Development of an in vitro vascular network using zebrafish embryonic cells

Ibrahim, M.

Development of an *in vitro* vascular network using zebrafish embryonic cells

Muhammad Ibrahim

Cover: development of vascular network-like structure from *kdrl:GFP*$^{+}$ endothelial cells in zebrafish embryoid body culture.

ISBN: 978-94-6182-805-7

Printed by Off Page, Amsterdam, www.offpage.nl

Development of an *in vitro* vascular network using zebrafish embryonic cells

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 13 juni 2017
klokke 10.00 uur

door

Muhammad Ibrahim
geboren te Mardan, Pakistan
17 February 1982

Promotiecommissie

Promotor

Prof. dr. Michael K. Richardson

Co-promotor

Dr. Anna Pavlina Haramis

Overige leden

Prof. dr. Gilles van Wezel
Prof. dr. Annemarie Meijer
Prof. dr. Robert E. Poelmann
Dr. Beerend P. Hierck (Leiden University Medical Center)
Dr. Neil Vargesson (University of Aberdeen)

The work described in this thesis was supported by the *faculty development* programme of Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar, Pakistan [scholarship number 06/SIBGE]; the *Smart Mix* programme of the Netherlands Ministry of Economic Affairs and the Netherlands Scientific Research Council (NWO) [grant number SSM06010]; and the *Generade* programme of the Centre of Expertise Genomics in Leiden, The Netherlands [grant number 2016_004].

For

Prof. Zahoor Ahmad Swati

Table of Contents

Chapter 1

General introduction
Culturing zebrafish vascular networks

Muhammad Ibrahim and Michael K. Richardson

Tissue engineering is one of the most important areas of biomedical research [1]. Strategies to develop complex tissues or organs *in vitro* will help our understanding of organ physiology and pathology [2-4]. Development of organ cultures could also have applications in organ transplantation and regenerative medicine [5]. Furthermore, these organ cultures can be used to test candidate drugs which might ultimately reduce the use of animals in research [6, 7].

One of the main issues in culturing complex organs is the lack of a vascular system [8]. In multicellular organisms, the vascular system allows the growth and function of organs by supplying nutrients and growth factors and by removing waste products [9]. The limited diffusion of nutrients and oxygen into the un-vascularized tissue mass, developed *in vitro,* hinders its growth and function into something resembling an organ [10]. To this end, several techniques have been developed to culture vascular networks, largely using mammalian cells and tissues.

The mammalian cells and tissues commonly used for this purpose are: (i) endothelial cell lines (most commonly human umbilical vein endothelial cells or HUVECs [11-22]); (ii) stem cells (embryonic stem cells [23], mesenchymal stem cells [24] or induced pluripotent stem cells [25]); and (iii) tissue explants [26]. However, there are certain limitations to these techniques. The endothelial cell lines are extensively adapted to growth and proliferation *in vitro*, and therefore vascular cultures derived from these cells does not truly represent the *in vivo* vasculature [26]. The use of embryonic stem cells from mammals, especially from humans, raises ethical issues [27]. Furthermore, adult stem cells (mesenchymal and induced pluripotent stem cells) possess technical challenges in their isolation and derivation procedures [28, 29]. Similarly, the isolation of tissue explants from mammals (in most cases, rodents) requires invasive surgical procedures and therefore raises ethical concerns [27, 30].

For these reasons, it is important to develop alternative models for studying vascular morphogenesis *in vitro*. The zebrafish is one such emerging model species in the field of vascular development and regeneration [31]. In contrast to rodents, zebrafish embryos are externally fertilized allowing easy access to large number of embryos [32]. The embryos are fast-developing and transparent in early life stages, allowing easy access to cells and tissues (for *in vitro* manipulation) at different developmental stages [33]. Furthermore, genome comparison studies have shown significant similarities in the functional domains of many protein-coding genes of zebrafish and humans, and have shown that many human disease genes are also present in the zebrafish genome [34]. For these and other reasons, there is growing interest in the zebrafish as a model for human disease [32].

Several factors have been identified which influence the formation of blood vessels. These factors include: (i) growth factors (such as vascular endothelial growth factors, fibroblast

growth factors, angiopoietins and transforming growth factors [35]); (ii) extracellular matrix components (such as collagen type I and IV, fibronectin and laminin [36]); (iii) supporting cell types (such as pericytes and smooth muscle cells [37, 38]); and (iv) haemodynamic forces caused by blood flow [39]. In order to develop a physiologically relevant and functional vascular network *in vitro*, endothelial or stem cells, or tissue explants are cultured in the presence of naturally derived vascular growth factors, supporting cell types and extracellular matrix [12, 40, 41].

Recently-developed microfluidic technology mimics the haemodynamic forces exerted by the blood flow *in vivo*, by culturing the cells in a closed system with circulating medium [42]. Using a combination of these factors, great advances have been made in recent years in developing a functional *in vitro* vascular network. In a landmark study, the vascular network developed inside a 3D scaffold connected to a microfluidic system allowed the growth and function of cardiac and hepatic tissues cultured on the outside of the scaffold [22].

Little is known about using zebrafish cells for culturing vascular network. The benefits associated with zebrafish cell culture makes it a model of choice for *in vitro* studies. The availability of large number of externally fertilized embryos allow easy access to primary embryonic cells and tissues [33]. Zebrafish cell cultures are maintained at relatively low temperatures (26-28 °C) and do not require extra CO_2 in the atmosphere for buffering the medium. In principle, this allows the zebrafish cells to be grown at room temperature, although the use of a simple incubator is recommended to maintain sterile conditions [43]. However, this can also be considered a disadvantage as these conditions are not ideal for human cells.

A further advantage is the availability of transgenic lines such as *fli:GFP* [44] and *kdrl:GFP* [45], expressing green fluorescence protein in endothelial cells. These transgenic lines allow direct observation of vascular development in living embryos and in cell cultures [46, 47]. In contrast to endothelial cell lines such as HUVECs, zebrafish primary embryonic cells are closer to the *in vivo* state. Furthermore, the development of vascular networks in zebrafish embryonic cell culture takes place in a complex environment of other cell types, which is difficult to achieve working with isolated cell lines. On the other hand, culturing a mixture of cell types allows less control over the cell culture environment compared to pure endothelial cell lines. In this thesis, I have studied the use of zebrafish embryonic cells and tissues as a complementary model to the mammalian cells and tissues used for vascular development *in vitro*.

In **Chapter 2** I have reviewed the current advances in the field of developing *in vitro* vasculature. The review includes a brief overview of vascular development and requirements of the process *in vivo*. This is followed by an extensive survey of the developed techniques using endothelial cell lines, stem cells and tissue explants for the

formation of vascular networks *in vitro*. Then I have argued the importance of zebrafish as a complementary model for such studies. Finally I have discussed the advances in the microfluidic technology making breakthroughs in developing functional vascular cultures.

In order to establish the basal requirements of zebrafish cell culture, in **Chapter 3** I have cultured primary blastocyst cells in media supplemented with different concentrations of fetal bovine serum and zebrafish embryo extract. The concentrations of these nutrients in the media showing optimal growth of the blastocyst cells were used in further experiments. Furthermore, the growth of putative endothelial cells (*fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells) was analysed in the blastocyst cell culture under basal conditions (without the additional growth factors).

In **Chapter 4** I have used different media compositions, growth factors and extracellular matrix components to analyse their effect on the generation of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cell culture. Different media compositions tested were LDF medium (commonly used medium for zebrafish cell culture) and endothelial growth medium (commonly used medium for mammalian endothelial cells). The effect of different substrates i.e. gelatin and collagen type-I was compared to the uncoated polystyrene substratum. Finally, the effect of different concentrations of recombinant zebrafish vascular endothelial growth factor in the media on the percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures was analysed.

In **Chapter 5**, I have analysed the effect of culturing blastocyst cells in suspension culture (to form embryoid body aggregates) compared to the adherent cultures on the generation of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells. The migration of *fli:GFP*$^{+}$ cells from the EB culture on collagen type-I, gelatin and fibrin substrates was analysed. The *kdrl:GFP* embryoid bodies showed the formation of vascular network-like structures. The dimensions of these networks varied on different substrates (collagen type-I and Geltrex™).

Finally, in **Chapter 6** I have developed a zebrafish EB model for sprouting vascular networks in 3D gel matrix. The effect of microfluidic flow on the growth of vascular sprouts in the 3D embryoid body cultures was examined. The results show an effect of microfluidic flow on the length and width of vascular sprouts. In addition, I have developed a technique for the sterile isolation and culture of liver and heart tissues from 5 days post fertilization zebrafish larvae. The isolated tissue explants developed vascular sprouts when cultured in a 3D gel matrix.

References

[1] Fisher MB, Mauck RL. Tissue engineering and regenerative medicine: recent innovations and the transition to translation. Tissue Eng Part B Rev 2013; 19(1): 1-13.

[2] Bhatia SN, Ingber DE. Microfluidic organs-on-chips. Nat Biotechnol 2014; 32(8): 760-72.
[3] Haraguchi Y, Shimizu T, Yamato M, Okano T. Concise review: cell therapy and tissue engineering for cardiovascular disease. Stem Cells Transl Med 2012; 1(2): 136-41.
[4] Ingram M, Techy GB, Ward BR, Imam SA, Atkinson R, Ho H*, et al.* Tissue engineered tumor models. Biotech Histochem 2010; 85(4): 213-29.
[5] Marx V. Tissue engineering: organs from the lab. Nature 2015; 522(7556): 373-7.
[6] Hansen A, Eder A, Bonstrup M, Flato M, Mewe M, Schaaf S*, et al.* Development of a drug screening platform based on engineered heart tissue. Circ Res 2010; 107(1): 35-44.
[7] Groeber F, Engelhardt L, Lange J, Kurdyn S, Schmid FF, Rucker C*, et al.* A first vascularized skin equivalent for as an alternative to animal experimentation. ALTEX 2016; 33(4): 415-422.
[8] Kannan RY, Salacinski HJ, Sales K, Butler P, Seifalian AM. The roles of tissue engineering and vascularisation in the development of micro-vascular networks: a review. Biomaterials 2005; 26(14): 1857-75.
[9] Levick JR. The microcirculation and solute exchange. In: Eds. An Introduction to cardiovascular physiology. 5 ed, Taylor & Francis 2010, pp. 166-187.
[10] Lovett M, Lee K, Edwards A, Kaplan DL. Vascularization strategies for tissue engineering. Tissue Eng Part B Rev 2009; 15(3): 353-70.
[11] Chen Y, Zhang Y, Deng Q, Shan N, Peng W, Luo X*, et al.* Inhibition of Wnt Inhibitory Factor 1 Under Hypoxic Condition in Human Umbilical Vein Endothelial Cells Promoted Angiogenesis *in Vitro*. Reprod Sci 2016; 23(10): 1348-58.
[12] Rohringer S, Hofbauer P, Schneider KH, Husa AM, Feichtinger G, Peterbauer-Scherb A*, et al.* Mechanisms of vasculogenesis in 3D fibrin matrices mediated by the interaction of adipose-derived stem cells and endothelial cells. Angiogenesis 2014; 17(4): 921-33.
[13] Kim S, Lee H, Chung M, Jeon NL. Engineering of functional, perfusable 3D microvascular networks on a chip. Lab Chip 2013; 13(8): 1489-500.
[14] Nguyen DH, Stapleton SC, Yang MT, Cha SS, Choi CK, Galie PA*, et al.* Biomimetic model to reconstitute angiogenic sprouting morphogenesis *in vitro*. Proc Natl Acad Sci U S A 2013; 110(17): 6712-7.
[15] Li H, Chang J. Bioactive silicate materials stimulate angiogenesis in fibroblast and endothelial cell co-culture system through paracrine effect. Acta Biomaterialia 2013; 9(6): 6981-6991.
[16] Zheng Y, Chen J, Craven M, Choi NW, Totorica S, Diaz-Santana A*, et al. In vitro* microvessels for the study of angiogenesis and thrombosis. Proc Natl Acad Sci U S A 2012; 109(24): 9342-7.
[17] Nakatsu MN, Sainson RC, Aoto JN, Taylor KL, Aitkenhead M, Perez-del-Pulgar S*, et al.* Angiogenic sprouting and capillary lumen formation modeled by human umbilical vein endothelial cells (HUVEC) in fibrin gels: the role of fibroblasts and Angiopoietin-1. Microvasc Res 2003; 66(2): 102-12.
[18] Stamati K, Priestley JV, Mudera V, Cheema U. Laminin promotes vascular network formation in 3D *in vitro* collagen scaffolds by regulating VEGF uptake. Exp Cell Res 2014; 327(1): 68-77.
[19] Boyd NL, Nunes SS, Krishnan L, Jokinen JD, Ramakrishnan VM, Bugg AR*, et al.* Dissecting the role of human embryonic stem cell-derived mesenchymal cells in human umbilical vein endothelial cell network stabilization in three-dimensional environments. Tissue Eng Part A 2013; 19(1-2): 211-23.

[20] Arnaoutova I, Kleinman HK. *In vitro* angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc 2010; 5(4): 628-35.
[21] Rao RR, Peterson AW, Ceccarelli J, Putnam AJ, Stegemann JP. Matrix composition regulates three-dimensional network formation by endothelial cells and mesenchymal stem cells in collagen/fibrin materials. Angiogenesis 2012; 15(2): 253-64.
[22] Zhang B, Montgomery M, Chamberlain MD, Ogawa S, Korolj A, Pahnke A*, et al.* Biodegradable scaffold with built-in vasculature for organ-on-a-chip engineering and direct surgical anastomosis. Nat Mater 2016; 15(6): 669-78.
[23] Hammoud L, Adams JR, Loch AJ, Marcellus RC, Uehling DE, Aman A*, et al.* Identification of RSK and TTK as modulators of blood vessel morphogenesis using an embryonic stem cell-based vascular differentiation assay. Stem Cell Reports 2016; 7(4): 787-801.
[24] Tancharoen W, Aungsuchawan S, Pothacharoen P, Markmee R, Narakornsak S, Kieodee J*, et al.* Differentiation of mesenchymal stem cells from human amniotic fluid to vascular endothelial cells. Acta Histochem 2016.
[25] Kusuma S, Gerecht S. Recent progress in the use of induced pluripotent stem cells in vascular regeneration. Expert Rev Cardiovasc Ther 2013; 11(6): 661-3.
[26] Staton CA, Reed MW, Brown NJ. A critical analysis of current *in vitro* and *in vivo* angiogenesis assays. Int J Exp Pathol 2009; 90(3): 195-221.
[27] Zou T, Fan J, Fartash A, Liu H, Fan Y. Cell-based strategies for vascular regeneration. J Biomed Mater Res A 2016; 104(5): 1297-314.
[28] Lin L, Bolund L, Luo Y. Towards Personalized Regenerative Cell Therapy: Mesenchymal Stem Cells Derived from Human Induced Pluripotent Stem Cells. Curr Stem Cell Res Ther 2016; 11(2): 122-30.
[29] Yamanaka S. Induced pluripotent stem cells: past, present, and future. Cell Stem Cell 2012; 10(6): 678-84.
[30] Rezzola S, Belleri M, Gariano G, Ribatti D, Costagliola C, Semeraro F*, et al. In vitro* and *ex vivo* retina angiogenesis assays. Angiogenesis 2014; 17(3): 429-42.
[31] Chavez MN, Aedo G, Fierro FA, Allende ML, Egana JT. Zebrafish as an emerging model organism to study angiogenesis in development and regeneration. Front Physiol 2016; 7: 56.
[32] Wilkinson RN, van Eeden FJ. The zebrafish as a model of vascular development and disease. Prog Mol Biol Transl Sci 2014; 124: 93-122.
[33] Grunow B, Mohamet L, Shiels HA. Generating an *in vitro* 3D cell culture model from zebrafish larvae for heart research. J Exp Biol 2015; 218(Pt 8): 1116-21.
[34] Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M*, et al.* The zebrafish reference genome sequence and its relationship to the human genome. Nature 2013; 496(7446): 498-503.
[35] Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature 2000; 407(6801): 242-8.
[36] Eble JA, Niland S. The extracellular matrix of blood vessels. Curr Pharm Des 2009; 15(12): 1385-400.
[37] Birbrair A, Zhang T, Wang ZM, Messi ML, Mintz A, Delbono O. Pericytes at the intersection between tissue regeneration and pathology. Clin Sci (Lond) 2015; 128(2): 81-93.
[38] Campbell GR, Campbell JH. Development of the vessel wall: overview. In: Mecham RP, Eds. The Vascular Smooth Muscle Cell. ed, San Diego, Academic Press 1995, pp. 1-15.

[39] Davies PF. Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. Nat Clin Pract Cardiovasc Med 2009; 6(1): 16-26.
[40] Shin Y, Jeon JS, Han S, Jung GS, Shin S, Lee SH*, et al. In vitro* 3D collective sprouting angiogenesis under orchestrated ANG-1 and VEGF gradients. Lab Chip 2011; 11(13): 2175-81.
[41] Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol 2005; 23(1): 47-55.
[42] Hasan A, Paul A, Vrana NE, Zhao X, Memic A, Hwang YS*, et al.* Microfluidic techniques for development of 3D vascularized tissue. Biomaterials 2014; 35(26): 7308-25.
[43] Choorapoikayil S, Overvoorde J, den Hertog J. Deriving cell lines from zebrafish embryos and tumors. Zebrafish 2013; 10(3): 316-25.
[44] Lawson ND, Weinstein BM. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 2002; 248(2): 307-318.
[45] Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Development 2005; 132(23): 5199-209.
[46] Tal T, Kilty C, Smith A, LaLone C, Kennedy B, Tennant A*, et al.* Screening for angiogenic inhibitors in zebrafish to evaluate a predictive model for developmental vascular toxicity. Reprod Toxicol 2016: http://dx.doi.org/10.1016/j.reprotox.-2016.12.004.
[47] Huang HG, Lindgren A, Wu XR, Liu NA, Lin SO. High-throughput screening for bioactive molecules using primary cell culture of transgenic zebrafish embryos. Cell Rep 2012; 2(3): 695-704.

Chapter 2

Beyond organoids: *in vitro* vasculogenesis and angiogenesis using cells from mammals and zebrafish

Muhammad Ibrahim and Michael K. Richardson

This chapter has been accepted for publication as following:

Ibrahim M, Richardson MK. Beyond organoids: *in vitro* vasculogenesis and angiogenesis using cells from mammals and zebrafish. Reproductive Toxicology (in press).

Abstract

The ability to culture complex organs is currently an important goal in biomedical research. It is possible to grow organoids *in vitro* and these small tissue masses can undergo a degree of histogenesis. However, a major limitation of organoids, and other 3D culture systems, is the lack of an *in vitro* vascular network. As a result, the growth and function of the organoid is constrained by the limited diffusion of nutrients, oxygen and growth factors. Protocols have been developed for establishing vascular networks *in vitro*, and they typically use human or rodent cells. The zebrafish is emerging as a complementary model to mammals in this regard. Its general advantages in developmental research and embryonic cell culture, and its specific advantages for studying vascular development (such as transgenic lines expressing green fluorescent in vascular cells) makes it of potential importance for developing *in vitro* vascular networks. The culture of functional (perfused) vascular networks is currently a major technical challenge. In this rapidly advancing field, some microfluidic devices are now getting close to the goal of an artificially perfused network in culture. One major hurdle is the need to sustain a microfluidic connection with a living vascular network that is growing rapidly in size, and therefore has an increasing demand for fluidic flow over time. In this review, we discuss the culture of endothelial cells and vascular networks from mammalian cells, and examine the prospects for using zebrafish cells for this objective. We also look into the future and consider how vascular networks *in vitro* might be successfully perfused using microfluidic technology.

Key words: Angiogenesis; *In vitro* vascular network; Microfluidics; Organ engineering; Vasculogenesis; Zebrafish

Introduction

In multicellular animals, nutrients and oxygen are carried by the cardiovascular system, and diffuse directly into the tissues [1]. Similarly, waste products are removed from the tissues by the same system. This allows the tissues to grow and develop into functional organs [1]. The cells in a living tissue are within 100-200 μm range of a blood capillary [2]. This is important for the survival of the cells as the oxygen and nutrients cannot diffuse through the tissue beyond this range [3]. A blood supply (vasculature connected to a pump) has therefore evolved to overcome the constraint on growth imposed by limited diffusion.

One area where blood vessel development is relevant is the tissue engineering for regenerative medicine and organ transplantation [4]. Currently, the lack of vascularization of tissues *in vitro* is a major hurdle in reaching this objective [5, 6]. This is unfortunate because cultured, vascularized tissues could not only have clinical applications [4], but could also be used as an alternative to whole animal models in research [7]. There are currently great efforts directed towards growing cells and tissues from a patient's own body (autologous transplantation), in order to overcome the potential danger of allogenic (from another individual) graft rejection, and graft-versus-host reactions [8, 9].

With current tissue culture techniques, tissues cannot be grown more than 100-200 μm in thickness, primarily because of the limited diffusion of nutrients and oxygen [10]. Tumor cells grown in non-adherent culture can develop into spherical masses (spheroids) up to 3mm in diameter, typically with a core of cells that are dead or dying due to diffusion limitation [11]. Similarly, masses of normal (non-malignant) cells grown *in vitro* are called organoids, and are currently the focus of great interest in biomedical research because they show some organization of tissues resembling *in vivo* organs [12]. We believe that the development of an *in vitro* vascular network could improve the culturing of spheroids and organoids, by allowing the tissues to grow and function in a way that is closer to the *in vivo* situation [13].

Other applications of vascular network culture could be fundamental studies of vascular development [14]; recapitulating disease conditions such as the retinal microvascular abnormalities seen in diabetes [15] or the abnormal angiogenesis in tumor development [16]; testing anti-angiogenic compounds in cancer research [17] or candidate drugs for their safe clinical application [18]; and studies in vascular regenerative medicine [19] (Figure 1).

It has long been known from the field of human and animal surgery, including transplant surgery, that tissue can become re-vascularized when grafted to a suitable site [20, 21]. Similarly, developmental studies have shown that embryonic tissues can also readily become re-vascularized, and continue to grow into functional organs, when transplanted to various locations in the embryo [22]. Furthermore, embryonic organ primordia can

become vascularized if transplanted not only to the embryo itself, but to the vascular network in the extra-embryonic membranes. A good example of this is the chicken embryo chorioallantoic membrane (CAM) system [23, 24]. In that model, organ primordia are placed onto the highly vascular CAM, the blood vessels first having been scratched to open them up. The organ primordia can then form a vascular connection with the CAM vessels, and undergo reasonable growth and morphogenesis. The CAM, however, is highly sensitive to environmental factors [25], therefore the development of the tissue graft is not perfect, possibly because it is not submerged in a supporting volume of fluid, but rather is exposed to the air. In a sense, therefore, the CAM and other developmental systems show that the growth of organs on vascular beds is a possibility. What is needed, however, is a vascular bed *ex vivo* that is perfused by some kind of microfluidic system.

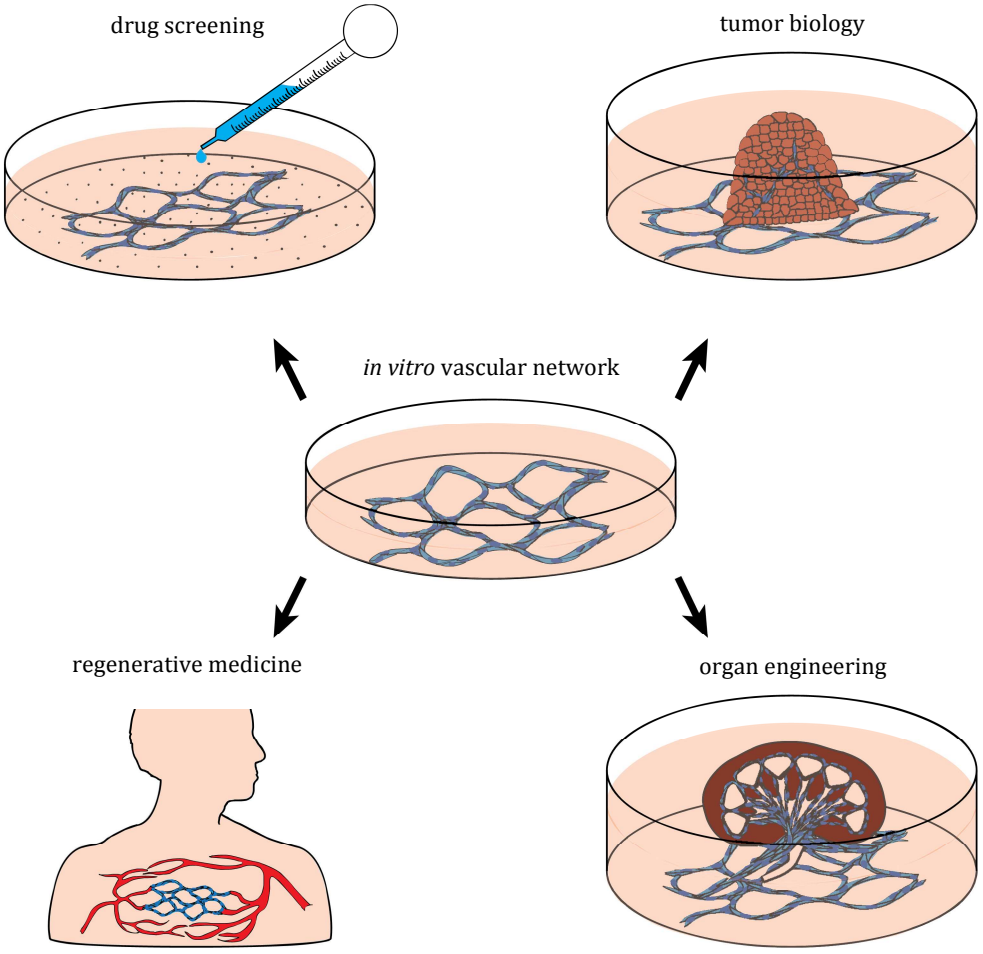

Figure 1: Potential applications of a vascular network culture.

Most of the current research describing vasculogenesis (de novo formation of blood vessels from progenitor cells) and angiogenesis (formation of blood vessels from existing blood vessels) uses mammalian models, mainly mice. However, these models are fairly expensive, time consuming and require ethical and other permissions [26]. Endothelial cell lines such as human umbilical vein endothelial cells (HUVECs) are commonly used for developing *in vitro* vascular networks [27]. Other sources for developing such cultures include embryonic or adult stem cells or tissue explants. The uses and limitations of these techniques are discussed in detail in the following sections.

Zebrafish can be an alternative to mammalian models [28]. The zebrafish produce a large number of fertilized eggs at low cost; the embryos are externally fertilized and therefore readily accessible for experiments [29]. In some jurisdictions, zebrafish embryos has fewer ethical restrictions. For example, in the European Union, the Directive 2010/63/EU on the protection of experimental animals allows zebrafish embryos to be used until 5 days post fertilization (dpf) without restriction [30]. Finally, the zebrafish genome has been sequenced and there is a high level of conservation between zebrafish and human protein coding genes [31]. This similarity supports the use of zebrafish to model various human diseases [32, 33]. Because of these advantages, the zebrafish is currently emerging as a model species to study vasculogenesis and angiogenesis *in vivo* [28]. Transgenic reporter lines are proving very useful in these studies [28].

In this review we give a general overview of vascular development *in vivo* and the role of various factors in the development of vasculature. Then, we review the current procedures used to culture vascular networks using mammalian endothelial cells and tissue explants. Then, we review the use of zebrafish to study various aspects of vasculogenesis and angiogenesis *in vivo*. Finally we look forward by summarizing the potential use of zebrafish model for *in vitro* studies of vascular development.

Development of vasculature *in vivo*

Formation of a vascular system is an essential process in embryonic development. Because multicellular tissues cannot survive without a blood supply, the cardiovascular system is one of the earliest systems formed during embryogenesis [34, 35]. The endothelial precursor cells (angioblasts) differentiate into endothelial cells and undergo the process of vasculogenesis in early embryos to form the primitive blood vessels [36]. Studies on zebrafish have shown that the angioblasts appear in the lateral mesoderm, migrate to the midline of the embryo and form the first blood vessels [37]. In adult mice and humans, endothelial progenitor cells reside in the bone marrow as multipotent adult progenitor cells, and contribute to the formation of new blood vessels [38].

Further development of blood vessels takes place by the extension of the pre-existing vascular network through the process of sprouting and non-sprouting angiogenesis [39].

During angiogenic sprouting, some endothelial cells within the existing blood vessel are selected as tip cells, and migrate in the direction of angiogenic stimuli [40]. The surrounding extracellular matrix is degraded by specific proteases released during the process [41]. Meanwhile, the stalk cells (endothelial cells following the tip cells) proliferate to extend the blood vessel [40]. Further in development the vascular network also extends through intussusceptive or non-sprouting angiogenesis [42]. The mature blood vessels attain arterial, venous and lymphatic differentiation types having different structures and functions [43]. Endothelial differentiation and blood vessel formation is a complex process which requires a number of growth factors, cell types and extracellular matrix (ECM) components, discussed in the following section.

Factors controlling vasculogenesis and angiogenesis *in vivo*

Exogenous protein factors influencing vascular development

The differentiation of endothelial cells and the formation of blood vessels is mainly controlled by several protein factors [44]. Some of these factors are released by the endothelial cells themselves, other factors are stabilizing signals released by other cell types [44]. The differentiation of angioblasts is induced mainly by fibroblast growth factor 2 (FGF2) and bone morphogenic protein 4 (BMP4) [43]. FGF2 induces the expression of vascular endothelial growth factor (VEGF) and other important chemokines required to control vascular morphogenesis [45]. The importance of FGF2 for vascular formation has been shown in studies on quail and zebrafish embryos [46, 47]. Similarly, BMP4 deficiency is associated with severe abnormalities in early mouse embryos, including the lack of a well-organized vasculature [48].

Among the endothelial growth factors, VEGFs play the predominant role in regulating the formation of blood vessels [49]. The VEGF family consists of several VEGF genes of which VEGF-A, which interacts with endothelial cells through VEGF receptor 2 (VEGFR2 also known as KDR or FLK1), is the main component responsible for the viability and proliferation of endothelial cells [50]. VEGFs also have important roles in the differentiation, migration and cell-cell adhesion of endothelial cells, as well as stimulating sprouting angiogenesis and the activation of tip cells [51]. Placental growth factor (PlGF), a member of VEGF family expressed in the placenta of early mammalian embryos, has a role in the activation of VEGFR2 and establishing interaction between VEGF-A and VEGFR2 [52]. PlGF has been demonstrated to increase the angiogenic potential of VEGF in ischemic myocardium in mouse [53]. PlGF expression is normally low in adult tissues, but high in pathological conditions, especially in cancer, where it promotes tumour angiogenesis [54].

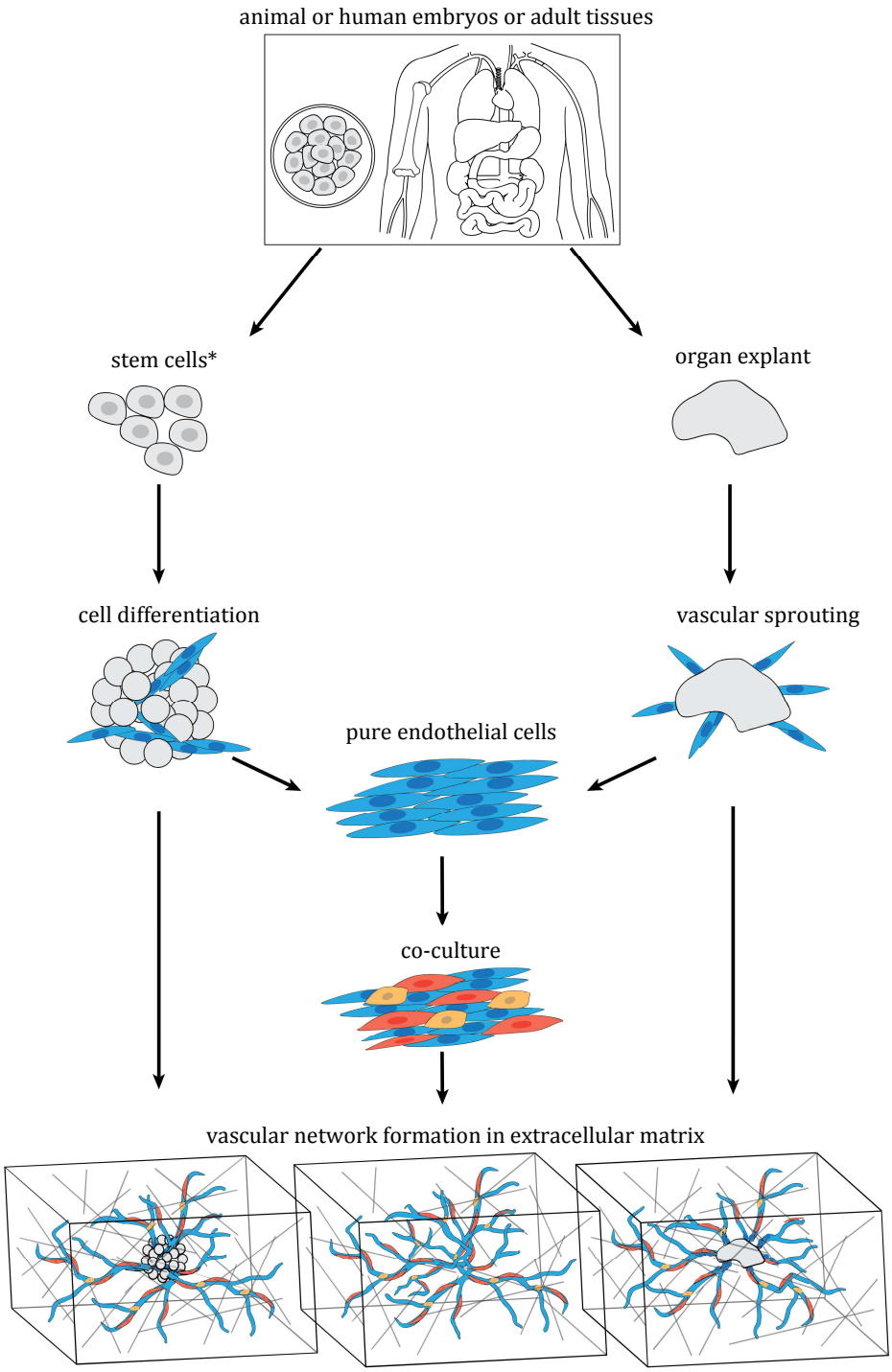

Figure 2. Schematic overview of three possible approaches to establishing cultures of vascular networks. *Stem cells, depending on their source, could be embryonic stem cells, mesenchymal stem cells, or induced pluripotent cells. Endothelial cells are in blue; diverse supporting cells are represented schematically by red and yellow.

Other growth factors involved in the spreading and maturation of blood vessels include angiopoietins (Ang-1 and Ang-2) [55], platelet-derived growth factor-B (PDGF-B) [56] and transforming growth factor β (TGF-β) [57], reviewed in Refs. [44, 49]. Many other transcription factors and signalling molecules have been identified to be involved in the differentiation of endothelial cells and the regulation of vascular development reviewed in Ref. [58]. In response to low oxygen levels in the tissues, Hypoxia inducible factors (HIFs) regulates the expression of a number of pro-angiogenic factors including VEGF, PlGF, Ang-1, Ang-2 and PDGF-B [59]. The HIFs are considered to be the principle mediators of *in vivo* vasculogenesis and angiogenesis at all developmental stages [59].

Role of membrane proteins and other cell types on vascular development

Membrane proteins on the surface of endothelial cells also play an important role in vascular morphogenesis. Examples of these membrane proteins include vascular endothelial cadherin (VE-cadherin) which functions to maintain endothelial cell-cell contact during VEGF-induced migration [60], epidermal growth factor like domain-7, which facilitates the formation of endothelial tubes [61], and delta like ligand-4 which specifies the tip cells for sprouting angiogenesis [62].

In addition to the soluble and bound protein factors, cell types other than endothelial cells also contribute to the formation of blood vessels. Pericytes and smooth muscle cells promote the proliferation and survival of endothelial cells and provide structural support to the blood vessels [63, 64]. Macrophages are reported to be involved in connecting two blood vessel sprouts in the process called anastomosis [65]. Under certain conditions (e.g. hypoxia), the parenchymal cells (neurons, hepatocytes, myocytes etc.) release angiogenic growth factors to initiate sprouting angiogenesis [66].

Role of extracellular matrix

Extracellular matrix (ECM) contributes to the formation and diversity of blood vessels in several ways including: (i) maintaining the histological structure and elasticity of the vessels, (ii) regulating the proliferation and differentiation of endothelial cells, and (iii) transporting, modifying or blocking the angiogenic growth factors [67]. The ECM is a complex network of macromolecules and its composition and properties are highly variable among different tissues, affecting the tissue specific differentiation of stem cells [68]. Research on the ECM of blood vessels have shown the presence of different ECM components at different stages of vascular development [69]. In the beginning of the process, the endothelial cells adhere to and migrate on a laminin-rich ECM which is later replaced by a collagen type-I rich ECM to support vascular tube formation [69].

Haemodynamic factors

Shear stress generated by blood flow on the luminal surface of endothelial cells is a mechanical factor that induces intracellular biochemical pathways resulting in gene expression changes and the modulation of the structure and function of blood vessels [70]. Heparin binding EGF-like growth factor is one such factor which is expressed in response to reduced blood flow and induces vessel narrowing [71]. Other molecular pathways involved in vascular remodelling are reported to be regulated by changes in shear stress leading to the expression of PlGF [72], Notch1 [73], and Smad6 (involved in TGB-β signalling) proteins [74].

Microfluidic culture of endothelial cells is currently an emerging technology which mimics the physiological shear stress on cultured cells to achieve the goal of culturing functional blood vessels for tissue engineering [13, 75]. A number of techniques for culturing vascular networks have been described in which endothelial growth factors, ECM components and microfluidics are combined; however, the development of fully functional blood vessels still remains a challenge [75].

Culture of vascular networks using endothelial cells

Pure endothelial cell populations can develop into vascular network-like structures in culture [27]. However, these networks are not sufficiently robust to be used for tissue engineering; they are mainly used to screen pro- and anti-angiogenic compounds for activity. Pure endothelial cell populations are derived from various sources including embryonic stem cells, induced pluripotent stem cells and adult tissues [76] (Figure 2). Human macro- and micro-vascular endothelial cells are commercially available (http://www.promocell.com/products-/human-primary-cells/) and have the ability to form vascular networks *in vitro*. The most commonly used endothelial cells in this regard are the HUVECs [27, 77-87], derived from the veins of the umbilical cord (Table 1). Other endothelial cell types such as bovine aortic endothelial cells [88] and rat aortic endothelial cells [89] have also been used to culture vascular networks.

By contrast, the culture of well-defined vascular networks with a lumen requires the co-culture of multiple cell types with endothelial cells [76]. The important supporting cell types, known to induce network formation by endothelial cells, include pericytes [90], mesenchymal stem cells [85], fibroblasts [79], hepatocytes [91], smooth muscle cells [92] and adipose-derived stem cells [78]. The importance of fibroblasts in enhancing angiogenesis has been shown in co-culture with HUVECs [81, 93]. The ECM components and angiogenic growth factors secreted by fibroblasts have been found to be critical for vascular tube formation from HUVECs [94].

Table 1. Endothelial cell cultures for vascular morphogenesis.

Interacting Cell types	Culture strategy	ECM Substrate	Medium additives	Main outcomes	Possible applications	Ref.
HUVECs hES-MCs	Static 3D	Col-1 + Fbn	DMEM/F12, VEGF, bFGF, HGF	hES-MC and HGF stabilize vascular network	Drug screening, regenerative medicine	[85]
HUVECs NHLF HPP	Flow 3D	Fibrin	EGM-2	Perfusable 3D vascular network	Drug screening; tissue engineering	[79]
HUVECs ASCs	Static 3D	Fibrin	EGM-2	Vasculogenesis is more stable in co-culture with ASC	Tissue engineering	[78]
HUVECs HDF	Static 2D		M199, ECGS, CS	HDF and bio-active silicate stimulate network formation	Tissue engineering	[81]
HUVECs	Flow 3D	Col-1	EGM-2	Directional neovessels growth in response to angiogenic signals	Drug screening	[80]
HUVECs HBMSCs	Static 3D	Col-1 + Laminin	EGM, DMEM	Laminin and HBMSCs promote vascular network formation	Tissue engineering	[84]
HUVECs MSCs	Static 3D	Col-1 + fibrin	EGM-2, DMEM	Increasing fibrin concentration increases vascular morphogenesis	Tissue engineering	[86]
HUVECs	Static 2D	BME	EGM-2	Vascular network formation	Drug screening	[27]
HUVECs HBVP	Flow 3D	Col-1	M199, ECGS, VEGF, bFGF	The vascular cells show an inflammatory response when stimulated	Modelling vascular diseases	[82]
HUVECs SF	Static 3D	Fibrin	EGM-2, VEGF, bFGF, Ang-1, TGF-β	vessel sprouting, lumen formation and vessel stability is regulated by different factors	Developmental studies	[83]
HUVECs HBMSCs OB	Flow 3D	Fibrin	EGM-2MV, VEGF, Ang1	Tumor cells introduced through the vessels escaped into the surrounding matrix	Drug screening, cancer research	[95]
HUVECs	Flow 3D	POMaC	EGM-2	The pre-formed vascular network allow the growth of tissues and show physiologically relevant responses	Drug screening, organ-on-a-chip technology	[87]
HMVECs	Flow 3D	Col-1	EGM-2MV, VEGF	Metalloproteinases modulate the length and diameter of vessels in 3D ECM	Developmental studies, tissue engineering	[96]
BAECs	Static 2D	Matrigel™	DMEM, VEGF, rGAL-8	GAL-8 promotes endothelial cell migration and capillary formation	Cancer research	[88]

Interacting Cell types	Culture strategy	ECM Substrate	Medium additives	Main outcomes	Possible applications	Ref.
RAECs	Static 2D	Matrigel™	DMEM, VEGF, roxarsone	Roxarsone promotes vascular formation *in vitro*	Cancer research	[89]

Abbreviations: Ang-1, angiopoietin-1; ASCs, adipose-derived stem cells; BAECs, bovine aortic endothelial cells; bFGF, basic fibroblast growth factor; BME, basement membrane extract (Trevigen); Col-1, collagen type-I; CS, calcium silicate; DMEM, Dulbecco's modified Eagle's medium; ECGS, endothelial cell growth supplement (Promocell); ECM, extracellular matrix; EGM, endothelial growth medium (Lonza or Promocell); EGM-MV, endothelial growth medium microvascular; F12, Ham's F-12 medium; Fbn, fibronectin; Flow, microfluidic flow-through culture; HBMSCs, human bone marrow-derived mesenchymal stem cells; HBVP, human brain vascular pericytes; HDF, human dermal fibroblasts; hES-MCs, human embryonic stem cell derived mesenchymal cells; HGF, hepatocyte growth factor; HMVECs, human dermal microvascular endothelial cells; HPP, human placental pericytes; HUVECs, human umbilical vein endothelial cells; M199, medium 199 (Lonza); MSCs, mesenchymal stem cells; NHLF, human normal lung fibroblasts; POMaC, poly(octamethylene maleate (anhydride) citrate); RAECs, rat aortic endothelial cells; rGAL8, recombinant galectin-8; SF, skin fibroblasts; Static, static replacement culture; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor.

The culture of vascular networks is usually established in synthetic matrices which mimic natural ECM; these matrices are particularly valuable for promoting successful vascular morphogenesis *in vitro* [97]. Examples of these matrices are Matrigel™ [98], Puramatix™ [99], collagen type-I [80], fibrin [78] and poly(ethylene glycol) hydrogel [100]. Combinations of these gel components can be used to mimic the complexity of natural ECM. The addition of laminin to collagen type-I scaffolds has been shown to increase network formation and VEGFR2 expression by HUVECs in culture [84]. Similarly, increased network formation from HUVECs was observed in composite collagen type-I/fibrin matrix compared to pure collagen [86].

Studies in which vascular networks are cultured from endothelial cells have revealed the important role of several cellular and molecular factors. In one study, adipose-derived stem cells (ASCs) were found to enhance vascular network formation from HUVECs in 3D fibrin gels [78]. That study showed that the expression of angiogenesis related genes (VE-cadherin, VEGFR2) and proteins (VEGF, FGF, Ang-1) was higher in ASCs/HUVECs co-culture compared to pure HUVECs culture [78]. The role of VEGF has been shown in specialization of the tip cells and directional migration of human micro vascular endothelial cells to form capillary-like structures [101]. Similarly, inducing the Notch signalling pathway upregulated VEGF-A, VEGF-B and VEGF-R1 expressions, and promoted vascular network formation in co-cultured mouse brain microvascular endothelial cells and ASCs in 3D collagen type-I gel [102].

Limitations of endothelial cell culture

Endothelial cell cultures are relatively easy to maintain. However, there are certain limitations which need to be considered while carrying out endothelial culture. A blood vascular network constitutes a number of vessel types, from large vessels to micro vessels,

and each vessel type has its own unique properties (including endothelial cell subtypes, as discussed below). Therefore it is challenging to attempt to recapitulate the formation of different vessel types using a homogeneous endothelial cell population [103]. Primary endothelial cell cultures are usually derived from terminally differentiated tissues; these cells have limited proliferative and regenerative capacity, and a short life span *in vitro* [104, 105].

Endothelial cells derived from different sites within the same tissue express different genes and respond differently to the same pro- or anti- angiogenic factors [106]. This supports the idea of functional subtypes among endothelial cells. Endothelial cells can be immortalised; however, this may change their behaviour and response to stimuli [107]. Immortalised endothelial cells may alter their gene expression and physiological properties with repeated passaging *in vitro*, resulting in loss of vasculogenesis efficiency [107]. The non-endothelial cell types that support *in vitro* vascular network formation from endothelial cells (e.g. fibroblasts), may represent an undesirable cell type if the resultant tissue is to be used for tissue engineering [76].

Use of stem cells for *in vitro* vasculogenesis

In recent years, stem cells have increasingly been used to develop vascular cultures; this is because stem cells have several advantages over terminally differentiated endothelial cells [19]. Stem cells are multipotent or pluripotent in nature, they show self-renewal, and their differentiation along various cell lineages can be manipulated by fine-tuning the culture conditions [19]. A few examples of the stem cells that can be used for endothelial, and ultimately vascular, differentiation are summarized in Table 2. Three main stem cell types used are: (i) embryonic stem cells (ESCs) [108], (ii) induced pluripotent stem cells (iPSCs) [109] and (iii) mesenchymal stem cells (MSCs) [110].

In addition, endothelial progenitor cells (EPCs), which originate in the bone marrow and contribute to the formation of new blood vessels in adults, are also useful in the study of *in vitro* vasculogenesis [105]. The differentiated endothelial cells arising from these stem cells directly undergo vasculogenesis because of the presence of other cell types that have also differentiated from the stem cells; alternatively, the endothelial cells can be isolated from the stem cell culture, without the unwanted additional cell types, and used for vascular morphogenesis (either in pure culture or co-culture with defined cell types) [111].

One of the advantages of using ESCs is that they can differentiate into multiple vascular cell lineages simultaneously in culture. In principle, these different lineages can contribute to the newly-formed vessels (neovessels) in a way that closely resembles the *in vivo* vasculogenesis in early embryos [19, 112]. Endothelial differentiation and vascular morphogenesis in ESCs is controlled by culture conditions (such as the presence of growth

factors in the medium and the use of feeder layers of stromal cells, or a substratum consisting of a synthetic hydrogel [112, 113]).

One approach to inducing the differentiation of ESCs in culture is to allow them to first aggregate into spherical cell masses, called embryoid bodies (EBs), in suspension culture [114]. The use of EBs as an intermediate step is common when ESCs are cultured for vascular differentiation (Table 2) [115]. In the absence of anti-differentiation factors (e.g. leukaemia inhibitory factor in mouse and feeder cell layer in human), ESCs differentiate into EBs consisting of mesodermal, ectodermal and endodermal lineages, similar to early embryogenesis [116]. In 2D (adherent) cultures the EB cells tend to proliferate and give rise to undesired cell types such as fibroblasts [114]. By contrast, in 3D culture (suspension or gels), the proliferation of EB cells is limited, allowing greater control of the differentiation of the desired cell type [114]. Significant effects of different factors, such as culture substrate (collagen type-IV or fibronectin), cell seeding density, concentration of VEGF and FGF in medium, and culture duration, have been observed on the endothelial differentiation in human, mouse and zebrafish ESC culture [117, 118]. Similarly, TGF-β has been identified to induce vascular differentiation in human ESCs [119].

Another important stem cell type, similar to ESCs in pluripotency and differentiation events, is the iPSCs [120]. An advantage of iPSCs is that they can be generated by genetic reprogramming of any adult somatic cell population, and therefore raise fewer ethical concerns compared to ESCs [19]. Endothelial differentiation in iPSCs can be induced by applying similar methods used for differentiation of ESCs [121]. Furthermore, gene expression in endothelial cells derived from ESCs and iPSCs is very similar [121]. MSCs are multipotent stem cells residing in adult tissues; they have limited differentiation potential compared to ESCs and iPSCs [19]. Endothelial differentiation in human amniotic fluid derived MSCs has been shown to be inducible by VEGF [110]. MSCs derived from various tissues (bone marrow, hair follicle, adipose tissue and muscles) have been used for vascular regeneration studies reviewed in Ref. [19]. In some studies the MSCs have been reported to promote and stabilize vascular network formation from HUVECs (Table 1).

In addition to the use of pluripotent and multipotent stem cells for endothelial differentiation and *in vitro* vasculogenesis, the unipotent EPCs also have the ability to differentiate into mature endothelial cells and form vascular tubes in culture [105]. The advantage of EPCs for culturing vascular networks is that these cells can be easily obtained from adult tissues such as peripheral blood [19]. *In vitro* studies have shown that the early EPCs do not directly undergo vascularization, but release factors to stimulate angiogenesis in distantly-cultured endothelial cells in a transwell [105]. Co-culture with MSCs has been proven to enhance vascular formation from EPCs both *in vitro* and after implantation *in vivo* [122, 123].

Table 2. The use of stem cell technology for endothelial differentiation and vascular development.

Stem cell type	Culture strategy	ECM Substrate	Medium additives	Main findings	Possible applications	Ref.
mESCs	EB static 3D	Col-1	IMDM, EPO, VEGF, bFGF,	RSK and TTK protein kinases modulate vascular formation	Drug screening, cancer research	[108]
mESCs	EB static 3D	Matrigel™	αMEM, VEGF	Reporter proteins in vascular cells allow track of vascular development	Developmental studies	[124]
mESCs	Static 3D	Col-1	IMDM, VEGF, bFGF	EB formation and angiogenic sprouting	Drug screening	[125]
mESCs	Static 2D	Gelatin	DMEM, VEGF	Endothelial differentiation, vascular network formation	Developmental studies, Drug screening	[126]
mESCs	EB static 2D	Col-1	DMEM, VEGF	VEGF receptors are involved in tip cell selection and sprouting	Developmental studies	[127]
mESCs	EB static 3D	Col-1	IMDM, EPO, VEGF, bFGF	Culture strategy, ECM substrate and growth factors effect vascular differentiation	Drug screening	[128]
hESCs	Static 2D	Matrigel™	EGM-2, BMP4	BMP4 increases vascular differentiation	Developmental studies, angiogenic therapy	[129]
hESCs	Static non-adherent		Knockout DMEM	Spontaneous endothelial differentiation and Vascular sprouting	Tissue engineering, regenerative medicine	[130]
miPSCs	EB static 3D	Col-1	DMEM, VEGF	TP73 gene regulate endothelial differentiation and vascular network formation	Cancer research	[131]
hiPSCs	Static 2D	Fbn	IMDM, VEGF, bFGF	Formation of vessel-like structures	Regenerative medicine, organ culture	[132]
hAFSCs	Flow 2D	Matrigel™	EGM-2	Shear stress promotes endothelial differentiation and vascular cord formation	Regenerative medicine	[133]
hAFSCs	Static 2D	Matrigel™	EGM-2, VEGF	EGM and VEGF promotes endothelial differentiation and vascular formation	Tissue engineering, angiogenic therapy	[110]
hTMSCs	Static 3D	Fibrin	EGM-2	TMSCs promote and stabilize vessel formation from endothelial cells	Regenerative medicine, tissue engineering	[134]

Abbreviations: αMEM, alpha-minimal essential medium (Cellgro); bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein-4; Col-1, collagen type-I; DMEM, Dulbecco's modified Eagle's medium; EB, embryoid body intermediate; ECM, extracellular matrix; EGM, endothelial growth medium (Cambrex or Clonetics); EPO, erythropoietin; Fbn, fibronectin; Flow, under flow of medium; hAFSCs, human amniotic fluid-derived stem cells; hESCs, human embryonic stem cells; IMDM, Iscove's modified Dulbecco's medium; mESCs, mouse embryonic stem cells; miPSCs, mouse induced pluripotent stem cells; RSK, ribosomal S6 kinase; Static, static replacement culture; TP73, tumor protein-73; TTK, threonine and tyrosine kinase; VEGF, vascular endothelial growth factor.

Issues and drawbacks with stem cell culture

Although stem cell technology has several advantages for vascular engineering and regenerative therapy, there are some limitations to its use [19]. Thus, while ESCs have been extensively studied in laboratory animals such as mouse and rats, and stable cell lines have been developed form these animals, the technique has been proven less successful for other species such as cattle, goat and dogs [120]. Furthermore, the very complexity of vascular differentiation from ESCs means that the growth factors necessary to support the generation and maintenance of multiple cell types need to be laboriously optimised [135]. Furthermore, the use of human ESCs for research raise ethical concerns [19].

ESCs and iPSCs are both pluripotent, and therefore it is challenging to direct the differentiation towards a specific lineage, and to obtain high quality pure cell cultures [120]. The iPSCs are developed by transfection of somatic cells with pluripotency genes; however, the efficiency of the process is very low (less than 1%) [136]. The iPSCs (in contrast to ESCs) are derived from adult differentiated cells by de-differentiation. Then, if re-differentiated into a specific cell type, they attain some of the characteristics of that cell type but are not identical to their normal counterparts [136]. Other issues with stem cells is that the isolation of MSCs from adult tissues requires invasive surgical procedures, and only yields small numbers of cells; the proliferation of these cells is also limited *in vitro* [137]. Furthermore, the MSCs isolated from different tissues or life stages are not the same, and therefore have different culture requirements and angiogenic potentials [138].

The isolation and culture methods for EPCs are only relatively recently developed (Asahara *et al.,* 1997 [139]). For this reason, there is no standard protocol among researchers. It should be noted that there are no specific markers for EPCs because many of the genes expressed by EPCs are also expressed in hematopoietic progenitors [140]. Similar to the iPSCs and MSCs, the number of EPCs found in isolated adult tissue cells is very low, and this greatly limits their study [19]. Finally, analysis of EPCs in long-term culture has shown that the late passage cells (45 days after the initiation of the primary culture) have changed morphology, reduced their proliferation rate, show high β-galactosidase expression and loss of vascular network formation ability, compared to the early passaged cells [141].

Use of tissue explants for *in vitro* angiogenesis

An important strategy for vascular morphogenesis *in vitro* is to stimulate the growth of the blood vessels existing in isolated sections or fragments of specific tissues [107]. The development of a well-defined blood vascular network requires the incorporation of multiple cell types both *in vivo* and *in vitro*, as discussed in the previous sections. The use of tissue explants is important in this context, because these explants already contain multiple cell types, and the angiogenesis stimulated in these cultures closely represents the corresponding process *in vivo* [107]. Furthermore, tissue explant experiments are relatively easy to perform and allow a large number of cultures to be derived from a single tissue sample [142].

Various tissue explants have been shown to have the ability to develop vascular sprouts *in vitro* (Table 3). Examples include cross sections of aorta called aortic rings [143]; metatarsal bones [144]; retina fragments [145]; choroid-sclera fragments [146]; and adipose tissue [147]. In most cases, the tissues for explant preparation are isolated from developing rodent embryos or neonates. Tissue explants from other species such as chick embryo aortic arch [148], rabbit aorta[149] and pig carotid artery [150] have also been adapted for sprouting angiogenesis. Furthermore, angiogenic sprouting has also been reported from human tissue explants e.g. adipose tissue [147], aortic explants from aborted embryos [151], placental explants [152, 153] and umbilical artery rings [154].

Explant cultures are usually established in a 3D gel matrix in the presence of angiogenic growth factors, and are examined for microvessel outgrowth (vascular sprouting)[107, 155]. The aortic ring model from various species is the most commonly used explant for studying *in vitro* angiogenesis (Table 3). The stimulatory effect of various factors, such as angiogenic growth factors (especially VEGF) and ECM components, on the growth of vascular sprouts from aortic ring have been extensively studied, reviewed in Ref. [156]. Recently developed explant cultures, using fetal metatarsals from mice, have shown advantages over the aortic ring model, in that they do not require a 3D matrix and exogenous growth factors for vascular sprouting [144]. In general, explant cultures can serve as an intermediate between the endothelial cell culture on the one hand, and *in vivo* models on the other. They are also thought to be more reliable for studying the mechanisms of angiogenesis and testing the role of regulatory factors [157].

Limitations of explant cultures

Besides the advantages of explant cultures, certain limitations need to be addressed before the technique can be fully accepted for research in tissue engineering and regenerative medicine. The mouse aortic ring model shows significant variability in microvessel sprouting from explants isolated from different age and strain of animals [158]. Variability in outcome has also been reported using explants isolated from different vessel types (artery or vein) of the same individual animal [159]. The vascular sprouts in

the aortic ring model regress over time in culture (with peak sprouting between days 6 and 7), and this limits the analysis time and increases variability in results with culture duration [143]. Furthermore, the aortic rings are derived from large vessels, and therefore do not truly represent *in vivo* angiogenesis, which is a microvascular process [107].

Table 3. Tissue explants used for sprouting angiogenesis *in vitro*.

Tissue explant	Culture strategy	ECM Substrate	Medium additives	Main findings	Possible applications	Ref.
mAR	Static 3D	Col-1	MCDB131, VEGF	Age of the mouse inversely affects vascular sprouting from explant	Drug screening	[160]
mAR	Static 3D	Col-1	ESFM	Endostatin inhibits vascular sprouting by modulating endothelial cell-ECM interaction	Cancer research	[161]
mAR	Static 3D	Col-1	Opti-MEM, VEGF	VEGF and collagen increase vessel sprouting	Drug screening	[143]
rAR	Static 3D	Matrigel™	EGM-200	Ascorbate inhibits sprouting angiogenesis	Cancer research	[162]
hUAR	Static 3D	BME	EGM-2	Capillary spouting upon VEGF stimulation	Drug screening, cancer research	[154]
cAA	Static 2D	Matrige™	bFGF, VEGF	Chemical compound releasing nitric oxide inhibits angiogenesis	Cancer research, drug screening	[163]
mAT	static 3D	Col-1	MCDB131, VEGF	Angiogenic sprouting	Drug screening	[164]
hAT	Static 2D	Matrigel™	EBM-2, EGM-2 MV	Angiogenic capacity reflects donor's physiology	Drug screening, angiogenic therapy	[147]
mRE	Static 2D	PTFE	DMEM, VEGF	VEGF stimulate vascular sprouting	Drug screening	[165]
mRE	Static 3D	Fibrin	DMEM, VEGF	VEGF stimulate sprouting angiogenesis	Drug screening	[166]
mMT	Static 2D	Gelatin OR Col-1	αMEM	Vascular sprouting occurs without additional growth factors	Developmental studies, Drug screening	[144]
mMT	Static 2D		αMEM, VEGF	VEGF recover the impaired vascular sprouting in endoglin deficient explants	Modelling vascular diseases, drug screening	[167]
mPE	Static 2D	Col-1	M199, VEGF, bFGF	Isoforms of VEGF differentially stimulate vascular sprouting	Developmental studies	[168]

Abbreviations: αMEM, alpha-minimal essential medium; bFGF, basic fibroblast growth factor; BME, basement membrane extract (BD Biosciences); cAA, chick aortic arch; Col-1, collagen type-I; DMEM, Dulbecco's modified Eagle's medium; EBM, endothelial basal medium (Lonza); ECM, extracellular matrix; EGM MV, endothelial growth medium microvascular; EGM-200, endothelial growth medium-200 (Cascade Biologics); ESFM, endothelial serum-free medium (Life Technologies); hAT, human adipose tissue explant; hUAR, human umbilical arterial ring; M199, medium 199 (Gibco); mAR, mouse aortic ring; mAT, mouse adipose tissue explant; MCDB131, basal medium (Invitrogen); mMT, mouse metatarsal explant; mPE, mouse proepicardium explant; mRE, mouse retinal explant; Opti-MEM, minimal essential reduced-serum medium (Gibco); PTFE, polytetrafluroethylene membrane; rAR, rat aortic ring; Static, static replacement culture; VEGF, vascular endothelial growth factor.

Tissues containing microvascular networks (e.g. adipose tissue and retina) can be used for explant preparation. However, these tissues are more difficult to isolate and, like the aortic explant, show variability between experiments [145]. The high levels of capillary sprouting observed in adipose tissue explant cultures are in many senses an advantage; however they do make it difficult to identify all the sprouts individually and interpret the results [147]. Similarly, angiogenic sprouts from fetal mouse metatarsal explants present microvascular features; however, their isolation and culture procedures also require advanced technical skills, which are key to the reproducibility of the research [144]. Finally, the metatarsal and chick aortic arch explants are isolated from developing embryos and have high proliferative capacity; therefore, angiogenesis in these models does not represent the *in vivo* situation in adults [107].

Zebrafish: a new model species for studying *in vivo* vasculogenesis and angiogenesis

The zebrafish is a freshwater teleost fish [169] that is emerging as a model of choice for studying vasculogenesis and angiogenesis [170]. The embryos and larvae are often used in these studies because of their external fertilization, optical transparency at early stages, and the ease of exposure to test substances (by simply adding the compound to the swimming water) [171]. Furthermore, the genome comparison study has revealed that there is at least one orthologue in zebrafish genome for more than 70% of human protein coding genes [31]. Vascular development and function in zebrafish are relatively conserved, compared to the same processes in other vertebrates [170].

The embryos develop a simple vascular system with circulating blood as early as 24 hours post fertilization (hpf) [37]. Vascular development can be directly observed non-invasively in the living, transparent embryo [28]. Enhanced visualization of vascular development can be achieved by injecting fluorescent micro particles into the blood stream, or by using transgenic lines such as *kdrl:GFP* and *fli:GFP* that express green fluorescent protein (GFP) in vascular cells [28].

For these and other reasons, vascular development in zebrafish — from early differentiation of angioblasts to the maturation of blood vessels — has been extensively

studied [32, 37, 172, 173]. Studies have shown similar angiogenic responses to the test substance irisin, in zebrafish embryos *in vivo*, and in HUVECs *in vitro* [174]. In another example, the genetic mutation (gridlock), which causes aortic malformations and congenital heart defects in humans, showed similar phenotypic effects in zebrafish [175]. Zebrafish have been successfully utilized to model several human vascular diseases reviewed in Ref. [32].

Similarly, a zebrafish *in vivo* xenograft model has been developed to study human carcinomas [176]. These studies have shown successful invasion, metastasis and extravasation of various human tumor cells in zebrafish embryos and adults [176]. It has been demonstrated that the transplantation of human WM-266-4 melanoma cells and breast adenocarcinoma cells in zebrafish embryos induced angiogenesis in the host vasculature; this led to the formation and infiltration of neovessels into the tumor masses [177, 178]. Other examples of human carcinomas studied in zebrafish include breast cancer bone metastasis [179], uveal melanoma [180] and retinoblastoma [181].

The zebrafish possesses remarkable regenerative capacity in several organs (including the caudal fin and heart [182, 183]) which makes it a useful model for studying regeneration [184]. The regeneration of organs also involves the regeneration of blood vessels, and therefore the regenerative capacity of zebrafish is also important for vascular regeneration studies [28].

Zebrafish transgenic reporter lines for vascular studies

Several transgenic lines have been developed for zebrafish which express fluorescent proteins under vascular cell specific promoters [28]. These transgenic lines allow the tracking of the differentiation, proliferation and migration of individual cells during vascular development *in vivo* and *in vitro* [28]. Moreover, different transgenes can be combined in the same embryo, as was done with a line (scl-PAC:GFP) expressing fluorescent proteins in both endothelial and blood cells; that line permitted the observation of the development of the vascular system and blood flow simultaneously [185].

The most important transgenic line that we are utilizing to study vascular development in zebrafish is the *kdrl:GFP* line (Figure 3A). This transgenic line is also known as *Tg(kdr:eGFP)* or *Tg(flk1:eGFP)* [186]. In this line, GFP is specifically expressed in endothelial cells under the control of the VEGFR2 or *kdr-like* gene [186]. The *kdrl:GFP* line allows high resolution analysis of single cell migration and vascular development in living embryos [186]. The utility of *kdrl:GFP* zebrafish embryos has been confirmed as a high-throughput toxicology screening model [187].

Other transgenic zebrafish lines are available for vascular studies, although they have some limitations. For example, In *Tg(Tie2:eGFP)* the GFP expression is relatively weak in the vascular cells [188]. Similarly, in *Tg(fli1:eGFP)* the GFP is expressed in certain non-

vascular cells which interferes with the results especially in the head region of the embryo [186]. Furthermore, studies on *Tg(fli1:eGFP)* have shown changes in the gene expression of a number of genes, compared to the wild type embryos, which may affect the results while using transgenic zebrafish for experiments [189].

In summary, zebrafish is a high-throughput, easily quantifiable, fast developing and relatively inexpensive *in vivo* model for vascular studies. However, there are some drawbacks associated with this model. The relevance of zebrafish embryo model to understand human angiogenesis is questioned, as there is a large evolutionary time difference between the two species [157]. Therefore, preclinical drug screening in zebrafish should always be followed by validation in mammalian models before going to clinical trials [28].

Future prospects for using zebrafish cells for *in vitro* vasculogenesis and angiogenesis

In principle, many of the techniques discussed in the previous sections for *in vitro* vasculogenesis and angiogenesis (using mammalian endothelial cells, ESCs and tissue explants), can also be adapted for use in the zebrafish model. With the availability of primary embryonic cells due to high fecundity of the species, and easy cell isolation procedures, the drawbacks associated with adapted cell lines can be avoided [190]. In addition to the above mentioned characteristics, zebrafish also possess specific desirable features for *in vitro* applications.

Cell culture techniques in zebrafish

The external fertilization and large number of fast developing embryos, allow easy harvesting of large numbers of cells and quantities of tissues from different developmental stages [191]. Zebrafish cells grow at a lower temperature (26-28 °C) than chick and mouse cells and do not usually require a CO_2-enriched atmosphere [191]. These properties allow zebrafish cells to be grown at room temperature, although the use of a simple incubator is recommended to help maintain sterile conditions [191]. The protective covering of the chorion, which is present until hatching at around 48 hpf, partly isolates the embryos from the environment [192]. This is important for *in vitro* studies because it maintains the embryos in an aseptic condition [193].

To harvest sterile cells or tissues from zebrafish embryos it is necessary to decontaminate the surface of the chorion. Using this approach, it is possible to isolate and culture sterile cells from blastula (3 hpf) or gastrula (24 hpf) stage embryos [191, 194]. In a recent study, we have shown that embryos with a chorion decontaminated at 24 hpf could be further cultured to 5 dpf under aseptic conditions [155]. The tissues and cells isolated from these embryos were successfully maintained free of contamination for eight days in culture.

Zebrafish embryonic stem cells

As is the case with mouse and human ESCs, it is possible to maintain zebrafish ESCs in a pluripotent state in long-term culture [195]. In zebrafish and medaka, the ESCs can be derived from the embryo before the blastocyst stage; these cells may possess a higher degree of pluripotency (and even totipotency), compared to mammalian ESCs derived from blastocysts [196]. When differentiation inhibition factors are depleted in the culture medium, the zebrafish ESCs have the ability to undergo differentiation into a range of specialized cell types [194]. The spontaneous differentiation of zebrafish ESCs into neuron-like cells, muscle-like cells, embryonic carcinoma-like cells and fibroblast-like cells has been demonstrated [191, 194, 197]. However, little is known about the condition needed to induce specific differentiation in ESCs from zebrafish.

The induction of myogenic differentiation in zebrafish primary ESCs has been shown by culturing these cells on a laminin substratum, in medium containing insulin [198], FGF [199] or sonic hedgehog protein [200]. In another study, the seeding density (between 1 and 2 × 10^4 cells/cm^2) of zebrafish primary ESCs, co-culture with a zebrafish fibroblast-like cell line (ZF4) and medium supplementation with insulin, were found to induce cardiomyocyte differentiation [201]. Similarly, an increase in the generation of cells resembling primordial germ cells was found in zebrafish blastocyst cell cultures after the addition of BMP4, EGF and retinoic acid to the medium [202]. Furthermore, the use of FGF and VEGF have been shown to increase differentiation towards the endothelial cell lineage in zebrafish blastocyst cells [203].

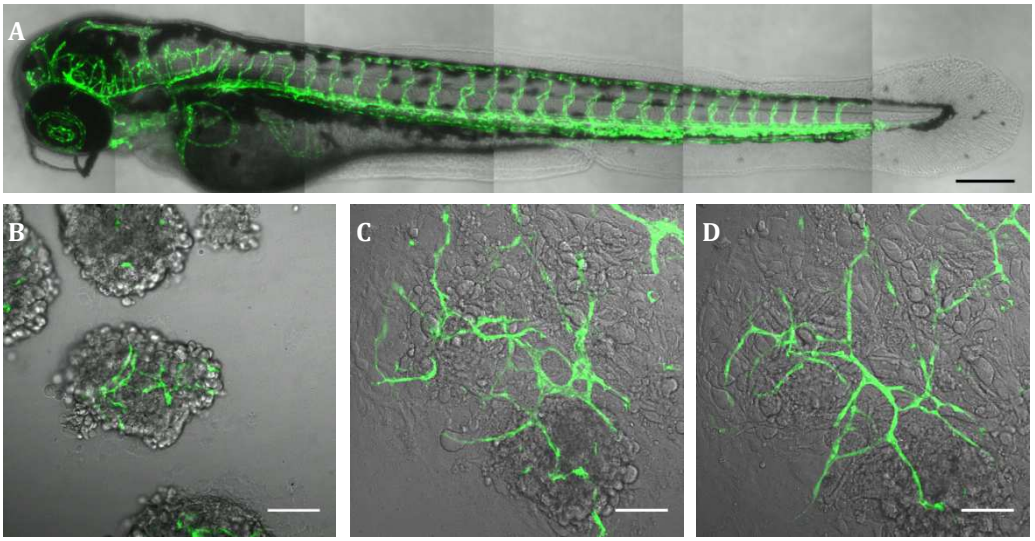

Figure 3: Confocal images of transgenic zebrafish *kdrl:GFP* whole embryo (A) and EB culture (B-D). (**A**) A 5 dpf *kdrl:GFP* embryo showing florescent endothelial cells forming blood vessels. Scale bar, 200 µm. (**B, C and D**) Developing embryoid body on subsequent days of culture (day 1, day 6 and day 8, respectively) on a mixture of collagen type-I, Geltrex™ and fibrin substratum, showing the development of vascular network-like structures from *kdrl:GFP*$^+$ cells. Scale bars, 100 µm.

Zebrafish blastocyst cells aggregate into EBs in culture. We have shown that the percentage of endothelial-like cells in EBs is increased by culturing them in suspension (hanging drop culture) rather than in adherent culture, and by adding endothelial growth supplements, including VEGF, to the medium [118]. We found that the endothelial cells in EB cultures form vascular network-like structures on hydrogel substrates (Figure 3B-D).

Cultures of specialised zebrafish cell types

Attempts have been made to develop cultures of specialised cell types using progenitor cells isolated from embryos. In one study, neural crest cells, isolated from dissociated 14 hpf (10-somite stage) zebrafish embryos, were maintained in culture [204]. These cells were able to proliferate, migrate and differentiate into neurons, chondrocytes and glial cells in the presence of specific factors [204]. Strategies have also been described for the culture of primary neurons from developing brain and spinal cord cells of zebrafish embryos [205]. We have found that the endothelial cells in dissociated hearts, isolated from 5 dpf zebrafish embryos, form colonies on a fibronectin substratum; however, these cultures could only be maintained for short periods, possibly because of low seeding-density [155].

These studies suggest that zebrafish embryonic cell culture can be an important model for studying endothelial differentiation and vascular morphogenesis *in vitro*. In principle, it could be possible to use zebrafish *kdrl:GFP* embryos for the isolation of endothelial cells using fluorescence activated cell sorting (FACS). The development of these cells into vascular networks in response to various signals could then be readily tracked in live cultures without the need to fix and stain them. Furthermore, tissues and organ explants from zebrafish embryos can be a promising model for sprouting angiogenesis, as we have shown using liver and heart explants from 5 dpf embryos [155].

In summary, the zebrafish allows easy access to large numbers of primary cells, and vascular development in cultures derived from these cells occurs in a complex environment of other cell types. In contrast, it is difficult to access primary cells and tissues in mammalian models, and the vascular culture using endothelial cell lines such as HUVECs does not reflect the complex process *in vivo*.

Disadvantages of zebrafish *in vitro* model

Zebrafish embryonic cell cultures usually combine cells or tissues from a large number of individuals due to the small size of embryos [198]. As a result, the cell population obtained is genetically heterogeneous, and may not be ideal for gene expression analysis [198]. Despite the simplicity of the required culture conditions (i.e. low incubation temperature and no requirement for a CO_2-enriched atmosphere), studies have shown that zebrafish primary embryonic cells require a complex medium containing FGF and fish embryo

extract for their growth [195]. The lower incubation temperature for zebrafish cells may not be ideal for human cells if a co-culture has to be established.

The zebrafish embryonic cells usually have to be cultured on a feeder layer of growth-arrested cells to maintain their pluripotency [193]. The development of vascular network in primary zebrafish embryonic cells and tissues in the presence of other supporting cell types may be considered as an advantages as it closely mimic the *in vivo* situation. However, this provides less control over the *in vitro* vascular development compared to endothelial cell lines. Because the zebrafish is a relatively new research model, the differentiation and culture conditions for its cells still needs to be optimised.

Beyond organoids: microfluidics and the development of a functional vascular network

In this final section we want to look at some future prospects in the field of *in vitro* vascular networks. These networks will always remain of limited value unless they support a functional blood flow. In a living animal, this flow is powered by the heart. But how can blood flow be initiated and maintained in a culture system? One obvious answer is to use microfluidic technologies to pump blood or some nutrient liquid through the vascular network.

Examples of microfluidic vascular culture systems

In one example of vascular culture in a microfluidic system, HUVECs and human lung fibroblasts were co-cultured in a perfused 3D fibrin gel [79]. The networks that developed allowed the transport of nutrients, molecules and cells, and also showed physiological responses to flow-induced shear stress [79]. In the same microfluidic device the authors showed that the presence of flow (regardless of flow direction) facilitates vasculogenesis by HUVECs, and that angiogenic sprouting occurs only in the direction opposite to the flow direction [206]. Similarly, angiogenic sprouting in 3D collagen type-I gel from HUVEC cultures in a microfluidic device showed morphological features resembling *in vivo* angiogenesis [80].

These studies employed a commonly-used microfluidic design containing a middle channel filled with ECM, and interconnected with two side channels which conduct the medium flow (Figure 4A). In this device the vascular network is connected to the media channels through the lumen openings [207]. This allows the vascular network to be perfused by chemical compounds or cells. The device allows rapid quantification of changes in vascular networks in response to test compounds [207]. Using the same design, Jeon et al. have shown the extravasation of cancer cells, introduced through the perfused vascular network, into the surrounding bone-mimicking matrix [95]. In a similar device, human colorectal or breast cancer cells co-cultured with endothelial cells in 3D

ECM in the middle channel have been shown to develop into vascularized tumor aggregates [208]. These tumor-like structures showed reduced growth, and sometimes even regression occurred, in response to standard vascular targeting therapies infused via the microvessels [208].

Another device (Figure 4B) mimics the tissue capillary interface by allowing the culture of endothelial cells on one side, and epithelial cells on the other, of a porous membrane inside a microfluidic channel [209]. An alternative approach is the pre-moulded hollow network (Figure 4C) in a 3D collagen type-I matrix with embedded supporting cells (in this case, human brain vascular pericytes [82]). The HUVECs are seeded into the cavities of cast. In this setup, the HUVECs first form a lining to the cast channels, and then form vascular sprouts into the surrounding matrix [82].

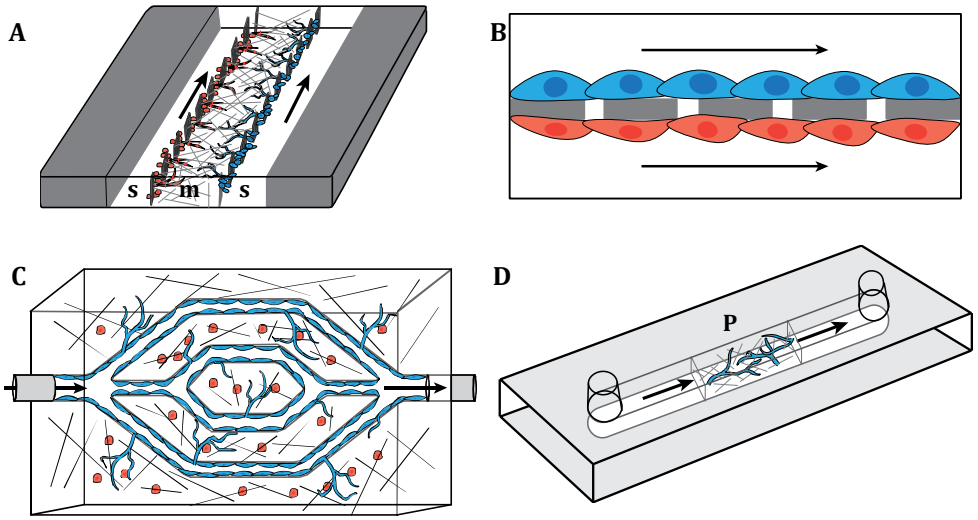

Figure 4. Microfluidic devices commonly used for culturing vascular-like networks. (**A**) Three channel microfluidic device [95, 206]. The middle channel (m) is filled with extracellular matrix with embedded cells. The two side channels (s) conduct the medium flow (arrows). (**B**) Microfluidic device mimicking capillary-tissue interface with endothelial cells on one side and epithelial cells on other side of a porous membrane [209]. The medium flow (arrows) can be established on the endothelial side only, or on both sides. (**C**) In this device, endothelial cells line the built-in channels cut into a hydrogel, with embedded supporting cells [82, 87]. (**D**) A microfluidic channel slide with a 3D matrix plug (P) in the middle of the channel [155]. The medium flows on either side of the plug (a small amount may penetrate the gel by diffusion). Note that (**C**) comes closest to a growing vascular bed connected to the microfluidic system. Arrows showing the direction of the medium flow. For further discussion of microfluidic devices in the field of vascular culture, see Refs. [13, 75, 210].

In a similar device, the luminized vasculature have been developed inside moulded channels in a biocompatible scaffold [87]. The network thus formed was able to vascularize cardiac and hepatic tissues cultured on the outside of the scaffold. The material used for the scaffold in that study was poly(octamethylene maleate (anhydride)

citrate) (POMaC; a biodegrade-able and biocompatible scaffold) [87]. In that setup, the micro pores incorporated into the scaffold allowed the uptake of nutrients, chemical compounds and cells from the vessels, and the release of metabolites into the vessels by the surrounding tissue [87].

In most cases, endothelial cell lines are used to culture vascular networks in a microfluidic system. The stem cells and explant cultures have been very little used in such studies. We have shown that zebrafish EBs embedded in 3D gel matrix, cultured in a microfluidic channel (Figure 4D) formed longer and wider vascular sprouts compared to the EBs cultured in conventional (static) 96-well plates [155].

Technical challenges for the future

As we have discussed above, scientists are beginning to realize the goal of a functional vascular network perfused by microfluidics *in vitro*. However, there are some severe technical challenges to be overcome. Presumably, some kind of synthetic interface or connector will be needed to connect the living vessels with microfluidic system. Another major challenge will be to maintain an increasing blood flow as the tissue explant, attached to the vascular network, grows in size. Thus, the growing tissue will require vessels of increasing diameter, and this in turn will require an expanding connection to the microfluidic system. Solutions to these problems will require intensive research.

Conclusions

In summary, the techniques developed for *in vitro* vascular network formation in mammals are producing rapid advances in our understanding. They can also be applied to zebrafish cells and tissues. For its benefits such as easy access to primary embryonic cells, availability of transgenic lines to visualize endothelial cells and conserved molecular pathways, the zebrafish can be a significant first-step model for studying *in vitro* vascular development. In recent years, microfluidic technology have shown great advances in developing *in vitro* vascular networks. Ultimately, the future of tissue culture and organ culture will be greatly extended if functional vascular networks with a fluidic flow can be grown *in vitro*. The major challenges in establishing such a system would be to recapitulate the key features of vascular system such as barrier function and vasoactivity, as wells as to compensate for the increasing demands of the growing tissue.

References

[1] Levick JR. The microcirculation and solute exchange. In: Eds. An Introduction to cardiovascular physiology. 5 ed, Taylor & Francis 2010, pp. 166-187.

[2] Kannan RY, Salacinski HJ, Sales K, Butler P, Seifalian AM. The roles of tissue engineering and vascularisation in the development of micro-vascular networks: a review. Biomaterials 2005; 26(14): 1857-75.
[3] Jain RK, Au P, Tam J, Duda DG, Fukumura D. Engineering vascularized tissue. Nat Biotechnol 2005; 23(7): 821-3.
[4] Marx V. Tissue engineering: organs from the lab. Nature 2015; 522(7556): 373-7.
[5] Kim JJ, Hou L, Huang NF. Vascularization of three-dimensional engineered tissues for regenerative medicine applications. Acta Biomater 2016; 41: 17-26.
[6] Bae H, Puranik AS, Gauvin R, Edalat F, Carrillo-Conde B, Peppas NA*, et al.* Building vascular networks. Sci Transl Med 2012; 4(160): 160ps23.
[7] Groeber F, Engelhardt L, Lange J, Kurdyn S, Schmid FF, Rucker C*, et al.* A first vascularized skin equivalent for as an alternative to animal experimentation. ALTEX 2016; 33(4): 415-422.
[8] Rezvani M, Grimm AA, Willenbring H. Assessing the therapeutic potential of lab-made hepatocytes. Hepatology 2016; 64(1): 287-94.
[9] Nayak DK, Saravanan PB, Bansal S, Naziruddin B, Mohanakumar T. Autologous and allogenous antibodies in lung and islet cell transplantation. Front Immunol 2016; 7: 650.
[10] Lovett M, Lee K, Edwards A, Kaplan DL. Vascularization strategies for tissue engineering. Tissue Eng Part B Rev 2009; 15(3): 353-70.
[11] Weiswald LB, Bellet D, Dangles-Marie V. Spherical cancer models in tumor biology. Neoplasia 2015; 17(1): 1-15.
[12] Clevers H. Modeling development and disease with organoids. Cell 2016; 165(7): 1586-97.
[13] Ibrahim M, Richardson MK. Microfluidic devices for cell, tissue and embryo culture. Rec Pat Regen Med 2013; 3(3): 249-263.
[14] Rosenfeld D, Landau S, Shandalov Y, Raindel N, Freiman A, Shor E*, et al.* Morphogenesis of 3D vascular networks is regulated by tensile forces. Proc Natl Acad Sci U S A 2016; 113(12): 3215-20.
[15] Shan K, Li CP, Liu C, Liu X, Yan B. RNCR3: a regulator of diabetes mellitus-related retinal microvascular dysfunction. Biochem Biophys Res Commun 2017; 482(4): 777-783.
[16] Roudsari LC, West JL. Studying the influence of angiogenesis in *in vitro* cancer model systems. Adv Drug Deliv Rev 2016; 97: 250-9.
[17] Azimi MS, Lacey M, Mondal D, Murfee WL. An *ex vivo* tissue culture model for anti-angiogenic drug testing. Methods Mol Biol 2016; 1464: 85-95.
[18] Therapontos C, Erskine L, Gardner ER, Figg WD, Vargesson N. Thalidomide induces limb defects by preventing angiogenic outgrowth during early limb formation. Proc Natl Acad Sci U S A 2009; 106(21): 8573-8.
[19] Zou T, Fan J, Fartash A, Liu H, Fan Y. Cell-based strategies for vascular regeneration. J Biomed Mater Res A 2016; 104(5): 1297-314.
[20] Antonopoulos IM, Yamacake KG, Oliveira LM, Piovesan AC, Kanashiro H, Nahas WC. Revascularization of living-donor kidney transplant with multiple arteries: long-term outcomes using the inferior epigastric artery. Urology 2014; 84(4): 955-9.
[21] Takebe T, Zhang RR, Koike H, Kimura M, Yoshizawa E, Enomura M*, et al.* Generation of a vascularized and functional human liver from an iPSC-derived organ bud transplant. Nat Protoc 2014; 9(2): 396-409.
[22] Rugh R. Intra-embryonic transplantations. In: Eds. Experimental embryology: techniques and procedures. ed, Burgess Pub. Co. 1962, pp. 443-448.

[23] Searls RL, Smith AA. Evidence that ectoderm influences the differentiation of muscle in the limb of the embryonic chick. J Exp Zool 1982; 220(3): 343-51.
[24] Rugh R. The methods of chorio-allantoic grafting. In: Eds. Experimental embryology: techniques and procedures. ed, Burgess Pub. Co. 1962, pp. 437-442.
[25] Ribatti D. The chick embryo chorioallantoic membrane (CAM). A multifaceted experimental model. Mech Dev 2016; 141: 70-7.
[26] Festing S, Wilkinson R. The ethics of animal research. Talking Point on the use of animals in scientific research. EMBO Rep 2007; 8(6): 526-30.
[27] Arnaoutova I, Kleinman HK. *In vitro* angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc 2010; 5(4): 628-35.
[28] Chavez MN, Aedo G, Fierro FA, Allende ML, Egana JT. Zebrafish as an emerging model organism to study angiogenesis in development and regeneration. Front Physiol 2016; 7: 56.
[29] Dahm R, Geisler R. Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Mar Biotechnol (NY) 2006; 8(4): 329-45.
[30] Strahle U, Scholz S, Geisler R, Greiner P, Hollert H, Rastegar S*, et al.* Zebrafish embryos as an alternative to animal experiments--a commentary on the definition of the onset of protected life stages in animal welfare regulations. Reprod Toxicol 2012; 33(2): 128-32.
[31] Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M*, et al.* The zebrafish reference genome sequence and its relationship to the human genome. Nature 2013; 496(7446): 498-503.
[32] Wilkinson RN, van Eeden FJ. The zebrafish as a model of vascular development and disease. Prog Mol Biol Transl Sci 2014; 124: 93-122.
[33] Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev Genet 2007; 8(5): 353-67.
[34] Ribatti D, Nico B, Crivellato E. The development of the vascular system: a historical overview. Methods Mol Biol 2015; 1214: 1-14.
[35] Richardson MK. Heterochrony and the phylotypic period. Dev Biol 1995; 172(2): 412-21.
[36] Schmidt A, Brixius K, Bloch W. Endothelial precursor cell migration during vasculogenesis. Circ Res 2007; 101(2): 125-36.
[37] Gore AV, Monzo K, Cha YR, Pan W, Weinstein BM. Vascular development in the zebrafish. Cold Spring Harb Perspect Med 2012; 2(5): a006684.
[38] Ribatti D. The discovery of endothelial progenitor cells. An historical review. Leuk Res 2007; 31(4): 439-44.
[39] Risau W. Mechanisms of angiogenesis. Nature 1997; 386(6626): 671-4.
[40] Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol 2007; 8(6): 464-78.
[41] Lu P, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harb Perspect Biol 2011; 3(12).
[42] Mentzer SJ, Konerding MA. Intussusceptive angiogenesis: expansion and remodeling of microvascular networks. Angiogenesis 2014; 17(3): 499-509.
[43] Marcelo KL, Goldie LC, Hirschi KK. Regulation of endothelial cell differentiation and specification. Circ Res 2013; 112(9): 1272-87.
[44] Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature 2000; 407(6801): 242-8.

[45] Murakami M, Simons M. Fibroblast growth factor regulation of neovascularization. Curr Opin Hematol 2008; 15(3): 215-20.
[46] Cox CM, Poole TJ. Angioblast differentiation is influenced by the local environment: FGF-2 induces angioblasts and patterns vessel formation in the quail embryo. Dev Dyn 2000; 218(2): 371-82.
[47] Nicoli S, De Sena G, Presta M. Fibroblast growth factor 2-induced angiogenesis in zebrafish: the zebrafish yolk membrane (ZFYM) angiogenesis assay. J Cell Mol Med 2009; 13(8B): 2061-8.
[48] Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, Zwijsen A. Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. Development 1999; 126(8): 1631-42.
[49] Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature 2011; 473(7347): 298-307.
[50] Ferrara N. VEGF-A: a critical regulator of blood vessel growth. Eur Cytokine Netw 2009; 20(4): 158-63.
[51] Phng LK, Gerhardt H. Angiogenesis: a team effort coordinated by notch. Dev Cell 2009; 16(2): 196-208.
[52] Newell LF, Holtan SG. Placental growth factor: what hematologists need to know. Blood Rev 2016.
[53] Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D*, et al.* Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat Med 2003; 9(7): 936-43.
[54] Schmidt T, Carmeliet P. Angiogenesis: a target in solid tumors, also in leukemia? Hematology Am Soc Hematol Educ Program 2011; 2011: 1-8.
[55] Zadeh G, Koushan K, Pillo L, Shannon P, Guha A. Role of Ang1 and its interaction with VEGF-A in astrocytomas. J Neuropathol Exp Neurol 2004; 63(9): 978-89.
[56] Kim J, Wu Q, Zhang Y, Wiens KM, Huang Y, Rubin N*, et al.* PDGF signaling is required for epicardial function and blood vessel formation in regenerating zebrafish hearts. Proc Natl Acad Sci U S A 2010; 107(40): 17206-10.
[57] Nguyen HL, Lee YJ, Shin J, Lee E, Park SO, McCarty JH*, et al.* TGF-beta signaling in endothelial cells, but not neuroepithelial cells, is essential for cerebral vascular development. Lab Invest 2011; 91(11): 1554-63.
[58] Park C, Kim TM, Malik AB. Transcriptional regulation of endothelial cell and vascular development. Circ Res 2013; 112(10): 1380-400.
[59] Krock BL, Skuli N, Simon MC. Hypoxia-induced angiogenesis: good and evil. Genes Cancer 2011; 2(12): 1117-33.
[60] Vestweber D. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. Arterioscler Thromb Vasc Biol 2008; 28(2): 223-32.
[61] Parker LH, Schmidt M, Jin SW, Gray AM, Beis D, Pham T*, et al.* The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation. Nature 2004; 428(6984): 754-8.
[62] Hellstrom M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P*, et al.* Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature 2007; 445(7129): 776-80.
[63] Birbrair A, Zhang T, Wang ZM, Messi ML, Mintz A, Delbono O. Pericytes at the intersection between tissue regeneration and pathology. Clin Sci (Lond) 2015; 128(2): 81-93.

[64] Campbell GR, Campbell JH. Development of the vessel wall: overview. In: Mecham RP, Eds. The Vascular Smooth Muscle Cell. ed, San Diego, Academic Press 1995, pp. 1-15.
[65] Schmidt T, Carmeliet P. Blood-vessel formation: bridges that guide and unite. Nature 2010; 465(7299): 697-9.
[66] Adair TH, Montani JP. Chapter 1. Overview of Angiogenesis. In: Eds. Angiogenesis. ed, San Rafael (CA), 2010.
[67] Eble JA, Niland S. The extracellular matrix of blood vessels. Curr Pharm Des 2009; 15(12): 1385-400.
[68] Gattazzo F, Urciuolo A, Bonaldo P. Extracellular matrix: a dynamic microenvironment for stem cell niche. Biochim Biophys Acta 2014; 1840(8): 2506-19.
[69] Davis GE, Senger DR. Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ Res 2005; 97(11): 1093-107.
[70] Davies PF. Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. Nat Clin Pract Cardiovasc Med 2009; 6(1): 16-26.
[71] Zhang H, Sunnarborg SW, McNaughton KK, Johns TG, Lee DC, Faber JE. Heparin-binding epidermal growth factor-like growth factor signaling in flow-induced arterial remodeling. Circ Res 2008; 102(10): 1275-85.
[72] Rashdan NA, Lloyd PG. Fluid shear stress upregulates placental growth factor in the vessel wall via NADPH oxidase 4. Am J Physiol Heart Circ Physiol 2015; 309(10): H1655-66.
[73] Jahnsen ED, Trindade A, Zaun HC, Lehoux S, Duarte A, Jones EA. Notch1 is pan-endothelial at the onset of flow and regulated by flow. PLoS One 2015; 10(4): e0122622.
[74] Topper JN, Cai J, Qiu Y, Anderson KR, Xu YY, Deeds JD*, et al.* Vascular MADs: two novel MAD-related genes selectively inducible by flow in human vascular endothelium. Proc Natl Acad Sci U S A 1997; 94(17): 9314-9.
[75] Hasan A, Paul A, Vrana NE, Zhao X, Memic A, Hwang YS*, et al.* Microfluidic techniques for development of 3D vascularized tissue. Biomaterials 2014; 35(26): 7308-25.
[76] Sun X, Altalhi W, Nunes SS. Vascularization strategies of engineered tissues and their application in cardiac regeneration. Adv Drug Deliv Rev 2016; 96: 183-94.
[77] Chen Y, Zhang Y, Deng Q, Shan N, Peng W, Luo X*, et al.* Inhibition of Wnt Inhibitory Factor 1 Under Hypoxic Condition in Human Umbilical Vein Endothelial Cells Promoted Angiogenesis *in Vitro*. Reprod Sci 2016; 23(10): 1348-58.
[78] Rohringer S, Hofbauer P, Schneider KH, Husa AM, Feichtinger G, Peterbauer-Scherb A*, et al.* Mechanisms of vasculogenesis in 3D fibrin matrices mediated by the interaction of adipose-derived stem cells and endothelial cells. Angiogenesis 2014; 17(4): 921-33.
[79] Kim S, Lee H, Chung M, Jeon NL. Engineering of functional, perfusable 3D microvascular networks on a chip. Lab Chip 2013; 13(8): 1489-500.
[80] Nguyen DH, Stapleton SC, Yang MT, Cha SS, Choi CK, Galie PA*, et al.* Biomimetic model to reconstitute angiogenic sprouting morphogenesis *in vitro*. Proc Natl Acad Sci U S A 2013; 110(17): 6712-7.
[81] Li H, Chang J. Bioactive silicate materials stimulate angiogenesis in fibroblast and endothelial cell co-culture system through paracrine effect. Acta Biomaterialia 2013; 9(6): 6981-6991.

[82] Zheng Y, Chen J, Craven M, Choi NW, Totorica S, Diaz-Santana A*, et al. In vitro* microvessels for the study of angiogenesis and thrombosis. Proc Natl Acad Sci U S A 2012; 109(24): 9342-7.
[83] Nakatsu MN, Sainson RC, Aoto JN, Taylor KL, Aitkenhead M, Perez-del-Pulgar S*, et al.* Angiogenic sprouting and capillary lumen formation modeled by human umbilical vein endothelial cells (HUVEC) in fibrin gels: the role of fibroblasts and Angiopoietin-1. Microvasc Res 2003; 66(2): 102-12.
[84] Stamati K, Priestley JV, Mudera V, Cheema U. Laminin promotes vascular network formation in 3D *in vitro* collagen scaffolds by regulating VEGF uptake. Exp Cell Res 2014; 327(1): 68-77.
[85] Boyd NL, Nunes SS, Krishnan L, Jokinen JD, Ramakrishnan VM, Bugg AR*, et al.* Dissecting the role of human embryonic stem cell-derived mesenchymal cells in human umbilical vein endothelial cell network stabilization in three-dimensional environments. Tissue Eng Part A 2013; 19(1-2): 211-23.
[86] Rao RR, Peterson AW, Ceccarelli J, Putnam AJ, Stegemann JP. Matrix composition regulates three-dimensional network formation by endothelial cells and mesenchymal stem cells in collagen/fibrin materials. Angiogenesis 2012; 15(2): 253-64.
[87] Zhang B, Montgomery M, Chamberlain MD, Ogawa S, Korolj A, Pahnke A*, et al.* Biodegradable scaffold with built-in vasculature for organ-on-a-chip engineering and direct surgical anastomosis. Nat Mater 2016; 15(6): 669-78.
[88] Delgado VM, Nugnes LG, Colombo LL, Troncoso MF, Fernandez MM, Malchiodi EL*, et al.* Modulation of endothelial cell migration and angiogenesis: a novel function for the "tandem-repeat" lectin galectin-8. FASEB J 2011; 25(1): 242-54.
[89] Zhu J, Cui W, Liu X, Ying J, Hu C, Zhang Y. *In vitro* and *ex vivo* angiogenic effects of roxarsone on rat endothelial cells. Toxicol Lett 2013; 223(2): 175-82.
[90] Geevarghese A, Herman IM. Pericyte-endothelial crosstalk: implications and opportunities for advanced cellular therapies. Transl Res 2014; 163(4): 296-306.
[91] Sudo R, Chung S, Zervantonakis IK, Vickerman V, Toshimitsu Y, Griffith LG*, et al.* Transport-mediated angiogenesis in 3D epithelial coculture. FASEB J 2009; 23(7): 2155-64.
[92] Ucuzian AA, Bufalino DV, Pang Y, Greisler HP. Angiogenic endothelial cell invasion into fibrin is stimulated by proliferating smooth muscle cells. Microvasc Res 2013; 90: 40-7.
[93] Costa-Almeida R, Gomez-Lazaro M, Ramalho C, Granja PL, Soares R, Guerreiro SG. Fibroblast-endothelial partners for vascularization strategies in tissue engineering. Tissue Eng Part A 2015; 21(5-6): 1055-65.
[94] Newman AC, Nakatsu MN, Chou W, Gershon PD, Hughes CC. The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. Mol Biol Cell 2011; 22(20): 3791-800.
[95] Jeon JS, Bersini S, Gilardi M, Dubini G, Charest JL, Moretti M*, et al.* Human 3D vascularized organotypic microfluidic assays to study breast cancer cell extravasation. Proc Natl Acad Sci U S A 2015; 112(1): 214-9.
[96] Wood LB, Ge R, Kamm RD, Asada HH. Nascent vessel elongation rate is inversely related to diameter in *in vitro* angiogenesis. Integr Biol (Camb) 2012; 4(9): 1081-9.
[97] Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol 2005; 23(1): 47-55.

[98] Lesman A, Habib M, Caspi O, Gepstein A, Arbel G, Levenberg S*, et al.* Transplantation of a tissue-engineered human vascularized cardiac muscle. Tissue Eng Part A 2010; 16(1): 115-25.
[99] Allen P, Melero-Martin J, Bischoff J. Type I collagen, fibrin and PuraMatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks. J Tissue Eng Regen Med 2011; 5(4): e74-86.
[100] Zanotelli MR, Ardalani H, Zhang J, Hou Z, Nguyen EH, Swanson S*, et al.* Stable engineered vascular networks from human induced pluripotent stem cell-derived endothelial cells cultured in synthetic hydrogels. Acta Biomater 2016; 35: 32-41.
[101] Shin Y, Jeon JS, Han S, Jung GS, Shin S, Lee SH*, et al. In vitro* 3D collective sprouting angiogenesis under orchestrated ANG-1 and VEGF gradients. Lab Chip 2011; 11(13): 2175-81.
[102] Zhao D, Xue C, Lin S, Shi S, Li Q, Liu M*, et al.* Notch signaling pathway regulates angiogenesis via endothelial cell in 3D co-culture model. J Cell Physiol 2016.
[103] Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N. Angiogenesis assays: a critical overview. Clin Chem 2003; 49(1): 32-40.
[104] Fernandez CE, Achneck HE, Reichert WM, Truskey GA. Biological and engineering design considerations for vascular tissue engineered blood vessels (TEBVs). Curr Opin Chem Eng 2014; 3: 83-90.
[105] Sieveking DP, Buckle A, Celermajer DS, Ng MK. Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. J Am Coll Cardiol 2008; 51(6): 660-8.
[106] Stewart EA, Samaranayake GJ, Browning AC, Hopkinson A, Amoaku WM. Comparison of choroidal and retinal endothelial cells: characteristics and response to VEGF isoforms and anti-VEGF treatments. Exp Eye Res 2011; 93(5): 761-6.
[107] Staton CA, Reed MW, Brown NJ. A critical analysis of current *in vitro* and *in vivo* angiogenesis assays. Int J Exp Pathol 2009; 90(3): 195-221.
[108] Hammoud L, Adams JR, Loch AJ, Marcellus RC, Uehling DE, Aman A*, et al.* Identification of RSK and TTK as modulators of blood vessel morphogenesis using an embryonic stem cell-based vascular differentiation assay. Stem Cell Reports 2016; 7(4): 787-801.
[109] Kusuma S, Gerecht S. Recent progress in the use of induced pluripotent stem cells in vascular regeneration. Expert Rev Cardiovasc Ther 2013; 11(6): 661-3.
[110] Tancharoen W, Aungsuchawan S, Pothacharoen P, Markmee R, Narakornsak S, Kieodee J*, et al.* Differentiation of mesenchymal stem cells from human amniotic fluid to vascular endothelial cells. Acta Histochem 2016.
[111] Kusuma S, Shen YI, Hanjaya-Putra D, Mali P, Cheng L, Gerecht S. Self-organized vascular networks from human pluripotent stem cells in a synthetic matrix. Proc Natl Acad Sci U S A 2013; 110(31): 12601-6.
[112] Tan KS, Tamura K, Lai MI, Veerakumarasivam A, Nakanishi Y, Ogawa M*, et al.* Molecular pathways governing development of vascular endothelial cells from ES/iPS cells. Stem Cell Rev 2013; 9(5): 586-98.
[113] Noghero A, Bussolino F, Gualandris A. Role of the microenvironment in the specification of endothelial progenitors derived from embryonic stem cells. Microvasc Res 2010; 79(3): 178-83.
[114] Kurosawa H. Methods for inducing embryoid body formation: *in vitro* differentiation system of embryonic stem cells. J Biosci Bioeng 2007; 103(5): 389-98.

[115] Jakobsson L, Kreuger J, Claesson-Welsh L. Building blood vessels--stem cell models in vascular biology. J Cell Biol 2007; 177(5): 751-5.
[116] Bratt-Leal AM, Carpenedo RL, McDevitt TC. Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. Biotechnol Prog 2009; 25(1): 43-51.
[117] Glaser DE, Turner WS, Madfis N, Wong L, Zamora J, White N, *et al.* Multifactorial optimizations for directing endothelial fate from stem cells. PLoS One 2016; 11(12): e0166663.
[118] Ibrahim M, Richardson MK. The growth of endothelial-like cells in zebrafish embryoid body culture. 2017: in prep.
[119] Wang Y, Qian DJ, Zhong WY, Lu JH, Guo XK, Cao YL, *et al.* TGF-beta1 induces the formation of vascular-like structures in embryoid bodies derived from human embryonic stem cells. Exp Ther Med 2014; 8(1): 52-58.
[120] Koh S, Piedrahita JA. From "ES-like" cells to induced pluripotent stem cells: a historical perspective in domestic animals. Theriogenology 2014; 81(1): 103-11.
[121] Taura D, Sone M, Homma K, Oyamada N, Takahashi K, Tamura N, *et al.* Induction and isolation of vascular cells from human induced pluripotent stem cells--brief report. Arterioscler Thromb Vasc Biol 2009; 29(7): 1100-3.
[122] Eman RM, Meijer HA, Oner FC, Dhert WJ, Alblas J. Establishment of an early vascular network promotes the formation of ectopic bone. Tissue Eng Part A 2016; 22(3-4): 253-62.
[123] Duttenhoefer F, Lara de Freitas R, Meury T, Loibl M, Benneker LM, Richards RG, *et al.* 3D scaffolds co-seeded with human endothelial progenitor and mesenchymal stem cells: evidence of prevascularisation within 7 days. Eur Cell Mater 2013; 26: 49-64; discussion 64-5.
[124] Glaser DE, Burns AB, Hatano R, Medrzycki M, Fan Y, McCloskey KE. Specialized mouse embryonic stem cells for studying vascular development. Stem Cells Cloning 2014; 7: 79-88.
[125] Hermant B, Desroches-Castan A, Dubessay ML, Prandini MH, Huber P, Vittet D. Development of a one-step embryonic stem cell-based assay for the screening of sprouting angiogenesis. BMC Biotechnol 2007; 7: 20.
[126] Li J, Stuhlmann H. *In vitro* imaging of angiogenesis using embryonic stem cell-derived endothelial cells. Stem Cells Dev 2012; 21(2): 331-42.
[127] Jakobsson L, Franco CA, Bentley K, Collins RT, Ponsioen B, Aspalter IM, *et al.* Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. Nat Cell Biol 2010; 12(10): 943-53.
[128] Feraud O, Prandini MH, Vittet D. Vasculogenesis and angiogenesis from *in vitro* differentiation of mouse embryonic stem cells. Methods Enzymol 2003; 365: 214-28.
[129] Boyd NL, Dhara SK, Rekaya R, Godbey EA, Hasneen K, Rao RR, *et al.* BMP4 promotes formation of primitive vascular networks in human embryonic stem cell-derived embryoid bodies. Exp Biol Med (Maywood) 2007; 232(6): 833-43.
[130] Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R. Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2002; 99(7): 4391-6.
[131] Fernandez-Alonso R, Martin-Lopez M, Gonzalez-Cano L, Garcia S, Castrillo F, Diez-Prieto I, *et al.* p73 is required for endothelial cell differentiation, migration and the formation of vascular networks regulating VEGF and TGFbeta signaling. Cell Death Differ 2015; 22(8): 1287-99.

[132] Kawamoto T, Kobayashi Y, Nakajima H, Yamagishi Y. Generation of robust vascular networks from cardiovascular blast populations derived from human induced pluripotent stem cells *in vivo* and *ex vivo* organ culture system. Biochem Biophys Res Commun 2013; 441(1): 180-5.
[133] Zhang P, Baxter J, Vinod K, Tulenko TN, Di Muzio PJ. Endothelial differentiation of amniotic fluid-derived stem cells: synergism of biochemical and shear force stimuli. Stem Cells Dev 2009; 18(9): 1299-308.
[134] Wang S, Mundada L, Johnson S, Wong J, Witt R, Ohye RG*, et al.* Characterization and angiogenic potential of human neonatal and infant thymus mesenchymal stromal cells. Stem Cells Transl Med 2015; 4(4): 339-50.
[135] Keller G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev 2005; 19(10): 1129-55.
[136] Yamanaka S. Induced pluripotent stem cells: past, present, and future. Cell Stem Cell 2012; 10(6): 678-84.
[137] Lin L, Bolund L, Luo Y. Towards Personalized Regenerative Cell Therapy: Mesenchymal Stem Cells Derived from Human Induced Pluripotent Stem Cells. Curr Stem Cell Res Ther 2016; 11(2): 122-30.
[138] Watt SM, Gullo F, van der Garde M, Markeson D, Camicia R, Khoo CP*, et al.* The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. Br Med Bull 2013; 108: 25-53.
[139] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T*, et al.* Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275(5302): 964-7.
[140] Yoder MC. Differentiation of pluripotent stem cells into endothelial cells. Curr Opin Hematol 2015; 22(3): 252-7.
[141] Bertelsen LB, Bohn AB, Smith M, Molgaard B, Moller B, Stodkilde-Jorgensen H*, et al.* Are endothelial outgrowth cells a potential source for future re-vascularization therapy? Exp Gerontol 2014; 58: 132-8.
[142] Kruger EA, Duray PH, Price DK, Pluda JM, Figg WD. Approaches to preclinical screening of antiangiogenic agents. Semin Oncol 2001; 28(6): 570-6.
[143] Baker M, Robinson SD, Lechertier T, Barber PR, Tavora B, D'Amico G*, et al.* Use of the mouse aortic ring assay to study angiogenesis. Nat Protoc 2011; 7(1): 89-104.
[144] Song W, Fhu CW, Ang KH, Liu CH, Johari NA, Lio D*, et al.* The fetal mouse metatarsal bone explant as a model of angiogenesis. Nat Protoc 2015; 10(10): 1459-73.
[145] Rezzola S, Belleri M, Gariano G, Ribatti D, Costagliola C, Semeraro F*, et al. In vitro* and *ex vivo* retina angiogenesis assays. Angiogenesis 2014; 17(3): 429-42.
[146] Shao Z, Friedlander M, Hurst CG, Cui Z, Pei DT, Evans LP*, et al.* Choroid sprouting assay: an *ex vivo* model of microvascular angiogenesis. PLoS One 2013; 8(7): e69552.
[147] Rojas-Rodriguez R, Gealekman O, Kruse ME, Rosenthal B, Rao K, Min S*, et al.* Adipose tissue angiogenesis assay. Methods Enzymol 2014; 537: 75-91.
[148] Auerbach R, Muthukkaruppan V. The chick embryo aortic arch assay. In: Zudaire E and F Cuttitta, Eds. The Textbook of Angiogenesis and Lymphangiogenesis: Methods and Applications. ed, Dordrecht, Springer Netherlands 2012, pp. 149-157.
[149] Chen CH, Cartwright J, Jr., Li Z, Lou S, Nguyen HH, Gotto AM, Jr.*, et al.* Inhibitory effects of hypercholesterolemia and ox-LDL on angiogenesis-like endothelial growth in rabbit aortic explants. Essential role of basic fibroblast growth factor. Arterioscler Thromb Vasc Biol 1997; 17(7): 1303-12.

[150] Stiffey-Wilusz J, Boice JA, Ronan J, Fletcher AM, Anderson MS. An *ex vivo* angiogenesis assay utilizing commercial porcine carotid artery: modification of the rat aortic ring assay. Angiogenesis 2001; 4(1): 3-9.
[151] Alessandri G, Girelli M, Taccagni G, Colombo A, Nicosia R, Caruso A*, et al.* Human vasculogenesis *ex vivo*: embryonal aorta as a tool for isolation of endothelial cell progenitors. Lab Invest 2001; 81(6): 875-85.
[152] Williams PJ, Bulmer JN, Innes BA, Broughton Pipkin F. Possible roles for folic acid in the regulation of trophoblast invasion and placental development in normal early human pregnancy. Biol Reprod 2011; 84(6): 1148-53.
[153] Jung SP, Siegrist B, Wade MR, Anthony CT, Woltering EA. Inhibition of human angiogenesis with heparin and hydrocortisone. Angiogenesis 2001; 4(3): 175-86.
[154] Seano G, Chiaverina G, Gagliardi PA, di Blasio L, Sessa R, Bussolino F*, et al.* Modeling human tumor angiogenesis in a three-dimensional culture system. Blood 2013; 121(21): e129-37.
[155] Ibrahim M, Richardson MK. *In vitro* development of zebrafish vascular networks. Reprod Toxicol 2017: DOI: 10.1016/j.reprotox.2017.02.008.
[156] Nicosia RF. The aortic ring model of angiogenesis: a quarter century of search and discovery. J Cell Mol Med 2009; 13(10): 4113-36.
[157] Irvin MW, Zijlstra A, Wikswo JP, Pozzi A. Techniques and assays for the study of angiogenesis. Exp Biol Med (Maywood) 2014; 239(11): 1476-88.
[158] Zhu WH, Iurlaro M, MacIntyre A, Fogel E, Nicosia RF. The mouse aorta model: influence of genetic background and aging on bFGF- and VEGF-induced angiogenic sprouting. Angiogenesis 2003; 6(3): 193-9.
[159] Nicosia RF, Zhu WH, Fogel E, Howson KM, Aplin AC. A new *ex vivo* model to study venous angiogenesis and arterio-venous anastomosis formation. J Vasc Res 2005; 42(2): 111-9.
[160] Reed MJ, Karres N, Eyman D, Vernon RB. Culture of murine aortic explants in 3-dimensional extracellular matrix: a novel, miniaturized assay of angiogenesis *in vitro*. Microvasc Res 2007; 73(3): 248-52.
[161] Li Q, Olsen BR. Increased angiogenic response in aortic explants of collagen XVIII/endostatin-null mice. Am J Pathol 2004; 165(2): 415-24.
[162] Mikirova NA, Casciari JJ, Riordan NH. Ascorbate inhibition of angiogenesis in aortic rings *ex vivo* and subcutaneous Matrigel plugs *in vivo*. J Angiogenes Res 2010; 2: 2.
[163] Kiziltepe T, Anderson KC, Kutok JL, Jia L, Boucher KM, Saavedra JE*, et al.* JS-K has potent anti-angiogenic activity *in vitro* and inhibits tumour angiogenesis in a multiple myeloma model *in vivo*. J Pharm Pharmacol 2010; 62(1): 145-51.
[164] Reed MJ, Damodarasamy M, Vernon RB. Angiogenesis *in vitro* utilizing murine vascular explants in miniaturized 3-dimensional collagen gels. Open Circ Vasc J 2011; 4: 12-17.
[165] Sawamiphak S, Ritter M, Acker-Palmer A. Preparation of retinal explant cultures to study *ex vivo* tip endothelial cell responses. Nat Protoc 2010; 5(10): 1659-65.
[166] Rezzola S, Belleri M, Ribatti D, Costagliola C, Presta M, Semeraro F. A novel *ex vivo* murine retina angiogenesis (EMRA) assay. Exp Eye Res 2013; 112: 51-6.
[167] Liu Z, Lebrin F, Maring JA, van den Driesche S, van der Brink S, van Dinther M*, et al.* ENDOGLIN is dispensable for vasculogenesis, but required for vascular endothelial growth factor-induced angiogenesis. PLoS One 2014; 9(1): e86273.
[168] Niderla-Bielinska J, Ciszek B, Jankowska-Steifer E, Flaht-Zabost A, Gula G, Radomska-Lesniewska DM*, et al.* Mouse proepicardium exhibits a sprouting

response to exogenous proangiogenic growth factors *in vitro*. J Vasc Res 2016; 53(1-2): 83-93.
[169] Spence R, Gerlach G, Lawrence C, Smith C. The behaviour and ecology of the zebrafish, *Danio rerio*. Biol Rev Camb Philos Soc 2008; 83(1): 13-34.
[170] Hasso S, Chan J. Chemical approaches to angiogenesis in development and regeneration. Methods Cell Biol 2011; 101: 181-95.
[171] Ali S, Champagne DL, Spaink HP, Richardson MK. Zebrafish embryos and larvae: a new generation of disease models and drug screens. Birth Defects Res C Embryo Today 2011; 93(2): 115-33.
[172] Schuermann A, Helker CS, Herzog W. Angiogenesis in zebrafish. Semin Cell Dev Biol 2014; 31: 106-14.
[173] Ellertsdottir E, Lenard A, Blum Y, Krudewig A, Herwig L, Affolter M*, et al.* Vascular morphogenesis in the zebrafish embryo. Dev Biol 2010; 341(1): 56-65.
[174] Wu F, Song H, Zhang Y, Zhang Y, Mu Q, Jiang M*, et al.* Irisin induces angiogenesis in human umbilical vein endothelial cells *in Vitro* and in zebrafish embryos *in vivo* via activation of the ERK signaling pathway. PLoS One 2015; 10(8): e0134662.
[175] Peterson RT, Shaw SY, Peterson TA, Milan DJ, Zhong TP, Schreiber SL*, et al.* Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation. Nat Biotechnol 2004; 22(5): 595-9.
[176] Konantz M, Balci TB, Hartwig UF, Dellaire G, Andre MC, Berman JN*, et al.* Zebrafish xenografts as a tool for *in vivo* studies on human cancer. Ann N Y Acad Sci 2012; 1266: 124-37.
[177] Haldi M, Ton C, Seng WL, McGrath P. Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish. Angiogenesis 2006; 9(3): 139-51.
[178] Nicoli S, Presta M. The zebrafish/tumor xenograft angiogenesis assay. Nat Protoc 2007; 2(11): 2918-23.
[179] Mercatali L, La Manna F, Groenewoud A, Casadei R, Recine F, Miserocchi G*, et al.* Development of a patient-derived xenograft (PDX) of breast cancer bone metastasis in a zebrafish model. Int J Mol Sci 2016; 17(8).
[180] van der Ent W, Burrello C, de Lange MJ, van der Velden PA, Jochemsen AG, Jager MJ*, et al.* Embryonic zebrafish: different phenotypes after injection of human uveal melanoma Cells. Ocul Oncol Pathol 2015; 1(3): 170-81.
[181] Chen X, Wang J, Cao Z, Hosaka K, Jensen L, Yang H*, et al.* Invasiveness and metastasis of retinoblastoma in an orthotopic zebrafish tumor model. Sci Rep 2015; 5: 10351.
[182] Poss KD, Keating MT, Nechiporuk A. Tales of regeneration in zebrafish. Dev Dyn 2003; 226(2): 202-10.
[183] Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. Science 2002; 298(5601): 2188-90.
[184] Shi W, Fang Z, Li L, Luo L. Using zebrafish as the model organism to understand organ regeneration. Sci China Life Sci 2015; 58(4): 343-51.
[185] Zhang XY, Rodaway AR. SCL-GFP transgenic zebrafish: *in vivo* imaging of blood and endothelial development and identification of the initial site of definitive hematopoiesis. Dev Biol 2007; 307(2): 179-94.
[186] Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Development 2005; 132(23): 5199-209.
[187] Tal T, Kilty C, Smith A, LaLone C, Kennedy B, Tennant A*, et al.* Screening for angiogenic inhibitors in zebrafish to evaluate a predictive model for developmental

vascular toxicity. Reprod Toxicol 2016: http://dx.doi.org/10.1016/j.reprotox.-2016.12.004.
[188] Motoike T, Loughna S, Perens E, Roman BL, Liao W, Chau TC*, et al.* Universal GFP reporter for the study of vascular development. Genesis 2000; 28(2): 75-81.
[189] Liu Z, Liu F. Cautious use of fli1a:EGFP transgenic zebrafish in vascular research. Biochem Biophys Res Commun 2012; 427(1): 223-6.
[190] Ciarlo CA, Zon LI. Chapter 1 - Embryonic cell culture in zebrafish. In: H. William Detrich MW and IZ Leonard, Eds. Methods in Cell Biology. ed, Academic Press 2016, pp. 1-10.
[191] Choorapoikayil S, Overvoorde J, den Hertog J. Deriving cell lines from zebrafish embryos and tumors. Zebrafish 2013; 10(3): 316-25.
[192] Henn K. Limits of fish embryo toxicity test with *Danio rerio* as an alternative to the acute fish toxicity test. PhD thesis, 2011. Center of Organismal Studies Heidelberg (COS), Ruperto-Carola University Heidelberg, Germany.
[193] Fan L, Collodi P. Zebrafish embryonic stem cells. Methods Enzymol 2006; 418: 64-77.
[194] Hong N, Schartl M, Hong Y. Derivation of stable zebrafish ES-like cells in feeder-free culture. Cell Tissue Res 2014; 357(3): 623-32.
[195] Alvarez MC, Bejar J, Chen S, Hong Y. Fish ES cells and applications to biotechnology. Mar Biotechnol (NY) 2007; 9(2): 117-27.
[196] Hong N, Li Z, Hong Y. Fish stem cell cultures. Int J Biol Sci 2011; 7(4): 392-402.
[197] Xing JG, Lee LEJ, Fan LC, Collodi P, Holt SE, Bols NC. Initiation of a zebrafish blastula cell line on rainbow trout stromal cells and subsequent development under feeder-free conditions into a cell line, ZEB2J. Zebrafish 2008; 5(1): 49-63.
[198] Myhre JL, Pilgrim DB. Cellular differentiation in primary cell cultures from single zebrafish embryos as a model for the study of myogenesis. Zebrafish 2010; 7(3): 255-66.
[199] Xu C, Tabebordbar M, Iovino S, Ciarlo C, Liu J, Castiglioni A*, et al.* A zebrafish embryo culture system defines factors that promote vertebrate myogenesis across species. Cell 2013; 155(4): 909-921.
[200] Norris W, Neyt C, Ingham PW, Currie PD. Slow muscle induction by Hedgehog signalling *in vitro*. J Cell Sci 2000; 113 (Pt 15): 2695-703.
[201] Xiao Y, Gao M, Gao L, Zhao Y, Hong Q, Li Z*, et al.* Directed differentiation of zebrafish pluripotent embryonic cells to functional cardiomyocytes. Stem Cell Reports 2016; 7(3): 370-82.
[202] Riesco MF, Valcarce DG, Alfonso J, Herraez MP, Robles V. *In vitro* generation of zebrafish PGC-like cells. Biol Reprod 2014; 91(5): 114.
[203] Huang HG, Lindgren A, Wu XR, Liu NA, Lin SO. High-throughput screening for bioactive molecules using primary cell culture of transgenic zebrafish embryos. Cell Rep 2012; 2(3): 695-704.
[204] Kinikoglu B, Kong Y, Liao EC. Characterization of cultured multipotent zebrafish neural crest cells. Exp Biol Med (Maywood) 2014; 239(2): 159-68.
[205] Chen Z, Lee H, Henle SJ, Cheever TR, Ekker SC, Henley JR. Primary neuron culture for nerve growth and axon guidance studies in zebrafish (*Danio rerio*). PLoS One 2013; 8(3): e57539.
[206] Kim S, Chung M, Ahn J, Lee S, Jeon NL. Interstitial flow regulates the angiogenic response and phenotype of endothelial cells in a 3D culture model. Lab Chip 2016; 16(21): 4189-4199.

[207] Chen MB, Whisler JA, Frose J, Yu C, Shin Y, Kamm RD. On-chip human microvasculature assay for visualization and quantification of tumor cell extravasation dynamics. Nat Protoc 2017; 12(5): 865-880.
[208] Sobrino A, Phan DT, Datta R, Wang X, Hachey SJ, Romero-Lopez M*, et al.* 3D microtumors *in vitro* supported by perfused vascular networks. Sci Rep 2016; 6: 31589.
[209] Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE. Reconstituting organ-level lung functions on a chip. Science 2010; 328(5986): 1662-8.
[210] Bhatia SN, Ingber DE. Microfluidic organs-on-chips. Nat Biotechnol 2014; 32(8): 760-72.

Chapter 3

Zebrafish blastocyst cell culture and differentiation of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells

Muhammad Ibrahim and Michael K. Richardson

Abstract

Different strategies have been developed to induce specific differentiation pathways in zebrafish embryonic cell culture. Our ultimate goal is to develop *in vitro* vascular networks using zebrafish embryonic cells. Therefore, we need to establish the basal, optimal conditions under which endothelial cells can be maintained in zebrafish embryonic cell culture. To achieve this aim, we describe here procedures for culture of zebrafish blastocyst cells and the quantification of putative endothelial cells in these cultures. To determine the basic nutrient requirements for zebrafish primary cells, we first cultured the blastocyst fragments (explants) in LDF medium (combination of Leibowitz's L-15, Dulbecco's modified Eagle's and Ham's F12 media) supplemented with different concentrations of heat-inactivated fetal bovine serum (FBS) or zebrafish embryo extract (ZEE). In the second experiment we analysed the development of putative endothelial cells (expressing green fluorescence protein), under basal culture conditions, in trypsinized blastocyst cell cultures derived from the transgenic zebrafish *fli:GFP* and *kdrl:GFP* lines. In the third experiment we analysed the characteristic and growth of blastocyst secondary cell cultures established by passaging primary cultures. The blastocyst explant cultures showed significant expansion in the area covered by cells in media supplemented with 15% FBS and 60 µg/mL ZEE. The trypsinized blastocyst cells formed embryoid body (EB) aggregates in culture which contained different cell types including *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells. The percentage of these GFP^{+} cells dropped in cultures after day 4, which is probably because of the thriving fibroblast-like cells emigrating from the EBs. These fibroblast-like cells were maintained for 8 passages with 12 population doublings. These cells showed a homogeneous morphology and higher growth rate compared to the primary blastocyst cells.

Introduction

In many vertebrates, embryonic stem cells (ESCs) are cells isolated from early embryos [1]. These ESCs may form, when cultured, embryoid bodies (EBs) containing endoderm and mesoderm cells which differentiate into different cell types including hematopoietic, endothelial, muscle and neural cells [2]. This differentiating property of ESCs can be used to study the differentiation and lineage commitment events in embryos [2]. Mouse ESCs have been extensively studied and used in various experiments [3]. Because of the their considerable potential, techniques for the culture of pluripotent ESCs are being developed in other mammals [4] including humans [5]. However, the use of these species in research is relatively expensive and raise ethical concerns [6]. As an alternative, the zebrafish can be used for ESC research because of it has advantages over mammalian cells as a culture system [7].

The manipulation of zebrafish is relatively simple and cell isolation procedures are well described [7]. Zebrafish are small, have a short generation time of about 2 to 3 months and one female produces about 200 eggs per week [8]. Zebrafish eggs are externally fertilized so there is no need to kill the mother for embryo isolation as in the mouse. Furthermore, zebrafish cells can be maintained at a lower temperature (26 °C) in atmospheric CO_2, in contrast to mammalian cells [7]. Being a relatively new research model, few cell cultures have been developed for zebrafish [9-17]. The methods used to culture zebrafish cells often vary between researchers. One of the important inconsistencies is the composition of the culture medium (Table 1). It will be important in this field to define a standard medium for efficient growth of zebrafish embryonic cell culture.

A commonly-used zebrafish cell culture medium is composed of LDF (combined Leibowitz's L-15, Dulbecco's modified Eagle's medium (DMEM) and Ham's F12) with added HEPES buffer (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), sodium bicarbonate, sodium pyruvate and antibiotics [18]. The medium is usually supplemented with fetal bovine serum (FBS), fish serum, fish embryo extract and basic fibroblast growth factor (bFGF) [7]. The concentrations of these ingredients, and the type of antibiotic used, may vary for different zebrafish cell culture experiments (Table 1). Primary cells from zebrafish embryos tend to require a nutrient-rich medium for their growth [7]. Therefore, the media are sometimes enriched with additional supplements and growth factors, including L-glutamine [19], bovine insulin [20], trout plasma, human epidermal growth factor (hEGF) [21] and murine epidermal growth factor (mEGF) [22].

When studying cellular differentiation in zebrafish ESC culture, transgenic zebrafish lines expressing green fluorescence protein (GFP) under *fli-1* [23] and *kdrl* [24, 25] promoters, may be used as a marker to observe real time differentiation of vascular progenitor cells.

Fli-1 (Friend leukemia virus integration site 1) is a transcription factor gene highly expressed in endothelial cells, hematopoietic cells and lymphoid tissue in mouse [26].

Table 1: Composition of various media used for zebrafish cell culture.

Culture	Units	Zebrafish cell type											
Constituents		EFB	EC	EC	EC	EC	SC	ESN	EMC	FB	EC	EFB	EC
Substrate		P	FC	P	FC	FC	P	L	L	P	PL	C1	G
Leibowitz's L-15	%	-	52	85	50	50	83	85.9	25	-	50	-	-
DMEM	%	45	21	-	35	35	-	-	25	85	35	84	85
Ham's F12	%	45	9	-	15	15	-	-	-	-	15	-	-
HBSS	%	-	-	-	-	-	-	-	50	-	-	-	-
HEPES	mM	15	15	-	-	15	-	4	15	-	15	-	20
Sodium bicarbonate	mg/mL	1.2	0.11	-	-	0.15	-	-	-	-	0.15	-	-
Sodium pyruvate	mM	0.5		-	-	-	-	-	12.5	-	-	-	1
Sodium selenite	nM	-	10	-	10	-	-	-	-	-	10	-	2
Calcium chloride	mM	-		0.8	-	-	-	0.16	0.8	-	-	-	-
2-mercaptoethanol	µM	-	-	-	-	-	-	-	-	-	-	-	100
Antibiotics													
Penicillin	µg/mL	-	72	50	-	120	-	-	60	60	120	-	-
Streptomycin	µg/mL	-	120	50	-	200	-	-	100	100	200	-	-
Ampicillin	µg/mL	-	15	-	-	25	-	-	-	-	25	25	-
Kanamycin	µg/mL	-	-	-	-	-	-	-	100	-	-	-	-
Gentamycin	mg/mL	-	-	10	-	-	-	-	0.05	-	-	-	-
Pen-strep	%	-	-	-	-	-	1	0.4	-	-	-	1	100#
Antimycotic mix*	%	-	-	-	-	-	1	-	-	-	-	-	-
Amphotericin	µg/mL	-	-	-	-	-	-	-	25	-	-	-	-
Serum	%												
FBS		10	14.5	15	5	5	15	2	10	15	1	15	15
Carp serum		-	-	-			-	-	5	-	-	-	-
Trout serum		-	-	-	0.4	1	-	-	-	-	0.5	1	1
Trout plasma	%	-	1	-	-	-	-	-	-	-	-	-	-
Embryo extract	µg/mL**												
Trout		-	-	-	50	50	-	-	-	-	40	-	-
Zebrafish		-	50	-	-	-	-	-	10	-	-	50	1##
Growth factors	ng/mL												
bFGF		-	50	-	50	50	-	-	-	-	-	50	10
hEGF		-	50	-	-	50	-	-	-	-	-	25	-
mEGF		-	-	-	50	-	-	-	-	-	-	-	-
l-glutamine	mM	2.5	-	-	-	-	-	-	2	3	-	-	2
Bovine insulin	µg/mL	-	10	-	10	10	-	-	5	-	10	10	-
NE amino acids	mM	-	-	-	-	-	-	-	-	-	-	-	1
Reference		[19]	[21]	[17]	[22]	[11]	[14]	[10]	[12]	[13]	[16]	[20]	[27]

Abbreviations: bFGF, basic fibroblast growth factor; C1, collagen type-I; DMEM, Dulbecco's modified Eagle's medium; EC, embryonic cells; EFB, embryonic fibroblasts; EMC, embryonic muscle cells; ESN, embryonic spinal neurons; FB, fibroblasts; FBS, fetal bovine serum; FC, growth arrested feeder cell line (RTS34st cells); HBSS, Hank's balanced salt solution; hEGF, human epidermal growth factor; HEPES, (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid); L, laminin; mEGF, murine epidermal growth factor; NE, non-essential; P, polystyrene; PL, poly-D-lysine; SC, spleen cells.
KEY: *, antimycotic mix from Gibco; **, concentration of embryo extracts means the amount of total proteins in the extract; #, concentration 100 U/mL; ##, concentration 1 embryo/mL; -, not added.

In zebrafish embryos *fli-1* is expressed during early vasculogenesis and the expression persists throughout vascular development [28]. The transgenic zebrafish (*fli:GFP*) has

been shown to express GFP in endothelial and hematopoietic cells, as well as in cells on the yolk sac and neural crest cells i.e. mesenchyme and jaw cartilages [23]. *Kdrl*, also known as *flk-1* or VEGFR2, is a vascular endothelial growth factor receptor, expressed particularly in endothelial cells during vascular development [25, 29].

Zebrafish embryos at the blastocyst stage of development (approximately 3.5 hours post fertilization or hpf) are usually dechorionated and trypsinized to isolate ESCs. Culturing blastocyst explants is equally important in studying cellular interactions, organization and lineage commitment in early embryos [30]. Embryo explant culture has also been applied to invertebrate embryos such as *Caenorhabditis elegans* [31] and the sea urchin [32]. However, in most of the procedures for ESC research, the embryos are dissociated (trypsinized) into single cells for culturing. These early embryonic cells are used to establish pluripotent ESC cultures which may be used for various applications e.g. genetic manipulation of cells and organisms and production of germ line chimeras [21]. By culturing these ESCs under differentiation conditions, specific cell lines can also be developed [12, 17].

In order to maintain the ESCs in a pluripotent, undifferentiated state, they may be grown on a feeder monolayer of growth-arrested cells [33]. Different feeder cell lines used to culture zebrafish embryonic cells include rainbow trout spleen cells (RTS34st) [11, 22], zebrafish spleen stromal cells (ZSSJ) [14], buffalo rat liver cells [34] and zebrafish embryonic fibroblasts [18]. For specific differentiation of ESCs, the feeder layer is replaced by coating the culture plate with adhesive molecules. Fibronectin, laminin, collagen and poly-D-lysine are the molecules known to promote attachment and differentiation of zebrafish cells [10, 12, 20]. Zebrafish embryonic cells have also been successfully cultured on polystyrene culture dishes without any feeder layer or substrate coating [17].

In this chapter we have optimized the culture conditions for zebrafish blastocyst cells in the following steps: (1) The optimization of FBS and zebrafish embryo extract (ZEE) concentrations in the media for the expansion of blastocyst explant cultures. (2) The quantification of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells under basic medium conditions, in the blastocyst cell culture developed from the transgenic zebrafish embryos. (3) The effect of different concentrations of FBS, ZEE and gelatin substratum on the growth and viability of blastocyst secondary cell cultures.

Materials and methods

Zebrafish rearing and mating setup for embryo isolation

All the animal experiments were performed according to the Netherland Experiments on Animals Act [35], based on guidelines laid by the Council of European Union [36]. Adult

zebrafish were maintained, as previously described [37], in 5 liter tanks having continuously circulating egg water ("Instant Ocean" sea salt 60 µg/mL demi water), on 14 h light: 10 h dark cycle. Temperature of the water and air was controlled at 26 and 23 °C, respectively. Wild type zebrafish embryos were used to characterize general growth of blastocyst cells. Transgenic *fli:GFP* and *kdrl:GFP* embryos were used to analyse differentiation of blastocyst cells into putative endothelial and hematopoietic cells. To obtain embryos, adult male and female fish, at a proportion of 1:1, were transferred to small breeding tanks in the evening. The zebrafish usually laid eggs when light turns on. The eggs were collected at the bottom of the tank, separated from adults using a cotton mesh to protect the eggs from being eaten.

Embryos were transferred to a temperature controlled room (28 °C) and were distributed in 9 cm Petri dishes at a final density of 100 embryos per dish, after removing dead and unfertilized eggs. The embryos were washed thoroughly with clean egg water to remove any debris.

Sterilization of embryos

Embryos were allowed to develop to the high blastula stage of Kimmel et al. [38] (approximately 3 hpf) at 28 °C. These embryos were then sterilized using 70% ethanol for 10 sec and 0.05% bleach solution (Table 2) for 2 × 5 min. Between the sterilization steps the embryos were washed with LDF medium (Table 2). The sterilization procedure described in Ref. [21] was followed. The embryos were finally left in 0.5 mL of LDF medium and any dead embryos or embryos with visible contamination inside the chorion were removed. These embryos were dechorionated using a pair of No. 5 watchmaker's forceps for the isolation of blastocyst cells.

Overview of experiments

The procedure of cell isolation and culture from embryos is shown in Figure 1. Three different experiments were performed (Figure 1). In the first experiment the wild type blastocysts were triturated gently with a P-200 Gilson micropipette (Gilson, B.V., Europe: Den Haag) without enzymatic digestion to form fragments (explants). These explants were cultured in LDF medium (Table 2) supplemented with different amounts of FBS (Invitrogen, 10500) and ZEE to optimize the concentration of these nutrients for zebrafish primary cell culture (see below, Experiment 1: Primary explant cultures). In the second experiment (see below, Experiment 2: Quantification of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures) trypsinized blastocyst cells isolated from *fli:GFP* and *kdrl:GFP* embryos were cultured to analyse the generation of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in these cultures. In the third experiment (see below, Experiment 3: Secondary blastocyst cell culture) the effect of FBS and ZEE supplementation and gelatin substratum was analysed on the growth and

viability of blastocyst secondary cell cultures, established after several passages from the primary cultures.

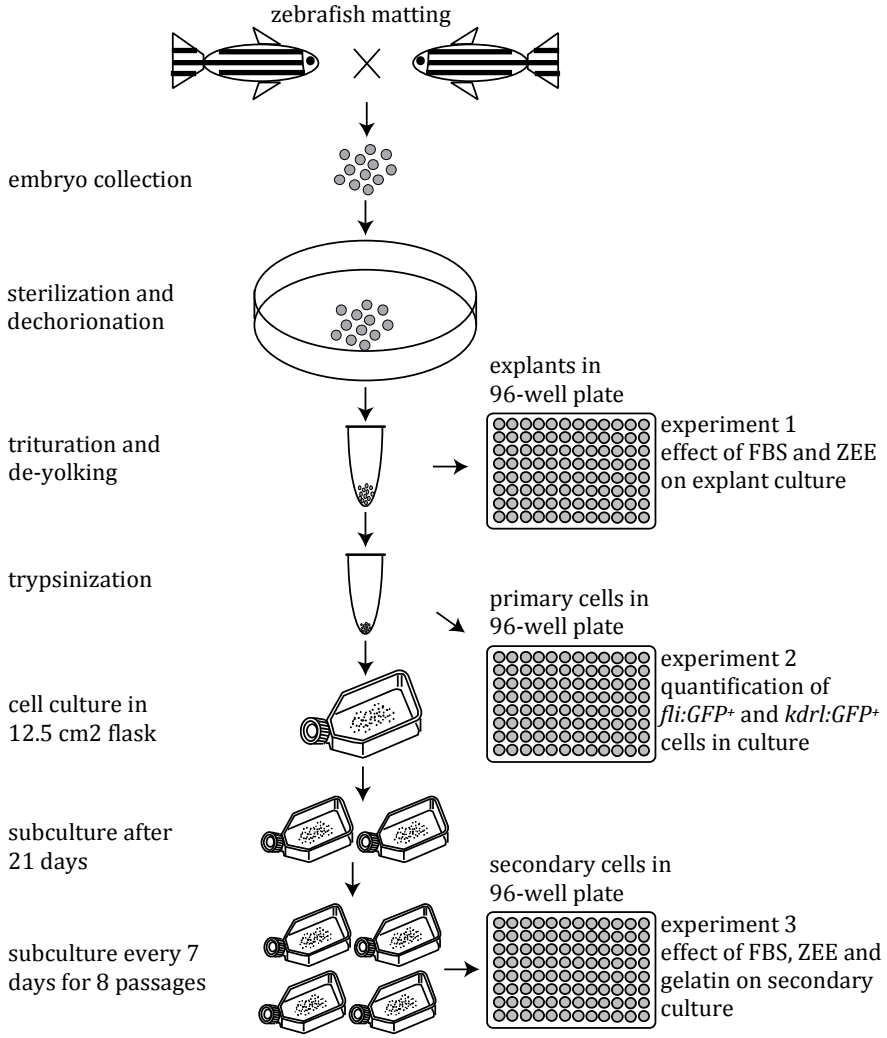

Figure 1: Schematic diagram of isolation and culture procedures of zebrafish blastocyst cells and the experiments performed.
Abbreviations: FBS, fetal bovine serum; ZEE, zebrafish embryo extract.

Medium preparation

A basic LDF medium was prepared as shown in Table 2. All of the solutions used in LDF medium were supplied from Invitrogen (Gibco). Media were reconstituted with different concentrations of FBS (5, 10, 15 and 20%) and ZEE (15, 30, 45 and 60 µg total protein/mL) in separate tubes, before adding into culture wells. FBS (10%) contained in all media having different concentrations of ZEE; similarly, ZEE (50 µg/mL) contained in

all media having different concentrations of FBS. Cells were cultured in each distinct medium in six wells (replicates) of the 96 well microplate. The FBS used in the experiments was qualified, heat inactivated, E.U.-approved, South America Origin from Invitrogen, catalog number 10500. ZEE was prepared using 36 hpf wild type zebrafish embryos using the following procedure.

Preparation of zebrafish embryo extract

The ZEE was prepared according to the procedure described in Ref. [21]. The 36 hpf wild type zebrafish embryos were washed thoroughly with egg water. About 500 embryos suspended in 0.5 mL LDF medium were transferred to a 2 mL Eppendorf tube having a 5 mm glass bead. The embryos were homogenized in a QIAGEN TissueLyser II at 30 oscillations/min for 1 min. After homogenization, 1 mL LDF was added to the embryo lysate. The tubes were then centrifuged at 20,000 g for 10 min at 4 °C. The supernatant was transferred to a new tube, and the total protein content of the extract was quantified using bovine serum albumin protein assay kit (Thermo scientific, product 23227). Finally, the extract was diluted to a final concentration of 1 mg total protein/mL, filter sterilized and stored in aliquots at -20 °C.

Experiment 1: Primary explant culture

The wild type embryos were dechorionated using sterile No. 5 watchmaker forceps and each set of 50 embryos was then transferred to a separate 1.5 mL sterile Eppendorf tube. The embryos were triturated using a P-200 Gilson micropipette, in the LDF medium, to fragmentize the blastocysts. The tubes were then centrifuged at 300 g for 2 min and the supernatant, including the yolk, was discarded. The blastocyst fragments (explants) were washed with CMF-PBS (calcium magnesium free – phosphate buffered saline) and then with LDF to remove any remaining yolk particles. Finally, the explants were re-suspended in 300 µL of LDF medium containing 10% FBS, and distributed in a 96-well plate such that, on average, the explants from 2 blastocysts were plated per well. Finally 200 µL LDF media, having varying concentrations of FBS and ZEE (as described above in Medium preparation) were added to the wells. The cultures were maintained in an incubator at 28 °C in atmospheric CO_2 until data collection.

Trypsinized blastocyst cell culture

The concentrations of FBS and ZEE in the medium optimized for explant culture in experiment 1 (above) was used to culture the completely dissociated (trypsinized) blastocyst cells. For this purpose, the embryos were sterilized, dechorionated and de-yolked as described above. Then, the blastocysts were given a rinse with CMF-PBS and incubated in 1 mL of 0.25% trypsin solution (Table 2) for 2 min. The trypsin-blastocysts solution was triturated several times using p-1000 Gilson micropipette, to facilitate

dissociation of cells. The trypsin was deactivated by adding 0.1 mL of FBS or 1 mL of trypsin inhibitor (Invitrogen, catalogue number R-007-100). The solution was centrifuged at 300 g for 3 min and the supernatant was discarded.

Table 2: Solutions and Media.

Reagent (supplier, catalog number)	Final Concentration
LDF medium	
Leibowitz's L-15 (Invitrogen, 11415) : DMEM (Invitrogen, 11966) : Ham's F12 (Invitrogen, 21765)	55 : 32.5 : 12.5
HEPES (Invitrogen, 15630)	15 mM
Antibiotic antimycotic mix (Invitrogen, 15240)	1%
$NaHCO_3$	0.015%
CMF-PBS (Invitrogen, 14190)	
KCl	2.7 mM
KH_2PO_4	1.5 mM
NaCl	138 mM
Na_2HPO_4-7H2O	8.06 mM
Trypsin solution for embryos	
Trypsin 2.5% (Invitrogen, 15090)	0.25%
CMF-PBS	99.75%
EDTA	1 mM
Trypsin solution for cultured cells	
Trypsin 10X (Invitrogen, 15400)	0.05%
CMF-PBS	99.95%
EDTA	1 mM
Bleach solution	
Sodium hypochlorite 10-15% (Sigma, 425044)	0.05%
Sterile distilled H_2O	99.95%
Gelatin solution	
Gelatin (Sigma, G1890)	2%
Sterile distilled H_2O	98%

Abbreviations: CMF-PBS, calcium magnesium free phosphate buffer saline; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

The cell pellet was washed three times with LDF medium containing 10% FBS and finally suspended in 300 µL of the same medium. The concertation of the cells in the isolates was determined by counting in a heamocytometer. The *fli:GFP* or *kdrl:GFP* blastocyst cells (for Experiment 2: Quantification of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in cultures, see below) were plated at a density of 17000 cells per well in a 96 well plate, with or without gelatin coating. Another batch of wild type blastocyst cells isolated using the same procedure was cultured in 12.5 cm^2 flasks (for Experiment 3: Blastocyst secondary cell culture, see below) at a density of 6×10^5 cells per flask for sub-culturing.

After allowing approximately 1 h for the cells to attach to the surface, 200 µL or 2 mL of LDF medium containing 15% FBS and 50 µg/mL ZEE was added per well of the 96 well plate or per 12.5 cm^2 flask, respectively. These cultures were maintained at 28 °C in 0.5% CO_2 until data collection or sub-culturing.

Media refreshment

To optimize the time for media refreshment, in a separate experiment, the zebrafish blastocyst explants were cultured without replacement of media. We changed to a refreshment interval of 4 days for the primary cultures when we observed a decrease in the surface area covered by the emigrating cells around the explants after day 4 in the non-replacement cultures. For the secondary cultures the medium was refreshed weekly.

Experiment 2: Quantification of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in culture

Two different cultures were established to analyse spontaneous differentiation in primary blastocyst cultures: one from the cells isolated from zebrafish *fli:GFP* embryos and the other from *kdrl:GFP* embryos. The cells cultured in the wells of 96 well plate, with or without gelatin coating, were isolated for the quantification of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ at successive time-points (days 2, 4, 6 and 8). The gelatin was used at a concentration of 0.1 mg/cm^2 (1.7 µL of 2% gelatin solution per well). For each of the above mentioned condition (i.e. transgene line, gelatin coating and time-points), the blastocyst cells cultured in six replicate wells were transferred to separate Eppendorf tubes (see below, Trypsinization of cultured cells). Each cells isolate was subjected to quantification of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ using the procedure described below (Data collection).

Experiment 3: Secondary blastocyst cell culture

The blastocyst cell culture in 12.5 cm^2 flasks was maintained for 21 days with media refreshment every four days. The cells were then isolated from the flask using 2mL of 0.05% trypsin solution (Trypsinization of cultured cells), and sub-cultured in a new flask in LDF medium containing 10% FBS. These secondary cells were then passaged every seven days. At least three 12.5 cm^2 flasks were platted, for the primary culture as wells as for the secondary cultures, to allow for statistical analysis of growth rates at successive passages. During transfer a small amount of cell isolate at each passage was used to determine the total number of cells harvested and the population doubling time (see below, Statistical analysis).

At passage eight the cells were isolated from the flask and distributed at 17000 cells per well in a 96-well plate (Figure 1). These cells were maintained in media supplemented with different concentrations of FBS (5, 10, 15 and 20%) or ZEE (15, 30, 45 and 60 µg/mL), or on different concentrations of gelatin substratum (see below, Gelatin coating of wells). The effect of FBS, ZEE and gelatin substratum was analysed by calculating the total number of cells harvested per well and the viability of the isolated cells on day 7 of culture (see below, Data collection).

Vimentin staining of secondary blastocyst cell culture

To identify possible fibroblasts, the blastocyst secondary cells at passage eight were stained with anti-vimentin antibody [39]. For this purpose, mouse anti-vimentin antibody, developed by Michael Klymkowsky and maintained by The University of Lowa, Department of Biology, was obtained from the Developmental Studies Hybridoma Bank. The culture was established in chambered coverglass plate for imaging. All of the following steps were performed at room temperature.

On day 7 of culture the medium was removed and the cells were washed twice with CMF-PBS. Then the cells were fixed for 20 min in 4% paraformaldehyde (PFA) solution. The fixing solution was removed and the cells were washed twice with washing buffer (0.1% bovine serum albumin or BSA in CMF-PBS). Then the cells were treated with blocking buffer (10% normal sheep serum, 1% BSA, 0.3% triton X-100 in CMF-PBS) for 45 min. The blocking buffer was removed and the cells were incubated for 3 h with primary anti-vimentin antibody at a concentration of 3 µg/mL in CMF-PBS containing 1% normal sheep serum, 1% BSA and 0.3% triton X-100. The cells were then washed with washing buffer and incubated for 1 h in 1 µg/mL of the secondary antibody (goat anti-mouse IgG Alexa Fluor 568) to visualize the stained cells. Finally, the cells were treated with 1 µg/mL of DAPI (4',6-diamidino-2-fenylindool) to stain the nuclei.

Gelatin coating of wells

The wells of a 96 well plate were coated with different concentrations (0.03, 0.06, 0.12, 0.24 and 0.48 mg/cm^2) of gelatin. Calculated volume of 2% gelatin solution (Table 2), to get the desired concentration of substratum, was added to the wells of a 96 well plate. Each concentration was replicated in six wells, in addition six wells were used as a control without gelatin coating. The plate was allowed to air dry at room temperature for one hour. The blastocyst secondary cells at passage eight were cultured in these wells in LDF medium containing 10% FBS and 50 µg/mL ZEE. The cells were isolated and counted on day 7 of culture (see below, Data collection).

Trypsinization of cultured cells

In order to isolate the cells from 96 well plate for counting or from 12.5 cm^2 flasks for sub-culturing, the medium was aspirated and the cells were washed with CMF-PBS. The cultures were then incubated in 0.05% trypsin solution (Table 2) at 28 °C. the solution was triturated several times using a p-1000 micropipette to detach remaining cells and to dissociate the cell clumps. The detachment of cells was observed under an inverted microscope. When the majority of cells were in the suspension (approximately after 2 min), the trypsin was inactivated by adding FBS to a final concentration of 10%. The suspensions were then transferred to separate Eppendorf tubes from each well or flask and centrifuged at 300 g for 3 min. The supernatant was discarded and the cells were

washed twice with LDF medium containing 10% FBS. Finally, the cells were re-suspended in the same medium for cell counting or re-plating.

Data collection

The blastocyst explant cultures were imaged using an inverted phase contrast light microscope, and the following measurements were made at each successive time-point (day 2, 4 and 6 of culture) with the software Image-J, version, 1.46r [40], using a pre-calibrated scale: (i) Area (mm^2) of initial explants (marked as 1 in Figure 2); (ii) area (mm^2) covered by the explant aggregates (marked as 2 in Figure 2); and (iii), the area (mm^2) covered by the flattening, fibroblast-like cells spreading at the periphery of the explants (marked as 3 in Figure 2). The percentages of growth in covered area was calculated as described in the section below (Statistical analysis).

The primary *fli:GFP* and *kdrl:GFP* blastocyst cell cultures were isolated from the 96 well plates at days 2, 4, 6 and 8 using 0.05% trypsin solution (see above, Trypsinization of cultured cells). The cell isolates from each well were transferred in a 5 µL droplet on a glass slide to the confocal microscope. Each drop was imaged with two channels; one showing GFP^+ cells and the other showing a phase contrast image of all the cells in the same field. For cell counting, both of the channels were combined, and the *fli:GFP*$^+$ or *kdrl:GFP*$^+$ and non-positive cells were counted in each image. From these counts the average percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells was calculated (Statistical analysis).

The secondary embryonic cells cultured in 96-well plate in different concentrations of FBS, ZEE or gelatin substratum were isolated and counted on day 7. In total six replicate wells were cultured for each condition. The cells were isolated from the wells using 0.05% trypsin solution (see above, Trypsinization of cultured cells). The total number of cells harvested per well was calculated using a heamocytometer. The viable cells were distinguished from non-viable cells using trypan blue dye exclusion (1:1 ratio of 0.4% trypan blue in CMF-PBS).

Statistical analysis

The percentage increase in the relative area of the blastocyst explant, at each successive time-point, compared to the area on day 0, and the area covered by the emigrating fibroblast-like cells as a percentage of the total covered area (explant + emigrating cells), was calculated from the above measurements (Data collection) using the following formulas (see Figure 2 for the number references with the equations).

Percent increase in area covered by cells in the explant culture:

(1) Area increased = $Total\ area\ covered\ by\ cells\ (2+3) - size\ of\ explant\ at\ day\ 0\ (1)$

(2) Percent increase in area = $\frac{Area\ increased}{Size\ of\ explant\ at\ day\ 0\ (1)} \times 100$

Area covered by flattened, fibroblast-like cells as a percentage of total covered area:

(3) Percent area covered by the flattened cells = $\frac{Area\ covered\ by\ flattened\ cells\ (3)}{Total\ covered\ area\ (2+3)} \times 100$

The percentage of *fli:GFP*+ or *kdrl:GFP*+ cells was calculated from cell counts in the confocal images of the cell isolates of the primary blastocysts cell cultures (Data collection) using the following formula.

(4) Percentage of GFP+ cells = $\frac{Number\ of\ GFP^{+}\ cells/field}{total\ number\ of\ cells/field} \times 100$

The total number of cells harvested per culture, cell viability and population doubling level of the secondary blastocyst cell cultures was calculated from the cell counts in the heamocytometer using the following formulas.

(5) Total cells harvested = $Number\ of\ cells/\mu L\ \times total\ volume\ of\ cell\ isolate$ in μL

(6) Cells viability = $\frac{Number\ of\ viable\ cells/\mu L}{Total\ number\ of\ cells/\mu L} \times 100$

(7) Population doubling level (PDL) = $3.32 \times (logNH - logNI)$

Where NH is the total number of cells harvested after a period of time, and NI is number of cells plated.

(8) Population doubling time(PDT) = $\frac{\frac{1}{PDL}}{total\ growth\ hrs}$

Arithmetic means of six replicates were calculated for all percentages under each condition, using the software SPSS 21.0. One-way analysis of variance (ANOVA) was performed to calculate p values to compare different culture conditions as well as different time-points of the same condition. Multiple mean values that varied significantly, were further analysed for pair comparisons using post-hoc Tukey's test.

Results

Blastocyst explant culture

Images of zebrafish blastocyst explant cultures are shown in Figure 2. By day 4 of cultures the fibroblast-like (flattened, elongated) cells, emerging from the explants, spread and covered the surrounding substratum (Figure 2B, dashed area marked with 3). Nearby blastocyst explants often fused to form larger bodies, and the area covered by fibroblast-like cells increased with time (Figure 2C).

On average, two blastocyst embryos yielded 8.3 ± 0.8 explant fragments (plated per well). The explants were of different sizes and the surface area covered by individual explant ranged from 0.01 to 0.29 mm^2. Combined all the explants per well covered an area of 0.27 ± 0.01 mm^2 of the substratum. Cultures observed on subsequent days (day 2, 4 and 6)

showed a gradual decrease in the total number of explants per well (6.2 ± 0.7, 4.4 ± 0.4 and 3.2 ± 0.3 respectively), due to fusion between adjacent explants (Figure 2C). The effect of fetal bovine serum (FBS) and zebrafish embryo extract (ZEE) on the explant cultures and the area covered by the emigrating fibroblast-like cells is discussed in the following sections.

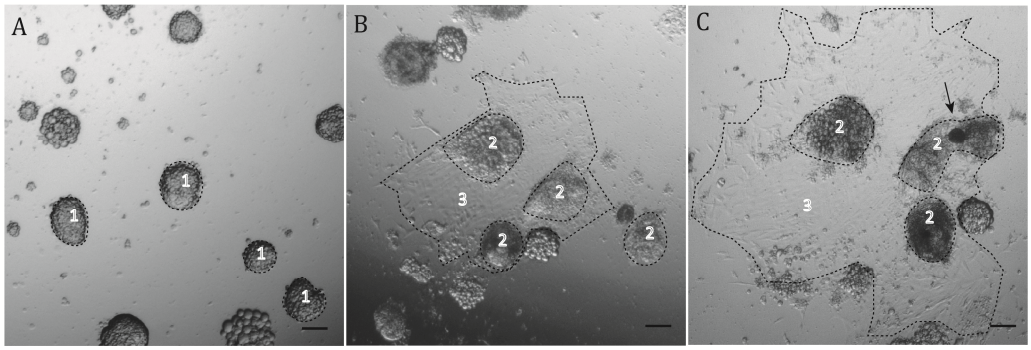

Figure 2: Culture of zebrafish blastocyst explants on day 0 (A), day 4 (B) and day 6 (C). The dotted lines represent (**1**) size of explant just after plating, (**2**) area covered by the rounded explant cells during culture, (**3**) area covered by elongated, fibroblast-like cells. In (**C**) the arrow showing the combination of two explants to form larger aggregate. Scale bar, 100 μm.

Effect of FBS on the blastocyst explant culture

FBS increases the spreading of cells around the explant

By day 6 the cells spreading around the explants covered 350-430% of the initial explant area on different FBS concentrations (Figure 3A). The increase in area covered by the cells around the explants was significantly lower in cultures without FBS compared to cultures with 5% FBS or above on day 4 and 6 of culture. Until day 2 the percent increase in covered area of explant and emigrating cells was similar in all media compositions including medium without FBS. After day 2, the covered area increased significantly faster in media supplemented with different FBS concentrations (Figure 3A). The explants cultured in medium without FBS did not show further increase in the area covered by cells after day 2. No significant differences were observed in area covered by cells cultured in different FBS concentrations.

FBS increases the percent area covered by flattened cells in explant culture

The percent area covered by the flattened, fibroblast-like cells was significantly higher in cultures supplemented with FBS (5-20%) compared to cultures without FBS (Figure 3B). The explants cultured in medium without FBS showed less extension of fibroblast-like cells, and remained similar with time. In the media with 5% or more FBS, the percent area covered by fibroblast-like cells increased significantly from day 2 till day 6. Cultures with

20% FBS had significantly higher percentage of area covered by the fibroblast-like cells on the day 4 and day 6, compared to cultures with 5% FBS. On day 2 the percent area covered by the fibroblast-like cells was similar in cultures with different FBS concentrations (5-20%).

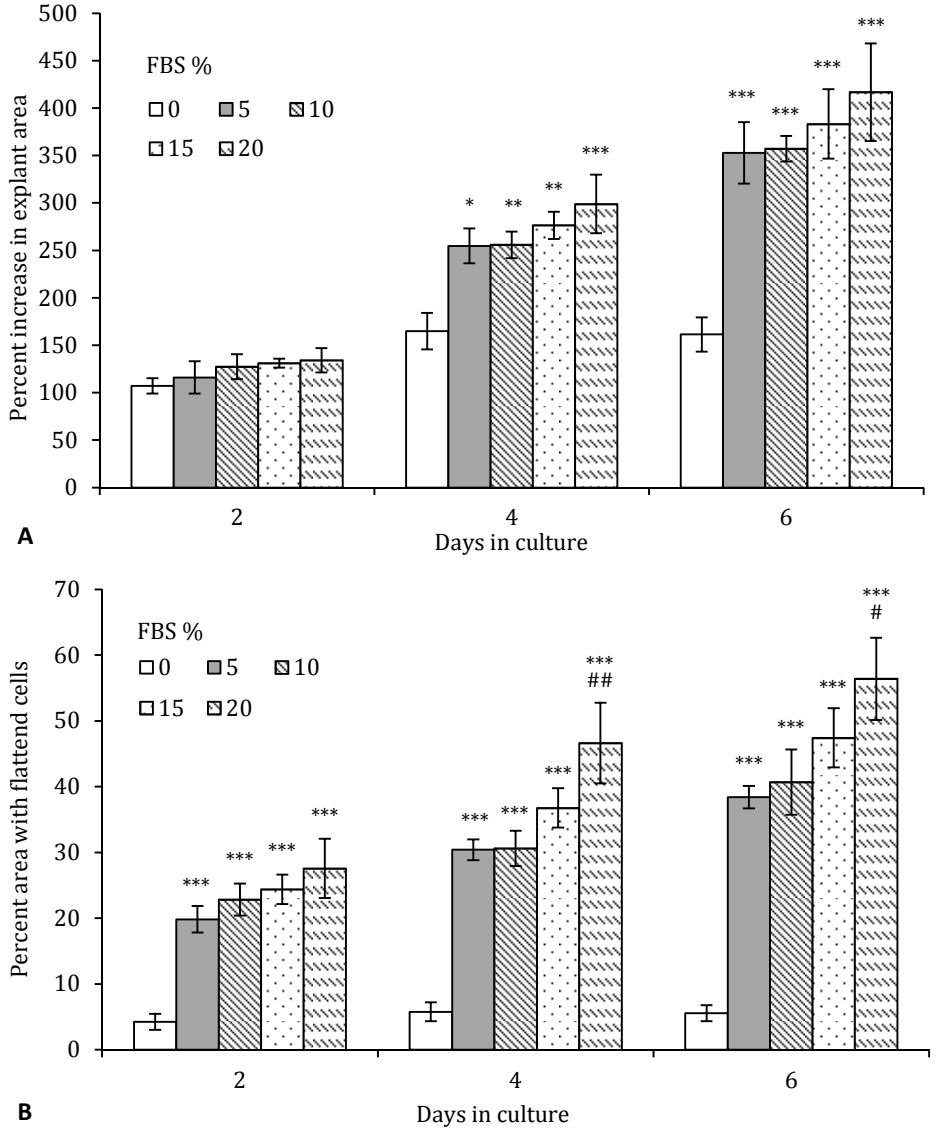

Figure 3: Blastocyst explant culture in media supplemented with different fetal bovine serum (FBS) concentrations. (**A**) Percentage increase in relative area of explant + outgrowth relative to area of initial explant (see equation 2 in Statistical analysis). (**B**) Percentage of area covered by flattening cells (area marked with number 3 in Figure 2; see equation 3 in Statistical analysis). Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to cultures with no FBS; ##, $p < 0.01$, #, $p < 0.05$ compared to cultures with 5% FBS).

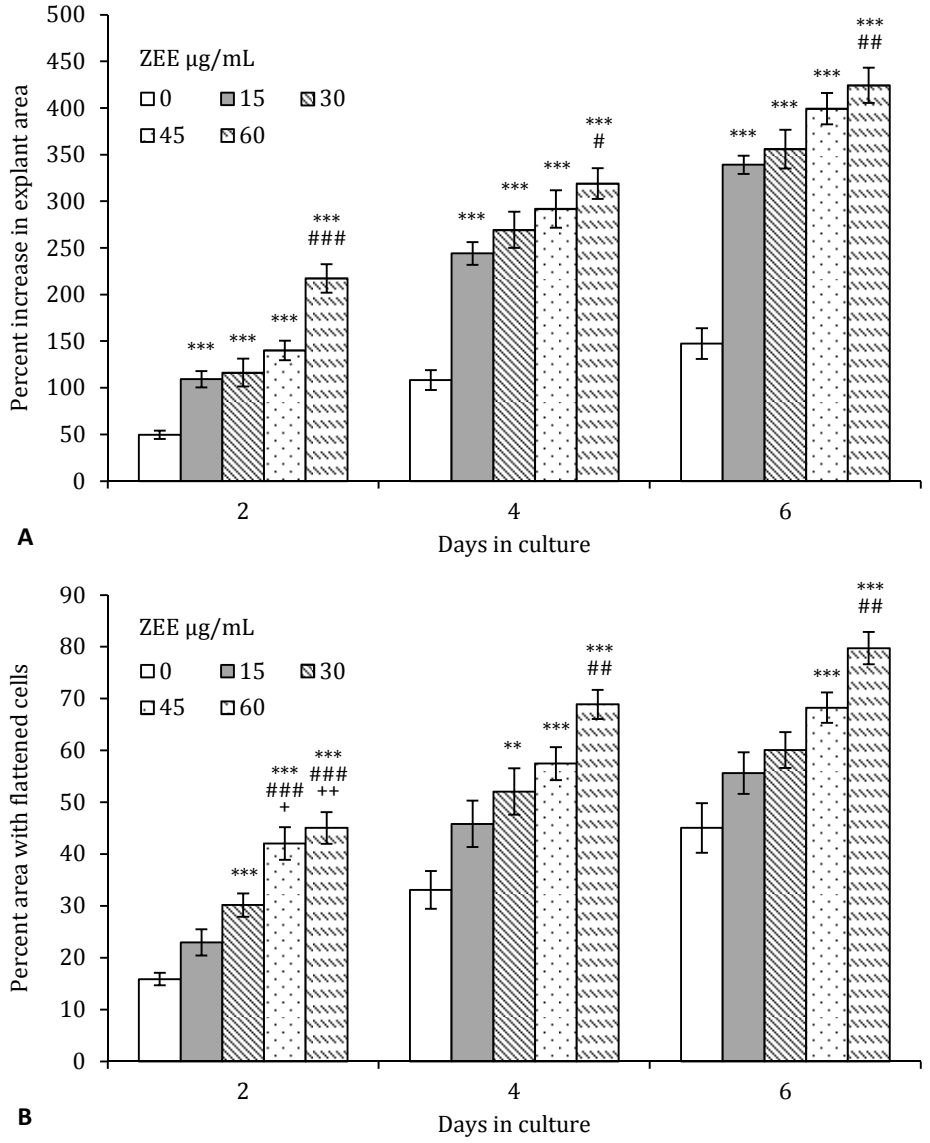

Figure 4: Effect of different zebrafish embryo extract (ZEE) concentration in medium on zebrafish blastocyst explant culture. (**A**) Percentage increase in relative area covered by cells around the explant compare to the initial area of explant (see equation 2 in Statistical analysis). (**B**) Percentage of area covered by flattening cells (area marked with number 3 in Figure 2; see also equation 3 in Statistical analysis). Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, compared to cultures with no ZEE; ###, $p < 0.001$, ##, $p < 0.01$, #, $p < 0.05$ compared to cultures with 15 µg/mL ZEE; ++, $p < 0.01$, +, $p < 0.05$ compared to cultures with 30 µg/mL ZEE).

Effect of zebrafish embryo extract on blastocyst explant culture

ZEE increases area covered by cells around blastocyst explant

Similar to FBS, ZEE also showed significant effect on the expansion in area covered by the emigrating cells from the blastocyst explants (Figure 4A). The increase in the area covered by cells around the explant was higher on day 2 in cultures with 15 μg/mL ZEE compared to cultures without ZEE in the medium. More expansion of the fibroblast-like cells around the explant was observed in cultures supplemented with 60 μg/mL compared to 15 μg/mL ZEE (Figure 4A). No significant differences between 15, 30 and 45 μg/mL ZEE concentrations was observed on the expansion of the cultures. Without ZEE in the medium the area of the explant remained similar from day 2 to day 6. In the presence of ZEE (15-60 μg/mL), significant increase was observed in the explant area from day 2 to day 6 ($p<0.001$).

Percent area covered by flattened cells is increased by ZEE

The area covered by the flattened cells, as a percentage of the total area covered by the explant (including the fibroblastic cells), was higher with 30-60 μg/mL ZEE concentration, compared to no ZEE in the medium (Figure 4B). Cultures maintained with 60 μg/mL ZEE showed higher percentage of area covered by fibroblast-like cells compared to cultures with 15 μg/mL ZEE. No significant differences were observed between cultures with zero and 15 μg/mL ZEE. The percent area covered by fibroblast-like cells increased significantly from day 2 to day 4 under all ZEE conditions ($p<0.01$). However, after day 4 to day 6 a significant increase was observed only with 60 μg/mL ZEE ($p<0.05$).

Trypsinized blastocyst primary cell culture

The trypsinized blastocyst cells formed aggregates known as EBs within the first 24 h of culture and then the elongated, fibroblast-like cells started to emigrate on the culture substratum as a monolayer from these EBs (Figure 5A-E). The primary cultures predominantly contained two types of cells: (i) flattened, elongated, fibroblast-like cells; (ii) round, EB cells. At this stage other cell types such as neuron-like (long thread-like) cells, and melanocytes (pigment cells; Figure 5F) were also visible in the EBs. The appearance of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells in the EBs was also observed in cultures (Figure 5B). By day 4 both the EBs and the proliferating fibroblast like cells contained cells expressing *fli:GFP* or *kdrl:GFP* marker (Figure 5C and G).

Expression of *fli:GFP*$^{+}$ cells in blastocyst primary cell culture

The *fli:GFP* blastocyst cell culture showed the *fli:GFP* expression in cells in culture medium without supplementation of endothelial/hematopoietic growth factors (Figure 5B-E). By day 2 of culture the *fli:GFP*$^{+}$ cells were observed on the periphery of the EBs (Figure 5B).

The percentage of *fli:GFP*$^{+}$ cells in cultures without surface coating (on tissue culture treated polystyrene), increased significantly from day 2 to day 4 ($p<0.001$) and then declined at the same rate from day 4 to day 6 ($p<0.01$; Figure 6B). The percentage of *fli:GFP*$^{+}$ cells was smallest in cells isolated from cultures on day 8. In contrast to polystyrene substratum, in cultures on gelatin-coated substratum the percentage of *fli:GFP*$^{+}$ cells remained unchanged overtime. On day 4 the percentage of *fli:GFP*$^{+}$ cells was significantly higher on polystyrene compared to gelatin substratum.

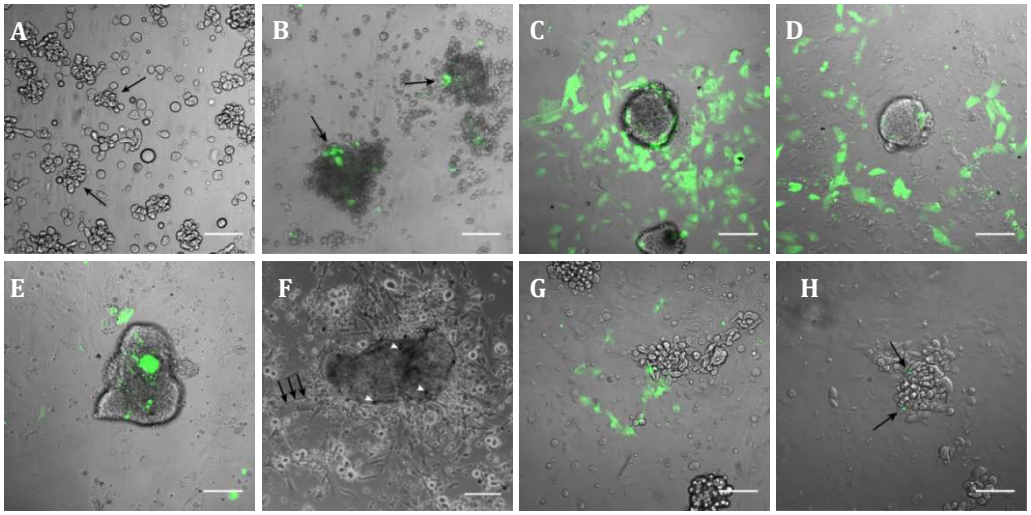

Figure 5: Culture of trypsinized blastocyst cells isolated from transgenic zebrafish embryos (B-E *fli:GFP*; G,H *kdrl:GFP*). (**A**) Image 1 h after cell plating, arrows showing the cells attaching each other to form aggregates. (**B**) Within the initial 24 h of culture the cells combine to form large aggregates called embryoid bodies (EBs). Arrows showing the appearance of *fli:GFP*$^{+}$ cells on the periphery of EBs. (**C**) More *fli:GFP*$^{+}$ cells appear and proliferate around the EBs by day 4 of culture. (**D**). By day 6 of culture the cells expressing GFP reduces. (**E**) Cells expressing *fli:GFP*$^{+}$ marker almost diminishes by day 8 of culture, while the non-GFP, fibroblast-like cell continue to grow. (**F**) phase contrast image of day 6 EB showing the extensions of fibroblast like cells (white arrow heads showing melanocytes; arrows showing neuron-like cell). (**G**) *kdrl:GFP* blastocyst cell culture at day 4 of culture with numerous *kdrl:GFP*$^{+}$ cells. (**H**) *kdrl:GFP* blastocyst cell culture at day 6 of culture. The *kdrl:GFP*$^{+}$ cells are completely disappeared on day 6 except few (arrows). Scale bar, 100 µm.

Expression of *kdrl:GFP*$^{+}$ cells in blastocyst primary cell culture

As with the *fli:GFP* cultures, the *kdrl:GFP*$^{+}$ cells were distributed at the periphery of the EB (Figure 5G). However, the overall percentage of *kdrl:GFP* $^{+}$ cells in cultures (1.8 ± 0.2%) was less than *fli:GFP*$^{+}$ cells (6.1 ± 0.4%). The percentage of *kdrl:GFP*$^{+}$ cells in blastocyst cell cultures was found to be 2.6 ± 0.3% on day 2 of culture (Figure 6C). The cells isolated from cultures had a rounded morphology and were readily counted without any need for nuclear staining (Figure 6A). No significant increase in the percentage of *kdrl:GFP*$^{+}$ cells was observed between days 2 and 4. Similar to *fli:GFP* cell cultures the percentage of *kdrl:GFP*$^{+}$ cells in the blastocyst cell cultures decreased significantly from day 4 to day 6

(p<0.001) and then remained the same until day 8. No significant differences were observed in the percentage of *kdrl:GFP*+ cells between cultures on polystyrene or gelatin.

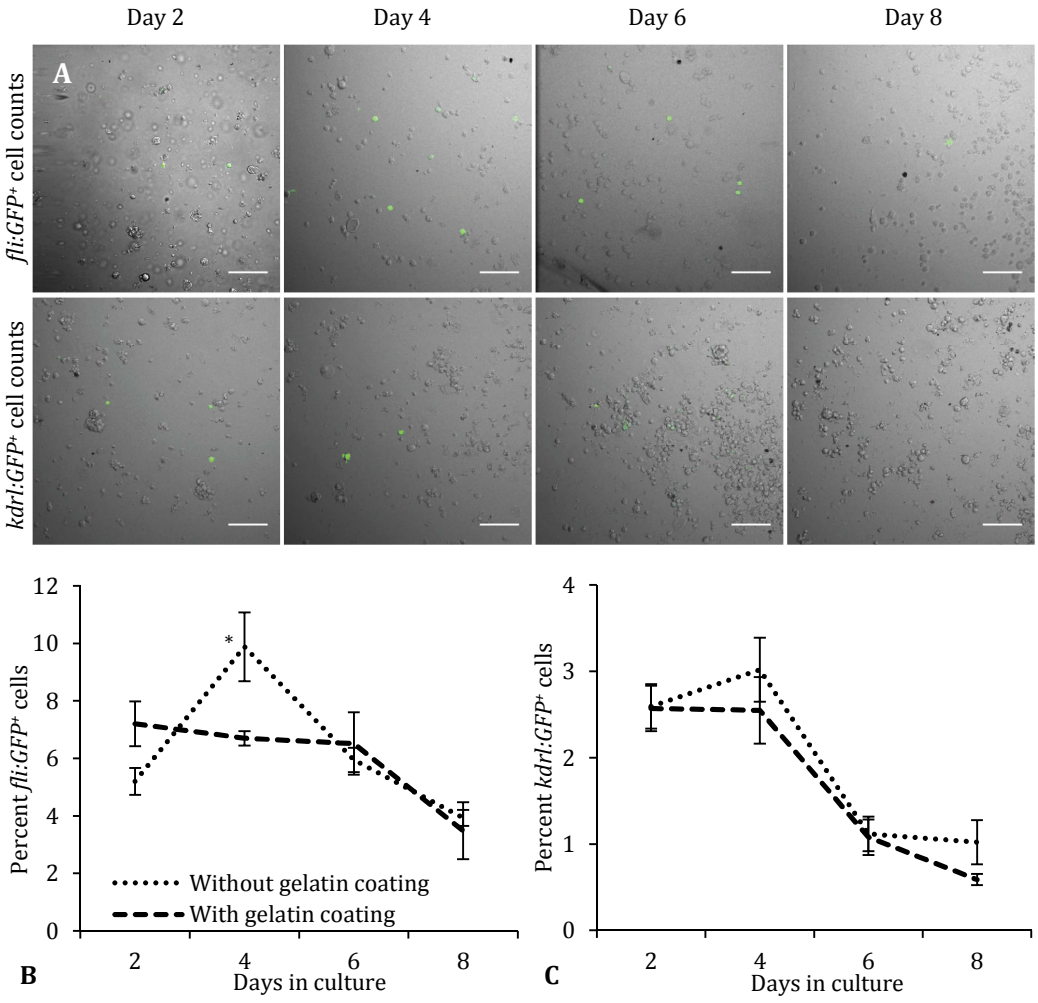

Figure 6: Percentage of *fli:GFP*+ and *kdrl:GFP*+ cells in cells isolated from blastocyst cell culture in LDF medium with 15% FBS. (**A**) Overlaid confocal and phase contrast images of cell isolates from cultures of different ages (days 2-8) for counting of *fli:GFP*+ or *kdrl:GFP*+ cells (cells illuminating green fluorescence) as a percentage of total cells (round non-fluorescence) in a microscopic field.. Scale bar, 100 μm. (**B**) Change in number over time of *fli:GFP*+ cells as a percentage of total cells sampled by trypsinization of cultures. (**C**) Change in number over time of *kdrl:GFP*+ cells as a percentage of total cells sampled by trypsinization of cultures (see equation 4 for B and C). Error bars represent standard error. (*, $p < 0.05$).

Blastocysts secondary cell culture

Subsequent sub-culturing of the primary blastocyst cells eliminated other cell types including the *fli:GFP*+ and *kdrl:GFP*+ cells, leaving a homogeneous population of fibroblast-like cells which expressed the fibroblast marker vimentin in some cells (Figure 7 B-D). The

primary cultures grow slower and took 21 days to reach sub-confluence. After passaging, the secondary cells showed a faster growth compared to primary cultures and reach to sub-confluence within seven days.

The population doubling time (PDT) was longer for primary cells then for the passaged cells (Figure 8A); however, the difference was not significant. Subsequent passaging did not have much effect on PDT. The time required for the cells to become sub-confluent was longer in primary cultures (3 weeks). The secondary cultures were sub-confluent within the first week. The viability of cells, as measured by trypan blue exclusion, was lower in primary cell culture (75.2 ± 4.2%) than in secondary culture (85 - 96%; Figure 8B). No significant differences were observed in the viability of primary and secondary cells up to six passages. However after passage seven the viability of cells was significantly higher than that of the primary cells ($p<0.01$). The fibroblast-like cells at passage 8 were used to determine the FBS, ZEE and gelatin concentrations for optimal growth of zebrafish embryonic cells in secondary culture.

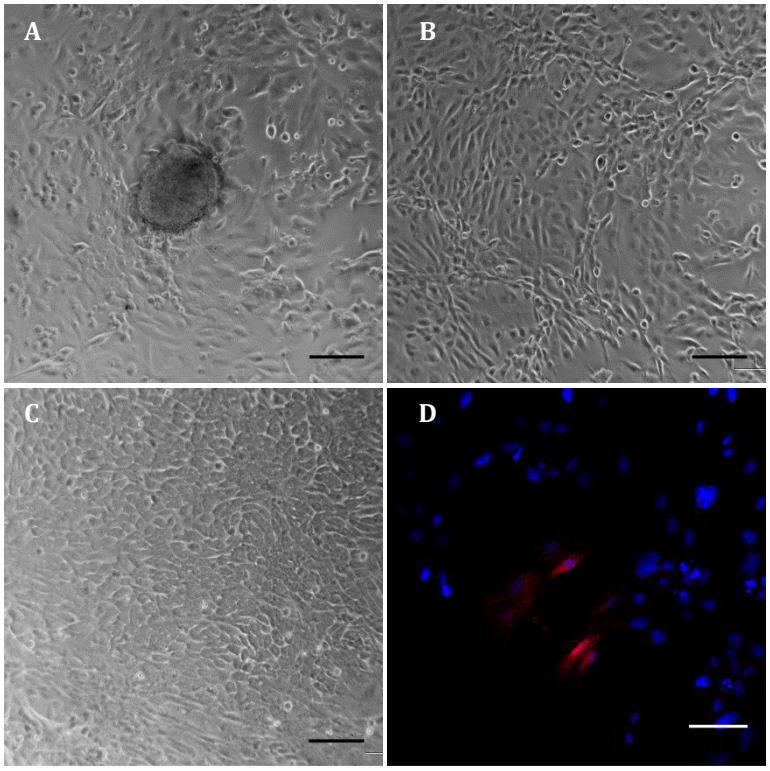

Figure 7: Zebrafish blastocyst cell culture at subsequent passages. (**A**) primary blastocyst cell culture (day 20), the flattened fibroblast-like cells grow around the periphery of EB. (**B**) Passage 1 (day 6), cells with similar elongated fibroblast-like morphology retain in culture. (**C**) passage 2 (day 6), Almost all cells have similar fibroblast-like morphology. (**D**) Cells at passage eight stained with fibroblast marker anti-vimentin (red), nuclei stained with DAPI (blue). Scale bar, A-C, 100 µm; D, 50 µm.

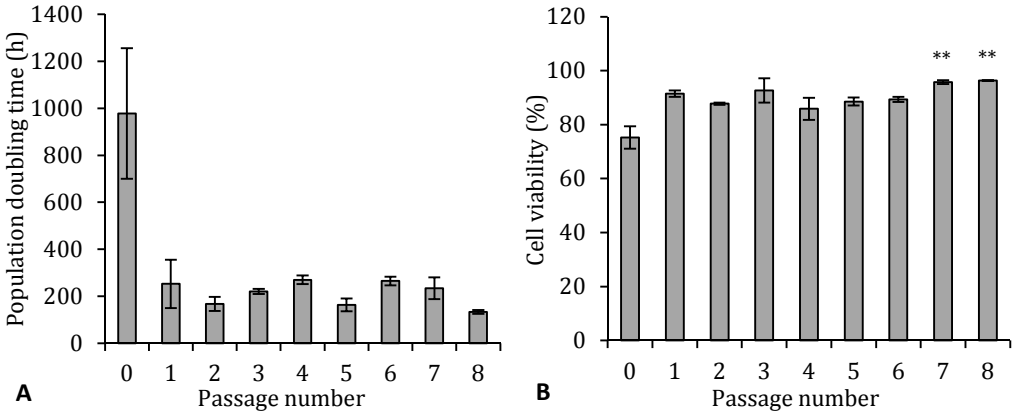

Figure 8: Growth curve of zebrafish blastocyst cells at subsequent passages. (**A**) Population doubling time (PDT) in hours based on heamocytometer counts of total number of cells harvested (see equation 7). (**B**) Viability of cells (as measured with trypan blue exclusion; see equation 5) at subsequent passages. Error bars represent standard error. (**, $p < 0.01$ compared to passage 0).

FBS and ZEE effect on secondary cell culture

With increasing concentrations of FBS, there was a significant increase in the proliferation of zebrafish embryonic secondary cell cultures. The total number of cells harvested per well, cultured for seven days in media with different FBS concentrations, was lowest in cultures with 5% FBS (Figure 9A). The number of cells harvested per well was significantly higher in 15% FBS compared to 5% FBS ($P<0.01$). Cell counts from cultures maintained with 10% FBS were intermediate between cell counts from cultures with 5 and 15% FBS. No significant difference in the number of cells harvested per well, was observed between cultures with 15 and 20% FBS supplementation.

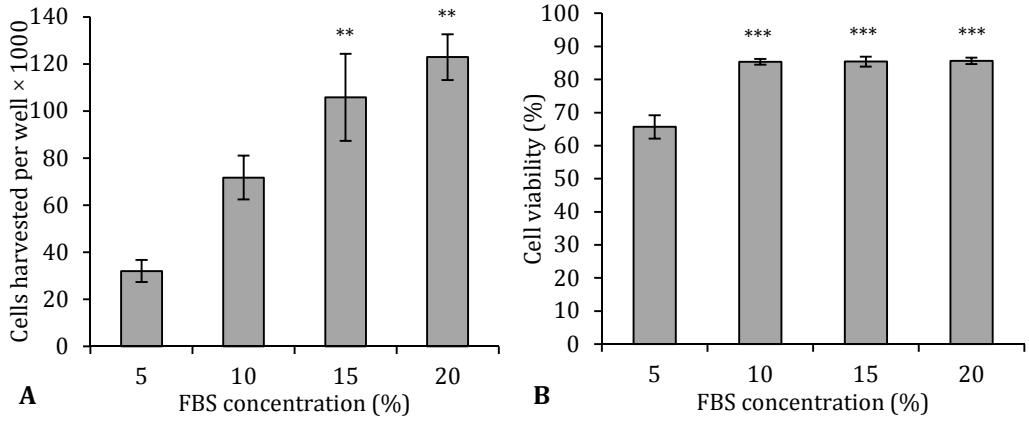

Figure 9: Effect of FBS concentration on secondary blastocyst cell culture. (**A**) Total number of cells harvested per well, from cultures maintained at different FBS concentrations. (**B**) Viability of isolated cells (trypan blue exclusion). Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$ compared to cultures maintained at 5% FBS).

The viability of cells, measured after isolation from culture by trypsinization, also varied with FBS concentration (Figure 9B). Lower cell viability was observed in cultures with 5% FBS supplementation compared to higher FBS concentrations ($P<0.001$). No significant differences were observed in the viability of cells cultured with 10, 15 and 20% FBS.

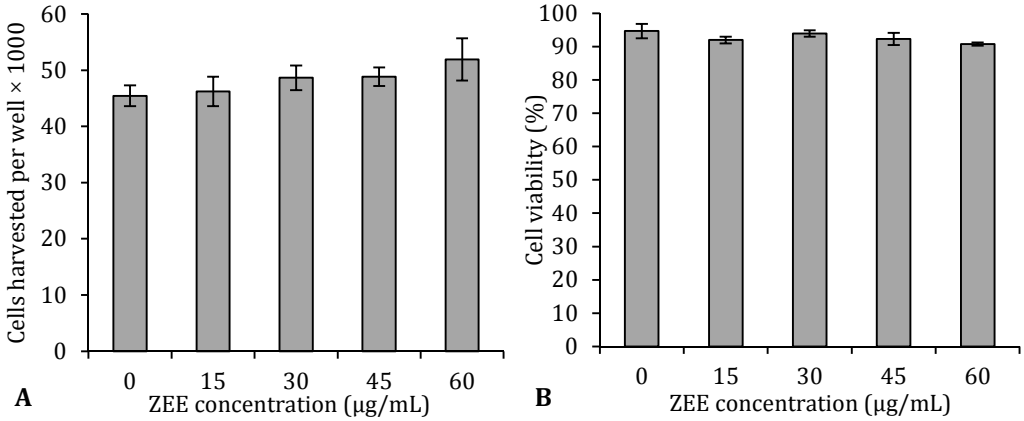

Figure 10: Total counts and viability of secondary blastocyst cell culture maintained for 7 days at different concentrations of zebrafish embryo extract. (**A**) Total number of cells harvested per well of a 96 well plate. (**B**) Viability of the isolated cells (trypan blue exclusion). Error bars represent standard error.

Different concentrations of ZEE showed no significant effect on cell counts or viability of blastocyst secondary cell culture. The total number of cells harvested per well, after seven days of culture in different ZEE concentrations, was 48213.7 ± 1128.9 on average. Cell counts were similar in all wells regardless of the ZEE concentration (range: 0-60 µg/mL; Figure 10A). The viability of the cell isolates were also similar between cultures maintained with different concentrations of ZEE (Figure 10B).

Effect of gelatin substratum on secondary cell culture

In this experiment, an increase in the concentration of gelatin substratum was correlated with a decrease in the total number of cells harvested per well (Figure 11A). Significantly higher numbers of cells were recovered from cells cultured on 0.03 mg/cm^2 of gelatin compared to higher gelatin concentrations (0.12, 0.24 and 0.48 mg/cm^2). No significant differences in total cell counts were observed between cells cultured without gelatin and with different concentrations of gelatin substratum. Higher gelatin concentrations also reduced the viability of the cells (Figure 11B). Viability of the cells cultured on polystyrene substrate ranged from 91 to 97%, which was significantly higher than the viability of cells cultured on 0.24 and 0.48mg/cm^2 gelatin substrate. The viability of cells cultured on lower gelatin concentrations (0.03 and 0.06mg/cm^2) was also higher than the viability of cells cultured on 0.48mg/cm^2 of gelatin.

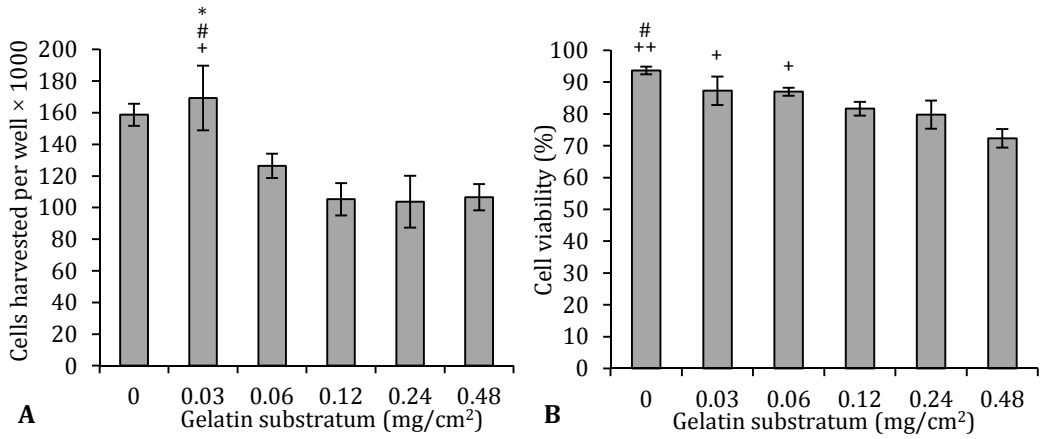

Figure 11: Effect of different concentration of gelatin substratum on growth and viability of secondary blastocyst cell culture. (**A**) Total number of cells harvested per well on day 7 of culture. (**B**) Viability of isolated cells. Error bars represent standard error. (*, $p < 0.05$ compared to cultures on 0.12 mg/cm^2 gelatin; #, $p < 0.05$ compared to 0.24 mg/cm^2 gelatin; ++, $p < 0.01$, +, $p < 0.05$ compared to 0.48 mg/cm^2 gelatin).

Discussion

Zebrafish blastocyst stage embryonic cells are usually used to develop ESC cultures, that have the potential to differentiate into a specific cell line [7]. These cells have been used to establish zebrafish embryonic fibroblast [19], embryonic neurons [10] and embryonic muscle cell lines [12]. Methods for culturing cells from zebrafish embryos are still developing. Depending on the research question, different conditions have been used for the culture of zebrafish embryonic cells (Table 1). Zebrafish embryonic cells are usually cultured on a feeder monolayer of growth-arrested cells [21, 22] or a gel substratum [10, 12, 20, 27]. In this study, we described a procedure for initiating zebrafish embryonic cell culture, using blastocyst stage embryos, without any feeder layer or gel substrate, an approach which is similar to that previously reported (Ref. [17, 19]). The media composition and culture procedures we used were modified from those used for zebrafish embryonic cell culture by Fan and Collodi [21].

Zebrafish blastocyst explant culture

Blastocyst explant cultures are usually performed to understand the early developmental and differentiation processes in zebrafish embryos [30, 41]. Historically, mouse embryonic blastocyst explants were used to develop pluripotent ESC cultures [42]. Similarly, cell lines can be developed using embryonic explants, such as mouse blastocyst cultures for the development of skeletal muscle cell line [43].

In this study, we analysed the emergence of fibroblast-like cells from zebrafish blastocyst explant cultures in media having different concentrations of FBS and ZEE. Higher

concentrations of FBS (15-20%) and ZEE (45-60µg/mL) were found to be optimum for zebrafish primary explant culture. Thus, at the concentrations indicated of FBS and ZEE, the fibroblast-like cells expanded to cover significantly more area at the periphery of the explant. We also noted that the fibroblast-like cells can be repeatedly sub-cultured. The number of blastocyst fragments (explants) per well decreased gradually overtime, because neighboring explants often fuse to form larger aggregates.

Blastocyst cell culture: *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cell differentiation

At the blastocyst stage, a zebrafish embryo consists of pluripotent cells [44] that can be induced to differentiate into a specific cell type, e.g. neurons, astrocytes [16] and myocytes [12], by the relevant *in vitro* manipulation. When the pluripotent blastocyst cells are cultured on a growth-arrested feeder cell layer, they retain their pluripotency [7]. When cultured in the absence of specific differentiation factors or feeder layer, blastocyst-derived cells differentiate spontaneously into EBs, composed of different cell types found in the early embryo [2]. The EBs formed in primary embryonic cell culture in our experiments contained different cell types including melanocytes, neuron-like cells, *fli:GFP*$^{+}$ cells, *kdrl:GFP*$^{+}$ cells and fibroblast-like cells. Similar populations of cells have been reported in EBs formed in mouse embryonic cell culture [2].

Studies have shown higher concentration of hematopoietic cells compared to other cell types in mouse EBs [45, 46]. This would be consistent with our finding of higher percentages of *fli:GFP*$^{+}$ cells compared to *kdrl:GFP*$^{+}$ cells in the blastocyst cell cultures. However, it should be remembered that *fli:GFP* include but is not specific for hematopoietic cells [23]. Culture substrata coated with different matrix-derived molecules have also been reported to increase the gene expression of specific cell types in human ESC cultures [47]. In that study, the EBs derived from human ESCs higher levels of neural and endodermal genes on different substrates (laminin and fibronectin, respectively) [47]. Zebrafish ESCs have also been reported to differentiate into fibroblast, neuron and epithelial cells on gelatin substrata [27], and into myocytes on laminin substrata [12]. Similarly, gelatin and collagen substrates have been used for differentiation of mouse ESCs into endothelial cells [48, 49]. In our experiments, in contrast to polystyrene substratum, the percentage of *fli:GFP*$^{+}$ cells was stable in cultures overtime on gelatin substratum.

Blastocyst secondary cell culture

In order to obtain a continuous cell line, zebrafish embryonic cells are cultured on a feeder layer of growth-arrested cells or on gelatin-coated culture dishes [50]. In this study, we established zebrafish embryonic cell cultures, with EB formation, on a tissue culture treated polystyrene surface without any feeder layer or gel coating. A similar strategy has previously been used for development of a fibroblast-like cell line from zebrafish embryonic cell culture [17]. The primary embryonic cells in our culture setup were slow

growing, being characterized by longer population doubling times, and lower cell viability. This may be because of the considerable amount of unattached cells observed in the primary cultures, which were washed away while refreshing the media.

Another reason for the slower growth rate of zebrafish primary cell cultures, could be the occurrence of cellular differentiation, because different cell types have different growth requirements [17]. In the present study, the most abundant fibroblast-like cells developed into a monolayer on the surface of the culture dish when passaged. The secondary cells, we obtained after passaging the primary blastocyst embryonic cells, were fast growing, mostly homogeneous and were similar in morphology to the fibroblast-like cell line derived from zebrafish embryos in ref. [17]. The mouse anti-vimentin antibody that we used to stain fibroblasts in the blastocyst secondary culture, has been previously reported to detect fibroblasts in zebrafish [39]. Similar cultures derived from zebrafish embryonic cells after several passages were referred to as a subpopulation of the primary cells that suited best or they have adapted themselves to the culture conditions [18].

Effect of FBS on blastocyst secondary cell culture

FBS is the most widely used supplement for cell cultures including zebrafish cell culture [21]. It contains growth factors, hormones, amino acids, vitamins, trace minerals as well as cell attachment, proliferating and binding factors [51]. In our study, the presence of higher than 15% FBS significantly increased the area covered by proliferating cells around the explant overtime. Similar FBS concentration (15%) have been used previously for zebrafish embryonic cell cultures [20, 21]. Secondary zebrafish embryonic cells we obtained after passaging the primary cells also showed higher growth rate with increasing FBS concentration. For these secondary cells, the use of 10% FBS in the medium was optimum, in our hands, for the growth of cultures. Similar to these findings, total cell counts of zebrafish embryonic cell lines (Z428 and ZEB2J) increased with increasing FBS concentrations in the culture media [52, 53]. Contrary to these findings, in early studies [18, 54], higher FBS concentrations were shown to inhibit the growth of zebrafish ESC culture. However, similar to our results, omitting FBS from the media significantly reduced the growth of these cells [18].

Effect of zebrafish embryo extract on blastocyst secondary cell culture

Fish embryo extract has been shown to support the attachment and growth of medaka [50, 55] and turbot [56] ESCs. Zebrafish embryo extract is particularly important for the growth of some of the zebrafish cell lines, e.g. in the case of zebrafish caudal fin cells [18]. However, this is not critical for most of the zebrafish cells cultures (Table 1). In our study, the emigrating cells around the blastocyst explants covered significantly more area in media supplemented with 60 μg ZEE protein per mL medium compared to lower ZEE concentrations. Similar ZEE concentrations have been used previously for zebrafish

embryonic cell cultures [20, 21]. Different concentrations of ZEE did not have significant effects on the growth rate and viability of the zebrafish secondary embryonic cells in our experiments. These results recommend the use of ZEE for zebrafish primary embryonic cell cultures. However, for secondary cell cultures ZEE is not important for population growth. Previous studies have shown significant mitogenic effect of medaka fish embryo extract on the zebrafish embryonic cell line ZF428 [52].

Effect of gelatin substratum on blastocyst secondary cell culture

Gelatin coating has been used as an alternative to a feeder cell layer for medaka [50], turbot [56] and zebrafish [52] embryonic cell cultures. In the current study, we cultured the zebrafish secondary embryonic cells on different concentrations of gelatin substrate. At higher gelatin concentrations lower number of cells were harvested, although the number was not significantly different compared to cells cultured without gelatin substrate. Cell viability was significantly higher in cultures without gelatin coating compared to cultures on 0.24 and 0.48 mg/cm^2 gelatin. Cells cultured on a range of gelatin substrates up to 0.12 mg/cm^2, or cultured without gelatin, had similar growth rates and viability. These results suggest that gelatin concentrations up to 0.12 mg/cm^2 may be used to coat the culture dish for the attachment and proliferation of zebrafish cells. Similar gelatin concentrations have been previously used for zebrafish cell culture [52]. A reason for lower cell growth and viability on high gelatin concentrations might because of the change in substrate after growing the cells for several passages on a polystyrene surface of the tissue culture flask without gelatin coating.

Conclusions

Zebrafish blastocyst cells differentiate *in vitro* into cell types including vimentin$^+$ fibroblastic cells, *kdrl:GFP*$^+$ cells, and *fli:GFP*$^+$ cells. However, in the absence of a selective medium, the latter two cell types decline in abundance over time in the primary cultures, and disappear with subsequent passaging. Only the fast growing fibroblast-like cells remain in the cultures. Zebrafish primary embryonic cells require a high FBS concentration (15%) in medium, while secondary fibroblast-like cell grow at a lower (10%) FBS concentrations. ZEE at a total protein concentration of 45-60 µg/mL medium is important for the maintenance of primary cell cultures. However, ZEE does not affect the growth of the zebrafish secondary cells. Gelatin can be used at low concentrations (0.03-0.12 mg/cm^2) as a substratum for zebrafish blastocyst cell culture.

References

[1] Bethesda MD. What are embryonic stem cells? In Stem Cell Information, 2015; Available from: http://stemcells.nih.gov/info/basics/pages/basics3.aspx.

[2] Keller GM. *In vitro* differentiation of embryonic stem cells. Curr Opin Cell Biol 1995; 7(6): 862-9.

[3] Evans M. Discovering pluripotency: 30 years of mouse embryonic stem cells. Nat Rev Mol Cell Biol 2011; 12(10): 680-6.

[4] Koh S, Piedrahita JA. From "ES-like" cells to induced pluripotent stem cells: a historical perspective in domestic animals. Theriogenology 2014; 81(1): 103-11.

[5] Tachibana M, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H*, et al.* Human embryonic stem cells derived by somatic cell nuclear transfer. Cell 2013; 153(6): 1228-38.

[6] Festing S, Wilkinson R. The ethics of animal research. Talking Point on the use of animals in scientific research. EMBO Rep 2007; 8(6): 526-30.

[7] Alvarez MC, Bejar J, Chen S, Hong Y. Fish ES cells and applications to biotechnology. Mar Biotechnol (NY) 2007; 9(2): 117-27.

[8] Barut BA, Zon LI. Realizing the potential of zebrafish as a model for human disease. Physiological Genomics 2000; 2(2): 49-51.

[9] Fan L, Crodian J, Collodi P. Culture of embryonic stem cell lines from zebrafish. Methods Cell Biol 2004; 76: 151-60.

[10] Chen Z, Lee H, Henle SJ, Cheever TR, Ekker SC, Henley JR. Primary neuron culture for nerve growth and axon guidance studies in zebrafish (*Danio rerio*). PLoS One 2013; 8(3): e57539.

[11] Fan L, Crodian J, Liu X, Alestrom A, Alestrom P, Collodi P. Zebrafish embryo cells remain pluripotent and germ-line competent for multiple passages in culture. Zebrafish 2004; 1(1): 21-6.

[12] Myhre JL, Pilgrim DB. Cellular differentiation in primary cell cultures from single zebrafish embryos as a model for the study of myogenesis. Zebrafish 2010; 7(3): 255-66.

[13] Paw BH, Zon LI. Primary fibroblast cell culture. Methods Cell Biol 1999; 59: 39-43.

[14] Xing JG, El-Sweisi W, Lee LE, Collodi P, Seymour C, Mothersill C*, et al.* Development of a zebrafish spleen cell line, ZSSJ, and its growth arrest by gamma irradiation and capacity to act as feeder cells. In Vitro Cell Dev Biol Anim 2009; 45(3-4): 163-74.

[15] Sander V, Sune G, Jopling C, Morera C, Izpisua Belmonte JC. Isolation and *in vitro* culture of primary cardiomyocytes from adult zebrafish hearts. Nat Protoc 2013; 8(4): 800-9.

[16] Ghosh C, Liu Y, Ma CG, Collodi P. Cell cultures derived from early zebrafish embryos differentiate *in vitro* into neurons and astrocytes. Cytotechnology 1997; 23(1-3): 221-230.

[17] Choorapoikayil S, Overvoorde J, den Hertog J. Deriving cell lines from zebrafish embryos and tumors. Zebrafish 2013; 10(3): 316-25.

[18] Collodi P, Kamei Y, Ernst T, Miranda C, Buhler DR, Barnes DW. Culture of cells from zebrafish (*Brachydanio rerio*) embryo and adult tissues. Cell Biol Toxicol 1992; 8(1): 43-61.

[19] He S, Salas-Vidal E, Rueb S, Krens SF, Meijer AH, Snaar-Jagalska BE*, et al.* Genetic and transcriptome characterization of model zebrafish cell lines. Zebrafish 2006; 3(4): 441-53.

[20] Badakov R, Jazwinska A. Efficient transfection of primary zebrafish fibroblasts by nucleofection. Cytotechnology 2006; 51(2): 105-10.
[21] Fan L, Collodi P. Zebrafish embryonic stem cells. Methods Enzymol 2006; 418: 64-77.
[22] Ma C, Fan L, Ganassin R, Bols N, Collodi P. Production of zebrafish germ-line chimeras from embryo cell cultures. Proc Natl Acad Sci U S A 2001; 98(5): 2461-6.
[23] Lawson ND, Weinstein BM. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 2002; 248(2): 307-318.
[24] Renaud O, Herbomel P, Kissa K. Studying cell behavior in whole zebrafish embryos by confocal live imaging: application to hematopoietic stem cells. Nat Protoc 2011; 6(12): 1897-1904.
[25] Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Development 2005; 132(23): 5199-209.
[26] Melet F, Motro B, Rossi DJ, Zhang L, Bernstein A. Generation of a novel Fli-1 protein by gene targeting leads to a defect in thymus development and a delay in Friend virus-induced erythroleukemia. Mol Cell Biol 1996; 16(6): 2708-18.
[27] Ho SY, Goh CW, Gan JY, Lee YS, Lam MK, Hong N*, et al.* Derivation and long-term culture of an embryonic stem cell-like line from zebrafish blastomeres under feeder-free condition. Zebrafish 2014; 11(5): 407-20.
[28] Thompson MA, Ransom DG, Pratt SJ, MacLennan H, Kieran MW, Detrich HW, 3rd*, et al.* The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. Dev Biol 1998; 197(2): 248-69.
[29] Bussmann J, Lawson N, Zon L, Schulte-Merker S, Committee ZN. Zebrafish VEGF receptors: a guideline to nomenclature. PLoS Genet 2008; 4(5): e1000064.
[30] Grinblat Y, Lane ME, Sagerstrom C, Sive H. Analysis of zebrafish development using explant culture assays. Methods Cell Biol 1999; 59: 127-56.
[31] Edgar LG, Goldstein B. Culture and manipulation of embryonic cells. Methods Cell Biol 2012; 107: 151-75.
[32] Sweet H, Amemiya S, Ransick A, Minokawa T, McClay DR, Wikramanayake A*, et al.* Blastomere isolation and transplantation. Methods Cell Biol 2004; 74: 243-71.
[33] Schmidt JV. Embryonic stem (ES) cell culture basics. Curr Protoc Toxicol 2001; Chapter 15: Unit15 1.
[34] Sun L, Bradford CS, Ghosh C, Collodi P, Barnes DW. ES-like cell cultures derived from early zebrafish embryos. Mol Mar Biol Biotechnol 1995; 4(3): 193-9.
[35] Experiments on Animal Act, 2014. The State Secretary of Health and Environmental Protection, Soestdijk. http://wetten.overheid.nl/BWBR0003081/2014-12-18
[36] Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance, 2010. The European Parliament and the council of the European Union, Official Journal of the European Union. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:32010L0063
[37] Westerfield M. The zebrafish book: a guide for the laboratory use of zebrafish (*Danio Rerio*). 4 ed, University of Oregon Press, Eugene 2000, pp.
[38] Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995; 203(3): 253-310.
[39] Chablais F, Veit J, Rainer G, Jazwinska A. The zebrafish heart regenerates after cryoinjury-induced myocardial infarction. BMC Dev Biol 2011; 11: 21.

[40] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012; 9(7): 671-5.
[41] Sagerstrom CG, Grinbalt Y, Sive H. Anteroposterior patterning in the zebrafish, *Danio rerio*: an explant assay reveals inductive and suppressive cell interactions. Development 1996; 122(6): 1873-83.
[42] Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981; 292(5819): 154-6.
[43] Smith J, Merrick D. Embryonic skeletal muscle microexplant culture and isolation of skeletal muscle stem cells. Methods Mol Biol 2010; 633: 29-56.
[44] Kimmel CB, Warga RM. Tissue-specific cell lineages originate in the gastrula of the zebrafish. Science 1986; 231(4736): 365-8.
[45] Keller G, Kennedy M, Papayannopoulou T, Wiles MV. Hematopoietic commitment during embryonic stem cell differentiation in culture. Mol Cell Biol 1993; 13(1): 473-86.
[46] Wang L, Cerdan C, Menendez P, Bhatia M. Derivation and characterization of hematopoietic cells from human embryonic stem cells. Methods Mol Biol 2006; 331: 179-200.
[47] Laperle A, Masters KS, Palecek SP. Influence of substrate composition on human embryonic stem cell differentiation and extracellular matrix production in embryoid bodies. Biotechnol Prog 2014.
[48] Blancas AA, Lauer NE, McCloskey KE. Endothelial differentiation of embryonic stem cells. Curr Protoc Stem Cell Biol 2008; Chapter 1: Unit 1F 5.
[49] Liu Z, Lebrin F, Maring JA, van den Driesche S, van der Brink S, van Dinther M*, et al.* ENDOGLIN is dispensable for vasculogenesis, but required for vascular endothelial growth factor-induced angiogenesis. PLoS One 2014; 9(1): e86273.
[50] Hong N, Li Z, Hong Y. Fish stem cell cultures. Int J Biol Sci 2011; 7(4): 392-402.
[51] Gstraunthaler G. Alternatives to the use of fetal bovine serum: serum-free cell culture. ALTEX 2003; 20(4): 275-81.
[52] Hong N, Schartl M, Hong Y. Derivation of stable zebrafish ES-like cells in feeder-free culture. Cell Tissue Res 2014; 357(3): 623-32.
[53] Xing JG, Lee LEJ, Fan LC, Collodi P, Holt SE, Bols NC. Initiation of a zebrafish blastula cell line on rainbow trout stromal cells and subsequent development under feeder-free conditions into a cell line, ZEB2J. Zebrafish 2008; 5(1): 49-63.
[54] Ghosh C, Collodi P. Culture of cells from zebrafish (*Brachydanio rerio*) blastula-stage embryos. Cytotechnology 1994; 14(1): 21-6.
[55] Hong Y, Schartl M. Isolation and differentiation of medaka embryonic stem cells. Methods Mol Biol 2006; 329: 3-16.
[56] Holen E, Hamre K. Towards obtaining long term embryonic stem cell like cultures from a marine flatfish, Scophtalmus maximus. Fish Physiol Biochem 2003; 29(3): 245-252.

Chapter 4

Influence of medium composition and substratum on the growth of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cell culture

Muhammad Ibrahim and Michael K. Richardson

This chapter is part of our following submission:

Ibrahim M, Richardson MK. The growth of endothelial-like cells in zebrafish embryoid body culture. Experimental cell research (in preparation).

Abstract

Embryonic stem cells (ESCs) are important tools to study lineage commitment and cellular differentiation in early embryos. The differentiation of ESCs can be directed towards a specific cell type by culturing them in appropriate conditions. In this study we analysed the effect of different media formulations and culture substrates on the quantification of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cell cultures. These cultures were derived from transgenic zebrafish (*fli:GFP* and *kdrl:GFP*) lines which express green fluorescent protein under the *fli1* and VEGFR2 promoters, respectively. The different media compositions used to culture the blastocyst cells were (i) LDF medium (a combination of Leibowitz's L-15, Dulbecco's modified Eagle's and Ham's F12 media); (ii) combined LDF and endothelial growth medium (LDF:EGM); and (iii) LDF medium supplemented with endothelial growth supplement mix (LDF:EGS). The blastocyst cells formed embryoid body aggregates within the first 24 h of culture containing *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells. The percentage of *fli:GFP*$^{+}$ cells was higher in cultures compared to *kdrl:GFP*$^{+}$ cells. This is presumably because the *kdrl:GFP* line is specific for endothelial cells, while the *fli:GFP* is expressed in multiple cell types. On day 6 of cultures, higher percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells were observed in LDF:EGS medium (31.8 ± 1.6% and 3.7 ± 0.4%, respectively) compared to LDF medium (21.6 ± 1.9% and 2.1 ± 0.2%, respectively). In LDF:EGM medium the percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells was comparable to that in LDF:EGS medium; however, the total cell yield was significantly less in LDF:EGM cultures. The percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells was higher in cultures on collagen type-I substratum compared to gelatin substratum. Recombinant zebrafish vascular endothelial growth factor protein was also found to increase the concentration of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures. Both *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells were no longer detectable in cultures after 14 days and eight days, respectively. Possible reasons for this may be the down regulation of the transgenes, change in endothelial identity and occurrence of cell death.

Introduction

Embryonic stem cells (ESCs) are cells, derived from blastocyst embryos, that have not started to differentiate yet [1]. By specific *in vitro* manipulation these ESCs can maintain their growth and pluripotency (the ability of cells to differentiated into multiple cell types) almost indefinitely [2]. ESCs are important tools for regenerative medicine [3], genome manipulation in animals [4], development of transgenic animals [5] and toxicity testing [6]. The ESCs have the ability to differentiated into the cells of three germ layers (ectoderm, endoderm and mesoderm), and further into a specific cell type by manipulating culture conditions [1]. Examples of differentiated cell types derived from ESCs *in vitro* include human cardiomyocytes [7], human neural progenitor cells [8], mouse hematopoietic progenitor cells [9], alveolar epithelial cells [10] and endothelial progenitor cells [11].

Research into endothelial cells is fundamental for understanding important processes regulated by these cells e.g. tissue homeostasis, blood cell activation and coagulation [12]. Endothelial cell culture can be used for important applications such as tissue regeneration. In one study, endothelial cells derived from ESCs modified into organ specific endothelial cells and helped regeneration of liver sinusoidal vessels in mice, after transplantation [13]. Similarly vascular networks cultured in 3D hydrogel matrix using endothelial cells derived from human pluripotent ESCs, were able to incorporate with the microvasculature of mouse and sustain blood flow after implantation [14].

Culturing endothelial cells alone is difficult to maintain, to overcome this problem co-culturing techniques have been developed, in which endothelial cells are cultured in the presence of supporting cells including fibroblasts, mural cells, pericytes and mesenchymal stem cells [15]. These endothelial co-culture techniques may be used to engineer vascularized organ culture. In one example, human umbilical vein endothelial cells (HUVECs) co-cultured with human mesenchymal stem cells, in a combination of endothelial growth medium (EGM) and osteogenic medium, formed an *in vitro* vascularized bone model [16]. It has been suggested that such vascularized organ cultures may one day be used for tissue transplantation [17].

Heamangioblasts, the common progenitors of endothelial and hematopoietic lineages, differentiate from the mesoderm during the early development of embryos [18]. The differentiation of heamangioblasts is initiated by various factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and bone morphogenic protein 4 (BMP-4) [19]. Haemangioblasts can be generated *in vitro* by treating ESCs with these various differentiation factors just mentioned, as well as others used in the differentiating media (Table 1; [20, 21]).

In vitro differentiation of ESCs into vascular cells have potential applications in studying vascular development, proposing vascular regenerative therapy, culturing vascularized

organs and developing endothelial cell line [22]. In order to induce differentiation the ESCs are usually cultured in differentiation media in a suspension culture to form embryoid bodies (EBs); on a feeder cell layer of stromal cell line; or on an artificial matrix e.g. collagen type-IV [23, 24].

Table 1: Culture substrates and media composition used for *in vitro* differentiation of ESCs into endothelial lineage.

Culture components	Units	Sources of ESCs						
		Mouse	Human	Human	Mouse	Mouse	Human	Mouse
Substrate		Col-I	MEF	Fbn	Col-IV	Gelatin	PEC	Gelatin
IMDM				-	-	-	-	-
Lv-EGM		-	-		-	-	-	-
α-MEM		-	-	-		-	-	-
SP-34 medium		-	-	-	-		-	-
DMEM/F12		-	-	-	-	-		-
DMEM (high glucose)		-	-	-	-	-	-	
Methyl cellulose	%	1	-	-	-	-	-	-
FBS	%	15	-	-	5	-	-	20
Knockout serum replacement	%	-	-	-	-	-	20	-
BIT 9500	%	-	20	-	-	-	-	-
Insulin	µg/mL	10	-	-	-	-	-	-
Penicillin	U/mL	50	50	-	-	-	-	-
Streptomycin	µg/mL	50	50	-	-	-	-	-
PS	%	-	-	-	1	1	1	100X
Monothioglycerol	µM	450	450	-	-	-	-	-
VEGF	ng/mL	50	50	-	-	20	10	50
FGF2	ng/mL	100	50	3	-	10	5	-
BMP4	ng/mL	-	50	-	-	5	10	-
hEGF	ng/mL	-	-	10	-	-	-	-
ActivinA	ng/mL	-	-	-	-	5	-	-
Erythropoietin	U/mL	2	-	-	-	-	-	-
Interleukin 6	ng/mL	10	-	-	-	-	-	-
BHT	µg/mL	-	-	-	-	200	-	-
L-glutamin		-	2mM	-	1%	1%	1%	2mM
NE amino acids		-	0.1mM	-	-	-	1%	100X
Hydrocortisone	µg/mL	-	-	1	-	-	-	-
Heparin	µg/mL	-	-	10	-	-	-	-
Ascorbic acid	mM	-	-	-	-	0.5	-	-
β-mercaptoethanol		-	-	-	50µM	0.1%	1%	50µM
ESC qualified nucleotides		-	-	-	-	-	-	100X
References		[25, 26]	[27]	[28]	[20]	[13]	[13]	[29]

Abbreviations: α-MEM, minimum essential medium (sigma); BHT, Bovine holo-transferrin; BMP, bone morphogenic protein; Col-I, collagen type-I; Col-IV, collagen type-IV; DMEM, Dulbecco's modified Eagle's medium; ESC, embryonic stem cell; F12, Ham's F12 medium; Fbn, fibronectin; FBS, fetal bovine serum, BIT, combined bovine serum albumin, insulin and transferrin (Stem Cell Technologies); FGF, fibroblast growth factor; hEGF, human epidermal growth factor; IMDM, Iscove's modified Dulbecco's medium; Lv-EGM, large-vessel endothelial growth medium; MEF, mouse embryonic fibroblasts; NE, non-essential amino acids; PEC, primary endothelial cells; PS, penicillin streptomycin mix; SP-34, Stem Pro-34 medium (Invitrogen); VEGF, vascular endothelial growth factor; grey boxes indicate the base medium; -, not added.

Mouse ESCs cultured on collagen type-IV substrate have been shown to differentiate along the mesodermal lineage with higher efficiency compared to EB culture [30]. In living embryos the mesodermal cells differentiate into endothelial and hematopoietic progenitor cells [12]. In *in vitro* studies, collagen type-IV has been successfully used for differentiation of endothelial cells from mouse ESCs [20]. As an alternative to collagen type-IV, gelatin has also been used as a substratum for mouse ESCs to induce endothelial differentiation [20]. The differentiated endothelial cells are identified using specific antibodies to stain $CD31^+$, $CD34^+$, or VE-cadherin$^+$ cells [31]. These cells are then enriched by florescence cells sorting or by other means e.g. magnetic beads, which isolates the stained cells from the rest of the cells [32]. Using the same strategy, vascular progenitor cells have been derived from mouse ESCs using *flk1* marker [33].

Various ESC cultures have been utilized to develop endothelial cell cultures using differentiating media (Table 1). ESCs from the mouse and other mammals are usually used for these studies [21]. However, it is desirable to develop alternative models in order to reduce the use of mammals in research. Zebrafish can be an excellent model for various cell culture applications because there is no need to sacrifice the mother to get embryos, as would be the case in mice. Also, the zebrafish embryo model provides easy and large-scale availability of embryos for cell isolation and comparatively simple conditions required for cell culture [34].

Beside the general advantages of zebrafish for cell culture applications, there are transgenic zebrafish lines that express green fluorescence protein (GFP) in a specific cell type. Two of the transgenic zebrafish reporter lines: (i) *Kdrl:GFP*, which expresses GFP under the promoter of VEGF receptor (VEGFR2), also known as *flk-1* (fetal liver kinase 1) or *KDR-like* (kinase insert domain receptor like) gene i.e. specifically expressed in endothelial cells [35]; and (ii) *fli:GFP*, which expresses GFP under the promoter of friend leukemia virus integration site 1, i.e. expressed in endothelial, hematopoietic and neural crest cells [36]. Being a relatively new research model, *in vitro* studies on zebrafish hematopoietic and endothelial cells are rare, except for a recently developed zebrafish embryonic stromal trunk cell line that was reported to support proliferation and differentiation of zebrafish hematopoietic stem cells [37].

In this chapter, we examine the development of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish blastocyst cell cultures. We analyse the yield of these cells under different culture conditions. We used different media compositions to test their potential to generate *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in blastocyst cell culture. The three media compositions used were: (i) LDF (combined Lebovitz-15, Dulbecco's modified Eagle's medium and Ham's F-12 medium) medium; (ii) 1:1 mixture of LDF and EGM (endothelial growth medium) medium; and (iii) LDF medium supplemented with endothelial growth supplement. Furthermore, we analyse the effect of different substrates (gelatin and collagen type-I),

and different concentrations of recombinant zebrafish vascular endothelial growth factor ($VEGF_{165}$), on the percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in culture.

Materials and methods

Embryo collection

All the animal experiments were performed according to the Netherland Experiments on Animals Act [38], based on the guidelines on the protection of experimental animals, laid by the Council of European Union [39]. Adult zebrafish were maintained as previously described [40]. Two different transgenic zebrafish lines *fli:GFP* and *kdrl:GFP* were used. To obtain embryos, adult male and female fish, at a proportion of 1 to 1, were transferred to small breeding tanks in the evening. The zebrafish usually laid eggs when the light comes on. The eggs were collected at the bottom of the tank, separated from adults using a cotton mesh.

Embryos were transferred to a temperature controlled room (28 °C) and were distributed in 9cm Petri dishes at a final density of 100 embryos per dish, after removing dead and unfertilized eggs. The embryos were washed thoroughly with clean egg water to remove any debris. These embryos were allowed to develop to the high blastula stage of Kimmel et al. [41] (approximately 3 h after fertilization) at 28 °C.

Sterilization of embryos

The embryos were transferred to a flow cabinet at room temperature for sterilization and cell isolation. For sterilization, the embryos, with the chorion intact, were immersed in 70% ethanol for 10 sec and then in two changes of 0.05% sodium hypochlorite (available chlorine 10-15%, Sigma, catalog 425044) for 4 min each. The sterilization was done according to the procedure described in Ref. [42]. After each immersion in ethanol or sodium hypochlorite, the embryos were rinsed with LDF medium (see below, Media combinations and composition). Finally the embryos were left in 0.5 mL of LDF medium for dechorionation. Before dechorionating, the embryos were observed under a dissecting microscope and any dead or abnormal embryos (with cloudiness in the perivitelline fluid) were removed.

Blastocyst cells isolation and culture

The following steps were all conducted at room temperature. The embryos were dechorionated in LDF medium using sterile No. 5 watchmaker's forceps and then transferred to Eppendorf tubes. The LDF medium was gently triturated with a P-200 Gilson micropipette (Gilson, B.V., Europe: Den Haag) to remove the yolk. The solution was then centrifuged at 300 g for 1 min and the supernatant was discarded. The blastocysts were washed once with CMF-PBS (calcium magnesium free phosphate buffered saline;

Invitrogen catalog 14190) and then dissociated with 1 mL of 0.25% trypsin solution (Invitrogen catalog 15090) containing 1 mM EDTA (ethylenediaminetetraacetic acid). The trypsin solution was gently triturated with a p-1000 Gilson pipette for 2min. The trypsin was inactivated with 0.1 mL FBS (Fetal bovine serum; Invitrogen, 10500) and the cells were isolated by centrifugation at 300 g for 3 min. The cells were washed three times with LDF medium with 10% FBS and re-suspended in 200 µL LDF medium containing 20% FBS. The cells were counted using a heamocytometer and distributed at 17,000 cells per well in a 96-well microtiter plates.

For each of the culture conditions (i.e. media compositions, substrates and vascular endothelial growth factor concentrations, described below) the blastocyst cells were cultured in 6 wells of the 96-well plate. Separate cultures were established for *fli:GFP* and *kdrl:GFP* blastocyst cells. For data collection at each time point (i.e. day 2, 4, 6 and 8), cultures were established in separate 96-well plates. The medium was refreshed on day 4 for cultures that were maintained till days 6 and 8. All the cultures were maintained at 28 °C in a humidified incubator in 0.5% CO_2.

Media combinations and composition

Different media combinations were used to analyse the effect of media composition on the quantification of GFP^+ cells in the cultures. All the media combinations contained LDF and EGM. LDF is a combination of different nutrient media commonly used for zebrafish cell culture [42-46], whereas EGM is usually used to culture human umbilical vein endothelial cells [47, 48]. EGM has also been used for human pluripotent stem cells to induce vascular endothelial cell differentiation [14]. In our preliminary experiments, zebrafish blastocyst cells did not grow well in EGM. Therefore, it was always subsequently used in combination with LDF medium. Three different media combinations (Table 2) were prepared to culture the zebrafish blastocyst cells. After allowing 1 h to the cells distributed in 96-well plate (as described above), each of the medium combination was added to separate wells. The cultures were maintained until data collection (see below)

Culture substrates

In this experiment different substrates namely gelatin from porcine skin (Sigma, Cat. No. G1890); and collagen type-I rat protein (Invitrogen, Cat. No. A1048301) were used to culture the *fli:GFP* or *kdrl:GFP* blastocyst cells. In addition, cultures were established on polystyrene surface of the tissue culture plate, without any extra substrate coating, for comparison. The percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells was evaluated in cell isolates from cultures established on these substrates on subsequent days. Gelatin was used at a concentration of 0.1 mg/cm^2 to coat the culture wells. Each well of the 96-well plate was coated with 1.7 µL of 2% gelatin solution and allowed to air dry for 1 h before the cells were plated.

Table 2. Media compositions used to optimize culture conditions for the growth of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cell culture.

Media composition (supplier, catalog number)	Final concentrations
LDF medium	
Lebovitz L-15 (Invitrogen, 11415) : DMEM (Invitrogen, 11966) : Ham's F-12 medium (Invitrogen, 21765)	55 : 32.5 : 12.5
HEPES	15 mM
Antibiotic/antimycotic mix (Invitrogen, 15240)	1%
$NaHCO_3$	0.015%
Fetal bovine serum (Invitrogen, 10500)	15%
Zebrafish embryo extract	50 µg/mL
Basic fibroblast growth factor (Invitrogen, PHG0024)	10 ng/mL
Recombinant zebrafish $VEGF_{165}$ (R&D systems, 1247-ZV)	10 ng/mL
LDF:EGM medium	
LDF medium	50%
Endothelial growth medium 2 (Promocell, C-22011)	50%
Recombinant zebrafish $VEGF_{165}$	5 ng/mL
FBS	7.5%
ZEE	25 µg/mL
LDF:EGS medium	
LDF medium	95.8%
Endothelial growth supplement mix (Promocell, C-39216)	4.1%
Recombinant zebrafish $VEGF_{165}$	10 ng/mL

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; HEPES, (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid); VEGF, vascular endothelial growth factor.

Collagen type-I was used at a concentration of 3 mg/mL. To coat the wells, the collagen type-I solution was neutralized using 7.5% sodium bicarbonate, and then added at 5 µL per well in a 96-well plate. The plate was incubated at 37 °C for 1 h to promote gel formation. The wells were then rinsed with 1X CMF-PBS before adding the cells. The blastocyst cells distributed in the pre-coated wells (with gelatin or Collagen type-I) were allowed to attach to the substrate for 1 h. The LDF:EGS medium was then added at 250 µL per well. The cultures were subjected to cell isolation and data collection at subsequent time points (see below).

Vascular endothelial growth factor

In this experiment the effect of recombinant zebrafish $VEGF_{165}$ was evaluated on the percentage of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells in blastocyst cell culture. The LDF:EGS medium was used as the basal medium for this experiment. This medium was further supplemented with different concentrations (0, 10, 20 and 40 ng/mL) of zebrafish $VEGF_{165}$. Media with different concentrations of VEGF was added to separate wells of the 96-well plate pre-distributed with blastocyst cells derived from *fli:GFP* or *kdrl:GFP* embryos. The cultures were isolated from the wells at days 2, 4, 6 and 8 and counted for GFP^{+} and GFP^{-} as described below.

Isolation of cultured cells for data collection

The percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells, total number of cells harvested per well and cell viability was calculated in cell isolates from the cultures at subsequent time-points. To isolate the cells, the medium was aspirated from each well. The wells were then rinsed with 200 µL of 1X CMF-PBS. Then 250 µL of 0.05% trypsin solution containing 1mM EDTA was added to each well. The solution was triturated in the wells several times and then the plate was incubated for 2 min at 28 °C. The degree of detachment was monitored under an inverted microscope. When most cells were rounded up, the trypsin was inactivated by adding 25 µL of FBS, and the cell suspension from each well was transferred to separate 1.5 mL Eppendorf tubes. The suspension was centrifuged at 300 g for 3 min and the supernatant discarded. The cell pellet was washed twice with LDF medium (without growth factors) and re-suspended in 15-20 µL of the same medium. These cells were used for further analysis.

Cell counts and data collection

For the cultures maintained in different media compositions (i.e. LDF, LDF:EGM and LDF:EGS media), the total number of cells harvested per well and the cell viability was calculated in addition to the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells. For other conditions (i.e. different substrates and VEGF concentrations) only the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells was calculated in cell isolates. The total number of cells harvested per well was estimated by counting the cells in a heamocytometer. Viability of the cells was determined by using trypan blue exclusion dye (0.4% trypan blue in CMF-PBS) to differentiate viable and non-viable cells.

The percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in the living cell suspensions was calculated using a confocal microscope. A 5 µL drop from each of the cell isolates was transferred to a cover glass slide. The slide was placed under the confocal microscope and the cells were allowed to settle for 30 s. An image was then captured from the center of each drop. Each image was taken in duplex, one fluorescence image showing GFP$^+$ cells and one phase contrast image showing all the cells. For both *fli:GFP* and *kdrl:GFP* cultures, number of GFP$^+$ and GFP$^-$ cells per microscopic field (image) were then counted in image J software. From these counts the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells was calculated for each sample.

Statistical analysis

Number of EBs formed, total number of cells harvested, percentage of viable cells and the percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells per well, collected from 6 replicate wells for each culture condition, were analysed for means and standard errors using SPSS software version 21.0. One way ANOVA was performed to calculate the probability values to analyse differences between different culture conditions, as well as differences at different

time-points at the same culture condition. Pair-wise comparison was performed using Post-Hoc Tukey's test.

Results

Effect of medium composition on zebrafish blastocyst cell culture

Embryoid body formation

The zebrafish blastocyst cells formed EB aggregates within the first 24 hours of culture, containing differentiated *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells. Later these EB aggregates become mature and the percentage of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells within it varied by maintaining the cultures in different media (following sections). The number of EBs formed was higher in LDF:EGM and LDF:EGS media compared to LDF medium (Figure 1A). The highest number of EBs per well were formed in LDF:EGS medium (12.5 ± 0.9) followed by LDF:EGM (10.1 ± 0.6) and LDF (7.1 ± 0.5) media. The average size per EB was smaller in LDF:EGM and LDF:EGS media compared to LDF medium (Figure 1B).

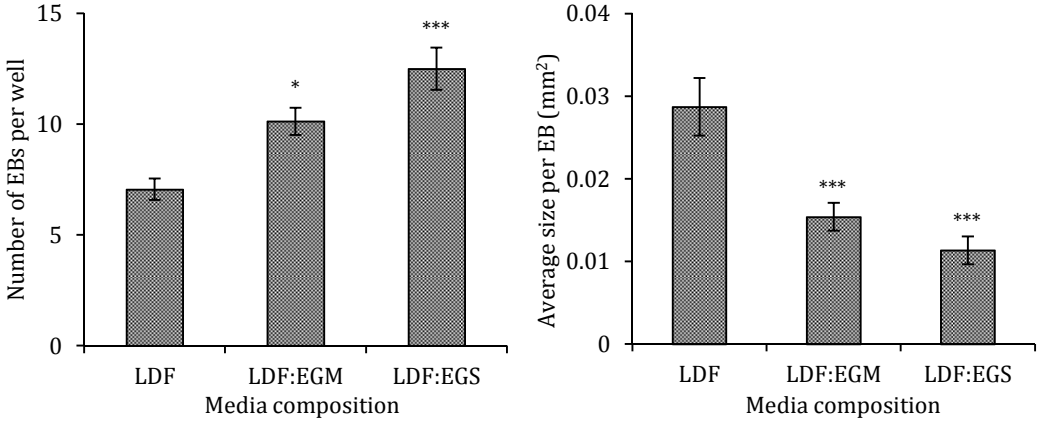

Figure 1: Formation of embryoid bodies (EBs) from zebrafish blastocyst cells cultured in different media. Data obtained at day 4 of culture. (**A**) Total number of EBs per well. (**B**) Average area (mm^2) covered by one EB formed in different media. Error bars represent standard error. (***, $p < 0.001$, *, $p < 0.05$ compared to LDF medium).

Quantification of *fli:GFP*$^{+}$ cells per medium

Different media compositions used to culture zebrafish blastocyst cells showed significant differences in the percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ in cultures (Figure 2). The percentage of *fli:GFP*$^{+}$ cells in cultures on LDF:EGM was significantly higher than cultures on LDF medium at each of the time-points (Figure 2C). No significant differences in the percentage of *fli:GFP*$^{+}$ cells were observed between LDF:EGM and LDF:EGS media except on day 6, where a higher percentage of *fli:GFP*$^{+}$ cells were found in LDF:EGM medium.

Compared to LDF medium, cells cultured in LDF:EGS medium showed significantly higher percentage of *fli:GFP*$^+$ cells on days 4 and 6. However, on days 2 and 8 the differences were not significant.

Quantification of *fli:GFP*$^+$ with duration of culture

In LDF medium the percentage of *fli:GFP*$^+$ cells increased gradually from day 2 to day 8 ($p<0.05$), with no significant increase until day 6. In LDF:EGM and LDF:EGS media the percentage of *fli:GFP*$^+$ cells increase significantly from day 2 to day 4 ($p<0.05$ and 0.01, respectively), with no significant variations after day 4 till day 8. The highest percentage of *fli:GFP*$^+$ cells (39.2 ± 1.1%) was found in cells cultured in LDF:EGM medium on day 6.

In a preliminary experiment blastocyst cell cultures maintained for 14 days in LDF:EGS medium contained 21.9 ± 2.3% of *fli:GFP*$^+$ cells. However, when these cells were sub-cultured, the number of *fli:GFP*$^+$ cells could be maintained in the secondary cultures.

Quantification of *kdrl:GFP*$^+$ cells per medium

Similar to *fli:GFP*$^+$ cells, a higher percentage of *kdrl:GFP*$^+$ cells was found in cultures maintained on LDF:EGS and LDF:EGM media, compared to LDF medium (Figure 2D). However, the percentage of *kdrl:GFP*$^+$ cells in culture was much less than *fli:GFP*$^+$ cells. The percentage of *kdrl:GFP*$^+$ cells was similar in different media composition until day 4 of culture. After day 4 the cultures maintained on LDF medium contained significantly lower percentage of *kdrl:GFP*$^+$ cells compared to cultures on LDF:EGS medium. The percentage of *kdrl:GFP*$^+$ cells in cultures maintained with LDF:EGS medium was slightly higher compared to LDF:EGM medium; however the differences were not significant.

Quantification of *kdrl:GFP*$^+$ cells with duration of culture

The quantification of *kdrl:GFP*$^+$ cells at subsequent time points showed a slight increase in percent *kdrl:GFP*$^+$ cells from day 2 to day 4 and then a decrease after day 4. In LDF medium the percentage of *kdrl:GFP*$^+$ cells dropped significantly from day 4 to day 6 ($p<0.01$) and then continued to decline until day 8. Similarly, in the LDF:EGM and LDF:EGS media, the percentage of *kdrl:GFP*$^+$ cells decreased significantly from day 4 to day 8 ($p<0.05$). The highest percentage of *kdrl:GFP*$^+$ cells was found in cultures maintained on LDF:EGS medium on day 4 of culture (4.2 ± 0.3%). In general, *kdrl:GFP*$^+$ cells could be observed in all different culture conditions for a maximum of 10 days.

Total number of cells harvested per medium

The total number of cells harvested from cultures grown in different media compositions were the lowest in LDF:EGM cultures (Figure 3A). On day 2 of culture, the total number of cells isolated from cultures maintained on LDF:EGS medium was higher than cultures on LDF and LDF:EGM medium. After day 2, the total number of cells declined until day 6 in all media compositions; it then increased again.

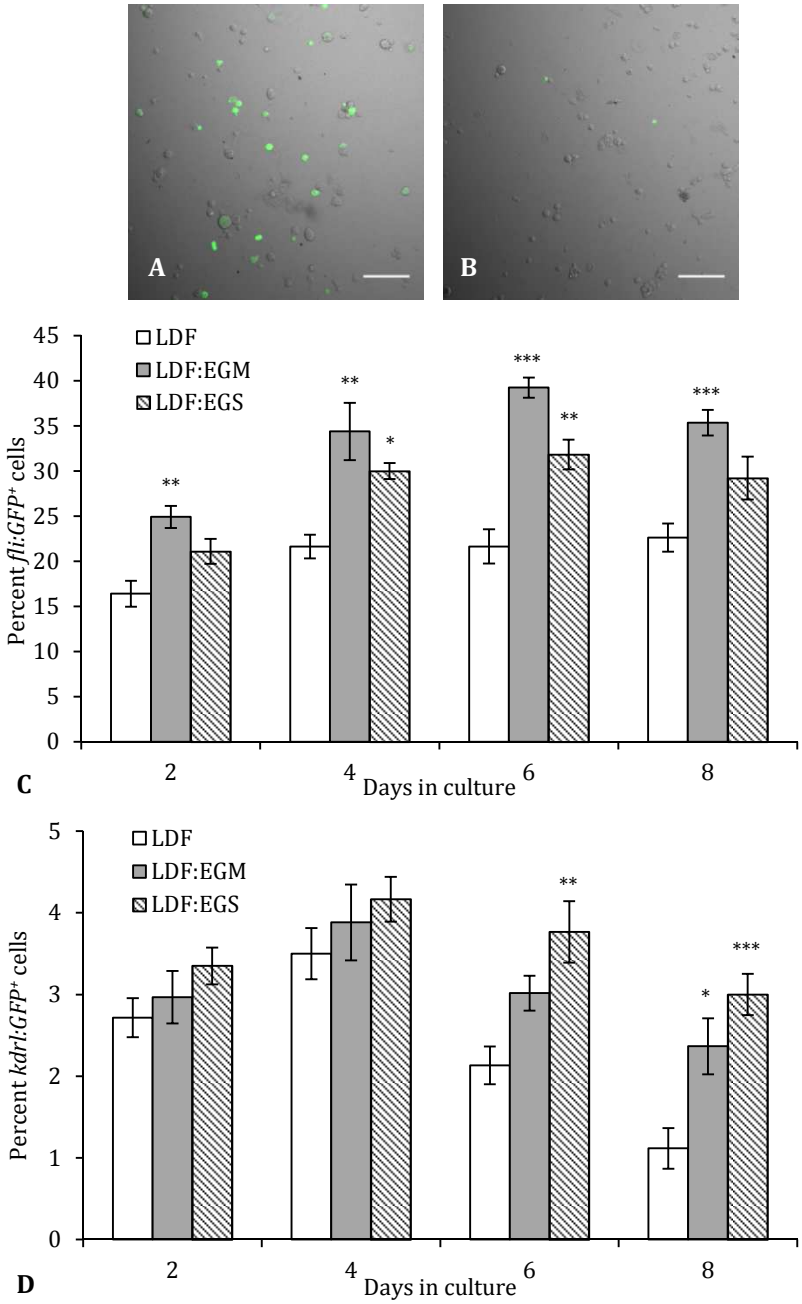

Figure 2: Percent quantification of *fli:GFP*⁺ and *kdrl:GFP*⁺ cells in blastocyst cell cultures maintained on different media. (**A and B**) Overlaid confocal and phase contrast images of cells isolated from *fli:GFP* (**A**) and *kdrl:GFP* (**B**) blastocyst cell culture. Cells in green shows *fli:GFP*⁺ or *kdrl:GFP*⁺ cells. Scale bar, 100 µm. (**C**) Quantification of *fli:GFP*⁺ cells in cells isolated from cultures, on subsequent days. (**D**) Quantification of *kdrl:GFP*⁺ cells in different media composition. All the media were supplemented with 15% FBS, 10 ng/mL FGF and 10 ng/mL zebrafish $VEGF_{165}$. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to LDF medium).

On day 4 the cell counts in LDF and LDF:EGS media were similar and both higher than LDF:EGM medium. No significant differences were found in cell counts isolated from cultures on day 6. After day 6 the cell counts in LDF medium increased significantly compared to LDF:EGM and LDF:EGS media. No significant differences were observed in the cell viability of cultures maintained in different media composition except day 6 (Figure 3B), where the cell viability was significantly higher in LDF:EGS medium.

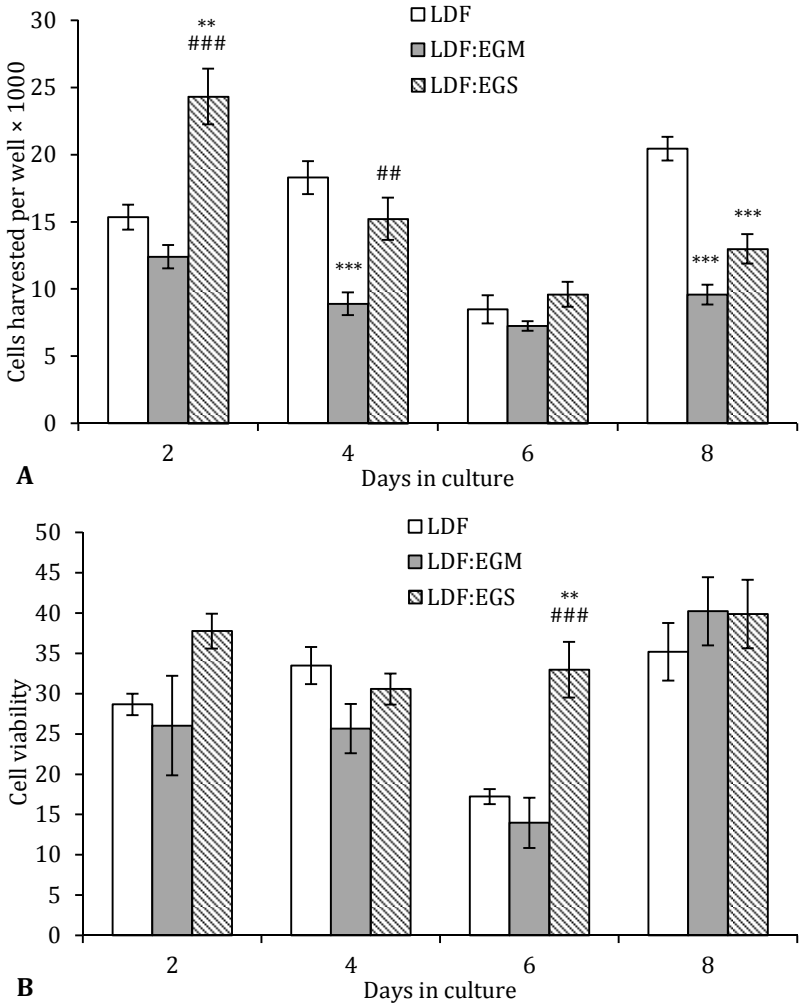

Figure 3: Total number and viability of isolated cells from cultures maintained on different media composition. (**A**) Total number of cells harvested per well at subsequent time-points. (**B**) Viability of isolated cells. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$ compared to LDF medium; ###, $p < 0.001$, ##, $p < 0.01$ compared to LDF:EGM medium).

Effect of substratum on blastocyst cell culture

Percentage of *fli:GFP*$^{+}$ cells in cultures on different substrate

The confocal images of cultures showed spreading out of *fli:GFP*$^{+}$ cells from EBs on collagen type-I and polystyrene substrate (Figure 4). However, on gelatin substratum the *fli:GFP*$^{+}$ cells remained on the periphery of the EBs. The percentage of *fli:GFP*$^{+}$ cells in cultures differed significantly between gelatin and collagen type-I substrates, such that collagen type-I was more favorable. Compared to the uncoated polystyrene surface, the percentage of *fli:GFP*$^{+}$ cells was slightly lower on gelatin substratum and slightly higher on collagen type-I substratum (Figure 5A). The percentage of *fli:GFP*$^{+}$ cells in cells isolated on day 8 of the cultures was significantly higher on collagen type-I (30.6 ± 2.0%) compared to gelatin (20.9 ± 1.6%) substratum (Figure 5A).

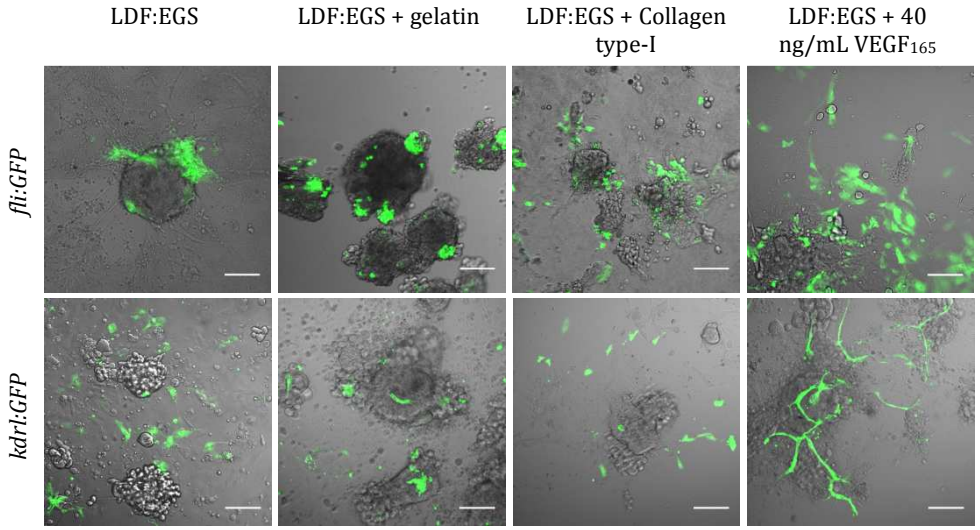

Figure 4: Induction of *fli:GFP*$^{+}$ cells and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cells cultured in different conditions. The morphological differences between *fli:GFP* and *kdrl:GFP* cells can be observed. The *fli:GFP*$^{+}$ cells develop in the form of a layer around the EBs in cultures. The *kdrl:GFP*$^{+}$ cells are more longitudinal and develop in the form of elongated structures around the EBs. In the presence of $VEGF_{165}$ the *kdrl:GFP*$^{+}$ cells form elongated cord-like structures. images taken on day 6 of cultures. Scale bar 100 µm.

Percentage of *kdrl:GFP*$^{+}$ cells in cultures on different substrate

Similar to *fli:GFP*, the *kdrl:GFP* blastocyst cells cultured on different substrate also showed differences in the percentage of *kdrl:GFP*$^{+}$ cells between gelatin and collagen type-I (Figure 5B). Migration of *kdrl:GFP*$^{+}$ cells from the EBs was observed on collagen type-I and polystyrene substrate, while on gelatin they remained mainly inside the EBs (Figure 4). The results showed significantly higher percentage of *kdrl:GFP*$^{+}$ cells in cultures maintained on collagen type-I substratum compared to gelatin substratum at different

time-point (day 2, day 4 and day 6; Figure 5B). No significant differences in the percentage of *kdrl:GFP*$^{+}$ cells was observed between cultures maintained on collagen type-I and polystyrene substratum. Compared to gelatin the percentage of *kdrl:GFP*$^{+}$ cells was higher in cultures on polystyrene substratum; however, the differences were not significant except on day 6 (Figure 5B).

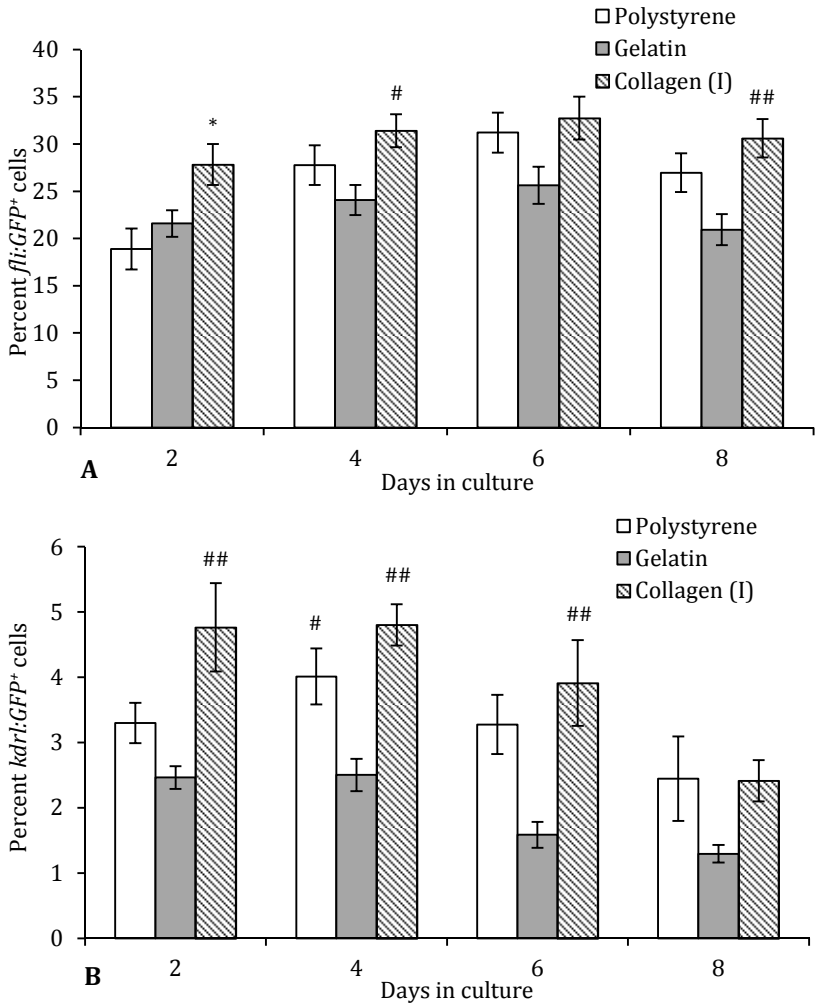

Figure 5: Percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures maintained on different substrates. (**A**) Percentage of *fli:*GFP^{+} cells in cells isolated from cultures on different substrates on subsequent days. (**B**) Percentage of *kdrl:GFP*$^{+}$ cells found in cultures on different substrates. Error bars represent standard error. (*, $p < 0.05$ compared to polystyrene surface; ##, $p < 0.01$, #, $p < 0.05$ compared to gelatin substratum).

Effect of zebrafish vascular endothelial growth factor

VEGF increases the percentage of *fli:GFP*$^+$ in culture

Zebrafish $VEGF_{165}$ protein showed significant effect on the percentage of *fli:GFP*$^+$ cells in the blastocyst cell cultures (Figure 6A). Cultures without $VEGF_{165}$ contained significantly less percentage of *fli:GFP*$^+$ cells on all time-points compared to cultures with $VEGF_{165}$. On day 2 no significant differences were observed in the percentage of *fli:GFP*$^+$ cells in cultures grown in media with different $VEGF_{165}$ concentrations (10, 20 and 40 ng/mL). On days 4, 6 and 8 the cultures maintained on 40 ng/mL $VEGF_{165}$ contained significantly higher percentage of *fli:GFP*$^+$ cells compared to cultures with 10 ng/mL $VEGF_{165}$. The only significant difference in the percentage of *fli:GFP*$^+$ cells between 10 and 20 ng/mL $VEGF_{165}$ was observed on day 8. No significant differences were observed between cultures maintained with 20 and 40 ng/mL $VEGF_{165}$ (Figure 6A).

In the absence of $VEGF_{165}$ the percentage of *fli:GFP*$^+$ cells increased from day 2 to 4 ($p<0.05$) and then decreased until day 8 ($p<0.01$). At 10 and 20 ng/mL $VEGF_{165}$ the percent *fli:GFP*$^+$ cells increased significantly from day 2 to 4 ($p<0.001$) and then remained similar until day 8 of culture. The percentage of *fli:GFP*$^+$ cells in cultures with 40 ng/mL $VEGF_{165}$ continued to increase until day 6 ($p<0.001$ for day 2 to 4; $P<0.05$ for day 4 to 6).

VEGF increases the percentage of *kdrl:GFP*$^+$ cells in culture

The *kdrl:GFP*$^+$ cells formed vascular cord-like structures in the presence of $VEGF_{165}$ in the culture medium (Figure 4). On day 2 the percentage of *kdrl:GFP*$^+$ cells was similar in cultures maintained with or without $VEGF_{165}$ in the medium (Figure 6B). An increase was observed in the percentage of *kdrl:GFP*$^+$ cells from day 2 to day 4 at all $VEGF_{165}$ concentrations. On day 4, cultures at 40 ng/mL $VEGF_{165}$ contained a significantly higher percentage of *kdrl:GFP*$^+$ cells compared to cultures without $VEGF_{165}$. No differences were observed in cultures with 10 and 20 and 40 ng/mL $VEGF_{165}$ in the medium on day 4 of culture.

After day 4 the percentage of *kdrl:GFP*$^+$ cells started to decrease in cultures. This decrease was significant in cultures without $VEGF_{165}$ from day 4 to day 6 ($p<0.01$). Similarly, the cultures with 10 ng/mL $VEGF_{165}$ in the medium showed a gradual decrease in the percentage of *kdrl:GFP*$^+$ cells from day 4 to day 8 ($p<0.05$). Cultures maintained with 20 and 40 ng/mL $VEGF_{165}$ showed a similar percentage of *kdrl:GFP*$^+$ cells after day 4 till day 8. On days 6 and 8 cultures with different concentrations of $VEGF_{165}$ showed significantly higher percentage of *kdrl:GFP*$^+$ cells compared to cultures without $VEGF_{165}$. Furthermore, the percentage of *kdrl:GFP*$^+$ cells was higher in cultures with 40 ng/mL $VEGF_{165}$ compared to cultures with 10 ng/mL $VEGF_{165}$ on days 6 and 8.

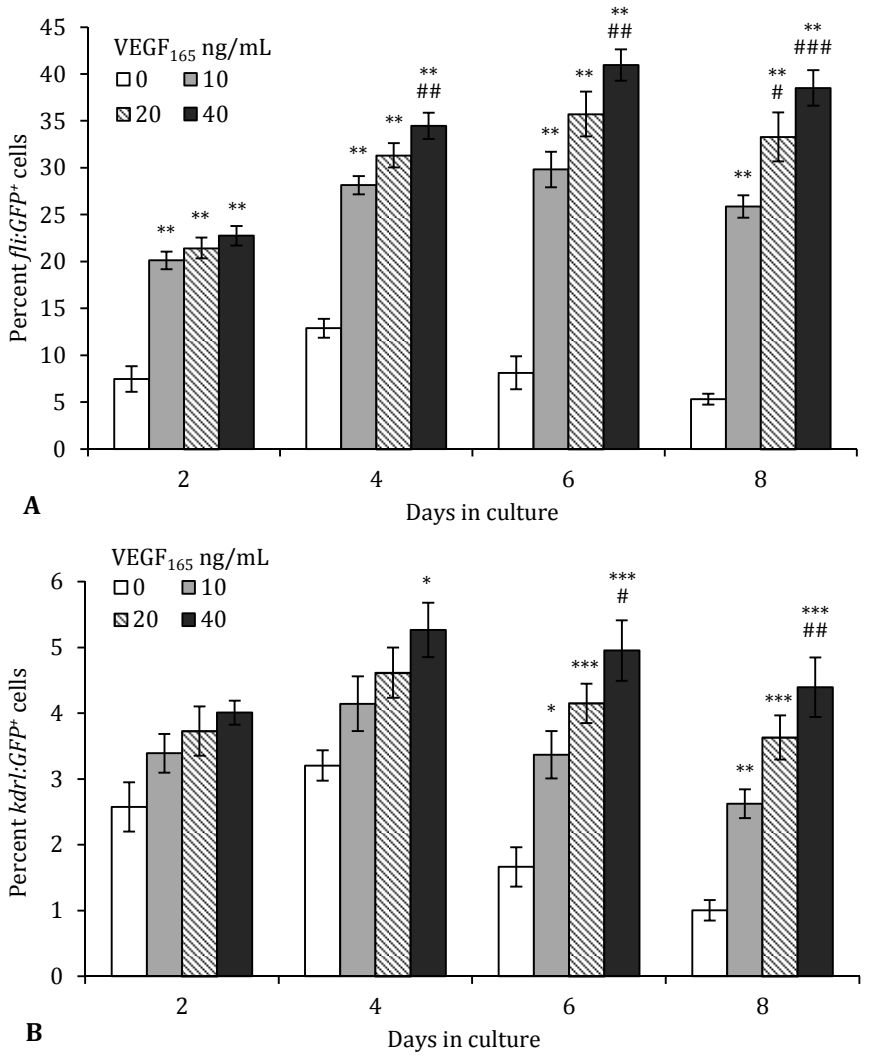

Figure 6: Effect of different concentrations of zebrafish $VEGF_{165}$ on the percentage of *fli:GFP*⁺ and *kdrl:GFP*⁺ cells in blastocyst cell culture. (**A**) Percentage of *fli:GFP*⁺ cells in cultures with different $VEGF_{165}$ concentrations at subsequent time points. (**B**) Percentage of *kdrl:GFP*⁺ cells in cultures with different $VEGF_{165}$ concentrations. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to culture without $VEGF_{165}$; ###, $p < 0.001$, ##, $p < 0.01$, #, $p < 0.05$ compared to culture with 10 ng/mL $VEGF_{165}$).

Discussion

Relationship of culture conditions, EB formation and *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cell quantification in culture

We have investigated different culture conditions for zebrafish blastocyst cells, with the objective to generate differentiated *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in relatively high numbers. The results showed important information for a controlled differentiation process in zebrafish blastocyst cell cultures. Studies have shown that without any anti-differentiation factors or feeder cells mouse and human ESCs undergo cellular differentiation (similar in some respects to embryogenesis) and form EBs [49]. In this study, we found that medium composition affects the development of EBs and the percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish blastocyst cell cultures.

Study on mouse ESC culture have shown that EBs developed in adherent cultures contained a higher number of total cells and a lower percentage of hematopoietic and endothelial cells, compared to the EBs grown in suspension cultures [50]. In our study, zebrafish blastocyst cells cultured in LDF medium developed a few, large-sized EBs, while cells cultured in LDF:EGM and LDF:EGS media developed more numerous, but smaller EBs with a higher percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells. These results suggest a direct relation between the number of EBs and the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells.

Previous studies have shown that the relative less cell-to-cell interaction, in the adherent cultures compared to 3D or suspension cultures, may favor the growth or differentiation of cell types other than hematopoietic and endothelial cells [50]. This conclusion is supported by our hanging-drop experiments in which there is by definition no outgrowth, but there is a high percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells (see Chapter 5). In our experiments described in this chapter, the supplementation of the LDF medium with endothelial growth supplement resulted in higher percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in cultures. This could be because the supplementation leads to increased cell-cell-interactions in the plated blastocyst cells as they are aggregating into EBs.

Differences between *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in culture

The blastocyst cell cultures initiated from *fli:GFP* embryos in our experiments expressed GFP in a higher percentage of cells compare to *kdrl:GFP*. This might be explained by the lineage-specificity of GFP expression in *kdrl:GFP* transgenic lines (endothelial cells only [51]), compared to the *fli:GFP* line (endothelial, lymphatic, hematopoietic and neural crest cells [36]). The *kdrl:GFP*$^+$ cells almost vanished by 8 days of culture, a similar phenomenon was recorded in a recent study using *kdrl:GFP* blastocyst cell culture for screening angiogenic compounds [52].

The *fli:GFP* signals by contrast, could be observed for up to 14 days. However, they also could not be maintained after that. This can be considered in the light of previous studies, describing *fli:GFP* expression persists in zebrafish embryos during early developmental stages [36]. Similarly, studies on mouse embryos and ESCs have shown significant reduction in the expression levels of *flk1/kdr* gene at later life stages [53]. These reports suggest as the cells matures in our zebrafish blastocyst cell cultures, they down regulate the transgenes, as these genes are expressed mainly in early stages of differentiation. However, other studies have reported that the expression of *fli1* and *kdrl* genes persists throughout vascular development in zebrafish [51, 54]. One possible explanation can be changes in gene expression and properties of endothelial cells, in our study, particularly *in vitro*, as previously described for other endothelial cells [55].

Studies have shown that primary endothelial cells have a short life span, which is usually overcome by immortalizing these cells using genetic manipulation [56]. Thus, an alternative explanation for the loss of *fli:GFP* and *kdrl:GFP* signals may be the occurrence of cell death in our primary blastocyst cell culture. This phenomena was observed as high number of floating cells in culture and long population doubling time of the primary blastocyst cell culture, discussed in chapter 3. A previous study also reported the loss of *kdrl:GFP* signal in zebrafish blastocyst cell culture due to apoptosis [57]. Another reason for this may be the development of fast growing fibroblast-like cells in our cultures, which depleted other cell types in subsequent passages (Chapter 3).

Media composition and endothelial growth supplementation

LDF is a commonly-used medium for zebrafish ESC culture [42-46]. It is also sometimes referred to as limiting dilution factor (LDF) medium, used to retain the homogeneity of a cell culture [43, 58]. The zebrafish primary blastocyst cells may have more complex nutrient requirements for their growth and attachment therefore supplements including FBS, fish embryo extract, fish serum and bFGF are usually added to the medium [59]. A nutrient rich medium is required to culture the primary blastocyst cells as the initial cell death is high in these cells because of bleaching the embryos [60]. The blastocyst cells are pluripotent in nature, therefore specific differentiation can be promoted by changing the culture conditions [34]. In some experiments additional supplementation or a substrate coating may be required to induce specific differentiation in these pluripotent ESCs. Examples include sonic hedgehog protein for myocyte differentiation [61], and poly-D-lysine coating for neurons and astrocytes differentiation [45].

Endothelial growth medium (EGM) is usually used to culture human umbilical vein endothelial cells (HUVECs; [47, 48]), as well as for the differentiation of human pluripotent stem cells into vascular endothelial cells [14]. The complete EGM is a combination of endothelial basal medium and endothelial growth supplement (EGS) mix. The EGS is composed of growth factors including human epidermal growth factor, bFGF,

insulin-like growth factor (IGF-1) and human VEGF. These components are usually used in differentiation media to induce endothelial differentiation in mouse [13, 25] and human [13, 27, 28] ESCs. Other components of EGS are heparin and hydrocortisone, which have been used for endothelial differentiation in human ESCs [28]. Similarly, ascorbic acid (also a component of EGS) have also been used in endothelial differentiation medium for mouse ESC culture [13].

In our experiments, the LDF medium increased the total cell counts after day 6 of the cultures. However, the percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells was significantly lower in this medium compared to LDF:EGM and LDF:EGS media. Previous studies on zebrafish blastocyst cell culture have shown the necessity of a feeder layer of growth arrested stromal cells in combination with LDF medium, to obtain a pluripotent zebrafish ESC culture [42, 44, 62]. Without the support of a feeder layer the blastocyst cells differentiate into EBs (that contain various cell types), and adherent fibroblast-like cells [59]. In further passages only the adherent cell type that suit best or adapt itself to the medium remains in culture [43, 58]. These studies suggest the suitability of LDF medium for growth and differentiation of cells other than *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells. However, we found that the addition of EGS and $VEGF_{165}$ to the LDF medium, increased the percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells compared to cultures in basic LDF medium.

When combined with the EGM, the LDF:EGM (1:1) medium increased the percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures. However, the total cells counts were significantly less in LDF:EGM medium compared to LDF medium. One of the reasons for this can be that EGM is an optimized medium for *human* endothelial cell lines [47, 48], and not for primary zebrafish cells. Therefore under an optimum medium condition (which is LDF for zebrafish cells) the cells grow at a normal rate. However, when combined with a non-specific medium (EGM in this case), the nutrient balance in the media changes and this then causes the slower growth rate of cultures in our study. On the other hand, EGM contains constituents to support endothelial cells, that may stimulate zebrafish blastocyst cells to differentiate into hematopoietic and endothelial lineages. This is why we obtained higher percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures with LDF:EGM compared to LDF medium.

LDF medium supplemented with endothelial growth supplement (EGS) significantly increased the percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in our experiments as expected. As noted above, LDF is defined as a standard medium in many zebrafish cell culture procedures [42-46]. By using the LDF:EGS medium for our experiments, the full strength of LDF medium was ensured for the optimum growth of the cell cultures, which we quantified by total cell counts. The EGS contains the necessary factors required for the growth of endothelial and hematopoietic cells as discussed above. Therefore, the combination of LDF medium and EGS, represents a medium that can support cell growth

and viability, while at the same time induces maximum differentiation of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures.

Culture substrate for endothelial differentiation

Extracellular matrix (ECM) is an important component of tissues *in vivo*, which directly interact with cells by cell receptors and support their growth and differentiation [63]. Different tissues possess ECM of differing composition and physical properties (stiffness, elasticity etc.), that influences the behavior and differentiation of cells in that tissue [63]. The same principle applies to cells *in vitro*. Different ECM substrates have been identified to direct differentiation of ESCs towards different cell lineages, as is reviewed in Ref. [64]. Some ECM substrates including collagen type-I [25, 26], collagen type-IV [20], and gelatin [13, 29], have been used to stimulate endothelial differentiation in mouse ESCs. Fibronectin has been used to promote the differentiation of human ESCs along endothelial lineage [28].

In our experiments the blastocyst cells cultured on collagen type-I substratum contained higher percentage of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells, compared to gelatin substratum. The percentages of these cells were slightly higher on collagen type-I compared to polystyrene substratum. The only significant difference in percentage of *fli:GFP*$^{+}$ cells between collagen type-I and polystyrene substrate was observed on day 2 of culture. A similar phenomena was observed in *kdrl:GFP* cultures. Where the percentage of *kdrl:GFP*$^{+}$ cells was higher on collagen type-I substratum compared to gelatin; however, the differences were not significant compared to polystyrene.

This may suggests that collagen type-I is not necessary for the differentiation of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish. However, based on our results collagen type-I is favorable compared to gelatin substratum for the differentiation of these cells. Previous research on zebrafish ECM have shown the production of fibronectin and laminin in early developing embryos, and the synthesis of collagen at later stages [65]. Similarly, other studies describe the role of collagen type-I in formation of tubular blood vessels from endothelial cells at advance developmental stages [66]. This may explain why collagen type-I did not show significant effect on the percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cell culture in our study.

Zebrafish vascular endothelial growth factor

VEGF is a known factor for the differentiation and growth of endothelial cells in early embryogenesis, as well as for the development of vascular networks in embryos and adult tissues [67]. VEGF has also been identified to increase endothelial differentiation in human ESC culture [68]. In our experiments, on day 6 of cultures the percentages of *fli:GFP*$^{+}$ cells was 5.0 fold higher, and *kdrl:GFP*$^{+}$ cells 2.9 fold higher, in cultures with 40 ng/mL $VEGF_{165}$ compared to cultures without $VEGF_{165}$ in medium. These results are

comparable with a previous study on human ESCs where VEGF at 50 ng/mL has been reported to increase endothelial differentiation by 4.7 folds [68].

Conclusions

The growth of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish blastocyst cell culture can be increased by manipulating culture conditions. Supplementation of cell culture medium with EGS (used as medium supplementation for human endothelial cell cultures) increases the percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish blastocyst cell culture. Collagen type-I substratum should be preferred over gelatin for the differentiation of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish blastocyst cell culture. However, compared to polystyrene substratum the effect of collagen is not significant for this purpose. Recombinant zebrafish $VEGF_{165}$ also increases the percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in culture.

References

[1] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS*, et al.* Embryonic stem cell lines derived from human blastocysts. Science 1998; 282(5391): 1145-7.

[2] Chen J, Zhou L, Pan SY. A brief review of recent advances in stem cell biology. Neural Regen Res 2014; 9(7): 684-7.

[3] Schroeder IS. Stem cells: are we ready for therapy? Methods Mol Biol 2014; 1213: 3-21.

[4] Rossant J, Bernelot-Moens C, Nagy A. Genome manipulation in embryonic stem cells. Philos Trans R Soc Lond B Biol Sci 1993; 339(1288): 207-15.

[5] Pirity M, Hadjantonakis AK, Nagy A. Embryonic stem cells, creating transgenic animals. Methods Cell Biol 1998; 57: 279-93.

[6] Yu GY, Cao T, Ouyang HW, Peng SQ, Deng XL, Li SL*, et al.* Development of human embryonic stem cell model for toxicity evaluation. Beijing Da Xue Xue Bao 2013; 45(1): 9-11.

[7] Lam AT, Chen AK, Li J, Birch WR, Reuveny S, Oh SK. Conjoint propagation and differentiation of human embryonic stem cells to cardiomyocytes in a defined microcarrier spinner culture. Stem Cell Res Ther 2014; 5(5): 110.

[8] Liao MC, Diaconu M, Monecke S, Collombat P, Timaeus C, Kuhlmann T*, et al.* Embryonic stem cell-derived neural progenitors as non-tumorigenic source for dopaminergic neurons. World J Stem Cells 2014; 6(2): 248-55.

[9] Thompson HL, McLelland BT, Manilay JO. Indirect immune recognition of mouse embryonic stem cell-derived hematopoietic progenitors *in vitro*. Exp Hematol 2014; 42(5): 347-359 e5.

[10] Sun H, Quan Y, Yan Q, Peng X, Mao Z, Wetsel RA*, et al.* Isolation and characterization of alveolar epithelial type II cells derived from mouse embryonic stem cells. Tissue Eng Part C Methods 2014; 20(6): 464-72.

[11] Nishikawa S. Embryonic stem cells as a source of hematopoietic and vascular endothelial cells *in vitro*. J Allergy Clin Immunol 1997; 100(6 Pt 2): S102-4.
[12] Risau W. Differentiation of endothelium. FASEB J 1995; 9(10): 926-33.
[13] Nolan DJ, Ginsberg M, Israely E, Palikuqi B, Poulos MG, James D*, et al.* Molecular signatures of tissue-specific microvascular endothelial cell heterogeneity in organ maintenance and regeneration. Dev Cell 2013; 26(2): 204-19.
[14] Kusuma S, Shen YI, Hanjaya-Putra D, Mali P, Cheng L, Gerecht S. Self-organized vascular networks from human pluripotent stem cells in a synthetic matrix. Proc Natl Acad Sci U S A 2013; 110(31): 12601-6.
[15] Costa-Almeida R, Granja PL, Soares R, Guerreiro SG. Cellular strategies to promote vascularisation in tissue engineering applications. Eur Cell Mater 2014; 28: 51-66; discussion 66-7.
[16] Correia C, Grayson WL, Park M, Hutton D, Zhou B, Guo XE*, et al. In vitro* model of vascularized bone: synergizing vascular development and osteogenesis. PLoS One 2011; 6(12): e28352.
[17] Rivron NC, Liu JJ, Rouwkema J, de Boer J, van Blitterswijk CA. Engineering vascularised tissues *in vitro*. Eur Cell Mater 2008; 15: 27-40.
[18] Marcelo KL, Goldie LC, Hirschi KK. Regulation of endothelial cell differentiation and specification. Circ Res 2013; 112(9): 1272-87.
[19] Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. Nature 2005; 438(7070): 937-45.
[20] Blancas AA, Lauer NE, McCloskey KE. Endothelial differentiation of embryonic stem cells. Curr Protoc Stem Cell Biol 2008; Chapter 1: Unit 1F 5.
[21] Bai H, Wang ZZ. Directing human embryonic stem cells to generate vascular progenitor cells. Gene Ther 2008; 15(2): 89-95.
[22] Descamps B, Emanueli C. Vascular differentiation from embryonic stem cells: novel technologies and therapeutic promises. Vascul Pharmacol 2012; 56(5-6): 267-79.
[23] Nishikawa S, Jakt LM, Era T. Embryonic stem-cell culture as a tool for developmental cell biology. Nat Rev Mol Cell Biol 2007; 8(6): 502-7.
[24] Noghero A, Bussolino F, Gualandris A. Role of the microenvironment in the specification of endothelial progenitors derived from embryonic stem cells. Microvasc Res 2010; 79(3): 178-83.
[25] Feraud O, Prandini MH, Vittet D. Vasculogenesis and angiogenesis from *in vitro* differentiation of mouse embryonic stem cells. Methods Enzymol 2003; 365: 214-28.
[26] Feraud O, Cao Y, Vittet D. Embryonic stem cell-derived embryoid bodies development in collagen gels recapitulates sprouting angiogenesis. Lab Invest 2001; 81(12): 1669-81.
[27] Wang ZZ, Au P, Chen T, Shao Y, Daheron LM, Bai H*, et al.* Endothelial cells derived from human embryonic stem cells form durable blood vessels *in vivo*. Nat Biotechnol 2007; 25(3): 317-8.
[28] Kane NM, Meloni M, Spencer HL, Craig MA, Strehl R, Milligan G*, et al.* Derivation of endothelial cells from human embryonic stem cells by directed differentiation: analysis of microRNA and angiogenesis *in vitro* and *in vivo*. Arterioscler Thromb Vasc Biol 2010; 30(7): 1389-97.
[29] Li J, Stuhlmann H. *In vitro* imaging of angiogenesis using embryonic stem cell-derived endothelial cells. Stem Cells Dev 2012; 21(2): 331-42.
[30] Tada S, Era T, Furusawa C, Sakurai H, Nishikawa S, Kinoshita M*, et al.* Characterization of mesendoderm: a diverging point of the definitive endoderm and

mesoderm in embryonic stem cell differentiation culture. Development 2005; 132(19): 4363-4374.
[31] Wilson HK, Canfield SG, Shusta EV, Palecek SP. Concise review: tissue-specific microvascular endothelial cells derived from human pluripotent stem cells. Stem Cells 2014; 32(12): 3037-45.
[32] Yoder MC. Differentiation of pluripotent stem cells into endothelial cells. Curr Opin Hematol 2015; 22(3): 252-7.
[33] Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T*, et al.* Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature 2000; 408(6808): 92-96.
[34] Alvarez MC, Bejar J, Chen S, Hong Y. Fish ES cells and applications to biotechnology. Mar Biotechnol (NY) 2007; 9(2): 117-27.
[35] Renaud O, Herbomel P, Kissa K. Studying cell behavior in whole zebrafish embryos by confocal live imaging: application to hematopoietic stem cells. Nat Protoc 2011; 6(12): 1897-1904.
[36] Lawson ND, Weinstein BM. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 2002; 248(2): 307-318.
[37] Campbell C, Su T, Lau RP, Shah A, Laurie PC, Avalos B*, et al.* Zebrafish embryonic stromal trunk (ZEST) cells support hematopoietic stem and progenitor cell (HSPC) proliferation, survival, and differentiation. Exp Hematol 2015.
[38] Experiments on Animal Act, 2014. The State Secretary of Health and Environmental Protection, Soestdijk. http://wetten.overheid.nl/BWBR0003081/2014-12-18
[39] Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance, 2010. The European Parliament and the council of the European Union, Official Journal of the European Union. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:32010L0063
[40] Westerfield M. The zebrafish book: a guide for the laboratory use of zebrafish (*Danio Rerio*). 4 ed, University of Oregon Press, Eugene 2000, pp.
[41] Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995; 203(3): 253-310.
[42] Fan L, Collodi P. Zebrafish embryonic stem cells. Methods Enzymol 2006; 418: 64-77.
[43] Collodi P, Kamei Y, Ernst T, Miranda C, Buhler DR, Barnes DW. Culture of cells from zebrafish (*Brachydanio rerio*) embryo and adult tissues. Cell Biol Toxicol 1992; 8(1): 43-61.
[44] Fan L, Crodian J, Liu X, Alestrom A, Alestrom P, Collodi P. Zebrafish embryo cells remain pluripotent and germ-line competent for multiple passages in culture. Zebrafish 2004; 1(1): 21-6.
[45] Ghosh C, Liu Y, Ma CG, Collodi P. Cell cultures derived from early zebrafish embryos differentiate *in vitro* into neurons and astrocytes. Cytotechnology 1997; 23(1-3): 221-230.
[46] Ma C, Fan L, Ganassin R, Bols N, Collodi P. Production of zebrafish germ-line chimeras from embryo cell cultures. Proc Natl Acad Sci U S A 2001; 98(5): 2461-6.
[47] Allen P, Melero-Martin J, Bischoff J. Type I collagen, fibrin and PuraMatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks. J Tissue Eng Regen Med 2011; 5(4): e74-86.

[48] Arnaoutova I, Kleinman HK. *In vitro* angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc 2010; 5(4): 628-35.
[49] Kurosawa H. Methods for inducing embryoid body formation: *in vitro* differentiation system of embryonic stem cells. J Biosci Bioeng 2007; 103(5): 389-98.
[50] Dang SM, Kyba M, Perlingeiro R, Daley GQ, Zandstra PW. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. Biotechnol Bioeng 2002; 78(4): 442-53.
[51] Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Development 2005; 132(23): 5199-209.
[52] Zhao H, Huang H, Lin S. Chemical approaches to angiogenesis in development and regeneration. Methods Cell Biol 2016; 134: 369-76.
[53] Kabrun N, Buhring HJ, Choi K, Ullrich A, Risau W, Keller G. Flk-1 expression defines a population of early embryonic hematopoietic precursors. Development 1997; 124(10): 2039-48.
[54] Thompson MA, Ransom DG, Pratt SJ, MacLennan H, Kieran MW, Detrich HW, 3rd*, et al.* The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. Dev Biol 1998; 197(2): 248-69.
[55] Jackson CJ, Nguyen M. Human microvascular endothelial cells differ from macrovascular endothelial cells in their expression of matrix metalloproteinases. Int J Biochem Cell Biol 1997; 29(10): 1167-77.
[56] Nisato RE, Harrison JA, Buser R, Orci L, Rinsch C, Montesano R*, et al.* Generation and characterization of telomerase-transfected human lymphatic endothelial cells with an extended life span. Am J Pathol 2004; 165(1): 11-24.
[57] Huang HG, Lindgren A, Wu XR, Liu NA, Lin SO. High-throughput screening for bioactive molecules using primary cell culture of transgenic zebrafish embryos. Cell Rep 2012; 2(3): 695-704.
[58] Choorapoikayil S, Overvoorde J, den Hertog J. Deriving cell lines from zebrafish embryos and tumors. Zebrafish 2013; 10(3): 316-25.
[59] Helmrich A, Barnes D. Zebrafish embryonal cell culture. Methods Cell Biol 1999; 59: 29-37.
[60] Myhre JL, Pilgrim DB. Cellular differentiation in primary cell cultures from single zebrafish embryos as a model for the study of myogenesis. Zebrafish 2010; 7(3): 255-66.
[61] Norris W, Neyt C, Ingham PW, Currie PD. Slow muscle induction by Hedgehog signalling *in vitro*. J Cell Sci 2000; 113 (Pt 15): 2695-703.
[62] Fan L, Crodian J, Collodi P. Culture of embryonic stem cell lines from zebrafish. Methods Cell Biol 2004; 76: 151-60.
[63] Gattazzo F, Urciuolo A, Bonaldo P. Extracellular matrix: a dynamic microenvironment for stem cell niche. Biochim Biophys Acta 2014; 1840(8): 2506-19.
[64] Wang H, Luo X, Leighton J. Extracellular matrix and integrins in embryonic stem cell differentiation. Biochem Insights 2015; 8(Suppl 2): 15-21.
[65] Jessen JR. Recent advances in the study of zebrafish extracellular matrix proteins. Dev Biol 2015; 401(1): 110-21.
[66] Whelan MC, Senger DR. Collagen I initiates endothelial cell morphogenesis by inducing actin polymerization through suppression of cyclic AMP and protein kinase A. J Biol Chem 2003; 278(1): 327-34.

[67] Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. Nat Rev Mol Cell Biol 2006; 7(5): 359-71.
[68] Nourse MB, Halpin DE, Scatena M, Mortisen DJ, Tulloch NL, Hauch KD*, et al.* VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering. Arterioscler Thromb Vasc Biol 2010; 30(1): 80-9.

Chapter 5

Zebrafish *fli:GFP* and *kdrl:GFP* embryoid bodies – a model for vasculogenesis and angiogenesis

Muhammad Ibrahim and Michael K. Richardson

This chapter is part of our following submission:

Ibrahim M, Richardson MK. The growth of endothelial-like cells in zebrafish embryoid body culture. Experimental cell research (in preparation).

Abstract

Embryoid body (EB) culture is a commonly used procedure to differentiate embryonic stem cells into the endothelial lineage. Under appropriate conditions the differentiating endothelial cells form a vascular network inside the EBs, that resembles vasculogenesis in early embryos. Here, we developed a method for culturing zebrafish EBs, as a low cost and promising model for vasculogenesis and angiogenesis research. Embryonic cells isolated from the high blastula stage of transgenic *fli:GFP* or *kdrl:GFP* zebrafish embryos, were cultured in hanging drops, in endothelial differentiating medium. Under these conditions the embryonic cells aggregated into EBs. These EBs contained *fli:GFP*$^{+}$ (putative hematopoietic, lymphatic, neural crest and endothelial) or *kdrl:GFP*$^{+}$ (endothelial precursor) cells, dependent on which transgenic zebrafish was used. When transferred to various substrates, the EBs showed spreading of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells. Different substrate molecules used in this study were gelatin, collagen type-I and Geltrex™. Significant decrease in the percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells was observed, after the EBs were transferred from hanging drops to an adherent culture. The development and morphology of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cell colonies in EB cultures, varied on different culture substrates, as observed at subsequent time-points. The *fli:GFP*$^{+}$ cells formed a monolayer around the EBs in culture. In contrast, the *kdrl:GFP*$^{+}$ cells formed network-like structures in culture. The network formation of the *kdrl:GFP*$^{+}$ cells was enhanced on a mixture of collagen type-I and Geltrex™ compared to pure substrates. These results suggest the importance of EB aggregates for differentiation of endothelial cells in zebrafish blastocyst cells. Furthermore, the choice of a complex substratum is critical for this particular differentiation event.

Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from blastocyst stage embryos [1]. The ability of ESCs to differentiate into vascular progenitor cells makes them of potential importance in regenerative medicine for treating cardiovascular disorders [2, 3]. Different techniques have been developed for the *in vitro* differentiation of ESCs into various cell types. These techniques include embryoid body culture (ESC aggregates formed in suspension culture); culture on a feeder cell layer; or culture on an extracellular matrix (ECM) substrate [4]. Embryoid bodies (EBs) are three-dimensional (3D) aggregates of ESCs [5]. These EBs are cultured using various non-adherent culturing strategies, reviewed in Ref. [6]. Inside the EBs the ESCs differentiate in the three germ layers i.e. ectoderm, mesoderm and endoderm, and then further in hematopoietic, endothelial, neuronal and muscle cells, mimicking cellular organization during early embryogenesis [7, 8].

Cellular differentiation within EBs can be controlled by regulating their size; by adding soluble growth factors or ECM components to the medium; and by enhancing or inhibiting cellular interactions within the EB [5]. The EB is an important model for studying the basis of endothelium differentiation and blood vessel formation [9], for screening angiogenic and antiangiogenic compounds [10-12], and for the development of an organ culture system [13]. The EB system as a model for vascular differentiation has advantages over other systems such as the possibility to control the microenvironment, easy access to the differentiated cells and the possibility to study vascular morphogenesis [14]. Using EBs as the intermediate, vascular endothelial differentiation systems have been developed for human [15, 16] and mouse [9, 17, 18] ESCs.

Formation of vascular networks starts at early stages of embryo development from the endothelial precursor cells called angioblasts through the process of vasculogenesis [19]. Mature blood vessels (lined with endothelial cells) are then formed from the existing vasculature by the process of angiogenesis via sprouting and non-sprouting mechanism [20]. Various growth factors are known to be involved in the differentiation of endothelial cells from angioblasts and the formation of blood vessels [21]. The most recognizable of which are vascular endothelial growth factors (VEGF) [22]. Isoforms of VEGF attaches to the extracellular matrix at different locations generating a VEGF gradient, producing chemical signals for the directional migration of newly forming capillaries [23]. Recombinant VEGF is an important medium constituent for differentiation of ESCs into endothelial lineage [17, 24-26].

Another important component required for the formation of vascular networks *in vivo* is the extracellular matrix [27]. ECM plays important roles in the binding and diffusion of growth factors, in the differentiation and proliferation of endothelial cells and in the maintenance of blood vessel shape [27]. For culturing complex tissues *in vitro*, several

naturally derived and synthetic biomaterials that mimic the natural ECM, have been developed [28]. Various ECM components used for the vascular differentiation of ESCs include collagen type-I [17], collagen type-IV [29] and gelatin [24, 25] for mouse ESCs; and fibronectin [30] for human ESCs. These materials promote the attachment, proliferation, migration and differentiation of ESCs in a physiologically relevant way [31].

In previous studies, the *in vitro* formation of vascular networks has been achieved by using various cells and tissues in the presence of growth factors and ECM, reviewed in Ref. [32, 33]. In most of the current techniques human umbilical endothelial cells, either alone or in co-culture with other cell types are used [34-40]. Other endothelial cell lines used to culture blood vessels *in vitro* include rat mesenteric microvascular endothelial cells [41], rat retinal capillary endothelial cells [42] and bovine aortic endothelial cells [43]. Although assays using endothelial cell lines are easy to perform, reproducible and easily quantifiable, these assays does not represent the complex biology of vascular formation *in vivo* [33]. Furthermore, these cell lines are relatively expensive and are subject to changes in their gene expression and behaviour with time in culture, which makes it difficult to control certain assays [33].

Alternatively, in an EB culture the endothelial cells differentiate and form blood vessels in a complex environment, which reflects the vascular formation in early embryos [32]. Unlike endothelial cell culture, multiple cell types are involved in vessel formation in EB culture [10]. The most commonly used culture method which allows control over the microenvironment and size of the EBs is the hanging drop (HD) method [6]. Vasculogenesis starts at day 3 of HD cultures in mouse EBs [44]. These EBs show sprouting of blood vessel-like structures, into the surrounding matrix, when transferred to two-dimensional (2D) or 3D collagen gel [44].

Mouse and human ESCs are commonly used to culture EBs for vasculogenesis and angiogenesis research. However, these models are relatively expensive, raise ethical concerns and may require special licenses [45]. For these reasons, the development of alternatives models is a matter of current interest. The zebrafish has a number of practical advantages as a model species [46]. For example, its eggs are externally fertilized so the embryonic cells can be easily isolated without harming the mother [46]. In contrast to mammalian cells, the zebrafish cells can be cultured at 26-28 °C in atmospheric air [47]. Furthermore, a genome comparison study has revealed a high level of sequence similarity in the majority protein coding genes of zebrafish and humans [48].

In addition to the above mentioned advantages as a model laboratory animal, there are several transgenic lines of zebrafish available such as *fli:GFP* and *kdrl:GFP* for vasculogenesis and angiogenesis research. The zebrafish *fli:GFP* and *kdrl:GFP* lines express green fluorescence protein (GFP) in putative endothelial cells, which allows real-time observation of the development of vascular system [49]. The *fli:GFP* zebrafish express GFP

under the *fli1* (Friend leukemia integration site 1) promoter which is expressed in endothelial, hematopoietic and neural crest cells [50]. The *kdrl:GFP* zebrafish express GFP under the *VEGFR2* (vascular endothelial growth factor receptor 2) promoter which is expressed in endothelial cells [51].

In summary, zebrafish embryonic cells can be a potential low-cost, high-throughput model to study *in vitro* vasculogenesis and angiogenesis. In this chapter, we describe a method for promoting the growth of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish EB cultures. The effect of different substrate molecules on the development of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in the EB cultures is evaluated. We demonstrate that the morphological characteristics and growth rate of *fli:GFP*$^+$ cells are different than the *kdrl:GFP*$^+$ cells *in vitro*.

Materials and methods

Embryo collection

All the animal experiments were performed according to the Netherland Experiments on Animals Act [52], based on the guidelines on the protection of experimental animals [53]. Adult zebrafish were maintained in continuously circulating egg water ("Instant Ocean" sea salt 60 µg/mL demi water), according to previously described [54]. Temperature of the water and air was controlled at 26 °C and 23 °C, respectively. Two different transgenic zebrafish lines *fli:GFP* and *kdrl:GFP* were used. To obtain embryos, adult male and female fish, at a proportion of 1:1, were transferred to small breeding tanks in the evening. The zebrafish usually laid eggs when the light came on. The eggs were collected at the bottom of the tank, separated from adults using a cotton mesh to protect the eggs from being eaten.

Embryos were transferred to a temperature controlled room (28 °C) and were distributed in 9 cm Petri dishes (100 embryos per dish), after removing dead and unfertilized eggs. The embryos were washed thoroughly with clean egg water to remove any debris. These embryos were allowed to develop to the high blastula stage of Kimmel et al. [55] (3.5 h after fertilization) at 28 °C.

Decontamination of embryos

The embryos were transferred to a flow cabinet at room temperature for sterilization and cell isolation. For sterilization, the embryos, with the chorion intact, were immersed in 70% ethanol for 10 sec and then in two changes of 0.05% sodium hypochlorite (available chlorine 10-15%, Sigma, Cat. No. 425044) for 4 min each. The sterilization was done according to the procedure described in Ref. [47]. After each immersion in ethanol or sodium hypochlorite, the embryos were rinsed with LDF medium (see below, Media composition). Finally the embryos were left in 0.5 mL LDF medium for dechorionation.

Before dechorionating, the embryos were observed under a dissecting microscope and any dead or abnormal embryos (with cloudiness in the perivitelline fluid) were removed.

Cell isolation

The sterilized embryos were dechorionated using a pair of sterile No.5 watchmaker's forceps. The dechorionated blastocysts were then transferred to an 1.5 mL Eppendorf tube. The blastocysts were triturated using a P-200 Gilson Pipette (Gilson, B.V., Europe: Den Haag) to remove the yolk. The blastocysts were then isolated by centrifugation (300 g for 1 min). The supernatant was discarded and the blastocysts were washed with CMF-PBS (calcium magnesium free phosphate buffered saline; Invitrogen Cat. No. 14190). After centrifugation the CMF-PBS was discarded and the blastocysts were dissociated into single cells by triturating in 1 mL of 0.25% trypsin solution (Invitrogen Cat. No. 15090) containing 1 mM EDTA (ethylene diamine tetra acetic acid). The trypsin was inactivated using 0.1 mL FBS (Fetal bovine serum). The cells were pelleted by centrifugation at 300 g for 3 min. The cell pellet was washed 3 times with the washing medium (see below, Media composition). Finally the cells were re-suspended in 200 µL of washing medium. The cell concentration in the suspension was calculated using a heamocytometer.

Media composition

For all the experiments described here we used LDF medium. The basic LDF medium is composed of Lebovitz L-15, Dulbecco's modified Eagle's and Ham's F-12 media (in a ratio of 55 : 32.5 : 12.5, respectively), supplemented with 15 mM of HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), 1% antibiotic/antimycotic mix (Invitrogen, 15240) and 0.015% $NaHCO_3$. The basic LDF medium without extra supplementation was used for rinsing and dechorionating the embryos. For further experiments with the blastocyst cells the LDF medium was enriched with three levels of supplementation as following.

1. **Washing medium:** for rinsing and re-suspension of blastocyst cells after trypsinization the LDF medium was supplemented with 15% FBS.
2. **EB differentiation medium:** for HD cultures (see below) the LDF medium was supplemented with 4.1% endothelial growth supplement mix (EGS; Promocell, Cat. No. C-39216), 20% FBS (Invitrogen, Cat. No. 10500), 50 µg/mL zebrafish embryo extract (ZEE), 50 ng/mL recombinant zebrafish vascular endothelial growth factor ($VEGF_{165}$; R&D systems, Cat. No. 1247-ZV) and 10 ng/mL recombinant human basic fibroblast growth factor (bFGF; Invitrogen, Cat. No. PHG0024).
3. **EB maturation medium:** for EBs culture on different substrates the LDF medium was supplemented with 15% FBS, 4.1% EGS, 50 µg/mL ZEE, 50 ng/mL $VEGF_{165}$ and 10 ng/mL bFGF.

Cell culture conditions

All cultures described here were carried out in a forced draft, humidified incubator at 28 °C in 0.5% CO_2. The media was refreshed on day 4, for the cultures that were maintained longer than four days.

Hanging drop culture

The blastocyst cells were re-suspended (at a final concentration of 50 cells/µL) in EB differentiation medium. All the cultures were established twice (one for *fli:GFP* and one for *kdrl:GFP* line) unless otherwise specified. The cell suspension was distributed in 20 µL droplets (containing 1000 blastocyst cells) onto the inside of the lids of 60 mm Petri dishes. The lids with the droplets were places inverted on the Petri dishes to initiate the HD cultures. To diminish evaporation from the droplets, CMF-PBS was added to each Petri dish. The cultures were maintained in the incubator for four days to allow the aggregation of cells in the drops to form EBs. These EBs were then further cultured on different substrates as described below.

Isolation of EBs

To isolate the EBs the Petri dish lid was carefully inverted and held at a 45° angle, the droplets containing the EBs were dropped to one side of the lid by gently tapping the lid. The EBs were transferred to a 1.5 mL Eppendorf tube using a p-1000 micropipette, and were allowed to settle to the bottom of the tube by gravity. The medium was removed and the EBs were washed with CMF-PBS and then with washing medium. Finally, the EBs were re-suspended at 1 EB per µL of EB maturation medium.

EB culture for quantification of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells

In order to quantify *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells, the EBs were sub-cultured in 96 well plates, without extra substrate coating. The EB suspension was diluted with EB maturation medium such that 250 µL of medium contain 20 EBs. This EB suspension was distributed at 250 µL per well in 96-well plates. For each transgenic line a total of four 96-well plates were seeded with the EBs, to analyse cell counts on four consecutive time-points (day 2, 4, 6 and 8; one plate for one time-point). For each time-point six wells of the 96-well plate were cultured with EBs. The cultures were maintained in EB maturation medium for a maximum of 8 days, with media refreshment on day 4. In addition to these time-points, the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells was also calculated in the EBs on day 0 (the time of harvesting of the EBs from the HD culture, that is day 4 of HD culture).

Cell isolation from EB culture

For day 0 counts, the EBs were distributed at 20 EBs per tube in six Eppendorf tubes. The EBs isolated on day 0 and the EB cultures at each time-point were washed with 250 µL of CMF-PBS, and trypsinized using 250 µL of 0.25% trypsin solution containing 1 mM EDTA,

for 2 min. Cell dissociation was observed in cultures using an inverted microscope. The trypsin was deactivated using FBS to a final concentration of 10%. The dissociated cells/trypsin suspension from the cultures were transferred from each well to an Eppendorf tube. The cells were isolated by centrifugation at 300 g for 3 min. The cells were washed twice with 200 µL, and finally re-suspended with 20 µL of washing medium. These cells were subjected to counting for the percentage of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells as following.

Quantification of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells in EB culture

To count the *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells, a 5 µL drop from each cell suspension (isolated cells per replicate) was transferred onto a cover glass under a confocal microscope. The cell population in each drop was imaged with 488 nm wavelength excitation light to visualize the *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells, and with phase contrast. For each image, the percentage of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells was calculated by counting the number of GFP^{+} and GFP^{-} cells. These values from six culture replicates were then used to calculate the mean percentages of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells on each time-point.

Quantification of *fli:GFP*$^{+}$ cells in EB secondary culture

For this experiment, the *fli:GFP* EBs cultures were sub-cultured for two passages and quantified at each passage for percentage of *fli:GFP*$^{+}$ cells. These cultures were established in three replicate wells of a 96-well plate. At each passage cells isolated from one well were sub-cultured in a single well of a new plate. On day 8 of primary cultures, the cells were isolated from each well by trypsinization as above (Cell isolation from EB cultures). The isolated cells were re-suspended in EB maturation medium for sub-culture (passage 1). These secondary cultures were maintained in EB maturation medium for 4 days. On day 4 the cells were isolated and sub-cultured in a fresh plate (passage 2). During transfer, a small volume of cell suspension from each replicate was withdrawn and used to count *fli:GFP*$^{+}$ cells in cultures (as described above; Quantification of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells in EB cultures). The passage 2 cells were cultured again for 4 days, then the cells were isolated and counted for the percentage of *fli:GFP*$^{+}$ cells.

Culture of *fli:GFP* EBs on different substrate

In order to observe the development and morphological appearance of *fli:GFP*$^{+}$ cells, the *fli:GFP* EBs were cultured on three different substrates: (i) gelatin (Sigma, Cat. No. G1890); (ii) collagen type-I (Invitrogen, Cat. No. A1048301); (iii) fibrin, made with bovine fibrinogen (Sigma, Cat. No. F8630). A 96-well format plate with a glass bottom (CS16-chambered coverglass plate; Grace Bio; Cat. No. 112358) was used to culture the EBs for imaging. To coat with 0.1 mg/cm^2 gelatin, 1.7 µL of 2% gelatin solution was added to a well and then allowed to air dry. The collagen type-I solution was prepared at 3 mg/mL and neutralized with 0.0125 mL/mL of 7.5% sodium bicarbonate. For coating, 5 µL of the

collagen type-I gel solution was added to a well and allowed to polymerize at 37 °C for 30 min.

The fibrin gel was prepared by mixing fibrinogen solution (at a final concentration of 2.5 mg/mL) with thrombin solution (Sigma, Cat. No. T4648; final concentration 3 Units/mL). A well of the chambered coverglass plate was coated with 5 µL of the mixture. The plate was incubated at 37 °C for 30 min. The coated wells were rinsed with washing medium before adding the *fli:GFP* EBs. The EBs isolated from HD cultures were distribute at 20 EBs per well with different substrate coating and maintained on EB maturation medium.

Culture of *kdrl:GFP* EBs on different substrate

The *kdrl:GFP* EBs were transferred from HD cultures to three different substrate coated wells: (i) collagen type-I; (ii) Geltrex™ (Invitrogen, Cat. No. A1413201); and (iii) collage type-I + Geltrex™. A well of chambered coverglass plate was coated with collagen type-I as above. Similarly, 5 µL Geltrex™ was used to coat another well. Geltrex™ has, according to the manufacturer's documentation, as its major components, laminin, collagen type-IV, entactin and heparin sulphate proteoglycans and has a total protein concentration 12-18 mg/mL. Collage type-I + Geltrex™ mixture was prepared by combining equal volumes of the pure gel solutions to make final concentrations of 1.5 mg/mL and 6-9 mg/mL, respectively. Sodium bicarbonate (7.5%) was added to the mixture at a final concentration of 0.0125 mL/mL of collagen type-I used. Five microliter of the gel mixture was used to coat one well of the plate. The plate was incubated at 37 °C for 30 min to allow the gels to polymerize. The wells were then rinsed with washing medium. Finally, 20 *kdrl:GFP* EBs in 250 µL of EB maturation medium were added to each well prepared with different substrate coating.

Image analysis

Selected EBs showing colonies of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells were imaged on consecutive days using a confocal microscope (Axio observer inverted microscope A1). The *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells were visualized with 488 nm wavelength excitation light for imaging. Image-J software, version 1.46r [56] was used to reconstruct the images for further analysis. The EBs were tracked from day one till the end of the experiment (day 12) to observe changes in the growth and morphology of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in culture. For all the measurements the pre-calibrated scale was used. The images were analysed for the following parameters:

Total area covered by *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells per EB

The area covered by *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells, for each EB, at each time-point, on different culture substrates, was measured from confocal images using image-J software. From these measurements, the percentage change in area covered by *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells at each time-point, compared to day one, was calculated for each individual EB.

Measurement of *kdrl:GFP*$^{+}$ cell networks in EBs

The *kdrl:GFP*$^{+}$ cell network formed in the EBs was measured from the confocal images at consecutive days using image-J software. The parameters for these measurements were: lengths of individual branches of the cell network in each EB, average width of the branches per EB, number of network branches per EB and total length of the network per EB.

Calculation of connectedness of the *kdrl:GFP*$^{+}$ cell network

The connectedness of the endothelial cell networks formed in *kdrl:GFP* EB cultures was calculated using the following formula:

$$Network\ c\text{onnectedness} = \frac{\text{Number of endpoints}}{Number\ of\ junctions}$$

Theoretically, for a well-connected network, the value obtained should be close to zero [57].

Statistical analysis

The percentages of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cell in EB cultures, increase in area covered by *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells per EB on different substrates and the measurements of *kdrl:GFP*$^{+}$ cell network per EB, were used to calculate means and standard errors per culture condition, using SPSS software version 21.0. One-way ANOVA was performed to calculate the p-values to analyse differences between different culture conditions and subsequent time-points. Pairs of different conditions were compared using the Post-Hoc Tukey's test.

Results

Development of *fli:GFP*$^{+}$ cells in EB culture

The percentage of *fli:GFP*$^{+}$ cells in EB cultures declined significantly over time (Figure 1). In total the percentage of *fli:GFP*$^{+}$ cells in the EBs dropped from 45% to 20% during eight days of culture. Results from the quantification of *fli:GFP*$^{+}$ cells in EB primary and secondary cultures, and migration of the *fli:GFP*$^{+}$ cells from EBs on different culture substrates, were as follows.

Percentage of *fli:GFP*$^{+}$ cells in EB primary culture

The *fli:GFP* EBs contained a high percentage of *fli:GFP*$^{+}$ cells on day 0 (45.0 ± 3.1%), directly after isolation from HD cultures. When transferred to a conventional 96-well plate, the percentage of *fli:GFP*$^{+}$ cells dropped gradually with time (Figure 1). In the first four days of the adherent culture no significant decrease in the percentage of *fli:GFP*$^{+}$ cells

was observed. However, on days 6 and 8, a significant decrease in the percentage of *fli:GFP*$^{+}$ cells was observed compared to day 2. The percentage of *fli:GFP*$^{+}$ cells in cultures on day 6 and 8 (22.3 and 20.1%, respectively) was less than half of the percentage (45.0%) found in EBs on day 0.

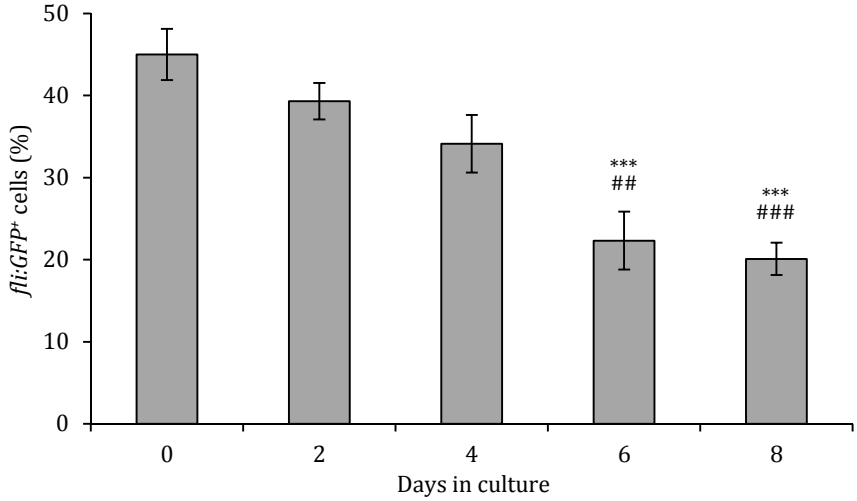

Figure 1: Change in the percentage of *fli:GFP*$^{+}$ cells over time in EB cultures. Day zero for EBs is the day four of hanging drop cultures. The EB Cells were isolated from cultures at each time-point (day 2, 4, 6, 8) to quantify the percentage of *fli:GFP*$^{+}$ cells. A decrease in the percentage of *fli:GFP*$^{+}$ cells can be observed at each time-point. The number of observations was six per time-point. Error bars represent standard error. (***, $p < 0.001$ compared to day 0; ###, $p < 0.001$, ##, $p < 0.01$ compared to day 2; +, $p < 0.05$ compared to day 4).

Percentage of *fli:GFP*$^{+}$ cells in EB secondary culture

In this experiment, the sub-cultures of *fli:GFP* EBs were used to estimate the percentage of *fli:GFP*$^{+}$ cells in secondary cultures. The passage 1 EB cultures contained 15.1 ± 1.9% *fli:GFP*$^{+}$ cells. In the next passage (passage 2) the percentage of *fli:GFP*$^{+}$ cells in cultures was 13.1 ± 0.8%. Although the percentage of *fli:GFP*$^{+}$ cells was more or less stable in sub-cultures, the intensity of the GFP signal from the cells greatly reduced in the second passage (Figure 2).

Migration of *fli:GFP*$^{+}$ cells from EBs on different substrate

The area covered by the *fli:GFP*$^{+}$ cells migrating from the EBs on different substrates (collagen type-I, gelatin and fibrin; Figure 3) was measured on each consecutive day (day 2, 4, 6, 8, 10 and 12). The percent increase in surface area covered by *fli:GFP*$^{+}$ cells, compared to the same value on day 1, was calculated at each time-point for individual EBs. On collagen type-I substratum a significant increase in area covered by *fli:GFP*$^{+}$ cells was observed between day 6 and day 8 ($p<0.05$). On gelatin substratum the area covered by

fli:GFP^{+} cells slowly increased from day 2 to day 6 ($p<0.01$). On fibrin substratum the area covered by *fli:GFP*$^{+}$ cells increased significantly from day 2 to day 4 ($p<0.001$). After day 8 no further increase in the area covered by *fli:GFP*$^{+}$ cells was observed on any of the three substrates.

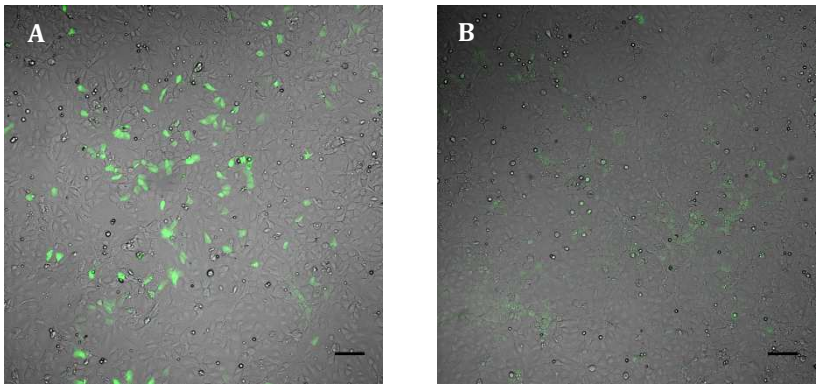

Figure 2: Confocal images showing *fli:GFP*$^{+}$ cells in EB secondary cultures. The EB cells not expressing GFP are shown in phase contrast. (**A**) Image from day 4 of Passage 1 EB cell culture showing *fli:GFP* expression in multiple cells. (**B**) In passage 2 the intensity of signal is visibly lower. Scale bar, 100 µm.

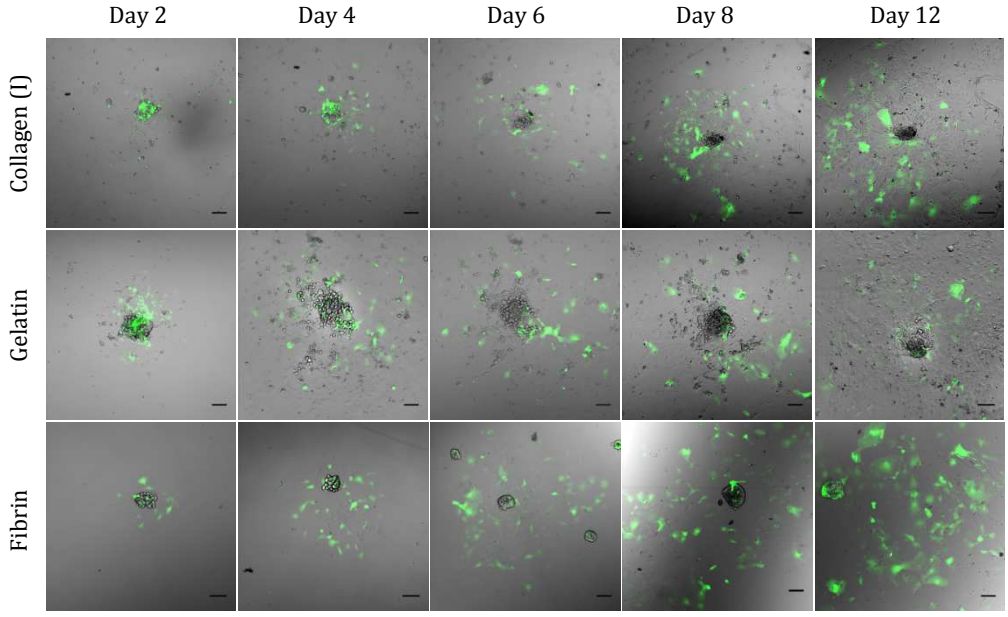

Figure 3: Time-lapse imaging of *fli:GFP* EB cultures on different substrate. The increase in area covered by *fli:GFP*$^{+}$ cells (green) around the EBs can be observed at subsequent time-points. In each horizontal row, the same field has been shown. Scale bar, 100 µm.

From day 2 to day 6 the differences in the percent increase in area covered by *fli:GFP*$^+$ cells were observed between different substrates (Figure 4). On days 2 and 4, the *fli:GFP*$^+$ cells covered significantly more area per EB on fibrin substratum compared to gelatin and collagen type-I. On gelatin substratum the percent increase in area covered by *fli:GFP*$^+$ cells on days 2 and 4 was higher than collagen type-I. On day 6 the increase in *fli:GFP*$^+$ area was higher on fibrin substratum compared to collagen type-I. No significant differences were observed between the three substrates on days 8, 10 and 12.

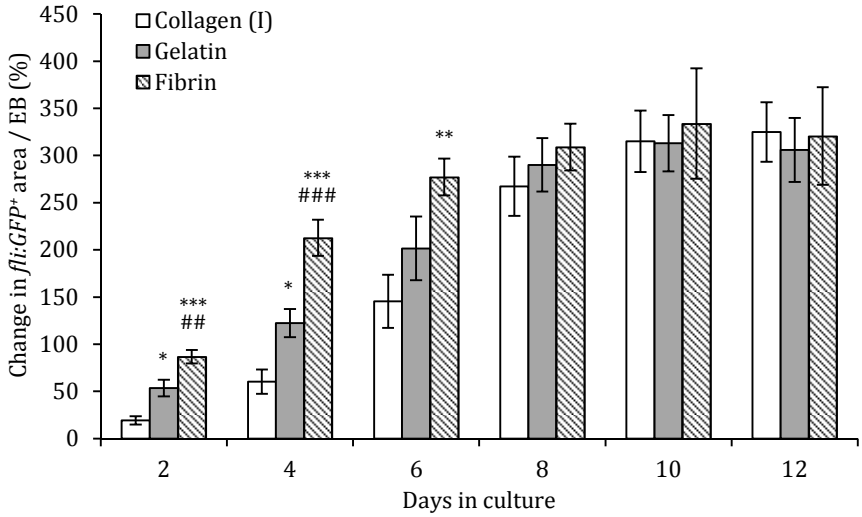

Figure 4: Percentage increase, compared to day 1, in surface area covered by *fli:GFP*$^+$ cells per EB on different culture substrate. Number of observations: 13 for collagen type-I, 12 for gelatin and nine for fibrin per time-point. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to collagen type-I; ###, $p < 0.001$, ##, $p < 0.01$ compared to gelatin).

Development of *kdrl:GFP*$^+$ cells in EB culture

The development of *kdrl:GFP*$^+$ cells was observed in the *kdrl:GFP* EB cultures at subsequent time-points. The quantification of *kdrl:GFP*$^+$ cells in cultures, changes in measurements of the *kdrl:GFP*$^+$ cell networks with time, and connectedness of the network on different substrates are presented below.

Percentage of *kdrl:GFP*$^+$ cells in EB culture

A comparatively high percentage of *kdrl:GFP*$^+$ cells was found in EBs on day 0 (8.7 ± 0.7%; Figure 5) i.e. directly after isolation from the HD cultures. These EBs were cultured in a 96-well plate on conventional substratum. Cell counts on subsequent days showed a significant decrease in the percentage of *kdrl:GFP*$^+$ cells on day 2 (down to 3.4 ± 0.5%) of the culture. From day 2 to day 8 a gradual decrease in the percentage of *kdrl:GFP*$^+$ cells in

EB cultures was observed (Figure 5). The percentage of *kdrl:GFP*$^{+}$ cells in cells isolated from the EB cultures on day 8 was significantly less than day 2.

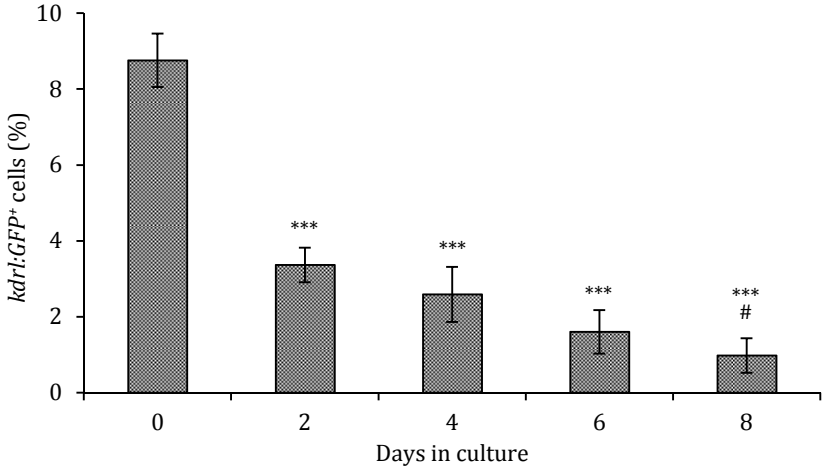

Figure 5: Percent quantification of *kdrl:GFP*$^{+}$ cells in EB cultures on subsequent days. Day zero for EBs is the day four of hanging drop cultures. An abrupt decrease from day 0 to day 2 and then a gradual decrease after day 2 can be seen. Number of observations: six per time-point. Error bars represent standard error. (***, $p < 0.001$ compared to day 0; #, $p < 0.05$ compared to day 2).

Development of *kdrl:GFP*$^{+}$ cells in EB culture on different substrate

The *kdrl:GFP* EBs, cultured on different substrates, were imaged at subsequent time-points to observe the development of *kdrl:GFP*$^{+}$ cells (Figure 6). Changes in total area and length of the *kdrl:GFP*$^{+}$ cell networks (Figure 7), and the dimensions of the branch-like structures of the network (Figure 8) per EB, are described in the following sections.

Percent changes in length and area of kdrl:GFP^{+} *cell network*

Percent changes in dimensions of *kdrl:GFP*$^{+}$ cell network per EB at consecutive time-points was calculated compared to day 1 (24 h after re-plating the EBs). A gradual decrease in the length and area of *kdrl:GFP*$^{+}$ cell network was observed on all three substrates (Figure 7). However, the rate of decline was different on different substrates. On Geltrex™ and on collagen type-I + Geltrex™ substrates the network length remained similar at subsequent time-points. On collagen type-I substratum a significant reduction in the network length was observed from day 2 to day 8 ($p<0.001$). The area covered by *kdrl:GFP*$^{+}$ cell network per EB also reduced with time on all the three substrates; however, the differences were not significant (Figure 7B).

On day 2 of cultures the length of *kdrl:GFP*$^{+}$ cell network per EB decreased by 10% on Geltrex™, which was significantly lower compared to an increase of 19% on collagen type-

I and 17% on collagen type-I + Geltrex™ substratum (Figure 7A). On day 8 of the cultures the decrease in the total length of *kdrl:GFP*+ cell network on collagen type-I substratum was significantly less than the other two substrates. These differences between the substrates were observed after day 8 until the end of the experiment (day 12).

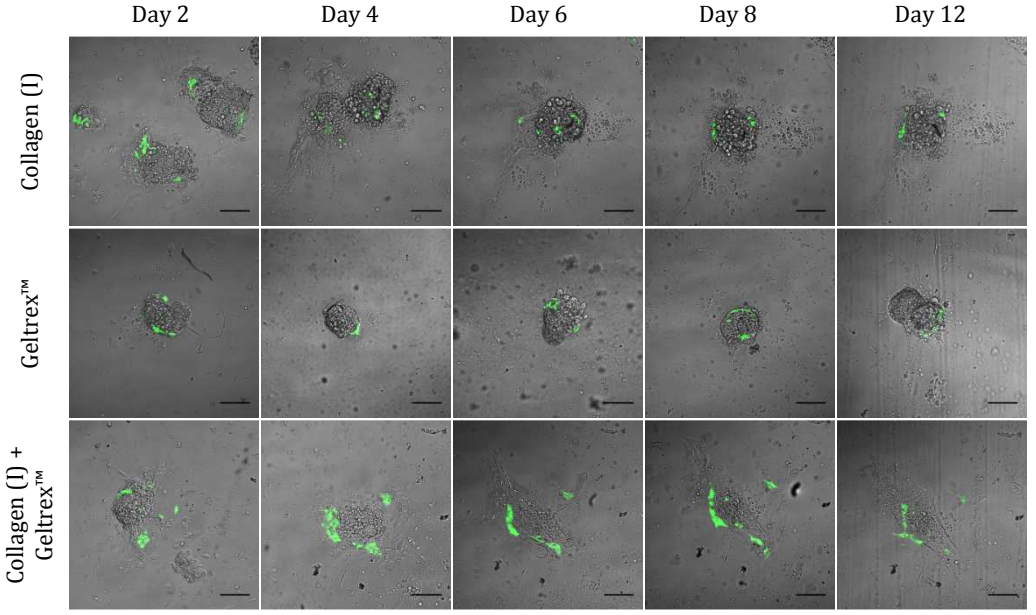

Figure 6: Time-lapse imaging of *kdrl:GFP* EBs cultures on different substrates. Each row shows the same field. Changes in the morphology of *kdrl:GFP*+ cell (green) networks can be observed on different time-points. As can be seen, Collagen (I) + Geltrex™ provides the most favourable substrate for *kdrl:GFP*+ cells, an observation that is supported by the quantitative data shown in (Figure 8 and 9). Scale bar, 100 µm

Morphometry of kdrl:GFP+ *cell network*

The number of branches, average branch length, branch width and total length and of the *kdrl:GFP*+ cell network per EB on different 2D substrates are presented in Figure 8. The results showed differences between collagen type-I and collagen type-I + Geltrex™ substrates in all parameters except branch width. Values obtained for Geltrex™ substratum were in between the collagen type-I and collagen type-I + Geltrex™ substrates, with no significant differences from either of the two, except at a few time-points.

The EBs cultured on collagen type-I substratum developed a low number of comparatively longer *kdrl:GFP*+ branches in the first two days of culture (Figure 8A and B). The branch length decreased significantly from day 2 to day 10 ($p<0.001$), while the number of branches remained similar on collagen type-I substratum. On Geltrex™ and on collagen type-I + Geltrex™ substrates both the branch length and number of branches remained similar at subsequent time-points. A higher number of *kdrl:GFP*+ branches per EB was

found on collagen type-I + Geltrex™ substratum compared to collagen type-I substratum (Figure 8A). On days 8, 10 and 12 the average branch length on collagen type-I substratum was significantly less than on collagen type-I + Geltrex™ substratum (Figure 8B).

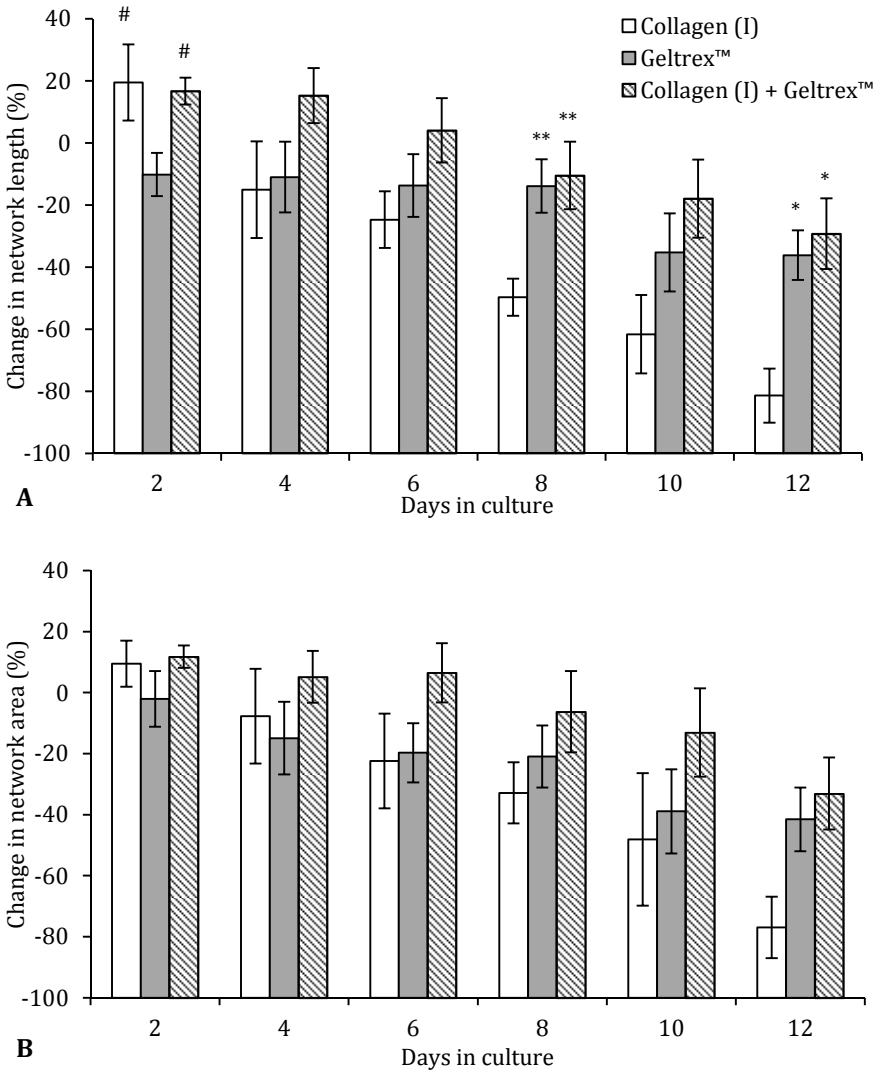

Figure 7: Percent changes compared to day one in *kdrl:GFP+* cell networks formed in EBs. (**A**) Percent change in total length of *kdrl:GFP+* cell network per EB. (**B**) Percent change in area covered by *kdrl:GFP+* cells per EB. Number of observations: eight for collagen type-I, 12 for Geltrex™ and 11 for collagen type-I + Geltrex™. Error bars represent standard error. (**, $p < 0.01$, *, $p < 0.05$ compared to collagen type-I substratum; #, $p < 0.05$ compared to Geltrex™ substratum).

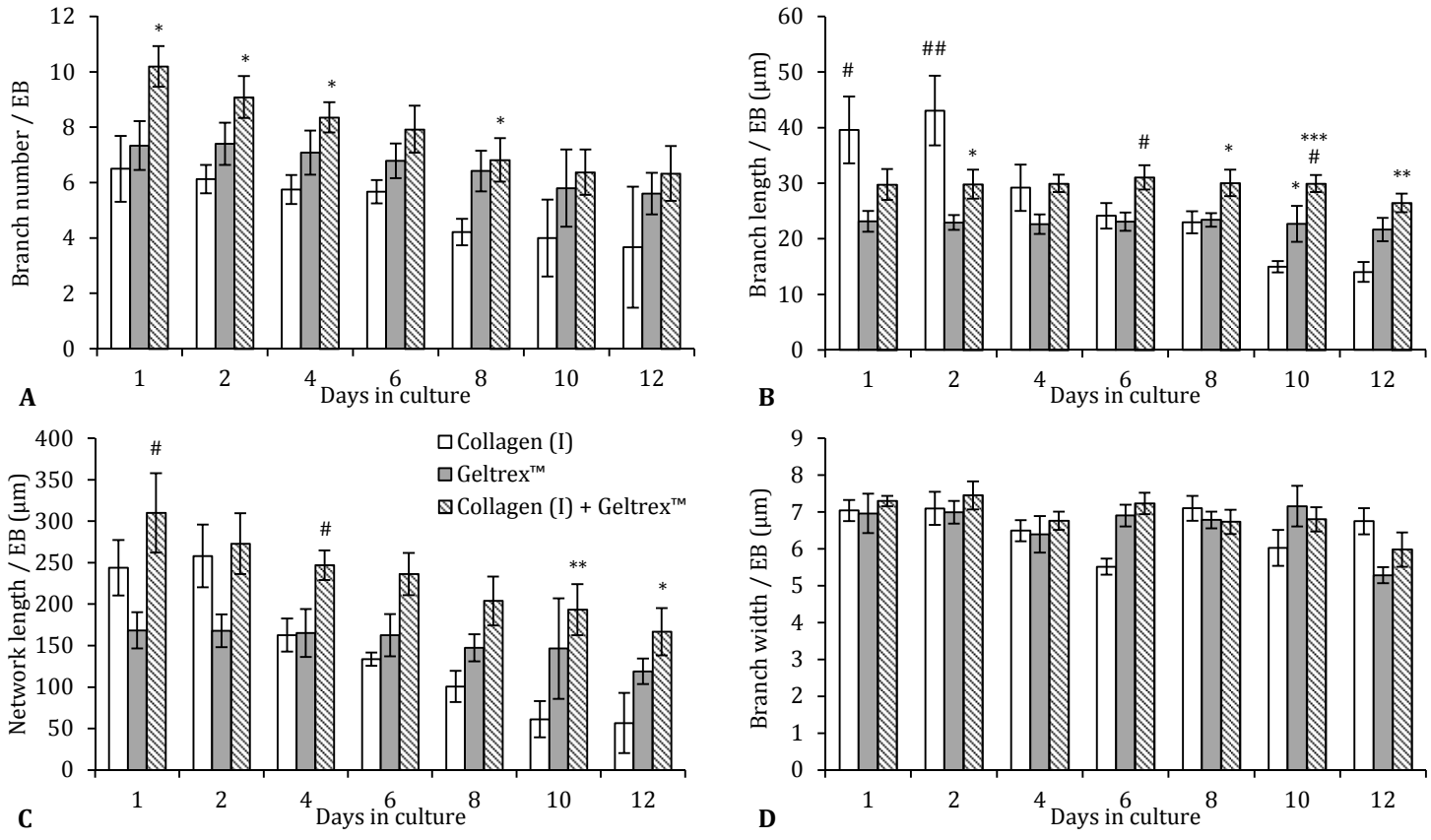

Figure 8: Changes with time in the parameters of *kdrl:GFP*⁺ cell networks on different substrates. (**A**) Number of *kdrl:GFP*⁺ cell network branches per EB on different time-points. (**B**) Average branch length of *kdrl:GFP*⁺ cell network per EB. (**C**) Total length of *kdrl:GFP*⁺ cell network per EB. (**D**) Average width of *kdrl:GFP*⁺ cell network branches per EB. The graphs show a higher network formation on collagen type-I + Geltrex™ substratum compared to single substrates used. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to collagen type-I substratum; ##, $p < 0.01$, #, $p < 0.05$ compared to Geltrex™ substratum).

In the first two days of culture, total length of the *kdrl:GFP⁺* cell network per EB was similar on collagen type-I and collagen type-I + Geltrex™ substrates. After day 2 the reduction in the individual branch length (Figure 8B) on collagen type-I substratum resulted in a significant reduction (from day 2 to day 8; $p<0.001$) in the total length of the network per EB on this substratum (Figure 8C). While on collagen type-I + Geltrex™ and on Geltrex™ substrates the length of the network remained similar over time. On Geltrex™ substratum the network length was less than collagen type-I + Geltrex™ substratum until day 4; however the difference was not significant after day 6 of culture. On days 10 and 12, the *kdrl:GFP⁺* network length per EB was significantly lower on collagen type-I compared to collagen type-I + Geltrex™ substratum (Figure 8C). The average width of the *kdrl:GFP⁺* branches was between 6 and 8 μm and remained similar on all the three substrates (Figure 8D).

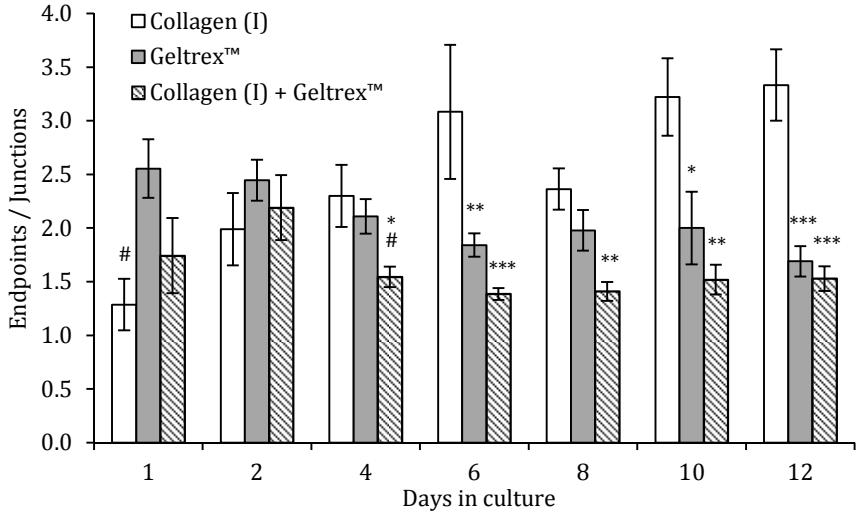

Figure 9: Connectedness of *kdrl:GFP⁺* cell network on different substrates. Values nearest to zero on the vertical axis shows a well-connected network. On collagen type-I substratum the network connectedness deteriorate with time, while on Geltrex™ and collagen type-I + Geltrex™ substrates a comparatively well-connected network is maintained until the end of culture. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to collagen type-I substratum; #, $p < 0.05$ compared to Geltrex™ substratum).

Connectedness of the kdrl:GFP⁺ *network*

The connectedness values of *kdrl:GFP⁺* cell networks on different substrates are given in Figure 9. On collagen type-I + Geltrex™ and on Geltrex™ substrates the *kdrl:GFP⁺* cells were more connected compared to collagen type-I substratum. The values obtained from dividing the network endpoints by junctions ranged from 1.4 ± 0.1 to 2.2 ± 0.3 and 1.7 ± 0.1 to 2.5 ± 0.3, respectively, for collagen type-I + Geltrex™ and Geltrex™ substrates. On

these substrates the connectedness values remained constant over the 12 days of culture. On collagen type-I substratum the *kdrl:GFP*$^{+}$ cell network per EB was well connected on day 1 of culture with lower endpoints divided by junctions value. However, the connectedness of the network on this substratum was lost with the duration of culture.

Discussion

EB culture in hanging drops

In this section the results from EBs cultures, in the current study, will be discussed in comparison with the blastocyst cell cultures in our previous experiments (Chapter 4). In studies on mouse ESCs, EBs grown in suspension culture have shown a higher percentage of hematopoietic and endothelial cells compared to EBs grown in adherent culture [58]. Our results from zebrafish cells are consistent with these findings. In our previous experiments (Chapter 4) zebrafish blastocyst cells in adherent culture showed fewer *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells compared to the EBs developed in HD suspension culture in this chapter.

In HD culture the dispersed cells collect by gravity in the bottom center of the drop, forming EB aggregates, where there is presumably more cell-to-cell contact than in the adherent culture, which is a monolayer. We suggest that the increased cellular contact in HD cultures may be favorable for the growth and differentiation of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells. previous studies have shown that in suspension cultures the proliferation of EB cells is limited compared to the adherent cultures, allowing greater control of the differentiation of specific cell types [6]. This would explain why the percentage of transgenic cells dropped significantly in our EB cultures after transferring to adherent culture. The medium composition used here for the EB culture was selected based on the optimization experiments described in chapter 4. In those experiments, this medium gave rise to a higher percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cell culture.

Development of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in EB culture

The percentage of *fli:GFP*$^{+}$ cells in EB culture was always higher than *kdrl:GFP*$^{+}$ cells. This is presumably because the *kdrl:GFP* transgene is expressed only in endothelial cells [51], whereas the *fli:GFP* is expressed in endothelial, lymphatic, hematopoietic and neural crest cells [50]. In contrast to the fibroblast-like morphology of *fli:GFP*$^{+}$ cells, the endothelial-like properties of *kdrl:GFP*$^{+}$ cells is supported by the fact that they form network-like structures in the EB culture developed here. This difference in morphology of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells also suggests that the *kdrl:GFP* is specific for endothelial cells.

In our previous experiments (Chapter 4) the *kdrl:GFP*$^{+}$ cells were no longer found after eight days of primary blastocyst cell culture. The numbers of *fli:GFP*$^{+}$ cells, on the other hand, were comparatively stable in the primary culture. However, these cells could also not be maintained in secondary culture (Chapter 4). The possible explanation for these findings could include apoptosis in *kdrl:GFP*$^{+}$ cells [59], or down regulation of *kdrl:GFP* [60] and *fli:GFP* [50] transgenes as previously suggested. In contrast to primary blastocyst cultures (Chapter 4), in the EB cultures described here, the *kdrl:GFP*$^{+}$ cell networks could be observed up to 12 days in culture. Similarly, the *fli:GFP*$^{+}$ cells could be maintained in secondary culture for up to three passages. Thus, under appropriate conditions the *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells can be maintained for a longer time *in vitro*.

Effect of substratum on *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in EB culture

In our previous experiments, the blastocyst cells cultured on collagen type-I substratum contained a higher percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells compared to those cultured on gelatin substratum (Chapter 4). In contrast, the increase in area covered by the outgrowing *fli:GFP*$^{+}$ cells from EBs in the current study was lower on collagen type-I, compared to gelatin and fibrin substrates. A reason for this may be the softness of collagen type-I gel, where the cells are in a more elongated shape and cover less area, compared to the more flattened cells on a gelatin and fibrin substrates. The area covered by *fli:GFP*$^{+}$ cells per EB was significantly higher on a fibrin substratum compared to gelatin and collagen type-I, in our study. These results are in comparison with the previous studies where fibrin substrata have been shown to enhance the formation of blood vessel-like structures from human dermal microvascular endothelial cells [61].

Similarly in our other experiments, the *kdrl:GFP*$^{+}$ cells formed network-like structures in 3D gel matrix containing fibrin (chapter 6), compared to the 2D substrates tested here. In those experiments (Chapter 6), the network length of *kdrl:GFP*$^{+}$ cells was higher compared to the network length on Geltrex™ and collagen type-I substrata described here. These results are in agreement with a previous study where a 3D fibrin scaffold enhanced network formation from human umbilical vein endothelial cells (HUVECs) compared to collagen type-I scaffolds [62]. Fibrin 3D matrices have also been demonstrated to increase angiogenesis during wound healing in pigs [63]. This suggest that fibrin could be an important part of the culture substrate for angiogenesis assays.

The selection of an appropriate substratum is critical for a particular assay, as different cell types may have different requirements for extra cellular matrix [64]. Of different substrate compositions used for the EB culture in the experiments in this chapter, longer and more number of *kdrl:GFP*$^{+}$ strands were observed on collagen type-I + Geltrex™ substratum, compared to pure substrates. The formation of *kdrl:GFP*$^{+}$ cell network was principally observed in the matrix composed of a combination of collagen type-I, Geltrex™ and fibrin, described in chapter 6. This may suggest the requirement of a complex

extracellular matrix substrate for the development of vascular networks in zebrafish EB culture. Similar to our findings, previous studies on HUVECs also recommend the use of composite 3D matrix containing collagen and fibrin, because it offers advantages over a pure matrix in the culture of vascular networks [65].

Fibronectin is a major constituent of zebrafish extracellular matrix (ECM) in early embryonic stages [66]. Furthermore, the cells in early zebrafish embryo start expressing membrane proteins of the integrin family, which bind fibronectin [67]. Studies in other animals and humans have shown that fibronectin cross-link to fibrinogen and form a clot which serve as a substrate for wound healing [68]. This may explain why an increased migration of *fli:GFP*$^{+}$ cells was observed on fibrin substratum in this study. Furthermore, the addition of fibrinogen to the 3D matrix favoured the development of *kdrl:GFP*$^{+}$ cell networks in our other experiments (chapter 6). Another possible cause of enhanced network formation or cell migration in fibrin containing matrices may be the vulnerability of fibrin to the proteases released from the EB cells. In previous studies, testing of pro-angiogenic compounds on zebrafish embryos has shown increased expression of matrix metalloproteinases MMP-2 and MMP-9 [69]. These metalloproteinases are suggested to be involved in the degradation of fibrin matrix [70].

Conclusions

Suspension culture of zebrafish blastocyst cells allows better control of cellular differentiation, compared to the adherent cultures. Using the EB intermediate (developed in suspension culture), the development of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells could be increased and maintained for longer duration in culture. Different substrate composition effect the spreading and development of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures. We recommend the use of a complex substratum for the growth of these cells in culture. Fibrin substratum has an effect on growth of *fli:GFP*$^{+}$ cells in cultures, as well as on the development of network-like structures from *kdrl:GFP*$^{+}$ cells. The *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells showed differences in their number and morphology, suggesting that these are diverse cell types.

References

[1] Bethesda MD. What are embryonic stem cells? In Stem Cell Information, 2015; Available from: http://stemcells.nih.gov/info/basics/pages/basics3.aspx.

[2] Descamps B, Emanueli C. Vascular differentiation from embryonic stem cells: novel technologies and therapeutic promises. Vascul Pharmacol 2012; 56(5-6): 267-79.

[3] Rumpold H, Wolf D, Koeck R, Gunsilius E. Endothelial progenitor cells: a source for therapeutic vasculogenesis? J Cell Mol Med 2004; 8(4): 509-18.

[4] Nishikawa S, Jakt LM, Era T. Embryonic stem-cell culture as a tool for developmental cell biology. Nat Rev Mol Cell Biol 2007; 8(6): 502-7.

[5] Bratt-Leal AM, Carpenedo RL, McDevitt TC. Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. Biotechnol Prog 2009; 25(1): 43-51.
[6] Kurosawa H. Methods for inducing embryoid body formation: *in vitro* differentiation system of embryonic stem cells. J Biosci Bioeng 2007; 103(5): 389-98.
[7] Desbaillets I, Ziegler U, Groscurth P, Gassmann M. Embryoid bodies: an *in vitro* model of mouse embryogenesis. Exp Physiol 2000; 85(6): 645-51.
[8] Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M*, et al.* Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 2000; 6(2): 88-95.
[9] Glaser DE, Burns AB, Hatano R, Medrzycki M, Fan Y, McCloskey KE. Specialized mouse embryonic stem cells for studying vascular development. Stem Cells Cloning 2014; 7: 79-88.
[10] Goodwin AM. *In vitro* assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. Microvasc Res 2007; 74(2-3): 172-83.
[11] Hermant B, Desroches-Castan A, Dubessay ML, Prandini MH, Huber P, Vittet D. Development of a one-step embryonic stem cell-based assay for the screening of sprouting angiogenesis. BMC Biotechnol 2007; 7: 20.
[12] Matsubara K, Mori M, Akagi R, Kato N. Anti-angiogenic effect of pyridoxal 5'-phosphate, pyridoxal and pyridoxamine on embryoid bodies derived from mouse embryonic stem cells. Int J Mol Med 2004; 14(5): 819-23.
[13] Ogawa S, Tagawa Y, Kamiyoshi A, Suzuki A, Nakayama J, Hashikura Y*, et al.* Crucial roles of mesodermal cell lineages in a murine embryonic stem cell-derived *in vitro* liver organogenesis system. Stem Cells 2005; 23(7): 903-13.
[14] Noghero A, Bussolino F, Gualandris A. Role of the microenvironment in the specification of endothelial progenitors derived from embryonic stem cells. Microvasc Res 2010; 79(3): 178-83.
[15] Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R. Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2002; 99(7): 4391-6.
[16] Boyd NL, Dhara SK, Rekaya R, Godbey EA, Hasneen K, Rao RR*, et al.* BMP4 promotes formation of primitive vascular networks in human embryonic stem cell-derived embryoid bodies. Exp Biol Med (Maywood) 2007; 232(6): 833-43.
[17] Feraud O, Cao Y, Vittet D. Embryonic stem cell-derived embryoid bodies development in collagen gels recapitulates sprouting angiogenesis. Lab Invest 2001; 81(12): 1669-81.
[18] Liu Z, Lebrin F, Maring JA, van den Driesche S, van der Brink S, van Dinther M*, et al.* ENDOGLIN is dispensable for vasculogenesis, but required for vascular endothelial growth factor-induced angiogenesis. PLoS One 2014; 9(1): e86273.
[19] Schmidt A, Brixius K, Bloch W. Endothelial precursor cell migration during vasculogenesis. Circ Res 2007; 101(2): 125-36.
[20] Risau W. Mechanisms of angiogenesis. Nature 1997; 386(6626): 671-4.
[21] Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature 2000; 407(6801): 242-8.
[22] Wang N, Zhang R, Wang SJ, Zhang CL, Mao LB, Zhuang CY*, et al.* Vascular endothelial growth factor stimulates endothelial differentiation from mesenchymal stem cells via Rho/myocardin-related transcription factor--a signaling pathway. Int J Biochem Cell Biol 2013; 45(7): 1447-56.

[23] Shin Y, Jeon JS, Han S, Jung GS, Shin S, Lee SH*, et al. In vitro* 3D collective sprouting angiogenesis under orchestrated ANG-1 and VEGF gradients. Lab Chip 2011; 11(13): 2175-81.
[24] Li J, Stuhlmann H. *In vitro* imaging of angiogenesis using embryonic stem cell-derived endothelial cells. Stem Cells Dev 2012; 21(2): 331-42.
[25] Nolan DJ, Ginsberg M, Israely E, Palikuqi B, Poulos MG, James D*, et al.* Molecular signatures of tissue-specific microvascular endothelial cell heterogeneity in organ maintenance and regeneration. Dev Cell 2013; 26(2): 204-19.
[26] Wang ZZ, Au P, Chen T, Shao Y, Daheron LM, Bai H*, et al.* Endothelial cells derived from human embryonic stem cells form durable blood vessels *in vivo*. Nat Biotechnol 2007; 25(3): 317-8.
[27] Eble JA, Niland S. The extracellular matrix of blood vessels. Curr Pharm Des 2009; 15(12): 1385-400.
[28] Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol 2005; 23(1): 47-55.
[29] Blancas AA, Lauer NE, McCloskey KE. Endothelial differentiation of embryonic stem cells. Curr Protoc Stem Cell Biol 2008; Chapter 1: Unit 1F 5.
[30] Kane NM, Meloni M, Spencer HL, Craig MA, Strehl R, Milligan G*, et al.* Derivation of endothelial cells from human embryonic stem cells by directed differentiation: analysis of microRNA and angiogenesis *in vitro* and *in vivo*. Arterioscler Thromb Vasc Biol 2010; 30(7): 1389-97.
[31] Wang H, Luo X, Leighton J. Extracellular matrix and integrins in embryonic stem cell differentiation. Biochem Insights 2015; 8(Suppl 2): 15-21.
[32] Vailhe B, Vittet D, Feige JJ. *In vitro* models of vasculogenesis and angiogenesis. Lab Invest 2001; 81(4): 439-52.
[33] Staton CA, Reed MW, Brown NJ. A critical analysis of current *in vitro* and *in vivo* angiogenesis assays. Int J Exp Pathol 2009; 90(3): 195-221.
[34] Arnaoutova I, Kleinman HK. *In vitro* angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc 2010; 5(4): 628-35.
[35] Kim S, Lee H, Chung M, Jeon NL. Engineering of functional, perfusable 3D microvascular networks on a chip. Lab Chip 2013; 13(8): 1489-500.
[36] Nguyen DH, Stapleton SC, Yang MT, Cha SS, Choi CK, Galie PA*, et al.* Biomimetic model to reconstitute angiogenic sprouting morphogenesis *in vitro*. Proc Natl Acad Sci U S A 2013; 110(17): 6712-7.
[37] Li H, Chang J. Bioactive silicate materials stimulate angiogenesis in fibroblast and endothelial cell co-culture system through paracrine effect. Acta Biomaterialia 2013; 9(6): 6981-6991.
[38] Zheng Y, Chen J, Craven M, Choi NW, Totorica S, Diaz-Santana A*, et al. In vitro* microvessels for the study of angiogenesis and thrombosis. Proc Natl Acad Sci U S A 2012; 109(24): 9342-7.
[39] Nakatsu MN, Sainson RC, Aoto JN, Taylor KL, Aitkenhead M, Perez-del-Pulgar S*, et al.* Angiogenic sprouting and capillary lumen formation modeled by human umbilical vein endothelial cells (HUVEC) in fibrin gels: the role of fibroblasts and Angiopoietin-1. Microvasc Res 2003; 66(2): 102-12.
[40] Stamati K, Priestley JV, Mudera V, Cheema U. Laminin promotes vascular network formation in 3D *in vitro* collagen scaffolds by regulating VEGF uptake. Exp Cell Res 2014; 327(1): 68-77.

[41] Ashley RA, Dubuque SH, Dvorak B, Woodward SS, Williams SK, Kling PJ. Erythropoietin stimulates vasculogenesis in neonatal rat mesenteric microvascular endothelial cells. Pediatr Res 2002; 51(4): 472-8.
[42] Rezzola S, Belleri M, Gariano G, Ribatti D, Costagliola C, Semeraro F*, et al. In vitro* and *ex vivo* retina angiogenesis assays. Angiogenesis 2014; 17(3): 429-42.
[43] Delgado VM, Nugnes LG, Colombo LL, Troncoso MF, Fernandez MM, Malchiodi EL*, et al.* Modulation of endothelial cell migration and angiogenesis: a novel function for the "tandem-repeat" lectin galectin-8. FASEB J 2011; 25(1): 242-54.
[44] Jakobsson L, Kreuger J, Claesson-Welsh L. Building blood vessels--stem cell models in vascular biology. J Cell Biol 2007; 177(5): 751-5.
[45] Festing S, Wilkinson R. The ethics of animal research. Talking Point on the use of animals in scientific research. EMBO Rep 2007; 8(6): 526-30.
[46] Alvarez MC, Bejar J, Chen S, Hong Y. Fish ES cells and applications to biotechnology. Mar Biotechnol (NY) 2007; 9(2): 117-27.
[47] Fan L, Collodi P. Zebrafish embryonic stem cells. Methods Enzymol 2006; 418: 64-77.
[48] Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M*, et al.* The zebrafish reference genome sequence and its relationship to the human genome. Nature 2013; 496(7446): 498-503.
[49] Chavez MN, Aedo G, Fierro FA, Allende ML, Egana JT. Zebrafish as an emerging model organism to study angiogenesis in development and regeneration. Front Physiol 2016; 7: 56.
[50] Lawson ND, Weinstein BM. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 2002; 248(2): 307-318.
[51] Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Development 2005; 132(23): 5199-209.
[52] Experiments on Animal Act, 2014. The State Secretary of Health and Environmental Protection, Soestdijk. http://wetten.overheid.nl/BWBR0003081/2014-12-18
[53] Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance, 2010. The European Parliament and the council of the European Union, Official Journal of the European Union. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:32010L0063
[54] Westerfield M. The zebrafish book: a guide for the laboratory use of zebrafish (*Danio Rerio*). 4 ed, University of Oregon Press, Eugene 2000, pp.
[55] Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995; 203(3): 253-310.
[56] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012; 9(7): 671-5.
[57] Moya ML, Hsu YH, Lee AP, Hughes CC, George SC. *In vitro* perfused human capillary networks. Tissue Eng Part C Methods 2013; 19(9): 730-7.
[58] Dang SM, Kyba M, Perlingeiro R, Daley GQ, Zandstra PW. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. Biotechnol Bioeng 2002; 78(4): 442-53.
[59] Zhao H, Huang H, Lin S. Chemical approaches to angiogenesis in development and regeneration. Methods Cell Biol 2016; 134: 369-76.

[60] Kabrun N, Buhring HJ, Choi K, Ullrich A, Risau W, Keller G. Flk-1 expression defines a population of early embryonic hematopoietic precursors. Development 1997; 124(10): 2039-48.
[61] Feng X, Tonnesen MG, Mousa SA, Clark RA. Fibrin and collagen differentially but synergistically regulate sprout angiogenesis of human dermal microvascular endothelial cells in 3-dimensional matrix. Int J Cell Biol 2013; 2013: 231279.
[62] Park YK, Tu TY, Lim SH, Clement IJ, Yang SY, Kamm RD. *In Vitro* Microvessel Growth and Remodeling within a Three-dimensional Microfluidic Environment. Cell Mol Bioeng 2014; 7(1): 15-25.
[63] Roy S, Driggs J, Elgharably H, Biswas S, Findley M, Khanna S*, et al.* Platelet-rich fibrin matrix improves wound angiogenesis via inducing endothelial cell proliferation. Wound Repair Regen 2011; 19(6): 753-66.
[64] Haycock JW. 3D cell culture: a review of current approaches and techniques. Methods Mol Biol 2011; 695: 1-15.
[65] Rao RR, Peterson AW, Ceccarelli J, Putnam AJ, Stegemann JP. Matrix composition regulates three-dimensional network formation by endothelial cells and mesenchymal stem cells in collagen/fibrin materials. Angiogenesis 2012; 15(2): 253-64.
[66] Latimer A, Jessen JR. Extracellular matrix assembly and organization during zebrafish gastrulation. Matrix Biol 2010; 29(2): 89-96.
[67] Jessen JR. Recent advances in the study of zebrafish extracellular matrix proteins. Dev Biol 2015; 401(1): 110-21.
[68] Bergmeier W, Hynes RO. Extracellular matrix proteins in hemostasis and thrombosis. Cold Spring Harb Perspect Biol 2012; 4(2).
[69] Wu F, Song H, Zhang Y, Zhang Y, Mu Q, Jiang M*, et al.* Irisin induces angiogenesis in human umbilical vein endothelial cells *in Vitro* and in zebrafish embryos *in vivo* via activation of the ERK signaling pathway. PLoS One 2015; 10(8): e0134662.
[70] Wang MF, Lai SC. Fibronectin degradation by MMP-2/MMP-9 in the serum of pregnant women and umbilical cord with Toxoplasma gondii infection. J Obstet Gynaecol 2013; 33(4): 370-4.

Chapter 6

In vitro development of zebrafish vascular networks

Muhammad Ibrahim and Michael K. Richardson

This chapter has been published as following:

Ibrahim M, Richardson MK. *In vitro* development of zebrafish vascular networks. Reproductive Toxicology (2017). DOI: 10.1016/j.reprotox.2017.02.008.

Abstract

A major limitation to culturing tissues and organs is the lack of a functional vascular network *in vitro*. The zebrafish possess many useful properties which makes it a promising model for such studies. Unfortunately, methods of culturing endothelial cells from this species are not well characterised. Here, we tried two methods (embryoid body culture and organ explants from transgenic zebrafish *kdrl:GFP* embryos) to develop *in vitro* vascular networks. In the *kdrl:GFP* line, endothelial cells expresses green fluorescent protein, which allows to track the vascular development in live cultures. We found that embryoid bodies showed significantly longer and wider branches of connected endothelial cells when grown in a microfluidic system than in static culture. Similarly, sprouting of *kdrl:GFP*$^{+}$ cells from the tissue explants was observed in a 3D hydrogel matrix. This study is a step towards the development of zebrafish vascular networks *in vitro*.

Key words: Angiogenesis; Embryoid bodies; Explant culture; Microfluidics; Vasculogenesis; Zebrafish.

Introduction

There are a number of reasons why it could be useful to develop culture systems containing functional vascular networks. For example, tissue engineering is a very important area of biomedical research that may have applications in regenerative medicine and organ transplantation [1]. The *in vitro* culture of complex tissues might also help our understanding of physiological aspects of organ function [2]; disease conditions such as cardiac disorders [3]; and drug screening [4]. *In vivo* the vascular system is essential for the growth and development of functional tissues and organs [5]. A major obstacle to engineering an organ *in vitro* with the current tissue culture procedures is the lack of a vascular network [6]. Development of three-dimensional (3D) culture systems with a functional capillary bed could overcome this problem [7, 8].

Development of an *in vitro* vascular network could also have other applications e.g. vascular regenerative therapy [9] and modelling diseases such as retinal microvascular abnormalities in diabetes [10] and abnormal angiogenesis in tumor development [11]. Vascular culture techniques are important in cancer research for the screening of compounds that inhibit angiogenesis [12]. Furthermore, *in vitro* vascular networks could also serve as a screening model for candidate drugs, as some of the drugs approved for clinical trials may disturb vascular development. An example of such a drug is thalidomide, whose teratogenicity is linked to anti-angiogenic effects [13].

Protocols for culturing vascular networks have been successfully developed using endothelial cell lines and embryonic tissues [14]. Commonly, human umbilical vein endothelial cells (HUVEC) are used in pure culture or in co-culture with other cells (Table 1). These cultures are established on biological matrices that mimic some of the properties of endogenous extracellular matrix [15]. Blood vessel sprouting has been shown to take place from beads coated with HUVECs, and cultured on a fibrin gel, in media supplemented with vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin-1 (Ang-1) and transforming growth factor beta (TGF-β) [16]. Endothelial cells from other species have also been adapted for *in vitro* vasculogenesis; examples include bovine aortic endothelial cells [17] and rat aortic endothelial cells [18].

Another method for culturing vascular networks is the embryoid body culture. Embryoid bodies (EBs) are three-dimensional (3D) aggregates of embryonic stem cells isolated from blastocyst stage embryos [19]. In an embryoid body culture the endothelial cells differentiate and form blood vessels in a complex environment, which reflects vascular formation in early embryos [20]. Unlike pure endothelial cell cultures, multiple cell types are involved in vessel formation in EB culture [21]. Vasculogenesis starts at day 3 of hanging drop cultures in mouse EBs [22]. These EBs show sprouting of blood vessel-like structures, into the surrounding matrix, when transferred to two-dimensional (2D) or 3D collagen gel [22].

Table 1: Culture conditions used to form *in vitro* vascular networks using endothelial cell lines.

Culture constituents	Units	Endothelial cell type										
		HUVEC	HEPC	HEVC	ECFC-EC	HUVEC	HUVEC	HUVEC	HUVEC	BAEC	HUVEC	HUVEC
Culture system	-	Static	Static	Static	Flow	Flow	Flow	Static	Flow	Static	Static	Static
Substrate	-	BME	Col-I Fbg Pmtx	HA-Hyg	Fbn	Fbn	Col-I		Col-I	MG	Fbn	Col-I
Supporting cell types	-	-	HMPC	-	NHLF	NHLF HPP	-	HDF	HBVP HUASMC	-	-	HBMSC
EGM-2	-							-	-	-		
DMEM	-	-	-	-	-	-	-		-		-	
M199	-	-		-	-	-	-	-		-	-	-
L-Gln	-	-	-	-	-	-	-	-		-	-	-
ECGS	-	-	-	-	-	-	-	-		-	-	-
FBS	%	-	20	-	-	-	-	10	16	1	-	1
bFGF	ng/mL	-	-	-	-	-	-	-	50	-	25	-
VEGF	ng/mL	-	-	-	-	-	-	-	50	50	25	-
L-AA	ng/mL	-	-	-	-	-	-	-	50	-	-	-
O_2	%	-	-	-	5	-	-	-	-	-	-	-
CS-ext	ratio	-	-	-	-	-	-	1/128	-	-	-	-
P/S	%	-	-	-	-	-	-	-	1	-	-	1
PMA	ng/mL	-	-	-	-	-	-	-	50	-	-	-
rGal-8	nM	-	-	-	-	-	-	-	-	5-20	-	-
References		[23]	[24]	[25]	[26]	[27]	[28]	[29]	[30]	[17]	[16]	[31]

Abbreviations: BAEC, bovine aortic endothelial cells; bFGF, basic fibroblast growth factor; BME, basement membrane extract (Trevigen); Col-I, collagen type-I; CS-ext, calcium silicate extract from bioceramics; DMEM, Dulbecco's modified Eagle's medium; ECFC-EC, human endothelial colony forming cell-derived endothelial cells; ECGS, endothelial cell growth supplement; EGM, endothelial growth medium; Fbn, fibrin; FBS, fetal bovine serum; Flow, microfluidic flow-through culture; HA-Hyg, hyaluronic acid based hydrogel; HBMSC, human bone marrow-derived mesenchymal stem cells; HBVP, human brain vascular pericytes; HDF, human dermal fibroblasts; HEPC, Human endothelial progenitor cells; HEVC, human early vascular cells; HMPC, human mesenchymal progenitor cells; HPP, human placental pericytes; HUASMC, human umbilical cord arterial smooth muscle cells; HUVEC, human umbilical vein endothelial cells; L-AA, L-ascorbic acid; L-Gln, L-glutamine; M199, medium 199 from Lonza; MG, Matrigel™; NHLF, human normal lungs fibroblasts; P/S, penicillin/streptomycin; PMA, phorbol-12-myristate-13-acetate; Pmtx, puramatrix; rGal-8, recombinant galectin-8; Static, static replacement culture; VEGF, vascular endothelial growth factor; grey boxes indicate the base medium; -, not added.

As an alternative, embryonic tissue explants are also used as precursors for culturing blood vessels (Table 2). Similar to the EBs, tissue explants contain multiple cell types required for the formation of blood vessels [14]. Furthermore, the cells in the explants are thought to be closer to the *in vivo* state, compared to repeatedly passaged endothelial cell lines [14]. A disadvantage of using tissue explants for the culture of vascular networks is that the growth rate of the cells in the explant is slower than in the cell lines [32]. One of the commonly-used tissue explants capable of developing blood vessel sprouts *in vitro* is the cross section of rat or mouse aorta called the aortic ring [33]. Other potential explants

include fragments of embryonic mouse metatarsal bones [34], mouse retina [35] and rat kidney [36] tissues.

Haemodynamic, or the mechanical forces produced by blood flow, influence the expression of several biochemical pathways in the endothelial cells; these in turn can modulate the structure and function of blood vessels [37]. In addition to the different techniques discussed above for culturing vascular networks, the role of haemodynamic factors has been studied using microfluidic or lab-on-a-chip technology [38]. By combining 3D culture in a hydrogel (which mimics the natural ECM) with microfluidics (which mimics the blood flow), an *in vitro* environment can be created which could be in principle, close to the *in vivo* environment for vascular morphogenesis [8, 39]. Advances in microfluidics and 3D culture technologies have greatly increased the possibilities for developing functional vascular models and vascularized tissues [39]. However, the challenges in selecting an appropriate microfluidic system and 3D matrix for culturing blood vessels, that can vascularize complex tissues *in vitro*, still need to be resolved [39].

Most of the current procedures for culturing blood vessels discussed above, involve cells or tissues from mouse, humans or other species. These techniques are associated with certain limitations. Human endothelial cell lines are not thought to closely represent the *in vivo* state of the endothelial cells [14]. Furthermore, these cell lines change their gene expression and physiological properties with repeated passaging *in vitro*, and may lose their ability to form vascular networks [14]. Mouse embryonic tissues are difficult to isolate because of the internal fertilisation and *in utero* development of the embryo. Techniques for the isolation of embryonic stem cells and organ explants from mammals are more costly, require invasive surgical procedures and can raise ethical concerns [9, 40]. Furthermore, mouse aortic explant cultures have shown significant variability between the experiments [41].

For these reasons, it is desirable to explore the possibilities offered by alternative models. The zebrafish is one such emerging model species [42]. In contrast to the mouse, the external fertilization in zebrafish allows easy access to a large number of embryos, as well as cells or tissues isolated from these embryos, for *in vitro* studies [43, 44]. Zebrafish early embryonic cells or adult stem cells have been used for *ex vivo* experiments; fewer have used cells from larvae [44, 45]. Zebrafish whole embryos and isolated cells are currently being developed as potential alternative screening models for toxicity analysis [46, 47].

Many of the organ primordia of zebrafish are formed during the first 72 h of embryo development [48]. There are practical advantages of zebrafish for cell culture e.g. they can be maintained in a simple incubator without additional CO_2 supply [49]. Zebrafish embryos are optically transparent until early larval stages [50]. Furthermore, the genetically-modified zebrafish line *kdrl:GFP* expresses green fluorescent protein (GFP) in its endothelial cells [51]. In this line, the development of blood vessels can be tracked

using confocal microscopy [52]. Zebrafish *kdrl:GFP* embryos and embryonic cell culture have been used for analysing the toxic effect of different compounds on vascular development [53, 54].

Table 2: Culture conditions used to form *in vitro* vascular networks using tissues explants.

Culture constituents	Units	Mouse tissues used for explant preparation						
		retina	AT	MT	MT	MT	AR	LV
Culture system		Static	Static	Static	Static	Static	Static	Static
Substrate		Fbn	MG	Col-I	Col-I	-	Col-I	Fbn
DMEM			-	-	-	-	-	
EBM-2		-		-	-	-	-	-
α-MEM		-	-				-	-
MCDB131		-	-	-	-	-		-
FBS	%	10	5	10	10	10	-	10
VEGF	ng/mL	100	0.5	50	-	-	-	5
hEGF	ng/mL	-	5	-	-	-	-	-
bFGF	ng/mL	-	10	-	-	-	-	10
PDGF-BB	ng/mL	-	-	-	-	-	-	10
R3-IGF	ng/mL	-	20	-	-	-	-	-
PS	%	-	-	1	1	1	-	-
GA	%	1	-	-	-	-	-	-
Penicillin	U/mL	-	-	-	-	-	100	-
Streptomycin	µg/mL	-	-	-	-	-	100	-
Rapamycin	nM	-	-	-	-	-	-	10
Ascorbic acid	µg/mL	-	1	-	-	-	-	-
Hydrocortisone	µg/mL	-	0.2	-	-	-	-	-
$NaHCO_3$	mM	-	-	-	-	-	25	-
Mouse serum	%	-	-	-	-	-	2.5	-
Glutamine	%	-	-	-	-	-	1	-
References		[35]	[55]	[56]	[57]	[34]	[58]	[59]

Abbreviations: α-MEM, minimal essential medium from Invitrogen; AR, aortic ring; AT, adipose tissue; bFGF, basic fibroblast growth factor; Col-I, collagen type-I; DMEM, Dulbecco's modified Eagle's medium; EBM-2, endothelial basal medium from Lonza; Fbn, fibrin; FBS, fetal bovine serum; GA, gentamycin amphotericin-B mix; hEGF, human epidermal growth factors; LV, left ventricle; MCDB131, basal medium life technologies; MG, Matrigel™; MT, metatarsal; PS, penicillin streptomycin mix; PDFG-BB, platelets derived growth factor; R3-IGF, insulin like growth factors; VEGF, vascular endothelial growth factor; grey boxes indicate the basal medium; -, not added.

Our ultimate goal is to develop an *in vitro* model of vascular networks using zebrafish embryonic cells, as an alternative to currently used mouse and human cell culture models. To achieve this aim, we describe here procedures for culturing zebrafish EBs and embryonic organ explants for sprouting angiogenesis. We first compare the growth of vascular network-like structures in *kdrl:GFP* EB cultures, maintained with or without microfluidic flow. We shall refer to the cultures without microfluidic flow as 'static' cultures. The EB cultures were derived from blastocyst stage zebrafish embryos at 3.5 h post fertilization (hpf). Second, we describe the culture of zebrafish organ explants (liver and heart) isolated from aseptically grown 5 days post fertilization (dpf) embryos for

sprouting angiogenesis. These explant cultures were developed in order to further optimize the culture conditions for these tissues, as cells derived from different tissues and at different developmental stage of embryo may have different culture requirements. Using the knowledge gained from these studies in zebrafish, we hope one day to extend the techniques to cells from other species.

Materials and methods

Zebrafish embryos

All the animal experiments were performed according to the Netherland experiments on Animals act [60], based on EU directives [61]. Adult *kdrl:GFP* zebrafish were maintained in circulating water according to previously described protocols [62]. Adult male and female fish, at a proportion of 1:1, were transferred to breeding tanks in the evening. The eggs were collected, next morning, at the bottom of the tank, separated from adults using a mesh to prevent the eggs from being eaten. Fertilized, healthy embryos were distributed in 9 cm Petri dishes (100 embryos per dish for EB culture and 50 embryos per dish for liver and heart isolation). The embryos used for EB culture were allowed to grow for 3.5 h, and the embryos for liver and heart isolation for 24 h in a temperature controlled room at 28 °C.

When zebrafish eggs are laid, they are exposed to a wide range of pathogens in the water including faecal pathogens from the adults [63]. The chorion represents a barrier to the entry of microorganisms into the perivitelline space and embryo [64]. Therefore, before isolating cells and tissues we decontaminated the eggs with their intact chorion.

Embryo sterilization

The embryos were surface decontaminated, with the chorion intact, using a procedure modified from Ref. [65]. Briefly, the embryos were transferred to a small net and immersed in 70% ethanol for 10 sec. The embryos were then washed with L15 medium (Table 3) to remove the ethanol. The embryos were then immersed twice, for 4 min each, in sodium hypochlorite solution (Table 3), with a change of L15 medium in between. After the last treatment with sodium hypochlorite the embryos were washed three times with L15 medium and finally left in 500 µL of L15 medium for dechorionation.

Embryo dechorionation

The embryos decontaminated in the previous step, still with their chorions intact, were subjected to manual dechorionation under a dissecting microscope using a pair of sterile No.5 watchmaker's forceps. The dead embryos, or embryos with cloudy perivitelline fluid were removed before dechorionation.

Embryoid body culture

The 3.5 hpf blastocyst stage embryos, after sterilization and dechorionation were transferred to Eppendorf tubes (100 blastocysts per tube) using a P-1000 Gilson pipette. The blastocysts were triturated using a P-200 Gilson pipette and then centrifuged at 300g for 1 min to remove most of the yolk. The supernatant was removed and the blastocysts were treated for two minutes with 1 mL trypsin solution (Table 3) with gentle trituration using a P-1000 pipette. The trypsinization was stopped by adding 100 µL of fetal bovine serum (FBS; Invitrogen, Cat. No. 10500), and the cells were pelleted by centrifuging the mixture at 300 g for 3 min. The cells were washed three times with 500 µL of LDF medium (Table 3) and then re-suspended in 200 µL of the same medium. The cell concentration was determined using heamocytometer and the suspension was cultured in hanging drops to initiate the formation of EBs.

Hanging drop culture

The blastocyst cell suspension was diluted to a final concentration of 50 cells/µL in LDF medium supplemented with 10% FBS, 4.1% endothelial growth supplement mix (EGS; Promocell; bio-connect B.V.; Cat. No. C-39216), 50 ng/mL recombinant zebrafish vascular endothelial growth factor ($VEGF_{165}$; R&D systems, Cat. No. 1247-ZV) and 10 ng/mL recombinant human basic fibroblast growth factor (bFGF; Invitrogen, Cat. No. PHG0024). This solution was distributed in 20 µL droplets (1000 cells per drop) onto the inside of the lid of 60 mm Petri dishes. Calcium- and magnesium-free phosphate buffered saline (CMF-PBS) was added to the Petri dishes to humidify the air and thereby reduce evaporation from the droplets. The lids with the droplets were carefully inverted on the Petri dishes and the cultures were left for four days in a humidified incubator at 28 °C and 0.5% CO_2, to allow the cells to aggregate and form EBs.

EB culture in 3D gel matrix

On day 4 of hanging drop culture, the EBs were collected from the hanging drops by inverting the lid and gently tapping it while holding it at 45° angle. The droplets with the EBs collected on one side of the lid and were then transferred to a 1.5 mL Eppendorf tube (100 EBs per tube) using a P-1000 pipette. The EBs were allowed to settle down to the bottom of the tube by gravity and were washed once with 500 µL of LDF medium. Finally, the EBs were re-suspended in 100 µL of LDF medium.

The EBs were then transferred to a 3D gel matrix composed of collagen type-I, Geltrex™ and fibrin (2.5 + 6-9 + 2 mg/mL). The 3D EB cultures were maintained under static replacement conditions in CS16-chambered coverglass plate (Grace Bio; Cat. No. 112358), or under microfluidic conditions in a microchannel slide (Ibidi, sticky-slide $VI^{0.4}$; Cat. No. 80608; Figure 1A). The gel was prepared by mixing the calculated volumes of collagen type-I (5 mg/mL; Ibidi, Cat. No. 50201), Geltrex™ (12-18 mg/mL; Invitrogen, Cat. No.

A1413201) and bovine fibrinogen (10 mg/mL; Sigma, Cat. No. F8630) on ice. The solution was diluted, to achieve the desired concentrations, using 10X CMF-PBS and LDF medium and was supplemented with $VEGF_{165}$ (50 ng/mL). Thrombin (final concentration 3 Units/mL; Sigma, Cat. No. T4648) was added to the gel mixture to polymerize the fibrinogen.

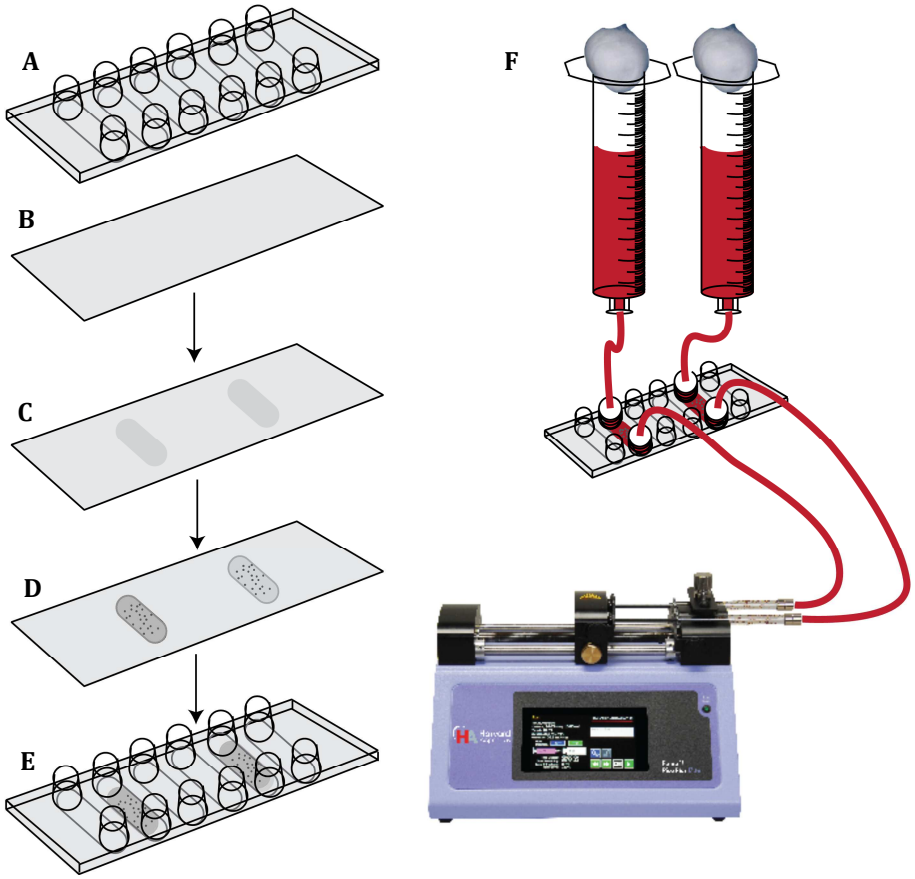

Figure 1. Microfluidic flow-through culture setup. (**A**) Ibidi six-microchannel sticky-slide. (**B**) Cover glass slide. (**C**) Applied gel mixture coating on the cover glass slide at the point where the slide will come in contact with the channels. (**D**) Embryoid bodies and another layer of gel mixture added to the coated area. (**E**) The cover glass with embryoid bodies embedded in gel is glued to the bottom of the slide. (**F**) The channels with embryoid bodies in 3D gel are connected to media reservoirs and Harvard syringe pump to start the media flow through the channels.

To initiate the cultures, the wells of the chambered coverglass plate or the bottom cover glass slide of the microchannel sticky-slide (Figure 1B and C) was coated with a thin layer of the gel mixture. The plate and slide with the gel coating were incubated at 28 °C for 30 min. The EBs were then plated in the coated well and on the coated area of the slide (20 EBs per well or per coated area on slide). The plates and slides with the EBs were again

incubated for 30 min at 28 °C. The excess medium was then removed and another layer of the gel was applied on the top of the EBs (Figure 1D).

After a third incubation at 28 °C for 30 min, 250 µL of the LDF medium supplemented with 5% FBS, 4.1% EGS, 50 ng/mL $VEGF_{165}$ and 10 ng/mL bFGF was added to the wells prepared for static cultures. The microchannel slide was sealed (Figure 1E) and connected to a medium reservoir with the same medium and a syringe pump (Pump 11 Pico Plus Elite; Harvard Apparatus; item No. 70-4506; Figure 1F). The medium was drawn through the culture chamber at a flow rate of 20 µL/min. The medium reservoirs were filled with 10 mL of the medium, which was enough for approximately eight hours of perfusion. Every eight hours, the reservoirs were refilled with the withdrawn medium. This was repeated until the end of the experiment. The cultures were maintained in the incubator at 28 °C and 0.5% CO_2. For static conditions the medium was refreshed at day 4 of culture.

Table 3: Preparation of media and solutions for experiments.

Reagents (supplier; catalogue number)	Final Concentration
L-15 medium	
Leibovitz's L-15 (Invitrogen; 11415)	99.75%
HEPES (Invitrogen; 15630)	15 mM
Antibiotic antimycotic mix (Invitrogen;15240)	1%
$NaHCO_3$	0.015%
LDF medium	
Lebovitz's L-15 : DMEM (Invitrogen; 11966) : Ham's F-12 (Invitrogen; 21765)	55 : 32.5 : 12.5
HEPES	15mM
Antibiotic antimycotic mix	1%
$NaHCO_3$	0.015%
FBS (Invitrogen; 10500)	10%
Zebrafish embryo extract	50 µg/mL
Embryo medium	
L-15 medium	10%
Antibiotic antimycotic mix	1%
FBS	1%
Sterile distilled H_2O	88%
Trypsin solution	
Trypsin 2.5% (Invitrogen; 15090)	0.25%
CMF-PBS	99.75%
EDTA	1 mM
Sodium hypochlorite solution	
Sodium hypochlorite, 10-15% (Sigma; 425044)	0.05%
Sterile distilled H_2O	99.95%

Abbreviations: CMF-PBS, calcium magnesium free phosphate buffer saline; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

Aseptic culture of embryos for liver and heart isolation

The embryos (24 hpf) decontaminated and dechorionated according to the procedure described above (Embryo sterilization and Embryo dechorionation) were raised for 5 days in Petri dishes in 25 mL of the embryo medium (Table 3). The Petri dishes were

sealed using 3M™ Micropore™ surgical tape to allow gas exchange while ensuring asepsis. These embryos were raised in a temperature-controlled room at 28 °C in 14 h light: 10 h dark cycle.

Isolation of embryonic liver and heart tissue

To isolate liver and heart tissues, each of the 5 dpf embryos was transferred to a 16 µL drop of L-15 medium containing 10% FBS and 0.16 mg/mL tricaine methane sulfonate (TMS) solution to anaesthetise them. The tissues were isolated by dissecting the embryo using a pair of sterile No.5 watchmaker's forceps. After they were dissected out, the liver and heart tissues were transferred to Eppendorf tubes (with the liver and heart tissue in separate tubes) in 100 µL of L-15 medium containing 10% FBS at room temperature. The tissues from 100 embryos were pooled in one Eppendorf tube.

Explant culture

The liver or heart explants were embedded in 3D hydrogels for culturing. Different substrate compositions were used to prepare the gel matrices. Liver explants were cultured in two different gel matrices: (i) Collagen type-I + Geltrex™ + fibrin (2.5 + 6-9 + 2 mg/mL), (ii) Geltrex™ (12-18 mg/mL). The heart explants were cultured in Collagen type-I + Geltrex™ + fibrin (2.5 + 6-9 + 2 mg/mL) matrix. The 3D cultures were prepared according to the procedure described above (EB culture in 3D gel matrix).

Culture of dissociated liver and heart cells

The liver and heart tissues isolated from 100 larvae were dissociated to make single-cell suspensions. The liver tissues were dissociated using trypsin. Briefly, the isolated livers were washed once with 500 µL CMF-PBS and then incubated for two min at room temperature with 1 mL of trypsin solution (Table 3) with gentle trituration using a P-1000 Gilson pipette. FBS (100 µL) was added to inactivate the trypsin. The heart tissues were dissociated into a single cell suspension using Liberase TL (Sigma, Cat. No. 05401020001) solution. Briefly, the hearts were incubated for 30 minutes at 28 °C, in 1 mL Liberase TL solution (0.4 mg/mL) with occasional trituration using a P-1000 pipette. The solutions were centrifuged at 300 g for 3 min to form a cell pellet, and the supernatant discarded. The cell pellet was washed three times with L-15 medium containing 10% FBS and then re-suspended in 100 µL of the same medium.

A 5 µL of the cell suspension was mixed at a 1:1 ratio with trypan blue dye (0.4% trypan blue in CMF-PBS), and loaded on a heamocytometer. The number of *kdrl:GFP*$^{+}$ and *kdrl:GFP*$^{-}$ cells inside the grid of the heamocytometer was counted under a confocal microscope. From these counts the number of *kdrl:GFP*$^{+}$ cells and total number of cells per microliter was calculated. The percentage of *kdrl:GFP*$^{+}$ cells and the total number of cells in the isolates from 100 tissues was calculated from these numbers. Finally, the cell

suspension was distributed at 20,000 cells per well in the pre-coated wells of the CS16-chambered coverglass plate.

The trypsinized liver cells were cultured on four different 2D substrates: (i) collagen type-I + Geltrex™ + fibrin (2.5 + 6-9 + 2 mg/mL), (ii) collagen type-I + Geltrex™ (2.5 + 6-9 mg/mL), (iii) Geltrex™ (12-18 mg/mL), (iv) tissue culture treated glass surface with no additional substrate added. The wells of the chambered coverglass plate were coated with 5 µL of the desired gel mixture per well and the plate was incubated at 28 °C for 30 min. The dissociated heart cells were cultured on Fibronectin (Invitrogen; Cat. No. 33010018) substratum (1 µg/cm^2). The fibronectin stock solution (1 µg/µL) was diluted using CMF-PBS and 5 µL of the diluted solution (containing 0.3 µg fibronectin) was added per well. The plate was incubated at 28 °C for 30 min and then air dried at room temperature. Before addition of cells, the wells coated with any of the above substrate were washed once with 200 µL of L15 medium.

The dissociated cells or explants cultures derived from liver and heart tissues were cultured in L-15 medium supplemented with 15% FBS, 50 µg/mL zebrafish embryo extract (ZEE), 4.1% EGS, 50 ng/ml $VEGF_{165}$ and 10 ng/ml bFGF. The cultures were maintained in incubator at 28 °C in atmospheric air. The medium was refreshed every second day.

Imaging of cultures

All the cultures were established in CS16-chambered coverglass plates (Grace Bio; Cat. No. 112358) or on coverglass slides for confocal imaging. The cultures were imaged every second day. The EB cultures were maintained until 12 days, while the liver and heart explant and dissociated cell cultures were maintained until six days. Excitation light of 488 nm wavelength was used to visualize the *kdrl:GFP*$^+$ (putative endothelial) cells in cultures. Live cultures at subsequent time-points were imaged to observe the development of vascular network-like structures from *kdrl:GFP*$^+$ cells in the EB and dissociated liver and heart cell cultures. Similarly, the explant cultures were imaged to observe changes in the existing vascular networks, and sprouting of the *kdrl:GFP*$^+$ cells from explants into the surrounding matrix overtime.

Data collection and analysis

The EB cultures were assessed for percentage change in the total *kdrl:GFP*$^+$ area per EB, total length and number of the *kdrl:GFP*$^+$ strands per EB, and average length and width of the *kdrl:GFP*$^+$ strands. The connectedness of the *kdrl:GFP*$^+$ cell network per EB was calculated by dividing the number of endpoints by the number of junctions of the network.

The images of the explant cultures were measured for total *kdrl:GFP*$^+$ area per explant on subsequent time-points of cultures. In addition, the number and average length of

kdrl:GFP^{+} branches was also calculated per explant. All the measurements were made in Image-J software version 1.46r [66].

The data were analysed for mean and standard error using SPSS software version 21.0. Variations in measurements at different time-points of culture and between different substrate conditions were assessed by calculating the p-value using a one-way ANOVA test with SPSS software.

Results

Development of *kdrl:GFP*$^{+}$ cell network in EB culture

The EBs isolated from hanging drop cultures showed a radial network of *kdrl:GFP*$^{+}$ cells inside the EBs (Figure 2, day 2). When these EBs were cultured in 3D gel matrix, the sprouting of *kdrl:GFP*$^{+}$ cells was observed in the surrounding matrix, so as to form a network-like structure (Figure 2). The sprouting appeared to be random; however, in some cases we did observe sprouting in the direction of a nearby EB. Network formation by *kdrl:GFP*$^{+}$ cells was observed only when a mixture of collagen type-I, Geltrex™ and fibrin gel was used as the matrix. The 3D gel matrices composed of a single gel type, or the combination of two (i.e. collagen type-I and Geltrex™) did not show any development of networks.

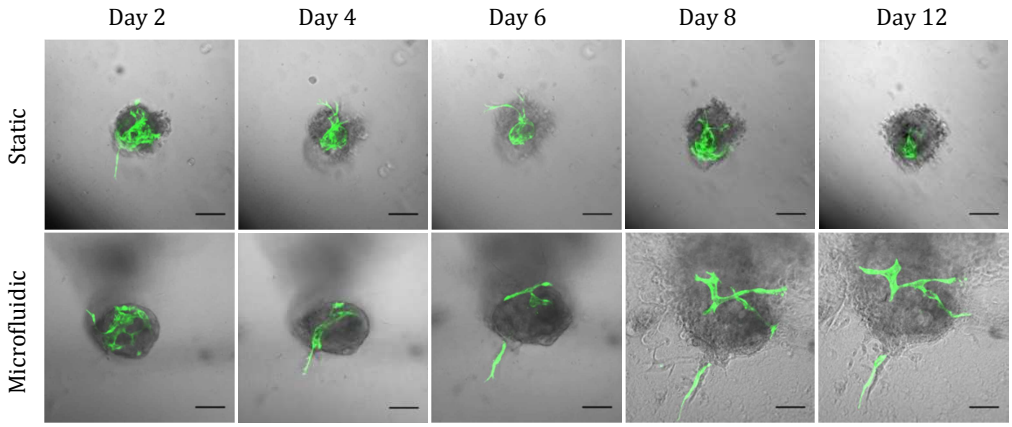

Figure 2: Time-lapse imaging of *kdrl:GFP* EB culture in 3D Collagen type-I + Geltrex™ + Fibrin matrix. Each horizontal row shows the same field. A radial network can be observed inside the EBs on day 2 of culture. A reduction in the *kdrl:GFP*$^{+}$ cells network (green) can be observed in static culture after day 6. In microfluidic culture the network can be observed until day 12. (See also Figure 3 and 4). Scale bar, 100 µm.

The minimum flow rate required for the formation of *kdrl:GFP*$^{+}$ cell networks in our microfluidic system was 20 µL/min. At lower flow rates (i.e. 2 and 10 µL/min) the *kdrl:GFP*$^{+}$ cells failed to form network-like structures in the microfluidic channel (data not

shown). Under these lower flow rates the *kdrl:GFP*$^{+}$ cells were mostly rounded in shape and did not attain the elongated shape as they do when forming a network. The 3D gel combination (collagen type-I, 2.5 mg/mL + Geltrex™ 6-9 mg/mL + fibrin 2 mg/mL) was found to be physically stable in the microfluidic culture at 20 µL/min flow rate. This gel combination was also used for 3D static culture of EBs for comparison (Figure 2). Microfluidic cultures with lower concentrations of the gel components could not be maintained because of tearing of the gel caused by medium flow (data not shown).

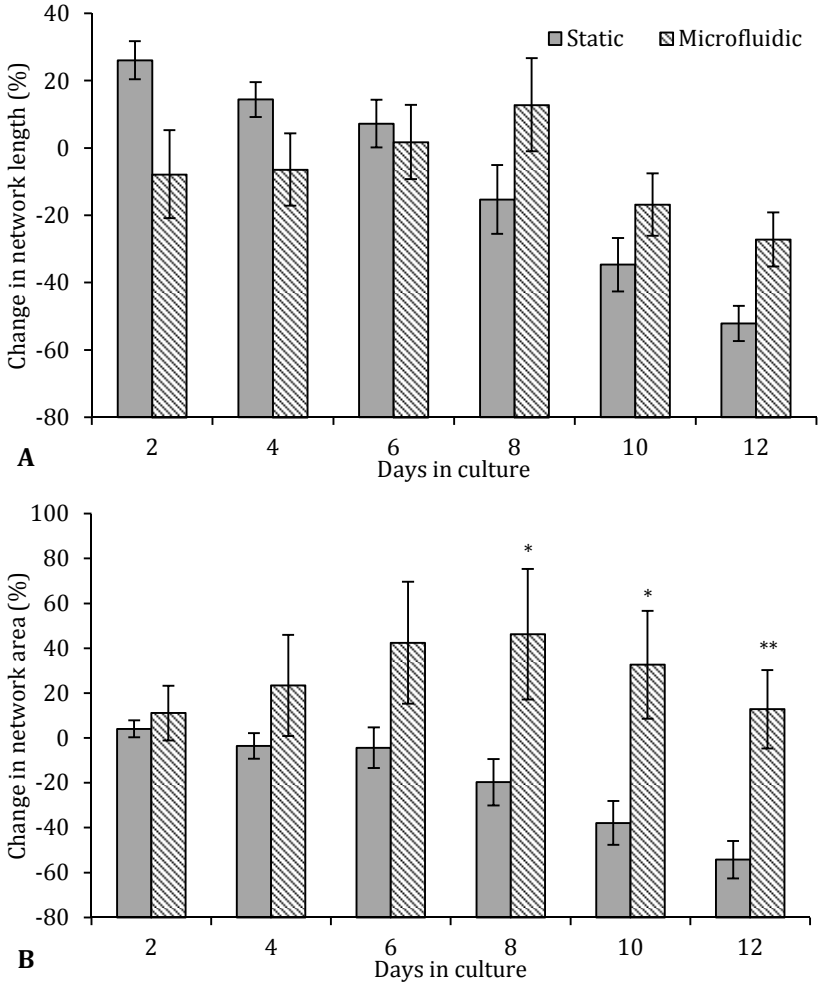

Figure 3: Percent changes, compared to day 1, in length (A) and area (B) of *kdrl:GFP*$^{+}$ cell network in 3D Collagen type-I + Geltrex™ + Fibrin matrix. In conventional (static) culture a decline in the *kdrl:GFP* expression can be observed, while in microfluidic culture the expression is more stable. (**A**) Percent change in total length of *kdrl:GFP*$^{+}$ cell network per EB. (**B**) Percent change in total area covered by *kdrl:GFP*$^{+}$ cells per EB. Number of observations were 12 for Static and eight for Microfluidic cultures. Error bars represent standard error. (**, $p < 0.01$, *, $p < 0.05$).

Changes in length and area of the *kdrl:GFP+* cell network in EB culture

Percent changes with time in the dimensions of *kdrl:GFP+* cell networks in 3D static and microfluidic culture are presented in Figure 3. The results show a gradual decrease in the length and area of the network under static culture conditions; both the length and area became significantly reduced between days 2 and 12 ($p<0.001$). In microfluidic culture the same measurements did not decline significantly with time. The change in length of *kdrl:GFP+* cell networks per EB was similar between static and microfluidic cultures at different time points (Figure 3A). However, the decrease in the total area of the network per EB in static culture over time resulted in significant differences between static and microfluidic cultures after day 8 (Figure 3B).

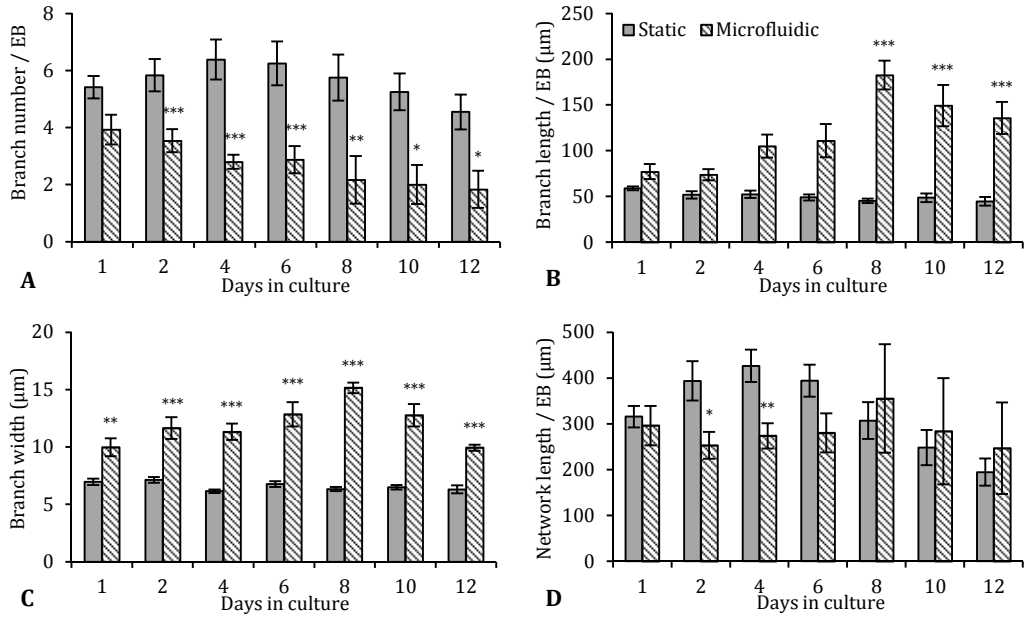

Figure 4: Parameters of *kdrl:GFP+* cell networks in 3D Collagen type-I + Geltrex™ + Fibrin matrix with or without microfluidic flow. (**A**) Number of *kdrl:GFP+* branches per EB at different time-points. (**B**) Average branch length per EB. (**C**) Average width of *kdrl:GFP+* branches per EB. (**D**) Total length of *kdrl:GFP+* cell network per EB. The graphs show significantly higher *kdrl:GFP+* branch length (**B**) and width (**C**) in microfluidic culture. Number of observations were 12 for static and eight for microfluidic culture. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$).

Morphometry of *kdrl:GFP+* networks in EB culture

The number of branches per EB was higher in static culture (Figure 4A). However, the EBs developed significantly longer and wider branches of connected *kdrl:GFP+* cells in microfluidic than in static culture (Figure 4B and C). A higher number of shorter branches in the static culture, and a lower number of longer branches in the microfluidic culture,

resulted in a similar total network length in both culture conditions (Figure 4D). However, on days 2 and 4 the total network length per EB was higher in static culture compared to microfluidic culture.

Under static culture conditions, the number of branches per EB, and the average branch length and width, was similar at different time-points. The total network length per EB in static culture became significantly reduced from day 4 to day 12 ($p<0.001$). Under microfluidic conditions an increase was observed in branch length ($p<0.001$) and width ($p<0.01$) between days 1 and 8. The number of branches and total network length per EB remained similar in microfluidic culture.

Connectedness of *kdrl:GFP*$^{+}$ cell networks in 3D EB culture

The connectedness of *kdrl:GFP*$^{+}$ cell networks in 3D culture was significantly higher under static conditions compared to microfluidic culture (Figure 5). The network connectedness remained similar from day 1 until day 8 in static culture; however, after day 8 the network started to break down, and connectivity became reduced ($p<0.001$). In microfluidic culture the network was less connected, having more end points compared to junctions. As the *kdrl:GFP*$^{+}$ average branch length in the microfluidic culture increased with time, the network connectivity gradually declined. On day 12 of culture no significant difference was observed in network connectedness between the static and microfluidic cultures.

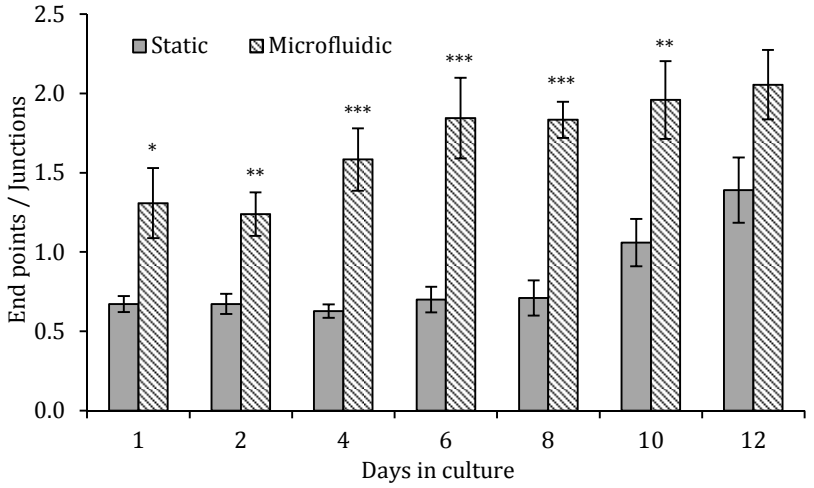

Figure 5: Connectedness of *kdrl:GFP*$^{+}$ cell network in 3D EB culture. Values near to zero on the vertical axis indicate a well-connected network. The graph shows that there is formation of a well-connected network in static culture compared to microfluidic culture. Number of observations were 12 for static and eight for microfluidic culture. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$).

General characteristics of liver explant culture

The liver explants isolated from 5 dpf zebrafish larvae already showed a *kdrl:GFP*$^{+}$ vascular network (Figure 6; day 0). This network changed over time in culture. The *kdrl:GFP*$^{+}$ cells covered the surface of the explant by day 2 of culture in collagen type-I + Geltrex™ + fibrin matrix (Figure 6). At this time-point short strands of *kdrl:GFP*$^{+}$ cells, sprouting from the explant were also observed. However, by day 4 these sprouts were retracted into the explant. By day 6 of culture the layer of *kdrl:GFP*$^{+}$ cells covering the explant formed a network-like structure on the surface of the explant (Figure 6). No *kdrl:GFP*$^{+}$ sprouts were observed from explants cultured in pure Geltrex™ substratum.

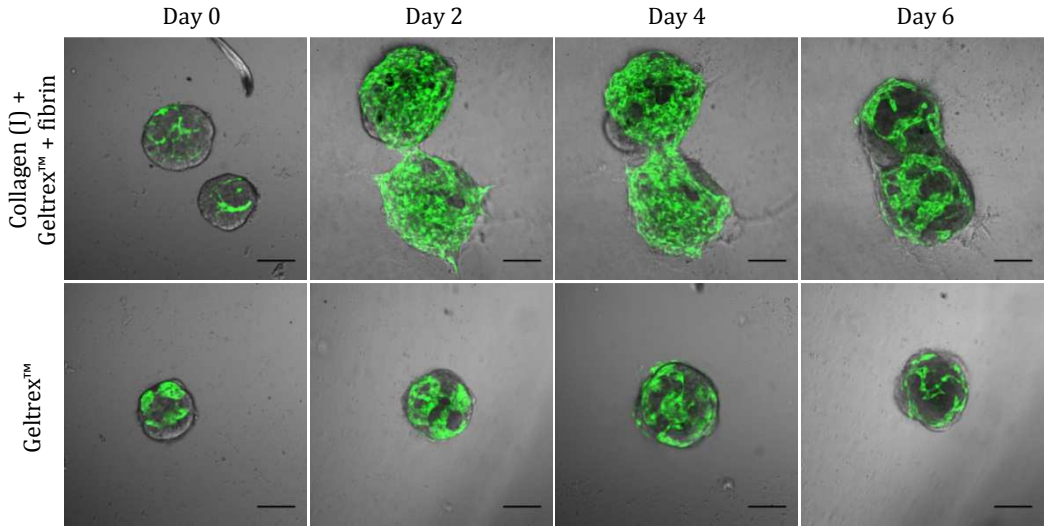

Figure 6: Culture of liver explants isolated from 5 dpf *kdrl:GFP* zebrafish larvae. Cells with green fluorescence are putative endothelial cells. Sprouting of endothelial cells from explant can be seen in collagen type-I + Geltrex™ + fibrin matrix on day 2 of culture. In Geltrex™ matrix the *kdrl:GFP*$^{+}$ cells remained inside or on the surface of the explant. Scale bar, 100 µm.

Measurements of *kdrl:GFP*$^{+}$ cell network in liver explant culture

The area covered by *kdrl:GFP*$^{+}$ cell networks per liver explant increased within the first two days of culture (Figure 7A) and decreased after day 4. No significant differences in the *kdrl:GFP*$^{+}$ area per explant were observed between the two substrates tested. The sprouting of the *kdrl:GFP*$^{+}$ cells was observed only in collagen type-I + Geltrex™ + fibrin substratum. On average, 1.2 sprouts were counted per explant on day 2, with an average length of 18.4 µm (Figure 7B and C). However, these sprouts gradually reduced in length and number with time and almost disappeared by day 6 of culture.

Liver cell culture on 2D substrate

On average 120,457 ± 5,571 cells were obtained by trypsinizing 100 livers. To establish cellular contact in culture 20,000 cells were plated per well of the CS16-chambered

coverglass plate (surface area of each well: 0.34 cm^2). Therefore, cells obtained from one batch of 100 livers were distributed in six wells, with each substrate composition replicated in two wells. The liver cells isolated contained 8.6 ± 0.6% *kdrl:GFP*$^+$ endothelial cells (Figure 8A). The *kdrl:GFP*$^+$ cells combined to form small colonies surrounded by *kdrl:GFP*$^-$ cells on collagen type-I + Geltrex™ + fibrin substratum, on day 2 of culture (Figure 8B).

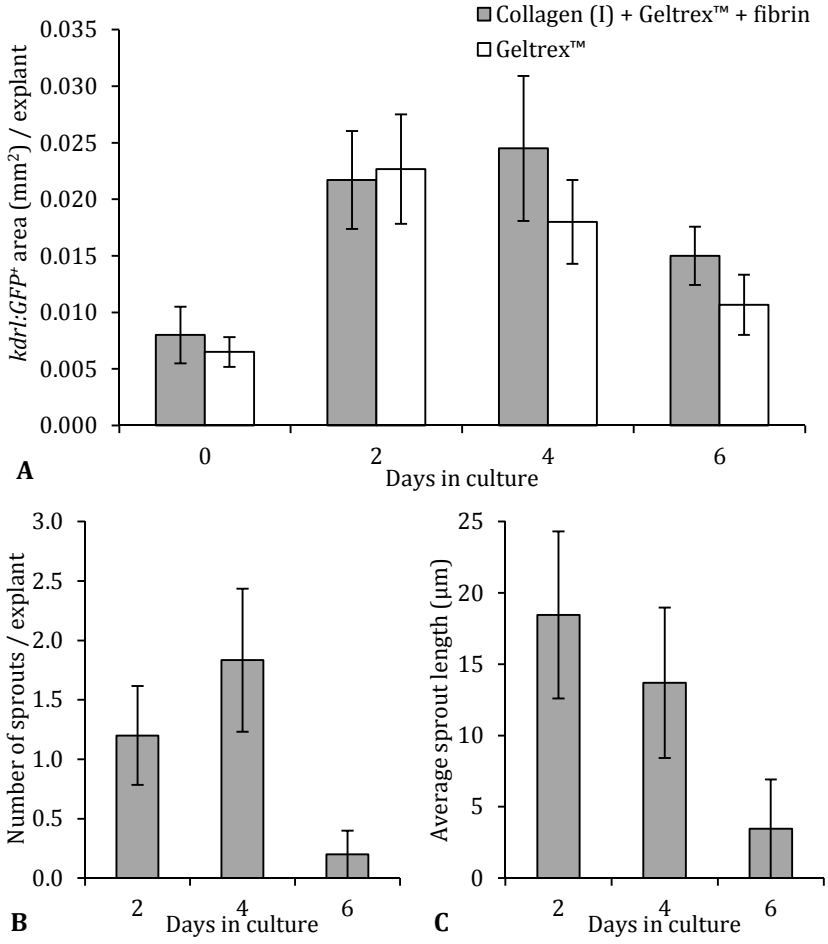

Figure 7: Quantification of area covered by *kdrl:GFP*$^+$ cells in liver explant culture overtime. (**A**) Change in total area covered by *kdrl:GFP*$^+$ cells per explant in the two gel matrices. (**B**, **C**) Data from cultures in collagen type-I + Geltrex™ + fibrin matrix. (**B**) The average number of *kdrl:GFP*$^+$ cell sprouts per explant. (**C**) Average sprout length per explant. Number of observations were six for collagen type-I + Geltrex™ + fibrin matrix and four for Geltrex™ matrix. Error bars represent standard error.

By day 3 of culture, the colonies of *kdrl:GFP*$^+$ endothelial cells appeared to increase in size (Figure 8C). These colonies connected to each other to form longer blood vessel-like structures on day 4 of culture (Figure 8D). These vessel-like structures could be observed

also on day 6 of culture (Figure 8E). However, on day 8 on collagen type-I + Geltrex™ + fibrin substratum, the culture formed a large clump of cells with a vascular network within (Figure 8F). This clump of cells was observed to form earlier when the cells had been plated onto collagen type-I + Geltrex™ (Figure 8G) and pure Geltrex™ (Figure 8H) substrates, or when plated on uncoated glass substrates (Figure 8I).

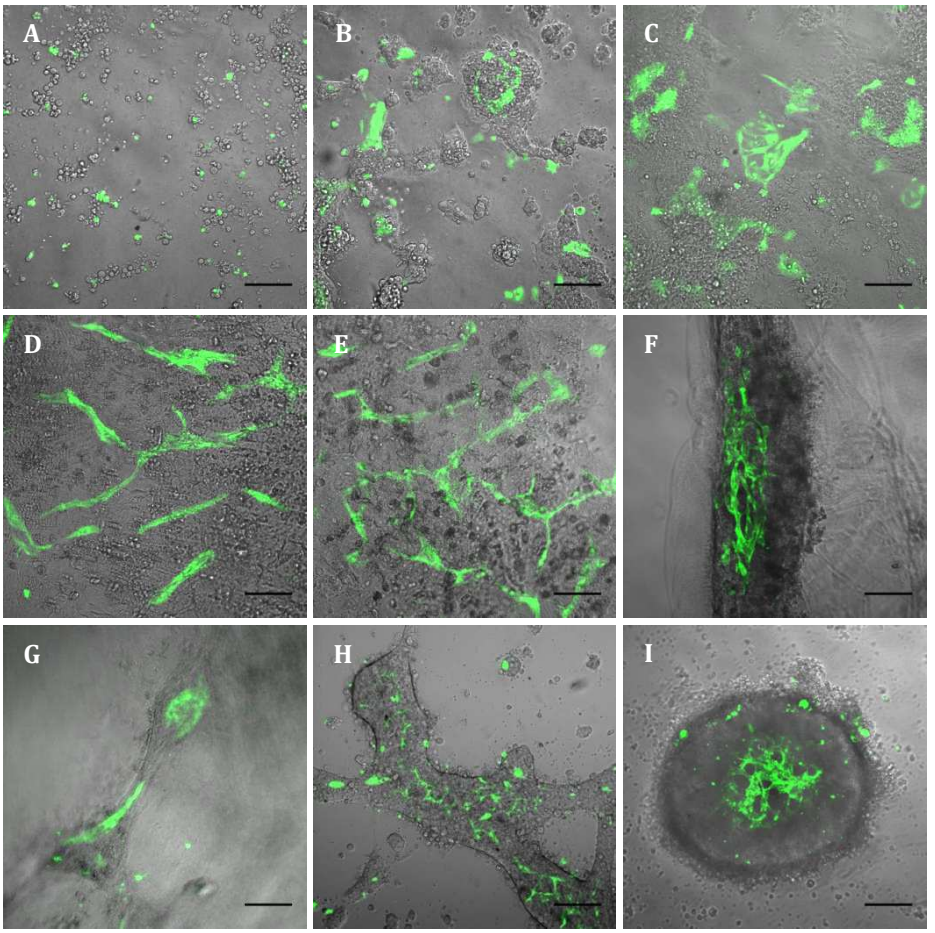

Figure 8: Development of vascular network-like structure in cultures derived from 5 dpf zebrafish liver cells. (**A-F**) Trypsinized liver cells cultured on collagen type-I + Geltrex™ + fibrin substratum. (**A**) Liver cells after plating on day 0 of culture, showing rounded *kdrl:GFP*$^{+}$ cells. (**B**) Day 2 of culture: The cells form colonies of *kdrl:GFP*$^{+}$ cells, surrounded by *kdrl:GFP*$^{-}$ liver cells. (**C**) Day 3 of culture: The *kdrl:GFP*$^{+}$ cells attain an elongated shape and the colonies appear to increase in size. (**D**) Network formation of *kdrl:GFP*$^{+}$ cells on day 4 of culture. (**E**) The *kdrl:GFP*$^{+}$ cell network on day 6 of culture. (**F**) The whole culture has started to condense into one large aggregate dragging with it the substratum, on day 8 of culture. The vascular network remains inside the aggregate. (**G**) On collagen type-I + Geltrex™ substratum the liver cells form large colonies, by day 4 of culture, with networks of *kdrl:GFP*$^{+}$ cells formed inside. (**H**) A similar colony formation of liver cells with *kdrl:GFP*$^{+}$ cell network on day 4 of culture on Geltrex™ substratum. (**I**) Without extra substrate coating, the liver cells form large 3D aggregates with a *kdrl:GFP*$^{+}$ cell network within 4 days of culture. Scale bar, 100 µm. **A** – **F** show the same field.

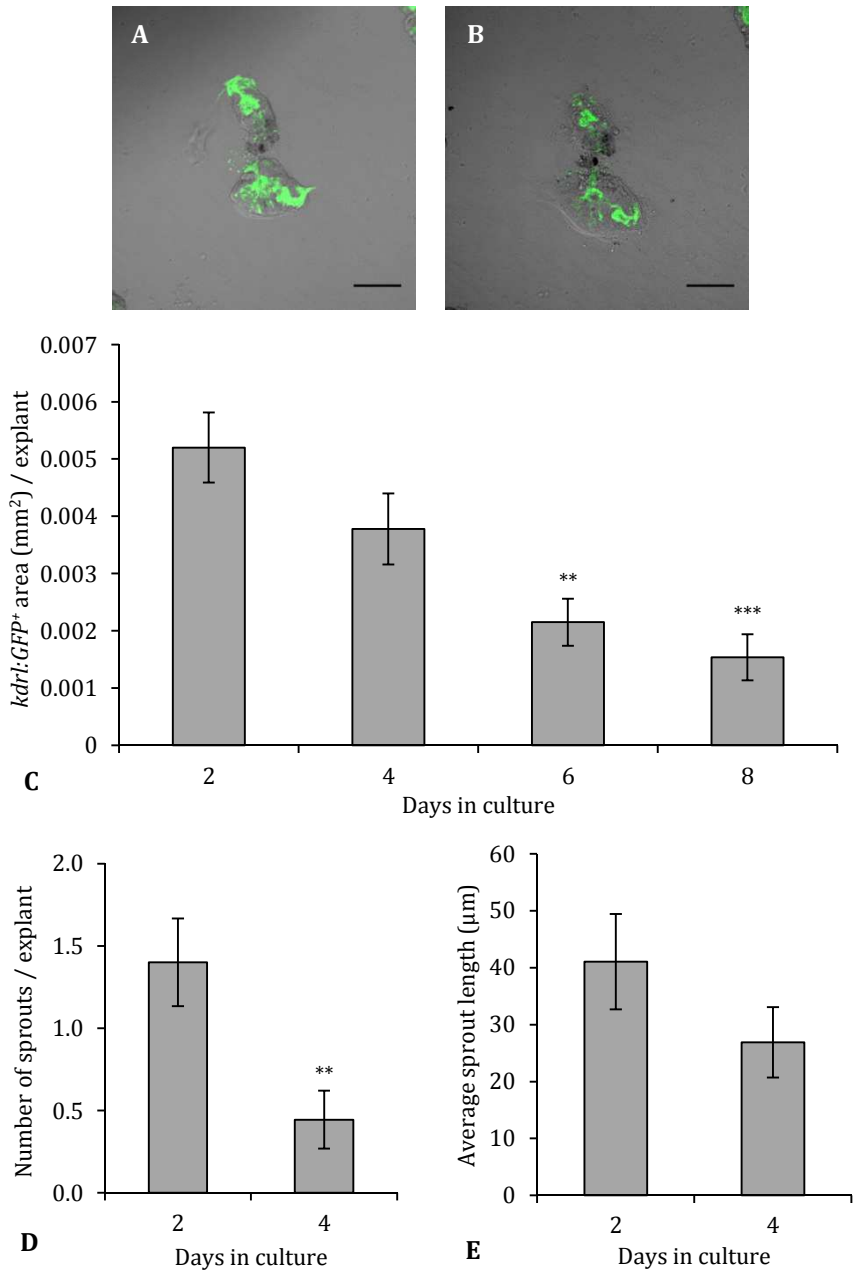

Figure 9: Sprouting of *kdrl:GFP+* cells from heart explant culture in collagen type-I + Geltrex™ + fibrin matrix. (**A**) The heart explant showing *kdrl:GFP+* sprouts in the surrounding matrix on day 2 of culture. (**B**) The sprouts retracted by day 4 of culture with *kdrl:GFP+* cells remaining inside the explants. (**C**) Total area covered by *kdrl:GFP+* cells per heart explant overtime. (**D**) Number of *kdrl:GFP+* sprouts per explant. (**E**) Average sprout length per explant. Scale bar for **A** and **B**, 100 µm. Number of observations were six for **C**, **D** and **E**. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$ compared to values on day 2).

Heart explant and dissociated cell culture

The heart explants cultured in 3D collagen type-I + Geltrex™ + fibrin matrix showed sprouting of *kdrl:GFP*$^{+}$ putative endothelial cells on day 2 (Figure 9A). However, these sprouts almost disappeared on day 4 of culture (Figure 9B). A quantitative analysis of the heart explant cultures showed a significant reduction in the total *kdrl:GFP*$^{+}$ area per explant on day 6 compared to day 2 (Figure 9C). The number of sprouts per explant (Figure 9D) and the average sprout length (Figure 9E) also became reduced from day 2 to day 4.

The embryonic hearts were difficult to trypsinize in the preliminary experiments; we therefore adopted liberase TL enzyme to dissociate them. On average, 42,353 ± 1,707 cells were isolated from 100 hearts, and the cell suspension contained 28.3 ± 1.0% *kdrl:GFP*$^{+}$ endothelial cells. On fibronectin substratum the *kdrl:GFP*$^{+}$ cells showed an elongated morphology 24 h after plating (Figure 10A). Other heart cells that were mostly rounded were removed from culture at this stage by washing with medium, and the colonies of *kdrl:GFP*$^{+}$ cells were maintained on fresh medium. However, these colonies could not be maintained longer and their size reduced with culture duration (Figure 10B and C). Only a few cells were found in culture on day 5 (image not shown).

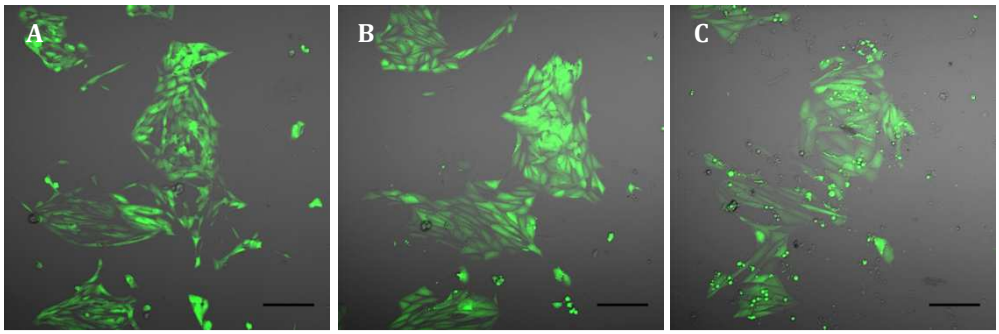

Figure 10: Colony formation of *kdrl:GFP*$^{+}$ cells in dissociated heart cell culture. (**A**) The *kdrl:GFP*$^{+}$ cells attached to the fibronectin substratum on day 1 of the culture. The unattached heart cells were washed out. (**B**) The colony of *kdrl:GFP*$^{+}$ cells appear to shrink in size by day 2 of culture. (**C**) Further reduction in size of the colony with rounded, detaching cells can be seen on day 4 of culture. scale bar, 100 µm.

Discussion

Choice of embryonic stage

For EB culture the cells isolated from blastocyst stage embryos (3.5 hpf) were used. The embryos at this stage contain pluripotent cells, and lineage segregation can be modulated by varying the culture conditions [67]. Techniques have been developed previously for the aseptic isolation and culture of zebrafish blastocyst cells [65]. However, little is known

about the differentiation events and culture strategies needed for the lineage-specific differentiation of these cells.

For embryonic liver and heart tissue culture, zebrafish embryos at an early larval stage (5 dpf) were used. At this stage, there is relatively little yolk remaining in the larva. This makes it easier to isolate the liver, which is more hidden by the yolk in earlier stages. We found that, at 5 dpf, the tissues isolated from 100 larvae contained sufficient numbers of cells to allow for replicates. Zebrafish larvae up to 5 dpf do not engage in exogenous feeding but rely instead on yolk nutrients; therefore, it is easier to keep them sterile in closed Petri dishes. Furthermore, at this stage the tissues presumably contain more precursor cells, with the potential to grow and differentiate, compared to the tissues of more advance stages.

EB culture in microfluidic setup

Mouse EB cultures can undergo the formation of blood islands and vascular morphogenesis [20]. In our study, the zebrafish EBs developed in hanging drop culture, also show some degree of vascular organization, i.e. a well-connected radial network inside the EBs. However, when the EBs were transferred to 3D culture, the radial pattern and connectivity of the network was lost due to the extension of the vascular sprouts into the surrounding matrix.

Vascular sprouting is a physiological process in which selection of endothelial tip cells, as well as migration and vascular extension, occurs in existing blood vessels in response to angiogenic stimuli [68]. The phenomenon of vascular sprouting has been previously reported in mouse EB cultures [56]. Similarly, we also observed angiogenic sprouting in our zebrafish EB cultures; however, the extent of sprouting was less compared to the mouse model. This may because we used primary blastocyst cells to establish EB cultures, while mouse embryonic stem cell lines, which are adapted to proliferation *in vitro*, have been used in the other studies. Previous studies on zebrafish primary blastocyst cell culture have reported high cell death rates and low proliferative capacity in these cells [69]; this may explain the low level of sprouting in our cultures.

Studies on mouse EBs *in vitro*, and zebrafish embryos *in vivo*, have shown the directional migration of vascular sprouts towards the highest concentration of VEGF [70]. In our EB cultures, the selection of tip cells and the direction of sprout extension appeared to be random. This may because of the presence of angiogenic stimuli (growth factors) dispersed throughout the medium. However, we observed in some cases the extension of sprouts from one EB in the direction of a nearby EB. This may correlate with the *in vivo* situation in which the release of angiogenic growth factors from a distant cell population directs the migration of vascular sprouting.

The EB cultures described here with *kdrl:GFP*$^{+}$ sprouts were maintained under microfluidic conditions for a maximum of 12 days. In our previous studies, the growth of EB cultures could be maintained for longer time in primary culture, as well as in subculture [71]. However, the percentage of *kdrl:GFP*$^{+}$ cells in those cultures dropped significantly because they became overgrown by fibroblast-like cells [71]. Furthermore, the regression of *kdrl:GFP*$^{+}$ sprouts was observed after day 6 in our static cultures described here. These results are in accordance with a study of angiogenic sprouting in mouse EB cultures, in which the cells continued to degrade the 3D matrix after 12 days of culture, and differentiate into a variety of cells, making it difficult to interpret the vascular sprouts [72].

The medium was refreshed in our static culture at four day intervals. This interval was chosen after our preliminary studies indicated that cell growth was hindered after four days in non-replacement cultures. By contrast, the microfluidic cultures continued to grow with recycling of the medium. Medium replacement at four day intervals in the static culture may not be ideal for screening drugs or molecules that have a short half-life. For those experiments, shorter interval between the medium refreshment may be needed.

It is possible that the microfluidic system described here can be adapted for toxicity screening. For this purpose, the molecules to be tested can be easily added to the medium reservoir. Depending on the exposure time, the final volume of the medium in the reservoir can be adjusted according to the flow rate (20 µL/min in our case). Once the medium is withdrawn through the culture chamber, the reservoir can be refilled with fresh medium and the exhausted medium discarded or used for further analysis.

In the experiments described here, we formed a 3D gel matrix containing zebrafish EBs in a microfluidic channel slide. The open design of the microfluidic channel allows direct contact at the interface between the matrix and the medium. This is different to the microfluidic systems currently used for 3D cell culture, in which the medium can only diffuse into the matrix [73-76]. One of the drawbacks with the latter systems is that they do not mimic the dynamic environment of the tissue, but represent a rather static condition [38]. The flow of medium around the 3D matrix in our system presumably exerted a shear stress on the EB cells inside the matrix and allowed the extension of *kdrl:GFP*$^{+}$ sprouts.

Under physiological conditions, endothelial cells produce secreted factors in response to the shear stress induced by the blood flow [37]. These factors are essential for the development, regulation and maintenance of the blood vessels [37]. In a microfluidic culture, the flow rate of the medium is critical for cell proliferation, viability and function [77]. This was observed in our microfluidic cultures, where the *kdrl:GFP*$^{+}$ cells failed to form networks at low flow rates (2 and 10 µL/min). One possible explanation for this could be poor viability of cells at such low flow rates. Thus, under these low flow rates the

kdrl:GFP^{+} cells were mostly rounded in shape compared to 20 µL/min flow rate, where the cells acquired an elongated shape and formed connected networks.

One of the challenges with our microfluidic culture was to find a balance between the flow rate of the medium and the mechanical stability of the 3D matrix. The gel matrices comprising lower concentrations of collagen type-I (1.5 mg/mL) and fibrin (1 mg/mL) were not mechanically stable at the 20 µL/min flow rate required for network formation. When the concentrations of the above mentioned substrates were increased to 2.5 mg/mL and 2 mg/mL, respectively, the matrix formed was stable at the desired flow rate. Beside the stability of the matrix of higher concentration, the stiffness of the 3D matrix itself is presumably important in the culturing of vascular networks; thus, previous studies have shown that stiffness of the matrix promotes the organization of endothelial cells into capillary networks *in vitro* [78].

In our experiments, the differences between the microfluidic and static cultures was seen mainly in the length and width of *kdrl:GFP*$^{+}$ strands. In general, network formation by *kdrl:GFP*$^{+}$ cells in static cultures was limited to the periphery of the EBs. In microfluidic culture by contrast, the *kdrl:GFP*$^{+}$ cells extended more into the matrix and formed longer branches. A possible explanation for this may be that the shear stress causes the cells to secrete factors (as discussed above) which modulate the surrounding matrix and allow the cells to grow further into the matrix. The wider diameter of *kdrl:GFP*$^{+}$ strands in microfluidic cultures may indicate the formation of blood vessel-like structures with a lumen. By contrast, in the static cultures, the thinner branches formed are more consistent with a solid chain of cells connected end-to-end than with a continuous vessel.

A network having fewer end points and more junctions is considered to be a well-connected network [26]. The connectedness of a network can be determined by dividing the number of endpoints by the number of junctions [26]. We find that the connectedness of the networks depends on whether the network is more confined (as in the static culture) or spread (as in the microfluidic culture). In principle, the more confined network will be well-connected compared to a network with longer branches. In the microfluidic cultures in our experiments, as the branch length increased, the network connectivity was lost.

Culture of embryonic liver and heart tissues

The zebrafish liver and heart explant cultures described here might be suitable for development as alternative to mouse aortic ring culture for sprouting angiogenesis. One possible application of these explants could be to test the stimulatory or inhibitory effect of various substances on angiogenesis. Mouse aortic ring cultures are currently being used for such studies [79, 80]. Variability between cultures remains an issue with the mouse aortic ring model [14]. Although we found variability in the explants isolated from zebrafish embryos, the zebrafish model can easily yield a large more number of explants

which may help minimise this issue The small size of zebrafish embryos makes it technically difficult to dissect the embryos for tissue isolation. However, with practice, we were able to isolate tissues from 100 embryos in approximately three hours.

Under standard conditions, zebrafish embryos hatch at 48 hpf [48]. We found it important to decontaminate the embryos before hatching (at 24 hpf), because preliminary experiments showed that explants, isolated from the embryos decontaminated after hatching at 5 dpf, could not be maintained free from contamination in culture. We also found that embryos treated with sodium hypochlorite at 24 hpf have greatly impaired hatching; it is therefore necessary to manually dechorionate these embryos before sealing them into the Petri dish. Using this procedure the tissues isolated from these embryos at 5 dpf were successfully maintained sterile in culture.

The sprouting of *kdrl:GFP*$^{+}$ cells from liver explants could only be observed in matrices composed of collagen type-I, Geltrex™ and fibrin. Explants cultured in pure Geltrex™ matrix did not show any sprouting. These results are in accordance with mouse aortic ring cultures which have been shown to produce higher microvessel sprouting in collagen type-I and fibrin matrices compared to Matrigel™ [81]. The Geltrex™ we used in our experiments is similar to Matrigel™, and is a mixture of laminin, collagen type-IV, entactin and heparin sulphate proteoglycans (manufacturer's documentation).

Similar to microvessel formation in mouse aortic ring culture [81], the dimensions of the network-like structure formed by *kdrl:GFP*$^{+}$ cells in trypsinized liver cells cultured on pure Geltrex™ substratum were thinner and different from the much broader networks formed on collagen type-I + Geltrex™ + fibrin substratum. The sprouting of *kdrl:GFP*$^{+}$ cells in our explant cultures was observed on day 2 which is earlier than in the mouse aortic ring culture, where the peak sprouting of microvessels is reported to take place on day 6 of culture [81]. Regression of the sprouts from the explants occurred on day 4 in our experiments, whereas in mouse aortic ring culture this phenomena occurs on day 9 of culture [81].

The average length of the *kdrl:GFP*$^{+}$ cell sprouts emerging from the heart explants was higher compared to the liver explants (41.1 ± 8.4 vs 18.4 ± 5.8 μm). This may be because of a higher percentage of *kdrl:GFP*$^{+}$ cells in the isolated hearts compared to the livers, as indicated by our quantification of the cell isolates of these tissues. The colonies of *kdrl:GFP*$^{+}$ cells in the dissociated heart cell culture could not be maintained longer, probably due to low seeding density.

Conclusions

Zebrafish embryoid body culture is a promising model for *in vitro* vasculogenesis and angiogenesis. Microfluidic flow seems to have an effect on the growth of blood vessels in

EB culture. The use of a complex extracellular matrix with fibrin as a crucial part was, in our hands, optimal for culturing vascular networks. The zebrafish liver and heart explant cultures are promising models for sprouting angiogenesis. More experiments are needed to optimize the substrate and medium composition for these cultures so that the cultures can be expanded and be maintained for longer periods. This goal may also require co-culture with other cell- or tissue-types. In principle, pure populations of viable *kdrl:GFP*$^{+}$ cells could also be isolated from these cultures using fluorescence activated cell sorting assay. These cells could then be used for further analysis (e.g. gene expression profiling), as wells as for co-culturing with the EBs to improve the formation of vascular networks.

Acknowledgements

We would like to thank Prof. Stefan Schulte-Merker (Institute for Cardiovascular Organogenesis and Regeneration, Westfalische Wilhelms University, Munster, Germany) for providing the zebrafish *kdrl:GFP* line for this research.

This work was supported by the *Smart Mix* programme of the Netherlands Ministry of Economic Affairs and the Netherlands Scientific Research Council (NWO)[grant number SSM06010]; and the Generade programme of the Centre of Expertise Genomics in Leiden, The Netherlands[grant number 2016_004].

References

[1] Fisher MB, Mauck RL. Tissue engineering and regenerative medicine: recent innovations and the transition to translation. Tissue Eng Part B Rev 2013; 19(1): 1-13.

[2] Bhatia SN, Ingber DE. Microfluidic organs-on-chips. Nat Biotechnol 2014; 32(8): 760-72.

[3] Haraguchi Y, Shimizu T, Yamato M, Okano T. Concise review: cell therapy and tissue engineering for cardiovascular disease. Stem Cells Transl Med 2012; 1(2): 136-41.

[4] Hansen A, Eder A, Bonstrup M, Flato M, Mewe M, Schaaf S*, et al.* Development of a drug screening platform based on engineered heart tissue. Circ Res 2010; 107(1): 35-44.

[5] Levick JR. The microcirculation and solute exchange. In: Eds. An Introduction to cardiovascular physiology. 5 ed, Taylor & Francis 2010, pp. 166-187.

[6] Kannan RY, Salacinski HJ, Sales K, Butler P, Seifalian AM. The roles of tissue engineering and vascularisation in the development of micro-vascular networks: a review. Biomaterials 2005; 26(14): 1857-75.

[7] Vunjak-Novakovic G, Scadden DT. Biomimetic platforms for human stem cell research. Cell Stem Cell 2011; 8(3): 252-61.

[8] Ibrahim M, Richardson MK. Microfluidic devices for cell, tissue and embryo culture. Rec Pat Regen Med 2013; 3(3): 249-263.

[9] Zou T, Fan J, Fartash A, Liu H, Fan Y. Cell-based strategies for vascular regeneration. J Biomed Mater Res A 2016; 104(5): 1297-314.

[10] Shan K, Li CP, Liu C, Liu X, Yan B. RNCR3: a regulator of diabetes mellitus-related retinal microvascular dysfunction. Biochem Biophys Res Commun 2017; 482(4): 777-783.
[11] Roudsari LC, West JL. Studying the influence of angiogenesis in *in vitro* cancer model systems. Adv Drug Deliv Rev 2016; 97: 250-9.
[12] Azimi MS, Lacey M, Mondal D, Murfee WL. An *ex vivo* tissue culture model for anti-angiogenic drug testing. Methods Mol Biol 2016; 1464: 85-95.
[13] Vargesson N. Thalidomide-induced teratogenesis: history and mechanisms. Birth Defects Res C Embryo Today 2015; 105(2): 140-56.
[14] Staton CA, Reed MW, Brown NJ. A critical analysis of current *in vitro* and *in vivo* angiogenesis assays. Int J Exp Pathol 2009; 90(3): 195-221.
[15] Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol 2005; 23(1): 47-55.
[16] Nakatsu MN, Sainson RC, Aoto JN, Taylor KL, Aitkenhead M, Perez-del-Pulgar S*, et al.* Angiogenic sprouting and capillary lumen formation modeled by human umbilical vein endothelial cells (HUVEC) in fibrin gels: the role of fibroblasts and Angiopoietin-1. Microvasc Res 2003; 66(2): 102-12.
[17] Delgado VM, Nugnes LG, Colombo LL, Troncoso MF, Fernandez MM, Malchiodi EL*, et al.* Modulation of endothelial cell migration and angiogenesis: a novel function for the "tandem-repeat" lectin galectin-8. FASEB J 2011; 25(1): 242-54.
[18] Zhu J, Cui W, Liu X, Ying J, Hu C, Zhang Y. *In vitro* and *ex vivo* angiogenic effects of roxarsone on rat endothelial cells. Toxicol Lett 2013; 223(2): 175-82.
[19] Bratt-Leal AM, Carpenedo RL, McDevitt TC. Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. Biotechnol Prog 2009; 25(1): 43-51.
[20] Vailhe B, Vittet D, Feige JJ. *In vitro* models of vasculogenesis and angiogenesis. Lab Invest 2001; 81(4): 439-52.
[21] Goodwin AM. *In vitro* assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. Microvasc Res 2007; 74(2-3): 172-83.
[22] Jakobsson L, Kreuger J, Claesson-Welsh L. Building blood vessels--stem cell models in vascular biology. J Cell Biol 2007; 177(5): 751-5.
[23] Arnaoutova I, Kleinman HK. *In vitro* angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc 2010; 5(4): 628-35.
[24] Allen P, Melero-Martin J, Bischoff J. Type I collagen, fibrin and PuraMatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks. J Tissue Eng Regen Med 2011; 5(4): e74-86.
[25] Kusuma S, Shen YI, Hanjaya-Putra D, Mali P, Cheng L, Gerecht S. Self-organized vascular networks from human pluripotent stem cells in a synthetic matrix. Proc Natl Acad Sci U S A 2013; 110(31): 12601-6.
[26] Moya ML, Hsu YH, Lee AP, Hughes CC, George SC. *In vitro* perfused human capillary networks. Tissue Eng Part C Methods 2013; 19(9): 730-7.
[27] Kim S, Lee H, Chung M, Jeon NL. Engineering of functional, perfusable 3D microvascular networks on a chip. Lab Chip 2013; 13(8): 1489-500.
[28] Nguyen DH, Stapleton SC, Yang MT, Cha SS, Choi CK, Galie PA*, et al.* Biomimetic model to reconstitute angiogenic sprouting morphogenesis *in vitro*. Proc Natl Acad Sci U S A 2013; 110(17): 6712-7.

[29] Li H, Chang J. Bioactive silicate materials stimulate angiogenesis in fibroblast and endothelial cell co-culture system through paracrine effect. Acta Biomaterialia 2013; 9(6): 6981-6991.
[30] Zheng Y, Chen J, Craven M, Choi NW, Totorica S, Diaz-Santana A*, et al. In vitro* microvessels for the study of angiogenesis and thrombosis. Proc Natl Acad Sci U S A 2012; 109(24): 9342-7.
[31] Stamati K, Priestley JV, Mudera V, Cheema U. Laminin promotes vascular network formation in 3D *in vitro* collagen scaffolds by regulating VEGF uptake. Exp Cell Res 2014; 327(1): 68-77.
[32] Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. Section 6.2 Growth of Animal cells in culture. In: Eds. Molecular cell biology. 4 ed, New York, Freeman, W. H. 2000.
[33] Nicosia RF. The aortic ring model of angiogenesis: a quarter century of search and discovery. J Cell Mol Med 2009; 13(10): 4113-36.
[34] Deckers M, van der Pluijm G, Dooijewaard S, Kroon M, van Hinsbergh V, Papapoulos S*, et al.* Effect of angiogenic and antiangiogenic compounds on the outgrowth of capillary structures from fetal mouse bone explants. Lab Invest 2001; 81(1): 5-15.
[35] Rezzola S, Belleri M, Ribatti D, Costagliola C, Presta M, Semeraro F. A novel *ex vivo* murine retina angiogenesis (EMRA) assay. Exp Eye Res 2013; 112: 51-6.
[36] Brodsky SV, Smith M, Kashgarian M, Goligorsky MS. A model for *ex vivo* renal angiogenesis. Nephron Exp Nephrol 2003; 93(1): e46-52.
[37] Davies PF. Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. Nat Clin Pract Cardiovasc Med 2009; 6(1): 16-26.
[38] Gupta N, Liu JR, Patel B, Solomon DE, Vaidya B, Gupta V. Microfluidics-based 3D cell culture models: Utility in novel drug discovery and delivery research. Bioengineering & Translational Medicine 2016; 1(1): 63-81.
[39] Hasan A, Paul A, Vrana NE, Zhao X, Memic A, Hwang YS*, et al.* Microfluidic techniques for development of 3D vascularized tissue. Biomaterials 2014; 35(26): 7308-25.
[40] Rezzola S, Belleri M, Gariano G, Ribatti D, Costagliola C, Semeraro F*, et al. In vitro* and *ex vivo* retina angiogenesis assays. Angiogenesis 2014; 17(3): 429-42.
[41] Zhu WH, Iurlaro M, MacIntyre A, Fogel E, Nicosia RF. The mouse aorta model: influence of genetic background and aging on bFGF- and VEGF-induced angiogenic sprouting. Angiogenesis 2003; 6(3): 193-9.
[42] Chavez MN, Aedo G, Fierro FA, Allende ML, Egana JT. Zebrafish as an emerging model organism to study angiogenesis in development and regeneration. Front Physiol 2016; 7: 56.
[43] Wilkinson RN, van Eeden FJ. The zebrafish as a model of vascular development and disease. Prog Mol Biol Transl Sci 2014; 124: 93-122.
[44] Grunow B, Mohamet L, Shiels HA. Generating an *in vitro* 3D cell culture model from zebrafish larvae for heart research. J Exp Biol 2015; 218(Pt 8): 1116-21.
[45] Yue MS, Plavicki JS, Li XY, Peterson RE, Heideman W. A co-culture assay of embryonic zebrafish hearts to assess migration of epicardial cells *in vitro*. BMC Dev Biol 2015; 15: 50.
[46] Selderslaghs IW, Van Rompay AR, De Coen W, Witters HE. Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. Reprod Toxicol 2009; 28(3): 308-20.

[47] Ciarlo CA, Zon LI. Chapter 1 - Embryonic cell culture in zebrafish. In: H. William Detrich MW and IZ Leonard, Eds. Methods in Cell Biology. ed, Academic Press 2016, pp. 1-10.
[48] Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995; 203(3): 253-310.
[49] Choorapoikayil S, Overvoorde J, den Hertog J. Deriving cell lines from zebrafish embryos and tumors. Zebrafish 2013; 10(3): 316-25.
[50] Simmich J, Staykov E, Scott E. Zebrafish as an appealing model for optogenetic studies. Prog Brain Res 2012; 196: 145-62.
[51] Li R, Beebe T, Jen N, Yu F, Takabe W, Harrison M*, et al.* Shear stress-activated Wnt-angiopoietin-2 signaling recapitulates vascular repair in zebrafish embryos. Arterioscler Thromb Vasc Biol 2014; 34(10): 2268-75.
[52] Harrison MR, Bussmann J, Huang Y, Zhao L, Osorio A, Burns CG*, et al.* Chemokine-guided angiogenesis directs coronary vasculature formation in zebrafish. Dev Cell 2015; 33(4): 442-54.
[53] Tal T, Kilty C, Smith A, LaLone C, Kennedy B, Tennant A*, et al.* Screening for angiogenic inhibitors in zebrafish to evaluate a predictive model for developmental vascular toxicity. Reprod Toxicol 2016: http://dx.doi.org/10.1016/j.reprotox.-2016.12.004.
[54] Huang HG, Lindgren A, Wu XR, Liu NA, Lin SO. High-throughput screening for bioactive molecules using primary cell culture of transgenic zebrafish embryos. Cell Rep 2012; 2(3): 695-704.
[55] Rojas-Rodriguez R, Gealekman O, Kruse ME, Rosenthal B, Rao K, Min S*, et al.* Adipose tissue angiogenesis assay. Methods Enzymol 2014; 537: 75-91.
[56] Liu Z, Lebrin F, Maring JA, van den Driesche S, van der Brink S, van Dinther M*, et al.* ENDOGLIN is dispensable for vasculogenesis, but required for vascular endothelial growth factor-induced angiogenesis. PLoS One 2014; 9(1): e86273.
[57] Song W, Fhu CW, Ang KH, Liu CH, Johari NA, Lio D*, et al.* The fetal mouse metatarsal bone explant as a model of angiogenesis. Nat Protoc 2015; 10(10): 1459-73.
[58] Masson VV, Devy L, Grignet-Debrus C, Bernt S, Bajou K, Blacher S*, et al.* Mouse aortic ring assay: a new approach of the molecular genetics of angiogenesis. Biol Proced Online 2002; 4: 24-31.
[59] Kiefer FN, Munk VC, Humar R, Dieterle T, Landmann L, Battegay EJ. A versatile *in vitro* assay for investigating angiogenesis of the heart. Exp Cell Res 2004; 300(2): 272-82.
[60] Experiments on Animal Act, 2014. The State Secretary of Health and Environmental Protection, Soestdijk. http://wetten.overheid.nl/BWBR0003081/2014-12-18
[61] Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance, 2010. The European Parliament and the council of the European Union, Official Journal of the European Union. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:32010L0063
[62] Westerfield M. The zebrafish book: a guide for the laboratory use of zebrafish (*Danio Rerio*). 4 ed, University of Oregon Press, Eugene 2000, pp.
[63] Pham LN, Kanther M, Semova I, Rawls JF. Methods for generating and colonizing gnotobiotic zebrafish. Nat Protoc 2008; 3(12): 1862-75.
[64] Henn K. Limits of fish embryo toxicity test with *Danio rerio* as an alternative to the acute fish toxicity test. PhD thesis, 2011. Center of Organismal Studies Heidelberg (COS), Ruperto-Carola University Heidelberg, Germany.

[65] Fan L, Collodi P. Zebrafish embryonic stem cells. Methods Enzymol 2006; 418: 64-77.
[66] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012; 9(7): 671-5.
[67] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS*, et al.* Embryonic stem cell lines derived from human blastocysts. Science 1998; 282(5391): 1145-7.
[68] Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol 2007; 8(6): 464-78.
[69] Myhre JL, Pilgrim DB. Cellular differentiation in primary cell cultures from single zebrafish embryos as a model for the study of myogenesis. Zebrafish 2010; 7(3): 255-66.
[70] Jakobsson L, Franco CA, Bentley K, Collins RT, Ponsioen B, Aspalter IM*, et al.* Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. Nat Cell Biol 2010; 12(10): 943-53.
[71] Ibrahim M, Richardson MK. The growth of endothelial-like cells in zebrafish embryoid body culture. 2017: in prep.
[72] Gu A, Tsark W, Holmes KV, Shively JE. Role of Ceacam1 in VEGF induced vasculogenesis of murine embryonic stem cell-derived embryoid bodies in 3D culture. Exp Cell Res 2009; 315(10): 1668-82.
[73] Zanotelli MR, Ardalani H, Zhang J, Hou Z, Nguyen EH, Swanson S*, et al.* Stable engineered vascular networks from human induced pluripotent stem cell-derived endothelial cells cultured in synthetic hydrogels. Acta Biomater 2016; 35: 32-41.
[74] Shin Y, Yang K, Han S, Park HJ, Seok Heo Y, Cho SW*, et al.* Reconstituting vascular microenvironment of neural stem cell niche in three-dimensional extracellular matrix. Adv Healthc Mater 2014; 3(9): 1457-64.
[75] Hsu YH, Moya ML, Abiri P, Hughes CC, George SC, Lee AP. Full range physiological mass transport control in 3D tissue cultures. Lab Chip 2013; 13(1): 81-9.
[76] Park YK, Tu TY, Lim SH, Clement IJ, Yang SY, Kamm RD. *In Vitro* Microvessel Growth and Remodeling within a Three-dimensional Microfluidic Environment. Cell Mol Bioeng 2014; 7(1): 15-25.
[77] Tehranirokh M, Kouzani AZ, Francis PS, Kanwar JR. Microfluidic devices for cell cultivation and proliferation. Biomicrofluidics 2013; 7(5): 51502.
[78] Sieminski AL, Hebbel RP, Gooch KJ. The relative magnitudes of endothelial force generation and matrix stiffness modulate capillary morphogenesis *in vitro*. Exp Cell Res 2004; 297(2): 574-84.
[79] Gopinathan G, Milagre C, Pearce OM, Reynolds LE, Hodivala-Dilke K, Leinster DA*, et al.* Interleukin-6 Stimulates Defective Angiogenesis. Cancer Res 2015; 75(15): 3098-107.
[80] Park SL, Won SY, Song JH, Lee SY, Kim WJ, Moon SK. Esculetin inhibits VEGF-induced angiogenesis both *in Vitro* and *in Vivo*. Am J Chin Med 2016; 44(1): 61-76.
[81] Baker M, Robinson SD, Lechertier T, Barber PR, Tavora B, D'Amico G*, et al.* Use of the mouse aortic ring assay to study angiogenesis. Nat Protoc 2011; 7(1): 89-104.

Chapter 7

Summary and conclusions

Summary

Development of an *in vitro* vascular network is currently one of the major challenges in tissue engineering and regenerative medicine [1]. Several techniques have been developed to culture vascular networks using mammalian cells and tissues [2-12]. However, it is desirable to develop alternative models which are cost effective, easily accessible and available in large numbers. The zebrafish possess these benefits and is emerging as a model laboratory animal in various fields of research. For *in vitro* studies the externally fertilized, abundant zebrafish embryos are an excellent source of primary embryonic cells, which are not always easy to access in other species [13]. The availability of a number of vascular transgenic lines and the regenerative capacity of zebrafish makes it of even greater importance in this field [14].

Development of blood vessels is a complex process which involves the differentiation, migration and arrangement of endothelial cells into a tubular form [15]. The process is complemented by supporting cell types [16, 17], protein factors [18], extracellular matrix molecules [19] and blood flow [20]. By combining these components *in vitro*, some of the researchers have recently developed cultures of perfusable vascular network [3, 10]; however, the formation of a functional vasculature which is close in resemblance to the physiological blood vessels remains a challenge [21].

The cultures that we have established in this study are a step towards helping our understanding of zebrafish *in vitro* vascular network development. The zebrafish embryoid body and tissue explant models we have developed are promising complementary models to mammalian embryonic stem cell and tissue explant models, for studying *in vitro* vascular development. The embryoid body cultures can easily be established in large numbers; however, the isolation of tissue explants from small embryos require technical expertise which can be acquired with practice.

Based on our results, zebrafish embryonic cells can be used to culture vascular networks. In its current state however, the zebrafish cannot completely replace the mammalian cell culture models, but can be used as a complementary model. The size of the vascular network formed in our cultures was smaller than previously reported for mouse and human embryonic cell and tissue culture. This may because of the limited availability of techniques for zebrafish in this regard. Further research in zebrafish model for this purpose should provide information that will improve the formation of *in vitro* vascular network in this species.

In **Chapter 2** we reviewed the available techniques for culturing vascular networks and considered their potential applications. There are very few studies using zebrafish cells for this purpose [22, 23]. However, in the light of general cell culture techniques developed in zebrafish, we believe that most of the techniques developed for culturing vascular networks using mammalian cells and tissues can be adapted for zebrafish. And,

because of its general benefits (such as low cost, external fertilization) the zebrafish can be a significant first step model for studying vascular development *in vitro*. Nevertheless, there are a few disadvantages with the zebrafish model such as combining cells from genetically diverse embryos in culture (the zebrafish is genetically polymorphic) [24], the requirement of a nutrient rich medium [25] and the need, reported in some studies [26], of a feeder layer of stromal cells to maintain an undifferentiated state of the primary embryonic cell cultures.

In **Chapter 3** we optimized the basic nutrient requirements for zebrafish blastocyst cell culture. We found that, for their optimal growth, the primary blastocyst cells require 15% fetal bovine serum (FBS) and 60 µg/mL zebrafish embryo extract (ZEE) in the medium. We found that endothelial cells differentiated spontaneously in zebrafish blastocyst cell cultures. Using transgenic lines (*fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells) we could track the further development of these cells until day 4 of culture under basal culture conditions (when they start to reduce in number).

During the course of culture, most of the differentiated cell types (we also observed neuron-like cells and pigment cells along with the *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells) diminished and a single subpopulation of cells appear to increase in number. By continuous passaging of the blastocyst cell cultures, the secondary cells attain a homogenous fibroblast-like morphology. These cells shows an optimal growth at a lower FBS concentration (10%) and without ZEE in the medium.

In **Chapter 4** we attempted to increase the *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cell populations in the blastocyst cell cultures by manipulating the culture conditions. We used different media compositions, substrate molecules and vascular endothelial growth factor concentrations to find optimum conditions for the growth of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures. We found that the LDF medium previously optimized for the zebrafish blastocyst cell culture [26-30], with added endothelial growth supplement mix (used in supplementation with endothelial growth medium for human endothelial cell culture) increases the percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures.

Collagen type-I substratum also increased the percentages of putative endothelial cells in culture; however, the effect was not significant compared to the uncoated polystyrene substratum. Furthermore, the recombinant zebrafish vascular endothelial growth factor also increased the percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells when added to the culture medium. With all these optimization steps we were able to maintain the *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells for longer duration (until eight days) in cultures.

In **Chapter 5** we utilised the possibilities of suspension culture of zebrafish blastocyst cells to see its effect on the development of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures. The blastocyst cells were cultured in hanging drops on the inside of the lid of a Petri dish in the zebrafish endothelial differentiation medium (optimized in the experiments described in

the previous chapters). The results were promising: we found significantly higher percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in the embryoid body (EB) aggregates of the blastocyst cells developed in hanging drop cultures compared to the adherent cultures. When transferred from hanging drops to a 2D substratum the percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in the EB cultures declined with time.

Under optimal conditions the *fli:GFP*$^{+}$ cells could be observed in the primary EB cultures as well as in secondary cultures until two passages. In contrast, the *kdrl:GFP*$^{+}$ cells could only be observed until 12 days of primary cultures. Different substrates tested for the EB cultures showed variations in the development of *fli:GFP*$^{+}$ of *kdrl:GFP*$^{+}$ cells. In the first six days of the EB adherent cultures, the *fli:GFP*$^{+}$ cells, migrating in a monolayer around the EBs, covered significantly more area on fibrin substratum compared to collagen type-I and gelatin substratum.

Similarly, the *kdrl:GFP*$^{+}$ cells developed significantly longer strands of connected *kdrl:GFP*$^{+}$ cells forming network-like structures on mixed collagen type-I and Geltrex™ substratum compared to pure substrates. These results suggest the requirement of a complex substratum including fibrin as a critical component for the development of endothelial-like cells in zebrafish blastocyst cell culture.

In **Chapter 6** we demonstrated the development of vascular network-like structures in zebrafish EB cultures in a 3D gel matrix composed of a mixture of collagen type-I, Geltrex™ and fibrin. We found that in the microfluidic culture the *kdrl:GFP*$^{+}$ cells in the EBs formed longer branches, and these branches were maintained for a longer period, compared to the static cultures. In addition, the sprouting of *kdrl:GFP*$^{+}$ cells was observed from zebrafish embryonic liver and heart explant cultures in 3D matrices. We found that the *kdrl:GFP*$^{+}$ cells in the dissociated liver cell cultures also form vascular network-like structures on 2D collagen type-I + Geltrex™ + fibrin substratum. These models are of potential importance to test the candidate drugs for their effect on vascular development.

Conclusions

- Zebrafish are a promising complementary model for studying vascular development *in vivo* as wells as *in vitro* (Chapter 2).
- The blastocyst cell cultures can be maintained without a feeder cell layer in basic LDF medium supplemented with 15% FBS and 60 µg/mL ZEE (Chapter 3).
- Under basic culture conditions the *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells can be observed in culture from day 1 until day 4 (Chapter 3).
- The growth of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cell culture can be enhanced by manipulating culture conditions (Chapter 4).

- The development of EB intermediate in suspension culture allows better control on the generation and development of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in blastocyst cell culture (Chapter 5).
- The *kdrl:GFP*$^{+}$ cells shows properties of endothelial cells by making network-like structures. In contrast, the *fli:GFP*$^{+}$ cells shows properties of fibroblast-like cells by growing in a monolayer around the EBs (Chapter 5).
- Culture in a 3D matrix allows the extension of *kdrl:GFP*$^{+}$ vascular sprouts emerging from the EBs (Chapter 6).
- The EBs cultured in 3D matrix developed longer and wider sprouts of *kdrl:GFP*$^{+}$ cells in microfluidic culture compared to static culture, suggesting the effect of microfluidic flow on vascular development (Chapter 6).
- The EB and organ explant models developed in this study showing vascular sprouting could be developed as screening platform for various chemical compounds and candidate drugs (Chapter 6).

Future prospects

The zebrafish embryo is currently emerging as a promising model for studies of vascular development. Much research has been done to develop transgenic lines for this species, and to study the *in vivo* development of its vascular system.

The small size of the zebrafish, and the relatively simple rearing conditions it requires, make this model of great interest for developmental studies using a whole animal model, although the species is still a relatively new model. However, for its general benefits (such as high fecundity, external fertilization) and specific benefits for cell culture applications (such as the requirement of simple culture conditions, easy access to a large number of primary embryonic cells for culture), the zebrafish can be developed as a promising *in vitro* model in several fields.

The availability of transgenic lines allows for the fluorescence activated cell sorting (FACS) of live endothelial cells; in principle, these could be developed as endothelial cell lines for *in vitro* studies of vascular development. However, the culture conditions for the differentiation or maintenance of zebrafish endothelial cells needs further optimization. In principle, the zebrafish cell culture systems developed in this study could also be subjected to FACS, in order to isolate and analyse pure endothelial cell population.

One of the experiments that can readily be performed with the zebrafish EB cultures developed here, is to study the effect of various chemical compounds that are known to inhibit or enhance vascular development *in vitro* in mammalian cell culture models. This would increase the validity of this model for future research on the screening of

compounds. Another advantage of the zebrafish is the ability to use mutant fish lines or genetically modified fish embryos to develop *in vitro* vascular networks. This could help uncovering the molecular processes involved in vascular development.

Microfluidic cultures mimicking the physiological dynamic environment of tissues are facilitating considerable contributions to the field of vascular engineering. The development of a microfluidic system connected to a perfused vascularized tissue construct would be an area of great interest in the future. The major challenge in establishing such a system would be to develop the ability to remodel the vascular-to-hardware connections in the microfluidic system, in order to accomplish the increasing demands of the growing tissue overtime.

References

[1] Kim JJ, Hou L, Huang NF. Vascularization of three-dimensional engineered tissues for regenerative medicine applications. Acta Biomater 2016; 41: 17-26.

[2] Rohringer S, Hofbauer P, Schneider KH, Husa AM, Feichtinger G, Peterbauer-Scherb A*, et al.* Mechanisms of vasculogenesis in 3D fibrin matrices mediated by the interaction of adipose-derived stem cells and endothelial cells. Angiogenesis 2014; 17(4): 921-33.

[3] Kim S, Lee H, Chung M, Jeon NL. Engineering of functional, perfusable 3D microvascular networks on a chip. Lab Chip 2013; 13(8): 1489-500.

[4] Nguyen DH, Stapleton SC, Yang MT, Cha SS, Choi CK, Galie PA*, et al.* Biomimetic model to reconstitute angiogenic sprouting morphogenesis *in vitro*. Proc Natl Acad Sci U S A 2013; 110(17): 6712-7.

[5] Zheng Y, Chen J, Craven M, Choi NW, Totorica S, Diaz-Santana A*, et al. In vitro* microvessels for the study of angiogenesis and thrombosis. Proc Natl Acad Sci U S A 2012; 109(24): 9342-7.

[6] Nakatsu MN, Sainson RC, Aoto JN, Taylor KL, Aitkenhead M, Perez-del-Pulgar S*, et al.* Angiogenic sprouting and capillary lumen formation modeled by human umbilical vein endothelial cells (HUVEC) in fibrin gels: the role of fibroblasts and Angiopoietin-1. Microvasc Res 2003; 66(2): 102-12.

[7] Stamati K, Priestley JV, Mudera V, Cheema U. Laminin promotes vascular network formation in 3D *in vitro* collagen scaffolds by regulating VEGF uptake. Exp Cell Res 2014; 327(1): 68-77.

[8] Arnaoutova I, Kleinman HK. *In vitro* angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc 2010; 5(4): 628-35.

[9] Rao RR, Peterson AW, Ceccarelli J, Putnam AJ, Stegemann JP. Matrix composition regulates three-dimensional network formation by endothelial cells and mesenchymal stem cells in collagen/fibrin materials. Angiogenesis 2012; 15(2): 253-64.

[10] Zhang B, Montgomery M, Chamberlain MD, Ogawa S, Korolj A, Pahnke A*, et al.* Biodegradable scaffold with built-in vasculature for organ-on-a-chip engineering and direct surgical anastomosis. Nat Mater 2016; 15(6): 669-78.

[11] Hammoud L, Adams JR, Loch AJ, Marcellus RC, Uehling DE, Aman A*, et al.* Identification of RSK and TTK as modulators of blood vessel morphogenesis using

an embryonic stem cell-based vascular differentiation assay. Stem Cell Reports 2016; 7(4): 787-801.
[12] Song W, Fhu CW, Ang KH, Liu CH, Johari NA, Lio D*, et al.* The fetal mouse metatarsal bone explant as a model of angiogenesis. Nat Protoc 2015; 10(10): 1459-73.
[13] Choorapoikayil S, Overvoorde J, den Hertog J. Deriving cell lines from zebrafish embryos and tumors. Zebrafish 2013; 10(3): 316-25.
[14] Chavez MN, Aedo G, Fierro FA, Allende ML, Egana JT. Zebrafish as an emerging model organism to study angiogenesis in development and regeneration. Front Physiol 2016; 7: 56.
[15] Schmidt A, Brixius K, Bloch W. Endothelial precursor cell migration during vasculogenesis. Circ Res 2007; 101(2): 125-36.
[16] Birbrair A, Zhang T, Wang ZM, Messi ML, Mintz A, Delbono O. Pericytes at the intersection between tissue regeneration and pathology. Clin Sci (Lond) 2015; 128(2): 81-93.
[17] Campbell GR, Campbell JH. Development of the vessel wall: overview. In: Mecham RP, Eds. The Vascular Smooth Muscle Cell. ed, San Diego, Academic Press 1995, pp. 1-15.
[18] Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature 2000; 407(6801): 242-8.
[19] Eble JA, Niland S. The extracellular matrix of blood vessels. Curr Pharm Des 2009; 15(12): 1385-400.
[20] Davies PF. Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. Nat Clin Pract Cardiovasc Med 2009; 6(1): 16-26.
[21] Hasan A, Paul A, Vrana NE, Zhao X, Memic A, Hwang YS*, et al.* Microfluidic techniques for development of 3D vascularized tissue. Biomaterials 2014; 35(26): 7308-25.
[22] Huang HG, Lindgren A, Wu XR, Liu NA, Lin SO. High-throughput screening for bioactive molecules using primary cell culture of transgenic zebrafish embryos. Cell Rep 2012; 2(3): 695-704.
[23] Ibrahim M, Richardson MK. *In vitro* development of zebrafish vascular networks. Reprod Toxicol 2017: DOI: 10.1016/j.reprotox.2017.02.008.
[24] Myhre JL, Pilgrim DB. Cellular differentiation in primary cell cultures from single zebrafish embryos as a model for the study of myogenesis. Zebrafish 2010; 7(3): 255-66.
[25] Alvarez MC, Bejar J, Chen S, Hong Y. Fish ES cells and applications to biotechnology. Mar Biotechnol (NY) 2007; 9(2): 117-27.
[26] Fan L, Collodi P. Zebrafish embryonic stem cells. Methods Enzymol 2006; 418: 64-77.
[27] Collodi P, Kamei Y, Ernst T, Miranda C, Buhler DR, Barnes DW. Culture of cells from zebrafish (*Brachydanio rerio*) embryo and adult tissues. Cell Biol Toxicol 1992; 8(1): 43-61.
[28] Fan L, Crodian J, Liu X, Alestrom A, Alestrom P, Collodi P. Zebrafish embryo cells remain pluripotent and germ-line competent for multiple passages in culture. Zebrafish 2004; 1(1): 21-6.
[29] Ghosh C, Liu Y, Ma CG, Collodi P. Cell cultures derived from early zebrafish embryos differentiate *in vitro* into neurons and astrocytes. Cytotechnology 1997; 23(1-3): 221-230.
[30] Ma C, Fan L, Ganassin R, Bols N, Collodi P. Production of zebrafish germ-line chimeras from embryo cell cultures. Proc Natl Acad Sci U S A 2001; 98(5): 2461-6.

Nederlandse samenvatting

De ontwikkeling van een *in vitro* vasculair netwerk is momenteel een van de grootste uitdagingen in weefselkweek en regeneratieve geneeskunde. Er zijn meerdere technieken ontwikkeld om vasculaire netwerken te kweken waarbij cellen en weefsel van zoogdieren worden gebruikt, maar het is wenselijk om alternatieve modellen te ontwikkelen die doeltreffend, toegankelijk en grootschalig beschikbaar zijn. De zebravis bezit deze voordelen en ontwikkelt zich tot een model proefdier in verschillende onderzoeksgebieden. Voor *in vitro* studies zijn de extern bevruchte grote hoeveelheden aan zebravisembryo's een uitstekende bron van primaire embryonale cellen, die in andere soorten minder makkelijk te verkrijgen zijn. Beschikbaarheid van een aantal vasculaire transgene lijnen en de regeneratieve capaciteit van de zebravis maakt dit model nog belangrijker in dit onderzoeksgebied.

De ontwikkeling van bloedvaten is een complex proces, wat onder andere bestaat uit differentiatie en migratie van endotheelcellen, die vervolgens een buis vormen. Het proces wordt verder gestuurd door ondersteunende celtypen, eiwitfactoren, extracellulaire matrix moleculen en de bloedstroom. Door deze componenten *in vitro* te combineren hebben onderzoekers recent een op celkweek gebaseerd vasculair netwerk ontwikkeld, inclusief doorbloeding. De vorming van een functioneel vasculair netwerk dat grote gelijkenis vertoont met fysiologische bloedvaten blijft echter een uitdaging.

De celculturen die we in deze studie hebben ontwikkeld zijn een stap in de richting van het beter begrijpen van de *in vitro* ontwikkeling van het vasculaire netwerk van de zebravis. De zebravis embryoid body (EB) en weefsel explantatie modellen die wij hebben ontwikkeld, zijn veelbelovende, aanvullende modellen naast de huidige zoogdieren embryonale stamcel en weefsel explantatie modellen, voor het bestuderen van *in vitro* vasculaire ontwikkeling. De EB celkweken kunnen gemakkelijk in grote aantallen worden opgezet. De isolatie van weefsel explantaten van kleine embryo's vereist echter technische expertise die door oefening moet worden verkregen.

Op basis van onze resultaten kunnen embryonale cellen van de zebravis worden gebruikt om vasculaire netwerken te groeien. In de huidige situatie kan de zebravis echter niet het zoogdier celcultuur model vervangen, maar wel als een complementair model worden gebruikt. De grootte van het vasculaire netwerk dat in onze culturen werd gevormd, was kleiner dan eerder beschreven voor muis en menselijke embryonale cel- en weefselkweek. Dit kan komen door de beperkte beschikbaarheid van technieken voor zebravissen in dit onderzoeksveld. Verder onderzoek naar het zebravismodel met betrekking tot dit onderwerp moet meer informatie verstrekken over de vorming van i*n vitro* vasculaire netwerken.

In **Hoofdstuk 2** hebben we een overzicht gegeven van de beschikbare technieken voor het kweken van vasculaire netwerken en hun mogelijke toepassingen onderzocht. Er zijn

maar weinig studies met dit doel waarin gebruik wordt gemaakt van zebravis cellen. Gebaseerd op de algemene celkweek technieken die ontwikkeld zijn voor de zebravis, geloven wij dat het merendeel van de technieken voor het kweken van vasculaire netwerken met behulp van zoogdiercellen en -weefsels kan worden aangepast voor de zebravis. Vanwege diens algemene voordelen (zoals lage kosten, externe bevruchting) kan de zebravis een belangrijke eerste stap zijn voor het bestuderen van *in vitro* vasculaire ontwikkeling. Er zijn echter enkele nadelen van het zebravismodel, zoals het combineren van cellen van genetisch diverse embryo's in de celcultuur (de zebravis is genetisch polymorf), een vereist voedselrijk medium en de vereiste van, zoals in sommige studies aangegeven, een voedingslaag van stromale cellen om de ongedifferentieerde toestand van de primaire embryonale celkweken te behouden.

In **Hoofdstuk 3** hebben we de minimale vereisten van de voedingsstoffen voor de zebravis blastocyst celkweek geoptimaliseerd. Wij vonden voor optimale groei, dat de primaire blastocyst cellen 15% foetaal runder serum (FBS) en 60 ug/mL zebravisembryo extract (ZEE) in het medium nodig habben. We vonden dat endotheelcellen spontaan differentieerden in de zebravis blastocyst celculturen. Met behulp van transgene lijnen (*fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen) konden we de verdere ontwikkeling van deze cellen volgen tot dag 4 van de kweek onder basale kweekcondities (wanneer ze in hoeveelheid afnemen).

Gedurende de kweek verminderden de meeste van de gedifferentieerde cel types (we namen ook neuron-achtige cellen en pigment cellen samen met de *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen waar) en een subpopulatie van cellen bleek in aantal toe te nemen. Door continue de blastocyst celculturen door te kweken, krijgen de secundaire cellen een fibroblast-achtige morfologie. Deze cellen vertonen een optimale groei bij een lagere concentratie FBS (10%) en zonder ZEE in het medium.

In **Hoofdstuk 4** hebben we geprobeerd om de hoeveelheid *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ celpopulaties in de blastocyst celculturen te verhogen door de kweekomstandigheden te manipuleren. We gebruikten verschillende media samenstellingen, substraten en vasculair endotheel groeifactor (VEGF) concentraties om optimale omstandigheden voor de groei van *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen te vinden. We vonden dat het LDF medium dat eerder is geoptimaliseerd voor de zebravis blastocyst celkweek aangevuld met een mix van endothele groeifactoren (in aanvulling met endotheel groeimedium voor humane endotheel celculturen) de percentages van *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen in de celkweek verhoogt.

Collageen type-I als substraat verhoogde ook de percentages van de mogelijke endotheelcellen in de celkweek; dit effect was echter niet significant in vergelijking met het ongecoatte polystyreen substraat. Ook de recombinante zebravis VEGF verhoogde de percentages van *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen indien toegevoegd aan het kweekmedium. Met

al deze optimalisatie stappen konden we de *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen voor een langere duur (tot acht dagen) kweken.

In **Hoofdstuk 5** hebben we gebruik gemaakt van de mogelijkheden van een suspensie cultuur van zebravis blastocyst cellen om het effect daarvan op de ontwikkeling van *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen in cultuur te onderzoeken. De blastocyst cellen werden gekweekt in hangende druppels op de binnenkant van de deksel van een petrischaal in het zebravis endotheel differentiatiemedium (geoptimaliseerd in de experimenten zoals die zijn beschreven in voorgaande hoofdstukken). De resultaten waren veelbelovend: we vonden significant hogere percentages *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen in de EB aggregaten van de in een hangende druppel opgegroeide blastocyst cellen vergeleken met de gehechte culturen. Wanneer de celculturen uit de hangende druppel worden overgebracht op een 2D-ondergrond dalen de percentages van *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen in de EB culturen met de tijd.

Onder optimale omstandigheden kunnen de *fli:GFP*$^{+}$ cellen worden waargenomen in de primaire EB celculturen en in secundaire kweken tot en met de tweede doorkweek. De *kdrl:GFP*$^{+}$ cellen konden daarentegen slechts worden waargenomen tot 12 dagen in de primaire celculturen. De verschillende onderzochte substraten resulteerden in variaties in de ontwikkeling van *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen. Gedurende de eerste zes dagen van de EB gehechte culturen, bedekten de *fli:GFP*$^{+}$ cellen, die migreren in een enkele laag rond de EBS, beduidend meer oppervlakte op fibrine substraat ten opzichte van het type-I collageen- en gelatine substraat.

Op een vergelijkbare manier ontwikkelden de *kdrl:GFP*$^{+}$ cellen significant langere strengen van aan elkaar verbonden *kdrl:GFP*$^{+}$ cellen, die zo een netwerk-achtige structuur vormden, op mixed collageen type-I en Geltrex ™ substraat in vergelijking met pure substraten. Deze resultaten suggereren dat een complex substraat, waarvan fibrine een essentieel onderdeel is, een vereiste is voor de ontwikkeling van endotheel-achtige cellen in de zebravis blastocyst celkweek.

In **Hoofdstuk 6** toonden we de ontwikkeling van vasculaire netwerkachtige structuren in zebravis EB celculturen in een 3D gelmatrix, bestaande uit een mengsel van collageen type I, Geltrex ™ en fibrine. We vonden dat in de microfluïdische cultuur *kdrl:GFP*$^{+}$ cellen in de EBs langere vertakkingen vormden en deze takken werden gedurende een langere periode onderhouden in vergelijking met de statische celkweken. Daarnaast hebben we het ontstaan van *kdrl:GFP*$^{+}$ cellen uit embryonale lever en hart explantatie culturen van zebravissen waargenomen in 3D matrices. We vonden dat de *kdrl:GFP*$^{+}$ cellen in de gedissocieerde levercelkweken ook vasculaire netwerkachtige structuren vormen op 2D collageen type-I + Geltrex ™ + fibrine substraat. Deze modellen zijn van mogelijk belang voor het testen van potentiele geneesmiddelen op hun effect op vasculaire ontwikkeling.

Conclusies

- Zebravissen zijn een veelbelovend aanvullend model voor het bestuderen van de ontwikkeling van bloedvaten *in vivo* en *in vitro* (Hoofdstuk 2).
- De blastocyst celkweek kan in stand gehouden worden zonder een voedende cellaag, in LDF medium gecomplementeerd met 15% FBS en 60 µg/mL ZEE (Hoofdstuk 3).
- Onder standaard groeicondities kunnen *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen *in vitro* waargenomen worden vanaf dag 1 tot dag 4 (Hoofdstuk 3)
- De groei van *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen in een zebravis blastocyst celkweek kunnen gestimuleerd worden door het manipuleren van de kweekcondities (Hoofdstuk 4).
- De ontwikkeling van EBs in een hangende kweek zorgt voor betere controle over de aanmaak en ontwikkeling van *fli:GFP*$^{+}$ en *kdrl:GFP*$^{+}$ cellen in een blastocyst celkweek(Hoofdstuk 5)
- *Kdrl:GFP*$^{+}$ cellen vertonen eigenschappen van endotheel cellen door het maken van netwerk-achtige structuren. *Fli:GFP*$^{+}$ cellen daarentegen vertonen fibroblast-achtige eigenschappen door in een cellaag rondom de EBs te groeien (Hoofdstuk 5).
- Het kweken in een 3D matrix faciliteert de uitgroei van *kdrl:GFP*$^{+}$ vasculaire strengen die ontstaan vanuit de EBs (Hoofdstuk 6).
- De EBs die gegroeid worden in een 3D matrix in een microfluïdisch kweek systeem, ontwikkelen langere en bredere vasculaire strengen van *kdrl:GFP*$^{+}$ cellen in vergelijking met statische kweek, wat duidt op een effect van de microfluïdische stroom op vatontwikkeling (Hoofdstuk 6)
- De EBs en orgaan explantatie modellen zoals ontwikkeld in deze studie laten zien dat het uitgroeien van vaatstructuren ontwikkeld zou kunnen worden als screening platform voor verschillende chemische stoffen en potentiële medicijnen (Hoofdstuk 6).

Toekomstperspectief

Het zebravis embryo is momenteel in opkomst als een veelbelovend model voor het bestuderen van (bloed)vat ontwikkeling. Veel onderzoek is gedaan om transgene lijnen te ontwikkelen voor deze soort, en de ontwikkeling van zijn bloedvaten *in* vivo te bestuderen.

De grootte van de zebravis en de relatief simpele behuizingscondities maken dit model zeer interessant voor studies in ontwikkelingsbiologie ondanks dat het nog een relatief nieuw model is. Door zijn vele voordelen (zoals hoge vruchtbaarheid en externe bevruchting) en specifieke voordelen voor celkweek toepassingen (zoals simpele kweek condities en grote aantallen primaire cellen om te kweken), kan de zebravis ontwikkeld worden als een veelbelovend *in vitro* model voor verschillende onderzoeksgebieden.

De beschikbaarheid van transgene lijnen maakt het mogelijk om fluorescent geactiveerde cel sortering (FACS) uit te voeren op levende endotheelcellen. Deze gesorteerde cellen kunnen vervolgens verder ontwikkeld worden tot cellijnen voor *in vitro* studies van bloedvatontwikkeling. Echter, de kweek condities voor de differentiatie en groei van zebravis endotheelcellen moet nog verder geoptimaliseerd worden. In principe zouden de zebravis celkweek systemen beschreven in deze studie met FACS gesorteerd kunnen worden om alleen de endotheel cellen te isoleren en analyseren.

Een experiment dat op dit moment uitgevoerd zou kunnen worden, met de in deze studie beschreven zebravis EB culturen, is het bestuderen van effecten van verschillende chemische stoffen waarvan bekend is dat ze een remmend of stimulerend effect hebben op bloedvatontwikkeling in *in vitro* zoogdier celcultuur modellen. Dit zou de potentie van dit model bevestigen voor het gebruik als screening platform in de toekomst. Een ander voordeel van de zebravis is de mogelijkheid van het gebruik van mutanten voor het ontwikkelen van een *in vitro* vasculair netwerk. Dit zou kunnen bijdragen aan het ontrafelen van de moleculaire processen die een rol spelen in de vasculaire ontwikkeling.

Microfluïdische culturen die de fysiologisch dynamische omgeving van weefsel nabootsen dragen bij aan het veld van bloedvatvorming. De ontwikkeling van een microfluïdisch systeem verbonden aan een weefsel met vaatstructuren zou een interessant onderzoeksgebied zijn voor de toekomst. De voornaamste uitdaging bij het ontwikkelen van een dergelijk system zouden liggen in de mogelijkheid om ‘vaatstructuur’ aan ‘hardware’ te verbinden en dit aan te passen aan de toenemende benodigdheden van het groeiende weefsel over de tijd.

Curriculum Vitae

Muhammad Ibrahim was born on 17th of February 1982, in Mardan, Pakistan. He completed his secondary school majoring in science from the Government Centennial Model School, Mardan in 1997. He did his intermediate studies in the pre-medical group at the Mardan Model School and College, Mardan in 1999. He obtained the title of Doctor of Veterinary Medicine from Gomal College of Veterinary Science, Gomal University, Dera Ismail Khan, Pakistan in 2005. Then he did an M.Phil in biotechnology from Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar, Pakistan in 2009. He was appointed in 2010 as Lecturer at the same institute where he obtained his M.Phil. degree. In 2012 he received a faculty development scholarship from The University of Agriculture Peshawar for his PhD studies. In August 2012 he started his PhD at the Institute of Biology Leiden, Leiden University, under the supervision of Prof. dr. Michael Richardson. After his PhD he intends to continue his career at The University of Agriculture Peshawar, Pakistan.

List of Publications

- Ibrahim M, Richardson MK. Beyond organoids: *in vitro* vasculogenesis and angiogenesis using cells from mammals and zebrafish. Reprod Toxicol (in press).
- Ibrahim M, Richardson MK. The growth of endothelial-like cells in zebrafish embryoid body culture. (in preparation).
- Ibrahim M, Richardson MK. In vitro development of zebrafish vascular network. Reprod Toxicol (2017) DOI: 10.1016/j.reprotox.2017-.02.008.
- Ibrahim M, Richardson MK. Microfluidic devices for cell, tissue and embryo culture. Rec Pat Regen Med (2013) 3(3): 249-263.
- Musavi SAA, Ahmad S, Ibrahim M. Documentation and morphology of Hazaragie sheep native to central Afghanistan. Indian J Anim Sci (2013) 83(9): 934-941.
- Musavi SAA, Ahmad S, Ibrahim M. Molecular characterization of Hazaragie sheep native to central Afghanistan. Indian J Anim Sci (2011) 81(7): 711-717.
- Ibrahim M, Ahmad S, Swati ZA, Ullah G. Fat Tailed Sheep Production Systems in the Khyber Pakhtunkhwa Province of Pakistan. Trop Anim Health Prod (2011) 43: 1395-1403.
- Ibrahim M, Ahmad S, Swati ZA, Khan MS. Genetic diversity in Balkhi, Hashtnagri and Michni sheep populations using SSR markers. Afr J Biotechnol (2010) 9(45): 7617-7628.