particles. The scaffolds were then placed at room temperature for 48 h and then soaked in distilled water for 48 h to leach out the NaCl particles. The scaffolds were sterilized in 70% ethanol, and finally rinsed five times in PBS over 24 h. Silk scaffolds were characterized by confocal and scanning electron microscopy, as subsequently described. The pore size of silk scaffolds was calculated to be slightly smaller than the salt particles used, as the salt is partially dissolved, having an average diameter of 370 ± 115 µm. Perfusion of the silk scaffold was tested at different flow rates (10–1000 µl/min) [25,26]. The total volume collected after each test corresponded to that injected in the system by the pump.

At day 13 of differentiation, $3\text{--}4 \times 10^5$ Mks were seeded into each silk sponge and kept at 37 °C and 5% CO₂. In some experiments, before being seeded, Mks were labeled with 0.5 µM CFSE for 15 min, at room temperature, washed 2 times with culture media and finally

dispensed into silk scaffold. During incubation, a cover top having an inner cavity to allow gas exchange sealed the chamber. After 24 h, the chamber was sealed and the outlet ports were connected to the outlet needles. Culture medium-filled syringes and tubing were connected to the inlet needles. The chamber was placed into the incubator (37 °C and 5% CO₂) and transfer bags for platelet collection containing acid-citrate-dextrose (ACD) were secured to the outlet ports. The syringes were placed into a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA) located outside the bioreactor and media was pumped for 8 h at a flow rate of 90 µl/min [16,18].

2.7. Scanning electron microscopy

Silk scaffolds were fixed in glutaraldehyde 2.5%, followed by a secondary fixation in 1% osmium tetroxide. Samples were then dehydrated in graded ethanol (10-20-50-95-100%) and freeze dried. The scaffolds were coated with platinum/palladium for 60 s before observation. Samples were then imaged by a Zeiss EVO MA10 scanning electron microscope (SEM) (Carl Zeiss, Oberkochen, Germany).

2.8. Immunofluorescence microscopy

For immunofluorescence imaging Mks were labeled with 0.5 µM CFSE as described above. Alternatively, samples were fixed in 4% paraformaldehyde (PFA) for 20 min and then blocked with 5% bovine serum albumin (BSA, Sigma) for 30 min, at room temperature. Samples were probed with anti-CD61 (1:100) or anti-fibronectin (1:100) over night at 4 °C, and then immersed in Alexa Fluor secondary antibody (1:500) for 2 h at room temperature. Nuclei were stained with Hoechst. Samples were imaged by a TCS SP2 or SP8 confocal laser scanning microscope (Leica, Heidelberg, Germany). For silk fibroin scaffolds imaging we took advantage of silk auto-fluorescence in UV light. In some experiments silk fluorescence was brighten by staining with Hoechst [27]. For all immunofluorescence imaging, the acquisition parameters were set on the negative controls. 3D reconstruction and image processing performed using Leica licensed software or Image J software.

2.9. Analysis of megakaryocyte and platelet morphology

For analysis of both culture Mk, peripheral blood and *ex vivo* collected platelet morphology, different approaches were used. First, Mks at the end of differentiation and platelets from peripheral blood or perfused medium were visualized by light microscopy with an Olympus IX53 (Olympus Deutschland GmbH, Hamburg, Germany). For the analysis of cytoskeleton components and granules samples, cells were stained as previously described [28]. Briefly, collected platelets were fixed in 4% PFA and centrifuged onto poly-L-lysine coated coverslip, peripheral blood smears were air-dried, while Mks at the end of differentiation were allowed to adhere overnight, at 37 °C and 5% CO₂, on glass coverslips previously coated with 25 µg/mL fibronectin and then fixed in 4% PFA. Proplatelet forming Mks were identified as cells displaying long filamentous structure ending with platelet-sized tips. All samples were permeabilized with 0.1% Triton X-100 for 5 min and blocked with 5% BSA for 30 min, at room temperature. In order to visualize microtubules organization samples were probed with anti-β1-tubulin (1:1000) or anti-α-tubulin (1:700) for 1 h at room temperature and then immersed in Alexa Fluor secondary antibody (1:500) for 2 h at room temperature. In order to visualize α granules samples were probed with anti-von Willebrand

Factor (1:100) for 1 h at room temperature and then immersed in Alexa Fluor secondary antibody (1:500) for 2 h at room temperature. Samples were mounted onto glass slides with ProLong Gold antifade reagent (Invitrogen, Milan, Italy) and then imaged by an Olympus BX51 fluorescence microscope (Olympus Deutschland GmbH, Hamburg, Germany) or TCS SP2 confocal laser scanning microscope (Leica, Heidelberg, Germany).

For analysis of platelet spreading 25 µg/mL type I collagen was coated onto glass coverslips at 4 °C, overnight. Washed peripheral blood platelets or *ex vivo* produced platelets were allowed to adhere for 30 min at 20 °C, as previously described [29]. Adherent platelets were fixed in 4% PFA, permeabilized with 0.1% Triton X-100 for 5 min, blocked with 5% BSA for 30 min, at room temperature and then probed with anti-β1-tubulin (1:1000) 1 h at room temperature and then immersed in Alexa Fluor secondary antibody (1:500) for 2 h, at room temperature. Samples were mounted onto glass slides with ProLong Gold antifade reagent (Invitrogen, Milan, Italy) and then imaged by an Olympus BX51 fluorescence microscope (Olympus Deutschland GmbH, Hamburg, Germany). For all immunofluorescence imaging the acquisition parameters were set on the negative controls, which were routinely performed by omitting the primary antibody.

2.10. Flow cytometry

Flow cytometry settings for analysis of Mks, peripheral blood and *ex vivo* generated platelets were established, as previously described [30–34]. Collected platelets produced *ex vivo* were analyzed using the same forward and side scatter pattern as human peripheral blood and identified as CD61⁺CD42b⁺ events. Isotype controls were used as a negative control to exclude non-specific background signal. Platelet number was calculated using a Tru-Count bead standard. For the analysis of platelet functionality, platelets were suspended in Tyrode's buffer and allowed to rest for 1 h at room temperature. Samples were then activated with 3 U/mL thrombin for 15 min at 37 °C, in presence of 2.5 mM CaCl₂ and 2.5 mM MgCl₂. Un-stimulated platelets were used to gate for non-activated platelets. All samples were probed with FITC conjugated PAC-1. Cells exhibiting PAC-1 binding were considered functional activated platelets. Importantly, PAC-1 binding was measured using the same fluorescence intensity of human peripheral blood platelets. All samples were acquired with a Beckman Coulter Navios flow cytometer. Off-line data analysis was performed using Beckman Coulter Navios software package.

2.11. Western blotting

In order to evaluate the expression of platelet-specific lineage markers and platelet activation, peripheral blood platelets and *ex vivo* produced platelets were washed in Tyrode's buffer and allowed to rest for 1 h at room temperature. Samples were then activated or not with 3 U/mL thrombin for 15 min or 100 ng/mL convulxin for 60 min, at 37 °C, in presence of 2.5 mM CaCl₂ and 2.5 mM MgCl₂. Samples were lysed with Hepes-glycerol lysis buffer (Hepes 50 mM, NaCl 150 mM, 10% glycerol, 1% Triton X-100, MgCl₂ 1.5 mM, EGTA 1 mM, NaF 10 mM, PMSF 1 mM, Na₃VO₄ 1 mM, 1 µg/mL leupeptin, 1 µg/mL aprotinin) in reducing conditions. For activation experiments platelets were pooled from multiple flow chambers perfused in parallel. Protein lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride

(PVDF) membrane (BioRad, Milan, Italy). Membranes were probed with antibodies against CD61, CD42b, fibronectin, thrombospondin, phosphotyrosine or β -actin following the conditions recommended by the manufacturers. Immunoreactive bands were detected by horseradish peroxidase-labeled secondary antibodies (BioRad), using enhanced chemiluminescence reagent (Merck-Millipore). Pre-stained protein ladders were from BioRad.

2.12. Statistics

Values were expressed as mean plus or minus the standard deviation (mean $\pm$ SD). Student's t-test was performed for paired observations. A value of $p < 0.01$ was considered statistically significant. All experiments were independently replicated at least three times.

3. Results

3.1. Design of the 3D modular chamber

In human adults the medullary cavity, which holds the hematopoietic bone marrow niche, is located inside flat bones and the proximal epiphyses of long bones, protected by a dense outer layer consisting of compact bone [3]. The bone marrow is served by blood vessels that branch out into a multitude of small thin-walled arterioles and sinusoids which allow mature blood cells to enter the bloodstream (Fig. 1A). Within this context, HSCs differentiate into Mks, under control of TPO, which release mature platelets into bone marrow sinusoids (Fig. 1A). In order to mimic this tissue structure, we have designed a modular chamber made of a supporting casket having a central cavity connected to gas-permeable tubing to allow perfusion of medium. A silk-based bone marrow can be patterned directly into the chamber by incorporating a silk 3D structure, prepared with salt leaching methods, to model a spongy scaffold with interconnected pores of 370 ± 115 μ m diameter mimicking bloodstream. Further, a cover, presenting two locks for chamber sealing, was utilized in order to ensure overall protection and sterility to the system (Fig. 1B).

We designed a negative mold of the chamber to be printed with a gypsum-based powder (VisiJet[®] PXL[™]) and a binder (VisiJet[®] PXL[™] Clear), and we used PDMS, an optically clear, inert and non-toxic polymeric organosilicon already employed in a wide range of medical applications, to finally shape the system (Fig. 2A). To test the functionality of the resulting chamber, a porous silk scaffold was assembled in the central cavity (Fig. 2B) and connected to heat-treatable stainless steel needles at the inlet and outlet ports (Fig. 3Ai). The cover top was secured with plastic tips (Fig. 3Aii) and the chamber connected to an electronic syringe pump to allow perfusion of the system (Fig. 3Aiii). The chamber was able to efficiently support diffusion of the perfused medium throughout the silk scaffold and outflow, from 10 up to 1000 μ l/min without affecting shape and integrity of the silk scaffold (Fig. 3B). Of note, the total volume collected after perfusion of the silk scaffold corresponded to that injected in the system by the pump, thus no restraints to the flow were exerted by the scaffold.

3.2. The functionalized silk scaffold supports 3D megakaryocyte culture

To test ability of the developed bone marrow model to support blood cell functions, we took advantage of our expertise in human Mk culture and analysis of platelet production [9,16,18]. Human cord blood-derived HSCs were differentiated in presence of TPO towards the megakaryocytic lineage, seeded at day 13 of differentiation within the system and sealed with a cover top having a hollow cavity, parallel to the silk sponge, to allow gas exchange into the system (Fig. 4A and Supplementary Fig. 2). The silk scaffold was functionalized with 25 $\mu\text{g/mL}$ fibronectin, an ECM component known to support Mk adhesion and platelet formation [20,35]. The porous structure of the resulting silk scaffold was not affected by functionalization (Fig. 4Aii, iii and Supplementary Video 1) compared to un-functionalized control (Fig. 3Aii, iii and Supplementary Video 2), as assessed by SEM and confocal microscopy. The 3D reconstruction of functionalized silk scaffold demonstrated that fibronectin was mainly distributed as silk surface (Fig. 4Bi–iv, Supplementary Fig. 3B and Supplementary Video 3,4) allowing efficient Mk adhesion upon seeding into the system (Fig. 4Bv). Importantly, the high pore interconnectivity of the functionalized silk scaffold efficiently supported rapid soaking of the structure, from the bottom of the sponge throughout the entire construct (Fig. 4Ci), thus supporting a homogeneous Mk distribution, as assessed 5 min after seeding by immunofluorescence confocal microscopy analysis of CFSE⁺ samples (Fig. 4Cii–v).

Supplementary videos related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2017.08.006>.

3.3. The perfusion of the modular chamber allows physiologic platelet formation and release

After 24 h incubation, the system was locked, connected to a syringe pump at the inlet and to a gas-permeable collection bag, containing acid citrate dextrose (ACD) as anticoagulant, at the outlet, and placed into an incubator at 37 °C and 5% CO₂ for additional 8 h (Fig. 5A). Confocal microscopy imaging of the 3D culture before starting perfusion demonstrated the presence of the characteristic cytoplasmic rearrangements of proplatelet forming-Mks (Fig. 5Bi), with the extension of multiple proplatelet shafts assembling nascent platelet at their terminal ends (Supplementary Fig. 4), thus demonstrating that the 3D culture conditions efficiently supported physiologic Mk function. When culture medium was perfused at ~90 $\mu\text{L/min}$ through the system, the flow allowed the detachment of platelet-like particles from the proplatelet shaft extending from Mk adhering to silk scaffold into the lumen of the silk pores (Fig. 5Bii). Imaging of the perfused scaffold at different time frames demonstrated the presence of released platelets dispersed within the lumen of the silk pores, while Mks remained adherent to the scaffold (Fig. 5Biii–iv). Accordingly, few Mks were present into the effluent with respect to the sample seeded in the silk sponge at the beginning of the experiment (Supplementary Fig. 5).

3.4. Ex vivo produced platelets present physiologically-relevant structure

The characterization of released particles by light microscopy (Fig. 6Ai–iv) and immunofluorescence staining (Fig. 6Av–vii) highlighted the presence of all known characteristic stages of platelet morphology after shedding from the proplatelet shaft,

namely big pre-platelets intermediates of $>4\ \mu\text{m}$ diameter, dumbbell-shaped platelets, and little disc-shaped platelets of $2\text{--}4\ \mu\text{m}$ diameter. Importantly, all platelets were showing the $\beta 1$ -tubulin coil at their periphery (Fig. 6Av–vii), typically present in peripheral blood platelets (Supplementary Fig. 6A). Further, the collected platelets were stained with anti-CD61 and anti-CD42b antibodies and analyzed by flow cytometry. Platelets exhibited expression of both lineage-specific markers (Fig. 6B), with same forward (FSC) and side scatter (SSC) of peripheral blood platelet control (Supplementary Fig. 6B). Western blot analysis of platelet lysates confirmed expression of surface antigens and demonstrated also the presence of the α -granules proteins fibronectin and thrombospondin (Fig. 6C), normally expressed by peripheral blood platelets, used as positive control (Supplementary Fig. 6C). α -granules were also imaged by immunofluorescence staining of von Willebrand Factor and compared to cultured Mks and peripheral blood platelets (Supplementary Fig. 7). Interestingly, the presence of α -granules in Mks and proplatelets demonstrated that they are delivered directly from Mk cytoplasm, along the proplatelet shaft, into nascent platelets [36].

Of note, pre-treatment with a selective inhibitor of AKT, a signaling molecule which is known to prevent proplatelet formation [18,35,37], significantly reduced the number of collected platelets, thus demonstrating that the observed platelet release and collection within the system could be attributed to active proplatelet formation and extension, rather than to passive detachment due to perfusion of medium (Fig. 6D).

3.5. Ex vivo collected platelets are physiologically functional

We next proceeded to analyze the functionality of *ex vivo* produced platelets. The $\beta 1$ -tubulin staining demonstrated no relevant cytoskeleton organization in resting platelets (Fig. 7Ai), while, after seeding onto immobilized type I collagen, platelets actively reassembled their micro-tubules and spread (Fig. 7Aii). Platelet function was further assessed by stimulation with 3 U/ml thrombin (THR). The treatment determined a marked increased activation of $\alpha_{\text{IIb}}\beta_3$ integrin, as assessed by flow cytometry analysis of PAC-1 binding (Fig. 7B). In parallel, activation of intracellular signaling was demonstrated by the increased phosphorylation of tyrosine residues, evidenced by Western blot analysis, in protein lysates of samples treated with 3 U/ml THR or 100 ng/mL convulxin (CVX), with respect to unstimulated platelets (Fig. 7C). For all functional tests, peripheral blood platelets were used as positive control (Supplementary Fig. 8).

4. Discussion

Bone marrow is a spongy tissue where HSCs support the physiologic homeostasis of all blood cells [4–6]. There is a critical need for bioengineering scaffold that are able to reproduce key features of the physiological bone marrow environment for functional blood cell generation and screening of therapeutic compounds *ex vivo* [1]. In this paper, we propose a simple design to recapitulate the microarchitecture of the bone marrow to sustain platelet production from Mks. We fabricated a flow chamber, reproducing the bone surrounding the marrow, with a central cavity connected to an inlet and an outlet to allow perfusion of the system. A silk sponge was made to fit the central cavity of the chamber mimicking the spongy structure of the bone marrow. Perfusion of the silk sponge was made

possible by the interconnection of the pores resulting from the salt leaching preparation. Moreover, silk was functionalized with ECM components that are expressed in the physiological bone marrow structure [7]. Importantly, it was previously shown that silk preserves the activity of the encapsulated molecules [13,15,16].

To demonstrate that this new system reproduces the basic features of the 3D bone marrow structure, *in vitro* differentiated Mks were seeded within the system for 24 h prior to perfusion and collection of released platelets into a transfusion bag positioned at the outlet of the chamber. Collected platelets showed the same morphology as peripheral blood platelets and comparable expression of the principal platelet receptors as demonstrated by both flow cytometry and Western blot analysis. Most importantly, the collected platelets expressed fibronectin, thrombospondin and vWF, proteins normally present in platelet α -granules. Consistently, an increase of tyrosine phosphorylation was observed upon thrombin and convulxin activation. Together, these data demonstrate that collected platelets displayed a physiological structure and function *in vitro*. Finally, inhibition of AKT prevented platelet generation in the silk sponge proving that Mk function within the system may respond to the addition of external soluble factors. Thrombocytopenia is one of the most frequent hematologic adverse events in different cancer treatments, including those involving Perifosine, an oral AKT inhibitor [38]. It is known that AKT inhibition prevents proplatelet formation without affecting Mk viability [8,39,40], however, the impact of such treatment on bone marrow niche has yet to be determined. Based on our results, we expect that the new multi flow chamber will provide a new technology for pharmacological studies aimed at predicting outcomes of therapies that may affect Mk functions.

The search for biomaterials that can support platelet formation and functions *in vitro* remains a major challenge for the field. Polydimethylsiloxane (PDMS) has been recently used to fabricate three different bioreactors based on different perfusion systems to promote platelet production. The first one consisted in a scaffold with two different media flows to maximize platelet production [41], while the second device consisted of two channels, separated by a series of columns to trap Mks entering the upper channel allowing proplatelet extensions and platelet release in the bottom chamber [42]. Both these devices enabled real-time visualisation of Mks during culture and released platelet collection. Finally, a third system, made by PDMS, was developed with a wide array of von Willebrand Factor-coated micropillars supposed to act as anchors on Mks, allowing them to extend proplatelets under a hydrodynamic shear [11]. Compared to these systems for platelet production, a critical feature of our flow chamber is the use of natural silk protein biomaterial to leverage its versatility such as programmable mechanical properties, and surface binding of growth factors and ECM components, while retaining their bioavailability [13,16]. In our previous BM model we used silk tubes and sponges to recreate the vascular niche where Mks release platelets [16]. In the current system the goal was to provide an easy to assemble and manageable tool to expose Mks to a 3D environment, functionalized with ECM components, and collect platelets through perfusion of the entire silk-sponge. Our hypothesis is that this system may be useful for scaling up platelet production as well as for studying drug responses and new therapeutic targets.

In conclusion, we developed a simple system to mimic the essential features of the 3D bone marrow structure and composition to support platelet generation from Mks. The combination of 3D printed scaffolds with silk biomaterial allows the development of a versatile and tailored platform for *ex vivo* platelet production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr. Rita Maccario and Dr. Margherita Massa for technical assistance with the flow cytometry analysis; Dr. Cesare Perotti for supplying human cord blood; “Centro Grandi Strumenti” of the University of Pavia, and Dr. Patrizia Vaghi for technical assistance with confocal microscopy; Dr. Alessandro Pecci and Dr. Valeria Bozzi for supplying samples for peripheral blood platelets imaging; Prof. Joseph Italiano for providing β 1-tubulin antibody; Dr. Daniela Parigi for helping in designing the 3D chamber; Dr. Michele Conti for technical assistance with 3D printer; Dr. Xuan Mu for helpful discussion on flow modeling. This paper was supported by Cariplo Foundation (2012-0529, 2013-0717), ERA-Net for Research Programmes on Rare Diseases (EUPLANE) and US National Institutes of Health (R01 EB016041-01). Christian A. Di Buduo fellowship was funded by Collegio Ghislieri, Pavia, progetto “Progressi in Biologia e Medicina”. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

- 1 Balduini A, Di Buduo CA, Kaplan DL. Translational approaches to functional platelet production *ex vivo*. *Thromb Haemost*. 2016; 115(2):250–256. [PubMed: 26353819]
- 2 Di Buduo CA, Kaplan DL, Balduini A. In vitro generation of platelets: where do we stand? *Transfus Clin Biol*. 2017 pii: S1246-7820(17)30094-0.
- 3 Travlos GS. Normal structure, function, and histology of the bone marrow. *Toxicol Pathol*. 2006; 34(5):548–565. [PubMed: 17067943]
- 4 Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature*. 2014; 505(7483):327–334. [PubMed: 24429631]
- 5 Wang LD, Wagers AJ. Dynamic niches in the origination and differentiation of haematopoietic stem cells. *Nat Rev Mol Cell Biol*. 2011; 12(10):643–655. [PubMed: 21886187]
- 6 Stegner D, vanEeuwijk JMM, Angay O, Gorelashvili MG, Semeniak D, Pinnecker J, Schmithausen P, Meyer I, Friedrich M, Dütting S, Brede C, Beilhack A, Schulze H, Nieswandt B, Heinze KG. Thrombopoiesis is spatially regulated by the bone marrow vasculature. *Nat Commun*. 2017; 8(1):127. [PubMed: 28743899]
- 7 Malara A, Currao M, Gruppi C, Celesti G, Viarengo G, Buracchi C, Laghi L, Kaplan DL, Balduini A. Megakaryocytes contribute to the bone marrow-matrix environment by expressing fibronectin, type IV collagen, and laminin. *Stem Cells*. 2014; 32(4):926–937. [PubMed: 24357118]
- 8 Abbonante V, Di Buduo CA, Gruppi C, De Maria C, Spedden E, De Acutis A, Staii C, Raspanti M, Vozzi G, Kaplan D, Moccia F, Ravid K, Balduini A. A new path to platelet production through matrix sensing. *Haematologica*. 2017; 102(7):1150–1160. [PubMed: 28411253]
- 9 Currao M, Malara A, Di Buduo CA, Abbonante V, Tozzi L, Balduini A. Hyaluronan based hydrogels provide an improved model to study megakaryocyte-matrix interactions. *Exp Cell Res*. 2016; 346(1):1–8. [PubMed: 26027944]
- 10 Thon JN, Mazutis L, Wu S, Sylman JL, Ehrlicher A, Machlus KR, Feng Q, Lu S, Lanza R, Neeves KB, Weitz DA, Italiano JE. Platelet bioreactor-on-a-chip. *Blood*. 2014; 124(12):1857–1867. [PubMed: 25606631]
- 11 Blin A, Le Goff A, Magniez A, Poirault-Chassac S, Teste B, Sicot G, Nguyen KA, Hamdi FS, Reyssat M, Baruch D. Microfluidic model of the platelet-generating organ: beyond bone marrow biomimetics. *Sci Rep*. 2016; 6:21700. [PubMed: 26898346]

- 12Omenetto FG, Kaplan DL. New opportunities for an ancient material. *Science*. 2010; 329(5991): 528–531. [PubMed: 20671180]
- 13Kluge JA, Li AB, Kahn BT, Michaud DS, Omenetto FG, Kaplan DL. Silk-based blood stabilization for diagnostics. *Proc Natl Acad Sci U S A*. 2016; 113(21):5892–5897. [PubMed: 27162330]
- 14Wray LS, Tsioris K, Gi ES, Omenetto FG, Kaplan DL. Slowly degradable porous silk microfabricated scaffolds for vascularized tissue formation. *Adv Funct Mater*. 2013; 23(274): 3404–3412. [PubMed: 24058328]
- 15Lu Q, Wang X, Hu X, Cebe P, Omenetto F, Kaplan DL. Stabilization and release of enzymes from silk films. *Macromol Biosci*. 2010; 10(4):359–368. [PubMed: 20217856]
- 16Di Buduo CA, Wray LS, Tozzi L, Malara A, Chen Y, Ghezzi CE, Smoot D, Sfara C, Antonelli A, Spedden E, Bruni G, Staii C, De Marco L, Magnani M, Kaplan DL, Balduini A. Programmable 3D silk bone marrow niche for platelet generation ex vivo and modeling of megakaryopoiesis pathologies. *Blood*. 2015; 125(14):2254–2264. [PubMed: 25575540]
- 17Rockwood DN, Preda RC, Yücel T, Wang X, Lovett ML, Kaplan DL. Materials fabrication from *Bombyx mori* silk fibroin. *Nat Protoc*. 2011; 6(10):1612–1631. [PubMed: 21959241]
- 18Di Buduo CA, Currao M, Pecci A, Kaplan DL, Balduini CL, Balduini A. Revealing Eltrombopag's promotion of human megakaryopoiesis through AKT/ERK-dependent pathway activation. *Haematologica*. 2016; 101(12):1479–1488. [PubMed: 27515246]
- 19Tenni R, Sonagere M, Viola M, Bartolini B, Tira ME, Rossi A, Orsini E, Ruggeri A, Ottani V. Self-aggregation of fibrillar collagens I and II involves lysine side chains. *Micron*. 2006; 37(7):640–647. [PubMed: 16714119]
- 20Balduini A, Pallotta I, Malara A, Lova P, Pecci A, Viarengo G, Balduini CL, Torti M. Adhesive receptors, extracellular proteins and myosin IIA orchestrate proplatelet formation by human megakaryocytes. *J Thromb Haemost*. 2008; 6(11):1900–1907. [PubMed: 18752571]
- 21Balduini A, Badalucco S, Pugliano MT, Baev D, De Silvestri A, Cattaneo M, Rosti V, Barosi G. In vitro megakaryocyte differentiation and proplatelet formation in Ph-negative classical myeloproliferative neoplasms: distinct patterns in the different clinical phenotypes. *PLoS One*. 2011; 6(6):e21015. [PubMed: 21698292]
- 22Kim UJ, Park J, Li C, Jin HJ, Valluzzi R, Kaplan DL. Structure and properties of silk hydrogels. *Biomacromolecules*. 2004; 5(3):786–792. [PubMed: 15132662]
- 23Li C, Vepari C, Jin HJ, Kim HJ, Kaplan DL. Electrospun silk-BMP-2 scaffolds for bone tissue engineering. *Biomaterials*. 2006; 27(16):3115–3124. [PubMed: 16458961]
- 24Lovett M, Cannizzaro C, Daheron L, Messmer B, Vunjak-Novakovic G, Kaplan DL. Silk fibroin microtubes for blood vessel engineering. *Biomaterials*. 2007; 28(35):5271–5279. [PubMed: 17727944]
- 25Mizuno S, Allemann F, Glowacki J. Effects of medium perfusion on matrix production by bovine chondrocytes in three-dimensional collagen sponges. *J Biomed Mater Res*. 2001; 56(3):368–375. [PubMed: 11372054]
- 26Porter B, Zauel R, Stockman H, Guldberg R, Fyhrie D. 3-D computational modeling of media flow through scaffolds in a perfusion bioreactor. *J Biomech*. 2005; 38(3):543–549. [PubMed: 15652553]
- 27Talukdar S, Nguyen QT, Chen AC, Sah RL, Kundu SC. Effect of initial cell seeding density on 3D-engineered silk fibroin scaffolds for articular cartilage tissue engineering. *Biomaterials*. 2011; 32(34):8927–8937. [PubMed: 21906805]
- 28Di Buduo CA, Alberelli MA, Glembocky AC, Podda G, Lev PR, Cattaneo M, Landolfi R, Heller PG, Balduini A, De Candia E. Abnormal proplatelet formation and emperipoiesis in cultured human megakaryocytes from gray platelet syndrome patients. *Sci Rep*. 2016; 6:23213. [PubMed: 26987485]
- 29Abbonante V, Chitalia V, Rosti V, Leiva O, Matsuura S, Balduini A, Ravid K. Up-regulation of lysyl oxidase and adhesion to collagen of human megakaryocytes and platelets in primary myelofibrosis. *Blood*. 2017; 130(6):829–831. [PubMed: 28592432]
- 30Cramer EM, Norol F, Guichard J, Breton-Gorius J, Vainchenker W, Massé JM, Debili N. Ultrastructure of platelet formation by human megakaryocytes cultured with the Mpl ligand. *Blood*. 1997; 89(7):2336–2346. [PubMed: 9116277]

- 31Fujimoto TT, Kohata S, Suzuki H, Miyazaki H, Fujimura K. Production of functional platelets by differentiated embryonic stem (ES) cells in vitro. *Blood*. 2003; 102(12):4044–4051. [PubMed: 12920021]
- 32Takayama N, Nishikii H, Usui J, Tsukui H, Sawaguchi A, Hiroshima T, Eto K, Nakauchi H. Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors. *Blood*. 2008; 111(11):5298–5306. [PubMed: 18388179]
- 33Nakamura S, Takayama N, Hirata S, Seo H, Endo H, Ochi K, Fujita K, Koike T, Harimoto K, Dohda T, Watanabe A, Okita K, Takahashi N, Sawaguchi A, Yamanaka S, Nakauchi H, Nishimura S, Eto K. Expandable megakaryocyte cell lines enable clinically applicable generation of platelets from human induced pluripotent stem cells. *Cell Stem Cell*. 2014; 14(4):535–548. [PubMed: 24529595]
- 34Abbonante V, Di Buduo CA, Gruppi C, Malara A, Gianelli U, Celesti G, Anselmo A, Laghi L, Vercellino M, Visai L, Iurlo A, Moratti R, Barosi G, Rosti V, Balduini A. Thrombopoietin/TGF- β 1 loop regulates megakaryocyte extracellular matrix component synthesis. *Stem Cells*. 2016; 34(4): 1123–1133. [PubMed: 26748484]
- 35Di Buduo CA, Moccia F, Battiston M, De Marco L, Mazzucato M, Moratti R, Tanzi F, Balduini A. The importance of calcium in the regulation of megakaryocyte function. *Haematologica*. 2014; 99(4):769–778. [PubMed: 24463213]
- 36Richardson JL, Shivdasani RA, Boers C, Hartwig JH, Italiano JE. Mechanisms of organelle transport and capture along proplatelets during platelet production. *Blood*. 2005; 106(13):4066–4075. [PubMed: 16118320]
- 37Björquist A, Di Buduo CA, Femia EA, Storey RF, Becker RC, Balduini A, Nylander S, Cattaneo M. Studies of the interaction of ticagrelor with the P2Y₁₃ receptor and with P2Y₁₃-dependent proplatelet formation by human megakaryocytes. *Thromb Haemost*. 2016; 116(6)
- 38Richardson PG, Eng C, Kolesar J, Hideshima T, Anderson KC. Perifosine, an oral, anti-cancer agent and inhibitor of the Akt pathway: mechanistic actions, pharmacodynamics, pharmacokinetics, and clinical activity. *Expert Opin Drug Metab Toxicol*. 2012; 8(5):623–633. [PubMed: 22512706]
- 39Balduini A, Di Buduo CA, Malara A, Lecchi A, Rebuzzini P, Currao M, Pallotta I, Jakubowski JA, Cattaneo M. Constitutively released adenosine diphosphate regulates proplatelet formation by human megakaryocytes. *Haematologica*. 2012; 97(11):1657–1665. [PubMed: 22689668]
- 40Badalucco S, Di Buduo CA, Campanelli R, Pallotta I, Catarsi P, Rosti V, Kaplan DL, Barosi G, Massa M, Balduini A. Involvement of TGF β 1 in autocrine regulation of proplatelet formation in healthy subjects and patients with primary myelofibrosis. *Haematologica*. 2013; 98(4):514–517. [PubMed: 23403314]
- 41Nakagawa Y, Nakamura S, Nakajima M, Endo H, Dohda T, Takayama N, Nakauchi H, Arai F, Fukuda T, Eto K. Two differential flows in a bioreactor promoted platelet generation from human pluripotent stem cell-derived megakaryocytes. *Exp Hematol*. 2013; 41(8):742–748. [PubMed: 23618622]
- 42Thon JN, Mazutis L, Wu S, Sylman JL, Ehrlicher A, Machlus KR, Feng Q, Lu S, Lanza R, Neeves KB, Weitz DA, Italiano JE. Platelet bioreactor-on-a-chip. *Blood*. 2014; 124(12):1857–1867. [PubMed: 25606631]

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2017.08.006>.

Fig. 1. Design of the flow chamber mimicking the bone marrow niche

(A) Bone marrow is a spongy tissue, contained in the hollow cavities of flat and long bones, composed by a network of sinusoids and ECMs, surrounding maturing blood cells. Within this context hematopoietic stem cells (HSCs) undergo self-renewal as well as differentiation into all committed lineages. Thrombopoietin (TPO) specifically promotes HSC commitment and differentiation toward megakaryocytes (Mks). Mature Mks are typically found close to endothelial cells lining bone marrow sinusoids, where they extend multiple long pseudopods, called proplatelets, that assemble platelets at their terminal ends. The release of mature platelets can be attributed to blood hydrodynamics which allow their final shedding.

(B) In order to mimic bone architecture, we designed a modular chamber made of a supporting casket having a central cavity connected to gas-permeable tubing to allow perfusion of medium and a cover top to ensure sterility to the system. The bone marrow model can be patterned directly into the chamber by incorporating a silk-based spongy scaffold with interconnected pores mimicking bone marrow microcirculation.

Fig. 2. Manufacturing of the bone marrow model

(A) The negative molds of the supporting scaffold and of the cover top were printed with a gypsum-based powder (VisiJet® PXL™) by a ProJet460 Plus printer based on binder jetting technology. Then, polydimethylsiloxane (PDMS) was used to finally shape the system (scale bars = 1 cm). (B) A silk sponge was prepared inside the chamber by dispensing an aqueous silk solution (i), mixed with salt particles (ii). After leaching out the salt, the resulting porous silk sponge can be sterilized (iii) (scale bars = 1 cm).

Fig. 3. Testing the functionality of the bone marrow model

(A) The flow chamber was connected to heat-treatable stainless steel needles at the inlet and outlet ports (i) and the cover top was secured with plastic tips (ii). Then the system was connected to an electronic syringe pump to allow perfusion of culture medium (iii) at different flow rate (scale bars = 2 cm). (B) The chamber supported diffusion of the perfused medium throughout the silk scaffold and outflow, from 10 up to 1000 $\mu\text{l}/\text{min}$ without affecting shape and integrity of the silk scaffold.

Fig. 4. Silk scaffold functionalized with human fibronectin supports megakaryocyte adhesion
 (A) To test ability of the bone marrow model to support human Mk function, the silk scaffold was functionalized with 25 $\mu\text{g/mL}$ fibronectin. During Mk culture the chamber was sealed with a cover top having a hollow cavity, parallel to the silk scaffold, to allow gas exchange into the system. (Ai) The resulting silk scaffold was analyzed by Scanning Electron Microscopy (SEM) (scale bar = 500 μm) and (Aii) immunofluorescence confocal microscopy (blue = silk; scale bar = 200 μm). (Bi) 3D confocal microscopy reconstruction of functionalized silk scaffold showing fibronectin distribution (blue = silk; red = fibronectin; scale bar = 200 μm). (Bii, iii) z-stacks of the functionalized silk scaffold analysis (blue = silk; red = fibronectin; scale bar = 200 μm). (Biv) Orthogonal cross-section of one representative z-stack from functionalized silk scaffold analysis revealed fibronectin staining at silk surface (blue = silk; red = fibronectin; scale bar = 200 μm). (Bv) Confocal microscopy analysis of CFSE⁺ Mks adhering onto functionalized silk scaffold (green = Mks; red = fibronectin; scale bar = 200 μm). (Ci) Representative picture of functionalized silk scaffold upon Mk-containing medium pipetting into the system (scale bar = 0.5 cm). (Cii-v) Confocal microscopy imaging of different fields of the silk scaffold analyzed immediately after CFSE⁺ Mks seeding (green = Mks; blue = silk; scale bar = 100 μm). Of note the porous structure of the silk scaffold allowed rapid soaking of the entire system and Mks were

homogenously distributed throughout the scaffold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5. Flow chamber perfusion and *ex vivo* produced platelets recovery

(A) After 24 h incubation, the system was locked, connected to a syringe pump at the inlet and to a gas-permeable collection bag, containing anticoagulant, at the outlet, and placed into an incubator at 37 °C and 5% CO₂ for additional 8 h (scale bars = 2 cm). (B) Confocal microscopy analysis of mature Mks seeded into the silk sponge. (Bi) 3D Mk culture imaged before starting perfusion. Boxes highlight Mks in adhesion to silk scaffold, elongating proplatelet shafts, which assemble nascent platelets at their terminal ends, within the hollow space of silk pores (green = Mks, blue = silk) (scale bars = 50 μm). (Bii) Confocal microscopy analysis of a Mk-forming proplatelet into the lumen of a silk pore during perfusion of the system at a flow rate of ~90 μl/min (scale bar = 10 μm). (Biii, iv) Confocal microscopy analysis of platelet particles released into perfused medium at different time frames after starting perfusion (2 and 5 h, respectively). The box highlights the detachment of platelet-like particles from the proplatelet shaft and platelets released into the silk pore lumen (scale bars = (iii) 50 μm and (iv) 20 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Characterization of *ex vivo* produced platelets

The modular flow chamber was perfused with culture media for 8 h and released platelets collected into gas-permeable bags. (Ai–iv) Light microscopy analysis of the collected medium demonstrated presence of large pre-platelets, dumbbells and little discoid platelets (scale bars = 10 μ m). (Av–vii) Immunofluorescence staining of β 1-tubulin (red) highlighted the presence of the microtubule coil typically present in resting platelets (scale bars = 5 μ m). (C) Flow cytometry analysis of expression of CD61 and CD42b surface markers. (D) Western blotting analysis of CD61, CD42b, fibronectin (FNC) and thrombospondin (TSP) in cell lysates from collected samples (MW = molecular weight). (D) Platelet count was assessed by flow cytometry by mixing samples with counting beads. Mk pre-treatment with 10 μ m AKT inhibitor (AKT inh) significantly reduced the number of collected platelets (scale bar = 2 cm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Analysis of *ex vivo* produced platelets functionality

(Ai) β 1-tubulin staining of resting platelets. (Aii) After adhesion on type I collagen platelets spread and showed the reorganization of microtubules. Magnification highlights β 1-tubulin assembly upon adhesion with respect to resting state (scale bars = 5 μ m). (B) Flow cytometry analysis of collected platelet activation before and after treatment with 3U/mL thrombin (THR) demonstrated increased PAC-1 binding indicating physiologic CD41 activation. (C) Parallel analysis of platelet lysates by western blotting evidenced increased phosphorylation of tyrosine residues in response to 3U/mL thrombin or 100 ng/mL convulxin (CVX), indicating activation of intracellular signaling upon exposure to activating stimuli (MW = molecular weight). β -actin has been used to ensure equal loading.