The Effects of Maternal Periconceptional Ethanol Exposure on the Development of the Pre-Implantation Embryo, Placenta, and the Influence of the Uterine Environment

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Abstract

Exposure of the embryo or fetus to perturbations in utero can result in intrauterine growth restriction (IUGR), a primary risk factor for the development of adult disease. One critical window that can result in developmental programming, is the periconceptional period which comprises maturation of the oocyte, conception and the formation of the blastocyst prior to embryo implantation. Alcohol is a common exposure during this period, as it is consumed prior to pregnancy recognition, and often ceases soon after. Maternal periconceptional alcohol exposure (PC-EtOH) in rat dams has been previously shown to cause fetal growth restriction and changes to the late gestation placenta. PC-EtOH also results in the development of adult onset disease, including insulin insensitivity, often in a sexually dimorphic manner. However, the mechanisms by which PC-EtOH can cause programming are relatively unexplored. This thesis aimed to characterise the effects of alcohol on 1. sex-specific pre-implantation development and trophoblast differentiation using in vitro (cell culture) and in vivo (rodent model) techniques, 2. alterations to the early uterine environment and 3. the interactions and communication between the embryo and uterus, and the resultant impacts on placental development across gestation.

The direct effect of EtOH on in vitro differentiation was assessed in mouse trophoblast stem cells (Chapter 4). Results demonstrated that doses of 0.2% and 1% EtOH reduced total cell count and expression of trophoblast subtype markers at terminal differentiation (day 6 of culture). Using our in vivo rat model, we also examined basal sex differences in blastocyst and placental development from pre-implantation to late-gestation in naturally cycling dams (Chapter 3). Although there were no significant differences between sexes in pre-implantation development (blastocyst cell numbers, trophoblast differentiation), by mid-gestation (E15) placentas from males were heavier and had increased blood space volume and surface area (to both maternal and fetal compartments) than those from females. This likely contributed to the greater fetal body weight in males at E20. This study confirmed the need to examine the effects of PC-EtOH in a sexually dimorphic manner across all of pregnancy.

To study the effects of PC-EtOH in vivo, Sprague-Dawley rat dams were given a liquid diet containing either control (0% v/v EtOH) or EtOH (12.5% v/v EtOH) from embryonic (E) day -4 to E4. The following day dams were returned to chow for the remainder of
gestation. Dams were sacrificed at E5 to determine cell number of the pre-implantation embryo and its capacity to differentiate into cells required for invasion (Chapter 5). Pre-implantation studies showed PC-EtOH altered inner cell mass count, trophoblast differentiation to trophoblast giant cells (TGCs) and trophoblast behaviour in a sexually dimorphic manner, with females showing the most deleterious outcomes. PC-EtOH females also showed reduced expression of Prl4a1, a gene exclusively expressed by TGCs for communication with decidual natural killer cells (dNK).

Maternal plasma and uterine samples were collected over the peri-implantation period (E5-E7) to assess the maternal hormonal environment, and uterine responses for implantation and maintenance of pregnancy in response to the invading PC-EtOH exposed embryo (Chapter 5). Whilst no changes to oestrogen or progesterone levels were observed, alterations to their receptors (Esr1 and Pgr), and downstream response genes (including those involved in uterine decidualisation, vasculogenesis and embryo attachment) were found, particularly at E7. Genes involved in dNK maturation and function were also markedly decreased by PC-EtOH at E7, suggesting that the uterine responses are altered by inappropriate communication by the embryo. By E11, a 25% increase in dNK cells was found in PC-EtOH exposed females, which may suggest that reduced timing of dNK cell homing has resulted from perturbed TGC communication.

Placental morphogenesis was further examined after PC-EtOH in the immature (E13), definitive (E15) and late gestation (E20) placenta (Chapter 6). Investigation of invasion of spiral artery trophoblast giant cells at E13 demonstrated a decrease in PC-EtOH females only. At E15, PC-EtOH, caused increased resorptions and reduced maternal blood space volume in both sexes. This suggests impaired or slowed development of the definitive placenta in mid-gestation by PC-EtOH, but was followed by compensatory growth of the late gestation E20 placenta. This ‘catch-up’ in placental growth, however, was not sufficient to prevent the fetal growth deficit.

This thesis has provided novel insights into sexually dimorphic placentation and the deleterious effects of early alcohol exposure on the developing embryo and placental morphogenesis. This study supports guidelines that abstinence from alcohol exposure when planning a pregnancy is the safest option.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Peer-reviewed publications


Conference abstracts

Kalisch-Smith, J.I., Simmons, D.G., Pantaleon, M., Moritz, K.M. Periconceptional alcohol exposure in the rat reduces trophoblast giant cell differentiation and outgrowth capacity in the rat. Fetal and Neonatal Physiological Society Meeting, 17-20 September 2016, University of Cambridge (Oral presentation)

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Contributions by others to the thesis

The majority of work completed throughout this thesis was undertaken by JI Kalisch-Smith. Help was provided for the conception and design of the project as well as interpretation of results and critical revision of work by KM Moritz, DG Simmons and M Pantaleon. S Anderson performed quantification of plasma estrogen and progesterone. JE Outhwaite assisted with cell culture experiments in Chapter 4.

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## Table of Contents

Abstract ........................................................................................................................................ 2  
Declaration by author .................................................................................................................. 4  
Publications during candidature ................................................................................................. 5  
Publications included in this thesis ............................................................................................. 8  
Contributions by others to the thesis .......................................................................................... 11  
Statement of parts of the thesis submitted to qualify for the award of another degree .......... 11  
Acknowledgements .................................................................................................................... 12  
Keywords ..................................................................................................................................... 13  
Australian and New Zealand Standard Research Classifications (ANZSRC) ......................... 13  
Fields of Research (FoR) Classification ....................................................................................... 13  
Table of Contents ........................................................................................................................ 14  
Chapters Index ............................................................................................................................... 14  
Figures Index ................................................................................................................................. 22  
Tables Index .................................................................................................................................. 25  
Equations Index ............................................................................................................................. 27  
Abbreviations ............................................................................................................................... 28

## Chapters Index

**Chapter 1 – Literature Review** .................................................................................................. 30  
1.1 Introduction to the developmental origins of health and disease ........................................ 30  
1.2 Evidence of fetal programming in humans ............................................................................ 32  
1.3 Influence of maternal alcohol consumption on fetal programming .................................. 33  
   1.3.1 Prevalence of maternal alcohol consumption ................................................................. 33  
   1.3.2 Alcohol – another agent of programming ......................................................................... 34  
1.4 Pre-implantation development: a summary .......................................................................... 34  
1.5 Environmental regulation of pre-implantation development ................................................ 35  
1.6 Uterine responses required for implantation ......................................................................... 36  
   1.6.1 Acquisition of receptivity and decidual response ............................................................. 37
Chapter 2 – General Materials and Methods .............................................. 77

2.1 Ethics ........................................................................................................... 77
2.2 Animal Handling and Liquid Diet Administration ..................................... 77
2.3 Post-mortem and tissue collection ............................................................. 78
    2.3.1 E5 cohort ............................................................................................. 78
    2.3.2 E6 and E7 cohorts ............................................................................... 79
    2.3.3 E11 and E13 cohorts .......................................................................... 80
2.3.4 E15 and E20 cohorts ................................................................. 80
2.4 Trophoblast outgrowth assays ...................................................... 81
2.5 Culture of trophoblast stem cells .................................................. 81
2.6 Plasma analyses ......................................................................... 82
   2.6.1 COBAS Integra analyses .......................................................... 82
   2.6.2 Hormone analyses .................................................................. 82
      2.6.2.1 Estrogen .......................................................................... 82
      2.6.2.2 Progesterone ................................................................. 83
2.7 Gene expression analyses .............................................................. 84
   2.7.1 RNA extraction ...................................................................... 84
      2.7.1.1 Column extractions ........................................................... 84
      2.7.1.2 TRIzol .............................................................................. 85
   2.7.2 RNA integrity .......................................................................... 85
   2.7.3 cDNA synthesis ...................................................................... 86
      2.7.3.1 Quantitek ........................................................................ 86
      2.7.3.2 Biorad iScript .................................................................. 86
   2.7.4 qPCR Quantification ............................................................... 86
      2.7.4.1 TaqMan qPCR ................................................................ 86
      2.7.4.2 SYBR qPCR .................................................................... 87
2.8 Genotyping for sex ...................................................................... 88
   2.8.1 Blastocyst and trophoblast outgrowths .................................... 88
   2.8.2 E11 to E20 placental samples ............................................... 89
2.9 Histology ...................................................................................... 89
   2.9.1 Haematoxylin and eosin .......................................................... 89
   2.9.2 Immunofluorescence for whole-mount pre-implantation embryos, trophoblast outgrowths and placenta cryo-sections ............. 90
   2.9.3 In situ hybridisation and fluorescent in situ hybridisation (FISH) ........ 90
      2.9.3.1 Construction of cRNA probes ........................................... 90
      2.9.3.2 In situ hybridisation protocol for paraffin sections .......... 92
      2.9.3.3 Modifications for fluorescent in situ hybridisation for paraffin and cultured cells ......................................................... 93
   2.9.4 Lectin histochemistry .............................................................. 94
   2.9.5 Immunohistochemistry ............................................................ 94
   2.9.6 Transmission electron microscopy ........................................... 95
Chapter 2. Imaging and Stereology of Implantation and Placental Tissues

2.10 Imaging parameters and analysis

2.10.1 Blastocysts
2.10.2 Determination of CDX2 TE nuclear fluorescence
2.10.3 Trophoblast outgrowths
2.10.4 Determination of nuclear DNA content of trophoblasts
2.10.5 Quantification of trophoblast invasion at mid-gestation
2.10.6 Quantification of FISH positive cells in culture

2.11 Stereology of placental and maternal tissues

2.11.1 Stromal and decidual volume at E7
2.11.2 Quantification of decidual natural killer cells
2.11.3 Estimation of placental volumes

2.12 Statistical analyses

Chapter 3. Sex differences in rat placental development: from pre-implantation to late-gestation

3.1 Abstract
3.2 Introduction
3.3 Methods

3.3.1 Ethics
3.3.2 Animal treatment, embryo collection and culture
3.3.3 Staining procedures for pre-implantation embryos
3.3.4 Blastocyst outgrowth assays
3.3.5 Confocal microscopy, image analysis and quantification
3.3.6 Genotyping for sex of pre-implantation embryos and blastocyst outgrowths
3.3.7 Collection of placental tissues at mid- and late-gestation
3.3.8 Stereology for placental volumes
3.3.9 Transmission electron microscopy
3.3.10 Gene expression studies
3.3.11 Statistical analysis

3.4 Results

3.4.1 Embryo studies
# Chapter 4. Alcohol exposure impairs trophoblast survival and alters subtype-specific gene expression *in vitro*

## 4.1 Abstract

## 4.2 Introduction

## 4.3 Methods

### 4.3.1 TS cell culture

### 4.3.2 RNA extraction and qRT-PCR

### 4.3.3 Histological assessment of trophoblast number

### 4.3.4 Fluorescent *in situ* hybridisation for localisation of trophoblast subtypes on cultured EGFP cells

### 4.3.5 Imaging and Analysis

### 4.3.6 Statistical analyses

## 4.4 Results and Discussion

### 4.4.1 Cell counts

### 4.4.2 Junctional zone cell markers

### 4.4.3 Labyrinth zone cell markers

## 4.5 Conclusions
5.1 Abstract ......................................................................................................................................... 147
5.2 Introduction ................................................................................................................................. 149
5.3 Methods ........................................................................................................................................ 151
   5.3.1 Ethics, animal treatment and sample collection ................................................................. 151
   5.3.2 Blastocyst viability and lineage allocation ............................................................................ 151
   5.3.3 Trophoblast outgrowth assays .............................................................................................. 152
   5.3.4 Immunofluorescence and image analysis ............................................................................. 152
   5.3.5 Genotyping for sex ................................................................................................................ 153
   5.3.6 In situ hybridisation and immunohistochemistry ............................................................... 153
   5.3.7 Stereology of uterine and placental samples ........................................................................ 154
   5.3.8 Plasma analyses ..................................................................................................................... 154
   5.3.9 RNA extraction, cDNA synthesis and qPCR ....................................................................... 155
   5.3.10 Statistical analyses .............................................................................................................. 157
5.4 Results ........................................................................................................................................... 158
   5.4.1 Effects of PC-EtOH on number of flushed embryos and cell counts .................................... 158
   5.4.2 Trophoblast outgrowth assays from PC-EtOH treated embryos ......................................... 163
   5.4.3 Effects of PC-EtOH on the peri-implantation uterine environment and early placenta ........ 166
5.5 Discussion ...................................................................................................................................... 172
   5.5.1 PC-EtOH exposure - a rodent model of early programming ................................................. 172
   5.5.2 PC-EtOH exposure on development of the pre-implantation embryo .................................. 172
   5.5.3 Influence of PC-EtOH exposure on trophoblast differentiation and invasive capacity ........ 174
   5.5.4 Influence of PC-EtOH on the maternal uterine responses for the establishment of pregnancy .......................................................... 176
   5.5.5 Future investigations ............................................................................................................. 179
5.6 Conclusions.................................................................................................................................. 179
Chapter 6. Periconceptional alcohol exposure: effects on the morphological
development of the placenta ................................................................. 181

6.1 Abstract .......................................................................................... 182
6.2 Introduction ..................................................................................... 184
6.3 Methods .......................................................................................... 186
  6.3.1 Ethics, animal handling and liquid diet administration ................. 186
  6.3.2 Post-mortem and tissue collection .............................................. 186
  6.3.3 Histology ................................................................................ 187
    6.3.3.1 Haematoxylin and eosin ..................................................... 187
    6.3.3.2 Immunofluorescence on cryo-sections ................................. 187
    6.3.3.3 In situ hybridisation ............................................................ 187
    6.3.3.4 Lectin histochemistry ........................................................ 188
  6.3.4 Imaging parameters and analysis ............................................... 188
  6.3.5 Stereology for placental volumes ............................................. 189
  6.3.6 Gene expression assays ............................................................ 189
  6.3.7 Statistical analyses ................................................................ 190
6.4 Results ............................................................................................ 191
  6.5 Discussion .................................................................................... 201
    6.5.1 PC-EtOH reduces fetal viability at E15 ...................................... 201
    6.5.2 PC-EtOH causes structural changes to the mid-gestation E15 placenta ...................................................................................... 201
    6.5.3 Structural changes in the labyrinth are preceded by reduced secondary
trophoblast invasion in PC-EtOH females .............................................. 203
    6.5.4 Physiological impacts of reduced spiral artery remodelling and reduced
  MBS..................................................................................................... 204
    6.5.5 Future investigations ............................................................... 205
6.6 Conclusions .................................................................................... 205

Chapter 7. General discussion................................................................. 206

7.1 Thesis Summary .............................................................................. 206
7.2 Summary of results ......................................................................... 208
7.3 PC-EtOH on programming of adult disease .................................... 209
Figures Index

Chapter 1

Figure 1.1. Trophoblast giant cell differentiation causes transition from mitotic cell
cycle to the endocycle to increase ploidy. ................................................................. 40
Figure 1.2. Trophoblast lineages that give rise to the placental and decidual zones.
........................................................................................................................................ 42
Figure 1.3. Critical windows during placental development which are susceptible to
perturbation. ....................................................................................................................... 48
Figure 1.4. Summary of the putative impact of ethanol consumption on
periconceptional programming. ....................................................................................... 71

Chapter 2

Figure 2.1. Implantation sites of rat at E7. ................................................................. 80
Figure 2.2. Gel electrophoresis of RNA samples to examine RNA integrity. .......... 85
Figure 2.3. Computer generated surface of DAPI channel enclosing cell nuclei using
Imaris. .............................................................................................................................. 96
Figure 2.4. Immunofluorescent image of E13 placenta to examine trophoblast invasion
and spiral artery remodelling. ....................................................................................... 97
Figure 2.5. Uterine histochemical markers of E7 implantation site. ....................... 99
Figure 2.6. Prf1 ISH for infiltrating maternal decidual natural killer cells (dNK) at E11
implantation sites. ......................................................................................................... 100

Chapter 3

Figure 3.1. Analysis of pre-implantation development at E5 for male and female in vivo
derived embryos. ........................................................................................................... 116
Figure 3.2. Gene profiles of trophoblast outgrowths after 6 days culture from in vivo-
derived dams. ............................................................................................................... 117
Figure 3.3. Sexually dimorphic outcomes of zonal volumes within the placenta from E13-E20 during gestation. ................................................................. 120
Figure 3.4. Quantification of labyrinthine blood spaces in males and females in the immature, definitive and late-gestation placenta. ....................................................... 121
Figure 3.5. Estimation of interhemal membrane thickness of the male and female labyrinth. ........................................................................................................ 122
Figure 3.6. Expression profiles of male and female placentas at E13, and the labyrinth at E15. ........................................................................................................ 123
Figure 3.7. Temporal gene expression profiles of male and female placentas from E13 to E15. ........................................................................................................ 124

Chapter 4

Figure 4.1. Trophoblast stem cell proliferation and expression profiles when exposed to EtOH over 6 days during differentiation. ...................................................... 141
Figure 4.2. Quantification of Prl7a2 and Tpbpa positive trophoblasts by fluorescent in situ hybridisation. ........................................................................................................ 142
Figure 4.3. Gene expression of pro-apoptotic marker Bax and anti-apoptotic marker Bcl2. ................................................................................................................... 143
Figure 4.4. Expression profiles of RS26 differentiated trophoblasts at day 6 after EtOH exposure. ........................................................................................................ 144

Chapter 5

Figure 5.1. In vivo PC-EtOH exposure does not alter cell counts of E5 blastocyst but does show effect of a liquid diet. ................................................................. 160
Figure 5.2. In vivo PC-EtOH exposure causes sex-specific alterations to pre-implantation blastocyst development. ................................................................. 161
Figure 5.3. In vivo PC-EtOH exposure reduces trophoblast outgrowth rate and sex-specific alterations to P-TGC differentiation and DNA content. ..................... 164
Figure 5.4. Expression profiles of trophoblast outgrowths exposed to PC-EtOH. . 165
Figure 5.5. PC-EtOH exposure on maternal hormone profiles of throughout gestation. ........................................................................................................ 167
Figure 5.6. Uterine expression profiles over the peri-implantation period at E5 and E7.

Figure 5.7. PC-EtOH does not alter stromal or decidual volume at E7.

Figure 5.8. PC-EtOH exposure causes sex-specific infiltration of maternal decidual natural killer cells at E11.

Chapter 6

Figure 6.1. PC-EtOH exposure reduces whole placental and zonal volumes in females only at E15.

Figure 6.2. PC-EtOH reduces labyrinthine MBS volume in both sexes.

Figure 6.3. PC-EtOH does not alter labyrinth blood space surface areas.

Figure 6.4. PC-EtOH does not alter interhaemal membrane thickness.

Figure 6.5. PC-EtOH exposure reduces trophoblast invasion into the mesometrial triangle in females only.

Figure 6.6. QPCR on placental tissues from whole placentas at E13.

Figure 6.7. QPCR on placental labyrinth tissues from E15.

Chapter 7

Figure 7.1 Summary of major findings of in vivo PC-EtOH exposure on sex-specific programming of pre-implantation and placental development.
Tables Index

Chapter 1

Table 1.1. Sex-specific outcomes of placental and fetal growth in human and animal studies. ................................................................. 52
Table 1.2. Effect maternal perturbations during the pre-implantation period on the development of adult-onset disease. ......................................................... 61
Table 1.3. Periconceptional diet modifications of pre-implantation development, placental development. ................................................................. 63

Chapter 2

Table 2.1. Liquid diet composition for control and ethanol treatment groups. ........ 78
Table 2.2. Hepes-KSOM embryo flushing and culture media .................................................. 79
Table 2.3. Conditioned media. ......................................................................................... 81
Table 2.4 Trophoblast stem cell media. .............................................................................. 82
Table 2.5. SYBR primers (mouse) and primer efficiencies. .............................................. 87
Table 2.6. SYBR primers (rat) and primer efficiencies. ....................................................... 88
Table 2.7 In situ hybridisation primers and product length. ............................................. 92

Chapter 3

Table 3.1. Fetal weight and placental weight and dimensions in males and females at E15 and E20. ................................................................. 119

Chapter 4

Table 4.1. Mouse trophoblast markers and associated primer sequences for SYBR qRT-PCR. ................................................................. 135
Chapter 5
Table 5.1 *In situ* hybridisation primers and product length. ........................................... 154
Table 5.2 TaqMan primers. .................................................................................................. 156
Table 5.3 SYBR green primer sequences .............................................................................. 156
Table 5.4. Flushing statistics of pre-implantation embryos derived from embryonic day 5. ......................................................................................................................... 159

Chapter 6

Table 6.1. Fetal and placental biometry at post-mortem at E15. ................................. 193
Equations Index

Equation 1. Relative gene expression calculation. ............................................. 87
Equation 2. Quantification of the percentage of inner cell mass cells to total cells of the E5 blastocyst. ......................................................................................................................... 95
Equation 3. Total volume estimation. ................................................................................................................................. 98
Equation 4. Estimation of surface areas of fetal capillaries and maternal blood spaces. ................................................................. 101
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Area of points or grid size</td>
</tr>
<tr>
<td>ART</td>
<td>Artificial reproductive technologies</td>
</tr>
<tr>
<td>aSMA</td>
<td>Alpha smooth muscle actin</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>Cdx-2</td>
<td>Caudal-like transcription factor 2</td>
</tr>
<tr>
<td>C-TGC</td>
<td>Canal-associated trophoblast giant cell</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary RNA</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle thresholds</td>
</tr>
<tr>
<td>DEC</td>
<td>Decidua</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DMO</td>
<td>5,5-dimethyl-2,4-oxazolidinedione</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid; Genomic DNA</td>
</tr>
<tr>
<td>Dnmt1</td>
<td>DNA methyltransferase 1</td>
</tr>
<tr>
<td>dNK</td>
<td>Decidual natural killer cell</td>
</tr>
<tr>
<td>DOHaD</td>
<td>Developmental origins of health and disease</td>
</tr>
<tr>
<td>DPX</td>
<td>Dibutylphthalate</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EPC</td>
<td>Ecto-placental cone</td>
</tr>
<tr>
<td>EGA</td>
<td>Embryonic genome activation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid;</td>
</tr>
<tr>
<td>Esr1</td>
<td>Estrogen receptor 1</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>EXE</td>
<td>Extraembryonic ectoderm</td>
</tr>
<tr>
<td>FAS</td>
<td>Fetal alcohol syndrome</td>
</tr>
<tr>
<td>FASD</td>
<td>Fetal Alcohol spectrum disorders</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal blood space</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridisation</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor family</td>
</tr>
<tr>
<td>Gcm-1</td>
<td>Glial cells missing homolog 1</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GlyT</td>
<td>Glycogen trophoblast</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HF</td>
<td>High fat</td>
</tr>
<tr>
<td>HFHS</td>
<td>High fat + high salt</td>
</tr>
<tr>
<td>HS</td>
<td>High salt</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic pituitary axis</td>
</tr>
<tr>
<td>IC</td>
<td>Intracellular</td>
</tr>
<tr>
<td>I$_{cap}$</td>
<td>Number of intersections between cycloid arcs and the fetal capillary endothelium</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>ICSI</td>
<td>Intracytoplasmic sperm injection</td>
</tr>
<tr>
<td>IDD</td>
<td>Insulin-dependent diabetes</td>
</tr>
<tr>
<td>IGF1/2</td>
<td>Insulin-like growth factor 1/2</td>
</tr>
<tr>
<td>Igfbp1</td>
<td>Insulin-like growth factor binding protein 1</td>
</tr>
<tr>
<td>IHM</td>
<td>Interhemal membrane</td>
</tr>
<tr>
<td>IIS</td>
<td>Inter-implantation site</td>
</tr>
<tr>
<td>IMS</td>
<td>Implantation site</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal (injection)</td>
</tr>
<tr>
<td>ISH</td>
<td>In situ hybridisation</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth restriction</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilisation</td>
</tr>
<tr>
<td>JZ</td>
<td>Junctional zone</td>
</tr>
<tr>
<td>KSOM</td>
<td>Potassium simplex optimization medium</td>
</tr>
<tr>
<td>LAB</td>
<td>Labyrinth</td>
</tr>
<tr>
<td>LAB TROPH</td>
<td>Labyrinth trophoblast</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>LF</td>
<td>Low fat</td>
</tr>
<tr>
<td>LOS</td>
<td>Large offspring syndrome</td>
</tr>
<tr>
<td>l(p)</td>
<td>Length of the test line</td>
</tr>
<tr>
<td>NHMRC</td>
<td>National health and medical research council</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>m-TE</td>
<td>Mural trophectoderm</td>
</tr>
<tr>
<td>MBS</td>
<td>Maternal blood space</td>
</tr>
<tr>
<td>N.D.</td>
<td>Not determined</td>
</tr>
<tr>
<td>NHMRC</td>
<td>National health and medical research council</td>
</tr>
<tr>
<td>NN</td>
<td>Nephron number</td>
</tr>
<tr>
<td>Oct-4</td>
<td>Octamer-3/4</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBT</td>
<td>Phosphate buffered saline with tween</td>
</tr>
<tr>
<td>( P_{\text{compartmnt}} )</td>
<td>Number of points within each section</td>
</tr>
<tr>
<td>PN</td>
<td>Postnatal</td>
</tr>
<tr>
<td>p-TE</td>
<td>Proximal trophectoderm</td>
</tr>
<tr>
<td>P-TGC</td>
<td>Parietal trophoblast giant cell</td>
</tr>
<tr>
<td>PC-EtOH</td>
<td>Periconceptional ethanol</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PN</td>
<td>Postnatal</td>
</tr>
<tr>
<td>Pgr</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>PW</td>
<td>Placental weight</td>
</tr>
<tr>
<td>PW:BW</td>
<td>Placental weight: body weight ratio</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RES</td>
<td>Resorptions</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SOF</td>
<td>Synthetic oviduct fluid</td>
</tr>
<tr>
<td>SpA-TGC</td>
<td>Spiral Artery trophoblast giant cell</td>
</tr>
<tr>
<td>S-TGC</td>
<td>Sinusoidal trophoblast giant cell</td>
</tr>
<tr>
<td>SpT</td>
<td>Spongiotrophoblast</td>
</tr>
<tr>
<td>Sv</td>
<td>Surface area of vessels</td>
</tr>
<tr>
<td>Syna/b</td>
<td>Syncytin a/b</td>
</tr>
<tr>
<td>SynT-1/2</td>
<td>Syncytiotrophoblast layer 1/2</td>
</tr>
<tr>
<td>T</td>
<td>Thickness</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris base, acetic acid and EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Trophectoderm</td>
</tr>
<tr>
<td>TE:ICM</td>
<td>Ratio of trophectoderm to inner cell mass</td>
</tr>
<tr>
<td>TGC</td>
<td>Trophoblast giant cell</td>
</tr>
<tr>
<td>TS</td>
<td>Trophoblast stem cell</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>( V_{\text{lab}} )</td>
<td>Total volume of the labyrinth</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>W</td>
<td>Watts</td>
</tr>
<tr>
<td>wks</td>
<td>Weeks</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight per weight</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
</tr>
<tr>
<td>WP</td>
<td>Whole placenta</td>
</tr>
<tr>
<td>( \Sigma )</td>
<td>Sum</td>
</tr>
<tr>
<td>( \Sigma P_{\text{lab.}} )</td>
<td>Sum of test points marking the labyrinth</td>
</tr>
<tr>
<td>% ICM</td>
<td>Percentage of inner cell mass to total cell count</td>
</tr>
</tbody>
</table>
Chapter 1 – Literature Review

1.1 Introduction to the developmental origins of health and disease

A “sub-optimal” maternal environment such as a result of poor diet, stress, or hypoxia during critical “developmental windows” has been shown to cause fetal intrauterine growth restriction (IUGR), (Barker, 1995, Gluckman et al., 2007). The developmental origins of health and disease hypothesis emphasises that adaptations in response to maternal perturbations in the short-term may ensure fetal survival; however, in the long-term they may become a disadvantage (Barker, 1995, 2000). Fetuses sense maternal nutrient and substrate availability to predict the postnatal environment and co-ordinate their growth trajectories accordingly (Bloomfield and Harding, 1998). Those that exhibit IUGR often undergo compensatory adaptations in the postnatal period when they experience an unanticipated environment. It is these later adaptations which are thought to be the most deleterious, and which act as independent risk factors for developing adult-onset cardiovascular disease, chronic kidney disease, and metabolic syndrome (Barker, 2000, Gluckman and Hanson, 2006).

It is now well established that the period around conception is a “critical window” during development that can cause programming of adult disease (Kwong et al., 2000, 2006). A poor maternal environment as a result of poor nutrition (Grieger et al., 2014), smoking (Homish et al., 2012), and alcohol exposure (Muggli et al., 2016), are likely perturbations during this period as many women are unaware of being pregnant. One major contributing factor to this is unplanned pregnancies, which accounts for approximately 50% of pregnancies in Australia and America (Colvin et al., 2007, Finer and Zolna, 2016).

Over the past two decades, the periconceptional period has been investigated for events occurring pre-conception prior to fertilisation of the oocyte by the sperm, including oocyte maturation and ovulation, and post-fertilisation in the pre-implantation period. The maturing oocyte relies on the surrounding granulosa cells within the preovulatory follicle for essential nutrients and other factors for growth and metabolism before it is released at ovulation. After fertilisation and prior to the establishment of the placenta for access to the maternal circulation, the pre-implantation embryo relies on
oviductal and uterine fluidal secretions for these same requirements. Maternally derived factors can thus affect early cell division and primary differentiative events in development that result in the formation of the mature blastocyst and progenitors to the embryo proper and the placenta. The pre-implantation embryo is thus highly susceptible to maternal perturbations not only because it has a unique metabolic profile and set of nutritional requirements to facilitate morphological development, but also because it undergoes genome wide epigenetic reprogramming after fertilisation and activation of its new genome (reviewed by Fleming et al. 2004). The female pre-implantation embryo in the extraembryonic (placental) lineage also receives during this time double dosage of X chromosome genes prior to X chromosome inactivation in the early post-implantation embryo (Hemberger, 2002). This may contribute to sexually dimorphic outcomes in morphogenesis and physiology of the early embryo in response to any adverse environment prior to placental formation (Kalisch-Smith et al., 2017a).

The pre-implantation embryo must also be “activated" to allow appropriate interactions with the uterine luminal epithelium, and become invasive to facilitate implantation into the maternal endometrium. Likewise, the endometrium must attain a hormonally regulated, transient state of “receptivity” for embryo implantation to be initiated. An intricately orchestrated embryonic-uterine dialogue is therefore essential for the dynamic morphological events of oocyte and embryonic growth, and to establish an appropriate growth trajectory in anticipation of the postnatal environment. Given that the cells of pre-implantation embryo act as a progenitor pool for all tissues required for development, any adaptive responses set during this phase will have lasting consequences both throughout gestation and into adult life. Not surprisingly perhaps, subtle changes to the maternal substrates such as glucose (Moley, 2001) or protein (Kwong et al., 2000, 2006), have been shown to cause alterations in early development, lead to IUGR and “programme” adult onset disease.

The placenta is also increasingly being recognised as a critical mediator of intrauterine programming during gestation. Placental adaptations are likely to depend on the type of stressor, duration, severity and the window of exposure during development. Regardless of the type of stressor, altered placental formation can result and contribute to impaired placental function and consequently, adaptation in fetal
development. This includes the periconceptional period, in which alterations to the trophectoderm, the progenitor pool to the placenta, can influence placentation days after the initial exposure (Kwong et al., 2000, Watkins et al., 2015, MacLaughlin et al., 2005, Tan et al., 2016a, Bloise et al., 2012). The placenta also can mediate sexually dimorphic outcomes, with perturbations in early development associated with female lethality (Tan et al., 2016b), while mid-late gestational exposures are largely associated with male-specific lethality (reviewed by Kalisch-Smith et al. 2017a).

The following literature review will focus on pre-implantation development, uterine responses for implantation, placental formation, as well as sexual dimorphism which can influence programming in response to maternal perturbations. This review will also highlight alcohol as another agent in the programming of adult disease, which is not as well explored in the periconception period as it is in other chronic gestational models.

1.2 Evidence of fetal programming in humans

Non-communicable diseases such as cardiovascular disease, stroke, chronic kidney disease and diabetes, continue to be the leading causes of premature death worldwide. In 2012, the World Health Organisation estimated these conditions were responsible for 38 out of 56 million deaths (WHO, 2014). In Australia, these conditions are estimated to cost the health system $7.7 billion for cardiovascular disease, and $1.52 billion for diabetes from 2008-2009 (AIHW, 2014). While prevention of these disorders is largely focused on lifestyle factors including smoking, poor diet (high fat, high sugar), and a lack of exercise, prenatal origins of disease outcomes are becoming increasingly realised.

The first evidence that an in utero environment could adversely affect the developing fetus and influence the onset of adult disease came from a study in Hertfordshire (United Kingdom), which associated low infant weight gain with coronary heart disease and type 2 diabetes (Barker et al., 1989). Since then, low birth weight has been confirmed as strong predictor and a contributor to these conditions. Other large cohorts of undernutrition including Helsinki (Eriksson et al., 2001) and the Dutch winter famine (Roseboom et al., 2001, 2011) have subsequently been associated with low birth weight. The field of developmental programming has arisen to investigate other
maternal stressors during pregnancy including glucocorticoid (stress), hypoxia, obesity, and diabetes, and their effects on fetal organ development. However, despite this increase in focus around other maternal perturbations, alcohol is still relatively unexplored for effects on the developing embryo, placentation and adult disease outcomes in different developmental windows.

1.3 Influence of maternal alcohol consumption on fetal programming

1.3.1 Prevalence of maternal alcohol consumption

Despite National Health and Medical Research (NHMRC) guidelines advocating abstinence, 47% of women continue to drink alcohol during pregnancy in Australia (NHMRC, 2009, Wallace et al., 2007). Alcohol consumption is more prevalent in the United States where a recent study showed that nearly 75% of women who were planning a pregnancy drank alcohol (Green et al., 2016). Current demographic analysis suggests that women who drink moderate-high levels during pregnancy are primarily from high income households with tertiary education (Muggli et al., 2016, Jansen et al., 2009).

One critical window in which women commonly drink alcohol is the periconception period. A population study from Australia showed that approximately 14% of women drank more than 5 standard drinks in 1 day of the 3 months prior to pregnancy (Colvin et al., 2007). A more recent study from Australia of 1570 participants showed that these rates have risen to 39% (Muggli et al., 2016). This same study also analysed the period prior to pregnancy recognition, in which 12% of women admitted to binge drinking regularly, with an additional 6% admitting to binge drinking on one occasion (Muggli et al., 2016). In total over the periconception period, 27% of women drank alcohol, with 87% ceasing alcohol consumption after pregnancy recognition (Muggli et al., 2016). This cessation after pregnancy recognition is a common finding (Parackal et al., 2013, Alvik et al., 2006), and is likely to be contributed by unplanned pregnancies, which are approximately 50% (Colvin et al., 2007, Finer and Zolna, 2016). This data then suggests that the early pre-implantation embryo may be highly exposed to alcohol during this periconceptional period.
1.3.2 Alcohol – another exposure capable of programming

Alcohol consumption during pregnancy is often associated with conditions such as fetal alcohol syndrome (FAS) and fetal alcohol spectrum disorders (FASD) (Jones and Smith, 1973). These disorders are the result of high dose alcohol exposure throughout gestation, and are mainly associated with deficits in the offspring’s brain and behaviour, as these are the most teratogenic and life-altering outcomes, reviewed by (Riley et al., 2011). Few studies however, investigate other postnatal outcomes that may be ‘programmed,’ including cardiovascular disease, hypertension or diabetes. Whilst the focus of this study is not to mimic the conditions of FAS or FASD, we acknowledge that outcomes in response to a periconceptional insult may contribute to pathogenesis of these conditions.

Like many different environmental exposure, maternal alcohol consumption can also result in low birth weight (Manning and Eugene Hoyme, 2007, O’Leary et al., 2009, Tai et al., 2016). Another study showed that even long-term alcohol exposure prior to conception could lead to low birth weight (Livy et al., 2004). Fetal abnormalities are also more prevalent, with alcohol shown to increase cardiac malformations (Kvigne et al., 2004) as well as kidney defects (Taylor et al., 1994). High doses of alcohol are also associated with reduced placental weight (Kaminski et al., 1978), preterm birth (O’Leary et al., 2009, Tai et al., 2016), still birth (Kaminski et al., 1978, Marbury et al., 1983), preeclampsia (Meyer-Leu et al., 2011, Salihu et al., 2011), placental abruption (Salihu et al., 2011) and miscarriage (Maconochie et al., 2007, Avalos et al., 2014). A recent human FAS study has also highlighted the role of the placenta in mediating these outcomes, which include defects to utero-placental perfusion (Tai et al., 2016).

In order to appreciate the potential effects of alcohol consumption around conception on early embryonic development, it is first necessary to review pre-implantation development.

1.4 Pre-implantation development: a summary

As the oocyte is released from the ovary, surrounded by its glycoprotein shell - the zona pellucida, it is swept up by the fimbrial projections of the infundibulum of the oviduct. Fertilisation occurs within the oviduct, with proteins on the zona pellucida acting as sperm receptors, to facilitate sperm entry (Wassarman and Mortillo, 1991).
Sperm entry activates the oocyte to complete meiosis II and begin mitosis (Maro et al., 1986). The first mitotic cell division results in the formation of two cells or blastomeres approximately 18-20 h following gamete fusion (Hogan, 1994). Maternal mRNAs are utilised for genetic control of growth and maturation of the oocyte, as well as co-ordination of the first cell cleavage until this point. A transition then occurs from maternal to embryonic genome control and the zygotic genome is activated following a wave of maternal mRNA degradation initiated during the first cell cycle. Embryonic genome activation (EGA) occurs by the 2-cell stage in mice and rats (Flach et al., 1982, Schultz, 1993), and the 4-cell stage in humans (Braude et al., 1988). Without this critical event, the embryo fails to develop further (Poueymirou and Schultz, 1990).

The embryo continues to divide within the zona pellucida whilst travelling along the oviduct towards the uterus. At the 32-cell stage, outer cells of the embryo become polarised and develop cell-cell junctions, giving them an epithelial-like character (Ducibella and Anderson, 1975, Johnson et al., 1986, McLaren and Smith, 1977, Schlafke and Enders, 1967). This first differentiative event produces two primary cell lineages within the mature blastocyst. The polarised epithelial-like sphere of outer cells called the trophectoderm (TE) assists in implantation of the blastocyst into the uterine wall and gives rise to the extra-embryonic tissues including the placenta. Conversely, the inner cell mass (ICM), located eccentrically within the blastocoel (fluid-filled cavity), gives rise to the embryo proper (Watson and Kidder, 1988). This differentiation is also orchestrated by distinct patterns of gene expression, with the TE expressing transcription factors Cdx2 (Beck et al., 1995), and the ICM expressing Oct3/4 (Palmieri et al., 1994). The mature blastocyst is formed by approximately embryonic day (E) 3 in the mouse, E4 in the rat, and E5 in humans (Brinster, 1963, Schlafke and Enders, 1967, Steptoe et al., 1971). It is interesting that while the gestation of rodents is quite short (18-21 days), compared to humans (approximately 9 months), the timing of pre-implantation development is relatively conserved. This emphasises the importance of these developmental events in establishing a viable fetus.

1.5 Environmental regulation of pre-implantation development

The oviduct and uterus provide a unique microfluidal environment necessary for embryonic growth through the secretion of nutrients, macromolecules (e.g. amino acids), electrolytes, hormones, growth factors, anti-oxidants and regulated pH,
reviewed by Leese et al. (2008). The complexity and significance of the oviductal/uterine microfluid environment is highlighted by experiments that demonstrated the in vitro culture of embryos in defined media resulted in reduced embryo proliferation and elevated apoptosis, relative to those grown in vivo, indicative of a stress response (Bowman and McLaren, 1970, Hardy, 1997).

Autocrine, paracrine and endocrine signals between the embryonic cell populations and the maternal environment regulate optimal growth and survival of the pre-implantation embryo prior to placental formation. Glucose (Pantaleon et al., 2010), insulin (Heyner et al., 1989), insulin-like growth factor (IGF)1 (Mattson et al., 1988) and IGF2 (Rappolee et al., 1992), are produced by the oviduct and uterus, and when coupled with appropriate nutrient signalling, act as mitogenic pro-survival factors to optimise pre-implantation embryo growth (to both the TE and the ICM) and further development (Harvey and Kaye, 1988, Murphy and Ghahary, 1990).

Maternal dietary perturbations during the periconception period, such as a low protein diet, have been shown to cause mild transient maternal hyperglycaemia and hypoinsulinemia (Kwong et al., 2000). This metabolic phenotype is a likely mechanism for the observed reductions in TE and ICM cell proliferation that accompany this change in maternal conditions. Alterations to either these lineages have significant implications for progenitor pools of the fetus or the placenta. Reductions in ICM number are suggested to result in reduced birth weight (Eckert et al., 2012), while alterations in the TE are also likely to mediate aberrant fetal growth indirectly through altered placental development. Nutrient and substrate modifications can thus have long lasting effects on cell metabolism, growth and ultimately, developmental competence. These changes not only impact development, but may also lead to disease in adulthood.

1.6 Uterine responses required for implantation

Embryo-uterine interactions are vital to co-ordinate implantation and establish appropriate interactions required for placentation and subsequent fetal growth. The uterine lumen provides a unique fluidal environment of secreted hormones, cytokines, substrates and growth factors, to facilitate blastocyst developmental competence, and create an epithelial surface to permit adhesion and attachment (Kennedy, 1980, Paria
et al., 1993). Alterations may occur to either the embryo from a periconception perturbation as discussed above, or may occur through alterations to the maternal uterine environment.

### 1.6.1 Acquisition of receptivity and decidual response

The process of implantation of the blastocyst into the uterine endometrium requires both the activation of the blastocyst and uterine “receptivity”. For the most part, the endometrium is not in a “receptive” state and does not allow embryo implantation. However, this changes in a spatiotemporal manner through a cascade of hormone-dependent changes to prime the uterus (Huet-Hudson et al., 1989, McLaren, 1971). In the mouse and rat, an estrogen surge at ovulation primes the uterine luminal epithelium to proliferate through FGF signalling (Quarmby and Korach, 1984, Li et al., 2011). Following, low levels of estrogen coupled with rising progesterone inhibits proliferation of the luminal epithelium (Finn and Martin, 1970, Finn and McLaren, 1967, Martin and Finn, 1968, Psychoyos, 1973), and switches to stromal proliferation (Huet-Hudson et al., 1989, Paria et al., 1993). A nidatory estrogen surge on E3 (mouse) and E4 (rat) sensitises the progesterone-primed uterus to secrete the cytokine leukemia inhibitory factor (LIF) by the uterine glands, which then aids in communication with the blastocyst to indicate the onset of receptivity and causes its activation (Bhatt et al., 1991, Song et al., 2000). The rise in progesterone also transforms the uterine epithelium in anticipation of the embryo, and primes the uterine stroma for decidualisation (Huet-Hudson and Dey, 1990). The beginning of nidation also correlates with the onset of receptivity at E4 (mouse) E5 (rat), lasting for approximately 24 hours (Psychoyos, 1973). This optimal time window permits embryo implantation, and afterwards transitions to a “refractory” uterine environment hostile to embryo survival within 48 hours (Paria et al., 1993, Psychoyos, 1973).

The physical interaction of the embryo with the uterine epithelium stimulates the underlying stromal cells to undergo “decidualisation”, a transformation into cuboidal, polyploid decidual cells (Finn and Martin, 1967, Psychoyos, 1973). These decidual cells undergo extensive proliferation and contribute to the maternal portion of the future placenta. Decidualisation also provides another means of cross-talk with the embryo as these cells secrete hormones, metabolites and growth factors which facilitate embryonic nutrient requirements prior to placentation, and adapt embryonic
development to ensure its survival (Dey et al., 2004, Huet-Hudson et al., 1989). One major secreted factor by the decidua is \textit{lgfbp1}, which aids in modulating IGF1 and IGFII bio-availability to the embryo, aiding in its growth and differentiation (Irving and Lala, 1995). Prolactin (\textit{Prl}) is another important secretory product of decidual cells which increases steadily after implantation (Wu et al., 1995). Here it is thought to stimulate endometrial gland secretions over the time of implantation to modulate the degree of trophoblast invasion into the endometrium (Yu-Lee, 1997). Alterations in both of these secretory products have been shown to alter trophoblast-uterine interactions, and lead to altered placental and fetal growth (Crossey et al., 2002).

Following blastocyst adhesion and just prior to decidualisation, the uterus undergoes a hormone-dependent and transient local oedema at the implantation site to increase microvascular permeability (Psychoyos, 1973, Rockwell et al., 2002). This further facilitates blastocyst implantation through angiogenesis of microvessels which connect the maternal blood supply of the implantation site to the developing conceptus. As implantation is initiated, a vast vascular network begins to be established which is required for placentation. Therefore, a shift or impairment of the uterine window of receptivity, decidualisation or alterations in the initiation of blastocyst invasion, may have deleterious downstream consequences for embryonic viability or placental development. Alterations to the maternal environment such as through consumption of a high fat (Whyte et al., 2007) or a low protein diet (Fernandez-Twinn et al., 2003) during gestation can alter hormone profiles, which may subsequently affect these events and may result in implantation failure (Ma et al., 2003). Although not investigated in pre-implantation perturbations, alterations to stromal decidualisation (Petit et al., 2007), secretion of decidual cell products (Crossey et al., 2002) or decidual-associated angiogenesis (Winterhager et al., 2013) have all been shown to affect downstream placental development. It is currently unknown whether alcohol consumption affects these uterine events in early pregnancy.

1.7 Formation of the placenta

Healthy maternal nutrition and normal placental development are crucial in the development of a viable fetus. The placenta provides a surface area for the exchange of nutrients and waste products between fetal and maternal blood supplies and is a key determinant of intrauterine growth. While the human placenta is largely different
to the rodent placenta a number of parallels and analogous structures can be drawn. Although this is not a major focus of this thesis, these differences are briefly mentioned in sections 1.5.1 primary differentiation of the parietal trophoblast giant cells, 1.5.3; spiral artery remodelling – establishing blood supply to the conceptus and 1.5.5; labyrinth formation. Despite these limitations, animal models including the rodent allow analysis of maternal perturbations without ethical concerns. This is particularly important for periconceptional models, as we will demonstrate, requires ontogeny analysis throughout gestation to examine placental outcomes.

The gross structure of the mature rodent placenta is essentially composed of three distinct layers. First is the maternal decidua which contains maternally derived uterine decidual cells, infiltrating immune cells and is the site of spiral arteries that supply the implantation chamber with maternal blood. The middle junctional zone consists of migratory trophoblast giant cells (TGCs) that interact with maternal spiral arteries to mediate blood flow, invasive glycogen trophoblasts (GlyT), and spongiotrophoblast (SpT) cells, which are thought to have structural and endocrine roles (Coan et al., 2004). Lastly, the inner labyrinth layer is the site of maternal-fetal exchange, and consists of trophoblast lined maternal sinusoids which are separated from the endothelial-lined fetal vasculature by two transporting syncytiotrophoblast (SynT) layers. Together these four cell layers make up the “interhaemal membrane” that facilitates the selective two-way exchange between the two blood compartments. The trophoblast cells of the placenta are derived from the TE of the blastocyst while the fetal endothelial cells lining the fetal circulation are derived from the ICM (Coan et al., 2004, Simmons and Cross, 2005).

1.7.1 Primary differentiation of the parietal trophoblast giant cells

As aforementioned, the placental lineages are largely derived from the TE. With the exception of the mural TE, which differentiates into primary parietal (P-) TGCs to line the implantation chamber and yolk sac and facilitate early implantation, the majority of cell types in the definitive placenta are derived from the proximal TE, cells which overlie the ICM (Cross et al., 1994). These P-TGCs are functionally analogous to the primary syncytium formed in humans (Knofler and Pollheimer, 2013). The P-TGCs are the first trophoblasts to differentiate (Cross et al., 1994), and like all TGCs become large polyploid cells, and with differentiation, increase their golgi apparatus and
endoplasmic reticulum (Bevilacqua and Abrahamsohn, 1988). Upon the onset of differentiation, they also secrete proteinases, cytokines, hormones and extracellular matrix to facilitate invasion (Cross et al., 1994).

The TGCs achieve an increase in nuclear ploidy through a specialised cell cycle termed endoreplication, which use continuing rounds of DNA replication without mitosis or cell division, and can obtain up to 1000 copies of DNA in the one cell (Zybina and Zybina, 1996), see Figure 1.1. Endoreplication is likely to be initiated through the Cdk inhibitor P57\textsuperscript{Kip2}, which prevents cell cycle entry into M phase (Ullah et al., 2008), as well as a decrease, and subsequent removal of mitotic cell cycle check points of G1 (p53 and Rb), and G2 (Snail) (Nakayama et al., 1998). A thorough analysis of TGC subtypes and the endocycle is reviewed by Hu and Cross. (2010).

![Diagram](https://via.placeholder.com/150)

**Figure 1.1. Trophoblast giant cell differentiation causes transition from mitotic cell cycle to the endocycle to increase ploidy.** M; mitosis, G1 checkpoint, S; DNA replication, G2 checkpoint. E; endocycle. Adapted from Hu and Cross (2010).

The endocycle of the TGCs also causes over-replication of selective parts of the TGC genome (Hannibal and Baker, 2016). Interestingly, these include expression of prolatin-like hormones, cathepsins (cysteine proteases), serpins (protease inhibitors), and genes in the NK/CLEC complex, involved with infiltrating maternal decidual natural killer cells (Hannibal and Baker, 2016). In response to stress, cells may enter into
endocycling to prevent apoptosis by altering cell cycle checkpoints (Mehrotra et al., 2008). For example, the aforementioned G1 check point p53, a tumour suppressor protein, regulates G1 arrest during genotoxic damage (Sherr, 2000). This may have long term consequences for pre-implantation exposures, such as shown by Yang et al. (2016), which demonstrate that hypoxic stress in culture to cause irreversible trophoblast differentiation despite application of FGF4 (promoting TS state), with 50% of trophoblasts examined being differentiated TGCs.

Early developmental perturbations such as in vitro fertilisation (Tan et al., 2016a, 2016b) which are known to alter TE differentiation may have the potential to form these invasive P-TGCs. Maintenance of the trophoblast stem cells is critical, as either excess or reduced P-TGC formation can lead to aberrant invasion into the uterine decidua, altered blood flow and implantation failure (Tompers et al., 2005, reviewed by Hu and Cross. 2010). This is particularly important in humans, as the human embryo is suggested to be highly susceptible to implantation failure, which is currently estimated between 30-45% of all pregnancies (Wilcox et al., 1988, Norwitz et al., 2001). In addition, early miscarriages have been associated with alcohol maternal exposure (Andersen et al., 2012, Avalos et al., 2014), low maternal body mass index, increased maternal or paternal age, stress, assisted conception, or change of partner (Maconochie et al., 2007). This highlights the importance of the P-TGCs in establishing the pregnancy. However, it is currently unknown how mild alterations in the P-TGCs may affect long-term placentation.

1.7.2 Trophoblast differentiation and lineage segregation

The proximal TE differentiates into two early structures of the placenta the EPC and the chorion. The EPC contributes to junctional zone trophoblast subtypes while the chorion gives rise to the labyrinthine layers (Coan et al., 2004). By E12.5 in the mouse and E14.5 in the rat, trophoblast subtypes are terminally differentiated, the structure of the definitive placenta is laid down and the onset of placental function is evident (Simmons, 2014). Quantification of these mature trophoblasts is becoming a useful tool in establishing their functions in the placenta, and the mechanisms behind placental perturbations (Jauniaux et al., 2001). For a summary of all the placental trophoblast subtypes and genetic markers, see Figure 1.2.
Figure 1.2. Trophoblast lineages that give rise to the placental and decidual zones. Markers of trophoblast subtypes are shown in red. Parietal (P-) trophoblast giant cells (TGCs) are derived from both the mural TE (primary TGCs) during early post-implantation development, as well as from the ecto-placental cone (EPC) in mid-gestation (secondary TGCs). C-TGCs; canal-associated TGCs. EXE; extra-embryonic ectoderm. ICM; inner cell mass. GlyT; glycogen trophoblasts. S-TGC; sinusoidal TGCs. SpA TGCs; spiral artery associated TGCs. SpT; spongiotrophoblast. Syn-T1/2; syncytiotrophoblast layer 1/2 cells. TE; trophectoderm. Adapted from Simmons and Cross, 2005, Simmons et al., 2007, Simmons, 2014.
1.7.3 Spiral artery remodelling – establishing blood supply to the conceptus

After the first invasive event by primary TGCs, one junctional zone-derived trophoblast subtype - the spiral artery trophoblast giant cells (SpA-TGCs), invade from the periphery of the EPC into the decidua at E10 (rodent), and with the cooperation of supporting maternal decidual cells and decidual natural killer (dNK) cells, remodel the spiral arteries into high flow, low-resistance vessels more suited to deliver high blood volume required for placental function in later gestation (Guimond et al., 1997, Chakraborty et al., 2011). These invasive SpA-TGCs are analogous in the human to extravillous trophoblasts, which through endovascular invasion, and interactions with dNK cells, similarly remodel the spiral arteries (reviewed by Kaufmann et al. 2003). This process is of particular importance in humans, as alterations in either the dNK cells or trophoblast invasions, and thus spiral artery remodelling and ongoing placental perfusion, has been associated with pregnancy complications including low birth weight and preeclampsia (Wallace et al., 2012, Ball et al., 2006). Animal models of excess oedema (Lu et al., 2013) and obesity (Hayes et al., 2013), have also associated alterations to either dNK or SpA-TGC invasion with improper spiral artery remodelling, reviewed by Croy et al. (2000). Although periconceptional insults are currently not investigated for aberrant spiral artery remodelling, considering the high expression of dNK cell-associated pathways by the P-TGCs, perturbation to P-TGC differentiation is one mechanism of programming which may have long term consequences for placentation.

1.7.4 Junctional zone formation

The junctional zone consists of mostly SpT cells, with the addition of small populations of TGC subtypes which line the maternal circulation entering the implantation site (spiral arteries) to the venous return (Simmons, 2014). Additionally, the EPC contains progenitors for another junctional zone trophoblast cell type, the glycogen trophoblasts (GlyT), analogous to the human interstitial extravillous trophoblast. GlyTs are a poorly characterised cell type which are vacuolated and accumulate glycogen (Coan et al., 2006). By E14.5, the intermediate SpT layer can be seen projecting into the labyrinth, defining and supporting the structure of this inner vascular layer (Tanaka et al., 1997). A vast expansion of GlyT cells occurs in mid-gestation and they begin migrating out of
the SpT layer into the maternal decidua through interstitial invasion (Adamson et al., 2002, Coan et al., 2004, 2006). By E16.5-18.5, their numbers halve (Coan et al., 2004), and are thought to act as late gestational energy source through the production of IGF2 and the breakdown of glycogen to glucose to facilitate fetal growth just prior to birth (Redline et al., 1993). Glycogen accumulation can appear in TE cells when grown in culture (Edirisinghe et al., 1984), EPC cells (Tesser et al., 2010), as well as in mature placenta after maternal perturbations (Barash et al., 1983, 1986, Gardebjer et al., 2014), which may be a stress response or adaptive mechanism to maximise fetal survival.

The junctional zone trophoblast subtypes, are relatively uncharacterised in function. However, all these cell types produce prolactin-like protein hormones, which may suggest a role in communication with the maternal physiology during pregnancy and regulation of fetal growth (Cross et al., 2002, Wiemers et al., 2003). This is supported by rodent models which show reduced area of the junctional zone to be associated with fetal growth restriction (Mark et al., 2011). Few studies however, have explored the mechanisms behind altered junctional zone function, such as through altered cell allocation from EPC progenitors, development of its trophoblast subtypes, or migration of the invasive subtypes to the maternal vasculature where they can influence maternal physiology and fetal growth.

1.7.5 Labyrinth formation

Labyrinth development requires both fetal and trophoblast-derived cell types to interact for formation of the villous tree and the interhaemal membrane, required for protection of the growing fetus and a mode for nutrient exchange (Sibley et al., 1997). The labyrinthine zone of the placenta begins to form mid-gestation (E7.5 mouse), with the development and attachment of the chorionic trophoblasts with the fetally-derived allantoic mesoderm (Watson and Cross, 2005). Chorionic trophoblasts differentiate into lineage restricted multinucleated syncytiotrophoblast cells which compose syncytiotrophoblast layers SynT-I and -II. Another perforated layer exists above these consisting of cuboidal-like mononuclear sinusoidal trophoblast giant cells (S-TGCs), currently of an unknown origin, which line the maternal blood sinusoids where they may have an endocrine role (Simmons et al., 2007, Simmons et al., 2008a). As the chorion and allantois begin to fold, they form primary villi which later extend into a vast
vascular tree via branching morphogenesis. Expression of the transcription factor Gcm1 begins at E7.5, marks future branching initiation sites along the leading edge of the flat chorionic disc. Later Gcm1 is confined to the branch tips, allowing it to be used as a marker for the degree of branching morphogenesis (Anson-Cartwright et al., 20000). Gcm1 can also be used as a marker of SynT-II cells, as it drives the expression of Synb which mediates the fusion of trophoblast cells into the SynT-II layer. This process consequently causes the up-regulation of Syna in cells at the distal edge of the chorion, which fuse into the SynT-I layer (Dupressoir et al., 2005, 2009). By E8.0, TS cell markers are down-regulated upon differentiation into labyrinthine trophoblasts (Chawengsaksophak et al., 1997, Luo et al., 1997). During this process, the fetal mesoderm also begins to differentiate into endothelial blood vessels, required to establish the fetal portion of the circulation. These endothelial cells then interdigitate with the maternal blood space for the exchange of nutrients, supplied by decidual sinuses and spiral arteries (Cross, 2000, Rossant and Cross, 2001). Involution of the chorionic S-TGCs, SynT-I and –II layers towards the maternal sinuses at E9.5, followed by ingrowth of the developing endothelial-lined fetal circulation, produces a barrier between the fetal and maternal blood spaces – the interhaemal membrane (Enders, 1965, Hernandez-Verdun, 1974, Simmons et al., 2008a). This is somewhat different to the human placenta which contains fetal endothelial cells, and one layer of syncytiotrophoblasts and a stem cell population that replenishes this outer layer; the cytotrophoblasts (Rossant and Cross, 2001).

Passive diffusion of the placenta plays a large part in facilitating growth of the fetus, so the surface area (fetal blood vessel density), permeability and distance of perfusion through the interhaemal membrane are critical in determining the capacity for nutrient transport (Sibley et al., 2004). Measurement of interhaemal membrane thickness and labyrinth surface area are thus used to assess this diffusion capacity (Hernandez-Verdun, 1974). Altered placental perfusion and consequent fetal grow can be caused by perturbations to chorionic patterning through allantoic budding and extension to reach the chorionic surface, trophoblast differentiation, chorioallantoic adhesion and attachment, subsequent branching morphogenesis (vascularisation), can all affect the labyrinthine architecture and the permeability of interhaemal membrane, and therefore the transport capacity of the placenta (Watson and Cross, 2005). In most extreme
cases, such as through inhibited allantoic budding, labyrinth development is arrested, becoming embryonic lethal by E9.5-10.5 (Zhadanov et al., 1999).

1.8 Potential placental perturbations from a periconceptional model

Perturbations may occur to numerous aspects of placental development to affect fetal growth and result in programming. If the growth trajectory of the placenta is unsynchronised with that of the fetus, it cannot meet the fetus’ required nutritional demands for rapid growth. There are many critical windows during development that can impact placentation, see Figure 1.3. However, a periconceptional insult may be able to affect all of these critical windows through programming of TE, epigenetic alterations, or through improper communication with the maternal environment, required for placental development. These changes may contribute to the development of:

- altered cell allocation to the TE, affecting the progenitor pool to the placenta and subsequent trophoblast lineages (Gundogan et al., 2013),
- poor trophoblast invasion into the decidua, impairing spiral artery transformation required for placental perfusion (Pijnenborg, 1994, Genbacev et al., 1996),
- aberrant formation and vascularisation of the labyrinthine villous tree which may affect the surface area for nutrient exchange (Anson-Cartwright et al., 2000), and finally,
- perturbed development the interhaemal membrane, or altered expression of its nutrient transporters (Sibley et al., 2004), which may both impair the transport capacity of the placenta.

Questions now arise to what aspects of placental formation are most susceptible to modification after a periconceptional perturbation, and do these modifications occur in a sexually dimorphic manner.
<table>
<thead>
<tr>
<th>Ovulation</th>
<th>Trophoblast differentiation</th>
<th>Definitive placenta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocyte maturation</td>
<td>Blastocyst, Invasive P-TGCs, Implantation, Chorioallantoic attachment</td>
<td>SpA-TGC invasion, GlyT invasion, Labyrinthine branching</td>
</tr>
<tr>
<td>Fertilisation</td>
<td>E(-)3.5</td>
<td>E3.5, E5.5, E8.5</td>
</tr>
</tbody>
</table>

Note: Figure 1.3 from Kalisch-Smith et al. (2017)
**Figure 1.3. Critical windows during placental development which are susceptible to perturbation.** The developmental timeline of mouse placentation is presented, with formation in the rat requiring an additional +1 day at the blastocyst stage, and +2 days post-implantation, with birth taking place at approximately day 22. All critical windows presented can alter placental growth and result in altered fetal development. Early exposures, prior to implantation, can alter development of the trophectoderm (TE), and may alter differentiation of the TE to form parietal trophoblast giant cells (P-TGCs), required to facilitate implantation into the maternal uterine epithelium. These may also alter formation of the ecto-placental cone (EPC) and chorion, progenitors to the two major placental compartments – the junctional zone and the labyrinth. Invasion of the spiral artery TGCs (SpA-TGCs – red) via endovascular invasion and glycogen trophoblasts (GlyT – black) by interstitial invasion are also rarely investigated, which have the potential to alter spiral artery remodelling, blood flow, or nutrient release.
1.9 Influence of genetic sex on placental programming

Sexual dimorphism arises in the timing and severity of disease outcomes, with males often displaying earlier onset and more severe disease than females (Barker, 1995). This can be traced back to the *in utero* environment, where male fetuses are more likely to be born preterm, experience neonatal complications or die *in utero* (Stevenson et al., 2000, Walker et al., 2012). This has led to the common view that males are more susceptible to developmental perturbations than females (Clifton, 2010). However, recent evidence suggests that female fetuses may be more affected by disorders during pregnancy such as maternal hypertension, preeclampsia, villous infarction, and in some studies, preterm birth (Walker et al., 2012, Shiozaki, et al. 2011, Vatten and Skjaerven, 2004, Verburg et al., 2016). These sexually dimorphic pregnancy outcomes make it essential that placental phenotypes be characterised in a sex-specific manner to allow further understanding into the mechanisms involved.

1.9.1 Epidemiological evidence of sexually dimorphic programming

Clinically, investigation of sexual dimorphism in the placenta is usually limited to analysis of the placenta at delivery and includes measurement of weight and gross placental structure. Recent studies have demonstrated the human term placenta shows sex-specific differences expression profiles for genes regulating cell proliferation, hormone function, maintenance of pregnancy and immune tolerance (Buckberry et al., 2014, Verburg et al., 2016). Males often have heavier placentas than females in uncomplicated pregnancies (Eriksson et al., 2010). Indeed, a recent study of nearly 80,000 deliveries in Japan demonstrated that males had a heavier placenta at every gestational age examined (week 24 to week 41) (Ogawa et al., 2016). Boys also show greater growth *in utero* than girls at any placental weight, suggesting that the male placenta is more efficient than the female (Eriksson et al., 2010). However, male fetuses are suggested to be at greater risk when nutrients are limited (Clifton, 2010, van Abeelen et al., 2011). For example, babies exposed *in utero* to the Dutch winter famine have shown sex specific adaptations in placental growth associated with offspring disease: in males, an oval placenta was associated with hypertension in men whilst in females, hypertension correlated with a small placental area (van Abeelen et al., 2011, Roseboom et al., 2011). Asymmetric growth of the placenta, therefore altering its shape, may perturb maternal blood flow and therefore placental perfusion.
(Kajantie et al., 2010), which may also affect the development of the placenta-heart axis (Thornburg et al., 2010). This is also suspected to be a result of altered implantation and/or spiral artery remodelling, affecting placental formation from very early in pregnancy (Thornburg et al., 2010). Restricted placental growth is most likely to affect nutrient exchange later in development during peak nutrient transfer, leading to a smaller fetus (Barker, 1995) and therefore programming of cardiovascular system by a different mechanism.

Examination of placental development and function in animal models is becoming increasingly important for understanding the mechanisms of developmental programming. Perturbations in animal models during discrete periods of development result in sex–specific alterations in placental structure in late gestation, and can be associated with fetal growth restriction (Table 1.1). Like the human, the majority of studies have investigated placentation late in gestation. Considering many of these maternal perturbations are applied from as early as maturation of the oocyte through to late gestation (see Figure 1.3), there remains an expanse of placental development relatively unexplored, especially in the context of sexual dimorphism. Characterisation of phenotypes during early placental development would allow further understanding into sex-specific embryonic viability, sex ratios at implantation, and stage specific malformations.
<table>
<thead>
<tr>
<th>Model</th>
<th>Species</th>
<th>Gestational Age at study</th>
<th>Placenta Weight (PW), Body Weight (BW), PW:BW Ratio</th>
<th>Structural Alterations / Pathologies in the Placenta</th>
<th>Resorptions (Res) and viability</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-Implantation Exposure</strong></td>
<td></td>
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</tr>
<tr>
<td><em>In vitro fertilisation, embryo transfer</em></td>
<td>Mouse</td>
<td>E7.5, E13.5, E19.5</td>
<td>$\leftrightarrow$ PW, $\downarrow$ BW (E13.5), $\uparrow$ PW:BW in males (E13.5, E19.5), $\uparrow$ PW (E19.5), $\leftrightarrow$ BW E19.5</td>
<td>$\uparrow$ Abnormal EPC (delayed, $\downarrow$ Volume, disorganised, E7.5 females $\downarrow$ LAB:PW area and vessel density (E13.5)</td>
<td>$\uparrow$ Res (19%) E13.5 $\uparrow$ Male:female ratio E13.5 and E19</td>
<td>(Tan et al., 2016a, 2016b)</td>
</tr>
<tr>
<td><strong>Periconceptional Exposure</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Ethanol, 12.5% v/v liquid diet, E-4-E4,</strong></td>
<td>Rat</td>
<td>E20</td>
<td>$\leftrightarrow$ PW, $\downarrow$ BW: males (8%), females (7%), $\uparrow$ PW:BW ratio (8%)</td>
<td>$\uparrow$ JZ and $\downarrow$ LAB area ratio in both sexes.</td>
<td>$\uparrow$ Res (7%)</td>
<td>(Gardebjør et al., 2014)</td>
</tr>
<tr>
<td><strong>Mid-Late Gestation Exposures</strong></td>
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<tr>
<td><strong>Hypoxia, 12% $O_2$, E14.5-E18.5</strong></td>
<td>Mouse</td>
<td>E18.5</td>
<td>$\leftrightarrow$ PW or PW:BW ratio: Males (7%), females (7%) $\downarrow$ BW: Males (7%), females (7%) $\downarrow$ LAB blood spaces in females</td>
<td>$\leftrightarrow$ Litter size</td>
<td></td>
<td>(Cuffe et al., 2014a)</td>
</tr>
<tr>
<td><strong>Corticosterone, 33ug/kg/h, E12.5 – E15</strong></td>
<td>Mouse</td>
<td>E14.5, E17.5</td>
<td>$\uparrow$ Placental length, depth (E14.5), $\leftrightarrow$ PW, BW or PW:BW (E14.5), $\uparrow$ PW and PW:BW in males (E17.5)</td>
<td>$\uparrow$ JZ volume in males (24%), $\downarrow$ LAB:JZ in males (E14.5)</td>
<td>$\leftrightarrow$ Res $\leftrightarrow$ Litter size</td>
<td>(Cuffe et al., 2012)</td>
</tr>
<tr>
<td><strong>Dexamethasone, 0.02 mg/kg/day, E12.5-E15</strong></td>
<td>Mouse</td>
<td>E14.5, E17.5</td>
<td>$\downarrow$ PW in females (19%, E14.5) $\downarrow$ BW: Male (15%), females (20%, E14.5) $\leftrightarrow$ PW:BW (E14.5) $\leftrightarrow$ PW, BW or PW:BW (E17.5)</td>
<td>$\downarrow$ Whole placental and JZ area.</td>
<td>Litter size N.D</td>
<td>(Cuffe et al., 2011)</td>
</tr>
<tr>
<td><strong>Dexamethasone, 125 ug/kg, E20-E23</strong></td>
<td>Spiny Mouse</td>
<td>E23, E37</td>
<td>$\leftrightarrow$ PW, BW or PW:BW (23) $\leftrightarrow$ BW, PW (E37)</td>
<td>$\uparrow$ JZ area and $\downarrow$ LAB:JZ at E23.</td>
<td>$\leftrightarrow$ Res Litter size N.D.</td>
<td>O’Connell et al., 2011 2013b,c</td>
</tr>
</tbody>
</table>

Note: Selected paragraphs for section 1.7 from Kalisch-Smith et al. (2017)
<p>| Exposure                                                                 | Species | Week | PW: N.D. BW: ↔ Male, ↓ Female (12%) | ↓ Capillary volume in males | ↑ Females total length of peripheral villi and % trophoblast | ↓↓ Chronic deciduitis and velamentous umbilical cord insertion in males | ↑ Villous infarctions in females | ↓ Blood spaces ↑ males, ↓ females (E37) | Mortality N.D. | Deaths N.D. (Mayhew et al., 2008, Murphy et al., 2003) | Deaths N.D. (Walker et al., 2012) | Lesions of chronic inflammation of males N.D. (Ghidini and Salafia, 2005) | Res, litter size or sex ratio ↔ | Litter size ↔ | Res % ↔ Litter size ↔ | Res size S % S ↔ |</p>
<table>
<thead>
<tr>
<th>Magnesium deficiency, moderate (0.02%), severe (0.005%) w/w Mg²⁺, (-) 4 weeks – E18.5.</th>
<th>Mouse</th>
<th>E18.5</th>
<th>Moderate: ↓ BW males (5%), females (5%) Severe: ↓ BW males (10%), females (9%) ↔ PW or dimensions</th>
<th>↑ LAB area ↓ JZ area</th>
<th>70% Res</th>
<th>(Schlegel et al., 2015)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein restriction, 6% reduction, E1- E21</td>
<td>Rat</td>
<td>E14, E18, E21</td>
<td>↓ PW (E14, E18), ↔ PW E21 ↓ BW (E14, E18, E21) ↑ PW:BW (E14), ↓ PW:BW (E18, E21)</td>
<td>↓ LAB weight (E14, E18), ↔ LAB weight (E21) ↓ JZ weight (E14, E21), ↔ JZ weight (E18)</td>
<td>Litter size N.D.</td>
<td>(Gao et al., 2012)</td>
</tr>
<tr>
<td>High fat (HF)/ high salt (HS) / high fat + high salt (HFHS) HS (4% NaCl); HF (45% kcal from fat), HFHS (HF+HS). E-21-E18</td>
<td>Rat</td>
<td>E18</td>
<td>HS: ↓ PW male, ↔ female, ↔ BW male, ↓ HF: ↓ PW male and female, ↔ BW male, ↓ female, HFHS: ↓ PW male, ↔ female ↔ BW male or female</td>
<td>LAB (%) HS: ↓ male, ↔ female, HF: ↔ male/ female, HFHS: ↓ male/ female, JZ (%) HS: ↑ male, ↔ female, HF: ↔ male, ↓ female, HFHS: ↑ male, ↔ female. DEC (%) HS: ↔ male, ↑ female, HF: ↔ male, ↑ female, HFHS: ↔ male, ↑ female</td>
<td>↔Litter size</td>
<td>(Reynolds, et al. 2015)</td>
</tr>
</tbody>
</table>

Table 1.1. Sex-specific outcomes of placental and fetal growth in human and animal studies. BW; body weight, DEC; decidua, E; embryonic day, EPC; ecto-placental cone, IUGR; intrauterine growth restriction, HF; high fat, HFHS; high fat + high salt, HS; high salt, JZ; junctional zone, LAB; labyrinth, LF; low fat, N.D; not determined, PW; placental weight, PW:BW; placental weight:body weight ratio, Res; resorptions, w/w; weight per weight, v/v; volume per volume. Modifications to fetal body weight or placental parameters occur in both sexes unless otherwise stated.
1.9.2 Sexually dimorphic programming of the labyrinth

The placental vasculature is essential for providing nutrients to facilitate fetal growth, and as such, this may be the principle structure that is affected in response to an in utero perturbation. The vasculature can be estimated by measurement of capillary density in humans (Mayhew et al., 2008), while animal models determine total labyrinthine area, volume, or surface area, and within the labyrinth - the fetal and maternal blood spaces (Coan et al., 2004). Approximately half of the studies in Table 1.1 which investigated the placental vasculature in complicated human pregnancies and animal models showed that it was reduced in response to maternal perturbations. These outcomes were often distinctly sexually dimorphic; males had less well-developed vasculature in response to maternal asthma in women (Mayhew et al., 2008), or following mid-late gestational corticosterone exposure in the mouse (Cuffe, 2012). In females, reductions in the vasculature of the mouse placenta occurred in response to mid-late gestation hypoxia (Cuffe et al., 2014a), and dexamethasone exposure (O’Connell et al., 2013b). Curiously, male spiny mice exposed to mid-gestation dexamethasone (O’Connell et al., 2013b), showed increases in the placental vasculature, suggesting species variations. Reduced labyrinthine formation may act to temporarily reduce energy expenditure, but may come at a cost of ongoing placental and fetal growth. Alternatively, upregulation or maintenance of labyrinth growth may aid in maintaining nutrient transfer to help ensure fetal survival. Changes to the labyrinthine vasculature are also likely to contribute to the observed sex specific alterations in gene expression of glucose and nutrient transporters, glucocorticoid responsive genes, growth factors, as well as inflammatory responses, see reviews by Clifton (2010) and Rosenfeld (2015). Further research into sexual dimorphism of labyrinthine differentiation, vasculogenesis and branching morphogenesis, interhemal membrane thickness, and functional nutrient transport assays may shed light on sex differences in placental efficiency and adaptation to maternal perturbations.

1.9.3 Sexually dimorphic programming of the junctional zone

Evident in many programming models in Table 1.1 are models which associate an increase in junctional zone with a reduction of labyrinth. These include periconceptional alcohol exposure (Gardebjer, 2014), glucocorticoid exposure (Cuffe et al., 2012, O’Connell et al., 2011, 2013b, 2013c), maternal protein restriction (Gao
et al., 2012) and magnesium deficiency (Schlegel et al., 2015). In some cases, (e.g. following periconceptional alcohol), these changes are sex specific. The majority of models in which maternal nutrients are limited, span early development of the placenta and may affect the formation and maturation of the ecto-placental cone and the immature definitive placenta. A subset of these models has also investigated one specialised trophoblast subtype that is present in the rodent placenta – the glycogen trophoblast (GlyT). Excess glycogen has been shown to accumulate in the GlyT cells of the junctional zone in late gestation, most often in placentas from females (Gardebjer et al., 2014, Schlegel et al., 2015, O’Connell et al., 2011, 2013b, 2013c). GlyTs originate from a common progenitor to spiral-artery associated trophoblast giant cells (Mould et al., 2012). Thus, sexual dimorphism in differentiation of these trophoblasts during early placental development may also perturb spiral artery remodelling in a sex-specific manner. As differentiation of junctional zone trophoblast subtypes like GlyT are regulated by a number of imprinted genes (Lefebvre, 2012), it is possible these lineages are particularly sensitive to perturbations that affect epigenetic modifications or chromosome dosage compensation mechanisms. By examining earlier stages of development from the oocyte, to early embryo and immature placenta, we may gain a deeper understanding of the function of these cells and how they contribute to structural or metabolic adaptations that can lead to placental programming.

1.9.4 Evidence of sexual dimorphism in the early embryo

Current knowledge of sexual dimorphism on early developmental structure is restricted to the blastocyst stage, where some studies suggest males develop faster than females, reviewed by Gardner et al., 2010). This is largely based on assessment of blastocyst size or cell number, including allocation to either the TE or ICM (Tan et al., 2016b, Valdivia et al., 1993, Peippo and Bredbacka, 1995, Perez-Crespo et al., 2005). Outcomes from these studies are conflicting in a variety of human and animal models with some studies demonstrating sex specific differences in preimplantation embryos whilst others detect no differences (Holm et al., 1998, Dumoulin et al., 2005). For example, the growth rates of human preimplantation embryos were found to be sex dependent after intracytoplasmic sperm injection (ICSI) but not after IVF (Dumoulin et al., 2005). Reasons for this may include the fact these experiments are conducted in vitro (utilising different types of culture media), and in some cases, use superovulation
to generate large quantities of embryos. These conditions are both known stressors to pre-implantation development (Giritharan et al., 2007, de Waal et al., 2012), which may potentially confound the determination of inherent sex differences, and should thus be interpreted with caution.

A subset of these *in vitro* pre-implantation experiments has additionally determined differences between males and females in their physiology, genetic profiles, and metabolic responses to stress. Firstly, male embryos exhibit a higher metabolic rate than females (Tiffin et al., 1991), and in response to a heat stressor, exhibit greater oxidative stress responses (Perez-Crespo et al., 2005). Another IVF model showed successful pregnancies were associated with higher glucose consumption, with viable females exhibiting higher levels than males (Gardner, 2010). This dimorphism in glucose metabolism is contrary to that in placentation which suggests that males and females show the same glucose uptake in response to stress, indicating greater regulation during mid-late gestation (Cuffe, 2011, 2014a). Other studies suggest female blastocysts to be more susceptible to apoptosis prior implantation (Tan et al., 2016b, Ghys et al., 2015). Expression profiles of male and female blastocysts show sexual dimorphism of both X-linked and autosomal genes (Bermejo-Alvarez et al., 2010b). These can include DNA methyltransferases; *Dnmt3a/3b*, stress-induced enzyme; *Ogt*, glucose handling; *G6pd*, and apoptosis; *Xiap, Hprt1* (Bermejo-Alvarez et al., 2008, 2010b). Interestingly, these are common pathways which are also upregulated during prenatal perturbations.

We can also gain insight into early placental programming from IVF and ICSI conducted in assisted reproduction as well as animal models. A human study by Dumoulin et al. (2005) investigated both of these embryo procedures in a sexually dimorphic manner and showed that while IVF did not change cell number, ICSI increased TE cell number in males only (Dumoulin et al., 2005). This is an interesting finding as ICSI is well known for causing placentomegaly in rodents – a large expansion of the junctional zone with increased accumulation of GlyT5s (Tanaka et al., 2001), further illustrating that perturbation of the early embryo alters the communication with the normal *in utero* environment, leading to altered placental formation. This also has implications for human pregnancies, as both IVF and ICSI can lead to disorders including preterm birth, stillbirth, and low birth weight (Helmerhorst et al., 2004). While sexual dimorphism in placental disorders and other
pathologies have currently not been investigated in detail, more males are born after blastocyst transfer (Kaartinen et al., 2015), further supporting the idea that males and females have different susceptibilities to perturbations in utero during different critical windows. While sex-specific adult outcomes of human offspring produced by IVF or ICSI remains to be evaluated, mouse models show that only female offspring from IVF and ICSI pregnancies have increased rates of glucose clearance, increased insulin secretion, higher peak insulin, as well as increased fat deposition (Scott et al., 2010, Feuer et al., 2014b).

Mouse models using IVF have also shown reduced cell count in the early blastocyst in both TE and ICM lineages, reduced embryonic viability, and when allowed to develop into late gestation, have shown increased placental weight, labyrinth and junctional zone size, as well as the development of fetal growth restriction (Giritharan et al., 2007, Bloise et al., 2014, Delle Piane et al., 2010). While much of this information hasn’t investigated fetal sex, another mouse model has shown female IVF embryos develop abnormal ecto-placental cones, leading to skewing of the sex ratio from 50% male at implantation to 57% male at birth (Tan et al., 2016a). This indicates increased female mortality similar to that seen in humans. While both sexes developed reduced placental vasculogenesis later in gestation, males developed the most placental overgrowth (Tan et al., 2016b). This further illustrates that females in particular may be most susceptible to perturbations in the pre-implantation period, as they may be most susceptible to changes in TE lineage commitment, differentiation and invasion of the trophoblast giant cells, and therefore, their capacity to form a placenta.

Further evidence for female specific TS differentiation comes from a mouse mutant for Eed, an X-lined gene involved in the PRC2 complex (Wang et al., 2001). Wang et al. (2001) showed null female embryos decreased trophoblast giant cell number and size at E7.5-E8.5. Other mouse mutants have also begun to investigate sexually dimorphic phenotypes in extraembryonic trophoblast and the placenta, where they consider the influence of the maternal or paternal derived X chromosomes, and X chromosome inactivation. Some of these models include the ATP-dependent helicase Atrx (Garrick et al., 2006), DNA methyltransferase Dnmt1 (McGraw et al., 2013), X-linked G6pd (Longo et al., 2002), and stress response Ndro1 (Larkin et al., 2014). Curiously, it is again the females which seem to show the most deleterious phenotypes in these models, often showing reduced placental weight, labyrinth and/or junctional zones, as
well as embryonic viability. Males seem to develop similar pathologies, albeit at reduced severity. The mechanisms behind the overlap and interaction between *in utero* nutrient or stress perturbations and placental development, remains an exciting area of research. Sex-specific adaptations during early development and differentiation such as during periconception or peri-implantation periods, may be a result of impaired X chromosome inactivation, as demonstrated in response to IVF (Tan et al., 2016a). Further sex-specific adaptations later in placental development, however, are more speculative, but may be due to ongoing alterations in X chromosome dosage, imprinting on the X-chromosome, or perhaps by some other mechanism, and is discussed in detail by others (Gabory et al., 2013, Hemberger, 2002).

In summary, while currently male fetuses are suggested to be more susceptible to prenatal perturbations through aberrant placental formation, we suggest that these models are mostly due to mid-late gestational exposures. Conversely, early gestational models of programming are likely to be more deleterious to female embryos. This is of particular interest as PC-EtOH exposure has shown female specific GlyT accumulation in late gestation, which may suggest other sexually dimorphic outcomes may be present earlier in pregnancy in this model.

### 1.10 Models of periconceptional programming

Many perturbations to development during the periconception period, such as maternal dietary modifications or alcohol consumption, are sufficient to cause fetal growth restriction and result in programming of adult disease, as viewed in Table 1.2. The most well characterised model is a pre-implantation low protein diet, which has been shown to cause IUGR, increased postnatal “catch up” growth in females, and increased blood pressure in adult males only (Kwong et al., 2000, 2007). Sex differences in adult phenotypes seems to be a common finding within periconceptional models, which also includes a methyl deficient diet (Sinclair et al., 2007) and *in vitro* culture (Fernandez-Gonzalez et al., 2004).

Few periconception models, however, explore placental phenotypes, but those that do, display zone- and sex-specific alterations in genes associated with fetal growth (*Igf-2, Grb-10*), and nutrient transporters (*Slc2a1, Slc38a4*) (Fowden et al., 2011), see
Table 1.3. Interestingly, periconception models do not explore potential sex differences when analysing pre-implantation phenotypes e.g. cell number. This is likely to be due to lack of genetic technologies in different animal models, technical limitations after staining procedures and the quantity required for genetic and protein analyses in which embryos are pooled per treatment.

Periconceptional models do show changes in pre-implantation embryo viability through altered cell number and cell allocation, as well as ongoing trophoblast differentiation, which may highlight its plasticity or that all aspects development can be compromised in response to an adverse environment, see Table 1.3. Alterations in blastocyst cell number seems to be a common finding between models, which suggests a common pathway may be perturbed. Maternal dietary modifications can alter substrates such as glucose, insulin and amino acids (Kwong et al., 2000), indicative of perturbed nutrient sensing pathways. One likely mediator is the hexosamine signalling pathway, which culminates multiple maternal substrates in influencing the degree of post-translational modifications to critical regulators of cell cycle, stress or apoptosis (Hanover et al., 2010). The mTOR pathway can also be altered by nutrients and growth factors to influence cell proliferation (Eckert et al., 2012). Maternal low protein (Kwong et al., 2000), vitamin and folate deficiency models (Sinclair et al., 2007) have also been shown to perturb the epigenetic environment, altering maternal serum homocysteine or choline levels, donor substrates which can influence the degree of DNA methylation and other epigenetic modifications. These epigenetic marks may be heritable through either the TE to effect placental development, or the ICM to influence fetal and postnatal growth, and may potentially mediate adult disease outcomes (Dean et al., 2003, Waterland and Jirtle, 2004). These pathways can thus signal a healthy or stressful maternal environment and modify pre-implantation development accordingly.
<table>
<thead>
<tr>
<th>Animal Model and Dose</th>
<th>Postnatal and Adult Phenotypes</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low Protein Diet</strong>, rat, <em>in vivo</em>, 9% casein, E0-E4.25</td>
<td>Male: ↓ Liver and ↑ kidney weights, ↑ PN BP Female: ↓ BW in females, ↑ PN weight</td>
<td>(Kwong et al., 2000)</td>
</tr>
<tr>
<td><strong>Low Protein Diet</strong>, mouse, <em>in vivo</em>, 9% casein, E0-E3.5</td>
<td>Male: ↔ BW or PN weight, ↑ BP Female: ↑ BW, ↑ PN weight, ↑ anxiety behaviour, ↑ BP</td>
<td>(Watkins et al., 2008a)</td>
</tr>
<tr>
<td><strong>Low Protein Diet</strong>, mouse, <em>in vivo</em>, 9% casein, E-3.5-E0</td>
<td>Male: ↑ BW in males, ↑ BP Female: ↓ Adult organ weights, ↑ NN, ↑ BP</td>
<td>(Watkins et al., 2008b)</td>
</tr>
<tr>
<td><strong>Undernutrition</strong>, ovine, <em>in vivo</em>, 70% of control diet, E-60 – E7</td>
<td>↓ BW (twins only)</td>
<td>(Edwards and McMillen, 2002)</td>
</tr>
<tr>
<td><strong>High Fat Diet</strong>, mouse, <em>in vivo</em>, -16 wks – E0, E4</td>
<td>Male: ↓ BW, ↑ PN cholesterol and fat mass, ↓ glucose tolerance Female: ↓ BW, ↑ PN fat mass</td>
<td>(Jungheim et al., 2010)</td>
</tr>
<tr>
<td><strong>pH</strong>, mouse, <em>in vitro</em>, superovulation, 2 mmol/l DMO, decreasing pH by &lt;0.2 pH units, zygotes-blastocyst</td>
<td>↓ BW</td>
<td>(Zander-Fox et al., 2010)</td>
</tr>
<tr>
<td><strong>Embryo culture</strong>, mouse, <em>in vitro</em>, 2-cell stage to blastocyst stage, superovulation</td>
<td>Male and Female: ↔ BW, ↑ BP</td>
<td>(Watkins et al., 2007)</td>
</tr>
<tr>
<td><strong>Embryo culture</strong>, ovine, <em>in vitro</em>, 2-5 days with granulosa cells and/or SOF medium</td>
<td>↑ BW and organ weights</td>
<td>(Rooke et al., 2007, Young et al., 2001)</td>
</tr>
<tr>
<td><strong>IVF or ICSI</strong>, epidemiological studies</td>
<td>↓ BW, ↑ birth defects</td>
<td>(Hansen et al., 2002, Schieve et al., 2002)</td>
</tr>
<tr>
<td><strong>IVF and ICSI</strong>, mouse, <em>in vitro</em></td>
<td>Male: ↑ BW, ↔ glucose clearance Female: ↑ BW, glucose clearance, insulin and fat deposition</td>
<td>(Scott et al., 2010)</td>
</tr>
</tbody>
</table>
Methyl-deficient diet, ovine, *in vivo*, -8wks-E6 | Male and Female: ↑ PN weight, insulin resistance, ↑ BP | (Sinclair et al., 2007)

Table 1.2. Effect maternal perturbations during the pre-implantation period on the development of adult-onset disease. Several maternal pre-implantation perturbations have been explored using various animal models and techniques (*in vivo* or *in vitro*), showing many similar adult onset phenotypes. BP; blood pressure, BW; body weight, DMO; 5,5-dimethyl-2,4-oxazolidinedione, E; embryonic day, EtOH; ethanol, HPA; hypothalamic pituitary axis, ICSI; intracytoplasmic sperm injection, IP; intraperitoneal (injection), IUGR; intrauterine growth restriction, IVF; *in vitro* fertilisation, NN; nephron number, LOS; large offspring syndrome, PN; postnatal, SOF; synthetic oviduct fluid, wks; weeks, w/v; weight per volume.
<table>
<thead>
<tr>
<th>Animal Model and Dose</th>
<th>Oocyte and Pre-Implantation Alterations</th>
<th>Placental Alterations</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low Protein Diet</strong>, rat, in vivo, 9% casein, E0-E4.25</td>
<td>↓ ICM and TE cell number, cell proliferation. ↓ H19 in male blasts only ↔ Igf2, Igf2R in male or female</td>
<td>↑ EPC proliferation and TGCs ↓ PW and PW:BW</td>
<td>(Kwong et al., 2000, 2006, Watkins et al., 2015)</td>
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<tr>
<td><strong>Undernutrition</strong>, ovine, in vivo, 70% of control diet, E-60 – 7 days</td>
<td></td>
<td>↑ PW (twin) ↔ Volume of placenta or fetal capillaries</td>
<td>(MacLaughlin et al., 2005)</td>
</tr>
<tr>
<td><strong>Undernutrition</strong>, ovine, in vivo, 60% of control diet, 8 weeks before oocyte collection, culture to blastocyst stage (8 days)</td>
<td>↓ Oocyte quality ↓ Cleavage rates and blastocyst formation</td>
<td></td>
<td>(Borowczyk et al., 2006)</td>
</tr>
<tr>
<td><strong>Hyperglycaemia</strong>, rat, in vitro, E5 embryos culture in 6 or 28mM glucose for 24h</td>
<td>↓ Cell number in blastocyst ↑ Apoptosis</td>
<td></td>
<td>(Hinck et al., 2003)</td>
</tr>
<tr>
<td><strong>Diabetes/Insulin resistance</strong>, mouse or rat, in vivo, streptozotocin or alloxin induced ~E-40/E-7 to E5, and/or in vitro for 72h</td>
<td>Altered oocyte maturation, ↓ blastocysts, total, TE and ICM number ↑ Morulae</td>
<td>↓ Outgrowth of P-TGC at 72h culture</td>
<td>(Diamond et al., 1989, Lea et al., 1996, Moley et al., 1991, Pampfer et al., 1994, Vercheval et al., 1990)</td>
</tr>
<tr>
<td><strong>High Fat Diet</strong>, mouse, in vivo, -16 weeks – E0 or E4, in vitro to E5</td>
<td>↓ Oocyte quality, ICM and blastocyst number, ↑ TE cells</td>
<td>↑ Igf2R expression</td>
<td>(Jungheim et al., 2010, Minge et al., 2008)</td>
</tr>
<tr>
<td><strong>Ethanol</strong>, mouse, in vitro, oocytes, 1-, 2- or 4-cell embryos exposed to 0-2% (w/v) EtOH on one day, cultured to blastocyst stage.</td>
<td><strong>High dose</strong> ↓ total cell number, blastocyst formation, hatching. ↑ Apoptosis</td>
<td>P-TGC outgrowth ↓ high dose, and ↑ low dose</td>
<td>(Leach et al., 1993, Huang et al., 2007)</td>
</tr>
<tr>
<td>Low dose</td>
<td>Blastocyst formation, cell number.</td>
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<tr>
<td><strong>Ethanol</strong>, mouse, <em>in vitro</em>, E3-4 embryos, 0.05-1.0% (w/v), 5 min or 24h to E5, embryo transfer</td>
<td><strong>↑ Cavitation</strong></td>
<td><strong>↓ P-TGC outgrowth (0.1-1.0% EtOH)</strong></td>
<td><strong>↑ Implantation (term)</strong></td>
</tr>
<tr>
<td><strong>Ethanol</strong>, rat or mouse, <em>in vivo</em>, 0-4 g/kg, 10-33% EtOH via gavage, drinking water, vapour, or pair fed E&gt;100-E5, embryo transfer</td>
<td><strong>↓ Cell division, cavitation, hatching, mitotic index, viability, and trophoblast outgrowth.</strong></td>
<td><strong>↓ Implantation (E19)</strong></td>
<td><strong>↑ PW</strong></td>
</tr>
<tr>
<td><strong>Ethanol</strong>, porcine, <em>in vitro</em>, 0.2% w/v, fertilisation + 7 days culture.</td>
<td><strong>↓ Blastocyst rate, expansion rate, mitochondrial function</strong></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Alcohols</strong>, mouse, <em>in vitro</em>, 2-cell in 0.05-1% butanol, propanol, isopropanol, propanediol, glycerol, or 0.1% EtOH for 1 day to E5</td>
<td><strong>↓ Blastocyst formation (except propanediol and glycerol)</strong></td>
<td><strong>EtOH ↑ Cavitation</strong></td>
<td>-</td>
</tr>
<tr>
<td><strong>Reduced pH</strong>, mouse, <em>in vitro</em>, superovulation, zygotes in 2 mmol/l DMO</td>
<td><strong>↓ ICM and total cell number</strong></td>
<td><strong>↑ Apoptosis</strong></td>
<td>-</td>
</tr>
<tr>
<td><strong>Embryo Culture and IVF</strong>, mouse, <em>in vitro</em>, oocyte to 2-cell stage to blastocyst stage, embryo transfer</td>
<td><strong>↓ Total, TE and ICM cells</strong></td>
<td><strong>↑ Abnormal EPC (delayed, ↓ volume, disorganised (E7.5) PW: ↓ E12.5, ↔ E13.5, ↑ E18.5 and E19.5, ↓ PW:BW (E15.5, E18.5)</strong></td>
<td><strong>↓ LAB vessel density E13.5</strong></td>
</tr>
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</table>
Table 1.3. Periconceptional diet modifications of pre-implantation development, placental development. Several maternal periconceptional perturbations have been explored using various animal models and techniques (*in vivo* or *in vitro*), showing similar pre-implantation modifications, but few have explored placental phenotypes. Ca^{2+}; calcium, DMO; 5,5-dimethyl-2,4-oxazolidinedione, E; embryonic day, EPC; ectoplacental cone, EtOH; ethanol, GlyT; Glycogen trophoblasts, IC; intracellular, ICM; inner cell mass, ICSI; intracytoplasmic sperm injection, IDD; insulin-dependent diabetes, IVF; *in vitro* fertilisation, LAB; labyrinth, JZ; junctional zone, PW; placental weight, PW:BW; ratio of placental weight to body weight, TE; trophectoderm/v; volume per volume, w/v; weight per volume.
1.11 Animal models of maternal alcohol exposure

Animal models have been effective in analysing dose and critical windows in programming of alcohol. As mentioned in section 1.9.3, chronic high dose maternal alcohol exposure is categorised into FAS and FASD. However, lower doses of EtOH and exposure across different critical windows have not been studies as extensively as chronic models. While chronic models simulating FAS, such as 37% v/v EtOH in the rat show reduced litter sizes, indicative of miscarriage (Gundogan et al., 2008), and significant fetal growth restriction (Gundogan et al., 2015), lower doses show more variability. A chronic low dose model in the rat of 8.2% EtOH from E6-E19 showed increased fetal weight, while 6% v/v EtOH from E1-E20 in the rat showed reduced fetal weight by 3% in males, and 8% in females (Probyn et al., 2013). Binge dosing (1g EtOH/kg maternal body weight) on E13.5 and E14.5 in the rat resulted in reduced body weight at PN30 (Gray et al., 2010), while repeated EtOH exposure (0.75 g EtOH/kg maternal body weight) in late gestation of the fetal sheep (E95-E124) showed no change in birth weight (Gray et al., 2008). In addition, reduced nephron number has been shown in the binge model in both sexes (Gray et al., 2010), as well as the late gestational sheep model (Gray et al., 2008), while hypertrophy in the heart occurred in the low dose 6% EtOH model (Nguyen et al., 2014). A subset of models has also investigated postnatal disease phenotypes, with chronic low dose EtOH associated with hyperinsulinemia in males (Probyn et al., 2013), and the binge model resulting in hypertension in both sexes (Gray et al., 2010). More recently, we have shown that PC-EtOH can also cause offspring insulin insensitivity (Gardebjjer et al., 2015). One organ already implicated in mediating programming of alcohol exposure, is through altered development and function of the placenta.

While few animal models of alcohol exposure investigate impacts on the placenta, those that do show dose-dependent effects. After mid-gestation at the onset of placental function through nutrient transport, alcohol can readily cross the placenta (Weinberg et al., 2008). A gestational model of 8% v/v EtOH exposure initiated on various day’s post E6, has also shown altered placental structure and function (Gundogan et al., 2013). Here they found reduced trophoblast stem cell pools, decreased TGCs, and reduced invasion of trophoblasts into the decidua. The invasion defect likely contributed to inhibited spiral artery remodelling and perturbed placental development.
blood flow. High dose (18-37% vol/vol) EtOH exposure from implantation until close to term (E6-E18) reduced invasion of trophoblast cells into the maternal decidua and caused more labyrinth disorganisation with increasing dose (Gundogan et al., 2015). However, it is unknown if placental defects are due to direct exposure of the trophoblasts of the post-implantation embryo to EtOH within the uterine cavity, or via other indirect mechanisms such as interactions with altered uterine cells.

1.12 Programming of ethanol on the pre-implantation embryo

The effects of ethanol during the periconceptional period, have been largely overlooked. While direct effects of ethanol have been explored on pre-implantation development, few examine possible indirect effects through altered maternal physiology, or the dialogue required for implantation and placentation. To date, no in vivo model other than our own has established whether PC-EtOH can result in fetal programming and lead to adult onset disease.

Ethanol has been shown to be able to enter the oviductal and uterine lumen, where it may be able to directly affect the developing pre-implantation embryo (Sandor et al., 1981). Ethanol was found both in the oviductal and uterine fluid (0.05-0.08%) following maternal administration, however, in lower concentrations than the blood (0.24%). This study however, used gas chromatography to measure ethanol concentrations, which is not considered the gold standard for substrate measurements. Several groups have thus studied the direct effects of ethanol on pre-implantation development in vitro. Interestingly, many studies show dose-dependent biphasic effects, with low doses (0.1% ethanol) shown to accelerate pre-implantation morphogenesis, through calcium-induced increases in cavitation, cell proliferation, blastocyst outgrowth, precocious differentiation (Stachecki et al., 1994a, 1994b, Rout et al., 1997, Mitchell, 1994, Leach et al., 1993), and increased implantation success after transfer (Stachecki et al., 1994a). This is in contrast to high doses (0.3-2% ethanol), which rather show perturbed development as indicated by increased apoptosis, reductions in total cell number, blastocyst formation and hatching (Leach et al., 1993, Huang et al., 2007), see Table 1.3.

In vivo models of PC-EtOH using moderate-high doses (10-33% v/v ethanol), show delayed pre-implantation development through reductions in cell division, hatching,
increased pathological embryos and resorptions, reduced implantation, and fetal growth restriction (Checiu, 1993, 1995, Huang et al., 2007, Pennington et al., 1984, Perez-Tito et al., 2014, Mitchell, 1994). With the exceptions of Padmanabhan and Hameed (1988) and Huang et al. (2007), all other in vivo models used methods of ethanol administration that may be confounded with other factors. When administered in drinking water, animals often reduce their calorie intake by 10-40% (Shankar et al., 2007), confounding the study with undernutrition, which in itself can cause programming, see Tables 1.2 and 1.3. Intravenous or intraperitoneal injection and gavage are also likely to cause further stress to the animal, activating glucocorticoid response pathways. A further limitation of these previous studies is that none have explored any sex effects, which may potentially mask phenotypes by combining both genders.

Ethanol may also perturb the maternal environment indirectly by altering critical maternal hormones associated with pregnancy such as estrogen (Gavaler and Rosenblum, 1987) and progesterone (Budec et al., 2002), nutrient substrates of glucose and insulin (Forney and Harger, 1969, Snyder and Singh, 1989), growth factor availability (IGF1) (Gundogan et al., 2008), acetaldehyde – a teratogenic downstream metabolite of ethanol (Sreenathan et al., 1982), reactive oxygen species – a cause cellular stress (de la Monte and Wands, 2001), or other pathways such as reduced retinoic acid synthesis (Gray et al., 2012). Recent unpublished work from our laboratory has found PC-EtOH exposure of 12.5% v/v ethanol to cause mild maternal hyperglycaemia over the treatment period (Gardebjer, unpublished). Many of these other indirect implications can also affect cellular viability, metabolism or differentiation in either the pre-implantation embryo, or the uterine stromal and decidual cells, which may be important when analysing their interaction. We have also recently shown increased resorptions at E20 in our model (Gardebjer et al., 2014), which adds precedence to exploring an implantation and placentation defect. Considering the importance of coinciding the windows of embryo activation and uterine receptivity to co-ordinating implantation and placentation, it is surprising that few periconceptional models explore altered implantation as a mode for fetal programming.

Ethanol can also increase homocysteine levels (Steegers-Theunissen et al., 2013), which may affect the degree of epigenetic modifications, especially to the embryo
during EGA. Given the importance of imprinting in the TE for placental development, alterations to the TE and subsequent trophoblast lineages could mediate differences in placental growth, efficiency and nutrient transport capabilities (Angiolini et al., 2006).

Our group has also reported increased Igf2 expression in the junctional zone of E20 placentas, further supporting a role for epigenetic modifications (Gardebjer et al., 2014).

Our laboratory has also reported structural and metabolic changes in the term placenta (Gardebjer et al., 2014). Structural changes included increased relative placental weight, increased junctional zone area to body weight ratio, and decreased labyrinth area ratio in PC-EtOH exposed fetuses. Alterations in both placental zones may indicate altered trophoblast cell allocation, and may explain other observed differential metabolic (altered glucose transport and glycogen accumulation) and vascular changes, as shown in other models of alcohol exposure (Gundogan et al., 2013). This evidence in addition to that from our own model, has led us to question whether PC-EtOH can alter trophoblast progenitor pool differentiation, allocation to downstream zones of the placenta, trophoblast invasion into the maternal decidua, and remodelling of spiral arteries. This will further inform us how the placenta is responsible for reducing fetal growth after PC-EtOH.
1.13 Rationale

Whilst a lot of focus has been directed on the impact of nutritional perturbations during this periconceptional period, maternal alcohol consumption, which is common prior to pregnancy recognition (Colvin et al., 2007, Muggli et al., 2016), has not received significant attention. Several studies have utilised in vitro models of ethanol exposure to explore the direct effects on pre-implantation development, however these studies overlook the indirect impact of perturbations to the maternal environment on the pre-implantation period, placentation, as well as any sexually dimorphic outcomes.

Our group has established a model of periconceptional alcohol exposure, shown to result in alterations to the late gestation placenta and result in fetal growth restriction (Gardebjer et al., 2014). These changes in development are associated with impaired insulin sensitivity during adulthood (Gardebjer et al., 2015) as well as renal and cardiac deficits (unpublished). However, we still have little understanding how PC-EtOH initiates these later placental and fetal abnormalities, or the full extent of the disruption to placental development. The placenta is critical in influencing fetal growth, so alteration in formation, function or adaptation can program other fetal, and therefore postnatal outcomes. Therefore, this study will explore the impact of PC-EtOH on the maternal uterine environment, its preparation for embryo implantation (uterine receptivity) and the development of the pre-implantation embryo in a sexually dimorphic manner. In addition, we will characterise the effects of PC-EtOH on the immature, mid- and late-gestational placenta (see Figure 1.4).
Figure 1.4. Summary of the putative impact of ethanol consumption on periconceptional programming. Alterations to pre-implantation events and/or changes to the uterine environment may adversely affect implantation and thus placental formation. These in turn can have additive impacts on growth of the fetus and its organ structures. These alterations may then impact further growth of these organs and may predispose adult onset diseases.
1.14 Scope of thesis

PC-EtOH exposure has been shown to cause fetal growth restriction and the development of adult insulin insensitivity. While some adaptations have been shown in the late gestation placenta, phenotypes present in earlier gestation, and the time points at which they arise, are currently unexplored. Therefore, this thesis aimed to further characterise the impacts of PC-EtOH exposure on placentation, and to examine whether impacts to either the pre-implantation embryo, uterine environment or a combination are implicated in programming by alcohol. It was also important to examine embryonic sex for all possible outcomes, as many phenotypes have been shown to be sexually dimorphic in this model.

1.15 Aims and hypotheses

1.15.1 Overall aims

To examine whether PC-EtOH exposure alters the development of the pre-implantation embryo and/or the uterine environment, and how this may cause sex-specific programming of placental development, and result in fetal growth restriction.

1.15.2 Overall hypotheses

It is hypothesised that in vivo PC-EtOH exposure affects pre-implantation development altering key events such as cell allocation and differentiation within the blastocyst. Furthermore, PC-EtOH exposure will impact embryo development, through changes to the maternal uterine environment, the acquisition of uterine “receptivity” required for embryo implantation, and the capacity of the uterine stromal cells to undergo decidualisation, and subsequent placental formation. All embryonic and placental outcomes will show sexual dimorphism.
1.16 Aims and hypotheses for chapter 3

It is well known that the male XY placenta is larger than the female XX to aid in the greater growth trajectory of the male fetus. Studies in the field of developmental programming highlight sexually dimorphic phenotypes in response to stress, particularly in placentation. However, studies showing inherent differences between male and female placenta, and tracing its cell lineage back to the pre-implantation embryo, currently have several limitations. Firstly, placentation has not been explored with appropriate numbers of animals, or sufficient developmental time points during gestation, while the pre-implantation field is currently confounded with inappropriate sample derivation (in vivo as opposed to in vitro, in addition to the utilisation of superovulation).

Specific aims addressed in chapter 3 were to determine basal sexual dimorphism from in vivo-derived samples. Sex differences will be examined in:

1. The pre-implantation blastocysts in respect to total cell number and allocation to either the TE or ICM lineages to determine developmental viability.
2. Trophoblast outgrowth capacity and differentiation into P-TGCs required for invasion of the uterine epithelium.
3. Morphogenesis of the placenta including its two compartments – the labyrinth and junctional zones, as well as the fetal and maternal blood spaces within the labyrinth (examined for volume and surface area), and separating them - the interhaemal membrane over gestation, which all contribute to ongoing placental development and appropriate nutrient exchange required for fetal growth.
4. Expression of genes involved in differentiation of the labyrinthine trophoblasts, growth, and vasculogenesis.

It is hypothesised that sexual dimorphism begins at the blastocyst stage with sex difference in the progenitor trophectoderm (TE) and its derived trophoblast lineages. As these cells populate the majority of cell types within the placenta during gestation, we will investigate sex specific differences in growth of the placental compartments by ontogeny.
1.17 Aims and hypotheses for chapter 4

Perturbations during pregnancy, including alcohol exposure (Gardebjer et al., 2014, Gundogan et al., 2015) often result in modifications to placental growth (Kwong et al., 2006, MacLaughlin et al., 2005) and zonal allocation (Gardebjer et al., 2014) in late gestation. However, few studies have determined whether alterations to trophoblast differentiation in early pregnancy may be mediating these effects. Here we utilise an in vitro model of differentiating mouse trophoblast stem (TS) cells to examine the direct effects of EtOH.

Specific aims addressed in chapter 4 are to determine whether in vitro EtOH exposure alters:

1. Cell proliferation,
2. Differentiation to downstream trophoblast subtypes,
3. Expression of genes involved in cell death pathways, and
4. Whether these outcomes occur in a dose-dependent manner.

It is hypothesised that in vitro culture with EtOH will reduce the proliferation and pluripotency of TS cells, causing premature differentiation into downstream cell types, assessed by expression of trophoblast subtype specific markers, and will occur in a dose-dependent manner, with higher doses showing the most severe phenotypes.

1.18 Aims and hypotheses for chapter 5

The periconceptional period is a critical window during development which is sensitive to maternal perturbations. Prior to placentation, the early embryo has limited capacity to regulate nutrients and substrates, facilitated by the oviductal and uterine environment. Perturbations to the uterine environment often result in reduced proliferation or an increase in cell death pathways of the early embryo. In addition, studies from mouse models of IVF suggest females to be most at risk of perturbed development and implantation failure, which may explain female bias in placental phenotypes after PC-EtOH exposure in late gestation. The uterine environment facilitates embryonic nutrition, including the supply of growth factors and other substrates, through the acquisition of uterine receptivity and the stromal cell reaction – decidualisation. The timing and extent of these important events can shift or reduce the window from implantation and can result in implantation failure, or can lead to
perturbation of uterine-embryo interactions, and therefore placental malformations. This chapter aimed to determine the effects of *in vivo* PC-EtOH exposure on pre-implantation development and the peri-implantation uterine environment.

Specific aims addressed in chapter 5 are to determine whether *in vivo* PC-EtOH exposure:

1. Alters cell viability (by assessing total cell number) and lineage allocation (to the ICM or TE) in the E5 blastocyst in a sexually dimorphic manner.
2. Results in sex specific alterations in trophoblast outgrowth capacity and differentiation into P-TGCs required for invasion of the uterine epithelium.
3. Alters maternal serum hormones (estrogen, progesterone) during the pre- and peri-implantation period.
4. Alters the window of receptivity, or decidual response within the early implantation site.
5. Alters the maternal immune response for implantation through dNK cell infiltration.

It is hypothesised that maternal PC-EtOH exposure will cause sex specific alterations in total cell number and cell allocation, affecting in turn trophoblast outgrowth capacity, the ability for the embryo to implant, and thus programming long-term development of the fetus and the placenta. Based on previous models of ethanol exposure (Gavalier and Rosenblum, 1987, Budec et al., 2002), PC-EtOH will elevate maternal estrogen levels, causing precocious uterine sensitisation and secretion of stromal products, altering the window of receptivity. Reduced progesterone levels will decrease the decidual cell reaction and their secretory products, diminishing the embryos ability for implantation. PC-EtOH will reduce the infiltration of dNK cells into uteri and SpA-TGCs into the decidua, which will reduce spiral artery transformation (further assessed in Chapter 6).
1.19 Aims and hypotheses for chapter 6

PC-EtOH exposure has been previously found to alter the late gestation placenta and result in fetal growth restriction (Gardebjer et al., 2014). This and other periconceptional models rarely examine earlier gestational time points, as placental nutrient transfer is at maximum capacity in late gestation, required to facilitate the late gestational fetal growth spurt. For this reason, this thesis chapter aims to explore earlier developmental events in placentation including differentiation of the placental trophoblasts, morphogenesis and function of the placenta by ontogeny. By doing so, precise timing of deleterious phenotypes can be determined.

Specific aims addressed in chapter 6 are to examine whether in *in vivo* PC-EtOH exposure causes sex specific alterations to:

1. The degree of invasion of junctional zone-derived trophoblast subtypes (SpA-TGCs) into the maternal decidua.
2. Formation of the labyrinthine vasculature; fetal and maternal blood spaces, and separating them the interhaemal membrane.
3. Differentiation of cells in the labyrinth
4. Expression of labyrinthine nutrient transporters, effecting nutrient exchange to the fetus.

It is hypothesised that based on prior placental characterisation of our model of PC-EtOH exposure Gardebjer et al. (2014) and gestational model of alcohol exposure by Gundogan et al. (2013), *in vivo* PC-EtOH exposure will reduce the invasion of SpA-TGCs into the maternal decidua, affecting spiral artery remodelling, and will reduce cell allocation to the labyrinth, reducing the volume of fetal vasculature and interhaemal membrane thickness. These changes in turn will reduce nutrient transfer of glucose and amino acids, leading to fetal growth restriction.
Chapter 2 – General Materials and Methods

2.1 Ethics
All animal experiments and procedures were approved by The University of Queensland Animal Ethics Committee (AEC approval number SBS/022/12/NHMRC) prior to commencement of this study.

2.2 Animal Handling and Liquid Diet Administration
Female Sprague Dawley rats were housed individually with controlled temperature, humidity and an artificial 12-hour light-dark cycle at the animal facility in the Australian Institute for Bioengineering and Nanotechnology at the University of Queensland. PC-EtOH exposure was administered in vivo to Sprague Dawley dams (*Rattus norvegicus*) in a liquid diet containing 12.5% v/v EtOH as previously described (Gardebjer et al., 2014, 2015). The proposed diets were based upon the established Lieber-DeCarli nutritionally complete diet (Lieber and DeCarli, 1982, 1989), see Table 2.1 for nutritional composition. A control liquid diet was given to all female rats prior to the experiment to avoid rejection. Dam weight was at least 230g before entering the protocol. Vaginal impedance was measured daily with an EC40 oestrous cycle monitor (Fine Science Tools), with $4.5 \times 10^3 \, \Omega$ or higher indicating oestrus and allocation to the diet regime. Dams were treated over two oestrus cycles, one prior to mating and the establishment of the pre-implantation period (denoted as embryonic day (E) -4 to E4). Dams were time-mated on E0 from 1200h to 1700h if impedance exceeded $4.5x10^3 \Omega$. If pregnancy was established by the presence of a seminal plug, the following day was considered E1. Timed mating’s were used in cohorts from E5-E13. Cohorts of E15 and E20 were allowed to mate overnight. Pregnant dams were then returned to individual cages and continued on the above-mentioned diets until the end of E4. Liquid diets were prepared fresh daily, administered at 1200h and were removed at 0900h the following day. Between 0900h-1200h, animals were given water *ad libitum*. On E5, dams were returned to laboratory chow until sacrifice. As this study aimed to determine the mechanisms behind ethanol exposure, it was important to allocate a separate cohort of dams to an additional control of standard rat chow which were not a part of the liquid diet regime to eliminate the possible effects of liquid diet procedures.
Table 2.1. Liquid diet composition for control and ethanol treatment groups. The ethanol diet was modified for isocaloric consumption to control dams. Sustagen hospital formula composition per 60g serving: protein (13.8g), fat (1.5g), carbohydrate (39g), sodium (160mg), vitamin A (210ug), thiamine (0.55mg), riboflavin (0.85mg), niacin (5.0mg), folate (100ug), vitamin B6 (0.8mg), vitamin B12 (1.0ug), biotin (5.0ug), pantothenic acid (0.8mg), vitamin C (20mg), vitamin D (3.6ug), vitamin E (5.0mg), vitamin K (27ug), calcium (400mg), chromium (27ug), copper (0.3mg), iodine (60ug), iron (3.6mg), magnesium (0.7mg), manganese (0.7mg), molybdenum (34ug), phosphorus (420mg), selenium (9.0ug), zinc (3.6mg), potassium (660mg), chloride (370mg).

2.3 Post-mortem and tissue collection

2.3.1 E5 cohort

At 0900h on E5, dams were rapidly killed via guillotine prior to dissection of the uterine horns. Maternal blood was collected into a 50mL falcon tube, and transferred into heparin-coated or ethylenediaminetetraacetic acid (EDTA)-coated tubes. Maternal tissues including liver, adrenals, ovaries and uterine segments were weighed and taken either snap frozen in liquid nitrogen for molecular analyses, or fixed in 4% paraformaldehyde (PFA) for histology. Oviducts and uteri were flushed in Hepes-KSOM media (Lawitts and Biggers, 1993), see Table 2.2, and embryos allocated to
either staining or culture procedures. All embryos were genotyped for sex as described below.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>MW (g/mol)</th>
<th>C (mmol)</th>
<th>C (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.44</td>
<td>95</td>
<td>5.5518</td>
</tr>
<tr>
<td>KCl</td>
<td>74.55</td>
<td>2.5</td>
<td>0.1683</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>136.09</td>
<td>0.35</td>
<td>0.0476</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>246.48</td>
<td>0.2</td>
<td>0.0493</td>
</tr>
<tr>
<td>Glucose</td>
<td>180.16</td>
<td>0.2</td>
<td>0.036</td>
</tr>
<tr>
<td>Penicillin G (1mg = 1670 IU)</td>
<td>356.37</td>
<td>100 IU/mL</td>
<td>0.0598</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>728.69</td>
<td>5mg/L</td>
<td>0.005</td>
</tr>
<tr>
<td>Sodium Lactate</td>
<td>112.06</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>84.01</td>
<td>5</td>
<td>0.4201</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>110.4</td>
<td>0.2</td>
<td>0.0221</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>147.02</td>
<td>1.7</td>
<td>0.2499</td>
</tr>
<tr>
<td>EDTA</td>
<td>372.24</td>
<td>0.01</td>
<td>0.0037</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>146.1</td>
<td>1</td>
<td>0.1461</td>
</tr>
<tr>
<td>Hepes</td>
<td>238.3</td>
<td>20</td>
<td>4.766</td>
</tr>
</tbody>
</table>

Table 2.2. Hepes-KSOM embryo flushing and culture media

2.3.2 E6 and E7 cohorts

Dams were sacrificed by guillotine with maternal blood and tissues collected as above (2.3.1). Left uterine implantation sites (IMS), and inter-implantation sites (IIS) were snap frozen in liquid nitrogen for molecular analyses, while IMS and IIS samples on the right uterine horn fixed in 4% PFA for histology.
Figure 2.1. Implantation sites of rat at E7. Panel A shows dissection of both left and right uterine horns with ovaries and oviducts dissected. Panel B shows magnified image of E7 IMS; implantation sites, and IIS; inter-implantation sites.

2.3.3 E11 and E13 cohorts

Dams were sacrificed by guillotine with maternal blood and tissues collected as above (2.3.1). Implantation sites were dissected individually in 1x PBS made in diethylpyrocarbonate (DEPC)-treated water. Implantation sites 1-5 (counting from right ovary) had uterine tissue removed, embryos separated from extraembryonic tissues and all segments frozen for molecular analyses. The remaining implantation sites were dissected for the embryo, and with uterus attached were fixed in 4% PFA for histology. All implantation sites were genotyped for sex from extraembryonic tissue.

2.3.4 E15 and E20 cohorts

E15 and E20 dams were heavily anaesthetised with 50:50 ketamine:xylazil (0.1ml/100g body weight, Lyppard Australia Ltd, QLD, AUS) as previously described (Gardebjør, 2014). Fetal and placental weights were taken at post-mortem for E15 and E20 cohorts, and placentas were separated from the uterus/decidua, with the junctional zone and labyrinth and weighed separately. Placental measurements were also taken for length, width and depth using digital callipers (LCD Display Calliper Vernier Micrometer, Scientrific Pty. Ltd., Yamba, NSW, AUS). A subset of placentas
was snap frozen in liquid nitrogen prior to RNA extraction. Other placental samples were cut in half with uterus and decidua attached and fixed in 4% PFA prior to processing into paraffin for stereology. A subset of labyrinth samples taken from the midline of E15 and E20 placentas were cut into 1mm³ and fixed into 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer until tissue processing for transmission electron microscopy (section 2.10.5). All cohorts collected fetal tissue for genotyping for sex as previously described (Gardebjer et al., 2014). Maternal blood was collected by cardiac puncture into heparinised or EDTA coated tubes.

2.4 Trophoblast outgrowth assays

A subset of flushed embryos at E5 were allocated to trophoblast outgrowth assays. Embryos were placed into preheated microdrops of trophoblast stem cell (TS) media (see Table 2.3 for composition) on tissue culture plates or gelatine coated coverslips and cultured in a humidifying chamber (Cook, Australia) at 37°C 5% CO₂ / 5% O₂ / 90% N₂ under paraffin oil. They were cultured for 6 days, with media changed every 2 days, and imaged with an inverted phase contrast microscope (Leica). Area occupied by outgrowths were traced in ImageJ using calibrated settings. A subset of outgrowths was allocated to staining procedures. The remaining were collected for RNA into TRIzol (Thermo Fisher Scientific).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditioned medium</td>
<td>70%</td>
</tr>
<tr>
<td>TS media (see below)</td>
<td>30%</td>
</tr>
<tr>
<td>FGF4</td>
<td>1:1000</td>
</tr>
<tr>
<td>Heparin</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 2.3. Conditioned media. The conditioned medium is created by culture with TS media on embryonic fibroblast feeders, described by (Quinn et al., 2006).

2.5 Culture of trophoblast stem cells

Murine TS cells (RS26 and EGFP lines) were raised in 70% conditioned media in TS media (Table 2.3 and 2.4), supplemented with FGF4 and heparin to maintain pluripotency as described by Tanaka et al. (1998) Oda et al. (2006). Cells were seeded at 5x10⁴ (EGFP) and 1x10⁴ (RS26), and differentiated in 0% (control), 0.2%, or 1% EtOH in TS media. To assess cell proliferation, cells were lifted off the culture
plates and counted using a hemocytometer on days 2, 4 and 6 of culture ($N=3/treatment$). Media was changed every 2 days including the addition of fresh EtOH. RNA was collected on day 6 ($N=9/treatment$, 3 technical replicates per set). Additional cultures on day 6 were collected fixed for staining procedures (see section 2.10.3.3).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640</td>
<td>192.5mL</td>
</tr>
<tr>
<td>Penicillin / streptomycin</td>
<td>2.5mL</td>
</tr>
<tr>
<td>B-mercaptoethanol</td>
<td>2.5mL</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>2.5mL</td>
</tr>
<tr>
<td>Fetal calf serum</td>
<td>50mL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>250mL</strong></td>
</tr>
</tbody>
</table>

Table 2.4 Trophoblast stem cell media.

**2.6 Plasma analyses**

At post-mortem, maternal blood was centrifuged at 3500 rpm for 15 minutes at 4°C. Plasma was collected and stored at -80°C or -20°C for future analyses.

**2.6.1 COBAS Integra analyses**

Maternal plasma was analysed using the COBAS Integra 400 Plus for glucose, triglycerides, sodium and chloride concentrations in singular. 100-120ul of plasma samples were assayed.

**2.6.2 Hormone analyses**

**2.6.2.1 Estrogen**

Plasma estradiol-17beta concentrations were measured using a commercially available radioimmunoassay (DSL4800, Oestradiol RIA, Beckman Coulter) following the manufacturer’s instructions. Briefly all standards, samples, and the quality control were prepared by dilution in charcoal-stripped fetal bovine serum (CS-FCS; #312676, Gibco ThermoFisher Scientific), known to not exhibit any cross-reactivity within the assay. 200 µl of standards or diluted samples were combined with 100µl of primary antibody (rabbit anti-estradiol) and incubated for 1 hour at room temperature (RT). 100µl of $^{125}I$-labelled estradiol tracer was then added and tubes further incubated for
2 hours RT. 1.0 ml of precipitating reagent (goat anti-rabbit gamma globulin in buffer with polyethylene glycol, sodium azide, and ProClin300) was added, before incubation for 20 mins RT, prior to centrifugation at 1500g for 20 mins. The supernatant was decanted by inversion and tubes drained before tubes were counted on a gamma counter (Perkin Elmer Wizard 2, model 2470) for 2 mins. Assay analysis was performed using AssayZap software (Biosoft). Reported values were corrected for dilution in assay procedure (4x). The sensitivity of the assay was 1.0 pg/ml and the intra- and inter-assay coefficients of variation were 0.6% and 6.2% respectively for a quality control of 62.3 pg/ml.

2.6.2.2 Progesterone

Progesterone concentrations in hexane extracts of the plasma samples were measured by radioimmunoassay as described by Curlewis et al. (1985), with the modification of progesterone antiserum C-9817 (Bioquest). Briefly, 20µl of plasma sample was added to 2ml HPLC grade hexane and vortexed for 5 minutes. The solution was then placed in an ethanol ice bath until aqueous fraction was frozen. The solvent extract was decanted into glass tubes and dried under a gentle stream of air using a heat block (45°C). The extract was then reconstituted in 200µl of assay buffer (PB with 0.9% saline, and 0.1% gelatine).

For the radioimmunoassay, 50µl of extracted sample, standard and quality control were combined with 100µl of primary antibody (sheep anti-progesterone-11alpha-hemisuccinate, C9817 at 1:30000 dilution) and 100µl of labelled progesterone tracer (1,2,6,7-H³-progesterone) and incubated overnight at 4°C. For separation 500µl of dextran-activated charcoal (0.125% Norit-A charcoal with 0.0125% dextran in PBS without gelatine) was added to each tube and incubated on ice for 10mins, before centrifugation at 4°C centrifuge for 10min at 2500rpm. The supernatant was decanted into scintillation vials and 1.5ml of scintillant (IGRA-Safe Plus, Perkin Elmer) added, before vials were counted on a beta counter (Perkin Elmer Tri-Carb TR, model 3110) for 2 mins. Assay analysis was performed using AssayZap software (Biosoft). Reported values were corrected for dilution in assay procedure (10x). Extraction efficiency of spiked samples was 75% and the values reported herein were not corrected for these losses. The sensitivity of the assay was 0.1 ng/ml and the intra-
and inter-assay coefficients of variation were 8.1% and 7.9% respectively on a quality control of 2.7 ng/ml.

2.7 Gene expression analyses

2.7.1 RNA extraction

RNA extraction techniques differed with gestational age. Trophoblast outgrowths, E11 and E13 samples were extracted using TRIzol (Thermo Fisher Scientific), while remaining E15 placenta samples utilised column extractions (Qiagen) with on column DNase digestion.

Note: outgrowths utilised gDNA wipe-out in the reverse transcription Quantitek kit (Qiagen), while E11 and E13 samples underwent DNase digestion with RNA clean-up on Qiagen columns.

2.7.1.1 Column extractions

RNA was extracted using the RNeasy Mini Kit protocol (Qiagen). 20-30mg samples were homogenised in 350μL lysate buffer (340 buffer RLT, 10ul B-mercaptoethanol) using a bead homogenizer (FastPrep-24 5G homogenizer, MP Biomedicals), and placed onto ice. Samples were centrifuged at 15000 rpm for 3 min. Supernatant was removed into a new tube without disturbing the pellet. An equal volume of RNase-free 70% ethanol was added and mixed well before transfer into a spin column. Samples were centrifuged at full speed for 15s. Waste was discarded, and 250ul RW1 buffer was added before centrifugation at full speed for 15s. 80 ul DNase solution (10ul DNase1 + 70ul buffer RDD) was applied to the spin column membrane and incubated for 15 min. RW1 wash was repeated. A wash of 500 ul of buffer RPE buffer was performed twice. A new flow-through tube was used and centrifuged for 2 minutes. 30μL RNase-free H2O was applied to the spin column with a new collection tube, and centrifuged for 1 minute. The purity of 1μL of each RNA sample was assessed using the NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). Readings produced absorbance ratios comparing A260:A280nm wavelengths. Values between 1.8 and 2.1 indicated pure RNA samples. Quantities of RNA greater than 500ng were used for complementary DNA (cDNA) synthesised and gene analysis.
2.7.1.2 TRIzol

As per manufacturers protocol, tissue was homogenised in 1mL of TRIzol (Thermo Fisher Scientific). 200ul chloroform was added and vortexed briefly before incubating 2-3 minutes. Samples were centrifuged at 4°C 20000 x g for 15 minutes. The aqueous phase was removed into a separate tube. 500ul of isopropanol was added and vortexed. Samples were incubated for 10 minutes RT. Samples were centrifuged at 4°C 20000 x g for 10 minutes to pellet RNA. Isopropanol was removed without disturbing the pellet, and subsequently washed with 1 mL 75% RNase-free EtOH. Samples were mixed gently prior to centrifugation at 4°C 7500 x g for 5 minutes. The pellet was airdried prior to resuspension in RNase-free H₂O. For E11 and E13 samples, RNA was passed through Qiagen spin columns and DNase procedures conducted as above in section 2.8.1.1.

Note: Trophoblast outgrowths also had additional 1ul of GlycoBlue (Ambion) added in the isopropanol step and precipitated overnight at -80°C due to low abundance of RNA and for clear localisation of the RNA pellet.

2.7.2 RNA integrity

To determine RNA integrity, 200ng of RNA was run on a 1% agarose gel made using RNase-free DEPC-treated H₂O. RNA was run at 100V for 20 minutes in a tank of 0.5% TAE buffer in DEPC-treated H₂O. 5ul of 1000kb+ DNA ladder (Invitrogen) was run with RNA samples.

![Figure 2.2. Gel electrophoresis of RNA samples to examine RNA integrity. The first lane contains a 1kb+ DNA ladder.](image-url)
2.7.3 cDNA synthesis

2.7.3.1 Quantitek

Trophoblast outgrowth RNA was reverse transcribed using Quantitek reverse transcriptase kit (Qiagen) as per manufacturers protocol. Briefly, 6ul RNA (due to low abundance) and 1ul 7x gDNA wipeout buffer were combined, incubated at 2 minutes at 42°C, and subsequently placed immediately on ice. A master mix of 0.5ul Quantscript reverse transcriptase, 2ul Quantscript 5x RT buffer and 0.5ul RT primer mix was added to total 10ul. Samples were incubated at 42°C for 15 minutes prior to inactivation of reverse transcriptase for 3 minutes at 95°C. Samples were placed immediately on ice prior to dilution to 200ng/ul with RNase-free H2O.

2.7.3.2 Biorad iScript

1ug of total RNA was transcribed into cDNA as per protocol specified by iScript reverse transcription supermix (Bio-Rad Laboratories). 2uL per reaction of iScript reverse transcription supermix were added to 1ug RNA and nuclease-free water up to a total volume of 10uL. The above procedure was carried out on ice before being incubated in a thermal cycler. A StepOne Plus Real-Time PCR System (Applied Biosystems) was used for priming at 25°C for 5 minutes, reverse transcription for 30 minutes at 42°C, and inactivation for 5 minutes at 85°C. cDNA was diluted 1:10 with RNase-free water.

2.7.4 qPCR Quantification

2.7.4.1 TaqMan qPCR

1uL (100-200ng) cDNA with 5ul TaqMan (Qiagen), 0.1ul TaqMan assay-on-demand primers (Thermofisher), and multiplexed with 18S (0.1ul). The remaining volume to 10ul was made up with ultrapure water. Samples were run either in duplicate or triplicate. Genes were compared for multiplex (requiring 18S), and single-plex (no 18S) reactions before analysis. Additional housekeepers Rpl0 and Rpl13a (SYBR) were used to create a geometric mean with 18S. The 96-well plates were run in a
StepOneTM Real-Time PCR System (Applied Biosystems), for 1 hour. Primers and product codes are provided individually in Chapters 3-6.

Results were used to calculate relative gene expression using the ΔΔCT method (Moritz et al., 2002). The difference between the cycle thresholds (C_T) of the gene of interest and the housekeeping gene (18S) were calculated for each sample (ΔC_T). The mean ΔC_T for control (for cell culture experiments), or control males (for when analysing treatment with sex) was used to standardise against the other groups, by subtracting C_T of each sample from ΔC_T of the control (or control males), to generate the ΔΔC_T. Relative expression was then calculated utilising Equation 1.

\[
\text{Relative Gene Expression} = 2^{-\Delta\Delta C_T}
\]

**Equation 1. Relative gene expression calculation.** Method uses ΔΔC_T to calculate relative expression levels compared to the average of the control male group.

### 2.7.4.2 SYBR qPCR

SYBR reactions utilised 5ul SYBR green reagent (Qiagen) with 3ul H2O, 1ul of 4uM forward and reverse primers, and 1ul (100-200ng) cDNA. Samples were run in duplicate or triplicate. Samples utilised 3 housekeepers (18S, Rpl0, Rpl13a) as above. SYBR primers were purchased from commercially available predesigned primer pairs from KiCqStart SYBR Green (Sigma). Melt curves were assessed to ensure amplification of a single product. Primer efficiencies were calculated from amplification data “Delta Rn” and were plotted using the LinReg PCR program (version 11) (Ruijter et al., 2009).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Primer Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prl7a2</td>
<td>GGGAGAATGTGGGCTCTGTA</td>
<td>TTGAGCTTCCAGGTTTT</td>
<td>2.062</td>
</tr>
<tr>
<td>Prl7b1</td>
<td>GGGAGGACGTGGCTCTGTA</td>
<td>TTTGGTGAGTTGGGCAA</td>
<td>1.988</td>
</tr>
<tr>
<td>Tbpba</td>
<td>AAGTTAGGCAACCGAGCGAAA</td>
<td>AGTGCAGGATCCCACCTGTC</td>
<td>2.023</td>
</tr>
<tr>
<td>Prl3d1 (Pl1)</td>
<td>CTGCTGACATTAAGGGCA</td>
<td>AAAAGAGACCATGGGCA</td>
<td>1.930</td>
</tr>
<tr>
<td>Prl2c (Plf)</td>
<td>TGTGTGCAATGAGAATGTT</td>
<td>TAGTGCTGAGCTTGGGT</td>
<td>2.045</td>
</tr>
<tr>
<td>Syna</td>
<td>ATGGGAGAACCCTTACGCT</td>
<td>TAGGGGTCTTTTGTGCCCTG</td>
<td>2.099</td>
</tr>
<tr>
<td>Ctsq</td>
<td>TTCATTGCGCCAATCCCTA</td>
<td>GAAGCTCCCAGAATTCACA</td>
<td>1.972</td>
</tr>
<tr>
<td>18S</td>
<td>GTAACCCTGTCGAGGTTT</td>
<td>CCACTCGAATCTGGTAGCG</td>
<td>1.976</td>
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</table>

**Table 2.5. SYBR primers (mouse) and primer efficiencies.**
Table 2.6. SYBR primers (rat) and primer efficiencies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Primer Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rpl13a</td>
<td>GCA CAA GAC CAA AAG AAG</td>
<td>CGC TTT TTC TTG TCA TAG GG</td>
<td>2.033</td>
</tr>
<tr>
<td>RplO</td>
<td>GAG TGA CAT CGT CTT TAA ACC</td>
<td>AAG CAT TTT GGG TAG TCA TC</td>
<td>2.023</td>
</tr>
<tr>
<td>Prl3d1</td>
<td>AGA CCT TAT ACA ACA GGA CTC</td>
<td>ATG GCA AAA GAT GAG TGT C</td>
<td>1.992</td>
</tr>
<tr>
<td>Hand2</td>
<td>CTC CAA AAT CAA GAC TCT GC</td>
<td>CAT TCA GCT CTT TCT TCC TC</td>
<td>1.963</td>
</tr>
<tr>
<td>Ncoa6</td>
<td>AAA AGA TCT TCT CGA CCT GC</td>
<td>GTA TCA AGT CAT CTT CCT GC</td>
<td>1.989</td>
</tr>
<tr>
<td>Usag1</td>
<td>ACT GGA TCG AAA TAG TCG AG</td>
<td>TCC AGT ACT TTG TCC GTG AG</td>
<td>1.945</td>
</tr>
<tr>
<td>Vdup1</td>
<td>CGT CAA TAC TCC TGA CTT AA TAT G</td>
<td>AAA TGT CAT CAC CTT CAC AG</td>
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<tr>
<td>Esr1</td>
<td>ATA TGA TCA ACT GGG CAA AG</td>
<td>CAT TTA CTT TCA TGG TCC GC</td>
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<tr>
<td>Pgr</td>
<td>TCT AAT CCT GAA TGA GCA GAG</td>
<td>GAC TTT CAT ACA GAG GAA CTC</td>
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</tr>
<tr>
<td>Fgf2</td>
<td>AAA CTC GGA TCC AAA ACG</td>
<td>TGT CTA AAG AGA GTC AGC TC</td>
<td>1.998</td>
</tr>
<tr>
<td>Fgf9</td>
<td>ACT ATA AAT GCT TCA TGC GG</td>
<td>CAA TAA ATC AAG CAA GTG GC</td>
<td>1.960</td>
</tr>
<tr>
<td>Igfbp-1</td>
<td>AAA CTG AAA GTT GTT TCC TCC G</td>
<td>ATA CAA ACC CAC TTG TAC ATC</td>
<td>1.969</td>
</tr>
<tr>
<td>Cyp26a1</td>
<td>CAA GCA GCG AAA GAA GGT GA</td>
<td>CTT CAG CAA TCA CTG GCA CG</td>
<td>1.940</td>
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<tr>
<td>Cnr1 (CB1)</td>
<td>CAC CCA TGG CTG AGG GTT CC</td>
<td>CCC ACG TAG AGG AGG TCT GT</td>
<td>1.985</td>
</tr>
<tr>
<td>Klf5</td>
<td>AAT CCA AAT TTA CCT GCC AC</td>
<td>TGC AAC CAT CAT AAT CAC AG</td>
<td>1.909</td>
</tr>
<tr>
<td>Ihh</td>
<td>AAA GAC GAG GAG AAC ACC</td>
<td>AAGATTCCTCTGAGTGG</td>
<td>1.924</td>
</tr>
<tr>
<td>Muc1</td>
<td>CCG AAG TCA ATG TGA ATG AG</td>
<td>CAA AAT ACA GAC CAG TAC CAG</td>
<td>1.955</td>
</tr>
</tbody>
</table>

2.8 Genotyping for sex

2.8.1 Blastocyst and trophoblast outgrowths

After staining procedures of the pre-implantation embryos or blastocyst outgrowths, they were removed mechanically from coverslips and digested overnight at 55°C in 5ul of lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM, MgCl2.6H2O, 0.1 mg/ml gelatin, 0.45% v/v Nonidet P40, 0.45% v/v Tween 20) with 1ul of 20mg/mL proteinase K. Proteinase K was then inactivated for 10 mins at 95°C. The total 6ul sample was used to amplify male determining gene Sry and B-Actin as a control. Amplification of both genes was carried out in the same reaction due to limited DNA content. Primers for Sry (317bp) and B-Actin (220bp) were derived from Miyajima et al. (2009) for use in the rat. Sry forward primer 5'-TACAGCCTGAGGACATATTA-3',
reverse primer 5’-GCACCTTAAACCCTCGATGA-3’. B-Actin forward primer 5’-
AGCCATGTACGTAGCCATCC-3’, reverse primer 5’-
TGTGTTGTTGAAGCTGTAGC-3’. PCR conditions were 95°C for 2 mins, followed by
35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min, and extension at 72°C for
5 mins. A 2% gel ran for 35 mins at 100V to separate bands. Double bands
represented male embryos and single bands represented female embryos. Samples
with no bands were eliminated from analysis. Male and female controls were amplified
from E20 fetal tail tissues.

2.8.2 E11 to E20 placental samples

DNA from extraembryonic tissues was collected at post-mortem and extracted using
QuickExtract (Epicentre) as per the manufacturers protocol. 50ul QuickExtract reagent
with a small 1-2mm³ piece of embryonic tail or extraembryonic membranes was added
and vortexed briefly. Extraction conditions were 65°C for 20 minutes and 95°C for 10
minutes. Samples were stored at -20°C prior to analysis. A qPCR run was set up for
Sry as per section 2.8.4.1 to detect gDNA. Cluster analysis showed E11 and E13 male
samples of extraembryonic tissues grouped in cycles 20-25, while E15 and E20
samples of fetal tail clustered from cycles 15-20. All other samples above these cycles
were assumed to be female.

2.9 Histology

2.9.1 Haematoxylin and eosin

Slides were dewaxed in xylene for 3 x 2 min. Slides were rehydrated in 2 x 100%
EtOH, and 1x 90% EtOH, 1x 70% EtOH for 2 min, and 1x dH₂O for 2 min. Slides were
incubated in Meyers haematoxylin for 2 mins prior to extensive washing in dH₂O. Slides
were incubated in 1% alcoholic eosin for 15 mins. Slides were dehydrated in 95%
EtOH, 2x 100% EtOH and 2x xylene washes prior to mounting in DPX mounting
medium (Ajax Finechem).
2.9.2 Immunofluorescence for whole-mount pre-implantation embryos, trophoblast outgrowths and placenta cryo-sections

Embryos were prepared for immunohistochemistry as previously described (Pantaleon et al., 2010). Briefly, embryos were fixed in 2% paraformaldehyde in 1x PBS for 20 minutes RT, washed in PBS, and immobilized on Cell-Tak (Collaborative Biomedical Products) coated coverslips. Embryos were permeabilised in 0.25% Triton in PBS and washed before blocking with 10% normal goat serum/BSA/Tween-20/PBS (PBT) for 1 h RT. Embryos were incubated in 1:50 anti-Mouse CDX2 (BiogenX, MU392A-UC) overnight at 4°C. Following, embryos were extensively washed in PBT prior to exposure of goat anti-mouse labelled secondary antibody Alexa 488 (1:1000, Molecular Probes) for 1 h RT. Embryos were counterstained with DAPI (Sigma Aldrich Inc, B2388) in PBS for 15 min and transferred through increasing concentrations of glycerol in PBS before mounting with Vectashield (Vector Labs, H-1000). Trophoblast outgrowths were treated above, incubating in anti-rabbit pan-cytokeratin (DAKO, Z0622) to distinguish cytoskeleton, goat anti-rabbit af568 secondary antibody, and DAPI (Sigma Aldrich Inc, B2388) to study number and ploidy of trophoblasts. Immunolocalization was carried out as above on E13 midline placental cryosections. Placental halves were embedded cut face down, and were cut at 10um, with every 10th section stained with 1:200 anti-rabbit pan-cytokeratin (DAKO, Z0622) to distinguish invasive placental trophoblasts, and 1:100 anti-mouse alpha smooth muscle actin (ab7817, Abcam) to distinguish uterine spiral arteries. No invasion was seen in all sections regardless of treatment after section 6. Goat anti-rabbit af555 and goat anti-mouse af488 secondary antibodies were used. All slides were counterstained with DAPI (Sigma Aldrich Inc, B2388), prior to image analysis.

2.9.3 In situ hybridisation and fluorescent in situ hybridisation (FISH)

2.9.3.1 Construction of cRNA probes

To construct ISH primers, firstly, gene sequences for the rat (Rattus norvegicus) were selected in the database Ensembl (Oxford). Primers were then designed with the program Primer3 (NIH) with specifications with oligo primer sequences between 18-24nt, product size between 400-600bp long, melting temperature (T_m) of primers between 57-60°C, and % GC content between 42-62. Primers were then checked
using Primer-Blast (NCBI) for sequence homology. Once forward and reverse primer sequences were selected, T3 sequence (AATTAACCCTCACTAAAGGG) was added to the beginning of the forward primer, and the T7 sequence (TAATACGACTCACTATAGGG) was added to the beginning of the reverse primer. Primers were then manufactured by Sigma, see Table 2.5 for genes and respective sequences. Oligonucleotides were created using standard PCR and run on a 1% agarose gel to check product sizes. DNA was then extracted using a QIAquick Gel Extraction Kit (Qiagen), as per manufacturer’s instructions. Briefly, agarose gel containing DNA oligonucleotides were weighed and added 3x volume of buffer QG per 100mg agarose and heated at 50°C for 10 minutes with samples vortexed every 2 min to aid dissolution. 1x volume of isopropanol was added and gently mixed before addition to a QIAquick spin column. Samples were centrifuged for 1 min RT at maximum speed and flow-through discarded. Washes then included 500ul buffer QG, and 750ul buffer PE. To elute DNA, 20ul buffer EB was added, and centrifuged. Single-stranded DNA was quantified using the NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). cRNA probes were then constructed using digoxigenin (DIG) labelling as per manufacturer’s instructions (Roche). 100-200ng DNA from PCR product was added to each reaction containing 2ul 10x transcription buffer (Roche), 2ul DIG reaction mix (Roche), 2ul T7 or T3 RNA polymerase, and RNase free H2O to 20ul. Reactions were incubated at 37°C for 2 hours prior to addition of 2ul 0.2M EDTA-DEPC to stop reactions. 2.5ul 4M LiCl4 and 75ul 100% EtOH was added to precipitate cRNA, prior to centrifugation at maximum speed for 20 minutes at 4°C to form a pellet. cRNA was washed with 70% EtOH and centrifuged again prior to air drying pellet, and resuspension in 20ul RNase-free H2O. 1ul of cRNA was run on a 1% agarose gel made in DEPC H2O for 20 min at 100V to check RNA integrity. cRNA was stored at -80°C prior to use.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence (5'-3')</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mest</td>
<td>Forward (T3)</td>
<td>CTGCTCTGCACCTCATGGAAG</td>
<td>478</td>
</tr>
<tr>
<td></td>
<td>Reverse (T7)</td>
<td>CCGTCTTTTAGAGGAGCTTTTG</td>
<td></td>
</tr>
<tr>
<td>Prf1</td>
<td>Forward (T3)</td>
<td>AGCCAGTGCTCAAGCGAAT</td>
<td>414</td>
</tr>
<tr>
<td></td>
<td>Reverse (T7)</td>
<td>CAGTCCCTGGTGTCGTTAGCC</td>
<td></td>
</tr>
<tr>
<td>Prl8a2 (dPRP)</td>
<td>Forward (T3)</td>
<td>TGCATCACTCTAGCCCTCTGTC</td>
<td>871</td>
</tr>
<tr>
<td></td>
<td>Reverse (T7)</td>
<td>TTTCCCTGTTATGCGACACAA</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.7 In situ hybridisation primers and product length.** T3; AATTAACCCTCACTAAAAGGG and T7; TAATACGACTCACTATAGGG sequences were added to the beginning of forward and reverse primers respectively. Note; Prl8a2 is also known as decidual prolactin-related protein (dPRP).

**2.9.3.2 In situ hybridisation protocol for paraffin sections**

Day 1 procedures all utilised solutions made in RNase-free DEPC-treated milliQ water. Firstly, paraffin slides were dewaxed with xylene (2 x 10 minutes), and rehydrated in ethanol (2x 100% 10 minutes, and 5 minutes in 95%, 70% and 50% EtOH), and into 1x PBS-DEPC for 5 minutes. Tissues were fixed in 4% PFA/1x PBS-DEPC for 20 minutes prior to 2 1x PBS-DEPC washes. Slides were treated in proteinase K (30 ug/ml) for 15 minutes prior to 1x PBS-DEPC wash and an additional 4% PFA/1x PBS-DEPC fixation, both for 5 minutes each. Slides were incubated in acetylation buffer (acetic anhydride, 0.25%; Sigma) for 2x 5 minutes, with an additional 500ul acetic anhydride added in the second wash. Following 1x PBS-DEPC wash, slides were incubated in 1:2000 DIG or FITC-labelled probes in hybridisation buffer overnight at 70°C in a humidified chamber of 1X salts (200 mM sodium chloride, 13 mM tris, 5mM sodium phosphate monobasic, 5 mM sodium phosphate dibasic, 5 mM EDTA)/50% formamide. Hybridisation buffer contained 1x salts, 50% formamide, 10% dextran sulfate, 1 mg/ml yeast tRNA (Roche), 1x Denhardt's (1% w/v bovine serum albumin, 1% w/v Ficoll, 1% w/v polyvinylpyrrolidone).

Day 2 procedures began with 2 x post-hybridisation (1x saline-sodium citrate (SSC) buffer, 50% formamide, 0.1% tween-20) washes at 65°C for 30 minutes. Two washes of 1x MABT (150 mM sodium chloride, 100 mM maleic acid, 0.1% tween-20, pH7.5) at 30 minutes RT, 1x RNA wash (400 mM sodium chloride, 10 mM tris pH7.5, 5 mM EDTA, 20 mg/ml RNase A) at 37°C for 10 minutes, 1x RNA wash with additional RNase A (10mg/ml) for 30 minutes at 37°C, 1x RNA wash for 5 minutes at 37°C and 1x MABT.
wash for 5 minutes RT. Slides were blocked for 1 hour in 1x MABT, 2% blocking reagent (Roche), 20% heat inactivated horse serum. Anti-DIG antibody was diluted 1:2500 in blocking and incubated overnight in a humidified chamber at 4°C.

Day 3 procedures were all conducted at RT and began with 4 washes in 1x MABT for 15 minutes, 1 wash in NTMT buffer (100 mM NaCl, 50 mM MgCl, 100mM tris pH 9.5, 0.1% tween-20) for 10 minutes, 1 wash in NTMT with 100mg of levamisole for 10 minutes prior to application of colour reagent (4.5 ul/mL NBT, 2.5ul/mL BCIP in NTMT) as per manufactures instructions (Promega). Development of colour reactions was conducted in a light protected box. Upon development of appropriate colour, slides were incubated in 1x PBS prior to counterstaining in nuclear fast red for 2 minutes, dehydrating through ethanol (2 minutes in 50%, 70% 90%, 2x 100%) and xylene (2x 2 minutes) washes, and mounting in Dibutylphthalate Polystyrene Xylene (DPX) mounting medium (Ajax Finechem).

2.9.3.3 Modifications for fluorescent in situ hybridisation for paraffin and cultured cells

Fluorescent in situ hybridisation (FISH) was used in paraffin and cell cultures as described by (Simmons et al., 2007). Fluorescein or DIG-labelled probes were made following manufacturer’s instructions (Roche) as described previously (Simmons et al., 2007, 2008b). In brief, cells were fixed in 4% PFA for 15 min and transferred to 0.4% PFA until analysis. Cells were not treated with proteinase K, but were acetylated for 5 minutes prior to administration of FITC- or DIG-labelled probes at 1:200 which were hybridised overnight at 65°C. The following day, cells were washed in 5xSSC pre-warmed to 65°C for 3 x 20 min, followed by TN buffer (100mM Tris-HCl ph7.5, 150mM NaCl) for 5 mins prior to incubation in block consisting of 1x MABT (150 mM NaCl, 100 mM maleic acid, 0.1% Tween-20, pH 7.5), 2% blocking reagent (Roche), and 20% heat inactivated horse serum for 1h. Samples were incubated in anti-DIG-POD or anti-FITC-POD antibody (1:500, Roche) overnight. The following day, cells were washed in TNT buffer containing 0.05% Tween20 in TN buffer for 3 x 15 min prior to probe detection with 1:50 FITC- or Cy5-Tyramide in amplification reagent (TSA kit, Perkin Elmer) for 10 min. Samples were washed in TNT buffer for 3 x 5 min and counterstained with DAPI (Sigma Aldrich Inc, B2388). Coverslips were mounted in
70% glycerol prior to imaging. Prior to use on cultured cells, probes were tested on either E12.5 or E14.5 mouse placentas fixed in 4% PFA overnight to confirm subtype-specific expression.

2.9.4 Lectin histochemistry

Paraffin slides were dewaxed and rehydrated as in ISH day 1 procedures (see section 2.10.3.2), and placed into dH$_2$O. Slides underwent antigen retrieval in sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0) was performed for 1.5 hours using a pressure cooker. Tissue was blocked for endogenous peroxides using 0.9% H$_2$O$_2$ in dH$_2$O for 10 minutes. Slides were incubated in 0.1% triton with 0.1mM ions (MgCl$_2$, CaCl$_2$, MnCl$_2$) for 10 minutes, prior to incubation of 1:200 IsolectinB4 (conjugated, Sigma, L5391, derived from Bandeiraea simplicifolia) in 1x PBS for 2h RT. A 1x PBS wash for 5 minutes followed prior to colour development with NovaRED peroxidase (Vector Labs, SK-4800). Slides were counterstained with haematoxylin for 2 minutes, and were dehydrated and mounted as per section 2.10.3.2.

2.9.5 Immunohistochemistry

Paraffin slides were dewaxed and rehydrated as in ISH day 1 procedures (see section 2.10.3.2), and placed into dH$_2$O. Slides underwent antigen retrieval in sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0) was performed for 2 hours using a pressure cooker. Slides were washed 3 times in 1x PBS for 5 minutes. Tissue was blocked for endogenous peroxides using 0.9% H$_2$O$_2$ in dH$_2$O for 10 minutes. Slides were washed 3 times in 1x PBS for 5 minutes. Slides were blocked in 10% normal goat serum/BSA/Tween-20/PBS (PBT) for 1 h RT. E7 samples were incubated 1:200 anti-rabbit vimentin (Thermofisher, PA5-27231) at 4°C overnight. The following day, slides were washed 3 times in 1x PBS for 5 minutes. Slides were incubated in 1:200 biotinylated anti-rabbit secondary antibody (Vector laboratories, PK-6101) for 1h at 37°C. Slides were washed 3 times in 1x PBS for 5 minutes. Slides were incubated in Avidin-Biotin complex (Vector laboratories, PK6100) for 1h at 37°C. Slides were washed 3 times in 1x PBS for 5 minutes prior to chromogen development utilised ImmPACT NovaRED Peroxidase Substrate (Vector Laboratories, SK-4805) as per manufacturers protocol.
2.9.6 Transmission electron microscopy

Labyrinth samples from E15 and E20 placentas were cut into 1mm³ and fixed into 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer until tissue processing. All samples were washed in 0.1M sodium cacodylate buffer at 250W and fixed further in 1% osmium tetroxide in cacodylate buffer at 80W under vacuum in a Biowave (Pelco). Samples were rinsed with water at 250W prior to counterstaining in 2% uranyl acetate\textsubscript{(aq)} at 150W under vacuum to enhance contrast. Samples were dehydrated in ethanol series (30%, 50%, 70%, 90%, 100%, 100%) at 250W and were infiltrated in increasing series of epon resin (1:3, 1:1, 3:1, 100%, 100%) under vacuum. Resin was polymerised for 48h at 60°C. Thick sections were cut at 500nm and stained with Toluidine blue to confirm localisation of the labyrinth. Thin sections of 100nm were post-stained with lead citrate prior to visualisation with an electron microscope (Jeol JEM-101). Two blocks per labyrinth samples were used to gather a minimum of 6 IHMs of which were averaged per block. See Chapter 3 Figure 3.5 for images.

2.10 Imaging parameters and analysis

2.10.1 Blastocysts

Embryos and outgrowths were visualised using an inverted microscope (Leica, DMi8). Pre-implantation embryos utilised a 40x air objective and captured in z stacks of 40. Excitation filters of 488nm (green), and 405nm (blue) fluorescence were imaged separately. CDX2 immunoreactivity (green) was localised to the TE of the E5 blastocyst. Cells devoid of CDX2 staining were assumed to be ICM. TE and ICM cells were counted individually using Image J (NIH). ICM proportion was calculated with Equation 2.

\[
ICM (%) = \left( \frac{\text{number of ICM cells}}{\text{number of total cells}} \right) \times 100.
\]

Equation 2. Quantification of the percentage of inner cell mass cells to total cells of the E5 blastocyst. ICM; inner cell mass.

2.10.2 Determination of CDX2 TE nuclear fluorescence

Nuclear fluorescent of CDX2 was determined using the imaging processing tool Imaris (version 7.7.2, Bitplane). Firstly, a surface (or mask) was created around the DAPI channel, creating a 3D object around the nucleus (see Figure 2.3 below). Surface area
detail was 0.568 (absolute intensity), and a threshold of 20.1 was use. To segregate adjoining nuclei, a seed point diameter of 5um was utilised. Additional manual processing was also used in cases in which some adjoining nuclei were not separated. CDX2 fluorescence intensity was then measured within these nuclei, with all nuclear fluorescence summed to create final counts.

Figure 2.3. Computer generated surface of DAPI channel enclosing cell nuclei using Imaris.

2.10.3 Trophoblast outgrowths

Outgrowths were imaged using an inverted microscope (Leica, DMi8) at 20x in z-stacks of 10 in a tile scan. Excitation filters 561nm (red) and 405 (blue) were imaged separately. For trophoblast outgrowths, Z-stacks of the DAPI channel were compressed using the average intensity for manual cell counts and ploidy analysis using ImageJ, see section 2.1.4. ICMs - indicated by negative staining of pan-cytokeratin, were edited out using Photoshop. Trophoblasts were considered if nuclear size exceeded 200um², and those above 1000um² were considered P-TGCs.

2.10.4 Determination of nuclear DNA content of trophoblasts

For trophoblast outgrowths, Z-stacks of the DAPI channel were compressed using the average intensity for ploidy analysis using ImageJ. Smallest trophoblasts with positive staining for pan-cytokeratin had nuclear sizes exceeding 200um². ICM cells (2N) were
found to be less than 200μm² in size. Nuclear sizes above 1000μm² were considered P-TGCs. DNA content, a surrogate marker of ploidy, was quantified from fluorescent intensity of DAPI, localising the nucleus. DNA content of all trophoblasts and P-TGCs were normalised to ICM cells of the same image to obtain relative ploidy to 2N.

2.10.5 Quantification of trophoblast invasion at mid-gestation
Placental cryo-sections were imaged on an upright fluorescence microscope (BX61 Olympus) using DAPI, GFP and Cy3 filters at 4x and 10x magnification using set parameters. Placentas were measured for distance of trophoblast invasion from the P-TGC layer (identified by large polyploid nuclei), to most distant pancytokeratin positive SpA-TGC.

Figure 2.4. Immunofluorescent image of E13 placenta to examine trophoblast invasion and spiral artery remodelling. Pan-cytokeratin (red) marks invading trophoblasts, alpha smooth muscle actin (green) and counterstained with DAPI (blue). White arrows show spiral arteries, yellow double-edged arrow shows distance of invasion from P-TGC layer, and red arrow shows invaded SpA-TGC cells. The dashed white line shows the barrier between placental decidua and the uterus. Images were captured at either 4x or 10x magnification.
2.10.6 Quantification of FISH positive cells in culture

Cells were counted using ImageJ (NIH). Z stacks were compressed with the maximum projections for DAPI and merged (DAPI and FISH marker). The greyscale DAPI image was converted to 8 bit, underwent watershed to separate adjoining nuclei, and counted with the automated “Analyse Particles” function. FISH positive cells were counted manually from merged images using “Cell Counter”.

2.11 Stereology of placental and maternal tissues

2.11.1 Stromal and decidual volume at E7

E7 implantation sites were serially sectioned at 5μm and collected. Sections were identified for midline invasion of the embryo. 5-10 equally spaced sections 1:10 were selected and underwent ISH for Prl8a2, to detect the primary decidual zone of the uterus (Dai et al., 2002), or immunohistochemistry for vimentin to localise uterine stroma in the rat (Glasser and Julian, 1986, Korgun et al., 2007), see Figure 2.5. A grid was superimposed over each section using ImageJ (NIH) and counted for Prl8a2 positive grid area. Total volume was estimated using the Cavalieri principle displayed in Equation 3.

\[
Volume = \Sigma P_{\text{compartment}} \times a_p \times T
\]

Equation 3. Total volume estimation. This equation sums (\(\Sigma\)) the number of points within each section (\(P_{\text{compartment}}\)), and multiplies this by the area of points or grid size (\(a_p\)), and the thickness (\(T\)); being the cutting thickness multiplied by the sampling fraction.
Figure 2.5. Uterine histochemical markers of E7 implantation site. A; Immunohistochemistry for vimentin marking all uterine stromal cells. B; Prl8a2 ISH for primary decidual cells (anti-senseT7). C; negative staining of vimentin. D; sense probe staining for Prl8a2 (T3).

2.11.2 Quantification of decidual natural killer cells

In situ hybridisation for the marker Prf1 was carried out on the E11 placenta. Similarly, for quantification of maternal decidua in section 2.12.1, E11 samples were quantified from 5 sections taken at 1:50. Using ImageJ (NIH), images were split into separate channels, with the red channel converted to black and white for counting with the “Analyse Particles” function. All samples were sections at 5um in paraffin, and utilised the same Equation 3 (section 2.13.1) for estimation of total number.
Figure 2.6. Prf1 ISH for infiltrating maternal decidual natural killer cells (dNK) at E11 and E15 implantation sites. A (E11) and B (E15) show positive staining of Prf1 with anti-sense probe (T7). C shows sense probe staining (T3).

2.11.3 Estimation of placental volumes

Placental halves were sectioned exhaustively at 5µm for the collection of 5 equally spaced sections, for E13 – 1:50, E15 – 1:100, and E20 – 1:150. Unbiased stereology for placental volumes was carried out using the Cavalieri principle, as described by Coan et al. (2004) using ImageJ (NIH). To identify structures within the placenta, E13 samples were stained with haematoxylin and eosin (as per section 2.10.1) to localise the nucleated fetal blood cells at E13, E15 placentas underwent in situ hybridisation for the fetal endothelial cell marker Mest (as per section 2.10.3.1-2), and E20 placentas were stained with the endothelial marker Isolectin B4 (as per section 2.10.4), also localising cells of the fetal blood space. See Chapter 3 - Figure 3.4, for representative images of each marker. Volumes of total placenta, labyrinth, junctional zone, and decidua were carried out at each age at low magnification (2x) along with FBS and MBS at high (40x) magnification within the labyrinth. Volume analyses also used Equation 3 in section 2.13.1, and doubled due to quantification of half the placenta. FBS and MBS were quantified in 3 small windows of the labyrinth, averaged, and multiplied by fractional volume for the whole labyrinthine zone. Surface areas of FBS capillaries and MBS spaces using randomly offset cycloid arcs as described by Baddeley (1986), and for use in the placenta by Coan et al. (2004). Estimation of these surface areas used Equation 4 (below).
\[ Sv = 2^* \Sigma I_{\text{cap}}/(l_{(p)} \,) \Sigma P_{\text{lab}} \, V_{\text{lab}} \]

Equation 4. Estimation of surface areas of fetal capillaries and maternal blood spaces. To determine surface area of vessels (Sv), the equation sums the number of intersections between cycloid arcs and the fetal capillary endothelium (Σ I_{cap}) and divides this by the length of the test line \( l_{(p)} \) and the sum of test points marking the labyrinth Σ P_{lab}. This is finally multiplied by the total volume of the labyrinth \( V_{\text{lab}} \).

2.12 Statistical analyses

For embryo studies in Chapter 3 analysing differences between males and females, statistically significant differences \((^*P<0.05; **^P<0.01)\) were analysed using student’s t tests. When student’s t tests showed unequal variances, Mann-Whitney’s non-parametric tests were used. For embryo studies in Chapter 5 analysing treatment (PC-EtOH, liquid diet control, and in some cases chow control) with sex, two-way ANOVA with Tukey’s multiple comparison tests were used. To analyse changing outgrowth area over time, repeated measures were additionally used. In cases in which numerous animals were utilised from one litter, litter averages were calculated. Cell culture experiments in Chapter 4 which analysed treatments of control, 0.2% and 1% EtOH were analysed by one-way ANOVA, with each treatment containing a mean of at least 3 replicates. For gene expression assays, mean relative expression of treatments were standardised to either the control group (in cell culture experiments), the control male group (if analysing treatment with sex), or liquid diet control (for maternal physiology experiments), with all data displayed as mean ± standard error of the mean. All graphs and statistics were performed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA).
Chapter 3. Sex differences in rat placental development: from pre-implantation to late-gestation

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Sample collection (100%)  
Interpretation of results (50%)  
Writing manuscript (100%) |
| Simmons, D.G.      | Experimental design (25%)  
Reviewing and editing manuscript (10%) |
| Pantaleon, M.      | Interpretation of results (25%)  
Reviewing and editing manuscript (5%) |
| Moritz, K.M.       | Experimental design (25%)  
Interpretation of results (25%)  
Reviewing and editing manuscript (75%) |

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Sex differences in rat placental development: from pre-implantation to late-gestation

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3.1 Abstract

**Background:** A male fetus is suggested to be more susceptible to *in utero* and birth complications. This may be due in part to altered morphology or function of the XY placenta. We hypothesised that sexual dimorphism begins at the blastocyst stage with sex differences in the progenitor trophectoderm (TE) and its derived trophoblast lineages, as these cells populate the majority of cell types within the placenta. We investigated sex specific differences in cell allocation in the pre-implantation embryo, and further characterised growth and gene expression of the placental compartments from the early stages of the definitive placenta through to late gestation. **Methods:** Naturally mated Sprague Dawley dams were used to collect blastocysts at embryonic day (E) 5 to characterise cell allocation; total, TE and inner cell mass (ICM), and differentiation to downstream trophoblast cell types. Placental tissues were collected at E13, E15, and E20 to characterise volumes of placental compartments, and sex-specific gene expression profiles. **Results:** Pre-implantation embryos showed no sex differences in cell allocation (total, TE, ICM), or early trophoblast differentiation, assessed by outgrowth area, number and ploidy of trophoblasts and P-TGCs, and expression of markers of trophoblast stem cell state or differentiation. Whilst no changes in placental structures were found in the immature E13 placenta, the definitive E15 placenta from female fetuses had reduced labyrinthine volume, fetal and maternal blood space volume, as well as fetal blood space surface area, when compared to placentas from males. No differences between the sexes in labyrinth trophoblast volume or interhaemal membrane thickness were found. By E20 these sex-specific placental differences were no longer present, but female fetuses weighed less than their male counterparts. Coupled with expression profiles from E13 and E15 placental samples may suggest a developmental delay in placental differentiation. **Conclusions:** Although there were no overt differences in blastocyst cell number or early placental development, reduced growth of the female labyrinth in mid gestation is likely to contribute to lower fetal weight in females at E20. These data suggest sex differences in fetal growth trajectories are due at least in part, to differences in placenta growth.

**Keywords:** Blastocyst, Trophectoderm, Placenta, Trophoblast, Sexual Dimorphism, Differentiation, DOHaD.
3.2 Introduction

Exposure of the fetus to a sub-optimal in utero environment such as poor maternal nutrition, stress or hypoxia can alter its development, potentially in a sexually dimorphic manner (Kalisch-Smith et al., 2016). Male fetuses are often reported to be at greater risk of preterm birth, neonatal complications and prenatal mortality (Peacock et al., 2012, Walker et al., 2012). For this reason, it is commonly viewed that males are more susceptible to developmental perturbations than females (Clifton, 2010). Critical to normal growth and viability of the fetus, is adequate development of the mature chorioallantoic placenta. Males often exhibit larger placental growth (weight) than females in uncomplicated human pregnancies at term (Eriksson et al., 2010). This is thought to help facilitate the greater growth trajectory of the male fetus. Sexual dimorphism in placental development is most often examined following prenatal stressors (O’Connell et al., 2013b, Cuffe et al., 2014a), but inherent differences have rarely been investigated. Study of the placenta is normally restricted to analysis at late gestation or term. Interestingly, sex specific changes have been observed in the late gestation placenta of the rat in a model of alcohol exposure which only occurred during the periconception, prior to implantation (Gardebjør et al., 2014). The question then arises to when does sexual dimorphism in growth of the placenta emerge, and can it be traced back to the pre-implantation embryo?

The placenta and its associated trophoblast subtypes derive from the trophectoderm (TE) of the pre-implantation embryo. In the rodent, a subset of cells from the TE differentiate into invasive trophoblast giant cells, which migrate into the maternal uterine compartment, while the remaining TE cells form later in gestation to populate either the labyrinth or junctional zone compartments (Cross et al., 1994, Simmons and Cross, 2005). The labyrinth contains fetal and maternal vasculature for the two-way exchange of nutrients and waste products, while the junctional zone is thought to have a primary endocrine role (Coan et al., 2004). Placentas from males and females can alter their growth in a sex-specific and zone-specific manner when confronted with maternal perturbations in different critical windows, which can lead to sex-specific alterations in fetal viability, recently reviewed by us in Kalisch-Smith et al., (2016). Investigations of sex differences in the early embryo have been largely restricted to analysis of in vitro models which use superovulation to generate large numbers of embryos. Both hormone induced ovulation and in vitro culture techniques are
implicated in causing stress to the early embryo, influencing its development (Giritharan et al., 2012, de Waal et al., 2012) and therefore may be potential confounders for the determination of basal sex differences. Current analysis in vitro, suggests male mouse (Tsunoda et al., 1985, Burgoyne, 1993, Valdivia et al., 1993, Peippo and Bredbacka, 1995 Perez-Crespo et al., 2005), and bovine (Avery et al., 1992, Xu et al., 1992, Ghys et al., 2015) embryos develop to the blastocyst stage at a faster rate than female embryos. Quantification of cell number in vitro supports this outcome, with males displaying increased total, TE or ICM count (Ghys et al., 2015, Tan et al., 2016b, Dumoulin et al., 2005). However, there is conflicting evidence in human (Dumoulin et al., 2005, Richter et al., 2006, Weston et al., 2009), bovine (Holm et al., 1998, Rizos et al., 2008, Bermejo-Alvarez et al., 2010a, porcine (Kaminski et al., 1996, Lechniak et al., 2000), and in vivo derived mouse model (Peippo and Bredbacka, 1995), which rather show equal developmental rates between the sexes. Indeed, culture in vitro has been shown to increase cell death in the early embryo, and therefore, may suggest that the reported sex differences are not in embryonic growth, but in differential survival from an artefact of culture. Sexual dimorphism is also suggested to exist in blastocyst physiology. Metabolic rate (Tiffin et al., 1991) and stress responses (Perez-Crespo et al., 2005) are suggested to be heightened in males, while females seem to more susceptible to cell death (Ghys et al., 2015, Tan et al., 2016b). Expression profiles of bovine male and female blastocysts show sexual dimorphism of both X-linked and autosomal genes (Bermejo-Alvarez et al., 2008, 2010b), whilst sexual dimorphism has also been shown in expression profiles as early as the 8-cell stage in the mouse (Lowe et al., 2015).

In this study, we use naturally cycling rat dams (without superovulation) to characterise sex differences in the pre-implantation embryo, through to trophoblast differentiation, and the formation of the placental architecture. This allowed us to examine inherent sex differences without prenatal stressors and highlights the importance of appropriately derived animals to investigate these outcomes. We hypothesised inherent sex differences exist in the blastocyst stage with differences in the progenitor trophectoderm (TE) and its derived trophoblast lineages which result in sex differences in growth of the definitive placenta.
3.3 Methods

3.3.1 Ethics

All animal experiments and procedures were approved by The University of Queensland Animal Ethics Committee (AEC approval number SBS/022/12/NHMRC) prior to commencement of this study.

3.3.2 Animal treatment, embryo collection and culture

Dam weight was at least 230g before entering the protocol. Sprague Dawley dams were maintained on standard laboratory chow. In addition, tissues (blastocysts and placentas) were available from dams fed either chow or a control liquid diet used previously as a control for ethanol fed dams (Gardebjer et al., 2014, 2015). Analysis of cell counts showed no differences between diet groups (data not shown). Dams were time-mated over a 5-hour window from 1200h to 1700h. The following day was considered E1. At 0900h on E5, a subset of dams (N = 17) were rapidly killed via guillotine prior to dissection of the uterine horns. Oviducts and uteri were flushed in Hepes-KSOM media (Lawitts and Biggers, 1993) and embryos allocated to either staining or culture procedures.

3.3.3 Staining procedures for pre-implantation embryos

Embryos were prepared for immunohistochemistry as previously described (Pantaleon et al., 2010). Briefly, embryos were fixed in 2% paraformaldehyde in 1x PBS for 20 minutes at room temperature (RT), washed in PBS, and immobilized on Cell-Tak (Collaborative Biomedical Products) coated coverslips. Embryos were permeabilised in 0.25% Triton in PBS and washed before blocking with 10% normal goat serum/BSA/Tween-20/PBS (PBT) for 1 h RT. Embryos were incubated in 1:50 anti-Mouse CDX2 (BiogenX, MU392A-UC) overnight at 4°C. Following, embryos were extensively washed in PBT prior to exposure to goat anti-mouse secondary antibody labelled with Alexa 488 (1:1000, Molecular Probes) for 1 h RT. Embryos were counterstained with DAPI (Sigma Aldrich Inc, B2388) in PBS for 15 min and transferred through increasing concentrations of glycerol in PBS before mounting with Vectashield (Vector Labs, H-1000).
3.3.4 Blastocyst outgrowth assays

E5 embryos were placed into preheated microdrops of trophoblast stem cell (TS) media on tissue culture plates or gelatine coated coverslips and cultured in a humidifying chamber (Cook, Australia) at 37°C 5% CO₂ / 5% O₂ / 90% N₂ under paraffin oil. They were cultured for 6 days, with media changed every 2 days, and imaged with an inverted phase contrast microscope (Leica). Areas occupied by outgrowths were traced in ImageJ using calibrated settings. A subset of outgrowths was immunolabelled as described for staining procedures above with anti-rabbit pan-cytokeratin (DAKO, Z0622) to distinguish cytoskeleton, goat anti-rabbit Alexa 568 secondary antibody, and DAPI (Sigma Aldrich Inc, B2388) to assess number and ploidy of trophoblast nuclei. The remaining outgrowths were collected and extracted for DNA and RNA with Trizol (Life Technologies), and cDNA synthesis from Quantitek reverse transcription kit (Qiagen) using as per manufacturer’s protocol.

3.3.5 Confocal microscopy, image analysis and quantification

Embryos and outgrowths were visualised using an inverted microscope (Leica, DMi8). Pre-implantation embryos utilised a 40x air objective and captured in z stacks of 40, while outgrowths were imaged at 20x in z-stacks of 10 using a tile scan. Excitation filters of 488nm (green), 561nm (red) and 405nm (blue) fluorescence were imaged separately. CDX2 immunoreactivity (green) was localised to the TE of the E5 blastocyst. Cells devoid of CDX2 immunoreactivity were assumed to be ICM. TE and ICM cells were counted individually using Image J (NIH). ICM proportion was calculated as: ICM (%) = (Number of ICM cells/ Number of total cells) x 100.

For trophoblast outgrowths, Z-stacks of the DAPI channel were compressed using the average intensity for manual cell counts and ploidy analysis using ImageJ. Smallest trophoblasts with positive staining for pan-cytokeratin had nuclear sizes exceeding 200um². ICM cells (2N) were found to be less than 200um² in size. Nuclear sizes above 1000um² were considered P-TGCs. DNA content, a surrogate marker of ploidy, was quantified from fluorescent intensity of nuclear DAPI. DNA content of all trophoblasts and P-TGCs were normalised to ICM cells of the same image to obtain relative ploidy to 2N.
3.3.6 Genotyping for sex of pre-implantation embryos and blastocyst outgrowths

After analysis, the pre-implantation embryos and blastocyst outgrowths were removed mechanically from coverslips and digested overnight at 55°C in 5μl of lysis buffer (50mM KCl, 10mM Tris-HCl pH 8.3, 2.5mM MgCl₂·6H₂O, 0.1mg/ml gelatin, 0.45% v/v Nonidet P40, 0.45% v/v Tween 20) with 1μl of 20mg/mL proteinase K. Proteinase K was then inactivated for 10 mins at 95°C. The total 6μl sample was used to amplify male determining gene *Sry* and *B-Actin* as a control. Amplification of both genes was carried out in the same reaction due to limited DNA content. Primers for *Sry* (317bp) and *B-Actin* (220bp) were derived from Miyajima et al. (2009), for use in the rat (see table below for primer sequences and PCR conditions). A 2% agarose gel was run for 35 mins at 100V to separate the bands. Double bands indicated male embryos and single bands represented female embryos. A number of samples (~10%) failed to result in clear bands and were eliminated from analysis.

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**Primer pair sequences for genotyping for sex.** PCR conditions were 95°C for 2 mins, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min, and extension at 72°C for 5 mins.

3.3.7 Collection of placental tissues at mid- and late-gestation

A subset of dams was maintained for collection of fetal and placental tissues at E13 (4 litters), E15 (9 litters) and E20 (14 litters). For E13, dams were sacrificed by guillotine as above. E15 and E20 dams were heavily anaesthetised with 50:50 ketamine: xylazil as previously described (Gardebjer et al., 2014). Fetal and placental weights were taken at post-mortem for E15 and E20 cohorts, and placentas were separated from the uterus, with the junctional zone and labyrinth and weighed separately. In addition, a ratio of placental weight to body weight was calculated to estimate placental efficiency. A subset of placentas was snap frozen in liquid nitrogen prior to RNA extraction. Other placental samples were cut in half with uterus and
decidua attached and fixed in 4% paraformaldehyde prior to processing into paraffin for stereology. It was assumed that tissue shrinkage was even between groups. A subset of labyrinth samples from E15 and E20 placentas were cut into 1mm³ and fixed into 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer until tissue processing for transmission electron microscopy. Fetal tissue for genotyping for sex was collected as previously described (Gardebjer et al., 2014).

3.3.8 Stereology for placental volumes

Placental halves were sectioned exhaustively at 5um for the collection of 5 equally spaced sections. Unbiased stereology for placental volumes and surface areas was carried out as described by Coan et al. (2004). To clearly localise fetal blood spaces within the labyrinth at each individual age, different histological procedures were used. At E13, nucleated fetal blood cells were easy to distinguish with haematoxylin and eosin staining, while this was not appropriate at either E15 or E20. At E15 the in situ hybridisation marker Mest clearly identified the fetal endothelial cells (see table below for primer sequences) (Simmons et al., 2008b), while at E20, the endothelial marker Isolectin B4 (Sigma Aldrich, L5391) was used as per manufacturer’s instructions. Neither of these markers were appropriate at other ages. By doing so, all compartments of the placenta (decidua, junctional zone, labyrinth) and the fetal (FBS) and maternal blood spaces (MBS) were quantified. One or two placentas of each sex from each litter were used for analysis. For volume analyses, we utilised 7 males and 6 females at E13 (4 litters), 9 males and 8 females from 6 litters at E15, and 12 males and 11 females from 6 litters at E20.

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Primer pair sequences for In Situ Hybridisation (ISH) probe synthesis. Forward primers included the addition of T3 - AATTAACCCTCACTAAAGG to the 5’ end, and likewise for T7 – TAATACGACTCACTATAGGG.

3.3.9 Transmission electron microscopy

Labyrinth samples from E15 and E20 placentas were cut into 1mm³ were collected and fixed into 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer until tissue processing. All samples were washed in 0.1M sodium cacodylate buffer at 250 Watts
(W) and fixed further in 1% osmium tetroxide in cacodylate buffer at 80W under vacuum in a Biowave (Pelco). Samples were rinsed with water at 250W prior to counterstaining in 2% uranyl acetate (aq) at 150W under vacuum to enhance contrast. Samples were dehydrated in ethanol series (30%, 50%, 70%, 90%, 100%, 100%) at 250W and were infiltrated in increasing series of epon resin (1:3, 1:1, 3:1, 100%, 100%) under vacuum. Resin was polymerised for 48h at 60°C. Thick sections were cut at 500nm and stained with Toluidine blue to confirm localisation in the labyrinth. Thin sections of 100nm were post-stained with lead citrate prior to visualisation with an electron microscope (Jeol JEM-101). Two blocks per labyrinth samples were used to gather a minimum of 6 interhemal membranes (IHM) of which were averaged per block, with a total average for each animal.

3.3.10 Gene expression studies

Total RNA from labyrinth samples at E13 and E15 were extracted using RNeasy kits with on column DNase digestion (Qiagen). 1ug RNA was reverse transcribed using the iScript cDNA kit (Bio-Rad). qRT-PCR was performed using TaqMan (Qiagen) or SYBR green (Qiagen) reagents, with 100ng cDNA used per reaction. Expression profiles of two replicates per sample were analysed relative to the geometric mean of 18S, Rplp0 and Rpl13a for outgrowths, while E13 whole placenta and E15 labyrinth cDNA used 18S and Rpl13a. Relative gene expression was determined in outgrowths for genes associated with trophoblast stem cell maintenance (Eomes), and early TGC differentiation (Ascl2, Prl3d1, Hand1). Placental labyrinth samples were examined for mRNA expression of trophoblast differentiation markers (Mest, Syna, Gcm1), and genes associated with placental growth and vasculogenesis (Igf1, Igf2, Vegfa, Pgf), their respective receptors (Igf1r, Igf2r, Kdr, s-Flt).

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SYBR primer sequences for qRT-PCR.
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TAQman primers for qRT-PCR.

### 3.3.11 Statistical analysis

For embryo studies, statistically significant differences (*P<0.05; **P<0.01) were analysed using student’s t tests to examine sex differences. All pre-implantation embryos were pooled for analyses. To analyse changing outgrowth area between sexes over time, repeated measures ANOVA was used with litter averages used as each litter was cultured separately. Subsequent outgrowth analyses also used litter averages for cell counts, ploidy, and gene expression assays. For analysis of placental morphology, a two-way ANOVA with sex and zone/compartment as factors was used, with Tukey’s post-hoc analysis used where appropriate. As expected, this revealed a strong effect of zone and thus subsequent analysis testing for sex differences was performed at each individual age using a student’s t-test. When student’s t tests showed unequal variances, Mann-Whitney’s non-parametric tests were used. Mean relative gene expression of treatments were standardised to the control male group, displayed as mean ± standard error of the mean. Gene expression studies from E13 and E15 were analysed by using a two-way ANOVA where ‘gene’ and ‘sex’ were factors. All graphs and statistics were performed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA).
3.4 Results

3.4.1 Embryo studies

To localise the distinct cell lineages of the pre-implantation embryo, CDX2 immunoreactivity was used to positively label the trophectoderm (TE), with all cell nuclei marked with DAPI as shown in Figure 3.1A. Cells negative for CDX2 staining were counted as ICM cells. No differences were observed between embryos from dams on a chow diet compared to those on a liquid diet. When male and female embryos were compared, no differences were found in total, ICM or TE cell numbers (Figure 3.1B-D), or when expressed as percentages or ratios (Figure 3.1E and F).

When E5 embryos from naturally cycling dams were cultured for 6 days, trophoblasts outgrowths showed no differences between sexes for outgrowth area, number of trophoblasts, P-TGCs, their nuclear area or DNA content (Figure 3.2 A-G). This result was also reflected in outgrowth gene expression profiles for markers of trophoblast stem cell maintenance and differentiation (Figure 3.2 H-K).

3.4.2 Placental biometry at E15 and E20

E15 and E20 fetal and placental weights and dimensions are shown in Table 3.1. Although all parameters increased in weight with age (PAge < 0.0001 for all parameters), there were no sex differences in placental length, width, or depth were found, nor weights of labyrinth or junctional zone compartments. Overall, placental weight at E15 and E20 was lower in females (PSex=0.05) being 12% lower at E15 and 5% lower at E20. Fetal body weight also differed between the sexes (PSex <0.05) but there was also an interaction with age (PInt<0.05). Post hoc analysis demonstrated no significant difference in fetal body weight at E15, but males were heavier than females by E20. No changes to placental efficiency (placental weight:body weight ratio) were exhibited at either age.

3.4.3 Placental morphology from early to late gestation

To further explore the placental compartments in more detail, stereological analysis was carried out at E13, E15 and E20. Placental tissues were stained as outlined in the methods, clearly distinguishing fetal and maternal blood spaces as well as the gross compartments of the labyrinth and junctional zones (see Figure 3.3B, D, F and Figure 3.4A, D, G for higher magnification). Analysis of total, labyrinth and junctional zone

113
volumes showed each compartment expanded with age (*Figure 3.3A*, C, E). At E13, there were no difference between the sexes in any placental compartment (*Figure 3.3A*). However, the E15 placenta showed marked sex differences; overall, the whole placenta tended to be smaller but this was not statistically significant (PSex = 0.15). However, the labyrinth volume was lower in females compared to males (P<0.05, *Figure 3C*). The LAB:WP ratio was similar between the sexes (data not shown).

Given growth of the labyrinth appeared to be sexually dimorphic, fetal and maternal blood compartments were analysed in more detail. Fetal and maternal blood spaces increased with age, but the placenta from females at E15 had a lower volume in both compartments (PSex<0.05, FBS P<0.01, MBS P<0.05), which was not present earlier (E13) or later (E20) in gestation (*Figure 4 B, E, H*). The placentas from females also showed reduced blood space surface area (PSex<0.01), primarily in the fetal compartment at E15 (P<0.05, *Figure 3.4F*). The volume of the labyrinth trophoblast was not different between sexes at any age (*Figure 3.4B, E, H*). Despite marked sex differences in fetal and maternal blood spaces at E15, further structural analysis of the interhaemal membrane showed no differences in thickness at either E15 or E20 between males and females (*Figure 3.5E, F*).

### 3.4.4 Labyrinthine expression profiles

The E15 labyrinth showed clear sexual dimorphism in structure, so next we determined whether there were alterations in expression profiles for genes involved in differentiation, vasculogenesis, growth factors and branching morphogenesis at both E13 and E15 (*Figure 3.6 A, B*). Firstly, we determined that expression profiles of many genes increased expression between E13 and E15 (*Figure 3.7*). Although there were no differences in expression of any individual genes between sexes at E13, there was a tendency for an increased in expression of genes overall (PSex=0.07, *Figure 3.6 A*). However, when genes were grouped into pathways (differentiation, growth, vasculogenesis), a significant increase in expression of genes related to vasculogenesis only was found (increases of 20-28%, PSex<0.05, *Figure 3.6 A*). Conversely, E15 female labyrinth samples showed a decrease in expression of the genes investigated (PSex<0.05, *Figure 3.6 B*), but this was not statistically significant for individual genes or when analysed for any particular pathway. Although not statistically significant, genes regulating differentiation; *Mest, Gcm1, Syna,* and
growth; *lgf2* and *lgf2r* at E15 were reduced by 20-34% in females. No changes were found in the Ct values of the geometric mean of the housekeepers for either E13 or E15 were found (Figure 3.6C, D).
Figure 3.1. Analysis of pre-implantation development at E5 for male and female in vivo derived embryos. A. Representative image of E5 blastocyst immunolocalised with CDX2 (green) marking the trophectoderm, and the counterstain for nuclei with DAPI (blue). B. Total cell count. C. Inner cell mass (ICM) count. D. Trophoderm (TE) count. E. Ratio of trophectoderm to inner cell mass (TE:ICM). F. Percentage of inner cell mass to total cell count (% ICM). Data shows mean ± SEM, analysed by student’s t test. Pooled embryos from $N = 22-27$ male, $N = 30-37$ female from 11-13 litters.
Figure 3.2. Gene profiles of trophoblast outgrowths after 6 days culture from *in vivo-derived dams*. All data are presented as mean ± SEM, analysed by student’s t test. White (male), black (female). A. Representative image of day 6 cultured trophoblast outgrowth from E5 stained with trophoblast specific marker pan-cytokeratin (red) and DAPI (blue) at 20x magnification. B. Trophoblast outgrowth area over time from time 0 (E5 derived embryo). Pmatch of two-way repeated measures ANOVA analyses subjects (matching). Assessed at day 6 of culture were C; number of trophoblasts (nuclear area >200um²), D; DNA content copy number (C) relative to 2N ICM cells, E; parietal trophoblast giant cell (P-TGC) number (nuclear area >1000um²), F; trophoblast nuclear area, G; P-TGC nuclear area and expression profiles H-K. H-K gene expression profiles displayed show the gene of interest relative to the geometric mean of 3 housekeeper genes (*Rplp0, Rpl13a, 18S*). Expression of *Prl3d1* (K) showed unequal variance and was analysed by Mann-Whitney non-parametric test. Data in (B) show litter averages from \( N = 4 \) litters, 2-9/sex/litter. Cell counts and DNA content (C-G) show litter averages of \( N = 4 \) litters, 1-3/sex/litter. QPCR (H-K) show \( N = 8/\text{sex} \) from 4 litters.
Table 3.1. Fetal weight and placental weight and dimensions in males and females at E15 and E20. All data are presented as mean ± SEM and analysed by two-way ANOVA for sex with age. † shows significant difference P<0.01 between sexes from Tukey’s post-hoc analysis. E15 parameters – N = 9 litters, E20 parameters – N = 12-14 litters.

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<tr>
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<th>Female E15</th>
<th>Male E20</th>
<th>Female E20</th>
<th>Statistics</th>
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<tr>
<td>Fetal weight (g)</td>
<td>0.208 ± 0.007</td>
<td>0.2062 ± 0.007</td>
<td>2.676 ± 0.038†</td>
<td>2.534 ± 0.035†</td>
<td>PSex &lt; 0.05 PAge &lt; 0.0001 PInt &lt; 0.05</td>
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<td>Placental weight (g)</td>
<td>0.247 ± 0.023</td>
<td>0.217 ± 0.006</td>
<td>0.561 ± 0.01221</td>
<td>0.536 ± 0.012</td>
<td>PSex = 0.05 PAge &lt; 0.0001 PInt = 0.86</td>
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<td>Placenta:BW ratio (g/gbw)</td>
<td>1.128 ± 0.059</td>
<td>1.127 ± 0.041</td>
<td>0.181 ± 0.010</td>
<td>0.188 ± 0.011</td>
<td>PSex = 0.92 PAge &lt; 0.0001 PInt = 0.89</td>
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<tr>
<td>Labyrinthine wet weight (g)</td>
<td>0.0863 ± 0.009</td>
<td>0.094 ± 0.006</td>
<td>0.317 ± 0.018</td>
<td>0.306 ± 0.018</td>
<td>PSex = 0.93 PAge &lt; 0.0001 PInt = 0.57</td>
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<tr>
<td>Junctional zone wet weight (g)</td>
<td>0.102 ± 0.008</td>
<td>0.096 ± 0.006</td>
<td>0.184 ± 0.019</td>
<td>0.196 ± 0.022</td>
<td>PSex = 0.88 PAge &lt; 0.0001 PInt = 0.62</td>
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<td>Placental length (mm)</td>
<td>12.090 ± 0.352</td>
<td>12.140 ± 0.232</td>
<td>14.080 ± 0.267</td>
<td>13.910 ± 0.196</td>
<td>PSex = 0.84 PAge &lt; 0.0001 PInt = 0.69</td>
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<tr>
<td>Placental width (mm)</td>
<td>11.410 ± 0.245</td>
<td>11.040 ± 0.187</td>
<td>12.650 ± 0.216</td>
<td>12.590 ± 0.241</td>
<td>PSex = 0.38 PAge &lt; 0.0001 PInt = 0.52</td>
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<tr>
<td>Placental depth (mm)</td>
<td>2.979 ± 0.104</td>
<td>2.833 ± 0.079</td>
<td>4.187 ± 0.086</td>
<td>4.056 ± 0.092</td>
<td>PSex = 0.16 PAge &lt; 0.0001 PInt = 0.94</td>
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Figure 3.3. Sexually dimorphic outcomes of zonal volumes within the placenta from E13-E20 during gestation. Volumes of placental zones - WP; whole placenta, LAB; labyrinth, JZ; junctional zone, DEC; decidua were estimated at E13 (A), E15 (C), and E20 (E). B; Haematoxylin and eosin stain of an E13 placenta, scale bar = 2mm. D; In situ hybridisation for Mest at E15, scale bar = 3mm and Isolectin B4 at E20 (F), scale bar = 4mm, both marking fetal endothelial cells of the fetal blood space of the labyrinth. See Figure 3.4 for higher magnification images of A, D, G. White (male), black (female). Data shows mean ± SEM, analysed by two-way ANOVAs for sex with zone. *P<0.05 post-hoc by student’s t test. For E13 N = 6-7 samples/sex from 4 litters, E15 N = 8-9 samples/sex from 6 litters, E20 N = 11-12 samples/sex from 6 litters.
Figure 3.4. Quantification of labyrinthine blood spaces in males and females in the immature, definitive and late-gestation placenta. A. Haematoxylin and eosin staining of the E13 labyrinth. D. In situ hybridisation for Mest marking the fetal endothelial cells of the fetal blood spaces in the E15 labyrinth. G. Isolectin B4 marking the fetal endothelial cells of the E20 labyrinth. White (male), black (female). Data shows mean ± SEM, analysed by two-way ANOVAs for sex with zone. *P<0.05, **P<0.01 from student’s t test. Fetal (FBS) and maternal (MBS) blood spaces, and labyrinth trophoblast (LAB TROPH) (*) were estimated for volume for E13 (B), E15 (E) and E20 (H). Similarly, surface areas for FBS and MBS were estimated for E13 (C), E15 (F), and E20 (I). For E13 N = 6-7 samples/sex from 4 litters, E15 N = 8-9 samples/sex from 6 litters, E20 N = 11-12 samples/sex from 6 litters.
Figure 3.5. Estimation of interhemal membrane thickness of the male and female labyrinth. Semi-thin sections stained with toluidine blue of the placental labyrinth at E15 (A) and E20 (B). Electron micrographs of the labyrinth at E15 (C) and E20 (D). White scale bars in D and E are 10μm. Interhaemal membrane thicknesses at E15 (E) and E20 (F). Data shows mean ± SEM, analysed by student’s t test. White (male), black (female). Data averaged 6 individual membranes from 2 blocks. E15 N = 5/sex from 3-4 litters. E20 N = 4/sex from 4 litter.
Figure 3.6. Expression profiles of male and female placentas at E13, and the labyrinth at E15. Expression profiles from whole placenta at E15 (A) and labyrinth samples at E15 (B). Relative expression profiles were calculated relative to the geometric mean of two housekeepers (Rpl13a, 18S) for E13 (C) and E15 (D), with Ct values displayed. White (male), black (female). Data shows mean ± SEM analysed by two-way ANOVA for sex with differing gene. Data was standardised to control male at each age. E13 - N = 8/sex from 4 litters, and E15 - N = 8/sex from 8 litters.
Figure 3.7. Temporal gene expression profiles of male and female placentas from E13 to E15. All data shows mean ± SEM, standardised to E13 control male. The geometric mean of 2 housekeepers (Rpl13a, 18S) were used.
3.5 Discussion

This comprehensive ontogeny study has assessed sexual dimorphism in cell number and differentiation of the pre-implantation embryo, and subsequent placental structure throughout gestation in the rat. E15, a time of rapid placental growth in the rat was identified as a critical point during development whereby reduced/slowed differentiation of the placental labyrinth and its blood compartments in females is likely to be a contributing factor to the lower weight of females by late gestation. This highlights the importance of assessing male and females separately in all developmental studies as differences exist in placental growth, morphology and function even in pregnancies without application of a stressor.

3.5.1 Pre-implantation development and differentiation

In the blastocyst, no sex differences were found. Cell counts were not different between sexes and were also similar to counts from Wistar rat embryos obtained at a similar time point (Master et al., 2015). Investigation of TE differentiation using culture of in vivo derived embryos also showed no changes to outgrowth rate, number of trophoblasts, P-TGCs, ploidy or expression of TS and trophoblast differentiation markers. This suggests that males and females have equal developmental potential to differentiate and implant. While this is contrary to the majority of literature which suggests that males are more developed than females, previous studies may be confounded by the use of in vitro fertilisation or superovulation. Indeed, as aforementioned, in vitro derived embryos show reduced cell counts due to an increase in apoptosis when compared to in vivo derived embryos (Hardy, 1997, Kamjoo et al., 2002, Giritharan et al., 2007). Tan et al. (2016b) recently demonstrated female specific perturbation of proliferation and differentiation is caused by the in vitro fertilisation (IVF) procedure. More specifically, they showed IVF in the mouse caused a reduced cell count and increased in apoptosis in female blastocysts during pre-implantation. This was associated with reduced ectoplacental cone formation and elevated mortality by late gestation, resulting in an altered sex ratio at birth (57% male) (Tan et al., 2016a, 2016b).

However, we do concede that models which do use these in vitro procedures also have shown no differences to cell count and allocation. These include studies in mouse (Bermejo-Alvarez et al., 2012), human (Dumoulin et al., 2005, Richter et al.,
2006, Weston et al., 2009), bovine (Holm et al., 1998, Rizos et al., 2008, Bermejo-Alvarez et al., 2010a), and porcine (Kaminski et al., 1996, Lechniak et al., 2000) models. Reasons for this could include choice of culture media, such as the addition of serum (Rizos et al., 2008, Bermejo-Alvarez et al., 2010a), or the addition (Bermejo-Alvarez et al., 2012) or removal (Peippo and Bredbacka, 1995) of glucose. In humans, as suggested by Richter et al. (2006), rather the differences in outcomes may be due to alternate selection criteria for embryo quality prior to transfer, which no longer prioritises the largest blastocysts, which are more likely to be male. Many studies also do not also report whether multiple embryos were cultured in the same microdrop, which could affect embryonic growth through the production of autocrine factors.

Artificial reproductive technologies (ART), in addition to IVF, use high, supraphysiological doses of gonadotropins to initiate ovulation. Adverse outcomes are also associated with these procedures, including reduced viability and imprinting disorders (DeBaun et al., 2003). The hormonal contribution to these disorders has been investigated primarily in mice, with gonadotropin administration in vivo being associated with altered genomic imprinting and DNA methylation in offspring (de Waal et al., 2012). Embryo transfer experiments show that when control derived embryos implant into a hormone-stimulated dam, fetal and placental weights are reduced at E18.5, and do not show any differences in placental DNA methylation profiles (Mainigi et al., 2014). This suggests that gonadotropin exposure can affect both embryo epigenetic reprogramming and the uterine responses for implantation by different mechanisms, with both affecting long term fetal and placental development. This highlights the importance of using naturally mated dams without additional hormonal intervention when investigating sex specific developmental outcomes.

### 3.5.2 Placental morphogenesis

To explain the reduced fetal weight in females which is only exhibited in late gestation (E20), the placenta was investigated for sexually dimorphic morphogenic differences at E13, a very early time point of definitive placental development and at E15, a time of rapid placental growth. Analysis of gross placental weight and stereology showed sex specific structural changes began in the rat at E15. At this age, placental weight was lower in females and this was associated with a smaller relative placental volume and reduced labyrinth vasculature. Further exploration into the labyrinthine blood
spaces showed both the FBS and the MBS were affected. Curiously, while both FBS and MBS showed reduced relative volume in females, only the FBS showed reduced surface area. Overall, this suggests that the female placenta has lower potential capacity for nutrient exchange to facilitate growth at this gestational age.

Investigation of the labyrinthine blood spaces are also of particular importance, as they are also affected in a sexually dimorphic manner in response to maternal perturbations. For example, exposure of mid-late gestational hypoxia shows a decrease in labyrinthine blood spaces in females only, without affecting placental weight in either sex as above (Cuffe et al., 2014a). Examination of blood spaces may therefore be a more accurate marker altered placental development. This may suggest other programming models which similarly show placental weight to be unaffected, but do show other changes to gross placental zones, may still have other subtler changes in the vasculature, e.g. glucocorticoid exposure (Cuffe et al., 2012). Sexually dimorphic phenotypes are also likely to be masked by the pooling of sexes in perturbation models, particularly when their affects are divergent.

3.5.3 Sex differences in placental gene expression

Placentas were investigated for the molecular mechanisms behind this altered labyrinth morphogenesis with selected gene expression assays conducted at E13 and E15. The E13 female placenta showed generally increased expression of the investigated genes, while the E15 female labyrinth generally decreased gene expression. This was without change to expression of housekeeper genes between males and females. The E13 data is consistent with microarray data on E12.5 mouse placenta, which showed that 183 genes were expressed at a higher level in females whilst only 35 genes were more highly expressed in males (Mao et al., 2010). The E15 data coincides with a mouse study at E15.5, which showed that females had greater global DNA methylation profile than males (3.3%) in unstressed conditions (Gallou-Kabani et al., 2010). However, the same laboratory has also shown greater up-regulation of genes in females than males at E15.5 (Gabory et al., 2011). Differences in the expression data at this latter time point are likely due the whole placenta being assayed by Gabory et al. (2011), while our study only utilised the labyrinth portion, allowing specific insight into labyrinth development. This is supported by a study in the human third trimester placenta, which showed unique expression of sexually
dimorphic genes between 4 different cell types; cytotrophoblasts, syncytiotrophoblast, arterial and venous endothelial cells (Cvitic et al., 2013). Collectively, with the structural data, this may suggest that the males may have already undergone the major rounds of proliferation and differentiation by E13, and therefore by E15 have rather switched to hypertrophic growth which continues to expand the placenta. Future analysis of gene expression profiles of male and placentas at E12-E12.5 would confirm this hypothesis.

3.5.4 Sexual dimorphism; initiated by the fetal adrenal?

The changes in placental morphogenesis in females only occurred at mid-gestation, which ruled out any contribution from double dosage of the X-chromosome, its inactivation, imprinting and so forth on cellular allocation in the early embryo. A likely candidate contributing to placental changes in mid-gestation, is the development and activation of the fetal adrenal gland. Development of the adrenal begins at E10 in the mouse, and its activation contributes to the divergence of ovary and testis developmental at E12.5-13.5, reviewed by Keegan and Hammer. (2002). This in turn upregulates androgen synthesis in XY males promoting further testis development, and reciprocal communication with the now masculinised adrenal, reviewed by Wen et al. (2016). Females, however, go down a default morphogenic pathway without hormonal contribution, reviewed by Biason-Lauber and Chaboissier. (2015). The growth and development of the male placenta therefore has the potential to react to circulating androgens within the implantation site. Conversely, other interesting research by Penaloza, et al. (2009) has shown that cultured male and female embryonic cells from the E10.5 embryo can also react independently of hormonal effects when exposed to stressors, and therefore may be explained by chromosomal differences.

3.6 Conclusion

This study has identified mid-gestation E15 is an important timepoint during gestation to analyse basal sex differences, which may not be present in early and late gestation. At this time, the male fetus may have greater metabolic demands than the female fetus, which may lead to the male placenta growing larger, and increasing the development and expansion of the fetal and maternal blood spaces in the labyrinth. This in turn, is likely to lead to greater nutrient delivery to the male fetus, and result a
greater fetal weight in late gestation. This greater metabolic demand of the male fetus may also render it more susceptible to subtle changes to the nutrient environment, and be more vulnerable to perturbation. As this study showed no basal sex differences in pre-implantation development, this highlights the use of unstressed controls to analyse sexual dimorphism prior to application of a treatment.
Chapter 4. Alcohol exposure impairs trophoblast survival and alters subtype-specific gene expression *in vitro*


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<td>Interpretation of results (50%)</td>
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Alcohol exposure impairs trophoblast survival and alters subtype-specific gene expression in vitro.

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Placenta short communication

4.1 Abstract
Maternal alcohol consumption is common prior to pregnancy recognition and in the rat results in altered placental development and fetal growth restriction. To assess the effect of ethanol (EtOH) exposure on the differentiation of trophoblast stem (TS) cells, mouse TS lines were differentiated in vitro for 6 days in 0%, 0.2% or 1% EtOH. This reduced both trophoblast survival and expression of labyrinth and junctional zone trophoblast subtype-specific genes. This suggests that fetal growth restriction and altered placental development associated with maternal alcohol consumption in the periconceptional period could be mediated in part by direct effects on trophoblast development.

3-5 Key Words: Ethanol, Placenta, Differentiation, Programming, Apoptosis
4.2 Introduction

Maternal perturbations during early pregnancy, including a low protein diet (Kwong et al., 2000, 2006, 2007), undernutrition (Edwards and McMillen, 2002, MacLaughlin et al., 2005), or alcohol (EtOH) exposure (Gardebjer et al., 2014, 2015), can result in fetal growth restriction and programming of adult disease. Alcohol is a common exposure during pregnancy, with current statistics being 47-58% of all pregnancies (Colvin et al., 2007, Wallace et al., 2007). Consumption of 5+ standard drinks has also been reported in the period prior to pregnancy recognition (14%) (Colvin et al., 2007, Wallace et al., 2007). In vivo rodent models of EtOH exposure during the periconception period (Gardebjer et al., 2014) and intermittently throughout gestation (Gundogan et al., 2013, 2015), have shown alterations to placental structure and expression of metabolic transporters. This is of interest as normal formation and function of the placenta is a critical determinant of fetal growth. Derived from the trophectoderm of the pre-implantation embryo, trophoblast cells contribute the majority of cells within the mature placenta and consist of a number of unique cell types with diverse morphologies and functions (Simmons et al., 2007). The definitive chorioallantoic placenta is organised into two zones containing specialised trophoblast cell types; the junctional zone which has a structural and endocrine role, while the labyrinth zone contains the fetal and maternal vasculature and is the location of nutrient exchange (Coan et al., 2004). Perturbations during pregnancy often result in modifications to placental growth (Kwong et al., 2006, MacLaughlin et al., 2005) and zonal allocation (Gardebjer et al., 2014) in late gestation, however few studies have determined whether alterations to trophoblast differentiation in early pregnancy may be mediating these effects. We have previously reported that periconceptional EtOH exposure prior to implantation in the rat causes an increase in glycogen trophoblasts (GlyT) in the junctional zone during late gestation (Gardebjer et al., 2014). In addition, high dose (18-37% vol/vol) EtOH exposure from implantation until close to term (E6-E18) reduced invasion of trophoblast cells into the maternal decidua and caused labyrinth disorganisation (Gundogan et al., 2015). However, it is unknown if placental defects are due to direct exposure of the trophoblast stem cells of the trophectoderm to EtOH within the uterine cavity, or via other indirect mechanisms such as interactions with altered uterine cells. Here we utilise an in vitro model of differentiating mouse trophoblast stem (TS) cells to examine the direct effects of EtOH on proliferation and differentiation.
4.3 Methods

4.3.1 TS cell culture

Murine TS cells (EGFP line) were maintained as previously described (Himeno et al., 2008). Cells were seeded at 5x10^4, and differentiated in 0% (control), 0.2%, or 1% EtOH in TS media. To assess cell proliferation, cells were lifted off the culture plates and counted using a hemocytometer on days 2, 4 and 6 of culture (N=3/treatment). Gene expression analysed on day 6 (N=9/treatment, 3 technical replicates per set). Media was changed every 2 days including the addition of fresh EtOH. The 0.2% EtOH dose was based on the maximum blood alcohol concentration from 12.5% v/v EtOH exposure in Sprague Dawley dams (Gardebjer et al., 2014), relating in women to the consumption of 5 standard drinks over 2 hours (Colvin et al., 2007). The 1% EtOH dose has been used previously in culture to demonstrate “proof of principle” using a supraphysiological dose (Chan and Chang, 2006). In culture, as much as 50% evaporation occurs by 8 hours and 80-90% evaporation by 24h in tissue culture plates (Rodriguez, 1992).

4.3.2 RNA extraction and qRT-PCR

RNA isolation and qRT-PCR were carried out as previously described (Moritz et al., 2002). Markers specific to labyrinth or junctional zone trophoblasts were analysed for gene expression relative to Rn18s using the ΔΔCt method (Moritz, 2002) (primer sequences in Table 4.1). Pro- and anti-apoptotic markers utilised TaqMAN gene expression assays (Qiagen), with assay on demand primers of Bax Mm00432051, Bcl2 Mm00477631, and 18S 4319413E (Thermofisher). Bax and Bcl2 were multiplexed to 18S.
<table>
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4.3.3 Histological assessment of trophoblast number

Fluorescent in situ hybridisation (FISH) localised Tpbpa and Prl7a2 positive trophoblasts (N=3-4/treatment) from day 6 cultures (Simmons et al., 2007). Fluorescein or DIG-labelled probes were made following manufacturer’s instructions (Roche) with primer sequences as described previously (Simmons et al., 2007, 2008b). Prior to use on cultured cells, RNA probes were tested on either E12.5 or E14.5 mouse placenta fixed in 4% paraformaldehyde overnight to confirm subtype-specific expression. Coverslips were imaged on an inverted microscope (Leica) in 20x20 frames, 20x magnification, with all cells being utilised within this area for calculation. Cells were counted using ImageJ (NIH).
4.3.4 Fluorescent in situ hybridisation for localisation of trophoblast subtypes on cultured EGFP cells

ISH for TS cell cultures were carried out as described (Simmons et al., 2007), with modifications for fluorescent localisation. Fluorescein or DIG-labelled probes were made for Tpbpa and Prl7a2 as per manufacturer's instructions (Roche) with primers sequences as described previously (Simmons et al., 2007, 2008b). In brief, cells were fixed in 4% PFA for 15 min and transferred to 0.4% PFA until analysis. Cells were not treated with proteinase K, but were acetylated for 5 minutes prior to administration of FITC- or DIG-labelled probes at 1:200 which were hybridised overnight at 65°C. The following day, cells were washed in 5xSSC pre-warmed to 65°C for 3x20 min, followed by TN buffer (100mM Tris-HCl ph7.5, 150mM NaCl) for 5 mins prior to incubation in block consisting of 1× MABT (150 mM NaCl, 100 mM maleic acid, 0.1% Tween-20, pH 7.5), 2% blocking reagent (Roche), 20% heat inactivated horse serum, for 1h. Samples were incubated in anti-DIG-POD or anti-FITC-POD antibody (1:500, Roche) overnight. The following day, cells were washed in TNT buffer containing 0.05% Tween20 in TN buffer for 3x15 min prior to probe detection with 1:50 FITC- or Cy5-Tyramide in amplification reagent (TSA™ kit, Perkin Elmer) for 10 min. Samples were washed in TNT buffer for 3x5 min and counterstained with Hoechst (Sigma). Coverslips were mounted in 70% glycerol prior to imaging. Prior to use on cultured cells, probes were tested on either E12.5 or E14.5 mouse placentas fixed in 4% paraformaldehyde overnight.

4.3.5 Imaging and Analysis

Coverslips were imaged in 20x20 frames in z stacks of 5 using a 20x air objective on an inverted microscope (Leica) using set parameters. Prl7a2-FITC was imaged with the 488nm laser, Tpbpa-DIG was imaged with the 561nm laser and Hoechst with the 405nm laser. Representative images were also taken of paraffin and cultured cells using either the 20x or 40x air objectives. Image analysis was carried out using ImageJ (NIH). Z stacks were compressed with the maximum projections for DAPI and merged (DAPI and FISH marker). The greyscale DAPI image was converted to 8 bit, underwent watershed to separate adjoining nuclei, and counted with the automated “Analyze Particles” function. FISH positive cells were counted from merged images using “Cell Counter”.
4.3.6 Statistical analyses

Mean relative gene expression of treatments were standardised to the control group for each line. Expression data, proliferation and FISH counts were analysed by one-way ANOVAs to compare treatments. Tukey’s post-hoc tests were used where appropriate, and when data was not normally distributed, a non-parametric Kruskal-Wallis test was used. Statistical differences: *P<0.05, **P<0.01.
4.4 Results and Discussion

To determine the mechanism by which EtOH exposure during early pregnancy may be mediating altered placental growth and function, we explored the direct impact of EtOH on TS cell proliferation and differentiation. To investigate EtOH exposure at a physiological level, 0.2% EtOH was chosen as this was the peak blood alcohol content found in a model of in vivo alcohol exposure in the rat at 30 minutes after initial consumption (Gardebjer et al., 2014). In addition, 0.2% EtOH is also the maximum dose that the TS cells would be exposed to when the media is applied, and due to alcohol evaporation (Rodriguez et al., 1992), would not be sustained at this level over the entire 6 days of treatment. In addition, TS cells express a well-validated set of genetic markers enabling identification of individual placental trophoblast cell types in the mouse (Simmons et al., 2007). This presents an opportunity to relate alterations in gene expression to potential changes in differentiation.

4.4.1 Cell counts

Proliferation rates were first characterised to determine the effects of EtOH over the course of TS cell differentiation. While no differences in cell count were found between treatments on day 2 or 4, marked reductions in cell counts in both the 0.2% (48% reduction) and 1% EtOH groups (44% reduction) were observed on day 6 (Figure 4.1A). This time point coincides with terminal differentiation of trophoblasts, as expression of stem cell markers are rapidly lost by day 2 of culture and expression of markers of mature cell types is just commencing on day 4 (Watson et al., 2011). This result is consistent with studies that demonstrate reduced proliferation in response to 20-40mM (0.09-0.18%) EtOH in cultured human cytotrophoblasts (first trimester villous explants and BeWo cells) (Lui et al., 2014). However, our observation of decreased cell numbers at day 6 of differentiation is unlikely the result of decreased proliferation, but more likely represents a loss of cell viability. This is supported by the observation of significant floating cells in the treatment but not control media on day 6 (data not shown). In addition, human cytotrophoblasts undergo apoptosis when exposed to EtOH (Clave et al., 2014, Bolnick et al., 2014). To determine whether this also occurs in mouse trophoblasts exposed to EtOH, we analysed the expression profiles of pro- and anti-apoptotic markers (Bax and Bcl2 respectively) from days 2-6 of culture. No changes were found to either gene on days 2 or 4 (data not shown).
However, on day 6, whilst no change was found to *Bax* expression, *Bcl2* expression was reduced by EtOH (see *Figure 4.3A, B*), suggesting a perturbed balance between pro- and anti-apoptotic signals. This may extend to an increase in apoptosis; however, to determine this definitively would require histological analysis. Collectively, these data indicate that placental trophoblasts are highly sensitive to EtOH during terminal differentiation.

4.4.2 Junctional zone cell markers

EtOH exposure caused dose-dependent decreases in gene expression of *Tpbpa* (*Figure 4.1B*), a marker of both fully differentiated junctional zone trophoblasts and their earlier ecto-placental cone progenitors (Adamson et al., 2002). Additionally, both *Prl7a2*, a marker of spongiotrophoblast (SpT) and parietal trophoblast giant cells (P-TGCs), and *Prl7b1* a marker of invasive glycogen trophoblast (GlyT) cells and spiral artery-associated TGCs (SpA-TGCs) were decreased by EtOH exposure (*Figure 4.1C, D*). Curiously, this suggestion of reduced GlyTs *in vitro* conflicts with increased accumulation of this cell type in the junctional zone of periconceptionally exposed placentas *in vivo* (Gardebjer et al., 2014). It is tempting to speculate that altered GlyT differentiation may also affect their invasive behaviour as seen in other EtOH exposure models (Gundogan, 2013). No differences were found in the 0.2% EtOH groups for *Prl7b1* or *Prl7a2* relative to control or 1% groups.

No alterations were found for *Prl3d1* (*Pl1, Figure 4.1E*), or *Prl2c* (*Plf, Figure 4.1F*), which are expressed by secondary TGCs *in vivo*, indicating that EtOH exposure does not alter expression of all trophoblast subtype specific gene markers. When this same experiment was performed on another cell line – RS26, similar expression profiles were seen at day 6 for all junctional zone trophoblast markers with the exception of *Tpbpa*, which did not change (*Figure 4.4A-G*). This difference between the TS lines may be either due to clonal differences between cell lines, or alternatively a sex-specific result, as the RS26 line is male and the EGFP line is female (data not shown). Maternal perturbations commonly result in sexually dimorphic impacts on placental structure, function, and differentiation - evidenced by GlyT cell accumulation in the junctional zone of females only following periconceptional EtOH exposure (Gardebjer et al., 2014). Further examination of multiple male and female cell lines would be required to determine this possibility.
The trophoblast subtype markers in this study have been used previously to infer effects of differentiation of specific trophoblast subtypes. However, it is also possible that EtOH may be directly altering gene expression levels, rather than the differentiation of the trophoblast subtypes per se. Therefore, to confirm whether EtOH reduces the number of differentiated trophoblast subtypes, FISH was carried out on cells collected from day 6 cultures to examine the number of Prl7a2 and Tpbpa positively labelled cells. Each marker was first localised in paraffin sections of mouse placentas to confirm reported trophoblast subtype specific labelling (Figure 4.2.A, D). Quantification of Prl7a2 positive (Figure 4.2.B, C, G) and Tpbpa positive (Figure 4.2.E, F, H) trophoblasts in vitro showed no difference between treatments although there was considerable variability in the cells cultured in 1% EtOH. This suggests that exposure to EtOH did not overtly affect the number of differentiated trophoblasts of each subtype, but may indicate either less maturity of trophoblasts or more simply, reduced expression of the genes themselves. Considering the heterogeneous cell populations marked by these genes, it is possible that subtle differences in number of trophoblast subtypes may still be apparent.

**4.4.3 Labyrinth zone cell markers**

Finally, we examined the impact of EtOH on expression of labyrinthine-specific trophoblast markers. While no alteration was found in Ctsq (Figure 4.1G) expression, representing sinusoidal TGCs (S-TGCs), EtOH exposure did cause a dose-dependent decrease in gene expression for Syna (Figure 4.1H), marking syncytiotrophoblast layer 1 (SynT-I). Syna expression was significantly decreased in the 1% group relative to the control whilst Syna expression in 0.2% EtOH exposed cells was intermediate and not significantly different to control or 1% EtOH groups. Exposure of the RS26 line to EtOH also revealed reduced Syna expression in the 1% EtOH group, also with no changes to Ctsq (Figure 4.4F, G). This demonstrates that EtOH has the potential to affect trophoblast viability and differentiation in multiple placental compartments. Furthermore, these observations indicate that a commonly used vehicle for dissolving drugs in itself has a significant impact on trophoblast behaviour, and must be properly controlled for in vitro experiments.
Figure 4.1. Trophoblast stem cell proliferation and expression profiles when exposed to EtOH over 6 days during differentiation. Panel A. Proliferation analyses showing control (circle), 0.2% EtOH (square), 1% EtOH (triangle), N=3/treatment. Panels B-H show gene expression profiles of trophoblast subtype markers for junctional zone (B-F) and the labyrinth (G and H), with control (white bars), 0.2% EtOH (striped bars), 1% EtOH (black bars). All data analysed by one-way ANOVA with Tukey’s post-hoc tests. N=9/treatment. All data represented by mean ± SEM. *P<0.05, **P<0.01 compared to CON.
Figure 4.2. Quantification of *Prl7a2* and *Tpbpa* positive trophoblasts by fluorescent *in situ* hybridisation. Panels A-C show representative images of *Prl7a2* (green) expression in SpT and P-TGCs in the E12.5 mouse placenta at low (10x) (A) magnification. B and C show representative images of EGFP cells cultured to day 6. Panel G shows percentage *Prl7a2* positive cell counts of total cultured cells at day 6 (N=3/trt). Panels D-F show representative images of *Tpbpa* (red) expression in SpT, GlyT, and junctional zone progenitors in the E14.5 placenta mouse placenta at low (D) magnification. E and F show representative images of cells cultured to day 6. Panel H shows percentage *Tpbpa* positive cell counts of total cultured cells at day 6 (N=3/trt). All nuclei stained with Hoechst (blue). All data represented by mean ± SEM. Data analysed by one-way ANOVA with Tukey’s or Kruskal-Wallis post-hoc test.
Figure 4.3. Gene expression of pro-apoptotic marker *Bax* and anti-apoptotic marker *Bcl2*. All data analysed by one-way ANOVA with Tukey’s post-hoc tests. *N=9/treatment*. All data represented by mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 compared to CON.*
Figure 4.4. Expression profiles of RS26 differentiated trophoblasts at day 6 after EtOH exposure. Panels A-G show gene expression profiles of trophoblast subtype markers for junctional zone (A-E) and the labyrinth (F and G), with control (white bars), 0.2% EtOH (striped bars), 1% EtOH (black bars). RS26 line was seeded at 10,000 cells per well to control for proliferative capacity. All data analysed by one-way ANOVA with Tukey’s post-hoc tests. N=9/treatment. All data represented by mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 compared to CON.
4.5 Conclusions

This study demonstrates that EtOH exposure can directly alter the viability of mature trophoblasts and the expression of trophoblast subtype markers for both labyrinth and junctional zones. Alterations to labyrinthine cell types may alter barrier thickness or labyrinth architecture potentially impacting appropriate nutrient exchange, whilst changes to invasive GlyT and SpA-TGCs may alter their invasive behaviour and lead to poor invasion. Future studies on how alterations of these cell types in vivo may lead to altered placental structure and function is now paramount in determining the impacts of EtOH exposure.
Chapter 5. Periconceptional alcohol exposure: effects on the pre-implantation embryo and the maternal environment.

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Note: The remaining animal treatment with liquid diets was performed by members of the Moritz laboratory PC-EtOH project, with all animals coordinated by Dr. Lisa Akison. Special thanks to Sarah Walton, Jenn Outhwaite and Moritz lab members who helped with euthanasia and collection of tissues at post-mortem.
5.1 Abstract

Periconceptional ethanol (PC-EtOH) exposure in the rat causes fetal growth restriction, increased resorptions, sex-specific changes to placental morphology in late gestation, and metabolic dysfunction in adult offspring. Previous models investigating environmental stressors such as IVF, suggest that females may be more susceptible to alterations in cell allocation and epigenetic modifications in the pre-implantation period. Therefore, this study investigated whether PC-EtOH exposure altered sex-specific development of the pre-implantation embryo, differentiation of the trophectoderm (TE) to invasive trophoblast giant cells, and the influence of the maternal environment in mediating these outcomes. Sprague Dawley rat dams were administered 12.5% v/v EtOH from 4 days prior (E-4) to 4 days after conception at embryonic day (E) 4 in a liquid diet and blastocyst were examined at E5.

PC-EtOH exposure decreased ICM cell number in female but not male embryos at E5, without changing total or TE counts. However, TE nuclear CDX2 fluorescence was reduced in PC-EtOH embryos of both sexes. Culture of E5 blastocysts in vitro showed PC-EtOH decreased outgrowth rate on day 6, and number of trophoblasts in both sexes. Investigation of primary parietal trophoblast giant cells (P-TGCs) showed only female PC-EtOH exposed embryos to have reduced cell count, suggestive of perturbed differentiation. Outgrowths from PC-EtOH females, however, also showed reduced expression of Prl4a1, a gene exclusively expressed by TGCs for communication with decidual natural killer cells (dNK).

Maternal hormone analysis showed no change to levels of plasma estrogen or progesterone over the peri-implantation period. However, hormone receptor mRNA expression in the uterus was altered by PC-EtOH; Esr1 was decreased at E5, while Pgr was reduced by PC-EtOH at E5, but increased at E7. Investigation of downstream estrogen response genes (Muc1, Hbegf, Vegfa, Lif) were unchanged by PC-EtOH. Uterine expression of progesterone response genes, however, showed reductions in Bmp2 and Coup-TFII at E5, but were unchanged at E7. No differences in expression of the remaining genes (Hand2, Ihh, Areg, Igfbp1, Cnr1) genes were found. As these alterations occur during implantation, they are likely to be a consequence of perturbed signalling from the embryo, leading to alterations in the uterine responses for implantation. Additionally, a reduction in decidual natural killer (dNK) genes Vdup1
and Ilng, involved in dNK maturation and function were also reduced at E5, but curiously without any change to dNK marker gene Prf1. By E11 in the immature placenta, a 25% increase in decidual dNK cells was found in PC-EtOH females, which may suggest that reduced dNK cell homing has resulted from perturbed TGC communication.

This study shows that PC-EtOH can alter trophoblast differentiation, with female embryos in particular, most susceptible to modification. These trophoblast defects may lead to reduced capacity for invasion, and perturbed communication with maternal immune cells required for ongoing placentation. These outcomes may contribute to placental defects, decreased embryonic viability, fetal growth restriction and adult onset disease associated with this model.
5.2 Introduction

The periconception period is increasingly being shown to be susceptible to maternal perturbations, leading to increased predisposition to adult disease in a sex specific manner (Kwong et al., 2000, 2006). Maternal nutrient perturbations often result in altered development of the early embryo, including changes to total cell number or cell allocation to the primary lineages of the blastocyst – the inner cell mass (ICM) and the trophectoderm (TE) (Kwong et al., 2000, Bloise et al., 2012, Tan et al., 2016a, 2016b). Alterations in either may lead to fetal growth restriction as the ICM provides a progenitor pool to the embryo proper, while the TE gives rise to the unique trophoblast subtypes to facilitate invasion into the uterine epithelium, and establish the definitive chorioallantoic placenta. Sexual dimorphism in programming of adult disease is becoming increasingly prevalent, and can be traced back to changes in the early embryo and formation of the placenta (Kalisch-Smith et al., 2017a). Studies by Tan et al. (2016a, 2016b), show that IVF can impact epigenetic reprogramming in the pre-implantation period, formation of the ectoplacental cone, and placenta, in a sex specific manner. Therefore, assessing the impacts of alcohol exposure on sex-specific pre-implantation phenotypes will offer further insight into periconceptional programming.

A common exposure which may be experienced by the early embryo is alcohol. Prior to pregnancy recognition, 47% of women have reported alcohol consumption, with 15-39% categorised as receiving high doses of 5+ standard drinks on one occasion (Colvin et al., 2007, Wallace, 2007, Muggli et al., 2016). Specifically, during the periconception period, approximately 27% of women drink alcohol, with 87% of these women ceasing their drinking activities after pregnancy recognition (Muggli et al., 2016). This cessation is a common finding (Parackal et al., 2013, Alvik et al., 2006), and is likely to be contributed by unplanned pregnancies, which are at approximately 50% in both Australia and the United States (Colvin et al., 2007, Finer and Zolna, 2016). Therefore, the early pre-implantation embryo may be highly exposed to alcohol during this periconceptional period.

We have recently characterised a model of PC-EtOH exposure in the rat and reported fetal growth restriction, sex-specific structural and metabolic alterations to the late gestational placenta, and compromised metabolic outcomes in adulthood (Garde...
et al., 2014, 2015). Of particular interest is the origin of the fetal growth restriction, and whether this derives from early alterations to pre-implantation development, and if they occur in a sex-specific manner. As discussed in Chapter 3, many in vitro models suggest males to develop at a faster rate than females, but this is likely due to an artefact of in vitro culture or superovulation techniques. This highlights the use of appropriate embryo derivation in determining sex-specific outcomes, especially in programming models.

Effects of maternal perturbations including alcohol exposure on the maternal physiology, hormone-dependent uterine environment and establishment of pregnancy are rarely investigated. There is some evidence from maternal low protein diets during pregnancy (Eckert et al., 2012) and diabetic models (Dehertogh et al., 1989), which have shown reduced estrogen levels during the peri-implantation period, may impact on the window of uterine receptivity and the capacity for implantation. Indeed, ovarian hyperstimulation in artificial reproductive technologies (ART) (Walton et al., 1982, Ertzeid and Storeng, 2001), can have dramatic impacts on implantation, as high estrogen levels can rapidly close the window of receptivity and reduce embryonic survival (Ma et al., 2003). Alcohol has been shown to alter both estrogen and progesterone levels after 4.8g/day EtOH for 2 weeks (Emanuele et al., 2001), and 30% v/v EtOH after 3h (LaPaglia et al., 1997). However, the more subtle impacts of an altered hormonal environment such as may occur with maternal alcohol consumption, have not been considered to their long-term impacts on implantation, and the post-implantation development.

This thesis chapter aims to quantify effects of PC-EtOH exposure on development of the early embryo, primary differentiation of the TE, the influence of the uterine environment, and the necessary communication between them for successful pregnancy progression.
5.3 Methods

5.3.1 Ethics, animal treatment and sample collection

All animal experiments and procedures were approved by The University of Queensland Animal Ethics Committee (AEC approval number SBS/022/12/NHMRC) prior to commencement of this study. PC-EtOH exposure was administered *in vivo* to Sprague Dawley dams in a liquid diet containing 12.5% v/v EtOH as previously described (Gardebjer et al., 2014, 2015). It was important to include a standard chow group to eliminate any possible effects of administration of ethanol in a liquid diet. Dam weight was at least 230g before entering the protocol. Vaginal impedance was measured daily with an EC40 oestrous cycle monitor (Fine Science Tools), with $4.5 \times 10^3 \Omega$ or higher indicating oestrus and allocation to the diet regime. Dams were treated over two oestrous cycles, one prior to mating and the establishment of the pre-implantation period (denoted as embryonic day (E) -4 to E4). Dams were time-mated on E0 from 1200h to 1700h if impedance exceeded $4.5 \times 10^3 \Omega$. If pregnancy was established, the following day was considered E1. At 900h on E5, dams were sacrificed via guillotine prior to dissection of the uterine horns and embryo collection. Oviducts and uteri were flushed in Hepes-KSOM media (Lawitts and Biggers 1993) and allocated to either staining ($N = 12$-$18$ dams/treatment) or culture procedures ($N = 4$ dams/treatment). All embryos were genotyped for sex as described in sections 2.9.1 and 3.3.6). E7, and E11 cohorts were similarly treated, with dams returning to standard laboratory chow on E5, and also sacrificed at 900h on the day of post-mortem. E5 uteri and E7 implantation sites ($N = 5$-$8$ litters/treatment) were collected for either molecular or histology procedures. E11 placentas ($N = 4$ litters/treatment) were dissected from the embryos, and placentas separated from uterine tissue for molecular experiments. A subset of placentas in each litter remained attached to the uterine tissue histology. Maternal plasma was collected at post-mortem from trunk blood into either heparin or EDTA coated tubes. Samples were spun at 3500 rpm for 10 minutes at 4°C, and plasma collected.

5.3.2 Blastocyst viability and lineage allocation

At post-mortem E5, embryos were flushed and scored for developmental progression and any abnormalities noted. Those that had achieved the correct developmental
stage at E5 (blastocysts), were prepared for immunohistochemistry as previously described (Pantaleon et al., 2010), and section 2.10.2. Briefly, embryos were fixed in 2% PFA in 1x PBS for 20 minutes at room temperature (RT), washed in PBS, and immobilized on Cell-Tak (Collaborative Biomedical Products) coated coverslips. Embryos were permeabilised in 0.25% Triton in PBS and washed before blocking with 10% normal goat serum/BSA/Tween-20/PBS (PBT) for 1 h RT. Embryos were incubated in 1:50 anti-Mouse CDX2 (BiogenX, MU392A-UC) overnight at 4°C. On the following day, embryos were extensively washed in PBT prior to exposure of goat anti-mouse labelled secondary antibody Alexa 488 (1:1000, Molecular Probes) for 1 h RT. Embryos were counterstained with DAPI (Sigma Aldrich Inc, B2388) in PBS for 15 min and transferred through increasing concentrations of glycerol in PBS before mounting with Vectashield (Vector Labs, H-1000).

5.3.3 Trophoblast outgrowth assays

E5 embryos were placed into preheated microdrops of TS media on tissue culture plates or gelatine coated coverslips and cultured in a humidifying chamber (Cook, Australia) at 37°C 5% CO<sub>2</sub>/ 5% O<sub>2</sub>/ 90% N<sub>2</sub> under paraffin oil. They were cultured for 6 days, with media changed every 2 days, and imaged with an inverted phase contrast microscope (Leica). Area occupied by outgrowths were traced in ImageJ using calibrated settings. A subset of outgrowths was immunolabelled as for staining procedures above with anti-rabbit pan-cytokeratin (DAKO, Z0622) to distinguish cytoskeleton, goat anti-rabbit af568 secondary antibody, and DAPI (Sigma Aldrich Inc, B2388) to assess number and ploidy of trophoblast nuclei. The remaining outgrowths were collected and extracted for DNA and RNA with Trizol (Life Technologies), and cDNA synthesis from Quantitek reverse transcription kit (Qiagen) using as per manufacturer’s protocol.

5.3.4 Immunofluorescence and Image Analysis

Embryos and outgrowths were visualised using an inverted microscope (Leica, DMi8). Pre-implantation embryos utilised a 40x air objective and captured in z stacks of 40, while outgrowths were imaged at 20x in z-stacks of 10 using a tile scan. Excitation filters of 488nm (green), 561nm (red) and 405nm (blue) fluorescence were imaged
separately. CDX2 immunoreactivity (green) was localised to the TE of the E5 blastocyst. Cells devoid of CDX2 immunoreactivity were assumed to be ICM. TE and ICM cells were counted individually using Image J (NIH). ICM proportion was calculated as: ICM (%) = (Number of ICM cells/ Number of total cells) x 100. LD control and PC-EtOH exposed embryos when E5 on the same day, were assessed for nuclear CDX2 fluorescence. A mask was created around the DAPI channel using Imaris (Bitplane), and the sum CDX2 within TE nuclei counted.

For trophoblast outgrowths, Z-stacks of the DAPI channel were compressed using the average intensity for manual cell counts and ploidy analysis using ImageJ. Smallest trophoblasts with positive staining for pan-cytokeratin had nuclear sizes exceeding 200um². ICM cells (2N) were found to be less than 200um² in size. Nuclear sizes above 1000um² were considered P-TGCs. DNA content, a surrogate marker of ploidy, was quantified from fluorescent intensity of nuclear DAPI. DNA content of all trophoblasts and P-TGCs were normalised to ICM cells of the same image to obtain relative ploidy to 2N.

5.3.5 Genotyping for sex

After analysis, the pre-implantation embryos and blastocyst outgrowths were removed mechanically from coverslips and digested overnight at 55°C in 5ul of lysis buffer with 1ul of 20mg/mL proteinase K. Proteinase K was then inactivated for 10 mins at 95°C. The total 6ul sample was used to amplify male determining gene Sry and B-Actin as a control. Amplification of both genes was carried out in the same reaction due to limited DNA content. Primers for Sry (317bp) and B-Actin (220bp) were derived from Miyajima et al. (2009) for use in the rat. A 2% gel was run for 35 mins at 100V to separate the bands. Double bands indicated male embryos and a single band represented a female embryo. A number of samples (~10%) failed to result in clear bands and were eliminated from analysis. E11 extraembryonic tissue was collected into QuickExtract (Epicentre) and DNA extracted as per manufacturer’s instructions, and genotyped as previously described by Gardebjør et al. (2014).

5.3.6 In situ hybridisation and immunohistochemistry

In situ hybridisation (ISH) was carried out as previously described (Simmons et al., 2007, 2008b), and explained in detail in section 2.10.3. Prf1 was used to identify
infiltrating decidual natural killer cells into maternal uteri (E7) and later, the placental decidua (E11), while Prl8a2, was used to identify the primary decidual zone of the early implantation site at E7. Primer sequences for probe synthesis of these two genes are shown in Table 5.1. cDNA probe templates were isolated by gel extraction (QIAquick Gel Extraction Kit, Qiagen). DIG-labelling of cRNA probes were carried out as per manufacturer’s instructions (Roche). Uterine and placental tissues were fixed in 4% PFA at 4°C for 2 days prior to processing into paraffin and sectioning at 5um. Probes (1:200) hybridisation was carried out overnight at 65°C in a humidified chamber and included negative antisense (T3) controls. All other steps were carried out as described in section 2.10.3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence (5'-3')</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prf1</td>
<td>Forward (T3)</td>
<td>AGCCAGTGCTCTCAAGCGAAT</td>
<td>414</td>
</tr>
<tr>
<td></td>
<td>Reverse (T7)</td>
<td>CAGTCCTGGTTGGGTGACCTT</td>
<td></td>
</tr>
<tr>
<td>Prl8a2 (dPRP)</td>
<td>Forward (T3)</td>
<td>TGCATCAGTCTCTCCTTGCTTGTC</td>
<td>871</td>
</tr>
<tr>
<td></td>
<td>Reverse (T7)</td>
<td>TTTCCCTGTATGCGACACA</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1 In situ hybridisation primers and product length. T3 ; AATTAACCCTCACTAAAGGG and T7 ; TAATACGACTCATATAGGG sequences were added to the beginning of forward and reverse primers respectively. Note; Prl8a2 is also known as decidual prolactin-related protein (dPRP).

5.3.7 Stereology of uterine and placental samples

Stereology for stromal volume, decidual volume, total placental volume and dNK total cell counts using the Cavalieri principle, as described for placental compartments (Coan et al., 2004). In brief, E7 samples embedded in paraffin were serially section at 5um, with 5 sections spaced equally apart at 1:10 taken for IHC or ISH and quantified. Similarly, for E11, 5 sections were collected 1:50. dNK cells were counted in ImageJ. The decidual-myometrial border was clearly demarcated in high resolution 20x images.

5.3.8 Plasma analyses

Maternal plasma samples taken from post-mortem were analysed for glucose concentrations using the COBAS Integra 400 Plus (Roche), and underwent radioimmunoassays for quantification of estrogen (Beckman Coulter, DSL4800),
protocol in section 2.7.2.1, and progesterone as described by Curlewis et al. (1985), using progesterone antiserum C-9817 (Bioquest), described in section 2.7.2.2.

5.3.9 RNA extraction, cDNA synthesis and qPCR

Outgrowth and E11 RNA were extracted with TRIzol (Thermo Fisher Scientific) as per manufacturers protocol (see section 2.8.1.2). E5 and E7 uteri (including the conceptus) were extracted with an RNeasy Mini Kit (Qiagen) as per manufacturer’s instructions. E11 samples underwent on column DNase digestion and RNA clean-up (Qiagen). Trophoblast outgrowth RNA was reverse transcribed using Quantitek reverse transcriptase kit (Qiagen) as per manufacturers protocol with a total of 6ul RNA with 1ul gDNA wipeout buffer. All other samples were reverse transcribed into cDNA using iScript (Biorad), using 1000ng RNA per reaction. Samples then underwent either TaqMan (Qiagen) or SYBR green (Qiagen) qPCR.

TaqMan reactions utilised 1uL (100-200ng) cDNA with 5ul TaqMan (Qiagen), 0.2ul TaqMan assay-on-demand primers (Thermofisher), and multiplexed with 18S (0.2ul), with the remainder to 10ul made up with H2O. SYBR reactions utilised 5ul SYBR green reagent (Qiagen) with 3ul H2O, 1ul of 4uM forward and reverse primers, and 1ul (100-200ng) cDNA. Samples were run in duplicate. The 96-well plates were run in a StepOneTM Real-Time PCR System (Applied Biosystems), for 1 hour. Primers and product codes are shown in Tables 5.2 and 5.3.

Results were used to calculate relative gene expression using the ΔΔCT method (Moritz et al., 2002). The difference between the cycle thresholds (C_T) of the gene of interest and the geometric mean of housekeepers (18S, Rpl0 and Rpl13a) were calculated for each sample (ΔC_T). Relative expression was generated compared to the mean ΔC_T for control, or control males (for when analysing treatment with sex). Preliminary analysis of expression of housekeeper genes showed no differences in expression when analysed for sex, time or treatment.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>AOD ID Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>4333760F</td>
</tr>
<tr>
<td>Rpl19</td>
<td>Rn00821265_g1</td>
</tr>
<tr>
<td>Eomes</td>
<td>Rn01746545_m1</td>
</tr>
<tr>
<td>Ascl2</td>
<td>Rn00580387_m1</td>
</tr>
<tr>
<td>Hand1</td>
<td>Rn00572139_m1</td>
</tr>
<tr>
<td>Prl4a1</td>
<td>Rn00566830_m1</td>
</tr>
<tr>
<td>Ifng</td>
<td>Rn00594078_m1</td>
</tr>
<tr>
<td>Prf1</td>
<td>Rn00569095_m1</td>
</tr>
<tr>
<td>Lif</td>
<td>Rn00573491_g1</td>
</tr>
<tr>
<td>Areg</td>
<td>Rn00567471_m1</td>
</tr>
<tr>
<td>Nr2f2 (CoupTF-II)</td>
<td>Rn00756179_m1</td>
</tr>
<tr>
<td>Hbegf</td>
<td>Rn01405658_m1</td>
</tr>
<tr>
<td>Bmp2</td>
<td>Rn00567818_m1</td>
</tr>
</tbody>
</table>

Table 5.2 TaqMan primers.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (3'-5')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rpl13a</td>
<td>GCA CAA GAC CAA AAG AAG</td>
<td>CGC TTT TTC TTG TCA TAG GG</td>
</tr>
<tr>
<td>Prl3d1</td>
<td>AGA CCT TAT ACA ACA GGA CTC</td>
<td>ATG GCA AAA GAT GAG TGT C</td>
</tr>
<tr>
<td>Hand2</td>
<td>CTC CAA AAA GAC GCA TCT GC</td>
<td>CAT TCA GCT CTT TCT TCC TC</td>
</tr>
<tr>
<td>Ncoa6</td>
<td>AAA AGA TCT TCT CGA CCT G</td>
<td>GTA TCA AGT CAT CTT CCT GC</td>
</tr>
<tr>
<td>Usag1</td>
<td>ACT GGA TCG AAA TAG TCG AG</td>
<td>TCC AGT ACT TTG TTC CGT AG</td>
</tr>
<tr>
<td>Vdup1 (Txnip)</td>
<td>CGT CAA TAC TCC TGA CTT AAT G</td>
<td>AAA TGT CAT CAC CTT CAC AG</td>
</tr>
<tr>
<td>Esr1</td>
<td>ATA TGA TCA ACT GGG CAA AG</td>
<td>CAT TTA CCT TGA TCC TGT TCC</td>
</tr>
<tr>
<td>Pgr</td>
<td>TCT AAT CCT GAA GAG GCA GAG</td>
<td>GAC TTT CAT ACA GAG GAA CTC</td>
</tr>
<tr>
<td>Fgl2</td>
<td>AAA CTC GGA TCC AAA ACG</td>
<td>TGT CTA AAG AGA GTC AGC TC</td>
</tr>
<tr>
<td>Fgf9</td>
<td>ACT ATA AAT GCT TCA TGC GG</td>
<td>CAA TAA ATC AGG CAA GTC GC</td>
</tr>
<tr>
<td>Igfbp-1</td>
<td>AAA CTG AAA GTT GTT TCC TCC TCC</td>
<td>ATA CAA ACC CAC TCT TCC TAT ATE</td>
</tr>
<tr>
<td>Cyp26a1</td>
<td>CAA GCA GCG AAA GAA GGT GA</td>
<td>CTT CAG CAA TCA CTG GCA CG</td>
</tr>
<tr>
<td>Cnr1 (CB1)</td>
<td>CAC CCA TGG CTG AGG GTT CC</td>
<td>CCC AGC TAG AGG AGG TCT GT</td>
</tr>
<tr>
<td>Klf5</td>
<td>AAT CCA AAT TTA CCT GCC AC</td>
<td>TGG AAC CAT CAT AAT CAC AG</td>
</tr>
<tr>
<td>Ihh</td>
<td>AAA GAC GAG GAG AAG ACC</td>
<td>AAAGATCTTCTGAGTGATGG</td>
</tr>
<tr>
<td>Muc1</td>
<td>CGG AAG TCA ATG TGA ATG AG</td>
<td>CAA AAT ACA GAC CAG TAC CAG</td>
</tr>
</tbody>
</table>

Table 5.3 SYBR green primer sequences.
5.3.10 Statistical analyses

For analysis of embryonic stage and number from flushing experiments, preliminary cell counts and uterine stereology QPCR, one-way ANOVAs were used to analyse statistically significant differences between treatments (chow control, liquid diet control, PC-EtOH). For embryo and trophoblast outgrowth analyses, data was analysed using two-way ANOVAs to examine the effects of treatment and sex. All pre-implantation embryos were pooled for analyses. Subsequent outgrowth analyses used litter averages for cell counts, ploidy and gene expression analyses. Maternal plasma analyses also utilised two-way ANOVAs to examine treatment over gestational age. Mean relative gene expression of treatments were standardised to the control or control male group (if more than one sex), displayed as mean ± standard error of the mean. All graphs and statistics were performed using GraphPad Prism 6 software (GraphPad Software, Inc).
5.4 Results

5.4.1 Effects of PC-EtOH on number of flushed embryos and cell counts

To examine the developmental competence of early embryos exposed in utero to PC-EtOH, developmental stage, and cell numbers for total, TE and ICM were recorded. Immunofluorescent labelling for CDX2 (see Chapter 3 Figure 3.1A) was carried out to localise TE cells, with cells showing negative staining assumed to be ICM. When all individual embryos (unsexed) were assessed, preliminary analysis showed the administration of a liquid diet alone had an effect on blastocyst development, causing a reduction in number of blastocysts (P<0.01), average number of blastocyst (P<0.01) and embryos (P<0.05) per litter, and blastocyst cell counts [total (P<0.01), TE (P<0.0001) and ICM cell number (P<0.05)], when compared to chow control (Table 5.4 and Figure 5.1 A-E). When compared to the liquid diet control (LD Control), PC-EtOH exposure had no statistically significant effect on the number of embryos reaching the blastocyst stage on E5 (Table 5.4). Importantly however, when taken as a litter average (Figure 5.1 F-J), analyses showed there was no effect of the liquid diet on any embryo parameter.

When a subset of embryos from LD control and PC-EtOH were subsequently sexed after cell counting, subsequent statistical analysis showed female embryos exposed to PC-EtOH had a reduced ICM count (Figure 5.2C). There were no differences in total cell number suggesting variation in TE counts in the PC-EtOH group. When LD control and PC-EtOH embryos were quantified for nuclear CDX2 fluorescence in the TE, an indicator of TE pluripotency, PC-EtOH exposure markedly reduced levels in both males and females (Figure 5.2F).
### Table 5.4. Flushing statistics of pre-implantation embryos derived from embryonic day 5.

Data was analysed using a one-way ANOVA with Tukey’s post-hoc tests. Morulae was analysed using a non-parametric one-way ANOVA for unequal variances (Kruskal-Wallis test). $N = 22-33$ litters/treatment. Data presented as percentages or mean ± SEM. Different letters (A, B) show significant differences (P<0.05) between treatments, with AB not being different to either A or B.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oocytes</th>
<th>Morulae</th>
<th>Aberrant</th>
<th>Blastocysts</th>
<th>Average Blastocysts / Litter</th>
<th>Average Embryos / Litter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow Control</td>
<td>13/473</td>
<td>1/473</td>
<td>14/473</td>
<td>452/473</td>
<td>12.91 ± 0.48 $^A$</td>
<td>13.52 ± 0.50 $^A$</td>
</tr>
<tr>
<td>Liquid Diet</td>
<td>13/274</td>
<td>1/274</td>
<td>15/274</td>
<td>240/274</td>
<td>10.91 ± 0.74 $^{AB}$</td>
<td>12.46 ± 0.91 $^{AB}$</td>
</tr>
<tr>
<td>PC-EtOH</td>
<td>4/285</td>
<td>2/285</td>
<td>8/285</td>
<td>271/285</td>
<td>10.54 ± 0.68 $^B$</td>
<td>10.96 ± 0.67 $^B$</td>
</tr>
<tr>
<td><strong>P Value</strong></td>
<td>$P = 0.10$</td>
<td>$P = 0.45$</td>
<td>$P = 0.29$</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.05$</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.1. *In vivo* PC-EtOH exposure does not alter cell counts of E5 blastocyst but does show effect of a liquid diet. A. Total cell count. B. Trophectoderm (TE) cell count. C. Inner cell mass (ICM) count. D. Ratio of trophectoderm to inner cell mass (TE:ICM). E. Percentage inner cell mass count of total cell count (% ICM). All data are presented as mean ± SEM and analysed by one-way ANOVA with Tukey’s post hoc tests, *P<0.05, **P<0.01, ***P<0.001. Total cell counts in panel A and F contain 12-18 dams per treatment, with 82-106 embryos. Panels B-E and G-J have 6-10 dams per treatment, with 49-62 embryos.
Figure 5.2. *In vivo* PC-EtOH exposure causes sex-specific alterations to pre-implantation blastocyst development. A. Total cell count. B. Trophectoderm (TE) cell count. C. Inner cell mass (ICM) count. D. Ratio of trophectoderm to inner cell mass (TE:ICM). E. Percentage inner cell mass count of total cell count (% ICM). F. Nuclear CDX2 fluorescence intensity. All data are presented as mean ± SEM and analysed by two-way ANOVA with post hoc tests, *P<0.05, **P<0.001. Embryos were pooled from each litter for analyses. Panels A-E *N* = 5-7 dams per treatment, 14-26 embryos. Panel F shows *N* = 2 dams per treatment, 6-8 embryos.
5.4.2 Trophoblast outgrowth assays from PC-EtOH treated embryos

To further examine the ongoing programming of the TE by alcohol, in vivo treated LD control and PC-EtOH embryos were cultured simultaneously to assess trophoblast outgrowth capacity. PC-EtOH exposure resulted in reduced embryo outgrowth area at day 6 and a reduction in the number of trophoblasts in both sexes. Interestingly, PC-EtOH females also showed reduced numbers of the large P-TGCs (Figure 5.3E). Trophoblast and P-TGC DNA content analysis, a marker of ploidy, showed males tended to increase ploidy of trophoblasts (P=0.07) and P-TGCs (P=0.09), while females remained unchanged. Gene expression analysis showed no alteration to trophoblast stem cell marker Eomes, P-TGC marker Prl3d1, or downstream EPC and chorion genes Ascl2, and Hand1 (Figure 5.4A-D) following PC-EtOH. However, PC-EtOH exposed female embryos showed a reduction in expression of Prl4a1 (P<0.05, Figure 5.4E), a gene exclusively expressed by TGCs for communication with maternal dNK cells.
Figure 5.3. *In vivo* PC-EtOH exposure reduces trophoblast outgrowth rate and sex-specific alterations to P-TGC differentiation and DNA content. Panel A. Control day 6 culture stained with pan-cytokeratin (red) and DAPI (blue). Control (white) and PC-EtOH (black) bars. All data are presented as mean ± SEM and analysed by two-way ANOVA with post hoc student’s t tests. Panel B; *N* = 4 litters/treatment, 2-10 per sex. Panels C-F *N* = 4 litters/treatment, 1-4 per sex.
Figure 5.4. Expression profiles of trophoblast outgrowths exposed to PC-EtOH.

A; *Eomes* (TS maintenance), B; *Prl3d1* (P-TGC marker), C; *Ascl2* (junctional zone marker), D; *Hand1* (TGC marker). Control (white) and PC-EtOH (black) bars. All data are presented as mean ± SEM and analysed by two-way ANOVA with post hoc student’s t tests. N = 7-10 from 4 litters. Gene expression is relative to control male group, and standardised to a geometric mean of 2 housekeepers (*18S* and *Rpl13a*). Gene expression assays were carried out in duplicate.
5.4.3 Effects of PC-EtOH on the peri-implantation uterine environment and early placenta

To further examine the influence of the maternal environment in mediating the effects of PC-EtOH, E5 uteri and E7 implantation sites were assessed for expression profiles for genes involved in receptivity and decidualisation, as both of these are critical events for the establishment and maintenance of pregnancy. Firstly, levels of reproductive hormones estrogen and progesterone were examined over the peri-implantation period (E5-E7), and were unaffected by PC-EtOH, although they did change with gestational age (Figure 5.5).

Estrogen and progesterone receptor expression was also investigated. Expression of \textit{Esr1} was decreased at E5 (P<0.01), but was unchanged at E7 (P<0.05) by PC-EtOH exposure (PInt<0.01, Figure 5.6A). Curiously, the majority of downstream estrogen response genes (\textit{Hbegf, Vegfa, Lif, Fgf2, Fgf9 Figure 5.6B-G}) were unchanged. One gene; \textit{Muc1}, showed an interaction with treatment and age when analysed at E5 and E7 (PInt<0.05). PC-EtOH did, however, cause reduced expression of \textit{Pgr} at E5 (P<0.01), and increased expression at E7 (P<0.01, Figure 5.6F). No significant changes to receptivity genes \textit{Lif, Ihh} or \textit{Usag1} were found, albeit a 46\% decrease in \textit{Usag1} was shown at E5 (P=0.19). Conversely, progesterone response genes \textit{Bmp2} and \textit{Coup-TFII} were decreased by PC-EtOH at E5 only (Figure 5.6K, L), with no other changes found for \textit{Ihh, Areg, Hand2} or \textit{Igfbp1} (Figure 5.6M-P). Some of the genes investigated however, did show temporal profiles with gestational age examined (Figure 5.6).

Further characterisation of histology at E7 implantation sites showed no gross changes to stromal or decidual volume (Figure 5.7). Expression of genes involved in dNK cell maturation; \textit{Vdup1} (P<0.05, Figure 5.6Q), and function; \textit{Ifng} (P<0.05 Figure 5.6R), were markedly decreased at E5 by PC-EtOH, but did not show any change to dNK cell function and common marker gene \textit{Prf1} (Figure 5.6S). At E11, no changes to implantation site volume, or dNK cells were found, although female PC-EtOH exposed implantation sites had 25\% more dNK cells than control females, although this was not statistically significant (P=0.16, Figure 5.8A,B). However, PC-EtOH did cause increased \textit{Prf1} expression at E11 in both sexes (Figure 5.8C).
Figure 5.5. PC-EtOH exposure on maternal hormone profiles of throughout gestation. Control (white), PC-EtOH (black). Data shows mean ± SEM, analysed by two-way ANOVA. E5 data $N = 9$/trt, E6 $N = 3$/trt, E7 $N = 3$/trt, E15 $N = 7-9$/trt, E20 $N = 7$/trt.
Figure 5.6. Uterine expression profiles over the peri-implantation period at E5 and E7. Panels A-E show estrogen response genes, J-O show progesterone response genes, and Q-S show genes involved in dNK maturation and function. Control (white) and PC-EtOH (black) bars. All data are presented as mean ± SEM and analysed by two-way ANOVA. Due to significant differences in age, treatments were analysed at each gestational age with student’s t tests. N = 6/trt (E5) and N = 10/trt (E7) from 5 litters per treatment. Gene expression is relative to control group at E5, and standardised to geometric mean of Rpl19 and B-actin.
Figure 5.7. PC-EtOH does not alter stromal or decidual volume at E7. A; Quantification of uterine stroma from sections localised with vimentin. B; Quantification of primary decidual zone (PDZ) volume from sections stained with Prl8a2. Data shows mean ± SEM, analysed by student’s t test. N = 4-7/treatment.
Figure 5.8. PC-EtOH exposure causes sex-specific infiltration of maternal decidual natural killer cells at E11. A; Quantification of total placental volume at E11. B; Quantification of decidual natural killer (dNK) cells at E11. dNK cells were marked with in situ hybridisation probe Prf1. C. QPCR of E11 placentas for Prf1. Data shows mean ± SEM, analysed by two-way ANOVAs for treatment with sex. N = 7-8 from 4 litters/treatment. Black bar shows scale of 2 mm, black dotted lines show barrier between decidua and uterus (myometrium), and red dotted lines show invading ecto-placental cone.
5.5 Discussion

5.5.1 PC-EtOH exposure - a rodent model of early programming

Historically in the field of developmental programming, the rat has been preferred over the mouse as a model system for investigation of the physiology of adult offspring. Conversely, the mouse has been well characterised for studying developmental biology. However, in more recent times, the rat has been used to investigate pregnancy outcomes including hormone profiles in the peri-implantation period (Kennedy, 1980), programming of the placenta (Mark et al., 2011, Gao et al., 2012, Gardebjer et al., 2014), as well as the development of the early embryo (Kwong et al., 2000, Master et al., 2015). The PC-EtOH project was thus extended to trace phenotypes from adulthood (Gardebjer et al., 2015) and the late gestation placenta (Gardebjer et al., 2014), back through earlier time points during development to determine the contribution of either the embryo or the uterus to programming of fetal growth restriction.

5.5.2 PC-EtOH exposure on development of the pre-implantation embryo

The model of PC-EtOH exposure was first established by Gardebjer, et al. (2014) for administration in a liquid diet. This study altered the ethanol containing diet to contribute equal energy percentages of protein, fat and calories compared to the control diet. This eliminated any potential effects of undernutrition which has been shown to alter pre-implantation development. To first assess the developmental competence of embryos exposed in vivo to PC-EtOH, flushed embryos were assessed for number and stage. No changes to formation of the blastocyst, or aberrant embryos were shown between treatments. Although a number of parameters investigated (number of blastocysts/ total embryos and cell counts), displayed an effect due to administration of a liquid diet, these effects were not significant when embryos form a single litter were averaged together suggesting any differences due to the LD were very subtle. Additionally, this first analysis of all embryos did not take into account embryo sex. In a recent review, we illustrated that regardless of the type of stressor, or the critical window exposed, that many programming models showed marked sex differences throughout placental development (Kalisch-Smith et al., 2017a). These included changes to blastocyst cell number, differentiation to TGCs, and placental formation; assessed by weight, volume stereology and gene profiles. We therefore
assessed embryo sex following analysis in a subset of pre-implantation embryos exposed to either the liquid diet control or PC-EtOH. This revealed that female PC-EtOH embryos had a reduced ICM count. This sex-specific outcome was an interesting finding as another pre-implantation perturbation of IVF, as well as mutant mouse models of X-linked genes, have shown female embryos to develop more deleterious phenotypes than males, including increased cell death and perturbed differentiation (Ghys et al., 2015, Tan et al., 2016a, 2016b, reviewed by Kalisch-Smith et al. (2017a). This is contrary to the current dogma that males appear to be more affected in response to developmental perturbations, as in models of undernutrition when assessed at pre-implantation (Kwong et al., 2006), or postnatal (Kwong, 2000, Jungheim et al., 2010). This suggests that at least in the early stages of development, female embryos may be most at risk of periconceptional perturbations, regardless of the type of stressor; be that genetic or environmental.

Although not investigated for embryonic sex, a decrease in cell number of the ICM is also seen in other perturbation models such as culture in vitro per se (reviewed by Hardy (1997) and maternal low protein diet (Kwong et al., 2000). In vitro, the ICM is considered more sensitive to cell death by apoptosis (10-20%) than the TE (3%), despite being only 30-40% of cells within the developing embryo (Hardy and Handyside, 1996, Brison and Schultz, 1997). The ICM is also suggested to be more metabolically active than the TE in terms of glucose utilisation, which may render it more sensitive to nutrient stress (Hewitson et al., 1996).

Compared to our PC-EtOH model, an in vivo model of a similar ethanol dose (10% EtOH) in the mouse, has shown reduced cell counts, hatching and increased apoptosis (Perez-Tito et al., 2014). While these outcomes were more deleterious than those observed in our model, alcohol was administered in their model in the drinking water, and thus is likely to be confounded by undernutrition, as food intake is often reduced in this model by 10-30% (Shankar et al., 2007). Other in vitro models of EtOH exposure on the developing embryo also show more deleterious outcomes than our in vivo PC-EtOH model, likely due to the added effects from suboptimal embryo culture (Kalisch-Smith, et al. 2017b). Doses of 0.2% EtOH, which are similar to the maximum dose in maternal plasma that PC-EtOH dams receive show reduced blastocyst development and expansion (Page-Lariviere et al., 2017), while 0.3% EtOH in culture is enough to induce apoptosis and cause reduced cell counts (Huang et al., 2007).
These higher doses of alcohol, however, are unlikely to be reached routinely in women, even when binge drinking raising questions regarding the clinical relevance of such studies.

5.5.3 Influence of PC-EtOH exposure on trophoblast differentiation and invasive capacity

Whilst no changes were found in TE cell count in PC-EtOH males or females, marked reductions in nuclear TE CDX2 fluorescence were found, regardless of sex. This suggests that PC-EtOH may result in reduced TE pluripotency (Strumpf et al., 2005). Trophoblast outgrowth experiments showed that both male and female PC-EtOH embryos had reduced outgrowth capacity and trophoblast number. However, it was only the females that showed reduced P-TGC number, suggestive of a differentiation defect. As the ICM also secretes various factors including FGF4 which aid in maintenance of TE stem cell fate (Tanaka et al., 2001), this may account for greater reductions in trophoblast differentiation in PC-EtOH females. Despite these changes in trophoblast number, however, no differences in expression profiles were found between control or PC-EtOH, suggesting that even though there are less trophoblasts/P-TGCs, they are likely at the same developmental stage as the control group.

We further investigated this by examining DNA content (ploidy) of all trophoblasts, and separately, the P-TGCs. Trophoblasts and P-TGCs from male embryos exposed to PC-EtOH tended to increase ploidy. This may suggest that trophoblasts from males can increase cell maturity after a perturbation, and that they may contain some sex-specific mechanisms which may be protective during this early stage. This would agree with the hypothesis there are male specific compensatory mechanisms that occur later in placental development (Clifton, 2010). Additionally, males may have greater influence over cell cycle machinery, as occurs in the divergent development of male and female germ cells (Bowles and Koopman, 2010). Currently, sexual dimorphic pathways of P-TGC differentiation in programming models have not been investigated. However, a mouse mutant of X-linked genes Eed, showed mutant females to show marked reductions in P-TGC numbers (Wang et al., 2001). Although not investigated, perturbation to the P-TGCs is also a likely mechanism to the phenotype of altered EPC formation in females after IVF (Tan, et al. 2016b).
P-TGC differentiation is known to be a default pathway, with TS cells preferentially differentiating in response to perturbations including *in vitro* administration of retinoic acid (Yan et al., 2001), HIF (hypoxic inducible factor) (Maltepe et al., 2005), TGFβ or activin (Erlebacher et al., 2004), and FGF4 removal (Tanaka et al., 2001). However, when TS cells are exposed to a poor environment in culture, such as hyperosmolar stress (Liu et al., 2009), or hypoxic stress (Yang et al., 2016), TGC differentiation is promoted, despite continued application of FGF4. This suggests that cellular stress promotes differentiation over that of TS renewal. Experiments in *Drosophila* show that cells may enter the endocycle instead of undergoing apoptosis, as a protection against genotoxic stressors (Mehrotra et al., 2008). However, these *in vitro* studies may not necessarily mimic what may be happening *in vivo*. Culture *in vitro* is a stressor in itself, and therefore when coupled to an additional perturbation, would be more deleterious. The *in vivo* models mentioned above, rather suggest that differentiation is reduced, leading to fewer P-TGCs (as in PC-EtOH), and smaller EPCs (as in IVF, Tan et al., 2016a). These alterations therefore may be acting through alternate mechanisms to *in vitro* experiments.

One such proposal is altered epigenetic profiles. IVF procedures including fertilisation and embryo culture have been associated with altered imprinted X chromosome inactivation (*Xist*), the exposed females of which exhibit reduced *Xist* and *Rnf12* expression in 8-cell and morulae, which both contribute to initiation and maintenance of X chromosome inactivation (XCI) (Tan et al., 2016a). They also show decreased H3K27me3 profiles in TE and EPCs, a marker of XCI and a known repressive modification to gene transcription (Tan et al., 2016a). The authors went on to rescue this phenotype in females with the morphogen retinoic acid (RA), as it is known to upregulate *Xist* expression in embryonic stem cells (Ahn and Lee, 2010). Significantly, alcohol has also been associated with global reductions in RA, as EtOH metabolism preferentially utilises the same enzymes required for RA synthesis from vitamin A (Duester, 1991, Yelin et al., 2005). Moreover, application of RA on TS cells shows giant cell differentiation (Yan et al., 2001), showing a reciprocal phenotype to that of PC-EtOH. RA may therefore be a common mechanistic conduit in the effects of PC-EtOH and IVF, as altered RA synthesis and secretion in the oviduct and/or uterus may compromise the epigenetic erasure and reprogramming of the female embryos as they proliferate and differentiate (Hore et al., 2016). As the early embryo resets its
epigenome, it removes 5-methylcytosine (methylation) and oxidises to 5-hydroxymethylcytosine, a process enhanced by retinol (Hore et al., 2016). Furthermore, a global reduction in retinol may cause a reduction in this oxidation, leading to global hypermethylation, which may be reflected by reduced CDX2 expression after PC-EtOH. A recent study in male and female induced pluripotent stem cell experiments (converting mouse embryonic feeder fibroblasts into embryonic stem cells) in vitro, has additionally shown females to undergo more pronounced global, untargeted demethylation during reprogramming, while targeted loss of methylation marks; (hypomethylated) genes required for cell identity (e.g. pluripotency factors) were similar between sexes (Milagre et al., 2017). This may further delineate sexually dimorphic phenotypes during the pre-implantation period, particularly to the ICM lineage, after an altered maternal environment. However, whether these outcomes are consistent with other nutritional perturbations such as a low protein diet, currently remains unknown.

Collectively, these results suggest females are at most risk of environmental perturbations during the periconception and pre-implantation periods, most likely due to their greater requirements for epigenetic reprogramming. Males, however, seem less affected, despite being exposed to the same in utero environment. However, given that in the postnatal environment, males and females experience the same disease susceptibility of altered glucose tolerance and insulin insensitivity (Gardebjer et al., 2015), this may suggest that other factors may be at play during placental formation and organ development. These early experiments also demonstrate that CDX2 fluorescence may be a more sensitive indicator of reduced developmental potential of the trophoblast lineage than TE cell count.

5.5.4 Influence of PC-EtOH on the maternal uterine responses for the establishment of pregnancy

As subtle changes in early embryonic development arose from PC-EtOH prior to implantation, the question now arises as to whether these changes are accounted for by either a direct effect of EtOH on the embryo, as assessed by in vitro experiments of TS cell differentiation (see Chapter 4), or rather – indirectly, through alterations to the uterine and/or maternal hormonal environment. To determine the impacts of PC-EtOH exposure on uterine responses required for the establishment of implantation
and pregnancy success, we first examined maternal hormone profiles for estrogen and progesterone over the peri-implantation period (E5-7), and later in gestation (E15 and E20). Similar to other studies in the rat investigating the peri-implantation period (following nidation), estrogen levels peaked at E7 and decreased thereafter. As aforementioned, a nidatory estrogen surge is usually present late on E4/early E5 (Shaikh, 1971, Singh et al., 1996) and is required for uterine sensitisation and marks the onset of receptivity. While this did not result in our animals at E5, this is likely due to the alternate light-dark cycle, which shifted the investigated window by 0.5 days. We thus may have not sampled our animals at the appropriate time. During the peri-implantation period, progesterone levels peaked at E6 at the onset of implantation, similar to other studies in the rat (Barkley et al., 1979, Garland et al., 1987), and declined until term. No changes were shown in either hormone profile during uterine receptivity (E5), or decidualisation (E6-7) in the PC-EtOH group.

Other studies investigating the effects of EtOH in the rat on hormones during pregnancy are varied. Lower doses of alcohol over a chronic period (60 days of 10% v/v EtOH) did not alter either estrogen or progesterone when assessed on the morning of estrus (Chuffa et al., 2013) nor did 4.8g/day EtOH after 2 months’ exposure (Emanuele et al., 2001). Short-term exposures, however, including 4.8g/day EtOH, increased estrogen without showing any change to progesterone after 2 weeks (Emanuele et al., 2001), while another model of high dose of 30% v/v EtOH (injected as a bolus) caused reduced estrogen and progesterone after 3h (LaPaglia et al., 1997). This suggests the effect of EtOH on hormonal profile is dosage and time dependent, with high doses eliciting a rapid change to hormone levels. This may be a limitation of our study at E5 as dams were not sampled during peak alcohol consumption but rather several hours after they had ceased consumption.

Although the short-term impact of EtOH on hormone levels was not investigated in this model, we were able to determine expression of their receptors which may reflect more chronic changes in hormone concentrations. These receptors control the major signalling pathways for receptivity and decidualisation. Of great interest, PC-EtOH reduced expression of estrogen receptor alpha (Esr1) and the progesterone receptor (Pgr) in the receptive E5 uterus. Conversely the Pgr was elevated at E7 following the PC-EtOH. A study by Chuffa et al. (2013) has also showed similar findings, with a similar dose of 10% EtOH. They showed reduced Pgr and Esr1 in the uterus after 60
days of alcohol administration. Associated with these changes to the hormone receptors, a select group of downstream hormone response genes were altered. At E5, progesterone response genes *Bmp2*, *Coup-TFII* (*Nr2f2*) were found to be downregulated. *Coup-TFII* is expressed in the sub-epithelial stroma, and is involved in the regulation of uterine implantation by controlling estrogen receptor activity (Kurihara et al., 2007). Knock out studies of *Coup-TFII* show embryo attachment and decidualisation are impaired (Kurihara et al., 2007), and this has adverse consequences for placental formation through trophoblast differentiation and labyrinth vascularisation, which leads to miscarriage by mid-gestation of pregnancy (Petit et al., 2007). *Bmp2* is also downstream of *Coup-TFII* and is involved in decidualisation and the initial attachment reaction (Paria et al., 2001). At E7 PC-EtOH caused reduced expression of *Klf5*, a hormone independent gene expressed in the uterine epithelial and stromal cells, which is involved in conferring uterine receptivity to implantation (Sun et al., 2012). Considering no changes to receptivity genes were apparent, the above results suggest that implantation may be slightly delayed, and is most likely due perturbed signalling to the luminal epithelium and stroma from an immature blastocyst.

One interesting outcome from the uterine expression profiles, was a reduction in *Vdup1* at E5 and E7 after PC-EtOH exposure. *Vdup1* has been shown as a marker of a refractory uterus, is expressed in the uterine stroma (Simmons and Kennedy, 2004), and is also involved in dNK cell maturation (Lee et al., 2005, Kim et al., 2007). Quantification of dNK cells at E11 by ISH for *Prf1* showed a tendency (25%) for elevated counts in PC-EtOH females and significantly increased *Prf1* expression in both sexes. Reduced signalling by the P-TGCs in PC-EtOH females through production of *Prl4a1*, could also affect the dNK cell homing, production of IFNg, and thus influence their maturation and survival (Ain et al., 2004, Muller et al., 1999). *Prl4a1* is also one of the most highly expressed genes on chromosome 13 in the prolactin cluster, and is the 29th most highly expressed gene in the whole P-TGC genome (Hannibal and Baker, 2016), which shows that it is highly endoreplicated and its importance in development. dNK cells are known to decrease around E11 (Paffaro et al., 2003), which suggests that PC-EtOH females may be slightly delayed in development. We hypothesise that reduced dNK cell maturation is the result of reduced embryo-uterine cross-talk. Conversely, these dNK cells may have greater activation after PC-EtOH, as can also occur in placental pathologies. Another
possibility is that the reduced decidualisation at E5 may also result in a
developmentally delayed uterus, also causing reduced dNK infiltration/maturation,
which may explain why males also have increased Prf1 expression at E11, but not
dNK cell count. However, the uterine genes investigated here at E7 did not support
this proposition. In addition to dNK cells producing cytokines such as Ilng (Ashkar et
al., 2000), they also secrete other pro-angiogenic factors that help attract invasive
TGCs into decidual spiral arteries (Chakraborty et al., 2011). The trophoblasts then
regulate spiral artery remodelling into low resistance, high flow vessels to facilitate the
late gestational fetal growth spurt through increased placental perfusion (Wallace et
al., 2012). This outcome of altered trophoblast differentiation and communication with
maternal factors, may be a primary mechanism by which periconceptional perturbation
models affect long term placentation.

5.5.5 Future investigations

Further investigation into the maternal hormonal environment will help establish
whether there are long term consequences to subtle changes in estrogen and
progesterone signalling, and whether these can contribute to changes in the uterine-
embryo cross-talk for implantation, and further placentation (see Chapter 6). Validation
of expression profiles also requires determination of protein levels where possible,
before definitive conclusions can be made. Future experiments should also investigate
X chromosome dosage and inactivation in females exposed to in utero perturbations
during early development and differentiation. This will aid in determining whether all
females show the most deleterious phenotypes during this period, or if this is only
specific to exposures of IVF and PC-EtOH exposure.

5.6 Conclusions

This chapter has shown that by E5, PC-EtOH exposure has programmed the
development of the pre-implantation embryo in a sexually dimorphic manner. Female
exposed embryos show alterations to both embryonic (ICM) and extraembryonic (TE)
compartments, while males only show mild changes to the TE. This suggests that
female embryos are more susceptible to implantation defects and placentation than
males following PC-EtOH exposure. Furthermore PC-EtOH resulted in a mildly
perturbed maternal environment indicated by altered expression of hormone receptor
genes within the uterus and some of their downstream constituents, suggesting there may be mild effects to uterine decidualisation, and embryo-uterine cross-talk. These results suggest PC-EtOH result in perturbations to both the embryo and the uterine environment which may contribute to altered implantation and placentation.
Chapter 6. Periconceptional alcohol exposure: effects on the morphological development of the placenta

<table>
<thead>
<tr>
<th>Contributions to Chapter 6</th>
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<tbody>
<tr>
<td>Contributor</td>
</tr>
</tbody>
</table>
| Kalisch-Smith, J.I. | Experimental design (20%)  
Animal treatment (10%)  
Sample collection (100%)  
Data collection (100%)  
Interpretation of results (40%) |
| Simmons, D.G. | Experimental design (35%)  
Interpretation of results (20%) |
| Pantaleon, M. | Experimental design (10%)  
Interpretation of results (5%) |
| Moritz, K.M. | Experimental design (35%)  
Interpretation of results (35%) |

Note: The remaining animal treatment with liquid diets was performed by members of the Moritz laboratory PC-EtOH project, with animals coordinated by Lisa Akison. Special thanks to Sarah Walton, Jenn Outhwaite and Moritz lab members who helped with post-mortem euthanasia.
6.1 Abstract

Periconceptional ethanol (PC-EtOH) exposure in the rat causes fetal growth restriction, increased resorptions, and sex-specific changes to placental morphology in late gestation (E20). This study continued to investigate the impacts of PC-EtOH exposure on structural morphogenesis and remodelling of the placenta from mid- to late-gestation in a sexually dimorphic manner. Sprague Dawley rat dams were administered 12.5% v/v EtOH from 4 days prior (E-4) to 4 days after conception at embryonic day (E) 4 in a liquid diet. Placental samples were collected to analyse the immature (E13), definitive (E15) and mature (E20) placenta. Placentas was assessed for volume and surface area of compartments (labyrinth and junctional zone), and a subset taken for gene expression analysis.

PC-EtOH caused an increase in resorptions at E15, which were not present at earlier ages (E11, E13). Similarly, at E15, PC-EtOH reduced labyrinthine wet weight and the placental depth. No other changes to weights of the whole placenta, junctional zone, or measurements of the length or width were found. Stereological analysis from E13-E20 showed differences only at the E15 time point to be significant between treatment groups. Whilst no change to volume of the whole placenta, decidua, labyrinth or junctional zone were found, PC-EtOH exposure reduced maternal blood space (MBS) volume in both sexes. Curiously, this was without any change to the MBS surface area. These changes were not observed at E13 or E20, suggesting the definitive placenta is most affected by PC-EtOH, and that by E20, compensatory growth has occurred. This growth of the placenta, however, was not able to ameliorate the fetal growth deficit which occurred at E20 but was not present at E13 or E15. Gene expression profiles of nutrient transporters showed that expression of the glucose transporter Glut3 in the labyrinth was reduced by PC-EtOH at E13 in both sexes whereas at E15, males tended to decrease placental Glut3 expression and females increased expression. No other changes in gene expression were found to occur following PC-EtOH for other nutrient transporters at either age (Slc38a1, Slc38a2, Slc38a4).

As a major alteration to placental structure was found at mid-gestational E15, we questioned whether this was due to altered remodelling of the uterine spiral arteries by inadequate trophoblast invasion earlier in gestation. When the E13 implantation
site was assessed, only PC-EtOH females showed reduced invasion, exhibiting little infiltration past the decidua into the mesometrial triangle. Considering the findings of reduced in MBS in both sexes, this perturbed invasion in females has most likely led to blunted or delayed spiral artery remodelling. We hypothesise this may have altered uterine blood flow overall, reducing the nutrients delivered to implantation sites of both sexes. This would explain why the placenta of both males and females exposed to PC-EtOH show reduced MBS expansion at E15. Overall, PC-EtOH led to a reduction is MBS at E15 which likely is the origin of fetal growth restriction in this model. This data will contribute to our knowledge on placental pathologies, the origins of fetal growth restriction, and the programming of adult disease.
6.2 Introduction

Appropriate formation and function of the placenta is crucial in meeting the nutritional demands of the fetus, to enable growth and viability. A suboptimal in utero environment is increasingly being established to program placental growth and development, having adverse outcomes for fetal growth, and leading to programming of adult disease. Insults to poor maternal nutrition such as a low protein (Gao et al., 2012), as well as exposure to alcohol (Gardebjer et al., 2014), glucocorticoids (Cuffe et al., 2011), hypoxia (Cuffe, 2014a) and embryo culture (Tan et al., 2016a), can all cause fetal growth restriction through modification to the structure and function of the placental zones. These adaptations to the placenta can also occur in a sexually dimorphic manner regardless of the critical window of exposure (Kalisch-Smith et al., 2017a). The placental zones carry out important functions, with the labyrinth housing the fetal (FBS) and maternal (MBS) blood compartments which mediate nutrient exchange across the interhemal membrane. The junctional zone has a primary endocrine role, and contains specialised trophoblast subtypes; spiral artery-associated giant cells (SpA-TGCs) and glycogen trophoblasts (GlyT), which invade into the decidua and uterine spiral arteries. SpA-TGCs are of particular interest, as they mediate remodelling of the uterine spiral arteries into low resistance, high flow vessels, and enable the late gestation fetal growth surge, through increased blood supply through the central canal, and subsequently, the MBS, allowing greater perfusion (Wallace et al., 2012). The placental trophoblast subtypes also express lineage-restricted markers which allows investigation into differentiation both in vivo and in vitro (as investigated in Chapter 4).

Despite considerable knowledge on the effects of poor of maternal nutrition on placental biology, alcohol consumption has not received as much attention. Alcohol consumption is widespread during pregnancy, especially in the periconception period prior to pregnancy recognition (Colvin et al., 2007, Wallace et al., 2007, Muggli et al., 2016). The majority of research to date has focused on a direct effect of alcohol on placentation during mid- to late-gestation, as alcohol can readily cross the placenta during nutrient exchange (Weinberg et al., 2008). However, few studies investigating alcohol exposure recognise the earlier periconceptional period in placental programming.
In the human, there is also evidence the periconceptional period may affect placentation: IVF studies have shown reduced endometrial vascularisation (Lai et al., 2014) and greater incidences of hypertension in mothers of IVF babies (Westerlund et al., 2014). Of the animal models that have investigated alcohol exposure, they show dose dependent effects including reduced vascularisation, with perturbed vascular disorganisation, trophoblast differentiation, invasion, and placental blood flow, along with fetal growth restriction (Gundogan et al., 2013, 2015). However, one more recent model of first trimester alcohol exposure in the rhesus macaque suggests that some of these outcomes may be related to perturbations to periconceptional events (Lo et al., 2017). Along with reduced fetal weight, placental blood flow and oxygen delivery were reduced after alcohol exposure (Lo et al., 2017). These outcomes may therefore contribute to placental pathogenesis of gestational hypertension and preeclampsia as seen in women who have consumed alcohol during pregnancy (Meyer-Leu et al., 2011, Salihu et al., 2011).

We have recently described how PC-EtOH in the rat causes a late gestation placental phenotype, consisting of altered labyrinth and junctional zone cross-sectional areas, increased resorptions and fetal growth restriction, with males and females showing reductions of 8% and 7% respectively (Gardebjer et al., 2014). As described above in Chapter 5, PC-EtOH can cause sex-specific alterations to the pre-implantation embryo and trophoblast differentiation, with females showing the most deleterious outcomes. Of particular interest, was the long-term impacts of reduced trophoblast pluripotency and invasive capacity in PC-EtOH exposed embryos, particularly females. This raises some key questions; including why PC-EtOH females don’t show greater growth restriction than PC-EtOH males by late gestation, why males and females have the same postnatal disease susceptibility for glucose tolerance and insulin sensitivity, and what is the role of the placenta in mediating these outcomes? As discussed in Chapter 5, PC-EtOH also tended to cause an increase in dNK cells in female exposed embryos, which may have long term consequences for the secondary wave of trophoblast invasion (Chakraborty et al., 2011).

This chapter therefore aims to characterise structural changes to the immature, mid- and late-gestation placenta after PC-EtOH exposure, and their influence on fetal growth.
6.3 Methods

6.3.1 Ethics, animal handling and liquid diet administration

All animal experiments and procedures were approved by The University of Queensland Animal Ethics Committee (AEC approval number SBS/022/12/NHMRC) prior to commencement of this study. PC-EtOH exposure was administered in vivo to Sprague Dawley dams in a liquid diet containing 12.5% v/v EtOH as previously described (Gardebjer et al., 2014, 2015). Dam weight was at least 230g before entering the protocol. Vaginal impedance was measured daily with an EC40 oestrous cycle monitor (Fine Science Tools), with 4.5 x 10^3 Ω or higher indicating oestrus and allocation to the diet regime. Dams were treated over two oestrous cycles, one prior to mating and the establishment of the pre-implantation period (denoted as embryonic day (E) -4 to E4). Dams were time-mated on E0 from 1200h to 1700h if impedance exceeded 4.5x10^3Ω. If pregnancy was established, the following day was considered E1. On E5, dams were returned onto standard laboratory chow until sacrifice.

6.3.2 Post-mortem and tissue collection

At 900h on E13 (N = 4-5 litters/treatment), dams were sacrificed via guillotine prior to dissection of the placental tissues. Dams in E15 (N = 9 litters/treatment) and E20 (N = 6 litters/treatment) cohorts were heavily anaesthetised with 50:50 ketamine:xylazil (0.1ml/100g body weight, Lyppard Australia Ltd, QLD, AUS) as previously described (Gardebjer et al., 2014). Fetal and placental weights were taken at post-mortem for the E15 cohort, and placentas were separated from the uterus/decidua, with the junctional zone and labyrinth and weighed separately. Placental measurements were also taken for length, width and depth using digital callipers (LCD Display Calliper Vernier Micrometer, Scientrific Pty. Ltd., Yamba, NSW, AUS). Placentas from all E13, E15 and E20 cohorts were collected for RNA and histological procedures. For histology, placentas were cut in half with uterus and decidua attached and fixed in 4% PFA prior to processing into paraffin for stereology. E13 placenta samples were processed as above into paraffin, or processed for cryo-sectioning. A subset of labyrinth samples from E15 and E20 placentalas were cut into 1mm^3 and fixed in 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer until tissue processing for
transmission electron microscopy (section 2.10.5). All cohorts collected fetal tissue for genotyping for sex as previously described (Gardebjer et al., 2014).

6.3.3 Histology

6.3.3.1 Haematoxylin and eosin

Slides were dewaxed in xylene for 3 x 2 min, rehydrated in 2 x 100% EtOH, and 1x 90% EtOH, 1x 70% EtOH for 2 min, and 1x dH₂O for 2 min. Slides were incubated in Meyer's haematoxylin for 2 minutes prior to extensive washing in dH₂O. Slides were incubated in 1% alcoholic eosin for 15 minutes. Slides were dehydrated in 95% EtOH, 2x 100% EtOH and 2x xylene washes prior to mounting in DPX mounting medium (Ajax Finechem).

6.3.3.2 Immunofluorescence on cryo-sections

Immunolocalisation was carried out as previously described (Pantaleon et al., 2010) and in section 2.10.2, but on E13 midline placental cryo-sections. Placentas were cut at 10um, with every 10th section taken for staining. Sections were blocked with 10% normal goat serum/BSA/Tween-20/PBS (PBT) for 1 h RT prior to incubation with primary antibodies; 1:200 anti-rabbit pan-cytokeratin (DAKO, Z0622) to distinguish invasive placental trophoblasts, and 1:100 anti-mouse alpha smooth muscle actin (ab7817, Abcam) to distinguish uterine spiral arteries. The following day, embryos were extensively washed in PBT prior to exposure of goat anti-rabbit af555 and goat anti-mouse af488 secondary antibodies. All slides were counterstained with DAPI (Sigma Aldrich Inc, B2388), and mounted in glycerol prior to image analysis.

6.3.3.3 In situ hybridisation

In situ hybridisation (ISH) was carried out as previously described (Simmons et al., 2007, 2008b), and explained in detail in section 2.10.3. Mest was used to identify fetal endothelial cells of the FBS in E15 tissue. Primer sequences for probe synthesis of these two genes are shown in the table below. cDNA probe templates were isolated by gel extraction (QIAquick Gel Extraction Kit, Qiagen). DIG-labelling of cRNA probes were carried out as per manufacturer’s instructions (Roche). Uterine and placental tissues were fixed in 4% PFA at 4°C for 2 days prior to processing into paraffin and
sectioning at 5um. Probes (1:200) hybridisation was carried out overnight at 65°C in a humidified chamber and included negative antisense (T3) controls. All other steps were carried out as described in section 2.10.3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence (5'-3')</th>
<th>Product (bp)</th>
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<tbody>
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<td>Mest</td>
<td>Forward (T3)</td>
<td>CTGCTCTGCACTCATGGAAG</td>
<td>478</td>
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<tr>
<td></td>
<td>Reverse (T7)</td>
<td>CCGTCTTGGAGAGCTTTTG</td>
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</table>

Primer pair sequences for In Situ Hybridisation (ISH) probe synthesis. Forward primers included the addition of T3 - AATTAACCCTCACTAAAGGG to the 5’ end, and likewise for T7 – TAATACGACTCACTATAGGG.

6.3.3.4 Lectin histochemistry

Paraffin slides were dewaxed and rehydrated as above, and placed into dH₂O. Slides underwent antigen retrieval in sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0) was performed for 1.5 hours using a pressure cooker. Tissue was blocked for endogenous peroxides using 0.9% H₂O₂ in dH₂O for 10 minutes. Slides were incubated in 0.1% triton with 0.1mM ions (MgCl₂, CaCl₂, MnCl₂) for 10 minutes, prior to incubation of 1:200 IsolectinB4 (conjugated, Sigma, L5391, derived from Bandeiraea simplicifolia) in 1x PBS for 2h RT. A 1x PBS wash for 5 minutes followed prior to colour development with NovaRED peroxidase (Vector Labs, SK-4800). Slides were counterstained with haematoxylin for 2 minutes, and were dehydrated and mounted as per section 2.10.3.2.

6.3.4 Imaging parameters and analysis

All chromogen slides were scanned with Aperio and snapshots taken with Imagescope. Placental cryo-sections were imaged on an upright fluorescence microscope (BX61 Olympus) using DAPI, GFP and Cy3 filters at 4x and 10x magnification using set parameters. Placentas were measured for distance of trophoblast invasion from the TGC layer, and number of invaded decidual and uterine arteries.
6.3.5 Stereology for placental volumes

Stereology for placental compartments; total, decidua, junctional zone, labyrinth, FBS and MBS were estimated using the Cavalieri principle as described by Coan et al. (2004). Placental halves were sectioned at 5um for 5-6 equally spaced sections with sampling fractions of 1:50 (E13), 1:100 (E15) and 1:150 (E20) used. E13 placentas utilised H&E staining, E15 placentas utilised ISH for Mest, and E20 samples utilised IsolectinB4, the latter two of which both marked fetal endothelial cells of the FBS. Volume and surface areas of placental compartments were estimated in ImageJ.

6.3.6 Gene expression assays

Total RNA from labyrinth samples at E13 and E15 were extracted using RNeasy kits with on column DNase digestion (Qiagen). 1ug RNA was reverse transcribed using the iScript cDNA kit (Bio-Rad). qRT-PCR was performed using TaqMan (Qiagen) or SYBR green (Qiagen) reagents, with 100ng cDNA used per reaction, see primer information below. Expression profiles of two replicates per sample were analysed relative to the geometric mean of 18S and Rpl13a.

<table>
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<tr>
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<td>Gcm1</td>
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<td>Glut3 (Slc2a3)</td>
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<td>Mest</td>
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<td>Sry</td>
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TAQman primer information

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SYBR primer information

189
6.3.7 Statistical analyses

To analyse treatment with sex, or zone, data was analysed using two-way ANOVAs with Tukey’s post-hoc tests. In cases where the sexes were analysed separately, student’s t-tests analysed significant differences between control and PC-EtOH. Stereological analyses utilised at most 2 samples per sex per litter. Mean relative gene expression of treatments were standardised to the control male group, displayed as mean ± standard error of the mean (SEM). All graphs and statistics were performed using GraphPad Prism 6 software (GraphPad Software, Inc).
6.4 Results

To characterise the placental biometry prior to E20, placental samples were collected at E13 and E15. Examination of litters at post-mortem showed PC-EtOH caused an increase in resorptions at E15 (P<0.05, Table 6.1), which were not exhibited earlier ages (E11, E13, data not shown). Analysis of sex ratios showed a greater percentage of male fetuses at E15 after PC-EtOH (P=0.05, Table 6.1). Similarly, at E15, PC-EtOH reduced labyrinthine wet weight and the placental depth (Table 6.1). There were no other gross differences between treatment groups in weight of the whole placenta, junctional zone, or measurements of the length or width were found at E15 (Table 6.1), nor ratios of labyrinth to junctional zone, labyrinth to whole placenta, or junctional zone to whole placenta (data not shown).

To determine whether any structural changes had occurred to the placenta, and the critical time points during gestation they may arise, E13 (immature), E15 (definitive), and E20 (mature) placentas were assessed by stereology for compartment volume and surface area of the vasculature. Stereological analysis showed significant differences between treatment groups at E15. Whilst no change to volumes of the whole placenta, decidua, labyrinth or junctional zone were found in males (Figure 6.1), females exposed to PC-EtOH showed markedly reduced placental volume, with all compartments contributing to this effect (P<0.01). Further examination of the placental vasculature for fetal and maternal blood compartments showed the placenta of females exposed to PC-EtOH to have significantly reduced MBS (P<0.05, Figure 6.2). When MBS volume was analysed with males and females together by two-way ANOVA, a treatment effect was found after PC-EtOH (PTrt<0.01). No change to MBS surface area was found between treatments for either sex (Figure 6.3). Along with MBS and FBS volume, PC-EtOH females also tended to reduce labyrinth trophoblast volume (PZone<0.05, Figure 6.3), but was not significant between treatments by post-hoc analysis. Despite the alteration in MBS, the thickness of the interhemal membrane remained unchanged (Figure 6.4).

As the alteration in placental development was found at mid-gestational E15, we questioned whether this was due to altered remodelling of the uterine spiral arteries by inadequate trophoblast invasion earlier in gestation. When the E13 implantation site was assessed by histological staining for trophoblast marker pan-cytokeratin,
there was a tendency for reduced maximum invasion in the placentas of females exposed to PC-EtOH (Figure 6.5, P=0.08), with little invasion past the decidua into the mesometrial uterine spiral arteries (Figure 6.5, P<0.05).

To investigate other changes to placental development from PC-EtOH exposure, E13 and E15 placentas were assessed by QPCR for markers of trophoblast differentiation (Mest, Ctsq, Gcm1, Syna), and nutrient transporters for glucose (Glut3), and amino acids (Slc38a1, Slc38a2, Slc38a4). At E13, PC-EtOH exposure reduced expression of Mest (PTrt<0.01, Figure 6.6); marking fetal endothelial cells, particularly in males (P<0.05). Expression of the nutrient transporter Glut3 was also decreased (PTrt<0.01), particularly in females (P<0.05). No changes were found in the remaining markers or genes at this age. At E15, although no statistically significant changes were shown in the abovementioned genes, several interactions were found for Mest (PInt<0.05), Syna (PInt<0.05) and Glut3 (PInt<0.05), suggesting that male PC-EtOH placentas decreased their expression, and female PC-EtOH increased expression of all genes.
Table 6.1. Fetal and placental biometry at post-mortem at E15.

All data are presented as mean ± SEM and N = 8-9 litters per treatment. *P < 0.05 for Bonferroni post-hoc compared with controls of same sex. Note: litter size includes resorptions.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Male Control</th>
<th>Male PC-EtOH</th>
<th>Female Control</th>
<th>Female PC-EtOH</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>0.208 ± 0.007</td>
<td>0.219 ± 0.006</td>
<td>0.206 ± 0.007</td>
<td>0.209 ± 0.005</td>
<td>PTrt = 0.27, PSex = 0.34, Pint = 0.49</td>
</tr>
<tr>
<td>Crown-rump length (mm)</td>
<td>12.405 ± 0.245</td>
<td>12.601 ± 0.048</td>
<td>12.226 ± 0.195</td>
<td>12.142 ± 0.092</td>
<td>PTrt = 0.74, PSex = 0.06, Pint = 0.40</td>
</tr>
<tr>
<td>Litter size (number)</td>
<td>Control: 16.330 ± 0.500</td>
<td>PC-EtOH: 17.000 ± 0.645</td>
<td>P = 0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resorptions (number)</td>
<td>Control: 0.440 ± 0.294</td>
<td>PC-EtOH: 1.889 ± 0.539</td>
<td>P &lt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex Ratio (Male:Female)</td>
<td>Control: 0.94</td>
<td>PC-EtOH: 1.41</td>
<td>P = 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male Fetuses (%)</td>
<td>Control: 44.26</td>
<td>PC-EtOH: 56.69</td>
<td>P = 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placental parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>0.247 ± 0.023</td>
<td>0.212 ± 0.015</td>
<td>0.217 ± 0.006</td>
<td>0.218 ± 0.009</td>
<td>PTrt = 0.23, PSex = 0.41, Pint = 0.22</td>
</tr>
<tr>
<td>Placenta:BW ratio (g/gbw)</td>
<td>1.128 ± 0.059</td>
<td>1.036 ± 0.111</td>
<td>1.127 ± 0.041</td>
<td>1.083 ± 0.060</td>
<td>PTrt = 0.37, PSex = 0.76, Pint = 0.75</td>
</tr>
<tr>
<td>Junctional zone wet weight (g)</td>
<td>0.102 ± 0.008</td>
<td>0.102 ± 0.007</td>
<td>0.096 ± 0.006</td>
<td>0.118 ± 0.008</td>
<td>PTrt = 0.27, PSex = 0.34, Pint = 0.49</td>
</tr>
<tr>
<td>Labyrinthine wet weight (g)</td>
<td>0.086 ± 0.009</td>
<td>0.078 ± 0.006</td>
<td>0.094 ± 0.006</td>
<td>0.075 ± 0.004</td>
<td>PTrt = 0.06, PSex = 0.75, Pint = 0.39</td>
</tr>
<tr>
<td>Placental length (mm)</td>
<td>12.087 ± 0.352</td>
<td>12.111 ± 0.212</td>
<td>12.144 ± 0.232</td>
<td>12.297 ± 0.155</td>
<td>PTrt = 0.15, PSex = 0.63, Pint = 0.80</td>
</tr>
<tr>
<td>Placental width (mm)</td>
<td>11.411 ± 0.245</td>
<td>11.027 ± 0.233</td>
<td>11.037 ± 0.187</td>
<td>11.191 ± 0.209</td>
<td>PTrt = 0.60, PSex = 0.64, Pint = 0.23</td>
</tr>
<tr>
<td>Placental depth (mm)</td>
<td>2.979 ± 0.104</td>
<td>2.590 ± 0.097</td>
<td>2.836 ± 0.079</td>
<td>2.653 ± 0.083</td>
<td>PTrt &lt; 0.01, PSex = 0.85, Pint = 0.26</td>
</tr>
</tbody>
</table>
Figure 6.1. PC-EtOH exposure reduces whole placental and zonal volumes in females only at E15. Data shows mean ± SEM, analysed by two-way ANOVA. *P<0.05. Control (white bars), PC-EtOH (black bars). E13; N = 5-7/sex, 4 litters/treatment. E15; N = 7-9/sex, 5-7 litters/treatment, E20; N = 10-12/sex, 6 litters/treatment.
Figure 6.2. PC-EtOH reduces labyrinthine MBS volume in both sexes. Data shows mean ± SEM, analysed by two-way ANOVA. *P<0.05. Control (white bars), PC-EtOH (black bars). E13; N = 5-7/sex, 4 litters/treatment. E15; N = 7-9/sex, 5-7 litters/treatment, E20; N = 10-12/sex, 6 litters/treatment.
Figure 6.3. PC-EtOH does not alter labyrinth blood space surface areas. Data shows mean ± SEM, analysed by two-way ANOVA. *P<0.05. Control (white bars), PC-EtOH (black bars). E13; N = 5-7/sex, 4 litters/treatment. E15; N = 7-9/sex, 5-7 litters/treatment, E20; N = 10-12/sex, 6 litters/treatment.
Figure 6.4. PC-EtOH does not alter interhaemal membrane thickness. Data shows mean ± SEM, analysed by two-way ANOVA. *P<0.05. Control (white bars), PC-EtOH (black bars). E13; \( N = 5-7/\text{sex}, 4 \text{ litters/treatment.} \) E15; \( N = 7-9/\text{sex}, 5-7 \text{ litters/treatment, E20; } N = 10-12/\text{sex, 6 litters/treatment.} \)
Figure 6.5. PC-EtOH exposure reduces trophoblast invasion into the mesometrial triangle in females only. Images show placental cryosections at E13 stained for pan-cytokeratin (red) marking invading trophoblasts, alpha smooth muscle actin (aSMA, green), and dapi (blue). White dashed line shows barrier between uterus and decidua below. Large images are at 4x, with smaller images at 10x magnification. Data shows mean ± SEM, analysed by two-way ANOVA. *P<0.05. Control (white bars), PC-EtOH (black bars). N = 6-12/sex/treatment.
Figure 6.6. QPCR on placental tissues from whole placentas at E13. Panels A-D show markers of placental cell types; A: Mest (fetal endothelial cells), B: Ctsq (S-TGCs), C: Gcm1 (SynT-II), D: Syna (SynT-I). Panels E-H show placental nutrient transporters. Data shows mean ± SEM, analysed by two-way ANOVA. *P<0.05. Control (white bars), PC-EtOH (black bars). N = 7-8/sex/treatment.
Figure 6.7. QPCR on placental labyrinth tissues from E15. Panels A-D show markers of placental cell types; A: *Mest* (fetal endothelial cells), B: *Ctsq* (S-TGCs), C: *Gcm1* (SynT-II), D: *Syna* (SynT-I). Panels E-H show placental nutrient transporters. Data shows mean ± SEM, analysed by two-way ANOVA. *P*<0.05. Control (white bars), PC-EtOH (black bars), *N* = 8/sex/treatment.
6.5 Discussion

Analysis of the effects of PC-EtOH on the early embryo, trophoblast differentiation and the uterine environment in Chapter 5 suggested that the female PC-EtOH embryo exhibited greater morphological defects than the male pre-implantation embryo, with reduced ICM number, P-TGC differentiation during trophoblast outgrowth experiments and increased dNK cells at E11. Both sexes however, showed reduced trophoblast pluripotency and outgrowth capacity. This study therefore aimed to investigate long term impacts of these early outcomes on the formation of the placenta by ontogeny analysis from E11, E13, E15 and E20 in a sexually dimorphic manner.

6.5.1 PC-EtOH reduces fetal viability at E15

Firstly, control and PC-EtOH litters were examined at post-mortem. E20 examination had already been carried out and published by Gardebjer et al. (2014), and showed reduced viability after PC-EtOH. Further investigation at earlier time points showed that PC-EtOH reduced the number of viable fetuses by 1-2 per litter at E15, which was not seen at earlier ages of E11 or E13. This suggests that E15 is a critical point during development which is susceptible to programming by PC-EtOH. Preliminary analyses suggest neither sex to be at more risk of resorption than the other (data not shown). E15 placentas also showed reduced gross labyrinthine weight after PC-EtOH. The placental compartments were next examined more rigorously for changes in volume and surface area of the placental compartments and vascular spaces.

6.5.2 PC-EtOH causes structural changes to the mid-gestation E15 placenta

When assessed by stereology, again E15 was the only significant time point affected by PC-EtOH, with no changes in any parameters investigated (volume, surface area) to E13 or E20. At E15, female fetuses were shown to have the most deleterious outcomes, exhibiting reduced whole placental volume, with all placental zones contributing to this effect. Males, conversely did not show any gross changes to volume. Basal sex differences were found between males and females at E15, with females showing reduced growth than males, and were investigated in depth in Chapter 3. The labyrinth was then investigated for FBS and MBS volume and surface area. Females again showed the most deleterious outcomes, with a significant reduction in MBS volume. When males were assessed with females for MBS volume, they too contributed to a reduction after PC-EtOH. Curiously, neither males nor
females showed alterations to blood space surface area, when analysed alone or with both sexes. As no changes to E20 parameters were found, this is suggestive of adaptive placental growth, which may occur both through maternal energy conservation from increased embryo resorption, and altered nutrient transport across the labyrinth.

To date, only one other study other than PC-EtOH has shown an MBS defect only; a PKBα/Akt1 deficiency study by Plaks, et al. (2011). Using contrast enhanced MRI with an albumin-based tracer, specific to maternal vasculature, they showed reduced enhancement of the maternal blood supply to the labyrinth. Similar to PC-EtOH, this was also without any changes to the interhemal membrane. Akt1 is highly expressed in TGCs where it is the predominant isoform, which may be a common pathway to PC-EtOH, as perturbed activation of AKT in trophoblast stem cells causes alterations in differentiation to invasive phenotypes (Kamei et al., 2002, Yang et al., 2003, Kent et al., 2010). Alterations in Akt1 are also associated with perturbed glycogen cell differentiation (Sharma et al., 2016), which is also shown in PC-EtOH (Gardebjer et al., 2014).

Reductions in MBS volume has also been shown by other programming models including hypoxia (Higgins et al., 2016), undernutrition (Coan et al., 2010), and a high-fat high-sugar diet (Sferruzzi-Perri et al., 2013). However, these models do not show reduced MBS alone, and also include additional reductions to the FBS vasculature. Adaptation of the labyrinth morphogenesis in this high-fat high-sugar model shows that reduced MBS at E19 is secondary to reduced fetal capillary volume, surface area and increased interhemal membrane at E16 (Sferruzzi-Perri et al., 2013), suggestive of reduced differentiation of the labyrinthine trophoblasts and branching of the vascular tree. Conversely, Coan et al. (2010) showed reduced placental weight and junctional zone volume at E16 preceded reduced volume and surface area of MBS and FBS at E19. Despite these marked changes in labyrinth vascular structure at E19, glucose uptake was maintained and system A amino acid transport was elevated, showing the labyrinth has the capacity to compensate for these structural defects. As PC-EtOH placentas at E13 and E20 showed volume and surface area to be unchanged, different mechanisms are suspected to occur to these other programming models. Importantly, in the models mentioned above, these perturbations occur directly during placental morphogenesis.
Adaptation of the placenta after PC-EtOH exposure is also likely as suggested by the alterations in the expression of nutrient transporters. At E13, reduced expression of Glut3 was found in both sexes after PC-EtOH. Curiously, this was without changes to other system A amino acid transporters. By E15, however, sexually dimorphic outcomes resulted, exhibiting reduced expression of Glut3 in PC-EtOH males, but increased expression in PC-EtOH females. Future investigations for placental function, through capacity of nutrient exchange will determine whether PC-EtOH placentas can adapt to reduced MBS volume to maintain fetal growth.

Considering that structural changes to the placenta (MBS) were only observed at E15, this suggested that an earlier structural malformation may have occurred. As the MBS only contributes to one side of the vasculature, this pointed us towards a maternal vascular defect. One major event of placentation which occurs during this time at E13 in the rat, is remodelling of the maternal spiral arteries, and were thus investigated in response to PC-EtOH.

6.5.3 Structural changes in the labyrinth are preceded by reduced secondary trophoblast invasion in PC-EtOH females

E13 placentas were analysed for the second wave of trophoblast invasion (specifically the SpA-TGCs) into the uterine mesometrial triangle through localisation with invasive trophoblasts marker pan-cytokeratin (Figure 6.5). Female PC-EtOH exposed embryos were again shown to have the most deleterious outcomes, exhibiting reduced depth of invasion and little infiltration into the uterine spiral arteries compared to control females. Conversely, male PC-EtOH placentas were unremarkable, showing similar measures to control males. As MBS volume was restored by E20, this may suggest that this reduction in invasion is only transient. The duration of this aberrant invasion is not clear, and would be achieved by analysis of E15 placental samples. Further histological investigation into replacement of vascular smooth muscle with elastin fibres would also contribute to our understanding of perturbed spiral artery remodelling in this model.

A delay in spiral artery remodelling in PC-EtOH females may also affect uterine blood flow, and potentially blood pressure, as multiple female conceptuses in one litter would amplify these effects. Indeed, reduced placental blood flow has already been shown in a first trimester model of alcohol exposure in the rhesus macaque (Lo et al., 2017),
which suggests at least a local effect on an individual conceptus. A maternal systemic effect may then explain why male PC-EtOH conceptuses too show reduced MBS volume, albeit not as severely as the PC-EtOH females, and why both PC-EtOH causes fetal growth restriction in both sexes, and postnatally, leads to the same disease susceptibility. These results also may suggest a role of the spiral arteries and maternal blood flow in expanding the MBS of the labyrinth.

6.5.4 Physiological impacts of reduced spiral artery remodelling and reduced MBS

Defects in utero-placental perfusion have long been suspected to originate at implantation (Roberts and Cooper, 2001, Kajantie et al., 2010, Burton et al., 2016), but until now have been unproven in animal models. Altered placental perfusion, as estimated in humans by “surface area” (in this case estimated by placental length, width and depth), has been shown to lead to maternal disorders in pregnancy including hypertension and preeclampsia (Barker et al., 2010, Kajantie et al., 2010). One mechanism in leading to these disorders is perturbation to spiral artery remodelling, which occurs in women experiencing hypertension with or without proteinuria, spontaneous abortion, preterm labor, and even uncomplicated pregnancies which show fetal growth restriction (Khong et al., 1986, Roberts, 1998, reviewed by Pijnenborg et al. 2006 and Brosens et al. 2011). Unsurprisingly, it has recently been shown that placental surface area is inversely related to uterine artery resistance (Salavati et al., 2016), which may be a phenocopy PC-EtOH. However, despite a major focus in pregnancy on preeclampsia, which occurs in 5% of pregnancies, an additional 30% of pregnancies experience hypertension, as shown by a study on Helsinki mothers (Kajantie et al., 2010). The influence of other nutritional perturbations, particularly during the periconceptional period, affecting trophoblast differentiation, invasion, spiral artery remodelling, and placental hemodynamics is now an important area of research. Furthermore, additional effects of placental malperfusion, such as causing oxidative stress, endoplasmic reticulum stress, infarction, damage to syncytiotrophoblast layers, hypoxemia and the roles they play in influencing fetal growth (Burton et al., 2016), also requires further exploration.
6.5.5 Future investigations

To validate that reduced MBS volume after PC-EtOH causes fetal growth restriction, *in vivo* functional experiments are required. Firstly, investigation of blood flow or perfusion to the placenta by doppler ultrasound or functional MRI and any sexually dimorphic outcomes, would determine if both male and female conceptuses are affected by the maternal circulation. Secondly, radiotracer experiments for glucose and system-A amino acid transport would determine whether there is reduced nutrient transport across the placenta to the fetus, or merely just changes in the expression of the nutrient transporters. Thirdly, ex-vivo placenta assays, as described by Goeden and Bonnin (2013), would determine the isolated effects of altered placental structure without any additive maternal effects on placental transport and consumption of oxygen and nutrients, secretion of proteins, and effects on the fetal circulation.

6.6 Conclusions

This thesis chapter has identified critical points during gestation after PC-EtOH exposure, during which the growth and morphogenesis of the placenta if affected. PC-EtOH reduced the secondary wave of trophoblast invasion, and may contribute to reduced or delayed spiral artery remodelling. Further into gestation, a reduction in MBS is likely to have led to reduced nutrient exchange within the labyrinth, and is suspected to be origin of fetal growth restriction in this model.
Chapter 7. General discussion

7.1 Thesis Summary

This thesis has focused on determining the effects of EtOH during the PC period; including the development of the pre-implantation embryo, TS differentiation, the maternal hormonal environment for implantation and structure of the developing placenta. All studies considered the potential for sexually dimorphic outcomes. The first goal was to characterise sex specific growth patterns of the early embryo and the placenta from in vivo derived embryos. This demonstrated that male placentas exhibited greater growth than females at mid-gestation (E15). We next examined a direct effect of EtOH on trophoblast differentiation, and showed reductions in cell count and expression of markers for several different trophoblast subtypes, which may indicate reduced cell maturity. Thirdly, we investigated the impacts of in vivo PC-EtOH exposure on the early embryo, maternal environment, and the programming of placental development. A major finding was that female PC-EtOH exposed embryos showed more deleterious outcomes throughout gestation than males, exhibiting perturbed cell allocation, differentiation, invasion and placental formation, (see Figure 7.1). Male fetuses exposed to PC-EtOH were also affected, albeit to a lesser extent. While we found little evidence that PC-EtOH was able to affect the maternal hormonal environment, we did show altered communication with maternal derived dNK cells following implantation, highlighting that perturbation to the trophoblast can have long term impacts during gestation through perturbed placental development. This data provides much needed evidence of the potential impacts of drinking alcohol around conception, and its long-term impacts on placentation, fetal growth and programming of adult disease. The following chapter will summarise the findings from this thesis, associate the data in a broader context of the literature, and other avenues for future research in this area.
Figure 7.1 Summary of major findings of *in vivo* PC-EtOH exposure on sex-specific programming of pre-implantation and placental development. Abbreviations: dNK; decidual natural killer cells, ICM; inner cell mass, JZ; junctional zone, LAB; labyrinth, MBS; maternal blood space, P-TGC; parietal trophoblast giant cells, SpA-TGC; spiral artery trophoblast giant cells, TE; trophectoderm, vol; volume.
7.2 Summary of results

Before determining the effect of alcohol on early growth of the embryo and placenta, it was first necessary to establish if there were underlying differences between males and females. Chapter 3 demonstrated the importance of utilising in vivo derived dams for investigating outcomes in pre-implantation development. Prior in vitro models had suggested males to grow at a faster rate than females. We subsequently found that this was likely to only be an effect of culture conditions, as no sexual dimorphism was present in the early embryo when assessed for cell count, and allocation to either TE or ICM lineages when derived in vivo. Sexual dimorphism did arise however, in the mid-gestation E15 placenta, with females showing reduced placental weight and volume, particularly to the labyrinth compartment. Females also showed reduced volume and surface area of the fetal and maternal blood spaces which is likely to contribute to reduced fetal growth by late gestational E20.

Next, to assess the ability of alcohol to directly affect the differentiation of trophoblast cells, we turned to in vitro cell culture. Chapter 4 demonstrated that alcohol could exert a direct effect on TS proliferation and differentiation. The physiologically relevant dose of 0.2% EtOH in addition to the supraphysiological 1% EtOH dose, showed marked reductions in cell count on day 6 of culture, without affecting those on day 2 or 4. This coincided with reduced expression of Bcl2, an anti-apoptotic gene, which may promote cells down the apoptotic pathway. Also on day 6, were reductions in expression of several trophoblast subtype specific markers, during terminal differentiation. While we could not conclude of which trophoblast subtypes may be specifically reduced, these results may point to reduced cell maturity.

The major aim of this thesis was to determine the effects of periconceptional alcohol exposure on the early embryo and subsequent placental development. Chapter 5 explored the effects of PC-EtOH exposure on morphological phenotypes in the pre-implantation embryo, trophoblast differentiation, and the influence of the maternal hormonal environment in mediating the uterine responses for pregnancy. At E5, PC-EtOH exposure reduced ICM cell count in females, and reduced TE pluripotency in both sexes. Trophoblast outgrowth experiments showed reduced outgrowth capacity, as well as reduced number of trophoblasts in both sexes on day 6 after PC-EtOH. Female PC-EtOH embryos additionally showed reduced
differentiation to the P-TGC lineages, and expression of Prl4a1, a gene known to communicate with dNK cells. These results are significant, as they provide critical evidence that PC-EtOH has reduced the capacity of the early embryo to differentiate and communicate with the uterus for implantation. This evidence also allowed this study to expand to show altered dNK cell signalling and hypothesise that the second wave of trophoblast invasion in the immature placenta may also be altered. Furthermore, the sexually dimorphic outcomes provide much needed evidence that males and females respond differently to maternal perturbations depending on the critical window of exposure.

Chapter 6 further investigated sexually dimorphic outcomes in placental structure after PC-EtOH exposure in the immature to late gestation placenta. Again, it was only in females that PC-EtOH resulted in markedly reduced invasion of secondary SpA-TGCs into the mesometrial triangle, and reduced infiltration around the maternal spiral arteries at E13. While the effects of PC-EtOH on maternal physiology, such as blood pressure, flow and perfusion to the placenta have not been investigated, both male and female PC-EtOH placentas showed markedly reduced MBS volume, suggestive of a greater maternal effect. Reduction in MBS volume is also likely to have affected capacity for nutrient exchange to the fetus, and is likely to have led to the growth restriction in PC-EtOH exposed fetuses in late gestation.

7.3 PC-EtOH on programming of adult disease

Our model of PC-EtOH has been associated with glucose intolerance and increased insulin insensitivity in adulthood (Gardebjer et al., 2015). Our laboratory is also exploring other post-natal outcomes that occur as a result of PC-EtOH, including alterations to brain and behaviour, cardiovascular and renal outcome, as well as influences to the hypothalamic-pituitary-adrenal axis, as occurs in other periconceptional exposures (Kwong et al., 2000, Watkins et al., 2008a, 2008b, Scott et al., 2010, Fernandez-Gonzalez et al., 2004). We are also interested in the effects on ovary development in female PC-EtOH offspring, potential impacts to their fertility, as well as their influence on programming the second F2 generation.
The data obtained from this PhD project may help to explain the pathways through which alcohol exerts its effects. *My data suggests there are two points of ‘stress’ during gestation in this model.* Firstly, the primary stressor during the pre-implantation exposure of the embryo to alcohol from E0-E5, which is likely to alter epigenetic reprogramming and expression of imprinted genes. Secondly, a sustained stress may also be programmed even when the alcohol has ceased through perturbed placental formation. Reduced MBS formation and nutrient transport capacity in the placenta, is likely to cause placental insufficiency and lead to nutrient stress in the fetus. This latter time point in mid-gestation, is more likely to lead to programming of adult disease through alterations to formation of the fetal organ structure and function. Further investigation of epigenetic modifications to both embryonic and extraembryonic tissues would gain much insight into the mechanisms (and time points) behind programming of PC-EtOH.

### 7.4 Sex specific programming of the early embryo

As discussed in Chapter 3, a major limitation of periconceptional and pre-implantation nutrient deficiency models is the lack of investigation into embryonic sex in pre-implantation outcomes. For this reason, procedures were optimised in this model to genotype for sex after fixation and imaging of immunolocalised blastocysts. A very significant finding of this project was the sexually dimorphic outcomes following PC-EtOH, which carried from the blastocyst stage, through to trophoblast differentiation and invasion of SpA-TGCs of the immature placenta. This may suggest that males have some resistance or adaptive capacity to an adverse maternal environment in the periconceptional window. However, while PC-EtOH and IVF (Tan et al., 2016a, 2016b) models show many female-specific effects, other periconceptional nutritional models rather show male specificity. *In vivo* undernutrition reduced expression of the imprinted gene *H19* in the male blastocyst, and reduced *H19* and *Igf2* expression in the male fetal liver at E20 in rats (Kwong et al., 2006). A model of a periconceptional methyl deficient diet in sheep found males in particular had altered methylation in fetal livers (Sinclair et al., 2007). Until other nutrient models assess blastocyst cell counts in a sexually dimorphic manner, we cannot determine whether these are deleterious or protective outcomes. However, in mid-gestation, if maternal blood flow is altered by
PC-EtOH, rather, this critical window may affect both males and females (Kalisch-Smith, et al. 2017a), which would explain the reduced MBS and fetal growth restriction in both sexes. This also increases the complexity of determining when during which critical window adult phenotypes may originate.

7.5 Mechanisms by which PC-EtOH can affect the early blastocyst

Epigenetic reprogramming

The early pre-implantation embryo is exquisitely sensitive to its environment, as it undergoes epigenetic and metabolic reprogramming as it progresses from the oviduct to the uterus (Fleming et al., 2004). The embryo undergoes genome wide reprogramming, whereby demethylation of the genome DNA continues to the blastocyst stage, where methylation levels are at their lowest stage. This sensitive period may render the embryo more susceptible to stressors, and may allow any adaptive epigenetic marks to be carried into post-natal life, affecting genome stability. Different models have begun to investigate sexually dimorphic epigenetic reprogramming in the pre-implantation period, but remains limited to X-chromosome inactivation in IVF (Tan et al., 2016a), and the influence on active and passive demethylation required to induce pluripotent stem cells in vitro (Milagre et al., 2017). Additional environmental perturbation models performed in vivo are now required to investigate the potential effects on DNA methylation and gene profiles in the blastocyst. While this is yet to be explored, evidence of altered epigenetic profiles in late gestation after PC-EtOH may suggest altered reprogramming during the pre-implantation period. Unpublished data from our laboratory suggests a marked decrease in global methylation (hypomethylation) of E20 female placentas following PC-EtOH (P<0.001). In addition, gene expression profiles for Dnmt1, Dnmt3a, and Dnmt3b were all increased after PC-EtOH in the E20 fetal liver (Gardebjer et al., 2015), which may indicate maintenance of a hypermethylated genome. These results may also suggest that this differential methylation pattern to the placenta and embryo may be traced back to TE and ICM lineages.
Evidence of imprinting

In the early embryo, imprinted regions escape the global DNA demethylation event, which enables gene expression from parent of origin alleles in embryonic and placental tissues (reviewed by Messerschmidt et al. 2014). Imprinted genes can direct growth of the fetus, with maternally expressed genes reducing fetal growth, and paternally expressed genes increasing fetal growth (Angiolini et al., 2006). Knock-out models of implicated genes associate these modifications to fetal growth, with alterations in the nutrient exchange capacity of the placental labyrinth, including changes to labyrinth surface area and interhemal membrane thickness (reviewed by Angiolini et al. 2006). Evidence of altered imprinted genes in E20 placentas after PC-EtOH includes increased expression of paternal Igf2 in the junctional zone of PC-EtOH females at E20, but no changes to paternally expressed Slc38a4, or maternally expressed Igf2r (Gardebjer et al., 2014). Further investigation of other imprinted genes in both maternal and paternal exposures is required to understand the influence on genomic imprinting.

Changes to maternal physiology

Triglycerides

Whilst not a major focus in this thesis, another curious finding at E5 was an increase in maternal plasma triglycerides, which also continued into late-gestation at E20. This is indeed a common finding after alcohol exposure, as shown in plasma of human males (Nestel and Hirsch, 1965, Mishra et al., 1991). Culture of hepatocytes in EtOH also shows increased lipid storage (Mahli et al., 2015). During pregnancy, an elevation in triglycerides, as occurs in maternal obesity, can cause increased lipid transfer through the placenta to the fetus, and result in fetal overgrowth (Di Cianni et al., 2005, Scifres et al., 2011). This increase in maternal triglycerides, may be a compensatory adaptation to minimise the fetal growth deficit after PC-EtOH, and also may hint to a potential pathway that can cause metabolic programming. Perturbation to serum triglycerides may also be indicative of other metabolic changes throughout pregnancy such as altered insulin and leptin, and are currently being investigated by our laboratory.
Glucose homeostasis

Additional unpublished data suggests that there may be mild hyperglycaemia in dams during alcohol administration (from E-4 to E4). In addition to glucose, further insight into maternal insulin levels and other metabolic growth factors (e.g. IGF1/IGF2), to plasma and/or uterine fluid, will aid in determining the mechanisms behind programming of alcohol exposure. In a different model of midgestational glucocorticoid exposure, our laboratory has associated elevated maternal glucose levels with alteration to the nutrient stress associated hexosamine signalling pathway in the placenta, and curiously in a sexually dimorphic manner (Pantaleon et al., 2017). Considering this pathway is also active in the early embryo (Pantaleon et al., 2010), this may be another pathway in which alcohol could exert sexually dimorphic effects to cell cycle or apoptosis, known downstream modifications of this pathway.

7.6 Additional periods of early life programming

Pre-conceptional oocyte

In this thesis, a major focus has been on structure and differentiation of the pre-implantation blastocyst. However, we have not investigated other mechanisms after PC-EtOH such as altered oocyte stress (oxidative, mitochondrial, endoplasmic reticulum), and their effects on oocyte quality, maturation and ovulation, as occurs in obesity (Wu et al., 2015), and diabetes (Wang et al., 2009). EtOH can affect the production of reactive oxygen species causing oxidative stress, endoplasmic reticulum stress, cellular signalling, and cell survival, regardless of the cell type (reviewed by Goodlett et al. 2005), and may also exert these effect on the oocyte.

Paternal exposure

We have also not determined potential effects of paternal alcohol exposure on sperm survival and competence, and their indirect actions on the female environment required for embryo development. For example, a model which excised the male seminal glands reduced female fertility and embryo development, due to oxidative injury in the female reproductive tract (Bromfield et al., 2014). Other models of paternal
obesity have additionally found delayed pre-implantation development, mitochondrial dysfunction, and altered cell allocation to the TE or ICM when assessed *in vitro*, as well as fetal growth restriction and reduced placental weight after embryo transfer (Mitchell et al., 2011, Binder et al., 2012a, 2012b).

### 7.7 Limitations and future directions

While this project has explored much into the effects of PC-EtOH on the developing embryo, placenta and maternal environment, functional and mechanistic experiments are currently lacking. Now that the characterisation of a periconceptional insult is apparent, it is now important to separate pre-conception and pre-implantation effects of EtOH. Assays of the uterine fluid are also important to determine the concentrations of EtOH within, and the nutrient profile in which the embryos are exposed. One possible mechanism that should be explored in more detail, is changes in maternal retinoic acid concentrations, and these may be behind the structural and differentiative changes after PC-EtOH. This could be explored by analysis of serum RA levels, or abundance of retinol binding protein 4 (analogous to retinol levels) over the PC period. If this is indeed the case, addition of RA during PC-EtOH exposure may rescue the phenotypes from this model. Single cell RNAseq of pre-implantation embryos would also provide an unbiased approach to investigate new unexplored pathways of PC-EtOH that may impact the development, differentiation potential and metabolism of the ICM and TE.

Embryo transfer experiments would also help to delineate the effects of PC-EtOH to the embryo separately to the maternal uterine environment. In addition, if male pre-implantation embryos were identified with transgenic fluorescent labelling, male or female embryos could be separately transferred into a single dam. This would determine whether the reduction in PC-EtOH male MBS volume was programmed from pre-implantation, or conversely, whether this was only due to a maternal systemic effect caused by defective PC-EtOH female embryos. We do concede, however, that the embryo transfer technique has an effect on embryonic survival (Tran et al., 2014), and would therefore be harder to investigate subtle phenotypes seen in PC-EtOH. In
addition, the dietary treatment regime (with control and PC-EtOH liquid diets) would also provide an additional level of complexity to completing these experiments.

Further investigation into the placental phenotypes would also confirm whether PC-EtOH causes a systemic hypertensive effect on the maternal physiology to placental blood flow, and consequently placental perfusion. This could be achieved through blood pressure telemetry or tail cuff, doppler ultrasound or functional MRI. Finally, to determine whether there is indeed a reduced capacity for nutrient exchange in the placenta after PC-EtOH exposure, functional experiments during placentation for radiolabelled nutrient tracers (e.g. C14-aminoisobutyric acid, C-14 glucose), would determine whether adequate nutrients are being transferred to the fetus, and would clarify if this is the cause of fetal growth restriction in this model.

7.8 Clinical implications

Despite NHMRC guidelines advocating abstinence, women continue to drink alcohol during pregnancy, especially around conception. This study has provided much needed evidence of the deleterious outcomes of alcohol exposure in this periconceptional period, showing that there is no safe window during pregnancy for consumption. The PC-EtOH model is relevant to humans in that the periconceptional window is conserved among species, with implantation taking place at day 5.5 in rats, and day 7 in humans. The PC-EtOH model was previously developed by Gardebjør, et al. (2014) to simulate moderate levels of alcohol consumption reported in an epidemiological study by Colvin et al. (2007), and more recently, levels are also supported in studies by Muggli et al. (2016) and Green et al. (2016). However, in the human, a major challenge in understanding the effects of periconceptional alcohol consumption on development, still results from women being unable to definitively report the amount of alcohol they have consumed. Surveys of women asking questions about their alcohol consumption usually occur many months into pregnancy or even after a child is born (Colvin et al, 2007), and many questionnaires do not ask women specifically about consumption around conception. This is despite epidemiological data consistently reporting that alcohol consumption is associated with higher miscarriage rates prior to 10 weeks (Avalos et al, 2007) suggesting early exposure has deleterious effects. More recent prospective studies where women are
asked about alcohol consumption in early pregnancy and then at regular intervals throughout pregnancy (Muggli et al, 2016) are likely to yield important information about exposure to alcohol around conception.

Future studies could also be developed to include reporting of alcohol exposure with or without pre-pregnancy supplements including folic acid. Folic acid, along with choline, are methyl donors in the one-carbon metabolism pathway, and are known to contribute to DNA methylation (reviewed by Zeisel and da Costa, 2009) which is known to be altered by alcohol (Refsum et al., 2006). Choline supplementation has been associated with prevention of brain deficits after alcohol exposure (Otero et al., 2012) and is increasingly becoming an intervention to prevent other developmental abnormalities. Epidemiological studies could therefore be used to gauge whether folic acid or choline may be able to reduce the adverse outcomes caused by PC-EtOH.

*Choline supplementation – a potential intervention after PC-EtOH*

In a preliminary study, PC-EtOH was investigated to use dietary choline as an intervention at mid-gestation E10, post-pregnancy recognition. Preliminary data at E5 firstly showed PC-EtOH to reduce maternal choline levels by approximately 50%. A cohort of dams was then investigated at E20 and showed choline supplementation was able to ameliorate the fetal growth deficit in PC-EtOH (unpublished). These results suggest that the outcomes of PC-EtOH are related at least in part to defects in DNA methylation and epigenetic reprogramming. Choline supplementation remains an exciting area of research and provides a possible treatment to the adverse effects of alcohol.

7.9 Final conclusions

This thesis has explored the effects of PC-EtOH on the developing pre-implantation embryo, placenta and maternal influences. We have shown PC-EtOH can affect the cell allocation of the blastocyst in a sexually dimorphic manner, and these changes persist into differentiation of the P-TGCs and further into formation of the placenta and the MBS compartment of the labyrinth. In turn, altered formation of the placental structure may impact of nutrient availability and influence growth of the fetus. This data is critical in providing evidence to support public health messages warning against the consumption of alcohol in the period around conception.
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