Tampere University

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Maturation and morphology of human pluripotent stem cell derived cardiomyocytes and vascular structures in 3D cardiovascular construct

Towards modelling myocardial ischemia

Faculty of Medicine and
Health Technology
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ABSTRACT

Sanna Koskimäki: Maturation and morphology of human pluripotent stem cell derived cardiomyocytes and vascular structures in 3D cardiovascular construct – Towards modelling myocardial ischemia
Master's thesis
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Cardiac diseases are the leading cause of mortality worldwide. In addition, cardiotoxicity is one of the major reasons for failure in drug development processes. Animal models cannot fully recapitulate the human pathophysiology alongside with high costs and ethical issues. There is an unmet need for 3D *in vitro* models that can truthfully represent human cardiac tissue, and which could be used for drug development, cardiotoxicity screening, disease modelling, as well as, for basic research. This project is part of the Centre of Excellence in Body-on-Chip research and aims to develop multicellular 3D construct modelling human heart tissue that could be used as a platform for modelling myocardial ischemia.

Human umbilical vein endothelial cells with fluorescent tag (GFP-HUVEC, Cellworks), human adipose stem/stromal cells (hASC) and human induced pluripotent stem cell derived -cardiomyocytes (hiPSC-CM) compose the 3D cardiovascular model. To provide 3D environment, cells are seeded inside and on top of novel hydrazone crosslinked gelatin-gellan gum hydrogel. Characterization methods include qPCR, immunocytochemical staining, and confocal imaging. Additionally, quantitative analysis of beating hiPSC-CMs and formation of vascular structures of HUVECs were conducted.

The results demonstrate that gelatin-gellan gum provides adequate two-parted 3D microenvironment for multicellular heart tissue model. On one hand, the hiPSC-CMs exhibited improved maturation and behaviour comparable to native cardiomyocytes, i.e. elongated morphology and sarcomere structures, as well as alignment with endothelial cells. On the other hand, vascular structures formed in the co-culture, supporting the maturation of hiPSC-CM, but co-culturing did not improve formation of vascular structures when compared to vascular structure alone.

3D cardiovascular model is shown to represent essential characteristics of human cardiac tissue and provides promising platform for modelling human cardiac diseases, such as, myocardial ischemia. In addition, co-culture of hASCs with hiPSC-CM exhibited interesting properties to be used as supportive cells in cardio co-culture and possibly as the only supporting cell type in cardiac co-culture studies.

Keywords: cardiomyocyte maturation, angiogenesis, hiPSC-CM, endothelial cell, human adipose stem/stromal cell, 3D *in vitro* -model, co-culture, hydrogel

TIIVISTELMÄ

Sanna Koskimäki: Ihmisen pluripotenttien kantasoluperäisten sydänlihassolujen ja verisuonirakenteiden maturaatio ja morfologia 3D sydän-verisuonimallissa - Kohti sydänlihasiskemian mallintamista
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Sydänsairaudet ovat johtava kuolinsyy maailmanlaajuisesti. Lisäksi kardiotoksisuus on yksi suurimmista syistä epäonnistumiseen lääkekehitysprosesseissa. Eläinmalleilla ei pystytä kattavasti mallintamaan ihmisen patofysiologiaa, ja lisäksi eläinmallien käyttäminen tutkimuksessa on kallista ja aiheuttaa eettisiä ongelmia. Ihmisen sydänkudosta totuudenmukaisesti edustavia 3D *in vitro* -malleja, joita voitaisiin käyttää lääkekehityksessä, kardiotoksisuusseulonnassa, sairauksien mallintamisessa sekä perustutkimuksessa, ei ole kattavasti saatavilla. Tämä projekti on osa Monikudosmallintamisen huippuyksikön tutkimusta, ja sen tavoitteena on kehittää monisoluinen 3D-rakenne ihmisen sydänkudoksesta, jota voitaisiin käyttää alustana sydänlihasiskemian mallintamiseen.

Solutyypit, jotka muodostavat 3D sydän-verisuonimallin, ovat ihmisen napanuoran laskimon endoteelisolut fluoresoivalla tunnisteella (GFP-HUVEC, Cellworks), ihmisen rasvakudoksen kantasolut (hASC) ja ihmisperäiset indusoiduista erittäin monikykyisistä kantasoluista johdetut kardiomyosyytit (hiPSC-CM). 3D-ympäristön aikaansaamiseksi solut viljellään hydratsonilla ristisilloitetun gelatiini-gellaanikumihydrogeelin sisässä ja päällä. 3D sydän-verisuonimallin karakterisointimenetelmiä ovat qPCR, immunosytokemialliset värjäykset ja konfokaalikuvantaminen. Lisäksi soluviljelmille suoritettiin kvantitatiivinen analyysi hiPSC-CM:ien toiminnallisista ominaisuuksista ja HUVEC:ien muodostamista verisuonirakenteista.

Tulokset osoittavat, että solutyypit ovat vuorovaikutuksessa keskenään yhteisviljelmissä, ja että gelatiini-gellaanikumi on soveltuva 3D-mikroympäristö monisoluiselle sydänkudosmallille. Yhteisviljelmistä havaittiin, että hiPSC-CM:t osoittivat parantunutta erilaistumista ja maturaatiota, joka oli verrattavissa natiiveihin sydänlihassoluihin, eli pidentynyttä morfologiaa ja sarkomeerirakenteita sekä linjautumista endoteelisolujen kanssa. Yhteisviljelmässä havaittiin verisuonirakenteita, jotka tukivat hiPSC-CM:n kypsymistä, mutta yhteisviljely ei parantanut verisuonirakenteiden muodostumista verrattuna verisuonikontrolliin.

3D-sydän- ja verisuonimallin on osoitettu edustavan ihmisen sydänkudoksen olennaisia ominaisuuksia ja tarjoavan lupaavan alustan ihmisen sydänsairauksien, kuten sydänlihasiskemian, mallintamiseen. Lisäksi hASC-solujen yhteisviljely hiPSC-CM:n kanssa osoittautui potentiaaliseksi vaihtoehdoksi sydän-verisuonimallille tarkasteltaessa solujen morfologiaa ja käyttäytymistä, ja hASC-soluja voitaisiinkin mahdollisesti käyttää ainoana tukisolutyyppinä yhteisviljelytutkimuksissa.

Avainsanat: sydänsolujen maturaatio, angiogeneesi, hiPSC-CM, endoteelisolu, rasvan kantasolu, 3D *in vitro* -malli, yhteisviljelmä, hydrogeeli

PREFACE

I want to ackowledge Adult stem cell group, Heart group and PIs Prof. Susanna Miettinen and Prof. Katriina Aalto-Setälä for the opportunity to work on this interesting and enlightening project. Big thanks to partner in crime, Emma Hovinen, with whom we shared many tricky situations as well as fun moments in the lab. Special thanks belong to our supervisors Hanna Vuorenpää (Postdoc, PhD), Kirsi Penttinen (PhD) and Mari Pekkanen-Mattila (PhD) for taking us along in this project and for your support, guidance, and know-how regarding the work.

Looking back my journey as a student in Tampere University, I want to praise all the professors, teachers, and mentors I've encountered for the high-level education and skills you have offered. I want to thank all the colleagues and friends at the university for your support, help and shared coffee breaks. Also, friends and family deserve praise for always lending me an ear and being there for me throughout this project. Thanks to Vertti for holding me and our house together in one piece and thanks to Terhi for the numerous study sessions in the library.

Tampere, April 29th, 2022

Sanna Koskimäki

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
3D co-culture	Cell culture containing HUVECs, hASCs and hiPSC-CMs in 3D environment
3D vascular culture	Cell culture containing HUVECs and hASCs in 3D environment
3D cardiac control	Cell culture containing CMs in 3D environment
2D cardiac control	Cell culture containing CMs in 2D environment
CD	Cardiac disease
cDNA	Complementary DNA
CM	Cardiomyocyte
CoEBoC	Centre of Excellence in Body-on-Chip Research
GFP-HUVEC	Green fluorescent protein -marked human umbilical vein endothelial cell
hASC	Human adipose stem/stromal cell
hiPSC	Human induced pluripotent stem cell
EB 5%	Embryoid body culture medium containing 5% serum
EC	Endothelial cell
ECM	Extracellular matrix
EGM-2	Endothelial growth medium
Gelatin (Gel) CHD	Carbohydrazide (CDH)-modified gelatin
GG	Gellan gum
ICC	Immunocytochemistry
MACS	Magnetic-activated cell sorting
mRNA	Messenger RNA
RT-qPCR	Real-time reverse transcription polymerase chain reaction
SDCM	Spinning disk confocal microscopy
VEGF	Vascular endothelial growth factor

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1. INTRODUCTION

The cardiac muscle tissue, or myocardium, is a highly vascularized muscle tissue type performing coordinated contractions that allow blood flow through-out circulatory system. The blood circulatory system is crucial in circulation of nutrients, vital gases, hormones, and body metabolites and thus in maintaining body homeostasis in vertebrates. (Hall, 2011; Saladin, 2011) Cardiac muscle tissue is composed of various cardiac cell types, including myocytes, fibroblasts, leukocytes, and cardiac vascular cells and of extracellular matrix (ECM) surrounding the cells (Guo & Pu, 2020). The study of human cardiovascular system requires efficient models replicating heart tissue and blood flow.

The models used in research vary from *in vivo* animal models to *ex vivo* tissue models and *in vitro* cell models. Precise and relevant *in vitro* constructs modelling human heart tissue are needed in order to study cardiac tissue and diseases related to cardiac functions, and in translation of research findings to clinical practise (Veldhuizen & Nikkhah, 2021). I*n vivo* -models and 2D *in vitro* -models have been used to study cardiac diseases and to develop drugs and therapeutics for cardiac diseases (CDs). However, these models fail to mimic the complex human pathophysiology (Veldhuizen & Nikkhah, 2021). 3D models focus on recapitulating more *in vivo* -like human tissue conditions and interactions (Jorba et al., 2021).

Cardiomyocyte maturation is the last step in the development of heart tissue that prepares it for the vital functional behavior, beating. This process is characterized by structural, functional, and metabolic specialization, as well as by a change in gene expression pattern. (Guo & Pu, 2020b) Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) have already demonstrated appropriate characteristics to be used in early toxicity screening, and current technology allow efficient differentiation of iPSC-derived cells into cardiomyocytes (Zuppinger, 2019).

Endothelial cells (ECs) interact with cardiomyocytes bidirectionally during heart development and through adult life and this interaction is necessary for proper maturation, function, and survival of the myocardium. ECs also take part in angiogenesis, which is described as formation of new blood vessels from pre-existing vessels, mainly in adult tissue. Concerning *in vitro* cultures, it is proven that human adipose stem/stromal cells can act as pericytes supporting the vasculature and enhance secretion of

angiogenic factors and cytokines including VEGF, hepatocyte growth factor and angiopoietin. (Vuorenpää et al., 2014) Therefore, by co-culturing HUVECs (human umbilical vein endothelial cell) and hASCs (human adipose stem/stromal cell) with hiPSC-CMs, the maturation state of CMs is improved and could be further enhanced with 3D cell culture conditions (Vuorenpää et al., 2017).

2. LITERATURE REVIEW

2.1 Cardiovascular system

The cardiovascular system consists of the heart and blood vessels. The cardiovascular system is a part of the blood circulatory system, divided in systemic and pulmonary circulation. The blood circulatory system is crucial in circulation of nutrients, vital gases, hormones, and body metabolites and thus in maintaining body homeostasis in vertebrates. Other important functions of cardiovascular system in maintaining homeostasis are stabilization of body temperature and pH. (Hall, 2011, Saladin, 2011)

2.1.1 Models to study cardiovascular system

The study of human cardiovascular system requires efficient models replicating heart tissue and blood flow. The complex tissue architecture, cellular diversity and distinct mechanical behaviour of cardiovascular system and cardiac tissue propose a demanding challenge for research and clinical translation of attempted models. The models used in research vary from *in vivo* animal models to *ex vivo* tissue models and *in vitro* cell models.

In vivo animal models have previously been the main platform to study myocardium functions and cardiac diseases (CD) and to develop drugs and therapeutics for CDs. However, these models fail to mimic the complex human pathophysiology. (Veldhuizen et al., 2019). *In vivo* -models are currently the primary choice and important for clinical translation of the research in biomechanical aspects of cardiac disease development (Jorba et al., 2021). Rodents and pigs are mainly used considering *ex vivo* tissue models modelling cardiac tissue. (Parry et al., 2018) Tissues are relatively easy, but expensive to harvest.

In vitro models aim to mimic the functions of myocardium with the usage of primary or pluripotent stem cells, and biological or synthetic matrix. Within *in vitro* models, the main issue lies in the source of cardiomyocytes (CMs) that are either adult or express adult cell and tissue phenotypes that can mimic the cellular complexity of myocardium. The lack of availability of patient derived human CMs and minimal proliferation capability of primary CMs have transitioned most *in vitro* research toward cardiac differentiation of human induced pluripotent stem cells (hiPSCs). (Veldhuizen & Nikkhah, 2021)

2.2 Cardiac *in vitro* models

2.2.1 Need for cardiac *in vitro* models

Cardiac diseases (CD) are the leading cause of mortality and morbidity world-wide, causing also major expenses for healthcare. Coronary heart disease (CHD) and stroke, two major causes of death globally, caused 12 million deaths alone per year in the late 20th century and the numbers are ever-growing with the addition of other CDs. (Beaglehole et al., 2001) By year 2020, CHD was associated with 17,8 million deaths annually. Prominent risk factors for CDs include hypertension, hyperlipidemia, diabetes mellitus type 2, obesity, and smoking. Often these risk factors occur coincidentally. (Brown et al., 2021)

Prevention and treatment of CDs require extensive research. *In vivo* -models and 2D *in vitro* -models have been used to study cardiac diseases and to develop drugs and therapeutics for CDs. However, these models fail to mimic the complex human pathophysiology. (Veldhuizen & Nikkhah, 2021) *In vivo* animal models are currently the primary choice and important for clinical translation of the research in biomechanical aspects of cardiac disease development and ECM remodelling. Another limitation of animal models is the lack of control over mechanical and structural cues from the cardiac environment, which is needed to understand the role of mechanobiology. (Jorba et al., 2021) There are also ethical issues considering the use of laboratory animals as part of *in vivo* and *ex vivo* studies (Ghasemi & Dehpour, 2009). 3D models focus on recapitulating more *in vivo* -like human tissue conditions and interactions (Figure 1). 3D *in vitro* models of cardiac tissue have applications both in compound screening and primary research, as well as disease modelling. (Jorba et al., 2021)

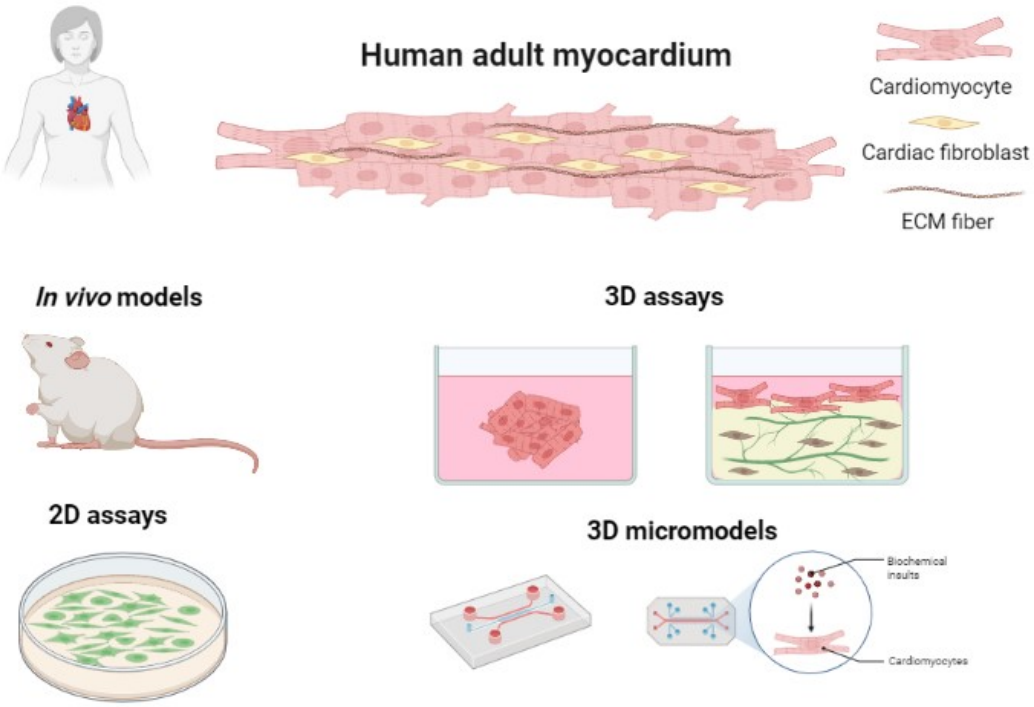

Figure 1. *Different modelling approaches for human cardiac tissue. Figure created in BioRender*

2.2.2 Current cardiac *in vitro* models

3D cardiac cultures aim to be used in studying basic properties of stem cells, in disease modelling using iPS cells, or contributing themselves as therapeutic agents (Kawaguchi et al., 2013). Current attempts on modelling cardiac tissue in *in vitro* 3D environment include various types of CMs and biomaterial from different origin. Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CM) are widely used, as well as mature rodent cardiomyocytes. Fibrin-based hydrogels and collagen scaffolds are used in various applications to provide 3D environment and structural support for the cardiac tissue constructs. (Wang et al., 2018),(Veldhuizen & Nikkhah, 2021) Microfluidic platforms provide alternatives for traditional static cell culture conditions in a shift towards recreating tissue-specific microenvironment (Visone et al., 2022). In addition, direct bioprinting has been tested with neonatal mouse ventricular cardiomyocytes (NMVCMs) in photocrosslinkable hydrogel (Liu et al., 2020). Multicellular organoids are also interesting option for constructing cardiac tissue *in vitro* (Kerr et al., 2021) Several previous *in vitro* applications utilize xenografted cells and/or biomaterials or show poor repeatability. Hence there remains a need for human cell derived platform that has potential for translation into clinical practise or disease modelling.

2.3 Myocardium and cardiomyocyte structure and maturation

Cardiac muscle tissue is composed of various cardiac cell types, including myocytes, fibroblasts, leukocytes, and cardiac vascular cells and of extracellular matrix (ECM) surrounding the cells. Specialized interconnected myocytes in cardiac tissue, cardiomyocytes (CM), provide cardiac tissue its structural and functional features. CMs, connected by intercalated discs that relay electrical impulses, drive heart contraction, and during maturation CMs undergo changes that permit the cells to sustain under the stress of multitudinous cycles of forceful contraction and relaxation (Guo & Pu, 2020). The term “cardiomyocyte maturation” *in vivo* refers to the repertory of changes affecting cell structure, metabolism, function, and gene expression, that transform fetal cardiomyocytes into adult cardiomyocytes.

Cardiac muscle cells have a striated or striped appearance under a microscope. These stripes occur due to alternating filaments comprising of myosin (anisotropic band, A-band) and actin (isotropic band, I-band) proteins. The dark stripes indicate thick filaments of myosin and thin, lighter filaments contain actin. During a cardiomyocyte contraction, the myosin filament and actin filaments move towards each other, causing the cell to shrink. A single myosin filament connects to two actin filaments on either side and forms a single unit of muscle tissue, called a sarcomere, which repeat throughout the cardiomyocyte. (Moo et al., 2016) Existence of sarcomeres in CM cells is a proof of myocyte identity, and the parallel alignment and organization of sarcomeres is a sign of maturation status (Bedada et al., 2016).

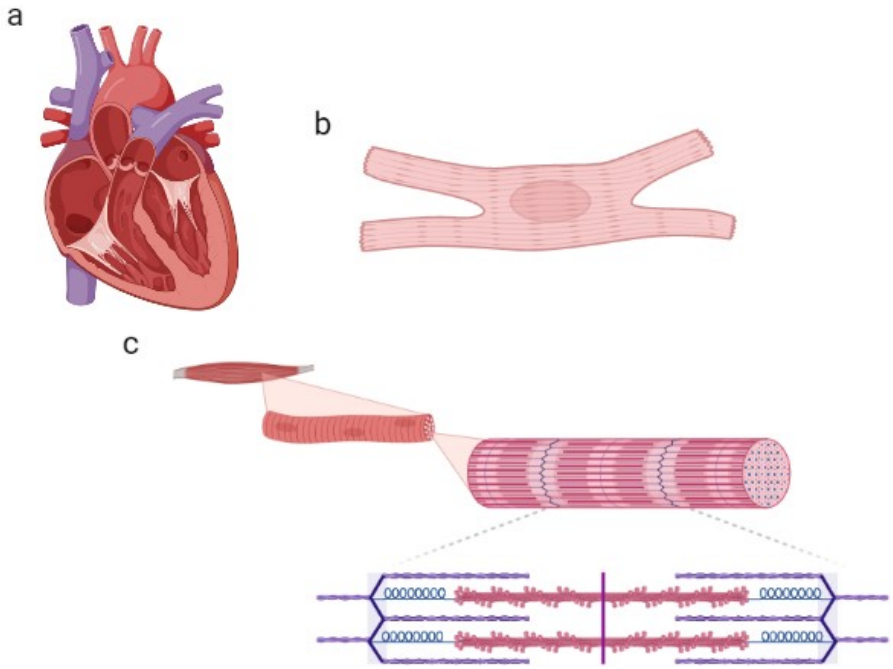

Figure 2. *a. Cardiac tissue b. Individual striated cardiac muscle cell (cardiomyocyte) c. Microstructure of cardiomyocyte, showing myofibrils consisting of sarcomeres. In the bottom figure is shown a sarcomere repeat with a myosin filament (pink) connecting two actin filaments (violet). Figure created in Biorender.*

Cardiomyocyte maturation is the last step in the development of heart tissue that prepares it for the vital functional behavior, beating. This process is characterized by structural, functional, and metabolic specialization, as well as by a change in gene expression pattern. (Guo & Pu, 2020) hiPSC-CMs have already demonstrated appropriate characteristics to be used in early toxicity screening and current technology allow efficient differentiation of iPSC-derived cells into cardiomyocytes (Zuppinger, 2019). However, iPSC-CMs express immature phenotypes that resemble more fetal- than adult tissue, which inevitably may affect the utilization of the results obtained and hinder development of *in vitro* -models for pathological, pharmacological, or therapeutic purpose (Guo & Pu, 2020).

Endothelial cells (ECs) interact with cardiomyocytes bidirectionally during heart development and through adult life. This interaction is necessary for proper maturation, function, and survival of the myocardium. Vascular ECs produce several essential auto- and paracrine agents including vascular endothelial growth factor (VEGF), angiopoietin, and nitric oxide. Concerning *in vitro* cultures, it is proven that human adipose stem/stromal cells can act as pericytes supporting the vasculature and enhance secretion of angiogenic factors and cytokines including VEGF, hepatocyte growth factor and angiopoietin. (Vuorenpää et al., 2014) Therefore, by co-culturing HUVECs (human

umbilical vein endothelial cell) and hASCs (human adipose stem/stromal cell) with hiPSC-CMs, the maturation state of CMs is improved and could be further enhanced with 3D cell culture conditions (Vuorenpää et al., 2017).

EB- differentiated hiPSC-CMs population can be heterogenous due to their background and differentiation method, which can give rise to cells in several stages of differentiation (Lahti et al., 2012). Before their usage, hiPSC-CMs are sorted using magnetic activated cell sorting, a method that takes advantage of surface markers on the target cells. In this application, cells not expressing surface markers specific for iPS-CM are bound to magnetic particles through antibody interactions and isolated from cell population. The throughput of this passive separation technique is high purity hiPSC-CM population. (Zeb et al., 2019)

2.4 Angiogenesis

Angiogenesis is described as formation of new blood vessels from pre-existing vessels. It is distinguished from vasculogenesis, which is the embryonic physiological process in which endothelial cells form from mesoderm precursor cells and from neovascularization. (Santulli, 2013) Thus, term angiogenesis is used when endothelial cells and pericytes form vasculature structures *in vivo* or *in vitro*. Angiogenesis is induced by specific set of secreted growth factors, including vascular endothelial growth factor A (VEGF-A), Fibroblast growth factor 2 (FGF-2), and angiopoietin 1 and 2 (Ang1 and Ang2). (Potente et al., 2011) Notch signalling pathway is proven to be crucial for arterial specification, sprouting angiogenesis, and vessel maturation (Adams & Alitalo, 2007). In newly forming vascular sprouts, Notch signalling is responsible for occurrences leading to distinction between the leading “tip” endothelial cell and the growing “stalk” cell, latter being part of the endothelial cells that eventually form a new capillary in sprouting angiogenesis (Figure 3) (Kofler et al., 2011).

Endothelial cells are heterogeneous cell population derived originally from the embryonic mesoderm. Mesodermal pluripotent cells are first differentiated into hemangioblasts (which form primitive blood islands) and then to angioblasts, which eventually give rise to endothelial cell types. (Adair & Montani, 2010) Vascular endothelial growth factor (VEGF) induces the angioblasts and newly formed endothelial cells to migrate on a matrix formed mainly of collagen and hyaluronan, which allows the fusion through the blood islands and remodelling of these cells into tubular structures, and eventually the formation of the first primitive vascular plexus. The remodelling of these tubules into larger vessels, by means of vasculogenesis, leads to vascularization of the embryo. In contrast to vasculogenesis described above that takes place during

embryogenesis, angiogenesis refers to the formation of new blood vessels from pre-existing ones, occurring mainly in adult tissue. (Lamalice et al., 2007)

Pericytes are mainly mesoderm derived mural cells that function as supportive cells in blood microvessels, embedded in the basement membrane and wrapped around endothelial cells. Pericytes participate in vascular development, maturation, remodelling, architecture, and permeability, and have an active role in regulating blood pressure and in immune functions through lymphocyte activation. (Birbrair et al., 2015) Bone-marrow-derived monocytic cells and their recruitment to the perivascular space is also an important part of adult angiogenesis.(Adams & Alitalo, 2007)

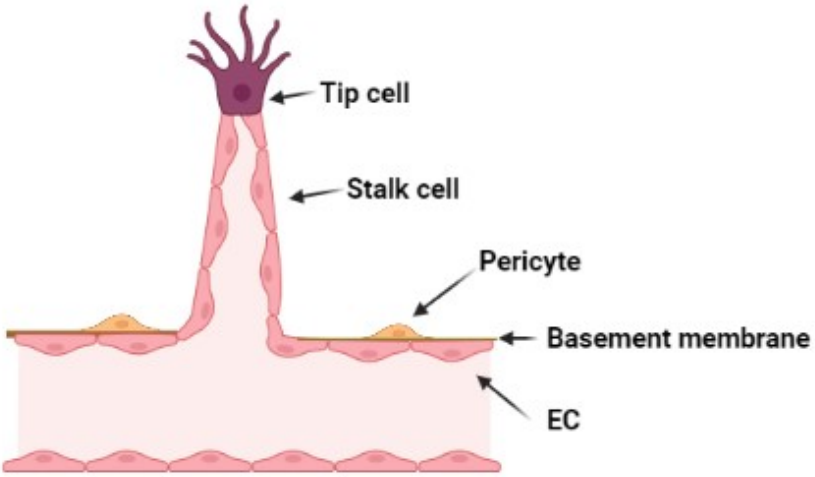

Figure 3. *Sprouting angiogenesis. Figure created in BioRender*

Angiogenesis in adult tissue typically takes place after tissue damage, as part of wound healing and formation of granulating tissue in regeneration. Tumour formation also induces dense sprouting of blood vessels into affected area. In addition, angiogenesis is also involved in ischemic and inflammatory diseases. (Birbrair et al., 2014) Although the ability of cardiac tissue to renew itself after injury is heavily debated topic, angiogenesis is thought to play an important role in regenerating heart tissue and myocardial regrowth after trauma, i.e., surgery or ischemic injury (Ingason et al., 2018). CMs in the cardiac tissue both produce VEGF and host target receptors, VEGFR1 and VEGFR2, on their cell surface. The relationship between VEGF-A and CMs is bilateral. On the one hand, VEGF-A can activate CM, inducing morphogenesis, contractility, and wound healing. On the other hand, CMs also produce VEGF-A during inflammation, mechanical stress, and cytokine stimulation to induce angiogenesis. (Braile et al., 2020) Induction of cardioprotective crosstalk between cardiomyocytes and endothelial cells could enhance angiogenesis after ischemia (Gladka et al., 2021).

2.5 ECM of myocardium and vascular endothelium

2.5.1 ECM structure

Cardiomyocytes in native tissue are surrounded with extracellular matrix (ECM). Cardiac ECM is a complex network of matrix proteins surrounding cardiac cells: myocytes, fibroblasts, leukocytes, and cardiac vascular cells. In addition to structural support, cardiac ECM hosts multiple crucial proteins with growth factor– and cell receptor–binding properties. The structural and non-structural proteins and sugars of cardiac ECM are further subdivided into glycoproteins, proteoglycans, and glycosaminoglycans, the main ones being collagens, serum albumin, non-collagenous glycoproteins (fibronectin and laminin), proteoglycans, glucosaminoglycans (GAGs), and elastins, presented in Figure 4. (Rienks et al., 2014), (Lindsey et al., 2018)

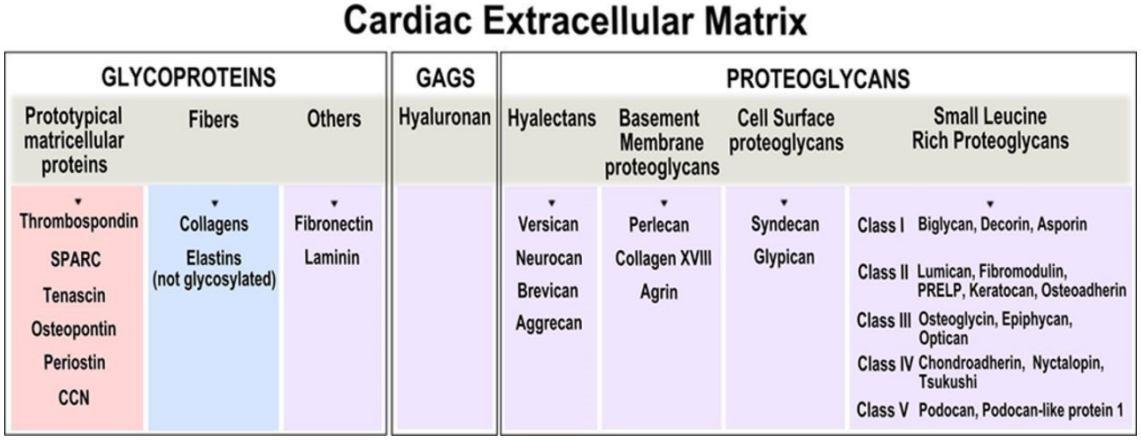

Figure 4. *Composition of cardiac extracellular matrix (Rienks et al., 2014).*

ECM in vascular structures provides crucial support for vascular endothelium that is essential for maintenance of organization when vascular ECs assemble into blood vessels. Vascular ECM functions mainly through adhesive interactions with integrins on the EC surface. These interactions are also needed for proliferation, migration, morphogenesis, and survival of ECs, and finally blood vessel stabilization, all of which are critical for neovascularization. The specific mechanisms which are utilized in ECM are complex and involve regulation of multiple signalling pathways within cell, as well as external structural support. Signalling pathways control e.g., apoptosis, proliferation, the cytoskeleton, and cell shape. Thus, by means of both mechanical and signalling roles of action, the ECM affects many fundamental aspects of CM and EC biology. (Davis & Senger, 2005)

2.6 3D cardiovascular model

2.6.1 Cells

The 3D vascular construct is based on previous work of (Sarkanen et al., 2012), conducted two-dimensionally on co-culture of HUVECs and hASCs. The 3D cardiovascular construct is based on previous work of Vuorenpää et al., 2017. They demonstrated that planar vasculature formed by HUVECs and human foreskin fibroblasts enhance orientation, maturation, and important physiological properties of the hiPSC-CMs in the construct, when compared to monoculture of cardiomyocytes. In order to shift the vascular structure into 3D environment, vasculature forming HUVECs and hASCs are seeded inside hydrogel. HUVECs have the ability to form tubular vascular structures and hASCs act as pericytes and support the vascular structures. The hydrogel structure provides support for the cardiomyocytes, as well as assumably affects the structural and functional maturation of the cardiomyocytes when compared to monolayer cardiomyocyte culture.

2.6.2 Biomaterial

Recent discoveries have shown the potential of the extracellular matrix (ECM) as an essential regulator in development, homeostasis, and restoration of the cardiac microenvironment (Almeida et al., 2021). The goal when designing biomaterial scaffold for 3D *in vitro* cell models is to find a material that mimics the functions of native ECM of the specific tissue type as accurately as possible. Both CMs and ECs propose specific requirements for used biomaterial, as the material needs to withstand biomechanical movement and shear stress. (Jorba et al., 2021),(Kawaguchi et al., 2013) The biomaterial also needs to enable phenotypical behaviour of the cell types (beating, elongation) and distribution of nutrition and biochemical cues in the culture.

GelatinCDH-gellan gum (GelatinCDH-GG) hydrogel has been developed and tested to be suitable for beating CM cultures (Koivisto et al., 2019). The material is proven to enable and support cell attachment, spreading and elongation in encapsulated 3D culture (Figure 5). Previous studies show that hiPSC-derived cardiomyocyte aggregates exhibit normal phenotypical beating behaviour when plated on top of the hydrogel. (Koivisto et al., 2019)

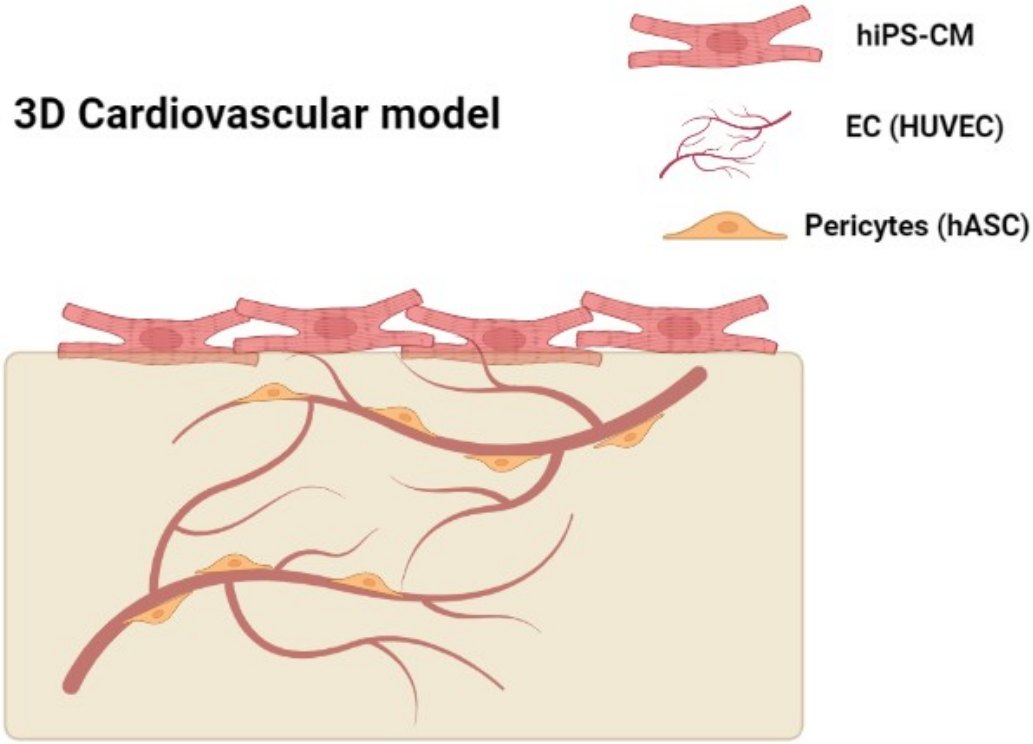

Figure 5. *3D cardiovascular construct with HUVECs and hASCs embedded in gelatinCDH-gellan gum hydrogel, with hiPSC-CMs seeded on top of the hydrogel structure. Figure created in BioRender*

2.7 Analysis techniques used to assess cardiovascular model

Immunocytochemical staining, video imaging, real time qPCR (RT-qPCR) and calcium imaging are used to study hiPSC-CM functionality, maturation, and morphology, as well as maturation and morphology of the vascular structure in the 3D cardiovascular model. Immunocytochemical staining, confocal imaging and RT-qPCR techniques are introduced in further detail in this thesis.

2.7.1 Immunocytochemical staining and confocal imaging

Immunocytochemistry (ICC) takes advantage of antibodies to analyse the existence of certain antigens (markers/proteins) in a sample of cells. The antibodies are usually linked to an enzyme or a fluorescent dye, as in this case. The secondary antibody bound to specific protein or antibody in the structure of the cell allows microscopic visualization of cell structures and to determine which sub-cellular compartments express the target antigen. (Im et al., 2019)

Immunocytochemical staining is used in this study to determine whether the vascular structures and hiPSC-CM cells express the specific maturation markers characteristic for the cell type and to observe the morphology of the cell cultures using fluorescence and confocal microscopy. Confocal microscopy is mainly used, due to the 3D identity of the co-culture cell samples. Confocal imaging allows imaging of the samples in z-stack planes and observation of cell structures in 3D. Spinning disk confocal microscopy (SDCM) takes advantage of hundreds of pinholes arranged in spirals on an opaque disk (Figure 6), rather than a single pinhole as in traditional laser scanning confocal microscopy. During imaging, the disk rotates at high speeds and the pinholes scan every part of the sample in rows, building up an image. Using a spinning disk greatly improves the speed of image acquisition and thus considerably reduces photo damage (photobleaching) of the sample (Oreopoulos et al., 2014).

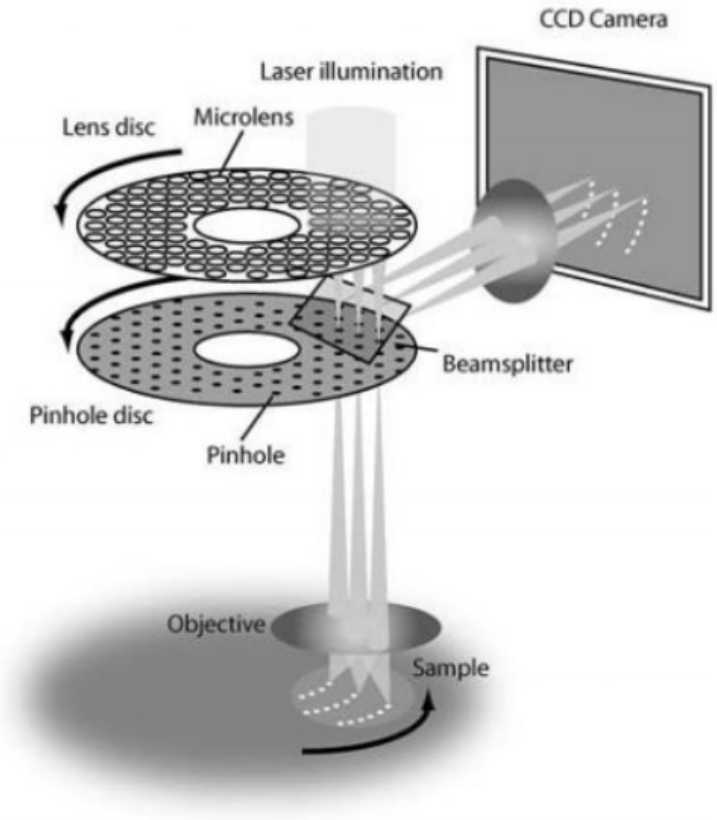

Figure 6. *The components of a spinning disk confocal microscope. The secondary microlens presented in the figure is optional. (Modified from Gräf et al., 2005)*

2.7.2 Real time qPCR and gene markers

Real-time reverse transcription polymerase chain reaction (RT-qPCR) method is used to analyse the presence of specific maturation gene markers in the cell cultures, as in the amount of messenger RNA (mRNA) in the sample. The method used is relative quantification, based on internal reference genes (endogenous controls) to assess fold-differences in expression of the target gene. The quantification is expressed as the change in expression levels of mRNA interpreted in the form of complementary DNA (cDNA, generated by reverse transcription of mRNA). (Nolan et al., 2006) Troponin T (*TNNT2)* is found solely in cardiac muscle and is used as a marker to study CM maturation. Cardiac TnT coded by *TNNT2* is the tropomyosin-binding subunit of the troponin complex, which is located on the actin filament of striated muscle and controls muscle contraction within sarcomere bundle in response to alterations in intracellular calcium ion concentration. (Bosè et al., 2019). *VEGF-A*, *FGF-2*, *Ang1* and *Ang-2* used to study maturation of vascular structures are angiogenesis related gene markers that code for angiogenic growth factors (Potente et al., 2011).

Previous study by Vuorenpää et al., 2017 proved that presence of HUVECs and human foreskin fibroblasts increase the expression of CM maturation marker *TNNT2* in hiPSC-CMs 2D co-culture when compared to hiPSC-CM monoculture. Expression of angiogenesis related gene markers remained relatively constant in 2D cardiovascular construct and vascular structures.

3. OBJECTIVES

Translation of preclinical data into clinical studies requires relevant *in vitro* models that can model essential structural and functional features of native human tissue types. This master's thesis work is conducted as a part of a Centre of Excellence in Body-on-Chip (CoEBoC) research project focusing on developing a multicellular 3D *in vitro* cardiovascular construct that aims to model human cardiac tissue in normal and ischemic conditions. Hypothesis of the study is that the differentiation and maturation of hiPSC-CMs, including structural and functional features, is improved in the co-culture with HUVECs and/or hASCs.

4. MATERIALS AND METHODS

4.1 Cells and Biomaterial

The 3D cardiovascular model consists of human umbilical vein endothelial cells (HUVEC), human adipose stem/stromal cells (hASC) and human induced pluripotent stem cells -derived cardiomyocytes (hiPSC-CM). In the model, hASCs and HUVECs are seeded inside the gelatin-gellan gum hydrogel and hiPSC-CMs are added on top of the vascular structure in 2D layer on day 7 (Figure 7). Vascular structure without CMs is used as angiogenesis control and CMs in monoculture are used as cardiomyocyte maturation control, correspondingly. In addition, hASCs in 3D hydrogel topped with hiPSC-CMs is plated and studied as alternative for 3D cardiovascular model to study the potential of hASCs acting as supportive cell type for hiPSC-CM co-culture. Experiments that have a qPCR endpoint are conducted in 48-well plates while the ones that will end in immunocytochemical staining and confocal imaging are done in ibidi 8-well plates.

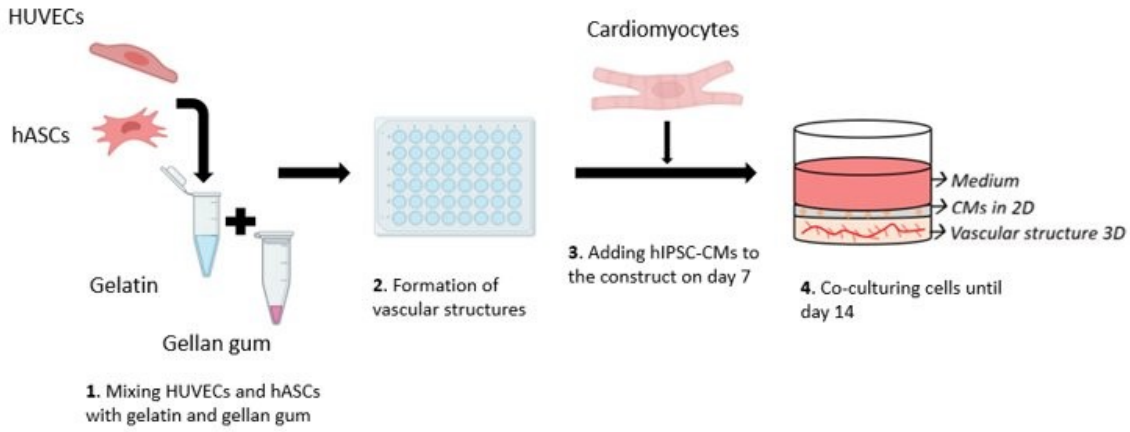

Figure 7. *Formation of 3D cardiovascular model. Figure created in BioRender*

The hASC lines used are derived from several donors, details are presented in Table 1. All hASC lines are FACS-characterized and tested for adipogenic and osteogenic differentiation (Mykuliak et al., 2022).

Table 1. *hASC lines and donor information*

hASC line	Donor age	Donor sex	Cell source
HFSC 3/19	56 years	Male	Subcutaneous
HFSC 4/19	31 years	Female	Subcutaneous
HFSC 6/19	33 years	Female	Subcutaneous

The HUVEC-line used is GFP-HUVEC line I.29715 (Cellworks) and hiPSC-CM cell line UTA 04602.WT. Establishment of patient-specific iPSC line UTA.04602.WT from healthy individual has been described earlier (Takahashi et al., 2007). Characterization of UTA.04602.WT cell line has been described earlier (Lahti et al., 2012).

The hydrogel in the vascular structure is composed of gelatin CDH (60mg/ml) and gellan gum [GG (40mg/ml)], (received from Prof. Kellomäki's Biomaterials and Tissue Engineering group, Tampere University), dissolved in EB5% medium. Gelatin-CDH (carbodihydrazide) was prepared as described previously (Koivisto et al., 2019) and GG was modified by NaIO4 oxidation according to previously reported method to produce GG-CHO at the modification degree of 25% (Karvinen et al., 2017). Hydrogel components are mixed in 1:1 ratio in +37 °C briefly prior plating. The hydrogel is cross-linked through covalent hydrazone bonds (Koivisto et al., 2019), which form as the hydrogel gelatinizes.

Ethical approval

This study conforms to the principles outlined in the Declaration of Helsinki. The use of human adipose stem/stromal cells (hASC), human umbilical vein endothelial cells (HUVEC) and induced pluripotent stem cells (iPSC) were approved by Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (Approval Numbers R15161, R08028 and R08070, respectively) and a written informed consent was obtained from all the participants.

4.2 Formation of cardiovascular construct

The cardiovascular construct is seeded inside and on top of hydrazone crosslinked gelatin-gellan gum hydrogel. Vascular structure containing HUVECs and hASCs embedded in hydrogel is formed on day 0 and hiPSC-CMs are seeded on top of vascular structure on day 7. The cardiovascular construct is cultured for 16 days total. The cell densities are presented in Table 2 and timeline for cell cultures in Figure 8.

Table 2. *Cell densities in 3D cell culture*

hASCs,	**3D: 0,22 x 10^6 cells/ml (3D in 150µl)**
HUVECs (GFP),	3D: 1,1 x 10^6 cells /ml (3D in 150µl)
iPSC-CMs **MACS sorted (Cell line: UTA.04602.WT),**	2D: 98 500 cells/cm^2 3D: 5.8mil. cells/ml (3D in 150µl)

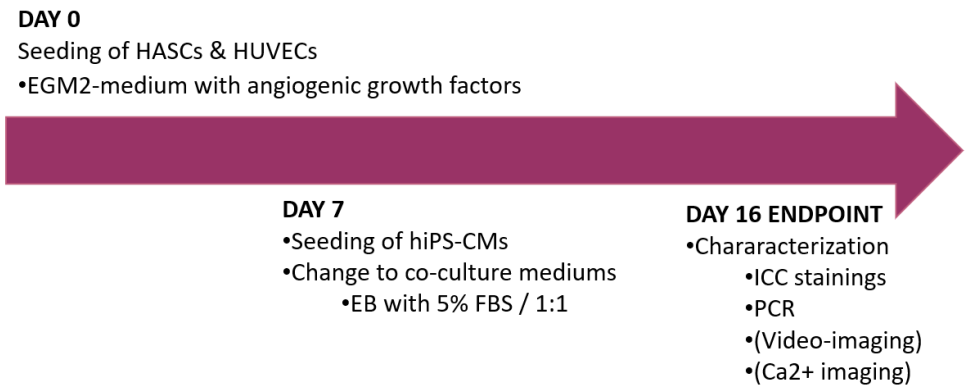

Figure 8. *Timeline for 3D cardiovascular culture*

4.2.1 Vascular structures

The cell composition of the vascular structures consists of human adipose stem/stromal cells (hASCs) and human umbilical vein endothelial cells (HUVECs) The ratio between hASC and HUVEC cells is 1:5 in each well, respectively.

The correct number of cells in a medium suspension is combined, centrifuged, and then suspended directly into gelatin CDH. Gelatin CDH suspension with cells is pipetted into bottom corner of the well while the plate is tilted. Gellan gum is added to the mixture and then the plate is tilted into opposite direction to let the suspension spread evenly. The hydrogel is let to gelatinize in an incubator (~1h) before addition of EGM-2 (500 µl/1,1 cm2). Vascular structure alone is used as angiogenesis control. Plate map displaying cell combinations, time points and medium compositions is shown below (Figure 9).

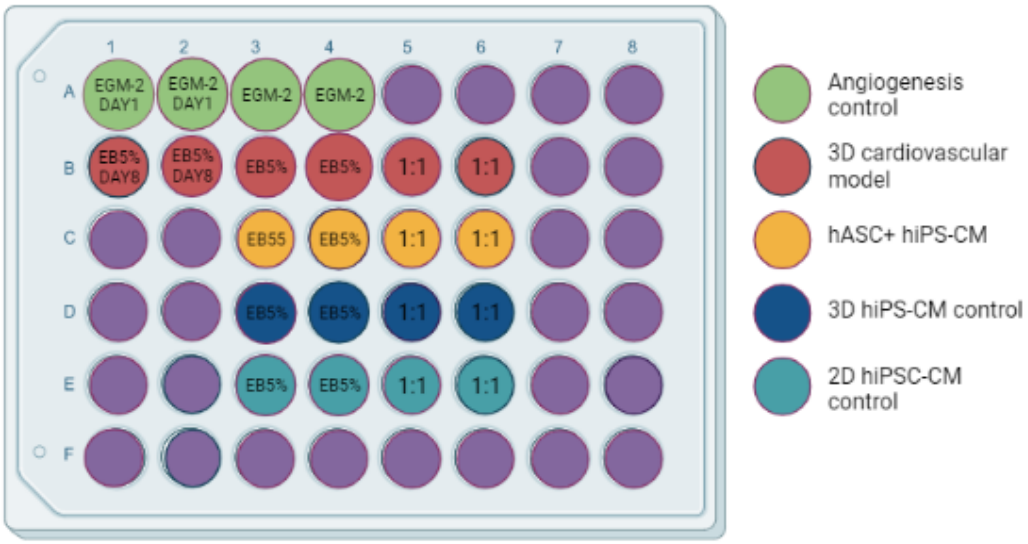

Figure 9. *Plate map and medium compositions of the cell cultures. Figure created in BioRender*

4.2.2 MACS-sorting of cardiomyocytes

Differentiation protocol for EB-differentiated hiPSC-CM cardiomyocytes has been described previously (Prajapati et al., 2021). Cardiomyocytes are hEB-differentiated for 17 days before MACS-sorting (Miltenyi Biotec, Germany). The differentiated hEBs are pooled together, washed with dPBS and dissociated with Multi Tissue Dissociation Kit 3 (Miltenyi Biotec). After dissociation, cells are pre-filtered and counted.

MACS-sorting protocol:

Cell suspension is centrifuged at 200 x g for 5 min in 4˚C and supernatant is aspirated

For less than 5 million cells, the cells are resuspended to 100 µl of mixture of non-CM depletion cocktail (20 µl) and MACS buffer (80 µl) and incubated at 4°C for 5 min. The mixture is multiplied for every 5 million cells.

1 ml MACS buffer is added, the sample is centrifuged at 200 x g for 5 min in 4°C and supernatant is aspirated.

For less than 5 million cells, the cells are resuspended in 100 µl of mixture of Anti-Biotin Microbeads (20 µl) and MACS buffer (80 µl) and incubated at 4 °C for 10 min.

The mixture is multiplied for every 5 million cells.

During incubation, LS column is prepared by attaching it to MACS magnet on the multistand and rinsing it with 3 ml of MACS buffer.

The cells are added to the LS column and the column is rinsed three times with 3 ml of MACS buffer. The throughput is collected to 15ml falcon containing CMs.

The cells are centrifuged at 200 x g for 5 min in 4°C and supernatant is aspirated. The cells are resuspended to EB 5% medium and then counted and plated within 30 minutes.

4.2.3 Plating of cardiomyocytes

Correct number of MACS-sorted cardiomyocytes (108 500 per well) are plated directly after MACS-sorting on top of the vascular structure (and on top of hASCs in gela-gg) in two-dimensional layer. Media (EB5%, 1:1 and EGM-2) is changed prior plating of the cardiomyocytes, according to figure 9. Cardiomyocyte controls include cardiomyocytes on top of gelatin-gellan gum hydrogel in two-dimensional layer and two-dimensional culture of cardiomyocytes in well coated with 0.1% gelatin, both controls with 108 500 CM per well.

4.2.4 Media composition

Angiogenic control with HUVECs and hASCs is cultured in EGM-2 medium the entire culture period. Cultures with cardiomyocytes are cultured in different media compositions after plating of the cardiomyocytes (Table 3), with doublets in EB5% and doublets in 1:1 (EB5% and EGM-2) of 4 parallel samples. The effect of media composition is observed with different analysis methods, including qPCR and confocal imaging.

Table 3. *Cell combionations and medium compositions*

Cell combination	Abbreviation	Culture medium
HUVEC+hASC+hiPSC-CM	3D model	EB5%
HUVEC+hASC+hiPSC-CM	3D model	EGM-2 / EB5% (1:1)
hASC+hiPSC-CM	hASC+CM	EB5%
hASC+hiPSC-CM	hASC+CM	EGM-2 / EB5% (1:1)
HUVEC+hASC	Angiogenesis control	EGM-2
hiPSC-CM 3D control	3D CM	EB5%
hiPSC-CM 3D control	3D CM	EGM-2 / EB5% (1:1)
hiPSC-CM 2D control	2D CM	EB5%
hiPSC-CM 2D control	2D CM	EGM-2 / EB5% (1:1)

Endothelial growth medium (EGM-2) (Lonza) includes:

EBM-2 Endothelial cell growth basal medium
2% Human serum (HS) (Serana)
0.5 ng/ml VEGF
5 ng/ml EGF
10 ng/ml bFGF
20 ng/ml long R3-IGF-1
22.5 µg/ml heparin
1 µg/ml ascorbic acid
0.2 µg/ml hydrocortisone
GA-1000

EB5% includes:

KnockOut DMEM/F-12
5% Fetal bovine serume (FBS) (Biosera Nuaille, France, FB- 1001)
2 mM GlutaMax
1% Non-essential amino acids (NEAA)
50 U/ml Penicillin/streptomycin (Pen/Strep)

4.3 Live imaging

During cell culture time the cell morphology and hydrogel integrity is observed using Zeiss Axio Vert.A1 phase contrast microscope and imaged using Zeiss AxioCam MRc5 (Carl Zeiss AG, Germany). EVOS™ FL Cell Imaging System (Thermo Fisher Scientific, US) is used to observe the formation of tubular structures formed by GFP-HUVECs and samples are imaged every two days throughout culture time immediately after medium change using 10x magnification. Nikon Eclipse TS100 (Nikon Corporation, Japan) microscope with 20X air objective and Bobcat B0620 (Imperix, Switzerland) camera mounted to the microscope is used for video-imaging of beating cardiomyocytes.

4.4 Endpoint analyses

The cell cultures are kept for total of 16 days (9 days with cardiomyocytes) and endpoint analyses are performed at that time point. Day 1 and day 8 samples are also collected and/or immunocytochemically stained, respectively, in addition to endpoint samples.

4.4.1 RT-qPCR

Endpoint samples are collected at day 16 and day 1 and day 8 at the specific timepoint, according to Figure 9.

RNA is isolated and purified using RNA isolation kit (Miltenyi Biotech, Germany) and converted to cDNA according to protocol provided by the manufacturer. RT-qPCR is conducted using TaqMan Fast Advanced master mix and TaqMan assay (Thermo Fisher Scientific, US) Equipment used for RT-qPCR is ABI QuantStudio 12K Flex System (Thermo Fisher Scientific, US).

Cardiac markers are presented in Table 4 and vascular markers in Table 5.

Table 4. *Cardiac markers used in RT-qPCR*

Gene	Protein
GJA1	Connexin-43
TNNT2	Cardiac type troponin T2
MYH6	Myosin Heavy Chain 6
MYH7	Myosin Heavy Chain 7
CACNA1C	Calcium Voltage-Gated Channel Subunit Alpha1 C
KCNJ2	Potassium Inwardly Rectifying Channel Subfamily J Member 2
SCN5A	Sodium Voltage-Gated Channel Alpha Subunit 5
ADRB1	Adrenoceptor Beta 1

Table 5. *Vascular markers used in RT-qPCR*

Gene	Protein
VEGF-A	Vascular endothelial growth factor A
FGF-2	Fibroblast Growth Factor 2(Basic)
Ang1	Angiopoietin 1
Ang2	Angiopoietin 2
ACTA	alpha smooth muscle actin

The focus of this thesis is in five gene markers in total: four angiogenesis gene markers and one cardiomyocyte maturation gene marker. These markers are presented in Table 6.

Table 6. *RT-qPCR markers studied in detail in this thesis*

Gene	Protein
VEGF-A	Vascular endothelial growth factor A
FGF-2	Fibroblast Growth Factor 2(Basic)
Ang1	Angiopoietin 1
Ang2	Angiopoietin 2
TNNT2	Cardiac type troponin T2

Cell combinations, media, abbreviations, and time points in the RT-qPCR results are presented in Table 7 and 8.

Table 7. *Cell combinations, media, abbreviations, and time points in the RT-qPCR results of vascular gene markers*

Cell combination	Medium	Abbreviation	Time point
HUVEC+hASC+hiPSC-CM	EB5%	3D day 8	Day 8
HUVEC+hASC+hiPSC-CM	EB5%	3D EB	Day 16
HUVEC+hASC+hiPSC-CM	EB5%&EGM-2 1:1	3D 1:1	Day 16
HUVEC+hASC	EGM-2	Ang Cont1	Day 1
HUVEC+hASC	EGM-2	Ang Cont	Day 16

Table 8. *Cell combinations, media, abbreviations, and time points in the RT-qPCR results of cardiac gene marker*

Cell combination	Medium	Abbreviation	Time point
HUVEC+hASC+hiPSC-CM	EB5%	3D day 8	Day 8
HUVEC+hASC+hiPSC-CM	EB5%	3D EB	Day 16
HUVEC+hASC+hiPSC-CM	EB5%&EGM-2 1:1	3D 1:1	Day 16
hiPSC-CM	EB5%	3D CM EB	Day 16
hiPSC-CM	EB5%&EGM-2 1:1	3D CM 1:1	Day 16

4.4.2 Statistical analysis

Statistical analysis is performed for RT-qPCR results, comparing the relative gene expression between target group and control group using independent t-test. Means of the target group and control group are compared in order to determine whether there is statistical evidence that the associated sample population means are significantly different. In the analysis, 3D cardiovascular construct is the target group, that is compared to either angiogenesis control (HUVEC+hASC) regarding vascular markers, or hiPSC-CM control in hydrogel regarding cardiac marker.

4.4.3 Immunocytochemical staining

Immunocytochemical stainings are performed after 16 days of culturing (9 days with cardiomyocytes). ICC stainings are performed according to protocol introduced by Mykuliak et al., 2022. Primary and secondary antibodies used are presented in Table 9.

Table 9. *Primary and secondary antibodies, concentrations and manufacturers*

Sample	Target	Primary antibody	Secondary antibody
3D model; hASC, HUVEC, CM	CM: Cardiac Troponin T	Anti-cardiac troponin T, goat, 1:1500, Abcam	Alexa 568, donkey, anti-goat, 1:500, Invitrogen
	GFP-HUVEC: CD31	Anti-human CD31, monoclonal mouse, 1:200, Dako	Alexa 568, goat, anti-mouse, 1:500, Invitrogen
hASC+CM	hASC: a-SMA	Anti-alpha smooth muscle actin, mouse [1A4], 1:200, Abcam	Alexa 488, donkey, anti-mouse, 1:1500, Invitrogen
	CM: Cardiac Troponin T	Anti-cardiac troponin T, goat, 1:1500, Abcam	Alexa 568, donkey, anti-goat, 1:500, Invitrogen
Cardiomyocyte controls	CM: Cardiac Troponin T	Anti-cardiac troponin T, goat, 1:1500, Abcam	Alexa 568, donkey, anti-goat, 3D 1:500, 2D 1:800, Invitrogen
Angiogenesis control	GFP-HUVEC	Anti-human CD31, monoclonal mouse, 1:200, Dako	Alexa 647, donkey, anti-mouse, 1:500, Life technologies

4.4.4 Confocal imaging

Confocal images are taken with Nikon Eclipse FN1 Upright Spinning Disk Confocal Microscope at Tampere Imaging Faculty with Nikon Plan Fluor 10x/0.3, WD 16.0 mm (Air) objective and NIS Elements AR 5.11.00 imaging software (Nikon Corporation, Japan). In addition, Leica Stellaris 8 confocal microscope with 10X dry objective (Leica Camera AG, German) are used for imaging cardiomyocyte microstructures. Upright microscopy is used due to the thickness of the 3D samples. Confocal images are taken with 10X magnification and focus on alignment and morphology of hiPSC cardiomyocytes and vascular structures individually and in co-cultures. The alignment and morphology of hiPSC cardiomyocytes are compared between 3D co-culture and 3D cardiomyocyte control group. Alignment and morphology of hiPSC cardiomyocytes and vascular structures are compared between 3D co-culture and 3D vascular culture.

Images are processed with ImageJ (Fiji) using "*Stacks → 3D project → Maximum intensity.* Imaging parameters are presented in Table 10.

Table 10. *Confocal imaging parameters*

Scale	**512 x 512 pixels (2,60 µm/px)**
Slice thickness	4,6 µm
Range	150 µm
Exposure time	2 s.
Voltage	100%

5. RESULTS

5.1 3D cardiovascular model

3D cardiovascular model composing of HUVECs, hASCs and hiPSC-CMs and hydrazone crosslinked gelatin-gellan gum hydrogel was formed, and all cell types stayed viable throughout culture period. Maximum time point for 3D cardiovascular model and control cultures was 16 days, counting from formation of vascular structure (plating of HUVECs and hASCs)

5.2 Vascular structure formation

GFP-HUVECs self-assemble into vascular structures and hASCs act as supporting cells in the co-culture with hiPSC-CMs. iPSC-derived cardiomyocytes are seeded on top of the vascular structure on day 7.

5.2.1 Modest vasculature was formed in the cardiovascular construct

GFP-HUVECs formed tubular structures through-out culture time. Vascular network formed mainly at the side of the wells, forming dense and consistent tubule structures (Figure 10), and cells migrated from the middle to the outer line of the well, causing the hydrogel to sink at the middle. The morphology of HUVECs changed from globular and “cobblestone” phenotype to branched and elongated.

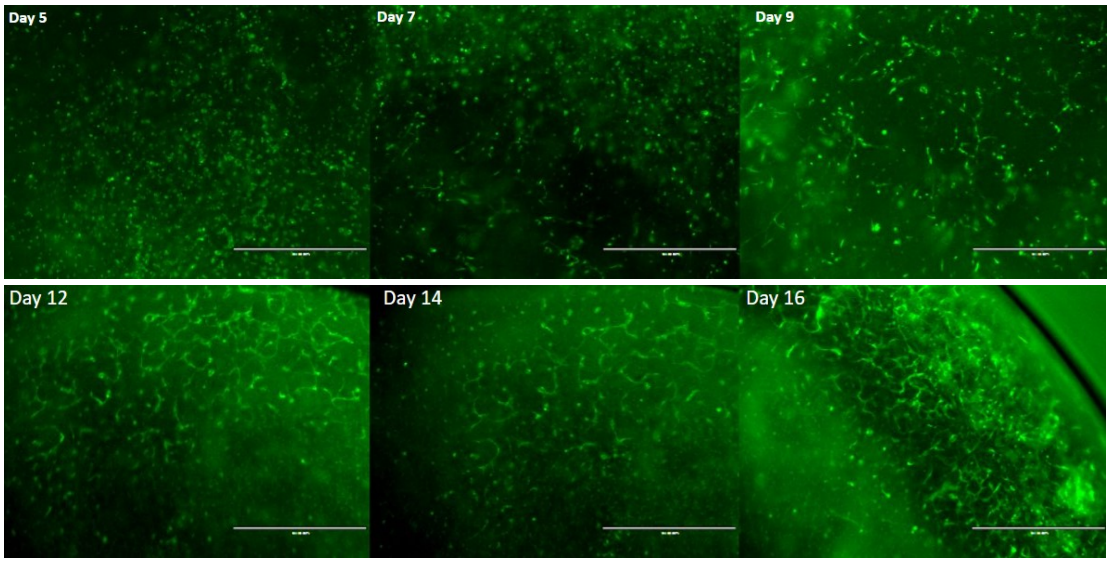

Figure 10. *GFP-HUVECs forming tubular vascular structures from day 5 to day 16 in the culture, taken with EVOS fluorescence microscope using 10X magnification. The scale bar is 1000 µm long.*

5.3 RT-qPCR results and statistical analysis of qPCR results

5.3.1 Vascular marker expression in cardiovascular construct

The studied vascular markers in this section are VEGF-A, FGF-2, Ang1 and Ang2. EEF1A1 was used as an endogenous control. The results are presented in Figure 11. All vascular markers show elevated gene expression ($p \leq 0,05$) in angiogenesis control group (Ang Cont) when compared to cardiovascular construct (3D EB and 3D 1:1), with the exception of VEGF-A, showing best gene expression in 3D cardiovascular construct cultured in EB5% medium.

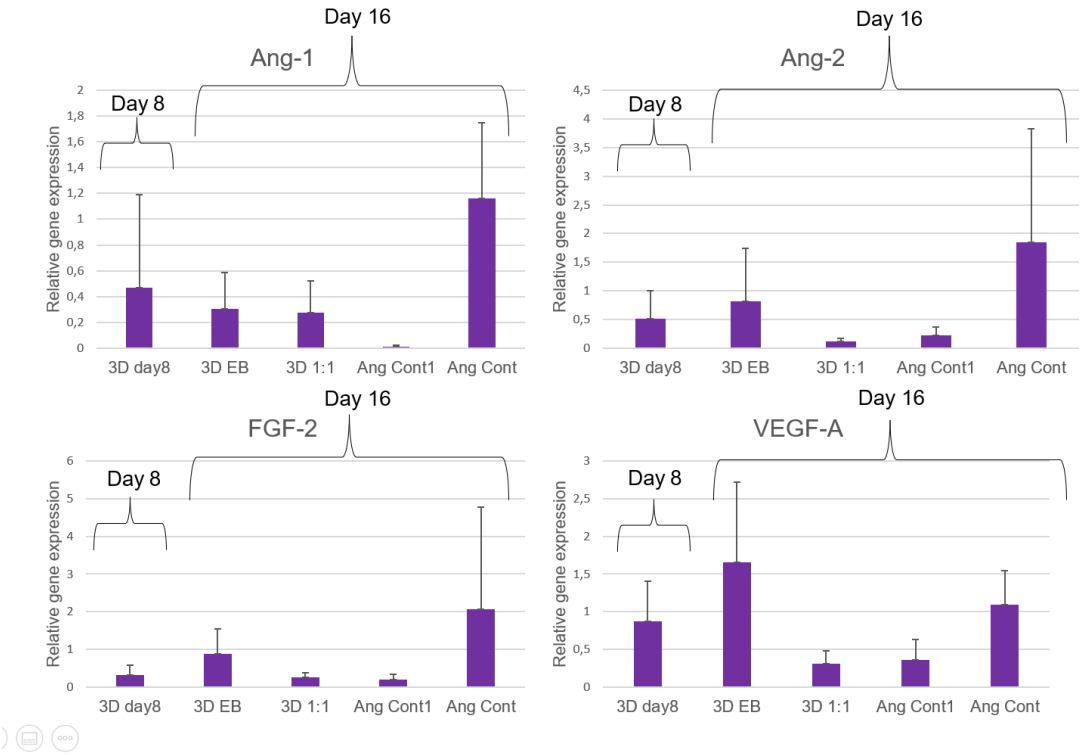

Figure 11. *Relative gene expression of angiogenic markers. 3D day8: HUVEC+hASC+hiPSC-CM at day8, 3D EB: HUVEC+hASC+hiPSC-CM at day 16 in EB5% medium, 3D 1:1: HUVEC+hASC+hiPSC-CM at day 16 in 1:1 medium, Ang Cont: HUVEC+hASC at day 1, Ang Cont: HUVEC+hASC at day 16. (Ang-1, Ang-2, FGF-2 p ≤ 0,05, VEGF-A p < 0,05.)*

Noticeable is that there is statistical difference between standard deviations of sample groups due to variations in gene expression status within sample group.

5.3.2 Cardiac marker expression in cardiovascular construct

The studied cardiac marker in this section is TNNT2 to represent the maturation of cardiomyocytes. Most elevated gene expression is present in hiPSC-CM monoculture in hydrogel, as presented in Figure 12.

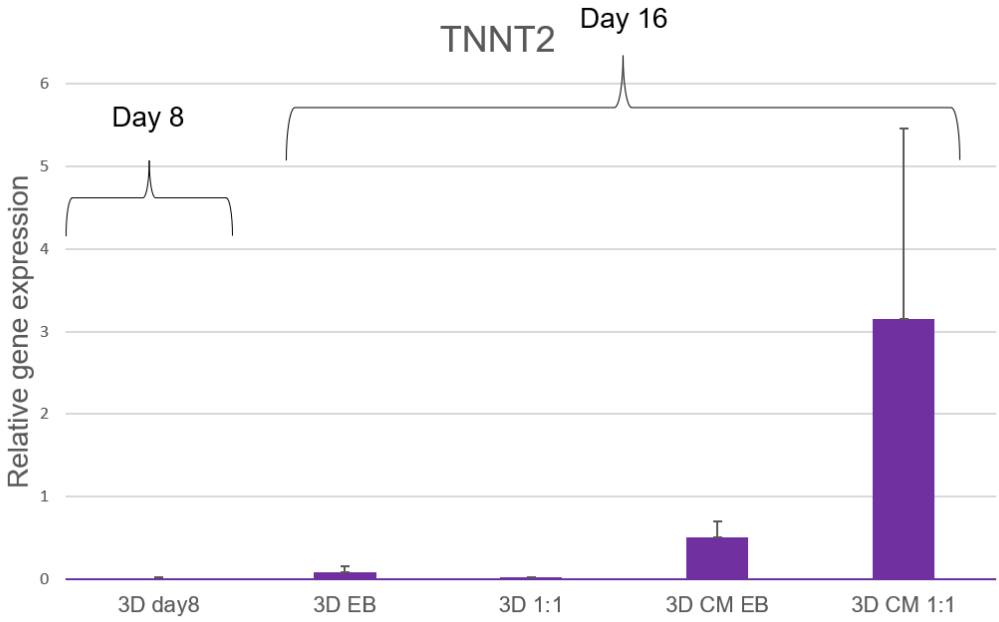

Figure 12. *Relative gene expression of TNNT2 Cardiac marker. 3D day8: HUVEC+hASC +hiPSC-CM at day8, 3D EB: HUVEC+hASC +hiPSC-CM at day 16 in EB5% medium, 3D 1:1: HUVEC+hASC +hiPSC-CM at day 16 in 1:1 medium, 3D CM EB: hiPSC-CM at day 16 in EB5% medium, 3D CM 1:1: hiPSC-CM at day 16 in 1:1 medium ($p \leq 0,05$).*

5.4 Confocal imaging

GFP-HUVECs formed tubular structures through-out culture time (Figure 13, a&b). A network of α-SMA positive hASCs was formed in the hASC+hiPSC-CM co-culture, aligning parallel with the hiPSC-CM network (Figure 13, c). The morphology of hiPSC-CMs changed from round and globular aggregates to elongated and oriented network of hiPSC-CMs in the co-cultures (Figure 13, c&d)

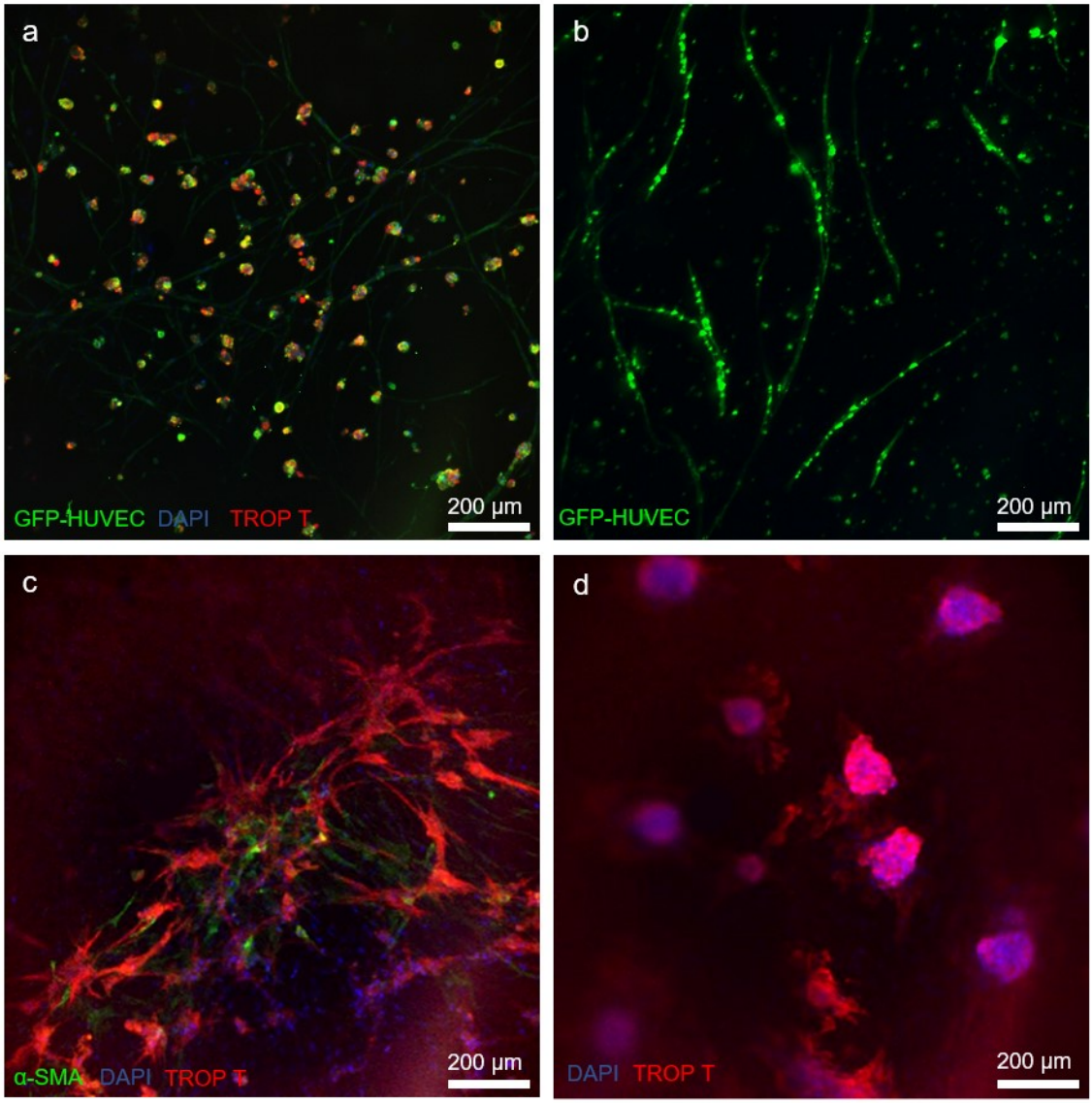

Figure 13. *a: 3D cardiovascular construct on day 8, cultured in EB5%, b: Angiogenesis control on day 16, in EGM-2 medium, c: CM+hASC 3D co-culture on day 16 in 1:1 medium, c: CM 3D culture on day 16, in 1:1 medium. Imaged using 10X magnification.*

Following confocal images present the microstructure of the hiPSC-CMs, including sarcomere structures, in co-culture with hASCs (Figure 14 and 15). Images are taken together with Leica Application specialist Dr. Daniel Smeets.

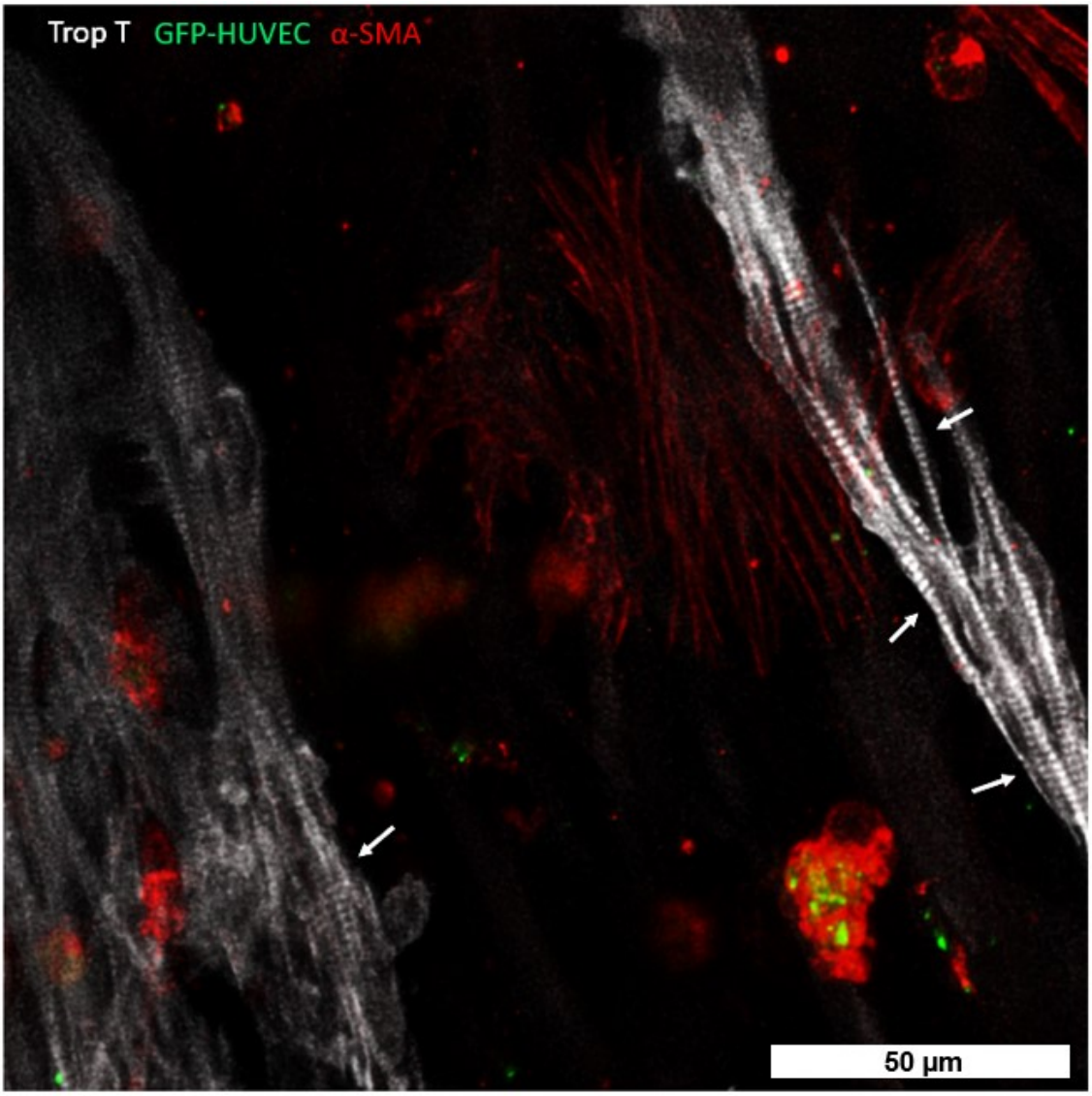

Figure 14. *Microstructures of hiPSC-CMs in hASC+hiPSC-CM culture at day 16, white arrows pointing on sarcomere structures. Image taken with 10X magnification.*

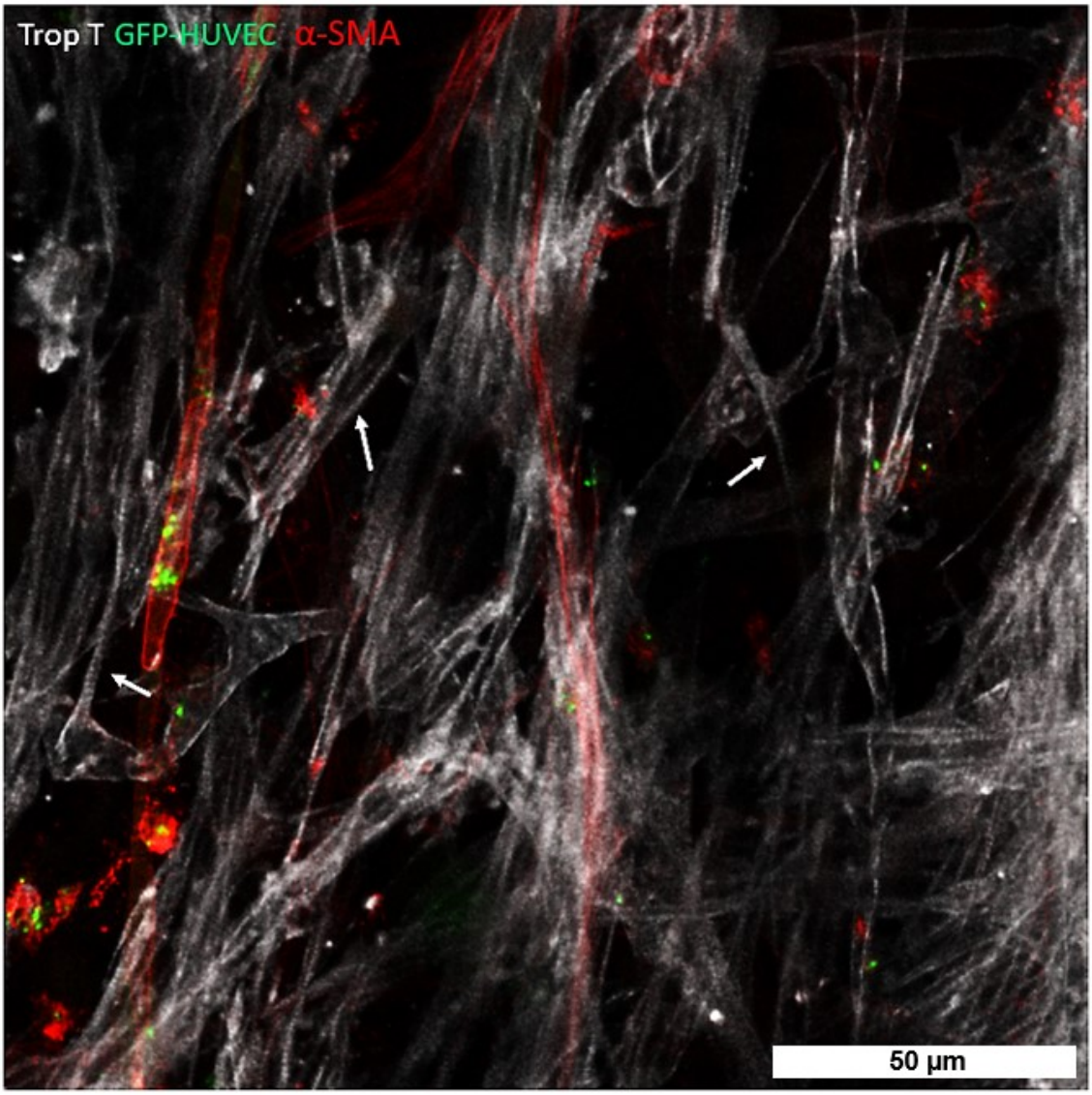

Figure 15. *Microstructures of hiPSC-CMs in hASC+hiPSC-CM culture at day 16, white arrows pointing on sarcomere structures. Image taken with 10X magnification*

6. DISCUSSION

Understanding of mechanisms regulating angiogenesis and interaction with cardiac cells may lead to novel therapies for ischemic disorders, including myocardial ischemia. The clear benefits of this model are the usage of human-derived cells and xeno-free biomaterial, and enduring 3D environment provided by the biomaterial. The 3D cardiovascular construct modelling myocardial ischemia was established based on previous studies of Vuorenpää et al., 2014 and Vuorenpää et al., 2017. Previously developed vascular structures and hiPSC-CM cultures were shifted to 3D environment, which more accurately mimics physiological tissue conditions. The model composes of HUVECs, hASCs and hiPSC-CMs in hydrazone crosslinked gelatin-gellan gum hydrogel, and the cells stayed viable throughout culture period, with culture time extending to 16 days for improved differentiation and maturation. Tubule formation, cellular morphology, orientation, and gene expression status were characterized after 8 to 16 days of co-culture, showing improved morphology and orientation of the cells in co-cultures.

Both HUVEC+hASC and hASC cultures support the maturation of hiPSC-CMs. Confocal images show morphology towards native tissue cardiomyocytes in the co-cultures when compared to hiPSC-CM monocultures. The hiPSC-CMs are elongated in the co-cultures and resemble more native cardiomyocytes than the globular and aggregated cells observed in hiPSC-CM monocultures. Additionally, hiPSC-CMs show alignment with HUVECs and hASCs. Especially close hiPSC-CM alignment with smooth muscle actin network formed by hASCs is observed from confocal images, with hASCs migrating towards hiPSC-CM network and both cell types aligning parallel, suggesting that there is crosstalk between the cell types. Concerning microstructures of cardiomyocytes, sarcomere structures of hiPSC-CMs can be observed from confocal images. The existence of sarcomere structures in cells is a proof of cardiomyocyte identity of hiPSC-CM, and a proof of maturation state. Biological matrix mimicking ECM enables the alignment of sarcomeres and organization of myofibrils of hiPSC-CMs in uniform direction, resembling microstructures in native cardiomyocytes (Bedada et al., 2016). Similar alignment and microstructures of hiPSC-CMs has been recently obtained using microfluidic platform by Veldhuizen & Nikkhah, 2021, however, the hiPSC-CM network cannot extend as vastly in microfluidic platform than within this construct.

The formation of vascular structures is relatively efficient in co-culture conditions. The gelatin-gellan gum hydrogel supports formation not only vasculature but of alpha smooth muscle-positive cell network formed by hASCs. However, gelatin-gellan gum hydrogel is

not optimal biomaterial for the formation and maturation of vascular structures by endothelial and adipose stromal/stem cells. As the cells migrate and proliferate forming tubule structures, the hydrogel tends to stretch, and eventually undesirable cavities can be formed in the hydrogel. In the vascular structures, the cells migrate from the middle of the gel to the outer lines of the wells, possibly seeking adhesion support from the well plate wall. As the cells migrate, the gel thins in the middle of the well. The gelatin-gellan gum hydrogel itself can withstand physical and shear stress relatively well, as observed from cell cultures with hiPSC-CMs plated on top of the hydrogel, and as previously proven (Koivisto et al., 2019). Therefore, a biomaterial with increased stiffness and viscoelastic properties could enhance proliferation and maturation of HUVECs and hASCs in the co-culture. In addition, culture period could it be expanded with more suitable hydrogel and consequently the maturation state of the cells could be improved.

Expression status of qPCR markers show relatively lower gene expression of cells and thus maturation in co-cultures when compared to hiPSC-CM monocultures and angiogenesis control. VEGF-A of the vascular gene markers show higher expression in the 3D cardiovascular model cultured in EB5% medium, but rest of the vascular markers show best expression in angiogenesis control, as well as cardiac TNNT2 marker in hiPSC-CM monoculture. Vascular gene markers show similar tendency in previous study by Vuorenpää et al., 2017, but the relative gene expression of TNNT2 was higher in co-culture with HUVECs and fibroblasts. The qPCR results could be explained with uneven number of cells between cultures. Although the cell number in the beginning is identical within all the cell cultures, proliferation status of the cells varies greatly when visually observed. When visually observed, especially the number of hiPSC-CMs seems to reduce when cultured with vascular structure, however the hiPSC-CM cells seem more mature in morphology when confocal images are observed. In addition, cell lysis and RNA isolation from cell cultures are challenging due to thick protein-rich biomaterial surrounding cells.

Medium composition does not seem to have a major effect on maturation of vascular structures and hiPSC-CMs in the co-cultures. In detail, vascular structure composing of HUVECs and hASCs and hiPSC-CMs seem to maturate better in EB5% medium. However, co-culture conditions with various cell types and medium compositions are a compromise between optimal conditions for angiogenesis and maturation of hiPSC-CMs in different time points.

This 3D cardiovascular model shows potential towards modelling myocardial ischemia. Combined model of angiogenesis and hiPSC-derived cardiomyocytes and the interaction between distinct cell types replicates the condition in myocardium after an injury or a stroke. Vascular structure composed of human umbilical vein endothelial cells

and human adipose stem/stromal cells support the maturation of hiPSC-CMs and vice versa, through biochemical signalling (Braile et al., 2020). Biochemical insults could be introduced to the co-culture to model the effect of hypoxia or drug molecules. A shift to microfluidic platform could enhance the maturation of vascular structures when interstitial flow of medium is introduced to the culture (Mykuliak et al., 2022). With appropriate biomaterial and microfluidic platform, the efficacy and accuracy of the model could be further improved.

7. CONCLUSION

Vascular structures form moderately in 3D co-culture with hiPSC-CMs. Also, hiPSC-CMs maturate in 3D co-culture, as proven in confocal imaging and qPCR results. Cultured hiPSC-CMs show elevated morphology towards morphology of native cardiomyocytes when cultured with hASCs and HUVECs or with hASCs alone in comparison with cardiomyocyte monoculture. Culture conditions face compromise concerning the optimal medium for angiogenesis and cardiac maturation, however, both hiPSC-CMs and vascular structures proliferate and maturate relatively well in both media compositions. Functional and morphological features of hiPSC-CMs in co-culture with HUVECs and hASCs, or hASCs alone, propose an interest for future research and for organ/body-on-chip research.

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