CHARACTERISING HUMAN PLURIPOTENT STEM CELLS-DERIVED ENDOTHELIAL CELLS

PhD thesis

dr. Edit Gara

Doctoral School of Basic Medicine
Semmelweis University

Supervisor: Dr. Gábor Földes PhD

Official reviewers: Dr. Melinda Pirity PhD
Dr. János Réthelyi PhD

Head of the Final Examination Committee:
Dr. Péter Ferdinandy PhD DSc MBA
Members of the Final Examination Committee:
Dr. Lívia Jánoskuti PhD
Dr. Ágota Apáti PhD

Budapest
2015

TABLE OF CONTENTS

1. Abbreviations

6MWT	six-minute walk test
ACh	acetylcholine
ACEi	angiotensin converting enzyme inhibitor
Ac-LDL	acetylated low density lipoprotein
AE	adverse event
Akt	protein kinase B
ALS	amyotrophic lateral sclerosis
BB	beta receptor blocker
BM	bone marrow
BNP	B-type natriuretic peptide
BOEC	Blood outgrowth endothelial cells
cDNA	complementary deoxyribonucleic acid
CGMP	current good manufacturing practice
CM	cardiomyocytes
CSC	cardiac stem cells
DAPI	4', 6-diamidino-2-phenylindole - dihydrochloride
DCM	dilated cardiomyopathy
DLL4	Delta-like ligand-4
DMSO	dimethyl-sulfoxide
EB	embryoid body
EC	endothelial cells
EBM	endothelial basal medium
ECM	extracellular matrix
EGM	endothelial growth medium
EHT	engineered heart tissue
ELISA	enzyme-linked immunosorbent assay
ET-1	endothelin-1

eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cells
ERC	endometrial regenerative cells
FACS	fluorescence-activated cell sorting
FDA	Food and Drug Administration
FOXO1A	Forkhead box O transcription factor 1A
FBS	foetal bovine serum
GABA	gamma amino butyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GVHD	graft-versus-host disease
HAEC	human aortic endothelial cells
HCAEC	human coronary arterial endothelial cells
hESC	human embryonic stem cells
hESC-EC	human embryonic stem cells -derived endothelial cells
hESC-CM	human embryonic stem cells-derived cardiomyocytes
HFpEF	heart failure with preserved ejection fraction
hiPSC	human induced pluripotent stem cells
hiPSC-EC	human induced pluripotent stem cells-derived endothelial cells
HLA	human leucocyte antigen
HOCM	hypertrophic obstructive cardiomyopathy
hPSC	human pluripotent stem cells
HUVEC	human umbilical cord endothelial cells
I-DCM	ischemic dilatative cardiomyopathy
iNOS	inducible nitric oxide synthase
ITGB1	integrin beta-1
IVF	*in vitro* fertilisation
LVEF	left ventricular ejection fraction
MI	myocardial infarction

MLHFQ	Minnesota living with heart failure questionnaire
MRI	magnetic resonance imaging
MSC	mesenchymal stem cells
MS	multiple sclerosis
NA	not applicable
NEC	non-enzyme control
nNOS	neural nitric oxide synthase
NOS	nitric oxide synthese
NTC	non-templates control
PBS	phosphate buffered saline
PCI	percutaneous coronary intervention
PCR	polymerase chain reaction
PDGFRA	platelet-derived growth factor receptor alpha
PI3K	phosphatidylinositol-4, 5-bisphosphate 3-kinase
PIK3R1	phosphoinositide-3-kinase regulatory subunit 1 beta
PIK3R2	phosphoinositide-3-kinase regulatory subunit 2 beta
PPP	platelet poor plasma
PRKCA	protein kinase C alpha
PRKCB	protein kinase C beta
PRKCZ	protein kinase C zeta
PRP	platelet rich plasma
PSC	pluripotent stem cells
PSC-EC	pluripotent stem cell-derived endothelial cells
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative real-time polymerase chain reaction
rh-bFGF	recombinant human basal fibroblast growth factor
rh-TGFβ	recombinant human transforming growth factor beta
RNA	ribonucleic acid

siRNA	small interfering RNA
SNP	sodium nitroprusside
SPECT	single-photon emission computed tomography
STEMI	ST segment elevation myocardial infarction
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
WBC	white blood cells
WSS	wall sheer stress

2. Introduction

2.1. Current applications and future challenges in cell therapy

Cell therapy is the focus of medical research for developing novel therapeutic strategies. Degenerative diseases affect large number of patients placing high burden on health and social care systems. Many patients suffer from severe neurodegenerative diseases (e.g. Parkinson's disease, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS)) and ischemic cardiovascular events (stroke or myocardial infarction (MI)). Cardiovascular diseases are the leading cause of death in industrialised countries. Pathophysiology of these diseases involves definitive necrosis of cells with a crucial role in the physiology of the human body (circulation, central or peripheral nervous system). The regenerative capacity of neurons is limited, similarly as cardiomyocytes [1, 2]. Many patients suffer severe decrease in quality of life due to a lack of definitive treatment. Insulin-dependent diabetes mellitus may develop in very young children and so far no definite therapy exists; treatment options focus on substitution of pancreatic β cell product, insulin. Preclinical research and early cell therapy trials are underway in these medical conditions. Regenerative medicine and tissue engineering may establish definitive treatments to cure these conditions.

Cell therapy is being routinely used in special clinical conditions. Bone marrow transplantation is performed frequently in haemato-oncological diseases, e.g. leukaemia and lymphomas. It is carried out through both autologous and allogeneic route.

In severe graft-versus-host disease (GVHD) mesenchymal stem cells (MSC)-derivatives are transplanted if the condition is resistant to steroid shot therapy. The Remestemcel-L® (*Prochymal, Osiris*) product is available in the US and Canada, and was approved by the FDA. Remestemcel-L® contains MSC from healthy adults, and applied intravenously in GVHD. Remestemcel-L® is a novel, of-the-shelf available cell therapy [3].

Umbilical cord blood contains multipotent stem cells (mononuclear haematopoietic stem cells and mesenchymal stromal cells), similar to MSC. Several commercial companies are offering collection and bio-banking of umbilical cord blood after birth. The treatment costs can be extremely high. These companies promote umbilical blood bio-banking as a procedure that could provide life-saving therapy in a theoretical future malignant haemato-oncological disease. However, the literature is lacking in data on autologous

transplantation of these precursor cells in malignant conditions. There is growing evidence supporting the theory that life-threatening hemato-oncological diseases are clonal diseases. This means that the multipotent precursor itself may carry malignant mutations. Albeit, collection of umbilical cord blood cells may serve public health purposes, HLA typization may enhance their widespread use. In the USA five umbilical cord blood products have received FDA approval so far for hemato-oncological therapeutic use. In Hungary, two allogeneic transplantations were performed using umbilical cord blood-derived precursor cells [4].

In recent years, new stem cell-derivatives have already been tested in various clinical conditions. Pluripotent stem cells show magnificent promises for tissue regeneration. Pieces of the mosaic in their future therapeutic palette involve recovery of motoneuron function after spinal cord injury or in neurodegenerative diseases, developing insulin producing β cells and regenerating the failing human heart.

Geron (Asterias Biotherapeutics) has initiated the first-in-human clinical trial with human embryonic stem cells (hESC) (*NCT01217008*) [5]. The phase I trial investigated the safety of hESC-derived oligodendrocytes implantation after spinal cord injury [6]. Administration of the stem cell product required continuous immunosuppression, as hESC-derivatives were allogenic. The trial recruited a small number of patients (n=10) due to large financial burden; the trial was terminated early for the same reason. Published safety results have not reported any surgical or neurologic adverse events (AE). Most AEs were as a result from immunosuppressive medication.

Another novel breaktrough in cell therapy was the development on donor type 0, Rh negative red blood cells from human pluripotent stem cells. A research group at Glasgow University has been working on this project [7]. Fulfilling the transfusion need with healthy blood donors is limited in many countries in the EU. The recently developed type 0, Rh negative red blood cells promise blood available off-the-shelf for transfusion, and researchers plan first-in-human transplantation in 2020 in the UK. The hESC-derived red blood cells function similarly to those isolated from bone marrow [8, 9]. However, directing the enucleation of cells and haemoglobin switch may be challenging during differentiation process. Large-scale engineering also promotes further development in differentiation procedure [10]. Immunological consideration is required, as these red blood cells are derived from hESC and may initiate immunological responses during allogeneic

transplantation. Human ESC derivatives may possess unexpected immunological properties [10-12].

Another field of medicine where cell therapy paves the way with novel therapeutic products, is ophthalmology. *Holoclar* contains limbal epithelial stem cells. These cells are isolated from the edge of the cornea and are responsible for corneal regeneration. *Holoclar* is transplanted autologous after chemical injury of the cornea, authorised by EU regulations. Pluripotent stem cell-derivatives are being used in several ophthalmological clinical trials. The eye is thought to be an immunologically special and protected organ in the human body. Human ESC derivatives transplanted into the macular region of retina are feasible without rejection or tumour formation complications. There are many clinical trials underway, collecting human samples for the generation of human induced pluripotent stem cells (hiPSC) in retinoblastoma (*NCT02193724*). Other trials are investigating putative beneficial effects of cell therapy in bestrophinopathy (*NCT02162953*), age-related macular degeneration (*NCT01674829*), macular dystrophy (*NCT01469832*) and myopic macular degeneration (*NCT02122159*).

Many clinical trials are enrolling patients in other special medical conditions (type 1 diabetes mellitus (*NCT02084407*), ALS (*NCT00801333*), ataxia teleangiectasia (*NCT02246491*), Barth syndrome (*NCT01625663*)) to collect human samples (especially fibroblasts) for hiPSC generation. Patients-derived hiPSC provide chance for investigating pathophysiology of diseases *in vitro*. Furthermore hiPSC offer personalised drug testing *in vitro*. The first clinical trial with hiPSC has already started in Japan [13]. Elderly patients, suffering from age-related macular degeneration receive autologous hiPSC-derived retinal pigment epithelial implantation. The hiPSC-derived retinal epithelial cells are grown in cell sheets to enable ophthalmic surgical procedures. The preclinical animal experiments demonstrated no tumorigenic potential of hiPSC-derived retinal pigment epithelial implantation on the long run [10, 14]. Altough first patient has already been treated without any significant adverse events so far [13] the trial was recenlty suspended. Single-nucleotid variations and copy-number variations were found in hiPSC of the second patient. These genomic abnormalities were not detected in the original fibroblasts. Impacct of these findings on the future of hiPSC clinical trials is not yet clear [15].

Following the early steps of cell therapy the clinical use still faces many challenges and open questions. My PhD thesis focuses on cardiovascular derivatives of human pluripotent

stem cells, especially on endothelial cells. Preclinical results and early clinical trials with cell therapy in cardiovascular field will be discussed in details later in section 2.3. Figure 1. summarizes cell therapy approaches in regenerative cardiac medicine.

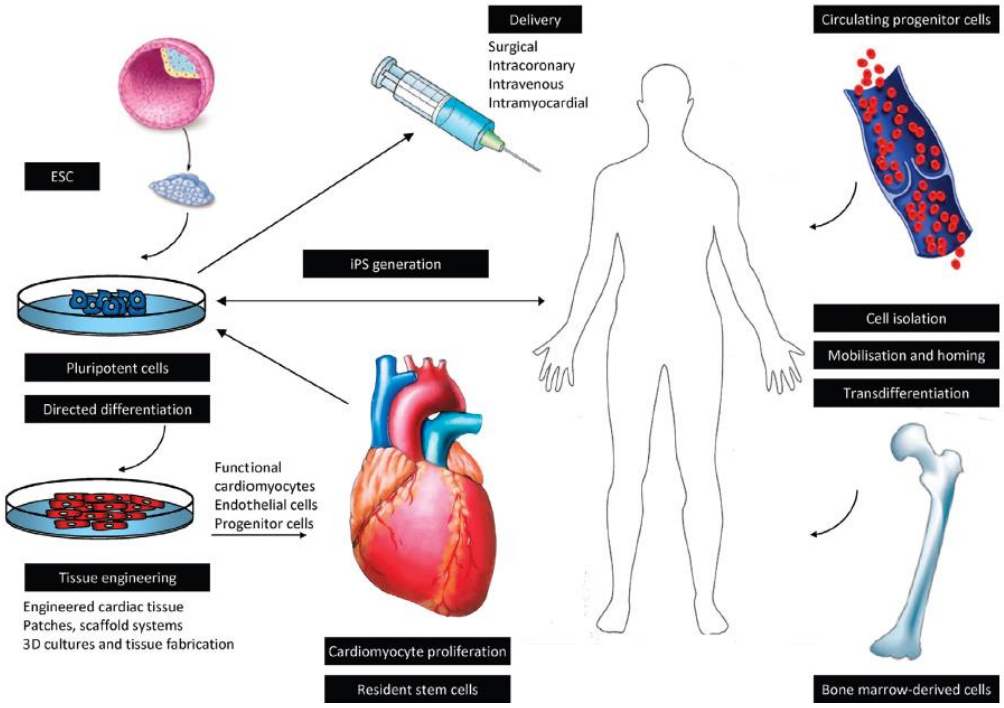


Figure 1. Experimental approaches to cell therapy in heart failure Figure shows experimental approaches, cell types and cell delivery routes for regenerative purposes in heart failure (Figure is from Kosztin/Gara et al. Stem cell therapy to treat heart failure Elsevier 2015, Reference Module in Biomedical Sciences)

The key aims of cardiovascular cell therapy are:

1) to prevent myocardial loss during myocardial tissue injury (ischaemic attacks, cardiomyopathy, valve diseases, etc.);
2) to decrease the ratio of definite necrosis after ischemic events and salvage stunned myocardium;
3) to decrease the rate of remodelling, and enhance reverse remodelling after myocardial injury;
4) to support angiogenesis during and after ischemic attack;
5) and to regenerate myocardial tissue in severe heart failure from different aetiologies.

Regarding cell therapy and translational research in the cardiovascular field many questions remain open:

1) *Which is the ideal cell type for cell therapy?*
   Circulating progenitor cells, multipotent stem cells, cardiac progenitor cells and pluripotent stem cell-derivatives are candidates.

2) *How to perform the cell implantation?*
   Applications: direct surgical implantation, surgical cardiac patches, local intra-myocardial injection, intracoronary injection, coronary sinus injection, systemic intravenous injection, use of bioscaffolds for cell engineering and implantation.

3) *What is the ideal number of cells for implantation?*
   Early clinical studies lack valuable information about the required amount of implanted cells. To build up a human adult heart, ~ 6 billion cells may be needed. The left ventricle is made up from ~ $4x10^9$ cardiomyocytes ($2x10^6$/g of tissue, the whole heart is ~300g) [16]. In a large MI ~ 10^9 cardiomyocytes suffer irreversible damage. State-of-the-art clinical imaging systems (e.g. SPECT-CT) may estimate the number of cells needed for regeneration before performing transplantation. Considering personalised regenerative therapy with hiPSC-derivatives, the amount of injected cell should also be adjusted individually.

4) *How to track injected cells after implantation to enable ability of following their fate in vivo?*
   The vast amount of transplanted cells seem to be washed out from the target tissue via venous and lymphatic circulation and imaging their homing *in vivo* is still a challenge. In most preclinical studies, the data are lacking about the amount of cells engrafted into the myocardium. Furthermore, no information exists about what happens to non-hosting cells. As clinical trials typically follow up the patients for a short period (two years), long term data is missing on attainable tumour formation or pro-carcinogenic considerations.

5) *What is the ideal timing for cell implantation?*

Clinical studies are inconclusive regarding the timing of cell therapy. Scientific knowledge is lacking regarding the best timing for anticipated beneficial regenerative effects. Some trials aim to treat in the acute phase of cardiovascular events, e.g. within one week after myocardial infarction. Others aim regeneration late after the myocardial event, e.g. in end-stage heart failure.

2.2. Types of stem cells

2.2.1. Circulating progenitor cells

In cardiovascular diseases the pathological steps are endothelial dysfunction and damage. Circulating endothelial progenitor cells (EPC) are in the focus of research for over a decade, providing possible source for therapeutic re-endothelisation and endothelial repair [17]. Furthermore, EPC were nominated to be biomarkers for cardiovascular risks and disease progression [18]. Many studies have also considered EPC to be putative biomarkers for pathological conditions [19]. The amount of circulating EPC is proposed to be biomarker for the progression of atherosclerosis and for coronary plaque characteristics [20, 21]. After promising early studies, recently published data have been controversial regarding the role of EPC as biomarkers. Data are also diverse regarding therapeutic considerations [17].

EPC were first characterised in 1997 [22] and early studies demonstrated them to be bone marrow derived, CD34-positive cells. Despite great efforts, no clear definition is available on these progenitor cells. In fact, data are confusing on titles and subgroups of EPC, naming them such as "circulating angiogenic cells", "endothelial colony forming cells" and "colony forming unit cells" [17]. EPC are mainly characterised by the presence of cell surface markers: CD34, VEGFR2, CD133, ICAM-1 and E-Cadherin, with flow cytometry. Isolation protocols are also available that group EPC to early and late outgrowth population [19, 23, 24]. In 2004 EPCs have been therefore redefined [25]. Population of cells from human peripheral blood were expanded *in vitro*, which expressed CD31 and vWF. In addition, these cells showed cobblestone morphology in culture which is characteristic for endothelial cells. It was proved that these human cells have the capacity to form chimeric human-murine vessels when implanted subcutaneously into mice [25]. Their role in adult organism was thought to be the enhancement of angiogenesis in adults [26]. Indeed, a number of clinical studies in humans show the activation or injection of these cells. Whether these cells contribute directly to new vessel formation or simply support that by cytokine release and the rate of beneficial effects remains unclear [19].

In a prominent phase II, randomised clinical trial patients with type 2 diabetes mellitus and hyperlipidaemia discontinued statin treatment for a short period and meanwhile changes in

EPC levels and angiogenic function were measured [27]. The results were impressive, as large increases were detected in the levels of circulating EPC and in their functional activity, after 5 days of statin discontinuation [27]. The data suggest that EPC may have an important role in atherosclerotic disease progression and therapeutic efforts for endothelial repair and re-endothelisation [27]. However, more investigations are needed to understand exact function of EPC and their role in vascular biology.

2.2.2. Mesenchymal stem cells

MSC are either derived from the bone marrow or from peripheral organs. MSC were first described in 1970 as nucleated cells in the bone marrow and were known to be responsible for steady-state circumstances in the bone marrow niche [28].
MSC are able to differentiate into multi-lineage derivatives and are able to perform self-renewal. Thus, they were named as mesenchymal *stem* cells, albeit recent publications rather call them mesenchymal *stromal* cells [29, 30]. The early clinical trials used many different types of MSC-like cells which were not precisely characterized. To enhance standardised characteristics of MSC in translational research and early phase clinical trials, strict requirements were set up for MSC definition [31]. In 2006, the International Society for Cellular Therapy published MSC definitions:

- Adherence to plastic surface in standard culture conditions
- Expression of the following cell surface markers: CD73, CD90, and CD105
- Absence of the following cell surface markers: CD34, CD45, HLA-DR, CD14 or CD11b and CD79a or CD19
- Ability to differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro* [31].

Sources of MSC isolation and collection are the bone marrow and peripheral organs. These include adipose tissue and many other organs like the liver, placenta, gut, lung, heart and even amniotic fluid, dental pulp, periodontal ligament or Wharton jelly of the umbilical cord. Many studies use adipose tissue-derived MSC, as their collection is easier and more feasible. Wharton jelly-derived MSC were also studied in many trials [32]. MSC from different sources possess many differences in genotype and phenotype. MSC derived from

the amniotic fluid express pluripotency marker SSEA4. MSC-like cells isolated from heart tissue express myogenic markers and tend to be myogenic precursor cells [33].

Despite the improvement of protocols, the isolation and expansion of MSC are still with low efficacy. For clinical therapeutic use the isolation and expansion requires therefore better and CGMP competent protocols. From 10ml of bone marrow aspirate 50-400 x 10^6 MSC can be isolated. Huge variation exists in elderly population and in case of co-morbidities.

MSC are elongated, fibroblast like cells which grow on plastic in MSC medium. MSC can differentiate into chondrocytes, osteoblast, myocyte precursor cells and adipocytes *in vitro*, although the differentiation rate is low. Beside direct use of cells for therapy, studies also focus on the indirect anti-inflammatory and paracrine effects of MSC. The favourable effect of MSC for therapeutic purposes may be their anti-inflammatory and anti-fibrotic role. These cells have been shown to secrete wide range of proteins which would promote regenerative angiogenesis and stimulate endogenous cardiac regeneration *in vivo* [33]. In post-MI failing myocardium, two major types of macrophages are present in the injured myocardium. Type M1is responsible for the production of pro-inflammatory proteins, debris degradation and apoptosis. Type M2 has anti-inflammatory role and supports early angiogenesis. After MI, in the presence of MSC, the number of type M2 macrophages increase [34, 35]. MSC have cardioprotective effects and can decrease size of scar after MI. For this, they may enhance the survival rate in stunned myocardium and decrease necrosis. Detailed studies showed, cardiac cytoprotection occurs by modulating key signalling pathways, which include the Wnt, IGF1-NF-kB, Akt1 and TGFβ pathways [36-39]. For cardiac regeneration and repair, MSC share pleiotropic mechanisms of action (Table 1.) [8]. However, limitations and controversial effects of MSC reside in formation of mesoderm-derived ectopic cells and enhancement in fibrosis may progress *in vivo* [40]. Clinical trials with MSC in cardiovascular field are discussed in details in section 2.3.2.

Table 1. Mechanisms of action of mesenchymal stem cells in cardiac regeneration Table shows putative effects and mechanisms of action of mesenchymal stem cells in cardiac regeneration (WBC: white blood cells, ECM: extracellular matrix)

Effect	Mechanism
Immunomodulation	Supressing WBC, enhancing M2 type macrophages
Antifibrosis	Modulation of ECM niche by paracrine effects
Pro-angiogenesis	Secretion of pro-angiogenic proteins and molecules
Pro-neomyogenesis	Activation of cardiac resident stem cells and progenitors
Cardiac cytoprotection	Modulating signalling pathways, anti-apoptotic effects
Direct cellular effects	Differentiation to cardiac myogenic precursor cells

2.2.3. Cardiac stem cells

Resident, adult stem cells occur in many organs and tissues of the human body. The main role of adult stem cells may be their participation in physiological turn-over of tissues as well as support of endogenous regeneration after tissue injury. In the cardiovascular system, endothelial and cardiac progenitor cells stand for adult stem cells. Adult, resident stem cells (CSC) in the myocardium are clonogenic, have the ability for self-renewal and may differentiate to any cardiovascular cell type (cardiomyocytes, endothelial cells and smooth muscle cells) [41, 42]. However, their exact role in cardiac regeneration and their potential to improve decreased cardiac function is still controversial. Scientific knowledge is diverse regarding the role of CSC in the physiologic turnover of the myocardium [41, 43-45]. Some resident CSC may re-enter cell-cycle and replace damaged or dysfunctional cardiomyocytes. The main characteristics of CSC cells are c-kit positivity; further characteristics can include positivity for islet-1, Nkx-2.5 and MEF-2c (cardiac progenitor markers). C-kit is a tyrosine kinase receptor (also known as CD117), which binds SCF (stem cell factor) and involved in many important signalling pathways, including the PI3K and ERK, mainly responsible for cell proliferation and migration [46-48]. The origin of c-kit positive CSC in the adult heart is not clear. Some studies showed their origin may be the cardiomyocytes and epicardial cells of the heart and CSC develop by dedifferentiation and epicardial-mesenchymal transdifferentiation [49, 50]. Other groups showed that CSC may arise from circulating progenitor cells and home to the adult heart [51, 52]. Further studies report that CSC preserved stem cell niche during embryonic cardiomyogenesis [53, 54]. Many studies have been investigating potential role of CSC in cardiac regeneration and repair. Similarly to MSC, CSC have multimodal mechanisms to enhance cardiac repair [53]. Their paracrine effects incluede secretion of pro-angiogenic, anti-apoptotic factors and supporting angiogenesis after ischemic injury. The direct cellular effects contain differentiation to early/late cardiac progenitors or cardiomyocytes (most controversial field).

Preclinical results and clinical trials investigating CSC in cardiovascular field are discussed in details in section 2.3.3.

2.2.4. Embryonic stem cells

Human ESC were first established in 1998, by James Thomson et al., in Wisconsin [55]. Inner cell mass of the blastocyst were isolated and embryonic stem cells were plated. Thomson used five blastocysts remaining from *in vitro* fertilisation (IVF) procedure, fourteen samples from inner cell masses were isolated and the first five hESC lines were established. Today over 1000 hESC lines exist [56, 57]. Ethical regulations are diverse regarding the development and use for research purposes of hESC (detailed in section 4.12.). Early protocols for inner cell mass isolation from the blastocyst resulted in the destruction of the embryo. Mechanical isolation, surgical isolation and even laser techniques have been developed [58-60]. Recent isolation procedures are enable to rescue the embryo, thus ethical concerns may be reduced [61]. Human ESC lines were successfully developed from single blastomeres by routine biopsies performed for preimplantation genetic testing during IVF [62-64]. These derivation protocols are facing a number of challenges, regarding the distinct phenotype and genotype of the developed hESC lines [65].

Human ESC can expand unlimitedly with asymmetric self-renewal while preserving normal karyotype and pluripotent state. During directed differentiation, hESC are able to give rise to all three germ layers (ectoderm, endoderm and mesoderm) and for germline, except extra-embryonic tissues of the placenta (trophoectoderm) (Figure 2.). Human ESC are able to differentiate into all tissue and cells in human organism: ectoderm derived neurons [66], Schwann cells [67], melanocytes [68], skin cells [69]; endoderm derived hepatocytes [70], intestinal cells [71], pancreatic cells [72], pneumocytes [73]; mesoderm derived blood cells [74], fibroblasts [75], adipocytes [76], smooth muscle cells [77], cardiomyocytes [78], endothelial cells [79], etc. Upon *in vivo* transplantation of hESC they spontaneously form teratomas. For *in vitro* colony formation, hESC require special cell culture conditions. Early protocols included xenogenic feeder cells and growth factors; later xeno-free feeder culture conditions developed. Recently hESC have been grown on ECM components in xeno-free conditions (eg. laminin, fibronectin, vitronectin, Matrigel) in commercially available predefined media for pluripotent cells [65]. Human ESC are characterized by cell surface markers and transcription factors associated with pluripotency. Briefly, these pluripotency markers are TRA1-60/81, TRA2-49/54, CD-

9/24/29/49f/324/338 and SSEA-1/3/4/5 (cell surface proteins) and transcription factors Oct-4, Nanog, Stat-3, FoxD3, c-Myc and Sox-2 [65]. Translational studies with hESC in the cardiovascular field are discussed in section 2.3.4. in details.

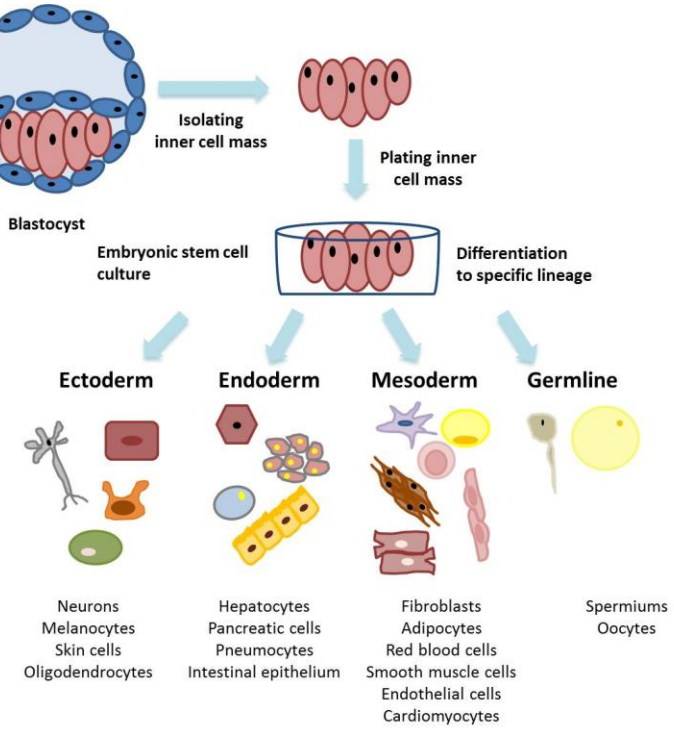


Figure 2. Human embryonic stem cells and derivatives Figure shows origin of human embryonic stem cells, these pluripotent stem cells are able to differentiate into any cell types of the three germ layers and germline (original figure from Edit Gara)

2.2.5. Induced pluripotent stem cells

In 2006 Japanese researcher Shinya Yamanaka, and his group successfully developed mouse pluripotent stem cells from adult somatic cells, by using genetic reprogramming [80]. The first hiPSC line was developed in 2007, parallel by two independent research groups: Yamanaka et al. and Thomson et al. [81, 82]. The idea of nuclear transfer itself, existed earlier, notably Dolly the sheep was born through the nuclear transfer cloning method [83]. Forced expression of specific genes in differentiated somatic cells has been shown to reverse the developmental program in adult somatic cells; this process is called reprogramming. When using reprogramming method, nuclear transfer or oocyte manipulation is not necessary to induce genome modification. During reprogramming, genes responsible for pluripotency are inserted into the genome of adult somatic cells by transfection. These pluripotency genes are named Yamanaka factors or master genes: Oct-3/4, Sox-2, Klf-4 and c-Myc [80]. Oct-3 and 4 play a key role in early embryonic development and in maintaining pluripotent fate is stem cells. Sox-2 is responsible for continuous self-renewal in pluripotent stem cells. Klf-4 is also involved in maintenance of pluripotency, proliferation and cell survival. Myc is known to be an oncogenic regulator, tumour suppressor and is affected in almost all human cancers. Myc plays a crucial role in physiologic operation of cell cycle and proliferation [84].

Human iPSC promise wide range of new possibilities in basic research, translational medicine and therapy. Human iPSC offer specific cell types for modelling diseases, testing drug toxicology and developing drugs *in vitro*, even in a patient-specific manner. Human iPSC have similar characteristics to those of embryonic stem cells, as they express same pluripotency markers and can differentiate into all three germ lines [85] (Figure 3.). Indeed, undifferentiated hiPSC were shown to form teratomas *in vitro* [80]. Human iPSC may later be the most efficient and feasible option for tissue engineering therapeutic perspectives. Ongoing preclinical studies with hiPSC will be discussed in details in section 2.3.5.

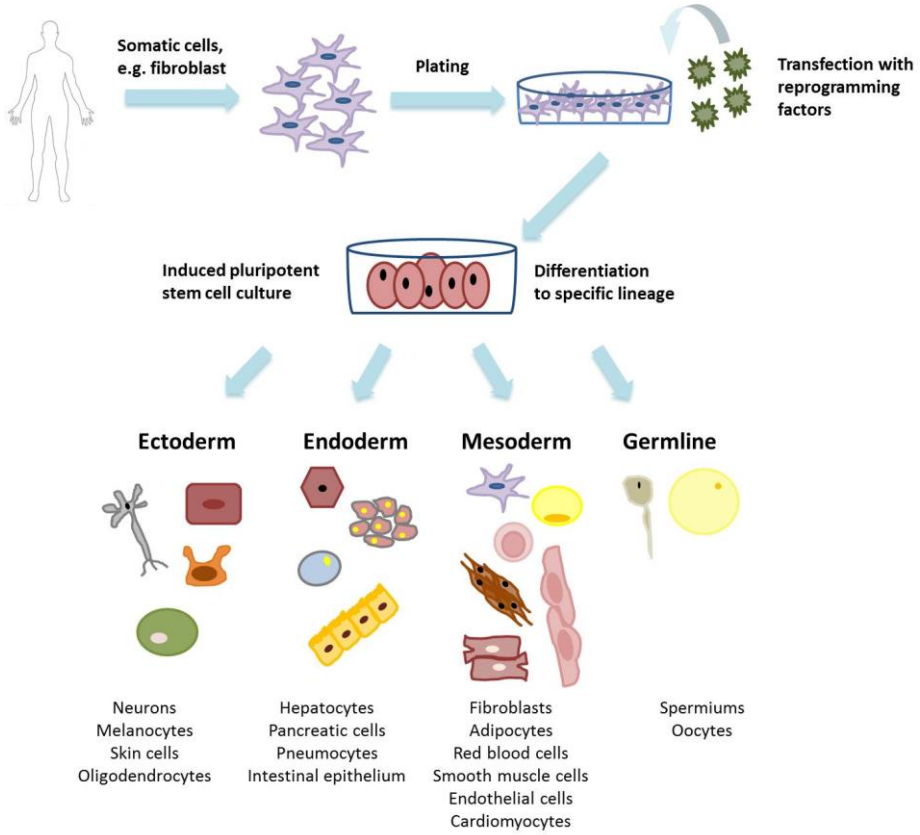


Figure 3. Induced pluripotent stem cells and derivatives Figure shows the derivation of human induced pluripotent stem cells. These pluripotent stem cells are able to differentiate to any cell types of the three germ layers and germline (original figure from Edit Gara)

Due to the great translational and clinical potential, hiPSC are in the focus of basic research. The reprogramming method itself determines the fate and phenotype of the developed pluripotent cells, also the donor cell line influences reprogramming. Several reprogramming techniques were established in the last decade with a large variability in safety and efficacy profile [86]. First techniques used fibroblast as somatic cells, however keratinocytes, lymphocytes, hepatocytes, neurons, or multipotent stem cells are also good sources for reprogramming [86]. Regarding availability and reprogramming efficiency, the most commonly used somatic cell type is still adult fibroblast [86]. Reprogramming methods mainly differ in the route of delivery of pluripotency genes. First protocols used retroviral, lentiviral or adenoviral vectors to integrate pluripotency genes into the genome of targeted cells. These methods may not be feasible for clinical use, as viral vectors mean infection of the cells with viruses and integration of viral genes into the human genome. Despite their risks integrating viral vector methods have high reprogramming efficacy. On the other hand, use of adenoviral vectors has very low transfection efficiency, although

adenoviruses do not integrate into human genome. To overcome feasibility issues, non-integrating viral methods have been developed, including the Sendai virus as a vector [87]. Sendai virus technique provides a safer and more efficient production of hiPSC, using RNA-based transfection of pluripotency genes. A special group of DNA-based integration technique is called the *Sleeping Beauty* transposon system [88]. *Sleeping Beauty* is a non-viral carrier system of the genetic information that can insert a gene into human chromosomes. The future clinical use of *Sleeping Beauty* transposon system, would be much safer than those vector systems that are viral-dependent. Ivics at al. found that the chromosomal distribution of *Sleeping Beauty* transposon insertions is random in the human genome. They also proved that insertions could occur at each chromosome. *Sleeping Beauty* transposons could be inserted at specific regions of the human genome, by modifying the insertion [89]. Furthermore, the third generation of virus-free/DNA-free iPSC was generated by direct delivery of small molecules, proteins and antibodies that remodel cellular epigenetic pattern [85, 90, 91]. Transgene-free non-integrating methods were developed, e.g. *piggyBac* system. The *piggyBac* transposon system enables insertion and then directed excision of a naked DNA segment from the genome, thus the constant modification of the human genome is avoided [88]. The transfection with a *piggyBac* transposon is completely under control, as the promoter region is induced by an antibiotic enhancer (e.g. doxycycline). Finally, modified messenger RNA delivery and microRNA-based transfection methods also exist. These viral-free/DNA-free, non-integrating methods partially eliminated the risk of modifying the human genome.

Recent studies have investigated the epigenetic effects of reprogramming and genomic stability of established hiPSC [92]. The genetic manipulation during hiPSC may be a critical issue in clinical scenario. Human iPSC seems to be superior among other pluripotent and multipotent stem cell lines, thus assessing and developing the safest and most efficient reprogramming method may be a critical issue for future therapeutic use. Short summary of reprogramming methods is shown in Figure 4.

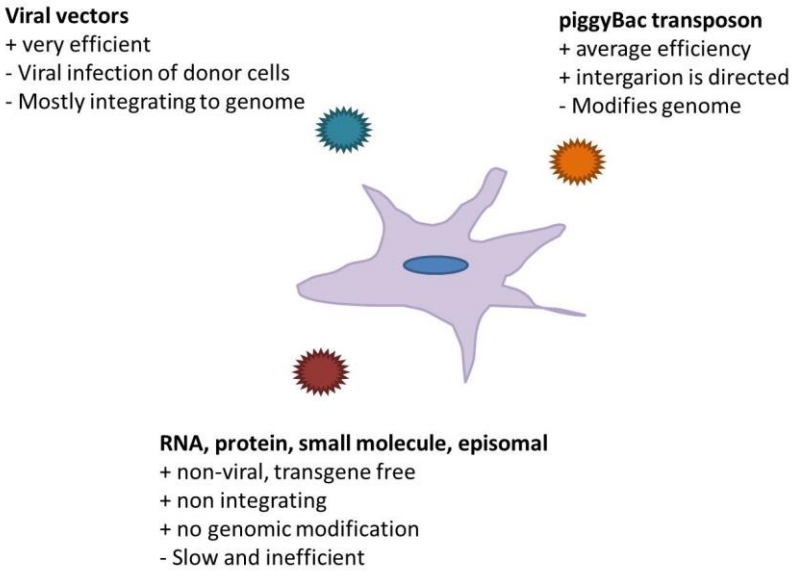


Figure 4. Advantages and disadvantages of reprogramming methods Figure shows main types of reporgramming methods to develop human induced pluripotent stem cells from adult, somatic cells. Advantages and disadvantages of main reprogramming methods are listed in figure (+ advantage, - disadvantage, original figure from Edit Gara)

2.3. Cardiovascular derivatives of stem cells, preclinical and early clinical results

2.3.1. Clinical studies investigating endothelial progenitor cells

After characterising EPC, translational research moved toward cardiac regeneration. First clinical trials assessed safety and feasibility of their activation *in vivo* and injection after exogenous boost [93-95]. Small clinical trials reported beneficial results and improvement in hemodynamic parameters. Injection of EPC into the injured myocardium resulted in increased left ventricular ejection fraction (LVEF) and inhibition of fibrosis after myocardial infarction [94, 96, 97]. To enhance stent function, anti-CD34 antibodies were applied onto coronary stents. The bioengineered stents can capture EPC, thus enhance endothelisation and defeat late in-stent restenosis. In **BO**ne Marr**O**w Transplant to enhance **ST**-elevation infarct regeneration (BOOST, *NCT00224536*) clinical trial patients received bone marrow-derived mononuclear cells (hematopoietic precursor). The autologous precursor cells were harvested and injected at the same day, after suffering ST segment elevation myocardial infarction, with preserved LVEF. The study reported no significant adverse event and significant increase in LVEF was found in the treatment group, after 6 months follow-up.

At the Heart and Vascular Centre, Semmelweis University, Budapest, the **C**xcr4 **A**n**T**agonism for **C**ell mobilization and **H**ealing in **AMI** (CATCH-AMI, *NCT01905475)* trial is investigating effects of endothelial progenitor/stem cells in acute ST segment elevation myocardial infarction (STEMI). I am taking part in this trial as a sub-investigator. This is a phase IIa, double-blind, placebo-controlled, randomised and multi-centre study of POL6326, a CXCR4 antagonist study drug. The objective of this study is to mobilise circulating endothelial progenitor cells by modulating the CXCR4/CXCL12 axis. Modulation of CXCR4/CXCL12 pathway results in homing of EPC into the injured myocardial tissues. The study drug inhibits CXCR4, thus mobilisation of EPC and their homing into the injured myocardium can occur. Enrolled patients received the study drug intravenously on day 5 and day 7 after STEMI. The primary end-point of the study is increase in LVEF at 4- and 12-month follow-up. The secondary end-point includes detailed hemodynamic parameters, mortality parameters and analysis of the level of activated EPC. Fluorescence cell sorting method is performed in a core lab, evaluating the exact amount of

activated CD31 positive, CD34 positive EPC. Outcome of this and other similar trials will reveal if EPC possess efficient regenerative capacity for cardiac repair. Mobilisation, characterisation and collection of EPC must improve to use them for regenerative cardiac repair.

2.3.2. Clinical results with mesenchymal stem cells for cardiac regeneration

The fate and characteristics of MSC were discussed in details in *section* 2.2.2. This section will introduce the clinical data of MSC treatment in cardiovascular diseases. As MSC have good availability and safety profile, most of the current clinical trials were performed with these cell types. Unfortunately, some of these clinical trials reported false results to enhance beneficial outcome. Comprehensive results of meta-analyses showed modest increases in cardiac functional parameters (e.g. LVEF, six-minute walk test (6MWT)). However, these significant beneficial effects diminished in longer-term follow up [98].

Major difficulty when comparing clinical results from MSC trials is the diversity in study designs. In most studies, intracoronary injections were performed after acute MI, using varying numbers of MSC (between $2x10^6$ and $2500x10^6$). However, these clinical trials can be described by their procedural heterogeneity, and a lack of standardization [99]. Some studies reported beneficial results, like a study from Lunde et al., which showed improvement in echocardiography, SPECT and MRI parameters after 6 months follow-up [100, 101]. The **R**einfusion of **E**nriched **P**rogenitor Cells **A**nd **I**nfarct **R**emodelling in **A**cute **M**yocardial **I**nfarction (REPAIR-AMI, *NCT00711542*) trial recruited a large number of patients (240) with acute phase STEMI. This study was a phase II, randomised, placebo controlled, and double-blinded one. Patients received autologous intracoronary injections of bone-marrow derived MSC (50ml) vs. placebo medium. Detailed coronary artery hemodynamics were measured to assess coronary microvascular function. At four months after MSC implantation, patients showed significant improvement in LVEF compared to placebo (+5.5 vs +3.0%, absolute difference +2.5%). However, detailed analysis revealed that substantial treatment effects of MSC were limited to areas with the greatest damage or extent of scarring [102].

Further studies investigated the effects of MSC implantation in stable coronary artery diseases. These studies showed improvement in left ventricular function, perfusion and relief of angina pectoris [94]. The **T**ransplantation **O**f **P**rogenitor **C**ells **A**nd **R**ecovery of Left Ventricular Function in Patients with non-ischemic **D**ilatative **C**ardio**M**yopathy (TOPCARE-DCM, *NCT00284713*) trial studied effects of MSC transplantation in non-ischemic heart failure. Patients with severe, idiopathic and non-ischemic heart failure received autologous implantation of bone marrow-derived progenitor cells into the LAD. MSC implantation was found to be safe and feasible in non-ischemic etiology. Furthermore, improvement was noted in clinical parameters such as left ventricular volume, LVEF and decrease in serum BNP levels.

In contrast to beneficial study results, some others published minor or no improvement in clinical parameters after MSC implantation. A study from Janssens and colleagues found no benefit in LVEF, but a significant reduction in infarct size and improved in regional left ventricular function [103].

Special type of MSC, the endometrial regenerative cells (ERC) is under investigation in congestive heart failure. Patients with NYHA III-IV functional status and decreased LVEF (<40%) receive ERC implantation via the coronary sinus. The study involves three cohort of cell dose: 50-100-200 million. The interim analysis provided beneficial safety results of ERC delivery [104].

In the recent years meta-analyses published large cohort of results with MSC. These trials [105-108] involving altogether 999 patients with acute MI or chronic ischaemic cardiomyopathy showed that transplantation of MSC improved LVEF by 5.4%, decreased infarct scar size by 5.5% and lowered left ventricular end-systolic volume by 4.8 ml [108]. Other meta-analyses [109] indicated that MSC treatment is beneficial; however, the typical modest increase in ejection fraction is of uncertain clinical significance. Recent meta-analysis from Gyöngyösi et al. suggested neutral effects of MSC treatment, especially in longer-term follow-up [98]. It was shown that intracoronary or direct intramyocardial delivery route may be the most beneficial. Other delivery routes (coronary sinus, systemic venous or surgical) have proven less success. The analysed data displayed an overall 0.9-6.1% improvement in LVEF; nevertheless these improvements diminished in half of the trials on the longer term follow-up. By now, arguments are clear that MSC are unable to transdifferentiate into cardiomyocytes [110]. Their beneficial effects may derive from

immunomodulation, pro-angiogenic, antiapoptotic and anti-fibrotic effects. Table 2. summarizes MSC advantages and disadvantages for cardiac regeneration. Preclinical and translational studies must improve to enhance MSC use for cardiac regeneration. State-of-the-art studies suggest utilizing MSC products, which may be responsible for their regenerative effects, e.g. transplantation of MSC-derived exosomes and microvesicles only [111, 112].

Table 2. Characteristics of mesenchymal stem cells in cardiac regeneration Table shows advantages and disadvantages of mesenchymal stem cells concerning cardiac regeneration (MSC: mesenchymal stem cells)

Advantages of MSC	Disadvantages of MSC
Easy to collect, isolate and expand	*Exact mechanism of action is unclear*
Low immunogenicity	*True myocardial differentiation is lacking*
Multipotent	*Large heterogeneity*
Beneficial safety reports from previous studies	*Diverse study designs aggravate clinical interpretation*

While unmodified MSC have shown some promise in cardiac repair, angiogenic and myogenic effect of these cells might be enhanced by phenotypic modification. To facilitate a standardized performance of MSC used for cardiac repair, a group at Mayo Clinic led by André Terzic has used proteomic and genomic analysis to identify critical factors in the pathways regulating cardiac differentiation. Their approach has yielded a suite of molecules that drive cells *in vitro* into cardiopoiesis. A company, Cardio3 Biosciences, (currently Celyad) completed a clinical study (*NCT00810238*) to apply this technology for the development of the cardiac cell lineage, named: C3BS-CQR-1 (C-Cure®) to treat heart failure, reporting a significant improvement of LVEF associated with reduction in LV end-systolic volume and improvement in the 6MWT [113]. The larger randomized study (CHART-1, *NCT01768702*) is currently recruiting patients with ischemic heart failure. This study is running at the Heart and Vascular Centre, Semmelweis University, I am currently working as a sub-investigator in the trial. Patients are treated with autologous bone-marrow-derived MSC (C3BS-CQR-1) product). MSC are collected from the iliac crest via posterior crista biopsy performed by haematologists. A large volume of BM is

harvested (65-85ml) in order to collect a sufficient quantity of MSC. Harvested BM is shipped to a core laboratory, where MSC are isolated. Cells are treated with a special cocktail containing growth factors to enhance development of cardiomyocyte progenitors. The differentiation procedure takes up to 7-15 weeks. When the differentiation part is completed, autologous cells are shipped back to local hospital sites, where C3BS-CQR-1 is injected intra-myocardially via femoral artery catheterisation. C3BS-CQR-1 are expanded into cell numbers of $600x10^6$ and injected into 20 sites of the left ventricular wall. The primary end-point is set up from a composite involving number of hospitalisations due to worsening heart failure, changes in Minnesota living with heart failure questionnaire (MLHFQ) (improvement at least ten points) and 6MWT (increase at least 40m), changes in LV parameters (15ml absolute change), and LVEF (4% absolute improvement). Secondary end-points include efficacy and safety measurements. Safety parameters include the number and cause of deaths and hospital re-admissions, the number of cardiac transplantations and myocardial infarctions and the number of strokes. Efficacy parameters entail time to all-cause mortality, time to worsening of heart failure and time to aborted sudden death. After promising results from C-Cure®, CHART-1 gives hope for beneficial outcome.

2.3.3. Clinical studies with cardiac stem cells for cardiac regeneration

Characteristics of cardiac stem cells are discussed in section 2.2.3. Here the preclinical results and clinical trials with CSC are discussed. The injection of human cardiosphere-derived cells into injured myocardium showed some benefit in animal models mainly by improving left ventricular function [114, 115]. The *cardiospheres* are obtained from myocardial biopsy and can give rise to cardiomyocytes, endothelial cells and smooth muscle cells. Human cardiospheres exhibit significant proliferation and differentiation capacity [114]. Isolated cell populations can be differentiated into spontaneously beating aggregates of cardiomyocytes [115]. In mouse model for myocardial infarction epicardium derived CSC could restore cardiac function, by reducing dilatation of the heart chambers and increasing ejection fraction [116].

The **CA**rdiosphere-**D**erived a**U**tologous stem**CE**lls to reverse ventric**U**lar dy**S**function (CADUCEUS, *NCT00893360*) trial is a completed phase I safety study, investigating the

effects of CSC in ischemic heart failure. During the study procedure by Marban et al. from the Johns Hopkins Hospital, small biopsy samples were taken from recruited patients' myocardium. Later 12.5x 10^6 or 25x 10^6 of autologous CSC were injected intracoronary. Results reported that CSC inplantation is safe after myocardial infarction [117]. Unexpected beneficial results showed in addition to decreased scar size, the increased amount of viable myocardium as measured by cardiac MRI [117].

Therapeutic perspectives of CSC are also investigated in rare and congenital cardiac diseases. The **T**ranscoronary **I**nfusion of **CA**rdiac **P**rogenitor Cells in Patients with Single Ventricle Physiology (TICAP, *NCT01273857*) and Cardiac Progenitor Cell Infusion to Treat Univentricular Heart disease (PERSEUS, *NCT01829750*) trials study the effects of cardiac progenitor cells in congenital heart diseases. In TICAP, cardiospehere-derived cells are implanted intracoronary, one month after Norwood, Glenn or Fontan procedures. Patients receive 0.3 million/kg autologous CSC. In phase I designed TICAP trial, safety results enhanced the running of phase II PERSEUS trial.

2.3.4. Preclinical and early clinical studies with embryonic stem cells in the cardiovascular field

Human ESC have emerged as one of the most promising sources of new cardiac cells for transplantation because of their capacity to efficiently undergo directed differentiation into genuine cardiomyocytes and supportive endothelial cells. A number of groups have successfully isolated cardiomyocytes or cardiac progenitor cells from differentiating ESC cultures [118, 119]. These *in vitro* derived cardiomyocytes have been characterized extensively. Structural, electrophysiological and contractility studies indicated that ESC-derived cardiomyocytes exhibit a phenotype reminiscent of foetal, rather than adult cardiomyocytes. In the animal transplantation models of cardiac disease, use of ESC-derived cardiomyocytes has resulted in a significant improvement in ventricular function and structure (Table 3.). The cells appear to form gap junctions with host cardiac tissue; however, formation of protective fibrotic tissue around the grafts can interfere with complete electrophysiological coupling [120]. The beneficial effects in MI have been reported one month after transplantation [121].

Table 3. Preclinical studies on embryonic stem cell derivatives in myocardial infarction models Table shows details of preclinical studies with human embryonic stem cells regarding cell number, animal models, timing and follow-up (Table is adapted from Kosztin/Gara et al. Stem cell therapy to treat heart failure Elsevier 2015, Reference Module in Biomedical Sciences)

Cell type	Number of injected cells	Animal model	Timing	Follow-up	Ref.
ESC (cardiac committed)	$5x10^7$	sheep	14 days after MI	4 weeks	[122]
hESC-CM	$0.03\text{-}0.1x10^6$	mouse	Day of MI	3-4 weeks	[123]
hESC-CM	10^6	mouse	Day of MI	12 weeks	[120]
hESC-CM	10^7	rat	4 days after MI	4 weeks	[121]
hESC-CM	10^8	guinea pig	10 days after MI	4 weeks	[124]
hESC-CM	10^9	monkey	14 days after MI	3 months	[125]

Murry et al. evaluated the effects of human cardiomyocyte implantation in non-human primate model of myocardial infarction. In their study design *Macaca nemestrina* non-human primates suffered myocardial infarction via balloon inflation in LAD. Two weeks later, hESC-derived cardiomyocytes were injected transepicardially into the myocardial scar and surrounding tissue. Animals received immunosuppression to avoid rejection of human cells. The injected CM cells expressed eGFP construct. This trial was the first to prove feasible and successful delivery of large human grafts in MI model. After follow-up for 14-84 days electromechanical coupling have been verified. Furthermore, GFP-positive human cells were successfully seeded among endothelial cells from host primate, suggesting enhancement in angiogenesis. Recently this study has used the largest number of injected cells. During the implantation procedure one billion hESC-CM were injected into the peri-infarct zone of the hearts. The commendable results postulate for further evaluation of post-transplantation expansion, maturation, survival, and long-term effects of grafted ESC-derived cardiomyocytes. In Murry's large animal model non-fatal, sustained ventricular and supraventricular tachyarrhythmias were observed after the implantation of cells. These pro-arrhythmogenic effects were not developed in small animal models, emphasizing importance of large animal studies [125, 126]. However, these cells have not been tested therapeutically since the problems of immunosuppression and the risk of

teratomas from residual undifferentiated ESC remain. The latest preclinical data suggest that co-transplantation of hESC-derived cardiovascular cells with MSC may enhance the beneficial effects of hESC derivatives. MSC may support the engraftment and survival of implanted hESC derivatives via anti-inflammatory and immunosuppressive effects [127].

The first clinical trials in cardiovascular disease are just launched. Menasche and colleagues have differentiated a population of CD15 and Isl1 positive cardiac progenitors from hESC. The Transplantation of Human embryonic Stem Cell-derived Progenitors in Severe Heart Failure (ESCORT, *NCT02057900*) phase I trial recruits patients with ischaemic heart failure with indication for coronary-artery bypass graft or valvular surgery. Eligible patients have NYHA III-IV functional status and decrease LVEF (<35%). During the operation a fibrin-based cardiac patch, seeded with CD15/Isl1 double-positive cardiopoietic cells, are placed epicardially onto the infarcted area with a pericardial flap [32, 128].

Table 4. shows the advantages and disadvantages of hESC for regenerative purposes.

Table 4. Advantages and disadvantages of hESC for cell therapy Table lists advantages and disadvantages of human embryonic stem cells for cell therapy in heart failure (hESC: human embryonic stem cells)

Advantages of hESC	Disadvantages of hESC
Unlimited availability	*Ethical and legal considerations*
Off-the-shelf-use	*Teratoma formation from residual stem cells*
Differentiate into functional CM	*Immunosuppression probably needed*

2.3.5. Preclinical results and future challenges with induced pluripotent stem cells

Induced pluripotent stem cell-derivatives promise considerable benefits for disease modelling and drug testing *in vitro*. As hiPSC are generally derived from adult somatic cells, new cell lineages may be patient specific. Thus, personalised drug testing and therapeutic strategies can be established. Commercially available hiPSC-derived cardiovascular cells, especially cardiomyocytes can be used in *in vitro* assays for testing cardiotoxicity, contractile properties, drug-induced early afterdepolarization or QT prolongation. First cardiac studies on hiPSC described long QT syndromes and catecholaminergic ventricular tachycardias [129-131]. Inherited disorders, like arrhythmogenic right ventricular cardiomyopathy or Brugada syndrome were also investigated by using hiPSC [132, 133].

Beside disease modelling *in vitro*, hiPSC provide platform for cardiovascular tissue engineering and cell therapy. A group developed differentiation methods to engineer cardiac tissue cell sheet, which contains cardiomyocytes, endothelial cells and pericytes [134]. This preclinical study involved implantation of hiPSC-derived cardiac cell sheets into rat model of myocardial infarction. The cell sheets were cultured on special thermo-sensitive polymers, and then the biopolymers were detached without enzymatic digestion. This method is promising, as non-enzymatic protocols avoid modification of cell phenotype or loss of cell number during enzymatic digestion. Results of this preclinical trial are in progress.

Human iPSC give opportunity to model cardiovascular diseases in vitro, to investigate inherited cardiovascular conditions in a dish, to assess cardiovascular drug toxicity and to move towards therapeutic options and cardiovascular tisseue engineering; even individually. A recent clinical trial, the Individualized Early Risk Assessment for Heart Disease (IndivuHeart, *NCT02417311*) clinical trial is recruiting patients from June 2015. The study is aimed to characterise in details phenotype of specific cardiac pathologies in engineered heart tissue (EHT) setting, *in vitro*. EHT enables high-throughput, comprehensive studies on hiPSC-CM, in 3D culture platforms [135, 136]. Fourty healthy volunteers, ten patients with DCM, ten patients with HOCM and ten patients with HFpEF will be recruited. Skin biopsies will be taken and EHT will be developed patient-specifically. The study design aims to characterise hiPSC-EHT function in basal and in

hemodynamic stress conditions. Furthermore, this study aims to define disease specific phenotype *in vitro*, to study pathophysiology steps of DCM and HOCM in EHT. These patient specific EHT constructs provide platform for individualised drug testing both on acute applications and long-term drug administrations.

Tracking cells via cell therapy and studying fate of the transplanted cell is one of the major challenges in preclinical trials with hiPSC. At Stanford University, Wu and colleagues work on hiPSC-derived cardiomyocytes to provide high scale results regarding prevention of donor cell loss during cell therapy [135, 137]. They aimed to establish high-throughput imaging techniques to track cells after implantation.

Advantages and disadvantages of hiPSC for therapeutic purposes are shown in Table 5. Advantages may translate into safety, cost-effectiveness and can terminate the ethical debates related to harvesting of stem cells from different sources. Immunocompatibility issues may be reduced with iPSC derivatives because the beginning cell line (e.g. skin fibroblasts) can be obtained patient-specificially. Albeit, the logistics of using them therapeutically may not be as simple as hoped [138]. Large-animal models using monkeys, dogs and pigs were demonstrated their feasibility and superiority to other pluripotent stem cells (ESC), due to low immunological complications with intramyocardial injection of autologous cells.

Efforts are now focused on the development of large hiPSC banks for a variety of diseases. Banking these cells, similarly to widespread umbilical cord blood banking, may serve public health advantages in the future. Characterizing the human leukocyte antigen (HLA) pattern of the banked cells may further enhance their future clinical use.

Table 5. Advantages and disadvantages of hiPSC for cell therapy Table lists advantages and disadvantages of human induced pluripotent stem cells for cell therapy in heart failure (hiPSC: human induced pluripotent stem cells)

Advantages of hiPSC	Disadvantages of hiPSC
Patient specific therapy	*Genome instability during and after reprogramming*
Patient specific disease modelling	*Epigenetic modulations during reprogramming*
Patient specific drug testing	*Development of product takes time*
Immunosuppression possibly not needed	*Teratoma formation from residual cells*

2.4. Tissue engineering for cardiovascular repair

In recent years many novel bioengineering methods and perspectives have been born, enabling cardiovascular tissue engineering. As cardiovascular diseases are the leading cause of death in industrialised countries, replacement of damaged cardiovascular tissue is in the focus of research. The number of cadaveric donor organs for cardiac transplantation is limited. In case of vascular diseases such as aortic aneurysm and aortic dissection, artificial tissue vascular grafts are available, but the number of biological donor vascular grafts is also limited. Thus, cardiovascular tissue engineering paves the way for novel therapeutic options concerning cardiovascular tissue regeneration. For cardiovascular tissue engineering, pluripotent stem cell derivatives and ECM components offer promising sources. Beside early clinical trials involving cell therapy *in vivo*, tissue engineering *in vitro* was also in focus in recent years. *In vitro* engineered cardiovascular tissues are developed for transplantation, to fill the gap between the availability of donor organs and their unmet need.

Tissue engineering requires special cell culture methods *in vitro*. To develop large number of cardiovascular cells, bioreactor systems seem to be ideal for scale-up. For tissue engineering purposes at least 10^6-10^9 cells (cardiomyocytes, endothelial cells and smooth muscle cells) are needed. Simple cell culture methods may be inefficient for such large number of cells. Bioreactor systems are capable for developing large capacity cultures with or without ECM components. Indeed, bioreactors provide cell culture techniques which

enhance maturation of stem cells-derived cardiovascular phenotype (e.g. wall sheer stress (WSS)), for the development of arterial endothelial cells) [139]. One of the major advantages of bioreactor systems is that cell culture environment is standardised and reproducible. In bioreactor systems temperature, the oxygen and carbon-dioxide pressure as well as pH are strictly regulated and monitored. Bioreactor systems enable culturing cardiovascular cells and ECM components in three dimension systems.

Beside cardiovascular derivatives of pluripotent stem cells, ECM components determine the product of tissue engineering procedure. The ideal ECM for tissue engineering should be biocompatible, biodegradable, matching with host environment and non-toxic. It should also be able to fulfil functional properties of the engineered tissue (e.g. electromechanical coupling and contractile function in case of the myocardium). So far, none of the investigated ECM have satisfied all requirements, although many ECM types have been established and studied for cardiovascular tissue engineering such as: fibrin [140], collagen [141], conductive biopolymers [142] and hydrogels [143]. The most promising are the decellularised biomatrices. Doris Taylor and her group have detailed, in depth research on cardiovascular tissue decellularisation. After decellularisation the biografts may be re-seeded with cardiovascular cells and thus biological cardiovascular tissue could be rebuilt [144-147]. This technique holds out for the utopian view of developing a whole new human heart. Weymann et al. succeeded to develop a human size whole tissue engineered heart in a bioreactor system on decellularised porcine hearts [148]. Decellularised whole heart ECM were recellularised with HUVEC and murine neonatal cardiomyocytes. Developing every human cell types in the heart (pacemaker cells, atrial cardiomyocytes, conductive tissue, ventricular cardiomyocytes, endothelial cells, fibroblasts, pericytes and smooth muscle cells) is a major challenge for cell culture and differentiational techniques.

Valvular tissue engineering is also on the way for regenerative purposes. Synthetic [149] and biological [150] ECM are being investigated to provide surface for valvular tissue engineering. These matrices are cellularised with endothelial cells from different sources [150-152]. Further, state-of-the-art platforms in tissue engineering offer bioprinting of biocompatible valvular structures [153]. Biomechanical and thrombogenic properties of decellularised valvular matrices are also studied [154]. Valvular tissue engineering may have the most benefits in paediatric heart surgery, while engineered biocompatible grafts

are able to change shape and grow within the host tissue, during aging of pediatric patiens [155-157].

Huge efforts have been made to optimise methods for tissue engineering: mechanical and electrical stimuli before cell seeding may enhance preconditioning of matrices for cell seeding. Furthermore, studying physical patterns (size, porosity, vulnerability, thickness, and rupture) enhances optimisation for tissue engineering. Some matrices enable encapsulation of growth factors and anti-apoptotic materials to improve cell survival and proliferation after seeding [158].

The groups of Thomas Eschenhangen and W.H. Zimmerman performed *in vitro* studies on EHT. EHT are created from fibrin polymers and hPSC-derived cardiovascular cells (cardiomyocytes and fibroblast) [159-162]. EHT provides unique platform for studying hPSC-derived cardiomyocytes function in three dimensional cell culture environment. In EHT fibrin matrices and cardiovascular cells are anchored to silicone stripes. In EHT contractile function of three dimensional structures can be accurately measured [163]. Therefore EHT provides platform for cardiovascular drug testing *in vitro*, even in patient-specific, personalised manner. Detailed inotropic, lusitropic and chronotropic effects can be investigated.

New preclinical perspectives include cellular transdifferentiation and *in vivo* gene therapy to enhance impaired cardiac function. During transdifferentiation, one mature cell type differentiates into another mature cell type. Despite our earlier knowledge on embryonic development, transdifferentiation can take place *in vivo*. After pathologic events transdifferentiation may be enhanced or forced to support tissue healing and prevent definite injury of cardiovascular cells. Transdifferentiation of fibroblast to cardiomyocytes or endothelial cells *in vivo* would be a cornerstone in prevention or reversion of ventricular remodelling. Thus, preclinical research focuses on transdifferentiation mechanisms [164, 165]. Detailed transcriptome analysis, epigenetic patterns and microRNA profiles are investigated to enhance *in vivo* cardiac regeneration [166, 167].

2.5. Endothelial differentiation and function

Endothelial cells have major role in physiology of the cardiovascular system. Their efficient operation is crucial for controlling vessels' tone and function of microcirculation. This chapter gives a short overview of endothelial physiology.

By sheathing the inner surface of the vessel wall, endothelial layer connects the flowing blood and the vessel tissue, at approximately 350 m^2 in the human body. It has been proven that the endothelium is not only a passive barrier, but it also plays an important role in physiological conditions: regulates vessel tone, vascular resistance and fluid flow through the capillaries; furthermore regulates metabolic functions [168]. Endothelial dysfunction plays a key role in cardiovascular disease, mainly in pathophysiologic steps of hypertension, atherosclerosis and diabetes mellitus [169, 170]. Endothelium is capable to communicate with smooth muscle cells, via released vasoactive factors [168]. Endothelium provides a semipermeable membrane between blood and vessel structure. To fulfil this barrier function endothelial cells form a tight monolayer, expressing wide range of cell adhesion molecules, like VCAM, ICAM, VE-cadherin, etc. Furthermore, endothelium also has a key role in orchestrating blood clotting, by producing mediators and inhibitors that regulate platelet activation. In endothelial injury on one hand endothelium enhances blood clotting. On the other hand endothelial derivatives inhibit overdrawn blood clotting and thrombotic events. Endothelial cells mimic monolayer structure when culturing *in vitro*, thus their morphology show cobblestone pattern [171]. Endothelial vasoactive factors have specific receptors on smooth muscle cells, resulting in vasodilation, or in vasoconstriction. Endothelial vasoactive agents regulate vessel tone and vascular resistance. Most important vasoactive factors in the point of endothelium (ACh, NO - endothelial derived relaxing factor (EDRF) and arachidonic acid metabolites) will be discussed.

Endothelium has the key role in flow induced dilation mechanism in the resistance arteries. The vasodilator response given to ACh is ceased after denuding the endothelium [172]. The EDRF, mediating smooth muscle relaxation is proven to be NO, is synthetized by nitric oxide synthase (NOS), from L-arginine in the vessel wall [173]. The NOS enzymes have three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). L-arginine is the physiological substrate of the family of NOS enzymes. nNOS and eNOS are expressed constitutively whereas iNOS is induced by inflammatory

cytokines. The production of NO in vascular beds is regulated by many haemodynamic actions such as WSS. Beside its role in flow dependent vasodilation, NO has other important functions in the microcirculation. NO is an endogenous modulator of leukocyte adherence and modulates platelet and leukocyte activation and adhesion to the vessel wall. Upon endothelial damage, subsequent inflammation causes an increase in leukocytes at the damaged site. The arachidonic acid-derived prostanoids are produced by COX enzymes. The COX enzymes have two isoforms, the COX1 and COX2 isoenzyme. COX1 is constitutively expressed in most tissues and produces mainly dilator prostaglandins, like PGI2. COX2 isoenzyme is thought to be an inducible enzyme; however it is also expressed constitutively. Proinflammatory conditions, inflammation, tissue damage, hypoxemia, ischaemic conditions and hyperalgesia induce COX2 enzyme [174]. Another important mediator is endothelin-1, which has major role in regulating arterial and venous tone. It has been shown as most potent vasoconstrictor agent in the circulation. Endothelin-1 is mostly secreted from endothelial cells, although during inflammatory responses it may also be produced by other cell types [175].

Arterial and venous subpopulations of endothelial cells differ not only in their localisation but also in their functional properties. In each organ and each level of vascular arch endothelial cells have specific functions. The main differences between arterial and venous endothelial cells are their functional characteristics. Arterial endothelial cells are responsible for regulating vessel tone and setting peripheral vascular resistance in the circulation by defining vascular tone of pre-capillary arteries. The vasoactive role is less important in venous endothelial cells; however it determines the preload of the left ventricle. Furthermore, post-capillary venulas and veins are the site of inflammatory reaction where white blood cell rolling, diapedesis and extravasation occur. Thus, venous endothelial cells must have different cell adhesion properties than those of arterial endothelial cells. Specific sites of the circulation, such as the blood-brain barrier, the renal glomeruli and liver sinusoids include endothelial cells with unique properties. It is well-known that environmental factors (paracrine signals, effects of WSS) may alter arterial and venous endothelial fate. Coronary grafts derived from saphenous veins function properly after CABG surgery. Recent studies have shown that beside the plasticity of arterial and venous fate, developmental potential may be regulated on transcription levels. Arterial and venous fate is genetically determined. As arteries and veins have far more different

functional role than lymphatic vessels, the development of the first arteries and veins is determined genetically; lymphatic vessels develop later from pre-developed veins [176]. The major regulatory pathway for arterial development is controlled by VEGF and Notch pathways [177]. Notch1, Notch2, Notch5, EphrinB2, DLL4 and Connexin40 are known to be regulators for arterial development [176]. EphB4 is responsible for venous development, as well as FLT4; the latter is more likely to be a lymphatic marker for endothelial phenotype. Other underlying regulatory pathways may also mediate endothelial development. A recent study has shown that transcription factor FoxC1 leads to development of arterial endothelial cells via upregulation of Notch1 [178]. Furthermore, activation of the PI3K/FOXO1A signalling pathway via Notch signals modulates endothelial development (Figure 5.).

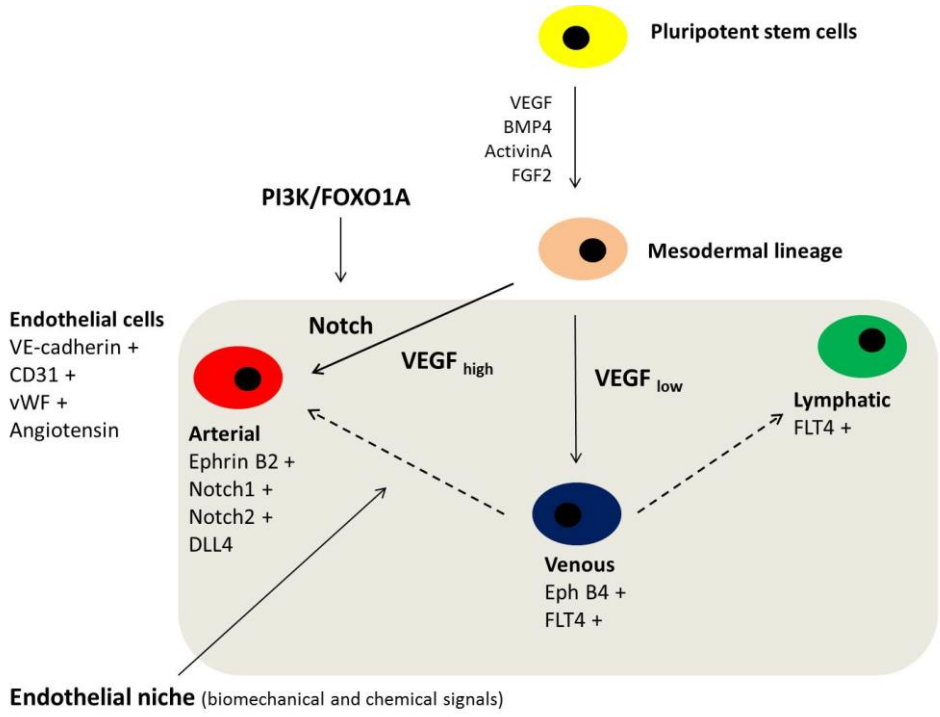


Figure 5. Differentiation of arterial, venous and lymphatic endothelial cells from human pluripotent stem cells Schematic drawing shows differentiational steps, crucial growth factors and cytokines responsible for endothelial development *in vitro*. General endothelial and specific arterial, venous and lymphatic markers are listed below each cell type. (Original figure is from Edit Gara)

2.6. Phosphatidylinositol 3-kinase (PI3K)-Forkhead box O transcription factor 1A (FOXO1A) signalling pathway

Phosphatidylinositol 3-kinase (PI3K), a lipid kinase, has been shown to play an important role in regulating cell proliferation, adhesion, DNA repair, senescence and stemness [179]. PI3K is highly suited for pharmacologic intervention, which makes PI3K pathway one of the most attractive therapeutic targets in cancer and diabetes [179]. The effects of PI3K in the therapy of cardiovascular diseases have not been tested yet. The members of the Forkhead box O (FOXO) transcription factor family are one of the main downstream components of PI3K pathway [180, 181]. FOXO transcription factors are critical proteins in the regulation of pluripotency of hESC and in modulation of cardiovascular development [182]. FOXO transcription factor family have almost 100 members, from FOXA to FOXR [183]. FOXO transcription factors are involved in response to oxidative stress, cell survival, cell cycle, angiogenesis, energy homeostasis and glucose metabolism [184-186]. Specific members of FOXO family have crucial role during embryonic development of the cardiovascular system, as Forkhead box O transcription factor 1A (FOXO1A)-deficient embryos die around embryonic day 11 because of their inefficient vascular and cardiac systems [187]. FOXO1A is also important in regulation of endothelial cell fate. Indeed, it has been found that mouse embryonic stem cell-derived endothelial cells lacking FOXO1A, are unable to respond to vascular endothelial growth factor (VEGF), an important angiogenic growth factor for normal vascular development [186]. A better understanding of PI3K-FOXO1A-related signalling in specification of human endothelial cells can provide a therapeutic advantage in cell transplantation and tissue engineering. Figure 6. shows schematic PI3K/FOXO1A signalling pathway in endothelial cells.

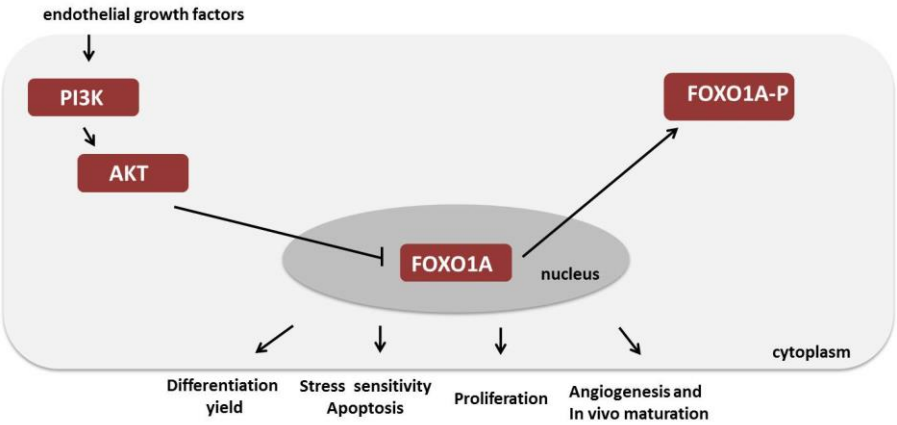


Figure 6. PI3K/FOXO1A signalling pathway in endothelial cells Schematic figure shows regulatory role of PI3K/FOXO1A signalling pathway in endothelial characteristics and function (Original figure is from Edit Gara)

3. Aims

The aim of this study was to develop and characterise endothelial cells from hESC and hiPSC. Gaining detailed information about endothelial differentiation and behaviour *in vitro* and *in vivo* increases a better understanding of cardiovascular diseases and development of new cell products which can be utilised in preclinical studies.

The specific aims of the project were:

1. To optimise endothelial differentiation protocols from human pluripotent stem cells.
2. To characterise endothelial fate in details, particularly arterial and venous subpopulations and angiogenesis-related properties.
3. To investigate the role of PI3K/FOXO1A signalling pathway in endothelial differentiation, proliferation, viability and angiogenesis.
4. To develop three dimensional endothelial cell culture systems for tissue engineering.
5. To define functional characteristics of endothelial cells, especially anticlotting activity.
6. To investigate endothelial maturation *in vivo* and to study effects of *in vivo* environment on endothelial characteristics.

4. Materials and methods

4.1. Human pluripotent stem cell culture

Stem cells were cultured under sterile conditions, using a biosafety cabinet (*ESCO Class II Type A2, Horsham, USA*). Human ESC used in these studies were commercially available. All ethical approvals had been acquired for research purposes, before purchase (Egészségügyi Tudományos Tanács, Humán Reprodukciós Bizottság, *ETT TUKEB*). Experiments were carried out on WA007 (H7) female hESC line (*WiCell Research Institute Bank, Madison, USA*). hESC culturing, maintenance and passaging followed protocols by the supplier.

Before thawing hESC, 6-well plates (*Greiner Bio One, Frickenhausen, Germany*) were coated with Matrigel (*BD Biosciences, San Jose, USA*). Matrigel is composed of extracellular matrix proteins and derived from Engelbreth-Holm-Swarm mouse sarcoma cells [188]. Matrigel is sufficient for supporting maintenance of pluripotent stem cells, as H7 stem cells were cultured in feeder-free conditions. 6-well plates were coated with the 1:30 dilution of Matrigel and KO-DMEM/F12 media (*Life Technologies Carlsbad, USA*) in ratio 1:1 (1ml/well). During thawing H7 hESC were gently warmed in water bath, after removing from liquid nitrogen. To avoid osmotic injury feeder medium was added drop wise. Cells were centrifuged (1100 RPM, 5 min), then pellet was re-suspended and stem cells were plated in Matrigel coated wells in mTeSR1 medium (*StemCell Technologies, Vancouver, Canada*). mTesR1 is a widely used predefined medium for human pluripotent stem cells, allowing feeder free conditions. mTeSR1 media is made from mTeSR1 basal medium and mTeSR1 supplements, containing wide range of factors (rh-bFGF, rh-TGFβ, lithium chloride, pipecolic acid and GABA) and other components: anorganic buffers (pH 7.2-7.4), carbohydrates, proteins, vitamins, fatty acids, bovine serum albumin; no further addition of proteins or growth factor is required for stem cell maintenance [55].

Human pluripotent stem cell cultures were observed daily by inverted light microscopy. Cells were fed daily by complete media change (2ml fresh mTeSR1/each well) and kept in 37°C, 5% CO_2 and 21% O_2 conditions. Combined (mechanical and enzymatic) passaging procedure was performed every 4-10 days, in 1:4 or 1:6 ratio with dispase (*StemCell*

Technologies) to remove spontaneously differentiated areas of the colonies. Stem cell colonies were scraped with mechanical scrapers (*Falcon, 353085*) or with 5ml pipette tip to standard size for further maintenance, freezing and for directed differentiation.

4.2. Generating human induced pluripotent stem cells

Human iPSC were generated by reprogramming adult somatic fibroblasts to pluripotent state (Figure 7.). Skin fibroblasts were derived from the members of our research group and were gained from skin biopsies, performed by our plastic surgeon collaborator (Dr. Béla Debreczeni). The skin biopsy procedure was carried out under sterile conditions and skin samples were immediately suspended in fibroblast medium (DMEM (*LifeTechnologies*) + 20% foetal bovine serum (FBS)). Penicillin-Streptomycin (P/S) was added to the medium to prevent contaminations from the normal flora of the skin. Skin samples were stacked onto gelatinised flasks. The cells were characterised by their phenotypic appearance in culture. Transfection of the fibroblast was performed with episomal plasmids:

-*Addgene Plasmid 27077*: pCXLE-hOCT3/4-shp53-F

-*Addgene Plasmid 27078*: pCXLE-hSK

-*Addgene Plasmid 27080*: pCXLE-hULpMXs-KLF4

-*Addgene Plasmid pCXLE-GFP* for control

The transfection procedure was carried out with electroporation (*BioRad Gene Pulser XCell, Gene Pulser Cuvette cat.no. 1652081*). During electroporation procedure cells receive electric stimuli in conductive media, resulting in temporary pores of the phospholipid layer on the cell membrane, thus transfection reagents can pass through it and reach cell nuclei. The transfected cultures were maintained in DMEM + 20% FBS + GlutaMax1 (*LifeTechnologies*), without P/S and in the presence of sodium butyrate (*Sigma-Aldrich, St. Louis, USA*). Changing media every other day was critical, until cells have grown 90% in confluence. Before overgrowing, the transfected cells were passaged and were seeded onto *Matrigel* surface and media was changed to *mTeSR1* to support proliferation of pluripotent cells. After 3 weeks pluripotent colonies were identified in culture. Pluripotent stem cell colonies were separated from culture mechanically and plated into feeder free conditions. The cell maintenance was the same as for hESC. The

fibroblast-derived iPS cells were characterised albeit have not been used in further experiments.

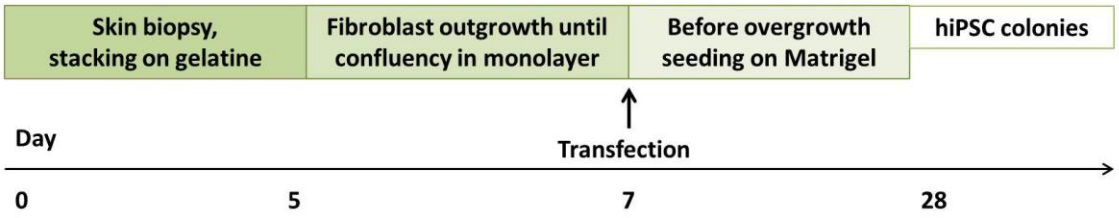


Figure 7. Generating human induced pluripotent stem cells from adult somatic cells Time line diagram shows development of hiPSC. Skin samples were obtained from biopsies and stacked on gelatine-coated plates to allow fibroblast outgrowth. Confluent fibroblast cultures were transfected with pluripotency genes. After 3 weeks pluripotent colonies appeared and were maintained on Matrigel.

4.3. Characterising pluripotency

The characterisation of pluripotency involved immunecytochemical analysis and gene expression analysis. Stem cell cultures were washed with PBS and fixed in 4% para-formaldehyde (PFA) to perform immunocytochemical staining. Fixation of the clusters in PFA lasted for 10 minutes in room temperature. Plates were then washed in PBS three times. The permeabilization of the cells were performed with 0.2% Triton X-100 (*Sigma-Aldrich*) for 10 minutes and then blocking was performed in 4% FBS in PBS for 1 hour at room temperature to avoid non-specific binding of primary antibodies. One of the most important pluripotency marker is SSEA4 (stage specific embryonic antigen 4). Stem cell clusters were stained with anti-SSEA4 antibody (*Abcam, Cambridge, United Kingdom*), overnight, at 4ºC. Secondary antibody was labelled with Alexa Fluor 488 for one hour. All applied antibodies are listed in Table 7. Cells were washed with PBS between incubations. Cell nuclei were counterstained with DAPI (*Life Technologies*). Plates were then stored in PBS at 4°C prior to imaging, using a Zeiss Observer Z1 microscope. Some wells were treated with secondary antibody only in order to determine levels of non-specific background staining.

To identify pluripotent state on gene expression level quantitative real-time polymerase chain reaction was performed (qRT-PCR). Pluripotent stem cell samples for RNA isolation were stored in Trizol at -80°C. Total RNA was isolated from cells with the *RNeasy Mini*

Kit (*Qiagen, Venlo, Netherlands*), according to manufacturer's protocols. In Spin-column equal volume of 70% ethanol and sample in Trizol were centrifuged at 10000g for 15 seconds. RNA was washed with buffers after centrifugation. Total RNA was eluted in 30µl of RNase-free water. RNA concentration was measured by micro-volume spectrophotometer, NanoDrop 2000c (*Thermo Scientific, Waltham, USA*). Complimentary-DNA (cDNA) was synthesized from extracted RNA using the *High Capacity cDNA Transcription Kit* (*Life Technologies*), according to manufacturer's protocols. 10µl of Master Mix cocktail (containing reverse transcriptase enzyme and buffers) and 10µl of equally attenuated RNA samples were added into *Microamp* fast reaction tubes and incubated for 10 minutes at 25°C – holding; 2 hours at 37°C – cycling then 5 second at 85°C holding. For quantifying mRNA levels pluripotency marker gene Oct4 qRT-PCR was succeeded, using *TaqMan® Gene Expression Assay* (*Applied Biosystems, Life Technologies*). As housekeeping gene control human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) endogenous control was used. Table 8. summarizes all assays used in PCR experiments. In every case non-templates control (NTC, without cDNA template) and non-enzyme control (NEC, without Master Mix were applied to detect contamination and non-specific amplification during PCR procedure. PCR was performed with real-time PCR instrument *(Applied Biosystem, StepOnePlus, Life Technologies or Mastercycler® ep realplex, Eppendorf)*: 2 minutes at 50°C – holding, 20 seconds at 95°C – holding, 1 second at 95°C – cycling; 20 second at 60°C – cycling; all together 40 cycles. Relative expression was determined by the ΔΔCt method in which fold increase = $2^{-\Delta\Delta Ct}$.

4.4. Endothelial differentiation

Endothelial differentiation was carried out by using four different protocols. Protocols involved either embryoid body formation or monolayer methods. In embryoid body method, H7 hESC were maintained in ultra-low attachment plates in order to form embryoid bodies (simulating the blastocyst stadium of the developing embryo). In monolayer methods, stem cells were maintained on Matrigel in monolayer. The differentiation protocols involved both normoxic and hypoxic conditions. To induce mesodermal development and endothelial differentiation H7 hESC colonies were seeded into EGM2 medium containing EBM2 media (*Lonza CC-3156, Basel, Switzerland*) and

SingleQuot supplements (*Lonza CC-4176*): growth factors, peptides, hormones and antibiotics. Table 6. shows detailed components of EGM2 medium.

To force differentiation into mesodermal lineage one differentiation protocol included extra growth factors and morphogenes in mTeSR1 media (Activin A *(R&D systems, Minnesota, USA, 10ng/ml*), FGF-2 (*Peprotech, New Jersey, USA, 10ng/ml*), $VEGF_{165}$ (*R&D systems, 10ng/ml*) and BMP4 (*R&D systems, 10 ng/ml*)). After 24 hours the media was removed and replaced with Stemline II complete media (*Sigma-Aldrich*) containing FGF-2, $VEGF_{165}$ and BMP4 (*all at 10ng/ml),* differentiating cells were maintained in Stemline II medium [171]. EBs were developed by mechanically breaking the hESC colonies as described before. EBs were cultured for four days in suspension on ultra-low attachment plates either in normoxic or hypoxic conditions. After four days, the EBs were seeded onto 0.5% gelatine (*Sigma-Aldrich*) coated 24-well plates or T25 flasks. At this point, one differentiation protocol included further $VEGF_{165}$ supplementation (1ng/ml *Peprotech*). After 13 days in EGM2 medium, CD31 positive endothelial cells were sorted from differentiating culture by fluorescence activated cell sorting method (FACS). Figure 8. shows endothelial differentiation methods used.

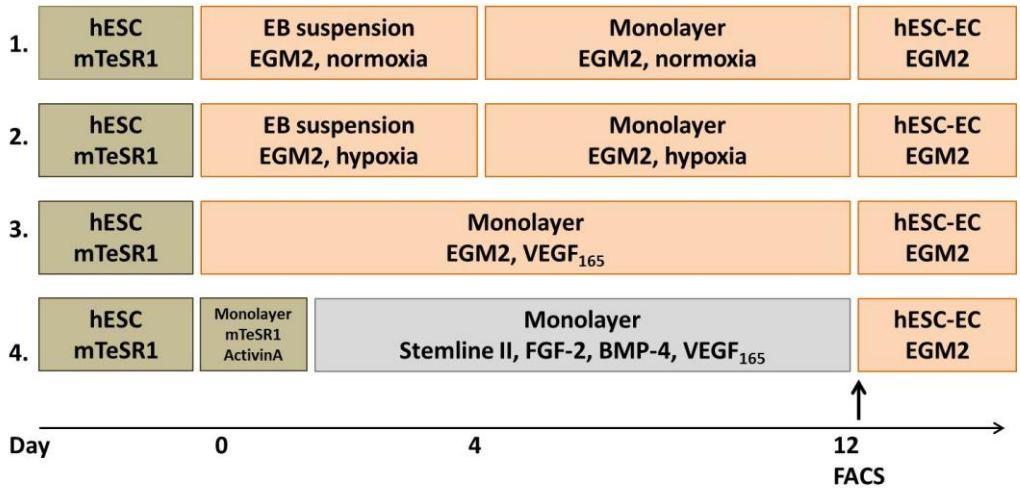


Figure 8. Endothelial differentiation methods Time line diagram shows steps of endothelial differentiation procedures. H7 hESC colonies were maintained in mTesR1 medium. Endothelial differentiation began with EB formation or in monolayer. Cells were differentiated both in hypoxic and normoxic conditions, with growth factor support. On the 13th day CD31 positive endothelial cells were sorted with fluorescence activated cell sorting method.

Table 6. Components of EGM2 medium. Table lists components of EGM2 medium

EGM2 BulletKit (*EBM2 + SingleQut Supplements*)	
EBM2	500 ml
Foetal bovine serum	10 ml
Human epidermal growth factor	500 µl
Vascular endothelial growth factor	500 µl
Human fibroblast growth factor B	500 µl
Insulin growth factor R3	500 µl
Hydrocortisone	200 µl
Heparin	500 µl
Ascorbic acid	500 µl
AmphotericinB/Gentamycin	500 µl

4.4.1. Flow cytometry and cell sorting

The hESC-derived differentiating culture was labelled with anti-CD31 antibody, conjugated with Alexa Fluor 488 fluorophore (*BD BioSciences, Minneapolis, USA*) for fluorescence activated cell sorting. CD31 is a cell surface endothelial marker protein. Labelling the transmembrane domain of CD31 protein allows sorting live cells. To perform cell sorting, differentiated cultures were trypsinized (0.25% Trypsin and 0.03% EDTA, *LifeTechnologies*), cell suspension was centrifuged (1100 RPM, 5 min) and the pellets were re-suspended in PBS, containing 1% FBS (FACS buffer). FACS buffer was also used for diluting antibodies. Cells were counted in hemocytometer before FACS. As controls, cells without any labelling and cells labelled with single channel CD31 IgG isotype control were used. Isotype control, labelled with Alexa Fluor 488 enabled avoiding false results from non-specific binding. Cell sorting was carried out on a FACS Aria II Cell Sorter (*BD BioSciences*). During FACS procedure cells pass through in a small nozzle that is vibrated

and produces small drops of the cell-suspension. In the nozzle cells are scanned by a laser light and cells gain electrical charge. The laser light excites Alexa Fluor 488 dye which emits a colour of light that is detected. By the light information the computer can determine which cells to separate and to collect according to their electrical charge. Figure 9. shows schematic figure of FACS procedure. FACS procedures were performed in collaboration with György Várady and Robert Sampson (Membrane Research Group, Hungarian Academy of Sciences and National Heart and Lung Institute, Imperial College, London, respectively).

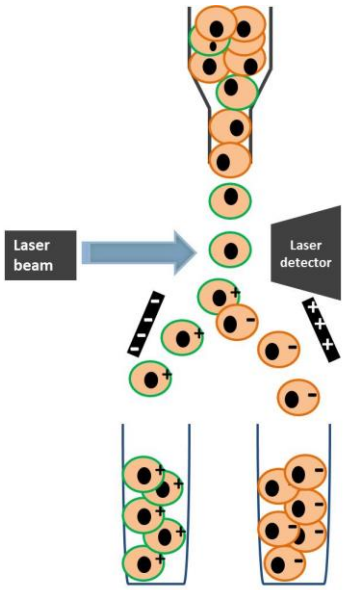


Figure 9. Schematic figure is representing fluorescence activated cell sorting (FACS) method During FACS procedure samples are scanned by laser light. The fluorescent, CD31 positive cells gain electronic charge, and separated by their electronic property (Original figure is from Edit Gara).

4.5. Endothelial cell culture and cell isolation techniques

The CD31 positive endothelial cells were cultured in 0.5% gelatinised T25 or T75 flasks. Cells were maintained in EGM2 medium and were feeded on every other day. Endothelial cells strictly proliferate in monolayer. Passages were needed on every 3-6 days according to proliferation rate. To characterise endothelial fate and for further experiments passages between 2 and 8 were used.

In addition to hESC-EC I have also studied hiPSC-derived endothelial cells (hiPSC-EC). Human iPSC were available from Osaka University, Japan (*ReproCell, Kanagawa, Japan*) and purchased from Wicell (*IMR-90-4*); endothelial differentiation steps were same as those with hESC. As endothelial control cells, human umbilical vein endothelial cells (HUVEC), human aortic endothelial cells (HAEC) and human coronary arterial endothelial cells (HCAEC) were used. HUVEC were a gift from the Royal Veterinary College London, HAEC and HCAEC were purchased from *Lonza*, and all were maintained in EGM2 medium in gelatinised T25 or T75 flasks. Endothelial cells were used for experiments between passages 2 and 6.

4.5.1. Isolation and culture of blood outgrowth endothelial cells

During my academic visit at National Heart and Lung Institute, Imperial College, London further human endothelial cell types were available for culture. Blood outgrowth endothelial cells (BOEC) were used as control endothelial cells in sheer stress studies to characterise endothelial fate. BOEC were isolated in collaboration with Daniel M. Reed at the NHLI. All ethical approval from local authorities and informed consent from BOEC donors were obtained. BOEC were isolated based on earlier protocols [189, 190]. Briefly, peripheral blood mononuclear cells were isolated from blood and expanded on tissue culture plates for 22 days in EGM2 medium with 10% FBS on collagen I (*Sigma-Aldrich*) coated plates (5.2 g/cm^2; *BD Biosciences*). Typically, endothelial cell colonies emerged between 5 and 22 days in culture and were expanded for use in experiments. Cells were used between passages 2–8.

4.5.2. Isolation of human umbilical vein endothelial cells

During my academic visit in Experimentelle Herzchirurgie, Ruprecht-Karls Universität, Heidelberg, I also learnt isolation protocol for HUVEC. Umbilical cords were collected in sterile conditions from Obstetrics Department under approval of local ethical authorities and after informed consent of the patients. All handling were performed in sterile biosafety cabinet. Umbilical cords were cannulated at both sides with 3 side syringe taps and were perfused with collagenase B solution (*Sigma Aldrich, 2.5mg/ml*). Umbilical cords were then filled up with collagenase B and incubated for 30 min in 37°C. After 30 min the umbilical cords were washed out with PBS solution (3 times). All enzyme and PBS solution were collected during the washing into 15ml tubes. Then the collected samples were centrifuged (1100 RPM, 10 min). The pellets (HUVEC) were re-suspended in EGM2 medium and plated onto gelatine coated plates or flask. These isolated HUVEC were not used further in my studies.

4.6. Three-dimensional culture system

Tissue engineering three-dimensional cell culture systems was used to study survival, viability and proliferation of pluripotent stem cell-derived endothelial cells (PSC-EC). The established 3D culture systems were based on bioscaffolds, hESC-EC and hiPSC-EC, using in small bioreactor system.

4.6.1. Bioscaffolds

Two types of biological ECM were used to develop 3D cultures: CorMatrix® and human aortic pieces. CorMatrix® (*CorMatrix ECM Technologies, Roswell, USA*) is a commercially available ECM for heart and vascular surgery (Figure 10. A). CorMatrix® is derived from porcine small intestinal submucosa. During the manufacturing process CorMatrix® is decellularised, thus only ECM proteins, such as collagen, elastin and adhesion proteins remain. Clinical use of CorMatrix® has already received FDA approval for vascular and cardiac repair. A clinical trial has started recently to study its feasibility

and safety in tricuspid valve plastic operations (*NCT023976689*). CorMatrix® samples used in our experiments were gift from the company.

Human aortic samples were used after decellularisation procedure. The aortic wall pieces or rings were derived from the Városmajor Biobank (*ETT TUKEB 7891/2012/EKU*), Heart and Vascular Centre and were collected during heart transplantations or other cardiac surgeries. The aortic pieces were stored in liquid nitrogen upon harvest and later thawed to perform decellularisation procedure. For this, aortic samples were washed in detergent solution for 48-72 hours, then in PBS + 1% P/S, until elimination of all detergent components (at least 72 hours) (Figure 10. B, C). Decellularisation protocols were adapted from collaborators at the Experimentelle Herzchirurgie at Ruprecht-Karls Universität, Heidelberg [191-195]. In our experiments decellularised aortic pieces were cut into 300 µm thin slices with a vibrating microtome system (*Campden Instruments, Lafayette, USA 7000smz*). Some slices were homogenized to perform DNA/RNA isolation. Decellularisation was confirmed by diminishing all DNA and RNA components, measured with *Nanodrop*. The aortic slices were pre-conditioned in EGM2 media prior to cell seeding.

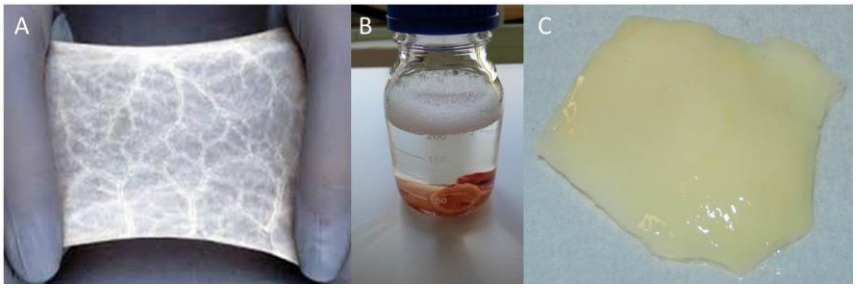


Figure 10. Bioscaffolds for 3D endothelial culture (A) CorMatrix® (B) Decellularisation procedure of human aortic samples in detergent solution (C) Decellularised human aortic sample

4.6.2. Bioreactor system

The small bioreactor system composed of scale-up spinner flask (*Corning, Basel, Switzerland*) (Figure 11.), endothelial growth media, endothelial cells and extracellular matrix as a carrier for endothelial growth (50000 endothelial cells/ 0.5cm^2 matrix). The small bioreactor system was kept under physiologic conditions (37°C, 85% humidity, 21% O_2) for endothelial growth. A magnetic stirrer is integrated into the spinner flask which enables shaking together endothelial cells and the scaffolds. Bioscaffolds along with hPSC-EC were seeded into the spinner flasks. Every other day fresh media was added to the flasks. After endothelial re-cellularisation vital imaging (Calcein AM (*LifeTechnologies*) and nuclear imaging with Hoechst-33342 or DAPI staining (*LifeTechnologies*) were performed. Confocal microscope and PCR analysis were performed to verify endothelial settlement on biomatrices. Microscope analysis visualised endothelial proliferation on the 3D vascular constructs.

Figure 11. Spinner flask

4.7. Endothelial characterisation

4.7.1. Immunocytochemistry

Applied immunocytochemistry were the same as earlier described in section 4.3. To label endothelial markers and FOXO1A wide range of immunostainings were used (Table 7.). Cell nuclei were counterstained with DAPI or Hoechst-33342. All antibodies were diluted in 3% bovine serum albumin in PBS. Plates were then stored in PBS, at 4°C prior to imaging acquired by Zeiss Observer widefield microscope or Cellomics® VTi HCS ArrayScanner (*Thermo-Fisher Scientific*). Some wells were stained with secondary antibody only in order to determine levels of non-specific background intensities. Cellomics® ArrayScanner is an automated fluorescence microscope. The system is able to automatically scan 96-well or 384-well plates and quantify the spatial and temporal distribution of fluorescence. Thus, the system provides detailed, high-throughput data about cell size, shape and nuclear size. Furthermore, detailed information can be obtained

about the cytosolic and nuclear fluorescence intensity, which allows quantification for translocation of transcription factors [196].

For live imaging to measure parameters of cell death, supernatant was removed, and hESC-EC and HUVEC were stained with TOTO-1 or TMRM (both 1:1000; *Life Technologies*) and Hoechst-33342 (1:200; *Life Technologies*) for 10 min in EGM2 medium. Fresh, serum-free medium was added after 1 h incubation. Change in nuclear morphology is a hallmark of the final stage in cell death. Nuclear events were recorded as additional toxicity readouts regardless of the cell death pathway involved.

H_2O_2 was used as a stable reactive oxygen species for induction of cell death in three different concentrations (high dose, 900 mM; medium dose, 600 mM; low dose, 300 mM) for 12 hours.

4.7.2. Immunohistochemistry

Three weeks after recellularisation procedure 3D vascular constructs were stored in formaldehyde upon harvest, to enable paraffin embedding and immunohistochemical analysis. Histological sections were analysed with 3D-HisTech software (*http://3dhistech.com*), which enables three dimensional analysis of whole slides in section, tissue segmentation and morphometric analysis. Furthermore, the software provides surface for human cell detection in surrounding tissue, cell filtering and merging to quantify angiogenetic activity. Endothelial cells were stained with anti-human CD31 antibody.

Table 7. Detailed characteristics of all applied antibodies (SSEA-4: stage specific embryonic antigen-4, vWF: von Willebrand factor, VE-cadherin: vascular-endothelial cadherin, ICC: immune-cytochemistry, M: mouse, Rb: rabbit, G: goat, D: donkey, N.A. not applicable, A546: Alexa Fluor 546, DAPI: 4',6-diamidino-2-phenylindole - dihydrochloride)

Target	Company/cat.no.	Species	Isotype	Dilution	Use
human SSEA-4	*Abcam/16287*	M	IgG3	1:100	ICC characterising pluripotent fate
human TRA-1-60	*Abcam/16288*	M	IgM	1:100	ICC characterising pluripotent fate
human CD31	*Abcam/24590/28364*	Rb	IgG1	1:100	ICC characterising endothelial fate
human vWF	*Dako/A0082*	Rb	IgG1	1:200	ICC characterising endothelial fate
human FOXO1A	*Millipore/AB4130*	Rb	IgG	1:200	ICC characterising FOXO pathway
human pFOXO1a	*Cell Signalling/94615*	Rb	IgG	1:100	ICC characterising FOXO pathway
Ki67	*Abcam 15580*	Rb	IgG	1:100	Endothelial proliferation assay
Mouse IgG	*LifeTechnologies/ A10036*	D	IgG	1:400	ICC secondary staining A546
Rabbit IgG	*LifeTechnologies/ A10040*	D	IgG	1:400	ICC secondary staining A546
Goat IgG	*LifeTechnologies/ A21085*	R	IgG	1:400	ICC secondary staining A546
DAPI/Hoechst-33342	*LifeTechnologies/ D21490/ V35118*	N.A.	N.A.	1:500	ICC nuclei staining
Human CD31	*BD Pharmigen/ 558068*	M	IgG1	Normalised to cell number	FACS, EC sorting
Mouse IgG1	*BD Pharmigen/ 345815*	M	IgG1	1:100	Isotype control for FACS

4.7.3. Matrigel tube formation assay

Matrigel tube formation assay was performed on endothelial cells. Matrigel was thawed overnight and handled on ice, as it is solidifies over 4°C. 24-well plates were coated with Matrigel; endothelial cells were seeded onto the solidified Matrigel. Endothelial cultures were imaged after 24h incubation.

4.7.4. Assessment of endothelial alignment under sheer stress

Endothelial cell alignment under shear stress was determined using *in vitro* sheer stress model [197]. Briefly, cells were grown to confluence in 6-well plates and placed on an orbital shaker. The movement of the shaker resulted in a wave of media that transversed around the well resulting in a complex pattern of shear stress with directional (laminar) shear towards the edge of the well, and non-directional (turbulent) shear at the centre. Alignment of cells was visualised by light microscopy. Quantification of cell alignment and elongation was carried out using a blind scoring system. Users were blinded to the identity of all images, captured at the centre and edge of wells from cells cultured under static and shear stress conditions and asked to score alignment (0–4) and elongation (0–4). Data were collected from 5 separate scorers. Elongation and alignment were defined and explained to blind scorers as follows: 'elongation' means how stretched the cells appear. A cell that looks like a square or cobblestone scores zero. An image with cells that look stretched/elongated = 4, with 1, 2 and 3 being intermediate. 'Alignment' means whether the cells are aligned in a similar direction. Remember elongated cells might not be aligned. Cells that are strongly aligned in one direction score 4, and cells not aligned and that are randomly arranged (regardless of elongation or not) score 0 with 1, 2 and 3 as intermediates. For shear stress protocols cells were plated on 6-well plates at a density of 100000 cells/ well for 48h under static conditions and allowed to reach confluence. Media was then replaced with fresh media and cells either exposed to shear stress by placing plates on the orbital shaker or incubated under control static culture conditions for a period of 4 days.

4.7.5. Ac-LDL uptake

Endothelial cells may be identified by their high level metabolism of Ac-LDL. To testify endothelial phenotype, cultures were incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate labelled with fluorescent probe (DiI-Ac-LDL, *LifeTechnologies*) for 4 hours, in EGM2 medium at 37°C and imaged by fluorescence microscopy.

4.7.6. Gene expression analysis, PCR

Details of PCR methods were mentioned previously in section 4.3. For quantifying mRNA levels of arterial, venous, lymphatic and universal endothelial genes qRT-PCR was performed, using *TaqMan Gene Expression Assay* (*Applied Biosystems, Life Technologies*). As housekeeping gene control human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) endogenous control was used. PCR was performed with real-time PCR instrument *(Applied Biosystem, StepOnePlus, Life Technologies)* and the relative expression was determined. Relative expression levels of genes of interest were detected. Table 8. shows list of studied endothelial genes.

Table 8. TaqMan® PCR primers applied in gene expression studies Table is listing primers, assays and their purposed use.

Target	Assay	Purpose
Oct4	*Hs00999634_gH*	pluripotency marker
Notch1	*Hs00384907_CE*	arterial EC marker
Notch2	*Hs00247288_CE*	arterial EC marker
EphrinB2	*Hs00341124_CE*	arterial EC marker
DLL4	*Hs00184092_m1*	arterial EC marker
EphB4	*Hs01822537_cn*	venous EC marker
FLT4	*Hs00270779_CE*	lymphatic/venous EC marker
VE-Cadherin	*Hs00170986_m1*	EC marker
NOS3	*Hs01574659_m1*	EC marker
CD31/PECAM1	*Hs00169777_m1*	EC marker
Angiopoietin-2	*Hs01048043_m1*	EC marker
Tie-2/TEK	Hs00945142_m1	EC marker
HIF1a	*Hs00153153_m*	hypoxia signalling
FOXO1A	*Hs00231106_m1*	PI3K signalling
GAPDH	*Hs02758991_g1*	housekeeping control

4.7.7. Proteome profiling assay

To identify protein production of endothelial cells angiogenesis and hematopoietic soluble receptor proteome profiling measurement was carried out on *R&D System* Proteome Profiler Human Angiogenesis Array Kit (ARY007) and Human Soluble Receptor Array Kit Hematopoietic Panel (ARY011). Both cell surface supernatant and cell lysates were used in these studies. Sample preparation and experimental setup followed the manufacturer's protocol. Pixel densities on assay membranes were assessed after multiple exposures under X-ray, then analysed by *ImageJ* software.

4.7.8. ELISA measurements

Human IL-6, IL-8, Rantes and ET-1 ELISA assays were performed on DuoSet ELISA Development System and Quantikine ELISA kits (*R&D Systems* DY206/DY208/DRN00B/DET100). The sample collection, preparation and experimental setup followed the manufacturer's protocol. IL-6, IL-8 and Rantes production of hESC-ECs and hiPSC-ECs were compared with HUVEC.

4.8. Modulation of PI3K/FOXO1A signalling pathway in hESC-EC

4.8.1. Cell treatments with PI3K inhibitor, FOXO1A small interfering RNA, and FOXO1A plasmid

Human ESC and hESC-EC were treated with LY294002 (10 μM, 24h; *Sigma-Aldrich*) during and after endothelial differentiation. As control, dimethyl sulfoxide (DMSO) was used in equal concentration. During endothelial differentiation EGM2 medium was changed in every 24 hours with LY294002. After sorting CD31 positive endothelial cells, hESC-EC were plated onto gelatinised 96-well plates (20000 cells/well) and were grown until confluency. Then, cells were treated with LY294002 for 24 hours.

For FOXO1A small interfering RNA (siRNA) knockdown on differentiating hESC and purified hESC-EC cultures, FOXO1A Flexitube siRNA Premix (10 nM, 6 hours and 24 hours; *Qiagen*) was performed as per manufacturer's instruction. Scrambled non-targeting (NT) siRNA (10 nM; *Qiagen*) was used as negative control. Differentiating hESC cultures were retransfected every 48h to maintain effects of siRNA.

Transfection of hESC-EC with plasmids encoding FOXO1A-eGFP or pmaxGFP (*Lonza*) used as DNA control was carried out by electroporation. Briefly, 10^6 hESC-EC were resuspended in 400 μL of EGM2 medium containing 5 μg plasmid DNA, electroporated on a Gene Pulser Xcell Total modular electroporation apparatus (*BioRad*), using time constant program (200 V, 25 ms). Overexpression efficiency was tested by by qRT-PCR and experiments were performed 48h later.

4.8.2. Measurement of cell viability

After modulating PI3K/FOXO1A signalling pathway either inhibition or overexpression of FOXO1A, cell viability was determined using the commercially available AlamarBlue® assay or cell counts. For cell counts, fixed cells were stained with DAPI (10µg/ml) and imaged using the Cellomics® VTi HCS Arrayscanner. Data for cell counts are expressed as average cells/field based on 49 fields.

4.8.3. Matrigel tube-formation assay

Matrigel was thawed on ice overnight and 24-well plates were coated with 200 µL Matrigel per well and then allowed to solidify at 37°C for 30 min. Control, LY294002, NT, or FOXO siRNA-pretreated hESC-EC (50000 cells/well) were plated onto Matrigel and cultures were photographed and quantitated after 24 hours of incubation. Cell nuclei were stained for Hoechst-33342 at day 0, 1, 2, and 3. The colony formation activity and the number of nuclei per colony were assessed by using a Colony Formation BioApplication on Cellomics platform.

4.8.4. PI3K/Akt PCR array

For the PCR array (Human PI3K-AKT Signalling RT2 Profiler PCR Array Kit, PAHS-058; *Qiagen*), cDNA was synthesized from 1 mg of total RNA and then hybridized in a 96-well format. PCR array data intensity values were normalized by gene-centered z score transformation. A modified z-score threshold of 3.5 was used as criteria defining outliers, and these samples were excluded from a rerun of the z-statistics. By calculating the gene expression stability measure M, which is the mean pairwise variation for a gene from all other tested genes, beta actin (ACTB) and 60S acidic ribosomal protein P13 (RPL13A) were accepted as being the most stable housekeeping genes in this assay setup. Normalized ΔCT values were derived as ΔCT (gene) - ΔCT (averaged geometric mean above housekeeping controls). Correlation plots and heatmaps were plotted for all samples and technical replicates were averaged. To reveal biologically relevant interactions for activated kinases, pathway analysis was performed by Ingenuity Pathways Analysis

software (*https://analysis.ingenuity.com*). Ingenuity Pathways are based on a curated database of published literature on gene functions and interactions. As one of the outputs Ingenuity identifies hub genes based on high degree of links to other genes in known pathways.

4.9. Antiplatelet functional assay

Assays were set up to characterise antiplatelet function of hPSC-EC. In the antiplatelet functional assays levels of Rantes/CCL5 chemokine were measured, which reveals level of platelet activation (*R&D Systems DRN00B*). During the experiments PSC-EC, control HUVEC cells, engineered 3D constructs and bioscaffolds alone were incubated with platelet rich plasma of healthy adults. The levels of Rantes/CCL5 were measured from the plasma with ELISA. The experimental groups designed as following:

1. hESC-EC/hiPSC-EC/HUVEC alone on gelatine + platelet rich plasma
2. hESC-EC on scaffold / hiPSC-EC on scaffold / HUVEC on scaffold + platelet rich plasma
3. Scaffold alone + platelet rich plasma
4. Platelet rich plasma alone

Schematic figure shows experimental setup of assays (Figure 12.). Assays were performed in 24-well plates.

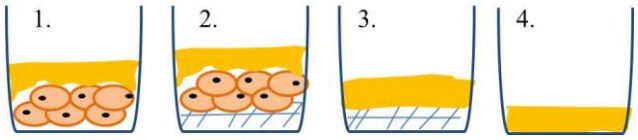


Figure 12. Experimental setup for antiplatelet functional assay 1. Endothelial cells alone + platelet rich plasma 2. Endothelial cells on bioscaffolds + platelet rich plasma 3. Bioscaffolds alone + platelet rich plasma 4. Platelet rich plasma alone

4.9.1. Preparation of platelet rich plasma

The platelet rich plasma (PRP) was derived from healthy adults, members of our research group. Blood samples were collected in trisodium-citrate tubes. Tubes were shaken gently immediately after blood sampling to enhance dilution of anticoagulant in blood. The blood processing was started within 30 minutes of sampling. First, samples were centrifuged (1000 RPM, 7 mins, 24°C), supernatant resulted in PRP. Then samples were further centrifuged (4000 RPM, 30 min, 15°C), the supernatant resulted platelet poor plasma (PPP). In PRP platelets were counted, samples were used in 300G/L concentration for antiplatelet functional assay experiment. To dilute PRP, PPP were used. On antiplatelet assay, samples were incubated with PRP for 30 mins at 37°C on slow rate stirrer. Finally, PRP was collected and centrifuged (4000 RPM, 15 min). ELISA measurements were performed from supernatant.

4.10. *In vivo* experiments

After characterisation of hESC-EC and hiPSC-EC *in vitro, in vivo* experiments were succeeded. The generated hESC-EC, hiPSC-EC and control endothelial HUVEC were implanted into athymic nude rats (*Crl:NIH-Foxn1^{rnu}, Charles River, Sulzfeld, Germany*), to investigate cell survivecell engraftment, capability of angiogenesis and development of vascular structures *in vivo*. Athymic nude rats are T-cell deficient and show depleted cell populations in thymus-dependent areas of peripheral lymphoid organs. These animals are suitable for transplantation and carcinogenesis research [198]. Animals were housed in microbe free conditions, care and handling fulfilled the Guide for Care and Use of Laboratory Animals, published by U.S. National Institutes of Health. Experimental protocols were approved by the Animal Use and Care Committee of Semmelweis University. Three months old, male, nude rats received general anaesthesia with Ketamine-Xylazine cocktail (80-100 mg/kg and 5-10mg/kg). During the transplantation procedure animals received standard laboratory feed. The generated hESC-EC, hiPSC-EC and HUVEC were mixed with Matrigel extracellular matrix (*BD Biosciences*) to provide extracellular matrix components for the transplanted cells. The injected suspension consisted of Matrigel (250µl), heparin (64U/ml), recombinant murine basic FGF (80ng/ml,

R&D Systems) and EGM2 (70µl). Matrigel and growth factors without cells were used as control. The cells were transplanted subcutaneously at four sites of the abdominal region of each rat. Two weeks after the implantation animals were harvested after overdosing anaesthetics, thus developing respiratory failure. Implantation sites and plugs were explanted for immunohistochemistry in order to study development of vascular structures from engraftment. Implanted cells were re-isolated from nude rats via enzymatic digestion of explanted plugs and sorting CD31 positive human endothelial cells. Further analysis (re-culturing and gene expression profile) were performed.

4.11. Statistical analysis

Statistical analysis was carried out using *GraphPad Prism 5* software. Data are presented as the mean ± standard error of mean (SEM). All experiments were carried out in least three biological replicates. Data were analysed by Student's t-test, paired t-test or using one-way ANOVA following Tukey post test detailed in each figure legend. In all cases significance was taken as $p<0.05$.

4.12. Ethical considerations

The use of hESC clearly raises ethical concerns regarding the destruction of the human embryo. EU has accepted that these cells can be used for approved research projects provided that they are sourced approximately and with full informed consent. Regulations regarding hESC differ in countries. In the USA development of hESC lines from IVF embryos is approved. In the EU some countries allow to develop new ESC lines (e.g. UK, Finland, Sweden, Netherland and Greece), others ban (e.g. Austria, Denmark, France, Ireland, Italy, Portugal and Germany). In Hungary generation of new ESC lines from IVF embryos is not authorised, whereas purchasing ESC lines from abroad for research purposes is approved.

Experiments with hESC were carried out with ethical approvals. H7 hESC line used in the Heart and Vascular Centre, Semmelweis University were purchased from WiCell Company and were approved for research purposes by Hungarian authorities (*ETT TUKEB* 11769-2/2010-1016EHR). All data from the donor have been anonymised before the lines reached Heart and Vascular Centre.

Generally the approval process on hESC line ensures that each of these cell lines: 1) Were created from embryos produced using *in vitro* fertilization for reproductive purposes and were no longer needed for this purpose; 2) The embryos were donated by donor(s) who gave voluntary written consent for the embryos to be used for research purposes; and 3) The research which resulted in the derivation of these cell lines was conducted under review of a local authorities.

Experiments with induced pluripotent stem cells and with their cardiovascular derivatives were performed at the National Heart and Lung Institute, Imperial College, London, approved by *UK Stem Cell Bank* (*Ref.No*. 20111230).

Collection and handling of human tissues were performed after informed consents were obtained from every patient. The human samples were collected in the Városmajor Biobank, Semmelweis University (*ETT TUKEB 7891/2012/EKU*).

For *in vivo* experiments using animals, the Ethical Committee of Hungary for Animal Experimentation and the Animal Use and Care Committee of Semmelweis University Budapest approved the experimental protocols (*Ref.no. 22.1/1098/3/2011*). The investigation conformed to the Guide for the Care and Use of Laboratory Animals.

5. Results

Results in this section are presented in the following order: characterisation of pluripotent stem cells; description of endothelial differentiation; characterisation of endothelial properties *in vitro* in 2D, 3D and *in vivo* circumstances. Therefore, this section will not everywhere follow the order of methods section.

5.1. Characterisation of human pluripotent stem cells

Human ESC (H7, *WiCell*) and hiPSC (*ReproCell*, IMR90-4 *WiCell*) showed pluripotent morphology *in vitro*; they formed colonies on Matrigel matrix surface. Human ESC and hiPSC both showed fast proliferation rate. Spontaneous differentiation rarely occurred in culture, those cells were discarded by enzymatic digestion or mechanical scraping. Figure 13. A shows pluripotent stem cell colonies *in vitro*. Immunocytochemistry analysis proved expression of pluripotency cell surface marker on the hESC. SSEA-4 is a cell surface marker on pluripotent stem cells (Figure 13. B).

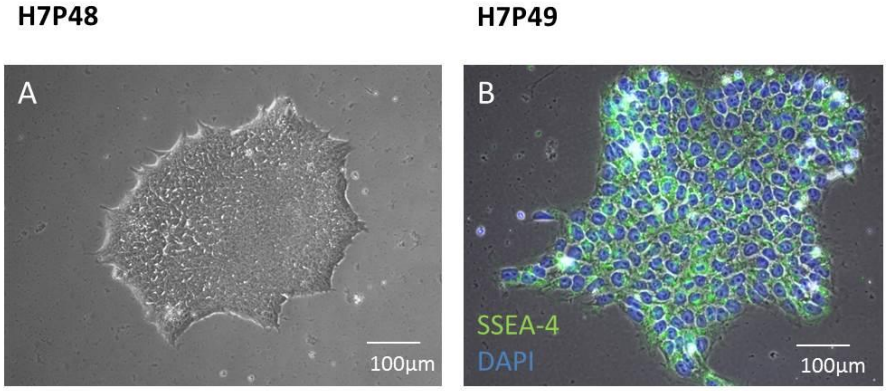


Figure 13. Phenotype *in vitro* and immunocytochemical properties of pluripotent stem cell colonies (A) Figure shows pluripotent stem cell colonies in culture, captured by inverted light microscope (H7 passage 48) (B) Pluripotency marker SSEA-4 is expressed on stem cell colony (H7 passage 49)

To assess expression of pluripotent marker genes, qRT-PCR analysis was performed. High mRNA levels of OCT4 pluripotency marker gene were found. Expression levels of OCT4 were normalised to GAPDH housekeeping control. Figure 14. shows OCT4 mRNA levels, compared to those in hESC-EB, hESC-EC and HUVEC. OCT4 mRNA levels decreased stepwise during differentiation procedure. Significantly lower OCT4 levels were found in

hESC-EB, hESC-EC and in control HUVEC than those in undifferentiated cultures. These results show that cells loose pluripotency during differentiation.

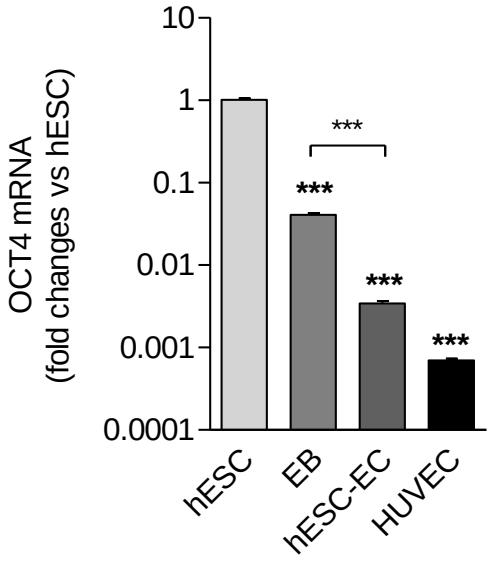


Figure 14. Changes in mRNA levels of embryonic stem cell marker OCT4 Human embryonic stem cells (hESC) were differentiated via embryoid body formation (EB) towards human embryonic stem cells-derived endothelial cells (hESC-EC). Human umbilical vein endothelial cells (HUVEC) were used as control endothelial cells. Data are expressed as fold changes vs. mRNA levels in undifferentiated hESC; ***$p<0.001$ vs. hESC, n=3 biological replicates. One-way ANOVA with Tukey post hoc test.

5.2. Reprogramming human adult fibroblasts to pluripotent state

Surgical harvest of human skin tissue was succeeded from healthy adults. Skin samples were attached to plastic surface and after one day fibroblasts appeared in culture. A semi-confluent fibroblast culture developed one week later, and skin samples were then removed. Keratinocytes spontaneously grew at the site of skin tissue on the plastic surface. Two weeks after skin harvest, confluent fibroblast cultures were achieved. Figure 15. shows steps of fibroblast culturing *in vitro*.

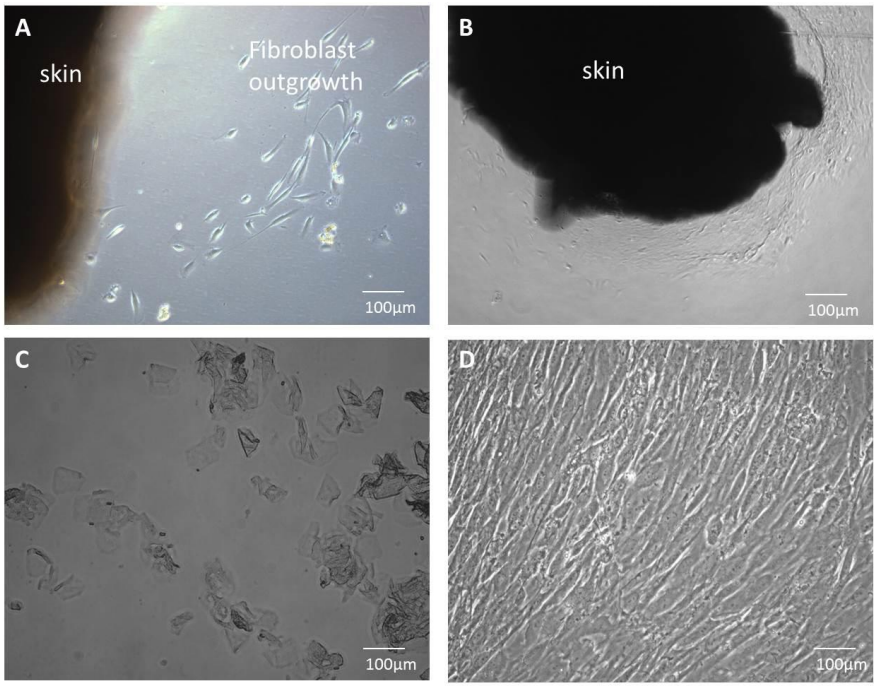


Figure 15. Generation of fibroblast culture from human skin samples (A, B) Human skin biopsy samples were attached to plastic surface, fibroblast outgrowth occurred. (C) Keratinocytes grew spontaneously from skin tissue. (D) Confluent fibroblast culture was achieved after 7-14 days.

After transfection of human adult fibroblasts eGFP positive cells were seen under fluorescent microscope after 2 days (Figure 16. A, B). Transfected fibroblasts were plated on Matrigel, colony-like clusters appeared in culture within 2-3 weeks. These colonies were recognized and observed daily in inverse light microscope (Figure 16. C, D). Colonies were then picked and plated on Matrigel for further expansion and banking.

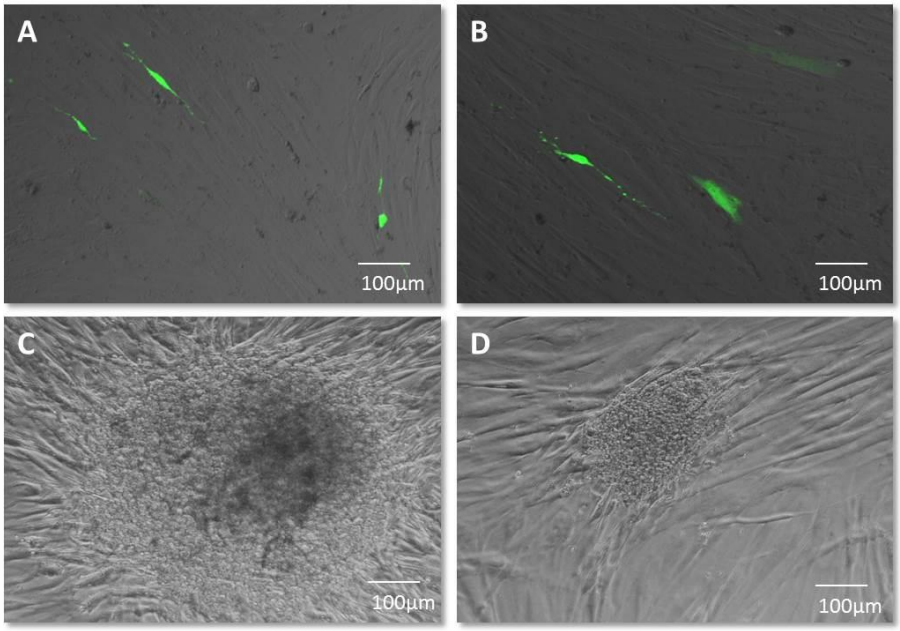


Figure 16. Development of human induced pluripotent stem cells from human adult fibroblasts (A, B) Adult fibroblasts express green fluorescent protein after transfection procedure. (C, D) Three weeks after transfection pluripotent colonies appeared in culture.

Newly generated hiPSC colonies showed expression of pluripotency markers. By confocal microscope we have seen expression of pluripotent stem cell markers such as TRA-1-60 and SSEA-4 (Figure 17.). Around passage 5, hiPSC were banked in liquid nitrogen.

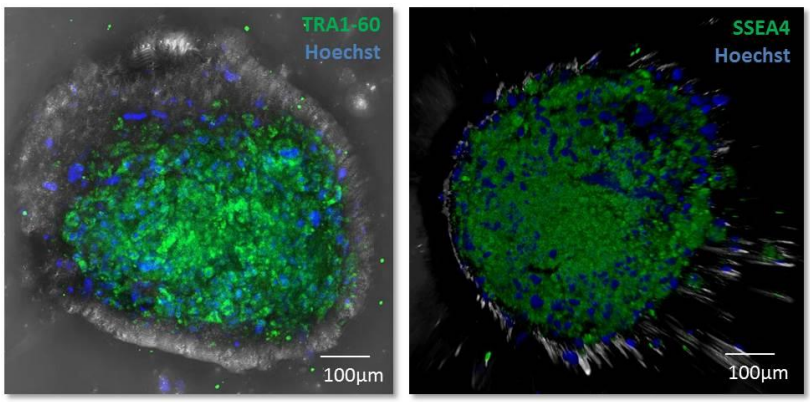


Figure 17. Expression of pluripotent cell surface markers on fibroblast-derived human induced pluripotent stem cells Pluripotency markers, TRA1-60 and SSEA-4 are expressed on human induced pluripotent stem cell colonies (Passage 4).

5.3. Characteristics of human pluripotent stem cells-derived endothelial cells

I have tested four different protocols for endothelial differentiation, as described earlier. CD31 positive endothelial cells were sorted from differentiating culture by FACS. Differentiation efficiency was calculated from total and CD31 positive cell numbers. Among the four endothelial differentiation protocols major, significant differences were not found in endothelial differentiation yield. Similarly to earlier protocols, three of those tested here were moderately successful in endothelial differentiation. The protocols using EB method and VEGF resulted in a generation of ~1-2% endothelial cells. Latest protocol includes strong triggers for mesodermal differentiation with an improvement in endothelial differentiation yield (~10-15%) [171].

After differentiation procedure endothelial cells were expanded until passage 5-7. Both hESC-EC and hiPSC-EC cultures showed cobblestone pattern *in vitro* (Figure 18. A). Analysis with Cellomics high content microscope revealed that CD31 positive endothelial cells were negative for haematopoietic marker CD45. Immunocytochemistry analysis showed that hESC-EC and hiPSC-EC are stained positive also for von Willebrand factor and CD31 (Figure 18. B, C). Endothelial cultures also showed high intensity of arterial endothelial marker DLL4 (Figure 18. D). Human ESC-EC and hiPSC-EC took up ac-LDL, and formed capillary-like structures in Matrigel tube formation assay (Figure 19. A, B).

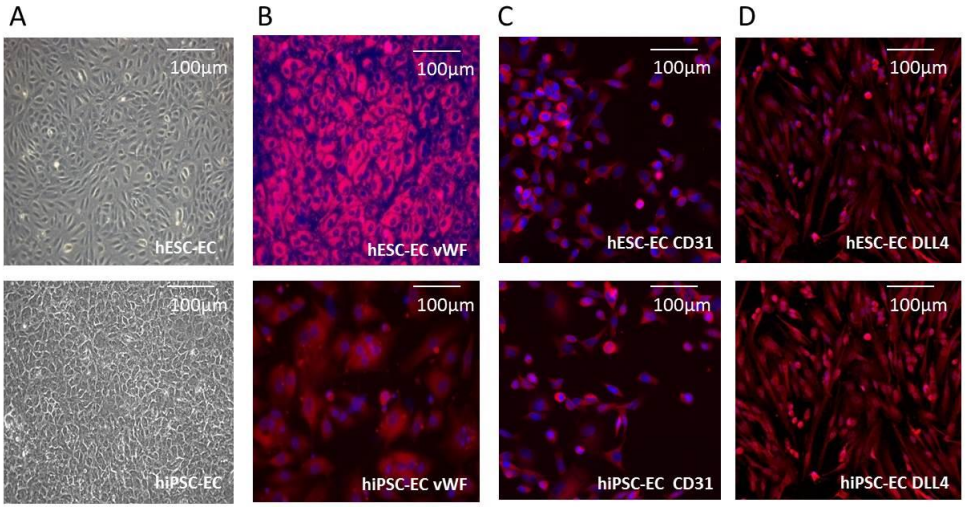


Figure 18. Characterization of pluripotent stem cells-derived endothelial cells (A) Human embryonic stem cells-derived endothelial cells (hESC-EC) and human induced pluripotent stem cells-derived endothelial cells (hiPSC-EC) formed cobblestone pattern *in vitro*. (B, C, D) Immunocytochemical characterization showed that cells are positive for von Willebrand Factor (vWF), CD31 and delta like 4 (DLL4) staining.

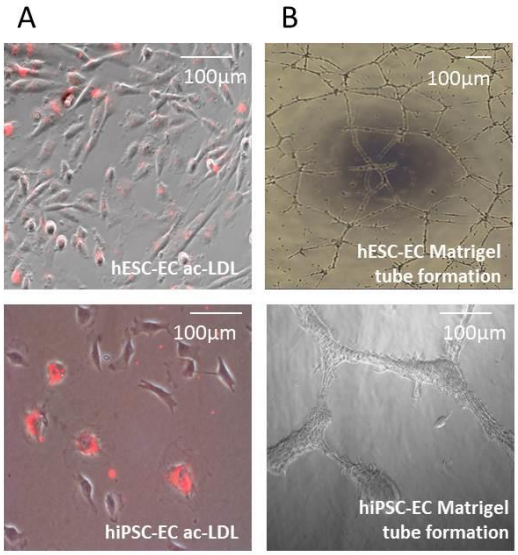


Figure 19. Acetylated low-density lipoprotein uptake and Matrigel tube formation assay on human embryonic stem cells-derived endothelial cells (A) Human embryonic stem cells-derived endothelial cells (hESC-EC) and human induced pluripotent stem cells-derived endothelial cells (hiPSC-EC) took up fluorescence (Alexa Fluor 546) labelled ac-LDL. (B) hESC-EC and hiPSC-EC formed tube-like structures on Matrigel.

Endothelial cells grown from the vasculature (HAEC) and endothelial cells from blood progenitors (BOEC) were cobblestone in appearance when grown under static culture conditions (Figure 20. A). BOEC and HAEC changed morphology when cultured under shear stress using a simple orbital shaker method for four days. Both BOEC and HAEC elongated and aligned when exposed to directional shear stress (Figure 20. B edge), but remained cobblestoned when exposed to non-directional, turbulent shear stress (Figure 20. B centre). Quantification of cell elongation and alignment by blind scoring showed statistically significant elongation and alignment of both HAEC and BOEC cultured under directional shear stress and that for all conditions hESC-EC did not appear to respond to shear stress (Figure 21.).

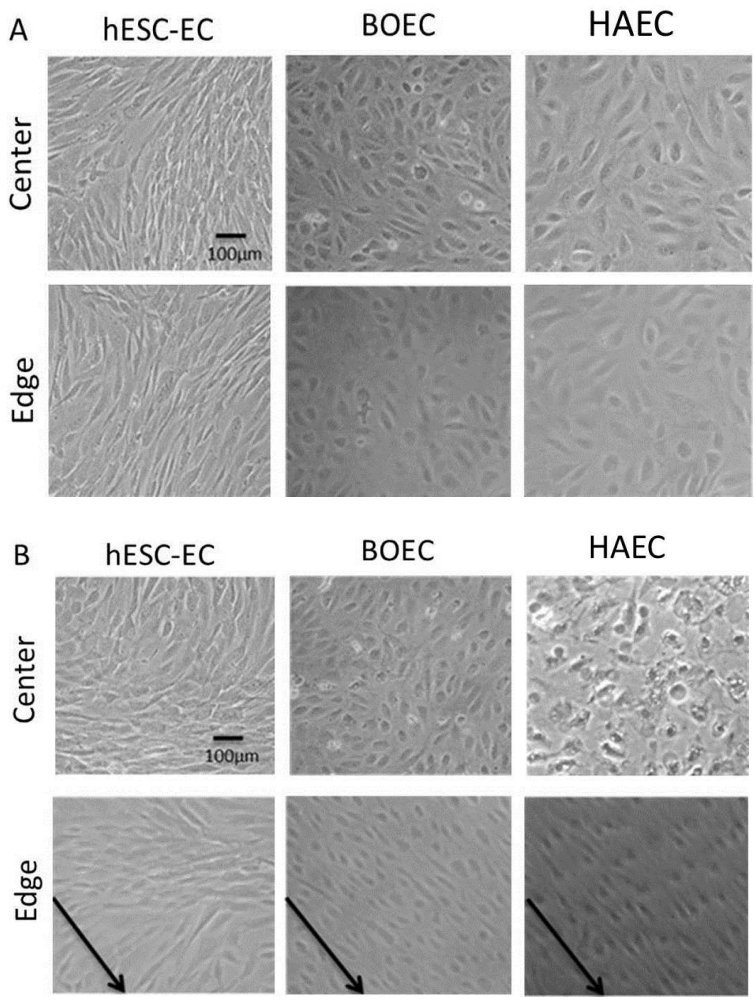


Figure 20. Responses of endothelial cells to shear stress Human embryonic stem cell-derived endothelial cells (hESC-EC), human aortic endothelial cells (HAEC) and blood outgrowth endothelial cells (BOEC) after 4 days cultured under either (A) static conditions or (B) under shear stress. Images were taken at the edge of the well, where shear stress is unidirectional and cells align; and at the centre of the well, where shear stress had no preferred direction. Black arrows on shear plate edge images indicate the direction of shear stress. Images are from cells of individual experiments and representative of observations made from n= 3–8 experiments.

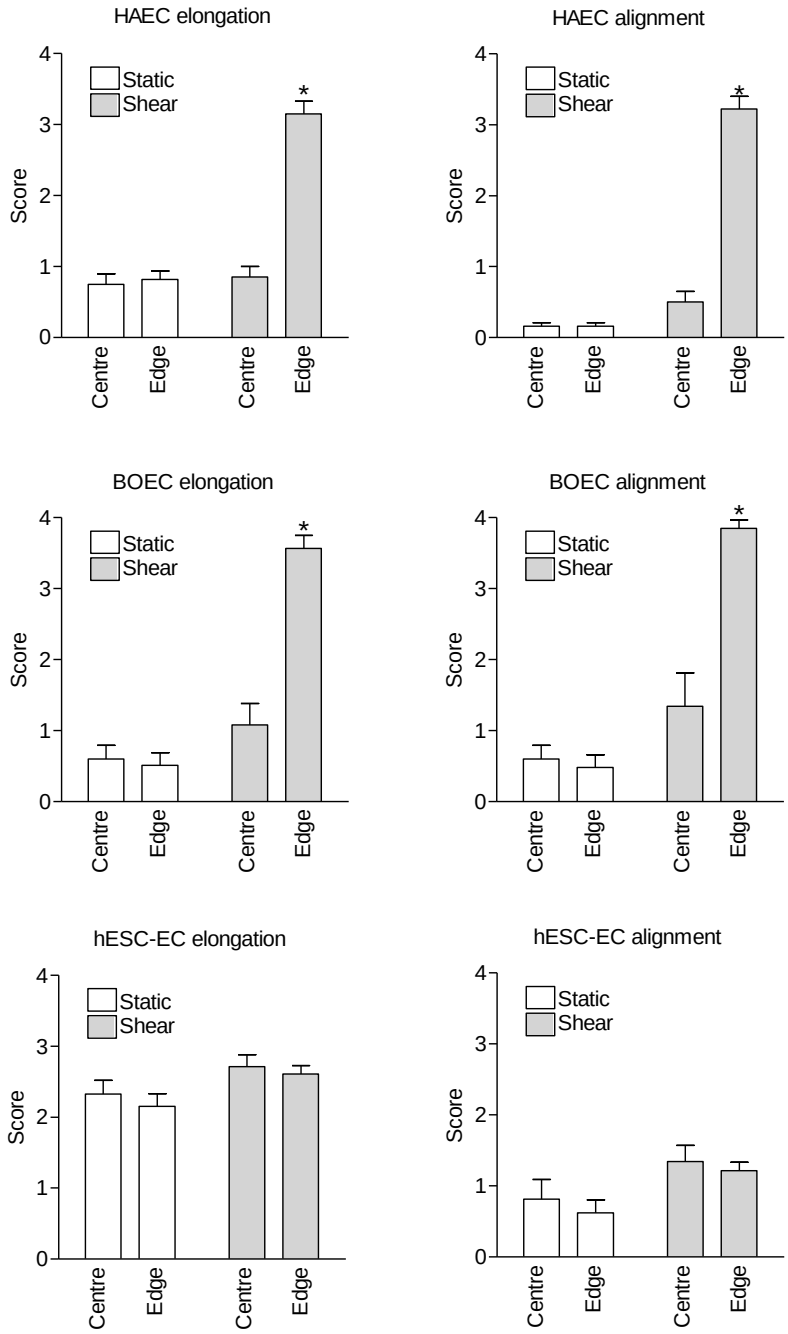


Figure 21. Quantification of elongation and alignment of endothelial cells from different sources Human aortic endothelial cells (HAEC), blood outgrowth endothelial cells (BOEC) and human embryonic stem cells-derived endothelial cells (hESC-EC) were scored for elongation and alignment from images at the centre and edge of the well under static and shear stress conditions for 4 days. Scoring (0–4) was carried out by using blind scoring system and is the average of five independent scores. Data are mean ± SEM (HAEC n = 6–7, BOEC n= 8, hESC-EC n= 6–7) derived from 3 to 8 separate experiments. Statistical significance between centre and edge scores for each cell and condition was determined by paired t-test ($p < 0.05$).

As assessed by qRT-PCR analysis a robust expression of endothelial and angiogenesis-related genes was present in hESC-EC and hiPSC-EC. We found a significant increase both in arterial (EphrinB2, Notch1, Notch2) (Figure 22. A-C) and venous (EphB4) (Figure 22. D) endothelial marker genes, as compared to those in undifferentiated (H7 hESC and IMR90-4 hiPSC) stem cell populations. However, no significant difference was found between arterial and venous endothelial gene expressions. Arterial endothelial marker genes had higher expression levels than venous marker genes. Lymphatic endothelial marker gene, FLT4 was not detectable in hESC-EC neither in hiPSC-EC populations. Marker genes for endothelial cells and angiogenesis, CD31 and vascular-endothelial cadherin showed significant increase in hESC-EC and hiPSC-EC as compared to those in hPSC (Figure 22. E, F). Comparing hESC-EC and hiPSC-EC showed similar gene expression pattern on arterial, venous and general endothelial marker genes.

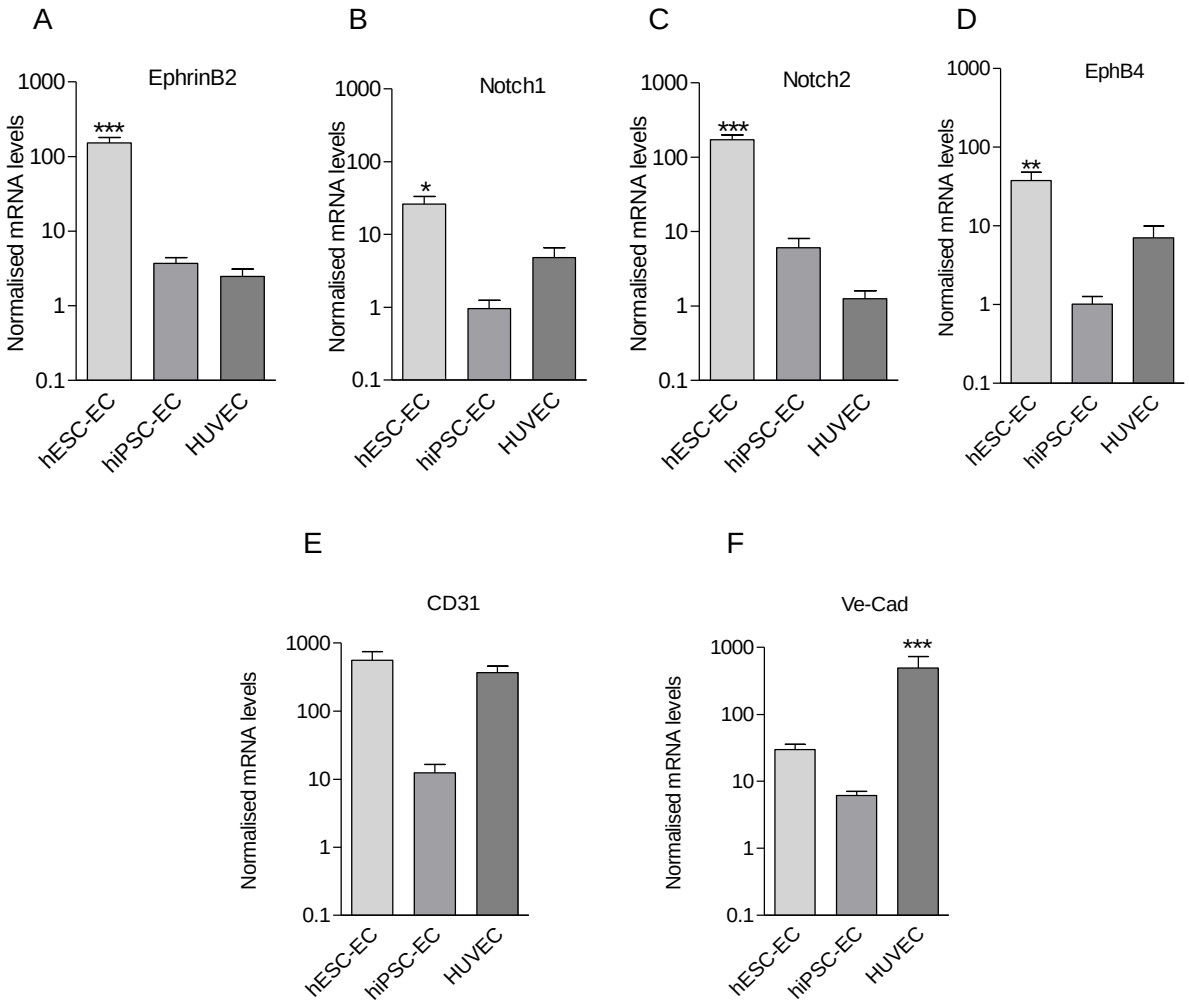


Figure 22. Characteristics of human pluripotent stem cells-derived endothelial cells with qRT-PCR Bar graphs show changes in endothelial gene expression levels; (ABC) arterial markers EphrinB2, Notch1 and Notch2, (D) venous marker EphB4, (EF) general endothelial marker genes: CD31 and vascular-endothelial cadherin were measured. mRNA levels are shown as fold changes vs. undifferentiated stem cells. n=3 biological replicates, p *<0.05, **<0.01, ***<0.001, One-way ANOVA and Tukey tests. (HCAEC: human coronary arterial endothelial cells, hiPSC-EC: human induced pluripotent stem cells-derived endothelial cells, hESC-EC: human embryonic stem cells-derived endothelial cells, HUVEC: human umbilical vein endothelial cells)

To study arterial endothelial subpopulation on hESC-EC and hiPSC-EC surface intensity of arterial endothelial cell surface marker EphrinB2 was further quantitated by automated microscopy. After staining hESC-EC, hiPSC-EC and human coronary arterial endothelial cells (HCAEC) for EphrinB2, intensity pattern was obtained by measuring fluorescence intensity (Figure 23. A). DLL4 is a pivotal marker for arterial endothelial cells and regulator in Notch signalling pathway. mRNA levels of DLL4 were significantly higher in hESC-EC and hiPSC-EC compared to those in undifferentiated hPSC (Figure 23. B). DLL4 levels were correspondingly increased in control endothelial HUVEC cells. EphrinB2/EphB4 ratio was analyzed to investigate relationship of EphrinB2/EphB4 bidirectional signalling pathway. EphrinB2/EphB4 ratio was significantly higher in hESC-EC and hiPSC-EC than those in HUVEC, suggesting that arterial phenotype is respresented in hPSC-EC cultures (Figure 23. C).

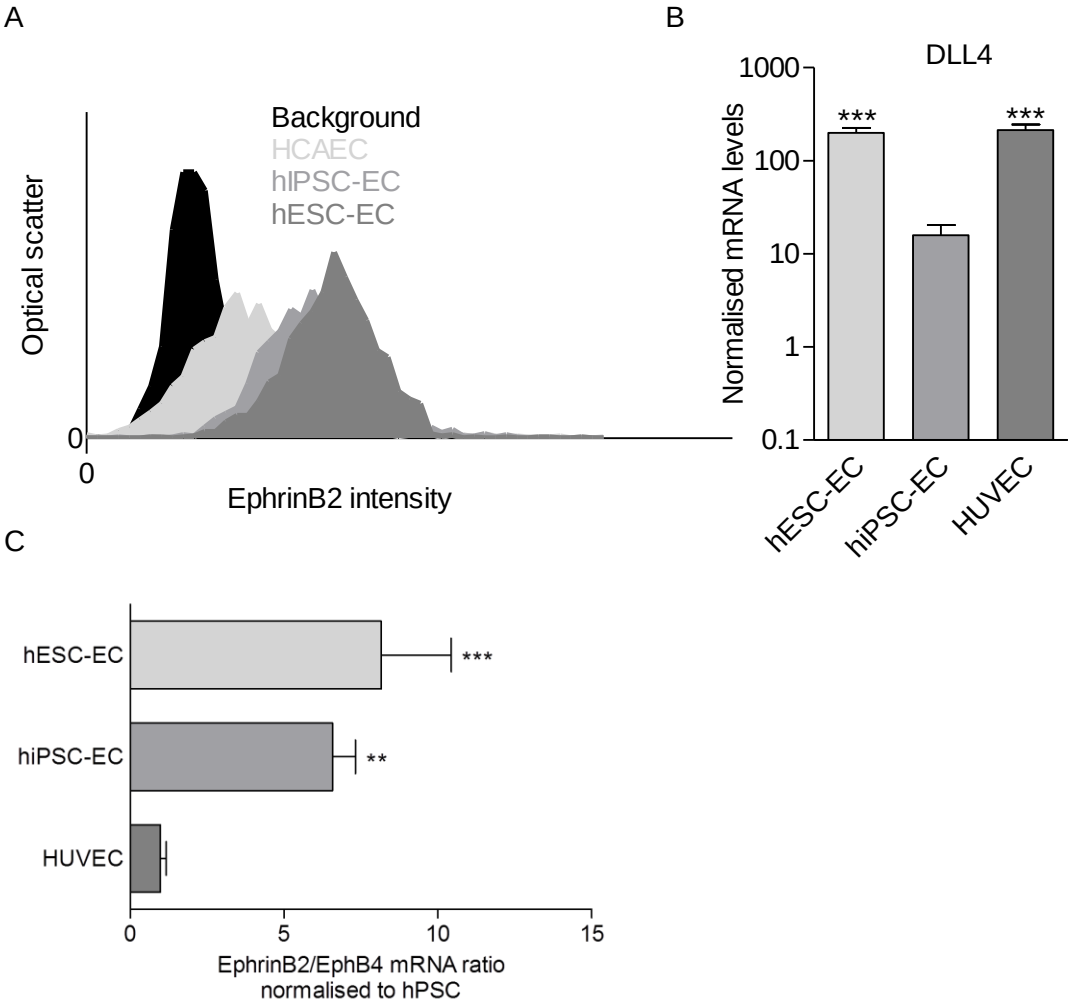


Figure 23. Characterization of arterial endothelial phenotype on hESC-EC and hiPSC-EC *in vitro* (A) Arterial cell surface marker, Ephrin B2 intensity scale is shown on histogram, compared to background control (stained with Alexa Fluor 488) and arterial control endothelial cells. (B) Bar diagrams show normalized mRNA levels of DLL4 on hESC-EC, hiPSC-EC and HUVEC. DLL4 expression levels are normalized to those in hPSC. (C) Bar diagram shows levels of EphrinB2/EphB4 ratio. mRNA levels are normalized to those in hPSC. n=3 biological replicates, p**<0.01, p***<0.001 one-way ANOVA with Tukey post hoc test. (HCAEC: human coronary arterial endothelial cells, hiPSC-EC: human induced pluripotent stem cells-derived endothelial cells, hESC-EC: human embryonic stem cells-derived endothelial cells, HUVEC: human umbilical vein endothelial cells)

Proteome profiling from endothelial cell lysates and supernatants showed the production of several angiogenesis-related proteins and cytokines (Figures 24. and 25.). The expression and secretion pattern of hESC-EC and hiPSC-EC were similar compared to those in human coronary arterial endothelial cells (HCAEC) (Figures 24. A and 25. B). Stem cells-derived endothelial cells express and produce factors such as VEGF isoforms, angiopoietin-1, angiopoietin-2, angiogenin, activin-A, endoglin, TIMP1, 2 and ADAM 9, 10 (Figure 25. A). Heat map analysis of expression levels were compared to those in HCAEC (Figure 25. B). Hematopoietic marker proteins (CD23, 49, 56, 58, 59,163) were not detected in hESC-EC and hiPSC-EC (Figure 24. B). CD105 is expressed both on endothelial and hematopoietic progenitors.

ELISA measurements assessed IL-6, IL-8 and ET-1 secretion in hESC-EC and hiPSC-EC and control endothelial HUVEC (Figure 26.). Results revealed significantly higher levels of IL-6 and IL-8 proteins in hESC-EC and hiPSC-EC than in undifferentiated stem cells (Figure 26.). Human ESC-EC and hiPSC-EC secreted IL-6 and IL-8 into supernatant in similar levels as in HUVEC cultures. Results from ELISA measurement were comparable with those in proteome profiling. Both revealed high expression and production of IL-8, furthermore both proved higher amount of these inflammatory proteins in hiPSC-EC than in hESC-EC. We found significantly lower ET-1 expression in hESC-EC than in control HUVEC and HCAEC cells.

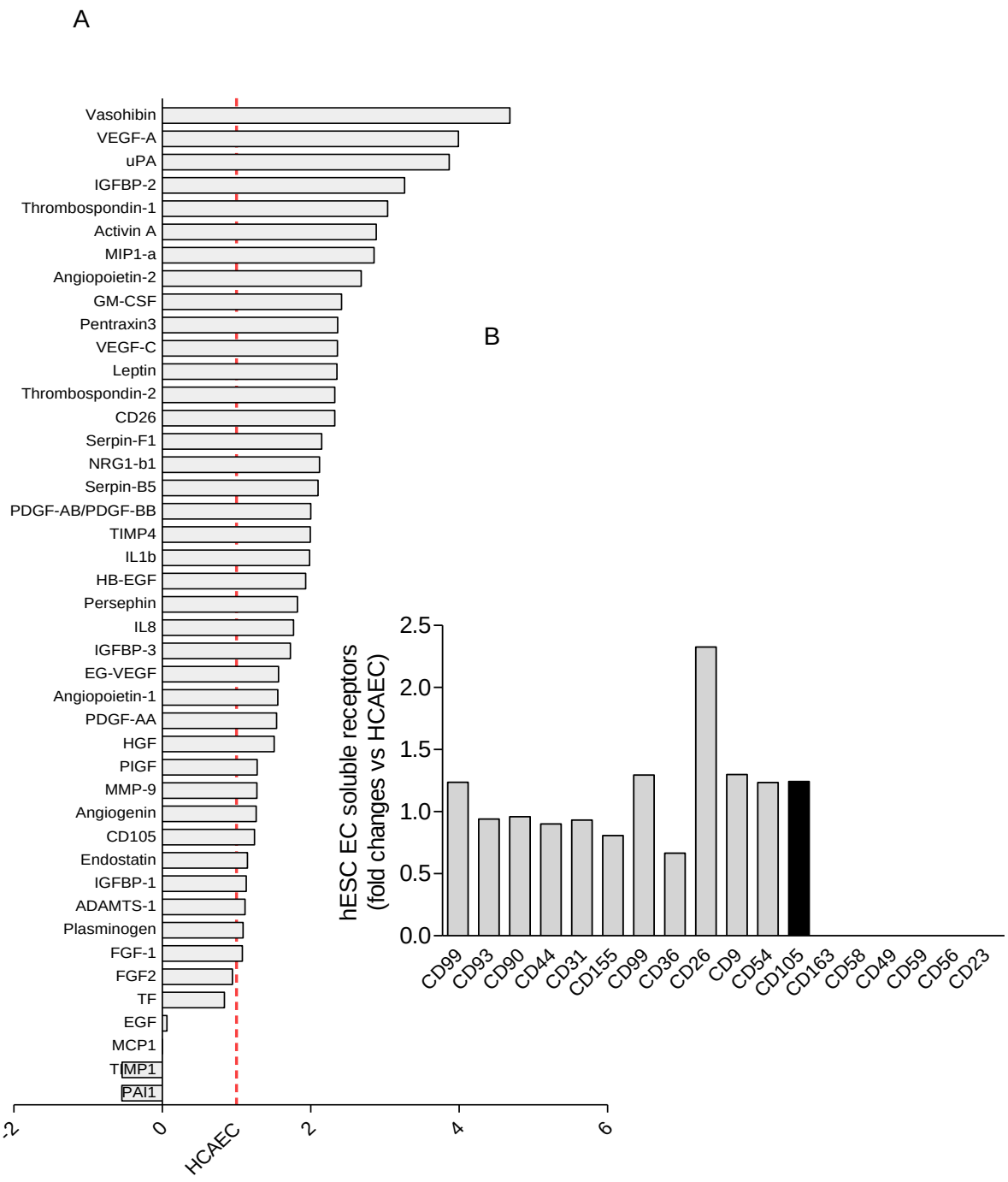
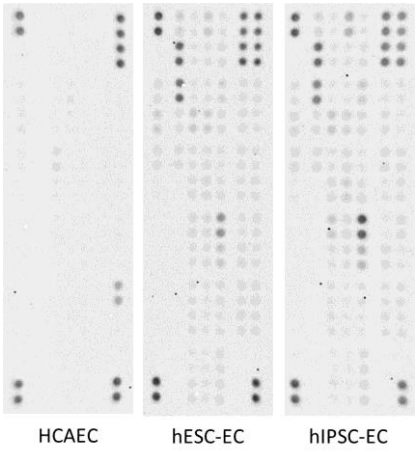


Figure 24. Proteome profiler analysis of pluripotent stem cells-derived endothelial cells (A) Bar graph shows angiogenesis proteome profiler analysis of human embryonic stem cells-derived endothelial cells (hESC-EC). Cell lysates of hESC-EC express many angiogenesis-related proteins. (B) Bar diagram shows expression of cluster of differentiation (CD) soluble receptors assessed by human soluble receptor proteome profiler array from cell culture supernatants. Data show expression levels of hESC-EC in fold changes compared to those in human coronary artery endothelial cells (HCAEC). Statistics are not available as n=2 biological replicates, 4 technical replicates.

A



B

1. hESC-EC	2. hiPSC-EC	
4,8	4,2	Platelet Factor 4
4,7	3,9	Vasohibin
4,0	5,5	VEGF-A
3,9	5,0	LAP (TGFb1)
3,9	4,7	Amphiregulin
3,9	3,5	uPA
3,3	3,9	IGFBP-2
3,0	3,4	Thrombospondin-1
2,9	2,0	Activin A
2,9	3,8	MIP-1a
2,7	2,7	Angiopoietin-2
2,5	2,1	FGF-4
2,4	2,4	GM-CSF
2,4	2,5	Pentraxin 3
2,4	3,1	VEGF-C
2,4	1,9	Leptin
2,3	2,2	Thrombospondin-2
2,3	2,4	DPPIV (CD26)
2,1	3,1	Serpin F1 (PEDF)
2,1	2,5	CXCL 16
2,1	2,7	NRG1-b1
2,1	2,0	Serpin B5
2,0	2,2	PDGF-AB/PDGF-BB
2,0	1,6	TIMP-4
2,0	1,6	IL-1b
1,9	2,2	HB-EGF
1,8	-0,2	Persephin
1,8	2,3	GDNF
1,8	2,0	IL-8
1,7	2,0	IGFBP-3
1,6	1,8	EG-VEGF (PK1)
1,6	1,8	Angiopoietin-1
1,5	1,9	PDGF-AA
1,5	-0,2	HGF
1,3	1,6	PlGF
1,3	3,1	MMP-9
1,3	1,8	Angiogenin
1,2	1,5	Endoglin (CD105)
1,1	2,0	Endostatin/Collagen XVIII
1,1	1,6	IGFBP-1
1,1	1,9	PD-ECGF
1,1	1,4	ADAMTS-1
1,1	1,9	Artemin
1,1	2,1	Angiostatin/Plasminogen
1,1	1,1	FGF-1
0,9	1,1	FGF-2
0,8	1,5	Tissue Factor (factor III)
0,1	0,0	EGF
0,0	3,6	MCP-1
-0,5	1,4	TIMP-1
-0,5	0,1	Serpin E1 (PAI-1)

Figure 25. Human pluripotent stem cells-derived endothelial cells produce angiogenesis-related proteins, cytokines and soluble receptors (A) Proteome profiling array membranes for HCAEC, hESC-EC and hiPSC-EC. (B) Heat map (colours) egy kis szinskala felirattal egyszerusiti az eletet shows levels of expression of angiogenesis related cytokines and soluble receptors in hESC-EC and hiPSC-EC. Fold changes (numbers) in expression levels are compared to those in human coronary arterial endothelial cells (HCAEC). Cell numbers were equalized in each experimental run. Statistics are not available as n=2 biological replicates, 4 technical replicates. (HCAEC: human coronary arterial endothelial cells, hiPSC-EC: human induced pluripotent stem cells-derived endothelial cells, hESC-EC: human embryonic stem cells-derived endothelial cells, HUVEC: human umbilical vein

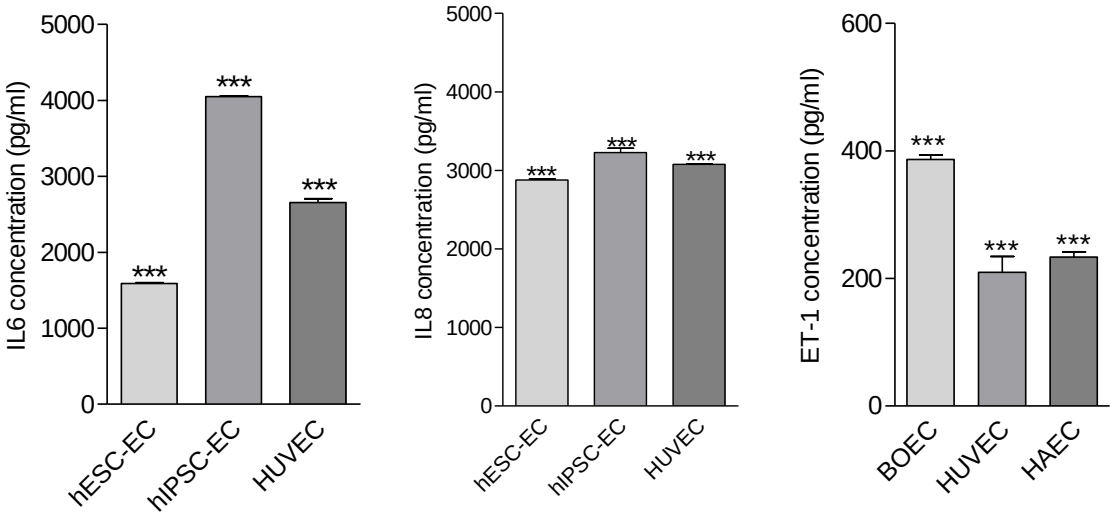


Figure 26. Stem cell-derived endothelial cells produce IL-6, IL-8 and ET-1 *in vitro*
Bar graphs show production of interleukin-6 (IL-6), interleukin-8 (IL-8) and Endothelin-1 (ET-1) proteins. IL-6, IL-8 and ET-1 production were measured from the supernatant of $5x10^5$ cells and normalized to those in hPSC. n= 6, p***<0. 001 from n=3 biological replicates, One-way ANOVA and Tukey post hoc test (BOEC: blood outgrowth endothelial cells, HAEC: human aortic endothelial cells, hiPSC-EC: human induced pluripotent stem cells-derived endothelial cells, hESC-EC: human embryonic stem cells-derived endothelial cells, HUVEC: human umbilical vein endothelial cells)

5.4. Role of differentiation conditions on endothelial marker genes and on arterial and venous endothelial subpopulations

Although differentiation conditions did not influence significantly the yield of generated CD31 positive endothelial cells, expression of general, arterial and venous endothelial marker genes were clearly modulated by cell culture conditions.

Further in-depth analysis of arterial and venous endothelial marker genes were carried out during endothelial differentiation protocols. Levels of arterial (EphrinB2, Notch1 and Notch2) and venous (EphB4) marker genes were measured during differentiation steps. In all differentiation protocols almost stepwise increases were observed in mRNA levels of endothelial marker genes via differentiation. No significant differences were found between endothelial differentiation protocols in terms of expression of arterial and venous markers. However EB/normoxia method resulted in the highest levels of these markers. Figure 27. shows levels of arterial and venous marker genes via differentiation in EB normoxia (Figure 27. A), EB hypoxia (Figure 27. B) and monolayer+VEGF (Figure 27. C) methods.

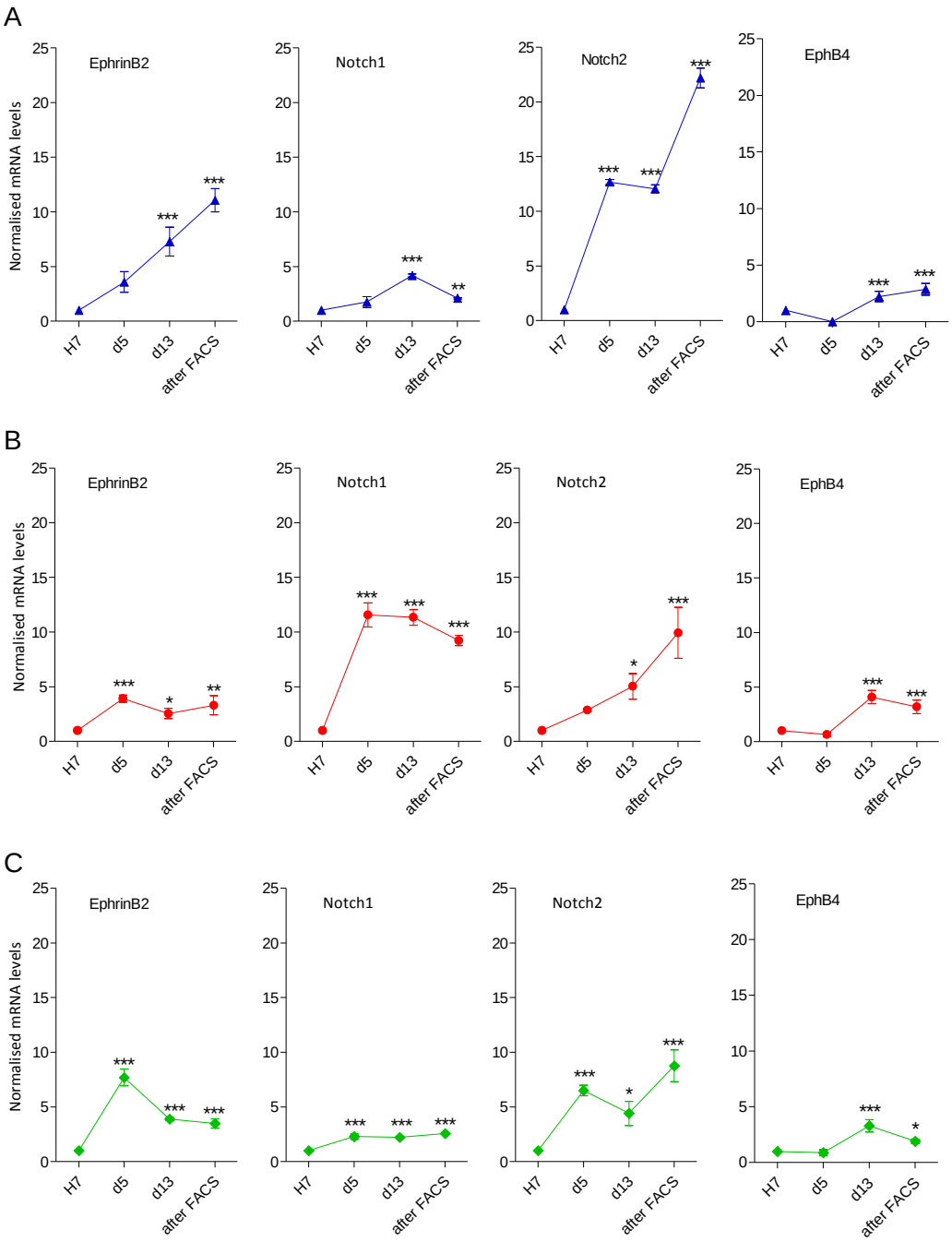


Figure 27. Differentiation conditions influence expression of arterial and venous endothelial marker genes during endothelial differentiation Line graphs show changes in mRNA levels of arterial (EphrinB2, Notch1, Notch2) and venous (EphB4) endothelial marker genes via differentiation (time points: undifferentiated hESC (H7), day 5, day 13 and after FACS). (A) Embryoid body in normoxia method (B) Embryoid body in hypoxia method (C) monolayer+VEGF method. n=3 biological replicates, p *<0.05, **<0.01, ***<0.001 One way ANOVA, Tukey post hoc test.

5.5. Role of PI3K/Akt/FOXO1A pathway on endothelial differentiation

Next we aimed to study role of PI3K/FOXO1A signalling pathway on endothelial development from hESC. As embryoid body in normoxia differentiation protocol resulted in highest levels of endothelial marker gene expressions we modulated PI3K/FOXO1A pathway on this differentiation protocol. To investigate the changes of genes related to PI3K pathway during endothelial differentiation of hESC, we analysed the expressions of main PI3K genes using a PCR array. Undifferentiated, differentiating hESC-EB and sorted CD31 positive hESC-EC showed different expression levels of most genes tested in this pathway (Figure 28.). Undifferentiated hESC expressed high mRNA levels of FOXO1A transcription factor; this critical downstream element of PI3K pathway was strongly down-regulated during differentiation. When compared to HUVEC, we found matching expression levels of most of the PI3K pathway elements in hESC-EC (Figure 29.). However, expression of FOXO1A was higher in HUVEC as compared to those in hESC-EC, suggesting that PI3K/FOXO1A may have distinct modulatory role in these endothelial cells (Figure 29. A). We also found that mRNA levels of PI3K-partners such as tyrosine-protein kinase BTK and CD14 were higher, whereas PIK3R1 and PDGFRA were lower in hESC-EC than those in HUVEC. To infer gene networks from expression profile of hESC-EC, Ingenuity pathway network analysis was performed. It suggested that FOXO1A expression is specifically linked to an indispensable cluster of angiogenesis- and vascular remodelling-related genes, including VEGF2 as well as PDK1 (pyruvate dehydrogenase kinase isoenzyme-1), cdc42, and PRKCB1 (Figure 29. B). Furthermore, analysis suggests that VEGF may signal through PTEN (phosphatase and tensin homolog) /PDGFRA pathway and modulate FOXO1A.

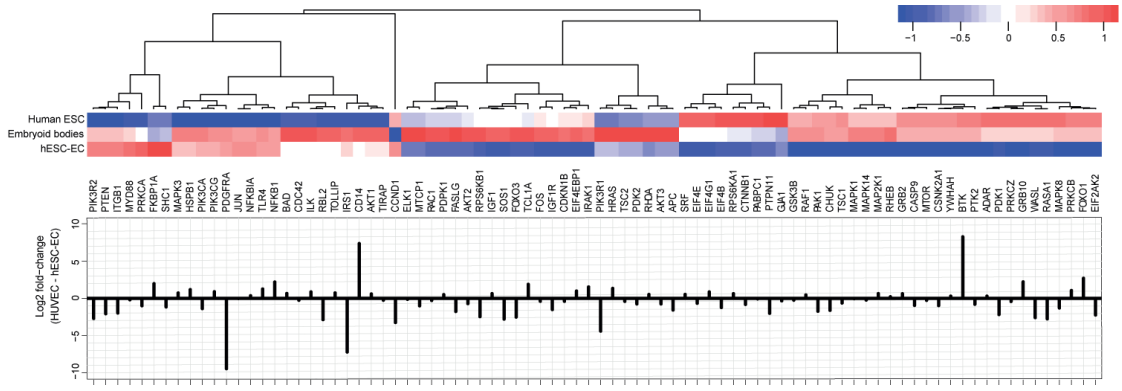


Figure 28. PI3K/FOXO1A signalling-related gene expression during hESC differentiation Heat map with agglomerative clustering shows changes in mRNA levels of PI3K/FOXO elements during different steps of endothelial differentiation: undifferentiated human embryonic stem cells (hESC), embryoid bodies (EB), human embryonic stem cells-derived endothelial cells (hESC-EC). Heat map shows modified z-score compared to expression levels in control endothelial human umbilical vein endothelial cells (HUVEC).

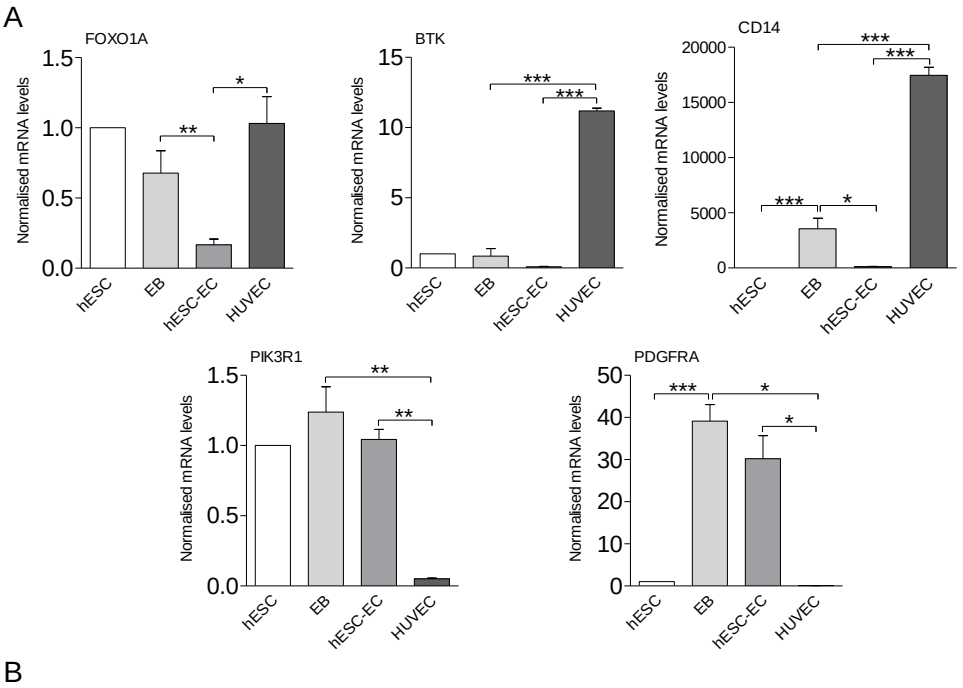


Figure 29. PI3K/FOXO1A signalling-related gene expression during hESC differentiation (A) Bar graphs show differences in essential elements of PI3K/FOXO1A signalling pathway in HUVEC vs. those in hESC-EC. Data are expressed as fold changes vs. mRNA levels in undifferentiated hESC; mean±SEM. *p<0.05, **p<0.01, ***p<0.01, from 3 biological replicates. **(**B**)** Schematic diagram of Ingenuity Pathway Analysis-mapped mechanistic interactions of various angiogenic modulator elements. Arrows between nodes represent direct (solid lines) and indirect (dashed lines) interactions between molecules as supported by information in the Ingenuity pathway knowledge base. hESC: human embryonic stem cells, EB: embryoid bodies, hESC-EC: human embryonic stem cells-derived endothelial cells, HUVEC: human umbilical vein endothelial cells, ITGB1: integrin beta-1, PRKCA: protein kinase C alpha, PRKCZ: protein kinase C zeta, PDGFRA: platelet-derived growth factor receptor alpha, PTEN: phosphatase and tensin homolog, PRKCB: protein kinase C beta, PIK3R2: phosphoinositide-3-kinase regulatory subunit 2 beta, SHC1: Src homology 2 domain containing transforming protein 1

Use of PI3K inhibitor/FOXO1A activator LY294002 increased the percentage of newly formed CD31 positive hESC-EC in the presence of EGM2 medium, containing endothelial growth factors (LY294002 0.62±0.04% vs. control differentiating hESC culture 0.24±0.1%). Silencing of FOXO1A by siRNA during differentiation had no influence on the percentage of CD31 positive cells (Figure 30.).

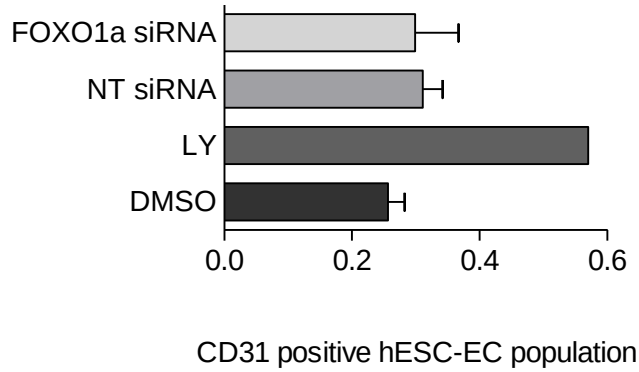


Figure 30. PI3K/FOXO1A signalling pathway modulates endothelial differentiation yield from undifferentiated human embryonic stem cells Bar graph shows CD31 positive hESC-EC population in the presence of FOXO1A siRNA or LY294002 (10μM). *p<0.05, ***p<0.001 vs. control group (DMSO for LY394002 control), 3 biological replicates. One-way ANOVA and Tukey post hoc test. hESC-EC: human embryonic stem cells-derived endothelial cells, NT siRNA: non-targeted siRNA, DMSO: dimethyl-sulfoxide

5.6. Modulation of PI3K/FOXO1A signalling pathway in sorted hESC-EC

To test the effects of PI3K/FOXO1A modulation in hESC-EC, we treated cells with LY294002 or FOXO1A siRNA (Figure 31. A, B). Nuclear translocation and mRNA levels of FOXO1A were markedly increased in hESC-EC in response to LY294002 (Figure 31. C, D). In contrast, silencing of FOXO1A with siRNA decreased both mRNA and nuclear translocation levels of FOXO1A as early as after 6 hours treatment in hESC-EC (Figure 31. E, F).

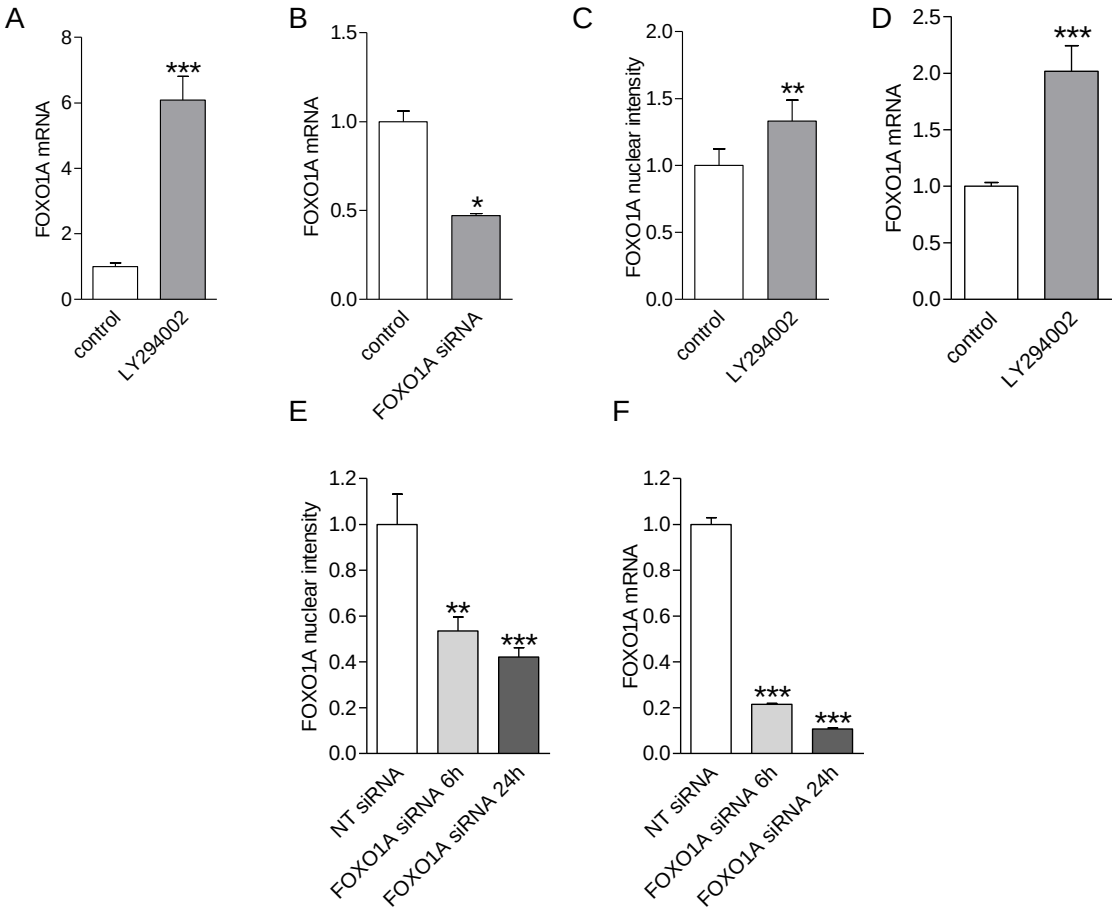


Figure 31. Modulation of FOXO1A expression and nuclear intensities (A) Changes of FOXO1A mRNA levels after treatment with the PI3K inhibitor/FOXO1A activator LY294002 (10μM) or (B) FOXO1A silencing using siRNA in differentiating hESC. (C) Changes of FOXO1A nuclear intensity and (D) mRNA levels in hESC-EC after treatment with the PI3K inhibitor/FOXO1A activator LY294002 (24 h). (E) Silencing of FOXO1A using siRNA caused decreased level of nuclear intensity or (F) FOXO1A mRNA levels.**p<0.01, ***p<0.001 vs. control, n=4 One-way ANOVA and Tukey post hoc test.

5.7. Differentiation conditions and PI3K/FOXO1A pathway modulates angiogenic activity of hESC-EC

Main angiogenic regulator angiopoietin-2 expression was modulated by endothelial differentiation conditions such as embryoid body formation or monolayer technique (Figure 32.). Normoxia (21% O_2) with embryoid body method was the most effective to increase angiopoietin-2 levels. Significantly higher levels of angiopoietin-2 were achieved via embryoid body technique. Embryoid body differentiation method in hypoxic condition had stronger induction on angiopoietin-2 levels than monolayer method (Figure 32. A). These results suggest that embryoid body method is more efficient to generate mature endothelial cells. To endorse hypoxic model, HIF1α levels were measured. HIF1α mRNA levels were significantly increased in hypoxic differentiation protocol, validating effective hypoxia generation in culture (Figure 32. B).

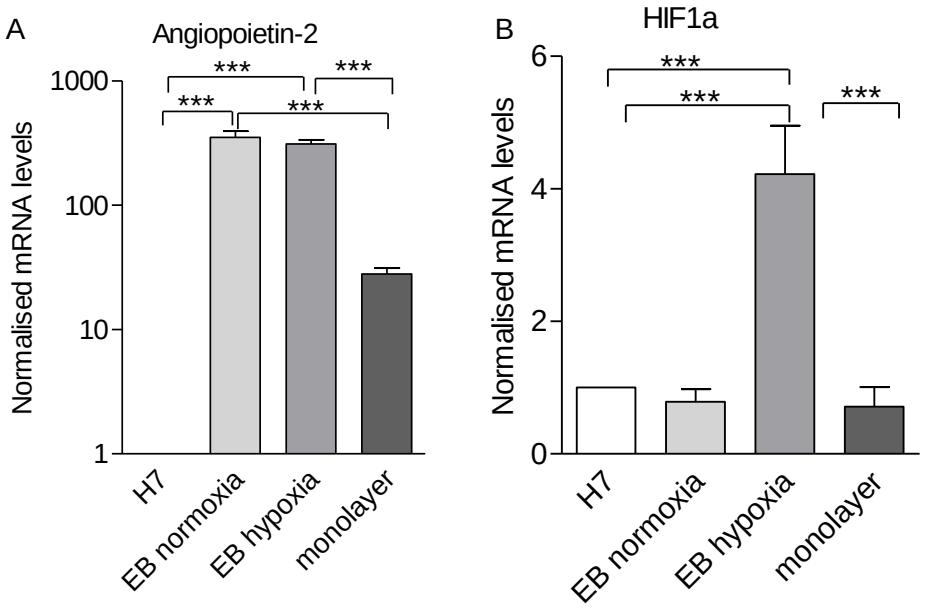


Figure 32. Hypoxia modulates expression of angiopoietin-2 and HIF1α (A) Bar graphs show changes in angiopoietin-2 levels modulated by differentiation conditions. mRNA levels are normalized to those in H7 hESC. (B) Bar graphs show changes in HIF1α levels modulated by normoxia and hypoxia. mRNA levels are normalized to those in H7 hESC. Cell numbers were equalized at each time point. n=3 biological replicates, p *<0.05, **<0.01, ***<0.001, One-way ANOVA and Tukey post hoc test.

As embryoid body formation method in normoxia resulted in highest levels of angiogenesis marker genes, further analyses were done on this endothelial differentiation method. Levels of endothelial marker CD31, angiogenesis marker Angiopoietin-2 and its receptor Tie-2 were characterised. Results proved that mRNA levels of CD31, Angiopoietin-2 and Tie-2 are increasing gradually during differentiation (Figure 33.). Expression levels on hESC-EC were comparable with those on control HUVEC.

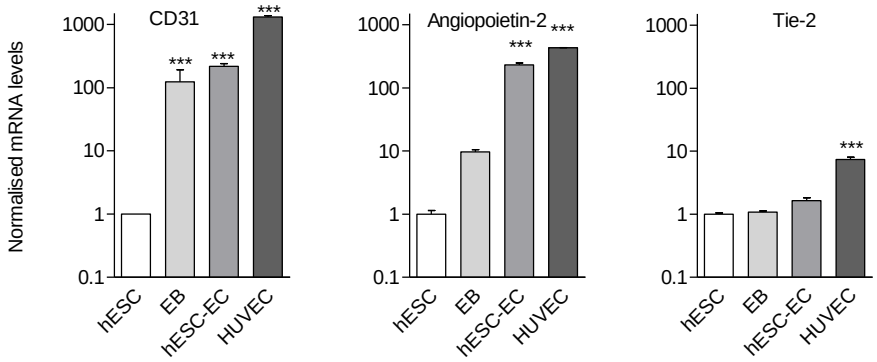


Figure 33. qRT-PCR characteristics of human pluripotent stem cells-derived endothelial cells differentiated via embryoid body, normoxia method Bar graphs show changes in gene expression levels of CD31, Angiopoietin-2 and Tie-2 levels. mRNA levels in EB, hESC-EC and HUVEC are shown as fold changes vs. undifferentiated stem cells. n=3 biological replicates, p *<0.05, **<0.01, ***<0.001, one-way ANOVA with Tukey post hoc test. hESC: human embryonic stem cells, EB embryoid bodies, hESC-EC: human embryonic stem cells-derived endothelial cells, HUVEC: human umbilical vein endothelial cells

Next we targeted PI3K/FOXO1A pathway to directly assess its role in endothelial differentiation. Administration of LY294002 during differentiation increased mRNA levels of CD31 and angiopoietin-2 in differentiating hESC (Figure 34. A, C). Cells treated with FOXO siRNA showed however decreasing tendency in mRNA levels of CD31 ($p=0.07$) and lower mRNA levels of angiopoietin-2 ($p<0.001$) as compared with non-targeting siRNA group (Figure 34. B, D). Treatment with FOXO siRNA resulted in increased mRNA levels of Tie-2 and VE-Cadherin (Figure 34. E, F).

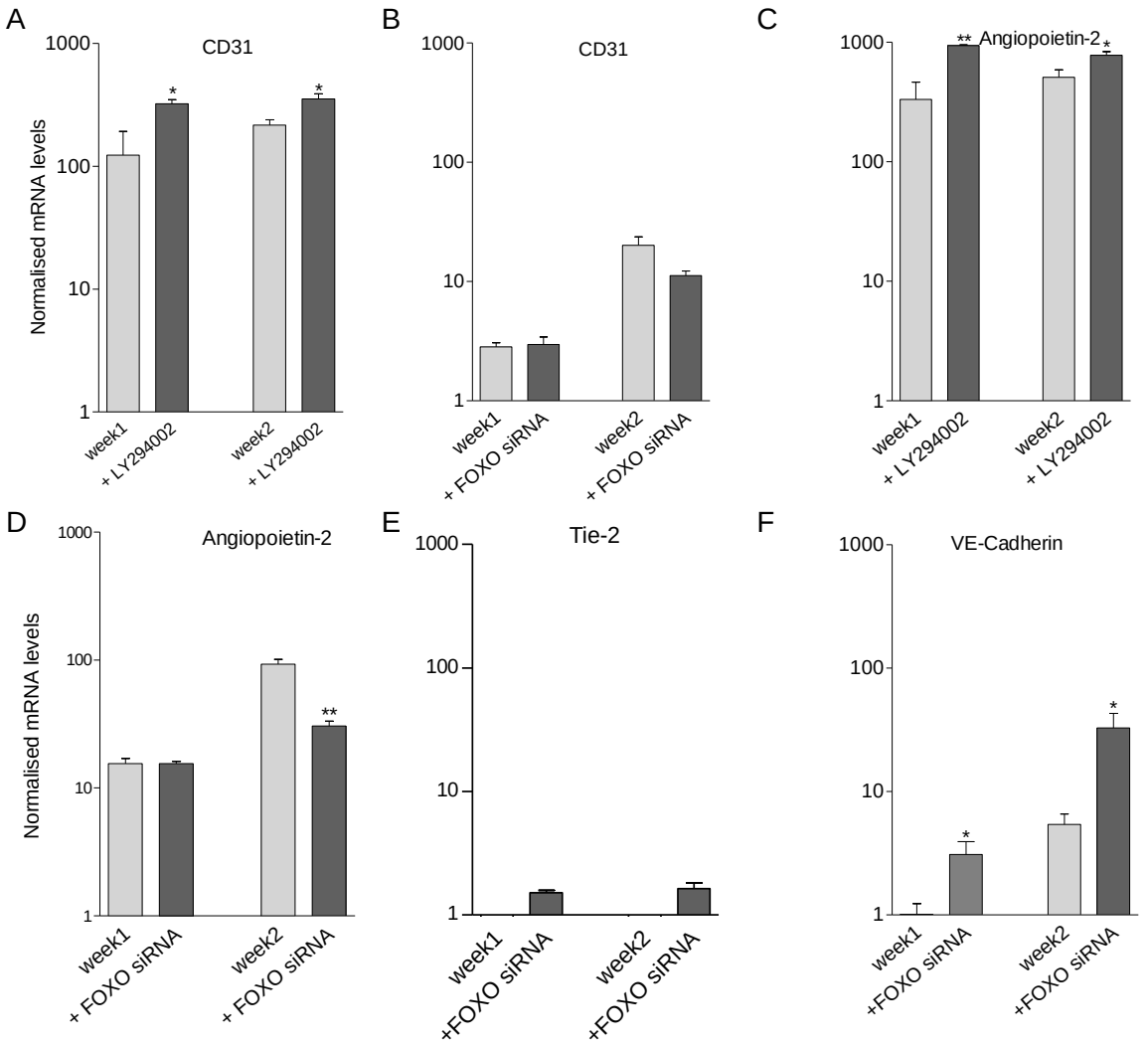


Figure 34. PI3K/FOXO1A signalling modulates endothelial differentiation of hESC (A-F) Bar graphs show mRNA levels of CD31, angiopoietin-2, Tie-2 and vascular-endothelial cadherin in differentiating hESC after (A, C) LY294002 treatment and (B, D, E, F) FOXO1A small interfering (si) RNA treatment. *p<0.05, **p<0.01 vs. control group, n=3 biological replicates. One-way ANOVA and Tukey post-hoc test.

To test the effects of PI3K/FOXO1A modulation in hESC-EC, we treated cells with LY294002 or FOXO1A siRNA. The mRNA levels of CD31 and angiopoietin-2 were increased in response to LY294002 in hESC-EC (Figure 35. A, B); on the other hand, FOXO siRNA resulted in lower mRNA levels of angiopoietin-2 (Figure 35. D, E). The mRNA levels of the angiopoietin-2 receptor, Tie-2 showed reverse regulation as angiopoietin-2 expression. Treatment with LY294002 decreased the mRNA levels of Tie-2, whilst treatment with FOXO siRNA resulted in increased mRNA levels of Tie-2 (Figure 35. C, F).

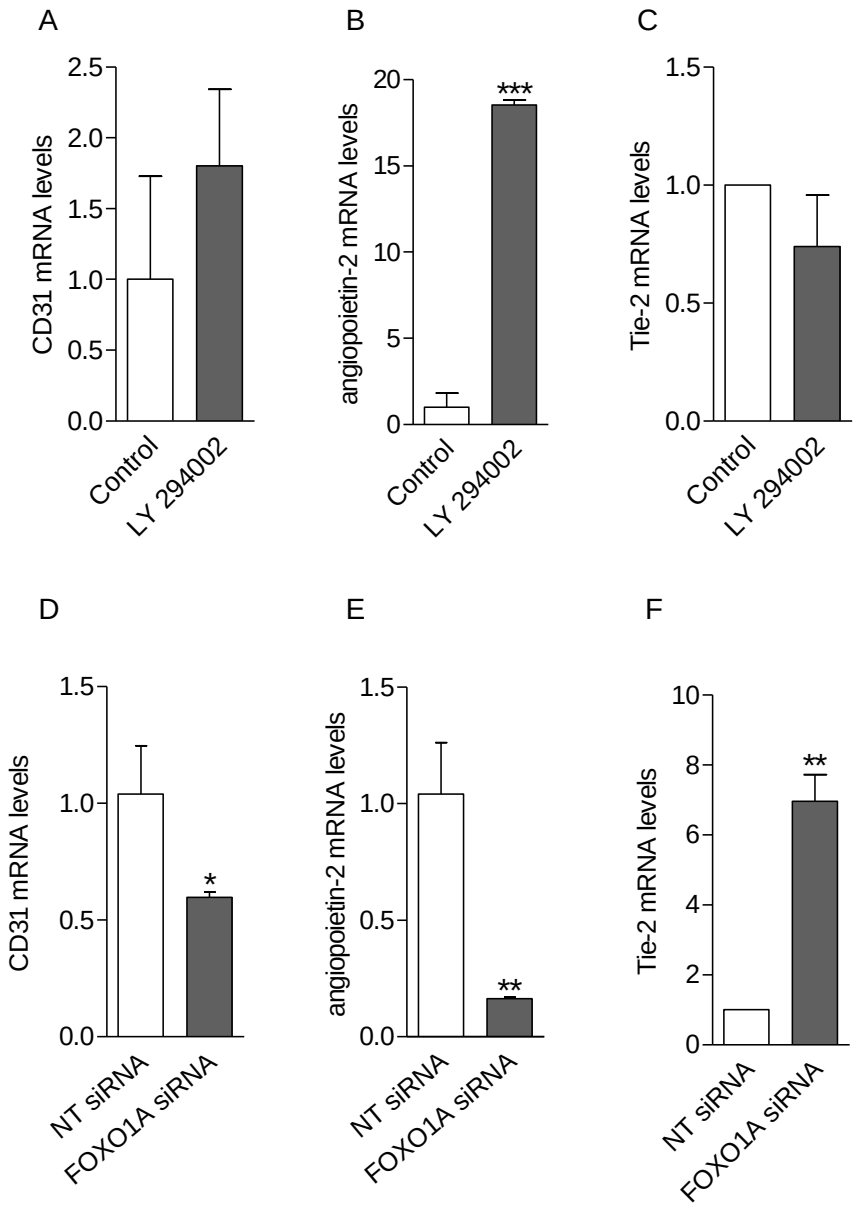


Figure 35. PI3K/FOXO1A signalling modulates angiogenesis-related gene expression activity of hESC-derived endothelial cells Bar graphs show changes in gene expression levels of CD31, angiopoietin-2, Tie-2 in differentiated hESC-EC in response to 24 hours treatment with (ABC) LY294002 or (DEF) FOXO1A small interfering (si) RNA. n=3 biological replicates. One-way ANOVA and Tukey post-hoc test

In vitro tube formation assay revealed that LY294002 inhibits hESC-EC migration and formation of tubes (Figure 36. A-D). On the other hand, silencing of FOXO1A by siRNA markedly increased *in vitro* tube formation activity of hESC-EC as shown by total tube length and node count (p<0.001, Figure 36. A, E, F, G).

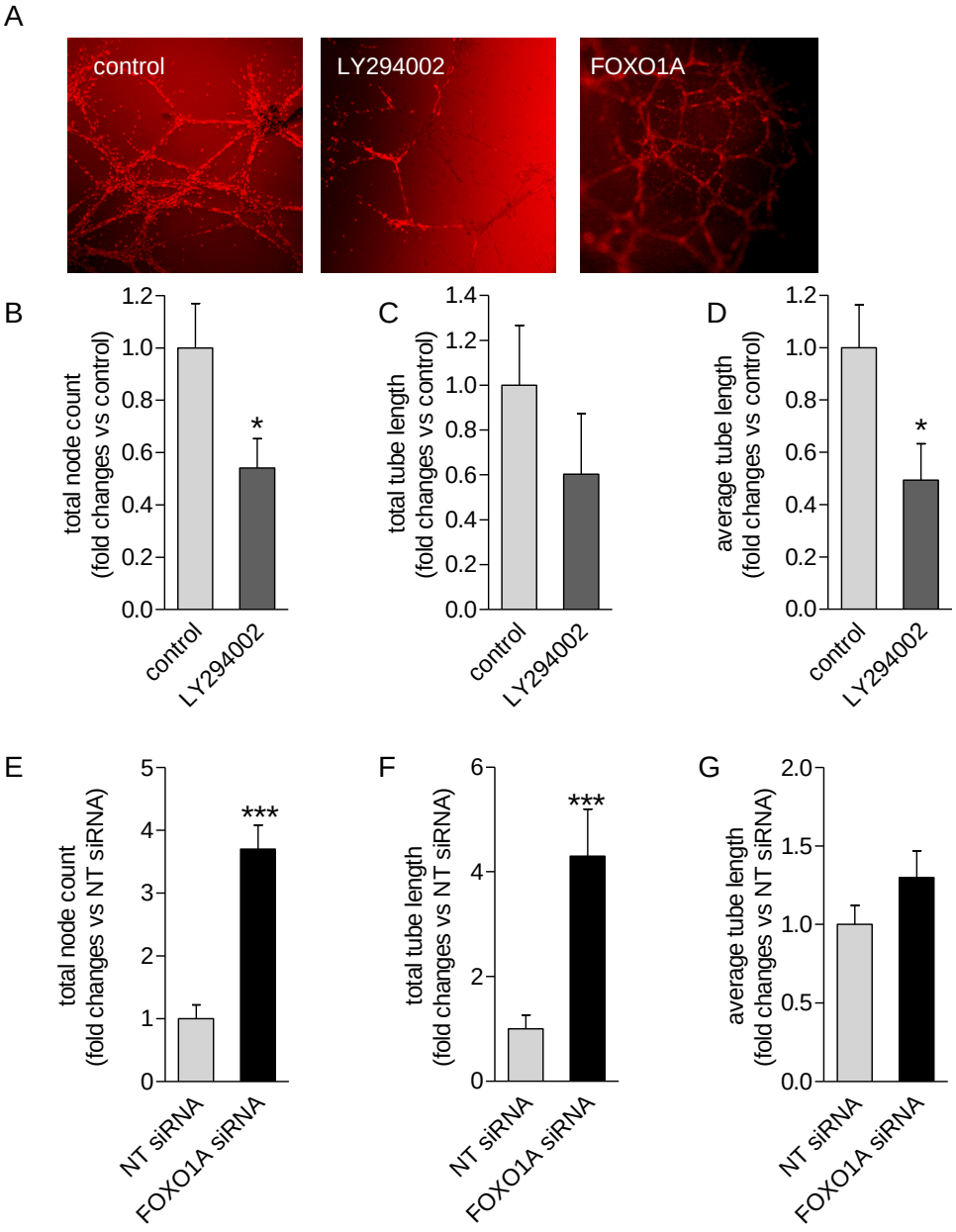


Figure 36. PI3K/FOXO1A pathway modulates *in vitro* tube formation of hESC-EC **(**A**)** Representative Matrigel tube formation images of hESC-EC pre-treated with LY294002 or FOXO1A small interfering (si) RNA. Cells were stained with vital dye TMRM (red). Bar graph show average and total tube lengths and number of formed nodes after **(**BCD**)** LY294002**, (**FGI**)** FOXO1A or non-targeting scrambled (NT) small interfering (si) RNA.***p<0.001 vs. control, n=3 biological replicates. One-way ANOVA and Tukey post test.

5.8. Effects of PI3K/FOXO1A pathway on arterial, venous and general endothelial gene expressions

To assess the modulatory effects of PI3K/FOXO1A signalling in arterial and venous endothelial subpopulations hESC-EC and hiPSC-EC were overexpressed with FOXO1A. After FOXO1A plasmid overexpression universal endothelial marker genes and special arterial and venous endothelial marker genes were studied. qRT-PCR revealed that FOXO1A overexpression downregulates expression of all endothelial genes investigated. Figure 37. shows that CD31, angiopoietin-2 and VE-Cadherin levels were significantly decreased. Arterial and venous marker genes were parallel decreased. FOXO1A overexpression had corresponding effects on hESC-EC and hiPSC-EC (Figure 37. C). EphrinB2/EphB4 ratio was calculated after FOXO1A overexpression, result shows huge decrease to those in control hESC-EC, hiPSC-EC (Figure 37. D).

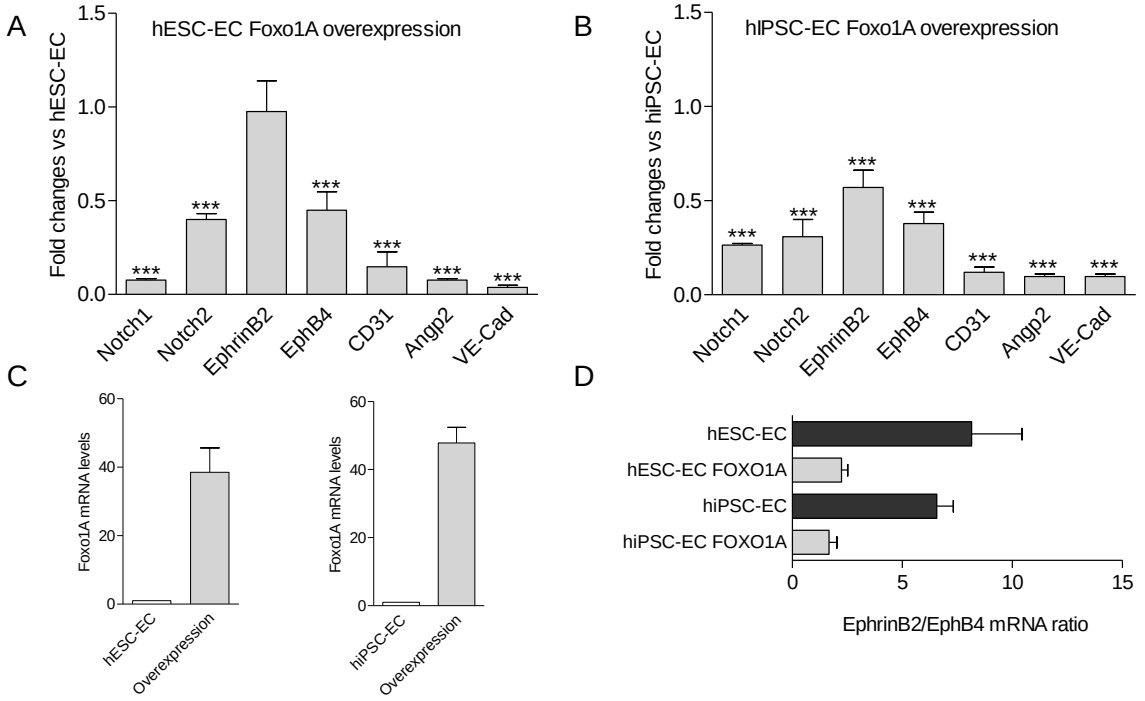


Figure 37. Effects of PI3K/FOXO1A modulation on endothelial marker genes in hESC-EC and hiPSC-EC (AB**)** Bar graphs show changes of expression of arterial (Notch1, Notch2, EphrinB2), venous (EphB4) and universal endothelial marker genes (CD31, angiopoietin-2 and VE-cadherin). (C) FOXO1A mRNA levels were increased via FOXO1A-overexpression (D) EphrinB2/EphB4 ratio after FOXO1A overexpression and in control human embryonic stem cells-derived endothelial cells (hESC-EC) and human induced pluripotent stem cells-derived endothelial cells (hiPSC-EC). ***p<0.001 vs control, n=3 biological replicates. One-way ANOVA and Tukey post hoc test.

5.9. Effects of PI3K/FOXO1A pathway on hESC-EC proliferation

Proliferative activity of sorted hESC-EC cultures were assessed by Ki67 marker and colony formation. Percentage of $Ki67^+$ cells and colony formation activity after 3 days were significantly decreased in response to LY294002 (Figure 38. B, C); in contrast, further downregulation of FOXO1A expression by siRNA treatment resulted in a modest increase in the ratio of $Ki67^+$ cells ($p<0.05$) with no effect on cell number (Figure 38. A, B, D).

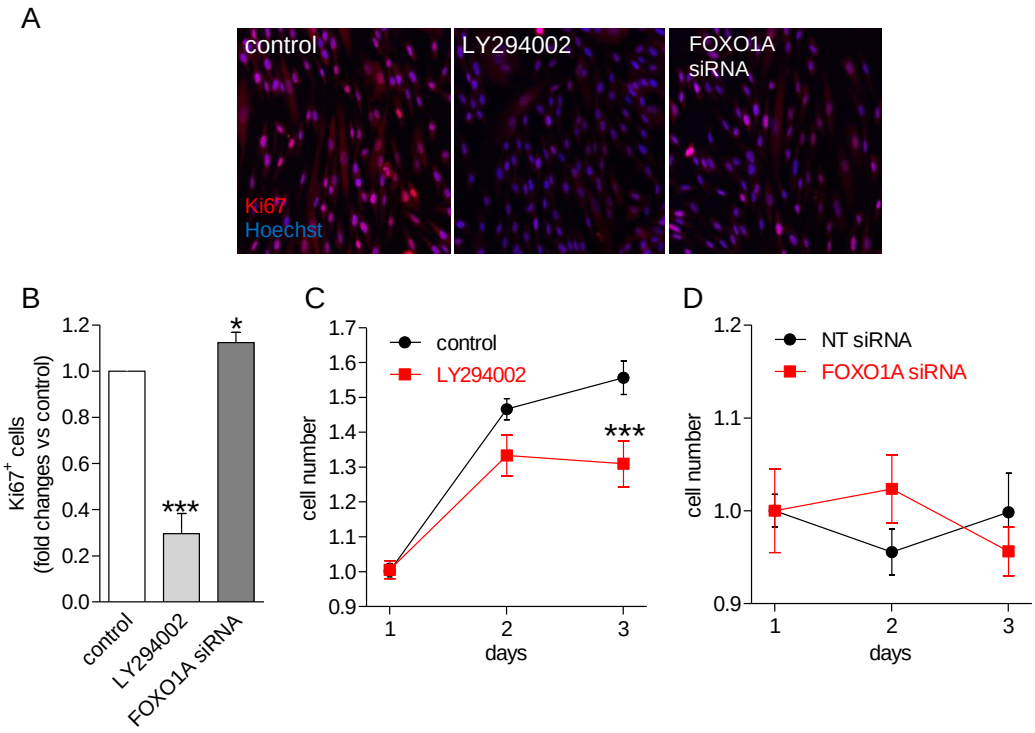


Figure 38. PI3K/FOXO1A pathway modulates proliferative activity of hESC-EC (A) Representative picture of $Ki67^+$ cells in hESC-EC population. (B) Changes in proliferation rate of the human embryonic stem cells-derived endothelial cell (hESC-EC) population ($Ki67^+$ cells) after 24 hours and in colony formation activity **(**CD**)** in response to LY294002 or FOXO1A small interfering (si) RNA treatments. (EF) Changes in proliferation rate of the hESC-EC population ($Ki67^+$ cells). Data are expressed as fold changes vs. percentage of $Ki67^+$ cells in control hESC-EC group, mean ± SEM. Fold changes in mRNA levels are shown vs. human embryonic stem cells (hESC). *$p<0.05$, ***$p<0.001$ vs. control, n=3 biological replicates. One-way ANOVA and Tukey post hoc test.

5.10. Effects of PI3K/FOXO1A pathway on hESC-EC viability

Next, role of PI3K/FOXO1A pathway on viability and cell survival of hESC-EC was tested. As assessed by high content microscopy, oxidative stress induced by H_2O_2 activated FOXO1A as shown by its increased nuclear density (Figure 39.). Using H_2O_2 as a danger signal caused necrosis and nuclear remodelling (expressed as decreased nuclear size) in a dose-dependent manner in hESC-EC (Figure 39. C, D). Silencing of FOXO1A by siRNA had no effect on levels of necrosis marker TOTO1 or nuclear remodelling in hESC-EC (Figure 39. B-D). Pre-treatment with LY294002 increased the pro-necrotic effects of H_2O_2 (at a dose of 600 μM, $P<0.001$) in hESC-EC (Figure 39. C).

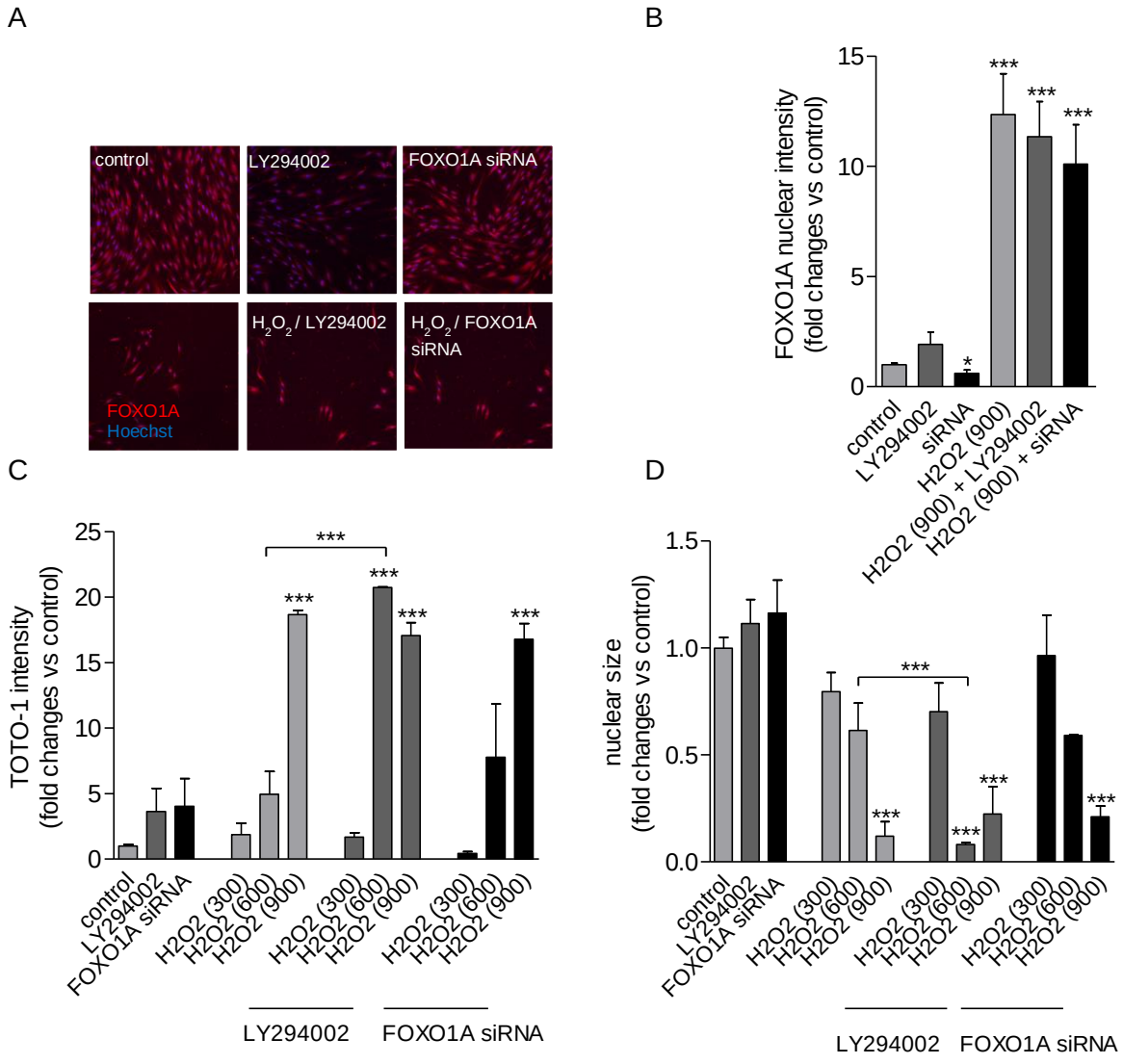


Figure 39. Cell death of hESC-derived endothelial cells is modulated by PI3K/FOXO1A signalling pathways H_2O_2 was used in different concentrations (300, 600, 900 μM) to induce oxidative stress. LY294002 (10μM, 24h) and FOXO1A-targeting small interfering (si)RNA (24h) were used to modulate FOXO1A activity. **(**A**)** Representative immunocytochemistry images of FOXO1A intensity during cell death experiments of human embryonic stem cells-derived endothelial cells (hESC-EC). Human ESC-EC were stained with anti-FOXO1A antibody and nuclei stained with Hoechst-33342. **(**B**)** Bar graph shows changes in FOXO1A nuclear translocation in hESC-EC pre-treated with LY294002 and FOXO1A siRNA after challenging with H_2O_2. **(**C**)** Bar graph shows changes in necrotic rate measured by TOTO-1 intensity of hESC-EC. **(**D**)** Changes in nuclear remodelling is calculated by measuring Hoechst-33342 positive nuclear size of hESC-EC. ***p<0.001 vs control, n=3 biological replicates. One-way ANOVA and Tukey post hoc test.

5.11. Modulation of endothelial gene expressions and anticlotting function in 3D cultures

Culturing hESC-EC and hiPSC-EC in 3D culture conditions aimed for scaling up vascular tissue engineering. Human ESC-EC, hiPSC-EC or HUVEC were cultured and expanded in spinner flasks and seeded on Cormatrix and decellularised human aortic slices. After 10 days in spinner flask, cultured endothelial cells seeded on bioscaffolds, proven by vital dyes (Figure 40. A). 3D Histech imaging showed that CD31 positive endothelial cells form closely monolayer structure on biomatrices (Figure 40. B). Anticlotting function of hESC-EC, hiPSC-EC and HUVEC was investigated by Rantes chemokine ELISA assay. Secretion of Rantes chemokine was measured in platelet rich plasma after incubating 3D vascular structures. Rantes is secreted from activated platelets, thus its level refers to clotting status in platelet rich plasma. Results showed similar antiplatelet activity in hESC-EC, hiPSC-EC and HUVEC. According to similar results in control and 'matrix alone' samples, the matrix itself was not thrombogenic (Figure 40. C-E). Endothelial cells decreased secretion of Rantes, suggesting lower platelet activation. The anticlotting effects of endothelial cells were stronger when they were cultured in 3D cultures (Figure 40. C-H). Results were comparable on two types of biomatrices, suggesting that hESC-EC and hiPSC-EC would possess stable phenotype on different biomatrices (i.e. variability of human ECM in vasculature). Anticlotting effects of hESC-EC and hiPSC-EC were similar as in HUVEC (Figure 40. C-H).

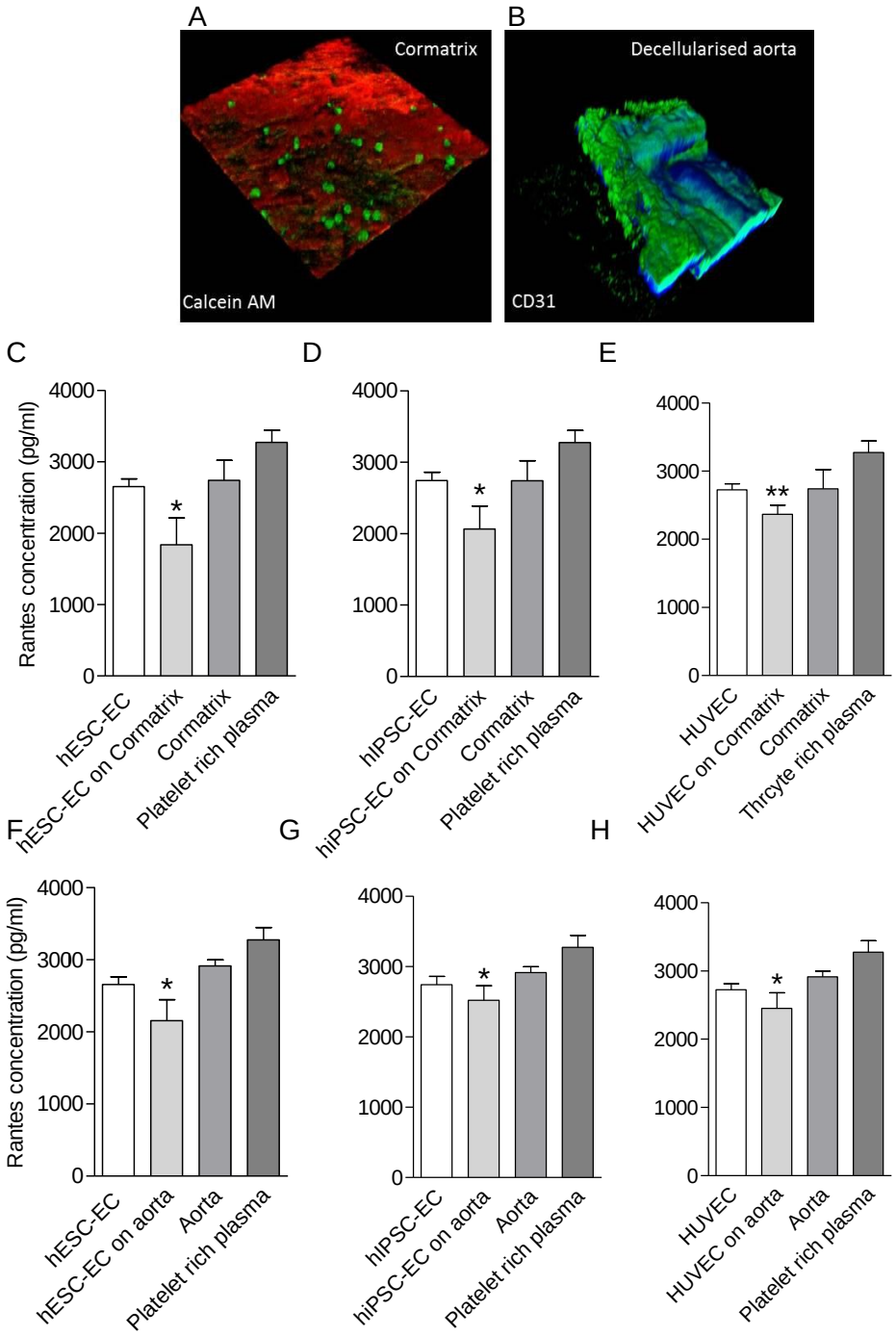


Figure 40. 3D culture and anticlotting effects of hESC-EC, hiPSC-EC and HUVEC Recellularised biomatrices were seeded with hESC-EC and hiPSC-EC (A) Calcein AM live cell imaging, 20X. (B) 3D Histech imaging analyses of CD31 positive hESC-EC and hiPSC-EC on decelluraised aortic slices. Bar graphs show changes in Rantes chemokine levels, measured from platelet rich plasma after incubation 3D vascular constructs. (CF) hESC-EC, (DG) hiPSC-EC and (EH) HUVEC showed similar anticlotting activity on (CDE) Cormatrix and (FGH) decellularised human aortic samples. n=3 biological replicates, One-way ANOVA with Tukey post test, hESC-EC: human embryonic stem cells-derived endothelial cells, hiPSC-EC: human induced pluripotent stem cells-derived endothelial cells, HUVEC: human umbilical vein endothelial cells

5.12. Transplantation of hESC-EC, hiPSC-EC and HUVEC *in vivo*

To test the viability and maturation of hESC-EC with low FOXO1A levels also *in vivo*, cells were transplanted into athymic nude rats. Human ESC-EC, hiPSC-EC and control endothelial HUVEC showed engraftment. After 21 days conditioning of cells, all general endothelial marker genes as well as arterial and venous marker genes were increased. General endothelial markers, Angiopoietin-2, Tie-2, CD31 and NOS3 mRNA levels were markedly increased in hESC-EC similar to those in control HUVEC (Figure 41. B-E). However, hESC-EC retain their low FOXO1A expression levels during the incubation period, whereas FOXO1A mRNA levels are further increased in HUVEC (Figure 41. A). Arterial (EphrinB2, Notch1 and Notch2) and venous (EphB4) endothelial marker mRNA levels were increased significantly in all studied endothelial cells (hESC-EC, hiPSC-EC and HUVEC) after engraftment (Figure 42.).

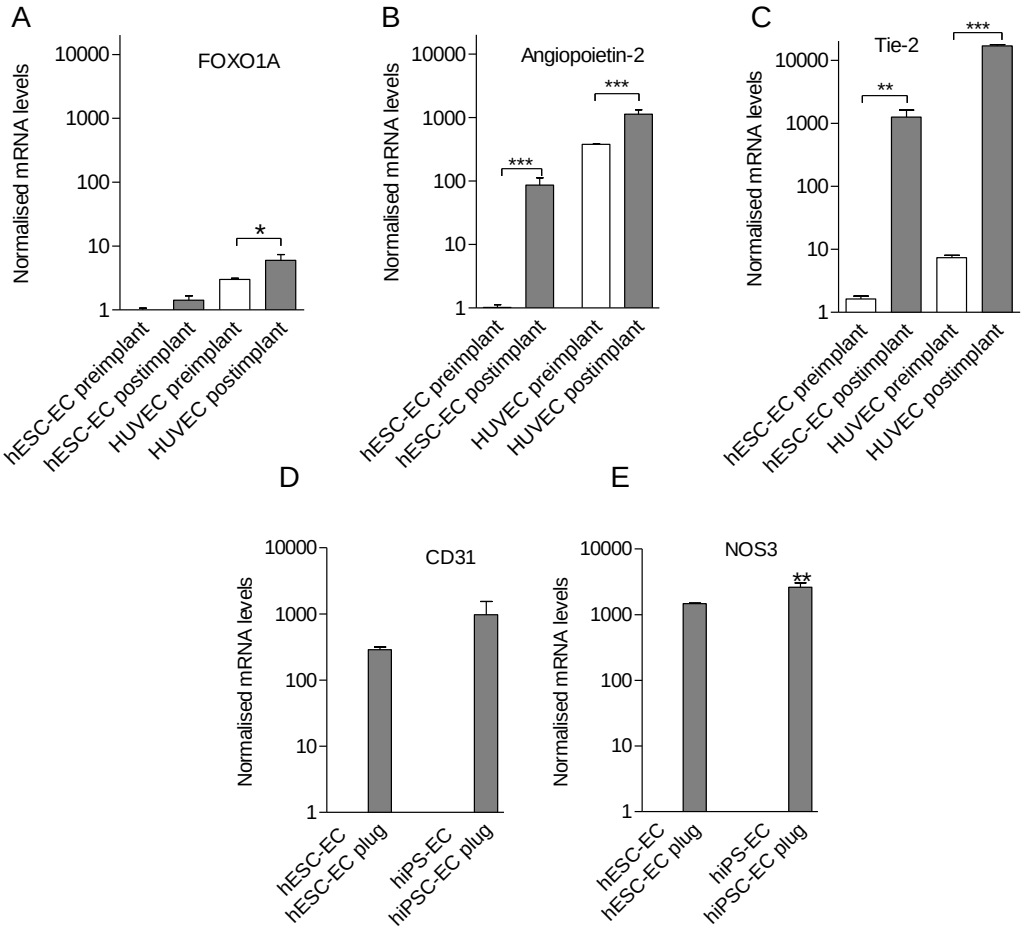


Figure 41. *In vivo* conditioning modulates mRNA levels of FOXO1A and universal endothelial marker genes Grouped bar graphs show (A) FOXO1A, (B) Angiopoietin-2, (C) Tie-2, (D) CD31 and (E) NOS3 endothelial marker mRNA levels before and after *in vivo* conditioning of hESC-EC, hiPSC-EC and HUVEC in Matrigel plugs, transplanted into athymic nude rats. (mRNA levels are normalised to those in preimplanted samples for each gene) n=12 from 3 biological replicates. * $p<0.05$ ** $p<0.01$ *** $p<0.001$ One-way ANOVA and Tukey post hoc test, hESC-EC: human embryonic stem cells-derived endothelial cells, hiPSC-EC: human induced pluripotent stem cells-derived endothelial cells, HUVEC: human umbilical vein endothelial cells

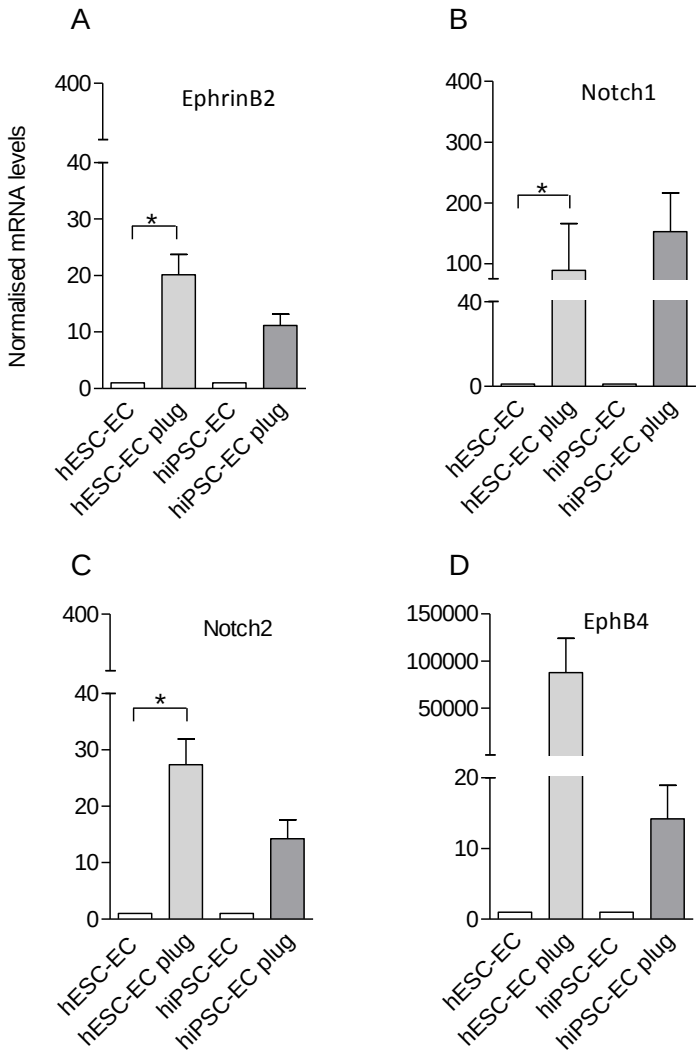


Figure 42. *In vivo* conditioning modulates mRNA levels of arterial and venous endothelial marker genes Grouped bar graphs show (ABC) arterial and (D) venous endothelial marker mRNA levels before and after *in vivo* conditioning of hESC-EC and hiPSC-EC in Matrigel plugs, transplanted into athymic nude rats. (mRNA levels are normalised to those in preimplanted samples for each gene) n=12 from 3 biological replicates. * $p<0.05$ ** $p<0.01$ *** $p<0.001$ One-way ANOVA and Tukey post hoc test, hESC-EC: human embryonic stem cells-derived endothelial cells, hiPSC-EC: human induced pluripotent stem cells-derived endothelial cells

6. Discussion

Cardiovascular ischemic diseases are leading cause of death worldwide. Peripheral arterial diseases and post infarction cardiomyopathy have high burdens on health and social care. In myocardial infarction ~1 billion cardiomyocytes suffer definite necrosis. After ischemic attack, only small amount of the injured tissue can regenerate from the stunned state. Regenerative capacity of the myocardium is inefficient to maintain left ventricular function. Myocardial inflammation, fibrosis, scar formation and remodelling occur. These pathological conditions result in vicious circle leading to severe ischemic cardiomyopathy with left ventricular remodelling and insufficiency. Albeit, pharmacological modulation of the renin-angiotensin-aldosterone system, beta receptor inhibition and statin therapy are beneficial on mortality outcomes and also have moderate effects on reverse remodelling. Definite therapy to improve reduced myocardial function and replace injured cardiomyocytes and endothelial cells is lacking. In the last decades many cell types were investigated as future candidate for cardiovascular cell therapy. Early investigation focused on cardiac resident stem cells, endothelial progenitor cells, bone marrow-derived mononuclear cells and MSC. First clinical trials ended with moderate or non-beneficial outcome; furthermore, many of those published conflicting data [98]. To eliminate doubts on the effects of bone marrow-derived mononuclear cells and MSC implantation in acute MI and heart failure, large, randomised clinical trials are underway (BAMI, CHART-1/2). Early studies with these cell types have reported mild or no increase in left ventricular function, exact transdifferentiation of these cells to cardiomyocytes is doubtful. Beneficial effects of MSC transplantation seem to lie in their paracrine, anti-inflammatory, anti-fibrotic and reverse remodelling effects. Large individual variability exists in these regenerative mechanisms. Thus, a cardiac index was recently set to characterise responder status of patients before MSC transplantation [110].

Human pluripotent stem cells have become a focus of regenerative medicine. Human iPSC seem to be promising cell type for cardiovascular regeneration. The ideal cell type for regenerative purposes would be easily available, easy to collect and expand, should have stable karyotype and phenotype, should be efficiently and feasibly differentiated towards the desired cell type, may not be immunogenic and must provide desired functional activity *in vivo*. In the early steps of cardiovascular regenerative medicine researchers first must

define the most appropriate differentiation protocols *in vitro*. Then *in vitro* studies must be completed to study phenotype, gene expression profile and functional activity of these cells. Preclinical *in vivo* experiments should demonstrate feasibility and safety of cell transplatation before human application.

Results from this project increase our knowledge on the characteristics and behaviour of cardiovascular derivatives of human pluripotent stem cells. Characterisation of hESC and hiPSC proved that these colonies possess pluripotent markers. Undifferentiated stem cells were successfully differentiated towards mesodermal and endothelial lineage. Preclinical small animal experiments call for large animal studies to move toward cardiovascular tissue engineering and human applications.

Recent studies focused on hiPSC which offer unique platform for patient specific disease modelling and drug testing [171]. Yamanaka developed first induced pluripotent stem cells from adult somatic fibroblasts. His publication was a clear breakthrough in regenerative medicine and stem cell research [80]. Since then, reprogramming methods have been continuously developing. Here we successfully developed hiPSC from human adult somatic fibroblasts. Cardiovascular derivatives of these cells offer platform for patient and disease specific investigations *in vitro*. In my studies healthy volunteers were fibroblast donors, but success of hIPSC development warrants further studies on patient specific induced pluripotent stem cells-derived.

6.1. Endothelial characteristics and functional activity

Human pluripotent stem cells-derived endothelial cells showed similar characteristics *in vitro* as mature endothelial cells. Their phenotype, immunocytochemical properties and gene expression profile were comperable with those in HUVEC. When endothelial cells were grown under shear stress, human aortic endothelial cells and blood outgrowth endothelial cells were elongated and aligned in the direction of shear. By contrast, hESC-EC did not align in the direction of shear stress. These observations show differences in endothelial cells derived from embryonic stem cells versus those from blood progenitor cells and large vessels. The differences in *in vitro* behaviour may suggest that endothelial cells from different sources and human embryonic stem cells not posses same phenotype *in vitro*. Human pluripotent stem cell derivatives may not have adult, mature phenotype *in vitro*, thus *in vivo* conditioning should be needed for further maturing.

Beside abundant expression of general endothelial markers, arterial and venous genes were also expressed in hESC-EC and hiPSC-EC. These results suggest that endothelial differentiation generated mixed endothelial cultures, without significant changes between arterial and venous marker gene expressions.

To study the functional properties of hESC-EC, hiPSC-EC and control endothelial cells proteome profiling assays and anticlotting assays were performed. Human ESC-EC and hiPSC-EC are able to fulfil therapeutic expectations only when they possess sufficient functional activity to support the physiological mechanisms of the vascular system. During tissue engineering, vascularisation and endothelialisation is a cornerstone towards therapeutic applications. Endothelial cells should provide an antithrombotic surface to line vessel walls. Furthermore, they play central role in inflammatory mechanisms, monocyte adhesion, extravasation and scar healing.

6.1.1. Proteomics

Proteome profiling showed that hESC-EC and hiPSC-EC express in cell lysates and secrete into the supernatant many angiogenesis-related proteins, cytokines, hormones and soluble receptors. Analysing the expression pattern of these proteins revealed differences in hPSC-EC and control HCAEC. Expression levels in hESC-EC and hiPSC-EC showed

strong parallel pattern, suggesting that endothelial cells from different human pluripotent stem cell lineages may have similar functional characteristics *in vivo*. A population of peptides was also recognized which showed marked differences to those in control, adult, mature HCAEC. Expression levels of EGF, FGF1 and FGF2 were lower than in control HCAEC. These factors are important regulators of angiogenesis, wound healing and endothelial proliferation. Distinct expression profile suggests varying regulatory pathways in hESC-EC and hiPSC-EC. Other important regulators on angiogenesis and endothelial behaviour showed higher expression levels in hESC-EC, hiPSC-EC. These are isoforms of VEGF, Angiopoietin1, 2 and Activin2. However, the level of Vasohibin, a strong negative regulator of angiogenesis, also showed a distinct expression pattern in hESC-EC and hiPSC-EC.

We have also found that hESC-EC do not produce significant amount of ET-1 which is central player in pulmonary hypertension and vascular inflammation [199]. Thus, hESC-EC may be favourable for these therapeutic concerns. Furthermore hESC-EC produces high amount of prostacyclin which regulates endothelium-dependent and independent vasoactive responses on resistance arteries. Earlier studies showed that reactive oxygen species act via prostacyclin-derivatives in post capillary veins [200].

These results suggest that developed endothelial cells are highly active in expressing and secreting angiogenesis-related and inflammatory proteins. Thus, they may act as underlying signalling elements in paracrine mechanisms *in vivo*. The signalling pathways in hESC-EC and hiPSC-EC show differences when compared to mature endothelial cells, this emphasises the need for detailed investigations on behaviour and function of hESC-EC and hiPSC-EC.

6.1.2. Anticlotting properties of hESC-EC and hiPSC-EC

Rantes ELISA assays revealed strong anticlotting effects of hESC-EC and hiPSC-EC in 3D cell culture conditions. Rantes, or so called CCL5 chemokine is secreted from activated platelets and also have functional role in inflammatory mechanism. We performed experiments to measure Rantes levels in hPSC and in their endothelial derivatives. Scientific data are lacking regarding Rantes production in hPSC. Here we found similar levels of Rantes in hPSC supernatant and those in hESC-EC, hiPSC-EC and

HUVEC, without any significant difference. Rantes may play crucial role in regulation of tumour angiogenesis and formation of metastases. Recent study emphasized its role in ovarian cancer metastasis and suggested targeting CCL5/NFkappaB signalling pathway as a therapeutic target to prevent metastasis formation [201]. Another study also suggested regulatory role for Rantes in neoangiogenesis in chondrosarcoma [202]. Here we showed that hESC-EC and hiPSC-EC decrease Rantes levels in platelet rich plasma, suggesting decreased amount of activated platelets and increased anticlotting effects. 3D culture conditions enhance anticlotting effects of hESC-EC and hiPSC-EC. This enhancement in functional activity may be favourable when considering scale-up for vascular tissue engineering. As Rantes is also related to angiogenic mechanisms, its modulation by hESC-EC and hiPSC-EC emphasises their strong angiogenic characteristics.

6.2. Arterial and venous endothelial subpopulations

Development of the vascular tree and the specification of arterial, venous and lymphatic endothelial cells are determined genetically [176, 203, 204]. However environmental factors may alter endothelial fate [203]. After embryonic development of vascular structures, organ-specific properties of endothelial cells are gained through environmental signalling, e.g. blood flow, vessel wall shear stress, cell junction molecules and paracrine factors from surrounding tissues [203]. Endothelial cells in brain-blood barrier, in hepatic sinusoids, renal tubule or in coronary arteries have far more different functional tasks. These special characteristics are gained via environmental influencing factors [203]. During endothelial development, arterial and venous fate is determined in the early stages before the development of the embryonic heart and blood flow. This is suggested by my results showing that no significant difference was found in arterial and venous gene expression with the use of different cell culture conditions during differentiation. However slight changes in arterial and venous marker gene expression were found in different developmental protocols, suggesting that cell culture conditions and modulation of key signalling pathways may alter endothelial phenotype in vitro. During endothelial development arterial and venous fate is determined in early steps before embryonic heart and blood flow develops [176]. EphrinB2/EphB4 ratio is regulating arterial and venous segregation in heterogeneous endothelial populations during embryonic

development [205]. In our studies EphrinB2/EphB4 ratio in hESC-EC and hiPSC-EC revealed that endothelial cells may stand closer to arterial phenotype. Although arterial endothelial markers were expressed also on HUVEC cells, their regulatory profile suggested venous phenotype, due to the low EphrinB2/EphB4 ratio. Lymphatic endothelial cells develop later from the venous network [176]. Lymphatic markers were never detected in my experiments, suggesting that hESC-EC and hiPSC-EC stand on early maturing steps of developmental procedure, as lymphatic endothelial cells develop only late from venous cells.

During vasculogenesis, tip arterial endothelial cells are the main regulators of arch development. It has so far been proven that the VEGF/Tie-2 and Notch1 signalling pathways trigger tip cells to initiate development of novel branches during sprouting angiogenesis [206]. Stalk cells are bystanders in this process. Arterial endothelial marker DLL4 is abundantly expressed in tip cells during vasculogenesis in the zebrafish [204]. After VEGF signalling on tip cells, DLL4 expression increases and activates Notch signalling in stalk cells to enhance development of functional branches. In my experiments strong expression of DLL4 on hESC-EC and hiPSC-EC suggested that endothelial specification occurs without environmental signal. Culturing pure arterial endothelial cells and using them for further tissue engineering experiments may further enhance the therapeutic applications of hESC-EC and hiPSC-EC. Since arterial endothelial cells are more atheroprotective and venous endothelial cells are more atheroprone, arterial endothelial cells would be preferable in many clinical scenarios. According to other studies VEGF signalling ought to induce arterial development *in vivo* [206]. In my endothelial differentiation studies, a strong external VEGF signal did not result in significantly higher levels of arterial endothelial cells compared to other differentiation protocols. These results suggest that signalling pathways responsible for this process have altered mechanisms of action *in vitro*. This finding proves on the one hand that HUVEC is a neonatal endothelial cell line with high plasticity. On the other hand, it suggests that altered environmental circumstances may influence HUVEC phenotype and gene expression during culturing *in vitro*. According to latest data from the literature, signalling through the VEGF-DLL4-Notch1 pathway has a pathological role on endothelial progenitor cells in preeclampsia [207]. Furthermore DLL4 is targeted in cancer therapy to prevent neoangiogenesis on immature vasculature of tumour tissue [208]. DLL4 inhibition results in EphrinB2

downregulation and inhibition of neoangiogenesis in mouse tumour models [209]. These findings enhance the potential therapeutic use of DLL4/EphrinB2 blockade in human malignant diseases. Understanding their regulatory role in hESC-EC and hiPSC-EC is crucial to evaluate their underlying role in future therapies.

6.3. Regulatory mechanisms of endothelial differentiation, behaviour and specification via the PI3K/FOXO1A signalling pathway

Earlier studies emphasised that the VEGF and PI3K/ERK/MAPK signalling pathway may have distinct regulatory roles in endothelial development [210]. Activation of the ERK pathway resulted in strong upregulation of arterial differentiation, while activation of the PI3K/Akt pathway resulted in downstream of arterial specification [211]. Here we showed that the PI3K/ERK/MAPK signalling pathway has regulatory role on human pluripotent stem cells-derived endothelial cells as well. Upregulation of FOXO1A, resulted in significant decrease in all endothelial marker genes. Levels of general endothelial markers and lineage specific arterial and venous endothelial markers were decreased. FOXO1A upregulation also resulted in robust decrease in arterial and venous endothelial ratio (EphrinB2:EphB4). Members of FOXO family have transcriptional role on regulation of vasculo- and angiogenesis and endothelial behaviour [176].

In this study I showed that PI3K pathway is one of the key intracellular signalling mechanisms which have wide-ranging effects on endothelial differentiation of hESC as well as cell death, proliferation and angiogenic activity of generated hESC-EC partially through FOXO1A transcription factor. The role of PI3K in generating new endothelial cells was supported by our observation that hESC differentiation towards endothelial lineage is accompanied with marked changes in expression of most of the genes related to PI3K/FOXO1A pathways. FOXO1A was shown to be the most abundant FOXO member at mRNA levels in undifferentiated hESC colonies and alteration of FOXO1A causes different expression of pluripotency genes in hESC, indicating that FOXO1A has a critical role in the regulation of hESC fate [212]. Indeed, here we showed that high FOXO1A expression was significantly downregulated during embryoid body formation and further differentiation into endothelial cells. Changes in expression also correlated with loss of pluripotency markers. By using a gene expression array of PI3K pathway elements,

Ingenuity Pathway Analysis with a curated database and subsequent clustering as an exploratory tool, we identified connections between FOXO1A and VEGF as well as various further angiogenic factors. Time development during differentiation modulated the expression of angiogenic factors clustered with FOXO1A such as PDK1 which play a role in vascular remodelling and endothelial differentiation [213]; cdc42, known to mediate tubulogenesis [214]; protein kinase C beta, being a stimulus for endothelial proliferation [215]; and activation of Rheb/mTOR for endothelial cell transformation [216]. As a potential result of these interactions, we found that inhibition of PI3K and consequent re-activation of FOXO1A by LY294002 resulted in an increased number of newly formed endothelial cells. Higher FOXO1A levels were accompanied with increasing CD31 and angiopoietin-2 expression levels in the differentiating culture. This suggests an indirect link between FOXO1A levels and the propensity of differentiating hESC towards endothelial lineage. However, the fact that the control endothelial HUVEC expressed FOXO1A at a higher level than hESC-EC suggests that post-transcriptional control of FOXO1A is more important in HUVEC than those in hESC-EC. The role of FOXO1A in endothelial development was further evidenced by siRNA silencing experiments. Directly targeting FOXO1A factor decreased expression of endothelial markers and angiogenesis genes. This also suggests that culture medium containing VEGF and other endothelial cytokines may be potent stimulus for endothelial differentiation where PI3K/FOXO1A pathway, at least in part, mediates these signals. This is similar to other studies, where VEGF was shown to increase the yield of adult endothelial cells in differentiated hESC culture [217] and can also favour endothelial cell survival, proliferation and cell cycle progression via the PI3K/Akt pathway during tissue regeneration and disease [218, 219]. Intact PI3K signalling as well as low but detectable FOXO1A levels may be prerequisite for endothelial differentiation. However, our data suggest a different regulatory role for PI3K/FOXO1A in differentiating ESC and ESC-derived endothelial cells where FOXO1A has mainly inhibitory feedback signal. We characterized the temporal expression of FOXO1A and angiopoietin-2. Angiopoietin-2 is important target gene for FOXO1A [220-222] and related to angiogenesis and vascular remodelling, during *in vivo* differentiation and maturation of hESC-EC. Three weeks after transplantation of hESC-EC into athymic nude rats, cells showed engraftment and were detectable with histology. As opposed to HUVEC, we detected an increase in angiopoietin-2 expression, with no significant change

in FOXO1A levels in hESC-EC. *In vitro* differentiation of hESC generated a unique endothelial cell type, where FOXO1A levels are significantly lower than those in control HUVEC, endothelial cells derived from human umbilical cord vein. This may suggest that these cells retain low FOXO1A levels and a controllable angiogenic activity even after *in vivo* conditioning. On contrary, we found that activation of FOXO1A by LY294002 blocked tube formation in culture. This activity may be further modulated by the component that inhibition of PI3K and consequent activation of FOXO1A by LY294002 was also found to block proliferation of hESC-EC. In line with this, earlier study showed that overexpression of FOXO1 inhibited endothelial tube formation and migration partly via direct inhibition of endothelial nitric oxide synthase [223]. FOXO proteins were shown to be involved in response to oxidative stress, in regulation of apoptosis and cell survival [223-225]. In line with these reports, here we showed that FOXO1A mediates danger signals such as oxidative stress by H_2O_2 in hESC-EC. Indeed, activation of FOXO1A nuclear translocation by H_2O_2 was accompanied with cell loss, necrosis and nuclear remodelling in a dose-dependent manner. Furthermore, overexpression of FOXO1A construct showed direct pro-necrotic effects and cell loss in hESC-EC cultures. The fact that FOXO1A expression levels stayed low during cell transplantation may suggests that these cells have lower responsiveness to *in vivo* danger signals. On the other hand, we found that silencing of FOXO1A had no protective effect on stress responsiveness of hESC-EC *in vitro*, which altogether suggests the presence of a broader regulation involving other, FOXO1A-independent pathways in stress-responsiveness in hESC-EC. LY294002 is one of the potent and specific cell-permeable inhibitor of PI3K [226]. We have found that inhibition of PI3K pathway by LY294002 further increased loss of hESC-EC in oxidative stress. This observation may be in line with earlier animal studies where development of dermal toxicity was an *in vivo* side effect of LY294002 in murine model [227]; together with low solubility and bioavailability prevented its use in clinical trials. However, several further compounds are being developed to inhibit different nodes of the PI3K pathway. These mainly PI3K/Akt/mTOR inhibitors showed no unexpected toxic effects [228]. It is still unclear whether downregulation of PI3K signalling will be sufficient to produce a clinical response. In conclusion, human embryonic stem cell-derived endothelial cells represent a unique endothelial population, with controllable proliferative and angiogenic activities. PI3K/FOXO1A pathway is one of the particular key

signalling elements for function and survival of hESC-EC but also in regulation of endothelial cell fate. We found that activated FOXO1A has various effects in hESC-EC: it mediates endothelial generation; on the other hand, FOXO1A, at least partly, inhibits proliferation and angiogenic activity, and plays a role in oxidative stress. To test whether using of cells along with direct pharmacological inhibition of FOXO to release negative feedback may be advantageous in cell therapy still need to be determined. Role of PI3K/FOXO1A on endothelial development and function is summarized on Figure 43.

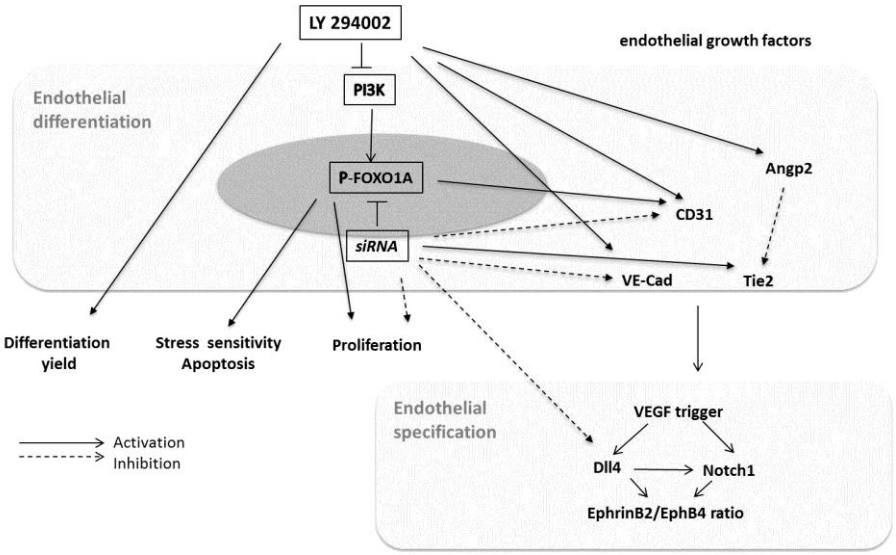


Figure 43. Summary figure of endothelial differentiation and specification Figure shows modulation of PI3K/FOXO1A pathway during endothelial differentiation and regulatory signals of arterial and venous endothelial specification. PI3K: phosphatidylinositol-4, 5-bisphosphate 3-kinase, P-FOXO1A: phosphorylated-FOXO1A, Angp2: Angiopoietin-2

6.4. Limitations

Limitations of my studies are including limited number of available human pluripotent stem cell lines. My experiments were performed on three pluripotent stem cell lines (hESC line H7 (WiCell) and hiPSC lines IMR90-40 (WiCell) and ReproCell line. A more robust understanding of endothelial development may require further pluripotent stem cell lines. Human IPS lines from patient with cardiovascular diseases may enhance detailed observations on pathophysiological steps *in vitro*.

Endothelial differentiation procedure was performed *in vitro* in 2D and 3D cell culture circumstances. Investigation of signalling pathways and endothelial subpopulations was performed in 2D and 3D endothelial differentiation conditions *in vitro*, which are unable to entirelly mimic *in vivo* conditions during embryonic development of vessels.

Use of hiPSC for research purposes solves ethical concerns about hESC. Although, hiPSC research need wise considerations, as genetic and epigenetic modifications may occur during reprogramming procedure.

Many studies reported that hPSC derivatives possess different characteristics than adult counterpart cell types. Thus, *in vitro* disease modelling and drug testing also need to be thoroughly overviewed.

According to our results, hPSC derivatives do not present an adult, mature cell type, albeit they get through maturation during *in vivo* conditioning. More complex transplantation procedure (e.g. into the hear)t would be informative to gain information on organotypic endothelial cell production.

Equivalence assessment of hESC-EC, hiPSC-EC, HUVEC, HAEC, HCAEC, BOEC and other endothelial cells from different sources is needed.

7. Conclusions

Cardiovascular derivatives of human pluripotent stem cells pave the way towards cell therapy in cardiovascular diseases.

During my PhD studies endothelial derivatives of hESC-EC and hiPSC-EC were studied. First pluripotent fate of hESC and hiPSC were featured. Reprogramming human adult fibroblast to pluripotent state was performed, resulting hiPSC. This result facilitate future patient specific disease modelling, drug testing *in vitro*. Next endothelial differentiation procedures were investigated to optimise endothelial differentiation in vitro. Efficient differentiation protocols are required to develop mature endothelial cells. In my studies cell culture conditions, effects of normoxia and hypoxia were investigated**.** Understanding endothelial differentiation conditions enhances establishment of efficient amount and functional of endothelial cell.

Detailed endothelial characteristics were obtained to study phenotype, immoncytochemical profile, secretion and expression of angiogenesis-related proteins and endothelial gene expression profile. General endothelial markers, arterial and venous endothelial subpopulations were also analysed. Further arterial and venous endothelial ratio (EphrinB2/EphB4) was studied. Understanding endothelial subpopulations enhance cell therapy efforts, regarding distinct functional activity of arterial and venous endothelial cells. Arterial and venous phenotype may differ on single cell properties, furthermore endothelial cells may behave different and form distinct subpopulations *in vitro*. Role of the PI3K/FOXO1A signalling pathway was studied on endothelial differentiation, proliferation, survival, angiogenesis, arterial and venous subpopulations. Results proved that the PI3K/FOXO1A pathway have significant role on these. Activation of FOXO1A resulted in increased CD31 positive endothelial cells yield after differentiation, decreased endothelial proliferation rate and inhibited tube formation on Matrigel. Inhibition of FOXO1A decreased CD31 and angiopoietin-2 mRNA levels, increased proliferative activity and increased tube formation on hESC-EC (Figure 44.).

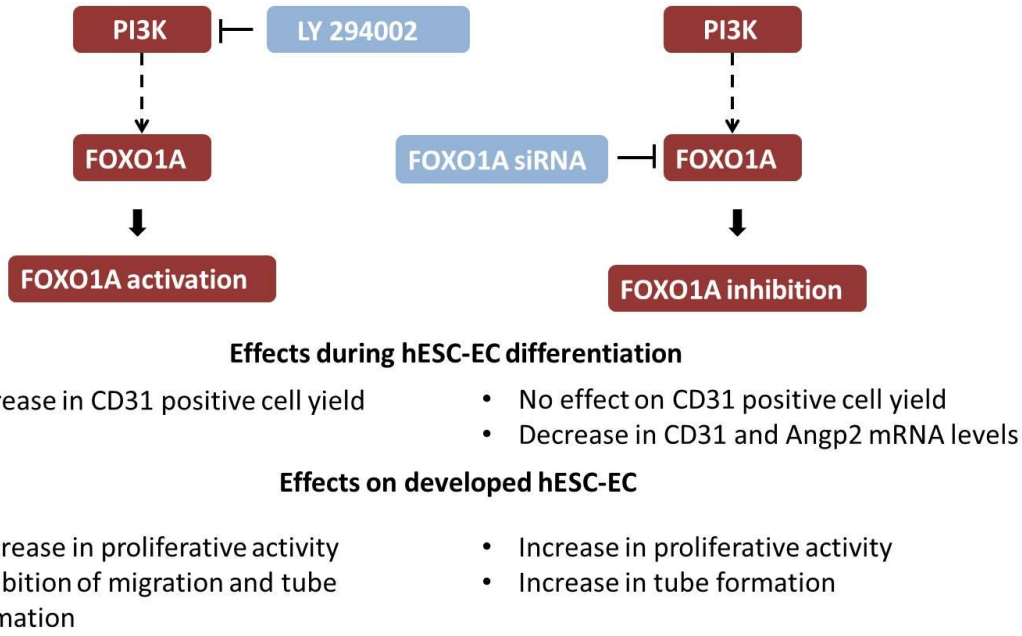


Figure 44. Modulation of PI3K/FOXO1A signalling pathway Summary figure shows effects of FOXO1A activation via LY 294002 and FOXOA1 inhibition via FOXO1A siRNA treatment on hESC-EC differentiation, proliferation and tube formation. PI3K: phosphatidylinositol-4, 5-bisphosphate 3-kinase
(original figure is from Edit Gara)

8. Summary

Endothelial derivatives of human pluripotent stem cells may offer regenerative treatments in ischemic cardiovascular diseases. We studied role of PI3K/FOXO1A pathways during differentiation, proliferation, maturation and cell death on hPSC-EC. Endothelial differentiation conditions were optimised. Both hESC-EC and hiPSC-EC showed mature endothelial characteristics such as cobblestone pattern, ac-LDL uptake, and tube formation *in vitro*. During differentiation expression of FOXO1A transcription factor was linked to the expression of a cluster of angiogenesis- and vascular remodeling-related genes. PI3K inhibitor (LY294002) induced formation of CD31 positive cells. In contrast, differentiating with silenced FOXO1A showed lower mRNA levels of CD31 and angiopoietin-2. LY294002 decreased proliferative activity of purified hESC-EC, while FOXO1A siRNA increased their proliferation. LY294002 inhibits migration and tube formation; in contrast, FOXO1A siRNA increased *in vitro* tube formation activity. Proteome profiling revealed high abundance of angiogenesis-related proteins in the cell lysates and supernatant. Expressions of arterial (EphrinB2, Notch1-2) and venous (EphB4) endothelial markers were increased, suggesting the presence of mixed endothelial population in culture. qRT-PCR analyses in transfected cells proved that universal and specific arterial and venous endothelial marker genes were downregulated in high FOXO1A group. For engineering 3D vascular constructs biomatrices were repopulated with hESC-EC and hiPSC-EC. Cells remained viable on engineered matrices. Imaging with Calcein AM live staining and 3D Histech analysis proved monolayer-like CD31 positive, live endothelial culture on biomatrices. After *in vivo* conditioning of cells in athymic nude rats, cells retain their low FOXO1A expression levels. Expression levels of general endothelial marker genes and arterial and venous endothelial markers increased during *in vivo* conditioning. PI3K/FOXO1A pathway is important for function and survival of hESC-EC/hiPSC-EC and in the regulation of endothelial cell fate. In-depth analyses of hESC-EC and hiPSC-EC, regarding phenotype specification and functional characteristics may enhance their application for preclinical vascular tissue engineering purposes.

Összefoglalás

A pluripotens őssejtek kardiovaszkuláris származékai ígéretes lehetőséget jelentenek az iszkémiás kardiovaszkuláris betegségek sejtterápiás gyógyításában. Munkám során a PI3K/FOXO1A jelátviteli út szabályozó szerepét vizsgáltam az őssejt eredetű endothelsejtek differenciációjában, proliferációjában, érésében és sejthalálozásban. A humán embrionális és indukált pluripotens őssejt eredetű endothelsejtek (hESC-EC, hiPSC-EC) felnőtt endothelsejtekre jellemző tulajdonságokkal rendelkeztek: utcakő rajzolat *in vitro,* ac-LDL felvétel és tubulus képzés. Szoros összefüggést találtunk a FOXO1A transzkripciós faktor és angiogenezishez kapcsolódó transzkripciós faktorok expressziójában az endothel differenciáció során. A PI3K gátlás (LY294002) növelte a CD31 pozitív sejtek arányát a differenciáció során. A FOXO1A csendesítés csökkentette a CD31 és angiopoietin-2 mRNS szinteket hESC-EC és hiPSC-EC sejtekben. LY294002 csökkentette a proliferációs képességet, FOXO1A siRNS kezelés ugyanakkor növelte a sejtek proliferációját. LY294002 gátolta a sejtmigrációt és tubulus formaló képességet, FOXO1A siRNS kezelés növelte a tubulus képző aktivitást. A sejtek fehérje profil vizsgálata igazolta, hogy a hESC-EC és hiPSC-EC sejtek nagy mennyiségben expresszálnak és szekretálnak angiogenezishez köthető fehérjéket. A hESC-EC és hiPSC-EC sejtek egyaránt expresszáltak artériás (EphrinB2) és vénás (EphB4, Notch1-2) endotheliális markereket. Eredményeim vegyes, artériás és vénás endothel populációk jelenlétére utalnak. FOXO1A transzfekció hatására az általános és artériás, vénás endothel markerek szintje szignifikánsan csökkent.

hESC-EC és hiPSC-EC sejteket 3D környezetben vizsgáltam extracelluláris mátrixon tenyésztve. A sejtek életképesek maradtak és funkcionális aktivitással rendelkeztek a 3D környezetben, így például thrombocyta aggregáció gátló hatásuk fokozódott a 3D sejttenyésztési környezetben. A hESC-EC és hiPSC-EC sejtek *in vivo* transzplantációja során emelkedtek az álalános és artériás, vénás endothel markerek szintjei. A PI3K/FOXO1A jelátviteli út jelentős szerepet játszik a hESC-EC és hiPSC-EC sejtek differenciációjában, fenotípusában és funkcionális tulajdonságaiban. A hESC-EC és hiPSC-EC sejtek tulajdonságainak részletes vizsgálata előmozdítja a sejtek jövőbeni terápiás felhasználását szövetépítési eljárások céljából.

9. Reference list

1. Behfar A, Terzic A. (2015) Stem cells versus senescence: the yin and yang of cardiac health. J Am Coll Cardiol, 65(2):148-150.
2. Fernandez-Hernandez I, Rhiner C. (2015) New neurons for injured brains? The emergence of new genetic model organisms to study brain regeneration. Neurosci Biobehav Rev, 56:62-72.
3. Chen GL, Paplham P, McCarthy PL. (2014) Remestemcel-L for acute graft-versus-host disease therapy. Expert Opin Biol Ther, 14(2):261-269.
4. Petranyi G, Masszi T, Timar L, Krivan G, Paloczi K, Nagy K,Fekete S, Reményi P, Torbágyi É, Dénes R, Kelemen E. (1996) The bone-marrow transplantation program in Hungary: report from the period 1990-1995. Orv Hetil, 137(40):2203-2208.
5. Alper J. (2009) Geron gets green light for human trial of ES cell-derived product. Nat Biotechnol, 27(3):213-214.
6. Chapman AR, Scala CC. (2012) Evaluating the first-in-human clinical trial of a human embryonic stem cell-based therapy. Kennedy Inst Ethics J, 22(3):243-261.
7. Mountford J, Olivier E, Turner M. (2010) Prospects for the manufacture of red cells for transfusion. Br J Haematol, 149(1):22-34.
8. Bartunek J, Behfar A, Vanderheyden M, Wijns W, Terzic A. (2008) Mesenchymal stem cells and cardiac repair: principles and practice. J Cardiovasc Transl Res, 1(2):115-119.
9. Mountford JC, Turner M. (2011) In vitro production of red blood cells. Transfus Apher Sci, 45(1):85-89.
10. Mountford JC, Olivier E, Jordanides NE, de SP, Turner ML. (2010) Red blood cells from pluripotent stem cells for use in transfusion. Regen Med, 5(3):411-423.
11. Schmitt J, Eckardt S, Schlegel PG, Siren AL, Bruttel VS, Mclaughlin KJ, , Wischhusen J, Müller AM. (2015) Human parthenogenetic embryonic stem cell-derived neural stem cells express HLA-G and show unique resistance to NK cell-mediated killing. Mol Med, 23;21:185-96.
12. Maynard KM, Arvindam U, Cross M, Firpo MT. (2014) Potentially immunogenic proteins expressed similarly in human embryonic stem cells and induced pluripotent stem cells. Exp Biol Med, 239(4):484-488.

13. Reardon S, Cyranoski D. (2014) Japan stem-cell trial stirs envy. Nature, 513(7518):287-288.
14. Sun J, Mandai M, Kamao H, Hashiguchi T, Shikamura M, Kawamata S, Sugita S, Takahashi M. (2015) Protective Effects of Human iPS-Derived Retinal Pigmented Epithelial Cells in Comparison with Human Mesenchymal Stromal Cells and Human Neural Stem Cells on the Degenerating Retina in rd1 mice. Stem Cells, 33(5):1543-1553.
15. Garber K. (2015) RIKEN suspends first clinical trial involving induced pluripotent stem cells. Nat Biotechnol, 33(9):890-891.
16. Olivetti G, Capasso JM, Sonnenblick EH, Anversa P. (1990) Side-to-side slippage of myocytes participates in ventricular wall remodeling acutely after myocardial infarction in rats. Circ Res, 67(1):23-34.
17. Madonna R, De CR. (2015) Circulating Endothelial Progenitor Cells: Do they live up to their name? Vascul Pharmacol, 67-69:2-5.
18. Alexandru N, Andrei E, Dragan E, Georgescu A. (2015) Interaction of platelets with endothelial progenitor cells in the experimental atherosclerosis: Role of transplanted endothelial progenitor cells and platelet microparticles. Biol Cell, 107(6):189-204.
19. Rafii S. (2000) Circulating endothelial precursors: mystery, reality, and promise. J Clin Invest, 105(1):17-19.
20. Sugimoto T, Hosomi N, Nezu T, Takahashi T, Aoki S, Takeda I, Mukai T, Ochi K, Kitamura T, Ohtsuki T, Matsumoto M. (2015) CD34+/CD144+ Circulating Endothelial Cells as an Indicator of Carotid Atherosclerosis. J Stroke Cerebrovasc Dis, 24(3):583-590.
21. Yao L, Heuser-Baker J, Herlea-Pana O, Barlic-Dicen J. (2013) Bone marrow endothelial progenitors in atherosclerotic plaque resolution. Organogenesis, 9(1):29-33.
22. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science, 275(5302):964-967.

23. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. (2003) Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med, 348(7):593-600.
24. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM, Dimmeler S. (2001) Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res, 89(1):E1-E7.
25. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D, Yoder MC. (2004) Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood, 104(9):2752-2760.
26. Aoki M, Yasutake M, Murohara T. (2004) Derivation of functional endothelial progenitor cells from human umbilical cord blood mononuclear cells isolated by a novel cell filtration device. Stem Cells, 22(6):994-1002.
27. Fadini GP, Rigato M, Boscari F, Cappellari R, Menegazzo L, Pilutti C, Iori E, Marescotti M Plebani M, Albiero M, Avogaro A. (2014) Short-term statin discontinuation increases endothelial progenitor cells without inflammatory rebound in type 2 diabetic patients. Vascul Pharmacol, 67-69:21-9.
28. Friedenstein AJ, Chailakhjan RK, Lalykina KS. (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet, 3(4):393-403.
29. Ren J, Wang H, Tran K, Civini S, Jin P, Castiello L, Feng J, Kuznetsov SA, Robey PG, Sabatino M, Stroncek DF. (2015) Human bone marrow stromal cell confluence: effects on cell characteristics and methods of assessment. Cytotherapy, 17(7):897-911.
30. Sabatino M, Ren J, David-Ocampo V, England L, McGann M, Tran M, Kuznetsov SA, Khuu H, Balakumaran A, Klein HG, Robey PG, Stroncek DF. (2012) The establishment of a bank of stored clinical bone marrow stromal cell products. J Transl Med, 10:23.
31. Dominici M, Le BK, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. (2006) Minimal criteria for defining

multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy, 8(4):315-317.

32. Wu KH, Tsai C, Wu HP, Sieber M, Peng CT, Chao YH. (2013) Human application of ex-vivo expanded umbilical cord-derived mesenchymal stem cells: enhance hematopoiesis after cord blood transplantation. Cell Transplant, 22(11):2041-51.
33. Karantalis V, Hare JM. (2015) Use of Mesenchymal Stem Cells for Therapy of Cardiac Disease. Circ Res, 116(8):1413-1430.
34. Frangogiannis NG. (2012) Regulation of the inflammatory response in cardiac repair. Circ Res, 110(1):159-173.
35. Majumdar MK, Keane-Moore M, Buyaner D, Hardy WB, Moorman MA, McIntosh KR, Mosca JD. (2003) Characterization and functionality of cell surface molecules on human mesenchymal stem cells. J Biomed Sci, 10(2):228-241.
36. Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, Noiseux N, Zhang L, Pratt RE, Ingwall JS, Dzau VJ. (2005) Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med, 11(4):367-368.
37. Herrmann JL, Wang Y, Abarbanell AM, Weil BR, Tan J, Meldrum DR. (2010) Preconditioning mesenchymal stem cells with transforming growth factor-alpha improves mesenchymal stem cell-mediated cardioprotection. Shock, 33(1):24-30.
38. Mirotsou M, Zhang Z, Deb A, Zhang L, Gnecchi M, Noiseux N, Mu H, Pachori A, Dzau V. (2007) Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. Proc Natl Acad Sci, 104(5):1643-1648.
39. Rogers TB, Pati S, Gaa S, Riley D, Khakoo AY, Patel S, Wardlow RD 2nd, Frederick CA, Hall G, He LP, Lederer WJ. (2011) Mesenchymal stem cells stimulate protective genetic reprogramming of injured cardiac ventricular myocytes. J Mol Cell Cardiol, 50(2):346-356.
40. Cieslik KA, Trial J, Entman ML. (2015) Mesenchymal stem cell-derived inflammatory fibroblasts promote monocyte transition into myeloid fibroblasts via an IL-6-dependent mechanism in the aging mouse heart. FASEB J, (8):3160-70.

41. Nigro P, Perrucci GL, Gowran A, Zanobini M, Capogrossi MC, Pompilio G. (2015) c-kit(+) cells: the tell-tale heart of cardiac regeneration? Cell Mol Life Sci, 72(9):1725-1740.
42. Nadal-Ginard B, Ellison GM, Torella D. (2014) The cardiac stem cell compartment is indispensable for myocardial cell homeostasis, repair and regeneration in the adult. Stem Cell Res, 13:615-630.
43. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisén J. (2009) Evidence for cardiomyocyte renewal in humans. Science, 324(5923):98-102.
44. Bergmann O, Zdunek S, Frisen J, Bernard S, Druid H, Jovinge S. (2012) Cardiomyocyte renewal in humans. Circ Res, 110(1):17-18.
45. Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu TD, Guerquin-Kern JL, Lechene CP, Lee RT. (2013) Mammalian heart renewal by pre-existing cardiomyocytes. Nature, 493(7432):433-436.
46. Lennartsson J, Blume-Jensen P, Hermanson M, Ponten E, Carlberg M, Ronnstrand L. (1999) Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-kit mediated activation of the Ras/MAP kinase pathway and c-fos induction. Oncogene, 18(40):5546-5553.
47. Lev S, Givol D, Yarden Y. (1992) Interkinase domain of kit contains the binding site for phosphatidylinositol 3' kinase. Proc Natl Acad Sci U S A, 89(2):678-682.
48. Trieselmann NZ, Soboloff J, Berger SA. (2003) Mast cells stimulated by membrane-bound, but not soluble, steel factor are dependent on phospholipase C activation. Cell Mol Life Sci, 60(4):759-766.
49. Di MF, Castaldo C, Nurzynska D, Romano V, Miraglia R, Bancone C, Langella G, Vosa C, Montagnani S. (2010) Epithelial-mesenchymal transition of epicardial mesothelium is a source of cardiac CD117-positive stem cells in adult human heart. J Mol Cell Cardiol, 49(5):719-727.
50. Zhang Y, Li TS, Lee ST, Wawrowsky KA, Cheng K, Galang G, Malliaras K, Abraham MR, Wang C, Marbán E. (2010) Dedifferentiation and proliferation of mammalian cardiomyocytes. PLoS One, 5(9):e12559.
51. Deb A, Wang S, Skelding KA, Miller D, Simper D, Caplice NM. (2003) Bone marrow-derived cardiomyocytes are present in adult human heart: A study of

gender-mismatched bone marrow transplantation patients. Circulation, 107(9):1247-1249.

52. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, Kajstura J, Leri A, Anversa P. (2002) Chimerism of the transplanted heart. N Engl J Med, 346(1):5-15.

53. Bolli R, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, Beache GM, Wagner SG, Leri A, Hosoda T, Sanada F, Elmore JB, Goichberg P, Cappetta D, Solankhi NK, Fahsah I, Rokosh DG, Slaughter MS, Kajstura J, Anversa P. (2011) Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet, 378(9806):1847-1857.

54. Cheng K, Malliaras K, Li TS, Sun B, Houde C, Galang G, Smith J, Matsushita N, Marbán E. (2012) Magnetic enhancement of cell retention, engraftment, and functional benefit after intracoronary delivery of cardiac-derived stem cells in a rat model of ischemia/reperfusion. Cell Transplant, 21(6):1121-1135.

55. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. (1998) Embryonic stem cell lines derived from human blastocysts. Science, 282(5391):1145-1147.

56. Allegrucci C, Young LE. (2007) Differences between human embryonic stem cell lines. Hum Reprod Update, 13(2):103-120.

57. Fraga AM, de Araujo ES, Stabellini R, Vergani N, Pereira LV. (2012) Establishment of new lines of human embryonic stem cells: evolution of the methodology. Methods Mol Biol, 873:1-12.

58. Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, Zucker JP, Wang S, Morton CC, McMahon AP, Powers D, Melton DA. (2004) Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med, 350(13):1353-1356.

59. Meng G, Liu S, Li X, Krawetz R, Rancourt DE. (2010) Derivation of human embryonic stem cell lines after blastocyst microsurgery. Biochem Cell Biol, 88(3):479-490.

60. Tanaka N, Takeuchi T, Neri QV, Sills ES, Palermo GD. (2006) Laser-assisted blastocyst dissection and subsequent cultivation of embryonic stem cells in a

serum/cell free culture system: applications and preliminary results in a murine model. J Transl Med, 4:20.

61. Johnson MH. (2008) Human ES cells and a blastocyst from one embryo: exciting science but conflicting ethics? Cell Stem Cell, 2(2):103-104.
62. Chung Y, Klimanskaya I, Becker S, Li T, Maserati M, Lu SJ, Zdravkovic T, Ilic D, Genbacev O, Fisher S, Krtolica A, Lanza R. (2008) Human embryonic stem cell lines generated without embryo destruction. Cell Stem Cell, 2(2):113-117.
63. Ilic D, Giritharan G, Zdravkovic T, Caceres E, Genbacev O, Fisher SJ, Krtolica A. (2009) Derivation of human embryonic stem cell lines from biopsied blastomeres on human feeders with minimal exposure to xenomaterials. Stem Cells Dev, 18(9):1343-1350.
64. Klimanskaya I, Chung Y, Becker S, Lu SJ, Lanza R. (2006) Human embryonic stem cell lines derived from single blastomeres. Nature, 444(7118):481-485.
65. Desai N, Rambhia P, Gishto A. (2015) Human embryonic stem cell cultivation: historical perspective and evolution of xeno-free culture systems. Reprod Biol Endocrinol, 13(1):9.
66. Adams KL, Rousso DL, Umbach JA, Novitch BG. (2015) Foxp1-mediated programming of limb-innervating motor neurons from mouse and human embryonic stem cells. Nat Commun, 6:6778.
67. Ma MS, Boddeke E, Copray S. (2015) Pluripotent stem cells for schwann cell engineering. Stem Cell Rev, 11(2):205-218.
68. Inoue Y, Hasegawa S, Yamada T, Date Y, Mizutani H, Nakata S, Matsunaga K, Akamatsu H. (2013) Analysis of the effects of hydroquinone and arbutin on the differentiation of melanocytes. Biol Pharm Bull, 36(11):1722-1730.
69. Tadeu AM, Lin S, Hou L, Chung L, Zhong M, Zhao H, Horsley V. (2015) Transcriptional profiling of ectoderm specification to keratinocyte fate in human embryonic stem cells. PLoS One, 10(4):e0122493.
70. Erdelyi-Belle B, Torok G, Apati A, Sarkadi B, Schaff Z, Kiss A, Homolya L. (2015) Expression of Tight Junction Components in Hepatocyte-Like Cells Differentiated from Human Embryonic Stem Cells. Pathol Oncol Res, 21(4):1059-70.

71. Sinagoga KL, Wells JM. (2015) Generating human intestinal tissues from pluripotent stem cells to study development and disease. EMBO J, 5;34(9):1149-63.

72. Teo AK, Tsuneyoshi N, Hoon S, Tan EK, Stanton LW, Wright CV, Dunn NR. (2015) PDX1 Binds and represses hepatic genes to ensure robust pancreatic commitment in differentiating human embryonic stem cells. Stem Cell Reports, 4(4):578-590.

73. Samadikuchaksaraei A, Cohen S, Isaac K, Rippon HJ, Polak JM, Bielby RC, Bishop AE. (2006) Derivation of distal airway epithelium from human embryonic stem cells. Tissue Eng, 12(4):867-875.

74. Piazzi M, Williamson A, Lee CF, Pearson S, Lacaud G, Kouskoff V,McCubrey JA, Cocco L, Whetton AD. (2015) Quantitative phosphoproteome analysis of embryonic stem cell differentiation toward blood. Oncotarget, 6(13):10924-39.

75. Hawkins F, Kotton DN. (2015) Embryonic and induced pluripotent stem cells for lung regeneration. Ann Am Thorac Soc, 12 S1:S50-S53.

76. Hafner AL, Dani C. (2014) Human induced pluripotent stem cells: A new source for brown and white adipocytes. World J Stem Cells, 6(4):467-472.

77. Zhao H, Wen G, Huang Y, Yu X, Chen Q, Afzal TA, Luong le A, Zhu J, Shu Y, Zhang L, Xiao Q (2015) MicroRNA-22 Regulates Smooth Muscle Cell Differentiation From Stem Cells by Targeting Methyl CpG-Binding Protein 2. Arterioscler Thromb Vasc Biol, 35(4):918-929.

78. Paige SL, Plonowska K, Xu A, Wu SM. (2015) Molecular regulation of cardiomyocyte differentiation. Circ Res, 116(2):341-353.

79. Merkely B, Gara E, Lendvai Z, Skopal J, Leja T, Zhou W, Kosztin A, Várady G, Mioulane M, Bagyura Z, Németh T, Harding SE, Földes G. (2015) Signaling Via PI3K/FOXO1A Pathway Modulates Formation and Survival of Human Embryonic Stem Cell-Derived Endothelial Cells. Stem Cells Dev, 24(7):869-878.

80. Yamanaka S, Takahashi K. (2006) Induction of pluripotent stem cells from mouse fibroblast cultures. Tanpakushitsu Kakusan Koso, 51(15):2346-2351.

81. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell, 131(5):861-872.

82. Smith-Arica JR, Thomson AJ, Ansell R, Chiorini J, Davidson B, McWhir J. (2003) Infection efficiency of human and mouse embryonic stem cells using adenoviral and adeno-associated viral vectors. Cloning Stem Cells, 5(1):51-62.
83. McLaren A. (2000) Cloning: pathways to a pluripotent future. Science, 288(5472):1775-1780.
84. Amati B, Alevizopoulos K, Vlach J. (1998) Myc and the cell cycle. Front Biosci, 3:d250-d268.
85. Patel M, Yang S. (2010) Advances in reprogramming somatic cells to induced pluripotent stem cells. Stem Cell Rev, 6(3):367-380.
86. Gonzalez F, Boue S, Izpisua Belmonte JC. (2011) Methods for making induced pluripotent stem cells: reprogramming a la carte. Nat Rev Genet, 12(4):231-242.
87. Ban H, Nishishita N, Fusaki N, Tabata T, Saeki K, Shikamura M, Takada N, Inoue M, Hasegawa M, Kawamata S, Nishikawa S. (2011) Efficient generation of transgene-free human induced pluripotent stem cells (iPSCs) by temperature-sensitive Sendai virus vectors. Proc Natl Acad Sci, 108(34):14234-14239.
88. Talluri TR, Kumar D, Glage S, Garrels W, Ivics Z, Debowski K, Behr R, Kues WA. (2014) Non-viral reprogramming of fibroblasts into induced pluripotent stem cells by Sleeping Beauty and piggyBac transposons. Biochem Biophys Res Commun, 450(1):581-587.
89. Grabundzija I, Wang J, Sebe A, Erdei Z, Kajdi R, Devaraj A, Steinemann D, Szuhai K, Stein U, Cantz T, Schambach A, Baum C, Izsvák Z, Sarkadi B, Ivics Z. (2013) Sleeping Beauty transposon-based system for cellular reprogramming and targeted gene insertion in induced pluripotent stem cells. Nucleic Acids Res, 41(3):1829-1847.
90. Driscoll CB, Tonne JM, El KM, Cattaneo R, Ikeda Y, Devaux P. (2015) Nuclear reprogramming with a non-integrating human RNA virus. Stem Cell Res Ther, 6(1):48.
91. Hu W, He Y, Xiong Y, Lu H, Chen H, Hou L, Qiu Z, Fang Y, Zhang S. (2015) Derivation, expansion, andmotor neuron differentiation of human-induced pluripotent stem cells with non-integrating episomal vectors and a defined xenogeneic-free culture system. Mol Neurobiol,12035-014-9084.

92. Hussein SM, Puri MC, Tonge PD, Benevento M, Corso AJ, Clancy JL, Mosbergen R, Li M, Lee DS, Cloonan N, Wood DL, Munoz J, Middleton R,Korn O, Patel HR, White CA, Shin JY, Gauthier ME, Lê Cao KA, Kim JI, Mar JC, Shakiba N, Ritchie W, Rasko JE, Grimmond SM, Zandstra PW, Wells CA, Preiss T, Seo JS, Heck AJ, Rogers IM, Nagy A. (2014) Genome-wide characterization of the routes to pluripotency. Nature, 516(7530):198-206.

93. Stamm C, Kleine HD, Choi YH, Dunkelmann S, Lauffs JA, Lorenzen B, David A, Liebold A, Nienaber C, Zurakowski D, Freund M, Steinhoff G. (2007) Intramyocardial delivery of CD133+ bone marrow cells and coronary artery bypass grafting for chronic ischemic heart disease: safety and efficacy studies. J Thorac Cardiovasc Surg, 133(3):717-725.

94. Losordo DW, Schatz RA, White CJ, Udelson JE, Veereshwarayya V, Durgin M, Poh KK, Weinstein R, Kearney M, Chaudhry M, Burg A, Eaton L, Heyd L,Thorne T, Shturman L, Hoffmeister P, Story K, Zak V, Dowling D, Traverse JH, Olson RE, Flanagan J, Sodano D, Murayama T, Kawamoto A, Kusano KF, Wollins J, Welt F, Shah P, Soukas P, Asahara T, Henry TD. (2007) Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina: a phase I/IIa double-blind, randomized controlled trial. Circulation, 115(25):3165-3172.

95. Vanderheyden M, Vercauteren S, Mansour S, Delrue L, Vandekerckhove B, Heyndrickx GR, Van Haute I, De Bruyne B, Timmermans F, Wijns W, Bartunek J. (2007) Time-dependent effects on coronary remodeling and epicardial conductance after intracoronary injection of enriched hematopoietic bone marrow stem cells in patients with previous myocardial infarction. Cell Transplant, 16(9):919-925.

96. Erbs S, Linke A, Adams V, Lenk K, Thiele H, Diederich KW, Emmrich F, Kluge R, Kendziorra K, Sabri O, Schuler G, Hambrecht R. (2005) Transplantation of blood-derived progenitor cells after recanalization of chronic coronary artery occlusion: first randomized and placebo-controlled study. Circ Res, 97(8):756-762.

97. Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schümichen C, Nienaber CA, Freund M, Steinhoff G. (2003) Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet, 361(9351):45-46.

98. Pavo N, Charwat S, Nyolczas N, Jakab A, Murlasits Z, Bergler-Klein J, Nikfardjam M, Benedek I, Benedek T, Pavo IJ, Gersh BJ, Huber K, Maurer G, Gyöngyösi M.

(2014) Cell therapy for human ischemic heart diseases: critical review and summary of the clinical experiences. J Mol Cell Cardiol, 75:12-24.

99. Dimmeler S, Burchfield J, Zeiher AM. (2008) Cell-based therapy of myocardial infarction. Arterioscler Thromb Vasc Biol, 28(2):208-216.

100. Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Forfang K. (2005) Autologous stem cell transplantation in acute myocardial infarction: The ASTAMI randomized controlled trial. Intracoronary transplantation of autologous mononuclear bone marrow cells, study design and safety aspects. Scand Cardiovasc J, 39(3):150-158.

101. Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Egeland T, Endresen K, Ilebekk A, Mangschau A, Fjeld JG, Smith HJ, Taraldsrud E, Grøgaard HK,Bjørnerheim R, Brekke M, Müller C, Hopp E, Ragnarsson A, Brinchmann JE, Forfang K. (2006) Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. N Engl J Med, 355(12):1199-1209.

102. Gyongyosi M, Wojakowski W, Lemarchand P, Lunde K, Tendera M, Bartunek J, Marban E, Assmus B, Henry TD, Traverse JH, Moyé LA, Sürder D, Corti R,Huikuri H, Miettinen J, Wöhrle J, Obradovic S, Roncalli J, Malliaras K, Pokushalov E, Romanov A, Kastrup J, Bergmann MW, Atsma DE, Diederichsen A, Edes I,Benedek I, Benedek T, Pejkov H, Nyolczas N, Pavo N, Bergler-Klein J, Pavo IJ, Sylven C, Berti S, Navarese EP, Maurer G; ACCRUE Investigators. (2015) Meta-Analysis of Cell-based CaRdiac stUdiEs (ACCRUE) in Patients With Acute Myocardial Infarction Based on Individual Patient Data. Circ Res, 116(8):1346-1360.

103. Janssens S, Dubois C, Bogaert J, Theunissen K, Deroose C, Desmet W, Kalantzi M, Herbots L, Sinnaeve P, Dens J, Maertens J, Rademakers F, Dymarkowski S, Gheysens O, Van Cleemput J, Bormans G, Nuyts J, Belmans A, Mortelmans L, Boogaerts M, Van de Werf F. (2006) Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. Lancet, 367(9505):113-121.

104. Bockeria L, Bogin V, Bockeria O, Le T, Alekyan B, Woods EJ,Brown AA, Ichim TE, Patel AN. (2013) Endometrial regenerative cells for treatment of heart failure: a new stem cell enters the clinic. J Transl Med, 11:56.

105. Hristov M, Heussen N, Schober A, Weber C. (2006) Intracoronary infusion of autologous bone marrow cells and left ventricular function after acute myocardial infarction: a meta-analysis. J Cell Mol Med, 10(3):727-733.
106. Lipinski MJ, Biondi-Zoccai GG, Abbate A, Khianey R, Sheiban I, Bartunek J, Vanderheyden M, Kim HS, Kang HJ, Strauer BE, Vetrovec GW. (2007) Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials. J Am Coll Cardiol, 50(18):1761-1767.
107. Martin-Rendon E, Brunskill SJ, Hyde CJ, Stanworth SJ, Mathur A, Watt SM. (2008) Autologous bone marrow stem cells to treat acute myocardial infarction: a systematic review. Eur Heart J, 29(15):1807-1818.
108. Abdel-Latif A, Bolli R, Tleyjeh IM, Montori VM, Perin EC, Hornung CA, Zuba-Surma EK, Al-Mallah M, Dawn B. (2007) Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. Arch Intern Med, 167(10):989-997.
109. Kang S, Yang YJ, Li CJ, Gao RL. (2008) Effects of intracoronary autologous bone marrow cells on left ventricular function in acute myocardial infarction: a systematic review and meta-analysis for randomized controlled trials. Coron Artery Dis, 19(5):327-335.
110. Crespo-Diaz R, Yamada S, Bartunek J, Perez-Terzic C, de WP, Mauen S, Terzic A, Behfar A. (2015) Cardiopoietic index predicts heart repair fitness of patient-derived stem cells. Biomark Med, 9(7):639-49.
111. Shabbir A, Coz A, Rodriguez L, Salgado M, Badiavas E. (2015) Mesenchymal stem cell exosomes induce the proliferation and migration of normal and chronic wound fibroblasts, and enhance angiogenesis in vitro. Stem Cells Dev, 24(14):1635-47.
112. Liang X, Ding Y, Zhang Y, Tse HF, Lian Q. (2014) Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. Cell Transplant, 23(9):1045-1059.
113. Bartunek J, Behfar A, Dolatabadi D, Vanderheyden M, Ostojic M, Dens J, El Nakadi B, Banovic M, Beleslin B, Vrolix M, Legrand V, Vrints C, Vanoverschelde JL, Crespo-Diaz R, Homsy C, Tendera M, Waldman S, Wijns W, Terzic A. (2013)

Cardiopoietic stem cell therapy in heart failure: the C-CURE (Cardiopoietic stem Cell therapy in heart failURE) multicenter randomized trial with lineage-specified biologics. J Am Coll Cardiol, 61(23):2329-2338.

114. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marbán E. (2007) Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation, 115(7):896-908.
115. Takehara N, Tsutsumi Y, Tateishi K, Ogata T, Tanaka H, Ueyama T, Takahashi T, Takamatsu T, Fukushima M, Komeda M, Yamagishi M, Yaku H, Tabata Y,Matsubara H, Oh H. (2008) Controlled delivery of basic fibroblast growth factor promotes human cardiosphere-derived cell engraftment to enhance cardiac repair for chronic myocardial infarction. J Am Coll Cardiol, 52(23):1858-1865.
116. Winter EM, Grauss RW, Hogers B, van TJ, van der Geest R, Lie-Venema H, Steijn RV, Maas S, DeRuiter MC, deVries AA, Steendijk P, Doevendans PA,van der Laarse A, Poelmann RE, Schalij MJ, Atsma DE, Gittenberger-de Groot AC. (2007) Preservation of left ventricular function and attenuation of remodeling after transplantation of human epicardium-derived cells into the infarcted mouse heart. Circulation, 116(8):917-927.
117. Malliaras K, Makkar RR, Smith RR, Cheng K, Wu E, Bonow RO, Marbán L, Mendizabal A, Cingolani E, Johnston PV, Gerstenblith G, Schuleri KH, Lardo AC, Marbán E. (2014) Intracoronary cardiosphere-derived cells after myocardial infarction: evidence of therapeutic regeneration in the final 1-year results of the CADUCEUS trial (CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction). J Am Coll Cardiol, 63(2):110-122.
118. Wang X, Wei G, Yu W, Zhao Y, Yu X, Ma X. (2006) Scalable producing embryoid bodies by rotary cell culture system and constructing engineered cardiac tissue with ES-derived cardiomyocytes in vitro. Biotechnol Prog, 22(3):811-818.
119. Zandstra PW, Bauwens C, Yin T, Liu Q, Schiller H, Zweigerdt R, Pasumarthi KB, Field LJ. (2003) Scalable production of embryonic stem cell-derived cardiomyocytes. Tissue Eng, 9(4):767-778.
120. van Laake LW, van Donselaar EG, Monshouwer-Kloots J, Schreurs C, Passier R, Humbel BM, Doevendans PA, Sonnenberg A, Verkleij AJ, Mummery CL. (2010)

Extracellular matrix formation after transplantation of human embryonic stem cell-derived cardiomyocytes. Cell Mol Life Sci, 67(2):277-290.

121. Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S, O'Sullivan C, Collins L, Chen Y,Minami E, Gill EA, Ueno S, Yuan C, Gold J, Murry CE. (2007) Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol, 25(9):1015-1024.

122. Menard C, Hagege AA, Agbulut O, Barro M, Morichetti MC, Brasselet C, Bel A, Messas E, Bissery A, Bruneval P, Desnos M, Pucéat M, Menasché P. (2005) Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study. Lancet, 366(9490):1005-1012.

123. Kolossov E, Bostani T, Roell W, Breitbach M, Pillekamp F, Nygren JM, Sasse P, Rubenchik O, Fries JW, Wenzel D, Geisen C, Xia Y, Lu Z, Duan Y, Kettenhofen R, Jovinge S, Bloch W, Bohlen H, Welz A, Hescheler J, Jacobsen SE, Fleischmann BK. (2006) Engraftment of engineered ES cell-derived cardiomyocytes but not BM cells restores contractile function to the infarcted myocardium. J Exp Med, 203(10):2315-2327.

124. Shiba Y, Fernandes S, Zhu WZ, Filice D, Muskheli V, Kim J, Palpant NJ, Gantz J, Moyes KW, Reinecke H, Van Biber B, Dardas T, Mignone JL, Izawa A, Hanna R, Viswanathan M, Gold JD, Kotlikoff MI, Sarvazyan N, Kay MW, Murry CE, Laflamme MA. (2012) Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts. Nature, 489(7415):322-325.

125. Chong JJ, Yang X, Don CW, Minami E, Liu YW, Weyers JJ, Mahoney WM, Van Biber B, Cook SM, Palpant NJ, Gantz JA, Fugate JA, Muskheli V,Gough GM, Vogel KW, Astley CA, Hotchkiss CE, Baldessari A, Pabon L, Reinecke H, Gill EA, Nelson V, Kiem HP, Laflamme MA, Murry CE. (2014) Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. Nature, 510(7504):273-277.

126. Chong JJ, Murry CE. (2014) Cardiac regeneration using pluripotent stem cells--progression to large animal models. Stem Cell Res, 13:654-665.

127. Rubach M, Adelmann R, Haustein M, Drey F, Pfannkuche K, Xiao B, Koester A, Udink ten Cate FE, Choi YH, Neef K, Fatima A, Hannes T, Pillekamp

F,Hescheler J, Šarić T, Brockmeier K, Khalil M. (2014) Mesenchymal stem cells and their conditioned medium improve integration of purified induced pluripotent stem cell-derived cardiomyocyte clusters into myocardial tissue. Stem Cells Dev, 23(6):643-653.

128. Menasche P, Vanneaux V, Hagege A, Bel A, Cholley B, Cacciapuoti I, Parouchev A, Benhamouda N, Tachdjian G, Tosca L, Trouvin JH,Fabreguettes JR, Bellamy V, Guillemain R, Suberbielle Boissel C, Tartour E, Desnos M, Larghero J. (2015) Human embryonic stem cell-derived cardiac progenitors for severe heart failure treatment: first clinical case report. Eur Heart J, 36(30):2011-2017.
129. Moretti A, Bellin M, Welling A, Jung CB, Lam JT, Bott-Flugel L, Dorn T, Goedel A, Höhnke C, Hofmann F, Seyfarth M, Sinnecker D, Schömig A, Laugwitz KL. (2010) Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N Engl J Med, 363(15):1397-1409.
130. Itzhaki I, Maizels L, Huber I, Zwi-Dantsis L, Caspi O, Winterstern A, Feldman O, Gepstein A, Arbel G, Hammerman H, Boulos M, Gepstein L. (2011) Modelling the long QT syndrome with induced pluripotent stem cells. Nature, 471(7337):225-229.
131. Matsa E, Rajamohan D, Dick E, Young L, Mellor I, Staniforth A, Denning C. (2011) Drug evaluation in cardiomyocytes derived from human induced pluripotent stem cells carrying a long QT syndrome type 2 mutation. Eur Heart J, 32(8):952-962.
132. Cerrone M, Lin X, Zhang M, Agullo-Pascual E, Pfenniger A, Chkourko GH, Novelli V, Kim C, Tirasawadichai T, Judge DP, Rothenberg E, Chen HS,Napolitano C, Priori SG, Delmar M. (2014) Missense mutations in plakophilin-2 cause sodium current deficit and associate with a Brugada syndrome phenotype. Circulation, 129(10):1092-1103.
133. Caspi O, Huber I, Gepstein A, Arbel G, Maizels L, Boulos M, Gepstein L. (2013) Modeling of arrhythmogenic right ventricular cardiomyopathy with human induced pluripotent stem cells. Circ Cardiovasc Genet, 6(6):557-568.
134. Masumoto H, Ikuno T, Takeda M, Fukushima H, Marui A, Katayama S, Shimizu T, Ikeda T, Okano T, Sakata R, Yamashita JK. (2014) Human iPS cell-engineered

cardiac tissue sheets with cardiomyocytes and vascular cells for cardiac regeneration. Sci Rep, 4:6716.

135. Eschenhagen T, Mummery C, Knollmann BC. (2015) Modelling sarcomeric cardiomyopathies in the dish: from human heart samples to iPSC cardiomyocytes. Cardiovasc Res, 105(4):424-438.

136. Vunjak NG, Eschenhagen T, Mummery C. (2014) Myocardial tissue engineering: in vitro models. Cold Spring Harb Perspect Med, 4(3).

137. Burridge PW, Diecke S, Matsa E, Sharma A, Wu H, Wu JC. (2015) Modeling Cardiovascular Diseases with Patient-Specific Human Pluripotent Stem Cell-Derived Cardiomyocytes. Methods Mol Biol, 1353:119-30.

138. Fairchild PJ. (2009) Transplantation tolerance in an age of induced pluripotency. Curr Opin Organ Transplant, 14(4):321-325.

139. Sivarapatna A, Ghaedi M, Le AV, Mendez JJ, Qyang Y, Niklason LE. (2015) Arterial specification of endothelial cells derived from human induced pluripotent stem cells in a biomimetic flow bioreactor. Biomaterials, 53:621-633.

140. Conradi L, Schmidt S, Neofytou E, Deuse T, Peters L, Eder A, Hua X, Hansen A, Robbins RC, Beygui RE, Reichenspurner H, Eschenhagen T,Schrepfer S. (2015) Immunobiology of Fibrin-Based Engineered Heart Tissue. Stem Cells Transl Med, 4(6):625-631.

141. Ghazanfari S, Driessen-Mol A, Strijkers GJ, Baaijens FP, Bouten CV. (2015)The evolution of collagen fiber orientation in engineered cardiovascular tissues visualized by diffusion tensor imaging. PLoS One, 10(5):e0127847.

142. Martins AM, Eng G, Caridade SG, Mano JF, Reis RL, Vunjak-Novakovic G. (2014) Electrically conductive chitosan/carbon scaffolds for cardiac tissue engineering. Biomacromolecules, 15(2):635-643.

143. Atluri P, Miller JS, Emery RJ, Hung G, Trubelja A, Cohen JE, Lloyd K, Han J, Gaffey AC, MacArthur JW, Chen CS, Woo YJ. (2014) Tissue-engineered, hydrogel-based endothelial progenitor cell therapy robustly revascularizes ischemic myocardium and preserves ventricular function. J Thorac Cardiovasc Surg, 148(3):1090-1097.

144. Robertson MJ, Dries-Devlin JL, Kren SM, Burchfield JS, Taylor DA. (2014) Optimizing recellularization of whole decellularized heart extracellular matrix. PLoS One, 9(2):e90406.

145. Sanchez PL, Fernandez-Santos ME, Costanza S, Climent AM, Moscoso I, Gonzalez-Nicolas MA, Sanz-Ruiz R, Rodríguez H, Kren SM, Garrido G,Escalante JL, Bermejo J, Elizaga J, Menarguez J, Yotti R, Pérez del Villar C, Espinosa MA, Guillem MS, Willerson JT, Bernad A, Matesanz R, Taylor DA, Fernández-Avilés F. (2015) Acellular human heart matrix: A critical step toward whole heart grafts. Biomaterials, 61:279-289.

146. Taylor DA. (2009) From stem cells and cadaveric matrix to engineered organs. Curr Opin Biotechnol, 20(5):598-605.

147. Taylor DA, Caplan AL, Macchiarini P. (2014) Ethics of bioengineering organs and tissues. Expert Opin Biol Ther, 14(7):879-882.

148. Weymann A, Patil NP, Sabashnikov A, Jungebluth P, Korkmaz S, Li S, Veres G, Soos P, Ishtok R, Chaimow N, Pätzold I, Czerny N, Schies C,Schmack B, Popov AF, Simon AR, Karck M, Szabo G. (2014) Bioartificial heart: a human-sized porcine model--the way ahead. PLoS One, 9(11):e111591.

149. Alavi SH, Kheradvar A. (2015) A hybrid tissue-engineered heart valve. Ann Thorac Surg, 99(6):2183-2187.

150. Lanuti P, Serafini F, Pierdomenico L, Simeone P, Bologna G, Ercolino E, Di Silvestre S, Guarnieri S, Canosa C, Impicciatore GG, Chiarini S,Magnacca F, Mariggiò MA, Pandolfi A, Marchisio M, Di Giammarco G, Miscia S. (2015) Human mesenchymal stem cells reendothelialize porcine heart valve scaffolds: novel perspectives in heart valve tissue engineering. Biores Open Access, 4(1):288-297.

151. Puceat M. (2013) Embryological origin of the endocardium and derived valve progenitor cells: from developmental biology to stem cell-based valve repair. Biochim Biophys Acta, 1833(4):917-922.

152. Simpson DL, Wehman B, Galat Y, Sharma S, Mishra R, Galat V, Sharma S, Mishra R, Galat V, Kaushal S. (2014) Engineering patient-specific valves using stem cells generated from skin biopsy specimens. Ann Thorac Surg, 98(3):947-954.

153. Jana S, Lerman A. (2015) Bioprinting a cardiac valve. Biotechnol Adv, S0734-9750(15)30019-7.
154. Wilczek P, Major R, Lipinska L, Lackner J, Mzyk A. (2015) Thrombogenicity and biocompatibility studies of reduced graphene oxide modified acellular pulmonary valve tissue. Mater Sci Eng C Mater Biol Appl, 53:310-321.
155. Duan B, Hockaday LA, Das S, Xu C, Butcher JT. (2015) Comparison of Mesenchymal Stem Cell Source Differentiation Toward Human Pediatric Aortic Valve Interstitial Cells within 3D Engineered Matrices. Tissue Eng Part C Methods, 21(8):795-807.
156. Reimer JM, Syedain ZH, Haynie BH, Tranquillo RT. (2015) Pediatric tubular pulmonary heart valve from decellularized engineered tissue tubes. Biomaterials, 62:88-94.
157. Zafar F, Hinton RB, Moore RA, Baker RS, Bryant R, III, Narmoneva DA, Taylor MD, Morales DL. (2015) Physiological growth, remodeling potential, and preserved function of a novel bioprosthetic tricuspid valve: tubular bioprosthesis made of small intestinal submucosa-derived extracellular matrix. J Am Coll Cardiol, 66(8):877-888.
158. Cantu DA, Hematti P, Kao WJ. (2012) Cell encapsulating biomaterial regulates mesenchymal stromal/stem cell differentiation and macrophage immunophenotype. Stem Cells Transl Med, 1(10):740-749.
159. Eder A, Vollert I, Hansen A, Eschenhagen T. (2015) Human engineered heart tissue as a model system for drug testing. Adv Drug Deliv Rev,96:214-24.
160. Hirt MN, Boeddinghaus J, Mitchell A, Schaaf S, Bornchen C, Muller C, Schulz H, Hubner N, Stenzig J, Stoehr A, Neuber C, Eder A, Luther PK,Hansen A, Eschenhagen T. (2014) Functional improvement and maturation of rat and human engineered heart tissue by chronic electrical stimulation. J Mol Cell Cardiol, 74:151-161.
161. Hirt MN, Hansen A, Eschenhagen T. (2014) Cardiac tissue engineering: state of the art. Circ Res, 114(2):354-367.
162. Vollert I, Seiffert M, Bachmair J, Sander M, Eder A, Conradi L, Vogelsang A, Schulze T, Uebeler J, Holnthoner W, Redl H, Reichenspurner H, Hansen

A,Eschenhagen T. (2014) In vitro perfusion of engineered heart tissue through endothelialized channels. Tissue Eng Part A, 20(3-4):854-863.

163. Zimmermann WH, Tiburcy M, Eschenhagen T. (2007) Cardiac tissue engineering: a clinical perspective. Future Cardiol, 3(4):435-445.
164. Jopling C, Boue S, Izpisua Belmonte JC. (2011) Dedifferentiation, transdifferentiation and reprogramming: three routes to regeneration. Nat Rev Mol Cell Biol, 12(2):79-89.
165. Sleep E, Boue S, Jopling C, Raya M, Raya A, Izpisua Belmonte JC. (2010) Transcriptomics approach to investigate zebrafish heart regeneration. J Cardiovasc Med, 11(5):369-380.
166. Hodgkinson CP, Kang MH, Dal-Pra S, Mirotsou M, Dzau VJ. (2015) MicroRNAs and Cardiac Regeneration. Circ Res, 116(10):1700-1711.
167. Thum T, Condorelli G. (2015) Long noncoding RNAs and microRNAs in cardiovascular pathophysiology. Circ Res, 116(4):751-762.
168. Harrison DG, Widder J, Grumbach I, Chen W, Weber M, Searles C. (2006) Endothelial mechanotransduction, nitric oxide and vascular inflammation. J Intern Med, 259(4):351-363.
169. Celermajer DS. (1997) Endothelial dysfunction: does it matter? Is it reversible? J Am Coll Cardiol, 30(2):325-333.
170. Zuurbier CJ, Demirci C, Koeman A, Vink H, Ince C. (2005) Short-term hyperglycemia increases endothelial glycocalyx permeability and acutely decreases lineal density of capillaries with flowing red blood cells. J Appl Physiol, 99(4):1471-1476.
171. Yoder MC. (2015) Differentiation of pluripotent stem cells into endothelial cells. Curr Opin Hematol, 22(3):252-257.
172. Furchgott RF, Zawadzki JV. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature, 288(5789):373-376.
173. Ignarro LJ, Byrns RE, Buga GM, Wood KS, Chaudhuri G. (1988) Pharmacological evidence that endothelium-derived relaxing factor is nitric oxide: use of pyrogallol and superoxide dismutase to study endothelium-dependent and nitric oxide-elicited vascular smooth muscle relaxation. J Pharmacol Exp Ther, 244(1):181-189.

174. Blobaum AL, Marnett LJ. (2007) Structural and functional basis of cyclooxygenase inhibition. J Med Chem, 50(7):1425-1441.
175. Thengchaisri N, Hein TW, Ren Y, Kuo L. (2015) Endothelin-1 impairs coronary arteriolar dilation: Role of p38 kinase-mediated superoxide production from NADPH oxidase. J Mol Cell Cardiol, 86:75-84.
176. De VS. (2011) Key transcriptional regulators of early vascular development. Arterioscler Thromb Vasc Biol, 31(7):1469-1475.
177. Sacilotto N, Monteiro R, Fritzsche M, Becker PW, Sanchez-Del-Campo L, Liu K, Pinheiro P, Ratnayaka I, Davies B, Goding CR, Patient R, Bou-Gharios G, De Val S. (2013) Analysis of Dll4 regulation reveals a combinatorial role for Sox and Notch in arterial development. Proc Natl Acad Sci , 110(29):11893-11898.
178. Rocha SF, Adams RH. (2009) Molecular differentiation and specialization of vascular beds. Angiogenesis, 12(2):139-147.
179. Engelman JA, Luo J, Cantley LC. (2006) The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet, 7(8):606-619.
180. Rodon J, Dienstmann R, Serra V, Tabernero J. (2013) Development of PI3K inhibitors: lessons learned from early clinical trials. Nat Rev Clin Oncol, 10(3):143-153.
181. Furman RR, Sharman JP, Coutre SE, Cheson BD, Pagel JM, Hillmen P, Barrientos JC, Zelenetz AD, Kipps TJ, Flinn I, Ghia P, Eradat H, Ervin T, Lamanna N,Coiffier B, Pettitt AR, Ma S, Stilgenbauer S, Cramer P, Aiello M, Johnson DM, Miller LL, Li D, Jahn TM, Dansey RD, Hallek M, O'Brien SM. (2014) Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. N Engl J Med, 370(11):997-1007.
182. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell, 96(6):857-868.
183. Pajvani UB, Accili D. (2015) The new biology of diabetes. Diabetologia, 58(11):2459-68.
184. Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM. (2002) Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature, 419(6904):316-321.

185. Nakamura T, Sakamoto K. (2008) Forkhead transcription factor FOXO subfamily is essential for reactive oxygen species-induced apoptosis. Mol Cell Endocrinol, 281(1-2):47-55.

186. Potente M, Urbich C, Sasaki K, Hofmann WK, Heeschen C, Aicher A, Kollipara R, DePinho RA, Zeiher AM, Dimmeler S. (2005) Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. J Clin Invest, 115(9):2382-2392.

187. Furuyama T, Kitayama K, Shimoda Y, Ogawa M, Sone K, Yoshida-Araki K, Hisatsune H, Nishikawa S, Nakayama K, Nakayama K, Ikeda K, Motoyama N, Mori N. (2004) Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. J Biol Chem, 279(33):34741-34749.

188. Hall CL, Dai J, van Golen KL, Keller ET, Long MW. (2006) Type I collagen receptor (alpha 2 beta 1) signaling promotes the growth of human prostate cancer cells within the bone. Cancer Res, 66(17):8648-8654.

189. Martin-Ramirez J, Hofman M, van den Biggelaar M, Hebbel RP, Voorberg J. (2012) Establishment of outgrowth endothelial cells from peripheral blood. Nat Protoc, 7(9):1709-1715.

190. Starke RD, Paschalaki KE, Dyer CE, Harrison-Lavoie KJ, Cutler JA, McKinnon TA, Millar CM, Cutler DF, Laffan MA, Randi AM. (2013) Cellular and molecular basis of von Willebrand disease: studies on blood outgrowth endothelial cells. Blood, 121(14):2773-2784.

191. Weymann A, Loganathan S, Takahashi H, Schies C, Claus B, Hirschberg K, Soós P, Korkmaz S, Schmack B, Karck M, Szabó G. (2011) Development and evaluation of a perfusion decellularization porcine heart model-generation of 3-dimensional myocardial neoscaffolds. Circ J, 75(4):852-860.

192. Weymann A, Patil NP, Sabashnikov A, Korkmaz S, Li S, Soos P, Ishtok R, Chaimow N, Pätzold I, Czerny N, Schmack B, Popov AF, Simon AR,Karck M, Szabo G. (2015) Perfusion-Decellularization of Porcine Lung and Trachea for Respiratory Bioengineering. Artif Organs, 39(12):1024-32.

193. Weymann A, Schmack B, Okada T, Soos P, Istok R, Radovits T, Straub B, Barnucz E, Loganathan S, Pätzold I, Chaimow N, Schies C, Korkmaz S, Tochtermann

U, Karck M, Szabó G. (2013) Reendothelialization of human heart valve neoscaffolds using umbilical cord-derived endothelial cells. Circ J, 77(1):207-216.

194. Weymann A, Radovits T, Schmack B, Korkmaz S, Li S, Chaimow N, Pätzold I, Becher PM, Hartyánszky I, Soós P, Merkely G, Németh BT, Istók R,Veres G, Merkely B, Terytze K, Karck M, Szabó G. (2014) Total aortic arch replacement: superior ventriculo-arterial coupling with decellularized allografts compared with conventional prostheses. PLoS One, 9(7):e103588.
195. Weymann A, Radovits T, Schmack B, Li S, Korkmaz S, Soos P, Istók R, Veres G, Chaimow N, Karck M, Szabó G. (2014) In vitro generation of atrioventricular heart valve neoscaffolds. Artif Organs, 38(7):E118-E128.
196. Mioulane M, Foldes G, Ali NN, Schneider MD, Harding SE. (2012) Development of high content imaging methods for cell death detection in human pluripotent stem cell-derived cardiomyocytes. J Cardiovasc Transl Res, 5(5):593-604.
197. Potter CM, Lundberg MH, Harrington LS, Warboys CM, Warner TD, Berson RE, Moshkov AV, Gorelik J, Weinberg PD, Mitchell JA. (2011) Role of shear stress in endothelial cell morphology and expression of cyclooxygenase isoforms. Arterioscler Thromb Vasc Biol, 31(2):384-391.
198. Jackson RS, Voss SG, Wilson ZC, Remmes NB, Stalboerger PG, Keeney MG, Moore EJ, Janus JR. (2015) An athymic rat model for mandibular osteoradionecrosis allowing for direct translation of regenerative treatments. Otolaryngol Head Neck Surg, 153(4):526-31.
199. Reed DM, Foldes G, Kirkby NS, Ahmetaj-Shala B, Mataragka S, Mohamed NA, Francis C, Gara E, Harding SE, Mitchell JA. (2014) Morphology and vasoactive hormone profiles from endothelial cells derived from stem cells of different sources. Biochem Biophys Res Commun, 455(3-4):172-177.
200. Debreczeni B, Gara E, Veresh Z, Marki A, Racz A, Matics R, Hamar J, Koller A (2013) Hydrogen peroxide via thromboxane A2 receptors mediates myogenic response of small skeletal muscle veins in rats. Clin Hemorheol Microcirc, 54(4):393-407.
201. Long H, Xiang T, Qi W, Huang J, Chen J, He L, Liang Z, Guo B, Li Y, Xie R, Zhu B. (2015) CD133+ ovarian cancer stem-like cells promote non-stem cancer cell

metastasis via CCL5 induced epithelial-mesenchymal transition. Oncotarget, 6(8):5846-5859.

202. Liu GT, Huang YL, Tzeng HE, Tsai CH, Wang SW, Tang CH. (2015) CCL5 promotes vascular endothelial growth factor expression and induces angiogenesis by down-regulating miR-199a in human chondrosarcoma cells. Cancer Lett, 357(2):476-487.

203. Corada M, Morini MF, Dejana E. (2014) Signaling pathways in the specification of arteries and veins. Arterioscler Thromb Vasc Biol, 34(11):2372-2377.

204. De VS, Chi NC, Meadows SM, Minovitsky S, Anderson JP, Harris IS, Ehlers ML, Agarwal P, Visel A, Xu SM, Pennacchio LA, Dubchak I, Krieg PA, Stainier DY, Black BL. (2008) Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. Cell, 135(6):1053-1064.

205. Lindskog H, Kim YH, Jelin EB, Kong Y, Guevara-Gallardo S, Kim TN, Wang RA. (2014) Molecular identification of venous progenitors in the dorsal aorta reveals an aortic origin for the cardinal vein in mammals. Development, 141(5):1120-1128.

206. Lanner F, Sohl M, Farnebo F. (2007) Functional arterial and venous fate is determined by graded VEGF signaling and notch status during embryonic stem cell differentiation. Arterioscler Thromb Vasc Biol, 27(3):487-493.

207. Liu X, Luo Q, Zheng Y, Liu X, Hu Y, Wang F, Zou L. (2015) The role of Delta-like 4 ligand/Notch-ephrin-B2 cascade in the pathogenesis of preeclampsia by regulating functions of endothelial progenitor cell. Placenta, 36(9):1002-10.

208. Liu YR, Guan YY, Luan X, Lu Q, Wang C, Liu HJ, Gao YG, Yang SC, Dong X, Chen HZ, Fang C. (2015) Delta-like ligand 4-targeted nanomedicine for antiangiogenic cancer therapy. Biomaterials, 42:161-171.

209. Yamanda S, Ebihara S, Asada M, Okazaki T, Niu K, Ebihara T, Koyanagi A, Yamaguchi N, Yagita H, Arai H. (2009) Role of ephrinB2 in nonproductive angiogenesis induced by Delta-like 4 blockade. Blood, 113(15):3631-3639.

210. Ren B, Deng Y, Mukhopadhyay A, Lanahan AA, Zhuang ZW, Moodie KL, Mulligan-Kehoe MJ, Byzova TV, Peterson RT, Simons M. (2010) ERK1/2-Akt1 crosstalk regulates arteriogenesis in mice and zebrafish. J Clin Invest, 120(4):1217-1228.

211. Deng Y, Larrivee B, Zhuang ZW, Atri D, Moraes F, Prahst C, Eichmann A, Simons M. (2013) Endothelial RAF1/ERK activation regulates arterial morphogenesis. Blood, 121(19):3988-3989.

212. Zhang X, Yalcin S, Lee DF, Yeh TY, Lee SM, Su J, Mungamuri SK, Rimmelé P, Kennedy M, Sellers R, Landthaler M, Tuschl T, Chi NW, Lemischka I, Keller G,Ghaffari S. (2011) FOXO1 is an essential regulator of pluripotency in human embryonic stem cells. Nat Cell Biol, 13(9):1092-1099.

213. Feng Q, Di R, Tao F, Chang Z, Lu S, Fan W, Shan C, Li X, Yang Z. (2010) PDK1 regulates vascular remodeling and promotes epithelial-mesenchymal transition in cardiac development Mol Cell Biol, 30(14):3711-3721.

214. Kesavan G, Sand FW, Greiner TU, Johansson JK, Kobberup S, Wu X, Brakebusch C, Semb H. (2009) Cdc42-mediated tubulogenesis controls cell specification Cell, 139(4):791-801.

215. Meier R, Alessi DR, Cron P, Andjelkovic M, Hemmings BA. (1997) Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bbeta. J Biol Chem, 272(48):30491-30497.

216. Sodhi A, Chaisuparat R, Hu J, Ramsdell AK, Manning BD, Sausville EA, Sawai ET, Molinolo A, Gutkind JS, Montaner S. The (2006) TSC2/mTOR pathway drives endothelial cell transformation induced by the Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor Cancer Cell, 10(2):133-143.

217. Nourse MB, Halpin DE, Scatena M, Mortisen DJ, Tulloch NL, Hauch KD, Torok-Storb B, Ratner BD, Pabon L, Murry CE. (2010) VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering Arterioscler Thromb Vasc Biol, 30(1):80-89.

218. Abid MR, Guo S, Minami T, Spokes KC, Ueki K, Skurk C, Walsh K, Aird WC. (2004) Vascular endothelial growth factor activates PI3K/Akt/forkhead signaling in endothelial cells. Arterioscler Thromb Vasc Biol, 24(2):294-300.

219. Ball SG, Shuttleworth CA, Kielty CM. (2007) Vascular endothelial growth factor can signal through platelet-derived growth factor receptors J Cell Biol, 177(3):489-500.

220. Daly C, Pasnikowski E, Burova E, Wong V, Aldrich TH, Griffiths J, Ioffe E, Daly TJ, Fandl JP, Papadopoulos N, McDonald DM, Thurston G, Yancopoulos

GD,Rudge JS. (2006) Angiopoietin-2 functions as an autocrine protective factor in stressed endothelial cells. Proc Natl Acad Sci, 103(42):15491-15496.

221. van der Vos KE, Coffer PJ. (2011) The extending network of FOXO transcriptional target genes. Antioxid Redox Signal, 14(4):579-592.

222. Hurley D, Araki H, Tamada Y, Dunmore B, Sanders D, Humphreys S, Affara M, Imoto S, Yasuda K, Tomiyasu Y, Tashiro K, Savoie C, Cho V, Smith S, Kuhara S,Miyano S, Charnock-Jones DS, Crampin EJ, Print CG. (2012) Gene network inference and visualization tools for biologists: application to new human transcriptome datasets. Nucleic Acids Res, 40(6):2377-2398.

223. Potente M, Urbich C, Sasaki K, Hofmann WK, Heeschen C, Aicher A, Kollipara R, DePinho RA, Zeiher AM, Dimmeler S. (2005) Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. J Clin Invest, 115(9):2382-2392.

224. Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM. (2002) Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature, 419(6904):316-321.

225. Nakamura T, Sakamoto K. (2008) Forkhead transcription factor FOXO subfamily is essential for reactive oxygen species-induced apoptosis. Mol Cell Endocrinol, 281(1-2):47-55.

226. Arcaro A, Wymann MP. (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem J, 296:297-301.

227. Hu L, Zaloudek C, Mills GB, Gray J, Jaffe RB. (2000) In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002). Clin Cancer Res, 6(3):880-886.

228. Rodon J, Dienstmann R, Serra V, Tabernero J. (2013) Development of PI3K inhibitors: lessons learned from early clinical trials. Nat Rev Clin Oncol, 10(3):143-153.

10. Publications

Publications directly related to the PhD dissertation:

Merkely B*, **Gara E***, Lendvai Z, Skopal J, Leja T, Zhou W, Kosztin A, Varady G, Mioulane M, Bagyura Z, Nemeth T, Harding SE, Foldes G. (2015) Signalling via PI3K/FOXO1A Pathway Modulates Formation and Survival of Human Embryonic Stem Cell-Derived Endothelial Cells. Stem Cells Dev, 24:(7) pp. 869-878.
** Equal contribution*
IF: 3.727

Reed DM, Foldes G, Kirkby NS, Ahmetaj-Shala B, Mataragka S, Mohamed NA, Francis C, **Gara E**, Harding SE, Mitchell JA. (2014) Morphology and vasoactive hormone profiles from endothelial cells derived from stem cells of different sources. Biochem. Biophys. Res. Commun, 455:(3-4) pp. 172-177.
IF: 2.300

Kosztin A*, **Gara E***, Harding SE, Földes G. (2015) Stem Cell Therapy to Treat Heart Failure Reference Module in Biomedical Sciences. London, Elsevier
** Equal contribution*
IF: 0.000

Gara E, Merkely B, Földes G. (2013) Őssejtek és érrendszeri megbetegedések. Hogyan készítsünk ereket? Élet és Tudomány, 68:(15) pp. 470-471.
IF:0.000

Publications not related to the PhD dissertation:

Kovacs A, Tapolyai M, Celeng C, **Gara E**, Faludi M, Berta K, Apor A, Nagy A, Tisler A, Merkely B. (2014) Impact of hemodialysis, left ventricular mass and FGF-23 on myocardial mechanics in end-stage renal disease: a three-dimensional speckle tracking study. International Journal of Cardiovascular Imaging, 30:(7) pp. 1331-1337.
IF: 2.539

Debreczeni B, Veresh Z, **Gara E**, Marki A, Racz A, Matics R, Hamar J, Koller A. (2013) Hydrogen peroxide via thromboxane A_2 receptors mediates myogenic response of small skeletal muscle veins in rats. Clin Hemorheol Microcirc, 54:(4) pp. 393-407.
IF: 2.242

Gara E, Gesztes E, Doroszlai R, Zacher G. (2014) Sürgősségi császármetszés szén-monoxid-mérgezésben. Orvosi Hetilap, 155:(22) pp. 871-875.
IF:0.000

11. Acknowledgements

First of all, I would like to say special thank for my supervisor Dr. Gábor Földes. His strict and wise supervision always encouraged me for enthusiastic and precise scientific work. His international reputation and experience let me reach unique opportunities to study and develop my skills and to build networks with other young researchers. I am grateful for his continuous motivation, encouragement, sage advices and support during my studies.

I am thankful for Professor Béla Merkely for providing unique opportunities to fulfil my PhD studies. The facilities of the Heart and Vascular Centre, Semmelweis University, Budapest offered special circumstances for cardiovascular basic research. Fruitful international collaborations advanced my experiences abroad. Professor Merkely was also my mentor in Kerpel-Fronius Program of Semmelweis University. I am grateful for his generous support and encouragement of my studies.

I am thankful for Dr. Judit Skopál, Dr. Violetta Kékesi and Dr. Tamás Radovits for their useful advices during my studies.

I am also thankful for all colleges from the Stem Cell and Experimental Research Laboratory: Zsuzsanna Lendvai, Dr. Annamária Kosztin, Dr. Kinga Lakatos, Regina Varga, Szilvia Király and Éva Szigetfű.

During my PhD studies I had the opportunity to collaborate in other Hungarian laboratories and abroad also. I am thankful for Professor Sian E. Harding for supporting my academic visits in the National Heart and Lung Institute, Imperial College, London. I am grateful for Professor Gábor Szabó to enable my academic visit at Ruprecht-Karls-Universität, Heidelberg. I am thankful to those who contributed to my studies: Dr. Domokos Máthé, Gábor Kiszler, Domonkos Papp, György Várady, Robert Sampson, Alexander Weymann, Orsolya Vittay and Viktória Gregor.

Last, but not least I am thankful for the support of my Family.

Financial support: European Society of Cardiology, Hungarian Society of Cardiology, Hungarian Research Fund OTKA, Journal of Cell Science, Boehringer Ingelheim Fonds, Kerpel-Fronius Program, Campus Hungary and Semmelweis Erasmus.