The fetal mouse metatarsal bone explant as a model of angiogenesis

Song, Weihua; Fhu, Chee Wai; Ang, Koon Hwee; Liu, Cheng Hao; Johari, Nurul Azizah Binte; Lio, Daniel; Abraham, Sabu; Hong, Wanjin; Moss, Stephen E; Greenwood, John; Wang, Xiaomeng

2015

Song, W., Fhu, C. W., Ang, K. H., Liu, C. H., Johari, N. A. B., Lio, D., et al. (2015). The fetal mouse metatarsal bone explant as a model of angiogenesis. *Nature Protocols*, 10(10), 1459-1473.

<https://hdl.handle.net/10356/82099>

<https://doi.org/10.1038/nprot.2015.097>

© 2015 Macmillan Publishers Limited, part of Springer Nature. This is the author created version of a work that has been peer reviewed and accepted for publication by *Nature Protocols*, Macmillan Publishers Limited. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at:
[<http://dx.doi.org/10.1038/nprot.2015.097>].

Downloaded on 26 Apr 2023 22:56:04 SGT

Title: The fetal mouse metatarsal bone explant as a model of angiogenesis

Authors: : Weihua Song^{1*} songweihua@ntu.edu.sg, Chee Wai Fhu^{1*} fcw@ntu.edu.sg, Koon Hwee Ang² khang@imcb.a-star.edu.sg, Cheng Hao Liu¹ LIUC0044@e.ntu.edu.sg, Nurul Azizah Binte Johari¹ nurul_azizah@ntu.edu.sg, Chin Shiuian Lio¹ daniel.lio@ntu.edu.sg, Sabu Abraham³ sabu.abraham@manchester.ac.uk, Wan Jin Hong² mcbhwj@imcb.a-star.edu.sg, Stephen E Moss⁴ s.moss@ucl.ac.uk, John Greenwood⁴ J.greenwood@ucl.ac.uk, Xiaomeng Wang^{1,2,4} wangxiaomeng@ntu.edu.sg

¹Lee Kong Chian School of Medicine, Nanyang Technological University, 50 Nanyang Drive, Research Techno plaza, Level 4, X-Frontiers Block, Singapore 637664. ²Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), 61 Biopolis Drive, Proteos, Singapore 138673. ³Institute of Cardiovascular Sciences, 3.20, Core Technology Facility (3rd floor) 46 Grafton Street, Manchester, UK, M13 9NT. ⁴Department of Cell Biology, Institute of Ophthalmology, University College London, 11-43 Bath Street, London, UK, EC1V 9EL. *equal contribution.

Correspondence should be addressed to X.M.W. (wangxiaomeng@ntu.edu.sg; telephone number: 65923840; Fax: 65150417).

Key words: Cell and Tissue Culture, Functional Analysis

Editorial Summary: This protocol describes how to set up an assay investigating vessel outgrowth from mouse foetal metatarsals. This assay is an *ex vivo* assay to investigate sprouting angiogenesis.

Abstract

The mouse foetal metatarsal provides a unique tool for studying angiogenesis. In comparison with other commonly used *in vitro* or *ex vivo* angiogenesis assays, vessel outgrowth from mouse foetal metatarsals is more representative of sprouting angiogenesis *in vivo*. It allows the analysis of blood vessel growth, and the mechanisms underpinning this process, in a multicellular microenvironment that drives the formation of a robust and complex vascular network in the absence of exogenous growth factors. By labelling different constituents of the vascular structure it is possible to perform 3D rendering of the spatial interplay between different cellular components and carry out quantitative analysis of vessel outgrowth. High-resolution imaging permits the visualization of fine structural and cellular details. As the assay involves the use of foetal tissues, it is possible to follow new blood vessel formation in genetically modified mice that are perinatally lethal. The entire process takes 9-13 days. A detailed description of how to set up and perform the assay is described here.

INTRODUCTION

Angiogenesis, a process involving the sprouting of new blood vessels from pre-existing capillaries, plays an important role in development as well as in physiological conditions such as wound healing and the menstrual cycle. In addition, angiogenesis is implicated in the development and progression of many disease conditions, including cancers, the exudative form of age-related macular degeneration, diabetic vascular disease, atherosclerosis, rheumatoid arthritis and cardiovascular complications. Targeting angiogenesis has therefore become a major therapeutic focus, and has also resulted in some clinical success. Nevertheless, additional strategies could add to the angiogenesis-related therapeutic armoury. *In vitro/ex vivo* model systems can be used to increase understanding of the processes of angiogenesis and enable identification of additional angiogenesis-related therapeutic targets. Subsequently they can be used as screens for modulators of angiogenesis. Many of these models of pathogenic angiogenesis, however, are of questionable value as they are reductionist or rely on added exogenous factors to drive the vessel growth. The mouse foetal metatarsal assay, first described by Deckers *et al.*, (2001)¹ offers a unique system in which to study sprouting angiogenesis in a physiologically relevant multicellular micro-environment that is more representative of *in vivo* blood vessel formation than many other models. Here, we present a step-by-step protocol for metatarsal dissection, culture, staining and imaging and microvasculature quantification methods.

Development of the protocol

To decipher angiogenic signalling cascades, validate potential therapeutic targets and evaluate the efficacy of novel pro/anti-angiogenic drugs, a reliable, (patho)physiologically relevant, and manipulatable assay is required. Numerous cell-based assays have been developed, however, their application as translational models is limited by the absence of a relevant, complex microenvironment. Although animal models provide the necessary complexity, *in vivo* assays are expensive, time consuming, unsuitable for high throughput studies, and restricted in their experimental flexibility. *Ex vivo* assays involving the use of intact organ explants bridge the gap between cell-based assays and *in vivo* assays.

The rat/mouse aortic ring assay is the most widely used organ-culture assay in angiogenesis research. However, in the absence of exogenous angiogenic factors the capacity of aortic rings to develop vascular sprouts is limited², restricting their use. Moreover, the fact that aortic rings are cultured in a three-dimensional matrix complicates vessel quantification. In 2001 Deckers *et al.*,¹ developed an *ex vivo* angiogenesis assay using foetal mouse metatarsal explants to study the effects of angiogenic and antiangiogenic compounds. Spontaneous blood vessel formation from metatarsals is the result of complex interactions between different types of cells leading to the development of an intricate vascular network on top of a fibroblast sheet. However, the dissemination of this technology has been hindered by technical challenges including the difficult dissection procedure, long experiment time (12-19 days) and reproducibility of vessel formation. Furthermore, no standardised quantification methods have been described to characterize the fine details of microvessel outgrowth. Here, we present a substantially optimized

protocol that greatly simplifies the dissection procedure, increases the viability of explants, shortens the experiment time and leads to consistent vessel formation between explants. We also provide immunostaining protocols for visualizing different types of vascular cells and quantification methods for characterising structural and cellular details of the emerging metatarsal microvasculature. By using different image analysis software, it is possible to carry out quantitative analysis of characteristic features of angiogenesis including tip cell and filopodia number, number of branch points, total vessel length, cell proliferation, and perivascular cell coverage. We have used this protocol to evaluate the role of a novel angiogenic factor LRG1 in sprouting angiogenesis³.

Applications of the assay

The mouse foetal metatarsal assay presents opportunities to address important vascular biology questions that are not readily testable in other *in vitro*, *ex vivo* or *in vivo* angiogenesis assays. As vessel outgrowth from metatarsals form a complex network in the absence of exogenous angiogenic factor stimulation, it is especially useful for evaluating the effects of exogenous agents on the angiogenic process (for example, small molecule inhibitors⁴⁻⁷, growth factors^{8,9}, antibodies⁸ and recombinant proteins^{1,3,10-14}). It can be used for deciphering angiogenic cascades or validating potential therapeutic targets by taking advantage of either global or cell type specific genetically manipulated mouse tissues^{8,9,13}. As foetal tissues are used for the metatarsal assay, it also provides a unique opportunity to study the mechanistic details of angiogenic defects that cause perinatal lethality. The assay is also a valuable tool for angiogenesis-related drug screening⁵ and provides an opportunity to distinguish between effects on angiogenesis and on established vasculature⁷. In addition, the microvasculature derived from metatarsals incorporates many of the cell types associated with angiogenesis including endothelial cells, mural cells, macrophages and fibroblasts. It is, therefore, possible to study the influence of drugs/treatments on specific cellular characteristics and the physical interactions between different types of cells as well as the influence of one cell type on the function of others. Protein and/or RNA can be extracted from the metatarsal and/or cells migrating out from metatarsals (including the microvasculature, macrophages and fibroblasts grown underneath) for further dissection of molecular mechanisms under different experimental conditions.

Advantages of the assay

The mouse foetal metatarsal angiogenesis assay provides many advantages over other commonly used models for studying sprouting angiogenesis:

- Vessel outgrowth from metatarsals recapitulates key features of angiogenesis observed *in vivo*, such as the formation of endothelial cell lumen, the development of tip cells and the recruitment of surrounding support cells. It permits the study of angiogenesis in a complex microenvironment involving interactions between different cell types, e.g.,

direct physical engagement or indirect effects exerted by one cell type on another.

- Angiogenesis is normally a microvascular event and the vessel outgrowth from metatarsals is derived from microvascular cells in the perichondrium unlike other *ex vivo* angiogenesis assays, including the aortic ring and choroid sprouting assays,¹⁵ that involve large blood vessels.
- Unlike the mouse aortic ring assay, a robust and complex vascular network develops from mouse foetal metatarsal bones without the requirement of exogenous growth factors (Figure 1). The assay, therefore, permits the evaluation of test compounds in an existing pro-angiogenic environment.
- By taking advantage of the transgenic mice carrying fluorescent reporter probes under the control of cell type-specific promoters, e.g., PDGFR β -RFP mice¹⁶ and Tie2-GFP mice (JAX mice no. 003658), it is possible to monitor blood vessel formation and the interaction between different types of vascular cells over time by time-lapse imaging.
- Imaging microvessels at high magnification using confocal microscopy allows the visualization of different types of vascular cells, fine details of cellular morphology and physiological activity, and spatial arrangement of multiple cell types, providing a comprehensive picture of angiogenic properties.
- Instead of being embedded in a gel matrix as with aortic rings and choroid explants, metatarsal bones are directly attached to the bottom of a cell culture plate. The ensuing vessel outgrowth occurs in a two dimensional plane which renders the metatarsal assay more amenable to quantitative analysis.
- It is possible to isolate the vessel outgrowth from the bones for protein and RNA extraction.
- The metatarsal assay can readily incorporate genetic manipulation and treatments with growth factors, drugs or antibodies. By taking the advantage of genetic mouse models with depletion of specific cell type such as macrophage¹⁷ and pericyte^{18,19}, it is possible to study the cell type-specific contributions to sprouting angiogenesis during drug treatment. The metatarsal assay is also useful for studying mouse mutants that are perinatally lethal.

In summary, the mouse foetal metatarsal assay provides a physiologically relevant model for sprouting angiogenesis involving key events similar to that observed *in vivo*.

Limitations of the assay

In the vascular system, the biomechanical force driven by the flow of blood influences the vascular cell phenotype. Unlike *in vivo* models of angiogenesis, there is no blood flow in newly formed microvasculature structures derived from metatarsals, a feature shared by all other *in vitro* and *ex vivo* models.

Dissecting mouse foetal metatarsals is technically demanding, and a standardized preparation procedure and analysis method is the key for subsequent reproducibility and data interpretation. However, with the optimized protocol described here, dissection time is greatly reduced which significantly improves explant viability and reproducibility of vessel formation.

A number of approaches have also been developed to carry out quantitative morphometric analysis of vessel outgrowth in metatarsal cultures and to analyse spatial information of neighbouring vascular cells by performing 3-dimensional rendering of z-stack confocal images. However, not all laboratories have access to high-end imaging analysis software necessary to perform such studies, and analysis can be time-consuming which limits its utility for large-scale screening studies. To overcome some of these limitations, we also provide here a method using free automated batch image processing software, which measures vascular area with high accuracy and can process 10 images per minute.

Experimental design

Overview of the procedure The general protocol for mouse foetal metatarsal assay involves dissecting (steps 2-12), seeding (steps 13-16) and feeding the metatarsals (steps 17-19), and staining (steps 20-29), imaging (step 30) and quantifying (step 31) the emerging microvasculature (Figure 2). The middle three metatarsals are dissected from each mouse hind leg. The 1st and 5th metatarsals are discarded as they are smaller and their inclusion introduces greater variability to the assay. Each metatarsal is then placed in the middle of a 24-well or a 35mm plate coated with a suitable matrix, for example, fibronectin, collagen I, or gelatin. Once seeded, the metatarsals are fed with growth medium every 2-3 days. This can be supplemented with various, peptides, recombinant proteins, growth factors, antibodies or small molecule inhibitors. At the end of the experiment, the metatarsal bone and its outgrowing cells (including endothelial cells, mural cells, macrophages, fibroblasts, etc.) can be harvested for RNA or protein extraction separately or be fixed for immunofluorescence staining using cell-type-specific markers to perform further microscopic quantification and analysis. Alternatively, cells may be dissociated, separated and analysed by flow cytometry.

Experimental animals This protocol is applicable to the study of sprouting angiogenesis in wild type mice or mice with either global or cell type specific genetic modifications. Each foetus can provide 6 metatarsals. As there is no significant difference in vessel outgrowths between metatarsals isolated from the same foetus or among different foetus within the same litter, metatarsals from the same litter are normally pooled before being seeded individually. In most cases, 6-8 metatarsals are used for each treatment to show statistically significant differences. The experiment needs be repeated in three independent assays using metatarsals isolated from different litters.

Choice of embryonic stage Foetuses at embryonic stage (E) 15.5-18.5 can be used for metatarsal preparation as they exhibit similar vessel outgrowth (Figure

3a). However, it is important to use metatarsals of the same age for the experiment as metatarsals isolated from fetuses at different embryonic stages are of different sizes, morphologies and structure. E15.5 metatarsal tissues are very fragile. Matrix mineralization of the bones has not started and the joints between phalanges, metatarsals and tarsals are not clearly visible making it difficult to keep a consistent amount of connective tissue around the bones during dissection. As a result, vessel outgrowth from E15.5 metatarsals is highly variable. At E16.5, the skin is stronger and more elastic, therefore, can be manipulated more easily. The mineralized zone of cartilage and joints between bones are readily visible. The digits are fanned out and metatarsals are not tightly connected to each other. As a result, metatarsals can be isolated more easily which leads to greater consistency in vessel formation. A prominent feature of E17.5 and E18.5 metatarsals is the secondary fusion of the epidermis between digits. The metatarsals are connected by tightly packed protein fibres of connective tissue, which makes metatarsal isolation and trimming more difficult to achieve. Furthermore, the attachment rate of E15.5 and E18.5 metatarsals is lower than that from E16.5 and E17.5 (Figure 3b). Therefore, E16.5 metatarsals are recommended for the assay. The middle three metacarpals from E17.5 and E18.5 can also be prepared in the same fashion. However, their size and angiogenic potential are different from corresponding metatarsals of the same age, therefore, need to be used as a separate group for the experiment (supplementary Figure 1).

Choice of culture medium Metatarsals grown in MEM α culture medium showed complex vessel formation with PECAM-1 positive endothelial cells. No vessel outgrowth occurs from metatarsals grown in DMEM, EBM2 or EGM2 culture medium (Figure 3c). Therefore, MEM α culture medium is ideal for metatarsal vessel formation.

Choice of Matrix Metatarsals grown on non-coated Iwaki and Greiner plate show significantly less sprouts compared to those grown on other plastic surfaces or extracellular matrix derivatives, such as gelatin, fibronectin or collagen I (Figure 3d and 3e). Coating culture plates with extracellular matrix derivatives can significantly improve the attachment rate of metatarsals (Figure 3f). As gelatin is less expensive than fibronectin or collagen I, we use it as the default coating substrate for the metatarsal assay.

Culture conditions Metatarsals with fibroblasts that migrate out from the explant on day 2 are considered viable. Blood vessels are visible from day 3 and a complex vascular network is completely established by day 7. Drug/experimental treatments normally start on day 2 with medium being changed every 2-3 days (this may be variable depending on the stability of the drug or other special requirements of the experiment). For metatarsals grown on 35mm culture plates, medium may be changed every 4-5 days.

Controls When studying sprouting angiogenesis from metatarsals isolated from genetically modified mice, wild type littermates from the cross between

heterozygous parents are ideal controls. If conditional knockout mice are used, metatarsals from littermates with floxed target genes, with or without Cre, are appropriate controls. 1 μ M 4OH-tamoxifen can be used to activate Cre-recombinase and delete the floxed target gene 3 days after seeding metatarsals³. In some instances it may be appropriate to use virally delivered Cre to perform *in situ* gene knock-out on the metatarsal. 2mm of tail tips from individual embryos are collected for DNA extraction and genotyping when mice with genetic modifications are used. For metatarsals treated with blocking antibodies, the pre-immune antibody (at the same concentration) is an ideal control. If that is not available, an isotype matched control antibody generated from the same species as the target antibody can be used as a negative control. For small molecule inhibitors, a control with the same volume of vehicle is required. Scrambled peptides are standard negative controls for small peptide drugs and the denatured form of the same protein is normally used as a negative control for recombinant proteins. Growth factors or molecules with known angiogenic/anti-angiogenic potentials such as VEGF, bFGF, LRG1 and Eylea could be used as positive/negative controls.

Immunofluorescence staining Fluorescent staining with platelet endothelial cell adhesion molecule (PECAM-1; CD31) (Figure 4a) and isolectin B4 (IB4) (Figure 4b) is normally used to visualize vessel outgrowth from metatarsals. NG2 (Figure 4a and 4c) and α SMA (Figure 4d) can be used to label perivascular mural cells. Macrophages can be double labelled with IB4 and F4/80-specific antibody (Figure 4b). Multiple staining with different antibodies or molecules allows the analysis of physical interactions between different types of cells. Ki67 antibody can be used to label proliferating cells (Figure 4e and supplementary figure 2). Cleaved Caspase 3 can be used to label cells undergoing apoptosis (Figure 4f). Podocalyxin is used to detect endothelial cell lumen in metatarsal vasculature (Figure 4g).

Imaging and quantification After immunofluorescence staining, an epifluorescence microscope can be used for visualizing the vessel outgrowth from metatarsals. Confocal microscopy maybe used to capture the detailed features of vascular cells (Figure 4h) and their interactions (Figure 4i). TRI2 software is useful for quick batch image processing, whereas Imaris software is an appropriate package for detailed analysis of vessel formation including vessel calibre, the number of branch points and total vessel length as well as to construct 3D images and videos. Image J is a freeware that can be used to calculate perivascular cell coverage, the number of tip cells, the number of filopodia and the percentage of proliferating cells.

MATERIALS

REAGENTS

- Pregnant C57/BL6 Mice from E15.5 to E18.5 ! **CAUTION** Adhere to all relevant ethics guidelines for animal care and experimentation. **CRITICAL** Other

strains like Swiss albino mice^{1,4,5}, Swiss 3T3 mice⁸, mice on 129/Ola and C57Bl/6J mixed genetic background^{3,6}, BALB/c²⁰ have also been used for metatarsal assay and are alternatives for the assay.

- DPBS, calcium, magnesium (Dulbecco's phosphate-buffered saline; Gibco, cat. no. 14040-133) **! CAUTION** Ca^{2+} , Mg^{2+} ions are required for cell-cell and cell-substrate adhesion. DPBS with CaCl_2 and MgCl_2 maintains the structural and physiological integrity of the explant.
- FBS (EU-approved foetal bovine serum; GE Healthcare, cat. no. A15-101).
- MEM α (1X), GlutaMAX™ (Minimum Essential Medium; Gibco, cat. no. 32561-037).
- DMEM, GlutaMAX™ (Dulbecco's Modified Eagle Medium; Gibco, cat. no. 10567-014).
- EGM-2 BulletKit (Lonza, cat. no. CC-3162).
- EBM-2 Basal Medium (Lonza, cat. no. CC-3162).
- Penicillin-Streptomycin Solution (100X; 10,000-U ml^{-1} penicillin, 10,000- $\mu\text{g ml}^{-1}$ streptomycin; Thermo Scientific HyClone, cat. no. SV30010).
- HBSS 1X (Hanks' balanced salt solution + CaCl_2 + MgCl_2 ; Gibco, cat. no. 24020-117).
- EmbryoMax Ultrapure water with 0.1% gelatin (Millipore, cat. no. ES-006-B).
- Collagen type I, rat tail, 100mg, 3mg/ml (Corning, cat. no. 354236).
- Fibronectin from bovine plasma (Sigma-Aldrich, cat. no. F1141-5MG).
- ALK1 inhibitors (LDN 193189; Axon Medchem BV, cat. no. 1509 - B5).
- Recombinant LRG1 (HLRG1-His; produced in house, Also available from Creative BioMart, cat. no. LRG1-2295H).
- Paraformaldehyde (PFA; Sigma-Aldrich, cat. no. 158127-500G) **! CAUTION** Harmful if inhaled or swallowed. It may sensitize skin upon contact and it is carcinogenic. Wear protective goggles, gloves and protective clothing. Work inside a fume hood.
- BSA (Bovine Serum Albumin; Sigma, cat. no. A5611).
- Triton X-100 (Bio-Rad, cat. no.161-0407) **! CAUTION** Harmful if swallowed and can cause damage to the eyes. Wear protective goggles, gloves and protective clothing.
- 10% Tween 20 solution (Bio-Rad, cat. no.161-0781).
- Sodium azide (MP, cat. no. 199869) **! CAUTION** Highly toxic; use in a fume hood, wear protective goggles, gloves and protective clothing.
- PECAM-1 antibody (Purified Rat Anti-Mouse CD31 Clone; BD Biosciences, cat. no. 553370) **CRITICAL** This protocol has been optimised using this antibody for PECAM-1 staining. PECAM-1 staining is critical for visualizing vessel outgrowth from metatarsals.
- Anti-AN2-PE (MACS Miltenyi Biotec cat. no. 130-100-468).
- GSL I-B4 Isolectin (Vector lab, cat. no. FL-1201) **▲ CRITICAL** Isolectin IB4 staining is optimized by this protocol and it is critical for visualizing tip cells and filopodia.
- Monoclonal anti- α -smooth muscle actin antibody (α -SMA; Sigma Aldrich, cat. no. A5228).

- Anti-F4/80 antibody (AbD Serotech, cat. no. MCA497G)
- Anti-cleaved caspase 3 antibody (Cell Signalling Technology, cat. no. 9664)
- Anti-Ki67 antibody (Abcam, cat. no. ab15580).
- Podocalyxin antibody (R&D, cat. no. AF1556).
- Alexa Fluor® 488 goat anti-rat IgG (Life technologies, cat. no. A-11006).
- Alexa Fluor® 488 donkey anti-goat IgG (Life technologies, cat. no. A-11055).
- Alexa Fluor® 594 goat anti-rat IgG (Life technologies, cat. no. A-11007).
- Alexa Fluor® 594 goat anti-mouse IgG (Life technologies, cat. no. A-11005).
- Alexa Fluor® 594 goat anti-rabbit IgG (Life technologies, cat. no. A-11037).
- Hoechst 34580 (Sigma-Aldrich, cat. no. 63493).
- Mowiol® 4-88 (Aldrich, cat. no. 81381).
- RNeasy® Mini Kit (Qiagen, cat. no. 74106).
- Tris base (Biorad, cat. no. 161-0719).
- Sodium Dodecyl Sulfate (Hoefer Inc, cat. no. GR126-500) **! CAUTION** Harmful; avoid direct contact.
- UltraPure™ glycerol (Invitrogen, cat. no. 15514-011).
- β-mercaptoethanol (Biorad, cat. no. 161-0710) **! CAUTION** Toxic; handle with care.
- Bromophenol blue (USB, cat. no. 12370).
- Hydrochloric acid (Fischer Scientific, A144C-212) **! CAUTION** Corrosive; Causes severe skin burns and eye damage; may cause respiratory irritation. Wear protective goggles, gloves and protective clothing.
- qScript™ 1-step SYBR green qRT-PCR kit (Quanta Biosciences, cat. no. 95088-050).
- iTaq™ Universal SYBR® Green supermix (Biorad, cat. no. 172-5124).
- MicroAmp® Fast Optical 96-well reaction plates (Applied Biosystems, cat. no. 4346906).
- RT-PCR machine (Applied Biosystems, StepOnePlus™ Real time PCR system).
- Distilled water.
- Absolute ethanol molecular grade (Fischer Scientific, BP2818-500) **! CAUTION** Flammable liquid and vapour. May cause central nervous system depression, irritation on eyes, respiratory tract and skin upon contact. May cause liver, kidney and heart damages. Wear protective goggles, gloves and protective clothing.

EQUIPMENT

- Tweezers, no 5 (Dumont, cat. no. 91150-20) **▲ CRITICAL** Keep forceps in 70% (vol/vol) ethanol during the procedure to maintain sterility. Air-dry forceps completely before dissection.
- Laminar flow tissue culture hood (NUAIRE Biosafety cabinet, Class 2, Model No. NU-440-400E).
- Filters (0.2 µm filter; Sartorius, cat. no. 16532).
- pH meter (Sartorius, Basic Meter PB-11).

- Fume hood (Thermo Scientific Hamilton Concept).
- Bench top centrifuge 4°C (Fischer Scientific, Sorvall Legend Micro 21R Centrifuge).
- Bench top analytical balance (Kern ABS).
- Bench top plate rocker (Stuart gyro-rocker SSL3).
- 37°C water bath (Grant OLS200).
- Culture dishes (10cm; TPP, cat. no. 93100).
- Culture plates (NUNC MultiDish 24 well, Thermo Scientific, cat. no. F142475).
- Culture plates (MultiDish 24 well, Iwaki, cat. no. 3820-024)
- Culture plates (MultiDish 24 well, TPP, cat. no. 92024)
- Culture plates (MultiDish 24 well, Greiner Bio One, cat. no. 662160)
- Culture plates (MultiDish 24 well; Corning, cat. no. 3526)
- Culture dishes (35mm; Corning, cat. no. 430165).
- Microscope round coverslips (13mm Ø; Marienfeld-Superior, cat. no. 0111530).
- Nunc™ Thermanox™ coverslips (13mm; Thermo Scientific, cat. no. 174950).
- RNase-free Microfuge Tubes, 1.5ml (Life technologies, cat. no. AM12400).
- Safe-lock Eppendorf tubes, 1.5ml (Eppendorf, cat. no. 22363204).
- Humidified cell culture incubator at 37°C and 5% CO₂ (NUAIRE, Model No. NU-5810E).
- Dissection microscope (Nikon, SMZ 745T).
- Falcon tube (50ml; BD Falcon, cat. no. 352070).
- Epifluorescence stereomicroscope (Olympus SZX16) equipped with a QImaging Retiga 6000R cooled monochrome CCD camera.
- Nikon eclipse Ti inverted microscope.
- Olympus IX81 motorized inverted microscope.
- Upright Leica TCS SP8 confocal laser scanning microscope.
- Image editing software (Photoshop CS6, Adobe).
- Imaris software (Bitplane AG, Zurich, Switzerland).
- TRI2 software (TRI2 software can be downloaded from https://www.assembla.com/spaces/ATD_TRI/wiki for free.)
- Image J software (free from <http://rsb.info.nih.gov/ij/download.html>).

REAGENT SETUP

- **Collagen I coating solution.** Prepare 0.6% of glacial acetic acid in distilled water. Filter sterile using a 0.2 µm filter. Dilute collagen I solution from bottle with 0.6% acetic acid solution to achieve a concentration of 0.3mg/ml. Add the mixture into HBSS in 1:20. Prepare immediately prior to use as long term storage may cause collagen I precipitation.
- **Fibronectin coating solution.** Dilute fibronectin in 1:10 using 1X DPBS (without Ca²⁺ and Mg²⁺). .
- **Dissection solution.** Mix FBS in DPBS (Ca²⁺ and Mg²⁺) to reach final concentration of 10%. .

- **Culture medium.** Add 10% (vol/vol) heat inactivated FBS in MEM α . Add Penicillin-Streptomycin to 1X (eg. 1:100 dilution of a 100X stock) and store at 4°C for up to 3 months.
- **Laemmli Sample Buffer (2X).** Mix 20% glycerol, 4% (wt/vol) SDS, 120mM Tris-Cl (pH6.8), 0.02% (wt/vol) bromophenol blue and 10% (vol/vol) β -mercaptoethanol in distilled water. Store at room temperature (25°C) and use within a week.
- **Paraformaldehyde (4%, wt/vol).** Dissolve 2g of PFA in 50ml of 1xPBS by heating the solution to 60°C in a water bath with shaking function. Store the solution in aliquots at -20°C for up to one year. Thaw aliquots at room temperature and store at 4°C for up to 1 month.
- **Ethanol (70%, vol/vol).** Dilute absolute ethanol in distilled water and store indefinitely at room temperature.
- **Blocking Buffer.** Mix 3% Triton X-100, 1% Tween 20, 0.5% BSA, 0.1% sodium azide in 1X PBS. Store at 4°C for up to 1 month.
- **Hoechst solution.** Dissolve 1 mg of Hoechst powder in 1 ml of distilled water and store at -20°C for at least 2 years when protected from light.
- **Mowiol® 4-88 mounting medium.** Dissolve 2.4 g of Mowiol® 4-88 to 6 g of glycerol. Mix well before adding 6 ml of H₂O. Stir at room temperature until Mowiol® 4-88 is completely dissolved. Add 12 ml of 0.2 M Tris-Cl (pH8.5) and heat to 50°C for 10 minutes with stirring. Centrifuge at 5000g for 15 min to clarify the solution. Aliquot in airtight containers and store at -20° for at least 2 years.

EQUIPMENT SETUP

Housing and husbandry of experimental animals

Mice should be housed in specific pathogen-free conditions with regulated-humidity, temperature and 12 hour light and dark cycle. Feed mice with sterilized chow and water. Carry out timed mating in order to obtain foetus at specific embryonic stage. In brief, check vaginal plugs every morning after placing a male and a female together. Once the plug is observed, put the female into a new cage and consider that date as E0.5. An indicator of pregnancy is enlargement at the centre of the body.

▲CRITICAL Ensure that, as we have done within our research programme, you comply with all the governmental and institutional guidelines for the care and the use of animals.

Surgical tools. Rinse tweezers and scissors under running water and wrap with foil individually before =autoclaving.

Pipette tips. Cut off a few millimetres of P1000 pipette tips using sterile scissors to facilitate metatarsal transfer between dishes.

Benchtop tube rocker. Place the rocker in the cold cabinet for primary antibody incubation at 4°C overnight.

PROCEDURE

Coating the plate (Day 0) • TIMING 5 minutes

1. Coat each well of a 24-well plate (with or without sterile microscope round coverslips) with 300µl of coating solution or a 35mm plate with 1ml of coating solution at 37°C for overnight.

▲ **CRITICAL** A 24-well plate is normally used for analysing total vascular area and vessel density. A 35mm plate is ideal for detailed characterisation of sprouting angiogenesis, such as the interaction between different types of vascular cells

Metatarsal isolation (day 1) • **TIMING** 20 minutes for each foetus

2. Kill the pregnant dam at the desired gestational stage using cervical dislocation.

▲ **CRITICAL STEP** This step must be performed by licensed, qualified individuals according to institutional and governmental ethics guidelines.

3. Surface-sterilize the mouse with 70% (vol/vol) ethanol.
4. Place the scarified dam back-down on a dissecting board in a laminar flow tissue culture hood to maintain sterility. Fix the legs and arms to the board with pins.
5. Open the abdominal cavity with a single cut to expose the uterus.
 6. Lift the uterine horns out of the abdominal cavity with a pair of sterile tweezers while cutting the connection to the mesometrium. Transfer the uterine horns into a 10cm culture dish containing ice-cold dissection medium and place the culture dish on ice.

■ **PAUSE POINT** the uterine horns can be stored in ice cold dissection medium on ice for up to 6 hours.

7. Cut between each sac to separate individual foetuses. Sever each sac with tweezers and separate foetuses from placentas.
8. Transfer the foetus under a dissection stereomicroscope, cut the feet off just above the ankle joint and transfer feet with tweezers to a fresh 10cm culture dish containing ice-cold dissection medium.

▲ **CRITICAL STEP** Work with one embryo at a time and keep the rest on ice.

9. Grasp the exposed fibula with one pair of tweezers. Gently insert the other pair of tweezers between calcaneus and skin and advance into the foot pad. Pull the skin off as per removing a glove. Phalanges will be removed together with the skin leaving metatarsals and tarsals intact (Figure 5a and Supplementary video 1).

▲ **CRITICAL STEP** It is critical to remove the skin from plantar instead of dorsal surface to ensure maximum removal of plantar fascia and phalanges.

? **TROUBLESHOOTING**

10. Hold tarsals with one pair of tweezers and remove excessive connective tissue left underneath the metatarsals. The connective tissue should come off easily at E16.5. It will be more difficult if the foetuses are older (Figure 5b-d).

▲ **CRITICAL STEP** Do not over-clean metatarsals as it may damage endothelial cells in the perichondrium.

11. Hold tarsals with one pair of tweezers and use the other pair to separate neighbouring metatarsals. Remove 1st and 5th metatarsals.

▲ **CRITICAL STEP** Gently pull metatarsals away with tweezers so that bones are separated at the tarso-metatarsal joint.

? **TROUBLESHOOTING**

12. Transfer isolated metatarsals to a fresh 10cm culture dish containing ice-cold dissection medium using a trimmed P1000 pipette tip. Keep metatarsals on ice before continuing dissecting the next foetus.

▲ **CRITICAL STEP** Carefully cut off the end of a blue pipette tip using sterile scissors within the laminar flow hood as this will prevent damaging the metatarsals.

Seeding and feeding (Days 1-7) • TIMING 5 minutes per foetus including culture plate washing time

13. Aspirate the coating solution from a plate set up in step 1 and keep the plate open in laminar flow hood to dry for 10 minutes

▲ **CRITICAL STEP** The coated plates can be wrapped in Parafilm and stored at 4°C for 3 days

14. Wash the plate with 250µl of culture medium

▲ **CRITICAL STEP** This step will prevent metatarsal detachment after feeding.

15. Transfer one metatarsal per well of a 24-well plate or a 35mm plate using the cut-off blue pipette tip. Place metatarsals at the centre of each well using a P2 pipette tip (Supplementary video 2).

16. Remove excessive dissection solution transferred with metatarsals and let metatarsals adhere for 5 minutes inside the cell culture hood.

▲ **CRITICAL STEP** Make sure metatarsals are covered with thin film of liquid throughout the 5-minute incubation. Putting the lid on will prevent metatarsals from over-drying. Over-dried metatarsals become darker and their sprouting potential will be significantly compromised. They should therefore be excluded from the experiment.

17. Carefully feed metatarsals with 200µl of culture medium.

▲ **CRITICAL STEP** Add medium by placing the pipette tip against the side wall of the well and dispense medium slowly to avoid washing metatarsals off the bottom.

? TROUBLESHOOTING

18. Carefully place the culture plate at the back of the incubator at 37°C/5%CO₂ overnight.

19. Change culture medium on day 2 and then approximately every other day until the experiment ends. Conditioned medium may be retained to measure levels of secreted factors, e.g. using ELISA. If required, protein and RNA levels can be checked at the end of the experiment as described in Box 1.

▲ **CRITICAL STEP** Fibroblasts start to migrate out from viable metatarsals on day 2 and drug treatment can start from day 2 in 250µl of culture medium. Vessel outgrowths are visible on day 3. Phase-contrast microscopy can be used to monitor vessel outgrowth during the experiment (Figure 4j and Supplementary figure 3). To definitively identify and image blood vessels and various cell types proceed to the next step (Step 20).

? TROUBLESHOOTING

Fixation • TIMING 30 minutes

20. Remove the culture medium and wash metatarsals with DPBS (Ca²⁺, Mg²⁺).

21. Remove DPBS and add 200µl of 4% (wt/vol) PFA per well for 20 minutes at room temperature.

▲ **CRITICAL STEP** PFA fixation is ideal for all antibodies used in this protocol. Alternative fixatives may be required for other antibodies.

22. Aspirate the fixative and wash the plate twice with DPBS (Ca^{2+} , Mg^{2+}).

■ **PAUSE POINT** Samples can be stored overnight in DPBS at 4°C.

Staining • TIMING 2 days

23. Incubate metatarsals in blocking buffer for 1 hour with gentle rocking at room temperature.

24. Prepare primary antibodies in blocking buffer and incubate 150µl of antibody mixture with metatarsals overnight with gentle rocking at 4°C. The dilution of all antibodies used in this protocol that we recommend can be found in table 1.

▲ **CRITICAL STEP** NG2-PE and IB4-FITC are incubated with primary antibody. After the addition of fluorochrome-conjugated antibodies, the plates should be kept in dark at all time to obtain optimal staining. This antibody incubation condition is ideal for all antibodies mentioned in this protocol. Optimization might be required for other antibodies.

25. Remove the antibody and wash metatarsals four times in blocking buffer each for 15 minutes

26. Prepare secondary antibody in blocking buffer and incubate the antibody mixture with metatarsals for 2 hours with gentle rocking at room temperature.

27. Remove the antibody and wash metatarsals four times in blocking buffer each for 15 minutes

28. Prepare Hoechst 34580 staining solution in DPBS (Ca^{2+} , Mg^{2+}): 10µg ml⁻¹ Hoechst 34580 and incubate metatarsal with Hoechst for 10 minutes at room temperature

29. Remove Hoechst and wash metatarsals once with DPBS (Ca^{2+} , Mg^{2+})

■ **PAUSE POINT** The samples can be stored in DPBS at 4°C for 2-3 days before imaging.

? **TROUBLESHOOTING**

Imaging • TIMING variable

30. After immunofluorescence staining, different microscopes can be used to visualize metatarsals grown on different types of culture plates. For metatarsals grown on a 24 well plate, follow option A. For metatarsals grown on a 35 mm plate follow option A or B. For metatarsals grown on coverslips, follow option C

(A) Imaging direct in the culture plate.

(i) Image metatarsals grown on 24-well and 35 mm plates directly in the culture plate using Olympus SZX16 stereomicroscope or Nikon eclipse Ti inverted microscope.

▲ **CRITICAL STEP** These two microscopes are ideal to take low magnification images for analysing parameters such as total vascular area, vessel length, branch points, pericyte coverage and proliferating cells *etc.*

(B) Imaging using a confocal laser scanning microscope.

(i) If you wish to visualize fine characteristics and the interaction between different types of vascular cells, image metatarsals grown on a 35mm plate with upright Leica TCS SP8 confocal laser scanning microscope .

(C) Imaging metatarsals grown on coverslips in 24-well plates

- i. Add a few drops of Mowiol® 4-88 mounting medium to the slide
- ii. Remove the coverslip from the well and invert it to mount on the slide
- iii. After the mounting medium has solidified, coverslips can be imaged.
■ **PAUSE POINT** Mounted slides should ideally be imaged as soon as possible, however can be stored at 4°C for up to 5 days before imaging.

? **TROUBLESHOOTING**

- iv. Visualize the detailed features of vascular cells including the number of tip cells and filopodia, and the spatial relationship between different vascular cells with Olympus IX81 motorized inverted microscope.

Quantification • **TIMING** variable

31. Quantify immune-stained metatarsals automatically or manually using the following options. Total microvessel number, length, area, calibre and branch points can be analysed using a software package such as Imaris filament tracing algorithm (option A). 3-D construction of neighbouring cells can be performed using Imaris volume rendering algorithm (option B). Fast and batch analysis of total vascular area can be achieved by using TRI2 software (option C). TRI2 software was initially set up to carry out automated microvessel area quantification for *ex vivo* aortic ring assay¹. As the pattern of vessel outgrowths from metatarsal is similar as that from aortic rings, this software is also suitable for batch analysis of metatarsal microvasculature. Supporting cell coverage, number of tip cells and filopodia, and vascular cell proliferation can be calculated by Image J (option D).

(A) Automated microvessel quantification by Imaris using filament tracing algorithm

• **TIMING** 2 minutes per image for 2-D images.

- i. Import image into Imaris.
- ii. Manually generate a contour surface to cover the metatarsal bone using Imaris surface rendering algorithm.
- iii. Mask the drawn area by selecting "set the voxels inside= 0".
- iv. Continue with the filament rendering algorithm by clicking on the button filament to create a new object, filament, in the object list. In the object properties area, select 'Autopath (no loops)' as filament algorithm setting. Check the option 'Calculate Diameter of Filament from Image'.
- v. Click next button and select the source channel for blood vessels. To define the size of vessels to be included in the analysis, the diameter of thinnest and thickest vessels can be measure under 'Slice View'.
- vi. Set the starting point threshold to minimum and place the starting point at a few locations around the edge of the masked metatarsal bones.
- vii. Adjust the seed point threshold to include all vessels but exclude any non-specific signals.
- viii. Adjust the spine threshold to include all the vessels.

- ix. Click the execute button. Value of the vessels length can be extracted from the statistical analysis (Figure 6).

! CAUTION Proper training and knowledge of scoring criteria reduces intra- and inter-observer variability to negligible levels.

(B) 3-D construction of neighbouring cells by Imaris using volume rendering algorithm.

- i. Import the Z-stack image set captured from confocal microscopy into Imaris.
- ii. Select volume rendering algorithm.
- iii. Adjust the contrast and brightness of individual channel to reduce noise.
- iv. Click the execute button to retrieve the rendered image (Figure 4i).

(C) Automated microvessel quantification by TRI2 programme • **TIMING** 1 minute for 10 images.

- i. Invert original grey scale image so that the metatarsal bones and vessel outgrowths are black.
- ii. Use File-open image batch to import multiple metatarsal images to TRI2 software.
- iii. Use macro-multiple image aortic ring assay processing option.
- iv. Choose the directory into which the analysis data and the binary images of the segmented vessels will be saved (Figure 7).
- v. Import the inverted image (step i) into Photoshop.
- vi. Use the 'magnetic lasso tool' to closely follow the boundary of metatarsal bone and select the entire bone area. Once the bone area is highlighted, the number of pixels in the selected area could be obtained using the 'histogram function'. Click the refresh icon (circular arrows in the upper right hand corner of the histogram window) and record the number of pixels in the bone area.
- vii. Normalise the total vascular area in pixels derived from step iv against the pixels of the metatarsal bone (step vi), expressing as arbitrary units.

! CAUTION As the metatarsal bones are not exactly of the same size, even for those isolated from the same foetus, the total vascularization area should always be normalised to the bone size.

(D) Manual quantification of vascular characteristics by Image J.

- i. Examine the extent of perivascular cell coverage using by Olympus IX81 Motorized Inverted Microscope at high power. Count the number of endothelial cells (PECAM-1 positive) and perivascular mural cells (NG2 or α -SMA positive) from 5 random vascular fields around metatarsals using cell counter function of Image J. Determine the Perivascular cell coverage index (PCI); this is the percentage of endothelial cells within the field.
- ii. Determine the percentage of proliferating or apoptotic cells by counting the number of proliferating (Ki67 positive) cells in 5 random vascular areas using Image J. Scored the percentage of endothelial cells or perivascular cells within the field.

Table 1 | Reagents used to stain different types of vascular cells, basement membrane, mitotic cells and apoptotic cells.

Cell type	Antibody/molecule	Dilution
ECs	Rat anti-mouse PECAM (CD31) (BD Pharmingen, cat. no. 553370)	1:150
ECs	Isolectin B4 (IB4) (Vector lab, cat. no. FL-1201)	1:100
Smooth muscle cells	Mouse anti-SMA (Sigma, cat. no. A5228)	1:200
Macrophages	Rat anti-mouse F4/80 (AbD Serotec, cat. no. MCA497G)	1:100
Pericytes	Anti-NG2-PE (MACS Miltenyi Biotec cat. no. 130-100-468)	1:100
Mitotic cells	Anti Ki67 (Abcam, cat. no. ab15580)	1:100
Apoptotic cells	Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb (Cell signalling technology. cat no. 9664)	1:400
Endothelial cell lumen	Mouse Podocalyxin Affinity Purified Polyclonal Ab, Goat IgG (R&D, cat. no. AF1556)	1:100

? TROUBLE SHOOTING

Trouble shooting advice is provided in table 2

Table 2 | trouble shooting table

Step	Problem	Possible reason	Possible solution
9	Cannot remove the skin as whole	The skin is damaged at multiple locations during dissection	Cut the dorsal skin open along the metatarsals first before inserting tweezers into the plantar side of the skin
10	Metatarsals are of hugely different sizes	Metatarsals are not separated from tarsals at the natural joints	Gently pull the metatarsals and tarsals instead of cutting or snapping the metatarsals off
11	Metatarsals are injured	Holding metatarsals too tightly with tweezers	Grab connective tissues around the metatarsal instead of the bone itself
17	Metatarsals float off substrate after feeding	Too much flow was generated during pipetting	Reposition floating metatarsals in the middle of the well and remove culture medium. Let metatarsals dry/adhere for a few minutes with lid on before adding fresh culture medium slowly.
19	Metatarsals do not sprout at all or sprout asymmetrically	Bones are damaged during dissection or over-dried after seeding	Avoid over-trimming of the metatarsal and use tweezers instead of surgical blades to remove excessive connective tissues as blades may damage endothelial cells in the perichondrium. Let surgical tools dry completely before dissecting metatarsals.

			Keep the culture plate covered while drying/adhering metatarsals in the cell culture hood.
			Metatarsals that do not have 360 degree fibroblast growth or relatively asymmetrical vessel outgrowth should be excluded from the analysis (Figure 8).
29	Metatarsals are detached during the immunostaining procedure	Too much flow was generated during pipetting	Position the pipette tip against the wall of the culture plate and release liquid slowly until metatarsals are fully covered.
			Do not add too much liquid in the well.
30C	Air bubbles in mounted sections	Air bubbles are created when adding the coverslip	Put a P2 pipette tip between the coverslip and the slide. Retreat the pipette tip slowly to avoid trapping air bubbles

Box1 | RNA and Protein extraction protocols • TIMING 30 minutes

This procedure can be used to check the influence of treatment on gene and protein expression or to check endogenous protein/mRNA levels in metatarsal and in vessel outgrowth from metatarsals (step 19). Pool 5 metatarsals from same treatment for RNA or protein extraction in a 1.5ml tube. Both RNazol®RT reagent and RNeasy mini kit can be used for total RNA extraction from metatarsal bones and their outgrowing cells. The yields of RNA are comparable using both methods. It takes slightly longer for RNA isolation using RNazol®RT reagent. However, RNazol®RT reagent is cheaper compared to RNeasy mini kit. Here, we described the protocol of RNA extraction using RNeasy mini kit.

1. At the desired time point, or the end of the treatment, aspirate the culture medium
2. Wash explants with 1ml ice-cold PBS and place the culture plate on ice
3. Aspirate as much PBS as possible
4. To extract protein, follow option A for extraction from metastarsal bones alone, or option B for extraction from metatarsal vessel outgrowths plus the fibroblasts grown underneath. To extract RNA, follow option C for extraction from metatarsal bones alone, and option D for extraction from metatarsal vessel outgrowths plus the fibroblasts grown underneath.

(A) Protein extraction from metatarsal bones alone (without vessels)

- i. Pick metatarsals from culture plate using tweezers and transfer them into a 1.5ml tube containing 50 µl of 2 x Laemmli Sample buffer. Crush the metatarsals with a 1.5ml tube pestle.
- ii. Spin in a bench top microcentrifuge at $\geq 10,000g$ for 5 min to pellet debris.
- iii. Transfer the supernatant to a fresh tube for Western blotting.

■ **PAUSE POINT** lysates can be stored at -20°C for later use

(B) Protein extraction from metatarsal vessel outgrowths plus the fibroblasts grown underneath

- i. Remove metatarsal bones for protein and RNA analysis as described in option A and C.
- ii. Lyse the vessels attached to the bottom of the plate by pipetting 50 µl of 2 x Laemmli Sample buffer up and down 4-6 times.
- iii. Transfer cell lysate to a 1.5ml tube and spin in a bench top microcentrifuge at $\geq 10,000g$ for 5 min to pellet debris.
- iv. Transfer the supernatant to a fresh tube for Western blotting

■ **PAUSE POINT** lysates can be stored at -20°C for later use

(C) RNA extraction from metatarsal bones (without vessels)

- i. Remove metatarsals as described in option A.
- ii. Transfer metatarsal bones to a 1.5ml RNase free microfuge tube containing 350 µl of buffer RLT containing β-mercaptoethanol from the RNeasy mini kit and crush the metatarsals with a 1.5ml tube pestle.
- iii. Follow the RNA extraction protocol according to manufacturer's instruction

(D) RNA extraction from metatarsal vessel outgrowths plus the fibroblasts grown underneath

- i. Remove metatarsals as described in option A.
- iv. Lyse the remaining vessels using 350 µl of buffer RLT containing β-mercaptoethanol from the RNeasy kit. Alternatively, RNeasy@RT could be used to extract total RNA from metatarsal bones.
- ii. Collect the lysate with a rubber policeman and transfer lysate into a 1.5ml RNase free microfuge tube.
- iii. Follow the RNA extraction protocol provided by the manufacturers.

END BOX

● TIMING

The entire procedure takes between 9-13 days to complete. Approximate timings are noted below, which apply to those new to the technique. The method will inevitably require more time when investigators are inexperienced with respect to metatarsal isolation, cleaning or seeding. Therefore, we recommend in the first instance working with no more than two litters each day and working with one animal each time. The time required will decrease as experience is gained.

Step 1, plate coating, 10 minutes

Step 2-12, metatarsal dissection: 20 minutes per foetus

Step 13-18, seeding: 5 minutes per foetus including washing time

Step 19, feeding: 15 minutes for metatarsals from 8 pups (a litter)

Step 20-22, fixation: 30 minutes

Step 23-29: staining: 2 days

Step 30, imaging: variable

Step 31, quantification: variable

Box1, RNA and protein extraction: variable depending on the number of treatments, ~30 minutes

ANTICIPATED RESULTS

The development of a microvascular network from metatarsals is not affected by the types of coating matrix (Figure 3d). However, metatarsals isolated from different embryonic stages do show differences in sprouting potential and attachment rate (Figure 3a and 3b). For example, E15.5 metatarsals demonstrate the highest degree of variability in sprouting and lowest attachment rate. The type of culture medium has a great influence on the sprouting potential of metatarsal bones (Figure 3c). No vascular sprouting occurs from metatarsals fed with EBM2, EGM2 and DMEM culture medium, whereas metatarsals grown in MEM-α lead to the formation of a complex vascular network.

The extent and time course of vessel formation depends primarily on the type of treatment and the genetic background. Analysing microvessel growth too early or too late may not reveal treatment-specific differences. One advantage of the metatarsal assay is that vessel formation can be monitored by phase-contrast light microscopy allowing vessel formation progress to be followed without terminating the experiment (Figure 4j and Supplementary figure 3). It is also possible to follow blood vessel formation and the interaction between different types of vascular cells by time lapse imaging using transgenic lines carrying fluorescent reporter probes under the control of cell type-specific promoters. For wild type metatarsals grown in MEM α culture medium, a complex vascular network is fully established by day 7. An example of microvessel sprouting from E16.5 metatarsals seeded on a gelatin coated 24-well plate with the supplementation of a pro-angiogenic factor, VEGF or an anti-angiogenic ALK1 inhibitor, LDN 193189, is shown in Figure 9. VEGF promotes vessel outgrowth from metatarsal bones, whilst inhibiting the pro-angiogenic type I TGF β receptor, ALK1, causes a significant inhibition of microvascular growth from metatarsals.

Different types of vascular cells can be visualized by using the antibodies described in table 1 but this list is not exhaustive. As shown in Figure 4, PECAM-1 staining visualised by epifluorescence microscopy at low power is ideal for analysing the overall microvascular morphology. High power images taken by either epifluorescence or confocal microscopy can reveal fine cellular details such as tip cells and filopodia. Where endothelial cells and pericytes are labelled simultaneously, the number of each cell type within a fixed area can be counted manually using Image J for calculating the PCI. In addition, co-localisation of cell types can be analysed. TRI2 software is suitable for quick batch analysis of the vascularised area whereas Imaris software can be used to carry out more detailed analysis of vascular characteristics such as total vessel length, branch points, calibre (Figure 6) as well as for rendering 3D images constructed from captured z-stack high power confocal images (Figure 4i). Ki67 can be used to label proliferating cells (Figure 4e and supplementary figure 2) and cleaved caspase 3 used to label apoptotic cells (Figure 4f). Following co-labelling with endothelial cell, pericyte and fibroblast markers the proliferation index of specific cell types after treatment can be calculated by using Image J.

Metatarsal bones or the cells migrating out from metatarsals (including the microvasculature, macrophages and fibroblasts etc.) can be used for mRNA and protein expression analysis separately at the end of the experiment (Figure 10). We have shown that the gene and protein expression profiles in the metatarsal bone and its outgrowing cells are very different (Figure 10). PECAM-1 conjugated Dynabeads® Magnetic Beads or flow cytometric sorting may be used for further separation of microvascular cells. Analysis of the expression profile of cells in an angiogenic microenvironment presents an opportunity to gain insight into the molecular mechanisms of sprouting angiogenesis *ex vivo*.

Figure legends

Figure 1: Blood vessel formation from aortic rings and metatarsals. **a-c**, Representative images of vessel outgrowth from aortic rings isolated from C57/BL6 mice at P7. Blood vessels were labelled by isolectin B4 staining. **d-f**, Representative images of vessel outgrowth from metatarsals isolated from C57/BL6 embryos at E16.5. Blood vessels were visualized by CD31 staining. **a**, Without treatment, there is only a limited vessel formation from mouse aortic rings. **d**, Vessel outgrowth from metatarsals leads to the formation of a complex network of vasculature. **b**, With the presence of angiogenic factor, LRG1 (20 ugml⁻¹), vessel outgrowths from aortic ring only show a few branches. **e**, There is a significant increase in total vessel length and the number of branch points in metatarsals upon LRG1 treatment. **c**, After the treatment with anti-angiogenic ALK1 inhibitor (LDN 193189, 100 nM), aortic rings show no significant difference from untreated controls. **f**, There is a significant decrease in vessel outgrowth from ALK1 inhibitor treated metatarsals. Scale bar, 180µm. Metatarsals were treated with respective drugs for 5 days and harvested for imaging 7 days after seeding. Aortic rings were treated with respective drugs for 12 days and harvested for imaging 12 days after seeding. Institutional ethical regulatory board permission was obtained at Nanyang Technological University.

Figure 2 Overview of the procedure

Figure 3. Optimization of the mouse foetal metatarsal assay. **a**, Vessel outgrowth from metatarsals isolated from different embryonic stages. There is no significant difference in total vascularization area between metatarsals isolated from different embryonic stages. Vessel outgrowth from E15.5 metatarsals shows highest variability. Scale bar, 240µm. **b**, The attachment rate of metatarsals isolated from different embryonic stages. The attachment rate of E15.5 metatarsal is much lower compared to that of metatarsals isolated from other embryonic stages. **c**, Vessel outgrowth from metatarsals grown in different culture medium. There is no vessel growing out from metatarsals cultured in EBM2, EGM, or DMEM medium. Metatarsals cultured in MEMα medium form a complex vascular network. Scale bar, 180µm. **d and e**, vessel outgrowth from metatarsals seeded on plastic and ECM derivatives-coated surfaces. Metatarsals grown on non-coated Iwaki and Greiner plate show significantly less sprouts compared to metatarsals grown on other plastic surfaces or ECM derivative coated cell culture plates. Scale bar, 240µm. **f**, Metatarsals grown on non-coated plates exhibit significantly lower attachment rate compared to those grown on coated plates. Scale bar, 320µm. n=3 independent experiments, n=8 metatarsals per treatment, data are mean±s.e.m. **P<0.01(Student's t-test). Institutional ethical regulatory board permission was obtained at Nanyang Technological University.

Figure 4. Immunofluorescence staining of metatarsals. Images of vessel outgrowth from metatarsals grown on gelatin coated plate in MEMα culture medium. PECAM-1 (green/red) and isolectin B4-FITC (green) label endothelial cells; F4/80 (red) labels macrophages; NG2-PE (red) and α-SMA (green) stain perivascular mural cells; Ki67 (red) stains proliferating cells; cleaved caspase 3

(red) labels apoptotic cells. Podocalyxin (green) labels endothelial cell lumen. Hoechst (blue) stains nuclei. **a**, Co-staining of endothelial cells (PECAM-1) and pericytes (NG2) of metatarsal vasculature 7 days after seeding. Scale bar, 320µm. **b**, Co-staining of endothelial cells (IB4) and macrophages (F4/80) 7 days after seeding. Scale bar, 10µm. **c and d**, Co-staining of endothelial cells (PECAM-1) and mural cells (NG2 or α -SMA) 7 days after seeding. Scale bar, 10µm for c and scale bar, 20µm for d. **e**, Co-staining of endothelial cells (PECAM-1) and proliferating cells (Ki6) 5 days after seeding. Scale bar, 10µm. **f**, Co-staining of endothelial cells (PECAM-1) and apoptotic cells (Cleaved Caspase 3) 3 days after ALK1 inhibitor treatment. Scale bar, 5µm. **g**, Endothelial cell lumen formation as indicated by podocalyxin (white arrows) staining 10 days after seeding. Scale bar, 5µm. **h**, Confocal images of metatarsals demonstrating tip cells and filopodia in metatarsal vessels. Images in the inset are enlarged tip cells. Scale bar, 20µm. **i**, 3D-rendered confocal image shows the interaction between endothelial cells (green) and perivascular mural cells (red). Scale bar, 20µm. **j**, Bright field phase contrast image showing vessel out growth from metatarsals 7 days after seeding (black arrow). Scale bar, 260µm. Institutional ethical regulatory board permission was obtained at Nanyang Technological University and UCL Institute of Ophthalmology.

Figure 5: Optimization of metatarsal dissection procedure. **a**, Schematic figure showing metatarsal dissection procedure. **b**, Removal of the skin as a whole can facilitate the maximum removal of connective tissue from metatarsals. Scale bar, 1.2mm. **c**, The size and morphological difference of metatarsals isolated from different embryonic stages. Scale bar, 1mm. **d**, There are excessive amounts of connective tissue around metatarsals except in those from E16.5 embryos. Scale bar, 1mm. Institutional ethical regulatory board permission was obtained at Nanyang Technological University.

Figure 6. Example of image rendering for quantification of vascular growth from metatarsals. Metatarsal vessel outgrowth stained with anti-PECAM-1 antibody (red) is captured digitally. After thresholding vessel configuration was quantified (white) using Imaris image analysis software (Bitplane AG, Switzerland). Scale bar, 100µm. Institutional ethical regulatory board permission was obtained at Nanyang Technological University.

Figure 7. Example of image processing for total vessel area analysis by TRI2 software. **a**, Original grayscale metatarsal image. **b**, Inverted metatarsal image, **c**, Exported binary image by TRI2 software. Scale bar, 320µm. Institutional ethical regulatory board permission was obtained at Nanyang Technological University.

Figure 8. Example of exclusion criteria. E16.5 metatarsals grown on gelatin coated plate 2 days after being seeded (day 3). **a**, A metatarsal with 360° fibroblast coverage. **b**, A metatarsal that is only partially surrounded with fibroblasts (red arrow) should be excluded from analysis. Red arrow indicates the area without

fibroblast coverage. Scale bar, 250µm. Institutional ethical regulatory board permission was obtained at Nanyang Technological University.

Figure 9. Treatment of metatarsal with pro-angiogenic and anti-angiogenic factors. **a**, Representative pictures showing that ALK1 inhibitor, LDN 193189, inhibits vessel outgrowth from metatarsals. VEGF treatment leads to significant vessel formation from metatarsals. **b**, Quantitative analysis of vessel outgrowth upon different treatment. n=3 independent experiments, n=6 metatarsals per treatment, data are mean±s.e.m. (Two-way ANOVA). Scale bar, 250µm. Institutional ethical regulatory board permission was obtained at Nanyang Technological University.

Figure 10. Gene and protein expression profile in metatarsal bones or cells migrating from metatarsals. **a**, Western blot analysis for the presence of phosphorylated Smad1/5 in metatarsal bones or cells migrating from metatarsals after treatment with rLRG1 and TGFβ. There is a slight decrease in pSmad1/5 level in metatarsal bones but there is an increase in pSmad1/5 level in cells after rLRG1 and TGFβ treatment. **b**, qRT-PCR analysis of Ang1 and VEGF expression in metatarsals bones and cells after rLRG1 and TGFβ treatment. rLRG1 and TGFβ inhibit Ang1 expression in cells but not metatarsal bones. rLRG1 and TGFβ treatment inhibit VEGF expression in both cells and bones. n=5 independent experiments, data are mean±s.e.m. * P<0.05, ***P<0.001 (Student's t-test). Institutional ethical regulatory board permission was obtained at Nanyang Technological University.

Supplementary information

Supplementary figure 1. Comparison of vessel outgrowth between metatarsals and metacarpals. Vessel outgrowths are labeled by PECAM-1 7 days after seeding. The total vascular area is normalized by bone size. Metacarpals lead to more vessel outgrowths compared to metatarsals. n=3 independent experiments, n=6 metatarsals per treatment, data are mean±s.e.m. **P<0.01 (Student's t-test). Scale bar, 320µm. Institutional ethical regulatory board permission was obtained at Nanyang Technological University.

Supplementary figure 2. Comparison of the number of proliferating cells in the metatarsal microvasculature over time. Proliferating cells are labelled by Ki67 antibody and blood vessels visualized by PECAM1 staining. The number of proliferating cells/50µm of blood vessels reaches the highest level 5 days after seeding and decrease dramatically from 8 days after seeding. n=3 independent experiments, n=6 metatarsals per treatment, data are mean±s.e.m. *P<0.05 (Student's t-test). Scale bar, 10µm. Institutional ethical regulatory board permission was obtained at Nanyang Technological University.

Supplementary figure 3. Visualization of vessel outgrowth and fibroblast migration from the metatarsals by phase contrast microscopy. Fibroblasts migrate out from metatarsals on day 2 and blood vessels are visible by phase contrast microscopy from day 4 onwards (red arrow). Scale bar, 250µm. Institutional ethical regulatory board permission was obtained at Nanyang Technological University.

Supplementary video 1. Dissection of E16.5 metatarsal bones.

Supplementary video 2. Seeding individual metatarsal bone in a 24-well plate.

Author contributions statements

W.H.S and X.M.W. optimized the protocol and designed the experiment. W.H.S., C.W.F., K.H.A., C.H.L, N.A.B.J, C.S.L, S.A. and X.M.W. performed the experiment. All authors contributed to the figure and manuscript preparation.

Acknowledgments

We thank D.L.B and L.E.M for their expertise in confocal microscopy. We thank S.C for his help with Imaris software. This work was sponsored by Singapore Ministry of Education Academic Research Fund Tier 1 (2013-T1-002-051) and Singapore Ministry of Education Academic Research Fund Tier 2 (MOE2014-T2-1-036) to X.M.W, National Medical Research Council - Cooperative Basic Research Grant (CBRG13nov094) to X.M.W and W.J.H, and Medical Research Council UK (Grant no. 002973) awarded to J.G, S.E.M and X.M.W.

Competing financial interests

The authors declare no competing financial interest.

References

- 1 Deckers, M. *et al.* Effect of angiogenic and antiangiogenic compounds on the outgrowth of capillary structures from fetal mouse bone explants. *Laboratory investigation; a journal of technical methods and pathology* **81**, 5-15 (2001).
- 2 Baker, M. *et al.* Use of the mouse aortic ring assay to study angiogenesis. *Nature protocols* **7**, 89-104, doi:DOI 10.1038/nprot.2011.435 (2012).

- 3 Wang, X. *et al.* LRG1 promotes angiogenesis by modulating endothelial TGF-beta signalling. *Nature* **499**, 306-311, doi:10.1038/nature12345 (2013).
- 4 Snoeks, T. J., Mol, I. M., Que, I., Kaijzel, E. L. & Lowik, C. W. 2-methoxyestradiol analogue ENMD-1198 reduces breast cancer-induced osteolysis and tumor burden both in vitro and in vivo. *Molecular cancer therapeutics* **10**, 874-882, doi:10.1158/1535-7163.MCT-10-0997 (2011).
- 5 van der Pluijm, G. *et al.* In vitro and in vivo endochondral bone formation models allow identification of anti-angiogenic compounds. *The American journal of pathology* **163**, 157-163, doi:10.1016/S0002-9440(10)63639-5 (2003).
- 6 Liu, Z. *et al.* VEGF and inhibitors of TGFbeta type-I receptor kinase synergistically promote blood-vessel formation by inducing alpha5-integrin expression. *Journal of cell science* **122**, 3294-3302, doi:10.1242/jcs.048942 (2009).
- 7 van Wijngaarden, J. *et al.* An in vitro model that can distinguish between effects on angiogenesis and on established vasculature: Actions of TNP-470, marimastat and the tubulin-binding agent Ang-510. *Biochem Bioph Res Co* **391**, 1161-1165, doi:DOI 10.1016/j.bbrc.2009.11.097 (2010).
- 8 Leenders, W. *et al.* Design of a variant of vascular endothelial growth factor-A (VEGF-A) antagonizing KDR/Flk-1 and Flt-1. *Laboratory investigation; a journal of technical methods and pathology* **82**, 473-481 (2002).
- 9 Liu, Z. *et al.* ENDOGLIN Is Dispensable for Vasculogenesis, but Required for Vascular Endothelial Growth Factor-Induced Angiogenesis. *Plos One* **9**, doi:ARTN e86273
DOI 10.1371/journal.pone.0086273 (2014).
- 10 Cackowski, F. C. *et al.* Osteoclasts are important for bone angiogenesis. *Blood* **115**, 140-149, doi:10.1182/blood-2009-08-237628 (2010).
- 11 Wan, L., Zhang, F., He, Q., Tsang, W. P. & Lu, L. EPO Promotes Bone Repair through Enhanced Cartilaginous Callus Formation and Angiogenesis (vol 9, e102010, 2014). *Plos One* **9**, doi:ARTN e111830
DOI 10.1371/journal.pone.0111830 (2014).
- 12 Chim, S. M. *et al.* EGFL7 Is Expressed in Bone Microenvironment and Promotes Angiogenesis via ERK, STAT3, and Integrin Signaling Cascades. *J Cell Physiol* **230**, 82-94, doi:Doi 10.1002/Jcp.24684 (2015).
- 13 Zhu, K. *et al.* ATF4 promotes bone angiogenesis by increasing vegf expression and release in the bone environment. *J Bone Miner Res* **28**, 1870-1884, doi:Doi 10.1002/Jbmr.1958 (2013).
- 14 Sun, H. L., Jung, Y. H., Shiozawa, Y., Taichman, R. S. & Krebsbach, P. H. Erythropoietin Modulates the Structure of Bone Morphogenetic Protein 2-Engineered Cranial Bone. *Tissue Eng Pt A* **18**, 2095-2105, doi:DOI 10.1089/ten.tea.2011.0742 (2012).
- 15 Shao, Z. *et al.* Choroid sprouting assay: an ex vivo model of microvascular angiogenesis. *Plos One* **8**, e69552, doi:10.1371/journal.pone.0069552 (2013).
- 16 Cooke, V. G. *et al.* Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met

- signaling pathway. *Cancer cell* **21**, 66-81, doi:10.1016/j.ccr.2011.11.024 (2012).
- 17 Goren, I. *et al.* A transgenic mouse model of inducible macrophage depletion: effects of diphtheria toxin-driven lysozyme M-specific cell lineage ablation on wound inflammatory, angiogenic, and contractive processes. *The American journal of pathology* **175**, 132-147, doi:10.2353/ajpath.2009.081002 (2009).
- 18 Lindblom, P. *et al.* Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. *Gene Dev* **17**, 1835-1840, doi:Doi 10.1101/Gad.266803 (2003).
- 19 Abramsson, A., Lindblom, P. & Betsholtz, C. Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. *J Clin Invest* **112**, 1142-1151, doi:Doi 10.1172/Jci200318549 (2003).
- 20 Yen, M. L. *et al.* Diosgenin induces hypoxia-inducible factor-1 activation and angiogenesis through estrogen receptor-related phosphatidylinositol 3-kinase/Akt and p38 mitogen-activated protein kinase pathways in osteoblasts. *Molecular pharmacology* **68**, 1061-1073, doi:10.1124/mol.104.010082 (2005).