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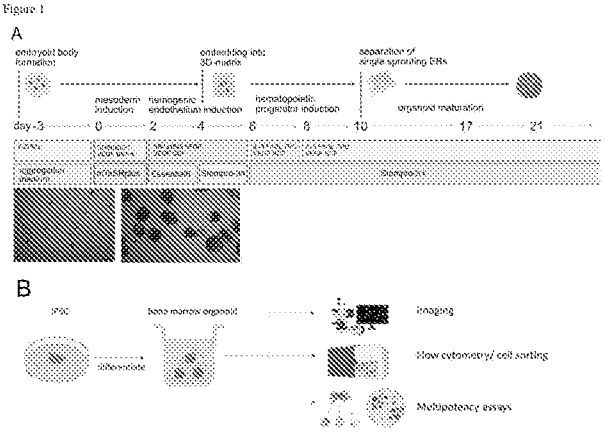
(54) **Bone marrow organoids produced from induced pluripotent stem cells and uses of these organoids.**

(57) The present invention relates to a method for producing mammalian vascular networks or mature mammalian bone marrow organoid organoids. Furthermore provided are uses of the vascular network or mammalian bone marrow organoids as produced for use in the treatment of bone marrow related diseases, for the in-vitro production of BMOs or mammalian blood cells, as a model system in the pathogenesis of a bone marrow related disease, and as a system for identifying and/or testing pharmaceutically effective compounds for treating or preventing of a bone marrow related disease.

Figure 1



Bone marrow organoids produced from induced pluripotent stem cells and uses of these organoids

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Background of the invention

Human postnatal hematopoiesis takes place in the bone marrow and involves a strictly regulated process of constant differentiation of hematopoietic stem cells (HSC) into mature blood cells while maintaining an HSC pool through self-renewal. The surrounding microenvironment is called the bone marrow niche and consists of a heterogeneous cell population, including mesenchymal cells (e.g. pericytes, adipocytes) and endothelial cells. The niche of the bone marrow plays an important role in regulating and maintaining hematopoiesis throughout the whole life (1).

A dense vascular network within the bone marrow is essential because it supplies other niche cells with nutrients, growth factors, and critical cell-cell-interactions. In addition, endothelial cells covered by perivascular PDGFRß+ mesenchymal cells (pericytes) directly promote hematopoietic homeostasis through the secretion of various factors; therefore, the HSCs are often located in the immediate vicinity of the blood vessels (2).

Impaired hematopoiesis can be caused by specific genetic mutations and manifests itself in the case of germline mutations in the form of congenital bone marrow failure (IBMFS), such as, for example, severe congenital neutropenia, or in somatic mutations in the form

of diseases such as myelodysplasia and leukemia. Interestingly, disruption of the bone marrow microenvironment has been shown to trigger myelodysplasia (3).

Human organoid models have been established in recent years as a model system for studying the development of diseases of various tissues. Organoids are self-organized 3D structures that mimic the most important functions and structural organization of organs and can be differentiated from induced pluripotent stem cells (iPSC). Organoids are partly superior to conventional 2D cultures, because they mimic the natural environment of certain cells through cell-cell interactions as well as cell-matrix interactions (7, Hofer, M., Lutolf, M.P. Engineering organoids. *Nat Rev Mater* **6,** 402–420 (2021). https://doi.org/10.1038/s41578-021-00279-y).

Organoid formation and maturation are preceded by a single cell or small cell-cluster expansion and reorganization. There are two main types of organoids based upon the choice of stem cells. The first is derived from PSCs that include both embryonic stem cells (ESCs) and iPSCs and the second type is derived from organ-specific adult stem cells (ASCs). A variety of workflows have been developed to generate organoids; however, specialized organoid types require unique culture methods, and not all general workflows are appropriate. The choices of cell culture conditions and the 3D matrix are critical for this complex organization.

WO2019122388A1 discloses co-cultures of organoids and immune cells, and methods of using these to identify agents for treating diseases.

Breunig et al. (Differentiation of human pluripotent stem cells into pancreatic duct-like organoids. STAR Protoc. 2021 Dec 8;2(4):100913. doi: 10.1016/j.xpro.2021.100913. PMID: 34917972; PMCID: PMC8669107) describe a scalable *in vitro* differentiation protocol to guide human pluripotent stem cells stepwise into pancreatic duct-like organoids. The protocol mimics pancreatic duct development and was successfully used to model the onset and progression of pancreatic ductal adenocarcinoma; the approach is suitable for multiple downstream applications. However, the protocol is cost- and time-intensive.

Vallmajo-Martin, Q., et al. (in: PEG/HA Hybrid Hydrogels for Biologically and Mechanically Tailorable Bone Marrow Organoids. Adv. Funct. Mater. 2020, 30, 1910282. https://doi.org/10.1002/adfm.201910282) disclose that bone marrow (BM) organoids provide powerful tools to study the vital interplay between the BM microenvironment and resident cells. A transglutaminase (TG) crosslinked system that seamlessly incorporates poly(ethylene glycol) (PEG) and hyaluronic acid (HA) into hybrid hydrogels for the formation of BM analogues is presented. Utility of the TG-PEG/HA hybrid hydrogels to maintain, expand, or differentiate human bone marrow-derived stromal cells and human hematopoietic stem and progenitor cells in vitro is demonstrated. TG-PEG/HA hybrid hydrogels are described as superior to currently used natural biomaterials in forming humanized BM organoids in a xenograft model. The engineered humanized BM organoids as presented may be effective tools for the study of this intricate organ.

Isern J, et al. (in Self-renewing human bone marrow mesenspheres promote hematopoietic stem cell expansion. Cell Rep. 2013 May 30;3(5):1714-24. doi: 10.1016/j.celrep.2013.03.041. Epub 2013 Apr 25. PMID: 23623496) discuss strategies for expanding hematopoietic stem cells (HSCs) include coculture with cells that recapitulate their natural microenvironment, such as bone marrow stromal stem/progenitor cells (BMSCs). Plastic-adherent BMSCs may be insufficient to preserve primitive HSCs. They describe a method of isolating and culturing human BMSCs as nonadherent mesenchymal spheres. Human mesenspheres were derived from CD45- CD31- CD71- CD146+ CD105+ nestin+ cells but could also be simply grown from fetal and adult BM CD45--enriched cells. Human mesenspheres robustly differentiated into mesenchymal lineages. In culture conditions where they displayed a relatively undifferentiated phenotype, with decreased adherence to plastic and increased self-renewal, they promoted enhanced expansion of cord blood CD34+ cells through secreted soluble factors. Expanded HSCs were serially transplantable in immunodeficient mice and significantly increased long-term human hematopoietic engraftment. They discuss the way for culture techniques that preserve the self-renewal of human BMSCs and their ability to support functional HSCs.

Janagama and Hui (in: 3-D Cell Culture Systems in Bone Marrow Tissue and Organoid Engineering, and BM Phantoms as In Vitro Models of Hematological Cancer Therapeutics-A Review. *Materials (Basel)*. 2020;13(24):5609. Published 2020 Dec 9. doi:10.3390/ma13245609) review the state-of-the-art in bone and marrow tissue engineering (BMTE) and hematological cancer tissue engineering (HCTE) in light of the recent interest in bone marrow environment and pathophysiology of hematological cancers. They focus on engineered BM tissue and organoids as in vitro models of hematological cancer therapeutics, along with identification of BM components and their integration as synthetically engineered BM mimetic scaffolds. In addition, the review details interaction dynamics of various BM and hematologic cancer (HC) cell types in co-culture systems of engineered BM tissues/phantoms as well as their relation to drug resistance and cytotoxicity. Interaction between hematological cancer cells and their niche, and the difference with respect to the healthy niche microenvironment narrated. Future perspectives of BMTE for in vitro disease models, BM regeneration and large-scale ex vivo expansion of hematopoietic and mesenchymal stem cells for transplantation and therapy are explained. They conclude by overviewing the clinical application of biomaterials in BM and HC pathophysiology and its challenges and opportunities.

Bessy T (in: Bioengineering the Bone Marrow Vascular Niche. *Front Cell Dev Biol.* 2021;9:645496. Published 2021 Apr 28. doi:10.3389/fcell.2021.645496) provide another comprehensive review with a focus on engineering vascularized BM niche models, and summarize current approaches including bioengineered microfluidic chips.

Cornelia Lee-Thedieck, et al. (in: The extracellular matrix of hematopoietic stem cell niches, Advanced Drug Delivery Reviews, Volume 181, 2022, 114069, https://doi.org/10.1016/j.addr.2021.114069) review that hematopoietic stem cells (HSCs) are the life-long source of all types of blood cells. Their function is controlled by their direct microenvironment, the HSC niche in the bone marrow. Although the importance of the extracellular matrix (ECM) in the niche by orchestrating niche architecture and cellular function is widely acknowledged, it is still underexplored. In the review, they provide a comprehensive overview of the ECM in HSC niches. For this purpose, they briefly outline HSC niche biology and then review the role of the different classes of ECM molecules in the niche one by one and how they are perceived by cells. Matrix

remodeling and the emerging importance of biophysics in HSC niche function are discussed. Finally, the application of the current knowledge of ECM in the niche in form of artificial HSC niches for HSC expansion or targeted differentiation as well as drug testing is reviewed.

Since mouse models often do not fully recapitulate the human phenotype due to differences in hematopoiesis between mice and humans, an alternative approach is needed to investigate the genetic causes and mechanisms that lead to bone marrow disease. Previous studies on in vitro modelling of the niche in the human bone marrow rely on the use of primary endothelial cells and mesenchymal cells, but these do not reflect the multicellular complexity of the natural niche system, and broader applications are limited by the supply and limited lifespan of these cells (4-6).

As seen from the above, there is an unmet need for new in vitro approaches that replicate hematopoiesis in a complex bone marrow-like niche system to study hematopoietic diseases and develop new therapies. It is therefore an object of the present invention, to provide respective approaches and methods that are used to establish suitable systems in order to study hematopoietic diseases and develop new therapies. Other objects and advantages will become apparent to the person of skill upon studying the present description of the invention at hand.

In a first aspect thereof, the present invention solves the above problem by providing a method for producing mature mammalian bone marrow organoids, comprising the steps of a) generating embryoid bodies from substantially single induced pluripotent stem cells (iPSCs) obtained from at least one mammal, comprising culturing single iPSCs in an aggregation medium in the presence of at least one Rho-associated protein kinase (ROCK)-inhibitor for about 1 day, followed by culturing the embryoid bodies in a suitable serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells without ROCK-inhibitor for about 48 hours; b) inducing mesoderm in said embryoid bodies as formed in step a), comprising culturing said embryoid bodies in a suitable serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells, wherein the medium is supplemented with 80 ng/ml Bone morphogenetic protein 4 (BMP4), 4 μM of

glycogen synthase kinase (GSK) 3 inhibitor, and 80 ng/ml Vascular Endothelial Growth Factor (VEGF), for about 48 hours, with resuspension of the mesoderm-induced embryoid bodies at about every 24 hours; c) replacing the medium of the culture of b) with Essential 6-medium, supplemented with 80 ng/ml VEGF, 25 ng/ml fibroblast growth factor (FGF)-2, 50 ng/ml stem cell factor (SCF), and 2 μM SB431542 for about 48 hours, with resuspension of the embryoid bodies at about every 24 hours and gentle shaking; d) embedding of the mesoderm-induced embryoid bodies of step c) into a suitable polymerized 3D collagen I/Matrigel® matrix followed by overlaying the matrix with StemPro®-34 medium supplemented with 80 ng/ml VEGF, 25 ng/ml FGF-2, 50 ng/ml SCF and 2 μM SB431542 for about 48 hours, followed by cytokine replacement with 50 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO for about 48 hours, and a cytokine boost to 25 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO for about 48 hours; e) extracting individual vascular networks that have been generated in step d) followed by culturing in StemPro®-34 medium, supplemented with 25 ng/ml VEGF, 50 ng/ml SCF, 50 ng/ml IL-3, 50 ng/ml Flt-3L and 5 ng/ml TPO, with refreshing the medium every 3 to 4 days for about 7 to 11 days, whereby the mature mammalian bone marrow organoids are produced. Preferably, the mammalian bone marrow organoids are mature organoids, i.e., they show the physiological main characteristics of the respective tissue(s) *in vivo*.

In a second aspect thereof, the present invention solves the above problem by providing a vascular network or mammalian bone marrow organoid, preferably a mature organoid, produced according to the method according to the present invention, or a pharmaceutical composition comprising the vascular network and/or mature mammalian bone marrow organoid according to the present invention. Furthermore provided are the vascular network or mammalian bone marrow organoid, preferably a mature organoid, produced according to the method according to the present invention, or a pharmaceutical composition comprising the vascular network and/or mature mammalian bone marrow organoid according to the present invention for use in the treatment of diseases.

In a third aspect thereof, the present invention solves the above problem by providing the use of the vascular network or mammalian bone marrow organoid or the pharmaceutical composition according to the present invention as a model system in the pathogenesis of

a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions.

In a fourth aspect thereof, the present invention solves the above problem by providing the use of the vascular network or mammalian bone marrow organoid or the pharmaceutical composition according to the present invention as a model system for identifying and/or testing pharmaceutically effective compounds for treating or preventing of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions. Another aspect of this embodiment is a method for screening a pharmaceutically effective compound for treating or preventing of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions, comprising the use of the vascular network or mammalian bone marrow organoid or the pharmaceutical composition according to the present invention as a model system.

In a fifth aspect thereof, the present invention solves the above problem by providing the use of the vascular network or mammalian bone marrow organoid or the pharmaceutical composition according to the present invention for the *in-vitro* production of BMOs or mammalian blood cells, in particular autologous BMOs or mammalian blood cells, in particular for transplantation purposes.

In a sixth aspect thereof, the present invention solves the above problem by providing the pharmaceutically effective amount of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical composition according to the present invention for use in the treatment of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers,

anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions, preferably for transplantation. Another aspect of this embodiment is a method for treating a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions, comprising administering to a subject in need thereof a pharmaceutically effective amount of the vascular network or mammalian bone marrow organoid or the pharmaceutical composition according to the present invention. Preferably, the vascular network or mammalian bone marrow organoid or the pharmaceutical composition according to the present invention is administered as transplant, e.g., an autologous transplant. Another aspect of this embodiment is a method for transplanting the vascular network or mammalian bone marrow organoid or the pharmaceutical composition according to the present invention into a mammalian subject in need thereof, preferably in order to treat a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and/or complications from chemotherapy or transfusions.

The inventors present a novel approach to generate bone marrow organoids (BMOs) (the term shall wherever possible include both bone marrow organoids and mature bone marrow organoids as produced) that mimic important structural and cellular features of the human bone marrow niche. These BMOs are generated exclusively from human induced pluripotent stem cells (hiPSCs) and have a hematopoietic, stromal and vascular compartment. The inventors show that this system induces the formation of mature blood cells of the myeloid lineage. Furthermore, the inventors can show that the stromal compartment consists of mesenchymal stem cells and progenitor cells, such as CXCL12-rich reticular (CAR) cells and perivascular cells, while the vascular compartment consists of self-assembling, connected and lumen-forming endothelial cells.

Khan et al. (in: Human bone marrow organoids for disease modelling, discovery and validation of therapeutic targets in hematological malignancies, bioRxiv

2022.03.14.483815; doi: https://doi.org/10.1101/2022.03.14.483815) describe a step-wise, directed-differentiation protocol in which organoids are generated from iPSCs committed to mesenchymal, endothelial and hematopoietic lineages. These 3-dimensional structures were reported to capture key features of human bone marrow - stroma, lumen-forming sinusoidal vessels and myeloid cells including pro-platelet forming megakaryocytes. The organoids were reported to support the engraftment and survival of cells from patients with blood malignancies, including cancer types notoriously difficult to maintain *ex vivo*. Fibrosis of the organoid occurred following TGFβ stimulation and engraftment with myelofibrosis but not healthy donor-derived cells. Khan et al. do not mention exact concentrations as used in their protocol. Furthermore, no Wnt- activator (e.g. CHIR99021) or Nodal-Inhibitior (e.g. SB431542) was used, which has been shown to be important for mesodermal patterning leading to induction of definitive hematopoiesis. Also, lineage-directed cytokines, such as EPO, IL-6 and G-CSF were used, which prevents the possibility to study intrinsic cytokine signaling within the bone marrow organoids, and likely presents an obstacle for modelling of genetic bone marrow failure syndromes, due to some possible rescue effect of these cytokines.

Therefore, the inventors developed an improved protocol that allows for a reliable production of mammalian bone marrow organoids, in particular mature mammalian bone marrow organoids derived solely from iPSCs and composed of blood cells and various niche cells such as endothelial cells and mesenchymal cells. The method according to the present invention comprises the steps of a) generating embryoid bodies from substantially single induced pluripotent stem cells (iPSCs) obtained from at least one mammal, comprising culturing single iPSCs in an aggregation medium in the presence of at least one Rho-associated protein kinase (ROCK)-inhibitor for about 1 day, followed by culturing the embryoid bodies in a suitable serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells without ROCK-inhibitor for about 48 hours; b) inducing mesoderm in said embryoid bodies as formed in step a), comprising culturing said embryoid bodies in a suitable serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells, wherein the medium is supplemented with 80 ng/ml Bone morphogenetic protein 4 (BMP4), 4 μM of glycogen synthase kinase (GSK)

3 inhibitor, and 80 ng/ml Vascular Endothelial Growth Factor (VEGF), for about 48 hours, with resuspension of the mesoderm-induced embryoid bodies at about every 24 hours; and gentle shakingc) replacing the medium of the culture of b) with Essential 6-medium, supplemented with 80 ng/ml VEGF, 25 ng/ml fibroblast growth factor (FGF)-2, 50 ng/ml stem cell factor (SCF), and 2 μM SB431542 for about 48 hours, with resuspension of the embryoid bodies at about every 24 hours; d) embedding of the mesoderm-induced embryoid bodies of step c) into a suitable polymerized 3D collagen I/Matrigel® matrix followed by overlaying the matrix with StemPro®-34 medium supplemented with 80 ng/ml VEGF, 25 ng/ml FGF-2, 50 ng/ml SCF and 2 μM SB431542 for about 48 hours, followed by cytokine replacement with 50 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO for about 48 hours, and a cytokine boost to 25 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO for about 48 hours; e) extracting individual vascular networks that have been generated in step d) followed by culturing in StemPro®-34 medium, supplemented with 25 ng/ml VEGF, 50 ng/ml SCF, 50 ng/ml IL-3, 50 ng/ml Flt-3L and 5 ng/ml TPO, with refreshing the medium every 3 to 4 days for about 7 to 11 days, whereby the mammalian bone marrow organoids are produced. Preferred is the method for producing mammalian bone marrow organoids according to the present invention, further comprising the step of culturing the mature organoids for a maximum of about 47 days.

The invention provides a vascularized BMO preparation or system derived exclusively from iPSCs (in particular hiPSCs), mimicking hematopoiesis in a multicellular context of native bone marrow, comprising blood cells and various bone marrow “niche cells”, such as endothelial cells and mesenchymal cells. The system allows the modeling of bone-marrow related diseases, i.e. the possibility of studying the interaction between niche cells and hematopoietic cells in the pathogenesis of hematological diseases. This can be furthermore achieved in the context of drug testing, i.e., studying the effects of drugs on the system, which allows the testing of new therapies in the inventive complex mammalian, e.g., human, in-vitro model system. Possible is also an implementation in a high-throughput format, for example in a 96-well-plate format. Furthermore, the “products” as generated can be used in therapeutic applications, i.e., bone-marrow related diseases, such as hematological diseases. The method provides the possibility of a transplantation of human BMOs as produced or the improved *in vitro* generation of

human blood cells from iPSCs, e.g., autologous patient-specific production, and subsequent infusion or transplantation.

Until now, the ex-vivo expansion of HSCs for stem cell transplantations was not possible. Advantageously, the vascular network or mature mammalian bone marrow organoid according to the present invention maintain stemness of hematopoietic stem cells (HSC), making it possible to keep HSC *in vitro* for further quality control studies and to sort only those cells for therapeutic application that show desired molecular changes without any unwanted side effects.

Preferred is the method for producing mammalian bone marrow organoids according to the present invention, further comprising the step of fixing the vascular networks in step e) and performing immunofluorescence analysis and/or dissociating the vascular networks into individual cells and performing flow cytometry analysis. This can be done in order to characterize the components of the networks as developed, and/or to study any effects of drugs to be tested (see also below).

Preferred is the method for producing mammalian bone marrow organoids according to the present invention, further comprising the step of fixing the (mature) organoids as produced, and performing immunofluorescence analysis and/or dissociating the mature organoids into individual cells and performing flow cytometry analysis. This can be done in order to characterize the components of the organoids (e.g. the maturation) as developed, and/or to study any effects of drugs to be tested (see also below).

In the first step of the method according to the present invention, embryoid bodies are generated from substantially single/individual induced pluripotent stem cells (iPSCs) obtained from at least one mammal. Individual cells can be obtained by several ways, for example by mechanical dissociation (e.g., using a pipette or the like). Nevertheless, preferred is the method for producing mammalian bone marrow organoids according to the present invention, wherein the single/individual iPSCs are provided by dissociating iPSC cells into single cells with Accutase® digestion, which is a more gentle procedure. Accutase® is gentle on cells and auto-inhibits at 37°C without the need for a neutralizing solution, like with trypsin. Accutase® is commercially available from Sigma, and works

for all mammalian iPSCs. The method further comprises culturing individual/single iPSCs in an aggregation medium in the presence of at least one Rho-associated protein kinase (ROCK)-inhibitor for about 1 day.

Preferred is the method for producing mammalian bone marrow organoids according to the present invention, wherein the aggregation medium is KnockOut DMEM/F12 (Thermo Fisher) with 20% serum substitute, 1% L-glutamine, 1% non-essential amino acids, 1% penicillin-streptomycin, and 100µM β-mercaptoethanol.

In the method for producing mammalian bone marrow organoids according to the present invention, in the first step, the cells are furthermore cultured in the presence of at least one Rho-associated protein kinase (ROCK)-inhibitor. ROCK inhibition enables maintenance of stem cell phenotype; its effects on metabolism are unknown.

Preferred is the method for producing mammalian bone marrow organoids according to the present invention, wherein the ROCK-inhibitor is selected from the group consisting of Y-27632 ((1R,4r)-4-((R)-1-aminoethyl)-N-(pyridin-4-yl)cyclohexanecarboxamide), fasudil, and a TS-f ROCK-inhibitor as disclosed in Shen et al. (Shen, M., Tian, S., Pan, P. *et al.* Discovery of Novel ROCK1 Inhibitors *via* Integrated Virtual Screening Strategy and Bioassays. *Sci Rep* **5,** 16749 (2015). https://doi.org/10.1038/srep16749, herewith incorporated by reference), in particular TS-f5 or TS-f22. Other suitable ROCK-inhibitors are known to the person of skill and are disclosed in the respective literature.

Usually and preferably, step a) is performed for 60 to 84 hours, preferably for about 72 hours, i.e., about 3 days, as mentioned above, the medium is changed to a suitable medium (e.g., TeSRplus) without Rock-inhibitor after about 24 hours.

In the context of the present invention, the term “about” shall mean a deviation form a given value of +/- 10%, unless indicated otherwise.

In the context of the present invention, culturing is generally done at about 37°C, unless indicated otherwise.

In the second step of the method according to the present invention, mesoderm is induced in said embryoid bodies as formed in step a). The step comprises culturing the embryoid bodies in a suitable serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells, and human iPSCs.

Preferably, the serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells is mTeSR® Plus medium (STEMCELL Technologies, Fisher Scientific). The medium is supplemented with 80 ng/ml Bone morphogenetic protein 4 (BMP4), 4 µM of glycogen synthase kinase (GSK) 3 inhibitor, and 80 ng/ml Vascular Endothelial Growth Factor (VEGF).

Inhibition of GSK3 e.g., by CHIR99021, promotes mESC self-renewal by stabilizing cytoplasmic β-catenin, an essential component of the canonical Wnt signaling pathway, abrogating TCF3-mediated transcriptional repression of pluripotency-associated genes including Oct4, Nanog, Tfcp2l1, and Esrrb. Therefore, preferred is the method for producing mammalian bone marrow organoids according to the present invention, wherein the glycogen synthase kinase (GSK) 3 inhibitor is CHIR99021 or NPD13432, an aurone derivative (Hiroki Kobayashi, et al. A novel GSK3 inhibitor that promotes self-renewal in mouse embryonic stem cells, *Bioscience, Biotechnology, and Biochemistry*, Volume 84, Issue 10, 2 October 2020, Pages 2113–2120, https://doi.org/10.1080/09168451.2020.1789445). The glycogen synthase kinase (GSK) 3 inhibitor may be combined with a MEK inhibitor (e.g., PD0325901, called "2i") which supports the long-term self-renewal of mouse embryonic stem cells (mESCs), while blockade of the MEK/ERK pathway by PD0325901 increases the expression of Nanog, Tfcp2l1, and Klf4 in mESCs, thereby promoting self-renewal.

Usually and preferably, step b) is performed for 36 to 60 hours, preferably for about 48 hours, i.e., about 2 days, with gentle mixing or resuspension of the mesoderm-induced embryoid bodies at about every 24 hours.

In the third step of the method according to the present invention, the medium of the culture of step b) is replaced with Essential 6-medium, supplemented with 80 ng/ml VEGF, 25 ng/ml fibroblast growth factor (FGF)-2, 50 ng/ml stem cell factor (SCF), and

a suitable inhibitor of the TGF-β/Activin/NODAL pathway, preferably 2 μM SB431542 (STEMCELL Technologies). SB431542 is a selective and potent inhibitor of the TGF-β/Activin/NODAL pathway that inhibits ALK5 (IC_{50} = 94 nM), ALK4 (IC_{50} = 140 nM), and ALK7 by competing for the ATP binding site. It does not inhibit the BMP type I receptors ALK2, ALK3, and ALK6. Essential 6 Medium (Thermo Fisher) is a feeder-free and xeno-free medium that supports the reprogramming of somatic cells and the spontaneous or directed differentiation of human pluripotent stem cells (PSCs).

Usually and preferably, step c) is performed for 36 to 60 hours, preferably for about 48 hours, i.e., about 2 days, with gentle mixing or resuspension of the embryoid bodies at about every 24 hours.

The fourth step of the method according to the present invention comprises the embedding of the mesoderm-induced embryoid bodies as generated in step c). Embedding the embryoid bodies into a suitable polymerized 3D matrix prevents the embryoid bodies from sinking to the bottom of the dish or well, which would hinder or impart the further development into mature embryoid bodies and vascular structures. The extracellular matrix (ECM) further provides biochemical cues and structural support, such as porosity and stiffness which mediates signaling for cell migration, cell behavior and polarization in organoid structures. In general, any suitable polymerized 3D matrix may be used. The present invention preferably uses a collagen I/Matrigel® matrix bottom layer in the culturing vessel (e.g., a vial, dish or well). For example, 500 μl/well of collagen I-Matrigel® mixture is first prepared and polymerized at 37° C. for about 1 hour as the lowest layer. Then, embryoid bodies were then resuspended in 500 μl/well of the same collagen-I-Matrigel mixture and a second layer was prepared, which was also polymerized at 37° C. for about 1 hour. The two layers were then overlayed with a suitable medium, such as, for example, StemPro®-34 medium, supplemented with 80 ng/ml VEGF, 25 ng/ml FGF-2, 50 ng/ml SCF and a suitable inhibitor of the TGF-β/Activin/NODAL pathway as above, preferably 2 μM SB431542 (STEMCELL Technologies). StemPro®-34 SFM (e.g., from Thermo Fisher) is a serum-free medium specifically formulated to support the development of human hematopoietic cells in culture (Burridge PW, et al. A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. PLoS One.

2011 Apr 8;6(4):e18293. doi: 10.1371/journal.pone.0018293. PMID: 21494607; PMCID: PMC3072973).

Usually and preferably, the first part of step d) is performed for 36 to 60 hours, preferably for about 48 hours, i.e., about 2 days. Then, a cytokine replacement followed in the same medium with 50 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO. Usually and preferably, the second part of step d) is performed for 36 to 60 hours, preferably for about 48 hours, i.e., about 2 days. Finally, a cytokine boost followed in the same medium to 25 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO, Usually and preferably, the third part of step d) is performed for 36 to 60 hours, preferably for about 48 hours, i.e., about 2 days. At the end of the fourth step of the method according to the present invention, vascular networks are generated.

In the fifth step of the method according to the present invention, individual vascular networks that have been generated in step d) are extracted/isolated, e.g., transferred into one well of a low-attachment 96-well-plate, followed by culturing vascular networks in StemPro®-34 medium, supplemented with 25 ng/ml VEGF, 50 ng/ml SCF, 50 ng/ml IL-3, 50 ng/ml Flt-3L and 5 ng/ml TPO for about 6 to 8 days, preferably about 7 days. The medium is refreshed every about 3 to 4 days.

Then, the mature mammalian bone marrow organoids according to the present invention are produced. The present method advantageously uses Wnt- activators (e.g., CHIR99021) and Nodal-Inhibitiors (e.g., SB431542) which are important for mesodermal patterning leading to induction of definitive hematopoiesis, and lineage-directed cytokines, such as EPO, IL-6 and G-CSF are avoided.

Preferred is the method for producing mammalian bone marrow organoids according to the present invention, wherein the ratio of the 3D collagen I to Matrigel® in the matrix as used is about 4:1, preferably at 4:1. The Matrigel®, in which the organoids are embedded, is derived from a mouse Engelbreth-Holm-Swarm sarcoma cell line, which hampers cGMP-compliant manufacturing of BMOs for the moment. Xenogeneic-free synthetic scaffold materials have been developed and tested for organoid differentiation (reviewed in Aisenbrey EA, Murphy WL. Synthetic alternatives to Matrigel. Nat Rev

Mater. 2020 Jul;5(7):539–51), which can be advantageously included in the method according to the present invention.

Further preferred is the method for producing mammalian bone marrow organoids according to the present invention, wherein the resuspension of the mesoderm-induced embryoid bodies comprises the use of a pipette.

Preferred is the method for producing mammalian bone marrow organoids according to the present invention, wherein the dissociating and/or extracting and isolating of individual vascular networks is mechanically, and preferably comprises the use of sterile dissection tools.

In the method for producing mammalian bone marrow organoids according to the present invention, the mammal is preferably selected from the group consisting of a human, a mouse, a monkey, a rat, a pig, a dog, a cat, a rabbit, a sheep, cattle, an equine, and a goat. Most preferred is a human, thus providing hiPSC.

Possible is also an implementation of the methods according to the present invention in a high-throughput format, for example in a 96-well-plate format.

The methods according to the present invention may furthermore comprise the step of testing and/or analyzing the BMOs as produced for their suitability as a pharmaceutical preparation for transplantation and other treatment purposes.

The methods according to the present invention may furthermore comprise the step of isolating cells and cell types from the BMOs as produced. In this context, the BMOs can be used as source of functional endothelial cells, pericytes, hematopoietic stem cells, and mesenchymal stem cells. Preferred is a method according to the present invention, further comprising the step of isolating mesenchymal stem cells ($CD45^-$ $CD31^-$ $CD34^-$ $CD90^+$ $CD105^+$ $CD271^+$ $CD73^+$) or HSCs. This can be preferably done by cell sorting, e.g., FACS. In the context of the present invention, it was found that in trilineage differentiation assays. FACS-sorted MSCs had the capacity to differentiate into osteogenic, chondrogenic and adipogenic cells, which was visualized by Alizarin-Red-S,

Alcian-blue and Oilred-O staining, respectively. Sorted BMO-derived MSCs expanded in culture and displayed serial replating capacity.

Yet another aspect of the present invention then relates to a preparation of HSCs or MSCs as produced.

Yet another aspect of the present invention then relates to a vascular network or mature mammalian bone marrow organoid, produced according to the method according to the present invention, or a pharmaceutical composition comprising the vascular network and/or mature mammalian bone marrow organoid. Furthermore provided are the vascular network or mammalian bone marrow organoid, preferably a mature organoid, produced according to the method according to the present invention, or a pharmaceutical composition comprising the vascular network and/or mature mammalian bone marrow organoid according to the present invention for use in the treatment of diseases.

Pharmaceutical compositions as used may optionally comprise a pharmaceutically acceptable carrier. The person skilled in the art knows suitable formulations for cells and cellular products and will readily be able to choose suitable pharmaceutically acceptable carriers or excipients, depending, e.g., on the formulation and administration route of the pharmaceutical composition.

Pharmaceutically acceptable carriers or excipients include diluents (fillers, bulking agents, e.g. lactose, microcrystalline cellulose), disintegrants (e.g. sodium starch glycolate, croscarmellose sodium), binders (e.g. PVP, HPMC), lubricants (e.g. magnesium stearate), glidants (e.g. colloidal SiO_2), solvents/co-solvents (e.g. aqueous vehicle, Propylene glycol, glycerol), buffering agents (e.g. citrate, gluconates, lactates), preservatives (e.g. Na benzoate, parabens (Me, Pr and Bu), BKC), anti-oxidants (e.g. BHT, BHA, Ascorbic acid), wetting agents (e.g. polysorbates, sorbitan esters), thickening agents (e.g. methylcellulose or hydroxyethylcellulose), sweetening agents (e.g. sorbitol, saccharin, aspartame, acesulfame), flavoring agents (e.g. peppermint, lemon oils, butterscotch, etc.), humectants (e.g. propylene, glycol, glycerol, sorbitol). Other suitable pharmaceutically acceptable excipients are inter alia described in Remington's Pharmaceutical Sciences, 15th Ed., Mack Publishing Co., New Jersey (1991) and Bauer

et al., Pharmazeutische Technologic, 5th Ed., Govi-Verlag Frankfurt (1997).

The pharmaceutical composition can be administered in any suitable way, e.g. in the form of solutions, syrups, emulsions or suspensions. Administration is preferably carried out by transfusion, e.g., in the form of injections or infusions.

In addition to the aforementioned products of the invention, the pharmaceutical composition can contain further customary, usually inert carrier materials or excipients. Thus, the pharmaceutical preparations can also contain additives, such as, for example, fillers, extenders, disintegrants, binders, glidants, wetting agents, stabilizers, emulsifiers, preservatives, sweetening agents, colorants, flavorings or aromatizers, buffer substances, and furthermore solvents or solubilizers or agents for achieving a depot effect, as well as salts for changing the osmotic pressure, coating agents or antioxidants. They can also contain other therapeutically active substances as also described herein.

The present invention provides a vascularized BMO preparation or system derived exclusively from iPSCs (in particular hiPSCs), mimicking hematopoiesis in a multicellular context of native bone marrow, comprising blood cells and various bone marrow "niche cells", such as endothelial cells and mesenchymal cells.

The preparations as produced can be generally used in two strategies; either as a research tool, for example to study the interaction between niche cells and hematopoietic cells in the pathogenesis of hematological diseases. The tool can also be used to study the effects of drugs on the system, and to use the effects as identified to develop and test new therapies. Furthermore, the system can be used as a tool to screen and identify new drugs against hematological diseases and other diseases as disclosed herein. In the second strategy, the "products" and compositions as generated can be used themselves as therapeutics, i.e., to prevent and/or treat bone-marrow related diseases, such as hematological diseases and other diseases as disclosed herein. This strategy involves the possibility of a transplantation of human BMOs as produced or the improved *in vitro* generation of human blood cells from iPSCs, e.g., autologous patient-specific production, and subsequent infusion or transplantation. The present invention has the advantage to maintain the stemness of the hematopoietic stem cells, which is usually lost when cultured

in vitro with cytokines. When stemness is preserved, one can perform single cell studies and select for stem cells that have the desired genetic modification.

Preferred is a vascular network or mature mammalian bone marrow organoid produced according to the present invention, which is a model for a bone marrow related disease, such as, for example a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions. In these cases, the model is generated on iPSCs that are mutated or modified in order to reflect the origin of the bone marrow related disease, and the cells are then used to generate the organoids. Nevertheless, also the organoids or networks or certain cellular components thereof may be modified in order to reflect the origin of the bone marrow related disease to be analyzed.

Yet another aspect of the present invention thus relates to a method for identifying a pharmaceutically active compound against a bone marrow related disease, comprising the steps of a) providing a vascular network or mature mammalian bone marrow organoid produced according to the present invention, which is a model for at least one bone marrow related disease, b) contacting said vascular network or mature mammalian bone marrow organoid according to step a) with at least one potentially pharmaceutically active compound, and c) identifying a physiological effect reflecting or indicating a treatment or amelioration of said bone marrow related disease in the presence of said at least one potentially pharmaceutically active compound, when compared to the absence of said at least one potentially pharmaceutically active compound, or to a control, wherein said effect identifies a pharmaceutically active compound against a bone marrow related disease. The bone marrow related disease may be, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusion.

Preferred is the method or use according to the present invention, wherein said contacting is in vivo or in vitro, in solution or comprises the candidate compound bound or

conjugated to a solid carrier. Respective formats are also described in the art and known to the person of skill.

In the context of the present invention, the anti-senescence candidate compound can be selected from any suitable molecule that is suitable, such as a chemical organic molecule, a molecule selected from a library of small organic molecules (molecular weight less than 500 Da), a molecule selected from a combinatory library, a cell extract, in particular a plant cell extract, a small molecular drug, a protein, a protein fragment, a molecule selected from a peptide library, an antibody or fragment thereof.

These candidate molecules may also be used as a basis to screen for improved compounds, (see below).

In the context of the present invention, any method that is suitable for detecting the effect of the compound may be used. Respective methods are known to the person of skill and are disclosed in the art. The components of the assays as disclosed herein may be labelled, for example with a radiolabel or fluorescent label or with an antigenic label.

The present invention further relates to identifying improved compounds that have been identified in a first round of screening/identification. Following the provision of a compound as identified, the compound can be modified. In general, many methods of how to modify compounds of the present invention are known to the person of skill, and are disclosed in the literature. Modifications of the compounds will usually fall into several categories, for example a) chemical modifications, e.g. through the addition of additional chemical groups, b) changes of the size, length and/or charge of the compound, and c) the attachment of additional groups to the molecule (including marker groups, labels, linkers or carriers, such as chelators). All these modifications present a new strategy wherein any one of these or a combination thereof finally leads to the “rational design” of improved molecules for use in the context of the present invention. The present invention also includes strategies in order to further improve compounds that have only partially undergone “directed evolution” or “directed mutagenesis”, i.e., the compounds can undergo several successive rounds of the above methods.

In a next step, the modified compound is tested for a change of the physiological effect reflecting or indicating a treatment or amelioration of said bone marrow related disease in the presence of said at least one potentially pharmaceutically active compound, when compared to the absence of said at least one potentially pharmaceutically active compound, compared to the non-modified pharmaceutically active compound or to a control.

Yet another aspect of the present invention thus relates to a pharmaceutically active compound against a bone marrow related disease as identified according to the present invention, or a pharmaceutical composition comprising the pharmaceutically active compound. This aspect also includes a method to produce a pharmaceutical composition comprising the pharmaceutically active compound against a bone marrow related disease as identified according to the present invention, comprising formulating the compound with a suitable diluent and/or carrier. In general, the same conditions apply to this pharmaceutical composition as mentioned above.

Yet another aspect of the present invention then relates to the use of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical compositions (including the pharmaceutically active compound as identified) according to the present invention as a model system for identifying and/or testing pharmaceutically effective compounds for treating or preventing of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions. The identifying and testing is generally outlined above.

Yet another aspect of the present invention then relates to the use of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical compositions (including the pharmaceutically active compound as identified) according to the present invention as a model system in the pathogenesis of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from

chemotherapy or transfusions. As mentioned above, in these cases, the model as used may be generated on iPSCs that are mutated or modified in order to reflect the origin of the bone marrow related disease, and the cells are then used to generate the organoids. Nevertheless, also the organoids or networks or certain cellular components thereof may be modified in order to reflect the origin of the bone marrow related disease to be analyzed. The model system may also be used to test the BMOs for their suitability as a pharmaceutical preparation for transplantation and other treatment purposes.

Yet another aspect of the present invention then relates to the use of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical composition according to the present invention for the *in vitro* production of BMOs or mammalian blood cells, in particular autologous BMOs or mammalian blood cells, in particular for transplantation.

Yet another aspect of the present invention then relates to the use of the vascular network or mature mammalian bone marrow organoid according to the present invention to maintain stemness of hematopoietic stem cells (HSC). It is advantageous to keep HSC in vitro for further quality control studies and to sort only those cells for therapeutic application that show desired molecular changes without any unwanted side effects (e.g. off-target editing, or activation of oncogenes, or the like). The present invention has the advantage to maintain the stemness of the hematopoietic stem cells, which is usually lost when cultured in vitro with cytokines. When stemness is preserved, one can perform single cell studies and select for stem cells that have the desired genetic modification.

Yet another aspect of the present invention then relates to a pharmaceutically effective amount of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical compositions (including the pharmaceutically active compound as identified) according to the present invention for use in the treatment or prevention of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions, preferably for use in transplantation.

Yet another aspect of the present invention then relates to a method for preventing or treating a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions in a subject, comprising administering to said subject an effective amount of a vascular network or mature mammalian bone marrow organoid or the pharmaceutical compositions (including the pharmaceutically active compound as identified) according to the present invention. Preferably, said method comprises transplantation of an effective amount of a vascular network or mature mammalian bone marrow organoid according to the present invention.

It is to be understood that the products, compounds and/or a pharmaceutical composition are for use to be administered to a human patient. The term "administering" means administration of a sole therapeutic agent or in combination with another therapeutic agent. It is thus envisaged that the pharmaceutical compositions of the present invention are employed in co-therapy approaches, i.e. in co-administration with other medicaments or drugs and/or any other therapeutic agent which might be beneficial in the context of the methods of the present invention. Nevertheless, the other medicaments or drugs and/or any other therapeutic agent can be administered separately from the compound for use, if required, as long as they act in combination (i.e. directly and/or indirectly, preferably synergistically) with the present compound for use.

Thus, the compounds of the invention can be used alone or in combination with other active compounds – for example with medication and therapy already known for the treatment of the aforementioned diseases, whereby in the latter case a favorable additive, amplifying or preferably synergistically effect is noticed.

As mentioned herein, the product or compound is administered to said subject in an effective dosage. This dosage can vary within wide limits and is to be suited to the individual conditions in each individual case. For the above uses, the appropriate dosage will vary depending on the mode of administration, the particular condition to be treated

and the effect desired. In general, however, satisfactory results are achieved at dosage rates are as above, e.g. of about 1 to 100 mg/kg animal body weight particularly 1 to 50 mg/kg. Suitable dosage rates for larger mammals, for example humans, are of the order of from about 10 mg to 3 g/day, conveniently administered once or in divided doses, e.g. 2 to 4 times a day, or in sustained release form. In general, a daily dose of approximately 10 mg to 100 mg, particularly 10 to 50 mg, per human individual is appropriate in the case of the oral administration. An effective concentration to be reached at the cellular level can be set at between 50 to 200 μM, preferably at about 100 μM.

Another aspect of the present invention then relates to a kit, for example a diagnostic kit comprising materials for performing a method according to the present invention. Materials are a set of media, such as the aggregation medium with Rho-associated protein kinase (ROCK)-inhibitor of step a) as herein, the serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells, supplemented in accordance with step b) as herein, Essential 6-medium supplemented in accordance with step c) as herein, and materials to produce the polymerized 3D collagen I/Matrigel® matrix as well as the StemPro®-34 medium supplemented in accordance with step d) as herein.

The kit may further comprise, relevant antibodies binding to cellular markers of the BMOs, dyes and other labels, as well buffers and matrices for performing the methods as above.

The kit may be used in the methods of the invention, i.e. for identifying and/or testing pharmaceutically effective compounds for treating or preventing of a bone marrow related disease, for use in a model system in the pathogenesis of a bone marrow related disease, and/or for the *in vitro* production of BMOs or mammalian blood cells, in particular autologous BMOs or mammalian blood cells, in particular for transplantation.

In summary, the present invention provides a vascularized BMO system derived exclusively from iPSCs and mimicking hematopoiesis in a multicellular context of native bone marrow. The generation of a BMO model derived solely from iPSCs and composed

of blood cells and various niche cells such as endothelial cells and mesenchymal cells has not been reported previously.

The present invention relates to the following items.

Item 1. A method for producing mammalian bone marrow organoids, comprising a) generating embryoid bodies from substantially single induced pluripotent stem cells (iPSCs) obtained from at least one mammal, comprising culturing single iPSCs in an aggregation medium in the presence of at least one Rho-associated protein kinase (ROCK)-inhibitor for about 1 day; followed by culturing the embryoid bodies in a suitable serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells without ROCK-inhibitor for about 48 hours; b) inducing mesoderm in said embryoid bodies as formed in step a), comprising culturing said embryoid bodies in a suitable serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells, wherein the medium is supplemented with 80 ng/ml Bone morphogenetic protein 4 (BMP4), 4 μM of glycogen synthase kinase (GSK) 3 inhibitor, and 80 ng/ml Vascular Endothelial Growth Factor (VEGF), for about 48 hours, with resuspension of the mesoderm-induced embryoid bodies at about every 24 hours; c) replacing the medium of the culture of b) with Essential 6-medium, supplemented with 80 ng/ml VEGF, 25 ng/ml fibroblast growth factor (FGF)-2, 50 ng/ml stem cell factor (SCF), and 2 μM SB431542 for about 48 hours, with resuspension of the embryoid bodies at about every 24 hours; d) embedding of the mesoderm-induced embryoid bodies of step c) into a suitable polymerized 3D collagen I/Matrigel® matrix followed by overlaying the matrix with StemPro®-34 medium supplemented with 80 ng/ml VEGF, 25 ng/ml FGF-2, 50 ng/ml SCF and 2 μM SB431542 for about 48 hours, followed by cytokine replacement with 50 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO for about 48 hours, and a cytokine boost to 25 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO for about 48 hours; e) extracting individual vascular networks that have been generated in step d) followed by culturing in StemPro®-34 medium, supplemented with 25 ng/ml VEGF, 50 ng/ml SCF, 50 ng/ml IL-3, 50 ng/ml Flt-3L and 5 ng/ml TPO, with refreshing the medium every 3 to 4 days for about 7 to 11 days, whereby the mammalian bone marrow organoids are produced.

Item 2. The method for producing mammalian bone marrow organoids according to Item 1, further comprising the step of fixing the vascular networks in step e) and performing immunofluorescence analysis and/or dissociating the vascular networks into individual cells and performing flow cytometry analysis.

Item 3. The method for producing mammalian bone marrow organoids according to Item 1 or 2, further comprising the step of fixing the organoids as produced, and performing immunofluorescence analysis and/or dissociating the organoids into individual cells and performing flow cytometry analysis.

Item 4. The method for producing mammalian bone marrow organoids according to any one of Items 1 to 3, further comprising the step of culturing the organoids for a maximum of about 47 days.

Item 5. The method for producing mammalian bone marrow organoids according to any one of Items 1 to 4, wherein the single iPSCs are provided by dissociating iPSC cells into single cells with Accutase® digestion.

Item 6. The method for producing mammalian bone marrow organoids according to any one of Items 1 to 5, wherein the ROCK-inhibitor is selected from the group consisting of Y-27632 ((1R,4r)-4-((R)-1-aminoethyl)-N-(pyridin-4-yl)cyclohexanecarboxamide), and fasudil.

Item 7. The method for producing mammalian bone marrow organoids according to any one of Items 1 to 6, wherein the aggregation medium is KnockOut DMEM/F12 with 20% serum substitute, 1% L-glutamine, 1% non-essential amino acids, 1% penicillin-streptomycin, and 100μM β-mercaptoethanol.

Item 8. The method for producing mammalian bone marrow organoids according to any one of Items 1 to 7, wherein the serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells is mTeSR® Plus medium.

Item 9. The method for producing mammalian bone marrow organoids according to any one of Items 1 to 8, wherein the glycogen synthase kinase (GSK) 3 inhibitor is CHIR99021.

Item 10. The method for producing mammalian bone marrow organoids according to any one of Items 1 to 9, wherein the ratio of the 3D collagen I/Matrigel® matrix is about 4:1.

Item 11. The method for producing mammalian bone marrow organoids according to any one of Items 1 to 10, wherein the resuspension of the embryoid bodies/ mesoderm-induced embryoid bodies comprises the use of a pipette.

Item 12. The method for producing mammalian bone marrow organoids according to any one of Items 1 to 11, wherein the dissociating or extracting of individual vascular networks comprises extraction with the use of sterile dissection tools.

Item 13. The method for producing mammalian bone marrow organoids according to any one of Items 1 to 12, wherein the mammal is selected from the group consisting of a human, a mouse, a monkey, a rat, a pig, a dog, a cat, a rabbit, a sheep, cattle, an equine, and a goat.

Item 14. A method for producing mesenchymal stem cells (MSCs) and/or hematopoietic stem cells (HSCs), comprising performing a method according to any one of Items 1 to 13, and suitably isolating the MSCs or HSCs from the vascular network or mammalian bone marrow organoid.

Item 15. A vascular network or mature mammalian bone marrow organoid, produced according to the method according to any one of Items 1 to 13, or a pharmaceutical composition comprising the vascular network and/or mature mammalian bone marrow organoid.

Item 16. The vascular network or mature mammalian bone marrow organoid according to Item 15, which is a model for a bone marrow related disease, such as, for example a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers,

anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions.

Item 17. Use of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical composition according to Item 15 as a model system in the pathogenesis of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions.

Item 18. Use of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical composition according to Item 15 as a model system for identifying and/or testing pharmaceutically effective compounds for treating or preventing of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions.

Item 19. Use of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical composition according to Item 15 for the in-vitro production of BMOs or mammalian blood cells, in particular autologous BMOs or mammalian blood cells, in particular for transplantation.

Item 20. A pharmaceutically effective amount of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical composition according to Item 15 for use in the treatment of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions, preferably for transplantation.

Item 21. MSCs or HSCs as produced according to ITEM 14 or a pharmaceutical composition comprising the MSCs or HSCs.

Item 22. A pharmaceutically effective amount of the MSCs or HSCs or the pharmaceutical composition according to Item 21 for use in the treatment of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions, preferably for transplantation.

The invention will now be further described in the following examples and with reference to the accompanying figures and the sequence listing, without being limited thereto. For the purposes of the present invention, all references as cited herein are incorporated by reference in their entireties.

Figure 1 shows a schematic illustration of the workflow of the invention. A. Schematic illustration of the preferred generation of BMOs in a 3-dimensional sequential cytokine induction system. Embryoid bodies are generated on day -3, and subsequently mesoderm is induced on day 0, hemogenic endothelium is induced on day 2 to 4, followed by the induction of hematopoietic fate on day 6. On day 4, mesoderm-induced embryoid bodies are embedded in a 3D matrix to promote vascular sprouting. On day 10, individual vascular networks are extracted and cultured in a 96wp format to promote self-assembly and maturation into mature BMOs. B. Schematic illustration of the experimental set up. The generated BMOs are analyzed by imaging, flow cytometry and cell sorting. Isolated blood progenitors and mesenchymal stem cells are tested for their multipotency potential via CFU and differentiation assays.

Figure 2 shows the characterization of the vessel-like structure of bone marrow organoids via immunofluorescence. A) 3D z-reconstruction of whole-mount organoid. Organoids stained for endothelial cells (CD31, red), pericytes (PDGFRβ, green) and DNA (DAPI, blue) on day 17 of differentiation. Scalebar = 200 μm, total depth: 128 μm, slice distance: 1 μm. B) Detailed depiction of vessel-like network composed of endothelial cells and perivascular mesenchymal cells. Organoids stained for pericytes (PDGFRβ, green), endothelial cells (CD31, red) and DNA (DAPI, blue). Upper right image: 3D z-reconstruction of whole mount organoid on day 17 of differentiation, total depth: 86μm, slice distance: 1μm. Upper left image and lower left image: single image slice with

selected area shows endothelial cells enclosed by pericytes. C) Higher magnification of pericyte-endothelial cell association on day 10 and day 17 of differentiation. Left: arrow indicates finger-like extensions of pericytes on day 10 of differentiation. Right: arrow indicates tight association of a pericyte with an endothelial cell on day 17 of differentiation. D) Detailed depiction of interconnected, lumen-forming vascular-like network. Organoids of day 17 of differentiation stained for endothelial cells (CD31, red / CD34, green) and DNA (DAPI, blue). Left image: Z-projection of whole-mount organoid. Note enveloped structure enclosing the whole organoid indicated by arrows. Upper middle image: Interconnectivity of vascular-like network computed with Angiotool. In total, 213 branching points are depicted as blue dots. Settings: Vessel diameter was set to 10 and vessel intensity high threshold was set to 211. Lower middle image: Orthogonal 2D z-projection of vessel structure in xz and yz direction. Note lumen formation indicated by arrows. Right image: Single slice of organoid stained for CD34 and DNA. Scalebar=200 µm. E) Depiction of the endothelial basement membrane. Organoid stained on day 17 of differentiation for endothelial cells (CD31, red), basement membrane (Col IV, green) and DNA (DAPI, bue). Scalebars: 50 µm. F) Depiction of CXCL12-abundant reticular mesenchymal cells (CAR cells) in close association to endothelial cells. Organoid stained on day 17 of differentiation for endothelial cells (CD31, red), CAR cells (CXCL12, green) and DNA (DAPI, blue). Scalebars: 20 µm.

Figure 3 shows the localization of hematopoietic cells within the bone marrow organoid. All organoids were stained on day 17 of differentiation. A) Staining of whole-mount organoid with DAPI (blue/grey) and the early hematopoietic marker runx1 (green). Selected area shows loose cluster of runx1+ hematopoietic cells. Scalebars in enlarged sections = 50 µm. B) Staining of whole-mount organoid with DAPI (blue/grey) and the pan-hematopoietic marker CD45 (green). Selected area shows loose clusters of CD45+ hematopoietic cells. Scalebars in zoom-in images = 50 µm. C) Staining of organoid slide with DAPI (blue) and runx1 (green). Selected area shows tight cluster of runx1+ hematopoietic cells. Scalebar = 200µm. D) Staining of organoid slide with DAPI (blue) and CD45 (green). Selected area shows tight cluster of CD45+ hematopoietic cells. Scalebar = 200 µm. E) Hematoxylin-Eosin stain of organoid slide depicted in different magnifications. Scalebars = 250 µm.

Figure 4 shows the localization of myeloid cells within the bone marrow organoid. All organoids were stained on day 17 of differentiation for DNA (DAPI, blue/grey) and the monocyte/granulocyte marker S100A8 (red) (A), S100A8/A9 (red) (B) and Myeloperoxidase (red) (C). Selected areas show loose clusters of myeloid cells within BMOs depicted in enlarged sections. Selected areas in-zoom in images show example of positively stained cells with banded nuclei (A), (B) and segmented nuclei (C). Scalebars zoom-in images = 50 µm.

Figure 5 shows the quantification of bone marrow organoid cell composition and assessment of changes in cell composition over time by flow cytometry.(A) Hematopoietic cells were defined as CD45+, (B) myeloid cells as CD45+ CD11b+, (C) endothelial cells as CD45− CD31+ and (D) mesenchymal cells as CD45− CD31− CD34− CD90+ CD105+ CD271+ CD73+. Left: representative flow cytometry plot of one differentiation experiment is shown for each cell type on day 17 and day 21 of differentiation. Flow cytometry analysis was done for four independent differentiation experiments with similar results. For each flow cytometry experiment several (>20) BMOs were pooled. Right: Frequencies of the different cell types, as identified by flow cytometry, visualized in a plot on day 17 and day 21 of differentiation. Frequencies of the different cell types refer to the living cell fraction of analyzed cells. Bar plot represents mean value of four independent differentiation experiments and individual measurements of each experiment are depicted as dots. Error bar represents S.E.M.

Figure 6 shows the analysis of multilineage potential of bone marrow organoid-derived hematopoietic progenitor cells and mesenchymal stem cells. Multipotency of hematopoietic progenitor cells was assessed by colony-forming unit assay (A, B) and multipotency of mesenchymal stem cells was assessed by a trilineage differentiation assay (C). (A) A colony-forming unit assay was performed with sorted bone marrow organoid-derived CD45+ CD34+ CD11b− hematopoietic progenitor cells. Colony counting was done after 14 days of cell seeding. Bar plot represents mean value of two independent experiments for each colony type. Each datapoint represents individual value of each experiment and symbols represent origin of datapoint from different experiments. Error bars represent standard deviation. (B) Left: Various colony types (CFU-GEMM, BFU-E and CFU-GM) were counted after 14 days of cell seeding in phase contrast

microscopy. Right: Identity of colony types were verified by colony picking followed by Wright-Giemsa stain. (C) Trilineage differentiation assay was performed with sorted bone marrow organoid-derived mesenchymal stem cells (CD45− CD31− CD34− CD90+ CD105+ CD271+ CD73+). MSCs were cultured in osteogenic differentiation medium, adipogenic differentiation medium or chondrogenic differentiation medium for at least 21 days. Osteogenic differentiation was visualized by Alizarin-Red-S stain (staining of calcium deposition). Adipogenic differentiation was visualized by Oilred-O-stain (staining of lipid droplets) and cell nuclei were counterstained with Haematoxylin stain. Chondrogenic differentiation was visualized by Alcian-Blue stain(staining of glycosaminoglycans) and cell nuclei were counterstained with nuclear fast red stain.

Figure 7 shows the modelling of VPS45 deficiency in bone marrow organoids (A) Schematic overview of experimental set-up. Patients carrying the Thr22Asn mutation in the VPS45 gene develop severe congenital neutropenia (SCN) and myelofibrosis. (B) Flow cytometric comparison of cell composition of wild type and mutant VPS45 bone marrow organoids. Hematopoietic cells are defined as CD45+, myeloid cells as CD45+ CD11b+, endothelial cells as CD45− CD31+ and mesenchymal cells as CD45− CD31−CD34− CD90+ CD105+ CD271+ CD73+. Bar plot represents frequencies of the different cell types on day 17 of differentiation from one flow cytometry experiment. For each clone several (>20) BMOs were pooled. For detailed gating strategy refer to Figure 6. Frequencies of the different cell types refer to the living cell fraction of analyzed cells. (C) Histological comparison of wild type and mutant VPS45 bone marrow organoids. Hematoxylin-Eosin and May-Grünwald-Giemsa stain show blood cell morphology and overall BMO architecture. Gomori stain shows reticulin fibers in dark-to-black color. Myeloperoxidase stain shows MPO+ myeloid cells. CD34 stain shows vascular structure and CD34+ blood cells. Scalebars =200μm.

EXAMPLES

Background

A 2D-system for the hematopoietic differentiation was described in 2011, wherein the blood precursor cells were generated from human induced pluripotent stem cells (hiPSCs) through the sequential induction of cytokines (8). At that time, a serum-free monolayer

culture that can trace the in vivo hematopoietic pathway from ES/iPS cells to functional definitive blood cells via mesodermal progenitors was established, and stepwise tuning of exogenous cytokine cocktails induced the hematopoietic mesodermal progenitors via primitive streak cells. These progenitors were then differentiated into various cell lineages depending on the hematopoietic cytokines present.

The protocol of (8) was modified considerably in order to differentiate the hematopoietic progenitors into functional neutrophils and macrophages. For the generation of a vascularized bone marrow organoid, the inventors implemented parts of a protocol for the generation of vascular organoids recently published by Wimmer and colleagues (9). These hiPSC-derived vascular organoids were not only composed of functional endothelial cells and pericytes, but also contained very small numbers of hematopoietic cells and mesenchymal stromal cells (10).

The inventors concluded that the association of the above hematopoietic differentiation protocol with a three-dimensional vascularized culture system would promote the formation of complex cell interactions, thus leading to the formation of a structurally organized microenvironment resembling the natural bone marrow niche., which surprisingly turned out to be correct.

Summary of the method according to the present invention

Bone marrow organoids were generated from iPSCs by mesoderm induction and subsequent induction of hematopoiesis in a 3D matrix cytokine induction system. Human iPSC embryoid bodies were cultured in low-adhesion plates with Y-27632 and grown for 2-3 days until aggregates reached a size of 0.5 µm (Fig. 1A). Mesoderm was induced on day 0 with bone morphogenic protein 4 (BMP4), the GSK-3 inhibitor CHIR99021 (Sigma), and vascular endothelial growth factor (VEGF) and patterned on day 2 and 4 with VEGF, TGF-β RI Kinase Inhibitor VI SB431521 (Sigma), fibroblast growth factor 2 (FGF2) and stem cell factor (SCF). On day 4, the embryoid bodies were embedded in a collagen I/Matrigel® (Sigma) solution to induce vascular sprouting and promote cell organization in 3-dimensional form. Thereafter, hematopoietic differentiation was induced with VEGF, SCF, Fms Related Receptor Tyrosine Kinase 3 Ligand (Flt-3L), Interleukin 3 (IL-3) and Thrombopoietin (TPO). On day 10, the vascular networks were

separated/extracted, and the individual networks transferred to a 96-well low attachment plate. From day 10 to day 17, the vascular networks formed into spherical BMOs and were collected on day 17, day 21 and day 45 for further analysis. The resulting BMOs were characterized by histological methods, confocal microscopy, flow cytometry and differentiation assays (Fig. 1B).

Detailed method for producing the bone marrow organoids

Bone marrow organoids (BMO) were generated in a 3D-culture system using the sequential addition of growth factors as follows.

As exemplary shown on Figure 1, on day -3 of the method, embryoid bodies were generated by dissociating iPSCs into single cells with Accutase® (Sigma) for 5 minutes at 37°C. Cell aggregates were mechanically disrupted using a P1000 pipette. The dissociation reaction was stopped with mTeSR® Plus (Fisher Scientific), and then the cells were collected at 300 g for 3 minutes at room temperature (RT).

The cells were resuspended in aggregation medium (KnockOut DMEM/F12 with 20% serum substitute, 1% L-glutamine, 1% non-essential amino acids, 1% penicillin-streptomycin and 100µM β-mercaptoethanol) and the cells were treated with a counted by hemocytometer. 2.5 - 3 x 10^6 cells were resuspended in aggregation medium supplemented with 50 µM Y-27632 and seeded in a low adherence petri dish.

Mesoderm was induced on day 0 with mTeSR® Plus supplemented with 80ng/ml BMP4, 4µM CHIR99021 and 80ng/ml VEGF. For the medium change, the embryoid bodies were collected in 15 ml canonical tubes by gravity (15-30 minutes). To avoid excessive fusion, the embryoid bodies were resuspended once a day. On day 2, the medium was changed to Essential 6 medium supplemented with 80 ng/mL VEGF, 25 ng/mL FGF-2, 50 ng/mL SCF, and 2 µM SB431542.

On day 4, 30-60 embryoid bodies were embedded in a 12-well plate with 1 ml/well of a 4:1 collagen I/Matrigel® mixture. Collagen I solution was prepared according to manufacturer's protocol (50 µl 10x DMEM, 137.5 µl ddH2O, 12.5 µl 7.5% sodium

bicarbonate, 235.9 µl Hams-F12, 9.45µl HEPES, 4.6µl Glutamax, 300 µl 5mg/ ml collagen type I) and 1N NaOH was added dropwise to bring the solution to pH 7.4.

For embedding, a layer of 500 µl/well of collagen I-Matrigel® mixture was first prepared and polymerized at 37° C. for 1 hour to prevent the embryoid bodies from sinking to the bottom of the dish. The embryoid bodies were then resuspended in 500 µl/well of the collagen-I-Matrigel mixture and a second layer was prepared, which was polymerized at 37° C. for 1 hour. Finally, the embedded embryoid bodies were overlaid with prewarmed StemPro®-34 medium supplemented with 80ng/ml VEGF, 25 ng/ml FGF-2, 50 ng/ml SCF and 2 µM SB431542.

On day 6, cytokines were replaced with 50 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO. On day 8, cytokines were changed to 25 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO.

On day 10, the vascular networks that had arisen from individual embryoid bodies were extracted with sterile dissection tools under the laminar flow unit with the lowest magnification of an inverted light microscope. Individual vascular networks were then transferred and further cultured in a 96-well low attachment plate. Thereafter, the organoids were cultured in StemPro®-34 medium (Fisher Scientific) supplemented with 25 ng/ml VEGF, 50 ng/ml SCF, 50 ng/ml IL-3, 50 ng/ml Flt-3L and 5 ng/ml TPO, with half of the medium every 3 4 days refreshed. At day 10, the vascular networks and at day $\geq$ 17 the mature organoids were either fixed for immunofluorescence analysis or dissociated into individual cells for flow cytometry analysis. In this case, the organoids were cultured until day 45.

Characterization and local distribution of niche cells and hematopoietic cells within the BMOs as produced

The present analysis shows that the BMOs consist of a hematopoietic compartment, a vascular compartment, and a stromal compartment. Confocal microscopy revealed a self-assembled, interconnected and partially lumen-forming vessel-like network composed of CD31+ endothelial cells covered by a Col IV+ basement membrane and PDGFR-β+ perivascular mesenchymal cells. In addition, flow cytometric analysis showed that the

relative composition of endothelial cells and mesenchymal stem cells is comparable to that in native bone marrow (11). Interestingly, the inventors found CXCL12+ cells in a network-like structure closely connected to the endothelial network, reminiscent of the native bone marrow architecture. This strongly suggests the formation of bone marrow-specific CAR cells in the present differentiation system. This is particularly remarkable since CAR cells are the main producers of CXCL12 and SCF, essential signaling molecules for the maintenance and homing of HSCs and the proliferation of lymphoid and erythroid progenitor cells (12). The inventors found that CD45+ hematopoietic cells are distributed in clusters evenly throughout the organoid. This partial clustering could be explained by the increased localization of certain niche cells and their regulatory cytokines in these areas. It is known that hematopoietic stem cells and progenitor cells are localized in the vicinity of blood vessels since growth factors and products produced by niche cells are important regulators for the maintenance of HSCs (11,13).

Differentiation of myeloid cells within the BMOs as produced

In addition, the inventors could show that the present differentiation protocol promotes the autologous differentiation of hematopoietic progenitors into myeloid cells within the BMO structure, indicating a facilitative interaction between hematopoietic progenitors and niche cells. Immunofluorescence showed cell populations positive for markers of monocytes and neutrophils (S100A8/A9, S100A8 and MPO). Interestingly, these cells also had bean-shaped and segmented nuclei, indicating the formation of monocytes and granulocytes at different stages of maturation. Wright-Giemsa staining of a FACS-sorted myeloid population (CD45+ CD34-CD11b+) also revealed the emergence of macrophages, monocytes and granulocytes. These results are particularly interesting because the differentiation of HSCs into granulocytes and macrophages depends on GCSF and GM-CSF signaling, but these cytokines are not included in the cytokine cocktail in the present differentiation system. Thus, these results indicate sufficient intrinsic production of these cytokines by niche cells within the BMO system. Although the inventors have not yet been able to demonstrate the emergence of other bloodlines such as lymphocytes, erythrocytes or platelets, CFU assays of FACS-sorted hematopoietic progenitor cells (CD45+ CD34+ CD11b-) showed the potential of these cells to differentiate into erythrocytes and megakaryocytes. Since the cytokines used in our system cause a shift in hematopoietic differentiation toward the myeloid cell lineages,

future adjustments in cytokine composition could allow differentiation of erythroid or even lymphoid cells.

Mesenchymal stem cells as obtained from BMOs fulfill the criteria for mesenchymal stem cells.

Mesenchymal stem cells are one of the major cellular components of the bone marrow niche. MSCs maintain bone marrow tissue homeostasis by differentiating into adipocytes, osteocytes, and chondrocytes, while primitive mesenchymal cells and their progeny directly regulate hematopoiesis through the secretion of cytokines (14). In other *in vitro* bone marrow niche studies, donor primary mesenchymal cells were used to generate the stromal compartment of the bone marrow niche, which is then co-cultured with donor hematopoietic cells to mimic the bone marrow hematopoietic compartment. In the present system, the iPSCs instead simultaneously differentiate into hematopoietic cells, endothelial cells, and most notably into mesenchymal progenitor cells such as PDGFRβ+ perivascular cells and CXCL12+ CAR cells. Furthermore, it was demonstrated that the present system induces the formation of functional bona fide mesenchymal stem cells, since BMO-derived MSCs meet the minimum criteria for mesenchymal stem cells proposed by the ISCT: MSCs were negative for the hematopoietic and endothelial surface molecules CD45, CD34, and CD31, but positive for CD90, CD105, CD271 and CD73. The MSCs adhered to plastic and could differentiate into osteoblasts, adipocytes and chondroblasts in vitro (15, 16).

Disease-related system generation

In order to test the suitability of the produced BMOs for modeling bone marrow-related diseases, the inventors generated VPS45-deficient BMOs in a proof-of-concept approach. Genetic VPS45 deficiency in patients results in disease associated with severe congenital neutropenia and myelofibrosis (17). Although there were no striking differences in the relative composition of hematopoietic cells and niche cells between WT and VPS45 BMOs, preliminary histological results of VPS45 BMOs indicated an increased formation of reticulin fibers as a sign of myelofibrosis in the VPS45-mutated BMOs.

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CLAIMS

1. A method for producing mammalian bone marrow organoids, comprising

a) generating embryoid bodies from substantially single induced pluripotent stem cells (iPSCs) obtained from at least one mammal, comprising culturing single iPSCs in an aggregation medium in the presence of at least one Rho-associated protein kinase (ROCK)-inhibitor for about 1 day; followed by culturing the embryoid bodies in a suitable serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells without ROCK-inhibitor for about 48 hours,

b) inducing mesoderm in said embryoid bodies as formed in step a), comprising culturing said embryoid bodies in a suitable serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells, wherein the medium is supplemented with 80 ng/ml Bone morphogenetic protein 4 (BMP4), 4 μM of glycogen synthase kinase (GSK) 3 inhibitor, and 80 ng/ml Vascular Endothelial Growth Factor (VEGF), for about 48 hours, with resuspension of the mesoderm-induced embryoid bodies at about every 24 hours;

c) replacing the medium of the culture of b) with Essential 6-medium, supplemented with 80 ng/ml VEGF, 25 ng/ml fibroblast growth factor (FGF)-2, 50 ng/ml stem cell factor (SCF), and 2 μM SB431542 for about 48 hours, with resuspension of the embryoid bodies at about every 24 hours;

d) embedding of the mesoderm-induced embryoid bodies of step c) into a suitable polymerized 3D collagen I/Matrigel® matrix followed by overlaying the matrix with StemPro®-34 medium supplemented with 80 ng/ml VEGF, 25 ng/ml FGF-2, 50 ng/ml SCF and 2 μM SB431542 for about 48 hours, followed by cytokine replacement with 50 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO for about 48 hours, and a cytokine boost to 25 ng/mL VEGF, 50 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL Flt-3L, and 5 ng/mL TPO for about 48 hours;

e) extracting individual vascular networks that have been generated in step d) followed by culturing in StemPro®-34 medium, supplemented with 25 ng/ml VEGF, 50 ng/ml SCF, 50 ng/ml IL-3, 50 ng/ml Flt-3L and 5 ng/ml TPO, with refreshing the medium every 3 to 4 days for about 7 to 11 days,

whereby the mammalian bone marrow organoids are produced, and
wherein preferably no lineage-directing cytokines, such as EPO, IL-6 and/or G-CSF, are used.

2. The method for producing mammalian bone marrow organoids according to claim 1, further comprising the step of fixing the vascular networks in step e) and performing immunofluorescence analysis and/or dissociating the vascular networks into individual cells and performing flow cytometry analysis.

3. The method for producing mammalian bone marrow organoids according to claim 1 or 2, further comprising the step of fixing the organoids as produced, and performing immunofluorescence analysis and/or dissociating the organoids into individual cells and performing flow cytometry analysis.

4. The method for producing mammalian bone marrow organoids according to any one of claims 1 to 3, further comprising the step of culturing the organoids for a maximum of about 47 days.

5. The method for producing mammalian bone marrow organoids according to any one of claims 1 to 4, wherein the single iPSCs are provided by dissociating iPSC cells into single cells with Accutase® digestion.

6. The method for producing mammalian bone marrow organoids according to any one of claims 1 to 5, wherein the ROCK-inhibitor is selected from the group consisting of Y-27632 ((1R,4r)-4-((R)-1-aminoethyl)-N-(pyridin-4-yl)cyclohexanecarboxamide), and fasudil.

7. The method for producing mammalian bone marrow organoids according to any one of claims 1 to 6, wherein the aggregation medium is KnockOut DMEM/F12 with 20% serum substitute, 1% L-glutamine, 1% non-essential amino acids, 1% penicillin-streptomycin, and 100μM β-mercaptoethanol.

8. The method for producing mammalian bone marrow organoids according to any one of claims 1 to 7, wherein the serum-free, stabilized cell culture medium suitable for the feeder-free maintenance and expansion of human embryonic stem cells is mTeSR® Plus medium.

9. The method for producing mammalian bone marrow organoids according to any one of claims 1 to 8, wherein the glycogen synthase kinase (GSK) 3 inhibitor is CHIR99021.

10. The method for producing mammalian bone marrow organoids according to any one of claims 1 to 9, wherein the ratio of the 3D collagen I/Matrigel® matrix is about 4:1.

11. The method for producing mammalian bone marrow organoids according to any one of claims 1 to 10, wherein the resuspension of the embryoid bodies/ mesoderm-induced embryoid bodies comprises the use of a pipette.

12. The method for producing mammalian bone marrow organoids according to any one of claims 1 to 11, wherein the dissociating or extracting of individual vascular networks comprises extraction with the use of sterile dissection tools.

13. The method for producing mammalian bone marrow organoids according to any one of claims 1 to 12, wherein the mammal is selected from the group consisting of a human, a mouse, a monkey, a rat, a pig, a dog, a cat, a rabbit, a sheep, cattle, an equine, and a goat.

14. A vascular network or mature mammalian bone marrow organoid, produced according to the method according to any one of claims 1 to 13, or a pharmaceutical composition comprising the vascular network and/or mature mammalian bone marrow organoid.

15. The vascular network or mature mammalian bone marrow organoid according to claim 14, which is a model for a bone marrow related disease, such as, for example a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers,

anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions.

16. Use of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical composition according to claim 14 as a model system in the pathogenesis of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions.

17. Use of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical composition according to claim 14 as a model system for identifying and/or testing pharmaceutically effective compounds for treating or preventing of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions.

18. Use of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical composition according to claim 14 for the in-vitro production of BMOs or mammalian blood cells, in particular autologous BMOs or mammalian blood cells, in particular for transplantation.

19. A pharmaceutically effective amount of the vascular network or mature mammalian bone marrow organoid or the pharmaceutical composition according to claim 14 for use in the treatment of a bone marrow related disease, such as, for example, a hematological disease, severe congenital neutropenia, myelofibrosis, blood cell cancers, anemia, thrombocytopenia, inborn errors of hematopoiesis and immunity, conditions related to HIV, sickle cell disease, and complications from chemotherapy or transfusions, preferably for transplantation.

Patentansprüche

1. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden, umfassend
a) Erzeugen von Embryoidkörpern aus im Wesentlichen einzelnen induzierten pluripotenten Stammzellen (iPSCs), die von mindestens einem Säugetier erhalten wurden, umfassend das Kultivieren einzelner iPSCs in einem Aggregationsmedium in Gegenwart mindestens eines Rho-assoziierten Proteinkinase (ROCK)-Inhibitors für etwa 1 Tag; anschließende Kultivierung der Embryoidkörper in einem geeigneten serumfreien, stabilisierten Zellkulturmedium, das für die Feeder-freie Erhaltung und Vermehrung humaner embryonaler Stammzellen ohne ROCK-Inhibitor geeignet ist, für etwa 48 Stunden,
b) Induzieren von Mesoderm in den wie in Schritt a) gebildeten Embryoidkörpern, umfassend das Kultivieren der Embryoidkörper in einem geeigneten serumfreien, stabilisierten Zellkulturmedium, das für die Feeder-freie Aufrechterhaltung und Expansion menschlicher embryonaler Stammzellen geeignet ist, wobei das Medium ergänzt ist mit 80 ng/ml Bone morphogenetic protein 4 (BMP4), 4 µM Glykogensynthasekinase (GSK) 3-Inhibitor und 80 ng/ml Vascular Endothelial Growth Factor (VEGF), für etwa 48 Stunden, mit Resuspension der Mesoderm-induzierten Embryoidkörper etwa alle 24 Stunden;
c) Ersetzen des Mediums der Kultur von b) durch Essential 6-Medium, ergänzt mit 80 ng/ml VEGF, 25 ng/ml Fibroblasten-Wachstumsfaktor (FGF)-2, 50 ng/ml Stammzellfaktor (SCF), und 2 µM SB431542 für etwa 48 Stunden, mit Resuspension der Embryoidkörper etwa alle 24 Stunden;
d)) Einbetten der Mesoderm-induzierten Embryoidkörper aus Schritt c) in eine geeignete polymerisierte 3D-Kollagen-I/Matrigel®-Matrix, gefolgt von Überschichten der Matrix mit StemPro®-34-Medium, ergänzt mit 80 ng/ml VEGF, 25 ng/ml FGF-2, 50 ng/ml SCF und 2 µM SB431542 für etwa 48 Stunden, gefolgt von Zytokinersatz mit 50 ng/ml VEGF, 50 ng/ml SCF, 50 ng/ml IL-3, 50 ng/ml Flt-3L und 5 ng/ml TPO für etwa 48 Stunden und einem Zytokin-Boost auf 25 ng/ml VEGF, 50 ng/ml SCF, 50 ng/ml IL-3, 50 ng/ml Flt-3L und 5 ng/ml TPO für ungefähr 48 Stunden;

e) Extrahieren einzelner Gefäßnetzwerke, die in Schritt d) erzeugt wurden, gefolgt von Kultivieren in StemPro®-34-Medium, ergänzt mit 25 ng/ml VEGF, 50 ng/ml SCF, 50 ng/ml IL-3, 50 ng/ml Flt-3L und 5 ng/ml TPO, mit Auffrischen des Mediums alle 3 bis 4 Tage für etwa 7 bis 11 Tage,
wodurch die Säuger-Knochenmark-Organoide produziert werden, und
wobei bevorzugt keine die Zelllinien-Entwicklung bestimmenden Zytokine, wie etwa EPO, IL-6 und/oder G-CSF, verwendet werden.

2. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach Anspruch 1, weiter umfassend den Schritt des Fixierens der vaskulären Netzwerke in Schritt e) und des Durchführens einer Immunfluoreszenzanalyse und/oder des Dissoziierens der vaskulären Netzwerke in einzelne Zellen und des Durchführens einer durchflusszytometrischen Analyse.

3. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach Anspruch 1 oder 2, weiter umfassend den Schritt des Fixierens der produzierten Organoide und des Durchführens einer Immunfluoreszenzanalyse und/oder des Dissoziierens der Organoide in einzelne Zellen und des Durchführens einer durchflusszytometrischen Analyse.

4. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach einem der Ansprüche 1 bis 3, weiter umfassend den Schritt des Kultivierens der Organoide für maximal etwa 47 Tage.

5. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach einem der Ansprüche 1 bis 4, wobei die einzelnen iPSCs durch Dissoziieren von iPSC-Zellen in einzelne Zellen mit Accutase®-Verdauung bereitgestellt werden.

6. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach einem der Ansprüche 1 bis 5, wobei der ROCK-Inhibitor ausgewählt ist aus der Gruppe bestehend aus Y-27632 ((1R,4r)-4-((R)-1-Aminoethyl)-N-(pyridin-4-yl)cyclohexancarboxamid) und Fasudil.

7. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach einem der Ansprüche 1 bis 6, wobei das Aggregationsmedium KnockOut DMEM/F12 mit 20 % Serumersatz, 1 % L-Glutamin, 1 % nicht-essentiellen Aminosäuren, 1 % Penicillin-Streptomycin und 100 µM β-Mercaptoethanol ist.

8. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach einem der Ansprüche 1 bis 7, wobei das serumfreie, stabilisierte Zellkulturmedium, das für die Feeder-freie Erhaltung und Vermehrung menschlicher embryonaler Stammzellen geeignet ist, mTeSR® Plus-Medium ist.

9. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach einem der Ansprüche 1 bis 8, wobei der Glycogen-Synthasekinase (GSK) 3 Inhibitor CHIR99021 ist.

10. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach einem der Ansprüche 1 bis 9, wobei das Verhältnis der 3D-Kollagen I/Matrigel®-Matrix etwa 4:1 beträgt.

11. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach einem der Ansprüche 1 bis 10, wobei die Resuspension der Embryoidkörper/Mesoderm-induzierten Embryoidkörper die Verwendung einer Pipette umfasst

12. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach einem der Ansprüche 1 bis 11, wobei das Dissoziieren oder Extrahieren einzelner Gefäßnetzwerke eine Extraktion unter Verwendung von sterilen Präparierwerkzeugen umfasst

13. Verfahren zur Herstellung von Säuger-Knochenmark-Organoiden nach einem der Ansprüche 1 bis 12, wobei das Säugetier ausgewählt ist aus der Gruppe bestehend aus einem Menschen, einer Maus, einem Affen, einer Ratte, einem Schwein, einem Hund, einer Katze, einem Kaninchen, einem Schaf, einem Rind, einem Pferd und einer Ziege.

14. Ein vaskuläres Netzwerk oder ein reifes Säuger-Knochenmark-Organoid, hergestellt gemäß dem Verfahren nach einem der Ansprüche 1 bis 13, oder eine pharmazeutische Zusammensetzung, die das vaskuläre Netzwerk und/oder das reife Säuger-Knochenmark-Organoid umfasst.

15. Gefäßnetzwerk oder reifes Säuger-Knochenmarkorganoid nach Anspruch 14, das ein Modell für eine mit dem Knochenmark zusammenhängende Krankheit ist, wie beispielsweise eine hämatologische Krankheit, schwere angeborene Neutropenie, Myelofibrose, Blutzellenkrebs, Anämie, Thrombozytopenie, angeborene Fehler der Hämatopoese und Immunität, Erkrankungen im Zusammenhang mit HIV, Sichelzellenanämie und Komplikationen durch Chemotherapie oder Transfusionen.

16. Verwendung des vaskulären Netzwerks oder reifen Säuger-Knochenmarkorganoids oder der pharmazeutischen Zusammensetzung nach Anspruch 14 als Modellsystem bei der Pathogenese einer mit dem Knochenmark in Zusammenhang stehenden Erkrankung, wie beispielsweise einer hämatologischen Erkrankung, schwerer angeborener Neutropenie, Myelofibrose, Blutzellenkrebs, Anämie, Thrombozytopenie, angeborene Störungen der Hämatopoese und Immunität, Erkrankungen im Zusammenhang mit HIV, Sichelzellenanämie und Komplikationen durch Chemotherapie oder Transfusionen.

17. Verwendung des vaskulären Netzwerks oder reifen Säuger-Knochenmark-Organoids oder der pharmazeutischen Zusammensetzung nach Anspruch 14 als Modellsystem zum Identifizieren und/oder Testen von pharmazeutisch wirksamen Verbindungen zum Behandeln oder Verhindern einer mit dem Knochenmark in Zusammenhang stehenden Erkrankung, wie zum Beispiel einer hämatologischen Erkrankung, schwerer angeborener Neutropenie, Myelofibrose, Blutzellkrebs, Anämie, Thrombozytopenie, angeborenen Störungen der Hämatopoese und Immunität, Zuständen im Zusammenhang mit HIV, Sichelzellenanämie und Komplikationen durch Chemotherapie oder Transfusionen.

18. Verwendung des vaskulären Netzwerks oder reifen Säuger-Knochenmark-Organoids oder der pharmazeutischen Zusammensetzung nach Anspruch 14 zur in-vitro-Produktion von BMOs oder Säuger-Blutzellen, insbesondere autologen BMOs oder Säuger-Blutzellen, insbesondere zur Transplantation.

19. Pharmazeutisch wirksame Menge des Gefäßnetzwerks oder des reifen Säuger-Knochenmark-Organoids oder der pharmazeutischen Zusammensetzung nach Anspruch 14 zur Verwendung bei der Behandlung einer mit dem Knochenmark zusammenhängenden Erkrankung, wie beispielsweise einer hämatologischen Erkrankung, schwerer angeborener Neutropenie, Myelofibrose, Blutzellkrebs, Anämie, Thrombozytopenie, angeborene Störungen der Hämatopoese und Immunität, Zuständen im Zusammenhang mit HIV, Sichelzellenanämie und Komplikationen durch Chemotherapie oder Transfusionen, vorzugsweise für Transplantationen.

Figure 1

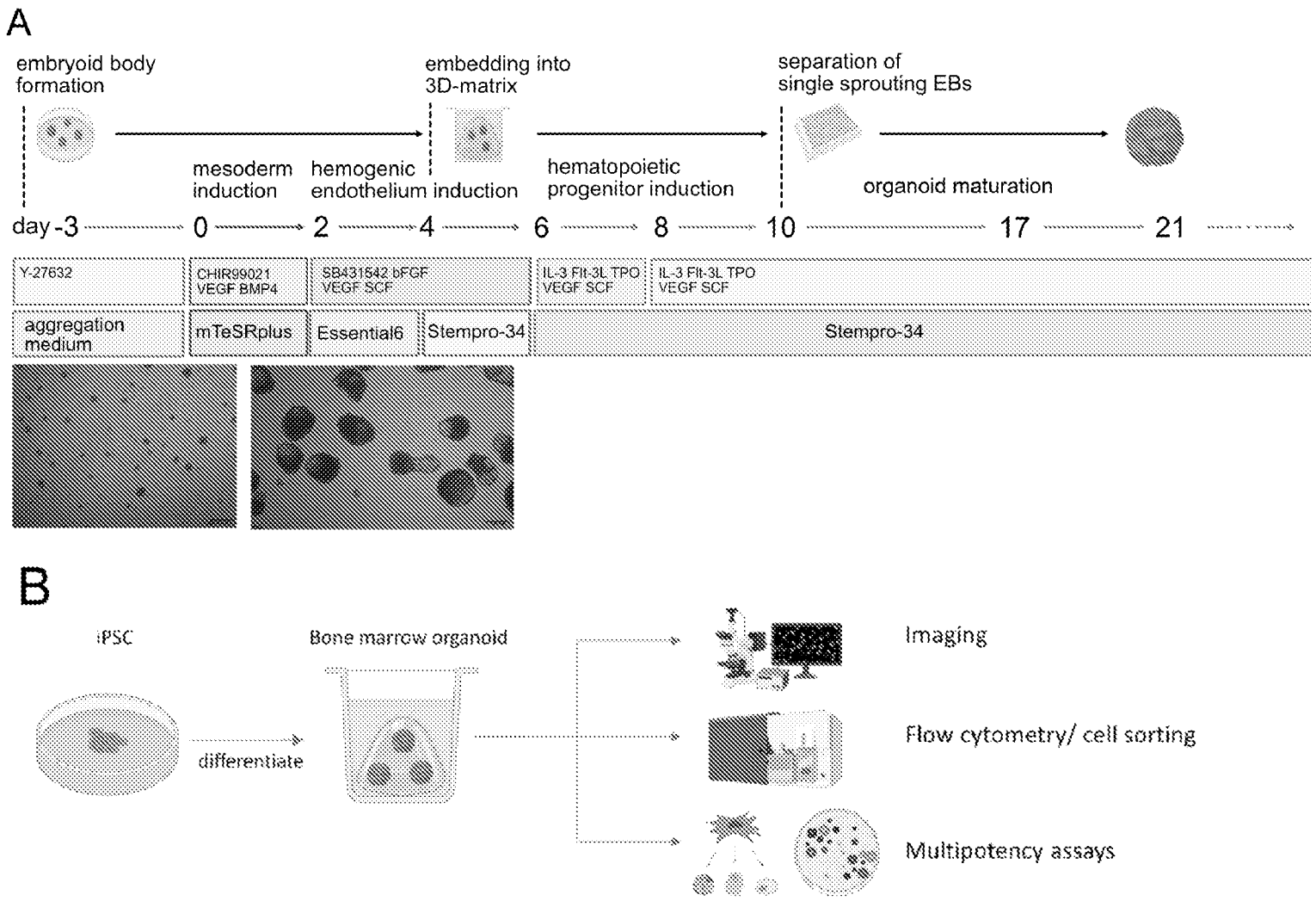
A
embryoid body formation
embedding into 3D-matrix
separation of single sprouting EBs
mesoderm induction
hemogenic endothelium induction
hematopoietic progenitor induction
organoid maturation
day -3
0
2
4
6
8
10
17
21
Y-27632
CHIR99021 VEGF BMP4
SB431542 bFGF VEGF SCF
IL-3 Flt-3L TPO VEGF SCF
IL-3 Flt-3L TPO VEGF SCF
aggregation medium
mTeSRplus
Essential6
Stempro-34
Stempro-34
B
iPSC
Bone marrow organoid
differentiate
Imaging
Flow cytometry/ cell sorting
Multipotency assays

Figure 2

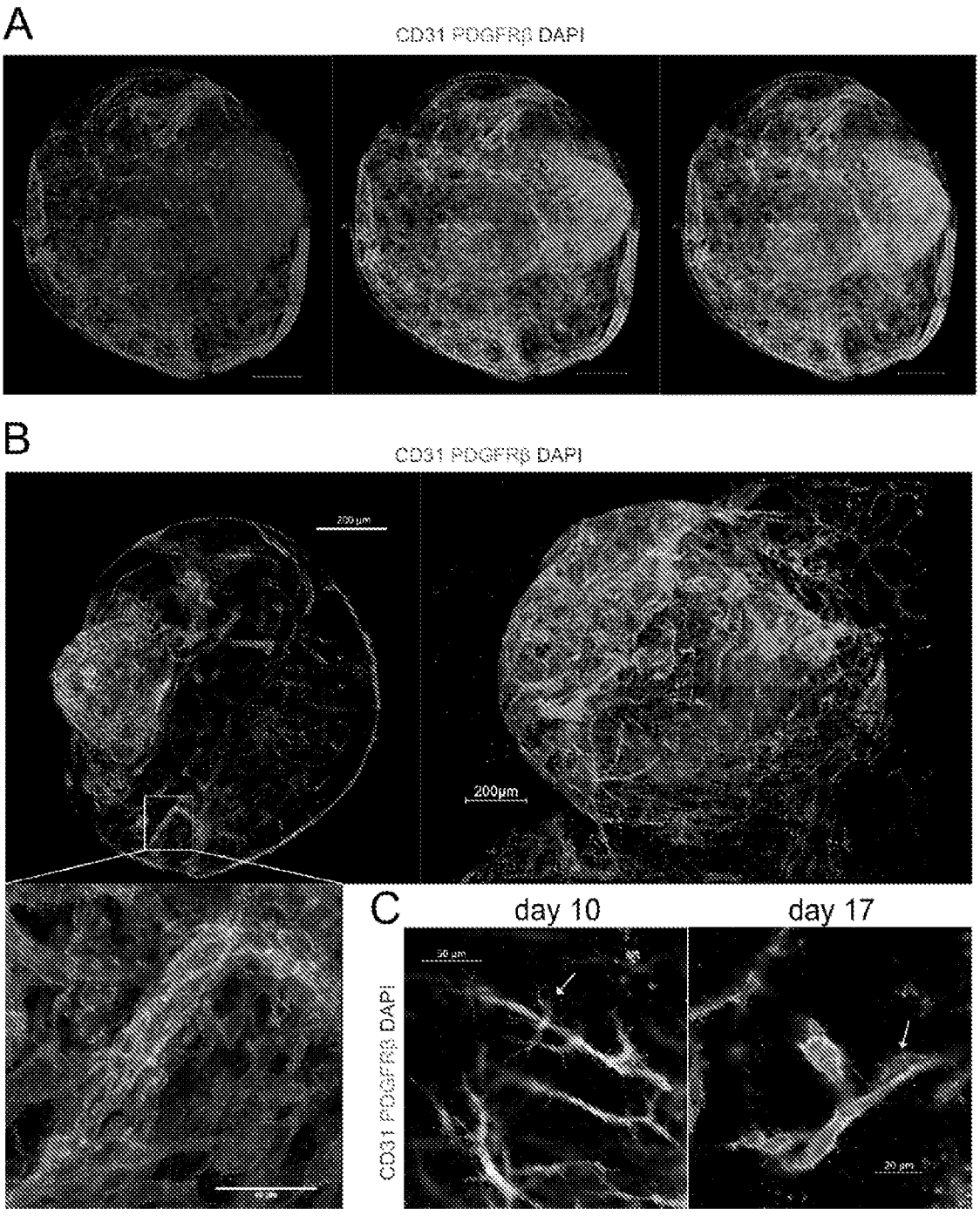
A
CD31 PDGFRβ DAPI
B
CD31 PDGFRβ DAPI
200µm
200µm
C
day 10
day 17
CD31 PDGFRβ DAPI
50 µm
20 µm

Figure 2 (continued)

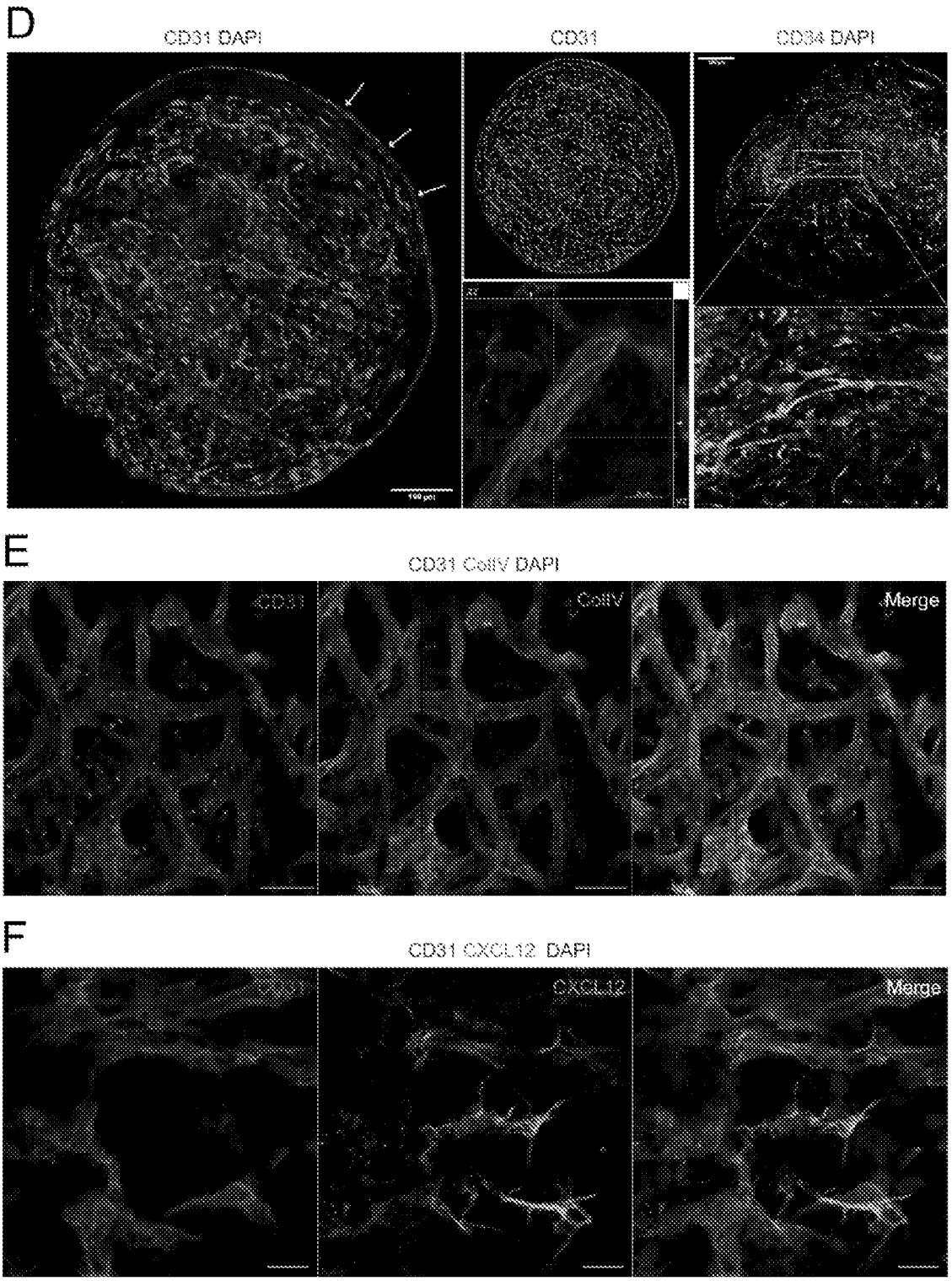

Figure 3

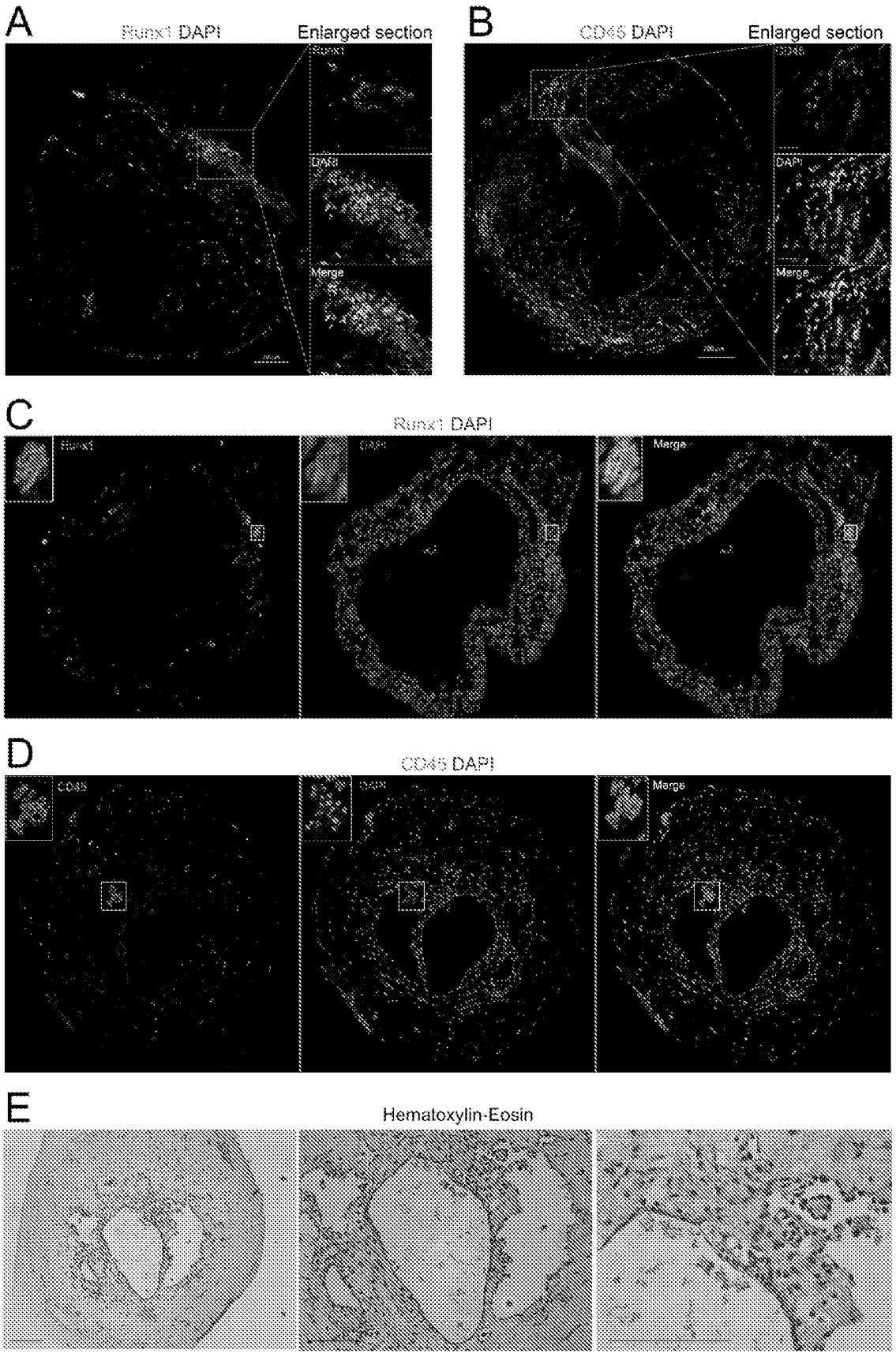
A
Runx1 DAPI
Enlarged section
Runx1
DAPI
Merge
B
CD45 DAPI
Enlarged section
CD45
DAPI
Merge
C
Runx1 DAPI
Runx1
DAPI
Merge
D
CD45 DAPI
CD45
DAPI
Merge
E
Hematoxylin-Eosin

Figure 4

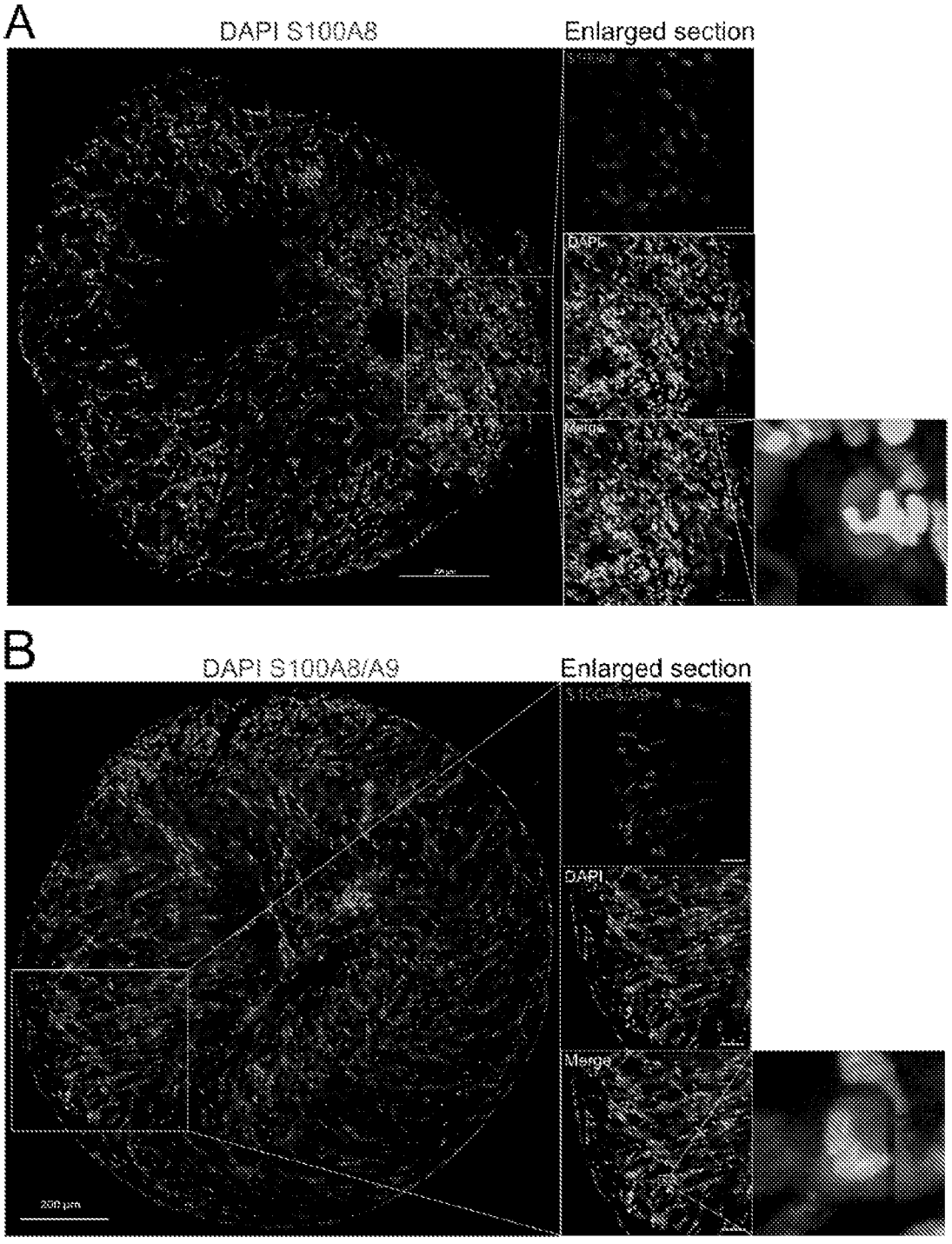
A
DAPI S100A8
Enlarged section
DAPI
Merge
B
DAPI S100A8/A9
Enlarged section
DAPI
Merge
200 μm

Figure 4 (continued)

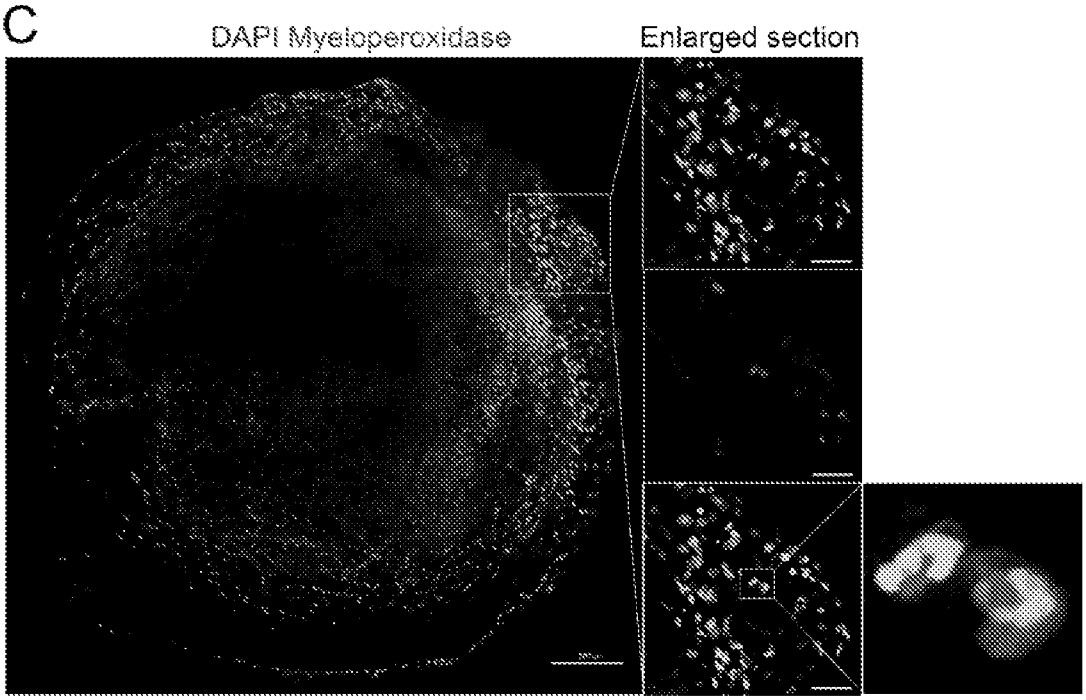

Figure 5

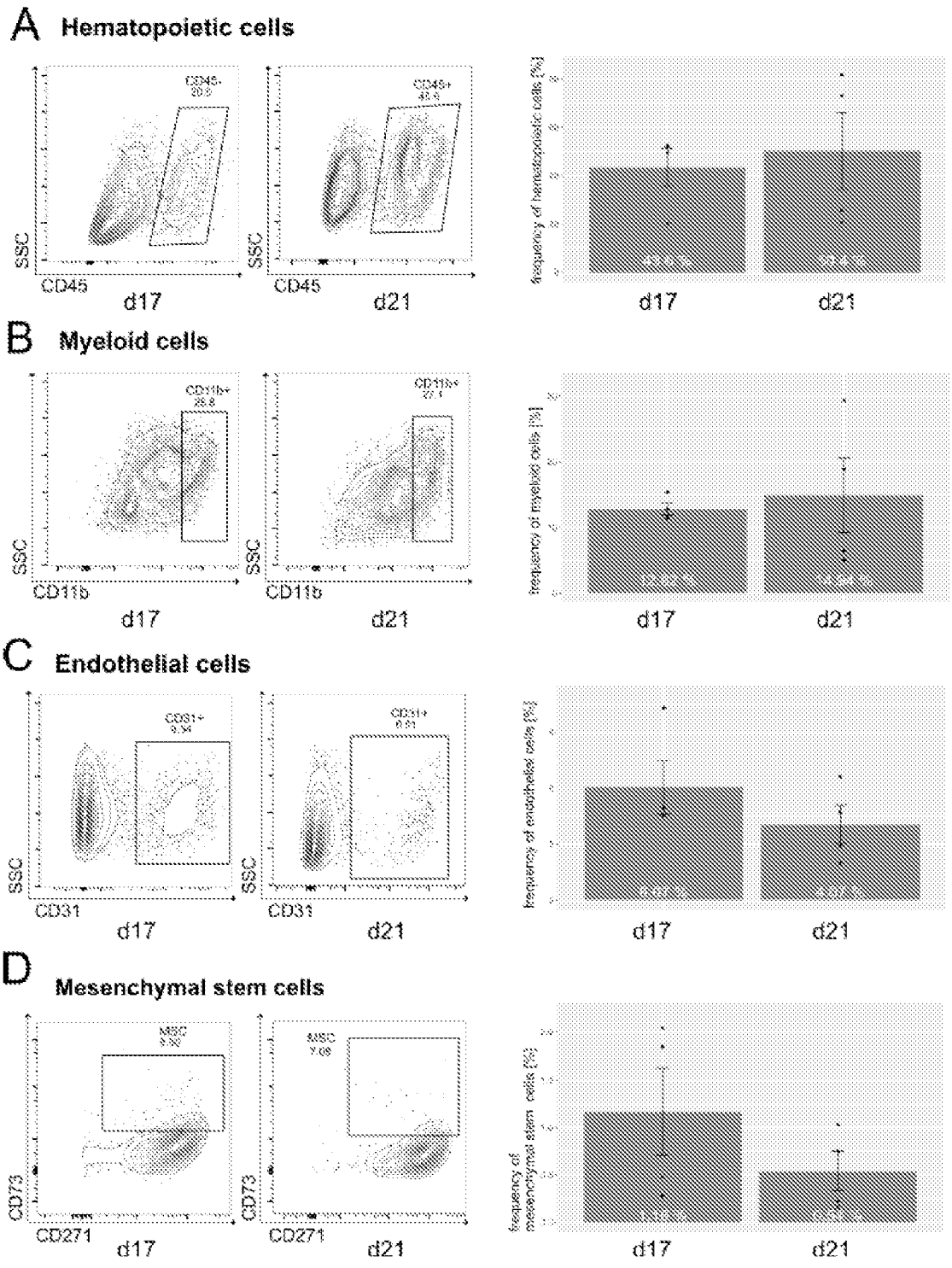
A
Hematopoietic cells
CD45+
SSC
CD45
d17
d21
frequency of hematopoietic cells [%]
B
Myeloid cells
CD11b+
CD11b
frequency of myeloid cells [%]
C
Endothelial cells
CD31+
CD31
frequency of endothelial cells [%]
D
Mesenchymal stem cells
MSC
CD73
CD271
frequency of mesenchymal stem cells [%]

Figure 6

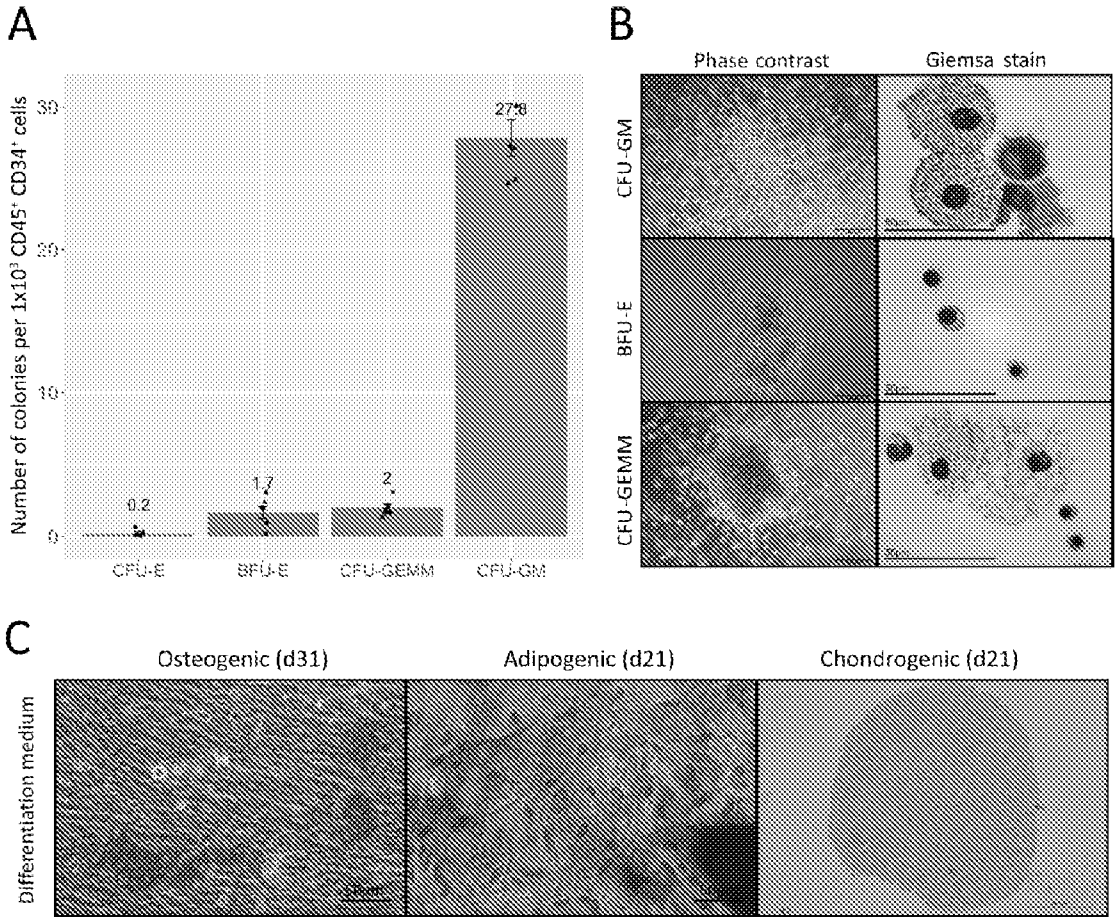
A
Number of colonies per 1x10³ CD45⁺ CD34⁺ cells
30
20
10
0
0.2
1.7
2
27.8
CFU-E
BFU-E
CFU-GEMM
CFU-GM
B
Phase contrast
Giemsa stain
CFU-GM
BFU-E
CFU-GEMM
C
Osteogenic (d31)
Adipogenic (d21)
Chondrogenic (d21)
Differentiation medium

Figure 7

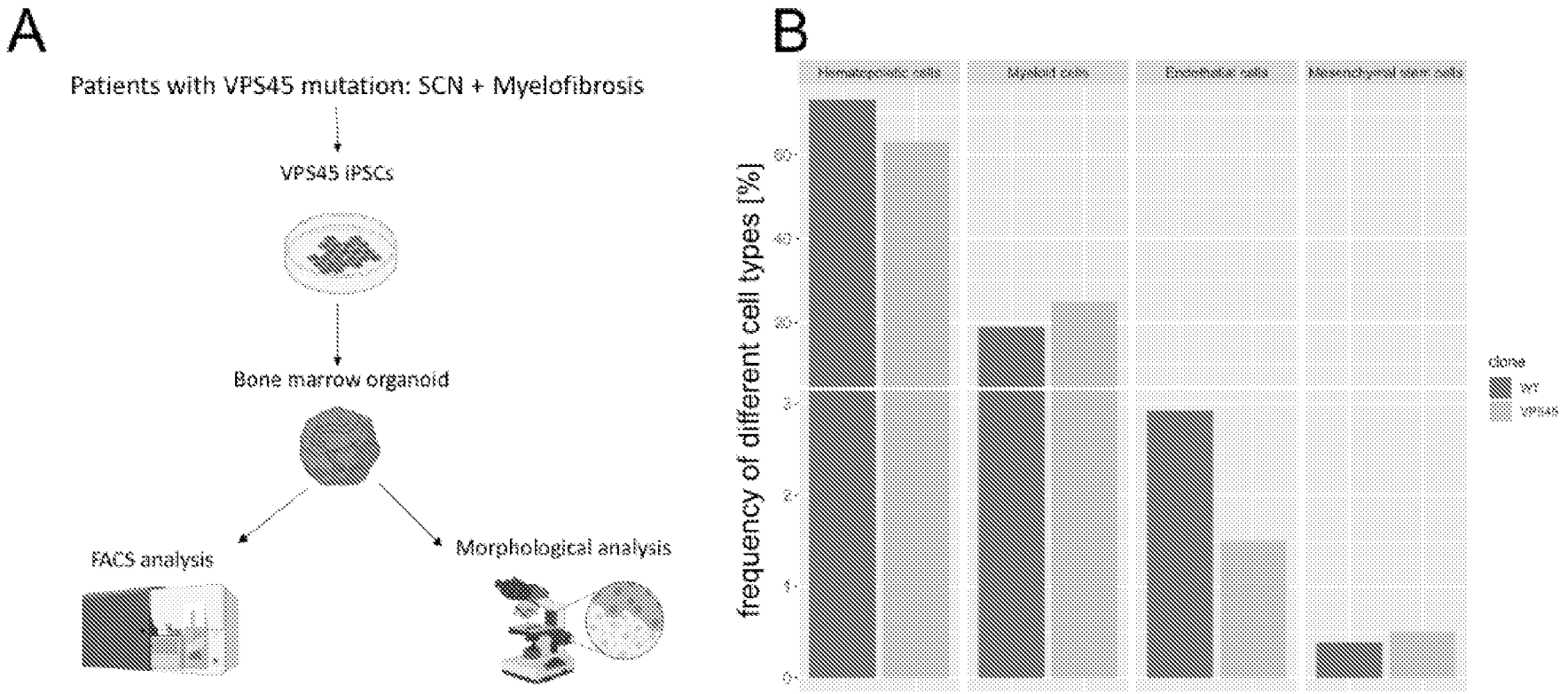
A
Patients with VPS45 mutation: SCN + Myelofibrosis
VPS45 iPSCs
Bone marrow organoid
FACS analysis
Morphological analysis
B
frequency of different cell types [%]
clone
WT
VPS45

Figure 7 (continued)