THE ROLE OF ENDOTHELIAL PROGENITOR CELLS IN THE UTERO-PLACENTAL VASCULATURE

A thesis written in the alternative format submitted to the University of Manchester for the degree of PhD in the Faculty of Medical and Human Sciences

2012

Péter István Sips

SCHOOL OF MEDICINE
CONTENTS

CHAPTER 1 INTRODUCTION

1.1 Adaptation of maternal and placental circulatory systems to pregnancy
  1.1.1 Maternal macrovascular adaptations
  1.1.2 Utero-placental microcirculatory adaptations
  1.1.3 Angiogenesis at the utero-placental interface
    1.1.3.1 Placental angiogenesis
    1.1.3.2 Uterine angiogenesis
  1.1.4 Endothelial Progenitor Cells in vascular adaptations and pregnancy disease

1.2 Published review article Endothelial progenitor cells: their potential in the placental vasculature and related complications
  1.2.1 Abstract
  1.2.2 Introduction
  1.2.3 Physiology of EPCs
  1.2.4 EPC-phenotypes
    1.2.4.1 Flow cytometry techniques
    1.2.4.2 Cell culture techniques
    1.2.4.3 Functional capabilities of endothelial progenitors
      1.2.4.3.1 (i) EPC cell-cell interactions and signals
      1.2.4.3.2 (ii) EPC mobilisation and recruitment
      1.2.4.3.3 (iii) EPC homing, migration, invasion, proliferation and differentiation.
  1.2.5 Endothelial progenitors and the developing fetus
  1.2.6 EPCs in pregnancy related conditions
    1.2.6.1 Clinical significance of EPCs in pregnancy
    1.2.6.2 Clinical significance of EPCs in the developing fetus
  1.2.7 Perspectives and possibilities
  1.2.8 Summary
1.3 Thesis considerations 54
1.3.1 ECFCs study focus 54
1.3.2 Is there a role for fetal ECFCs at the fetoplacental interface? Overall thesis hypothesis. 56
   1.3.2.1 Chapter 2: Fetal derived ECFCs play a physiological role in placental vasculogenesis 56
      1.3.2.1.1 Hypothesis 57
      1.3.2.1.2 Aims 57
   1.3.2.2 Chapter 3: The role of fetal ECFCs in the vasculogenesis of the maternal uterus 58
      1.3.2.2.1 Hypothesis 58
      1.3.2.2.2 Aims 58
   1.3.2.3 Chapter 4: Involvement of fetal ECFCs in the pathogenesis of Intrauterine Growth Restriction 59
      1.3.2.3.1 Hypothesis 60
      1.3.2.3.2 Aims 60

2 CHAPTER 2 A PHYSIOLOGICAL ROLE FOR HUMAN ENDOTHELIAL COLONY FORMING CELLS 62
2.1 Abstract 63
2.2 Introduction 64
2.3 Materials and methods 66
   2.3.1 Sample collection 66
   2.3.2 Flow cytometry 66
   2.3.3 ECFC expansion 67
   2.3.4 Lentiviral vector production and transduction of ECFCs and HUVECs 68
   2.3.5 Verification of ECFC-characteristics after genetic modification 68
   2.3.6 In vivo characterisation of fetal ECFCs 69
   2.3.7 Ex vivo perfusion of chorionic plate arteries with cord blood ECFCs 70
   2.3.8 Transplantation of ECFCs into immuno-compromised mouse fetuses 70
   2.3.9 Statistics 74
   2.3.10 Study approval 74
2.4 Results 75
   2.4.1 Umbilical arterio-venous gradient favours placental uptake of ECFCs and CACs 75
   2.4.2 Morphologic and functional phenotype of cells used for xenotransplantation 76
   2.4.3 Human fetal ECFCs incorporate into placental vessels ex vivo 78
   2.4.4 Xeno-transplanted fetal ECFCs preferentially migrate from the fetus to placental vessels 79
   2.4.5 ECFCs contribute to vessel formation in murine placental tissue 81
2.5 Discussion 83
2.6 Study approval 86
2.7 Acknowledgements 86
2.8 Author contributions 86
2.9 Supporting information 87
CHAPTER 3  UTERINE VASCULATURE REMODELLING IN HUMAN PREGNANCY INVOLVES FUNCTIONAL MACRO-CHIMERISM BY ENDOTHELIAL COLONY FORMING CELLS OF FETAL ORIGIN

3.1 Author contributions: 91
3.2 Acknowledgements: 92
3.3 Keywords: 92
3.4 Abstract 93
3.5 Introduction 94
3.6 Methods 96
   3.6.1 Ethical Approval 96
   3.6.2 Sample collection 96
   3.6.3 Transmigratory model of fetal cells to the mouse uterus 96
   3.6.4 Culture expansion of ECFCs 97
   3.6.5 Lentiviral vector transduction of ECFCs 97
   3.6.6 Characterisation of fetal ECFC 98
   3.6.7 Mouse transplantation of human ECFCs into immuno-compromised fetuses 98
   3.6.8 RT-QPCR of maternal uterine microvessels 99
   3.6.9 Sex chromosome FISH 100
   3.6.10 Statistics 100
3.7 Results 101
   3.7.1 Fetal endothelial-like cells are found in maternal uterine vessels in the mouse 101
   3.7.2 Human fetal ECFCs transmigrate the murine placenta and exhibit vasculogenic function 103
   3.7.3 Fetal derived endothelial cells located in the human uterine vasculature 105
3.8 Discussion 109
3.9 Supplementary information 114

CHAPTER 4  ENDOTHELIAL COLONY FORMING CELLS DERIVED FROM PREGNANCIES COMPLICATED BY INTRAUTERINE GROWTH RESTRICTION ARE FEWER AND HAVE REDUCED VASCULOGENIC CAPACITY

4.1 Abstract 116
4.2 Introduction 117
4.3 Methods 118
4.4 Online supplementary methods 119
   4.4.1 Patients 119
   4.4.2 ECFC isolation, culture and phenotyping 119
   4.4.3 Flow cytometry 120
   4.4.4 Cell migration and chemotaxis assays 121
   4.4.5 Gelatin zymography for MMP-2 activity 121
   4.4.6 Hypoxic treatments 122
   4.4.7 In vivo vasculogenesis bioassay 122
4.5 Results 124
   4.5.1 Patient demographics 124
   4.5.2 Blood from IUGR neonates has fewer and slower proliferating ECFCs 125
   4.5.3 ECFCs from IUGR-babies have impaired vasculogenesis capacity 128
5 CHAPTER 5  OVERALL DISCUSSION OF THESIS STUDIES: CRITICISMS, POTENTIAL CLINICAL BENEFITS AND DIRECTIONS OF FUTURE WORK 132

5.1 Criticism of techniques 133
5.1.1 Critique of investigations on placental uptake of EPCs (Chapter 2) 133
5.1.1.1 Potential weaknesses of flow cytometry 133
5.1.1.1.1 Optimisation of flow cytometry technique 133
5.1.1.1.2 Points related to mode of normalisation 135
5.1.1.1.3 Differentiating ECFCs from circulating mature endothelial cells in flow cytometry 135
5.1.1.2 Comments on the ex vivo perfusion of ECFCs through placental arteries 136
5.1.1.3 Considerations in regards of the murine fetal transplantation model 137
5.1.1.3.1 Coincidental co-localisation of injected cells with murine endothelium and graft versus host reaction 137
5.1.1.3.2 Cytoplasmic fusion 138
5.1.1.3.3 Lentiviral labelling of transplanted human ECFCs 140
5.1.2 Critique of techniques to examine feto-maternal transmigration of ECFCs (Chapter 3) 140
5.1.2.1 Critique of murine experiments 140
5.1.2.2 Critique of human observations 141
5.1.2.2.1 SRY-specific RT-QPCR of maternal uterine microvessels 141
5.1.2.2.2 Y-chromosome-specific FISH of cross sections of maternal uterine microvessels 142
5.1.3 Critique of methods to compare normal and growth restricted ECFCs (Chapter 4) 142
5.1.3.1 Flow cytometry 142
5.1.3.2 Vasculogenic bioassay 143

5.2 Potential clinical use 145
5.2.1 Development of new diagnostic tools 145
5.2.1.1 Background 145
5.2.1.2 Early diagnosis of IUGR based on fetal ECFCs 146
5.2.2 Treatment of IUGR by targeting fetal ECFCs 147

5.3 Future work 149
5.3.1 Basic research into the role of ECFCs in IUGR 149
5.3.1.1 Identification of targets for drug therapy 149
5.3.1.2 Identification of targets for viral therapy 150
5.3.2 Translational research into ECFCs in IUGR 150
5.3.2.1 The COMT (−/−) mouse model 150
5.3.2.2 The Reduced Uterine Perfusion Pressure model of IUGR 151
5.3.2.3 Other animal models 152
5.3.3 Research into IUGR-related conditions 153
5.3.3.1 Preeclampsia 153
5.3.3.2 Fetal ECFCs and Barker’s hypothesis of fetal programming 155
5.3.3.3 Fetal ECFCs and cardiovascular health of the mother 156

5.4 Overall summary 157
6 APPENDIX    MINOR CORRECTIONS  159

6.1 Appendix to chapter 1  160
  6.1.1 Literature update  160
    6.1.1.1 Superior capacities of fetal ECFCs in the fetomaternal unit.  160
    6.1.1.2 ECFCs in fetal life and pathologies of placentation  161
  6.1.2 Expansion of methodology in Chapter 1.  162
    6.1.2.1 Methods used for determination of EPC-phenotypes  162
      6.1.2.1.1 Immunofluorescent staining  162
      6.1.2.1.2 Ac-LDL uptake test  163
      6.1.2.1.3 Flow cytometry for determining surface marker expression of outgrowth cells  163
      6.1.2.1.4 Angiogenesis test on Matrigel  164
    6.1.2.2 Flow cytometry for counting CACs and ECFCs  164
  6.1.3 Statistics used  165
  6.1.4 Appendix to Chapter 2  166

REFERENCES  167

APPENDIX 2 (DIGITAL MEDIUM)  A DVD CONTAINING SUPPLEMENTARY VIDEO FILES  185

FINAL WORD COUNT: 48 531
LIST OF ILLUSTRATIONS

CHAPTER 1

Figure I-1. A schematic of uterine–driven placental pathology. 25
Figure I-2 Bright-field images of outgrowth cells from human fetal peripheral blood mononuclear cells (PBMNCs). 32
Figure I-3. Illustration of flow-cytometry technique for surface marker distribution of ECFCs in fetal peripheral blood. 37
Figure I-4 Illustration of flow-cytometry technique for surface marker distribution of CACs in fetal peripheral blood. 38
Figure I-5 A comparison of CAC and ECFC characteristics. 40
Figure I-6. A schematic of EPC-mobilisation. 42
Figure I-7. A schematic of EPC-recruitment. 43
Figure I-8. A schematic summarising integrins and their ligands involved in homing of EPCs. 44
Figure I-9. A schematic summarising the role of ECFCs in fetal vascular development. 47
Table I-1 Pharmaceutical agents known to influence EPC number or function. 53

CHAPTER 2

Figure II-1. Illustration of ultrasonographic visualisation of intra-cardiac transplantation process of human fetal ECFCs (expressing eGFP or LacZ) into the NOD/SCID murine fetus (E15.5 pregnancy). 73
Figure II-2. Concentration of EPC-subtypes in umbilical arterial and corresponding venous blood from uncomplicated pregnancies at term. 75
Figure II-3. The characterisation of fetal-derived ECFCs isolated from cord blood mononuclear cells. 79
Figure II-4. The incorporation of fetal-derived ECFCs into ex-vivo perfused human chorionic plate arteries. 78
Figure II-5. The homing of human fetal eGFP-ECFCs (xenotransplanted in NOD/SCID fetuses on E15.5) to the murine placental vasculature. Representative images taken by fluorescent imager. 80
Figure II-6. Cross-sectional images of murine placentas following fetal transplantation with human transgenic ECFCs and HUVECs. 84
Video II-1. A clip showing the unsuccessful search for apparent fluorescent cell aggregates in the body of an NOD/SCID fetus, which had received an intra-cardiac injection of eGFP-ECFC during intrauterine life (still image). 87
Video II-2. An adjunct to Figure 6d, showing the placenta circulation in a NOD/SCID fetus transplanted with eGFP-ECFC (still image). 88
Supplement figure II-1. Flow cytometry data for determining ECFC-identity. 91
Supplement figure II-2 Efficiency of lentiviral transfection. 90
CHAPTER 3

Figure III-1. Fetal cells of endothelial characteristics cross the mouse placenta and colonize the uterine vasculature in a transgenic murine model with eGFP-expressing offspring. 102

Figure III-2. Transplanted fetal-derived human ECFCs traverse the mouse placenta and home to the pregnant uterus. 104

Figure III-3. The presence of fetal-derived male cells within maternal human uterine vessels. 108

Figure III-4. Endothelial location of fetal-derived cells within uterine vessels. 108

Video III-1. Ultrasound-based in vivo injection of human fetal ECFCs into the mouse fetal circulation. 114

Video III-2. A series of real-time in vivo optical sequences of the mouse uterine microvasculature infiltrated by transmigratory fetal cells. 114

Video III-3. In vivo images of pregnant uteri of NOD/SCID mice whose fetuses were transplanted with human fetal eGFP-ECFCs. 114

CHAPTER 4

Table IV-1 Patient information at the time of delivery 124

Figure IV-1 ECFC and CAC are fewer in cord-blood obtained from IUGR-complicated pregnancies. 125

Figure IV-2. ECFCs obtained from pregnancies complicated by IUGR have altered proliferation and migration in culture. 127

Figure IV-3. Vasculogenic capacity is reduced in IUGR-derived fetal ECFCs. 129

CHAPTER 5

Figure V-1. A schematic summarising speculations derived from this thesis. 161

APPENDIX 1

Appendix Figure 1. A control to insert II-3Cii. 166
ABSTRACT

Fetal growth in utero depends on nutrient and oxygen reaching the fetus through the uterine and placental microcirculations, both undergoing massive expansion during pregnancy. Aberrations of the placental vasculature are associated with Intrauterine Growth Restriction (IUGR), a common pathological outcome of pregnancy; however, the cellular components responsible for vessel formation in the placenta and the uterus remain unknown.

Endothelial Progenitor Cells (EPC) are a group of morphologically and functionally varied bone marrow derived vasculogenic cell types, divided into two major subsets: (i) Circulating Angiogenic Cells (CACs), which promote vessel formation by interfering with the extracellular matrix and (ii) Endothelial Colony Forming Cells (ECFCs), which provide the source for new endothelium. This role has been demonstrated in pathophysiological studies, but not in normal physiological events in vivo. Fetal ECFCs are more proficient than their adult counterparts, but it is unclear in what specific fetal or maternal physiological situations fetal ECFCs are involved. Based upon these considerations, it was hypothesised that: (i) fetal-derived ECFCs play a role in placental vasculogenesis, (ii) these cells transmigrate the placenta and home to loci of vessel formation in the pregnant uterus, and that (iii) intrinsic alterations in their capabilities are associated with fetal growth restriction during intrauterine life.

To support these hypotheses the following experiments were performed;
(i) EPCs in blood from pairs of human umbilical arteries and veins were counted by flow cytometry. Numbers of EPCs in these samples showed an arterio-venous gradient suggesting their placental sequestration. Furthermore, ECFCs were isolated from human umbilical blood using established culture techniques. Labelled human fetal ECFCs were transplanted into the circulation of murine fetuses using an ultrasound-guided intra-cardiac injection. Using a fluorescent imager and microscopy these cells were shown to home to the murine placenta and participate in vasculogenesis.
(ii) Male mice ubiquitously expressing eGFP were crossbred with native females, and fetal (eGFP-positive) endothelial-like cells integrated into the uterine microvasculature. Human fetal ECFCs injected into murine fetuses were shown to migrate to the maternal uterus and became functionally involved with the microvasculature. In humans, microvessels were isolated from uterine biopsies of mothers with male offspring. Copies of the male specific SRY gene (quantified by RT-QPCR) indicated that cells of fetal origin constituted 12% of the endothelium in these vessels. In cross-sections, hybridisation of the Y-chromosome demonstrated the presence of fetal cells in the maternal endothelium of the human uterus.
(iii) Using flow cytometry, fewer EPCs were defined within the peripheral circulation of growth-restricted babies. Functional assays showed that ECFCs derived from these growth-restricted cases had intrinsically impaired proliferation, migration, matrix-metalloproteinase (MMP-2) production, and generated fewer blood vessels in a murine vasculogenic bioassay.

These results demonstrated the vasculogenic capacity of human fetal ECFCs in vivo and established them as key players in human placental vasculogenesis and uterine vessel expansion. Notably, these results also showed a link between impaired function of fetal ECFCs and IUGR, which is associated with increased cardiovascular risk of both the fetus as an adult, and mother in later life. From these findings it could be speculated, that intrinsic changes in ECFC-biology may be the causative link between IUGR and fetal and maternal cardiovascular susceptibility. Insight into these processes may contribute to early diagnosis, prevention and treatment of IUGR and associated conditions.
DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning, other than Supplement figure II-1., which has been included in the MRes thesis (University of Manchester, 2011) of Ms. Alice Ridgeway, a co-author of the manuscript included in Chapter 2.

With the permission of The Faculty of Medical and Human Sciences Graduate Office, this PhD Thesis is written and submitted in the Alternative Format. As laid out in Section 7 of The Presentation of Thesis Policy of The University of Manchester “The Alternative Format thesis allows a postgraduate doctoral or MPhil student to incorporate sections that are in a format suitable for submission for publication in a peer-reviewed journal” and “materials included in the alternative format thesis may include those which are solely and/or partly authored by the student and may be already published, accepted for publication, or submitted for publication in externally refereed contexts such as journals and conference proceedings”. The introduction of this thesis consists of a published review paper, and the subsequent three results chapters constitute of manuscripts prepared and submitted for peer-reviewed publication. All papers are presented in this thesis in their original formats. Dr Peter Sipos is first author of these articles sharing first authorship of the manuscript presented herein as Chapter 4.

Published papers and submitted manuscripts have been modified from their original version to better fit the structure of this thesis. Roman thesis chapter numbers have been added to headings of papers and manuscripts, as well as to figure numbers. The following changes have been made to references: individual reference lists have been removed, references were reformatted from the original journal specific format, and all references are included in a single list to be found at the end of the thesis.
COPYRIGHT STATEMENT

The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and he has given the University of Manchester certain rights to use such Copyright, including for administrative purposes. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

Further information on the conditions under which disclosure, publication and commercialization of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://www.campus.manchester.ac.uk/medialibrary/policies/intellectualproperty.pdf), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.manchester.ac.uk/library/aboutus/regulations) and in the University’s policy on presentation of Theses.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-AG</td>
<td>2-Arachidonyl-Glycerol</td>
</tr>
<tr>
<td>7-ADD</td>
<td>7-Amino-Actinomycin D</td>
</tr>
<tr>
<td>ADMA</td>
<td>Asymmetric Dimethylarginine</td>
</tr>
<tr>
<td>AEA</td>
<td>Arachidonyl-Ethanol-Amide</td>
</tr>
<tr>
<td>AFP</td>
<td>α-Feto-Protein</td>
</tr>
<tr>
<td>Ang</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>APC</td>
<td>Allo-Phyco-Cyanin</td>
</tr>
<tr>
<td>AT1R</td>
<td>Angiotensin Type 1 Receptor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAC</td>
<td>Circulating Angiogenic Cell</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine Receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CMFDA</td>
<td>5-Chloro-Methyl-Fluorescein Diacetate</td>
</tr>
<tr>
<td>ct</td>
<td>Cycle Threshold</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyanin</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Di-Amidino-2-Phenyl-Indole</td>
</tr>
<tr>
<td>Dil-AcLDL</td>
<td>Acetylated Low Density Lipoprotein, labelled with 1,1' Dioctadecyl-3,3,3',3'-Tetramethyl-Indocarbocyanine Perchlorate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>dNK</td>
<td>Decidual Natural Killer Cell</td>
</tr>
<tr>
<td>EBM</td>
<td>Endothelial Basal Media</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>ECC</td>
<td>Endothelial Cell Cluster</td>
</tr>
<tr>
<td>ECFC</td>
<td>Endothelial Colony Forming Cell</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-Diamine-Tetra-Acetic acid</td>
</tr>
<tr>
<td>Egfl</td>
<td>Endothelial Growth Factor Like Domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EGM</td>
<td>Endothelial Growth Medium</td>
</tr>
<tr>
<td>EMAP</td>
<td>Endothelial Monocyte Activating Polypeptide</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial Progenitor Cell</td>
</tr>
<tr>
<td>Epo</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>EVT</td>
<td>Extravillous Trophoblast</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FcR</td>
<td>Fragment Crystallisable Receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent In Situ Hybridisation</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Iso-Thio-Cyanate</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HMGP</td>
<td>High-Mobility Group Box</td>
</tr>
<tr>
<td>HOX</td>
<td>Homebox Transcription Factor</td>
</tr>
<tr>
<td>HPP-ECFC</td>
<td>High Proliferative Potential Endothelial Colony Forming Cell</td>
</tr>
<tr>
<td>II</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intra Uterine Growth Restriction</td>
</tr>
<tr>
<td>KDR</td>
<td>Kinase Insert Domain Receptor</td>
</tr>
<tr>
<td>KitL</td>
<td>Kit Ligand</td>
</tr>
<tr>
<td>LPP-ECFC</td>
<td>Low Proliferative Potential Endothelial Colony Forming Cell</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metallo-Proteinase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide-Adenine-Dinucleotide-Phosphate</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH-Oxidase</td>
</tr>
</tbody>
</table>
NP        Neurophillin
NP        Neurophilin
OCT       Optical Cutting Temperature Compound
PAPP-A    Pregnancy-Associated Plasma Protein-A
PAR       Protease Activated Receptor
PBMNC     Peripheral Blood Mononuclear Cells
PBS       Phosphate Buffer Saline
PCR       Polymerase Chain Reaction
PDGF      Platelet Derived Growth Factor
PE        Phycoerythrin
PECAM     Platelet Endothelial Cell Adhesion Molecule
PerCP     Peridinin-Chlorophyll Protein
PI3K      Phosphatidylinositol-3-Kinase
PI3K/Akt  Phosphatidylinositol-3-Kinase and Protein Kinase B
PIH       Pregnancy Induced Hypertension
PIGF      Placental Growth Factor
PPAR      Peroxisome-Proliferator Activated Receptor
ROS       Reactive Oxygen Species
RT-QPCR   Real Time Qualitative Polymerase Chain Reaction
SCF       Stem Cell Factor
SDF       Stromal Cell Derived Factor
SEM       Standard Error of the Mean
sFlt-1    Soluble Vascular Endothelial Growth Factor Receptor 1
SRY       Sex-determining Region Y
SSc       Side Scatter
SSC       Standard Sodium Citrate
TAL       T-cell Acute Lymphocytic Leukaemia
TGFb1     Transforming Growth Factor Beta 1
tGFP       turbo Green Fluorescent Protein
TNF       Tumor Necrosis Factor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>TU</td>
<td>Transducing Unit</td>
</tr>
<tr>
<td>UEA</td>
<td>Ulex Europaeus-1 Agglutinin</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGF-R</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrandt Factor</td>
</tr>
</tbody>
</table>
THE AUTHOR

Outcome arising directly from the work described in this thesis.

Publications:


Publications in preparation:


Sipos PI, Bourque S, Wareing M, Hubel C, Baker PN, , Sibley CP, Davidge ST, Crocker IP. Endothelial colony forming cells derived from pregnancies complicated by intrauterine growth restriction are fewer and have reduced vasculogenic capacity (Manuscript under review by Journal of Pathology)

Podium Presentations


Sipos P. The role of Endothelial Progenitor Cells in placental vasculogenesis and pathogenesis of IUGR. Annual Conference of the Chilean Physiological Society. Santa Cruz, October 2010.

Sipos P. Fetal endothelial progenitor cells are sequestered by the placenta. Tox(emia) Talks. Mill Valley, June 2009.

Accepted conference abstracts/poster presentations


Awards:


The Pfizer President's Presenter's Award. Annual Meeting of the Society for Gynecologic Investigation. 2011.


Manchester Biomedical Research Centre Laboratory Placement Scheme, 2010.

The Giorgio Pardi Foundation President’s Presenter’s Award, Society of Gynecological Investigators, 2010.
CONTRIBUTION OF COLLABORATORS

All experiments were performed by Peter Sipos with the exception of:

Chapters 2 & 3 where Xia Fan provided help with viral transfection.

Chapter 3 where RT-QPCR was conducted by Helene Schlecht with assistance of Peter Sipos; Willem Rens provided help with FISH experiments.

Chapter 4 where vasculogenesis assay was conducted by Peter Sipos together with Stephane Bourque, and zymography was conducted by Stephane Bourque with assistance of Peter Sipos.
ACKNOWLEDGEMENTS

The author of this thesis would like to express his gratitude to his direct (Dr. Ian Crocker) and indirect supervisors (Prof. Philip N. Baker, Prof. Colin P. Sibley, Dr. Mark Wareing and Prof. Carl Hubel) for their careful guidance throughout the research period and writing of the thesis. The author would also like to thank the co-authors of the papers and manuscripts incorporated into the Introduction (Ian P. Crocker, Carl A. Hubel, Philip N. Baker), Chapter 2 (Xiaohu Fan, Stephane L. Bourque, Joanne L. Stanley, Irene J. Andersson, Alice L. Ridgway, Mark Wareing, Carl A. Hubel, Philip N. Baker, Sandra T. Davidge, Colin P. Sibley, Ian P. Crocker), Chapter 3 (Willem Rens, Helene Schlecht, Xiaohu Fan, Christina Hayward, Mark Wareing, Philip N. Baker, Sandra T. Davidge, Colin P. Sibley, Ian P. Crocker) and Chapter 4 (Stephane L. Bourque, Carl A. Hubel, Philip N Baker, Colin Sibley, Sandra T. Davidge, Ian P. Crocker) for their contributions.

The scientific work presented was supported by the Wellcome Trust UK, the Manchester NIHR Biomedical Research Centre, National Institutes of Health grant (P01HD030367), the Canadian Institutes for Health Research (CIHR), and the Women’s and Children’s Health Research Institute.
DEDICATION

For my parents, grandparents and my brother who always believed in me and gave me the chance to improve throughout my life.
CHAPTER 1

INTRODUCTION
1.1 ADAPTATION OF MATERNAL AND PLACENTAL CIRCULATORY SYSTEMS TO PREGNANCY

The fast development and growth of the human fetus requires an enormous metabolic rate with corresponding high demands for nutrients and oxygen. The backbone of feto-maternal exchange is the human haemochorial placenta [1]. This transient organ provides the conduit for the interaction of maternal and fetal blood. In this type of placentation the part of blastocyst in contact with myometrium develops into so-called trophoblast, the two-cell layer membrane of the placenta [2]. The outer layer is multinucleated and it is called syncytiotrophoblast, while the underlying inner layer contains highly proliferative cytotrophoblast cells [3]. These form branch-like structures called chorionic villi. These are in direct contact with maternal blood, supplied by the adapted terminal arterioles of the placental bed termed spiral arteries. The adaptive capacity of spiral arteries is further increased through invasion by extrachorionic trophoblasts [4]. The placenta is perfused on the maternal side through the uterine microvasculature and fetal aspect through the fetoplacental microvasculature. In order to cope with the growing demands of the fetus, the uteroplacental circulation, on the fetal and maternal side, undergo significant changes during the course of pregnancy. Some of these changes are systematic, but the focus of adaptation lies in the macro- and microvascular system, including dilation, expansion and arboration of vessels [5].

1.1.1 Maternal macrovascular adaptations

Accommodating the growing fetus in utero during pregnancy requires extensive maternal adaptation of a wide range of physiological parameters, including elementary functions of metabolism, respiration, immunology and locomotion [6-11]. Nevertheless, the most apparent and profound changes occur in the circulatory system, in which blood volume increases significantly, and there are marked alterations in blood counts and cardiac function [12,13]. Plasma volume reaches its maximum by 34-36 weeks gestation, increasing by 1250-1300mls, corresponding to a 50% increase compared to the pre-pregnant state. Red cell mass increases by 250mls (18%), however in the event of iron
supplementation, which is common clinical practice, this elevation rises further, up to 400-450mls by term. These changes result in a haematocrit drop from 40 to 33% [14].

Changes in plasma volume are primarily mediated by an oestrogen-driven renin activity, which ultimately results in high angiotensin-II levels. This stimulates a 7-8 fold increase in aldosterone-production, but angiotensin-II also has vasoconstrictive effects. Aldosterone then leads to sodium retention and secondary retention of water. Increased levels of prolactin, placental lactogen, growth hormone and prostaglandins are also likely to contribute to this process [12]. Greater blood volume is associated with 50% increase in cardiac output and 20% increase in heart rate, whilst mean arterial pressure and central venous pressure remain unaltered. This surprising continuity in blood pressure can be attributed to a wholesale, three-fold reduction in systemic and pulmonary vascular resistance [15], which in turn can be attributed to systemic vasodilation.

To achieve this dilatation several vasodilatory mechanisms are up regulated during gestation. Of these, serum levels of Angiotensin-(1-7), a vasodilatory component of the renin-angiotensin-aldosterone system, is elevated by 50-200% during pregnancy [16,17]. As a consequence, the ratio of this vasodilatory agent and the primarily elevated, but vasoconstrictive angiotensin II remains unaltered. The interaction between these two components of the renin-angiotensin system is suggestive of constituting a compensatory mechanism, which balances out the intrinsic vasoconstrictive effects of angiotensin II, the major driver of essential plasma volume expansion during pregnancy.

Increase in the systemic activity of Nitric Oxide (NO), the most direct vasodilator, has been demonstrated during gestation in rats. [18]. High levels of the NO-inhibitor asymmetric dimethylarginine (ADMA) are associated with aberrant uterine blood flow in humans [19], suggesting the importance of NO-dependent vasodilation in pregnancy.

Prostacyclin is the main vasodilatory prostanoid. Its systemic level is elevated five-fold by term, whilst alternative vasoconstrictive prostanoids, such as the thromboxanes, remain at baseline levels [20].

The up regulation of the vasodilatory kalikrein-kinin system is a genuine observation in both rat and human, but the exact role of this remains elusive; notwithstanding, changes are prominent from early stages of pregnancy [21,22].
The overall purpose of these maternal circulatory changes is to make blood volume available for redirection to the pregnant uterus [23]. To accommodate this, low resistance is maintained in the uteroplacental unit, as shown by direct pressure measurements in the uterine vasculature of various species [24,25]. This helps achieve high blood flow through the uterine system and the mechanisms by which low resistance is maintained in the pelvic circulation in pregnancy will be elucidated in further detail.

1.1.2 Utero-placental microcirculatory adaptations

Similar to systemic maternal changes the local vasculature of the placental bed (spiral arteries) and their immediate supplier vessels undergo dramatic modifications during pregnancy in order to accommodate exaggerated blood flow [23,26-28]. A dramatic decrease in the impedance of spiral arteries is characterized by the atony of these vessels secondary to their remodelling, which results from them being invaded by extravillous trophoblasts [4] (Figure I-1).

Further changes are driven by the placenta and result in maximal vasodilation of both the immediate microvessels proximal to the placental bed and microvasculature of the placental villi, as vasodilatory mechanisms are readily activated on the feto-maternal interface. Endothelial Nitric Oxide Synthase (eNOS) activity is high in extravillous and syncytiotrophoblast, and this plays a key role in priming the uterine spiral arteries for trophoblast invasion [29,30]. Kallikrein and its vasodilatory receptor (B2R) are highly expressed by villous fetal endothelium, syncytio- and cytotrophoblasts [31,32]. Similarly, the vasodilatory angiotensin-(1-7) is highly expressed in trophoblasts, placental endothelium and perivascular smooth muscle [33]. So is the vasodilatory receptor of angiotensin IV (AT-4-R) [34]. Although these vasodilatory mechanisms are numerous they ultimately are limited in the vasodilation they can afford. Given these shortcomings, additional vascular modifications are needed based on vessel expansion and remodelling of mature endothelial cells, i.e. angiogenesis.
Figure I-1. A schematic of uterine–driven placental pathology.

In human placentation, extra-embryonal trophoblast cells remodel maternal uterine spiral arteries by (i) eliminating perivascular smooth muscle cells causing significant increase in their vessel bores and decrease in their resistance, resulting in sufficiently high flow for fetal supply (third panel). In some pathological situations, trophoblast invasion is insufficient and spiral arteries do not achieve optimal increase in calibre, potentially resulting in underperfusion (central panel). (ii) Extra-embryonal trophoblasts also eliminate the endothelial layer of spiral arteries and, the endothelium is replaced by transdifferentiated trophoblast cells or, as recently suggested, it is re-endothelialised. (With permission of Dr. Ian Crocker)

1.1.3 Angiogenesis at the utero-placental interface

1.1.3.1 Placental angiogenesis

While the blood flow increases through the uterus and placenta in a linear fashion during pregnancy, the placenta grows in its entirety from conception and the uterus expands greatly in size throughout gestation [35,36]. This implies that sufficient reduction of vascular resistance in these organs is not primarily achieved by vasodilation, but to achieve both these transformations, a strong angiogenic component is necessary [37,38]. In humans angiogenesis in the placenta commences on the 5th week of pregnancy. Initially, it consists of branching vessel growth associated with initial arboration of villi. This type of angiogenesis continues until week 24 gestation. This process is mainly driven by cytotrophoblast derived Vascular Endothelial Growth Factor-A (VEGF-A), and it is associated with a high expression of Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) in placental endothelium [39,40]. After this stage (associated with settling of the
villous branching process) non-branching angiogenesis then dominates, as characterised by increased endothelial proliferation. This process is primarily stimulated by placental growth factor (PIGF), an alternative member of VEGF-family, and is associated with the increased expression of VEGFR-1 [41,42]. Completion of the vascular structure by recruitment of pericytes and smooth muscle cells is driven by angiopoietins 1 and 2 which, along with their receptor (Tie-2), are highly expressed by the endothelium of newly formed villi and its surrounding cells [43-45].

Placental angiogenesis is a hypoxia driven process, mediated by oxygen-level dependent differential production of members of the VEGF family, controlled by the transcription factor hypoxia inducible factor (HIF-1) [46]. The change between the two major angiogenic factors (VEGF-A and PIGF) at around the end of the second trimester, seems to be associated with improvements in oxygenation of the placental tissue, as VEGF-A and VEGFR-2 are more prominent in severe hypoxia while PIGF and VEGFR-1 are more characteristic of low to moderate ischemia [47,48].

1.1.3.2 Uterine angiogenesis

In the adult circulation, unlike that of the placenta, angiogenesis is kept to a minimum, with the dominance of angio-static factors (angiostatin, endostatin, and thrombospondin) [49]. The main exceptions are the female reproductive organs, associated with menstrual cycle and pregnancy.

During human pregnancy, uterine angiogenesis occurs in an oxygen dependent manner, driven by the angiogenic agents produced by immune cells migrating to the site of implantation. Examples are decidual Natural Killer Cells (dNK cells), which excrete VEGF and PIGF in response to fetal-derived stimulation by interleukin-15 (IL-15) [50-52], and dendritic cells, which co-localize with the uterine microvasculature and excrete VEGF [53], further stimulating endothelial cells in the endometrium via the PI3K/Akt pathway [54]. These cells also produce pro-angiogenic Transforming Growth Factor β1 (TGFβ1) and soluble VEGFR1 (SFlt-1), which are modulators of decidual maturation, specifically that of the microvasculature [55]. Dendritric cells also stimulate giant cells of the trophoblast and in particular they stimulate their production of various angiogenic hormones including the
cell proliferation stimulant proliferin [56]. Regardless of these changes in uterus and decidua, from a gestational perspective angiogenesis is a relatively slow process, and involvement of some more effective mechanisms might be crucial.

1.1.4 *Endothelial Progenitor Cells in vascular adaptations and pregnancy disease*

Angiogenesis is based on cellular remodelling of mature endothelial cells, which have a low proliferative capacity and the vascular volume they produce is limited [57,58]. It could therefore be argued, that angiogenesis *per se* in the decidua or placenta, is insufficient in the extraordinarily labile environment of the pregnant uterus. Different cellular mechanisms for vascular expansion could therefore have a role; although not as comprehensively characterised, fast proliferating progenitor cells might be one component necessary to cope with such demands.

Within the last two decades, novel more productive mechanisms of vessel formation have been defined, driven by highly proliferative, so-called Endothelial Progenitor Cells (EPC) [59]. The reason why these mechanisms are more effective in producing high quantities of new endothelium is that EPCs have a much higher propensity for proliferation than mature cells, providing substantially larger endothelial surfaces given the higher number of daughter cells [60,61]. As angiogenesis is a volume-limited process, this higher potential of vessel formation may be important to help achieve sufficient vascular density in placental villi and the placental bed.

Satisfactory vascularisation of these key areas has major clinical importance. Insufficient vascularisation and aberrations of the placental vasculature are associated with some late pregnancy complications of proposed placental origin. These include IUGR and pre-eclampsia, characterised by suboptimal fetal size and my maternal high blood pressure, proteinuria and end-organ failure respectively, both associated with fetal and maternal morbidity and mortality [62-65]. In both cases, inadequate perfusion of the placenta has been implicated. In one scenario, insufficient expansion of maternal uterine microvasculature results in suboptimal perfusion of the placental bed, decreasing fetal supply, and potentiating fetal growth restriction [28]. Under such circumstances, maternal compensatory mechanisms are invoked to maintain optimal placental perfusion. As a
result, systemic blood pressure rises and pre-eclampsia ensues. In an alternative case, these compensatory mechanisms remain ineffective and fetal supplies remain insufficient reflecting in reduced fetal growth development.

As common obstetric complications, IUGR and pre-eclampsia are not only associated with short term fetal and maternal morbidity and mortality [65-68], but also with long term sequelae with deleterious effects on cardiovascular risk in particular [69-79]. Despite the recognised health implications of IUGR, [80-82], very little is known about the cellular biology that underscores aberrant vessel formation in the human placenta, and inadequate vascular transformation of the pregnant uterus that might pre-empt it. As a consequence this thesis set out to provide a greater insight into the cellular biology of vessel formation in the human placenta and pregnant uterus, specifically examining a physiological and pathophysiological role for fetal EPCs.

Presently the general study of EPCs is a novel scientific area with quick progress. As such controversy and misconceptions frequently pervade the field. The following section summarises available knowledge of EPCs. It is a published review article in its entirety, the remaining text of the introduction rationalises this knowledge to generate the specific hypotheses and aims for the study reported in this thesis.
1.2 PUBLISHED REVIEW ARTICLE

ENDOTHELIAL PROGENITOR CELLS: THEIR POTENTIAL IN THE PLACENTAL VASCULATURE AND RELATED COMPLICATIONS

Placenta, Volume 31, Issue 1, Pages 1-10, January 2010

Peter I. Sipos\textsuperscript{a}, Carl A. Hubel\textsuperscript{b}, Ian P. Crocker\textsuperscript{a}, Philip N. Baker\textsuperscript{b},
\textsuperscript{a}Maternal and Fetal Health Research Centre, University of Manchester, Manchester
\textsuperscript{b}Academic Health Science Centre, UK
\textsuperscript{b}Magee-Women’s Research Institute and Dept. Obstetrics, Gynecology & Reproductive Sciences, University of Pittsburgh, PA, USA

Keywords: Endothelial progenitor cells; Embryogenesis; IUGR; Pre-eclampsia; Placenta

Corresponding author:
Dr Peter Sipos, Maternal and Fetal Health Research Centre, The University of Manchester,
St Mary’s Hospital, Hathersage Road, Manchester, M13 0JL, UK
Tel: +44 7931254176, Email: peter.sipos@manchester.ac.uk
1.2.1 Abstract

Endothelial progenitor cells (EPCs) have received significant attention in recent times. A role for EPCs has been suggested in a range of pathologies and some recent studies of EPCs in pregnancy have been published. This review provides a guide to the confusing field of EPCs. Attention is paid to their phenotyping, as although elementary this remains a highly debated topic. The current understanding of different subtypes and physiological role of EPCs in the placenta, fetus and adult are also considered. An overview is given as to role of EPCs in the pathophysiology of different disease states and the possible therapeutic and diagnostic applications expected from EPC-related research in obstetrics.

1.2.2 Introduction

In 1997, Asahara et al. [59] proposed a new concept of neovasculogenesis and suggested that a cell type exists in the adult, which resides in the bone marrow and migrates to ischaemic sites to form new endothelium. They also proposed a culture technique for the isolation of such cells. These outgrowth cells were referred to as putative Endothelial Progenitor Cells (EPCs). Since then, more than 2000 EPC-related articles have been published and a relationship has been found between endothelial progenitors and dozens of disease states from pulmonary hypertension [83] to moya-moya disease [84]. EPCs are thought to be diagnostic of cardiovascular risk [85-89] [90,91] and are therapeutic in animal ischaemia models [92-95]. In hoping to find a cure for atherosclerosis, the main stream of EPC-related research has been conducted in the field of adult cardiovascular medicine, despite the fact that the presence of these cells has been known for much longer in the fetus and that vessel-related complications represent a significant clinical problem in pregnancy. This article provides a critical overview of the current literature and discusses avenues for further obstetric research. In doing so, we propose a potential role for EPCs in the pathophysiology of pre-eclampsia, intra-uterine growth restriction (IUGR), gestational diabetes and increased life-long cardiovascular risk associated with these conditions. The potential for EPCs to be used as diagnostic tools or therapeutic agents in pregnancy-related complications is also briefly considered.
1.2.3 Physiology of EPCs

Vascularisation is a new described model of vessel formation - as opposed to the previously recognised vasculogenesis and angiogenesis. This model was created once the presence of EPCs in the adult circulation was suspected. Vasculogenesis refers to the de novo formation of blood vessels from progenitor cells. Angiogenesis, in contrast, means the sprouting of new vessels or elongation from existing ones, through the remodelling of differentiated endothelial cells. Vascularisation in many ways resembles angiogenesis; however, in this case, EPCs contribute to neovessel formation by their incorporation into vessel walls, their secretion of paracrine hormones and subsequent angiogenic stimulation. Endothelial and blood cell precursors originate from the same ancestor: the haemangioblast [96,97]. EPCs were originally thought to be derivates of the haemangioblast and were defined as cells capable of proliferation, incorporation into forming blood vessels, and differentiation into mature endothelial cells. They were thought to reside in the bone marrow, leave after recruitment, and have the capacity to migrate and participate in vasculogenesis and vascularisation.

The expression “putative EPC” is rather historical, and the EPC population consists of two distinct sub-populations with very different phenotypes and functional capabilities, both closely involved in vessel formation. The first group is a haemopoietic subpopulation, the so called Circulating Angiogenic Cells (CACs), which have strong paracrine and hormonal activities and stimulate cell migration and proliferation [98-101]. The second, Endothelial Colony Forming Cells (ECFC), have more endothelial-like characteristics and are profoundly influenced by CACs. They are highly proliferative and migrate to sites of vessel formation, before differentiating into mature endothelial cells, thus forming de novo endothelium [61,102,103].

In isolating EPCs and defining these subtypes, cell culture techniques have been widely used. In some protocols, fibronectin coated dishes are employed and adherent cells are typically harvested within one week after seeding. These cells are frequently referred to as “early outgrowth cells”. The most common approaches include: the Asahara technique [59], Hill-Colony Forming technique [85] and Vasa-technique [104]. A different approach, using collagen-coated dishes and adherent cells harvested several weeks later, generates the so
called “late outgrowth cells”. This technique was originally described by Lin et al. in 2000 [102] (Figure I-2).

As the phenotypes and functions of early and late outgrowth cells are now clarified, cells obtained with early outgrowth techniques are practically considered CACs, while those produced with late outgrowth protocols are deemed ECFCs. In accord with current opinion, these terms will be used throughout the rest of this article: using the term EPC, where the actual sub-population is not determined, or where the comment applies equally to both CAC and ECFC phenotypes.

Figure I-2 Bright-field images of outgrowth cells from human fetal peripheral blood mononuclear cells (PBMNCs).
A: CFU-Hill technique. Round cells in the centrum of the colony, spindle shaped cells in the periphery. The colonies appear to be smaller than in adult and spindle shaped cells migrate away from the centre rapidly. (Scale bar: 50µm) B: Vasa technique; monolayer of round adherent cells. (Scale bar: 100µm) C: Lin-technique: Colony formed by a monolayer of cells with cobble stone appearance. The colonies are larger and appear earlier than those in the adult. (Scale bar: 50µm) Unpublished images by the authors.
1.2.4 EPC-phenotypes

Although ECFCs and CACs can be isolated by the above culture techniques and by flow-cytometry, no standardised and accepted means of phenotyping has yet been defined, and there are several different, partly contradictory, approaches found in the literature. Consequently, confirming cell-phenotype is a core agenda and remains a major issue. The identity of outgrowth cells or those acquired by flow-cytometry is determined by the expression of tissue-specific surface markers. In addition, functional assays can contribute to the verification of endothelial or progenitor progeny.

1.2.4.1 Flow cytometry techniques

To date no EPC-specific antigen has been defined. Instead, various combinations of haematopoietic and endothelial stem-markers are proposed. It has been postulated that at the angioblast stage of development, i.e. before the loss of haematopoietic markers, cells start expressing endothelial antigens. The three antigens most commonly considered are CD34, CD133 and KDR/VEGFR-2.

(i.) CD34 is a single pass transmembrane sialomucin. As a cell-cell adhesion factor, it may mediate the attachment of stem cells to bone marrow extracellular matrix or directly to stromal cells. It is expressed on numerous cell types of mesodermal progeny, including blood cells, endothelial and fibroblast cells, epithelial and cancer stem cell populations. It is expressed by all haematopoietic stem cells, but it is subsequently lost upon differentiation [105,106]. It is also expressed by many cells of endothelial progeny, including differentiated endothelial cells in the adult [107].

(ii.) CD133 or prominin-1 is a 5-transmembrane domain cell-surface glycoprotein. It is localised on membrane protrusions of epithelial, haematopoietic and cancer stem cells. It is strongly expressed on haematopoietic stem cells, but also disappears during haematopoietic differentiation [108].
(iii.) KDR or VEGFR-2 (Human) is a tyrosine-kinase transmembrane receptor of vascular endothelial growth factor (VEGF). It is expressed by both early haemopoietic stem cells and endothelial cell-lines, but its expression also ceases with haematopoietic differentiation [109,110].

It was Asahara et al. that showed that CD34 enriched KDR+ cells localise to sites of vasculogenesis [59], and Peichev et al. that suggested the addition of CD133 to differentiate EPCs from mature endothelial cells. They concluded that circulating CD34+/KDR+/CD133+ cells represent a distinct population with a role in neo-angiogenesis and CD34+/KDR+/CD133+ represent a more mature population of progenitors [111]. In 2006, Friedrich et al. identified a new CD34+/CD133+/KDR+ EPC sub-population which differentiated into CD34+/133+/KDR+-defined EPCs in culture [112]. Currently, most available research is based on these phenotypes; however direct clonal evidence is not usually provided and these techniques notably fail to distinguish CACs and ECFCs. More recently the use of Fluorescence Activated Cell Sorting (FACS) and PCR has shown that ECFC from peripheral or umbilical blood are inevitably CD34 positive and CD45 negative, and that they also express KDR but not CD133 [113]. This population represents approximately 2% of all CD34+ mononuclear cells. Timmermans et al. also showed that CACs belong to the CD14+ monocytic sub-population of CD34+/CD45+ cells and although they co-express CD133 they never express KDR [113]. This CD34+/KDR+/CD133+ triple-positive population has since been shown to be neither CAC, nor ECFC in origin, but nevertheless they are primitive haematopoietic progenitors [114], and some suggest they fit with a more broader definition of CACs [115,116].

In summary, ECFCs can be characterised by the surface marker-combination of CD31+/CD34+/CD45+/KDR+/CD133+, but it must be noted that this combination does not differentiate ECFCs from circulating endothelial cells. Alternatively, CACs can be characterised by the surface marker-combination of CD31+/CD34+/CD45+/CD14+/CD133+/KDR+. However, some suggest that CD34+/KDR+/CD133+ may also be considered CACs [115,116]. It should also be noted that the specificity of these flow cytometry-techniques, as based on these antigen-
combinations, can be significantly improved by excluding false-positive events, i.e. those of non-vital cells and CD3 positive T-lymphocytes. **Figures I-3 and I-4** show typical gating strategies in flow cytometry, and they demonstrate the current understanding of surface antigen expression patterns of two EPC subpopulations, CACs and ECFCs.
Figure I-3. Illustration of flow-cytometry technique for surface marker distribution of ECFCs in fetal peripheral blood.

A: Typical Forward Scatter-Side Scatter Histogram of lysed blood; Gate 1 set over mononuclear leucocytes. B: 7AAD-monohistogram. Gate 1 applied. Gate 2 set over 7AAD-negative (viable) cells. Non-viable cells have the tendency to non-specifically bind antibodies. Exclusion of non-vital cells helps reduce the number of false-positive events. C: CD45 monohistogram. Gates 1 and 2 applied. Gate 3 set over the CD45-negative population. D: CD31 monohistogram. Gates 1, 2 and 3 applied. Gate 4 set over the CD31-bright population. E: X-axis represents CD133; Y-axis represents CD34. Gates 1, 2, 3 and 4 are applied. Gate 5 set over CD34+ and CD133- populations, while Gate 6 segregates CD34+ and CD133+ dual positive cells. Gate 5 highlights CD45-/CD31Bright/CD133-/CD34+ events, consistence with an ECFC/CEC identity. Non-vital cells are excluded. Unpublished images of the authors. Flow cytometry technique modified from Duda et al. 2007 [230]. (For methods see 6.1.2.2)
Figure I-4 Illustration of flow-cytometry technique for surface marker distribution of CACs in fetal peripheral blood.

A: Typical Forward Scatter-Side Scatter Histogram of lysed blood; Gate 1 set over mononuclear leucocytes. B: 7AAD-monohistogram. Gate 1 applied. Gate 2 set over 7AAD-negative (vital) cells. Non-vital cells may non-specifically bind antibodies. Exclusion of non-vital cells helps reduce false-positive events. C: CD45 monohistogram. Gates 1 and 2 applied. Gate 3 set over CD45-dim population. D: CD31 monohistogram. Gates 1, 2 and 3 applied. Gate 4 set over CD31-positive population. E: X-axis represents CD133; Y-axis represents CD34. Gates 1, 2, 3 and 4 applied. Gate 5 set over CD133\(^{+}\) and CD34\(^{\text{Bright}}\) population. This population is CD45\(^{\text{Dim}}\)/CD31\(^{-}\)/CD133\(^{+}\)/CD34\(^{\text{Bright}}\). Non-vital cells are excluded. These features are characteristic to CACs. Unpublished images of the authors. Flow cytometry technique modified from Duda et al. 2007 [230]. (For methods see 6.1.2.2)
1.2.4.2 Cell culture techniques

The confirmation of the phenotype of outgrowth cells in culture is another essential validation process. Differentiating CACs from ECFCs requires distinct methods and many authors fail to achieve this sufficiently or even differentiate EPCs from epithelial or mononuclear cells. Originally it was believed that a combination Dil-AcLDL-uptake and positive Ulex europaeus-1 Agglutinin binding test [117,118] (two probes traditionally used to identify vascular tissues) was sufficient to confirm EPC-identity. However, the specificity of this dual-positivity test has now been discredited [119-123].

Further confirmation is therefore essential, and this is typically achieved by surface marker recognition. ECFCs grown by the Lin-technique express endothelial surface markers, such as CD31, CD141, CD105, CD146, CD144, von Willebrand Factor (vWF), and KDR, but are invariably negative for haemopoietic surface markers such as CD45 and CD14. Morphologically they resemble endothelial cells with a cobblestone appearance. They form capillary-like tubes in three-dimensional culture, a feature unique to endothelial cells, and also incorporate into newly forming blood vessels both in vitro [103] and in animal models of xenograft implantation [124].

CACs obtained from culture are monocytic cells, positive for CD45 and CD14 and the classic EPC markers CD34 and CD133; they are also negative for KDR. They readily incorporate Dil-AcLDL and stain with UAE-1 and unlike ECFCs, have a monocytic morphology in culture (Figure I-5).
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Angiogenesis assay</th>
<th>Dil AC-LDL uptake</th>
<th>CD31</th>
<th>CD34</th>
<th>CD133</th>
<th>CD45</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECFC</td>
<td>![Image H]</td>
<td>![Image I]</td>
<td>![Image J]</td>
<td>![Image K]</td>
<td>![Image L]</td>
<td></td>
</tr>
</tbody>
</table>

Figure I-5 A comparison of CAC and ECFC characteristics.
ECFCs form tubes on matrix, while CACs fail to do so. Both CACs and ECFCs ingest AC-LDL, and express CD31 and CD34. CACs are CD45 and CD133 positive; whilst ECFCs fail to express these antigens. (Scale bar: 100µm (A-H, K-M); Scale bar: 10µm (I-J)) Unpublished images of the authors. (For methods see 6.1.2.1)
1.2.4.3 Functional capabilities of endothelial progenitors

EPCs are thought to behave similarly to previously known progenitor cells, i.e. (i) they interact with each other and other cell types, (ii) they are recruited and mobilised mainly from the bone marrow, (although residing ECFCs have been isolated from human large vessel walls [125] and cells with CAC-characteristics are also isolated from the spleen [126], liver [127] and adipose tissue [128], and equally, it cannot be excluded that EPCs are produced in the placenta itself, as it is a site of fetal haematopoiesis [129-131]), and (iii) they migrate to sites of vessel formation, invade locally, proliferate and differentiate into mature endothelial cells. Each of these basic functions is elucidated below:

1.2.4.3.1 (i) EPC cell-cell interactions and signals

EPCs have dominant hormonal activity and a main consideration is the co-operative work that occurs between ECFC and CAC subpopulations. As mentioned, CACs are thought to play an essential role in stimulating ECFCs. However, feedback mechanisms also exist. Vascular Endothelial Growth Factor (VEGF), Stromal Cell Derived Factor-1 (SDF-1) and Interleukin-8 (Il-8) [132,133] are secreted by CACs and these act on ECFCs as stimulants and chemoattractants [134]. Furthermore, Il-8 is also released by ECFCs, which is chemoattractant to CACs [135], and mitogenic to ECFCs in an autocrine/paracrine fashion [136]. The activation of the thrombin receptor, Protease Activated Receptor 1 (PAR-1), increases ECFC-proliferation and their organisation into tubal structures [137,138]. Moreover, stimulation of PAR-1 significantly increases the amount of Il-8 excreted by ECFCs [133]. These findings suggest a complex stimulatory positive feedback mechanism which helps maintain and enhance the proliferative capability of ECFCs in situ.

The mobilisation of progenitor cells from the bone marrow requires the degradation of extracellular matrix. In this context, Matrix metalloproteinase-9 (MMP-9) is secreted by CACs and Matrix metalloproteinase-2 (MMP-2) is produced by ECFCs, and assists in the breakdown of collagen-IV [132]. In response to VEGF and Il-8, ECFCs increase MMP-2 production and CACs increase MMP-9 secretion [132]. This may partly explain the
increased invasiveness and vasculogenic activity of both cell types in co-culture, as the interaction between the MMP subtypes may also be significant [139].

In general terms, fat-soluble substances are less volatile in the circulation and their concentrations remain higher in the local micro-environment, therefore their effect on neighbouring cells may be more pertinent. Consequently, fat-soluble substances like endocannabinoids, produced by EPCs, may represent important paracrine signals. EPCs produce and release two sub-types of endocannabinoids: anandamide (arachidonylethanolamide; AEA) and 2-arachidonoylglycerol (2-AG). CACs secrete higher levels of 2-AG, while ECFCs secrete higher levels of anandamide. Adult blood derived ECFCs secrete more anandamide than cord blood ECFCs [140].

Although endocannabinoid secretion-patterns of EPC sub-populations show marked differences, the significance of this pattern and the exact role of different endocannabinoids in various cell types remain unclear. What is known is that endocannabinoids suppress the induction of the pro-inflammatory Vascular Cell Adhesion Molecule 1 (VCAM-1), which is a facilitator of leukocyte-endothelium adhesion. In this respect, these EPC derivatives may act as anti-inflammatory agents. For cord blood ECFCs pro-inflammatory Tumor Necrosis Factor-α (TNF-α) increases anandamide-excretion, but decreases endocannabinoid-release from CACs [140]. In as yet undefined ways, this differential affect may hold significance in the pathogeneses of certain pregnancy-related conditions.

1.2.4.3.2 (ii) EPC mobilisation and recruitment

Progenitor cells reside in stem cell niches of the bone marrow from which they leave and differentiate in response to micro-environmental changes. As suggested, MMP-9 has a crucial role in mobilising progenitor cells from this stem cell niche. Kit Ligand (also called Stem Cell Factor: KitL or SCF) is an adhesive molecule, which keeps EPCs bound to the osteoblastic niche of the bone marrow. MMP-9 catalyses the proteolysis of KitL from the cell membrane and a soluble form is released. The proteolysis of KitL improves the mobility of progenitors and their transfer to the vascular-enriched niche, which is a favourable environment for further differentiation and egress into the peripheral circulation [141]. The expression of proMMP-9 and active MMP-9 is nitric oxide (NO)-dependent [142], and
VEGF also activates the enzyme [141]. Therefore, the mobilisation of EPCs is considered NO-dependent and stimulated by VEGF. This pathway is similarly exploited by estradiol, which likewise augments the mobilisation and proliferation of bone marrow–derived EPCs and their incorporation into a recovering endothelium [143,144] (Figure I-6).

**Figure I-6. A schematic of EPC-mobilisation.**

EPCs are tied in the osteoblastic niche by Kit Ligand, which is broken down into soluble KitL by MMP-9 via an NO-dependent VEGF and oestrogen-driven mechanism, allowing the mobilisation of EPCs from the osteoblastic niche. SDF-1 promotes ECFC progression into the haemopoietic niche, while Endothelial Monocyte Activating Polypeptide 2 (EMAP-2) promotes progression into the blood stream in the event of hypoxia.

Other stimuli for the recruitment of EPCs are Stromal Cell-Derived Factor-1 (SDF-1), a significant chemoattractant released by platelets in large quantities [145], and reactive oxygen species (ROS), end products of NOX-2 activity - the enzymatic core of NADPH-oxidase. In reply to endothelial activation, ROS stimulate diverse signalling pathways leading to angiogenic-related responses. Numerous stimuli including angiogenic growth factors, cytokines, shear stress, hypoxia, and G protein–coupled receptor agonists are implicated [146]. Specifically, VEGF has an important role in signal-induced migration,
proliferation and reparative angiogenesis [147] whilst, NOX-2 derived ROS are involved in the homing, chemotaxis, invasion, and actin-polarisation of bone marrow derived-EPCs [148] (Figure I-7).

Figure I-7. A schematic of EPC-recruitment.

SDF-1 is released in response to hypoxia, mechanical forces, exposure to extracellular matrix inflammatory (Infl) mediators, and also by platelets when recruited to sites of vessel injury. In addition to VEGF and hypoxia, SDF-1 is responsible for the recruitment of EPCs.

1.2.4.3.3 (iii) EPC homing, migration, invasion, proliferation and differentiation.

Once in the vicinity of ongoing vasculogenesis, progenitor cells interact with the pre-existing endothelial monolayer. The adhesion molecules P-selectin and E-selectin mediate the initial steps of this interaction, with the activation of Eph-B4 receptor by Ephrin B2 increasing EPC-affinity [149]. Integrins on the surface of EPCs further mediate adhesion and facilitate transmigration [149]. In this context, high-mobility group box 1 (HMGB1) activates β1 and β2 integrins, encouraging homing to hypoxic areas [150], whilst α-4 integrin promotes recruitment to sites of tissue remodelling [151] (Figure I-8).
Target-to-ECFC contact is initiated by the Ephrin-B2 secreted by the target cells. Ephrin-B2 stimulates the expression of P-selectin Glycoprotein Ligand-1 (PSGL-1) on the surface of ECFC, which then binds to P-and E-selectins found on target cells. Hypoxia stimulates SDF-1 secretion by the target via the Integrin Linked Kinase (ILK) pathway. SDF-1 then stimulates ECFCs to express α-integrin, which binds to the Intracellular Adhesion Molecule-1 (ICAM-1) on target cells. As a response to tissue necrosis, cells produce fibronectin, which β1 and β2 integrin bind to. Vascular Cell Adhesion Molecule-1 (VCAM-1) binds to α4β1-integrin.

To achieve transmigration, extracellular matrix degradation by EPCs must occur. In this context, ECFC derived matrix metalloproteinase-2 (MMP-2), secreted in response to increased VEGF, plays a role in the degradation of collagen-IV [139] and Cathepsin L is an essential protease in CAC invasion [152].

For resident EPCs, VEGF, SDF-1 and mechanical forces may initiate differentiation, with Homebox transcription factor A9 (HoxA9) acting as a master switch in the expression of endothelial-committed genes, i.e. endothelial nitric oxide synthase (eNOS), VEGFR-2, and VE-cadherin [153,154]. Peroxisoma-proliferator activated receptor-δ (PPAR-δ) is also considered to induce proliferation of EPCs and has reported anti-apoptotic effects [155].
Overall, EPCs not only have complicated cell-cell interactions, but also have well defined functional roles. Impairment in any of these functional steps could result in defected vasculogenesis. These steps can be influenced by agents targeting the messengers or ligands known to be involved in these processes. Testing of these functionalities is the basis of currently used EPC assessments, i.e. combinations of migration, invasion, tube forming assays and xenograft models of implantation.

1.2.5 Endothelial progenitors and the developing fetus

Haemangioblasts reside in the blood islands of the early embryo [96] and during embryogenesis undergo differentiation, with those in the periphery giving rise to endothelial progenitors, and those in the centrum of the blood islands generating haematopoietic precursors - having potentially undergone a prior haemotogenic endothelial stage [156]. Although no direct evidence is available to confirm their origin, the angioblasts observed in the fetus are identical with ECFCs, and their phenotype and capacity to proliferate and differentiate are undoubtedly analogous. These angioblasts express Vascular Growth Factor Receptor (VEGFR), Vascular Endothelial-cadherin (endothelial junction molecule, CD144), but not CD45 (pan-haemopoietic) surface markers. While conversely, haemopoietic progenitors (haematoblasts) express CD45 and c-Kit (a cytokine receptor on the surface of haematopoietic stem cells, also known as CD117). With the diverging point in development of haematoblast and angioblast at the VEGFR+/VE-cadherin-/CD45- stage [97].

During the maturation of blood vessels, the expression of surface markers follows a specific sequence: TAL1 (T-cell acute Lymphocytic Leukaemia-1) and VEGFR are expressed first, followed by the Platelet Endothelial Cell Adhesion Molecule (PECAM; CD31), CD34, VE-cadherin, and later Tie-2. Subsequently, TAL1 is down regulated in the endothelial cells of mature vessels [157]. Loose aggregations of cells expressing endothelial markers have been observed throughout the entire embryonic mesoderm, except on the notochord and precordial plate, and these aggregations have been shown to form definitive blood vessels [158]. Some endothelial lineages may also develop directly
from the mesothelium without passing through the haemangioblast stage. However, this mode of vessel formation is primarily present in the somites [159].

Although several signals are known to control vasculogenesis in the fetus, none have yet been defined for the induction of angioblasts or haemangioblasts. Whilst, endodermal signals (e.g. those which influence the Indian Hedgehog and Wnt signalling pathways) have been recognised in the formation of tubal structures [160], Fibroblast Growth Factor (FGF) and Bone Morphogenetic Proteins 2 and 4 (BMP 2 and 4) are proposed modulators of haemato-endothelial differentiation [161] and early vasculogenensis [162]. The most proximal signalling molecule in endothelial development is Vascular Endothelial Growth Factor (VEGF) [163], which forms a complex with neurophilin-1 (NP1), a semaphorin receptor, that enhances its binding to VEGF receptor-2 (VEGFR-2) resulting in anti-apoptotic signals, and the promotion of endothelial progenitor survival [164].

Additional intracellular and extracellular signals for vessel formation are multiple and include, Hypoxia Inducible Factor-1 (HIF-1), Endothelial growth factor-like-domain multiple 17 (Egfl-17), neurophilins, plexins and Ephrin-B2. Of these, HIF-1 induces blood vessel growth at sites of hypoxia [165], whilst Egfl-17 is essential in the linear arrangement of angioblasts. Neurophilins and plexins, two classes of semaphorin receptors, direct microvessel growth and branching to their targets [166], whilst Ephrin-B2, a membrane ligand exclusive to arterial cells, matches the arterial and venous ends of growing capillaries, through reciprocal signalling with its opposing receptor, Eph-B4, which is uniquely expressed on the venous endothelia [167].

Finally, platelet derived growth factor (PDGF) and tumor necrosis factor (TNF) act as chemoattractants, recruiting pericytes and smooth muscle cells, which ultimately form into complex vascular structures [168] (Figure I-9).
Mesodermal stem cells give rise to haemangioblasts, a process controlled by wnt, Hedgehog, Fibroblast Growth Factor (FGF). Angioblasts/ECFCs develop from haemangioblasts, similarly to haematoblasts, according to classical embryology (driven by Bone Morphogenic Proteins 2 and 4 (BMP2 and 4)). According to more recent understanding haemopoietic cells pass endothelial stage prior to becoming committed haematoblasts. Following differentiation (VEGF-Neuropilin complex, HIF-1, BMP), assembly of complex arteriovenous vascular structures takes place, stimulated by the factors illustrated in the schematic diagram.
1.2.6 EPCs in pregnancy related conditions

Aberrant vasculature and abnormal endothelial function on both the maternal and fetal side are thought to play a role in the pathogenesis of certain pregnancy related complications and in the pathogenesis of pre-eclampsia and intrauterine growth restriction in particular. The pathogenesis of aberrant vessel-formation may be the impaired bioavailability or function of the EPCs forming them. Therefore, altered EPC-function may contribute to both these pregnancy complications.

It is clear from the literature that many authors have worked with different hypotheses regarding the role of EPCs, and that the EPC-phenotypes investigated have been often wrongly identified and insufficiently verified. Therefore, many past studies should be interpreted and compared with caution.

1.2.6.1 Clinical significance of EPCs in pregnancy

There have been limited reports of EPC number and function in normal pregnancy and in pregnancies complicated with pre-eclampsia, and the results obtained have been somewhat contradictory. Sugawara et al 2005, found that the number of outgrowing EPCs increased gradually with gestation in normal pregnancy and correlated with the levels of serum estradiol [169], whilst Buemi et al 2007 showed similar results by flow cytometry [170]. In the latter, the antibodies to detect EPCs were inconsequently selected and results expressed as a percentage of the total white cell counts (instead of absolute numbers), something inappropriate given the physiological variations between pregnant and non-pregnant states. In opposition to these findings, Savvidou et al. found that EPC numbers are decreased in normal and multiple pregnancies compared to non-pregnant individuals. They also found a gradual decrease across gestation. EPC numbers in this study were determined as a ratio of UEA/Ac-LDL double positive cells compared to all fibronectin-adherent peripheral blood mononuclear cells (PBMNCs), but no further verification was attempted [171].

One of the first studies monitoring maternal EPCs hypothesised a role in the pathogenesis of preeclampsia. Sugawara et al. found that the number of circulating EPCs was decreased
in women with preeclampsia as defined by the CFU-HILL count. The rate of cellular senescence was also significantly increased in these pre-eclamptic patients [172]. Nevertheless, a more recent study failed to distinguish a difference between EPC numbers in peripheral blood in normal and pre-eclamptic pregnancies, but the proliferation of pre-eclampsia-derived EPCs in culture was significantly exaggerated [173]. Overall, it could be said that the assessment of EPCs in normal pregnancy and pre-eclampsia requires significant input, as even the basic issues regarding their number and function remain unanswered.

1.2.6.2 **Clinical significance of EPCs in the developing fetus**

It would seem biologically plausible that progenitor cells are more readily available in fetal life and more active than those in the adult. Ingram et al. performed a head to head comparison of umbilical and adult ECFCs. They found that EPC concentrations were 15 times higher in cord blood than in the adult peripheral circulation. In culture, fetal colonies emerged a week earlier than adult colonies and they were consistently larger, while the cells forming them were smaller in size. Cord blood ECFC-populations doubled at least 100 times without any signs of senescence, while adult ECFC did not exceed more than 20 to 30 population doublings. The population doubling time was 2.5 fold shorter in cases of fetal ECFCs. Moreover, they responded with more active DNA-synthesis to endothelial mitogenic stimulants [61].

These results not only confirm that fetal ECFCs are more abundant and more active, but also suggest that fetal and adult ECFCs may not be clonally identical entities. This was further elucidated in a single-cell culture model, where three different cell populations with different phenotypes have been established. The first cell population forms large colonies, which after re-plating form secondary colonies and tertiary colonies in 9% of cases. One single cell gives rise to as many as $10^7$-$10^{12}$ descendent cells and their telomerase-activity is high, but they are not immortalised. The second cell population forms colonies larger than 50 cells, but they cannot be successfully re-plated. The third population forms colonies of less than 50 cells. The terms High Proliferative Potential Endothelial Colony
Forming Cell (HPP-ECFC), Low Proliferative Potential Endothelial Colony Forming Cell (LPP-ECFC) and Endothelial Cell Cluster (ECC) were suggested to describe these identities. Notably, HPP-ECFC differentiate into LPP-ECFC and ECC, while LPP-ECFC differentiate into ECCs. Strikingly, the most active form - HPP-ECFC - is not present in the adult, and is unique to umbilical blood.

Fetal ECFC numbers appear to change during pregnancy, gradually increasing with advancing gestation. Javed et al. found that ECFC numbers - determined as ECFC colonies per umbilical blood volume - are constant between 24 and 31 weeks of gestation, increase to double this between 32 and 36 weeks, and triple this between 37 and 40 weeks [174]. Whether this reflects an increase in production or reduced consumption in the advanced stages of pregnancy is still unclear.

In addition, Case et al. [175] evaluated the effect of oxidative stress on the proliferative potential of different classes of ECFCs in a single cell culture assay. Oxidative stress reduced the proliferative potential of each sub-population, but the proliferative potential of HPP-ECFCs was affected more significantly than those of LPP-ECFC and EC clusters. Oxidative stress also reduced the vasculogenetic capacity of ECFCs in tubulogenesis assays in vitro and in murine xenograft implantation models. Surprisingly, adult ECFCs were more sensitive to oxidative stress in this respect.
1.2.7 Perspectives and possibilities

Considering the available evidence, it could be envisaged that EPCs have a yet unrecognised role in the patho-mechanistic origins of placental-derived pre-eclampsia and IUGR, and perhaps also the associated increase in long-term cardiovascular risk. In fact, EPCs could pose a plausible explanation linking these pregnancy complications and Barker’s hypothesis [70], i.e. the associated connection between sub-optimal intrauterine growth and adult chronic disease.

In the first instance, it could be envisaged that anomalies in either CAC numbers of recruitment to the early placenta, or the secondary attraction and function of ECFCs, may impair formation of normal placental vessels and influence the embryonic endothelium. In this respect, the significant reduction in proliferative potential of HPP-ECFCs in response to oxidative stress may play an important role. Similarly, functional irregularities could impact on the maternal side, affecting uterine vessels, restricting or attenuating intrauterine blood flow and thus perpetuating placental disease. In this regard, the potential mechanisms are still unclear, but may involve hypoxia or oxidative stress, two proposed utero-placental features of pre-eclampsia and IUGR. In both cases, EPCs (most likely CACs) would be first attracted via HIF-mediated signals, followed by the secondary attraction of ECFCs. In pathological conditions, i.e. under excessive hypoxia, EPC numbers and function may be impaired; whilst in conditions of excessive ROS, peroxynitrite formation could reduce the bioavailability of NO, and thereby influence both EPC mobilisation and recruitment. Given these possibilities, further research is undoubtedly warranted.

In a study by Ingram et al. [124], in vitro hyperglycaemia and the diabetic intrauterine environment resulted in impaired ECFC-colony formation, reduced proliferative capacity and functional irregularities both in angiogenic and xenograft transplant assays. The sensitivity of ECFCs to the hyperglycaemic state may be one explanation for the increased risk of pre-eclampsia in diabetic pregnancies and again may link the diabetic environment to increased life-long cardiovascular risk.
1.2.8 Summary

The precise phenotype of endothelial progenitor cells remains to be determined, and different approaches make it difficult to compare previous studies. There is indirect evidence that both EPC subtypes play a role in the vasculogenesis of the human placenta. However, there is limited reliable literature about fetal EPCs and their role in the pathogenesis of pregnancy-related complications. Nevertheless, the existing evidence would suggest a potential role and both diagnostic and therapeutic clinical tools might be developed through a better understanding between EPCs and placental vascular complications.

It could be envisaged that a significant amount of clinical benefit would be expected from this field: (i) methods designed to assess EPC numbers and function in pregnancy may be developed into diagnostic tools, (ii) EPCs could be direct targets of medical interventions - medications known to improve EPC function are listed in Table I-1, (iii) EPCs could be developed as therapeutic tools themselves, for either autologous or donor cell therapies in the pregnant women or fetus, (iv) autologous ECFCs may be utilised to form artificial blood vessels, which can bypass occlusions in the adult or replace larger vessels in cases of congenital vascular abnormalities in the fetus or new-born, and (v) in the case of pathologies associated with excessive vessel formation, such as malignancies, EPCs may be targets of therapy that impair their function or carry anti-tumor agents directly to sites of tumorigenesis. Time will tell whether these potential benefits will be realised.
<table>
<thead>
<tr>
<th>Pharmaceutical agent</th>
<th>EFFECT on EPCs</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR-δ agonists [176]</td>
<td>Stimulates CAC-proliferation and mobilisation. Antiapoptotic.</td>
<td>PI3K/Akt pathway.</td>
</tr>
<tr>
<td>Nifedipine [177]</td>
<td>Increases CAC-number, function, resistance to oxydative stress.</td>
<td></td>
</tr>
<tr>
<td>Angiotensin-II [178]</td>
<td>CAC senescence.</td>
<td>Angiotensin type 1 receptor (AT1R)</td>
</tr>
<tr>
<td>Angiotensin-II inhibitors losartan [179], candesartan [180], olmesartan and ilbesartan [181]</td>
<td>Increased EPC-numbers, migration.</td>
<td>C-kit-expression through anti-oxidative mechanisms.</td>
</tr>
<tr>
<td>Erythropoietin [182,183]</td>
<td>Increased CAC, mobilisation, antiapoptotic. NO-synthesis.</td>
<td>NO-dependent</td>
</tr>
<tr>
<td>Statins</td>
<td>Elevated EPC number in bone marrow and circulation [184] Integrin-upregulation [185].</td>
<td>PI3K/Akt pathway.</td>
</tr>
<tr>
<td>Sildenafil [186]</td>
<td>Increased EPC-numbers.</td>
<td></td>
</tr>
<tr>
<td>Estradiol</td>
<td>Mobilisation and proliferation [143].</td>
<td>NO and MMP-9-dependent [144,187]</td>
</tr>
<tr>
<td>Physical exercise [187,188]</td>
<td>Increased EPC number, capacity and inhibited apoptosis.</td>
<td>NO-dependent increase of VEGF level</td>
</tr>
<tr>
<td>Prostaglandin E 1 [189]</td>
<td>EPC mobilisation and improved function.</td>
<td>NO-dependent, Increased CXCR4 expression.</td>
</tr>
</tbody>
</table>

Table I-1 Pharmaceutical agents known to influence EPC number or function.
The pharmaceutical agents included are accompanied by effects on EPCs and modes of action, where known.
1.3 THESIS CONSIDERATIONS

The review above highlighted two relevant points: (i) The outcome of extensive research with regards to the physiological importance and cell identity of true EPCs - which resulted in the consensus, that ECFCs are true EPCs and they have the highest direct impact on vessel formation and (ii) the limited literature on EPCs in the mother and fetus, suggesting the existence of a link between pathological conditions in intrauterine life and EPCs. Based upon these considerations, this thesis is specifically concerned with fetal ECFCs and their involvement in optimal and suboptimal fetal growth and pregnancy. The next section will briefly expand on the reasons for this ECFC focus and crystallise a hypothesis regarding the role of fetal ECFCs in IUGR.

1.3.1 ECFCs study focus

As indicated, debates regarding the identity of cells responsible for and carrying out EPC function have been long ongoing. Different authors proposed several different methods of isolation for a wide range of cell phenotypes as putative EPCs. The common characteristic of these cells was that they all appeared after a relatively short period of culturing, thus they were summarised as early outgrowth cells [59,85,104]. The details used for identification of these cells have significantly varied and in many cases the phenotyping attributed to the specific cell type proposed was not sufficiently rigorous to identify one single cell type. All of these proposed poorly classified cells were referred to as EPCs. The cell characteristics were often contradicting and unclear. Initially, dual staining with UEA-lectin and uptake of Ac-LDL on its own were considered sufficient criterion for peripheral blood derived cells to be named EPCs [59,85,104,190,191]. Even at later stages there was no clear definition of an EPC-specific phenotype, and practically any blood-borne cell found co-localised with vessels in a vessel injury model was accredited as an EPC, without further insight into their cellular origin or function [92,126,143,144,185,188,192-194]. Likewise, crude flow cytometric techniques were initially used to calculate EPC numbers using only two non-specific discriminators (usually combinations of KDR, CD133 or CD34) [87,90,115,170,173,186,187]. With hindsight these measurements would have been
hampered by false-positive recognition of dead cells, thus outcomes and published results were unsurprisingly confusing [91,115].

More detailed work demonstrated that the various cell types considered as EPCs by early investigators are in fact haemopoietic cells [112,113,195-197]. This too added to the general confusion, when these cells were shown to be monocytes [198,199]. Some authors suggested that progenitors of endothelium develop by trans-differentiation of these monocytes. [86], but this seems highly unlikely, considering recent evidence that haemopoietic cells pass an endothelial stage prior to committing to a haemopoietic lineage, rather than developing in parallel with endothelial lineages from a common haemangioblast precursor [156].

Together these results suggested that putative EPCs, considered as early outgrowth cells, are incapable on their own of providing the cellular basis for new vascular endothelium [200]. Nevertheless, it has since transpired that these cells in fact co-localize with new vessels for a particular, but different reason. Although they do not directly provide the new endothelium, as previously thought, they are closely involved in the process of vessel formation, significantly augmenting the process [201]. Specifically, they are involved in altering the extracellular matrix in preparation of growth of the vessel, interacting with pre-existent endothelial cells to target vessels, and attracting and stimulating those progenitors truly responsible for endothelial formation [201]. Considering this function, early outgrowth cells, have been subsequently summarised as Circulating Angiogenic Cells (CAC) [101,103,132,201].

Whilst the definition of CACs was being forged, Lin et al isolated a new blood borne endothelial cell type, whose origins from the bone marrow were proven in a human using a bone marrow transplantation model [102]. These cells took several weeks before appearing in culture and were therefore labelled late outgrowth cells. The Yoder/Ingram group established a distinct set of morphological and in vitro as well as in vivo functional criterion, by which this cell type can be precisely phenotyped [202]. They introduced the term Endothelial Colony Forming Cells (ECFCs) as the currently accepted nomenclature and established their vasculogenic activity when implanted into mice. Sieveking, Opitz and others elegantly demonstrated in vitro, how the primary vasculogenic function of ECFCs is
augmented by CACs [103,132,140]. As such it is now a widely accepted consensus that ECFCs function as true endothelial progenitor cells, providing new endothelium, while they are assisted by CACs in this role, stimulating them and interacting with the intercellular matrix [201].

From all respects, ECFCs can now be seen as the centre point for vasculogenesis, and their adequate proliferation the primary determinant of sufficient vessel formation. For this reason the role of ECFCs, over CACs, becomes of principle interest in placental physiology and pathophysiology, and thus forms the main focus of this thesis.

1.3.2 Is there a role for fetal ECFCs at the fetoplacental interface? Overall thesis hypothesis.

As previously summarised, the extent of expansion of the vascular endothelium, both within the human placenta and uterus during pregnancy, is extraordinary. Angiogenesis, based on mature endothelial cells, might be insufficient to provide adequate cells to resource this expanding endothelium. Consequently, it is plausible that a major source is circulating fetal ECFCs. This possibility had not been explored at the start of the study reported in this thesis. Therefore I examined the hypothesis that fetal-derived ECFCs contribute to (i) placental and (ii) uterine vessel formation in physiological conditions, and (iii) that their intrinsic failure is associated with inadequate fetal growth in pathological conditions, i.e. IUGR. A detailed description and rationale behind these hypotheses is delineated along with the associated aims and experiments. Each constitutes results Chapters 2, 3 and 4, respectively, and considerations leading to these experiments are as follows.

1.3.2.1 Chapter 2: Fetal derived ECFCs play a physiological role in placental vasculogenesis

ECFCs are principally derived from the bone marrow in the adult; however the source of ECFCs during intrauterine life is unknown. Primarily, haemopoietic stem cells pass an endothelial stage before engaging to the haemopoietic cell line [156]. Therefore,
endothelial stem production in haemopoietic fetal organs cannot be unreservedly excluded. Although less likely, ECFCs might not originate from the fetal bone marrow as they do in adults, but from the placenta, which also exhibits active haemopoiesis during fetal life. [203-207].

1.3.2.1.1 **Hypothesis**

ECFCs originating from the fetus actively migrate to the placenta through the umbilical circulation and become sequestered by the developing organ. Once in the vicinity of the placenta fetal-derived ECFCs adopt an active vasculogenic role and contribute to the vasculogenesis necessary for placenta expansion.

1.3.2.1.2 **Aims**

To test this hypothesis the following aims were devised:

(i) To define the likely fetal source and possibility of placental sequestration of fetal ECFCs (and CACs) in blood from pairs of umbilical arteries and veins. These cells were counted in uncomplicated pregnancies by accepted flow cytometry techniques, and arterio-venous gradients determined, suggestive of EPC-migration.

(ii) To test the potential of human fetal ECFC to play a role in the vascularisation of placental microvessels. For this, cultured ECFCs were perfused into chorionic plate microarteries, and their subsequent migration was examined.

(iii) To further elucidate the role of fetal ECFCs in placental vascularisation in its full complexity. Assessing their function in a novel *in vivo* murine model of placental vessel formation was undertaken by tracking human ECFCs in murine placentas following ultrasonographic guided intra-cardiac injection into the murine fetal heart.
The work related to this hypothesis is summarised in Chapter 2, in the format submitted to Angiogenesis.

1.3.2.2 Chapter 3: The role of fetal ECFCs in the vasculogenesis of the maternal uterus

To reiterate, vascular expansion in pregnancy is not exclusive to the fetal side of the placenta, as circulatory adaptations are also extensive within the maternal pelvic vasculature. Similarly to the placenta, extensive vessel growth may not be driven solely by angiogenesis, but may require additional EPC-driven vasculogenesis and vascularisation. In vitro observations suggest that fetal ECFCs have a more substantial capacity for vessel expansion than their adult counterparts [61,208]. Although EPCs have not been directly implicated in chimaeras, fetal cells have long been known to survive in the maternal organism for several years following pregnancy [209-223]. Mouse models showed specifically that fetal derived endothelial-like cells homed to the vasculature in some maternal pathologies, including skin injury and skin tumours [214,224]. Most recently the presence of fetal stem cells in maternal endocardium was detected in physiological circumstances [225].

1.3.2.2.1 Hypothesis

High potential fetal ECFCs transmigrate the placenta, circulate in the maternal blood and home to loci where vessel formation is required. As the uterus is a prime location for exaggerated vessel formation in pregnancy, it could be further suggested that these fetal cells home to expanding uterine vessels, and actively contribute to its physiological expansion.

1.3.2.2.2 Aims

This hypothesis was tested with the following aims:

(i) To examine the presence of fetal-derived endothelial cells in the maternal uterine endothelium in the murine system. For this, eGFP-labelled cells
originating from fetuses conceived from transgenic fathers were tracked in the maternal tissue.

(ii) To define which specific endothelial cells transmigrate the placenta. To determine this, eGFP-labelled human fetal ECFCs and mature human fetal endothelial cells (HUVECs) were tracked to the murine maternal uterine endothelium, following their fetal intra-cardiac injection. To determine complete integration into the host tissue, the presence of tight and gap junctions was histologically examined.

(iii) To investigate the quantity of fetal cells in the maternal uterine vasculature in the human. For this, the number of copies of a fetus-specific gene (SRY) in microvessels isolated from the uterine tissue of pregnancies with male offspring was determined by RT-QPCR.

(iv) To determine the location of fetal cells within the various structures of maternal uterine microvessels in the human. Cross sections of vessels were hybridised in situ for the detection of a fetus-specific Y-chromosome. Thus highlighting the vascular location of fetal-derived cells.

The results of these investigations are described in Chapter 3, and have been published by Stem Cells in this format.

1.3.2.3 Chapter 4: Involvement of fetal ECFCs in the pathogenesis of Intrauterine Growth Restriction

The physiological role of fetal derived ECFCs in placental vasculogenesis and vascularisation was hypothesised above in Section 1.3.2.1.1. If this is correct, reduced numbers or the impaired function of these cells could ultimately lead to suboptimal placental vasculogenesis, resulting in an under-developed placental villus vasculature, limiting nutrient and oxygen supply to the fetus and encouraging IUGR. Indeed, it has been shown by this group and others that aberrant placental vasculature is associated with IUGR [62,63,226,227].
In addition, the role of fetal ECFCs in uterine vascular adaptations to pregnancy was likewise proposed above (Section 1.3.2.2.1). Conceivably, the reduced availability or damaged function of fetal ECFCs could equally influence the development of the uterine vasculature if upheld. Like its effects on the placenta, abnormalities in fetal ECFCs could equally lead to suboptimal perfusion of the placental bed, causing reduced fetal nutrient supply and secondarily IUGR.

1.3.2.3.1 **Hypothesis**

The numbers of ECFCs available and taken up by the placenta may be reduced, and their intrinsic function may be impaired in IUGR.

1.3.2.3.2 **Aims**

(i) To compare the availability and placental uptake of ECFCs between growth restricted and normal human fetuses. This was achieved by evaluating ECFC (and CAC) counts in the umbilical blood of IUGR affected neonates. Again, placental uptake was calculated as the arterio-venous gradient.

(ii) To further assess and compare the number of circulating viable ECFCs between these two patient groups. For this, the numbers of primary ECFC-colonies were established in culture.

(iii) To compare the proliferative capacities of ECFCs derived from IUGR and control neonates. To achieve this, the population doubling times in culture and the time elapsed before first observation of colonies, were also determined.

(iv) To assess the functional capacities of IUGR and normal ECFCs in vitro, their migration. For this, chemotaxis and MMP-2 production were investigated by established techniques.
(v) To determine the vasculogenic capacities of ECFCs from uncomplicated and IUGR pregnancies. To examine this, their effective vasculogenesis was determined in artificial tissue blocks in vivo transplanted in the mouse. The outcome of these investigations is described in Chapter 4, as submitted to Journal of Pathology.
2 CHAPTER 2

A PHYSIOLOGICAL ROLE FOR HUMAN ENDOTHELIAL COLONY FORMING CELLS

Peter I. Sipos\textsuperscript{a}, Xiaohu Fan\textsuperscript{a}, Stephane L. Bourque\textsuperscript{c}, Joanne L. Stanley\textsuperscript{c}, Irene J. Andersson\textsuperscript{c}, Mark Wareing\textsuperscript{a}, Carl A. Hubel\textsuperscript{d}, Philip N. Baker\textsuperscript{c}, Sandra T. Davidge\textsuperscript{c}, Colin P. Sibley\textsuperscript{a}, Ian P. Crocker\textsuperscript{a}

\textsuperscript{a}Maternal and Fetal Health Research Centre, University of Manchester, Manchester Academic Health Science Centre, UK
\textsuperscript{b}Department of Pediatrics and Pharmacology, University of Alberta, Edmonton, AB, Canada
\textsuperscript{c}Women and Children’s Health Research Institute, University of Alberta, Edmonton, AB, Canada
\textsuperscript{d}Magee-Women’s Research Institute and Dept. Obstetrics, Gynecology & Reproductive Sciences, University of Pittsburgh, PA, USA

Conflict of interest statement: The authors have no conflicts of interest.

Corresponding author:
Dr. Peter Sipos and Dr Ian Crocker, Maternal and Fetal Health Research Centre, School of Biomedicine
Faculty of Medical and Human Sciences, University of Manchester, Reception, 5th Floor (Research), St Mary's Hospital, Oxford Road, Manchester, M13 9WL, UK
Tel: +44(0)161 701 6973
Email: Peter.Sipos@manchester.ac.uk, Ian.Crocker@manchester.ac.uk
2.1 ABSTRACT

Endothelial Colony Forming Cells (ECFCs) are thought to produce newly formed endothelium during vascular formation and repair. Their vasculogenic potential has been shown in vitro, and in vivo in artificial tissues, but their direct contribution to human physiology remains unclear. Here, for the first time, we establish their physiological role using the human placenta. Through cord blood measures of ECFCs, and through their ex vivo perfusion into placental chorionic arteries, we define their sequestration and incorporation into pre-existing human placental vessels. Subsequently, by transplantation of retro-viral transfected human fetal ECFCs (expressing eGFP or LacZ) into the circulation of immuno-deficient murine fetuses, we demonstrate that these cells preferentially migrate to the placenta, where they actively participate in vasculogenic activity. This ability, which was notably absent in differentiated endothelial cells, provides the first evidence, that human ECFCs non-fusogenically contribute to de novo endothelium and vessel formation in vivo. With this confirmed role, we predict that aberrations in the number or function of fetal ECFCs would have profound implications for human vascular development, firstly in the fetus and placenta, impacting upon fetal growth, and subsequently in cardiovascular repair in adult life.
2.2 INTRODUCTION

Vascular integrity is a pre-requisite for cardiovascular health and is dependent upon appropriate equilibrium between endothelial injury and repair. Before more potent bone marrow-derived circulating cells were discovered, repair was considered to rely solely upon differentiated endothelial cells. These bone marrow-derived cells, including lineage-committed Endothelial Colony Forming Cells (ECFCs) of high proliferative potential, were thought to migrate to sites of hypoxia or vessel injury and contribute to endothelium formation or neovascularisation [102]. As the identity of ECFCs cannot be accurately verified by surface markers, confirmation necessitates culture-based functional assays, the most important being positive outgrowth from single cell beginnings and the active formation of vascular structures in animal implanted artificial tissue blocks [202,228]. Given these criteria, experimental design must be based unequivocally on ECFCs functional verification. In situ these functions are promoted by a different group of bone marrow derived angiogenic cells of haematopoietic lineage, called Circulating Angiogenic Cells (CACs) [112,229]. These supportive cells typically locate subjacent to the forming endothelium, appearing first in response to hypoxic signals and secondarily, through paracrine and autocrine intermediates, attracting and stimulating ECFCs for true vasculogenic activity.

Although the endotheliogenic capacity of ECFCs is repeatedly suggested, it is not fully elaborated, given the issues of function based ECFC-recognition. The vasculogenic capacity of ECFCs is known in vitro and in artificial tissues, along with their involvement in pathological conditions (see [228] for review). However, their role in normal physiological processes in vivo, although anticipated, has yet to be confirmed. Consequently, their true physiological potential and direct contribution to human vascular development remain unrecorded.

In correcting this oversight, we have exploited the unique anatomical position of the human placenta, whose blood supply is exclusively provided by the readily accessible arteries and vein of the umbilical cord. In these studies, the net placental uptake of ECFCs and CACs was calculated from the arterio-venous gradient, using accepted combinations of
phenotypic markers [230]. Additionally, the vasculogenic potential of verified ECFCs was investigated in the human placenta by their ex vivo perfusion into human chorionic plate arteries. Through transplantation of characterised human fetal ECFCs into the immunodeficient mouse fetus, and subsequent tracking to the developing placenta, migration through the umbilical cord and direct involvement in placental vascularisation was confirmed. In this context, isolated human fetal ECFCs were labelled by lentiviral delivery of eGFP or LacZ, and then transplanted in vivo into the intact circulation of the murine fetus under ultrasonographic guidance. Their subsequent distribution, integration and placental vasculogenic involvement were determined by fluorescent optical imager and immunohistochemistry. Where necessary, human umbilical vein endothelial cells (HUVECs) were used as differentiated endothelial cell controls.
2.3 MATERIALS AND METHODS

2.3.1 Sample collection

In total, 20 pregnancies within St Mary's Hospital, Manchester, UK were considered. All pregnancies were normotensive and uncomplicated, with no fetal disorders or abnormalities diagnosed before newborns were discharged. The mean gestational age of participants was 39.6 weeks (±1.4, SD), maternal age 30.4 years (±5.8), BMI 27.0 (± 7.9), and parity 0.8 (±0.76). One of the women smoked in pregnancy and 50% underwent an elective caesarean section for obstetric reasons unrelated to placental or fetal disease. Birth weights, placental weights and individualized birth weight ratios [231,232] were all within normal range (mean ± SD, 3652 ± 341g, 679.2 ± 114.9 and 70.1 ± 20.6, respectively).

For flow cytometry, a minimum of 500 µl of arterial and venous umbilical blood samples were collected using 20G hypodermic needles (Brown, Sheffield, UK) from double-clamped umbilical cords immediately after delivery. For cell culture, a minimum of 15ml mixed cord blood was collected from unclamped cords. Blood was collected into Vacutainer tubes containing EDTA as anticoagulant (Becton, Dickinson, Franklin Lakes, NJ, USA). Samples for flow cytometry were kept on ice, while samples for culturing were maintained at room temperature with continuous shaking and processed within 30 minutes (see below).

2.3.2 Flow cytometry

The counting of ECFCs and CACs in both arterial and venous umbilical samples was based on the protocol of Duda et al. [230]. Modifications were made to accommodate differences between adult and fetal blood. These included, (i) the addition of a viability stain to exclude non-viable cells, a frequent cause of false positive results [233], (ii) immediate processing and avoidance of cell fixation, to permit viability recognition, and (iii) alterations in lysis reagent (Pharmalyse Lysing Buffer, BD Biosciences) and time to successfully lyse resistant nucleated fetal erythrocytes (20min at room temperature). Further minor modifications were made following optimisations. Venous and arterial umbilical blood was
obtained from double-clamped umbilical cords at birth. In detail, 125µl of umbilical whole blood, lysed within 30 min of placental delivery, was pre-treated with 25µl FcR-blocking reagent (Miltenyi Biotec Ltd. Bisley, UK) for 10 min at room temperature and the following combinations of antibodies added for detection of CACs: (i) CD31-FITC (4µl, BD Biosciences, Oxford Science Park, UK), CD133-PE (5µl, Miltenyi Biotec Ltd.), CD45-APC-H7 (10µl, BD Biosciences), CD34-APC (3µl BD Biosciences), and ECFCs: (ii) CD31-FITC (4µl, BD Biosciences), KDR-PE (5µl, Miltenyi Biotec Ltd.), CD45-APC-H7 (10µl, BD Biosciences), CD34-APC (3µl, BD Biosciences). Relevant IgG isotype controls were included. Ten minutes prior to analysis the viability stain 7-Amino-actinomycin D (7AAD) (5µL, BD Biosciences) was also added.

All flow cytometry readings were made against isotype controls on a CyAn-ADP flow cytometer (DAKO A/S, Glostrup, Denmark), analyzing all cells in each sample. Data were analyzed using Summit 4.3 flow cytometry analysis software (DAKO A/S). The gating strategy for CACs involved the selection of a 7AAD negative population of mononuclear cells [228]. These live cells were subdivided into a CD31+/CD45+ dual positive fraction, and displayed on a CD34/CD133 histogram. In turn this quadruple positive population (CD31+/CD45+/CD34+/CD133+) were considered CACs, and their number expressed relative to CD45 positive mononuclear cells. For ECFCs, CD31bright/CD45- mononuclear cells were displayed on a CD34/KDR histogram. The resulting CD31bright/CD45-/CD34+/KDR+ cells were similarly related to the total CD45+ mononuclear population [228].

2.3.3 ECFC expansion

In accordance with published protocol [202], ECFCs were expanded from fresh umbilical blood mononuclear cells isolated by density gradient cell separation using Hystopaque-1077 (Sigma-Aldrich Ltd, Gillingham, UK). These cells were expanded under standard culture conditions on rat-tail collagen-I (BD Biosciences) using an EGM-2 bullet kit for media and supplementation (Lonza Verviers, S.p.r.l. Verviers, Belgium). Direct observations on proliferative capacity were made on the outgrowth cells. After no more than three passages, cells were cryopreserved and subsequently used for experimentation.
Cell identity was verified by cellular phenotype, characterized by a range of endothelial and leukocyte markers (UEA-lectin, VEGFR-2, CD14, CD31, CD34, CD45, CD105, CD146) and endothelial-specific functions (Ac-LDL uptake, tube formation on Matrigel), in accordance with standard techniques [202].

2.3.4 Lentiviral vector production and transduction of ECFCs and HUVECs

By screening multiple HIV-1-based lentiviral vectors for optimal transduction efficiency [234], a vesicular stomatitis virus (VSV)-pseudotyped lentiviral vector was selected, harboring eGFP under the control of the ubiquitous EF1-α promoter. Alternatively, pLenti6/V5-GW/lacZ from the ViraPower™ lentiviral expression system (Invitrogen, Carlsbad, CA) was used, with β-galactosidase (LacZ) under the control of the CMV promoter. To package these lentiviral vectors, 293T cells were co-transfected with pDelta 8.74 expression plasmid and vesicular stomatitis virus envelope expression plasmid pMD2G, using polyethyleneimine (PEI)-based transfection [235].

The vector-containing supernatants were harvested 48 hours after transfection and filtered through 0.22µm pore-size cellulose acetate filters. The viral supernatants were serially diluted and titrated on 293T cells. Ultracentrifugation yielded titers of up to 5x10⁸ transducing units (TU) per ml. The lentiviral stock was used immediately or stored at -80°C. For ECFC/HUVEC transductions, 1x10⁷ cells were incubated with 2x10⁷ TU of lentiviral vector in the presence of 7µg/ml protamine sulfate. After 6 hours incubation, supernatants were replaced with fresh media and cells harvested 72 hours later. Persistent gene expression was confirmed by flow cytometry and fluorescent microscopy through 10 subsequent passages.

2.3.5 Verification of ECFC-characteristics after genetic modification

Pooled HUVECs (Lonza, Basel, Switzerland) at passage 4 were used as mature endothelial controls. Where necessary they were tracked with 5-chloromethylfluorescein diacetate (CMFDA) according to manufacturer’s instructions (Molecular Probes Cell,
Invitrogen Life Technologies Ltd, Paisley UK). For single-cell cultures - the most rigorous test for clonogenic potential in progenitor cells [61] - ECFCs and eGFP-ECFCs (see above) were detached from culture plates and cell-sorted (FACSAria, BD, Toronto, Ca) to single cells into each well of a collagen-I coated 96-well culture plate. These single cell ECFC/eGFP-ECFC cultures were incubated in EGM-2 media/supplements with additional 1.5%(v/v) amphotericin-B (Sigma-Aldrich Ltd), 1%(v/v) penicillin/streptomycin (Sigma-Aldrich Ltd) and 0.15%(v/v) gentamycin (Sigma-Aldrich Ltd). As non-progenitors, HUVECs and CMFDA-HUVECs were plated as single cells into collagen-I coated dishes or at a standard re-plating density of 5x10^3 cells per cm^2 into wells coated with gelatine and cultured in classic DMEM with 20%(v/v) fetal bovine serum and aforementioned antimicrobials.

2.3.6 In vivo characterisation of fetal ECFCs

The in vivo angiogenic capacities of these cells were determined in the mouse using artificial tissue blocks as previously described [202]. In brief, eGFP-ECFCs or HUVECs (1x10^6) and Adipose Derived Stem Cells (2.5x10^5) (Lonza) were detached from culture and suspended in a (1:1) mix of collagen-I (BD Biosciences) and fibronectin (Fisher Scientific Ltd. Ottawa, ON, Canada) in a total volume of 500μl reagent mix (HEPES, sodium-bicarbonate, fetal bovine serum and EBM-2 (pH 7.4)). Once gelled in a 48-well culture plate, the cell-containing block was covered with EGM-2 media and incubated overnight. The resulting contracted gels (approximately 2-3mm in diameter) were subcutaneously implanted into the flanks of NOD/SCID immunodeficient mice (Strain 005557, Jackson Laboratory, Sacramento, USA) under isoflurane inhalation anaesthesia through a parasagittal skin incision in the lumbar area [202]. Once the wound was closed with silk sutures, pain relief from subcutaneous ketoprofen was provided (5mg/kg) and the mice were treated for 14 days with Augmentin (amoxicillin/clavulanate) prophylaxis in their drinking water to prevent post-operative infections. After this period, the implants were harvested by dissection under stereomicroscope, following isoflurane euthanasia in accordance with local protocols. Implants were examined in vivo under a small animal imager (OV-110, Olympus, Hamburg, Germany) and 10μm thick cross sections were
examined under a confocal fluorescent microscope (X81, Olympus, Hamburg, Germany). To examine continuity between murine circulation and neovessels of the implants, thoracic aortas were dissected, incised, cannulated and perfused with heparin in normal saline (10ml, 50 iu/ml, 1ml/min), followed by Microfil radiopaque compound (3mls/min) (MV-122, FlowTech Inc. Carver, MA) using a syringe driver, until cast in the mesogastric veins was visually confirmed.

2.3.7 *Ex vivo perfusion of chorionic plate arteries with cord blood ECFCs*

Culture-expanded ECFCs from umbilical blood were detached with trypsin-EDTA (0.5 %v/v), labelled with CMFDA and 5x10^6 cells were suspended in 100µl EGM-2 media. Dissected chorionic plate placental arteries of approximately 150µm diameter isolated from uncomplicated pregnancies at term were mounted on cannulae in a perfusion chamber (Living Systems Instrumentation Inc, VT, USA) and perfused with CMFDA-ECFC in suspension [236]. The vessel ends of the artery segments were subsequently tied to prevent leakage and the perfused arteries, containing cells, were co-incubated for 72 hours in EGM-2 media. After snap freezing in OCT for storage, vessel cross-sections (10µm) were stained for VEGFR-2 (using mouse anti-human VEGFR-2 primary (R&D Systems) and phycoerythrin-conjugated rabbit anti-mouse secondary (Invitrogen) antibodies) to highlight the vascular surface. These were microscopically analyzed (Leica TCS SP5 X, Leica Microsystems GmbH Wetzlar, Germany) for the presence of ECFCs in the pre-existent endothelial lining.

2.3.8 *Transplantation of ECFCs into immuno-compromised mouse fetuses*

This protocol was designed to investigate whether ECFCs migrate from the fetus to the placenta *in vivo*. Transgenic eGFP-ECFCs and LacZ-ECFCs were included as test cells, and eGFP-HUVECs and fluorescent bright CMFDA-HUVECs as mature endothelial fetal controls.
Pregnant NOD/SCID mice were anesthetized by isoflurane on D15, as determined from their mating plug. They were placed on a heating pad and abdominal fur was removed and skin disinfected in accordance with local guidelines and standard operating procedures (Animal Care Facility, University of Alberta). Using a VS 40 high frequency high-resolution ultrasound imaging platform (VisualSonics, Toronto, ON, Canada), with 30MHz probe, the ongoing pregnancy was confirmed and the position of each fetus defined in each uterine horn. Live fetuses, with their abdomens optimally positioned, were chosen and their hearts sonographically visualized and centred for needle insertion (Figure II-1A).

For test animals, $1 \times 10^7$ eGFP-ECFC or LacZ-ECFC were diluted in 70µl EBM-2, whilst for controls $1 \times 10^7$ eGFP-HUVEC or CMFDA-HUVECs were suspended in 70µl DMEM. For negative control mock injections of 70µl EGM-2 were used. In all cases, cells were taken into a sterile 250µl GasTight luer tip syringe (Hamilton AG, Bonaduz, Switzerland) maintained at 37°C. Preliminary testing showed that the injection system retains 35µl in the luer, hence the number of injected cells was estimated at $5 \times 10^6$ per fetus. A 32G x 12mm needle (Brown, Sheffield, UK) was subsequently attached and guided with ultrasound through the maternal skin, abdomen and uterine wall and into the amniotic cavity. The needle was manoeuvred manually throughout to maintain it in the plane of the ultrasound probe. Within the amniotic cavity, the needle was re-angled and aimed at the fetal heart. After piercing the fetal thoracic skin, the tip of the needle was guided into the cardiac cavity, the correct placement of the needle sonographically confirmed (Figure II-1B), and the contents of the syringe injected over 4-5s (Figure II-1C). When complete, the needle was withdrawn and the injected solution within the heart cavity observed. Each fetus was monitored for several minutes for their normal heart rate to re-establish and checked for bleeding in the amniotic cavity. Once fetal well being was confirmed and transplantation complete, anaesthesia was withdrawn and the murine pregnancy continued for a further 3.5 days with prophylactic oral amoxicillin/clavulanate antibiotics in the drinking water.
Figure II-1. Illustration of ultrasonographic visualisation of intra-cardiac transplantation process of human fetal ECFCs (expressing eGFP or LacZ) into the NOD/SCID murine fetus (E15.5 pregnancy).

A Typical trans-sagittal image of a murine fetus in the amniotic sac (purple arrow = ocular lens, yellow square = fetal heart), B horizontal plane showing needle tip (green arrows) within the fetal heart cavity (yellow arrow), C oblique plane with cardiac chamber (purple arrow) and needle (green arrow). A bright echo-dense area is observed with the introduction of ECFCs (yellow arrow).
On pregnancy D18.5, mice were euthanized by halothane overdose. Following confirmation of maternal death, feto-placental units were subsequently delivered through uterotomy and placental disks blunt dissected from the uterus and examined with a small animal imager (OV-100, Olympus, Tokyo, Japan). The entire surface of both maternal and fetal sides were scanned with 4x magnification to identify fluorescent patches and at least four randomly selected fluorescent areas were examined with high (8-16x) magnification.

Placentas of fetuses transplanted with eGFP-ECFCs were bisected in the midline and randomly selected. Half was snap frozen in OCT, whilst the remainder was fixed in paraformaldehyde (4% w/v, pH 7.4) for 20 minutes. To confirm full integration of graft cells, immunohistochemistry for connexin-40 and claudin-5 was performed on cryopreserved and paraffin-embedded sections respectively, using primary antibodies cross-reacting between human and mouse. These demonstrated gap and tight junctions between graft and host cells, respectively. Cryosections (7µm) were stained with rabbit anti-connexin 40 primary antibodies (Invitrogen) and biotin-conjugated porcine anti-rabbit secondary antibodies (DAKO). Paraffin embedded sections (5µm) were stained with rabbit anti-claudin 5 primary antibodies (Invitrogen) and biotin-conjugated porcine anti-rabbit secondary antibodies (DAKO), using standard immunohistochemical techniques.

Placentas of fetuses transplanted with LacZ-ECFC were fixed and stained with LacZ Tissue Staining Kit (Invivogen, San Diego, CA, USA) in accordance with manufacturer’s instructions, then bisected, embedded and similarly stained for connexin 40 and claudin 5. Cross-sections (six sections from 2 independent, randomly selected locations), extending to all three layers of the murine placenta, were examined with confocal fluorescence and light microscopy (Nikon Eclipse 80i, Kingston Upon Thames, UK) to show the presence of integrated, labelled, transplanted cells either incorporated into pre-existent vessels or forming de novo capillaries, i.e. evidence of human fetal ECFC involvement in placental vascularisation and vasculogenesis, respectively.
2.3.9 Statistics

In the absence of comparable studies, sample sizes were empirically determined. Normality of distribution was determined by D’Agostino-Pearson and Smirnoff-Kormogoroff tests, and parametric, non-parametric tests applied accordingly. Prism V5.0a (Graphad Software Inc., La Jolla, CA) was used for data analysis. Further information is supplied in 6.1.3.

2.3.10 Study approval

Approval for human samples was obtained from the UK Department of Health Local Research Ethics Committee. Protocols complied with the guidelines and policies of the University of Manchester and Declaration of Helsinki. All individuals provided informed consent. All animal protocols were conducted at the University of Alberta, Edmonton with approval from the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines.
2.4 RESULTS

2.4.1 Umbilical arterio-venous gradient favours placental uptake of ECFCs and CACs

From flow cytometry analysis, the numbers of circulating CACs in human umbilical cord blood (determined as percentage of mononuclear cells) were shown to be significantly higher in human umbilical arteries than in their paired veins (Figure II-2a, median [interquartile range] 0.55% [0.27-0.83] vs. 0.40% [0.19-0.71], respectively, n=12, p=0.0001, paired t-test). Similarly, ECFC numbers were higher in arterial-derived samples than venous counterparts (Figure II-2b, 0.00305 [0.00072-0.00701] vs. 0.00050 [0.0-0.00244], respectively) (n=17, p=0.0008, Wilcoxon’s matched pairs test). This disparity between veins and arteries suggests placental uptake of EPCs from cord-blood for both CAC and ECFC subtypes.

Figure II-2. Concentration of EPC-subtypes in umbilical arterial and corresponding venous blood from uncomplicated pregnancies at term.

A comparison of EPC-subtype concentrations in umbilical arterial and corresponding venous blood samples, determined within the mononuclear cell population of uncomplicated term pregnancies. Both CACs (A) and ECFCs (B) show a higher prevalence in the umbilical artery than vein, suggesting placental sequestration (paired t-test (A), Wilcoxon’s matched pairs test (B)).
2.4.2 Morphologic and functional phenotype of cells used for xenotransplantation

Following an established protocol, ECFCs were derived from cord-blood mononuclear cells in culture [202]. These cells differentially expressed endothelial specific CD31, CD34, CD105, CD146 and VEGFR-2 antigens and stained positively with UEA-lectin, ingested Ac-LDL, and formed tube-like structures on Matrigel (Fig. II-3a, Supplemental Fig. II-1). These positive markers were coupled with negative recognition for mononuclear cell CD14 and pan-haemopoietic CD45 (Fig. II-3a, Appendix Figure 1).

From single cell beginnings, eGFP-labelled ECFCs (eGFP-ECFCs) readily proliferated with clonal subtypes as described by Ingram et al. [61] (Fig. 3b). No harmful effects of genetic manipulation were recorded and progenitor capability was confirmed. Fourteen days after seeding, single ECFCs and eGFP-ECFCs formed large colonies of cells which could be replated several times (High Proliferative Potential ECFC (HPP-ECFC, 29%), smaller colonies which could be replated only once (Low Proliferative Potential ECFC (LPP-ECFC, 31%), and non-replateable colonies containing fewer than 50 cells (Endothelial Clusters, 20%). HUVECs, and their trackered equivalents, CMFDA-HUVECs, failed to proliferate in single cell culture, but were expanded on gelatine-coated plates when seeded at standard densities.

Using artificial tissue blocks implanted subcutaneously into NOD/SCID mice (n=22), transduced human eGFP-ECFCs were shown to contribute to de novo vessel formation (Fig. II-3c); thereby confirming their vasculogenic credentials. Confirmation was provided by (i) imaging the implants with a fluorescent optical imager, demonstrating intact fluorescent vascular structures supplied by macroscopically visible arteries and veins, (ii) exposing tissue sections to fluorescence microscopy, localising eGFP-ECFC within the vascular walls, (iii) using light microscopy to show murine erythrocytes perfusing the de novo-generated artificial vessels, and (iv) demonstrating the presence of intraluminal cast in de novo vessels, demonstrating continuity of vessels with the murine vascular system, following introduction of cast agent into the murine aorta.
Figure II-3. The characterisation of fetal-derived ECFCs isolated from cord blood mononuclear cells.

A. In addition to recognised surface markers, ECFCs displayed typical cobblestone morphology, formed tube-like structures on Matrigel and digested Ac-LDL (Red stain: PE/Dil, Green stain: FITC, Blue stain: DAPI, scale bars: 50µm). B. Single cell cultures of ECFCs confirmed progenitor capacity, generating three subpopulations; High Proliferative Potential ECFCs (HPP-ECFCs), Low Proliferative Potential ECFCs (LPP-ECFCs) and Endothelial Clusters. D = days in culture. Scale bars 10µm. C. Fetal ECFCs within artificial tissue blocks implanted into NOD/SCID mice (n=25 implants), formed complex vessels with intact blood flow: (i) Human eGFP- ECFCs (green cells, yellow arrows) forming true vascular structures with lumina (absent fluorescence, red arrows), (ii) fluorescent and light microscopy of implants, showing vascular cross-sections (L = lumen, human eGFP-ECFCs = yellow arrows, Red = nuclei), (iii) haematoxylin-eosin stained sections showing de novo human vessels (L) containing red cells from the intact murine circulation (arrows), (iv) cast perfused through the murine aorta appears within the neovascularure of the implant, demonstrating continuity with the murine vascular system. (Control image to II-1Cii found at 6.1.4)
2.4.3 Human fetal ECFCs incorporate into placental vessels ex vivo

Cord blood ECFCs, expanded in culture and perfused into the lumens of chorionic plate arteries ex vivo (n=3 vessels perfused), showed localization into the pre-existent endothelial layer of the perfused vessels, demonstrating their potential for incorporation into the human placental vasculature (Fig. II-4).

![Image of incorporation of fetal-derived ECFCs into ex-vivo perfused human chorionic plate arteries.](image)

**Figure II-4.** The incorporation of fetal-derived ECFCs into ex-vivo perfused human chorionic plate arteries.

CMFDA-labelled ECFCs (yellow arrow) shown to infiltrate the pre-existing endothelial lining (red arrow) (Red: VEGFR-2, Blue: DAPI, 72 hours incubation). **A.** Exogenous ECFC with retained lateral VEGFR-2, evidencing the absence of lateral junctions with pre-existent endothelial cells, suggesting a coincidentally co-localised, but not integrated CMFDA-ECFC. **B.** Fully integrated ECFC with only apical VEGFR-2 remaining; continuous with resident endothelium (observed in n=3 perfused vessels). **C:** Isotype control.
2.4.4 Xeno-transplanted fetal ECFCs preferentially migrate from the fetus to placental vessels

Human fetal eGFP-ECFCs were introduced into the E15.5 mouse fetus by intra-cardiac injection (n=14 in total). Following 3.5 additional gestational days, the distribution of exogenous eGFP-ECFCs was investigated by fluorescent optical imager in isolated fetoplacental units delivered through hysterotomy. As opposed to mock injection alone (Fig. II-5a-c), numerous large fluorescent patches of eGFP-ECFCs were detected on the maternal facing aspect of the placental disk (Fig. II-5e), with fewer, smaller patches on the fetal facing side (Fig. II-5d). Fluorescent colonies were undetected in the intact fetus (Video II-1), perhaps through limited resolution. Nevertheless, transplanted cells were concluded to favour the murine placenta. In the control group, i.e. those injected with CMFDA-HUVEC (n=3) and eGFP-HUVEC (n=4), a different pattern of distribution was defined. In these cases, fluorescent areas of transplanted cells were restricted to fetal-facing aspect of the placenta, i.e. the chorionic plate. Unlike injected ECFCs, HUVECs failed to form patches and populated the large vessel lumina only, predominantly on the fetal side (Fig. II-5g,h). Upon enlargement small groups of cells were characteristically observed subjacent to the vascular lumina of placentae from eGFP-ECFC injected fetuses (Fig. II-5f). These cell clusters were discerned forming tube-like structures, reminiscent of immature capillaries. Video shots illustrated how these adherent cells remained static in the presence of moving blood, suggesting full attachment or integration (Video II-2). For fetuses transplanted with CMFDA-HUVECs, high magnifications supported the dominance of individual cells randomly scattered within the vascular lumina - the tube-like entities and mural arrangements seen with eGFP-ECFCs were never observed with transplanted HUVECs (Fig. II-5i).
Figure II-5. The homing of human fetal eGFP-ECFCs (xenotransplanted in NOD/SCID fetuses on E15.5) to the murine placental vasculature. Representative images taken by fluorescent imager.

A-C. Placentas of mock injected fetuses (x4 (A, B) and 10x (C) magnification). Vascular lumina (red arrows) are characterised by low auto-fluorescence, positive signals are absent (n=3 placentas observed, representative images included). D-F. Placentas of fetuses injected with human eGFP-ECFCs. At low magnification, eGFP-ECFCs (yellow arrows) were observed in distinct colonies on the fetal (D) and maternal (F) facing aspects of each placenta. Greater magnification (F) revealed ECFCs located (yellow arrows) subjacent to the vessel lumina, unperturbed by vascular flow (observed in n=14 placentas). G-I. Placental uptake of xenotransplanted HUVECs. Green CMFDA-HUVECs (yellow arrow) were observed in the lumina of chorionic plate vessels (red arrow, G), but were absent on the maternal-facing placental surface (H, n=3 placentas). Under high magnification, they were randomly disseminated or forming emboli (yellow arrow) within the smaller vessels (red arrow, I). J: Schematic of two imaging approaches.
2.4.5 ECFCs contribute to vessel formation in murine placental tissue

Using light and fluorescence microscopy, placentas from fetuses transplanted with eGFP-ECFC (n=14) and LacZ-ECFC (n=11), as well as control CMFDA-HUVEC and eGFP-HUVEC were examined. To determine the incorporation of ECFCs into an existing or newly formed endothelium, antibody recognition of endothelial tight (claudin-5) and gap junctions (connexin 40) were undertaken (Fig. II-6) [237,238]. Corresponding to the findings of the optical imager, within all ECFC transplanted fetuses, large round or triangle shaped patches of ECFCs were distributed within the spongiotrophoblast and labyrinth of the placentas, whilst smaller elongated patches were frequently found in the giant cell zones. Within the labyrinth zone, i.e. the main regions of materno-fetal exchange, transplanted ECFCs were found juxtaposed to the pre-existing endothelium, and under higher magnification (x100) shown to express Claudin-5 and Connexin-40, suggestive of full integration and neovascularisation; (Fig. II-6i and II-6ii). In opposition, control HUVECs were exclusively observed within vascular lumina and failed to express these junctional markers (Fig. II-6(iii-v)). In addition, placental derived human xenotransplanted ECFCs also frequently formed cytoplasmic vacuoles (Fig. II-6vi), a feature typical of endothelial cells undergo initial vasculogenesis, as described by others [239].
Figure II-6. Cross-sectional images of murine placentas following fetal transplantation with human transgenic ECFCs and HUVECs.

(i) Merged light and fluorescent image showing integration of eGFP-labelled ECFCs (arrows) within vessel endothelium (confirmed by claudin-5 expression (brown staining) and (ii) intra-mural eGFP-ECFCs – fluorescence only. (iii) Merged light and fluorescent image showing differentiated endothelial cells (eGFP-HUVECs) resigned to the vessel lumen (L) (nuclei = red). (iv) fluorescence only micrograph of intra-lumen restricted eGFP-HUVECs. (v-vi) Placental vascularisation and vasculogenesis following fetal intra-cardiac injection. (v) LacZ-labelled ECFCs (blue punctate cytoplasmic stain) integrated into the surrounding vascular tissue, as demonstrated by connexin-40 expression (brown staining). (vi) Xenotransplanted LacZ-ECFCs (yellow arrow), forming a vacuole and lumen (L), giving rise to de novo lumina (observed in n=12 placentas, 3 sections/placenta). (vii) Immunohystochemistry
2.5 DISCUSSION

These data are consistent with the hypothesis that fetal ECFCs play a role in placental vessel formation. To our knowledge, this is the first confirmatory evidence that ECFCs participate in non-pathologic vessel formation, with functional involvement in human physiological vasculogenesis.

Our flow cytometric observations of CACs and ECFCs in the umbilical circulation showed that cell numbers travelling to the placenta, through the umbilical artery, were consistently higher than those departing in the venous circulation. Two conclusions can be drawn: (i) the placenta is an unlikely source of ECFCs/CACs, and (ii) the placenta retains these cells on passage through the organ, even in late pregnancy when vasculogenic/angiogenic events are curtailed [240], but nonetheless persistent [241]. ECFCs may equally fulfil their role of vascular repair, maintaining and replacing eroded placental endothelium throughout gestation.

Our ECFCs, isolated from cord blood, were morphologically and functionally characterised [60,61,102]. In addition to progenitor and angiogenic capabilities in vitro [61] and in vivo [200] these homogenous cultures (Supplement Fig. II-2) exhibited solely endothelial characteristics, with no haemopoietic markers. Although ECFCs are more abundant and active in the fetus than their adult counterparts [61,202], without functional examination [200] they remain indiscernible from differentiated endothelial cells, based exclusively on the presence/absence of surface markers. As a consequence, our five-colour flow cytometry technique, although thorough, was unable to distinguish ECFCs from these differentiated endothelial cells. Notwithstanding, our cord blood endothelial cells, when expanded in culture, showed no signs of differentiated endothelial cell colonies, had their progenitor characteristics confirmed by single cell culture, and their authenticity supported through de novo vessel formation in collagen-fibronectin implants; all in accordance with current guidelines [60,202]. We therefore consider the majority of circulating endothelial-like cells in the fetus to be fetal-derived ECFCs and not differentiated alternatives.

More advanced cytometric approaches of defining ECFCs than those employed are now recognised [242], but not available at the time of study. Nevertheless, our protocol did
accommodate issues of artefact, including the exclusion of dead cells and erythrocytes, but the presence of anucleate particles were not accounted; a caveat applicable to our flow cytometric assessments, but not our ex vivo/in vivo findings.

In defining the fate of fetal-derived vasculogenic cells in the placenta, our ex vivo and in vivo studies have predominantly focused on ECFCs, i.e. those cells believed to have a primary role in endothelium formation [200]. With the function of haemopoietic CACs previously established, i.e. facilitation of vessel expansion by attracting and stimulating ECFCs in a paracrine fashion [101,243], there is no reason to assume that this role is distorted in the human placenta. Certainly our presented evidence of their placental uptake is in line with migration of ECFCs from the fetus to the placenta and although the mechanistic interactions between CACs and ECFCs were not studied in this context, our ECFC results should be interpreted with the knowledge that CACs may also be present and active.

In the past, in vivo attempts to demonstrate a role for ECFCs in vessel formation have been exclusively conducted in studies of pathology, employing various models of vascular injury [59,100,244-246] and cardiovascular disease [85,91,186,247]. Notably, data to establish ECFCs in the physiological, non-pathological processes of vessel formation are lacking. In previous transplantation models, the precise phenotype of putative progenitors, as introduced into the host has remained unverified [245,246,248-250], with the possibility that these cells instigate endothelial attachment through graft-versus-host reactions [251,252]. Thus, although shown to localize to vessel walls, their complete and functional integration has never been previously confirmed. In our murine intrauterine transplantations, we have tracked (fully characterised) human fetal ECFCs from the fetal circulation to the placenta, where the presence of tight [253] and gap junctions [254] have confirmed their true integration. We have additionally verified their capacity for de novo vessel formation (with circulation), and confirmed their endothelial lineage; precluding a graft-versus-host involvement. These data thus confirm the previously assumed role of ECFCs in vessel formation, and importantly have demonstrated their formerly unproven physiological involvement in human vasculogenesis and vascularisation.
Within our xenotransplantation studies, HUVECs, as differentiated endothelial cells of fetal origin, failed to integrate and populate the mural tissue of placental microvessels and likewise were unable to form vessels *de novo* in artificial tissue blocks implanted in NOD/SCID mice (negative images not shown). We speculate that their incapacity for expansion *in vivo* [61], is one explanation for this vasculogenic impotence, whilst their recorded inferior expression of urokinase-like plasminogen-activator (uPA), uPA-receptor (uPAR), MMP-2 and VEGFR-2 limit their ability to integrate [255,256], and their inferior CD-144 expression limits their migratory capacity [256,257]. In addition, as opposed to ECFCs, HUVEC fail to deposit components of extracellular matrix [258].

In theory, our culture generated ECFCs could result from differentiation or trans-differentiation of cells triggered through favourable endothelial-specific culture conditions. Nevertheless, recent evidence, combining FACS and magnetic beads, has confirmed ECFC presence in peripheral and cord blood [103,113,242]. Notwithstanding, we acknowledge that the quantities, qualities and behaviour of our externally generated, fetal-injected ECFCs may differ from those of native cells, resident in the fetal circulation.

In human pregnancy, besides abnormal remodelling of maternal spiral arteries [259], an aberrant placental vasculature is associated with frequent and potentially severe pregnancy complications, such as preeclampsia and fetal growth restriction (FGR) [62-64]. By virtue of fetal programming, babies affected by these complications are not only unable to fulfil their growth potential, but also have an increased risk of cardiovascular disease and diabetes in adult-life [71,73,75,78,260-265]. Having established a role for ECFCs (and perhaps CACs) in placental vasculogenesis, subsequent research may elaborate on the causative correlation between ECFC/CAC deficits or dysfunction, impaired placental size/function, FGR and pre-eclampsia, and the life-long sequelae of vascular risk in these babies. In this regard, the recent article by Ligi et al [266], reporting proliferative and angiogenic impairments of ECFCs in preterm low-birthweight neonates, would certainly support this notion.
2.6 STUDY APPROVAL

Approval for studies on human samples was obtained from the UK Department of Health Local Research Ethics Committee. Protocols complied with the guidelines and policies of the University of Manchester and Declaration of Helsinki. All individuals provided informed consent. All animal protocols were conducted at the University of Alberta, Edmonton with approval from the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines.

2.7 ACKNOWLEDGEMENTS

The authors would like to thank the Wellcome Trust, UK (Grant: 083765/B/07/Z), Canadian Institutes for Health Research and NIH P01/HD030367 for supporting these studies.

2.8 AUTHOR CONTRIBUTIONS

Conception and design: PB, SB, IC, SD, CH, MW, PS

Financial support: PB, IC, SD, CH, CS, MW

Provision of study material or patients: IA, SB, XF, AR, JS, MW, PS

Collection and/or assembly of data: IA, SB, AR, PS

Data analysis and interpretation: IC, SB, SD, CS, PS

Manuscript writing: PB, IC, SD, CH, MW, CS, PS

Final approval of manuscript: PB, SB, IC, SD, CH, MW, CS, PS
2.9 SUPPORTING INFORMATION

Video II-1. A clip showing the unsuccessful search for apparent fluorescent cell aggregates in the body of an NOD/SCID fetus, which had received an intra-cardiac injection of eGFP-ECFC during intrauterine life (still image).

By changing the centre of focus of the small animal imager (Olympus OV-110), all layers between the two flanks were visualized. The skin of the fetus was removed surgically to prevent artefacts caused by strong auto-fluorescence of the skin tissue and by eGFP-ECFCs coincidentally seeded on the skin during the injection process. (See Video II.1 on attached DVD)
Video II-2. An adjunct to Figure 6d, showing the placenta circulation in a NOD/SCID fetus transplanted with eGFP-ECFC (still image).

Three-and-a-half days after transplantation, fluorescent ECFCs (yellow arrows) are seen to associate with the placental microvessels. Free-flowing blood (red arrows), within these vessels, suggests that incorporated eGFP-ECFCs reside outside the vascular lumina within the vessel wall, unperturbed by the force of vascular flow. Images generated in real time by small animal imager (OV-100, Olympus) at 16x magnification. (See Video II.2 on attached DVD)
Supplement figure II-1. Flow cytometry data for determining ECFC-identity.

Gate A: ECFCs defined for analysis. B and C: IgG1 isotype controls. D: Cells stained positive for CD31 (98.6% gated). E: CD144 (98.8% positive cells gated). Cells had weak positive staining for VEGFR2 (F: 50.1% positivity). FITC and PE denote fluorescein isothiocyanate and phycoerythrin conjugated antibodies, respectively. (Image of Ms. A. Ridgeway, co-author of the manuscript.)
Supplement figure II-2 Efficiency of lentiviral transfection.

Fluorescent image of live confluent fetal ECFCs following two sequential replatings after transfection with (VSV)-pseudo-typed lentiviral vector, harboring eGFP under the control of the ubiquitous EF1 α. This image shows that gene expression is adequate and maintained.
CHAPTER 3

UTERINE VASCULATURE REMODELLING IN HUMAN PREGNANCY INVOLVES FUNCTIONAL MACRO-CHIMERISM BY ENDOTHELIAL COLONY FORMING CELLS OF FETAL ORIGIN

Peter I. Sipos, Willem Rens, Hélène Schlecht, Xiaohu Fan, Mark Wareing, Carl A. Hubel, Philip N. Baker, Sandra T. Davidge, Colin P. Sibley, Ian P. Crocker

Maternal and Fetal Health Research Centre, University of Manchester, Manchester Academic Health Science Centre, U.K. Department of Veterinary Medicine, Cambridge Veterinary School, University of Cambridge, UK. National Genetics Reference Laboratory (Manchester), St Mary’s Hospital, Manchester, UK. Department of Pediatrics and Pharmacology, University of Alberta, Edmonton, AB, Canada. Magee-Women’s Research Institute and Dept. Obstetrics, Gynecology & Reproductive Sciences, University of Pittsburgh, PA, USA. Women and Children’s Health Research Institute, University of Alberta, Edmonton, AB, Canada.

3.1 AUTHOR CONTRIBUTIONS:

Peter Sipos: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing.
Willem Rens: Collection of data
Helene Schlecht: Collection of data
Xiaohu Fan: Provision of study material
Mark Wareing: Financial support, final approval of manuscript
Christina Hayward: Provision of study material
Carl A. Hubel: Financial support, final approval of manuscript
Stephane Bourque: Collection of data
Phillip N. Baker: Financial support, design, final approval of manuscript
Sandra T. Davidge: Final approval of manuscript
Colin P Sibley: Financial support, final approval of manuscript
Ian P. Crocker: Financial support, design, data analysis and interpretation, manuscript writing, final approval of manuscript.
**Corresponding author:**
Dr. Peter Sipos and Dr Ian Crocker, Maternal and Fetal Health Research Centre, Institute of Human Development, Faculty of Medical and Human Sciences, University of Manchester, 5th Floor (Research), St Mary’s Hospital, Oxford Road, Manchester, M13 9WL, UK. Tel: +44(0)161 701 6973, Email: peter.sipos@manchester.ac.uk, Ian.Crocker@manchester.ac.uk

**Conflict-of-interest disclosure:** None.

### 3.2 ACKNOWLEDGEMENTS:

The work was funded by the Wellcome Trust, UK (Grant: 083765/B/07/Z), Canadian Institutes for Health Research and NIH (P01/HD030367).

### 3.3 KEYWORDS:

Neovascularization, Physiologic
Maternal-Fetal Exchange
Chimerism
Circulation, Uteroplacental
3.4 ABSTRACT

The potency of adult-derived circulating progenitor Endothelial Colony Forming Cells (ECFCs) is drastically surpassed by their fetal counterparts. Human pregnancy is associated with robust intensification of blood flow and vascular expansion in the uterus, crucial for placental perfusion and fetal supply. Here we investigate whether fetal ECFCs transmigrate to maternal bloodstream and home to locations of maternal vasculogenesis, primarily the pregnant uterus. In the first instance, endothelial-like cells, originating from mouse fetuses expressing paternal eGFP, were identified within uterine endothelia. Subsequently, LacZ or eGFP-labelled human fetal ECFCs, transplanted into immuno-deficient (NOD/SCID) fetuses on D15.5 pregnancy, showed similar integration into the mouse uterus by term. Mature endothelial controls (HUVECs), similarly introduced, were unequivocally absent. In humans, SRY was detected in 6/12 myometrial microvessels obtained from women delivering male babies. The copy number was calculated at 175 [IQR 149-471] fetal cells/mm² endothelium, constituting 12.5% of maternal vessel lumina. Cross-sections of similar human vessels, hybridized for Y-chromosome, positively identified endothelial-associated fetal cells. It appears that through ECFC donation, fetuses assist maternal uterine vascular expansion in pregnancy; potentiating placental perfusion and consequently their own fetal supply. In addition to fetal growth, this cellular mechanism holds implications for materno-fetal immune-interactions and long-term maternal vascular health.
3.5 INTRODUCTION

Pre-eclampsia and intrauterine growth restriction (IUGR) are late pregnancy complications associated with insufficient uterine vascular density and suboptimal placental perfusion. These pathologies affect up to 5 and 10% of pregnancies, respectively [80, 267, 268], and have immediate and long-term health implications for mother and fetus [71, 79]. The cellular source of optimal vascular development within the pregnant uterus is unexplored. Human vascular development relies upon endothelial expansion achieved by angiogenesis, remodelling of mature endothelial cells, and by progenitor-driven types of vessel formation; i.e. vascularisation and vasculogenesis, distinguished by the presence or absence of pre-existent vessels. For angiogenesis, vessel formation is limited by the non-proliferative nature of mature endothelial cells. This mechanism maybe insufficient for the exaggerated vascular expansion of pregnancy.

For vasculogenesis and vascularisation, vessel expansion is boosted by highly proliferative Endothelial Progenitor Cells (EPCs). From the many putative EPCs, Endothelial Colony Forming Cells (ECFCs) are of classic endothelial and true progenitor characteristics [269]. These cells are actively recruited in humans from the bone marrow [102] and we have demonstrated their physiological vasculogenic capabilities in vivo [271]. Putative EPCs have a suggested, but unconfirmed role for physiological endometrial angiogenesis in the mouse and human [272, 273]. Thus, the participation of ECFCs in the pregnant uterus would likewise be anticipated.

In the maternal circulation, most studies report an increase in ECFC number (or their equivalents) through the second half of pregnancy [169], with cells significantly elevated in the last trimester [274]. Whether this reflects increased production or decreased consumption hampers interpretation. As the rate of fetal development declines, functional capacities of fetal ECFCs decrease with gestational age [266], however, the course of changes in the number of fetal ECFCs is debated [228, 275].

The clonogenic classification of ECFCs by Ingram et al. defined three sub-sets of cells, with varying proliferative capacity [61]. Of these, the most efficient, High Proliferative Potential ECFCs (HPP-ECFC), have high telomerase activity and can duplicate over a hundred times. It is of note that these cells are exclusive to the fetus and absent in the adult [61, 208]. Moreover, there is greater abundance in general of EPCs in the fetal circulation [202]. With our previous results showing an age-related decline in function, as specified by 6-o-sulphation of heparan sulphate proteoglycans [276] (Williamson, 2013 #865), these observations portend that adult EPCs fall short of their fetal counterparts, both numerically and functionally, regarding vessel formation and vascular repair.

Throughout human pregnancy the uterus undergoes extensive adaptations to accommodate the growing fetus [277]. Perhaps the most striking is the rapid expansion of the uterine microvasculature [28], supporting an increase in uterine blood flow from 20-50ml/min in the non-pregnant state, to around 900ml/min at term [278]. To accommodate,
uterine spiral arteries undergo extensive remodelling by placental-derived extravillous trophoblasts (EVT). These invasive cells interact with the arterial smooth muscle and endothelium, transforming vessels into wide-bore, high-flow conduits [13,279]. Although originally conceived that EVT trans-differentiated to replace this liberated endothelia [280-283], recent evidence supports a practice of re-endothelialisation within these spiral arteries [284], and an active process of de novo endothelial replacement.

We have previously demonstrated active migration and uptake of ECFCs from the fetus to the placenta, and shown their role in normal placental vasculogenesis [271]. Here we considered whether these cells, with their superior potency, traverse the placenta; circulate in the maternal blood and home to sites of intensive vessel formation, including the uterine microvasculature. Although fetal cells are found in maternal blood and can survive in a range of tissues after pregnancy [285], true confirmation of chimeric EPCs (including ECFCs) in the maternal circulation is lacking. To investigate, we have (i) tracked genetically labelled cells of the mouse fetus, ubiquitously expressing enhanced Green Fluorescent Protein (eGFP), to the maternal uterine endothelium; (ii) introduced culture-derived human fetal ECFCs into the circulation of immuno-deficient NOD/SCID fetuses during intact mouse pregnancy, confirming the subsequent integration into the maternal uterus, and (iii) identified male cells within microvessels of human uterine myometrial biopsies, estimating their relative abundance by RT-QPCR, and confirming their localisation within vessel walls by fluorescence in situ hybridization (FISH).
3.6 METHODS

3.6.1 Ethical Approval

All patients gave consent for participation and the study was approved by the North West Research Ethics Committee, UK and the Ethics Committee of Central Manchester Foundation Trust Hospitals, UK. All protocols related to animal experiments were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines.

3.6.2 Sample collection

Neonates (n=20) born at St Mary's Hospital, Manchester, UK were considered. All pregnancies were uncomplicated, with no fetal or neonatal illness detected before discharge. Babies were born at 39.6 weeks (±1.4, SD), maternal age was 30.4 years (±5.8), BMI 27.0 (± 7.9), and parity 0.8 (±0.76). For non-related reasons 50% of these babies were born by caesarean section. One mother smoked in pregnancy. Birth weights, placental weights and individualized birth weight ratios [231,232] were normal (mean ± SD, 3652 ± 341g, 679.2 ± 114.9 and 70.1 ± 20.6, respectively).

For flow cytometry, 500µl of arterial and venous umbilical blood was collected using 20G needles (Brown, Sheffield, UK) from double-clamped sections of the umbilical cords. For cell culture, 15ml mixed cord blood was used. Blood was stored in Vacutainer tubes containing EDTA (Becton, Dickinson, Franklin Lakes, NJ, USA). Blood intended for flow cytometry was cooled on ice, while blood for culture was shaken at room temperature, until processed within one hour of delivery.

3.6.3 Transmigratory model of fetal cells to the mouse uterus

Virgin wild-type C57 female mice (Strain: C57BL/6NJ, Jackson Lab, Sacramento, CA) were mated with transgenic male mice, heterozygous to the eGFP gene, under the control of a chicken beta-actin promoter and cytomegalovirus enhancer (Strain: CByJ.B6-Tg(CAG-eGFP)1Os/J, Jackson Lab). Prior to mating, eGFP was absent in the maternal organism.
After mating, a portion of generated pups were shown to ubiquitously express eGFP as a result of paternal inheritance. Using a small animal imager (Olympus, Tokyo, Japan), eGFP expressing cells of fetal origin were localised within maternal uterus and its arterial system. Cross-sections of tissue (10µm) were immunofluorescently stained, using standard protocols [286] for von Willebrand-factor (Millipore, Billerica, MA, USA), as a marker for endothelial cells, and connexin 40 and claudin 5 (Invitrogen, Carlsbad, CA, USA), for gap and tight junctions respectively, confirming true graft cell incorporation.

3.6.4 Culture expansion of ECFCs

ECFC were expanded from fresh cord blood as previously described by Mead et al. [202], using rat-tail collagen-I coated plates (BD, Oxford, UK) and EGM-2 growth media (Lonza, Basel, Switzerland).

3.6.5 Lentiviral vector transduction of ECFCs

For detection within maternal organism, fetal ECFCs have been labelled by reporter genes. A fluorescent label (eGFP) has been used for better detection of rare fetal cells, and LacZ has been used with the aim of providing more detailed anatomical observations under light microscopy.

By screening multiple HIV-1-based lentiviral vectors for optimal transduction efficiency, a vesicular stomatitis virus (VSV)-pseudotyped lentiviral vector, harboring eGFP under the control of the ubiquitous EF1α promoter was selected. An additional lentiviral vector, harboring β-galactosidase (LacZ) under the CMV promoter, was also used: pLenti6/V5-GW/lacZ from ViraPower™ (Invitrogen, Carlsbad, CA). To package vectors, 293T cells were co-transfected with pDelta 8.74 and pMD2G plasmid using polyethyleneimine (PEI). For transduction of ECFCs, 1x10⁷ cells were incubated overnight with 2x10⁷ TU lentiviral vector, in the presence protamine sulfate (7µg/ml).
3.6.6 Characterisation of fetal ECFC

Using a FACS Aria (BD, Toronto, CA), unlabelled ECFCs, eGFP-ECFCs, human umbilical vein endothelial cells (HUVECs) and CMFDA-HUVECs (HUVECs labelled with the green Cell Tracker® CMFDA in accordance with manufacturer’s instructions (Molecular Probes Cell, Invitrogen Life Technologies Ltd, Paisley UK)) were single cell sorted into 96-well culture plates, pre-coated with collagen-I (BD, Oxford, UK) (ECFCs) or 1% gelatine (HUVECs). Further cultures were prepared from seeding densities of 5000 cells/cm². ECFCs were cultured in EGM-2 media (Lonza Vervieres, S.p.r.l. Verviers, Belgium), supplemented with 1.5% (v/v) amphotericin-B, 1% (v/v) penicillin/streptomycin and 0.15% (v/v) gentamycin, HUVECs were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with similar supplements and 20% (v/v) fetal bovine serum (FBS).

To measure in vivo angiogenic capacity, ECFCs mixed with Adipose Derived Stem Cells (ADSCs) at 4:1 were suspended in a collagen-fibronectin matrix and allowed to develop processes overnight [202]. Contracted gels were implanted subcutaneously into the flanks of NOD/SCID immunodeficient mice (Strain 005557, Jackson Laboratory, Sacramento, USA) under isoflurane anaesthesia and mice were allowed to recover for 14 days, with continuous amoxicillin/clavulanate prophylaxis. Harvested implants were examined under a small animal visualiser and fluorescent microscope.

3.6.7 Mouse transplantation of human ECFCs into immuno-compromised fetuses

On D15.5 pregnancy, as determined by plug test, NOD/SCID mice under isoflurane anaesthetic were monitored on heating pads whilst abdominal fur was removed and skin disinfected. Pregnancy and fetal position were confirmed using a VS 40 high frequency, high-resolution ultrasound platform with a 30 MHz probe (VisualSonic, Toronto, ON, Canada) (Video III-1).

Live fetuses, in an optimal position with their abdomens facing the maternal abdominal wall, were selected for intervention (Video III-1a). Hearts were centred for needle insertion. 10⁷ eGFP-ECFCs, LacZ-ECFCs or CMFDA-HUVECs diluted in 70ml supplemented EGM-2
or DMEM, were taken into a 250ml GasTight luer type syringe (Hamilton AG, Bonaduz, Switzerland) with 32Gx12mm needle. By ultrasonic guidance, the needle was inserted sequentially through the maternal skin, abdominal and uterine walls into the amniotic cavity and fetal heart. After piercing the thoracic skin, the tip was guided into the cardiac cavity (Video III-1b). Here the syringe was injected over 4-5s and gently withdrawn (Video III-1c). The fetus was monitored for several minutes to confirm a re-established heart rate and to observe potential bleeding within the amniotic cavity. As the syringe routinely retains 35ml in the luer, the amount of injected cells was estimated at $5 \times 10^6$ per fetus. With transplantation complete, the mother was allowed to recover and the pregnancy to continue for a further 3.0 days, continually receiving prophylactic antibiotics (amoxicillin/clavulanate in the drinking water). The maternal uterus and broad ligaments were subsequently examined with small animal imager (Olympus OV-100, Tokyo, Japan) and dissected uteri were bisected and one half was fixed in Z-FIX (Anatech Ltd., Battle Creek, MI USA), while the other half was cryopreserved, and sectioned for histology immunohistochemistry, highlighting Claudin 5 and Connexin 40 components of endothelial tight and gap junctions, respectively.

3.6.8 RT-QPCR of maternal uterine microvessels

Human microvessels, approximately 100µm diameter and 6mm length, were isolated from muscular tissue biopsies taken at caesarean section from the upper uterine segment. Pregnancies with male babies were primarily considered, however, microvessels from women with female infants and placentas of newborn males were also used as external negative and positive controls, respectively. DNA was extracted using the Qiagen (Valencia, CA) DNA-extraction kit according to manufacturer’s instructions. DNA-concentration and purity were determined by Nanodrop ND-1000 (Thermo Fisher, Wilmington, DE) using spectrophotometric absorbance at 260nm and absorbance ratio at 260/280nm, respectively. After normalisation of the DNA-concentration (2ng/µl, DNase-free water), samples underwent RT-QPCR. The male-specific SRY gene (8 replicates) and, for internal control, the endogenous CCR5 gene (2 replicates), were amplified. (SRY and
CCR5 assay (20x), TaqMan® Universal PCR Master Mix, No AmpErase® UNG, ABI Prism7900 Sequence Detection System (Applied Biosystems, Carlsbad, CA)). PCR settings were: 50°C 2min, 95°C 10min, 45 cycles 95°C 15s, 60°C 1min [287]. Experiments were considered successful if all samples had cycle thresholds (ct) < 40 for CCR5, while negative controls had ≥45 and positive controls <38 ct values for SRY. Test samples were considered positive for the presence of SRY in the event of at least one replicate having repeated ct value <40 in two independent experiments. Standard curves were generated using human male genomic DNA (20ng/µl-0.02ng/µl, Promega, Southampton, UK) and resultant ct values used to determine male cells per vessel extract (ABI SDS software). This technique, in our hands, has specificity of 100% and 98% sensitivity [287].

3.6.9 Sex chromosome FISH

Similar vessels were used as for RT-QPCR above. Samples, initially embedded in OCT, were first sectioned (10µm thick) then fixed with 4% (w/v) paraformaldehyde. Slides were baked at 64°C, tissues were permeabilised by a sequence of citrate buffer treatment, immersion into saponin (1.5% (w/v)) and Triton-X (1.5% (w/v)) with added glycerol (20%(v/v)), followed by five freeze-thaw cycles in liquid nitrogen and glycerol (20%(v/v)). The process of permeabilisation was complete by immersion into pepsin 1%(w/v) in10mM HCl. DNA was denatured at 72°C and slides incubated with denatured Cy-3 labelled Y-chromosome specific, and FITC-labelled chromosome 20 or 15 specific, whole-chromosome paints (STARFISH, Cambridge, UK). Slides were counterstained with DAPI and analyzed using a cytogenetics-workstation (Leica, Peterborough, UK). Chromosomal signals were determined as intranucleal fluorescent events within the focal plane of nuclei, having identical shape, size and calculated fluorescent brightness equal to male placental controls.

3.6.10 Statistics

Given the novelty of the field, no comparable studies have been found in the literature, and thus sample sizes were determined empirically. Normality of distribution was determined by
D'Agostino-Pearson and Smirnoff-Kormogoroff tests, and parametric, non-parametric tests applied accordingly using Prism V5.0a software (Graphad Software Inc., La Jolla, CA).

3.7 RESULTS

3.7.1 Fetal endothelial-like cells are found in maternal uterine vessels in the mouse

Native virgin female mice mated with transgenic males, heterozygous for eGFP, resulted in litters of eGFP-positive and negative pups (Figure III-1a). In control experiments, all pups derived from mating native mice were homozygous eGFP-negative (n=3, Figure III-1b).

Using optical imager at D18.5, fluorescent cells, derived from eGFP-expressing fetuses, were observed within uteri of pregnant dams (n=6, Figure III-1c) and also in their broad ligaments, a peritoneal fold, which includes the vascular supply of the uterus (Figure III-1d). These cells were found distal to placental implantation sites, and thus potential regions of extravillous trophoblast invasion. Close inspection (10-16x magnification) showed fluorescent fetal cells lining-up in colonies subjacent to vascular lumina. Video recordings showed these chimeric cells to be unaffected by circulating erythrocytes, suggesting complete vascular infiltration (Video III-2). In controls, no fluorescent signals resembling uterine or broad ligament colonisations were detected (n=3; Figure III-1e and f, respectively). Dual fluorescence from intrinsic eGFP-expression and staining for Von Willebrand Factor, a recognised endothelial marker, confirmed endothelial lineage for these transmigrated fetal cells (Figure III-1g). These observations, demonstrating fetal-derived cells of endothelial progeny (but not unequivocally ECFCs) within the murine uterus, prompted utilization of a further model, designed to more specifically investigate human ECFC-behaviour.
Figure III-1. Fetal cells of endothelial characteristics cross the mouse placenta and colonize the uterine vasculature in a transgenic murine model with eGFP-expressing offspring.

Images taken with small animal imager demonstrated eGFP-positive offspring (yellow arrows) in dissected intact uterine horns of eGFP-negative mothers (n=6), supplied by vessels of broad ligaments (red arrows, A). Pups (yellow arrows) did not express eGFP in the event of control non-transgenic mouse mating (n=3, B) The adjunct to (B) shows the structure of murine anatomy of reproductive organs as seen under fluorescent imager after delivery of fetuses and placentae by hysterotomy. White arrow points at the cervix, orange arrows point at both uterine horns. The broad ligament is a duplication of peritoneum connecting the cervix and horns, and it contains the arcuate and uterine vessels. (Typical location for images C and E are marked with Ho (horn), typical location for images shown D and F are marked with Bl (Broad ligament).) Fluorescent cells of fetal origin were detected by small animal imager at D18.5 within maternal uteri (C) and broad ligaments (D). These cells (yellow arrows) were aligned along lumina of arterial and venous branches (red arrows), and frequently formed obvious vascular colonies, while eGFP signals were undetected in uteri (E) and broad ligaments (F) of controls. For cross mating, immunofluorescence of uterine cross-sections showed co-expression (amber, yellow arrows, G) of fetus-associated eGFP (green) and the endothelial marker Von Willebrand Factor (red) by cells surrounding vessel lumina (L), confirming both fetal origin and endothelial character. Blue: Nuclei. Scale bars (unless indicated) = 10µm.
### 3.7.2 Human fetal ECFCs transmigrate the murine placenta and exhibit vasculogenic function

Human fetal ECFCs, isolated from neonatal umbilical cord blood by using established culture techniques [202], were phenotyped for authenticity before transfection by retroviral gene delivery systems transducing the eGFP or LacZ reporter genes for ubiquitous labelling (eGFP-ECFCs or LacZ-ECFCs). They were found to express CD31, CD105, CD146, CD34, VEGFR-2, stain with UEA-lectin, take up Ac-LDL and form tubes on Matrigel, suggestive of endothelial lineage, while they did not express CD14, CD45 and CD133 excluding mononuclear, haematopoietic origin. Both untreated and modified cells proliferated in single cell cultures and showed optimal and unaffected angiogenic capabilities when subcutaneously implanted within adipose-derived stem cell, collagen and fibronectin combined artificial tissue blocks into immuno-deficient NOD/SCID mice, two features unique to ECFCs and essential for their full characterization [269]. Expression of reporter genes was confirmed in these fetal ECFCs preceding subsequent use for transplantation.

In pregnant NOD/SCID mice, identical cells were intra-cardiac injected under sonographic guidance into murine fetuses at D15.5 (Video III-1). After injection and animal recovery, human eGFP-ECFCs (n=7 mouse pregnancies) or LacZ-ECFCs (n=5) were tracked to the maternal mouse uteri on D18.5. Within these uteri, groups of genetically labelled cells were observed associating with arterial walls (Figure III-2a). Real-time video (Video III-3), demonstrated their retention within vessels despite visible and contiguous blood flow. Parallel studies, with injected eGFP-HUVEC (n=2), CMFDA-HUVEC (n=2) or EGM-2 (n=2) medium alone, failed to demonstrate evidence of transmigration and uterine relocation of fluorescent cells (Figure III-2b). Tissue staining for tight and gap junction markers, claudin 5 and connexin 40, confirmed mural integration of eGFP/LacZ-ECFCs (Figure III-2c and d, respectively). Graft cells were observed forming complex vascular structures (Figure III-2c) and de novo lumina by vacuolisation (Figure III-2d), providing combined evidence for cross-species capacity for vasculogenesis and vascularisation.
Figure III-2. Transplanted fetal-derived human ECFCs traverse the mouse placenta and home to the pregnant uterus.

On D15.5, eGFP (n=7) and LacZ (n=3) expressing culture-propagated human cord blood ECFCs were transplanted by ultrasound guided intra-cardiac injection into NOD/SCID mouse fetuses. Using a small animal imager, eGFP-fluorescent cells (yellow arrows) were subsequently located along the vascular lumina (red arrows) within the pregnant mouse uterus (A). Although detectable in the blood of mouse placentas [271], fluorescent-tracked mature endothelial cells (HUVECs) could not be defined in the maternal uterine microvasculature (n=2, B). Combined fluorescence and light microscopy of uterine tissues (red nuclei), showed transplanted eGFP-ECFCs (yellow arrows) undertaking de novo lumen (L) formation (C). Positive staining for claudin-5, a tight junction marker (brown stain), confirms their integration, while non-integrated ECFCs fail to express tight junctions (red arrow). Transplanted human LacZ-ECFCs (punctate blue stain, yellow arrows) similarly demonstrated vasculogenic activities, including vacuole formation (L, insert), and expressed the gap junction marker connexin 40 (brown), confirming full vascular integration (D).
3.7.3 Fetal derived endothelial cells located in the human uterine vasculature

For human observations, micro-arteries (60-140µm internal diameter x 4-8mm length) were dissected from uterine muscle biopsies obtained from elective caesarean section of 12 uncomplicated pregnancies with singleton male babies. The presence of male specific SRY gene and Y chromosome were explored by RT-QPCR and FISH, respectively. The presence of SRY of fetal origin was detected in the extracted DNA of these vessels in at least one in eight replicate reactions in two independent sets of experiments. Overall, the SRY gene was detected in 6 of 12 pregnant women (Figure III-3a). Uterine microvessels from mothers with female babies were used as external negative controls (Figure III-3b, n=3) and placental chorionic plate arteries of a male baby for positive confirmation. Using a dilution curve and the total quantity of DNA extracted, the SRY-copies in positive vessels were successfully quantified in 4/6 positive samples as 331 [IQR 283-892] (Figure III-3c). Using an estimated length (6mm) and diameter of vessel (100µm) a crude calculation yields an internal surface area of 1.89mm², giving 175 [IQR 149-471] fetal cells/mm² endothelium. Assuming a single endothelial cell has a diameter of 30µm; 1mm² vascular lining is covered by 1411 endothelial cells in total. The proportion of fetal-derived cells within the pregnant uterus therefore approximates to 12.4% of the total microvascular endothelium at term.
Figure III-3. The presence of fetal-derived male cells within maternal human uterine vessels.

RT-QPCR amplification plots of the male-specific SRY gene in microvessels from biopsies of the upper uterine segments showed gene presence in 6/12 cases of mothers with male offspring (A, arrow points at positive SRY-amplification, fluorescence higher than threshold (red line). Gene copies were absent in women with female babies (B, n=3, arrow points at intact detection threshold). Horizontal axes: PCR cycle numbers, vertical axes: ΔRn (arbitrary units (log)). In 4/6 positive cases the copies of SRY were successfully quantified (C).
To further assess the exact location of fetal cells within the various anatomical structures of vascular walls, vessel cross-sections embedded in uterine biopsy samples were hybridized in situ using fluorescent-conjugated (Cy-3) Y-chromosome specific whole chromosome paint probes. FITC-conjugated probes for chromosomes 20 or 15 were simultaneously used as internal controls. Typical intra-nuclear fluorescent events with luminescence equivalent to external controls were considered as genuine chromosome-associated signals. Y-chromosome-containing fetal cells were detected in at least one from three sections of uterine microvessels obtained from 8 of 11 pregnancies studied (Figure III-4a-c). Individual fetal cells and colonies were observed in the endothelium of larger arteries (Figure III-4a), arterioles (Figure III-4b), and veins (Figure III-4c). Detachment of endothelial cells from the basal membrane resulted in spherical remodelling of originally elongated nuclei, allowing superior chromosome-detection in these cells (Figure III-4a), compared to the, otherwise more informative, case of intact vascular structure (Figure III-4.b-c). As opposed to spherical shape, chromosomes in elongated nuclei were distributed through several focal planes with the consequential decline in the probability of simultaneous visualisation of control and Y-chromosomes. Additionally, with preserved mural nuclear position, proximity of exceptionally high autofluorescence from arterial wall collagen resulted in further impairment of chromosome recognition. Fetal cells were not observed in non-endothelial vascular components, although recognition of chromosomal signals in arterial adventitia was intricate, given the high autofluorescence of this layer. Placental chorionic plate arteries, associated with male neonates (n=4), were used as confirmatory positives (Figure III-4.d), while uterine biopsies from pregnancies eventuating female offspring (n=2) were used as negative controls (Figure III-4e).

Surprisingly, the incidence of positive findings was infrequent which did not correspond to RT-QPCR results. This discrepancy is not only explained by the technical difficulties involved in imaging elongated endothelial nuclei, but also by the poor penetration of FISH-probes into myometrium compared to control placental tissue. This resulted in generally dimmer chromosomal fluorescence in these samples, but Cy-3 signals weaker than in controls were invariably disregarded in order to ensure maximal specificity of the test, even at the cost of overlooking fetal cells contributing to the inconsistency with RT-QPCR.
Figure III-4. Endothelial location of fetal-derived cells within uterine vessels.

Y-chromosome specific FISH in uterine tissue cross-sections detected male cells in the endothelium in 8/11 mothers with male babies, as demonstrated by prominent Y-signals in detached endothelial cells of a larger arterial branch (A), a colony of fetal cells in the intact endothelial lining of an arteriole (B) and a fetal cell in a venous endothelium (C). Male placentas were used as positive controls (D, n=4), and uterine tissue of mothers with female offspring was used for negative control (E, n=2, magenta arrow points at an artefact resembling Y-signals, but outside nuclear margin). Somatic chromosomes were hybridized for internal control. (Red colour and arrows: Cy3-conjugated Y chromosomes, Green colour and arrows): FITC-conjugated chromosomes 20/15, Grey: DAPI, L: Lumen, Scale bars: 5µm, 20 µm (inserts), 50 µm (insert A)).
3.8 DISCUSSION

From initial transgenic matings, eGFP-expressing cells of fetal origin were identified as resident within the pregnant mouse uterus. These cells, of confirmed endothelial lineage, showed positive transmigratory capacity, in line with past maternal models of mouse vascular injury [224]. However, ECFCs are solely distinguished from mature endothelial cells by their functional characteristics [200]. It was therefore imperative to combine our search for fetal-derived cells in the uterus, with a mouse model of chimerism incorporating functional human ECFCs. The further tracking of fetal-derived human ECFCs, cardiac-injected within the fetus, showed equal infiltration of the pregnant mouse uterus, with additional de novo vessel formation. This transmigration and vascularisation was peculiar to fetal ECFCs and absent with mature endothelial cells (HUVECs), similarly transplanted. Although the mechanism by which fetal ECFCs migrated across the feto-maternal barrier and integrated the maternal uterus was undefined in this study. For progression of the cells, a passive, accidental movement cannot be excluded, as elicited by haemodynamic disturbances within the placental intervillous spaces [288]. Chemotaxis to agents which stimulate ECFC passage may be another mechanism, as a number of confirmed chemotactic agents, i.e. EMAP-2, SDF-1, IL-8, VEGF and Gro-alpha, are reported in both uterine and placental tissues [289-293]. For transmigrating the fetomaternal barrier, their previous characterisation proposes a matrix metalloproteinase-2 (MMP-2) dependent process, for which these cells are highly regarded [132]. Although HUVECs also respond to these agents, and produce MMPs, their migratory capacities and the expression of the MMP-2 and collagen binding receptor CD44 are inferior [256,257]. Irrespective of placental integration, ECFC survival is favoured in the hostile environment guarded by maternal immunity, as these cells are not allogenic, for they do not express major histocompatibility complex II (MHC-II) and their level of MHC-I is minimal, even after INFg-stimulation [294]. The further dominance of ECFCs over HUVECs is also apparent at the time of vessel invasion. ECFC integration depends upon active urokinase-like Plasminogen Activator Receptor (uPAR), preferentially found in cellular caveolae [295]. uPAR and caveolae are both up-regulated by vascular endothelial growth factor (VEGF) [295] [296]. With ECFCs in general having higher expression of uPA, uPAR and VEGFR-2, along with greater MMP-2
activity than HUVECs [255,256,296], ECFCs again hold the clear advantage. For *de novo* vasculogenesis, ECFCs actively excrete components of the vascular basal membrane and extracellular matrix. Differentiated endothelial cells can also produce laminin, collagen and fibronectin, but they fail to deposit these agents extracellularly [258]. Furthermore, their restricted proliferation, inability to form colonies and incapacity for integration [271], would limit maternal vasculogenesis by HUVEC. The fact that not only incorporated but even free HUVECs were absent in any uterine tissue type of both mouse and human, and they failed to integrate into placent al vessels in our past transplantation studies, where ECFCs succeeded ([271]), strongly implies that mature endothelial cells, unlike fetal ECFCs, fail at the first hurdle in placental transmigration, unable to infiltrate placent al vessels, let alone traverse the placental for eventual uterine residency.

Within our studies, the incorporation of human fetal ECFCs in the mouse uterus, although patchy, must be taken in the context of the *in vivo* period of incubation, i.e. 3.5 days, as compared to 280 days of human pregnancy. Given that these fetal ECFCs can proliferate for at least 200 days in culture, a single transmigratory cell could yield 1023 daughter cells within second and third trimesters [61]. Considering this, and also the observed absence of fetal-introduced HUVECs in the maternal vasculature, it is perhaps not surprising that the frequency of fetal cells within human uterine vessels was as high as 12.4% at term, as these cells are likely ECFCs and not mature endothelial cell equivalents. This level of mass migration, or in situ expansion, supersedes that of microchimerism reported in other maternal tissues [210,297]. This frequency also hints at a physiological role in the human and cannot be explained by coincidental inoculation.

Beyond the previously mentioned low MHC-expression of ECFCs and endothelial cells derived from them, it is reasonable to believe that upon full maturation of subsequent generations of daughter cells, MHC-expression may notably increase over time. Within the maternal circulation and entrenched within the uterus, it is difficult to envisage how these daughter cells avoid immune detection, or instigate maternal tolerance. Nonetheless, this same conundrum befits the fetus, and more specifically the placenta, and although hotly debated, appears to have basis in unresponsive T-lymphocytes, with a direct requirement for Regulatory T cells [298]. Perhaps the most important question is how this acceptance
can be maintained in a past-pregnant woman, sometimes decades post-delivery. There is no doubt that after normal pregnancy, or pregnancy loss, fetal cells remain distributed widely in maternal tissues, differentiated into numerous cell phenotypes. Murine models of persistent microchimerism demonstrate clonal deletion in the thymus of self-reactive T cells which could prolong this transitory tolerance [299]. Nevertheless, cytotoxic T cells, specific for fetal cells, can be continually isolated in women post-delivery [300], suggesting a more delicate balance between tolerance and active immunity. The maternal impact of protracted microchimerism is also undecided. It is speculated that harbouring fetal cells could benefit or disadvantage maternal health and future pregnancies, contributing to tissue repair and tumor surveillance on one-hand, or encouraging autoimmune disease and secondary miscarriage on the other.

By producing and releasing potent ECFCs into the umbilical circulation, the fetus contributes to placental vascularisation [271]. From this study it looks feasible that similar ECFCs assist in maternal perfusion of the human placenta, expanding uterine microvasculature by being actively involved in formation of de novo endothelium during pregnancy. From these results it is noteworthy that fetal-derived endothelial cells were identified in abundance in myometrial arteries distal to the utero-placental bed, i.e. the point of placental origin. In these distal sites it could be envisaged that infiltrating ECFCs have a more classical role in vasculogenesis and vascular expansion. Nevertheless, their additional importance in vascular repair cannot be discounted, especially within the placenta-bed and more specifically proximal, transformed spiral arteries. Given the likelihood of re-endothelisation of these arteries following trophoblast remodelling [284], it is tantalizing to consider that this restored endothelium is wholly re-colonized and re-established by fetal ECFCs. Although this suggestion is hard to confirm, given the practicalities of obtaining placenta-bed biopsies and undoubted presence of contaminating trophoblast, this finding would place fetal-ECFCs on a par with invading trophoblasts with regard to human placentation, placing fundamental importance on their integrity for successful pregnancy.

As both adult and fetal ECFCs have vasculogenic capacities, and endothelial cells derived from EPCs are not subject to a maternal immune response [294], ECFCs of fetal or
maternal origin may, in theory, concomitantly contribute to vascular expansion in the pregnant uterus. However, with fetal ECFCs superior to the adult in both their vasculogenic and proliferative capabilities [61,301,302], it is anticipated that contributions would favour fetal cells, particularly given the infrequency of adult ECFCs in the peripheral circulation (1 cell/10ml) [202]. Nevertheless, without substantive data, the relative contribution of maternal and fetal ECFCs within the pregnant uterus remains undecided.

For a number of obstetric complications, utero-placental irregularities are considered pathogenic cornerstones. Thus, within pre-eclampsia and intrauterine growth restriction, suboptimal conditions are often forewarned by sonographically defined elevations in uterine artery resistance. As a surrogate for downstream arboration and vessel distensibility, these uterine artery measurements arguably highlight suboptimal microvascularisation. Given the abundance of fetal ECFCs in these affected microvessels, restricted numbers or function could constitute a realistic pathogenic phenomenon. In pre-eclampsia, vascular inflammation and maternal endothelial dysfunction have been linked to restricted maternal EPC numbers and proficiency [172], although phenotypic definition and quantification challenge this suggestion [228]. Whether these changes in EPCs (inclusive of ECFCs) precede the clinical condition is unknown. Two recent reports, highlighting a decrease in cord-blood EPCs in pre-eclamptic women with unclassified and severe disease [303,304], certainly tally with a maternal dependence on these fetal cells. Our new finding links these observations, perhaps shifting importance away from circulating adult-derived EPCs in pre-eclampsia, to the far more efficient ECFCs of the fetus, residing within, but perhaps not restricted to, the maternal vascular uterus.

Within the fetal circulation recent studies have associated aberrant EPC numbers and function with preterm birth and fetal growth restriction [266,305]. These anomalies may impact upon placental vascular development, restricting nutrient supply. If these same abnormalities persist in chimeric ECFCs, then a concomitant effect on maternal vascular adaptations would be predicted, and indeed this notion is upheld with the clinical overlap between IUGR and early onset pre-eclampsia [306]. Moreover, given that vascular repair is a perquisite for cardiovascular health [115], it would also be predicted that fetal ECFCs that underperform in the fetus and maternal uterus in pregnancy could equally underperform in
later-life. Epidemiological evidence supports this, with both mothers and babies affected by IUGR and pre-eclampsia showing increased susceptibility to adult cardiovascular disease [71,79]. In summary, this is the first confirmation of fetal ECFC passage across the human placenta and first description of fetal cells maintaining active function following transmigration. Their frequency and relative activity in comparison to native cells, would propose a profound role in uterine vascular expansion and optimal placentation, and speculations regarding their pathogenic role in pregnancy seem warranted. For description of this novel phenomenon, we propose the term functional feto-maternal macro-chimerism. Their subsequent involvement in maternal autoimmune disease and long-term cardiovascular risk are likewise intriguing propositions.
3.9 SUPPLEMENTARY INFORMATION

Video III-1. Ultrasound-based in vivo injection of human fetal ECFCs into the mouse fetal circulation.

After anaesthesia, with full vital monitoring, target fetuses (arrow) were identified using a high frequency ultrasound system (A). A 32G needle (arrow) attached to a syringe containing ECFCs was sonographically guided through the maternal skin, peritoneum, uterine wall and amniotic sac to perforate the sternal surface of the fetal chest and wall of the cardiac ventricle (B). Once in position, the aortic arch was visualized in the oblique plane and the content of the syringe slowly injected into the ventricle. Following ventricular contractions, the echo-dense (yellow arrow) cell suspension is visible jetting into the aortic arch (blue arrow) on its way to the fetal circulation (C). (See Video III-1a-c on attached DVD.)

Video III-2. A series of real-time in vivo optical sequences of the mouse uterine microvasculature infiltrated by transmigratory fetal cells.

The eGFP-expressing fetal cells (bright green), generated by transgenic/native cross-matings, are shown as clusters/colonies (yellow arrows) aligning the microvascular lumina. The colonies are fixed within the vessel walls, unperturbed by maternal blood flow (red arrows). (See Video III-2 on attached DVD.)

Video III-3. In vivo images of pregnant uteri of NOD/SCID mice whose fetuses were transplanted with human fetal eGFP-ECFCs.

Transmigratory cells are subsequently observed at different magnifications, singularly or in clusters, aligning the maternal uterine microvascular lumina (yellow arrows). These cells, fixed within the vascular walls, remain static in the presence of moving maternal blood (red arrows). (See Video III-3 on attached DVD.)
4 CHAPTER 4

ENDOTHELIAL COLONY FORMING CELLS DERIVED FROM PREGNANCIES COMPLICATED BY INTRAUTERINE GROWTH RESTRICTION ARE FEWER AND HAVE REDUCED VASCULOGENIC CAPACITY

Peter Sipos¹,⁴, Stephane L. Bourque²,⁴, Carl A. Hubel³, Philip N Baker², Colin Sibley¹, Sandra T. Davidge², Ian P. Crocker¹.

1. Maternal and Fetal Health Research Centre, Manchester Academic Health Science Centre, University of Manchester, Central Manchester University Hospitals NHS Foundation Trust, Oxford Road, Manchester, M13 9WL, UK.
2. Department of Obstetrics and Gynecology, University of Alberta, Edmonton, Alberta, T6G 2S2
4. These authors contributed equally to this work

Short Title: Dysfunctional fetal ECFCs in IUGR

Word count (total): 2718

Corresponding Author:
Dr. Ian Crocker,
Maternal and Fetal Health Research Centre,
Manchester Academic Health Science Centre,
University of Manchester,
Central Manchester University Hospitals NHS Foundation Trust,
Oxford Road, Manchester, M13 9WL, UK.
Email: ian.crocker@manchester.ac.uk
Ph: +44(0)-161-701-6973; Fax: +44(0)-161-701-6971
4.1 ABSTRACT

**Rationale:** Endothelial colony forming cells (ECFC) are a subset of endothelial progenitor cells capable of vasculogenesis and endothelial repair, and their dysfunction may represent a risk factor for cardiovascular disease. Intrauterine growth restriction (IUGR) is a pregnancy-related disorder, which is associated with long-term vascular dysfunction.

**Objective:** To determine whether ECFCs derived from pregnancies complicated by IUGR exhibit altered vasculogenic potential.

**Methods and Results:** Arterial and venous cord blood ECFCs were counted by flow-cytometry and morphologically and functionally characterized following expansion in cell culture. Vasculogenic capacity was investigated *in vivo* by measuring vessel formation from ECFCs in artificial tissue implants in adult immuno-deficient (NOD/SCID) mice. ECFC numbers, as a percentage of mononuclear cells, were markedly lower in arterial cord blood of IUGR pregnancies, compared to controls (median and interquartile ranges: 0.0 [0.0-0.0009], n=10 vs. 0.0031 [0.0007-0.0070], n=17, P=0.002). These cells exhibited longer doubling times in culture than those from normal pregnancies (3.4 [3.0-4.2] days, n=8 vs. 1.5 [1.1-3.1] days, n=10, P=0.01), indicating a reduced proliferation rate. *In vivo*, there was a significant reduction in blood vessel (4.1 [3.0-8.6], n=8 vs. 32.0 [14.9-46.9] vessels per field, n=8, P<0.001) and capillary formation (3.2 [2.5-4.6], n=8 vs. 16.8 [9.3-30.6] capillaries per field, n=7, P=0.001) in artificial tissue blocks populated with IUGR-derived ECFCs.

**Conclusions:** These findings suggest that ECFCs derived from IUGR cord blood are rarefied and dysfunctional, resulting in diminished vasculogenic potential. This could be one of the causes of placental dysfunction in IUGR and have long-term postnatal implications for circulatory function in offspring.

**Key Words:** Endothelial colony forming cells, progenitor cells, intrauterine growth restriction, pregnancy, vasculogenesis.
4.2 INTRODUCTION

Endothelial progenitor cells (EPCs) are a subset of bone-marrow derived cells central to developmental and postnatal vasculogenesis (reviewed in [228][307]). Two subsets of EPCs have been identified; these play distinct but complementary roles in vascular formation. Endothelial colony forming cells (ECFCs) are a well-defined homogeneous cell population phenotypically identical to mature endothelial cells in most respects, but display true vasculogenic and progenitor properties [102]. They are actively involved in vasculogenesis and vascularisation by directly populating the new endothelial surface. In contrast, “circulating angiogenic cells” (CACs) are of haematopoietic lineage and are recruited to areas of nascent vessel formation, and promote neovascularisation principally via modifying the intracellular matrix and orchestrating interactions between various cell types (including ECFCs) via secretion of paracrine factors (reviewed in [228]). Collectively, EPCs are thought to play a prominent role in forming and maintaining the vasculature, and as such, their dysfunction is a proposed risk factor for cardiovascular disease [308].

Intrauterine growth restriction (IUGR) is a complication in pregnancy in which the fetus fails to achieve its genetically determined growth potential [80]. Epidemiological and animal studies have shown that smaller size at birth predisposes to increased cardiovascular disease in adult life [309,310]. Currently the mechanisms underlying this “developmental programming” of cardiovascular disease remain unclear. Here we tested the hypothesis that ECFCs derived from pregnancies complicated by IUGR exhibit altered vasculogenic potential.
4.3 METHODS

An expanded Methods section is available in the Online Data Supplement (Section 4.4). Ethical approval and subject written informed consent were obtained. Term neonates (gestational age ≥ 37 weeks) in the study were born from uncomplicated pregnancies (control; n=23) or those with intrauterine growth restriction (IUGR; n=13) defined by an individualized birth weight ratio (IBR) ≤5. ECFCs and CACs were counted in both umbilical arterial and venous blood. ECFCs were isolated from the umbilical blood using established culture techniques [202], and their growth characteristics were recorded. The identity and purity of ECFCs expanded from cord blood was rigorously verified (see details in supplement). ECFCs were subsequently exposed to 21% O\textsubscript{2} or 1% O\textsubscript{2} (hypoxia) over 72 hours for in vitro functional studies. Vasculogenic capacity of ECFCs was established by analysing the presence of de novo vasculature in artificial tissue blocks containing these cells 14 days after implantation into NOD/SCID mice.

Data are presented as medians and interquartile ranges. Distribution was examined with D’Agostino-Pearson and Smirnoff-Kormogoroff tests, and compared by parametric or non-parametric tests or two-way ANOVA. Data were analysed with Prism 5 software package (Graphpad Software Inc., La Jolla, CA, USA).
4.4 ONLINE SUPPLEMENTARY METHODS

4.4.1 Patients

Women with normal (n=23) and IUGR-complicated pregnancies (n=13) at gestational ages more than 36 weeks were included in the study. Intrauterine growth restriction was defined as term neonates with an individualised birth weight ratio less than 5 [231,232]. Exclusion criteria included: medical or obstetric complications of pregnancy other than IUGR, use of any medication or adverse medical history. For flow cytometry, >500µL of arterial and venous umbilical blood samples were collected using 20G hypodermic needles (Brown, Sheffield, UK) from double-clamped umbilical cords immediately after delivery. For cell culture, >15mL mixed cord blood was collected from unclamped sections of cords. Blood was collected into Vacutainer tubes containing EDTA as anticoagulant (Becton, Dickinson, Franklin Lakes, NJ, USA). Samples for flow cytometry were kept on ice, while samples for culturing were maintained at room temperature with continuous shaking.

Consent was obtained from all participants and the study was approved by the North West Research Ethics Committee, UK and the Ethics Committee of Central Manchester Foundation Trust Hospitals, UK. Protocols of animal experiments were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines.

4.4.2 ECFC isolation, culture and phenotyping

Mononuclear cells were isolated and expanded from whole fetal blood according to established protocols [202]. Briefly, whole blood was diluted (3:4 with phosphate buffered saline) and subjected to density gradient centrifugation (711xG for 30 minutes) using Histopaque-1077 (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). The mononuclear cell layer was then collected and placed into collagen-I coated dishes (60 million Cells per well in a 6-well cell-culture plate) using fully supplemented EGM-2 media (Lonza Group Ltd,
Basel, Switzerland). In this setting, adherent single layers of cobble stone shaped, late outgrowth cells are suggestive of ECFCs, and their colonies are clones of one single ECFC. Colonies were defined as discrete clusters consisting of more than 50 cells. Culture plates were observed daily for their appearance. The time elapsed between seeding and first appearance of visible colonies, and population doubling times [311], as determined by live cell count during first three passages were recorded. The identity of isolated cells was confirmed by a range of stringent phenotype criteria and extensive functional assays. The cells were shown to display typical cobble stone morphology, to express several endothelial markers (CD31, CD 34, CD 144, CD105, CD 146, Ulex Europaeus lectin) and to lack any haematopoietic (CD45) or mononuclear (CD14) markers. In vitro, these cells were capable of Ac-LDL (BD Biosciences) uptake and formed tubes on Matrigel (BD Biosciences, Oxford, UK). When single cell sorted using a FacsAria III Cell Sorter (BD Biosciences), they were shown to grow from single-cell cultures, a proof of true progenitor progeny [61]. Their vasculogenic capacity was demonstrated by evidence of ECFC-based de novo vessel formation within subfascial murine implantations of artificial tissue blocks. Implanted human ECFC were retrovirally transduced to express eGFP (eGFP-ECFC) to label and distinguish them from cells of murine origin and confirm their role in neovascularisation [312].

4.4.3 Flow cytometry

Flow cytometry was used to calculate the quantity of both subsets of EPCs sequestered by the examined placentas, by determining the circulating ECFC and CAC numbers in both the umbilical arteries and corresponding veins as percentages of mononuclear cells and calculating the placental uptake. Venous and arterial umbilical blood samples were obtained from the umbilical cord at birth of the placenta. Following blockage of non-specific binding sites with 20µL of FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany), conjugated antibodies were added to 125mL of tested blood directly in optimised quantities (4µL CD31-FITC (BD Biosciences, Franklin Lakes, NJ, USA), 5µL CD133-PE (Miltenyi Biotec), 5µL KDR-PE (R&D Systems, Minneapolis, MN, USA), 10µL CD45-APC-H7 (BD Biosciences), 3µL CD34-APC (BD Biosciences), lysed in non-fixing
lysis buffer (Pharmalyse lysis Buffer (BD Biosciences). Prior to running on CyAn flow cytometer (DAKO, Glostrup, Denmark) 5uL of the viability stain 7AAD (BD Biosciences) was added. Flow cytometer was calibrated using Sphero Rainbow fluorescent particles (BD Biosciences), while gain, compensation and autofluorescence was accounted for by measurements with corresponding isotype controls. Data were analysed by Summit Flow Cytometry Analysis Software (DAKO) and ECFCs were determined in the mononuclear gate and characterised by the discriminators 7AAD-/CD31bright/CD45-/KDR+/CD34+, while CACs were determined as 7AAD-/CD31-/CD45+/CD133+/CD34+ [230].

4.4.4 Cell migration and chemotaxis assays

For migration assays, ECFCs were detached from culture, suspended in phenol red-free and serum-free DMEM (Invitrogen, Carlsbad, CA, USA). 3x10⁵ cells were seeded over the porous membrane of 24-well fluorescent cell migration assay kit (Millipore, Billerica, MA, USA), and phenol red-and serum-free DMEM was added to the lower chamber. For chemotaxis assays, the lower chamber was supplemented with chemotactic factors: SDF1α (20ng/mL), IL-8 (100ng/mL) or VEGFβ (2µg/mL).

Plates of migration and chemotaxis assays were incubated in humidified environment at 5% CO₂ for 24 hours. Chambers were handled and stained according to manufacturer’s recommendations and microplates read on SpectraMax Gemini XS plate reader (Molecular Devices, Sunnyvale, CA, USA) and data analysed using Soft Max Pro 4.8 software (Molecular Devices). Numbers of migrated cells were calculated from measurements of the intensity of fluorescence using a standard curve. Experiments were carried out in duplicate.

4.4.5 Gelatin zymography for MMP-2 activity

Activity of MMP-2 was assessed by gelatine zymography. Conditioned medium (EBM-incubated for 12Hr with cells, then spun at 12,000Xg to remove cell debris) from ECFC were loaded on 8% sodium dodecyl sulfate-polyacrylamide gels copolymerized with gelatine (2 mg/mL, type A, from porcine skin; Sigma Aldrich). After 1 hour electrophoresis,
gels were washed with 2.5% Triton X-100 at room temperature for 1 hour, during which time the solution was changed every 20 minutes. Gels were then incubated for 18 hours at 37°C in incubation buffer: 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L CaCl2, and 0.05% NaN3 (pH 7.6). After incubation, gels were stained with 0.05% Coomassie Brilliant Blue (Sigma Aldrich) in a mixture of methanol-acetic acid-water (2.5:1:6.5 v/v) and de-stained in aqueous 4% methanol-8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against a dark blue background. Digital images were obtained with a high-resolution scanner (Expression 1680, Epson, Nagano Japan), and band intensities were quantified using Quantity One 4.3.1 software (BioRad Laboratories Inc., Berkley, CA, USA).

Bands were normalized to lysate protein concentration. For protein lysates, confluent monolayers of cells were washed with PBS, and each well of a 6-well plate was treated with 400mL/well of cell lysis buffer (25mM Tris, 0.5% Triton X100), supplemented with HaltTM Protease Inhibitor Cocktail (Thermo Fisher Scientific Inc., Rockford IL, USA). Plates were scraped, and centrifuged at 12,000xg to remove residual cell debris. Protein content was assayed using the Pierce BCA Protein Assay (Thermo Fisher) according to the manufacturer’s instructions.

4.4.6 Hypoxic treatments

Cells were treated with EGM-2 media that was pre-incubated in hypoxic conditions (1%O2) for 24 hours prior to experimentation. Cells were incubated in media in an oxygen-controlled (21% O2 or 1% O2) incubator for 72 hours. Following incubation, cells were removed, rinsed with PBS, and used for gelatine zymography and beta-galactosidase assays, as described above.

4.4.7 In vivo vasculogenesis bioassay

Artificial collagen-based plugs were prepared according to established procedures [202]. Briefly, 10^6 ECFC and 2.5x10^5 adipose derived stem cells (ADSC) (Invitrogen), grown
separately, were detached from culture and suspended in a collagen gel matrix consisting of a (1:1) mix of collagen-1 (BD Biosciences) and fibronectin (Fisher Scientific Ltd. Ottawa, ON, Canada) in a total volume of 500µl reagent mix (HEPES, sodium-bicarbonate, fetal bovine serum and EBM-2 (pH 7.4)). Once gelled in a 24-well culture plate, the cell-containing block was covered with EGM-2 media and incubated overnight. The resulting contracted gels (approximately 2-3mm in diameter) were subcutaneously implanted into the flanks of female adult virgin immunodeficient NOD/SCID mice (Strain 005557, Jackson Laboratory, Sacramento, USA) under isoflurane inhalation anesthesia (induction: 5%; maintenance: 1.5%) through a parasagittal skin incision in the lumbar area. Pairs of artificial tissue blocks containing ECFCs from growth restricted pregnancies and control pregnancies were bilaterally implanted into each animal. Once the wound was closed with silk sutures, pain relief from subcutaneous ketoprofen was provided (5mg/kg) and the mice were treated for 14 days with Augmentin (amoxicillin/clavulanate) prophylaxis in their drinking water to prevent post-operative infections. After this period, the implants were harvested by dissection under stereo-microscope, following halothane euthanasia in accordance with local protocols and cleaned of extraneous connective tissue, and fixed in 4% z-fixative overnight. Dissected implants were then paraffin embedded, and cut at 5 µm sections at increments of approximately 50-70µm such that 10 sections represented the entire tissue block. Tissue sections were stained with hematoxylin and eosin. Three non-consecutive sections were examined under light microscopy (Eclipse 80i, Nikon, Tokyo, Japan) and assessed for vascular plugs, with a minimum of 6 fields per slide at both 10x and 60x. Numbers and surface areas of cross sections of complex vascular structures were measured at x10, while counts of capillary structures were assesses at x60 magnification, using Image J image analysis software (NIH, Bethesda, MA, USA). Capillaries were determined as tubular structures with a diameter less than 7µm, formed by the vacuolisation of not more than one endothelial cell, while vessels are determined as more complex, wider than 7µm structures formed by more than one ECFCs.
4.5 RESULTS

4.5.1 Patient demographics

There were no differences between groups in gestational age, maternal BMI, smoking status or ethnicity (Table IV-1). Mothers of neonates in the IUGR group were younger and were more frequently primigravidas.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>IUGR</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>23</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Individualized birth ratio</td>
<td>66.7 ± 20.3</td>
<td>1.5±2.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Birth Weight (g)</td>
<td>3503 ± 341</td>
<td>2237 ± 535</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Placental Weight (g)</td>
<td>661 ± 119</td>
<td>363 ± 98</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Gestational Age (wk)</td>
<td>39.5 ± 1.5</td>
<td>38.1 ± 2.2</td>
<td>NS</td>
</tr>
<tr>
<td>Maternal BMI</td>
<td>27.1 ± 8.6</td>
<td>26.1 ± 4.3</td>
<td>NS</td>
</tr>
<tr>
<td>Parity (n)</td>
<td>0.87 ± 0.8</td>
<td>0.12 ± .35</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Maternal Age (y)</td>
<td>30.5 ± 6.1</td>
<td>23.5 ± 2.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>6.2%</td>
<td>11.1%</td>
<td>NS</td>
</tr>
<tr>
<td>c-section (%)</td>
<td>50</td>
<td>68</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table IV-1 Patient information at the time of delivery

P values were determined with Mann-Whitney test.
4.5.2 Blood from IUGR neonates has fewer and slower proliferating ECFCs

In both control and IUGR pregnancies, ECFCs were more abundant in umbilical arterial blood than venous counterparts (Figure IV-1). Umbilical arterial blood from IUGR pregnancies had fewer ECFCs than uncomplicated controls (Figure IV-1A). However, this difference was not observed in venous samples (Figure IV-1B). By extension, the net difference between arterial and venous ECFC content, taken as the number of EPCs sequestered by the placenta, was markedly increased in control pregnancies compared to IUGR (Figure IV-1C). Consistent with these observations, circulating CAC-counts in umbilical arterial blood of IUGR fetuses were also lower (Figure IV-1D), and again no difference was found in venous blood (Figure IV-1E), corresponding to a smaller arterial-venous gradient in growth restricted babies (Figure IV-1F).

Figure IV-1 ECFC and CAC are fewer in cord-blood obtained from IUGR-complicated pregnancies.

Number of cells in (A, D) arterial and (B, E) venous blood are expressed as a percentage of total blood mononuclear cells; arterio-venous gradients of (C) ECFCs and (F) CACs were taken as representation of placental sequestration (A-C: n=17(ctrl) / 10(IUGR), D-F: n=13 (ctrl) / 10(IUGR, Mann-Whitney test.)
Growth characteristics of ECFCs (derived from plating 6x10^6 cells from the density gradient layer corresponding to mononuclear leukocytes of cord blood) were observed in primary culture. The time elapsed from plating the mononuclear cells to appearance of first colonies, assessed by daily observations, was longer in the case of ECFCs from growth restricted pregnancies (Figure IV-2A). The population doubling time of ECFCs, assessed by live cell counts during the first 2-3 passages, was 2.3 times longer in IUGR cases (Figure IV-2B). Furthermore, the number of ECFC colonies that appeared in primary culture before confluence was fewer from IUGR compared to control samples (Figure IV-2C). Taken together, these observations are indicative of a lower proliferative rate.

Non-directional migration was found to be lower in IUGR derived ECFCs (Figure IV-2D), and chemotaxis in response to SDF-1 was significantly attenuated (Figure IV-2E). Migratory responses to VEGF-B (Figure IV-2F) and IL-8 (data not shown) were unaltered. The release of MMP-2 from IUGR-derived ECFCs was higher than control cells (Figure IV-2G), and hypoxia caused an exaggerated release in controls compared to IUGR derived cells (Figure IV-2H).
Figure IV-2. ECFCs obtained from pregnancies complicated by IUGR have altered proliferation and migration in culture.

The number of ECFCs identified in culture after plating $6 \times 10^6$ mononuclear cells and their proliferative capacity was assessed by (A) the minimum number of days before colonies were identified in culture, (B) average calculated doubling time and (C) number of colonies per well (see methods for details). (D) Non-directional migration, and chemotaxis in response to (E) SDF-1α (20ng/mL) and (F) VEGFβ (2µg/mL) in culture conditions. MMP-2 release by cultured ECFCs after 72h incubation in either (G) normoxia or (H) hypoxia (1% O2); data in (H) are shown as a % increase over MMP-2 release under normoxic (G) condition. (A: n=13 (ctrl) / 12(IUGR), B: n=10 (ctrl) / 8(IUGR), C: n=12 (Ctrl) / 8(IUGR), D-F: n=12 (ctrl) / 12(IUGR), G-H: n=6 (ctrl) / 6(IUGR), Mann-Whitney test (A-F), Two-way ANOVA (G-H)).
4.5.3 **ECFCs from IUGR-babies have impaired vasculogenesis capacity**

Vasculogenic capacity of ECFCs was evaluated using an *in vivo* murine vasculogenic bioassay, based on assessing the extent of vasculogenesis within collagen/fibronectin blocks containing ECFCs (with adipose-derived stem cells acting as pericytes) while implanted into the mouse. We observed reduced blood vessel formation in implants populated with IUGR-derived ECFCs. Specifically, compared to controls (Figure IV-3A and Figure IV-3C), IUGR ECFC populated implants had 8-fold fewer large blood vessels (consisting of complex multi-cellular make-up) (Figure IV-3B; data summarized in Figure IV-3E), and had 6-fold fewer capillaries (Figure IV-3D; data summarized in Figure IV-3F) formed *de novo*. Compared to controls, vessels formed by ECFCs from IUGR pregnancies had 2-fold narrower bores, expressed as an average and maximal cross-sectional area of the vascular lumen (Figure IV-3G-H).
Figure IV-3. Vasculogenic capacity is reduced in IUGR-derived fetal ECFCs.

Results of vasculogenesis bioassay. ECFCs expanded from the circulation of growth restricted fetuses have a strikingly reduced capacity to form both simple capillaries and complex vascular structures when sub-fascially implanted into adult, virgin NOD/SCID mice, in pairs with normal controls, suspended in collagen-fibronectin matrix mixed with human adult adipose derived stem cells (ADSC). A and B: Representative images of complex vascular structures (arrows) formed by ECFCs derived from control neonates (A) and those born with IUGR (B) taken at 10 x magnification. (Scale bar 100um, stained with HE). C and D: Representative images of simple capillaries (Arrows) formed by ECFCs from normal (C) and growth restricted newborns (D) taken at 60x magnification. (Scale bar 20 um, stained with HE) E: Comparison of quantity of complex vascular structures per microscopic view (n=7, Mann-Whitney test). E: Comparison of numbers of vacuoles representing newly formed capillaries (n=7, Mann-Whitney test) F: Comparison of the total vascularized surface per microscopic view (n=7, Mann-Whitney test). G: Comparison of the largest vessel bore observed ((n=7, Mann-Whitney test).
4.6 DISCUSSION

Here we show several differences in ECFC number and function between normal pregnancies and those complicated by IUGR, implying a severe functional deficit. Importantly, we excluded known pregnancy-associated complications that have been shown to contribute to ECFC dysfunction, such as gestational diabetes [124], and pre-term birth [266]. To our knowledge, this is the first study to demonstrate alterations in ECFC number and function in pregnancies strictly characterised as isolated IUGR.

IUGR fetuses are predisposed to chronic illness in later life, including endothelial dysfunction [313,314], which is an important predictor of cardiovascular disease [315]. However, the mechanisms linking abnormal intrauterine environment to long-term endothelial dysfunction and vascular damage remain elusive. As EPC dysfunction is a risk factor for cardiovascular decline [308], reduced circulating numbers of CACs and ECFC, as well as reduced vasculogenic capacity of these latter cells, may provide a tentative link between IUGR and increased susceptibility to CVD.

IUGR is a pregnancy complication with many etiologies, therefore multiple mechanisms may underlie the observed ECFC dysfunction. We have identified several cellular changes that could explain, at least in part, the reduced ECFC vasculogenic capacity associated with IUGR. The finding that ECFCs from IUGR pregnancies have reduced proliferative capacity is potentially important, since vascular formation to the extent observed in the control collagen/fibronectin implants undoubtedly requires extensive ECFC proliferation. The number of IUGR ECFCs populating the collagen matrices would be lower than controls after a few days, thereby limiting vascular formation. Diminished non-directional migratory and chemotactic behaviour may also be indicative of diminished vasculogenesis capacity. SDF-1 plays a particularly important role in EPC homing to areas of vessel formation, as well as cell polarity and morphology, thereby influencing migration [316]. Consequently, reduced responsiveness to this chemokine may impede the capacity of IUGR ECFC to migrate and orient appropriately for vascular formation.

Hypoxia increased MMP-2 secretion in control ECFCs, consistent with the notion that MMPs are important in mediating the tissue responses to hypoxia, including
neovascularisation [317]. Recently, MMP-2 has been shown to be indispensable for EPC mediated vessel formation [318]. The failure of IUGR derived ECFCs to increase release of MMP-2 under hypoxic conditions may therefore represent an important mechanism underlying the diminished vasculogenesis in these cells. Interestingly, we also observed that basal (non-hypoxic) release of MMP-2 was higher in IUGR-derived ECFCs. The significance of this observation is unclear, although excessive endothelial gelatinase activity has been reported in models of vascular disease [319].

The present work does not distinguish whether ECFC dysfunction is a cause or consequence of IUGR. It remains to be seen whether prior disturbance in ECFC function results in defective placental vasculogenesis; conversely, inadequate placentation might occur for other reasons, and the ensuing hypoxia and nutrient deprivation could have lasting effects on ECFC function. Due to the multifaceted etiologies underlying IUGR, clearly defined animal models may be required to address these issues.

In summary, ECFCs from pregnancies affected by IUGR are reduced in number and are dysfunctional. This may prove to be important in understanding the cause of placental dysfunction in IUGR and the link between small size at birth and programming of CVD in later life.

4.7 SOURCES AND FUNDING

The study was supported by the Wellcome Trust UK (WT), the Manchester NIHR Biomedical Research Centre, National Institutes of Health grant (P01HD030367), the Canadian Institutes for Health Research (CIHR), and the Women’s and Children’s Health Research Institute. PS is funded by fellowships from WT. SLB is funded by fellowships from CIHR and Alberta Innovates Health Solutions (AIHS). STD is an AIHS-funded Scientist and Canada Research Chair in Women’s Cardiovascular Health.

4.8 DISCLOSURES

None
CHAPTER 5
OVERALL DISCUSSION OF THESIS STUDIES: CRITICISMS, POTENTIAL CLINICAL BENEFITS AND DIRECTIONS OF FUTURE WORK
5.1 CRITICISM OF TECHNIQUES

In the introduction of this thesis, the current knowledge regarding the identity of Endothelial Progenitor Cells was described, and it was concluded that the topic is surrounded by a large number of controversies and inconsistencies, resulting in serious difficulties when interpreting results of studies relating EPCs and clinical conditions. To avoid such difficulties in this thesis, the most stringent strategies were adopted when working with EPCs. Here, the potential weaknesses of these studies are considered.

5.1.1 Critique of investigations on placental uptake of EPCs (Chapter 2)

It was shown in Chapter 2 that ECFCs derived from the fetus are sequestered by the placenta and contribute to its vasculogenesis. By using (i) flow cytometry of umbilical arterial and venous blood, (ii) perfusion of human fetal ECFCs through placental arteries, and (iii) their injection into murine fetuses, their true endothelial progenitor-like function was demonstrated in physiological processes.

5.1.1.1 Potential weaknesses of flow cytometry

5.1.1.1.1 Optimisation of flow cytometry technique

Flow cytometry was used to count ECFC-numbers in pairs of human umbilical arteries and veins to determine the arterio-venous gradient, which is suggestive of placental sequestration. Although, in the literature there is a wide range of flow cytometry techniques for counting EPCs, choosing an adequate method was challenging, because of the inaccuracies associated to these published protocols. Many authors suggested that KDR+/CD34+ or KDR+/CD133+ cells in the mononuclear gate are EPCs. They proposed the examination of 10-20,000 flow cytometry events. [112,115,173,187,320]. During trial of these protocols a flow-cytometer restricted to two colours and a maximal event count of 10,000 had been used. It was recognised that gating strategies exclusively based on rare markers (KDR, CD133, CD34) -without a pre-selection by frequent markers (such as CD45
and CD31)- resulted in the relatively high number of false positive events caused by non-specific binding. For more accurate measurements a higher number of events was to be examined due to the extremely low frequency of circulating EPCs. In addition, these protocols did not differentiate between CACs and ECFCs. Following some pilot studies it was concluded that the adoption of a more advanced flow cytometry approach (one which involves both frequent and rare markers, counts a high number of events, and differentiates ECFCs from CACs) was required.

The four-colour protocol of Duda et al. [230] fulfils these requirements. In this protocol, those cells considered CACs are CD45+/CD31+, and of the rare markers they express KDR, and CD133, while those considered ECFCs are CD45-/-CD31bright and express KDR and CD34. To avoid non-specific staining and significant auto-fluorescence by non-vital cells and insufficient haemolysis, the protocol was optimised for ideal use in fetal blood. This included (i) optimising the FcR-blocking reagent, (ii) addition of a viability-stain (7-AAD) to the protocol, and alterations (iii) in certain antibody-conjugates, as well as (iv) haemolysis. The following are details of the changes made:

(i) A higher dose of FcR (20µl instead of 10µl) successfully prevented non-specific binding.

(ii) Using 7-AAD allowed exclusion of non-viable cells characterised by high autofluorescence.

(iii) To allow room for the fifth fluorescent colour of 7-AAD and minimise overlaps between various emission spectra, the conjugates of some antibodies were changed from what was originally suggested by Duda et al. Using FITC and the novel fluorescent stain ACP-H7 for the frequent antibodies (CD31 and CD45 respectively), allowed the brightest PE and APC conjugates to highlight the rare events of CD133, CD34 and KDR, while 7-AAD could also be safely detected.

(iv) The haemolytic agent was changed from ACK (Invitrogen) to Pharmalyse (BD Biosciences) because ACK contains a fixative component which destroys suspended cells. This renders the concomitant use of ACK and a viability stain
incompatible. In addition, with optimisation of incubation time, Pharmalyse haemolysed the resistant nucleated red cells in fetal blood more effectively, than ACK.

These efforts resulted in the most stringent flow cytometry technique we could apply to determine ECFC and CAC numbers in fetal blood. Despite such attention to detail there are certain aspects in relation to the use of this technique which will be considered next.

5.1.1.1.2 Points related to mode of normalisation

EPC numbers were not determined as absolute numbers as per unit of volume, instead the ratios of EPCs related to mononuclear cells were defined. These ratios between arterial and venous umbilical samples were then compared. This approach is based on the assumption that cellular content between the arterial and venous umbilical blood is more constant, compared to the volume of serum. This is due to the fact that feto-maternal fluid exchange is definite, while placento-maternal migration of leukocytes is unknown. The examined placentas might potentially sequester some mononuclear cells or release them into the fetal circulation, however this was not examined. In the view of these facts and the high potential of increased risk of false measurements by further complicating the flow cytometry technique by adding fluorescent counting beads, the only reliable method of volume-analysis in flow cytometry, and causing potential false readings by wavelength interactions, we strongly suggest that determining EPC-counts as percentages of mononuclear cells is more accurate than attempting to determine them as percentages per blood volume.

5.1.1.1.3 Differentiating ECFCs from circulating mature endothelial cells in flow cytometry

No combinations of markers are currently known which differentiate ECFCs from mature endothelial cells (ECs) [228]. Indeed, mature ECs do express the same combination of surface markers as those used to define ECFCs by flow cytometry [230]. This means that
the ECFC count derived from the flow cytometry used in this study includes circulating EC. The frequency of circulating endothelial cells in the umbilical circulation is hard to estimate. It was assumed that in the absence of placental uptake or release of EC, the frequency of circulating EC is constant between arterial and venous samples. Should this assumption be false, it may exert an impact on the results.

5.1.1.2 Comments on the ex vivo perfusion of ECFCs through placental arteries

In our ex vivo perfusion model we perfused CMFDA-labelled fetal ECFCs through chorionic plate arteries and showed that ECFCs were incorporated into the endothelial layer of these vessels. Ex vivo experiments do not account for circulatory, endocrine, neural and other factors, and therefore may not fully mimic physiological circumstances. This is an intrinsic limitation of these experiments, and results might be difficult to interpret, although the subsequent fetal injection experiments provided in vivo confirmation. Additionally, there are some technical points to consider. According to the product information of the green fluorescent cell tracker CMFDA, which was used to label perfused ECFCs, it continues fluorescing for 72 hours and four cell divisions. This suggests that the amount of cytoplasmic CMFDA taken up by one cell during staining is sufficient for detection of daughter cells, even if the dye is distributed in the cytoplasms of several cells. Similarly, the dye diluted in the two fused cytoplasms would be at detectable concentrations in case of cytoplasmic fusion, furthermore, green fluorescent signals in the endothelial layer may not be genuine, but they may result from potential fusion between perfused fetal ECFCs and endothelial cells. Exclusion of potential cell fusion is a challenge and the potential approaches will be discussed in section 5.1.1.3.2, as the same problem occurs in subsequent models. We used VEGFR-2 staining to highlight the luminal surface of the endothelium and to aid differentiation of incorporation and coincidental co-localisation of free, labelled cells and pre-existent endothelium. Free cells express VEGFR-2 on their entire cell surface, while those incorporated only express it on their luminal surface, given their involvement with tight and gap junctions laterally. The limitations of this approach lay in the potential failure
of the staining technique; incompletely stained, free cells which are co-localised with the endothelium may appear as if only the luminal surface expresses VEGFR-2. This may be falsely misinterpreted as incorporation (See Figure II.4).

5.1.1.3 Considerations in regards of the murine fetal transplantation model

To further elucidate the fate of fetal derived ECFCs sequestered by the placenta an in vivo mouse model was used. Human fetal ECFCs were injected into the hearts of NOD/Scid fetuses on E15.5 and these cells were tracked in the placenta. This is the first model proposed to examine the vasculogenic properties of ECFCs in the placenta in vivo. However, certain considerations regarding this model exist, including co-localisation of graft cells with pre-existent endothelium and cytoplasmic fusion.

5.1.1.3.1 Coincidental co-localisation of injected cells with murine endothelium and graft versus host reaction

This model is a xenograft; the recipient is a double knock out (NOD.Cg-Prkdc<sup>scid</sup>I2rg<sup>m1Wj</sup>/SzJ) immuno-deficient murine organism, which has diminished cellular and humoral immune systems, given the absence of T, B and NK cells as well as deficient gamma-chains of II-2 receptors [321,322]. The purpose of using this strain in the model was to mimic the lack of immune-interaction between migrating cells and different target organs of the same individual. However this design might give rise to some further considerations. Although the injected ECFC populations were pure, the presence of leukocytes in the injected matter could not be fully excluded. Leukocytes could potentially participate in graft versus host reactions in the immunodeficient mouse. In this process fluorescent graft leucocytes may attach to placental endothelial cells. Given their green fluorescence they could be mistaken with ECFCs, thus potentially influencing the results. Similarly, ECFC might be coincidentally co-located with the endothelium making the same false impression.
To avoid misinterpretation of such artefacts we used immuno-histochemistry to demonstrate integration of graft cells by showing the presence of tight and gap junctions as indicated by claudin 5 and connexin 40, building units of these junctions, found in endothelia [237,238,254,323]. These intercellular junctions do not form during immune reactions or in case of coincidental co-localisation and we considered their development as signs of complete integration into the recipient organism.

5.1.1.3.2 Cytoplasmic fusion

Staining for tight and gap junctions does not fully exclude the possibility of some other artefacts, namely those potentially rising from the fusion of graft cells with pre-existent murine endothelial cells – as briefly mentioned above (Chapter 5.1.1.2). The only definitive way of excluding the possibility of cell fusion would be to show the presence of human DNA and the lack of murine DNA in these labelled cells.

Given the limitations of the technique, examining the DNA-content of labelled cells during optical visualization in the samples is not feasible. In cross sections, two approaches can be theoretically considered to analyze the DNA content of cells.

(i)  *In situ* hybridization of human and murine chromosomes in cross sections.

The absence of murine with concomitant presence of human chromosomes in examined cells would be a proof against cell fusion. Two chromosomes of both species should be hybridized to avoid misinterpretation of artefacts. Essentially, four-colour FISH is a challenge, especially when green colour cannot be used, as cells are labelled with eGFP. In addition, tissue sections are exposed to very harsh permeability processes before the actual hybridization to allow the probes to contact nuclear DNA. Previously used methods include exposing the tissue to acids, alcohols, and heat which would bleach the fluorescent colour produced by eGFP, making the detection of injected cells impossible. Optimising the permeability process, in order that eGFP-fluorescence is
preserved is a major challenge, which could not be undertaken during the course of this PhD.

(ii) The second approach to determine the DNA content of labelled cells is by using laser capture, followed by PCR of certain selected human and murine genes. Consequently, the lack of murine genes in fluorescent integrated cells would exclude cell fusion. Due to the relative low numbers of graft cells available, capturing sufficient amount of labelled cells is not feasible.

In both cases, potential contamination from the relative high number of surrounding murine cells could cause false positive results, while low sensitivity of the assays might result in false negative results, necessitating further verification. Therefore, excluding cell fusion involving rare, tagged cells in recipient tissues remains an unresolved problem.

In the context of stem cell development, cell fusion was first described to provide an alternative explanation to trans-differentiation of stem cells, when co-cultured with certain mature cell types. In co-culture, labelled stem cells seemingly expressed features of the mature cell type they were co-cultured with, and authors suggested their ability of trans-differentiation. Subsequently, it was shown that appearance of mature features are caused by stem cells fusing with the mature cell type forming syncytia, which contained the DNA of both cells and adopted the characteristics of the mature cell type [324,325]. Co-culture is a specific environment where two cell lines are permanently in contact in artificial circumstances. Such circumstances are not found in vivo, where a large variety of cell types do come into contact. In opposition to the enduring cell-to-cell contact in co-culture, contact in vivo is very transient in the case of circulating cells. In this model the progenitor and mature cells are not derived from the same species, which further reduces the likelihood of cell fusion. It is therefore unlikely that cell fusion was the cause of the results found.
5.1.1.3.3 Lentiviral labelling of transplanted human ECFCs

Three different lentiviral vectors (LV), two of them carrying the eGFP gene under THM and EF-1α ubiquitous promoters and one carrying the turbo Green Fluorescent Protein (tGFP) gene under the CMV promoter, were tested for transfection rates, gene expression rate, fluorescence and cytotoxicity of fetal ECFCs. Carrier solution was used for control. A range of viral titres (0.5; 1; 2; 4 TU/cell) were trialled in the presence of the transfection reagents, 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (Polybrene, Millipore, 7µg/ml) and protamine-sulfate (7µg/ml). LV-EF-1α and LV-CMV had equally high gene expression rates (100%) at 2 TU-cell concentrations without showing signs of cytotoxicity. Cells expressing eGFP had stronger fluorescence, as measured by plate reader. Therefore LV-EF-1α (eGFP) was used for fluorescent labelling. LV-CMV was used for transfection of LacZ for economical reasons. Although LV-CMV(LacZ) was not separately tested on ECFCs, the transfection efficiency and cytotoxicity of the vector were evaluated in the LV-CMV(eGFP) form.

These reporter genes were purely used for labelling and not to report on expression of certain genes. The use of empty viruses as control were therefore unnecessary and not performed.

5.1.2 Critique of techniques to examine feto-maternal transmigration of ECFCs (Chapter 3)

5.1.2.1 Critique of murine experiments

In Chapter 3 it was examined whether fetal ECFCs transmigrate the placenta and contribute to its vasculogenesis. To demonstrate labelled human fetal ECFCs were injected into the hearts of NOD/Scid fetuses during intact pregnancy. These cells were then tracked in the maternal uterus and detected by small animal imager and microscopy, confirming their full integration by demonstrating the presence of tight and gap junctions. In the second model, cells of transgenic murine fetuses, ubiquitously expressing paternally inherited eGFP, were tracked in the maternal uterus; their endothelial character was also
examined. In the case of these models, coincidental co-localisation and cytoplasmic fusion must be considered. This topic was discussed in section 5.1.1.3.2 in detail.

In addition, the potential pollution of maternal tissues by labelled cells may be considered. Pollution might occur either during the process of injection or dissection. However, the microscopic structure of tissues examined is markedly different from what could result from contamination. In the event of injections misplaced into the maternal peritoneal cavity or the spread of fluorescent fetal cells over maternal tissues during dissection, the labelled cells would form a layer over the visceral peritoneum of the examined uterus. They would be superficially attached to or seeded on the peritoneum, they would not be found inside the muscular layer, neither would they be organized in colonies in vessel walls, nor would they show signs of integration, as observed in these studies.

5.1.2.2 Critique of human observations

5.1.2.2.1 SRY-specific RT-QPCR of maternal uterine microvessels

To show the presence of fetal cells in the human uterine endothelium, maternal uterine microvessels were examined for male fetal cells containing Y-chromosome. An optimised, verified, internally and externally controlled RT-QPCR technique was used to accurately determine the number of Y-chromosomes in the maternal tissue samples [287]. The number of fetal cells was normalised to the total number of endothelial cells in each sample, however, no correct way was found to determine the number of endothelial cells in the samples. In this aspect a crude estimate was used based on the average internal vessel surface divided by the average cellular surface area. As a consequence, the study was unable to accurately quantify the proportion of fetal endothelial cells in maternal tissue. However, the results did show that the prevalence of fetal cells in maternal endothelium is biologically significant, and it is at a higher magnitude than previously defined in microchimerism.
To show the exact topography of fetal cells in maternal uterine vessels, their cross sections were hybridised in situ to highlight the fetal Y-chromosomes. Somatic chromosomes (Chromosomes 15 or 20) were used as positive internal controls, however, the ideal external positive control was impossible, as there is no uterine tissue in the male. As a substitute, a different male tissue type (placenta) was used. Given the unequal microstructure of myometrium and placenta, some sample-dependent variations in permeability of probes and signal strengths were experienced. Chromosome paints penetrated more effectively into the placental compared with uterine tissue, and signal strengths of control chromosomes (Chromosome-15/20) were often stronger in placentas than in uteri. It was therefore expected that signal strengths of the test chromosomes (Y-chromosome) are dimmer in test samples compared to the uterine tissue used as external control. To avoid misinterpretation of artefacts, only intra-nuclear Y-associated signals were considered whose signal strength was equal to the Y-chromosomes in the control tissue. Dimmer than control signals were invariably ignored. As a consequence, these tests have high specificity, but low sensitivity, suggesting that the assay is not appropriate for quantification; however it is robust enough to prove the principle that fetal cells are found in human maternal uterine endothelium.

5.1.3 Critique of methods to compare normal and growth restricted ECFCs (Chapter 4)

5.1.3.1 Flow cytometry

The techniques used in the fourth chapter partially overlap with some of those used in earlier chapters. The flow cytometry technique, which is used in this case to compare numbers of EPCs in the umbilical blood of growth restricted and control fetuses, is essentially identical to the one used and criticised in previous chapters. In contrast to comparing EPC numbers in pairs of umbilical arteries and veins, samples from unrelated
individuals are assessed in this case. Interpreting EPC-counts determined as proportion to mononuclear cells in independent sources is further complicated by potential variations of white cell counts between individuals. Although white cell counts are altered in IUGR when associated with preterm labour [326], flow cytometry data showed no differences in counts of mononuclear cells in blood from normal and growth restricted fetuses at term [327]. This suggests that mononuclear cell count is not a confounding factor, and it is therefore feasible to compare IUGR and control EPC numbers, when they are determined as percentages of mononuclear cells.

5.1.3.2 Vasculogenic bioassay

The most advanced assay to compare capacities of ECFCs from groups of growth restricted and control offspring is the vasculogenic bioassay. It compares the quantities and qualities of newly formed vessels in artificial tissue blocks implanted into immunodeficient NOD/SCID mice. This assay models and examines in vivo vessel assembly by ECFCs in its full biological complexity. This is composed of various steps, which include; proliferation, invasion, migration and remodelling. ECFCs are the single known cell type with true vasculogenic capacity; in consequence quantification of vasculogenic capacity is therefore a very robust measurement of the unique function of these cells. As a bioassay, results can be influenced by factors determined by the recipient carrier organism. Variations potentially caused by different characteristics of individual mice were excluded by comparing pairs of plugs implanted into the same individual animal at identical anatomical locations on either side of the mouse.

Cross sections of implants were imaged by light microscopy, and numbers as well as sizes of vessels analysed. The high number of systematically chosen sections and images helped ensure that human error was kept to a minimum and data remained robust, and these demonstrated robust multifold differences between functional capacities of ECFCs from normal and IUGR pregnancies. It was attempted however, to advance the assay, to make it independent of human interference with data collection and analysis to avoid human error or bias. A tomographic data collection and analysis step was trialled, which
included perfusing the vasculature of the implants by x-ray contrast media, imaging the vascular lumina highlighted by the contrast media using nano-CT technology and digital quantification of vascular structures formed within the implants. Due to the limited time and other resources, the perfusion step was not successfully optimised to allow the production of accurate nano-CT images. In the absence of electronic data collection and analysis, microscopic data analysis was performed as described in section 4.4.7.
5.2 POTENTIAL CLINICAL USE

5.2.1 Development of new diagnostic tools

5.2.1.1 Background

Involvement of placental and uterine vasculatures in the pathophysiology of IUGR has long been suspected. Morphological and stereological observations have shown that, beyond anomalies of the trophoblast, the microvasculature of chorion is underdeveloped in IUGR [62,63,328-330]. In the absence of innervation, placental resistance vessels are nearly fully dilated even under non-stress conditions, allowing a low-impedance flow through the placenta [331]. The fetus then has limited ability to compensate for placental hypoperfusion by further vasodilation. Vascular density is therefore the single most important determinant of placental vascular resistance. Chorionic hypovascularity results in high impedance and underperfusion of the placenta, which is observed in growth restriction, and it is associated with anomalies of Doppler measurements in the umbilical artery [332].

Similar to the placenta, high resistance resulting from suboptimal vascular density in the uterine system also results in underperfusion of the uterine vasculature. This is associated with atypical waveforms of uterine artery Doppler ultrasound analysis, frequently seen in fetal growth restriction [333,334].

Diagnostic tools to identify placental hypovascularisation prior to manifestation of IUGR are crucial for the development of effective therapies. Clinical tools to diagnose developing fetal growth restriction before apparent clinical manifestation, have been limited to the analysis of the uterine artery waveforms, placental shape and placental hormones or fetal proteins crossing the placenta [334-341]. High pulsatility index (>95 centile) or bilateral notching predicts IUGR with 24.4% sensitivity and 89.3% specificity at 23 weeks of gestation. In addition, they predict IUGR associated with pre-eclampsia with 83.3% sensitivity and 88.5% specificity [334]. The test is even less sensitive if carried out in the first trimester, predicting only 27% of IUGR-cases [342]. In pre-eclampsia related IUGR particularly, the sensitivity of screening is marginally improved by considering risk factors and measuring certain biomarkers, including some angiogenesis-related factors (VEGF,
PIGF, sFlt-1 and placental protein 13 [335-337]), α-feto protein (AFP), and pregnancy-associated plasma protein-A (PAPP-A) [340,341]. Measurement of spiral artery velocity in the first trimester was not found predictive of IUGR [338]. For placental shape, low (<10 centile) placental quotient (determined as the dividend of placental volume and the crown-rump length) was found to predict IUGR with 25% sensitivity and 90% specificity [339]. These however have not become effective clinical tools of identification of fetuses with placental hypoperfusion prior to the physical growth retardation, due to the poor insight into the mechanisms involved. The current description of the mechanism of placental and uterine vasculogenesis and their association with IUGR opens new avenues to develop novel diagnostic tools.

5.2.1.2 Early diagnosis of IUGR based on fetal ECFCs

Many of the assays developed and optimised in conjunction with this thesis can potentially form the bases of new diagnostic tools. Association between low numbers of fetal ECFCs and IUGR were demonstrated. As a consequence, circulating numbers of these cells in either maternal or fetal blood could be diagnostic for IUGR. Future clinical assays based on determining ECFCs by surface markers, could be similar to the flow cytometry technique described in Chapter 2. It was demonstrated that the functionality of these cells is detrimentally impaired in IUGR, and determining the capacity of fetal ECFCs could have clinical consequences. The in vitro functional assays described in Chapter 4 yield some potential and the MMP-2 activity or expansion rate in hypoxia could become diagnostic to threatening growth restriction. Fetal cells could become available for testing by chordocentesis (ultrasound-guided puncture of the umbilical vein), in case benefits outweigh associated risks (including fetal loss and infection), or potentially by isolating circulating microchimeric fetal cells from maternal blood, if this becomes technically feasible.
5.2.2 Treatment of IUGR by targeting fetal ECFCs

In the absence of understanding the process of pathological vasculogenesis in the uterus and placenta, therapies of growth restriction are very limited. The single clinically available treatment of IUGR is delivery of the fetus. This is prophylactically induced close to term or prematurely in the event where fetal wellbeing cannot be verified by Doppler studies of the umbilical artery and ductus venosus [343-345]. Current experimental therapeutic approaches target the consequences of pathological vasculogenesis, i.e. they attempt to revert hypoperfusion of the placenta by vasodilation, rather than preventing it by stimulating vasculogenesis. In particular, this group and others have shown beneficial effects of the phosphodiesterase-5-inhibitor sildenafil [346-348]. In a study by Stanley et al. pregnant catechol-O-methyl transferase (COMT) knock out mice (COMT (−/−)), which normally have growth restricted fetuses, were administered sildenafil. The drug reversed the intrauterine growth restriction and normalised the umbilical artery Doppler waveforms by re-sensitizing uterine arteries to metacholine, an endothelium-dependent vasodilator agent [346]. Provisional data showed that oral sildenafil has improved the growth velocity of severe, early onset IUGR in the human [348]. In contrast, sildenafil did not display any beneficial effects in a single uterine artery ligation IUGR-model in sheep, but it increased hypoxia-related fetal morbidity due to redistribution of maternal circulation [347]. This warrants the possible side effects of simple pelvic vasodilation as a therapeutic approach without stimulating vessel formation in that region. This highlights the potential benefits of stimulating pelvic vessel expansion in the preference to using simple vasodilators.

Indeed, local over-expression of VEGF-A in the uterine arteries of pregnant sheep (achieved by adenoviral gene therapy) increased angiogenesis in these arteries. A combination of these effects resulted in improved feto-placental blood flow associated with enhanced fetal weight [349,350].

In the view of the results in this thesis, new approaches to the intrauterine therapy of placental failure can be envisaged targeting fetal ECFCs – the driving force of more efficient types of vessel formation. These approaches could include (i) drug, (ii) cell and (iii) gene therapies.
(i) Several different therapeutic agents can stimulate ECFCs by either affecting the function of ECFCs directly, or by indirect effects mediated by CACs. A range of relevant agents and their target mechanisms are listed in Table 1-1. Many of these agents, including, prostacyclins, nifedipine, sildenafil and estradiol can be administered safely in pregnancy and could be considered for therapeutic use to prevent or revert uterine hypovascularisation. In addition, by crossing the placenta some of these drugs may assist to restore normal placental vascular density by medically stimulating the activity of placental ECFCs. These agents however, are non-selective to ECFCs, suggesting a great array of side effects. These could be minimised by cell-specific drug delivery or by development of novel, narrow-spectrum substances.

(ii) Cell therapy can be carried out by fetal transfusion of external or artificial ECFCs or by autotransfusion of externally propagated fetal ECFCs. Initial source of ECFCs undergoing external propagation can be obtained from the umbilical vein or by isolation from the maternal blood, as described in section 5.2.1.2.

(iii) Fetal ECFCs have been found to be susceptible for viral gene delivery. Viruses, binding to (yet to be identified) ECFC-specific receptors, may target ECFCs and deliver genes exclusively to this cell type. These genes could have a beneficial effect on ECFC-capacities by increasing expression of receptors to stimulating agents, such as SDF-1, whose role was demonstrated in Chapter 4. It remains a concern, however, whether stimulation of ECFCs conveys the risk of escalated augmentation of vessel expansion outside target areas, which may include the rise of vessel-borne malignancies.
5.3 FUTURE WORK

This thesis provided some initial data, which may contribute towards development of novel diagnostic and therapeutic strategies for the treatment of IUGR. A substantial amount of additional work must be done for this vision to be realised in the form of clinical trials. This data form the basis of several questions in both basic cell biology and translational research. The following proposals are made using the working hypothesis that IUGR is the consequence, not the cause of impaired ECFC-function.

5.3.1 Basic research into the role of ECFCs in IUGR

5.3.1.1 Identification of targets for drug therapy

For further development of novel, ECFC-specific medical treatment options, it is pertinent that the aetiology of the unique characteristics of ECFCs is identified. Understanding the cause of their superior qualities compared to other endothelial cell types, even adult ECFCs, is fundamental. This would help uncover targets for drug therapy. To explore the differential gene expression between these cell types, a possible experimental design is discussed as follows. The materials would include human umbilical cord, cord blood (preserved at birth) and adult blood. HUVECs would be obtained from the umbilical vein, while fetal and adult ECFCs would be isolated from the umbilical and adult blood, respectively. Using microarray analysis, differentially expressed candidate genes could be identified. From the results currently generated, receptors of angiogenic growth factors, metalloproteinases and telomerase would be considered as primary candidates. Their differential gene expression could be verified by RT-QPCR at the mRNA level. Some tools developed in conjunction with this thesis could be used in the validation steps of these examinations. Following knocking out of candidate genes in ECFCs, then examining their function by the murine fetal transplantation model (described in Chapters 2 and 3) or by the artificial tissue implant model (described in Chapter 4) would confirm the significance of these candidate genes.
5.3.1.2 Identification of targets for viral therapy

For ECFC-specific gene delivery, discovery of a unique marker, which could function as the binding site of viral vector, would be a significant step forward. In the absence of such a marker, genes could be inserted under novel, ECFC-specific promoters.

5.3.2 Translational research into ECFCs in IUGR

In the search for human therapies, the effects of experimental treatment of IUGR (cell therapy or ECFC-stimulation), cardiovascular status of the fetus as adult, and maternal cardiovascular risk could be pre-clinically explored. Principles of therapeutic approaches could be tested by known animal models of IUGR. The benefits and disadvantages of using various IUGR-models will be discussed as follows.

5.3.2.1 The COMT (-/-) mouse model

When pregnant, COMT (-/-) mice show signs of pre-eclampsia including high blood pressure, proteinuria and associated growth restriction [346], although it is not certain that the pathogenesis of IUGR is correctly duplicated in this model. This is suggested by contradicting results in COMT (-/-) and other models. [346,347]. Experimental cell therapy of COMT (-/-) fetuses can be envisaged in the form of ultrasound-guided intra-cardiac injection (described in Chapters 2 and 3). Following ECFC-injection, uterine artery Doppler wave-forms and fetal growth velocity can be assessed, alongside with assessment of uterine vascular function in vitro. Nevertheless, considerations regarding such experimental design are many fold. Given the insufficient amount of blood available in mouse fetuses for isolating ECFCs, the experimental use of such cells is not feasible and the use of donor, maybe cross-species, ECFC would be required. For injection of allogenic graft cells, immuno-tolerance of the recipient organism is a pre-requisite. In this thesis the NOD/Scid immunodeficient mouse strain was used. Currently, no strain is available which is sufficiently immunodeficient and is also concomitantly COMT (-/-). In theory, injection of allogenic cells could be enabled by either generating an immunodeficient COMT (-/-) strain
or by medical immunosuppression of the currently known COMT (−/−) strains. Genetic engineering of triple knockout mice (COMT, NOD/Scid) might be a challenge, while results may be influenced by immunosuppressants.

Another disadvantage of murine models is the short duration of pregnancy (18.5 days). This allows only 3.5 days for the potential effects of treatment to take place, which might be insufficient for detection.

Therapeutic substances, however, may be administered from the beginning of pregnancy, as opposed to injections given at a later stage. Given the significantly longer impact period, their effects may be easier to demonstrate. Additionally, issues around host versus graft reactions would also be obsolete. Among others, sildenafil is one of the agents found to stimulate ECFCs. Indeed, Stanley et al. administered sildenafil to COMT(−/−) mothers with beneficial effects. Regulation of ECFC numbers or activity was not examined in that study, and authors exclusively explained their observations by the vasodilatory effect of sildenafil [346]. ECFC-stimulant role of sildenafil, however, might be important, and certainly merits examination in this context.

5.3.2.2 The Reduced Uterine Perfusion Pressure model of IUGR

Besides the COMT (−/−) mice, the other established animal model of IUGR is the Reduced Uterine Perfusion Pressure (RUPP) model in the pregnant rat. In this model the aorta is obliterated above the iliac bifurcation on day 14 of pregnancy, resulting in blockage of the blood flow through the uterine arteries, which arise from the internal iliacs. The pregnant uterus, however, is not solely supplied by the uterine arteries; in the event of failure of uterine arteries, anastomoses dilate and provide sufficient blood supply to the uterus. The most significant of these shunts is found between the ovarian arteries and the uterine arch. This shunt is also clipped in the RUPP model. Using the standard size of clips described by Crews et al. the blood flow through the placental bed is reduced by 40% [351]. Rats seem to be more appropriate to study compared to mice, as ECFCs have already been successfully isolated in this species [352], while physiological role of various EPC types are strongly debated in the mouse [249]. The RUPP-operation can be performed on nude rats
providing an immunodeficient, growth restricted system, without having to produce new transgenic rodents or administering immunosuppressant drugs.

This system is based upon hypoperfusion of the individual placental beds. In addition to donor ECFC-driven increase in placental vascular density, the fetus may also benefit from promoting formation of anastomoses on the maternal side. This would potentially bypass the obliterating clips by assisting in recovery of the artificially reduced flow through the uterine arch.

Investigating long term effects in the rat is more suitable, given the more extensive adult life expectancy of this animal, which allows a longer observation for adult health hazards associated with IUGR [69,353-358].

These considerations may render the RUPP-model superior to the COMT (+/-) model in testing ECFC-based cell therapies of IUGR.

In contrast, the effects of being exposed to two separate invasive procedures (the RUPP-operation and the fetal intracardiac injection) might be intolerable for these pregnant animals. The ultrasound-guided fetal intracardiac injection was technically challenging in the mouse, and when piloting, even more so in the rat. This was due to the mobile uterine horns in the larger abdominal cavity. Although the duration of rat pregnancy is somewhat longer than in the mouse, it is still short, as it lasts only 23 days. Again, it is unclear, whether any differences in fetal growth will become apparent within such short period of time. Alternatively, secondary observations and observations of long-term effects of growth restriction are necessary to discover potential effects of cell therapy.

Testing both drug and gene therapies would be feasible in rats. Data from these animals might be more relevant in the aspect of human conditions, compared to murine data, as ECFCs possess an established role in rats [352], while their status in the mouse is unclear [249].

5.3.2.3 Other animal models

Primates have the most similar reproductive system to humans, and they have substantially longer pregnancies compared to rodents. This allows sufficient time for the
potential effects of experimental therapies to become apparent. Additionally, their sonographic scanning and fetal injection is relatively straightforward compared to rodents, given their size. The RUPP model has been successfully applied in primates [359] [360], and ECFCs have also been isolated from these animals [361], unlike from mice [249]. These considerations suggest the potential benefits of working with primate-models when performing trials of ECFC-targeted therapies. Some, including ethical and financial, considerations prevent primate models from being used commonly. Other commonly used models of placentation include guinea pig and sheep. Their placentation is similar to the human and their gestational period is substantially longer than mice and rats, which allows them to serve as superior models. However, their use in these types of experiments, is inhibited by the fact that ECFCs have not been isolated from these animals and the absence of readily available immunodeficient strains.

5.3.3 Research into IUGR-related conditions

Low numbers and impaired function of fetal ECFCs could be the cause rather than the consequence of fetal growth restriction by underperforming in the vasculogenesis of both the placenta and the placental bed. This results in suboptimal vascular density in these key areas causing placental failure and secondarily IUGR. In addition to the direct involvement in pathogenesis of growth restriction, fetal ECFCs may play a role in conditions associated with IUGR, including preeclampsia, adult and maternal cardiovascular disease. Research addressing these issues is pertinent.

5.3.3.1 Preeclampsia

Preeclampsia has been associated with alterations in EPC function [172,173,303,304,362]. Fetal ECFCs could play a role in the development of pre-eclampsia syndrome, especially when preeclampsia is associated with signs of placental insufficiency. Preeclampsia is thought to be triggered by hypoxic events in the placenta caused by insufficient remodeling of spiral arteries, including endothelial transdifferentiation of extravilious trophoblast [363-366]. Recently it has been suggested that the new endothelium of spiral arteries is
produced by re-endothelialisation, rather than transdifferentiation [284]. In any case, this process is incomplete in pre-eclampsia and high impedance is maintained in the spiral arteries, causing hypoperfusion and excessive hypoxia in the placenta, an organ, which is exposed to moderate hypoxia even in normal conditions [367]. According to common working hypothesis, in response to reperfusion-related oxidative stress the placenta releases soluble factors, interfering with angiogenic balance [368,369]. These factors initiate a pathological process predominantly characterised by production of increased levels of sFlt-1 via the HIF1-pathway [370-372]. Free VEGF in the circulation permanently binds to sFLT-1, which competes cellular VEGF-receptors, resulting in reduced bioavailability of free circulating VEGF, which is a main stimulant of ECFCs.

It is reasonable to believe that closer to the placental bed the frequency of fetal ECFCs may be higher than was found elsewhere in the uterus remote from the placenta; therefore their role in re-endothelialisation of the spiral arteries seems feasible. Inadequate fetal ECFCs like those found in IUGR may cause incomplete re-endothelialisation of the spiral arteries. This would start a perpetual circle by causing placental hypoxia with subsequent reduction in release of placental angiogenic factors, resulting in impaired ECFC-lead uterine vasculogenesis ultimately causing pre-eclampsia.

Potentially, this physiological role for fetal ECFCs could be demonstrated in the human, by using placental bed biopsies and examining the presence of fetal ECFCs in the spiral arteries, using methods similar to the Q-PCR and FISH techniques described in Chapter 3. Alterations of frequency of fetal ECFCs in these samples may indicate their pathophysiological role in these processes.

VEGF is a stimulant of ECFCs, and in preeclampsia decreased levels of active circulating VEGF caused by increased s-Flt-1 levels could result in impaired ECFC-activity in the uterus, both in the spiral arteries and further away, as well as in the placenta. This may result in suboptimal vasculogenesis, ultimately insufficient vascular density in these key organs would lead to failure of the placenta and placental bed. These phenomena could provide a potential explanation for the common case of simultaneous pre-eclampsia and IUGR. Testing ECFCs in the relevant patient group of pre-eclampsia complicated with
IUGR using the functional assays described in Chapter 4 could likewise answer some of these questions.

5.3.3.2 Fetal ECFCs and Barker’s hypothesis of fetal programming

The demographic observations of Barker et al were the first to show an increase in cardiovascular risk of individuals born with low birth weight [70-78,262,373]. These observations lead to the theory of fetal programming, suggesting that conditions in utero predispose the fetus too maladies in their adult lives. Currently, no specific cellular targets or proposed pathomechanisms have been suggested in the explanation for this phenomenon. The association between ECFCs and a wide range of cardiovascular conditions has emerged in recent time [83,84,86,90,104,180,186]. A role of CACs and ECFCs in repair of vascular damage and injury has been demonstrated in several animal models and even in humans to some extent [92-95,112,132,143,176,189,193,245,246,250,374,375]. In conjunction, these observations place ECFCs at the centre point of fetal programming, as a potential common causative agent. ECFCs reside dormant in the stem cell niche and are recruited to loci of vasculogenesis whether in utero or in the adult. We propose that the same genuine, genetically inherited or obtained damage to the function of ECFCs is responsible for sub-optimal vasculogenesis in the placenta, placental bed and during endothelial repair in adulthood. Vascular function was found impaired in the adult life of growth-restricted rats [69,353,354]. Whether the cause of these observations is ECFC-related, could be examined by transplanting normal, labelled bone marrow into growth restricted rat offspring and observing the effects in the adult lifespan. Greatest benefit would be gained by using bone marrow from a strain, which expresses a fluorescent reporter gene (e.g. eGFP) under an endothelium-specific promoter (VEGFR, CD31, etc.). As a consequence, endothelium derived from donor ECFCs would be highlighted by eGFP and could be readily quantified in the following manner. After pepsinising the endothelial lining [376], the proportion of endothelial cells derived from donor ECFCs could be determined by flow cytometry, based upon their inherited autologous fluorescence.
Examining the correlation of endothelial function in adulthood and the extent of bone marrow derived endothelialisation would highlight the significance of ECFCs in fetal programming.

Long term follow up of human neonates born from pregnancies complicated with IUGR would enable observations regarding association between birth weight, cardiovascular risk in adulthood and ECFC function at birth, as well as in the adult life.

5.3.3.3 Fetal ECFCs and cardiovascular health of the mother

Recent studies showed that nulliparous, postmenopausal women have increased cardiovascular risk compared to their multiparous counterparts [377]. Epidemiological studies have also demonstrated an association between intrauterine growth restriction of the fetus and cardiovascular risk of their mothers in later life [79,378]. The migration of fetal ECFCs from the fetus to the mothers was shown in Chapter 4. These ECFCs have a robust proliferative phenotype compared to their adult counterparts [61,208]. It has also been shown that fetal cells survive in the maternal organism for several decades after pregnancy. This raises the question whether fetal ECFCs survive in maternal stem cell niches following pregnancy, and whether they become available for recruitment and contribute to subsequent vascular repair. As such, this could be an explanation for the above epidemiological observations. Furthermore, given their superior qualities, the active assistance of high potential fetal ECFC would be robust. This would provide a potential explanation for differences between cardiovascular risk of nulliparous versus multiparous women. Differences between cardiovascular risk of mothers with growth restricted offspring and mothers of children with normal birth weight, could possibly be explained by the differential capacities of IUGR versus normal ECFCs.

Examining these hypotheses would involve determining the various maternal localisations of fetal ECFCs in the mouse and human, other than the uterus. Major vessels and bone marrow would be of particular interest. To investigate, the murine fetal eGFP-ECFC-injection tool could be an ideal model, as it overcomes the difficulty that adequate identification of ECFCs requires expansion in culture. For major blood vessels, small
animal imager and microscopy would allow detection of potentially resident injected ECFCs. For bone marrow, injected eGFP-ECFCs could be isolated and purified using culture techniques, potentially enhanced by cell sorting methods (magnetic beads or FACS), which could be based on surface markers, or the intrinsic fluorescence of injected cells.

In the human, biopsies from major vessels of patients who were previously pregnant with male offspring could be tested for the presence of male cells containing the Y-chromosome, using our SRY-specific RT-QPCR and Y-specific FISH techniques, as described in Chapter 3. The proportion of fetal cells in ECFC-isolates from maternal bone marrow could be determined with SRY-specific RT-QPCR. Alternatively, FACS-based sorting of hybridised chromosomes of ECFCs cultured from maternal bone marrow could be considered. The benefit of cell culture based approach, as compared to others,, is that isolated cells are available for full functional characterisation to confirm their ECFC-identity. For determining potential differences in the function of normal and IUGR fetal ECFCs in the mother, long-term follow-up studies are required. The quantities of fetal ECFCs from maternal bone marrow could be compared between patient groups, using RT-QPCR or chromosome sorting. The functional capacities of these ECFCs could be compared using the functional assays included in Chapter 4. In addition, a potential association with maternal cardiovascular disease could be monitored in the longer term.

5.4 OVERALL SUMMARY

In summary, we have shown that fetal ECFCs play a role in placental and uterine vasculogenesis and that their dysfunctional abilities are associated with IUGR. We suggest that this association is causative and, in conjunction, we propose a complex hypothesis where these dysfunctions are not solely associated with IUGR, but may be the common cause of IUGR and its associated sequelae, including pre-eclampsia, and cardiovascular disease in the fetus as an adult, and the mother (Figure V-1.).
In normal circumstances, fetal ECFCs contribute to both placental and uterine vasculogenesis. In depleted fetal ECFC-function placental and uterine vasculature are consequently aberrant, resulting in IUGR of the fetus and pre-eclampsia. Aberrant ECFCs continue providing pathological vascular repair both in the adulthood of the fetus and in later life of the mother, resulting in cardiovascular disease of both.
6 APPENDIX
MINOR CORRECTIONS
6.1 APPENDIX TO CHAPTER 1

6.1.1 Literature update

6.1.1.1 Superior capacities of fetal ECFCs in the fetomaternal unit.

Since the time of publication of the review article incorporated in the introduction of this thesis a substantial amount of work has been done, which provide mechanistic explanation to the observations published here. These include information on the unique capacities of fetal ECFCs in vasculogenesis in general, and in the fetal and maternal environment.

In the vicinity of vessel formation, integration of ECFCs depends on active urokinase-like Plasminogen Activator Receptor (uPAR) in their caveolae. This ensures efficient enzyme activity at the location, where the cell is in contact with the surface to be invaded. Both caveole formation and uPAR expression are VEGF-driven [295]. ECFCs fail to incorporate in the absence of VEGFR [296]. In regard to de novo vasculogenesis, ECFCs actively excrete components of the vascular basal membrane and extracellular matrix, while mature endothelial cells, although also producing laminin, collagen and fibronectin, fail to deposit these proteins into the extracellular matrix [258]. Production of extracellular components is regulated by HIF-1α and -2β.

CD44 is a hyaluronic acid receptor, which also binds to collagen, MMPs and plays a role in cell migration and adhesion [379]. The expression of CD44 by ECFCs is 6-fold higher [257], and migratory capacities of ECFCs also higher than those of HUVEC [256]. Similarly the expression of the receptor of the chemokine fractalkin is also higher in ECFCs. [380]. Placental transmigration requires breaking of the placental barrier, a process similar to incorporation. ECFCs have higher uPA, uPAR expression levels and MMP-2 activity than HUVEC [255]. Their expression of VEGFR-2, the main regulator of this process, is also higher [256].

As opposed to differentiated endothelial cells, ECFCs do not express major histocompatibility complex II (MHC-II) and their level of MHC-I expression is minimal. Their upregulation of MHC-expression upon INFγ-stimulation is also restricted. Thus they escape immune reactions, mediated by the complement-system and cytotoxic T lymphocytes, CD4 cells and ultimately rejection by the maternal immune system [294].
Several mechanisms are in place to ensure that circulating progenitors are recruited to the areas where they are required and they home to the specific tissue type of interest. Endothelial P-selectin and E-selectin play a role in mediation of the initial steps of homing of EPCs to endothelial structures. Upon activation by EphB4, EPCs increase their affinity to P-selectin and E-selectin by increasing expression of the selectin ligand PGSL-1 [381]. EPC express β2-integrins, which mediate their firm adhesion and transmigration to pre-existent endothelial cells [149]. High-mobility group box 1 (HMGB1) facilitate EPC adhesion and homing to hypoxic areas by activating β1 and β2 integrins[150]. Over expression of the hypoxia-responsive integrin linked kinase (ILK) induces SDF-1 expression and ICAM-1 activation in endothelial cells, which contribute to EPC recruitment to ischaemic areas and homing to pre-existent endothelium [382,383]. VCAM-1 ligand α-4 integrin promotes homing of circulating endothelial progenitors to the endothelium of tissues undergoing active remodelling[151,384], a process, which might be of exceptional importance in the pregnant uterus.

Fetal ECFCs proliferate with a high rate and provide a high number of daughter cells. In culture their proliferation is more effectively induced by VEGF, FGF-2 and PlGF [256]. The proliferative capacities of fetal ECFCs are explained by higher than adult expression of several proliferation related genes, including the nuclear proliferative antigens Ki-67 and survivin, as well as controllers of the cell cycle topoisomerase 2A, Nek-2 and Aurora-A [302].

Overall fetal ECFCs possess a wide range of capacities, which equip them with ability of vascular incorporation, migrating, transmigrating basal membranes, homing, and proliferation in both placental and maternal tissues and survival in the hostile maternal environment.

6.1.1.2 ECFCs in fetal life and pathologies of placentation

Recently, Ligi et al. reported that low birth weight infants have lower numbers of circulating ECFC, as determined by the numbers outgrowing colonies, they have lower proliferative rate in culture and they are less angiogenic in vivo [266]. They found that the reason in the differences of angiogenic capacities lays in differential expression of a set of anti-
angiogenic genes, incl. thrombospondin 1, whose pathogenomic role was confirmed by reversal of angiogenic capacities on inhibition of thrombospondin. However, the patient demographics of their sources clearly showed that these babies were premature, not only growth restricted.

Baker et al. detected in vitro confirmation of these findings [275]. They also found increased circulating and colony numbers of ECFCs in preterm infants. However, they also reported that ECFCs from preterm infants are more susceptible to damage caused by hypoxia. Chronic hypoxia is well established in pathologic placentae, suggesting that ECFCs from preterm infants might suffer even more profound damage from placental hypoxic states, as suggested by Ligi et al.

In line with one of our clinical speculations, suggesting that EPCs may be a causative factor in the correlation between IUGR and increased cardiovascular risk of their mothers, King et al. detected inverse correlation between IUGR, maternal EPC count, migratory capacities and maternal cholesterol levels [385].

6.1.2 Expansion of methodology in Chapter 1.

6.1.2.1 Methods used for determination of EPC-phenotypes

In the review article embedded in Chapter 1, previously unpublished images of the authors were used to illustrate principles of EPC biology. As these images were made using common techniques and served purely as illustration, the review article did not include methodology to these images. This methodology is presented below.

6.1.2.1.1 Immunofluorescent staining

For immunofluorescent staining EPCs (CAC grown by Hill-technique and ECFCs) were plated on 5 wells of a 24-well plate with appropriate coating, fibronectin and collagen-1 respectively. When colonies or monolayers were formed, the culture media was aspirated, cells were washed twice with PBS, before incubation for 30 minutes with 200µl of PBS containing 20µl FcR blocking reagent (Miltenyi Biotec). Subsequently, the following conjugated antibody combinations were added to each well and incubated for 30 minutes
in the dark over ice:

A., 20 µls IgG1-FITC (BD Bioisciences) and 20 µls IgG1-PE (BD Bioisciences)

B., 20 µls IgG2-FITC (BD Bioiscinces)

C., 20 µls CD31-FITC (BD Bioscinces) and 20 µls CD146-PE (BD Bioisciences)

D., 20 µls CD45-FITC (BD Bioisciences) and 10 µls CD105-PE (Invitrogen, Carlsbad, CA)

E., 20 µls CD14-FITC (BD Bioisciences) and 10 µls CD144-PE (eBioscince, San Diego, CA)

Cells were examined unfixed in vivo under the fluorescent microscope, and staining levels compared to the isotype control.

6.1.2.1.2  Ac-LDL uptake test

CACs and ECFCs were plated and grown on a well of 24-well plate coated with fibronectin and Collagen-I, using EndoCult (Stem Cell Technologies Inc., Grenoble, France) and EGM-2 media, respectively. When Hill-clones or cobblestone monolayer were observed, culture media was aspirated, cells washed, and 500 µls of serum-free maintenance media (Media-199 (Sigma Aldrich) in case of Hill culture, Endothelial Basal Medium-2 (Lonza) in case of Lin culture) containing 5 µl of Dil-labelled Ac-LDL (Invitrogen, Eugene, OR) was added. After 5 hours of incubation, supernatant was aspirated and cells washed and covered with maintenance media again. Cultured cells were examined in vivo by fluorescence microscopy.

6.1.2.1.3  Flow cytometry for determining surface marker expression of outgrowth cells

Cells were gently trypsinized, washed twice in PBS and resuspended in PBS with a density of 100,000 cells per 100 µl PBS. Five 100 µl aliquots of cell suspension were incubated with 10 µl FcR blocking reagent (Miltenyi Biotec) for 20 minutes. After incubation the following conjugated antibody combinations were added and incubated in the dark over ice for 30
minutes:

A., 10 µls IgG1-FITC (BD Bioisciences) and 10 µls IgG1-PE (BD Bioisciences)
B., 10 µls IgG2-FITC (BD Bioisciences)
C., 10 µls CD31-FITC (BD Bioisciences) and 10 µls CD146-PE (BD Bioisciences)
D., 10 µls CD45-FITC (BD Bioisciences) and 5 µls CD105-PE (Invitrogen)
E., 10 µls CD14-FITC (BD Bioisciences) and 5 µls CD144-PE (eBioscince)

Cells were washed twice and taken to a volume of 500µl. Cells were run on an ExCalibur Flow cytometer (BD Biosciences) and the frequency of cells staining with each antibody determined against the relevant isotype control (Supplement Figure II-1).

6.1.2.1.4 Angiogenesis test on Matrigel

The basement membrane extract Matrigel (BD Biosciences) was thawed over ice. Pipettes and plates were pre-cooled to prevent early polymerization. Wells of 96-well plates were pre-coated with 40µl Matrigel. Extra care was taken to avoid bubbles. Cells were trypsinized, washed and resuspended in the appropriate basal medium with a cell density of 5000 cells per 100µls. 200µls of the cell suspension was layered over pre-coated wells, resulting in 10000 cells seeded per well. Plates were incubated for 24 hours and fixed with paraformaldehyde 2%(w/v), stained with haematoxylin. Plates were examined under light microscope.

6.1.2.2 Flow cytometry for counting CACs and ECFCs

For the counting of ECFCs and CACs in 550µl fresh fetal blood the protocol of Duda et al. [230], was used with some modifications, including addition of viability stain and optimised red cell lysis (Pharmalyse Lysing Buffer, BD Biosciences for 20min at room temperature). Four aliquots of 125µl of fetal blood, were lysed, treated with 25µl FcR-blocking reagent (Miltenyi Biotec Ltd. Bisley, UK) for 10min at 25C and the following combinations of
antibodies added for the detection of CACs and ECFCs along with relevant IgG isotype control:

A., CD31-FITC (4µl, BD Biosciences, Oxford Science Park, UK), CD133-PE (5µl, Miltenyi Biotec Ltd.), CD45-APC-H7 (10µl, BD Biosciences), CD34-APC (3µl BD Biosciences),

ECFCs

B., CD31-FITC (4µl, BD Biosciences), KDR-PE (5µl, Miltenyi Biotec Ltd.), CD45-APC-H7 (10µl, BD Biosciences), CD34-APC (3µl, BD Biosciences)

C., IgG-FITC (4µl, BD Biosciences), IgG-PE (5µl BD Biosciences), IgG-APC-H7 (10µl BD Biosciences), IgG-APC (3µl BD Biosciences)

D., CD31-FITC (4µl BD Biosciences), IgG-PE (5µl BD Biosciences), CD45-APC-H7 (10µl BD Biosciences), IgG-APC (3µl BD Biosciences)

The viability stain 7-Amino-actinomycin D (7AAD) (5µL, BD Biosciences) was also included ten minutes prior to analysis.

All flow cytometry readings were made against isotype controls on a CyAn-ADP flow cytometer (DAKO A/S, Glostrup, Denmark). Data were analyzed using Summit 4.3 flow cytometry analysis software (DAKO A/S). The gating strategy is outlined in Section 1.2.4.1.

6.1.3 Statistics used

Given the novelty of the field and lack of comparable studies involving advanced flow cytometry or in vivo vasculogenesis of pathological and normal fetal ECFC in the literature, prior sample size analysis was not performed, likewise . experimental repetitions were determined empirically. Normality of distribution before further analysis was determined by D’Agostino-Pearson and Smirnoff-Kormogoroff test, [386,387]. Parametric and non-parametric paired and non-paired tests were applied accordingly using Prism V5.0a software (Graphad Software Inc., La Jolla, CA). For multi-variant analysis two-way ANOVA was used.
6.1.4 Appendix to Chapter 2

Appendix Figure 1. A control to insert II-3Cii.

Neovessels formed by cord blood ECFCs within artificial tissue implants do not fluoresce spontaneously. autofluorescence equal to neighbouring tissue, as opposed to eGFP-ECFC expressing genuine green fluorescence in insert II-C3ii.
REFERENCES

25. Moll W, Kunzel W. The blood pressure in arteries entering the placenta of guinea pigs, rats, rabbits, and sheep. Pflugers Arch 1973;338:125-131


54. Yoshie M, Miyajima E, Kyo S, Tamura K. Stathmin, a microtubule regulatory protein, is associated with hypoxia-inducible factor-1alpha levels in human endometrial and endothelial cells. Endocrinology 2009;150:2413-2418


122. Graziano M, St-Pierre Y, Potworowski EF. UEA-I-binding to thymic medullary epithelial cells selectively reduces numbers of cortical TCRalphabeta+ thymocytes in FTOCs. Immunol Lett 2001;77:143-150


cells: the role of angiogenic cytokines and matrix metalloproteinases. Circulation 2005;112:1618-1627


179. Pardanaud L, Dieterlen-Lievre F. Does the paraxial mesoderm of the avian embryo have hemangioblastic capacity? Anat Embryol (Berl) 1995;192:301-308.


201. **Prater DN, Case J, Ingram DA, Yoder MC.** Working hypothesis to redefine endothelial progenitor cells. Leukemia 2007;21:1141-1149
210. **Khosrotehrani K, Johnson KL, Cha DH, Salomon RN, Bianchi DW.** Transfer of fetal cells with multilineage potential to maternal tissue. JAMA 2004;292:75-80


240. Mayhew TM. Fetoplacental angiogenesis during gestation is biphasic, longitudinal and occurs by proliferation and remodelling of vascular endothelial cells. Placenta 2002;23:742-750.


258. Kusuma S, Zhao S, Gerecht S. The extracellular matrix is a novel attribute of endothelial progenitors and of hypoxic mature endothelial cells. FASEB J 2012;26:4925-4936


320. Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003;107:1164-1169
328. Rainey A, Mayhew TM. Volumes and numbers of intervillous pores and villous domains in placentas associated with intrauterine growth restriction and/or pre-eclampsia. Placenta 2010;31:602-606
329. Mayhew TM, Wijesekara J, Baker PN, Ong SS. Morphometric evidence that villous development and fetoplacental angiogenesis are compromised by intrauterine growth restriction but not by pre-eclampsia. Placenta 2004;25:829-833


357. Dolinsky VW, Rueda-Clausen CF, Morton JS, Davidge ST, Dyck JR. Continued postnatal administration of resveratrol prevents diet-induced metabolic syndrome in rat offspring born growth restricted. Diabetes 2011;60:2274-2284

358. Rueda-Clausen CF, Morton JS, Dolinsky VW, Dyck JR, Davidge ST. Synergistic effects of prenatal hypoxia and postnatal high fat diet in the development of cardiovascular pathology in young rats. Am J Physiol Regul Integr Comp Physiol 2012


369. Noori M, Donald AE, Angelakopoulou A, Hingorani AD, Williams DJ. Prospective study of placental angiogenic factors and maternal vascular function
before and after preeclampsia and gestational hypertension. Circulation 2010;122:478-487


376. Lang I, Schweizer A, Hiden U, et al. Human fetal placental endothelial cells have a mature arterial and a juvenile venous phenotype with adipogenic and osteogenic differentiation potential. Differentiation 2008;76:1031-1043


381. Lee SP, Youn SW, Cho HJ, et al. Integrin-linked kinase, a hypoxia-responsive molecule, controls postnatal vasculogenesis by recruitment of endothelial progenitor cells to ischemic tissue. Circulation 2006;114:150-159


APPENDIX 2 (DIGITAL MEDIUM)

A DVD CONTAINING SUPPLEMENTARY VIDEO FILES

A DVD attached to this thesis contains the following supplementary documents in .mov format:

CHAPTER 2

- **Video II.1.** A clip showing the unsuccessful search for apparent fluorescent cells in the body of an NOD/SCID fetus, which received an intra-cardiac injection of eGFP-ECFC during intrauterine life.
- **Video II.2.** An adjunct to Figure 6d, showing the placenta circulation in a NOD/SCID fetus transplanted with eGFP-ECFC.

CHAPTER 3

- **Video III.1.** Ultrasound-based in vivo injection of human fetal ECFCs into the mouse fetal circulation.
  - **Video III.1a** Visual of murine fetus
  - **Video III.1b** Needle in the fetal what?
  - **Video III.1c** Evident ejection of injection-material into the aortic arch
- **Video III.2.** A series of real-time in vivo optical sequences of the mouse uterine microvasculature infiltrated by transmigratory fetal cells.
- **Video III.3.** In vivo images of pregnant uteri of NOD/SCID mice whose fetuses were transplanted with human fetal eGFP-ECFCs.