Development of an in vitro vascular network using zebrafish embryonic cells

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Chapter 1

General introduction
Culturing zebrafish vascular networks

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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.