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EXISTENCE OF ENDOTHELIAL PROGENITOR CELLS WITH SELF-RENEWAL AND CLONOGENIC POTENTIAL IN THE NORMAL HUMAN PLACENTA AND PREECLAMPSIA

By

Ioana Garbacea

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Dedication

I dedicate this thesis to my husband Alex and
my soon to be born son

ABSTRACT

Preeclampsia (PE) is the leading cause of maternal death in developing countries. PE is defined as hypertension and proteinuria after 20 weeks of gestation. The only curative treatment is placental delivery. Evidence suggests that placental vasculature is disturbed with shallow invasion of maternal arteries.

We hypothesize that the function of a particular type of EPCs - endothelial colony forming cells (ECFCs) are impaired in PE.

ECFCs were isolated from macro vasculature of normotensive and PE placentas. ECFCs robustly expressed CD31, CD105, CD144, CD146 and were negative for CD14 and CD45. PE-ECFCs formed less complex structures on Matrigel and gave rise to lower numbers of high proliferative potential colonies upon single cell plating compared to normotensive controls.

ECFCs exist in the human placenta. The function of PE-ECFCs is disturbed. Our data provides new insight into the pathophysiology of PE and the role of ECFCs.

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List of Symbols, Abbreviations and Nomenclature

Symbol	*Definition*
ANOVA	Analysis of variance
CAC	Circulating angiogenic cells
CFU	Colony forming unit
Dil-Ac-LDL	1,1\'-dioctadecyl – 3,3,3\',3\'-tetramethyl-indocarbocyanine perchlorate
ECFC	Endothelial colony forming cell
EPC	Endothelial progenitor cell
FACS	Fluorescence activated cell sorting
HPP	High proliferative potential
HUVEC	Human umbilical vein endothelial cell
IFN	Interferon
LPP	Low proliferative potential
MNC	Mononuclear cell
NT	Normotensive
P #	Passage number
PBS	Phosphate buffer solution
PE	Preeclampsia
SEM	Standard error mean
UEA-1	Ulex Europaeus Agglutinin -1

Chapter 1.

Introduction

1.1. Preeclampsia – a major health care problem

Preeclampsia complicates approximately 8% of all pregnancies worldwide, causing significant maternal and neonatal mortality. It claims the lives of more than 60,000 mothers each year in developing countries making it the leading cause of maternal death[1]. Preeclampsia (PE) is defined as *de novo* hypertension with proteinuria after 20 weeks of gestation, with reversal of hypertension and proteinuria after delivery. PE is further classified into early onset, between 20 and 34 weeks, and late onset, after 34 weeks[2]. Severe cases can develop in both early and late PE and can lead to the development of HELLP syndrome (Hemolysis, increased Liver Enzymes, Low Platelet levels), seizures and fetal growth restriction. However, regardless of the clinical picture, the placenta of term pregnancies has minimal histo-pathological changes compared to premature deliveries[3].

The exact etiology of this pregnancy-specific disease is still unclear. Possible associations with PE are autoimmune disorders, blood vessel problems, diet and genes. The presence of certain risk factors like first pregnancy, multiple gestation, obesity, age greater than 35 years old, personal history of diabetes, high blood pressure and kidney disease can increase the rate of development. Infants born to affected mothers face a 5-fold increase in death rate and have a greater risk of developing cardiovascular disease later in life, as do their mothers[4-6]. Women with chronic hypertension have an additional 15-25% increase in the risk

of developing PE during their pregnancies[7]. A positive maternal and/or paternal family history of preeclampsia also increases the likelihood of developing this complication[8].

Delivery of the placenta is the only known cure for PE and is indicated when maternal risks outweigh risks to the fetus, thereby increasing the incidence of prematurity. Some extremely premature babies do not survive, and the ones that do, need prolonged mechanical ventilation to help them survive. Another major complication is fetal growth restriction due to poor placental perfusion[9].

Further research in the pathophysiology of PE is crucial in order to develop a cure and decrease the high mortality and morbidity risks that lie with the mother and the infant.

1.2. Normal placental development

Birth of a healthy fetus is dependent upon normal placental development. Placenta is the organ that connects the developing fetus to the uterine wall, allowing nutrient uptake, waste elimination and gas exchange via the mothers blood supply. As a consequence, any disturbance in placental formation may lead to a premature birth and increase in perinatal morbidity and mortality.

The morfo-functional unit of the placenta is the cotyledonary villi that later form a branching villous structure lined by two layers of cells, cytotrophoblast and syncytiotrophoblast. The latter is a multinucleated, external, continuous layer of cells that covers the entire surface of the villous placenta and

provides an impermeable barrier to maternal blood. The cytotrophoblast is the inner layer of mononuclear cells that eventually can expand and unite with neighboring villi forming the cytotrophoblast shell, from which they migrate and later remodel the maternal spiral arteries. During the remodeling process, the cytotrophoblastic cells replace the vascular wall smooth muscle cells, releasing the vessel from neuro-vascular control. Vascular tone mediators, like nitric oxide and prostaglandins, do not control the vessel dynamic anymore and thus high-resistence, narrow blood vessels become dilated, high-capacitance vessels. This process is the result of uterine embryonic implantation and increased demand of the growing fetus. The main changes taking place are vasodilatation, increased vascular permeability, trophoblast invasion of maternal spiral arteries, and uterine angiogenesis[10].

Establishment of the placental vascular system is comprised of cell proliferation and differentiation along major steps that lead to the formation of placental villi. At first, the primary villi appear around day 13 post-conception and are formed of a massive core of cytotrophoblast covered by a thick layer of syncytiotrophoblast. At day 15, connective tissue cells start to appear beneath the cytotrophoblast layer and hemangiogenic progenitor cells start forming string-like aggregates called “hemangiogenic cords”, completing the secondary villi stage. Finally, endothelial tube formation takes place between day 21 to 32 and blood vessels can be noticed within the villous stroma, resulting in the formation of tertiary villi. This is the time when vasculogenesis is first observed and the

endothelial cell surface marker CD31 is detected within the villi. In close relation, angiogenesis follows vasculogensis and both lead to the final formation of the villous tree[11,12]. Vasculogenesis, formation of *de novo* blood vessels, is responsible for the initial connection between the placental and embryonic vascular beds and continues until 10-12 weeks of gestation. Angiogenesis, formation of new blood vessels from already existing ones, is responsible for expanding the placental vascular system, first through elongation and then by lateral sprouting of the capillaries.

At present, only ubiquitous angiogenic factors have been described. From those, major players are the vascular endothelial growth factor (VEGF) and the placental growth factor (PIGF)[13]. The role of progenitor cells, and especially endothelial colony forming cells, during vasculogenesis and angiogenesis in the placental development is still not fully understood.

1.3. Pathophysiology of preeclampsia

Research studies referring to PE, even though extensive, have not improved the methods of prediction, prevention and treatment of this disorder [14-16]. The exact etiology of PE to date remains unknown and several pathophysiologic processes have been investigated: immune response, poor placentation, systemic endothelium dysfunction, and inefficient placental growth factors [17].

Some studies support the theory of poor placentation with inadequate blood supply to the fetus. PE develops in women predisposed to this complication by hormonal release, endothelium dysfunction and alterations in inflammation process[18].

Another theory is the abnormal maternal immune response and the insufficiency in the gestational immune tolerance. One of the main changes in PE is the switch towards a pro-inflamatory Th-1 response and production of IFN-γ[19]. In addition, there is a theory of a micro-chimerism process, a cell migration process between mother and fetus. Fetal cells were shown to be increased in the maternal circulation in PE women compared to normotensive ones. The immune theory supports the idea of a preliminary placental injury like hypoxia that leads to increased passage of fetal material into the maternal circulation. Defective immune and inflammatory response in combination with increased number of fetal cells in the maternal circulation leads to the development of PE[19].

Major players that can also contribute to hypoxia and placental growth restriction are soluble fms-like tyrosine kinase-1 (sFLT-1 or sVEGFR-1), vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) antagonist[20]. In PE there is an imbalance between these circulatory factors with decreased VEGF levels and increased anti-VEGF factors (sFLT-1 and soluble endoglin. [21]) which guides the vascular development against angiogenesis and potentially inhibit uterine arteries invasion. Thus, the placental vascular tree ends up having low numbers of vessels, with a narrow caliber. These changes potentially lead to development of PE later in pregnancy. sFLT-1 and soluble

endoglin even if both are up-regulated during normal pregnancy, they have an increased level in PE and in addition, they increase with the severity of PE. These are just few of the factors implicated in the development of PE, as placenta has a wide range of organ-specific hormones and growth factors.

Placenta has a central and crucial role in the development and remission of PE, as the disorder resolves once the placenta is delivered. For example, women with hydatidiform mole are at the same risk of developing PE as normal pregnant women, even though there is no fetus present and PE remitted with curettage and removal of the mole[22]. In postpartum cases with eclampsia where there was placental tissue retained into the uterine cavity, the condition resolved with uterine curettage[23]. In addition, in a case of ectopic pregnancy complicated by PE, the disorder did not resolve after removal of the fetus and symptoms persisted until the placenta was delivered[24].

Severe PE is associated with pathologic evidence of placental hypo-perfusion and ischemia. Findings include acute atherosis, a lesion of diffuse vascular obstruction that includes fibrin deposition, intimal thickening, and necrosis, atherosclerosis and endothelial damage. Placental infarcts, probably due to occlusion of spiral arteries, are also commonly observed in pathological analysis of the placenta[25].

Defective placentation supports the most prominent theory of development of PE, the vascular hypothesis. In PE the transformation from high-capacitance, narrow vessels to large caliber capacitance is incomplete and is limited to the

superficial decidual area of the placenta. During normal placental development, cytotrophoblasts adopts an endothelial cell surface adhesion phenotype undergoing a vascular mimicry of the endothelium phenotype, in order to replace it[17]. In PE, cytotrophoblasts do not undergo this switch of cell-surface molecules and thus are unable to invade the myometrial spiral arteries effectively[26,27]. And thus the uterine vessels remain under central nervous system control leading to a fluctuating blood flow, and whilst deficiency of oxygen to the fetus[28]. As a consequence, a "hypoxia-reperfusion injury" results, revealing at macroscopic level small placentas, chronic placental ischemia, placental infarcts and thin umbilical cords.

Regardless of the PE pathophysiologic theory, when taken individually, neither of them was proven to constantly cause PE. This makes PE a multi factorial pregnancy complication and that much challenging to diagnose. This also stresses the need for a better diagnostic method and a treatment option in the clinical setting, with less risk on the life of the fetus and the mother.

1.4. Stem cells in human placenta

Regenerative medicine is a new research field that aims at developing strategies to maintain, enhance or restore the function of tissues or organs compromised by disease or injury. One of the most prominent strategies is the use of stem cells. Although they hold great promise they have several limitations, which would need to be addressed in order for effective therapies to be developed. For example, embryonic stem cells are ethically charged as they require the usage

of a human embryo for their processing and they are also associated with an increased risk of tumor formation. Mesenchymal stromal cells from bone marrow have an increased risk of viral infection[29] and their potency decreases with donor age[30].

Recently, the attention for obtaining easily available stem cells has been turned towards the placenta. As placenta is a temporary organ that originates during the first steps of embryological development it is thought that it contains cells that retained their plasticity from the early embryonic cells and that also have some immunomodulatory characteristics.

To date there are several studies on stem cells isolated from the human placenta. However, the different names and characterization methods used, make it hard to compare these studies. “Placenta derived multi-potent cells” were shown to exhibit mesechymal surface markers like CD105/endoglin/SH-2, SH-3, and SH-4, as well as embryonic stem cell markers like SSEA-4, TRA-1-61, and TRA-1-80 [31]. In another study, maternal origin placenta derived mesenchymal stem cells showed an increased and prolonged proliferation potential, maintaining their stemness for several passages[32]. The anatomical origin of placenta isolated stem cells varies across the board from amnion[33] and amniotic fluid[34], to decidua basalis[35] and chorionic membrane[36]. No consensus has been reached to date on a clear definition or characterization of placenta derived stem cells.

1.5. Endothelial progenitor cells (EPCs): an enigmatic population with therapeutic potential

Recent insights into the biology of EPCs that have the capacity to form new blood vessels and the potential to contribute to vascular repair, open new therapeutic avenues for the understanding of placental vascular development.

The main process during vascular embryonic development is vasculogenesis. It implies spontaneous de novo blood vessel formation under the stimulation and contribution of angioblasts[37], endothelial cell precursors. Once the vascular system is matured, new blood vessels arise by sprouting of endothelial cells from post capillary venules (angiogenesis), or by maturation and de novo development of collateral arteries from vessels with a large diameter (arteriogenesis)[38] .

In vasculogenesis "blood islands" are formed and upon long-term culture these island give rise to vascular structures in vitro[39]. Analysis of angioblasts and hematopoietic stem cells (HSC) revealed that they shared several antigenic markers, among which, the most important ones are CD34, Flk-1 and Tie2. CD34 is a glycol-phospho-protein expressed on stem cells and "lineage-specific progenitor cells" suggesting a role in early blood cell differentiation[40]. Flk-1 is a receptor of vascular endothelial growth factor (VEGF).Flk-1 and CD34 are expressed by all HSC, but lost in their differentiation process[41].

Angiogenesis is a process characteristic of mature organisms that contributes to growth and development, wound healing and granulation tissue formation, as well as tumor formation. Vasculogensis, on the other hand, is a

process that until 1997 was thought to pertain exclusively to intrauterine development. Asahara et al. challenged this theory, when they purified a population of circulating cells that displayed properties of both endothelial cells (EC) and progenitor cells[42]. Termed “endothelial progenitor cells” (EPCs), they would give rise to differentiated ECs in a process called “post natal vasculogenesis”. The ability to isolate a circulating cell that appears endothelial-like *in vitro* and has the potential to incorporate at sites of neoangiogenesis *in vivo*, spawned a new field of investigation.

In the process of isolating EPCs from peripheral blood there have been identified three sub-populations: colony-forming units (CFU), circulating angiogenic cells (CACs) and endothelial colony a forming cells (ECFCs).

CFU cells are cultured from a heterogeneous population of peripheral mononuclear cells (MNCs) enriched in CD34 or VEGF receptor -2 (VEGFR-2)[42]. Colonies are composed of round cells in the center and spindle-shaped cells in the periphery.

As well as CFUs, CACs are isolated from peripheral blood MNCs [43]. CACs promote neovascularization under ischemic conditions, such as myocardial ischemia and hind limb ischemia. Their number and migration potential is decreased in hypertension, diabetes and in smokers[44]. CACs do not form spindle-shaped colonies as CFUs. However, because both sub-populations give rise to colonies within 3-7 days in culture they were grouped into the “early outgrowth EPCs”. In addition, both sub-populations express surface markers typical for monocyte/macrophage lineage and endothelial markers[45-47].

The least studied of EPCs are the endothelial colony forming cells (ECFCs). They display a late outgrowth pattern, with colonies appearing after 10-21 days of culture[48]. Colonies have a specific cobblestone-like appearance on phase-contrast microscope. This EPC sub-population has an increased potential to give rise to clonal outgrowth of cells after single cell plating[49]. ECFCs are phenotypically indistinguishable from mature endothelial cells, but in culture ECFCs have a robust proliferative potential. ECFCs are able to form secondary and tertiary colony upon re-plating, and have the ability to form de novo blood vessels in vivo when transplanted into immune-deficient mice[48-50]. Because of these abilities, ECFCs were proposed to be THE true endothelial progenitor cells.

1.5.1. Potential role of EPCs in normal pregnancy

Few studies have been conducted on circulating EPCs in normal pregnancy. One of the studies concluded that pregnancy is a condition that promotes the mobilization of maternal origin EPCs. Early outgrowth EPCs were present in both pregnant and non-pregnant women, whereas the late-outgrowth EPCs were only identified in 60% of pregnancies[51]. A study done on 20 pregnant women showed that CFU numbers increase with progressing pregnancy[52]. In contrast a study of 36 CFU peripheral blood samples from pregnant women concluded that circulating EPCs decreased with progressive gestational age[53]. The fetal or maternal origin of EPCs was not consistently assessed in these two last studies.

One recent study focusing on placental resident ECFCs, found that there was no difference in *in vitro* function compared to umbilical cord blood ECFCs. However, upon *in vivo* blood vessel formation, resident ECFCs were significantly more potent[54].

Because there is still no definite characterization method for these EPC sub-population, the studies used different methods to identify and quantify EPCs, thus preventing a rigorous comparison between them.

1.5.2. Role of EPCs in preeclampsia

EPCs have been analyzed in several pregnancy related conditions. In gestational diabetes, circulating early outgrowth EPCs are decreased compared to matched controls[55-57]. In the case of fetal intra-uterine growth restriction, not associated with any other pregnancy condition, umbilical cord blood early outgrowth EPCs were decreased in number and had a longer differentiation time and increased senescence[58].

The EPC impairment in PE follows a similar pattern. Peripheral blood CFUs are decreased in number and they have an increased senescence rate compared to matched controls[59]. A study supported these findings with CFUs isolated from the umbilical cord blood. They showed that the proliferation, migration and vasculogenic abilities of PE - CFUs were decreased compared to controls[60]. In addition, early outgrowth EPCs from umbilical cord of preeclamptic pregnancies had a longer differentiation time and fewer colonies in vitro. Another study found an increased EPC proliferation capacity but no

significant difference in the number of peripheral blood EPCs of normal and preeclamptic pregnancies.

In more recent studies, EPCs were quantified by flow cytometry. EPCs co-expressing CD34/VEGFR-2 or CD133/VEGFR-2 were increased and positively correlated with advancing pregnancy[61,62].

However, the isolation and characterization methods were not consistent between studies and thus it makes it difficult to rigorously compare them.

1.6. Overall hypothesis and significance

The main objective of this thesis is to explore whether the late outgrowth EPCs, ECFCs, exist in the normal human placenta. Further, in chapter 3, we investigate the function of ECFCs in preeclamptic placentas compared to normotensive ones as means to develop new insights into the normal and abnormal development of human placental vasculature.

To date, no study looked into the presence of ECFCs in the micro and macro vasculature of normal human placenta. In addition, the function of resident placental PE-ECFCs has not been analyzed.

The present study brings forward a novel approach to the impaired placental vascular development in PE and the potential role late outgrowth EPCs might have in this pregnancy-specific complication.

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Chapter 2

Functional differences between placental micro and macro vascular endothelial colony-forming cells

- The present chapter will soon be submitted for publication

2.1. INTRODUCTION

The placenta is the organ that connects the developing fetus to the uterine wall, allowing nutrient uptake, waste elimination and gas exchange via the mothers blood supply. Vascular adaptation to pregnancy is comprised of maternal uterine arteries remodeling and fetal placental development. The later takes place through different processes. Vasculogenesis, the process of *de novo* blood vessel formation, takes place in the first weeks of pregnancy and by the 4^{th} week a defined lumen can be identified[63]. Angiogenesis, the process of blood vessel formation by sprouting of preexisting vessels, succeeds vasculogenesis and continues to take place untill term, helping with the constant vascular adaptation to fetal needs[12]. Any impairment in the vascular development can lead to poor placentation and a decreased blood flow to the fetus. Two prominent pregnancy complications associated with poor placentation and hypoxia are gestational diabetes and preeclampsia.

Preeclampsia is a pregnancy complication defined as *de novo* hypertension and proteinuria arising after 20 weeks of gestation, with reversal of hypertension and proteinuria after delivery. Preeclampsia complicates 8 % of all pregnancies worldwide. It significantly increases maternal and neonatal morbidity and mortality. Preeclampsia causes more than 60,000 maternal death each year in developing countries[1,6]. It is also associated with low birth weight and intrauterine fetal growth restriction[64]. It thus becomes of major concern, as there is no definitive prediction, prevention or treatment strategy to date[14,15].

Thus, more insight into the components of placental vascular development

is necessary in order to identify and influence major playing factors in preeclampsia and also to gain further understanding of the normal physiology of placenta.

Although vasculogenesis was thought to pertain to the embryonic development stage, in 1997 a new population of adult circulating cells was described to have properties of both endothelial and progenitor cells. Termed endothelial progenitor cells (EPCs), they were proven to give rise to differentiated endothelial cells in vivo and incorporate at sites of injury and repair[42,65]. In the process of isolating EPCs from peripheral blood, 2 subpopulations of EPCs can be identified: early outgrowth EPCs that contain colony forming units (CFUs) and circulating angiogenic cells (CACs); and late-outgrowth EPCs – namely endothelial colony forming cells (ECFCs)[48]. CFUs are cultured from a peripheral MNC population and display colonies with round cells in the center and spindle-shaped at the periphery. The early outgrowth EPCs express both macrophage and endothelial surface markers and upon long-term culture they do not develop into mature endothelial cells. However, the late – outgrowth EPCs do have this ability. They form specific endothelial cobblestone-like colonies after 10-21 days in culture and have an increased clonogenic potential [49,50]. When transplanted to immune-deficient mice ECFCs are able to form *de novo* blood vessels [49,50,66].

Few studies have explored the role of circulating EPCs during normal pregnancy and the use of different characterization methods

makes it difficult to compare their results. The existence and role of placental resident EPCs is still unknown.

We hypothesize that late outgrowth EPCs exist in the normal human placenta and that their function differs with location within the placenta.

2.2. MATERIALS AND METHODS

All procedures were approved by the Human Research Ethics Board of the University of Alberta.

2.2.1. Study population

Term pregnant women were recruited at the moment of admission to the hospital. Written informed consent was obtained before collection of placenta. We further selected the samples by collecting cesarean sections placentas. For experimental analysis 16 placental samples were used- 8 for isolation of micro-vasculature and 8 for isolation of macro-vasculature. The clinical characteristics of the two groups are shown in *Table 2.1*.

We excluded pregnant women who were known to have a history of chronic hypertension, diabetes, renal disease, cardiovascular disease, hepatic disease, infections (defined as fever and premature rupture of membranes and/or established bacterial infection), autoimmune disorders or other significant pre-existing metabolic disorders, recent history of illicit drug use, and current multiple gestation and fetal malformation.

2.2.2. Collection of placental micro-vasculature

Immediately after delivery, placentas were sent to the laboratory for isolation within 90 minutes post-partum. The amnion was removed and placenta placed with the maternal side up. Three blocks of tissue were collected from 3 random cotyledons by cutting out 1/3 of the total placental thickness. The 3 blocks were placed in sterile 1x Phosphate Buffered Saline (PBS) and set aside.

2.2.3. Collection of placental macro-vasculature

Within 10 minutes after delivery placenta was collected and placed with the maternal side up. After removal of amnion, 1/3 of the placental depth was removed using scissors. Then, placenta was turned with the fetal side up. Through the umbilical cord, 50 mL normal saline was injected into the placenta in order to flush the proximal vessels. Using a scalpel 3 blocks of tissue were removed from 3 random places. The tissue samples were placed in sterile normal saline and transported to the laboratory where the placental vessels were cleaned from the adjacent tissue and put in sterile 1x PBS. The isolation protocol was started within 90 minutes post-partum (*Figure 2.1*)

2.2.4. ECFC isolation and culture

After tissue collection, both micro and macro vasculature were finely chopped using the McIlwain Tissue Chopper. The tissue was then suspended in collagenase/dispase digestive solution (0.1 U collagenase & 0.8 U dispase/mL) (Roche Applied Science, Laval, OC) at 37^{o} C for 1 hour with intermittent shaking. Equal volume of Dulbecco's Modified Eagle Medium (DMEM) with 10 % fetal

bovine serum (FBS) was added and the digested sample was strained through 100 μm and 70 μm sterile cell strainers. The obtained cell suspension was centrifuged at 300 g, 10 minutes , at 4^{o} C and re-suspended in sterile 1x PBS with 0.1 % bovine serum albumin (BSA). CD31 coated dynabeads were added and the mix was rotated for 30 minutes at 4^{o} C, followed by magnetic separation. The positive selected cells were plated in 6 well plates previously coated with rat tail collagen type I at different plating densities- starting at 400,000 cells/ well and down to 1500 cells/well. Endothelial basal medium-2 (EBM-2) was supplemented with EGM-2 singlequots (Lonza, Basel, Switzerlad), 10% FBS and 1% penicillin/streptomycin and amphotericin B (PSF) and this mix was used as the endothelial growth medium for the entire culture process. Media was changed every day for the first 7 days and every other day for the rest of the culture from this point on. ECFC colonies were identified morphologically at day 14-21 and isolated using glass rings. The colonies were further plated in T25 flask and later in T75 flasks.

2.2.5. Seeding density

For both micro and macro vascular ECFCs, upon plating them at passage 0, we used decreasing seeding density. We started at 400,000 cells/ well and continued down to 1500 cells/well using the limited dilution method.

2.2.6. ECFC characterization

2.2.6.1. Immunophenotyping by FACS.

Once expanded, at passage 4-6, ECFCs were characterized by identifying endothelial specific surface markers, previously described for ECFC[66].

Cells were trypsinized and then washed twice with flow buffer (1x PBS containing 0.05% sodium azide and 0.1% BSA). Later, using 150,000 cells/tube, ECFCs were incubated with appropriate concentrations of primary antibody, for 1 hour, in dark at 4^{o}C. Cells were washed twice and re-suspended in 300 µL flow buffer and analyzed by FACS (FACS Canto, BD Biosciences) using the BD Biosciences DIVA software. Antibodies used were : CD14 (BD Pharmingen FITC mouse anti-human CD14, Cat. No. 555397), CD45 (BD Pharmingen FITC mouse anti-human CD45, Cat. No. 555482), CD31 (BD Pharmingen PE mouse anti-human CD45, Cat. No. 555446), CD105 (Abcam PE mouse anti-human CD105, Cat. No. ab91238, CD144 (E-Bioscience PE anti-human CD144, Cat. No. 12-1449-80), CD146 (BD Pharmingen PE mouse anti-human CD45, Cat. No. 550315)

2.2.6.2. Endothelial specific staining

Further, both micro and macro vascular ECFCs were assessed for their capacity to incorporate endothelial specific staining: Dil-Acetylated Low Density Lipoprotein (Dil-acLDL) and *Ulex Europaeus Agglutinin-1* (UEA-1), as previously described[66].

2.2.6.3. ECFCs clonogenic assay

Pure micro- and macro-vascular ECFCs, from passages P5-8, were used to assess and compare their capacity to form colonies when plated at a single cell density. Cells were sorted and plated in 96 well plates in triplicates using FACS Aria cell sorter (BD Biosciences, Mississauga, ON). Media was changed every other day and wells were analyzed at day 14. The number of cells/well that a single cell was able to generate classified the wells according to a previously decribed hierarchy[48,67] into low proliferative potential (LPP) colonies with 2-50 cells, 50-500 cells and 500 -2000 cells/well sub-groups, and high proliferative potential (HPP) colonies, with more than 2000 cells/ well, A high proliferative well was subsequently trypsinized and used for the second-generation clone formation – where cells were plated again at a single cell level in 96 well plates. Media was changed every 2 days and wells were analyzed at day 14 as described above. Cells were kept at 37^{o}C, 5% CO_2 in a humidified incubator.

2.2.6.4. Cord formation in vitro

For each micro- and macro- vascular ECFCs, 3 wells were used in a 96 well plate. After plating 40μL Matrigel in each well, 10,000 cells were seeded in each well and placed in incubator. Cells were analyzed at 6-8 hours for their cord-like formation. At this point, using a 10x magnification, 3 different fields were chosen randomly and used for cord length and intersection measurement using OpenLab software (Quorum Technologies Inc., ON, Canada)

2.2.6.5. ECFC - genetic analysis

In order to establish the origin of micro- and macro- vascular ECFCs, we used ECFCs coming from pregnancies with male fetuses. We looked for the presence of the Y chromosome using the following markers: sY127, sY84, sY255, sY86, SRY, sY134, sY254 and for the presence of the X chromosome using the ZFY marker. We did 3 multiplex PCR reactions, using fluorescently labelled primers. The pooled PCR products were run on the AB 3130 Genetic Analyzer. A normal male, a normal female and a no template control were run.

Statistical Analysis. Values are expressed as the mean ± SE. Statistical comparisons were made with the use of ANOVA. A value of $p<0.05$ was considered statistically significant.

2.3. RESULTS

2.3.1. Limited dilution assays determines optimal seeding density.

In order to establish the seeding density that yields the most number of viable ECFC colonies, we used the limited dilution assay. At day 14 we observed that by seeding 100,000 cells/well, we could get well-defined colonies, with low number of contaminating cells. Conversely, higher seeding densities gave rise to over confluent wells and lower densities to fewer or no ECFC colonies (*Figure 2.2*).

2.3.2. Both micro and macro vascular ECFCs were positive for endothelial surface antigens.

Both ECFC populations strongly expressed endothelial specific cell surface antigens: CD31, CD105, CD144, CD146 and neither of them expressed the hematopoietic cell specific CD45 or monocyte/macrophage marker CD14 (*Figure 2.3-A 2.3-B*). The robust expression (>95%) of the positive antigens and extremely low expression (<0.5 %) of CD45 and 14, also confirmed the presence of a pure ECFC population with no contaminating cells.

2.3.3. ECFCs incorporated Dil-acLDL and UEA-1.

Both populations of micro- and macro-vascular endothelial cells showed basic endothelial cell characteristics such as ingestion of Dil-acLDL and binding to *Ulex europaeus*-lectin (*Figure2.4-A, 2.4-B*).

2.3.4. Macro vascular endothelial cells gave rise to highly proliferative colonies.

14 days after single cell plating in 96 well plates we classified the wells as low proliferative potential (LPP) or high proliferative potential (HPP) wells. Wells with 2-2000 cells were split in 3 sub-groups (2-50, 50-500 and 500-2000 cells) and considered to have a LPP, whereas the wells with more than 2000 cells were considered to have a HPP. In the first and second generation, we compared HUVECs, our control cells, with macro and micro ECFCs. HUVECs had significantly lower percentages of HPP wells compared to macro vascular ECFCs

and no difference compared with micro vascular ECFCs. With a 40% HPP wells, macro vascular ECFCs were significantly more potent compared to micro vascular ECFCs. (*Figure 2.5-A*). Upon re-plating, the second generation ECFCs gave rise to HPP colonies following the same pattern. HUVECs were less proliferative compared to macro vascular ECFCs, but not significantly different compared to micro vascular ECFCs. In addition, macro vascular ECFCs maintained their increased proliferation potential compared to micro vascular ECFCs. (*Figure 2.5-B*).

2.3.5. Macro vascular ECFCs are able to form more complex networks in vitro.

When plated on Matrigel, both on gross examination and on quantitative study, HUVECs formed significantly more intersections compared to micro vascular ECFCs, but with no significant difference from macro vascular ECFCs. However, macro vascular ECFCs had markedly more intersections than micro vascular ECFCs (*Figure. 2.6-A*). By measuring cord length on matrigel, we noticed that HUVECs formed significantly lengthier cords compared with both macro and micro vascular ECFCs, with there being no difference between the last two groups (*Figure 2.6-B*)

2.3.6. Macro vascular ECFCs are of fetal origin, whereas the micro vascular ECFCs are of maternal origin.

In order to analyze the origin of the two isolated ECFC populations, we used ECFCs from pregnancies with male fetuses. We noticed that macro vascular ECFCs expressed the X chromosome marker (ZFY) as well as the Y chromosome markers (sY127, sY84, sY255, sY86, SRY, sY134, sY254) leading us to the conclusion that ECFCs from the macro-vasculature are of fetal origin (*Figure 2.7*). Conversely, the micro-vascular ECFCs only expressed the X chromosome marker and none of the Y chromosome, showing that these cells are of maternal origin.

2.4. DISCUSSION

ECFCs were present in both the micro and macro vasculature of human placenta. Isolated from two distinct locations in the placenta and having different origin, they displayed different functional abilities. Fetal, macro vascular ECFCs were more potent as they gave rise to the highest percentage of HPP wells and more complex networks on Matrigel compared to the maternal, micro vascular ECFCs.

EPCs have been identified and isolated for more than a decade now. From the three subpopulations of EPCs (CFUs, CACs and ECFCs) the ECFCs have been the least analyzed. This could be due to the fact that they are present in low number especially in the peripheral blood of healthy adults, and also because they do not have a definite characterization to date. However, because ECFCs are

considered to be the true progenitor cells and following a previously proposed ECFC characterization protocol, we proposed to investigate their presence and role in the vasculature of normal human placenta.

During the isolation process, the micro and macro vascular ECFCs were always processed from different placentas. Even though this might constitute a selection bias, technically we were not able to process both isolations at the same time. This is considering the fact that we established a 2-hour deadline for placental processing and that the initial handling was slightly different for the two isolation protocols.

In designing our experiment, we proposed to analyze the placental micro and macro vasculature. Upon performing the genetic analysis of the macro and micro vascular ECFCs, we showed that they have different origins. Macro vascular ECFCs pertain to the fetus with a XY phenotype and micro vascular ECFCs to the mother with a XX phenotype. Considering the fact that human placenta develops from fetal cells[68], we can speculate that these XX-ECFCs could have been isolated from the maternal blood trapped around the placental villi. Another theory, would be the micro-chimerism where there is transfer of maternal cells to the fetus.[69]. However, the reverse cell migration from the fetus to the mother's circulation is more frequent[70-72].

By redefining the groups as maternal ECFCs and fetal ECFCs we investigated their phenotype and function. Both groups showed comparable surface antigen expression and endothelial specific stain incorporation. During the functional assays, clonogenic assay and capillary like formation on Matrigel, we

showed that fetal ECFCs are more potent. Through both first and second generation, fetal ECFCs maintained their increased proliferative potential by generating mostly colonies with more than 2000 cells. The significantly increased number of intersections and cord length compared with the maternal ECFCs further proved the increased potency of fetal ECFCs. One speculation would be the higher reproductive state and potency of fetal cells as they are creating and sustaining the developing fetus. Another would be that the resource of ECFCs lies in the bigger vessels, upstream, and when signaled they can travel at the tip of the capillaries and branch the existing vessel or create new ones.

The few studies conducted on EPCs in pregnancy focused mainly on the early outgrowth EPCs. Two studies showed opposite results referring to an increasing or decreasing number of circulating early outgrowth EPCs with gestational age[52,53]. However, results could not be compared because of the different characterization method used. One study shows that the late outgrowth EPCs, the ECFCs are present in the peripheral blood of pregnant women and absent in non-prenancy, without analyzing their function[51]. A more recent study comparing placental resident ECFCs with umbilical cord blood ECFCs showed that there was no functional difference *in vitro*. However, upon *in vivo* blood vessel formation, the resident ECFCs were significantly more potent compared to the umbilical cord blood ECFCs[54]. This group used a similar method of characterization and functional assessment *in vitro* as our present study, thus making them comparable. In addition to this recent study, we showed the fetal

origin of the resident placental ECFCs and the fact that they are more potent compared to maternal origin ECFCs.

In summary, we showed that resident ECFCs exist in the human placenta and that by isolating them from two different anatomical parts we obtained two different ECFC populations – fetal and maternal. The fetal ECFCs being more potent are the cells to be looked at in the future studies. Because pregnancy complications like preeclampsia or gestational diabetes have a vascular etiology, it might be that these ECFCs are impaired and prevent the proper development of the fetal villi that in consequence influences the maternal-fetal exchange barrier. Similarly, the maternal ECFCs could be impaired as well, by influencing the uterine arteries remodeling.

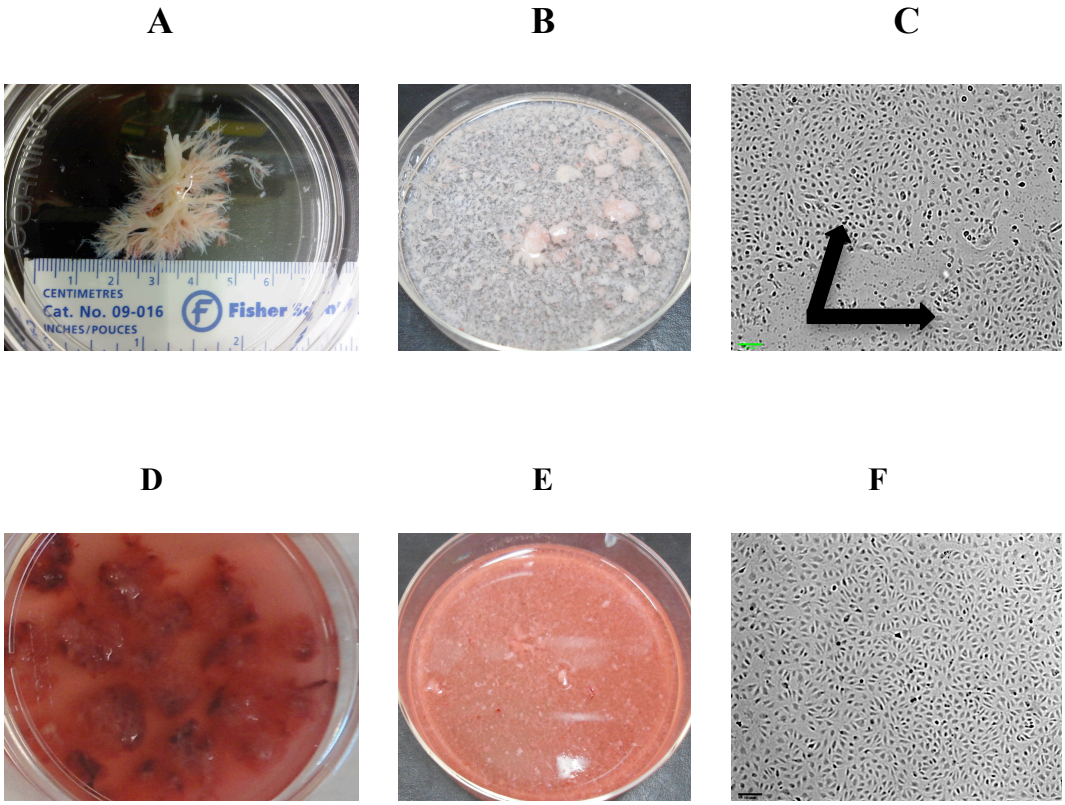
In conclusion, highly proliferative ECFCs with self-renewal capacity exist in the normal human placenta. ECFCs from the macro vasculature are of fetal origin and ECFCs from the microvasculature are of maternal origin. Investigation of these ECFCs during placental pathology such as preeclampsia may lead to a better understanding of the disease process in order to develop new therapies.

Table 2.1. Clinical characteristics of macro and micro vascular ECFC groups

	Macro vascular ECFCs n=8	**Micro vascular ECFCs n=8**	**Significance**
Maternal age (years)	31 ± 4.3	29 ± 4.1	NS
Gestational age at delivery (weeks)	38.9 ± 0.6	39 ± 1.1	NS
Infant birth weight (grams)	3641.2 ± 828.5	3513.7 ± 369.7	NS

*Values are the mean ± SE; NS= not significant

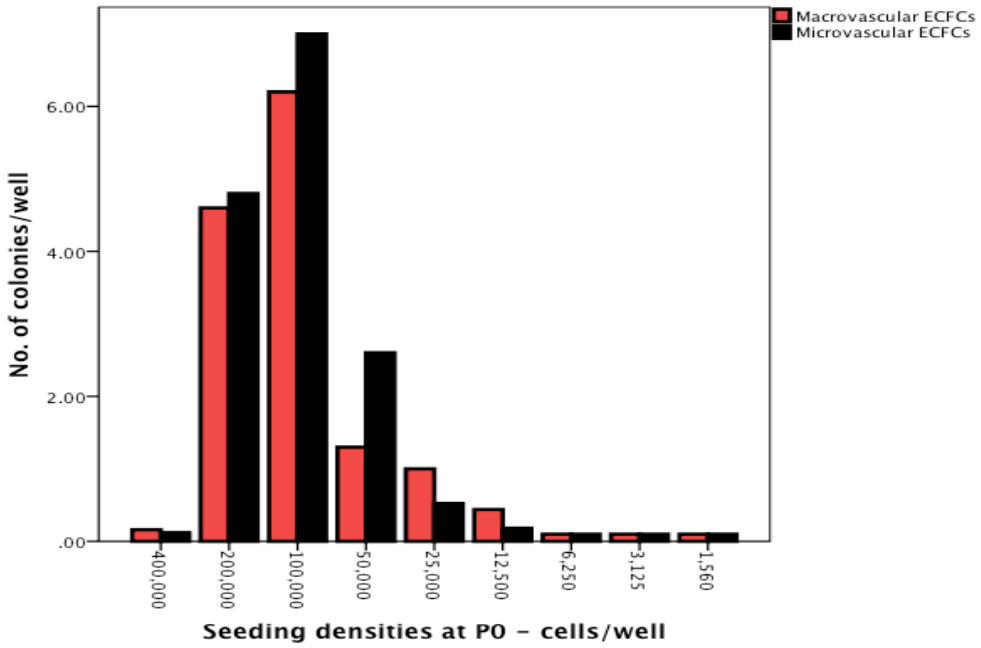
Figure 2.1. Isolation process of macro and micro vascular ECFCs



A, B, C - Macro vascular ECFCs: isolation: (A) placental villi that was previously flushed with normal saline and cleaned of adherent tissue. (B) chopped villi placed in collagenase/dispase for digestion; (C) ECFC colonies with cobblestone appearance at day 14 in culture (10X magnification);

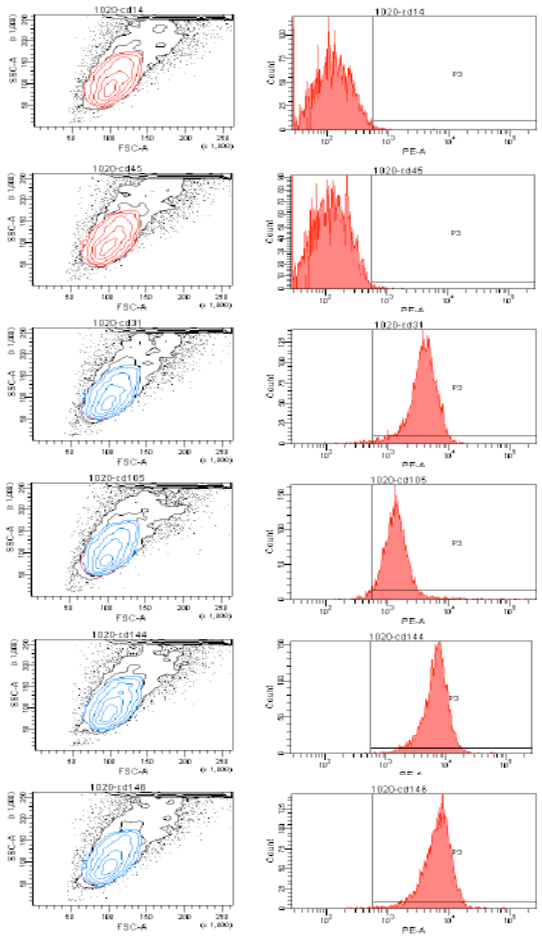
D, E, F - Micro vascular ECFC isolation: (D) 1 cm^2 blocks from the 1/3 distal placental tissue, previously washed in PBS; (E) Chopped tissue in collagenase/dispase for digestion; (F) ECFC colonies with cobblestone appearance at day 14 in culture(10X magnification)

Figure 2.2. – Limited dilution assays determines optimal seeding density



100,000 cells yielded the most number of colonies. We counted only colonies that can be isolated for further passage, excluding the ones at the periphery of the well, as well as the contaminated colonies.

Figure 2.3-A – Representative FACS analysis of macro vascular ECFCs



CD 14 (-)

CD 45 (-)

CD 31 (+)

CD 105 (+)

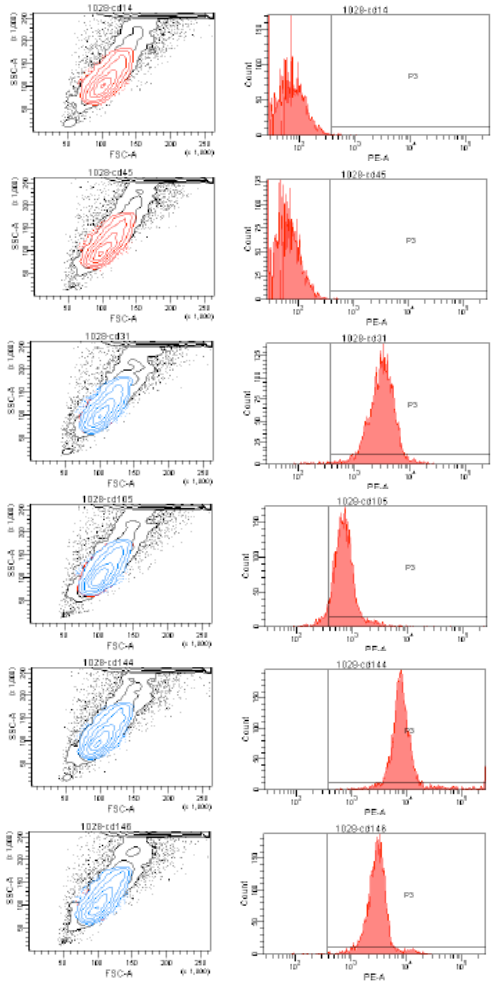
CD 144 (+)

CD 146 (+)

Representative data from 8 different ECFC isolations

ECFCs stained positive for the endothelial specific cell surface antigens CD31, CD105, CD144, CD146 and negative for the hematopoietic antigen CD45 and the macrophage/monocyte CD14.

Figure 2.3-B - Representative FACS analysis of micro vascular ECFCs



CD 14 (-)

CD 45 (-)

CD 31

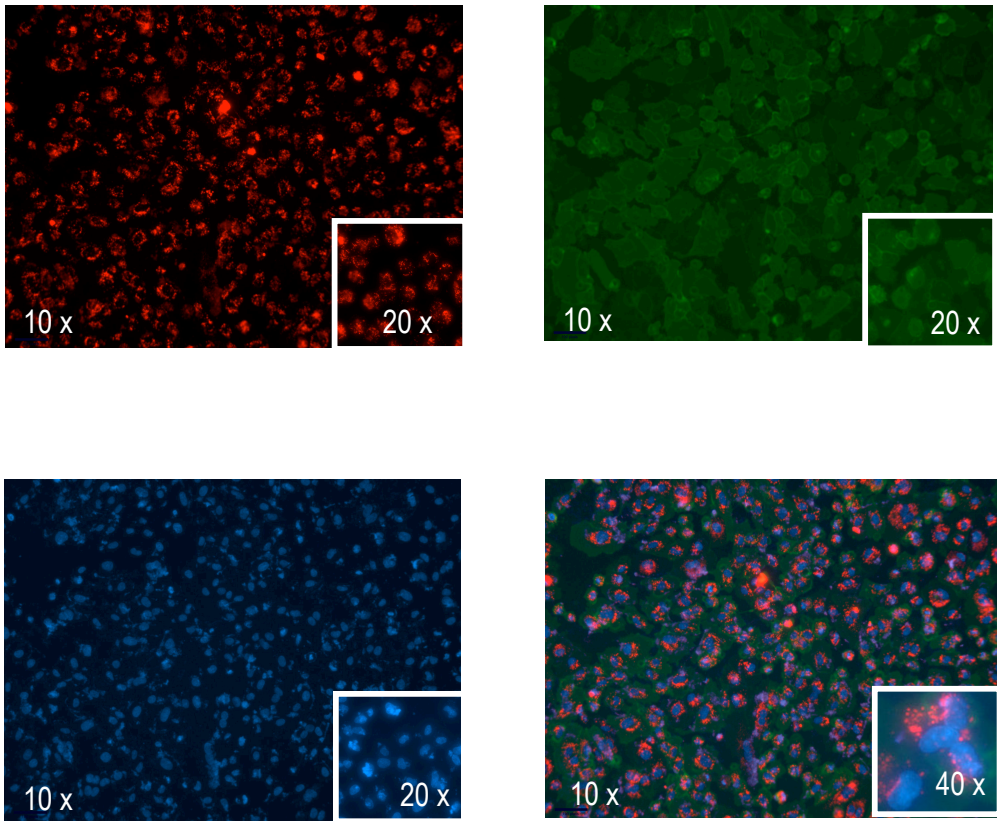
CD 105 (+)

CD 144 (+)

CD 146 (+)

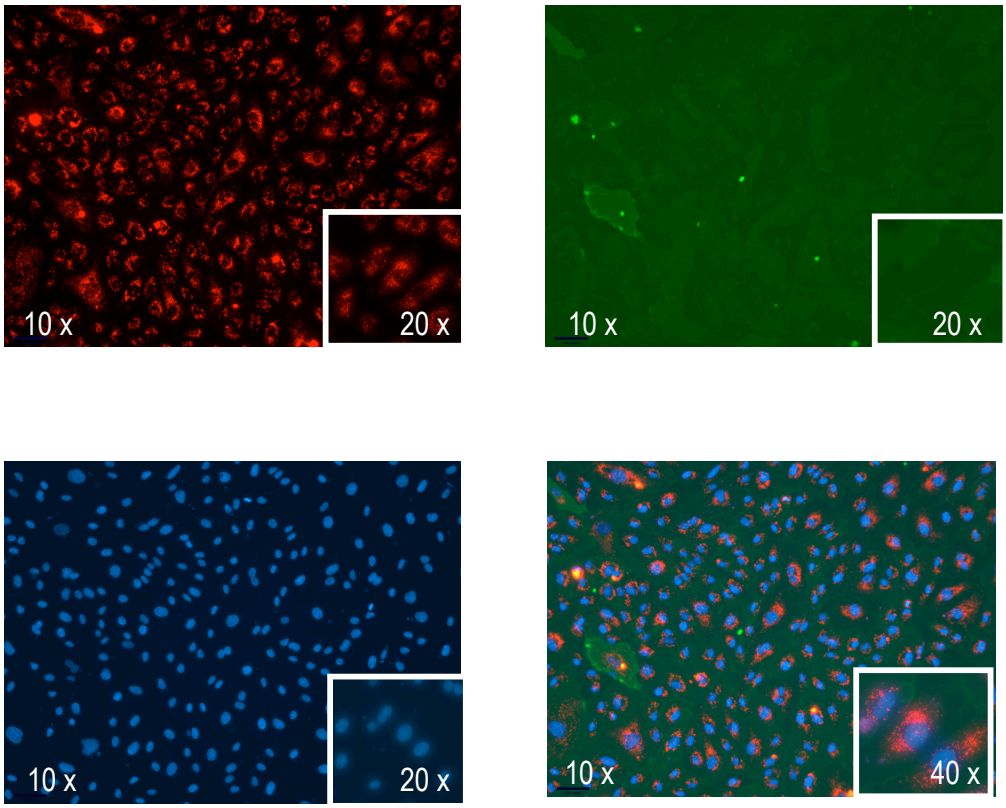
ECFCs intensely express endothelial markers CD31, CD105, CD105, CD 144 and do not express CD45 and CD14.

Figure 2.4-A – Macro vascular ECFCs incorporated Dil-acLDL and UEA-1



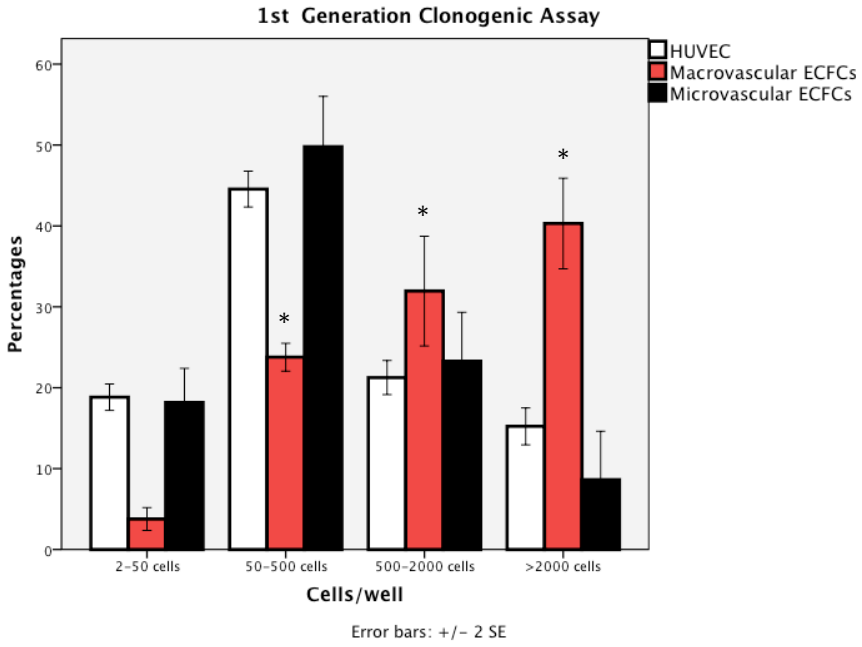
Cells were observed under phase contrast microscopy at 10X magnification. Macro vascular ECFCs incorporated the typical endothelial stains DiL-Ac-LDL and UEA-1 (red and green stain) as well as Hoechst (blue stain)

Figure 2.4-B – Micro vascular ECFCs incorporated Dil-acLDL and UEA-1



Micro vascular ECFCs stained similar to macro vascular ECFCs – triple staining for DiL-Ac-LDL, UEA-1 and Hoechst. Pictures were captured with 10x, 20x and 40 x magnification.

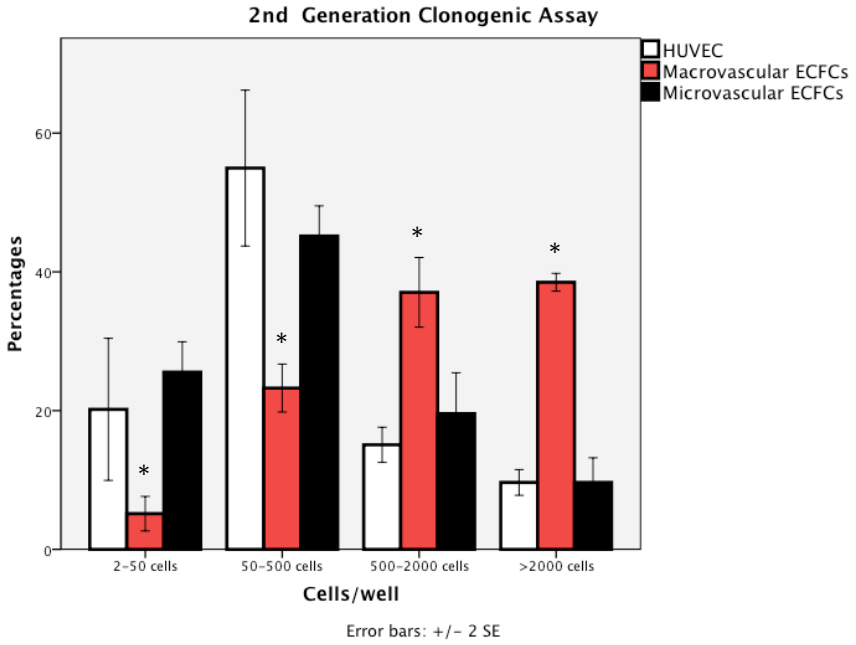
Figure 2.5-A - Functional assessment of clonogenic potential-1st generation



First clone generation at day 14 from single cell plating. Macro vascular ECFCs were consistently and significantly more potent than HUVECs and micro vascular ECFCs in generating HPP colonies. There was no significant difference between HUVECs and micro vascular ECFCs, both generating the most number of colonies in the range of 50-500 cells from the LPP group.

Results represent the mean value ± SE of 8 individual experiments. * $p<0.001$- compared to both HUVEC and microvacular ECFCs in that particular category (by Univariate Anova test)

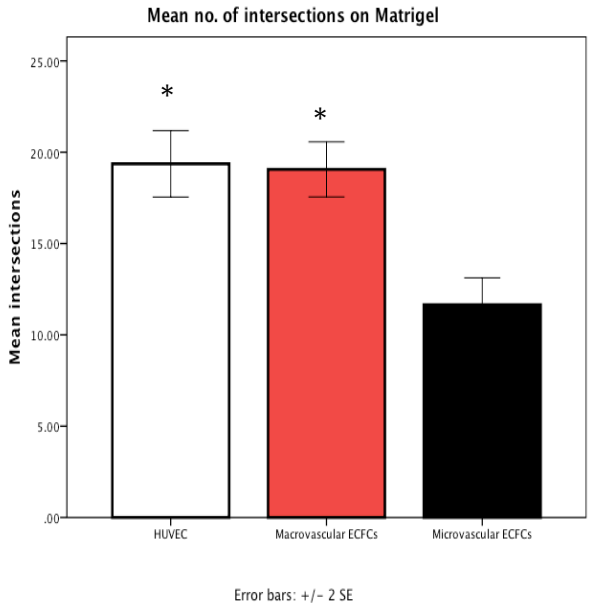
Figure 2.5-B - Functional assessment of clonogenic potential – 2nd generation



Second generation of ECFC clones maintained the pattern of the first generation with the macro vascular ECFCs generating significantly mote HPP colonies from one single cell at day 14. Still with no significant difference, HUVECs and micro vascular ECFCs generated mostly 50-500 cell colonies.

Results represent the mean value ± SE of 8 individual experiments. * $p<0.001$- compared to both HUVEC and micro vascular ECFCs in that particular category (by Univariate Anova test)

Figure 2.6 – A - Capillary like formation in vitro - intersections

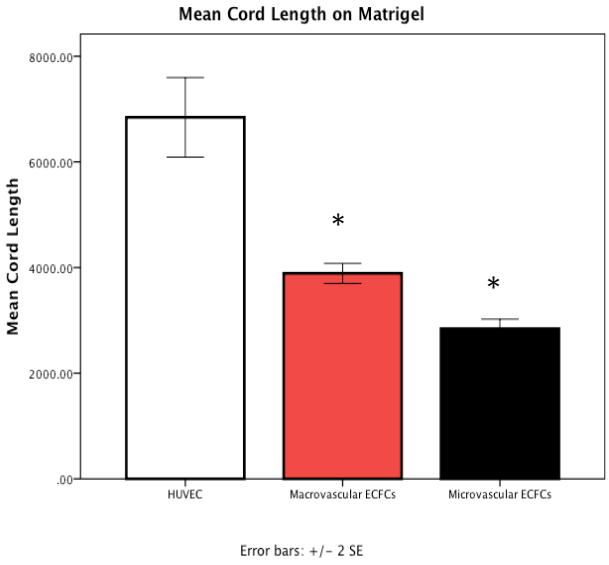


HUVECs and macro vascular ECFCs form significantly more intersections on Matrigel compared to micro vascular ECFCs. There was no difference in intersection formation between HUVEC and macro-vascular ECFCs**.**

Results represent the mean ± SE of 8 individual experiments.

* $p<0.001$- compared to micro vascular ECFCs (by Univariate Anova test)

Figure 2.6-B - Capillary like formation in vitro – cord length



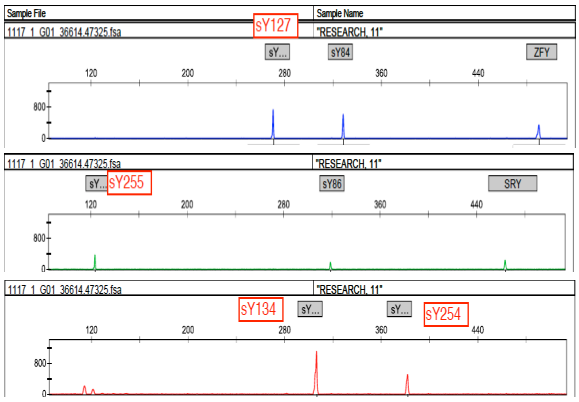
Upon counting of the cord length, HUVECs had significantly longer cords compared with both macro and micro vascular ECFCs. There was no significant difference between the macro and micro vascular ECFCs.

Results represent the mean ± SE of 8 individual experiments.

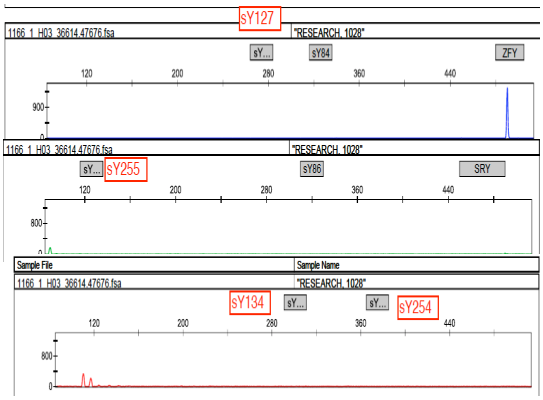
* $p < 0.001$ compared to HUVECs (by Univariate Anova test)

Figure 2.7 - Genetic analysis of macro and micro vascular ECFCs

Macro vascular ECFCs



Micro vascular ECFCs



Gene legend:

X- Chromosome gene: SFY

Y- Chromosome genes: sY127, sY84, sY255, sY86, SRY, sY134, sY254

Macro vascular ECFCs demonstrated a positive signal for both the X the Y chromosome genes, which shows their XY phenotype and thus the fetal origin.

Micro vascular ECFCs had a positive signal only for the X chromosome gene, proving their XX phenotype and the maternal origin.

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Chapter 3

Existence of endothelial progenitor cells with self-renewal and clonogenic potential in the normal human placenta and preeclampsia

3.1. INTRODUCTION

Pregnancy induced hypertension is a common finding. About 10% of pregnant women will have an abnormal blood pressure recording before delivery. Mild hypertension alone yields the same outcome as women with normal blood pressure. Once blood pressure becomes very high and/or proteinuria develops, the outcome deteriorates. Preeclampsia (PE) is defined as hypertension that develops after 20 weeks of gestation and is associated with proteinuria[73]. PE affects 2-8 % of pregnancies worldwide[74] and is a major cause of maternal and fetal death[1]. It can lead to maternal liver, brain or kidneys disorders and it can affect the blood clotting system. Serious but rare complications of PE include seizures, stroke, hemolysis with high liver enzymes and low platelets (HELLP syndrome). The fetus is at risk of intrauterine fetal growth restriction, as a consequence of poor placental blood flow, and of prematurity either because a spontaneous premature labor or because of an early delivery to protect the mother. In addition to these short-term PE complications, mothers and infants carry a long-term risk for developing cardiovascular disease later in life and an increased death rate[4-6]. The only current available treatment for PE is placental delivery. Fetal delivery only will not clear PE[22-24].

Placental vascular development starts at day 13 post conception with the primary villi and by day 32 tertiary villi are formed and first blood vessels are noticed. At this time vasculogenesis, the formation of *de novo* blood vessels is the main vascular process. Angiogensis, sprouting of blood vessels from already

existing ones, follows closely and defines the final vascular branching system of the placenta[11,12,38].

The exact etiology of PE is still poorly understood. From the proposed pathophysiologic theories- immune, genetic and vascular, the last one is the most prominent. The vascular hypothesis of PE supports the idea of poor placentation, shallow invasion of maternal arteries, and as such an overall poor vascular development of placenta[25]. An improvement of the vascular branching could prevent or treat PE.

Endothelial progenitor cells (EPCs) are an understudied cell population. Until 1997 it was thought that vasculogenesis is a process restricted to intrauterine development. However, since then, EPCs were isolated from both adult peripheral blood and umbilical cord blood [42,65,66,75]. Upon isolation, EPCs yields 3 sub-populations: early outgrowth EPCs comprised of colony forming units (CFUs) and circulating angiogenic cells (CACs); and late outgrowth EPCs named endothelial colony forming cells (ECFCs). ECFCs are thought to be the true progenitor cells, as they are the only ones with the capacity to give rise to clonal cells and to form new blood vessels *in vivo* and *in vitro*. In addition ECFCs, in contrast with CFUs and CACs, develop into mature endothelial cells.

Limited studies conducted on EPCs in normal pregnancies showed a proportional increase of peripheral early outgrowth EPCs with gestational age[52]. However, there was a study that concluded the opposite[53]. Because of different methods used to characterize and measure the cell number, it is hard to compare these studies.

The main findings associated with preeclampsia were that early outgrowth EPCs are decreased and are more senescent compared with matched controls. In addition, they had a longer differentiation time and formed fewer colonies in culture[59,60].

To date there is no study conducted on the late outgrowth EPCs, the ECFCs, in either normal human placenta or in PE. Considering that ECFCs are the true progenitor cells, we hypothesized that their function is impaired in PE placentas compared with normotensive controls.

3.2 MATERIALS AND METHODS

All procedures were approved by the Human Research Ethics Board of the University of Alberta.

3.2.1 Study population

Pregnant women going for caesarian section were recruited at the moment of admission to hospital. We excluded women that had a history of chronic hypertension, diabetes, renal disease, cardiovascular disease, hepatic disease, infections (defined as fever and premature rupture of membranes and/or established bacterial infection), autoimmune disorders or other significant pre-existing metabolic disorders, recent history of illicit drug use; and current multiple gestation and fetal malformation.

We considered for the control group, women that had a normal range blood pressure (normotensive-NT) during their pregnancy and that had none of

our exclusion criteria. As our study groups, we included women that developed high blood pressure after 20 weeks of gestation and that also had proteinuria of at least 2+. Headaches and vision problems were not considered exclusion criteria if they developed in association with preeclampsia.

For experimental analysis we compared 3 NT with 3 PE placental ECFCs. Clinical profile of the two study populations is presented in *Table 3.1*.

3.2.2. Collection of placental vessels

Placentas were collected immediately after delivery in a sterile container. They were washed with normal saline on both fetal and maternal sides. With the maternal side facing up, we cut off the superficial tissue layer in order to facilitate the following step. After turning the placenta with the fetal side up, we flushed 50 mL of normal saline through the umbilical vessels. Next, we removed 3 blocks (of 1.5 cm^3 each) close to the umbilical cord insertion. For transportation, we used normal saline with 1% penicillin/streptomycin and amphotericin B (PSF). Placentas were processed in a 2-hour time interval from delivery.

HUVECs were collected from the same placentas and isolated according to a previously described protocol[76].

3.2.3. ECFC isolation and culture

Each of the 3 blocks was washed several times with sterile 1x phosphate buffer solution (PBS). Using a scalpel blade, the visible vessels from each block were cleaned of the adherent tissue. Isolated vessels were washed again several

times with sterile PBS 1x. Next, vessels were finely chopped using the McIlwain Tissue Chopper. The resultant minced tissue was mixed with collagenase/dispase digestive solution (0.1 U collagenase & 0.8 U dispase/mL) (Roche Applied Science, Laval, OC) and placed in water bath at 37^{o}C. After 1 hour, Dulbecco's Modified Eagle Medium (DMEM) with 10 % fetal bovine serum (FBS) in a 1:1 dilution with the enzyme. The suspension was then strained through 100 μm and 70 μm strainers followed by centrifugation at 300 g, 10 minutes , 4^{o}C. The supernatant was discarded and the sediment was re-suspended in 1x PBS with 0.1 % bovine serum albumin (BSA) mixed previously with CD31 coated dynabeads. After 30 minutes on rotor at 4^{o}C, we conducted a magnetic separation process. We discarded the negative selected products and plated the CD31 positive cells in 6 well plates at different seeding densities. Endothelial basal medium-2 (EBM-2) supplemented with EGM-2 singlequots (Lonza, Basel, Switzerlad), 10% FBS and 1% penicillin/streptomycin and amphotericin B (PSF) was used as a culture media that was changed every day for the first 7 days and every 2 days afterwards. ECFC colonies started to grow around day 10 and at day 14 we conducted a ring isolation step by extracting for expansion only the ECFC colonies. Cells were further expanded into T25 flask and later into T75 flasks. For characterization and functional assays we used pure ECFCs at passages 5-8.

3.2.4. Seeding density

We used decreasing seeding densities for both NT and PE groups. We started with 400,000 cells/well and using limited dilution went down to 1500

cells/well. We plated one 6 well plate for each of the densities. At day 14, before performing ring isolation, we analyzed the wells under 5x microscope magnification and counted the ECFC colonies in each well. We excluded from our counts contaminated colonies or the side-of-well colonies that were impossible to isolate for further expansion.

3.2.5. ECFC characterization

Immunophenotyping by FACS and Endothelial specific staining methods – as described in Section 2.2.6.1. and 2.2.6.2.

Functional assays: - Clonogenic assay and In vitro cord formation methods – as described in Sections 2.2.6.3. and 2.2.6.4.

ECFC - genetic analysis – as described in Section 2.2.6.5.

3.3. RESULTS

Gestational ages of the three PE-ECFC isolations were 37, 38 and 39 weeks. We used matched controls for the functional analysis of these PE-ECFCs. Representing the NT group, we used ECFCs isolated and characterized in the previous chapter (the macro vascular ECFCs). We used as control cells normotensive HUVECs (n=3) and added preeclamptic HUVECs (n=2). One HUVEC isolation failed because of a punctured umbilical cord vein and the impossibility to seize the enzyme in the vein for endothelial cell detachment.

The clinical characteristics of the two groups are presented in *Table 3.1*.

3.3.1. Limited dilution

We used the limited dilution method in order to determine the best seeding density that will yield the most number of colonies suitable for expansion. After counting the colonies at day 14 in culture, we noticed that PE-ECFCs had a markedly higher yield of viable colonies upon initial plating of 200,000 cells/well compared with lower seeding. This is in contrast with the NT-ECFCs where 100,000 cell/well was the best seeding density. (*Figure 3.1*)

3.3.2. Endothelial specific antigens – immunofluorescence characterization by FACS

After ECFC expansion, cells at passage 5 or 6 were characterized by the presence of endothelial surface antigens. Both NT and PE groups intensely expressed CD31, CD105, CD144 and CD146, and were negative for CD14 and CD45. This proves that the ECFCs have an endothelial origin and that neither pertains to a leucocyte cell family. The strongly positive expression of endothelial markers reinforces the fact that we analyzed a pure ECFC population (*Figure 3.2.*)

3.3.3. Endothelial specific staining

We stained the cells with Dil-ac-LDL first, then with Hoechst and ultimately with UEA-1. We noticed that both NT and PE ECFCs stained intensely for the endothelial specific markers Dil-ac-LDL and UEA-1. This is an additional proof that both ECFC populations have an endothelial origin (*Figure3.3-A, 3.3-B*)

3.3.4. Self-renewal and clonogenic potential assay

We considered low proliferative potential (LPP) wells that grew 2-2000 cells (sub-divided in 2-50, 50-500 and 500-2000 cells) and high proliferative potential (HPP) wells the ones with more than 2000 cells at day 14 after plating one single cell in each well.

On examination of both first and second generation we noticed a similar pattern of proliferation. When compared with normotensive HUVECs, the NT-ECFCs had significantly lower numbers of LPP, but markedly more HPP wells. There was no significant difference between PE-ECFCs and normotensive HUVECs (*Figure 3.4*)

On examination of PE-HUVEC, they had significantly fewer HPP wells compared with NT-ECFCs, with there being no difference compared to PE-ECFCs.

When comparing the two study groups, NT and PE ECFCs, we noticed that in both generations, NT-ECFCs had significantly more HPP wells. PE-ECFCs excelled the NT-ECFCs in the LPP sub-group 500-2000 cells.

3.3.5. In vitro capillary-like network formation

We used the same groups and cells that we used for the clonogenic assay. We analyzed the cord and intersection formation at the 8-hour time point. When reporting to the NT-HUVECs we noticed that they formed significantly longer *cords* compared to all the other groups-NT-ECFCs, PE-ECFCs and PE-HUVECs.

There was no significant difference in cord length between the NT-ECFCs and PE-ECFCs.

However, the number of *intersections* was markedly higher for NT-ECFCs compared to PE-ECFCs. There was also a significant difference between NT-HUVEC and PE-ECFCs. No difference was noticed in intersection formation between the NT-HUVECs and the PE-HUVECs, or between NT-HUVEC and NT-ECFCs (*Figure 3.5*)

3.3.6. Genetic analysis of NT-ECFCs and PE-ECFCs

We examined one ECFC population from each group that came from a male fetus pregnancy. We looked into X and Y chromosome genes, ZFY and respectively sY127, sY84, sY255, sY86, SRY, sY134, sY254. Both ECFC groups pertain to a fetal origin, as they express both genes from the X and Y chromosome (*Figure3.6*)

3.4. DISCUSSION

PE is a multifactorial pregnancy complication that resolves after placental delivery. In the present study we showed that HUVECs are not affected by the presence of PE and that they have a limited proliferation capacity. In addition, the data points towards a functional impairment of ECFCs, as there was a significant difference between NT- and PE-ECFCs.

3.4.1. HUVECs do not display HPPs

From a developmental point of view HUVECs are considered to be mature endothelial cells. On clonogenic analysis, HUVECs were able to form colonies from 2 to more than 2000 cells/well. This suggests that even if HUVECs are mature, differentiated cells, they have different proliferation rates and potential and are able, to some extent, to generate a hierarchy. However, only 20 % were HPP colonies, the rest being LPP with the 50-500 cells/well category being the most prevalent.

In addition, there was no significant difference between the function of HUVECs isolated from PE or NT placentas, as shown by the clonogenic and the cord-formation assays. This shows that there may be no need in the future to distinguish between these 2 groups and NT – HUVECs can be used as control cells for studies on endothelial cells isolated from PE placentas.

3.4.2. ECFCs are impaired in PE compared to NT matched controls

Genetic analysis showed that both NT-ECFCs and PE-ECFCs are of fetal origin with an XY phenotype. Although a two - way exchange of cells can take place between the mother and her fetus, there is still not enough evidence to support this process. Thus, we can speculate that the ECFCs isolated in the present study are “resident/local placental ECFCs”. To date the only studies conducted on placental EPCs are on peripheral blood samples and are considered circulatory EPCs and their maternal origin was proved in only one study.

Considering this, we proposed to analyze the function of fetal ECFCs in NT and PE placentas.

Although they show the same phenotype in terms of surface markers and endothelial specific staining, PE-ECFC's and NT-ECFC's function is significantly different. As shown by the single cell assay PE-ECFCs display low numbers of the HPP colonies (5%) in contrast with NT-ECFCs where 40% of the wells had more than 2000 cells, and another 40% between 500-2000 cell/well. A similar functional impairment was observed on Matrigel, where PE-ECFCs formed less complex networks. In addition to this significant functional difference we noticed a decreased proliferation capacity of PE-ECFCs. Upon initial plating, they needed double the seeding density of NT-ECFCs in order to generate comparable number of viable colonies at day 14.

Considering this functional discrepancy and the fact that there was no difference between the NT- and PE –HUVECs, we show that the impairment lies at the level of the true endothelial progenitor cells than at the level of the more mature HUVECs.

A limitation of the present study is the low number of samples analyzed (*n=3*). However, we were able to show a proof of concept that a true endothelial progenitor cell with self-renewal and clonogenic potential is impaired in PE. We speculate that there might be a multitude of factors contributing to this abnormality in PE. The impairment could lie within the ECFCs, but there could also be an outside component. A paracrine effect can influence the proliferation

rate, the functional abilities and the ECFC's responsiveness to the distress present in PE.

The innovation of our study relies on the fact that resident placental EPCs have not been studied to date in PE. Moreover, by showing that ECFCs are impaired in PE we bring new insight into the placental vascular development and the role that ECFCs might play in PE.

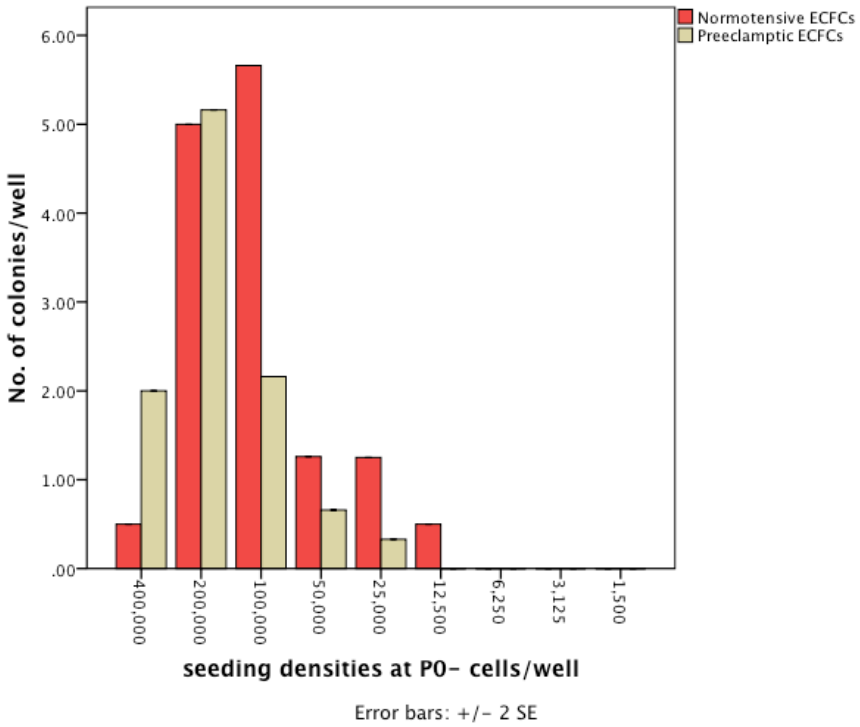
Table 3.1. Clinical characteristics of normotensive and preeclamptic groups

	Normotensive n=3	**Preeclampsia n=3**	**Significance**
Maternal age (years)	30 ± 1	35 ± 6	NS
Gestational age at delivery (weeks)	38 ± 1	37.9 ± 1.4	NS
Infant birth weight (grams)	3376.6 ± 558.9	3153 ± 505	NS
Systolic blood pressure (mmHg)	115.3 ± 3.2	151.3 ± 15	$p < 0.01$
Diastolic blood pressure (mmHg)	77.3 ± 8	97.3 ± 3.2	$p < 0.01$

* Values are expressed as mean ± SE; NS= not significant

Blood pressure values included in the study were recorded in the hospital before delivery

Figure 3.1. – Limited dilution assay – NT- vs PE - ECFCs

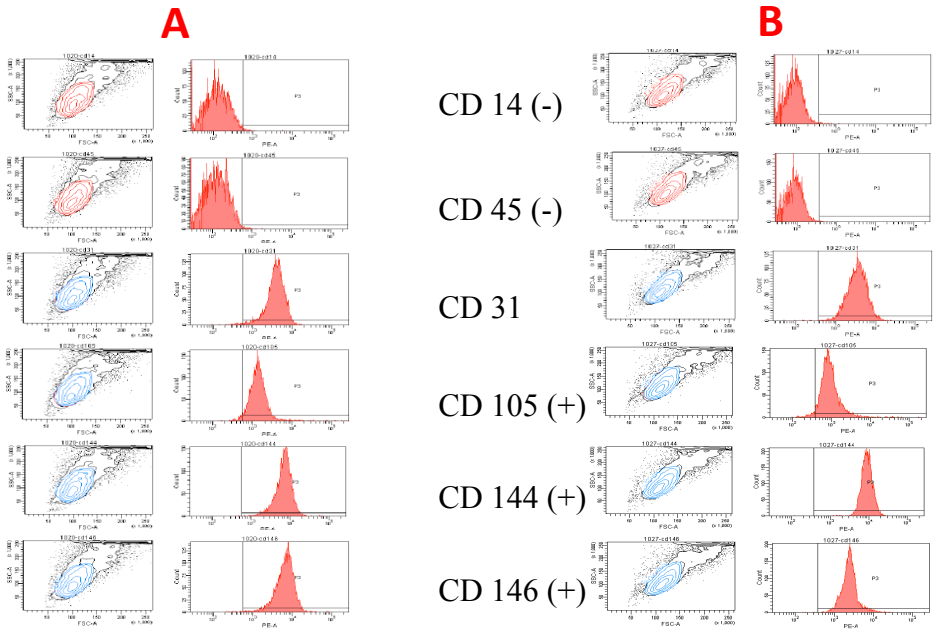


Seeding densities at passage zero. The colony count was based on the cobblestone appearance and the colony's viability for further expansion.

Red bar = NT-ECFCs;

Brown bar = PE-ECFCs.

Figure 3.2. - Phenotypic characterization by FACS of NT-ECFCs

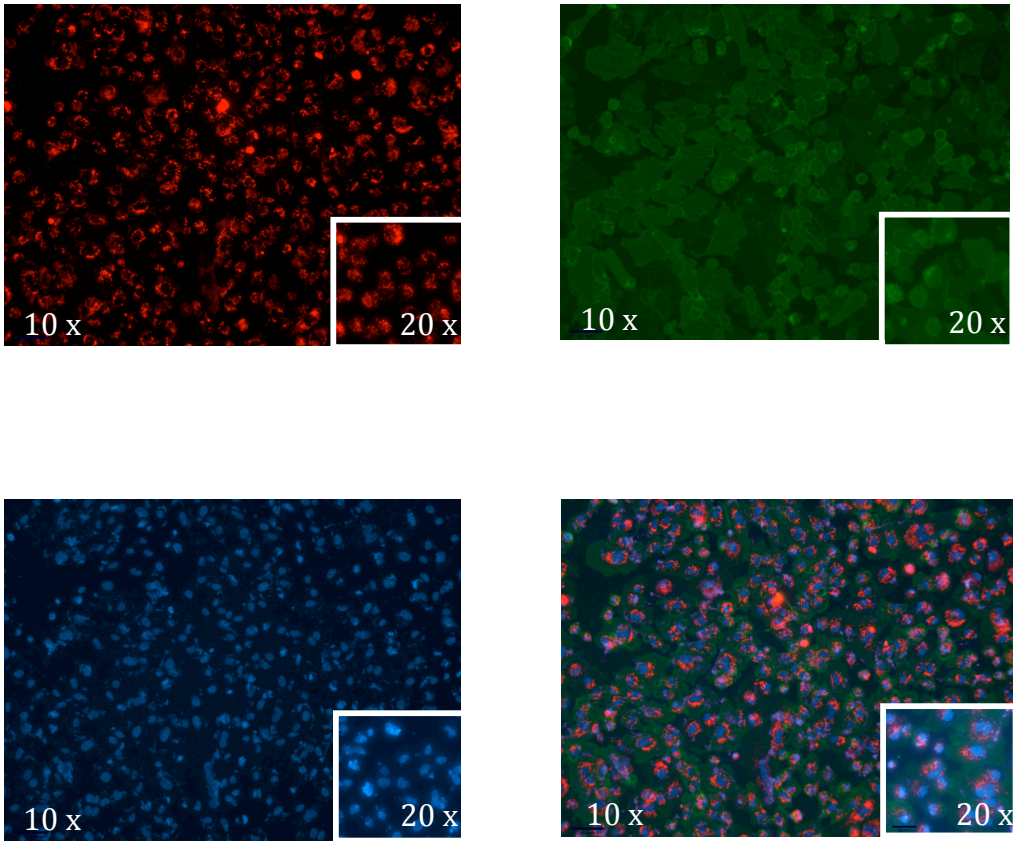


ECFCs Immunophenotyping through FACS Canto – representative data from 3 samples NT-ECFCs and 3 samples PE-ECFCs. The surface markers showed similar expression for both groups: intensely expressing CD31, CD105, CD144, CD146 and neither expressing CD14 or CD45;

(A) NT-ECFCs;

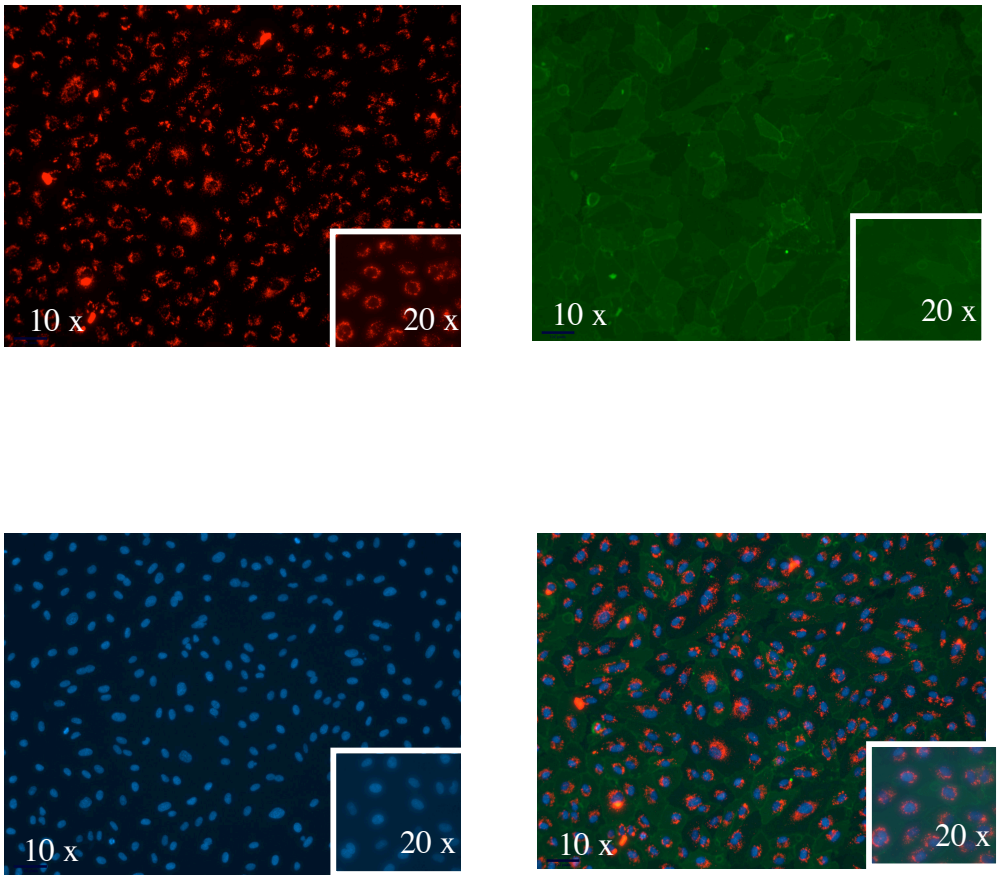
(B) PE-ECFCs

Figure 3.3-A: Typical endothelial staining of NT- ECFCs



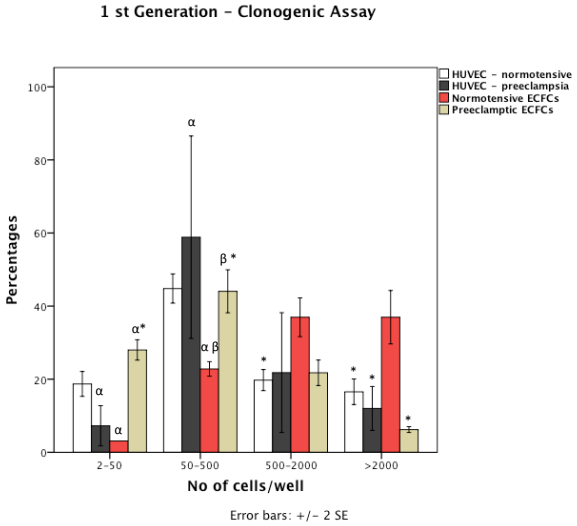
NT-ECFCs intensely stained for endothelial specific markers – Dil-Ac-LDL (red stain) and UEA-1 (green stain). Cells were also stained with Hoechst (blue stain).

Figure 3.3-B: Typical endothelial staining of PE - ECFCs



PE – ECFCs showed similar staining pattern as the NT-ECFCs. They stained for Dil-Ac-LDL and UEA-1, Both endothelial stains and were also positive for Hoechst stain.

Figure 3.4-A- Self-renewal and clonogenic potential assay- 1st generation

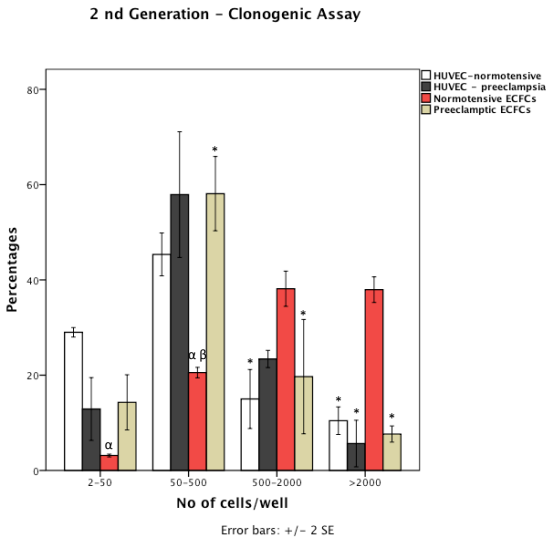


Symbol legend: α = p<0.05 when compared with NT-HUVECs; β = p<0.05 when compared to PE-HUVECs; * = p<0.05 when compared to NT-ECFCs. The statistical analysis was conducted using Univariate Anova within each individual group of the 2-50 cells/well, 50-500 cells/well, 500-2000 cells/well and with more than 2000 cells/well.

We considered LPP wells – 2-2000 cells/well groups and HPP wells with more than 2000 cells/well. Results represent 3 individual experiments. Values are expressed as mean ± SE.

NT-ECFCs had significantly more HPP colonies compared to NT-HUVECs, PE-HUVEC and PE-ECFCs There was no statistical significance between NT-HUVECs and PE-HUVECs in HPP formation.

Figure 3.4-B - Self-renewal and clonogenic potential assay – 2nd generation



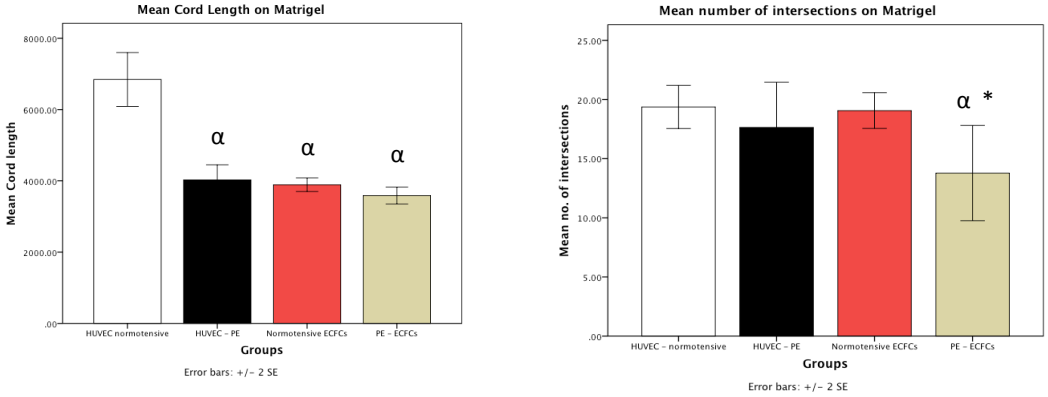
Symbol legend: α = $p<0.05$ when compared with NT-HUVECs; β = $p<0.05$ when compared to PE-HUVECs; * = $p<0.05$ when compared to NT-ECFCs. The statistical analysis was conducted using Univariate Anova within each individual group of the 2-50 cells/well, 50-500 cells/well, 500-2000 cells/well and with more than 2000 cells/well.

We considered LPP wells – 2-2000 cells/well groups and HPP wells with more than 2000 cells/well. Results represent 3 individual experiments.

Values are expressed as mean ± SE.

Growth pattern was maintained in the second generation. NT-ECFCs yielded significantly more HPP colonies compared to the other groups.

Figure 3.5. - In vitro capillary-like network formation of NT and PE – ECFCs



Cord formation potential in vitro – Matrigel Assay, *n=3 in duplicates*

α = $p<0.05$ compared to NT-HUVEC; * = $p<0.05$ compared to NT-ECFCs;

Values are expressed as mean ± SE.

Cord length measurement - significant difference between NT-HUVECs and the rest of the groups PE-HUVEC, NT-ECFCs and PE-ECFCs; No difference between NT-ECFCs and PE-ECFCs;

Intersection number – PE-ECFCs formed significantly less intersections compared with both NT-HUVEC and NT-ECFCs. There was no difference between NT-HUVECs and PE-HUVECs.

NT – ECFCs **PE - ECFCs**

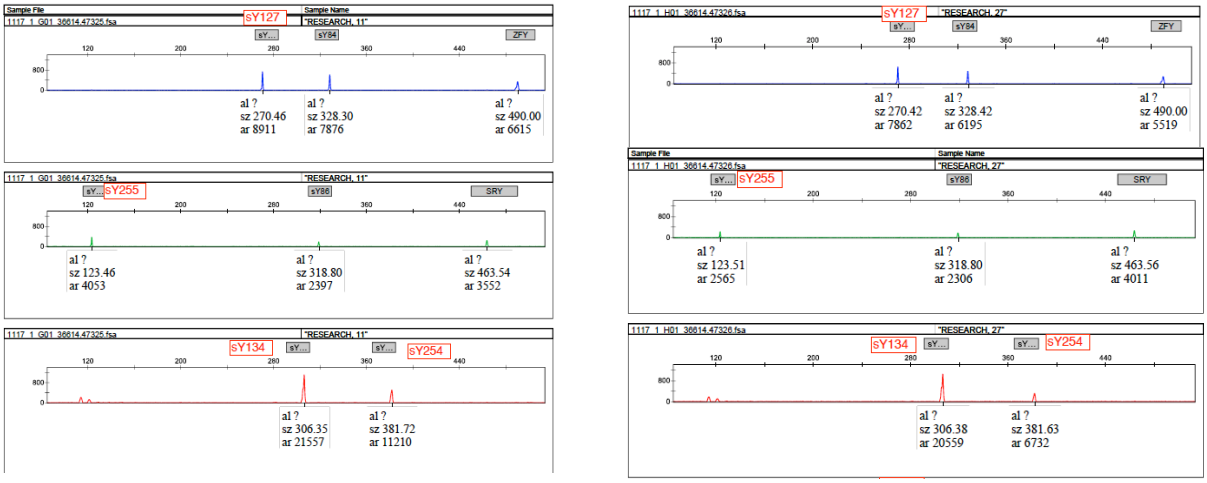


Figure 3.6. - Genetic analysis of NT-and PE – ECFCs

Gene legend:

X- Chromosome gene: SFY

Y- Chromosome genes: sY127, sY84, sY255, sY86, SRY, sY134, sY254

The genetic analysis showed similar results for both NT- and PE – ECFCs.

There was a positive signal for both the X and the Y chromosome genes, which proves that these cells are of XY phenotype. Thus, the ECFCs are of fetal origin.

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Chapter 4.

General summary and future directions

4.1. Overview

PE is a life-threatening pregnancy complication with no effective treatment that affects the health and outcome of both mother and infant[1,4-6]. A better understanding of the pathophysiology will give new insight into developing treatment strategies.

In Chapter 2 we reported a novel finding. We showed that ECFCs, a promising endothelial progenitor population, exists in the vasculature of the human term placenta. This proves that besides circulating progenitors, vessel walls carry resident progenitors as well. The relation between the circulatory and local EPCs is not known. However, our speculation is that there are niches along the blood vessels that lodge progenitor cells and that at certain time intervals and under certain conditions some of these cells are released into the blood stream. With the help of other signaling cells through paracrine effect EPCs later migrate at sites of injury or repair.

Following our observations that placental macro-vasculature contains fetal ECFCs with self-renewal and clonogenic potential, we investigated the function of these cells in preeclamptic placentas.

In Chapter 3, we provided proof of concept that the function and proliferation ability of PE-ECFCs is impaired compared to the NT-ECFCs. This finding brings new light into the potential implications of progenitor cells in the

vascular development of the human placenta. Further, it opens new research avenues towards an effective treatment for PE.

4.2. Limitations

There are several limitations that should be mentioned.

In Chapter 3 we used a small sample size of three placentas for each group. We will increase the sample size in order to confirm our current findings.

Although EPCs have been isolated almost 2 decades ago[42,65], there is still no definite consensus over their characterization. ECFC, the least studied of the three EPC subpopulations, seems to be the most controversial. Its name varies across literature from late-outgrowth EPCs, to ECFCs or simply EPCs, as they are thought to be the true progenitor cells. In our study, we used previously described isolation methods, terminology and characterization methods [48,66] in order to keep the consistency for future research studies.

As we showed in the present study, ECFCs can be isolated from the placental tissue, in contrast to previous studies that isolated EPCs from the peripheral blood[51-53]. The circulating EPCs are easily accessible and can be used as potential predictor biomarkers of PE. In addition the peripheral blood EPCs can be isolated at any time during pregnancy. However, the resident ECFCs identified in our study can only be isolated upon placental delivery. Thus, the difficulty of isolating cells from the same gestational age placentas and matching them with normal controls.

4.3. Future directions

The placenta is the connection between the mother's blood supply and the fetus. It is the placental function that is absolutely necessary in order for the fetus to survive. Our lab concentrates on diseases of the newborn, especially on bronchopulmonary dysplasia, a prevalent disease in premature infants that have been mechanically ventilated.

Because PE is the leading cause of maternal death in developing countries[1],and it is associated with high rates of prematurity, we decided to look deeper into potential disruptions that might take place in the placental vascular development. As we showed that fetal ECFCs are impaired in function, we speculate that there might be several reasons. One could be that the fetal and maternal ECFCs are inherently impaired. In the case of maternal ECFCs, their impairment leads to narrow uterine arteries with low blood volume delivery to the fetus causing hypoxia. Maternal ECFCs can be impaired long before pregnancy and manifest under pregnancy conditioning. This is supported by the idea that even if PE resolves after placental delivery, it is considered a prediction factor for developing cardiovascular disease later in life[77]. Another reason could be that the created hypoxic placental environment damages or negatively affects the function of fetal ECFCs. Even if these resident ECFCs are signaled that there is a blood supply deficit, the fact that there is not enough oxygen delivery to the cells

themselves, they are not able to function properly and thus will not generate the growth of new blood vessels or sprouting of the already existing ones.

There are studies that classify PE in early onset, between 20 and 34 weeks of gestation, and late onset, with diagnosis after 34 weeks[1]. Considering the potential ECFC implication in placental vascular development, we speculate that the ECFC's functionality in early PE is further hindered compared to late PE. This is to say that in late PE, ECFCs were able to compensate for the blood vessel demand up to a point after which PE developed. In early PE, because ECFCs are more impaired, they cannot compensate for the need of new blood vessels and in turn PE develops. In addition, early PE is most likely associated with predisposing factors, whereas late PE can be an isolated pregnancy event. Thus, further research needs to be conducted in order to establish the degree of ECFC impairment in early onset PE compared to late onset PE.

Our observations bring light on the potential role the EPCs play in vascular maturation and maintenance. Looking into the mechanism of action with more focus on the molecular level of ECFCs could help develop a cure for PE, and in the same time prevent a frequent cause of prematurity and broncho-pulmonary dysplasia.

This thesis reports findings that are innovative to both EPCs and PE research fields. Further studies need to be conducted in order to establish the therapeutic effects of ECFCs in PE. Potential animal studies, protein and gene analysis would be just a few prospects that will help regenerative medicine move forward.

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