Investigation of human embryonic stem cell differentiation towards endothelial lineages.

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Authors Declaration

I declare that this thesis has been written entirely by myself and is a record of work performed by myself with the exception of H9 hESC RNA used to determine ROBO4 expression during hESC-EC differentiation, and generation of the SFFV-GFP control lentivirus (both Dr E. Scott, University of Glasgow). This thesis has not been submitted previously for a higher degree. The research was carried out in the Institute of Cardiovascular and Medical Sciences, University of Glasgow under the supervision of Professor Andrew H. Baker, Dr Joanne Mountford and Dr Stuart Nicklin.

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Summary

Cardiovascular disease represents a significant socio-economic burden and minimally invasive therapies that address the needs of patients suffering with peripheral arterial disease and critical limb ischemia are needed. Cell therapies have been proposed as an alternative to pharmacological and surgical treatments, yet, have demonstrated somewhat limited efficacy. However, the unlimited capacity for self-renewal, and the ability to differentiate into cell types from all three germ layers, including endothelial cell (EC) forming mesoderm, make human embryonic stem cells (hESC) and human induced pluripotency stem cells a promising source for well-defined differentiated cell populations with high angiogenic capacity.

Numerous endothelial differentiation protocols have been published with high differentiation efficiencies achieved recently. However, most of these approaches are not optimised for clinical purposes due to the use of poorly defined, non cGMP compatible reagents, or require additional processing steps, such as cell sorting, complicating the clinical approval of these therapies. Therefore, here it was aimed to develop and optimise a clinically compatible hESC-EC differentiation protocol that avoids using poorly defined reagents, and yields high percentages of cells expressing EC markers without the use of cell sorting.

A novel, serum free hESC-EC differentiation protocol was developed in the lab. The use of Pluronic F-127 well coating was demonstrated as a low cost alternative to low adherence wells. Furthermore, inhibition of TGFB signalling during hESC-EC differentiation to increase the differentiation efficiency was evaluated and did not reveal any additional benefits and thus was not included in the optimised protocol. The optimised protocol consists of embryoid body based mesodermal induction phase, followed by plating and monolayer culture for vascular specification. By day 7, approximately 30% of cells express endothelial markers CD31 and CD144. In addition, transient induction of mesodermal gene, followed by induction of endothelial progenitor and endothelial gene expression was demonstrated, following the expected gene expression patterns.

It was proposed that high throughput screening using hESC lines carrying fluorescent reporter constructs could be used to optimise the differentiation protocol for increased efficiency, thus, avoiding the need of cell sorting prior to therapeutic use. Here, reporter constructs where ETV2, ROBO4 and CDH5 promoter sequence fragments were cloned upstream from florescent reporter sequences were generated and preliminary validation was attempted in NCI60, HUVEC and HSVEC cell lines, and during the hESC-EC differentiation. Reporter gene expression was not observed in any of the validation experiments, suggesting that these constructs were not functional. Similarly, previously published CDH5 and commercially sourced ETV2 reporter constructs were validated during the hESC-EC differentiation. Preliminary testing of these reporter constructs showed non-specific reporter gene expression, therefore, the work with reporter constructs work was not pursued further.

Next, rational targeting of novel signalling pathways that may contribute to the hESC-EC differentiation was employed as an alternative approach for the optimisation of hESC-EC differentiation. Firstly, it was hypothesised that intracellular cAMP levels could be targeted pharmacologically to increase the differentiation efficiency, and to induce expression of arterial and arterial phenotype associated genes, which could reduce the need for cell sorting and deliver arterial cell populations with a superior angiogenic profile. Forskolin treatments induced increased intracellular cAMP levels during the hESC-EC differentiation, yet, this did not result in increased arterial or arterial phenotype associated gene expression. However, an increase in the percentage of cells expressing EC markers was observed in Forskolin treated differentiations, mainly mediated via an increase in the $CD144^{low}$ CD31+ cell population.

Additionally, it was hypothesised that angiotensin II (Ang II) signalling may play a role in hESC-EC differentiation and may be exploited to increase the endothelial differentiation efficiency. Indeed, differential renin angiotensin system receptor expression was demonstrated during hESC-EC differentiation, supporting a role for Ang II signalling in endothelial development. However, no significant differences in the differentiation efficiency and total cell numbers were observed when Ang II and AT_1R antagonist Losartan treatments, in combination

or alone, were applied during the hESC-EC differentiation. In contrast, a significant reduction in total cell numbers and a trend of reduced differentiation efficiency was observed when AT_2R antagonist PD-123319 was used in combination with Ang II. These observations highlight the negative effects of AT_1R signalling during hESC-EC differentiation and show that signalling via AT_2R counterbalances these effects.

In summary, a novel endothelial differentiation protocol was developed and rational selection of signalling pathways for the optimisation of the hESC-EC differentiation was employed. Here, the role of cAMP and Ang II signalling during hESC-EC was demonstrated, highlighting the contribution of various signalling systems to endothelial differentiation. Both of these signalling systems can be easily manipulated in a clinically compliant manner, and therefore represent an attractive target during clinically compatible hESC-EC differentiation. Further research is needed to investigate the underlying mechanisms of the observed effects and to evaluate other, novel signalling pathways that may be targeted to enhance endothelial differentiation. The work described has highlighted the difficulties of establishing efficient, clinically compatible hESC-EC differentiation methods, which are needed to provide highly defined cell populations for future cell therapies and tissue engineering.

Abbreviations

ACE	Angiotensin converting enzyme
AKT	Protein kinase B
ALK	Activin receptor like kinase
Ang II	Angiotensin II
Ang-(1-7)	Angiotensin-(1-7)
ANOVA	Analysis of variance
APC	Allophycocyanin
AT_1R	Angiotensin receptor type 1
AT_2R	Angiotensin receptor type 2
bFGF	Basic fibroblast growth factor, FGF-2
BM-MNC	Bone marrow mononuclear cells
BMP	Bone morphogenic protein
bp	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of Differentiation
CDH5	Vascular endothelial cadherin, CD144
cDNA	Complimentary deoxyribonucleic acid
cGMP	Current good manufacturing practice
CLI	Critical limb ischemia
CMV	Cytomegalovirus
CRE	cAMP response element
CREB	cAMP response binding protein
Ct	Cycle threshold
CVD	Cardiovascular disease
DAPI	4',6-diamidino-2-phenylindole

DLL4	Delta like ligand 4
DMEM	Dulbecco's modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EB	Embryoid body
EC	Endothelial cells
ECC	Embryonic teratocarcinoma cells
EFNB2	Ephrin B2
eGFP	Enhanced green fluorescent protein
EGM-2	Endothelial growth medium 2
eNOS	Endothelial nitric oxide synthase
EPAC	Exchange protein directly activated by cAMP
EPC	Endothelial progenitor cells
EPHB4	Ephrin type B receptor 4
ERG1	ETS related gene 1
ERK	Extracellular signal regulated kinases
ETS	E twenty six transcription factors
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FC	Flow cytometry
FCS	Foetal calf serum
FGF	Fibroblast growth actor
FITC	Fluorescein isothiocyanate
FLI1	Friend leukaemia integration 1 transcription factor
FN	Fibronectin
GFP	Green fluorescent protein
GSK3B	Glycogen synthase kinase-3β
hESC	Human embryonic stem cells

HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
hIPSC	Human induced pluripotent cells
HIV	Human immunodeficiency virus
hPSC	Human pluripotent stem cells
HSVEC	Human saphenous vein endothelial cell
HUVECs	Human umbilical vein endothelial cells
ICC	Immunocytochemistry
ICM	Inner cell mass
IGF	Insulin like growth factor
iPSC	Induced pluripotent stem cells
Kbp	Kilobase pair
KDR	Kinase insert domain receptor, CD309
LDL	Low density lipoprotein
LIF	Leukaemia inhibitory factor
MAPK	Mitogen activated protein kinases
MEF	Mouse embryonic fibroblasts
MEM	Minimal essential medium
mESC	Murine embryonic stem cells
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
NO	Nitric oxide
NRP	Neuropillin
OCT4	Octamer binding transcription factor 4
PAD	Peripheral arterial disease
PB-MNC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDEs	Phosphodiesterases
PDGFB	Platelet derived growth factor B
PE	Phycoerythrin
PECAM	Platelet endothelial cell adhesion molecule, CD31
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase a
PLGF	Placental growth factor
PS	Primitive streak
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RAS	Renin angiotensin system
RBPJ	Recombination signal binding protein for immunoglobin kappa J region
RFP	Red fluorescent protein
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
ROCKi	Rho-associated protein kinase inhibitor
RQ	Relative quantification
RT	Room temperature
SEM	Standard error of the mean
SFFV	Spleen focus forming virus
SHH	Sonic hedgehog
SOX2	Sex determining region Y box 2
SSEA3	Stage specific embryonic antigen 3
TF	Transcription factor
TGFB	Transforming growth factor beta
TLR3	Toll like receptor 3

UBC	Ubiquitin protein C
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSMC	Vascular smooth muscle cell
vWF	von Willbrand factor
WHO	World Health Organisation

Chapter 1: Introduction

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) includes various diseases of the heart and circulation, and has four main types: coronary heart disease, stroke, peripheral arterial disease and aortic disease. According to the World Health Organisation (WHO), CVD is the main cause of deaths worldwide (WHO, 2015). In the United Kingdom (UK), CVD causes more than a quarter of all deaths with yearly costs relating to lost productivity and disease treatment estimated at £19 billion (BHF, 2015).

One of the fundamental processes in CVD is atherosclerosis, which is a slow disease process driven by endothelial dysfunction and low grade inflammation, leading to formation of a plaque, consisting of accumulated low-density lipoprotein, oxidized lipids, debris, immune cells and apoptotic cells (Weber and Noels, 2011). This leads to narrowing of arteries (Figure 1) and reduced blood supply to tissues, also known as ischemia. Ischemic tissues do not receive sufficient oxygen, glucose and other nutrients as well as have an accumulation of cellular waste products, leading to altered cell metabolism, remodelling and even tissue atrophy.

Although, targeting behavioural risk, current pharmacological and surgical interventions have reduced CVD death rates in the UK by a third since 1961 (BHF, 2015), there is still a need for novel therapies to address the needs for those patients who have had limited success with pharmacological treatments and don’t have an option of surgical interventions.

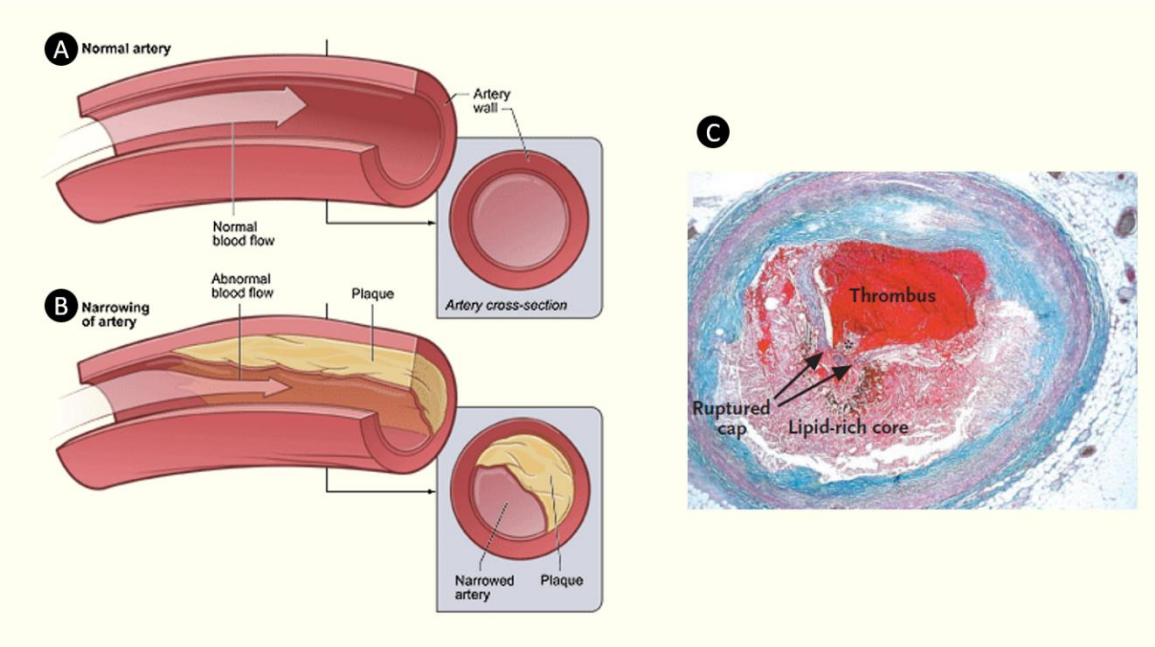


Figure 1: Atherosclerosis and blood flow. A. *In a normal artery, blood flow is not limited and tissues receive adequate blood supply.* ***B.*** *Formation of a plaque can result in narrowing of an artery and subsequently abnormal blood flow and reduced blood supply to the tissues as seen in PAD and CLI.* ***C.*** *Cross section of a coronary artery where lipid rich atherosclerotic plaque can be seen. This blood vessel was blocked completely by a thrombus (in red) that formed due to the ruptured atherosclerotic plaque. Adapted from: Hansson (2005) and National Institutes of Health (2015).*

1.2 Peripheral arterial disease

Peripheral arterial disease (PAD), also known as peripheral vascular disease, is an ischemic disease associated with systemic atherosclerosis resulting in decreased tissue perfusion pressure and comprised capillary function as shown in Figure 2 (Lambert and Belch, 2013). PAD can affect arteries in different parts of the body, however, in the majority of cases PAD affects lower limbs. Intermittent claudication, which is pain and stiffness in calf muscle usually attenuated by rest, is a symptom of early stage PAD. Severe form of PAD is known as critical limb ischemia (CLI). PAD is associated with age, with estimated incidence increasing to 150 - 250 cases per 100 000 of over 85s population per year, depending on the classification system used (Howard et al., 2015).

According to the National Institute for Health and Care Excellence (NICE) guidelines (2012), current treatments of PAD rely on behavioural changes, exercise programs, pharmacological treatments targeting disease cofactors, for example diabetes, high blood pressure or cholesterol levels, and invasive approaches, for example, endovascular therapy to restore blood flow to the tissues. In addition to pain management, the only pharmacological treatment offered for PAD in the UK is vasodilator naftidrofuryl oxalate, used only if revascularization is not considered.

Two meta-analysis have shown that naftidrofuryl oxalate significantly improves pain free and maximum walking distances (Squires et al., 2011; Stevens et al., 2012). However, these improvements are not deemed statistically significant in Cochrane analysis of eight clinical trials and it was also noted that amputation rates remained high with naftidrofuryl oxalate treatment (Smith et al., 2012). Other drugs used for their vasodilatory effects are naftidrofuryl and cilostazol, however, there is limited evidence for their efficacy in CLI (Lambert and Belch, 2013).

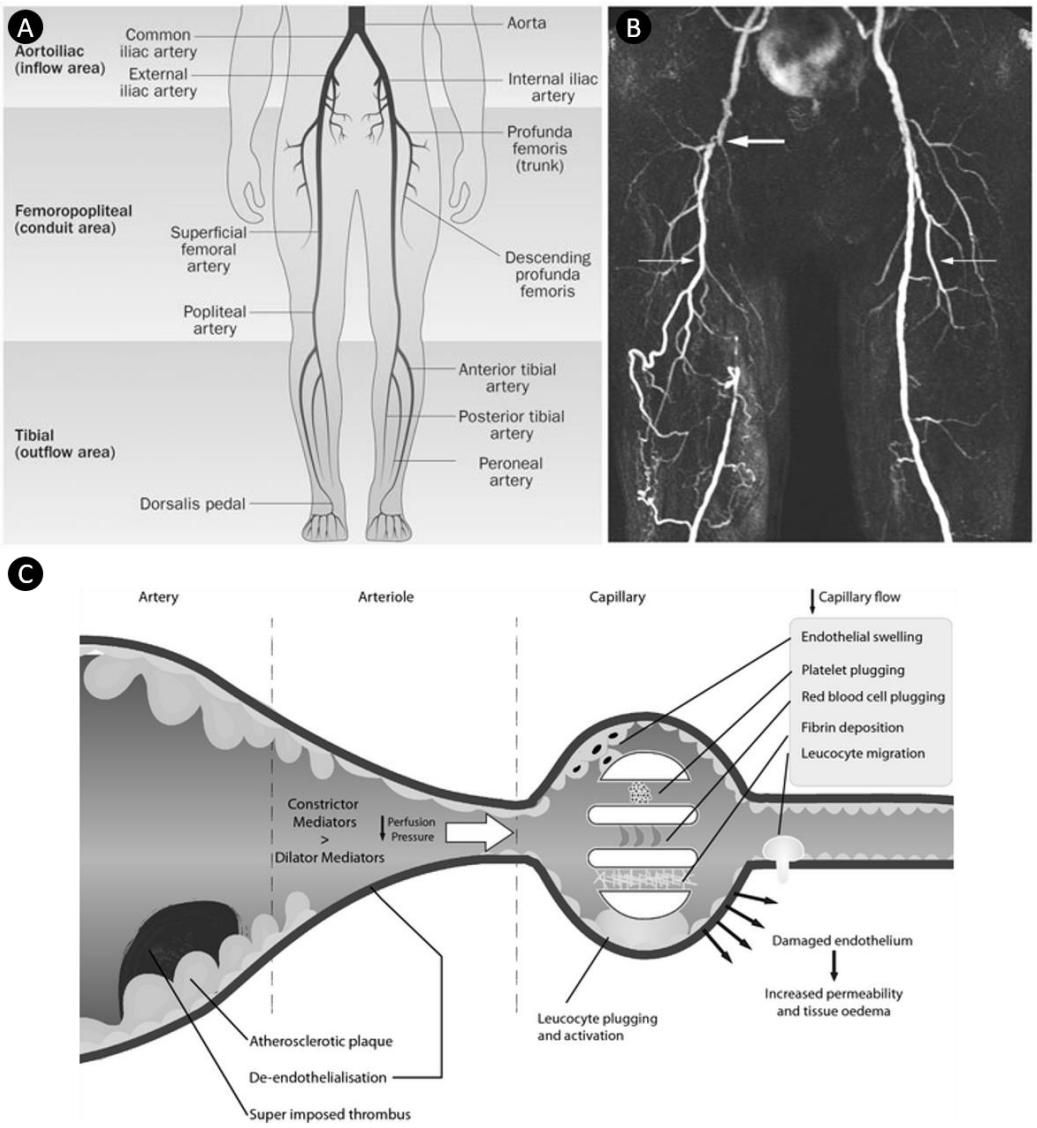


Figure 2: CLI pathology. A. *In the majority of cases PAD affects lower limbs. Severe form of PAD is known as CLI* ***B.*** *Magnetic resonance angiogram where near complete occlusion of the right superficial femoral artery can be seen as indicated by the thick arrow).* ***C.*** *Systemic atherosclerosis results in decreased tissue perfusion pressure and comprised capillary function. Adapted from: Annex et al. (2013) and Lamberth and Belch (2013).*

Another pharmacological approach is the use of prostanoids for their antiplatelet and vasodilatory properties. Due to lack of evidence showing efficacy, it is not currently not recommended in the UK (NICE, 2013). A recent meta-analysis by Vitale et al. (2015) presented some evidence that prostanoids were associated with a lower major amputation rate while total amputation and healing rate was not significantly affected by prostanoid treatment. However, they were unable to draw conclusions or recommend their use.

Approximately in one in five patients with PAD, the disease progresses to a point where the blood supply of limbs is severely restricted resulting in CLI. CLI represents a significant clinical and socioeconomic burden, especially when considering the consistently reported mortality and amputation rates of over 20% (Abu Dabrh et al., 2015; Henry et al., 2011; Howard et al., 2015). In addition, a recent UK based population study has estimated the five year CLI mortality rate of 29% and amputation rate exceeding 40% (Howard et al., 2015).

It is clear that current drug therapies are insufficient for treating PAD and CLI, and surgical revascularisation strategies have been receiving increased clinical focus as additional approaches for increasing limb salvage rates and reducing PAD related mortality.

1.3 Current revascularization strategies for PAD

There are two main revascularization strategies for PAD. Surgical bypass surgery is more suitable for younger patients with longer life expectancy and requires a bypass vessel, usually an autologous vein. In contrast, endovascular revascularization (angioplasty, stenting and atherectomy) is less invasive and costly and has become the preferred treatment (Goodney et al., 2009; Slovut and Sullivan, 2008).

Both methods offer increased limb salvages rates. However, there is a lack of consensus on which is the best therapeutic approach. An attempt to address this was made in BASIL study (2005) which followed 452 patients with CLI and severe limb ischemia randomly assigned to either angioplasty or bypass surgery. The study reported similar medium term outcomes between the treatment groups with amputation-free survival at 3 years of 57% in bypass surgery group and 52% in angioplasty group. However, evidence was also provided suggesting that bypass surgery might have a favourable long term amputation and survival rate using post-hoc analysis (Bradbury et al., 2010). However, there have been multiple criticisms of the BASIL study, for example regarding to cohort and clinical endpoint (amputation free survival) selection (Conte, 2010) and a new BEST-CLI study has been designed to address these issues and evaluate the current CLI revascularization therapies (Farber et al., 2014).

Another approach is hybrid revascularization which combines open surgery with endovascularization techniques promising similar early and long term efficacy to surgical bypasses but with reduced morbidity and hospitalization times (Huynh and Bechara, 2013). A recent, long term 57 patient study demonstrated no significant differences in limb salvage rates, major procedure-related complications, systemic complications and mortality between patients undergoing combined iliac endovascular therapy and infra-inguinal bypass, even though, preoperative limb ischemia was more severe in patients undergoing combined iliac endovascular therapy (Miyahara et al., 2015). This is in line with observations by Zhou et al. (2014) and one of the largest studies to date reporting 125 procedures by Dougherty et al. (2003). However, long term analysis with higher patient numbers is required to further explore any potential benefits of hybrid revascularisation.

While endovascular revascularization is an alternative for those patients who are not eligible for bypass surgery, there's a significant patient population, for example patients with complex lesions, who are not suitable for either of the treatments. Therefore, minimally invasive therapies that address the needs of this patient group are needed. In addition, novel approaches, for example gene therapy, to revascularization have the potential to complement current therapies, offering limb salvage, quality of life and survival improvements.

1.4 Gene therapy for PAD

Gene therapy aims target disease processes by introducing exogenous genes and has attracted increasing attention since the first therapeutic trials were performed in the beginning of 1990s. Even though the majority of the gene therapy trials focus on cancer treatment, cardiovascular gene therapy is the second largest area of interest (Edelstein et al., 2007, 2004; Ginn et al., 2013).

There are two main approaches to gene delivery: viral and non-viral. Viral gene delivery can be further classified, depending on the use of non-integrative vectors, for example adenoviruses, or integrative vectors like retroviruses which offer higher transduction efficiencies and stable transgene expression. After initial promising proof of concept trials using retroviral vectors, concerns regarding preferential viral integration near oncogenes (Edelstein et al., 2007) became an important obstacle in the clinical progression of the viral gene therapies. Indeed, multiple high profile adverse effect reports have highlighted that additional precautions are required when considering viral gene therapy vectors (Check, 2005; Gansbacher and European Society of Gene Therapy, 2003; Hollon, 2000; Hughes, 2007; Marshall, 1999).

The development of new human immunodeficiency (HIV) based lentiviral vectors (Dull et al., 1998; Naldini et al., 1996) has minimised these risks. In addition, methods have been developed for clinical grade lentiviral vector production (Ausubel et al., 2012) and, unsurprisingly, a large number of clinical trials have used lentiviral vectors for treatments of a wide range of diseases in the past years (Ginn et al., 2013). Yet, some risks of genotoxicity and immunogenicity remain, thus, non-viral gene delivery methods remain important alternative tools for gene therapy (Baum, 2014; Nayak and Herzog, 2010).

Direct gene transfer in vivo was first demonstrated in mouse skeletal muscle by Wolff et al. (1990) and recently plasmid based vectors have gained more attention as an alternative to viral vectors with reduced adverse event risk, larger capacity of therapeutic deoxyribonucleic acid (DNA) and cheaper manufacture, albeit lower efficiency and concerns regarding the presence of antibiotic resistance genes (Edelstein et al., 2007; Vandermeulen et al., 2011). Plasmid mini-circles lacking antibiotic resistance gene have been developed to

overcome this (Mayrhofer et al., 2009; Vandermeulen et al., 2011), making plasmid based approaches highly suitably for clinical setting.

Angiogenesis has been well characterised both in health and disease as reviewed by Carmeliet (2003) and a role for various signalling molecules, for example, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hypoxia inducible factor (HIF) and platelet derived growth factor (PDGF), has been well documented. Several of these cytokines have attracted interest as potential therapies for CLI and numerous gene therapy trials using plasmids or adenoviruses have been completed (Cooke and Losordo, 2015; Ko and Bandyk, 2014).

There are 4 FGF receptor genes and 18 members in the FGF ligand family with several members expressed both on endothelial and vascular smooth muscle cells (Yang et al., 2015). Interestingly, FGF is involved both in vascular homeostasis, atherosclerosis, early and late angiogenesis and thus has received increased attention as a potential target for gene therapy.

TAMARIS is the highest profile clinical trial to date and was set up after initial positive findings in an open label Phase I trial and a double-blind, randomized, placebo-controlled Phase II trial (Comerota et al., 2002; Nikol et al., 2008). TAMARIS followed 525 patients over a period of 12 months and evaluated the efficacy and safety of FGF1 encoding plasmid NV1FGF for CLI (Belch et al., 2011). Unfortunately, NV1FGF did not achieve any improvement in major death or amputation rates, mainly due to lower than expected placebo major amputation or death rate when comparing Phase II versus Phase II trial (33% vs >50%). Safety was comparable to placebo at the end of the TAMARIS trial and subsequent follow up at 36 months confirmed these observations. Two cases of cancer were reported in the NV1FGF treated patient group, however, no conclusions could be drawn from this observation due to the small sample size (Belch et al., 2011; Niebuhr et al., 2012; Prokosch et al., 2014).

HIF1 is the main transcriptional regulator expressed in response to hypoxia and is able to activate a range of genes via binding hypoxia response elements in gene promoters or enhancers (Zimna and Kurpisz, 2015). HIF1 upregulates gene families associated with cytokines and growth factors, including VEGF and PDGFB

and can support all stages of angiogenesis. Manalo et al. (2005) has shown that HIF1 carrying adenoviral vector is sufficient to induce endothelial tubule formation *in vitro*. In addition, it has been reported that low density lipoprotein (LDL) reduces hypoxia induced expression of *HIF1A* and *HIF2A in vitro* (Yao et al., 2015), this suggests that *HIF* expression might be reduced in patients with CLI and could be targeted in CLI therapies.

The largest clinical study of HIF1 to date has been reported by Creager et al. (2011) and was a randomised, double blind, placebo controlled trial following 289 patients with intermittent claudication over a 12 month period and tested adenovirus vector encoding constitutively active *HIF1A* gene (Ad2/HIF1/VP16). No improvement in any of the quality of life indicators was observed in the Ad2/HIF1/VP16 treated group. Safety was comparable between the groups, but it was noted that four cases of stroke were observed in the Ad2/HIF1/VP16 treated group, while no cases were observed in the control group. Nevertheless, the small sample size limits the conclusions that can be drawn from this observation. It was speculated that the treatment might benefit patients with more severe PAD, or that another vector is needed for increased efficiency, however, this is yet to be tested in a clinical setting.

Other, smaller clinical trials have evaluated VEGF, hepatocyte growth factor (HGF) and developmental endothelial locus-1 gene therapies with mixed results (Cooke and Losordo, 2015). While overall the data from clinical trials suggest acceptable safety of gene therapy in PAD and CLI, there is an overall trend of limited efficacy in larger, clinically relevant patient populations.

Therefore, there is a clear need for alternative therapy strategies to complement or replace pharmacological and gene therapy approaches for the treatment of CLI and PAD. Cell therapies represent a novel approach for therapeutic angiogenesis and have been attracting more and more interest. Multiple clinical trials have attempted to use adult cell sources for therapeutic angiogenesis, which will be discussed in the following section.

1.5 Cell therapy for PAD

Bone marrow contains not only hematopoietic stem cells but also a variety of other progenitor cells which have gained increased attention as potential sources for treatments of non-hematopoietic disorders (Prockop, 1997). Further research has led to multiple adult cell types being trialled in a clinical setting for PAD – bone marrow derived or granulocyte colony-stimulating factor mobilized peripheral blood derived mononuclear cells (BM-MNC and PB-MNC), mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPC).

The vast clinical experience with bone marrow and hematopoietic cell transplantation was seen as advantage as it promised simplified road to clinical setting with faster development and approval for these therapies. This was somewhat restricted by 2011 recommendations of Committee for Advanced Therapies of the European Medicines Agency which classified the use of BM-MNC and cell products derived from BM-MNC as advanced therapy medicinal products when used in non-hematopoietic applications (Cuende et al., 2013, 2012; Pirnay et al., 2013). However, this has not diminished the interest in bone marrow derived cells as clinically relevant therapies for PAD and multiple clinical studies have been undertaken as summarised in Table 1 and discussed in the upcoming sections.

Study	Phase	Patients	Cell Type	Follow up	Outcomes
JUVENTAS (Teraa et al., 2015)	I/II	160	BM-MNC	9 months	No significant differences in major amputation rate when compared to placebo.
Prochazka et al. (2010)	n/a	96	BM-MNC	4 months	A reduction in major limb amputation in BM-MNC treated patient group.
RESTORE-CLI (Powell et al., 2012)	II	86	BM-MNC derived *Ixmyelocel-T*	12 months	No significant differences in amputation free survival when compared to placebo. Significantly prolonged time to treatment failure (major amputation of injected leg, all-cause mortality, doubling of total wound surface area, *de novo* gangrene).
Li et al. (2013)	n/a	58	BM-MNC	6 months	No difference in major amputation rate when compared to placebo. Improved rest pain, skin ulcers and ABI.
Huang et al. (2007)	n/a	150	BM-MNC vs PB-MNC	12 weeks	No difference in amputation rates. Improvement of ABI, skin temperature, and rest pain in PB-MNC treated group.
TACT (Tateishi-Yuyama et al., 2002)	n/a	47	BM-MNC vs PB-MNC	24 weeks	ABI, rest pain and pain free walking time was significantly improved in BM-MNC treated group compared to PB-MNC. Amputation rate comparison not reported.
Zhang et al. (2016)	n/a	53	PB-MNC: CD133+	18 months	No statistically significant differences in amputation rates when compared to placebo. Improved ulcer healing and ABI.
Raval et al. (2014)	n/a	10	PB-MNC: CD133+	12 months	No differences in overall survival, freedom from amputation or freedom from hospitalization at 12 months compared to placebo.

Table 1: Key clinical studies evaluating cell therapies for PAD. *Continued on the next page.*

Study	Phase	Patients	Cell Type	Follow up	Outcomes
Kirana et al. (2012)	n/a	24	BM MSC vs CD90+	45 weeks	Comparable safety and efficacy between the treatment groups.
Perin et al. (2011)	I	21	MSC: ALDH+ vs BM-MNC	24 weeks	Improvement in Rutherford ischemic limb classification system score in ALDH+ treated patients when compared to BM-MNC.
Gupta et al. (2013)	I/II	20	MSC: CD90+ CD73+ CD166+ CD106+	6 months	No difference in serious adverse effects (death, infected gangrene, amputations) compared to placebo.
Losordo et al. (2012)	I/IIa	28	EPC: CD34+	12 months	No difference in major amputation rates when compared to placebo.
Kawamoto et al. (2009)	I/IIa	17	EPC: CD34+ dose escalation	12 weeks	Demonstrated safety and improvement in efficacy score (total walking distance, TBI, and pain), placebo control not used.
Skóra et al. (2015)	n/a	32	BM-MNC + VEGF expressing plasmid	12 weeks	Increase in ABI and reduction of pain observed in BM-MNC + VEGF expressing plasmid treated group when compared to control group receiving pentoxifylline.

Table 1 (cont.): Key clinical studies evaluating cell therapies for PAD.

1.5.1 BM-MNC and PB-MNC

BM-MNC are a heterogeneous population of cells, including hematopoietic stem cells and MSCs, amongst other cell types. Multiple clinical trials of BM-MNC therapies for PAD have been conducted as reviewed by Liu et al. (2015) and Raval and Losordo (2013), often reporting positive findings. However, these studies enrolled small numbers of patients and lack statistical power, so the positive effects need to be validated in larger patient populations.

This was attempted in a recent randomized, double-blind, placebo-controlled JUVENTAS trial which is the largest study to date, enrolling 160 patients (Teraa et al., 2015). JUVENTAS compared repeated intra-arterial infusion of BM-MNC versus placebo and did not find any significant differences in major amputation rates at 6 months and the extended follow up at 9 months between the patient groups. Safety of BM-MNC was comparable to placebo.

Other large scale studies have provided conflicting evidence, both supporting the clinical efficacy of BM-MNC and showing a reduction in major limb amputation (Procházka et al., 2010) and showing no benefit in amputation free survival (Powell et al., 2012). However, an updated meta-analysis of the effect of cell therapy on major amputation rates in CLI (Teraa et al., 2015) reported that, when non-blinded studies were excluded, the positive effect of cell therapies was lost. While differences in patient population or doses used or cell isolation methods, which can be detrimental to BM-MNC (Pösel et al., 2012), could contribute to the observed discrepancies in treatment efficiency between blinded and non-blinded studies, this highlights the need for double-blind, placebo controlled studies with sufficiently large patient populations to determine the efficacy of BM-MNC treatments for PAD.

Furthermore, DAMASCENE, a comprehensive evaluation of autologous bone marrow stem cell trials for treatment of ischemic heart disease (Nowbar et al., 2014), has demonstrated a correlation between the number of discrepancies in trial reports and the reported clinical efficiency. Indeed, while studies without discrepancies did not report an enhancement of ejection fraction, studies with increasing numbers of discrepancies reported an increasing benefit of bone marrow stem cell therapies. The possible explanations for these observations –

pressure for results to match expectations, reporting of exciting results before full checking, consistency in applying the treatment or measuring the response, also apply to cell therapies for PAD. Therefore, the quality of the clinical studies requires thorough evaluation to exclude possibility of non-reproducible positive study results.

Stem and progenitor cells are released from bone marrow in the circulation after stimulation with granulocyte colony-stimulating factor (Johnsen, 2001) and become a part of the PB-MNC together with lymphocytes and monocytes. Similarly to BM-MNC, some positive effects on ABI and amputation rates have been reported in clinical trials using PB-MNC (Y. Liu et al., 2015), however, only limited conclusions can be drawn these studies due to limited patient numbers. There doesn't seem to be a clear benefit of using PB-MNC over BM-MNC as studies comparing these two therapies have had conflicting results. Huang et al. (2007) reported an advantage of using PB-MNC in the largest comparative trial to date with 150 patients, while Tateishi-Yuyama et al. (2002), reported ABI, rest pain and pain free walking time improvements in BM-MNC treated patient population when comparing to PB-MNC, in a study with 47 patients. Yet, both of these studies failed to demonstrated improved amputation rates in any of the treatment groups.

Furthermore, neither of these studies included comparisons to placebo and, thus, it is difficult to evaluate the efficacy of these treatments. It can be speculated that both BM-MNC and PB-MNC therapies suffer from similar shortcomings. For example, Li et al. (2010) has shown that bone marrow cell angiogenic potency (for example, implantation and VEGF production) was significantly reduced in aged patient populations, which represent the majority of CLI cases. In addition, both approaches use somewhat variable and undefined populations that might not represent the best approach for therapeutic angiogenesis.

This has been attempted to address in studies using prominin-1 (CD133) expressing cells isolated from PB-MNC, which are thought to possess high regenerative capacity (Burt et al., 2010; Raval et al., 2014), however, the efficacy of the treatments was not demonstrated, perhaps due to the small number of patients enrolled in these studies. More recently, autologous

peripheral blood sorted CD133+ cell transplants were demonstrated to improve ulcer healing and ABI in a larger study enrolling 53 patients with diabetic critical PAD (Zhang et al., 2016). However, while reduced amputation rates were reported, these results were not statistically significant and, thus, the clinical efficacy for prevention of amputations cannot be claimed.

Therefore, it can be proposed that therapies utilizing other cell types would be more suitable as treatment strategy for PAD. Cell types of interest include mesenchymal stem cells, due to their paracrine and immunomodulatory effects, and endothelial progenitor cells for taking part in angiogenesis directly, as discussed in the following sections.

1.5.2 MSCs

Mesenchymal stem cells (MSCs), also known as marrow stromal cells, are multipotent adherent cells that possess capacity to self-regenerate and undergo osteogenic, chondrogenic and adipogenic differentiation (Conget and Minguell, 1999; Jiang et al., 2002; Pittenger et al., 1999). Originally, MSCs were isolated from bone marrow, however, they can be found in most vascularised tissues (Crisan et al., 2008) and have been isolated from various sources, including adipose tissue (Eirin et al., 2012), peripheral blood (Tondreau et al., 2005) and skeletal muscle (Uezumi et al., 2010).

MSCs are defined as cells expressing CD105, CD73, CD90, but not CD45, CD34, CD14, CD11b, CD79a, CD19 and HLA-DR (Dominici et al., 2006). Various stemness markers have been suggested to identify MSCs, for example CD146, SSEA-3, CD237, however, it is becoming increasingly clear that no single MSCs identifying marker exists (Álvarez-Viejo et al., 2015; Lv et al., 2014). Indeed, the phenotypes of MSCs differ depending on the cell source with both marker expression and functional differences reported as reviewed by Lv et al. (2014).

While MSCs have also been reported to undergo endothelial differentiation and support neovascularisation (Oswald et al., 2004; Silva et al., 2005), the major effects of MSCs are mediated via paracrine immunomodulatory mechanisms (Liang et al., 2014; Murphy et al., 2013). Indeed, MSCs have a secretory phenotype and express various cytokines and growth factors, including ones that are highly relevant to angiogenesis – VEGF and bFGF (Tang et al., 2005). Furthermore, MSCs upregulate VEGF expression in response to hypoxia (Mayer et al., 2005), secrete factors that attract endothelial cells (L. Chen et al., 2008) and protect endothelial cells under hypoxic conditions (Bader et al., 2014).

Another major advantage of using MSCs for cell therapies is presence of multiple potential MSCs sources, lack of associated ethical issues and their immunomodulatory properties which permits them to avoid immune clearance (Aggarwal and Pittenger, 2005). In addition, whereas growth rate and differentiation capacity of MSCs is affected by the age of the donor (Stolzing et al., 2008; L. W. Wu et al., 2014; Zaim et al., 2012), a report by Gremmels et al.

(2014) suggested that MSCs growth factor secretion and angiogenic capacity is not.

Taken together, it can be speculated that the paracrine nature of MSCs is a crucial advantage over conventional BM-MNC and PB-MNC therapies, as it might provide a strong stimulus for angiogenesis in ischemic tissues and translate into superior clinical efficacy. Furthermore, the immunomodulatory properties and robustness of MSCs in older patients, support the rationale for clinical studies evaluating the efficiency of MSCs as a cell therapy for PAD.

Various MSCs have been used in a clinical setting - Perin et al. (2011) sorted progenitor cells based on aldehyde dehydrogenise expression, Kirana et al. (2012) used CD90+ cells, while Gupta et al. (2013) selected population positive for CD90, CD73, CD166, CD106 markers. In these studies, MSCs therapies were shown to be safe and of a better or comparable efficiency than BM-MNC therapies. However, amputation rates were similar between the treatment groups, and therefore the advantage of MSCs therapies is not clear. The small scale of these clinical studies further limits the conclusions that can be drawn. Additionally, it is essential to design large scale double blind studies comparing MSCs therapies to placebo to comprehensively evaluate the efficacy of MSC based PAD therapies.

While therapies using MSCs are attracting significant interest due to their paracrine and immunomodulatory effects, there is lack of comprehensive clinical data supporting their efficacy. In addition, it can be argued that the need for a cell type that can directly contribute to therapeutic angiogenesis directly remains unmet. Thus, a range of other cells have been investigated, including circulating EPC which are obvious candidates for targeting angiogenesis.

1.5.3 EPC

Circulating EPC were first reported by Asahara et al. (1997) who used CD34 and CD309 surface markers to isolate EPC from human peripheral blood. These cells acquired endothelial phenotype *in vitro* and contributed to angiogenesis in mouse and rabbit *in vivo* models of hind limb ischemia (Asahara et al., 1999). Another study using early EPC demonstrated that human EPC expanded *ex vivo* but not human microvascular endothelial cells (EC) restored blood flow in murine hindlimb ischemia model (Kalka et al., 2000), providing evidence for potential clinical benefit of early EPC cell therapies.

The strong evidence presented by these publications led to extensive research into circulating EPC and it was proposed that circulating EPC originate from bone marrow and contribute to endothelialisation processes. This was further supported by evidence from Dacron graft endothelium studies by Shi et al. (1998) and later research using sex mismatched bone marrow donors by Li et al. (2000).

However, the exact identity of EPC is still under a discussion, which is further complicated by various selection and culture methods described. Originally, two types of EPC were identified depending on their appearance in culture – early EPC (Asahara et al., 1997) and late EPC (Shi et al., 1998), and differences in their surface marker expression profiles, proliferative and angiogenic capacity have been demonstrated (Cheng et al., 2013; Hur et al., 2004; Medina et al., 2010).

While EPC endothelial marker expression has been reported (Asahara et al., 1999; Kalka et al., 2000), their endothelial identity has been questioned. Indeed, cell selection by plating on fibronectin coated tissue culture plates carries a risk of contaminating cultures with platelets (Prokopi et al., 2009) and monocytes (Rehman et al., 2003; Schmeisser et al., 2003, 2001) which obtain similar phenotype as EPC and can express CD144, CD31 and eNOS. It has been proposed that EPC therapies benefit angiogenesis via paracrine secretion of various growth factors, for example *VEGF*, *HGF*, *interleukin 6* and others (Gnecchi et al., 2005; Kinnaird et al., 2004; Kwon et al., 2014).

Other progenitor cell types have been described by Hill et al. (2003) who obtained adherent colony forming unit EC and showed that the *in vitro* characteristics correlated with endothelial function *in vivo* and cardiovascular event risk. However, the endothelial identity of these cells is in question as well and evidence has been presented demonstrating their myeloid properties (Yoder et al., 2007). Meanwhile, prolonged culture of adherent cells from umbilical cord blood also has been reported to give rise to cells with mature endothelial phenotypes (Ingram et al., 2004), while retaining some immature stem cell line characteristics (Guillevic et al., 2016). These cells can also be further specified into arterial or brain microvascular endothelial cells (Boyer-Di Ponio et al., 2014). However, the therapeutic utility of these cells remains to be explored.

Various culture methods do not guarantee creation of a pure EPC population, therefore selection using FACS can be used to obtain a more defined cell population. However, the marker profile of EPC is still debated. CD133 has been proposed in addition to CD34 and CD309 (Peichev et al., 2000; Povsic et al., 2009). However, Case et al. (2007) demonstrated that CD34+ CD133+ CD309+ cells form hematopoietic progenitor but not endothelial cells. Aldehyde dehydrogenese expression has been proposed as another EPC marker (Povsic et al., 2007), while more recently Mund et al. (2012) has proposed using a CD34+ CD31+ CD146+ CD133- CD45- phenotype and provided evidence that these cells do not form hematopoietic colonies while retaining endothelial colony forming potential.

Slight benefits in quality of life have been reported in clinical trials using CD34+ selected or a mix of CD34+/CD133+ cell therapies (Kawamoto et al., 2009; Losordo et al., 2012), however, clear placebo controlled data demonstrating reduction of amputation rates is lacking. In addition, the proliferative and angiogenic capacity can vary significantly between the different cell populations (Hur et al., 2004), thus, it can be proposed that well defined highly proliferative and secretory endothelial populations could offer superior efficacy for therapeutic angiogenesis.

1.5.4 Combination treatments

With gene therapy or adult cell treatments alone having limited efficacy in PAD, it has been proposed that combining these approaches could enhance the efficacy of these therapies. Research in animal models has demonstrated higher efficiency of umbilical cord derived MSCs (Li et al., 2015) and EPC expanded *ex vivo* (Wang et al., 2015) in combination with a VEGF expressing plasmid.

A trial reported by Skóra et al. (2015) randomised 32 patients to receive BM-MNC transfected with VEGF plasmid or pharmacological treatment with pentoxifylline. Statistically significant reduction of pain and increase of ABI was reported. A comparison with non-transfected cells was not made and, therefore, it's hard to evaluate the contribution of transplanted cells and speculate about the benefits of combination therapy.

Therefore, currently there is little research and a lack of evidence suggesting that combination therapies could overcome the lack of efficacy observed in adult cell therapies for PAD. It can be argued that additional complexity of combination treatments further complicates clinical testing of such therapies, and, given the limited efficiency of adult cell therapies alone, combination therapies are unlikely to resolve the issues observed with adult cell therapies. Therefore, other approaches focusing on other cell types need to be considered for clinical angiogenesis.

1.5.5 Summary - cell therapy for PAD

In summary, while there is a strong rationale supported by preclinical studies for the use of cell therapies to target angiogenesis in PAD, clinical studies have failed to clearly demonstrate a robust improvement in critical clinical endpoints (prevention of major adverse effects of the limb) and currently none of the cell therapies for PAD have reached Phase III clinical trials.

It is clear that alternative approaches are needed for targeting ischemia clinically. It can be speculated that adult cell therapies lack robustness for clinical angiogenesis, perhaps due to their mixed phenotypes, reduced secretory or proliferative activity with aging, or lack of capability to directly integrate in vascular networks. Thus, only novel approaches would deliver the significant increase in treatment efficacy needed for successful treatment of CLI and PAD.

One such approach would be using human embryonic stem cells (hESC) to differentiate a large number or well defined endothelial cell populations. It can be proposed that the differentiation methods could be optimised to generate cells with high angiogenic capacity that can both contribute to angiogenesis directly, as well as aid angiogenesis via paracrine mechanisms. This would represent a significant shift in the mechanisms underlying the treatment and is likely to be more efficient than the use of pharmacological, surgical or adult cell treatment approaches alone.

1.6 Stem cells

There are two characteristics that set stem cells apart from other cell types. The first is capacity to differentiate into mature functional cell types. The second is unlimited self-renewal via cell division. Interestingly, the first cells to fulfil these criteria and establish the field of stem cell research were derived from teratocarcinomas and teratomas. Kleinsmith and Pierce (1964) derived *in vivo* clonal cell lines from teratocarcinoma and showed that a single transplanted cell was able to differentiate in tissues representing all three main germ layers – endoderm, mesoderm and ectoderm.

This led to attempts to isolate and grow these cells in *in vitro* and further refinements, most notably use of a feeder cell layer, led to large scale creation of pluripotent *in vitro* cell lines named embryonic teratocarcinoma cells (ECC) (Martin and Evans, 1974). The similarities between the behaviour of ECC and cells in developing embryos were quickly noted, and ECC were proposed as a tool to study early mammalian development (Martin, 1980, 1975).

Shortly after, the observations and techniques developed with ECC, enabled the derivation of first murine embryonic stem cell (mESC) lines by two groups simultaneously (Evans and Kaufman, 1981; Martin, 1981). After deriving primate embryonic stem cells from rhesus monkey, Thomson et al. (1998, 1995) were the first group to report derivation of human embryonic stem cell (hESC) lines. Almost a decade later, generation of induced pluripotent stem cells (iPSC) was reported by Takahashi and Yamanaka (2006). The derivation of hESC and iPSC not only offered novel scientific models for human development, disease modelling and toxicology studies but also established the field of regenerative medicine.

The ability to confirm the pluripotency of these cells is critical for stem cell work. Currently, there are multiple approaches to test stem cell pluripotency, both *in vitro* and *in vivo*. Firstly, a single transplanted undifferentiated stem cell into an immunodeficient mouse model should form teratoma consisting of all three germ layers. Alternatively, spontaneous differentiation to all germ layers can also be induced *in vitro* by culturing pluripotent stem cells in 3D spheroid embryoid body (EB) culture. Ideally, the ability of stem cells to form chimeras

and to contribute to germ lines should be tested, however, this testing is not performed with hESC due to ethical reasons.

High placental type alkaline phosphatase activity was demonstrated in undifferentiated carcinoma cell lines (Bernstine et al., 1973) and is still commonly used for pluripotent stem cell identification (Singh et al., 2012). In addition, a range of markers for identifying pluripotency and differentiation using antibodies have been described. Stage specific embryonic antigens SSEA-1, SSEA-3 and SSEA-4, and human embryonic carcinoma antigens TRA-1-60, TRA-1-80 were the first ones described (Andrews et al., 1984; Kannagi et al., 1983; Solter and Knowles, 1978) and are still used today.

1.6.1 Human embryonic stem cells

Human embryonic stem cells (hESC) are derived from the inner cell mass of a preimplantation blastocyst and fulfil the basic requirements that define pluripotency – hESC are able to self-renew and differentiate towards into other cell types from all three germ layers - ectoderm, mesoderm, endoderm as well as germline (Figure 3). The first hESC lines were derived by Thomson et al. (1998) from inner cell mass (ICM) of donated cleavage stage embryos produced for clinical purposes. Five hESC lines were established from five separate embryos – H1, H13 and H14 with a normal XY karyotype and H7 and H9 with a normal XX karyotype.

They remained pluripotent after cryopreservation and prolonged culture, expressed pluripotency markers SSEA–3, SSEA-4, TRA-1-60, TRA-1-81, and had high telomerase and alkaline phosphatase activity. These cell lines formed teratomas and were able to differentiate in cell types from all three germ layers. The ability of these cells to form chimeras and contribute to germ lines was not tested due to ethical considerations. However, hESC ability to engraft into mouse blastocyst has been demonstrated (James et al., 2006). In addition, Amit et al. (2000) demonstrated pluripotency and stable karyotype in clonal cell lines derived from H9, demonstrating that H9 are highly stable and thus suitable for *in vitro* studies. The original hESC lines derived by Thomson et al. are still widely used and have become the ‘gold standard’ of hESC lines.

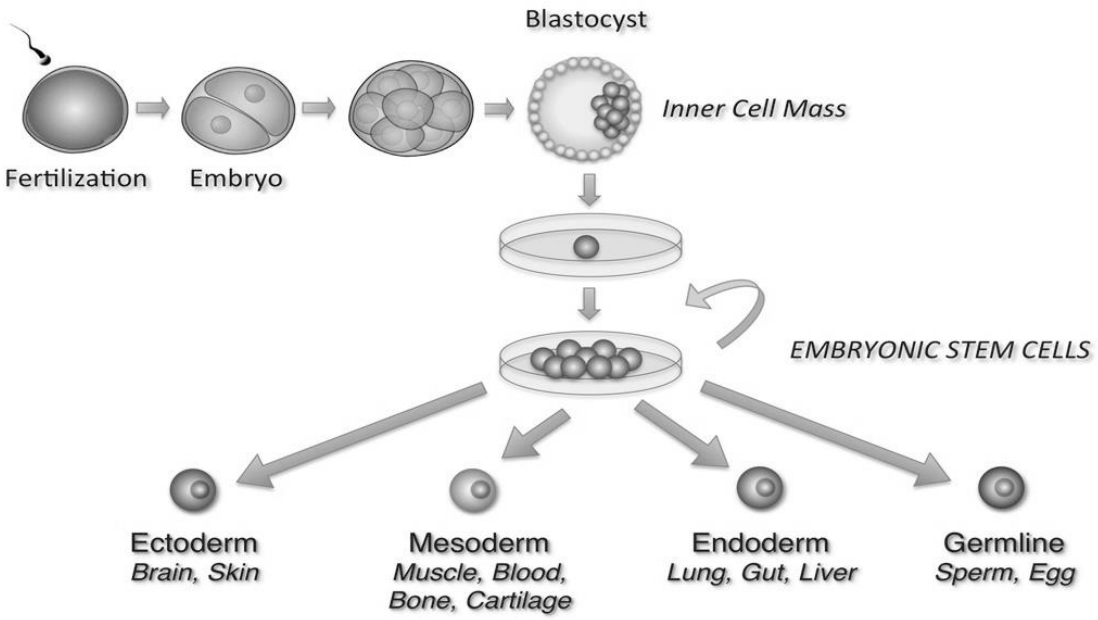


Figure 3: hESC derivation and differentiation. *hESC are derived from the inner cell mass of a blastocyst and are able to self-renew indefinitely as well as differentiate towards adult cell types. Adapted from: Yabut and Bernstein (2011).*

Initially, hESC were derived using immunosurgical methods and cultured on irradiated mouse embryonic fibroblasts in media supplemented with 20% bovine serum (Thomson et al., 1998). Whereas this is acceptable for research purposes, a fully defined and preferably xeno free derivation and culture methods are needed for clinical applications.

Early observations with mESC uncovered myeloid leukaemia inhibitory factor (LIF) as one of the main pluripotency factors produced by the feeder layer and further research showed that supplementing the media with bone morphogenic protein 4 (BMP4) in addition to LIF allows a serum free culture without a fibroblast feeder layer (Smith et al., 1988; Williams et al., 1988; Ying et al., 2003). However, these factors were not sufficient to maintain undifferentiated hESC (Thomson et al., 1998), highlighting the differences between hESC and mESC pluripotency signalling networks.

Initially, it was shown that bovine serum can be replaced with a knock out serum replacement (Amit et al., 2000). In addition, successful use of human fibroblasts was reported (Richards et al., 2002). Xu et al. (2001) reported successful feeder

free culture of hESC on mouse sarcoma cell derived matrigel or laminin in medium conditioned by MEF and later culture in non-conditioned media supplemented with bFGF and BMP signalling inhibitor noggin (Xu et al., 2005).

First feeder and xeno free derivation and culture of hESC lines was reported by Ludwig et al. (2006) using mTeSR1 media, however, they did not maintain a stable karyotype. In addition, a comparison of various xeno free culture media (Rajala et al., 2007) showed that various xeno free media, including mTeSR1 had limited success in maintaining pluripotent hESC cultures.

Soon after, generation of six clinical grade hESC lines was reported (Crook et al., 2007). These cell lines were derived adhering to current good manufacturing practice (cGMP) standards, which define critical control points and procedures to ensure the quality, reliability and safety of medicinal products (Hewitt et al., 2007). However, clinical grade human foreskin fibroblasts and cGMP bovine serum albumin serum replacement was used and, thus, these hESC lines were not derived and cultured in fully defined, xeno free systems.

Nevertheless, it can be argued that xeno derived reagents are crucial for the reliability of hESC derived products and thus such reagents could be cGMP qualified after strict selection and testing criteria are met (Hewitt et al., 2007). Further refinements, most importantly the creation of cGMP compliant human fibroblast lines (Prathalingam et al., 2012), led to hESC lines derived in xeno free, cGMP conditions (Tannenbaum et al., 2012) which are highly suitable for future clinical applications. However, the work towards completely feeder and serum free hESC derivation and maintenance procedures still is ongoing.

In addition to H1 and H9 hESCs, here we also use clinical grade cell lines RC-9 and RC-11 (Roslin Cells, UK) which are derived and cultured in compliance with laws set out by Human Fertilisation and Embryology Authority (“Human Fertilisation and Embryology Act 2008,” 2008) and cGMP guidelines as set out in Rules and Guidance for Pharmaceutical Manufacturers and Distributors (GOV.UK, 2015).

1.6.2 Human induced pluripotent stem cells

There are some concerns regarding the use of hESC – firstly, ethical considerations due to their embryonic origin, and secondly the requirement of patient matched hESC lines to avoid immune rejection. The generation of mouse and subsequently human induced pluripotent stem cells (iPSC) by Takahashi et al. (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) is arguably the most important development in the stem cell field in the last decade as it offers potentially unlimited, patient matched source of iPSC without a destruction of an embryo.

These cells were generated from adult cells by transduction of OCT4, SOX2, KLF4 and cMYC. They had similar morphology, surface marker expression profile and telomerase activity to hESC, expressed pluripotency factors, for example, *OCT3/4, SOX2, NANOG*, were able to differentiate to all three germ layers *in vitro* and form teratomas. Experiments with mouse iPSC have also demonstrated their capability to form chimeras and contribute to germ lines (Okita et al., 2007).

In addition, iPSC are a valuable tool for disease modelling as patient specific iPSC can be differentiated in somatic cell types for *in vitro* disease analysis. Park et al. (2008) reported generation of iPSC from patients with various genetic diseases, including Duchenne muscular dystrophy and Huntington syndrome, and confirmed the disease specific genotype. More complex disorders can also be successfully modelled using iPSC, for example, iPSC derived neuronal cells have been used to study molecular mechanisms underlying schizophrenia (Brennand et al., 2011), Alzheimer's disease (Israel et al., 2012) and bipolar disorder (Chen et al., 2014).

There are still multiple concerns regarding the use of iPSC. Firstly, even though, these cells are not derived from embryos, their pluripotent nature still presents a range of ethical issues (de Miguel-Beriain, 2015). Secondly, the use of viral vectors for the generation of iPSC carries a risk of unwanted genetic mutations or oncogene activation, however, a wide range of non-viral iPSC generation strategies have been developed (Deng et al., 2015). In addition, concerns have been raised regarding primed state and “memory” of iPSC, yet, the discussion of

naïve vs primed pluripotency states also applies to hESC (De Los Angeles et al., 2015; Hackett and Surani, 2014; Hu et al., 2016) and only recently naïve hESC have been directly derived from blastocyst (Ware et al., 2014). Finally, there's still an ongoing discussion regarding the "aging signature" of human IPSC cells (L. Rohani et al., 2014) and very recently evidence has been presented showing accumulation of mutations in mitochondrial DNA in human iPSC derived from elderly adults (Kang et al., 2016).

The ability to generate pluripotent stem cells from adult cells represents a significant advance for disease modelling and regenerative medicine. Yet, there are multiple obstacles that need to be overcome before we can utilise the full potential of hIPSC.

1.6.3 Core pluripotency transcriptions factors

Multiple pluripotency factors have been described and studied and three of these factors – OCT4, SOX2 and NANOG are seen as the core pluripotency regulators that work closely with other TF, for example cMYC, to form a pluripotency network (Boyer et al., 2005). They will be introduced in this section, while their interactions in pluripotency networks will be described in section 1.6.4 .

Octamer-binding transcription factor 4 (OCT4) is encoded by *POU5F1* gene and belongs to POU (Pit-Oct-Unc) transcription factor family. It is expressed in pluripotent and germ line cells and downregulated during differentiation (Rosner et al., 1990; Schöler et al., 1990). While mice heterozygous for *POU5F1* are viable and fertile, Nichols et al. (1998) observed an absence of homozygous *POU5F1* deficient pups and midgestation embryos, and subsequently showed that ICM did not develop in *OCT4* deficient blastocysts *in vitro* and *in vivo*.

Sex determining region Y box 2 (*SOX2*) gene has a similar phenotype to *OCT4*. It is expressed in ICM, mice heterozygous for *SOX2* are viable and fertile, however, homozygous *SOX2* deficient blastocysts have abnormal ICM development and fail to survive shortly after implantation (Avilion et al., 2003).

NANOG is a divergent homeodomain transcription protein that is expressed in mammalian ICM, epiblast and pluripotent cells (Chambers et al., 2003; Mitsui et al., 2003). As with the other core pluripotency TF, heterozygous *NANOG* deficient mice are viable and fertile, however, ICM of homozygous *NANOG* deficient blastocysts fails to proliferate and undergoes differentiation (Mitsui et al., 2003). Hyslop et al. (2005) demonstrated the expression of NANOG in human blastocyst ICM but not earlier stages, suggesting a role in the maintenance of hESC pluripotency, which is in contrast to observations in mESC where NANOG is not required for the maintenance pluripotency (Chambers et al., 2007).

In addition, MYC family of basic helix-loop-helix leucine zipper transcription factors works closely with the core pluripotency factors. It has three members cMYC, nMYC and lMYC, which usually heterodimerize with MAX protein when binding DNA (Blackwood and Eisenman, 1991). Of these, cMYC, nMYC and MAX are required for late embryonic development (Charron et al., 1992; Davis et al., 1993; Hatton et al., 1996). The role of cMYC in ESC pluripotency and renewal was first observed by Cartwright et al. (2005) and later research showed that cMYC and its targets formed a somewhat independent pluripotency regulating cluster from core pluripotency factors (X. Chen et al., 2008; Kim et al., 2008; Young, 2011).

In summary, cMYC and the core pluripotency factors OCT4, SOX2 and NANOG are crucial for development of pluripotent cells and, therefore, embryonic development. The expression of these appears to be tightly regulated and interdependent, forming pluripotency networks as described in the following section.

1.6.4 Regulation and maintenance of pluripotency

The core pluripotency factors OCT4, SOX2 and NANOG are tightly regulated and interact with large number of other proteins and genes. Understanding these interactions not only gives us insight into mammalian development, but also provides a framework for understanding hESC characteristics and generating hIPSC (as introduced in section 1.6.2), this way advancing the field of cell therapies and regenerative medicine.

NANOG binds promoters of *SOX2* and *OCT4* (Boyer et al., 2005), while OCT4 and SOX2 function as a heterodimer, promoting expression of *SOX2* (Avilion et al., 2003; Catena et al., 2004; Chew et al., 2005; Tomioka et al., 2002), *POU5F1* (Chew et al., 2005; Okumura-Nakanishi et al., 2005) and *NANOG* (Rodda et al., 2005). This way the core pluripotency factors form an auto-regulatory loop, as shown in Figure 4, which functions to establish and maintain pluripotent cell identity.

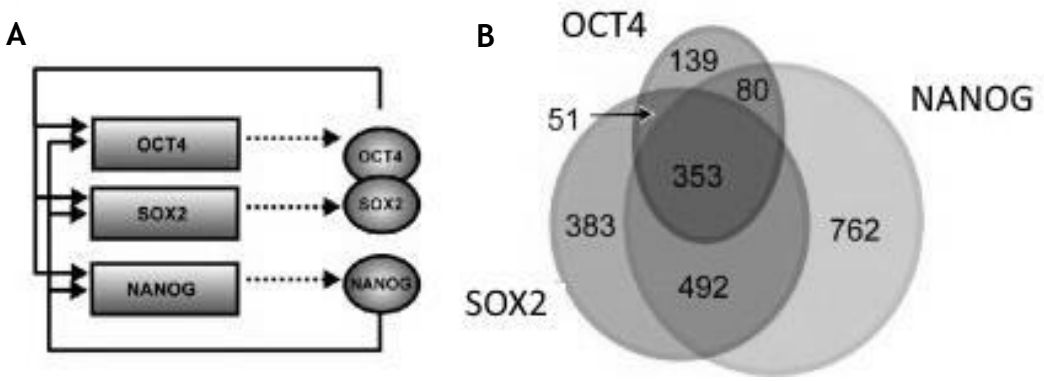


Figure 4: Core pluripotency factor network. A. *The core pluripotency factors (OCT4, SOX2 and NANOG) form an auto-regulatory loop responsible for establishing and maintaining pluripotency.* ***B.*** *Venn diagram illustrating genes regulated by the core pluripotency factors. Most of the target genes are regulated by multiple core pluripotency factors as shown by the overlapping areas. Adapted from: Boyer et al (2005).*

Analysis of core pluripotency factor transcription networks has revealed a wide range of genes regulated by OCT4, SOX2 and NANOG, including other pluripotency related TF, as well as protein complexes involved in histone modification, DNA methylation and chromatin remodelling (Huang and Wang, 2014; Young, 2011). Most of the target genes are regulated by multiple core pluripotency factors as shown in Figure 4 (Babaie et al., 2007; Boyer et al., 2005; Loh et al., 2006). In addition, most of the genes bound by core pluripotency factors are also bound other pluripotency related transcription factors, cMYC being the most frequent one, while only 2% of genes bound by core pluripotency factors not binding any of the closely associated pluripotency

factors (Young, 2011). This way, a gene switch is formed, where binding of few factors represses gene expression, while binding of multiple TF activates gene expression (Kim et al., 2008).

The core pluripotency factors not only promote pluripotency gene expression but also suppress gene programs that initiate differentiation. An unifying lineage fate regulation model has been proposed by Wang et al. (2012), suggesting that NANOG represses neuroectoderm, SOX2 represses primitive streak formation, while OCT4 represses neuroectoderm, trophectoderm and primitive endoderm fates.

In summary, the three core pluripotency factors OCT4, SOX2 and NANOG establish and maintain pluripotency via three complimentary processes – auto-regulatory loop, activation of other TF and genes involved in pluripotency or differentiation, and suppression of genes responsible for differentiation. Together these processes not only maintain the pluripotent state, but also enable rapid differentiation towards any of the germ layers and later cell types, including endothelial cells which are the focus of this project.

1.7 Endothelial Development.

Endothelial development has been studied both *in vivo* and *in vitro*, and more recently significant insights have been gained from studies using hESC differentiation models. During human embryonic development, ICM from blastocyst gives rise to epiblast cells. In the third week of development, gastrulation is initiated and primitive streak forms from the epiblast population followed by an appearance of mesodermal cells expressing brachyury (Wilkinson et al., 1990) as illustrated in Figure 5.

Some of these cells start expressing kinase insert domain receptor (KDR, CD309) and later develop into endothelial cells expressing endothelial markers platelet endothelial cell adhesion molecule (PECAM, CD31) and vascular endothelial cadherin (CDH5, CD144). Similar differentiation patterns are observed during hESC differentiation towards endothelial lineages and have been used to evaluate and optimise hESC differentiation methods.

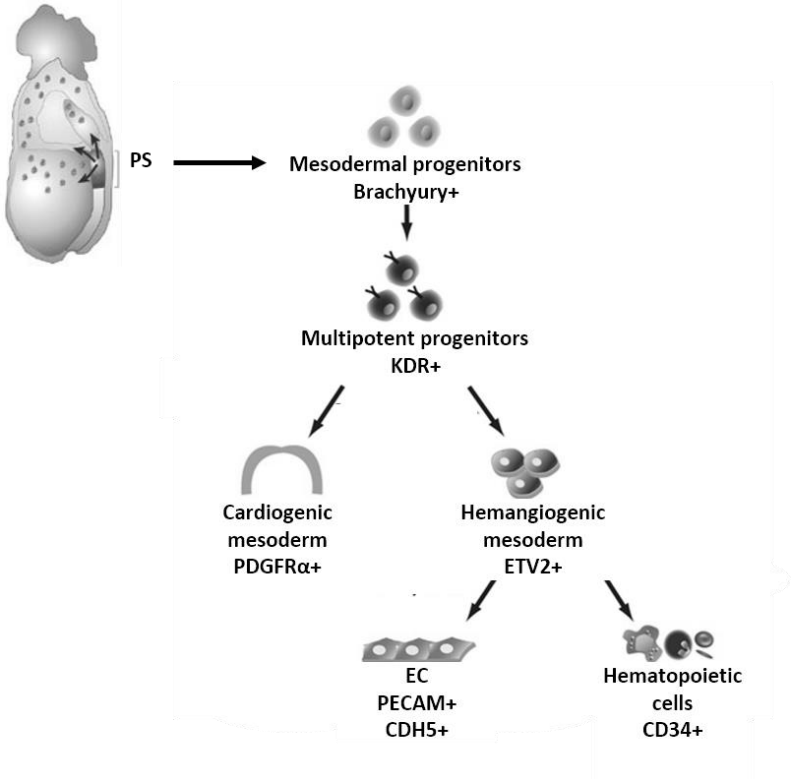


Figure 5: Overview of the development of endothelial cells (EC). *Primitive streak (PS) forms in the epiblast population of the developing embryo, followed by appearance of mesodermal cells expressing brachyury. These cells then undergo further differentiation and form PECAM and CDH5 expressing cells via multipotent progenitor and hemangiogenic mesoderm intermediates. Adapted from: Coultas et al. (2005) and Park et al. (2013).*

1.7.1 Development of mesoderm.

First step in the differentiation towards endothelial lineages is specification of mesoderm – one of the three primary germ layers, that gives rise to a range of tissues, including smooth muscle cells, blood cells and endothelium.

Mesodermal cells are marked by expression of brachyury (Wilkinson et al., 1990) which is required for normal progression of gastrulation (Wilson et al., 1995). More recently, $CD326^{-}$ $CD56^{+}$ surface marker expression profile has been proposed for identifying early mesoderm cells which have upregulated mesoderm

but not endoderm or ectoderm germ layer gene expression patterns (Evseenko et al., 2010).

Various members of transforming growth factor beta (TGFB) family have been implicated in the development, maintenance and patterning of mesoderm. Nodal subfamily of TGFB factors is expressed during gastrulation in mouse embryos (Zhou et al., 1993) and induce mesoderm formation (Jones et al., 1995), while nodal disruption abolishes primitive streak formation (Conlon et al., 1994; Iannaccone et al., 1992). Other members of TGFB family described to be involved in mesoderm formation and demonstrated in mouse development models are activins (Matzuk et al., 1995; Song et al., 1999), BMP-4 (Winnier et al., 1995), and VG1 and its mammalian homologs (Andersson et al., 2007).

WNT3A is expressed before gastrulation and is required for primitive streak formation and subsequent expression of nodal (Liu et al., 1999). Recent work by Yoon et al. (2015) using *WNT3A* visceral endoderm knockout models suggests that visceral endoderm derived WNT3A induces *WNT3A* gene and brachyury gene expression in the primitive streak. Taken together, this establishes WNT3A as one of the key players in mesodermal development.

In addition, basic fibroblast growth factor (bFGF, also known as FGF-2) has been extensively described to have an important role in mesoderm formation. Indeed, Bursdal et al. (1998) demonstrated that *in vitro* treatment of mouse epiblast cells with bFGF induced expression of brachyury and vimentin. Expression of a dominant negative FGF receptor in *Xenopus* embryos results in abnormal gastrulation, however, mesoderm can still be detected (Amaya et al., 1991). In addition, subsequent research in zebrafish and *Xenopus* models has provided evidence that FGF signalling is required for mesoderm induction by TGFB family factors (Mathieu et al., 2004; Mitchell and Sheets, 2001). Thus, FGF signalling is regarded as synergetic and complimentary to TGFB signalling.

1.7.2 Development of endothelium.

A subset of mesoderm cells expressing brachyury, upregulate *KDR* (Fehling et al., 2003) and VEGF co-receptor neuropillin-1 (Cimato et al., 2009) expression, and these are regarded as the earliest angioblast precursors or multipotent cardiovascular precursor cells.

KDR is a key receptor for normal endothelial and hematopoietic development and *KDR* deficient mice display embryonic lethality (Shalaby et al., 1995). Analysis of translational regulation of the *KDR* gene has revealed an enhancer that is activated by BMP, WNT and FGF (Ishitobi et al., 2011). In addition, it also contains various TF binding sites, including ETV2 which is expressed transiently before the expression of *KDR* and acts downstream of BMP, NOTCH, and WNT signalling (Lee et al., 2008).

These KDR+ cells form vascular plexus which subsequently undergoes extensive remodelling and establishes closed circulatory loop. Vascular endothelial growth factor (VEGF) has been shown to drive endothelial specification of the KDR+ precursor cells (Giles et al., 2005; Kawasaki et al., 2008; Yamashita, 2004) and expression of endothelial markers, for example, *CDH5* which initially can be detected in yolk sac during murine development, followed by an appearance in the more general embryonic vasculature (Alva et al., 2006). Downstream VEGF signalling through phospholipase Cγ1 is thought to mediate the endothelial differentiation and survival of the KDR+ cells (Sase et al., 2009), while phosphatidylinositol-3 kinase (PI3K) downstream signalling acts as a negative regulator (Merkely et al., 2015).

Furthermore, KDR signalling is negatively regulated by TGFB (Ginsberg et al., 2012; Mandriota et al., 1996), which also can interfere with endothelial specification by inducing endothelial-to-mesenchymal transition (Zeisberg et al., 2007), or promoting alternative mesodermal, for example, smooth muscle cell fates (Kurpinski et al., 2010).

Additionally, other signalling pathways contributing to endothelial differentiation are of interest for this project. Firstly, e-twenty six (ETS) family transcription factors, particularly ETS variant 2 (ETV2), play a key role in

endothelial specification (Oh et al., 2015) and can be regulated by intracellular cyclic adenosine monophosphate (cAMP) mediated activation of protein kinase a (PKA) (Yamamizu et al., 2012b). Secondly, the renin angiotensin system (RAS) has also been reported to contribute to endothelial differentiation and specification (Ishizuka et al., 2012; Zambidis et al., 2008). Yet, the role of cAMP and the RAS signalling in endothelial development requires further investigation.

There is, however, some controversy regarding the development of endothelial and hematopoietic lineages and multiple models have been proposed as reviewed by Bautch et al. (2011) and illustrated in Figure 6. Initial observations in lineage tracing experiments suggested that KDR+ cells form hemangioblasts – a common precursor of endothelial and hematopoietic lineages (Choi et al., 1998; Nishikawa et al., 1998). In contrast, later *in vivo* studies demonstrated emergence of hematopoietic cells from endothelial cells expressing endothelial markers Cd31 and CD144 (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010; Zovein et al., 2008) and additional supporting evidence has been provided using hESC differentiation models where a CD34+ CD31+ CD144+ population with hemogenic potential has been described (Bai et al., 2016).

These observations can be merged into a developmental model where hemangioblast cells give rise to hemogenic endothelium in addition to hematopoietic stem cells (Lancrin et al., 2009) as shown in Figure 6c. Recently, Ditadi et al. (2015) proposed that CD73 and CD184 marker expression profile can be used to distinguish between vascular and hemogenic endothelium.

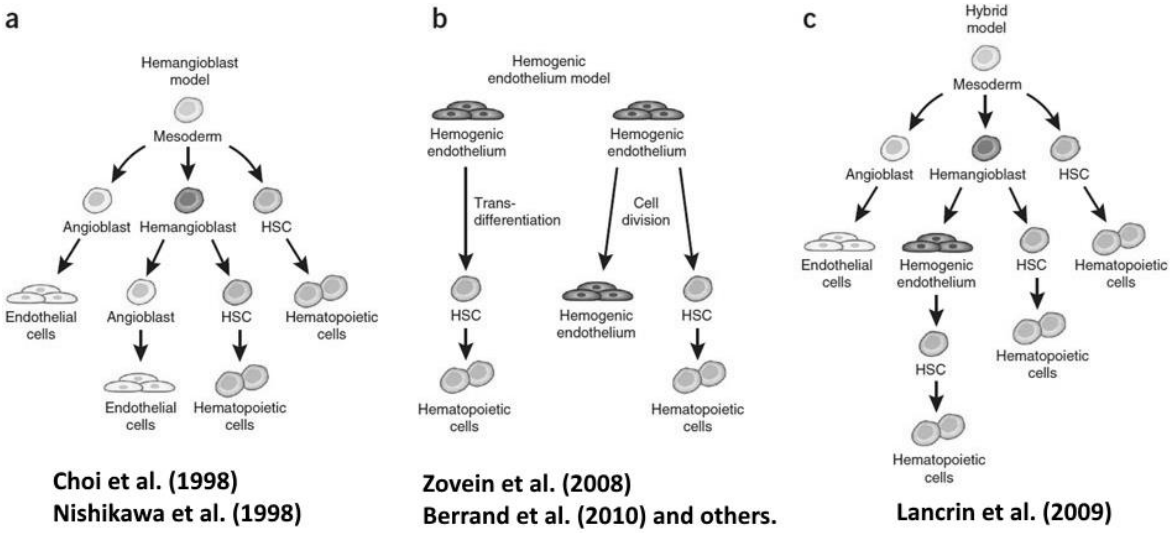


Figure 6: Three models proposed for the developmental relationships of endothelial and hematopoietic lineages. A. *Hemangioblasts give rise to both endothelial and hematopoietic stem cells.* ***B.*** *Hemogenic endothelium gives rise to hematopoietic stem cells and subsequently hematopoietic cells.* ***C.*** *Hemangioblasts give rise to hematopoietic stem cells and hemogenic endothelium, which is distinct from endothelium that arises from angioblasts. Adapted from: Bautch (2011).*

Taken together, the current evidence suggests that KDR+ cells give rise to various endothelial subpopulations, some of which commit to vascular endothelial fate, while others give rise to hematopoietic progenitors.

1.7.3 Specification of arteries and veins.

Primitive vascular plexus undergoes extensive remodelling, forming larger and smaller vessels and eventually establishing closed circulatory loop. Originally, it was believed that arterial and venous fates were induced in response to physical stimuli caused by blood circulation, however, soon after arterial (Ephrin B2, also known as EFNB2) and venous specific (Ephrin type-B receptor 4, EPHB4) markers were observed prior to establishment of blood flow (Wang et al., 1998), highlighting the genetic contribution of endothelial specification.

NOTCH1 and *NOTCH4* genes are specifically expressed in arteries, however, only *NOTCH1* homozygous mutant mice embryos fail to reorganize vascular plexus and show embryonic lethality (Krebs et al., 2000; Swiatek et al., 1994). In addition, NOTCH delta like ligand 4 (*DLL4*) heterozygous embryos display similar

phenotype – failure to reorganize vascular plexus and embryonic lethality, and NOTCH signal transcriptional mediator recombination signal binding protein for immunoglobulin kappa J Region (RBPJ) null mutants do not express arterial markers (Krebs et al., 2004). The requirement for precise *DLL4* expression, suggest that NOTCH-DLL4 signalling axis is the key player for arterial specification.

Crosstalk between VEGF and NOTCH signalling pathways is at the core of current arterial specification model (Lawson et al., 2001) as illustrated in Figure 7, however, VEGF can induce both arterial and venous specification, depending on downstream signalling via PI3K or mitogen-activated protein kinases / extracellular signal regulated kinases (MAPK/ERK) respectively (Hong et al., 2006; Ren et al., 2010). Interaction of these two pathways during VEGF signalling has been previously demonstrated in human umbilical vein endothelial cells, HUVECs (Blum et al., 2001; Deng et al., 2013), and observations in microvascular endothelial cells have led to a proposal that MAPK signalling is VEGF dose dependent, while PI3K activation is not enhanced by increasing VEGF concentrations (Akeson et al., 2010). This allows for differential signalling for endothelial specification and, indeed, VEGF signalling via MAPK, induces both *DLL4*, and *NOTCH4* expression and NOTCH downstream target genes hairy/enhancer of split related with YRPW motif protein 1 and 2 (*HEY1* and *HEY2)*, hairy and enhancer of split 1 and 2 (*HES1* and *HES2*) (Deng et al., 2013; Wythe et al., 2013; Yang et al., 2013).

However, it is also important to note that PI3K signalling has been also shown to induce *NOTCH1* and *DLL4* expression (Liu et al., 2003), activate Fox transcription factors FOXC1 and FOXC2, and promote arterial specification in cultured endothelial cells (Hayashi and Kume, 2008). Therefore, it appears that complex interactions are formed between NOTCH and VEGF with downstream signalling pathways contributing to endothelial specification via various mechanisms.

Other regulators that contribute to arterial specification via activating *DLL4* are ETS factors (Wythe et al., 2013), catenin beta-1 (Yamamizu et al., 2010), and SOXF family (Corada et al., 2013; Sacilotto et al., 2013). With the onset of blood flow, arterial endothelium is exposed to various physical forces that are not present in the venous system. These can also contribute to arterial phenotype

via VEGF-NOTCH arterial induction axis, for example, shear stress induces *VEGF* expression and *KDR* upregulation (dela Paz et al., 2012), upregulates arterial *EFNB2* gene expression (Masumura et al., 2009), and downregulates *EPHB4* expression levels in venous cells (Model et al., 2014).

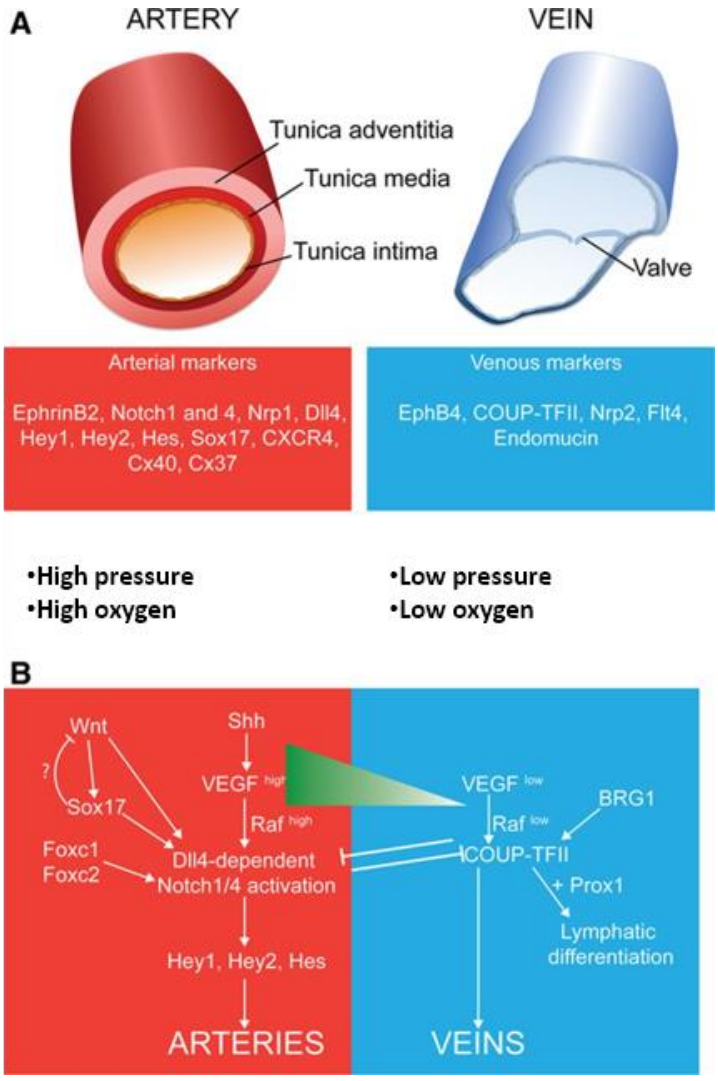


Figure 7: Specification of arterial and venous endothelium. A. *Endothelium forms tunica intima in arteries and veins, and expresses arterial (red box) or venous (blue box) endothelial markers accordingly.* ***B.*** *VEGF induced NOTCH signalling at the core of current arterial specification model to establish venous or arterial endothelial cell identity. High* VEGF *signalling results in NOTCH1/4 activation leading to expression of arterial genes, for example HEY2 and HES, while low* VEGF *signalling induces COUP-TFII which prevents the arterial gene expression, leading to a development of venous phenotype. Adapted from: Corada et al. (2014)*

In contrast, venous specification has been less researched and was thought to occur mostly in absence of NOTCH signalling. However, a venous specification TF factor, chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), has been identified, is expressed in venous endothelial cells and acts via inhibiting NOTCH signalling (Chen et al., 2012; You et al., 2005). In addition EPHB4 has been described to mark venous endothelium (Wang et al., 1998) and is regulated by VEGF (Yang et al., 2013). These transcription factors highlight the genetic contribution of venous specification, directly contrasting actions of arterial transcription factors.

Venous endothelium gives rise to lymphatic vasculature as recently reviewed by Semo et al. (2016). Venous COUP-TFII contributes to lymphatic differentiation by inducing Prospero Homeobox 1 expression (Srinivasan et al., 2010) which is the main determinant of lymphatic phenotype (Wigle et al., 2002; Wigle and Oliver, 1999). VEGF-C and VEGF-D regulates proliferation and migration of the lymphatic endothelium via VEGFR3 receptor (Mäkinen et al., 2001), expression of which is progressively limited to lymphatic endothelium during development (Kaipainen et al., 1995).

It also is important to appreciate that endothelial phenotypes also differ depending on the tissues and various highly specialised endothelial cell subtypes can be identified, for example, endothelium forming blood brain barrier, bone marrow endothelium and various endothelium types in kidneys (Garlanda and Dejana, 1997; Nolan et al., 2013). The specification of these endothelial subtypes is thought to be guided by interactions with neighbouring cells and various tissue specific signals, including soluble factors and extra-cellular matrix composition. Indeed, brain microvascular endothelium has been derived using neural cell coculture differentiation systems (Lippmann et al., 2012; Minami et al., 2015) and angiocrine VEGF-A has been shown to drive multipotent heart progenitor specification toward cardiac EC fates (Lui et al., 2013).

1.7.4 Angiogenesis

After establishment of the major blood vessels during development and, to a lesser extent, in adults, new blood vessels form from the pre-existing vasculature through various processes – sprouting, intussuception, elongation/widening and through recruitment of circulating EPC (Carmeliet and Jain, 2011; Chung and Ferrara, 2011) as illustrated in Figure 8a. Together these processes are known as angiogenesis which is defined as new blood vessel development from the existing vasculature. Angiogenesis is of a great clinical interest both as a target during disease processes, for example cancer progression and ocular disease (Carmeliet and Jain, 2000; Gariano and Gardner, 2004), as well as potential therapy for hypoxic disorders (Annex, 2013).

VEGF is the key signalling molecule involved in angiogenesis, however, it is important to appreciate the contribution of other VEGF family members, VEGF subtypes and receptors. There are five members of the VEGF family – VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor PLGF (Shibuya, 2013). Expression of *VEGF-A* requires precise regulation and mice lacking a single *VEGF-A* allele display abnormal blood vessel development and embryonic lethality (Carmeliet et al., 1996; Ferrara et al., 1996) demonstrating the importance of VEGF-A in the development of circulation system.

Hypoxia induces the expression of *VEGF* (Liu et al., 1995; Namiki et al., 1995) and alternative splicing creates VEGF-A isoforms – $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, which differ in heparin binding affinities and signal transduction pathways (Fearnley et al., 2016, 2015; Houck et al., 1991; Tischer et al., 1991). Mice expressing only $VEGF_{164}$ (murine VEGF proteins are single amino acid shorter than human ones) develop vasculature normally (Maes et al., 2004), while mice expressing only $VEGF_{120}$ or $VEGF_{188}$ display developmental abnormalities (Carmeliet et al., 1999b; Maes et al., 2004; Ruhrberg et al., 2002). $VEGF_{206}$ does not have a mouse equivalent due to a frameshift resulting in frame stop codon (Shima et al., 1996) and is thought to be involved in inflammatory processes (Grützkau et al., 1998).

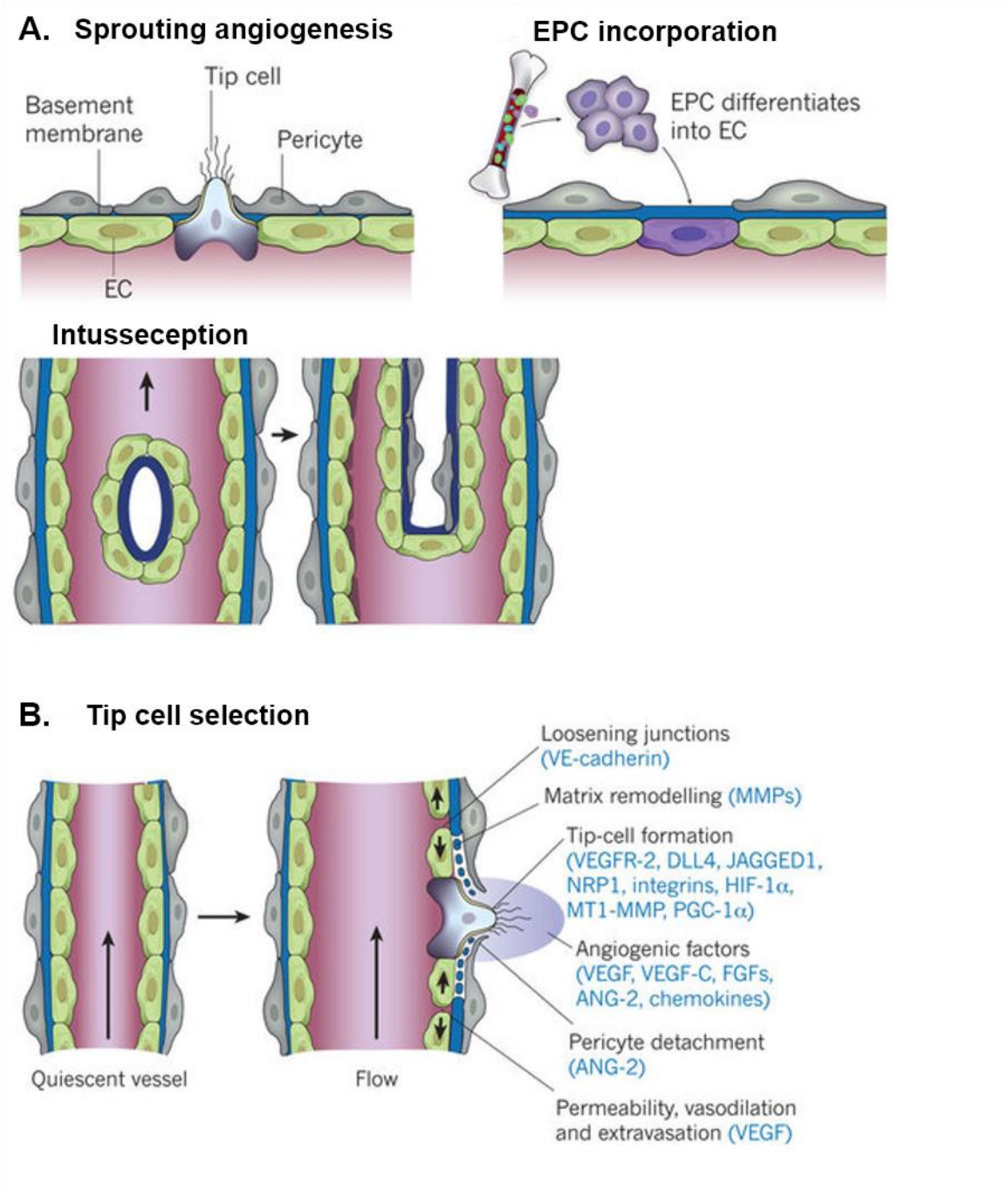


Figure 8: Overview of angiogenesis. A. *New blood vessels can form from the pre-existing vasculature through various processes – sprouting, intussuception, elongation, widening and through recruitment of circulating EPC.* ***B.*** *During sprouting, endothelial tip cell is selected through interaction of VEGF and NOTCH signalling pathways. These cells extend fillipodia which follow VEGF gradient, while a range of other signalling molecules contribute to angiogenesis indirectly – via altering pericyte and endothelial cell adhesion, stimulating breakdown of extracellular matrix and guiding tip cell migration. Adapted from: Carmeliet et al. (2003).*

Novel anti-angiogenetic VEGF isoforms have also been reported (Eswarappa and Fox, 2015; Pritchard-Jones et al., 2007) and, interestingly, $VEGF_{165b}$ isoform has also been investigated in PAD and shown to be induced by WNT5A signalling and to supress angiogenesis in mouse hindlimb ischaemia models, explaining the paradox of increased $VEGF_{165}$ levels, yet impaired angiogenesis, in general PAD patient population (Kikuchi et al., 2014). More recently, also a highly angiogenic VEGF isoform has been reported (Danastas et al., 2015), however, with somewhat limited role in physiological angiogenesis.

VEGF-A can bind both FLT-1, also known as VEGFR1 (de Vries et al., 1992; Shibuya et al., 1990), and KDR, also known as VEGFR2, FLK-1 (Terman et al., 1992), in addition to co-receptors neuropillin 1 and neuropillin 2 (NRP1 and NRP2) which potentiate the signalling of $VEGF_{165}$ or $VEGF_{165}$ and $VEGF_{145}$ accordingly (Gluzman-Poltorak et al., 2000; Soker et al., 1998). Binding of VEGF-A to KDR mediates endothelial cell migration and proliferation (Gerhardt et al., 2003; Hiratsuka et al., 2005). Signalling via FLT-1 appears not to be required during embryogenesis, as mice expressing FLT-1 without tyrosine kinase domain display normal development (Hiratsuka et al., 1998). However, FLT-1 knockout mice display embryonic lethality due to abnormal endothelial cell growth and organisation (Fong et al., 1995). Given that FLT-1 has a higher affinity for VEGF-A and lower kinase activity, it is proposed to act as a regulator of VEGF signalling (Hiratsuka et al., 1998), with an important role in normal vessel sprouting (Kearney et al., 2004).

However, it is important to appreciate the interplay between VEGF and TGFB during angiogenesis. TGFB induces angiogenesis *in vivo* (Roberts et al., 1986). And even though TGFB induces VEGF expression in EC (Ferrari et al., 2006) and VEGF signalling mediates EC proliferation and migration as described above, TGFB induces EC apoptosis *in vitro* (Pollman et al., 1999) which is VEGF dependent (Ferrari et al., 2009). With the arrival of small interfering RNA mediated downregulation of specific p38 MAPK isoforms, it was shown that this discrepancy is due to shift from pro-survival p38 MAPK kinase ß to pro-apoptotic p38 MAPK kinase α VEGF signalling in the presence of TGFB (Ferrari et al., 2012). Crucially, this interplay with VEGF and TGFB provides a coordinated mechanism for capillary formation and angiogenetic sprouting.

Sprouting is led by a tip cell, which is selected by genetic determinants, namely FLT-1 and KDR expression in addition to NOTCH signalling (Jakobsson et al., 2010). Metabolism also contributes to tip and stalk cell balance, for example, glycolysis regulator Phosphofructokinase-2/fructose-2,6-bisphosphatase enzyme deficiency impairs tip cell formation (De Bock et al., 2013) as well as cell proliferation and migration (Xu et al., 2014). More recently, presence of multiple tip cells in a developing sprout has been suggested (Pelton et al., 2014), thus, it is possible that more complex interactions during angiogenic sprouting will be uncovered in the close future.

Endothelial tip cells extend fillipodia which follow VEGF gradient (Gerhardt et al., 2003; Ruhrberg et al., 2002) as illustrated in Figure 8b. In tip cells, VEGF binding to KDR activates via PI3K/AKT signalling pathway to upregulate expression of DLL4, this in turn activates NOTCH signalling in nearby stalk cells, inhibiting formation of additional tip cells, downregulating KDR expression (Liu et al., 2003; Lobov et al., 2007; Suchting et al., 2007) and upregulating phosphatase and tensin homolog protein which limits stalk cell proliferation (Serra et al., 2015). In contrast, stalk cells express jagged1 which limits NOTCH signalling, counterbalancing the effect of DLL4 (Benedito et al., 2009). In addition, stalk cells also produce non membrane bound FLT-1 which sequesters VEGF-A, further refining angiogenic response (Chappell et al., 2009; Kappas et al., 2008; Kearney et al., 2004).

Stalk cells proliferate and multiple mechanisms have been proposed to underlie lumen formation in the newly developed sprout, while the exact mechanisms are still being uncovered, changes in cell polarisation, CDH5 expression patterns, cell shape and vacuole formation are all likely to contribute to lumen formation (Charpentier and Conlon, 2014).

Angiogenesis is an important process both in health and disease and can be upregulated or downregulated in pathologic processes, for example in diabetic retinopathy, ocular disease and cancer biology. Understanding the processes and mechanisms underlying angiogenesis not only increases our understanding of human development but also can potentially provide multiple therapeutic targets. Given the essential role of endothelial cells during angiogenesis, cell therapies are an attractive approach for therapeutic angiogenesis for various

vascular diseases, including PAD. Given the large number of cells required for such therapies, hESC differentiation towards endothelial lineages has been proposed as a highly perspective approach for therapeutic angiogenesis.

1.8 Endothelial differentiations from hESC and iPSC.

Endothelial differentiations from hESC and iPSC can be used to generate large numbers of EPC and EC populations and, thus, attract significant attention as potential approaches for clinical cell therapy angiogenesis. Numerous publications have described various approaches to endothelial cell differentiation as summarised in an extensive review by Descamps and Emanueli (2012). There are three main differentiation strategies, as illustrated in Figure 9 – differentiations in three dimensional stem cell aggregates (embryoid bodies, EBs), differentiations using co-cultures with adult cell types, and monolayer based differentiations. More recently, the focus has been on developing protocols that can generate large numbers of endothelial cells in a relatively short period of time using, if possible, chemically defined, clinical grade reagents. In addition, transdifferentiation of adult cell types can also be used to generate endothelial cells. All these approaches will be discussed in the following subsections.

1.8.1 Endothelial differentiation of hESC and iPSC – main approaches.

Initially, spontaneous appearance of cells expressing endothelial markers was observed in mESC derived EBs (Vittet et al., 1996). Later, these observations were confirmed in spontaneously differentiating hESC derived EBs by Levenberg et al. (2002) who sorted and cultured CD31 expressing cells from day 13 EBs. These cells expressed endothelial markers CD31, CD144 and vWF, were capable of taking up Dil-Ac-LDL, formed tubules in matrigel and integrated with host vasculature when transplanted in immunodeficient mice. Similarly, Wang et al. (2004) observed CD144+ CD31+ CD45- cells as early as Day 7 during spontaneous EB differentiation, and showed that they had both endothelial and hematopoietic potential.

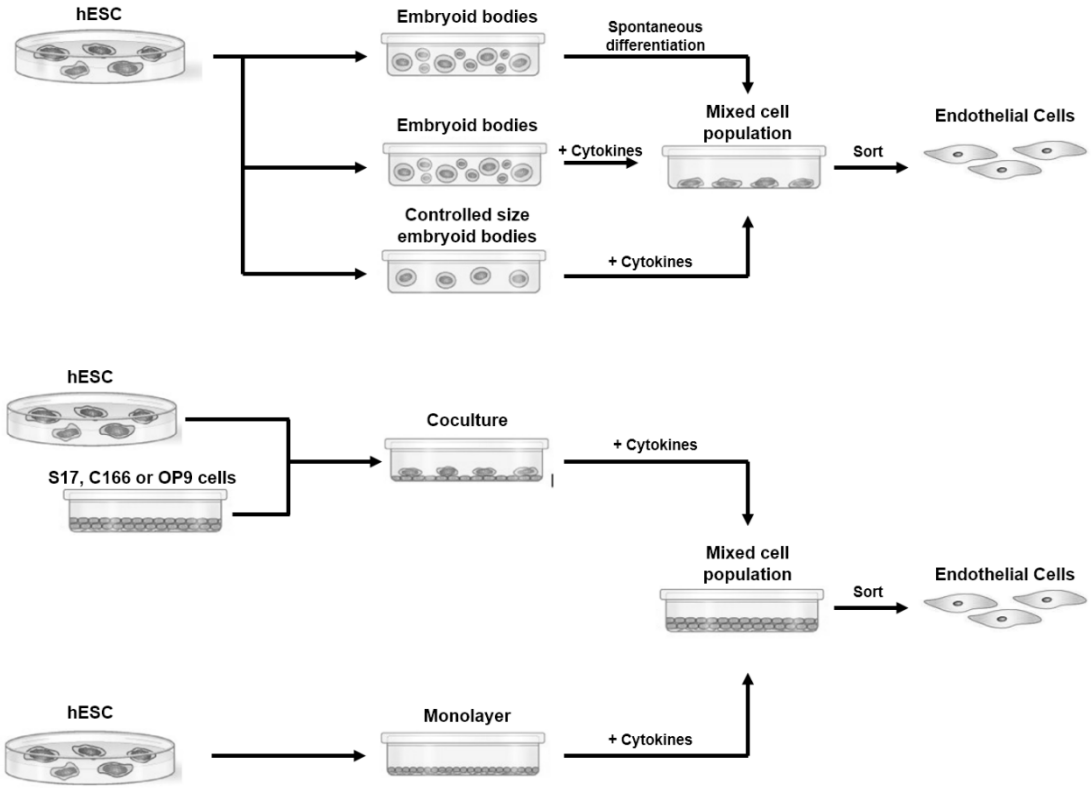


Figure 9: Endothelial differentiation approaches. *Three main approaches have been described for endothelial differentiations of hESC. Initially, hESC were differentiated in embryoid body based systems, while later methods using co-culture with other cell types or monolayer culture were described. These differentiation approached yield mixed cell populations with 2-80% of cells expressing endothelial markers (for example, CD31 or CD144). These cells then can be sorted and cultured further to obtain near pure endothelial populations. Adapted from: Mummery et al. (2012).*

While co-culture differentiation methods have been mostly focused on differentiation of hematopoietic cells, differentiation of endothelial cells has also been reported. For example, Kaufman et al. (2001) demonstrated differentiation of hematopoietic precursor cells after hESC co-culture with murine bone marrow cell line S17 or the yolk sac endothelial cell line C166. In these experiments, CD34+ CD31+ cells were also observed and suggested to identify endothelial progenitor population. Similarly, CD34+ KDR+ population was observed in a co-culture system with OP9 stromal cells by Vodyanik et al. (2005). However, co-culture differentiation methods have not gained popularity for

endothelial differentiations, perhaps due to low efficiencies and presence of other cell types in the differentiation system.

Later, first feeder and serum free endothelial monolayer differentiation system was reported by Kane et al. (2012, 2010) which was further optimised to use cGMP compliant reagents (Kaupisch et al., 2012). Extensive testing of these cells showed downregulation of pluripotency marker expression as well as upregulation of mesodermal and endothelial marker expression. Over 80% CD31+ CD144+ yield was reported after 21 day of differentiation and the differentiated cells produced NO, formed tubules *in vitro* and contributed to blood flow recovery in hindlimb ischemia model. Interestingly, downregulation of anti-angiogenic and upregulation of angiogenic micro-RNAs was observed (Kane et al., 2010) and, in addition, novel micro-RNAs regulating endothelial differentiation were identified (Kane et al., 2012).

1.8.2 Endothelial differentiation of hESC and iPSC – recent developments.

Interest in developing efficient methods for hESC endothelial differentiation has remained high and recently there have been numerous publications describing novel approaches to endothelial differentiation and providing further evidence for the utility of these cells for therapeutic angiogenesis.

Sahara et al. (2014) employed a CDH5 GFP reporter hESC line to develop and optimise a novel, two step endothelial differentiation protocol. Firstly, mesoderm was induced using BMP4 and GSK3B inhibitor; followed by treatment with VEGF, γ-secretase inhibitor DAPT, PLGF and HGF for vascular specification. This combination is reported to generate over 40% of CD144+ CD31+ cells, yielding 2.5 x 10^6 CD144+ CD31+ from 10^6 of hESCs. Further selection and expansion of CD144+ CD31+ cells increased their number to 20 million by day 14. The ability of these cells to form blood vessels *in vivo* was also confirmed, however, it was noted that cells isolated before expansion demonstrated more than three times higher efficacy of vascular tube formation in *in vivo* Matrigel plugs, when compared to HUVECs and differentiated cells expanded up to day 14. This suggests that early differentiated endothelial cells might be superior for

therapeutic angiogenesis, and thus methods for expanding these cells, while retaining their angiogenic capacity, are needed for future cell therapies.

A potential solution to this problem could be the approach taken by Prasain et al. (2014) who optimised differentiation for increased yield of cells expressing VEGF co-receptor NRP1, speculating that NRP1+ CD31+ population would represent a highly proliferative EPC population with a stable phenotype. Treatment of 10^4 hESC cells with Activin a, bFGF, VEGF and BMP4 yielded 3 x 10^4 NRP1+ CD31+ cells on day 12 of the differentiation. These cells were highly proliferative, maintained stable phenotype over prolonged culture and could be expanded to over 10^{12} cells by day 61 of culture. The ability of these cells to contribute to vascular repair *in vivo* of both ischemic retina and limb was demonstrated, however, comparison of angiogenic activity between early and expanded endothelial cells was not performed.

More recently, a significant advance was reported by Patsch et al. (2015) who screened various GSK3B inhibitors for mesodermal specification and optimised vascular specification phase to establish a high yield endothelial differentiation protocol. GSK3B inhibitor screen showed that CP21R7 induced the highest levels of mesoderm gene expression when used together with BMP4 for three days. Subsequently, both Forskolin and VEGF were shown to be required for highly efficient vascular specification and generation of CD144+ cells. Over 60% differentiation efficacy was reported, generating over 25 x 10^6 CD144+ cells from 10^6 hESC in six days.

In addition to demonstrating formation of vascular structures using commonly used *in vitro* tubule formation assay and *in vivo* matrigel plug assay, a range of other assays were performed providing a unique insight into the characteristics of derived endothelial cells (Patsch et al., 2015). Transcriptional signature of the derived cells was compared to primary cells and showed that after day 4 of the differentiation the differentiated cells lost their pluripotent stem cell signature and upregulated genes related to endothelial cell differentiation, angiogenesis, endothelium and blood vessel development, becoming highly similar to the respective primary cells. Transcriptome and metabolomic profile analysis was performed and showed signatures similar to primary endothelial cells. Additionally, the day 6 differentiated cells formed a tight monolayer and

this barrier function was responsive to thrombin, as well as various cytokines, for example VEGF, tumour necrosis factor alpha, interleukin 1B. Taken together, these cells closely resembled primary ECs both phenotypically, functionally and on a metabolic level, providing strong evidence for the suitability of such cells for clinical purposes.

Meanwhile, a low yield but high efficiency method has been reported by Wu et al. (2015). They showed that anti-adsorptive agents can be used to selectively plate out cells that give rise to almost pure (>90%) CD144+ CD31+ cultures within 5 days. Impressively, this was achieved using a minimal set of growth factors, namely, ACT-A and WNT agonist for mesoderm specification, and VEGF for endothelial specification, which on their own yielded less than 4% of CD144+ CD31+ cells. It can be speculated that this selection method could be applied to the more efficient differentiation protocols described above to obtain near-pure endothelial cell cultures without the need of cell sorting.

Currently, there is a vast amount of data demonstrating that endothelial cells derived from hESC acquire endothelial phenotype and are capable of contributing to angiogenesis, as demonstrated by a range of *in vitro* and *in vivo* assays. It can be speculated, that it would be possible to obtain clinically relevant numbers of almost pure differentiated endothelial cells with a high proliferative and angiogenic capacity by combining approaches described above. This would represent a significant advance for future CLI and PAD cell therapies, and the field of regenerative medicine.

1.8.3 Endothelial differentiation using other cell sources.

To avoid ethical and clinical issues associated with the use of hESC and iPSC, endothelial differentiations using other cell courses have been explored. These methods aim to induce or exploit plasticity of various adult cell types to generate endothelial cells without pluripotent intermediates.

Initial reports used viral vectors to partially dedifferentiate fibroblasts followed by endothelial differentiation (J. Li et al., 2013; Margariti et al., 2012). An alternative approach was employed by Kurian et al. (2013) who demonstrated that episomally delivered pluripotency factors, increase fibroblast plasticity

without inducing pluripotency and allow for differentiation of multipotent CD34+ cells. The obtained CD34+ cells were sorted and differentiated towards endothelial lineages where endothelial, arterial and venous marker expression was observed. In addition, generated endothelial cells formed tubules *in vitro* and integrated in host vasculature *in vivo*. While fibroblasts exposed to pluripotency factors did not form teratomas, the use of pluripotency factors remains an important limitation.

A direct transdifferentiation approach was reported by Veldmann et al. (2013) who demonstrated skeletal muscle transdifferentiation to endothelial lineages *in vivo* in zebrafish and *in vitro* mouse myofibroblast cultures. This established ETV2 as the main orchestrator of endothelial transdifferentiation and recently there have been multiple publications using multiple TF in addition to ETV2 for direct endothelial transdifferentiation of fibroblasts (Han et al., 2014; Van Pham et al., 2016; Wong and Cooke, 2016).

In contrast, Morita et al. (2015) screened a panel of TF factors for direct conversion of human fibroblasts into ECs and demonstrated that transient *ETV2* expression alone was able to efficiently induce endothelial phenotypes. The generated cells which formed vascular *in vitro* and *in vivo,* as well as contributed to blood flow recovery in hindlimb ischemia model. However, this method generated 30-40% of cells expressing CD309, while CD144 expression levels were not reported. Thus, it's not clear whether this approach generated stable cells expressing CD144 or just a more immature endothelial progenitor cells.

Ginsberg et al. (2015, 2012) speculated that lineage-committed amniotic fluid-derived cells might be a cell type allowing for more efficient transdifferentiation to endothelial lineages due to higher plasticity and more similar phenotype to early endothelial cells than fibroblasts. They were able to generate >80% cells expressing CD144+ by Day 21 of the differentiation by using vectors expressing ETS factors ETS related gene 1 (*ERG1*) and friend leukaemia integration 1 transcription factor (*FLI1*) constitutively in addition to transient *ETV2* expression and TGFB signalling inhibition. Transcriptome analysis revealed a full induction of endothelial gene expression, and the generated endothelial cells formed tubules in Matrigel *in vitro* and *in vivo* assays, as well as integrated

in the host vasculature. They also demonstrated that the cells were genetically stable, however the use of lentiviral vectors as well as the long differentiation protocol limits the suitability of this method for clinical setting.

The use of viral vectors should be avoided when generating cells for therapeutic angiogenesis. A novel method was reported by Sayed et al. (2015) who hypothesised that innate immunity takes part in nuclear reprogramming by increasing cell plasticity. Indeed, they were able to transdifferentiate human and murine fibroblasts to endothelial lineages, by using a toll-like receptor 3 (TLR3) agonist and endothelial differentiation media. Further evidence of the role of TLR3 was provided showing that *TLR3* knockout cells resisted transdifferentiation. Expression of pluripotency genes *NANOG* and *OCT4* was not detected. Endothelial identity and functionality was confirmed in a range of tests, including transcriptome analysis, NO production and *in vivo* hindlimb perfusion assays. This represents a significant advance for therapeutic angiogenesis as endothelial cells were generated from adult cells without the use of viral or other gene vectors, or pluripotent intermediates. However, the reported yields endothelial cell yields were low (2-4%) so currently this is only a proof of principle study and further optimisation is required to take advantage innate immunity mechanisms for therapeutic reprogramming of adult cell types.

The described methods generate endothelial cells without the use of hESC or iPSC potentially circumventing the ethical and clinical issues associated with pluripotent cells. However, most of these methods use viral vectors or suffer from limited efficacy which limits their suitability for therapeutic uses. In addition, while some adult cell sources are widely available the scale up required for angiogenetic therapies would still pose a significant challenge. Thus, endothelial differentiations of hESC or iPSC remain the most attractive approach for future clinical therapies.

1.9 Summary

In summary, CVD represents a significant clinical and socioeconomic burden. Atherosclerosis, one of the fundamental processes in CVD, can lead to PAD which has high amputation and mortality rates. Currently, there are no effective pharmacological treatments for preventing CLI and aiding angiogenesis. While the current surgical revascularisation strategies for PAD and CLI offer increased limb salvage rates, these procedures are invasive and there is a large patient population, who are not suitable for these treatments. Recent trials with gene therapy or adult cell therapies for PAD have yielded mixed results, highlighting the need for novel approaches for clinical angiogenesis therapies.

One of such approaches is the use of human embryonic or induced pluripotent stem cells. These cells are capable of differentiating towards endothelial phenotypes and, theoretically, it is possible to obtain unlimited number of pure endothelial populations that are highly suitable for clinical use. Endothelial development has been widely researched *in vivo*, providing insight into the key mechanisms driving endothelial specification. Similarly, research of angiogenesis has demonstrated the role of endothelial cells and suggests that endothelial cell based therapies could vastly benefit PAD.

Whereas, multiple endothelial differentiation approaches have been published, there's scope for further research and optimisation differentiation protocols, particularly focusing on endothelial cell yields and purity and well as clinical compatibility. Research of hESC differentiation towards endothelium *in vitro* also offers an opportunity to investigate other signalling pathways contributing to endothelial development and specification.

Development of clinically relevant endothelial differentiation protocols is crucial for progression of differentiated endothelial cell progression towards clinical therapies and offers hope to meet a significant clinical need.

1.10 Project aims.

The aims of this project were:

- To develop and optimise a clinically relevant, serum-free endothelial differentiation (hESC-EC) protocol.

- To characterise hESC-EC differentiation during the differentiation.

- To generate hESC cell lines reporting markers of interest for optimisation of hESC-EC differentiation.

- To investigate potential signalling pathways that could be exploited to increase the efficacy or yield of hESC-EC differentiations.

Chapter 2: Materials and Methods

2.1 General laboratory practice

All chemicals were supplied by Sigma-Aldrich, UK, unless otherwise stated. Control of Substances Hazardous to Health (COSHH) guidelines were followed when handling hazardous substances. Powder free nitrile gloves and laboratory coat was worn during work at the laboratory. Laboratory spectacles, facemask and fume hood was used, if appropriate.

2.2 Tissue culture

All tissue culture was performed in standard biological safety class II vertical laminar flow cabinets under sterile conditions. Cells were cultured at 37°C in a humidified incubator atmosphere maintained at 5% CO_2.

2.2.1 hESC culture

Human embryonic stem cell (hESC) lines H1 and H9 (Thomson et al., 1998) supplied by WiCell, USA and cGMP clinical grade lines RC-11 and RC-9 (Roslin Cells Ltd, Edinburgh) used with approval of the UK Stem Cell Bank steering committee, were cultured in StemPro media (Thermo Fisher Scientific, UK) (Table 2) in 6 well format tissue culture plates coated with CellStart matrix (Thermo Fisher Scientific, UK) and later Vitronectin (Thermo Fisher Scientific, UK) as shown in Table 3. Cultures received daily media exchange six times a week. Cultures were allowed to reach 90% confluence before passaging to avoid spontaneous differentiation. For passage, cultures were washed with Phosphate Buffered Saline (PBS) (Thermo Fisher Scientific, UK), supplemented with fresh StemPro media, cut to small colonies using StemPro EZ-Passage tool (Thermo Fisher Scientific, UK) and seeded in a 1:2 to 1:6 ratio in new culture plates. Cells were obtained from cell stocks tested for normal karyotype prior to freezing. As the risk of genetic changes increases with prolonged culture (Lund et al., 2012), hESCs were not cultured above passage 50.

Component	Concentration
DMEM F12 + GlutaMAX	
StemPro hESC supplement	1x
BSA	1.8%
Mercaptoethanol	0.1mM
bFGF	20ng/ml

Table 2: Components of StemPro media.

ECM	Supplier	Volume and concentration per well	Incubation
Cellstart	Thermo Fisher Scientific, UK	750µL	2h at 37°C
Vitronectin	Thermo Fisher Scientific, UK	500µL, 25µg/mL	1h at RT
Gelatin	Sigma-Aldrich, UK	1mL 0.1%	1h at RT

Table 3: Extracellular matrices used for hESC culture and differentiation.

2.2.2 hESC cryopreservation and recovery

For cryopreservation, hESC cultures were allowed to reach 90% confluency, washed with PBS, supplemented with 0.9mL fresh StemPro media and cut to small colonies using StemPro EZ-Passage tool. 0.9mL of freezing solution (60% KOSR, 20% DMSO, and 20% DMEM F12 + GlutaMAX) was added. The suspension was transferred to a cryovial, placed in a Mr. Frosty Freezing Container with isopropanol (Thermo Fisher Scientific, UK) and stored at -80°C. This ensures controlled rate freezing of approximately 1°C/min, which is optimal for cell preservation. The following day, cryovials were transferred to liquid nitrogen for prolonged storage.

To recover frozen hESC cultures, cryovials were placed in a 37°C waterbath. Before complete thawing, prewarmed StemPro media was added dropwise and cells were transferred to a 15mL Falcon, centrifuged at 300 x g for 3 minutes and resuspended in StemPro supplemented with 10μM Y-27632 (Tocris, UK) to reduce apoptosis after thawing (Watanabe et al., 2007).

2.2.3 NCI60 cancer cell line culture

NCI60 cancer cell lines (IGROV-1, K562, OVCAR3) were cultured in RPMI1640 media (Thermo-Fischer, UK) supplemented with 10% foetal bovine serum (FBS, Thermo Fischer, UK), 100U/mL penicillin, 100μg/mL streptomycin, 10μg/mL gentamicin (Thermo Fisher Scientific, UK) and 1μg/mL Amphotencin B (Thermo Fisher Scientific, UK). Cells were cultured in tissue culture treated T75 or T150 flasks and received 100% feeds every 2-3 days. Cultures were allowed to reach near confluence, dissociated using 0.05 % Trypsin, 0.53mM EDTA and split in a 1:8 ratio.

2.2.4 Human embryonic kidney 293T cell line culture

Human embryonic kidney 293T cells (HEK293T, ATCC, UK) were cultured in MEM media (Thermo Fisher Scientific, UK) supplemented with 100μg/mL penicillin, 100μg/mL streptomycin, 2mM L-Glutamate and 1mM sodium pyruvate (Thermo Fisher Scientific, UK). HEK293T were passaged 1:6 using 1x citric saline (10mg/ml potassium chloride, 4.4mg/ml sodium citrate) after reaching 70% confluence.

2.2.5 Human saphenous vein endothelial cell (HSVEC) and human umbilical vein endothelial cell (HUVEC) culture

Human saphenous veins were obtained from patients undergoing coronary bypass graft at the Golden Jubilee National Hospital, or patients undergoing elective varicose vein stripping at the Glasgow Gartnavel General. The procedure was approved by Glasgow West Ethics committee. Written informed consent was required from the patients prior to participation.

HSVECs and HUVECs were cultured in Cascade Biologics Medium 200 (Thermo-Fischer, UK) supplemented with Low Serum Growth Supplement Kit (Thermo-

Fischer, UK), 20% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2mM L-Glutamine. Cells were cultured in tissue culture treated T25 or T75 flasks and received 100% feeds every 2-3 days. Cultures were allowed to reach near confluence, dissociated using 0.05 % Trypsin, 0.53mM EDTA and seeded at 2,500-5,000 cells per cm^2.

2.3 hESC-EC differentiation

For hESC differentiation towards endothelial cell lineages (hESC-EC differentiation), hESCs were grown to full confluence, cultures were dissociated with TrypLE select (Thermo Fisher Scientific, UK) and controlled size embryoid bodies (10 000 cells per EB) were formed using round bottom tissue culture 96 well plates (Corning, UK) coated with 5% Pluronic F-127, following the Spin-EB protocol as described by Ungrin et al. (2008) in 100µl Stemline media (Sigma-Aldrich, UK) with 10ng/ml BMP4, 10ng/ml VEGF, 5ng/ml Activin A, (all Peprotech, US), 10ng/ml Wnt3a (R&D Biosystems, UK) and 10µM Y-27632 (Tocris, UK) as described by Olivier et al. (2016). This is Day 0 of the differentiation.

The cultures were further supplemented with 20ng/ml BMP4, 30ng/ml VEGF, 10ng/ml WNT3a and 5ng/ml Activin A on Day 2 of the differentiation. On Day 3, the formed embryoid bodies were plated out on wells coated with 0.1% Gelatin solution (Table 3) and cultured in Day 3 media, consisting of EBM-2 media (Lonza, UK) supplemented with EGM-2 SingleQuots (Lonza, UK). FBS and VEGF from the kit were excluded and the media was supplemented with 50ng/ml VEGF (Peprotech, US). Cultures were given 100% media exchange on Day 5. Day 7 is the end of the differentiation.

2.3.1 Intracellular cAMP level quantification during hESC-EC differentiation

To determine optimal Forskolin concentrations for increasing intracellular cAMP levels during hESC-EC differentiation, Promega cAMP Glo Max kit (Promega, UK) was used as per manufacturer's instructions. Briefly, Day 3 embryoid bodies were incubated in Day 3 media supplemented with 100µM IBMX and Forskolin at concentrations ranging from 0.1µM - 50 µM (induction buffer). DMSO was used as a negative control.

After 1 hour stimulation, each EB was plated in a well of a 96 well plate in 37.5µL of the induction buffer. 2.5 µl of 1M $MgCl_2$ and 10µl of cAMP Detection Solution with PKA were added to each well and the plate was mixed by shaking for 1 minute. During the incubation, cAMP stimulates PKA activity, depleting

ATP. After 20 minute incubation, 50 µl of Kinase-Glo® Reagent was added to each well and plate was mixed by shaking for 1 minute.

Kinase-Glo® Reagent contains Luciferase which requires ATP for production of oxyluciferin and subsequently light. In this coupled PKA-Luciferase reaction, increase of cAMP levels, decreases bioluminescent signal. Luminiscence was measured after 10 minute incubation with Victor X3 plate reader (PerkinElmer, UK).

2.4 Generation of reporter construct plasmid vectors

Eukaryotic expression plasmids were used to generate reporter constructs where promoter of a gene of interest drives expression of either eGFP or RFP reporter protein.

2.4.1 Transformation of chemically competent bacteria

DH5α Max (Thermo Fisher Scientific, UK) or JM109 (Promega, UK) chemically competent *E.Coli* bacterial strain was used to produce plasmids. For each transformation 5ng for DH5α or 25ng for JM109 of plasmid of interest or ligation product was added to thawed 50µL of cells kept on ice and carefully mixed. After 30 minute incubation on ice, heat shock was used to induce uptake of the plasmid by placing tubes in a 42^{o}C water bath for a 40 seconds, after which, tubes were returned on ice for further two minutes. 900µL for DH5α or 450µL for JM109 of Super Optimal broth with Catabolite repression (SOC) medium (Thermo Fisher Scientific, UK), consisting of 2% tryptone, 0.5% yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride, 10mM magnesium sulphate and 20mM glucose, was added to the tubes, followed by incubation at 37˚C for 1 hour in a shaking incubator set to 180 oscillations per minute. 20-50µL of the resulting mixture was plated on to Luria-Bertani agar plates containing 100µg/mL ampicillin or 50µg/mL Kanamycin, as required. After 5 minutes, plates were inverted, incubated and checked for colony growth the next day.

2.4.2 Low volume plasmid DNA purification

Purification of plasmid DNA from low volume *E.Coli* bacterial cell culture was performed using Wizard® Plus SV Minipreps DNA Purification System (Promega, UK). A single well isolated colony was picked to inoculate 12mL of Luria broth media with added 100µg/mL ampicillin or 50µg/mL Kanamycin, as required. After 6 - 8 hour incubation at 37˚C in a shaking incubator set to 180 oscillations per minute, 2mL of the solution was stored at 4^{o}C and bacterial cells were harvested by spinning the remaining solution at 6000 × g for 15 minutes. The pellet was resuspended in 250µl of Cell Resuspension Solution with RNAse A by pipetting up and down thoroughly and transferred to an eppendorf tube. 250µl

of Cell Lysis Solution, containing SDS to solubilize cell membrane and denature proteins and NaOH to denature DNA, was added, the tube was inverted few times to mix and incubated in room temperature until the solution cleared. 10µl of Alkaline Protease Solution was added to the solution, tube was inverted to mix and incubated for 5 minutes. Alkaline protease lyses various proteins, including endonucleases. 350µl of Neutralization Solution containing guanidine hydrochloride and glacial acetic acid was added to the solution, making the solution acidic, rendering alkaline proteases inactive and renaturing plasmid DNA. Renatured plasmid DNA dissolves in the solution while genomic DNA and proteins form precipitate. Tube was inverted few times and centrifuged at 16 000 x g for 10 minutes.

The cleared lysate was carefully transferred to the spin column, followed by centrifugation at 16 000 x g for 1 minute. Plasmid DNA from the cleared lysate is bound to the spin column membrane. This was followed by two washes with column wash solution of 750µl and 250µl, 1 and 2 minute centrifugation respectively at 16 000 x g. Any remaining contaminants, including RNA, are washed away during this step. To prevent any carryover of the column wash solution and to remove any residual ethanol, the spin column was placed in a fresh eppendorf tube and another spin was performed. Plasmid DNA was eluted by adding 100µl of Nuclease-Free Water to the Spin Column and centrifuging at 16 000 g for 1 minute. Restriction digests were performed to confirm identity of the plasmid and the presence of an insert as per 2.4.6 . If required, the plasmid DNA solution was stored at -20^{o}C.

2.4.3 High volume plasmid DNA purification

High volume plasmid DNA purification was performed using PureLink® HiPure Plasmid Filter Purification Maxi Kit (Thermo Fisher Scientific, UK). Bacterial solution from the low volume plasmid DNA purification (2.4.2) was used to inoculate up to 500mL of LB broth followed by overnight incubation at 37^{o}C in a shaking incubator set to 180 oscillations per minute. Bacterial cells were harvested by centrifugation at 6000 × g for 15 minutes. The pellet was resuspended in 10mL Resuspension Buffer containing RNase A. 10 mL of Lysis Buffer was added, the solution was mixed by inverting and incubated at room temperature for 5 minutes. 10 mL of Precipitation Buffer, containing potassium

acetate to renature plasmid DNA, was added. The solution was inverted to mix and centrifuged at 12 000 × g for 10 minutes.

The supernatant was transferred to an anion exchange column that was previously equilibrated with 30 mL Equilibration Buffer, and allowed to drain by gravity flow. The negatively charged phosphates on the backbone of the DNA bind the positively charged surface of the resin. 60mL of Wash Buffer (W8) was added to the column and allowed to drain by gravity flow. This buffer has moderate NaCl content and washes away RNA, proteins and carbohydrates, while DNA remains bound to the column resin. 15mL of high NaCl content Elution buffer (E4) was added to the column and allowed to drain by gravity flow. This elutes DNA from the resin.

DNA was precipitated from the elution buffer by adding 10.5mL of isopropanol, mixed and centrifuged at 12 000 × g for 30 minutes at 4°C. Supernatant was discarded and the pellet was washed in 5mL of 70% ethanol. The tube was centrifuged at 12 000 × g for 5 minutes at 4°C, the supernatant was discarded, tube was inverted and pellet was allowed to air dry for 10 minutes at room temperature. DNA was resuspended in 200µL of nuclease free water. If required, the plasmid DNA solution was stored at -20ºC.

2.4.4 PCR

Phusion High-Fidelity PCR Kit (NEB, UK) was used for amplification of fragments of interest from plasmid or genomic DNA as per manufacturer's instructions, using PCR reaction components as shown in Table 4 and cycling conditions as shown in Table 5. Primers were designed to include endonuclease restriction digestion sites, if required. Annealing temperature was based on average calculated annealing temperature (T_m) of both primers and was tested prior to PCR.

Component	Concentration
5X Phusion HF Buffer	1X
10mM dNTPs	200µM
10µM Forward Primer	0.5µM
10µM Reverse Primer	0.5µM
Template DNA	< 250ng
DMSO (optional)	3%
Phusion DNA Polymerase	1 unit/50µl PCR

Table 4: PCR reaction components.

Cycle Step	Cycles	Temperature	Time
Initial denaturation	1	98˚C	30 seconds
Denaturation		98˚C	10 seconds
Annealing	34	45-72˚C	30 seconds
Extension		72˚C	30 seconds
Final extension	1	72˚C	10 minutes
Hold	1	4˚C	∞

Table 5: Cycling conditions for a routine PCR.

2.4.5 PCR purification

PCR clean-up was performed using QIAquick PCR Purification Kit (Qiagen, UK). Briefly, 5 volumes of Buffer PB were added to 1 volume of PCR product, the solution was applied to QIAquick spin column and it was centrifuged at 16 000 x g for 1 minute. The flow through was discarded, 750µL of Buffer PE containing ethanol was added to the spin column followed by another spin at 16 000 x g for 1 minute. The flow through was discarded, the column was placed in the empty collection tube and centrifuged at 16 000 x g for 2 minutes to

remove any residual ethanol. The spin column was placed in a new eppendorf tube, 30µL of nuclease free water was applied to the membrane followed by a 1 minute incubation and centrifugation at 16 000 x g for 1 minute.

2.4.6 Restriction digestion

Double restriction digestions were performed as per manufacturer's instructions using endonucleases manufactured by NEB, UK, unless otherwise stated. When small amounts of DNA were required, 20µL reactions were used, containing 100-200ng of DNA, 0.5µL of restriction endonucleases and 2µL of 10x buffer. The reactions were incubated for 1-2 h at 37°C. For larger amounts of DNA, 50µL reactions were used, containing up to 10µg of DNA, 5µL of restriction endonucleases and 5µL of 10x buffer. The reactions were incubated overnight at 37°C.

2.4.7 Agarose gel electrophoresis

Agarose gel eletrophoresis was used to confirm DNA fragment sizes after PCR or endonuclease digestion, as well as to separate successfully cut plasmids. Current is applied to an the gel immersed in 1x Tris/Borate/EDTA (TBE) solution (10mM Tris, 10mM boric acid, 10mM EDTA, pH 8.3) and negatively charged DNA migrates through the gel towards the positive electrode. Varying the percentage of the agarose gel changes the pore size with higher percentages resulting in smaller pores, limiting migration of larger DNA fragments, this way separating the fragments by size.

0.8 - 1.5% w/v agarose gels were used (Thermo Fisher Scientific, UK) with 0.2-0.5µg/mL ethidium bromide for DNA band visualisation using ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories, UK). 6x blue/orange loading dye (Promega, UK) was added to the DNA solution at the time of loading the DNA and constant voltage of 30V - 100V was applied. 1Kbp and 100bp ladders (both Promega, UK) were used to estimate DNA fragment size.

2.4.8 DNA gel extraction

To separate plasmids after endonuclease restriction digestion, the digestion product was run on agarose gel as described in 2.4.7 and bands were visualized

under trans UV illumination using a DNA ladder (100bp and 1Kbp, both Promega, UK) as a reference. The band of interest was cut with a clean scalpel blade and gel extraction was performed using Wizard® SV Gel and PCR Clean-up System (Promega, UK) as per manufacturer's instructions. 10µL of Membrane Binding Solution (4.5M guanidine isothiocyanate, 0.5M potassium acetate, pH 5.0) was added per every 10µg of gel, followed by incubation in a 65^{o}C waterbath until the gel was fully dissolved. The solution was incubated on the provided silica gel column for 1 minute, followed by centrifugation 16 000 x g for 1 minute. Two washes, 700µL followed by 500µL, with membrane wash solution (10mM potassium acetate pH 5, 80% ethanol, 16.7µM EDTA pH 8.0) were performed. This was followed by centrifugation at 16 000 x g for 5 minutes to remove any remaining ethanol. The spin column was placed in a new eppendorf tube, 50µL of nuclease free water was applied to the membrane followed by a centrifugation at 16 000 x g for 1 minute.

2.4.9 Dephosphorylation of plasmid DNA

To prevent spontaneous recirculation of plasmid DNA after restriction digestion, the plasmid DNA was treated with Antarctic Phosphatase (NEB, UK). Briefly, appropriate volume of 10X Antarctic Phosphatase Reaction Buffer was added to up to 5µg plasmid DNA in nuclease free water. 1 µl of Antarctic Phosphatase was added, the solution was gently mixed, followed by 15 minute incubation at 37°C. Antarctic Phosphatase was inactivated by incubation at 70°C for 5 minutes.

2.4.10 Ligation of insert into plasmid backbone

Inserts were ligated into plasmid backbone using Quick Ligation Kit (Promega, UK). Molar ligation ratios were calculated using formula below and 1:3, 1:1 and 3:1 molar ratios were used:

$$Insert\ ng = Vector\ ng \times \frac{Insert\ fragment\ size\ Kbp}{Vector\ fragment\ size\ Kbp} \times ratio\ \frac{Insert}{Vector}$$

For each ligation reaction, 50ng of Vector plasmid was put in an eppendorf tube and volume was adjusted to 9µL with nuclease free water. 10µL of 2x Quick Ligase buffer and 1µL of quick T4 ligase was added to the tube and mixed. The

solution was incubated at room temperature for 5 minutes, followed by refrigeration at 4^{o}C or storage at -20^{o}C.

2.4.11 DNA sequence analysis

DNA sequences were analysed by dideoxy sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, UK). Sequencing PCR reaction was set up with reagents as shown in Table 6 and made up to the final volume with nuclease free water. PCR reaction consisted of 20 cycles of 96˚C for 50 seconds, 50˚C for 20 seconds, 60˚C for 2 minutes.

Reagent per reaction	
DNA	200 – 500ng
BigDye Sequencing buffer 5x	4µL
Ready Reaction Mix	1µL
Sequencing Primer (2µM)	2µL
Final volume	20µL

Table 6: Reagents used in sequencing PCR reactions.

Sequencing PCR reactions were purified using Agencourt CleanSeq (Beckman Coulter Ltd, UK). Briefly, 10µL of CleanSeq reagent and 62µL of 85% ethanol was added to each well followed by brief vortexing and centrifugation. The reagent contains paramagnetic beads coated with carboxyl molecules which bind DNA in the presence of polyethylene glycol and salt. The plate was put on a magnetic block for two minutes, and put upside down to remove ethanol while beads with bound DNA remain in plate. This was followed by 150µL of 85% ethanol wash, excess ethanol was removed by brief upside-down centrifugation while keeping plate on the magnet block, after which the plate was allowed to air dry. Plate was removed from the magnet block, 40µL of nuclease free water was added to each well and the DNA was resuspended by pipetting up and down. Plate was returned to magnet block for two minutes, 20µL of the cleaned up sequencing product was loaded into optically clear 96 well plates and DNA sequencing was

performed using Applied Biosystems 3730 Genetic Analyser (Applied Biosystems, UK).

2.4.12 Plasmid transfections

Generated reporter plasmids were transfected in HUVEC and HSVEC using cationic lipid Lipofectamine LTX (Thermo Fisher Scientific, UK) following manufacturer's instructions. Briefly, 4 x 10^4 cells were seeded per well in a 24 well format. For each well, 0.5µg of plasmid DNA were diluted in 100µL of Optimem reduced serum media, 0.5µL of Plus reagent was added and the solution was incubated for 10 minutes. 1.5µL of Lipofectamine LTX was added and the solution was incubated for 25 minutes. Cultures were supplemented with fresh 0.5mL of media as per 2.2.5 and the solution containing DNA-Lipofectamine complexes was added.

For transfections of NCI60 cell lines, Lipofectamine 2000 (Thermo Fisher Scientific, UK) was used following manufacturer's instructions. Briefly, 2 x 10^5 cells were seeded per well in a 24 well format in 0.5mL of OptiMem reduced serum media. For each well, 0.5µg of plasmid DNA were diluted in 25µL of Optimem, and 1.5µL of Lipofectamine 2000 were diluted in separate 25µL of Optimem. These two solutions were combined, incubated for 5 minutes and added to cells. After 6 hour incubation, media was replaced to fresh NCI60 culture media as per 2.2.3 .

2.5 Generation of reporter construct lentiviral vectors

2.5.1 Production of lentivirus

Second generation, self-inactivating lentiviral vectors were used. HEK293T cells were cultured in T150 flasks and passaged in a 1:2 ratio the day before transfection. The following day, triple transient transfection was performed using polyethylenimine (PEI). PEI is a cationic polymer that forms dense particles with DNA that accumulate next to negatively charged cell membranes and are subsequently endocytosed. PEI containing endosomes are susceptible to lysis, releasing the DNA in cell cytoplasm (Boussif et al., 1995; Sonawane et al., 2003).

Briefly, per each T150 flask, 32.5µg of packaging plasmid Int, 17.5µg of plasmid encoding the vesicular stomatitis virus envelope (pMDG) (Plasmid Factory, Bielefeld, Germany) and 50µg of the generated reporter plasmid were added to 5mL Optimem reduced serum media with Glutamax (Thermo Fisher Scientific, UK) and combined with 5mL of the same media with 2µM PEI. The combined reagents were incubated for 20 minutes at room temperature in dark to allow formation of PEI-DNA polyplexes.

Each flask of HEK293T cells was washed with 10 mL of Optimem media and 10mL of polyplex Optimem solution was added per flask. The cells were incubated for 4 hours at 37°C in 5% CO_2. After the incubation, the solution was replaced with 20mL of HEK293T medium. Transfected cultures were incubated at 37°C in 5% CO_2 and media containing lentiviral particles was removed after 48 hours. Another 10mL of HEK293T medium was added and cultures were incubated for further 24 hours after which media was removed. The collected media was sterile filtered through a 0.22µM filter and stored at 4°C in dark prior to concentration.

2.5.2 Concentration of lentivirus

Ultra-clear centrifuge tubes (Beckman Coulter Ltd, Buckinghamshire, UK) were washed with 70% ethanol and sterile PBS. Collected media with lentiviral particles was put in tubes and centrifuged in Optima L-80 XP Ultracentrifuge (Beckman Coulter) at 23 000rpm at 4ºC for 1 hour. The media was poured off

carefully, and tubes were topped up with the remaining collected media. Spins were repeated, until all the collected media with lentiviral particles was processed. After the final spin, media was poured off and the tubes were drained by placing them upside down on a layer of tissue paper. After 1-2 minutes, 100µl of Optimem media was added to each tube and the tubes were placed on ice for 20 minutes. The media was mixed by pipetting up and down several times and aliquoted in individual single use aliquots that were stored at -80^{o}C.

2.5.3 Determination of functional lentivirus titre

Functional lentivirus titre was determined using qPCR based assay as described by Butler et al. (2001). Viral genomic RNAs are reverse transcribed to yield linear viral cDNA. This method uses a pair of primers and probes to detect late reverse transcription products. The primer and probe sequences are as follows:
forward: 5'-TGTGTGCCCGTCTGTTGTGT-3';
reverse: 5'- 75 GAGTCCTGCGTCGAGAGAGC-3';
probe: 5'-(FAM)-CAGTGGCGCCCGAACAGGGA- (TAMRA)-3').

HEK293T cells were seeded at the density of 5 x 10^4 per well in a 12 well format. After 24 hours, the media was replaced with 1mL fresh media and serial dilutions from 10^{-2} to 10^{-6} were made from a thawed concentrated lentivirus aliquot and added to the wells. 72 hours later, cells were washed with PBS and 200µL of PBS was added to each well. Cells were snap frozen followed by DNA extraction as per 2.6.1 and DNA was eluted in 50µL of nuclease free water. The DNA concentration was estimated using Nanodrop (ND-1000 spectrophotometer, Labtech International, UK) and values were kept for later calculations. All the samples were diluted to a DNA concentration of 250ng/µL. A standard curve was generated using diluted expression plasmid corresponding to 10^{13} to 10^4 plasmid copies.

The amount of plasmid required for 10^{13} copies was determined as follows:

1. Molecular weight of a single expression plasmid was calculated:

$$\frac{size\ of\ plasmid\ (bp)\ \times 660\ daltons}{6.023\ \times 10^{23}} = g\ per\ molecule$$

2. Plasmid copy number in 1mL was calculated:

$$\frac{concentration\ of\ plasmid\ (g/mL)}{g\ per\ molecule} = plasmid\ copy\ number$$

3. Volume of plasmid stock for $1x10^{13}$ copies for top standard:

$$\frac{10^{13}}{plasmid\ copy\ number} \times 1000 = \mu L\ of\ plasmid\ stock$$

The qRT-PCR reaction was run as per Table 7 with reactions set up as shown in Table 7.

Reagent per reaction	
DNA samples or standard curve	1µL
Taqman universal master mix 2x	6.25µL
Primer/probe mix	3.125µL
H20	2.125µL

Table 7: qRT-PCR reaction for determination of lentivirus titre.

Lentivirus titre was calculated as follows:

1. Standard curve was obtained by plotting obtained cycle threshold (Ct) values vs logarithmic plasmid copy number per sample in Excel and the best fit formula was used calculate total number copies of viral DNA per each of the infected culture wells.

2. Total amount of DNA in ng collected from each well was calculated by multiplying individual DNA concentration readings by elution volume of 50µL.

3. Percentage of total DNA used per reaction was calculated:

$$\frac{250ng\ DNA\ used\ per\ reaction}{total\ DNA\ collected\ ng} = \%\ total\ DNA\ used$$

4. Percentage of total DNA used was multiplied by cells seeded per well (5×10^4) to estimate the number of cells used for 250ng of DNA.

5. Values obtained in step 1 and 4 were used to calculate number of viral copies per cell:

$$\frac{total\ copies\ of\ viral\ DNA}{estimated\ cell\ number} = viral\ DNA\ copies\ per\ cell$$

6. And finally PIU/mL was calculated:

$$\frac{dilution\ factor}{volume\ added} \times 1000 \times viral\ DNA\ per\ cell = PIU/mL$$

This calculation was performed for every infected HEK293T well and the average PIU/mL between all the wells was used to calculate volume of concentrated viral solution required in experiments to achieve desired multiplicity of infection (MOI) which is a ratio between virus and cells.

2.5.4 Lentiviral Transductions

For lentiviral transductions of pluripotent hESC, cells were dispersed using Accutase (Thermo Fisher Scientific, UK) and 1 x 10^4 to 3 x 10^4 cells were seeded onto tissue culture treated 24 wells coated with vitronectin as per 2.2.1 . The following day, media was exchanged and supplemented with 4µg/mL of polybrene (hexadimethrine bromide). Polybrene is a cationic polymer that enhances lentiviral transduction efficiency by increasing receptor independent uptake of the virus via cell membranes (Davis et al., 2002). Lentivirus was added at the appropriate MOI and cultures were incubated for 24 hours, followed by two PBS washes and addition of fresh media to the wells.

For lentiviral transductions of HSVEC and HUVEC or NCI60 cancer cell line cells, 2 x 10^4 cells were seeded per well in a 48 well format. The following day, media was exchanged and supplemented with 8µg/mL of polybrene. Lentivirus was added at the appropriate MOI and cultures were incubated for 24 hours, followed by two PBS washes and addition of fresh media to the wells.

2.6 Molecular Biology

2.6.1 Extraction of DNA from cells

Extraction of total DNA from cell cultures was performed using QIAamp DNA mini and blood mini kit (Qiagen, UK) following manufacturer's instructions. Cultures were washed in PBS and 200µl of PBS was added to each well. Cell scraper or vigorous pipetting was used to detach the cells and the resulting suspension was transferred to a microcentrifuge tube containing 20 µl of Qiagen Proteniase K. 200µL of buffer AL, containing guanidine hydrochloride that disrupts DNA and RNA, was added and the sample was thoroughly mixed by pulse vortexing. The suspension was incubated at 56^{o}C for 10 minutes for efficient lysis. Then, 200µL of ethanol was added, the samples was further mixed by pulse-vortexing and applied to the spin column. QIAamp spin column silica membrane selectively binds DNA and RNA, while protein and other contaminants are washed away in the two following washes, 500µl each. First wash buffer AW1 contains guanidine hydrochloride and ethanol, while the second was buffer contains only ethanol to wash away any residual salts. This was followed by DNA elution in 50µL room temperature sterile nuclease-free water. The DNA solution was stored at -20^{o}C.

2.6.2 Extraction of total RNA from cells

Extraction of total RNA from cell cultures was performed using miRNeasy kit (Qiagen, UK) following manufacturer's instructions. Cell monolayer was washed with PBS and 700µL QIAzol Lysis Reagent was added, followed by vigorous pipetting to dislocate the cells. The solution was transferred to a microcentrifuge tube and mixed by pulse vortexing for 1 minute followed by 5 minute incubation. QIAzol Lysis Reagent is a phenol / guanidine based solution that effectively lyses tissues and preserves RNA by inhibiting RNases. 140 µL of chloroform was added followed by mixing of the sample and further 2 minute incubation at room temperature. After 15 minute centrifugation at 12 000 x g at 4^{o}C, the sample separates in three phases. In low pH conditions, the upper aqueous phase contains RNA and is separated by a white, DNA containing interphase from lower red organic phenol-chloroform phase with proteins and lipids (Chomczynski and Sacchi, 2006; Rio et al., 2010). The upper phase was transferred to a new microcentrifuge tube and mixed with 1.5x volumes 100%

ethanol. This enhances the binding of larger RNA molecules (>18 nucleotides) to the silica membrane of the RNeasy mini spin column. The solution was loaded onto the column and centrifuged at 8000 x g for 30 seconds, followed by a 700µL wash with guanidine hydrochloride and ethanol containing Buffer RWT. DNAse I treatment was performed to remove any residual DNA. This was followed by another wash with RWT buffer and two 500 µL washes with ethanol based Buffer RPE. Second wash was followed by a prolonged centrifugation to remove any residual ethanol from the column membrane. The RNA was eluted in 30 - 50 µL of nuclease free water added directly to the membrane and passed through twice to increase the RNA concentration. The concentration of RNA was estimated by Nanodrop (ND-1000 spectrophotometer, Labtech International, UK). The RNA was stored at -80°C, if required.

2.6.3 cDNA synthesis

To synthesise cDNA from the extracted RNA, Taqman reverse transcription kit (Thermo Fisher Scientific, UK) was used as per manufacturer's instructions in 20µL reactions as described in Table 8 and Table 9.

Reagent per reaction	
10x buffer	1x
$MgCl_2$	5.5mM
dNTP mix	2mM
Random hexamers	2.5µM
RNAse inhibitor	0.4U/µL
Multiscribe	1.25U/µL
RNA	200 – 500ng

Table 8: cDNA synthesis reaction.

Cycle Step	Cycles	Temperature	Time
Primer annealing	1	25˚C	10 minutes
Reverse transcription	1	48˚C	30 minutes
Inactivate multiscribe	1	95˚C	5 minutes
Hold	1	4˚C	∞

Table 9: cDNA synthesis cycling conditions.

2.6.4 qRT-PCR analysis

Gene expression analysis was performed using Taqman gene expression assays (Thermo Fisher Scientific, UK) as shown in Table 10 in 10µL reactions in 384-well format with each reaction containing 1 x Taqman Master Mix, 1 x TaqMan gene expression probe, 10-20ng cDNA and nuclease free H_2O as required. ABI Prism Applied Biosystems 7900HT or QuantStudio™ 7 Flex System real time PCR system was used with cycling conditions as shown in Table 11.

Gene name	Probe ID
Housekeeper genes	
UBC	Hs01871556_s1
GAPDH	Hs02758991_g1
Endothelial genes	
PECAM-1 (CD31)	Hs00169777_m1
CDH5 (VE-Cadherin, CD144)	Hs00901465_m1
KDR	Hs00911700_m1
PDGFB	Hs00966522_m1
ROBO4	Hs00219408_m1
Arterial genes	
HEY2	Hs00232622_m1
EFNB2	Hs00970627_m1
DLL4	Hs00184092_m1
Venous genes	
EPHB4	Hs00174752_m1
NRP2	Hs00187290_m1

Gene name	Probe ID
Pluripotency genes	
NANOG	Hs04399610_g1
POU5F1 (OCT4)	Hs00999632
SOX2	Hs01053049_s1
Other genes	
ETV2	Hs01012850_g1
*T (*Brachyury*)*	Hs00610080_m1
APLN	Hs00936329_m1
APLNR	Hs00270873_s1
NFATC1	Hs00542678_m1
SEMA3D	Hs00380877_m1
DACH1	Hs00362088_m1
TAL1	Hs01097987_m1

Table 10: List of Taqman gene expression assays used.

Cycle Step	Cycles	Temperature	Time
Initial denaturation	1	95°C	10 minutes
Denaturation	40	95°C	10 seconds
Annealing and Extension		60°C	60 seconds

Table 11: qRT-PCR cycling conditions.

Data was analysed by using the comparative Ct (cycle threshold) method (Schmittgen and Livak, 2008). Ct is the PCR cycle at which the fluorescent signal reaches a threshold level set in the exponential phase of the PCR amplification. For each sample, an internal gene expression control is analysed in addition to genes of interest. The obtained internal control Ct value was subtracted from Ct value for gene of interest:

$$Ct\ gene\ of\ interest - Ct\ internal\ control = \delta Ct$$

To compare difference in gene expression between two samples, the obtained δCt values were subtracted:

$$\delta Ct\ sample\ 1 - \delta Ct\ sample\ 2 = \delta\delta Ct$$

And the fold change (relative quantification, RQ) was calculated:

$$RQ = 2^{-\delta\delta Ct}$$

RQ_{min} and RQ_{max} values were used to plot error bars, showing the range of possible RQ values with a confidence interval set at 95%. SEM of δδCT values was calculated and preRQ max and min values were obtained:

$$preRQ = 2^{-(RQ \pm SEM\ of\ \delta\delta CT)}$$

Then RQ_{max} and RQ_{min} values were calculated as follows:

$$RQmax = preRQmax - RQ$$

$$RQmin = RQ - preRQmin$$

2.7 Immunocytochemistry (ICC)

For Immunocytochemistry, cells in a 12 well format were washed with PBS and fixed with either cold 4% paraformaldehyde or 1:1 acetone and methanol mix cooled to -20^{o}C. Cells were washed with PBS three times and permeabilised, if required, with 0.5% Tween (Tween 20 diluted in PBS) for 30 minutes. Cells were washed with 0.1% Tween and incubated with 20% Normal Goat Serum (NGS) for 1 hour. This was followed by another wash with 0.1% Tween and cells were incubated overnight at 4^{o}C with primary antibodies diluted in 2% NGS. After the incubation, cells were washed three times with 0.1% Tween, 5 minutes per wash. Diluted secondary antibodies were added as appropriate and cells were incubated in dark for 1 hour at room temperature. This was followed by 2 washes with 0.1% Tween and two washes with PBS, 5 minutes each. Finally, 15µl of ProLong Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fischer Scientific, UK) was added per well and coverslip was applied, avoiding formation of bubbles. Imaging was performed with Axiovert 200M microscope (Carl Zeiss Microscopy, Germany) using Axiovision software (AxioVs40).

Antigen	Species Raised in	Conjugate	Dilution	Supplier
CD31	Mouse	-	1:40	Dako
ETV2	Rabbit	-	1:500	Abcam
GFP	Mouse	-	1:500	Abcam
Anti-Rabbit	Goat	Alexa Fluor® 546	1:500	ThermoFisher
Anti-Mouse	Goat	Alexa Fluor® 488	1:500	ThermoFisher

Table 12: Antibodies used for ICC.

2.8 Flow Cytometry

For flow cytometry (FC) analysis, cultures were dissociated using TrypLE Select (Thermo Fisher Scientific, UK), resuspended in FC buffer consisting of PBS with 1% knock out serum replacement (Thermo Fisher Scientific, UK) and 2nM EDTA (Thermo Fisher Scientific, UK). For each test, $1x10^5$ cells were used in 100µl FC buffer with antibodies, as required, as shown in Table 13. Cells were incubated for 20 minutes – 1 hour, washed twice using PBS and pelleted using 300 x g centrifugation for 3 minutes and analysed using FACSCanto II flow cytometer or LSR II cytometer (both BD, UK) with BD FACSDiva Software v.6.1.3 (BD, UK). Forward and side scatter gating was used to exclude dead cells, forward scatter height and width gating was used to exclude cell doublets as illustrated in Figure 10. Subsequent analysis was performed using FlowJo 10 software (Treestar, US).

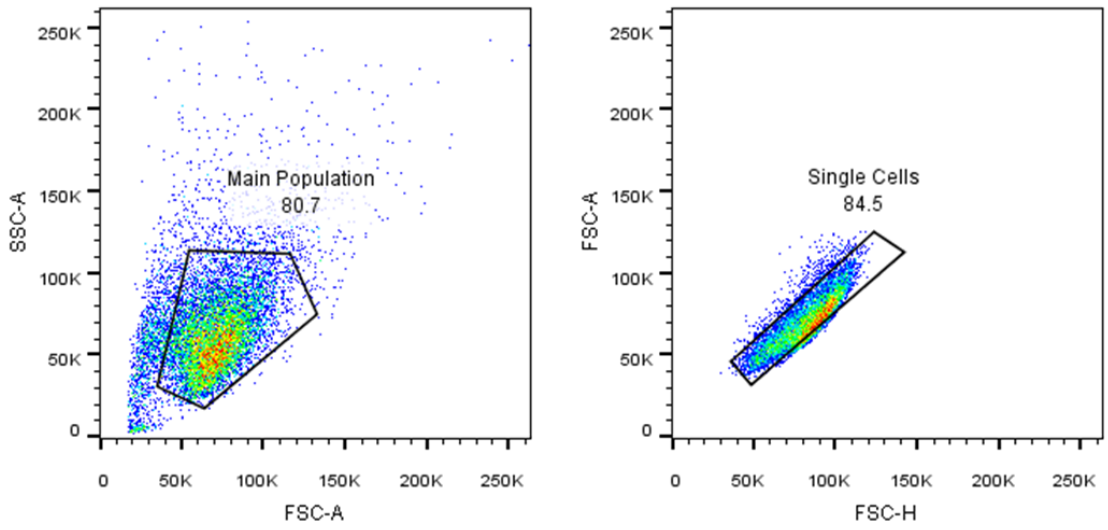


Figure 10: Example of gating strategy for FACS analysis. *Forward and side scatter gating was used to exclude dead cells (left), forward scatter height and width gating was used to exclude cell doublets (right).*

Antigen	Species Raised in	Conjugate	Dilution	Supplier
SSEA3	Rat	Alexa fluor® 647	1:100	BD Bioscience
SSEA3	Mouse	PE	1:50	BD Bioscience
Tra-1-60	Mouse	PE	1:50	BD Bioscience
CD56	Mouse	PE	1:50	BD Bioscience
CD326	Mouse	APC	1:100	BD Bioscience
CD326	Mouse	FITC	1:25	BD Bioscience
CD309	Mouse	PE	1:50	BD Bioscience
CD34	Mouse	APC	1:100	BD Bioscience
CD31	Mouse	APC	1:100	R&D Systems
CD31	Mouse	PE	1:50	BD Bioscience
CD31	Mouse	FITC	1:25	BD Bioscience
CD144	Mouse	APC	1:100	eBioscience
CD144	Mouse	PE	1:50	BD Bioscience
CD144	Mouse	FITC	1:25	BD Bioscience
CD43	Mouse	PE	1:50	BD Bioscience

Table 13: Antibodies used for FC analysis.

2.9 Statistical analyses

Statistical analysis was performed on non-normalised values using GraphPad Prism software suite 5 (GraphPad Software, US). Data values were presented as mean ± standard error of the mean (SEM) to demonstrate the accuracy of the estimated population mean (Altman and Bland, 2005). Data values were presented as averages, if only two data points were available.

Student t-test was used for analysis of two treatment groups. For two or more treatment groups, analysis of variance (ANOVA) was performed. If required, two way ANOVA analysis was used to account for variability between independent experiments as described by Lew (2007). ANOVA p-value is displayed above the figures. If ANOVA p-value was below 0.05, indicating statistically significant differences between the means, statistical significance of the differences between the individual groups was determined by Tukey post-hoc test. Differences were considered significant at $p<0.05$.

Chapter 3: Development and characterisation of hESC-EC differentiation protocol.

3.1 Introduction

To date, there have been a large number of publications describing hESC differentiation towards endothelial lineages and, more recently, there has been an increased focus in developing high efficiency methods that yield a high number of endothelial like cells. However, while significant advances have been made in increasing the yield of these differentiation protocols, often the reagents used limit their suitability for clinical purposes due to being poorly defined, as is the case with various serum derived products or not being xeno free, for example, Matrigel. Indeed, the recent methods reported by Sahara et al. (2014), Prasain et al. (2014) and Patsch et al. (2015) all use Matrigel in their differentiation protocols.

These poorly defined reagents raise multiple concerns in relation to clinical use. Firstly, they carry a risk of contaminant or pathogen transmission and, whereas screening can be undertaken to minimise this risk, it is impossible to screen for novel or currently unknown pathogens. Secondly, as these reagents are not chemically defined, it cannot be excluded that they contribute to cell signalling during the differentiation and therefore batch to batch variability of these reagents can consequently alter the differentiation process.

Other groups have reported differentiation protocols that have been developed keeping clinical relevance in mind. Kaupisch et al. (2012) reported optimisation of a serum-free derivation of endothelial cells under cGMP compatible conditions that yielded approximately 30% of CD144+ cells. The endothelial progenitor (CD133+ CD56+) differentiation was demonstrated in multiple hESC lines, however, the generation of CD144+ cells was only performed with RC-13 hESC. The efficacy of this protocol in other cell lines remains to be demonstrated.

Wu et al. (2015) used a minimal set of growth factors to induce endothelial differentiation in a clinically relevant manner as neither Matrigel or serum was used. However, the reported differentiation efficacy before selection using anti-absorptive agents was below 4% and thus a relatively low amount of endothelial like cells was generated which limits the clinical usefulness of this approach.

In contrast, Sriram et al. (2015) reports a promising, clinically relevant xeno and serum free five day endothelial differentiation protocol that yields over 90% of EPC expressing CD31 and CD34. However, CD144 expression was not reported after the five day differentiation, only after cell sorting and further culture. Even though the differentiation is performed both using hESC H9 and H1, no data is provided on repeats and reproducibility. In addition, the pluripotent hESC cells were cultured on Matrigel which contains various unspecified growth factors that may alter the cell behaviour (Hughes et al., 2010; Ojala et al., 2012). Overall, this protocol represents a significant advance towards clinically relevant hESC-EC differentiation. However, more refinement and data is needed to validate the findings and make this method truly clinically relevant.

Initially, the endothelial differentiations performed in this chapter followed a protocol, where hESC are mechanically cut with a StemPro® EZ-tool and cultured in a low adherence plate to form EBs for mesodermal induction. The method, however, was not robust and displayed high variation between experiments and cell lines. This can be explained by the variability in the EB size and morphology. Cell to cell and extracellular matrix interactions play an important role in stem cell differentiation (Chen et al., 2007) and while 3D culture can aid mesodermal hESC fate during spontaneous differentiation (Bauwens et al., 2008; Hsiao et al., 2014), the differences in size and morphology can also introduce additional variation. EB size has been shown to influence the efficiency of cardiac (Mohr et al., 2010) and hematopoietic (Ng et al., 2005) differentiation. Size can also affect EB growth profiles (Mohr et al., 2010), as well as oxygen and cytokine concentrations at the core of the EBs (Van Winkle et al., 2012). Therefore, it was hypothesised that controlled size EB generation could be used to minimise the variability and increase the robustness of endothelial lineage differentiation.

Conventional methods for EB generation are liquid suspension culture, methylcellulose culture and hanging drop formation (Dang et al., 2002; Kurosawa, 2007). Only hanging drop formation allows for control of EB size, however, this technique is technically challenging and does not allow for media exchange and thus an alternative methods need to be considered. More recently, various other approaches have been established for controlled size EB

generation, for example, using agarose coated micro-wells (Pettinato et al., 2015) or commercially available Aggrewell™ dishes (Stover and Schwartz, 2011).

Alternatively, low attachment, round bottom 96 well plates can be used for controlled size EB formation (Ng et al., 2005), and this approach has been further refined to use v-shaped 96 wells coated with Pluronic (Ungrin et al., 2008). Initially, hESC-EC differentiation was optimised in our lab using low attachment, round bottom 96 well plates. However, the cost of these plates was a serious limitation of this method. In contrast, Pluronic coating of tissue culture 96 wells plates is a low cost alternative and presents a simple, high throughput way for generating controlled size EBs. Therefore, it was evaluated if hESC-EC differentiations can be undertaken in Pluronic coated instead of low adherence 96 well tissue culture plates.

Further optimisation of the endothelial differentiation protocol also requires evaluation of potential signalling pathways that enhance or interfere with endothelial specification, survival and proliferation. As described in section 1.7.4, TGFB plays a role in angiogenesis and modulates VEGF signalling, and thus is a prime candidate for optimisation. TGFB signals via type I and type II receptor complexes with activin receptor-like kinase 1 (ALK1) and ALK5 type I receptors expressed in EC as illustrated in Figure 11 (Cunha and Pietras, 2011; Oh et al., 2000). ALK1 and ALK5 receptor complexes signal via SMAD1/5/8 or SMAD2/3 TF accordingly, often having opposing effects on cell apoptosis, proliferation and migration (Goumans et al., 2002). In addition, endoglin, a type III receptor expressed on EC (Jonker and Arthur, 2002), promotes TGFB signalling via ALK1 (Lebrin et al., 2004) and TGFB also has a range of SMAD independent effects via direct modulation of various signalling mediators forming complex signalling networks (Moustakas and Heldin, 2005).

Observations during endothelial hESC differentiations have shown that inhibition of TGFB signalling can increase endothelial yield (James et al., 2010; Sahara et al., 2014), as well as preserve endothelial phenotype and proliferation (James et al., 2010). This is in line with previous reports demonstrating that TGFB signalling inhibits endothelial cell proliferation (Castañares et al., 2007) and destabilises Fli1 (Asano and Trojanowska, 2009), which is one of the ETS factors which contributes to the maintenance of EC phenotype (Ginsberg et al., 2012;

Israely et al., 2013). This led to hypothesis that TGFB inhibition could further enhance hESC-EC differentiation yield via increasing differentiation efficacy during the differentiation, maintaining endothelial cell phenotype and aiding EC expansion during prolonged culture.

This was followed by preliminary analysis of marker expression during the optimised hESC-EC protocol and more detailed gene expression analysis to confirm the expected progression of hESC-EC differentiation – loss of pluripotency markers, induction of mesoderm, followed by endothelial progenitor and endothelial gene expression. This supporting data is essential for understanding and designing future experiments using hESC-EC differentiation protocol.

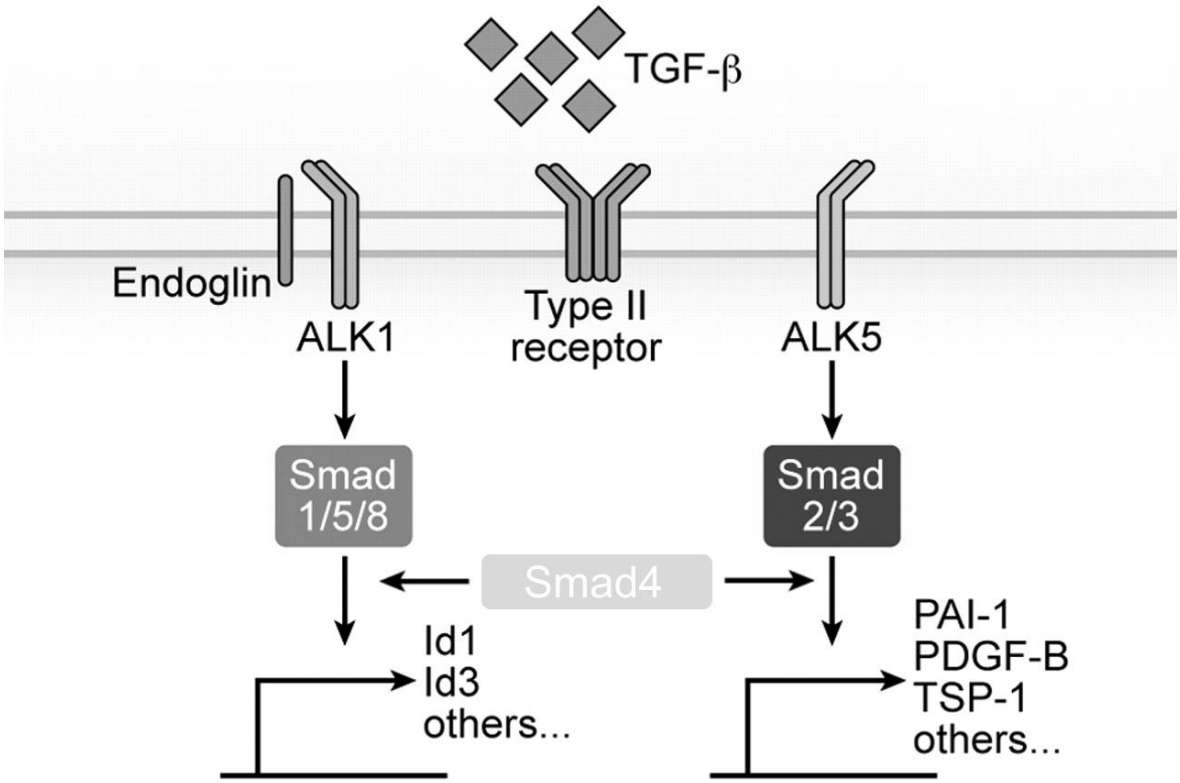


Figure 11: Overview of TGFB signalling. *TGFB signalling is mediated via TGFB type II receptor complexes with type I receptors ALK1 or ALK5 and downstream SMAD 1/5/8 or 2/3 TF accordingly. Receptor activated SMADs form a complex with SMAD4 and activate a range of genes. Type II receptor Endoglin which is expressed on endothelial cells enhances signalling via ALK1. Adapted from Cunha and Pietras (2011).*

3.2 Aims

The aims of this chapter were:

- To develop a hESC-EC differentiation protocol:
    - To test whether controlled size EB generation in 96 well plates is a suitable low cost alternative to low adherence 96 well plates for hESC-EC differentiation.
    - To evaluate if inhibition of TGFB signalling is beneficial for endothelial specification and expansion during hESC-EC differentiation.
- To characterise hESC differentiation during differentiation using hESC-ECs differentiation protocol.

3.3 Results

3.3.1 hESC differentiation towards endothelial lineages via 6-well embryoid body culture intermediate.

Initially, multiple hESC lines (H1, H9, and clinical RC-9 and RC-13) were differentiated in a monolayer differentiation system towards endothelial lineages (Kane et al., 2010; Kaupisch et al., 2012). Differentiations with RC-13 yielded 1.1% cells expressing CD144 and CD31 at the end of the differentiation, while no expression of CD144 and CD31 was detected with H1, H9 and RC-9 differentiations (all n=1, data not shown). As the monolayer differentiation system did not differentiate hESCs towards endothelial lineages, further repeats were not performed and work was undertaken in order to develop an alternative differentiation method via 6-well embryoid body culture intermediate.

Two hESC lines – H9 and RC-11 were differentiated towards endothelial lineages following differentiation protocol using 6-well embryoid body culture for mesoderm induction. As presented in Figure 12, initial differentiations with H9 yielded 32.1 ±2.3 % cells expressing both endothelial markers CD31 and CD144, and over 33.8 ±1.4 % cells expressing endothelial progenitor marker CD309. However, negligible % of cells expressed CD144 and CD31 (0.6 ±0.6 %), or CD309 (1.3 ±0.8 %) after differentiations using hESC line RC-11. Additionally, repeated H9 differentiation attempts also failed to generate cells expressing CD31 and CD144 (personal communication, Dr Peter Burton, University of Glasgow). Therefore, a novel protocol was developed using controlled size EBs generated in low adherence 96 well plates for mesodermal induction.

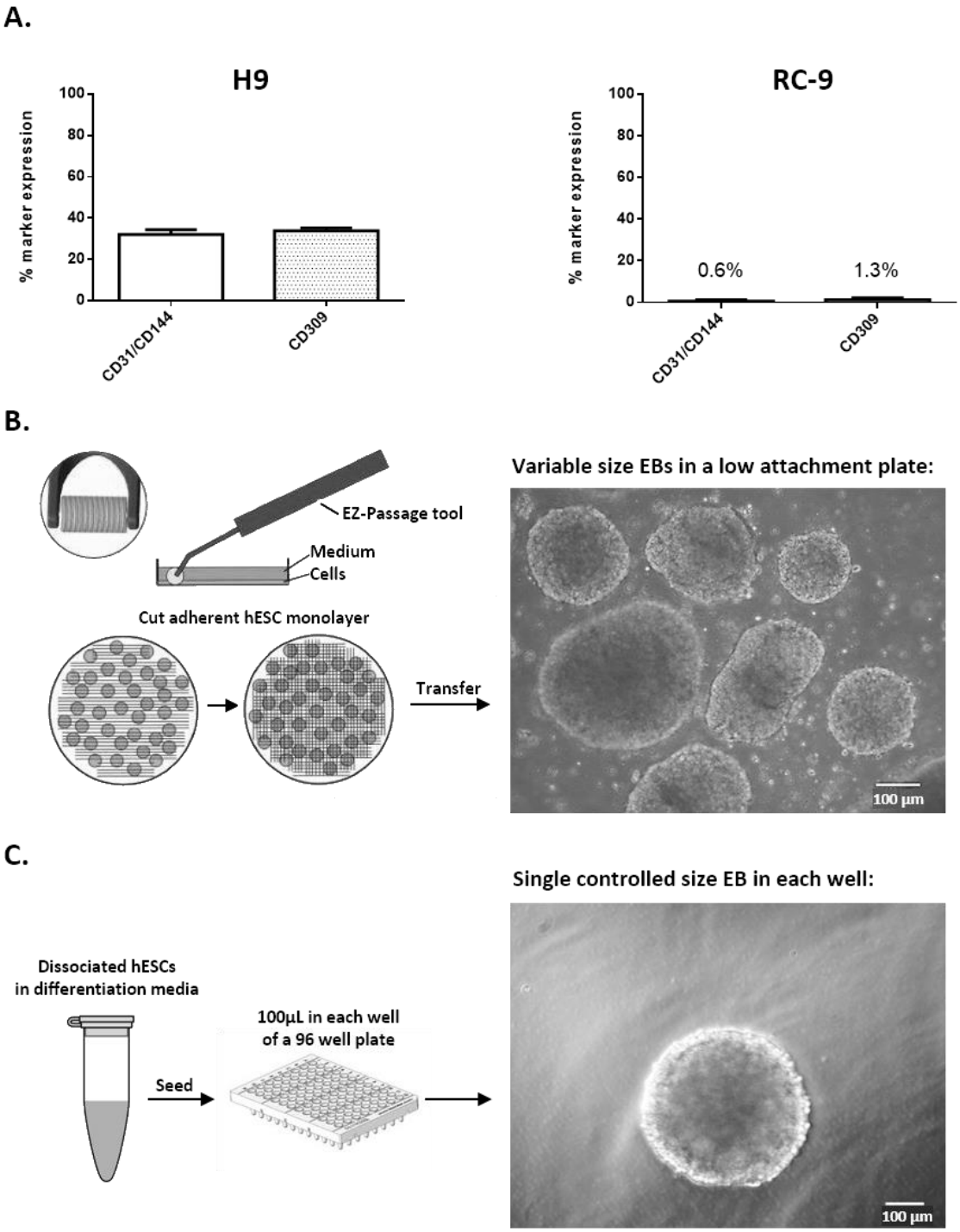


Figure 12: Endothelial marker expression at the end of differentiation towards endothelial lineages via 6-well embryoid body culture intermediate. A. *H9 and RC-9 were differentiated towards endothelial lineages (n=3, independent experiments) following differentiation protocol using embryoid body culture for mesoderm induction and marker expression was assessed using FC at the end of the differentiation. Data shown as mean ± SEM.* ***B.*** *Generation of variable size EBs.* ***C.*** *Generation of controlled size EBs. Scale bars 100µm. Adapted from: ThermoFisher (2007).*

3.3.2 Optimisation of a novel hESC-EC differentiation protocol: Pluronic F-127 well treatments.

Seeding a set number of cells in low adherence 96 well plates allows for controlled size EB generation for hESC-EC differentiation. However, the high cost of such plates is a serious limitation for routine use and, thus, Pluronic F-127 (Pluronic) treatments were evaluated as a low cost alternative for controlled size EB generation (Ungrin et al., 2008). It was proposed that hESC-EC differentiations using Pluronic coated 96 well plates would display comparable efficacy to differentiations performed in low adherence plates. Two hESC cell lines – H9 and RC-9 were differentiated towards endothelial lineages using hESC-EC differentiation protocol. The pluripotent hESC cultures were enzymatically dissociated and 10 000 cells were seeded per well in either low adherence 96 well plates with round wells (control) or 96 well plates with round or v-shaped wells coated with Pluronic F-127 for the generation of EBs. EC and pluripotency marker expression was evaluated on day 7 of the differentiation by FC.

The data presented in Figure 13 shows that differentiations with RC-9 successfully generated cells expressing EC markers, with 27.9 ±3.6 % cells expressing CD144 and over 43.5 ±5.5 % cells expressing CD31 in control. Pluronic well treatments did not significantly change CD144 marker expression with 28.4 ±6.3 % and 28.2 ±3.0 % positive cells in Pluronic coated round and v-shaped wells respectively. Similarly, CD31 expression remained comparable with 44.6 ±5.3 % and 37.9 ±2.6 % CD31+ cells in differentiations using Pluronic treated round or v-shaped wells respectively. There were no statistically significant changes in pluripotency marker TRA-1-60, or SSEA3 expression.

Differentiations with H9, as shown in Figure 14, also successfully generated cells expressing EC markers, with 21.3 ±3.7 % cells expressing CD144 and over 44.6 ±7.9 % cells expressing CD31. Pluronic well treatments did not significantly change EC marker expression with 21.3 ±3.7 %, 25.7 ±7.7 %, 28.1 ±5.4 % cells expressing CD144 when comparing low adherence round wells with Pluronic coated round and v-shaped wells respectively. There were no statistically significant changes in EC marker CD31 and pluripotency marker TRA-1-60, SSEA3 expression.

To sum up, hESC enzymatic dissociation and generation of controlled size EBs of 10 000 cells per EB in Pluronic coated 96 well plates for hESC-EC differentiation was comparable to differentiations set up in low adherence 96 well plates. Given the significantly lower cost of Pluronic coated 96 well plates, the future differentiations were performed using Pluronic coated u-shaped 96 well plates.

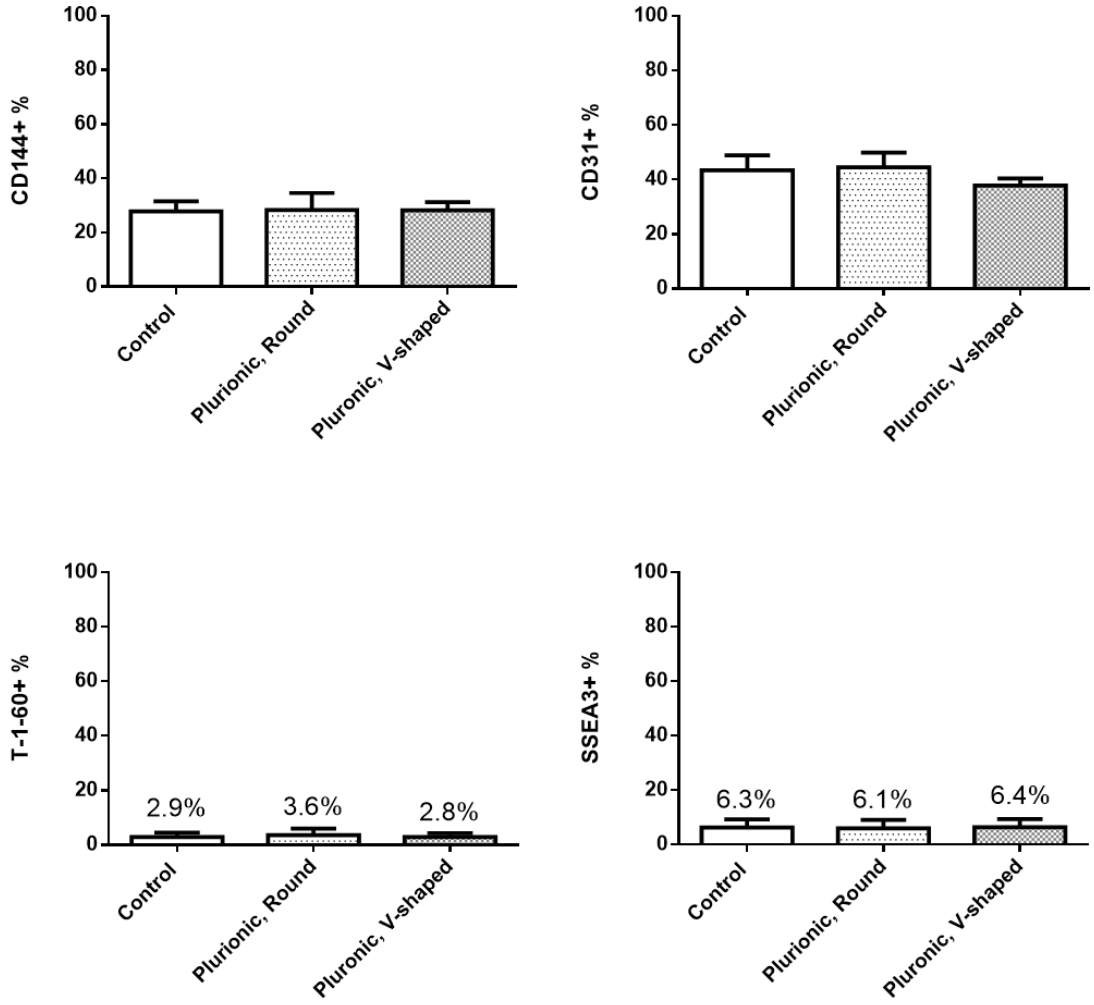


Figure 13: Effect of Pluronic well treatments on EC and pluripotency marker expression during hESC-EC differentiation in RC-9. *Pluronic coated round and v-shaped wells were compared to low adherence round wells (control) during RC-9 hESC-EC differentiation (n=3, independent experiments). Data shown as mean ± SEM, statistical significance determined using one-way ANOVA. All non-significant, ANOVA p>0.05.*

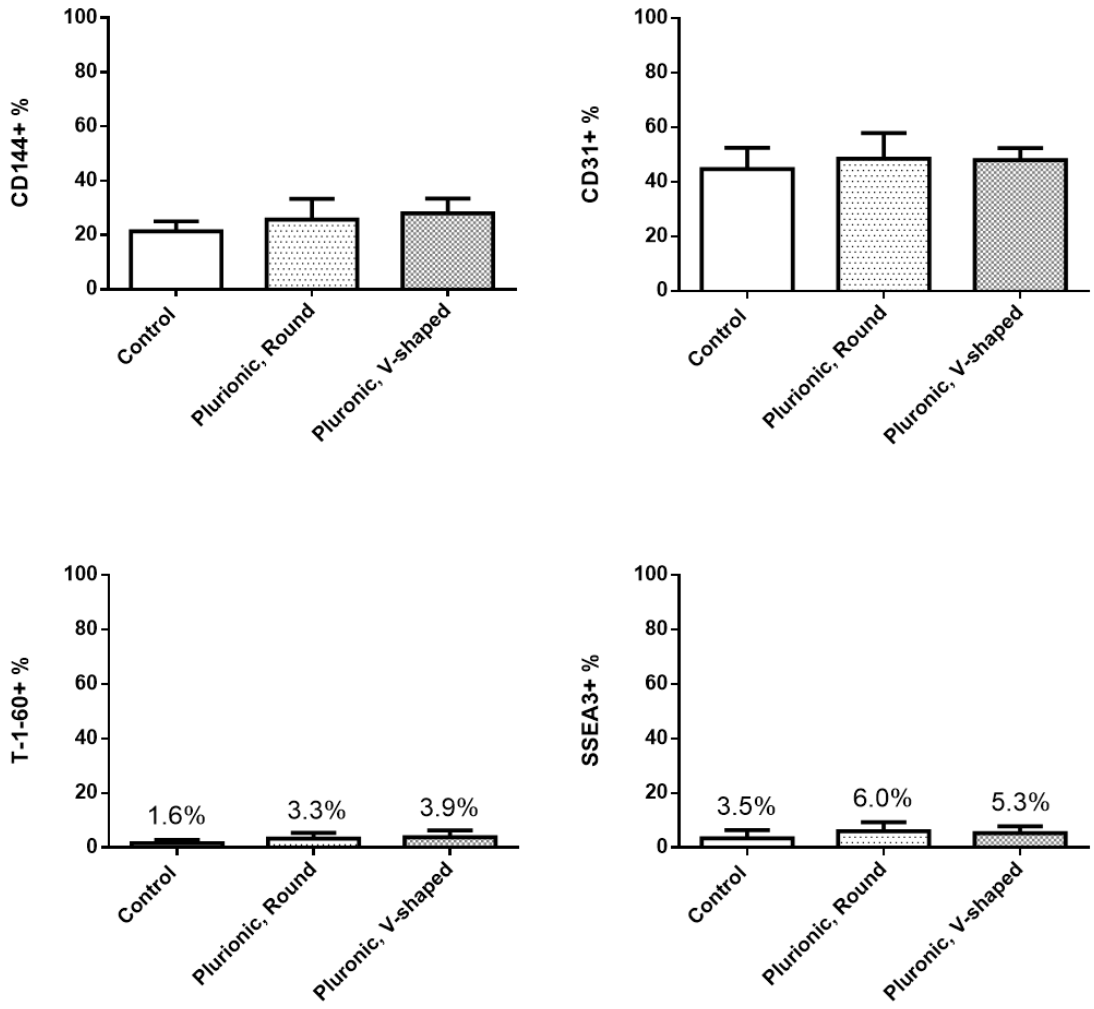


Figure 14: Effect of Pluronic well treatments on EC and pluripotency marker expression during hESC-EC differentiation in H9. *Pluronic coated round and v-shaped wells were compared to low adherence round wells (control) during H9 hESC-EC differentiation (n=3, independent experiments). Data shown as mean ± SEM, statistical significance determined using one-way ANOVA. All non-significant, ANOVA p>0.05.*

3.3.3 Optimisation of a novel hESC-EC differentiation protocol: TGFB signalling inhibition.

To evaluate, whether TGFB signalling inhibition would increase the percentage of cells expressing EC markers or total cell numbers at the end of hESC-EC differentiation, 10μM SB431542 was added after mesodermal and vascular specification as described by James et al. (2010), from day 5 of hESC-EC differentiation. Expression of EC markers CD31 and CD144 as well as pluripotency markers TRA-1-60 and SSEA3 was evaluated on day 7, and, in addition, the differentiation culture was continued for additional analysis on day 10 and day 14 of the differentiation.

The marker expression remained comparable (data not shown, n=1 each cell line) between the SB431542 and control dimethyl sulfoxide (DMSO) treated H9 cultures on day 7, while a reduction in CD144 (34.5% vs 21.2%) and CD31 (52.0% vs 41.8%) expression was observed in RC-11 SB431542 treated cultures. The EC marker expression remained lower in SB431542 treated cultures on day 14 both in H9 and RC-11. Pluripotency marker SSEA3 expression remained comparable in treated and control cultures both in H9 and RC-11. There was a trend of higher total cell counts in SB431542 treated differentiations on day 7 and day 10 of the differentiation, with larger differences observed on day 14 (3.7×10^6 vs 1.9×10^6 cells in H9 and 3.0×10^6 vs 1.9×10^6 cells in RC-11).

The data described above suggests that there was no added benefit of TGFB inhibition on EC differentiation and expansion during hESC-EC differentiation. Therefore, this experiment was not repeated and characterisation of hESC-EC differentiation was performed.

3.3.4 hESC-EC differentiation protocol.

The final differentiation protocol, as illustrated in Figure 15, takes eight days, during which EBs are formed from hESC on day 0 and cultured in mesodermal induction media as described by Olivier et al. (2016). The EBs are then plated out on in six well plates on day 3 for endothelial induction, with an appearance of spindle shaped cells between day 3 – 5 and elongated cell islands on day 7 (Figure 16). Analysis of H9 hESC-EC differentiation demonstrated that pluripotency marker expression is gradually lost during the differentiation (Figure 17), however, residual SSEA3+ expression could still be observed in 4.4 ±1.5 % of the cells on day 7. Mesodermal population of CD326^{low} CD56+ cells (Boulberdaa et al., 2016; Evseenko et al., 2010) was detected on day 3 (43.0 ±10.7 %), followed by the appearance of CD309+ CD34+ cells (27.4 ±5.0 %) and CD31+ CD144+ cells (22.6 ±3.0 %) as shown in Figure 18. Differentiations yielded 22.3 ±1.9 % cells co-expressing CD31 and CD144 on day 7.

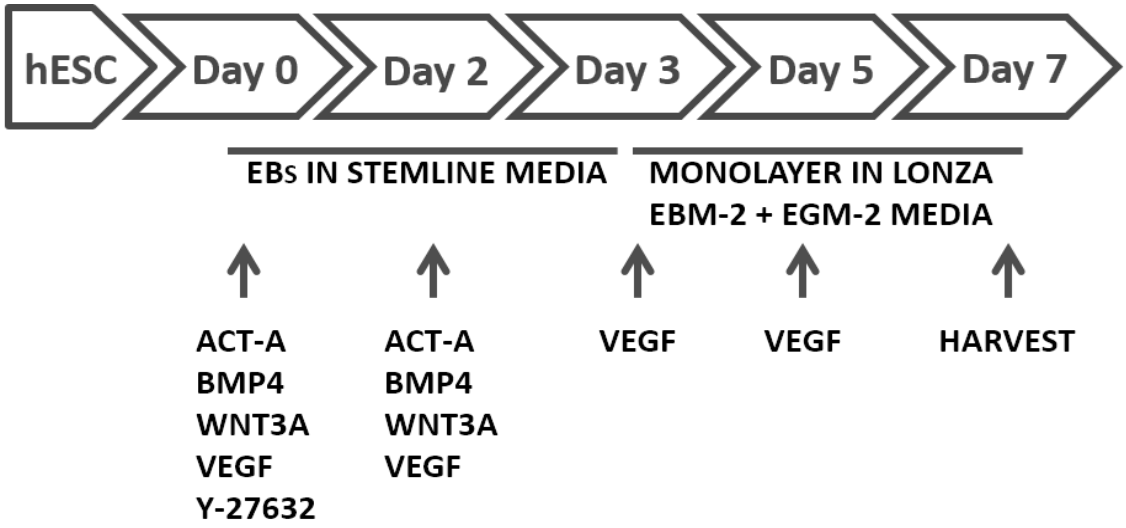


Figure 15: Schematic representing hESC-EC differentiation. *Embryoid bodies (EBS) are made from monolayer culture of hESC for mesoderm induction and on day 3 of the differentiation, the EB are plated in 6-well plates for further monolayer culture in vascular induction phase. Differentiation finishes on day 7.*

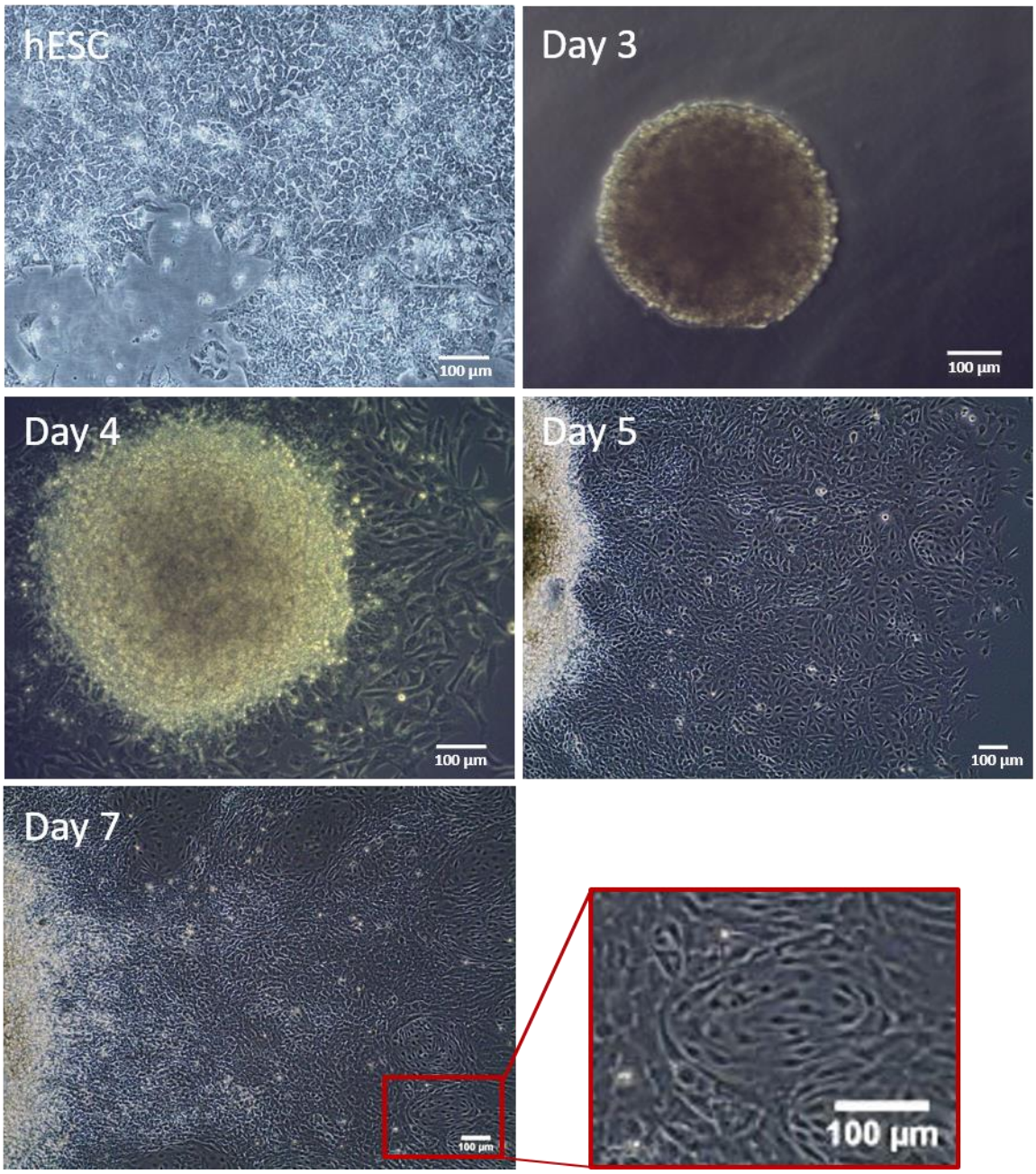


Figure 16: Morphology changes during hESC-EC differentiation. *Monolayer hESC cultures are used to make EB, which remain in EB culture till day 3. Then these are plated out in 6-well plates, where they attach and cells start migrating and proliferating on the well surface, forming spindle shaped cells as seen on day 5 and elongated cells on day 7, as shown in the inlay. Scale bars 100µm.*

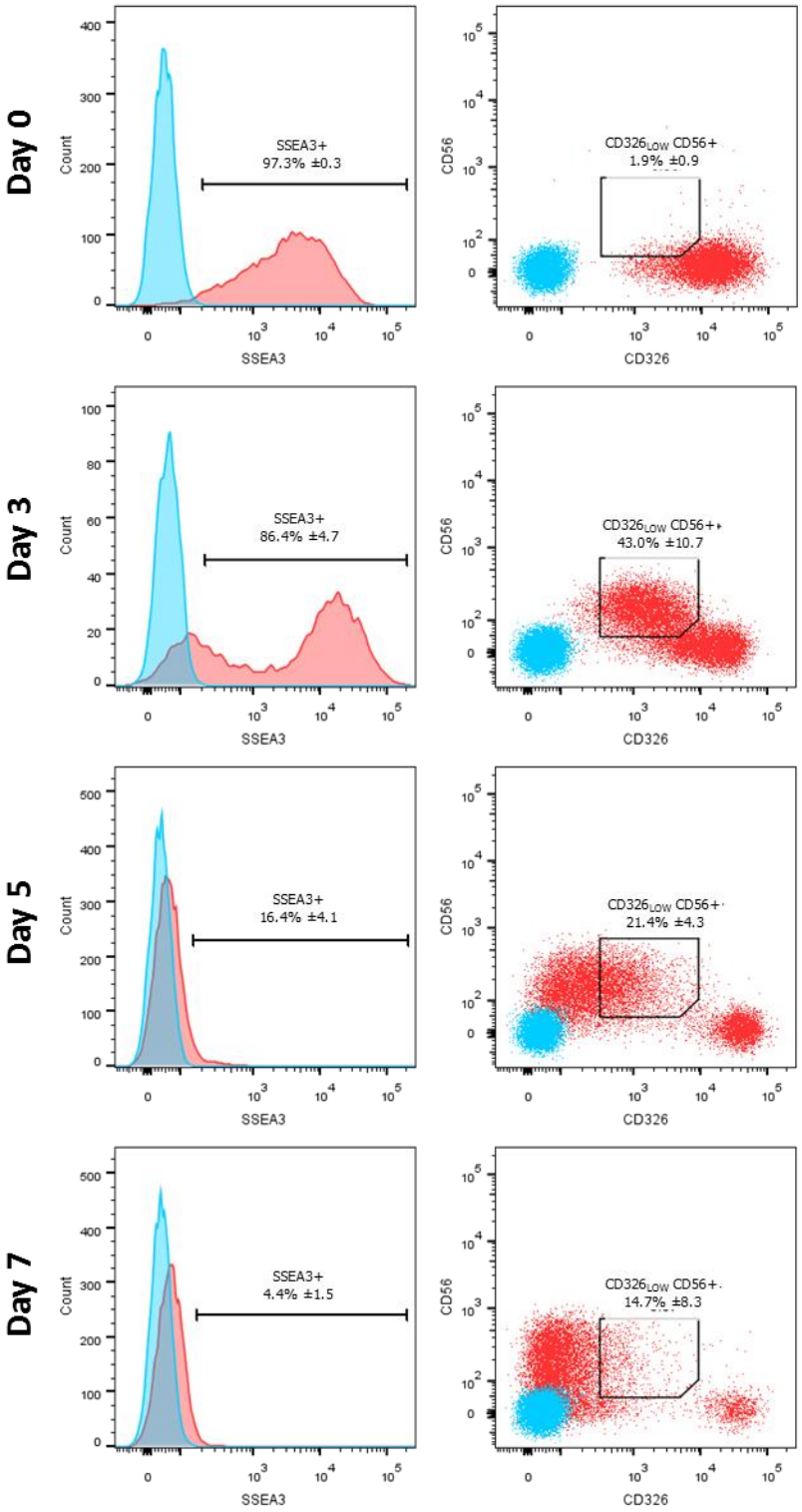


Figure 17: Analysis of pluripotency and mesodermal marker expression during hESC-EC differentiation. *Pluripotency marker SSEA3 expression and mesodermal population ($Cd326_{low}$ CD56+) was measured using FC on day 0, 5, 3 and 7 of H9 hESC-EC differentiation (n=3, independent experiments, representative shown). Isotype control – blue; Markers of interest – red. Percentages of expression displayed: marker staining %.*

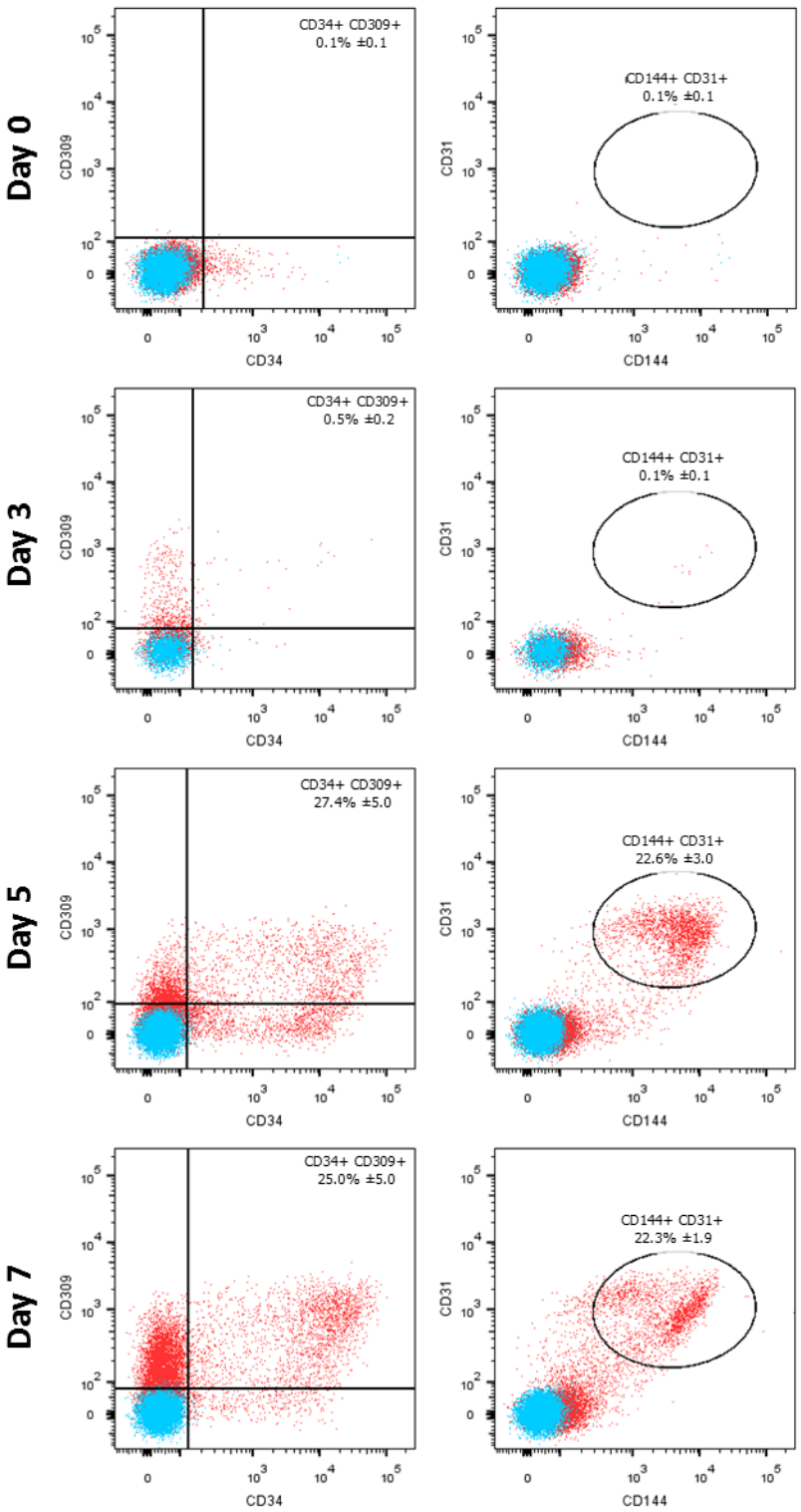


Figure 18: Appearance of endothelial progenitor and endothelial marker expressing populations during hESC-EC differentiation. *Endothelial progenitor marker (CD34 and CD309) and EC marker (CD31 and CD144) expression was measured using FC on day 0, 5, 3 and 7 of H9 hESC-EC differentiation (n=3, independent experiments, representative shown). Isotype control – blue; Markers of interest – red. Percentages of expression displayed: marker staining %.*

3.3.5 Gene expression changes during hESC-EC differentiation.

For gene expression analysis during hESC-EC differentiation, H1 hESC and RC-11 hESC were differentiated as described in Methods section 2.3, and RNA was collected on day 0, and Days 2 - 7 of the differentiation. All differentiations yielded >20% CD31+ CD144+ cells on day 7 of the differentiation. Statistical significance was evaluated using δCT values.

In H1 cells, a two fold increase in *NANOG* gene expression was unexpectedly observed on day 3 ($p<0.05$ vs day 0). This was followed by a trend of downregulation, yet, on day 7 the expression of *NANOG* remained comparable to expression levels observed on day 0 with a Ct value of 23.1 ±0.2 ($p>0.05$). In contrast, *SOX2* was rapidly downregulated 50-fold by day 2 ($p<0.001$ vs day 0) and was further downregulated during the differentiation reaching a Ct value of 34.2 ±0.3 on Day 7 ($p<0.001$ vs day 0).

Brachyury was significantly upregulated more than 300-fold on day 2 ($p<0.001$ vs day 0), followed by downregulation on day 3 ($p<0.001$ vs day 2) and day 4 ($p<0.01$ vs day 3) matching the expected pattern of mesodermal induction.

KDR expression was upregulated 3-fold on day 2 ($p<0.01$ vs day 0) and further upregulated on day 3 ($p<0.001$ vs day 2) to 15-fold increase, compared to day 0 ($p<0.001$) reaching Ct of 26.1 ±0.2. *CDH5* was slightly upregulated as early as day 2 ($p<0.001$ vs day 0) and highly upregulated from day 4 onwards ($p<0.001$ vs day 3) reaching Ct of 25.8 ±0.3 on Day 7 of hESC-EC differentiation, in line with the expected endothelial specification.

In RC-11, *NANOG* expression was downregulated by day 6 ($p<0.05$ vs day 0) and reached 30-fold decrease on day 7 ($p<0.001$ vs day 0) with Ct of 29.6 ±0.6, which is a smaller decrease than expected. *SOX2* gene expression was rapidly downregulated 12-fold on day 2 ($p<0.001$ vs day 0), with expression levels decreasing further during the differentiation and reaching Ct of 35.1 ±0.3 on Day 7. Brachyury expression was highly upregulated on day 2 ($p<0.001$ vs day 0), and this was followed by downregulation on day 4 ($p<0.05$ vs day 3), following a similar trend as seen in hESC-EC differentiations with H1. *KDR* was upregulated 8-fold on day 2 ($p<0.001$ vs day 0) and further upregulated by day 5 ($p<0.001$ vs

day 3), reaching Ct of Ct 21.1 ±0.2. *CDH5* was slightly upregulated on day 3 ($p<0.001$ vs day 0) and highly upregulated during endothelial specification phase as expected from day 4 onwards ($p<0.001$ vs day 3) reaching Ct value of 25.6 ±1.0 on Day 7 of hESC-EC differentiation.

In summary, in both hESC lines a higher than expected pluripotency gene *NANOG* expression was observed, while *SOX2* expression was rapidly downregulated as expected. Brachyury gene expression peaked on Day 2, indicating mesodermal induction, followed by upregulation of *KDR* and *CDH5* expression indicating EC specification during day 3 -7 of hESC-EC differentiation as expected.

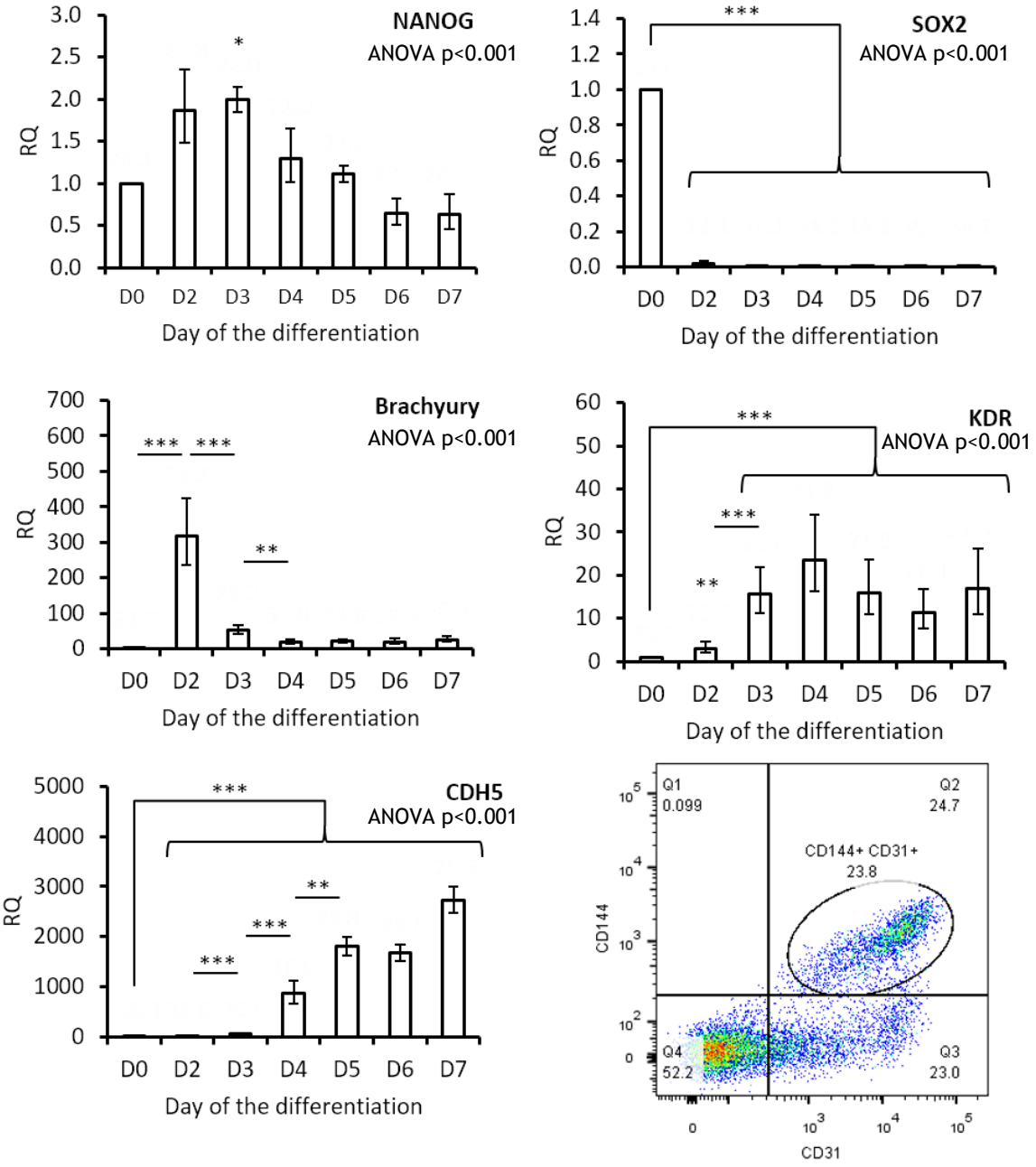


Figure 19: Gene expression changes during hESC-EC differentiation in H1. *RNA was collected on day 0, and Days 2 - 7 of hESC-EC differentiation (n=3, independent experiments). Expression of pluripotency genes NANOG and SOX2, mesoderm associated gene Brachyury, multipotent progenitor gene KDR, and endothelial gene CDH5 was quantified using qRT-PCR. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05, ** p<0.01, *** p<0.001 when compared to d0 hESC control, unless indicated otherwise. Data shown is RQ ± RQ max and min. Ct values displayed above bars. All differentiations yielded >20% cells expressing CD144 and CD31 on day 7, representative FC plot shown.*

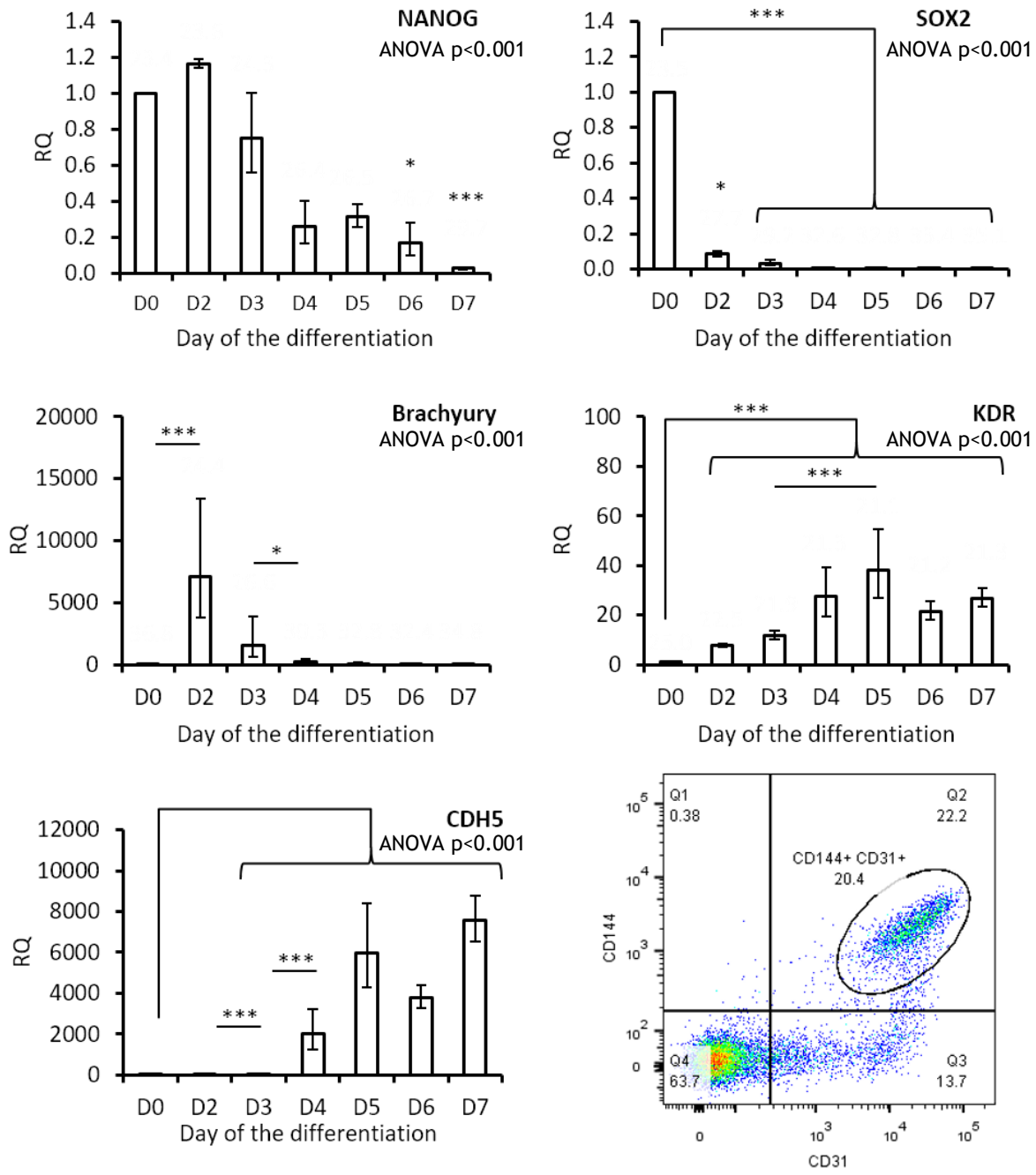


Figure 20: Gene expression changes during hESC-EC differentiation in RC-11. *RNA was collected on day 0, and Days 2 - 7 of hESC-EC differentiation (n=3, independent experiments). Expression of pluripotency genes NANOG and SOX2, mesoderm associated gene Brachyury, multipotent progenitor gene KDR, and endothelial gene CDH5 was quantified using qRT-PCR. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05, ** p<0.01, *** p<0.001 when compared to d0 hESC control, unless indicated otherwise. Data shown is RQ ± RQ max and min. Ct values displayed above bars. All differentiations yielded >20% cells expressing CD144 and CD31 on day 7, representative FC plot shown.*

3.4 Discussion

In summary, the data presented in this chapter demonstrated a novel protocol for differentiation of hESC towards endothelial lineages, using controlled size EB culture for mesodermal induction and monolayer culture for endothelial specification. This method was more robust for endothelial lineage differentiation than the previous 6-well based protocol and could be reproduced in H1, H9, RC-9 and RC-11. Further steps were taken to optimise hESC-EC differentiation, showing that Pluronic coated wells could be used as a low cost alternative for hESC-EC differentiation and suggesting that TGFB signalling inhibition did not confer additional benefit for this differentiation method. Finally, expression of pluripotency, mesoderm, endothelial progenitor and endothelial genes during hESC-EC differentiation was profiled.

The initial monolayer endothelial differentiation protocol did not generate cells expressing endothelial markers CD144 and CD31, in contrast to the observations previously reported by Kaupisch et al. (2012). Given that multiple cell lines were tested, it appears that the monolayer differentiation protocol does not sufficiently activate cell signalling pathways directing endothelial differentiation. In addition, cell to cell interactions contribute to the differentiation processes (Bratt-Leal et al., 2009; Itskovitz-Eldor et al., 2000) and might be lacking in a monolayer culture, therefore, an EB based endothelial differentiation protocol was developed and initially appeared promising with efficient endothelial differentiations with H9.

However, further testing revealed that it lacked robustness and it was speculated that this is due to the heterogeneous nature of the EBs generated using EZ-Passage Tool. Therefore, it was proposed that generation of controlled size EB using one of the previously described 96-well EB generation methods (Ng et al., 2005; Ungrin et al., 2008) could increase the reliability of the protocol. Here data are presented showing that generation of controlled size EBs for mesoderm induction during hESC-EC differentiation increases the reliability of the protocol with efficient differentiation in two cell lines - H9 generating >25% CD31+ CD144+ cells, and RC-11 generating >20% CD31+ CD144+ cells.

In addition, the use of Pluronic well coating was evaluated to create a low cost alternative to commercially available low adherence 96-well plates (Ungrin et al., 2008). No significant differences could be seen in CD31, CD144, TRA-1-60 and SSEA3 marker expression on day 7 of the differentiation. However, it cannot be excluded that Pluronic could alter signalling either directly or indirectly. Indeed, Pluronic has been reported to induce expression of VEGF and TGFB in wound healing assays (Kant et al., 2013) and possibly alter gene expression profiles during early hESC-EC differentiation (personal communication, Dr Elizabeth Scott, University of Glasgow). However, *in vitro* evaluation reports are lacking, and in depth marker comparison was not performed in the experiments described in this chapter, therefore conclusions cannot be drawn.

One of the reported disadvantages of using round bottom wells is the formation less compact cell pellet and multiple separate aggregates (Ungrin et al., 2008), however, this was not observed during hESC-EC differentiation. ROCK inhibitor Y-27632 is used on day 0 during hESC-EC differentiation to reduce cell apoptosis due to dissociation to single cells. However, it is also reported to aid formation of hESC agglomerates (Horiguchi et al., 2014; Watanabe et al., 2007) and this could explain the observed robust EB formation in round bottom wells.

Taken together, the observations suggest that both low adherence round shape and Pluronic coated round or v-shaped wells can be used for the differentiations, with no significant differences observed in pluripotency and endothelial marker expression at the end of hESC-EC differentiations. Pluronic coated round wells were used for the following experiments.

To investigate whether TGFB signalling inhibition would benefit EC differentiations and increase EC yield during hESC-EC differentiation, SB431542 was applied to differentiation cultures from day 5 onwards. The preliminary observations presented here show comparable EC marker expression between the SB431542 treated and control H9 differentiations, and reduced CD31 and CD144 expression in RC-11. As this suggested that TGFB inhibition did not enhance hESC-EC differentiation, additional repeats were not performed.

The lack of beneficial effect of TGFB inhibition on EC differentiation can be explained by multiple differences between previous reports and the

experimental approach reported in this chapter. TGFB signalling has been reported to have a detrimental effect on KDR signalling and ETS factors (Asano and Trojanowska, 2009; Ginsberg et al., 2012). Here, however, TGFB inhibitor was applied from day 5 during hESC-EC differentiation, while KDR expression is significantly upregulated as early as day 2. Thus, earlier inhibition of TGFB signalling might be required to limit the negative effects of TGFB earlier during hESC-EC differentiation.

Another possible explanation for the observed discrepancies is the differences in EC differentiation efficacies. Both reports showing the beneficial effect of TGFB inhibition for vascular specification, used low efficiency differentiations with James et al. (2010) reporting 1.8% CD31+ CD144+ yield and Sahara et al. (2014) reporting up to 8.7% efficacy. HESC-EC differentiations reported in this chapter were more efficient and generated over 30% CD144+ cells. The higher efficacy of hESC-EC differentiation protocol might limit the additional benefit of TGFB inhibition as any detrimental effects of TGFB might be overcome by other cytokines and growth factors present in hESC-EC differentiation mix.

In addition, the expression of TGFB was not evaluated during hESC-EC differentiation. It cannot be excluded that the levels of paracrine TGFB secretion during hESC-EC differentiation method were too low to have a significant detrimental effects by day 7 of the differentiation. Measuring the concentrations of TGFB in the media during hESC-EC differentiations would be necessary to clarify this. Furthermore, inhibition of TGFB signalling by SB431542 should be evaluated to confirm the efficacy of the used compound.

A trend of increased cell numbers in SB431542 treated differentiations was seen with the largest differences observed after prolonged culture on day 14. Indeed, TGFB signalling is known to suppress growth and proliferation of many cell types, including endothelial and hematopoietic cells, while it can also stimulate some mesenchymal cell types (Huang and Huang, 2005). This is also consistent with observations by James et al. (2010) who reported that TGFB inhibition stabilises endothelial phenotype and increases endothelial proliferation.

Here, however, a reduction in CD31 and CD144 marker expression in SB431542 treated differentiations was observed on day 14. Given the large increase in cell

numbers, it appears that SB431542 non-specifically increased proliferation in multiple cell types which remained in culture after day 7. Similarly, some loss of endothelial phenotype and increase in CD31- cell numbers was observed in the original paper after culturing CDH5 sorted cells in the presence of SB431542 (James et al., 2010). Therefore, it can be suggested that TGFB inhibition can be used to expand carefully selected cultures, while there's little benefit in mixed differentiation systems.

Taken together, it can be speculated that TGFB inhibition is beneficial only with the first appearance of KDR+ cells but not during later endothelial specification. It could also be beneficial to prevent the anti-proliferative and pro-apoptotic effects of TGFB signalling after sorting the cells of interest, however, there appears to be no added benefit in using TGFB inhibitor in differentiations with mixed cell populations. Therefore, TGFB inhibition was not used in the following hESC-EC differentiation experiments. However, as additional repeats were not performed, no conclusions can be drawn from these observation.

The final protocol uses controlled size EB culture in media supplemented with BMP4, WNT3A, activin A and VEGF to induce mesoderm lineages as described by Olivier et al. (2016). Of these, BMP4, WNT3A and activin A are the core signalling molecule required for mesodermal induction (Nostro et al., 2008) and later for inducing gene expression patterns required for endothelial specification, for example, via inducing Etv2 (Lee et al., 2008) and KDR expression (Ishitobi et al., 2011). VEGF not required for mesodermal induction, however, it plays a supporting role by contributing to activation of Etv2 (Rasmussen et al., 2012) and increasing angioblast and early endothelial cell proliferation (Gentile et al., 2013).

ROCK inhibitor is used on day 0 to aid hESC survival after dissociation to single cells (Watanabe et al., 2007) and the media is not changed until day 3, thus residual effects Y-27632 may persist during mesoderm induction. Interestingly, Yung et al. (2011) has observed a detrimental effect of Y-27632 on differentiation of single or dual positive cells for CD34, CD31 or CD309 in a hESC hematopoietic differentiation system, while in contrast Joo et al. (2012) has reported increased KDR+ cell expansion in Y-27632 treated mESC endothelial differentiation cultures. Therefore, a more detailed evaluation of the use of

ROCKi in this hESC-EC differentiation system should be undertaken to evaluate any possible detrimental or beneficial effects on differentiation.

After mesoderm induction, EBs are plated out and grown in endothelial growth medium containing multiple growth factors and cytokines, including bFGF, IGF, EGF and VEGF. Of these, VEGF is the key growth factor for endothelial specification and expansion (Kawasaki et al., 2008; Yamashita, 2004) and was optimised for use with this protocol (personal communication, Dr Peter Burton, University of Glasgow). The concentrations of the other growth factors were not optimised and were used as supplied with the endothelial media kit (Lonza EBM-2 + EGM-2 as described in 1.3), using the proprietary concentrations. It can be suggested, that further optimisation could be undertaken to pinpoint the best growth factor concentrations for vascular specification and to minimise any differentiation and growth signals for other lineage precursor cells present in the heterogeneous differentiation culture.

Once plated, cells migrate and proliferate on the gelatin coated surfaces, forming a monolayer where spindle shaped cells can be observed, consistent with endothelial morphology. However, gelatin can support attachment and growth of a range of cell lineages and therefore other surface treatments might be beneficial for maximising endothelial cell yield. Indeed, treatments reducing well surface adherence can be used to select endothelial progenitor like cells, obtaining nearly pure endothelial cultures, as reported by Wu et al. (2015).

However, given that EBs remain in suspension during mesodermal differentiation in Pluronic treated wells, it is not clear whether low adherence wells could be used for plating out the EBs during hESC-EC differentiation. It can be speculated that the mesodermal population observed during preliminary surface marker expression analysis on day 3 needs further maturation towards endothelial lineages, if low adherence treatments are to be used with the hESC-EC protocol. Further research is needed to evaluate if alternative well treatments could be used with hESC-EC differentiation method to select and support cell populations of interest.

Gene expression and marker expression was profiled during the optimised hESC-EC differentiation. As expected, most pluripotency markers were progressively

downregulated during the differentiation both on gene and marker expression level. Interestingly however, *NANOG* expression was upregulated during hESC-EC differentiations with H1. NANOG is part of the core pluripotency network and is downregulated during hESC differentiation (Bhattacharya et al., 2005), however, expression in adult tissues, for example heart and muscle, has also been reported (Hart et al., 2004). While expression of *NANOG* in mature endothelial cells is not reported, it has been shown to be induced during EC dedifferentiation during angiogenesis and possibly represents more immature cells with higher proliferative potential (Kohler et al., 2013, 2011).

It has also been proposed that NANOG supresses differentiation to endoderm but not mesoderm lineages (Wang et al., 2012). Furthermore, AKT/NANOG axis has been implied in mesodermal differentiation (Bertero et al., 2015) and WNT/NANOG axis has been shown to upregulate KDR expression in EC (Kohler et al., 2011). As both AKT and WNT3A is used from day 0 – day 3 of hESC-EC differentiation, it is tempting to speculate that during the initial differentiation NANOG expression is sustained by AKT and WNT3A signalling and contributes to hESC differentiation to endothelial lineages. This is in line with the observed trend of reduced NANOG expression during days 4 – 7 when KDR expression stops increasing and remains steady.

While less pronounced, NANOG expression also remained high in RC-11, and a trend of reduced NANOG expression was observed on day 4 and became significant on day 6. KDR expression remained steady between Days 4 – 7 and this therefore mimics the pattern seen in H1 and suggests that NANOG marks immature cell population and possibly takes part in inducing the expression of KDR. It can also be speculated that further downregulation would be observed in both in RC-11 and H1 as the cells acquire more mature phenotype after day 7. However, further research on gene expression in prolonged culture is required to confirm this.

Given that strong downregulation could be observed in gene expression of SOX2 both in H1 and RC-11 the expression of NANOG appears to be due to immature phenotype of the cells, not maintained pluripotency. The link between the expression of NANOG and KDR needs further validation, possibly by using RNA interference or other knockdown models. Additionally, in order to ascertain the

suitability of hESC-EC differentiation products for use in clinical practice, residual pluripotency has to be evaluated, using *in vitro* and *in vivo* pluripotency assays, for example teratoma formation.

Expression of Brachyury showing mesodermal specification peaked at day 2 both in RC-11 and H9, followed by a rapid downregulation and upregulation of KDR, indicating appearance of multipotent cardiovascular progenitor cells. Surface marker expression analysis in H9, showed appearance of $Cd326_{low}$ CD56+ mesodermal population (Evseenko et al., 2010) on day 3 which is in line with upregulation of Brachyury observed a day earlier. CDH5 was strongly upregulated after the appearance of KDR+ cells on day 3 both in H1 and RC-11. This follows the expected gene expression patterns for differentiations towards endothelial lineages.

While the surface marker and gene expression profiles confirm the commitment towards endothelial phenotypes, it would also be also necessary to confirm the endothelial lineage of the cells using a range of function tests, for example, using *in vitro* tubule formation assays, AcLDL uptake assays and NO production. While preliminary functional testing was done (personal communication, Dr Peter Burton and Dr Elizabeth Scott, University of Glasgow), a full range of tests is required as these would indicate the clinical potential of the differentiated cells and, thus, are crucial part of evaluation of hESC-EC differentiation.

Further testing, both on gene level and on surface marker expression level, is required to identify and characterise the cell population that does not express EC or EPC markers at the day 7 of the differentiation. While it can be speculated that this cell population represents a mixture of other differentiated and progenitor cell types of mesodermal lineage, this was not evaluated. Staining for other mature cell markers, for example alpha smooth muscle actin for identifying smooth muscle cell phenotypes, would give additional insight into this. Additionally, western blot protein expression analysis should be performed to confirm the expression of proteins of interest, and as a control for the antibodies used as expected protein molecular weight could be confirmed.

Finally, here multiple hESC cell lines (H1, H9, RC-9 and RC-11) were used for the experiments. While this confirms that the developed hESC-EC differentiation

generates cells expressing EC markers CD31 and CD144 robustly in a range of cell lines, it also complicates the evaluation of the experimental results, due to the heterogeneity of hESC cell lines (Abeyta et al., 2004; Cahan and Daley, 2013; Osafune et al., 2008). Here, it was attempted to use one of the gold standard hESC lines (H1 and H9) for initial experiments, followed by validation of the findings in at least one of the Roslin Cells (RC-9, RC-11 or RC-13) hESC lines. For consistency, it would have been beneficial to use the same set of cell lines for all experiments, however, here it was not always possible due to varying hESC cell line availability over the course of the experimental work for this thesis.

In summary, optimisation of a novel, serum free hESC-EC differentiation protocol was undertaken and it was demonstrated that the use controlled size EB increases the reliability of the protocol and that TGFB inhibition does not further increase EC % at the end of the differentiation. The final hESC-EC differentiation protocol was then validated in RC-11 and H1 and gene expression analysis was undertaken, showing stepwise activation of mesodermal, endothelial progenitor and endothelial genes, and reduction of pluripotency marker expression. This protocol represents an advance over current methods as it achieves high EC yield using only defined reagents suitable for clinical applications.

Chapter 4: Generation of reporter cell lines for monitoring of hESC-EC differentiation.

4.1 Introduction

The previous chapter described the work undertaken to develop and optimise a novel, serum free, two step differentiation protocol that generated up to 30% CD31+ CD144+ cells. While this represents a significant advance when compared to the differentiation methods published previously (Descamps and Emanueli, 2012), there is still scope for further optimisation with focus on the use of small molecule drugs to increase the differentiation efficiency and generate large numbers of CD31+ CD144+ cells. In particular, high throughput live cell monitoring could provide the needed insight in the effects of various compounds on cell differentiation during hESC-EC differentiation.

There are multiple approaches that can be employed for high throughput screening of endothelial differentiation. Use of antibody based approaches for labelling proteins of interest has been widely used in stem cell research and long term monitoring of various cell surface marker expression changes has been reported in a range of cell lines (Eilken et al., 2011). More importantly, successful live cell immunofluorescence staining has also been reported in human pluripotent cells (Manos et al., 2011). However, there are multiple limitations of immunofluorescence based screening approaches – highly specific and stable antibodies are required, the detection is limited to the proteins expressed on cell surface, and antigens can be masked when tight cell junctions are established (Manos et al., 2011). And, while methods for delivering antibodies intracellularly have been described (Canton et al., 2013; Raz-Prag et al., 2010), their suitability for monitoring of hESC differentiation remains unclear with concerns regarding potential toxicity and effects on the differentiation processes.

Another approach is the use reporter cell lines, where reporter protein sequence is inserted after the endogenous gene sequence in the genome, allowing for a simultaneous expression of the gene of interest and the reporter (Rojas-Fernandez et al., 2015). Alternatively, a reporter construct can be created where an extrinsic cognate promoter drives expression of a reporter gene, such as green or red fluorescent protein (GFP or RFP accordingly) and the construct can be delivered into the cells using electroporation or, for example, lentiviral

vectors can be used to for the delivery and random integration of the reporter construct.

Indeed, *NANOG* (Fischer et al., 2010) and *OCT4* (Huangfu et al., 2008) extrinsic reporter construct cell lines have been used to monitor stem cell pluripotency and reprogramming, while reporters of endothelial specific genes, for example *CDH5* (Sahara et al., 2014) and *PECAM* (Zeng et al., 2007), have been used to investigate endothelial biology and differentiation. Such cell lines allow for efficient real time monitoring of gene regulation and have been widely used in stem cell research and, therefore, this approach was selected for the purposes of the hESC-EC optimisation.

As discussed in the previous chapter, hESC-EC differentiation protocol can be divided in two phases – the mesodermal induction phase and the endothelial specification phase. Thus, it is clear that a cell line with a single reporter gene would not be sufficient to monitor the efficacy of both differentiation phases and the use of multiple reporter constructs has to be considered. Additionally, it can be proposed that cell lines with multiple reporter constructs linked to non-overlapping fluorescent proteins (for example, GFP and RFP) could be an invaluable tool for the optimisation of the endothelial differentiation process.

The mesoderm induction phase of hESC-EC differentiation ends on day 3 of the differentiation, after the expression of mesodermal gene Brachyury peaks on day 2 as demonstrated in 3.1.6. To optimise this phase of the differentiation, a reporter for a gene expressed after mesodermal induction but prior to induction of the full endothelial program would be required. There are multiple candidate genes which match this requirement. For example, friend leukaemia integration 1 TF (FLI1) has been demonstrated to be a key TF for endothelial development (Liu et al., 2008) and transdifferentiation (Ginsberg et al., 2012), and to regulate CDH5 expression (Asano et al., 2010). However, *FLI1* is expressed early during differentiation and is also expressed in *cloche* zebrafish mutant lacking endothelial cell development (Brown et al., 2000) and, thus, an alternative gene reporting endothelial specification would be preferable.

The zinc finger TF GATA2 is another key player in endothelial and hematopoietic development (Lugus et al., 2007; Shi et al., 2014). However, in later

development it becomes restricted to hematopoietic lineages (Brown et al., 2000) and hESC can be differentiated towards endothelial lineages in the absence of *GATA2* (Huang et al., 2015), limiting the usefulness of *GATA2* reporters for the optimisation of the endothelial differentiation.

In contrast, ETV2, a member of the E-twenty six TF factor family, plays a central role in endothelial development. Indeed, ETV2 induces vascular mesoderm (Kataoka et al., 2011) and is required for endothelial and hematopoietic development (Ferdous et al., 2009; Shi et al., 2014). Overexpression of ETV2 induces endothelial gene expression in hESC (Elcheva et al., 2014), increases efficiency of endothelial differentiation (Lindgren et al., 2015) and can be exploited for endothelial transdifferentiation of fibroblasts (Morita et al., 2015), skeletal muscle (Veldman et al., 2013) and amniotic cells (Ginsberg et al., 2015, 2012). Given the overwhelming evidence for the role of ETV2 in early endothelial development, it was selected as the best candidate gene for the early differentiation reporter construct.

It is also necessary to select a gene indicating endothelial commitment. Here, *CDH5* was used, as it has been widely used for monitoring of endothelial differentiation before (James et al., 2010; Sahara et al., 2014; Schmeckpeper et al., 2009). Additionally, *ROBO4*, a member of the roundabout family, was chosen as a complimentary, endothelium specific gene (Huminiecki et al., 2002; Huminiecki and Bicknell, 2000) for more robust monitoring of endothelial differentiation.

This chapter describes the work undertaken for the generation and validation of hESC reporter cell lines for hESC-EC differentiation optimisation. Firstly, *ETV2* and *ROBO4* expression was profiled during hESC-EC differentiation, this was followed by generation of *CDH5*, *ROBO4* and *ETV2* reporter constructs. The functionality of the constructs was tested in NCI60 cancer cell lines, followed by generation of lentiviruses carrying the reporter constructs and further validation attempts in primary cell lines and during hESC-EC differentiation. Finally, alternative *CDH5* and *ETV2* reporter constructs were outsourced and tested during hESC-EC differentiation.

4.2 Aims

The aims of this chapter were:

- To assess the expression of *ETV2* and *ROBO4* during hESC-EC differentiation

- To generate *ETV2*, *CDH5* and *ROBO4* virally delivered reporter constructs and test them during hESC-EC differentiation.

- To validate the outsourced *ETV2* and *CDH5* reporter constructs.

4.3 Results

4.3.1 *ETV2* and *ROBO4* expression during hESC-EC differentiation.

To assess *ETV2* expression during hESC-EC differentiation, H1 and RC-11 were differentiated as described in Methods section 2.3, and RNA was collected on day 0, and days 2 - 7 for RT-PCR analysis. For *ROBO4* expression analysis, RNA was collected on day 0, 3, 5 and 7 of hESC-EC differentiations with H9.

As shown in Figure 21, *ETV2* was expressed both in pluripotent RC-11 and H1 with Ct values of 30.6 ±1.2 and 31.3 ±0.2 accordingly. In RC-11, the expression of *ETV2* was upregulated 8-fold on day 3 ($p<0.001$ vs day 2) and further upregulated on day 4 ($p<0.001$ vs day 3) reaching Ct values of 26.4 ±1.0. This was followed by rapid downregulation on day 5 ($p<0.001$ vs day 4) and return to baseline levels by day 7.

In contrast, *ETV2* expression was rapidly upregulated and peaked on day 3 during H1 hESC-EC differentiation ($p<0.001$ vs day 0 and day 2, Ct of 26.3 ±0.2). This was followed by downregulation on day 5 ($p<0.001$ vs day 3, $p<0.01$ vs day 4). By day 7, *ETV2* remained expressed at a slightly higher level than on day 0 with Ct values of 30.7 ±0.1.

In addition to the gene expression analysis, *ETV2* expression was also assessed at protein level between days 4 - 7 of hESC-EC differentiation with RC-11 (Figure 23). Staining was not performed before day 4 due to cells being differentiated in EBs which are not suitable for ICC. As shown in Figure 23, staining for *ETV2* could be observed with the first appearance of CD31+ cells on day 4 and co-localised with cell nuclei, as expected. *ETV2* expression was not observed after day 5 of hESC-EC differentiation.

ROBO4 was expressed at a low level in pluripotent H9 hESC (Figure 22) with an average Ct value of 33.8 ±0.2. As expected, the expression of *ROBO4* was upregulated 19-fold on day 5 of hESC-EC differentiation ($p<0.05$ vs day 0) and further upregulated over 120-fold on day 7 ($p<0.01$, vs day 0) reaching Ct values of 25.8 ±0.1.

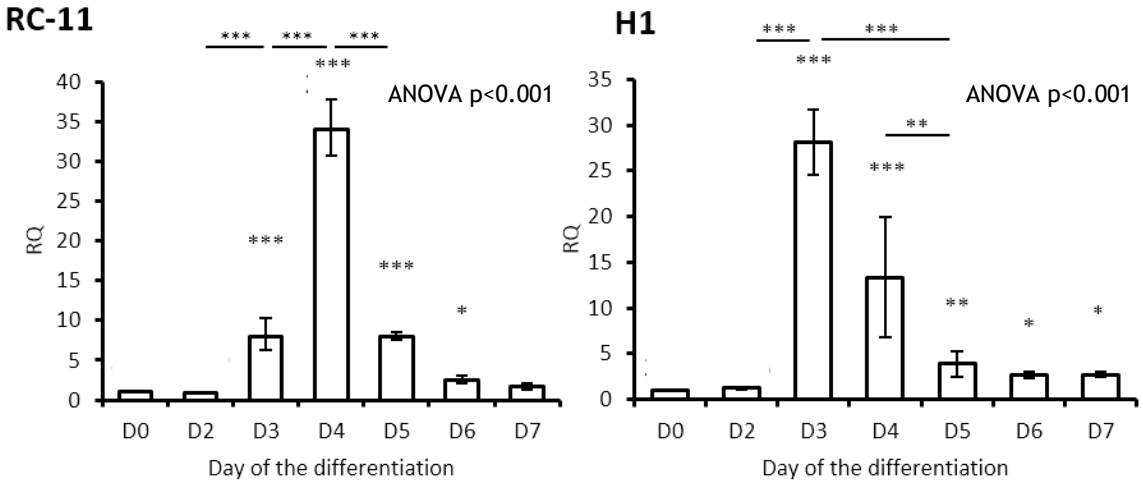


Figure 21: Expression of ETV2 during hESC-EC differentiation. *RNA was collected on day 0 (D0), and days 2 – 7 (D2-D7) of hESC-EC differentiation with RC-11 (n=3, independent experiments) and H1 (n=3, independent experiments). Expression of ETV2 was quantified using qRT-PCR. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05, ** p<0.01, *** p<0.001 when compared to d0 hESC control, unless indicated otherwise. Data shown is RQ ± RQ max and min.*

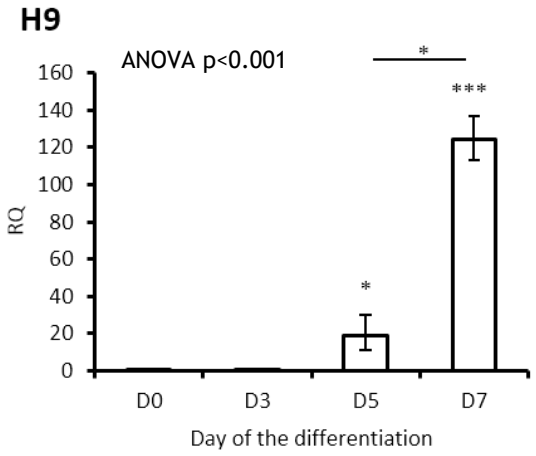


Figure 22: Expression of ROBO4 during hESC-EC differentiation. *RNA was collected on days 0, 3, 5 and 7 (D0, D3, D5 and D7) of hESC-EC differentiation with H9 (n=3, independent experiments). Expression of ROBO4 was quantified using qRT-PCR. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05, ** p<0.01, *** p<0.001 when compared to d0 hESC control, unless indicated otherwise. Data shown is RQ ± RQ max and min.*

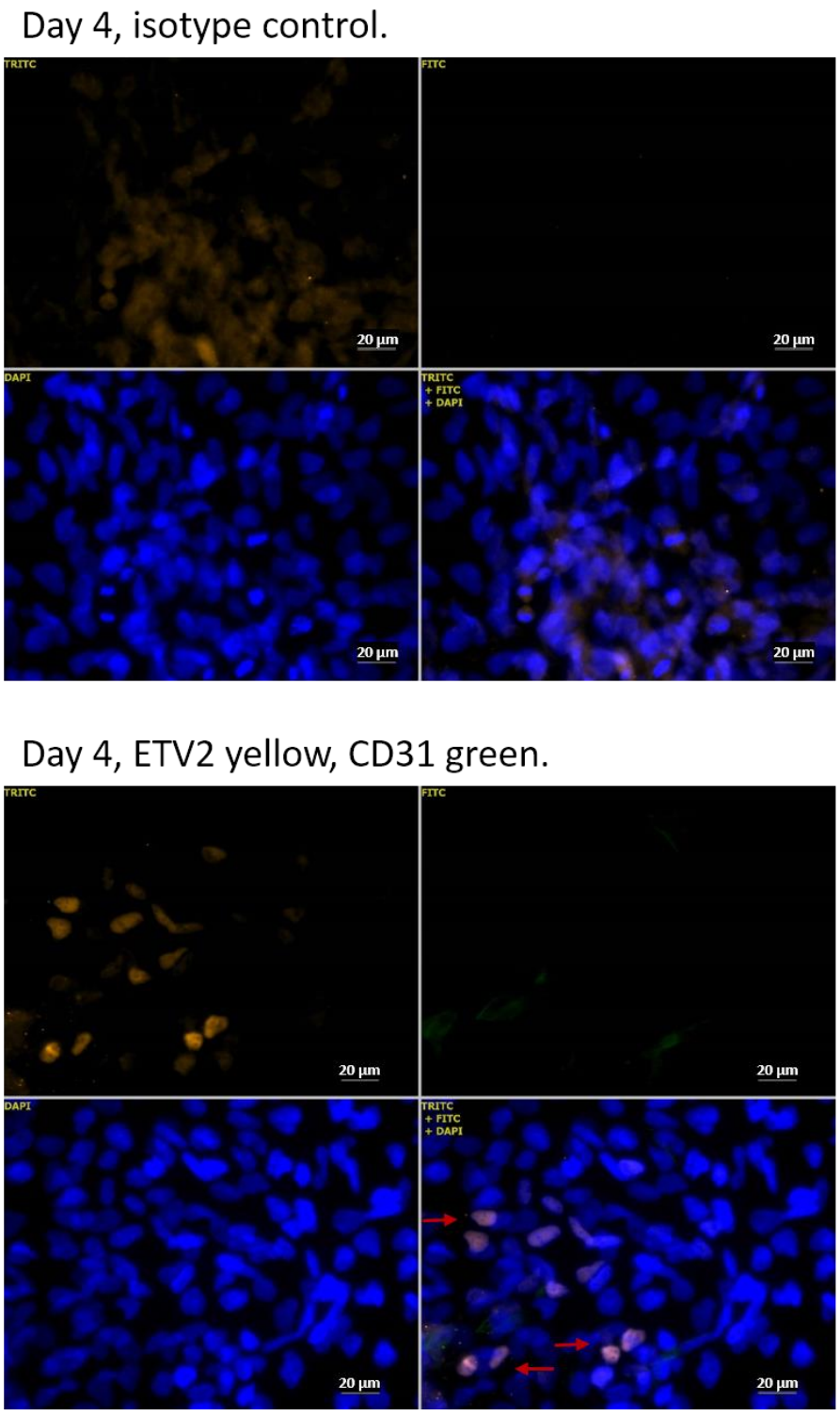


Figure 23: Immunocytochemistry showing the expression of ETV2 during hESC-EC differentiation. *RC-11 were differentiated towards endothelial lineages (n=3, independent experiments, representative images shown) and fixed between days 4 and 7 of hESC-EC differentiation. ETV2 staining shown in yellow, CD31 in green, DAPI in blue. Red arrows show overlap of ETV2 and DAPI staining. Scale bar 20µm. Continued on page 140, 141 and 142.*

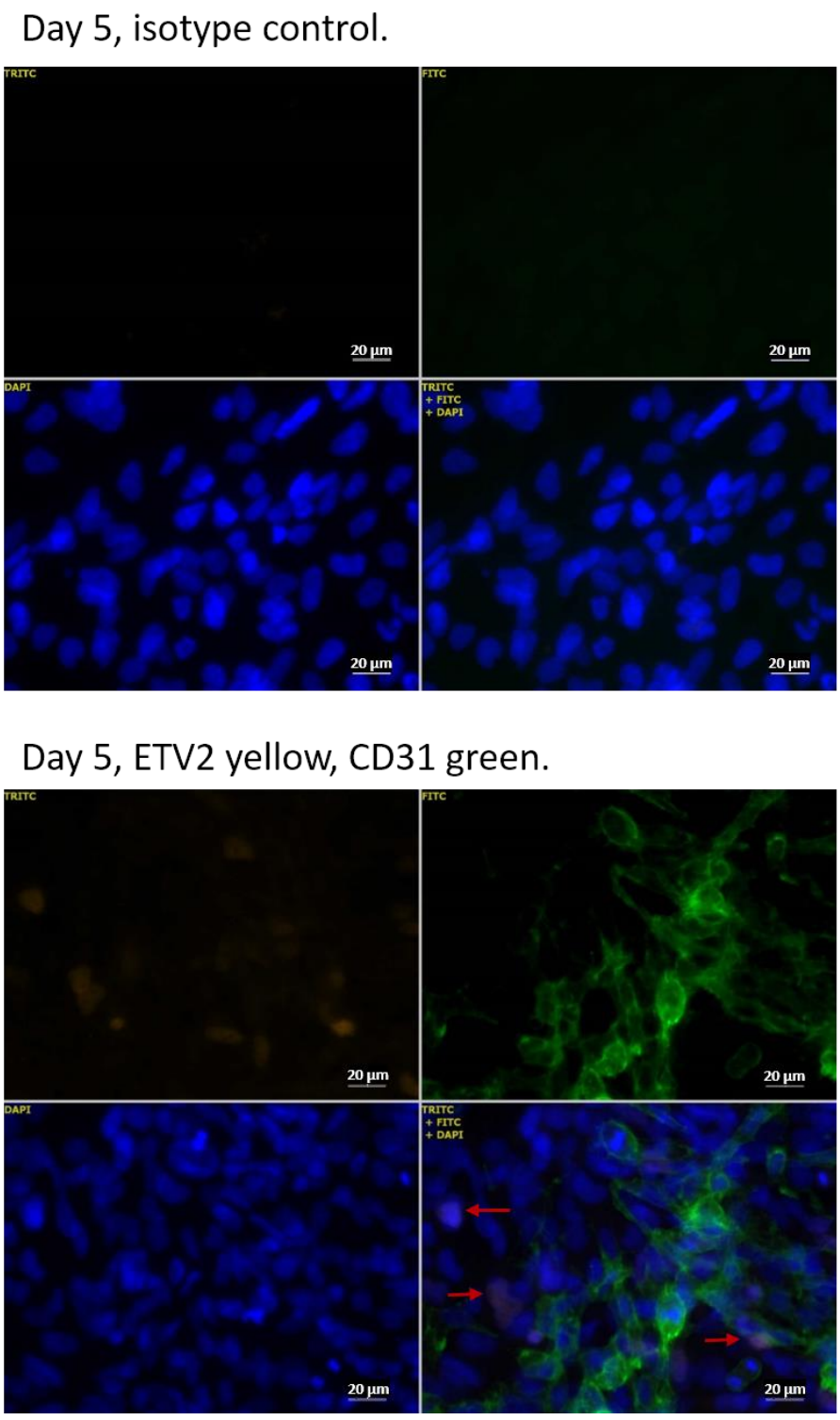


Figure 23 (cont.): Immunocytochemistry showing the expression of ETV2 during hESC-EC differentiation with RC-11. *RC-11 were differentiated towards endothelial lineages (n=3, independent experiments, representative images shown) and fixed between days 4 and 7 of hESC-EC differentiation. ETV2 staining shown in yellow, CD31 in green, DAPI in blue. Red arrows show overlap of ETV2 and DAPI staining. Scale bar 20µm. Continued on pages 141 and 142.*

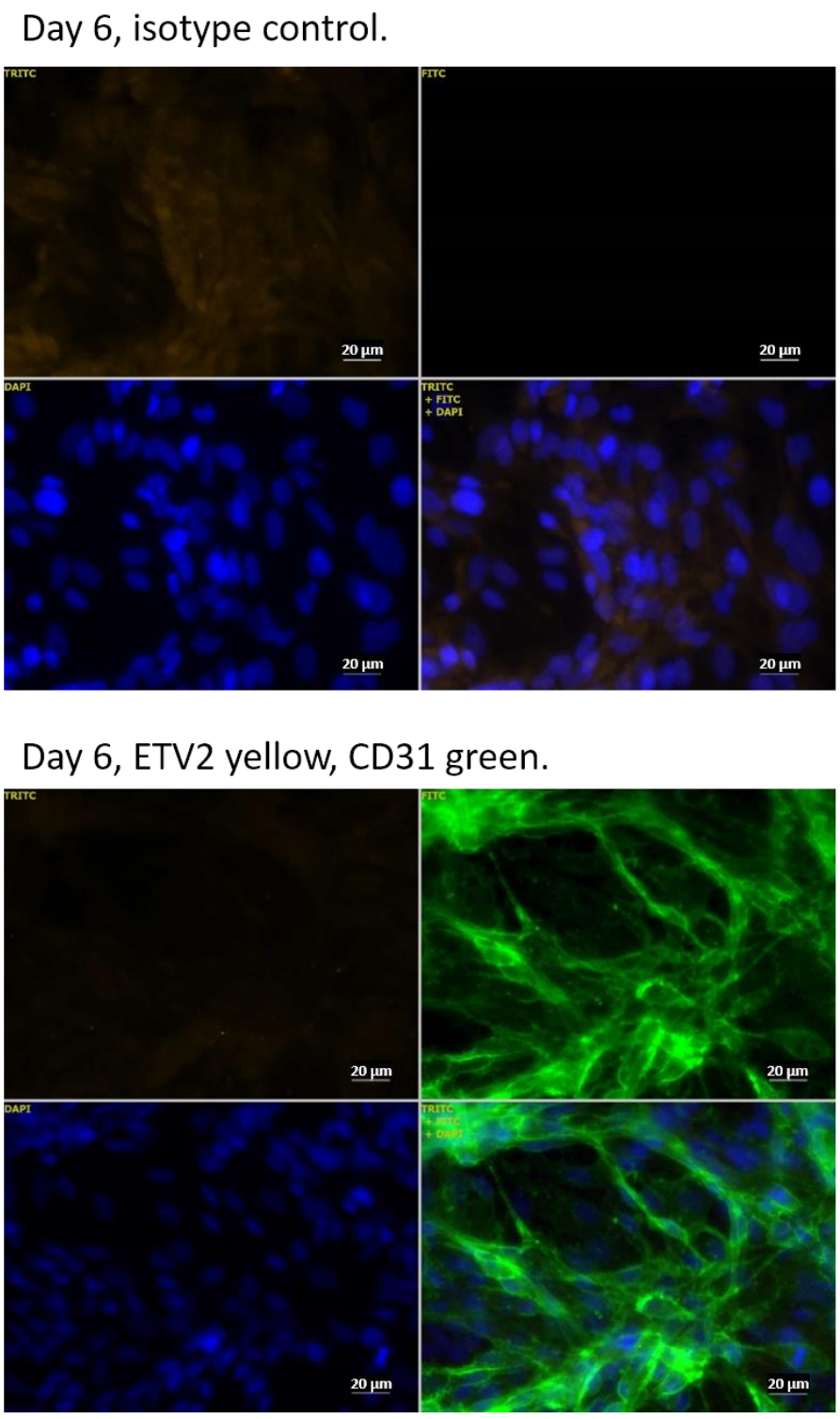


Figure 23 (cont.): Immunocytochemistry showing the expression of ETV2 during hESC-EC differentiation with RC-11. *RC-11 were differentiated towards endothelial lineages (n=3, independent experiments, representative images shown) and fixed between days 4 and 7 of hESC-EC differentiation. ETV2 staining shown in yellow, CD31 in green, DAPI in blue. Red arrows show overlap of ETV2 and DAPI staining. Scale bar 20µm.* *Continued on page 142.*

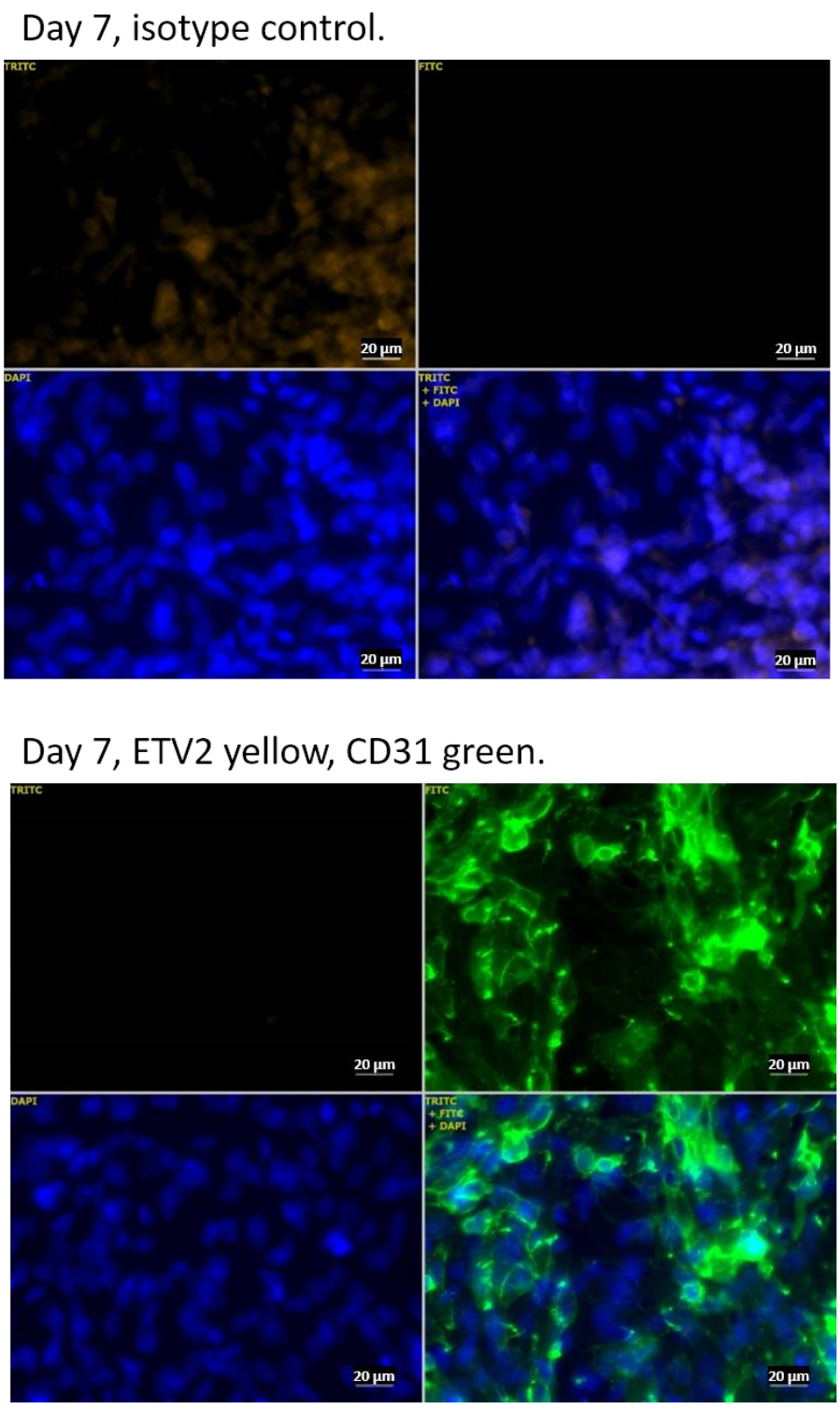


Figure 23 (cont.): Immunocytochemistry showing the expression of ETV2 during hESC-EC differentiation with RC-11. *RC-11 were differentiated towards endothelial lineages (n=3, independent experiments, representative images shown) and fixed between days 4 and 7 of hESC-EC differentiation. ETV2 staining shown in yellow, CD31 in green, DAPI in blue. Red arrows show overlap of ETV2 and DAPI staining. Scale bar 20µm.*

4.3.2 Generation and preliminary validation of reporter constructs for hESC-EC differentiation.

Two lentiviral plasmid backbones were outsourced (ABMgood, US) for generation of the hESC-EC reporter constructs - pLenti promotorless (pLenti) and pLenti promotorless GFP (pLenti-GFP). The plasmid maps, including multiple cloning sites are shown in Figure 24.

Primers with added restriction sites of interest as shown in Table 14, were used to amplify the promoters of interest from purified H9 hESC DNA, and to clone the fragments of interest using the respective restriction sites in plasmids, as shown in Table 15 and described in section 1.4. In addition, monomeric RFP (mRFP) sequence was amplified from pMXs-mRFP1 plasmid (Addgene, US) and ligated in the pLenti backbone. Overview of the steps taken for generation of *CDH5* and *ETV2* RFP reporter constructs can be seen in Figure 25, and a similar overview is given for the generation of CDH5 and ROBO4 GFP reporter constructs in Figure 26.

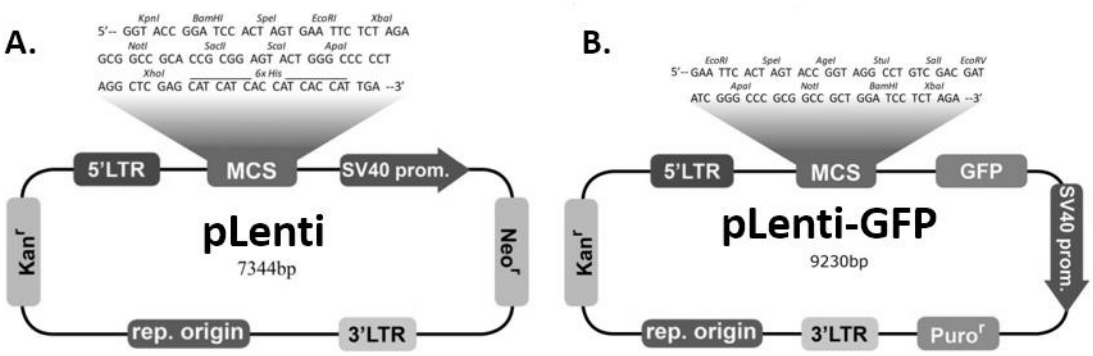


Figure 24: Schematic of the two outsourced plasmid backbones used for generation of hESC-EC differentiation reporter constructs. Adapted from: Promoterless GFP Lentiviral Vector, and Promoterless Lentiviral Vector product manuals (ABMgood, US).

Fragment		*Restriction sites*	*Primer sequence*
mRFP	FW	ApaI	ATTA**GGGCCC**GCCACCATGGCCTCCT
	RV	XhoI	GCGC**CTCGAG**TTCTCAGTTATGTATTTTTCCATGC
Promoters			
CDH5	FW	NotI	ATTA**GCGGCCGC**TCTCTCCTGGTCAGCAG
	RV	SacII	ATTA**CCGCGG**CTGTGGGCTGAGGGATG
CDH5	FW	SpeI	GCGC**ACTAG**TTTCTCAGGGTCTCTGCT
	RV	NotI	ATTAT**GCGGCCG**CATCTTGGGCGCAGGG
ROBO4	FW	SalI	CGCG**GTCGAC**GCTGACATTGTAGGCTC
	RV	ApaI	ATTA**GGGCCC**GGAGGCTGTCTCCTCC
ETV2	FW	NotI	CC**GCGGCCGC**ACAGGCTGATCTAGAACTCC
	RV	SacII	CG**CCGCGG**CTGGGAGAAGTTTACGG

Table 14: Primers used for polymerase chain reaction. *FW – Forward primer, RV - Reverse primer. Sequences in bold indicate restriction sites.*

	Restriction Sites	*Insert*
pLenti		
	ApaI / XhoI	mRFP
	NotI / SacII	CDH5
	NotI / SacII	ETV2
pLenti-GFP		
	SalI / ApaI	ROBO4
	SpeI / NotI	CDH5

Table 15: Restriction sites used to insert the amplified inserts into pLenti and pLenti-GFP plasmids.

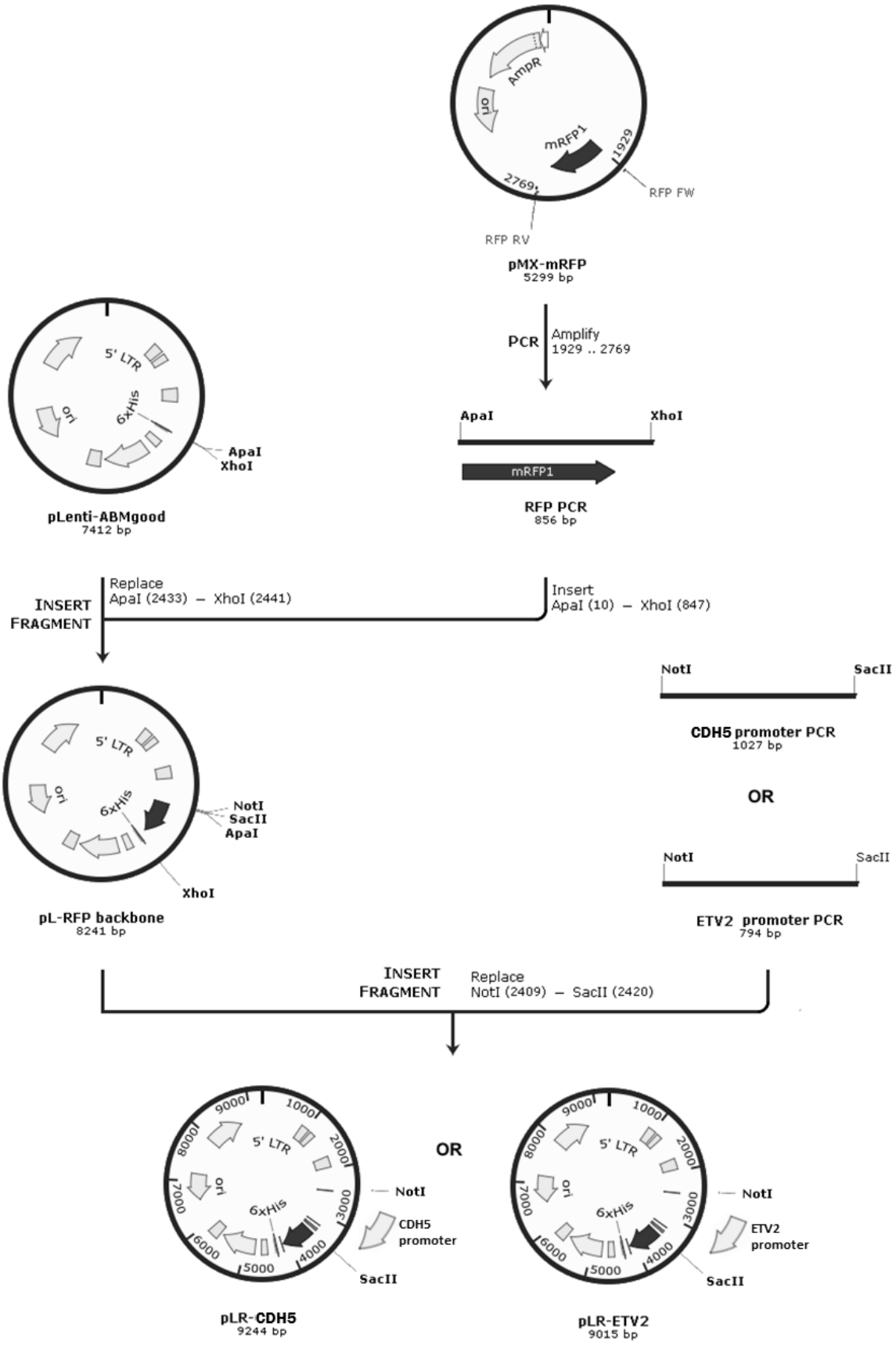


Figure 25: Schematic of steps taken to generate pLR-CDH5 and pLR-ETV2 reporter constructs. *The generated constructs contain an RFP sequence driven by CDH5 or ETV2 promoter respectively.*

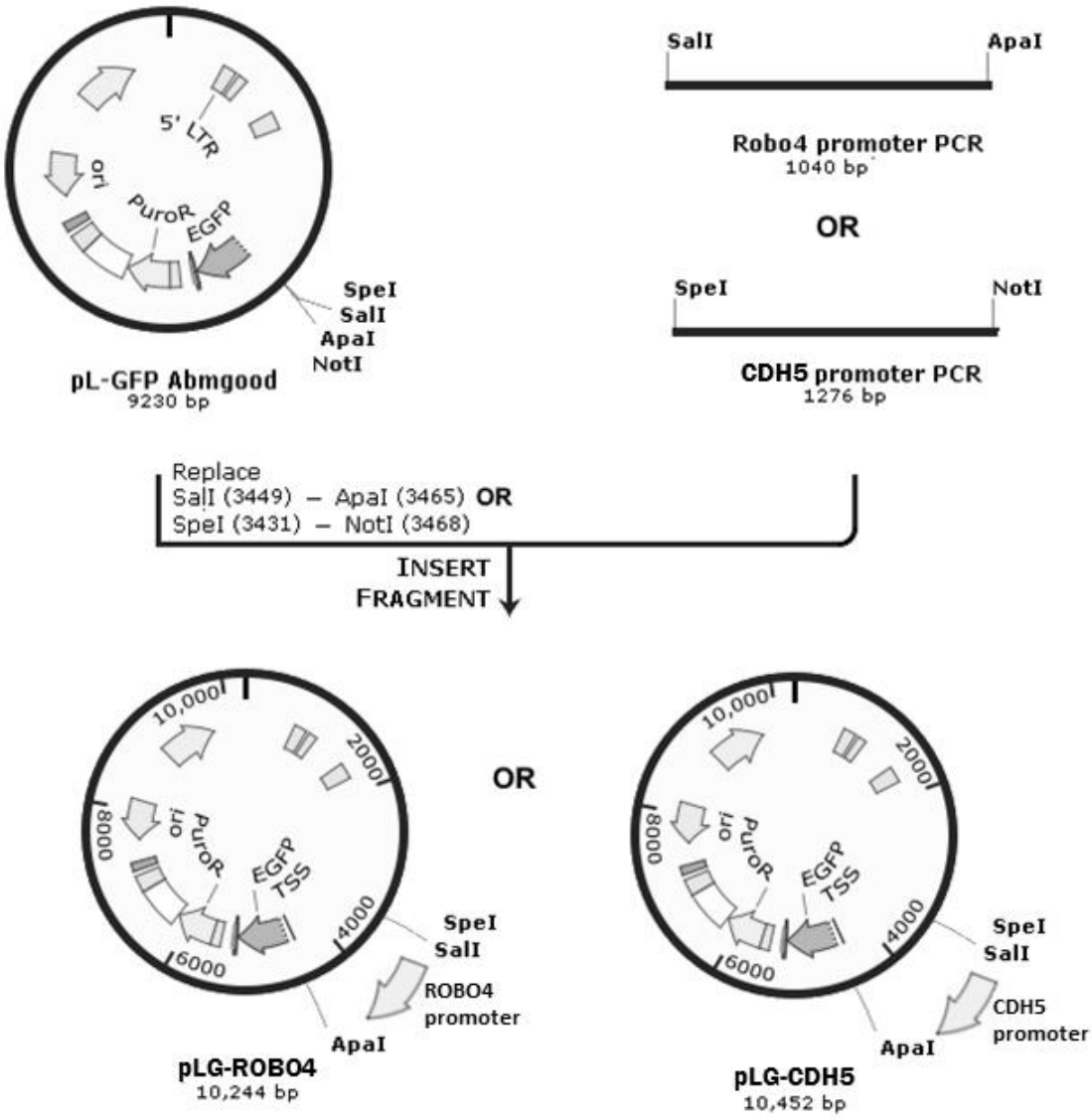


Figure 26: Schematic of steps taken to generate pLG-ROBO4 and pLG-CDH5 reporter constructs. *The generated construct contains an enhanced GFP (eGFP) sequence driven by ROBO4 or CDH5 promoter respectively.*

Control digests of the generated reporter plasmids were performed to confirm the presence of the promoter inserts. As shown in Figure 27a, control restriction digestion generated fragments of the expected sizes. The digest of pLG-ROBO4 (column A) with SalI and ApaI generated 9.2 kbp and 1 kbp fragments; pLG-CDH5 (column B) SpeI and NotI digest generated 9.2 kbp and 1.2 kbp fragments; pLR-ETV2 (column C) XhoI and NotI digest resulted in 7.4 kbp and 1.6 kbp fragments; while pLR-CDH5 (column D) XhoI and NotI digest generated 7.4 kbp and 1.9 kbp fragments, as expected. In addition, the generated plasmids were sequenced and the presence of the promoter constructs was confirmed.

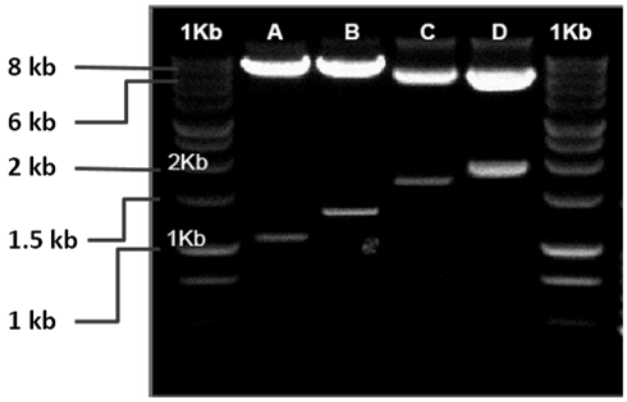


Figure 27: Control digests of generated reporter. *Control restriction enzyme digests of the generated plasmids confirm presence of the inserts. Column A: pLG-ROBO4 SalI and ApaI digest. Column B: pLG-CDH5 SpeI and NotI digest. Column C: pLR-ETV2 XhoI and NotI digest. Column D: pLR-CDH5 XhoI and NotI digest. 1Kb. Promega 1Kb DNA ladder, fragments of interest indicated on the left hand side.*

Initially, it was attempted to validate the generated plasmids using transfections in NCI60 cell lines expressing the markers of interest. While there was visible GFP expression in cell lines transfected with a plasmid expressing enhanced GFP (eGFP) under constitutively active cytomegalovirus (CMV) promoter (a kind gift from Dr Laura Denby, University of Glasgow), GFP reporter expression was not observed in the cell lines transfected with the reporter constructs (n=1, data not shown). Therefore, it was decided to attempt validation using lentiviral vectors

carrying the constructs as this would allow for validation using primary cell lines or during hESC-EC differentiation.

Lentiviral vectors were generated using pLR-ETV2, pLR-CDH5, pLG-CDH5 and pLG-ROBO4 plasmids as described in Methods section 1.5. The concentrated lentivirus solution was titred in HEK293T cells and, in addition, particle counts and sizes were estimated using Nanosight (Malvern, UK) with the results shown in Table 16.

To validate the reporter constructs, HUVEC, HSVEC and IGROV cell lines were transduced using the generated lentiviruses carrying the CDH5 and ROBO4 reporter constructs. Furthermore, H9 and RC-11 hESC were transduced with lentiviral vectors carrying pLR-ETV2 or pLG-CDH5 construct accordingly, followed by hESC-EC differentiation. Reporter expression was observed visually and evaluated using FC on day 5 and day 7, as appropriate. While control cell lines transduced with lentiviral vectors carrying constitutively active spleen focus-forming virus (SFFV) eGFP (a kind gift from Prof. Adrian Thrasher, Institute of Child Health, University College London, UK) induced the expression of eGFP in the majority of cells across the cell lines, expression of GFP in the cell lines transduced with reporter constructs was not observed (all n=1, data not shown). Due to the lack of any reporter expression in these experiments, additional repeats were not performed and alternative reporter constructs were outsourced.

Vector	*Titre, PIU/mL*	*Particle count, P/mL*	*Particle size, nm*
pLR-CDH5	4×10^9	3×10^{11}	123
pLG-ROBO4	9×10^9	1.3×10^{12}	134
pLR-ETV2	8×10^7	1.5×10^{12}	120
pLG-CDH5	2.5×10^{10}	*	*
ETV2-GFP *(Genecopoeia)*	4.5×10^8	*	*
CDH5-GFP *(Published)*	9×10^8	*	*

Table 16: Titres of the generated lentiviruses. *Infectious particle counts were obtained by titring in HEK293T cells. In addition, total viral titre and particle sizes were measured using Nanosight, unless indicated by *.*

4.3.3 Preliminary validation of an outsourced CDH5 reporter

CDH5 reporter construct is crucial for the optimisation of the endothelial differentiation as it indicates mature endothelial commitment. As the *CDH5* reporter constructs described in the previous section could not be validated, a construct where a 2.5kb *CDH5* promoter fragment drives eGFP expression (SM-CDH5-GFP reporter) was obtained (a kind gift from Sahara et al. (2014)). Control restriction digests were performed and yielded expected fragment sizes, as shown in Figure 28. The generated lentiviral vectors were titrated using HEK293T cells and a titre of 9×10^8 PIU/mL was obtained as shown in Table 16.

To validate the SM-CDH5-GFP reporter, RC-11 and H9 hESC were infected on day 0 of hESC-EC differentiation (both n=1, separate experiments, data not shown). In both of these cell lines, large percentage of cells expressing GFP but not staining positive for CD144 was observed. Furthermore, reporter GFP expression was observed in CD144- pluripotent H9 hESC. While additional repeats are required to draw conclusions, these observations suggested that this construct does not function correctly in this differentiation system and more work is require to generate reliable CDH5 reporter hESC lines. Therefore, further repeats and validation experiments were not performed and it was decided to focus on alternative reporter constructs for optimisation of the hESC-EC differentiation.

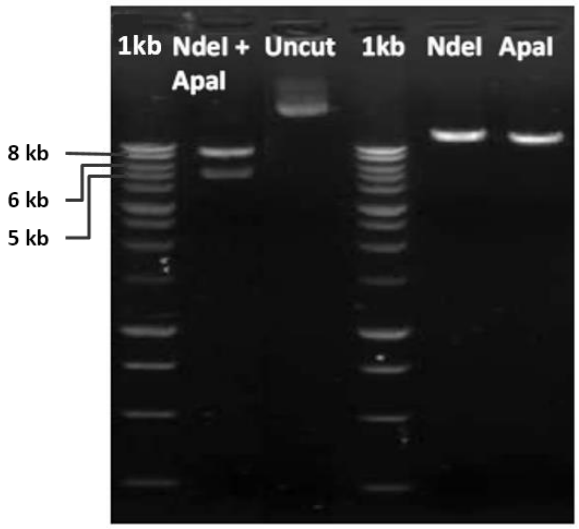


Figure 28: Control digests of the outsourced SM-CDH5-GFP reporter construct. *A single restriction site for NdeI and ApaI present in the plasmid. Double digest generated 5kb and 8kb fragments as expected. 1Kb shows Promega 1Kb DNA ladder, fragments of interest indicated on the left hand side.*

4.3.4 Validation of an outsourced ETV2 reporter.

The analysis of *ETV2* expression, as described in section 4.3.1 suggests that expression of *ETV2* indicates early endothelial commitment. Therefore, the use of an *ETV2* reporter construct represents a novel approach for the optimisation of the early endothelial differentiation. As the generated *ETV2* reporter constructs did not validate, a construct where 1.5 kbp ETV2 promoter fragment drives eGFP expression (GC-ETV2-GFP) was acquired from Genecopoeia (US). Control restriction digests were performed and yielded expected fragment sizes of 5.7kb, 2.2kb and 1.6kb as shown in Figure 29. The generated lentiviral vectors were titrated using HEK293T cells and a titre of 4.5 x 10^8 PIU/mL was obtained as shown in the previous table (Table 16).

To validate the GC-ETV2-GFP reporter construct, RC-11 hESC were transduced with the GC-ETV2-GFP lentivirus at a MOI of 10, grown to confluence and differentiated towards EC lineages (n=1, data not shown). When evaluating the GC-ETV2-GFP transduced hESC-EC differentiations, GFP expression was observed on day 4 and to a higher level on day 7 of hESC-EC differentiation, not matching the expected expression pattern of ETV2 from the gene expression data reported in 4.3.1 .

To ensure that all the cells carry the reporter gene construct, polyclonal reporter cell lines were generated by transducing RC-11 with lentiviral vectors carrying ETV2-GFP reporter construct, followed by culture in a selection media containing 2 µg/ml puromycin, and hESC-EC differentiation (n=3). Reporter expression was analysed by FC on days 0, 4 and 7 of the differentiation and by IC on day 4. CD144+ expression was evaluated on day 7 using FC.

Unexpectedly, the majority of cells (73.2% ±8.6) expressed GFP on day 0 of hESC-EC differentiation and the expression levels decreased over time with, on average, 24.3% (±7.7) expressing GFP on day 7, as shown in Figure 30. On average, 22.5% (±5.9) of cells expressed CD144 at the end of the differentiation suggesting successful hESC-EC differentiation. IC on day 4 of the differentiation (Figure 31) further confirmed the observation of high GFP expression that was not confined to cells expressing ETV2.

This suggests that the GC-ETV2-GFP reporter construct does not specifically report *ETV2* expression in hESC-EC differentiation system. Therefore, the work with this reporter construct was not continued.

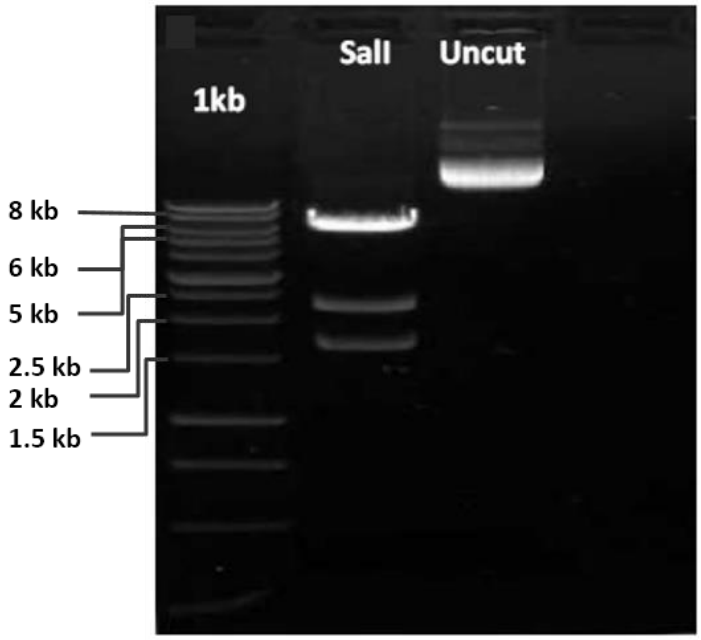


Figure 29: Control digests of the outsourced GC-ETV2-GFP reporter construct. *SalI digest generated 5.7kb, 2.2kb and 1.6kb fragments observed as expected. 1Kb shows Promega 1Kb DNA ladder, fragments of interest indicated on the left hand side.*

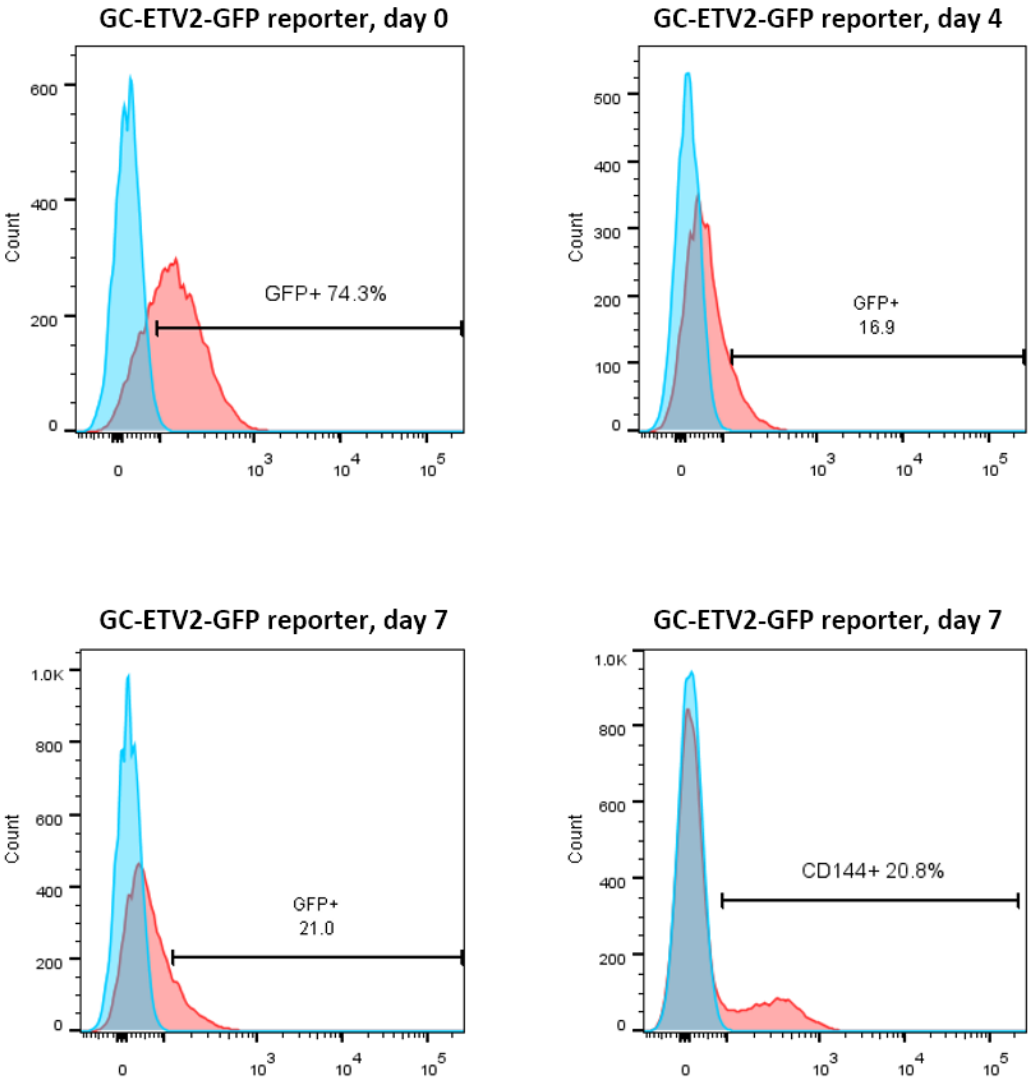


Figure 30: Validation of the outsourced GC-ETV2-GFP reporter construct. *RC-11 (n=3, independent experiments, representative data shown) were transduced with lentiviral vectors carrying GC-ETV2-GFP reporter construct, grown in a selection media containing puromycin, followed by hESC-EC differentiation. Reporter expression was analysed by FC on days 0, 4 and 7 of the differentiation, and CD144+ expression was evaluated on day 7 using antibody staining and FC. Uninfected hESC control differentiation or isotype controls, as appropriate, shown in blue.*

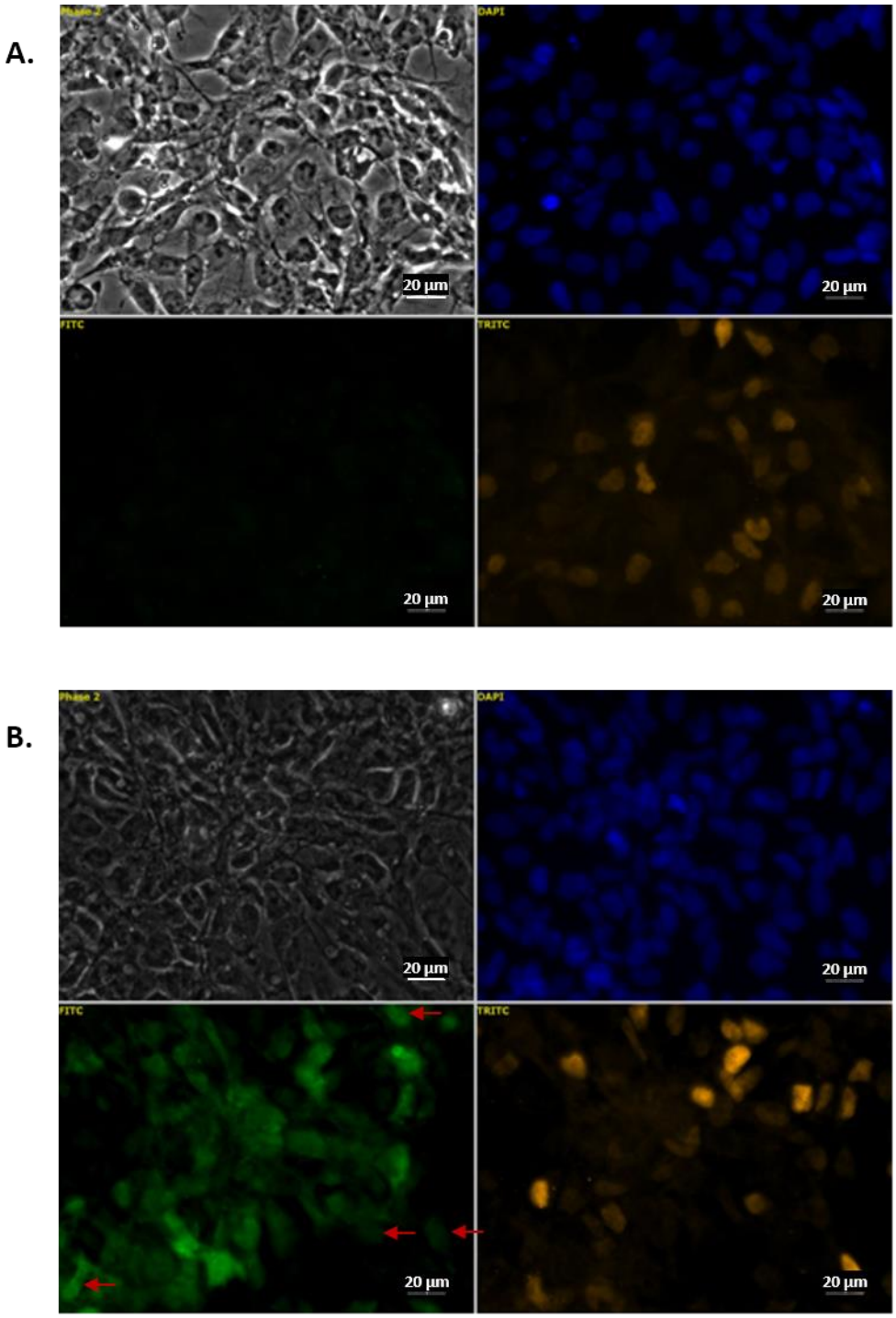


Figure 31: Immunocytochemistry showing the expression of ETV2 and GFP on day 4 of hESC-EC differentiation with RC-11 carrying the GC-ETV2-GFP construct. *RC-11 (n=3, representative images shown) were differentiated towards endothelial lineages, fixed and stained on day 4 of the differentiation. ETV2 staining shown in yellow, GFP in green, DAPI in blue. Red arrows indicate cells expressing GFP but not ETV2.* ***A.*** *Non-infected control.* ***B.*** *RC-11 infected with a lentiviral vector carrying the outsourced GC-ETV2-GFP reporter construct, followed by culture in selection media.* ***C.*** *Isotype control.* *Continued on page 156.*

C.

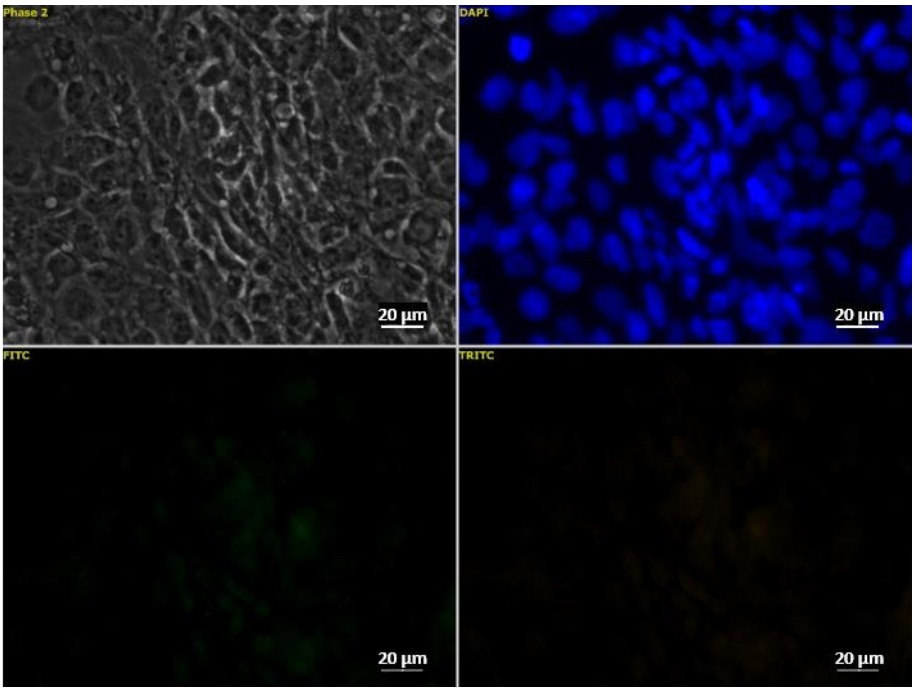


Figure 31 (cont.): Immunocytochemistry showing the expression of ETV2 and GFP on day 4 of hESC-EC differentiation with RC-11 carrying the GC-ETV2-GFP construct. *RC-11 (n=3, representative images shown) were differentiated towards endothelial lineages, fixed and stained on day 4 of the differentiation. ETV2 staining shown in yellow, GFP in green, DAPI in blue. Red arrows indicate cells expressing GFP but not ETV2.* ***A.*** *Non-infected control.* ***B.*** *RC-11 infected with a lentiviral vector carrying the outsourced GC-ETV2-GFP reporter construct, followed by culture in selection media.* ***C.*** *Isotype control.*

In summary, the data presented in this chapter showed that ETV2 is transiently upregulated at the end of the mesodermal induction phase of hESC-EC differentiation and, therefore, is a suitable gene for the monitoring of the early differentiation. CDH5, ROBO4 and ETV2 reporter constructs were generated using pLenti and pLenti-GFP plasmid backbones. Preliminary validation of the constructs in NCI60, HSVEC and HUVEC cell lines, as well as during hESC differentiation, suggested lack of reporter functionality and, thus, the work with these constructs was discontinued to focus on other reporter constructs. This was followed by preliminary validation of outsourced CDH5 and ETV2 reporter constructs. Here, the preliminary analysis showed non-specific reporter gene expression when either of these constructs were transduced in hESC. While further repeats are required to confirm these observations, it was decided to discontinue to work with these constructs and other approaches for optimisation of the hESC-EC differentiation were employed.

4.4 Discussion

The described hESC-EC differentiation protocol efficiently generates cells expressing EC markers CD31 and CD144, without the use of cell sorting during the differentiation process. However, there is scope for further optimisation to both increase the percentage of cells expressing EC markers at the end of the differentiation and to aid EPC survival and proliferation during the differentiation. The use of reporter cell lines allows monitoring gene expression changes in real time during the differentiation and, thus, is highly suitable for the purposes of screening small molecule drugs during hESC-EC differentiation. Firstly, multiple reporter constructs for monitoring hESC-EC differentiation were generated and validation was attempted. This was followed by validation of outsourced CDH5 and ETV2 reporter constructs, as described in the following discussion.

Optimisation of the hESC-EC differentiation protocol – choice of genes for monitoring of the EPC and EC commitment.

HESC-EC differentiation consists of two phases – mesoderm induction and vascular specification phase. It is clear that monitoring expression of a single gene would not be sufficient for the optimisation of both differentiation phases and, thus, a gene indicating early endothelial progenitor commitment is required in addition to endothelial specification reporters. Here, *ETV2*, a member of the E-twenty six TF factor family, expression was used for monitoring early endothelial commitment. ETV2 is crucial for blood and vascular development and *ETV2* knock-out mice display embryonic lethality with vascular defects and significantly reduced KDR+ mesoderm formation (Ferdous et al., 2009; Lee et al., 2008; Wareing et al., 2012). *ETV2* is expressed transiently during development (Ferdous et al., 2009), initiates the specification of vascular mesoderm (Kataoka et al., 2011) and facilitates endothelial and hematopoietic differentiation (Shi et al., 2014). Indeed, ETV2 binds and activates multiple key endothelial genes, including *KDR*, *TAL1*, *CDH5* and *PECAM* (De Val et al., 2008; Ferdous et al., 2009; Prandini et al., 2005).

Furthermore, a range of experimental approaches have confirmed the role of ETV2 in the induction of endothelial phenotype. A key paper by Ginsberg et al. (2012) demonstrated that transient *ETV2* expression transdifferentiated amniotic c-kit+ cells to endothelial cells. These cells were highly proliferative and showed similar transcriptome profiles to HUVECs and human adult liver sinusoidal ECs. Later, Veldman et al. (2013) reported that ETV2 induced endothelial transdifferentiation of zebrafish fast muscle cells to endothelial cells *in vivo* and showed that it was not dependent on VEGF signalling. The ability of ETV2 to induce EC phenotypes has also been confirmed in murine (Koyano-Nakagawa et al., 2012) and human stem cell differentiation models (Elcheva et al., 2014; Lindgren et al., 2015). Therefore, *ETV2* is a highly suitable gene for monitoring of early endothelial differentiation and it can be proposed that the hESC-EC protocol can be optimised for increased ETV2+ cell generation during the first phase of the differentiation.

Firstly, the expression of *ETV2* was quantified during hESC-EC differentiation with RC-11 and H1. *ETV2* expression was upregulated after the downregulation of brachyury expression (see gene expression data in 3.3.6), and peaked on day 3 in H1 and day 4 in RC-11, followed by downregulation. This is in line with the expected expression patterns as well as with observations by Lindgren et al. (2015) where *ETV2* expression was also upregulated at the end of mesodermal specification phase, after the peak of brachyury expression. Similarly, *ETV2* expression was upregulated by day 3 and downregulated after day 5 of hESC-EC differentiation with H9 (Boulberdaa et al., 2016).

When protein expression levels were compared, ETV2 expression was localised to cell nuclei, as expected, and transiently visible on days 4 and 5 of the differentiation, in line with the RNA expression data. However, here no relationship between ETV2 expression levels and the differentiation efficiency was established. While ETV2 overexpression has been reported to induce endothelial phenotypes (Elcheva et al., 2014; Lindgren et al., 2015), evidence from fibroblast endothelial transdifferentiation suggests that ETV2 expression has to be precisely regulated for optimal endothelial differentiation (Morita et al., 2015). Thus, currently it is not clear whether increased ETV2 expression in

hESC-EC differentiation system would result in higher CD31+ CD144+ cell percentage at the end of the differentiation.

In addition, ETV2 protein expression levels were not analysed prior to day 4 of hESC-EC differentiation due to EB culture system being unsuitable for ICC staining. However, western blotting could be used instead to validate the ETV2 antibody, quantify ETV2 protein levels during mesodermal induction phase and to gain further insight into ETV2 expression patterns during hESC-EC differentiation. This would further confirm the suitability of ETV2 for monitoring of early endothelial differentiation.

For monitoring of the vascular specification phase, reporter constructs for *CDH5* and *ROBO4* expression were generated. CDH5 is the major protein forming endothelial adherens junctions (Dejana et al., 1999), has been widely used in endothelial reporter constructs (James et al., 2010; Sahara et al., 2014; Schmeckpeper et al., 2009) and is progressively upregulated during hESC-EC differentiation as described in section 3.3.6. *ROBO4* has gained attention as another gene specifically expressed in mature endothelium (Huminiecki et al., 2002; Huminiecki and Bicknell, 2000) and, as expected, *ROBO4* mRNA expression was upregulated from day 5 of hESC-EC differentiation with the appearance of CD31+ CD144+ cells. Therefore, the use of these two reporter constructs should allow for robust monitoring of endothelial phenotype induction during hESC-EC differentiation.

Preliminary validation of the generated CDH5, ETV2 and ROBO4 reporter construct plasmids.

Four reporter constructs were generated (pLR-CDH5, pLR-ETV2, pLG-ROBO4 and pLG-CDH5) by cloning promoters of interest upstream from reporter protein GFP or RFP sequences. Control digests and plasmid sequencing were performed and confirmed the presence of the promoter inserts in the generated plasmids. Preliminary validation used NCI60 cell lines, selected for their expression of genes of interest using BioGPS NCI60 cell line gene expression database (Wu et al., 2009) and transfected with the generated constructs. The plasmids were

successfully transfected in the NCI60 cell lines, as suggested by the observed expression of eGFP in the control cultures transfected with the CMV-eGFP plasmid. However, the expression of reporter genes was not observed in any of the cell lines transfected with the reporter constructs.

While more repeats are needed to draw reliable conclusions, this suggests that either the reporter constructs were not functional. Generally, GFP transcript and fluorescence levels display a good correlation, and as little as <100 GFP transcripts are sufficient for GFP detection by FC allowing for monitoring of genes with weak expression (Bloom et al., 2014). Therefore, FC analysis or the more sensitive Proximity Ligation Assay qPCR (Ståhlberg et al., 2012) should be performed to confirm the lack of reporter gene expression. However, this experiment was not repeated and validation was attempted using lentiviral vectors which would allow for reporter gene validation in a range of cell lines and also during hESC-EC differentiation, when strong gene of interest induction is observed.

Preliminary validation of lentiviral vectors carrying the generated CDH5, ETV2 and ROBO4 reporter constructs.

Lentiviral vectors carrying pLR-CDH5, pLR-ETV2, pLG-ROBO4 and pLG-CDH5 constructs were generated and titred in HEK293T cells. In addition, pLR-CDH5, pLR-ETV2 and pLG-ROBO4 lentiviral vector particle size was estimated and matched the expected lentiviral size of approximately 120nm (Perletti et al., 2004).

To validate the *CDH5* or *ROBO4* reporter constructs, HSVEC, HUVEC and IGROV cell lines, and hESC lines subjected to hESC-EC differentiation were transduced with the generated viruses and reporter gene expression was observed visually and evaluated by FC. Lentiviral vector carrying SFFV-GFP construct was used as a control and demonstrated high transduction levels. However, reporter expression was not observed in any of these validation experiments. While this data suggests lack of the reporter construct functionality, only single repeats

were performed of these experiments and, thus, this data should be regarded as preliminary and more repeats are needed to confirm these observations.

Promoter fragments used in the CDH5, ETV2 and ROBO4 reporter constructs.

Another possible explanation for the lack of the reporter functionality is the use of an incomplete promoter fragment. Indeed, previously described *CDH5* promoter constructs (Alva et al., 2006; Sahara et al., 2014) have used longer promoter sequences, while here the promoter fragment size was limited to approximately 1 kbp for optimal virus production and transduction (Canté-Barrett et al., 2016; Kumar et al., 2001). However, *CDH5* promoter analysis by Gory et al. (1999) suggests that the main transcriptional machinery binding region is within +24 base pair (bp) and -139 bp, in relation to transcription start site, followed by the major specific inhibitory region to -289 bp, and both of these promoter regions were included in the promoter construct as shown in Figure 32. Similarly, promoter analysis performed by Prandini et al. (2005) demonstrated the presence of two critical endothelium specific promoter region within -1135/-744 bp and -166/-5 bp and these regions were also included in the promoter constructs and have been used in other published *CDH5* reporter constructs of a similar size (Schmeckpeper et al., 2009).

Previously published murine *ETV2* GFP reporter construct used 5 kbp upstream region (Wareing et al., 2012), while here 0.8 kbp fragment was used rationalising that it would include the previously described fragments required for the promoter activity. Analysis of the murine *ETV2* promoter by De Haro and Janknecht (2005) demonstrated promoter activity when -466/+51 bp promoter sequence was used, furthermore basal transcription was driven by a shorter promoter sequence of -85/+51 bp. These sequences were also present in the generated *ETV2* reporter construct (Figure 32) and, thus, should ensure functionality of the promoter region. However, two cAMP response elements have been described as a regulatory sequences for PKA mediated *ETV2* induction (Yamamizu et al., 2012b) and only one of these sites was included in the promoter reporter construct. In murine undifferentiated ESC, absence of the second cAMP response element significantly reduced the expression of a

luciferase reporter gene (Yamamizu et al., 2012b). Therefore, it cannot be excluded that the absence of this regulatory fragment contributed to the lack of pLR-ETV2 reporter construct functionality.

Similarly, a 3 kbp sequence is required for full *ROBO4* promoter functionality (Okada et al., 2007), while approximately 1 kbp fragment was used in the pLG-ROBO4 construct. The shorter promoter fragment does not include enhancers present near -2.5kbp (Figure 32) and, thus, two fold reduction is promoter activity is expected (Okada et al., 2007). While this can potentially explain the lack of functionality of the *ROBO4* reporter construct, the observed lack of *CDH5* and *ETV2* reporter functionality suggests an issue with the plasmid backbone used for the generation of reporter constructs and requires further investigation.

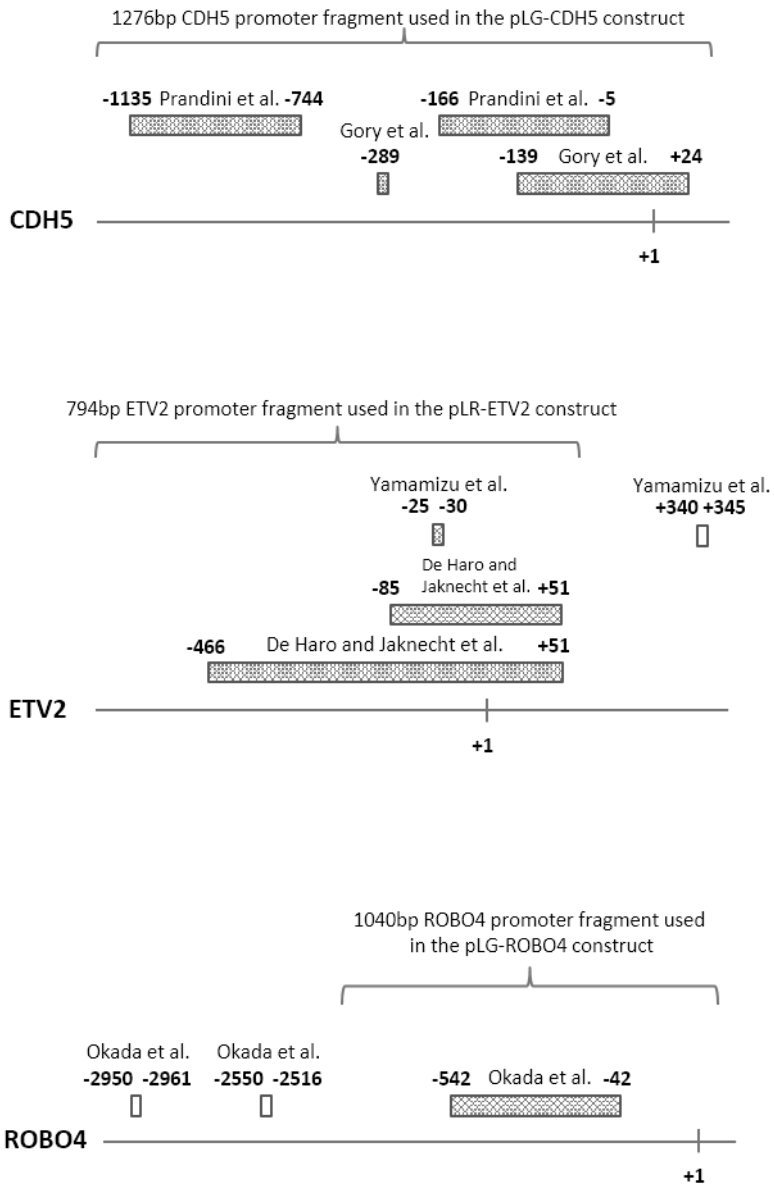


Figure 32: Promoter structures of CDH5, ETV2 and ROBO4 promoters. *The key regulatory fragments reported in literature (De Haro and Janknecht, 2005; Gory et al., 1999; Okada, 2012; Prandini et al., 2005; Yamamizu et al., 2012b) are shown, shaded for those included and in white for those omitted from the generated constructs.*

Limitations of the employed experimental approach for the validation of the generated reporters.

There are multiple other shortcomings which need to be addressed. The successful titration of the generated viruses confirms the presence of late reverse transcription products (Butler et al., 2001) but not integration of the lentivirus in the genome. To confirm the reporter integration in the genome, qPCR using an integration specific set of primers (Butler et al., 2001) or sequencing of the infected cell lines is required. It would also be important to use RFP+ in addition to used GFP+ controls, in order to exclude any technical errors during FC analysis. Alternatively, the reporter gene mRNA expression could be confirmed using qRT-PCR.

Furthermore, not all cells are successfully transduced, thus, selection of cells carrying the reporter constructs might be required. Similarly, generation and selection of monoclonal cell lines might be required to obtain high quality reporter lines. Additionally, GFP may provide insufficient signal for low gene expression levels, and additional quantification using GFP antibodies or proximity ligation qPCR assay may be required (Ståhlberg et al., 2012; Swenson et al., 2007), however, these approaches are not suitable for live cell imaging.

In addition, thorough testing of the plasmid backbone should be performed to ensure functionality of the reporter gene and other plasmid components. In order to validate the functionality of the plasmid backbones, a constitutively active promoter, for example SFFV or CMV, should be cloned in the plasmid and reporter gene expression should be confirmed *in vitro*. This would clarify if the plasmid backbone is fully functional and indicate whether there are any issues with the chosen promoter fragments. Extensive control restriction digests performed using the original pLenti plasmids revealed unexpected fragments (personal communication, Dr Raquel Garcia, University of Glasgow), further implicating issues with the plasmid backbone. Therefore, work with the generated reporter constructs was not continued.

Preliminary validation of the outsourced CDH5 reporter construct.

The optimisation of hESC-EC differentiation protocol could also be performed using only two reporter constructs – *ETV2* reporter and *CDH5* reporter construct. It was speculated that these reporters could be outsourced and used for the optimisation of hESC-EC differentiation. Therefore, an *ETV2* (HPRM12894-LvPF02, GeneCopoeia, US), here GC-ETV2-GFP, and a *CDH5* reporter (Sahara et al., 2014), here SM-CDH5-GFP, constructs were obtained. Control digests generated fragments of expected sizes and this was followed by generation of lentiviral vectors carrying the constructs of interest.

Preliminary validation of the SM-CDH5-GFP construct was attempted during hESC-EC differentiation with RC-11 and H9 hESC and showed a large percentage of cells expressing GFP but not staining positive for CD144+. Furthermore, when H9 hESC were infected with the SM-CDH5-GFP lentivirus, expression of GFP in the absence of CD144 staining was observed in pluripotent hESC. While transient GFP expression upon lentiviral integration cannot be excluded, expression of GFP after prolonged culture suggests non-specific GFP expression. More repeats, if possible using monoclonal cell lines created after selection in antibiotic containing media, are required to confirm these observations and this is only preliminary validation, therefore, no reliable conclusions can be drawn.

In the original publication (Sahara et al., 2014), multiple clonal cell lines were created and one of these cell lines was selected for further experiments after testing reporter gene expression during spontaneous differentiation. Interestingly, a population of cells expressing GFP but not staining positive for CD31 can be observed in the published FC plots (Sahara et al., 2014). It can be speculated that the CD31- cells also were CD144-. If so, this is in line with the non-specific GFP expression reported here. It is also likely that CD144+ GFP- cells reported here were not been transduced with the construct. Thus, it can be speculated that selection in antibiotic containing media followed by creation of clonal reporter lines would result in a more accurate reporter expression. However, the non-specific GFP expression remains a significant limitation of this construct and alternative *CDH5* reporter constructs should be considered. Therefore, the work with the SM-CDH5-GFP was not continued to focus on the outsourced ETV2 reporter construct.

Preliminary validation of the outsourced ETV2 reporter construct.

For validation of the GC-ETV2-GFP construct, RC-11 hESC were transduced on day 0 of hESC-EC differentiation. GFP reporter expression was observed on day 4 of the differentiation and, unexpectedly, increased further by day 7. This is in contrast with the expected expression pattern, and suggests that the reporter construct was not specific for *ETV2* expression. Infection on day 0 of hESC-EC differentiation might have a negative effect on hESC-EC differentiation, as suggested by the low CD144 expression at the end of the differentiation. In addition, not all cells will be transduced with the reporter construct, further complicating monitoring of ETV2 expression early in hESC-EC differentiation. Thus, this experiment was not repeated and an alternative experimental setup was selected for the following experiments.

To ensure that all cells carry the construct and to prevent any detrimental effects of lentiviral transduction on hESC-EC differentiation, pluripotent RC-11 hESC were transduced with lentiviral vectors carrying GC-ETV-GFP construct, followed by selection in puromycin containing media before hESC-EC differentiation. Here, a majority of cells expressed GFP on day 0. This might be explained by the low basal *ETV2* expression levels, as seen in the RT-PCR data. However, the percentage of GFP+ cells decreased during hESC-EC differentiation, once again not matching the expected expression pattern of increased *ETV2* expression by day 3 of hESC-EC differentiation. The lack of reporter specificity was further confirmed by immunostaining, where cells expressing GFP but not staining positive for ETV2 could be identified. Taken together, this indicates that GC-ETV2-GFP construct does not specifically report *ETV2* expression and, thus is not suitable to be used for optimisation of hESC-EC differentiation.

It is difficult to speculate about the mechanisms underlying non-specific expression of the reporter gene without further analysis. As above, it is likely that testing of multiple clonal cell lines carrying the construct could be used to select a cell line with more specific *ETV2* reporter expression. Interestingly, while this construct included a larger (1.5 kbp) *ETV2* promoter fragment, it did not contain the second cAMP response element binding site implicated in expression regulation during endothelial differentiation (Yamamizu et al.,

2012b) discussed previously. This might explain the lack of reporter upregulation during hESC-EC differentiation. Therefore, it can be proposed that a reporter construct including both cAMP response element binding sites, in addition to the basal expression sequences (De Haro and Janknecht, 2005), would be required for efficient monitoring of early endothelial differentiation. However, the presence of other, yet not described, negative regulatory elements in the promoter of ETV2, cannot be excluded. Therefore, further characterisation of the *ETV2* promoter is necessary.

Limitations of the chosen strategy for the optimisation of hESC-EC differentiation.

It is clear that there are multiple possible complications with the chosen approach for reporter cell line generation. Firstly, it is difficult to capture the complexity of gene expression regulation within a relatively short promoter fragment. Additionally, the use of lentiviral vectors further complicates generation of the reporter cell lines as the exact integration site and copy number cannot be easily controlled (Charrier et al., 2011; Sakuma et al., 2012). Various methods for precise genome editing have been developed, for example, zinc finger nucleases (Urnov et al., 2010), TALEN - transcription activator-like effector nucleases (Hockemeyer et al., 2011; Joung and Sander, 2013) and CRISPR - clustered regularly interspaced short palindromic repeats systems (Cong et al., 2013; Mali et al., 2013). These systems allow precise insertion of a fluorescent reporter sequence in the genome. The main advantage of such approach is that all the regulatory regions controlling gene expression would be preserved and, thus, the reporter would be highly specific and expressed at near native levels.

However, while fluorescent proteins are highly suitable for live cell imaging and can be easily coupled to the protein of interest, the large size of fluorescent proteins may interfere with the protein function (Toseland, 2013). Therefore, a thorough comparison of wild type and cells carrying the fusion protein construct should be undertaken, or alternatively bicistronic vectors can be used to obtain separate proteins (Sakuma et al., 2012). Additionally, various fluorescent

proteins should be carefully considered (Shaner et al., 2007, 2005), and if possible, a fluorescent protein with minimised oligomerization should be used to minimise the risk of any alteration of protein function.

Generation of reporter cell lines for monitoring of hESC-EC differentiation - summary.

In summary, the data presented in this chapter shows temporary ETV2 expression upregulation during the mesodermal induction phase and progressive ROBO4 expression upregulation as hESC-EC differentiation progresses. This suggests that reporter constructs of these two genes would be useful in addition to CDH5 reporter for the optimisation of hESC-EC differentiation. Here, ETV2, ROBO4 and CDH5 reporter constructs were generated and preliminary validation was attempted in NCI60 cancer cell lines, HUVECs and HSVECs, and during hESC-EC differentiation. No reporter expression was observed, possibly due to issues with the plasmid backbone used. However, these experiments were not continued and thus this data remains preliminary and no reliable conclusions can be drawn. This was followed by preliminary validation of outsourced CDH5 and ETV2 reporter constructs during hESC-EC differentiation. Non-specific reporter gene expression was observed in cell lines carrying either of the constructs and, thus, the work with these constructs was discontinued. Similarly, further research is required for robust conclusions and this remains preliminary data. However, there's still scope to optimise hESC-EC differentiation. Therefore, the following chapters investigate the role of cAMP signalling and angiotensin signalling during hESC-EC differentiation.

Chapter 5: Manipulation of cAMP signalling to increase hESC-EC differentiation yield and induce arterial phenotype.

5.1 Introduction

A range of methods have been published describing endothelial differentiations of hESCs and hIPSCs as introduced in 1.8. While the angiogenic properties of these cells have been demonstrated and significant advances have been made in developing differentiation protocols suitable for clinical use, further specification of the differentiated cells has received relatively little attention.

Indeed, the recent research has focused on generating cells expressing EC markers CD144 and/or CD31 (Orlova et al., 2014, 2013; Patsch et al., 2015; Prasain et al., 2014; Sahara et al., 2014; Wu et al., 2015), while only some have attempted differentiating arterial endothelium (Rufaihah et al., 2013; Sivarapatna et al., 2015; Sriram et al., 2015). Given that evidence has been presented suggesting that arterial EC have superior angiogenetic capacity compared to venous EC (Rufaihah et al., 2013; Sriram et al., 2015), it became apparent that it is important to investigate further endothelial specification during hESC-EC differentiation and consider approaches for increasing arterial commitment of the differentiated cells.

Arterial specification is mostly mediated via NOTCH-VEGF signalling axis, as described in 1.7.3, however, manipulation of cyclic adenosine monophosphate (cAMP) signalling is another promising approach for arterial specification as described by Yurugi-Kobayashi et al. (2006). cAMP is a second messenger molecule involved in various cellular processes in mature endothelial cells, including proliferation (Aslam et al., 2014; Favot et al., 2004), apoptosis (Kumar et al., 2009), CDH5 junction formation (Fukuhara et al., 2005; Kooistra et al., 2005) and angiogenesis (Namkoong et al., 2009).

As illustrated in Figure 33, the intracellular levels of cAMP are increased after activation of transmembrane adenylyl cyclases which are regulated by various G-protein coupled receptors and, additionally, soluble adenylyl cyclases generate intracellular cAMP pools (Kamenetsky et al., 2006). The intracellular cAMP gradients are further regulated by phosphodiesterases (PDEs), which are responsible for cAMP hydrolysis (Mika et al., 2012). More importantly, spatially anchored cAMP effectors protein kinase A (PKA) (Walsh et al., 1968) and exchange protein directly activated by cAMP (EPAC) (de Rooij et al., 1998)

produce specific downstream signals and gene expression changes (Baillie, 2009; Du and Montminy, 1998).

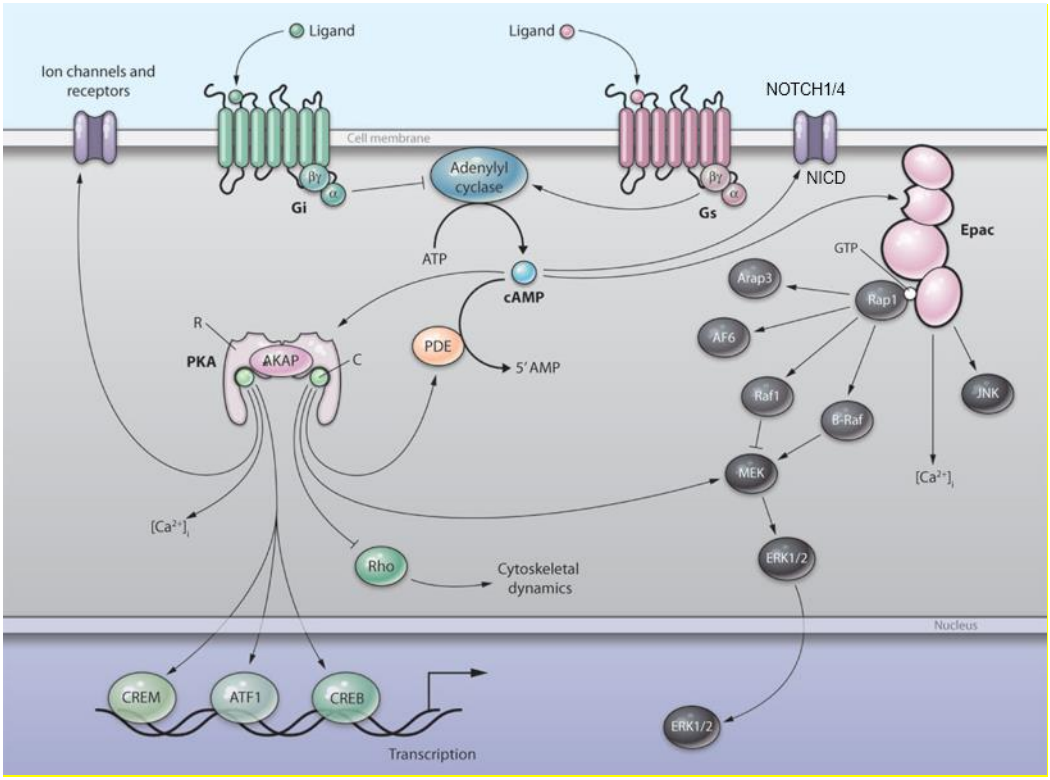


Figure 33: Overview of cAMP signalling. *Intracellular cAMP pools are generated after activation of adenylyl cyclases. The two main effectors of cAMP are PKA and EPAC, however, other targets have been described, for example, NICD. PDE are responsible for hydrolysis of cAMP, further regulating cAMP signalling. Adapted from: Murray (2008).*

While the role of cAMP signalling has been extensively researched in mature endothelial cells, the role of cAMP in endothelial cell development and specification remains to be explored. One of the regulatory targets of PKA is cAMP response element binding protein also known as CREB (Du and Montminy, 1998). Mice homozygous for truncated form of CREB show decrease in CD31+ cell numbers and lack of vascular networks (Oike et al., 1999). Additionally, CREB has been shown to alter CD144+ cell phenotypes *in vivo* (Kim et al., 2015). Therefore, it can be speculated that cAMP also may play a role in EC development and specification.

Indeed, early observations using mESC endothelial differentiation models have uncovered a range of processes that are modulated by cAMP. Yurugi-Kobayashi et al. (2006) demonstrated that 8-bromo-cAMP aided endothelial specification of CD309+ cells and induced arterial specification. It was speculated that this may be due to direct activation of NOTCH signalling, which is at the core of current arterial specification model (Corada et al., 2014).

Later observations suggested that cAMP activated PI3K and subsequently suppressed GSK3B, thus, proposing ß-catenin as the complimentary signal for cAMP induced arterial specification (Yamamizu et al., 2010). Indeed, a complex consisting of NICD, RBPJ and ß-catenin was purified from both embryonic and adult murine arteries but not veins, providing further evidence for the role of cAMP and ß-catenin axis in arterial specification (Yamamizu et al., 2010). Further research also demonstrated that cAMP contributed to endothelial differentiation via PKA and ETV2 axis (Yamamizu et al., 2012b) and later CREB interaction was also demonstrated to be crucial for ETV2 activation (Shi et al., 2015).

Moreover, research has provided preliminary evidence that cAMP can induce NOTCH signalling and arterial phenotypes in sorted CD31+ cells derived from hiPSC (Rufaihah et al., 2013; Sivarapatna et al., 2015). However, both reports used low efficiency hIPSC differentiation protocols that were not optimised for clinical purposes, thus, these observations needs to be validated using clinically relevant differentiation protocols and "gold standard" hESC lines.

Aranguren et al. (2013) have proposed a set of 8 transcription factors (AFF3, HEY2, SOX17, MSX1, EMX2, NKX2-3, TOX2, PRDM16) which act complimentary to induce full arterial gene expression program in addition to NOTCH signalling. Out of these, we decided to focus on EMX2, PRDM16, MSX1 and TOX2 as these TF induced the highest levels of phenotype changes when expressed in HUVECs (Aranguren et al., 2013). Interestingly, promoter analysis data reported by Zhang et al. (2005) revealed a presence of a full, conserved CREB binding sequence in the promoter of *EMX2* and an incomplete CREB binding sequence in the promoter of *MSX1*. Thus, the transcription of some or all of these genes might be activated by cAMP (Mayr and Montminy, 2001), while other targets could be regulated by cAMP indirectly via mechanisms downstream from PKA or EPAC.

Therefore, we hypothesised that an increase in intracellular cAMP levels would promote endothelial differentiation by potentiating signalling pathways already targeted in our hESC-EC differentiation, for example ETV2 activation, and might also drive arterial specification by inducing NOTCH signalling and expression of TF contributing to arterial phenotype. Here, Forskolin was used to activate transmembrane adenylyl cyclases (Kamenetsky et al., 2006; Seamon and Daly, 1981) and increase intracellular cAMP levels in hESC-EC differentiation system, followed by endothelial, arterial and arterial phenotype associated gene expression analysis by qRT-PCR, as well as EC and EPC marker expression analysis by FC.

5.2 Aims

The aims of this chapter were:

- To evaluate arterial and venous marker expression during hESC-EC differentiation.

- To test a range of Forskolin doses for increasing intracellular cAMP levels during hESC-EC differentiation.

- To assess endothelial, arterial and arterial phenotype related gene expression changes during hESC-EC differentiation on Day 5 and Day 7 after Forskolin treatments.

- To assess EC and EPC surface marker expression changes in Forskolin treated differentiations on Day 5 and Day 7 of hESC-EC differentiations.

5.3 Results

5.3.1 Arterial and venous gene expression during hESC-EC differentiation.

Firstly, endothelial commitment during hESC-EC differentiation was evaluated. For gene expression analysis during hESC-EC differentiation, H1 hESC and RC-11 hESC were differentiated as described in the Methods chapter, section 1.1, and RNA was collected on Day 0, and Days 2 - 7 of the differentiation. All differentiations yielded >20% CD31+ CD144+ cells on Day 7 of the differentiation.

As shown in Figure 34a and 1b, expression of venous gene *EPHB4* remained steady throughout the differentiation both in H1 and RC-11 with Ct values of 26.5 ±0.5 and 28.2 ±2.5 on day 7 respectively. In contrast, arterial gene *HEY2* expression was slightly upregulated as early as Day 3 ($p<0.05$ vs Day 0) in H1, with two fold upregulation between Days 4 to 6 ($p<0.001$ vs Day 0) and more than 4-fold upregulation on Day 7 ($p<0.001$ vs Day 0) with Ct values of 25.7 ±0.5. In RC-11, *HEY2* was upregulated two fold on Day 6 ($p<0.01$ vs Day 0), reaching three fold upregulation on Day 7 ($p<0.001$ vs Day 0), with Ct values of 28.6 ±1.7 respectively.

5.3.2 Changes in the intracellular cAMP levels during hESC-EC differentiation in response to Forskolin treatments.

In order to evaluate effects of intracellular cAMP changes during differentiations, it was necessary to determine the appropriate Forskolin concentrations to be used for low, submaximal and maximal intracellular cAMP level increase. To evaluate this, Forskolin was applied to Day 3 EBs during hESC-EC differentiation, followed by analysis using Promega cAMP-Glo kit. A dose dependent increase in intracellular cAMP levels was observed as expected (Figure 34c). Maximal intracellular cAMP levels were reached at 5μM Forskolin concentration and three concentrations were selected for further experiments - 1μM Forskolin for low increase in intracellular cAMP levels, 2.5μM Forskolin for submaximal increase in intracellular cAMP levels and 10μM Forskolin for a full response.

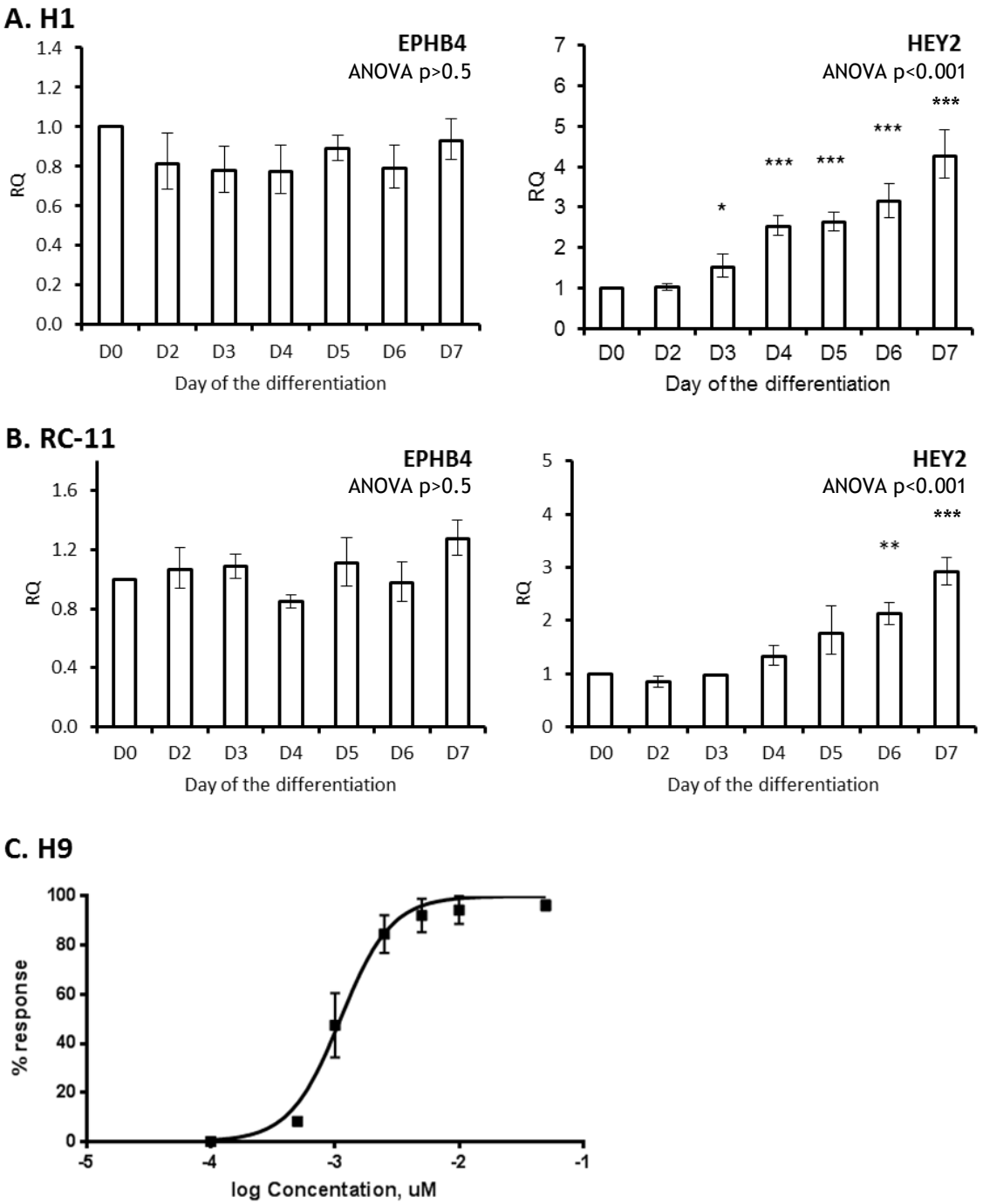


Figure 34: Arterial and venous gene expression and Forskolin dose response curve during hESC-EC differentiation. *RNA was collected on Day 0, and Days 2 - 7 of* ***A.*** *H1* ***B.*** *RC-11 hESC-EC differentiation (n=3, independent experiments). Expression of venous gene EPHB4 and arterial gene HEY2 was quantified using qRT-PCR. Statistical significance was measured using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05, ** p<0.01, *** p<0.001 when compared to d0 hESC control. Data shown as RQ ± RQ max and min.* ***C.*** *Day 3 EBs were incubated with 0.1μM - 50 μM of Forskolin (n=3, independent experiments) and relative intracellular cAMP levels were measured. Response expressed as % Max response observed.*

5.3.3 Gene expression profiles during hESC-EC differentiation after Forskolin treatments.

To assess endothelial, arterial and arterial phenotype related gene expression changes after Forskolin treatments, hESC-EC differentiation media was supplemented with 1μM, 2.5μM or 10μM Forskolin on Day 3 and 5 of the differentiation. RNA was collected on Day 0, 3, 5 and 7 of the differentiation for qRT-PCR analysis.

Expression of EC and EPC markers *CD34*, *PECAM* and *CDH5* was not detected in pluripotent Day 0 H9 cultures. As expected, expression of all these markers was upregulated as early as Day 3 during hESC-EC differentiation ($p<0.001$ vs Day 0 control). Further increase of expression was observed as the differentiations progressed and on Day 7 Ct of *CD34* was 33.6 ±0.4 ($p<0.01$ vs Day 0 control), Ct of *PECAM* was 31.0 ±0.7 and Ct of *CDH5* was 28.3 ±0.8. *KDR* was expressed on Day 0 (Ct 28.6 ±0.5) and its expression increased significantly 13-fold on Day 3 ($p<0.001$ vs Day 0, Ct 26.2 ±0.9) and remained steady till the end of the differentiation.

Genes used as markers for arterial commitment were detected in pluripotent cultures on Day 0 with Ct values of 27.4 ±0.2 for *HEY2*, 34.7 ±0.6 for *DLL4*, and 28.1 ±0.3 for *EFNB2*. In contrast to the H1 and RC-11 differentiation data presented in the previous section, *HEY2* expression remained comparable throughout the differentiation. *DLL4* expression increased approximately 100-fold on Day 5 and 200-fold on Day 7 with Ct values decreasing to 27.8 ±0.9. *EFNB2* expression increased 6-fold on Day 3 ($p<0.001$ vs Day 0) and remained steady till Day 7 with Ct values of 27.2 ±0.2. In contrast, venous commitment gene *EPHB4* expression increased only slightly after day 0 of hESC-EC differentiation with Ct values between 30.1 ±0.4.

The data presented in this chapter shows that expression of *MSX1* was not detected in pluripotent cells and was strongly upregulated more than 200-fold on Day 3 of hESC-EC differentiation ($p<0.01$ vs Day 0, Ct of 31.1 ±0.5) and remained upregulated till Day 7 albeit at lower levels with Ct of 33.7 ±0.7. In contrast, both *PRDM16* and *TOX2* had low expression levels in pluripotent H9, and their expression was further reduced 4-fold ($p<0.01$ vs Day 0) and 2-fold

($p<0.05$ vs Day 0) accordingly during mesoderm induction on Day 3. *PRDM16* was upregulated on Day 7 4-fold ($p<0.001$ vs Day 0, Ct 32.3 ±0.9), while *TOX2* was upregulated 3-fold on Day 3 ($p<0.001$ vs Day 0) and 8-fold on Day 7 ($p<0.001$ vs Day 0) reaching Ct of 28.0 ±0.3.

Overall, Forskolin treatments did not significantly alter *PECAM*, *CDH5*, *KDR*, *DLL4*, *EFNB2*, *EPHB4*, *PRDM16*, *TOX2* and *NANOG* gene expression levels on Day 5 and 7 of the differentiation. A trend of increased *CD34* expression was observed in Forskolin treated differentiations with Ct values of 31.7 ±1.0 vs 30.8 ±1.0 on Day 5 and 33.6 ±0.4 vs 32.0 ±0.6 on Day 7 in control vs 10μM Forskolin treated differentiations accordingly). *HEY2* expression was steady during the differentiation, which is in contrast to observations in H1 and RC-11, and was significantly reduced two-fold on Day 7 in differentiations treated with 10μM Forskolin (Ct 28.1 ±0.5 vs 29.6 ±0.6, $p<0.05$ vs control).

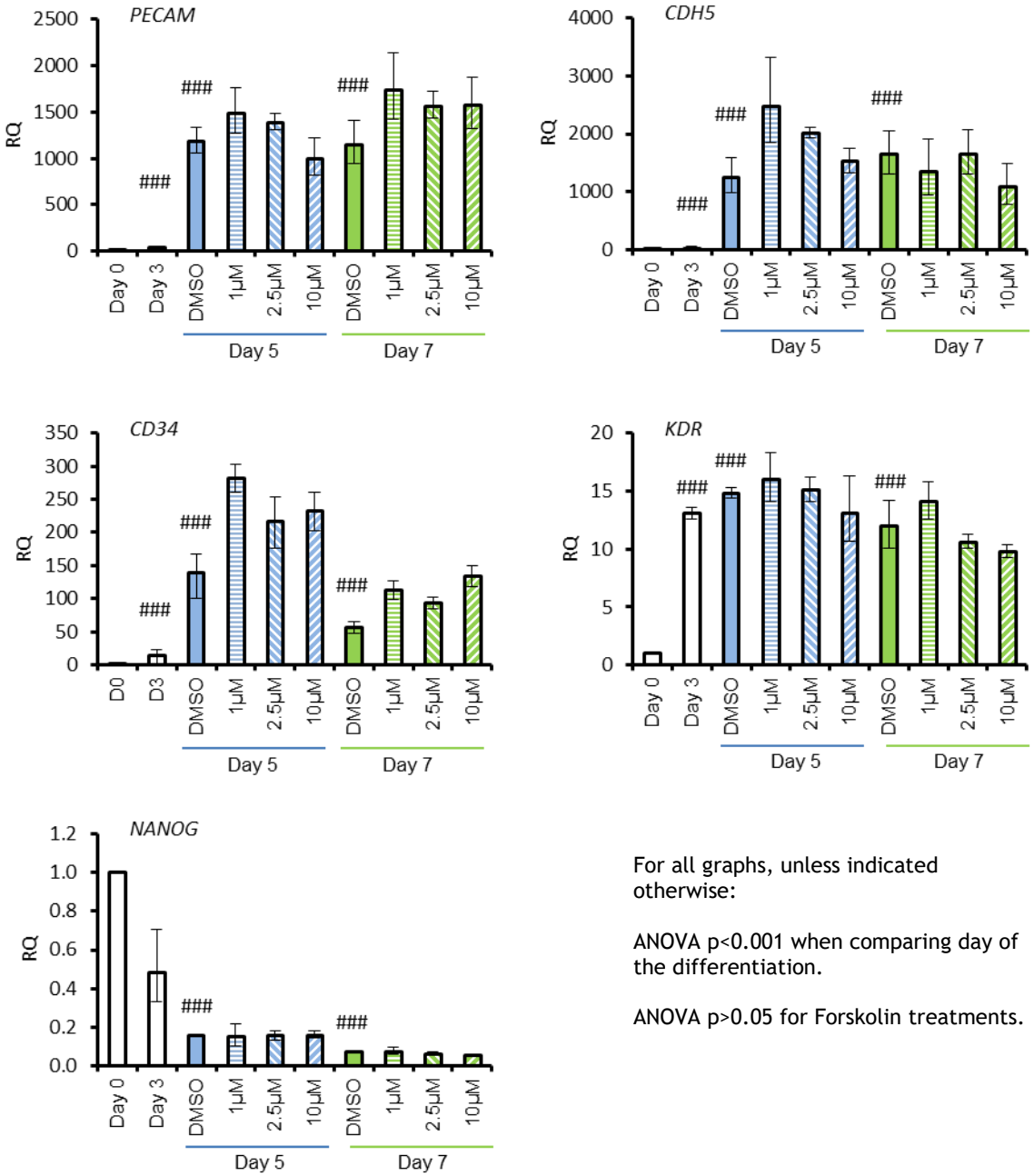
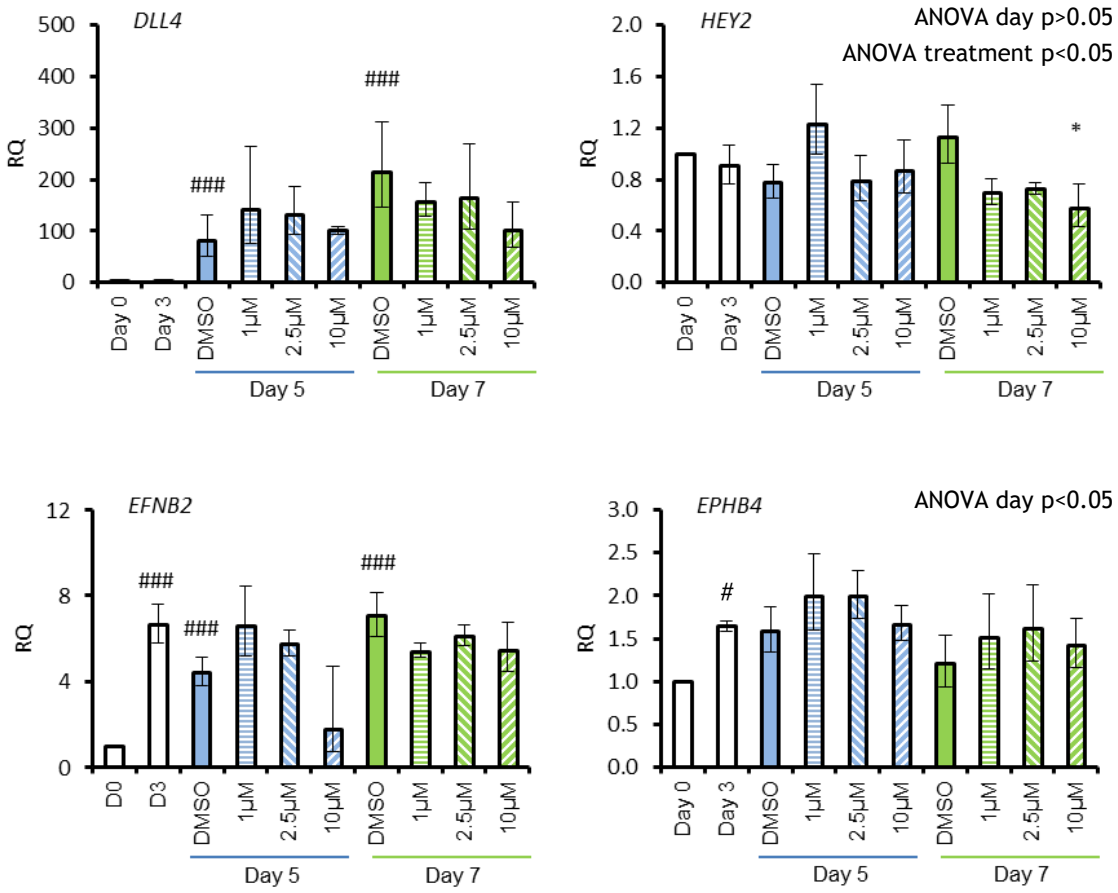


Figure 35: Gene expression changes in response to Forskolin treatment in H9. *The differentiation media was supplemented with 1µM, 2.5µM or 10µM Forskolin on Day 3 and 5 of the differentiation. RNA was collected on Day 0, 3, 5 and 7 of the differentiation, followed by qRT-PCR analysis. Statistical significance was measured using repeated measures ANOVA with Tukey's post hoc comparisons, # p<0.05, ## p<0.01, ### p<0.001 when compared to D0, all non-significant when compared to D5 or D7 control, as appropriate. Data shown as RQ to Day 0 ± RQ max and min. Continued on the next page.*

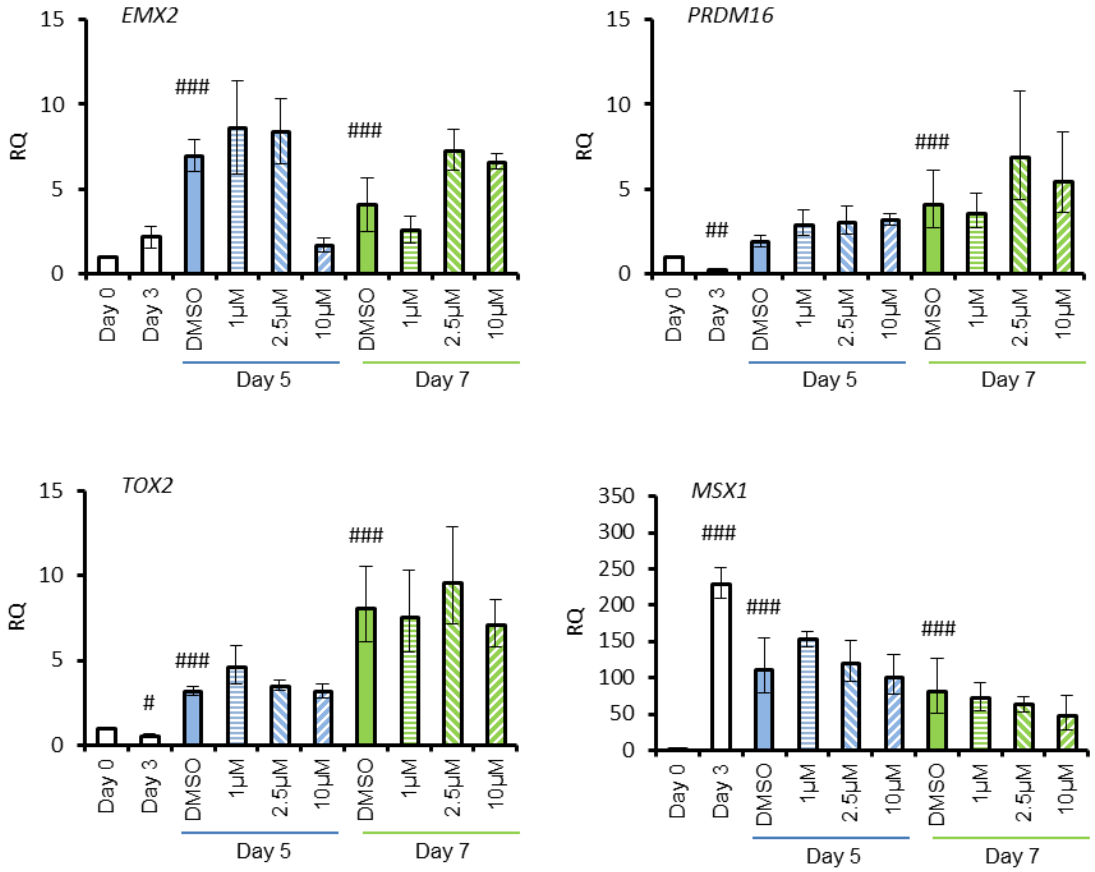


For all graphs, unless indicated otherwise:

ANOVA p<0.001 when comparing day of the differentiation.

ANOVA p>0.05 for Forskolin treatments.

Figure 35 (cont.): Gene expression changes in response to Forskolin treatment in H9. *The differentiation media was supplemented with 1µM, 2.5µM or 10µM Forskolin on Day 3 and 5 of the differentiation. RNA was collected on Day 0, 3, 5 and 7 of the differentiation, followed by qRT-PCR analysis. Statistical significance was measured using repeated measures ANOVA with Tukey's post hoc comparisons, # p<0.05, ## p<0.01, ### p<0.001 when compared to D0, * p<0.05, ** p<0.01, *** p<0.001 when compared to D5 or D7 control, as appropriate. Data shown as RQ to Day 0 ± RQ max and min. Continued on the next page.*



For all graphs, unless indicated otherwise:

ANOVA $p<0.001$ when comparing day of the differentiation.

ANOVA $p>0.05$ for Forskolin treatments.

Figure 35 (cont.): Gene expression changes in response to Forskolin treatment in H9. *The differentiation media was supplemented with 1µM, 2.5µM or 10µM Forskolin on Day 3 and 5 of the differentiation. RNA was collected on Day 0, 3, 5 and 7 of the differentiation, followed by qRT-PCR analysis. Statistical significance was measured using repeated measures ANOVA with Tukey's post hoc comparisons, # p<0.05, ## p<0.01, ### p<0.001 when compared to D0, * p<0.05, ** p<0.01, *** p<0.001 when compared to D5 or D7 control, as appropriate. Data shown as RQ to Day 0 ± RQ max and min.*

5.3.4 Effect of Forskolin on EC marker expression during hESC-EC differentiation.

While no gene expression changes in response to Forskolin treatment were observed, it cannot be excluded that hESC-EC differentiation cell phenotypes were altered via posttranscriptional mechanisms. Therefore, EC and EPC surface marker expression changes in response to increased intracellular cAMP levels were assessed. In these experiments, hESC-EC differentiation media was supplemented with 1μM, 2.5μM or 10μM Forskolin on Day 3 and 5 of the differentiation. Surface marker expression was analysed by FC on Day 5 and 7 of the differentiation.

On Day 5, 19.9 ±3.1 % of cells stained double-positive for EPC markers CD34 and CD309 and 19.9 ±2.0 % cells expressed EC markers CD31 and CD144 (Figure 36). Forskolin treated cultures showed a trend of increased % of cells expressing EPC markers, reaching 30.6 ±1.2 % CD34+ CD309+ cells in 10μM Forskolin treated differentiations ($p=0.07$ vs DMSO). EC marker expression was significantly increased in 2.5μM and 10μM Forskolin treated cultures, reaching 32.0 ±1.8 % cells expressing both CD31 and CD144 (10μM Forskolin, $p<0.01$ vs DMSO).

As shown in Figure 37a, around 18.1 ±5.0 % of cells expressed EPC markers in control and the marker expression was comparable between each of the experimental conditions on Day 7. In contrast, increased EC marker expression was observed with Forskolin treatments and reached 31.4 ±3.9 % CD31+ CD144+ cells in 10μM Forskolin treated differentiations, compared to 19.4 ±0.8 % in DMSO control ($p<0.01$). It was observed that the increase in CD31+ CD144+ % was mainly due to increase in cells expressing CD144 at a low level as illustrated in Figure 38. The analysis of this population (Figure 37b) confirmed a dose dependent Forskolin effect increasing % of $Cd144^{low}$ CD31+ cells (4.4 ±0.5 % control to 12.8 ±2.4 % 10μM Forskolin, $p<0.01$). However, 10μM Forskolin treatments, but not lower concentrations, also showed a trend of increased $Cd144^{high}$ CD31+ % (15.7 ±0.4 % vs 21.1 ±3.5 %, $p=0.15$ vs control).

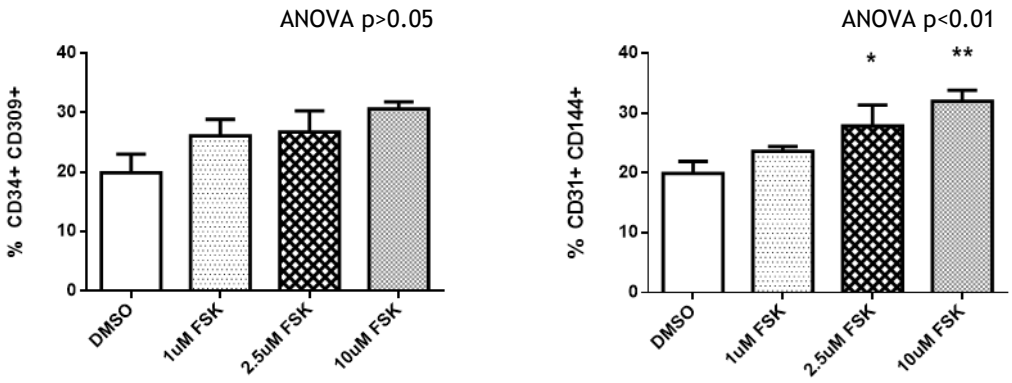


Figure 36: Endothelial marker expression changes in response to Forskolin treatments in H9 on Day 5 of hESC-EC differentiation. *The differentiation media was supplemented with 1µM, 2.5µM or 10µM Forskolin (FSK) on Day 3 of the differentiation. Surface marker expression was analysed by FC on Day 5 (n=3, independent experiments) of the differentiation.*

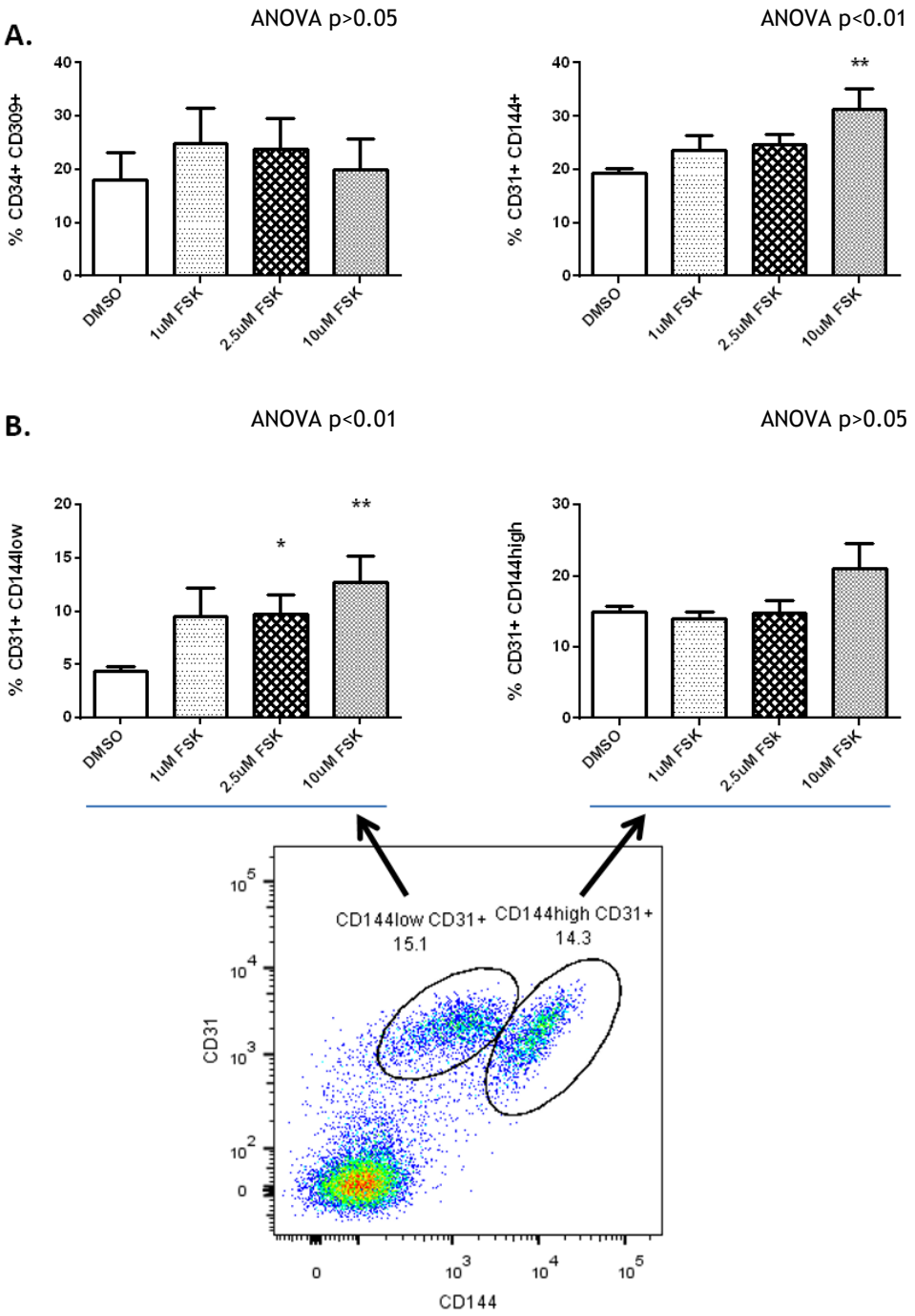


Figure 37: Endothelial marker expression changes in response to Forskolin treatments in H9 on Day 7 of hESC-EC differentiation. *The differentiation media was supplemented with 1µM, 2.5µM or 10µM Forskolin (FSK) on Day 3 and 5 of the differentiation.* ***A.*** *EC (n=5, independent experiments) and EPC (n=3, independent experiments) surface marker expression was analysed by FC on Day 7 of the differentiation.* ***B.*** *CD144+ CD31+ population observed on Day 7 can be subdivided in two populations with low CD144 expression (CD144low) and high CD144 expression (CD144high) as illustrated. Statistical significance was measured using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05, ** p<0.01, *** p<0.001 when compared to DMSO control.*

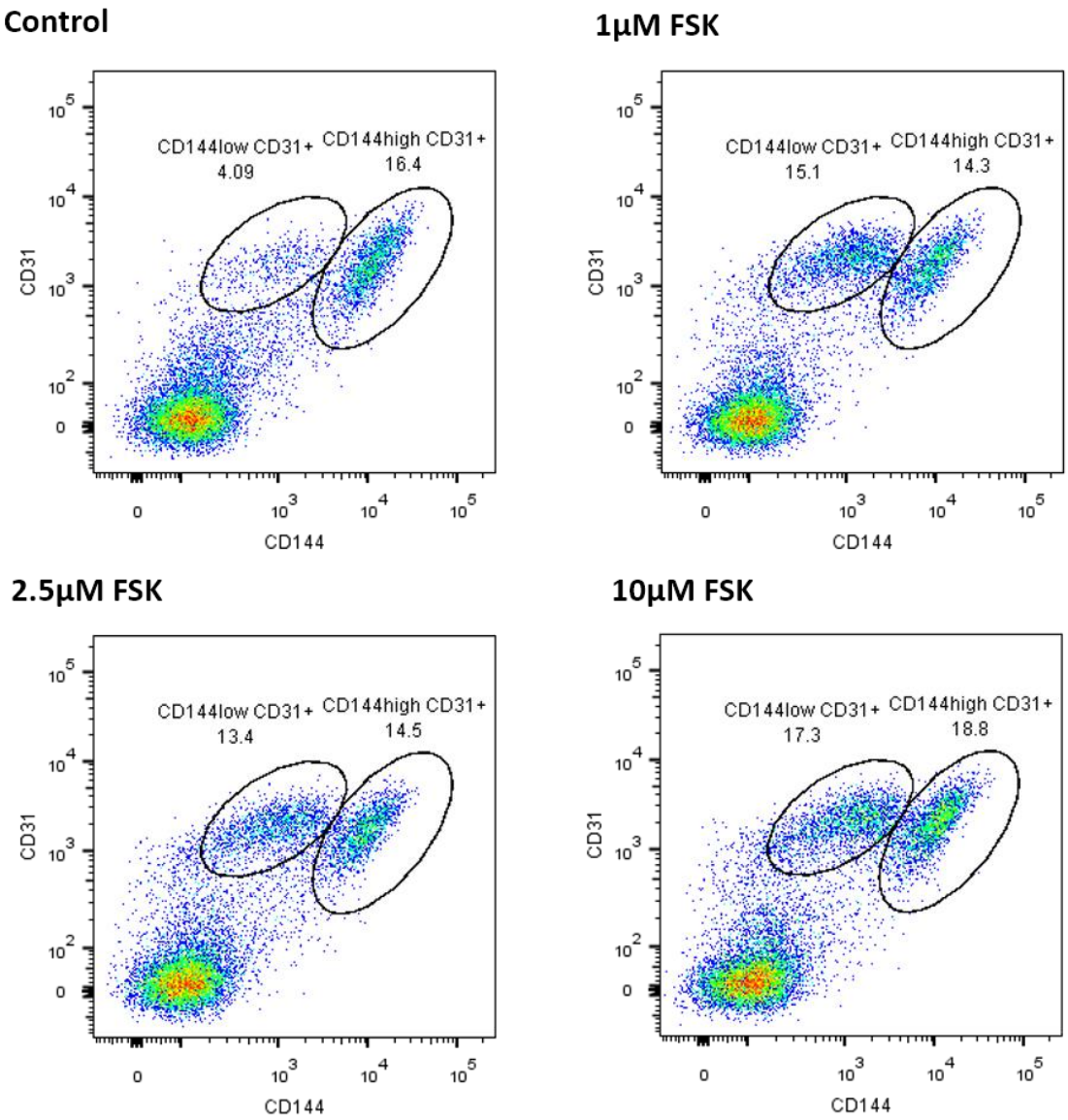


Figure 38: Endothelial marker expressing populations observed on Day 7 in H9 hESC-EC differentiations treated with Forskolin. *The differentiation media was supplemented with 1µM, 2.5µM or 10µM Forskolin (FSK) on Day 3 and 5 of the differentiation. Surface marker expression was analysed by FC on Day 7 of the differentiation (n=3, independent experiments) as shown in Figure 37, representative plots shown. Two populations were observed with low CD144 expression (CD144low) and high CD144 expression (CD144hi) as illustrated.*

5.4 Discussion

In summary, the data presented in this chapter showed that arterial marker HEY2 but not venous marker EPHB4 expression was upregulated during hESC-EC differentiations in H1 and RC-11. Increase in intracellular cAMP levels after Forskolin treatments was evaluated in H9. This was followed by gene expression analysis, which showed that Forskolin treatments did not increase endothelial, arterial or arterial associated gene expression during hESC-EC differentiations with H9. Finally, surface marker comparison between Forskolin treated and untreated differentiations was performed and showed that Forskolin treatments increased % cells expressing EC markers, mainly via increase in $Cd144_{low}$ CD31+ population.

HESC-EC differentiation protocol generates cells expressing EC markers CD144 and CD31 in clinically relevant conditions. It has been speculated, that these cells would be superior to mixed adult cell populations currently trialled for therapeutic angiogenesis. However, EC further sub-specify in arterial and venous phenotypes, which differ in their gene expression profiles (Aranguren et al., 2013; Chi et al., 2003), responses to vascular growth factors (Blebea et al., 2002) and secretory profiles (Sriram et al., 2015). It can be proposed that some endothelial phenotypes might be superior for therapeutic angiogenesis. This is further supported by previous observations showing that arterial EC outperform venous EC in *in vitro* scratch wound assays (Sriram et al., 2015), and form denser capillary networks than mixed EC populations in *in vivo* Matrigel plug assays (Rufaihah et al., 2013). Therefore, inducing arterial phenotypes during hESC-EC differentiation represents an attractive approach for therapeutic angiogenesis.

NOTCH signalling is essential for arterial specification and mice mutant for components of NOTCH signalling pathway display abnormal vascular development and lack arterial marker expression (Krebs et al., 2010, 2000; Swiatek et al., 1994). HEY2 acts downstream from NOTCH1 in vascular remodelling (Fischer et al., 2004) and activates arterial gene expression (Chi et al., 2003). Additionally, Aranguren et al. (2013) has showed that HEY2 is one of the key TF for restoring arterial phenotype lost upon *in vitro* culture. A preliminary analysis of arterial gene HEY2 expression was performed in H1 and RC-11, and EPHB4 expression was used as a control measure for venous

differentiation (Gerety et al., 1999). In both cell lines, EPHB4 expression remained steady throughout differentiation. In contrast, three to four fold increase in HEY2 expression was seen on Day 7. This suggests induction of arterial phenotype and activation of NOTCH signalling, however, it is not known how the HEY2 expression level compares to expression levels in tissue, and further confirmation using a panel of arterial probes, both at gene and protein level is required.

While the role of cAMP signalling has been extensively researched in mature endothelial cells, the role of cAMP in endothelial cell development and specification remains to be explored. A role for PKA has been suggested in germ layer differentiation and downregulation of pluripotency (Yamamizu et al., 2012a) and cAMP signalling has been reported to contribute to endothelial differentiation via ETV2 activation (Shi et al., 2015; Yamamizu et al., 2012b). In addition, Yurugi-Kobayashi et al. (2006) presented evidence showing that 8-bromo-cAMP was required in addition to VEGF for activation of NOTCH signalling and subsequent arterial induction during mESC differentiation towards endothelial lineages. Later observations proposed catenin beta-1 as the complimentary signal for cAMP induced arterial specification (Yamamizu et al., 2010) and demonstrated a complex consisting of NICD, RBPJ and ß-catenin in both embryonic and adult murine arteries but not veins (Yamamizu et al., 2010).

Therefore, it was hypothesised that intracellular cAMP level manipulation could be exploited to enhance endothelial differentiation and also drive arterial specification in hESC-EC differentiation system. To test this, increase in intracellular cAMP levels was evaluated after Forskolin treatments levels during hESC-EC differentiation and three Forskolin concentrations (1µM, 2.5µM and 10µM) were selected for low, submaximal and maximal increase in intracellular cAMP. A panel of genes was chosen to investigate pluripotency, endothelial, arterial and arterial phenotype related gene expression by qRT-PCR.

The analysis of gene expression changes during hESC-EC differentiations with H9 shows that endothelial gene CD31, CD144, CD34 and CD309 expression was upregulated during the differentiation, while pluripotency gene NANOG expression was strongly downregulated by Day 5, in line with observations reported in Chapter 3.

In contrast to preliminary data in H1 and RC-11, HEY2 expression remained steady in H9 throughout the differentiation. Somewhat unexpectedly, HEY2 was expressed in pluripotent hESC in all three cell lines. However, NOTCH pathway genes have been reported to be active in pluripotent hESC (Walsh and Andrews, 2003) and HEY2 expression has also been previously observed in pluripotent H9 (Yu et al., 2008). It can be speculated that the increase in HEY2 expression was lacking in H9 due to higher baseline expression (Ct of 27), when compared to RC-11 and H1 (Ct of 28 and 29 accordingly), however, it would be important to have a comparison to gene expression levels in primary arterial cells. In addition, expression of other arterial (EPHB4, DLL4) and venous (EFNB2) markers was quantified to gain further insight into endothelial specification during hESC-EC differentiation.

The receptor tyrosine kinase EPHB4 and its ligand ephrinB2 (EFNB2 gene) can be used to distinguish between arterial and venous endothelium in the earliest stages of the development (Wang et al., 1998). Expression of both EFNB2 and EPHB4 was detected in the pluripotent H9. Previously, expression of ephrin receptors and ligands has been reported in pluripotent mESCs (Nunomura et al., 2005), however, the expression and role of EFNB2 and EPHB4 in hESC is unclear and was not investigated further as this was not within the scope of this project.

The expression of EFNB2 was upregulated before plating out on Day 3 of the differentiation, while EPHB4 expression increased non-significantly. These increases in expression by Day 3 are in line with the reported roles of EFNB2-EPHB4 axis in EB formation during stem cell differentiations (Li et al., 2009; Z. Wang et al., 2004), and primary germ layer separation (N. Rohani et al., 2014).

Neither the expression of EFNB2 nor EPHB4 was further upregulated later during the differentiation with the appearance of the first EC like cells. It is likely that EC like cells remain of an embryonic phenotype, indeed, expression of both ENFNB2 and EPHB4 has been reported in other differentiation systems (Aranguren et al., 2007; Orlova et al., 2013). Additionally, Orlova et al. (2013) also observed expression of both EFNB2 and EPHB4 in both cultured HUVECs and human umbilical artery endothelial cells, therefore, it can be suggested that EFNB2 and EPHB4 expression patters alone are not sufficient to determine endothelial specification in *in vitro* differentiation systems.

Observed changes in DLL4 expression might provide additional insight into arterial commitment during hESC-EC differentiation. DLL4 marks arterial endothelium (Shutter et al., 2000) and is the first NOTCH ligand detected in early arterial cells (Chong et al., 2011). DLL4 was strongly upregulated during vascular specification phase, when high levels of VEGF are present in the differentiation media and this is in line with the proposed role of VEGF in inducing DLL4 expression (Wythe et al., 2013). Yet, the increase in DLL4 expression was not followed by an increase in HEY2 and EFNB2 expression as expected (Iso et al., 2006; Li et al., 2007; Rufaihah et al., 2013).

As discussed above, this could be due to the somewhat higher than expected baseline expression of HEY2 and early upregulation of EFNB2. Perhaps the heterogeneous nature of the differentiation populations limits the conclusions that can be drawn from these observations. To gain a better insight in the endothelial specification during hESC-EC differentiation, arterial marker expression should be evaluated in a selected population of interest (CD144+). Alternatively, it could indicate that the differentiated cells remain embryonic and uncommitted to a particular phenotype. Analysis of other markers such as SOX17 and NRP-1 for arterial and COUP-TFII and NRP-2 for venous commitment (Corada et al., 2013; Herzog et al., 2001; You et al., 2005) is required to confirm this.

Culture *in vitro* alters endothelial cell phenotype (Müller et al., 2002), and this was further confirmed by Aranguren et al. (2013) who demonstrated loss of arterial marker expression when comparing freshly isolated and cultured ECs. In addition, a range of TFs with altered expression profiles were also identified and EMX2, PRDM16, TOX2 and MSX1 were shown to act complimentary to induce near full arterial phenotype mimicking freshly isolated cells (Aranguren et al., 2013). Therefore, expression of these arterial phenotype associated TF was also analysed.

The data presented in this chapter shows that expression of MSX1 was not detected in pluripotent cells and was strongly upregulated on Day 3 of hESC-EC differentiation. This is in line with observations describing MSX1 as being expressed in mesoderm with roles in extracellular matrix organisation and blocking cardiac differentiation (Rao et al., 2016). EMX2 was expressed at a low

level on Day 0, was upregulated on Day 5 and this expression was maintained on Day 7. The observed expression pattern suggests a role for EMX2 during vascular specification. While previously, EMX2 has been described to regulated by WNT and BMP signalling in neuronal development (Theil et al., 2002), the role of EMX2 in vascular development remains to be investigated.

In contrast, both PRDM16 and TOX2 were expressed in pluripotent H9, and their expression was reduced 4-fold and 2-fold accordingly during mesoderm induction on Day 3. Roles of PRDM16 and TOX2 in germ layer development have not been described, and this data suggests that they might be negatively regulated during mesodermal commitment. However, further research into the role of Prmd16 and TOX2 during mesodermal differentiation was not undertaken as this was not within the scope of this project. Expression of both PRDM16 and TOX2 was upregulated on Day 5 and 7 of the differentiation, suggesting maintained arterial associated phenotype.

To sum up, upregulation of arterial phenotype associated TF expression was observed during hESC-EC differentiation without requiring Forskolin treatments. Taken together with the observed DLL4, EFNB2 and HEY2 expression, it can be suggested that this indicates an inclination towards a full arterial phenotype, however, a more thorough phenotype analysis would be needed to investigate this. Additionally, a direct comparison to freshly isolated arterial cells, as well as arterial cells after prolonged culture would be needed to confirm that these expression levels are relevant to *in vivo* arterial phenotype described previously (Aranguren et al., 2013).

When evaluating the effects of Forskolin treatments on hESC-EC differentiations, the gene expression data reported here shows that 10μM Forskolin treatments reduced EMX2 expression 4-fold on Day 5. The presence of a conserved CRE sequence in EMX2 gene (Zhang et al., 2005) suggests transcriptional activation by cAMP. In addition, EMX2 expression has been reported to be activated by BMP and WNT signalling (Theil et al., 2002), both of which are positively regulated by cAMP (Hino et al., 2005; Ohta et al., 2008). In contrast, the data reported in this chapter shows no positive effect of Forskolin on EMX2 expression levels. This suggests that cAMP alone is not sufficient to induce EMX2 expression during hESC-EC differentiation. Given that EMX2 expression is comparable between

treated and untreated differentiations on Day 7, it can be suggested that the observed decreased expression on Day 5 is not biologically relevant. Further research is required to evaluate the mechanisms underlying this and confirm this observation in other hESC cell lines.

In addition, 10μM Forskolin treatments reduced HEY2 expression two-fold on Day 7. Interestingly, a similar trend was observed in 1μM and 2.5μM treated differentiations. Taken together with the lack of EFNB2 upregulation and EPHB4 downregulation, it appears that NOTCH signalling by cAMP and did not induce arterial phenotype in hESC-EC differentiation. This is in contrast to previous reports showing increased expression of NOTCH1 in response to cAMP stimulation (Rufaihah et al., 2013; Sivarapatna et al., 2015), yet, expression of HEY2, which is downstream from NOTCH1 (Fischer et al., 2004), was not reported. Additionally, cAMP has been reported to activate NOTCH signalling in mature human monocytes (Larabee et al., 2013), suggesting that cAMP-NOTCH axis is conserved across multiple cell types.

The observed differences can be explained by the differentiation systems used. Indeed, serum was used both in human (Rufaihah et al., 2013; Sivarapatna et al., 2015) and murine (Yamamizu et al., 2012a; Yurugi-Kobayashi et al., 2006) differentiation systems reporting cAMP induced NOTCH activation. Recently it has been demonstrated that expression of HEY1 and HEY2, but not other NOTCH target genes such as EPFNB2 and DLL4, is induced by serum via activation of BMP signalling in endothelial cells (Wöltje et al., 2015). This raises an interesting possibility (Figure 39) that cAMP simulation acts in addition to BMP to induce HEY2 expression and promote arterial phenotype and, possibly, activate NOTCH signalling (Hermkens et al., 2015; Rowlinson and Gering, 2010). As serum was not used in hESC-EC differentiation system (Figure 39a), such effect would be absent, explaining the lack of NOTCH and arterial phenotype gene upregulation.

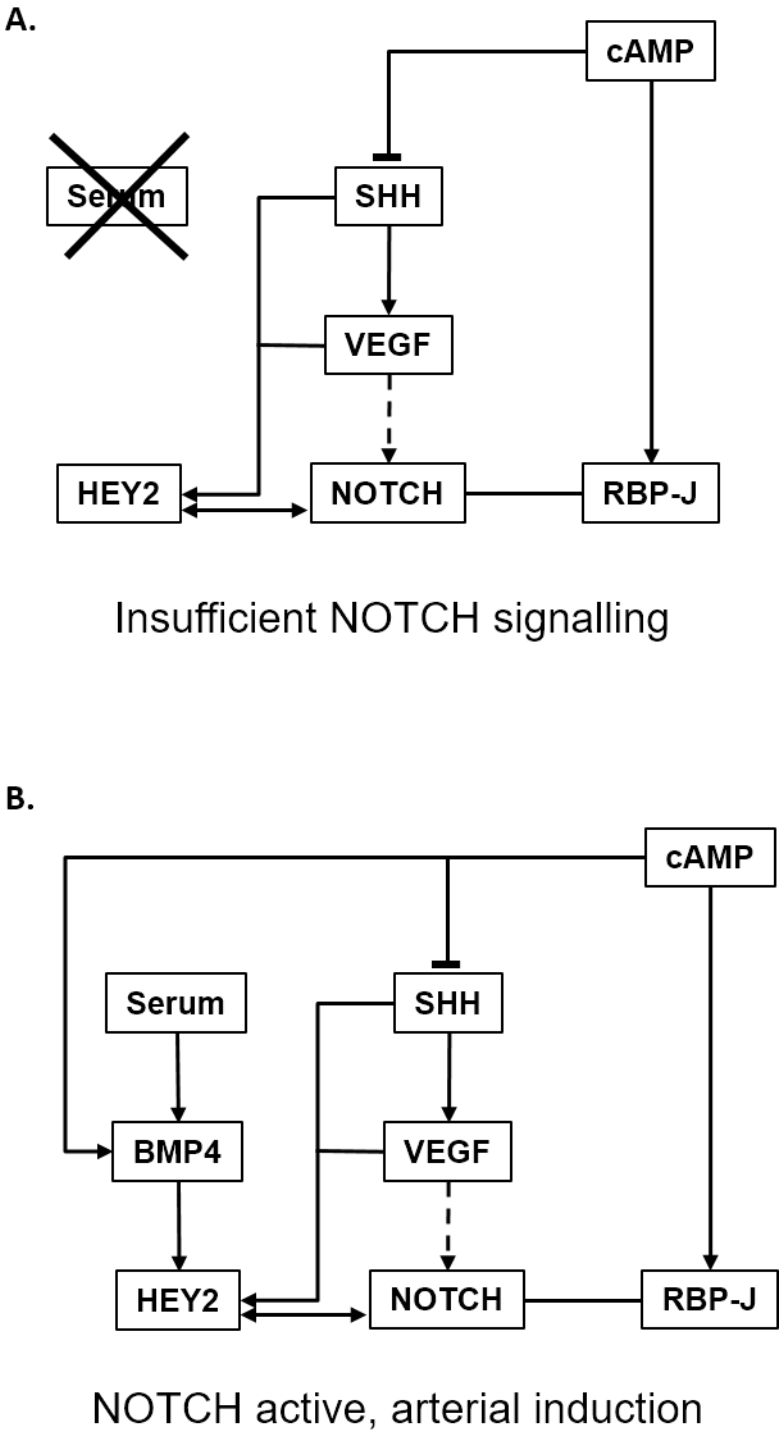


Figure 39: Proposed relationship between intracellular cAMP and induction of NOTCH signalling and subsequently arterial phenotypes. A. *In the absence of serum cAMP suppresses SHH signalling, inhibiting HEY2, resulting in insufficient NOTCH signalling activation for induction of arterial phenotypes.* ***B.*** *When serum is present in the differentiation media, expression of HEY2 is upregulated, possibly via BMP4 signalling, subsequently contributing to NOTCH signalling activation and induction of arterial phenotype.*

Indeed, sonic hedgehog (SHH) - VEGF axis activates NOTCH signalling during vascular development in zebrafish (Lawson et al., 2002) and VEGF signalling also contributes to the expression of HEY2 directly by activating HEY2 promoter (Hayashi and Kume, 2008). It has been previously demonstrated that increased cAMP levels can inhibit SHH signalling (Noveen et al., 1996), thus, it can be proposed that, in the absence of serum, increased intracellular cAMP levels reduce HEY2 expression levels via inhibition of SHH and subsequently VEGF signalling. In contrast, in the presence of serum (Figure 39b), cAMP potentiates BMP4 signalling (Ohta et al., 2008; Wöltje et al., 2015) which induces Hey2 expression that can subsequently induce NOTCH as seen in hematopoietic differentiation systems. In addition, cAMP can also increase RBPJ expression (Larabee et al., 2013), potentially further aiding induction of arterial phenotype together with NOTCH and VEGF signalling (Yamamizu et al., 2010).

In contrast, a trend of increased *CD34* expression was observed in Forskolin treated cultures, however, this did not reach statistical significance and surface marker expression data is needed to draw any conclusions. No other significant differences were seen in endothelial progenitor (*KDR*), endothelial (*PECAM*, *CDH5*), arterial (*HEY2* and *EFNB2*) and arterial phenotype associated (*DLL4*, *EMX2*, *PRDM16*, *TOX2*) gene expression. Venous (*EPHB4*) and pluripotency (*NANOG*) gene expression was also comparable between the experimental conditions. Taken together, this suggests that an increase in intracellular cAMP levels does not induce NOTCH signalling and arterial phenotype, nor activate expression of arterial associated genes in this differentiation system with H9.

This differs from observations using mESC differentiation models (Yamamizu et al., 2012b, 2010; Yurugi-Kobayashi et al., 2006) and a report using hIPSC cells, where Rufaihah et al. (2013) demonstrated that high concentrations of VEGF upregulate arterial gene expression and downregulate venous gene expression during endothelial hIPSC differentiation, and that this effect was amplified in 8-bromo-cAMP treated cultures. More recently, Sivarapatna et al.(2015) reported similar observations in another hIPSC differentiation model and showed that 8-bromo-cAMP increased KDR, EFNB2 and expression while reducing the expression of EPHB4.

There are multiple differences between the experimental approaches that could explain this. First and foremost, both reports evaluated sorted CD31+ cells that had been differentiated for at least 14 days. These cells represent a homogenous cell population that might exhibit a more mature phenotype and different gene expression profiles, potentially including expression of NOTCH pathway related genes. In addition, the mixed nature of hESC-EC differentiation increases the variability and background noise in qRT-PCR experiments, possible masking any effect that Forskolin had on the endothelial marker expressing cells present in the differentiation culture. To draw conclusions from these observations, changes in gene expression would need to be evaluated in sorted cells after Day 7 of hESC-EC differentiation.

In addition, both differentiation approaches used serum in the differentiation media. Serum can contribute to signalling during the differentiation and has been shown to interact with NOTCH signalling in endothelial cells (Wöltje et al., 2015). It can be speculated that cAMP acts complimentary to growth factors present in serum to induce arterial phenotype. As serum was not used during hESC-EC differentiations, the increased intracellular cAMP levels alone would not be sufficient for such effect.

Alternatively, it has to be considered that Forskolin activates adenylyl cyclase to increase intracellular cAMP levels and, thus, depends on its expression and activity, while cell membrane soluble cAMP analogues used in the previous publications can active downstream effectors, for example PKA and EPAC, directly (Insel and Ostrom, 2003; Lane-Ladd et al., 1997). Absolute intracellular cAMP levels here were not measured after Forskolin treatments during hESC-EC differentiation, therefore, it cannot be excluded that the intracellular cAMP levels were lower than in the previously reported experiments and not sufficient to induce arterial phenotype gene expression. Repeating the experiments using comparable 8-bromo-cAMP concentrations to the ones reported in the previous publications would be necessary to confirm this. Additionally, to account for any off target effects of Forskolin, a biologically inactive Forskolin analogue 1,9-Dideoxyforskolin should also be included as an additional control.

An increase in endothelial surface marker expression was observed in Forskolin treated cultures. To gain a better insight, additional analysis of Day 5 and 7

surface marker expression was performed. A trend of increased EPC marker CD34 and CD309 expression was observed on Day 5 and, in contrast to gene expression data discussed above, increased EC marker CD144 and CD31 expression was observed on both Day 5 and Day 7. The CD144+ CD31+ population observed on Day 7 could be further subdivided in two separate populations, depending on CD144 expression levels. The increase in CD31+ CD144+ cell % was mainly due to increase in $Cd144_{low}$ cell %, even though a trend of increased $CD144_{high}$ cell % was observed increase in the 10μM Forskolin treated differentiations.

A non-significant increase EPC marker CD34 and CD309 expression was observed in Forskolin treated hESC-EC differentiation cultures both at gene and protein level. This is in line with the gene expression data reported in this chapter and previously described role of cAMP response element in ETV2 activation (Shi et al., 2015), which subsequently induces expression of endothelial gene expression programmes (Elcheva et al., 2014; Lee et al., 2008; Morita et al., 2015; Shi et al., 2014). In addition, Negrotto et al. (2006) have shown that cAMP acts via PKA and PI3K to prevent apoptosis of human umbilical blood derived CD34+ progenitor cells, in line with the observed increase in CD34+ CD309+ % on Day 5 of hESC-EC differentiation.

Previously, Forskolin treatments have been reported to increase EC gene *PECAM* and *CDH5* mRNA expression in hIPSC differentiation model, however, surface marker expression was not reported (Rufaihah et al., 2013). More recently, Forskolin has also been used to facilitate highly efficient EC differentiation, however, the underlying mechanisms were not investigated (Patsch et al., 2015). Interestingly, the data reported in this chapter does not show an increase in *PECAM* and *CDH5* gene expression levels, while increase in % cells expressing these markers was observed using FC. This suggests that increase in the intracellular cAMP levels alter post-transcriptional processes altering cell surface marker expression.

The previously described VEGF, CD144 and cAMP interactions could be used to explain this observation. High levels of VEGF have been reported to disrupt endothelial adherens junctions (Mirzapoiazova et al., 2006) and stimulate CD144 endocytosis (Gavard and Gutkind, 2006), without altering intracellular cAMP levels (Mirzapoiazova et al., 2006). Forskolin mediated activation of adenylyl

cyclase stimulates both PKA and EPAC (de Rooij et al., 1998), and EPAC aids endothelial CD144 junction formation (Fukuhara et al., 2005; Kooistra et al., 2005). Thus, it can be proposed that the increased intracellular cAMP levels countered the negative effects of high VEGF concentrations on CD144 cell surface expression resulting in higher CD144 surface expression as observed in Forskolin treated differentiations both on Day 5 and Day 7 of the differentiation. It can be speculated that the observed $Cd144_{low}$ population becomes CD144- in the absence of Forskolin.

In addition, CD144 plays a critical role in VEGF mediated endothelial cell survival (Carmeliet et al., 1999a). Thus, cAMP mediated CD144 surface expression and stabilised junctions could contribute to explaining the higher % of CD31+ and CD144+ cells observed at the end of hESC-EC differentiation. This is also in line with very recent observations by Saxena *et al.* (2016) who demonstrated that EPAC inhibition reduces endothelial cell yield in a hematopoietic hIPSC differentiation system. To confirm the role of EPAC in these observations, these experiment should be repeated using EPAC specific cAMP analogues, and ICC staining should be undertaken to evaluate CD144 internalisation and cell apoptosis.

However, VEGF-CD144-cAMP axis can also have a detrimental effect on mature EC proliferation (Caveda et al., 1996; D'Angelo et al., 1997). Whether this applies to immature hESC-EC remains unknown, yet, the observed increase in CD31+ CD144+ % cells suggests that increased intracellular cAMP levels did not inhibit cell expansion. However, cells counts and proliferation should be evaluated to confirm this.

Responses to intracellular cAMP levels are further regulated by PDEs (Mika et al., 2012). Given the positive effects of Forskolin on EC marker expression in hESC-EC differentiation system, it would be interesting to investigate which PDEs are involved in regulating this response. It can be speculated that selectively inhibiting PDE isoforms could enhance the differentiation process either in addition to or independently from Forskolin stimulation.

In summary, the data reported in this chapter shows expression of arterial markers *HEY2*, *EFNB2* and *DLL4* during hESC-EC differentiation. Increase in

intracellular cAMP levels did not further enhance the expression of these arterial markers, nor TF associated with arterial phenotype. It is speculated that this was due to lack of serum in the differentiation media and BMP signalling is proposed as a complimentary signal provided by serum required for induction of NOTCH signalling and arterial phenotype.

However, a trend of increase EPC gene *CD34* and *KDR* expression was observed and was confirmed by FC. Interestingly, increase in intracellular cAMP levels during hESC-EC differentiation, resulted in higher % of cells expressing both EC markers CD31+ CD144+. It was observed that this was mostly due to increase in Cd144low population. Here it is proposed that cAMP-CD144-VEGF axis could underlie the observed effect. While other methods to induce arterial specification in a clinically relevant manner are required, manipulation of intracellular cAMP levels represents an attractive approach in enhancing the efficacy of this clinically relevant hESC-EC differentiation protocol.

Chapter 6: The role of the renin angiotensin system in hESC differentiation towards endothelial lineages.

6.1 Introduction

The description of a pressor compound present in renal extracts marks the discovery of the first renin angiotensin system (RAS) component more than 100 years ago (Basso and Terragno, 2001; Tigerstedt and Bergman, 1898). Years of research has uncovered the complexity of the RAS and established it as a central circulating hormone system controlling blood pressure, fluid homeostasis and cardiovascular disease pathogenesis, that has been successfully targeted pharmacologically in various disease processes, reviewed in (Bader, 2010).

Angiotensin II (Ang II) is the main effector in the RAS and is generated in successive steps starting from angiotensinogen, which is constitutively produced in liver (Matsusaka et al., 2012). Reduction in fluid volume and subsequent changes in sodium chloride concentration are sensed by juxtaglomerular apparatus in the kidney and, in response, renin is secreted (Schnermann and Briggs, 2013). The enzymatic cleavage of angiotensinogen by renin produces angiotensin I which is then converted to Ang II by angiotensin converting enzyme (ACE) expressed on the EC surface (Skeggs et al., 1956).

The majority of the effects of Ang II, such as fluid retention and constriction of vascular smooth muscle, are mediated via the classical and predominant angiotensin II receptor type 1 (AT_1R), a G protein coupled receptor (Figure 40). An alternative receptor, the angiotensin type 2 receptor (AT_2R) has a low sequence homology (Mukoyama et al., 1993) to the AT1R, yet, has Ang II has a similar binding affinity to both AT2R and the AT_1R (Dasgupta and Zhang, 2011). The AT_2R is expressed in the embryo during development and rapidly reduces to negligible levels in terminally differentiated adult tissues, however it can be re-expressed in adults during pathological processes (de Gasparo et al., 2000; Kaschina and Unger, 2003; Santos et al., 2013). The AT2R is reported to counteract the actions of AT_1R signalling, for example AT2R signalling is vasodilatory and also mediates anti-growth effects (Chow and Allen, 2016; Nakajima et al., 1995).

Alongside the classical RAS a counter-regulatory axis has been identified, centred on an ACE homologue, angiotensin converting enzyme 2 (ACE2) (Tipnis et al., 2000). ACE2 cleaves Ang I generating Angiotensin-(1-9) which is then

converted to Angiotensin-(1-7) [Ang-(1-7)] by ACE (Donoghue et al., 2000). Alternatively ACE2 can also convert Ang II directly to Ang-(1-7) (Vickers et al., 2002). While the reports of the biological activity of Ang-(1-7) are more than two decades old (Schiavone et al., 1988), Mas, the receptor for Ang-(1-7), has been identified only more recently (Santos et al., 2003). The ACE2/Ang-(1–7)/Mas axis not only counters the effects of Ang II/AT_1R signalling, but also has a range of independent cardiovascular, renal and metabolic effects (Santos et al., 2013).

Given the central role of the RAS in regulation of cardiovascular homeostasis, it is not surprising that pharmacological approaches targeting the RAS have been widely successful. ACE inhibitors were the first to be introduced clinically with the discovery of captopril (Ferguson et al., 1977) and still remain the first line choice for the treatment of hypertension. Later, Losartan was the first AT_1R antagonist introduced (Duncia et al., 1992), and more recently the first non-peptide renin inhibitor, aliskiren, has been developed (Stanton et al., 2003). These drugs have been widely used for treatment of hypertension and have been shown to reduce the mortality and morbidity in congestive heart failure and chronic renal diseases (Bader, 2010; Williams, 2016). Furthermore, new generation angiotensin receptor blockers offering higher potency and longer effect duration have reached the clinic, for example fimasartan which is a derivative of Losartan (Lee and Oh, 2016), and novel therapeutic approaches exploiting the RAS are being actively pursued with novel compounds, for example dual-acting angiotensin receptor-neprilysin inhibitors, holding a lot of promise for future therapies (Gori et al., 2016).

RAS components have also been described in various organs and tissues, for example, brain, kidney and heart, defining a local RAS as reviewed by Bader et al. (2010) and might underlie some of the observed therapeutic benefits of ACE inhibitors and AT_1R blockers (Campbell, 2014; De Mello and Frohlich, 2014). Indeed, the efficacy of treatments targeting cardiac RAS has been demonstrated in a clinical setting supporting the use of ACE inhibitors in the treatment of myocardial infarction (Düsing, 2016; Pfeffer et al., 1992).

Additionally, it can be suggested that a local RAS also is formed in the vasculature as angiotensinogen has been described to be expressed in the rat aorta (Campbell and Habener, 1986), the presence of renin has been

demonstrated in endothelial cells (Lilly et al., 1985) and is closely associated with angiogenesis during development (Rider et al., 2015), while ACE has been described as an early marker for lymphatic, hematopoietic and endothelial cell development (Jokubaitis et al., 2008; Sinka et al., 2012; Zambidis et al., 2008). Additionally, intracellular synthesis of Ang II has been described in endothelial cells (Kifor and Dzau, 1987), therefore, it is likely that the RAS is active both during endothelial development, as well as during endothelial differentiation *in vitro*.

Ang II receptors are differentially expressed during development. In general, the AT_2R is expressed during development in cardiovascular and renal tissues and gets downregulated after birth, while AT_1R expression appears in late foetal development and is further upregulated during maturation (Vinturache and Smith, 2014). This also holds true during the development of vasculature and expression of the AT_2R has been demonstrated in foetal but not adult microswine and rat aorta, while AT_1R expression progressively increased with age (Bagby et al., 2002; Shanmugam et al., 1996).

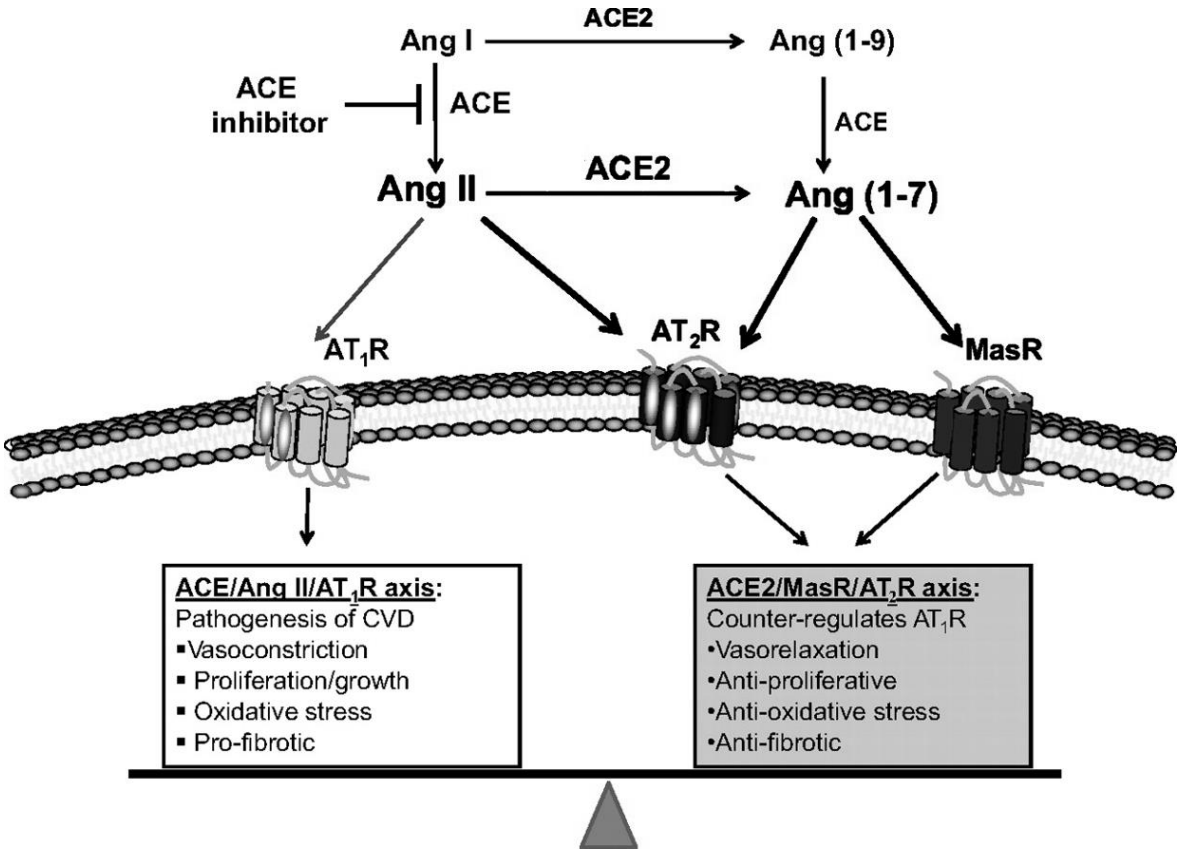


Figure 40: Overview of the renin angiotensin system. *The majority of Ang II effects are mediated via Angiotensin II receptor type 1 (AT_1R), however Angiotensin II receptor type 2 (AT_2R) and Mas receptor (MasR) are expressed in various developmental and pathological processes and form a counter-regulatory angiotensin signalling axis. Adapted from: Gaspari et al. (2012).*

The presence of an active RAS in blood vessels and differential regulation of Ang II receptor expression during development suggests that the RAS may also regulate endothelial developmental processes. While no abnormalities in vascular development and angiogenesis have been reported in AT_1R and AT_2R knockout mice (Biermann et al., 2012; Tsuchida et al., 1998), a role for the RAS has been described in mesodermal development, with Ang II increasing KDR expression in a murine iPSC differentiation model (Ishizuka et al., 2012), and hematopoietic lineage development, with ACE inhibition resulting in diminished erythropoiesis (Savary et al., 2005). In addition, Zambidis et al.(2008) have also reported that the local RAS can be manipulated in a hESC haematopoietic differentiation system to induce endothelial phenotypes. They showed that a majority of re-plated hemangioblast colonies generated endothelial colonies after AT_2R antagonist PD-123319 treatment, while the AT_1R antagonist Losartan

generated hematopoietic colonies in treated cultures (Zambidis et al., 2008). However, further research is necessary to confirm and expand on these observations.

Observed effects of a local RAS on EPC in bone marrow provide further evidence for a role in progenitor cell biology. RAS components have been demonstrated in bone marrow (Strawn et al., 2004) and the effects of angiotensin signalling on hematopoietic and EPC have been receiving increasing attention (Durik et al., 2012). Signalling via AT_1R appears to have a dual effect on the EPC, depending on the duration of the stimulation. Acute activation of the AT_1R in addition to VEGF signalling has been reported to increase proliferation, reduce apoptosis and upregulate KDR expression in bone marrow-derived EPC, mostly mediated via PI3K/AKT and nitic oxide signalling (Fujiyama et al., 2001; Imanishi et al., 2004; Yin et al., 2008).

However, chronic AT_1R stimulation induces apoptosis and senescence, reduces EPC outgrowth from isolated progenitor cells, and reduces the angiogenic potential of the EPC, mediated via increased oxidative stress and activation of apoptosis signalling, for example Caspase-3 (Endtmann et al., 2011; Imanishi et al., 2008, 2005). Taken together, it can be speculated that a local RAS may mediate similar effects on endothelial progenitors during development or hESC differentiation and, thus, acute activation of the AT_1R could aid hESC-EC differentiation by upregulating KDR expression and increasing EPC proliferation, while intrinsic autocrine Ang II production should be limited to minimise oxidative stress and apoptosis.

Similarly, members of the counter-regulatory axis of the RAS may also contribute to endothelial differentiation. This is supported by recent observations by Ikhapoh *et al.* (2015) who reported that signalling via AT_2R potentiates VEGF signalling and induces endothelial phenotypes in microswine bone marrow derived MSCs. However, signalling via AT_2R has been shown to decrease AKT and eNOS phosphorylation in endothelial cells and exert anti-growth effects (Benndorf et al., 2003; Kou et al., 2007; Stoll et al., 1995).

Furthermore, Ang-(1-7) signalling via the Mas receptor and downstream signalling via PI3K/AKT has been shown to increase CD34+ cell proliferation and

survival, and counteract the negative effects of Ang II by reducing oxidative stress and restoring nitric oxide production (Heringer-Walther et al., 2009; Jarajapu et al., 2013; Papinska et al., 2015; Singh et al., 2015; Y. Wang et al., 2010; Xiao et al., 2015). Therefore, it can be proposed that both AT_2R and Mas receptor signalling can also be exploited to enhance hESC-EC differentiation.

In summary, the observations described above suggest that a local RAS is active during endothelial development and can potentially alter the differentiation of EPC and EC. Therefore, we hypothesised that targeting components of the RAS could be exploited during hESC-EC differentiation to increase endothelial cell yields and give further insight into its role during endothelial development. Here, we evaluated the expression of RAS components during hESC-EC differentiation and compared endothelial marker expression and cell numbers after the use of Ang II, Ang-(1-7) and the AT_2R agonist CGP-42112A (Hines et al., 2001), the AT_1R inhibitor Losartan and the AT_2R inhibitor PD-123319 (Dudley et al., 1990) during hESC-EC differentiation.

6.2 Aims

The aims of this chapter were:

- Measure the expression of AT_1R, AT_2R and Mas RNA levels during hESC-EC differentiation.

- Evaluate the effects of Ang II, Ang 1-7 and CGP-42112A treatments during hESC-EC differentiation.

- Examine the role of the AT_1R and AT_2R during hESC-EC differentiation using the receptor-specific antagonists losartan, PD-123319 and PD-12377.

6.3 Results

6.3.1 Expression of the RAS receptors during hESC-EC differentiation.

To assess the RAS component gene expression during hESC-EC differentiation, hESC were differentiated as described (Methods section 2.3), and RNA was collected for RT-PCR analysis on day 0, and days 2 - 7 for H1 and RC-11, and on day 0 and days 3, 5 and 7 for H9.

In RC-11, AT_1R expression was detected at a low level (Figure 41a). In contrast, the expression of AT_1R was not detected on day 0 of the differentiation in both H1 and H9 (Figure 41b and Figure 41c respectively). Low expression of AT_1R was detected on day 2 in H1 and day 3 in H9. During the hESC-EC differentiation, AT_1R expression was progressively upregulated reaching statistical significance on day 3 in H9 (7-fold upregulation, $p<0.05$ vs day 0), day 5 in H1 (12-fold upregulation, $p<0.05$ vs day 0) and day 6 in RC-11 (6-fold upregulation, $p<0.05$ vs day 0). By day 7 of the differentiation, AT_1R was expressed in all three cell lines, reaching 36-fold upregulation in RC-11 (Figure 41a, Ct 32.0 ±0.3), 460-fold upregulation in H1 (Figure 41b, Ct 31.8 ±1.3) and 150-fold upregulation in H9 (Figure 41c, Ct 33.4 ±0.3), all these changes were statistically significant when compared to day 0 ($p<0.001$).

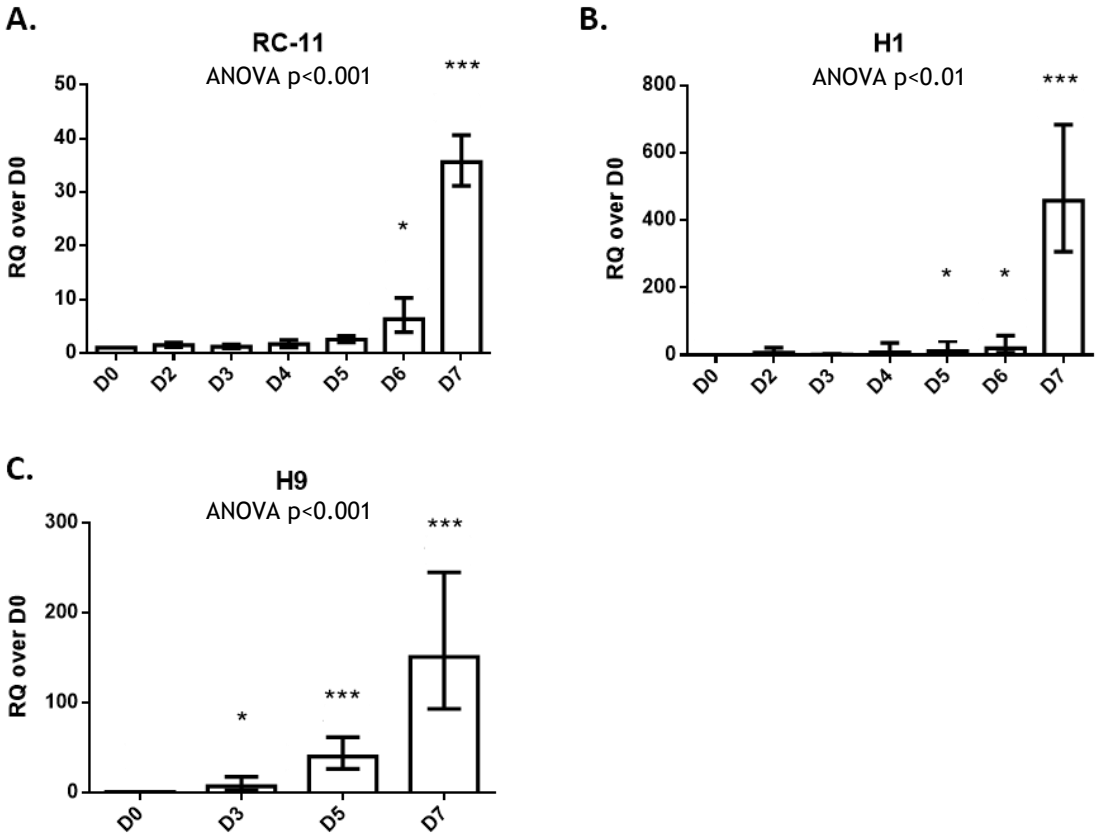


Figure 41: Expression of the AT_1R during hESC-EC differentiation. *RNA was collected on day 0, and days 2 - 7 of hESC-EC differentiation with RC-11 (n=3, independent experiments) and H1 (n=3, independent experiments), and on day 0, 3, 5 and 7 of hESC-EC differentiation with H9 (n=3, independent experiments). Expression of the AT_1R was quantified using qRT-PCR. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05, ** p<0.01, *** p<0.001 when compared to d0 hESC control. Data shown is RQ ± RQ max and min.*

AT_2R expression was detected in all three cell lines at a low level. The expression of the AT_2R was strongly upregulated 15-fold on day 3 in RC-11 (Figure 42a, $p<0.001$ vs day 0) and 60-fold on day 3 in H9 (Figure 42c, $p<0.001$ vs day 0) reaching Ct values of 34.4 ±1.1 and 31.6 ±0.7 respectively. This was followed by a rapid downregulation on day 4 in RC-11 ($p<0.01$ vs day 3) and day 5 in H9 ($p<0.01$ vs day 3) returning to baseline expression levels which remained stable till the end of the differentiation. In contrast, in H1 (Figure 42b) AT_2R expression was strongly upregulated 30-fold on day 2 (Ct 32.2 ±0.4, $p<0.01$ vs day 0) and, although slightly lower, upregulation was observed on day 7 (Ct 33.5 ±0.5, $p<0.05$ vs day 0).

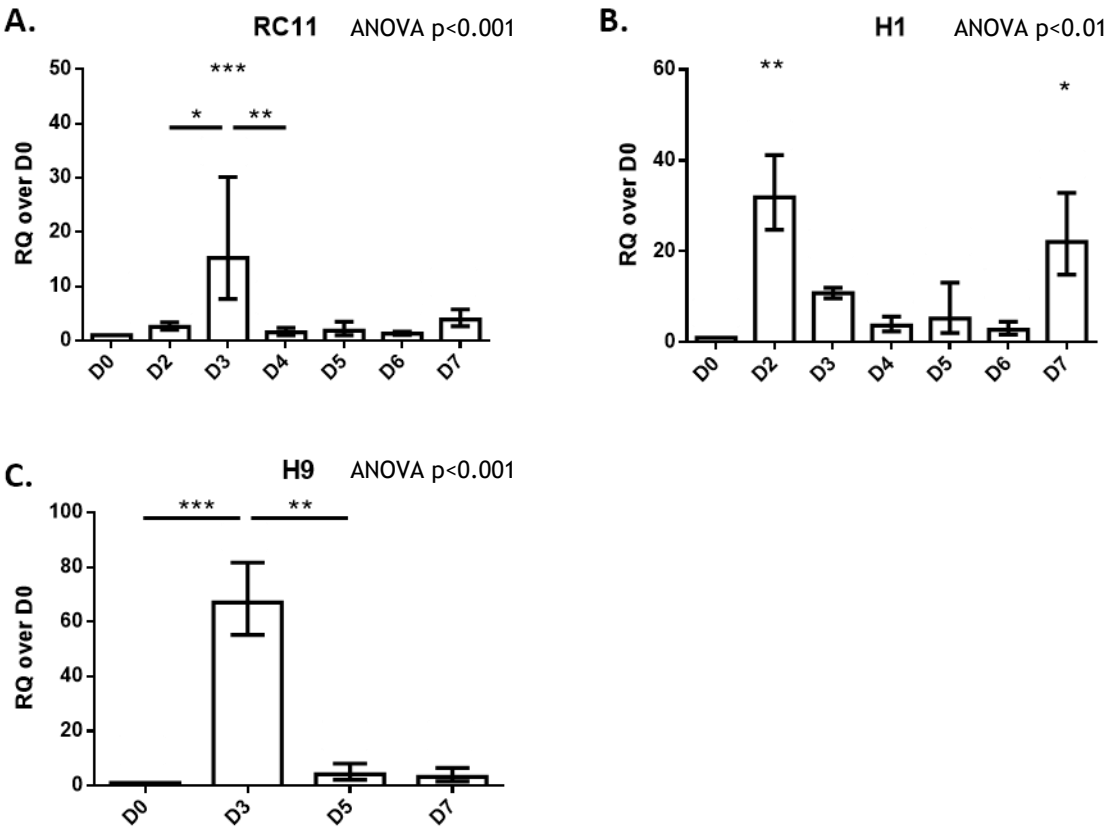


Figure 42: Expression of the AT_2R during hESC-EC differentiation. *RNA was collected on day 0, and days 2 - 7 of hESC-EC differentiation with RC-11 (n=3, independent experiments) and H1 (n=3, independent experiments), and on day 0, 3, 5 and 7 of hESC-EC differentiation with H9 (n=3, independent experiments). Expression of the AT_2R was quantified using qRT-PCR. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05, ** p<0.01, *** p<0.001 when compared to d0 hESC control, unless indicated otherwise. Data shown is RQ ± RQ max and min.*

Mas was expressed in all three hESC cell lines with Ct values of 31.9 ±0.1 in RC-11 (Figure 43a), 24.7 ±0.3 in H1 (Figure 43b), and 31.7 ±0.1 in H9 (Figure 43c). The expression of Mas remained comparable throughout the differentiation.

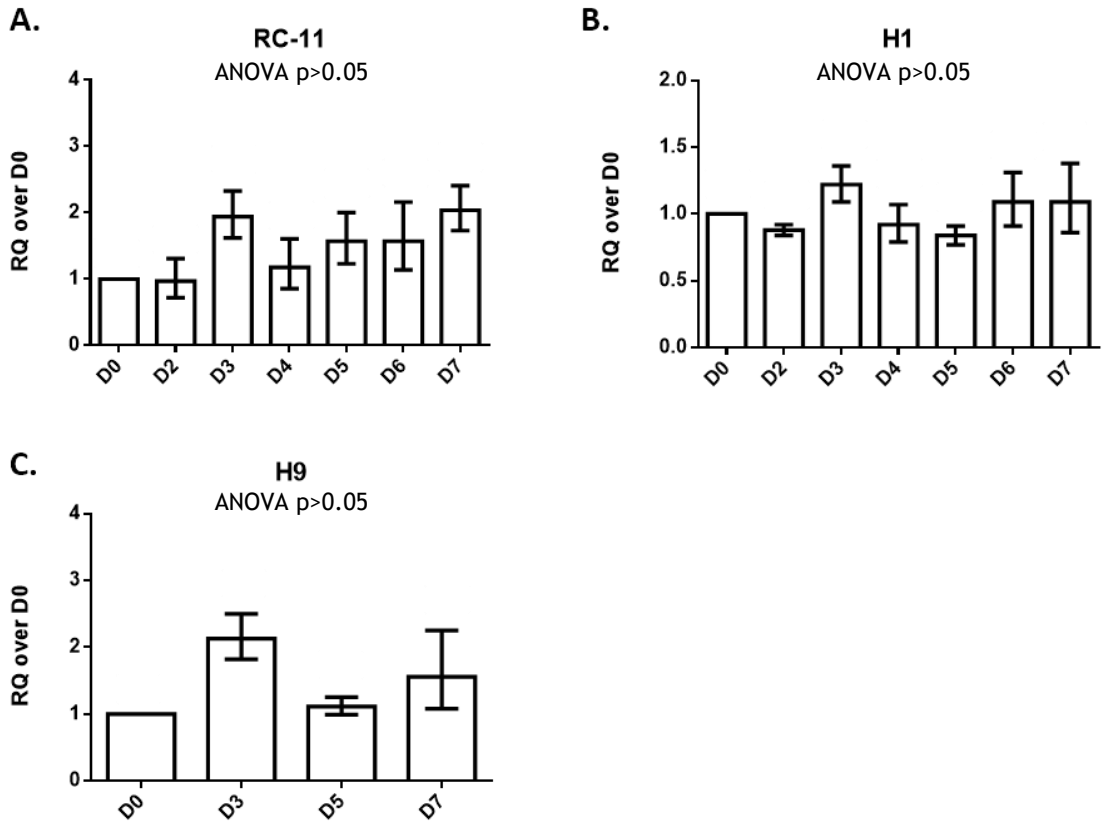


Figure 43: Expression of the Mas receptor during hESC-EC differentiation. *RNA was collected on day 0, and days 2 - 7 of hESC-EC differentiation with RC-11 (n=3, independent experiments) and H1 (n=3, independent experiments), and on day 0, 3, 5 and 7 of hESC-EC differentiation with H9 (n=3, independent experiments). Expression of the Mas receptor was quantified using qRT-PCR. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, all non-significant when compared to d0 hESC control. Data shown is RQ ± RQ max and min.*

6.3.2 Angiotensin peptide stimulation during hESC-EC differentiation.

To evaluate effects of RAS receptor activation during hESC-EC differentiation, H9 were differentiated towards endothelial lineage (n=3, independent experiments) and Ang II (100nM or 1μM, as indicated) or Ang-(1-7) (1μM) treatments were applied on days 3 and 5 of hESC-EC differentiation, followed by EC (CD31 and CD144) and EPC (CD34 and CD309) surface marker expression analysis using FC on day 7. Total cell counts and viability were also assessed.

On average, 28.8 ± 8.0 % cells expressed both CD144 and CD31 at the end of the control differentiations. Data is presented normalised to control differentiations to account for the variability between the experiments. There were no significant differences in EC, EPC surface marker expression or total counts between the treated and control differentiations (Figure 44a-c).

Additionally, Ang II (100nM) or, to study the role of AT_2R, the AT_2R agonist CGP-42112A (Hines et al., 2001) (100nM) treatments were applied on days 0, 2, 3, 5 of H9 hESC-EC differentiation (n=3, independent experiments), followed by surface marker expression analysis using FC on day 7 (Figure 44d-f). It was observed that 36.9 ±5.1 % of cells expressed EC markers CD31 and CD144 in control differentiations on day 7. EC, EPC marker expression and total cell numbers were not different between control and Ang II or CGP-42112A treatment.

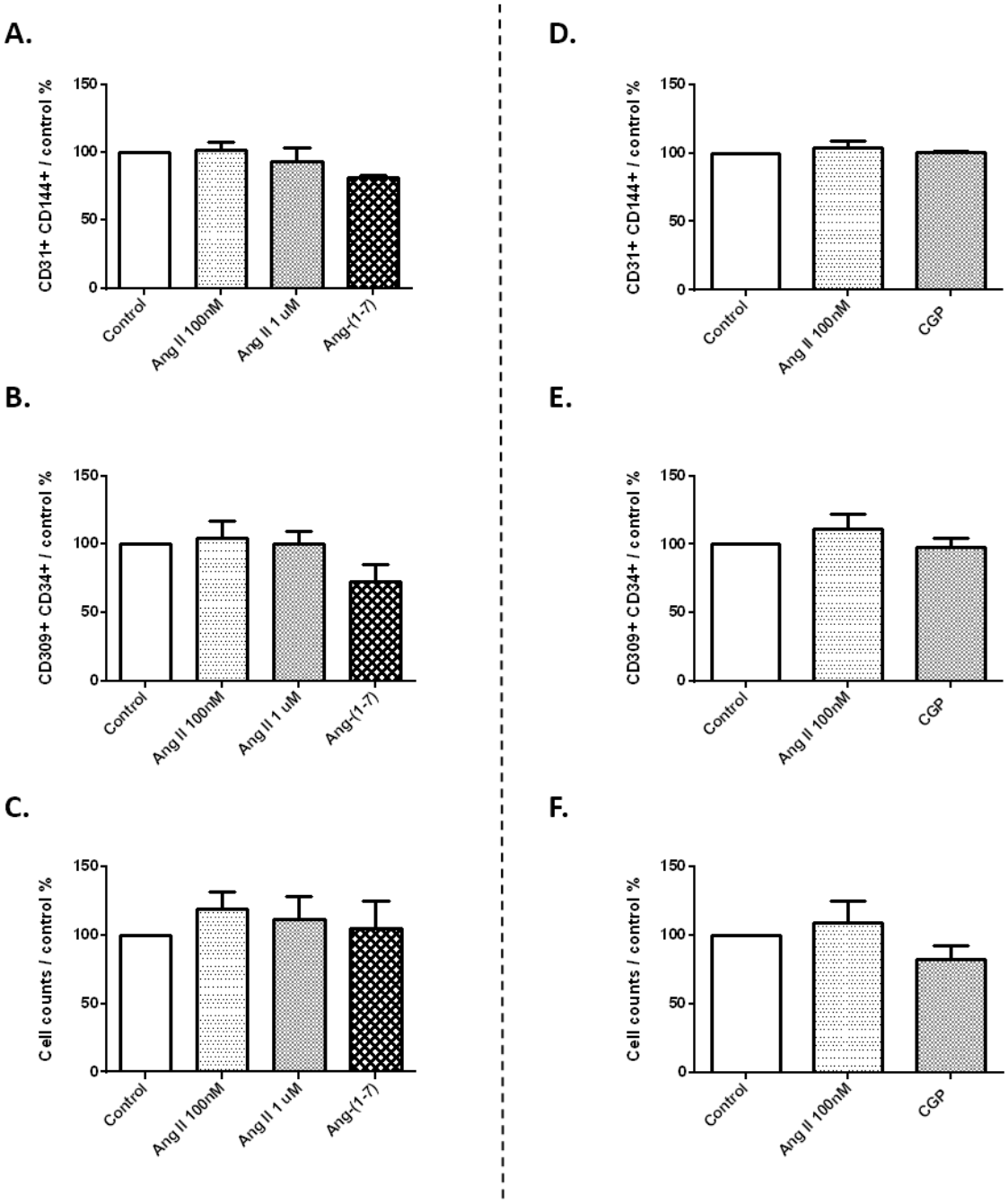


Figure 44: Assessment of Ang II, Ang-(1-7) and CGP-42112A treatments during hESC-EC differentiation. A-C. *Angiotensin II (Ang II, 100nM or 1μM) or angiotensin-(1-7) (Ang-(1-7), 1μM) treatments were applied on days 3 and 5 of hESC-EC differentiation with H9 (n=3, independent experiments) or* ***D-F.*** *Angiotensin II (Ang II, 100nM) or the selective AT_2R agonist CGP-42112A (CGP, 100nM) treatments were applied on days 0, 2, 3, 5 of hESC-EC differentiation with H9 (n=3, independent experiments). Populations expressing both EC markers CD31 and CD144 or EPC markers CD309 and CD34 were compared using FC on day 7 of the differentiation. Statistical significance was evaluated using repeated measures ANOVA, all non-significant (ANOVA p>0.05) when compared to d0 hESC control.*

6.3.3 AT_1R inhibition during hESC-EC differentiation.

To examine the role of the AT_1R during hESC-EC differentiation, Ang II and the AT_1R antagonist Losartan (Duncia et al., 1992) alone or in combination were added to the differentiation media from day 0 onwards (Figure 45a). Total cell counts, viability and EC and EPC surface marker expression was evaluated on day 5 and day 7 of the differentiation by FC.

Addition of Ang II did not change EPC/ EC marker expression, total cell counts or viability, when compared to the control (Figure 45b). Incubation with Losartan only did not change total cell counts and viability compared to controls. However, a reduction in the percentage of cells expressing EPC markers CD309 and CD34 on day 5 in Losartan treated differentiations was observed (10.9 ±1.6 % vs 16.9 ± 2.1 %, $p<0.05$ vs Control, Figure 45c). No differences in expression of the EC markers CD144 and CD31 was observed on day 7 (Figure 45b), when comparing Losartan treatment to control (24.6 ± 2.1 % vs 28.1 ± 4.9 %, $p=0.76$).

Incubation of the cultures with Ang II and Losartan also reduced the percentage of cells expressing EPC markers on day 5 in comparison to Ang II alone (15.9 ±3.4 % vs 10.1 ±0.8 %, $p<0.05$ vs Ang II only, Figure 45c), and a similar reduction in the percentage of cells staining positive for EC markers CD144 and CD31 was observed on day 7 (18.3 ±2.3 % vs 24.6 ±2.8 %, $p<0.05$ vs Ang II only, Figure 45b). Total cell counts and viability remained comparable between the conditions.

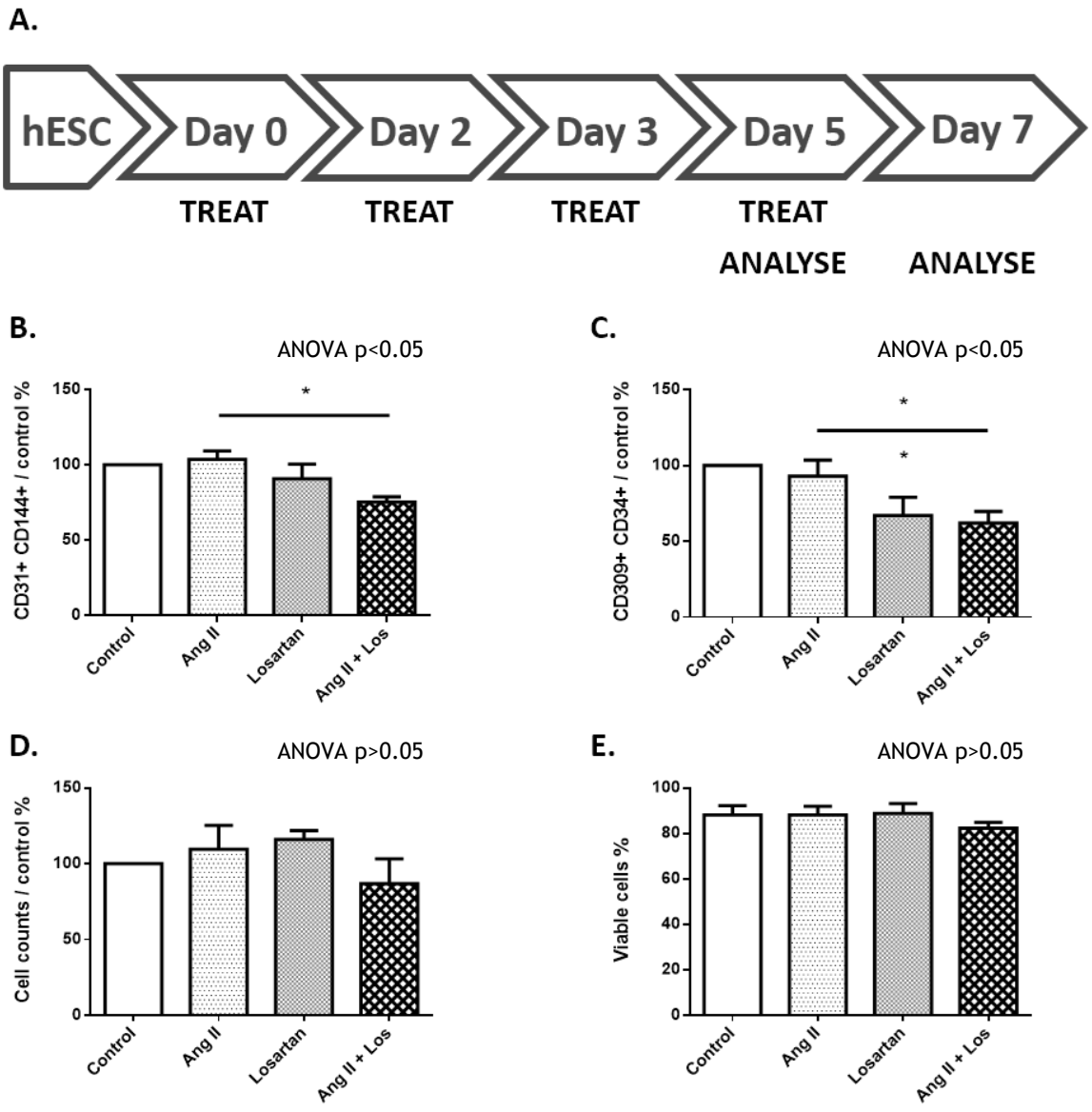


Figure 45: Assessment of the effects of blocking the AT_1R during hESC-EC differentiation. A. *Treatment schematic.* ***B-E.*** *Losartan (10μM) and angiotensin II (Ang II, 100nM) treatments were applied on days 0, 2, 3, 5 of hESC-EC differentiation with H9 (n=3, independent experiments). Populations expressing EPC markers CD309 and CD34 were compared using FC on day 5 of the differentiation. Total cell counts, viability and populations expressing EC markers CD31 and CD144 were compared on day 7 of the differentiation. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05 when compared to control, unless indicated otherwise.*

To confirm the observed effect of the AT_1R antagonist Losartan on EPC marker expression and to investigate the effect of addition of Losartan earlier during differentiation Losartan was added at various time points, followed by EPC and EC marker analysis on day 7 (Figure 46a). On average, 34.5 ±4.3 % cells expressed EC markers CD31 and CD144 and 30.6 ±1.1 % cells expressed EPC markers CD309 and CD34 on day 7 of the control differentiations. In contrary to the observed reduction in EPC (day 5) and EC (day 7) marker expressing cell percentages in the previous experiment (Figure 45), no other significant differences were observed in EC and EPC marker expression or total cells counts and viability (Figure 46b-e).

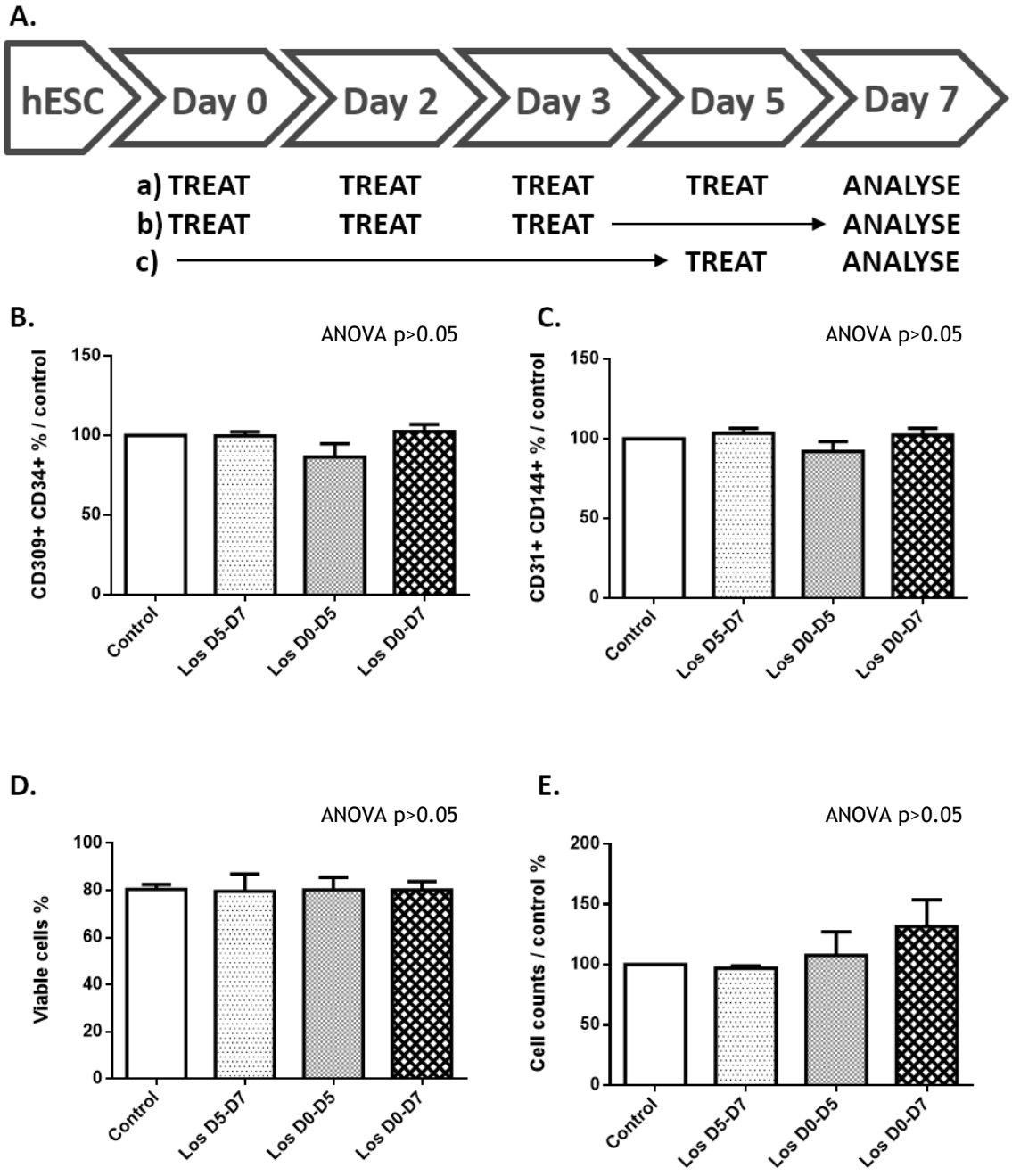


Figure 46: Assessment of the effects of blocking the AT_1R during various stages of hESC-EC differentiation. A. *Treatment schematic.* ***B-E.*** *Losartan (Los, 10μM) treatments were applied as indicated during hESC-EC differentiation with H9 (n=3, independent experiments). D0 – day 0, D5 – day 5, D7 – day 7 of the hESC-EC differentiation. Total cell counts, viability and populations expressing EPC markers CD309 and CD34 and EC markers CD31 and CD144 were compared on day 7 of the differentiation. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, all non-significant.*

It has been previously suggested that when AT_1R is blocked, Ang II may stimulate AT_2R (Chow and Allen, 2016). To investigate if the observed reduction in the percentage of cells expressing EC and EPC markers in differentiations treated with Losartan and Ang II was due to signalling via AT_2R, an AT_2R antagonist PD-123319 (Dudley et al., 1990) was added to the differentiation media in addition to Ang II and Losartan from day 0 of the differentiation.

Expression of EC markers CD144 and CD31 was reduced by a fifth on day 7 from 20.5 ±2.8 % to 16.5 ±3.2 % in differentiations treated with Ang II and Losartan ($p<0.05$ vs Ang II, Figure 47c), and was not significantly different when compared to the combination of Ang II, Losartan and PD-123319 (18.3 ±2.7 %, $p=0.18$ vs Ang II and Losartan, Figure 47c). Percentage of cells expressing EPC markers, total cell counts and viability remained comparable between the conditions (Figure 47b, d-e).

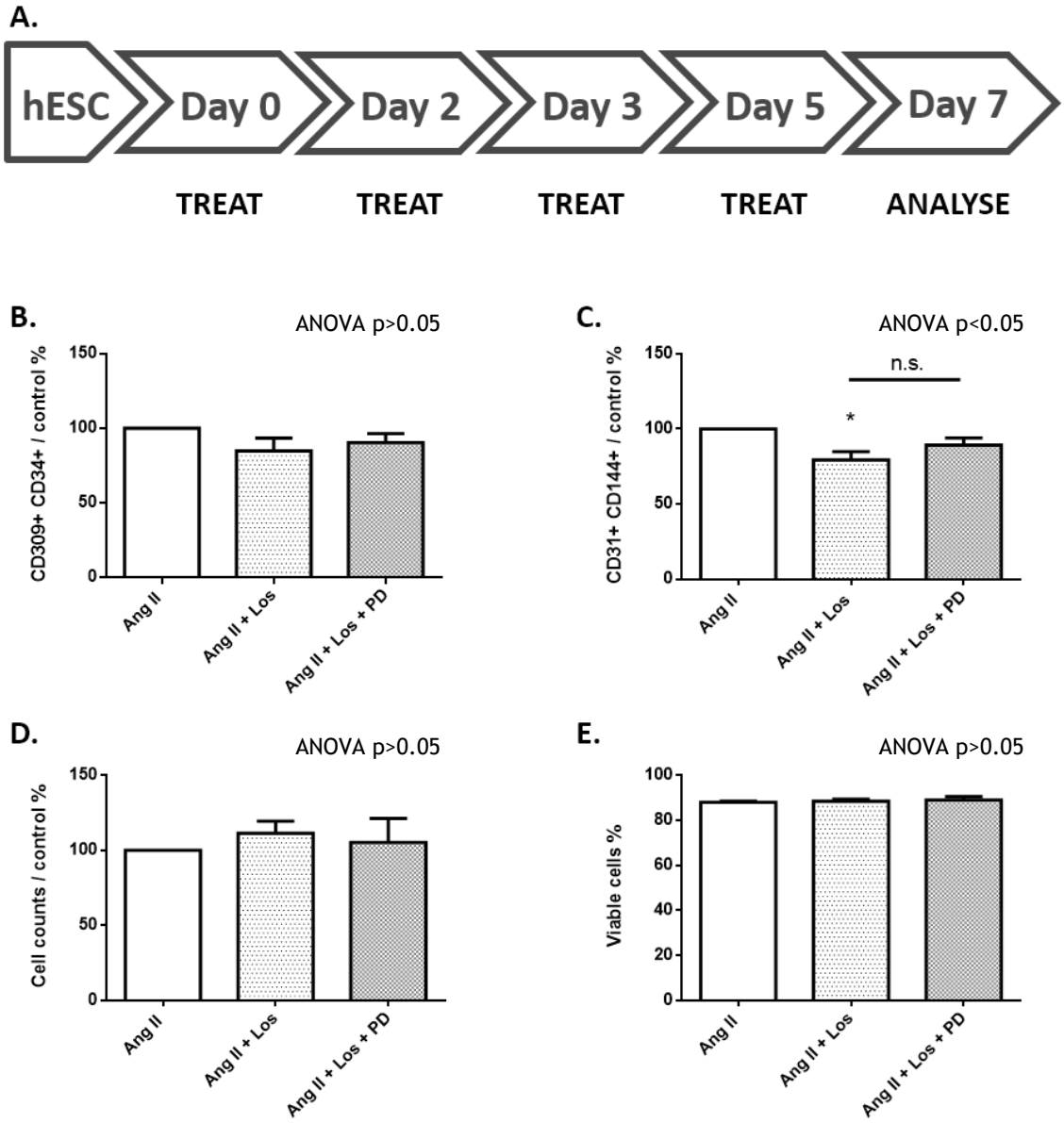


Figure 47: Effect of antagonism of the AT_1R and the AT_2R during hESC-EC differentiation. A. *Treatment schematic.* ***B-E.*** *Losartan (Los, 10μM), angiotensin II (Ang II, 100nM) and PD-123319 (PD, 500nM) treatments were applied as indicated during hESC-EC differentiation with H9 (n=3, independent experiments). Total cell counts, viability and populations expressing EPC markers CD309 and CD34 and EC markers CD31 and CD144 were compared on day 7 of the differentiation. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05 when compared to control.*

To account for possible differences in the half-life of the added drugs in cell culture, differentiations were repeated, with the exception that each drug was replenished every 24hr (Figure 48a). A comparable percentage of cells expressing EC markers was observed in Ang II and Losartan treated differentiations on day 7 (20.4% ±2.4 vs 23.7% ±2.4, p=0.2 vs Ang II only, Figure 48c). In contrast, the percentage of cells expressing EC markers was significantly reduced in differentiations where PD-123319 was added in addition to Ang II and Losartan when compared to Ang II treated differentiation (17.4% ±0.7 vs 23.7% ±2.4, $p<0.05$ vs Ang II only, Figure 48c). However, the difference was not significant in comparison to Ang II and Losartan treated differentiations (17.4% ±0.7 vs 20.1% ±2.4, p=0.25). Percentage of cells expressing EPC markers, total cell counts and viability were comparable between conditions (Figure 48b, d-e).

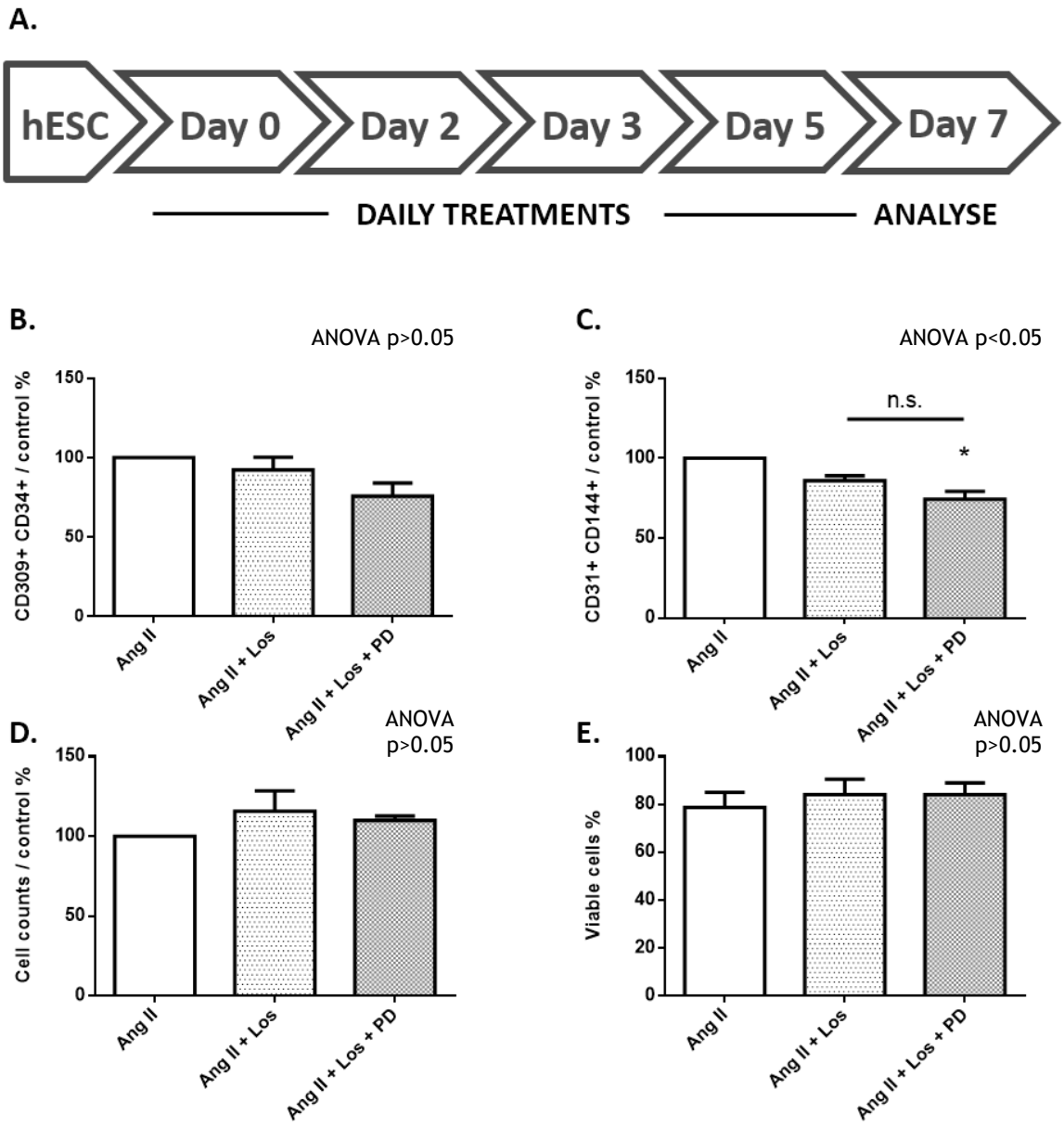


Figure 48: Effect of daily antagonism of the AT_1R and the AT_2R during hESC-EC differentiation. A. *Treatment schematic.* ***B-E.*** *Losartan (Los, 10μM), angiotensin II (Ang II, 100nM) and PD-123319 (PD, 500nM) treatments were applied daily as indicated during hESC-EC differentiation with H9 (n=3, independent experiments). Total cell counts, viability and populations expressing EPC markers CD309 and CD34 and EC markers CD31 and CD144 were compared on day 7 of the differentiation. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05 when compared to control.*

6.3.4 AT_2R inhibition during hESC-EC differentiation.

To investigate the role of the AT_2R during hESC-EC differentiation, the AT_2R antagonist PD-123319 and Ang II, alone or in combination, were added to the differentiation media from day 3 onwards (Figure 49a). Total cell counts, viability, EC and EPC surface marker expression was evaluated on days 5 and 7 of the differentiation by FC.

Analysis on day 5, as shown in Figure 49b, revealed that PD-123319 treatment did not significantly alter EPC surface marker expression on day 5, with 12.4 ±0.4 % and 13.5 ±2.0 % cells staining positive for CD309 and CD34 in control and PD-123319 treated differentiations respectively. In addition, a slight trend for increased total cell numbers was observed on day 5 in Ang II treated differentiations (2.1×10^6 in Control differentiations vs 2.7×10^6 in Ang II treated differentiations, Figure 49c), however only two cell counts were taken so more repeats are needed to confirm this and determine the statistical significance.

On day 7, 17.4 ± 3.2 % cells expressed CD144 and CD31 in control (Figure 49d). In contrast to the observations on day 5, a significant reduction of total cell numbers was observed in Ang II and PD-123319 treated differentiations on day 7 ($1.3 \pm 0.3 \times 10^6$ vs $2.7 \pm 0.3 \times 10^6$ cells, $p<0.05$ vs Ang II only, Figure 49e). Thus, the total EC yield at the end of the protocol was reduced from $0.5 \pm 0.1 \times 10^6$ cells in Ang II treated differentiations to $0.2 \pm 0.04 \times 10^6$ cells in Ang II and PD-123319 treated differentiations. The viability was 94.0 ±0.8 % in the control and remained comparable between the conditions.

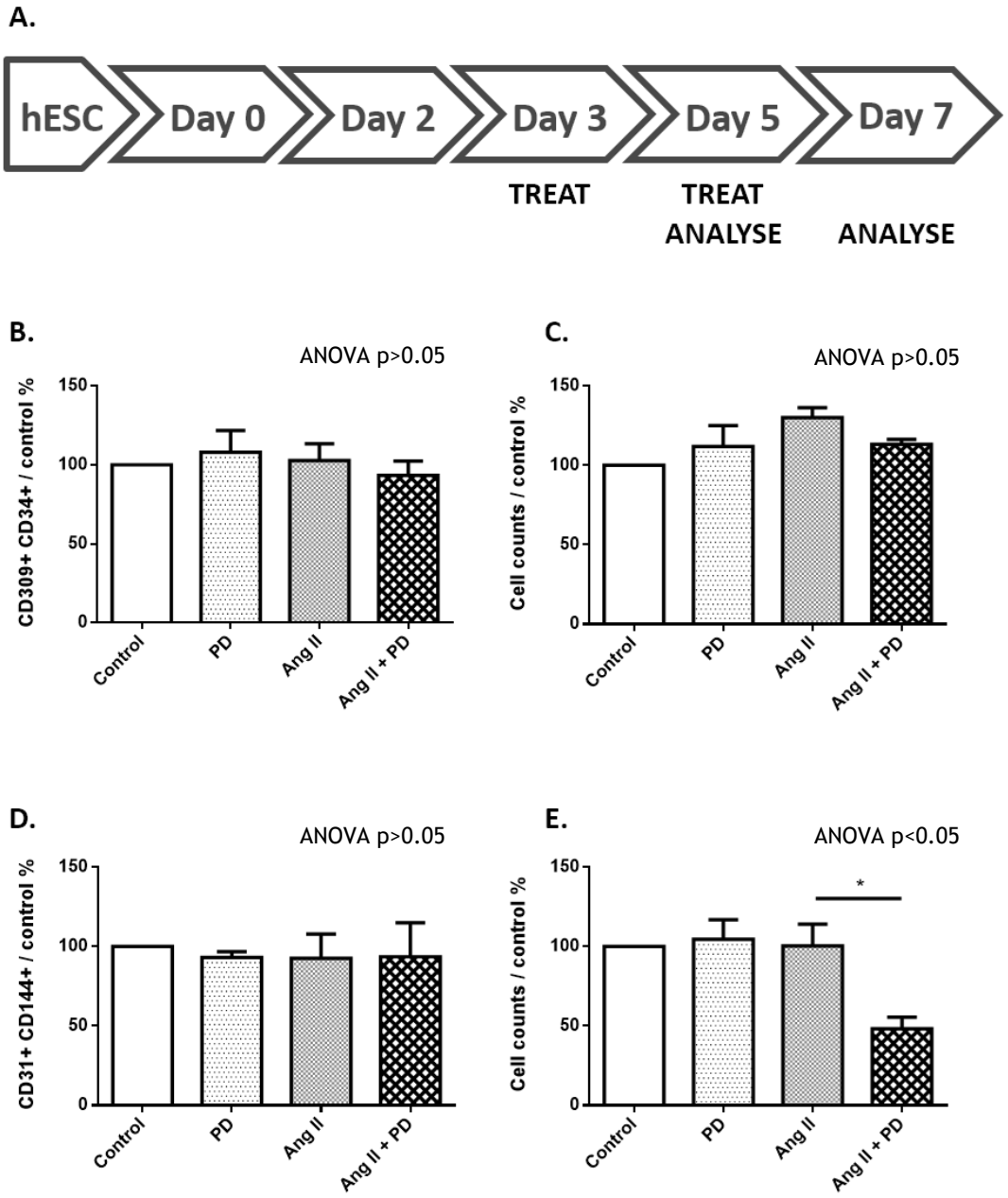


Figure 49: PD-123319 treatments during hESC-EC differentiation. A. *Treatment schematic. PD-123319 (PD, 500nM) and angiotensin II (Ang II, 100nM) treatments were applied on days 3 and 5 of hESC-EC differentiation with H9 (n=3, independent experiments).* ***B-C.*** *Total cell counts[#] and populations expressing EPC markers CD309 and CD34 were compared using FC on day 5 of the differentiation.* ***D-E.*** *Total cell counts and populations expressing markers CD31 and CD144 were compared on day 7 of the differentiation. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05 when compared to control, unless indicated otherwise. [#]n=2 for cell counts on day 5 only as first count was not taken.*

To account for possible off-target effects of PD-123319 (Daugherty et al., 2013; Lautner et al., 2013; Tetzner et al., 2016), total cell counts, viability and EC and EPC marker expression was compared between differentiations treated with Ang II and PD-123319 as above and differentiations treated with Ang II and the alternative AT_2R antagonist PD-123177 (Wong et al., 1990) (Figure 50a).

Total CD144+ CD31+ cell counts were significantly reduced on day 7 of the differentiation from 0.8 ±0.1 $x10^6$ in Ang II treated differentiations to 0.4 ±0.1 $x10^6$ in Ang II and PD-123177, and to 0.4 ±0.004 $x10^6$ Ang II and PD-123319 treated differentiations (both $p<0.05$ vs Ang II only, Figure 50b). This was mainly due to a significant reduction in total cell counts was observed in both Ang II and PD-123319 treated differentiations (4.2 ± 0.6 $x10^6$ vs 3.0 ± 0.4 $x10^6$, $p<0.05$ vs Ang II only) and Ang II and PD-123177 treated differentiations (2.9 ± 0.5 $x10^6$ vs 4.2 ± 0.6 $x10^6$, $p<0.05$ vs Ang II only) (Figure 50c). Viability was comparable between the conditions and was 92.8% ± 2.2 in control. EC marker expression remained comparable with 20.4 ± 4.1 % cells expressing CD144 and CD31 in Ang II only control differentiations, compared to 14.5 ±1.0 % ($p=0.37$) and 14.9 ±1.8 % ($p=0.32$) in Ang II and PD-123319 or Ang II and PD-123177 treated differentiations respectively (Figure 50d). Similarly, EPC surface marker remained comparable with 25.0±7.8 % CD309+ CD34+ cells in Ang II treated differentiations compared to 17.4 ±3.1 % ($p=0.48$) and 15.3 ±0.9 % ($p=0.33$) CD309+ CD34+ in differentiations treated with Ang II and PD-123319, or Ang II and PD-123177 respectively (Figure 50e).

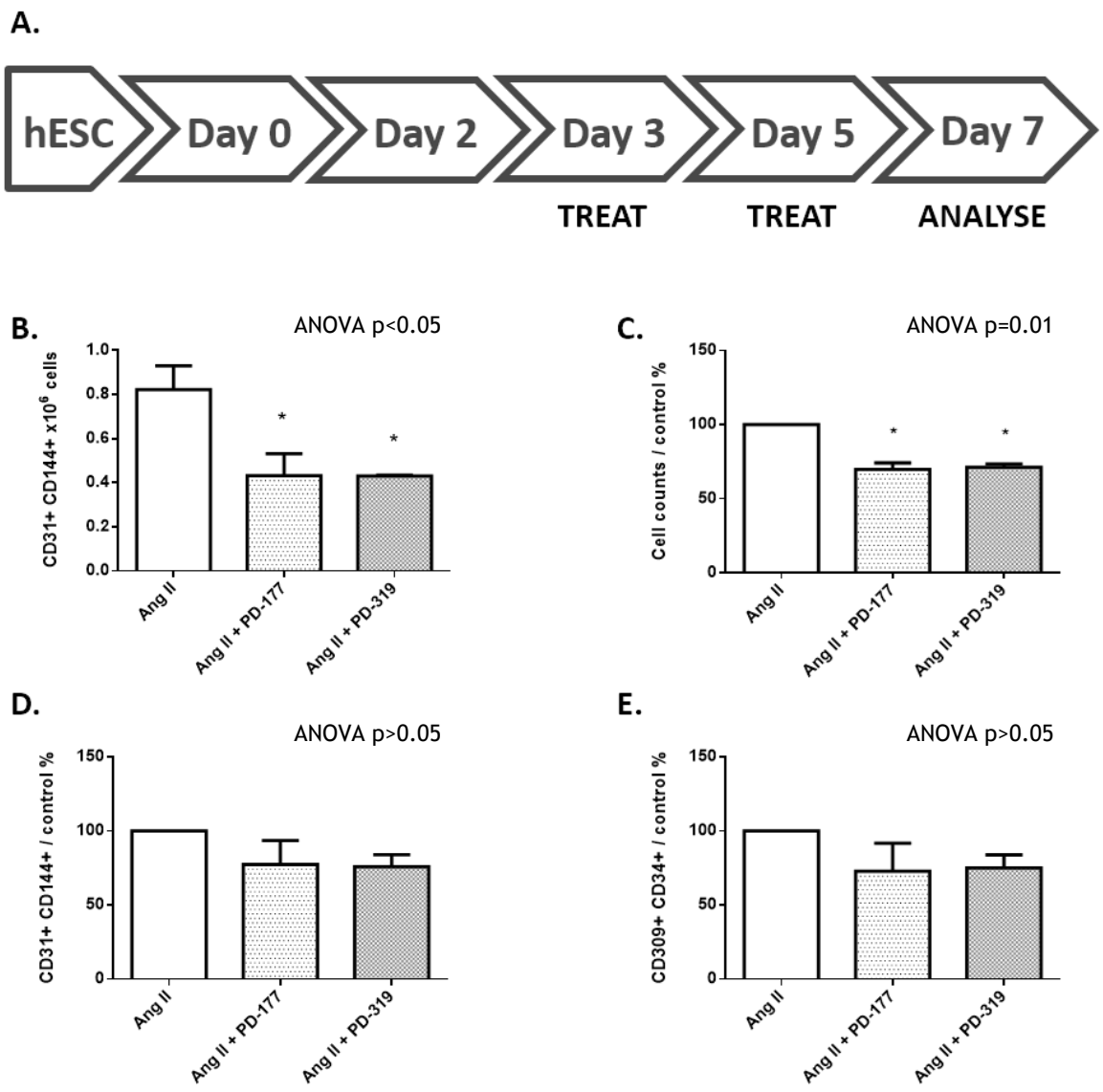


Figure 50: Comparison of PD-123319 and PD-123177 treatments during hESC-EC differentiation in the presence of added Ang II. A. *Treatment schematic.* ***B-E.*** *PD-123319 (PD-319, 500nM), PD-123177 (PD-177, 1μM) and angiotensin II (Ang II, 100nM) treatments were applied on days 3 and 5 of hESC-EC differentiation with H9 (n=3, independent experiments). Total cell counts, viability and populations expressing EC markers CD31 and CD144 and EPC markers CD309 and CD34 were compared on day 7 of the differentiation. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, * p<0.05 when compared to Ang II only control.*

Next, the experiment was repeated with replenishment of the drugs every 24hrs to account for any reduced effects through breakdown of the added drugs. In contrast to the data previously described (Figure 49 and Figure 50), CD144+ CD31+ cell yield remained comparable between the conditions and was 1.1 ±0.1 $x10^6$ cells in the Ang II treated control differentiations, 0.9 ±0.2 $x10^6$ cells in the And II and PD-123319 treated differentiations (p=0.33 vs Ang II only) and 1.1 ±0.1 $x10^6$ cells in the Ang II and PD-123177 treated differentiations (p=0.83 vs Ang II only) (Figure 51b). Total cell numbers were not decreased by PD-123319 or PD-123177 treatments in combination with Ang II, with 4.5 ±0.6 $x10^6$ cells in Ang II, 4.1 ±0.6 $x10^6$ in Ang II and PD-123319, and 4.6 ±0.7 $x10^6$ in Ang II and PD-123177 treated differentiations (Figure 51c).

Both EC marker CD31 and CD144 expression remained comparable between the conditions with 25.4 ±0.9 % in Ang II, 23.1 ±1.3 % in Ang II and PD-123319 and 23.6 ±2.7 % in Ang II and PD-123177 treated differentiations (Figure 51d). Similarly, EPC marker CD309 and CD34 expression was comparable between each experimental condition with 25.4 ±0.69 %, 23.6 ±2.7 % and 23.1 ±1.3 % CD309+ CD34+ cells in Ang II, Ang II and PD-123319, and Ang II and PD-123177 treated differentiations respectively (Figure 51e). The viability was comparable between the each experimental condition and was 87.9 ±1.0 % in Ang II treatment group.

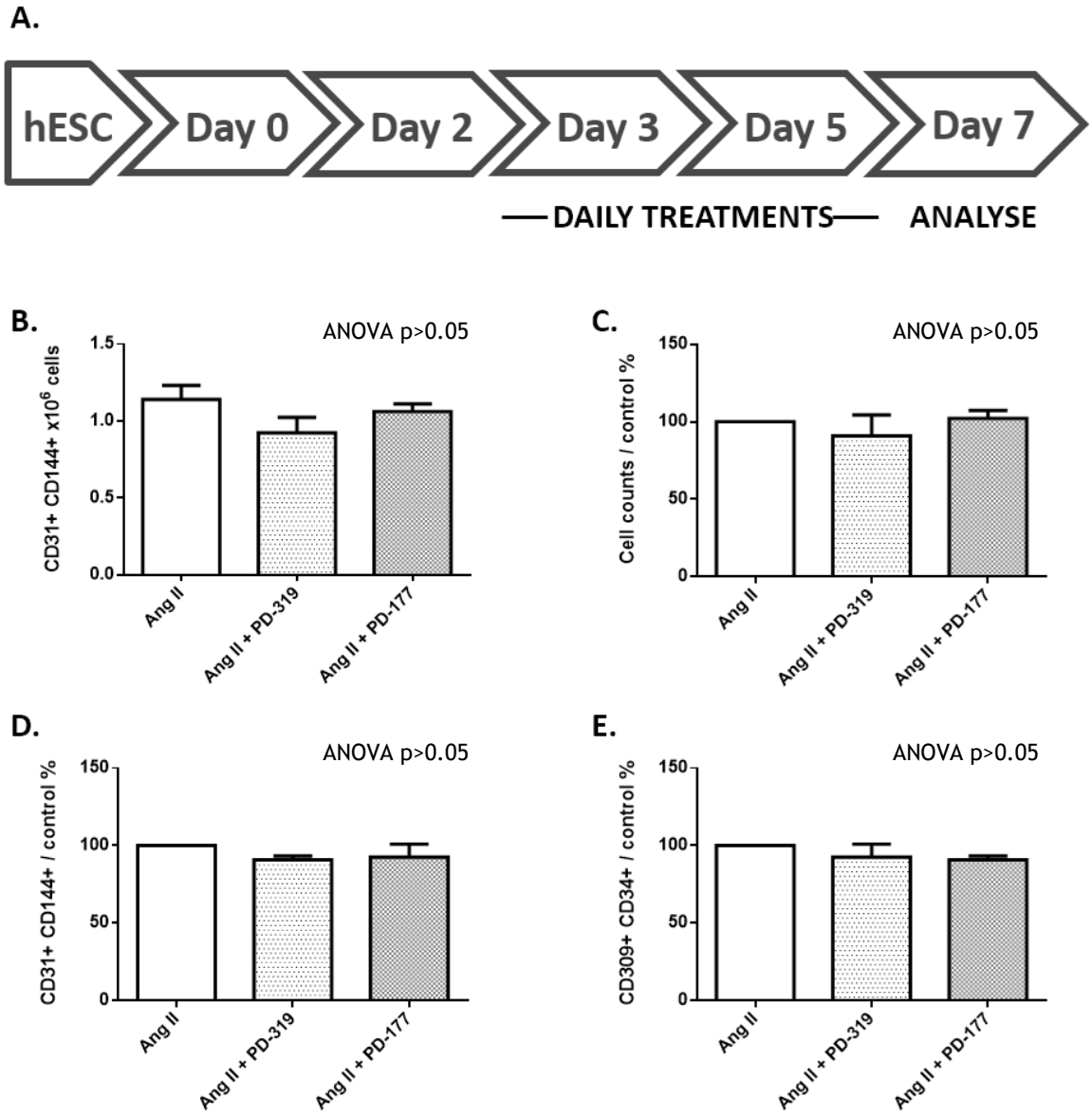


Figure 51: Comparison of daily PD-123319 and PD-123177 treatments during hESC-EC differentiation in the presence of added Ang II. A. *Treatment schematic.* ***B-E.*** *PD-123319 (PD-319, 500nM), PD-123177 (PD-177, 1μM) and angiotensin II (Ang II, 100nM) treatments were applied daily between days 3 to 7 of hESC-EC differentiation with H9 (n=3, independent experiments). Total cell counts, viability and populations expressing EC markers CD31 and CD144 and EPC markers CD309 and CD34 were compared on day 7 of the differentiation. Statistical significance was evaluated using repeated measures ANOVA with Tukey's post hoc comparisons, all non-significant when compared to Ang II only control.*

6.4 Discussion

In summary, the data presented in this chapter revealed upregulated AT_2R expression between day 2 and 3 of hESC-EC differentiation and progressive AT_1R upregulation, suggesting that components of the RAS may play a role in endothelial differentiation. However, addition of exogenous Ang II at two different concentrations to the differentiation media did not alter total cell numbers and/ or the differentiation efficiency. Utilising the AT_1R antagonist Losartan from day 0 of the hESC-EC differentiation had a limited effect on hESC-EC differentiation and thus was not investigated further. Blocking the AT_2R from day 3 of the differentiation using PD-123319 (Dudley et al., 1990) also did not have an effect on hESC-EC differentiation. However, when PD-123319 was added in the presence of Ang II, reduced differentiation efficiency and reduced total cell numbers at the end of the hESC-EC differentiation were observed. Taken together, it can be suggested that, in the absence of AT_2R signalling, AT_1R signalling is detrimental to hESC-EC differentiation.

Ang II receptors are differentially regulated during endothelial development with the AT_2R expressed in the foetal cardiovascular and renal tissues and the AT_1R upregulated during maturation (Bagby et al., 2002; Shanmugam et al., 1996; Vinturache and Smith, 2014). Additionally, ACE is regarded as one of the early markers of endothelial development (Jokubaitis et al., 2008; Sinka et al., 2012; Zambidis et al., 2008). However, while the observations during hematopoietic differentiation suggest a role for Ang II signalling in establishing endothelial phenotypes (Zambidis et al., 2008), the effect of Ang II signalling during endothelial development remains unclear.

First, expression of the RAS receptors was quantified during hESC-EC differentiation with H1, H9 and RC-11. Expression of the AT_1R could not be detected in pluripotent H1 and H9 hESC, and was detected at low levels in RC-11. However, progressive upregulation and high AT_1R expression levels were observed in all three cell lines by day 7 of the hESC-EC differentiation. The AT_2R expression was low in hESC and peaked during mesoderm induction on day 2 in H1, and day 3 in H9 and RC-11. This is in line with the reported AT_1R and AT_2R expression patterns during development (Bagby et al., 2002; Shanmugam et al.,

1996; Vinturache and Smith, 2014) and suggests that Ang II signalling may be exploited during endothelial differentiation.

In contrast, expression of the Mas receptor remained comparable throughout the hESC-EC differentiation – it was highly expressed in H1, while expression levels in H9 and RC-11 were lower. Currently there are no reports on the role of Mas receptor in pluripotency. Underlying reasons for the high Mas expression were not investigated further as no effect of added Ang-(1-7), the natural Mas agonist (SANTOS 2003), could be detected. Therefore, although it would be interesting to further study the role of Mas in hESC-EC differentiation in the future, the work here focused on the role of Ang II and the AT1R and AT2R.

To confirm the RNA expression data, protein expression levels of AT_1R and AT_2R could be evaluated. Previously, antibodies that can reliably discriminate between the receptor subtypes (Gao et al., 2012; Liles et al., 2015) have been described and could be used to investigate AT_1R and AT_2R expression levels. The interpretation of RNA expression data is further complicated by the presence of other cell types in the mixed differentiation system. For example, it cannot be excluded that fibroblasts, which express angiotensin receptors (Gray et al., 1998), may also be present in the mixed cell population and contribute to the observed receptor expression patterns.

Ideally, the expression of AT_1R and AT_2R should be measured in sorted cell populations of interest, for example the CD309+ population. To confirm the receptor expression in mesodermal and early endothelial progenitors, CD87+ (Drukker et al., 2012) or CD56+ CD326- (Evseenko et al., 2010) populations could be analysed. Fluorescence or magnetic activated cell sorting could be used to obtain 90%+ pure populations of interest which would provide a more accurate insight in the RAS receptor expression profiles.

To investigate whether Ang II signalling had an effect on the endothelial differentiation, Ang II treatments were applied from day 0 or day 3 of the hESC-EC differentiation. Ang II treatments did not alter EC and EPC marker expression and total cell counts during the differentiation. The lack of effect suggests that either the hESC-EC differentiation system was already saturated by Ang II produced by the differentiating cells, that Ang II activated both AT_1R and

the AT_2R which can antagonise each other (Nakajima et al., 1995), or that Ang II does not alter the hESC-EC differentiation process. It would be critical to measure Ang II levels in the differentiation media, using mass spectrometry or commercially available enzyme-linked immunosorbent assay kits (Chappell, 2015), to clarify this. Here, receptor-specific antagonists were used to evaluate the role of the AT_1R and the AT_2R during the endothelial differentiation.

While observations from human peripheral blood EPC studies, suggest that Ang II signalling via the AT_1R has been induces oxidative stress and accelerates EPC senescence (Endtmann et al., 2011; Imanishi et al., 2008, 2005). Treatments with the AT_1R antagonist Losartan (Duncia et al., 1992) from day 0 of the hESC-EC differentiation did not have significant effects on the total cell counts, or percentage of cell expressing EPC an EC markers. However, oxidative stress and cell senescence was not measured in this experiment.

When Ang II was added to the differentiation media in addition to Losartan, a significant reduction in the percentage of cells expressing EPC markers on day 5 and EC markers on day 7 was observed, however no changes in total cell counts were observed. To confirm whether the observed effect was due to AT_1R inhibition alone or AT_2R activation in the presence of the AT_1R blockade, the AT_2R inhibitor PD-123319 (Dudley et al., 1990) was added to the differentiation media in addition to Losartan and Ang II. A reduction in the percentage of cells expressing EC markers was seen in Losartan and Ang II treated differentiations, as observed in the previous experiment. This was also not significantly different from the Losartan, Ang II and PD-123319 treated differentiations, implying that the observed effect was due to AT_1R blockade, not preferential AT_2R signalling.

To account for possible loss of the drug potency due to degradation during the time course following a single treatment, the experiment was modified to include daily treatments. No significant differences were seen in EC marker expression at the end of the differentiation in Ang II and Losartan treated differentiations. Given that the effect of Losartan treatments, alone or in combination with Ang II, on the hESC-EC differentiations was limited, further experiments focused on investigating the effects of AT_2R signalling during hESC-EC differentiation instead.

To examine the role of the AT_2R during hESC-EC differentiation, the AT_2R agonist CGP-42112A (Hines et al., 2001) was added from the day 0 of the hESC-EC differentiation. No significant differences were seen in EC and EPC marker expression, while a non-significant reduction in total cell numbers was seen and needs to be confirmed in additional experimental repeats. Signalling via the AT_2R has been shown to exert anti-growth effects in endothelial cells via decrease of AKT and eNOS phosphorylation (Benndorf et al., 2003; Kou et al., 2007; Stoll et al., 1995), which is in line with this observation. To confirm this, changes in nitric oxide production could be measured using the commercially available nitric oxide kits. In contrast, Ikhapoh *et al.* (2015) suggested that signalling via the AT_2R potentiates VEGF signalling and induces endothelial phenotypes in microswine bone marrow derived MSCs, while no changes in cell numbers were reported. This discrepancy might be explained by cell type-specific differences. Alternatively, as the role of the AT_2R was inferred from experiments using Ang II in combination with PD-123319 (Ikhapoh et al., 2015), it could be suggested that AT_1R activation in the absence of AT_2R, or blocking of Mas or MrgD receptor signalling (Lautner et al., 2013; Tetzner et al., 2016) could also be responsible for the reported effects.

To investigate the effect of blocking the AT_2R during the hESC-EC differentiation, PD-123319 was added from day 3 of the hESC-EC differentiation when AT_2R expression was upregulated. PD-123319 treatments alone did not alter the percentage of cells expressing EC and EPC markers and total cell counts. This suggests that AT_2R signalling does not have a beneficial effect during hESC-EC differentiation. However, when Ang II was added to the differentiation media in combination with PD-123319, a significant reduction in total cell counts was observed, while viability remained unchanged. As Ang II treatments in the previous experiments were not detrimental to the hESC-EC differentiation, it can also be suggested that AT_1R activation in the absence of compensatory AT_2R signalling may be responsible for the observed decrease in total cell numbers. A similar pattern was observed in Ang II and PD-123177 treated differentiations, supporting the conclusion that this was a specific effect of AT_2R blockade and not an off target effect of PD-123319 (Daugherty et al., 2013; Lautner et al., 2013; Tetzner et al., 2016).

Taken together, this data supports the observation that AT_1R signalling in the absence of AT_2R signalling during hESC-EC differentiation is detrimental to the total cell numbers and the efficiency of EC differentiation. This also mimics the observations in the MSC differentiation system, where combined VEGF, Ang II and PD-123319 treatments significantly reduced EC marker expression (Ikhapoh et al., 2015), however, the data presented here suggests that this is due to detrimental effects of AT_1R signalling, when compensatory AT_2R-mediated effects are absent.

Additionally, while signalling via the AT_2R does not appear to have any beneficial effects on its own, as suggested by the experiments utilising the specific AT2R agonist CGP-42112A, it seems to be required to counterbalance the negative effects of the AT_1R as the detrimental effects were not observed in Ang II only treated differentiations. The mechanisms underlying this were not investigated due to time constraints. However, as the cell viability remained comparable, it's likely that the reduced cell growth is due to AT_1R effects on cell proliferation or senescence as discussed above.

Furthermore, one of the potential targets of Ang II signalling is VEGF signalling, which forms a central signalling axis during endothelial differentiations as described in section 1.7.2, with a range of effects on endothelial differentiation and cell survival. Ang II signalling via AT_1R and PKC has been demonstrated to increase VEGF receptor CD309 expression in EPCs (Imanishi et al., 2004) and retinal microcapillary endothelial cells (Otani et al., 1998). Signalling via AT_1R has also been shown to aid differentiation of CD309+ mesodermal cells in mouse iPSC (Ishizuka et al., 2012) and haemangioblast colonies preferentially differentiate towards endothelial progenitor cells after PD-123319 treatment (Zambidis et al., 2008), suggesting that similar effects may be observed in hESC endothelial differentiation.

However, here Ang II treatment alone did not increase the percentage of cells expressing EPC and EC markers, and Losartan treatments alone, did not consistently have a detrimental effect on the EPC and EC populations. In contrary, when Ang II was added in addition to PD-123319, a trend for a reduced percentage of cells expressing EC and EPC markers was observed, perhaps due to the detrimental effects of AT_1R signalling on EPC growth.

Interestingly, when this experiment was repeated with daily replenishment of the agonists/ antagonists this effect was abolished. While direct comparison of these experiments cannot be made as only two of the three experimental runs were done in parallel, this observation suggests that AT_1R activation in the absence of AT_2R activation may induce paracrine secretion of a growth factor affecting cell proliferation and survival which is then removed during media changes. In order to identify the growth factor responsible for the observed effects, growth factor RNA expression and protein level expression should be determined.

Shi et al. (2009) reported that Ang II signalling via AT_1R and ERK/AKT induces paracrine secretion of VEGF in a MSC differentiation system. Additional evidence has been provided from *in vivo* observations showing Ang II and AT_1R dependent upregulation of VEGF expression (Amaral et al., 2001; Tamarat et al., 2002). However, increased VEGF signalling would be a positive regulator for hESC-EC differentiation. Furthermore, while here changes in VEGF expression were not evaluated, it is unlikely that changes in the VEGF secretion would have significant effects on the hESC-EC differentiation due to the high concentrations of VEGF added to the differentiation media.

While, without further experimental evidence, it is impossible to be certain of the growth factors responsible for the observed effects, TGFB is an obvious candidate. Indeed, TGFB signalling can negatively affect both EC proliferation, viability and specification (Asano and Trojanowska, 2009; Castañares et al., 2007; James et al., 2010; Sahara et al., 2014) as described previously in section 3.1. In addition, Ang II acts on fibroblasts to induce TGFB secretion in myofibroblast cultures (Campbell and Katwa, 1997; Gray et al., 1998) and also induces TGFB in vascular smooth muscle cultures (Gibbons et al., 1992; Itoh et al., 1993). It remains unclear, whether TGFB expression is also induced in endothelial progenitor cells, however, given the mixed nature of hESC-EC differentiation, the presence of other secretory cell types is likely.

To confirm that the detrimental effects are indeed mediated via the AT_1R, it should be confirmed that adding Losartan, in addition to Ang II and PD-123319 reversed the observed effects. Previous experiments using combined Ang II, Losartan and PD-123319 reported in this chapter demonstrated a trend for

reduced percentage of EC cells, but not reduced total cell numbers. However, treatments were applied from day 0 of the differentiation in these experiments, and thus are not directly comparable.

Alternatively, AT_1R or AT_2R-specific knock out cell lines could be used to gain insight into the role of these receptors during hESC-EC differentiations. To do this, clustered regularly interspaced short palindromic repeats systems (Cong et al., 2013; Mali et al., 2013) could be employed for precise genome editing, and inducible gene knockout systems have also been described (Chen et al., 2015) allowing for differentiation stage specific gene editing. The knock out cell lines could be differentiated towards endothelial lineages in the presence or absence of added Ang II and the cell phenotypes throughout the differentiation could be evaluated and compared with wild type cells.

Additionally, it is required to confirm these observations in a range of cell lines and further research is required to investigate the underlying mechanisms using high efficiency differentiation cultures, if possible. The activation of downstream signalling pathways, for example ERK, AKT/eNOS, and oxidative stress should also be evaluated to gain further insight into the downstream effectors of RAS signalling during the endothelial differentiation.

Here the functional effects of RAS manipulation were not evaluated, however, it is likely that the RAS could alter the functionality of the differentiated cells without obvious changes in EC and EPC marker expression in line with the previously described effects of the RAS on nitric oxide production. For example, AT_1R mediated eNOS induction has been extensively reported to enhance angiogenesis *in vitro* and *in vivo* (Imanishi et al., 2004; Otani et al., 1998; Tamarat et al., 2002), with the AT_2R reported to have opposing actions (Benndorf et al., 2003; Kou et al., 2007). Alternatively, more recently Ang II pretreatments have been reported to enhance BM-MNC angiogenic capacity via the AT_2R induced eNOS induction (Xu et al., 2013) and MSC angiogenic capacity via increased paracrine VEGF secretion (C. Liu et al., 2015) in *in vivo* myocardial infarction models, thus, any beneficial or detrimental effects of RAS signalling on the functional characteristics of the derived hESC-EC cells remain to be clarified.

In summary, the data presented in this chapter demonstrated differential expression of RAS receptors during hESC-EC differentiation, suggesting that Ang II signalling may be active during endothelial differentiation. Ang II treatments alone did not alter cell numbers and EC marker expression at the end of the differentiation. To investigate the roles of the AT_1R and the AT_2R during hESC-EC differentiation, receptor specific antagonists were used alone or in combination with Ang II. The AT_1R antagonist Losartan had a limited effect on the hESC-EC differentiation and thus was not investigated further. When PD-123319 was added to the differentiation system alone, no significant changes were observed in the total cell numbers and marker expression profiles. However, when PD-123319 was used in addition to Ang II, a significant reduction in the total cell numbers and EC marker expression was observed. This effect was consistent when the alternative AT_2R antagonist PD-123177 was used, suggesting that unopposed AT_1R activation is detrimental to hESC-EC differentiation. Additionally, the data also demonstrates that the effect was abolished by daily treatments, suggesting that a secreted growth factor might mediate the observed effects. Further research is required to investigate this and identify the secreted factor. This highlights the complex role of the RAS during endothelial differentiation and suggests that Ang II signalling should be limited during hESC-EC differentiation.

Chapter 7: General Discussion.

7.1 Discussion

Cardiovascular disease represents a significant socio-economic burden and minimally invasive therapies that address the needs of patients suffering with PAD and CLI are needed. Cell therapies have been proposed as an alternative to pharmacological and surgical treatments, yet, have demonstrated somewhat limited efficacy. Therefore, it has been speculated that differentiation of hESC towards endothelial lineages, could provide well defined cell populations with superior angiogenic profile.

While numerous endothelial differentiation protocols have been published (Descamps and Emanueli, 2012) and, more recently, high differentiation efficiencies have been achieved (Patsch et al., 2015; Prasain et al., 2014; Sahara et al., 2014), most of these approaches are not optimised for clinical purposes due to the use of poorly defined, non cGMP compatible reagents, or require additional processing steps, such as cell sorting to remove undefined cell populations which can pose a teratoma risk. This complicates the clinical approval and increases the therapy cost, representing a significant hurdle that needs to be overcome in order to use differentiated endothelial cells for the future angiogenesis therapies.

Therefore, here it was aimed to develop and optimise a clinically compatible hESC-EC differentiation protocol that avoids using poorly defined reagents, and yields high percentages of cells expressing EC markers without the use of cell sorting. While not all reagents used in the experimental work were cGMP grade due to cost implications, for example Lonza EGM-2 and VEGF, clinical grade versions of these reagents are available, if required. Use of cGMP compatible reagents simplifies the bench to bedside translation of this differentiation protocol, reducing the time and cost of bringing hESC-EC cell therapies to clinic.

Initially, as described in Chapter 3, the hESC-EC differentiation protocol was optimised to reduce the cost of the differentiation by using Pluronic F-127 (Ungrin et al., 2008) coated instead of low adherence wells, while retaining the differentiation efficiency. Inhibition of TGFB signalling using SB431542 was evaluated as a cGMP compatible approach for increasing EC marker expressing cell yields (James et al., 2010; Sahara et al., 2014), yet, no additional benefit

was observed. Thus, TGFB inhibitors were not included in the hESC-EC differentiation protocol described here. The optimised protocol consists of dissociating pluripotent hESC on day 0 of the differentiation and generation of controlled size EB in 96 well tissue culture plates coated with Pluronic F-127 (Ungrin et al., 2008) in mesodermal specification media developed in the group (Olivier et al., 2016). On day 3 of the differentiation, the EBs are plated in Gelatin coated 6 well tissue culture plates in vascular specification media supplemented with VEGF. Gene expression profiling showed a peak of mesodermal gene Brachyury expression on day 2 of the differentiation, followed by upregulation of endothelial gene KDR and CDH5 expression. By day 7, cells with endothelial morphology can be observed and approximately 30% of the differentiated cells express both endothelial markers CD31 and CD144.

While gene and surface marker expression patterns showed endothelial commitment of the differentiated cells, the functional characteristics, and the *in vitro* and *in vivo* angiogenic potential of the differentiated cells was not evaluated in this thesis. However, hESC-EC differentiated cell *in vitro* Matrigel tubule formation and AcLDL uptake assays have been performed in the group (personal communication, Dr Alison Condie, University of Glasgow) and confirmed the functionality of the differentiated cells. It would be beneficial to also perform the widely used nitric oxide production and matrigel plug *in* vivo assays, in addition to other functional tests suggested for extensive EC function testing, for example, vascular structure formation in coculture with pericytes, and incorporation in the vasculature of zebrafish xenografts (Orlova et al., 2014). Such testing would provide the much needed insight of the functionality of the generated cells and would allow for comparison to other endothelial cell types, for example NRP1+ CD31+ cells (Prasain et al., 2014) or EC expressing arterial markers (Rufaihah et al., 2013), reported to have superior *in vivo* vessel-forming ability.

The developed hESC-EC protocol yields a reasonable percentage of cells coexpressing EC markers CD31 and CD144 after 7 days of differentiation, without out the use of cell sorting or undefined reagents, such as Matrigel and serum, which sets it apart from other endothelial differentiation approaches. Yet, it is clear that there is scope for further optimisation with focus on the use of small

molecule drugs to enhance the differentiation efficiency and avoid the need of sorting prior to the clinical use. Therefore, it was hypothesised that high throughput screening could be used to optimise the hESC-EC differentiation protocol.

While various approaches can be employed for high throughput optimisation of the endothelial differentiation, the use of fluorescent reporter cell lines offers a highly specific and flexible way to monitor the progression of the differentiation. Therefore, as described in Chapter 4, work was undertaken for generation of reporter cell lines suitable for monitoring both mesodermal induction and vascular specification phase of the hESC-EC differentiation. *ETV2* plays a central role in endothelial development - it induces vascular mesoderm (Kataoka et al., 2011) and is required for endothelial and hematopoietic development (Ferdous et al., 2009; Shi et al., 2014). ETV2 expression profiling revealed transient ETV2 mRNA expression at the end of the mesodermal specification phase of the hESC-EC differentiation. Therefore, ETV2 was selected as an early endothelial commitment reporter gene. For monitoring of the endothelial commitment during the vascular specification phase of the differentiation, CDH5 (Dejana et al., 1999) and ROBO4 (Huminiecki et al., 2002; Huminiecki and Bicknell, 2000) genes were selected as both of these genes are specifically expressed in mature endothelium and were shown to be progressively upregulated during the hESC-EC differentiation.

Reporter constructs where cognate ETV2, CDH5 or ROBO4 promoter fragments drive the expression of GFP or RFP fluorescent proteins were generated. Preliminary validation was attempted both using the generated plasmids, and, in addition, lentiviral vectors carrying the constructs were generated and used for validation in HUVECs, HSVECs and during the hESC-EC differentiation. Reporter gene expression was not observed during the validation, suggesting that the generated reporter constructs were not functional, however, further validation is required. Instead, it was decided to obtain a commercially available ETV2 reporter construct and a previously published CDH5 reporter construct (Sahara et al., 2014), and use these for the optimisation of hESC-EC differentiation.

Preliminary validation of the lentiviruses carrying either of the obtained reporter constructs suggested non-specific reporter gene expression. This highlights the

complexity of gene expression regulation and suggests that there are additional regulatory elements that were not included in the chosen promoter sequences. While reporter cell line generation for high throughput screening was not pursued further, the use of reporter cell lines for high throughput screens remains an attractive approach for the optimisation of the hESC-EC differentiation. It can be suggested that the recent advances in precise genome editing, for example TALEN (Hockemeyer et al., 2011; Joung and Sander, 2013) and CRISPR (Cong et al., 2013; Mali et al., 2013), should be considered as an alternative strategy for reporter generation, allowing for creation of endogenous reporter constructs. However, here it was proposed that rational targeting of novel signalling pathways could be employed as an alternative approach for the optimisation of hESC-EC differentiation.

Furthermore, it became clear that arterial endothelial cells may offer higher efficacy for clinical angiogenesis (Rufaihah et al., 2013; Sriram et al., 2015). Indeed, limited efficacy has been one of the key issues with the adult BM-MNC therapies (Teraa et al., 2015) and, thus, the prospect of differentiating EC populations with superior angiogenic characteristics is highly appealing. Preliminary gene expression analysis during the hESC-EC differentiation suggested induction of arterial gene HEY2 but not venous gene EPHB4 expression during the hESC-EC differentiation. However, there is scope for more robust induction of arterial phenoytpes. Therefore, the potential targets to induce arterial phenotypes and to increase the arterial marker expressing cell percentages during the hESC-EC differentiation were investigated.

The central role for NOTCH and VEGF signalling in arterial specification is well established (Corada et al., 2014; Lawson et al., 2001), however, research using mESC differentiation models suggests that intracellular cAMP levels also contribute to arterial phenotypes, possibly via activation of NOTCH signalling (Yurugi-Kobayashi et al., 2006), and forming of NICD, RBPJ and ß-catenin complexes (Yamamizu et al., 2010). Additionally, targeting cAMP signalling has also been reported to increase EC differentiation efficiency via targeting ETV2 (Shi et al., 2015; Yamamizu et al., 2012b). Therefore, in Chapter 5, it was hypothesised that intracellular cAMP levels could be targeted pharmacologically to increase the differentiation efficiency, and to induce complete arterial

phenotype, including the expression of arterial phenotype associated genes (Aranguren et al., 2013). This could not only reduce the need for cell sorting before clinical use, but also could potentially deliver a clinically superior cell population for therapeutic angiogenesis.

Forskolin treatments increased intracellular cAMP levels during the hESC-EC differentiation, however, no significant increases in arterial or arterial associated gene expression were observed. The lack of arterial gene expression was in contrast to the previous observations using mESC differentiation systems (Yamamizu et al., 2012b; Yurugi-Kobayashi et al., 2006) and more recent publications using hESC differentiation models (Rufaihah et al., 2013; Sivarapatna et al., 2015). Here, it was speculated that this discrepancy could be due to use of serum in the previously published differentiation protocols, which may supplement the differentiation media with BMP4 (Wöltje et al., 2015) that, in the presence of increased intracellular cAMP levels, may induce arterial phenotype. Further research is required to confirm this, and comparing the effects of increased cAMP levels during hESC-EC differentiation in presence and absence of serum would provide a valuable validation of this hypothesis. However, serum products are not well defined, can display batch variability and, thus, present unnecessary complications for clinical translation of cell therapies. Therefore, it would be required to identify and validate the active compound in serum that is essential in addition to cAMP for the induction of arterial phenotype and use it for hESC-EC arterial differentiation instead.

However, a trend of increased EC marker CD31 and CD144 expression was observed and was shown to be mediated mostly via higher percentage of $CD144^{low}$ CD31+ cells. This supports the use of Forskolin in recently developed endothelial differentiation methods (Patsch et al., 2015; Sahara et al., 2014), however, the effects and mechanisms of Forskolin treatments were not investigated in these publications. ETV2 mediated effects may contribute to the increased EC percentage at the end of the differentiation (Shi et al., 2015; Yamamizu et al., 2012b). However, here it was also observed that the increased differentiation efficiency was mediated via increase in CD31+ $CD144^{low}$ cell population. Therefore, it was proposed that higher intracellular cAMP levels may contribute to the stabilisation of the CD144 junctions via EPAC (Fukuhara et al.,

2005; Kooistra et al., 2005) and, thus, increasing CD144 expression levels and promoting EC survival (Carmeliet et al., 1999a). To confirm this, these experiments should be repeated using EPAC specific cAMP analogues (Carmeliet et al., 1999a) and a detailed CD144 expression analysis should be performed. The observed increase in the differentiation efficiency highlights the value of using small molecule drugs for the optimisation of hESC-EC differentiation. Such treatments can be easily added to the differentiation protocols without significantly increasing the differentiation cost, and are compatible with the clinical requirements for cell therapies.

The renin angiotensin system (RAS) may play a role in the hESC-EC differentiation, yet, that has not been described in publications investigating endothelial differentiation of hESC. Indeed, evidence suggests a presence of a local RAS in the vasculature (Campbell and Habener, 1986; Lilly et al., 1985; Rider et al., 2015), ACE has been described as an early marker for lymphatic, hematopoietic and endothelial development (Jokubaitis et al., 2008; Sinka et al., 2012; Zambidis et al., 2008), and AT_1R and AT_2R signalling manipulation has been shown to induce endothelial phenotypes in a hESC hematopoietic differentiation system (Zambidis et al., 2008). Additionally, active Angiotensin II (Ang II) signalling via AT_1R may also mediate a range of detrimental effects on EPC growth and proliferation (Endtmann et al., 2011; Imanishi et al., 2008, 2005). Therefore, in Chapter 6, it was hypothesised that Ang II signalling is active during the hESC-EC differentiation and that this axis may be exploited to enhance the hESC-EC differentiation.

Differential RAS receptor expression was demonstrated during hESC-EC differentiation, supporting the hypothesis and suggesting a role for the Ang II in the differentiation. Ang II and Losartan treatments, alone or in combination, did not consistently alter the percentage of endothelial cells and total cell counts at the end of the differentiation. However, when PD-123319 was used together with Ang II, a significant reduction in total cell numbers and a trend of reduced percentage of cells expressing EC markers CD31 and CD144 at the end of the differentiation was observed. This suggests that unopposed AT_1R activation is detrimental for hESC-EC differentiation. Further research is required to confirm the mechanisms underlying this during the hESC-EC differentiation. In particular,

the role of AT_1R induced oxidative stress and senescence, and the role of AT_2R in counteracting these effects needs to be investigated. While here AT_1R blocking did not have a robust beneficial effect, it cannot be excluded that in other differentiation systems AT_1R signalling mediated effects hinder EC differentiation efficiency. Thus, the use of clinically available AT_1R antagonists may be an attractive, low cost and cGMP compliant way to enhance the differentiation efficiency of clinical grade endothelial differentiation protocols.

Taken together, the work described in this thesis has contributed to the current knowledge of processes underlying endothelial differentiation and has suggested approaches for development and optimisation of clinically compatible endothelial differentiation protocols. Indeed, while hESC and hIPSC derived endothelial cell therapies for therapeutic angiogenesis remain an area of active research and higher differentiation efficiencies have been achieved (Patsch et al., 2015; Prasain et al., 2014; Sahara et al., 2014), there is a lack of focus on novel approaches to increase endothelial differentiation efficiency and most of the published protocols employ highly similar differentiation strategies. Here it was shown that the intracellular cAMP levels can be manipulated to increase the percentage of cells expressing endothelial markers at the end of the differentiation and suggests mechanisms responsible for these effects. Furthermore, the negative effects of the AT_1R signalling during the hESC-EC differentiation were demonstrated. Both of these signalling systems have received little attention and research focus during hESC-EC differentiation, and therefore represent novel targets for optimisation. Therefore, it can be proposed that evaluation of other signalling pathways, for example sphingosine 1-phosphate signalling (Schuchardt et al., 2011), may reveal additional targets that can be used to further enhance the endothelial differentiation in a clinically compatible manner.

Furthermore, while the developed hESC-EC differentiation protocol is clinically compatible, as it does not use poorly defined reagents, there is scope for additional optimisation to ensure the reliability, cGMP compatibility and affordability of the differentiated endothelial cell therapies. Firstly, optimisation of pluripotent hESC and hIPSC derivation and culture conditions is required. Various methods for cGMP compatible hESC and hIPSC derivation have

been described (Baghbaderani et al., 2015; Tannenbaum et al., 2012), and therefore, if possible, clinical grade hESC and hIPSC should be used. Here research grade hESC cell lines H1 and H9, as well as clinical grade hESC cell lines RC-11 and RC-9 were cultured in StemPro media in tissue culture vessels coated with CellStart or Vitronectin XF. While this culture method is clinically compatible, other commercially available, simplified and highly defined culture media, for example, Essential 8 medium (Chen et al., 2011; Wang et al., 2013) and L7 hPSC medium (Baghbaderani et al., 2016), may be more suitable for clinical grade hESC culture. More recently, human serum derived protein inter-α-inhibitor (IαI) has been identified as supporting feeder free, hESC and hIPSC culture on uncoated tissue culture plastic (Pijuan-Galitó et al., 2016), if recombinant IαI can be developed, it would represent a significant advance for future large scale, simplified, clinically compatible hESC culture systems.

Undefined reagents which may contribute to cell signalling, for example Matrigel, are often used for hESC and hIPSC culture in the recently reported endothelial differentiation methods (Patsch et al., 2015; Prasain et al., 2014; Sahara et al., 2014). Ideally, the developed endothelial differentiations protocols, including the hESC-EC differentiation protocol described here, should be developed, optimised and evaluated using multiple hESC and hIPSC lines derived and cultured following the latest clinically compatible methods. This would ensure the reproducibility of the developed differentiation methods and also would simplify the clinical translation.

Safety of the proposed cell therapies is another key aspect that has to be considered during preclinical development. The use of hESC and hIPSC derived cell therapies carries a tumorigenicity risk due to undifferentiated cells remaining in the final cell therapy product and due to genetic instability of the cells (Knoepfler, 2009). Such concerns represent an important roadblock in getting stem cell therapies to clinical practice and, indeed, unexpected mutations have been observed in the first hIPSC clinical trial where differentiated retinal pigment epithelium cells were used to treat age-related macular degeneration, resulting in temporary suspension of the trial (Garber, 2015). These safety concerns can be addressed by using extensive cell therapy quality control testing for genetic abnormalities and ensuring the absence of any

residual pluripotency cells. To address the concerns of residual pluripotency, characterisation of the non-EC marker expressing cells present after hESC-EC differentiation has been performed in our group (personal communication, Dr Alison Condie, University of Glasgow) and confirmed lack of pluripotent cells, which is crucial for translation of this method to clinical therapy.

Other methods for ensuring differentiated hESC cell therapy safety include isolating the cells from the rest of the body, as done with the insulin producing islet replacement product developed by Viacyte (Agulnick et al., 2015), however, this would not be a suitable approach for therapeutic angiogenesis, where the therapeutic cells support angiogenesis and integrate in the vasculature. Alternatively, introducing inducible suicide genes mediating apoptosis by caspase-9 (Ando et al., 2015; C. Wu et al., 2014; Yagyu et al., 2015) has been proposed as an additional safety feature for hESC and hIPSC cell lines which, if required, would allow for efficient clearance of the transplanted cells. However, such approaches require genetic manipulation of the cell lines, which introduces additional risks and further complicates clinical approval of such cell products.

With the key signalling pathways for endothelial differentiation being well described and widely targeted, it can be suggested that future work should also evaluate using small molecule drugs to enhance these signalling pathways, and screen for non-peptide cytokine mimetics. Generally, small molecule drugs are more stable and easier to quantify, thus benefiting the robustness and reproducibility of the differentiation, and offer a cost advantage, when compared to recombinant proteins (Feng et al., 2016). Indeed, small molecule GSK3B inhibitors have been used instead of WNT in multiple published endothelial differentiation methods (Patsch et al., 2015; Sahara et al., 2014). And, while the complex nature of cytokine receptor signalling poses significant challenges for pharmaceutical targeting of growth factor receptors (Boger and Goldberg, 2001), a small molecule sensitizer of BMP signalling has been shown to contribute to mesodermal induction (Feng et al., 2016) and induction of one of the BMP4 effectors (ID1) by small molecule flavonoids has been shown in human cervical carcinoma cell line (Vrijens et al., 2013), providing a proof of principle for this approach.

Once highly efficient, robust and cGMP compliant endothelial cell derivation methods are established, further research will be required to evaluate various other clinical aspects of endothelial cell therapies. For example, the required cell dose and the best administration route for therapeutic angiogenesis currently remains unclear. Indeed, the grafting efficiency is the key determinant of the required dose and insights from adult cell therapies suggest that the engraftment efficiencies can vary significantly depending on the route of the administration (de Silva and Lederman, 2004; Golpanian et al., 2016; Scudellari, 2009). While intravenous injection is the least invasive method of cell delivery, other methods, for example, direct injection in the affected areas, may offer superior cell grafting (Freyman et al., 2006). Furthermore, the use of hydrogel based vehicles for cell therapy delivery allow for additional control of cell fate and behaviour (C. Wang et al., 2010) and perhaps the previously described hydrogel based local VEGF delivery systems (Silva and Mooney, 2007) could be combined with cell therapy products to enhance cell grafting and angiogenic activity.

While currently the delivery of endothelial cells capable of directly contributing to therapeutic responses receives the majority of research focus, it can also be argued that hESC derived endothelial cells are crucial for the advancement of biotechnology and tissue engineering fields. Indeed, tissue vascularisation remains a key limitation for tissue engineering (Lovett et al., 2009; Mao and Mooney, 2015) and endothelial cell can be used to prevascularise the generated tissues before transplantation. For example, EC coated collagen rods have been used to create perfusable vessel networks for tissue engineering (McGuigan and Sefton, 2006), while endothelial cell coculture with osteoblasts (Unger et al., 2007), myoblasts and fibroblasts (Levenberg et al., 2005), and keratinocytes and fibroblasts (Black et al., 1998) has been used to generate tissues containing capillary networks. The large number of endothelial cells required for vascularisation limits the use of adult cell types, thus, the prospect of large scale clinically compatible endothelial cell differentiation from hESC and hIPSC is particularly attractive.

In summary, endothelial cell therapies hold a lot of promise not only for therapeutic angiogenesis, but also are a cornerstone for future developments in

tissue engineering. While adult cell sources can be used to obtain and expand *in vitro* endothelial cultures, these methods cannot provide the large number of cells required for therapeutic purposes and tissue engineering. Therefore, well characterised, highly efficient, robust, and cGMP compliant endothelial methods are required to facilitate the development of novel clinical therapies. The work described in this thesis contributes to the development of clinically compatible endothelial differentiation methods and suggests areas of future research for further advancement of endothelial differentiation methods. While multiple significant hurdles concerning cGMP compatibility and efficiency of the described endothelial differentiation methods remain to be overcome, the recent advances in hESC derivation and culture, and endothelial differentiation promise large scale, clinically compatible endothelial cell differentiation methods in the near future, representing an important milestone towards clinical cell therapies for angiogenesis and tissue engineering.

7.2 Concluding remarks

In conclusion, it was aimed to develop a clinically compatible endothelial differentiation protocol and optimise the developed differentiation method using reporter cell lines indicating early and late endothelial commitment. The reporter constructs could not be validated and, thus, high throughput optimisation was not performed. However, the use of reporter cell lines is a viable approach to screen for compounds increasing the yields of hESC-EC differentiation, this way avoiding the need for cell sorting prior to clinical use of the differentiated cells. Additionally, cAMP signalling was exploited during hESC-EC differentiation to increase the percentage of the EC at the end of the differentiation. Furthermore, the negative effects of AT_1R signalling during hESC-EC differentiation were demonstrated. Both of these signalling systems can be easily manipulated in a clinically compliant manner, and therefore represent an attractive target during clinically compatible hESC-EC differentiation. Taken together, these studies have highlighted the difficulties of establishing efficient, clinically compatible hESC-EC differentiation methods, which are crucial for future therapeutic angiogenesis cell therapies and tissue engineering.

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