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FUNCTIONALITY AND MORPHOLOGY OF IPSC-DERIVED CARDIOMYOCYTES IN MULTICELLULAR 3D CARDIOVASCULAR CONSTRUCT
Towards 3D *in vitro* model of human myocardium

ABSTRACT

Cardiovascular diseases are major death leading cause in the world, and with aging people the socio-economic burden caused by these diseases exponentially grows. In addition, cardiotoxicity is most common reason behind drug withdrawal from the market, as toxicity is usually detected after longer clinical use. Animal models can't truthfully recapitulate human pathophysiology, therefore the results obtained are not often accurate. Also, they are hampered by ethical issues. There is unmet need for *in vitro* models of human heart , that could be used for modeling cardiovascular diseases, for drug development and for cardiotoxicity screening. The main aim of this work is to create functional multicellular three-dimensional model of human myocardium, that could be used for modeling cardiovascular diseases. The project is part of Center of Excellence in Body-on-Chip research.

In this project, induced pluripotent stem cells -derived cardiomyocytes (iPSC-CM) were cultured together with human umbilical vein endothelial cells (HUVEC) and human adipose stem cells (hASCs) to form 3D cardiovascular construct, supported with gelatin-gellan gum hydrogel. HUVECs and hASCs formed vascular structures in the model, as well as were supporting iPSC-CMs maturation, functionality, and viability in the construct. Additionally, also construct composed of hASCs and iPSC-CMs was built, to see if hASCs could be used alone as supportive cell type in co-culture. Cells were co-cultured together for 9 days, in two different culture mediums. During the co-culture, iPSC-CMs beating was video imaged in two separate days, to investigate if there were any changes in cells function in different environments. CellVisus software was used to transform the data into quantitative form. In addition, cells were characterized with immunocytochemical staining and confocal imaging.

The results showed that iPSC-CMs were functional and viable in the co-culture. The used culture media did not have effect on the cells' viability, nor function. However, the beating frequency was higher in co-culture and iPSC-CMs showed transformed and more mature morphology. The shape was more elongated, iPSC-CMs were aligned and attached to each other, and organized and elongated sarcomere structures could be seen in co-culture. In addition, similar results were gotten from hASCs and iPSC-CMs co-culture, showing that hASCs function well as supporting cells in the construct.

Cardiomyocytes showed typical characteristics when cultured in 3D environment together with HUVECs and hASCs, thus this model provide potential platform for cardiovascular disease research and development in the future.

Keywords: iPSC-CMs, 3D *in vitro* model, co-culture, functionality, beating analysis, human adipose stromal cells, endothelial cells

TIIVISTELMÄ

Maailmanlaajuisesti sydänsairaudet ovat yksittäinen suurin kuolemaan johtava ryhmä sairauksia. Väestön ikääntyessä, terveydenhuollon taakka kasvaa. Kardiotoksisuus on yleisin syy lääkkeiden poisvetämiseen markkinoilta, sillä lääkeaineen kardiotoksisuus usein havaitaan vasta kliinisessä käytössä pidemmän ajan jälkeen. Eläinmallit eivät totuudenmukaisesti kuvaa ihmisen fysiologiaa, ja eläinkokeista saadut tulokset ovat usein epätarkkoja. Lisäksi niitä varjostavat eettiset ongelmat. Tarve ihmisen sydänkudosta mallintaville kudosmalleille on suuri, jotta kardiovaskulaarisia sairauksia ja niiden mekanismeja voidaan luotettavasti tutkia. Kudosmalleja tarvitaan myös lääkekehityksen tarpeisiin, seulomaan potentiaalisia yhdisteitä prekliinisissä vaiheissa, nopeuttamaan lääkkeiden kehitystä ja vapautusta markkinoille. Tämän työn tarkoituksena on kehittää toiminnallinen, eri solutyypeistä koostuva, kolmiulotteinen malli jäljittelemään ihmisen sydänlihaskudosta, jota voitaisiin hyödyntää sydänsairauksien mallintamiseen. Työ on toteutettu osana Monikudosmallintamisen Huippuyksikön tutkimusta.

Tässä työssä, indusoiduista pluripotenteista kantasoluista erilaistettuja kardiomyosyyttejä viljeltiin yhdessä ihmisen napanuoran laskimosta peräisin olevien endoteelisolujen, sekä rasvakudoksesta peräisin olevien kantasolujen kanssa, tavoitteena luoda kolmiulotteinen malli sydänlihaskudoksesta. Kolmiulotteinen ympäristö luotiin gelatiinista ja gellaanikumista peräisin olevaan hydrogeeliin. Endoteelisolujen ja rasvakudoksen kantasolujen tarkoitus yhteisviljelmässä on muodostaa verisuonimaisia rakenteita, ja tukea kardiomyosyyttien toiminnallisuutta ja kypsymistä. Kardiomyosyyttejä viljeltiin lisäksi myös pelkkien rasvan kantasolujen kanssa, jotta kantasolujen potentiaalia tukisoluina voitiin tutkia. Endoteelisolut, rasvakudoksen kantasolut sekä kardiomyosyytit olivat yhteisviljelmässä 9 päivää. Soluja viljeltiin kahdessa erityyppisessä kasvatusliuoksessa. Tänä aikana kardiomyosyyttien sykintää videoitiin kahtena erillisenä päivänä, jotta toiminnallisuutta eri ympäristöissä voitaisiin laskennallisesti tutkia. Videot prosessoitiin CellVisus ohjelmistolla. Lisäksi suoritettiin immunosytokemialliset värjäykset, ja soluja kuvattiin konfokaalisella mikroskoopilla rakenteiden tutkimiseksi.

Tulokset osoittivat, että kardiomyosyytit ovat eläviä ja toiminnallisia yhteisviljelmässä. Eri mediumien välillä ei havaittu eroja solujen elävyydessä tai toiminnassa. Sykintä oli voimakkaampaa yhteisviljelmissä, ja konfokaalikuvantaminen osoitti kardiomyosyyttien muuttunutta ja kypsynyttä rakennetta. Muoto oli pidentynyt, solut olivat linjautuneet ja kiinnittyneet toistensa kanssa, sekä järjestäytyneitä ja linjautuneita sarkomeerirakenteita voitiin havaita kardiomyosyyteissä yhteisviljelmässä. Samankaltaisia tuloksia saatiin myös yhteisviljelmästä, jossa oli vain rasvakudoksen kantasoluja sekä kardiomyosyyttejä. Rasvakudoksen kantasolut toimivat yksinään hyvin tukisoluina kardiomyosyyteille.

Kardiomyosyytit osoittivat niille tyypillisiä ominaisuuksia yhteisviljelmässä kolmiulotteisessa ympäristössä. Sydänkudosmallia voitaisiinkin mahdollisesti hyödyntää tulevaisuudessa alustana erilaisten sydänsairauksien tutkimiseen, ja lääkkeiden kehitykseen.

Avainsanat: kardiomyosyytti, indusoidut pluripotentit kantasolut, yhteisviljelmä, rasvan kantasolut, endoteelisolut, toiminnallisuus, sykintäanalyysi, kolmiulotteinen solumalli

PREFACE

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CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
BSA	bovine serum albumin
BPM	beats per minute
CDH	carbodihydrazide
CM	cardiomyocyte
CVD	cardiovascular diseases
GelMA	UV crosslinkable methacrylated gelatin
GG	gellan gum
DAPI	4'6-diamidino-2-phenylindole
EB	embryonic body
EBM	endothelial basal medium
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGM-2	endothelial cell growth media
ESC	embryonic stem cells
DMEM	Dulbecco's Modified Eagle Medium
F	female
FBS	fetal bovine serum
FGF	fibroblast growth factor
GFP	green fluorescence protein
Klf4	Kruppel-like factor 4
iPSC	induced pluripotent stem cell
HUVEC	human umbilical vein endothelial cell
hASC	human adipose stem cell
M	male
MACS	magnetic-activated cell sorting
MEM	Minimum Essential Medium
MMP	matrix metalloproteinase
MSC	mesenchymal stem cell
NEAA	non-essential amino acids
NDS	normal donkey serum
NO	nitric oxide
Oct3/4	octamer binding transcription factor ¾

PFA	paraformaldehyde
PBS	phosphate buffered saline
PDMS	polydimethylsiloxane
Sox2	sex determining region Y-box 2
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
RT	room temperature

1. INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of mortality worldwide. Every year, almost 18 million deaths occur globally due to different cardiovascular diseases, the most common one being myocardial infarction. The death rate is expected to increase during upcoming years, due to the aging people and the COVID-19 pandemic, which adverse effects on cardiovascular system provide extra burden to the emerging problem. (Basara *et al.*, 2022) After prolonged ischemia, tissue suffers of lack of oxygen, which leads into cell death. Once happened, cardiomyocytes loss is irrevocable, as they have minimal regenerative capabilities, which often is followed by scar tissue formation in the muscle, leading almost inevitably to heart failure. (Basara *et al.*, 2022) Currently available therapy options slow down the fibrosis and scar formation, and ease the symptoms, but don't repair the cardiac tissue itself. Therefore, currently the only completely disease curing treatment is organ transplant. (Parrotta *et al.*, 2019)

The major challenge in finding new treatment solutions for CVDs, has been the mismatch between pre-clinical findings and the final clinical outcome (Basara *et al.*, 2022). Traditionally used animal models are not seen as relevant as they used to, as they fail to recapitulate human cells' pathophysiology in addition to relatively high costs related to maintenance and ethical concerns. Differences between humans and rodents in e.g., beating frequency, heart size and ion channel protein expression, lead into results that often can't be adequately clinically translated. (Milani-Nejad and Janssen, 2014; Tani and Tohyama, 2022)

In addition to animal models, traditionally used two-dimensional (2D) cell culture models do not truthfully represent cells as in their natural complex three-dimensional (3D) environment, thus results obtained can be inaccurate. This discrepancy hampers the discovery of new drugs, as well as research and development of treatments for CVDs. (Langhans, 2018) Moreover, cardiotoxicity remains one of the most common reasons for drug withdrawal from the markets, as the prediction has been incomplete with available *in vitro* and animal models (Basara *et al.*, 2022). Novel solutions, for treating cardiovascular diseases and for developing new drugs needs to be discovered to overcome this problem.

Emerging field of induced pluripotent stem cells (iPSC), and cardiomyocytes (CMs) differentiated from them could be potential solution to this problem. It has been shown

that iPSC-CMs can recapitulate important functional and molecular properties of human cardiomyocytes and they have shown to have accurate response to drugs, thus they can be used for disease modelling, drug development and toxicity screening. Possibility to create patient specific cell lines provides change for understanding disease mechanisms better and for creating personalized treatments for individuals. (Tani and Tohyama, 2022)

However, there remains still one major obstacle that needs to be overcome before the fully potential of iPSC-CMs can be utilized, that being relatively immature phenotype of the differentiated cells as CMs derived from iPSCs resemble more fetal than adult cell type. Maturation has been shown to be improved, when CMs have been cultured in 3D environment, with different biochemical cues and co-culturing them with other cell types. This provides not only more clinically relevant results, but more complex tissue models that can recapitulate the myocardial environment with cell-cell and cell-extracellular matrix (ECM) crosstalk better *in vitro.* (Tani and Tohyama, 2022)

Solutions to improve cardiotoxicity testing and shift from animal testing to *in vitro* models, is needed. Furthermore, there is requirement for easily constructable platform for drug development, and disease modelling. This thesis project aims to develop 3D cardiovascular construct, where iPSC-CMs are co-cultured with vascular structures forming endothelial cells and human adipose stem/stromal cells (hASCs). In addition, construct containing only hASCs and iPSC-CMs was built, to see if hASCs could be used alone as supportive cell type in co-culture. The model could potentially be used for cardiovascular diseases research, drug development and cardiotoxicity screening. In this project, previous work of Vuorenpää *et al.,* (2014,2017) is continued, and previously established cardiovascular model is transferred to 3D hydrogel environment. iPSC-CMs functionality is characterised by beating analysis with video microscopy and morphology changes are studied with confocal imaging.

2. LITERATURE REVIEW

2.1 Cardiac muscle tissue

2.1.1 Cardiomyocytes

Cardiac myocytes are the cells responsible for the continuous beating of the heart muscle tissue throughout the whole human life. The heart is the first organ to form in embryo and during development cardiac muscle cells differentiate into mature CMs that are connected to each other by gap-junctions to allow the synchronized contraction. (Woodcock and Matkovich, 2005) Mature CMs contain two nuclei, organised sarcomere structures, t-tubules, cellular machine responsible of the beating, and aligned cells to form the muscle tissue and allow contraction (Pagliarosi *et al.*, 2020; Tani and Tohyama, 2022). After birth there is a loss in CMs ability to proliferate, and myocardial growth is therefore mostly related to increasing the size of the CMs (Woodcock and Matkovich, 2005).

The contractile function of the heart relies in continuous fluctuation of intracellular Ca^{2+} controlled by ion channels. Cardiomyocytes' contraction is triggered by action potential, electrical stimulus that is created by specialized pacemaker cells located in sinoatrial node in the heart. Through gap junctions between CMs, the signal is then spread throughout the whole tissue by depolarization of the sarcolemma followed by action potential. (Woodcock and Matkovich, 2005; Gilbert *et al.*, 2020) The normal development and function of CMs is highly dependent on the crosstalk between cells around them, including endothelial cells and supportive cells (Perbellini *et al.*, 2018).

2.1.2 Endothelial cells

Cardiac muscle needs constant supply of oxygen, glucose, and other essential molecules due to high metabolic activity that continues throughout the whole human life. This is possible due to vascular and capillary network that spreads through heart muscle tissue and supplies blood to cardiac cells. (Segers, Brutsaert and De Keulenaer, 2018) In adult myocardium, every cardiomyocyte has at least one capillary contact (Perbellini *et al.*, 2018).

In addition to forming the microvasculature and supplying myocardium with blood, endothelial cells also affect CMs development and function by communicating with nearby cells by exchanging growth factors, extracellular vesicles, proteins, peptides, and

other small molecules. This cross-talk is essential for CMs normal development, growth and function. (Brutsaert, 2003; Colliva *et al.*, 2020) For example, nitric oxide (NO) secreted by endocardial endothelium modulates contraction of CMs (Brutsaert, 2003). This communication also occurs other way around and CMs too secrete growth factors affecting endothelial cells and control angiogenesis. During development, CMs secrete vascular endothelial growth factor (VEGF) which mostly affects the formation of cardiac microvasculature. (Díaz-Trelles *et al.*, 2016; Colliva *et al.*, 2020)

2.1.3 Pericytes, smooth muscle cells and fibroblasts

In addition to endothelial cells that form the inner layer of vascular tubes, blood vessels contain also mural cells that surround the endothelial tubes. Mural cells are further classified to pericytes and vascular smooth muscle cells (VSMCs) that are associated with capillaries and, arteries and veins, respectively. (Alex *et al.*, 2022)

Pericytes encircle capillaries and they are responsible for regulating perfusion and vascular permeability. Together with endothelial cells, pericytes can regulate blood flow in the heart. (Avolio and Madeddu, 2016; Lee, Khakoo and Chintalgattu, 2021) In addition, pericytes can also have a role in regulation of angiogenesis, maturation and stability of vascular structures and inflammatory responses. (Avolio and Madeddu, 2016; Alex *et al.*, 2022)

A layer of smooth muscle cells forms the majority of the vascular wall in bigger blood vessels. They are responsible for regulating blood pressure by being highly sensitive for mechanical and molecular cues. VSMCs also produce extracellular matrix during development and are involved in repair processes after injuries in adult life. (Lacolley *et al.*, 2012)

Cardiac fibroblasts are the main cell type in the heart that are responsible for producing majority of the extracellular matrix (ECM) proteins and therefore providing structural support for the beating CMs (Hall *et al.*, 2021). In addition to producing ECM proteins, fibroblasts control the composition of the ECM by regulating matrix metalloproteinases (MMPs) and their inhibitors that are responsible for degradation of collagen, which is the main component of the cardiac ECM (Hall *et al.*, 2021).

2.1.4 Extracellular matrix

Extracellular matrix plays crucial role in cardiac development and in maintaining normal function of cardiac cells in adult myocardium (Pagliarosi *et al.*, 2020). The ECM is composed of glycoproteins, proteoglycans, cytokines and growth factors and forms 3D environment that enable connection of cell structures and provides support for all the cell

populations in the myocardium (Baudino *et al.*, 2006). The ECM in myocardium is rigid and stable, since the beating CMs are anchored to it, however it is also elastic to allow the constant movement. The ECM in myocardium is mostly composed of collagens, in addition there are also laminin, fibronectin, fibrillin and elastin. (Schwach and Passier, 2019) ECM has significant role in distributing mechanical force and propagating impulse trough myocardium and transmitting mechanical signals to individual cells (Baudino *et al.*, 2006; Schwach and Passier, 2019). Three dimensional organization of the ECM components significantly affect alignment of the CMs, blood flow and stiffness of the tissue (Baudino *et al.*, 2006). In addition, ECM proteins are involved in regulation of many growth factors such as VEGFs and fibroblast growth factors (FGFs) that are important for maintaining homeostasis in myocardium. By signaling, ECM has effects on cardiac cells migration, adhesion, and differentiation. It also has a role in regulating angiogenesis in the tissue. (Kim *et al.*, 1999; Pagliarosi *et al.*, 2020) The composition of the ECM is remodeled throughout human life, from embryonic state to adulthood. These changes are mostly related to maturation of the tissue, regarding increase of stiffness of the muscle tissue. (Pagliarosi *et al.*, 2020) The highly organized structure of human myocardium, is shown in Figure 1.
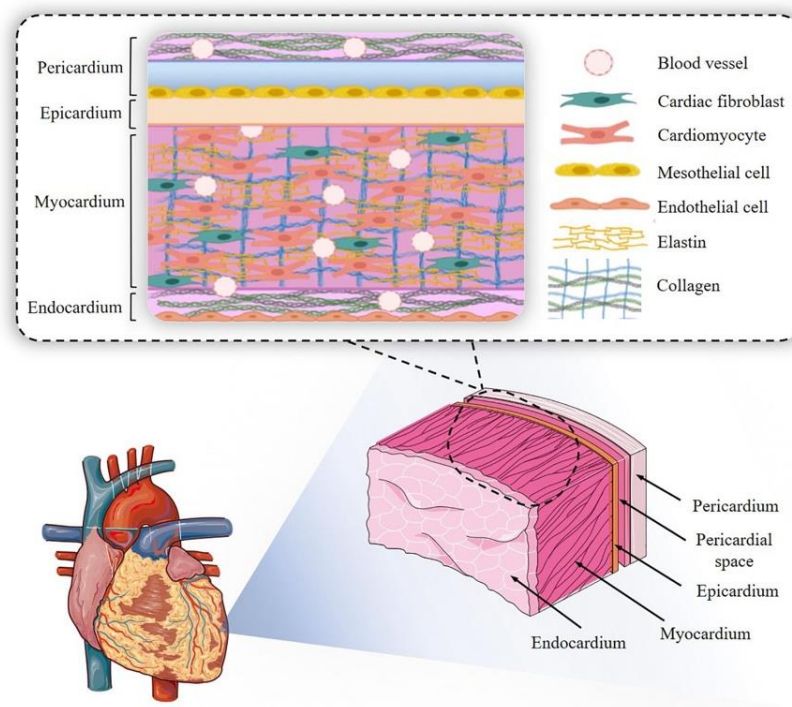
Figure 1. *Organized structure of human myocardium. Image modified from* Montero *et al.*, 2020*.*

2.2 Stem cells as cell source for cardiac research and development

Stem cells have been used for cardiac research for decades and the first attempt of pre-clinical trials of stem cell therapy was done already 20 years ago when bone marrow stem cells were injected to the infracted heart of mice (Orlic *et al.*, 2001). With novel stem cells techniques, researchers have been able to overcome the obstacles related to primary cardiac cells, which are challenging to get from heart tissue, and keep alive in cell culture conditions to do any examination nor develop complex *in vitro* models (King *et al.*, 2022). Stem cells have proved themselves to be a great tool for understanding the molecular mechanisms behind many cardiovascular diseases and to develop novel solutions for therapeutics (Samak and Hinkel, 2019).

The different stem cells that have been intensively studied during the years have been adult stem cells, mesenchymal stem cells (MSCs), embryonic stem cells (ESC) and finally, induced pluripotent stem cells (iPSC). Great efforts to find potential from adult stem cells, including cardiac stem cells, have been shown during last decades, however unfortunately, adult stem cells have not been shown to be potentially trustworthy treatment option for cardiovascular diseases, at least not yet. (Samak and Hinkel, 2019) MSCs have been under research for half of a century (Bianco, Robey and Simmons, 2008) and their impact for cardiac research and for this project are reviewed later in this text.

Pluripotent stem cells have been shown to have a more possibilities to answer to the need of reliable source of native-resembling CMs. Pluripotent embryonic stem cells (ESC) were firstly generated already in 1998 by Thomson et al. (Thomson, 1998) and these cells can be nowadays easily differentiated into functional CMs and therefore they provide really good source for variety of research applications, drug screening, regenerative medicine, and cell therapy. (Rikhtegar *et al.*, 2019) Compared to human embryonic stem cells, iPSCs provide major advantages over them. Using iPS cells overcomes the significant ethical issues related to the usage of ESC and their use as cell therapy will not cause risk of unwanted immune response. (Parrotta *et al.*, 2019)

In next two chapters, iPS cells and mesenchymal stem cells are discussed further on the point of view of this thesis project.

2.2.1 Induced pluripotent stem cells as cardiomyocyte source

The stem cell research world was revolutionized in 2006 when Takahashi and Yamanaka (Takahashi and Yamanaka, 2006) first introduced induced pluripotent stem

cells, stem cells with similar properties to embryonic stem cells regarding morphology, proliferation, and gene expression, to name a few. They reprogrammed successfully mouse somatic cells into pluripotent state with using four transcription factors, called Yamanaka factors, which are c-Myc, octamer binding transcription factor 3/4 (Oct3/4), sex determining region Y-box 2 (Sox2) and Kruppel-like factor 4 (Klf4). One year later, the same four factors were used to reprogram human fibroblasts into iPS cells that resembled human embryonic stem cells by their characteristics and had ability to differentiate into three different germ layer cell types. (Takahashi *et al.*, 2007)

First cardiac differentiation from murine iPS cells was reported in 2008 from two groups simultaneously (Mauritz *et al.*, 2008; Narazaki *et al.*, 2008) and during the past decade, iPS cells have been turned out to be good source of CMs for research and development. They have demonstrated similar characteristics as native CMs with spontaneous beating and contractility (Zhang *et al.*, 2009). Furthermore, it has been shown that CMs differentiated from iPSCs truthfully express genes that are specific for CMs; sarcomere proteins coding genes (e.g., cardiac troponin, alfa and beta-myosin heavy chains), ion channel proteins (calcium and sodium channels) and cardiac specific transcription factors (Khan, Lyon and Harding, 2013). In addition, accurate drug response have been demonstrated with iPSC- derived CMs, from tens of different drugs, that have previously known effect on human heart (Dick *et al.*, 2010).

Moreover, iPSC derived CMs that are differentiated from cells of affected donors have great potential to recapitulate truthfully functional and molecular aspects of the heart diseases. Potentially they could show and detect dysfunctions and arrhythmias as well as other irregularities and therefore could be applied to drug development processes in cardiotoxicity testing and to clinical trials. (Tani and Tohyama, 2022) CMs differentiated from iPSCs maintain the patient specific genomic and phenotype features through the differentiation process and therefore provide really good opportunities for disease modelling, cardiotoxicity testing and personal treatments development of cardiovascular diseases (Khan, Lyon and Harding, 2013; Tani and Tohyama, 2022).

Despite of the great future prospects of iPSC-derived CMs, there is still few major obstacles that needs to be unraveled, before their fully potential can be utilized (Khan, Lyon and Harding, 2013). The differentiated cells resemble their prenatal form, rather than adult phenotype and it can be seen in morphology, gene expression and functionality. Immature CMs are round shaped, they lack t-tubules and other organized structures related to organized contractions, and due to differences in ion channels, they have different action potential leading to different electrophysiology. (Tani and Tohyama, 2022) Immature and mature CMs and existing methods to improve maturation are presented in Figure 2.
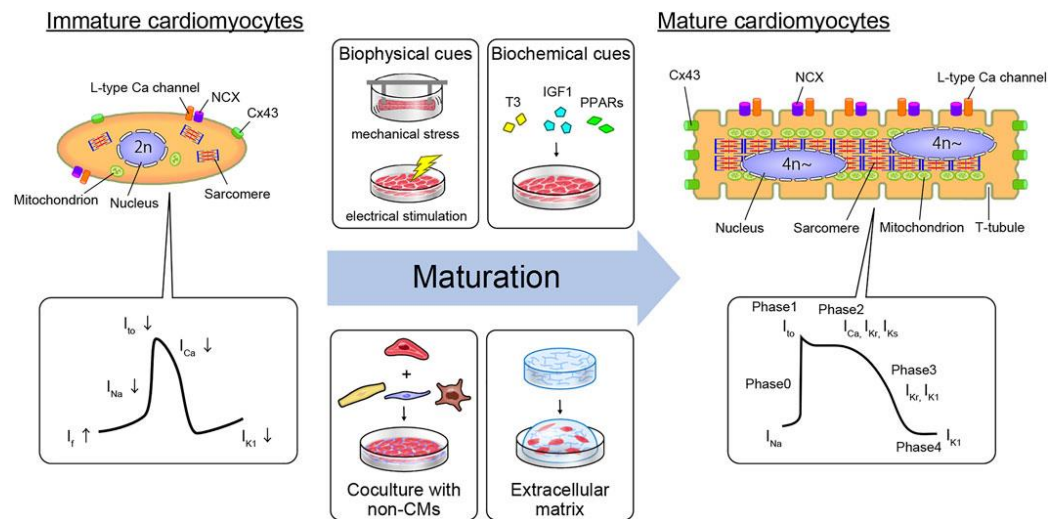
Figure 2. *Differences between immature and mature CM. The maturation state affects in the action potential of the cells. Maturation can be enhanced with different biophysical and -chemical cues, co-culturing CMs with non-myocytes and culturing cells in 3D environment. (Image modified from:* (Tani and Tohyama, 2022)

2.2.2 Mesenchymal stem cells

After first report of findings in the 1970s, mesenchymal stem cells have been widely studied for regenerative and healing purposes for damaged tissues and organs (Marion and Mao, 2006). The research has truly prevalently spread, and in addition to research in lab, MSCs have been used in hundreds of clinical trials during last two decades in attempts to find cure for conditions such as infarcts and immune system disorders (Gomez-Salazar *et al.*, 2020). MSCs are relatively easy to get access, they can be easily harvested from several different tissues, such as subcutaneous adipose tissue and umbilical cord, and they can be passaged several times without losing their stem cell capabilities. It has been shown, that MSCs are capable of differentiating into cells resembling tissue specific cell types, including endothelial cells and CMs. (Marion and Mao, 2006)

Rather than differentiating into other cell types and providing regenerative potential that way, MSCs are nowadays thought to act through their immunomodulate properties and by secreting growth factors, cytokines and extracellular vesicles to enhance surrounding cells' and tissue's well-being (Gomez-Salazar *et al.*, 2020). For example, MSCs promote angiogenesis by paracrine signaling, which has been reported both *in vitro* and *in vivo* experiments (Mabotuwana *et al.*, 2022). In co-cultures together with iPSC-CMs and endothelial cells, it has been shown that MSCs can decrease apoptosis, and improve proliferation and formation of vascular structures in hypoxic conditions. This

has also been shown in ischemic hearts, where injection of MSCs enhanced angiogenesis and thus protected the tissue from the ischemic injury. (Mabotuwana *et al.*, 2022)

2.3 Modelling cardiac tissue *in vitro*

Improvements during recent decades in stem cell technology, in addition to tissue engineering and microfluidics have made possible to develop more native like tissue models from which more clinically relevant results could be obtained (Basara *et al.*, 2022). Ideally, the components in cardiac tissue models should have similar characteristics with the building blocks that human heart naturally is comprised of, including cells, ECM, and biophysical properties, as described in Figure 3 below.
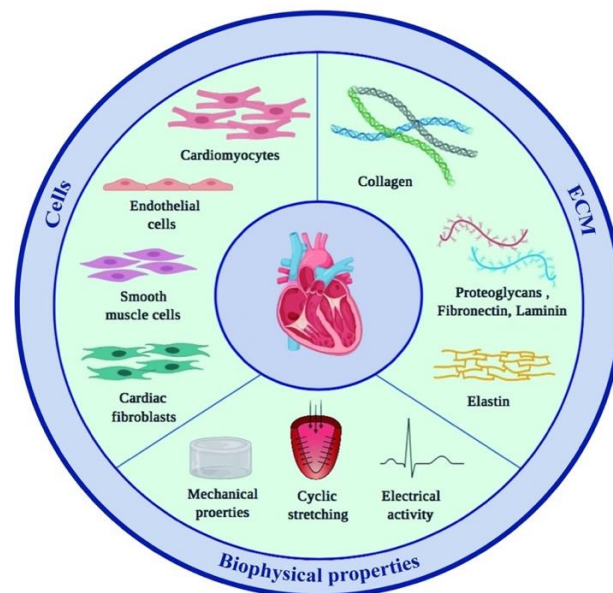
Figure 3. *When designing in vitro models, three aspects that should be considered carefully are cells, biophysical properties, and ECM. (Image modified from* Montero *et al.*, 2020*)*

In the following chapters, some of the approaches to achieve native tissue like environment are discussed.

2.3.1 2D *in vitro* models

Traditional two dimensional (2D) cell culture models are still very much used in cardiac research, for instance in studies of ischemia and perfusion (Sebastião *et al.*, 2019; Häkli *et al.*, 2021), as there are long history and standardized protocols with them. 2D culture systems are good when particular mechanism in specific conditions is needed to study

as they allow characterization of the cells better, when compared to more complex 3D culture systems (Basara *et al.*, 2022). Furthermore, when cell lines needs to be expanded 2D cultures are optimal choice. 2D cultures are well established and there are lot of literature to compare results with, they are easy to maintain and changes to the test set-up can be made, not to mention cost effectiveness. (Zuppinger, 2019; Basara *et al.*, 2022) Therefore, they have made their way up to the standard culture model in the field over the years (Zuppinger, 2019).

However, in human heart, there is constant stream of mechanical, biochemical, and electrical cues which affect normal cell function, growth and survival (Tani and Tohyama, 2022). This dynamic environment can't be modelled in 2D conditions truthfully, in a way that research results could be adequately clinically translated (Zuppinger, 2019; Basara *et al.*, 2022). In addition iPSC -derived CMs cultured in 2D environment represent immature state with lacking t-tubule structures and gene expression similar to fetal type (de Lange *et al.*, 2021). With 3D models, the gap between 2D models and human tissue could be enclosed, and truly clinically relevant results could be obtained (Beauchamp *et al.*, 2015).

2.3.2 Benefits of using 3D cell culture

As previously described, one of the biggest obstacle in developing clinically relevant tissue models is the lack of maturation of the iPSC-CMs. In order to fully release the potential of iPSC derived CMs, the issue should be eventually overcome. With 3D culture systems, more tissue like environment with appropriate biomechanical cues can be created. (King *et al.*, 2022) It has been shown that the maturation of the CMs could be enhanced in 3D environment, with help of different pharmacological, nutritional, and electromechanical cues. Thus more clinically relevant results could be obtained (Fleischer *et al.*, 2019; de Lange *et al.*, 2021)

Commonly used methods to create 3D environment for cardiac *in vitro* tissue models include cardiac spheroids and organoids, bioprinting, different scaffolds, and microfluidic systems (Basara *et al.*, 2022). With these novel 3D technologies, tissue models have demonstrated qualities that are comparable to human myocardium, thus could be used for platform for early drug safety testing and disease research (de Lange et al. 2019). These qualities include for instance enhanced cardiac specific protein expression, developing t-tubular structures and accurate responsiveness to physiological stimuli (Fong *et al.*, 2016; Ellis *et al.*, 2017; Fleischer *et al.*, 2019; de Lange *et al.*, 2021). In the table 1 some recent 3D cardiac models are represented in more detail.

Table 1. *Recent iPSC-derived 3D cardiac models. (UV crosslinkable methacrylated gelatin = GelMA, polydimethylsiloxane = PDMS, MOC = myocardium on chip, EC = endothelial cell)*

Model	Cells used	Platform	Biomaterial/ scaffold	Main results	Reference
iPSC - derived engineered cardiac construct	iPSC - derived CMs and iPSC - derived cardiac fibroblasts	20 × 3-mm cylindrical mold of FlexCell Tissue Train culture plate	Fibrinogen and thrombin mixture, to which cells were mixed to	Improved CM maturation T-tubules presence Improved Ca^{2+} handling	De Lange *et al.*, 2019
iPSC - derived myocardium-on-chip (MOC)	iPSC - derived CMs and iPSC - derived endothelial cells, from same iPSC donor cell line	MOC - three channel microfluidic device.	CMs in GelMA, ECs on top of fibronectin coating	CMs retained ability to beat in MOC ECs aligned with the flow, forming tube-like structures to CM channel	Ellis *et al.*, 2017
iPSC - derived CM spheroids in long term culture	iPSC - derived CMs	Long term culturing in U-bottomed 96-well plate	Cell spheroid in culture media	Long term (+100 days) stable 3D culture Improved maturation in 3D	Fleischer *et al.*, 2018
iPSC - derived CMs in hydrogel	iPSC - derived CMs, donor cell line	PDMS retention rings, 8mm ∅	Hydrogel made from adult bovine ECM, fibrinogen and thrombin to which cells were mixed to	Enhanced expression of maturation related genes Improved Ca^{2+} handling Clearer responsiveness to drugs	Fong *et al.*, 2016

Despite of all the great efforts made towards 3D culture systems, there still remains universal challenges that should be overcome to fully release their potential. Firstly, when compared to 2D systems, the lack of standardized models and protocols, that would allow automated high-throughput processes, is distinct. The results obtained from 3D constructs are more relevant when compared to ones from 2D, however, the models tend to be more unique solutions developed by each group individually, rather than mass

production. Models can't be easily reproduced, and traditional assay methods can't be sometimes used with 3D models. (Langhans, 2018) Secondly, the visualization of the cells embedded in sometimes complex 3D structures is challenging, due to limitations related to imaging devices. Cells and their detailed protein structures located in the middle of the structures are hard to capture with current imaging systems. (Langhans, 2018) Thirdly, improvements towards long-term cultures need to be achieved before *in vitro* models can be fully utilized for long term cardiotoxicity and repeated dose studies of candidate drugs (de Lange *et al.*, 2021).

In next chapters there are further discussion about the role of hydrogels, relevance of co-culturing in developing these models and the importance of added vascularization in developing novel cardiac tissue models.

2.3.3   Hydrogels for 3D environment in cardiac *in vitro* models

As previously described, ECM plays important role in normal cell function in myocardium, during the development and in adulthood. Cardiac function is depending on seamless function of cells embedded in extracellular matrix that provides strong support and platform for cells to adhere, but allows elastic movement coming from the beating cells. (Baudino *et al.*, 2006)   The biomaterial that should mimic the native myocardium ECM *in vitro* needs to be considered carefully, as the environment plays big role in regulating cell behavior (Basara *et al.*, 2022).

One scaffold-based way to construct 3D environment for modelling myocardium *in vitro*, is with hydrogels which are water-insoluble biomaterial, composed of cross-linked polymers with high water content (Li and Guan, 2011). Hydrogels can be of biological origin, completely synthetic or hybrid with mixture of both (Langhans, 2018). Often used biomaterials to compose the hydrogels used *in vitro* models are collagen, fibrinogen, gelatin and Matrigel, which are all biological origin (Basara *et al.*, 2022). They are biocompatible; thus, cells naturally easily adhere to them. In addition, when cultured in natural hydrogels, cells often can retain their natural specific functionality and resemble more their *in vivo* phenotype. With modifications and combining these natural origin hydrogels with synthetic ones, appropriate and native tissue resembling environment can be created. (Langhans, 2018)

Hydrogels have been shown to resemble needed qualities for mimicking native myocardium environment accurately, therefore being suitable environment for CMs (Li and Guan, 2011). It has been shown, that when iPSC-CMs are cultured on hydrogel, they restore the ability to beat and stay viable during the culturing (Koivisto *et al.*, 2019).

As described earlier on Table 1 the structural and functional maturity can be improved when iPSC-CMs are cultured in hydrogels.

2.3.4 Relevance and possibilities of co-culturing cells

In addition to 3D systems, it has been noticed that by culturing pluripotent stem cells derived CMs together with other cell types, both non-cardiac and cardiac origin, improvements in cell functionality and maturity towards more native myocardial environment has been achieved (Ravenscroft *et al.*, 2016; Kahn-Krell *et al.*, 2022). In addition, when drug toxicity studies have been performed, more accurate responses have been obtained from co-cultures than from monocultures (Zuppinger, 2019). As previously discussed, the signaling between CMs, endothelial cells, stromal cells, and other tissue specific cells is necessary for normal tissue development and function, and tissue homeostasis maintaining. Endothelial cells provide CMs oxygen and nutrients, while stromal cells contribute by producing ECM components, and supporting signal transduction throughout the tissue. (Giacomelli *et al.*, 2020; Kahn-Krell *et al.*, 2022) In this light, it is clear that co-culture models are essential for capturing the complex multicellular environment with all relevant cell-cell crosstalk.

During recent years, very promising results from different co-cultures have been presented. Khan-Krell et al., recently introduced cardiac spheroids composed of one cell line iPSC-derived CMs, endothelial cells, smooth muscle cells and fibroblasts, that showed improved maturation and stable long-term viability, compared to only CM containing spheroids (Kahn-Krell *et al.*, 2022). Similar results were reported by Giacomelli et al in 2020, when they combined iPSC-derived CMs with cardiac endothelial cells and fibroblasts. CMs formed improved t-tubule structures in sarcomeres, and showed more mature-like electrophysiology, when compared to monocultures. (Giacomelli *et al.*, 2020) In addition, iPSC-CMs maturation can be improved by co-culturing them with mesenchymal stem cells. As described earlier, MSCs have been under intensive research for decades, and they have been shown to be advantageous when applied to *in vitro* models. Yoshida et al., demonstrated that when iPSC -derived CMs were cultured together with mesenchymal stem cells, iPSC-CMs structural and electrophysiological maturation was improved, and CMs cell-cell interactions were enhanced. (Yoshida *et al.*, 2018)

There are some obstacles that needs to be overcome before the full potential of these co-culture models in 3D environment can be released. Co-culturing cells in 3D environment provides some additional challenges to already described ones, related to the 3D models. Naturally, the preparation of biomaterial and individual cell types requires

lot of professional skills, equipment and is time consuming. These complex individual processes might limit the broad adaption of these models, and their high throughput usability. (Hofbauer, Jahnel and Mendjan, 2021) Unavoidable, the complexity of the models increases when more elements are added, which can cause difficulties in predicting and detecting cause-effect relations. In addition, some challenges can be faced when choosing the most appropriate environment for the different cell types, including for example culture medium, for which different cells have their own individual requirements. (Zuppinger, 2016; Vis, Ito and Hofmann, 2020)

2.3.5 Adding vascularization to cardiac tissue models

As discussed earlier, vasculature, formed by endothelial cells play essential role in maintaining normal cardiac functions, during development and in adulthood. In order to recapitulate vascular functions and CM-EC crosstalk in tissue models to obtain clinically relevant results, vasculature forming cells, i.e., endothelial cells should be included to the constructs, preferably together with supportive pericytes. (Osaki, Sivathanu and Kamm, 2018) Furthermore, adding vasculature to the tissue constructs is needed for gas and nutrients delivery and for waste removal that is otherwise handled with diffusion, and to mimic natural state of constant blood flow in myocardium (Osaki, Sivathanu and Kamm, 2018; Pollet and den Toonder, 2020).

The best characterized and widely used primary endothelial cells are human umbilical vein endothelial cells (HUVECs). They have been used for studies of angiogenesis and vasculature for decades, and wide range of protocols to isolate and maintain the cells have been developed since first successful isolation in the 70s, by Jaffe et al. (Jaffe *et al.*, 1973; Kocherova *et al.*, 2019).

Recent cardiac models where CMs have been co-cultured with vasculature and exciting results have been gotten. Vuorenpää et al., (Vuorenpää *et al.*, 2017) showed that culturing iPSC-derived CMs with human fibroblasts and HUVECs, that formed vascular structures, improved ion channel gene expression, as well as more mature structure of the CMs, when compared to monocultures. Cells also responded accordingly to adrenaline, in multicellular model. More recently Koivisto et al., (Koivisto *et al.*, 2022) demonstrated that iPSC-CMs cultured together with vascular structures originating from HUVEC and hASC co-culture (Sarkanen *et al.*, 2012), provided tissue model capable of predicting drug effects with high accuracy, when it was compared to human data. Also, in 2022 King et al. introduced 3D co-culture of iPSC-CMs, cardiac microvasculature endothelial cells and fibroblasts within a heart-on-a-chip. They showed beating

myocardium with perfusable microvasculature, and with live-imaging they were able to demonstrate flow of red blood cells through the vasculature. (King *et al.*, 2022)

3. OBJECTIVES

The main objective of this thesis is to build a multicellular 3D construct that can represent human cardiac muscle tissue, and that could be used to model e.g., cardiac ischemia. There are three main research questions that this thesis aims to answer:

1) How the medium composition affects the cells in co-culture? - Can differences be found between cells that were cultured in two different medium?
2) How the co-culturing affects the CMs morphology when compared to CM monoculture controls?
3) Can the CM functionality be enhanced by co-culturing?

4. MATERIALS AND METHODS

4.1 Cell lines

This study conforms to the principles outlined in the Declaration of Helsinki. Commercial cell line of green fluorescence protein (GFP) expressing human umbilical vein endothelial cells (HUVECs) (l.29715) (CellWorks) and donor based, anonymized human adipose stem cell (hASC) lines obtained from three donors were used for this project. The iPS cell line used for CMs was UTA.04602.WT. The use of the hASC and iPSC cell lines was approved by Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (approval numbers R15161 and R08070, respectively). Signed informed consent has been obtained from all the participants. More details of each cell line can be found in Table 3.

4.2 Setting up the vasculature for the construct 3D model

HUVECs and hASCs were thawed five days prior to setting up the base for the construct 3D model and CM+hASC (Table 2). Culture medias that were used in thawing of HUVECs and hASCs and also further on this project, were endothelial cell growth media (EGM-2) containing EBM™-2 Basal Medium (Lonza) and EGM™-2 SingleQuots™ Supplements, supplemented with 2% human serum (Serana), as well as Minimum Essential Medium (MEM) Alpha Medium (gibco) supplemented with 5% human serum (Serana) and 1% penicillin/streptomycin (Lonza), respectively.

Prior to the seeding of hASCs and HUVECs to the hydrogel, the cell amount was calculated with Bürker chamber. Cells were detached with Tryple (Lonza), centrifuged at 200 x g for 5 minutes, and resuspended in appropriate medium in volume of 4 ml. A sample was taken from each cell suspension and dilution of 1:10 was made with Trypan Blue (Biorad) to calculate the cell amount. The total amount of cells needed per well was 0,22 x 10^6 cells/ml for hASCs and 1,1 x 10^6 cells/ml for HUVECs, 1:5 ratio.

To provide 3D environment for the cells, hydrogel containing carbodihydrazide (CDH) -modified gelatin (60mg/ml in Dulbecco's Modified Eagle Medium (DMEM)) and gellan gum (GGox(60), 40mg/ml in DMEM) was used. More about the properties of the hydrogel are described in detail by Gering *et al.*, 2022. In each well, the volume of each gel component was 75µl, so that the final gelatin-GG hydrogel volume was 150µl.

The right amount of each cell suspension, based on the calculations, was mixed together and then centrifuged at 200 x g for 5 minutes. After removing the supernatant, the pellet containing both cells, was suspended to warm (37°C) gelatin. Additionally, suspension of gelatin and only hASCs was made for construct hASC + CM (Table 2). The volume of 75µl of the appropriate gelatin-cell suspension was then added to the bottom of the wells one at time and 75µl of warm gellan gum was added on top immediately after and mixed carefully, in order to get evenly mixed hydrogel. The hydrogel gelatinized approximately for one hour in +37°C and then cell culture media EGM-2 was added on top of each construct. The plate was kept in incubator, and culture media was changed three times per week. Depending on the endpoint of the experiment, the cell culture was done either in 48-well plate or in µ -Slide 8 Well [high] ibiTreat (ibidi) plate. Summary of different sample types and cells in each are presented in Table 2 and the timeline is described in Figure 5.

Table 2. *The different sample types and cells within each*

Sample	Cells
3D model	Human umbilical vein endothelial cells, human adipose stem/stromal cells, iPSC-CMs
hASC + CM	Human adipose stem/stromal cells, iPSC-CMs
Ang. ctrl	Human umbilical vein endothelial cells, human adipose stem/stromal cells
3D CM ctrl	iPSC-CMs on top of hydrogel
2D CM ctrl	iPSC-CMs on top of 0,1% gelatin coating

4.3 Magnetic-activated cell sorting and seeding of iPSC-CMs

EB-differentiated embryonic bodies (EBs), from patient specific cell line 04602.WT.UTA were magnetically sorted (MACS) before adding them to the construct. The EB differentiation protocol is described in detail previously by (Prajapati *et al.*, 2021). Cells were maintained according to normal procedures of the research group. In total, four 6-well plates containing differentiated cells were used for each experiment. Prior to the start of the protocol, MACS-buffer of phosphate buffered saline (PBS) containing 0,0370g ethylenediaminetetraacetic acid ((EDTA) Sigma) and 0,5% of 10% bovine serum albumin ((BSA) Sigma) in PBS was made.

EBs were first dissociated with Multi Tissue Dissociation Kit 3 (Miltenyi Biotec). Briefly, after combining cells from 6-well plates, cells were washed with PBS (Lonza) and enzyme mix containing Enzyme T (Miltenyi Biotec) and Buffer X (Miltenyi Biotec) in ratio 1:10 was made for appropriate amount; 1ml for each well containing cells. The cells were then incubated for 10 minutes at 37°C. After incubation, 1ml of KnockOut ™ DMEM (gibco), supplemented with 20% fetal bovine serum ((FBS) Lonza), Non-Essential Amino Acids ((NEAA) gibco), Glutamax (gibco) and PenStrep (Lonza) was added to the wells to stop the effects of the enzyme. Then, cells were gently detached by pipetting and collected to 15ml falcon tube. Finally, cells were filtered with separation filter (Miltenyi Biotec) and counted with Neubauer chamber.

Cell suspension was centrifuged at 200 x g for 5 minutes in 4°C and the supernatant was resuspended to 1:4 mixture of Non-CM depletion cocktail (Miltenyi Biotec) and MACS-buffer, of which volume was depending on the number of cells. If the cell number was less than 5 million, total volume of reagents was 100µl and in each 5 million cell - mark, the total volume was amplified by 100µl. After 5min incubation in 4°C, adding 1ml of MACS-buffer and centrifuging at 200 x g for 5 minutes in 4°C, solution of Anti-biotin Microbeads and MACS-buffer made by same manner than Non-CM depletion solution was added to the falcon tube and let incubate for 10 minutes in 4°C. After incubation, cells were rinsed through magnetic column. Since all the non-CMs were labeled and therefore would stick in the column, CMs could be collected from the fraction. The solution containing MACS-buffer and CMs were then centrifuged at 200 x g for 5 minutes in 4°C and the pellet was resuspended to DMEM/F-12 (1:1, gibco) supplemented with 5% FBS, (Lonza), NEAA (gibco), Glutamax (gibco) and PenStrep (Lonza). The cell amount was then calculated using Neubauer chamber. The needed number of CMs was 108 500 cells per each well.

The correct amount of cell suspension was added then on top of the hydrogels containing already seeded HUVECs and hASCs. In addition, same number of CMs were added on top of plain gelatin-GG hydrogel and on 0,1% gelatin coated wells, according to the plate map presented in Figure 4. Co-culture medias, EB 5% and 1:1 mixture of EB5% and EGM-2 was changed to cells as shown in the Figure 4. The media was then changed as previously, in every other day and the multicellular construct were maintained until day 16. The scheduling of the project is presented in Figure 5.
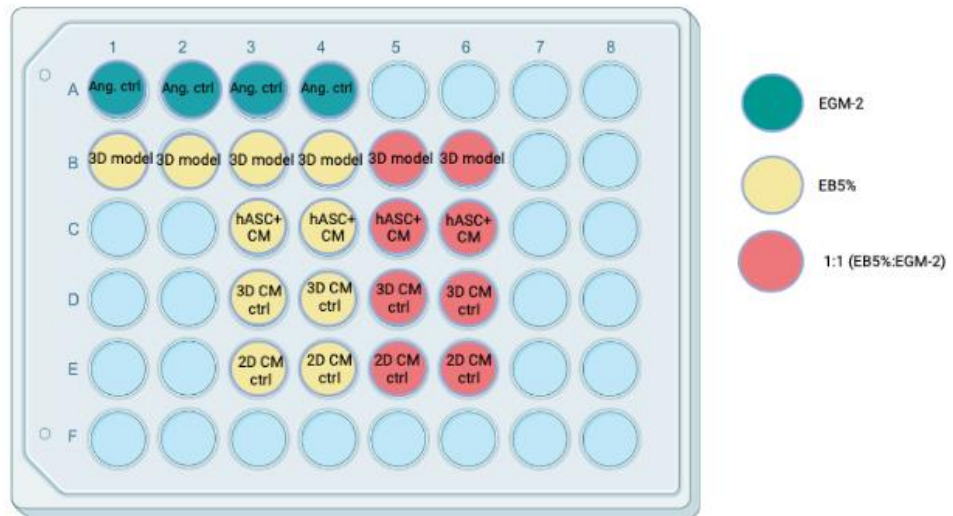
Figure 4. *Plate map. In each experiment there were two parallels of each medium composition within each sample type. Image created with Biorender.*
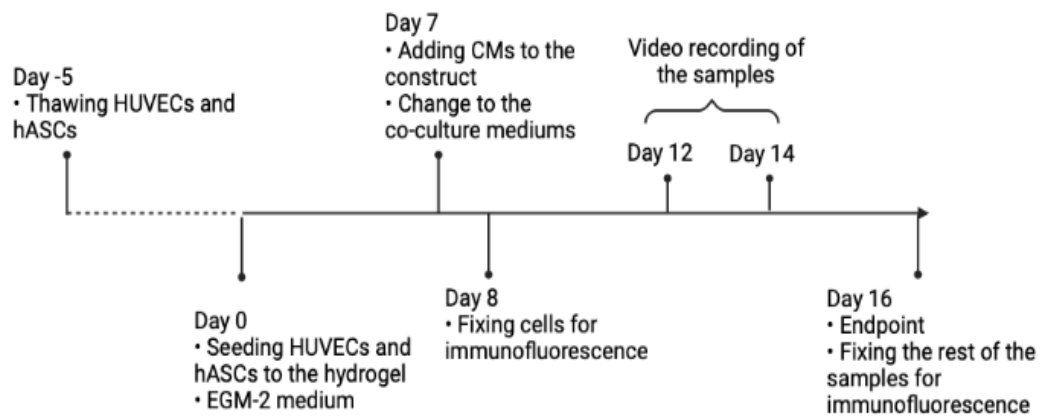
Figure 5. *Summarizing timeline of each experiment. From thawing HUVECs and hASCs to the endpoint, each experiment lasted for 21 days. Image created with Biorender.*

Used cell lines and platforms in each experiment are presented in Table 3.

Table 3. *Details of cell lines used in each experiment. p. = passage, M = male, F = female*

Experiment	HUVECs	hASCs	iPSC-CMs	Platform
	750 000 cells/	**150 000 cells/**	**108 500 cells/**	
1.	GFP-HUVEC (l.29715) p.4 & p.5	hASC 3/19 p.2, donor M, 56yrs, cell source subcutaneous	UTA.04602.WT HEB p.31	48-well plate
2.	GFP-HUVEC (l.29715) p.5	hASC 6/19 p.2, donor F, 33yrs, cell source subcutaneous	UTA.04602.WT HEB p.34	48-well plate
3.	GFP-HUVEC (l.29715) p.5 & p.6	hASC 4/19 p.2, donor F, 31yrs, cell source subcutaneous	UTA.04602.WT HEB p.39	48-well plate
4.	GFP-HUVEC (l.29715) p.4	hASC 6/19 p.2, donor F, 33yrs, cell source subcutaneous	UTA.04602.WT HEB p.42	µ-Slide 8 Well high ibiTreat
5.	GFP-HUVEC (l.29715) p.4	hASC 4/19 p.4, donor F, 31yrs, cell source subcutaneous	UTA.04602.WT HEB p.46	µ-Slide 8 Well high ibiTreat

4.4   Video microscopy to characterize cardiomyocyte function

In order to analyze functional properties of the iPSC-CMs in different environments 30 seconds long video clips were taken of each well containing spontaneously beating iPSC-CMs, at days 12 and 14. The appropriate culture media (Figure 4) was first

changed to the cells for approximately 1 hour before imaging in order to enhance the beating of the iPSC-CMs. Well plate was kept on warming plate in +37 C during the imaging.

The video recording was done with Nikon Eclipse TS100 (Nikon Corporation) microscope with 20x air objective (Nikon Corporation) and Bobcat B0620 (Imperix) camera mounted to the microscope. 30 second video clips with 60 frames per second rate with 640 x 480 resolution were taken. The software used for the recording was JAI Control Tool.

4.4.1 Video analysis of iPSC-CM functionality with CellVisus

Analysis of the recordings were done with CellVisus (v.1.108) software. The principle of operation of the software is described in detail by Ahola et al. (2014). The videos were processed without choosing any region of interest by hand, however if there were clearly two or more beating centers, more minor was cut out of the processing to avoid errors in the software.

CellVisus measured three different phases for individual beat and times (ms) for those phases, which are shown and described in more detail in Figure 6. In addition to contraction duration (1), duration contracted (2) and relaxation duration (3) -values, beat per minute (BPM) -value, i.e., the frequency of the beats, was measured. These measured values from 30 second recording time were then exported to Excel and means were then taken from each sample's measured values for further analysis.
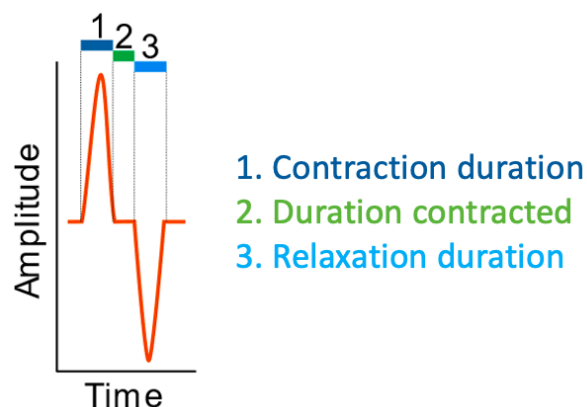
Figure 6. *Schematic representation of three phases of heart beat that CellVisus - software measures. It detected 1. Contraction duration = time of the contraction, 2. Duration contracted = time spent in contracted state and 3. Relaxation duration = time of relaxation phase*

4.4.2 Statistical analysis of CellVisus results

Before further analysis, the calculated means for each sample were grouped together with same sample types including 3D model, hASC + CM, 3D and 2D controls in separate mediums. The final number of samples used in statistical analysis is shown in Table 4. As the beating of the CMs and movement of the hydrogel was so frequent in some samples, all of the recorded videos couldn't be processed due to reasons related with the software.

Table 4. *Number of processed video recordings got from each sample type from each recording day. More detailed list of different samples and cells in them can be seen in Table 2.*

Sample type	Medium	N (day12)	Total per type (day 12)	N (day14)	Total per type (day 14)
3D model	EB5%	7	13	8	15
3D model	1:1	5		7	
hASC+ CM	EB5%	2	9	6	11
hASC + CM	1:1	7		5	
3D CM ctrl	EB5%	5	10	4	9
3D CM ctrl	1:1	5		5	
2D CM ctrl	EB5%	3	7	5	9
2D CM ctrl	1:1	4		4	
	In total		**38**		**44**

First, the effect of medium composition (EB5% or 1:1) on beating of CMs was studied with Independent-samples Mann-Whitney U Test. Then, Independent-Samples Kruskal-Wallis one-way analysis of variance and pairwise comparisons was used to compare all samples with each other. Significance values were adjusted by the Bonferroni correction for multiple tests. Then, same Kruskal-Wallis test was used to compare samples that were grouped only according to the cells within a sample and medium compositions were ignored. All differences were considered statistically significant if p-value was <0.05. Statistical analysis was conducted with IBM SPSS Statistics (v.27).

4.5 Immunofluorescence

In addition to video microscopy, constructs were also analyzed with confocal microscope. For that, cells were stained according to the workflow presented in Table 5. Cells were fixed in 4% paraformaldehyde (PFA) for 60 minutes at RT. Samples collected

at day 8 were fixed similarly and washed with dPBS and stored at +4°C until fixing was done also to the endpoint samples. In each sample 250 µl of reagents was used.

Table 5. Immunofluorescence protocol used in this project. (NDS = normal donkey serum, BSA = bovine serum albumin, PBS = phosphate buffered saline, DAPI = 4'6-diamidino-2-phenylindole, RT = room temperature)

Stage	Blocking	1st wash	Primary antibodies	2nd wash	Secondary antibodies	Final wash	DAPI
Reagents	10% NDS, 0,1% TritonX-100, 1% BSA in PBS	1% NDS, 0,1% TritonX-100, 1% BSA in PBS	Primary antibodies (table X) in 1st wash solution	1% BSA in PBS	Secondary antibodies (table X) in 2nd wash solution	PBS	DAPI (1mg/ml in H2O, Sigma) in PBS (1:5000)
Time and temperature	90 minutes in RT	Quick wash	Two days at 4°C on shaker (~7 rpm)	One quick wash following two washes of two days (PBS changed twice per day)	Overnight at 4°C on shaker (~7 rpm)	One quick wash following two washes of one hour	3 hours on shaker (~7 rpm) at RT

After completing primary and secondary antibody staining, 4′,6-diamidino-2-phenylindole (DAPI) staining, two 15 minutes PBS wash were done, and samples were left in second PBS for imaging. In Table 6 the used first and second antibodies and their dilutions are presented.

Table 6. Primary and secondary antibodies used for immunofluorescence. Sample types and their immunostaining targets are listed in the table.

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

After immunostaining samples were imaged with Nikon Spinning disk confocal microscope (Nikon Corporation) with Nikon Plan Fluor 10x/0.30, WD 16.0 mm air objective (Nikon Corporation). In addition, one image presented in the results was taken with Leica STELLARIS 8 FALCON by Dr. Daniel Smeets.

5. RESULTS

5.1 Cardiomyocytes remained viable and functional in all experimental settings

The iPSC-CMs were viable and functional for 9 days in multicellular construct in two different culture medias, i.e. CM-specific EB5% and 1:1 mixture of EB5% and angiogenic EGM-2 in all experiments. CMs beating was clearly visible in co-cultures, regardless of the culture media. In addition, iPSC-CMs were as viable and functional in co-culture with hASCs, as they were in co-culture with HUVECs and hASCs. Beating could be seen also in both monoculture control samples in 3D and 2D environment, however it was not so frequent as it was in co-culture samples. Below, the results of video microscopy are discussed in more detail. Of the results gotten from video microscopy, means, standard deviations, minimum and maximum values for each sample type group for examination days 12 and 14 are listed in Appendix 1.

5.1.1. Culture media does not affect on iPSC-CMs function

The results of beating analysis from video microscopy showed that the culture media does not affect to the function of the iPSC-CMs in 3D model construct. No significant differences in BPM or duration contracted (ms) values were detected when data from samples cultured in two different media were compared as shown in data in Figure 7 (ABCD). At day 12 (A) and day 14 (B), the measured results of BPM range from 0 to 100, and higher BPM values were measured at day 14. In addition, duration contracted values varied from 0 to 50 ms at day 12, and from 0 to 200 ms at day 14. On both days, higher separate values were detected (C,D).

The medium composition did not have effect on the iPSC-CMs function in CM + hASC co-culture samples There were no significant differences between BPM or duration contracted when the data from two groups were compared, as shown in the data presented in figure 7 (EFGH). The range of measured BMP results within 1:1 medium group was 50 to 150 on both recording days (E,F), furthermore higher BMP value was measured from EB5% samples at day 14 (F). The duration contracted values measured from 1:1 samples, were below 100ms at day 12 (G) and below 20ms at day 14 (H). Most

of recorded duration contracted values of EB5% cultured samples were below 40ms at day 14 (H). Both group had single separate higher values measured.

The effects of the culture media on iPSC-CMs function were examined using Mann Whitney U test. The effect was tested for each beating phase (Figure X) and also for BPM in each sample group, on day 12 and day 14 time points. Here statistics for BMP and Contraction duration (ms) are shown. In Figure 7, effect of culture media composition to iPSC-CMs in 3D model and CM + hASC are shown, respectively.
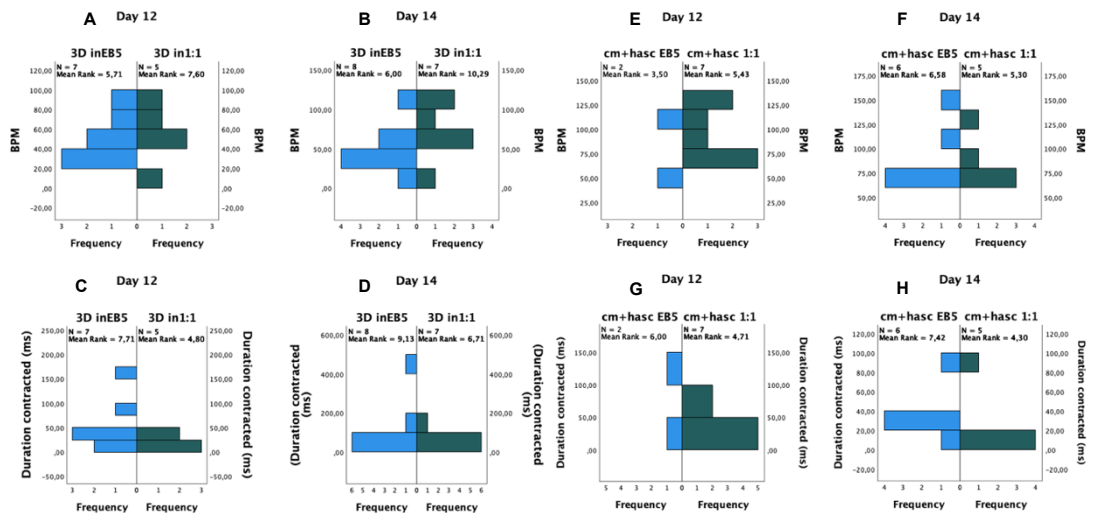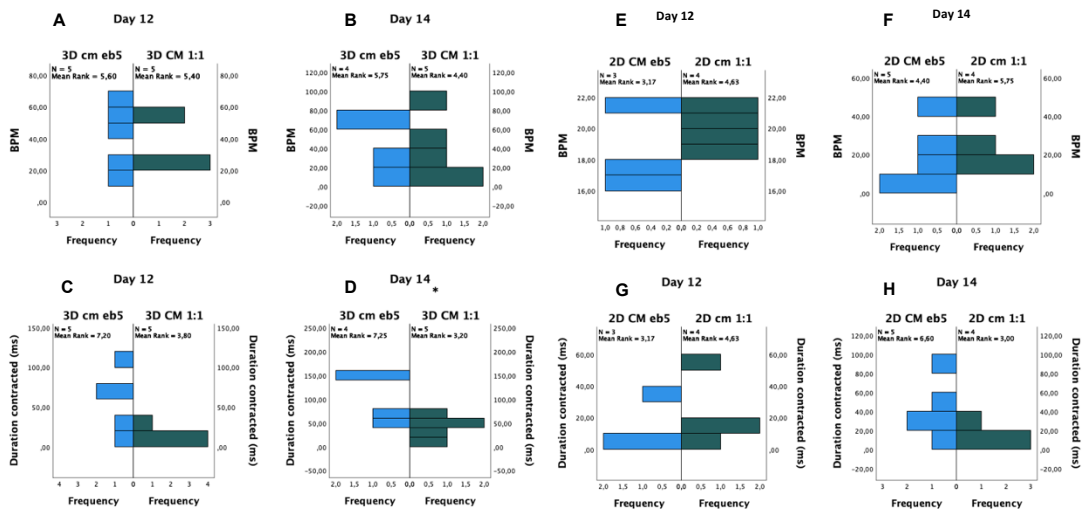

Figure 7. *No significant (p > 0.05) differences in iPSC-CMs function was detected, when data from samples cultured in two different media were compared. In left, graphs of 3D model are shown, and in right graphs of CM + hASC. Statistics according to the Mann Whitney U test.*

The culture media did not affect the iPSC-CMs function in monoculture control samples in 3D nor 2D environment. No significant differences could be seen between samples cultured in two different medias, when the BPM and contraction duration (ms) values from sample groups were compared, as shown in data in Figure 8 (ABCD for 3D, EFGH for 2D).

The BPM results from 3D control samples vary at both days 12 (A) and 14 (B), from 0 to 80, except for one separate higher value at day 14 (B). When looking at results of Duration contracted (C,D), EB5% sample group at day 12 (C) shows higher measured results, ranging from 0 to over 100ms, when all measured values from 1:1 samples were under 50ms. At day 14 (D), significant difference (p < 0.05, *) between two sample groups when comparing duration contraction results is shown. At day 14 (D), there are higher measured results in EB5% group, that range between 50ms and 150ms, when compared to 1:1 samples, in which the result range from 0ms to 100ms.

The recorded BPM values from 2D control are between 16 to 22 at day 12 (E), from both sample groups. Day 14 (F) BPM results vary between 0 and 60 BPM. Of measured values of duration contracted, the majority of measured results are under 20ms in both groups at day 12 (G). From day 14 (H), higher values were measured from samples cultured in EB5% media, ranging from 0ms to 100ms. Results measured from 1:1 cultured samples are all below 40ms at day 14 (H).

Here statistics for BMP and Contraction duration (ms) are shown. In Figure 8, effect of culture media composition to iPSC-CMs in monoculture controls in 3D environment and in 2D environment are shown, respectively.

Figure 8. *There was significant (p<0.05, *) difference between sample groups when Duration contraction values from day 14 recordings were compared. No other significant differences could be seen in function of iPSC-CMs in monoculture, according to the Mann Whitney U test.*

5.1.1 Co-culture affects iPSC-CMs beating

The data obtained from video microscopy showed that the co-culture affected the function of the iPSC-CMs, as the difference in beating frequency was significant between co-culture samples and monocultures at both recording days 12 and 14, as shown the data presented in Figure 9 and 10. Significant difference was also detected on Contraction duration (ms) between 3D model and 3D control samples at both days.

There are significant differences between 3D model and 2D CM, as well as between hASC + CM and both 3D and 2D CM controls. Highest beating frequency has been recorded from hASC + CM samples, 137,5 BPM. For 3D model highest recorded value was 95,7 BPM, for 3D control 61,9 BPM and for 2D control 21,5 BPM.

The effect of co-culturing for iPSC-CMs function was examined and the data from different sample groups were compared with each other using Kruskal Wallis one-way analysis of variance. It was decided to group samples only according to the cells and not take into account the culture media, since the medium composition did not affect the iPSC-CMs function significantly. Significance values were adjusted by the Bonferroni correction for multiple tests and difference was considered to be significant if p-value was smaller than 0.05. Due to small size of sample groups, outliers nor extreme outliers have not been removed from the graphs, as they have been included in the statistical analysis. They are marked by individual * and °, respectively.
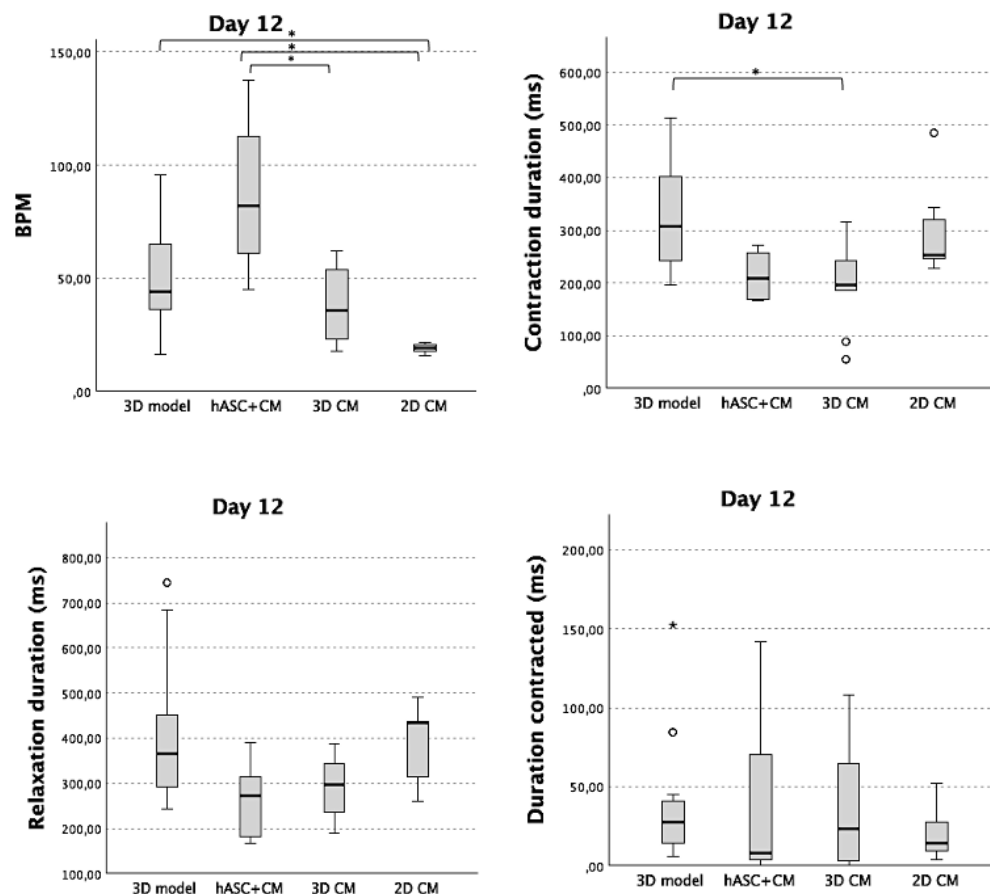
Figure 9. *Significant (p> 0.05, *) difference was detected in beating frequency and contraction duration, when compared co-culture samples and monoculture ones at day 12, according to the Kruskal-Wallis one-way analysis.*

Significant differences could be seen between co- and monoculture samples when BPM values at day 14 were compared, as shown in data in Figure 10. There are significant differences between 3D model and 2D CM samples, as well as between hASC + CM and both 3D and 2D CM controls. The highest measured BPM value was from

hASC + CM, 145,4 BMP. For 3D model the highest recorded value was 122,8 BPM, for 3D control it was 85,1 BPM and for 2D control 47,6 BPM. In addition, there are also significant difference in measured results of contraction duration between 3D model and 3D CM control samples. Overall, results from day 14 show similar pattern with the results recorded on day 12, and all the measured highest BPM values are higher than at day 12.
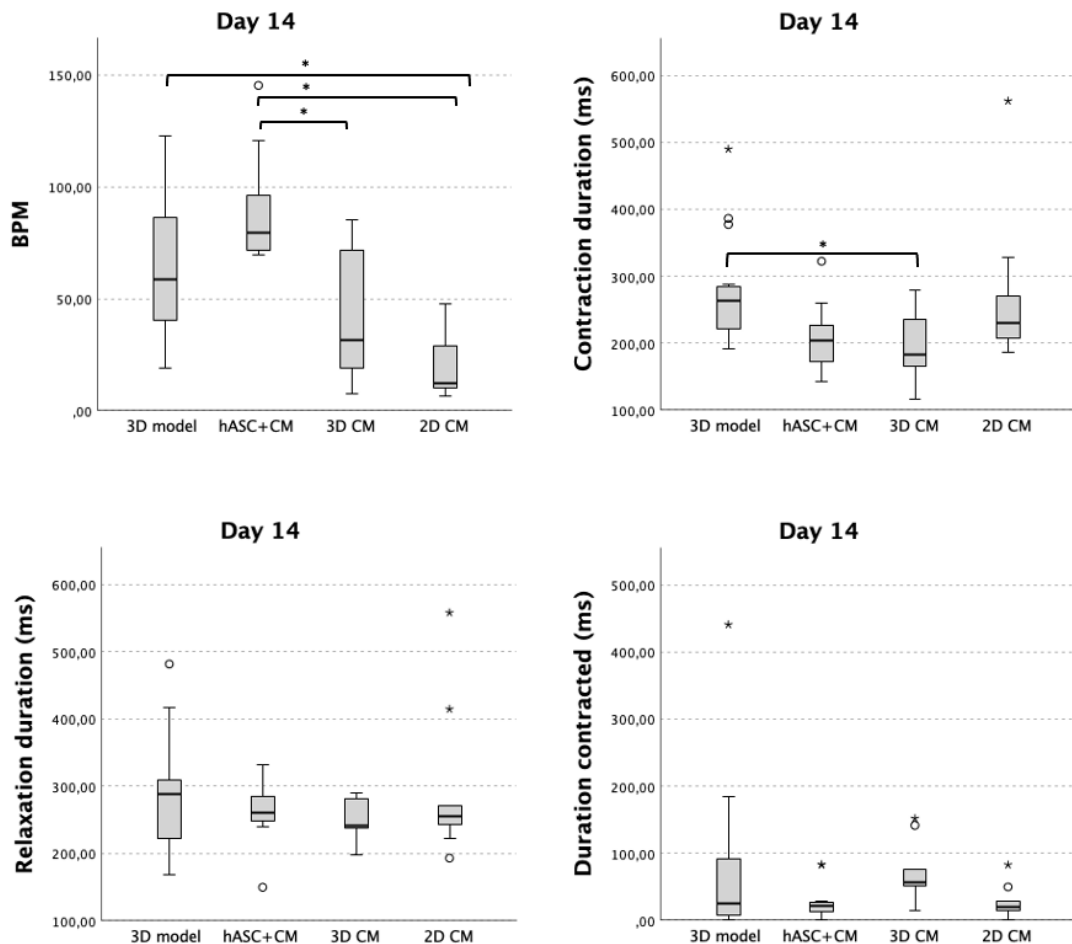
Figure 10. *There was significant (p>0.05,*) difference in beating frequency and contraction duration, when data from co-cultured samples and monoculture ones at day 14 were compared, according to the Kruskal-Wallis one-way analysis.*

5.2 iPSC-CMs align and change morphology in co-culture

iPSC-CM morphology changed in co-culture towards more adult-like phenotype. The small, round shaped morphology transforms into more elongated, rod-like type and the cells are connecting with each other in the endpoint. In addition, clear alignment between

hASCs and iPSC-CMs could be seen from the beginning of co-culture. The cells were characterized with immunostaining and confocal imaging, and the results are discussed more detail in next chapters.

When comparing the morphology of iPSC-CMs in the beginning of both co-cultures to the endpoint, clear difference in their morphology can be seen between cells imaged at different time points. After one day of co-culturing iPSC-CMs are separately in the 3D model, and they are small and round-shaped, as shown in Figure 11A. iPSC-CMs showed similar small and globular morphology in co-culture with only hASCs, however interestingly, in hASC+CM co-culture, iPSC-CMs are aligned and attached already with hASCs after one day of culturing, as shown in Figure 11B.

At the endpoint in both co-cultures clearly grown and elongated morphology of iPSC-CMs can be seen. In addition, iPSC-CMs are clearly attached to each other and are forming framework, as shown in Figure 11C and D. In hASC co-culture (D), iPSC-CMs are still aligned and connected with alfa smooth acting (a-SMA) network formed by hASCs. In hASCs and iPSC-CMs co-culture (Figure 11D), iPSC-CMs show more thin and elongated structures compared to 3D model (Figure 11C). The a-SMA network formed by hASCs is denser at day 16 when compared to the day 8.
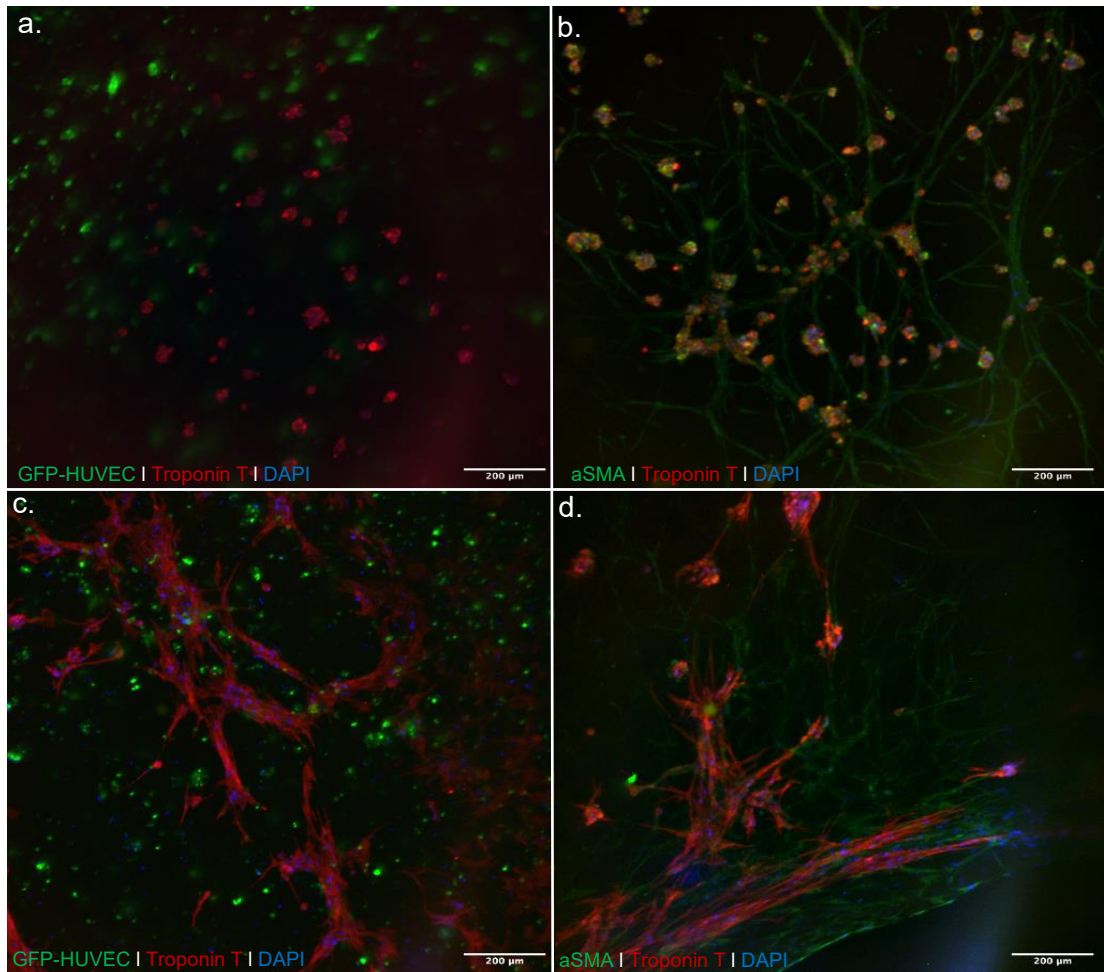
Figure 11. Representative immunofluorescence images of iPSC-CMs in co-culture with HUVECs and hASCs (A, C) and with only hASCs (B,D) cultured in EB5% media, after 1 day of co-culture (A,B) and after 9 days of co-culture (C,D). *CMs are stained against Cardiac Troponin T, which is shown as red in every image. GFP-HUVECs are shown in green in A and C, and alpha smooth muscle actin (a-SMA) for hASCs is shown in green in B and D. DAPI for nuclei is shown in blue in all of the images. In all images scale bar is 200μ.*

Organized and aligned sarcomere structures were formed in iPSC-CMs during the co-culturing, as shown in Figure 12.
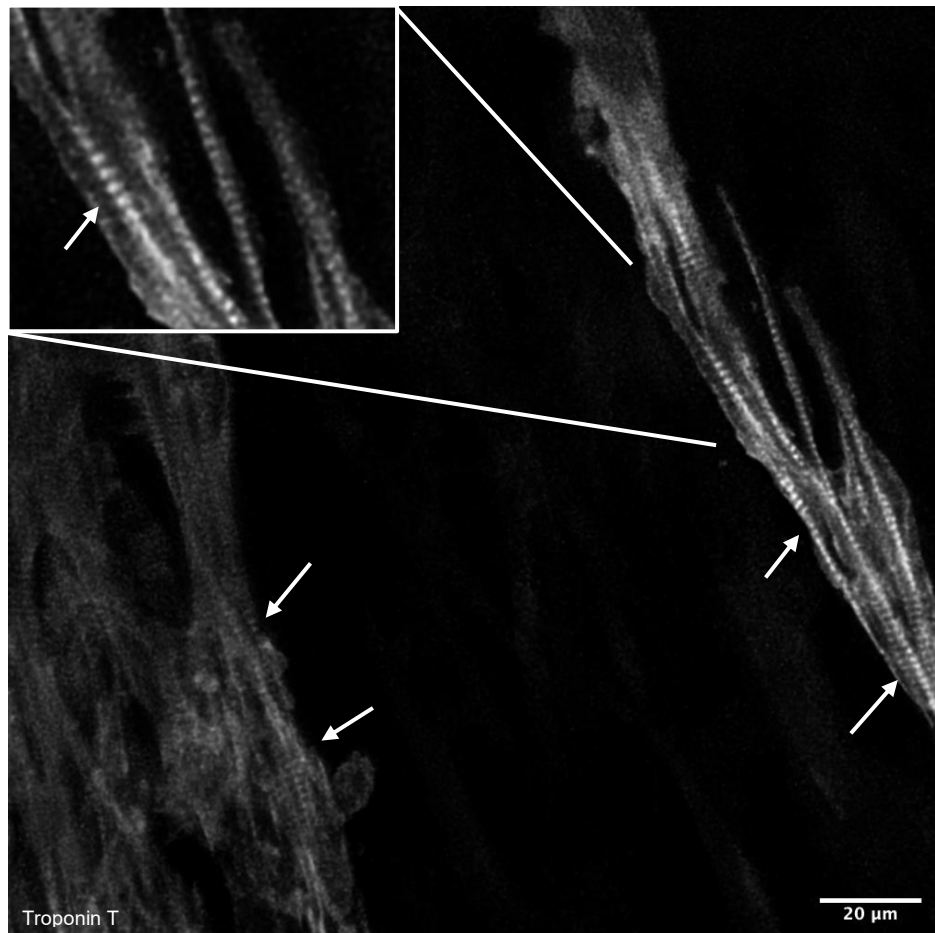
Figure 12. Representative image of cardiomyocytes' organised and aligned sarcomere structures in 3D model sample, cultured in EB5% media and fixed after nine days of co-culture. *Image taken by Dr. Daniel Smeets with Leica STELLARIS 8 FALCON with HC PL APO CS2 10x/0.40 DRY objective. Scale bar is 20 μm.*

The iPSC-CMs showed clear difference in morphology, when comparing co-culture and monoculture endpoint. In 3D model and CM + hASC constructs, iPSC-CMs are attached with each other and are forming organized network, as shown in Figure 13A and B, respectively. Especially, the network formed by iPSC-CMs and a-SMA formed by hASCs is aligned and organized, and elongated and thin structures can be seen in the Figure 13B. Differences in the iPSC-CMs morphology are similar than shown previous Figure 12, in 3D model (Figure 13 A), it is not so elongated or thin when compared to iPSC-CMs cultured only with hASCs (Figure 13 B)

When iPSC-CMs were in monoculture they gathered together, and individual structures or cells couldn't be detected similarly than they can be from the co-culture, shown in Figure 13C. In addition, iPSC-CMs formed aggregates in monoculture as shown in Figure 13D.
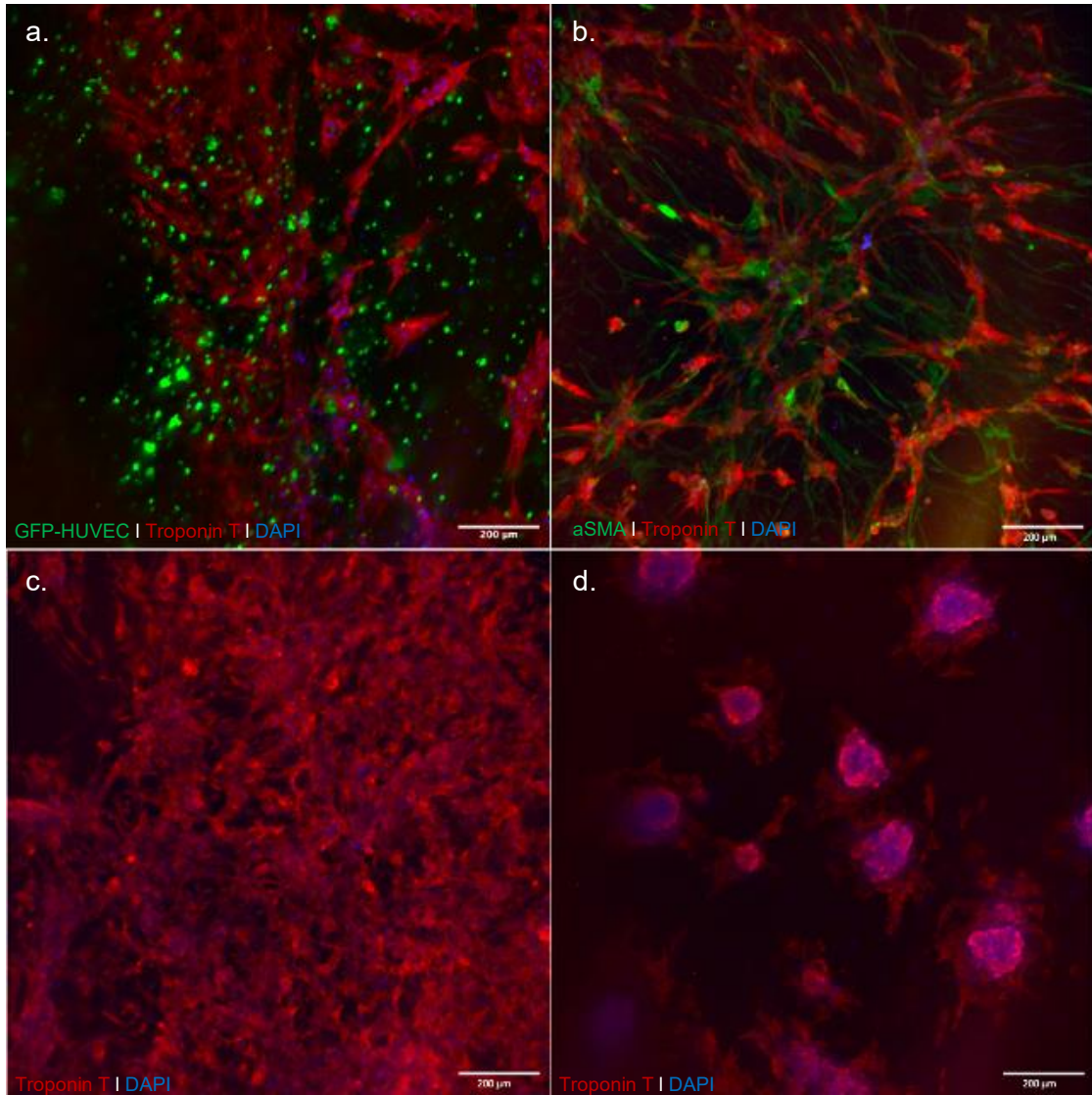
Figure 13. Representative fluorescence images of 3D model (CM, HUVEC, hASC) (A), CM + hASC (B) and monoculture control in 3D (C,D), after nine days of culture. *CMs are stained against Cardiac Troponin T, which is shown as red in every image. GFP-HUVECs are shown in green in A, and alpha smooth muscle actin (a-SMA) for hASCs is shown in green in B. DAPI for nuclei is shown in blue in all of the images. In all images scale bar is 200um.*

6. DISCUSSION

There is currently inevitable need for trustworthy, high-throughput *in vitro* models of myocardium. Firstly, to find novel solutions for cardiovascular research, to overcome the socio-economic burden caused by heart diseases. And secondly, to enhance the process of releasing new drugs to the markets, as cardiotoxicity is one of the most common reasons for drug withdrawal. (Basara *et al.*, 2022) This project was based on previous work of Vuorenpää et al., (Vuorenpää *et al.*, 2014, 2017), and the previously developed multicellular model of myocardium was established in 2D environment. The current results showed that iPSC-CMs, co-cultured in 3D environment provided by gelatin-gellan gum hydrogel, could provide potential platform for cardiovascular diseases research, as iPSC-CMs express improved characteristics towards more mature phenotype in 3D multicellular construct, similarly, to previously demonstrated results by Vuorenpää et al., (2014, 2017). This thesis focused on studying the viability and functionality of the iPSC-CMs. Sanna Koskimäki's thesis, "Maturation and morphology of human pluripotent stem cell derived cardiomyocytes and vascular structures in 3D cardiovascular construct, 27.5.2022" provides further results and discussion about the maturation, growth and functionality of vasculature forming cells in addition to iPSC-CMs.

There were not differences in the function or in structure of the cardiomyocytes between samples that were cultured in different culture mediums. The co-culture media which was used for half of the samples was made with 1:1 ratio of CM specific EB5% media and angiogenic growth media (EGM-2), when another half was cultured in only EB5% medium. The paracrine signaling between endothelial cells and cardiomyocytes is well documented and goes both ways. (Brutsaert, 2003; Colliva *et al.*, 2020) Endothelial cells secrete growth factors and other molecules that are essential for normal development and function of cardiomyocytes, and cardiomyocytes in the other hand secrete for example VEGF-A, which is important growth factor for angiogenesis and formation of microvasculature. (Vuorenpää *et al.*, 2017) Also, the factors secreted by MSCs have been shown to affect the functionality and enhance maturation of iPSC-CMs. (Yoshida *et al.*, 2018) Our study suggest that the paracrine signaling between HUVECs and cardiomyocytes together with hASCs that functioned as supportive cells for both, leads in environment where the importance of culture media diminishes as the cells secrete necessary growth factors and other molecules by themselves. Thus, cells can be viable and functionable regardless of the culture media. (Vis, Ito and Hofmann, 2020)

The morphology of CMs clearly transformed in co-culture, in both 3D model and hASC + CMs constructs. The iPSC-CMs were more elongated, compared to the round and small shape captured in the first day of the culturing period. In addition, cardiomyocytes were not in contact with each other in the beginning of the co-culture, however at the endpoint they were clearly attached. Also, more organised and aligned sarcomere structures could be seen at the endpoint in 3D model, which indicates of structural maturation of the iPSC-CMs. (Ahmed *et al.*, 2022) Morphology of iPSC-CMs is different in the end of experiments between monoculture controls and co-culture samples. In the monoculture, similar alignment can't be distinguished, that can be seen in co-culture. In addition, the morphology of iPSC-CMs seems to be different also when compared the images between HUVEC and hASC co-culture, to the samples where only hASCs were cultured with iPSC-CMs. Clear alignment between cardiomyocytes and hASCs' a-SMA (alfa smooth muscle actin) network can be seen in the images, already after one day of co-culturing. This suggests of crosstalk between cardiomyocytes and hASCs.

The video microscopy showed that the beating frequency of the iPSC-CMs is enhanced in the co-culture when compared to controls. In addition, cardiomyocytes monocultured in 3D environment had higher beating frequencies than CMs cultured in 2D environment. Interestingly, the highest recorded BPM values were from hASC + CM co-culture. The results demonstrate that the spontaneous beating of the iPSC-CMs were enhanced in 3D environment, and in co-culture with HUVEC and hASCs. Also, hASCs alone can support iPSC-CMs functionality, as the results from hASC + CM construct are similar with co-culture with HUVEC and hASCs**.** Similar results were reported by Yoshida et al. recently, when they showed that iPSC-CMs functionality was improved regarding the beating, electrophysiological properties, and myofibril structures maturation when they were co-cultured with human MSCs. Also, they demonstrated that co-culture with human MSCs enhanced interactions between iPSC-CMs as well as formation of organised framework, which was also demonstrated in our project. (Yoshida *et al.*, 2018)

Co-culture of hASCs and iPSC-CMs is definitely one path that should be discovered further. As the results showed, cardiomyocytes were functionable and viable in co-culture with hASCs, as much as together with endothelial cells which are essential for cardiomyocytes normal development and function. (Colliva *et al.*, 2020) This indicates that hASCs can be used instead of endothelial cells in co-culture and it opens up new possibilities for developing *in vitro* model of myocardium. In addition, the co-cultures could be next constructed on microfluidics device, so that the formation and functionality of vasculature together with beating cardiomyocytes could be examined, similarly to study of Ellis *et al.*, (2017) and King *et al.* (2022). The perfusion itself can improve iPSC-CMs maturation, which would help reaching the goal of fully adult-like iPSC-CMs.

(Kolanowski *et al.*, 2020) As it has been shown that iPSC-CMs respond accurately to drugs (Koivisto *et al.*, 2022) when cultured together with HUVECs and hASCs, it would be good to study further the effects of different environments, such as hypoxia, to the iPSC-CMs functionality in co-culture and how for example hASCs could function as supportive cells in such conditions.

As discussed previously, one of the biggest challenges to truly release the potential of 3D culture systems is to overcome the visualization difficulties, which were present in this project as well. Due to the thickness of the gel, and limitations of the imaging system, more detailed structures of the cells could not be captured. In addition, due to gel surface arising towards the corners of square shaped wells of Ibidi plate, there were challenges in imaging of the cells and for example showing alignment between CMs and HUVECs, as HUVECs migrated towards the sides of the wells. This alignment have been however demonstrated before (Vuorenpää *et al.*, 2017). The two different culture formats could have some effect on the cardiomyocytes' behavior, as the beating frequency seemed to be higher in Ibidi plates, than in round shaped wells. The effect of the platform should be further studied and should be carefully considered in follow up studies, as it might have effect on the cardiomyocytes' functionality.

Some limitations were faced also in characterization with using the in house built CellVisus software, which initially have been developed to analyze single cells. (Ahola *et al.*, 2014) The problems were highlighted with the samples where the beating was the most frequent, in the hASC and CM co-culture samples. As the beating of the cardiomyocytes in these samples were so strong and frequent, the hydrogel was moving along as fast, which further hampered the recording of the beating as the software might have detected the movement of the gel rather than the cells. This might have affected to the rather low number of processed video recordings of CM + hASC, that we were able to get from the software to be used in further analysis. No doubt, this project and material recorded will help to further develop the software to meet the need for easy and efficient tool for analyzing data from live imaging of cardiomyocytes in 3D environment.

The gelatin-gellan gum hydrogel which have been previously used and demonstrated to be suitable culture environment for iPSC-CMs (Koivisto *et al.*, 2019), supported the co-culture for 16 days in total, from which nine with more load from beating iPSC-CMs. Cardiomyocytes were viable and functionable in the hydrogel and were able to migrate, which indicates that being suitable environment for culturing iPSC-CMs with other cell types. However, towards the end of each experiment the gel was detaching from the walls, and holes were formed within the gel. Similar detachment did not happen in monocultures, so the hydrogel couldn't bear the load from interaction and migration of cells in co-culture. For long-term co-culture, the hydrogel properties should be modified,

or the material should be reconsidered as it did not bear the physical stress from several cells cultured within, in addition to the beating of the cardiomyocytes. With improvements in the biomaterial and thus longer culture periods, improvements in the maturation of iPSC-CMs could be achieved. (Fleischer *et al.*, 2019)

7. CONCLUSIONS

The main aim of this thesis project was to build 3D construct, to be used to model human myocardium *in vitro* by combining iPSC-derived cardiomyocytes, human adipose stem cells and human umbilical vein endothelial cells. iPSC-CMs functionality in construct was studied based on analysis of the beating. In addition, the changes in iPSC-CMs morphology in co-culture was characterized with immunostainings and confocal imaging. The morphology of the iPSC-CMs was compared between co-culture and monoculture, as well as between the starting and endpoint, to see the effects of the co-culture on the cells. With video microscopy, we aimed to find out if the function could be improved by co-culturing, and if the culture media composition would affect the cells.

The experiments were repeated five times in total, and in each time, cardiomyocytes were viable at the endpoint, and visibly beating. The medium composition does not affect the iPSC-CMs behaviour in co-culture. The morphology of CMs transforms from small, round shaped to more elongated and rod-like, with more organised sarcomere structures, from the start to the endpoint in co-culture samples. In addition, iPSC-CMs align and attach to each other in co-culture, forming structural framework. Furthermore, the morphology of iPSC-CMs is different at the end of experiments between monoculture controls and co-culture samples. Per video microscopy, beating is enhanced in the co-culture samples compared to monoculture controls as the measured BMP values of the iPSC-CMs were significantly higher in co-culture, when compared to monoculture controls. Interestingly, hASCs alone could provide enough support for cardiomyocytes as the results from beating analysis were similar from the co-culture where HUVECs and hASCs were both in.

The project showed that this model could be potentially used in the future for cardiovascular diseases research, drug development and cardiotoxicity screening.

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APPENDIX 1

Day 12	Sample	N		Mean	Std. Deviation	Min.	Max.
	3D model in EB5%	7					
			Contraction duration (ms)	367,6	97,5	237,7	512,7
			Duration contracted (ms)	53,4	49,7	11,0	152,4
			Relaxation duration (ms)	467,4	182,7	287,0	744,6
			BPM	47,8	22,7	22,4	92,4
	3D model in 1:1	5					
			Contraction duration (ms)	262,9	50,6	195,8	314,6
			Duration contracted (ms)	19,8	12,2	5,6	35,9
			Relaxation duration (ms)	324,8	63,3	244,0	410,5
			BPM	54,2	29,9	16,4	95,7
	CM+hASC in EB5%	2					
			Contraction duration (ms)	240,7	45,3	208,6	272,7
			Duration contracted (ms)	73,0	97,4	4,11	141,8
			Relaxation duration (ms)	276,1	131,6	183,0	369,1
			BPM	78,2	46,6	45,2	111,1
	CM+hASC in 1:1	7					
			Contraction duration (ms)	205,6	45,6	167,5	256,3
			Duration contracted (ms)	27,1	36,7	0,0	89,4
			Relaxation duration (ms)	260,3	80,5	167,0	390,3
			BPM	92,6	32,9	61,1	137,5
	3D CM in EB5%	5					
			Contraction duration (ms)	236,7	51,4	186,4	315,6
			Duration contracted (ms)	55,1	40,0	3,33	107,8
			Relaxation duration (ms)	332,2	57,5	250,5	387,5
			BPM	39,8	18,4	17,9	61,9
	3D CM in 1:1	5					
			Contraction duration (ms)	154,2	78,3	55,2	240,0
			Duration contracted (ms)	10,92	13,1	0,0	30,9
			Relaxation duration (ms)	245,5	59,5	189,3	319,3
			BPM	36,5	16,8	22,0	55,8
	2D CM in EB5%	3					
			Contraction duration (ms)	319,7	143,4	227,8	485,0
			Duration contracted (ms)	16,1	17,0	3,75	35,6
			Relaxation duration (ms)	319,3	100,6	261,1	435,5
			BPM	18,2	2,9	16,0	21,5
	2D CM in 1:1	4					
			Contraction duration (ms)	284,2	44,9	246,4	343,0
			Duration contracted (ms)	23,6	19,3	9,09	52,0
			Relaxation duration (ms)	433,6	50,0	369,0	491,1
			BPM	19,8	1,4	18,2	21,5

Day 14	Sample	N		Mean	Std. Deviation	Min.	Max.
	3D model in EB5%	8					
			Contraction duration (ms)	281,5	66,2	215,8	386,5
			Duration contracted (ms)	109,2	142,3	0,0	441,1
			Relaxation duration (ms)	308,0	102,7	180,9	482,0
			BPM	51,1	26,6	18,9	107,9
	3D model in 1:1	7					
			Contraction duration (ms)	269,5	103,6	191,7	490,0
			Duration contracted (ms)	38,4	64,9	3,6	184,3
			Relaxation duration (ms)	256,4	53,3	167,7	315,5
			BPM	79,8	35,4	23,3	122,8
	CM+hASC in EB5%	6					
			Contraction duration (ms)	233,9	54,3	172,9	322,5
			Duration contracted (ms)	32,9	24,9	18,0	83,1
			Relaxation duration (ms)	258,5	61,9	150,0	332,8
			BPM	93,8	29,1	69,7	145,4
	CM+hASC in 1:1	5					
			Contraction duration (ms)	176,5	28,1	143,0	206,7
			Duration contracted (ms)	22,9	34,3	1,3	83,1
			Relaxation duration (ms)	267,2	25,5	239,2	308,3
			BPM	83,0	21,5	69,7	120,7
	3D CM in EB5%	4					
			Contraction duration (ms)	238,2	40,8	183,3	280,0
			Duration contracted (ms)	106,8	47,2	57,1	152,0
			Relaxation duration (ms)	274,4	22,6	241,0	290,6
			BPM	50,1	29,2	19,0	78,0
	3D CM in 1:1	5					
			Contraction duration (ms)	156,9	29,3	116,7	182,7
			Duration contracted (ms)	43,9	18,8	15,0	63,3
			Relaxation duration (ms)	237.7878	29,2	198,5	280,0
			BPM	37,9	33,1	7,7	85,1
	2D CM in EB5%	5					
			Contraction duration (ms)	319,8	142,9	208,3	562,0
			Duration contracted (ms)	40,2	27,0	15,0	82,9
			Relaxation duration (ms)	241,8	29,3	193,3	271,4
			BPM	20,5	17,5	6,7	47,6
	2D CM in 1:1	4					
			Contraction duration (ms)	219,7	34,0	185,7	264,6
			Duration contracted (ms)	10,3	10,1	0,0	20,0
			Relaxation duration (ms)	365,9	152,5	221,9	558,5
			BPM	22,8	14,6	11,4	42,4