Isolation and characterization of human endothelial colony forming cells (ECFCs) and effect of oxidative stress on their differentiation

Thesis submitted in partial fulfillment of the requirement of the degree of Doctorate of philosophy

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January 2014

Endothelial dysfunction plays a pivotal role in the development and progression of atherosclerosis. Atherogenic risk factors harbor the potential to injure the endothelial layer by promoting apoptosis, preceded by an inflammatory reaction in the vessel wall. The maintenance of the endothelial layer is therefore crucial to ensure its integrity. More recent studies suggest an important role of endothelial progenitor cells (EPCs) in contributing towards reendothelialization after vascular injury. These EPCs home to sites of hypoxia or ischemia and take part in vascular repair. Recent studies have shown that a rare population of EPCs called endothelial colony forming cells or ECFCs can be derived from peripheral and cord blood and can be transplanted into immunodeficient mice that have demonstrated the ability to form chimeric blood vessels. Hyperoxia has been shown to be a model of mild oxidative stress, and this oxidative stress is characterized by an increase in reactive oxygen species (ROS) levels that can lead to a wide range of chemical reactions resulting in cellular necrosis and apoptosis through lipid peroxidation and DNA damage. We therefore proposed that by growing endothelial cells *in vitro* in hyperoxic conditions can in turn lead to cellular damage and activation of pro inflammatory markers in these cells. PGC-1α belongs to a small family of transcriptional coactivators which have been shown to regulate reactive oxygen species generation and apoptosis in mature endothelial cells. The mechanisms through which PGC-1α activates gene expression are poorly understood. This study aims at identifying a molecular signature of human endothelial progenitor cells (ECFCs) so that they can be distinguished from mature endothelial cells (HUVEC) and identifying the role of PGC-1α and its downstream targets when these endothelial progenitors and mature endothelial cells are exposed to oxidative stress. In order to achieve that goal we isolated endothelial colony forming cells and mature endothelial cells from the same donor. This was followed by their molecular profiling at mRNA level using qPCR and at protein level using immunofluorescence and FACS analysis. Cells were grown till late passages to study the growth kinetics of ECFCs and HUVEC in normoxia and hyperoxia. Telomerase activity was measured using TRAP assay and telomere length measurement was done using flow FISH. Low density array was carried out in order to distinguish between ECFCs and HUVEC in terms of gene expression analysis. The results showed that ECFCs were distinguished from early EPCs and HUVEC. PGC-1 alpha levels were up regulated in ECFCs when exposed to hyperoxia and this in turn activated several other genes that are involved in angiogenesis, oxidative phosphorylation and electron transport chain. ECFCs and HUVEC have shown to behave differently under conditions of oxidative stress and we found ECFCs to be more resistant to stress than HUVEC as shown in their growth kinetics.

Abbreviations

ABC–me	ATP-binding cassette–mitochondrial erythroid
AcLDL	acetylated low-density lipoprotein
ALA	amino levulinic acid
AMI	acute myocardial infarction
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANG-1	angiopoeitin-1
ANG-2	angiopoeitin-2
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
CAC	circulating angiogenic cells
CAT	catalase
CECs	circulating endothelial cells
CFU	colony forming unit
DMSO	dimethyl sulfoxide
DRP1	dynamin-related protein-1
EBM-2	endothelial basal medium-2
ECs	endothelial cells
ECFCs	endothelial colony forming cells
ECM	extracellular matrix
ECSCR	endothelial restricted cell surface receptor

EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
ER	endoplasmic reticulum
FBS	foetal bovine serum
FIS1	fission 1
FISH	fluorescence in situ hybridization
FSB	first strand buffer
GPx	glutathione peroxidase
G-CSF	granulocyte colony stimulating factor
GH	growth hormone
GnRH	gonadotropin releasing hormone
ips	induced pluripotent stem cells
HSCs	hematopoietic stem cells
HPP-ECFCs	high proliferative potential colony-forming cells
hTERT	human telomerase reverse transcriptase
HUCBC	human cord blood mononuclear cells
HUVEC	human umbilical vein endothelial cells
HPRT1	hypoxanthine phosphoribosyl transferase
IGF-1	insulin like growth factor-1
IIS	insulin and IGF-1 signalling
IL	interleukins
LC3	microtubule-associated protein 1 light chain 3

LDA	low density array
LPS	lipopolysaccharide
LVEF	left ventricular ejection fraction
MAM	mitochondria-associated membrane
MAP Kinase	mitogen-activated protein kinase
MAO	monoamine oxidase
MCAM	melanoma cell adhesion molecule
miR	microRNA
mitoKATP	mitochondrial ATP-sensitive potassium channel
MnSOD	manganese superoxide dismutase
MSCs	mesenchymal stem cells
MPT	mitochondrial permeability transition
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
NO	nitric oxide
NOX	NADPH oxidase
NYHA	New York heart association
OECs	outgrowth endothelial cells
PECAM-1	platelet endothelial cell adhesion molecule-1
PGC-1α	peroxisome proliferator–activated receptor-γ coactivator-1α
PACs	proangiogenic cells
PAD	peripheral artery occlusive disease
PBS	phosphate buffer saline

PBMNCs	peripheral blood mononuclear cells
Pen/strep	Penicillin/Streptomycin
PDGF-A	platelet derived growth factor-A
PDGF-B	platelet derived growth factor-B
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
SCID	severe combined immunodeficiency
SABG	senescence-associated β-galactosidase
SIRT1/3	Sirtuin 1/3
SDF-1	stromal derived factor-1
TBP	TATA box binding protein
TERC	telomerase RNA component
TGF-β	transforming growth factor-β
TNF-α	tumour necrosis factor-α
VEGF	vascular endothelial growth factor
vWF	Von Willibrand factor
WBCs	white blood cells
UCP	uncoupling protein

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Chapter 1. Introduction

1.1 Origin of the human vascular system

The formation of a human vascular network can occur through vasculogenesis or angiogenesis, which are two mutually non-exclusive processes (Jain, 2003). Angiogenesis refers to sprouting of vessels from pre-existing vessels and plays a vital role in wide variety of pathological and physiological conditions in post natal life (Folkman, 1995; Morin and Tranquillo, 2013). This process is crucial to the maintenance of many physiological and pathological phenomenon that includes development of an embryo, carcinogenesis, wound healing, diabetes and several ocular disorders (Carmeliet and Jain, 2000; Carmeliet, 2005; Carmeliet and Jain, 2011; Zheng *et al.*, 2013). These newly formed capillaries lead to the formation of a complex vascular network that eventually penetrates the tissue and provides direct oxygen and nutrient supply. Angiogenesis is characterized by three successive events before blood supply to the tissue ensues. These include initiation, extension and maturation, (Mantzaris *et al.*, 2004) a brief description of which is presented below.

In fully mature blood vessels, endothelial cells (ECs) are encapsulated by mural cells that include pericytes. These ECs are maintained in a resting state by continuous secretion of angiopoietin-1 (Augustin *et al.*, 2009). In pathological conditions such as vascular injury this quiescent state is subjected to alteration by cell surface binding of proangiogenic growth factors mainly vascular endothelial growth factor (VEGF). This binding of VEGF results in production and release of angiopoietin-2 which in turn leads to activation of endothelial cells by detachment of mural cells so that they can move towards source of VEGF (Hegen *et al.*, 2004; Matsushita *et al.*, 2005; Jang *et al.*, 2009).

Endothelial cells migrate through the extra cellular matrix (ECM) and remain attached to each other forming a new capillary bed. The tip cells perform the function of extending the capillary, while the stalk cells continue to proliferate adding cells to the growing sprout (Gerhardt *et al.*, 2003). Endothelial cell migration and proliferation are therefore considered to be two of the most important steps in capillary extension (De Smet *et al.*, 2009).

Endothelial cells release platelet derived growth factor-B (PDGF-B) during advanced stages of vascular development that leads to the proliferation of pericytes and their migration (Gaengel *et al.*, 2009). This is followed by pericyte production of angiopoetin-1 and as a

result of that, ECs switch back towards the resting state and the capillary is stabilized (Augustin *et al.*, 2009).

On the other hand, vasculogenesis is the process in which primitive vascular networks are formed from angioblasts or endothelial progenitor cells (EPCs) that differentiate and give rise to endothelial cells (Jain 2003). During embryonic development, haematopoietic stem cells (HSCs) form blood whereas the endothelial progenitor cells form blood vessels in a highly coordinated manner. There is strong evidence shown in mammals, as shown in figure 1.1, that HSCs originate from a common precursor, the hemangioblast or from hemogenic endothelium by a process termed as endothelial-haematopoietic transition (Tavian *et al.*, 1996; Kattman *et al.*, 2006; Eilken *et al.*, 2009; Lancrin *et al.*, 2009; Bertrand *et al.*, 2010; Boisset *et al.*, 2010; Kissa and Herbomel, 2010). Despite extensive studies, the steps involved in lineage development from endothelial progenitor cells to mature endothelial cells and their site of origin in humans postnatally still remain less well-defined (Yoder and Ingram, 2009). EPCs are found in umbilical cord blood, peripheral blood, bone marrow and vessel walls. Umbilical cord blood is known to be a rich source of these cells, and show high levels of CD133 and CD34 positive cells, same as in the peripheral blood of adults (Ingram *et al.*, 2004).

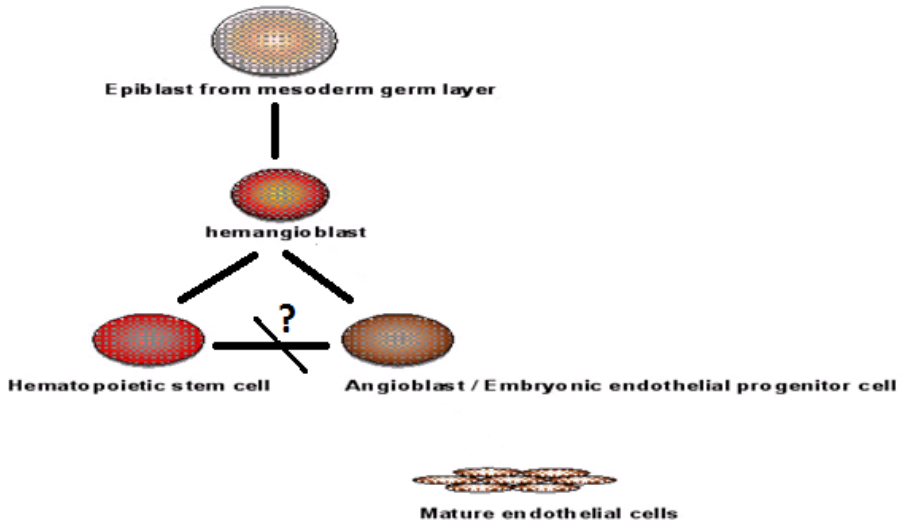


Figure1.1. Illustrating the proposed development programme of endothelial cells. Endothelial and hematopoietic stem cells originating from a common precursor, the hemangioblast.

1.2 Postnatal vasculogenesis in humans

Postnatal vasculogenesis was always believed to occur by angiogenesis, until this dogma was challenged by Asahara and colleagues (1997). They showed for the first time that peripheral blood mononuclear cells (PBMNCs) could be differentiated into potential endothelial cells and can become incorporated at neovascularisation sites in ischemic animal models. After this, several studies indicated that these putative human EPCs could be differentiated *in vitro* into endothelial cells and could be incorporated at sites of active angiogenesis in *NOD/SCID* bone marrow transplantation models (Kalka *et al.*, 2000a). These human EPCs were also shown to be mobilized in to the peripheral blood in response to ischemia by cytokines (Takahashi *et al.*, 1999). Lin *et al*. showed that in allogeneic bone marrow-transplanted patients, the $CD14^{-}$ cell population led to the production of late-outgrowth endothelial cell colonies in culture and proposed that EPCs could originate from existing vessel walls or may be from transplanted marrow itself (Lin *et al.*, 2000). Endothelial precursor cells derived from bone marrow that were positive for Tie-2 showed postnatal induction of revascularization in an *in vivo* graded model of ischemia (Tepper *et al.*, 2005). This proposed that cells of monocytic lineage could be the source of endothelial progenitors or of proangiogenic cells that were named as circulating angiogenic cells (CACs).

Hill *et al.* proposed a clonogenic colony- forming unit 'endothelial cell' (CFU-EC or CFU-Hill) assay and demonstrated that there exists an inverse relationship between cardiovascular risk factors and number of CFU-Hill in human peripheral blood (Hill *et al.*, 2003). But these cells again were classified as EPCs on the basis of expression of biomarkers such as CD31, CD105, CD144, CD146, CD309 and vWF, which are not specific for endothelial cell lineage. For instance, CD144 shows expression on foetal HSCs in both mouse and human (Fleming, 2005; Oberlin *et al.*, 2010). Recently, it has been shown that $CD34^{+}$ $CD45^{+}$ $CD144^{+}$ cells in human foetal liver were able to produce hematopoietic cells but failed to give rise to endothelial cells (Oberlin *et al.* 2010). The EPCs derived by the Hill method also showed the expression of specific hematopoietic markers like CD14 and CD45, and demonstrated the uptake of AcLDL (acetylated low-density lipoprotein), a known function of macrophages and some endothelial cells (Yoder *et al.*, 2007). These human cells did not form secondary colonies on replating and did not form chimeric vessels when transplanted into mice. They were shown to be clonally distinct from the endothelial colony forming cells (ECFCs) as shown by JAK-2 mutational analysis of cell sets (Yoder *et al.* 2007). Several recent studies

have confirmed that CFU-Hill derived cells are of monocytic/hematopoietic origin, and might also contain T cells (Critser and Yoder, 2010; Steinmetz *et al.*, 2010).

Elsheikh *et al*. proposed that a common monocytic-endothelial progenitor might exist. They identified a subset of CD14 and CD309/VEGF-2 expressing human peripheral blood monocytes (2% of CD14 expressing cells) and showed that they could contribute to revascularization in an *in vivo* tissue damage model (Elsheikh *et al.*, 2005). Similarly, CD202b/Tie-2 and VEGF-2 expressing cells in monocytes of the peripheral blood were identified as proangiogenic cells and were recruited to tumours, where they lead to enhancement of vessel growth in these tumours but failed to differentiate in to endothelial cells (De Palma *et al.*, 2007; Venneri *et al.*, 2007; Patel *et al.*, 2013). In summary, bone marrow derived monocytic subsets of cells that express biomarkers found on endothelial cells can promote neovascularisation, but their transcriptomic and proteomic analysis clusters them more with the monocytic cells, and they or their myeloid progenitors can never differentiate towards endothelial lineage (Watt and Fox, 2005; Rohde *et al.*, 2006; Purhonen *et al.*, 2008; Pearson, 2009; Critser and Yoder, 2010; Fantin *et al.*, 2010; Medina *et al.*, 2010b).

1.3 Human endothelial progenitor cells (EPCs)

Endothelial progenitor cells (EPCs) are progenitor cells that are considered to be unipotent cells, in contrast to other stem cells that are usually pluripotent. EPCs have been a matter of debate for a long time, and their role in postnatal vasculogenesis and angiogenesis still remains controversial, simply because of discrepancies in their identification. To this date, there is no unique marker that can identify EPCs, and it has been widely recognized now that the bio-markers that have been used over the years for characterizing EPCs have not been exclusive to the endothelium and have shown expression on other cell lineages (Crister and Yoder 2010).

There has always been some controversy regarding the nomenclature used to define EPCs. Richardson and Yoder have recently suggested a revised terminology for different types of cells based on the expression or absence of certain well known markers that have been used to define endothelial cells (Richardson and Yoder, 2011). A brief description of these cell types along with the revised nomenclature is described below.

1.3.1 Isolation of proangiogenic or early endothelial progenitor cells (EPCs)

Human circulating endothelial progenitor cells called CECs, CACs or CEPs or collectively as EPCs mainly represent the mature non-proliferative endothelial cells that in response to tissue damage are sloughed-off from the blood vessels (CECs) or proangiogenic cells of myeloid or monocytic lineage (CACs, CEPs, EPC). Both these varieties of cells are not considered the true EPCs and are mostly considered to be part of hematopoietic lineage. CECs have been reported to be increased in blood of patients with burns or cancers (Duda *et al.*, 2007; Fox *et al.*, 2008b; Lowndes *et al.*, 2008; Strijbos *et al.*, 2009). Phenotypically they are supposed to be similar to ECFCs, but they are non-proliferative mature viable endothelial cells. They express CD34, CD31, CD105, and CD146 and are negative for CD45 (Mancuso and Bertolini, 2010). Similarly, proangiogenic haemopoietic cells are defined as those that are positive for CD34, CD31, VEGF2, Tie2, CD45 and AcLDL uptake, but negative for CD14 (Hirschi *et al.*, 2008). They represent the cells for which the term EPC has been used before by several groups (Asahara *et al.* 1998; Kalka *et al* 2000). They also include previously described CFU-Hill cells that are positive for CD31, VEGF2 and Tie2, formed myeloid colonies *in vitro* and could be distinguished from ECFCs by lack of expression of BMP2 and BMP4 (Hill *et al.*, 2003; Smadja *et al.*, 2008). Similarly, cells expressing CD34, CD45, CD133 and CD31 have been shown to belong to hematopoietic lineage, and most likely represent the cells previously classified as CACs (Estes et al. 2010).

1.3.2 Isolation of human endothelial colony forming cells (ECFCs)

Endothelial colony forming cells (ECFCs) represent EPCs with differing proliferative potential, and although they are believed to be synonymous with the late outgrowth endothelial cells, their relationship with endothelial stem cells still remains unclear. Yoder *et al.* (2007) were the first group to analyse the proliferative potential of human ECFCs in clonogenic assays *in vitro.* This assay included plating of ECFCs in collagen-coated plates and the number of colonies developed over 14 days were enumerated. The developing cells displayed a typical cobblestone morphology shown in figure 1.2, and showed differing proliferative potential, with high proliferative potential colony-forming cells (HPP-ECFCs) giving rise to low proliferative potential colony-forming cells (LPP-ECFCs) and finally to mature non-dividing endothelial cells. The LPP-ECFCs formed colonies of less than 2000 cells, and failed to form colonies on replating, whereas HPP-ECFCs formed colonies containing greater than 2000 cells and formed at least secondary colonies. Those forming tertiary colonies were proposed to have much higher proliferative potential (Melero-Martin *et*

al., 2007; Melero-Martin *et al.*, 2008; Reinisch *et al.*, 2009; Melero-Martin *et al.*, 2010). The cells that showed high proliferative potential possessed the ability to form vascular tubes in vitro (Zhang *et al.*, 2009a) had high levels of telomerase activity (Yoder et *al.* 2007) and could be incorporated in to the vascular endothelium or in matrigel *in vivo* immunodeficient vasculogenic models (Kung *et al.*, 2008; Yoder and Ingram, 2009). Several phenotypic studies combined with culture assays have indicated that ECFC levels are several fold higher in human umbilical cord blood at birth than in adult peripheral blood from a normal donor, suggesting the former to be a much richer source of ECFCs (Watt *et al.*, 1980; Estes *et al.*, 2010). According to the revised criteria ECFCs are now classified as cells that are proliferative and form day 14 endothelial colonies with more than 50 cells and are positive for CD34, CD31, CD105, CD146 and negative for CD133, CD45, CD14, CD41a and CD235a (Richardson and Yoder 2010).

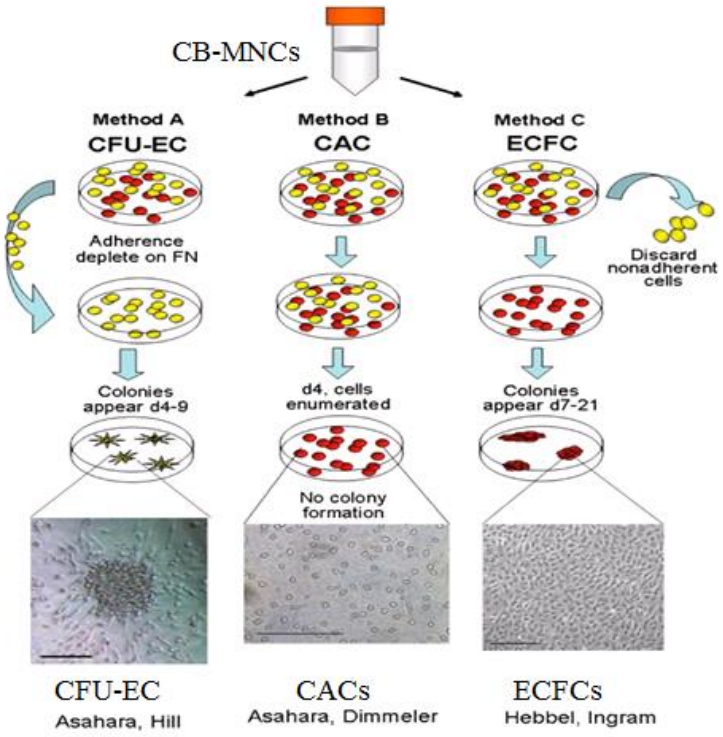


Figure 1.2. Schematic representation of different approaches used to isolate EPCs. Adapted from (Prater *et al.*, 2007).

1.4 Role of EPCs and angiogenic factors in neovascularization in humans

It has been shown that human blood consists of a small proportion of circulating hematopoietic stem cells (HSCs) and much more hematopoietic progenitor cells which are already committed to erythroid lineage (Ho *et al.*, 1998; Cheung *et al.*, 2007). It has been proposed that these circulating hematopoietic progenitors are remnants of stem/progenitor cell pool formed during embryonic development. Another view is that these circulating progenitors have the ability to rapidly enter the tissues or bone marrow in response to haematological stress, thereby allowing their maturation leading to oxygen delivery to tissues when required (Watt *et al.*, 2010). Similarly, human non hematopoietic stem/progenitor cells including endothelial colony forming cells (ECFCs) and mesenchymal stem cells (MSCs) are found in adult peripheral blood in low counts under homeostatic conditions (He *et al.*, 2007; Salem and Thiemermann, 2010; Yoder, 2010). It has also been shown that these ECFCs along with MSCs and HSCs reside in the bone marrow postnatally and are associated with vascular niche and can be released into the blood in response to ischemia or under the influence of angiogenic growth factors (Ergün *et al.*, 2007; Watt and Forde, 2008; Campagnolo *et al.*, 2010; Klein *et al.*, 2010; Lymperi *et al.*, 2010).

1.5 Proangiogenic factors and their role in vascular inflammation

One of the most important angiogenic factors that play a vital role in angiogenesis is vascular endothelial growth factor (VEGF). It has been shown that patients with coronary artery bypass grafting and burn injuries show an increased level of VEGF in plasma for 6-12 hr which is linked with a concomitant mobilization of proangiogenic cells in the blood stream along with circulating late out growth endothelial cells (Watt *et al.*, 2010). It has recently been demonstrated that in normal adult blood, levels of proangiogenic growth factors including CXCL-12 (also known as stromal derived factor-1) and VEGF which are ligands for the chemokine receptors CXCR-7 and CXCR-4 respectively, are proportional to the number of proangiogenic cells (Smythe *et al.*, 2008; Thelen and Thelen, 2008; Watt and Forde, 2008).

The release of VEGF in the plasma of burns patients is associated with an increase in mRNA level of expression of endothelial specific genes mainly *ECSM2* which is also related to the severity of burns in terms of total surface area of the patient (Fox *et al.*, 2008a; Fox *et al.*, 2008b). ECSM2 which is also referred to as endothelial restricted cell surface receptor

(ECSCR) is known to increase the sensitivity of VEGF during vasculogenesis (Verma *et al.*, 2010; Kilari *et al.*, 2013). This suggests that in response to vascular injury and an increase in VEGF and CXCCL-12 circulating levels, both proangiogenic and endothelial progenitor cells are mobilized into the blood. As expected there is also an increase in pro- inflammatory markers as a result of vessel injury that include MCP-1, MIP-1α, IL4, IL6, IL8, IL10, IL13, IL15, IL17 and IFN-γ (Finnerty *et al.*, 2008). The relationships between these factors and ECFCs and proangiogenic levels in the blood have still not been studied in great detail. It has been shown that IL-8 is a cytokine that is found in abundance in ECFCs but not in HUVEC and could act in a paracrine manner to promote angiogenic activity of these cells as shown by enhanced capillary tube formation (He *et al.*, 2005; Nagano *et al.*, 2007).

1.6 Angiogenic growth factors and related cardiovascular disorders

There has been considerable amount of work done on studying the role of mobilizing factors and their role in cardiovascular disorders, but till now it has been a challenge to distinguish between the ECFCs, circulating mature endothelial cells and proangiogenic cells. The term EPCs has been used widely to describe these mobilizing cells collectively. First evidence for a relationship between cardiovascular diseases and EPCs was demonstrated by Hill *et al*. They showed that patients who are at risk of developing cardiovascular diseases have a low EPC (CFU-Hill) count in their circulation and the number of these cells was inversely proportional to the Framingham cardiovascular risk score (Hill *et al.*, 2003). There have been differences in the number of cells in patients with coronary artery diseases and conflicting data has been demonstrated (Leone *et al.*, 2009). As earlier documented with severe burn injuries, acute myocardial infarction (AMI) can also lead to mobilization of these EPCs (Proangiogenic cells) into the circulation with a concomitant increase in circulating angiogenic factors. Enhanced VEGF levels have been shown in patients with post MI (Shintani *et al.*, 2001). This is in correlation with an increase in circulating levels of cells that can be identified as either circulating angiogenic cells (CACs), circulating endothelial cells (CECs) or endothelial colony forming cells (ECFCs). However, there was no significant change in expression levels of IL-3, IL-6, GM-CSF, G-CSF and b FGF in these patients post-MI, measured for a period of over 4 weeks (Shintani *et al.*, 2001). Other studies have shown a rapid increase in circulating levels of proangiogenic cells post-MI that reached a peak at around 7 days and the levels then gradually declined over a period of two months (Massa *et*

al., 2005). This increase in level of progenitor cells was linked with an increase in VEGF levels but no significant change was seen in the plasma levels of CXCL-12 and SCF. Some studies have reported an increase in VEGF, CXCL-12 and G-CSF in patients after MI (Leone *et al.*, 2009). It has been suggested that VEGF shows two peaks during MI, the first in acute phase which is within 48hrs and the other is in the sub-acute phase which is within 7 days, although this requires further investigation (Pannitteri *et al.*, 2006). Similarly, in patients with heart failure, cells with proangiogenic capacity and forming CFU-Hill were found to be increased in number along with an increase in VEGF and CXCL-12 at early stages of heart failure (NYHA I and II) and reduced in number during later stages of heart failure, (NYHA III and IV) (Valgimigli *et al.*, 2004). It has also been shown that endothelial colony forming cells (ECFCs) have increased levels in peripheral blood following myocardial infarction (MI) and they are correlated with the number of $CD34^{+}$ $CD45^{-}$ and $CD34^{+}$ $VEGFR\text{-}2^{+}$ cells, but are not related to $CD133^{+}$ $CD34^{+}$ cell population in the blood (Massa *et al.*, 2009). Finally, apart from above mentioned factors angiopoeitins, PDGF, IGF-1 and some cell surface receptors such as CD146, N-cadherin, Notch, VEGFR-2 and EphB4 are involved in endothelial cell proliferation and control the mobilization of proangiogenic cells in to the circulation (Al Haj Zen and Madeddu, 2009; Leone *et al.*, 2009; Azam *et al.*, 2010; Harhouri *et al.*, 2010; Kebir *et al.*, 2010; Padfield *et al.*, 2010; Saharinen *et al.*, 2010; Sheldon *et al.*, 2010). This further confirms that following an acute cardiovascular insult both circulating angiogenic cells (CACs) and ECFCs are mobilized in to the peripheral blood and play their respective roles.

1.7 Diagnostic and therapeutic potential of EPCs

Endothelial damage following vascular injury or tissue damage requires wound healing, which is divided in to four phases. It starts with haemostasis which involves deposition of extracellular matrix followed by inflammation and proliferation in which blood vessel formation takes place (Nguyen *et al.*, 2009). The remodelling phase is also known as cellular phase which involves proangiogenic cells including fibroblasts and monocyte/macrophages and smooth muscle cells. Many studies have recently focused on demonstrating the relationship between circulating angiogenic cells, angiogenic growth factors and progression of diseases and clinical outcomes. It has been suggested by Estes et al, that a ratio between circulating proangiogenic cells and non-angiogenic cells of less than 1 is indicative of vascular disorders in humans (Estes *et al.*, 2010). On the other hand, ECFCs are present in

human blood in low numbers and are not as easily identified as their proangiogenic and non-angiogenic counterparts which are readily detected. Very few studies have been reported that have analysed these recently identified subsets in the blood of patients suffering from vascular injury and therefore their importance as diagnostic tools to predict vascular disease requires more patient groups. Studies from various groups have shown that the levels of circulating endothelial cells or circulating endothelial progenitors change in several cancers and in response to chemotherapy (Strijbos *et al.*, 2008). Proangiogenic hematopoietic cells and CACs levels in peripheral blood can be used as a diagnostic tool as certain advanced cancers show increased levels of these cells which are reduced to normal with cancer therapy. Similarly, higher CAC levels in blood of breast cancer patients which were undergoing chemotherapy was a positive predictor of increased survival rate, and these CAC levels have been used as markers for anti-angiogenic drug therapy and for detecting vascular toxicity following long term chemotherapy (Bertolini, 2009; Watt *et al.*, 2010).

1.8 Cell therapies for cardiovascular repair

Several studies in animal models have shown that blood or bone marrow derived endothelial progenitor cells can be used for vascular repair. This was instrumental in rapid translation of this research into clinical scenario and clinical research settings (Watt *et al.*, 2010). These included use of mononuclear cells or $CD34^{+}/CD133^{+}$ selected cells from peripheral blood or bone marrow. Several groups have carried out randomized clinical cellular therapy trials for treating acute myocardial infarction and ischemia performing intracoronary or intramyocardial infusion of mononuclear cells (Brunskill *et al.*, 2009; Martin-Rendon *et al.*, 2009). In a more recent study it was shown that late outgrowth endothelial cells were much more effective than mesenchymal stem cells (MSCs) in cardiac revascularization in a swine model of AMI (Stevens *et al.*, 2009; Dubois *et al.*, 2010). Other strategies that have been employed include application of induced pluripotent stem (iPS) and ES-derived cells in scaffolds, or use of drugs to assess the effects on vascular repair and cardiac revascularization (Jujo *et al.*, 2010; Kong *et al.*, 2010a; Maltais *et al.*, 2010; Qian and Srivastava, 2010; Shrivastava *et al.*, 2010; Evans *et al.*, 2013).

1.8.1 Clinical trials for cardiovascular disorders using EPCs

Several randomized clinical trials targeting cellular therapy for acute myocardial infarction and ischemic heart diseases have been carried out recently. These trials were limited to intra-coronary or intra- myocardial infusion of MNCs derived from peripheral blood or bone marrow. This resulted in an improvement in left ventricular ejection fraction (LVEF) particularly in those cases where $\geq 10^8$ cells were infused into the heart. This requires further follow up in patients to study the persistence of this positive effect over a longer period of time (Martin-Rendon *et al.*, 2008a; Martin-Rendon *et al.*, 2008b; Brunskill *et al.*, 2009; Assmus *et al.*, 2010; Chavakis *et al.*, 2010). In another study, intracoronary cellular therapy after percutaneous coronary intervention for acute myocardial infarction showed signs of improvement in patients (Lipinski *et al.*, 2007). In cases of non-randomized clinical trials some adverse effects of the cellular therapy have been observed. High number of cases with in-stent restenosis or denovo stenosis following intracoronary infusion of granulocyte colony stimulating factor (G-CSF) mobilized MNCs or $CD133^+$ progenitor cell population derived from bone marrow suggested an increased risk of progression of atherosclerosis (Bartunek *et al.*, 2005; Mansour *et al.*, 2006). Therefore, further analysis of randomized clinical trials is required to assess the efficiency of these therapies. One of the consequences of atherosclerosis which affects lower extremities and often referred to as peripheral artery occlusive disease (PAD) has been attempted to treat with these cellular therapies using mobilized PBMNCs and bone marrow derived cells and applying them directly to the injured site (Martin-Rendon *et al.*, 2009).

1.8.2 Limitations of cell therapies used for cardiovascular repair

These studies have been carried out in non-randomized clinical trials and long term pain relief along with some adverse effects being reported. Randomized clinical trials, along with well-defined and sorted cell populations are needed to confirm the safety and efficacy of these therapies for PAD. All these cell therapies that have been attempted so far have some limitations. The trials that have been conducted in animal models of MI indicate that some of the cell populations derived from bone marrow such as mesenchymal stem cells do not remain in the heart and the improvement that occurs is due to hormonal or paracrine effects that limit inflammation and also prevents scar formation. This effect is also responsible for increased neovascularization and promoting the proliferation of stem/progenitor cells (Stuckey *et al.*, 2006; Carr *et al.*, 2008; Martin-Rendon *et al.*, 2008c; Quevedo *et al.*, 2009;

Sato *et al.*, 2010). It has also been shown that ECFCs contributed more towards neovascularization than mesenchymal stem cells (MSCs) in a swine model of acute myocardial infarction (Dubois *et al.*, 2010).

1.9 Clinical applications of ECFCs

There are three strategies that can be used once ECFCs are successfully isolated from either peripheral or umbilical cord blood.

1.9.1 ECFCs as neovascularizing agents for cell therapy

The first strategy involves using ECFCs as neovascularization agents for cellular therapy, which in a clinical scenario can be implicated for the promotion of wound healing or rescuing of critical ischemia in patients or may be as a source for delivering angiogenic factors. Intracoronary infusion of VEGF and bFGF (basic fibroblast growth factor) has been attempted that led to an improved blood flow but the effects were transient and patients presented with hypotension (Al Haj and Madeddu 2009). Gene therapy techniques have also been tested to deliver angiogenic factors. Adenoviral vector encoding VEGF injected in to ischemic tissue led to an increase in systemic VEGF. Similarly gene transfer of naked DNA encoding VEGF resulted in improved lower extremity blood flow demonstrated by magnetic angiography (Isner and Asahara, 1999; Critser and Yoder, 2010; Dubois *et al.*, 2010; Medina *et al.*, 2010a). Conversely, an anti-angiogenic strategy to suppress excess proliferation of endothelial cells as required in some ocular diseases has been attempted successfully (Anderson *et al.*, 2010; Rajappa *et al.*, 2010). Cells can also be used as vehicles for gene delivery. It has been shown that retrovirally induced myoblasts resulted in VEGF productions which in turn lead to 30 fold increase in the capillary density with in few days (Padfield *et al.*, 2010). It has been suggested that the cells involved in vascular regeneration including proangiogenic and mesenchymal cells can be used for controlled delivery of growth factors (Padfield *et al.*, 2013).

1.9.2 ECFCs as progenitor cells for vascular repair

The second strategy involves using capacity of colony derived cells/ECFCs to differentiate into endothelial cells that can be used as a tool to re-endothelialize damaged blood vessels and maintain an intact endothelium. Prosthetic vascular grafts or stents that are coated with endothelial cells have been proposed as an approach to decrease the incident of neointimal

formation and premature graft failure and drug-eluting stents have shown to be more effective in that regard (Padfield *et al.*, 2010). The implantation with cells of left ventricular assist devices and grafts in human and animal models have been reported recently (Yoder, 2010). As shown earlier, it has been suggested that proangiogenic cells that are mobilized from bone marrow in response to vascular injury also promote the mobilization of ECFCs and their proliferation and re-endothelialization of stents (Watt *et al.*, 2010). This endothelialization can also be enhanced by use of proangiogenic factors that can mobilize these cells from bone marrow and peripheral blood and include statins, G-CSF and peroxisome proliferator activator antagonists. Cellular therapies involving infusion of ECFCs and proangiogenic cells directly to the affected area and stent-based treatments have been tested before and include VEGF gene-eluting stents and CD34 cell capture stents (Padfield *et al.*, 2010; Watt *et al.*, 2010).

1.9.3 ECFCs for tissue engineering applications

Third strategy involves using the ECFCs for tissue engineering applications, and as a source of endothelium in forming a microvasculature. The scarcity of transplantable tissues and organs has led researchers to think in terms of creating an organ or a tissue that can be a suitable replacement for human transplantation. For this to happen, a source of vascular supply is the most important issue along with specific progenitor cells, since *in vivo*, oxygen and nutrient supply is essential for maintaining a three-dimensional tissue construct. In an *in vitro* system various attempts have been made to form a vasculature which mainly involved culturing endothelial cells in collagen gels, which formed tubular structures spontaneously. Similarly, human umbilical vein endothelial cells have been co-cultured with fibroblasts and keratinocytes resulting in a graft containing properly developed capillaries with a basement membrane (Black *et al.*, 1998). Endothelial cells, when cultured together with mesenchymal cells have been shown to play a role in stabilizing the micro vessels by recruiting pericytes to their luminal surface. Human umbilical vein endothelial cells have also been used after transfection with bcl2 in fibrinogen or collagen gels in order to achieve long term survival with the formation of HUVEC lined micro vessels (Zheng *et al.*, 2004). Human umbilical vein endothelial cells have also been cultured together with mouse myoblasts along with embryonic fibroblasts to produce skeletal muscle constructs in an *in vitro* system. This construct was implanted *in vivo* and it was observed that the developed vasculature was successfully maintained (Levenberg *et al.*, 2005). It has been reported that HUVECs along

with 10T1/2 mesenchymal progenitors when seeded in fibronectin type I collagen gel and implanted in to SCID (severe combined immunodeficiency) mice resulted in the formation of blood vessels that became connected to the circulation and survived *in vivo* for more than a year (Koike *et al.*, 2004). The use of human bone marrow derived mesenchymal stem cells (hMSCs) and EPCs have been attempted by Au and colleagues who showed that these cells (MSCs) can be successfully used as vascular progenitors and played an important role in stabilizing newly formed blood vessels by functioning as perivascular progenitors (Au *et al.*, 2008b). The blood vessels formed remained stable *in vivo* for more than four months. More recently, it has been shown that blood derived ECFCs and human dermal fibroblasts can lead to the formation of micro vessels in dermal substitutes *in vitro* (Hendrickx et al., 2010). They further extended this study to produce an *in vivo* model of wound healing (Hendrickx *et al.*, 2010; Watt *et al.*, 2010). ECFCs and hMSCs have been shown to be cultured in animal serum free cultures in an *in vitro* system producing a microvasculature in immunodeficient mice (Reinisch *et al.*, 2009). More recently, it has been shown that human cord blood derived ECFCs when administered together with MSCs resulted in an increase in *in vivo* vasculogenic response and ECFCs were found to be incorporated into neovessels (Schwarz *et al.*, 2012).

1.9.4 Limitations in using ECFCs for vascular repair

As reviewed by kirton and Xu most of the studies conducted so far for vascular repair have been conducted in animal models or have used ECFCs in human clinical trials which have been difficult to obtain in large numbers (Kirton and Xu, 2010). This is important in the context that cellular therapies for cardiovascular repair that have used blood or bone marrow derived mononuclear cells, and $CD34^+$ and $CD133^+$ cells have resulted in very low level of improvement in left ventricular ejection fraction (Martin-Rendon *et al.*, 2009). This is therefore essential to study in detail the source of origin of ECFCs. As till now, this is unclear whether ECFCs that reside within vessel wall and close to the damaged vasculature take part in the repair or the cells that are mobilized from the bone marrow in response to vascular injury and home into sites of damaged endothelium take part in this process.

One of the approaches that have been suggested are to use autologous ECFCs and support proangiogenic cells with or without scaffolds to promote neovascularization, as this will decrease the risk of transplant rejections by overcoming human leukocyte antigen systems.

Another challenge is that the ECFCs occur in low counts postnatally, and it is important to generate sufficient proliferating ECFCs *in vitro*. These ECFCs have been expanded in specialized and defined protein conditions and have maintained their phenotypic characteristics. More recently, an exciting attempt with varying degree of success has been made to generate endothelial cells from induced pluripotent cells (Taura *et al.*, 2009). These cells and factors that are involved in the regulation of revascularization and maintenance of an intact endothelium can be used as a future source of autologous cell therapy or in drug discovery. Grafting of a human donor trachea that was precolonized with chondrogenic and epithelial cells in to a patient suffering from tuberculosis has been shown to produce a functional trachea that was vascularized by patients own cells (Hollander *et al.*, 2009).

1.10 The role of oxidative stress and endothelial progenitor cells

Oxidative stress has been shown to be involved in wide range of pathogenic diseases due to weakened antioxidative mechanisms or overproduction of free radicals that overcome body scavenging mechanisms. To study cardiovascular diseases mainly atherosclerosis several experimental models have been proposed that support the fact that reactive oxygen and nitrogen species play a role in atherosclerosis and other vascular diseases. The main models that have been used include using extracellular sources of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), normobaric hyperoxia (elevated oxygen levels) and oxidized low-density lipoprotein induction. All these methods have been used to induce oxidative stress in cell culture systems. Induction of oxidative stress can be achieved by two different approaches, either by increasing the free radical load or by disrupting anti oxidative defence mechanism.

Hydrogen peroxide can be added directly to the culture medium that results in rapidly decreasing concentration of H_2O_2. It is mainly stable in most culture systems but in the presence of cells its concentration declines quickly depending upon cell density and presence of catalase enzyme. Its main advantage is that it readily penetrates the cellular envelope. Superoxide can also be added directly in the culture medium in the form of potassium superoxide. It also leads to rapidly decreasing concentrations but is converted to hydrogen peroxide by enzymatic reaction which can also produce hydroxyl ion. The effects are similar to hydrogen peroxide but it does not readily penetrate the cellular envelope. Also, catalase needs to be added to the media in order to avoid hydrogen peroxide and subsequent hydroxyl formation. Exposure to hyperoxia is mainly performed by incubating cells for several days

and the main effect is produced by direct interaction of oxygen with the cells instead of oxygen reacting with the media producing substances that can affect the cells. The main difference between extracellular hydrogen peroxide and superoxide in comparison to hyperoxia is that the later requires prolonged exposure. We, therefore chose hyperoxia as we required to grow cells for long term culture and with hydrogen peroxide there is this issue of rapid decrease in concentration.

It has been shown recently that endothelial progenitor cells play an important role in postnatal neovascularization and are involved in re-endothelialization of disrupted endothelium (Asahara *et al.*, 1999; Griese *et al.*, 2003; Zampetaki *et al.*, 2008; Wang *et al.*, 2013). These EPCs have been isolated from several sources that include bone marrow, peripheral blood and cord blood derived mononuclear cells (Murohara *et al.*, 2000; Kawamoto *et al.*, 2001). Animal models of hind limb ischemia and myocardial infarction have shown improved blood flow as a result of endogenous mobilization of EPCs (Takahashi *et al.*, 1999; Kawamoto *et al.*, 2003). Several clinical studies have mentioned that EPC numbers have been affected by various risk factors that also account for coronary artery diseases thus causing a challenge for promotion of neovascularization by these EPCs (Vasa *et al.*, 2001; Taniyama and Griendling, 2003; Werner and Nickenig, 2006). It has been proposed and shown that oxidative stress that leads to an increase in reactive oxygen species (ROS) production and a decrease in antioxidant enzyme expression, can promote vascular senescence and is considered to be one of the main factors involved in coronary artery disease (CAD) along with smoking, aging, diabetes and hypertension (Cai and Harrison, 2000). Oxidative stress has been implicated in a wide range of vascular disorders leading to DNA damage, decreased nitric oxide (NO) availability and change in redox state as a result of increased ROS production (Sugamura and Keaney Jr, 2011; Higgins *et al.*, 2012; Williamson *et al.*, 2012). Oxidative stress has been shown to be involved in the progression of atherosclerosis as ROS has shown to directly result in endothelial dysfunction (Cai and Harrison, 2000; Taniyama and Griendling, 2003; Touyz, 2004). It is suggested that ROS plays a key role in the progression of atherosclerosis, as oxidative stress affects EPC survival and mobilization (Torsney *et al.*, 2005; Yao *et al.*, 2006). ROS has been shown to directly affect the endothelium and increased superoxide accumulation decreases EPC activity and survival (Griendling and FitzGerald, 2003a; Thum *et al.*, 2007). Treatment of EPCs with high levels of hydrogen peroxide has shown induced oxidative stress and progression towards apoptosis and reduced EPC count (Kao *et al.*, 2001; Hung *et al.*, 2003; Urbich *et al.*, 2005). In an animal model of myocardial infarction,

increased ROS production has been associated with reduced EPC level (Thum *et al.*, 2006). Oxidative stress has also led to the mobilization of dysfunctional EPCs which has reduced capacity to migrate and home to sites of vascular injury (Schatteman *et al.*, 2000; Tepper *et al.*, 2002). This oxidative damage can be linked to vascular aging, as this cellular damage due to increased aging can reduce EPC function resulting in an increased risk of vascular pathologies. It has been shown that both early and late EPCs are more resistant to this oxidative stress than mature endothelial cells (HUVEC) as they express higher level of antioxidant enzymes (Dernbach *et al.*, 2004; He *et al.*, 2004a; Cai *et al.*, 2006; Williamson *et al.*, 2012). This suggested that these EPCs can be more efficient cells involved in vascular repair as a result of an ischemic insult, owing to this improved resistance to oxidative stress. However, it has been shown recently that early EPCs or circulating angiogenic cells (CACs) are cells of myeloid origin and express certain monocyte-macrophage lineage markers including CD45, CD14 and CD115 and are proangiogenic but do not take part in vascular repair directly, whereas late EPCs or endothelial colony forming cells (ECFCs) have been shown to be the EPCs involved in vascular repair although they themselves are not shown to be completely resistant to oxidative stress, but are better suited to these conditions as compared to mature endothelial cells (Ingram *et al.*, 2007; Yoder *et al.*, 2007). Hydrogen peroxide induced oxidative stress has shown to impair EPC function although they are known to have better equipped intracellular mechanisms to combat oxidative stress but they are still not resistant to this stress (Dernbach *et al.*, 2004; He *et al.*, 2004a; Ingram *et al.*, 2007). However, this antioxidative capacity of EPCs has been shown to diminish with aging, and early EPCs isolated from young subjects have shown higher levels of antioxidant enzyme glutathione peroxidase (GPx-1) as compared to old ones (He *et al.*, 2009) and were found to be more resistant to stress induced apoptosis. There has been some conflicting data in terms of effect of oxidative stress on EPCs, but it is mainly due to the fact that these EPCs are better classified as early and late EPCs depending upon their appearance in an *in vitro* culture system. These two EPC types are different from each other morphologically and in terms of clonogenic and proliferative capacity (Hur *et al.*, 2004; Deschaseaux *et al.*, 2007). These early and late EPCs have been used by researchers for testing the effect of oxidative stress by exposing these cells to hydrogen peroxide after culturing in an *in vitro* system. It has been reported that early EPCs have shown to express both endothelial and monocyte/macrophage cell surface antigens and were found to be tolerant to oxidative stress, whereas late EPCs or ECFCs have shown to be sensitive to oxidative stimuli, do not express monocyte/macrophage markers and show much higher proliferative potential (Dernbach *et al.*, 2004; Ingram *et al.*,

2004; Ingram *et al.*, 2007). In a more recent study, late EPCs have been used to test the effect of hydrogen peroxide induced oxidative stress and it has been shown that it leads to a decrease in cell survival and causes increased apoptosis which was dependent on hydrogen peroxide dosage (Wang *et al.*, 2013).

Aging also leads to an up regulation of Angiotensin II which is known to promote atherosclerosis by enhancing ROS production and associated cell death (Baylis *et al.*, 1997; Wang *et al.*, 2003; Imanishi *et al.*, 2005). Similarly, it has been shown recently that EPC number was increased in a coronary artery disease patient when treated with an angiotensin II receptor antagonist (Endtmann *et al.*, 2011). It has been shown that forkhead hemeobox type O (FoxO) family of transcription factors 1, 3a and 4 are involved in apoptosis and cell survival (Puig and Mattila, 2011). The activity of foxOs is mainly dependent on their nuclear localization and are phosphorylated by Akt kinase in the presence of serum and growth factors leading to their nuclear removal and deactivation (Accili and Arden, 2004; Zhang *et al.*, 2011). However, in the presence of oxidative stress localization of the foxOs to the nucleus occurs resulting in activation of foxO target genes that include a pro-apoptotic gene Bim that promotes apoptosis (Dijkers *et al.*, 2000; Storz, 2011). More recently, role of foxO3a has been studied and it has been shown that oxidative stress reduces tube forming ability of EPCs and this occurs through a foxO3 dependent mechanism (Wang *et al.*, 2013). It has been shown that in mammals all three above mentioned foxO proteins are present in EPCs performing different functions (Potente *et al.*, 2005; Marchetti *et al.*, 2006; Spinetti *et al.*, 2013). There have been studies that reported insights into mechanisms involving oxidative stress induced EPC dysfunction and revealed that foxOs transcription factors mainly work by inhibition of cell growth eventually leading to apoptosis (Dejana *et al.*, 2007).

1.11 Redox control mechanisms and angiogenesis

Redox control and signalling remains a topical area in vascular research. Endothelial cells mediate ROS production in the form of superoxide and hydrogen peroxide which act as messengers leading to a growth factor dependent response, promoting angiogenesis (Case *et al.*, 2008). NADPH oxidase is considered to be the most important source of ROS in vasculature (Griendling *et al.*, 2000; Lassègue *et al.*, 2012). NADPH oxidase (NOX) derived ROS has been shown to be implicated in several cardiovascular diseases and increased ROS

levels have been found in both human and animal models (Lacy *et al.*, 2000; Touyz and Schiffrin, 2001; Álvarez *et al.*, 2008; Brandes *et al.*, 2010; Lassègue and Griendling, 2010). Interestingly, it has been shown that two important angiogenic factors vascular endothelial growth factor (VEGF) and angiopoeitin-1(ANG1) promote endothelial cell migration via activation of NOX2 expressing NADPH oxidase (Ushio-Fukai *et al.*, 2002; Harfouche *et al.*, 2005). It has been shown that increased expression of *Nox2* is linked with enhanced production of ROS in murine angiogenic models of hind limb ischemia and retinopathy (Al-Shabrawey *et al.*, 2005; Tojo *et al.*, 2005). Similarly, *Nox2* null mice have shown inhibition of angiogenesis in response to ischemia and VEGF signalling.

ASK1 protein is another important regulator of angiogenesis that is controlled by redox signalling. This protein belongs to the family of mitogen-activated protein kinase (MAP kinases) and is involved in activation of P38 kinase pathway. ASK1 is known to play a role in regulating oxidative stress induced apoptosis and cellular senescence (Tobiume *et al.*, 2001). ASK1 is also involved in cytokinesis and proliferation and a hyperglycaemia induced ASK1 activation leads to cell senescence in endothelial cells (Izumi *et al.*, 2003; Matsuzawa *et al.*, 2005; Yokoi *et al.*, 2006). ASK1 is also involved in activation of plasminogen activator inhibitor-1 (PAI-1) expression in endothelial cells. PAI-1 is an important protein involved in fibrinolysis and aging-related thrombosis (Juhan-Vague *et al.*, 1989). ASK1 deficient mice has also been shown to be involved in endothelial dysfunction caused by nitric oxide (NO) deficiency (Yamashita *et al.*, 2007). ASK1 is therefore, proposed to be a therapeutic target in patients to combat vascular aging (Case *et al.*, 2008).

Glutathione peroxidase 1 (GPx-1) is another important protein involved in redox signalling that plays a role in vascular homeostasis. GPx-1 has been shown to play a protective role in preventing oxidative damage to the endothelium by reducing both lipid peroxides and hydrogen peroxide (Raes *et al.*, 1987; Maiorino *et al.*, 1995). Vascular injury has been demonstrated to be linked with a reduction in GPx-1 activity and atherosclerotic plaques isolated from carotid arteries have shown decreased expression of GPx-1 (Lapenna *et al.*, 1998). More importantly, over expression lead to restoration of endothelial function in endothelial cells treated with high levels of homocysteine (Weiss *et al.*, 2001). Several mice studies have demonstrated that loss of GPx-1 is implicated in endothelial dysfunction and several other vascular abnormalities and renders them vulnerable to ischemic insult (Forgione *et al.*, 2002a; Forgione *et al.*, 2002b).

1.12 Tumour angiogenesis and its role in carcinogenesis

Tumour progression requires orchestration of a micro vascular network and in order to achieve that goal tumour cells secrete wide range of cytokines and angiogenic growth factors thus promoting angiogenesis and in turn tumorigenesis (Folkman, 1971; Hanahan and Folkman, 1996; Nör and Polverini, 1999; Naumov *et al.*, 2006). There are several signalling pathways and growth factors that are involved in coordinating and regulating this complex process, but three main growth factor/receptor systems play a central role that include VEGF/VEGFR2, ANG1/TIE2 and PDGF-B/PDGFR-β (Cébe-Suarez *et al.*, 2006; Naumov *et al.*, 2006; Augustin *et al.*, 2009; Gaengel *et al.*, 2009). Role of VEGF as an angiogenic growth factor is well documented as it is expressed by most tumour cell types and has shown to promote endothelial cell migration and proliferation (Dvorak, 2002; Ferrara, 2005). VEGF has also shown involvement in EC survival by up regulation of Bcl-2 expression mediated through its receptor VEGFR-2 (Gerber *et al.*, 1998). VEGF along with angiopoeitins (1 and 2) seem to play a coordinated and complementary role in the development of blood vessels. The ANG/TIE2 pathway is considered to be the gateway of angiogenesis, as the endothelial cell phenotype is determined by regulation of the balance between ANG1 and ANG2 expression (Tait and Jones, 2004). When ANG1 levels are increased with respect to ANG2 endothelial cells shift to quiescent state and maturation and stabilization of blood vessels take place along with promotion of interaction between endothelial cells and supportive cells mainly pericytes (Zheng *et al.*, 2013). On the other hand, overexpression of ANG2 relative to ANG1 leads to active migration and proliferation of ECs in the presence of VEGF. Pericytes are mural cells that play a supportive role in angiogenesis by extending cytoplasmic projections over ECs surface so that they become interconnected. This interaction between pericytes and endothelial cells is essential for the maturation and remodelling of the vascular system. PDGF-B is involved in proliferation of pericytes and also acts as a chemotactic factor that promotes the migration of pericytes to the site of blood vessel formation (Gaengel *et al.*, 2009).

1.13 Hallmarks of aging

Aging is normally defined as a gradual and time-dependent decline in body functions associated with cellular damage that affects every living organism (Kirkwood, 2005; Vijg and Campisi, 2008; Gems and Partridge, 2013). In addition to cancer, there are several clinically important pathologies including vascular inflammation and atherosclerosis which manifest themselves as age-related diseases and are characterized by uncontrolled cell growth and hyperactivity (Blagosklonny, 2008). More recently, effort has been attempted at characterizing the molecular and cellular hallmarks of aging and nine candidate processes have been categorized as contributors towards aging. These include as shown in figure 1.3, genomic instability, telomere dysfunction, epigenetic modifications, stem cell exhaustion, altered intercellular communications, cellular senescence, mitochondrial dysfunction, abnormal nutrient regulation and loss of proteostasis (López-Otín *et al.*, 2013). The processes which have been relevant to our area of research are discussed in detail below.

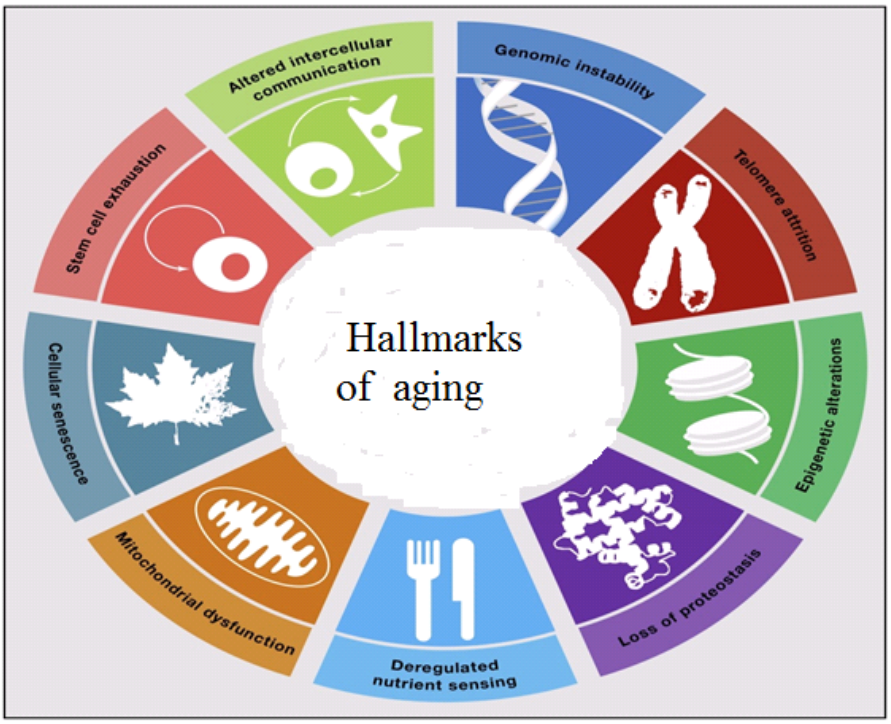


Figure 1.3. Schematic representation of hallmarks of aging. Adapted from (López-Otín *et al.*, 2013).

1.13.1 Telomere attrition and dysfunction

There is strong evidence that aging encompasses genomic damage and factors that promote this damage can lead to accelerated aging. It has been shown recently that enhancement of chromosomal segregation can lead to extended longevity in mammals (Baker *et al.*, 2013). In cases of progerias which are linked nuclear defects there is enough evidence to show that treatments can prevent premature aging (Fong *et al.*, 2006; Varela *et al.*, 2008; Gregg *et al.*, 2012; Kane *et al.*, 2013). This suggests that exploring ways to stabilize nuclear and mitochondrial genome by having a better control on aspects that govern them such as DNA repair mechanisms can have a positive effect on normal aging (López-Otín *et al.*, 2013). DNA damage that accumulates with normal aging seems to affect the genome randomly, but there are specific region known as telomeres that are more susceptible to these age-related changes (Blackburn *et al.*, 2006).

DNA polymerases do not have the capacity to completely replicate the terminal ends of the linear DNA molecule, and this function is the characteristic of a specialized DNA polymerase known as telomerase. The telomerase is however, not expressed by most mammalian somatic cells causing gradual loss of telomere-protective sequences from chromosome ends. This explains the phenomena of replicative senescence, which allows limited proliferative potential of cells grown in an *in vitro* culture system, also termed as Hayflick limit (Hayflick and Moorhead, 1961; Olovnikov, 1996). Overexpression of telomerase has been shown to confer immortality to cells without even leading to oncogenic transformation (Bodnar *et al.*, 1998). Telomere shortening is therefore considered as a part of normal aging process in both mice and humans (Blasco, 2007). Telomeres are bound by a multiprotein complex known as shelterin which prevents the access of DNA repair proteins to them and blocks the repair of telomeres when DNA stands break, resulting in fusion of chromosomes (Palm and De Lange, 2008). As a result of this restricted DNA repair, when telomeres are succumbed to DNA damage, this usually is persistent and leads to senescence and apoptosis (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). Human telomerase deficiency is associated with premature disease progression, mainly in pulmonary fibrosis, dyskeratosis congenita and also aplastic anaemia all of which are related to reduced regenerative capacity of the tissues (Armanios and Blackburn, 2012).

1.13.2 Mitochondrial dysfunction and reactive oxygen species (ROS)

The relationship between mitochondrial dysfunction and aging has been discussed and studied for a long time but still most aspects are not well understood. It has been proposed that as cells undergo aging, the respiratory chain loses its efficiency and leads to electron leakage causing reduced ATP production (Green *et al.*, 2011). These findings stem from the mitochondrial free radical theory of aging, first proposed by Harman et al.1965. In the recent past, there has been a lot of development in re-evaluating this free radical theory and considerable amount of work has been done to investigate the links between ROS and mitochondrial damage (Hekimi *et al.*, 2011). Several studies have come up in last few years that have reported interesting but conflicting data in terms of the effect of ROS production and aging in different species. This includes the observation that increased ROS production plays a role in prolonging the life span of yeast and *C. elegans* (Doonan *et al.*, 2008; Van Raamsdonk and Hekimi, 2009; Mesquita *et al.*, 2010). In mice however, genetic alterations causing an increase in ROS production and subsequent mitochondrial damage did not prevent aging (Van Remmen *et al.*, 2004; Zhang *et al.*, 2009b). Similarly, an increase in antioxidative defence mechanisms did not cause an extension of life span in mice (Pérez *et al.*, 2009) and interestingly, genetic alterations that damaged mitochondrial activity but did not increase ROS levels lead to accelerated aging (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005; Vermulst *et al.*, 2008; Edgar *et al.*, 2009; Hiona *et al.*, 2010). These studies have resulted in researchers moving towards re-evaluating the role of ROS in aging and several studies have recently reported that intracellular signalling mechanisms have shown enough evidence that ROS activation occurs in response to physiological stress and result in cell proliferation and survival (Ristow and Schmeisser, 2011; Sena and Chandel, 2012). Hekimi *et al*, 2011 have recently proposed a conceptual framework to redefine the role of ROS in aging. According to this hypothesis, as the chronological age increases the cellular stress and damage increases as well and ROS functions to balance this damage in order to maintain the survival. This is referred to as a compensatory homeostatic response, but after reaching a certain limit this counteracting mechanism becomes weak and instead of preventing this damage it causes aggravation of this age-associated destruction (Hekimi *et al.*, 2011).

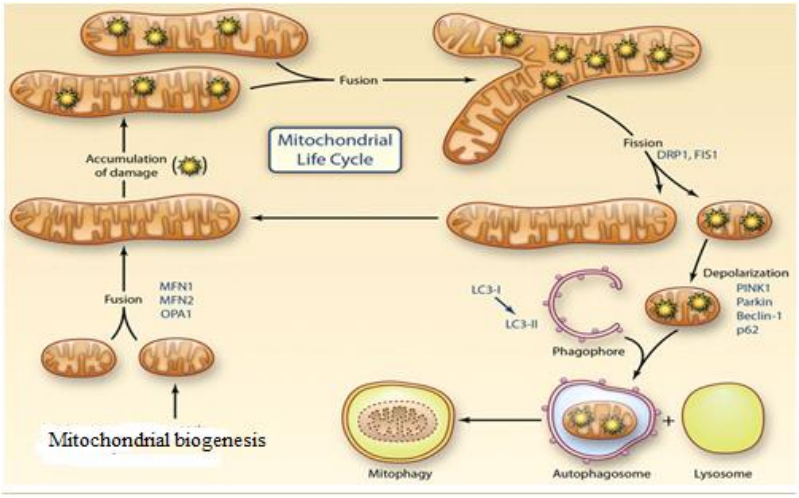


Figure 1.4. Mitochondrial life cycle involving biogenesis and mitophagy. Adapted from (Kluge *et al.*, 2013).

It has been shown recently that in DNA polymerase γ deficient mice, mitochondrial dysfunction can accelerate the aging process on its own and independently of any damage caused by ROS production (Edgar *et al.*, 2009; Hiona *et al.*, 2010). Several mechanisms have been proposed for this to occur which include disrupted apoptotic signalling due to mitochondrial dysfunction resulting in permeabilization of mitochondria in response to physiological stress (Kroemer *et al.*, 2007) and promoting inflammatory response by triggering ROS or permeabilization mediated activation of inflammatory cytokines (Green *et al.*, 2011). Another implication of mitochondrial dysfunction is its impact on cell signalling pathways by affecting the links between endoplasmic reticulum and outer mitochondrial membrane (Raffaello and Rizzuto, 2011). Telomere dysfunction in telomerase deficient mice has been shown to be a consequence of p53-mediated reduction in the expression of PGC-1α and β resulting in repression of mitochondrial biogenesis (Sahin and DePinho, 2012). This mitochondrial compromise also occurs in physiological aging but can be partially reversed by activation of telomerase, as shown recently in wild type mice (Bernardes de Jesus *et al.*, 2012). The NAD^+-dependent histone deacetylase SIRT1, which is the human ortholog of Sir2 (Silent information regulator), has a potential role in longevity and metabolic functions in mammals and interacts with PGC-1α and deacetylates it at 13 lysines in different domains of the protein (Rodgers *et al.*, 2005). SIRT1 has also been shown to play a role in modulation of mitochondrial biogenesis through regulation of this transcriptional coactivator, PGC-1α and is also involved in regulating autophagy that removes degraded mitochondria (Rodgers *et al.*, 2005; Lee *et al.*, 2010). Similarly, SIRT3 which is a recently identified downstream target of

PGC-1 alpha (Kong *et al.*, 2010b) is a mitochondrial deacetylase (In *et al.*, 2008) that targets several important enzymes which play a key role in energy metabolism (Lombard *et al.*, 2007). SIRT3 has been shown to be involved in regulating enzymes that form part of the respiratory chain, β oxidation of fatty acids, TCA cycle and ketogenesis (Giralt and Villarroya, 2012). More importantly, SIRT3 is involved in controlling the rate of ROS production by deacetylating an important antioxidative enzyme MnSOD in response to stress (Qiu *et al.*, 2010; Tao *et al.*, 2010). This suggests that telomeres along with sirtuins are involved in regulation of age associated diseases by controlling mitochondrial function. Mitochondrial biogenesis and metabolism can also be altered by various other mechanisms that include mtDNA mutations, mitochondrial protein oxidation, derangement of respiratory chain complexes and alterations in mitochondrial membrane lipid composition and defective functioning due to improperly managed fission and fusion events (Wang and Klionsky, 2011). Defective mitophagy also leads to impaired mitochondrial biogenetics resulting in low mitochondrial turn over contributing towards aging, although it has recently been shown that alternate day fasting can improve health by reducing mitochondrial degradation (Castello *et al.*, 2011; Safdar *et al.*, 2011). It is interesting to note that this increase in health span occurs in part due to activation of autophagy which is triggered by fasting and endurance training (Rubinsztein *et al.*, 2011). Activation of autophagy however, is not the only pathway that can improve health span and counteract aging and additional mechanisms promoting longevity play a role (Kenyon, 2010). More recently a concept has evolved that has gained wide interest explaining mitochondrial dysfunction during aging process termed as mitohormesis. This suggests that toxic treatments trigger a beneficial compensatory response and this overcomes the existing mitochondrial damage and over repairs the organelle leading to improvement in overall cellular fitness much more than that of the predamaged condition (Calabrese *et al.*, 2011). This further explains the earlier observation that mild respiratory deficiencies can help in improving the lifespan of an individual if they do not lead to severe mitochondrial damage that can be pathogenic (Haigis and Yankner, 2010). As shown in *C. elegans* these hormetic responses can ensue this defence mechanism in tissues that have defective mitochondria as well as in those distant tissues which do not have this mitochondrial dysfunction (Durieux *et al.*, 2011). There is strong evidence that compounds such as resveratrol and metformin act as triggers leading to mild mitochondrial damage causing low energy state by activation of AMPK and increasing adenosine monophosphate(AMP) levels (Hawley *et al.*, 2010). Metformin has shown to increase the lifespan in *C. elegans* by activation of AMPK mediated compensatory stress response that

proved to be beneficial and also involved antioxidant regulator NRF2, which is considered to be an important player in regulating and combating oxidative stress (Onken and Driscoll, 2010). More recently, it has been shown that metformin promotes longevity in worms by inhibiting methionine and folate metabolism of their intestinal flora (Cabreiro *et al.*, 2013). Regarding the effects and impact of metformin usage in mammals not a lot has been shown in terms of life span improvement, although early administration of metformin has been reported to extend the life span in mice (Anisimov *et al.*, 2011). Resveratrol and an important sirtuin activator SRT1720 have been shown to activate PGC-1 alpha and improve mitochondrial function and prevent the metabolic damage (Baur *et al.*, 2006; Lagouge *et al.*, 2006; Feige *et al.*, 2008; Minor *et al.*, 2011). Resveratrol unlike metformin has shown to have no effect in promoting longevity in mice under normal dietary conditions (Pearson *et al.*, 2008; Strong *et al.*, 2013). The role of PGC-1 alpha in improving mitochondrial activity has further been confirmed, as PGC-1 alpha overexpression leads to an extension of life span in *Drosophila* (Rera *et al.*, 2011). Increase in lifespan of flies and mice has also been achieved recently by chemical administration of uncoupler 2-4- dinitrophenol and by genetically engineered overexpression of potent uncoupler protein UCP1 (Gates *et al.*, 2007; Caldeira Da Silva *et al.*, 2008; Fridell *et al.*, 2009; Mookerjee *et al.*, 2010). Finally, this has now been confirmed by several studies that there exists an important relationship between mitochondrial activity and aging and mitochondrial damage can lead to acceleration in the progression of aging. This still remains to be seen if by improving mitochondrial activity, an extension in the lifespan of mammals can be achieved successfully and already several evidence exists that provide a link to that possibility (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005; Vermulst *et al.*, 2008; Ristow and Schmeisser, 2011; Ivanova and Yankova, 2013).

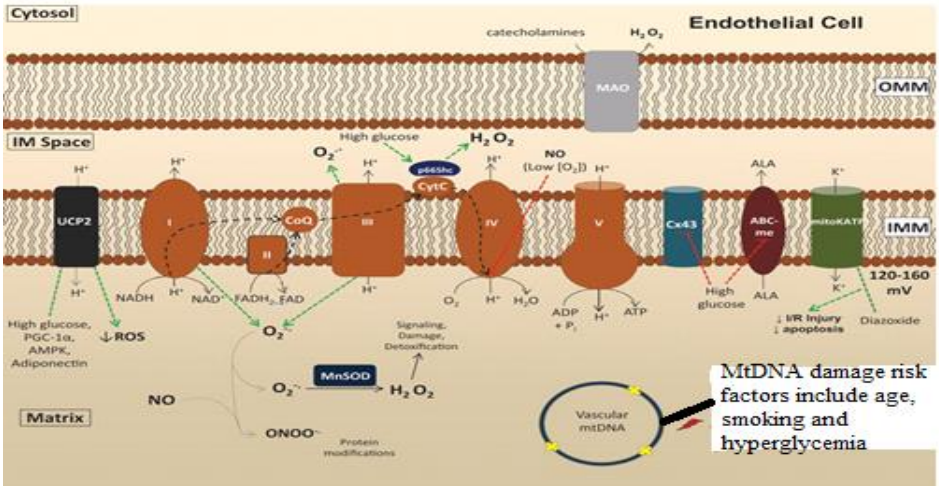


Figure 1.5. Illustration of mitochondrial contents in endothelial cells. Electron transport chain complexes are shown in relation to reactive oxygen species generation. Adapted from (Kluge *et al.*, 2013).

1.13.3 Cellular senescence and age progression

Cell senescence is simply referred to as the cell cycle arrest which is linked to well defined phenotypic changes (Campisi and D'Adda Di Fagagna, 2007; Collado *et al.*, 2007; Kuilman *et al.*, 2010; López-Otín *et al.*, 2013). This term was first coined by Hayflick who cultured human fibroblasts *in vitro* for several passages (Hayflick and Moorhead, 1961). It has now been shown that this senescence occurs as result of telomere shortening (Bodnar *et al.*, 1998). More recently, it has been suggested that apart from telomere shortening there are other factors that promote cell senescence that include non telomeric DNA damage and derepression of *INK4/ARF* loci both of which are key players involved in chronological aging and induce aging (Collado *et al.*, 2007). DNA damage is a good indicator of accumulation of senescent cells in tissues undergoing aging process and several studies have shown that these cells can be identified using senescence-associated β-galactosidase (SABG) staining that has been confirmed in young and old mice liver (Dimri *et al.*, 1995; Wang *et al.*, 2009). Several other tissues showed similar result patterns with staining for senescent cells including lung, spleen and skin and involved detailed quantification of DNA damage and β-gal staining showing high percentage of senescent cells in old mice as compared to young ones (Wang *et al.*, 2009). It was interesting to note that no change was observed in heart, muscle and kidney tissues suggesting that cell senescence is not a feature of all aged

tissues and as shown in tumour senescent cells, an extremely efficient immune system regulates removal of senescent cells regularly by phagocytosis (Wang *et al.*, 2009; Hoenicke and Zender, 2012). It has been proposed for a long time that as the number of senescent cells keeps on increasing with advancing age there has to be some relationship between aging and cell senescence. This could be due to a decrease in rate of elimination of senescent cells as a result of attenuated immune system, a consequence of aging or due to an increase in generation of senescent cells because of aging (Xue *et al.*, 2007; Kang *et al.*, 2011). More recently it has been suggested that as senescence mainly serves the purpose of preventing the propagation and promoting the clearance of damaged cells by maintaining a strict surveillance of immune system, an efficient cell replacement system is required that eliminates senescent cells and at the same time mobilizes progenitor cells to compensate for the number of lost senescent cells and re-establishing the cell count (Kuilman *et al.*, 2010; Rodier and Campisi, 2011). It has recently been shown that senescent cells acquire a specific type of secretome which is a mixture of proinflammatory cytokines and metalloproteinase referred to as senescence-associated secretory phenotype and promotes aging process (Kuilman *et al.*, 2010; Rodier and Campisi, 2011). Apart from DNA damage there are several other important factors that can lead to senescence, and mitogenic signalling is one such factor that plays a vital role in progression of senescence (Gorgoulis and Halazonetis, 2010). A recent study has mentioned that more than fifty oncogenes and mitogens can induce cell senescence as a result of the compensatory response to this form of stress and mainly involve on of these two pathways namely p16INK4a/Rb and p19ARF/p53 (Serrano *et al.*, 1997). These two important pathways that are involved in aging are related to the chronological age of tissues as shown in humans and in mice (Krishnamurthy *et al.*, 2004). Levels of $p16^{INK4a}$ and $p19^{ARF}$ have been used to assess the age of tissues in both mice and humans (Ressler *et al.*, 2006). To date there has not been any other gene or protein identified that is so closely associated with chronological aging as these two genes. This relationship has been so robust that it has been tested across species and tissues and proves to be the most validated means to distinguish between young and old cells (Jeck *et al.*, 2012). Both these genes are encoded by the same locus which is INK4a/ARF locus and a recent analysis has revealed that genome wide association study of more than 300 has identified this locus to be genetically linked and involved in several important age-associated pathologies namely Alzheimer's disease, diabetes mellitus and most of the cardiovascular diseases (Jeck *et al.*, 2012). This confirms the status of this gene as the most well documented gene locus that is involved in regulation of human aging and associated age-related human pathologies. The role of $p16^{INK4a}$ and p53

in promoting senescence has been studied in detail and it has been proposed that senescence induced due to these two can lead to physiological aging. In several mouse models, mutant mice that show signs of premature aging due to persistent cell damage undergo further deterioration when either $p16^{INK4a}$ or p53 are eliminated (Varela *et al.*, 2005). Similar results have been obtained with mice having chromosomal aberration due to BubR1 mutation (Baker *et al.*, 2011). It has been shown that overexpression of either $p16^{INK4a}$, $p19^{ARF}$, or p53 in mice can lead to extended longevity (Matheu *et al.*, 2007; Matheu *et al.*, 2009). Similarly, amelioration of progeroid phenotypes have been observed in mice that are p53 deficient (Calderwood *et al.*, 2009; Ruzankina *et al.*, 2009). To summarize this, the induction of p53 and *INK4a/ARF* occurs as a compensatory response to prevent accumulation of damaged cells and progression of aging and cancer, but this can in certain advance conditions exhaust tissue regenerative capacity and might result in accelerated aging (Matheu *et al.*, 2007; Matheu *et al.*, 2009; Baker *et al.*, 2011).

1.13.4 Energy sensing network and role of AMPK and sirtuins

Growth hormone (GH) produced from anterior pituitary gland is the main somatotrophic axis in mammals and its most important mediator is insulin like growth factor-1(IGF-1) which is secreted in response to different cell types, most specifically by hepatocytes in the liver. As the name suggests IGF-1 acts in the same manner as insulin and uses the same intercellular signalling pathway informing cells of the presence of glucose. Due to this reason, insulin and insulin like growth factor-1signalling is collectively known as "insulin and IGF-1 signalling (IIS) pathway" (Kluge *et al.*, 2013). This IIS pathway is considered to be the most important and evolutionary conserved aging-controlling pathway and mainly targets two of the aging associated complexes including FOXO family of transcription factors and mTOR complexes (Fontana *et al.*, 2010; Kenyon, 2010; Barzilai *et al.*, 2012). It has been interesting to note that genetic mutations or polymorphisms that affect growth hormone (GH) or its mediator IGF-1 and any of its downstream targets including Akt, FOXO or mTOR results in longevity, and again emphasizing the impact of energy pathways on extension of life span (Colman *et al.*, 2009; Fontana *et al.*, 2010; Mattison *et al.*, 2012). As deranged nutrient signalling is considered to be a sign of aging, dietary restriction has therefore shown to have a positive impact on health and life span of all eukaryotes from yeast to humans (Fontana *et al.*, 2010).

Genetic alterations that affect the IIS signalling pathway at different levels has been consistently shown to improve longevity in worms, flies and mice and the most important effector system has been FOXO signalling (Kenyon *et al.*, 1993; Slack *et al.*, 2011). In mice, FOXO1 is responsible for carrying out the tumour suppressive effect of dietary restriction, but if this effect leads to the extension of life span still remains to be seen (Yamaza *et al.*, 2010). It has been shown recently that mice that are treated with tumour suppressor *PTEN* have undergone down regulation of IIS pathway and show an increase in oxidative metabolism with enhanced activity of brown adipose tissue (Garcia-Cao *et al.*, 2012; Ortega-Molina *et al.*, 2012). Increased lifespan has been observed in these *PTEN*-induced mice coupled with decreased IIS activity. Similar results have been reported with hypomorphic PI3K mice (Ortega-Molina *et al.*, 2012; Foukas *et al.*, 2013). Growth hormone and IGF-1 levels are known to decline as part of normal aging as well as in mouse progeroid models, therefore decreased activity of IIS is considered to be a hallmark of both physiologic and accelerated aging and extends lifespan (Garinis *et al.*, 2008; Schumacher *et al.*, 2008). When IIS activity declines to an extremely low level it can become lethal as has been reported in cases of mice with null mutations of PI3K and Akt kinases, and alternatively supplementation with IGF-1 has shown to improve symptoms of premature aging (Renner and Carnero, 2009; Mariño *et al.*, 2010).

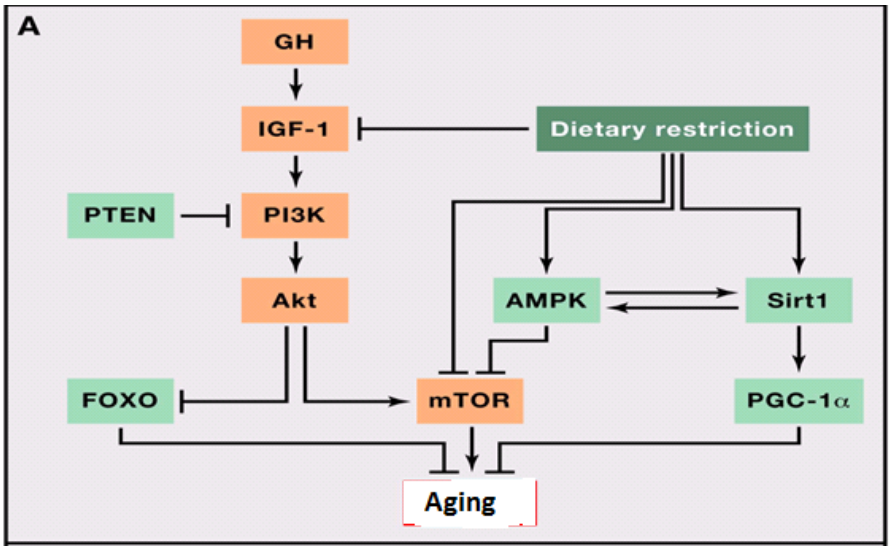


Figure 1.6. Schematic representation of energy sensing network. A) Showing nutrient regulation mechanisms involving mTOR, AMPK and Sirtuins**.** Adapted from (López-Otín *et al.*, 2013).

Apart from IIS pathway which mainly focuses on glucose sensing mechanisms, there are three important nutrient sensing pathways. Increased amino acid levels are instantly detected by mTOR signalling, whereas low energy states which mainly manifest as high AMP concentrations are detected by AMPK signalling mechanisms and finally sirtuins, which sense low energy status by high NAD^+ levels (Houtkooper *et al.*, 2010; Houtkooper *et al.*, 2012).

The mTOR kinases are involved in the regulation of anabolism and consists of two protein complexes, namely mTORC1 and mTORC2 (Laplante and Sabatini, 2012). Genetic mutations resulting in down regulation of mTORC1activity has shown to increase lifespan of worms, flies and mice (Johnson *et al.*, 2013). Rapamycin treatment in mice which involves binding to mTORC1complex has shown to extend longevity and still remains the drug of choice that has enhanced life span in mammals (Harrison *et al.*, 2009). It has been interesting to note that mice that are genetically altered to have low levels of mTORC1but have normal mTORC2 levels show an increase in longevity and a genetic knockout of S6K1, which is an mTORC1substrate results in mice having increased life span (Selman *et al.*, 2009; Lamming *et al.*, 2012). These findings further validate that a decrease in expression of mTORC1 or S6K1 can signal a progression towards longevity in mammals in relation to mTOR (Yang *et al.*, 2012). This suggests that mTORC1and associated pathways are promoters of aging process and inhibition of TOR activity can have beneficial effects on aging, although certain undesirable effects have been observed in mice as a result of this mTOR inhibition that include insulin resistance, testicular degeneration, development of cataract and abnormal wound healing (Wilkinson *et al.*, 2012; López-Otín *et al.*, 2013).

The remaining two nutrient sensing networks including sirtuins and AMPK mainly respond to nutrient deprivation and works through catabolism as opposed to the anabolic approach used by IIS and mTOR signalling pathways and therefore, up regulation of the sirtuins and AMPK promotes health span (Alers *et al.*, 2012). AMPK activation has multiple consequences and most importantly it leads to deactivation of mTORC1signalling and has also shown to improve life span in worms and mice following metformin treatment (Onken and Driscoll, 2010; Anisimov *et al.*, 2011; Mair *et al.*, 2011). SIRT1 is involved in deacetylation of PGC-1 alpha leading to its activation which in turn encompasses a wide range of metabolic responses, including mitochondrial biogenesis, increased fatty acid metabolism and improved antioxidant mechanisms (Rodgers *et al.*, 2005; Fernandez-Marcos and Auwerx, 2011). Both AMPK and SIRT1 are activated by nutrient scarcity and when there

is high energy demand, so they are sometimes unified in their response creating a positive feedback loop and activating PGC-1 alpha through phosphorylation and deacetylation respectively (Price *et al.*, 2012).

1.13.5 Inflammatory response involving altered intercellular communications

Age progression is linked with several other changes that take place simultaneously and can be attributed towards signs of aging and include intercellular communications disruptions at endocrine, neuroendocrine and neuronal level (López-Otín, 2013). It has been shown that neurohormonal signalling declines as aging occurs and secretion of proinflammatory cytokines from senescent cells leads to inflammatory response causing attenuation of the immune response and decreased defence mechanisms against pathogens creating an overall change in extracellular environment (Russell and Kahn, 2007; Zhong *et al.*, 2010; Rando and Chang, 2012). Inflammaging is the term that has been used recently to describe age related alterations in the intercellular communication which manifests itself as having a proinflammatory phenotype (Salminen *et al.*, 2012; López-Otín *et al.*, 2013). This inflammation can result from previously accumulated damaged tissue that has been affected by proinflammatory cytokines or a failure of immune system to eliminate senescent cells and pathogens allowing senescent cells to keep on secreting proinflammatory secretome or due to reduced autophagy response and finally inflammaging can also occur due to activation of NF κB transcription factor (Green *et al.*, 2011; Salminen *et al.*, 2012). These changes result in the activation of several important proinflammatory pathways that result in the production of inflammatory interferons TNFα and IL-1β (Green *et al.*, 2011). Human aging is closely linked and correlated with two inflammation associated pathologies namely obesity and diabetes mellitus in which inflammation plays a major role in acceleration of aging (Barzilai *et al.*, 2012). Impaired inflammatory response is the hallmark of atherosclerosis and Inflammaging has recently been shown to disrupt epidermal stem cell function and has led to gradual decline in adaptive immune system (Tabas, 2010; Deeks, 2011; Doles *et al.*, 2012). This associated immunosenescence further aggravates aging process due to the failure of elimination of dead and infected cells promoting their malignant transformation (Davoli and De Lange, 2011; Senovilla *et al.*, 2012). Several large scale studies have confirmed the importance of inflammatory processes in the progression of aging and overexpression of NFκB signalling mechanism is a vital sign of aging (de Magalhães *et al.*, 2009; Lee *et al.*, 2012). Transgenic mice have shown reversal of aging symptoms to varying degree of extent

when NFκB inhibition has been achieved through conditional expression in aged skin tissue (Adler *et al.*, 2007). Similar results have been obtained recently by inhibition of NFκB signalling activity in mouse models through genetic and pharmacologic interventions (Osorio *et al.*, 2012; Tilstra *et al.*, 2012). More recently, it has been suggested that hypothalamus is involved in mediating aging, as inflammation activates NFκB in hypothalamus which responds by decreasing the production of gonadotropin releasing hormone (GnRH) by neurons which results in muscle weakness, skin atrophy, reduced neurogenesis and other age-related symptoms (Zhang *et al.*, 2013). Recent studies on mRNA decay factor AUF1 has further revealed links between inflammatory response and aging, as a deficient mice shows signs of premature aging coupled with cell senescence (Pont *et al.*, 2012). AUF1 has been shown to promote cytokine mRNA degradation thereby promoting inflammatory response and has been involved in maintaining telomere length by the activation of TERT so a deficiency of this factor can accelerate aging through different pathways (Pont *et al.*, 2012) Sirtuins have long been shown to have an impact on inflammation associated aging. SIRT 1has shown to down regulate inflammation related genes by deacetylation of histones and inhibition of NFκB signalling (Xie *et al.*, 2013). Similarly, a decrease in expression of SIRT1 results in progression of several inflammatory pathologies and mice studies have confirmed the anti-inflammatory response to pharmacologic activation of SIRT1 (Zhang *et al.*, 2010; Gillum *et al.*, 2011; Yao *et al.*, 2012). SIRT2 and SIRT6 are the other two sirtuins that have shown to reduce inflammatory response by deacetylation of NFκB (Kawahara *et al.*, 2009; Rothgiesser *et al.*, 2010). It has been reported recently that age-associated changes can be transferred from one tissue to another referred to as inter organ communication in which senescent cells are able to induce senescence to adjacent cells via gap junction mediated signalling that also involves reactive oxygen species (Nelson *et al.*, 2012). Conversely, it has been shown that life span enhancing alterations attempted in one tissue can have an adverse and retarding effect in the other (Tomás-Loba *et al.*, 2008; Durieux *et al.*, 2011; Lavasani *et al.*, 2012).

1.13.6 Summary and perspectives

Studying pathways involved in aging in different cell types can help in building a structured format to investigate molecular mechanisms underlying aging and can help in interventional research aimed at improving human health (Martin, 2012). Next-generation sequencing technologies have recently established a special impact in aging research by making it

possible to evaluate genetic and epigenetic changes in individual cells of an aged subject (de Magalhães *et al.*, 2010; Gundry and Vijg, 2012; Miller, 2012). More recently, there has been great advancement in aging research and whole genome sequencing analysis has been carried out in individuals having exceptional longevity and comparative genome studies along with in depth study of age-related epigenetic alterations have been underway (Kim *et al.*, 2011; Heyn *et al.*, 2012; Sebastiani *et al.*, 2012). This is essential to have more in vivo studies and analysis with gain or loss of function experimental models to validate the importance of these hallmarks of aging and understand the mechanics involved in progression of aging and age-related diseases (Kirkwood, 2008; Gems and Partridge, 2013).

1.14 Role of PGC-1 alpha coactivators in biological systems

Coactivators are considered as proteins that are involved in regulation of gene expression but they do not directly interact with the DNA, instead they regulate this by interacting with transcription factors. Peroxisome proliferator activated receptor gamma coactivator 1- alpha (PGC-1α) belongs to a family of transcriptional coactivators that has been shown to bind with numerous transcription factors and includes most nuclear receptors (Handschin and Spiegelman, 2006; Rowe *et al.*, 2010; Patten and Arany, 2012). These transcriptional coactivators have recently been shown to be involved in regulation of mitochondrial biology in different tissues including heart, liver and brain (Handschin and Spiegelman, 2006). PGC-1 alpha has shown reduced expression in various models of failing heart and considered to be important contributors towards abnormal oxidative metabolism and as oxidative phosphorylation mainly occurs in mitochondria, heart and all these tissues maintain high mitochondrial content (Rowe *et al.*, 2010). Apart from being a master regulator of mitochondrial activity, PGC-1 alpha is also involved in the regulation of several biological functions in different tissues that includes angiogenesis in skeletal muscle, gluconeogenesis and fatty acid metabolism (Patten and Arany, 2012). Reduced PGC-1 alpha expression has recently been implicated in neurodegenerative disorders like Parkinson's disease and in transgenic mouse model of Huntington's disease (Chaturvedi *et al.*, 2010; Zheng *et al.*, 2010; Shin *et al.*, 2011).

1.14.1 PGC-1 alpha regulation and its role in cardiovascular system

Mitochondrial biogenesis and mitophagy which refers to the elimination of damaged mitochondrial content by means of a strict immunosurveillance mechanisms are two processes that work in tandem and maintain a balance so that the mitochondrial content remains in check. It has been shown that peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) plays a central role in coordinating this mitochondrial biogenesis which is an extremely complex process and several aspects of which are still not completely understood (Nisoli *et al.*, 2003; Patten and Arany, 2012). This formation of new mitochondria involves replication of mitochondrial DNA (mtDNA) along with expression and activation of several mitochondrial and nuclear genes. PGC-1 alpha being a transcriptional coactivator activates mitochondrial factors A and B commonly known as transcription factor A mitochondrial (TFAM) and transcription factor B mitochondrial (TFBM) which in turn activate the mitochondrial genes encoded by mitochondrial DNA (mtDNA) and include genes involved in regulation of electron transport chain (Handschin *et al.*, 2003; Rowe *et al.*, 2010).

PGC-1 alpha is known to be activated by several stimuli which have high energy demands and in order to achieve that they require mitochondrial synthesis. These stimuli include exercise, cold exposure, caloric restriction and hypoxia (Nisoli *et al.*, 2003; Patten and Arany, 2012). On the other hand, the expression of PGC-1 alpha is regulated by several factors that include nitric oxide (NO), activation of sympathetic nervous system, cyclic adenosine monophosphate (cAMP), AMP-activated protein kinase (AMPK), calcineurin, calcium/calmodulin -dependent protein kinase and tumour suppressor p53 (Li *et al.*, 2007; Trausch-Azar *et al.*, 2010). In addition to that, PGC-1 alpha is also regulated through posttranslational modifications. Most important of these modification include deacetylation by sirtuins (SIRT1) which increases PGC-1 alpha activity and acetyltransferase GCN5 which leads to a decrease in PGC-1 alpha expression (Olson *et al.*, 2008; Dominy Jr *et al.*, 2010). Phosphorylation of PGC-1 alpha by AMPK, p38 mitogen-activated protein kinase, Akt and glycogen synthase kinase-3 causes an increase in expression of PGC-1 alpha (Li *et al.*, 2007; Miura *et al.*, 2007; Pogozelski *et al.*, 2009). More recently identified modifications include *O*-GlcNAc and phosphorylation by S6 and Clk2 kinases which mainly modify PGC-1 alpha activity in liver (Housley *et al.*, 2009; Rodgers *et al.*, 2010; Lustig *et al.*, 2011).

PGC-1 alpha is considered to be a master regulator and in addition to mitochondrial biogenesis, it controls the expression of several genes that are involved in regulation of

glucose and fatty acid metabolism in cardiac myocytes as well as many other cell types. (Leone and Kelly, 2011; Riehle *et al.*, 2011). Mitochondrial content is much lower in endothelium as compared to cardiac myocytes, the latter being highly oxidative and has high energy demands. It has been shown that in rats mitochondria covers nearly 5% of the cytoplasmic volume in endothelial cells, whereas more than 30% is occupied by mitochondria in cardiac myocytes (Oldendorf *et al.*, 1977; Barth *et al.*, 1992). This further explains that PGC-1 alpha is mainly activated in conditions that require more energy in the form of ATP production. PGC-1 alpha is known to play an important role in angiogenesis by regulating the expression of vascular endothelial growth factor-1(Valle *et al.*, 2005; Arany *et al.*, 2008). It also plays a role in regulating metabolism in cardiac myocytes and genetic deletion of PGC-1 alpha can contribute towards heart failure. Diabetes patients show an increase in cardiac PGC-1 alpha expression which again promotes heart failure (Leone and Kelly, 2011; Riehle *et al.*, 2011).

1.14.2 PGC-1 alpha and its role in endothelial cells

Mitochondrial content is much lower in endothelial cells as compared other cell types with high energy demands including oxidative tissues of heart, lung, brain and liver. Mitochondrial distribution within a cell has also been very interesting to note, as active endothelial cells at blood-brain barrier show much higher mitochondrial content as compared to endothelial cells in other capillary beds (Oldendorf *et al.*, 1977; Park *et al.*, 2011). In normal conditions, energy requirements are much lower in endothelial cells therefore, PGC-1 alpha is suggested to play a different role in endothelial cells although it is well known that it is expressed in endothelial cells and regulates mitochondrial synthesis (Nisoli *et al.*, 2003). PGC-1 alpha regulates reactive oxygen species and provides defence against oxidative stress. It has been shown that induction of PGC-1 alpha by viral transfection leads to an increase in expression of antioxidants including catalase, MnSOD and thiroredoxin-2 along with uncoupling protein-2 (UCP-2) all of which are involved in combating oxidative stress mechanism. PGC-1 over expression has been shown to decrease inflammatory activity, increase NO bioavailability and prevent endothelial cell apoptosis (Valle *et al.*, 2005; Schulz *et al.*, 2008). It has recently been reported that PGC-1 alpha promotes antioxidative mechanisms by inducing mitochondrial synthesis and providing fresh and undamaged mitochondria that are prone to produce much less reactive oxygen species (Twig *et al.*, 2008).

1.14.3 PGC-1 alpha and its clinical significance

Recent studies have focused on using PGC-1 alpha as a therapuertic strategy to overcome atherosclerosis, as it imparts positive effects on endothelial phenotype, but how effective this endothelial specific PGC-1 alpha overexpression can prove still remains to be seen. Interestingly, it has been shown recently that PGC-1 alpha null mice with an apolipoprotein $E^{-/-}$ background showed decreased lesion formation, but this could be due to the fact that PGC-1 alpha null mice are thin and show signs of hyperactivity that could naturally limit athersosclerosis progression (Stein *et al.*, 2010). Similarly, PGC-1β has shown a compensatory increase in expression due to loss of PGC-1α (Mitra *et al.*, 2012). It has been suggested to have mouse models having double knockouts of PGC-1α and β so that issues can be sorted (Patten and Arany, 2012). Endothelial –specific overexpression of PGC-1 alpha has recently been shown to have protective effect against hypertension.

In a clinical setting, it is well known that PGC-1 alpha expression and associated mitochondrial content are lower in patients suffering from diabetes mellitus manifested by insulin resistance and disrupted energy metabolism (Kelley *et al.*, 2002; Mootha *et al.*, 2003; Petersen *et al.*, 2004). In patients diagnosed with pulmonary hypertension lower mitochondrial mass in endothelial cells has beeen reported (Xu *et al.*, 2007). A biopsy taken from subcutaneous tissue of a diabetic patient has revealed lower than normal mitochondrial content in the arterioles (Kizhakekuttu *et al.*, 2012). Epidemiological data suggests that PGC-1 alpha is closely asssociated with vascular pathologies and linkage analysis confirm that PGC-1 alpha polymorphisms are linked with coronary artery disease, carotid atherosclerosis and hypertensive disorders (Oberkofler *et al.*, 2003; Iglseder *et al.*, 2006; Zhang *et al.*, 2008). As a result of this convincing data, more research is now directed towards developing drugs that can activate PGC-1 alpha which in turn would improve mitochondrial biogenesis and provide defence against cardiovascular diseases (Kitami *et al.*, 2012).

Chapter 2. Aims and objectives

Aim 1: To characterise the phenotype of endothelial colony forming cells (ECFCs) isolated from cord blood compared to human umbilical vein endothelial cells (HUVECs) isolated from the same donor.

In order to achieve these goals human umbilical cord mononuclear cells (day 0 MNCs) were isolated from cord blood using ficoll based lymphoprep. They were differentiated towards endothelial lineage by growing in endothelial specific EBM- 2 media. This resulted in isolation of early EPCs from these cord blood mononuclear cells (day 7). The same mononuclear cells were grown in macrophage specific media (RPM1 + Human monocyte colony stimulating factor) and were differentiated towards macrophage lineage (day 7). This was done to distinguish between early and late EPCs as the former are known to be related to monocyte-macrophage lineage. Cells were frozen down and RNA was extracted. The remaining mononuclear cells were further cultured under normoxic conditions (20% O_2) for 2-3 weeks resulting in isolation of late EPCs also known as endothelial colony forming cells (ECFCs) or out growth endothelial cells (OECs). Once an endothelial colony was observed, it was picked and grown under normal conditions for 4-6 weeks in order to get late EPCs or ECFCs. In order to have a better comparison all these early and late EPCs and macrophages were isolated from same mononuclear cells (day 0 MNCs) derived from the cord blood of the same donor. Once isolated, morphological analysis of the isolated cell types was carried out which was followed by molecular profiling of these cells. RNA extraction was carried out from all these cell types at different point times followed by qPCR analysis. The mRNA expression level of cell surface markers including CD34, CD31, CD14, CD45, CD105, CD144, CD146 and vWF was carried out. In order to validate these results and to differentiate them from mature endothelial cells (HUVEC) at protein level, FACS analysis with cell surface markers including CD34, CD14, CD146, and CD144 and direct immunofluorescence analysis with vWF antibody was performed comparing early and late ECFCs and HUVEC.

Aim 2: To elucidate the effects of hyperoxia as a model of oxidative stress on ECFCs vs HUVECs, focusing on telomere length, telomerase activity and PGC-1 alpha expression.

One of the aims of this project was to study the effect of mild oxidative stress on the expression of PGC-1α and its downstream targets in human endothelial progenitor cells (ECFCs) and mature endothelial cells (HUVEC). Hyperoxia has already been established as a model of mild oxidative stress, as it leads to increased intracellular generation of hydrogen peroxide and reactive oxygen species (ROS) accelerating the accumulation of DNA single strand breaks causing cellular senescence. In addition to that, hyperoxia increases MCP-1, a crucial molecule which is involved in the initiation and development of atherosclerosis (Harrington, 2000).

Recently, it has been shown that telomere dysfunction in the nucleus leads to deactivation of PGC-1 proteins in the mitochondria via activation of p53 (Sahin *et al.,* 2011). They showed that a mice null in either *Tert* or *Terc* genes leads to p53- mediated apoptosis and growth arrest in several tissues and repression of PGC genes and their downstream networks leading to impaired mitochondrial biogenesis, oxidative phosphorylation and gluconeogenesis (Sahin *et al.*, 2011). We, therefore, hypothesized that mild oxidative stress would lead to telomere dysfunction in human endothelial cells, and as a consequence of that a decrease in the expression of PGC-1α in endothelial progenitor cells (ECFCs) and mature endothelial cells (HUVEC). As PGC-1α is a key regulator of mitochondrial biogenesis and oxidative metabolism, a decrease in its expression would lead to an impairment of mitochondrial activity in these cells. This could in turn lead to a decrease in the angiogenic potential of these cells as mitochondrial deregulation has already been linked with several vascular dysfunctions.

In order to achieve this goal, we isolated late endothelial progenitor cells (ECFCs) and mature endothelial cells (HUVEC) from human cord blood and human umbilical vein, respectively. Following subculture of cells for 2 passages, we exposed them to conditions of mild oxidative stress (40% O_2). This was be followed by extraction of RNA at different time points from these cells followed by cDNA synthesis. Real time quantitative PCR was used to assess the mRNA expression level of PGC-1α and its downstream targets including ERR-alpha and G6- Phosphatase. Our initial results indicated that ECFCs grow much better than HUVEC under conditions of mild oxidative stress (40% O_2). HUVEC grew at a slower rate and did not grow well after few passages in hyperoxic conditions, and we therefore hypothesized that ECFCs are much more resistant to stress than HUVEC. In order to test this,

we isolated ECFCs from human umbilical cord blood and HUVEC from the umbilical cord. Cells were grown for 2 passages in normoxia (20% O_2) and then split and cultured simultaneously in both normoxic and hyperoxic conditions (40% O_2). This was followed by comparing the growth kinetics of both these cell types and measurement of telomerase activity and telomere length under normal and hyperoxic conditions.

Aim 3: To determine the effect of oxidative stress on the expression of PGC-1 alpha and its downstream targets involved in regulating reactive oxygen species (ROS), oxidative metabolism and angiogenesis in ECFCs and HUVEC

Cellular stress has been shown to be one factor leading to increased ROS production resulting in activation and up regulation of PGC-1 alpha (Wareski *et al.*, 2009). The cellular response to this oxidative stress is an increased ATP production and availability by transcription of oxidative phosphorylation (OXPHOS) regulated genes (Pascual *et al.*, 2005; Stravodimou *et al.*, 2012). We therefore, proposed that exposing these endothelial progenitor cells to mild oxidative stress (40% O_2) would lead to an increase in ROS production and an increase in PGC-1 alpha expression. This in turn would lead to ROS regulation by PGC-1 alpha and increased expression of genes involved in oxidative phosphorylation.

Similarly, PGC-1 alpha coactivators have been shown to be involved in regulation of wide range of metabolic programs including gluconeogenesis and responses to fasting in liver, fatty acid oxidation and import mechanisms and angiogenesis in skeletal muscle (Yoon *et al.*, 2001; Chinsomboon *et al.*, 2009). We therefore investigated the role that oxidative stress could play in ECFCs and HUVEC when grown in hyperoxic conditions. It has been shown before that PGC-1 alpha is activated by lack of nutrients and oxygen and regulates VEGF expression and angiogenesis by co activating nuclear receptor ERR alpha (Arany *et al.*, 2008). Hypoxia has been known to induce VEGF expression and angiogenesis, but role of hyperoxia in angiogenesis has not been well understood and is an emerging area of research. Our earlier results showed that ECFCs were much more resistant than HUVEC when exposed to oxidative stress (40% O_2). We proposed that ECFCs would behave differently in terms of gene expression analysis and both these cell types might be using different pathways to counteract stress environment. In order to test this hypothesis, we carried out gene expression analysis using low density array in ECFCs and HUVEC grown in normoxia and hyperoxia and checked for genes involved in oxidative phosphorylation, genes involved in antioxidative mechanisms, genes involved in DNA damage and inflammatory response, oxidative

metabolism and angiogenesis. This was followed by qPCR validation for genes that showed significant change in expression when ECFCs or HUVEC were grown in hyperoxia.

Hypothesis:

We, therefore hypothesized that endothelial colony forming cells (ECFCs) that have been derived from an endothelial colony would behave differently in conditions of oxidative stress as compared to mature endothelial cells (HUVEC). As they have shown to be more resistant to oxidative stress they would up regulate genes involved in different pathways as compared to HUVEC. The difference would be evident both in terms of gene expression analysis and functional assays.

Chapter 3. Materials and Methods

3.1 Isolation of human endothelial progenitor cells (early EPCs) from cord blood mononuclear cells

Before starting the isolation, 6 well plates were coated with fibronectin in order to provide an attachment surface for the cells. 1 ml of fibronectin which was diluted in phosphate buffer solution (PBS) 1:100 was used per well of a 6 well plate. Fibronectin was removed after 30 min, and the plates were allowed to dry under the hood.

Once collected, the cord blood was mixed with PBS (1:1) and the mix was layered on to equal volume of ficoll solution in a way that the blood was not mixed with the ficoll but it continued to swim on it. This was allowed to centrifuge for 20 min without brakes at 800 × *g*. After the centrifugation was complete, three different phases could be seen as shown in the figure 3.1. The white blood cells were carefully removed using a pipette along with some plasma which could not be avoided. It was important that no ficoll was taken along with the WBCs as this could damage the cells. This mix of WBC and serum was transferred to a new 50 ml falcon and PBS was added to make it to 50 ml. This was subjected to centrifugation at 800 x *g* for 10 min with brakes. The supernatant was aspirated and the pellet was resuspended in 50 ml of PBS. At this point cells were counted with a dilution of 1:20 and then cells were resuspended in EBM- 2 media containing single Quots, 10% FBS and Pen/Strep at a concentration of around 8 million cells/ml. 2 ml of this cell suspension was added to each well of the 6 well plate. Some cells were frozen down the same day as human cord blood mononuclear cells (HUCBC) also known as day 0 MNCs at -80˚C.

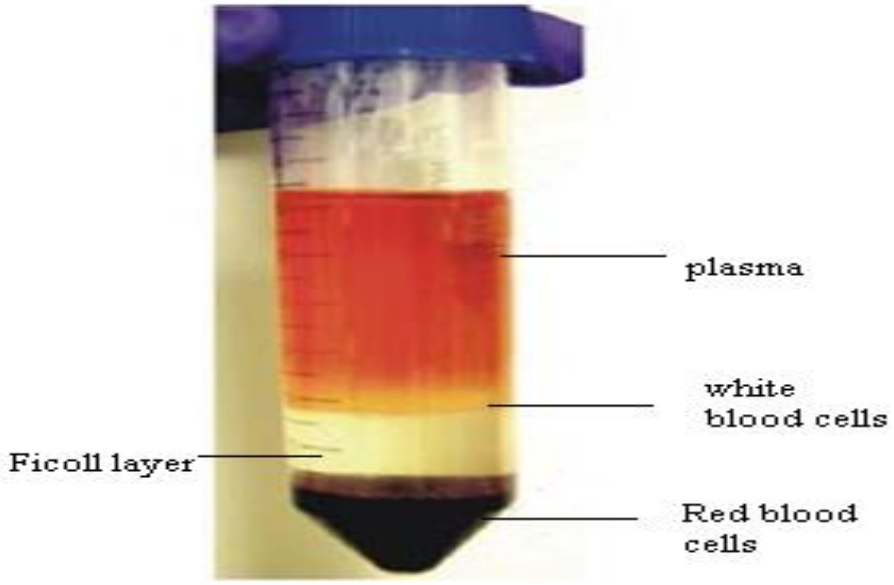


Figure 3.1. Showing lymphoprep preparation using ficoll. Adapted from (current protocols.com).

3.2 Isolation and differentiation of human cord blood mononuclear cells (HUCBC) in to macrophages

Macrophages are the resident phagocytic cells found in lymphoid and nonlymphoid tissues and are derived from hematopoietic stem cells. Cord blood was received from donors from *Queen Elizabeth Hospital Gateshead* and was subjected to lymphoprep separation using ficoll. WBC layer was obtained and PBS was added to that, followed by centrifugation at 800 x *g* for 20 min without brakes. Supernatant was removed and the cells were again resuspended in 50 ml of PBS. Cells were counted at this stage and around 15 million cells were plated per well of a 6 well plate. The plates did not require coating with fibronectin. The cells were grown in RPMI media and human monocyte colony stimulating factor was added in the media so that the mononuclear cells could be differentiated towards macrophage lineage. After 3 days cells started to change their morphological appearance and resembled macrophages. RNA was extracted from cells at day 7 and kept frozen at -80°C.

3.3 Isolation and culture of Human endothelial colony forming cells (ECFCs) derived from cord blood

Human endothelial colony forming cells (ECFCs) were derived from human cord blood. Before starting the isolation, well plates were coated with Type 1 rat tail collagen (BD Biosciences), which was diluted in 0.02N acetic acid, as collagen is insoluble at neutral PH. 0.02 N acetic acid was prepared by adding distilled water to glacial acetic acid. Plates were coated with collagen for an hour at room temperature and then the solution was aspirated, and the plates were washed once with PBS, so that the acid was completely removed which could damage the cells. Plates were allowed to air dry for 30 min.

Fresh cord blood (50-120ml) once received from the hospital, was diluted with PBS 1:1, and then a lymphoprep separation of blood: PBS was performed as explained earlier. The mononuclear cells collected as a result were transferred to a new 50 ml falcon and PBS was added to make it a 50 ml suspension. This mix was subjected to centrifugation at 800 × g for 5 min without brakes to gently pellet the cells. Supernatant was removed and dilution with PBS and centrifugation was repeated. Again the supernatant was aspirated and cells were resuspended in 50 ml PBS. At that point, cells were counted using a haemocytometer with 1:20 dilution and also using Vi-Cell XR 2.03 (Beckman Coulter) using 1:10 dilution. In all, 400-700 million cells were obtained depending upon the volume of cord blood received. The supernatant was removed and the cells were then resuspended in 12 ml of complete EBM- 2 media (with single Quotes) that additionally contained 20% FBS and 2% Pen/Strep. 2 ml of this suspension was added to each well of the collagen-coated 6 well plate. Around 60 million cells were seeded per well. After 48-72 hr non adherent cells were removed by changing the culture media. The media was changed on alternate days and at around 3 days of culture big round cells started to appear, which were proposed to be early EPCs or circulating angiogenic cells (CACs). An endothelial colony was observed after 3-4 weeks of culture, and this was picked up using a pipette tip and grown in T25 flask. Once confluent enough, the cells were split and the cells were grown for several passages till P5 and kept frozen down and stored at -80˚C.

3.4 Isolation and culture of human umbilical vein endothelial cells (HUVEC)

Human umbilical vein endothelial cells (HUVEC) were isolated from the umbilical cord of the new born. Once received, the umbilical cord was washed with 1× PBS using a 50 ml syringe several times so that the cord was clean and there was no blood or clot visible. The two ends of the cord were tidily cut with a sterile scalpel and a cannula was introduced at one end of the vein, which was the widest vessel having a thick lumen as shown in figure 3.2 B. Once inside the vein, the cannula was tightly maintained by stitching around the cord using surgical needle. Cord was then washed from inside with PBS using a 50 ml syringe. This was followed by injection of collagenase (0.2% in PBS) at the end of the vein that contained the cannula, and leakage at the other end was stopped by using a clamp with a surgical clip. The cord was then incubated for 30 min at 37°C. After incubation, the cord was gently squeezed and the cells were collected in a sterile falcon tube by washing with 40 ml of PBS. This was followed by a centrifugation at 500 × g for 5 min. The supernatant was carefully discarded and the pellet was resuspended in 12 ml of culture media containing M199 Earle 1×, 0.2M Glutamine, 1M HEPES, 7.5% $NAHCO_3$, Penicillin/Streptomycin (10000/10000) and 20 % foetal bovine serum (FBS). The cells were dissociated by aspiration and repulsion using a pipette. 2ml of the cell suspension was added to each well. Cells were incubated at 37°C in humidified air (95%) with 5% CO_2. After 48 hr non-adherent cells were removed by changing the culture medium. The culture medium was changed on alternate days and attached endothelial cells were visible after 48 hrs. These cells reached confluence at around 3-5 days of culture and showed "cobblestone" appearance on observation under microscope. They were split when around 70-80% confluent, using Trypsin-EDTA and then regularly passaged till P5.

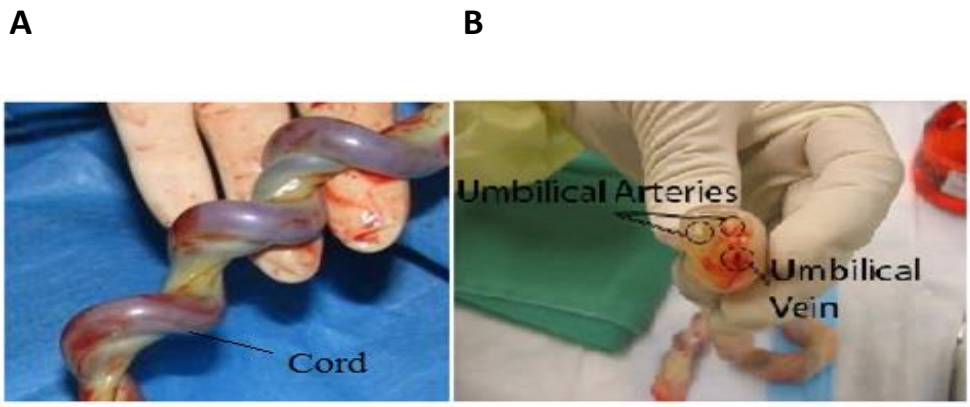


Figure 3.2. Showing human umbilical cord and human umbilical vein. Adapted from www.mothersofchange.com.

3.5 Passaging of endothelial cells

The cells were examined routinely under the microscope for sub confluence. Once the cells reached a confluence of around 70-80%, normally after 3-5 days, they were split. Cells were split using Trypsin-EDTA in a ratio of 1:4. Medium was aspirated, and the cells were washed with PBS. Trypsin was added to the flask and incubated for 5 minutes and the flask was tapped with both hands in order to detach the cells. A scraper was used to detach the cells that were firmly attached. An equal volume of medium containing FBS was added to neutralize the Trypsin and the resulting mixture was transferred to a 15 ml falcon tube. Centrifugation was carried out at 500 × *g* for 5 min. The supernatant was sucked and the cell pellet thus obtained was resuspended in fresh medium and transferred to each of the four T25 flasks. The flasks were put in an incubator at 37˚C, with 5% CO_2 in humidified conditions (>95%).

3.6 Culture of ECFCs and HUVEC in conditions of normoxia and hyperoxia

Normoxia (20%O_2):

ECFCs were isolated from cord blood and for the purpose of having a better comparison mature endothelial cells were isolated from the umbilical vein of the same donor. ECFCs were derived from cord blood by differentiation of Human cord blood mononuclear cells (HUCBC) by growing them in endothelial specific media (EBM-2). Cells were grown on collagen coated plates. These mononuclear cells were allowed to differentiate in endothelial media and cells were washed with PBS regularly followed by change of media every alternate day. It took 3-4 weeks before an endothelial colony appeared and in some cases there was no colony formation observed even after 1 month of cell culture and for some isolations more than one colony was obtained from the same isolation. Once a colony was observed, it was picked using a pipette tip and allowed to grow in smaller volumes either in a T25 flask or in 1-2 wells of a 6 well plate. The cells obtained from these colonies were grown again in EBM-2 media and were named as endothelial colony forming cells (ECFCs) as shown before. These ECFCs were sub cultured and split regularly using Trypsin-EDTA or some times by using cell scraper when cells became too adherent. Cells were split in a ratio of 1:3. ECFCs were grown till late passages (P16-P18) in normoxia (20% O_2). Cells were frozen down at -80°C in freezing media containing DMSO. Cells were used for downstream applications for experiments including RNA extraction followed by qPCR, Telomerase

activity measurement using TRAP assay, measurement of telomere length across passages using flow-FISH, direct immunofluorescence analysis, multicolour flow cytometry and gene expression analysis using low density array (LDA) followed by qPCR validation (same donor analysis). 1million cells were replated and two million cells were frozen down with each passage. Cells were split every 3-7 days and passage 10 corresponds to around 8 weeks of culture.

Mature endothelial cells (HUVEC) were derived from the umbilical cord of the same donor in some cases (n=6). Apart from that, most HUVEC were isolated and grown but for several samples no endothelial colony was obtained from the same cord blood sample. HUVEC were grown in 6 well plates coated with human fibronectin. 1ml of fibronectin diluted in PBS (1:100) was used to coat the plates. Plates were allowed to dry down after coating for 30 min. HUVEC were grown till late passages (P16-P18) and cells were regularly passaged using Trypsin-EDTA in a ratio of 1:3. HUVEC were initially used as a positive control for the study to differentiate endothelial progenitor cells from mature endothelial cells. HUVEC were isolated from umbilical vein of the cord using collagenase. Cells were grown in normoxia (20% O_2) and cells were regularly washed with PBS and media was changed on alternate days. HUVEC were grown in endothelial specific media (EBM-2) and showed no further change in morphology. 1 million cells were plated each time, both for ECFCs and HUVEC. Once they were around 70% confluent they were split using trypsin in a ratio of 1:3. 1 million cells were replated and 2 million cells were frozen down with each passage. This was carried out till passage 16-18. Cells were passaged after every 3-5 days and passage 10 corresponds to around 8-10 weeks of culture.

Hyperoxia (40%O_2):

Hyperoxia can lead to oxidative stress and this has been shown before. We therefore exposed the cells to hyperoxia in order to study their behaviour under stress conditions and to test if it was different for ECFCs and HUVEC. Both ECFCs and HUVEC were grown in normoxia till P2 and then were split and incubated in hyperoxic conditions (37°C with 5% CO_2 and 40%O_2). ECFCs were grown in either T25 flasks or 6 well plates coated with rat tail collagen in hyperoxia and as shown in growth curves grew well. HUVEC were grown in either T25 flasks or 6 well plates and showed much less resistance to stress conditions. Cells were split using Trypsin-EDTA in a ratio of 1:3. 2 million cells were frozen down with each passage and stored at -80°C in freezing media containing 20 % DMSO.

3.7 RNA extraction from EPCs, macrophages, ECFCs and HUVEC

RNA isolation was carried out using Trizol method in 1 ml volume. Cells were trypsinized using 0.1% Trypsin-EDTA. An equal volume of EBM-2 media containing 20 % FBS was added to neutralize the trypsin. This was followed by centrifugation at 1000 rpm for 5 min. Cells were washed twice with Phosphate Buffer Solution (PBS without Ca^{++} and Mg^{++}). After washing, the supernatant was removed and the cell pellet was resuspended in 1 ml of Trizol, and left for incubation at room temperature for 5 min. This suspension was then transferred to an eppendorf tube and 1/5 Volume (200 µL) of chloroform was added and the suspension was shaken vigorously for 15s. After incubation at room temperature for 2-3 min, cells were centrifuged at 12000 × g for 15 min, and the upper aqueous layer was then transferred to another eppendorf tube. A half volume (500 µL) of isopropanol was added and the cell sample was incubated at room temperature for 10 min. This was followed by another centrifugation at 12000 × g for 15 min, and a small pellet was obtained as a result. The supernatant was removed and 1 volume of 70% ethanol was added. A vortex for 30s was followed by centrifugation at 7500 × g for 5 min. Ethanol was removed and the pellet was allowed to air dry for about 15 min. Around 30 µL RNase free water was added and then incubated for 10 min at 60˚C. Eluted RNA was quantified using Nanodrop and then kept at -80˚C.

RNA extraction was also carried out using the RNeasy Mini kit (Qiagen). Frozen cell pellet was taken from -80˚C and then subjected to homogenization in 600 µL of Buffer RLT. The lysate was centrifuged for 3 min at high speed and the supernatant was carefully removed by means of a pipette and transferred into a new eppendorf tube. An equal volume of 70% ethanol was added to the cleared lysate, and mixed well by gentle pipetting. The mixture thus produced along with any precipitate formed was transferred into an RNeasy spin column which was placed in a 2 ml collection tube. This was subjected to Centrifugation at 13000 rpm for 15s. This was followed by DNase digestion to eliminate any genomic DNA contamination. 350 µL of Buffer RW1 was added to the spin column. After centrifugation 80 µL DNase (10 µL DNase mixed with 70 µL Buffer RDD) was directly added to the column membrane, and incubated for 15min at room temperature. 350 µL of Buffer RW1 was added to wash the spin column. After the DNase digestion the spin column was twice washed by 500 µL of Buffer RPE. Finally the RNA was eluted in around 20-25 µL of RNase-free water. The eluted RNA was quantified using the Nanodrop and kept frozen at -80°C.

Table 3.1 Primer sequences and annealing temperatures designed for qPCR

Gene Name		Primer Sequence		Product Size	Annealing Temp
1	CD31 NM_000442.4	F R	AACAGTGTTGACATGAAGAGCC TGTAAAACAGCACGTCATCCTT	148 bp	55˚C
2	CD34 NM_001773.2	F R	GCGCTTTGCTTGCTGAGTTT GCCATGTTGAGACACAGGGT	183 bp	55˚C
3	CD144 NM_001795.3	F R	GATCAAGTCAAGCGTGAGTCG AGCCTCTCAATGGCGAACAC	114 bp	55˚C
4	vWF NM_000552.3	F R	AGCCTTGTGAAACTGAAGCAT GGCCATCCCAGTCCATCTG	154 bp	55˚C
5	CD 133 NM_001145852.1	F R	TGGCTGGGTGGCCTGGTCAT GACACAGCCTCGGGTGGTCG	123 bp	60˚C
6	CD146 NM_006500.2	F R	TCCCGCAGCCCCTGAGAGAC CAGCGATAGCCGCCTCCTGC	174 bp	60˚C
7	CD105 NM_000118.2	F R	GCCGTGCTGGGCATCACCTT CACTGTGGGGGCCTGGGGTA	107 bp	60˚C
8	CD309 NM_002253.2	F R	GCGGGCCAATGGAGGGGAAC AAGGCACCACGGCCAAGAGG	165 bp	60˚C
9	CD14 NM_001174105.1	F R	CGGCGGTGTCAACCTAGAG GCCTACCAGTAGCTGAGCAG	142 bp	60˚C
10	CD115 NM_005211.3	F R	TCCAAAACACGGGGACCTATC TCCTCGAACACGACCACCT	133 bp	60˚C

11	**CD45** **NM_002838.3**	**F** **R**	**CTCCGCCGCCAATGCAAAACT** **GAGCTGTGGTGTGCAAGGCTGAG**	**144 bp**	**61˚C**
12	**ACTA 2** **NM_001613.2**	**F** **R**	**TGCTGAGCGTGAGATTGTCCGG** **AGGGTCTCTCTGGGCAGCGGAA**	**176 bp**	**60˚C**
13	**BMP4** **NM_130851.2**	**F** **R**	**GCGGGACTTCGAGGCGACAC** **GCCGGGCGCTCAGGATACTC**	**171bp**	**63˚C**
14	**TBP** **NM_001172085.1**	**F** **R**	**TCAGTGCCGTGGTTCGTGGC** **GCCACGCCAGCTTCGGAGAG**	**184 bp**	**60˚C**
15	**PGC-1 α** **NM_013261.3**	**F** **R**	**TGTTTTTGACGACGAAGCAG** **AATGAATAGGATTGCGTGCC**	**183 bp**	**60˚C**
16	**CAT** **NM_001752.3**	**F** **R**	**GCTTCAGGGCCGCCTTTTTGC** **ATCGGGCCGTCACGCTGGTA**	**126 bp**	**60˚C**
17	**SOD3** **NM_003102.2**	**F** **R**	**GCCTCCTGCACTCGCGCTAA** **GAGTCGGGCACCTTTCCAGCTC**	**123 bp**	**60˚C**
18	**GPX1** **NM_000581.2**	**F** **R**	**TGGGCATCAGGAGAACGCCA** **CGCACCGTTCACCTCGCACT**	**118 bp**	**60˚C**
19	**G6PC** **NM_000151.3**	**F** **R**	**TGTGAGACTGGACCAGGGAGCC** **AGCTGCACAGCCCAGAATCCC**	**167 bp**	**60˚C**
20	**ERRα** **NM_004451.3**	**F** **R**	**AGGACCATCCAGGGGAGCATCG** **TTGAGCATGCCCACCCGCAG**	**125 bp**	**60˚C**
21	**SIRT1** **NM_012238.4**	**F** **R**	**CTCCAAGGCCACGGATAGGTCCA** **TCAGGTGGAGGTATTGTTTCCGG CA**	**112 bp**	**60˚C**

22	**PGC-1β** **NM_001172699.1**	**F** **R**	**CTCACTGGCGGACAGCACCC** **GGGAGAGGCTGGAGCTGGCT**	**217 bp**	**60˚C**
23	**GLUT4** **NM_001042.2**	**F** **R**	**GGGGCCTGCCAGAAAGAGTCTG** **AGCTGCAGCACGACCGCAAT**	**177bp**	**60˚C**
24	**CD105** **NM_001278138.1**	**F** **R**	**GGTGACCCGCGCTTCAGCTT** **CTTGCTGGGG A CGCGTGTG**	**291 bp**	**60˚C**
25	**CD241** **NM_000324.2**	**F** **R**	**TGGGCGCCTCCAACACGTCTAT** **GGTTCCAGCTGGCTGTGGTCA**	**226 bp**	**60˚C**
26	**CD235** **NM_002099.6**	**F** **R**	**CGGGACACATATGCAGCCACTCC** **GGCAAGTTGTACCCTTTCTCCGGT**	**105 bp**	**60˚C**
27	**TEK/TIE2** **NM_000459.3**	**F** **R**	**TCACTTCGCTGCCGACGTGG** **CGCACTGGGAGCCTTCCCATT**	**183 bp**	**60˚C**
28	**SYK** **NM_001174167.1**	**F** **R**	**TGTGCCTGCTGCACGAAGGG** **GGGGAGGACGCAGGATGGGA**	**241 bp**	**60˚C**
29	**CD146** **NM_006500.2**	**F** **R**	**AACGTCAACGGCACGGCAAGT** **CCGGCTCCGGCAGCTTTCTC**	**274 bp**	**60˚C**
30	**SIRT3** **NM_001017524.2**	**F** **R**	**GCTTGGCGGCAGGGACGATTA** **CCCCGGCGATCTGAAGTCTGG**	**221 bp**	**60˚C**
31	**PGC-1 α** **NM_013261.3** **NM_013261.3**	**F** **R** **F** **R**	**ACTGCAGGCCTAACTCCACCCAC** **AACTCGGATTGCTCCGGCCC** **AGACGTCCCTGCTCGGAGCTT** **TGGGTGGAGTTAGGCCTGCAGT**	**191 bp** **283 bp**	**60˚C** **60˚C**

3.8 Primer optimization

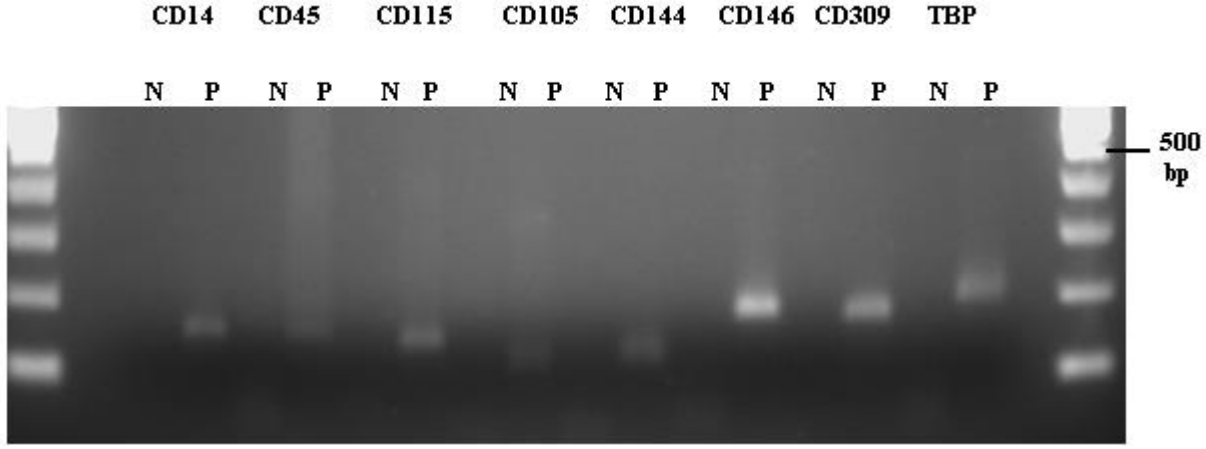


Figure 3.3. Showing RT-PCR results for primer testing. N stands for negative control where H_20 was used instead of cDNA. P stands for the positive control used which for macrophage markers (CD14, CD115) was day 7 macrophage cDNA, and for all other markers was HUVECS. TBP was used as a house keeping gene. 100 bp DNA ladder was used.

3.9 Reverse Transcription PCR

RT-PCR allows the formation of cDNA fragments from RNA. These fragments are then amplified using specific set of primers. Up to 500 ng of RNA was mixed with 1 µL oligo dt primer and 10mM of dNTP, and dH_2O was added to adjust the total volume to 13 µL. This mixture was incubated at 65˚C for 5 min, and then immediately put on ice for 1 min. This chilling was followed by addition of 4 µL of 5 x FSB (first strand buffer) along with 2 µL of DTT, and the mixture was allowed to incubate at 42˚C for 2 min. 1 µL reverse transcriptase (Superscript III) was added. Incubation was carried out at 42ºC for 1 hr to synthesize the cDNA, followed by incubation at 70˚C for 15 min in order to inactivate the enzyme. PCR was then carried out using a reaction setup with a final volume of 50 µL, and the annealing temperature was set specifically for each primer. The duration of extension was set according to the expected size of the product.

3.10 Quantitative polymerase chain reaction (qPCR)

Quantitative PCR refers to a laboratory technique based on PCR, which is used to amplify and at the same time quantify a targeted DNA molecule. This leads to both the detection and quantification of one or more sequences in a DNA sample. There are two main methods used for detection of PCR products in real-time PCR. These include, use of non-specific fluorescent dyes that intercalate with any double-stranded DNA and use of sequence-specific

DNA probes that have a fluorescent reporter tagged to them that allows detection only when the probe hybridizes with its complementary DNA target.

The gene expression analysis for the comparison between early and late EPCs and mature endothelial cells (HUVEC) was done using fast SYBR Green fluorescent chemistry. The method followed was real-time reverse-transcription PCR which is often referred to as qRT-PCR. RNA was extracted from the cells at different point times and was reverse transcribed to synthesize cDNA. For all the cDNA preparations, 100- 500 ng of RNA was used. Expected product size was around 100-200 bp. SYBR Green master mix was used and once the samples were prepared they were put in the light cycler (7500 Fast Real–time PCR system Applied Biosystems). Each sample contained 33 µL of fast SYBR Green master mix, 24.75 µL of dH_2O, .875 µL of each of the primer and 3.3 µL of cDNA prepared from total RNA. All the samples were run in triplicates. An increase in DNA product during the PCR lead to an increase in fluorescence intensity and was measured at each cycle allowing DNA concentrations to be quantified. As shown in the figure 3.4, the results are shown like this plot, where cycles are shown against ΔRn. Rn value or the normalized reporter value represents the fluorescence signal from SYBR Green normalized to the signal of the passive reference dye for a given reaction. ΔRn represents the difference between Rn values of an experimental reaction and Rn values of the baseline signals generated by the machine. As shown in the figure 3.5, a higher gene expression shows signals at earlier PCR cycles, whereas the negative control used for the reaction showing no expression of the gene is shown at higher cycles having high CT value. Quantitative PCR was carried out in triplicates for each sample and n represents the number of donors.

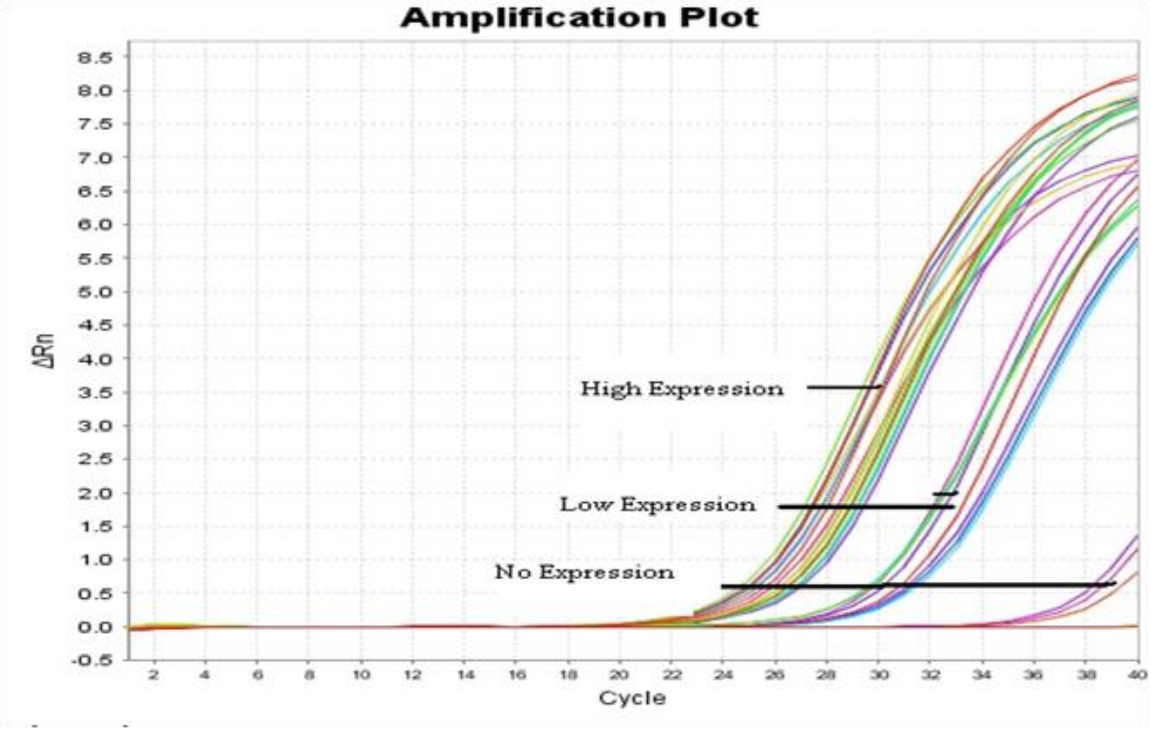


Figure 3.4. An amplification plot for a qPCR showing gene expression in terms of number of cycles and corresponding CT values.

In order to confirm the specificity of the primers that were designed for the PCR and to make sure that the SYBR Green is not detecting any nonspecific double-stranded DNA including primer dimers and contaminating DNA and PCR product from misannealed primer, a melt curve was analysed for all the primers. Melt curve analysis is a tool to assess the dissociation-characteristics of double-stranded DNA during heating. As more than one peak on a melt curve would indicate that the primers are producing nonspecific products. As shown in the figure there was only one peek seen in the melt curve, confirming that the primers were specific for the DNA target.

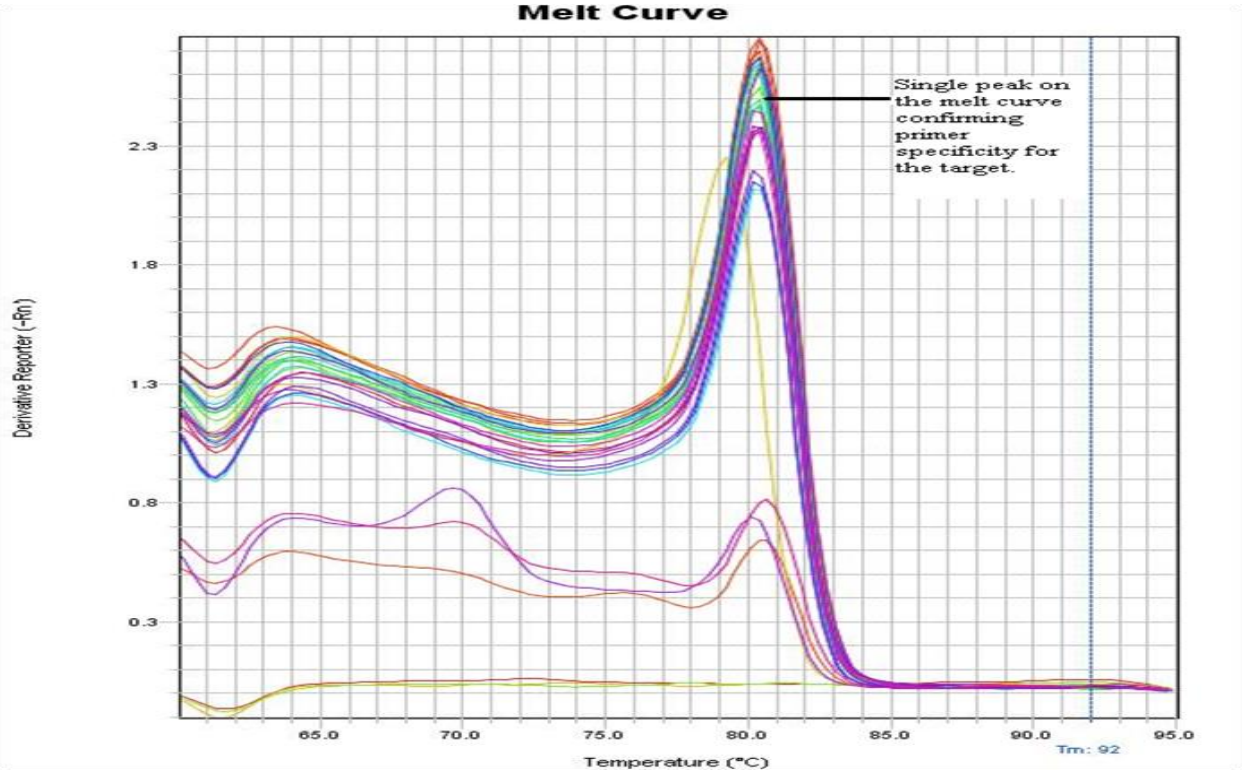


Figure 3.5. Showing melt curve stage during qPCR reaction.

3.11 Agarose gel electrophoresis

The concentration of agarose and applied electrical field depends upon the size of DNA sequences. Usually 3gm of agarose was added in 150 ml of 1 × TBE Buffer, and allowed to mix thoroughly by heating in microwave for around 2-3 min. The mixture was then allowed to cool down at around 60˚C, and then 150 µL of Gel Red (10 mg/ml) was added. This agarose mixture was poured into the gel chamber and allowed to solidify. 1× TBE was used as the electrophoresis buffer. 6× loading buffer was added to the samples and these were then loaded in to the gel. Electrophoresis was carried out at a voltage of around 80-110v, current of 400 mA for about 60-120 min. Sizes of the DNA fragments were determined by comparison with a 100 bp DNA ladder. The gel was observed under UV light.

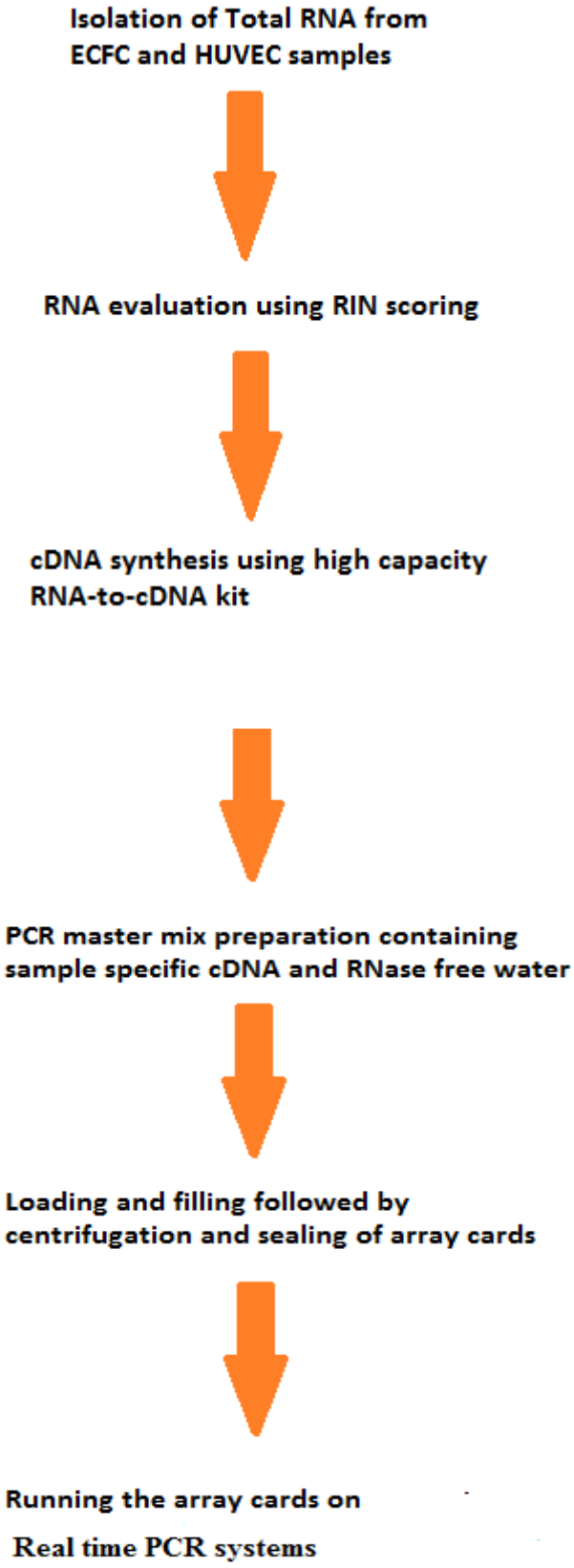
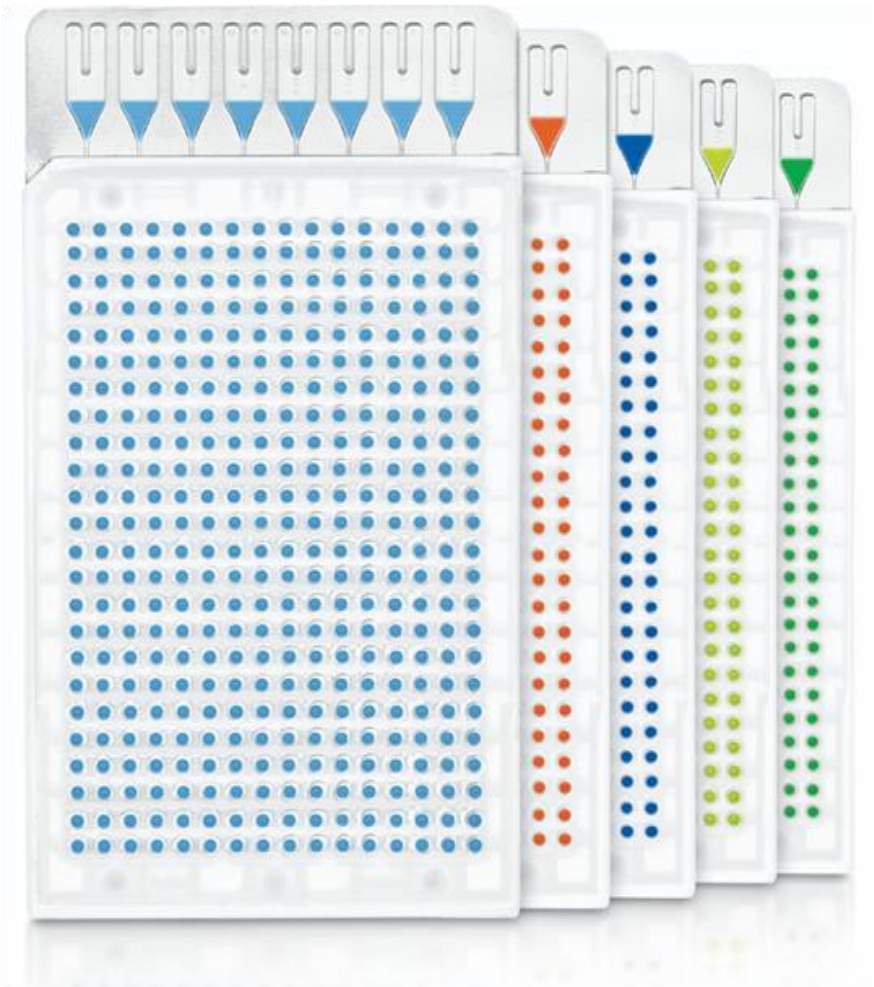


Figure 3.6. Schematic representation of the methods used for low density array (LDA).

3.12 Taqman array micro fluidic cards background

Taqman array micro fluidic cards are 384-well cards which are loaded with dried down gene expression assays. These cards allow gene expression measurement using comparative ΔΔCT method and using relative quantification for gene expression analysis. These cards are provided in different formats and the format used included using one sample per card and 192 genes to be analysed together including endogenous controls. Taqman array custom microfluidic cards were used that allowed selecting target genes (Taqman assays) and appropriate endogenous controls.

A



B

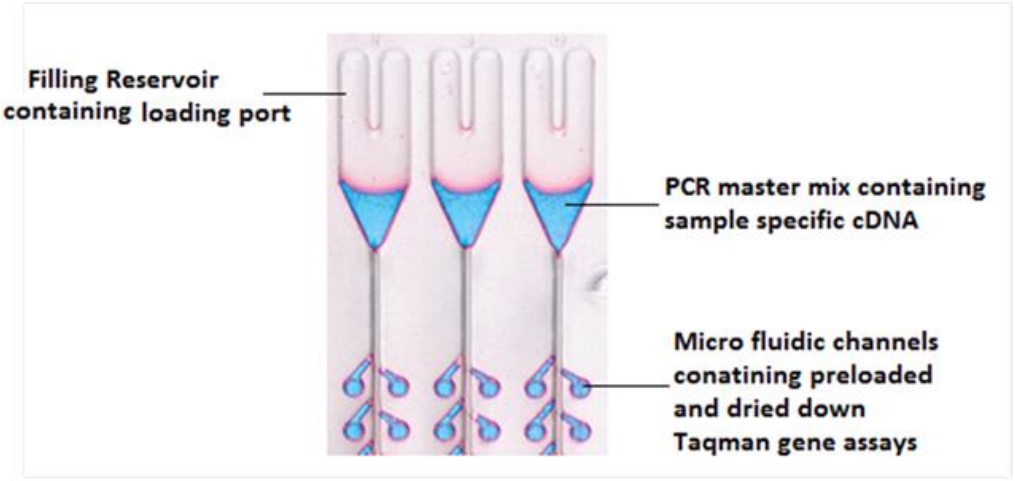


Figure 3.7. A), B) Showing Taqman array micro fluidic card design and channels containing Taqman assays in dried down form. (Adapted from applied Biosystems).

3.12.1 Assay selection strategy

The assays were selected based on three different criteria. We proposed that ECFCs are the endothelial progenitor cells that must be at an earlier stage of differentiation as compared to mature endothelial and as there is still no marker that can distinguish between these cells and mature endothelial cells (HUVEC). Therefore, several genes were picked that were expected to show expression in progenitor cells and absence in mature endothelial cells and vice versa.

We needed to test whether we can find genes that show high and low level of expression in ECFCs and HUVEC and could differentiate these cells on the basis of gene expression analysis.

Secondly, as our earlier results indicated that there was an up regulation of antioxidative genes in ECFCs and HUVEC when grown in hyperoxia (40% O_2) and also there was an up regulation of PGC-1 alpha and its downstream targets. As PGC-1 alpha is a transcriptional co activator we needed to investigate its downstream targets to establish if growing these cells in hyperoxia can have an effect on its downstream transcriptional factors that are known to be

involved in angiogenesis, electron transport chain assembly and mtDNA transcription as shown in figure below.

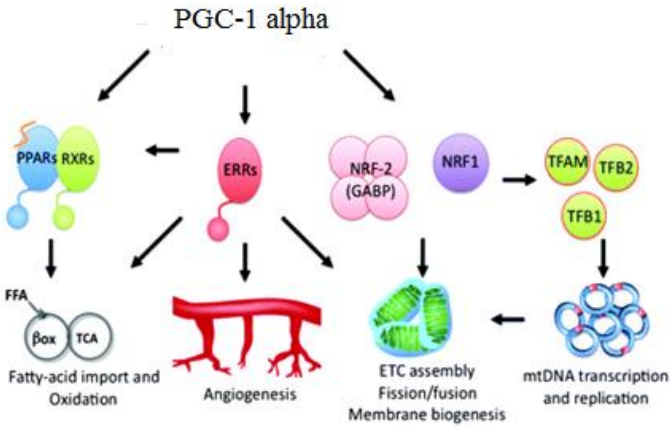


Figure 3.8. Showing schematic representation of PGC-1α and its downstream targets. Adapted from (Arany *et al*. 2010).

Thirdly, as we proposed that oxidative stress (40% O_2) could be used as a model to study atherosclerosis in ECFCs so we checked for genes that were involved in DNA damage, telomere dysfunctioning and inflammatory response and angiogenesis and if this leads to change in expression as a result of exposure to oxidative stress and if this response was different in mature endothelial cells grown in hyperoxia.

3.12.2 Endogenous controls used for the array

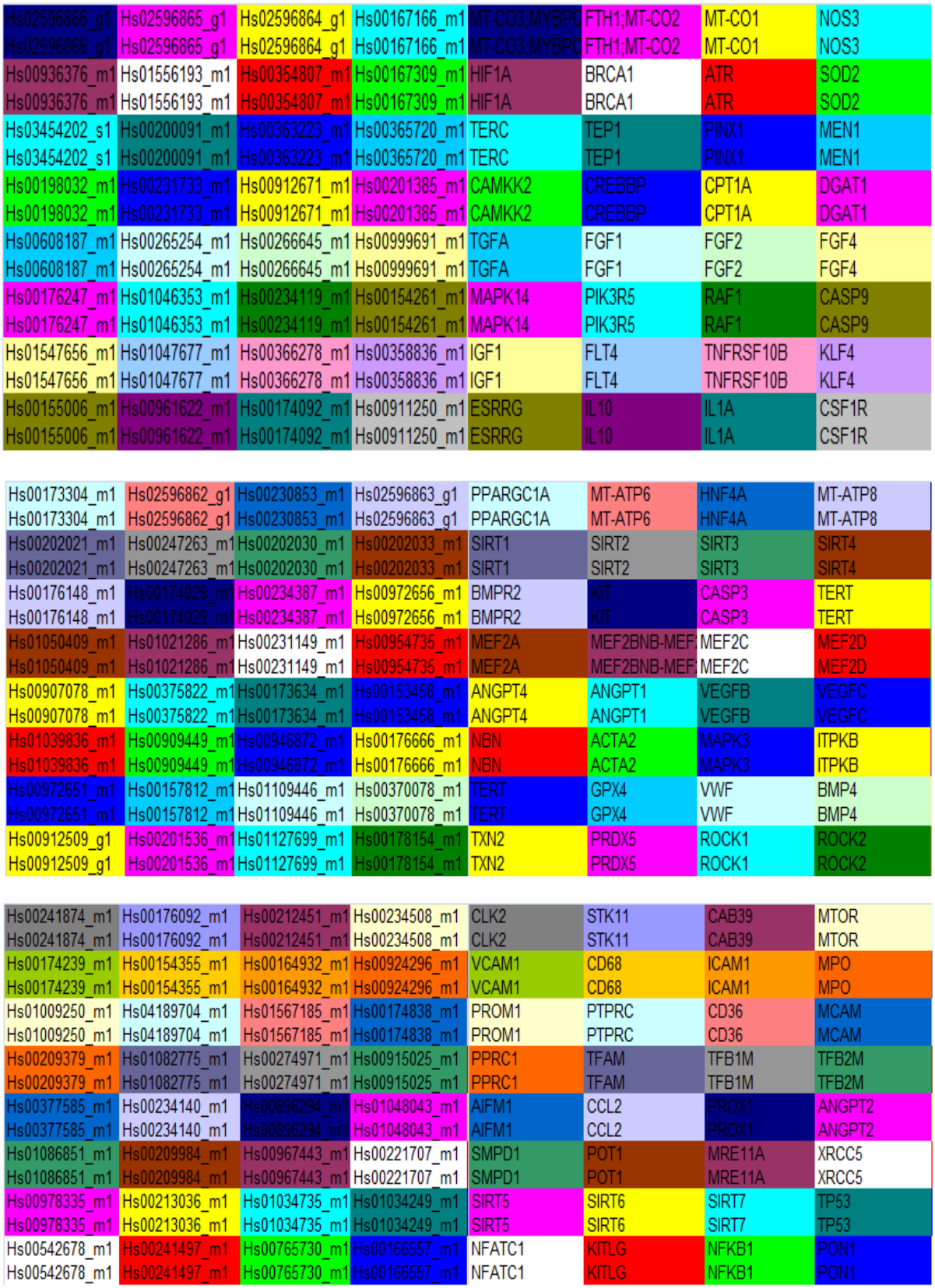
All the data were analysed using relative quantification method (ΔΔCT) and the data were normalized to three housekeeping genes that were used an endogenous control. These included:

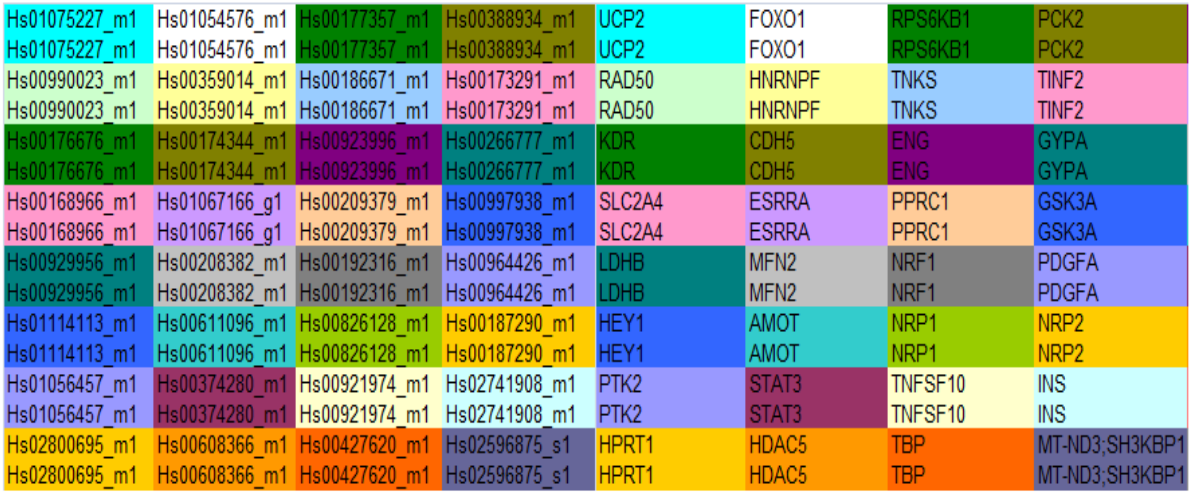
18s rRNA

Hypoxanthine phosphoribosyl transferase (HPRT1)

TATA box binding protein (TBP)

3.12.3 Low density array (LDA) gene map





Schematic representation of the low density array (LDA). In all, 192 assays were used. 3 assays were used as endogenous controls. The assays were run in duplicates.

3.12.4 Isolation of total RNA from ECFC and HUVEC samples and DNase treatment

RNA was extracted from ECFC and HUVEC samples using Qiagen RNeasy mini kit. Cells were kept frozen down at -80°C in freezing media containing DMSO. Cells were taken out from the freezer and quickly thawed and then transferred to a new eppendorf tube. This was followed by centrifugation at 800 x *g* for 5 min. Supernatant was removed and the cell pellet was disrupted by adding buffer RLT. The volume of lysis buffer used depended upon the no of cells:

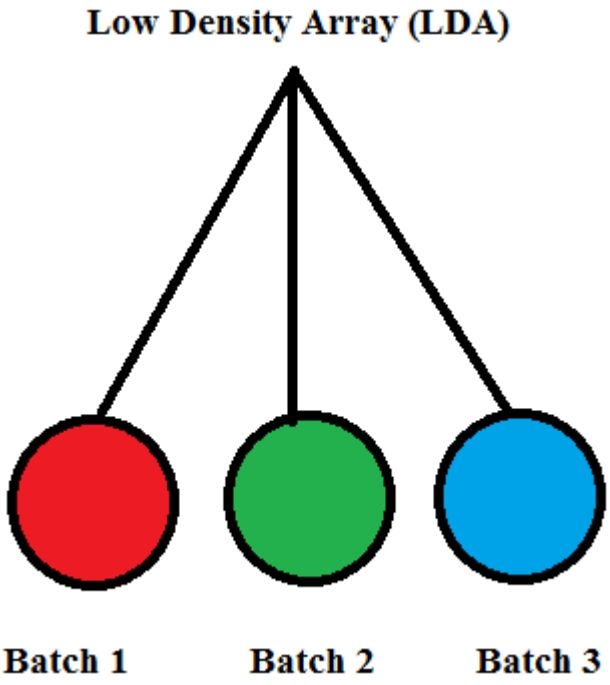
Table 3.2. Showing volume of lysis buffer to be used for extraction of total RNA

Number of cells	**Volume of RLT Buffer**
1-5 x 10^6	350 µL
5 x 10^6-1 x 10^7	600 µL

Cells were mixed well by gentle vortex or pipetting and the resulting cell lysate was directly transferred into a QIA shredder spin column which was placed in a collection tube. The column was subjected to centrifugation at 13000 x *g* for 2 min. This step was performed in order to homogenize the lysate as improper homogenization can lead to reduced RNA yield. Equal volume (350 µL) of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. The resultant 700 µL of the sample along with some precipitates that were formed were transferred to the RNeasy spin column which was placed in a collection tube. This was allowed to centrifuge for 15s at 13000 x *g*. The flow-through was discarded. The next step was to treat the samples with DNase in order to avoid genomic DNA contamination. 350 µL of wash buffer RW1 was added to the RNeasy spin column and lid was gently closed. This was followed by centrifugation for 15s at 13000 x *g*. Flow-through was discarded. DNase mix was prepared separately by adding 10 µL of DNase 1 from the stock to 70 µL of RDD buffer followed by gentle mixing. This DNase mix was added to the RNeasy column and incubated at room temperature for 15 min. After this incubation, 350 µL of buffer RW1 was added to the column and centrifuged at 13000 x *g* for 15s. Flow-through was discarded. This was followed by addition of 500 µL of buffer RPE to the RNeasy spin column and centrifugation at 13000 x *g* for 15s. This step was repeated with an additional centrifugation for 2 min at 13000 x *g* so that no residual ethanol is carried over that can affect downstream reactions. An optional step was performed by placing the RNeasy spin column in a separate

and new 2ml collecting tube and centrifugation for 1 min at full speed. This was done to avoid any carryover of buffer RPE. The spin column was finally placed in a 1.5 ml eppendorf tube and 10-20 µL of RNase free water was added to the column membrane directly. This was followed by a final centrifugation for 1 min at 13000 x *g*. RNA was eluted and quantified using Nanodrop. The quality of RNA was assessed by measuring A260/A280 ratio which was found to be around 1.7 - 2.1.

3.13.5 Samples used for Taqman low density array (LDA)



Same donor analysis

Batch 1: ECFC 1, ECFC H1, HUVEC 1 and HUVEC H1

Batch 2: ECFC 2, ECFC H2, HUVEC 2 and HUVEC H2

Batch 3: ECFC 3, ECFC H3, HUVEC 3 and HUVEC H3

ECFC 1, 2, 3 = ECFCs grown in normoxia (20%O_2) till P5.

ECFC H1, H2, H3 = ECFCs grown in normoxia till P2 and then grown in hyperoxia (40%O_2) till P5.

HUVEC 1, 2, 3 = HUVEC grown in normoxia till P5.

HUVEC H1, H2, H3 = HUVEC grown in normoxia till P2 and then grown in hyperoxia (40%O_2) till P5.

LDA samples were run in duplicates and n represents the number of donors. Three donors each (n=3) for ECFC normoxia, ECFC hyperoxia, HUVEC normoxia and HUVEC hyperoxia. One day 0 MNC sample was used that was a calibrator or reference sample and all the gene expression analysis was plotted relative to that. In all, thirteen array cards were used.

Table 3.3. Showing RNA purity analysis

LDA Sample	A260/280 Ratio
ECFC 1	2.07
ECFC H1	2.04
HUVEC 1	2.08
HUVEC H1	2.07
ECFC 2	2.06
ECFCH 2	1.71
HUVEC 2	2.08
HUVEC H2	2.07
ECFC 3	2.11
ECFC H3	2.07
HUVEC 3	2.06
HUVEC H3	1.96

3.12.6 RNA integrity analysis using bioanalyzer

After RNA extraction and quantification it was important to evaluate the integrity of RNA samples that would be used for the low density array (LDA).

RNA evaluation is the first and most important step involved in gene expression analysis. This RNA evaluation was carried out using Agilent RNA 6000 Pico kit. By using electrophoretic separation, the RNA samples were separated on micro fabricated chips and detected through laser via fluorescence detection. The bioanalyzer provides an electropherogram for the RNA sample along with a gel image and gives the RNA concentration and the ribosomal ratio (28s/18s). RNA integrity number (RIN) was developed to assess the RNA quality and grades from 1 -10 in increasing order of integrity.

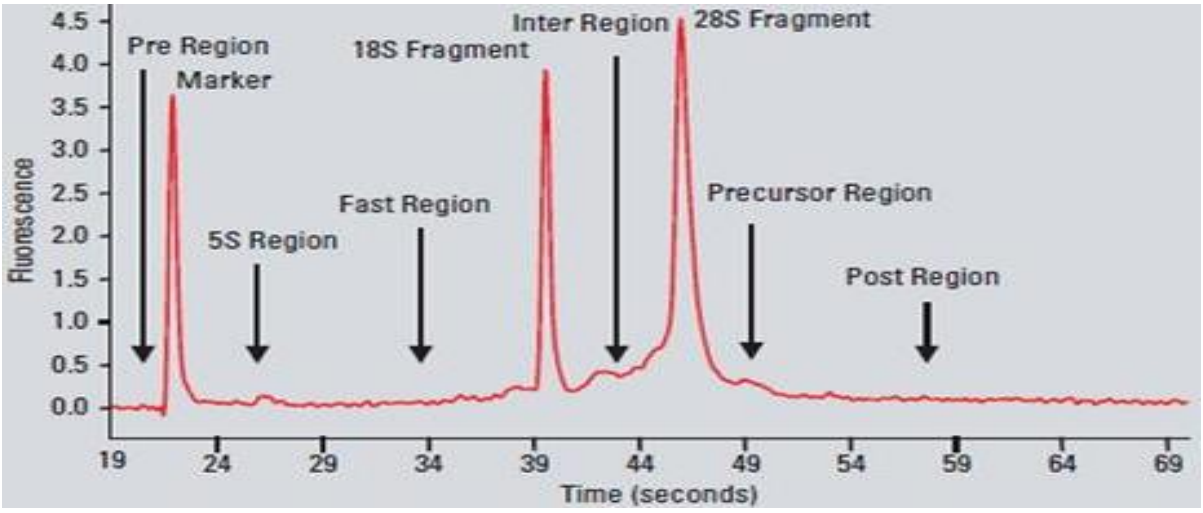


Figure 3.9. Showing an electropherogram with the regions indicative of RNA quality. (Adapted from Agilent technologies).

3.12.7 Preparation of RNA ladder

RNA ladder was spun down and allowed to heat denature for 2 min at 70°C. It was immediately put on ice to get cooled down. 90 µL of RNase-free water was added to the ladder and thoroughly mixed. Once prepared, the ladder was aliquoted into several RNase free vials and kept at -80°C.

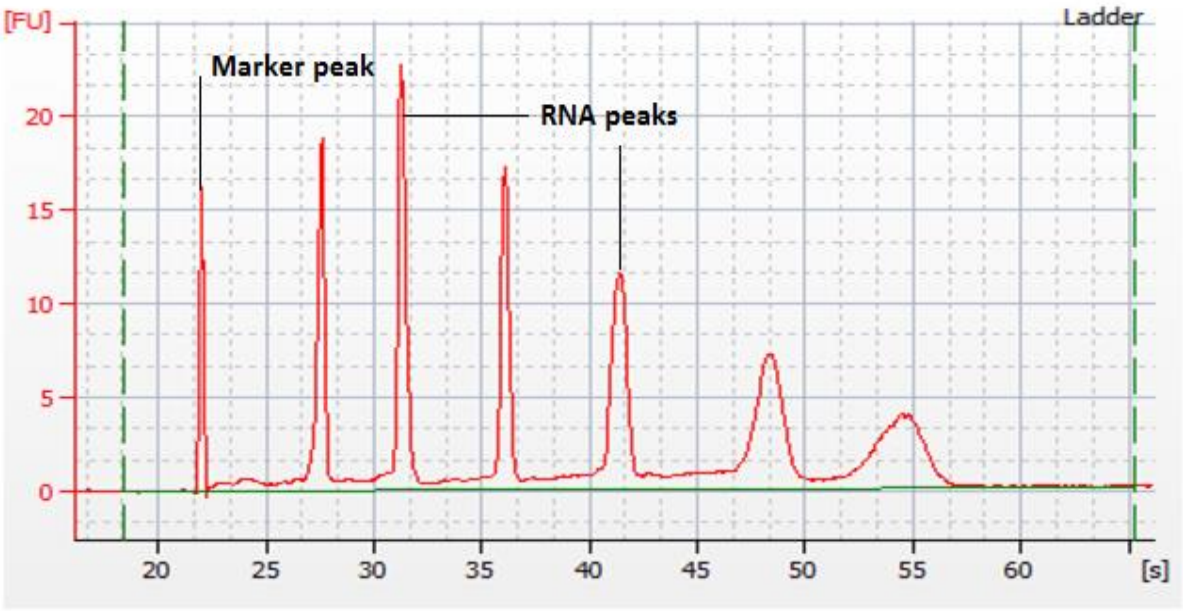


Figure 3.10. Showing RNA 6000 Pico ladder. Seven well resolved peaks are illustrated including the first marker peak and the six RNA peaks.

Table 3.4. Showing gene names and assay ID information used for LDA

Gene Symbol	Assay ID	Location on Array Card
PPARGC1A	Hs01016719_m1	A01
PRKAA1	Hs01562315_m1	A02
PPARGC1B	Hs00991677_m1	A03
UCP1	Hs00222453_m1	A04
UCP2	Hs01075227_m1	A05
FOXO1	Hs01054576_m1	A06
RPS6KB1	Hs00177357_m1	A07
PCK2	Hs00388934_m1	A08
G6PD	Hs00166169_m1	A09
AKT1	Hs00178289_m1	A10
AKT3	Hs00987350_m1	A12
CLK2	Hs00241874_m1	A13
STK11	Hs00176092_m1	A14
CAB39	Hs00212451_m1	A15
MTOR	Hs00234508_m1	A16
PPARGC1A	Hs00173304_m1	A17
MT-ATP6	Hs02596862_m1	A18
HNF4A	Hs00230853_m1	A19
MT-ATP8	Hs02596863_m1	A20
MT-CO3	Hs02596866_g1	A21
MT-CO2	Hs02596865_g1	A22
MT-CO1	Hs02596864_g1	A23
NOS3	Hs00167166_m1	A24
THPO	Hs00171249_m1	C01
TEK	Hs00176096_m1	C02
TERF1	Hs00744634_s1	C03
TERF2	Hs00194619_m1	C04
RAD50	Hs00990023_m1	C05
HNRNPF	Hs00359014_m1	C06
TNKS	Hs00186671_m1	C07
TINF2	Hs00173291_m1	C08
FIGF PIR-FIGF	Hs00189521_m1	C09
TGFB1	Hs00171257_m1	C10
TGFB2	Hs00234244_m1	C11
CCL2	Hs00234244_m1	C12
VCAM1	Hs00174239_m1	C13
CD68	Hs00154355_m1	C14
ICAM1	Hs00164932_m1	C15
MPO	Hs00924296_m1	C16
SIRT1	Hs00202021_m1	C17
SIRT2	Hs00247263_m1	C18
SIRT3	Hs00202030_m1	C19
SIRT4	Hs00202033_m1	C20
HIF1A	Hs00936376_m1	C21

BRCA1	Hs01556193_m1	C22
ATR	Hs00354807_m1	C23
SOD2	Hs00167309_m1	C24
CAT	Hs00156308_m1	E01
AIFM1	Hs00269879_m1	E02
GPX1	Hs00829989_gH	E03
ABCG2	Hs00184979_m1	E04
KDR	Hs00176676_m1	E05
CDH5	Hs00174344_m1	E06
ENG	Hs00923996_m1	E07
GYPA	Hs00266777_m1	E08
ITGA2B	Hs01116228_m1	E09
SRXN1	Hs00607800_m1	E10
CD34	Hs00990732_m1	E11
PECAM1	Hs00169777_m1	E12
PROM1	Hs01009250_m1	E13
PTPRC	Hs04189704_m1	E14
CD36	Hs01567185_m1	E15
MCAM	Hs00174838_m1	E16
BMPR2	Hs00176148_m1	E17
KIT	Hs00174029_m1	E18
CASP3	Hs00234387_m1	E19
TERT	Hs00972656_m1	E20
TERC	Hs03454202_s1	E21
TEP1	Hs00200091_m1	E22
PINX1	Hs00363223_m1	E23
MEN1	Hs00365720_m1	E24
PPARG	Hs01115513_m1	G01
RFC1	Hs01099126_m1	G02
POLE2	Hs00160277_m1	G03
SMC6	Hs00226378_m1	G04
SLC2A4	Hs00168966_m1	G05
ESRRA	Hs01067166_g1	G06
PPRC1	Hs00209379_m1	G07
GSK3A	Hs00997938_m1	G08
GSK3B	Hs01047719_m1	G09
PPARGC1A	Hs00173304_m1	G10
PPARGC1A	Hs01016722_m1	G11
PPARGC1A	Hs01016721_m1	G12
PPRC1	Hs00209379_m1	G13
TFAM	Hs01082775_m1	G14
TFB1M	Hs00274971_m1	G15
TFB2M	Hs00915025_m1	G16
MEF2A	Hs01050409_m1	G17
MEF2B	Hs01021286_m1	G18
MEF2C	Hs00231149_m1	G19
MEF2D	Hs00954735_m1	G20
CAMKK2	Hs00198032_m1	G21

CREBBP	Hs00231733_m1	G22
CPT1A	Hs00912671_m1	G23
DGAT1	Hs00201385_m1	G24
FGF21	Hs00173927_m1	I01
DNM1L	Hs00247147_m1	I02
FOXO3	Hs00818121_m1	I03
GPAM	Hs01573680_m1	I04
LDHB	Hs00929956_m1	I05
MFN2	Hs00208382_m1	I06
NRF1	Hs00192316_m1	I07
PDGFA	Hs00964426_m1	I08
PDK4	Hs01037712_m1	I09
PPARA	Hs00947539_m1	I10
TNF	Hs99999043_m1	I11
ZFYVE9	Hs00245109_m1	I12
AIFM1	Hs00377585_m1	I13
CCL2	Hs00234140_m1	I14
PROX1	Hs00896294_m1	I15
ANGPT2	Hs01048043_m1	I16
ANGPT4	Hs00907078_m1	I17
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VEGFB	Hs00173634_m1	I19
VEGFC	Hs00153458_m1	I20
TGFA	Hs00608187_m1	I21
FGF1	Hs00265254_m1	I22
FGF2	Hs00266645_m1	I23
FGF4	Hs00999691_m1	I24
CXCL12	Hs00171022_m1	K01
PTN	Hs00383235_m1	K02
CXCL2	Hs00601975_m1	K03
LECT1	Hs00993254_m1	K04
HEY1	Hs01114113_m1	K05
AMOT	Hs00611096_m1	K06
NRP1	Hs00826128_m1	K07
NRP2	Hs00187290_m1	K08
PDGFRA	Hs00998018_m1	K09
PDGFRB	Hs01019589_m1	K10
CCL3	Hs00234142_m1	K11
PLA2G4A	Hs00233352_m1	K12
SMPD1	Hs01086851_m1	K13
POT1	Hs00209984_m1	K14
MRE11A	Hs00967443_m1	K15
XRCC5	Hs00221707_m1	K16
NBN	Hs01039836_m1	K17
ACTA2	Hs00909449_m1	K18
MAPK3	Hs00946872_m1	K19
ITPKB	Hs00176666_m1	K20
MAPK14	Hs00176247_m1	K21

PIK3R5	Hs01046353_m1	K22
RAF1	Hs00234119_m1	K23
CASP9	Hs00154261_m1	K24
BAD	Hs00188930_m1	M01
PXN	Hs01104424_m1	M02
SOS1	Hs00362308_m1	M03
MTMR12	Hs00539666_m1	M04
PTK2	Hs01056457_m1	M05
STAT3	Hs00374280_m1	M06
TNFSF10	Hs00921974_m1	M07
INS	Hs02741908_m1	M08
BCL2	Hs00608023_m1	M09
TAF6	Hs00425763_m1	M10
VEGFA	Hs00900055_m1	M11
FLT1	Hs01052961_m1	M12
SIRT5	Hs00978335_m1	M13
SIRT6	Hs00213036_m1	M14
SIRT7	Hs01034735_m1	M15
TP53	Hs01034249_m1	M16
TERT	Hs00972651_m1	M17
GPX4	Hs00157812_m1	M18
VWF	Hs01109446_m1	M19
BMP4	Hs00370078_m1	M20
IGF1	Hs01547656_m1	M21
FLT4	Hs01047677_m1	M22
TNFRSF10B	Hs00366278_m1	M23
KLF4	Hs00358836_m1	M24
CXCR4	Hs00237052_m1	O01
HMOX1	Hs01110250_m1	O02
PTGS2	Hs00153133_m1	O03
EDN1	Hs00174961_m1	O04
HPRT1	Hs02800695_m1	O05
HDAC5	Hs00608366_m1	O06
TBP	Hs00427620_m1	O07
MT-ND3	Hs02596875_s1	O08
MT-CYB	Hs02596867_m1	O09
OGT	Hs00269228_m1	O10
PRMT1	Hs01587651_m1	O11
NAT10	Hs01120371_m1	O12
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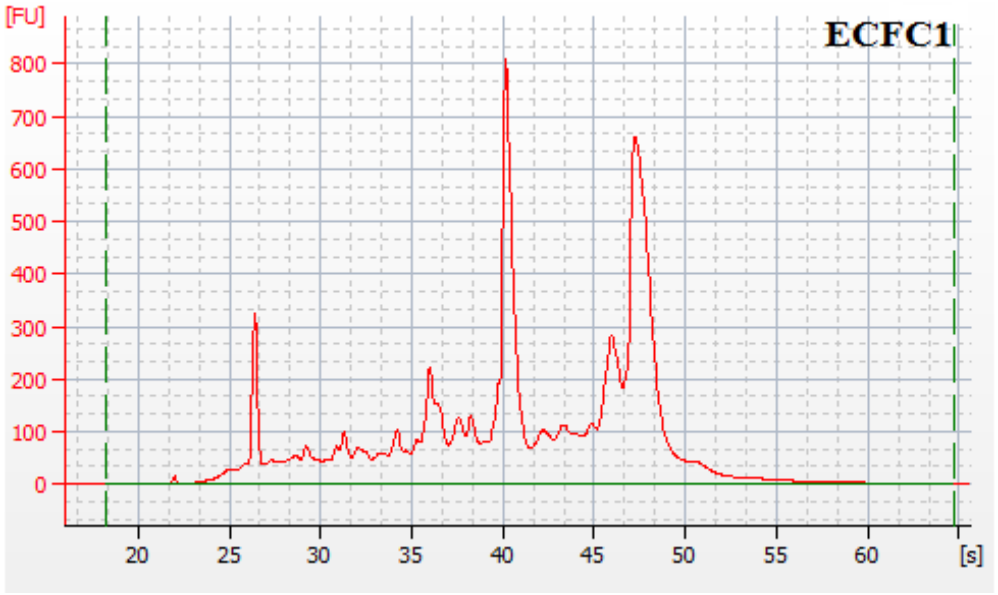
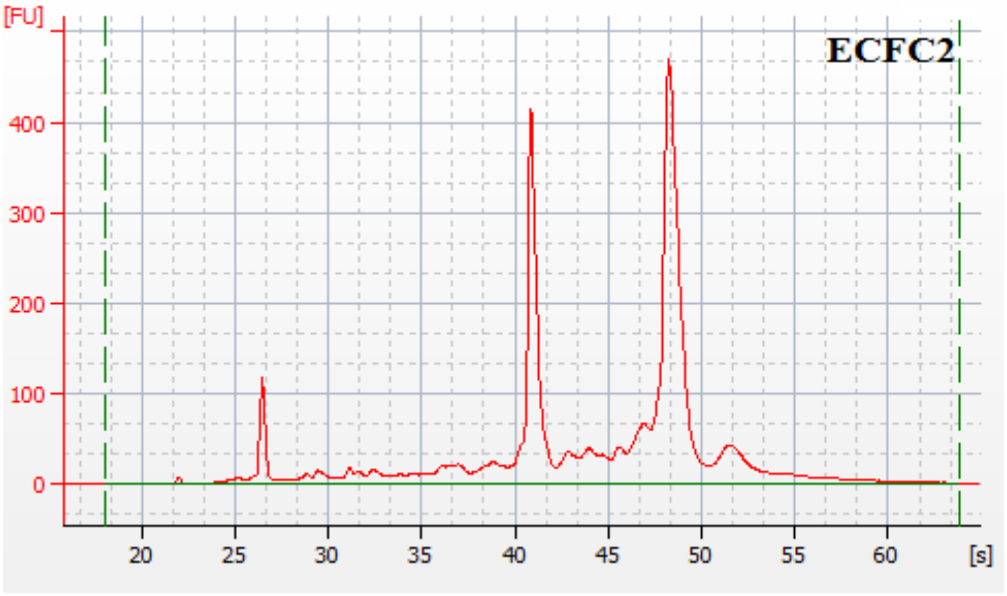
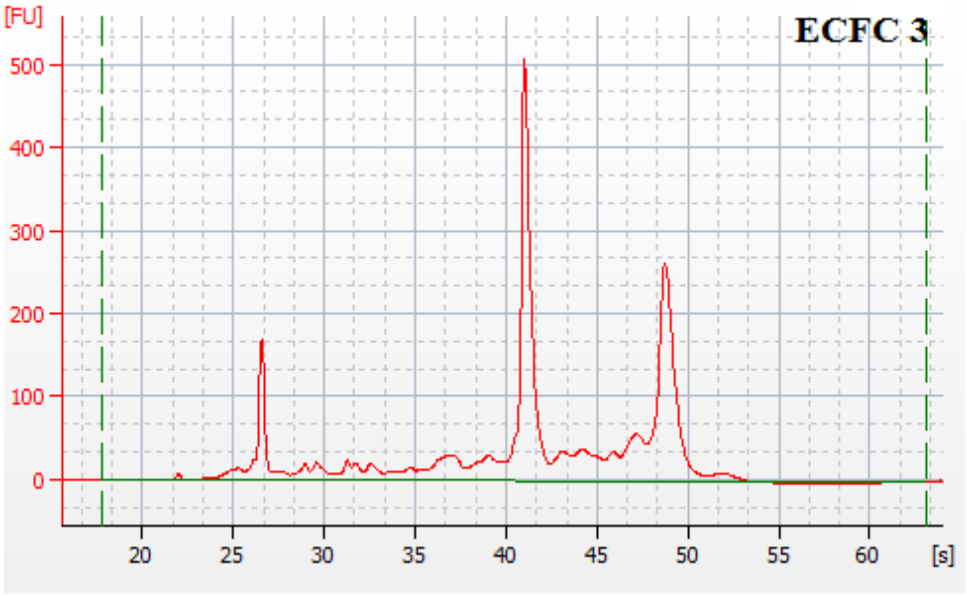
NFKB1	Hs00765730_m1	O15
PON1	Hs00166557_m1	O16
TXN2	Hs00912509_g1	O17
PRDX5	Hs00201536_m1	O18
ROCK1	Hs01127699_m1	O19
ROCK2	Hs00178154_m1	O20
ESSRG	Hs00155006_m1	O21

1L10	Hs00961622_m1	O22
1L1A	Hs00174092_m1	O23
CSF1R	Hs00911250_m1	O24

3.12.8 Electropherogram showing RNA integrity analysis of samples used for LDA



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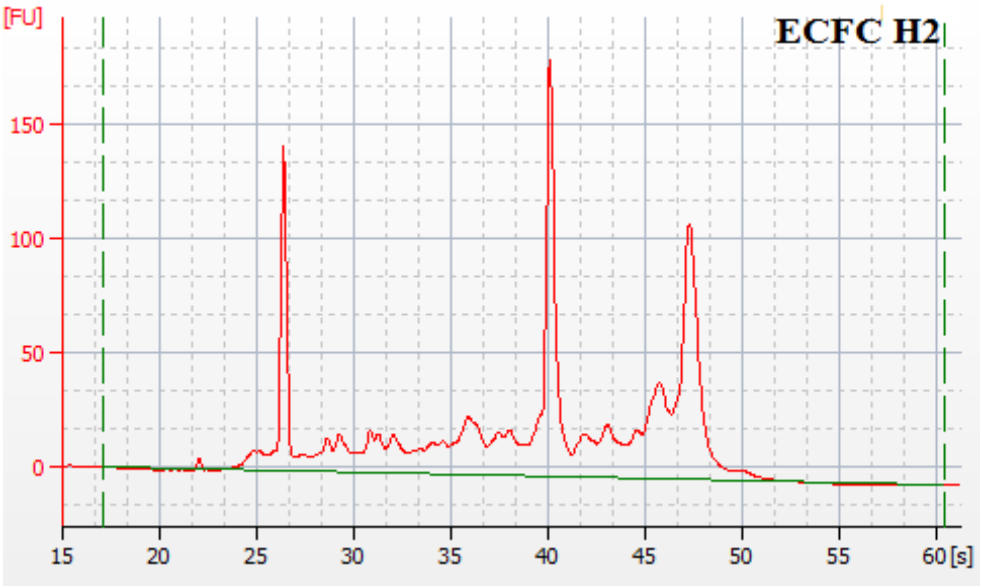
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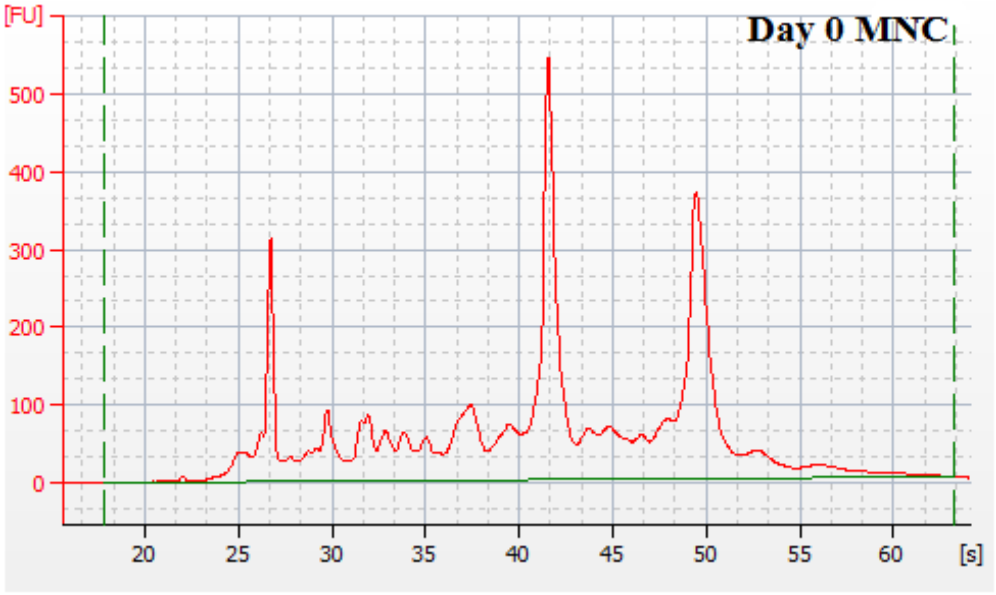
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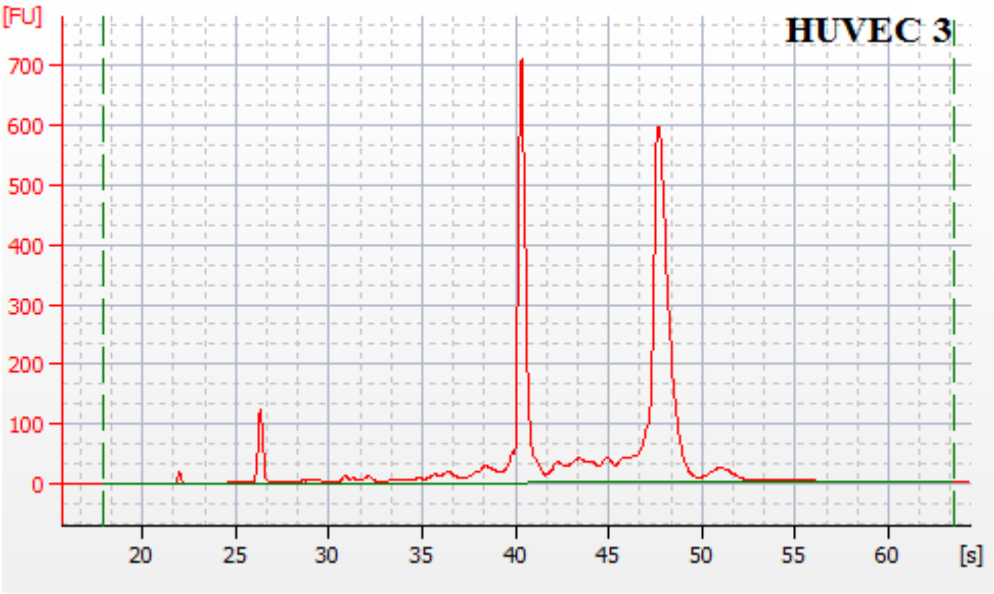
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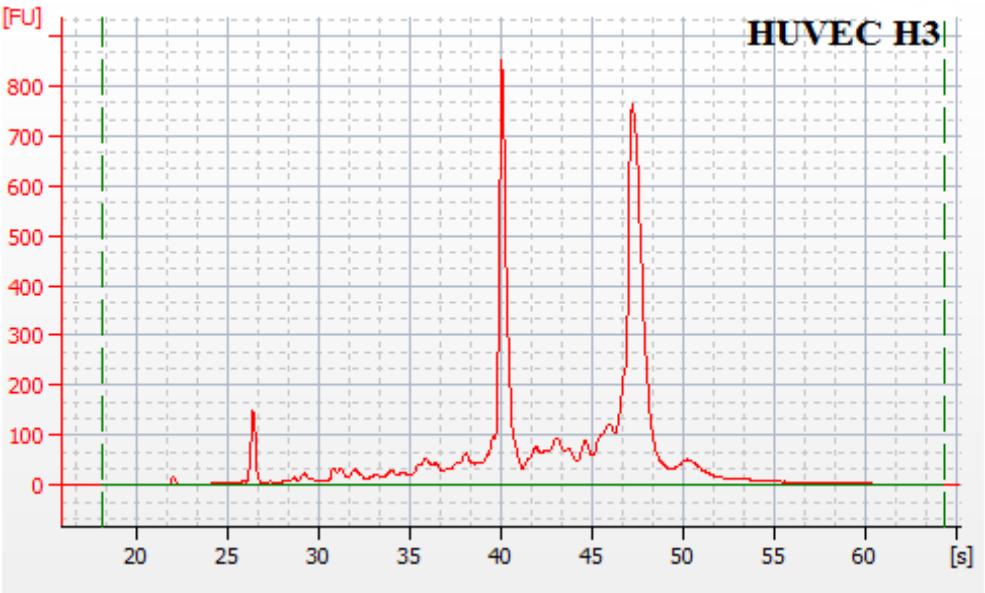
HUVEC H3
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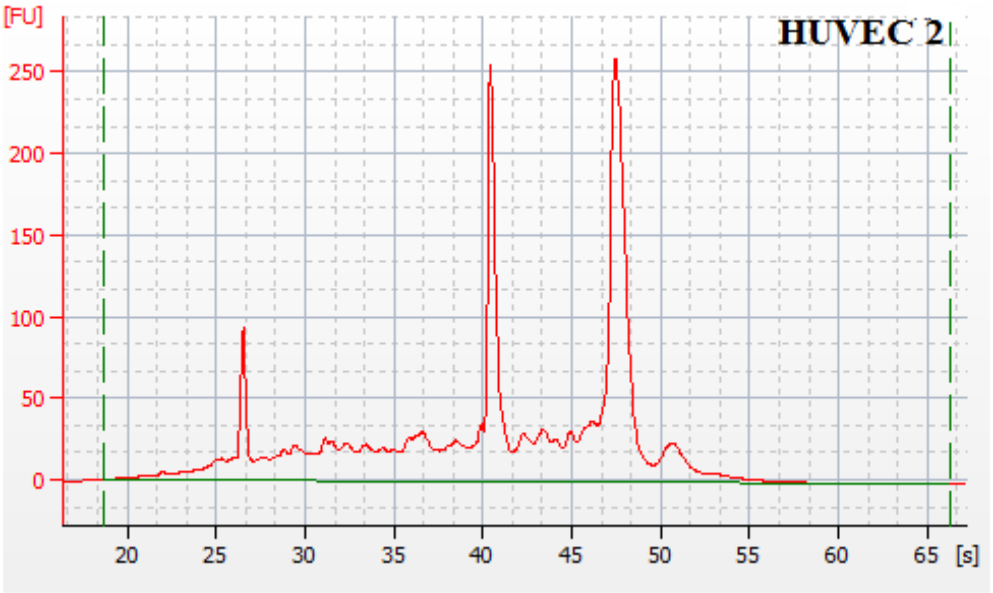
[s]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100
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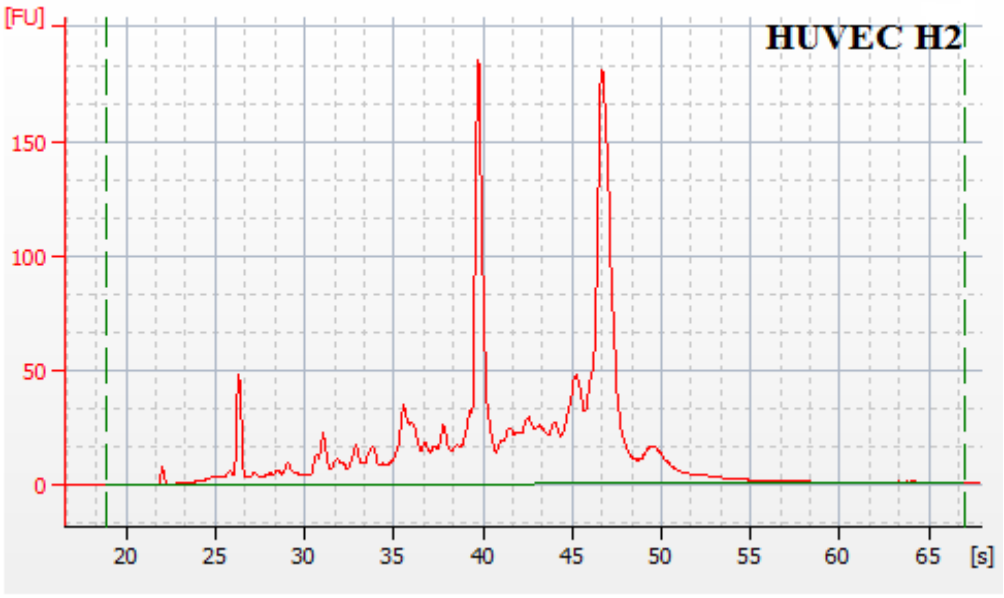
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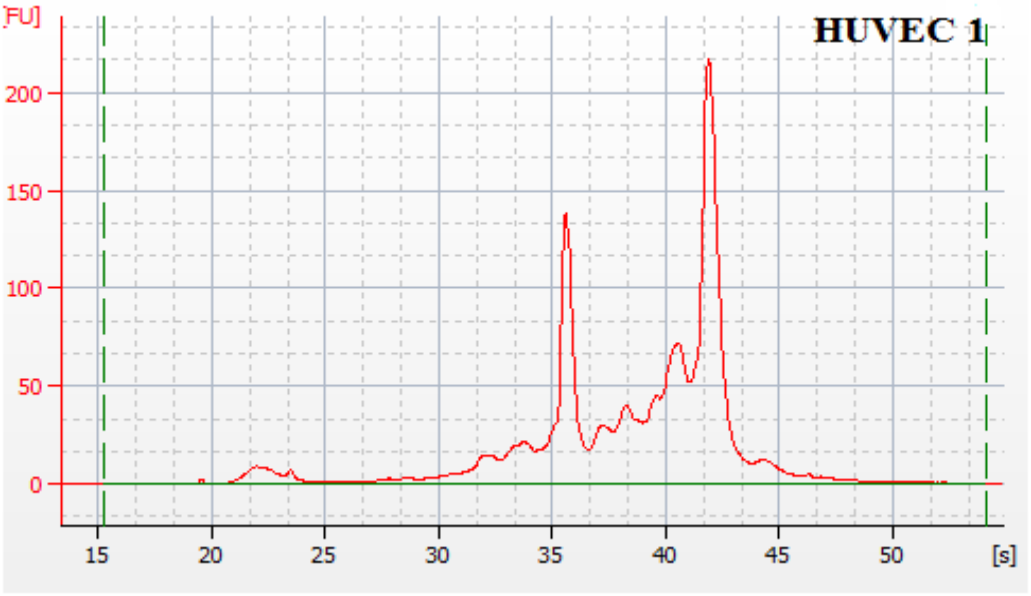
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[s]

3.12.9 cDNA synthesis using high capacity RNA-to-cDNA Kit

After extraction of total RNA from ECFC and HUVEC samples next step was to reverse transcribe it to produce complementary cDNA. High capacity kit was used to do this synthesis. The kit contained RT buffer mix (2X) that was stored at 4°C and RT enzyme mix (20X) that was kept at -20°C.

Preparation of RT reaction:

All the kit components were thawed on ice. RNA samples were taken out from -80°C and kept on ice. The following table was used to calculate the exact volume of the components to be used for the desired number of reactions. In all, 13 samples and array cards were used for a total of 8 reactions per card making it a total of 104 reactions for this LDA experiment.

Table 3.5. Showing reverse transcription preparation

Components	Volume/Reaction
2 X RT Buffer	10 x 8 = 80 µL
20 X RT Enzyme mix	1x8 =8 µL
Nuclease-free H_2O	To make it to 400 µL
RNA Sample	Up to 9 µL
Total per reaction	400 µL

The reaction was mixed well by pipetting and kept on ice.

Reverse transcription of the samples:

RT reaction mix was aliquot in to Eppendorf tubes and the tubes were sealed with appropriate caps. In order to remove the air bubbles, the tubes were briefly centrifuged. Reverse transcription was started by incubating the tubes at 37°C for 60 min. The reaction was stopped by heating at 95°C for 5 min and immediately put on ice. The cDNA samples were kept at -20°C until they were used to perform the PCR.

Preparation of PCR mix:

Taqman fast advanced master mix was used for this purpose. cDNA samples that were kept at -20°C were taken out and thawed by at room temperature. Sample tubes were briefly

vortexed followed by centrifugation. The following table represents the calculations that were used for this array.

Table 3.6. Showing final calculations for the PCR to be loaded in fill reservoir

Component	Volume per fill reservoir
cDNA Sample (50 ng) + nuclease-free H_2O	50 µL
Taqman Fast advanced master mix	50 µL
Total volume	100 µL

3.12.10 Loading the PCR reaction mixes in to array card reservoirs

For each card one cDNA sample was used. Taqman array card was removed from 4°C and kept at room temperature for about 30 min. Sample was removed from -20°C and thawed at room temperature. Each sample contained 400 µL of cDNA and nuclease free water. Equal volume of Taqman fast advanced master mix was added to the sample and mixed well by pipetting. 100 µL of sample specific master mix was loaded in to each reservoir.

3.12.11 Centrifugation of array cards

After loading the PCR master mix to all 8 fill reservoirs, the next step was to centrifuge the cards so that the cDNA sample could be distributed evenly across the wells. Centrifugation was carried out using Heraeus centrifuge machine and bucket type was set to 15679. The card was centrifuged using settings shown below in the table.

Table 3.7. Showing centrifugation settings used for LDA

Parameter	QUIKSet (Knob-operated)
Up Ramp rate	3
Down Ramp rate	N/A
Rotational speed	1200 rpm
Centrifugation time	2 x 1 min

Once the centrifugation was completed, the card was removed and checked properly if filling was complete. It was confirmed that the amount of cDNA sample left in each fill reservoir was uniform and consistent. If it was not found to be like that, an additional 1 min centrifugation was performed.

3.12.12 Sealing of array cards

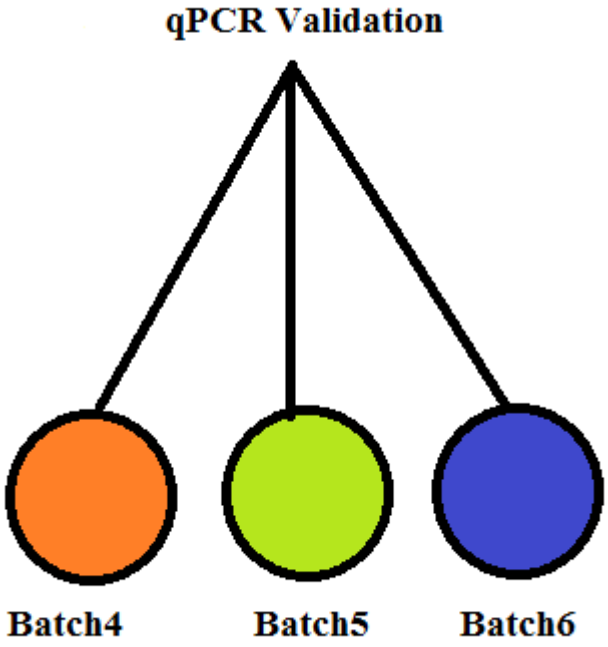
After centrifugation was complete, the next step was to seal the array cards. This was a very important step because sealing of the card allows the isolation of wells after cDNA samples were evenly distributed following centrifugation. The sealer works by using a carriage to seal the fluidic channels of the array. It was important to check that the sealer's carriage was in starting position which could otherwise damage the card if allowed to seal in that position. Taqman array card was inserted in to the sealer in such a way that the end with fill reservoir was closest to the arrows at the base of the sealer. Array card was gently pushed until it was fixed securely in the insert plate. The carriage was pushed across the sealer's base in the direction of the arrows indicated on the sealer. It was important to perform this step slowly and steadily to finish the sealing smoothly.

Once the card was sealed the last step was to trim the filling reservoirs from the array card using scissors. The card was run on ABI prism 7900 sequence detection system.

3.12.13 Real time data analysis

Data analysis was carried out using SDS 2.3 software and RQ manager version 1.2.

3.13 qPCR Validation for low density array



Batch 4: ECFC4, ECFC H4, RNA extracted from samples from Batch 2 but at different passages. P4 for normoxia and P2 for hyperoxia samples. HUVEC4, HUVEC H4 RNA extracted from samples from an additional batch at P5 and P3.

Batch 5: ECFC5, ECFC H5, HUVEC5 and HUVEC H5. RNA extracted from different samples from Batch 1 but at same passages. P5 for normoxia and P3 for hyperoxia.

Batch 6: ECFC 6, ECFC H6, HUVEC 6, HUVEC H6. RNA extraction from an additional batch. All from same donor. P5 for normoxia and P3 for hyperoxia.

3.13.1 Rationale for qPCR validation

After finishing the LDA experiments, the next step was to validate the results. There were several genes that showed up regulation in ECFCs when grown in hyperoxia (40% O_2) and several genes that showed down regulation in HUVEC when gown in hyperoxia (40% O_2). So it was important to see if this difference persists with different samples and if this changes across passages. In order to do that we picked the genes that showed statistically significant difference amongst samples and used Taqman gene expression assays to validate these results

across different samples. Samples were run in triplicates and n represents the number of donors.

Taqman gene expression assays are pre-optimized primer and probe based assays designed for qPCR analysis. The primers are formulated at a concentration of 20 µm. Each assay contains three target-specific oligonucleotides. They include:

Unlabelled PCR Primer pair

Taqman probe with FAM dye label and minor groove binder (MGB) moiety on the 5′ end non fluorescent quencher (NFQ) on the 3′ end. Taqman gene expression assays are used to amplify cDNA in the qPCR step in RT–PCR. We used two step RT-PCR approach using total RNA isolated from samples. RNA was quantified using Nanodrop.

Table 3.8. Showing RNA purity analysis

qPCR Sample	A260/280 Ratio
ECFC 4	**2.03**
ECFCH4	**2.18**
HUVEC 4	**2.12**
HUVEC H4	**2.03**
ECFC 5	**2.03**
ECFC H5	**1.93**
HUVEC 5	**1.91**
HUVEC H5	**1.95**
ECFC 6	**2.07**
ECFC H6	**1.96**
HUVEC 6	**2.12**
HUVEC H6	**2.10**

3.13.2 qPCR using Taqman gene expression assays

These assays are based on 5′ nuclease activity of *Taq* DNA polymerase. Taqman probes hybridize to the target DNA between two unlabelled primers. Signal from the fluorescent dye on the 5′ end of the probe is quenched by NFQ on its 3′ end. This occurs through fluorescence resonance energy transfer (FRET). During PCR, *Taq* polymerase extends the unlabelled primer using template as a guide. When the polymerase reaches the Taqman probe, it cleaves the molecule, separating the dye from the quencher and allowing it to fluoresce. This fluorescence is detected by the PCR machine using nonquenched FAM dye.

With each PCR cycle, there is a release of dye molecules which results in an increase in fluorescence intensity that is proportional to the amount of amplicon synthesized.

Table 3.9. Showing Taqman gene expression assay IDs and transcript accession information

Gene Symbol	Gene ID	Assay ID	Transcript Accession	Location on NCBI Genome Assembly	Location on Transcript or Gene	Size of Amp (bp)
ITPKB	3707	Hs00176666_m1	NM_002221.3	226819391	2583	71
PRDX5	25824	Hs00201536_m1	NM_181651.2	64085560	299	128
PPARGC1A	10891	Hs01016721_m1	NM_013261.3	23793644	2413	80
MT-ATP8	4509	Hs02596863_g1	NC_012920.ATP8.0	NA	38	120
MT-CYB	4519	Hs02596867_s1	NC_012920.CYB.0	NA	692	151
MT-ND3	4537	Hs02596875_s1	NC_012920.ND3.0	NA	118	150
VEGFC	7424	Hs00153458_m1	NM_005429.2	177604691	1140	126
GPX4	2879	Hs00157812_m1	NM_001039847.1	1103936	197	123
PON1	5444	Hs00166557_m1	NM_000446.5	94927669	1010	122
PECAM1	5175	Hs00169777_m1	NM_000442.4	NA	2376	65
CXCL12	6387	Hs00171022_m1	NM_199168.3	44872506	273	77
KIT	3815	Hs00174029_m1	NM_001093772.1	55524095	154	64
IL1A	3552	Hs00174092_m1	NM_000575.3	113531488	1578	69
KDR	3791	Hs00176676_m1	NM_002253.2	55944426	2567	84
ABCG2	9429	Hs00184979_m1	NM_004827.2	89011416	1027	92
NRP2	8828	Hs00187290_m1	NM_018534.3	206547224	863	81
SIRT4	23409	Hs00202033_m1	NM_012240.2	120740124	559	106
FGF2	2247	Hs00266645_m1	NM_002006.4	123747863	649	82
ANGPT1	284	Hs00375822_m1	NM_001199859.1	108261710	925	74
PCK2	5106	Hs00388934_m1	NM_001018073.1	24563483	405	73
TBP	6908	Hs00427620_m1	NM_001172085.1	170863421	578	91
TGFA	7039	Hs00608187_m1	NM_001099691.2	70674410	614	70
PROX1	5629	Hs00896294_m1	NM_002763.3	214161860	2301	74
VEGFA	7422	Hs00900055_m1	NM_001025366.2	43737946	1352	59
CDH5	1003	Hs00901463_m1	NM_001795.3	66400525	1994	63
ACTA2	59	Hs00909449_m1	NM_001141945.1	90694831	455	64
TEK	7010	Hs00945146_m1	NM_000459.3	27109147	3742	123
PDGFRA	5156	Hs00998018_m1	NM_006206.4	55095264	2481	84
PROM1	8842	Hs01009250_m1	NM_002609.3	15969849	2864	75
PDGFRB	5159	Hs01019589_m1	NM_002609.3	149493400	3374	62
TP53	7157	Hs01034249_m1	NM_001126112.2	7571720	1301	108
PDK4	5166	Hs01037712_m1	NM_002612.3	95212807	1098	74
FLT1	2321	Hs01052961_m1	NM_001159920.1	28959688	1387	72
PPARG	5468	Hs01115513_m1	NM_138711.3	12330436	812	90
NOS3	4846	Hs01574659_m1	NM_001160109.1	150690892	266	107
HPRT1	3251	Hs02800695_m1	NM_000194.2	133594175	297	82
18S	HSRN	Hs99999901_s1	X03205.1	109078	604	187
ANGPT2	285	Hs01048043_m1	NM_001118888.1	6357172	973	113
MT-C01	4512	Hs02596864_g1	NC_012920.CO1.0	NA	613	94
VWF	7450	Hs01109446_m1	NM_000552.3	6058040	308	56

Table 3.10. Showing statistical analysis for the samples used for LDA and qPCR validation using two way ANOVA

Gene Name	ECFC 20%Vs40%	HUVEC 20%Vs40%
ABCG2	*P < 0.05	ns P > 0.05
ACTA 2	***P < 0.001	ns P > 0.05
ANGPT1	***P < 0.001	ns P > 0.05
ANGPT2	***P < 0.001	* P < 0.01
CDH5	*P < 0.05	ns P > 0.05
CXCL12	***P < 0.001	ns P > 0.05
FGF2	*P < 0.05	ns P > 0.05
FLT1	**P < 0.01	ns P > 0.05
GPX4	*P < 0.05	ns P > 0.05
IL1A	*P < 0.05	**P < 0.01
ITPKB	*P < 0.05	ns P > 0.05
KDR	***P<0.001	ns P > 0.05
KIT	*P < 0.05	ns P > 0.05
MT-ATP8	***P<0.001	ns P < 0.05
MT-C01	*P < 0.05	ns P > 0.05
MT-CYB	*P < 0.05	ns P > 0.05
MT-ND3	*P < 0.05	ns P > 0.05
NOS3	*P < 0.05	ns P > 0.05
NRP2	ns P > 0.05	ns P > 0.05
PCK2	ns P > 0.05	* P < 0.05
PDGFRA	*P < 0.05	ns P > 0.05
PDGFRB	*P < 0.05	ns P > 0.05
PDK4	ns P > 0.05	*P < 0.05
PECAM1	*P < 0.05	ns P > 0.05
PPARG	**P<0.01	ns P > 0.05
PPARGC1A	*P < 0.05	ns P > 0.05
PRDX5	ns P > 0.05	ns P > 0.05
PROM1	***P<0.001	ns P > 0.05
PROX1	*P < 0.05	ns P > 0.05
SIRT4	ns P > 0.05	***P<0.001
TEK	ns P > 0.05	ns P > 0.05
TGFA	ns P > 0.05	***P<0.001
TP53	ns P > 0.05	*P < 0.05
VEGF A	***P<0.001	ns P > 0.05
VEGF C	***P<0.001	*P < 0.05
VWF	ns P > 0.05	***P<0.001

3.14 Telomerase activity measurement using telomeric repeat amplification protocol (TRAP) assay

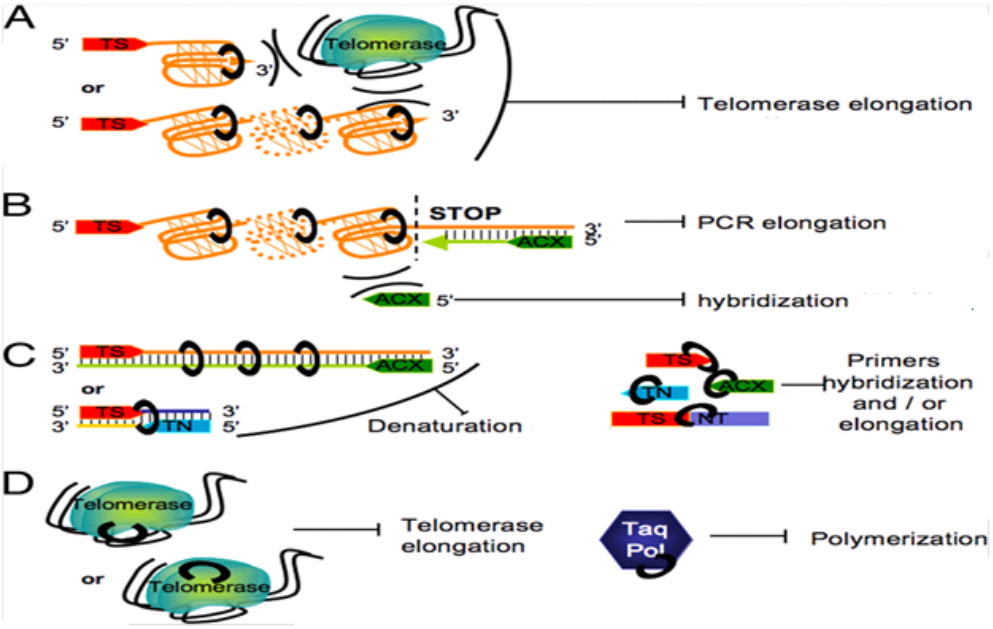


Figure 3.11. Showing TRAP assay used for the measurement of telomerase activity. (Adapted from De Cian *et al.*, 2007).

TRAP assay also known as telomeric repetition amplification protocol is used to measure telomerase activity in desired cell types. Telomerase is an enzyme, belonging to ribonucleo protein family. It acts as a reverse transcriptase and has its own RNA molecule which is used as a template for telomere elongation, which is shortened with each replicative cycle. The main function of telomerase is to add DNA sequence repeats (TTAGGG) at 3′ end of the telomeric DNA strands located in regions at the extreme ends of eukaryotic chromosomes called telomeres.

Human telomerase consists of two main catalytic subunits, a protein component that has reverse transcriptase activity called human telomerase reverse transcriptase (TERT or hTERT), being encoded by the same gene and the telomerase RNA (TERC), that acts as a template for this telomere repeats. Telomerase therefore elongates telomeres in DNA strands allowing senescent cells to exceed the Hay flick limit (number of times a cell population can divide before the cell division stops) and potentially becomes immortal, as seen in cancer cells.

ACX (HPLC purified) 100ng/ µL 5'- GCGCGGCTTACCCTTACCCTTACCCTAACC-3'
TS (HPLC purified) 100ng/ µL 5'- AATCCGTCGAGCAGAGTT-3'

TRAP assay was carried out on two cell types endothelial progenitor cells ECFCs and mature endothelial cells HUVEC while cord blood mononuclear cells (HUCBC) were used as reference samples to which relative telomerase activity was measured.

Endothelial colony forming cells (ECFCs) were obtained from umbilical cord blood and allowed to differentiate in endothelial specific media (EBM-2). The cells were grown in normoxia (20% oxygen) and hyperoxia (40% oxygen). In normoxia cells were grown till passage 10 (P10) and were passaged regularly once they became around 70-80% confluent. Trypsin–EDTA was used to split the cells. Once the cells were grown for passage 2 (P2) in normoxia they were incubated in hyperoxia for an additional passage (P1). Cells were frozen down with each passage in freezing media containing DMSO. Mature endothelial cells HUVEC were isolated from human umbilical vein and were grown in normoxia (20% oxygen) till passage 10 (P10). The cells were split regularly once they grew to 70-80% confluence.

Frozen cells were thawed quickly and spun down at 800 x *g* for 5 min. Supernatant was aspirated and the cell pellet was resuspended in NP40 cell lysis buffer to produce 50,000 cells/ µL suspensions. The resultant cell suspension was incubated for 30 min on ice. This was followed by a centrifugation at 16000 x *g* at 4°C for 20 min. The supernatant as transferred to a pre-cooled eppendorf and the sample was ready to use for the assay.

No of cells used for telomerase activity measurement (TRAP assay)

100,000 cells/triplicate

300,000 cells/sample

Table 3.11. Showing calculations used for telomerase activity measurement

Material	Volume per tube in µL	Volume per triplicate in µL
Sybr Green PCR Master mix	12.5	37.5
ACX primer	1	3
TS primer	1	3
dH_2O	8.25	24.75
EGTA	.25	.75

Final volume used per tube was 75 µL, so 69 µL of this master mix was used and 6 µL of sample was added. The resultant mixture was divided in to three triplicates of 25 µL each and samples were placed in the PCR machine rack and the TRAP assay programme was run.

There were five controls used for the TRAP assay, to make sure that it has worked well. These controls included the following as shown in the table below:

Table 3.12. Showing controls used for TRAP

1	Distilled water (dH_2O)
2	No primers (ACX and TS)
3	NP40
4	R8 1: 100
5	R8 1: 1000

The PCR programme used for the TRAP assay was as follows:

Table 3.13. Showing PCR programme used for the assay

Elongation	25°C for 30 min
Denaturation	95° C for 10 min
Denaturation	95° C for 10 sec
Annealing	60 ° C for 1 min
PCR cycles	40

3.15 Direct immunofluorescence analysis

Immunofluorescence refers to the labelling of antigens by using fluorescent dyes. This technique is widely used to detect subcellular distribution of specific biomolecule targets with in a cell. Immunofluorescence-stained cells or tissue sections can be studied in detail using light microscopy, confocal microscopy and flow cytometry analysis. The first step is to prepare cells and making them attached to a solid support. The next step is to fix and permeabilize the cells in order to ensure proper access of the antibody to its specific antigen. The third step involves incubation with the antibody. Antibody which is not bound is removed by washing and unbound antibody is detected either directly (if primary antibody is fluorochrome-labelled) or indirectly using a secondary antibody, labelled with a fluorochrome. Finally, the staining is evaluated by using fluorescent microscopy.

3.15.1 Experimental design

We needed to test whether endothelial colony forming cells (ECFCs) were at an earlier stage of differentiation than mature endothelial cells (HUVEC). As it was shown in our earlier data that mRNA expression analysis confirmed that the ECFCs showed much lower expression of vWF transcripts than mature endothelial cells which had much higher expression of vWF. In order to test this, ECFCs and HUVEC were grown in special chamber slides (Lab-TEK II Cat # 154526). Around 10,000 cells were plated in each well. For a better comparison early (P5) and late passage (P15) cells were used both for ECFCs and HUVEC. IgG matched isotype control (Human IgG (Normal) Life technologies (Molecular Probes) Cat # 12000C) was used as a negative control.

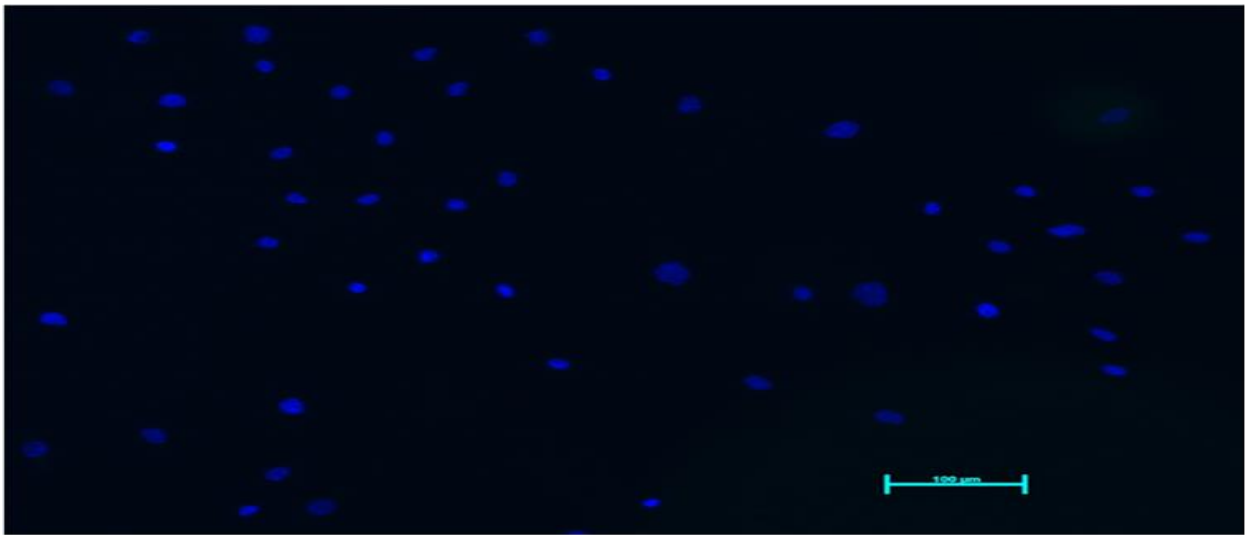


Figure 3.12. Showing cells stained with human IgG antibody used as an isotype control for the direct Immunofluorescence analysis. Images taken at 10 X magnification. Scale on the bar represents 100 µm. DAPI was used to stain the nuclei.

3.15.2 Staining procedure

Cells were grown in chambered slides and incubated at 37°C at 5% CO_2 in normoxia. Cells were checked for attachment the next day. Cells were attached well and washed briefly with PBS. Fixation of cells was carried out using methanol (Sigma Aldrich Cat # 179337). 500 µL methanol was added and cells were kept at -20°C for 10 min. Methanol was removed using a sucking pump. Permeabilization was carried out by adding 500 µL cooled acetone (Sigma Aldrich Cat # W332607) and cells were kept at -20°C for 1 min. Cells were rinsed twice in wash buffer (0.05% Tween 20 in PBS) for 2 min. This was followed by incubation in blocking buffer (2% BSA diluted in PBS) for 30 min in order to avoid nonspecific binding of the immunoglobulin. Primary antibody (Sheep PAb to vWF, Applied Biosciences Cat #8820) was diluted in antibody buffer (2% BSA in PBS). The final concentration used was 1:2000. Cells were incubated with primary antibody diluted in buffer for 60 min at room temperature in a humidified chamber. After this incubation cells were washed 5-6 times with the wash buffer. Excess buffer was removed and cells were counterstained with mounting media containing DAPI (Vectashield Cat # H-1200). Chambers were removed and slides were mounted using glass cover slips.

Axio imager fluorescent microscope B162 was used to inspect the slides. Images were taken at 10 X magnification and Image J software (Fiji) was used for analysing and counting the FITC positive cells required for statistical analysis that was done using unpaired *t* tests.

3.15.3 Limitation of the Experiment

Human IgG (Normal) was used as a negative control for the experiments. A better control would have been Human IgG (FITC).

3.16 Flow FISH (Fluorescent *in situ* hybridization)

Flow FISH is rapidly emerging as a powerful tool to measure length of telomere repeats in cells. This technique allows using fluorescent *in situ* hybridization (FISH) in conjunction with a labelled peptic nucleic acid (PNA) probe which is specific for those telomere repeats along with measurement of fluorescence using flow cytometry. This method to measure the lengths is working on the fact that, at decreased ionic strength, PNA can anneal to complementary single stranded DNA sequences. Hybridized PNA probe used $(C_3TA_2)_3$ (FITC-conjugated) for these experiments was based on the principal that low levels of fluorescent signals could be detected by a laser flow cytometer (FACS Canto II in this case) and Propidium Iodide was used a counter stain. So, only those cells were gated which took

up propidium iodide as shown in figure 3.13. Only those samples were included in which at least 1000 events were obtained with the desired gate. Figure 3.1 shows the gating strategy and the control used for the experiment for measuring telomere lengths ECFCs and HUVEC across passages. Thymocytes were used as a control and a reference telomere length.

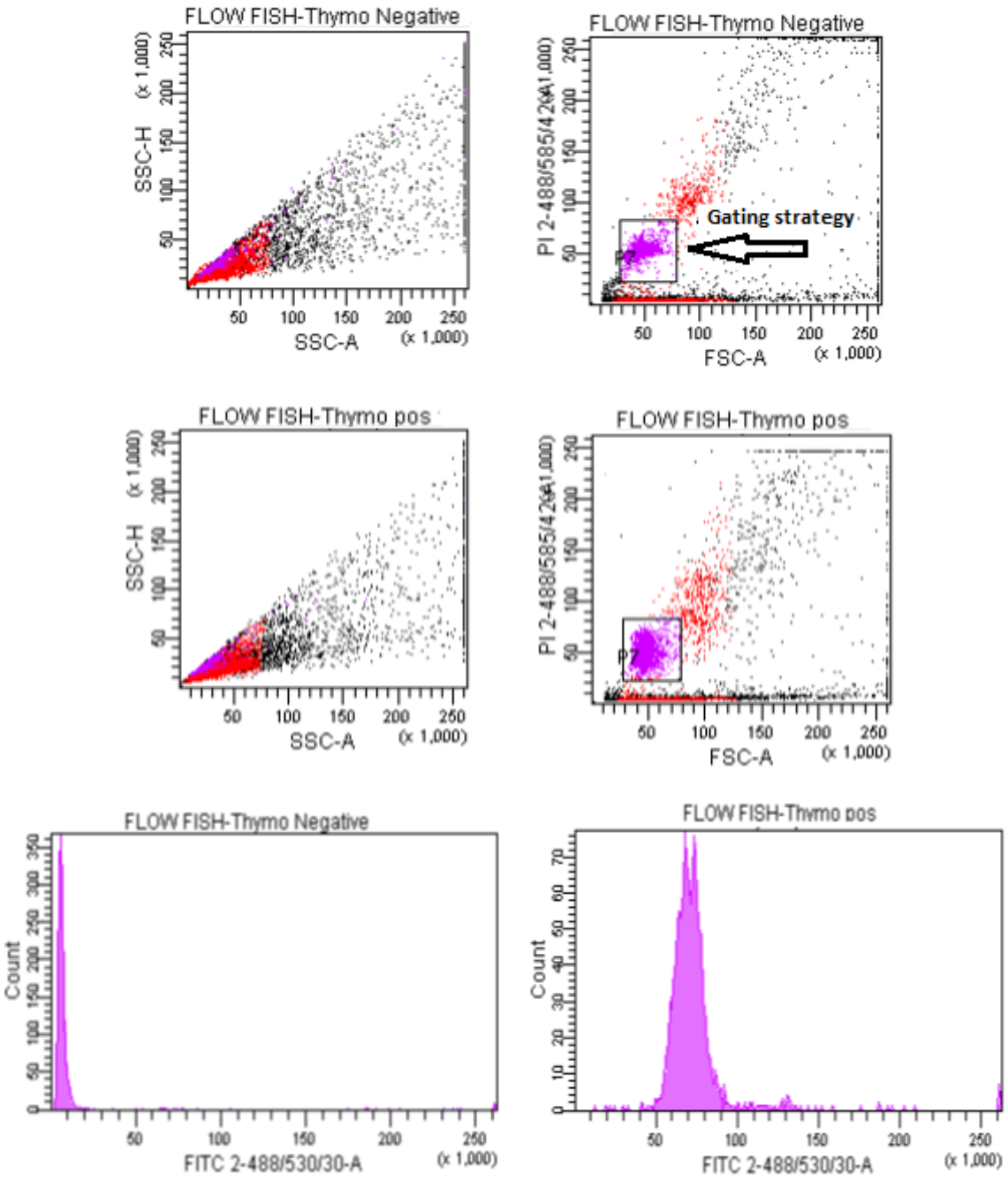


Figure 3.13. Showing histogram of the analysis of thymocytes which were used a control for flow FISH experiments used to measure the telomere length of ECFC and HUVEC samples across passages. Thymocytes were used as reference telomere length which was nearly 1.5 times longer than found in human endothelial cells.

3.16.1 Telomere length measurement using flow FISH

After measuring the telomerase activity of ECFC and HUVEC samples in both normoxia and hyperoxia it was needed to measure telomere lengths of ECFC and HUVEC samples across passages. This was to test whether telomere length is decreasing with each replicative cycle in both these cells. Secondly, to check if these progenitor cells (ECFCs) are different from mature endothelial cells in terms of preserving their telomere lengths and if there was a difference in hyperoxia conditions. ECFCs were derived from cord blood and cells were passaged regularly using Trypsin-EDTA. Cells were split in a ratio of 1:3. ECFC samples used were grown in normoxia (20% O_2) and early to late passage samples were used (P2-P16). HUVEC were isolated from umbilical vein of the cord and cells were grown in normoxia. Cells were split regularly in a ratio of 1:3. Measurement of telomere lengths was done across passages in HUVEC samples (P2-P16). Bovine thymocytes were used as an internal control for the study, and used as a reference being a sample with known telomere length.

3.16.2 Flow FISH Assay

Bovine thymocytes were frozen down at -80°C. They were thawed at room temperature. 500 µL of FBS was added to the cells and freezing media. This suspension was mixed well by pipetting and transferred to an eppendorf tube. This was subjected to centrifugation at 300 x *g* for 5 min. Supernatant was sucked down and the pellet was resuspended in 1 ml of PBS containing 5% FBS and mixed well by pipetting. This cell mix was transferred to a 15 ml falcon tube and 2 ml of this PBS was added. This was kept on ice till other samples were ready for cell count.

HUVEC samples were frozen down at -80°C. Cells were taken out and thawed at room temperature. 2 ml of PBS containing 5 % FBS was added to the cell mix containing freezing media. Cells were counted using Vi-Cell XR 2.03 (Beckman Coulter). 300,000 cells were used per triplicate for PNA^+ samples. 300,000 cells were used for 1 PNA^- sample.

ECFC samples were kept at -80°C. Cells were thawed by rolling fingers and 2 ml of PBS with 5% FBS was added. Cells were counted and 300,000 cells were used per duplicate.

Viable cell count was used for calculations for all cell types. Cell mix was centrifuged for 10 min at 2000 x *g*. Supernatant was aspirated and cells were resuspended in 300 µL of hybridization mix in separate tubes.

3.16.3 Preparation of hybridization mix

Ultra-pure formamide was taken out from -20°C and thawed at room temperature. Water bath was set at 87°C. Hybridization mix was prepared using following calculations.

Table 3.14. Showing hybridization mix preparation for flow FISH

Material	End Concentration	Volume for 6 PNA^- Tubes	Volume for 12 PNA^+ Tubes
1M Tris PH 7.1	20mM	50 µL	90 µL
1M NaCl	20mM	50 µL	90 µL
BSA 10%	1%	250 µL	450 µL
Ultra pure Formamide	75%	1876 µL	3376 µL
dH_2O		249 µL	449 µL
PNA Probe 30µg/ml	0.3µg/ml		45 µL
Negative Control (TE Buffer)		45 µL	

Cells were incubated in dark for 10 min after resuspension in hybridization mix. This was followed by 15 min of placement in water bath at 87°C. Cells were removed from water bath and kept at room temperature in dark for approximately 2 hrs.

After this incubation, cells were washed using two different washing solutions. Cells were vortexed and centrifuged for 5 min at 2000 x *g.* Supernatant was sucked till around 100 µL still remained. This wash was repeated for two additional times. Table below shows the content and concentration of wash solution I.

Table 3.15. Showing wash buffer I preparation

Materials	End Concentration	Volume for 12 Tubes
Formamide	75%	45 ml
1M Tris PH 7.1	10 mM	600 µL
BSA 10%	1%	6 ml
Tween20 10%	1%	6 ml
dH_2O		2.4 ml

This wash was followed by a second wash and table below shows the content and concentrations of the materials used.

Table 3.16. Showing wash buffer II preparation

Materials	End Concentration	Volume for 12 Tubes
BSA 10%	1%	1.5 ml
Tween20 10%	1%	1.5 ml
1M HEPES	10mM	150 µL
Glucose		11.9 ml

1 ml of wash solution 2 was added to each tube and cells were centrifuged for 10 min at 2000 x *g*. Supernatant was sucked till 50 µL remained.

Table 3.17. Showing DNA counterstain mix preparation

Material	End Concentration	12 Tubes
Propidium Iodide	0.06 µg/ml	45 µL
RNase A	10 µg/ml	4.5 µL
BSA 10%	0.1 %	45 µL
PBS		4405.5 µL

Telomere length measurement calculation:

Relative telomere length was measured using following method.

Mean fluorescence measurements were obtained for both PNA^+ and PNA^- samples. An average was taken for the positively stained samples used in duplicates (ECFC) and triplicates (HUVEC). This value was subtracted from the PNA^- mean fluorescence values obtained. Thymocytes were used as a reference telomere length and telomere length measurements were calculated relative to the mean fluorescence values obtained for thymocytes.

3.17 Multicolour flow cytometry

Multicolour flow cytometry involves using several antibodies together each having a different fluorochrome to stain them followed by analysis using a flow cytometer. In order to confirm the expression of cell surface markers at protein level, several important markers were checked. This included FACS analysis with antibodies for markers that showed expression at mRNA level in earlier experiments in human cord blood mononuclear cells, ECFCs and HUVEC samples. Unstained cells were used for gating and used as a negative control. The analysis was carried out using antibodies as shown in the table below.

Table 3.18. Showing set up used for Multicolour flow cytometry

Antibodies	Fluorochrome	Volume Used
Mouse Anti Human CD34	APC Conjugate	5 µL
Mouse Anti Human CD144	FITC	10 µL
Mouse Anti Human CD14	Pacific Blue Conjugate	5 µL
Mouse Anti Human CD146	PE	10 µL

One million cells were used per sample. Cells were resuspended in 500 µL of PBS and transferred to a labelled FACS tube. Antibodies were added and cells were incubated at room temp for 20-30 min. This was followed by a wash and then cells were analysed for FACS using FACS Canto II. Gating strategy for this experiment was devised using unstained samples. All the gates were set using that as shown in figure below. For all the samples including cord blood mononuclear cells (MNCs), ECFCs and HUVEC a negative (unstained) sample was used to set up the gates.

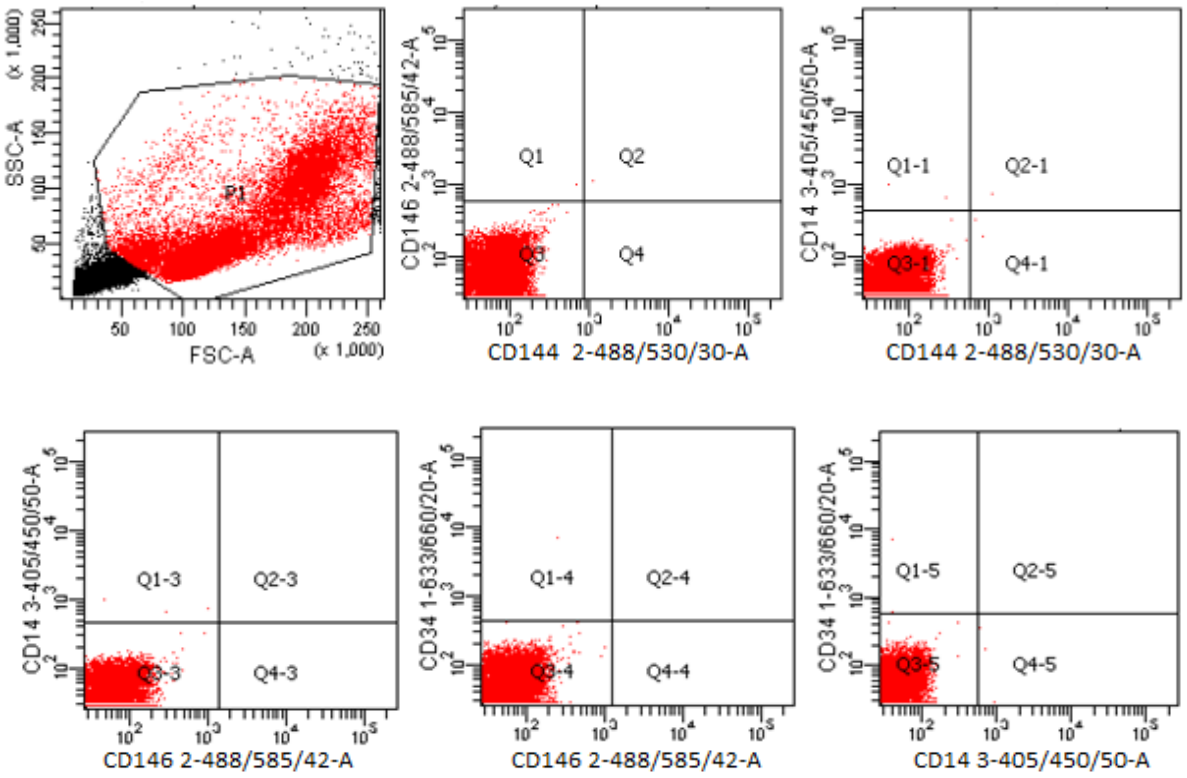


Figure 3.14. Gating strategy of unstained cells used for the detection of ECFCs and HUVEC. These unstained cells were used for setting gates for the positive and stained samples.

Table 3.19. Showing list of materials and associated information used for the study

Product	Manufacturer	Catalogue no
Rat Tail Collagen	BD Biosciences	#354236
Human Plasma Fibronectin	Chemicon (Millipore)	#FC010
EBM-2 media	Clonetics	#CC 3156
Single Quotes	Clonetics	#CC4176
Foetal Bovine Serum	Clonetics	#CC 4101A
Hydrocortisone	Clonetics	#CC 4112A
h FGF	Clonetics	#CC 4113 A
VEGF	Clonetics	#CC 4114 A
R3- IGF	Clonetics	# CC 4115 A
Ascorbic Acid	Clonetics	# CC 4116 A
GA -1000	Clonetics	# CC 4381 A
Heparin	Clonetics	#CC 4396 A
RPMI 1640	Gibco (Invitrogen)	#21875
Medium M-199	Sigma- Aldrich	#M 4530
HEPES 1M	Gibco (Invitrogen)	#15630
L- Glutamine	Gibco (Invitrogen)	#25030
Trypsin-EDTA	PAA	#L11-004
Penicillin/Streptomycin	Gibco (Invitrogen)	#15070
TRizol	Invitrogen	#15596-026
Taq DNA Polymerase	Fermentas	# EP0402
Random Hexamer Primer	Fermentas	#S0142
Fast SYBR Green	Applied Biosciences	# 4385618
6 X Loading Dye	Promega	#G1881

RNase H	Fermentas	#EN0201
dNTP - mix	Invitrogen	#10297-117
First Strand Buffer 5 X	Invitrogen	#10812-014
0.1M DTT	Invitrogen	#18080-044
RNase Inhibitor	Fermentas	#EO0381
Superscript Reverse Transcriptase	Invitrogen	#18064-022
100 bp DNA Ladder	Invitrogen	#10488-058
Agarose	Lonza SeaKem	#50004
Hematocytometer	ROTH	#T728.1
Phosphate Buffer Solution	PAA	#H15-002
DMSO	Sigma-Aldrich	#D2650
25 mM $MgCl_2$	Fermentas	#R0971
Collagenase	Gibco (Invitrogen)	# 17101-015
Ficoll-Hypaque	Biochrom	#L6115
Pipette Tips 0.1-10µl	Star Lab	#S1111-3700
Pipette Tips 1-200µl	Star Lab	#S1111-1700
Pipette Tips 101-1000µl	Star Lab	#S1111-2721
Pipette Tips 101-1000µl	Star Lab	#S1122-1830
RNeasy Mini Kit	Qiagen	#74104
Glucose	Sigma-Aldrich	#G5400
Formamide ultra-pure	Invitrogen	#15515-026
Hydrochloric acid (HCl)	Sigma-Aldrich	#H1758
Sodium chloride	Sigma-Aldrich	#S6191
TE buffer	Qiagen	#11910

Tris (Trizma base)	Sigma-Aldrich	#T-1503
Tween 20	Sigma-Aldrich	#P-1379
Propidium iodide (PI)	Molecular Probes	#P-1304MP
PNA probe $(C_3TA_2)_3$ (FITC-conjugated)	Panagene	Custom order
Mouse anti human CD 34 APC conjugate	BD Pharmingen	#560940
Human CD 14 Pacific Blue Conjugate	Invitrogen	#MHCD 1428
PE Mouse Anti-Human CD146 Monoclonal	BD Pharmingen	#561013
Sheep pAb vWF	Applied Biosciences	#8822
Human IgG	Molecular Probes	#12000C
Methanol	Sigma- Aldrich	# 179337
Chamber slides	Lab-TEK II	# 154526
Acetone	Sigma- Aldrich	# W332607
Mounting media containing DAPI	Vectashield	# H-1200
RNase A (100 mg/ml)	Qiagen	#19101
FITC Mouse Anti-Human CD144	BD Pharmingen	#560874

Chapter 4. Isolation and characterization of human endothelial progenitor cells (ECFCs) and comparison with mature endothelial cells (HUVEC)

4.1 Introduction

The human vasculature provides a dynamic network in the body for gaseous exchange and delivery of nutrients and circulation of cells. Blood vessel formation is considered as a fundamental aspect of development and any abnormality or irregularity in that can lead to fatal disease and therefore making it a therapeutic target. It has been proposed for long that endothelial progenitor cells (EPCs) hold great promise in the field of vascular biology, as their ability to differentiate in to endothelial cells can be used as a tool to re-endothelialize damaged blood vessels and facilitate the process of maintaining an intact endothelium. Similarly, low EPC count has been associated with coronary heart diseases (CHD). But till now, the identification of an endothelial progenitor cell has been a matter of debate, as there is no unique marker that can distinguish these cells from mature endothelial cells. They are therefore, identified on the basis of the expression of several cell surface markers. The first aim of our study was to isolate endothelial progenitor cells (ECFCs) from cord blood and differentiate them from early EPCs and macrophages derived from the same cord blood and compare them with mature endothelial cells which were derived from human umbilical vein of the same donor. This was followed by characterization of all these cell types isolated from the same donor, at mRNA and protein level and comparison with mature endothelial cells. In order to carry out this study our first goal was to isolate the correct endothelial progenitor cells as there has been so many discrepancies associated with their identification. To provide a better comparison we decided to isolate the cells from the same donor. This meant that the umbilical cord blood used to isolate the endothelial progenitor cells was from the same donor whose umbilical cord was used for the isolation of human umbilical vein endothelial cells (HUVEC). The study was designed in such a way that human umbilical cord mononuclear cells (HUCBC) also known as day 0 mononuclear cells (MNCs) were used as a starting population for the comparison and they were isolated from cord blood using ficoll based lymphoprep separation. Macrophages were used as a control for the study for two reasons. Firstly, it has been shown that the early EPCs are related to monocyte-macrophage lineage and the endothelial progenitor cells (ECFCs) should be distinguished from them on the basis of expression of monocyte-macrophage markers (Yoder et *al.* 2007). Secondly, macrophages are known to be resistant to oxidative stress (Dernbach et *al.* 2004) and therefore it was

important to make sure that the endothelial progenitor cells isolated for testing further hypothesis were not composed of a mix of cell populations like monocyte and macrophages**.**

4.2 Results

4.2.1 Isolation and morphological analysis of cell types isolated from cord blood and human umbilical vein of the same donor

The human cord blood mononuclear cells (HUCBC) also known as MNCs were isolated from cord blood using lymphoprep. A cell pellet was obtained after centrifugation and around 5-10 million cells were frozen down for carrying out RNA extractions. These were designated as day 0 MNCs or the starting population from which all the cell types were derived apart from mature endothelial cells (HUVEC), which were derived from the umbilical vein of the same donor. Morphologically, the MNCs appeared as small rounded cells as shown in figure 4.1A. After freezing down cells, the remaining MNCs were divided into three cultures to isolate early EPCs, macrophages and ECFCs from the same donor. Around 10-15 million cells per well were plated in order to isolate early EPCs also known as circulating angiogenic cells (CACs) and the cells were allowed to differentiate in endothelial specific media in normoxia. Cells were washed and the media was changed regularly. After 3-5 days of culture early EPCs were seen visible and were found to be the adherent cells. They appeared as rounded cells as shown in figure 4.1B. All cells that were not attached were washed away and the supernatant was removed. After 7 days of culture early EPCs were frozen down and RNA extraction was performed. Around 10-15 million MNCs were allowed to differentiate in macrophage specific media and grown under the same culture conditions. After 3-5 days of culture in macrophage specific media these MNCs started to differentiate towards macrophage lineage and became elongated fibroblast shaped cells as shown in figure 4.1C. These cells were adherent and were washed regularly and the media was changed on alternate days till day 7. Cells were then frozen down at this stage and RNA extraction was carried out. In order to derive ECFCs, around 60-70 million MNCs were plated per well and cells were allowed to differentiate in endothelial specific media. Cells were washed regularly and after 14-21 days, adherent cells forming clusters and resembling an endothelial colony were observed under the microscope. Figure 4.1D, shows the edge of the colony that distinguishes it from confluent cells that cover the entire well plate. The colony was picked and the cells were grown separately for a period of around 6-8 weeks, and were classified as endothelial colony forming cells or ECFCs as shown in figure 4.1E. Mature endothelial cells were

derived from human umbilical vein and were morphologically similar to ECFCs as shown in figure 4.1F. HUVEC demonstrated typical cobble stone appearance. The isolation of all these cell types from the same donor was difficult because not all isolations lead to colony formation and ECFCs have been shown to be a rare population of cells.

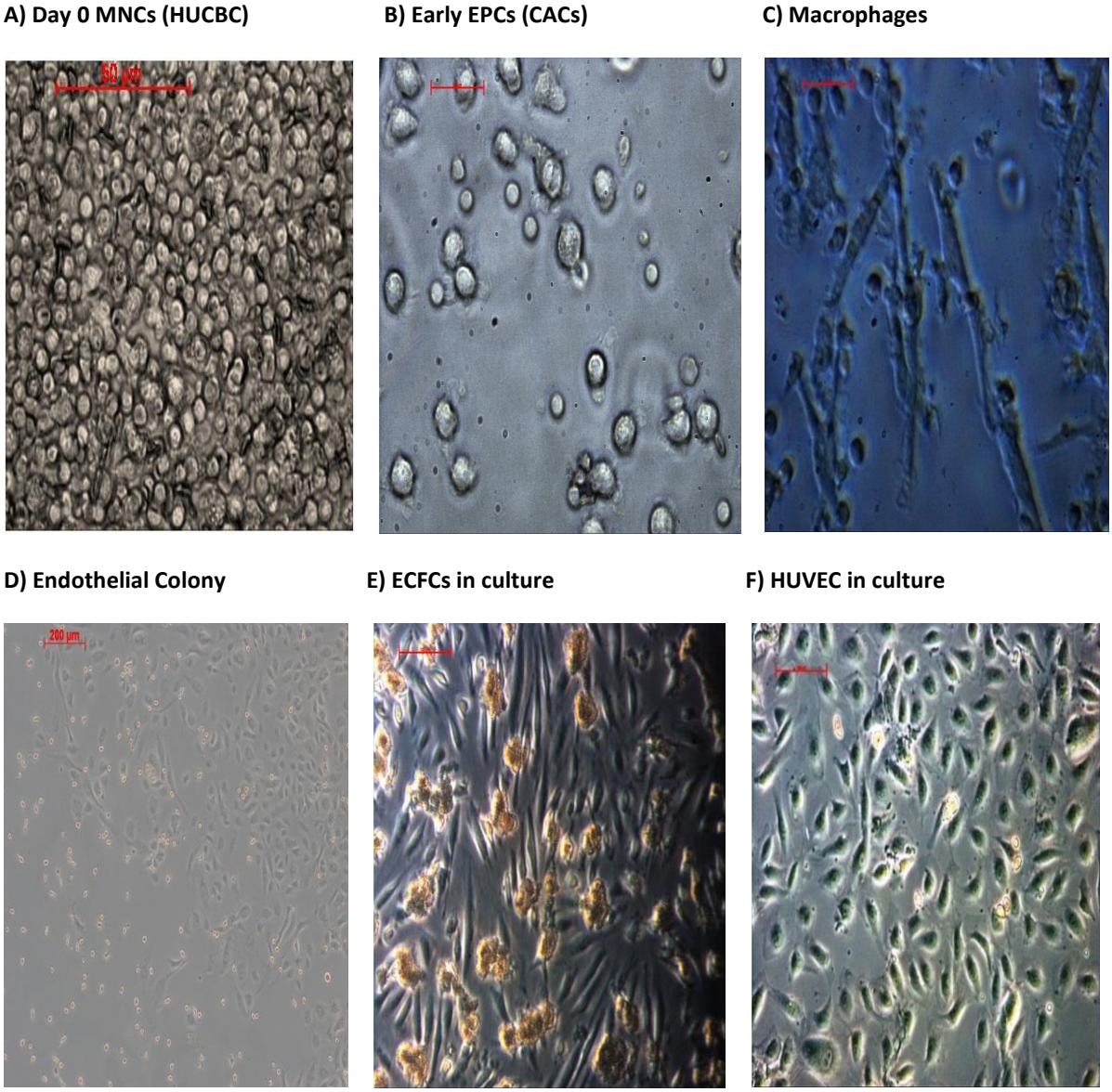


Figure 4.1. Morphological analysis of different cell types isolated from human umbilical cord blood and human umbilical vein. A) MNCs were isolated from cord blood after ficoll based separation. Scale = 50µm**. B)** Early EPCs were isolated from cord blood by differentiating MNCs in endothelial specific media. Scale = 50µm. **C)** Macrophages were derived from cord blood by differentiating MNCs in macrophage specific media. **D)** Showing the edge of an endothelial colony derived from cord blood. Scale = 200µm **E)** ECFCs derived from an endothelial colony after 6 weeks of culture. Scale =100µm. **F)** Mature endothelial cells derived from human umbilical vein. Scale = 50µm. Images taken at 10 x magnification.

4.2.2 Gene expression analysis of hematopoietic stem cell and macrophage specific markers in early EPCs, macrophages, late EPCs (ECFCs) and mature endothelial cells (HUVEC) derived from the same donor

After successful isolation and culture of early EPCs or circulating angiogenic cells (CACs), macrophages and late EPCs or ECFCs from cord blood and mature endothelial cells (HUVEC) from the same donor, the next step was to carry out the characterization of these cells at mRNA level. RNA extraction was carried out from frozen samples including day 0 MNCs, day 7 EPCs, day 7 macrophages and early passage ECFCs (P4) and HUVEC (P4). Specific primers were designed for cell surface markers for qPCR analysis. There were several important markers that were chosen to be tested for expression in these cell types.

These included the *CD34* gene that encodes CD34 protein and has been used as a common marker for EPC identification, and has shown to be expressed in hematopoietic stems cells, hematopoietic progenitor cells and circulating endothelial cells. The results as depicted in figure 4.2A, showed high expression of CD34 in early EPCs which is consistent with the earlier findings, but there was no CD34 expression seen in macrophages. As EPCs differentiate there is a gradual decline in the levels of CD34 but it still showed some level of expression in ECFCs which are known to retain low expression of this cell surface marker. HUVEC did not show any expression of the marker at mRNA level. CACs showed more than 2 fold higher mRNA abundance of CD34 in comparison to ECFCs and HUVEC and the difference was found to be statistically significant. Gene expression was plotted relative to day 0 MNCs that were taken as 0 or no expression.

The next gene to test was *PECAM-1* that encodes CD31 protein also known as platelet endothelial cell adhesion molecule-1 (PECAM-1) and is a type 1 trans membrane glycoprotein, belonging to immunoglobulin gene super family (Stockinger *et al.*, 1990). CD31 shows a wide range of cellular distribution with the highest level of expression shown in endothelial cells (Woodfin *et al.*, 2007). The results showed that there was much higher expression of CD31 in the endothelial colony forming cells (ECFCs) obtained from the umbilical cord blood as shown in figure 4.2B, and there was also expression seen in mature endothelial cells (HUVEC). Early EPCs and macrophages showed much lower levels of CD31 as compared to ECFCs and HUVEC. ECFCs showed a 2 fold increase in expression as compared to HUVEC, but the difference was not found to be statistically significant.

In order to differentiate early EPCs from ECFCs and mature endothelial cells it was important to confirm the expression of certain monocyte-macrophage markers which are known to be expressed in early EPCs and macrophages and are not expressed in ECFCs and HUVEC. Human CD14 gene encodes a protein that is a well-known marker to identify the cells of monocyte-macrophage lineage, and was first identified as a receptor for the endotoxin lipopolysaccharide (LPS) (Wright *et al.*, 1990). ECFCs showed no expression of *CD14*, as shown in figure 4.2C, and there was much higher expression of the gene seen in macrophages. There was some expression seen in early EPCs which is consistent with what has been known before and EPCs are known to gain the expression of CD14 as they differentiate (Estes *et al.*, 2010). As shown in figure 4.2, early EPCs showed more than 3 fold increase in mRNA level of expression in comparison to ECFCs and HUVEC and the difference was found to be statistically significant.

Protein tyrosine phosphatase receptor type C also known as CD45 is differentially expressed in subsets of leucocytes, and on all differentiated hematopoietic stem cells. It is a human enzyme encoded by *PTPRC* gene. mRNA level of expression of CD45 was tested as it is not expressed by endothelial cells or erythrocytes (Baldwin *et al.*, 2000). It is a leukocyte marker and as the MNCs differentiate, they lose the expression of CD45 as shown in figure 4.2D.

There was no expression of *PTPRC* seen in ECFC or HUVEC, as shown in figure 4.2D, which is consistent with the previous findings and further confirmed that these colony derived cells are the cells of interest, as the ECFCs are known to be negative for this leukocyte marker (Zhang *et al*. 2009).

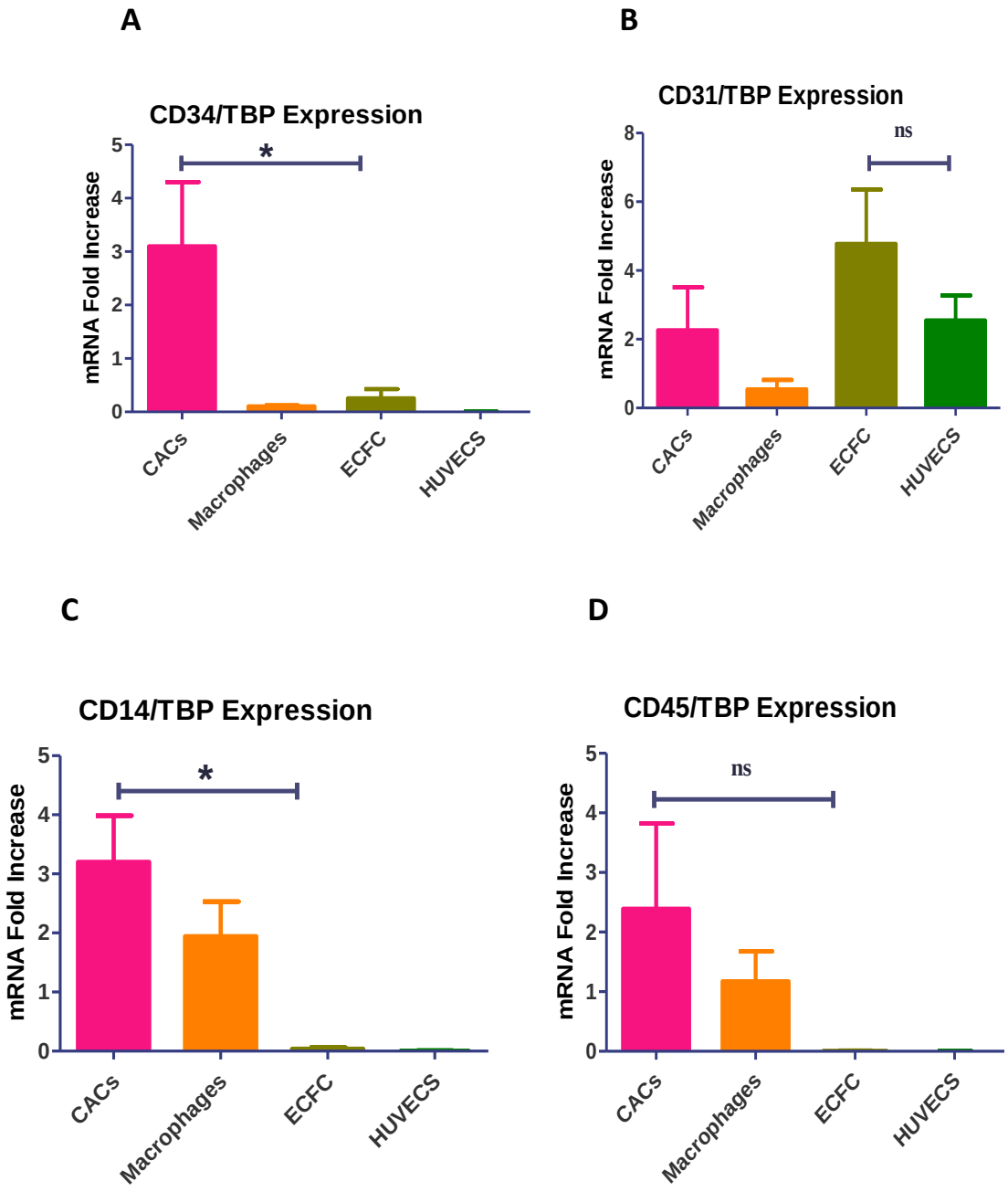


Figure 4.2. Gene expression analysis of hematopoietic stem cell and monocyte-macrophage specific markers in early and late endothelial progenitor cells and macrophages derived from cord blood and mature endothelial cells derived from the umbilical cord of the same donor. A) Gene expression analysis of cell surface marker CD34 in early and late EPCs and HUVEC showing higher expression in early EPCs (day7) and ECFCs still retaining CD34 expression**. B)** Gene expression profile of endothelial cell surface marker PECAM-1 and comparison between early and late EPCs showing that both cell types express this marker with higher level of expression in ECFCs and HUVEC**. C)** Gene expression analysis of macrophage specific marker CD14 in cell types isolated from cord blood and umbilical vein confirming the expression of CD14 in macrophages and in early EPCs suggesting that these cells belong to monocytic-macrophage lineages. ECFCs and HUVEC did not show CD14 expression. **D)** Gene expression analysis of cell surface marker CD45 showing expression in early EPCs and macrophages and absence in ECFCs and HUVEC. All data are normalized to TBP (TATA box binding protein) which was used as a housekeeping gene. Gene expression is plotted relative to cord blood mononuclear cells. All values are calculated as ± SEM, n=3-5. * $P < .05$, ** $P < .01$, *** $P < 0.001$, ns stands for not statistically significant.

4.2.3 Gene expression analysis of endothelial markers in early EPCs, macrophages, ECFCs and HUVEC derived from cord blood and umbilical cord of the same donor

After the confirmation of expression of monocyte-macrophage markers in early EPCs and macrophages and absence of these markers in endothelial progenitor cells (ECFCs) and mature endothelial cells (HUVEC), it was important to check the mRNA level of certain endothelial markers in these isolated cell types. Endothelial progenitor cells (ECFCs) and mature endothelial cells were expected to show the expression of endothelial markers; whereas early EPCs and macrophages should not be showing these markers. In order to test this, we carried out gene expression analysis for certain markers that included CD105, CD144, CD146 and vWF. Early EPCs, macrophages and ECFCs were derived from cord blood samples (n=3 for EPC and macrophages, n=5 for ECFCs) and mature endothelial cells (HUVEC n=5) were derived from umbilical veins of the same donors. RNA was extracted from frozen down cells at day 7 for early EPCs (CACs) and macrophages and early passage ECFCs (P4) and HUVEC (P4).

The human endoglin gene encodes endoglin protein also known as CD105, which is a type I membrane glycoprotein located on the surface of endothelial and haematopoietic cells, forming part of the TGF-β complex. It is required for extra embryonic angiogenesis and plays a role in cardiovascular development in mammals (Arthur *et al.*, 2000; Conley *et al.*, 2000). As shown in the figure 4.3A, there was no expression of CD105 gene in early EPCs and macrophages, but there was high level of expression seen in ECFCs and mature endothelial cell also showed expression of endoglin. There was more than 2000 fold increase in expression of CD105 in ECFCs in comparison to early EPCs and the difference was found to be statistically significant. There was significant difference between expression level of CD105 in ECFCs and HUVEC.

CDH5 is a human gene that encodes a member of cadherin family of proteins namely CD144 also known as VE-cadherin that plays an important role in maintaining newly formed blood vessels (Corada *et al.*, 2001). It mainly shows expression in endothelial cells. As shown in the figure 4.3B, there was no expression of CDH5 seen in early EPCs and in macrophages as expected, but there was higher expression seen in early EPCs and ECFCs as compared to HUVEC.

MCAM is a human gene that encodes the protein CD146 also known as the melanoma cell adhesion molecule (MCAM). CD146 is a member of Ig super family and mainly shows the expression in endothelial cells including ECFCs and CECs. The results were consistent with

the previous findings and showed high expression of CD146 in ECFCs as compared to early EPCs (Yoder *et al*. 2007). There was more than 50 fold increase in mRNA level of expression of CD146 in ECFCs as compared to early EPCs and the difference was found to be statistically significant. As expected, macrophages showed no expression of CD146 as shown in figure 4.3C.

Von Willibrand factor (vWF) is a blood glycoprotein encoded by vWF gene that is exclusively found in endothelial cells and megakaryocytes and is produced and stored in weibel-palade bodies in endothelial cells and platelet granules (Mannucci, 1995). As shown in figure 4.3D, there was no expression of vWF gene in early EPCs and macrophages. ECFCs showed low level of gene expression of vWF and mature endothelial cells HUVEC strongly expressed this endothelial marker. Statistical analysis was carried out that suggested that vWF could be used as a marker that could distinguish between endothelial progenitor cells (ECFCs) and mature endothelial cells (HUVEC). ECFCs showed low level of gene expression and as these cells differentiate towards endothelial lineage they would start strongly expressing this specific endothelial marker. There was several thousand fold increase in expression of vWF in ECFCs as compared to HUVEC. The difference between mRNA expression level of ECFCs and HUVEC was found to be statistically significant.

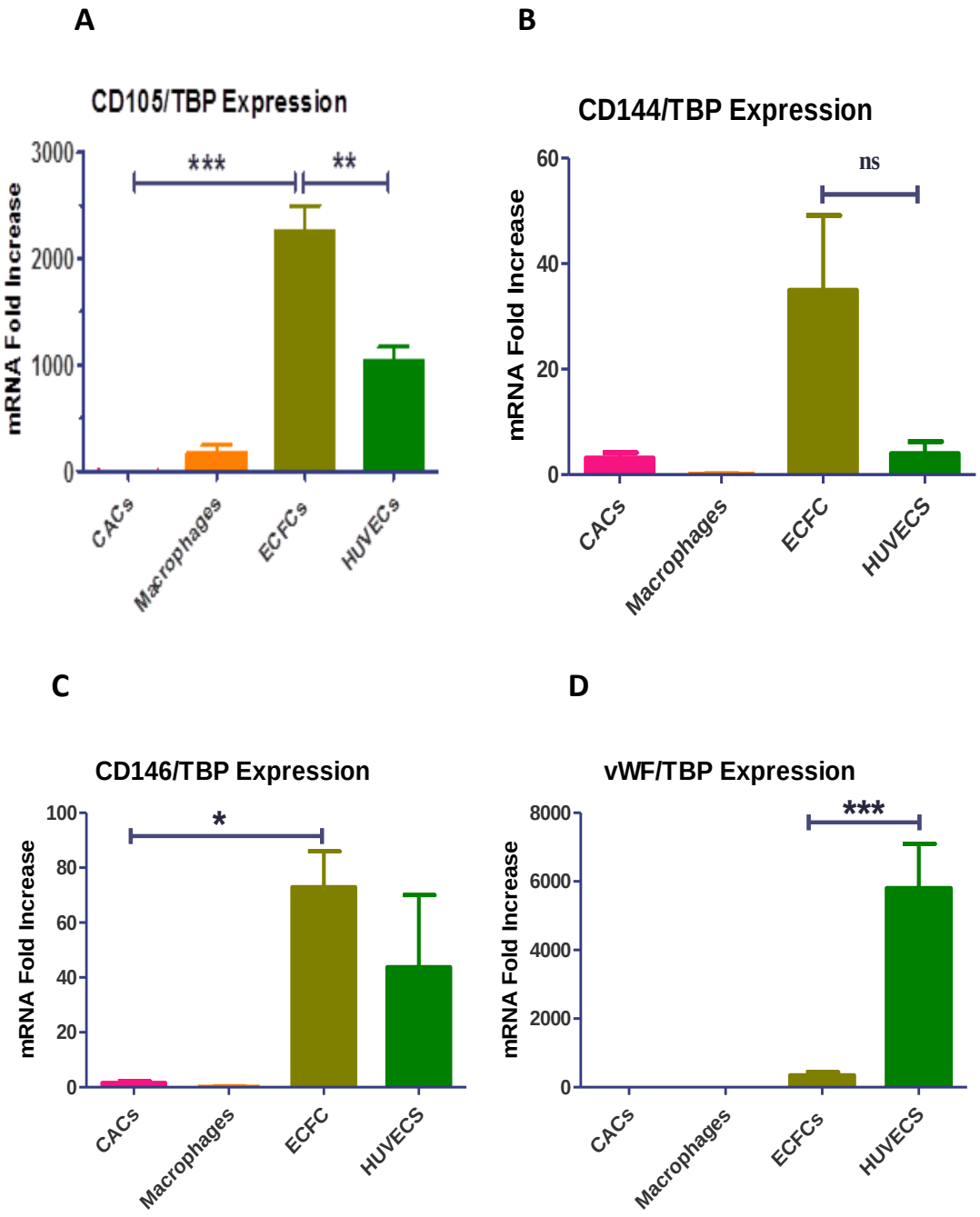


Figure 4.3. Gene expression analysis of endothelial markers in early and late endothelial progenitor cells and macrophages derived from umbilical cord blood and mature endothelial cells derived from the umbilical cord of the same donor. A) Gene expression analysis of cell surface marker endoglin (CD105) in early EPCs (day7) and macrophages showing no expression, and ECFCs showing high expression levels of CD 105. **B)** Gene expression profiling of endothelial specific marker vWF, showing a high level of expression in mature endothelial cells and a very low level of expression in ECFCs. There was no vWF expression seen in early EPCs and macrophages. **C)** Gene expression analysis for CD144 (VE-cadherin), showing high expression in ECFCs and low expression in mature endothelial cells. There was no expression seen in macrophages. **D)** Gene expression analysis of cell surface marker CD146 (MCAM), showing high expression in endothelial cells and no expression in early EPCs. All data are normalized to TBP (TATA box binding protein) which was used as a housekeeping gene. Gene expression is plotted relative to human cord blood mononuclear cells (HUCBC). All values are calculated as ± SEM, n=3 (CACs and macrophages) and n=5 (ECFCs and HUVEC). * $P < .05$, ** $P < .01$, *** $P < 0.001$

4.2.4 Multicolor flow cytometry analysis for the expression of cell surface markers CD14, CD34, CD144 and CD146 in endothelial colony forming cells (ECFCs)

As shown in figures 4.2 and 4.3 there was confirmation of the expression of endothelial markers in ECFCs and HUVEC and no expression in early EPCs. There was expression of markers like CD14 and CD45 which are known to be monocyte-macrophage specific and leukocyte markers in early EPCs and absence in ECFCs and HUVEC. The next step was to validate these results and confirm the expression and absence of these markers at protein level. Antibodies against cell surface marker CD14, CD34, CD144 and CD146 (Mouse-anti human) were used to stain the cells each linked to a different fluorochrome. ECFCs were isolated from cord blood and were derived from an endothelial colony which was observed at around 3 weeks of culture and differentiation of cord blood mononuclear cells in endothelial specific media. Cells were passaged and split regularly and fresh cells were used for staining. All four antibodies having different fluorochrome were used together to stain the cells and analysis was carried out using FACS. As shown in figure 4.4, ECFCs showed low level of expression of hematopoietic stem cell marker CD34, and very low level of expression of monocyte-macrophage marker CD14. ECFCs have been known to retain the expression of CD34 and are distinguished from early EPCs on the basis of absence of expression of monocyte-macrophage markers like CD14. Early EPCs retained high expression of CD14. As shown in figure 4.4, ECFCs showed high level of expression of CD146 also known as MCAM, which is known to be expressed in endothelial progenitors and endothelial cells. ECFCs also expressed CD144 also known as VE-cadherin which is mainly present in endothelial cells and known to play an important role in maintaining newly formed vessels.

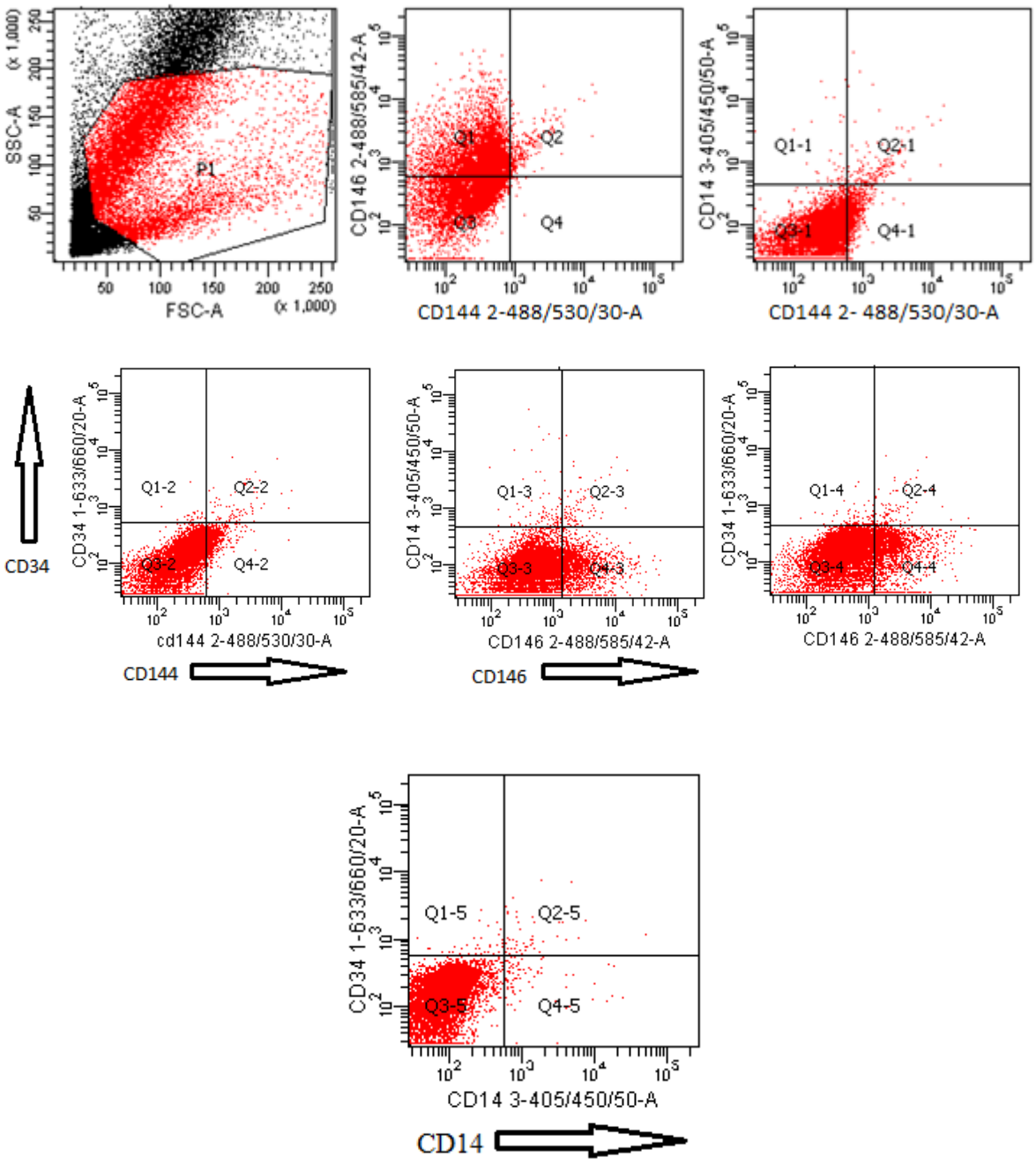
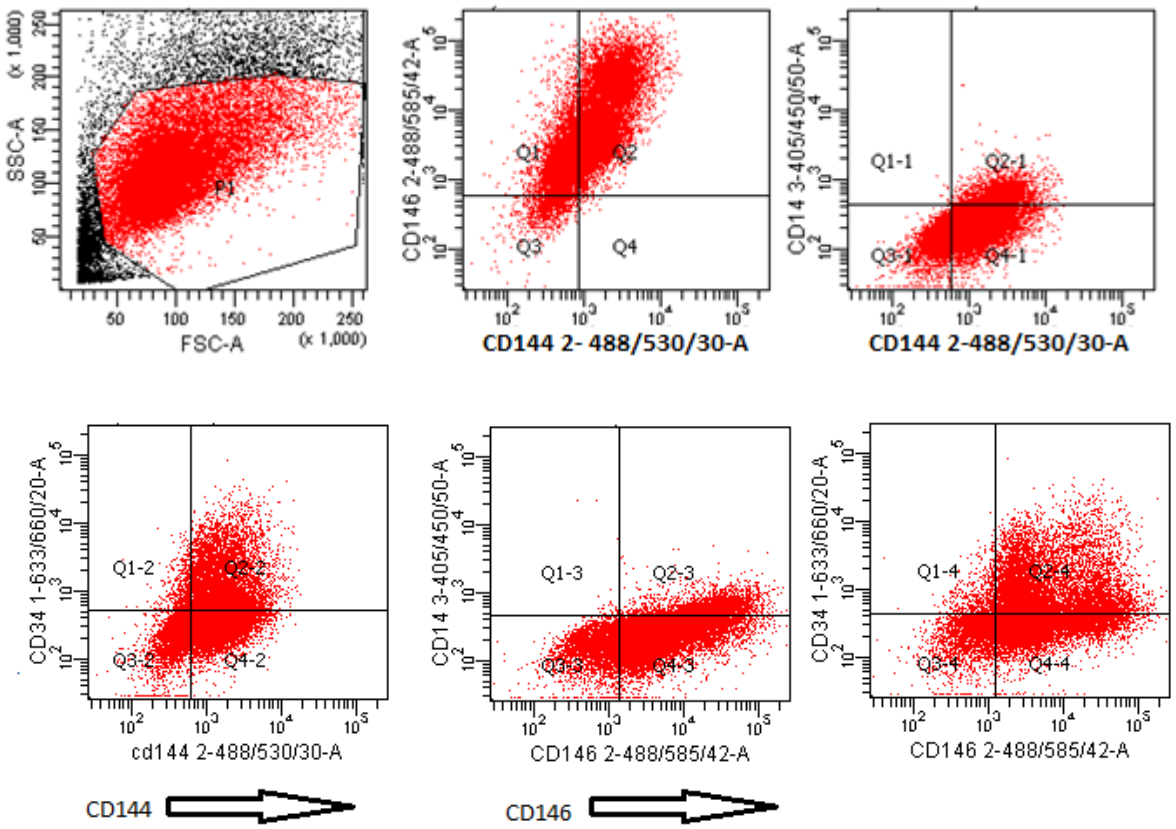


Figure 4.4. Multicolour flow cytometry analysis showing cell surface markers in ECFCs. The analysis was carried out using antibodies against cell surface markers, each having a different fluorochrome. The ECFCs were isolated from cord blood and the cells were passaged once they reached confluence. Fresh cells were used for flow cytometry analysis. The figure shows the confirmation of the expression of endothelial markers CD146 and CD144 and absence of monocyte-macrophage marker CD14 in endothelial colony forming cells (ECFCs). The ECFCs as shown above retained low level of CD34 expression. n=3 n represents number of donors.

4.2.5 Multicolor flow cytometry analysis for the protein expression of cell surface markers CD14, CD34, CD144 and CD146 in mature endothelial cells HUVEC.

Mature endothelial cells (HUVEC) were derived from the umbilical vein. Cells were grown in endothelial specific med and regularly passaged. After the confirmation of expression of endothelial markers at mRNA level, as shown in figures 4.2 and 4.3, it was important to validate the expression at protein level. Cells were stained with antibodies for cell surface markers including CD14, CD34, CD144 and CD146. Cells were stained with four antibodies together each having a different fluorochrome. Analysis was carried out using FACS canto II. As shown in figure 4.5, HUVEC strongly expressed endothelial markers CD144 and CD146. They showed absence of CD14 expression which is known to be a monocyte-macrophage marker. HUVEC also retained high level of expression of CD34.



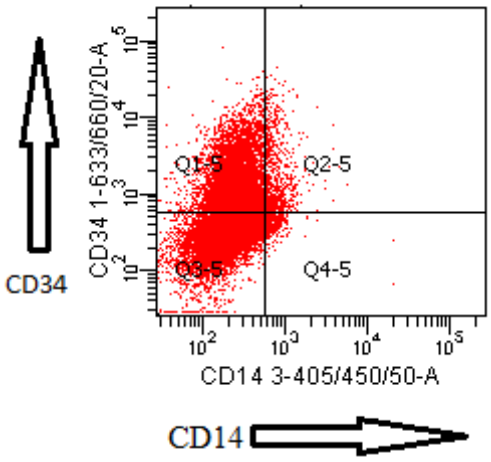


Figure 4.5. Multicolour flow cytometry analysis in HUVEC. The analysis was carried out using antibodies against cell surface markers, each having a different fluorochrome. HUVEC are mature endothelial cells which were isolated from human umbilical vein. Fresh cells at early passages were used for this analysis. The figure shows the confirmation of the expression of endothelial cell surface markers CD146 and CD144 and absence of macrophage marker CD14 in mature endothelial cells (HUVEC). HUVEC have shown to retain CD34 expression. n=3, where n represents number of donors.

4.2.6 Protein expression analysis for cell surface markers CD14, CD34, CD144 and CD146 in cord blood mononuclear cells (day 0 MNCs), endothelial colony forming cells (ECFCs) and HUVEC.

After confirmation of expression of endothelial markers in ECFCs and HUVEC and absence of monocyte-macrophage markers CD14 and CD45, the next step was to confirm the expression at protein level. As shown in figure 6, FACS analysis was carried out using antibodies for CD14, CD34, CD144 and CD146. Cells were stained with all these antibodies together and a comparison was made between MNCs, ECFCs and HUVEC based on the percentages of cells positive for these cell surface markers. As shown in figure 6, cord blood mononuclear cells showed the greatest percentage of $CD14^+$ cells, whereas ECFCs and HUVEC showed almost no expression of this monocyte-macrophage marker. Mature endothelial cells HUVEC showed highest percentage of $CD34^+$ cells, and ECFCs still maintained CD34 expression. MNCs did not show any expression of $CD144^+$ and $CD146^+$ cells as was expected, as these markers are expressed in endothelial progenitors and mature endothelial cells. ECFCs showed low expression of CD144 protein as compared to HUVEC, which has been shown before. CD146 which is known to be expressed in mature endothelial cells showed the highest percentage of positive cells in both ECFCs and HUVEC.

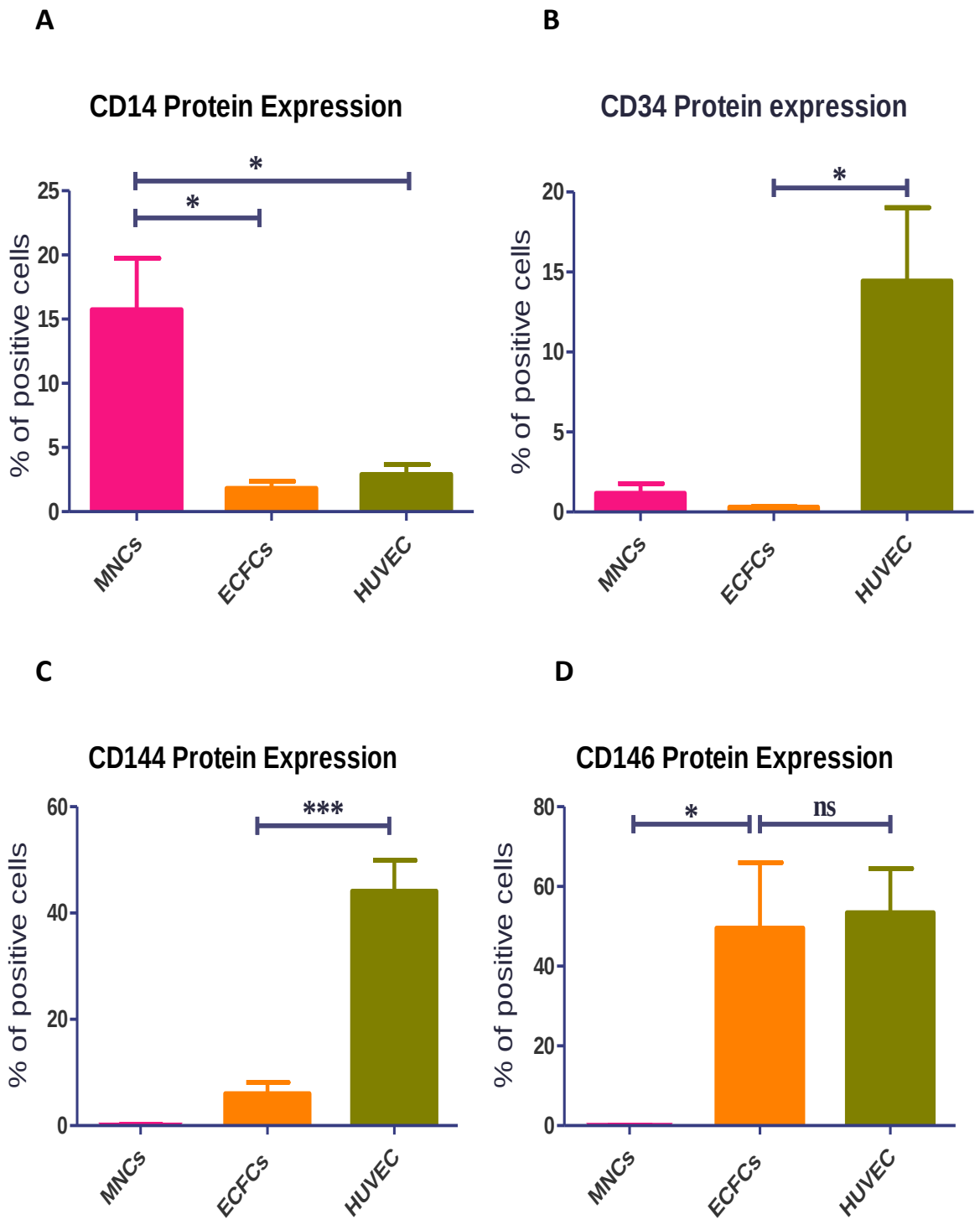
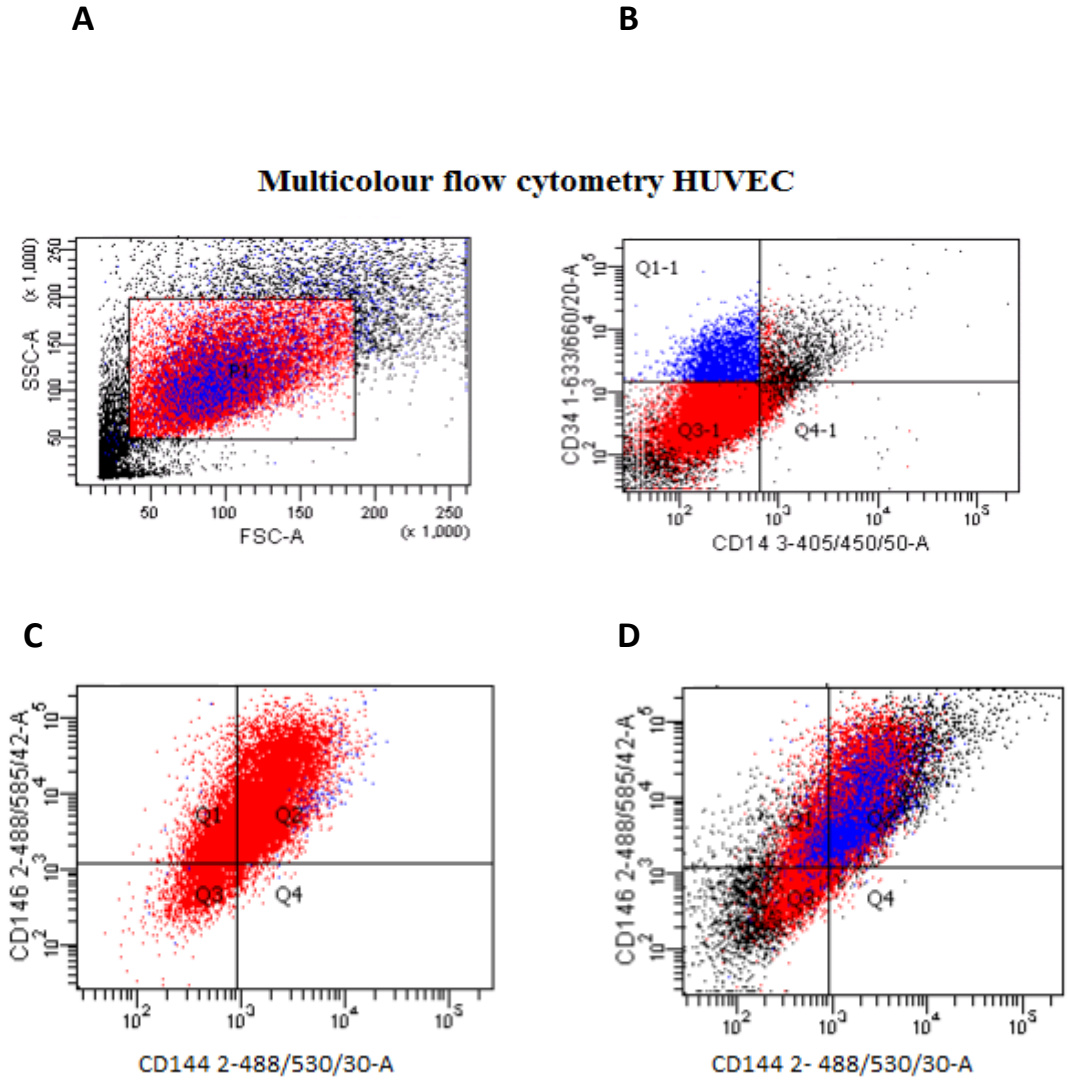
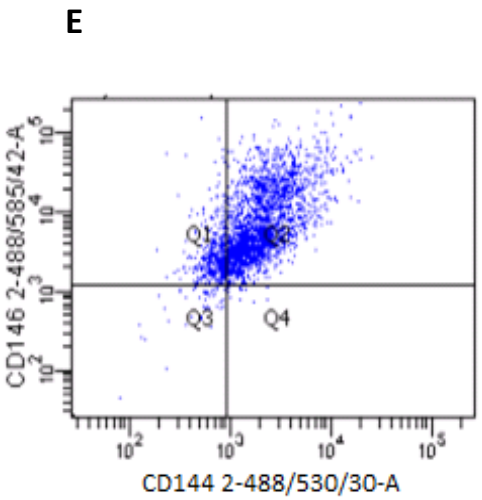


Figure 4.6. Protein expression analysis of the haematopoietic stem cell and endothelial markers in cell types derived from cord blood and umbilical cord. A) The figure shows the expression of monocyte-macrophage marker CD14 in cord blood mononuclear cells, whereas there was no protein expression of CD14 seen in ECFCs and HUVEC. ECFCs were derived from a colony that was observed at day 14 to day 21 of culture **B)** Protein expression analysis of haematopoietic stem cell marker CD34 showing high expression in mature endothelial cells HUVEC. **C)** Protein expression analysis of CD144 (VE-cadherin) in day 0 MNC and ECFCs and HUVEC confirming expression of CD144 in endothelial progenitors and mature endothelial cells and no expression in MNCs. **D)** Protein expression analysis of cell surface marker CD146 confirming the expression in ECFCs and HUVEC and absence in MNCs. Statistical analysis was carried out using one way ANOVA, followed by Tukeys multiple comparison tests. All values are calculated as ± SEM, n=3. * $P < .05$, ** $P < .01$, *** $P < 0.001$.

4.2.7 Cell surface marker expression analysis of $CD34^{+}/CD14^{-}$ cell population in mature endothelial cells HUVEC

CD34 is considered to be a marker of hematopoietic cell lineage, but has been known to show expression in endothelial cells and endothelial progenitor cells. Our earlier FACS analysis showed high percentage of CD34 population in HUVEC. Therefore, we further analysed this cell population and as shown in figure 4.7, HUVEC were stained with antibodies for CD14, CD34, CD144 and CD146 and all of all them were tagged with a different fluorochrome. Cells were analysed using FACS and $CD34^{+}/CD14^{-}$ cell population was isolated. This population was further analysed and as shown in figure 4.7 E, double positives and double negative ($CD144^{+}/CD146^{+}$ and $CD144^{-}/CD146^{-}$) cell populations were obtained and statistical analysis was carried out.





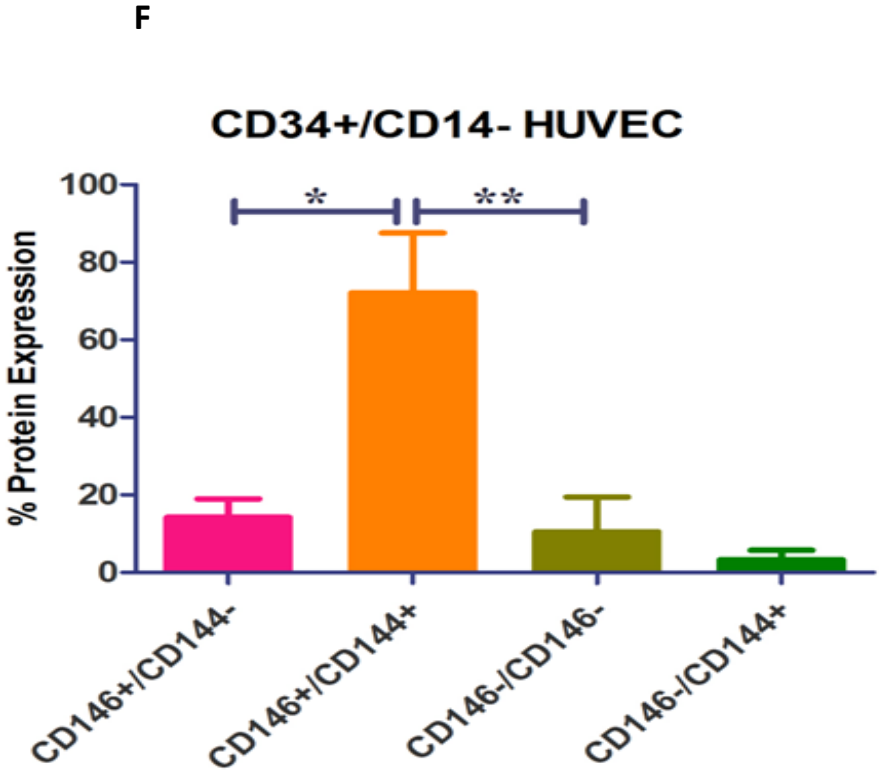
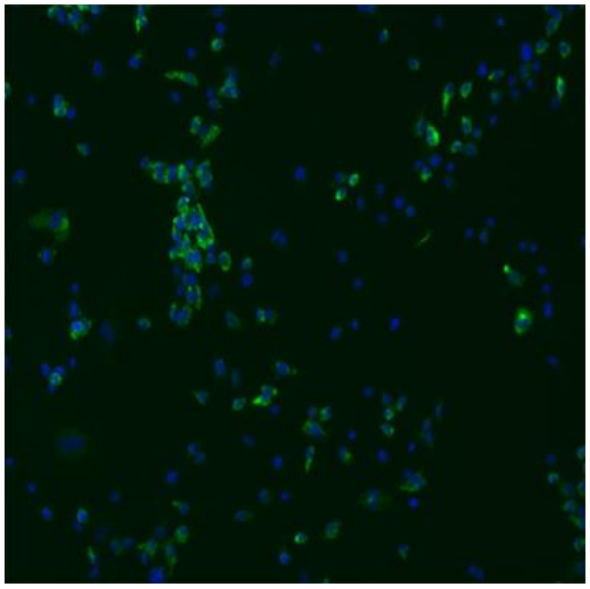


Figure 4.7. Protein expression analysis of CD34^{+}/CD14^{-} cell population sorted by FACS analysis. The analysis was carried out on mature endothelial cells (HUVEC) isolated from human umbilical vein. FACS analysis was done using antibodies for cell surface markers including heamtopoietic stem cell marker CD34, macrophage specific marker CD14, and endothelial markers CD144 and CD146. Fresh cells were used for the analysis. More than 70% of this sorted poulation was found to be double positive for CD144 and CD146 which are both endothelial markers. Less than 10% was double negative for CD146 and CD144. The difference between double positives and double negatives was found to be statistically significant. Statistical analysis was carried out using one way ANOVA, followed by Tukeys multiple comparison tests. All values are calculated as ± SEM, n=3. * $P < .05$, ** $P < .01$, *** $P < 0.001$.

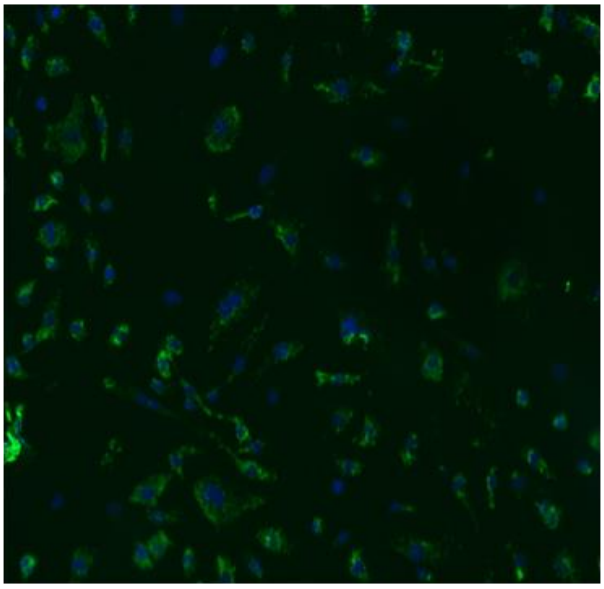
4.2.8 Immunofluorescence analysis for vWF expression in ECFCs and HUVEC comparing early and late passages for the protein expression of vWF

Von willibrand factor (vWF) is a blood glycoprotein involved in platelet adhesion. Our earlier results indicated that at mRNA level vWF showed higher expression in mature endothelial cells than in endothelial progenitors (ECFCs) and the difference was found to be statistically significant. In order to confirm and validate this at protein level we performed direct immunofluorescence analysis. ECFCs were isolated from cord blood and derived from an endothelial colony. Cells were passaged regularly and early passage (P5) and late passage (P15) ECFCs were used for comparison so that the level of expression of vWF in early and late ECFCs could be observed. The next comparison was between HUVEC grown at same passages. HUVEC were isolated from umbilical vein and early (P5) and late passage (P15) cells were used. Cells were grown in special chamber slides and stained with vWF antibody and observed under microscope. As shown in figure 4.8, early and late ECFCS showed much lower level of expression as compared to HUVEC at same passages which was determined by counting FITC positive cells for both ECFCs and HUVEC. Statistical analysis was carried out and the difference was found to be significant. This further confirmed that ECFCs which are endothelial progenitors showed low level of expression of vWF as compared to mature endothelial cells HUVEC and could be used as a marker to distinguish between these two cell types.

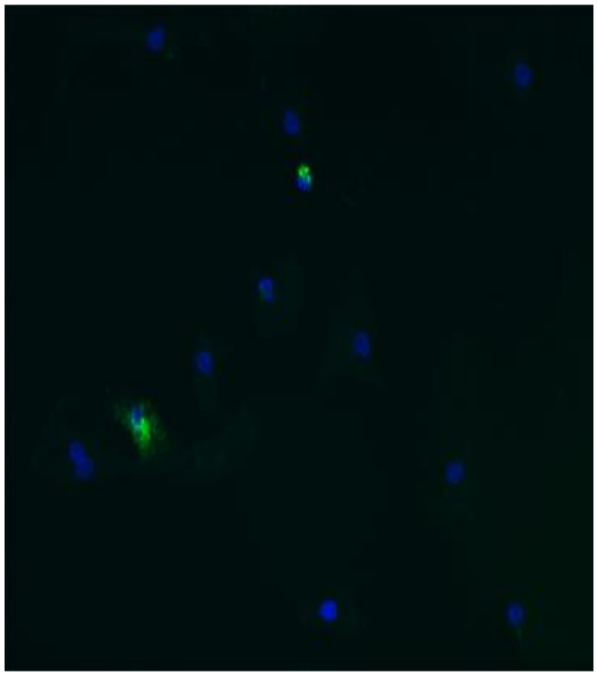
A HUVEC P5



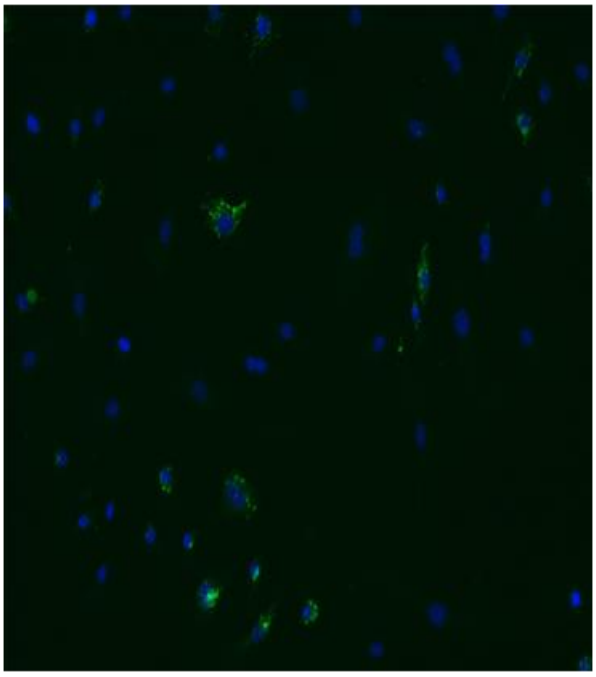
B HUVEC P15



C ECFC P5



D ECFC P15



E

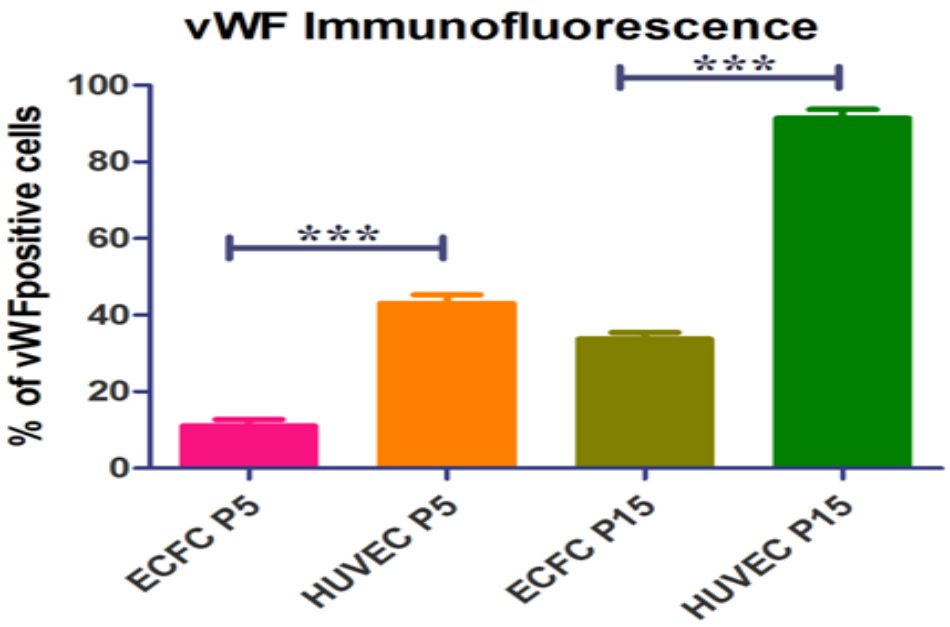


Figure 4.8. Immunofluorescence analysis for the protein expression of vWF (von Willibrand factor) in endothelial progenitor cells (ECFCs) and mature endothelial cells (HUVEC). vWF antibody was used for staining ECFCs and HUVEC having FITC tagged as a fluorescent marker. Human IgG isotype control was used as a negative control for the experiments. Figure **A and B** showing mature endothelial cells which were obtained from the human umbilical vein. Cells were passaged regularly and grown till late passage P15. Immunofluorescence was done using vWF antibody on early (P5) and late passage (P15) HUVEC. Figure **C and D** showing ECFCs which were derived from an endothelial colony obtained by differentiation of mononuclear cells from cord blood. Cells were passaged regularly and immunofluorescence was carried out on early (P5) and late passage (P15) ECFCs. Figure E shows lower expression of vWF in early passage ECFCs as compared to early passage HUVEC and late passage ECFCs as compared to late passage HUVEC. vWF positive cells were counted in these cells and statistical analysis was done using unpaired *t* test. n=3, where n represents number of donors. The difference was found to be statistically significant and the results as shown in the figure indicate that the ECFCs are at an earlier stage of differentiation as compared to HUVEC.

4.3 Discussion

There has been a lot of debate regarding the identification of human EPCs as there has not been a single marker that can be used for their prospective isolation from peripheral or cord blood. The term human EPC has been used for different population subsets and cell types depending upon the expression of several cell surface antigens. It has now been shown that most of these EPCs actually belonged to hematopoietic subsets that support blood vessel formation but were not directly involved in the process of blood vessel formation. These EPCs are broadly divided in two main categories, proangiogenic haemopoietic cells for which the term EPC has been used, since Asahara *et al*. for the first time reported prospective isolation of these cells from peripheral blood (Asahara *et al.*, 1997). These cells showed the expression of cell surface markers including CD31, CD34, CD202b (Tie2), CD309 (VEGFR-2/KDR), CD62E, CD45, UEA-1, and the uptake of acetylated LDL. These cells lacked the expression of monocyte lineage marker CD14. These findings were further validated by several groups (Shi *et al.*, 1998) and it has been shown that these EPCs can be isolated from bone marrow and other sources (Takahashi *et al.*, 1999; Kalka *et al.*, 2000b). Similarly, $CD34^+$, $CD133^+$, $CD309^+$ cell populations have been isolated from peripheral blood and named as circulating endothelial precursors (Peichev *et al.*, 2000; Gill *et al.*, 2001). $CD31^+$ $CD202b^+$ $CD309^+$ cells have been isolated that form myeloid colonies in an *in vitro* system also termed as CFU-Hill and have been named as EPCs (Hill *et al.*, 2003; Smadja *et al.*, 2008). It has been shown that $CD45^+$ $CD133^+$ $CD34^+$ $CD144^+$ and $CD309^+$ cell populations have been isolated and termed as EPCs (Fox *et al.*, 2008b; Smythe *et al.*, 2008). It has recently been shown that the early EPCs belong to the monocytic-macrophage lineage and are termed as proangiogenic cells and express several cell surface antigens including CD31, CD45 and in some cases CD309 and CD202b. They are further differentiated on the basis of additional expression of CD133, CD34 and absence of CD14 expression and are classified as immature progenitors (Estes *et al.*, 2010). Circulating angiogenic cells (CACs) with further differentiating potential start expressing CD14 and loose the expression of CD133 and are termed as cells of mature monocytic lineage (Estes *et al.*, 2010).

Table 4.1. Showing analysis of the phenotypic properties of CACs and ECFC

Early EPCs/CACs	ECFCs
$CD45^{+}$	$CD45^{-}$
$CD14^{+}$	$CD14^{-}$
$CD115^{+}$	$CD115^{-}$
$CD31^{+}$	$CD31^{+}$
$CD146^{low}$	$CD146^{high}$
$CD144^{+/-}$	$CD144^{++}$
$CD105^{+}$	$CD105^{+}$
$VWF^{+/-}$	VWF^{+}
$CD34^{+/-}$	$CD34^{+/-}$
$CD133^{+}$	$CD133^{-}$
CD117 $(KIT)^{+}$	CD117 $(KIT)^{+/-}$
$VEGFR1^{+}$	$VEGFR1^{+}$
$VEGFR2^{+}$	$VEGFR2^{++}$
AcLDL $uptake^{+}$	AcLDL $uptake^{+}$
$ALDH^{+}$	$ALDH^{+}$

They are identified as supportive cells that promote but are not directly involved in vascular repair whereas late EPCs which are derived from an endothelial colony and also known as endothelial colony forming cells (ECFCs) are true endothelial progenitors that can be transplanted in immunodeficient mice and have the ability to form vascular structures in vivo (Yoder *et al.,* 2007). These cells have shown to have the capacity to form chimeric blood vessels in a xenograft model of vessel formation (Yoder *et al.,* 2007). Yoder *et al.* (2007) were the first group to analyse the proliferative potential of human EPCs in clonogenic assays *in vitro.* This assay included plating of single EPCs in collagen-coated plates and the number of colonies developed over 14 days were enumerated. The developing cells displayed a typical cobblestone morphology and showed differing proliferative potential, with high proliferative potential colony-forming cells (HPP-ECFCs) giving rise to low proliferative potential colony-forming cells (LPP-ECFCs) and finally to mature non-dividing endothelial cells. The LPP-ECFCs formed colonies of less than 2000 cells, and failed to form colonies on

replating, whereas HPP-ECFCs formed colonies containing greater than 2000 cells and formed at least secondary colonies. Those forming tertiary colonies were proposed to have much higher proliferative potential (Reinisch *et al.*, 2009).

It was therefore important to isolate the true EPCs. The first step was to isolate both early and late EPCs and distinguish them on the basis of expression of cell surface markers as these EPCs have been characterized on the basis of the expression or lack of expression of certain biomarkers used for their isolation. The second step was to distinguish between endothelial progenitor cells (ECFCs) and mature endothelial cells (HUVEC). The study was designed in such a way that human umbilical cord mononuclear cells (HUCBC) also known as day 0 MNCs were used as a starting population. They were differentiated to produce early EPCs also known as circulating angiogenic cells (CACs) when grown in endothelial specific media and to macrophages when grown in macrophage specific growth media. Human umbilical cord mononuclear cells (HUCBC) were obtained from the umbilical cord of normal newborn deliveries. They are known to be a mixed population of cells including cells of hematopoietic, endothelial stem cell and mesenchymal origin (Henning *et al.*, 2012). These cells have recently been used to overcome cell depletion in the bone marrow caused by treatment of several hematological malignancies and disorders including acute lymphoid and myeloid leukemia and aplastic anemia (Broxmeyer *et al.*, 2011). These HUCBC lack the expression of the major histocompatibility complex antigen MHC II which minimizes the risk of transplant rejection from the recipient. Morphologically, they appeared as rounded cells as shown in figure 4.1A. Early EPCs also known as circulating angiogenic cells (CACs) were derived from these HUCBC (day 0 MNCs). They were allowed to differentiate in endothelial specific media and were found to be non-proliferative cells that did not form endothelial colony after 14 days of culture. They appeared as rounded cells as shown in figure 4.1B.

Macrophages were also derived from the same day 0 mononuclear cells which were allowed to differentiate in macrophage specific media. The macrophages were seen after around 5-7 days of culture and resembled elongated fibroblast like structures as shown in figure 4.1C. Macrophages were used as a control for two reasons. Firstly, it has been shown that the early EPCs are related to monocyte-macrophage lineage and the endothelial progenitor cells should be distinguished from them on the basis of expression of macrophage markers (Yoder et *al.* 2007) Secondly, macrophages are known to be resistant to oxidative stress (Dernbach *et al.*, 2004) and therefore it was important to make sure that the endothelial progenitor cells isolated for testing further hypothesis are not composed of a mix of cell populations like monocyte and macrophages.

We characterized them first on the basis of gene expression analysis for cell surface markers including PECAM-1, CD34, CD14, PTPRC, MCAM, CDH5, END and vWF.
PECAM-1 has shown varied level of expression in several hematopoietic cells and human early EPCs (Rehman *et al.*, 2003). Our results showed that there was a decreased expression of this gene in early EPCs in comparison to HUVEC which is consistent with the previous findings (Furuhata *et al.*, 2007).

Strong expression of *PECAM-1* in ECFCs suggests that these might be the endothelial progenitors that are involved in post-natal vasculogenesis, as shown before CD31 forms part of a mechanosensory complex that mediates endothelial cell response to shear stress (Tzima *et al*. 2005), and a recent study indicates that CD31 has a role in arteriogenesis and collateral remodelling (Chen *et al*. 2010).

The results showed that early EPCs (CACs) derived from umbilical cord blood showed the expression of hematopoietic markers like CD34 and CD31and also expressed macrophage specific markers like CD14 and CD45 as shown in figure 4.2, and could be clearly distinguished from late EPCs or ECFCS and mature endothelial cells and fulfilled the criteria of being proangiogenic cells as has been shown before (Estes *et al.*, 2010).

The expression of CD34 and CD31decreases gradually as the EPC differentiation occurs, but ECFCs still maintain the expression of both CD34 and CD31 (Richardson and Yoder 2010). The results indicated that both ECFCs and HUVEC did not express macrophage specific markers CD14 and CD45, as shown in figure 4.2. The difference again was found to be statistically significant. Figure 4.3 shows the differential gene expression analysis of endothelial markers in these cell types. The results indicated that early EPCs and macrophages did not show the expression of endothelial markers as expected. There were several markers that showed statistically significant difference in the gene expression analysis of ECFCs and HUVEC. Endoglin and *CDH5* can be used as markers to distinguish between endothelial progenitor cells and mature endothelial cells but vWF showed much higher expression in HUVEC as compared to ECFCs.

The expression of cell surface markers was confirmed at protein level by using FACS analysis, as shown in figure 4.4 and 4.5. This was based on using multicolor flow cytometry. The cell surface markers CD34, CD14, CD144 and CD146 were all tagged with different fluorochrome, so that the expression level of each could be quantified at the same time. The results further confirmed that colony derived endothelial cells (ECFCs) showed the

expression of cell surface markers CD144 and CD146 and lacked the expression of monocyte-macrophage marker CD14 and could be used to function as true EPCs. We found that mRNA expression of CD34 and CD144 was different as compared to protein expression mainly in HUVEC. This could be due to the fact that different donors were used for the FACS analysis using multicolour flow cytometry and RNA and protein expression does not always match. FACS analysis was carried out on fresh cells.

The results confirmed that we had successfully isolated the true EPCs also known as endothelial colony forming cells (ECFCs). These cells can be defined as proliferative cells that were first isolated from peripheral blood but this is no more considered a rich source of ECFCs. They have since then been isolated from cord blood, human aorta and umbilical vein and also pulmonary aorta of the rat (Ingram *et al.*, 2004; Ingram *et al.*, 2005; Alvarez *et al.*, 2008). They were identified in search of a true endothelial progenitor but till now there has been no specific marker that can distinguish them from other endothelial cells. ECFCs have shown to express cell-surface markers including CD34, CD31, CD146 and CD105 but still it remains unclear if these cells belong to uni or multipotent stem cell lineage or are endothelial cells with high level of proliferative potential as they have been shown to achieve over 100 population doublings *in vitro* (Ingram *et al.*, 2004; Ingram *et al.*, 2005). Our results indicate that ECFCs can be further distinguished from mature endothelial cells by the expression level of vWF. It has been shown before that vWF stains for both ECFCs and mature endothelial cells (Doung *et al*., 2011). But as shown in figure 4.8 we compared early and late passage ECFCs with early and late passage mature endothelial cells (HUVEC) and we found that there was statistically significant difference between ECFCs and HUVEC compared at same passages (P5 and P15) in terms of number of vWF positive cells demonstrated by direct immunofluorescence analysis that stains for vWF intracelluarly.

The results as shown in this section clearly indicate that we have successfully isolated the true EPCs from cord blood. These cells are derived from an endothelial colony and fulfil the criteria of being ECFCs as recently proposed by Yoder *et al*,. 2010. As shown in figure 4.1 these cells are morphologically different from early EPCs also known as CACs. The molecular characterization of these cells showed that they are distinguished from CACs and also from mature endothelial cells on the basis of expression of several cell surface markers. There have been several studies before that have characterized these ECFCs using different strategies and our results further validate them. Cord blood and peripheral blood derived $CD34^+$ cells have been used to generate ECFCs and it has been shown that its $CD34^+/CD45^-$

cell population that produces ECFCs whereas $CD34^{+}/CD45^{-}$ and $CD133^{+}$ cells failed to generate ECFCs (Timmermans *et al.*, 2007). Our results also showed that the ECFCs were $CD34^{+}/CD45^{-}$. Protein expression analysis was carried out using FACS and showed the expression of these cell surface markers in ECFCs and HUVEC. There was higher protein expression of CD34 seen in HUVEC and also CD144 had higher expression in HUVEC as compared to mRNA expression and this could be due to the fact that the FACS analysis was carried out in different donors and also mRNA level of expression does not always match with protein expression. Also, FACS analysis was carried out on fresh cells, whereas qPCR was performed on samples that were frozen down. Immunofluorescence analysis was performed with vWF and it was shown that ECFCs can be distinguished from mature endothelial cells (HUVEC) both at mRNA and protein level by the expression of vWF.

Chapter 5. Investigating the effect of oxidative stress on PGC-1 alpha expression and growth kinetics and telomere dynamics of ECFCs and HUVEC

5.1 Introduction

In eukaryotes, mitochondrial activity is responsible for controlling the cellular and systemic metabolism. Similarly, the energy homeostasis is maintained through regulation of tissue-specific metabolic pathways, and at molecular level main hormonal and nutrient pathways depend upon the genes encoding for metabolic enzymes. One of the major players involved in this are the PGC transcriptional complexes. PGC-1α is one of the members of this small family of transcriptional coactivators, that also includes PGC-1β and PGC-related coactivators (PRC), all of which are promoters of mitochondrial biogenesis and oxidative metabolism (Wu *et al.*, 1999). Although, apart from PRC which shows ubiquitous expression, both PGC1- α and PGC1-β are mainly expressed in oxidative tissues including heart, brain, liver, muscle, kidney, pancreas and brown adipose tissue (Uldry *et al.*, 2006). PGC-1α also regulates reactive oxygen species (ROS) and apoptosis in mature endothelial cells (St-Pierre *et al.*, 2006). Dysregulation of the gene encoding PGC-1α leads to a wide variety of pathological conditions and therefore pharmacological regulation of its expression and activity can be used as a novel approach to treat several diseases.

It has been shown that PGC-1α can be induced by lack of nutrients and oxygen; leading to VEGF expression and promoting angiogenesis in cultured muscle cells and also skeletal muscle *in vivo* (Arany et al., 2008). Similarly, it has been shown that PGC-1α knockout mice fails to show neovascularisation after an ischemic insult, which in normal circumstances is carried out by the stimulation of VEGF and other angiogenic factors in response to hypoxia, leading to vascularisation as a protection against ischemic injury .

Telomeres are DNA repeats at the ends of chromosomes, helping to maintain their integrity. They are widely regarded as the internal biological clock of a living organism, and shorten by a few base pairs with every cell division (Blasco, 2005). Telomere shortening can be compensated or slowed down by concomitant activation of telomerase, a DNA polymerase and specialized ribonucleoprotein. The catalytic core of telomerase is composed of an RNA subunit (TERC) serving as a template for sequence addition and a reverse transcriptase (TERT) subunit that facilitates the replication of telomeres. Oxidative stress is an important

factor that contributes to telomere attrition (Von Zglinicki, 2002). Correlative evidence from human population studies suggests an association of short telomeres with conditions of increased oxidative stress, including smoking, obesity and coronary heart disease (CHD).

It has been shown recently, that telomerase is essential in maintaining a healthy human lifespan. Accumulating evidence from premature ageing (progeroid) syndromes, such as Werner syndrome or dyskeratosis congenita, both of which show mutations in either TERT or TERC, paralleled by short telomeres are a proof of this (Sahin *et al.*, 2011). Similarly, a third-generation telomerase knockout mouse displays an age-related phenotype with shortened lifespan. The reactivation of telomerase in aged telomerase-deficient mice even leads to a reversal of tissue degeneration, suggesting that telomere rejuvenation strategies for age-associated diseases might prove a therapeutic alternative, especially those driven by accumulating genotoxic stress. Recently it has been shown that telomere abnormality in the nucleus leads to deactivation of PGC1 proteins in the mitochondria via activation of p53. Sahin et al 2011 showed that a mice null in either *Tert* or *Terc* genes leads to p53 mediated apoptosis and growth arrest in several tissues and repression of PGC genes and their downstream targets leading to impaired mitochondrial biogenesis.
We, therefore, hypothesized that mild oxidative stress would lead to telomere dysfunction in endothelial cells, and as a consequence of that a decrease in the expression of PGC-1α in endothelial progenitor cells (ECFCs) and mature endothelial cells (ECs). As PGC-1α is a key regulator of mitochondrial biogenesis and oxidative metabolism, a decrease in its expression would lead to an impairment of mitochondrial activity in these cells. This would in turn lead to a decrease in the angiogenic potential of these cells.

Telomeres are complex DNA-protein structures located at each end of the chromosomes. Telomeres are shortened with each cycle of cell replication, and can be a predictor of organismal age (Blackburn, 2001). Telomere preservation requires intact telomerase enzyme activity and maintenance of telomere length itself (Edo and Andrés, 2005). Recently, it has been shown that critically shortened telomeres are linked to age-related cardiovascular diseases and promote apoptosis and cellular senescence (Samani *et al.*, 2001; Brouilette *et al.*, 2003; Brouilette *et al.*, 2008; De Meyer *et al.*, 2008; Samani and Van Der Harst, 2008; Butt *et al.*, 2010; Aviv, 2012). Similarly, it has been proposed that endothelial cells within an atherosclerotic plaque show signs of cell senescence, whether this is linked to shortening of telomere length and does this differ between endothelial progenitor cells and mature endothelial cells remains to be seen. Our initial results indicated that endothelial progenitor

cells (ECFCs) could be grown for a longer period of time and grow at a faster rate in comparison to mature endothelial cells (HUVEC) in conditions of oxidative stress. It has already been shown that chronic oxidative stress leads to telomere dysfunctioning and promotes cell senescence in human endothelial cells (Kurz *et al.*, 2004). We therefore proposed that ECFCs are better equipped to counteract this ROS mechanism and could be the cells that take part in repairing damaged endothelium.

It has been shown that oxidative stress is one of the factors associated with telomere dysfunction, and as a result of that cells grown in hyperoxia show telomere shortening (Von Zglinicki, 2002).Telomere shortening can be compensated or slowed down by concomitant activation of telomerase, a DNA polymerase and specialized ribonucleoprotein. The catalytic core of telomerase is composed of an RNA subunit (TERC) serving as a template for sequence addition and a reverse transcriptase (TERT) subunit that facilitates the replication of telomeres (Von Zglinicki, 2002).

We had hypothesized that oxidative stress would lead to telomere dysfunction and a decrease in the level of PGC-1α as shown in mice recently (Sahin *et al.* 2011), but again instead of a decrease in the levels of PGC-1α, its mRNA transcripts were increased, as shown in figure 5.5C. Also, several downstream targets of PGC-1α showed an increase in gene expression as shown in figure 5.5 D.

5.2 Results

5.2.1 Growth kinetics of ECFCs and HUVEC grown in normoxia and hyperoxia

Endothelial colony forming cells (ECFCs) were isolated from human cord blood. Cord blood mononuclear cells were plated at a density of 60-70 million cells per well of a 6 well plate. They were allowed to differentiate in endothelial media so that they could differentiate towards endothelial lineage. Once an endothelial colony was observed usually after 14-21 days of culture, it was picked and grown in the same endothelial media. ECFCs were derived from these endothelial colonies which were observed in the culture dishes. Cells were grown and split once they became 70-80% confluent. 1 million cells were plated to start the ECFC culture and that was taken as P0 and day 0. Cells were split regularly in a ratio of 1:3 and 1 million cells were replated and the rest were frozen down each time cells were passaged. ECFCs were grown till late passages in normoxia (20% O_2) as shown in figure 5.1A, indicated by red circles. ECFCs were grown in normoxia till P2 and then cells were split and

1 million cells were plated and cells were placed in hyperoxic incubator (40% O_2). All the conditions and cell culture media was same for the cells grown in hyperoxia and the only difference was oxygen concentration. This was counted as starting day for ECFC hyperoxia cell culture and cells were passaged regularly once they became confluent.1 million cells were plated each time and cells were split in a ratio of 1:3. One million cells were plated again and the rest were frozen down. As shown in the growth kinetics of ECFCs in figure 5.1A, ECFCs grew in hyperoxia at a rate that was slightly slower than that of cells grown in normoxia but the difference was not found to be statistically significant.

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical vein. Cells were plated and allowed to grow and observed under microscope regularly for a cobble stone appearance known for these mature endothelial cells. Once these cells became around 70-80% confluent they were split and counted using a haemocytometer. 1 million cells were replated and this started HUVEC cell culture and this was taken as P0 as shown in figure 5.1B, and indicated by red circles. Cells were allowed to grow in normoxia (20% O_2) and split again once they became confluent, in a ratio of 1:3. 1 million cells were plated again and the rest were frozen down. Cells were passaged regularly and cells were grown over a period of 8 weeks as shown in figure 5.1B.

Cells were split after P2 and 1 million cells were plated and placed in hyperoxia (40% O_2). This was counted as P0 and then cells were allowed to grow in hyperoxic conditions. HUVEC grew at a much slower rate in hyperoxia as compared to cells grown in normoxia and were found to be much less resistant than ECFCs to oxidative stress (40% O_2). The difference as shown in growth kinetics was found to be statistically significant. As shown in the figure ECFCs were found to be growing at a faster rate after 7 days than HUVEC grown in hyperoxia and the cells grown in normoxia almost showed similar growth pattern till day 40 as shown in the figure.

For both ECFCs and HUVEC, 1 million cells were plated to start the culture that was taken as P0. Cells were grown till they were around 70% confluent. At this stage the cells were split using trypsin in a ratio of 1:3. 1 million cells were replated and the rest were frozen down at -80°C in freezing media containing DMSO to be used in later experiments. The cells were regularly passaged in around 3-7 days as shown in figure 5.1. Early passage cells grew faster and were split at around 3days in culture. Late passage cells took nearly 7 days to be 70% confluent. Cells at P10 correspond to around 8 weeks of culture as shown in figure 5.1.

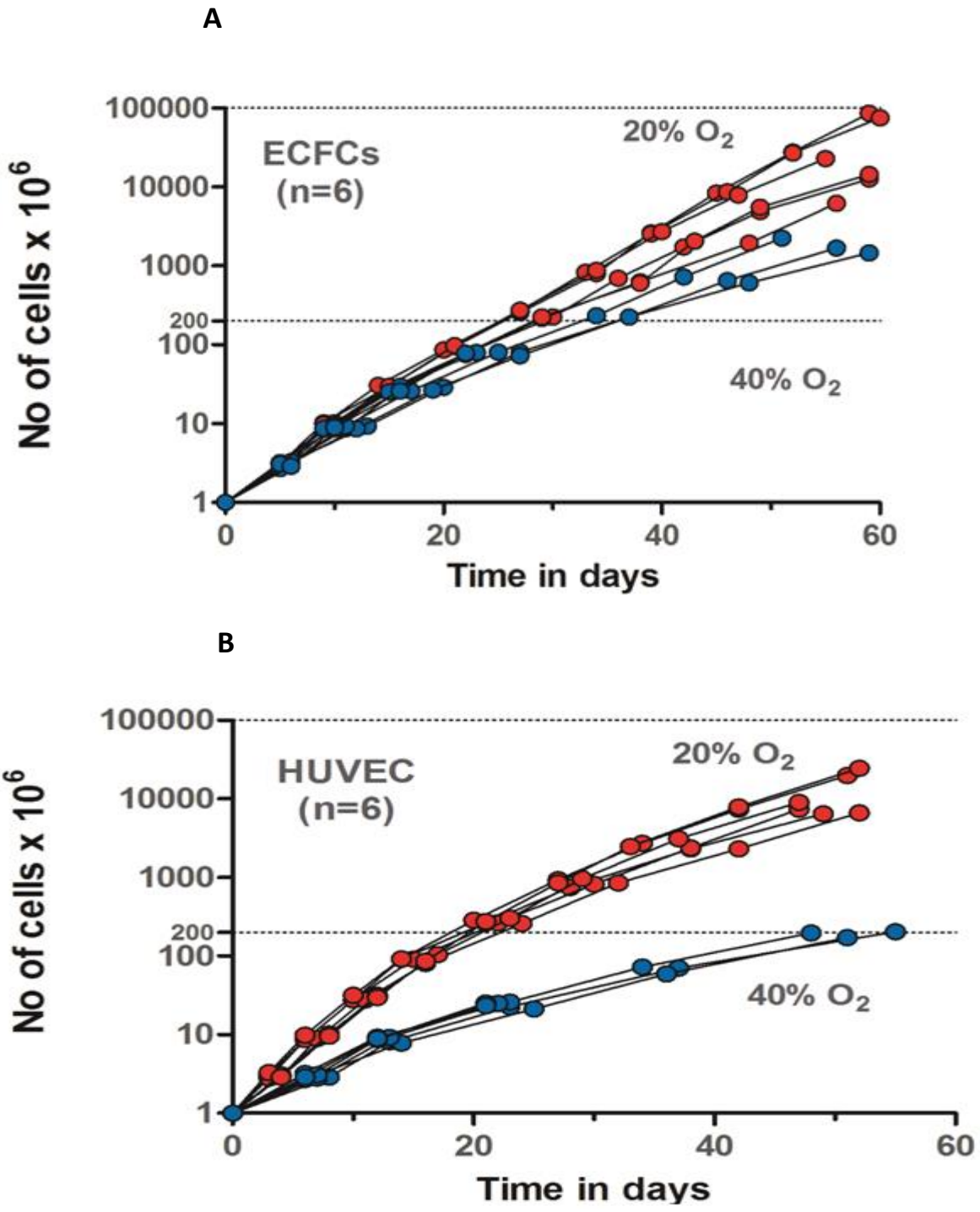


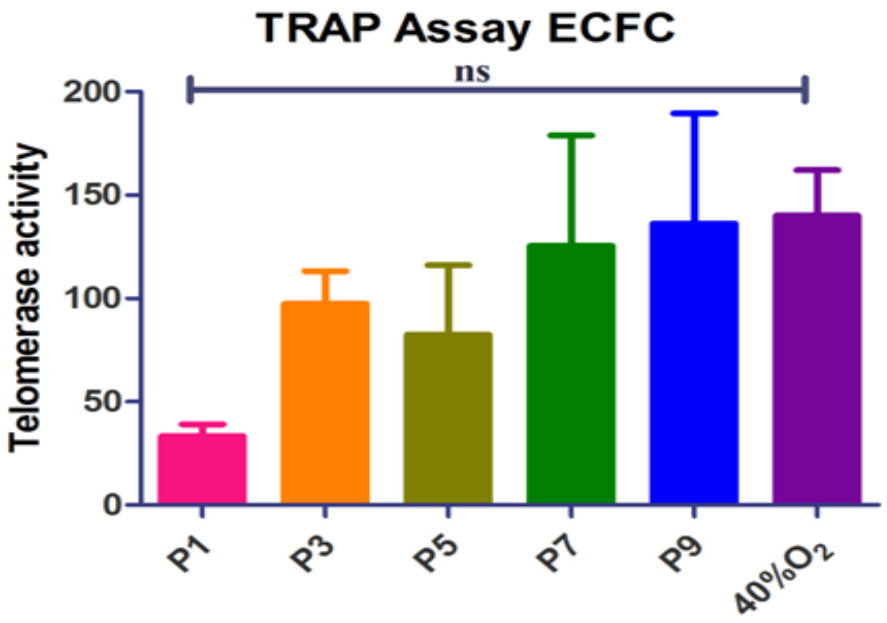
Figure 5.1. Illustrating the growth kinetics of endothelial colony forming cells (ECFCs) and mature endothelial cells (HUVEC) derived from cord blood and human umbilical vein of the same donor. Figure **A and B** showing the comparison between growth behaviour of ECFCs and HUVEC grown in normoxia (20% O_2) and hyperoxia (40% O_2). Statistical analysis was carried out using two sample *t* tests between the slopes of the normoxia and hyperoxia curves for both ECFCs and HUVEC. The difference was found to be statistically significant with a *p* value of .001 for the comparison between HUVEC grown in normoxia and hyperoxia, and a *p* value of .02 for the comparison between ECFCs and HUVEC grown in hyperoxia. The difference between ECFCs grown in normoxia and hyperoxia and ECFCs and HUVEC grown in normoxia was not found to be statistically significant with a *p* value of ≥ .05 in both cases.

5.2.2 Telomerase activity measurement using TRAP assay in ECFCs and HUVEC grown in normoxia and hyperoxia

As shown in growth kinetics of ECFCs and HUVEC in figure 5.1, we proposed that as endothelial progenitor cells (ECFCs) grow much better in hyperoxia as compared to mature endothelial cells (HUVEC), they might show a higher level of telomerase activity when grown in hyperoxia that could be compensating for a shortening of telomere length due to oxidative stress. In order to test this, ECFCs were isolated from umbilical cord blood and derived from an endothelial colony. ECFCs were grown in normoxia till late passage (P9) so that a change in telomerase activity could be measured and to test whether these cells would show a change in telomerase activity across passages. Telomerase activity was measured using TRAP assay and as shown in figure 5.2, there was no statistically significant difference found between early and late passage ECFCs and also there was no change in telomerase activity in ECFCs grown in hyperoxia. We expected to have higher telomerase activity in ECFCs grown in hyperoxia, but the difference found was not statistically significant.

Similarly, mature endothelial cells HUVEC were isolated from human umbilical vein and grown over a period of 2-3 weeks. The cells were then split and grown in normoxic and hyperoxic conditions, and cells were frozen down at different passages, early and late passage till P11 and telomerase activity was measured using a sensitive TRAP assay as shown in figure 5.2. There was no change in telomerase activity in mature endothelial cells (HUVEC) grown in normoxia across passages and also there was no significant change in telomerase activity of cells grown in hyperoxic conditions, and again the difference shown did not achieve statistical significance.

A



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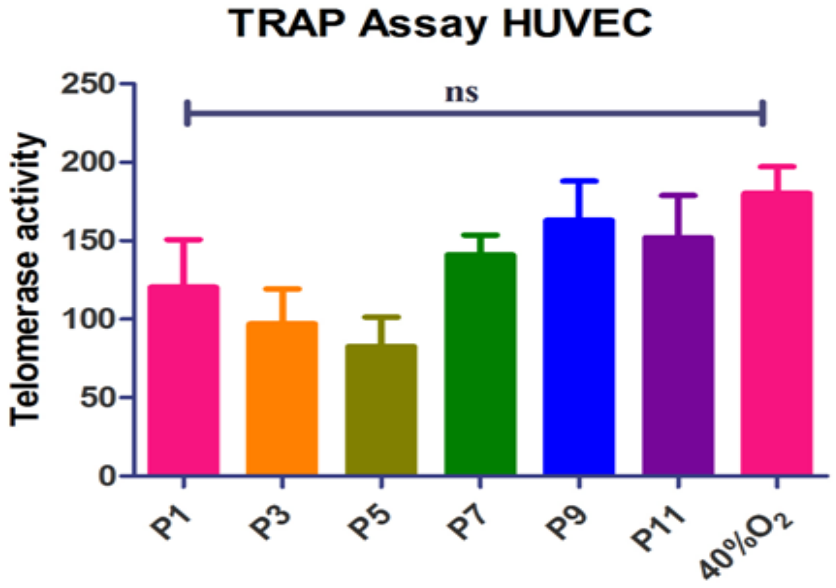
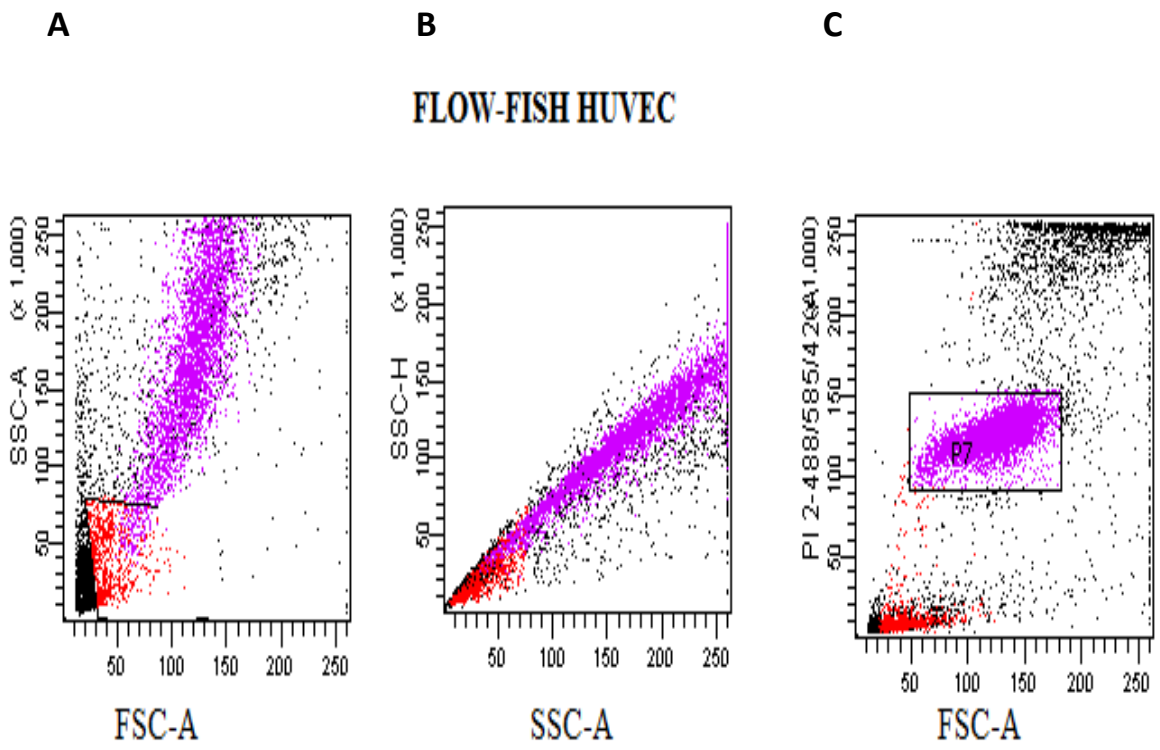


Figure 5.2. Longitudinal comparison of the measurement of telomerase activity in endothelial colony forming cells (ECFCs) and mature endothelial cells using a TRAP assay. Figure **A** showing the changes in telomerase activity in HUVEC grown across passages in normoxia and compared to HUVEC grown in hyperoxia. Figure **B** showing the changes in telomerase activity across early and late passages in endothelial colony forming cells (ECFCs) derived from cord blood and compared to ECFCs grown in hyperoxic conditions (40%O_2). The cells were cultured over a period of 6- 8 weeks. Statistical analysis was carried out using one way ANOVA, followed by Tukeys multiple comparison tests. The telomerase activity seems to go up across passages and in hyperoxia but the difference was not statistically significant with a *p* value of >.05 in all cases. ns stand for not statistically significant. n=6.

5.2.3 Telomere length measurement analysis of HUVEC grown in normoxia and comparison between early and late passage cells

After measuring the telomerase activity of HUVEC samples across passages, as shown in the figure 5.3, we measured telomere length of HUVEC from early to late passages (young and old cells). Mature endothelial cells HUVEC were isolated from umbilical vein. Cells were grown in normoxia (20% O_2) and once around 70-80% confluent they were split and 1 million cells were plated to start the culture taken as P0. Cells were passaged regularly and split in a ratio of 1:3. 1 million cells were replated and the rest were frozen down. Cells were grown till late passage P16 (10-12 weeks of culture) so that any change in telomere length as the cells grow older could be detected. As shown in figure 5.3, the results showed that the telomere length goes down from early passage to late passage and the difference was found to be statistically significant. This could be as a result of cellular senescence as telomere length is shortened with each replicative cycle. In order to compensate for this attrition of telomere length, we proposed that the telomerase activity would increase and compensate for this loss, but as shown in figure 5.3, the telomerase activity did not increase across passages and also showed no significant increase in the activity under oxidative stress conditions (40% O_2).



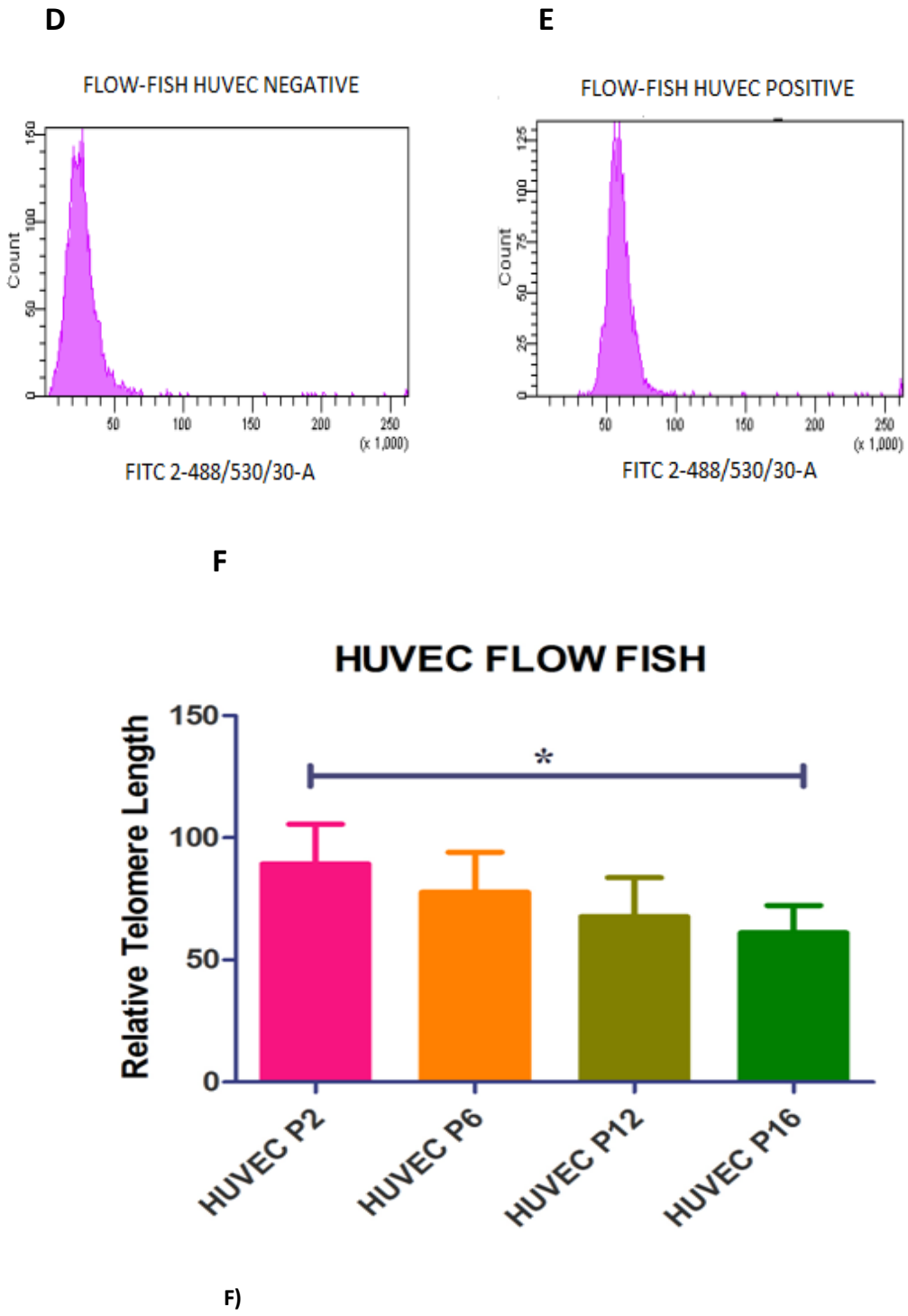
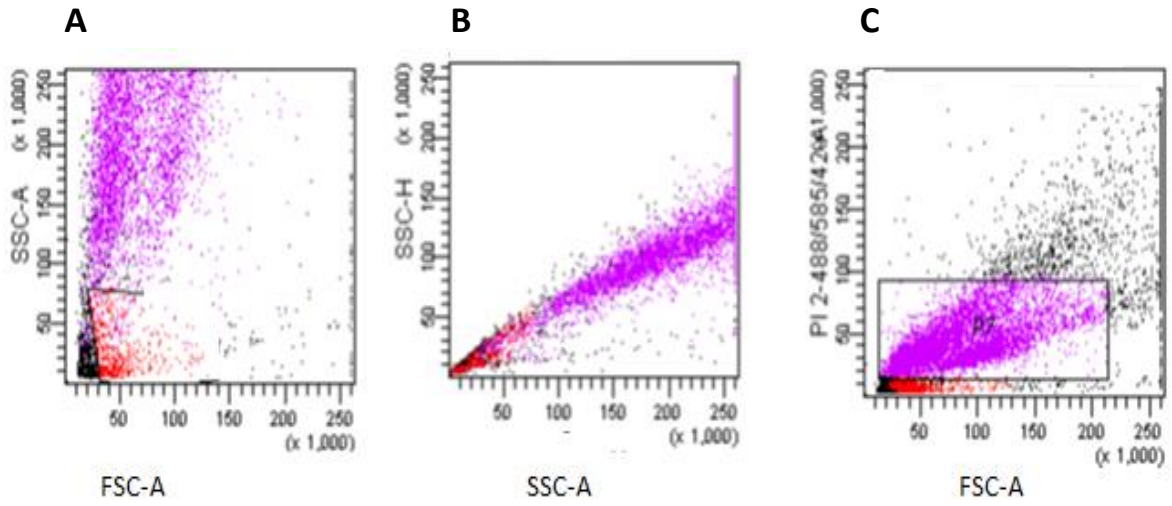


Figure 5.3. Flow FISH data analysis for the measurement of telomere lengths in early and late passage mature endothelial cells (HUVEC). Flow FISH is a PNA probe based assay in which mean fluorescence based on FITC signal is used to measure the telomere lengths. Thymocytes were used as an internal control and as a reference telomere length for telomere length measurements in HUVEC. Mature endothelial cells HUVEC were isolated from human umbilical vein and were grown in normoxia. Cells were passaged till P16 over a period of 5-6 weeks to compare telomere lengths of early and late passage endothelial cells. Propidium Iodide was used as a counter stain and cells were gated based on PI uptake. Telomere length seems to go down across passages, and the difference was found to be statistically significant. Statistical analysis was carried out using one way ANOVA followed by Dunn's multiple comparison tests. n=3.

5.2.4 Telomere length measurement analysis of ECFC grown in normoxia and comparison between early and late passage cells

After successful measurement of telomerase activity in ECFCs grown in both normoxia and hyperoxia, the next step was to measure the telomere length of ECFCs grown over a period of 8-10 weeks till passage 16. ECFCs were obtained from umbilical cord blood by differentiation of cord blood mononuclear cells in endothelial specific media. Cells were grown till an endothelial colony was seen which was observable at around 3-4 weeks of culture. Once a colony was seen it was picked up and grown in culture. The cells were grown in normoxia and were regularly passaged. Cells were frozen down at each passage and 1 million cells were plated each time the cells were split using trypsin-EDTA. As shown in figure 5.5, there was a decrease in telomere length of ECFCs when grown from early to late passages. This further confirms that these ECFCs were not stem cells and have committed to endothelial progeny. This was shown in less than 3 samples so the statistical analysis was not carried out.



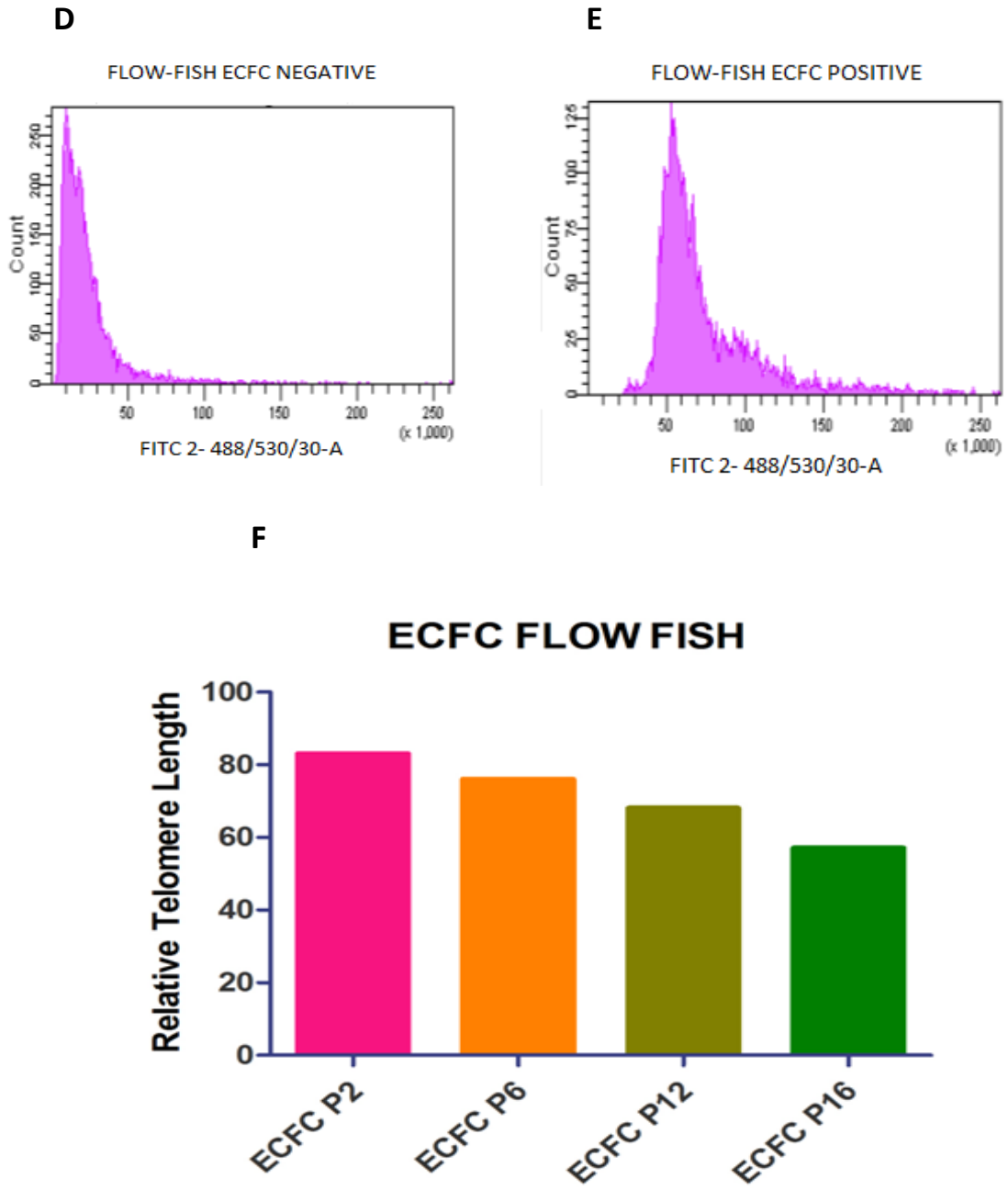


Figure 5.4. Flow FISH data analysis for the measurement of telomere lengths in early and late passage endothelial progenitor cells (ECFCs). Flow FISH is a PNA probe based assay in which mean fluorescence based on FITC signal is used to measure the telomere lengths. Thymocytes were used as an internal control. ECFCs were isolated from cord blood derived endothelial colony. Cells were grown in normoxia and passaged till P16. As shown in the figure the telomere length decreases from early to late passage. n=1.

5.2.5 Gene expression analysis of PGC-1 alpha and its downstream targets in ECFC and HUVEC grown in normoxia and hyperoxia

Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 alpha) belongs to a family of transcriptional coactivators that orchestrate a wide range of genetic programs. PGC-1 alpha induction occurs as a result of coactivation of certain transcription factors including estrogen- related receptor alpha (ERR alpha), nuclear respiratory factor-1 and 2 (NRF-1, 2) and many others (Patten and Arany, 2012). PGC-1 alpha promotes angiogenesis by inducing the expression of vascular endothelial growth factor (VEGF) along with other angiogenic factors (Arany *et al.*, 2008). This induction of VEGF occurs through co activation of ERR alpha and is independent of the well-studied hypoxia- inducible factor (HIF-1 alpha) pathway (Arany *et al.*, 2008). As shown in figure 5.5, we investigated the effect of oxidative stress (40% O_2) on the gene expression analysis of PGC-1 alpha and its downstream targets including ERR alpha and Glucose 6 phosphate dehydrogenase (G6PD). We first compared the expression of PGC-1 alpha in early EPCs derived from cord blood and late EPCs or ECFCs derived from the cord blood to mature endothelial cells (HUVEC) derived from the umbilical vein of the same donor. RNA extraction was carried out followed by quantitative PCR analysis. As shown in figure 5.5A, there was a decrease in the expression level of PGC-1 alpha in ECFCs as compared to early EPCs and the difference was found to be statistically significant. The difference between the gene expression level of PGC-1 alpha in ECFCs and HUVEC was not statistically significant. As shown in fig 5.5B, we compared the expression level of PGC-1 alpha in ECFCs and HUVEC grown in normoxia (20% O_2) and in conditions of oxidative stress (40% O_2). The cells were grown in normoxia till passage 2 (P2) and then exposed to oxidative stress by incubating in hyperoxia. We found that there was an up regulation of PGC-1alpha gene expression level in both ECFCs and HUVEC, although the difference was not found to be statistically significant. We then investigated, if this increase in gene expression level caused any change in the expression level of any of the downstream targets of PGC-1 alpha. As shown in figures 5.5C and D, we compared the gene expression level of glucose 6 phosphatase and ERR alpha in both ECFC and HUVEC. The cells were grown in normoxia till P2 and then exposed to oxidative stress, by growing in 40% O_2. It was found that there was an up regulation of both these genes in ECFCs and HUVEC suggesting that this oxidative stress has led to an activation of PGC-1 alpha, which showed a slight but insignificant increase in expression which in turn co activated other transcription factors involved in other biological functions.

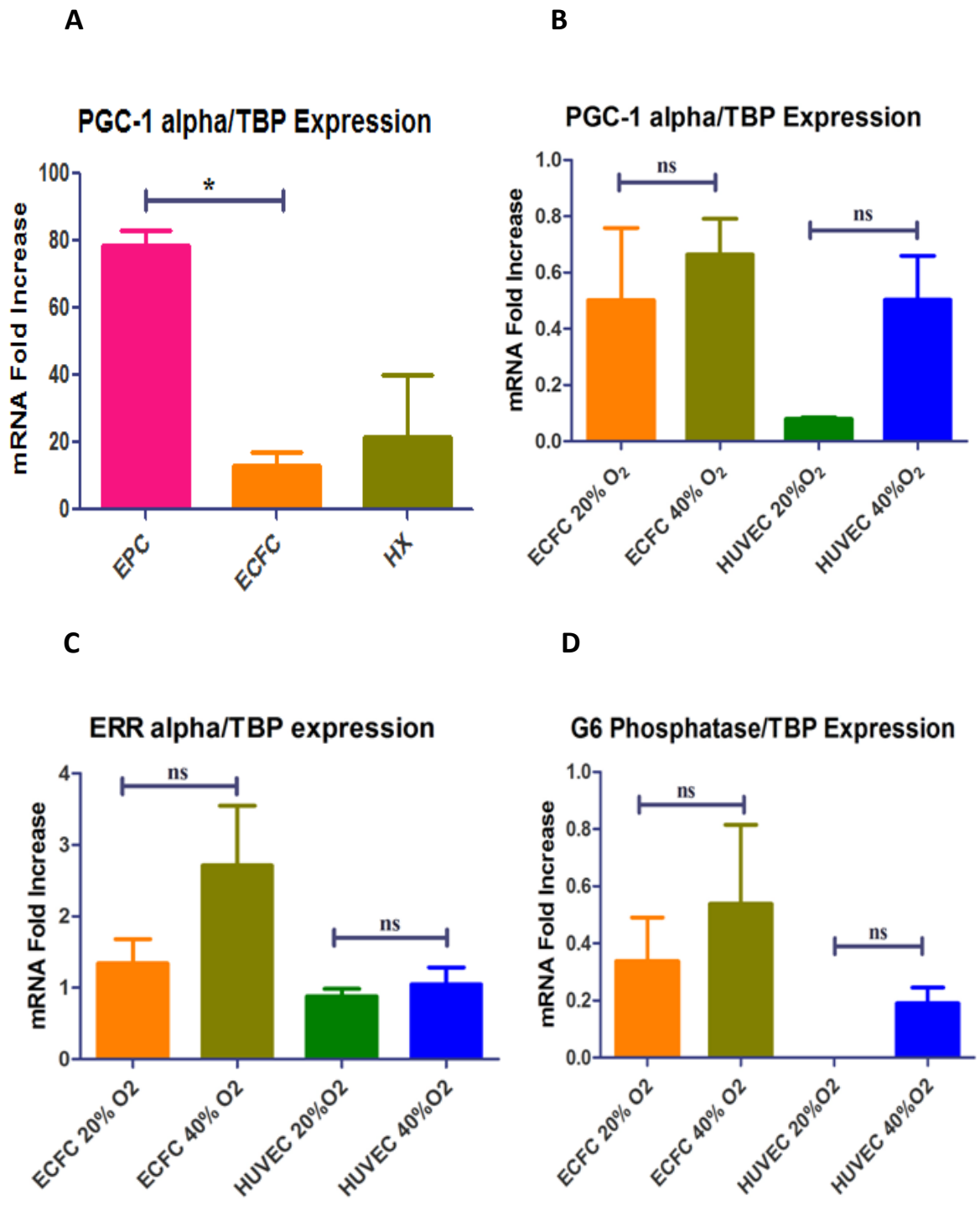


Figure 5. Gene expression analysis of PGC-1 alpha and its downstream targets in normal and hyperoxic conditions. The figure shows that PGC-1 alpha expression level goes down as the EPC differentiation occurs. The cells were exposed to hyperoxia till passage 1 (P1) that corresponds to 3-5 days of culture. Statistical analysis was carried out using one way ANOVA followed by Tukeys multiple comparison tests. The figure further shows the gene expression analysis of PGC-1 alpha and its downstream targets in ECFCs and HUVEC grown in normal (20% O_2) and hyperoxic conditions (40% O_2). The expression increases for both targets in hyperoxia but the difference was not statistically significant. n=3 (HUVEC 20% and 40% O_2) and n=5 (ECFC 20% and 40% O_2). All values are calculated as ± SEM. * $P < .05$, ** $P < .01$, *** $P < 0.001$.

5.2.6 Gene expression analysis of antioxidative genes in HUVEC grown in hyperoxia

Cardiovascular risk factors are responsible for increased ROS production in the vascular wall which eventually leads to oxidative stress. This occurs when ROS production exceeds anti oxidative defence mechanism which in the vessel wall is composed of a set of antioxidative enzymes that can reduce this ROS damage to the endothelium. These enzymes include Catalases (CAT), Glutathione peroxidases (GPx), superoxide dismutase (SOD), Heme oxygenase (HO) and periredoxins. We therefore proposed that exposing endothelial cells to oxidative stress (40% O_2) would lead to an increase in gene expression of these antioxidative genes. As shown earlier, our results indicated that there was an up regulation of PGC-1 alpha expression in ECFCs and HUVEC grown in hyperoxia (40% O_2). We therefore investigated the time points when this up regulation begins and alongside PGC-1 alpha is there any change in level of expression of these anti oxidative genes. We chose two antioxidative genes namely CAT and GPx-1 that encode respective antioxidant enzymes catalase and glutathione peroxidase. CAT gene encodes catalase which is an important antioxidant enzyme that prevents the body against reactive oxygen species by degrading hydrogen peroxide to oxygen and water. Catalase overexpression has been shown to have positive effects in cardiovascular system and has been shown to play a role against atherosclerosis and angiotensin induced hypertrophy of the aortic wall (Zhang *et al.,* 2005; Li *et al.,* 2013).

GPx-1 has been shown to play a protective role in preventing oxidative damage to the endothelium by reducing both lipid peroxides and hydrogen peroxide (Raes *et al.,* 1987; Maiorino *et al.,* 1995). Vascular injury has been demonstrated to be linked with a reduction in GPx-1 activity and atherosclerotic plaques isolated from carotid arteries have shown decreased expression of GPx-1 (Lapenna *et al.,* 1998).

Mature endothelial cells HUVEC at P0 were grown in normoxia and were split once the cells became confluent. 1 million cells were plated and cells were passaged till P2 and then the cells were exposed to hyperoxic conditions. Cells were not split till day 17 and cells were frozen down at specific time points as shown in figure 5.6. Cells were frozen down and RNA was extracted followed by gene expression analysis of PGC-1 alpha and antioxidative genes that we proposed would be up regulated in these cells. There was an up regulation of antioxidative genes CAT and GPx-1 in HUVEC when exposed to hyperoxia as shown in figure 5.6 B and C suggesting that HUVEC have responded to this exposure to stress

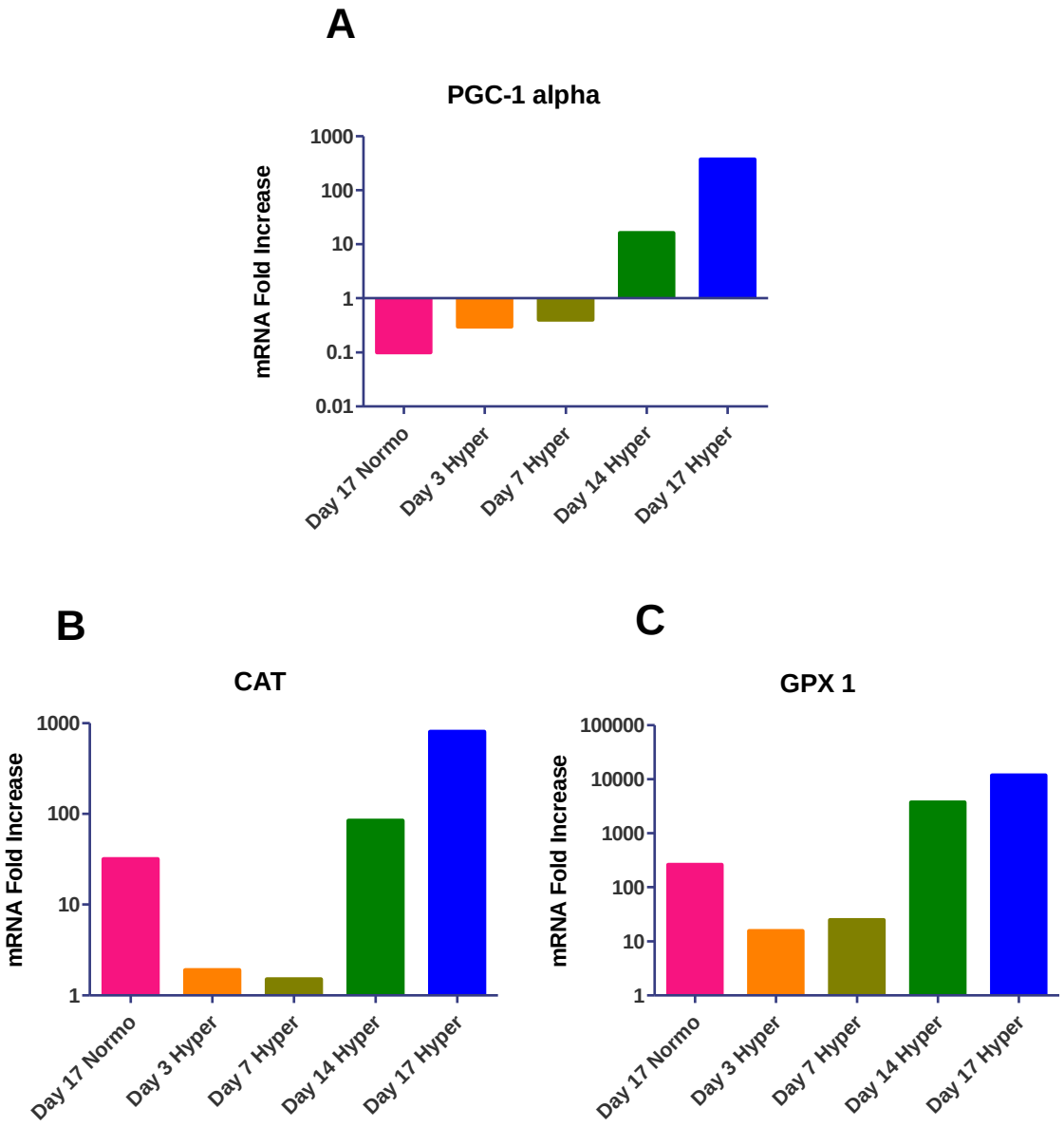


Figure 5.6. Hyperoxia time point experiment showing up regulation of PGC-1 alpha and anti-oxidative genes in HUVEC grown in 40% Oxygen (n=1). Figure showing an increase in the level of expression of antioxidative genes in HUVEC. PGC-1 alpha showed a significant increase in expression of HUVEC grown in hyperoxia. Catalase and GPx-1 are two antioxidants and increase in their mRNA level of expression as the incubation period of HUVEC exposure to oxidative stress was increased suggests that antioxidative mechanisms are up regulated in HUVEC when they are grown in 40% O_2.

5.3 Discussion

Peroxisome proliferator -activated receptor γ coactivator-1α (PGC-1 α) is a transcriptional coactivator that is considered to be a master regulator of mitochondrial biogenesis and also known to suppress reactive oxygen species (ROS), thus combating oxidative stress (St-Pierre *et al.*, 2006). Cell senescence has been shown to be linked with oxidative stress and associated telomere and mitochondrial dysfunction. Therefore, it is more than likely that there exists a relationship between all these factors promoting senescence and PGC-1 alpha plays an important role in preventing this by centrally coordinating all these processes (Xiong *et al.*, 2013). It has been shown recently, that PGC-1 alpha deletion leads to increased mitochondrial reactive oxygen species (ROS) production as a consequence of vascular inflammation and dysfunctioning (Kröller-Schön *et al.*, 2013). It was therefore important to find this link and investigate the effect that oxidative stress can have on ECFCs and HUVEC in terms of PGC-1 alpha gene expression and associated telomere dysfunction.

Telomeres are complex DNA-protein structures located at each end of the chromosomes. They are shortened with each cycle of cell replication, and can be a predictor of organismal age (Blackburn, 2001). Telomere preservation requires intact telomerase enzyme activity and maintenance of telomere length itself (Edo and Andrés, 2005). Recently, it has been shown that critically shortened telomeres are linked to age-related cardiovascular diseases and promote apoptosis and cellular senescence (Samani *et al.*, 2001; Brouilette *et al.*, 2003; Brouilette *et al.*, 2008; De Meyer *et al.*, 2008; Samani and Van Der Harst, 2008; Butt *et al.*, 2010; Aviv, 2012). Similarly, it has been proposed that endothelial cells within an atherosclerotic plaque show signs of cell senescence, whether this is linked to shortening of telomere length and does this differ between endothelial progenitor cells and mature endothelial cells remains to be seen. As shown in figure 5.2, we measured the telomerase activity of ECFCs and HUVEC grown in normoxia across passages (early to late passage) and there was no significant change in telomerase activity. We exposed the mature endothelial cells oxidative stress but still there was no significant increase in telomerase activity that could be observed using this sensitive TRAP assay. Similarly for ECFCs as shown in figure 5.2, there was no statistically significant difference seen in telomerase activity when these cells were exposed to 40% O_2. These results suggested that may be this stress has not induced telomere dysfunction in either ECFCs or HUVEC and there was no telomere shortening and as a consequence of that no concomitant increase in telomerase activity.

This now is well established that there exists a correlation between oxidative stress and inflammation, and several pathological conditions showing signs of both oxidative stress and chronic inflammation are characterized by low EPC count and activity (Guzik *et al.*, 2003; Tousoulis *et al.*, 2008; Hulsmans *et al.*, 2011). It has been shown that early EPCs including CACs and late EPCs (ECFCs) are more resistant to oxidative stress in comparison to mature endothelial cells (HUVEC), due to the expression of high levels of antioxidant enzymes (Dernbach *et al.*, 2004; Cai *et al.*, 2006; He *et al.*, 2009). Recent studies have shown that there exists a strong interplay between EPCs, reactive oxygen species (ROS) and inflammation, and it has been suggested that these endothelial progenitor cells can be used as a tool to repair the vasculature in these atherogenic conditions (Rabelink *et al.*, 2004; Tousoulis *et al.*, 2008; Badimon *et al.*, 2011).

Our initial results indicated that endothelial progenitor cells (ECFCs) could be grown for a longer period of time and grow at a faster rate in comparison to mature endothelial cells (HUVEC) in conditions of oxidative stress (40% O_2). It has already been shown that chronic oxidative stress leads to telomere dysfunctioning and promotes cell senescence in human endothelial cells (Kurz *et al.*, 2004). We therefore proposed that ECFCs are better equipped to counteract this ROS mechanism and might have been the cells that take part in repairing damaged endothelium and as shown in figure 5.1, the growth kinetics of ECFCs and HUVEC confirms that ECFCs are much more resistant to oxidative stress.

We had hypothesized that oxidative stress would lead to telomere dysfunction and a decrease in the level of PGC-1α as shown in mice recently (Sahin *et al.* 2011), but again instead of decreasing the levels of PGC-1α mRNA transcripts were increased as shown in figure 5.5. We also investigated two downstream targets of PGC-1α that included *ESSRA* gene that encodes ERR- α which is involved in angiogenesis and G6-Phosphatase gene that encodes the protein which is part of gluconeogenesis.

Estrogen- related receptor alpha (ERR-α) also referred to as NR3B1 is an orphan nuclear receptor that has no endogenous ligand identified till date. In humans it is encoded by *ESSRA* (Estrogen related receptor alpha) gene which was initially cloned due to DNA sequence homology to estrogen receptor alpha but has been confirmed that it is not regulated by estrogens (Giguere *et al.*, 1988; Deblois and Giguère, 2011). This gene is involved in the regulation of mitochondrial biogenesis, oxidative phosphorylation and glucose and fatty acid

metabolism and is activated by transcriptional coactivator PGC-1 alpha (Wu *et al.*, 1999; Yoon *et al.*, 2001; Huss *et al.*, 2004; Mootha *et al.*, 2004).

Glucose 6- phosphatase dehydrogenase (G6Pase) encodes an enzyme that belongs to the catalytic sub family of proteins involved in hydrolysis of glucose-6 phosphate which is an important step in gluconeogenesis and glycolysis and essential for regulation of glucose levels in blood. PGC-1 alpha is known to promote insulin resistance and is involved in inducing gluconeogenic genes including G6Pase (Yoon *et al.*, 2003). PGC-1- deficient mice have reduced fasting glucose levels and reduced mRNA expression of genes encoding for gluconeogenic enzymes including glucose 6-phosphatase (G6Pase) and phospheonolpyruvate carboxy kinase (PEPCK) (Koo *et al.*, 2004). Similarly, telomere dysfunction induced repression of PGC-1 alpha and its downstream targets lead to reduced expression of genes involved in gluconeogenesis including G6Pase (Sahin *et al.*, 2011).

The results showed a slight but insignificant increase in gene expression of both these downstream targets of PGC-1 alpha at mRNA level in ECFCs and as shown in figure 5.5C and D they were consistent with the previous findings in other cell types but this has not been shown before in ECFCs. This further confirmed that the hypothesis as it worked in mice was not followed in human cells and as seen in figure 5.5B, exposing the cells to 40% O_2 did not lead to a repression in PGC-1 alpha mRNA level of expression in both ECFCs and HUVEC when compared with cells grown in normoxia, although the difference was statistically insignificant. Also, both ERR-α and G6Pase mRNA level expression was increased although the difference found was not statistically significant.

It has been proposed and shown that oxidative stress that leads to an increase in reactive oxygen species (ROS) production and a decrease in antioxidant enzyme expression can promote vascular senescence. Oxidative stress has been implicated in a wide range of vascular disorders leading to DNA damage, decreased nitric oxide (NO) availability and change in redox state as a result of increased ROS production (Sugamura and Keaney Jr, 2011; Higgins *et al.*, 2012). This oxidative damage can be linked to vascular aging, as this cellular damage due to increased aging can reduce EPC function resulting in an increased risk of vascular pathologies. It has been shown that both early and late EPCs are more resistant to this oxidative stress than mature endothelial cells (HUVEC) as they express higher level of antioxidant enzymes (Dernbach *et al.*, 2004; He *et al.*, 2004a; Cai *et al.*, 2006). This suggested that these EPCs can be more efficient cells involved in vascular repair as a result of

an ischemic insult, owing to this improved resistance to oxidative stress. However, it has been shown recently that early EPCs or circulating angiogenic cells (CACs) are cells of myeloid origin and express certain monocyte-macrophage lineage markers including CD45, CD14 and CD115 and are proangiogenic but do not take part in vascular repair directly, whereas late EPCs or endothelial colony forming cells (ECFCs) have been shown to the EPCs involved in vascular repair although they themselves are not shown to be completely resistant to oxidative stress, but are better suited to these conditions as compared to mature endothelial cells (Ingram *et al.*, 2007; Yoder *et al.*, 2007). However, this antioxidative capacity of EPCs has been shown to diminish with aging, and early EPCs isolated from young subjects have shown higher levels of antioxidant enzyme glutathione peroxidase (GPx-1) as compared to old ones (He *et al.*, 2009) and were found to be more resistant to stress induced apoptosis. Glutathione peroxidase 1 (GPx-1) is another important protein involved in redox signalling that plays a role in vascular homeostasis. More importantly, over expression lead to restoration of endothelial function in endothelial cells treated with high levels of homocysteine (Weiss *et al.*, 2001). Several mice studies have demonstrated that loss of GPx-1 is implicated in endothelial dysfunction and several other vascular abnormalities and renders them vulnerable to ischemic insult (Forgione *et al.*, 2002a; Forgione *et al.*, 2002b). As shown in figure 5.6, there was an up regulation of both the antioxidative genes CAT and GPx-1 in HUVEC and suggested that by exposing these endothelial cells to 40% O_2 meant that they were subjected to oxidative stress and these genes were up regulated to counteract this stress.

In conclusion, we found endothelial progenitor cells (ECFCs) to be more resistant to oxidative stress as compared to mature endothelial cells (HUVEC) and they could be grown at a faster rate and over longer periods when exposed to hyperoxic conditions (40% O_2). Exposing both these cell types to oxidative stress did not lead to telomere dysfunction and as we had proposed that a shortening of telomere length would be compensated by an increase in telomerase activity in these cells. We measured telomere lengths for both ECFCs and HUVEC across passages (early and late passage cells) using flow FISH which has not been shown before using this technique and found that the telomere length goes down for HUVEC (n=3) but the difference was detected at very late passage (P16). ECFCs as we expected were not stem cells and they were not able to preserve their telomere length and it was shortened as well as cells grew older (late passage P16) but this was done with one sample (n=1) and no statistical analysis could be carried out. There was no reduction in PGC-1 alpha gene

expression and instead there was an up regulation along with an increase in mRNA level expression for both of its downstream targets when grown in hyperoxia. There was statistically significant difference seen in PGC-1 alpha expression in early EPCs as compared to ECFCs and the former showed high level of PGC-1 alpha expression. We investigated the expression levels of antioxidative genes CAT and GPx-1 in cells grown in hyperoxia so that we could confirm that exposing cells to 40% O_2 can lead to oxidative stress and an up regulation of these genes after 2 weeks of culture confirmed that this much time period is required for these endothelial cells to get affected by stress.

The results in this section indicate that the hypothesis which we had proposed that oxidative stress would lead to telomere dysfunction and as a result of that repression of PGC-1 network has not worked in ECFCs and mature endothelial cells (HUVEC). The work done by Sahin *et al.,* 2011 was done in mice and telomere dysfunction was induced by producing mice that were null for either telomerase reverse transcriptase (*Tert*) or telomerase RNA component (*Terc*) genes. This resulted in an activation of p53 in the nucleus causing a decrease in PGC-1 alpha expression and subsequent reduced mitochondrial biogenesis, decreased gluconeogenesis and increased reactive oxygen species. The activation of p53 resulted in cellular growth arrest, apoptosis and senescence. Our results showed that there was no increase in telomerase activity which we proposed that would occur due to telomere dysfunction induced due to exposure to hyperoxia. As shown later in figure 6.14 there was insignificant decrease in expression of *p53* in ECFCs grown in hyperoxia. Although, there was a significant increase in *p53* expression in HUVEC grown in hyperoxia and this suggests that may be HUVEC could show signs of senescence and apoptosis due to its activation. In summary, the results suggest that exposure to 40% O_2 did not lead to telomere dysfunction in these cells and as a result of that there was no concomitant increase in telomerase activity. PGC-1 alpha levels were not decreased and this could be further confirmed by functional assays evaluating cell senescence and measurement of extracellular ROS.

Chapter 6. Investigating the effect of oxidative stress on genes involved in mitochondrial biogenesis, oxidative metabolism and angiogenesis in ECFCs and HUVEC

6.1 Introduction

Vascular endothelium is the key that maintains vascular homeostasis and endothelial dysfunction or injury is the first step towards the progression of atherosclerosis (Chen *et al.*, 2006b; Lin *et al.*, 2009; Egido *et al.*, 2011; Liu *et al.*, 2012). Several recent studies have shown that endothelial progenitor cells constitute an important endogenous mechanism that is instrumental in maintaining endothelial integrity and vascular homeostasis (Urbich and Dimmeler, 2004). In most forms of cardiovascular diseases, inflammation acts as a mediator of oxidative stress, endothelial dysfunction and endothelial cell senescence and inappropriate homage of EPCs to the site of vascular injury promotes the progression of vascular diseases (Imanishi *et al.*, 2005; Mikirova *et al.*, 2009; Ribeiro *et al.*, 2010; Ungvari *et al.*, 2010). As reendothelialization is considered to be a proinflammatory process, it has been proposed that it works independently and it has been shown more recently that this occurs exclusively in response to oxidative stress mainly due to a localized or a systemic inflammatory response (Reinders *et al.*, 2006; Case *et al.*, 2008; Watson *et al.*, 2008). This response is aided by and involves vascular and immune systems as well as several signaling mechanisms, mediators and various cell types. This cascade of events can initiate due to changes in microcirculation, starting from vessel impairment to endothelial migration of leukocytes, endothelial dysfunction and changes in vascular permeability finally leading to new blood vessel formation, a process known as angiogenesis. We, therefore proposed that in this *in vitro* system where endothelial progenitor cells (ECFCs) and mature endothelial cells (HUVEC) are grown in normal conditions, will be exposed to mild oxidative stress (40% O_2), and this stress would lead to an inflammatory response in these cells and we would be able to study the response of these two cell types under stress conditions.

Mitochondria are considered to be the power houses of the cell and are involved in energy production mainly in the form of ATP. Although, endothelial cells are mainly glycolytic and do not use oxidative phosphorylation as a main source of ATP production, we proposed that oxidative stress (40% O_2) would lead to an increase in oxidative phosphorylation and as a

result of that, an increase in expression level of genes involved in electron transport chain (ETC).

A link between mitochondrial dysfunction leading to endothelial damage and initiating the process of atherosclerosis is now an emerging area of research and there is enough evidence that suggest that mitochondrial damage plays an important role in the progression of vascular diseases including atherosclerosis (Madamanchi and Runge, 2007; Victor *et al.*, 2009; Harrison *et al.*, 2011; Hulsmans *et al.*, 2012; Xiong *et al.*, 2013; Yu *et al.*, 2013). PGC-1 alpha has been shown to be a master regulator of mitochondrial biogenesis and along with PPAR gamma, which is transcriptionally co activated by PGC-1 alpha plays a role in maintaining energy metabolism (Liu and Lin, 2011). cAMP response element binding proteins (CREB) and Nuclear respiratory factor (NRF-1 and 2) are also involved in this regulation of mitochondrial biogenesis in response to an external stimuli (Jones *et al.*, 2012). Reactive oxygen species (ROS) are continuously produced by mitochondria and are balanced by cellular defense mechanism in the form of antioxidative enzymes including catalase, glutathione peroxidase, superoxide dismutase and peroxiredoxins present throughout the cell. If this balance between ROS and antioxidative defense system is disrupted, it results in the production of oxidative stress.

The significance of mitochondrial ROS production in endothelial cells is difficult to assess because ROS is also produced by other sources as well as other cell types within the myocardium. Although, it has been shown that superoxide is produced at both complex I and III in mitochondria obtained from bovine aortic endothelial cells (O'Malley *et al.*, 2006), this needs further investigation. Similarly, how does oxidative stress affect the pathways that utilizes ROS as a signal transducers, still remains to be seen in endothelial cells.

6.2Results

6.2.1 Gene expression analysis of ECFCs and HUVEC grown in normoxia using low density array (LDA)

In order to carryout gene expression analysis of ECFCs and HUVEC, cells were isolated from umbilical cord blood and cord of the same donor. ECFCs were isolated from differentiation of cord blood mononuclear cells (day 0 MNCs) in endothelial specific media. An endothelial colony was observed at around 3 weeks of culture that was picked and grown separately. This culture was grown for a period of around 4-6 weeks and cells were regularly passaged. Cells were grown till passage 5 (P5) and then cells were frozen down for RNA extraction. RNA extraction was followed by cDNA synthesis and low density array was performed. HUVEC were isolated from the cord of the same donor and cells were grown till passage 5 (P5). Both ECFCs and HUVEC were grown in normoxic conditions (20% O_2). As shown in figure 6.1 a comparison was carried out between ECFCs and HUVEC grown in normoxia in terms of gene expression analysis. The data were normalized to three housekeeping genes individually that included TBP, HPRT1 and 18s RNA. As shown in figure 6.1B, ECFCs showed a down regulation of certain genes in comparison to HUVEC. These included genes involved in angiogenesis like VEGF A and C, PDGFRB, FGF2 and TGF. Genes showing down regulation in ECFCs also included antioxidative gene SOD2 which mainly combats against oxidative stress. As shown in figure 6.1B there have been several genes that showed an up regulation in ECFCs in comparison to HUVEC. These included pro inflammatory markers IL1A and CXCL12 and cell surface markers like PECAM1, KDR and KIT. There was also an up regulation of PON1 and BRCA1that was found in HUVEC when grown in normoxia in comparison to ECFCs grown in normoxic conditions. For the purpose of having a better comparison both ECFCs and HUVEC were derived from the same donor and cells at same passage (P5) were used.

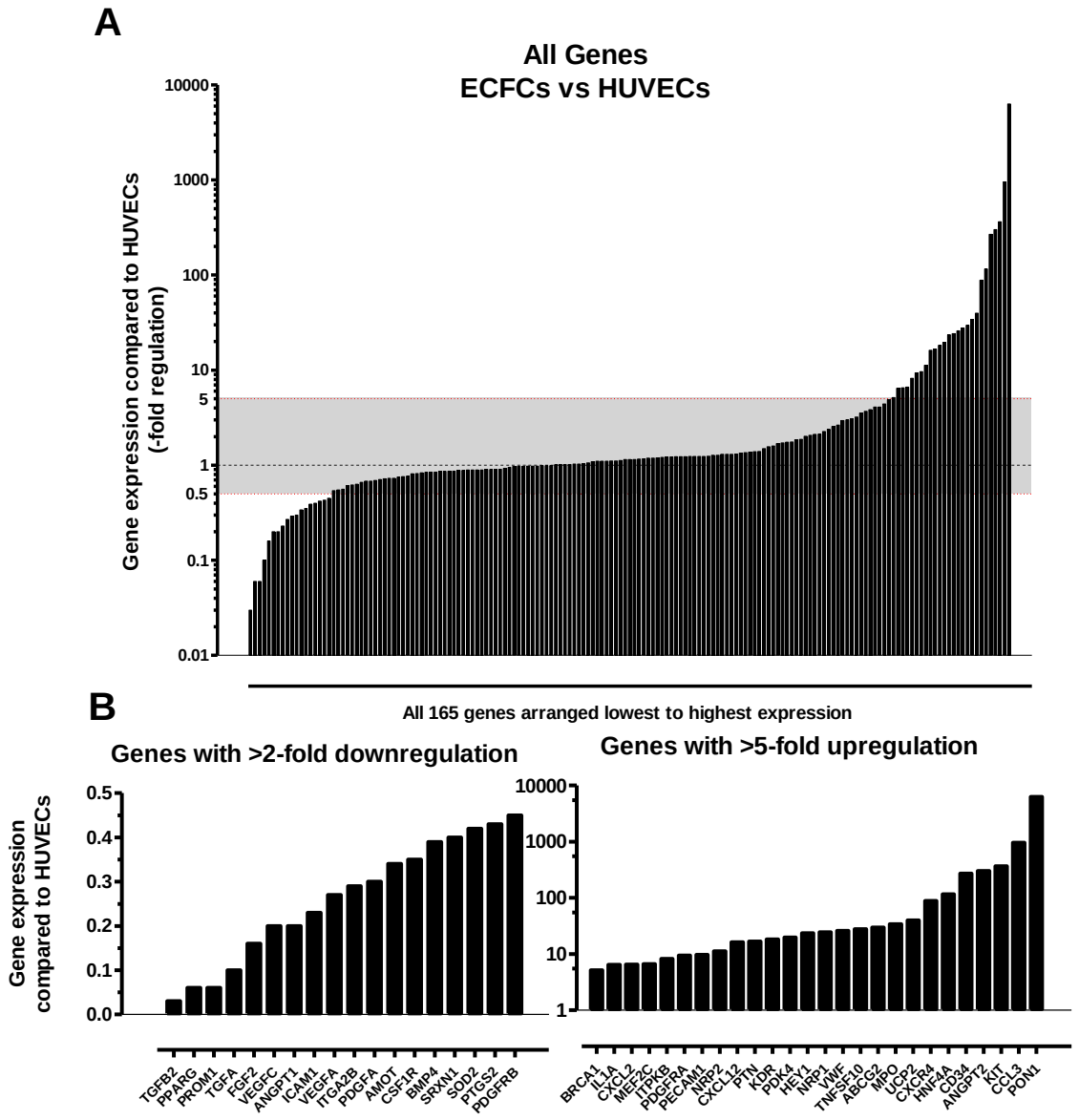


Figure 6.1. An overall representation of the gene expression analysis carried out using low density array. ECFCs were isolated from cord blood obtained from the umbilical vein and HUVEC were isolated from the walls of the umbilical vein of the same donor. The comparison is carried out between ECFCs and HUVEC grown in normoxia conditions (20% O_2). Gene expression is plotted relative to MNCs. The data were normalized to three housekeeping genes 18s RNA, TBP and HGPRT1. n=2 (ECFC 20% and 40% O_2, HUVEC 40% O_2) and n=3 (HUVEC 20% O_2).

6.2.2 Gene expression analysis of ECFCs grown in normoxia and comparison with cells grown in hyperoxia

After the gene expression analysis of ECFCs and HUVEC grown in normoxia, the next step was to analyse the expression pattern in ECFCs grown in normoxia to ECFCs incubated in hyperoxic conditions (40% O_2). ECFCs were obtained from cord blood and derived by the differentiation of cord blood mononuclear cells. ECFCs were isolated from an endothelial colony that was observed and picked at around 3 weeks of growing the cells in endothelial specific media. The cells were cultured in normoxic conditions (20% O_2) for several passages and grown till P5 to get the cells required for ECFCs normoxia. For the array analysis, the comparison was made between ECFCs grown in normoxia till P5 and in order to get hyperoxic cells, ECFCs were grown in normoxia till P2 and then the cells were split using trypsin-EDTA and 1 million cells were plated in hyperoxic conditions (40%O_2). These cells were grown in hyperoxia till P3 and then frozen down for RNA extractions. This was followed by cDNA synthesis and low density array was carried out. As shown in figure 6.2B there have been several genes that showed down regulation in ECFCs when grown in hyperoxia. These included some genes that are involved in angiogenesis like ANG2, NOS3, CXCR4 and endoglin (CD105). The results showed a decrease in expression of several cell surface markers including PECAM1 (CD31), CDH5 (CD144), MCAM (CD146) and vWF when ECFCs were grown in hyperoxia. UCP2 showed a reduced expression when cells were grown in 40% O_2. There have been some markers that showed higher expression when ECFCs were grown in hyperoxia and these included PGC-1α that showed significant up regulation in ECFCs grown in hyperoxic conditions. It is interesting to note that as cells were exposed to hyperoxia till P3 there was a significant increase in PGC-1 alpha expression in ECFCs whereas earlier data showed no statistically significant difference when cells were exposed to hyperoxia till P1. Other markers showing an increase in expression when ECFCs were grown in hyperoxia included angiogenic markers ANG1, FGF2, VEGF A and C, CXCL12, ACTA2 and PDGFRA and B.

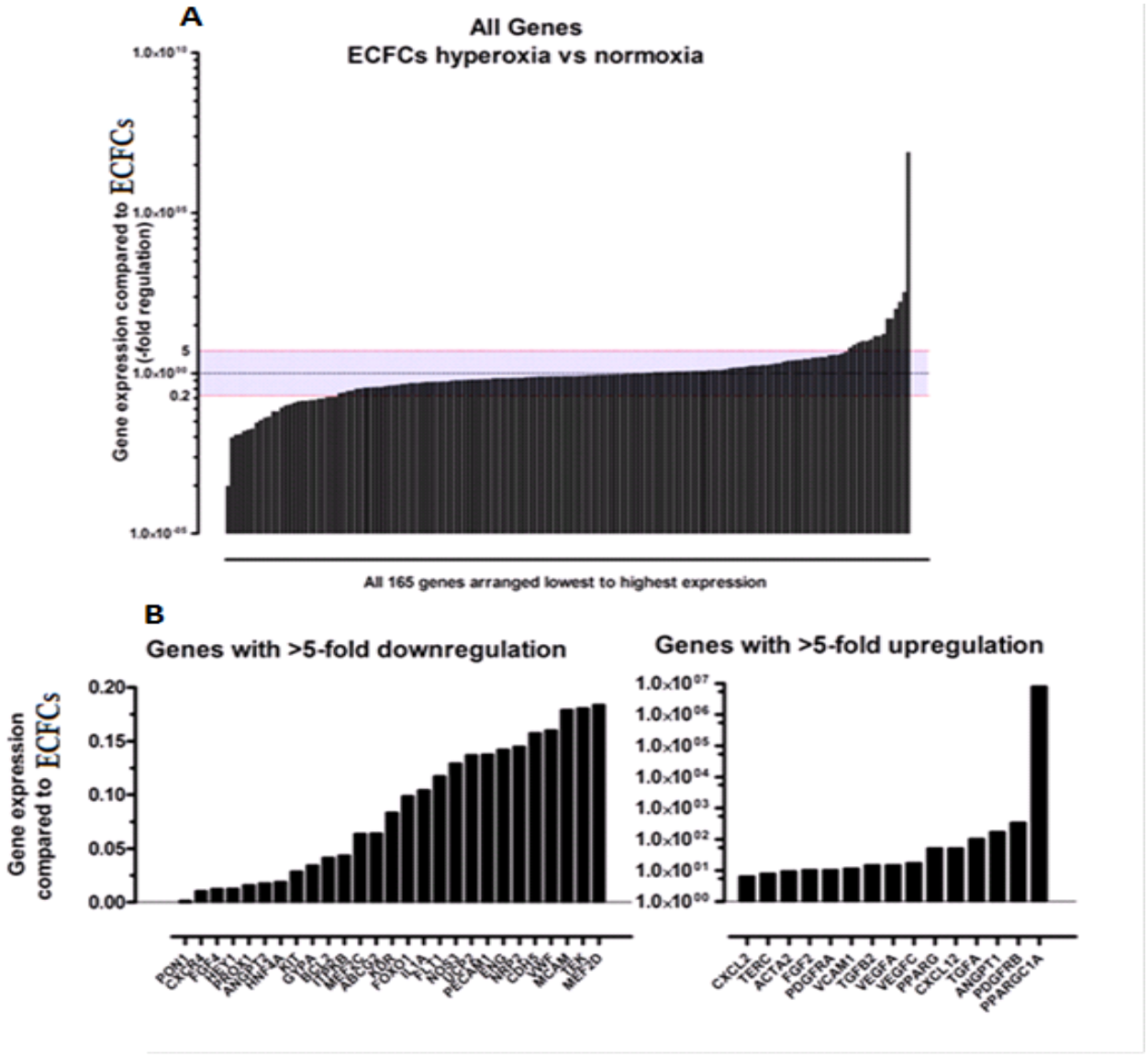


Figure 6.2. An overall representation of the gene expression analysis carried out using low density array. ECFCs were obtained from the cord blood. The comparison is made between ECFCs grown in normoxia (20% O_2) to the ECFCs grown in hyperoxia (40% O_2). Gene expression is plotted relative to MNCs. The data were normalized to three housekeeping genes 18s RNA, TBP and HGPRT1. n=2 (ECFC 20% and 40% O_2, HUVEC 40% O_2) and n=3 (HUVEC 20% O_2).

6.2.3 Gene expression analysis of HUVEC grown in normoxia and comparison with HUVEC grown in hyperoxia using low density array (LDA)

In order to compare the expression analysis of HUVEC grown in normoxia with HUVEC cultured in hyperoxia, HUVEC were obtained from the umbilical cord. Cells were grown in normoxic conditions for several passages and regularly washed and split using PBS and trypsin-EDTA respectively. Cells were frozen down at passage 5 (P5) and RNA extraction was carried out. Cells were passaged at P2 and split into two cultures. 1 million cells were plated and cells were incubated and grown in hyperoxic conditions (40% O_2). The comparative analysis was carried out between HUVEC grown in normoxia and HUVEC grown in hyperoxia. As shown in figure 6.3, there have been some genes that are down regulated in HUVEC grown in hyperoxia and mainly include mitochondrial genes that form part of electron transport chain namely MTCO1, MTND3, MTCYB, MTCO3 and MTATP6. There have been some genes that are involved in angiogenesis which are down regulated and included ANG1, VEGFB, NOS3, TGF and PDGFRB. As shown in figure 6.3, there were several genes that showed an up regulation in HUVEC grown in hyperoxia. These included mitochondrial genes PGC-1 alpha, SIRT4 and PON1. Cell surface markers including PROM1 and CD34 showed an up regulation in HUVEC grown in hyperoxia. Genes involved in angiogenesis like ANG2 and FLT4 were also up regulated in HUVEC exposed to hyperoxia.

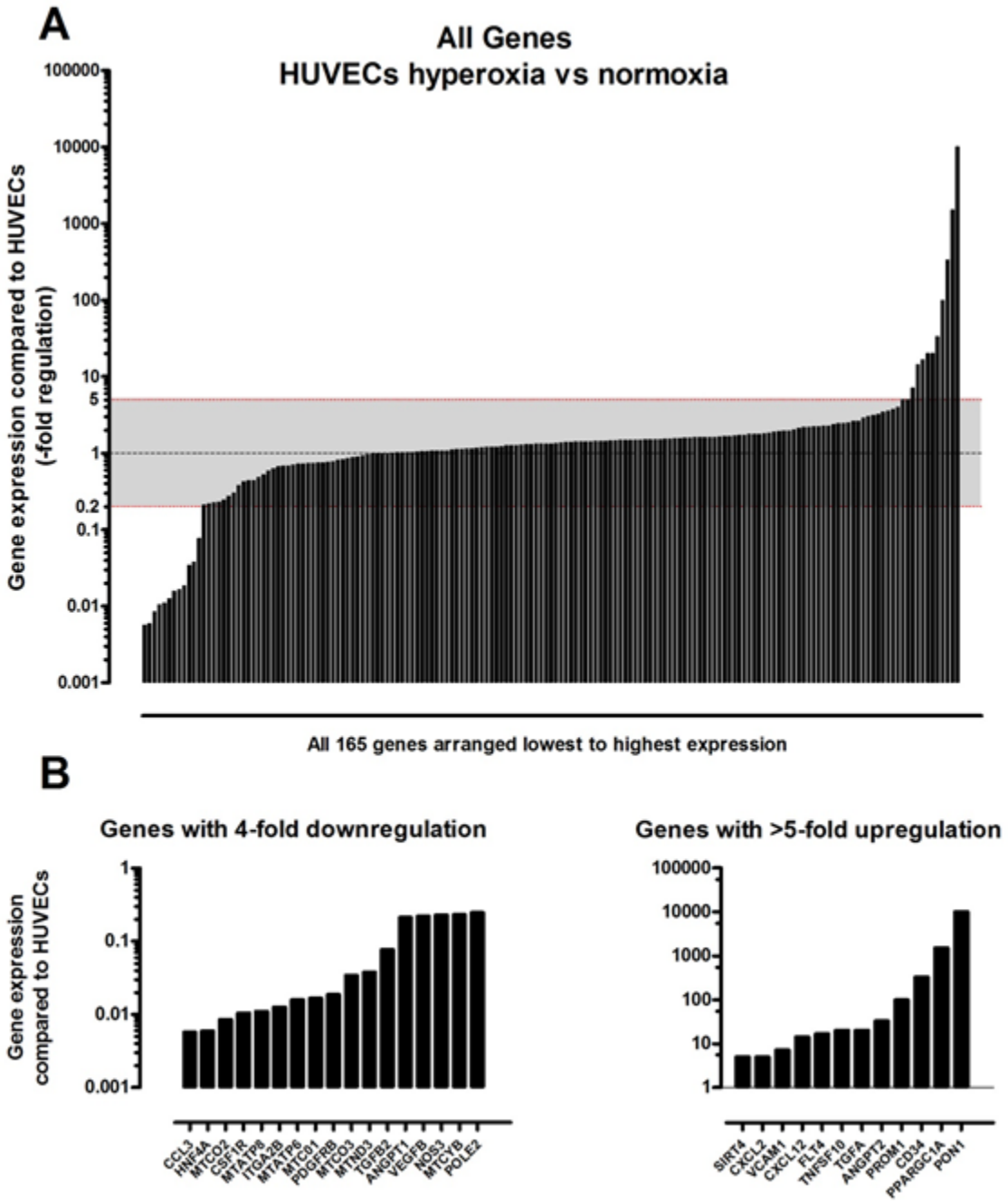


Figure 6.3. An overall representation of the gene expression analysis carried out using low density array. HUVEC were derived from the umbilical vein. The comparison is made between HUVEC grown in normoxia (20% O_2) to the HUVEC grown under hyperoxic conditions. Gene expression is plotted relative to MNCs. The data were normalized to 18s RNA, TBP and HGPRT1. n=2 (ECFC 20% and 40% O_2, HUVEC 40% O_2) and n=3 (HUVEC 20% O_2).

6.2.4 Gene expression analysis and qPCR validation of genes involved in mitochondrial biogenesis, oxidative metabolism and angiogenesis in ECFCs and HUVEC grown in normoxia

After analysing low density array (LDA) data, the next step was to validate the genes that showed statistically significant difference in ECFCs and HUVEC grown in normoxic and hyperoxic conditions. The validation was carried out on different samples and ECFCs were obtained from cord blood and HUVEC from the umbilical cord of the same donor. ECFCs were derived from an endothelial colony which was picked after 3 weeks of culture of cord blood mononuclear cells (HUCBC) in endothelial specific media. Once a colony was isolated, it was grown in normoxia for 4-6 weeks and the cells were termed as endothelial colony forming cells (ECFCs). Cells were passaged regularly till P5 and at this stage cells were frozen down for carrying out RNA extractions. HUVEC were isolated from the umbilical cord of the same donor and cells were grown in normoxic conditions and regularly passaged as soon as they reach around 80% confluence. This was followed by RNA extractions of frozen down HUVEC samples at P5. Validation was done using qPCR. As shown in figure 6.4, the comparative analysis was carried out between ECFCs and HUVEC grown in normoxia (20% O_2). All data were normalized to three housekeeping genes namely 18s RNA, HPRT1 and TBP. LDA and qPCR data were analysed together. Cells at same passage were used for the comparison (P5). There have been several genes that showed down regulation in HUVEC as compared to ECFCs grown in normoxia. These included angiogenic genes like ANG2, NOS3 and CDH5. Other genes showing decreased expression were PECAM1, PROX1, KIT and NRP2. There were genes that showed up regulation in HUVEC grown in normoxia in comparison with ECFCs and they included genes involved in angiogenesis like ANG1, TGFα, VEGFA and C, FGF2 and PDGFRB. Other genes showing up regulation included PCK2, PPARG and TEK.

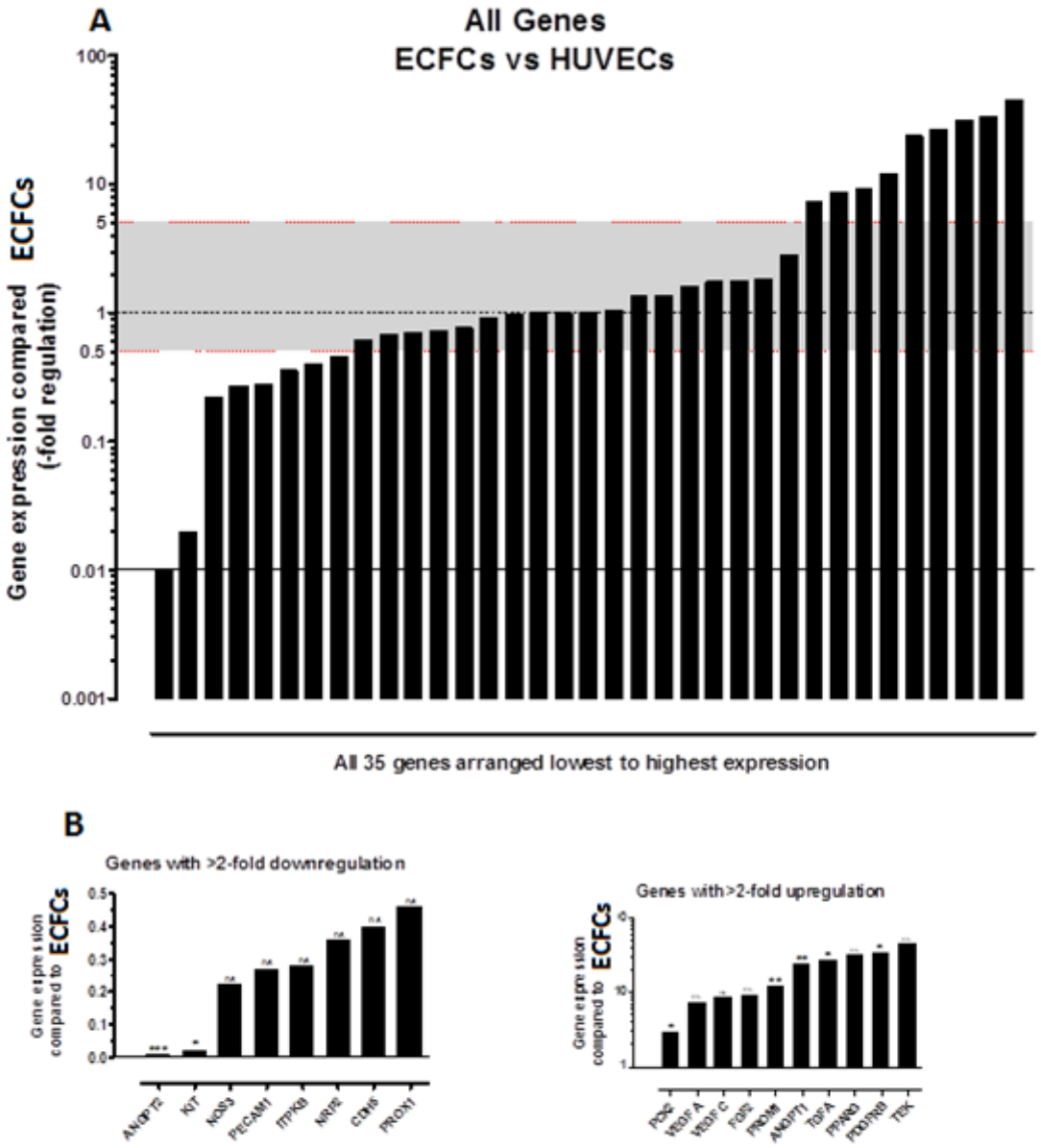


Figure 6.4. Gene expression analysis of qPCR validation done on LDA genes showing significant up regulation or a decrease in expression. ECFCs and HUVEC were obtained from the cord blood and umbilical vein of the same donor. Comparative analysis is carried out on ECFCs and HUVEC grown in normoxia conditions (20% O_2). Gene expression is plotted relative to MNCs. The data were normalized to 18s RNA, TBP and HGPRT1. n=4 (ECFC 20% and 40%O_2) and n=5 (HUVEC 20% and 40% O_2).

6.2.5 Gene expression analysis and qPCR validation of genes involved in mitochondrial biogenesis, oxidative metabolism and angiogenesis in ECFCs grown in normoxia and hyperoxia

In order to validate the array results qPCR validation was carried out. ECFCs were derived from cord blood by differentiation of cord blood mononuclear cells grown in endothelial specific media. An endothelial colony was observed at around 3weeks of culture that was picked and grown separately. The cells derived from the colony were named as endothelial colony forming cells (ECFCs) and were allowed to grow for 4-6 weeks in endothelial specific media. The cells were grown in normoxia and regularly passaged till P5 and then the cells were frozen down for RNA extractions. Cells were also required to be grown in hyperoxia and to do so, cells were cultured in normoxic conditions till P2 and then cells were split and 1 million cells were plated in hyperoxia (40% O_2) and cells were grown in these conditions for three additional passages (P3) so that they are compared with normoxic cells at P5. As shown in figure 6.5, the comparison was carried out between ECFCs grown in normoxia to ECFCs grown in hyperoxic conditions and cells at same passage were used for RNA extractions. This was followed by cDNA synthesis and qPCR. As shown in figure 6.5, all the data were normalized to three housekeeping genes including 18s RNA, HPRT1 and TBP. The LDA and qPCR data were analysed together. There were several genes that showed down regulation in ECFCs grown in hyperoxia and included cells surface markers PECAM1, CDH5, KDR and KIT. Several angiogenic genes also showed down regulation in ECFCs cultured in hyperoxia included ANG2, NOS3, FLT1 and TP53. As shown in figure 6.5, there have been many genes that showed an up regulation in ECFCs grown in hyperoxia and mainly included mitochondrial genes involved in electron transport chain like MT-CYB, MT-ND3, MT-CO1and other mitochondrial genes mainly PGC-1 alpha, PPARG and SIRT4. There were several angiogenic genes that showed up regulation in ECFCs cultured in hyperoxia and included ANG1, VEGF A and C, CXCL12, ACTA 2, PDGFR A and B and FGF2.

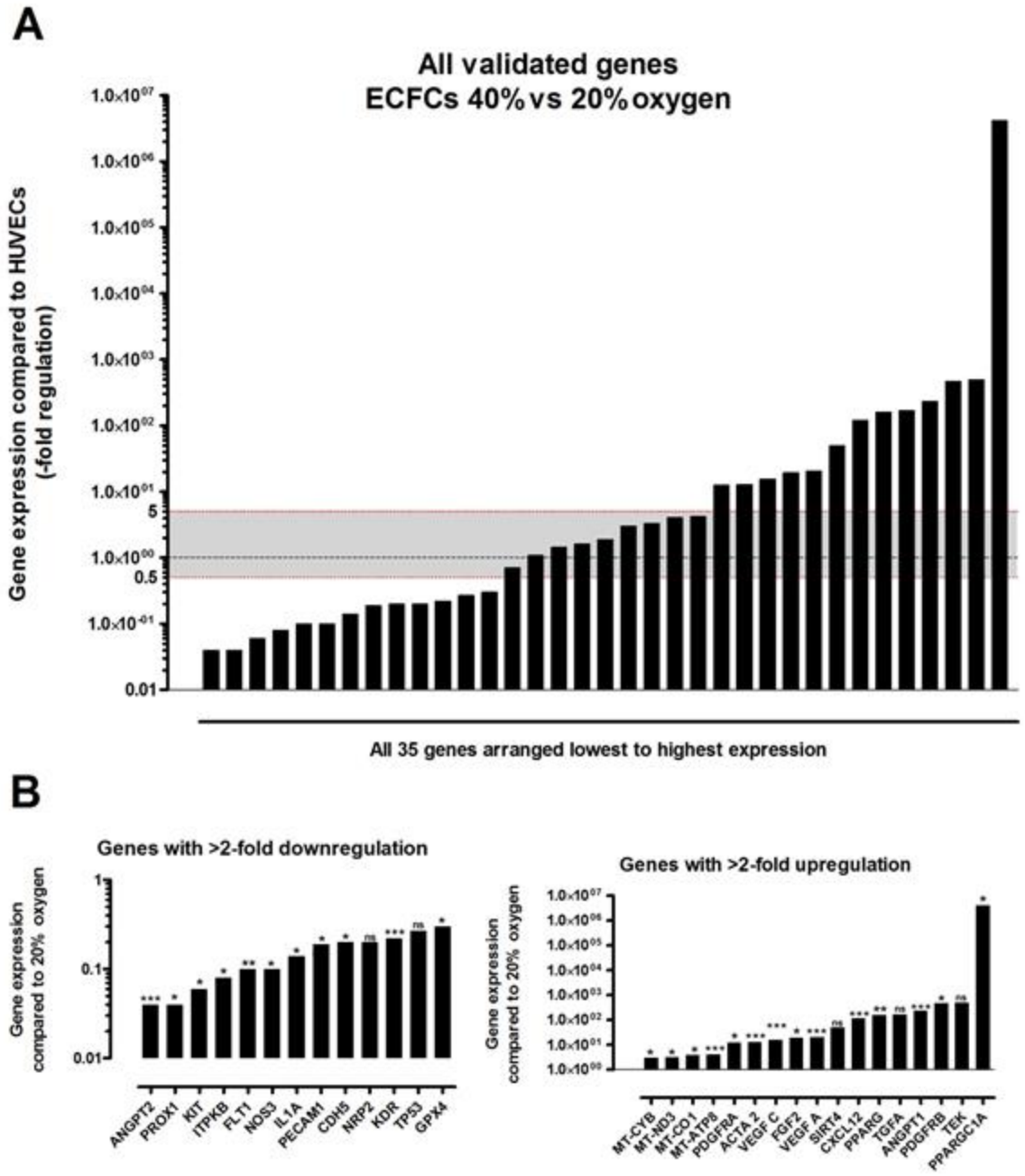


Figure 6.5. Gene expression analysis of qPCR validation done on LDA genes that showed significant difference in expression. ECFCs were derived from the cord blood. Comparative analysis was carried out between ECFCs grown in normoxia to the ECFCs grown in hyperoxic conditions. Gene expression is plotted relative to MNCs. The data were normalized to 18s RNA, TBP and HGPRT1. n=4 (ECFC 20% and 40% O_2) and n=5 (HUVEC 20% O_2 and HUVEC 40% O_2).

6.2.6 Gene expression analysis and qPCR validation of genes involved in mitochondrial biogenesis, oxidative metabolism and angiogenesis in HUVEC grown in normoxia and hyperoxia

After the low density array (LDA) analysis the next step was to validate the genes that showed significant difference in HUVEC grown in hyperoxia. HUVEC were isolated from the umbilical cord and cells were grown in normoxia. The cells were regularly passaged when around 80% confluent. Cells were grown till passage 2 (P2) and then culture was split in to two. 1 million cells were allowed to grow in normoxia and 1 million cells were incubated in hyperoxic conditions. The cells were allowed to grow till passage 5 (P5). As shown in figure 6.6 the comparative analysis was done between HUVEC grown in normoxia and hyperoxia. All the data were normalized to three housekeeping genes. Both LDA and qPCR data were analysed together. As shown in figure 6.6 there have been several genes that showed down regulation in HUVEC grown in hyperoxic conditions and mainly included mitochondrial genes that are part of electron transport chain namely, MT-CYB, MT-ND3, MTCO1 and MT-ATP8. Genes showing down regulation also included angiogenic markers ANG1 and VEGFC. As shown in figure 6.6, there were several genes that showed up regulation in HUVEC grown in hyperoxia and included mitochondrial genes SIRT4, PCK2 and PGC-1 alpha. Other genes showing up regulation included cell surface markers and genes involved in angiogenesis including ACTA2, CDH5, NOS3, KDR, TGFα, PDK4, PECAM1, ANG2, CXCL12, PDGFR A and B and IL1A.

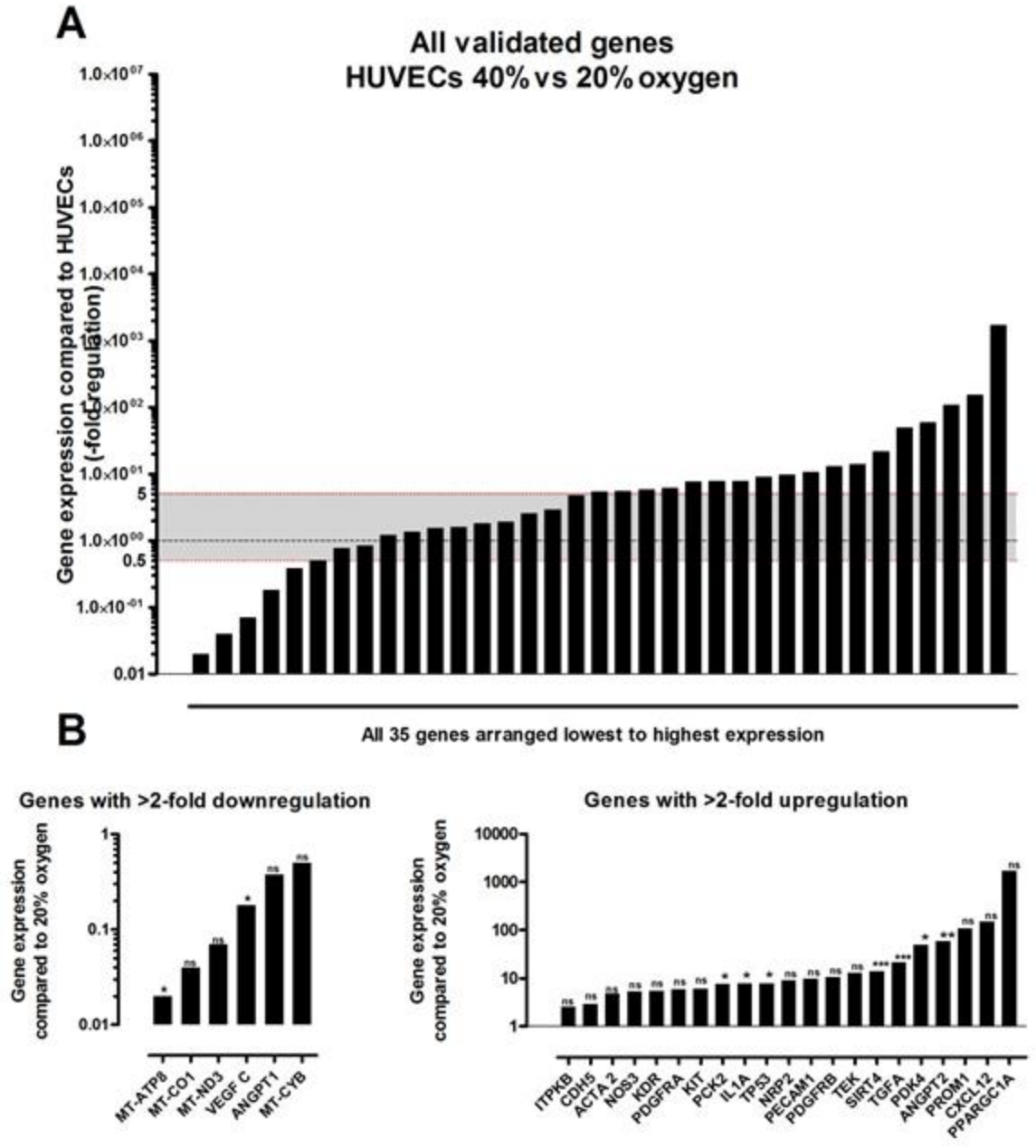


Figure 6.6. Gene expression analysis of qPCR validation done on LDA genes that showed significant difference in expression. HUVEC were isolated from the umbilical vein. Gene expression is plotted relative to MNCs. The comparison is made between HUVEC grown in normoxia to the HUVEC grown in hyperoxic conditions. The data were normalized to three housekeeping genes 18s RNA, TBP and HGPRT1. n=4 (ECFC 20% and 40% O_2) and n=5 (HUVEC 20% O_2 and HUVEC 40% O_2).

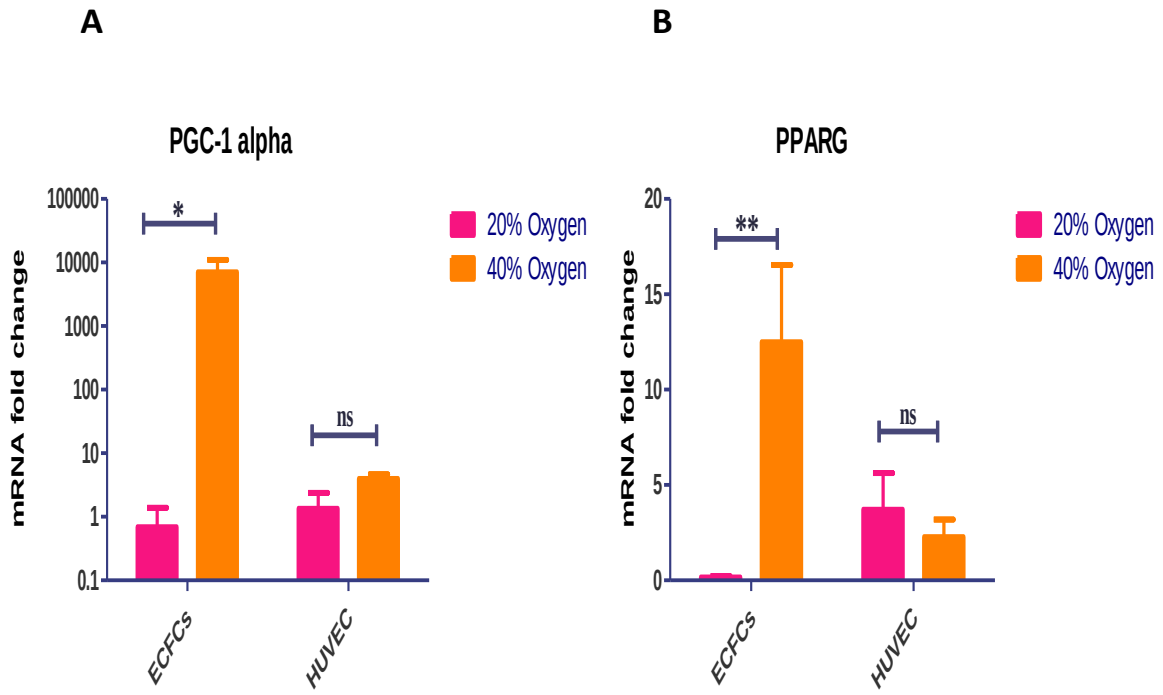
6.2.7 Gene expression analysis of genes involved in regulating mitochondrial biogenesis and comparing ECFCs and HUVEC grown in normoxia and hyperoxia

PGC-1 alpha belongs to a family of transcriptional coactivators that are involved in mitochondrial biogenesis, gluconeogenesis and angiogenesis. We therefore wanted to investigate what effect oxidative stress can have on the gene expression levels of PGC-1 alpha and other related mitochondrial genes. Peroxisome proliferator receptor gamma (PPARG) is known to be activated by PGC-1 alpha in brown fat and cardiomyocytes where it promotes fatty acid oxidation, so we investigated the effect in endothelial cells. The cells were cultured till passage 5 (P5) in normal oxygen conditions (20% O_2). Cells were frozen down and RNA extraction was performed. To compare the gene expression analysis these cells were also grown in conditions of oxidative stress. RNA was isolated from these cells and quantitative PCR analysis was carried out. The results as shown in figure 6.7 showed that there was a significant up regulation of PGC-1 alpha levels in ECFCs when grown in hyperoxia and the difference was found to be statistically significant. There was an increase in PPARG expression in ECFCs when exposed to oxidative stress and this could be due to PGC-1 alpha co activation. In HUVEC, PGC-1 alpha levels did not show any significant change in expression when grown in hyperoxia. It also shows interestingly that there was no significant change in expression of PPARG in HUVEC and this suggests that in ECFCs the up regulation of PPARG was due to co activation by PGC-1 alpha. This shows that these two cell types namely endothelial progenitor cells (ECFCs) and mature endothelial cells (HUVEC) respond and behave differently when exposed to oxidative stress.

PROX -1gene encodes prospero homeobox protein 1. PROX 1 is a transcription factor which is involved in the development of various tissues (Wigle and Oliver, 1999; Sosa-Pineda *et al.*, 2000; Dyer *et al.*, 2003). It plays an important role in the development of lymphatic endothelial cells and it has been shown before that *prox-1* null embryos failed to form lymphatic vasculature and died in utero (Johnson *et al.*, 2008). PROX-1 is known to regulate activity of nuclear receptors including estrogen related receptor (ERRα) and interact with PGC-1 α, influencing its transcriptional activity (Charest-Marcotte *et al.*, 2010). PROX 1 has also been shown to interact with hepatocyte nuclear factor 4α (HNFα) and human liver receptor homolog-1 (LHR-1) and reducing the transcription of the cholesterol 7-alpha-hydroxylase gene, suggesting its role in fatty acid oxidation and gluconeogenesis in liver (Qin *et al.*, 2004; Song *et al.*, 2006). As shown in figure 6.7D, there was a down regulation of around 200 fold in the mRNA expression level of PROX1 when ECFCs were grown in hyperoxia and as it is known to interact with PGC-1 alpha it could be possible that activation

of PGC-1 alpha has led to this decrease in expression of PROX1. HUVEC did not show a significant decrease in expression of PROX1.

PRDX5 is a protein which in humans is encoded by mitochondrial gene *PRDX5*. It belongs to peroxiredoxins family of antioxidant enzyme whose main function is to reduce hydrogen peroxide (Yamashita *et al.*, 1999). It is known to play a protective antioxidative role in tissues during normal and also inflammatory conditions (Knoops *et al.*, 1999). As shown in figure 6.7C there was no statistically significant change in mRNA expression level of PRDX5 in either ECFCs or HUVEC when grown in hyperoxic conditions.



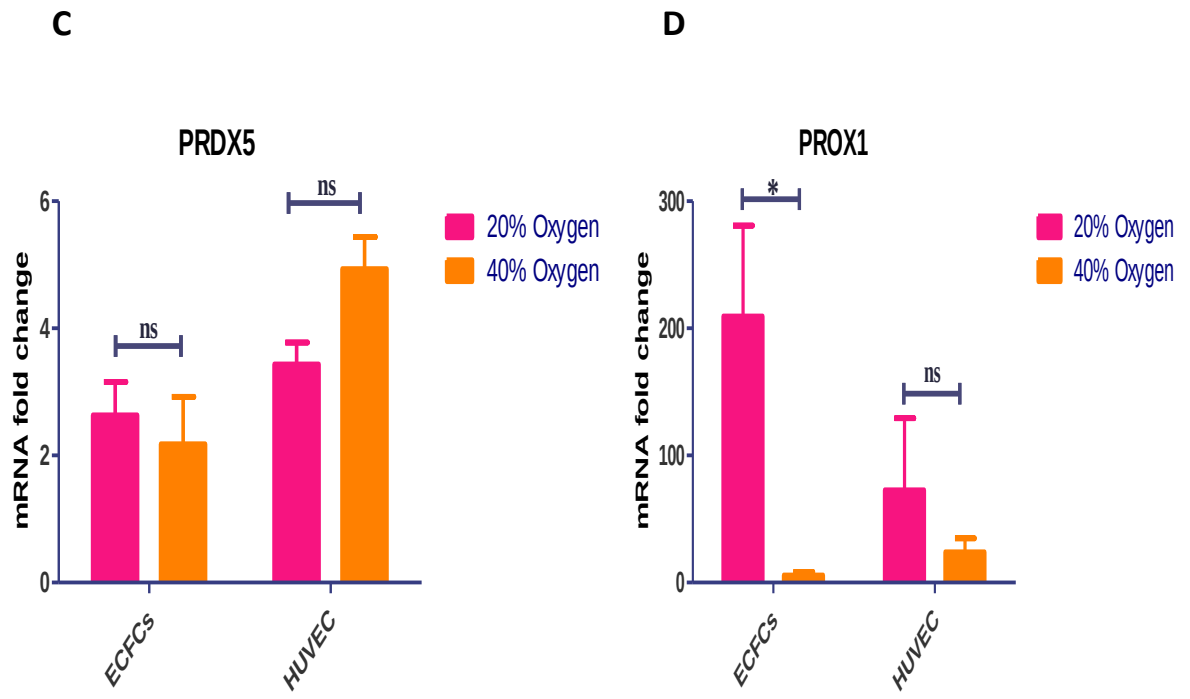
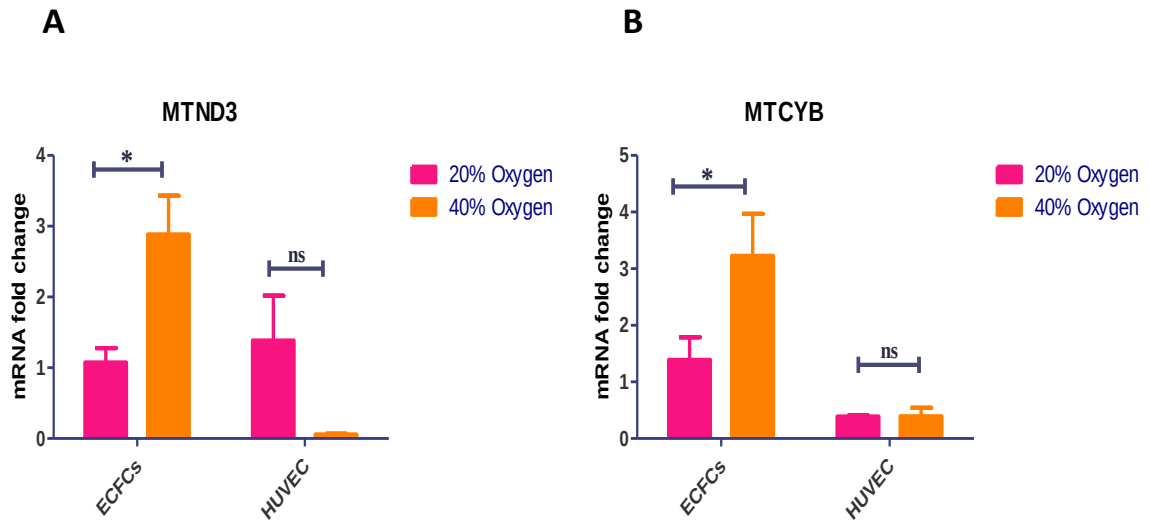


Figure 6.7. Gene expression analysis of genes involved in mitochondrial gene regulation and fatty acid and glucose metabolism. Endothelial colony forming cells (ECFCs) were derived from cord blood and mature endothelial cells (HUVEC) were derived from the umbilical cord of the same donor. Both ECFCs and HUVEC were grown in conditions of normoxia (20% O_2) and hyperoxia (40% O_2). Figure **A and B** shows an up regulation of PPARGC1A and PPARG genes in ECFCs when grown in hyperoxia and the difference was found to be statistically significant. Figure **C and D** shows down regulation of antioxidative gene PRDX5 in ECFCs although the difference was not statistically significant. The data were normalized to 18s rRNA, HPRT1 and TBP which were used as endogenous controls. Gene expression is plotted relative to cord blood mononuclear cells. Statistical analysis was carried out using two way ANOVA followed by Bonferroni posttests. All values are calculated as ± SEM. n=4 (ECFC 20% and 40% O_2) and n=5 (HUVEC 20% and 40% O_2). * $P < .05$, ** $P < .01$, ***$P < .001$ and ns stands for not significant.

6.2.8 Gene expression analysis of electron transport chain genes involved in oxidative phosphorylation (OXPHOS) and comparison between ECFCs and HUVEC grown in normoxia and hyperoxia

We investigated one protein-coding gene from each of the steps involved in ETC of oxidative phosphorylation. MT-ND3 belongs to a group of genes that encode for NADH dehydrogenase enzyme complex also known as complex I which is the largest component of the mitochondrial respiratory chain. MT-CYB is a mitochondrially encoded gene that encodes cytochrome b protein which forms part of coenzyme Q- cytochrome c reductase also known as complex III which is involved in biochemical production of ATP (oxidative phosphorylation). MT-CO1 also known as cytochrome c oxidase 1(COX1) encodes a protein that forms part of mitochondrial DNA (mtDNA) encoded enzyme subunit of respiratory complex IV which is the final enzyme of the ETC of oxidative phosphorylation. MT-ATP8 encodes a protein that forms a subunit of enzyme mitochondrial ATP synthase and important enzyme required for ATP generation. We cultured the cells till passage 5 (P5) under normal oxygen conditions (20% O_2). Cells were frozen down and RNA extraction was carried out. The cells were grown in hyperoxia till passage 3 (P3) and RNA was isolated from them. RNA extraction was followed by quantitative PCR. The results as shown in figure 6.8 indicate that as a result of exposure to oxidative stress there was an increase in gene expression level of the genes involved in ETC of oxidative phosphorylation in ECFCs when grown in hyperoxia and the difference was found to be statistically significant. The expression level of ETC genes in mature endothelial cells (HUVEC) either remained same or there was a down regulation, although the difference was not found to be statistically significant. This suggests that endothelial progenitor cells and mature endothelial cell behave differently when exposed to oxidative stress.



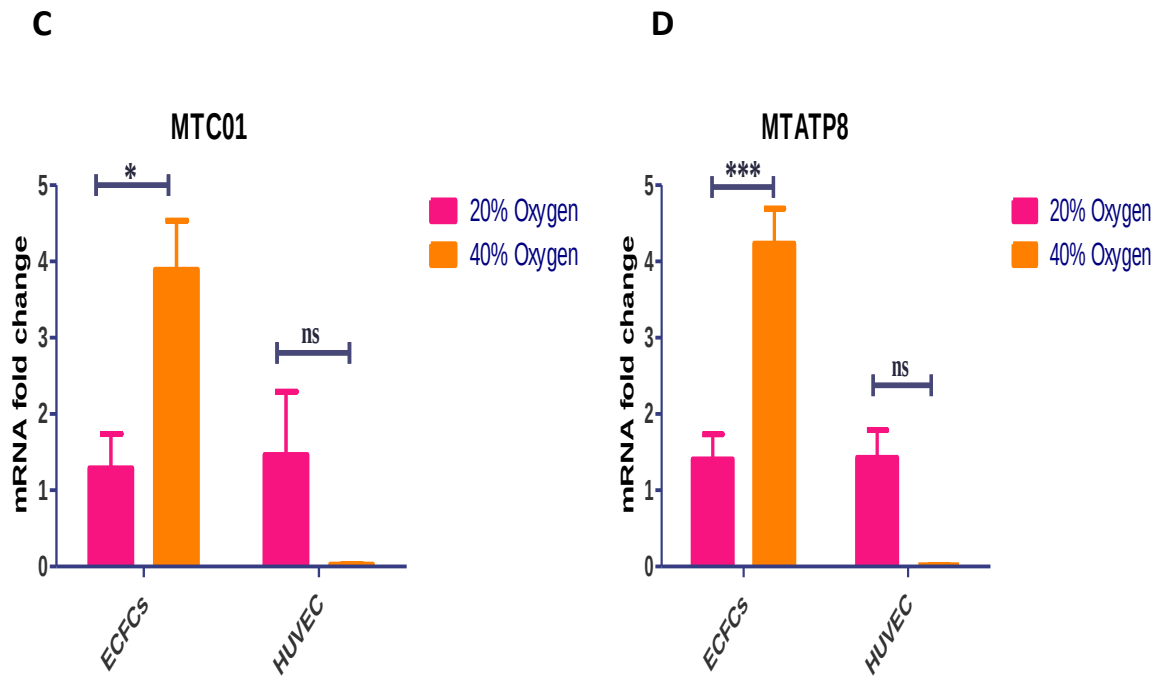


Figure 6.8. Gene expression analysis of human mitochondrial genes which are part of the electron transport chain and are involved in oxidative phosphorylation. Endothelial colony forming cells (ECFCs) were derived from cord blood and mature endothelial cells (HUVEC) were derived from the umbilical cord of the same donor. Both ECFC and HUVEC were grown in conditions of normoxia (20% O_2) and hyperoxia (40% O_2). Figure shows an up regulation of the mitochondrial genes in ECFCs grown in hyperoxia, and the difference was found to be statistically significant. HUVEC showed down regulation of these genes under hyperoxic conditions although the difference was not found to be statistically significant. The data were normalized to 18s rRNA, HPRT1 and TBP which were used as endogenous controls. Gene expression is plotted relative to cord blood mononuclear cells. Statistical analysis was carried out using two way ANOVA followed by Bonferroni posttests. All values are calculated as ± SEM. n=4 (ECFC 20% and 40% O_2) and n=5 (HUVEC 20% and 40% O_2). * $P < .05$, ** $P < .01$, *** $P < .001$ and ns stands for not significant.

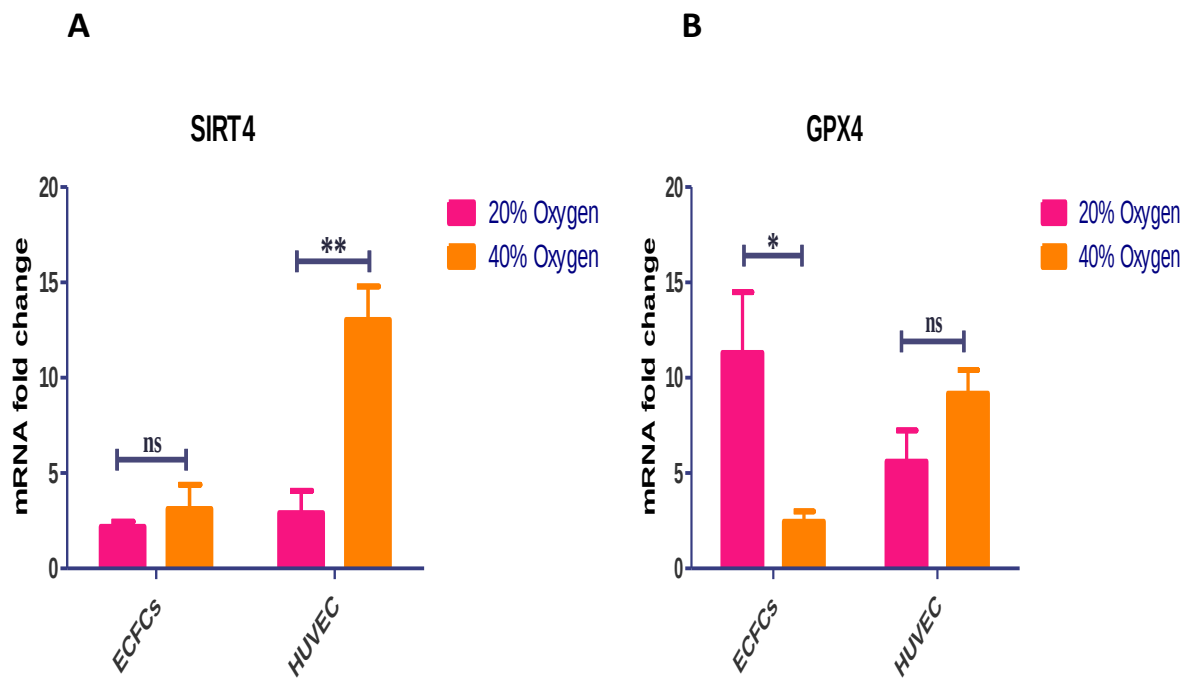
6.2.9 Gene expression analysis of mitochondrial genes involved in antioxidative mechanisms and oxidative metabolism and comparing ECFCs and HUVEC grown in normoxia and hyperoxia

Phospheonolpyruvate carboxykinase also known as PEPCK or PCK2 is a human mitochondrial gene that encodes a protein that acts as an enzyme involved in a rate limiting step in gluconeogenesis. PEPCK gene transcription is induced by glucagon, retinoic acid and cAMP and is down regulated by insulin (O'Brien *et al.*, 1990).

SIRT 4 is a mitochondrial gene which encodes a member of sirtuins family of proteins that function as intracellular regulatory proteins and belong to a group of nicotinamide adenine dinucleotide (NAD) - dependent enzymes which are involved in stress responses, longevity and metabolism. SIRT4 is known to be involved in regulation of mitochondrial gene expression and fatty acid oxidation in muscle and liver cells (Nasrin *et al.*, 2010). It has been shown that SIRT4 inhibits mitochondrial glutamate dehydrogenase activity resulting in a decrease in insulin secretion (Haigis *et al.*, 2006). It has been shown recently that mTORc1 pathway is involved in regulation of amino acid glutamine metabolism and it does that by repressing SIRT4 transcription resulting in an increase in glutamine metabolism (Csibi *et al.*, 2013). Both PCK2 and SIRT4 mRNA expression did not show any statistically significant difference in ECFCs grown in normoxia and hyperoxia. As shown in figures 6.9A and C, there was an increase in gene expression level of mitochondrial genes PCK2 and SIRT 4 in HUVEC grown in hyperoxia, and the difference was found to be statistically significant. This suggests that oxidative stress lead to an increase in fatty acid oxidation in mature endothelial cells.

Pyruvate dehydrogenase lipoamide kinase isozyme 4 is a mitochondrial enzyme, which in humans is encoded by *PDK4* gene. PDK4 has been shown to contribute towards regulation of glucose metabolism by inhibiting pyruvate dehydrogenase complex. The gene is induced by retinoic acid, insulin and glucocorticoids and is activated in response to fasting in muscle and liver (Wu *et al.*, 1999; Pilegaard *et al.*, 2000; Hildebrandt *et al.*, 2003). It has been shown that PGC-1 α regulates the expression of PDK4 by coactivation of ERR α and PPARα and also directly induces PDK4 gene expression in skeletal muscle (Wu *et al.*, 2001; Wende *et al.*, 2005). This leads to increased mitochondrial fatty acid oxidation and a simultaneous decrease in glucose oxidation. As shown in figure 6.9D there was a 4 fold increase in mRNA expression level of PDK4 in HUVEC grown in hyperoxia suggesting that it could be due to PGC-1 alpha activation and hyperoxia leads to increased mitochondrial fatty acid oxidation.

Glutathione peroxidase 4, also known as GPx4 is an enzyme which is encoded by *GPX4* gene in humans. It is a phospholipid peroxidase and protects cells against lipid peroxidation. It protects the cells against oxidative stress by catalyzing the reduction of hydrogen peroxide at the expense of glutathione and is essential for embryonic development (Yant *et al.*, 2003; Muller *et al.*, 2007). As shown in figure 6.9B, there was a 10 fold decrease in the expression of GPX4 in ECFCs grown in hyperoxia and the difference was found to be statistically significant.



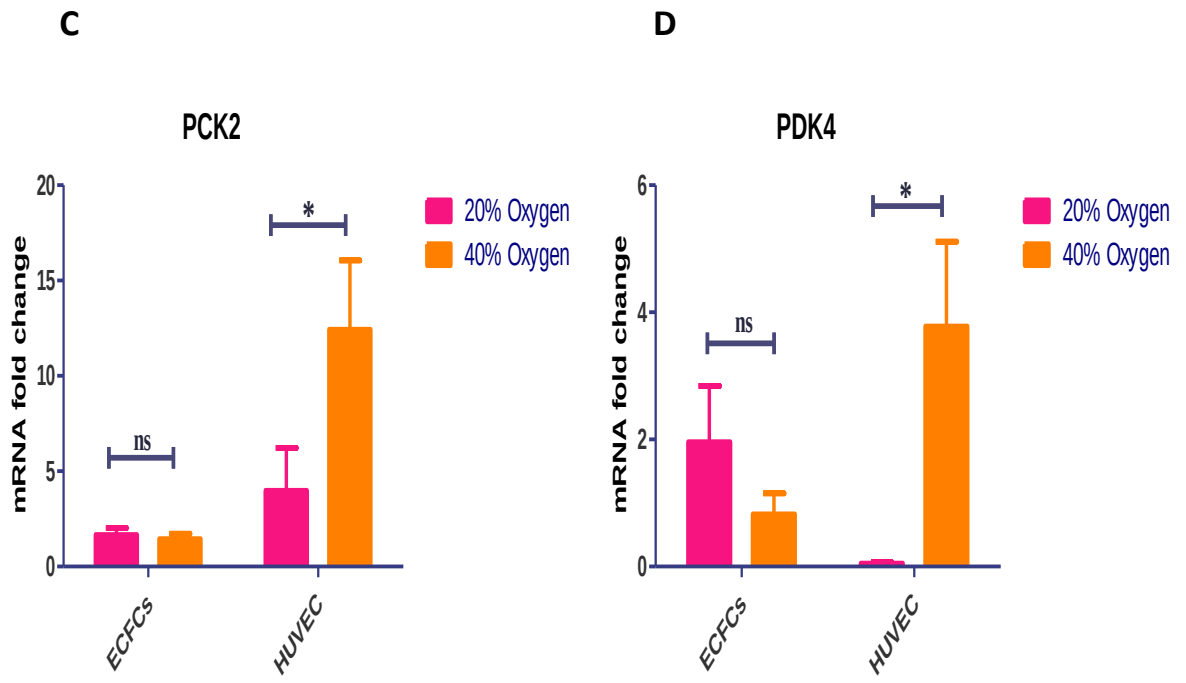


Figure 6.9. Gene expression analysis of human mitochondrial genes which are part of fatty acid and glucose metabolism and antioxidative mechanism. Endothelial colony forming cells (ECFCs) were derived from cord blood and mature endothelial cells (HUVEC) were derived from the umbilical cord of the same donor. Both ECFC and HUVEC are grown in conditions of normoxia (20% O_2) and hyperoxia (40% O_2). Figure shows an up regulation of the mitochondrial genes in ECFCs grown in hyperoxia, and the difference was found to be statistically significant. HUVEC showed down regulation of these genes under hyperoxic conditions although the difference was not found to be statistically significant. The data were normalized to 18s rRNA, HPRT1 and TBP which were used as endogenous controls. Gene expression is plotted relative to cord blood mononuclear cells. Statistical analysis was carried out using two way ANOVA followed by Bonferroni posttests. All values are calculated as ± SEM. n=4 (ECFC 20% and 40% O_2) and n=5 (HUVEC 20% and 40% O_2). * $P < .05$, ** $P < .01$, *** $P < .001$ and ns stands for not significant.

6.2.10 Gene expression analysis of cell surface endothelial markers involved in angiogenesis and comparison between changes in expression in ECFCs and HUVEC grown in normoxia and hyperoxia

Von willibrand factor (vWF) is a plasma glycoprotein encoded by vWF gene which is involved in adhesion of platelets and plays an important role in maintaining haemostasis (Cheresh, 1987). It acts as a carrier for coagulation factor VIII and its deficiency can lead to von Willibrand disease, which is a common congenital bleeding disorder (Huang *et al.*, 2009). Endothelial vWF has been shown to regulate inflammation via an independent mechanisms including leukocyte adhesion and increased levels of vWF are implicated in acute coronary thrombosis and is considered to be a clinical marker of atherosclerotic progression (Spiel *et al.*, 2008). As shown in figure 6.10, there was no significant change in expression of vWF in ECFCs when grown in normoxia and hyperoxia. Whereas, there was a significant increase in expression of vWF in HUVEC and this suggests that oxidative stress lead to an increase in this expression and this can resemble an inflammatory and pro atherosclerotic environment.

VE-Cadherin is a type of cadherin also known as CD144, and encoded in humans by *CDH5* gene. It has been shown to be involved in the maintenance of newly formed blood vessels and is essential for endothelial permeability. CD144 deficiency has shown to induce haemorrhage in vivo and increased monolayer permeability in an *in vitro* culture system (Carmeliet *et al.*, 1999; Corada *et al.*, 2002; Crosby *et al.*, 2005). As shown in figure 6.10, there was several thousand fold decrease in the expression of CDH5 in ECFCs grown in hyperoxia suggesting it is playing its role in angiogenesis. HUVEC did not show any significant change in expression of CDH5, although it showed an up regulation.

Platelet cell adhesion molecule 1(PECAM-1) also called CD31 is a protein which in humans is encoded by *PECAM-1*gene. It is involved in removing aged neutrophils, leukocyte migration and angiogenesis (Newman *et al.*, 1990; Gumina *et al.*, 1996; Xie and Muller, 1996). CD31 is mainly expressed in endothelial cells and forms part of intercellular junctions. It has shown the expression in several vascular tumours. As shown in figure 6.10 there was a statistically significant difference in the expression of CD31 in ECFCs grown in hyperoxia and showed more than 5 fold change in mRNA expression level. HUVEC showed an increase in expression but the difference was not found to be statistically significant.

PROM1 is a glycoprotein also known as CD133 or AC133. It is mainly expressed in hematopoietic stem cells, early endothelial progenitor cells and glial stem cells (Horn *et al.*,

1999; Corbeil *et al.*, 2000; Sanai *et al.*, 2005). As shown in figure 6.10 there was an increase in expression of PROM1 in HUVEC grown in hyperoxia. ECFCs did not show a significant change in expression.

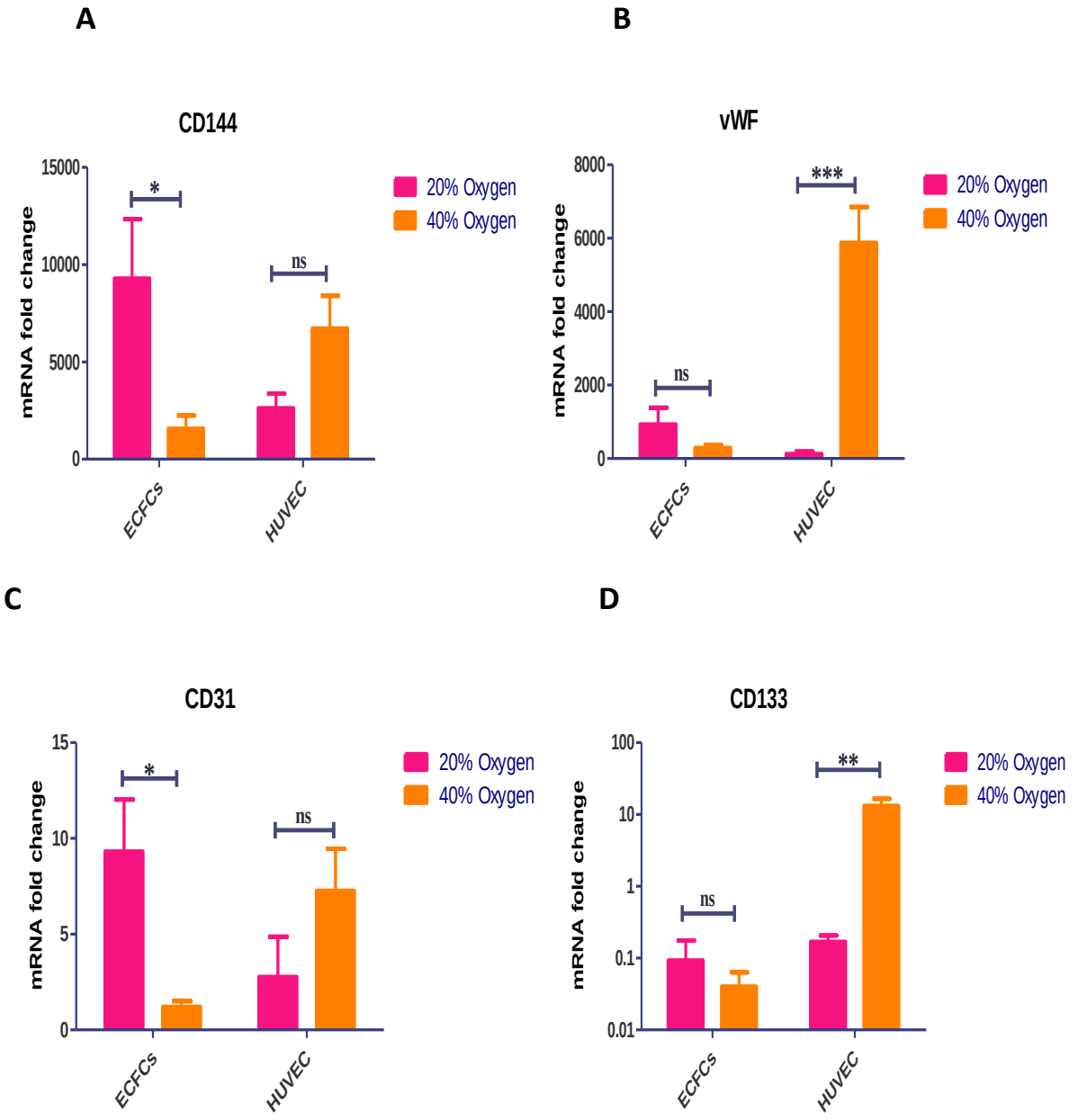


Figure 6.10. Gene expression analysis of endothelial cell surface markers. Endothelial colony forming cells (ECFCs) were derived from cord blood and mature endothelial cells (HUVEC) were derived from the umbilical cord of the same donor. Both ECFCs and HUVEC were grown in conditions of normoxia (20% O_2) and hyperoxia (40% O_2). Figure shows down regulation of endothelial markers PECAM 1 and CDH5 (VE-cadherin) in ECFCs grown in hyperoxia, and the difference was found to be statistically significant. HUVEC showed an increase in expression of endothelial marker vWF and an up regulation of PROM 1. The data were normalized to 18s rRNA, HPRT1 and TBP which were used as endogenous controls. Gene expression is plotted relative to cord blood mononuclear cells. Statistical analysis was carried out using two way ANOVA followed by Bonferroni posttests. All values are calculated as ± SEM. n=4 (ECFC 20% and 40% O_2) and n=5 (HUVEC 20% and 40% O_2). * $P < .05$, ** $P < .01$, *** $P < .001$ and ns stands for not significant.

6.2.11 Gene expression analysis of angiogenic factors involved in promoting angiogenesis and comparison between changes in expression in ECFCs and HUVEC grown in normoxia and hyperoxia

VEGFA gene encodes the protein vascular endothelial growth factor A in humans. The gene is a member of PDGF/VEGF family and has been studied in detail in vascular endothelium. VEGFA has been known to play many important functions in angiogenesis involving migration and mitosis of endothelial cells and creation of blood vessel lumen (Creuzet *et al.*, 2002; Stockmann *et al.*, 2008; Mackenzie and Ruhrberg, 2012). As shown in figure 6.11 there was more than 2 fold increase in mRNA level of expression of VEGFA in ECFCs that were cultured in hyperoxic conditions. This shows that ECFCs promote angiogenesis when they are exposed to oxidative stress. HUVEC showed no significant change in expression of VEGFA when grown in hyperoxia.

VEGF C encodes vascular endothelial growth factor C in humans. VEGFC belongs to the PDGF/VEGF family of proteins and is involved in several important functions including angiogenesis, endothelial growth, blood vessel permeability and lymphangiogenesis (Paavonen *et al.*, 1996; Schoppmann *et al.*, 2002). As shown in figure 6.11 there was several thousand fold increase in the expression of VEGFC in ECFCs grown in hyperoxia as compared to cells grown in normoxia. HUVEC showed a decrease in expression and the difference was found to be statistically significant. This suggests that HUVEC do not take part in angiogenesis when they are exposed to hyperoxia.

Vascular endothelial growth factor (VEGF) is a key signaling protein which is involved in both vasculogenesis and angiogenesis. There are three main types of receptors to which VEGF binds which are all tyrosine kinase receptors. Two main receptors include VEGFR1 also known as FLT-1 and VEGFR2 which is also known as FLK-1 or KDR (Holmes *et al.*, 2007; Stuttfeld and Ballmer-Hofer, 2009). VEGFA binds to both FLT-1 and KDR receptors but mainly KDR receptor mediates the cellular responses (Fujita *et al.*, 2008). VEGFC binds mainly to KDR. As shown in figure 6.11 both FLT-1 and KDR showed several fold decrease in expression when ECFCs were exposed to oxidative stress. This could be due to receptor filling as both VEGFA and C showed higher expression in ECFCs grown in hyperoxia. HUVEC did not show any significant change in mRNA level of expression of any of these receptors again suggesting that these cells do not take part in angiogenesis as opposed to ECFCs which show up regulation of angiogenic genes when grown in hyperoxic conditions.

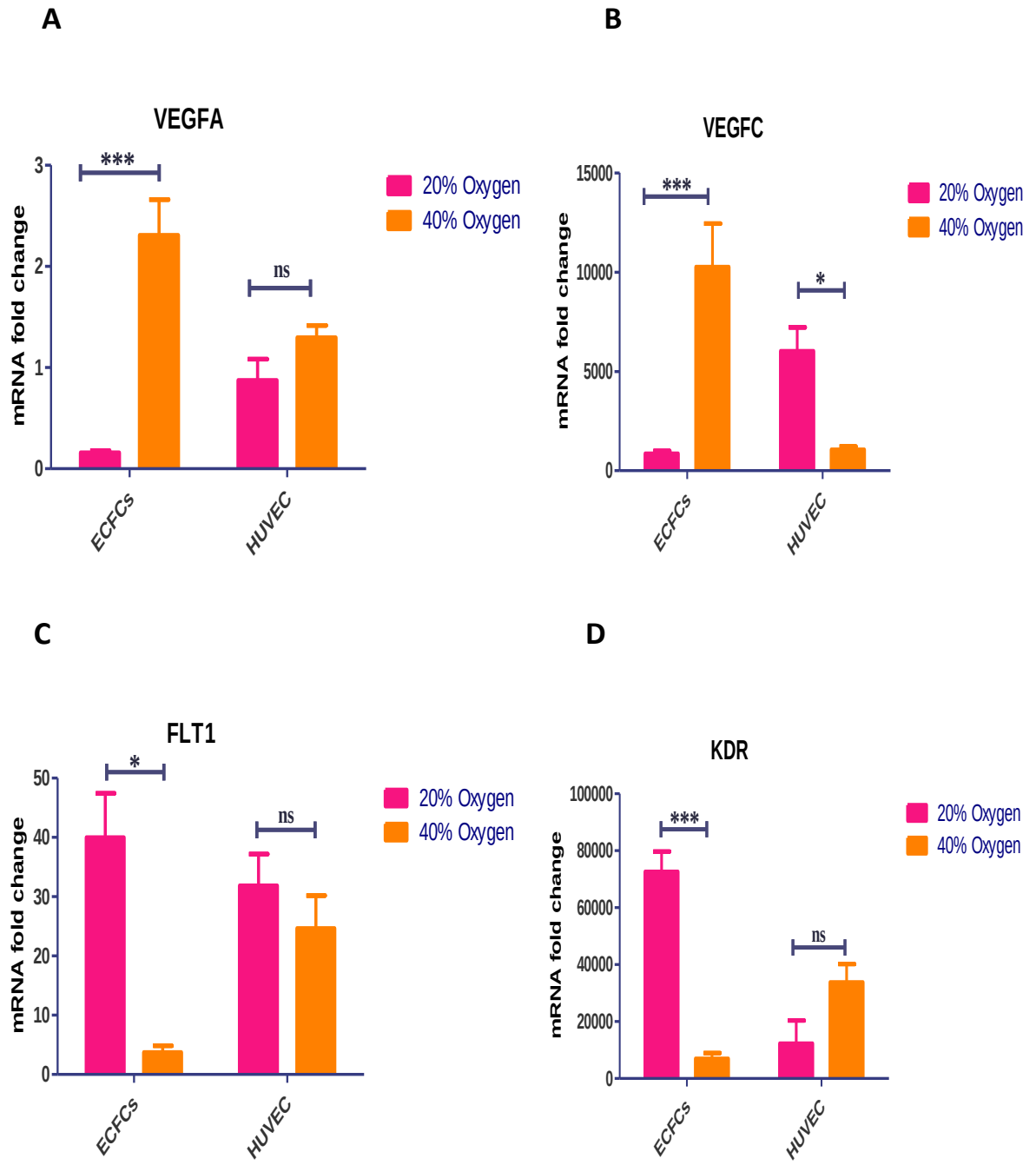


Figure 6.11. Gene expression analysis of angiogenic growth factors and angiogenesis inhibitor FLT1. Endothelial colony forming cells (ECFCs) were derived from cord blood and mature endothelial cells (HUVEC) were derived from the umbilical cord of the same donor. Both ECFC and HUVEC were grown in conditions of normoxia (20% O_2) and hyperoxia (40% O_2). Figure A and B showing an increase in expression of angiogenic growth factors VEGF A and C in ECFCs when exposed to hyperoxia and HUVEC showed no significant difference in gene expression. Figure C and D showing down regulation of angiogenic inhibitor FLT1 gene in ECFCs in hyperoxia, and the difference was found to be statistically significant. There was no significant difference seen in HUVEC for VEGF A expression, but VEGF C expression decreased under hyperoxia and the difference was found to be statistically significant. The data were normalized to 18s rRNA, HPRT1 and TBP which were used as endogenous controls. Gene expression is plotted relative to cord blood mononuclear cells. Statistical analysis was carried out using two way ANOVA followed by Bonferroni posttests. All values are calculated as ± SEM. n=4 (ECFC 20% and 40% O_2) and n=5 (HUVEC 20% and 40% O_2). * $P < .05$, ** $P < .01$, ***$P < .001$ and ns stands for not significant.

6.2.12 Gene expression analysis of pro-angiogenic factors and angiogenic targets involved in angiogenesis and comparison between changes in expression in ECFCs and HUVEC grown in normoxia and hyperoxia

As shown in figure 6.12, we investigated the genes that we thought would be involved in an inflammatory response. This included as shown in figure 6.12 A, a cytokine belonging to chemokine family, CXCL12 also known as stromal cell-derived factor-1(SDF-1). This gene is involved in hematopoietic stem cell migration and blood vessel formation during early embryogenesis. It has recently been shown to be involved in EPC recruitment from bone marrow via CXCR4 which is its binding receptor, thus contributing towards blood vessel formation (Zheng *et al.,* 2007). CXCL12 gene has been shown to be a pro-inflammatory genetic marker associated with atherosclerosis and encodes a chemokine which is involved in vascular repair (Linsel-Nitschke *et al.*, 2010; Mehta *et al.*, 2011). The results showed that ECFCs when exposed to hyperoxia show an up regulation of CXCL12 gene suggesting that these cells are responding to this stress environment and could be the cells involved in vessel repair. The difference between ECFCs grown in normoxia and hyperoxia was found to be statistically significant. As shown in figure 6.12 A, the mature endothelial cells HUVEC did not show any significant change in CXCL12 gene expression.

Fibroblast growth factor 2 (FGF2) is a potent angiogenic growth factor involved in blood vessel formation, which is dependent on heparin sulphate (HS) (Ferreras *et al.*, 2012). HS degrading enzymes activate FGF2 during wound healing and tumorigenesis. As shown in figure 6.12 B, ECFCs grown in hyperoxia show a 200 fold increase in gene expression of FGF2. This suggests that these cells are responding to oxidative stress and promoting angiogenesis. The difference was found to be statistically significant. Mature endothelial cells (HUVEC) did not show any significant change in expression of FGF2, when grown in hyperoxia.

PDGFRA gene encodes a protein in humans which is known as alpha platelet derived growth factor receptor (PDGFRA). It encodes cell surface tyrosine kinase receptors for PDGF family of proteins. It has shown interactions with PDGFRB and both of them have been involved in growth and maturation being mitogens for cells of mesenchymal origin (Seifert *et al.,* 1989; Rupp *et al.,* 1994). As shown in figure 6.12 both genes showed more than 5 fold increase in mRNA expression level in ECFCs grown in hyperoxia. This suggests that hyperoxia environment provided an atherosclerotic and proinflammatory condition and ECFCs respond

by up regulation of angiogenic markers. HUVEC did not show any significant change in expression of either of these genes.

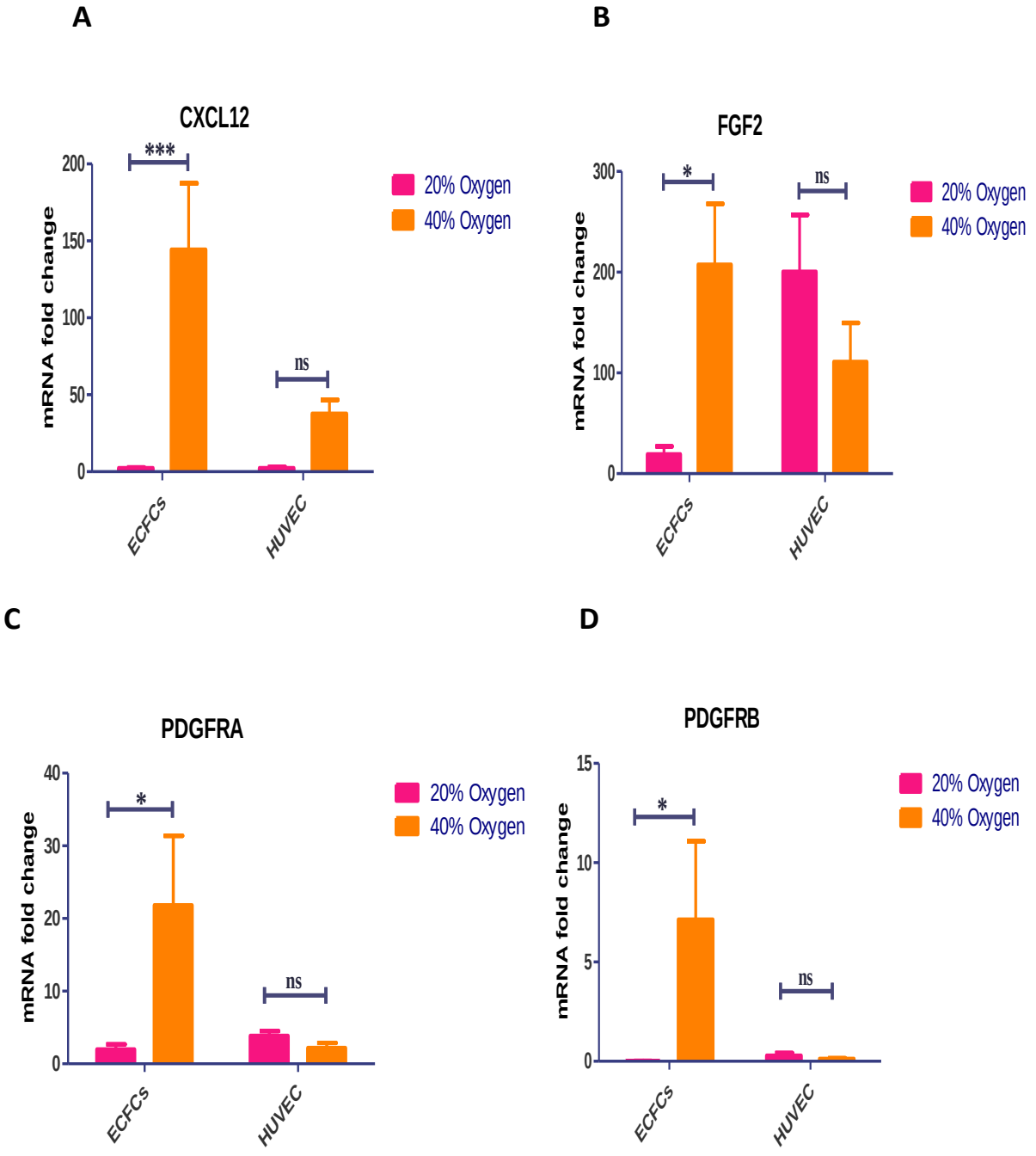


Figure 6.12. Gene expression analysis of angiogenesis target genes. Endothelial colony forming cells (ECFCs) were derived from cord blood and mature endothelial cells (HUVEC) were derived from the umbilical cord of the same donor. Both ECFC and HUVEC were grown in conditions of normoxia (20% O_2) and hyperoxia (40% O_2). Figure shows an up regulation of these genes in ECFCs in hyperoxia, and the difference was found to be statistically significant. The data were normalized to 18s rRNA, HPRT1 and TBP which were used as endogenous controls. Gene expression is plotted relative to cord blood mononuclear cells. Statistical analysis was carried out using two way ANOVA followed by Bonferroni posttests. All values are calculated as ± SEM. n=4 (ECFC 20% and 40% O_2) and n=5 (HUVEC 20% and 40% O_2). * $P < .05$, ** $P < .01$, ***$P < .001$ and ns stands for not significant.

6.2.13 Gene expression analysis of angiogenic factors involved in promoting angiogenesis and comparison between changes in expression in ECFCs and HUVEC grown in normoxia and hyperoxia.

Endothelial nitric oxide (eNOS) is the main source of NO, which plays an important role in maintaining vasodilation and vasoconstriction by regulating smooth muscle cells activation (Ignarro *et al.*, 1987). More importantly, NO is a competitive inhibitor of electron transport chain at complex IV (Moncada and Erusalimsky, 2002), thus altering the rate of oxidative metabolism in both cardiomyocytes and endothelial cells (Clementi *et al.*, 1999), which is suggested to be inversely related to oxygen levels. As shown in figure 6.13 there was a 20 fold down regulation in mRNA expression level of NOS3 when ECFCs were exposed to hyperoxic conditions and suggests that this is ECFCs response to oxidative stress.

Alpha -actin-2 which also known as alpha smooth muscle actin is a protein that in humans is encoded by *ACTA2* gene. It is considered to be a highly conserved protein and is involved in cell motility, integrity and shaping of cell structure. It forms the bulk of cells contractile machinery and has also been used as a marker of myoblast formation and known to be activated by p53 (Tomasselli *et al.*, 1991; Comer *et al.*, 1998). As shown in figure 6.13 there was a 40 fold increase in expression of ACTA 2 in ECFCs exposed to oxidative stress suggesting this environment promotes angiogenesis in ECFCs. HUVEC showed no significant change in expression of ACTA2.

Angiopoeitin1is a protein that is encoded by *ANGPT1* gene in humans. It is an important growth factor that plays a vital role in promoting angiogenesis and vascular development. It binds to and interacts with endothelial specific TEK tyrosine kinase receptor and is known to be involved in maintaining blood vessel maturity and stability (Maisonpierre *et al.*, 1997).

ANGPT2 is a gene which encodes angiopoeitin 2 proteins in humans. It mainly acts as an antagonist to ANG1 and TEK tyrosine kinase and is mainly expressed at vascular remodelling sites (Maisonpierre *et al.*, 1997; Cheung *et al.*, 1998; Sato *et al.*, 1998; Fiedler *et al.*, 2003). As shown in figure 6.13 ANG1 mRNA expression level was increased 40 fold and as ANG2 acts as an antagonist to ANG1, its level of expression was found to be several thousand fold decreased in ECFCs that were exposed to oxidative stress. This shows that during hyperoxia angiogenesis is activated and ECFCs take part in this vascular repair. HUVEC did not show a significant change in expression of these angiogenic genes.

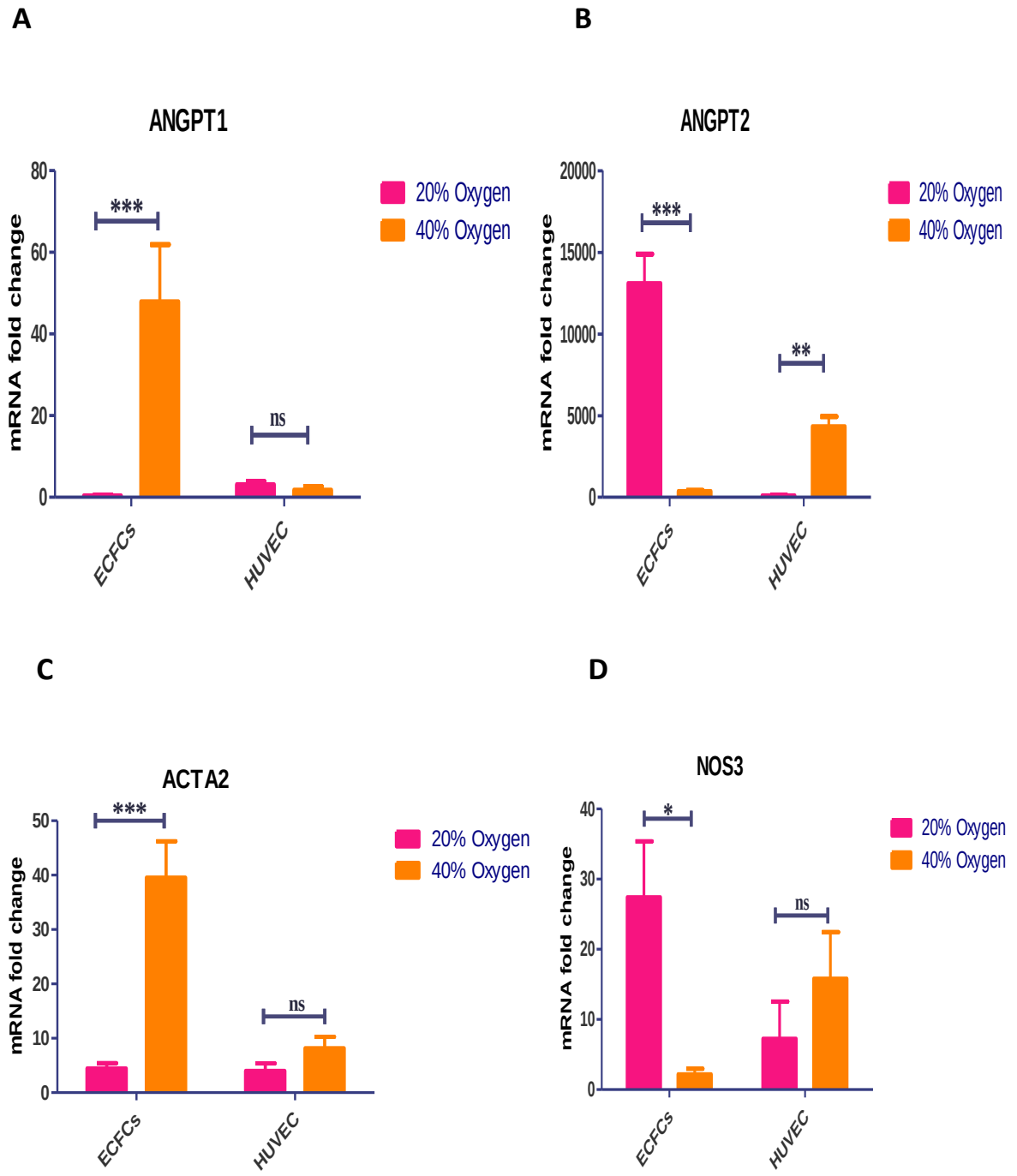


Figure 6.13. Gene expression analysis of pro angiogenic target genes and genes involved in regulation of blood vessel formation. Endothelial colony forming cells (ECFCs) were derived from cord blood and mature endothelial cells (HUVEC) were derived from the umbilical cord of the same donor. Both ECFC and HUVEC were grown in conditions of normoxia (20% O_2) and hyperoxia (40% O_2). Figure shows an up regulation of these proangiogenic genes in ECFCs grown in hyperoxia, and the difference was found to be statistically significant. Figure shows reduced expression of ANGPT2 and endothelial NO (eNOS) in ECFCs grown in hyperoxia and the difference was found to be statistically significant. HUVEC did not show any statistically significant change in expression of these angiogenic markers. The data were normalized to 18s rRNA, HPRT1 and TBP which were used as endogenous controls. Gene expression is plotted relative to cord blood mononuclear cells. Statistical analysis was carried out using two way ANOVA followed by Bonferroni posttests. All values are calculated as ± SEM. n=4 (ECFC 20% and 40% O_2) and n=5 (HUVEC 20% and 40% O_2). * $P < .05$, ** $P < .01$, *** $P < .001$ and ns stands for not significant.

6.2.14 Gene expression analysis of angiogenic and anti angiogenic factors involved in regulating angiogenesis and comparison between changes in expression in ECFCs and HUVEC grown in normoxia and hyperoxia

Transforming growth factor alpha (TGF-α) is a marker that has been found to be up regulated in several cancers and is mainly produced by macrophages and keratinocytes and known to take part in the development of epithelium. It has shown to be involved in neural cell development in cases of adult brain injury (Fallon *et al.*, 2000). ECFCs showed the expression of TGF- α and the expression level decreased when cells were grown in hyperoxia, but the difference was not statistically significant as shown in figure 6.14. HUVEC on the other hand, showed statistically significant increase in the expression of TGF-α when grown in conditions of oxidative stress.

TEK tyrosine kinase also known as angiopoeitin 1-receptor is a protein which in humans is encoded by *TEK* gene. It is exclusively expressed in endothelial cells and is known to interact with both ANG1 and ANG2 (Davis *et al.*, 1996; Sato *et al.*, 1998; Fiedler *et al.*, 2003). As shown in figure 6.14 TEK levels did not show a significant change in expression of either ECFCs or HUVEC when these cells were exposed to oxidative stress.

TP53 is a tumor suppressor protein in humans and is encoded by *TP53* gene (Isobe *et al.*, 1986; Kern *et al.*, 1991). It has been known to play key anticancer roles and is involved in apoptosis and inhibition of angiogenesis (Teodoro *et al.*, 2006; Assadian *et al.*, 2012). It is known to be activated by oxidative stress and DNA damage. As shown in figure 6.14 there was a down regulation of the mRNA expression level of the gene in ECFCs grown in hyperoxia, as these cells promote angiogenesis when exposed to oxidative stress. HUVEC showed a statistically significant increase in the expression of TP53. This again suggests that HUVEC do not take part in vascular repair when exposed to oxidative stress.

NRP2 is a gene which in humans encode for neuropilin-2 protein. It plays a role in cardiovascular development and is known to interact with VEGF (Chen *et al.*, 1997; Soker *et al.*, 1998). As shown in figure 6.14 there was a decrease in expression of NRP2 in ECFCs grown in hyperoxia, but the difference was not found to be significant. HUVEC also did not show any significant change in expression of the gene.

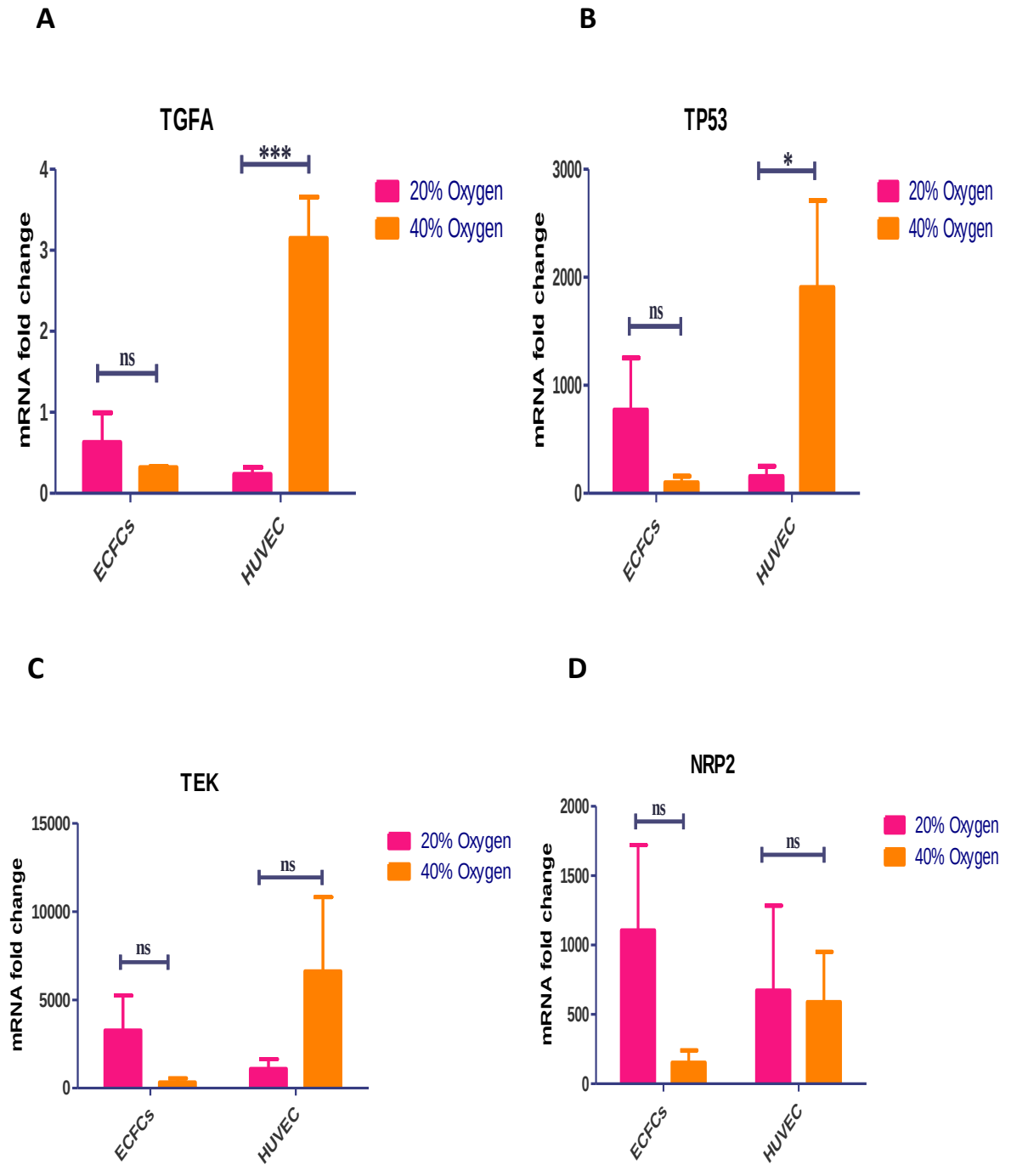


Figure 6.14. Gene expression analysis of angiogenic growth factors and target genes. Endothelial colony forming cells (ECFCs) were derived from cord blood and mature endothelial cells (HUVEC) were derived from the umbilical cord of the same donor. Both ECFC and HUVEC were grown in conditions of normoxia (20% O_2) and hyperoxia (40% O_2). Figure shows reduced expression of these proangiogenic genes in ECFCs grown in hyperoxia, but the difference was not found to be statistically significant. HUVEC showed an up regulation of some of these angiogenic growth factors and the difference was found to be statistically significant. The data were normalized to 18s rRNA, HPRT1 and TBP which were used as endogenous controls. Gene expression is plotted relative to cord blood mononuclear cells. Statistical analysis was carried out using two way ANOVA followed by Bonferroni posttests. All values are calculated as ± SEM. n=4 (ECFC 20% and 40% O_2) and n=5 (HUVEC 20% and 40% O_2). * $P < .05$, ** $P < .01$, *** $P < .001$ and ns stands for not significant.

6.2.15 Gene expression analysis of factors involved in proinflammatory response for angiogenesis and comparison between changes in expression in ECFCs and HUVEC grown in normoxia and hyperoxia

Interleukin -1alpha (IL-1α) is an inflammatory cytokine that in humans is encoded by *IL1A* gene. It is known to play a central role in the regulation of immune response and is produced mainly by macrophages and endothelial cells (March *et al.*, 1985). It binds to interleukin-1 receptor and has shown to activate tumor necrosis factor (TNF- α) in endothelial cells (Bankers-Fulbright *et al.*, 1996; Hu *et al.*, 2003). As shown in figure 6.15 HUVEC showed a 20 fold increase in mRNA level expression of IL1A and shows that exposing cells to hyperoxia can activate pro inflammatory markers. ECFCs showed a decrease in expression that was found to be statistically significant.

c-kit or Stem cell factor receptor (SCFR) also known as CD117 is a protein which in humans is encoded by KIT gene (Andre *et al.*, 1997). It is a proto-oncogene mainly expressed in hematopoietic stem cells and myeloid progenitors and bind to stem cell factor playing a role in cell survival and proliferation (Anzai *et al.*, 2002). There was a 20 fold decrease in expression of KIT in ECFCs grown in hyperoxic conditions as shown in figure 6.15. HUVEC showed no significant change in expression.

ABCG2 is a gene that in human encodes the protein ATP- binding cassette subfamily G receptor 2 also known as ABCG2 (CD338). This protein belongs to a family of ATP- binding cassette (ABC) transporters, which are mainly involved in transport of substances across cell membranes by using energy derive from ATP hydrolysis. ABCG2 has been shown to be a marker of progenitor cells and unique amongst this family of transporters to have this expression pattern. It also shows expression (mRNA and protein) in cancer stem cells, epithelial cells, placenta, blood brain barrier and many other organs including liver, lung and brain (Gutmann *et al.*, 2005; Visvader and Lindeman, 2008). This gene has been shown to be suppressed during DNA methylation (Turner *et al.*, 2006). As shown in figure 6.15 ECFCs when cultured in hyperoxic conditions showed more than 5 fold decrease in expression of the gene suggesting that oxidative stress induced this change in expression.

The *ITPKB* gene in humans encodes the protein inositol –triphosphate 3-kinase B which is an enzyme that regulates inositol metabolism by phosphorylation and mediates cell signaling (Erneux *et al.*, 1992). The gene is involved in immune cell function and promotes T and B cell maturation (Sauer and Cooke, 2010). As shown in figure 6.15 there was statistically

significant difference in mRNA level expression of ITPKB gene in ECFCs. HUVEC showed no significant change in expression of the gene.

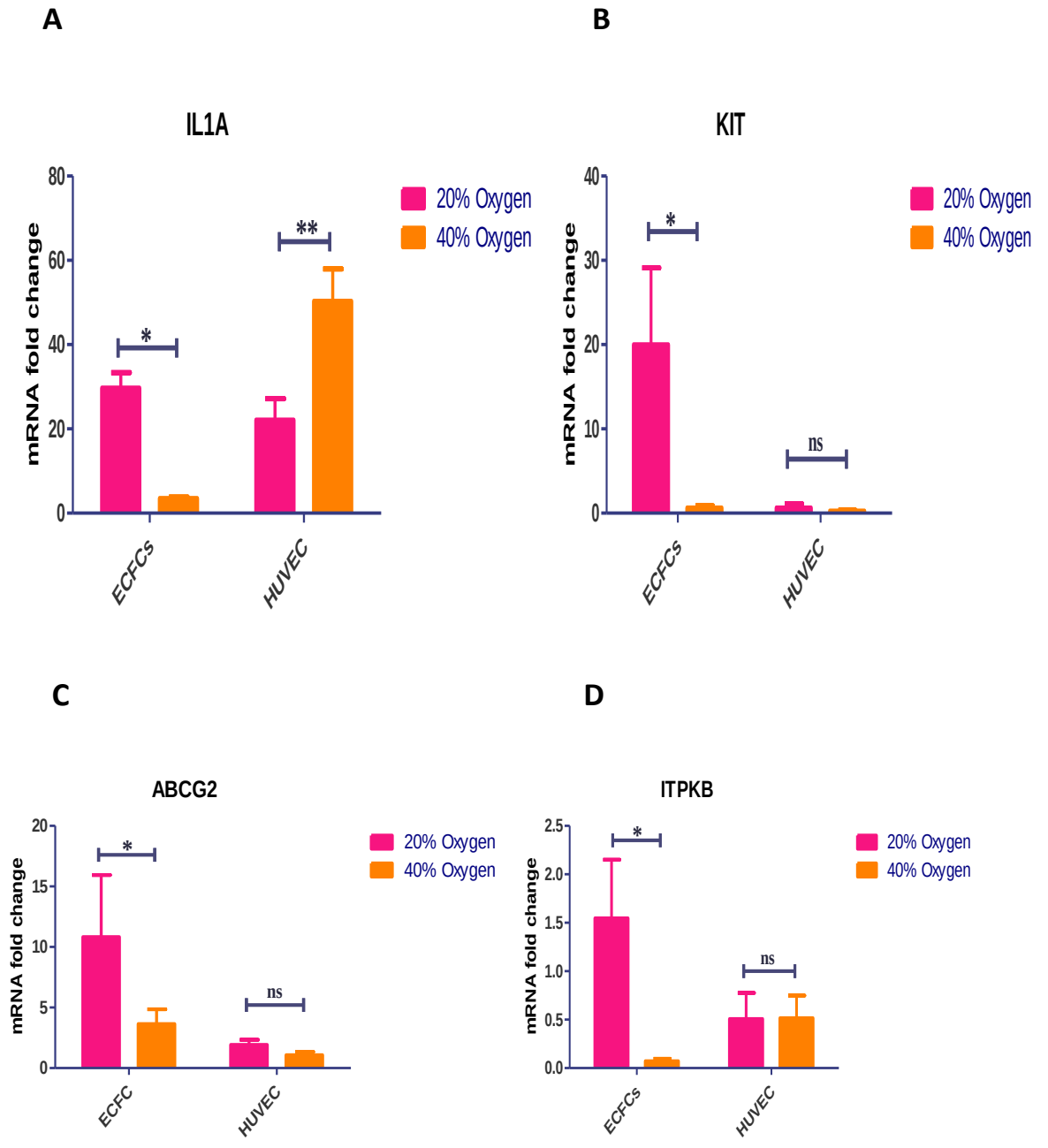


Figure 6.15. Gene expression analysis of proinflammatory and immunoregulatory markers and angiogenesis target genes. Endothelial colony forming cells (ECFCs) were derived from cord blood and mature endothelial cells (HUVEC) were derived from the umbilical cord of the same donor. Both ECFC and HUVEC were grown in conditions of normoxia (20% O_2) and hyperoxia (40% O_2). Figure shows down regulation of these proangiogenic genes in ECFCs grown in hyperoxia, and the difference was found to be statistically significant. HUVEC did not show any statistically significant change in expression of these markers. The data were normalized to 18s rRNA, HPRT1 and TBP which were used as endogenous controls. Gene expression is plotted relative to cord blood mononuclear cells. Statistical analysis was carried out using two way ANOVA followed by Bonferroni posttests. All values are calculated as ± SEM. n=4 (ECFC 20% and 40% O_2) and n=5 (HUVEC 20% and 40% O_2). * $P < .05$, ** $P < .01$, *** $P < .001$ and ns stands for not significant.

6.3. Discussion

We carried out gene expression analysis of mitochondrial, antioxidative, proangiogenic and proinflammatory genes and those involved in DNA damage due to oxidative stress. In order to understand the role of PGC-1 alpha in ECFCs and HUVEC we also analysed several downstream targets of PGC-1 alpha so that we could study the pathways PGC-1 alpha monitors when cells are grown in conditions of oxidative stress. We proposed that exposing cells to 40 % O_2 would lead to oxidative stress, and endothelial damage will lead to an atherosclerotic and inflammatory environment in an *in vitro* system. Inflammation refers to a series of complex but well conserved processes that forms part of an organism's response to a harmful stimulus, including cell damage and irritants and leads to repair mechanisms following this tissue injury. It has been shown recently that there exists an interplay between oxidative stress and inflammation which results in the initiation and progression of cardiovascular diseases and this combination of oxidative stress and inflammation leads to mobilization of EPCs (Stenvinkel *et al.*, 2003; Haubitz and Woywodt, 2004).

Endothelial cells mainly derive their energy from anaerobic glycolysis and it has been shown that more than 70 % of ATP synthesis occurs via glycolysis in porcine aortic endothelial cells (Spahr *et al.*, 1989; Culic *et al.*, 1997; Quintero *et al.*, 2006). Endothelial mitochondria are thought to be involved in regulating interplay between ROS, NO and calcium ions. This is suggested to be important in maintaining endothelial function, and a disruption to endothelial mitochondria can progress towards endothelial dysfunction leading to atherosclerosis.

An important endothelial marker Von Willibrand factor (vWF) was investigated in order to differentiate between endothelial progenitors and mature endothelial cells. vWF has recently been shown to play a regulatory role in angiogenesis in endothelial cells (Starke *et al.*, 2011). Angiogenic factors mainly, Angiopoeitins (Ang) and vascular endothelial growth factor (VEGF) mediate cell signaling pathways involved in endothelial cell proliferation and migration leading to blood vessel formation. VEGF- A is known to be a key player involved in angiogenesis (Grothey and Galanis, 2009) and acts mainly on Kinase insert domain receptor (KDR) also known as the VEGFR-2 (CD309/FLK1), which is a type 3 tyrosine kinase receptor (Petrova *et al.*, 1999). This leads to endothelial cell proliferation, migration and early angiogenic sprouting (Gerhardt, 2008). Vascular endothelial growth factor C also known as VEGF- C has been known to be involved in endothelial cell proliferation and angiogenesis (Meyer *et al.*, 1999). It is also involved in lymphangiogenesis and mediates its signaling through both VEGFR-2 (KDR) and VEGFR-3 (FLT4) receptors (Joukov *et al.*,

1996; Dias *et al.*, 2002). Angiopoeitins play an important role in advanced stages of blood vessel formation. ANG-1 and ANG-2 binding to endothelial TEK receptor (Tie2) maintain vascular maturity and intact endothelium (Thomas and Augustin, 2009). It was initially considered that ANG-1 and ANG-2 act as antagonists. ANG-1 playing a role in vessel wall maturation and promoting endothelial cell migration and adhesion and ANG-2 breaking the links between perivascular cells and endothelium and leading to vascular regression and permeability (Suri *et al.*, 1996; Lobov *et al.*, 2002).

Endothelial cells mediate ROS production in the form of superoxide and hydrogen peroxide which acting as messengers lead to a growth factor -dependent response promoting angiogenesis (Case *et al.*, 2008). NADPH oxidase is considered to be the most important source of ROS in vasculature (Griendling *et al.*, 2000; Lassègue *et al.*, 2012). NADPH oxidase (NOX) derived ROS has been shown to be implicated in several cardiovascular diseases and increased ROS levels have been found in both human and animal models (Lacy *et al.*, 2000; Touyz and Schiffrin, 2001; Álvarez *et al.*, 2008; Brandes *et al.*, 2010; Lassègue and Griendling, 2010).

Reactive oxygen species (ROS) are substances produced as a result of cellular metabolism, and are chemically active molecules that contain oxygen. ROS produced at normal levels, as a byproduct of oxygen metabolism are involved in maintaining homeostasis and cell signaling mechanisms (Dickinson and Chang, 2011). An increase in ROS production can lead to significant cell damage causing oxidative stress (Mancardi *et al.*, 2004). In order to counteract this ROS damage, cells defend themselves by activating certain enzymatic and nonenzymatic pathways leading to up regulation of anti-ROS genes (Lomri, 2008). The effects of oxidative stress on endothelial progenitor cells are well known and it has been shown that oxidative stress affects EPC activity and mobilization. ROS has been shown to directly damage the vascular endothelium, suggesting that it plays a role in the progression of atherosclerosis (Griendling and FitzGerald, 2003b; Torsney *et al.*, 2005; Tigges *et al.*, 2013). Reduced EPC levels and impaired EPC function have been implicated with increased superoxide production (Thum *et al.*, 2007; Qiao *et al.*, 2010). It has been shown that oxidative stress leads to increased ROS production which in turn causes Nitric oxide (eNOS) inactivation and DNA damage resulting in altered redox state (Laufs *et al.*, 2004; Fike *et al.*, 2013). EPC migration is an essential step during angiogenesis following vascular injury or endothelial dysfunction. Cell migration occurs in response to chemotactic stimuli, and there is strong evidence to show that proangiogenic factors including VEGF and Angiopoeitin- 1are

involved in directing cell migration in response to ROS production (Yamaoka-Tojo *et al.*, 2004; Chen *et al.*, 2006a; Kim *et al.*, 2006; Xia *et al.*, 2007).

Nitric oxide is considered to be an important regulator of mitochondrial dynamics and also promoter of oxidant production, although its effects are dependent upon the availability of oxygen and associated redox cellular state (Erusalimsky and Moncada, 2007). NO is known to be an inhibitor of cytochrome c oxidase (complex IV) of the electron transport chain and competes at oxygen binding site and results in reduction of mitochondrial respiration leading to an increase in ROS signaling (Dranka *et al.*, 2010; Widlansky and Gutterman, 2011). In conditions of oxidative stress NO inhibits complex I that further blocks mitochondrial ROS production (MacMillan-Crow *et al.*, 1998). Acute inflammatory response leads to an increase in NO levels that shows that the capacity of endothelial cells to respond to cellular stress has diminished. It is therefore proposed that conditions that reduce endothelial nitric oxide (eNOS) bioavailability can promote mitochondrial ROS production (Doughan *et al.*, 2008). This reduction in availability of endothelial NO results in the formation of peroxynitrite that affects complex I and III in such a way that it promotes ROS production and also inhibits antioxidants like MnSOD causing further mitochondrial damage (Wang *et al.*, 2012).

Mitochondrial ROS production and understanding the mechanisms regulating their activity has gained immense importance recently, as they play an important role in cell signaling and pathogenesis of vascular diseases (Sena and Chandel, 2012). As has been shown before the bulk of oxygen consumption occurs at complex IV (cytochrome c oxidase) of the electron transport chain and complex I and III contribute to less than 2% of the total consumption, although these analysis are based on studies in isolated mitochondria and an *in vivo* analysis of ROS production in endothelial cell still remains to be seen (Chance *et al.*, 1979; Murphy, 2009; Widlansky and Gutterman, 2011). In addition to that there are several other mitochondrial ROS sources that have been identified in endothelial cells and include nicotinamide adenine dinucleotide phosphate oxidase (NOX4) which shows high expression in endothelial cells, but mitochondrial localization is still uncertain whereas in most other tissues it is mainly present in mitochondria (Chen *et al.*, 2012). NOX4 serves as an important regulator of ROS signaling mechanisms in endothelial cells and has been shown to play a vital role in contributing towards endothelial cell senescence, angiogenesis and migration and combating inflammatory response as a result of oxidative stress and hypoxia (Lassègue *et al.*, 2012).

An important indicator of increased mitochondrial ROS production is an altered mitochondrial membrane potential. It has been shown that a decrease in membrane depolarization can increase the activity of complexes I and III of the ETC leading to an increase in ROS production (Freed and Gutterman, 2013). Mitochondrial membrane hyperpolarization can also lead to an increase in ROS mainly in metabolic states where there is nutrient excess and no more ATP synthesis is required. This causes an increase in NADH/NAD+ ratio resulting in a decrease in electron flow leading to accumulation of reactive metabolites at complexes I and III of the electron transport chain causing reduction of oxygen and superoxide ions. Several metabolic disorders including diabetes mellitus and obesity that are characterized by increased glucose and fatty acid levels have been shown to use this mechanism resulting in increased production of mitochondrial ROS (Brownlee, 2001).

As shown before, availability of excess nutrients in cardiac myocytes results in alteration of mitochondrial metabolism and can lead to heart failure, but in endothelial cells where energy demands are not as higher this excess nutrient supply provides a signal that increases mitochondrial ROS production that leads to a change in phenotype of endothelial cells (Lopaschuk *et al.*, 2010; Algahim *et al.*, 2012). Endothelial cells when exposed to high glucose or fatty acid levels leads to a decrease in eNOS activity, resulting in activation of transcription factor NFκB and protein kinase c (Nishikawa *et al.*, 2000; Brownlee, 2001). This up regulation requires counteractive mechanisms which includes over expression of uncoupling protein (UCP1), mitochondrial membrane depolarization and inhibition of electron transport chain which bring membrane potential to lower limits (Nishikawa *et al.*, 2000).

Vascular diseases are characterized in most cases by ROS- induced damage to mitochondrial membrane and mtDNA, lipid and proteins which further alleviate ROS damage (Algahim *et al.*, 2012; Hill *et al.*, 2012). Mitochondrial DNA (mtDNA) damage in particular has long term consequences as it causes either a decrease in the expression of ETC components or results in the production of defective components generating more ROS (Ballinger *et al.*, 2000). It is interesting to note that all the important risk factors that eventually progress towards cardiovascular diseases including smoking, hypertension, hyperglycaemia and hypercholesterolemia are associated with mitochondrial DNA damage (Knight-Lozano *et al.*, 2002). More importantly, it has been shown in several studies both in mice an in human tissues that the severity of atherosclerosis is directly proportional to the associated mtDNA

damage (Ballinger *et al.*, 2002). A recent study has shown that glycation of mitochondrial proteins can increase ROS production in diabetic patients (Pun and Murphy, 2012).

Mitochondrial antioxidant enzymes are instrumental in combating oxidative stress and there have been several of these that are involved in this counteracting mechanism. Manganese superoxide dismutase (MnSOD) is considered to be the most potent antioxidant against mitochondrial superoxide and takes part in conversion of superoxide ion to hydrogen peroxide. This antioxidative enzyme is known to play a protective role in endothelium and $MnSOD^{+/-}$ mice has shown to have impaired vasodilation (Brown *et al.*, 2007). In addition to that, mice with double knockout of apolipoprotein E along with $MnSOD^{+/-}$ has been shown to have more mitochondrial DNA damage and as a consequence of that more severe atherosclerosis in comparison with same double knockout mice with $MnSOD^{+/+}$, suggesting a vital role of MnSOD in maintaining the integrity of the endothelium (Ballinger *et al.*, 2002). Catalase (CAT) along with other cytosolic peroxidases including glutaredoxin-2, peroxiredoxins-3and thioredoxin-2 is involved in maintaining the levels of hydrogen peroxide within normal limits. Glutathione peroxidase (GPx-1) is present in cytosol as well as mitochondria in the endothelial cells (Griendling and FitzGerald, 2003a). PGC-1 alpha regulates this antioxidative response and sends this signal as soon as oxidative stress in the form of hydrogen peroxide or as we proposed by exposure to 40% oxygen affects the endothelial cells. These antioxidative genes are up regulated and in turn promotes the translation of antioxidant proteins to protect the endothelium against these form of stress (Valle *et al.*, 2005; Schulz *et al.*, 2008). In several studies performed in mice and other experimental models it has been shown that loss of any of these antioxidant enzymes can make the endothelium vulnerable to various forms of stress leaving the endothelium prone to injury and progression to atherosclerosis with associated mitochondrial damage (Forgione *et al.*, 2002a; Brown *et al.*, 2007). On the other hand, overexpression of these antioxidant enzymes have shown to have a protective role against forms of vascular diseases (Widder *et al.*, 2009).

Uncoupling proteins (mainly UCP1 and UCP2) are localized inside inner mitochondrial membrane and play an important role in ROS production. The main function of UCPs is to promote the uncoupling of electron transport chain leading to reduction of mitochondrial membrane potential and ATP production (Duval *et al.*, 2002; Mailloux and Harper, 2011). UCP1 is mainly expressed in brown fat, whereas UCP2 is the main endothelial isoform that regulates the mitochondrial membrane potential when they get activated by oxidative stress in

the form of superoxide ion and function to reduce the ROS (Echtay *et al.*, 2002; Echtay *et al.*, 2003). There is strong epidemiological evidence that UCP2 is related to human cardiovascular pathologies. 866 G > A variant is linked with overexpression of UCP2 causing a reduction in cardiovascular disease occurrence, and a polymorphism at this locus has been shown to be linked with carotid artery atherosclerosis (Oberkofler *et al.*, 2005; Cheurfa *et al.*, 2008). There has been no study so far that has examined the role of UCP2 in the regulation of endothelium-dependent vasodilation in humans, but several experimental studies have confirmed the importance of UCP2 in endothelial cells. UCP2 overexpression has been shown to decrease mitochondrial ROS and inhibit inflammatory response in cultured endothelial cells and improved vasodilation in rat aorta (Lee *et al.*, 2005). UCP2 expression is regulated by PGC-1 alpha and AMPK mediated stress response which up regulates several other anti-ROS genes (Valle *et al.*, 2005). It has been reported that UCP2 null mice has shown to have impaired and defective vasodilation and increased incidence of atherosclerosis (Moukdar *et al.*, 2009; Haines *et al.*, 2010; Tian *et al.*, 2012).

Sirtuins are involved in longevity and regulation of metabolism with control over mitochondrial function mainly through PGC-1 alpha deacetylation, and have been studied recently as potential therapeutic targets (Abdellatif, 2012; Nogueiras *et al.*, 2012). Sirtuins are NAD^+ dependent deacetylases and mainly regulate gene expression through this activity. Mammals have seven sirtuins 1-7. SIRT 3,4 and 5 are mitochondrial, SIRT 1,6 and 7 are nuclear and SIRT 2 is cytoplasmic (Guarente, 2013). Sirtuin 1 (SIRT1) deacetylates PGC-1 alpha affecting glucose and fatty acid metabolism along with the expression of several important mitochondrial genes (Vega *et al.*, 2000; Rodgers *et al.*, 2005; Gerhart-Hines *et al.*, 2007; Abdellatif, 2012). Sirtuin 3 (SIRT3) is a mitochondrial sirtuin which is specifically expressed in mitochondrial matrix and involved in regulation of mitochondrial function by deacetylating mitochondrial enzymes and importantly MnSOD, thereby decreasing superoxide levels in mitochondria and contributing towards antioxidative mechanisms (Onyango *et al.*, 2002; Tao *et al.*, 2010). Sirtuins have been known to play an important role in cardiovascular system. SIRT1 has been shown to prevent ischemic heart injury and hypertrophy of cardiac muscle and promotes angiogenesis by deacetylating and activating eNOS in the endothelium (Mattagajasingh *et al.*, 2007; Potente *et al.*, 2007). It has been interesting to note that endothelial SIRT1 expression was found to be decreased in older subjects progressing towards aging and was correlated with reduced endothelium dependent vasodilation (Donato *et al.*, 2011). SIRT1has been shown to regulate glucose metabolism by

controlling insulin secretion, gluconeogenesis and glycolysis (Abdellatif, 2012). It inhibits the transcription of uncoupling protein 2 (UCP2) thus regulating insulin secretion from pancreatic β -cells (Moynihan *et al.*, 2005; Bordone *et al.*, 2006). SIRT3 is a recently identified downstream target of PGC-1 alpha and although no studies on humans or animal models have so far examined the role of SIRT3 activation on endothelial phenotype, there have been reports that up regulation of SIRT3 is found in vascular tissues where inhibition of transcription factor NFκB has shown to reduce inflammatory activity and improved mitochondrial biogenesis and life span (Hasegawa *et al.*, 2012). In our study, we investigated for the mitochondrial SIRT, SIRT4 and found that it showed expression in ECFCs as well as HUVEC. ECFCs did not show a significant change in its expression when cells were grown in hyperoxia, whereas HUVEC showed a significant change in its expression when grown in hyperoxic conditions. This suggests that SIRT4 is activated in HUVEC and these cells undergo fatty acid oxidation in response to oxidative stress.

In conclusion, the results obtained in this section showed that ECFCs and HUVEC behaved differently when exposed to oxidative stress and as shown by the gene expression analysis, different genes are activated in ECFCs as compared to HUVEC. As shown in figure 6.7, PGC-1 alpha showed a significant increase in expression in ECFCs when exposed to hyperoxia, whereas HUVEC did not show a significant change in expression. This suggests that HUVEC use a different pathway to combat stress conditions. PPARG has been shown to be co-activated by PGC-1 alpha in cardiomyocytes and here we show that in ECFCs there was a significant up regulation of PPARG in ECFCs that could be linked to the activation of PGC-1 alpha. HUVEC did not show a significant change and it could be due to no significant change in PGC-1 alpha expression in HUVEC grown in hyperoxia. PROX1 has been shown to be playing a role in the development of lymphatic endothelial cells and is known to be a negative modulator of ERR-α/PGC-1 alpha pathway. Our results showed that there was a down regulation of PROX1 gene in ECFCs grown in hyperoxia and this suggests that this could be related to an increase in PGC-1 alpha gene expression. HUVEC did not show a significant change in expression. PRDX5 is a mitochondrial gene that has been shown to be involved in reduction of hydrogen peroxide playing a role in antioxidative mechanisms. Our results did not show any significant change in expression of this gene in either ECFCs or HUVEC suggesting this gene is not activated in oxidative stress in these cells.

As shown in figure 6.8, we investigated the genes involved in electron transport chain of oxidative phosphorylation. We checked for a gene from each step of this process and the

results showed that there was an up regulation of all these oxidative genes namely MT-ND3, MT-CYB, MT-CO1 and MT-ATP8 in ECFCs grown in hyperoxia. This suggests that this could be due to the activation of PGC-1 alpha causing an increase in mitochondrial biogenesis and oxidative phosphorylation. HUVEC showed an insignificant decrease in expression of all these genes suggesting they are not activating electron transport chain of oxidative phosphorylation.

As shown in figure 6.9, we investigated several mitochondrial genes that are involved in fatty acid oxidation and gluconeogenesis in liver and muscle cells namely SIRT4 and PCK2. We found that there was significant up regulation of these genes in HUVEC that were exposed to hyperoxia suggesting that these cells activate metabolic pathways in stress conditions. ECFCs did not show a significant change in expression of these genes. Our results showed that there was an up regulation of PDK4 gene in HUVEC grown in hyperoxia. This gene is involved in glucose metabolism and did not show a significant change in ECFCs grown in hyperoxia. GPX4 gene is involved in protecting cells against oxidative stress by the reduction of hydrogen peroxide. We found a down regulation of this gene in ECFCs exposed to hyperoxia suggesting that may be other isoforms of GPX are activated in response to oxidative stress in ECFCs. HUVEC did not show a significant change in expression.

As shown in figure 6.10, we investigated the effect of oxidative stress on several cell surface markers. This included vWF which as shown earlier showed high expression in HUVEC and is involved in platelet adhesion. High endothelial vWF expression suggests atherosclerotic progression and as seen in the figure HUVEC showed significant increase in its expression when grown in hyperoxic conditions. ECFCs did not show any significant change in expression. CD144 is involved in maintaining vascular permeability and shows decreased expression in early stages of angiogenesis which is in correlation with increased vascular permeability observed in angiogenesis (Corada *et al.*, 2001). As shown in the figure, ECFCs showed a significant decrease in its expression when exposed to hyperoxia suggesting that these cells are angiogenic and exposure to oxidative stress leads to inflammatory and atherosclerotic environment promoting vascular repair. HUVEC did not show a significant change in the expression. CD31 mainly shows expression in endothelial cells and as shown in figure 6.10 there was a down regulation of its expression seen in ECFCs grown in hyperoxia. HUVEC did not show a significant change in expression of CD31. CD133 is mainly expressed early endothelial progenitors and hematopoietic stem cells. ECFCs showed insignificant change in its expression when grown in hyperoxia. HUVEC showed a

statistically significant change in CD133 expression and this could be further investigated as till now the function of CD133 is not known.

As shown in figure 6.11, we investigated several genes that encode growth factors involved in promoting angiogenesis. These included VEGF A and C that are known to be involved in angiogenesis, mitosis of endothelial cells and their migration. The results showed that there was a significant increase in the expression of both VEGFA and C in ECFCs exposed to hyperoxia suggesting that these cells become angiogenic when exposed to oxidative stress. HUVEC did not show a significant change in expression of VEGFA but showed a significant decrease in VEGFC expression suggesting that these cells do not promote angiogenesis. FLT1 and KDR are VEGF receptors and both showed a significant decrease in expression in ECFCs grown in hyperoxia and it could be due to the filling of receptors as both VEGFA and C showed a significant increase in expression. HUVEC did not show any significant change in expression of either FLT1 or KDR.

As shown in figure 6.12, we investigated some genes involved in proinflammatory response including CXCL12 (SDF-1) that has been shown to be a proinflammatory genetic marker that is up regulated in atherosclerosis (Linsel-Nitschke *et al.*, 2010). Our results showed that there was a significant increase in expression CXCL12 in ECFCs grown in hyperoxia suggesting that oxidative stress has led to an atherosclerotic environment. HUVEC did not show any significant change in expression. FGF2, PDGFRA and PDGFRB were the other genes that we investigated and the results showed that there was an up regulation of all these markers in ECFCs grown in hyperoxia suggesting that these cells promote angiogenesis when exposed to oxidative stress. HUVEC did not show any significant change in expression.

As shown in figure 6.13, we investigated several pro angiogenic target genes involved in blood vessel formation. This included ANGPT1which encodes a growth factor involved in vascular development. ECFCs showed a significant increase in its expression when grown in hyperoxia suggesting that these cells are promoting angiogenesis. HUVEC did not show any significant change in expression. ANPT2 acts as an antagonist to ANGPT1 and shown in figure ECFCs showed a significant decrease in its expression when grown in hyperoxia. This suggests that the oxidative stress has led to an atherosclerotic environment and ECFCs are promoting vascular repair as shown by up regulation of ANGPT1 and down regulation of ANGPT2. We also investigated for ACTA2 which is involved cell motility and forms the contractile machinery of the cell. ECFCs showed a significant increase in its expression and

HUVEC did show any significant change in expression. NOS3 is considered to be the main source of NO which is involved in smooth muscle regulation. It is also an inhibitor of electron transport chain at complex IV which is inversely related to oxygen levels. Our results showed that exposing cells to hyperoxia resulted in decreased NOS3 expression in ECFCs. HUVEC did not show a significant change in expression.

As shown in figure 6.14, we checked for the gene expression of transforming growth factor alpha (TGF-α) and the results showed no significant change in expression in ECFCs grown in hyperoxia. HUVEC showed a significant increase in its expression when exposed to hyperoxia. This requires further investigation as TGF alpha is known to be up regulated in several cancers. TP53 showed an insignificant decrease in ECFCs grown in hyperoxia but showed a significant up regulation in HUVEC exposed to hyperoxia. This gene is up regulated in oxidative stress and DNA damage and inhibits angiogenesis. Up regulation in HUVEC again confirm that HUVEC do not take part in angiogenesis when exposed to oxidative stress. TEK encodes angiopoeitin 1 receptor. It is mainly expressed in endothelial cells but both ECFCs and HUVEC did not show any significant change in the expression of this gene when grown in hyperoxia. NRP2 is known to interact with VEGF and is known to play a role in cardiovascular development. Our results showed that both ECFCs and HUVEC showed insignificant change in expression of this gene.

As shown in figure 6.15, we checked for the gene expression of IL-1alpha which encodes an inflammatory cytokine mainly produced by endothelial cells. ECFCs showed a significant decrease in its expression and HUVEC showed statistically significant increase in the expression of IL-1 alpha. This again suggests that both ECFCs and HUVEC behave differently when exposed to oxidative stress. KIT is a proto-oncogene expressed in hematopoietic stem cells and myeloid progenitors and our results showed that ECFCs expressed KIT but it was down regulated when these cells were exposed to oxidative stress. This suggests that may be ECFCs grown in hyperoxia were differentiated towards a more mature lineage and lost their stem cell characteristics. As expected, HUVEC did not show the expression of KIT gene and there was no significant change in expression when grown in hyperoxia. ABGC2 (CD338) has been shown to be suppressed during DNA methylation and ECFCs showed significant down regulation in ECFCs grown in hyperoxia. This could be the effect of oxidative stress on these cells. HUVEC did not show any significant change in expression. ITPKB gene is involved in immune cell functions and our results indicate that

there was a significant down regulation seen in ECFCs grown in hyperoxia. HUVEC did not show any significant change in expression.

The results in this section clearly indicate that ECFCs and HUVEC behave differently when exposed to oxidative stress. ECFCs mainly promote angiogenesis and up regulate angiogenic markers suggesting that this stress environment resembles that of inflammatory and pro atherosclerotic conditions that promotes these cells to become angiogenic. HUVEC did not show up regulation of angiogenic markers suggesting that these cells do not become angiogenic on exposure to oxidative stress and do not take part in vascular repair.

Chapter 7. General discussion and limitations

The research work carried out in the study includes successful isolation of mononuclear cells (day 0 MNCs) from umbilical cord blood also known as human umbilical cord mononuclear cells (HUCBC), based on ficoll based separation referred to as lymphoprep (n >15). These MNCs were used in subsequent experiments including qPCR and FACS analysis. They were used as a calibrator or reference sample for low density array and validation experiments. We have been able to isolate early EPCs or CACs both from peripheral blood and umbilical cord blood from more than 15 samples. Macrophages have been successfully isolated from both peripheral blood and cord blood n >10. Endothelial colony forming cells (ECFCs or late EPCs) have been successfully derived from umbilical cord blood (n >10) by differentiating cord blood mononuclear cells (day 0 MNCs) in an *in vitro* system. Mature endothelial cells (HUVEC) have been successfully isolated from over 15 human umbilical vein samples. We have successfully attempted the isolation of all the cell populations from the same donor. Cord blood mononuclear cells (day 0 MNCs), early EPCs (proangiogenic cells), ECFCs (endothelial colony forming cells) and macrophages all were derived from the umbilical cord blood of the donor whose umbilical cord was used for the isolation of mature endothelial cells (HUVEC) from the umbilical vein n=6. This has therefore provided us with a longitudinal comparison of the m RNA expression of hematopoietic stem cell and endothelial cell surface markers from mononuclear cells (day 0 MNCs) through to mature endothelial cells of the same donor for the first time. It was difficult to get an endothelial colony from each cord blood isolation and the success ratio was less than 50%. There could be different reasons to explain this low success rate and include age of the donor, anaesthetics used in the delivery, circadian influences, (early morning births or night deliveries), nutritional influences, fasting, caffeine levels, vitamin D status; ethnicity of donor and many more. We have carried out the molecular profiling of these cells based on the expression of cell surface markers including hematopoietic stem cell, macrophage specific and endothelial markers. The results showed that early EPCs can easily be distinguished from ECFCs and mature endothelial cells (HUVEC). The difference found was statistically significant. Early EPCs were found to be related to monocyte-macrophage lineage and showed expression of macrophage specific markers. These findings have been confirmed at both RNA and protein level and were consistent with the previous findings as shown in earlier studies. Our results further showed that endothelial colony forming cells (ECFCs) derived from cord blood and mature endothelial cells (HUVEC) derived from the umbilical vein of the same donor can

also be distinguished on the basis of expression of cell surface markers namely CD144, CD105 and vWF, and the difference was found to be statistically significant (n=3-5). The difference was investigated further at protein level, and we performed direct immunofluorescence analysis with vWF antibody as it is an internal marker. The results confirmed that ECFCs and HUVEC derived from the same donor showed statistically significant difference (n=3) in terms of number of vWF positive cells when compared at same passages (P5 for both ECFCs and HUVEC and P15 for both ECFCs and HUVEC). FACS analysis was carried out for cell surface marker including CD144, CD146, CD14 and CD34. This way protein expression was confirmed and compared in MNCs to the expression in ECFCs and HUVEC based on the percentage of positive cells. This comparison at mRNA and protein level clearly distinguished early and late EPCs and we narrowed down our approach to use of late EPCs (ECFCs) which have been used for vascular repair and compared with mature endothelial cells. The early EPCs were distinguished from ECFCs at mRNA and protein level and addition of a functional assay like *in vitro* matrigel assay could have further validated the results.

The second part of the study involved studying the behavior of these late EPCs (ECFCs) and mature endothelial cells (HUVEC) under hyperoxic conditions (40% O_2) which has been shown to be a model of mild oxidative stress. We proposed that oxidative stress would lead to telomere dysfunction and as a result of that a decrease in the expression of PGC-1α that has been shown recently in mice cells. We proposed that PGC-1 alpha being a key metabolic regulator would be involved in mitochondrial biogenesis and oxidative metabolism in endothelial cells, and a decrease in its expression would lead to an impairment of mitochondrial activity in these cells. This would in turn lead to a decrease in the angiogenic potential of these cells. We first studied the growth kinetics of these two cell types in conditions of normoxia and hyperoxia. The results showed that ECFCs were found to be much more resistant to stress conditions as compared to mature endothelial cells (HUVEC). They grew at a faster rate and were able to survive in oxidative stress conditions (40% O_2) for longer period of times. The next step was to investigate the mRNA level expression of PGC-1 alpha and its downstream targets in both ECFCs and HUVEC under normoxic and hyperoxic conditions. The results showed that instead of going down PGC-1 alpha levels increased in both ECFCs and HUVEC and also there was an increase in expression of its downstream targets G6- phosphatase and ERR-alpha which are known to be involved in glucose metabolism and angiogenesis respectively. The difference found was not statistically

significant (n=3-5). This suggested that oxidative stress has led to an increase in PGC-1 alpha expression and this in turn would activate antioxidative mechanisms by upregulting antioxidant genes. The next step was to confirm if oxidative stress has resulted in telomere dysfunction in these cells. The cells were grown till late passages in an *in vitro* system and were regularly split so that they are not overgrown. We first measured the telomerase activity in ECFCs and HUVEC using a sensitive TRAP assay no significant difference was found in either ECFCs or HUVEC as cells grew older and progressed towards aging. We expected to have a higher telomerase activity in these cells under conditions of oxidative stress, but again the difference found was not statistically significant. This was done to see if there was a concomitant increase in telomerase activity as a result of telomere shortening that could be the result of exposure of these endothelial cells to oxidative stress. In order to see the effect of aging on telomere length of ECFCs and HUVEC cells were grown till late passages (P2-P16) and as expected it was observed that telomere length was shortened as a result of growing these cells for long-term. The telomere length was measured using flow-FISH analysis which is an extremely sensitive and one of the most accurate methods of measuring the telomere lengths. This is a newly developed technique and we have used this assay to measure telomere lengths in these cells ECFCs and HUVEC for the first time as it has mostly been used to measure lengths of fresh blood cells mainly leukocytes. The results indicate that flow-FISH can be used as a technique to measure telomere lengths of frozen cells and cells that are old and aged and have been cultured for a longer period of time in an *in vitro* system.

The results obtained clearly showed that true endothelial progenitor cells have been isolated. They are easily distinguished from early EPCs on the basis of expression of macrophage marker CD14 and leukocyte marker CD45. This was important as the EPCs isolated and cultured were not a mixture of monocyte-macrophage lineage cells as these cells are resistant to oxidative stress and would have made it difficult to analyse the results. The results also indicated that ECFCs are better equipped to combat oxidative stress as compared to mature endothelial cells and could be grown for longer periods. On the basis of these findings we designed a low density gene array that could differentiate these cells further on the basis of expression of angiogenic or antioxidative markers or genes that are involved in DNA damage repair and electron transport chain complexes so that we can conclude on the basis of expression these gene if these two cell types behave differently under conditions of oxidative stress and perform different functions when exposed to stress.

The array results confirmed that these endothelial progenitors which are known to take part in vascular repair are much more resistant to stress environment as they home in to the sites of vascular injury and take part in vessel repair. There are several antioxidative genes that are up regulated in ECFCs when exposed to stress conditions and increased ROS production as evident from up regulation of genes involved in electron transport chain (ETC). ECFCs show increased mRNA expression of several genes that are involved in angiogenesis, including several growth factors that are important in initiating and maintaining new blood vessel formation. Similarly, in terms of metabolism ECFCs and HUVEC show differential gene expression analysis when compared with normoxic conditions. The overall results further validate our earlier findings that these are two different cell types that behave differently under conditions of oxidative stress. This is confirmed at both levels with functional assay including the growth kinetics and gene expression analysis at mRNA level using LDA and protein analysis using immunofluorescence and FACS.

Limitations of the study:

The main part of the research study was carried out in cells that were derived from the same donor. This provided a better comparison, so that the mononuclear cells (MNCs) from the same donor were differentiated in to early EPCs and macrophages by growing in different media but under same culture conditions. The ECFCs were isolated once a colony was observed which normally takes around 3 weeks to appear and in certain cases there was no colony formation from the culture so the results were excluded from the final analysis and only those cultures were included in which all cell types were isolated from the same donor. This reduced the sample size to around n=3-6 as all the mRNA gene expression level experiments were carried out as same donor analysis.

Similarly, for a comparison between early and late passage cells for telomerase activity and telomere length measurements the cells were grown for a period of around 2-3 months in order to achieve significant results. This again was time consuming but was inevitable.

In the earlier qPCR experiments, the data were normalized to one housekeeping gene (TBP) but later on for the LDA and array validation experiments this was compensated by data normalization to three housekeeping genes (18s RNA, TBP and HGPRT).

In direct immunofluorescence analysis with vWF (FITC) antibody, isotype matched control was used but that was not tagged with a fluorochrome. The negative control used was human IgG (normal) which was a good control, but a better control would have been IgG (FITC).

There was lack of senescence assay to evaluate endothelial cell senescence and SA-β-gal staining or crystal violet staining could have been done to see if hyperoxia has led to vascular aging and senescence.

The characterization of early and late EPCs was done at mRNA and protein level but the addition of *in vitro* tube forming assays and *in vivo* matrigel plug assays for evaluating in vivo angiogenesis would have differentiated both types of EPCs and would have added strength to the gene expression studies.

8. Future work

In my opinion, ECFCs are one of the best candidates for regenerative vascular therapy. At the first place, they have this capacity to differentiate in to endothelial cell progeny and secondly they are highly proliferative cells. Human ECFCs have been successfully injected into immunodeficient mice that took part in blood vessel formation (Au *et al.*, 2008a; Melero-Martin *et al.*, 2008). More importantly, ECFCs have been shown to incorporate in damaged vasculature in vivo in various animal models including rabbit carotid artery injury, hind limb ischemia, murine retinal ischemia and porcine myocardial infarction (He *et al.*, 2004b; Hur *et al.*, 2004; Dubois *et al.*, 2010; Medina *et al.*, 2010a). This makes it more important to design and develop preclinical methods to study and evaluate the angiogenic and vasculogenic potential of these ECFCs. A detailed study of experimental models of revascularization can become a better tool to further define these cells and the pathological conditions that can be explored with this. Recent studies have shown the involvement of ECFCs in various pathological diseases including vonwillibrand disease and myeloproliferative disorders. ECFCs obtained from vonwillibrand disease patients have shown to have increased angiogenic activity (Starke *et al.*, 2011). There is a growing need for improving the methods to culture these ECFCs, as so far one of the limitations in autologous usage of these cells is their paucity in peripheral blood. More studies are required focusing on *in vitro* growth of these ECFCs and co-culturing them with other cell types mainly mesenchymal stem cells.

Endothelial senescence is an important feature of vascular aging and contributes to the vascular dysfunction, therefore, SA-β-gal and crystal violet staining can be performed to evaluate cellular senescence and proliferation in these cells under hyperoxia and that can be compared with mature endothelial cells.

Western blot analysis for SIRT1 and PGC-1α, from early to late passages, specifically enhancement of SIRT1 phosphorylation has been shown in senescent cells.

Measurement of telomere length under oxidative stress in ECFCs and HUVEC using flow-FISH can be done to establish if there is a difference between these two cell types.

Telomerase activity (TERT/TERC subunit) of ECFCs and HUVEC can be measured under oxidative stress.

Another downstream organelle of PGC-1α, the mitochondria, can be assessed under oxidative stress by quantifying mitochondrial activity and measurement of ROS production. The measurement of extracellular ROS production in an *in vitro* culture of ECFCs can be achieved by the use of compounds such as luminol and lucigenin using techniques such as electron spin resonance spectroscopy and liquid scintillation counting.

Cellular ATP production can be measured under conditions of oxidative stress by ELISA and mitochondrial membrane potential can be measured by flow cytometry using JC-1.

9. Acknowledgements

First of all, I would like to thank higher education commission of Pakistan (HEC) for giving me this opportunity to carry out this research project. I would also like to thank Newcastle University for supporting my studies for the later part of this project. I would also like to thank my supervisors for being supportive and helpful in guiding me through the difficulties of the project. Ian Dimmick provided very good support with use of FACS facilities and I would like to thank him as well.

In the end, I would like to specially thank my parents for being a constant source of inspiration and my sisters for being extremely caring and supportive.

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