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**OPTIMIZATION OF CO-CULTURING  
CONDITIONS FOR HIPS CELL-DERIVED  
CARDIOMYOCYTES AND HUMAN  
MESENCHYMAL STEM CELLS**

Lääketieteen ja terveysteknologian tiedekunta  
Syventävien opintojen kirjallinen työ  
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# TIIVISTELMÄ

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Iskeemiset sydänsairaudet ovat yleisin kuolinsyy maailmanlaajuisesti. Lukuun ottamatta sydänsiirtoa, myokardiumia vaurioittaviin sairauksiin ei ole parantavaa hoitoa. Kantasoluteknologiasta etsitäänkin uusia hoitovaihtoehtoja.

Ihmisen uudelleenohjelmoiduista pluripotenteista kantasoluista erilaistetuilla kardiomyosyyteillä (hiPSC-kardiomyosyytit) voitaisiin tulevaisuudessa korvata menettyä sydänlihaskudosta. Ne ovat kuitenkin vielä epäkypsiä, häiriöherkkiä, eivätkä ne kiinnity tehokkaasti kohdekudokseen. Ihmisen mesenkymaalisisilla kantasoluilla on lukuisia parakriinisia ja immunomoduloivia ominaisuuksia, jotka voisivat ratkaista näitä ongelmia. Tavoitteenamme oli kasvattaa kyseisiä soluja yhdistelmäviljelmissä ja optimoida tähän soveltuvat kasvatusolosuhteet. Lisäksi selvitimme yhdistelmäviljelyn vaikutuksia soluille, liittyen erityisesti kardiomyosyyttien maturaatioon.

HiPSC-kardiomyosyyttejä viljeltiin kaksi viikkoa yhdessä mesenkymaalisten kantasolujen kanssa tai näiden elatusliuoksessa. HiPSC-kardiomyosyyttikontrolleja kasvatettiin yksin EB-liuoksessa sekä testiryhmissä käytetyssä mesenkymaalisten kantasolujen kasvatusliuoksessa. Viljelyistä otetuille mRNA-näytteille suoritettiin qPCR-analyysi, jonka lisäksi valomikroskooppi- ja fluoresenssikuvat analysoitiin.

Tutkimuksemme osoitti, että hiPSC-kardiomyosyyttejä ja mesenkymaalisia kantasoluja voidaan viljellä onnistuneesti yhdessä. Mesenkymaaliset kantasolut proliferoituvat voimakkaasti, mikä tulisi huomioida solumäärissä. Kardiomyosyyttien morfologia, tumalukumäärät tai merkkigeeniekspressio eivät viitanneet tehostuneeseen kypsymiseen testiryhmissä. Toisaalta qPCR-tulokset viittasivat EB-kasvatusliuoksen soveltuvan paremmin kardiomyosyyttien viljelyyn.

Avainsanat: kantasolututkimus, sydänlihaskudon vaurio

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# 1. INTRODUCTION

Ischemic heart diseases remain as the leading cause of death worldwide (1). Ischemia damages the myocardium which is then replaced with non-contractile scar tissue. Myocardium itself has an insufficient regeneration capacity. Whereas majority of the postnatal cardiomyocytes are at post-mitotic state, the presence of stem or progenitor type of cells have been witnessed. Also, non-myocardial cells can modulate the regenerative process. (2)

In extensive myocardial damage, the high-risk and otherwise problematic heart transplantation remains as the only option. Cell transplantation involving the use of different stem cell-based strategies could provide a future option. These stem cells include mesenchymal stem cells, endothelial progenitor cells, cardiac progenitor cells and pluripotent stem cells (3). However, clinical trials on stem cell therapy are still at their baby stages thus clinical applications in myocardial repair are still lacking. The main challenge in cell transplantation is its insufficiency. (3)

Cardiomyocytes can be derived from induced pluripotent stem cells *in vitro* (iPSC-CMs) and could further be implanted into a recipient's heart. Problematically, these cardiomyocytes still have an immature phenotype and insufficient integration into the heart. The use of pluripotent stem cells also possesses a tumorigenic risk. (3)

Mesenchymal stem cells (MSCs) of different origin have been used in pre-clinical and clinical trials with promising results. The MSCs' paracrine qualities are believed to assist the already existing endogenous factors in myocardial tissue repair and improve cardiomyocytes' survival in ischemic conditions. MSCs may even have the potential to differentiate into cardiomyocytes *in vivo*. Hence MSCs could help to overcome the problems mentioned with iPSC-CMs. (3)

We hypothesized that hMSCs and hiPSC-CMs are suitable for co-culturing. We also assumed that MSCs might improve these cardiomyocytes' maturation. Thorough *in vitro* studies are required to evaluate the interaction and potential beneficial outcomes when combining human MSCs with human iPSC-CMs. At the beginning of this study no other publications of co-cultures involving hiPS-CMs and hMSCs were discovered.

## 2. LITERATURE REVIEW

### 2.1 The role of stem cell research in cardiac injury

Stem cells have proven themselves as a promising tool in tissue engineering for myocardial repair and therefore have been widely studied both *in vitro* and *in vivo*. Stem cells have the ability to self-renewal and differentiation into various cell types. Pluripotent stem cells, and to some extent mesenchymal stem cells, have the potential to differentiate into cardiomyocytes (3).

Mesenchymal stem cells can also stimulate reparation through paracrine signaling and may create other cardiac tissues *in vivo* as described later (3).

Early clinical trials on stem cell therapy have been conducted using primarily bone marrow-derived MSCs (BOOST, POSEIDON) but also cardiac stem cells (SCIPIO), cardiosphere derived stem cells (CADUCEUS), embryonic stem cells (ESCORT), adipose tissue-derived stem cells and endothelial progenitor cells have been studied (3). As decreased left ventricular ejection fraction (LVEF) is associated with heart failure, LVEF is commonly used to assess the impact of stem cell therapy on cardiac function. Scar tissue formation and the amount of viable tissue are also often evaluated for being the underlying cause of cardiac failure and the target of stem cell therapy.

The BOOST trial showed an improvement in the LVEF in patients who received BM-MSCs (4). Although this improvement was also present in the POSEIDON trial, it was statistically non-significant. However, clinical improvement (improved NYHA classification, 6-minute walk test and MLHFQ scores) was evidenced in patients with ischemic cardiomyopathy after BM-MSC treatment. (5) In the ESCORT trial cardiac progenitor cells were generated from embryonic stem cells *in vitro* prior to administration. The LVEF increased by 10 % alongside with clinical improvement (from NYHA III to NYHA I) but this study involved only one patient. (6) The SCIPIO trial also demonstrated cardiac magnetic resonance results with significant increase in the LVEF accompanied by decrease in the infarct size in patients treated with c-kit<sup>+</sup> cardiac stem cells (7). Consistently, MRI analysis revealed reduction in scarring and increase in viable cardiac tissue in the CADUCEUS trial (8). APOLLO, the first clinical trial with AdMSCs, demonstrated improvements in cardiac function, perfusion and reduction in scar formation (9).

Despite the promising results, stem cell therapy has not progressed past early clinical trials. Main challenge has been the inadequate efficiency of treatment. Ineffective delivery methods and challenging conditions at the injured target site restrict engraftment of the cells. Additionally, the therapeutic potential of delivered cells varies depending on the donor and cell type itself. (3) It becomes evident that more *in vitro* trials are required to overcome these issues.

## 2.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent adult stem cells. Depending on the signaling pathway, MSCs can differentiate into various mesodermal tissues such as bone, cartilage, adipose tissue and muscle. According to the International Society for Cell Therapy (ISCT) MSCs are defined by the following properties: adherence to plastic in standard culturing conditions, specific surface antigen expression and multipotent differentiation potential. MSCs express CD105, CD73 and CD90 while CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR are absent. (10) As MSCs lack class 2 MHC molecules and express class 1 MHCs at intermediate levels, immune rejection is nearly absent (3). MSCs have several endogenous functions. They participate in wound healing and successful aging process but also maintain stem cell niches, especially hematopoietic niches. MSCs have been typically isolated from bone marrow, adipose tissue, umbilical cord blood, peripheral blood and the connective tissue of dermis and skeletal muscle. However, MSCs can be found in almost every tissue type. (11,12)

Adipose tissue derived mesenchymal stem cells (AdMSC) have multiple advantages. AdMSCs are abundant and relatively easy to isolate, they have immunosuppressive properties and lack rejection especially when autologous cells are used (13). In culturing conditions, AdMSCs are highly proliferative and resemble fibroblasts (3). AdMSCs are also able to form cardiovascular structures (13).

MSCs migrate effectively through chemotaxis. *In vitro* chemotactic factors such as insulin-like growth factor-1 (IGF-1), platelet-derived growth factor-AB, macrophage derived chemokine (MDC) and RANTES attract the MSCs. Supporting their role in endogenous repair, inflammatory cytokine TNF- $\alpha$  amplifies this effect. Stromal derived factor-1 (SDF-1) and HGF seem to guide the MSCs to the site of injury. (12) However, it seems that different subtypes have different migration potential

and the cell culture itself affects the phenotype (14). Therefore, culturing conditions could also provide a tool to modify MSCs' properties.

## **2.3 hiPS cells and cardiac differentiation**

Pluripotent stem cells such as embryonic stem cells are able to proliferate while retaining their ability to differentiate into all human cell types and have therefore created countless opportunities for regenerative medicine (15,16). Yamanaka and his research group succeeded to create human induced pluripotent stem cells (iPSC) by reprogramming human adult's fibroblasts (17). There are fewer ethical dilemmas concerning the use of iPSC cells as there are with embryonic stem cells. This technology also enables the use of donor specific cells in medical treatment.

Just as embryonic stem cells, iPSC cells can be differentiated into cardiomyocytes (18–20). Today a few alternative methods are prevalent. In the spontaneous embryoid body (EB) method aggregation of the cells into a three-dimensional cluster containing three germ layers causes the differentiation process. The EB method is rather simple to execute yet the yield of cardiomyocytes is quite low, usually less than 10 %. Other alternative involves culturing of the cells with cardioinductive cells or their conditioned medium. Visceral endoderm-like cell line (END-2) originating from mouse P19 embryonal carcinoma cells is frequently used for these purposes. Lastly, the differentiation process can also be conducted by using cardiomyogenesis stimulating growth factors adopted from the natural cardiac differentiation signaling pathways such as BMP4, Activin A, bFGF and Wnt3. This so-called guided approach can be applied directly to undifferentiated iPSC cells or after the formation of the embryoid bodies as to sustain the development process. The advantage with this approach is that it yields over 30 % cardiomyocytes. However, timing is crucial in this method as the effect of the growth factors depend on the step of differentiation. (20,21)

## **2.4 hiPS-cardiomyocytes and their properties**

The generation of hiPS-derived cardiomyocytes (hiPS-CMs) has created several opportunities for regenerative medicine. The hiPS-CMs could enable the use of autologous cells in stem cell therapy. They have also provided information as *in vitro* models for monogenic cardiac diseases. Other

possible applications involve the use of hiPS-CMs in cardiotoxicity screening and drug development. (21)

Even though exhibiting cardiac specific structures and biophysiological properties, the phenotype of hiPS-derived cardiomyocytes (hiPS-CMs) has proven rather immature. Morphologically hiPS-CMs resemble more fetal cardiomyocytes than their adult counterparts. Adult cardiomyocytes appear rod-like with oriented sarcomere structures, whereas hiPS-CMs are often round or cubic in shape and their sarcomeres are fairly disorganized and shorter. (20,22,23) Cardiomyocytes differ from most cell types by being partially polynuclear. Around 20% of adult cardiomyocytes are multinucleated whereas less than 1 % of hiPS-CMs express this feature (20,22). In an adult heart different cardiac subunits such as atrial, ventricular and pacemaker cells have been organized into a functional structure which is yet to be achieved *in vitro*. (20)

Also, the biophysiological properties of hiPS-CMs differ somewhat from mature cardiomyocytes. As mature cardiomyocytes' metabolism relies mainly on aerobic  $\beta$ -oxidation of fatty acids, in immature cells this is replaced by glycolysis (23,24). This theory is supported by the low count of mitochondria in immature cells (23). Although hiPS-CMs are able to contract spontaneously, their upstroke velocity, conduction velocity and contractile force are inferior to their adult counterparts (23). Although these contractions are initiated by intracellular calcium, the calcium handling in hiPS-CMs isn't as developed as in adult cardiomyocytes, most likely due to immature sarcoplasmic reticulum (20). There are many applications to evaluate these electrophysiological functions. To name a few, multielectrode array (MEA) is used to measure field potentials whereas patch clamp mechanism detects ion currents. The contractility, on the other hand, can be estimated by video microscopy although the non-linear shape of the cells affect the analysis. (22)

Associated with the morphology and biophysiological properties, hiPS-CMs' expression of structural, junctional and regulatory proteins is typically lower or varies compared to mature cardiomyocytes. The presence of these proteins can be indirectly evaluated by the expression of the corresponding encoding genes. Cardiac sarcomeric components such as  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC),  $\beta$ -myosin heavy chain ( $\beta$ -MHC),  $\alpha$ -actinin and cardiac isoform of Troponin-T (cTnT) are encoded by MYH6, MYH7, ACTN and TNNT2 genes, respectively. (20,24) However, these components can be present even at early stages of maturation. Luckily, the developing cardiomyocytes undergo myofibrillar isoform switching, which can be utilized to further assess the

maturation. The relation of Troponin I indicates maturation as immature cardiomyocytes express slow skeletal troponin I (ssTnI/TNNI1) but only adult cardiomyocytes express cardiac troponin I (cTnI/TNNI3). (22) Increase in the MYH7/MYH6-ratio is also associated with cardiac maturation since the human heart expresses more  $\beta$ -MHC than  $\alpha$ -MHC but there is relatively more  $\alpha$ -MHC present in the fetal heart (23). The expression of various proteins related to ion passing on the sarcolemma and sarcoplasmic reticulum has also proven lower in hiPSC-CMs than in mature cardiomyocytes (23). From the regulatory perspective, hiPSC-CMs often express transcription factors GATA-4, Nkx2.5, Isl-1, Tbx-20 and Mef2c which again are related to cardiogenesis (20,24).

Along with inadequate function of immature cells, undifferentiated cells could result in teratomas or non-cardiac cells (25). *In vivo* trials in turn have demonstrated that the retention rate of hiPSC-CMs in the injured heart is low, most likely due to ischemic conditions (3,25). Infarcted myocardium is a hostile environment and the delivered cells need to endure hypoxic and inflammatory conditions, attach to the host tissue and survive.

Various of strategies have been described to improve these cells' survival including cell conditioning prior to transplant, utilization of anti-apoptotic pathways, inflammation inhibition, angiogenic and homing factors and co-transplantation with a supporting cell type. Cell conditioning can be used to induce endogenous cellular survival mechanisms while suppressing apoptotic pathways. It can also be used to adapt the cells to hypoxia. Angiogenesis is vital for reparation of damaged myocardium. Angiogenic factors can be induced during cell conditioning albeit pre-treating the host myocardium with angiogenic growth factors such as VEGF could also be utilized. Pro-inflammatory cytokines, especially interleukin-1, which are abundant in ischemic myocardium mediate cardiomyocyte apoptosis and fibrosis. Co-transferring cytokine inhibiting cells like skeletal myoblasts could therefore modulate inflammation and assist cell survival. Co-transplantation could also facilitate implantation as it is believed to be the case with supporting stromal cells. (25)

## 2.5 Co-culturing of MSCs and hiPSC-CMs

### 2.5.1 MSCs' properties regarding cardiac reparation

#### *Paracrine mechanisms*

MSCs are known for secreting various paracrine factors which impacts regarding cardiac repair are diverse. Pro-survival cytokines and growth factors such as basic fibroblast growth factor (bFGF) and insulin-like growth factor 1 (IGF-1) inhibit apoptosis and support proliferation, survival and differentiation. Fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF) on the other hand are crucial for angiogenesis. Metalloproteinases remodel the extracellular matrix whereas hepatocyte growth factor (HGF), interleukin 6 (IL-6) and prostaglandin E2 (PGE2) modulate the immune system, especially by T-cell downregulation. (3,11)

*In vitro* studies on MSCs have demonstrated they secrete growth factors IGF-1, HGF, VEGF and FGF-2. These factors have been upregulated in hypoxic MSCs overexpressing pro-survival gene Akt1 and have since improved cardiac function in rat models. In addition, akt-MSCs have produced frizzled related protein (Sfrp2) which supports the survival of ischemic myocardium. (11) There's evidence that IGF-1 can enhance PCS-CMs' cardiac gene expression, Ca<sup>2+</sup> handling and  $\beta$ -adrenergic response by activating Akt-kinase signaling. VEGF and FGF can also activate this signaling pathway. In addition, FGF increases formation of gap junctions between CMs which improves their electrical functions. (24)

Besides soluble factors, MSCs excrete exosomes and related extracellular vesicles (EVs) that contain nucleic acids such as microRNAs. These microvesicles are involved in intracardiac communication and possess cardioprotective effects *in vivo* animal trials. Furthermore, hypoxia induces the release of EVs by the MSCs. (26) Microvesicles and soluble factors mentioned above have shown to enhance cardiomyocyte survival, recruit endogenous cardiac stem cells and reduce scarring mechanisms leading eventually to cardiac repair and functional improvements *in vivo* models. (3,11,26)

We hypothesized that the paracrine factors secreted by MSCs might also support the survival of hiPS-cardiomyocytes and even further stimulate their maturation process via conditioned culturing medium. A recent study on co-culture of hiPS-cardiomyocytes and human mesenchymal

stem cells has shown that MSCs' paracrine properties have supported the maturation of hiPS-cardiomyocytes (27).

#### *Cell-to-cell contacts*

It has been demonstrated that MSCs and cardiomyocytes can also communicate through cell-to-cell interactions (28,29). Junction formation via connexin and N-cadherin has been studied for their importance in electromechanical coupling (28–30). It has also been shown that MSCs are able to couple with cardiomyocytes through partial cell fusion and thin membrane channels, nanotubes. Nanotubes allow a cytosolic connection and transfer of proteins or even organelles between cells. These tubes and gap junctions allow the transfer of energy producing mitochondria from MSCs to cardiomyocytes. (28,29) An *in vitro* study on rat cells has shown that direct cell-to-cell contact induces MSCs to differentiate into cardiomyocytes (31). In addition, cell-to-cell interaction plays a role in downregulation of NK-activity and therefore has an immunoregulatory aspect (26).

#### *Differentiation potential*

Although *in vivo* and *in vitro* studies on animal cells indicate that MSCs can differentiate into cardiomyocytes, the question whether this can be executed in fully human models remains controversial. Different approaches have been experimented in order to differentiate MSCs into cardiomyocytes: DNA methyltransferase inhibitor 5-azacytidine, modified cardiomyogenic medium, TSA (histone deacetylase inhibitor trichostatin A) treatment and co-culturing with cardiomyocytes. (11,31–33).

5-azacytidine (5-aza) treatment has its limitations as repeated treatment with 5-aza prevents human cell growth, causes apoptosis and it has created cardiomyocyte-like cells only with animal cells. As for human AdMSCs, TSA treatment has increased cardiac actin mRNA expression significantly. The expression of cardiac myosin heavy chain,  $\alpha$ -actin, cardiac troponin I and connexin 43 were also increased but only direct co-culturing with rat cardiomyocytes has produced beating cells with gap junctions to native myocytes. (32)

Other potential mechanisms for cardiomyogenesis are cytokine signaling agents such as growth factors and interleukins, different microenvironmental factors such as culturing medium, electrostatic fields and culturing substrates (34). Some studies implicate that transdifferentiation

requires cell-to-cell contact with native myocytes while others conflict with these findings (11,31). Therefore, it could be assumed that both physical and paracrine mechanisms play a role.

The differentiation of mesenchymal stem cells is a complex process regulated mainly by TGF- $\beta$  superfamily, especially BMP, and Wnt signaling pathways. BMPs induce cartilage, bone, tendon and adipose lineages as Wnt signaling suppresses adipocyte and chondrocyte formation directing the differentiation process towards osteogenesis. Platelet derived growth factor (PDGF) pathway directs MSCs towards angiogenesis alongside with vascular endothelial growth factor (VEGF). Cardiogenic gene expression relies on cell-to-cell relayed transmembrane receptor Notch signaling pathway. (35)

### **2.5.2 Previous studies**

A co-culture study on murine cells indicated that MSCs improved morphological integration of hiPS-CMs to nonvital and vital ventricular slices. Additionally, co-culture involving MSCs or MSC conditioned medium with hiPS-CMs improved electrical integration with the vital ventricular slices. (36) A recent study on human cells discovered that BM-MSCs improved hiPS-CMs' expression of cardiac troponin T (cTnT) and other cardiac markers as well as structural, electrophysiological and metabolic development, motility and maturity. The maturation process seemed to be linked to soluble factors secreted by the MSCs but the exact mechanism remains unclear. (27)

Due to the lack of previous research our aim is to describe the effect of hMSC and hiPS-CM co-culture on the cells. Our additional goal is to determine the optimal medium and ratio of the cells in this co-culture. In an ideal situation MSCs would have beneficial effects on the cardiomyocytes maturation and survival without taking over the cultures. The cultures also have to be sparse enough for analysis and cell survival.

## **3. MATERIALS AND METHODS**

### **3.1 Ethical considerations**

The preparation and use of the iPS-cells in this study has been approved by the Ethical Committee of Pirkanmaa Hospital District. The donors of the skin fibroblast used to regenerate these cells have given their written consents for this purpose. These consents have since been filed according to the EU-directive regarding the use of human organs, tissues and cells in medical use.

### **3.2 Materials**

The study material consists of hiPS-cell line UTA.04602.WT prepared in advance by Tampere University's Heart Group. Reprogramming of the cells was performed using retroviral vectors, SOX-2, OCT-3/4, KLF4 and CMYC. (37) Additionally, human mesenchymal stem cells derived from adipose tissue (hMSC, 6/11 p.4) were used. The derivation of adipose tissue derived hMSCs have been described previously by Tirkkonen L et al. (38).

### **3.3 Culturing of the hiPS cells**

iPS cells were cultured for 7 to 14 days in two feeder-free Geltrex™-matrix (Geltrex was diluted in 1:100 ratio to DMEM/F-12 basal medium, Gibco Life Technologies) coated 6-well plates. mTeSR™1 (StemCell Technologies) medium including 0,5 % Pen-Strep-antibiotic (Sigma-Aldrich) was used. Half of the medium was replaced every two to three days.

### **3.4 Differentiation of the hiPS cells into cardiomyocytes**

The differentiation process shown in Table 1 was carried out using a method modified from the differentiation protocol created by Karakikes et al. (39). This method involved the spontaneous embryoid body formation combined with the use of growth factors. In the first batch approximately fourth of the targeted coverage of the wells was reached. Culturing medium was aspirated, and the cells dissociated by adding 1ml of versene (Thermo Fisher Scientific) per well for 10 minutes. All the cells were resuspended in one well containing 3ml of mTeSR™1 (StemCell Technologies) supplemented with 5µM Blebbistatin (Sigma-Aldrich). Finally, the cells were divided into a 6-well low-attachment plate containing the same medium as previously. For differentiation the medium was changed for eleven days according to Table 1. Hence RPMI + B27 medium (Gibco, Life Technologies) with insulin was used and replaced every two to three days until the co-culture.

*Table 1. The growth factor induced cardiac differentiation protocol of embryoid bodies.*

Day	Medium	Basal medium	Growth factors
0	mTeSR™1	mTeSR	rh bFGF rh TGFβ Blebbistatin
1	RPMI + B27 - insulin	RPMI	5μg/ml Ascorbic acid 10ng/ml BMP4 25ng/ml Activin A
3	RPMI + B27 - insulin	RPMI	5μg/ml Ascorbic acid
4	RPMI + B27 - insulin	RPMI	2.5μM IWP-4
8	RPMI + B27 - insulin	RPMI	-
11	RPMI + B27	RPMI	Insulin

The cells were dissociated by using MACS Multi Tissue Dissociation Kit 3 (Miltenyi Biotec) according to the manufacturer's instructions. After the dissociation the cells were counted by using a hemacytometer. In the first batch cell number was low, approximately 510 000 cells in total.

For isolation of the cardiomyocytes MACs PSC-Derived Cardiomyocyte Isolation Kit (Miltenyi Biotec) and its isolation protocol was used. The first isolation yielded 163 000 cells with a purity up to 97 % according to Miltenyi Biotec. This amount determined the use of 4000 or 8000 hiPS-cardiomyocytes per well depending on the desired proportion in relation to Mesenchymal stem cells. In a 24-well plate this resulted in a density of 2000 and 4000 cardiomyocytes/cm<sup>2</sup>.

### **3.5 The co-culture of hiPSC-cardiomyocytes and human adipose tissue derived mesenchymal stem cells**

In total three separate batches were prepared in 24-well plates. Four different co-cultures were created with the percentage of mesenchymal stem cells (MSC) being 3 %, 6 %, 10 % and 20 %. Practically 120 MSCs were added into cultures of 2000 and 4000 cardiomyocytes which resulted in a ratio of 6 % and 3 % of MSCs, respectively. 10 % and 20 % well were made in the same manner with the MSC count being 400. In addition, hiPSC-cardiomyocytes were cultured in MSCs' conditioned media and in two different control groups which contained no MSCs. The culturing medium used was DMEM/F12 (Thermo Fisher Scientific) with 10 % FBS (PAA Laboratories GmbH),

1 % glutamax (Invitrogen) and 1 % Penicillin/Streptomycin (Lonza). Half of this medium was replaced with MSC-culture conditioned medium for the conditioned medium samples. For the other control group EB medium was used and it consisted of: KO-DMEM (Thermo Fisher Scientific) with 20 % FBS (PAA Laboratories GmbH), 1 % non-essential aminoacids (NEAA, Cambrex Bio Science), 1 % glutamax and 0,5 % Penicillin/Streptomycin. In each well 1ml of medium was used, half of which was replaced every two to three days.

The wells were plated in three different manners in order to examine the effect of the plating strategy. Each well was coated with Geltrex. Additionally, in the first batch the wells were covered with glass slips and the cardiomyocytes were plated in a droplet onto the gel. In the second batch glass slips weren't used and cardiomyocytes were plated into the media. In the third batch both of the previous methods were used but the cardiomyocytes were also plated in a droplet without the slips. The different plating strategies are illustrated in the following diagram 1 which represents the third plate.

<b>8000 CMs</b>	<b>8000 CMs</b>	<b>4000 CMs</b>	<b>8000 CMs</b>	<b>4000 CMs</b>	<b>4000 CMs</b>
<b>Control</b> Droplet + slip	<b>3% MSC</b> Droplet + slip	<b>6% MSC</b> Droplet + slip	<b>10% MSC</b> Droplet + slip	<b>20% MSC</b> Droplet + slip	<b>Conditioned</b> Droplet + slip
<b>Control</b> Droplet	<b>3% MSC</b> Droplet	<b>6% MSC</b> Droplet	<b>10% MSC</b> Droplet	<b>20% MSC</b> Droplet	<b>Conditioned</b> Droplet
<b>EB control</b> Droplet + slip	<b>3% MSC</b> -	<b>6% MSC</b> -	<b>10% MSC</b> -	<b>20% MSC</b> -	<b>Conditioned</b> -
<b>EB control</b> Droplet	<b>3% MSC</b> -	<b>6% MSC</b> -	<b>10% MSC</b> -	<b>20% MSC</b> -	<b>Conditioned</b> -

*Diagram 1. Example of the 24-well plate representing the third batch. The different MSC concentrations were achieved by altering the number of plated hiPSC-CMs (8000 or 4000). Control stands for MSC medium control.*

### 3.6 Microscopy imaging of the co-cultures

The co-cultures were observed for 14 days by using Nikon Eclipse TS100 phase contrast microscope (Instruments Europe B.V. Amstelveen, The Netherlands). Images were captured every two to three days of every culturing condition.

### 3.7 Fixation and immunocytochemical staining

The cultures were fixed and dyed according to our Heart Group's double-fluorescence protocol (table 2). In brief, the cells were washed with PBS then fixed with 4 % PFA and blocked. After blocking and washing, a mixture of primaries Anti-Cardiac Troponin T (Abcam) and Vinculin (LifeTechnologies) or Vimentin (Abcam) was introduced. After an overnight incubation the cells were washed again and a mixture of secondaries (Alexa Fluor) was introduced and incubated for an hour. After multiple of washes samples were dried and mounted with DAPI containing vectashield (Vector) and stored light protected. The last two batches were dyed simultaneously, and the primaries were incubated only briefly.

*Table 2. The double-fluorescence protocol*

<i>Step</i>	<i>Reagents</i>
<b>Wash 2 x 5 min</b>	PBS (0.01M, pH 7.4)
<b>Fixing 20 min</b>	4% PFA
<b>Wash 2 x 5 min</b>	PBS (0.01M, pH 7.4)
<b>Blocking 45 min, room temperature</b>	10% NDS (Biowest), 0.1% TritonX-100 (Sigma), 1% BSA (Sigma) in PBS
<b>Wash</b>	1% NDS, 0.1% TritonX-100, 1% BSA in PBS
<b>Mixture of primaries, overnight +4°C</b>	<ol style="list-style-type: none"> <li>1. Troponin T (Abcam), origin: goat dilution 1:2000 OR</li> <li>2. Troponin T (Abcam), origin: mouse dilution 1:500</li> <li>1. Vinculin Ab (LifeTech.), origin: rabbit dilution 1:100 OR</li> <li>2. Vimentin (Abcam), origin: goat dilution 1:300</li> </ol> in 1% NDS, 0.1% TritonX-100, 1% BSA in PBS
<b>Wash 3 x 5-7 min</b>	1% BSA (Sigma) in PBS
<b>Mixture of secondaries 1h, room temperature</b>	<ol style="list-style-type: none"> <li>1. Anti-goat 568(Alexa Fluor) origin: donkey dilution 1:600 OR</li> <li>2. Anti-mouse 568 (Alexa Fluor) origin: donkey dilution 1:600</li> <li>1. Anti-rabbit 488 (Alexa Fluor) origin: donkey dilution 1:600 OR</li> <li>2. Anti-goat 488 (Alexa Fluor) origin: donkey dilution 1:600</li> </ol>
<b>Wash 3 x 5 min</b>	PBS (0.01M, pH 7.4)
<b>Wash 2 x 5 min</b>	PB
<b>Dry and mount + coverslips</b>	Vectashield mounting medium with DAPI (Vector)

### 3.8 Fluorescence imaging

The IX 51 Inverted Microscope (Olympus) was used for fluorescence imaging.

Individual cardiomyocytes were counted from these images and categorized by their nucleus count, uni- or multinucleated, in the following groups: all control samples and all co-cultures with 6 %, 10 % and 20 % of MSCs. The statistical analysis of dependence was performed with SPSS release 23.0 (IBM Corp, Armonk, NY using the Pearson  $\chi^2$ -test. Statistical significance was set to  $p < 0.05$ .

### 3.9 Quantitative polymerase chain reaction

RNA was isolated from the samples and then converted into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific). 2 x RT Master Mix was prepared from the following reagents: 20 % 10 x RT Buffer, 8 % 25 x dNTP Mix (100mM), 20 % 10 x RT Random Primers, 10 % Multiscribe Reverse Transcriptase, 10 % RNase inhibitor and 32 % nuclease free H<sub>2</sub>O. In addition, a Reverse Transcriptase-free control mixture was prepared, and its volume was corrected with nuclease free H<sub>2</sub>O. RNA samples were mixed into 2 x RT Master Mix in a 1:2 ratio with a final volume of 20  $\mu$ l. Two 2 x RT Master Mix controls without RNA samples and two RT-free Master Mix controls were prepared. In total four 8-tube strips were created as illustrated in diagram 2. cDNA conversion was executed using Mastercycler Pro 5341's (Eppendorf AG, Hamburg) thermal cycle program (table 3).

8-tube strips							
D3% <sub>1</sub>	D3% <sub>2</sub>	D6% <sub>1</sub>	D6% <sub>2</sub>	D10% <sub>1</sub>	D10% <sub>2</sub>	D20% <sub>1</sub>	D20% <sub>2</sub>
S3% <sub>1</sub>	S3% <sub>2</sub>	S6% <sub>1</sub>	S6% <sub>2</sub>	S10% <sub>1</sub>	S10% <sub>2</sub>	S20% <sub>1</sub>	S20% <sub>2</sub>
DC <sub>1</sub>	DC <sub>2</sub>	SC <sub>1</sub>	SC	EB <sub>1</sub>	EB <sub>2</sub>	MSC <sub>1</sub>	MSC <sub>2</sub>
H <sub>2</sub> O <sub>1</sub>	H <sub>2</sub> O <sub>2</sub>	-RT + H <sub>2</sub> O	-RT + EB				

**Diagram 2. The converted cDNA samples.**

Code

D: Plated as a droplet

S: Spread, plated in the medium

C: MSCs' conditioned medium

EB: Embryoid body medium control

MSC: MSC medium control

H<sub>2</sub>O: 2 x RT Master Mix control, no sample

-RT: RT-free Master Mix control without sample and with EB medium sample

%: the percentage of MSCs in the sample

**Table 3. Thermal cycle program used for cDNA conversion.**

	Step 1	Step 2	Step 3	Step 4
Temperature	20°C	37°C	85°C	4°C
Time	10min	120min	5min	>>>

The cDNA concentration in each sample was 5ng per 10µl after 1:2 dilution assuming all of the RNA had converted. Four qPCR Master Mixes were prepared using the following reagents: for each reaction 5µl TaqMan Gene Expression Master Mix (AppliedBiosystems, Thermo Fisher Scientific), 0,5µl 20xTaqMan Assays pool (GAPDH, TNNT2, Myh6 or Myh7) and 3,5µl nuclease free H<sub>2</sub>O. cDNA samples were added in a 1:10 ratio into two 96-well plates according to diagram 3. Each plate contained qPCR Master Mix with a different primer: GAPDH, TNNT2, Myh6 or Myh7. As to the last plate, only one droplet plated 3 % co-culture sample was created.

D3% <sub>1</sub>	D6% <sub>1</sub>	D10% <sub>1</sub>	D20% <sub>1</sub>	S3% <sub>1</sub>	S6% <sub>1</sub>	S10% <sub>1</sub>	S20% <sub>1</sub>	DC <sub>1</sub>	SC <sub>1</sub>	EB <sub>1</sub>	MSC <sub>1</sub>
D3% <sub>1</sub>	D6% <sub>1</sub>	D10% <sub>1</sub>	D20% <sub>1</sub>	S3% <sub>1</sub>	S6% <sub>1</sub>	S10% <sub>1</sub>	S20% <sub>1</sub>	DC <sub>1</sub>	SC <sub>1</sub>	EB <sub>1</sub>	MSC <sub>1</sub>
D3% <sub>1</sub>	D6% <sub>1</sub>	D10% <sub>1</sub>	D20% <sub>1</sub>	S3% <sub>1</sub>	S6% <sub>1</sub>	S10% <sub>1</sub>	S20% <sub>1</sub>	DC <sub>1</sub>	SC <sub>1</sub>	EB <sub>1</sub>	MSC <sub>1</sub>
D3% <sub>2</sub>	D6% <sub>2</sub>	D10% <sub>2</sub>	D20% <sub>2</sub>	S3% <sub>2</sub>	S6% <sub>2</sub>	S10% <sub>2</sub>	S20% <sub>2</sub>	DC <sub>2</sub>	SC <sub>2</sub>	EB <sub>2</sub>	MSC <sub>2</sub>
D3% <sub>2</sub>	D6% <sub>2</sub>	D10% <sub>2</sub>	D20% <sub>2</sub>	S3% <sub>2</sub>	S6% <sub>2</sub>	S10% <sub>2</sub>	S20% <sub>2</sub>	DC <sub>2</sub>	SC <sub>2</sub>	EB <sub>2</sub>	MSC <sub>2</sub>
D3% <sub>2</sub>	D6% <sub>2</sub>	D10% <sub>2</sub>	D20% <sub>2</sub>	S3% <sub>2</sub>	S6% <sub>2</sub>	S10% <sub>2</sub>	S20% <sub>2</sub>	DC <sub>2</sub>	SC <sub>2</sub>	EB <sub>2</sub>	MSC <sub>2</sub>
						-RT <sub>1</sub>	-RT <sub>1</sub>	-RT <sub>1</sub>	H <sub>2</sub> O <sub>1</sub>	H <sub>2</sub> O <sub>1</sub>	H <sub>2</sub> O <sub>1</sub>
						-RT <sub>2</sub>	-RT <sub>2</sub>	-RT <sub>2</sub>	H <sub>2</sub> O <sub>2</sub>	H <sub>2</sub> O <sub>2</sub>	H <sub>2</sub> O <sub>2</sub>

**Diagram 3. Samples for qPCR.** Four comparable plates with a different primer were created. Primers used were TNNT2, GAPDH, MYH6 and MYH7.

The quantitative PCR was carried out in 7300 Real Time PCR System (AppliedBiosystems) as shown in table 4.

**Table 4. qPCR program.**

Temperature	time	cycles
50°C	2min	1
95°C	10min	
95°C	15s	40
60°C	1min	

To evaluate relative quantification the  $2^{-\Delta\Delta Ct}$  method was used (40). GAPDH was set as the internal control gene and MSC medium control was chosen as the calibrator. The fold change of the target genes TNNT2, MYH6 and MYH7 was evaluated in the EB medium control, conditioned medium and co-cultures with 6 % and 10 % of MSCs. The number of samples was 6 for the MSC and EB medium controls and 12 for the conditioned medium and co-cultures. Each culturing condition was compared with the MSC medium control. The statistical analysis of the differences was conducted using Kruskal-Wallis test for independent samples in SPSS release 23.0 (IBM Corp, Armonk, NY). Statistical significance was set to  $p < 0.05$ .

## 4. RESULTS

### 4.1 Microscopy and culture appearance

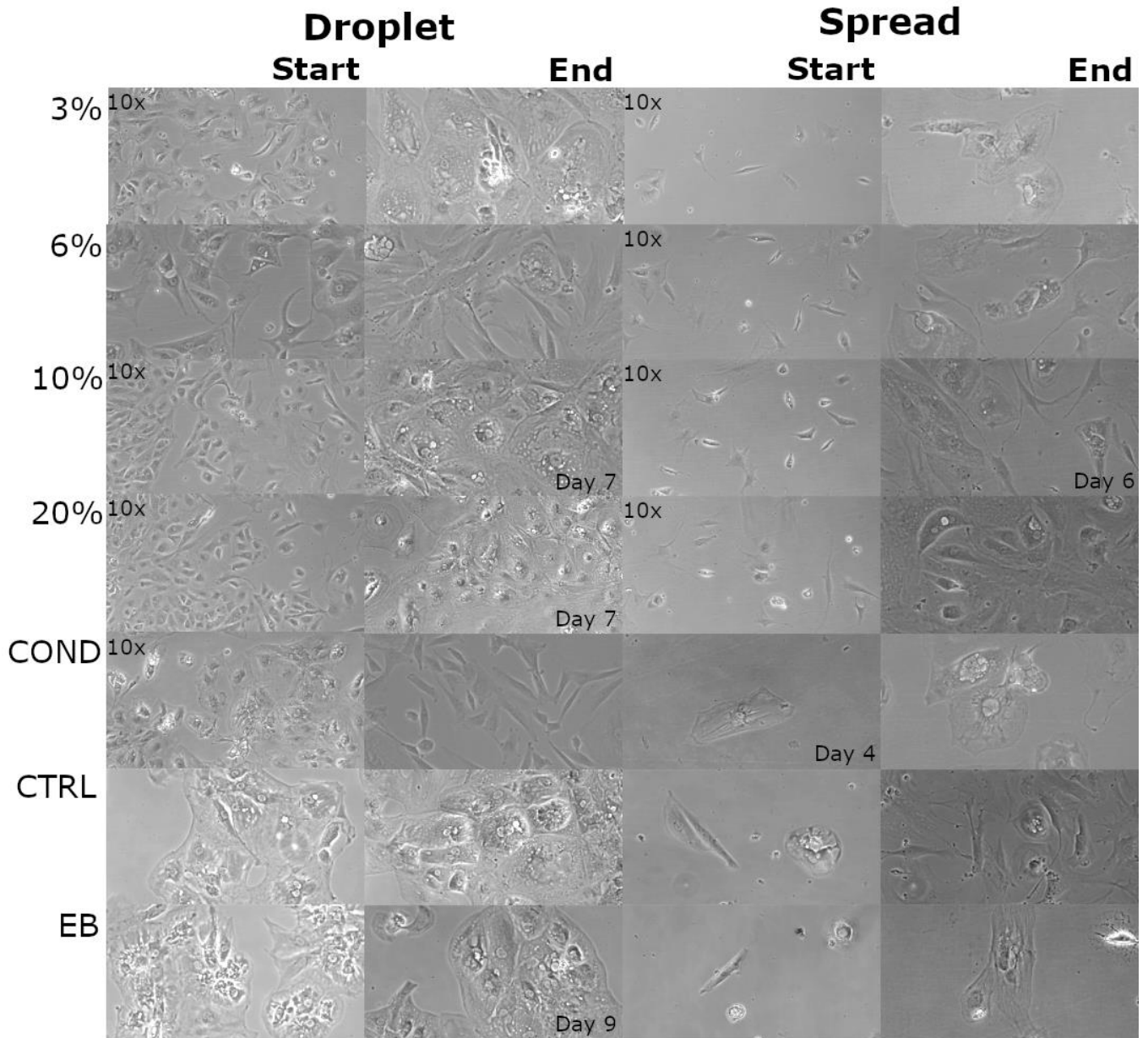
Panel 1 shows the difference between the used plating strategies as well as the test and control cultures at the beginning and end of culturing. Samples in which hiPSC-cardiomyocytes were plated onto the gel in a droplet will be referred as the “droplet group” as the samples in which hiPSC-CMs were plated directly into the culturing medium will be referred as the “spread group”.

As seen in panel 1, the cultures were already dense in the droplet group while being sparse in the spread group during day 1 or 2 of culturing. Individual hiPSC-CMs are clearly visible throughout the spread cultures and within the edges of the cell clusters in the droplet group. Panel 1 also shows that the cultures grew in all culturing conditions in the droplet group. Co-cultures with 10 % and 20 % of hMSCs were most dense. In the spread group the EB controls and conditioned cultures were still sparse at this point. Co-cultures became increasingly full complying with the ratio of hMSCs.

hMSCs appeared in all co-cultures and were detected increasingly according to their plated ratio (Panel 1). At the beginning of the co-culturing individual hMSCs were observed amidst the hiPSC-CMs but most of them appeared within the margins of the cultures (image 1) in the droplet group, whereas hMSCs situated randomly amongst the hiPSC-CMs (panel 1) in the spread group. Proliferation of the hMSCs was rapid and more evident in spread group (image 3). As the cultures grew in it became difficult to distinguish the cell types. hMSCs seemed to adhere to one another with long projections (image 2). Only hiPSC-CMs were present in the conditioned samples and controls apart from the MSC-medium control in the spread group which was full of fibroblast-like cells at the end of the trial.

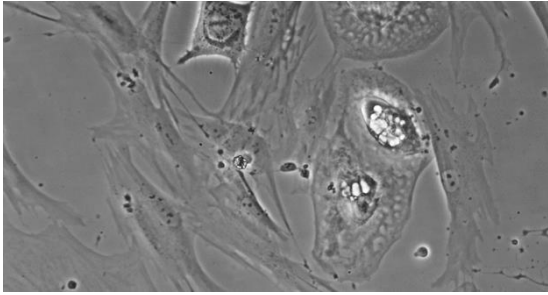
It becomes evident from panel 1 that the hiPSC-CMs appeared similar in the test and control samples throughout culturing. Beating cells were detected in all culturing conditions but weren't analyzable. In the droplet group, hiPSC-CMs were mainly round or cubic in shape in all culturing conditions in the beginning of the trial whereas multiple elongated hiPSC-CMs appeared in the spread group. By the end of the culturing, most of the hiPSC-CMs in the spread group appeared

also round or cubic in shape. Lace-like structuring or intracellular vesicles of the hiPSC-CMs appeared after culturing in all culturing conditions. In panel 1 this can be seen best in the droplet group co-culture with 10 % of hMSCs at day 7. Enlarged hiPSC-CMs (image 4) were present in areas sparse with cells, that is in the spread cultures and within the edges in the droplet group.

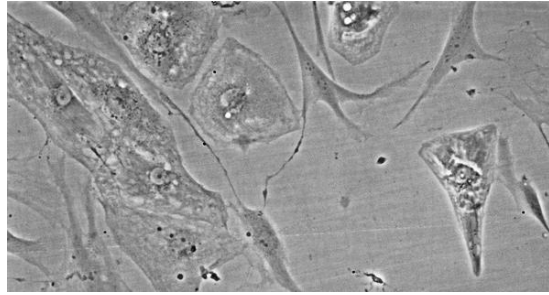


Start: day 1 or 2 unless marked otherwise  
 End: day 11 or 14 unless marked otherwise

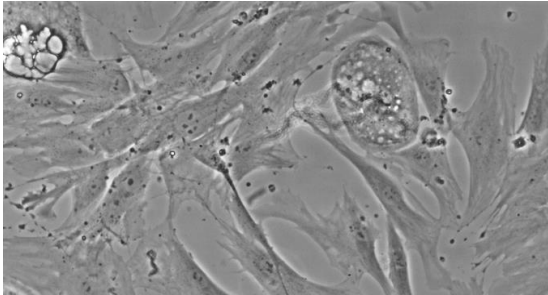
**Panel 1. Light microscope images captured during two weeks of culturing.** Spread and droplet plated hiPSC-CMs are presented separately. Wells with droplet plated hiPSC-CMs are already dense at the beginning of culturing whereas individual hiPSC-CMs are abundant in “the spread group” even by the end of culturing. hMSCs appear in all co-cultures and increase according to their plated ratio. hMSCs are easier to detect amidst the hiPSC-CMs and seem to expand more rapidly in “the spread group”. Multiple elongated hiPSC-CMs are seen in “the spread group” at the beginning but not at the end of culturing.



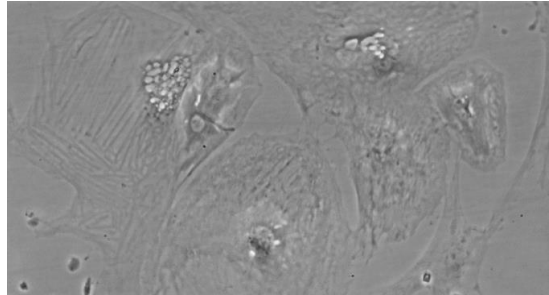
*Image 1. hMSCs in the margins of the well.*



*Image 2. Projections between adjacent hMSCs.*



*Image 3. Multiple hMSCs in spread 6% MSC co-culture.*



*Image 4. Enlarged hiPSC-CMs.*

## 4.2 Fluorescence images

As seen in panel 4, the appearance of the hiPSC-cardiomyocytes seems similar throughout all culturing conditions and is mainly round or cubic despite the plating strategy or the test setup. Few cells with sarcomeres and ladder structures are detected by eye but no culturing condition seems to exhibit this more than others.

In general, cultures in the droplet group and the spread co-cultures and are highly abundant with cells as the EB-control and conditioned medium cultures in the spread group are relatively sparse (Panel 4). Cultures with hiPSC-CMs plated in a droplet created hiPSC-CMs clusters whereas there were few adjacent hiPSC-CMs in the spread group (Panel 2). Only hiPSC-CMs are present in the control and conditioned medium cultures, except for the MSC-medium control of the second trial in which fibroblast-like cells are abundant in all samples (Panel 4).

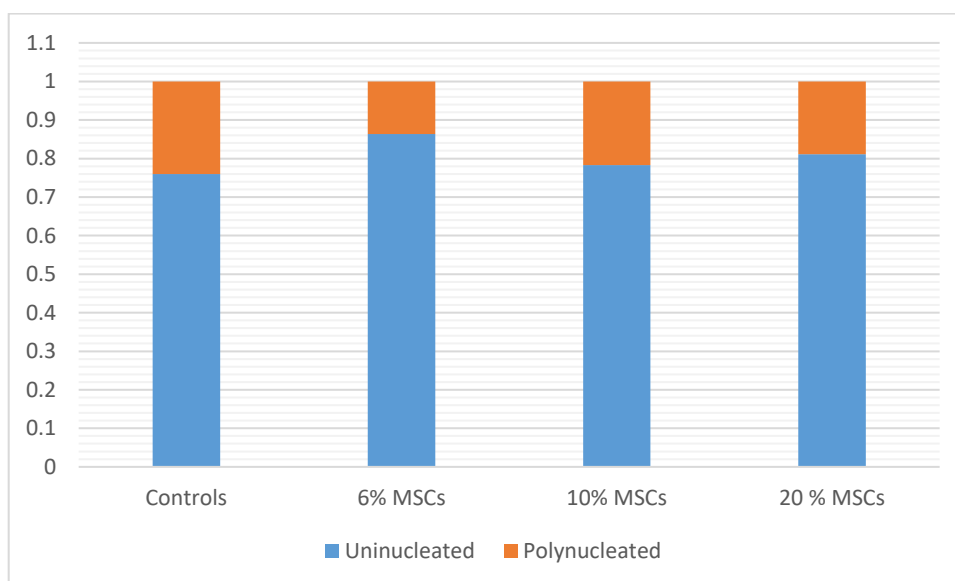
Vimentin or vinculin stained cells (green), presumably hMSCs, can be found in all co-cultures. These cells seem increasingly abundant in higher hMSC concentrations as expected. All the samples, droplet or spread, with 20 % of hMSCs are packed with these cells. The count of hMSCs seems to increase when the hiPSC-CMs are scattered, that is, in the spread group and in the margins of the droplet cultures (Panel 2 and 3). hMSCs seem to mainly surround the hiPSC-CMs when the hiPSC-

CMs are clustered. However, hMSCs begin to overlap cardiomyocytes in high hMSC concentrations and when the hiPSC-CMs are more scattered (Panel 2 and 3).

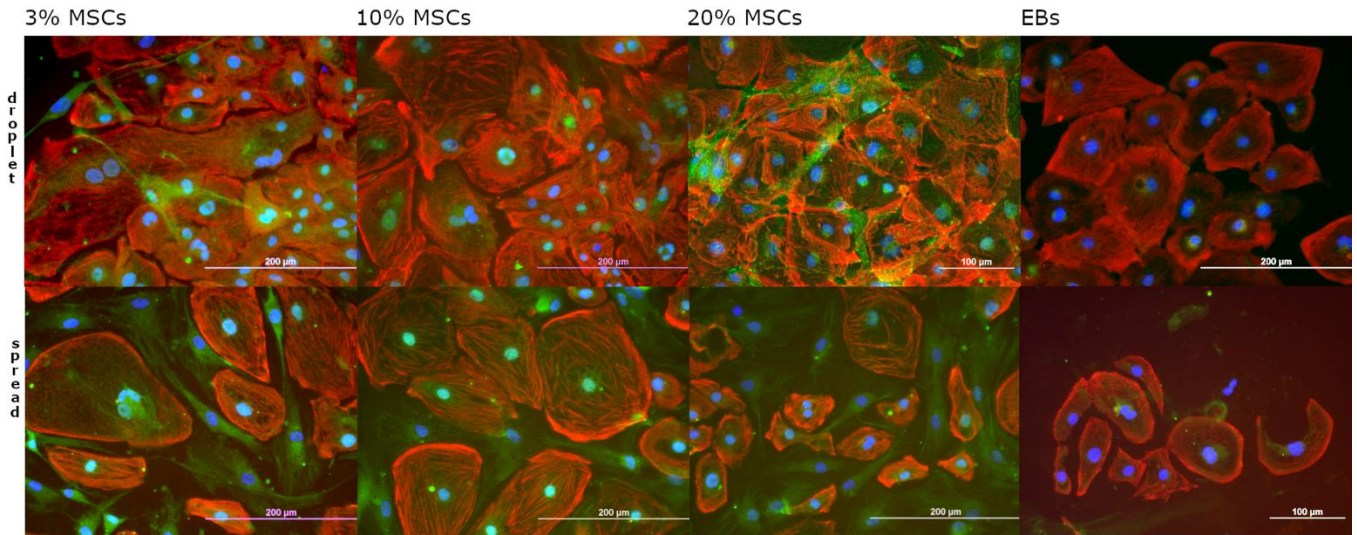
As seen in diagram 4, the nucleus count did not depend on the presence of hMSCs,  $\chi^2(3, N = 381), = 3,538, p = 0,316$ . The ratio of multinucleated cells was highest (24 %) in the control group and in the co-culture with 10 % of hMSCs (22 %). Co-culture with 6 % of hMSCs had the lowest count of multinucleated cells (14%).

**Table 5. The number and portion of uni- and polynucleated cells in different culturing conditions. There was no significant difference in the amount of polynucleated hiPSC-CMs between the groups.**

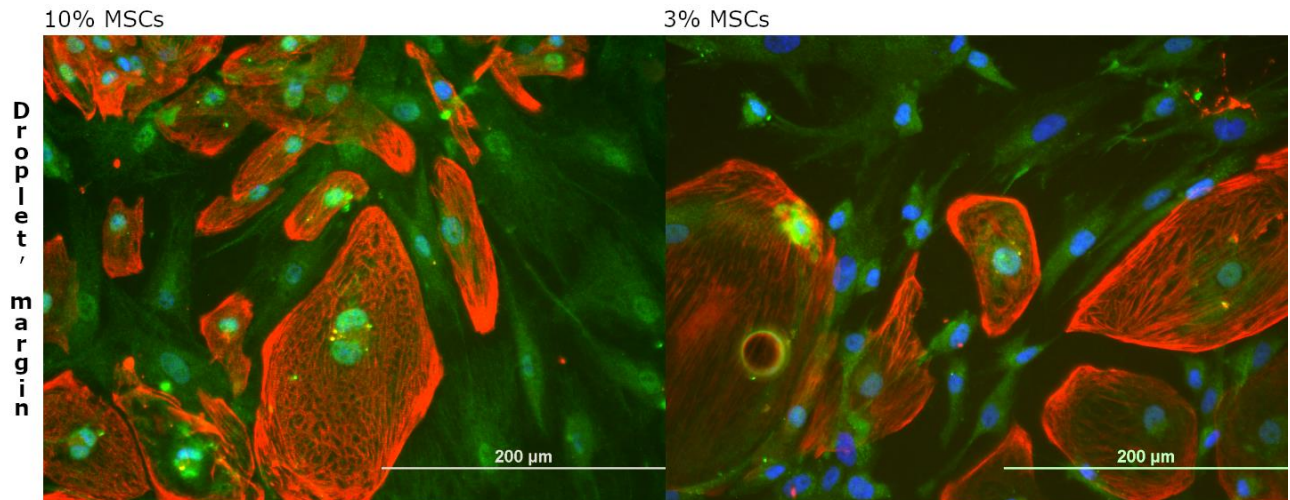
Culturing condition	Cell count (CMs)			Portion	
	Uninucleated	Polynucleated	Total	Uninucleated	Polynucleated
Controls	79	25	104	0,75961538	0,24038462
6% MSCs	76	12	88	0,86363636	0,13636364
10% MSCs	65	18	83	0,78313253	0,21686747
20 % MSCs	86	20	106	0,81132075	0,18867925



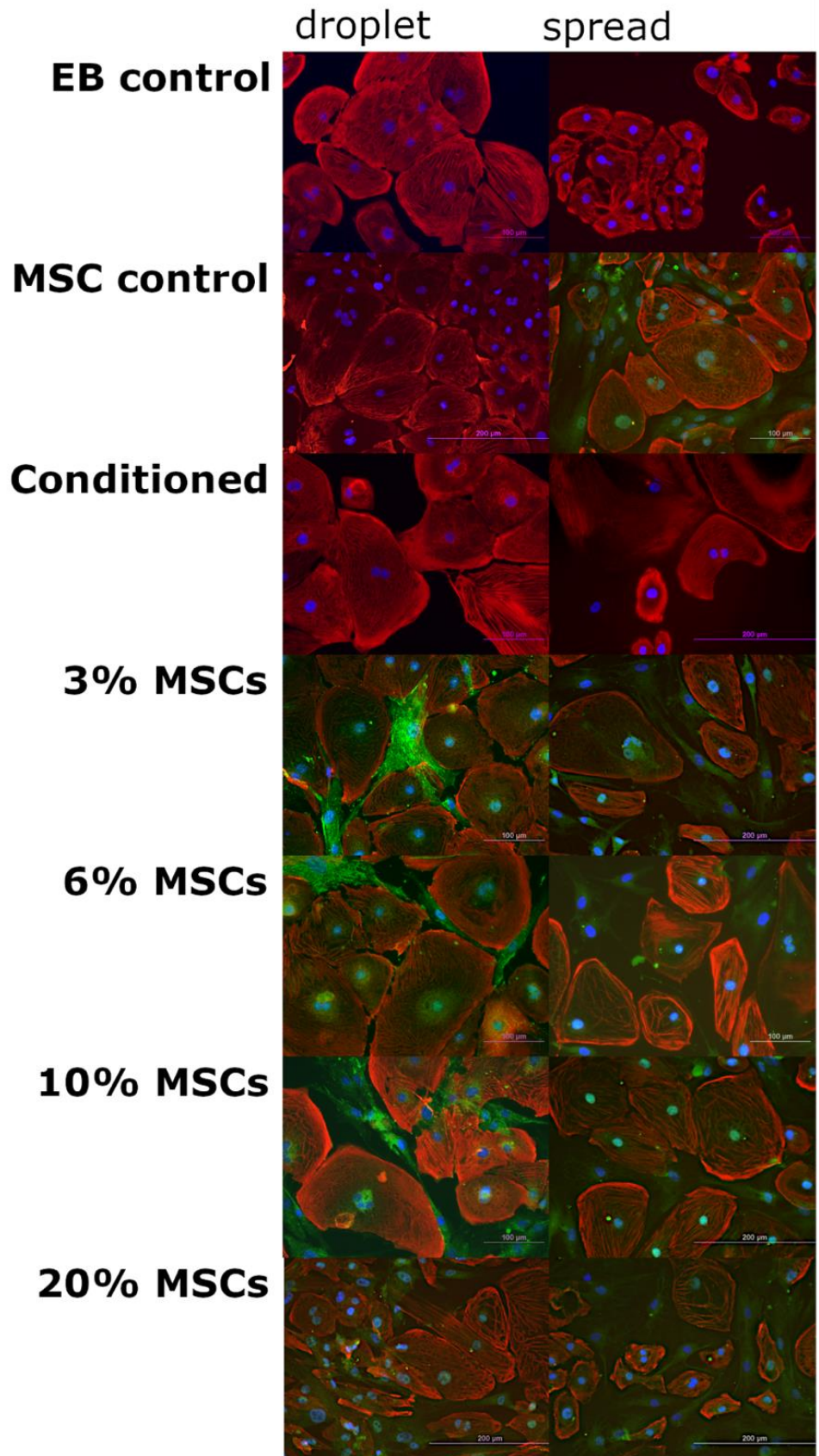
**Diagram 4. Cell distribution according to the nucleus count.**  
hMSCs did not affect the number of polynucleated hiPSC-CMs



**Panel 2. Coverage of the wells and cell distribution in the “droplet group” compared to the “spread group”.** hiPSC-CMs plated in a droplet created cell clusters as there are fewer adjacent cells in the “spread group”. hMSCs are more abundant in the spread group and intrude the space between hiPSC-CMs. hMSCs overlap hiPSC-CMs in higher concentrations (20 % MSCs, droplet).



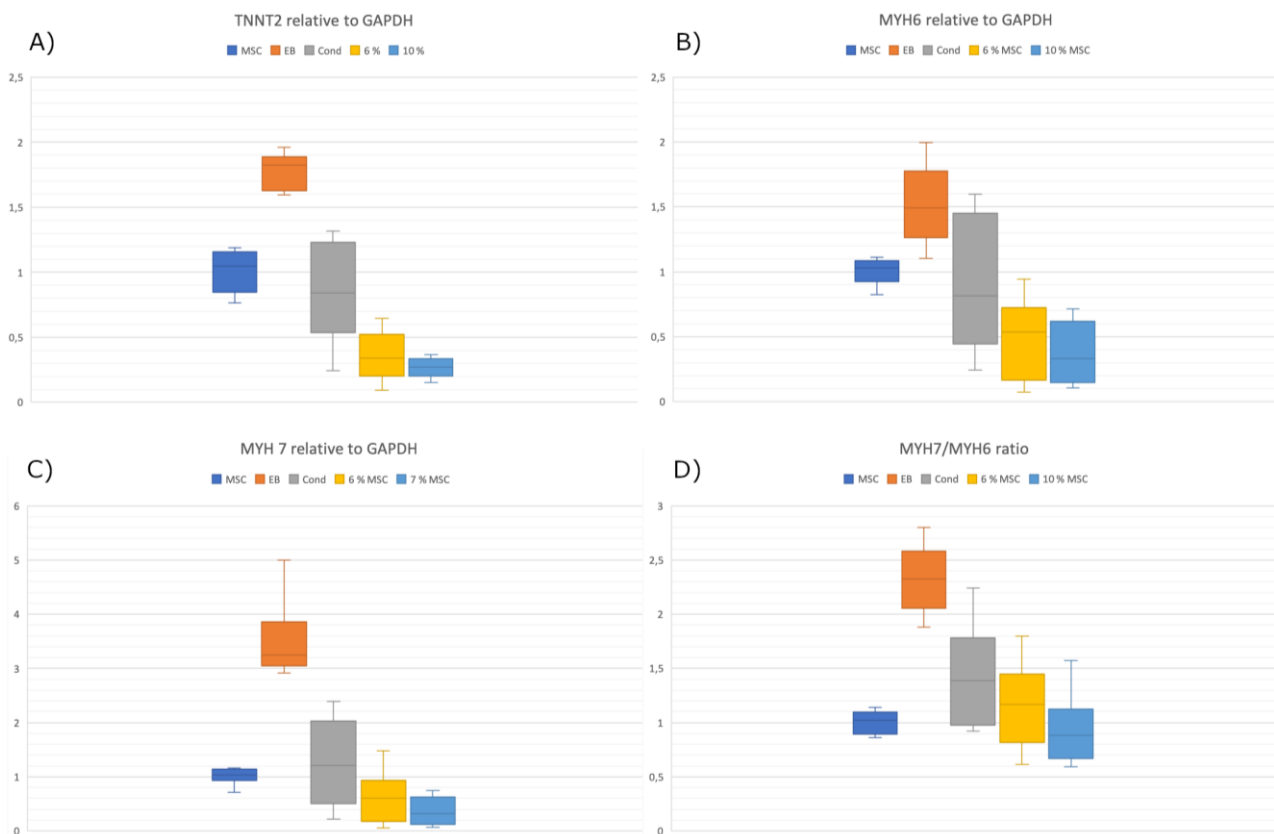
**Panel 3 hMSCs in the well margins.** hMSCs are highly abundant in areas sparse with hiPSC-CMs and being to overlap these cells.



**Panel 4. General appearance of the cultures.** hiPSC-CMs appear similar in all cultures. Cultures are dense in the "droplet group" and co-cultures in the "spread group". Fibroblast-like cells are present in the spread MSC medium control.

### 4.3 qPCR data

The relative expression of TNNT2, MYH6 and MYH7 was highest in the EB medium compared to the co-cultures which had the lowest overall cardiac specific gene expression (Diagram 5A-C). Similar results were obtained within the MYH7/MYH6-ratio alongside with EB medium outdoing the MSC medium (Diagram 5D). Compared to the 10 % MSC co-culture, TNNT2 and MYH6 was elevated in MSC medium and TNNT2 in conditioned samples (Diagram 5A&B).



**Diagram 5. Quantitative PCR results with MSC medium control as the calibrator.** MSC medium and EB controls ( $n = 6$ ), Conditioned, 6 % MSCs and 10 % MSCs ( $n = 12$ ). Error bars display the standard error of the mean (SEM). **A)**  $p = 0,001$  in EB vs. 10 % and 6 %,  $p = 0,01$  in MSC vs. 10 % and Cond vs. 10 %. **B)**  $p < 0,01$  in EB vs. 10 % and EB vs. 6 %,  $p < 0,05$  in MSC vs. 10 %. **C)**  $p < 0,001$  in EB vs. 10 % and 6 %. **D)**  $p < 0,001$  in EB vs. 10%,  $p = 0,01$  in EB vs. 6% and EB vs. MSC. Other differences were statistically non-significant.

## 5. DISCUSSION

These results show that human iPSC-CMs can be successfully co-cultured with human MSCs. This study also suggests that the hMSCs had no hazardous impact on the iPSC-CMs although they proliferate faster during culturing and should therefore be plated in lesser extent than the hiPSC-CMs. However, there was no evidence of any beneficial effects the hMSCs might have on the maturation or survival of the cardiomyocytes.

It was unclear whether the same culturing medium was suitable for both hiPSC-CMs and hMSCs. Various protocols exist in culturing of the adipose tissue derived MSCs. These protocols usually involve a DMEM based medium with 10 % FBS but its eligibility for the hiPSC-CMs and co-culturing purposes was uncertain (41,42). Differentiation medium for the hiPSC-CMs on the other hand is typically composed of a DMEM-F12 basal medium with 20 % FBS although other basal mediums such as KO-DMEM can also be applied (43). Increase in the presence of FBS can enhance the MSCs' proliferation rate but is more likely to depend on the overall amount of growth inducing supplements (42). Solutions with less than 5 % FBS have also shown to reduce the size of the hiPSC-CMs (44). The visual assessment indicated that the MSC-medium was suitable for the hiPSC-CMs and co-culturing as the hiPSC-CMs appeared similar in all cultures. However, the expression of cardiac markers was higher in the EB-medium which suggests its superior performance. Although being an essential tool in cell differentiation and culture survival, animal originating serum is a problematic supplement for it rules out *in vivo* purposes and its content varies due to poor reproducibility (45).

Based on our findings hiPSC-CMs survival was increased when plated in a droplet. However, some analyzing methods require solitary cardiomyocytes which were more abundant in the spread group (44). The hMSCs had a high proliferation rate which was most evident when the hiPSC-CMs were sparse. A possible explanation for this is the lack of free surface limiting the hMSCs' proliferation and migration. The hMSCs also began to overlap with the hiPSC-CMs in the higher hMSC/hiPSC-CM-ratios and was present even within dense hiPSC-CM clusters in the highest hMSCs concentrations. This might implicate to a competitive situation. It can therefore be

concluded that the droplet group tolerates a higher hMSC concentration than the spread group. The less restricted propagation of the hMSCs in the latter group could also further limit the hiPSC-CMs cell-to-cell contacts but would on the other hand increase the likelihood of hMSC-hiPSC-CM cell-to-cell contacts. The projections observed between the MSCs likely refer to cell-to-cell interactions. Lastly, due to their proliferation capacity the concentration of the hMSCs should be kept at the minimum in order to restrain the overpopulation of the cultures.

Despite some variation in the shape and size of the cells only few cells displayed an elongated morphology. Contrary to our findings, Yoshida's group which shared a similar research design with our study, reported a significant decrease in cell sphericity in their co-cultures ( $p < 0.001$ ) but the reported number of cells used for this analysis was only seven (27). The co-culturing neither increased multinucleated cells in the analyzed groups which also speaks against improved maturation. The individual hiPSC-CMs seemed to expand in low hiPSC-CM-densities which is in line with previous studies (46). The cause of the blistering phenomenon of the hiPSC-CMs in the microscope images remains unknown but is unlikely caused by the hMSCs as it also occurred in both controls. We hypothesized this phenomenon could implicate cell stress or even apoptotic blebs but these hiPSC-CMs were clearly beating which conflicts with this theory.

It should be noted that the presence of MSCs in co-cultures increase the amount of GAPDH which can distort qPCR results when used as the internal control. Therefore, this could partially explain the EB medium's superior cardiac specific gene expression. However, this medium appeared to perform better compared to MSC medium although the difference was statistically non-significant. Moreover, the MYH7/MYH6-ratio which does not rely on GAPDH was also compatible with these results. Yoshida et al. excluded the direct co-cultures from their qPCR analysis and discovered an increase in the mRNA expression of a cardiac maturation marker myosin heavy chain 7 ( $p = 0.0272$ ) in the conditioned samples whereas the expression of myosin heavy chain 6 did not differ significantly (27). This finding was supported by western blotting analysis which revealed an increase in the myosin heavy chain- $\beta$  (MHC- $\beta$ ) to MHC- $\alpha$  ratio in this group ( $p = 0.0455$ ) (27). Although our results did not agree with these findings, they could be affected by the less suitable culturing medium.

Our study's advantage was the long 14-day culturing period compared to Yoshida's group's 3-day culturing providing a better outlook on the long-term impacts of the co-culturing process. Their short culturing period could further explain the use of relatively high concentrations of the hMSCs being 25 % and 50 % in the indirect and 1:1 in the direct co-cultures. Our study showed that even hMSC concentrations of 3 % expanded significantly during the two-week culturing. Contrary to our study Yoshida et al. utilized a semipermeable membrane instead of pre-conditioned medium. (27)

Other previous studies on co-culturing MSCs with cardiomyocytes focus mainly on the MSCs' ability to differentiate into cardiomyocytes (31,32,47). Studies involving rat cardiomyocytes and rat MSCs or human adMSCs demonstrated cardiac marker expression such as  $\alpha$ -actin, cTnT and cardiac myosin heavy chain by the MSCs in direct co-culture with cardiomyocytes (31,32). Similar results were obtained when human MSCs were in direct co-culture with human cardiomyocytes (47). These hMSCs in direct co-culture with hCMs expressed MYH7,  $\beta$ -actinin, troponin-I and troponin-T while only  $\beta$ -actinin was expressed in conditioned medium treated samples (47). Additionally Choi YS et al. discovered beating adMSCs with gap junctions with adjacent cardiomyocytes (32).

### **Study limitations**

A major challenge in this study was the differentiation of the two cell types when analyzing the data. The presence of hMSCs in the co-cultures prevented the reliable use of GAPDH as a reference gene. It would have also been impossible to identify the cause of possible marker gene expression variations solely by qPCR considering the hypothesis of hiPSC-CMs maturation and hMSC differentiation. Although the hMSC pre-conditioned medium samples suited this analysis, the preservation and therefore the potency of this medium was questionable. Fluorescence images would have enabled computerized cell-shape analysis but was ruled out due to inadequate number of individual cells. In addition, user-based selection in the imaging process exposes to inherent bias.

## Conclusions

Both culturing mediums used were suitable for culturing of hiPSC-CMs and co-cultures although EB medium performed better with the hiPSC-CMs. Nonetheless, FBS-free alternatives should be discovered. The plating strategy affects the distribution of the hiPSC-CMs and should therefore be considered while selecting the analyzing methods (48). Additionally, hMSCs proliferate more efficiently in sparse hiPSC-CM cultures and thus require lower hMSC quantities. Eligible analyzing methods for evaluating direct co-cultures should be explored. There was no indication that the hMSCs would affect the hiPSC-CMs' maturation but further studies on this topic are still required.

## Acknowledgements

This study was supported by The Finnish Medical Foundation.



## Attachments

<b>Stock solutions used</b>
Roswell Park Memorial Institute (RPMI 1640), Gibco ThermoFischer Scientific
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), Gibco ThermoFischer Scientific
KnockOut™ D-MEM (KO-DMEM), Gibco ThermoFischer Scientific
mTeSR™1, components: mTeSR™1 Basal Medium and mTsR™1 5X Supplement (StemCell Technologies)
Phosphate-buffered saline (PBS), Gibco ThermoFischer Scientific
Phosphate-buffer (PB)
Bovine serum albumin (BSA), Sigma
Donkey serum (NDS), Biowest
PFA (paraformaldehyde)
<b>Abbreviations</b>
bFGF basic fibroblast growth factor
TGFβ transforming growth factor beta
BMP4 bone morphogenic protein 4
IWP-4 inhibits Wnt signaling
KLF4 Kruppel-like factor 4

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