IMPRINTED GENES IN THE PLACENTA AND OBSTETRICAL COMPLICATIONS

by

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ABSTRACT

Each year, many pregnancies are associated with obstetrical complications such as maternal pre-eclampsia (PET) and fetal intrauterine growth restriction (IUGR). Poor placentation is thought to contribute to these complications, but specific causes are largely unknown. Mouse models suggest that epigenetic mechanisms, in particular genomic imprinting, that alter gene regulation may help regulate placental development and embryonic growth. The first goal of this thesis is to examine if epigenetic modifications (i.e. DNA methylation) and altered expression of imprinted genes in the human placenta are contributing factors to PET and IUGR. The second goal of this thesis is to identify imprinted loci that are useful in the diagnosis of placental pathologies that associated with abnormal imprinting, including triploidy, hydatidiform moles, and placental mesenchymal dysplasia.

I found that DNA methylation at the imprinting control region 1 (ICR1) on chromosome 11p15.5 was significantly decreased in IUGR placentas ($p < 0.001$), but not in those associated with pre-eclampsia. Methylation at ICR2 (KvDMR1) was not significantly altered in PET or IUGR. No significant changes in expression levels were observed in the genes controlled by these ICRs. There were no significant methylation changes observed in any candidate imprinted gene evaluated by the Illumina array. LINE-1 methylation, a marker of whole genome methylation, was also similar in all groups. The establishment of biomarkers that could be used to accurately identify those women at an increased risk for pre-eclampsia or IUGR would be a major step forward in antenatal care.

All placental pathologies (triploidy, hydatidiform moles or placental mesenchymal dysplasia) were associated with altered ICR2 (KvDMR1) methylation. Pyrosequencing assays for *SGCE*, *SNRPN*, and *MEST* were also compared for their utility in diagnosing parental genomic imbalance in placental samples. *SGCE* showed the clearest separation between groups. The combined use of KvDMR1 and *SGCE* assays could provide a potentially valuable diagnostic tool in the rapid screening of methylation errors in placental disorders. These results also demonstrate the maintenance of imprinting status at these loci in the human placenta, even in the presence of abnormal pathology.

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AFP	alpha fetoprotein
ART	assisted reproductive technologies
ATP	adenosine triphosphate
β-hCG	beta human chorionic gonadotropin
BP	blood pressure
BW	birth weight
BWS	Beckwith-Wiedemann syndrome
CHM	complete hydatidiform mole
CpG	cytosine-phosphate-guanine dinucleotide
DMR	differentially methylated region
DNA	deoxyribonucleic acid
EVT	extravillous trophoblast
GA	gestational age
HELLP	hemolytic anemia, elevated liver enzymes, low platelet count
ICR	imprinting control region
ICSI	intracytoplasmic sperm injection
IUGR	intrauterine growth restriction
Ms-SNuPE	Methylation-sensitive Single Nucleotide Primer Extension
PCR	polymerase chain reaction
PET	pre-eclampsia/pre-eclamptic toxaemia
PHM	partial hydatidiform mole
PMD	placental mesenchymal dysplasia
PPi	pyrophosphate
RNA	ribonucleic acid
SAM	Significance Analysis of Microarray
SGA	small for gestational age
STBM	syncytial trophoblast microvillous membranes
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
TNDM	Transient Neonatal Diabetes Mellitus
uE3	unconjugated estriol
UPD	uniparental disomy

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CO-AUTHORSHIP STATEMENT

I conducted the literature review and wrote Chapter 1 in its entirety. Dr. Wendy Robinson provided input and edited the chapter.

Manuscript 1. Placentas were recruited by the Robinson lab clinical research coordinator from either the EMMA Clinic (run by Dr. Peter von Dadelszen) or from hospital postings. Luana Avila and Ruby Jiang sampled the placentas and extracted DNA and RNA. I ran all the methylation and expression experiments and analyzed the data. Luana also assisted with the analysis of the microarray data. Dr. Maria Penaherrera prepared the DNA for microarray analysis in collaboration with the lab of Dr. Michael Kobor. She also provided technical assistance and assisted with data analysis. I wrote this manuscript. Dr. Wendy Robinson provided guidance, aided with experimental design and manuscript editing.

Manuscript 2. Beckwith-Wiedemann syndrome samples were obtained from Dr. Rosanna Weksberg in Toronto or were referred by Dr. Margot van Allen and other clinicians at the BCCW Medical Genetics Clinic. Dr. Deborah McFadden provided placental samples from triploids, CHMs and some PMDs. DNA was extracted by Ruby Jiang and other lab personnel. I designed all the assays outlined in Manuscript 2 and I tested the controls with the various assays and the BWS cases and abnormal placentas with the KvDMR1 assay. Ryan Yuen helped with pyrosequencing assay design. Dr. Maria Penaherrera and Ruby Jiang also ran some pyrosequencing assays on the abnormal placentas. I wrote the original draft of the manuscript. Dr. Wendy Robinson aided with experimental design and manuscript revisions.

I wrote Chapter 4 in its entirety.

CHAPTER 1. INTRODUCTION

1.1. Overview

Each year in British Columbia, many pregnancies are associated with obstetrical complications and poor outcomes. Two of these complications are pre-eclampsia (PET), which affects approximately 5% of pregnancies, and low birth weight or intrauterine growth restriction (IUGR), which also affects approximately 5% of pregnancies [1]. Pre-eclampsia is a form of pregnancy-induced hypertension. While there is no universally accepted definition of IUGR, it can be described as a failure of a fetus to reach its growth potential [2].

Pre-eclampsia is the number one cause of maternal death and it can be detrimental, and even fatal, for the baby. For example, babies born to mothers with pre-eclampsia are more likely to be premature and have low birth weight compared to babies from uncomplicated pregnancies. These babies are also more likely to have high blood pressure and body mass [3] and be at an increased risk for atherosclerosis and insulin-resistance syndrome in later life compared to adults from unaffected pregnancies [4]. In the mother, complications from pre-eclampsia include pulmonary edema and renal failure [5]. Pre-eclampsia has also been identified as a predictor of future cardiovascular disease in affected women [6].

The presence of IUGR often correlates with increased perinatal morbidity and mortality, as well with long-term consequences in post-natal health [7]. Multiple complications are known to be associated with IUGR, including oxygen deprivation, ischemic encephalopathy, polycythemia, hypoglycemia, and other metabolic abnormalities

[8]. Adults affected by IUGR as a baby may continue to show long-term sequelae such as development of coronary heart disease, insulin resistance, and hypertension [7]. Other long-term problems include decreased IQ and learning difficulties [9], seizures, and attention problems [10]. Both PET and IUGR may result in serious long-term health problems and are a significant burden on the health care system [11].

Poor placentation, a deficiency in migration and differentiation of cells at the maternal-fetal interface, is thought to contribute to obstetrical complications [12], but specific causes are largely unknown. The placenta is a complex organ that is composed of many cell types that have distinct functions [13]. The variety of cell types that compose the placenta, each with different gene expression patterns and changing distribution throughout pregnancy, makes it difficult to diagnose specific causes of placental failure.

Although the underlying causes of placental insufficiency are still unknown, mouse models have suggested that epigenetic changes, both globally and at the level of specific genes, that alter gene regulation may be involved. In particular, genomic imprinting in the placenta may have a key function in the regulation of placental development and embryonic growth [14,15]. Imprinted genes show parent-of-origin dependent expression and are disproportionately expressed in the placenta. Methylation of the DNA is a main mechanism of establishing and maintaining genomic imprinting during development.

Disruption of imprinted genes in the mouse often results in abnormal placental development and fetal growth [16]. Imprinted gene expression, as well as DNA methylation, appears to be variable in the early mouse embryo and in the placenta and may help the placenta respond to environmental stimuli; however, it is also possible that epigenetic errors can arise spontaneously in early development and contribute to placental insufficiency and abnormal fetal development. Preimplantation culture of mouse embryos can lead to loss of

placental imprinting at multiple genes and is affected by the culture media used [17,18]. If epigenetic errors confined to the placenta may result in placental insufficiency, they may also lead to an increased risk of pre-eclampsia and IUGR [19,20].

There is remarkable variability in placental structure among different mammals [21,22] and thus animal models are limited in their direct application to the human situation. So, despite the information obtained from mouse models, very little is currently known about the role of imprinting in the regulation of normal and pathological human placental development.

The goal of this thesis was to investigate if the disruption of normal patterns of DNA methylation associated with imprinted gene expression in early development is a significant cause of human placental insufficiency leading to maternal pre-eclampsia and fetal IUGR. The first objective of this project was to characterize methylation and imprinted gene expression in term placental whole villous cells. The second part of the project was to identify abnormal patterns of methylation and imprinting errors in chorionic villi from placentas that are from pregnancies associated with PET and/or IUGR and to identify if these changes are restricted to the chorionic villi. The final objective of this project was to design new methylation assays that may aid in the diagnosis of various placental pathologies.

1.2. The placenta

1.2.1. Placental structure and function

The placenta is a multifunctional organ that serves as a lifeline for the developing fetus. Although the placenta is situated outside of the fetal body and has a limited lifespan, it is of critical importance to the fetus in marsupials and placental mammals. It provides contact to the mother and can act as: lungs, kidneys, gut, endocrine system, liver, bone marrow, and immune system [23].

In humans, implantation of the embryo occurs approximately six days after conception [24]. During this time, there is a decidualization of the uterine endothelium. The decidua is involved in establishment and maintenance of pregnancy and will also have paracrine, nutritional, and immunoregulatory roles during pregnancy. Ultimately, normal implantation and placentation is a balance between regulatory gradients created by both the trophoblasts and endometrium. Shallow implantation has been reported in pre-eclampsia and IUGR [25] while excessive invasion is associated with trophoblast disease and placenta acreta.

The early blastocyst must develop a mechanism to invade the uterine endothelium to anchor the embryo and to establish a connection for nutrient exchange with the mother. Early placentation is a highly regulated process as foreign placental cells must be allowed to invade the uterus deeply enough for secure implantation but not so deeply as to cause harm to the mother's body [24]. A subpopulation of cells, trophoblasts, is involved in implantation into the endothelium. These cells are extremely invasive and must be tightly controlled. The

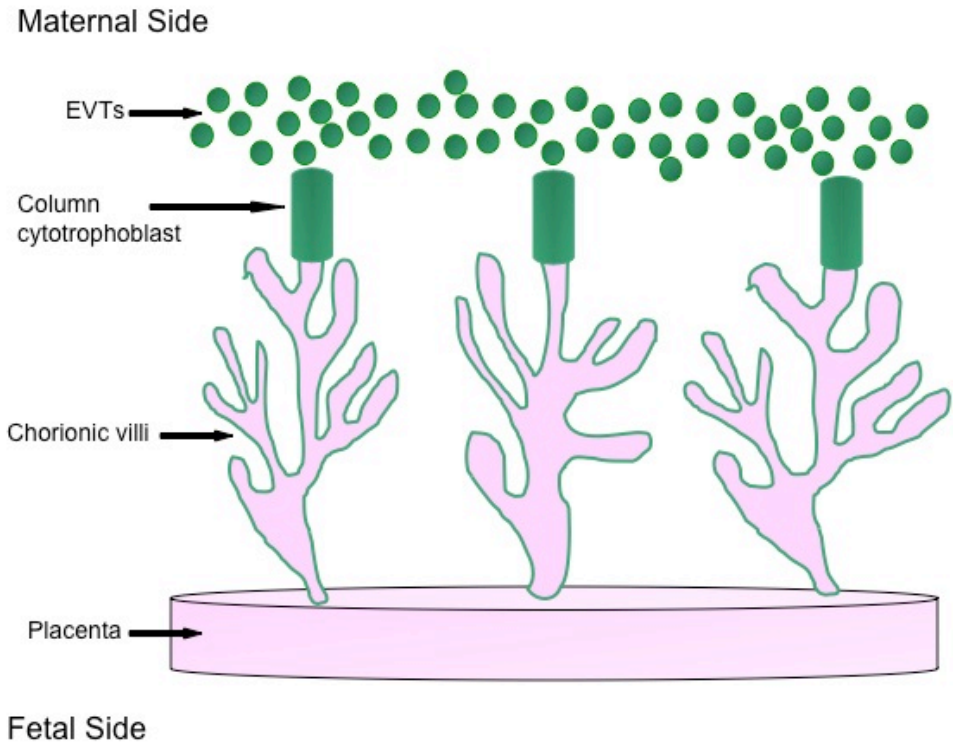
endometrium must control the trophoblast invasion by secreting locally acting factors (cytokines and protease inhibitors).

Trophoblast cells are found in the outer layer of the chorionic villi (the inner layer is a mesenchymal core composed of villous stroma and fetal blood vessels) [26]. Cytotrophoblasts are the undifferentiated, mitotically active, mononucleate type of trophoblasts. They are progenitor cells that give rise to three differentiated forms of trophoblasts in response to various hormonal stimuli.

The first of these are the villous syncytiotrophoblasts – a layer of differentiated, multinucleate cells that arise from the post-mitotic fusion of cytotrophoblasts [27]. (Figure 1.1) These cells comprise the outer layer of the chorionic villi. The syncytiotrophoblasts are involved in the initial stages of blastocyst invasion and placentation, maternal-fetal exchange of nutrients and wastes, and secretion of placental hormones (e.g. human chorionic gonadotropin). During trophoblast development, cavities (lacunar spaces) begin to appear in between the syncytiotrophoblasts and will eventually form the spaces into which maternal blood flows [24].

The second type of trophoblasts includes the column or anchoring trophoblasts [24]. They are found in columns at the junction between the chorionic villi and the maternal endothelium. These cells produce a compound (trophouteronectin) that helps the placenta attach to the uterus.

a)



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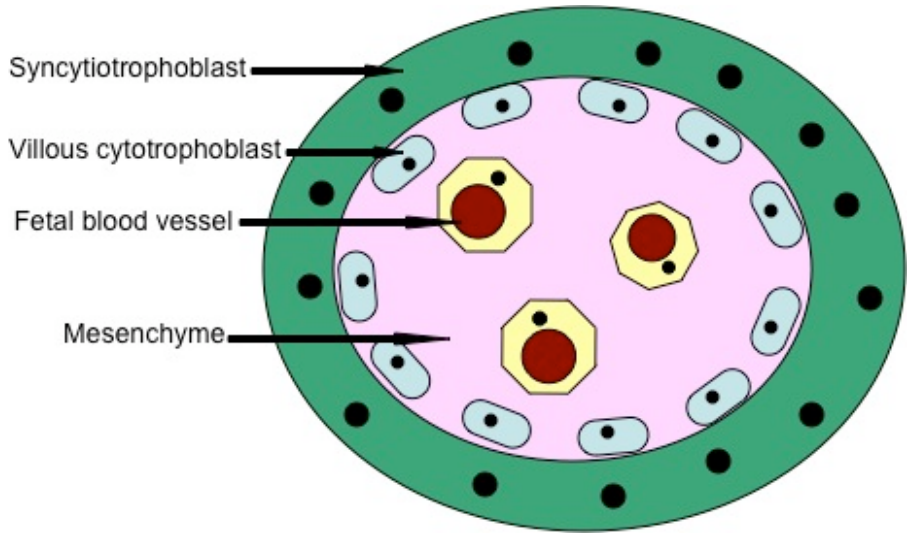

Figure 1.1. Structure of the human placenta. a) Relative locations of the various forms of trophoblasts during placental development (not to scale; EVTs are extra-villous trophoblasts); b) A cross-section through a chorionic villus.

The third and final type of trophoblasts is the intermediate invasive trophoblasts. These cells will invade deeply into the maternal decidua, forming columns and differentiate into extra-villous trophoblasts (EVTs). The EVTs are important in remodeling the maternal spiral arteries to help the placenta acquire a blood supply sufficient to sustain a developing fetus [28].

In areas of low oxygen (e.g. near the uterine surface) cytotrophoblasts continue to proliferate, but in areas of high oxygen (e.g. near the uterine spiral arteries) cytotrophoblasts differentiate. These low oxygen conditions stimulate the placenta to grow more quickly than the embryo it is supporting. Once the placenta has obtained an adequate blood supply, the growth of the placenta slows; as the cytotrophoblasts begin to differentiate, embryonic growth accelerates [29]. Proliferation in response to hypoxic conditions is somewhat unique to the trophoblasts as the majority of cell types found in the body tend to decrease cellular activity in response to hypoxia [30]. While the cause of the trophoblast response to hypoxia is still unknown, it has been suggested that the response may be mediated by epigenetic factors as DNA methylation and histone modifications in the mouse placenta appear to be different from that in the embryo [19].

By four weeks post-conception, the basic structure of the mature placenta has been achieved. The placenta now includes a fetal circulation that terminates in the capillary loops of the villi. By this point, the fetal circulation interacts with the lacunar spaces, which in turn are supplied by the maternal arteries and veins. The villi closest to the maternal blood supply will become the placenta proper, while the villi farthest from the maternal blood supply will develop into the chorion [24,31]. During this time, the amnion develops to surround and protect the developing embryo within the fluid-filled amniotic sac.

Between the fetus and the placenta, there are two fetal membranes – the amnion and the chorion [32]. The closest membrane to the fetus is the amnion, which encloses the amniotic sac and amniotic cavity. Just outside the amnion is the chorion, which developed from the chorionic villi farthest from the maternal blood supply. Moving closer to the maternal side, the next layer is the chorionic villi that developed close to the maternal blood supply [26]. These villi are composed of mesenchymal cells and trophoblast cells. Within the villi there are lacunar spaces to allow maternal blood to enter the placenta and to allow nutrient transfer through the placenta to the fetus. The nutrients are absorbed into the fetal blood in the placental vessels and eventually the nutrients make it to the fetus via the umbilical cord. The final layer of the placenta is the maternal contribution, also known as the decidua. Fetal invasive trophoblast cells (EVTs) penetrate into the decidua and help the placenta attach to the uterus.

1.2.2. Gene expression in placental development

Placental development is a series of highly regulated, coordinated processes that are under the control of multiple placentally expressed genes. Individual genes and genome wide transcriptional programs of the placenta have been studied in both mice [33] and humans [34]. While mice are an informative model system, the murine placenta is structurally different from the human placenta and as such, gene expression patterns may not be identical to those in the human placenta. Despite this, mice have helped us understand the processes involved in placental development and have allowed identification of genes that merit further studies in humans [35].

From the limited number of studies that have been performed to date, it appears that all mouse placental cell types and genes have human counterparts. The knowledge of which genes are expressed in different placental cell types and the expression patterns of these genes in the mouse has allowed the identification of orthologous cells in humans [36]. If the human orthologues to all mouse placental genes can be identified, the mouse will serve as an even more informative model organism for human placental dysfunctions. Understanding the genetic contribution to mouse placental failure will help direct future studies in human placental dysfunction and its outcomes – particularly pre-eclampsia and intrauterine growth restriction [36].

1.3. Pre-eclampsia (PET)

Pre-eclampsia is a form of pregnancy-induced hypertension; it is the number one cause of maternal death and it can be detrimental, and even fatal, for the baby. Pre-eclampsia is defined as hypertension above 140/90 mm Hg and proteinuria above 0.3g/24 hours [37]. Symptoms appear after 20 weeks gestation and subside after delivery of the baby. It has been suggested that there are two forms of pre-eclampsia: early onset (<34 weeks) and late onset (>34 weeks). Early onset pre-eclampsia is thought to be a more severe form of the disease and is associated with abnormal uterine artery Doppler (increased resistance), HELLP syndrome, intrauterine growth restriction and prematurity [38]. Late onset pre-eclampsia is associated with better neonatal outcomes, in part due to onset being nearer to term. As pre-eclampsia is a heterogeneous disorder, it is thought that the two forms of pre-eclampsia may have different etiologies [37]. However, in both forms the underlying cause of pre-eclampsia appears to be reduced placental perfusion caused by abnormal

placentation and trophoblast invasion [39]. The reduced perfusion can cause the fetus to outstrip the placental and maternal supplies [40]. Delivery of the fetus, and consequently the placenta, is sufficient to resolve the disease. It has been suggested that maternal (e.g. an inflammatory response to the poorly functioning placenta [41]) and genetic factors (e.g. confined placental trisomy 16 [42]) may also play a role in the development of pre-eclampsia.

There are two syndromes involved in pre-eclampsia: maternal and fetal. The hallmark feature of the maternal syndrome is hypertension; however, many organ systems may be affected (e.g. respiratory, hepatic) [41]. The fetal syndrome of pre-eclampsia may manifest as IUGR. Even though normotensive IUGR may appear on its own in the absence of pre-eclampsia, the two complications are often considered to be variations in the manifestation of the same underlying placental pathophysiology [43].

1.4. Intrauterine growth restriction (IUGR)

Intrauterine growth restriction is the failure of a fetus to reach its growth potential [2]. The presence of IUGR often correlates with increased perinatal morbidity and mortality, as well with long-term consequences in post-natal health [7].

There is no universally accepted definition of intrauterine growth restriction (IUGR). Some groups [44] use the term small for gestational age (SGA) which is based on a strict cut off for weight or length, such as less than the 5^{th} or 10^{th} percentile. Using a criterion like this may exclude babies who were destined to be large, but failed to meet their growth potential and may include babies that are constitutionally small, but otherwise healthy. Other groups have also used additional measures when defining IUGR, for example a longitudinal

decrease in abdominal circumference [45] or alterations in fetal and placental metabolism and transport [46] in addition to birth weight. In this work, IUGR was defined using a definition which required the presence of either of two pediatric criteria: 1) less than the 3rd percentile for gestational age corrected length and weight; 2) less than the 10th percentile for gestational age corrected length and weight and the presence of at least one obstetrical factor (measured by ultrasound): (a) persistent uterine artery notching at 22+0-24+6 weeks gestation, (b) absent or reversed end diastolic velocity on umbilical artery Doppler, and/or (c) oligohydramnios (amniotic fluid index <50mm).

Multiple non-placental causes (e.g. fetal infection [2], maternal smoking [47]) for IUGR have been suggested, but abnormal trophoblast invasion, decreased maternal spiral artery remodeling, and consequent reduction in blood flow between the mother and placenta appear to be involved in many cases [39]. Trisomy mosaicism, the presence of cells with one extra chromosome in the placenta, has been reported at an increased frequency in placentas associated with IUGR compared with placentas from babies with normal birth weight, further supporting the hypothesis that problems with the placenta may lead to IUGR, at least in some cases [48].

1.5. The placenta in pre-eclampsia and intrauterine growth restriction

Abnormal placental pathology is common in PET and IUGR and the placenta itself may be in part responsible for the development of pre-eclampsia and IUGR [49]. Shallow implantation and poor remodeling of the maternal spiral arteries can lead to a reduction in blood flow from the maternal circulation to the lacunar spaces of the placenta and

consequently a reduction in nutrient transfer to the fetus, possibly leading to pre-eclampsia in the mother and poor growth of the fetus [50].

There are two main theories for the development of pre-eclampsia. The first theory proposes that poor trophoblast invasion and inadequate remodeling of the maternal spiral arteries during the first trimester of pregnancy can lead to pre-eclampsia. Poor invasion can lead to a hypoxic placenta that suffers oxidative stress and eventually to the endothelial vascular (maternal) syndrome of pre-eclampsia [5]. With poor invasion it is also possible that the fetus may not receive adequate nutrition and oxygen from the mother and will suffer from growth restriction. Secondly, the development of pre-eclampsia in the mother may result from an inflammatory reaction to the fetus and placenta. Shedding of trophoblast debris into the maternal circulation is increased in pre-eclampsia and may cause an inflammatory reaction [4]. A third model combines the above theories and proposes that a hypoxic and dysfunctional placenta may release factors to which the mother has an immune response (e.g. vascular endothelial growth factor-1, *VEGF-1*) [12]. It has also been suggested that the inflammatory response differentiates pre-eclampsia from isolated fetal growth restriction [41].

Often the cause of IUGR is not known, but in some cases of isolated IUGR where decreased maternal blood flow to the fetus has been suggested to be the underlying cause, vascular changes including impediment of normal blood flow between villi (e.g. maternal floor infarction) and chronic inflammation of the villi (e.g. villitis) may be involved [49]. Defects in placental transport due to underlying vascular problems can lead to nutritional deficiencies in the fetus which may in turn lead to IUGR [51].

1.6. Imprinting

The study of epigenetics in the placenta is a rapidly advancing field. Epigenetics is the study of heritable, but reversible, changes to DNA that do not affect the sequence of the DNA directly but may alter gene expression. These changes include DNA methylation and histone methylation or acetylation. One particularly interesting type of epigenetic process is genomic imprinting that marks the parental genomes as non-equivalent and allows genes to be expressed in a parent-of-origin specific manner.

1.6.1. Early evidence that parental genomes are not equivalent

In the 1980s, scientists noticed that uniparental mouse embryos failed to develop to term [52,53]. Gynogenetic embryos were created from two maternal contributions by removing the paternal pronucleus from a fertilized egg and transferring a female pronucleus from a second egg in to the manipulated egg. The embryos were then implanted in to a pseudopregnant mouse [53]. The gynogenetic embryos suffered from severe growth restriction and failed to develop extra-embryonic tissues. In contrast, androgenetic embryos are created from two paternal contributions by extracting the female pronucleus from a fertilized egg and replacing it with a second male pronucleus, and implanting the resultant embryo in a pseudopregnant mouse [52]. The androgenetic embryos arrested at an extremely early phase in development but had overgrown extra-embryonic tissues (the murine equivalent of a hydatidiform mole in humans). From these early studies, it is clear that both male and female contributions are required for normal development.

Early studies using mice presenting with uniparental disomy of individual chromosomes confirmed that maternally and paternally derived chromosomes can produce different phenotypes in the resultant offspring [54]. The two most common outcomes of these uniparental disomies were alterations in growth (both enhanced and repressed) and abnormal behaviour. However, not all chromosomes produce an abnormal phenotype when present as a uniparental disomy, which indicates that specific regions or genes are involved in this phenomenon. These findings are consistent with the idea that DNA is modified differentially during male and female gametogenesis. If the male and female gametes were functionally non-equivalent, there would be a need for both parental genomes in normal development.

Additionally, abnormal placental pathology can result from genomic imbalances, highlighting the importance of having both parental genomes in normal development. Of particular interest are placental mesenchymal dysplasia (PMD), complete hydatidiform moles (CHM), and partial hydatidiform moles (PHM). Placental mesenchymal dysplasia manifests as enlarged, cystic villi and is caused by the presence of androgenetic cells in the placenta in addition to biparental cells [55]. The androgenetic cells arise after a failure of the maternal genome to undergo DNA replication during the first post-fertilization cell division, forming 2 daughter cells – one diploid biparental cell and one haploid androgenetic cell [56]. The androgenetic daughter cell then undergoes endoreduplication to become diploid. The severity of the PMD is dependent on the degree of androgenetic cells. A wide degree of variation in the percentage of androgenetic cells can be seen between different cases of PMD. Complete hydatidiform moles usually arise by the fertilization of an empty ovum with one or more sperm [57]. When one sperm fertilizes an empty ovum, it must undergo endoreduplication to have the required 46 chromosomes. PHMs are caused by androgenetic

triploidy (two paternal contributions but only one maternal contribution). This can occur following fertilization of one haploid egg with one (and endoreduplication) or two sperm [58]. Digynic triploids do not manifest as PHMs, but as a growth-restricted fetus with a very small placenta.

1.6.2. The role of DNA methylation in genomic imprinting

DNA methylation has been suggested to be one of the mechanisms that mark the maternal and paternal genomes as functionally different [59]. Methylation occurs primarily on the cytosine residue of CpG dinucleotides. When present at the promoter of certain genes, methylation can be associated with transcriptional silencing of that gene; however, many other factors (e.g. histones, transcription factors) may also play a role. Methylation is heritable and reversible, which means that it can be stably passed on from cell to cell, but can be erased when needed (i.e. before establishing the correct pattern of imprints during gametogenesis). In the case of imprinted genes, only the maternal or paternal copy of the gene is expressed and the other copy is silenced by DNA methylation (often in the promoter region).

Shortly after fertilization, there is a global demethylation of the genome and methylation must be reestablished very early in embryonic development [60]. DNA methylation marks, including those of imprinted genes, are established during gametogenesis by enzymes known as de novo DNA methyltransferases – particularly DNMT3a. Deletion of these enzymes in mice results in an embryonic lethal phenotype. In the germ cells, all imprints are erased and replaced with the correct marks (i.e. female germ cells acquire a female pattern of imprints, and vice versa for male germ cells). In contrast, in

somatic cells DNA methylation imprints are maintained to reflect the imprinting status acquired from the parents. Once, the initial methylation marks are set down, maintenance of methylation ensures methylation and imprints are not lost during cell division. This function is performed by another DNA methyltransferase – DNMT1 [60].

Imprinted genes are often found clustered together on chromosomes, which can result in coordinated expression of the genes under the control of an imprinting control region (ICR) [61]. An ICR is often a differentially methylated region (DMR) that has a parent-specific pattern of DNA methylation inherited directly from the gametes. One example is ICR1 on chromosome 11p15.5 (Figure 1.2) that controls the expression of *H19* (coding for a non-translated RNA) and *IGF2* (coding for a growth factor) [62]. *H19* is only expressed from the maternal chromosome and *IGF2* is only expressed from the paternal chromosome (Figure 1.2). When the ICR is unmethylated, as on the maternal chromosome, a protein called CTCF binds to the ICR. The bound CTCF protein inhibits *IGF2* from interacting with the enhancer and thus *IGF2* is not expressed. On the paternal chromosome, the ICR is methylated. This blocks CTCF from binding to the ICR. Without CTCF bound, *IGF2* can interact with its downstream enhancer and is gets expression (*H19* is methylated and repressed at this time).

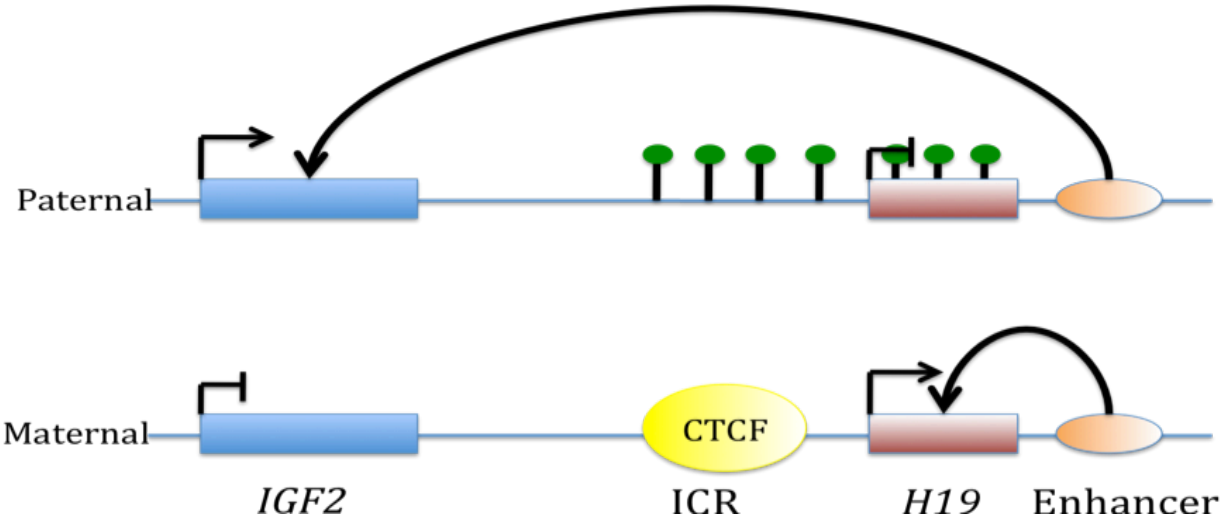

Figure 1.2. The structure of the ICR that controls *H19* and *IGF2* on chromosome 11p15.5. The "lollipops" on the paternal chromosome represent DNA methylation marks. When the ICR is methylated, CTCF is unable to bind to it, and the downstream enhancer can interact with *IGF2*, resulting in the expression of *IGF2*. When the ICR is unmethylated, CTCF binds, blocking the interaction between the enhancer and *IGF2*, resulting in the expression of *H19*.

1.6.3. Methods to assess DNA methylation

a) Traditional bisulfite sequencing

In bisulfite sequencing, genomic DNA is treated with a compound known as sodium bisulfite, which converts all unmethylated cytosine nucleotides to uracil, while preserving the methylated cytosine residues. The locus of interest is subject to amplification by polymerase chain reaction (PCR). The PCR step pairs the uracil residues with adenine, which then pair with thymine residues (thereby replacing the uracils with thymines) [63]. The resulting product may be sequenced directly, but often it is cloned in to a plasmid vector

and transformed in to bacteria which gets plated on selective agar. Traditional dideoxy, gel-based Sanger sequencing is used to quantify the level of methylation by comparing the ratio of cytosine/thymine. Multiple CpG sites can be assayed at one time with this method. The gold standard for methylation analysis has traditionally been bisulfite sequencing; however newer technologies may offer advantages over this technique. In particular, the cloning step is labour intensive and is not suitable for high throughput analysis.

b) Methylation-sensitive Single Nucleotide Primer Extension (Ms-SNuPE)

Methylation-sensitive Single Nucleotide Primer Extension (Ms-SNuPE) also uses sodium bisulfite to convert unmethylated cytosine residues to uracil, PCR to amplify the DNA, and primer extension with dideoxynucleotides; however, it differs from traditional bisulfite sequencing in two ways [63]. The first difference is that a cloning step is not required, but in some cases an additional nested PCR step may be required to achieve ample product for analysis. The second way in which it differs is that it only measures one CpG site at a time – it is the equivalent of genotyping. A primer is laid down next to the site of interest and that primer is extended by one nucleotide only. The ratio of C genotype to T genotype reflects the degree of methylation at that site. At the beginning of this project, Ms-SNuPE was used to assay methylation; however, due to decreased time and cost associated with pyrosequencing, the lab has moved away from Ms-SNuPE.

c) Pyrosequencing

Pyrosequencing also makes use of bisulfite conversion, PCR amplification and primer extension; however, it does not use traditional dideoxy Sanger sequencing [64]. Instead, an enzyme cascade is used to produce light when nucleotides are incorporated into the sequence. Each time a nucleotide is added to the growing sequence, pyrophosphate (PPi) is released. An enzyme called ATP-sulfurylase converts the PPi to ATP; ATP then powers a luciferase-catalyzed reaction that produces light. The amount of light produced is directly related to the number of nucleotides incorporated; therefore, the degree of methylation (i.e. the ratio of the light produced by a C nucleotide to a T nucleotide) is proportional to the light emitted. As with traditional bisulfite sequencing, multiple sites can be assayed at one time; however, the sequence of interest must be known ahead of time. Pyrosequencing is quite high throughput and cost effective.

d) Illumina GoldenGate Methylation Array

The techniques described above all have one thing in common – they can only assay one area at a time. The Illumina GoldenGate Methylation array (Cancer Panel 1) measures methylation at 1,505 CpG sites in 807 genes (70 of which are found in imprinted regions) [65]. Genomic DNA is treated with sodium bisulfite and amplified. CpGs are assayed as single nucleotide polymorphisms using allele specific extension followed by ligation to locus specific DNA sequences. The ligation adds universal priming sites and a bead "address" to the target DNA. The target regions are then amplified with fluorescently labelled universal primers and hybridized to the array beads. The degree of fluorescence (red

is methylated, green is unmethylated) is the measure of methylation. There are two major drawbacks to this method: 1. the cost is substantial; 2. it may overestimate methylation differences. This, and other methylation arrays, function well as a tool to help identify candidate genes that merit further study. The next generation of Illumina is the Infinium Array that measures methylation at over 27,000 sites in over 14,000 genes.

1.6.4. The Placenta and Genomic Imprinting

The emergence of the placenta in mammals has been suggested to be a driving force for the evolution of genomic imprinting. With the development of placentation, new challenges were imposed on the mother. The placental mammals had to ensure a delicate balance was maintained during reproduction - the female had to allow for the growth and development of a new organism within her (half of which was foreign) while restraining the growth of this new organism so her own wellbeing was not sacrificed [66]. When the zygote implants itself in the uterus, there must be some limitation on the ability of the placenta to invade the maternal tissues or the mother might not survive her pregnancy. In this instance, it is likely that paternally expressed genes would promote growth of the fetus and increase the maternal invasion by the placenta, whereas maternally expressed genes would restrict these functions [67]. In keeping with this hypothesis, the majority of imprinted genes are found in eutherian mammals and these genes tend to influence growth.

Knockouts of some genes in mouse models have been shown to affect the structure and function of the placenta; paternally expressed genes decrease placental function when knocked out [68] and vice-versa for maternally expressed genes [69]. One such imprinted gene that is critically important in normal embryonic and placental development is *Igf2*. For

example, complete loss of *Igf2* expression in mice (fetus and placenta) resulted in 40% growth retardation at birth [70]; in contrast, biallelic expression can result in overgrowth of up to 160% [71] and cancer development [72]. High levels of *Igf2* have also been associated with various malformations and intrauterine death [73]. Complete loss of *Igf2* expression in the placenta alone results in decreased placental nutrient transfer [74], severe placental growth retardation, loss of glycogen cells, and ultimately in fetal growth restriction [14].

Although the placenta plays an important role in coordinating fetal growth and development, there is some evidence to suggest that epigenetic regulation of gene expression appears to be less stable and more environmentally responsive in the placenta than in the fetus itself. Doherty and Mann [17,18] have both demonstrated that preimplantation culture of mouse placental cells can result in the loss of imprinting at a variety of genes (including *H19, Snrpn, Ascl2, Xist*). In addition to culture effects, methylation at imprinted regions, including ICR1, has been shown to be responsive to outside influences such as, environmental toxins (e.g. TCDD [75]), tamoxifen treatment [76], and prenatal ethanol exposure [77]. One recent study identified periconceptional maternal folic acid as being a factor in decreased birth weight and increased methylation at *IGF2* in young children [78]; however, despite progress in understanding the role of epigenetics in mouse development, current knowledge of imprinting in human development is still limited [79].

In the past few years, interest in the role of imprinting in placentation and obstetrical disorders has grown substantially. Several groups have used whole genome arrays to try to better understand the role of imprinted genes. McMinn *et al.* [80] have shown that eight imprinted genes (*PHLDA2, MEST, MEG3, GATM, GNAS, PLAGL1*, *IGF2* and *CDKN1C*) are differentially expressed in normal human and IUGR placentas. More recently, Diplas *et al.* [81] found that nine imprinted genes were differentially expressed in IUGR placentas –

five were up-regulated (*PHLDA2, ILK2, NNAT, CCDC86, PEG10*) and four were down-regulated (*PLAGL1, DHCR24, ZNF331, CDKAL1*).

At the single gene level, Guo *et al*. [44] reported a reduction in *IGF2* expression in small for gestational age human placentas; however, they did not find a concomitant alteration in methylation at the differentially methylated region that controls *IGF2* expression. *IGF2* has also been shown to play a role in placental transport [68]. Loss of imprinting of *PHLDA2* has also been shown to be associated with IUGR [82]. Loss of placental *Cdkn1c* expression has been shown to produce pre-eclampsia-like symptoms in mice [83], while *STOX1* has been suggested to be involved in the pathogenesis of pre-eclampsia in humans [84]. Biallelic expression of H19 in the human placenta has recently been associated with severe forms of pre-eclampsia [85]. These findings suggest abnormal expression of imprinted genes may be involved in the development of IUGR and pre-eclampsia, but to date, it has not been well studied in humans.

1.6.5. Selected imprinted genes and associated disorders

a) H19/IGF2 (and ICR1)

The genes for *H19* and insulin-like growth factor 2 (*IGF2)* are found on chromosome 11p15.5 (Figure 1.3). The maternally expressed *H19* gene codes for a non-translated RNA of currently unknown function and the paternally expressed *IGF2* has been implicated in several growth disorders, primarily Beckwith-Wiedemann (BWS) [86] and Silver-Russell (SRS) [87] syndromes. The two genes are coordinately regulated by a differentially methylated CTCF binding region known as imprinting control region 1 (ICR1), located

between the genes [88]. When ICR1 is unmethylated (as on the maternal allele), CTCF (an insulator protein) binds and blocks the enhancer (downstream of *H19)* from interacting with *IGF2* and *H19* gets expressed. When the DMR is methylated (as on the paternal allele), the enhancer can interact with *IGF2* resulting in its expression (and the repression of *H19*) (Figure 1.2).

IGF2 plays a role in multiple aspects of embryonic, fetal growth and postnatal growth. Loss of *Igf2* expression in the mouse placenta results in severe placental and fetal growth restriction [70]. In humans, a decrease in *IGF2* expression was recently reported in small for gestational age placentas as compared to control placentas [44]. Additionally, biallelic expression of *H19* (which occurs when the enhancer can't interact with *IGF2*) in early gestation human placentas has been correlated with severe forms of pre-eclampsia [85].

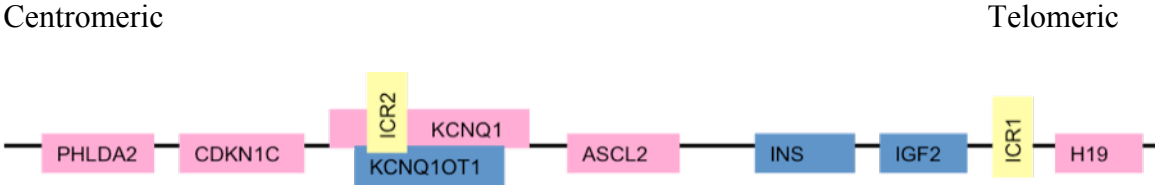

Figure 1.3. Map of chromosome 11p15.5 showing selected imprinted genes. Pink boxes represent maternally expressed genes; blue boxes represent paternally expressed genes; yellow boxes represent imprinting control regions (ICR) whose methylation status controls the expression of nearby genes.

Changes to the methylation status of ICR1 are associated with two clinical syndromes: Beckwith-Wiedemann (BWS) and Silver-Russell (SRS). ICR1 hypermethylation can lead to an increase in expression of *IGF2* and the resulting pre- and post-natal overgrowth in BWS [89,90]. Children with BWS also have large tongues, omphalocoeles, and severe hypoglycemia. Biallelic expression of *IGF2* is also involved in the development of Wilms' tumour that is seen at increased frequency in children affected by BWS. Approximately 10% of BWS cases are caused by hypermethylation of ICR1 [90,91]. ICR1 hypomethylation and consequent repression of *IGF2* expression occurs in approximately one-third of patients with Silver Russell syndrome (SRS) [87]. SRS is characterized by pre- and postnatal growth deficiency, feeding difficulties, body asymmetry, failure to thrive and developmental delay.

b) CDKN1C (and ICR2)

Imprinting control region 2 (ICR2, KvDMR1), located centromeric to ICR1 on 11p15.5, is normally methylated on the maternal allele and controls the expression levels of several nearby genes, including *CDKN1C* (Figure 1.3). Loss of methylation at ICR2 accounts for approximately 50% of all BWS cases [90,92] and a maternally inherited duplication with gain of methylation of this region has been reported in a patient with SRS [93].

Loss of methylation at ICR2 results in decreased cyclin-dependent kinase inhibitor 1C (*CDKN1C)* expression (maternally expressed). *CDKN1C* encodes for a negative regulator of the cell cycle and overexpression of *CDKN1C* can cause cells to arrest in G1. In contrast, loss of *Cdkn1c* expression in fetal mice correlates with some phenotypes of BWS

and pre-eclampsia in humans (e.g. placentomegaly and trophoblastic hyperplasia) [83] and approximately 40% of BWS familial cases involve loss of function mutations in *CDKN1C* [61].

c) PHLDA2/IPL/TSSC3

PHLDA2 (found on chromosome 11p15.5) codes for a cytoplasmic protein with a pleckstrin-homology (PH) domain; the protein may play a role in apoptosis and growth inhibition [94]. It is expressed from the maternal allele in both the placenta and the liver. In mice, knockouts of the maternal allele result in placental overgrowth without obvious effects on the fetus [95]. In contrast, over-expression of *Phlda2* from a transgene causes a reduction in placental development indicating that *Phlda2* may be a negative regulator of placental growth [96]. In humans, elevated expression of *PHLDA2* has been reported in the placentas of babies born with low birth weight [82].

d) PEG1/MEST

Paternally expressed gene 1 (*PEG1*) is also known as mesoderm-specific transcript (*MEST*). It is located at 7q32 – a region that may be associated with SRS. Uniparental disomy (UPD, a situation in which both copies of a gene, region, or chromosome come from one parent) of this region has been reported in SRS [97,98] and hypermethylation of this gene has been reported in SRS following assisted reproduction [99], although MEST's involvement in SRS is hotly contested [100,101]. The gene codes for an α-hydrolase enzyme and is known to be imprinted in a variety of fetal tissues [102]. In mice, paternal

transmission of a knockout results in placental and embryonic growth retardation. Post-natally, these mutants continue to suffer from reduced growth and survival. Additionally, parthenogenetic embryos do not develop past day 10, coinciding with the highest expression of murine *Mest* [103]. Female mice that are deficient for *Mest* show a reduction in normal maternal behaviours (e.g. impaired placentophagia, deficient nest building and young gathering) [104]. *MEST* has also been shown to be imprinted in human placentas [103] and may play a role in placental development and angiogenesis [105].

e) PEG3

Paternally expressed gene 3 (*PEG3*) is found at 19q13.4 and codes for a zinc finger protein that may a play a role in the tumour necrosis factor pathway (mediates cell proliferation, differentiation and apoptosis). In mice, a *Peg3* knockout has been created through insertion of a *βgeo* cassette into the fifth exon of the gene [106]. The *Peg3* deficient mice are viable, but the placentas of mutant embryos are smaller than normal and infants may suffer from growth restriction. In addition, mutant females are deficient in maternal behaviours (e.g. nest building, keeping pups warm) and because of this lack of maternal care there is decreased infant feeding and growth and consequently an increase in infant mortality. Expression of human *PEG3* is considerably higher in the villous cytotrophoblast than in the corresponding decidua (expression has not been reported in other placental cells). As *PEG3* is expressed in proliferating cells, it may play a role in placental growth. This hypothesis is corroborated with the finding that *Peg3* deficient mice have small placentas [107].

f) PLAGL1/ZAC/LOT1

PLAGL1 is a paternally expressed gene located on chromosome 6q24 originally identified as an imprinted gene in 2000 [108]. Uniparental disomy for 6q24 has been reported in patients with transient neonatal diabetes (TNDM), which is associated with IUGR, dehydration, hyperglycemia and failure to thrive; it usually resolves by six months of age [109]. In patients with no chromosomal changes, loss of methylation at an imprinting control region near the gene has also been implicated in TNDM [110,111]. Additionally, *Plagl1* has also been reported to be a member of an imprinted gene network (along with *Igf2, H19*, *Cdkn1c*, *Kcnq1ot1* and *Dlk1*) that is involved in the regulation of mouse embryonic growth [112]. Decreased expression of murine *Plagl1* can result in IUGR, bone anomalies, and neonatal mortality. In humans, this imprinted gene network may also be involved in BWS as PLAGL1 interacts with *LIT1* (*KCNQ1OT1*) on 11p15.5 to influence the expression of *CDKN1C*, a gene known to be involved in the pathogenesis of some cases of BWS [113].

g) STOX1

STOX1 is maternally expressed from chromosome 10q22 in early placental development, particularly in extravillous trophoblast cells (EVTs). It was first thought to play a role in pre-eclampsia when it was discovered that, in Dutch families, pre-eclampsia segregated with a susceptibility locus on chromosome 10q22 [114]. Development of pre-eclampsia has been linked to various missense mutations within the gene, but pre-eclampsia only develops when the mutation is passed through the maternal allele. This loss of function

can result in the failure of extravillous trophoblast cells to take on an invasive phenotype that leads to placental insufficiency and then to pre-eclampsia. There is ongoing conflict however as to whether the gene is indeed imprinted [115] and/or involved in pre-eclampsia [116].

h) CTNNA3

CTNNA3 is found on chromosome 10q21.3 and codes for alpha T-catenin, which is necessary for cell-cell adhesion complexes. Transcription of the gene in the placenta is high in normal first trimester development. The maternal allele is preferentially expressed in the placenta, but imprinting is actually limited to the villous cytotrophoblasts and biallelic expression is seen in extravillous cytotrophoblasts [117]. The transition from proliferating, anchoring villi to motile, invasive extravillous cytotrophoblasts correlates with a decrease in alpha T-catenin levels [84]. Abnormal transcription of cellular adhesion molecules in invasive cytotrophoblasts is thought to be an early sign of pre-eclampsia and increased expression of *CTNNA3* in invasive trophoblasts could result in their decreased ability to invade the uterine wall and could potentially lead to pre-eclampsia [118].

1.7. The goals of this thesis

1.7.1. Research questions

Are epigenetic modifications (i.e. DNA methylation) and altered expression of imprinted genes in the human placenta a contributing factor to maternal pre-eclampsia

(PET) and fetal IUGR? Also, what methylation-based assays for imprinted regions could be used to facilitate the diagnosis of placental pathologies that are associated with abnormal imprinting (i.e. triploidy, hydatidiform moles, and placental mesenchymal dysplasia)?

1.7.2. Hypothesis

Given that DNA methylation and imprinted gene expression appear to be important in the regulation of embryonic growth and placental development in the mouse, I hypothesize the disruption of normal patterns of DNA methylation associated with imprinted gene expression in early development is a significant cause of human placental insufficiency leading to maternal pre-eclampsia and fetal IUGR.

1.7.3. Objective 1 – Characterization of imprinting in normal placentas

The first objective of this project was to characterize methylation and imprinted gene expression in term placental whole villi. Methylation-sensitive Single Nucleotide Primer Extension (Ms-SNuPE) and pyrosequencing were used to assess methylation of functionally important CpG sites within or near the *H19*, *CDKN1C, PLAGL1, MEST, PEG10,* and *SNRPN* imprinted genes. These genes were chosen for their important role in placental development in murine studies. Illumina GoldenGate Methylation arrays were used to assay methylation at the whole genome level. Illumina HumanRef-8 v2 Bead Chip arrays were used to measure imprinted gene expression levels.

1.7.4. Objective 2 – Identification of abnormal patterns of imprinting in placentas associated with PET and/or IUGR

The second part of the project was to identify abnormal patterns of methylation and imprinting errors in chorionic villi from placentas that are from pregnancies associated with PET and/or IUGR and to identify if these changes are restricted to the chorionic villi. As with the controls, MS-SNuPE and pyrosequencing were used to assess CpG sites within or near individual genes and Illumina GoldenGate Methylation arrays were used to assay methylation at the whole genome level and to identify candidate genes that merit further study by identifying major epigenetic changes between normal and affected placentas. Samples were also assessed with the Illumina HumanRef-8 v2 Bead Chip expression array to measure imprinted gene expression levels.

1.7.5. Objective 3 – Design of imprinted gene methylation assays for the diagnosis of placental conditions

The final part of this project was to design new methylation assays that may aid in the diagnosis of various conditions. To help diagnose conditions such as IUGR and PET, loci that have variable methylation may be most useful as they allow the placenta to adapt to changing environmental conditions. In contrast, the diagnosis of hydatidiform moles or identification of parent of origin in triploidy, would be best served by studying loci that are less variable and maintain their imprinting status in a variety of situations.

1.7.6. Introduction to Manuscript 1

Manuscript 1 addresses the main objectives of the thesis (Objectives 1 and 2): whether abnormal regulation (both methylation and expression) of imprinted genes is associated with PET and/or IUGR. This manuscript has been published in *Placenta* [119].

1.7.7. Introduction to Manuscript 2

Manuscript 2 originally outlined a new pyrosequencing assay designed to measure methylation at KvDMR1 (found on chromosome 11p15.5) and its application to the study of fetal and placental disorders. It has since evolved to address Objective 3 by presenting a comparison of methylation assays for imprinted loci to determine which may be the most useful in the diagnosis of hydatidiform moles (complete and partial) and parent of origin in triploidy. This manuscript has been accepted for publication in *Clinical Genetics*.

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CHAPTER 2. MANUSCRIPT 1: Decreased placental methylation at the *H19/IGF*2 imprinting control region is associated with normotensive intrauterine growth restriction but not preeclampsia.

2.1. Introduction

Abnormal placental development is responsible for a wide-range of pregnancy complications including infertility, miscarriage, maternal pre-eclampsia (PET) and intrauterine growth restriction (IUGR). Pre-eclampsia affects approximately 5% of pregnancies and can be fatal for both the mother and fetus. The fetal syndrome of pre-eclampsia may manifest as IUGR [1] and both normotensive IUGR and pre-eclampsia are associated with a deficiency of extravillous trophoblast (EVT) invasion leading to incomplete remodeling of the maternal spiral arteries [2,3]. This in turn limits the transfer of nutrients and wastes between the fetus and mother. Placentas from pregnancies associated with maternal pre-eclampsia also have areas of syncytial knots (clusters of preapoptotic/apoptotic nuclei) and areas of necrosis associated with loss of the syncytial trophoblast microvillous membranes (STBM). Excess STBM are observed in early-onset pre-eclampsia but not in normotensive IUGR [4]. Although confined placental trisomy can contribute to placental insufficiency in some cases [5,6] the initiating cause for abnormal trophoblast development in most cases is largely unknown and likely heterogeneous in etiology.

Many imprinted genes, those exhibiting parent-of origin differences in gene expression, are intimately involved with the regulation of embryonic growth and placental

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development [7,8] and disruption of imprinting in mouse models can result in abnormal placental development and fetal growth [9]. Although the placenta plays a critical role in coordinating fetal growth and development, regulation of imprinted gene expression appears to be less stable in the placenta than in the fetus itself. Preimplantation culture of mouse embryos can lead to loss of placental imprinting at multiple genes and is affected by the culture media used [10,11]. This instability and relaxation of methylation may aid the placenta in adapting to changing physiological conditions. It has thus been hypothesized that sporadic loss-of-imprinting errors could also occur in human placentas and contribute to abnormal fetal growth. However, it has also been suggested that imprinting may be less maintained in human, as compared to mouse placentas [12].

Over 50 imprinted genes that are distributed in distinct clusters that are regulated by a common imprinting control region (ICR) have been identified in humans [13]. Two clusters of imprinted genes within chromosome 11p15.5, each regulated by a separate imprinting control region (ICR1 and ICR2), have been implicated in fetal and placental growth. The paternally expressed insulin-like growth factor 2 (*IGF2)* and maternally expressed *H19* genes are coordinately regulated by a differentially methylated CTCF binding region known as imprinting control region 1 (ICR1) [14]. The *H19* gene codes for a non-translated RNA of currently unknown function, while *IGF2* has been implicated in several growth disorders. ICR1 is hypomethylated, leading to repression of *IGF2* expression in approximately one-third of patients with Silver Russell syndrome (SRS), a syndrome associated with pre- and postnatal growth deficiency [15], whereas it is hypermethylated leading to an increase in expression of *IGF2* in some cases of pre- and post-natal overgrowth diagnosed as Beckwith-Wiedemann syndrome (BWS) [16,17]. Complete loss of *Igf2* expression in the mouse placenta results in severe placental and fetal

growth restriction. Recently, a decrease in *IGF2* expression was reported in small for gestational age placentas as compared to control placentas [18].

Imprinting control region 2 (KvDMR1), located centromeric to ICR1, is normally methylated on the maternal allele. Loss of methylation at this region has been reported in BWS patients with normal ICR1 methylation [19], and a maternally inherited duplication with gain of methylation of this region has been reported in a patient with SRS [20]. Loss of methylation at ICR2 can result in decreased cyclin-dependent kinase inhibitor 1C (*CDKN1C)* expression. Loss of *Cdkn1c* expression in fetal mice correlates with some phenotypes of BWS and pre-eclampsia in humans [21], and approximately 40% of BWS familial cases involve loss of function mutations in *CDKN1C* [22].

Despite improved understanding of the fundamental role that is played by genomic imprinting in the regulation of placental function in the mouse, current knowledge of imprinting in human placental development is poor [23]. The goal of the present study is to assess the role of aberrant DNA methylation associated with imprinted genes, particularly involving ICR1 and ICR2 within 11p15.5, in human placentas from pregnancies associated with pre-eclampsia and/or IUGR.

2.2. Methods

2.2.1 Sample Ascertainment

Pregnancies were prospectively ascertained through poster recruitment (hospital, midwives and doctors' offices) or through referral to the EMMA (Evaluating Maternal Markers of Acquired Risk of Pre-eclampsia) Clinic, BC Women's Hospital. Women are

seen in this clinic if they have at least one of: past history of pre-eclampsia (severe, early onset and/or associated with perinatal loss), pre-existing hypertension, unexplained low first trimester PAPP-A (<0.60 multiples of the median [MoM]), unexplained elevated second trimester alpha-fetoprotein (AFP; >2.5 MoM) or human chorionic gonadotrophin (HCG; >3.0 MoM).

Ethics approval for this study was obtained through the University of British Columbia and the BC Children's & Women's Hospital ethics boards. Consent was obtained during pregnancy. Placentas were obtained at birth and assigned to control (N=22, mean gestational age, GA=39.0 weeks), intrauterine growth restricted only (IUGR, N=13, mean GA=35.4 weeks), pre-eclampsia (PET, N=17, mean GA=35.9 weeks), and PET with IUGR (P+I, N=21, mean GA=32.5 weeks) outcomes. Intrauterine growth restriction was defined as either (1) birth weight <3rd percentile for gender and gestational age using Canadian charts [24], or (2) birth weight <10th percentile with either of the following ultrasound findings: (a) persistent uterine artery notching at 22+0-24+6 weeks gestation, (b) absent or reversed end diastolic velocity on umbilical artery Doppler, and/or (c) oligohydramnios (amniotic fluid index <50mm). Pre-eclampsia was defined as: (1) at least two of the following: hypertension (sBP ≥140mmHg and/or dBP ≥90mmHg, twice, >4h apart) after 20 weeks, and proteinuria defined as ≥0.3g/d or ≥2+ dipstick proteinuria after 20 weeks [25], (2) non-hypertensive and non-proteinuric HELLP syndrome, using Sibai's criteria [26], or (3) an isolated eclamptic seizure without preceding hypertension or proteinuria, using the British Eclampsia Survey Team (BEST) criteria to define eclampsia [27] (see Supplementary Table 1 for additional clinical information).

Two distinct placental samples of ~1cm^3 were biopsied (one near the cord insertion and one near the placental periphery) from the fetal side of each placenta for DNA

extraction. From each site, the surface layers of amnion and chorion were removed before DNA and RNA extraction. Extraction of DNA and RNA was performed using standard techniques. For methylation studies, approximately 300ng of DNA was treated with sodium bisulfite (EZ DNA Methylation-Gold™, Zymo Research, Orange, CA, USA).

All placentas were evaluated for the presence of placental trisomy using comparative genomic hybridization [28] and any identified trisomy was confirmed using microsatellite markers. Trisomy was observed in four placentas (Control PM65: 47,XXX; IUGR PM41: 47,XX,+7 and PM72: 46,XX/47,XX,+13; and PET + IUGR PM60: 47,XX,+2) [29]. Trisomy was also present in the amnion for case PM65 but not for cases PM41, PM72 or PM60, indicating that in the latter three cases the trisomy was likely confined to the placenta.

2.2.2. Whole Genome Methylation Arrays

GoldenGate Methylation Cancer Panel 1 arrays (Illumina Inc., San Diego, CA) were used to identify candidate imprinted genes that merit further study. Two independent villous samples were analyzed from each placenta (control, N=5 placentas; IUGR, N=5; and PET, N=4) for a total of 28 analyzed samples. The BeadChip array was processed in the Centre for Molecular Medicine and Therapeutics (CMMT) BioAnalyzer Core Facility (Vancouver, BC, Canada). Output was analyzed using Illumina's BeadStudio software (v3.2.7, 2007). Parameters for differential methylation analysis were as follows: normalization=average; reference group=control placentas; error model=t-test. The BeadStudio software uses diffscores to represent methylation differences between groups. Samples with a diffscore of greater than ±13 correspond to a nominal (uncorrected) significance $p<0.05$ (diffscore = |10

log pval|). Negative diffscores reflect hypomethylation compared to the control group and positive diffscores reflect hypermethylation compared to the control group. The Benjamini-Hochberg correction was used to correct for multiple comparisons and results were then further analyzed using the Significance Analysis of Microarrays (SAM) software [30].

2.2.3. Methylation-sensitive Single Nucleotide Primer Extension (Ms-SNuPE) for ICR1

Bisulfite converted DNA was PCR amplified using primers F6005 and R6326 and PCR as previously reported [31]. Each 10μL PCR contained: 1X Rose Taq buffer (including 2mM $MgCl_2$), 0.125mM dNTP, 3pmol of each primer, 0.1U Rose Taq (Rose Scientific, Edmonton, AB, Canada), and 2μL bisulfite converted DNA, with an initial denaturation at 94°C, 10 min; 30 cycles of 94°C for 45s, 61°C for 45s, 72°C for 1 min; a final extension at 72°C for 10 min. The first PCR was followed by a 20μL semi-nested PCR of 35 cycles using primers F6115 and R6326 using 1 μL of PCR product from the first reaction. PCR products were cleaned using DNA Clean & Concentrator™-5 (Zymo Research, Orange, CA, USA).

Methylation-sensitive Single Nucleotide Primer Extension (Ms-SNuPE) was used to assess methylation at two CpGs (C10 and C12) within the 6th CTCF binding site of ICR1 that were previously identified as being differentially methylated by parental origin and representative of the region [32,33]. SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA, USA) was used to according to manufacturer's directions. The reaction was terminated by dephosphorylation using 1U of calf intestinal phosphatase (Invitrogen, Carlsbad, CA, USA) and incubation at 37°C for 1 hour followed by deactivation of the enzyme at 72°C for 15min. Products were sized and quantified on an ABI Prism 310

Genetic Analyzer. Using this method, we previously observed a strong correlation between estimated methylation values for the independent PCR assays of C10 and C12 in blood samples ($r=0.95$, $p<0.0001$, $N=87$) and for repeat estimates from distinct bisulfite conversions ($r=0.8$, $p<0.0001$, $N=93$) [34] and have applied this assay to diagnosis of hypomethylation in Silver-Russell Syndrome patients. Assessment of methylation at ICR1 by pyrosequencing was not performed as a published assay for this region [35] was found to span the rs2107425 polymorphism, and further assays we attempted showed an amplification bias in some individuals based on a SNP within the amplified region (rs10732516). No amplification bias or association of methylation with local sequence variation was detected with the Ms-SNuPE assay (data not shown).

2.2.4. Pyrosequencing for ICR2, candidate gene and LINE-1 methylation

Pyrosequencing was used to assess methylation at seven CpGs within ICR2 including the differentially methylated *NotI* site that is often altered in BWS[19,36] and is used in diagnostic testing for BWS.[17] Bisulfite converted DNA was amplified by PCR (see Supplementary Table 2 for primer sequences). Each 25 µL PCR contained: 1X HotStarTaq buffer (including 1.5mM $MgCl_2$), 0.2mM dNTP, 5pmol of each primer, 1.0U HotStarTaq DNA Polymerase (QIAGEN Inc., Mississauga, ON, Canada), and 2µL bisulfite converted DNA. Thermocycling conditions included: an initial denaturation at 95 °C for 10 min; 40 cycles of 95 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s; a final extension at 72 °C for 7 min. Sequencing of PCR products (10µL) using a PyroMark™ MD (Biotage AB, Uppsala, Sweden), available in the lab of Dr. Angela Devlin, was performed according to

manufacturer's directions. Pyro Q-CpG software (v1.0.9, 2006, Biotage AB) was used to analyze results.

Methylation at 13 CpG sites within the *CDKN1C* promoter was assessed using a pyrosequencing assay available from the Biotage PyroMark™ Assay Database. Pyrosequencing assays for the following candidate genes were also developed: *H19* promoter, *PEG10* promoter, *PLAGL1* promoter, *SNRPN* promoter, *MEST* exon 1 (see Supplementary Table 2 for primer sequences). The PCR and thermocycling conditions were identical to those described above. Methylation at 7 CpG sites from a consensus sequence found within LINE-1 elements was also preformed according to manufacturer's directions (PyroMark™ LINE-1 Kit, Biotage AB).

2.2.5. Whole genome expression arrays

HumanRef-8 v2 BeadChip gene expression arrays (Illumina Inc., San Diego, CA) were used to correlate methylation patterns with gene expression. Total mRNA was extracted from a subset of the placentas using an RNeasy® kit (QIAGEN). Two independent villous samples were analyzed from each placenta (control, N=5 placentas; IUGR, N=5; and PET, N=4) for a total of 28 analyzed samples. The samples were chosen because RNA was available and the placentas were processed soon after delivery. The BeadChip array was processed in the Centre for Molecular Medicine and Therapeutics (CMMT) BioAnalyzer Core Facility (Vancouver, BC, Canada). Output was analyzed using Illumina's BeadStudio software (v3.2.7, 2007). Parameters for differential expression analysis were as follows: normalization=average; reference group=control placentas; error model=t-test. Samples with a diffscore of greater than ±13 correspond to a nominal

(uncorrected) significance $p<0.05$ (diffscore = |10 log pval|). Negative diffscores reflect underexpression compared to the control group and positive diffscores reflect overexpression compared to the control group. The Benjamini-Hochberg correction was used to correct for multiple comparisions and results were then further analyzed using the SAM software [30].

2.3. Results

2.3.1. Illumina methylation results

Although 1,505 CpG sites are assayed on the GoldenGate Methylation Cancer Panel, only 70 are located within the promoter region of imprinted genes. None of the differentially methylated ICRs are included on this array. After analysis with SAM and correction for multiple comparisons, no CpGs in either the IUGR or PET groups were found to be significantly altered.

2.3.2. ICR1 Methylation by MS-SNuPE

Methylation at ICR1 (associated with *H19* and *IGF2* expression) was quantified at two CpG sites (C10 and C12) from each of two sampling sites within each placenta. There was a strong correlation between C10 and C12 methylation levels from a single placental sample ($r=0.82$, $p<0.0001$, Figure 2.1). However, this correlation was weaker than the C10-C12 correlation observed in blood samples analyzed in the same laboratory by this method ($r=0.95$, $p<0.0001$ N=87) [34] which suggests that methylation of these sites is more

variable in the placenta than in peripheral blood. The within-placenta between-site correlation was r=0.47 for C10, r=0.48 for C12 and was r=0.56 when comparing the average C10 and C12 methylation for each site ($p<0.0001$ for each correlation). To obtain a methylation value representative of the whole placenta, it is thus important to average data from multiple sites. For subsequent comparisons, methylation values were averaged across the two CpGs and two sampling sites to obtain a single methylation value for each placenta.

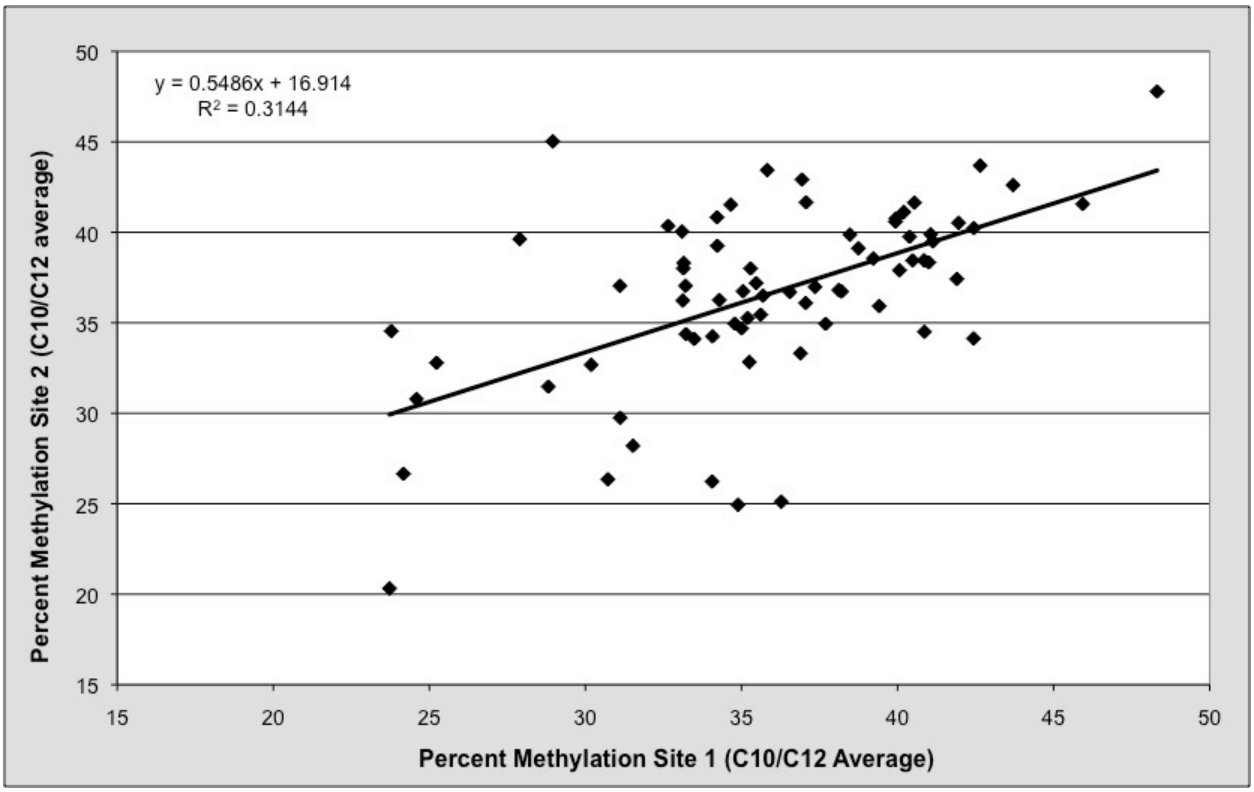

Figure 2.1. Intra-placental correlation for percent methylation at the *H19/IGF2* ICR. The methylation values as measured by SNuPE from two separate sampling sites from one placenta were correlated (r=0.56, $p<0.0001$). Percent methylation at C10 and C12 were averaged to obtain a single methylation value for each sample.

The mean ICR1 methylation values for each clinical group were: controls (N=22), 36.7%; IUGR (N=13), 30.8%; PET (N=17), 38.3%; PET+IUGR (N= 21), 37.6% (Figure 2.2, Table 2.1). Methylation at ICR1 showed significant between group differences ($p<0.001$, one-way ANOVA). This effect was due to a reduction of methylation in the IUGR group compared to all other groups ($p<0.0001$, compared to controls) and 7 of the 13 placentas in the IUGR group had methylation values at least 2 SD below the mean of the control group. These seven placentas were: PM30, PM35, PM41, PM47, PM123, PM128, PM120. There was no difference between mean methylation in PET, with or without IUGR, and the control group. There were also no significant differences in ICR1 methylation when considering early onset (N=6) or late onset (N=11) pre-eclampsia separately (not shown). There was no correlation between methylation and sex, gestational age, time to placental sampling after birth, mode of delivery, oligohydramnios, symmetrical vs. asymmetrical IUGR, maternal gestational diabetes mellitus or the presence of placental trisomy. There was a significant correlation between methylation and gestational age corrected birth weight (measured in SD relative to the mean) ($r=0.29$, $p=0.015$), which was more pronounced when the cases with pre-eclampsia were excluded from the analysis ($r=0.62$, $p<0.0001$). Nonetheless, a similar correlation between methylation and GA-corrected birth weight was present within the pre-eclampsia (with or without IUGR) group analyzed separately ($r=0.29$, $p=0.038$). It appears that a greater average methylation in the pre-eclampsia group overall is confounding the association with IUGR in comparisons with control placentas.

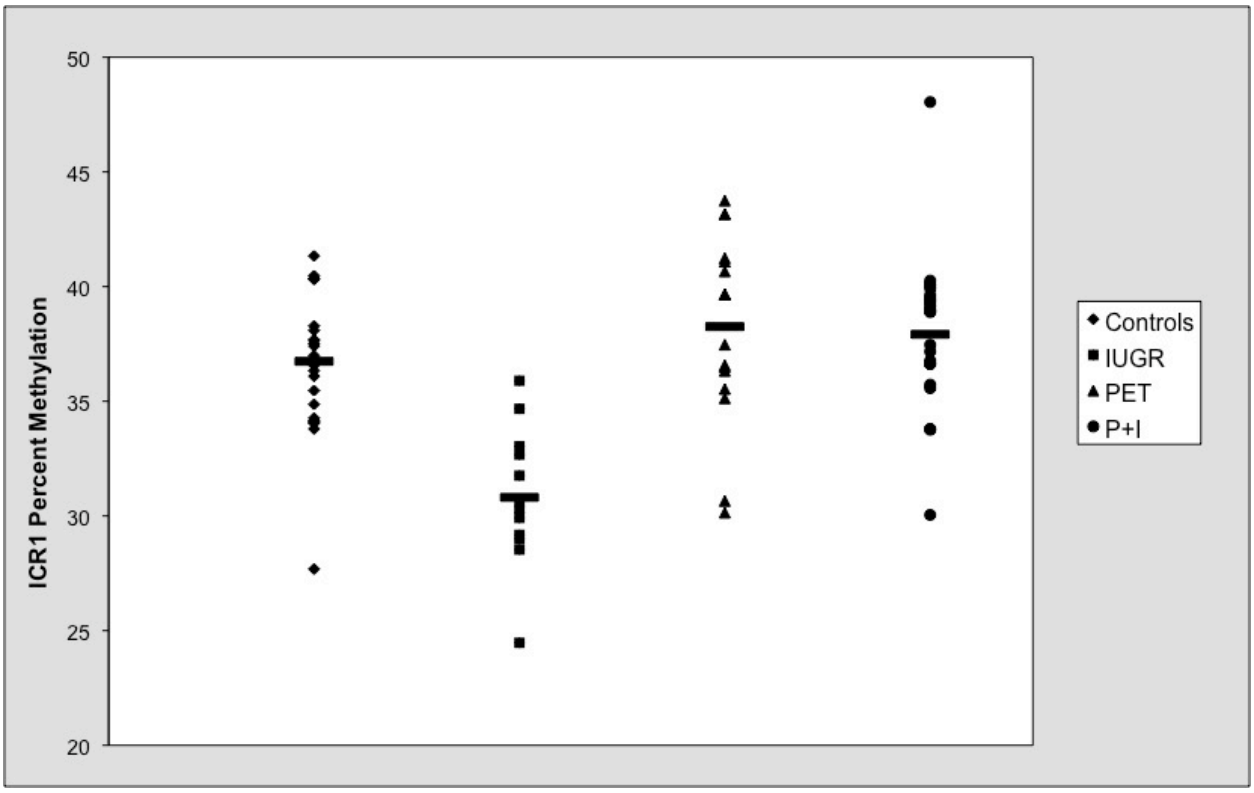

Figure 2.2. Inter-group comparisons for percent methylation at the *H19/IGF2* ICR. There is a significant reduction in mean methylation (average of two sites measured by SNuPE) in placental villi associated with normotensive IUGR compared to controls ($p<0.01$), PET ($p<0.01$), and PET+IUGR ($p<0.01$) pregnancies.

Table 2.1. Summary of mean methylation values obtained using MS-SNuPE (ICR1) and pyrosequencing (ICR2, *H19*, *CDKN1C*, *PEG10, PLAGL1, SNRPN, MEST*, LINE-1). ICR1 methylation was significantly different between groups (P<0.0001) due to reduced methylation in villi from IUGR placentas compared to each of the other groups. No other groups were significantly altered.

	Control	**IUGR**	**PET**	**PET+IUGR**
VILLI	N=22	**N=13**	N=17	N=21
ICR1	36.7 ± 3.0% (S.E. 0.6%)	**30.8 ± 3.2% (S.E. 1.0%)***	38.3 ± 4.0% (S.E. 1.0%)	37.6 ± 3.8% (S.E. 0.8%)
ICR2	65.4 ± 3.4% (S.E. 0.7%)	64.8 ± 3.2% (S.E. 0.9%)	65.2 ± 3.0% (S.E. 0.7%)	64.7 ± 3.3% (S.E. 0.7%)
H19 promoter	49.5 ± 4.4% (S.E. 0.9%)	48.9 ± 3.3% (S.E. 0.9%)	49.3 ± 4.2% (S.E. 1.0%)	48.5 ± 6.8% (S.E. 1.5%)
CDKN1C promoter	5.2 ± 2.8% (S.E. 0.7%)	4.5 ± 0.6% (S.E. 0.6%)	7.0 ± 5.5% (S.E. 1.7%)	6.9 ± 3.3% (S.E. 0.9%)
PEG10 promoter	57.1 ± 5.8% (S.E. 1.2%)	56.6 ± 11.9% (S.E. 3.3%)	57.9 ± 8.0% (S.E. 1.9%)	57.9 ± 6.4% (S.E. 1.5%)
PLAGL1 promoter	52.6 ± 2.6% (S.E. 0.5%)	53.0 ± 2.1% (S.E. 0.9%)	54.2 ± 6.6% (S.E. 1.8%)	53.7 ± 3.9% (S.E. 0.8%)
SNRPN promoter	46.3 ± 3.0% (S.E. 0.6%)	50.7 ± 4.8% (S.E. 1.3%)	47.8 ± 4.1% (S.E. 1.0%)	51.4 ± 5.8% (S.E. 1.3%)
MEST exon 1	58.9 ± 9.5% (S.E. 2.0%)	60.1 ± 8.4% (S.E. 2.3%)	59.3 ± 11.0% (S.E. 2.7%)	59.7 ± 8.8% (S.E. 2.0%)
LINE-1	49.6 ± 2.0% (S.E. 0.6%)	50.0 ± 2.0%, (S.E. 0.7%)	48.5 ± 2.4% (S.E. 0.7%)	51.0 ± 4.6% (S.E. 1.3%)
AMNION	N=5	N=5	N=5	N=4
ICR1	35.8 ± 2.9% (S.E. 1.3%)	36.4 ± 3.4% (S.E. 1.7%)	34.5 ± 3.3% (S.E. 1.5%)	37.2 ± 1.4% (S.E. 0.7%)

*p<0.001 compared to control (t-test)

To determine if reduced methylation was restricted to chorionic villi, ICR1 methylation was assessed in a subset of amnion samples. There was no significant difference between methylation at ICR1 within the amnion in any of the clinical groups and specifically no reduction in methylation in the IUGR group as compared to the control group (Table 2.1). In addition, chorionic villi from five control and five IUGR group placentas were separated into trophoblast and mesenchyme by enzymatic digestion. Mean ICR1 methylation was similar between cell-types in both groups and there was not a significant correlation between methylation level of trophoblast and mesenchyme from a single site (r=0.14), though sample size was likely too small to evaluate such an effect.

2.3.3. ICR2, candidate gene, and LINE-1 methylation by pyrosequencing

Methylation at ICR2 was quantified at seven adjacent CpG sites, including those in the BWS diagnostic *NotI* site. There was a correlation in methylation levels between different CpGs from the same sample and for average methylation between placental sites (r=0.38, p=0.001). As for ICR1, methylation across the CpGs and the two placental sampling sites was averaged to give one measurement per placenta. No significant differences in ICR2 methylation were detected between any of the clinical groups or between early and late onset preeclampsia (Table 2.1).

Methylation level at 13 CpG sites within the *CDKN1C* promoter was very low and no significant differences were detected between any of the groups. Whole blood and saliva were also tested with this method and also showed very little methylation; however, promoter regions associated with CpG islands are frequently unmethylated [37]. None of the other candidate genes were significantly altered between the groups (Table 2.1). As an

indirect marker of genome-wide methylation, methylation status was also evaluated at a consensus sequence within LINE-1 elements, which make up about 15% of the genome. The mean methylation values for each clinical group were all approximately 50% (Table 2.1). It thus appears that the altered methylation at ICR1 does not stem from a general hypomethylation of the genome.

2.3.4. Illumina expression results

Although over 22,000 transcripts are assayed on the Illumina HumanRef-8 v2 BeadChip, for the present study we only considered the expression level of 44 reported imprinted genes (59 transcripts). Among these, only *IGF2* (transcripts NR_003512.1, NM_001007139.4, NM_00612.4) was significantly underexpressed in placental villi associated with IUGR compared to controls (average expression level of 16700 vs 35000, $p<0.0001$). Other imprinted genes had decreased expression (e.g. *CDKN1C* in PET) or increased expression (*MEST* in IUGR and PET, SNRPN in IUGR); however, these were not significant when analyzed by SAM. In addition, only *IGF2* was significantly altered after using the Benjamini-Hochberg correction. This result was not supported by quantitative real time PCR using either β-actin or β-2-microglobulin as an endogenous control; however, other groups have reported both increases [38] and decreases [18] in *IGF2* expression in IUGR placentas. Unlike DNA methylation, which is relatively stable, placental RNA degrades quite rapidly after birth. Most of our placentas were >6 hours post-birth at the time of sampling, and thus the mRNA data must be interpreted cautiously.

2.4. Discussion

The placenta is a remarkably adaptive organ that mediates the exchange of nutrients between two genetically distinct individuals, the mother and the fetus, which may have conflicting needs [39]. Many imprinted genes studied in the mouse appear to regulate fetal growth in a manner that maintains a balance between maternal nutrient supply and fetal growth [8,39,40]. Altered expression of both *IGF2* and *PHLDA2* have been reported in pathological human placentas [18,41,42]; however, methylation provides an independent, potentially more stable, assessment of the placental genome.

Although both IUGR and PET associated placentas show similar deficiencies in trophoblast invasion, only normotensive IUGR associated placentas showed reduced methylation at ICR1 in this study as compared to controls. This reduced methylation may reflect an adaptive process serving to adjust placental and fetal growth in response to poor placental perfusion and prevent maternal pre-eclampsia. Consistent with this possibility, average methylation in pre-eclampsia tended to be higher than controls, particularly in the absence of IUGR. This supports failure to limit fetal growth in the presence of poor placental perfusion could in turn contribute to the development of maternal pre-eclampsia. The presence of high levels of trisomy in two of the IUGR associated placentas, which is likely the initial cause of placental dysfunction, is consistent with the hypothesis that reduced methylation at ICR1 may be a consequence of other placental abnormalities rather than a spontaneous defect. Methylation at ICR1 has been shown in a number of studies to be particularly responsive to environmental influences such as culture media [10,11], environmental toxins (e.g. TCDD) [43], and prenatal ethanol exposure [44].

Previous studies have reported a reduction in *IGF2* expression in placentas from pregnancies associated with IUGR or SGA [18,41] and complete loss of placental *Igf2* expression is associated with fetal growth restriction in mice [7]. Furthermore, selective deletion of the placental specific form of *Igf2* from murine placentas also leads to a significant decrease in fetal weight, with pups being 69% of normal weight at birth [45]. Reduced placental *Igf2* expression leads to a reduction in size of all placental layers and alters the diffusional exchange characteristics of the placenta [46]. In human pregnancies, reduced exchange surface area, and likely reduced transfer capacity of the placenta, has been noted in IUGR [47]. Altered placental transfer to the fetus may also be a mechanism involved in the pathogenesis of IUGR, as the developing fetus will not be able to receive adequate nutrition to allow for normal growth.

Although a reduction in *IGF2* expression was observed in SGA placentas in a recent report, loss of methylation at ICR1 was not observed in the same placentas [18]. The differences in methylation values between the two studies may reflect differences in patient ascertainment or sampling procedures. An increase in average methylation of ICR1 in preeclamptic placentas may confound the relationship of decreased methylation with IUGR, if these placentas are not analyzed separately. While there is much overlap between cases diagnosed as SGA or IUGR, these are different diagnostic criteria, and in our study cases with low birth weight were required to show other prenatal indicators of poor placental function to be classified as IUGR. Furthermore, we removed the amnion and chorion from the villous sample prior to DNA extraction. Including amnion could dilute the methylation effect, as we observed normal methylation in amnion even when reduced in the placental villi. Another study found biallelic expression and loss of imprinting at *H19* in placentas from pre-eclamptic women [48]; however, our results do not support these findings.

In the present study we did not find evidence for altered methylation at ICR2 (KvDMR1), nor specifically at *CDKN1C,* a gene associated with altered growth in BWS. Very little methylation (~5%) was detected at *CDKN1C* despite being reported to be differentially methylated in murine placenta [49], and some imprinted genes, including those within the ICR2 cluster, may not be imprinted in the human placenta [12]. The mouse knockout of *Cdkn1c* displays some phenotypes of preeclampsia and BWS [21], and a modest reduction in *CDKN1C* expression was observed in both the IUGR and PET group.

Measurement of DNA methylation may provide a useful diagnostic tool for indirectly detecting altered gene expression, due to its increased stability over RNA. Placental RNA in particular degrades extremely rapidly and may be affected by labour duration and delivery method. Further studies will be necessary to determine if altered methylation at ICR1 is an early or late event in IUGR and thus could provide any prognostic value. If reduction of *IGF2* expression is a compensatory response to other factors such as poor placental perfusion, it may be a beneficial response for the mother. A full understanding of the genetic and or environmental conditions leading to reduced *IGF2* in some pregnancies with abnormal trophoblast invasion and not in others (i.e. those with preeclampsia) will be important before any therapies attempting to improve fetal growth are initiated.

2.5. Supplementary Tables

Please see Appendix A for the following Supplementary Tables.

2.5.1 Supplementary Table 1.

Additional clinical information for the placentas used in this study.

2.5.2 Supplementary Table 2.

Primers and sequences to analyze for pyrosequencing assays.

2.6. References

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CHAPTER 3. MANUSCRIPT 2: The utility of quantitative methylation assays at imprinted genes for the diagnosis of fetal and placental disorders

3.1 Introduction

Abnormal expression of imprinted genes has been implicated in many growth disorders of the placenta and/or fetus [1,2]. These genes tend to be distributed in distinct clusters that are epigenetically regulated by one or more imprinting control regions (ICRs) [3]. Quantification of DNA methylation at differentially methylated regions (DMRs) located at either ICRs or promoters associated with specific genes is commonly used for the diagnosis of imprinting disorders including Beckwith-Wiedemann syndrome (BWS) [4], Russell-Silver syndrome [5,6], and Prader-Willi and Angelman syndromes [7,8]. While imprinting is recognized as important in the genesis of a variety of disorders affecting the placenta including complete hydatidiform mole (CHM), partial hydatidiform mole (PHM), and placental mesenchymal dysplasia (PMD), DNA methylation has not traditionally been applied to their diagnosis. Distinguishing between CHM, PHM, and hydropic abortuses (HA) continues to pose problems in diagnosis [9-11]. It is imperative that accurate diagnosis be made as CHM is associated with an increased risk of gestational trophoblastic neoplasia and patient management differs from that in the other placental conditions. Recurrence risks can also differ depending on the underlying etiology. Triploidy rarely is recurrent, however familial recurrent biparental CHMs can occur in female carriers of mutations in *NALP7* [12,13]. We hypothesize that methylation-based assays at imprinted regions would be useful to help distinguish these phenotypically overlapping entities.

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Chromosome 11p15.5 contains two clusters of imprinted genes each regulated by a different ICR. The centromeric ICR, known as KvDMR1, is located within an intron of the *KCNQ1* gene, and contains the promoter for the *KCNQ1OT1* non-coding RNA [14]. It is normally methylated on the maternal allele and unmethylated on the paternal allele and this methylation is associated with the regulation of several imprinted genes, including *CDKN1C, KCNQ1, KCNQ1OT1,* and *PHLDA2*. Roughly half of BWS patients show loss of methylation at KvDMR1 [4,14] and diagnosis typically utilizes a differentially methylated *Not1* restriction enzyme cutting site at this locus and Southern blotting. Loss of KvDMR1 methylation is associated with decreased expression of the maternally expressed *CDKN1C,* mutations in which can also cause BWS [15,16].

CHMs are typically of androgenetic origin [2] (i.e. there is no maternal contribution to the genome) and show decreased *CDKN1C* expression [17]. Thus immunostaining of CDKN1C (also known as $p57^{KIP2}$) has been used in the diagnosis of CHMs and gestational trophoblastic disease [1,11]. However, some CHMs have stained positively for CDKN1C as a result of retention of a maternal chromosome 11 [11,18], confounding the interpretation of some cases. Methylation changes at KvDMR1 or other DMRs associated with imprinted genes may provide an alternative method of diagnosis. Furthermore, placentas affected with placental mesenchymal dysplasia (PMD) may also exhibit a paternally biased methylation pattern, despite positive CDKN1C staining, as PMD is usually caused by the presence of chimeric androgenetic cells in the placenta [19]. PMD has also been reported with mosaic deletion of the maternal copy of 11p15.5 [20] and there appears to be an increased incidence of prenatally diagnosed BWS in association with PMD [21], though the etiology in those cases is unclear [20]. PHMs are due to diandric triploidy [22,23] and will also stain positively for CDKN1C, but have a 2:1 ratio of paternal to maternal haploid genomes.

Methylation assays of imprinted sites have not been used in clinical placental pathology possibly because it has been suggested that regulation of imprinted gene expression may not be well-maintained in the human placenta [24-26]. Furthermore, differentially methylated sites that may be used in clinical diagnosis of imprinting disorders in blood samples, such as assays for the *CDKN1C* and *IGF2* promoters, are not useful in placental samples as they are not methylated in this tissue. It is thus important that a potential methylation-based assay for detecting genomic imbalance in placental tissue is evaluated for stability across a variety of normal and abnormal placental samples. In this study we evaluate the utility of a pyrosequencing assay for KvDMR1 to assess 11p15.5 abnormalities in blood and placenta to identify imprinting errors and distinguish between those placental abnormalities characterized by parental genome imbalances, such as in triploidy and PMD. We then compare this assay to similar assays involving other imprinted DMRs of clinical relevance to determine which ones can be reliably applied to assist in the diagnosis of a variety of placental disorders.

3.2 Methods

3.2.1 Study samples

Ethics approval for this study was obtained through the University of British Columbia and the BC Children's & Women's Hospital ethics boards. Genomic DNA was extracted using standard protocols. Blood (N=11) and saliva (N=8) samples were collected from healthy adults, thirteen cases of BWS (six with known abnormal KvDMR1 methylation status), and epidermal cells (from both sides of the body) from one case of left

side hemihypertrophy. In addition, DNA was extracted from chorionic villi of placentas from healthy pregnancies (N=22), placentas with triploidy of known parental origin (N=8 diandric triploidy, N=13 digynic triploidy) based on previous microsatellite testing [27,28], placentas affected with PMD (N=11 samples taken from 6 placentas) confirmed previously to be due to androgenetic chimerism [19] and trophoblast cells from one CHM. The CHM was confirmed by microsatellite testing to be homozygous at all tested polymorphic markers from multiple chromosomes, consistent with an origin from endoreduplication of the haploid genome present in a single sperm.

3.2.2 Pyrosequencing assays

Pyrosequencing was used to assess methylation at seven CpGs within KvDMR1 including the differentially methylated *NotI* site that is often altered in BWS [14,29] and is used in diagnostic testing for BWS [4]. PCR conditions were as outlined previously [30]. The two CpGs in the *Not1* site were compared to the other five CpGs in the region and were highly correlated ($r=0.94$, $p<0.0001$, $N=142$). Thus, methylation values at the seven CpG sites were averaged to get a single methylation value per sample. Hypomethylation was defined as more than 2SD below the mean of controls while hypermethylation was defined as more than 2SD above the mean of controls.

Because methylated and unmethylated strands show different sequences after bisulfite conversion, there may be differences in the efficiency of each strand to be amplified by PCR [31]. To test for linearity of amplification of the methylated allele, a standard curve comparing serial dilutions of methylated : unmethylated DNA with relative proportions of 0:100, 25:75, 33:66, 50:50, 66:33, 75:25, 100:0 was prepared using DNA from two cell

lines obtained from the NIGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, Camden NJ, USA). Somatic cell hybrid (SCH) cell line NA10482 (GM10482A) contains a single derivative chromosome 11 with a paternal methylation imprint, and SCH NA07300 (GM07300) contains a single chromosome 11 with a maternal methylation imprint [32]. While the results fit well with a linear model ($y= -0.92*x+101.86$, $R^2=0.977$), a closer fit was obtained with a slight curvilinear model ($y=-0.0043*x^2-0.49x+95.3$ $R^2=0.998$).

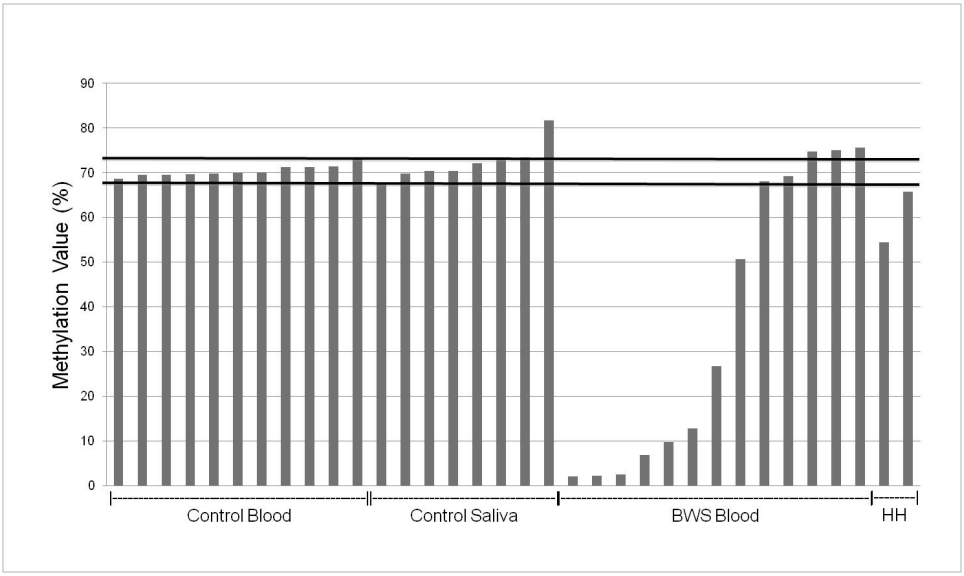
Other DMRs were selected based on being 1) associated with clinically relevant genes; 2) approximately 50% methylated in control placental samples; and 3) highly reproducible. These included the telomeric chromosome 11p15.5 ICR, located proximal to the *H19* promoter and shown previously to be useful in the diagnosis of 11p15.5-associated imprinting abnormalities in Russell-Silver syndrome [33]. Methylation at the promoter regions for the *H19* and *CDKN1C* genes was also tested by pyrosequencing but these sites were excluded because the average level of methylation was <10% in control placental samples. Non-chromosome 11 assays included those for the promoter of i) *SNRPN* [30], a maternally methylated region used in diagnosis of abnormalities of 15q11.2 in Prader-Willi and Angelman syndromes; ii) *SGCE*, a maternally methylated locus on chromosome 7q21 which we previously showed displayed increased methylation in cases of maternal uniparental disomy 7 in Silver–Russell syndrome patients [5]; and iii) *MEST* [34], another similarly methylated chromosome 7 imprinted gene.

3.3. Results

3.3.1. Chromosome 11p15.5 assays (KvDMR1 and *H19*-ICR)

Repeat measurements of 13 samples (including blood, saliva and placental samples) assayed in two independent reactions were highly correlated ($r=0.98$, $p<0.0001$), thus demonstrating high reproducibility of the KvDMR1 assay. Values for blood and saliva from controls were similar (70%±1.2% and 72%±4.3%, respectively). Six cases of BWS, known to have reduced KvDMR1 methylation status based on clinical testing using a Southern blot assay, also showed extremely reduced methylation (2-12%) by the pyrosequencing assay (Figure 3.1.a), and the two results were highly correlated ($r=0.99$, $p<0.0001$). Of the seven cases of BWS not previously tested at this site by Southern, two had a reduction in methylation of 20% or more below the control blood mean (27% and 50%), while the others had methylation levels within the normal range (68-75%). Epidermal cells from a case of asymmetric hemihypertrophy had reduced methylation on the left side compared to the right (55% and 66%, respectively), which was consistent with the restriction of hypertrophy to the left side of the body (Figure 3.1.a).

a)



b)

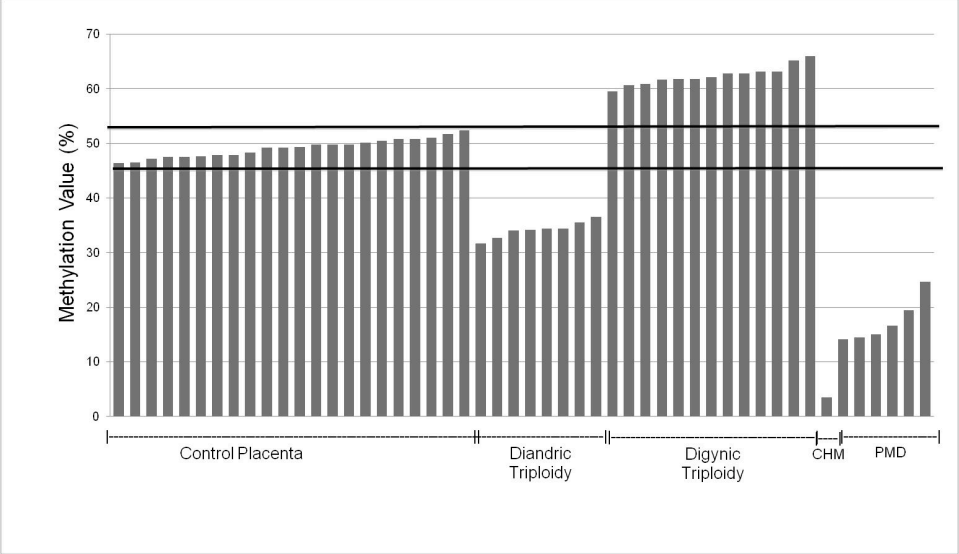


Figure 3.1. Pyrosequencing results for KvDMR1 and *SGCE* methylation assays. a) KvDMR1 methylation values for control blood, control saliva, BWS samples, and left and right epidermal samples from a case of hemihypertrophy (HH). b) *SGCE* methylation values for control placental villi, diandric triploidy, digynic triploidy, trophoblast of one CHM (CHM), and six PMD samples with more than 60% androgenetic cells. Black bold lines indicate ±2 S.D. of control placental villi.

Control placental villi were slightly less methylated (65%±3.4%) than control blood and saliva ($p<0.01$, ANOVA). Twelve of thirteen placentas with triploidy of maternal origin were hypermethylated compared to control placentas (mean=77%, $p<0.0001$, t-test) with the remaining case being at the high end of normal. All eight placentas with triploidy of paternal origin were hypomethylated compared to controls (mean=45%, $p<0.0001$, t-test). The difference in KvDMR1 methylation between the two types of triploids was also highly significant ($p<0.0001$, t-test), and the distinct non-overlapping range of values observed within each group suggests this test can be used to determine the parental origin of triploidy.

An assay for the *H19*-ICR useful in the diagnosis of hypomethylation in Russell-Silver syndrome [33] was also tested. In this case the paternal allele is methylated and thus the digynic triploids exhibit lower levels of methylation for this site than the diandric triploids (Table 3.1).

Table 3.1. Comparison of methylation assays at imprinted genes for the ability to distinguish digynic triploidy (N=13) from diandric triploidy (N=8).

Methylation		Control		Digynic triploid		Diandric triploid	
Assay	Locus	Mean (Range)	s.d.	Mean (Range)	s.d.	Mean (Range)	s.d.
KvDMR1	11p15.5	65.4 (57.7-70.8)	3.4	77.1 (69.6-81.3)	2.8	47.4 (40.9-53.1)	3.0
H19 ICR	11p15.5	49.6 (46.5-53.3)	2.0	37.4 (33.8-40.8)	2.1	60.3 (51.0-64.0)	4.0
SGCE	7q21	49.1 (46.3-52.3)	1.7	62.4 (59.5-66.0)	1.8	34.2 (31.7-36.5)	1.5
MEST	7q32	65.3 (61.3-68.0)	2.3	74.4 (68.8-81.2)	4.4	47.7 (38.7-52.3)	3.9
SNRPN	15q11.2	46.1 (38.8-63.9)	5.4	67.6 (63.0-73.4)	3.0	38.5 (36.0-44.2)	3.2

3.3.2. Non-chromosome 11 pyrosequencing assays

While an assay for KvDMR1 is of value to the diagnosis of BWS, any imprinted DMR may be used to evaluate parental origin of triploidy. Table 3.1 shows the results for additional assays tested in the triploid samples. All showed highly significant ($p<0.0001$) differences between the diandric and digynic triploids with non-overlapping ranges. The *SGCE* assay showed the smallest standard deviation of values within each group and the largest between group difference, with 36.5% methylation being the highest observed level of methylation in a digynic triploid and 59.5% methylation being the lowest observed methylation value for a diandric triploid (Figure 3.1.b). Furthermore, neither triploid group overlapped with the range of values observed in control placentas.

3.3.3. Utility of assays for estimation of paternal:maternal genomic ratios

Placental mesenchymal dysplasia is characterized genetically by a mix of androgenetic and biparental cells that varies from sample to sample within a single placenta. Using the assay for KvDMR1, there was a significant inverse correlation ($r=0.93$, $p<0.0001$) between the estimated percent paternal contribution (based on microsatellite data) and methylation level of the same DNA sample (data not shown). However, the best linear correlation was achieved with the assays for the *H19*-ICR and *SGCE* ($r^2=0.98$, $P<0.0001$ for both) (Figure 3.2). As the site assayed within the *H19*-ICR is methylated on the paternal allele, while the assayed *SGCE* site is methylated on the maternal allele, a reciprocal relationship between methylation at these sites and relative genomic imbalance (excess paternal contribution) is observed.

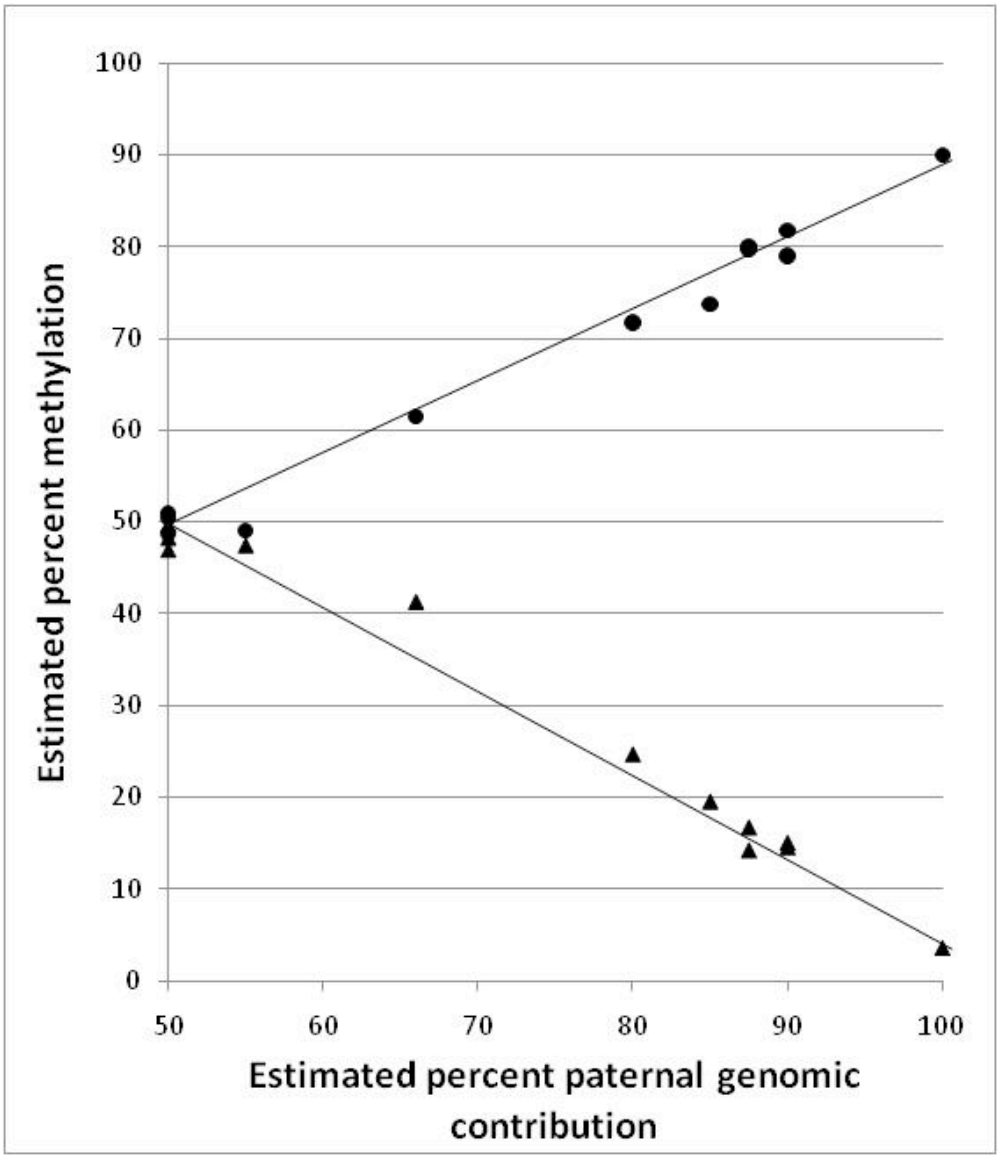


Figure 3.2. Among 11 samples of placental mesenchymal dysplasia (PMD) and one complete hydatiform mole (CHM), estimated percent methylation shows a significant linear relationship with the relative paternal genomic contribution (as estimated by allelic dosage at microsattelite loci) for both the *H19*-ICR (●) and *SGCE* promoter(▲) (R^2=0.98, p<0.0001 for each).

3.4. Discussion

Rapid and reliable diagnosis of methylation abnormalities at the KvDMR1 is useful for many clinical conditions. While methylation at this site has been extensively studied in the past in cases of BWS, pyrosequencing offers an inexpensive, rapid and high-throughput alternative approach. The level of KvDMR1 methylation measured in control blood is somewhat higher than expected (70% methylation instead of 50% methylation) which is in part due to a slight amplification bias in the PCR. The CHM showed 8% methylation, despite no evidence for any maternal contribution, which suggests either that some methylation can occur on the paternal allele or that some CHMs have biparental cell populations in low numbers. Further studies of a large number of CHMs are needed to fully establish the range of methylation values expected in complete androgenetic moles, as well as in biparental moles.

Methylation at this and other imprinted DMRs can be used to distinguish between diandric triploid PHM, CHM, and hydropic normal placentas based on methylation profile. Furthermore, the presence of triploidy and its parental origin can be suspected based on the methylation value, which cannot be determined from immunostaining alone. The methylation assays used here are relatively inexpensive and rapid, but would require access to molecular diagnostic testing facilities.

SGCE provided the clearest separation of diandric and digynic triploids and demonstrated an inverse linear relationship with level of methylation and relative paternal contribution. However, the combination of a chromosome 11 DMR assay (e.g. KvDMR1 or *H19*-ICR) and non-chromosome 11 assay (e.g. *SGCE*) would be indicated in some cases. CHMs of androgenetic origin that retain a maternal chromosome 11 show positive

immunostaining of CDKN1C [11,17]. Such cases would be expected to display an abnormal methylation profile consistent with diandric triploidy at chromosome 11 markers but absent methylation for maternally methylated sites from other chromosomes. Such a result could help distinguish such cases from a PHM (diandric triploidy). PMD is characterized by site-to-site variability in the levels of androgenetic cells, tending to be absent in trophoblast samples and highest in the characteristic enlarged vessels [19]. Thus a corresponding site-to-site variability in methylation is expected in such cases. The presence of methylation levels between that expected for CHMs and diandric triploidy combined with such within placenta variability would this suggest the diagnosis of androgenetic chimerism.

Regulation of imprinted gene expression has been hypothesized to be less stable in the placenta than in the fetus itself [24]. Furthermore, it has been suggested that methylation may be less important for the maintenance of imprinted gene expression in the placenta [25], and imprinting in general may be less maintained in human, as compared to mouse, placentas [26]. While there are indeed promoters of some imprinted genes for which methylation is not maintained in the placenta (such as the promoters for 11p15.5 genes *H19* and *CDKN1C*) [30], the parent-of-origin specific methylation at the sites utilized here all appear to be stably maintained in the placenta even in the presence of abnormal pathology. Thus, parent-of-origin specific methylation appears to be important for the regulation of many imprinted genes in the placenta.

3.5. References

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CHAPTER 4. DISCUSSION AND CONCLUSION

4.1. DNA methylation as a biomarker

DNA methylation may fulfill the requirements for a useful biomarker of the development of pre-eclampsia (PET) and intrauterine growth restriction (IUGR) for several reasons. First, DNA methylation is implicated in the control of gene expression [1]. This can be achieved either directly (e.g. promoter methylation) or indirectly (e.g. recruited factors to change histone modifications). Gene expression itself can be difficult to measure due the need to measure expression at a physiological relevant time, whereas DNA methylation may be more stable and could be measured at a later time point. Second, DNA methylation is stable and heritable in the sense that it is stably passed from mother to daughter cells during cell division [1]. This means that DNA methylation can be measured from the placenta after birth and possibly still reflect the epigenetic landscape during the pregnancy. Finally, from a technical perspective, the stability of DNA methylation makes it easier to work with than mRNA in the laboratory setting. As is true for mRNA from other sources [2], placental mRNA degrades quickly and may be affected by mode of delivery, duration of labour, and processing time [3] and obtaining samples expeditiously can prove challenging. Additionally, quantitative real-time PCR analysis of gene expression requires comparison of the expression levels of the gene of interest to a reference gene that is ideally expressed at a similar level as the target and degrades at a similar rate as the target gene. In the case of the placenta, normalization to several reference genes may be recommended due to mRNA degradation [4]. Measurement of DNA methylation does not require comparison to a reference gene and provides an absolute measure of the percent methylation.

Measurement of DNA methylation has disadvantages and challenges associated with it as well. DNA methylation status may not directly reflect expression levels due to the presence of other factors, such as histone modifications. Methylation may be influenced by a variety of factors including cell type and gestational age. Finally, you must know which CpG sites are relevant to assay when assessing DNA methylation; this is in contrast to assessing gene expression where any part of the transcript may be used as a target.

Manuscript 1 describes DNA methylation changes that are found at ICR1 on chromosome 11p15.5 from placentas associated with normotensive IUGR. Several sites (ICR2 and other 11p15.5 genes) were also assayed; however, ICR1 was the only site to exhibit altered methylation. Other groups have failed to show a similar methylation decrease in ICR1 methylation in small for gestational age (SGA) placentas [5]. This discrepancy may be due to the different criteria used to define SGA and IUGR. While SGA may be defined as simply $<10^{th}$ percentile for weight (gestational age corrected), our definition of IUGR requires the presence of other prenatal indicators of poor placental function. Specifically, IUGR was defined as either (1) birth weight <3rd percentile for gender and gestational age using Canadian charts [6], or (2) birth weight <10th percentile with one ultrasound finding: (a) persistent uterine artery notching at 22+0-24+6 weeks gestation, (b) absent or reversed end diastolic velocity on umbilical artery Doppler, and/or (c) oligohydramnios (amniotic fluid index <50mm). While there is much overlap between cases diagnosed as SGA or IUGR, these are different diagnostic criteria.

Comments received during the submission and revision process of Manuscript 1 at *Placenta* indicate that some researchers are hesitant to accept methylation data without concomitant gene expression and protein data. However, I feel that I must address these objections. Messenger RNA is unstable [2] and may degrade quickly after birth. As DNA

methylation tends to be more stable than expression, it is easier to assess it accurately post delivery. It is quite possible that gene expression levels at term do not reflect expression earlier in gestation; to understand the gene expression and protein levels at the relevant time of gestation (i.e. at the onset of IUGR or pre-eclampsia) placental samples must be taken at that time. Needless to say, this is practically impractical and unethical to do from a human pregnancy. Also, gene expression may vary between placental sampling sites, possibly reflecting local environmental influences (such as hypoxia) [7]. As such, methylation data may be more reliable and easy to assess than gene expression for certain areas (e.g. ICR1 and ICR2 on 11p15.5); however, I found no methylation at the promoter of *CDKN1C* which highlights that not all genes are controlled directly by DNA methylation. In these cases, assessment of histone modifications may also prove helpful.

Several groups have previously reported a decrease in *IGF2* expression placentas from pregnancies associated with IUGR or SGA [5,8]. Although I reported a decrease in *IGF2* expression with the Illumina gene expression array, I was unable to detect the same decrease with qRT-PCR despite using two endogenous controls. This is likely caused by one of two factors. The first is the length of time between delivery and sampling. This could result in a general degradation of mRNA. Secondly, work in the Robinson laboratory has shown that mRNA from some genes may degrade at a faster rate than others (unpublished). In light of this, the Illumina expression data may actually be more accurate than the qRT-PCR data. Unlike in qRT-PCR, where expression is measured relative to a single endogenous control, the Illumina gene expression array normalizes gene expression relative to all the other probes on the plate (in this case over 22,000 other probes). Unfortunately, without good quality RNA taken from placentas quickly after delivery, it will be difficult to

truly establish whether the change in methylation we have observed at ICR1 is associated with a decrease in *IGF2* expression in placentas from pregnancies affected by IUGR.

4.2. Imprinting in the placenta

Additional points brought up by a reviewer of Manuscript 1 highlight the possibility that imprinting may not be as well maintained in the human placenta as in the mouse placenta and as such, our data may not be relevant. This claim is based on a paper published in 2006 [9] that reported some genes, while known to have imprinted expression in murine placentas, are actually biallelically expressed in human placentas. While some of my data do support this claim (for example, we show a lack of methylation present at the promoter of *CDKN1C* which is controlled by ICR2 on 11p15.5), I show here that many genes and ICRs do retain their imprinted status in human placentas.

Work presented in Manuscript 2 uses cases of hydatidiform moles, placental mesenchymal dysplasia and triploidy to further reinforce that some genes maintain their imprinted status in the placenta and that methylation may actually be useful in differentiating various placental pathologies at the molecular level. Macroscopic and histological analysis may not be able to differentiate the various types of abnormal placental pathologies and diagnosis is subject to inter-observer variability [10]. Molecular analysis currently involves immunostaining for CDKN1C; however, this method may be influenced by retained maternal chromosomes in some cases of complete hydatidiform moles [11,12].

Multiple studies performed by other groups also support that (at least some) imprinted genes are imprinted in the human placenta [5,13-15]. Additionally, McMinn *et al.* [8] have used expression arrays to show that eight imprinted genes (*PHLDA2, MEST,*

MEG3, GATM, GNAS, PLAGL1, *IGF2* and *CDKN1C*) are differentially expressed in normal human and IUGR placentas. These studies, as well as the ones ongoing in the Robinson laboratory, highlight the importance of continuing to undertake studies of imprinting in the human placenta.

4.3. Methods to assess DNA methylation

A variety of methylation methods were used during the course of this thesis. The original studies performed in our lab used Methylation-sensitive Single Nucleotide Primer Extension (Ms-SNuPE) to assess methylation at ICR1 of 11p15.5; however, a pyrosequencing assay for this region has recently been developed by the Weksberg lab [16]. Due to the availability of this new assay, the Robinson lab has made the switch from SNuPE to pyrosequencing for this region. Pyrosequencing offers several advantages over SNuPE: 1. It is less expensive, less labour intensive, and more high-throughput than SNuPE; 2. It is not dependent on gel analysis; and, 3. It allows analysis of multiple CpG sites in a single assay. As outlined in Manuscripts 1 and 2, I have developed a variety of pyrosequencing assays that will continue to be used in future projects.

The gold standard in methylation analysis has traditionally been the clone and sequence technique in which bisulfite treated DNA is cloned in to a vector, transformed in to bacteria, and plated on selective agar. Pyrosequencing may be a more quantitative method than bisulfite sequencing as it does not require cloning and selection steps, which may reduce the accuracy of standard bisulfite sequencing. One group has reported that while the methods were relatively equal in their sensitivity, they observed greater variability in methylation measured by bisulfite sequencing, which they attribute to the cloning and

selection step [17]. Additionally, the cloning step is both time and labour intensive and is not well suited for high throughput analysis.

There are some drawbacks to using pyrosequencing. To design assays for pyrosequencing, the sequence target of interest must already be known. In areas with multiple CpG sites, it can be difficult to find appropriate areas to place primers, which may lead to some amplification biases. Additionally, work in the Robinson laboratory has shown there to be approximately five percent variation within a given assay.

I also made use of Illumina GoldenGate methylation array. It covers 1505 CpG sites across the human genome, with 70 probes located in imprinted genes. I found that the GoldenGate methylation array had a tendency to overestimate methylation differences between genes. Additionally, as the Illumina array measures many sites at one, there is a high likelihood of false positive findings (which must be controlled for by correcting for multiple comparisons). In spite of this, I feel that it is still an important tool to identify genes that merit further investigation with pyrosequencing.

4.4. Future directions

4.4.1. Assisted reproductive technologies (ART)

In recent years, the use of assisted reproductive technologies (ART) has increased dramatically. The umbrella of ART encompasses procedures such as hormonal stimulation to induce superovulation, in vitro fertilization (IVF), and intracytosplasmic sperm injection (ICSI), culture and preimplantation genetic diagnosis. This increase in prevalence is in part due to women postponing child bearing until later in life. ART pregnancies are more often

associated with congenital anomalies (although this is a small increase above those conceived naturally) and obstetrical complications, primarily low birth weight, than are pregnancies conceived naturally.

Given that many genes are expressed very early in development, and imprinted genes, may be sensitive to environmental influences and culture [18], it stands to reason that ART may have an effect on the resulting pregnancy. There is a growing body of evidence pointing to possible epigenetic changes in pregnancies that have been conceived with the help of ART. Animal studies have suggested that both genome-wide [19] and locus specific [20] changes can occur following embryo manipulation. There is also some evidence to suggest that underlying infertility may affect DNA methylation [21,22] or that abnormal DNA methylation could lead to reduced fertility.

Methylation changes in human pregnancies have also been reported. Katari *et al.* used the same methylation array used in our study (GoldenGate Methylation Array from Illumina) to investigate genome wide differences between pregnancies conceived in vivo or in vitro [23]. They found some modest methylation differences between the groups and found alterations in the imprinted genes on the array. They also found some concomitant gene expression changes. At the single-locus level, Gomes *et al.* found the same KvDMR1 methylation changes seen in Beckwith-Wiedemann syndrome in 3 out of 18 clinically normal children conceived through ART (2 IVF and 1 ICS1) [24].

There is a clear need for additional studies of methylation and expression of imprinted regions in ART pregnancies. A better understanding of the underlying reasons why these pregnancies are at a higher risk for complications and the ability to tease apart the effects of infertility and ART may aid in the prevention of such complications. As the use of

ART continues to increase, so will the importance of better understanding the effects, both short and long term, of these technologies on the resulting pregnancies.

4.4.2. Diet and environmental influences

As diet, specifically folate and vitamin B12, plays a role in imprinted gene methylation in mice [25-28], it may also be involved in imprinted gene methylation in humans. While it was not possible within the confines of this project, an analysis of diet prior to and during pregnancy and the subsequent effects on imprinted gene methylation and obstetrical outcome could provide insight in to the role of diet on pregnancy outcomes. Also, as a variety of environmental factors are known to influence methylation (ethanol [29], tamoxifen [30], TCDD [31], bisphenol A [32-34]), measuring exposure levels prior to and during pregnancy may shed some light on the role of environmental influences on pregnancy outcome. These studies might better help us understand if imprinting is maintained in the placenta to ensure that it can react accordingly to changing conditions and to allow the pregnancy to continue.

4.4.3. Utility of this work

The new methylation assays presented in this thesis will hopefully lead to better, and more reliable, diagnoses of hydatidiform moles, placental mesenchymal dysplasia, and parent of origin in triploidy. Although some groups feel that there is no or limited retention of imprinting status in the human placenta [9], my work with these pathologies in Manuscript 2 shows that they reliably maintain their imprinting status. The triploids for

which parent of origin has been identified could then help confirm or find additional imprinted genes as these regions would have significantly different patterns of methylation from normal placentas. Additional pyrosequencing assays and the Illumina GoldenGate methylation array would prove to be very useful in this respect.

The techniques presented in this thesis will hopefully be able to be applied to the study of whether altered methylation in intrauterine growth restriction is a cause of abnormal placental pathology or a consequence of an underlying placental pathology. I attempted to use Ms-SNuPE to look at placentas with confined placental trisomy with the idea that the placental trisomy, not the methylation, should be the cause of growth restriction. Unfortunately, the poor quality DNA from these trisomic placentas was unable to be amplified with the Ms-SNuPE assay and did not produce any useable data. With the development of an *H19* ICR pyrosequencing assay [16], future studies might be able to better understand if the change in methylation at ICR in IUGR is a cause or a consequence of an underlying placental problem.

4.5. Significance

This project is part of a larger initiative to develop a strategy for improved diagnosis and management of pregnancies associated with pre-eclampsia and/or IUGR. Pre-eclampsia accounts for up to 20% of maternal mortality in developed countries [35] and is associated with a significant number of perinatal deaths and IUGR [36]. Both pre-eclampsia and IUGR are associated with many long term health risks and even a small reduction in their incidence can result in a significant reduction in health care costs [37]. Medical intervention for pre-eclampsia is most useful early in pregnancy, thus early diagnosis is important.

One of the major goals of this initiative is to correlate molecular data with clinical course and severity to identify clinical subsets of pre-eclampsia/IUGR of distinct etiology. The epigenetic changes that correlate with clinical sub-populations can be then used to identify patients that differ in terms of recurrence risk and long-term outcomes of their babies. The establishment of biomarkers that could be used to accurately identify those women at an increased risk for pre-eclampsia or IUGR would be a major step forward in antenatal care. The work presented in this thesis is a preliminary step towards achieving these goals.

4.6. References

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APPENDIX A.

Supplementary Table 1. Additional clinical details from the placentas in Manuscript 1. GA=gestational age; BW=birth weight; AFP=alpha-fetoprotein; uE3=unconjugated estriol; B-hCG=human chorionic gonadotropin; Bal=Balanced; GD= gestational diabetes; IUFD= intrauterine fetal demise; EoPET= early onset preeclampsia; LoPET= late onset preeclampsia; HELLP= hemolytic anemia, elevated liver enzymes, low platelet count; REDF= reversed end diastolic flow.

Placenta	Karyotype	GA (weeks)	BW (g)	SD from mean	Mat. Age (years)	AFP (MoM)	uE3 (MoM)	B-hCG (MoM)	Other
CONTROLS									
PM5	46, XY	37 +5	3270	0 SD	37	N/A	N/A	N/A	
PM8	46, XY	40 +2	3570	(-0.2) SD	32	1.63	1.49	1.22	
PM10	46, XY	38 +5	2950	(-1.2) SD	33	0.64	1.25	0.40	
PM17	46, XX	36 +	2305	(-1.3) SD	35	1.15	1.13	1.12	
PM20	46, XY	36 +5	3335	(+0.8) SD	32	0.72	1.22	0.90	
PM57	46, XX	41 +4	2925	(-1.5) SD	36	N/A	N/A	N/A	Severe oligohydramnios
PM59	46, XY	40	3635	0 SD	26	N/A	N/A	N/A	
PM65	47, XXX	41 +3	3250	(-0.8) SD	30	N/A	N/A	N/A	Severe oligohydramnios
PM68	46, XY	39	4615	(+3.0) SD	36	0.77	1.02	0.71	
PM70	46, XX	39	3915	(+1.8) SD	34	N/A	N/A	N/A	
PM73	46, XY	38 +1	3510	(+0.5) SD	30	N/A	N/A	N/A	
PM74	46, XY	37 +6	3460	(+0.5) SD	36	1.20	0.98	0.55	
PM77	46, XY	36 +3	2975	(+1.6) SD	33	0.84	0.68	1.09	
PM78	46, XY	38	3610	(+0.7) SD	34	0.79	1.05	0.48	
PM82	46, XX	41 +3	3890	(+0.6) SD	35	N/A	N/A	N/A	
PM84	46, XX	39 +6	3635	(+0.4) SD	33	1.31	1.14	1.31	
PM87	46, XX	37 +2	3470	(+1.1) SD	34	1.04	1.16	0.90	
PM89	46, XX	41 +5	4140	(+1.7) SD	25	0.88	0.50	1.78	
PM94	46, XX	40 +2	3580	(+0.2) SD	36	N/A	N/A	N/A	
PM96	46, XY	40	3900	(+0.8) SD	33	0.85	0.74	0.94	
PM101	46, XY	38	2885	(-0.88) SD	34	1.75	0.93	1.20	
PM104	46, XY	40 +5	3360	(-0.37) SD	30	0.84	0.74	0.46	
IUGR									
PM4	46, XX	37	2340	(-1.2) SD	37	N/A	N/A	N/A	
PM29	46, XX	36	2600	(-0.4) SD	29	2.53	0.74	3.03	GD, oligohydramnios
PM30	46, XX	36 +2	2240	(-0.8) SD	32	0.92	0.50	3.10	
PM35	46, XX	37	2345	(-1.2) SD	28	2.33	0.36	0.58	
PM41	47, XX,+7	37 +1	1725	(-2.4) SD	43	0.62	0.73	0.50	Oligohydramnios
PM42	46, XX	26	450	(-2.1) SD	33	N/A	N/A	N/A	IUFD, severe oligohydramnios, retrognathiat
PM47	46, XX	38	2645	(-1.5) SD	35	N/A	N/A	1.75	Oligohydramnios
PM72	46, XX/47, XX,+13	34	1445	(-2.2) SD	38	0.91	0.24	1.11	Severe oligohydramnios
PM121	46, XY	40	2930	(-1.2) SD	35	1.01	0.09	3.42	

PM123	46, XX	35 +3	1565	(-1.9) SD	26	N/A	N/A	N/A	
PM128	46, XX	31	1390	(-1.11) SD	33	1.70	1.57	1.02	Severe oligohydramnios
PM130	46, XY	36 +6	2090	(-1.7) SD	37	N/A	N/A	N/A	Asymmetric IUGR, oligohydramnios
PM139	46, XY	36	1740	(-2.5) SD	30	2.16	0.49	2.38	Asymmetric IUGR, severe oligohydramnios

PET

PM36	46, XX	37 +2	3170	(+0.4) SD	31	N/A	N/A	N/A	EoPET
PM44	46, XY	39 +2	2730	(-1.34) SD	27	1.32	0.67	1.34	LoPET
PM46	46, XX	40	3385	(-0.1) SD	40	N/A	N/A	N/A	LoPET
PM50	46, XX	38 +2	2935	(-0.8) SD	30	N/A	N/A	N/A	LoPET, HELLP
PM53	46, XX	38 +4	4400	(+2.5) SD	35	0.70	0.55	1.38	LoPET, GD
PM54	46, XX	34 +4	2270	0 SD	41	N/A	N/A	N/A	LoPET
PM55	46, XX	40	4095	(+1.3) SD	26	1.11	1.06	1.37	LoPET
PM56	46, XX	34	2615	(+2.2) SD	27	0.92	1.52	1.00	LoPET
PM58	46, XX	37	3010	(+0.2 SD)	37	1.00	1.17	0.73	LoPET, GD
PM64	46, XX	33 +2	1728	(-1 SD)	27	N/A	N/A	N/A	EoPET, HELLP, oligohydramnios
PM71	46, XX	38 +6	2675	(-1.5) SD	39	N/A	N/A	N/A	LoPET
PM80	46, XY	28 +4	1095	(-1) SD	35	2.10	0.88	2.31	EoPET
PM98	46, XY	37 +3	3310	(+0.6 SD)	34	1.39	1.66	1.51	LoPET
PM99	46, XY	26 +6	N/A	N/A	37	N/A	N/A	N/A	EoPET
PM100	46, XY	36	3385	(+3.0) SD	34	N/A	N/A	N/A	EoPET
PM119	46, XY	37	2530	(-1.2 SD)	33	N/A	N/A	N/A	LoPET
PM138	46, XY	34	3685	(+6.7) SD	38	0.88	0.91	0.89	EoPET, GD

PIH + IUGR

PM6	46, XY	32 +5	1160	(-3.4) SD	42	N/A	N/A	N/A	EoPET, asymmetric IUGR
PM15	46, XX	32 +6	1480	(-2.0) SD	36	1.65	1.19	2.00	EoPET, HELLP
PM21	46, XY	33	1650	(-1.4 SD)	34	N/A	N/A	N/A	EoPET, heart defects, omphalocoele
PM26B	46, XX	31 +5	940	(-3.2) SD	36	2.14	1.94	2.52	EoPET
PM31	46, XX	36	1480	(-3.3) SD	31	N/A	N/A	N/A	LoPET, asymmetric IUGR
PM32	46, XX	35	1630	(-1.8) SD	36	N/A	N/A	N/A	LoPET, oligohydramnios
PM37	46, XY	24 +4	360	(-2.6) SD	35	1.49	0.74	5.57	EoPET, IUFD, severe oligohydramnios, REDF
PM38	46, XY	36	2225	(-0.8) SD	31	N/A	N/A	N/A	LoPET
PM39	46, XY	32	1700	(+0.3) SD	19	N/A	N/A	N/A	EoPET, mild asymmetric IUGR
PM40	46, XX	38 +2	2565	(-1.7) SD	33	0.81	0.90	1.36	LoPET
PM43	46, XX	31 +5	1440	(-0.9) SD	32	0.86	1.26	0.81	EoPET
PM51	46, XX	34	1400	(-2.9) SD	42	N/A	N/A	N/A	EoPET, asymmetric IUGR, oligohydramnios
PM52	46, XY	35 +3	1840	(-1.3) SD	23	1.41	0.76	3.95	LoPET, GD, oligohydramnios

PM60	47, XX,+2	33 +2	1465	(-2.6) SD	39	N/A	N/A	N/A	EoPET, severe asymmetric IUGR, severe oligohydramnios, REDF
PM62	46, XY	27 +1	480	(-2.5 SD)	40	N/A	N/A	N/A	EoPET
PM66	46, XY	35	1790	(-1.4) SD	38	N/A	N/A	N/A	LoPET, mild IUGR
PM67	46, XY	33 +6	1560	(-2.2) SD	40	N/A	N/A	N/A	EoPET, GD
PM86	46, XY	25	545	(-1.6) SD	35	2.02	1.17	3.24	EoPET
PM97	46, XY	26	440	(-2.2) SD	23	1.77	0.39	1.09	EoPET,chr.3 variant, IUFD
PM116	46, XY	32 +3	1480	(-0.7) SD	26	0.64	0.79	0.78	EoPET
PM129	46, XY	37 +2	1840	(-2.2) SD	37	9.55	0.68	3.11	EoPET, asymmetric IUGR

Supplementary Table 2. Primers and sequences to analyze for pyrosequencing assays.

Location	Primers
KvDMR1	KvDMR1-F2 (5'-TTAGTTTTTTGYGTGATGTGTTTATTA-3')
	KvDMR1-R (5'-Biotin/CCCACAAACCTCCACACC-3').
	KvDMR1-S (5'-TTGYGTGATGTGTTTATTA-3').
Seq. to Analyze	TTTYGGGGTGATYGYGTGAGGATAGYGGTYGTATTTYGATATTGTTGTGGGTTTTTYG
H19 Promoter	H19-F (5'-Biotin/ATTGTGGGAGGGGTTAGTATAGGA)
	H19-R (5'-CTCCACRCTCAAAAATCATCAC-3')
	H19-S (5'-ATTTACCCACAAATATTCC-3')
Seq. to Analyze	CCRTACCTACRCATTACTAACAACACRACCRAATCCT
PEG10 Promoter	PEG10-F (5'-TTGGTTTTGGTTTTTGGAAATAG-3')
	PEG10-R (5'-Biotin/TTTCCCCCTCTTACTAAATACATTTCT-3')
	PEG10-S (5'-TTGTTTAGTTTTTAGTATTTTATGA-3')
Seq. to Analyze	TTTYGTTTTTTTGTTTYGTAAAATYGAAGAAAATYGAGATTTTYGTTATYG
PLAGL1 Promoter	PLAGL1-F (5'-Biotin/GAYGGGTTGAATGATAAATGGTAGATG-3')
	PLAGL1-R (5'-TCRACRCAACCATCCTCTTAACTAC-3')
	PLAGL1-S (5'-ACRCAACCATCCTCTTA-3')
Seq. to Analyze	TTTYGTTTTTTTGTTTYGTAAAATYGAAGAAAATYGAGATTTTYGTTATYG
SNRPN Promoter	SNRPN-F (5'-Biotin/TATGTTTAGGYGGGGATGTGTG-3')
	SNRPN-R (5'-AAAAACCACCRACACAACTAACCTTAC-3')
	SNRPN-S (5'-CAAACAAATACRTCAAACATCT-3')
Seq. to Analyze	CCRACRACCRCTCCACTCTACRCCAAACTCRCTACAACAAC
MEST Exon 1	MEST-F (5-Biotin/GGGTTTTTTTTGGGAATAGGGTGAA-3')
	MEST-R (5'-CRCCTCTTACCTAATTCAAATAAAACCTT-3')
	MEST-S (5'-CCTTACCTACAAAACTCCAT)
Seq to. Analyze	ATTTCRAAAAACCRATTACRCATACRCTTCCT

APPENDIX B.

UBC C&W CREB Approval

CHILDREN'S & WOMEN'S HEALTH CENTRE OF BRITISH COLUMBIA

University of British Columbia – Children's & Women's Health Centre of BC
Research Ethics Board
(UBC C&W REB)

UBC C & W Research Ethics Board
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Email: reb@cw.bc.ca
Website: www.cfri.ca/Research Ethics
RISe: https://rise.ubc.ca

Nov 05 2009

C&W Institutional Certificate of Approval
-- RENEWAL --

PRINCIPAL INVESTIGATOR	DEPARTMENT	NUMBER
Robinson, Wendy	Medical Genetics	CW04-0173 / H04-70488

CO-INVESTIGATORS:
Von Dadelszen, Peter; Langlois, Sylvie;

C&W DEPARTMENTS, PATIENT BASED PROGRAMS AND ADMINISTRATIVE JURISDICTIONS IMPACTED BY THIS STUDY:
Pathology and Laboratory Medicine; none;

SPONSORING AGENCIES:
Canadian Institutes of Health Research;

TITLE
Trisomy Mosaicism and the placenta

TERMS OF RENEWAL	
Nov 05 2009 - Nov 04 2010	

CERTIFICATION:

Ethical approval has been granted for the above-referenced research project. I am pleased to inform you that all necessary hospital program/resource approvals and institutional agreements/contracts are now in place and that you have permission to begin your research.

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Dr. Stuart MacLeod
Vice President, Academic Liaison and Research Coordination,
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This Certificate of Approval is valid for the above term provided there is no change in the research protocol