THE ROLE OF IMPRINTED GENES IN MOUSE MODELS OF IUGR

by

Kang Yun Connie Lee

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Abstract

Each year in Canada, 5% of ongoing pregnancies are affected by intrauterine growth restriction (IUGR), a condition diagnosed when a baby's birth weight is less than the bottom 5th percentile. Placental dysfunction is thought to be the main contributor to IUGR and many genetic aberrations can lead to problems in the placenta. The epigenetic phenomenon, genomic imprinting, has evolved with placentation and gene knockout studies of several imprinted genes in the mouse result in IUGR. The main goal of this thesis is to examine how gene expression of all imprinted genes is affected in mouse models of IUGR (*Mmp2-/-*, *Mest+/-* and Surgical). The first step is to find suitable IUGR mouse models by comparing the embryonic weights of potential models to normal mouse embryos. Next, I assessed gene expression using genome-wide assays and looked at how expression of imprinted genes is altered in the IUGR mouse model.

Amongst the three models, only the surgical model was identified as having IUGR and RNA samples from this model were used in genome-wide expression assays. We found 68 candidate IUGR genes, 42 genes had a 2-fold difference in IUGR embryos or placentae, with 26 genes altered in both tissue. Genes that function in the transport of substances are the most altered in both tissue. The genes that are involved in the development of anatomical structures were affected more in the IUGR embryos whilst stress response genes were more affected in the IUGR placentae. For imprinted genes, only 4 genes in the embryo (*H19, Igf2, Slc38a4*, and *Dlk1*) and 6 genes in the placenta (*Slc38a4, Sfmbt2, Slc22a3, Phlda2, Cdkn1c*, and *Dlk1*) exhibited significant difference in gene expression between wild-type and IUGR. The majority of these imprinted candidates have been linked to IUGR in either mouse and/or human studies. Overall, imprinted genes as a whole are not more affected in IUGR samples than would be expected by chance based on the chi-square test. These results illustrate that though individual

imprinted genes may be important regulator of IUGR, genes regulated by genomic imprinting as a whole are not more affected in this pregnancy complication.

Preface

All animal experiments were performed under certificate A08-0454 from the UBC Animal Care Committee and complied with the Canadian Council on Animal Care guidelines on the ethical care and use of experimental animals.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AS	Angelman Syndrome
BWS	Beckwith-Wiedemann Syndrome
EB	Empirical Bayes
ER	endoplasmic reticulum
EtOH	ethanol
DMR	differentially-methylated region
DNA	deoxyribonucleic acid
ICR	imprinting control region
IGN	imprinted gene network
ISH	*in situ* hybridization
IUGR	intrauterine growth restriction
KO	knockout
LOI	loss of imprinting
MeAIB	methyl-alpha aminoisobutyric acid
MeV	MultiExperiment Viewer
PCA	Principal Component Analysis
PCR	polymerase chain reaction
PWS	Prader-Willi Syndrome
RNA	ribonucleic acid
SAM	Significant Analysis of Microarray
SD	standard deviation
SGA	small for gestational age
SRS	Silver-Russell Syndrome
UPD	uniparental disomy
VST	Variance-stabilized Transformation
WT	wild-type

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Chapter 1:

Introduction

1.1 Overview

Pregnancy complications occur in approximately 20% of pregnancies every year in Canada (BC Vital Statistics, 2004). These can lead to spontaneous abortions as well as other harmful effects to the mother. Preeclampsia, a maternal hypertensive disorder, and intrauterine growth restriction (IUGR) account for most adverse perinatal outcomes. IUGR is defined as having fetal weight of <5 percentile for the gestational age, though having weight of <10 percentile has been found to be clinically relevant. IUGR babies have varying degrees of perinatal pathology such as decreased organ size, brain hypoxia, and hypoglycemia (Cox and Marton, 2009). Long-term effects including diabetes and abnormal psychological profile have also been associated with IUGR (Nicholls et al., 1998; Sebire and Fisher, 2005; van der Smagt et al., 2006).

The placenta is the tissue that lies at the fetal-maternal interface and is the site of gas and nutrient exchange between the mother and the fetus during development. Therefore, problems in the placenta, such as abnormal vasculature, can directly impact the growth of the developing embryo(s) and possibly lead to IUGR. In fact, it is suggested that the majority of IUGR-only pregnancies have underlying problems in the placenta (Cox and Marton, 2009). A major epigenetic contributor to IUGR may be genomic imprinting. Many mouse knockout studies of imprinted genes have documented IUGR in null mutant mice, indicating that proper expression of imprinted genes is necessary for normal development of the embryo. Several of these genes have high expression in the placenta. Interestingly, there are more genes that are only imprinted in the placenta than in the embryo. The link between imprinting in the placenta and how

imprinted genes contribute to IUGR is the main premise of this thesis. We wish to assess if imprinted genes as a group are affected more than other groups of genes in IUGR.

1.2 Intrauterine growth restriction

There are many different causes for IUGR. Chromosome and genetic abnormalities often lead to syndromes in which IUGR is one of the phenotypes observed. For instance, Silver-Russell Syndrome (SRS) is characterized by IUGR, postnatal growth restriction, and craniofacial features. The known causes of the syndrome are maternal uniparental disomy (UPD) of chromosome 7 as well as aberrations to the epigenetic modification of *H19* and *IGF2* on chromosome 11p15, though these causes only account for <50% of SRS cases (Bliek et al., 2006; Eggerding et al., 1994; Gabory et al., 2009; Kozak et al., 1997). Fetuses with trisomies also exhibit IUGR as a pathology (Cox and Marton, 2009). Development of IUGR has been linked to fetal infections by cytomegalovirus or rubella viruses (Choong et al., 2000; Schuster et al., 1993). However, the majority of causes of IUGR are due to infarctions in the placenta, the tissue that lies at the fetal-maternal interface (Cox and Marton, 2009).

Though the clinical diagnosis for IUGR is based on the birth weight or fetal weight, assessment using biometric parameters of the developing fetus are also utilized for research purposes. Ultrasound typically is used to estimate fetal weight parameters such as abdominal circumference, femur length, and head size (Hadlock et al., 1985). Gestational age of the pregnancy is also estimated by ultrasound during the first trimester. The two estimates together are compared to the normal fetal growth percentiles to determine if growth restriction has occurred (Neilson, 1984; Platz and Newman, 2008). Another method to diagnose fetal growth restriction is to use Doppler ultrasound to measure blood flow of major vessels in and out of the

placenta (Rigano et al., 2001). This diagnostic is suggested to differentiate IUGR due to placental insufficiency from other potential causes in the fetus; this delineation is important since placental-based IUGR is more manageable (Miller et al., 2008).

1.3 Placental development

In order to understand how placental insufficiency can lead to IUGR, we must first understand the connection between the uterus, the placenta, and the fetus. Insights into human placental development are largely based on mouse mutants. Comprehensive comparison between the human and mouse placenta has been reviewed (Georgiades et al., 2002). After fertilization and the first several rounds of cleavage, the zygote develops into a blastocyst where the trophectoderm cells overlie the inner cell mass, which will become the embryo proper. This occurs at around three to five days into mammalian gestation. Trophoblast cells develop from trophectoderm (TE) to mediate the invasion of the uterus and initiate the necessary uterine responses for implantation (Cross et al., 1994). The trophectoderm also gives rise to structures that will fuse with the embryonic mesoderm-derived allantois. This area of fusion eventually becomes the site where fetal and maternal vasculature interact to mediate exchange of gases, nutrients and waste (Rossant and Cross, 2001).

There are differences between the structure of mouse and human placenta, but key developmental events are similar. Initial invasion of the uterus by trophoblast cells must occur, resulting in implantation. Secondary invasion of maternal and fetal blood vessels into the placenta results in the formation of the fetal-maternal interface, where the exchanges of gases, nutrients and waste between the mother and the embryo occur in the chorioallantoic placentae (Rossant and Cross, 2001).

The layer where fetal-maternal exchange occurs is known as the labyrinth in the mouse. Fetal as well as maternal circulation can be found in this layer. The fetal capillaries in the placenta are lined by trophoblast cells and are connected to the embryo by the umbilical vessels. The exact organization of the fetal vessels differs between human and the mouse. Fetal capillaries in the mouse placenta have a maze-like pattern whereas in the human the fetal capillaries form villous trees. There are also slight differences in the arrangement of trophoblast cells but the result is the same; the maternal and fetal circulation come into close contact with only intervening trophoblast cells for the exchange of gases, nutrients, and waste (Georgiades et al., 2002). Effective circulation is not established in the human placenta until approximately 12 weeks gestation, perhaps to prevent the fetus from being exposed to high levels oxygen and blood pressure from maternal blood flow before the formation of a functional placenta (Jauniaux et al., 1995). Similarly, maternal blood is not observed in the labyrinth until 10.5 days (E10.5) into mouse gestation (Georgiades et al., 2002; Muntener and Hsu, 1977).

In humans, the villous trees of the monochorial placenta are covered by a single multi-nucleated trophoblast layer known as the syncytiotrophoblast (Gaunt and Ockleford, 1986; Huppertz et al., 1998). The mouse has three trophoblast layers (trichorial placenta) in between the maternal blood sinus and the fetal endothelial cells that encompass fetal circulation (Fig. 1.1). Though the anatomy of the mouse "syncytium" is different, these layers behave like the syncytiotrophoblast in the human placenta. These cells have structures to increase the surface area to allow for more absorption to occur (Enders, 1965).

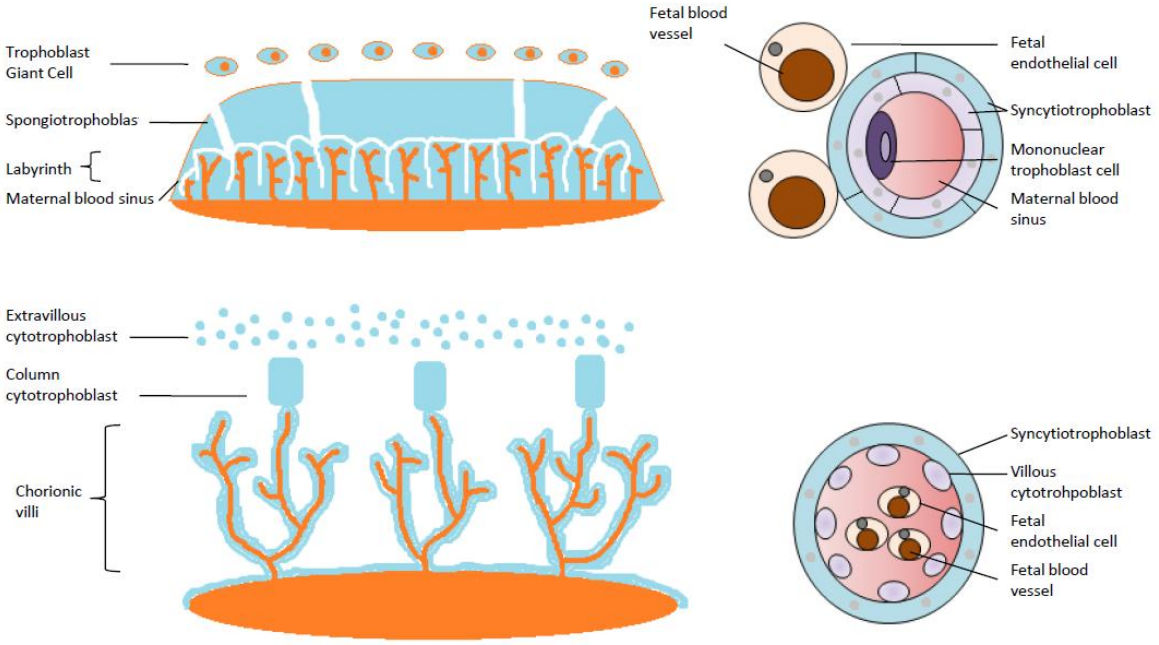


Fig. 1.1 Comparison of mouse and human placenta. Three-layer mouse placenta with depiction of fetal-maternal interaction. Villous tree-shaped human placenta with cross section of the chorionic villi.

1.4 Oxygen and nutrient exchange in IUGR

The availability of oxygen governs the proliferation or differentiation of trophoblast cells. Prior to 10 weeks in human gestation, there is little secondary invasion, resulting in minimal amounts of maternal blood flow reaching the placenta (Genbacev et al., 1997). Cytotrophoblasts initially invade and plug the uterine spiral arterioles at 8 weeks gestation, resulting in low oxygen tension in the interstitial space (2.5% oxygen) (Jauniaux et al., 2000; Rodesch et al., 1992). Therefore, the placenta is placed in a relatively hypoxic environment at this point (Jauniaux et al., 2003). In mice no uterine arteriole plug is observed, but the murine placenta during early embryogenesis is also left in a hypoxic environment (Pringle et al., 2007). This hypoxia promotes the proliferation of trophoblast cells and consequently, the placenta increases rapidly in size in comparison to the embryo (Genbacev et al., 1997). It also promotes

angiogenesis by increasing the expression of vascular-endothelial growth factor (VEGF) , a well-studied molecule that promotes the proliferation of endothelial cells (Phillips et al., 1995). The regulation of angiogenic factors depend on hypoxia-inducible factors (HIF). Mouse knockouts (KO) of *Hif1a* die around midgestation (normal gestation in the mouse is approximately 20 days) due to a vascularisation defect in the labyrinth and spongiotrophoblast or failure in chorioallantoic fusion (Kozak et al., 1997). With the onset of secondary invasion and subsequent angiogenesis, the supply of oxygen also increases after 10 weeks of gestation, resulting in trophoblast cells that differentiates into a more invasive form (Genbacev et al., 1997). Low oxygen culture condition (1% oxygen) has been found to reduce the extent of outgrowths of ectoplacental cultures and decrease expression of *Hif1a* (Pringle et al., 2007). *Hif1a* is regulated by pro-inflammatory cofactors in addition to hypoxia. Cytokines such as TNF-α and IL-1 were discovered to be elevated in placental villous explants exposed to low oxygen environment (Benyo et al., 1997). Restricting blood flow to IUGR placentae results in a larger increase in TNF-α as compared to controls (Holcberg et al., 2001). This reduction of TNF-α in IUGR may be placental-specific since it is not observed in fetal lymphocytes derived from IUGR pregnancies (Iruloh et al., 2009). Moreover, it has been demonstrated that trophoblast cultures exposed to hypoxic condition demonstrate a marked decrease in system A amino acid transport (Nelson et al., 2003). System A amino acid transport is amongst one of the most important nutrient exchange system in the developing placenta. These experiments looking at hypoxia in the placenta have outlined how this condition can lead to pregnancy complications such as preeclampsia and/or IUGR (Genbacev et al., 1996; Genbacev et al., 1997; Gerretsen et al., 1981).

Nutrient transfer in the placenta involves the transfer of glucose, amino acids and fatty acids from maternal circulation to fetal circulation. Different transport proteins and receptors are

present on the maternal-facing plasma membrane versus those on the fetal-facing basal membrane (Jones et al., 2007). Amino acid transport appear to be the most affected in IUGR pregnancies as concentrations of many amino acids are decreased in fetal circulation with corresponding elevation of maternal concentrations of these amino acids (Cetin et al., 1996). The sharpest decrease in the fetomaternal ratio was observed for leucine, taurine, cationic amino acids, and system A amino acids (serine and glycine). Factors that regulate amino acid transport are also affected in IUGR. Additionally, there is a decrease in the levels of fetal insulin and lower expression of placental insulin receptor (Chellakooty et al., 2004; Economides et al., 1989; Potau et al., 1981). Maternal serum levels of insulin-growth factors (IGF1 and IGF2) are also lower in IUGR pregnancies (Holmes et al., 1997). Both insulin and IGF1 have been demonstrated to affect system A transport (Fang et al., 2006; Karl, 1995; Karl et al., 1992; Masuyama et al., 1996; Sferruzzi-Perri et al., 2006; Sferruzzi-Perri et al., 2007). System A transport is the major sodium-dependent amino acid transport system. In the human placenta it regulates transport of alanine, serine, and glycine on the microvillous membrane and directs the uptake of methyl-alpha aminoisobutyric acid (MeAIB) on the basal membrane (Hoeltzli and Smith, 1989; Johnson and Smith, 1988). The mTOR signalling system, which is made up of serine/threonine kinases important for sensing placental oxygenation and directing transport of leucine, has also been found to be down-regulated in IUGR placentae (Roos et al., 2007). This is indicative that proper nutrient transfer is important in determining fetal growth.

1.5 Genomic imprinting

The first indication of the importance of equal maternal and paternal contribution for the proper development of the embryo and extraembryonic tissue comes from experiments in the

1980's that succeeded in generating bi-maternal (gynogenetic) and bi-paternal (androgenetic) mouse embryos. Both types of uniparental embryos exhibit gross abnormalities and die around mid-gestation. Parthenogenetic (and gynogenetic) conceptuses exhibit growth restriction with very limited and abnormal development of the extraembryonic tissue whereas the androgenetic conceptuses have overgrown extraembryonic tissues with poor development of the embryo proper (McGrath and Solter, 1984; Surani et al., 1984). It has also been observed in human diseases that deletion of the same chromosomal region can result in different syndromes depending on the parental inheritance of the deletion (Kagami et al., 2008; Ledbetter and Engel, 1995). Based on these results, it was postulated that the mammalian genome might contain developmentally important genes expressed only from one of the two parental alleles. To explain such a mode of expression, these genes were postulated to be differentially epigenetically-marked in the two parental germlines, a model which led to the hypothesis of genomic imprinting.

Genomic imprinting is a type of gene regulation that results in monoallelic gene expression. During gametogenesis, all epigenetic modifications are erased and re-set according to the sex of the parent. Consequently, differential DNA methylated regions (DMR) and/or histone modifications exist between oocytes and sperm. These epigenetic marks, or imprints, are maintained after fertilization and govern the monoallelic expression of these genes. Imprinted genes are often found in clusters in large chromosomal domains and are regulated by non-coding RNAs, histone modifications, or both (Koerner et al., 2009; Nagano et al., 2008; Wagschal et al., 2008).

Imprinted genes have been associated with embryonic development ever since their identification in the early 1980s. They are especially important to the proper growth of the

embryo as will be outlined in the following paragraphs. *Igf2* is the earliest gene discovered to be imprinted and several other factors affected by IGF-II are also imprinted, including *Slc38a4*, which codes for one of the System A transporters, SNAT4 (Mackenzie and Erickson, 2004).

1.6 Genomic imprinting and the placenta

The presence of a placenta distinguishes mammals from the rest of the animal kingdom with exceptions only in some reptilian species and in egg-laying mammals (monotremes). It has been implied that the emergence of genomic imprinting is closely associated with the evolutionary development of the placenta. Indeed, genomic imprinting has been suggested to have evolved as a result of a competition for resources between the mother (maternal genome only) and the fetus (both maternal and paternal genomes) (Mochizuki et al., 1996; Moore and Haig, 1991). As an extreme example, the complete paternal human conceptus manifests as a large mass of placental tissue with overgrowth of the trophoblast known as the hydatidiform mole. Since the placenta is the site of nutrient extraction for the embryo, this suggests that the paternal genome has evolved to maximize the chance of it being passed onto future generations by maximizing the chance of survival of the embryo. Conversely, bi-maternal conceptuses in mouse have a very small placenta, indicative of the role the maternal genome plays in restricting nutrient extraction of the embryo to ensure the mother's survival (McGrath and Solter, 1984; Surani et al., 1984).

Several mouse knockouts have demonstrated the necessity of some imprinted genes in the placenta. The genes *Peg10, Rtl1, Igf2, Phlda2,* and *Ascl2* all directly affect the structure of the placenta. *Peg10* and *Ascl2* are essential for the development of the spongiotrophoblast of the mouse placenta whereas *Rtl1* is needed for development of the fetal capillaries in the labyrinth;

absence of these factors leads to failure to thrive by midgestation (Gabory et al., 2009; Guillemot et al., 1995; Guillemot et al., 1994; Ono et al., 2006; Sekita et al., 2008). *Phlda2* is suggested to regulate glycogen storage of glycogen-containing cells of the spongiotrophoblast, which is necessary for the continual invasion of the maternal tissue during mouse gestation (Tunster et al., 2010). *Igf2* P0 KO (*Igf2* $P0^{+/-}$) is a placental-specific knockout of *Igf2* and *Igf2* $P0^{+/-}$ conceptuses have growth-restricted placenta beginning E14, accompanied by later reduction in fetal growth (Constancia et al., 2002).

Recent studies have demonstrated the importance of *Igf2* in regulating nutrient exchange in the placenta (Coan et al., 2010; Constancia et al., 2005; Constancia et al., 2002). It was shown that nutrient diffusion capacity is reduced in the *Igf2* $P0^{+/-}$ placenta, which eventually affects nutrient uptake by the embryo despite an initial compensatory effect (Constancia et al., 2002; Sibley et al., 2004). Constancia and colleagues (2005) have conducted physiological assays looking at nutrient transfer in the *Igf2* $P0^{+/-}$ mutants. They injected the mother with radioisotope-labelled glucose and MeAIB, an amino acid analogue of the System A transporters. These injections allowed them to deduce the amount and direction of nutrient transfer into the fetus. By comparing the nutrient transfer levels between the mother and *Igf2* $P0^{+/-}$ embryos, they have concluded that there is a transient increase in nutrient exchange around E16, which is partially caused by an increase in expression of glucose transporter GLUT2 genes (*Slc38a4* and *Slc3a2*).

1.7 Imprinted genes and fetal growth

Mice with uniparental disomy (UPD) or knockouts of imprinted genes tend to exhibit overgrowth, undergrowth, and/or behavioural abnormalities. The null mice of paternally-expressed *Mest* and *Peg3* exhibit embryonic growth restriction and stunting of the placenta

(Lefebvre et al., 1998; Li et al., 1999). Interestingly, null females display abnormal maternal behaviour (Lefebvre et al., 1998; Li et al., 1999). One of the most important growth factors for embryonic development is located directly downstream to *H19*. Insulin-like growth factor 2 (*Igf2*) is amongst one of the first genes identified to be imprinted (DeChiara et al., 1991). This gene is expressed from the paternal allele and deletion of *Igf2* leads to fetal growth restriction as well as stunting of the placenta. Conversely, the over-expression of *Igf2* results in fetal overgrowth and placentomegaly (DeChiara et al., 1991; Ferguson-Smith et al., 1991). The maternally expressed imprinted gene *Igf2r* codes for a receptor that sequesters IGF2. Mouse knockout of *Igf2r* shows overgrowth (Lau et al., 1994). *Grb10* codes for an adaptor protein that binds to insulin receptor, whilst *Dlk1* codes for a ligand that functions in the notch signalling pathway (Baladron et al., 2005; Giovannone et al., 2003; Moore and Haig, 1991). *Grb10* and *Dlk1* have reciprocal expression, with the former predominantly expressed from the maternal allele and the latter from the paternal allele. These two genes also have intriguing reciprocal phenotypes when knocked out in mice. Newborn *Grb10*-null mice are 30% heavier than their wild-type littermates but the weight differences between null and wild type diminish postnatally (Smith et al., 2007). On the other hand, mice inheriting the null paternal allele of *Dlk1* are dwarf at birth and exhibit postnatal catch-up growth (Moon et al., 2002). The embryonic and placental phenotypes of these mouse knockout studies suggest that genomic imprinting plays a key role in the proper development of the mouse embryo.

A few human disorders have also been associated to abnormalities of specific imprinting regions. For example, patients with Prader-Willi (PWS) and Angelman Syndrome (AS) have maternal or paternal microdeletions of 15q11-q13, respectively (OMIM 17620, OMIM 105830). Though the patients of these two syndromes are missing the same chromosomal region, they exhibit different abnormalities due to the effect of parent-of-origin specific imprinting. Patients

of these two syndromes suffer from mental retardation as well as other congenital physical and behavioural abnormalities. Moreover, changes in both DNA methylation and histone modifications have been observed in UPD(14) syndromes, Beckwith-Wiedermann syndrome (BWS), PWS and AS. Hypomethylation of the paternal imprints at the DLK-GTL2 DMR leads to the development of UPD(14)mat-like phenotypes since this region is usually methylated on the paternal chromosome. Loss of methylation at this DMR leads to an epigenetic switch and this region of paternal chromosome 14 now behaves more like the maternal chromosome. Conversely, hypermethylation of maternal chromosomes will result in UPD(14)pat-like syndrome.

The most relevant syndromes linking genomic imprinting to growth are BWS and Silver Russell Syndrome (SRS). Patients with BWS exhibit pre- and postnatal overgrowth whereas SRS patients exhibit IUGR and postnatal growth restriction (Abu-Amero et al., 2008; Temple, 2007). BWS is caused by genetic and epigenetic aberrations at H19 DMR or KvDMR1, the two separate imprinting control regions on human chromosome 11, and maternal loss of CDKN1C (Lim and Ferguson-Smith, 2010). Some BWS patients exhibit loss of imprinting at the *IGF2* locus due to improper methylation at the H19 DMR (Bliek et al., 2001). Half of BWS patients exhibit aberrant methylation at KvDMR1, the promoter of the non-coding RNA *KCNQ1OT1* (Bliek et al., 2001). Imprinting of CDKN1C is maintained through the KvDMR1, and hypomethylation at this site causes biallelic *KCNQ1OT1* expression and *CDKN1C* silencing. Maternally inherited mutations in the coding sequence of *CDKN1C* can also contribute to the development of BWS phenotypes in a subset of patients (Hatada et al., 1996; Romanelli et al., 2009). Additional methylation differences have been discovered in BWS patients at imprinting control regions of *PLAGL1*, *IGF2R*, *MEST*, and *GNAS* (Bliek et al., 2009). SRS is also associated with mutations as well as hypomethylation at *H19* (loss of *IGF2*) and

hypomethylation at KvDMR1 (biallelic CDKN1C) on chromosome 11 (Bliek et al., 2006; Eggermann et al., 2006; Gicquel et al., 2005; Guo et al., 2008; Penaherrera et al., 2010).

These human disorders combined with mouse knockout experiments affirm the involvement of genomic imprinting in embryonic growth and development. It has also been demonstrated in mice and humans that some imprinted genes are only imprinted in the placenta, and many of these genes are highly expressed in the placenta during embryogenesis (Coan et al., 2005). Therefore, imprinted genes found in the placenta have been designated as the top candidates in many IUGR studies (Bourque et al., 2010; Diplas et al., 2009; Guo et al., 2008; Jager et al., 2009; McMinn et al., 2006).

1.8 Imprinted genes and IUGR

Though imprinted genes have seemed the most likely candidates for the regulation of fetal growth, the associations between them and IUGR are still under debate. One study has specifically looked at the differential expression of imprinted genes between normal and IUGR placentae in humans by microarray (McMinn et al., 2006). The authors discovered that 7% of all differentially expressed genes were imprinted genes. *PHLDA2* and *CDKN1C* were found to be upregulated, whilst *MEST, MEG3, GATM, GNAS, PLAGL1,* and *IGF2* were found to be downregulated. *PHLDA2* and *PLAGL1*, were also found to be affected in a different IUGR study using quantitative Real-Time PCR (qRT-PCR) to look at imprinted genes' expression (Diplas et al., 2009). In contrast, *IGF2* was found to be downregulated in studies looking at methylation and expression differences in small-for-gestational placentae (SGA) (Guo et al., 2008; Lo et al., 2002). Moreover, Bourque et al. (2010) assessed 44 imprinted genes' expression using the expression microarray and has found *IGF2* to be differentially expressed in IUGR placental

villous samples. In their study, they mentioned that since they obtained their samples several hours after birth, expression analyses of placental tissues may be affected by rapid RNA degradation. In addition to tissue handling differences, varying methods of assaying gene expression, tissue sampling, as well as determination of statistical significance may be the cause of these conflicting results.

In addition to looking at expression differences, some groups have also looked at DNA methylation differences between normal and growth restricted placental samples (Bourque et al., 2010; Guo et al., 2008). Guo et al. (2008) only found one out of 24 SGA placentae to have loss-of-imprinting at the imprinting control region of *H19/IGF2* (ICR1). In the study conducted by Bourque and colleagues (2010) using genome-wide methylation array technology, 7 of the 13 IUGR placentae have significant hypomethylation at ICR1 when compared to normal controls. These results indicate that though imprinted genes appear to be excellent candidates for IUGR, only the well-characterized *IGF2* is associated with the complication. Nevertheless, several imprinted genes have been reported to regulate *IGF2* expression (Abu-Amero et al., 1998; Cattanach et al., 2004; Gabory et al., 2009; Varrault et al., 2006). Slight alteration in their expression may have an impact on *IGF2*, leading to impaired fetal growth. It is even suggested that a group of imprinted genes may function in a network to facilitate embryonic growth (Arima et al., 2005; Varrault et al., 2006).

1.9 Imprinted gene network

This idea of an imprinted gene network (IGN) was explored by Varrault and colleagues (2006) by looking for coexpressed genes with *Plagl1*/*Zac1* in multiple mouse array datasets. They have observed a significant over-representation of imprinted genes coexpressed with

Plagl1, which lead them to further promote the theory of an IGN that is first suggested in Arima et al. (2005). Varrault et al. (2006) have confirmed a change in expression levels of a few of the imprinted genes (*Igf2, H19, Cdkn1c, Dlk1*) from the IGN in *Plagl1*-transfected cell line as well as the livers of the *Plagl1*-null mice. Furthermore, they demonstrate that *Plagl1* is a regulator of the *H19/Igf2* locus due to its ability to bind to a shared enhancer.

This observation is not the first demonstration of imprinted genes working together, nor is it likely to be the last. The imprinted gene *Igf2r* codes for a receptor that binds and sequester insulin growth factor II (Igf2), which is produced from the imprinted *Igf2* (Czech et al., 1989; Filson et al., 1993). The decline of gene expression of the genes in IGN corresponds to the growth deceleration of multiple organs in postnatal somatic tissues (Lui et al., 2008). Gabory et al. (2009) have shown that the H19 RNA levels affects the expression level of five of the genes in the IGN. Our group has uncovered a curious relationship between *Phlda2* and *Ascl2* expression in the developing mouse placenta (R.O.-M., A. B. B. and L.L, submitted). Tunster et al. (2010) have also observed this interplay between these two genes.

1.10 Rationale and experimental approach

The research thus far on the effect of imprinted genes perturbation suggests that if there is an IGN, it most likely affects embryonic growth. In conjunction with the effects of some genes in the placenta, we propose the idea that genes within this IGN are affected in terms of their expression level in mouse models of IUGR. Our research is part of a larger collaboration that is looking at perturbation of epigenetic modifications in complicated pregnancies in humans. Recent studies looking at imprinted gene perturbations have indicated that in humans, *IGF2* is the main gene that shows a difference in expression in SRS as well as idiopathic IUGR (Bourque

et al., 2010; Guo et al., 2008). There is more uncertainty as to whether or not epigenetic modifications are affected at that locus. Using mouse models of IUGR in my research, I hope to provide a more stringent look at the effects of IUGR on imprinted gene expression in the placenta. My IUGR models will be in a more controlled environment than studies done with the human population as well as address the contribution of genetic variations to the effects of IUGR.

1.10.1 Research hypothesis

Idiopathic IUGR is mainly caused by disruption of proper placental function and the main group of genes that are affected by this complication are the imprinted genes. Expression levels of imprinted genes are different in IUGR when compared to normal mouse placental samples.

1.10.2 Research objectives

My research is aimed at assessing whether or not imprinted genes are affected by IUGR and identifying those that are implicated. The goal was to explore multiple models of IUGR to find the common genes that are affected. Three potential mouse models of IUGR were studied: mouse KO of *Mmp2* (non-imprinted gene) and *Mest* (imprinted gene), and surgically-induced IUGR mouse model. The main method of analysis of gene expression is microarray as well as qRT-PCR. We also wish to characterize placental phenotypes that are previously uncharacterized to determine the potential cause of IUGR in some of these mouse models.

Through my research I hope to identify candidate genes implicated in the etiology of IUGR, which will provide some insight into human studies in IUGR. Some of these candidates may become important diagnoses of IUGR in complicated pregnancies. I also wish to assess the

role of imprinted genes in IUGR and whether or not they are the most affected group of genes in the disease.

Chapter 2:

Materials and methods

2.1 Animal work

The *Mmp2* knockout (*Mmp2*^{tm1Itoh}) was created by Itoh et al. (1997) (MGI:2386252). Two *Mmp2+/-* males and two *Mmp2+/-* females were transferred from the colony room of Dr. Chris Overall and the line was expanded in our mouse facility. The *Mest* knockout line (*Mest*^{tm1Lef}) was already available in-house (Lefebvre et al., 1998) (MGI:2181803). These two lines of mice were housed in a windowless room in one of the animal facility (D. H. Copp) at University of British Columbia. Approximately four mice occupied each polycarbonate cage (floor surface area 500cm^2) with stainless steel cage tops. The C57BL/6J mice used for the surgical model were obtained from The Jackson Laboratory and maintained at the Centre for Disease Modelling at UBC. The mice were housed in ventilated cages (floor surface area 610cm^2). The on-site animal technicians provided food and water as needed. The cages were changed once a week.

Mice were randomly mated with discovery of vaginal plug noted as E0.5. *Mmp2*-deficient conceptuses were obtained by heterozygous crosses, whereas loss of *Mest* was obtained in the progeny of wild-type CD-1 outbred females (UBC Animal Care Centre) crossed with *Mest* KO heterozygous males. Embryonic dissections were made on E14.5 for *Mmp2* and *Mest* lines. The pregnant female was removed to a separate cage that was placed in a polycarbonate chamber that could attach to the CO_2 tank and sacrificed as per UBC Animal care SOP 009E4. The animal remained in the chamber for 15 minutes and then removed. The two uterine horns were dissected from the female and conceptuses were removed. Embryos and placentae were weighed using an analytical balance. Newborn pups were weighed using a bench-top balance at postnatal day 1,

around 20 days after discovery of vaginal plug. Individual embryo and placenta were preserved differently depending on the uses.

2.2 Genotyping

Yolk sac was used for genotyping the mice. A small piece of yolk sac was placed in a 1.5mL microtube.1.25µL of 20mg/mL Proteinase K (Roche) and 50µL ProK solution (50mM KCl, 10mM Tris-HCl pH 8.3, 2mM $MgCl_2$, 0.1mg/mL gelatin, and 0.45% Tween). 1.5µL of yolk-sac lysate in 15µL of dH_2O was incubated at 95°C for 5min, then held at 85°C for loading of master mix. Cycling was programmed for 35 cycles of 95°C 30s, 59°C (Tm) 30s, 72°C 30s. Each reaction contained 2.5µL of 10X PCR buffer, 2.5µL of $MgSO_4$, 2.0µL of 2.5mM dNTPs, 0.1µL of *Tsg* DNA Polymerase, and 2µL of 10mM genotyping primers in a volume of 25µL. The PCR buffer, magnesium, and *Tsg* DNA Polymerase were from BioBasics. Primers used for genotyping or sexing the *Mmp2*, *Mest* and C57Bl/6J embryos are listed in Table 2.1.

Table 2.1 Primers for genotyping, qRT-PCR, and *Mmp2* ISH probe construction. All primers are listed in the 5' to 3' orientation and are obtained from Integrated DNA Technologies.

Genotyping	
Mest+/+ Mest-/-	0702a: GAA ACC GAG AAA CAG ATT GGA 0702c: TCC CAG TGG ATC ACC TGA GC 0702a and IRES1: AGA CCG CGA AGA GTT TGT CCT C
Mmp2+/+ Mmp2 +/-	Mmp2 F2: CTG GCG CTT AGG AAA CAC TC Mmp2 R2: AGC TAG GAG TTC CGG CTT CT F2 and PGK4: CC AAA GAA CGG AGC CGG TTG
Male	Zfy1a: GAC TAG ACA T GTC TTA ACA TCT GTCC Zfy1b: CCT ATT GCA TGG ACA GCA GCT TAT G
Mmp2 ISH probe	Mmp2 F3: ATG GCC CCG ATC TAC ACC TA Mmp2 R3: TTC CAA ACT TCA CGC TCT TGA
qRT-PCR	
Cdkn1c	Cdkn1c F1: CGA ACA GGC AGG CAA GCT Cdkn1c R1: GCT GTT CTG CTG GCT GAT TG
H19	H19 F1: CGT ATG AAT GTA TAC AGC GAG TGT G H19 R1: ACA CGG CCA CAC CCA GTT
Igf2	Igf2 F: GCT TGT TGA CAC GCT TCA GTT TG Igf2 R2: CCG GAA GTA CGG CCT GAG AG
Igf2r	Igf2r F: GCA CAG AAT CCA GAC TAG CAT TAC A Igf2r R: CCT CCT TAT CAG CCT TAA ATA TGT CTT TCT T
Peg3	Peg3 F: GCC GAG TCA TAC CAG AAT GTT Peg3 R: ACC TCG ATG AGT GGC CTT G
Phlda2	Phlda2 F2: TCA GCG CTC TGA GTC TGA AA Phlda2 R2: CAG CAA GCA CGG GAA TAT CT
Slc38a4	Slc38a4 F1: TTT GAT ACG GCT CTT CTC ATG GT Slc38a4 R1: CAG CAG TGT GAT CAC CGA AGT AC
Gapdh	G3pdh F: ACC ACA GTC GCC ATC AC G3pdh R: TCC ACC ACC CTG TTG CTG
Ppia*	Ppia F1: CGC GTC TCC TTC GAG CTG TTT G Ppia R1: TGT AAA GTC ACC ACC CTG GCA CAT
Gm5155	Gm5155 3' end F2: CCT TTT GTG CGA GTG ACT GAC A Gm5155 3' end R2: TGA AGA GCC AAC GGA TGG A
D93	D93 F1: GAG CAA GTT TCA GGA CTC AAG GA D93 R1: GAG GAC CCA AAA GCC TGT CA
Loc802	LOC802 F1: GTG ACA AAT GGC ACC AAT GC LOC802 R1: GTG CTC AGA AGG CGC AAT T
633Rik	633RIK F1: TAC GAG GGC CTG TTC GAT AAG 633RIK R1: TCA AAC GCC GAC CAG ATT TCC
Vstm2l	Vstm2l F1: TGG GAC AAC CAC GTC TCC G Vstm2l R1: CTG GTT GGA GGC CCA CGT
Prl2c1	Prl2c1 F1: AGA CAA AAG CCC CAC GAG AT Prl2c1 R1: TCC TGA TTT CAG AAG AGC TTC ATA G

* Primer sequences obtained from Mamo and colleagues (Mamo et al., 2007).

2.3 RNA extraction

For RNA collection, embryos and placentae were preserved on dry ice then transferred to -70°C. Invitrogen's TRIzol Reagent was used to extract RNA from whole placenta or embryo according to the manufacturer's protocol. The tissues were homogenized using motorized pestles in 1mL of TRIzol reagent. 500µL of chloroform was added to separate the organic layer from the aqueous layer. The aqueous layer contained the RNA and was removed into a new tube. 100% isopropanol was used to precipitate the RNA, which was subsequently washed with 75% ethanol (EtOH). Pellets were allowed to air-dry and re-suspended in 100µL of DEPC-treated distilled water (DEPC-dH_2O). Total RNA samples were stored in -70°C freezer until further uses.

2.4 Hematoxylin and Eosin staining

Placentae were fixed in 4% paraformaldehyde (PFA) in scintillation vials and left at 4°C overnight. The tissues were rinsed in 0.85% NaCl solution for 50min, then in 100% Nacl/100% EtOH for 35min. Two 35min of 70% EtOH washes followed. All of the steps were done on ice. The vials were stored in fresh 70% EtOH overnight at 4°C. The tissues were subjected to five 30min EtOH washes (85%, 90%, 95%, 100%, 100%) at room temperature. Then two 30min xylene washes were used to harden the tissues for sectioning. The vials were filled with paraffin using Shandon Histocentre 3 (Thermo Electron Corporation) and were incubated at 65°C for three hours. Fresh paraffin was changed every hour. The tissues were embedded in silicone molds (VWR, 1560-215) according desired orientation and sectioned after the molds had hardened. Paraffin blocks were stored at room temperature until sectioning. Paraffin sections were made using Leica's RM2255 microtome. The microtome was set for automatic sectioning until the blade was close to the centre of the placenta, then 10µm sections were made by hand.

The paraffin sections were placed on Fisherbrand Superfrost slides and dried overnight in the 37°C room.

The sections were re-fixed in xylene for 2 x 10min, then re-hydrated going from 100% EtOH (100%, 95%, 90%, 80%, 70%, 50%, 30%) to water at 2min intervals. The slides were stained in hematoxylin (Sigma-Aldrich) for 15min, then they were placed under running water for 15min. The slides were dehydrated at 2min intervals using the same increase in EtOH concentrations, going from 30% to 100%. The slides were stained in eosin (Sigma-Aldrich) for 30sec and then washed twice with 100% EtOH. Lastly, the slides were placed in xylene for 2 x 10min and then coverslips were mounted onto the slide with Entellan (Harleco). Subsequent analyses of the tissues were made using a light microscope (Leica MS5). Pictures were taken at 0.65X magnification using the QImaging colour camera (Micropublisher) and the QCapture software.

2.5 *In situ* hybridization

All solutions used are listed at the end of this section.

2.5.1 Probe preparation

Mmp2 probes were RT-PCR amplified from E14.5 placental total RNA using *Mmp2* F3 and R3 primers then cloned into the pGEM-T vector (Promega) (Suppl Fig. 3). The method in which cDNA was generated is listed in Section 2.6. The orientation of the insert was determined by digesting with *SacI*, then the correctly oriented plasmids were sent for sequencing to the Sanger DNA sequencing services (McGill University and Genome Quebec). One of the plasmid preparations had no mismatches and it was linearized with *NotI* (for sense probe) and *NcoI* (for antisense probe). 22µg of plasmid were linearized with 50 units of enzymes overnight. 100µL of phenol chloroform (Invitrogen) was added and centrifuged. 9µL of 3M sodium acetate (NaAc)

and 270µLof EtOH were added to the top 90µL of digestion reaction, washed with 70% EtOH, and resuspended in 20µL DEPC water, making the concentration ~1µg/µL. 1µL of the linearized plasmid and 40 units of RNA Polymerase (Roche) were added to 17µL of the labelling master mix and incubated at 37°C for two hours. T7 and SP6 RNA polymerases were used for creation of sense and anti-sense probes, respectively. A 15 minute incubation at 37°C followed the addition of 1µL of RNase-free DNase (Promega). 100µL of 4M LiCl mix and 40µL of DEPC water was added to the labelling reaction and incubated in -20°C overnight. The RNA was pelleted, washed, and resuspended in 100µL of DEPC water. 1µL was run on a RNase-free agarose gel and the plasmid concentration was determined to be approximately 1µg/µL. The probe was stored at -80°C until use.

2.5.2 Tissue preparation and cryosectioning

The placentae were fixed with 4% PFA overnight then transferred to a 30% sucrose solution for another overnight incubation at 4°C. The placentae were embedded in silicone molds using O.C.T. (Tissue-Tek) and frozen on dry ice. The molds were wrapped in Saran Wrap and aluminium foil and stored in -80°C until sectioning. 10µm sections were made using the Leica CM 3050S cryostat and adhered onto Fisherbrand Superfrost slides. Sections were placed at the bottom of the slides. The cryosections were immediately used in the following fixation step.

20% PFA was thawed a day prior to the first day of ISH. Cryosections were thawed in a Wheaton dish at 50°C for 20 minutes. The sections then went through the following sequence of PFA fixes and 1X PBS washes: 4% PFA Fix (20 min), PBS (2 x 5 min), TE/ProK (1 min) to increase porosity of the sections, PBS (5min), 4% PFA Fix (5 min), DEPC water (1 min), TEA/AA (10 min) adds acetyl groups to the functional groups on the tissue and slides to reduce background, and three 5-minute washes in 1X PBS.

2.5.3 Prehybridization and hybridization

Sections for sense and antisense probes were placed in different slide mailers (Fisher Scientific). 4mL of hybridization buffer covered the placental sections at the bottom of the slides in a full slide mailer. The mailers were incubated overnight at 55°C. Separate slides used for different probes into different mailers and 15µL of probes were added to 5mL of hybridization buffer, heated at 80°C for 5 min, and iced for 5 min.. Additional hybridization buffer was added to cover the slides and left in 55°C for hybridization reaction overnight.

The slides were then subjected to a series of SSC and 1X RNA washes: 5X SSC (15 min), 0.2X SSC (60 min), 1X RNA at 37°C (10 min), 400µL of boiled 10mg/mL RNaseA in 1X RNA (30 min), 1X RNA (5 min), 2X SSC (10 min), and 0.2X SSC (10 min). The RNase A was used to digest away the endogenous single-stranded RNA to increase the signal strength. The RNA probes that were bound to *Mmp2* sequences would not be affected since the nucleic complexes are double stranded. RNase A was boiled to inactivate contaminating DNases.

2.5.4 Antibody reaction and mounting

The antibody reaction occurred in the following steps in a slide mailer: NT at 20°C (5 min), 1% blocking in NT at 37°C (60 min), addition of 4µL of 1:2000 dilution of anti DIG-AP antibody to blocking (60 min), NT washes at 20°C (3 x 20 min), NTMT wash (10 min), NTMTL wash (5 min), and addition of 37.75µL of 100mg/mL NBT (Roche) and 35µL of 50mg/mL BCIP (Roche) for the colour reaction. The mailers were placed in a box overnight and stored in a cabinet to prevent light from entering the slide mailers.

The slides were washed 3X 15 min with 1X PBS at 20°C and fixed in a Formaldehyde Mounting fix for 2 hours at room temperature. Slides were stained for a few seconds using Nuclear Fast Red (Sigma-Aldrich), then dehydrated with 70%, 90%, and 3X 100% EtOH for a minute each. The slides were transferred to xylene, then mounted with Entellan (Harleco).

Table 2.2 ISH Solutions

DEPC water	0.5mL of diethylpyrocarbonate in 500mL ddH_2O
10X PBS	2g KCl, 2g KH_2PO_4, 80g NaCl, 1.45g Na_2HPO_4 Up to 1L dH_2O
20% PFA	Dissolve 100g paraformaldehyde in 1.9mL 10N NaOH by stirring the solution on a hot plate in the fumehood and make up to 500mL DEPC water
10N NaOH	12g in 30mL DEPC water
10mg/mL Yeast tRNA	100mg of yeast tRNA (Roche) in 10mL of DEPC water
0.5M EDTA, pH 8.0	186.1g EDTA in 1L of dH_2O 1mL DEPC can be added before autoclaving
1M TRIS pH 7.0, 8.0, and 9.5	121.1g in 1L of dH_2O
4M LiCl	3.39g in 20mL DEPC dH_2O
3M NaAc pH 5.2	24.61g of sodium acetate in 100mL dH_2O 100µL DEPC before autoclaving
Denhardt's	2g Ficoll 400, 2g PVP, 2g BSA Up to 200mL DEPC dH_2O
20X SSC pH 7.0	175.3g NaCl, 88.2g sodium citrate Up to 1L Can treat with DEPC
30% sucrose	15g in 50mL 1X PBS and syringe filtered with 45mm Stericups
Labelling master mix	2µL of 10X transcription buffer (Roche) 1µL of 40 units/µL of Ribonuclease inhibitor (Promega) 2µL of 10X DIG-RNA labelling mix (Roche) 12µL of DEPC dH_2O 17µL per reaction
4% PFA Fix	20mL of 10X PBS, 40mL 20% PFA, 140mL dH_2O
TE/ProK	10mL of 1M TRIS pH 8.0, 2mL of 0.5M EDTA pH 8.0, 800µL of 10mg/mL Proteinase K, 188mL dH_2O
TEA/AA	3.72g Triethanolamine, 448µL 10N NaOH, 625 µL to 200mL with dH_2O
Hybridization buffer	50mL of 100% Formamide 25mL of 20X SSC 10mL of Denhardt's 2.5mL of Yeast tRNA 5mL of 10mg/mL single stranded fish sperm DNA 7.5mL DEPC dH_2O
10X RNA wash	118.4g NaCl, 50mL of 1M Tris pH 7.5, 50mL 0.5M EDTA in 500mL
NT	100mL of 1M Tris pH 7.5 and 30mL of 5M NaCl in 870mL of dH_2O
1% blocking	2g of blocking (Roche) in 200mL of NT
NTMT	5mL of 5M NaCl 25mL of 1M TRIS pH 9.5 12.5mL of 1M MgCl 250µL of Tween 20
NTMTL	0.03g in 200mL of NTMT
FA Mounting fix	20mL of 37% formaldehyde and 20mL of 10X MEM buffer in 160mL dH_2O
10X MEM buffer	20.9g MOPS, 0.76g of EGTA, 1mL of 1M $MgSO_4$, 2 pellets of NaOH Up to 100mL dH_2O then filter sterilized
1M $MgSO_4$	2.64g $MgSO_4$ in 10mL of ddH_2O

2.6 Quantitative real-time polymerase chain reaction

10µL of total RNA was used in a 20µL DNase treatment solution based on Promega's protocol. The reaction contained 2.5µL 10X RQI Buffer, 1µL of DNaseI, and 1µL of RNasin (Promega). The reaction was placed in a 37°C thermomixer for 1hr and the DNase was inactivated at 65°C for 30min. DNase-treated RNA (D+ RNA) was checked against crude RNA on RNase-free agarose gel to ensure that the treatment was successful.

The D+ RNA was used in first-strand complementary DNA synthesis. The first solution, containing 2µL of N15 (10ng/µL), 2µL of 10mM dNTPs, 8µL of D+RNA, and 7µL of DEPC-dH_2O, was incubated at 65°C for 5min. Then the second solution, which contained 6.5µL of DEPC-dH_2O, 8µL 5X FirstStrand Buffer (Invitrogen), 4µL of 0.1mM DTT, and 0.5µL RNasein (Promega), was added to the first solution. The solutions were mixed by pipetting and 19µL was removed to another PCR tube as a negative control for reverse transcriptase (RT-). The final cDNA mix (RT+) was incubated at 42°C for 2min before the addition of 1µL of SuperScript II (Invitrogen) reverse transcriptase to RT+ tubes. RT+ and RT- tubes were incubated at 42°C for another 50min. Both the RT+ and RT- reactions were checked by qRT-PCR and melt-curve analysis using endogenous control gene prior to running differential expression assays.

The standard 25µL PCR reaction volume was used for the qRT-PCR. The reaction contained 2.5µL of 10X PCR buffer, 2.5µL of $MgSO_4$, 2.0µL of 2.5mM dNTPs, 1.0µL of 10X SyberGreen, 0.2µL of *Tsg* DNA Polymerase, 1µL of 10mM gene-specific primers, and 1µL of cDNA. The cycling program used was set at 95°C for 5min and 95°C 30s, 55-60°C (Tm) 30s, 72°C 30s, 82-86°C (reading temperature) 1s for 35 cycles using Bio-Rad's Opticon II. The sequences for gene-specific primers are listed in Table 2.2.

The amplification data was exported as an Microsoft Excel file for Ct and amplicon's efficiency analysis using the LinRegPCR ver11.3 software (Ruijter et al., 2009). The Cts from

the technical triplicates were averaged. The fold change was determined using the following formulas:

Corrected Ct (cCt) = Ct x log(efficiency of amplicon)

Fold change = $2^{(\text{amplicon cCt} - \text{endogenous cCt})}$

The fold changes of the samples from each cohort were averaged and the standard deviation was plotted as the error bar.

2.7 Unilateral ovariectomy

C57BL/6J females (from The Jackson Laboratory) were given subcutaneous injection of 5mg/kg ketoprofen and anesthetized with isoflurane after an hour. After the females reached a surgical plane of anesthesia, the area around the incision site was shaved and cleaned with 70% EtOH. A small longitudinal incision (<1cm) was made in the skin at the dorsal midline of the last rib with fine dissection scissors. The incision was positioned over the ovary and another small incision in the body wall was made with forceps. A loop of absorbable suture was tied between the oviduct and the ovary and the ovary was excised. The incision in the body wall was closed with a single stitch of absorbable suture and the incision in the skin was closed with non-absorbable suture. The females were monitored for a week for signs of stress before mating.

2.8 Illumina microarray

Approximately 40µg of crude RNA was cleaned using the Qiagen RNeasy Mini Kit. The cleaned RNA was re-suspended in 50µL of RNase-free water. Concentration of the RNA was estimated using the Nanodrop Spectophotometer ND-1000. The final concentration that was sent for microarray analysis was ~200ng/µL. The samples were sent to the Functional Genomics Platform at the Innovation Centre at McGill University

(http://www.gqinnovationcenter.com/service). The quality of the RNA was assessed using the BioAnalyzer, then cDNA was synthesized using the TotalPrep RNA amplification kit. The platform used for expression profiling was the Illumina MouseRef8.0 that could evaluate 8 samples on a single BeadChip. A total of 14 samples were profiled on two BeadChips.

The raw signal, bead detection p-value and bead standard deviation results were collected into a text file by McGill and made available for download on Nanuq. All of the statistical analyses on the Illumina data were completed using the microarray analysis software FlexArray, developed by Michal Blazejczyk from McGill. Flexarray utilized the *lumi* package, which transforms the raw data using variance-stabilizing transform (VST) into expression values similar to but superior than a log2 transformation (Du et al., 2008; Lin et al., 2008). Significance of differential expression between WT versus IUGR samples was determined using the Empirical Baysian methods: Wright & Simon and Cyber-T (Murie et al., 2009). The p-values from these two methods were corrected for false discovery rate (Benjamini and Hochberg, 1995).

2.9 Statistical analysis

The Student's *t* test function in Microsoft Excel 2007 was used to find significance of differential expression by qRT-PCR and to find significance of weight differences between WT and IUGR cohorts. Hierarchal clustering of gene expression data from Illumina MouseRef8.0 BeadChip was completed using the MultiExperiment Viewer program (MeV) (Chu et al., 2008). The principal component analysis was a built-in application of FlexArray that generates a PCA plot for viewing the clustering pattern of data generated from the different microarrays (in our case different biological replicates) on the same BeadChip.

Chapter 3:

Exploration and characterization of IUGR mouse models

3.1 Introduction

There are three mouse models of IUGR in which I was interested in looking for gene expression differences in the placenta: *Mmp2* knock out (KO), *Mest* KO, and surgically-induced IUGR.

Mmp2 is a member of a very large family of matrix metalloproteinases. It is mainly expressed from implantation in the mouse to E16.5. The site of *Mmp2* mRNA is in decidual cells on the maternal side. Its expression pattern is reciprocal to a close relative *Mmp9*, which has been identified as being expressed early in trophoblast giant cells (Alexander et al., 1996). Mice null for *Mmp2* exhibit a significant postnatal growth restriction starting from day 10, as well as small size at birth (Itoh et al., 1997). This is an interesting candidate for IUGR since these proteinases are thought to digest matrix of maternal uterine cells, which eventually results in successful implantation of the conceptuses into the uterine wall (Bischof and Campana, 2000). In the case of improper implantation, it is possible that maternal blood supply may be affected, contributing to growth restriction. In addition, *Mmp2* has also been implicated in skeletal development as well as neovasculogenesis (Itoh et al., 1998; Mosig et al., 2007). *Mmp2* expression has often been used as an assay to indicate angiogenesis in cancer metastasis studies (Foda and Zucker, 2001). It is also a downstream effector of VEGF, which relates the gene to a hypoxia response (Garzetti et al., 1999; Sounni et al., 2002). All of these findings suggest that *Mmp2* may impact placental development. The *Mmp2* KO mice were kindly provided to us from a researcher at the Life Sciences Institute, Dr. Chris Overall.

Mest/Peg1 is a paternally-expressed gene with a differentially-methylated region in its promoter (Lefebvre et al., 1997). Expression of *Mest/Peg1* highlights the fetal capillaries in the

labyrinthine (Mayer et al., 2000; Oh-McGinnis et al., 2010). The PEG1 protein is a hydrolase of unknown function. Our group has made the mouse KO of *Mest/Peg1* and documented IUGR in the mutant conceptuses (Lefebvre et al., 1998). The KO embryos (*Mest*+/-) exhibit the growth phenotype from E15.5 to birth with corresponding small placentae. Other than this growth restriction, no other obvious organ or placental defects are observed in the KO embryos. This model is of particular interest to us since *Mest* mutants and the *Plagl1* KO display similar embryonic IUGR, and the authors have discovered changes in imprinted gene expression in in the null mutants (Varrault et al., 2006).

The surgical model is a hemiovariectomy model of IUGR. This model is based on the observation that if blood supply is restricted to the conceptus, then the embryos will develop an IUGR phenotype. Uterine ligation animal models, which is a method to restrict blood supply to the placenta by ligating the uterine vessel, have been demonstrated to exhibit IUGR in mouse, rat, and sheep (Andersen et al., 1988; Coe et al., 2008; Newnham et al., 1986; Vileisis et al., 1982; Vuguin, 2007). In our case, we will not ligate the artery that directly feeds the placenta to induce IUGR, but create a crowded situation where the conceptuses in the middle of the horn will receive less blood supply than those on the outside edges of the horn (Fig. 3.1) (Coe et al., 2008; Vom Saal and Dhar, 1992). The reason behind the reduced blood supply is twofold. First, the mouse uterus is divided into two horns, with a membrane separating the horns; thus, removal of one ovary means that the remaining ovary will ovulate every cycle, resulting in all the conceptuses to be implanted in one horn. Second, the uterine blood supply is bidirectional with the main artery splitting into two large branches supplying each end of the horn. These vessels join in the middle of the horn forming a loop, with smaller branches coming off the loop to provide blood to each conceptus. In a crowded situation, the conceptuses implanted in the middle

of the horn will be subjected to lower blood pressure and receive less blood (Coe et al., 2008; Vom Saal and Dhar, 1992).

Uterine position	Placental blood flow (ml/min./g)
O 1	31.5
O 2	33.1
O 3	20.6
M–2	20.8
M–1	9.3
M 0	18.8
M 1	27.3
M 2	29.7
C 3	31.3
C 2	43.5
C 1	36.6

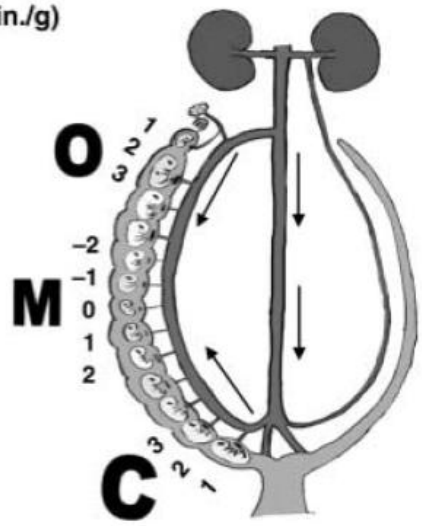


Fig. 3.1 Basis of the "Crowded Uterine Horn" mouse model of IUGR. Taken from Coe et al., 2008 with permission from John Wiley & Sons.

3.2 Results

3.2.1 *Mmp2* expression in the developing placenta

There have been several studies documenting the function of MMP2 in the adult as well as during early embryogenesis (prior to E8.5). In short, *Mmp2* expression in the peri-implantation uterus is restricted to the site of the developing maternal decidua (Alexander et al., 1996; Das et al., 1997). Only one group has looked at *Mmp2* expression in the placenta at later stages. Teesalu et al. (1999) validated the presence of *Mmp2* in the maternal decidua and further demonstrated the emergence of its expression in the labyrinthine at E16.5. Interestingly, their Northern blot data shows that *Mmp2* expression actually begins to decline from E13.5 and is nonexistent by E18.5 (Teesalu et al., 1999).

Here, I wished to further elucidate the role of *Mmp2* in the late stage placenta by examining its expression pattern in more detail. For this, a colorimetric *in situ* hybridization (ISH) was performed on E14.5 wild-type placental sections using an anti-sense *Mmp2* riboprobe (Fig. 3.2). I was able to detect sparse staining in a few fetal vessels in the labyrinthine of the placenta (Fig. 3.2B) and distinct staining in the yolk sac remnants of the sections (Fig. 3.2C,D). However, there was no staining in the maternal decidua near the top of the sections (Fig.3.2A).

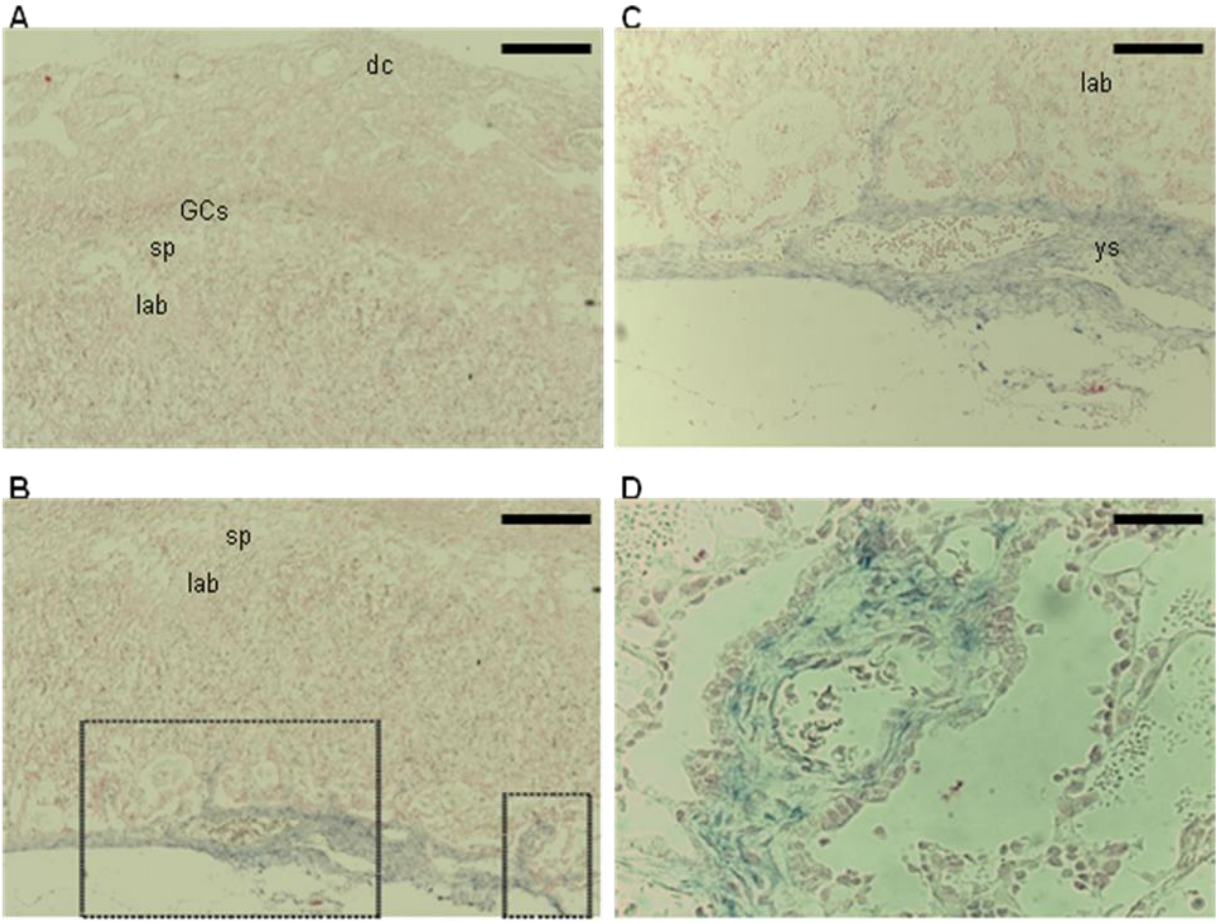


Fig. 3.2 Expression of *Mmp2* mRNA in E14.5 *Mmp2*+/+ placentae by ISH. (A) Upper half of the placenta. (B) Lower half of the placenta. Scale bar: 0.25mm for (A) and (B). (C) Yolk sac. Scale bar: 0.13mm (B). (D) Fetal endothelial cell. Scale bar: 0.062mm. Sense probe not shown. dc: decidua, GCs: giant cells; sp: spongiotrophoblast layer; lab: labyrinthine layer.

3.2.2 *Mmp2*-deficient placentae exhibit normal morphology

Since *Mmp2* has been postulated to be involved in early implantation, specifically in the digestion of the matrix that surrounds uterine cells, it would be expected to observe an abnormal placental morphology in *Mmp2*-deficient conceptuses (Alexander et al., 1996; Bischof and Campana, 2000; Teesalu et al., 1999). To assess the morphology of the placenta, H&E staining was performed on paraffin sections of E14.5 placentae. There was also no observable difference in the size of each placental layer nor gross disorganization of blood vessels in the labyrinthine between the *Mmp2*+/+, *Mmp2*+/-, and *Mmp2*-/- (Fig. 3.3).

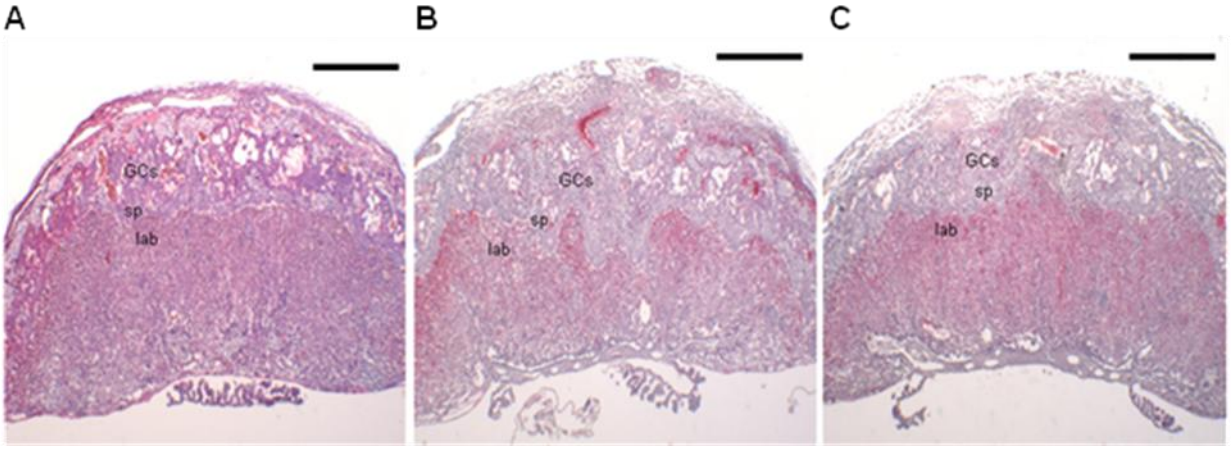


Fig.3.3 Placental morphology of E14.5 *Mmp2* placentae by H&E. (A) *Mmp2*+/+ (B) *Mmp2*+/-. (C) *Mmp2*-/-. n=2 for each genotype. All scale bars = 1mm.

3.2.3 *Mmp2*-/- conceptuses do not exhibit IUGR

Itoh *et al*. (1997) have demonstrated that the *Mmp2*-/- mice exhibit a clear postnatal growth restriction. They also observed that the *Mmp2*-null mice have low birth weights. However, they did not present any data showing this growth difference between the null mice and their WT littermates at birth. I needed to confirm whether the *Mmp2*-/- mice are in fact growth restricted during development in order to validate the *Mmp2* KO mice as a model for

IUGR to test for differential gene expression. Therefore, I set up intercrosses between *Mmp2* heterozygotes and measured the weights of *Mmp2*+/+, *Mmp2*+/-, and *Mmp2*-/- mice at E14.5 and postnatal day 1 (P1). I found that *Mmp2*-/- conceptuses exhibited no significant weight difference when compared to WT at either stage (Fig.3.4A and Fig. 3.4B). There was also no difference between WT and null placental weights at E14.5 (Fig.3.4C).

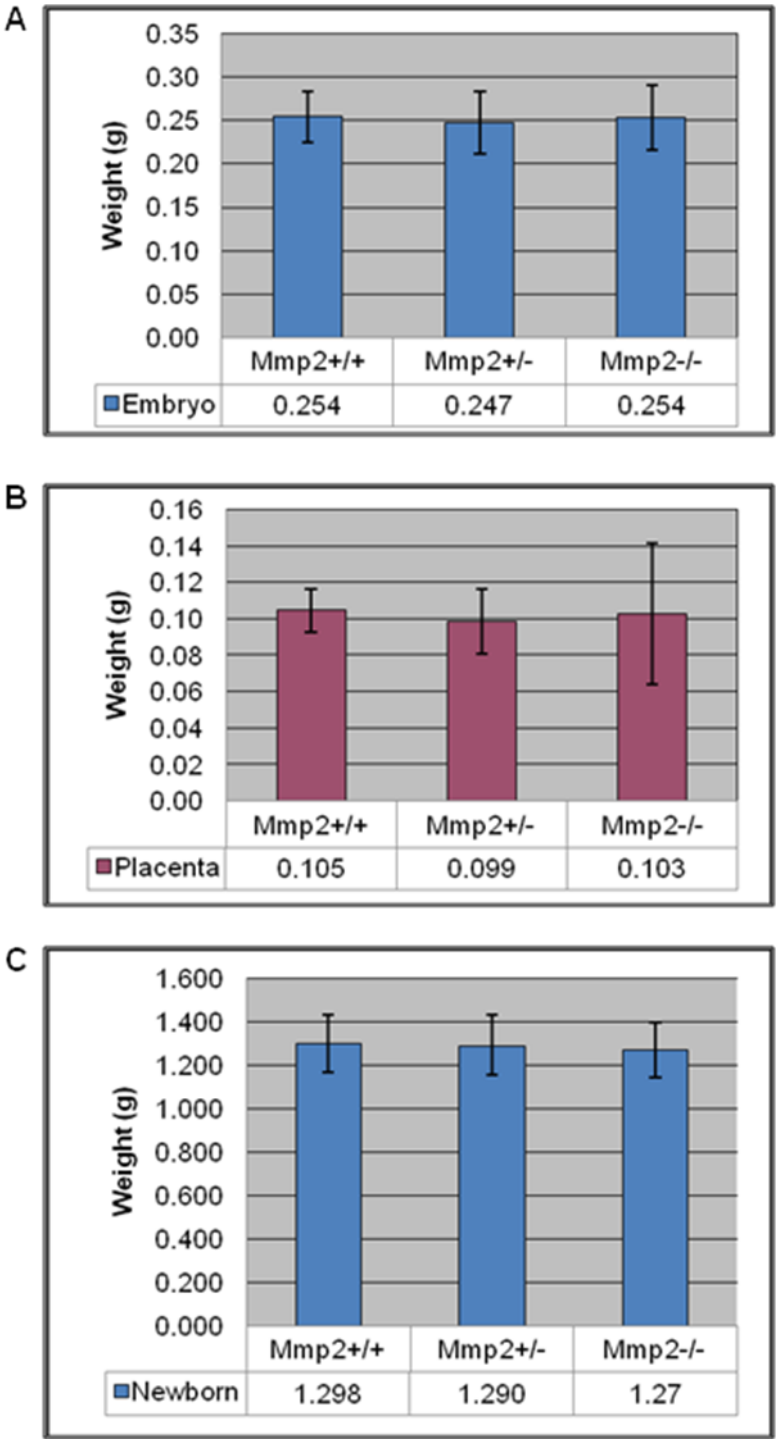


Fig. 3.4 Weight comparisons between *Mmp2+/+, Mmp2+/-, and Mmp2-/-*. (A) Embryonic weights of E14.5 conceptuses (*Mmp2+/+*: n = 25; *Mmp2+/-*: n = 42; *Mmp2-/-*: n = 27) from 11 litters . P = 0.85. (B) Placental weights of E14.5 conceptuses (*Mmp2+/+*: n = 7; *Mmp2+/-*: n = 17; *Mmp2-/-*: n = 10) from 4 litters. P = 0.84. (C) Newborn pup weights at postnatal day 1 (*Mmp2+/+*: n = 8; *Mmp2+/-*: n = 24; *Mmp2-/-*: n = 10) from 5 litters . P = 0.89. All weight comparisons were subjected to Student's *t* test.

3.2.4 Imprinted gene expression in *Mest* mutants

The IUGR phenotype of the *Mest*-deficient mice has been previously described (Lefebvre et al., 1998). Thus I analyzed the gene expression of imprinted genes by quantitative real-time PCR (qRT-PCR) of E14.5 placental samples to see if there is differential expression between WT and *Mest+/-* littermates.

The imprinted genes that were first tested for differential expression between *Mest+/+* and *Mest+/-* placentae are genes that have been implicated in human studies of IUGR or regulators of those genes. *H19, CDKN1C,* and *IGF2* have been directly involved in syndromes such as Beckwith-Wiedemann and Silver-Russell Syndrome (Bliek et al., 2006; Gicquel et al., 2005; Lam et al., 1999; Romanelli et al., 2009; Zhang et al., 1997). *Igf2r* is a direct regulator of *Igf2* (Czech et al., 1989; Filson et al., 1993). *Slc38a4* is one of the genes that code for a system A transporter, which is crucial for fetal-maternal nutrient exchange (Mackenzie and Erickson, 2004). In a placental-knockout of *Igf2*, researchers only identified increased expression of *Slc38a4* out of the three System A transporters (Constancia et al., 2005). *PEG10* is located in the region on human chromosome 7 and UPD of chromosome 7 is implicated in 10% of SRS patients (Kozak et al., 1997; Penaherrera et al., 2010). The mouse knockout of *Peg3* exhibit similar IUGR phenotypes as *Mest+/-*, though *PEG3* specifically has not been implicated in human growth-related syndromes (Li et al., 1999).

I assessed the imprinted gene expression of four maternally-expressed genes (*H19, Cdkn1c, Phlda2, Igf2r*) and four paternally-expressed genes (*Peg10, Igf2, Peg3, Slc38a4*). None of the genes showed significant difference in expression between the WT cohort (n=3) and the *Mest+/-* cohort (n=3) but *H19, Cdkn1c*, *Igf2r,* and *Slc38a4* appear to be decreased, whereas *Phlda2* appears to be increased in the *Mest+/-* placentae (Fig.3.5).

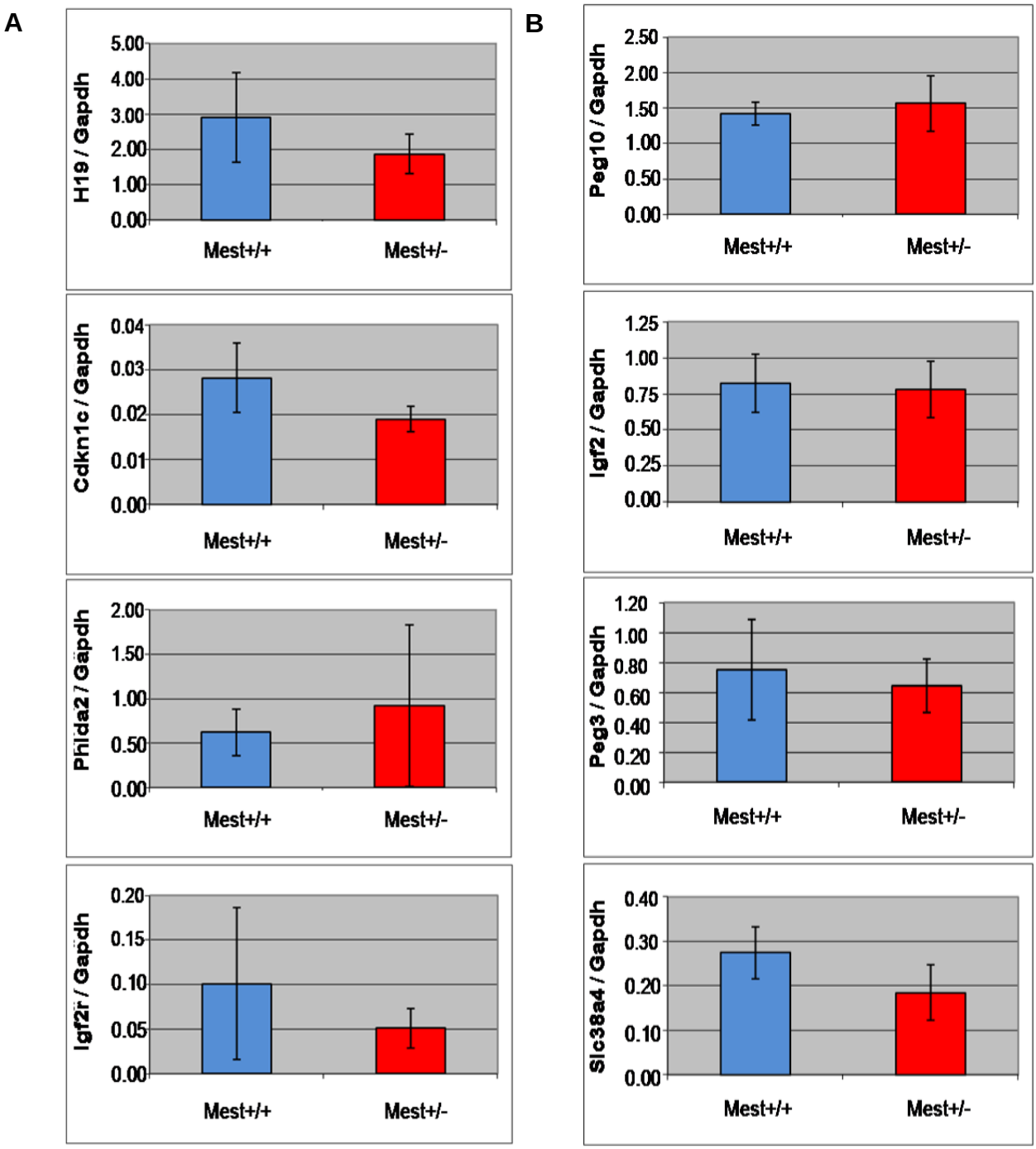


Fig. 3.5 Comparison of candidate imprinted gene expression between E14.5 WT (*Mest*+/+) and IUGR (*Mest*+/-) placentae by qRT-PCR. (A) Gene expression of maternally-expressed genes. (B) Gene expression of paternally-expressed genes. All qRT-PCR was performed on three different placentae for each genotype with three technical replicates per placenta. Expression is represented as a ratio of imprinted gene Ct over *Gapdh* Ct. The value of each cohort (*Mest*+/+ and *Mest*+/-) is the average of the three biological replicates. Error bars represent the standard deviation between the biological replicates in each cohort ($P > 0.5$ for all genes).

3.2.5 Variability in gene expression by qRT-PCR

Despite a trend for difference between the WT and *Mest-/-* cohorts, these differences were not significant due to the variability in gene expression between biological replicates within the same cohort. Most notably, *Phlda2* and *Igf2r* exhibited the greatest variability of expression within a particular cohort (Fig.3.5A). Looking more specifically at the expression level of individual replicate, it is apparent that *Igf2r* expression of one of the WT replicates is very different from the rest and *Phlda2* expression is variable in the *Mest+/-* cohort (Fig. 3.6A). The small sample size (n = 3) makes it difficult to determine what is the actual level of gene expression in *Phlda2* since each replicate has an expression level very different from one another.

Expression differences between biological replicates is common, but we have particularly noticed it in the placenta (Fig.3.5B). Pidoux et al. (2004) have reached the same conclusion in human placental samples. It is possible that differences in gene expression in IUGR may be masked by this variability in gene expression. Therefore, we decided to eliminate some of the causes of variability and re-assess gene expression. We were also interested in seeing if this variability we observed in the placenta is manifested in the embryo. I analyzed WT C57BL/6J samples and compared the variability of expression of two control genes (*Gapdh* and *Ppia*) and imprinted IUGR candidates (*H19, Cdkn1c, Phlda2, Igf2r, Igf2,* and *Slc38a4*) between embryo and placenta and found that, with the exception of *Igf2* and *Cdkn1c*, the rest of the imprinted genes and *Gapdh* have more variable expression in the placenta (Fig.3.5C).

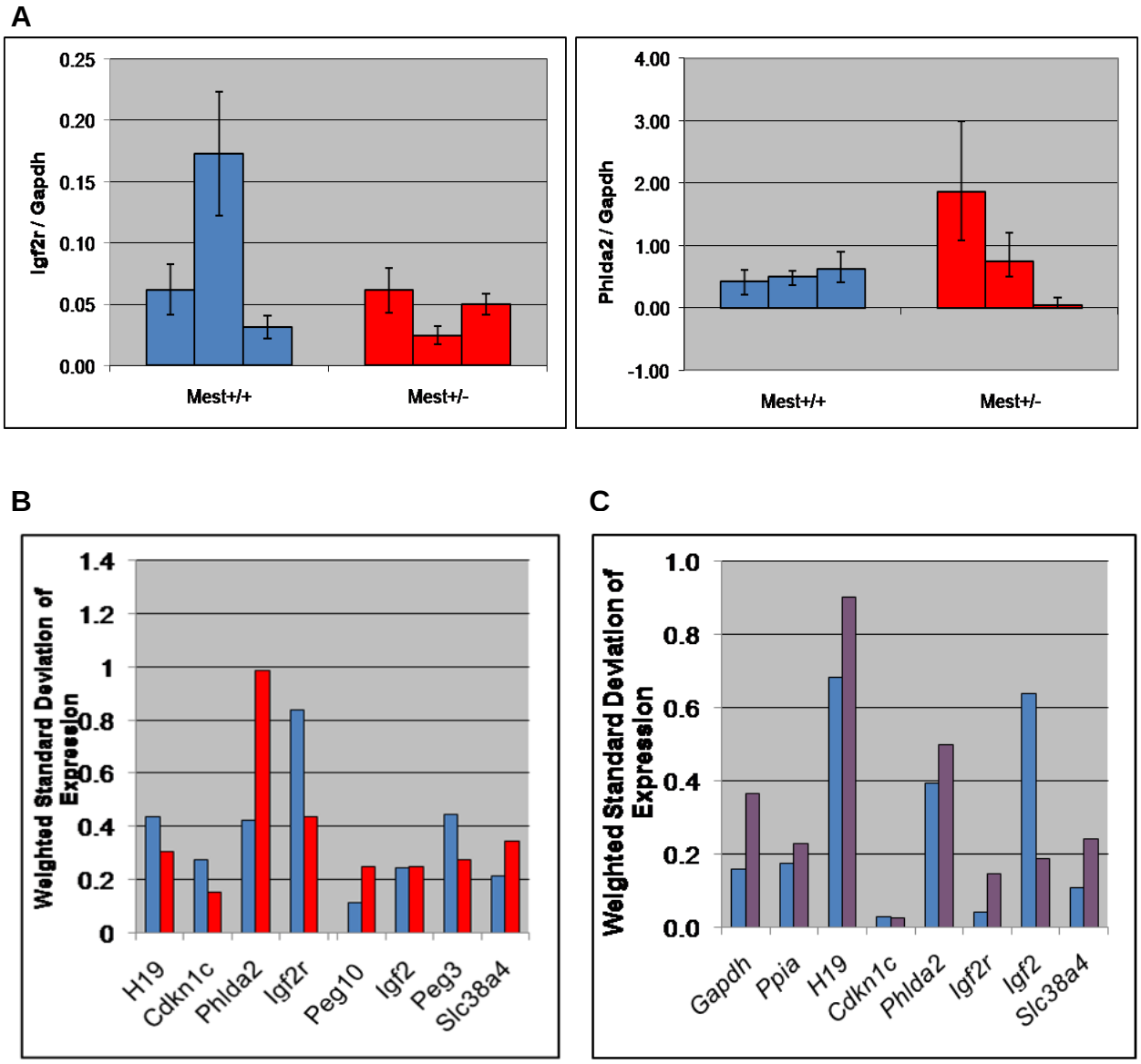


Fig. 3.6 Variability in gene expression. (A) *Igf2r* and *Phlda2* expression in placental replicates (n = 3 for each genotype). Error bar represents the standard deviation of three technical triplicates of each biological replicate. (B) Variation of imprinted gene expression in *Mest*+/+ (blue) and *Mest*+/- (red) placentae. The *Mest* IUGR mouse model is on the outbred CD-1 background. (C) Gene expression variation of control genes and imprinted genes in the embryo (blue) and the placenta (purple) of WT C57BL/6J conceptuses. Variation is presented using weighted standard deviation of gene expression (SD/expression). All of the genes' expression level is relative to a new qRT-PCR control, *Ppia*, which was determined to be a better qRT-PCR control than *Gapdh* (Mamo et al., 2007). *Ppia*'s expression level is relative to *Gapdh*.

3.2.6 Crowded uterine horn

Two C57BL/6J females had one ovary removed in order to induce IUGR in the embryos via the crowded uterine horn method (Coe et al., 2008). Female 1 was plugged by a C57BL/6J male twice. She did not appear pregnant on the day of the first planned dissection (E15.5) and was allowed to go to term. She delivered one abnormal pup on E23.5 with the aid of on-site veterinarian. The pup was large and had an elongated neck. It had no discernible facial features but limbs and digits were present. Female 1 was dissected 15.5 days after the second plug and had a litter of ten embryos, all located in one uterine horn (Fig. 3.7A). Weight data indicated that all except one of the embryos and placentae were smaller than the previous normal C57BL/6J litter (Fig. 3.7B). Average weight of the crowded embryos was 0.397 ± 0.025g, which was significantly different ($p < 0.001$) from the average weight of WT embryos at E15.5 (0.452 ± 0.026g). The bottom 5^{th} percentile of normal Bl6 was <0.409g (n = 15), therefore five embryos were deemed as IUGR. These IUGR embryos did not exhibit gross morphological difference when compared to WT embryos (data not shown).

Female 2 was plugged by a C57BL/6J male twice. She appeared pregnant on the day of her first planned dissection (E15.5) but was not dissected because she had pregnancy bulges on both sides, which indicated that she was carrying pups in both horns. She delivered a litter of 7 pups on E20 or postnatal day 1 (P1). Pups had an average weight of 1.26 ± 0.07g and none had observable difference in size (Suppl. Fig. 1B). IUGR cut-off was pre-determined to be <1.1g, which represented the bottom 10^{th} percentile of P1 pups (n=41) and none of the pups in this litter was found to be under 1.1g. Female 2 carried a litter of 10 conceptuses for her second pregnancy and all of the conceptuses appeared to be implanted in one horn. The average embryonic weight was 0.235 ± 0.015g. This weight average was closer to E14.5 embryonic

weight (*Mmp2*+/+), which was 0.254 ± 0.030g. Due to the uncertainly of embryonic stage, the second litter from Female 2 was not used.

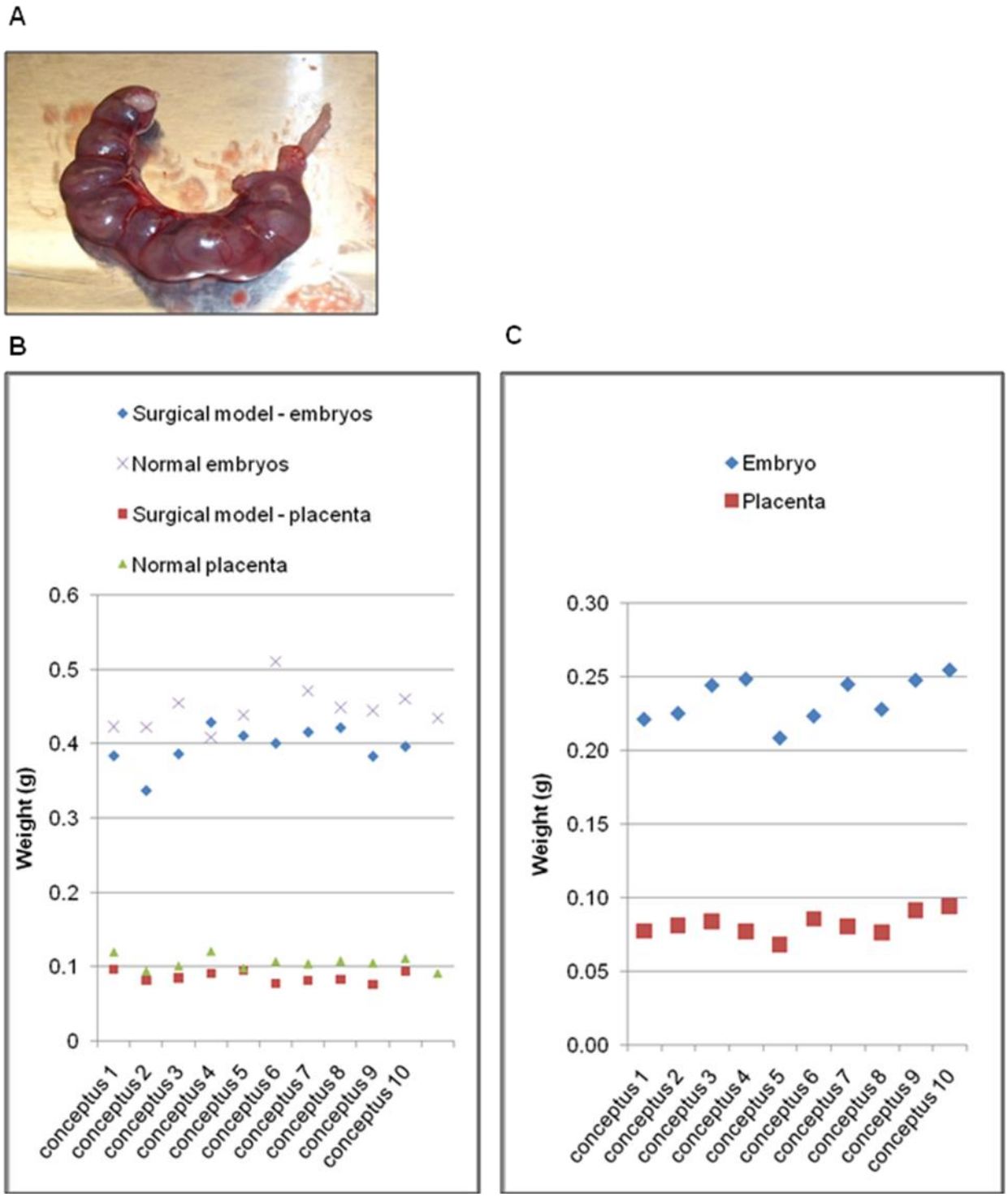


Fig. 3.7 Growth phenotype of surgical model. (A) All implantations were located in the uterine horn that is still attached to the ovary. Uterine horn was dissected from Female 1 at E15.5 (n=10). (B) Comparison of the embryonic and placental weights from crowded uterine horn versus normal E15.5 conceptuses (n = 11). (C) Embryonic and placental weight of E14.5 litter from hemiovariectomized Female 2. Implantations were also found only in one horn.

3.3 Discussion

3.3.1 First IUGR model: *Mmp2 -/-* conceptuses do not exhibit IUGR

The selection of this model was based on an extensive search throughout the Mouse Genome Informatics for mouse models that exhibit prenatal growth defect. *Mmp2* was amongst one of the five mouse knockouts selected that would have live mice with an IUGR phenotype and that were purchasable from repositories. It became the first candidate when we realized we could easily obtain live *Mmp2* knockout mice for breeding from a fellow researcher at UBC. *Mmp2*'s role in implantation and the growth phenotype observed by Itoh et al. (1997) further suggested that this model may be a suitable model of IUGR due to placental dysfunction. However, Itoh et al. (1997) did not conduct any prenatal analysis on the *Mmp2-/-* conceptuses. This omission lead me to confirm the status of IUGR and placental dysfunction in the KO mice. At the end of ten litters, there was no difference in embryonic weights between the three genotypes (Fig. 3.4).

At the same time as I was collecting embryos for weight measurements, I also conducted morphometric analyses by H&E on *Mmp2* placentae to assess if placental dysfunction was the cause of the supposed IUGR, as noted by Itoh et al. (1997). I did not observe any gross morphological differences between *Mmp-/-* and its littermates (Fig. 3.3). There is the possibility that differences may exist if I assess size and amount of branching of fetal vessels in the labyrinthine by doing placental casts, but in conjunction with the lack of IUGR, any findings would not be worthwhile for our purpose.

It is possible that since there are sixteen other matrix metalloproteinases in the mouse, there may be a compensatory effect from these other proteases, or their inhibitors. For example, the target proteases of *Timp3*, which includes multiple MMPs, show no change in enzyme activity in *Timp3-/-* tissue-derived culture (Fogarasi et al., 2008).

The observation of *Mmp2* being expressed in the maternal decidua during early embryogenesis suggests that instead of looking for IUGR in *Mmp2*-/- embryos, it is more likely that breeding *Mmp2*-/- females may lead to IUGR. I had collected one litter from *Mmp2*-/- females and did not observe any difference in weight measurements when compared to litters born from *Mmp2*+/- females. Nevertheless, there is still a possibility of IUGR as one litter is statistically insignificant. An issue with studying IUGR effects in *Mmp2*-/- female pregnancies is that both of the *Mmp2*+/- and *Mmp2*-/- conceptuses may be affected if the null mutant mother does not the produce the metalloproteinase important for implantation, then there will be no phenotypically normal embryos within the same litter to act as WT control. For gene expression in the mouse system, the best method for comparison is to compare IUGR placenta with phenotypically normal littermates since there may be a greater amount of variation in gene expression between litters (Pidoux et al., 2004). This expression variation may mask slight differences in gene expression between IUGR and WT placentae that may be important in IUGR. It is possible to still use this strain if *Mmp2*-/- conceptuses from *Mmp2*-/-female mother are more affected than their *Mmp2*+/- littermates. However, it will be more cost effective to obtain a strain of mice that has well-documented IUGR such as $Akt1^{tm1Mbb}$, which is suggested to be caused by a lack of glycogen cells important for continual invasion of the labyrinthine leading to placental insufficiency. (Cho et al., 2001; Yang et al., 2003). In this case IUGR would not have to be confirmed before doing a differential expression assessment.

3.3.2. Second IUGR model: *Mest/Peg1* IUGR model has high variation in gene expression

Several mouse KO of imprinted genes exhibit IUGR. One of these is the *Mest/Peg1* mouse knockout. There is a 10% reduction in the *Mest*+/- embryo by E18.5 and it is one of the genes in the imprinted gene network proposed by Varrault et al. (2006), therefore making this a

suitable IUGR model to study. All the three layers of the mouse placenta appear to retain proper thickness in the *Mest*+/- placenta, though these placentae are smaller in comparison to their WT littermates (Lefebvre et al., 1998). The 10% reduction in placental size reflected the 10% growth restriction in the *Mest*+/- embryos. The cause of the IUGR observed in the *Mest*+/- embryos is unclear. The gene codes for a hydrolase, but it has an unknown function. The gene is highly expressed in all mesoderm derivatives (Kaneko-Ishino et al., 1995). Placental expression of *Mest* at E14.5 is restricted to the fetal blood vessels. This may indicate a function for *Mest* in the development of fetal vasculature though no obvious disturbance in vasculature is observed in the *Mest*+/- placenta with *in situ* hybridization (personal communication). It may be necessary to construct *Mest*+/- placental casts in order to see if there are differences in branching of fetal blood vessels in the labyrinthine. If there is reduction in branching in these placentae, it may explain the IUGR observed in the embryo as one of the main cause of IUGR in human. Alternatively, the placental vasculature might not show any morphological abnormalities in the *Mest* mutants but rather could exhibit a functional defect in transport properties.

Out of the eight imprinted genes assessed, none of them had significant expression difference between the *Mest*+/+ and *Mest*+/- placentae as determined by Student's *t*-test. This may be explained by the small sample size used as well as the accompanied variation in expression between biological replicates. There was only a sample size of three for each cohort (*Mest*+/+ and *Mest*+/-). Therefore, if there is even one outlier in the cohort, the overall expression would be affected. This was the case for the *Igf2r* assay that showed WT sample #2 was an outlier (Fig.3.6A). Placental sample 2 was a clear outlier in all three cases. If it was removed then *Igf2r* expression between WT and IUGR cohort would be comparable. An increase in sample size would be necessary for accurate assessment of differential expression and subsequent determination of role of some imprinted genes in IUGR. In the *Mest*+/- IUGR

cohort, *Phlda2* exhibits an increase in expression that is similarly observed by three other studies (Apostolidou et al., 2007; Diplas et al., 2009; McMinn et al., 2006). But the variation of gene expression in *Phlda2* between biological replicates was substantial in my study, especially in the IUGR placental samples (Fig.3.6A). Nonetheless, my microarray study has also identified *Phlda2* to be over-expressed in a separate IUGR model, indicating its involvement as a general regulator of growth (Apostolidou et al., 2007).

3.3.3 Placenta has greater variation in gene expression than the embryo

With the previous discovery that gene expression between biological replicates can vary significantly, we became interested in the issue of expression variation. It turned out that our concurrent study of imprinting expression in human IUGR was also plagued by this situation. Bourque et al. (2010) documented that their expression study done on placental tissues had great amount of variation in expression. They were concerned with the difference in the amount of RNA degradation for each placenta than the amount of time for labour in each individual (Avila et al., 2010). Another group had also documented that different sampling sites within the same human placenta was a contributor to variability as well. They also noted that variability between placentae from different pregnancies was greater than the variability from sampling the same placenta (Pidoux et al., 2004).

The great inter-individual variability in human placental expression may be attributed to genetic heterogeneity in the human population. In our case, the *Mest* KO is on an outbred background (CD1). This means that embryos of the same litter will also be genetically heterogeneous. This may have explained partly for the variability I observed in my imprinted gene analyses between *Mest*+/+ and *Mest*+/- cohort though I observed similar variability in expression in WT C57BL/6J samples (Fig.3.). Another cause of variation can be due to pipetting

error during qRT-PCR, but the technical triplicates generally have standard deviation of less than 0.5% of the expression Ct (data not shown). A more interesting idea is that expression variability may be an intrinsic characteristic of the placenta. Development in the embryo is specific at each stage since organs need to properly form in order for it to be able to survive throughout development and adulthood; this may require a tighter control in gene expression. Comparatively, the placenta is a tissue that is discarded after birth and regulation do not need to be as stringent. This can be seen in the observation of normal pregnancy outcome with polyploid placenta whereas polypoloidy has never been observed in fetus that survives (Kalousek, 1994). Considering the function of the placenta is to provide nutrients for the embryo, each embryo may have different needs due to the amount of blood supply it obtain or other factors. Thus programming in the placenta may need to be more relaxed to reflect this difference in nutrient demand. This can result in a difference in gene expression between biological replicates.

3.3.4 Third IUGR model: crowded uterine horn can lead to development of IUGR

The average litter size at E14.5 or E15.5 of C57BL/6J females was eight embryos in my hands. Removal of one ovary in a female would result in the conceptuses to be all implanted in one uterine horn (Coe et al., 2008). The average embryonic weight of crowded litters at E14.5 and E15.5 were reduced by 8% and 12.5% respectively when compared to WT litters. This suggested that crowding does affect embryonic weights, most likely affecting the entire litter. For the litter collected at E15.5, some of the embryos did exhibit IUGR. The other litter that was collected at E14.5 instead E15.5 and only had one IUGR embryo, even though it carried the same number of conceptuses. This suggests the development of IUGR in the surgical model may be just beginning at E14.5, which is a similar time for the development of IUGR for other mouse mutants (Constancia et al., 2005; Constancia et al., 2002; Lefebvre et al., 1998). The growth

phase in mouse development begins at E14.5, as signalled by significant invasion of the labyrinthine vasculature into the maternal decidua. This invasion will result in an increase in surface area for nutrient exchange in the placenta. The embryos increase in size significantly between this point until birth. It is possible that the embryos collected at E14.5 may become more growth restricted by E15.5, as the fetal-maternal blood flow becomes increasingly important.

A key difference between our surgical-induction of IUGR is that the embryos on the side of the uterine horn are smallest, not the ones in the middle as had been suggested by other studies (Coe et al., 2008; Vom Saal and Dhar, 1992). In fact, this is true even for normal litters (Fig. 3.7). Coe et al. (2008) actually have depicted this in their figure of the crowded model (Fig. 3.1). I hypothesize that these embryos are smaller because they are the ones that get pushed further up in the body cavity during mouse gestation. This may physically prevent those embryos from growing normally. This is supported by the consistent observation of the smaller embryos occupying the ends of the horn in normal C57BL/6J litters.

The crowded horn phenomenon would be affected by the number of conceptuses in a single litter. The second litter from Female 2 showed that the conceptuses were implanted in one horn, indicating that the surgery was successful. Nevertheless, the first litter from Female 2 did not contain any IUGR pups. This inconsistency could be explained by the litter size between litter one and litter two. The average litter size for C57BL/6J is 6.2±0.2 (Nagasawa et al., 1973). Litter one had a litter size of seven, whilst litter two had a litter size of ten. Consequently, I considered the second litter from Female 2 to be crowded since there were ten conceptuses all in one horn and this was reflected in their decreased weights (Fig. 3.7). It may be that blood pressure difference within the less-crowded horn (litter 1 from Female 2) was not enough to

result in weight differences. More litters would need to be collected and weighed in order to observe how litter size may contribute to the degree of IUGR in this surgical model.

The next chapter presents the results from whole-genome expression studies done to compare the expression levels between normal C57BL/6J litter and the litter from female 2 that exhibited IUGR (Fig. 3.7A,B).

Chapter 4:

Effects of surgically-induced IUGR on whole-genome expression

4.1 Introduction

We constructed a mouse model of IUGR by performing hemiovariectomy in C57BL/6J females. All the conceptuses were implanted in one horn and the IUGR phenotype results due to a reduction of blood supply to the crowded embryos (Coe et al., 2008; Vom Saal and Dhar, 1992). We wished to observe the effects of crowding on gene expression in the embryo and the placenta. We utilized the Illumina BeadChip Array technology to obtain a profile of gene expression at the desired embryonic stage. Normally IUGR in the mouse is observed from E15.5 onwards, as examplified in the *Mest* KO (Lefebvre et al., 1998). This is the stage we chose to obtain RNA from the embryo and the placenta to conduct microarray gene expression analysis.

Illumina uses a probe-cDNA hybridization technique often use in spotted microarrays, except that probes are attached to microbeads. The probes are 50 bases and are linked to an "address" sequence for identification (Kuhn et al., 2004). The probes hybridize to biotin-labelled cDNA derived from total RNA from samples of interest. Each transcript is represented by an average of 30 beads with attached probes placed randomly on the array, and each gene is usually represented by an average of 2-3 transcripts. The background controls are probes of random sequences that are not complementary to any location in the mouse genome. The raw signals are then normalized to the background signal (Kuhn et al., 2004).

The Illumina BeadChip technology allows for the simultaneous assessment of whole-genome expression for up to eight biological replicates per beadchip. Since we have observed expression variation between biological replicates by qRT-PCR, increasing the number of biological replicates is desirable (www.illumina.com). By utilizing the Illumina platform, we can assess differential expression between WT and IUGR samples using multiple biological

replicates at a lower cost as compared to other microarrays that only allow one sample to be assessed per slide.

We have completed two MouseRef8 Beadchips for this project. The first Beadchip slide contained normal C57BL/6J E15.5 embryo and placental samples, with four biological replicates per tissue. The second Beadchip slide was used to assess expression of three biological replicates of surgically-induced IUGR samples. Results from the Illumina array are interpreted using the FlexArray software, developed by Michael Blazejczyk at McGill Innovation Centre (http://genomequebec.mcgill.ca/FlexArray). The FlexArray software employs the lumi package that normalizes the raw signal obtained from the Illumina Beadchips. The lumi package uses a method called variance stabilization transformation (VST), which is an enhanced version of a Log2 transformation of the raw signal (Lin et al., 2008). FlexArray has built-in statistical analyses to assess the significance of differential expression between WT and IUGR samples; it also generates different plots to visualize the data.

4.2 Results

4.2.1 Gene expression in wild type C57BL/6J embryos and placentae

As expected, gene expression patterns between E15.5 embryo and the placenta are really different (Fig. 4.1). Out of the 18,138 genes assessed, there are 9031 genes that exhibit significant differential expression ($p<0.05$) between the placenta and the embryo at E15.5. Fold change is expressed as the ratio of a gene's expression in the placenta to its expression in the embryo. Since microarray technology is comparative, even genes that are not expressed (not detected by PCR) will have a raw signal. Therefore, traditional methods have used an arbitrary 2- to 10-fold to distinguish gene expression differences. The genes with fold change differences of over 10 are defined as placenta-specific genes, and under 0.1 to be embryo-specific genes

(Tanaka et al., 2000). According to these criteria, our analysis uncovered 60 placental-specific genes and 39 embryo-specific genes (Suppl. Table 1).

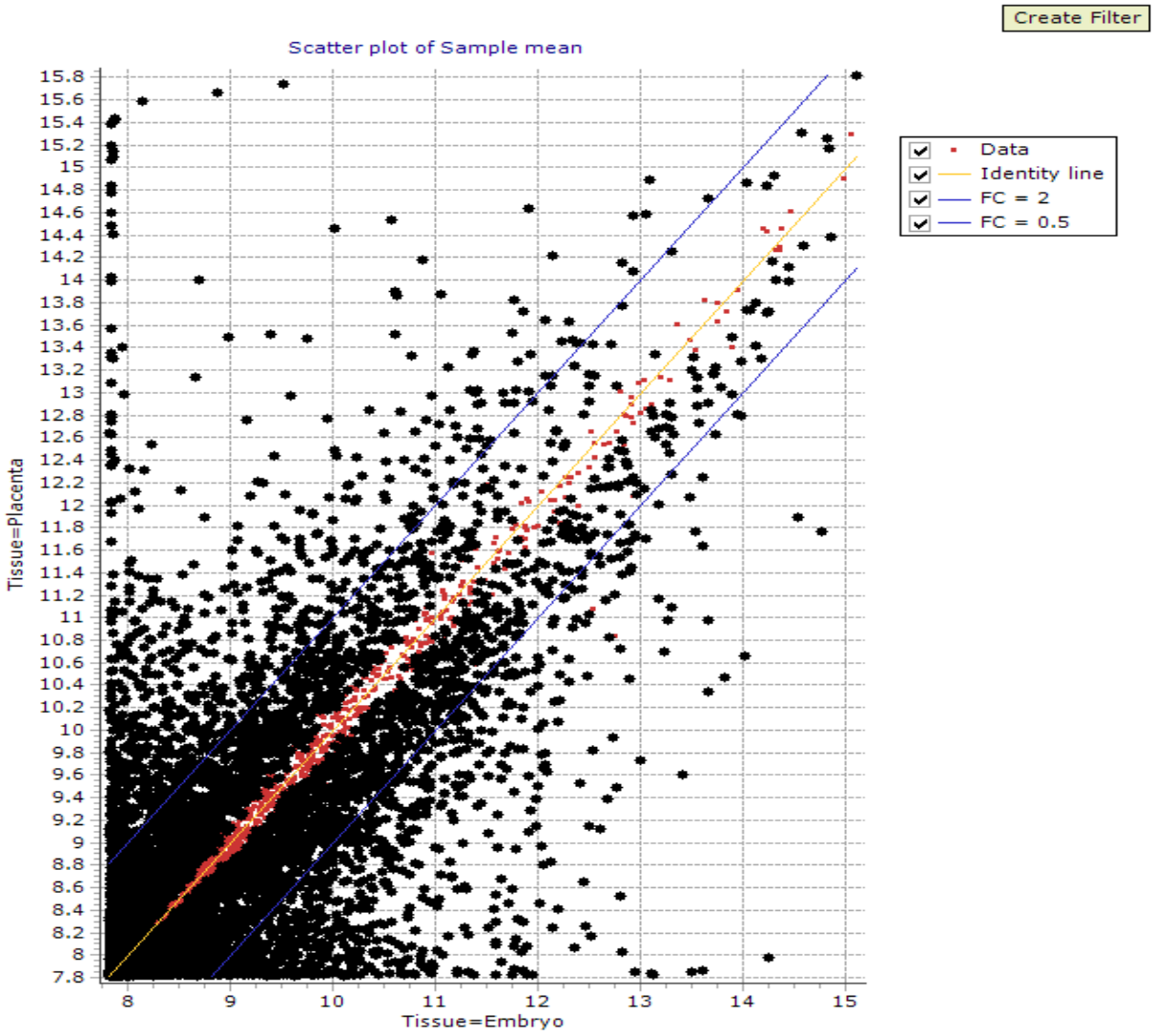


Fig. 4.1 Gene expression in E15.5 WT C57BL/6J embryo and placenta by Illumina expression profiling system. Scatter plot of mean expression generated by Flexarray software. Illumina dot plot of gene expression of a subset of genes. The y- and x-axis shows the VST-transformed value of gene expression signal from the placenta and the embryo, respectively. Samples size = 4 for each tissue. The genes that exhibit significant difference in expression between the embryo and the placenta are represented by the black dots, whilst the red dots represent the genes that exhibit similar expression in the embryo as well as the placenta.

4.2.2 Variability in gene expression in the placenta vs in the embryo

The intriguing observation of higher variations of gene expression in the placenta introduced in 3.2.5 prompted us to pursue this question of whether or not gene expression is truly more variable in the placenta than in the embryo on a genome-wide scale. We used the Illumina platform to compare genome-wide expression between the placenta and the embryo. After normalization against background signal control and data transformation, most genes (over 10,000) have expression values between 7.8 and 8 in embryo and/or placenta (Fig.4.2A), and they have relatively low expression at this developmental stage. The genes were binned into categories based on their expression level. The Illumina data delineates a non-linear positive correlation between expression and variance (Fig.4.2B). It is observed that the variance in gene expression is higher in the placenta than in the embryo in almost all categories. Large difference in the variance between the two tissues are only present in the expressions bins 10-11, 11-12, 12-13, and >13 (Fig.4.2B). The placenta exhibits a minimum of 1.4-fold higher variance in these categories. We weighted the variance against expression level to prevent a mathematical confound: since one particular gene has higher expression, leaving more room for variance. I then filtered for genes that have expression level > 10 and almost no difference in expression between the embryo and the placenta (fold change ~ 1) (Fig.4.2C). The number of genes per expression bin was less than 40 genes; thus, only three expression bins were created with a minimum of 15 genes per bin (Fig.4.2C). Since there was almost no difference in fold change between the embryo and the placenta, it was not necessary to divide the variance by the expression. The placenta exhibits a minimum 1.25-fold increase in variance using this filtering method.

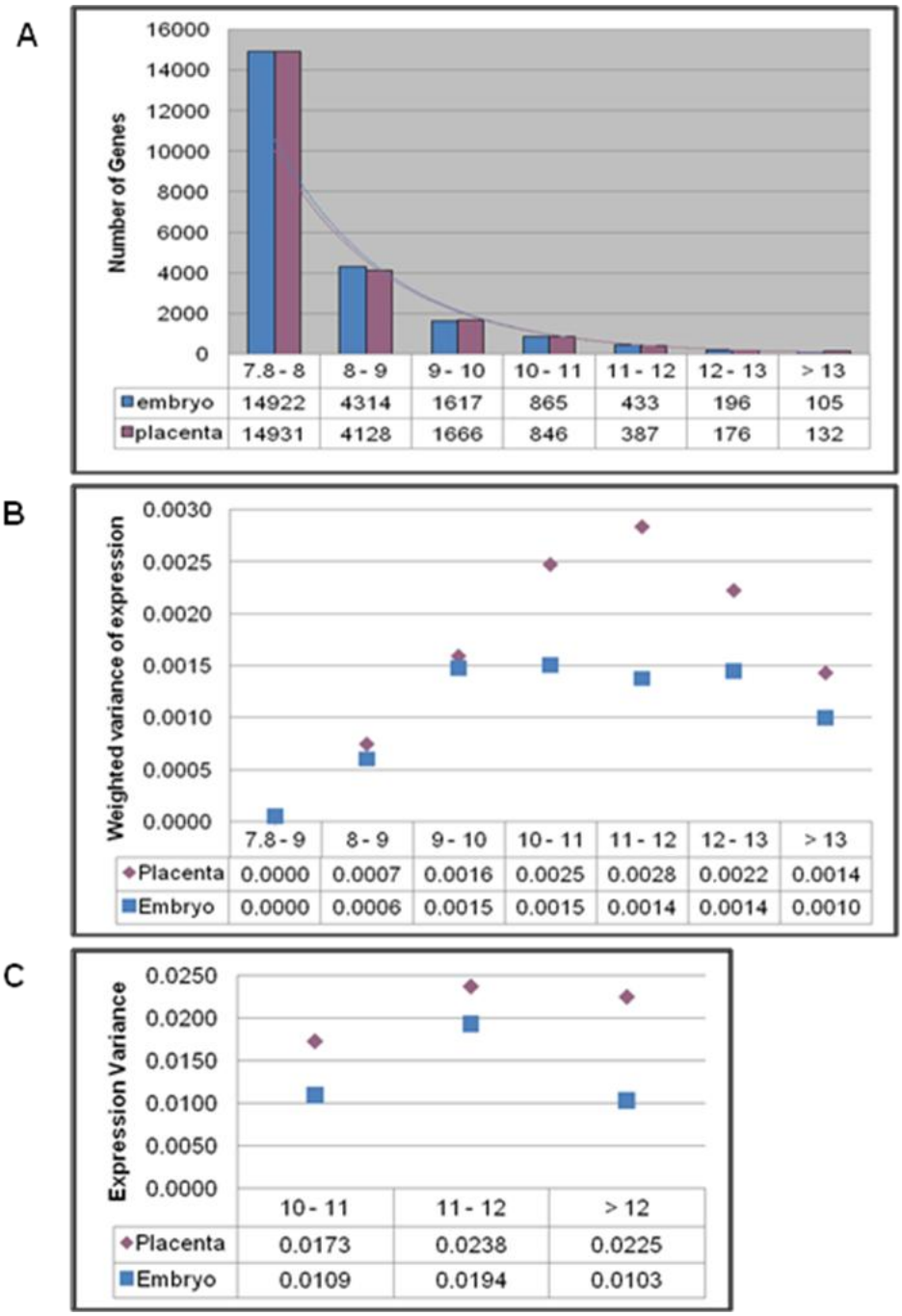


Fig. 4.2 Comparison of gene expression variation between E15.5 WT embryos and placentae. (A) The number of genes in each expression level. Expression level ranges from 7.8 to 15.5. (B) Variances in gene expression increase with an increase in expression level. The variance in the embryo plateaus when expression level reaches 10 but the placenta continues to exhibit increased variance at higher expression levels. Expression variance is weighted by dividing the expression variance of a gene with its expression level. (C) Variance is assessed for genes that have similar expression (> 10) in the embryo and the placenta. Both (B) and (C) show a higher variance in the placenta than in the embryo.

4.2.3 Most variable genes in the placenta and embryo

Even though the placenta appears to have more variability in gene expression, both tissues have highly variable genes (Table 4.1). The arbitrary cut-off for high variance was having expression variance > 0.2 (Fig. 4.3). There are 29 genes that exhibit the highest variance in the placenta, which represent 0.1% of the total number of genes included on the Illumina array. In the embryo, the most variable genes represent 0.066% (12 genes) of the total number of genes. The variances of these genes were tested for equality of variance (F Test) to determine if the variance of a gene in the placenta is significantly different from the variance of the same gene in the embryo. Specifically genes that have variance ratio of placenta variance/embryo variance >1 as placenta-variable genes and <1 as embryo-variable genes. Out of the 29 placenta-variable genes, only 17 have significant difference in variance between the placenta and the embryo: *Gzmg, Cxcl1, Prl8a6, Aqp1, Rnu6, Prlpn/Prl7b1, Apom, Apoa4, Gzmd, Eraf/Ahsp, Slc4a1, Spp2, Spink3, Apoa1, Afp, Apoa2,* and *Ttr*. Most of these genes code for proteins that are located in the extracellular compartment. They function in transport of substances, regulation of protein activity, and regulation of blood vessel size. Out of the 12 embryo-variable genes, 9 genes have significant difference in variance: *Myl7, Asprv1, Nppa, Lor, Krt10, Myl2, Car3, Akr1b7,* and *Pnliprp1*. These embryo-variable genes play a role in metabolism or code for proteins that make up the cytoskeleton.

Table 4.1 Genes with the most variability in expression in E15.5 C57BL/6J embryos and placentae. Variable genes characterized by their biological functions and components, and their chromosomal locations. Exprs. = VST-transformed expression level. Var. = Variance of expression. U = chromosome location unknown.

Tissue	Gene	Exprs.	Var.	mChr	GO Component	GO Process
Embryo	*Myl2*	11.74	0.277	5	Cytoskeleton - Myosin	Cell differentiation - muscle
	Asprv1	9.529	0.211	6	Membrane	Cell differentiation - skin
	Myl7	8.991	0.204	11	Cytoskeleton - Myosin	Cytoskeletal function
	Krt10	11.58	0.273	11	Cytoskeleton - intermediate filaments	Formation of keratin
	Lor	11.85	0.228	3	Membrane	Keratinization of the skin
	Clp2	8.942	0.224	17	Extracellular space	Lipid catabolism
	Pnliprp1	10.95	0.485	19	Extracellular space	Lipid catabolism
	Akr1b7	8.966	0.405	6	Cytoplasmic	Lipid metabolism
	Nppa	10.41	0.212	4	Cytoplasmic	Modulate size of blood vessel
	Car3	9.513	0.394	3	Cytoplasmic	Single-carbon metabolism
	Hbb-Y	12.91	0.283	7	Hemoglobin	Transport of oxygen
	Hba-X	10.35	0.388	11	Hemoglobin	Transport of oxygen
Placenta	*Prl8a6*	9.807	0.214	13	Extracellular Space	(placental hormone)
	Prlpn	12.63	0.284	13	Extracellular Space	(placental hormone)
	Apoa1	10.83	1.53	9	Extracellular Space	Angiogenesis
	Spp2	9.364	0.800	1	Extracellular Space	Bone metabolism
	Eraf/Ahsp	10.97	0.389	U	Cytoplasmic	Cell differentiation -blood cells
	S100a9	8.583	0.405	3	Unknown	Cell movement - leukocyte
	Fgg	8.478	0.211	3	Extracellular Space	Clot formation
	Gzmg	8.919	0.202	14	Unknown	Cytolysis
	Gzmd	10.13	0.333	14	Unknown	Cytolysis
	Prl8a2	12.99	0.251	13	Extracellular Space	Hypoxic response
	Apoa4	8.940	0.330	9	Extracellular Space	Immune response - innate
	Serpina1b	8.650	0.201	12	Extracellular Space	Immune response - non-humoral
	Cxcl1	8.491	0.212	5	Extracellular Space	Inflammation
	Kng1	9.263	0.218	16	Extracellular Space	Modulate size of blood vessel
	Alas2	11.67	0.397	X	Mitochondria	Regulate formation of hemoglobin
	Ttr	11.64	1.92	18	Extracellular Space	Thyroid hormone synthesis
	Slc4a1	10.66	0.418	11	Membrane	Transport - anions
	Tfrc	12.19	0.560	16	Membrane	Transport - vesicle mediated
	Afp	11.08	1.56	5	Extracellular Space	Transport of copper
	Apom	8.916	0.303	17	Extracellular Space	Transport of lipids
	Apoa2	10.84	1.84	1	Extracellular Space	Transport of lipids and beta fatty acids
	Hba-X	9.666	0.439	11	Hemoglobin	Transport of oxygen
	Hbb-Y	12.09	0.683	7	Hemoglobin	Transport of oxygen
	Aqp1	9.759	0.246	6	Membrane	Transport of water
	Rnu6	10.86	0.249	U	Unknown	Unknown
	Loc674706	10.39	0.257	U	Unknown	Unknown
	Wfdc2	11.67	0.258	2	Extracellular Space	Unknown
	Stfa1	8.321	0.270	16	Cytoplasmic	Unknown
	Spink3	10.58	1.306	18	Extracellular Space	Unknown

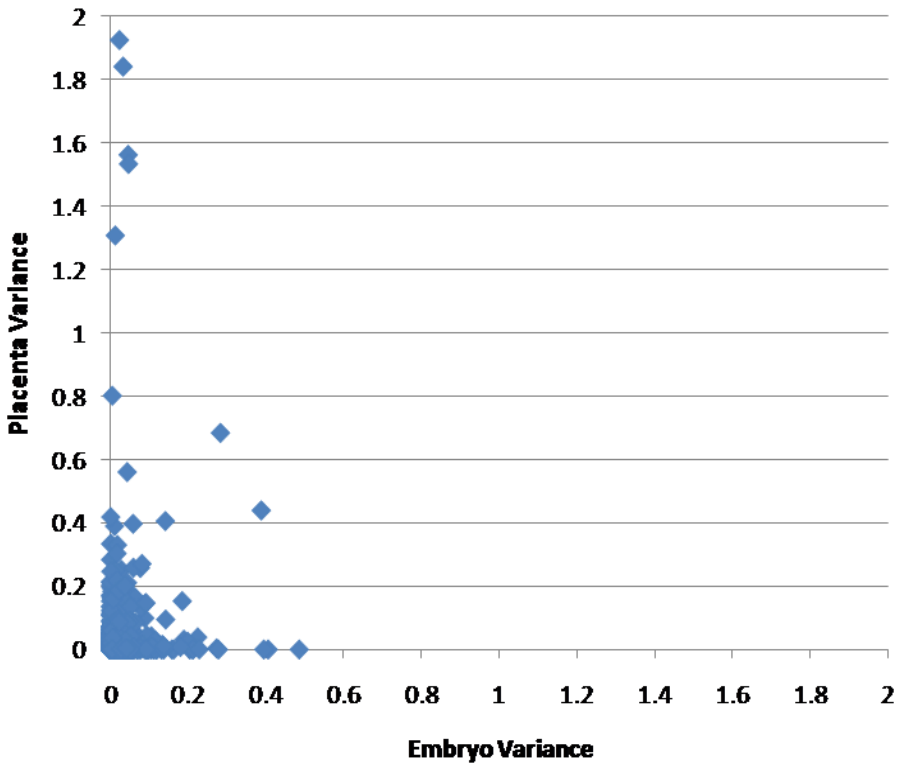
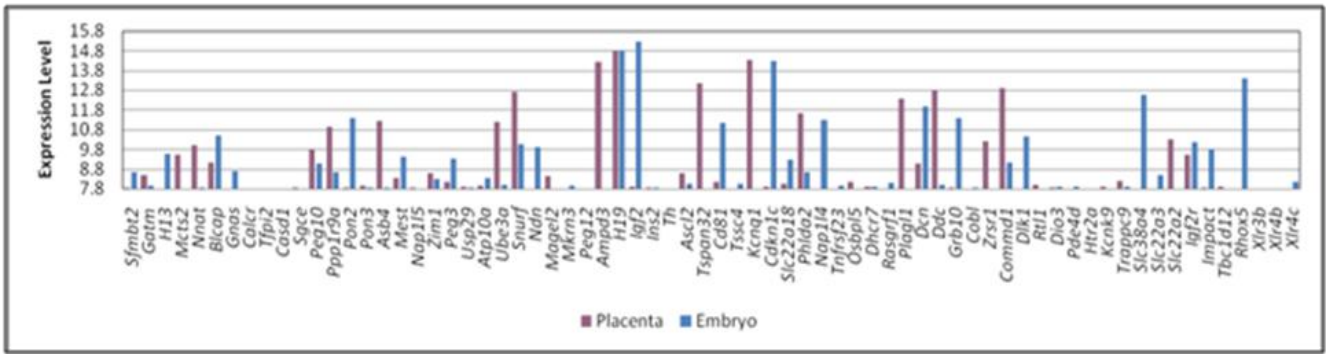


Fig.4.3 Whole-genome comparison of variance in gene expression between E15.5 C57BL/6J embryo and placenta. Scatter plot of expression variance illustrates that the maximal variance is under 0.5 in the embryo and under 2 in the placenta.

4.2.4 Imprinted gene expression and variability in C57BL/6J embryos and placentae

The Illumina platform assessed the expression of 18,138 genes, including 71 known imprinted genes. Many imprinted genes are important regulators of embryonic development, as suggested by their high expression level during development. *Blcap, Pon2, Snurf, H19, Igf2, Cd81, Cdkn1c, Nap1l4, Dcn, Grb10, Dlk1,* and *Igf2r* have relatively high expression (expression >10) in both the embryo and the placenta. *Rhox5* and *Slc38a4* are only highly expressed in the placenta whereas *Mest, Ndn,*and *Commd1* are predominantly highly expressed in the embryo (Fig. 4.4A). The variability in imprinted gene expression is also higher in the placenta than in the embryo, though none of the imprinted genes have variance > 0.08 (Fig. 4.4B).

A



B

	Embryo	Placenta
7.8 - 9	39	35
8 - 9	10	14
9 - 11	10	12
> 11	11	10

C

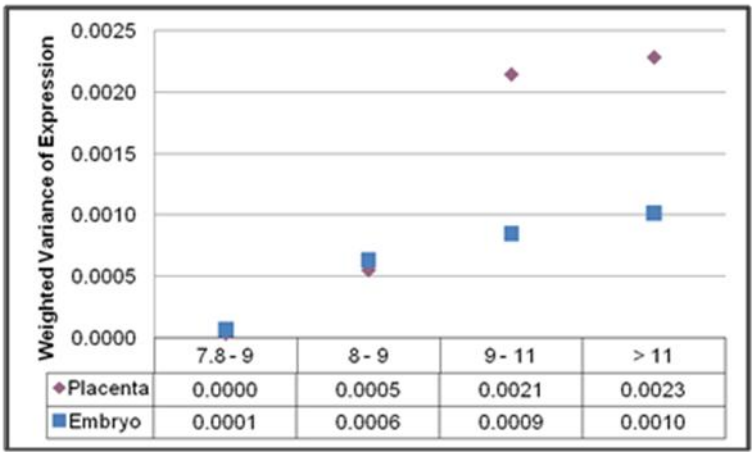


Fig. 4.4 Imprinted genes' expression and variability in E15.5 WT embryo and placenta. (A) Expression profile of 71 imprinted genes, organized by chromosomes from left to right. Expression level is the VST-transformed raw signal. (B) Variance of imprinted genes was weighted against the expression. The table represents the number of genes in each expression bin on the x axis.

4.2.5 Differential expression between wild-type and IUGR

Though the placenta seemed to exhibit more variance in gene expression between biological replicates than in the embryo, overall the variance is relatively small (10^{-2}) using the Illumina platform. Thus we profiled the gene expression of the embryonic and placental RNA samples derived from E15.5 conceptuses from the surgical IUGR model. There were five embryos that exhibited IUGR but only two of them were males (Fig. 3.7A,B). There was one other male that did not meet the IUGR cut-off but we believed that the crowding effect would affect all of the conceptuses, which prompted us to include that male sample as well. The samples were sent to McGill for expression profiling using the Illumina MouseRef8.0 BeadChip.

Clustering analyses of microarray data group together samples (biological replicates) with similar properties. Four clusters of the samples are clearly displayed using a hierarchal clustering method to group the different RNA samples (Fig. 4.5). As expected, the embryonic samples cluster independently from the placental samples. Furthermore the IUGR samples cluster separately from the WT samples. The IUGR embryo cluster exhibits larger within-group variation than the other clusters. One of the IUGR placental samples (LLE052) actually clustered with the IUGR embryonic samples (Suppl. Fig. 2). The tissue type of LLE052 may have been labelled incorrectly when I was preparing the RNA samples to send to McGill for expression profiling. LLE049 is the corresponding embryo sample to LLE052. LLE049 and LLE052 are not used when looking for differential expression between WT and IUGR cohorts.

Two Empirical Bayesian methods (Wright & Simon and cyber-T) and Benjamini-Hochberg false discovery rate were used to find genes that have significant differential expression ($p<0.05$) between E15.5 wild-type (n=4) and IUGR samples (n=2). 1770 genes were found to have differential expression in the IUGR placenta cohort. In the embryo, 2039 genes have differential expression in the IUGR embryo cohort. There are 799 genes that are

differentially expressed in both tissues, with 84 genes exhibiting > 2-fold difference between WT and IUGR samples. In the placenta, only 16 genes have a > 2-fold difference (Fig. 4.6C, Suppl. Table 2). 42 genes have > 2-fold difference only in the embryo (Fig. 4.6B, Suppl. Table 2), while 26 genes have > 2-fold difference in both the embryo and the placenta (Fig. 4.6A,D, Suppl. Table 2). These genes are involved in transport of proteins or ions, intracellular signalling, cellular processes (differentiation, proliferation, death, and metabolism), anatomical structural development, nucleotide-associated activities, immune or stress response, and cell adhesion (Fig.4.6B-D).

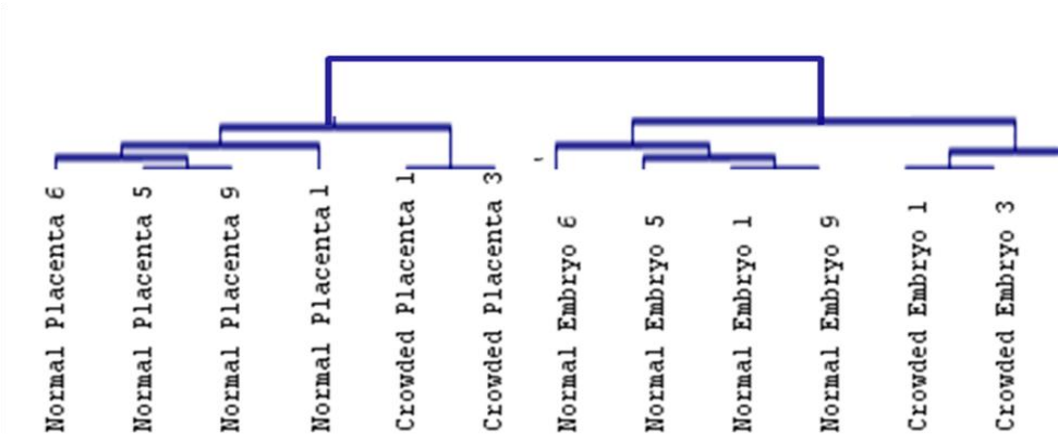


Fig. 4.5 Clustering of WT and IUGR samples. Four independent clusters are illustrated using the hierarchal clustering function on MeV. The embryo samples group significantly from the placental samples and accounts for the majority of the differences between the four groups.

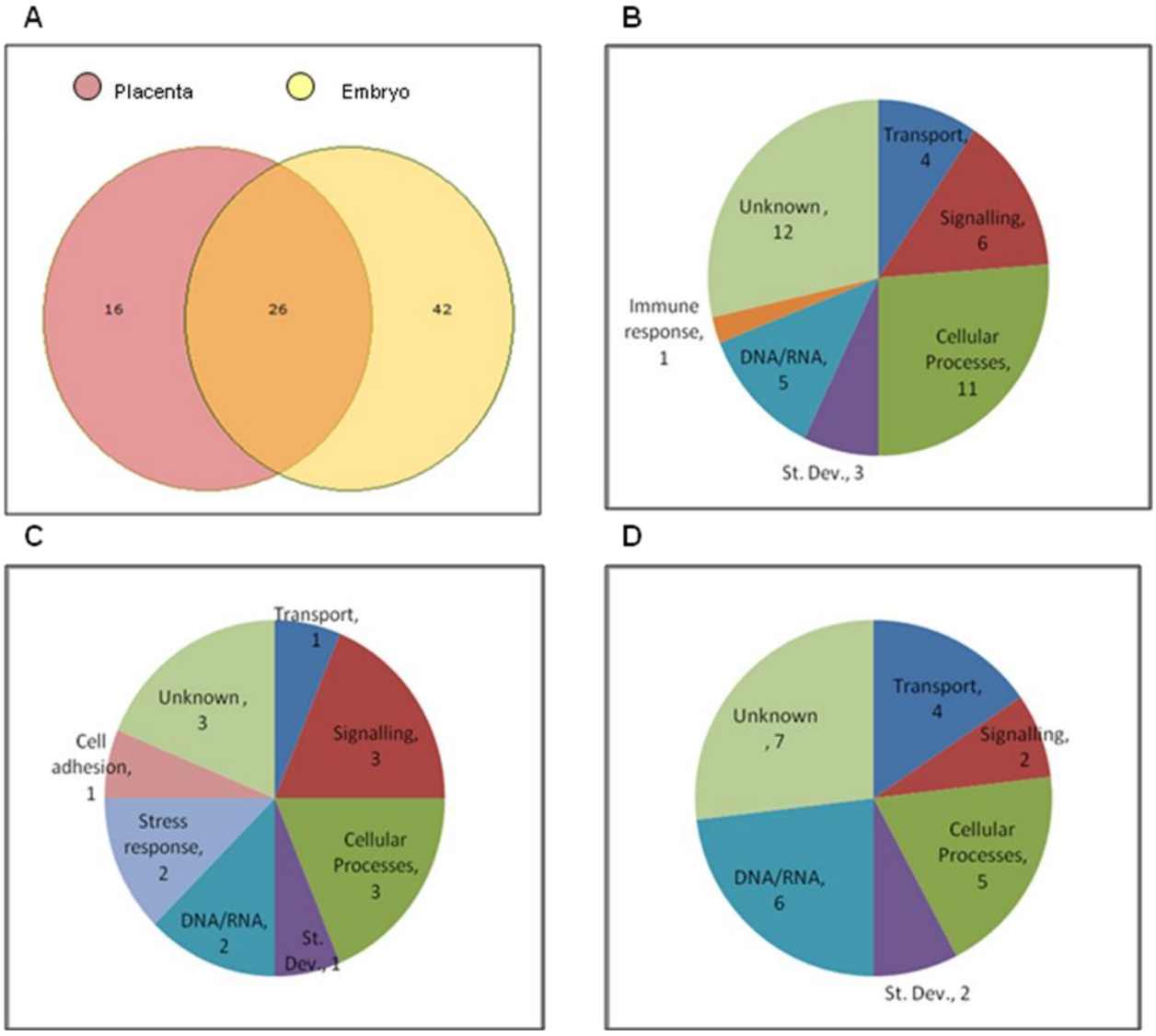


Fig. 4.6 Differentially expressed genes with > 2-fold different in IUGR. (A) Venn diagram illustrating the common genes that are differentially expressed in both IUGR embryo and placentae. (B) The functional categories of the 68 genes that are differentially expressed only in the embryo. The categories are based on gene ontology of the biological processes of the genes involved. The unknown category include genes with known molecular function but unknown biological process. (C) The functional categories of the 42 genes differentially expressed only in the placenta. (D) The functional categories of the 26 genes that are differentially expressed in both tissue. The functional categories of genes differentially expressed only in the placenta. IUGR samples size = 2 for each tissue. St. Dev. = Structural development.

4.2.6 Imprinted gene expression in IUGR samples

The majority of imprinted genes analyzed do not exhibit significant differential expression between WT and IUGR samples. The genes that are differentially expressed in the placenta are *Sfmbt2, Phlda2, Cdkn1c, Cobl, Zrsr1, Dlk1, Slc38a4, Slc22a3,* and *Xlr4c* (Table 4.2). In the embryo, *H19, Igf2, Dlk1, Pde4d,* and *Slc38a4* are differentially expressed between WT and IUGR samples. The fold change ratio of IUGR to WT and gene ontology of these imprinted genes are illustrated in Table 4.2.6. *Dlk1* exhibits the greatest increase in expression in both IUGR embryos and placentae for imprinted genes. It is also the imprinted gene that has the highest increase in expression in IUGR placentae (Suppl. Table 2).

71 genes previously demonstrated to be imprinted in mice are assayed on Illumina MouseRef8.0. We have hypothesized that imprinted genes as a whole will be more affected in IUGR because many of them have been shown to be essential to embryonic growth and development. Therefore I have conducted a chi-square test to see if imprinted genes are over-represented in the group of genes that are differentially expressed in IUGR (Table 4.3). Moreover, I have also done the chi-square test on potential candidates that have recently been suggested to be imprinted in the mouse (Gregg et al., 2010). In both categories, imprinted genes are not over-represented out of the differentially expressed genes in IUGR embryo cohort, but the number of imprinted genes that are actually differentially expressed are slightly more than expected in the IUGR placenta cohort. However, the chi-squared test shows that this slight difference in the IUGR placenta cohort is not statistically significant.

Table 4.2 Differentially expressed imprinted genes in IUGR samples. The imprinted genes are specified by gene ontologies of biological and molecular function.

Tissue	Gene	mChr	WT Exprs.	IUGR Exprs.	Fold change	GO Biological Process	GO Molecular Function
Embryo	*H19*	7	14.99	14.52	0.7214	Regulate expression of Igf2	ncRNA that interacts with DNA
	Igf2	7	15.68	15.28	0.7575	Promote growth	Growth factor activity
	Slc38a4	15	8.340	8.059	0.8662	Transport of amino acid	Uptake amino acid
	Pde4d	13	7.982	8.133	1.055	Generate force in smooth muscle	Hydrolase activity
	Dlk1	12	13.45	14.07	1.534	Embryonic skeletal development	Binding to calcium
Placenta	*Slc38a4*	15	12.96	11.34	0.3253	Transport of amino acid	Uptake amino acid
	Sfmbt2	2	8.811	8.235	0.6709	Regulation of transcription	Unknown
	Slc22a3	17	8.617	8.126	0.7116	Transport	Transfer of quaternary ammonium group
	Xlr4c	X	8.273	8.034	0.8472	Unknown	Unknown
	Cobl	11	7.964	7.996	1.023	Neural tube closure	Protein interaction
	Zrsr1	11	7.942	8.002	1.043	Unknown	Interact with zinc
	Phlda2	7	8.797	9.300	1.418	Glycogen storage	Unknown
	Cdkn1c	7	14.77	15.36	1.509	Cell cycle arrest	Cyclin-dependent cell cycle inhibitor
	Dlk1	12	10.75	12.25	2.841	Embryonic skeletal development	Binding to calcium

Table 4.3 Chi-square test of known imprinted genes and imprinting candidates. The statistical test compares the number of imprinted genes or candidates that are actually differentially expressed in IUGR samples versus the expected number of imprinted genes or candidates.

Confirmed or candidate imprinted genes	Embryo (actual)	Embryo (expected)	Placenta (actual)	Placenta (expected)
Imprinted Genes	5	7.98	9	6.93
Gregg et al. (2010) Candidates	27	28.9	33	25.1
Chi square (p-value)	0.533		0.208	

4.3 Discussion

4.3.1 Genome-wide expression analysis using Illumina MouseRef8.0 Beadchip

My goals to conduct genome-wide analysis were two-fold: 1) Provide wild-type (WT) control for comparison with IUGR samples and 2) address the issue of variability of gene expression in the embryo and the placenta. Another advantage was that we could use the expression data to uncover unknown genes that may play a role in either the embryo or the placenta at E15.5. We employed an arbitrary cut-off value for a gene as expressed at 9 after variance-stabilized transformation of raw intensity level since the majority of the genes with expression < 9 did not exhibit any ISH staining in E14.5 embryo sections submitted to GenePaint (www.genepaint.org).

As expected, the gene expression pattern in the placenta is very different from that of the embryo (Fig.4.1). Several members of the prolactin and cathepsin families are amongst the highest-expressed genes in the placenta. Prolactins are hormones that bind to maternal targets, which result in alteration of maternal physiology (Lin et al., 2000). The cathepsin family mostly functions in the hydrolysis of peptide bonds. These proteases are thought to likely function in placental vasculature remodelling (Simmons et al., 2007; Varanou et al., 2006). These genes are only highly expressed in the placenta and not in the embryo (Suppl. Table 1). Ribosomal genes are the highest-expressed genes in the embryo. The high levels of ribosomal expression suggests that protein anabolism occurs extensively in the embryo, probably due to the metabolic requirements of rapidly dividing cells during the growth phase. These genes also exhibit high expression in the developing placenta. The embryo-specific genes (Fold change > 10) code for actin, myosin, and troponin proteins that compose the cytoskeleton. Some of these molecules like ACTA1 and MYLPF, are important for skeletal development, which only occurs in the embryo (Garner et al., 1989; Wang et al., 2007). Expression pattern of imprinted genes in our

array data correlate well with previously known expression levels (Fig. 4.4A) (Schulz et al., 2008).

4.3.2 Variability of gene expression is more significant in the placenta than in the embryo

Many studies have indicated that gene expression is not consistent from one biological replicate to another. Pidoux and colleagues (2003) demonstrated that in the placenta there was greater variation of expression between placentae from different individuals than within the same placenta. In our study, the variance observed in both the embryo and the placenta was on the order of 10^{-2}. From the principal component plot, the largest difference (97%) of the WT samples was between the embryo cohorts versus the placental cohort. It was advantageous to see that variation between biological replicates was not as large as qRT-PCR of the *Mest* samples, since it could mask the difference in expression between normal and IUGR samples.

Fig.4.2B shows a positive correlation between expression and variance. This is expected since genes at lower expression level (Expression < 8) were likely not expressed or had a very low level of expression. If the gene was not expressed, then its expression from one biological replicate to another would not alter. Interestingly, the placenta cohort displayed a higher variance even for these genes, indicating that gene expression in the placenta is more variable. Moreover, all of the most variable genes have expression level < 12, indicating that the highest expressed genes do not necessarily have the highest variance (Table 4.1). Though the difference in variances between the embryo and placenta cohort were small, I consistently found the placenta to have expression just slightly more variable than that of the embryo, especially at higher levels of expression (Fig.4.2E-F). This result is in agreement with qRT-PCR data (Fig.3.6B)

The function of the placenta can explain the observation of greater variability in gene expression. We have used littermates as biological replicates but even if the littermates are

within the same environment, there may be differences in the amount of nutrient exchange between the littermates. Several studies have indicated that blood flow to each conceptus may be different depending on their location in the uterine horn (Coe et al., 2008; Vom Saal and Dhar, 1992). The variation in blood flow may be due to the bi-furicated nature of the blood flow in the uterine artery, which branches out to supply each placenta in the murine uterine horns. Vom Saal and Dhar (1992) have found that the blood pressure supplying the conceptuses at the ends of each uterine horn is higher than the blood pressure supplying those implanted in the middle of the horn. Accordingly, the number of conceptuses and their position in the horn can affect the level of blood each placenta receives causing it to be different. So the placenta of the middle conceptuses may show altered gene expression level as a result of compensation for the lower level of blood supply (Vom Saal and Dhar, 1992).

It has been demonstrated that the placenta can respond to the altered intrauterine environment by changing gene expression. For example, gene expression in the placenta is altered in response to hypoxia (Genbacev et al., 1996). Moreover, alteration of solute carriers' gene expression to meet embryonic nutritional demand has been demonstrated in the placental-specific mouse knockout of *Igf2*, which codes for an essential embryonic growth factor (Constancia et al., 2005). Therefore, gene expression in the placenta may be altered to meet the needs of the embryo even in regular pregnancy, which can lead to variability in gene expression between littermates. This is supported by our finding that 7/29 of the most variable genes in the placenta have transport function when only 2 genes would be expected given the number of transport genes represented on the array (Table 4.1).

The next question to ask is why the embryo does not display the same variability. The major developmental events in the embryo require very specific levels of signalling between different cell types. For example, neural tube closure is governed by specific signalling between

neural crest cells and neighbouring cell types. Perturbation of signalling at this stage in development can result in openings along the dorsal midline, which impacts the survival and welfare of the embryo (Copp et al., 2003; Detrait et al., 2005). Differences in gene expression in an embryo can lead to malformation or even death. Due to this sensitivity embryonic gene expression may be more specific than that of the placenta, as the placenta may possibly tolerate volatile gene expression since it will be disposed after birth.

It is also possible that the placenta evolved to have more adaptive gene regulatory mechanism to specifically address nutrient demands (Coan et al., 2008; Coan et al., 2010). Genomic imprinting is a mechanism that is believed to have evolved to regulate nutritional demands of the embryo (Wolf and Hager, 2006). In the placenta, there are more genes that have been identified with imprinted expression than in the embryo. Moreover, imprinted gene regulation by histones, which is more volatile than gene regulation by DNA methylation has been suggested to play a more significant role in the placenta than in the embryo (Lewis et al., 2004; Umlauf et al., 2004; Wagschal et al., 2008). We found that 23/71 (33%) imprinted genes have significant difference in variance between placenta and embryo, 15 of which exhibit more variance in the placenta. This signifies that the overall trend of higher placental variance is also seen for imprinted genes (Fig. 4.4). It will be interesting to see if the genes that have high variability will be confirmed with expression profiling of additional WT litters.

4.3.3 Comparison of whole-genome expression between normal C57BL/6J and IUGR samples

Performing hemiovariectomy on C57BL/6J females resulted in all the conceptuses being implanted in one horn (Fig. 3.7A). The decreased size of placenta created a crowding effect

where some of the embryos had lower embryonic weights than embryos in a normal pregnancy. There were only two E15.5 male embryos from the crowded horn that had weights in the bottom 5^{th} percentile. I believed that all the embryos were subjected to the crowding effect, thus we included the last E15.5 male in our second microarray.

Plate-to-plate variation in older microarray studies created problems for researchers since it was difficult to assess if the differences observed between normal and diseased samples were real or due to technical artifact. It was suggested that all microarray studies use three to five biological replicates for each cohort (http://discover.nci.nih.gov/microarrayAnalysis). Modern array equipment the plate-to-plate variation has been reduced. In our study, we conducted two Illumina Beadchips, each contained eight samples. All the WT samples were on one Beadchip whilst all the crowded samples were on the second Beadchip. However, though the normalization procedures used by the Flexarray software enables me to directly compare the expression levels between the WT and crowded samples, there may still be chip-to-chip variation that can influence the current findings presented in this thesis.

Principal component analysis was used to quickly visualize clustering of the samples (Suppl. Fig. 2). Four clusters were observed: WT embryo, WT placenta, crowded embryo, and crowded placenta. One difficulty encountered was that one of the crowded placental sample (LLE052) actually clustered with the crowded-embryo samples. This was perhaps due misplacement or labeling error. Fortunately LLE052 was the male placentae whose embryonic weight was not in the bottom 5^{th} percentile. Interestingly, the corresponding male embryo (LLE049) also clustered slightly further from the two IUGR embryos. Since the purpose of this study was to look for genes differentially expressed in IUGR, both samples LLE049 and LLE052 were not used in subsequent statistical analyses for assessing significance of differential expression between WT and IUGR samples.

One statistical method used to determine the significance of differential expression between WT and IUGR samples may return a list of genes that differs from the list of differentially-expressed genes generated when another statistical method was used. The best approach to find genes that would be more representative of real differential expression was to use more than one statistical method. Thus the data was subjected to the Empirical Baysian tests EB (Wright & Simon) and cyber-T tests, two of the most common tests used on microarray data (Murie et al., 2009). The Significance Analysis of Microarray (SAM) was also conducted but this test did not return any genes that showed differential expression. This was most likely due to the small sample size of the IUGR cohort (n=2) which does not meet the stringency of SAM. Nevertheless, it was still worthwhile to identify candidate genes so when more samples are used, I could compare those findings that would be analyzed using SAM with the ones I have identified in the current study.

Overlapping the results of the two tests showed 1770 genes to be differentially expressed in the IUGR placentae, while 2039 genes were differentially expressed in IUGR embryos. The maximum fold difference was slightly less than 6 in the embryo, and less than 3.5 in the placenta. The majority of the genes exhibited fold difference of less than 1.5. Considering more genes are expressed (Expression > 9) in the placenta than in the embryo (3595 versus 3569), it was surprising to find more genes to be differentially expressed in the embryo. Looking at the clustering of the IUGR placental cohort versus the IUGR embryo cohort provided some insight. The two IUGR placental samples cluster closer than the two IUGR embryos, which indicated that the overall variance in the IUGR placental cohort was less. With a sample size of two, it is impossible to determine if one of the IUGR embryo sample used was an outlier. If an outlier was present, then the accuracy of the differential expression data would decrease. Indeed the limited samples size was a major issue in this study. Nonetheless, the goal of the microarray study was

to find candidates that may play a role in IUGR. All of the interesting candidates would need to be further re-evaluated by qRT-PCR, or even quantitative sequencing. The sample size would need to be increased during these validations.

To narrow down the candidate genes that are affected in IUGR, an arbitrary 2-fold cut-off was employed. This reduced the number of genes altered in IUGR to less than 100. The major categories that are implicated were transport of substances, cellular metabolism, signalling, and nucleotide metabolism (Fig. 4.6). It was found that 12% of genes (5/42) exhibit differential expression in the IUGR placenta cohort are involved in transport, whereas 11% of differentially expressed genes in the IUGR embryo cohort are involved in transport. This was not surprising since transport molecules are the most affected because at this stage of development, transport genes encompass the largest class of genes that are expressed in our WT microarray data (13% in embryo and 15% in the placenta). Though transport genes were not particularly enriched, there was still a candidate that was of particularly interest: *Npc2*. *Npc2* is highly expressed in both the embryo and the placenta and exhibits a 2-fold under-expression in IUGR embryos, and a 2.7-fold under-expression in IUGR placentae. The *NPC2* mutation in humans represents the cause of 5% of the lipid storage disease known as Niemann-Pick disease (Millat et al., 2001). NPC2 functions together with NPC1 to facilitate intracellular transport of lipids in lysosomes (Sleat et al., 2004). It has also been documented that IUGR is a phenotype in a subset of individuals who experience fetal onset of the disease (Spiegel et al., 2009).

A couple of interesting candidates in IUGR embryos function in cellular differentiation: *Gap43* and *Stmn1*. *Gap43* exhibits a 2.3-fold down regulation in IUGR embryos. It codes for a growth-associated protein whose main function is the development of the optic nerve (Strittmatter et al., 1995). *Stmn1* exhibits a 6-fold under-expression in IUGR embryo cohort. It is found that down-regulation of *Stmn1* affects cell motility (Jin et al., 2004; Ozon et al., 2002). It

has been suggested that the expression of *Stmn1* is associated with early migration of trophoblast cells and differentiation into syncytiotrophoblasts (Yoshie et al., 2008).

Two collagen-encoding genes, *Col1a1* and *Col3a1*, are over- and under-expressed in IUGR embryos, respectively. These collagen proteins contribute to structural integrity of the extracellular matrix and both contribute to blood vessel development (Liu et al., 1997; Rahkonen et al., 2004). A mutation in human *COL3A1* has been linked to aortic rupture. Homozygous mouse mutants that survive have reduced body size and most die within six months due to rupturing of blood vessels (Liu et al., 1997). *Col1a1* also functions in bone development and mutations in *COL1A1* are thought to be the main cause of Osteogenesis Imperfecta in humans (OMIM 166200, OMIM 166210).

None of the non-imprinted genes found to be differentially expressed in human studies comparing normal versus IUGR term placentae were significantly differentially expressed in this study. A few genes (*IGFBP1, IGF-I, CRH,* and *LEP*) were consistently shown to have different expression level in human IUGR placentae (Economides et al., 1989; Lee et al., 2010; McMinn et al., 2006; Struwe et al., 2010). Other *Igfbp* genes (*Igfbp3* and *Igfbp4*) do exhibit differential expression in IUGR embryos but do not meet the 2-fold cut-off.

In the IUGR placental cohort, *Prl8a9* shows a 3.3-fold down-regulation when compared to WT cohort. Expression of *Prl8a9* is the highest in the spongiotrophoblast from E12.5 until birth (Simmons et al., 2008). Not much is known about the specific function of *Prl8a9,* but it is likely to aid in the invasion of maternal decidua since it is highly expressed in the spongiotrophoblast, the placental layer suggested to house the precursors of the invasive glycogen cells in the murine placenta (Georgiades et al., 2002; Rossant and Cross, 2001). On a separate note, this gene is murine-specific so it may not play a very significant role in human IUGR.

Three other genes that are of interest are *Vhl* (FC = 0.3), *Hspa5* (FC = 0.4), and *Gpx5* (FC = 2.2). Gene ontologies indicate that they are involved in stress response. *Vhl* has been found to code for a protein product that regulates oxygen-dependent degradation of hypoxia-induced factor 1 (HIF-1) (Maxwell et al., 1999). Absence of the VHL protein results in defective vasculature in the labyrinthine of the murine placenta (Gnarra et al., 1997; Tang et al., 2006). The connection of IUGR to regulators of placental vasculature indicates that impaired vasculogenesis may be involved in IUGR. HSPA5 is an endoplasmic reticulum (ER) chaperone protein that is involved in signalling pathways activated upon ER stress (Kaufman, 1999). *Hspa5* exhibits an increase in expression upon ER stress and has been suggested to attenuate apoptosis (Oyadomari et al., 2002). GPX5 is a gluthathione peroxidase that is implicated in oxidative stress response (MGI Gene Ontology J:72247). Researchers have found that disrupting *Gpx5* in the mouse results in abnormal sperm production. The abnormal sperm exhibit an increase in DNA fragmentation upon treatment with hydrogen peroxide, which indicates that the null mutants have problems when responding to oxidative stress (Chabory et al., 2009).

Though some of the candidates discussed here have functions that may contribute to IUGR, a very important issue must be addressed before we can conclude if these genes are truly candidates for IUGR. Pidoux et al. (2003) has found that there is more variation in expression between different human placenta than within the same placenta. In the mouse system littermates have been shown to have relatively low variance in expression (Fig. 4.2). The best approach to solve this problem is to conduct expression profiling of additional WT samples from multiple different litters than the ones used in our current study. Genes that are found to be differentially expressed between WT litters can then be cross-referenced to the list of IUGR candidates and be excluded from the candidate list.

In addition, more IUGR samples are also needed to provide a samples size that is much greater than two as in the current study to perform the Significance Analysis of Microarray (SAM) to find candidate IUGR genes. I propose to start with 8 biological replicates for each cohort (32 samples in four categories: WT embryo and placenta, IUGR embryo and placenta) and analyze those first to see if there are any commonalities between those findings and the results presented in this thesis. Once those candidates have been identified then I can pursue the interesting candidates using qRT-PCR, which enables me to analyze more samples due to the lower cost. Only after all these steps are taken can we present the final list of genes differentially expressed in IUGR. If structural developmental genes and stress response genes still play a role in IUGR embryo and placenta, then it suggests that a different mechanism may be contributing to IUGR in the two tissues. It is not clear whether or not the changes in expression level of these genes are causing the IUGR or an adaptive response to the stress. It may be a combination of both since our study only takes a "snapshot" of gene expression at a specific stage in development.

4.3.4 Imprinted candidates of IUGR

Recently the topic of epigenetics in IUGR has been explored in several studies (Apostolidou et al., 2007; Bourque et al., 2010; Diplas et al., 2009; Guo et al., 2008; McMinn et al., 2006; Penaherrera et al., 2010). Given the involvement of genomic imprinting in embryonic growth and development, imprinted genes are thought to be the most likely candidates that are affected in IUGR. Indeed several studies have looked at differential expression of imprinted genes in human IUGR placentae (Apostolidou et al., 2007; Bourque et al., 2010; Diplas et al., 2009; Guo et al., 2008; McMinn et al., 2006). We have found four imprinted genes to exhibit significant fold change in the embryo of the crowded IUGR model. *H19* (FC = 0.72), *Igf2* (FC =

0.76), and *Slc38a4* (FC = 0.87) are under-expressed in IUGR embryos and *Dlk1* (FC = 1.5) is over-expressed.

IGF2 has been repeatedly reported to be under-expressed and hypomethylated in human IUGR (Abu-Amero et al., 1998; Antonazzo et al., 2008; Bourque et al., 2010). *Igf2* codes for an insulin-like growth factor that is important in regulating embryonic growth. Mouse KO of *Igf2* (*Igf2*+/-) has been found to exhibit a 40% reduction in birth weight (DeChiara et al., 1990). The function of *Igf2* has largely been explored in the placenta. The placental-specific knockout of *Igf2* (*Igf2* $P0^{+/-}$) display a milder phenotype than *Igf2*+/-, the null mutant of *Igf2* since the gene is paternally expressed. Comparison of these two mouse mutants has increased the understanding of the function of the fetal *Igf2* and placental *Igf2*. The fetal *Igf2* functions in labyrinthine cells from both the trophoblast and epiblast lineage (fetal endothelial cells), whilst the placental *Igf2* only functions in the labyrinthine cells from the trophoblast lineage (Constancia et al., 2000; Redline et al., 1993). *Igf2* $P0^{+/-}$ is constructed through deleting the placental-specific promoter of *Igf2*. Constancia and colleagues (2005) has also demonstrated that in *Igf2* $P0^{+/-}$, *Slc38a4* expression is up-regulated in the placenta to increase amino acid transport which they suggest is an adaptive response to the need to maintain nutrient transport to the embryos. Accordingly, since *Igf2* is under-expressed in IUGR, then *Slc38a4* should increase. This differs from the decreased expression observed for both IUGR embryo and placenta in this study. Though *Slc38a4* shows differential expression, there is a 10% reduction in IUGR embryos. This result needs to be verified with increased sample size. It is also possible that *Igf2* may not affect *Slc38a4* in the embryo, or affect *Slc38a4* expression in a different manner.

Though *H19* has not shown differential expression in IUGR in previous studies, it is found to be biallelically expressed indicating a loss of imprinting (LOI) in IUGR placentae (Diplas et al., 2009; Guo et al., 2008). *H19* codes for a non-coding RNA that directly regulates

the expression of *Igf2* through epigenetic mechanisms (Drewell et al., 2002; Leighton et al., 1995). Maternally-inherited double KO of *H19* and *Igf2* has shown a compounded effect that leads to embryonic lethality (Eggenschwiler et al., 1997). Furthermore, targeted disruption to *H19* only affects embryonic and placental growth. Depending on whether the mutant is inherited maternally or paternally, overgrowth or IUGR will occur (Drewell et al., 2000; Thorvaldsen et al., 1998). The inter-relatedness of *H19, Igf2,* and *Slc38a4* supports the idea of an imprinted gene network (IGN) (Arima et al., 2005; Varrault et al., 2006). Though the fold change difference of these genes in IUGR is not greater than 2-fold, the compound effect of irregular gene expression can lead to the development of IUGR.

Human studies cannot look at differential gene expression in IUGR fetus except using cord blood, so our study is the first study to look at the genes that may be altered in expression using whole IUGR embryos. As mentioned, the genes that contribute to IUGR will not necessarily coincide with those that are differentially expressed in the placenta. For imprinted genes, only *Slc38a4* and *Dlk1* exhibit differential expression in both the embryo and the placenta. *Igf2* in our IUGR placental cohort is over-expressed by 1.2-fold, though the difference was not significant ($p < 0.5$).

Dlk1 exhibits increased expression in both IUGR embryo (FC = 1.5) and placenta (FC > 2.8). *Dlk1* is normally highly expressed in both the embryo and the placenta (Schmidt et al., 2000; Yevtodiyenko and Schmidt, 2006). Its expression in the embryo is high at E12.5 in most mesodermally-derived tissues, as well as in the pituitary, adrenal gland, and pancreas. This trend changes to high expression only in the pituitary, adrenal gland, and skeletal muscle by E16.5. In the placenta, *Dlk1* is expressed in endothelial linings of labyrinthine vessels (Yevtodiyenko and Schmidt, 2006). The expression pattern signifies its role in embryonic development, as well as its role in nutrient exchange in the placenta. *Dlk1-/-* mouse mutants exhibit IUGR, perinatal

mortality, and skeletal defects involving the rib. *Dlk1* is expressed from the paternal allele, yet paternal inheritance of the null mutation only results in postnatal growth restriction (Moon et al., 2002). This suggests that both the parental alleles of *Dlk1* are essential for normal development in the mouse. *DLK1* is one of the genes from the imprinting region (IG-DMR) on human chromosome 14. Uniparental disomy of chromosome 14 (UPD14), which exhibit various congenital phenotypes including growth restriction, is attributed to aberration of epigenetic features of IG-DMR (Kagami et al., 2008; Temple et al., 2007). Recently *DLK1* has been discovered to exhibit loss of imprinting (LOI) in Silver-Russell Syndrome or IUGR (Azzi et al., 2009; Guo et al., 2008). The over-expression of *Dlk1* in our IUGR samples may occur through LOI. This could be evaluated in the mouse by doing bisulphite sequencing on F1 offspring of C57BL6/J and *Mus musculus* castaneus mice for instance.

Two other genes were found to be under-expressed by greater than 1.4-fold in the IUGR placental cohort. The gene *Sfmbt2* (FC = 0.67) is a newer addition to the imprinting family in the mouse. It is the first gene discovered in a known imprinted region on mouse chromosome 2 (mChr2); the region is considered imprinted since maternal duplication of the proximal region result in early embryonic lethality (Cattanach et al., 2004; Kuzmin et al., 2008). Not much is known about the function of this gene except it is part of the polycomb group and is hypothesized to be important in maintenance of trophoblast stem cells during early embryonic development (Kuzmin et al., 2008). *Slc22a3* (FC = 0.71) belongs to the organic cation transporter family. Along with *Igf2r* and *Slc22a2*, *Slc22a3* is regulated in *cis* by the Air non-coding RNA in the murine placenta (Nagano et al., 2008). The organic cationic family is important in controlling the amount of neurotransmitters like norepinephrine or epinephrine in the extracellular matrix. No obvious defects are observed in *Slc22a3*-null mice except for reduced activity of this uptake system in the adult heart as well as in the embryos (Zwart et al.,

2001). The uptake system also exhibits high activity in the placenta, and *Slc22a3* shows high expression in the labyrinthine (Verhaagh et al., 1999; Zwart et al., 2001). It is suggested that redundancy of other transporters in the family (*Slc22a1* and *Slc22a2*) is the reason why reduced uptake activity is not observed in other organs of *Slc22a3*-null mice (Verhaagh et al., 1999; Zwart et al., 2001). *SLC22A3* expression is also observed in first-trimester and term human placenta (Verhaagh et al., 1999). No association between embryonic growth has been demonstrated with *Slc22a3*, but considering its high expression in the labyrinthine, it is likely to function in nutrient exchange.

Slc38a4 is under-expressed in IUGR placentae by 3-fold when compared to WT. As previously mentioned this is different from my expectation since in the IUGR *Igf* $P0^{+/-}$, *Slc38a4* was observed to increase at E16 (Constancia et al., 2005). Another study has also found an inverse relationship between birth weight and this System A amino acid transporter activity in human (Godfrey et al., 1998). SLC38A4 is an essential transporter responsible for amino acid transport that is required since amino acids account for 50% of all nitrogen and carbon required for fetal growth (Fowden and Forhead, 2004). Considering the function of this protein, it is possible to interpret that the decreased expression is contributing to growth restriction. Interestingly, *Slc38a4* expression in the *Mest* KO model, though statistically insignificant, do exhibit a decreased expression in the *Mest*+/- cohort (Fig. 3.7). In contrast to the *Igf2* $P0^{+/-}$ mice that do not exhibit IUGR until E16, our crowded embryos have already begun to be growth restricted by E14.5 (Suppl. Fig. 1A). It is possible that earlier on there may have been an adaptive increase in expression of *Slc38a4* to compensate for the reduced blood supply of the crowded IUGR placenta. Constancia et al. (2005) has documented that by E19 of *Igf2* $P0^{+é-}$, *Slc38a4* expression is not different. This does not completely explain the decrease in *Slc38a4*

expression but the discovery of differential expression of this gene does signify that it may be an important regulator of growth.

Cdkn1c (FC = 1.5) and *Phlda2* (FC = 1.4) display over-expression in the IUGR placentae. *CDKN1C* is involved in the overgrowth syndrome known as Beckwith-Wiedemann Syndrome (BWS) (OMIM 130650). The function of CDKN1C is to inhibit the cell cycle so that when it is down regulated abnormal overgrowth of different tissues will result (Chellappan et al., 1998; Zhang et al., 1997). Mouse mutants homozygous for null mutation of the gene exhibit growth restriction as well as some phenotypes characteristic of BWS (Zhang et al., 1997). However, other groups have not observed the same BWS-related phenotypes (Takahashi and Nakayama, 2000). Only a subset of BWS patients have maternally inherited mutations in *CDKN1C*, indicating the cause of BWS is multifactorial (Lam et al., 1999). Additionally several groups of researchers have found an association between *CDKN1C* and preeclampsia (Enquobahrie et al., 2008; Kanayama et al., 2002; Romanelli et al., 2009). Preeclampsia is a maternal hypertensive disorder that if present in the mother, the fetus has a higher probability of having IUGR.

PHLDA2 is the only imprinted gene that has consistently been found to be differentially expressed in human IUGR placentae (Apostolidou et al., 2007; Diplas et al., 2009; McMinn et al., 2006). The function of PHLDA2 has been recently suggested to regulate glycogen storage in the mouse placenta (Tunster et al., 2010). Glycogen-containing cells are important in late mouse gestation for the expansion of the labyrinthine into maternal decidua, an important event for maximization of nutrient transfer (Georgiades et al., 2002). Mouse KO of *Phlda2* have placentomegaly as well as a 13% decrease in fetal size (Frank et al., 2002). The over-expression observed in our IUGR placental samples also agreed with the pattern of these studies (Fig. 3.7 and Table 4.2.5). McMinn and colleagues (2006) have suggested under-perfusion in the placenta

leading to IUGR may induce activation of genes to restrict placental growth to compensate for the lack of blood.

Even though all of the data we have obtained regarding differential expression needs to be verified, our differentially expressed imprinted genes correlate well with their roles in IUGR. The candidate that is thought to play the largest role in IUGR, *Igf2*, did not appear altered in our data. The one imprinted gene that warrants further functional studies to identify its role in the placenta is *Sfmbt2* since its function is unknown. Also further analysis of *Phlda2* in human IUGR samples may prove to be fruitful as a good candidate for screening for IUGR since it has repeatedly been reported to be differentially expressed.

Though several of these imprinted genes are interesting candidates for IUGR, our main purpose was to evaluate the role of imprinted genes as a whole in IUGR. We hypothesized that imprinted genes would be over-represented in those genes that are differentially expressed in IUGR. We approached this question by conducting a chi-square test which compared the number of imprinted genes that were found to be differentially expressed versus the expected number of imprinted genes that should be differentially expressed based on the percentage of imprinted genes assayed on the Illumina array. Aside from the genes that were known to be imprinted, we included the imprinting candidates that came out of the recent research from Gregg et al. (2010). Based on the chi-square test we could not conclude that imprinted genes are over-represented (Table 4.3). However, there was a trend that suggested that imprinted genes may be more important in the placenta as the actual number of differentially expressed imprinted genes were more than expected. This agreed with the function of the placenta as a tissue that mediates embryonic growth, as well as the observation that many imprinted genes only demonstrate parent-of-origin expression in the placenta (Lewis et al., 2006; Mizuno et al., 2002).

Chapter 5:

General discussion

The topic of intrauterine growth restriction has been explored for decades due to its relationship with many perinatal diseases and mortality. The recent interest in exploring how epigenetics contributes to pregnancy complications has prompted many researchers to assess the extent of epigenetic aberration, which can lead to changes in gene expression in cases of IUGR (Bourque et al., 2010; Enquobahrie et al., 2008; Guo et al., 2008). Our work has looked at genome-wide expression, with particular emphasis on imprinted genes, and how it is affected in IUGR using the mouse as a model system. Besides studying differential expression in IUGR placenta, our work is the first to explore differential expression in the embryo during IUGR.

There are many advantages to studying gene expression in the murine system. The major cause of IUGR has been attributed to placental dysfunction. In particular, vascular problems in the placenta can lead to disruption in blood flow, subsequently affecting fetal-maternal nutrient and gas exchange (Cetin and Alvino, 2009; Cox and Marton, 2009; Jansson et al., 1993; Jansson et al., 2002; Roos et al., 2007). Compared to human IUGR studies where the samples have a variety of causes resulting in IUGR, we can know the cause of IUGR in the mouse. This thesis has outlined a surgical procedure where blood flow to some conceptuses in the uterus is restricted, resulting in IUGR. Another benefit with using the mouse system is that variation due to heterogeneity in the population can be avoided. Many lab strains of mice are inbred, which means their genetic composition will be almost identical, therefore minimizing variation due to differences in their genetic code. This is particularly useful in gene expression studies since smaller sample size can have the same power as human studies that require a large sample size just to be representative of the genetically heterogenous population. At least one study has indicated large variation in gene expression between placental samples from different individuals

(Pidoux et al., 2004). Human placental RNA samples are also subject to rapid RNA degradation due to indeterminate amount of time before RNA preservation procedures can be done postpartum (Avila et al., 2010). This can create false positives for differential expression in human IUGR studies. Studies involving human placentae also have sampling issues where they can only take a "core" of the placenta for RNA extraction, which may not be representative of the condition of the entire human placenta. In our study we extract RNA from the entire mouse placenta without any sampling bias. Another advantage of studying IUGR in the mouse is that we can explore how IUGR impacts embryonic gene expression, whereas human studies cannot due to ethical concerns.

Diagnoses of pregnancy complications have often divided cases into early-onset or late-onset. In general, the earlier the onset , the more severe the maternal and/or fetal phenotypes will be. Early obstruction in placental function often results in more severe fetal phenotype (Cox and Marton, 2009). The majority of these cases will also result in the appearance of additional phenotypes other than IUGR (Cox and Marton, 2009; Genbacev et al., 1996). Since the focus of our study is on IUGR specifically and not in conjunction with other malformations, we have focused on late-onset IUGR. The final growth phase of human fetal development occurs in the last trimester (>27 weeks), but late-onset IUGR is defined as after 32 weeks gestation. Mouse gestation differs from human gestation in that mouse gestation is divided into two terms, whilst in humans there are three terms. Even so the mouse system still has a comparable growth phase that begins around E14.5. Similarly mouse mutants that exhibit only IUGR, the phenotype is often not observed until E15.5 in mouse gestation (Constancia et al., 2002; Lefebvre et al., 1998).

Gene expression profiles in IUGR studies may provide insight to the factors involved in the regulation of placental function and embryonic growth. In particular, imprinted genes are

shown to be crucial to proper embryonic growth and development. Growth-related disorders such as Silver-Russell Syndrome (SRS) and Beckwith-Wiedemann Syndrome are linked to mutations or epigenetic aberration of several imprinted genes (Frost and Moore, 2010; Lim and Ferguson-Smith, 2010). Some of these same imprinted genes have also been identified to be differentially expressed in human IUGR studies (Abu-Amero et al., 1998; Antonazzo et al., 2008; Bourque et al., 2010; Guo et al., 2008). These observations suggest that some of these imprinted genes may work in concert to regulate development (Arima et al., 2005; Gabory et al., 2009; Varrault et al., 2006). Moreover, differentially-expressed genes implicated in IUGR can potentially be used in the diagnosis of IUGR, possibly through procedures similar to the Triple Screen Test (http://www.sogc.org/health/pregnancy-prenatal_e.asp#triple). Earlier monitoring and intervention in complicated pregnancies have been successful at alleviating disease symptoms and preventing mortality, which in turn reduce costs to our healthcare system in the long run.

The next section summarizes the findings of our study of IUGR in the mouse. Three proposed models of IUGR were explored in the hope of identifying genes that contribute to the etiology of IUGR, but only samples from the surgical model are used in the microarray screen to identify candidates that contribute to IUGR in the murine embryo and placenta. This study has identified non-imprinted IUGR candidate genes and discussed the relevance of their function to embryonic growth and development. Finally, we explored in depth the involvement of genomic imprinting to IUGR.

5.1 Summary of results

The grant this project was funded under hypothesizes that epigenetic abnormalities in the placenta contribute to pregnancy complications (preeclampsia and IUGR). The original

involvement of our lab in the grant was to study the function *in vivo* of the gene candidates pulled out from array-based screens. However, we decided to go straight ahead to study the relationship of IUGR and imprinting in the mouse model. The basis for my project stemmed from the idea of the imprinted gene network (IGN) first proposed in Arima et al. (2005). An actual network was drawn out through meta-analysis of co-expression of mouse genes using publicly available microarray data (Varrault et al., 2006). This proposed imprinted gene network included 15 imprinted genes: *Gnas, Dcn, H19, Igf2, Igf2r, Plagl1, Sgce, Cdkn1c, Mest, Ndn, Peg3, Gatm, Grb10, Meg3,* and *Dlk1*. Varrault et al. (2006) suggested these genes may function in a network to regulate embryonic growth and development. Subsequently we decided to explore imprinted gene expression in three mouse models of IUGR: one non-imprinted mouse knockout *Mmp2*, *Mest* mouse KO, and surgically-induced model. *Mmp2-/-* was suggested by the authors to exhibit IUGR, but no birthweight data was shown (Itoh et al., 1997). Therefore, I decided to characterize placental phenotype and weight differences in *Mmp2-/-* conceptuses. There was no significant difference in weight between *Mmp2+/+* and *Mmp2-/-* embryos and placenta, nor was there any obvious morphological difference in the null placenta. This suggested that we could not use *Mmp2* as a model for IUGR.

Since the *Mest* mouse knock-out was previously demonstrated to exhibit IUGR, we started looking at differential expression of some imprinting candidates in *Mest+/-* placental samples. However, we did not find any significant differences between WT and IUGR placental samples. Moreover we observed significant variation in gene expression between littermates. Variation in gene expression was expected, but not to the extent that we had observed. In accordance to theories suggesting the adaptive trait of the placenta, we proposed to that there could be more variation in gene expression in the placenta than in the embryo (Coan et al., 2008; Coan et al., 2010; Constancia et al., 2005). Illumina expression profiling (MouseRef8.0) enabled

us to assess expression variation in four biological replicates of WT inbred C57BL/6J embryos and placentae. Through various techniques that binned genes by expression level, we were able to demonstrate that gene expression variation was slightly higher in the placenta than in the embryo.

Even though the variance in expression was higher in the placenta, the embryonic and placental samples still formed independent clusters. The variance observed was also on the order of 10^{-2} by Illumina, which indicated that we could still look for candidates of IUGR by microarray. Therefore, we decided to induce IUGR in C57BL/6J females via hemiovariectomy. Weight measurements of this surgical model indicated that some of the embryos did exhibit lower weight than embryos from WT females, indicating the presence of IUGR. Subsequently, we conducted analysis of differential gene expression by Illumina using IUGR samples from the surgical model. Comparison between WT and IUGR cohort indicated that transport genes were the most affected in both the embryo and the placenta. This was expected because at this developmental stage, nutrient transport activity is essential for growth. There were also differences in the gene functions between the genes found to be differentially-expressed in IUGR embryo versus those found differentially-expressed in IUGR placenta. In the embryo, developmental and cellular differentiation genes were found to be differentially expressed, while more stress response genes were differentially expressed in the placenta. The involvement of these genes correlated well with the function of the embryo and the placenta. Development of organs through cellular differentiation occurred mainly in the embryo. Several studies have demonstrated that stress response contributes to pre-eclampsia and IUGR (Burton et al., 2009).

Four and six imprinted candidates of IUGR were identified in the embryo and the placenta, respectively. We could not compare the results from IUGR embryos with other human IUGR studies since there was no gene expression analyses on human fetus affected only by

IUGR. Nevertheless the genes that were found to be differentially expressed in the embryo (*H19, Igf2, Slc38a4,* and *Dlk1*) have all been demonstrated to be involved in embryonic growth (Antonazzo et al., 2008; Bliek et al., 2006; Bourque et al., 2010; Constancia et al., 2005; Moon et al., 2002) In the placenta, *Phlda2* emerged as a promising candidate that may be used as a diagnostic tool in the future as it was repeatedly reported to be over-expressed in IUGR placentae in human studies (Apostolidou et al., 2007; Diplas et al., 2009; McMinn et al., 2006). The polycomb group gene *Sfmbt2* was found to be under-expressed by 1.5-fold in IUGR placenta and had not been previously associated with IUGR. This gene may be an interesting candidate to study for growth-related phenotypes since its function has not been characterized. Verifications of the IUGR candidates still need to be completed via qRT-PCR. The IUGR sample size can be increased with three additional female IUGR samples. Hopefully with increased sample size, the natural variation between biological replicates will be smaller, which may allow us to detect even small changes between WT and IUGR samples. In contrast to our hypothesis that genomic imprinting is the most important group of genes involved in IUGR, we found that imprinted genes were not over-represented amongst those genes found to be differentially expressed in IUGR for both the embryo and the placenta.

5.2 Future directions

Immediate experiments can be done to evaluate the onset of IUGR and the extent of growth restriction in the surgical model used here. This may prove to be useful in separating cause from consequence. Assuming that we can pinpoint the onset of IUGR, we can conduct another microarray analysis to see which genes are differentially expressed between WT and IUGR samples. Then we can cross-reference to our list of candidate genes to see if there is any overlap. Genes that are found on both lists may likely be the cause of the IUGR phenotype in the

surgical model. Morphometric and blood flow assessment of the IUGR placentae in the surgical models can aid in understanding the physiological cause of IUGR. Moreover, assessing the genes we have already found to be differentially expressed at E15.5 just prior to birth (E18.5) may further identify those genes that are responding to IUGR. Constancia and colleagues (2005) have compared the amount of nutrient transfer between two embryonic stages (E15 and E19) in the placental KO of *Igf2*. They wanted to know what is contributing to the observed increase in placental efficiency, which they suggest is an adaptive response to meet the nutrient demands of the embryo in *Igf2* $P0^{+é-}$ (Constancia et al., 2005). The genes that we may identify to be responding to E18.5 may also be involved in adapting to adverse developmental conditions, though we will need to know the gene functions relatively well to make that supposition. We can also design experiments to assess the gene function of some of the candidates we have found. For example, *Sfmbt2* currently has not been mutated in mouse, thus we could knockout the gene and see if there are any placental and/or embryonic abnormalities.

We can also determine some of the general regulators of IUGR by assessing expression difference of our IUGR genes in other mouse models of IUGR. We can begin with looking at differential expression in the *Mest* knockout. To address the issue of expression variability due to heterogeneity of the littermates, we can increase the sample size or breed the KO mice back onto the inbred C57BL/6J background.

Lastly, we can explore the molecular mechanism that causes differential expression of imprinted genes in IUGR. As an example, we can determine if the observed increase in *Dlk1* expression is due to it being biallelically expressed or due to an increase in monoallelic expression. This can be examined by inducing IUGR using C57BL/6J x *Mus musculus castaneus* females and look to see if both alleles are present in polymorphisms within the gene.

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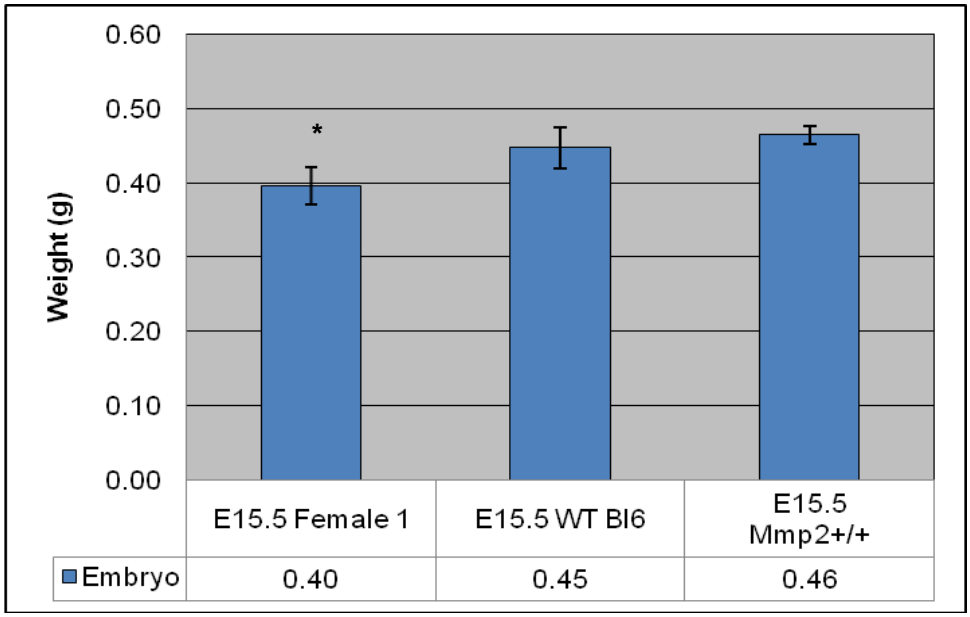
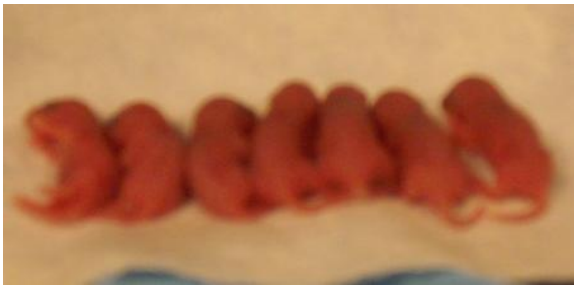
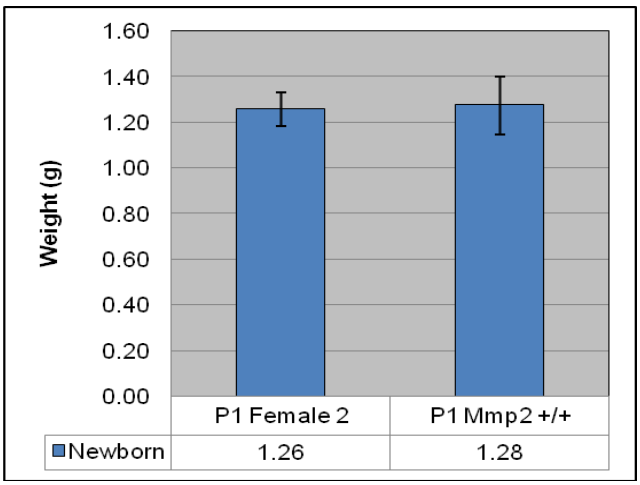
Appendix A: Supplementary information

A

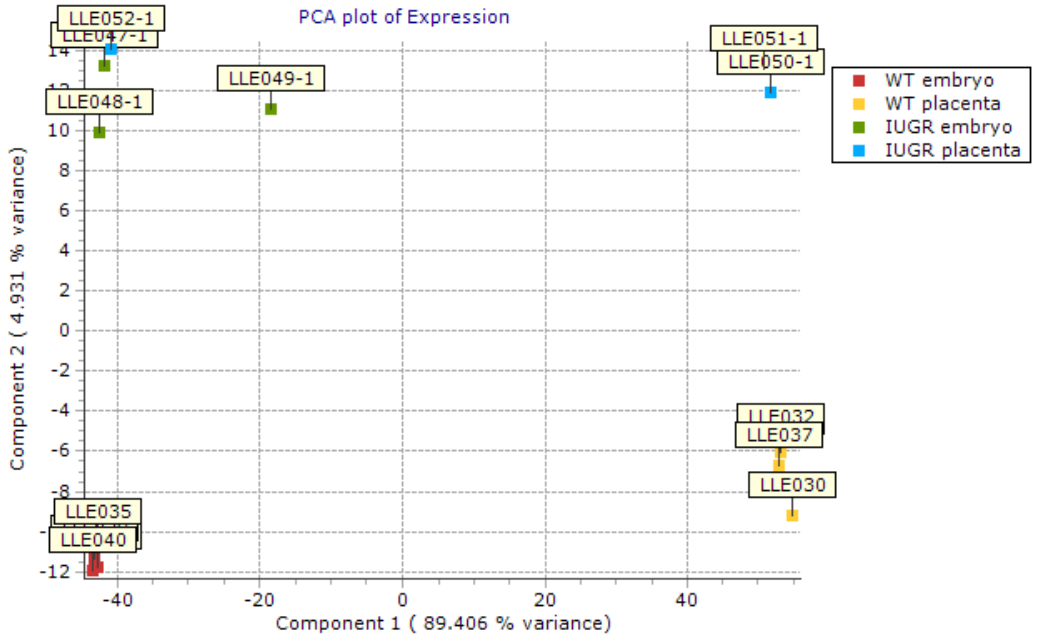


B

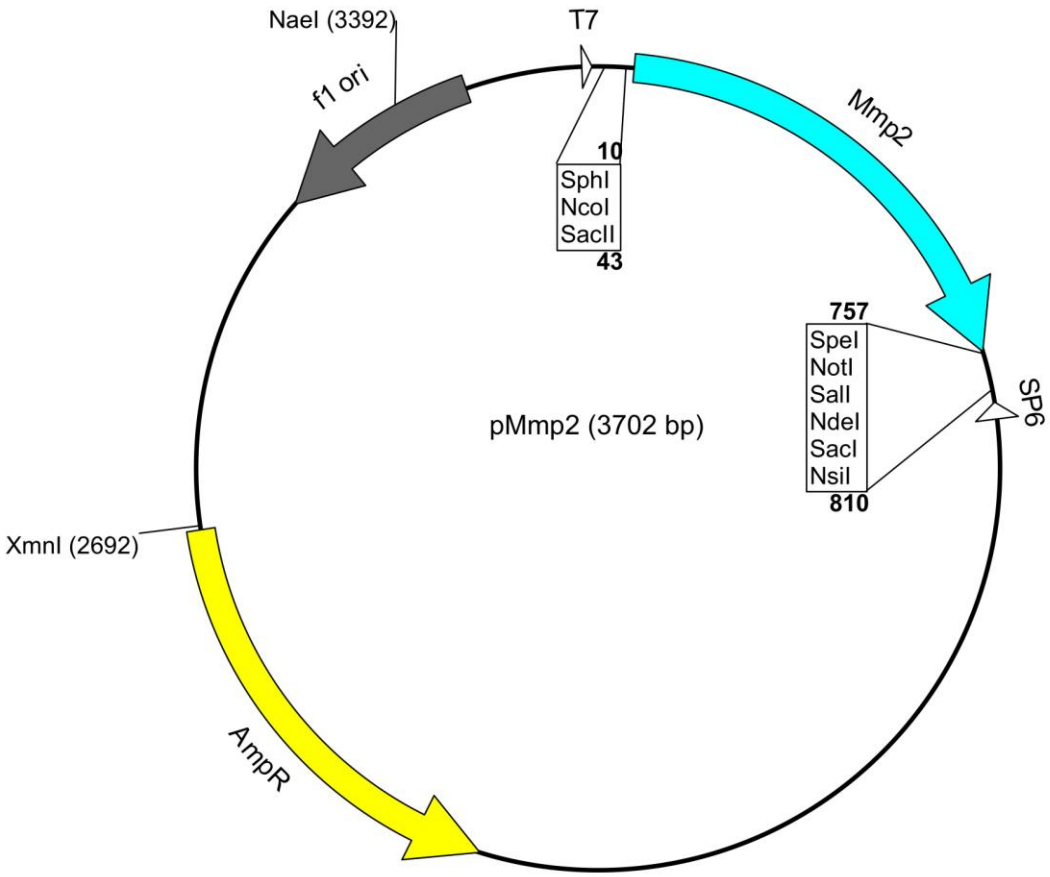




Suppl Fig. 1 Weight and size measurements of Surgical IUGR model. (A) Weight comparisons between embryos from E15.5 WT C57BL/6J, hemi-ovariectomized Female 1, and *Mmp2*+/+. The embryonic weight litter from Female 1 was assessed to be significantly different by *t* test ($p < 0.001$) from normal C57BL/6J litter of the same stage. Error bars represent standard deviation of weights of littermates. (B) Comparison of size and weight difference between littermates of P1 litter from hemi-ovariectomized Female 2 and *Mmp2*+/+.



Suppl Fig. 2 Grouping of WT and IUGR samples. Four independent clusters are illustrated using the principal component analysis (PCA) function on FlexArray. The embryo samples group significantly from the placental samples. The only exception is LLE052-1, which is IUGR placental sample that has grouped with the IUGR embryo samples. The IUGR samples also group different from WT samples.



Suppl Fig. 3 Plasmid map of pGEM-T vector with *Mmp2* ISH probe as insert. The map of the plasmid is generated using A Plasmid Editor software (ApE) made by M. W. Davis (http://biologylabs.utah.edu/jorgensen/wayned/ape/).

Suppl Table 1. Genes differentially expressed in embryo versus placenta at E15.5

(Empirical Bayes Wright & Simon adjusted with Bejamini-Hochberg false discovery rate = 0.05)
Only genes with 10-fold difference between the embryo and the placenta are listed.

Symbol	Fold change Placenta /Embryo	Adjusted p-value	Definition
Embryo-specific (FC < 0.1)			
Acta1	0.01299186	4.62E-08	actin, alpha 1, skeletal muscle.
Myl1	0.0188905	9.03E-08	myosin, light polypeptide 1.
MYLPF	0.01997901	2.73E-08	myosin light chain, phosphorylatable, fast skeletal muscle.
TNNC2	0.02548014	1.28E-07	troponin C2, fast.
ACTC1	0.02595083	9.29E-08	actin, alpha, cardiac muscle 1.
MYH8	0.02930698	4.56E-07	myosin, heavy polypeptide 8, skeletal muscle, perinatal.
TNNI2	0.03616861	3.02E-07	troponin I, skeletal, fast 2.
IGFBP5	0.04921925	2.40E-09	insulin-like growth factor binding protein 5.
AMBP	0.05152062	1.98E-07	alpha 1 microglobulin/bikunin.
TUBB2B	0.05169867	1.47E-08	tubulin, beta 2b.
TNNT1	0.05451234	5.17E-07	troponin T1, skeletal, slow.
SLN	0.05826783	2.45E-07	sarcolipin.
ACTN2	0.06011932	5.38E-08	actinin alpha 2.
LOR	0.06353214	1.04E-05	loricrin.
MYL2	0.06610431	1.74E-05	myosin, light polypeptide 2, regulatory, cardiac, slow.
PTN	0.0673354	1.85E-08	pleiotrophin.
GAP43	0.06804696	3.19E-09	growth associated protein 43.
SERPINF1	0.07127633	2.13E-07	serine (or cysteine) peptidase inhibitor, clade F, member 1.
D0H4S114	0.07186939	1.64E-08	DNA segment, human D4S114.
SBK	0.07198077	1.77E-08	SH3-binding kinase 1.
SERPINA1B	0.07279541	1.43E-05	serine (or cysteine) preptidase inhibitor, clade A, member 1b.
CAPN6	0.07284341	1.42E-08	calpain 6.
LOC100045403	0.07464686	5.38E-08	PREDICTED: similar to orthologue of H. sapiens chromosome 21 open reading frame 102 (C20orf102), misc RNA.
6330403K07RIK	0.07644952	1.42E-08	RIKEN cDNA 6330403K07 gene.
COL6A1	0.07763579	5.66E-08	procollagen, type VI, alpha 1.
COL1A1	0.08120836	3.16E-07	collagen, type I, alpha 1
COL1A2	0.08505238	1.13E-07	collagen, type I, alpha 2.
ATP2A1	0.08513667	4.41E-07	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1.
COX6A2	0.08569458	5.23E-07	cytochrome c oxidase, subunit VI a, polypeptide 2, nuclear gene encoding mitochondrial protein.
AHSG	0.08896152	6.79E-06	alpha-2-HS-glycoprotein.
COL5A1	0.08924741	9.63E-07	procollagen, type V, alpha 1.

Symbol	Fold change Placenta /Embryo	Adjusted p-value	Definition
Embryo-specific (FC < 0.1)continued			
STFA1	0.09011926	5.70E-05	stefin A1.
TNNT3	0.09072438	3.55E-07	troponin T3, skeletal, fast.
MYL3	0.09089174	1.26E-05	myosin, light polypeptide 3.
EMID2	0.09700852	1.47E-08	EMI domain containing 2.
STMN2	0.09783124	3.96E-08	stathmin-like 2.
HIST1H2AH	0.09786976	4.59E-06	histone cluster 1, H2ah.
HIST1H2AF	0.09847035	2.65E-06	histone cluster 1, H2af.
SLC4A1	0.09848848	1.09E-04	solute carrier family 4 (anion exchanger), member 1.
Placenta-specific (FC > 10)			
GNS	10.49764	1.80E-07	glucosamine (N-acetyl)-6-sulfatase.
LOC100046802	10.56654	1.73E-07	PREDICTED: similar to Inhbb protein.
D930020E02RIK	10.9562	4.26E-07	RIKEN cDNA D930020E02 gene.
SERPINB6B	11.11392	9.28E-07	serine (or cysteine) peptidase inhibitor, clade B, member 6b.
LOC100041103	11.169	2.73E-07	PREDICTED: hypothetical protein LOC100041103.
ADA	11.58439	3.10E-07	adenosine deaminase.
GPX3	12.13485	1.03E-05	glutathione peroxidase 3, transcript variant 2.
PCGF5	12.33669	7.83E-09	polycomb group ring finger 5.
FABP3	13.35281	5.33E-08	fatty acid binding protein 3, muscle and heart.
GKN2	14.43306	6.18E-06	gastrokine 2.
TCFAP2C	14.76052	7.61E-09	transcription factor AP-2, gamma.
KRT8	15.74381	1.03E-07	keratin 8.
SLCO2A1	16.80767	3.45E-08	solute carrier organic anion transporter family, member 2a1.
SERPINB9G	17.28993	1.68E-06	serine (or cysteine) peptidase inhibitor, clade B, member 9g.
GM2A	17.53276	6.70E-08	GM2 ganglioside activator protein.
SLC6A12	17.58351	5.70E-08	solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12.
GJB2	17.97248	1.27E-07	gap junction protein, beta 2.
TNFRSF9	18.27959	8.92E-08	tumor necrosis factor receptor superfamily, member 9, transcript variant 1.
CTSM	18.50106	9.18E-09	cathepsin M.
SLC13A4	20.04887	1.40E-06	solute carrier family 13 (sodium/sulfate symporters), member 4.
SLC38A4	20.11131	5.80E-08	solute carrier family 38, member 4.
LGALS3	21.73318	1.92E-07	lectin, galactose binding, soluble 3.
KRT18	22.2426	6.34E-08	keratin 18.
PRL4A1	22.9778	2.34E-06	prolactin family 4, subfamily a, member 1.
PLAC8	23.09103	3.78E-08	placenta-specific 8.
CAR4	23.13739	2.65E-07	carbonic anhydrase 4.
PSG16	23.19711	1.69E-07	pregnancy specific glycoprotein 16.

Symbol	Fold change Placenta /Embryo	Adjusted p-value	Definition
Placenta-specific (FC > 10) continued			
RHOX6	24.72437	3.55E-07	reproductive homeobox 6.
PRL2C5	25.37251	2.73E-08	prolactin family 2, subfamily c, member 5.
PRLPN	27.94913	7.03E-06	prolactin family 7, subfamily b, member 1.
PRL2A1	28.20164	3.55E-07	prolactin family 2, subfamily a, member 1.
TAF7L	30.31489	1.21E-11	TAF7-like RNA polymerase II, TATA box binding protein (TBP)-associated factor.
CEACAM14	31.02065	2.76E-08	CEA-related cell adhesion molecule 14.
TPBPB	31.63772	9.82E-08	trophoblast specific protein beta.
PRL8A2	32.65128	5.57E-06	prolactin family 8, subfamily a, member 2.
CTS3	38.49477	1.88E-07	cathepsin 3.
RARRES2	39.80894	2.93E-07	retinoic acid receptor responder (tazarotene induced) 2.
CTSR	43.80073	2.71E-08	cathepsin R.
RHOX5	44.52213	6.39E-10	reproductive homeobox 5.
PSG19	44.61459	1.31E-10	pregnancy specific glycoprotein 19.
OTTMUSG00000000651	46.145	1.88E-07	predicted gene, OTTMUSG00000000651.
PSG18	53.50965	5.77E-08	pregnancy specific glycoprotein 18.
PSG25	71.48119	2.36E-09	pregnancy-specific glycoprotein 25.
CEACAM12	73.25169	7.93E-09	CEA-related cell adhesion molecule 12.
SCT	74.85725	2.87E-08	secretin.
GHRH	94.85656	4.78E-08	growth hormone releasing hormone.
CTS6	100.2826	1.25E-10	cathepsin 6.
PSG26	109.1666	1.22E-08	pregnancy-specific glycoprotein 26.
PSG23	111.8458	1.32E-09	pregnancy-specific glycoprotein 23.
RHOX9	123.7153	2.40E-09	reproductive homeobox 9.
PRLPC3	129.8168	2.36E-09	prolactin-like protein C 3.
PRL2B1	149.9389	2.56E-09	prolactin family 2, subfamily b, member 1.
PSG27	153.0084	4.03E-12	pregnancy-specific glycoprotein 27.
PRL2C4	158.1028	1.12E-08	prolactin family 2, subfamily c, member 4.
LOC381852	164.0082	4.03E-12	similar to carcinoembryonic antigen-related cell adhesion molecule 3.
PRL3B1	176.3235	2.92E-08	prolactin family 3, subfamily b, member 1.
PRL2C3	187.1391	3.01E-08	prolactin family 2, subfamily c, member 3.
CTSQ	187.901	0	cathepsin Q.
CEACAM11	189.0166	2.13E-09	carcinoembryonic antigen-related cell adhesion molecule 11.
CTSJ	190.1107	3.58E-09	cathepsin J.

Suppl Table 2. Differentially expressed genes in E15.5 IUGR samples (EB Wright & Simon and cyber-T both adjusted with Benjamini-Hochberg false discovery rate = 0.05). Fold change ratio is presented as raw signal intensity of WT Bl6 samples over the raw signal intensity of IUGR samples.

Symbol	mChr	Fold change IUGR vs non-IUGR	GO Biological Process
Embryo (> 2-fold difference)			
BC030476	15	0.291509	Unknown
DEK	13	0.345345	(Interact with DNA)
OTTMUSG00000007855	4	0.346944	Unknown
PTPRE	7	0.351035	Dephosphorylation; signalling
ATP5A1	18	0.351811	Cellular biosynthesis
VPS26B	9	0.376267	Transport of proteins
COL1A2	6	0.376994	Signalling
GAP43	16	0.379969	Nervous system development
ATP5E	2	0.397288	ATP synthase; cellular metabolism
SETX	2	0.405491	DNA damage
LOC100044087	10	0.415231	Unknown
2210412D01RIK	7	0.415548	Unknown
NDUFB9	15	0.42548	ETC
DUSP7	9	0.443806	Dephosphorylation; Signalling
SDHD	9	0.44595	Transport of iron
CRYGA	1	0.456604	Development of the eye
PRF1	10	0.457993	Cell death
ATP6V1A	16	0.460898	ATP synthase; cellular metabolism
COL3A1	1	0.461502	Blood vessel formation; Digestive system development
GNB2L1	11	0.464736	Phosphorylation; Signalling
EIF2S3Y	Y	0.469798	Translation
NDUFB5	3	0.472128	ETC
1110002B05RIK	12	0.472291	Unknown
AFP	5	0.472334	Transport of copper
AI314180	4	0.478028	Unknown
THSD4	9	0.478174	Hydrolysis of carbon bonds
CCNG2	5	0.482467	Cell cycle
ZC3H15	2	0.483429	Signalling
TOMM70A	Unknown	0.48619	Signalling
ALDH6A1	12	0.487987	Cellular metabolism
LOC100048622	Unknown	0.490724	Unknown
9130005N14RIK	5	0.491016	Unknown
NDUFB2	6	0.498272	ETC
GLTP	5	2.003372	Transport of glycolipid
KRT2-1	Unknown	2.018775	Unknown
HIST1H2BF	13	2.018933	DNA packaging

Symbol	mChr	Fold change IUGR vs non-IUGR	GO Biological Process
Embryo (> 2-fold difference) continued			
EG433923	5	2.043626	Unknown
LOC100046918	Unknown	2.120295	Unknown
PTMS	6	2.161152	Immune reponse
HIST1H2BH	13	2.170868	DNA packaging
TACSTD2	6	2.274034	Unknown
DMKN	7	3.163002	Cell Differentiation
Placenta (> 2-fold difference)			
PRL8A9	13	0.307782	Signaling (hormone)
ALDH1A3	7	0.313594	Anatomical Structure Development
SLC38A4	15	0.325339	Transport of amino acids
1600029D21RIK	9	0.400365	Unknown
HSPA5	2	0.404304	ER stress response
SERPINB6B	13	0.425737	Unknown
CEACAM13	7	0.437663	Unknown
PRL3C1	13	0.449103	Signalling (hormone)
PTPRA	2	0.4509	Dephosphorylation; signalling
GKN1	6	0.464278	Cell proliferation
MGAT4A	1	0.465189	Carbonhydrate synthesis
SRP14	2	0.48053	Repress translation
DDX6	9	0.496593	DNA unwinding
NID2	14	2.054392	Cell adhesion
GPX3	9	2.190024	Cellular metabolism
DLK1	12	2.841104	Embryonic skeletal development
> 2-fold difference in both			
Target ID	mChr	Fold change in IUGR embryo	Fold change in IUGR placenta	GO Biological Process
VAPA	17	0.401791	0.418898	(Structural molecule)
COL1A1	11	2.089283	2.154194	Skeletal development
VHL	6	0.465995	0.289318	Angiogenesis; blood endothelial migration; hypoxic response
PTGES3	10	0.315851	0.38242	Cell proliferation; Fatty acid synthesis
ACADSB	7	0.326914	0.38917	Fatty acid β-oxidation
IDH2	7	0.460251	0.472428	TCA Cycle
STMN1	4	0.169451	0.495621	Cellular component organization;
HUWE1	X	0.278906	0.400182	Cellular metabolic process
H1F0	15	0.301818	0.463918	DNA packaging
GKAP1	13	0.400632	0.38971	Signalling
KLF6	13	0.407415	0.349256	Signalling
BACH1	16	0.411779	0.347331	Regulate transcription

Symbol	mChr	Fold change IUGR vs non-IUGR		GO Biological Process
> 2-fold difference in both continued				
TRRAP	5	0.49338	0.441657	Chromatin modification
POLR2G	19	0.402921	0.422382	Transcription
PAIP2	18	0.421414	0.485043	Repress translation
CALR	8	2.022648	2.568425	Actin organization; repress translation; regulate meiosis
POM121	5	0.357	0.47367	Transport of protein
KPNA3	14	0.320142	0.392815	Transport of protein
KCTD3	1	0.43465	0.418345	Transport of potassium ion
NPC2	12	0.430276	0.374513	Transport of cholesterol
RP23-297J14.5	11	0.461336	0.370887	Unknown
LOC100041703	2	0.367259	0.406665	Unknown
LOC668837	14	0.205998	0.336597	Unknown
ZBED4	15	0.411543	0.431737	Unknown
DDX3X	X	0.299251	0.327402	Unknown
LOC100044779	Unknown	0.33	0.341703	Unknown