PLACENTAL PHENOTYPES ASSOCIATED WITH ABNORMAL GENOMIC 
IMPRINTING ON DISTAL MOUSE CHROMOSOME 7

by

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Abstract

Imprinted genes are expressed either from the maternal or paternal allele during development and tend to be found in clusters throughout the mammalian genome, suggesting they may be regulated by long-range mechanisms. Many of them have important roles in placental development. The Beckwith-Wiedemann Syndrome (BWS) region on human chromosome 11p15.5 contains two imprinted subdomains each regulated by their own differentially methylated regions, known as imprinting centres (IC1 and IC2). These two imprinted subdomains are separated by an evolutionarily conserved region of about 300 kilobases. Distal mouse chromosome 7 (MMU7) shares syntenic homology with the human BWS region. Since the mechanisms by which imprinting occurs are unclear, we sought to characterize this region further using two mouse lines carrying deletions within the BWS imprinted region. The first mouse line, called DelTel7/IC2KO, allows us to dissect out the role of imprinting centre 2 in the silencing of imprinted genes. We demonstrate that all of the distal MMU7 imprinted genes implicated in placental function are silenced by IC2 and the noncoding RNA Kcnq1ot1. The second mouse line, called Del7AI, allows us to determine whether placental imprinting is perturbed when the region between IC1 and IC2 is deleted. We found that maternal inheritance of Del7AI leads to partial loss of the gene Ascl2, and we show that this affects all three layers of the mature mouse placenta. We found that paternal inheritance of Del7AI leads to partial loss of Ascl2 imprinting. Detailed investigation of the underlying mechanisms of imprinting and phenotypes in these mouse lines provides us with new fundamental insights into placental biology and the regulation of gene expression by imprinting centres on distal mouse chromosome 7.
Preface

For Chapter 2, Rosemary Oh-McGinnis performed the experiments and wrote the manuscript. Aaron Bogutz and Rosemary Oh-McGinnis performed the placental corrosion cast experiments. Kang Yun Lee analyzed the microarray data. Michael Higgins provided us with the IC2KO mouse line. Louis Lefebvre conceived the study, helped with experimental design and revised the manuscript. A version of Chapter 2 has been published. Oh-McGinnis R., Bogutz A., Lee K.Y., Higgins M.J. and Lefebvre L. (2010) Rescue of placental phenotype in a mechanistic model of Beckwith-Wiedemann syndrome. *BMC Dev Biol*, 10, 50.

For Chapter 3, Rosemary Oh-McGinnis performed the experiments and wrote the manuscript. Aaron Bogutz and Rosemary Oh-McGinnis performed the placental corrosion cast experiments. Aaron Bogutz performed the IHC analyses at E7.5. Louis Lefebvre designed and directed the study and revised the manuscript. A version of Chapter 3 has been published. Oh-McGinnis R., Bogutz A.B. and Lefebvre L. (2011) Partial loss of *Ascl2* function affects all three layers of the mature placenta and causes intrauterine growth restriction. *Dev Biol*, 351, 277-286.

For Chapter 4, Rosemary Oh-McGinnis performed the experiments and wrote the manuscript. Rita Ho performed the allele-specific assays for *Ascl2* and *Tssc4*. Initial in utero and live rescue breeding experiments were done in the laboratory of Andras Nagy by Louis Lefebvre. Louis Lefebvre conceived the study, directed the study and revised the manuscript. A version of Chapter 4 will be submitted for publication.

All animal experiments were performed under certificate A07-0160 from the UBC Animal Care Committee and complied with the Canadian Council on Animal Care guidelines on the ethical care and use of experimental animals.

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List of Abbreviations

bHLH  basic helix-loop-helix
bp    base pairs
BAC   bacterial artificial chromosome
BWS   Beckwith-Wiedemann syndrome
Chr7  mouse chromosome 7
CpG   cytosine-phosphate-guanine dinucleotide
Del7Al deletion allele of region between Ascl2 and Ins2
DelTel7 deletion allele of distal mouse chromosome 7 containing IC2 domain
DMR   differentially methylated region
E     embryonic day
EPC   ectoplacental cone
GC    trophoblast glycogen cells
IC1   imprinting centre 1
IC2   imprinting centre 2
IC2KO imprinting centre 2 knockout
ICM   inner cell mass
IHC   immunohistochemistry
ISH   in situ hybridization
IUGR  intrauterine growth restriction
kb    kilobases
KI    knockin
KO    knockout
LOI   loss of imprinting
Mb    megabases
MBD   methyl binding protein
ncRNA noncoding RNA
orf   open reading frame
PAS   periodic acid Schiff
PGK   phosphoglycerate kinase (promoter)
qRT-PCR quantitative reverse transcription polymerase chain reaction
RBC   red blood cell
SEM   scanning electron microscope
SNP   single nucleotide polymorphism
TE    trophectoderm
TS    trophoblast stem (cell)
TGC   trophoblast giant cell
UPD   uniparental disomy
WT    Wilms’ Tumor
wt    wild type
XCI   X-chromosome inactivation
YAC   yeast artificial chromosome
3C    chromosome conformation capture
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Chapter 1: Introduction

1.1 The mouse placenta

The developing mouse blastocyst consists of two major components: the inner cell mass (ICM) and the trophectoderm (TE). The ICM forms the embryonic ectoderm which in turn forms the embryo and also the extraembryonic mesoderm that forms the fetal vasculature of the mouse placenta. The TE cells that overlay the ICM are referred to as the polar TE and these cells continue to proliferate after implantation and make the extraembryonic ectoderm that consists of trophoblast stem (TS) cells and the diploid ectoplacental cone (EPC). The TE cells not in contact with the ICM are referred to as mural TE cells. At implantation, they cease division and form the primary trophoblast giant cells (TGCs) of the placenta. Differentiation of the extraembryonic ectoderm gives rise to the rest of the trophoblast lineages that eventually make up the mouse placenta (1,2).

The mouse placenta is a fascinating organ that acts as the interface between the fetus and mother and is required for the exchange of nutrients, gases and waste products, as well as the ultimate survival of the fetus (1). The mouse placenta is functionally mature by E10 (about mid-gestation) and is basically made up of three main layers: TGC layer, spongiotrophoblast layer (also known as junctional zone), and labyrinth. TGCs are terminally differentiated polyploid cells comprising the outermost layer of the placenta (1). The spongiotrophoblast originates from the EPC and forms the middle layer between the TGCs and labyrinth layer. The labyrinth is the innermost layer of the placenta and consists of fetal villi and maternal blood spaces known as sinusoids. In the mouse, proper function of all three layers is required to produce viable progeny.

Each layer of the mouse placenta specifically serves one or more unique functions in the placenta and consists of several different types of cells. TGCs play a variety of essential roles...
both in early and late gestation. Initially, they mediate blastocyst implantation and invasion into the uterine epithelium (3). They are subsequently responsible for the secretion of several hormones including steroid hormones and placental lactogens that ensure proper maternal adaptation to the pregnancy (3). There are at least four different types of TGCs: parietal trophoblast giant cells (P-TGCs), spiral artery-associated trophoblast giant cells (SpA-TGCs), maternal blood canal-associated trophoblast giant cells (C-TGCs) and sinusoidal trophoblast giant cells (S-TGCs) (4). There is considerable overlap regarding the origin of the trophoblast giant cells. The mural TE gives rise to P-TGCs which line the site of implantation and are in direct contact with decidual and immune cells in the uterus and are implicated in facilitating implantation (1,3). The polar TE also gives rise to P-TGCs, but in addition will give rise to the chorion and the ectoplacental cone which in turn give rise to the rest of the other TGC cell types (1,3). It has previously been thought that the other TGC cell types all arose from 4311+ EPC precursors, but it is now known that the inner EPC and chorion (4311-negative cells) can give rise to C-TGCs and S-TGCs (3). The SpA-TGCs line the maternal spiral arteries that bring blood into the placenta and are implicated in regulating blood flow into the placenta (3,4). The C-TGCs and S-TGCs are both found in the labyrinth and line canals that bring maternal blood to the base of the labyrinth and the maternal blood sinusoids of the labyrinth, respectively (3). The precise functions of these cell types are not known but probably play a role in regulating maternal physiology or nutrient exchange.

Although the precise mechanism is not known, the spongiotrophoblast is known to arise from the EPC. Cells of the spongiotrophoblast express unique genes such as 4311 (also known as Tphpa) (5). The spongiotrophoblast has been implicated in a variety of functions including endocrine secretion (6), limiting the growth of maternal endothelium into the fetal placenta (7,8),
and providing structural support to the placenta (1,9). At least one other known function is that a few of the cells of the spongiotrophoblast give rise to TGCs (9). Moreover, at about E12.5, specialized trophoblast cells of unknown origin called glycogen cells arise from the spongiotrophoblast and migrate towards the maternal decidua (6). The role of glycogen cells in the mouse placenta is unknown but it is possible that they act as an energy store for either the placenta or the growing fetus since they contain glycogen (6). The 4311 gene is also expressed in glycogen cell populations, while the gene Pcdh12 (protocadherin 12) is known to be exclusively expressed in glycogen cells (10).

The first step in the development of the labyrinth is chorioallantoic fusion, whereby the chorionic plate trophoblast cells (which is formed by the extraembryonic ectoderm and mesoderm) and the allantoic mesoderm and underlying fetal vessels fuse to form branches (11). The next step after chorioallantoic fusion is branching morphogenesis, whereby primary villi develop across the chorionic surface and blood vessels fill in the villous folds (9,12). Within the labyrinth, the extraembryonic mesoderm gives rise to the fetal vascular endothelium which lines the fetal blood vessels. The chorion gives rise to three different cell types, the first being the previously described S-TGCs which are commonly referred to as mononucleated trophoblast cells that line the maternal blood spaces in the labyrinth. The second and third cell types both form what is known as the syncytiotrophoblast bilayer, which make up the rest of the spaces between the maternal and fetal blood cells. These cell types are referred to as Syncytiotrophoblast layer I and Syncytiotrophoblast layer II (Syn-I and Syn II) and are thought to play important roles in nutrient transport (13). All of these cell types of each of the layers of the mouse placenta have been summarized in Figure 1.1.
Figure 1.1 Different cell types of the mature mouse placenta.

P-TGC: parietal giant cell; SpA TGC: spiral artery-associated trophoblast giant cells; C-TGC: maternal blood canal-associated trophoblast giant cell; S-TGC: sinusoidal trophoblast giant cell; GC: glycogen cell; Syn-I: syncytiotrophoblast layer I; Syn-II: syncytiotrophoblast layer II; end: fetal vascular endothelium; RBC: red blood cell. Inset rectangle is enlarged to demonstrate the cell types of the labyrinth. Nuclei are denoted in blue. Adapted from (3,12).
1.2 Comparative overview of the human and mouse placenta and developmental outcomes associated with abnormal placentation

The mouse and human placenta have several analogous cell types in common, making the mouse a useful model organism in which to study human placental development and placental disorders (9,12). The innermost layer of the human placenta is made up of the chorionic villi which are composed of syncytiotrophoblast, villous cytotrophoblast, blood vessels and stroma (9); this is fully analogous to the function of the labyrinth in the mouse. The middle section of the human placenta consists of column cytotrophoblasts, which is comparable to the spongiotrophoblast in the mouse (9). The outermost component of the human placenta is composed of extravillous cytotrophoblast, which can become polyploid (9), similar to the polyploid TGCs found in the mouse. In the mid-90s, few genes were known to be essential for placental development in human and mouse, but more recently, well over one hundred have been identified (12,14). Transgenic and knockout approaches in the mouse have significantly increased our understanding of the function of genes early in the trophoblast lineage pathway as well as in the maturing placenta. Space limitations do not allow us to discuss the phenotype of every gene knockout in the mouse, but key genes and their functions are highlighted in the following paragraphs.

Failure of the blastocyst to segregate the ICM and TE lineages will lead to implantation failure and early lethality at around E3.5-4.5 in the mouse. Several genes have been identified that play a role in trophectoderm fate in the mouse. Of these, notably, \textit{Cdx2}, a caudal-type homeodomain transcription factor, is vital because it is the earliest transcription factor identified to specify TE fate (15). \textit{Cdx2} knockout embryos fail to implant and fail to maintain trophoblast differentiation (15). These mutants also fail to downregulate \textit{Oct4} and \textit{Nanog} in the blastocyst,
two critical transcription factors that are involved in self-renewal of undifferentiated embryonic stem cells, resulting in subsequent death (15). Another gene, *Eomes (eomesodermin)*, a T-box transcription factor, has also been found to specify TE fate (16). *Eomes* knockouts form blastocysts and correctly express both *Oct4* in the ICM and *Cdx2* in the TE, but the trophoblast fails to differentiate any further (15,16). Finally, *Fgf4 (fibroblast growth factor 4)* knockouts have been found to implant but not to develop, subsequently resulting in early embryonic death (17). *Fgf4* and about twenty other genes have been identified as playing a role in trophoblast stem cell specification and maintenance (please refer to (3)). Failure to make TS cells results in lethality at implantation. However, if TS cells are made but not maintained, mutants can survive further, but not usually past about E9.5, and they are usually accompanied by placental irregularities of each of the layers at this stage (3).

There are several problems associated with abnormal development of each of the placental layers. Although little is known about the development of the spongiotrophoblast, several key knockout experiments have revealed that it is essential for placental development and embryonic survival. Perhaps the most well known gene that is essential for the development of the spongiotrophoblast is the imprinted basic helix loop helix (bHLH) transcription factor, *Ascl2 (achaete-scute complex homolog 2)*, which will be discussed at length in this thesis. *Ascl2* knockouts mutants have an expanded TGC layer and die at midgestation due to a lack spongiotrophoblast (18).

Several gene mutations are known to affect the production of glycogen cells or the production of glycogen within glycogen cells (please refer to (6,19,20,21,22)). For example, knockout of the imprinted gene *Cdkn1c (cyclin-dependent kinase inhibitor 1C)* leads to a lack of the production of glycogen cells (6). These studies point to the observation that although the role
of glycogen cells is largely unknown, they may be important for optimal placental development, but their presence is not essential for embryonic survival. Lack of glycogen cells is often accompanied by fetal and placental growth restriction (19,23). The origin of this cell lineage is still unclear but recent work based on the expression of *Pcdh12* in glycogen cells suggest that these cells might already be set aside in the E7.5 EPC (10).

Currently, only a couple of genes have been identified that affect TGC differentiation, *Hand1* (*heart and neural crest derivatives expressed 1*) and *I-mfa* (*myoD family inhibitor*), a bHLH transcription factor and an inhibitor of bHLH, respectively. *Hand1* knockouts die at about E7.5 due to defects in TGC differentiation (24). *I-mfa* knockouts die at about E10.5 due to a lack of TGCs, depending on the background of the mouse strain used (25). Interestingly, *Ascl2* is also thought to inhibit TGC formation, since the TGC layer becomes expanded when *Ascl2* is knocked out (18). *I-mfa* has been shown to interfere with *Ascl2* activity but not *Hand1* activity (25).

Over twenty gene mutations affecting allantoic development, chorionic development and chorioallantoic attachment have been described (please refer to (12)). In each of these cases, because of either allantoic failure or failure of chorioallantoic fusion, the labyrinth fails to form, resulting in embryonic lethality at midgestation. Over sixty gene mutations affecting branching initiation or branching morphogenesis have been described (12). In almost all of these cases, the resulting placental phenotype that occurs is what is known as a “small labyrinth”, or insufficient vascularization of the labyrinth and overall reduction in the labyrinth layer. A reduction in the labyrinth layer can result in viable pups, but they are usually growth restricted (26). Further, this is associated with vascularization problems in several mouse models of preeclampsia (26,27), a
common human placental disorder believed to cause high blood pressure in the mother due to inadequate maternal blood flow to the fetus (28).

1.3 Evolution of genomic imprinting and the placenta

Genomic imprinting is an epigenetic phenomenon resulting in the restricted expression of either the maternal or paternal allele of a gene, rather than the biallelic or silenced state that occurs for most genes. Imprinted genes can have a variety of functions (placentation, growth promotion and restriction, behaviour, and brain function) and a variety of epigenetic mechanisms have been implicated in the regulation of their monoallelic expression (methylation acquisition, histone modification, and noncoding RNA-mediated silencing). The imprinting field has sought a single unifying hypothesis to explain the evolution of genomic imprinting. However, in human aberrant genomic imprinting is associated with several syndromes and phenotypes including a number of placental abnormalities such as those found in placentae associated with intrauterine growth restriction (IUGR) (29)(30), an increased risk of childhood tumors (31), and mental retardation (32), obscuring a “unifying hypothesis”. Nonetheless, a few plausible theories exist that can help us understand the origin of this complex phenomenon.

The most highly favoured theory to date to help explain why genomic imprinting evolved is the kinship theory (33,34) (also referred to as the conflict theory). This theory was initially formed to address the mother/fetus relationship and refers to the asymmetrical contribution of paternal and maternal gene expression whereby the father’s best interests are served by increased fetal growth and those of the mother are served by smaller fetal size. Thus paternally inherited genes are programmed to extract as much as they can from the mother as possible through the placenta since this has little or no impact on the father (35). In contrast,
maternally expressed genes have evolved to prevent draining maternal resources prematurely, instead guaranteeing that maternal resources are handed out evenly to all of their offspring (35). The kinship theory is supported by several key examples, including imprinted genes involved in growth promotion (Igf2) and growth restriction (Cdkn1c), and placentation in mice (such as Ascl2, Phlda2, and Cdkn1c).

One limitation of the kinship theory is that it does not account for the genomic imprinting that occurs in phyla that lack placentae. Moreover, a prediction of the kinship theory is that paternal uniparental disomy (pUPD) should cause fetal overgrowth while maternal UPD (mUPD) should cause fetal undergrowth. While this has been observed in several cases such as in the Beckwith-Wiedemann syndrome region on chromosome 11 and the Dlk1-Gtl2 region on chromosome 14, other paternal UPDs are associated with a reduction in growth (36) or no phenotype at all (37). Another prediction of the kinship theory is that many maternally imprinted genes should be involved in post-weaning care, but to date, only a few have been found, such as Mest (38), Gnas1 (39) and Peg3 (40). However, an increasing number of imprinted genes are continuously being found to be consistent with this theory.

Another well established theory referred to as the ovarian time bomb (OTB) hypothesis states that imprinting evolved because it protects females from potential damage from the trophoblast (41). To support this, ovarian teratomas frequently arise from parthenogenetically activated oocytes and have the capacity of forming malignant trophoblast (41). Thus it may be possible that an allele that favoured imprinting would become predominant because it would increase fitness while reducing the risk of cancer in females (41). However, the OTB hypothesis has limitations as a general hypothesis because it cannot account for: 1) the large number of imprinted genes that do not have a specific role in trophoblast development; 2) the genomic
imprinting that occurs in phyla that lack placentae; and 3) imprinted genes silenced in the male germline.

The placenta is predicted to be the organ most affected in the kinship and OTB theories described above. Both theories support the concurrent evolution of genomic imprinting and origin of the mammalian placenta. It should be noted that some evidence suggests that genomic imprinting extends beyond placental mammals. Several organisms that lack placentae completely can exhibit parent of origin effects (42). In other cases, certain fish (43), reptiles (44), amphibians (45), and plants (46,47) have independently developed organs of analogous function to the placenta. For example, it has been shown that placentae evolved multiple times in the fish *Poeciliopsis* and that they are closely related to taxa that have either no placentae or “intermediate” placentae (43). It is unknown yet if imprinting occurs in these fish or organs. Whether genomic imprinting occurs in organisms closely related to placental mammals has also been studied. Imprinting has not been observed in monotremes which are egg-laying mammals, such as the platypus (48), but has rarely, if at all, been observed in birds (49). Paternal *Igf2* expression in two marsupial species has been observed (50). Marsupials are unique mammals in that although they have fully functional placentae, placental attachment and intrauterine life are relatively short.

### 1.4 Mechanisms of genomic imprinting in the mouse placenta

Currently there are over one hundred genes known to be clustered in twenty five genomic regions in the mouse that are subject to the phenomenon of genomic imprinting (please refer to the: Web atlas of murine genomic imprinting and differential expression (WAMIDEX) at https://atlas.genetics.kcl.ac.uk/). Imprinted genes tend to be imprinted in the developing embryo
and the placenta, and are often not expressed in adult tissues, suggesting they are primarily required for proper development and growth. Many imprinted genes in the embryo are not necessarily imprinted throughout the entire embryo but can also exhibit tissue-specific imprinting (51). To date, all imprinted regions are associated with the presence of differentially methylated regions (DMRs) (also referred to as imprinting centres in this thesis). Imprinted DMRs are DNA segments rich in CpG dinucleotides that are methylated on the chromosome from one parent but not methylated on the other parental chromosome. These parent-specific differential methylation patterns acquired during oogenesis or spermatogenesis are referred to as primary or gametic DMRs, and those acquired during embryogenesis are referred to as secondary or somatic DMRs.

Many genes that are not imprinted in the embryo are imprinted in the placenta, leading to the concept that different mechanisms have evolved to regulate genomic imprinting in extraembryonic tissues. In this section, we summarize what is currently known about mechanisms of genomic imprinting in the mouse placenta. There are two imprinted domains in the mouse that contain placental-specific imprinted genes: the \textit{Igf2r} domain and the \textit{Kcnq1} domain (also referred to as the imprinting centre 2 or IC2 domain in this thesis).

In the \textit{Igf2r} domain on mouse chromosome 17, there are at least four known imprinted genes (from proximal to distal orientation): \textit{Igf2r} (\textit{insulin-like growth factor 2 receptor}), \textit{Slc22a1}, \textit{Slc22a2}, and \textit{Slc22a3} (\textit{solute carrier family 1, 2, 3}). The antisense noncoding RNA (ncRNA) \textit{Airn} is expressed from the unmethylated paternal allele of the imprinting centre within intron 2 of \textit{Igf2r}. This ncRNA silences the paternal allele of \textit{Igf2r}, \textit{Slc2a3} and \textit{Slc22a2}. \textit{Slc22a1} remains biallelic in the placenta by an unknown mechanism. \textit{Airn} is thought to be about 108kb in length (52). The silencing is unidirectional since all of these genes silenced by \textit{Airn} only flank
one side of Igf2r. In the embryo, although Airn is not expressed, all of the Slc22a1, 2, 3 genes are biallelically expressed (53).

In the IC2 domain on mouse chromosome 7, there are at least nine known imprinted genes (from proximal to distal orientation): Ascl2, Tspan32, Cd81, Tsse4, Kcnq1, Cdkn1c, Slc22a18, Phlda2, and Osbpl5 (Figure 1.2). The antisense ncRNA Kcnq1ot1 arises from intron 10 of the paternal Kcnq1 allele and silences the paternal allele all of these protein-coding genes in the placenta. The silencing is bidirectional, similar to the Xist ncRNA on the X chromosome that coats the inactive X. While many of the genes in the IC2 domain are imprinted in both the placenta and embryo, about half of them are exclusively imprinted in the placenta (Ascl2, Cd81, Tsse4, and Osbpl5) (Figure 1.2). With the exception of Ascl2 which is silenced in the embryo, Cd81, Tsse4, and Osbpl5 are biallelic in the embryo (54).

Perhaps the role of the ncRNAs in the placenta in both cases is to recruit chromatin modifying machinery, which may not necessarily occur in the same manner as in the embryo. In the Igf2r domain, both Airn and the repressive histone 3 lysine 9 trimethylation (H3K9me3) mark have been shown to accumulate at the promoter of Slc22a3 (55). Further, a knockout of G9a, which is a histone methyltransferase (HMTase) that plays a role in H3K9 modification (56), results in biallelic Slc22a3 (55), suggesting that the interaction between Airn and G9a is important for silencing in the placenta at least in this domain. The imprinted genes in the IC2 domain have been shown to be marked by repressive histone marks H3K9me2 and H3K27me3 in an allele-specific manner on the paternal allele in the placenta, demonstrating that imprinted regulation in extraembryonic tissues differs from embryonic tissues which rely more heavily on DNA methylation (57,58). Although histone modifications are remarkably important in placental-specific imprinting, it has been difficult to pinpoint the HMTases implicated in
Figure 1.2 Distal mouse chromosome 7 and structures of the alleles.

The genes of the IC1 and IC2 domains are shown from left to right in the telomeric to centromeric orientation. Genes in grey are maternally expressed, genes in black are paternally expressed and genes in white are biallelicly expressed. Deletion alleles used in this thesis are shown above. DelTel7 is shown open-ended to indicate that the entire IC2 domain to the telomere was deleted. Figure not drawn to scale. The interval between IC1 and IC2 domains that extends from Ascl2 to Ins2 and contains the non-imprinted gene, Tyrosine hydroxylase (Th) is approximately 300kb. The IC2 domain from Osbpl5 to Ascl2 is about 800kb. The IC1 domain is approximately 90kb. The Kcnq1ot1 ncRNA (curvy line) is transcribed from the unmethylated paternal IC2. Genes that exhibit placental-specific imprinting are denoted with an asterisk.
imprinted silencing for the IC2 domain. G9a was found to have only a modest impact on the genes in the IC2 domain and therefore is not considered essential in maintaining silencing (59). EZH2 is a HMTase with a predominant role in H3K27 modification that forms part of Polycomb repressive complex 2/3 (PRC2/3), and also includes SUZ12 and EED. EED-EZH2 Polycomb complexes have been shown to be recruited to the genes in the IC2 domain on the paternal allele (57). EED-EZH2 complexes do not associate with IC2 but only preferentially associate with particular regions, such as Ascl2 and Cdkn1c, suggesting that other proteins or candidate regions are required for paternal repression along the distal paternal genes (57,60). Further, allele-specific histone modifications in all the IC2-regulated genes are no longer seen in placentae with a paternal inheritance of the IC2KO allele compared to wild type (58), suggesting that IC2 and Kcnq1ot1 are required in cis to establish repressive chromatin. This does not, however, exclude the possibility of the existence of IC2-independent mechanism of silencing on the paternal chromosome.

1.5 Genes in distal mouse chromosome 7 and the placenta

The distal region of mouse chromosome 7 (MMU7) provides an excellent model system to study the role of imprinted genes in the function of the mouse placenta for several reasons. First, several well known genes play critical roles in placental development in this region and mutants of these genes have marked impacts on placental development. Second, this is one of two imprinted regions that contains several of the genes that have been shown to contain placental-specific imprinted genes (the other is the Igf2r domain). Aberrant imprinting at IGF2 and CDKN1C has been implicated in the imprinted disorders BWS and Silver-Russell syndrome (SRS), which are both associated with abnormal placentation.
To date, the entire distal region of mouse chromosome 7 (Chr 7) contains at least twelve known imprinted genes, most of which are expressed from the maternal homologue in the placenta. This region is further divided into two subdomains, each regulated by its own differentially methylated region. In the imprinting centre 1 (IC1) domain, there are two well-known reciprocally imprinted genes, *H19* and *Igf2* (*insulin-like growth factor*), as well as the paternally expressed *Ins2* (*insulin 2*) gene.

The *H19* gene codes for a ncRNA of unknown function and is abundantly expressed in the developing embryo and placenta. It also codes for a microRNA called miR-675 (61) that has recently been shown to regulate the tumour suppressor retinoblastoma (RB) gene (62). *H19* knockout mice are 30% larger than wild type littermates (63), however, this is thought to be most likely due to the increase in *Igf2* expression also observed in these mice rather than the loss of *H19* expression. In addition, placentae from *H19* knockout mice exhibit placentomegaly, have increased numbers of glycogen cells and an increase in glycogen content than wild type littermates (64). *H19* is expressed throughout the mouse placenta with highest localization in the spongiotrophoblast.

IGF2 is a powerful fetal growth factor and mitogen and is differentially expressed in development and in specific tissues (65). It is expressed throughout the mouse placenta with highest localization in the spongiotrophoblast (66) (Oh-McGinnis et al, 2011, to be submitted). Paternal inheritance of a knockout of *Igf2* results in severe growth restriction (67) and in human, a decrease in IGF2 is also implicated in growth restriction (30). The placentae of these mice are also known to possess less glycogen (20). Knockout of a placental labyrinth trophoblast specific *Igf2* transcript (*Igf2* P0) has shed some light into the mechanism by which *Igf2* knockouts are growth restricted, since the PO transcript is the predominant isoform of *Igf2* expressed in the
placenta. These mutants exhibit placental growth restriction and a decrease in nutrient transfer to the fetus which ultimately affects fetal size (68).

There are actually two insulin genes in the mouse, \texttt{Ins1} and \texttt{Ins2}, which have redundant functions in the mouse (69). \texttt{Ins1} is not imprinted and \texttt{Ins2} has been found to be biallelically expressed in all tissues except that it is paternally expressed in the yolk sac (70). Whether \texttt{Ins2}, the most distal gene in IC1 domain, is imprinted in the placenta is unclear.

In the IC2 domain, the maternally expressed genes (from proximal to distal orientation) are: \texttt{Ascl2}, \texttt{Tspan32}, \texttt{Cd81}, \texttt{Tssc4}, \texttt{Kcnq1}, \texttt{Cdkn1c}, \texttt{Slc22a18}, \texttt{Phlda2}, and \texttt{Osbpl5} (Figure 1.2). \texttt{Ascl2}, \texttt{Tssc4}, \texttt{Cd81} and \texttt{Osbpl5} are maternally expressed in the placenta, while the rest of the genes in this domain are maternally expressed in both the placenta and embryo (Figure 1.2) (54). The corresponding paternal alleles of these genes are silenced in \textit{cis} in both the placenta and embryo by a poorly understood mechanism involving the ncRNA \texttt{Kcnq1ot1} initiated from IC2. \texttt{Kcnq1ot1} is the only paternally expressed transcript known to date in the IC2 domain and is strictly paternally expressed. It is currently estimated to be about 91kb in length (71), although some groups believe it may be even longer (M. Mann, unpublished results). It is localized to the nucleus, transcribed by RNA Polymerase II, and unspliced (72).

Not much is known about the roles of most of the genes in the placenta in the IC2 domain. Perhaps the gene with one of the most dramatic consequences to the placenta in this domain is \texttt{Ascl2}, since \texttt{Ascl2} knockouts die at midgestation due to a lack of spongiotrophoblast (18). In addition, they exhibit an expanded TGC layer. In this thesis, additional roles that \texttt{Ascl2} plays in the placenta will be discussed at length in chapters 3 and 4.

\texttt{Cd81} (\textit{cluster of differentiation 81}) is a member of the \textit{Tetraspanin} family and is known to play a variety of roles in human immune response. \texttt{Cd81} knockouts are viable, but \texttt{Cd81}
homozygous knockout females exhibit reduced fertility (73), while the impact in the placenta is unknown. Although it is known to be imprinted in the mouse placenta (54), its localization and function in the placenta is unknown.

The function of Tspan32 (tetraspanin 32) in the placenta is unknown. Members of the Tetraspanin superfamily are implicated in a variety of functions in human, including acting as anchoring proteins for a variety of other proteins to one area of the cell membrane. A knockout of Tspan32 results in problems associated with proper platelet function (74). It has been shown to be expressed in embryogenesis in primitive blood cells and later in hematopoietic organs in adult mouse (75). Thus perhaps the localization of Tspan32 is in the placental blood and its role in the placenta is associated with placental platelet function. Tspan32 was originally reported to be biallelic (76,77), but is now reported to be imprinted in the mouse placenta (57).

The IC2 domain was originally identified as a candidate tumor suppressing region because of the link this region has to Wilms’ tumor (WT) incidences and the large number of tumor suppressing candidate genes in the domain that have since been characterized to have other functions. One of these, Tssc4 (tumor suppressing subtransferable candidate 4), has an unknown function in the placenta. To the best of our knowledge, a knockout of Tssc4 has not yet been generated and its localization in the placenta is unknown. Tssc4 was previously reported to be biallelic (76), but has recently been shown to be imprinted, albeit in a leaky manner since a small amount of paternal Tssc4 expression can be detected in the mouse in the wild type setting (78) (Oh-McGinnis et al, 2011, to be submitted).

Kcnq1 (potassium voltage-gated channel, KQT-like subfamily, member 1) is known to play a role in human long QT and Jervell and Lange-Nielsen syndromes causing cardiac arrhythmia and congenital deafness (79), but is not itself implicated in the etiology of BWS (80),
although \textit{Kcnq1ot1} happens to be transcribed from this gene. \textit{Kcnq1} homozygous knockout mice are viable, but display a number of deficiencies such as deafness and gastric hyperplasia\cite{80}, while the impact in the placenta is unknown and has not been studied.

\textit{Cdkn1c} (\textit{cyclin-dependent kinase inhibitor 1C}) is a powerful growth inhibitor. Maternal inheritance of a \textit{Cdkn1c} knockout allele results in perinatal lethality, a reduced labyrinth, and an expanded spongiotrophoblast\cite{81}. \textit{Cdkn1c} knockout mice exhibit many features of preeclampsia\cite{26,27}, and therefore have been used as a model to study this disorder. More detail on this particular model will be given in section 1.7. \textit{Cdkn1c} is expressed in all cell types of the mouse placenta with highest expression in glycogen cells. In human, a decrease in CDKN1C has been associated with cases of BWS that also exhibited preeclampsia\cite{82}, while an increase in CDKN1C is also implicated in growth restriction\cite{30}.

\textit{Slc22a18} (\textit{solute carrier family 22, member 18}) is known to be imprinted in mouse placenta and codes for a multi-membrane-spanning protein similar to bacterial and eukaryotic metabolite transporters\cite{83}. Its role in the placenta is unknown. Although a \textit{Slc22a18} knockout has not been generated, transgenics in conjunction with other genes have been made in a few mouse models that will be discussed in section 1.6, with minimal to no known consequence to the placenta\cite{84,85}.

\textit{Phlda2} (\textit{pleckstrin homology-like domain, family A, member 2}) codes for a protein that is thought to play a role in regulating glycogen stores\cite{19}. Maternal inheritance of a \textit{Phlda2} knockout allele results in viable mice with placentomegaly, reduced labyrinth and expanded spongiotrophoblast\cite{86}, suggesting that one of its main roles is to restrict placental growth and increase labyrinthine proliferation. \textit{Phlda2} is exclusively expressed in the syncytiotrophoblast bilayer I of the labyrinth\cite{86}.
Osbpl5 (oxysterol binding protein-like 5) is the most distal gene on distal MMU7 that is known to be under the regulation of IC2 (87). It is exclusively imprinted in the placenta, but its function and localization in the placenta has not been established. To the best of our knowledge, a knockout of Osbpl5 has not yet been generated.

The Th (tyrosine hydroxylase) gene in the IC1-IC2 interval is the only protein-coding gene in the interval, although several transcripts of unknown function are thought to be expressed in this region (88). Further, recent preliminary work on Th reveals that a few retrotransposon-driven isoforms of Th exist in the rodent placenta that are maternally expressed and are under the regulation of IC2, extending the action of Kcnq1ot1 to an area that is much larger than what has been previously reported (88,89).

Genes that are not imprinted that are found in the IC2 domain are Trpm5 (transient receptor potential cation channel, subfamily M, member 5), Cars (cysteinyl-tRNA synthetase), Tnfrsf26 (tumor necrosis factor receptor superfamily, member 26), and Tnfrsf22 (tumor necrosis factor receptor superfamily, member 22). Genes with conflicting imprinted status that are found in the IC2 domain are Nap1l4 (nucleosome assembly protein 1-like 4) and Tnfrsf23 (tumor necrosis factor receptor superfamily, member 23) (78,90). Due to the difficulty of studying genes with conflicting reports of their imprinted status, many groups including ours have altogether avoided analysis of these genes when looking at IC2-regulated genes and have focused on the other more reliably imprinted genes. It is possible that these genes are not strictly maternally expressed but preferentially maternally biased, with some paternal expression, which may cause confusion as to their imprinted status. It is not clear how a gene(s) escapes imprinting from the Kcnq1ot1 ncRNA.
1.6 Imprinted genes in other domains and their functions in the placenta

Generally speaking, maternally expressed genes tend to restrict growth and paternally expressed genes tend to promote placental growth (91). Specifically, it seems that most imprinted genes studied to date tend to play roles in either: 1) placental growth and morphology, or; 2) fetal growth via nutrient transfer capacity of the placenta. In this section, we focus on several key imprinted genes found in other domains that play a significant role in each of these areas.

Several maternally expressed genes in the placenta have a significant impact on placental growth and morphology, many of which have already been described such as Ascl2, Cdkn1c, and Phlda2. Other maternally expressed genes that are critical for proper placentation are Grb10 and Esx1. Interestingly, with the notable exception of Ascl2 and Cdkn1c, defects in most of these imprinted genes still result in viable pups. But these pups are often growth restricted. Further, placentae of imprinted genes that have been knocked out at first glance may appear normal, but there are subtle size and morphological differences in these placentae that ultimately affect the well-being and growth of the fetus.

Grb10 (growth factor receptor-bound protein 10) encodes for a signaling adaptor protein (92). It is located on mouse chromosome 11 and has been linked in humans to Silver-Russell syndrome. In the mouse placenta, it is highly expressed in the labyrinth (93). Maternal inheritance of a Grb10 knockout allele results in viable mice with a variety of phenotypes including placentomegaly and a reduced labyrinth (93,94). Grb10 has been implicated to negatively regulate the insulin signaling pathway and the Igf1 (insulin-like growth factor 1) signaling pathway (95), but not the Igf2 signaling pathway (94), suggesting that more than one imprinted pathway than the well-known Igf2 pathway affects fetal and placental growth.
*Esx1 (Esx homeobox 1)* encodes for a homeobox gene and is located on the distal region of the X chromosome. The *Esx1* mutant phenotype includes growth retardation due to abnormal labyrinth morphogenesis and vascularization defects (96). This phenotype establishes the importance of not just imprinting on autosomes for proper placental development but also the contribution of the X chromosome in placental development, when the paternally inherited X chromosome is preferentially inactivated during X-chromosome inactivation (XCI) in the mouse trophoblast lineage.

In addition to *Igf2*, there are a number of paternally expressed genes in other imprinted domains in the developing mouse placenta that play key roles in placental growth, morphology, and nutrient transfer. The well known ones that have been identified to date include *Mest*, *Peg3*, *Peg10*, *Peg11*, *Dlk1*, and *Gnas1*. *Mest* (*mesoderm specific transcript homolog*, also known as *Peg1*) encodes an enzyme that has alpha/beta hydrolase activity and is located on proximal mouse chromosome 6 (38). Paternal inheritance of the *Mest* knockout allele results in growth restriction of the embryo and placenta, however there appear to be no other apparent abnormalities in these mutant placentae (38). Decreased MEST in human appears to be associated with growth restriction (30), although more recent evidence suggests no correlation (97). *Mest* has also been shown to play an important role in maternal behaviour, since mothers who have inherited a knockout allele from their fathers do not end up taking proper care of their pups (38) and it has also been implicated in angiogenesis (98).

*Peg3 (paternally expressed gene 3)* is a putative zinc finger transcription factor (99) and is located on proximal mouse chromosome 7. Paternal inheritance of the *Peg3* knockout allele results in growth retardation and lack of proper maternal behaviour (40), similarly to the *Mest* knockout phenotype. Both *Mest* and *Peg3* are highly expressed and imprinted in both the
developing and adult brain, which helps explain their roles in monitoring maternal nurturing behaviour and provides insight into the role of imprinted genes in the evolution of maternal care.

Retrotransposon-derived imprinted genes can also play a role in the proper development of the placenta and allows us to gain some insight into placental evolution. Such as is the case for Peg10 (paternally expressed gene 10) (100) and Peg11 (paternally expressed gene 11, also known as retrotransposon-like 1) (101), located on mouse chromosomes 6 and 12, respectively. Paternal inheritance of the Peg10 knockout allele results in mid-gestational lethality due to lack of spongiotrophoblast and labyrinth development (100). Paternal inheritance of the Peg11 knockout allele results in growth retardation, neonatal lethality and abnormalities of the fetal capillaries caused by incomplete material transport between the maternal and fetal blood (101). Peg11 is part of the Dlk1-Gtl2 imprinted domain which includes other key genes such as Dio3 (deiodinase, iodothyronine, type III), a regulator of thyroid hormone metabolism, and the noncoding maternally expressed Gtl2 (now known as maternally expressed gene 3 (Meg3)). The function of Gtl2 is unknown, but maternal inheritance of a knockout of the IG-DMR that is responsible for imprinting of Gtl2 and the rest of the genes in the domain results in perinatal lethality and the placental phenotype has not been described (102). Paternal inheritance of the Dlk1 (delta-like 1) knockout allele results in a number of abnormalities including pre- and post-natal growth retardation, skeletal abnormalities, and obesity (103), but no placental abnormalities were reported. Dio3 null mice possess hypothyroidism (104), also with no reported placental abnormalities. The placental phenotype of inheritance of mUPD of chromosome 12 is growth retardation (105), thus it may be the case that the majority of the placental phenotype seen in conceptuses with mUPD of chromosome 12 may be due to LOI of Peg11, and not the rest of the genes in this imprinted domain, whereas the majority of the embryonic phenotype observed in
mUPD of chromosome 12, as well as the orthologous human chromosome 14, may be due to LOI of *Dlk1*.

*Gnas1* (guanine nucleotide binding protein, alpha stimulating 1) encodes for a signaling protein called XLα and is located on mouse chromosome 2. Paternal inheritance of the *Gnas1* knockout allele results in a number of abnormalities including postnatal growth restriction, and problems with postnatal behaviour such as suckling (39), although no placental abnormalities have been reported. It would be of interest to determine if the placentae of these mutant mice are also growth restricted and abnormal since this could be predicted from the observed phenotype. The phenotypes described here caused by disruption of various imprinted genes support the importance of proper imprinted regulation in placental development.

1.7 Mouse models of the Beckwith-Wiedemann syndrome imprinted region

Originally described by Dr. Bruce Beckwith in 1963 followed independently by Dr. Hans Wiedemann in 1964, Beckwith-Wiedemann syndrome (BWS) is a rare pre- and post-natal overgrowth disorder affecting roughly 1 in 14 000 children at birth in the United States (106). The BWS and WT region in human has been mapped to human chromosome 11p15.5 and is orthologous to distal mouse chromosome 7 (MMU7). BWS is thought to be caused by deregulation of the paternally expressed *IGF2* (to biallelic) and/or the maternally expressed *CDKN1C* (to silenced). Here we examine key mouse models in the BWS imprinted region generated to date that have sought to address the mechanisms of imprinting on distal MMU7 and the roles of imprinted genes in the aetiology of BWS. With the generation of such mouse models has come a clearer understanding of the functional independence of IC1 and IC2 in regulating imprinting in this region, the critical role of IC2 in coordinating gene expression through long-
range epigenetic silencing, and the emergence of a model system of imprinting that has broader implications for understanding human imprinted disorders. Of course, many mouse models of the BWS imprinted region do not recapitulate aspects of the human disease, but nonetheless, they have greatly increased our understanding of the mechanisms of the ICs in this region.

The first mouse models generated to study BWS sought to overexpress Igf2 to confirm its central role in the human disorder. Igf2 transgenic mice overexpress Igf2 and mimic many of the human features of BWS such as pre- and post-natal overgrowth, macroglossia, organomegaly, some skeletal abnormalities, and neonatal lethality (107). This Igf2 mouse model is perhaps the mouse model that most closely resembles the wide spectrum of symptoms observed in the human disorder. Several other key Igf2 mouse models were generated prior to this that also provided insight into Igf2 function. Igf2 receptor (Igf2r) binds Igf2 and targets it for degradation. Igf2r knockout mice possess increased levels of Igf2 mRNA and thus display fetal overgrowth (108,109). Another Igf2 mouse model, Igf2-pUPD, which has paternal uniparental disomy (pUPD) of the distal mouse chromosome 7 region, results in biallelic expression of Igf2 (110). These mice exhibit features such as placental abnormalities and midgestational lethality. Similar to the model from Sun et al (1997) (107), when McLaughlin et al (1997) (110) generated androgenetic mouse chimeras, they observed whole body overgrowth and perinatal lethality. These mouse models collectively laid the groundwork for understanding the function of Igf2 in disease.

Defects at IC1 have been shown to directly impact Igf2 mRNA levels. A mouse line with paternal inheritance of point mutations introduced in certain CpGs of IC1, while leaving CTCF-binding and enhancer-blocking activity intact, results in biallelic expression of H19 and in 40% growth retardation in the pups (111). Maternal inheritance of these mutations does not affect H19
and Igf2 imprinting and does not result in any obvious phenotype. This study established the critical CpGs in IC1 required for maintaining imprinting of H19 and Igf2.

It has also been of notable interest to knock out Cdkn1c and assess the resulting effect on growth and whether there are any BWS-like symptoms that can be attributed to this deficiency, since about 50% of BWS cases are associated with epigenetic defects at IC2 leading to biallelic KCNQ1OT1 expression and CDKN1C silencing (112). Several groups have independently generated Cdkn1c knockout mice (113,114,115), all reporting slightly different phenotypes. The phenotypic differences observed in these models have not been addressed but may possibly be due to genetic background effects. The Cdkn1c knockout model generated by Zhang et al (1997) (113) exhibit organomegaly and abdominal wall defects, two classical features of BWS, in addition to neonatal lethality. However, other Cdkn1c knockout models do not exhibit any symptoms of BWS and had a 10% survival rate past the neonatal stage (114,115). One key difference between the Cdkn1c knockout models and the Igf2 transgenic models is that overgrowth has not been observed in the Cdkn1c knockout mouse, although it is unclear why this is the case.

To address the distinct, but overlapping roles of Igf2 and Cdkn1c in the development of BWS and to mimic BWS patients with paternal uniparental disomy of 11p15.5 exhibit both Igf2 overexpression and lack of Cdkn1c expression, a double mutant that possessed both a null mutation in Cdkn1c and Igf2 overexpression (loss of Igf2 imprinting) were bred together (116). These mice display many of the same features as the Igf2 transgenic model with additional symptoms such as placentomegaly and dysplasia, cleft palate, kidney dysplasia and polydactyly (116), and thus have been useful in helping to dissect the roles of each of the implicated genes in this disorder.
The first evidence that Cdkn1c imprinting was controlled by IC2 came from a bacterial artificial chromosome (BAC) mouse model used to study Cdkn1c regulation, which demonstrated that an element at least over 260kb away was controlling Cdkn1c expression (117). Shortly afterwards, a translocation of a few genes in the IC2 domain adjacent to Kcnq1 (Cdkn1c, Slc22a18, and Phlda2) was generated to determine if this would impact imprinting in this region (84). Cdkn1c expression was misregulated in the translocation mutants. At a time when it was unclear how Cdkn1c and the rest of the genes in the IC2 domain were regulated, both the BAC and translocation lines confirmed that an element(s) within or at least close to Kcnq1 was responsible for regulating the neighbouring genes.

To determine the role of IC2 in the expression of neighbouring genes, a targeted deletion of a 2.8kb region of intron 10 in the Kcnq1 gene was generated, referred to as the IC2KO mouse model (118). Paternal inheritance of the IC2KO deletion causes reactivation of at least six maternally expressed genes on distal MMU7, located proximal or distal of IC2 (118). +/-IC2KO mice are growth retarded by about 20-25% presumably due to the overexpression of growth inhibitors usually expressed only from the maternal allele, while maternal inheritance has no obvious phenotype (118). Although BWS is caused by loss, not increase, of distal gene expression, the IC2KO model has nevertheless established that the long-range bidirectional effect, requiring an unmethylated IC2, is critical for transcriptional silencing over 800kb. The silencing mechanism remains unknown, and the role of the ncRNA in this epigenetic mechanism is still unclear.

To determine if not only production but elongation of Kcnq1ot1 was also required for gene silencing, a transcriptional stop signal 1.5kb downstream of the Kcnq1ot1 transcription start site was inserted to prematurely truncate the Kcnq1ot1 transcript, referred to as the TermΔ mouse
model (51,119). This results in a derepression of the IC2-regulated genes, demonstrating that the entire full-length Kcnq1ot1 transcript is necessary for cis-silencing. Paternal inheritance of the Kcnq1ot1 premature transcript, consistent with the IC2KO mouse model, results in about 10-20% growth retardation, while maternal inheritance has no obvious phenotype (119).

To test whether IC1 and IC2 are functionally independent or if there are any long-range epigenetic interactions between the two domains, an 800kb YAC transgene carrying the entire IC2 domain was generated, referred to as the IC2 YAC mouse model (120). Most of the genes in this region are still appropriately imprinted outside of the distal MMU7 context, demonstrating that both IC1 and IC2 can function independently at ectopic sites. When the YAC is inherited paternally, there is no obvious phenotype and the progeny show normal body weights at birth compared to wild type (120), suggesting that the transgene is silenced upon paternal transmission, irrespective of context. However, maternal transmission causes growth retardation, suggesting that the genes in the transgene are expressed and thus the overall expression of Cdkn1c and other growth inhibitors are the cause of the defect in growth (120).

To confirm the independent function of IC1 in the absence of IC2 in the context of MMU7, we engineered a chromosomal truncation that resulted in a deletion of IC2 and the 2.7Mb domain, distal to the IC1 subdomain, replacing it with an artificial telomere, referred to as the DelTel7 mouse model (121). When this truncation is paternally inherited, there is no obvious phenotype. However, maternal transmission of DelTel7 leads to a mid-gestational lethality, consistent with the deletion of several maternally expressed genes in the distal IC2 sub-domain, and is also accompanied by abnormal placentation. Maternal inheritance of this truncation can be rescued by a paternally inherited IC2KO allele which has been previously described (118). Chapter 2 of my thesis will go into this in more depth. This demonstrates that all of the imprinted
genes in the distal IC2 sub-domain required for development are maternally expressed and normally under IC2 silencing on paternal MMU7. These results also imply that there are no additional paternally expressed genes in the mouse distal to the \textit{Ins2} region.

To determine if the 300kb intergenic region between the IC1 and IC2 interval plays a role in regulating imprinting in this domain, we generated a deletion of this interval called the $\text{Del}^{7\text{AI}}$ allele (122). Initial findings demonstrate that the ICs appear to behave normally whether the deletion is paternally or maternally inherited (124). Chapters 3 and 4 of my thesis will discuss new findings from studying this deletion allele.

The phenotypes of the mouse models of the BWS imprinted region described in this section are summarized in Table 1.2. In summary, to date, the studies described in this section have established: 1) human \textit{IGF2} LOI and/or \textit{CDKN1C} LOI as the main culprits in the etiology of BWS and that these genes have parallel effects in the mouse; 2) independent functions of the two ICs in the mouse; and 3) the role of \textit{Kcnqlot1} as the regulator of genomic imprinting in a large domain. As this thesis will demonstrate, we hope to further contribute to this field by investigation into the mechanism of IC2 and the IC1-IC2 interval.

\section*{1.8 Thesis theme and objectives}

The overarching objectives of this thesis are to understand the regulation of genomic imprinting using two mouse lines of the imprinted distal mouse chromosome 7 region that have recently been developed, called the DelTel7/IC2KO and $\text{Del}^{7\text{AI}}$ mouse lines. We have extensively outlined the Cre-\textit{loxP} recombination technology used to generate the DelTel7 and $\text{Del}^{7\text{AI}}$ alleles (123), both of which were made in Toronto by L. Lefebvre in the laboratory of A. Nagy (123), while the IC2KO allele was a gift from M. Higgins. The structures of all three
alleles are summarized in Figure 1.2 and are highlighted in more detail in Figures 2.1A, 2.2A, and 3.1A. In my second chapter, we determined the impact of paternal inheritance of the IC2KO allele, which acts as a promoter for the ncRNA Kcnq1ot1, and its ability to rescue the embryonic lethality associated with a maternal DelTel7 allele. Since Kcnq1ot1 is thought to be the key regulator of silencing in the IC2 region, the analysis of DelTel7/IC2KO placentae offers a unique opportunity to address whether all developmentally important genes are reactivated from the IC2KO allele. In my third and fourth chapters, we asked what happens upon both maternal and paternal inheritance of the Del7AI allele, respectively, and describe their impact. Thus, the objectives that will be studied in this thesis are:

i) To determine the consequences of rescuing a truncation of the distal MMU7 region (DelTel7/IC2KO) for placentation and development; and

ii) To determine the consequences on the regulation of imprinting in the distal MMU7 region upon inheritance of the Del7AI deletion.
<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Description of targeted region</th>
<th>Observed phenotype</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td><em>Igf2</em> transgenic</td>
<td><em>Igf2</em> expression constructs introduced in ES cells resulting in overexpression of endogenous <em>Igf2</em></td>
<td>overgrowth, macroglossia, organomegaly, skeletal abnormalities, neonatal lethality</td>
<td>[107]</td>
</tr>
<tr>
<td>IC1</td>
<td>Introduction of point mutations in CpGs of IC1</td>
<td>Maternal inheritance: no phenotype, Paternal inheritance: 40% growth retardation</td>
<td>[111]</td>
</tr>
<tr>
<td><em>Cdkn1c</em> KO</td>
<td>Targeted deletion of <em>Cdkn1c</em></td>
<td>neonatal lethality, organomegaly, abdominal wall defects, bone ossification defects</td>
<td>[113, 114, 115]</td>
</tr>
<tr>
<td><em>Cdkn1c</em> + <em>Igf2</em></td>
<td><em>Cdkn1c</em> deficiency and loss of <em>Igf2</em> imprinting</td>
<td>placentomegaly and dysplasia, kidney dysplasia, macroglossia, cleft palate, omphalocele</td>
<td>[116]</td>
</tr>
<tr>
<td><em>Cdkn1c</em> BAC</td>
<td>85kb BAC containing only <em>Cdkn1c</em></td>
<td>LOI of <em>Cdkn1c</em></td>
<td>[117]</td>
</tr>
<tr>
<td>Translocation</td>
<td>Translocation of <em>Cdkn1c</em>, <em>Scl22a18</em>, and <em>Pldha2</em> in the IC2 domain adjacent to <em>Kcnq1</em></td>
<td>LOI of telomeric genes, imprinting of centromeric genes maintained</td>
<td>[84]</td>
</tr>
<tr>
<td>IC2KO</td>
<td>2.8kb deletion of intron 10 of <em>Kcnq1</em> gene resulting in no <em>Kcnq1ot1</em> production</td>
<td>Maternal inheritance: no phenotype, Paternal inheritance: 20-25% growth retardation</td>
<td>[118]</td>
</tr>
<tr>
<td>TermΔ</td>
<td>Premature truncation of <em>Kcnq1ot1</em> transcript resulting in lack of full-length <em>Kcnq1ot1</em></td>
<td>Maternal inheritance: no phenotype, Paternal inheritance: 10-20% growth retardation</td>
<td>[51, 119]</td>
</tr>
<tr>
<td>IC2 YAC</td>
<td>800kb YAC transgene spanning entire IC2 domain</td>
<td>Maternal inheritance: growth retardation, Paternal inheritance: no phenotype</td>
<td>[120]</td>
</tr>
<tr>
<td>DelTel7</td>
<td>2.7Mb chromosomal truncation of entire IC2 domain</td>
<td>Maternal inheritance: mid-gestational lethality, placental abnormalities, Paternal inheritance: no phenotype</td>
<td>[121]</td>
</tr>
<tr>
<td>Del7Δ</td>
<td>280kb deletion between IC1 and IC2 domain</td>
<td>Maternal inheritance: 30% growth retardation, placental abnormalities, Paternal inheritance: no phenotype, partial <em>Ascl2</em> LOI</td>
<td>[23, 122]</td>
</tr>
</tbody>
</table>
Chapter 2: Rescue of placental phenotype in a mechanistic model of Beckwith-Wiedemann syndrome

2.1 Introduction

Genomic imprinting is the mechanism by which haploid maternal and paternal genomes carry different epigenetic marks, resulting in monoallelic transcription of a subset of genes which are expressed exclusively from either the maternal or paternal allele (124). To date, over eighty imprinted genes have been identified in humans and mice (90). Many of these genes are known to have critical roles in embryonic development and placentation (125) and have also been implicated in the regulation of postnatal behaviour (38,39,40). The imprinted region on distal mouse chromosome 7 (Chr 7) shares syntenic homology with human chromosome 11p15.5, a region associated with Beckwith-Wiedemann syndrome (BWS) and Wilms tumor. BWS is an imprinted disorder that is commonly characterized by macroglossia, organomegaly, abdominal wall defects, unusual facial features, hemihypertrophy, hypoglycemia, exomphalos, ear and renal anomalies (126). Roughly 5-10% of BWS patients are predisposed to a variety of childhood tumours, including hepatoblastoma, rhabdomyosarcoma, neuroblastoma, adrenal carcinoma, with Wilms tumor occurring most frequently (31,126).

Distal mouse Chr7 contains several key imprinted genes required for fetal development, two of which are also implicated in the etiology of BWS, the paternally expressed insulin-like growth factor-2 (Igf2) gene regulated by imprinting centre 1 (IC1, also known as H19 DMR) located upstream of the maternally expressed H19 gene and the maternally expressed cyclin-
dependent kinase inhibitor 1C (Cdkn1c) which is regulated by imprinting centre 2 (IC2, also known as KvDMR1) located within intron 10 of the Kcnq1 gene (127,128). In addition to Cdkn1c, the IC2 cluster contains at least eight other maternally expressed genes (Ascl2, Cd81, Tscc4, Kcnq1, Slc22a18, Phlda2, Osbpl5, and Dhcr7) (78,79,83,89,129,130,131,132,133). Of these, Ascl2, Cd81, and Osbpl5 have been shown to be expressed and exclusively imprinted in the placenta (57,58). The precise functions, if any, of Cd81, Osbpl5, Tscc4, Kcnq1 and Slc22a18 in the placenta remain to be elucidated. However, the remaining genes in the cluster (Ascl2, Phlda2, and Cdkn1c) have well-documented roles in placentation since knockouts of each of these genes result in drastic placental phenotypes. Ascl2 null mice fail to form a spongiotrophoblast layer, a defect leading to embryonic lethality at E10 (18,131); Phlda2 null mice are viable but show an expanded spongiotrophoblast and placentomegaly (86); Cdkn1c mutants possess varying phenotypes including a reduced labyrinth layer, expanded spongiotrophoblast, placentomegaly and perinatal lethality (113,114,115).

The mechanism of silencing of several genes both centromeric and distal to the position of the paternal IC2 over a large domain is not completely understood, though production and elongation of the ncRNA Kcnq1ot1 is thought to play a critical role in this process (51,119). Kcnq1ot1 production is accompanied by recruitment of Polycomb group complexes (134) and acquisition of repressive histone modifications (57,58). These observations suggest similarities with the action of other ncRNAs such as the regulation of Igf2r by the ncRNA Airn (135) and X-inactivation by Xist (136). The deletion of the paternal IC2 leads to activation of IC2-regulated genes on the paternal chromosome and +/-IC2KO mice possess a growth restriction phenotype at least in part because of biallelic expression of Cdkn1c (118,137). The placentae of these animals were also reported to be smaller than their wild type litter mates (85). Recent studies have also
shown that IC2 contains CTCF-binding sites which are occupied only on the unmethylated paternal allele however it is still unclear what role these CTCF binding sites might play in silencing the paternal alleles of IC2-regulated genes (138). It is also not known whether the methylated maternal IC2 allele is important for the activation of nearby genes.

We previously described an engineered truncation of distal Chr 7 with a breakpoint upstream of the Ins2 gene (121). The resultant 2.7-Mb deletion, called DelTel7, removes the entire IC2-regulated domain along with the telomeric end of the Chr 7 domain, replacing it with an artificial telomere. When the DelTel7 allele is paternally inherited (+/DelTel7 hemizygotes), there is no obvious phenotype, showing absence of paternally expressed imprinted genes required for development or of a haploinsufficiency effect (121). Maternal transmission of DelTel7 (DelTel7/+ hemizygotes) however, leads to a mid-gestational lethality, consistent with the deletion of all maternally expressed genes in the IC2-regulated sub-domain, and is also accompanied by abnormal placentation including the lack of spongiotrophoblast and an expanded trophoblast giant cell layer (121). Despite a difference in phenotypic outcome, possibly attributable to the absence of imprinting of essential genes such as ASCL2 in humans (139,140), maternal inheritance of DelTel7 provides a mechanistic model for BWS, which is often associated with loss of expression of maternally expressed genes (127). Remarkably, we also demonstrated that the lethality observed upon maternal inheritance of this truncation can be rescued in trans by a paternally inherited IC2KO allele, which was previously shown to abolish Kcnq1ot1 transcription and its associated epigenetic silencing (118). The compound heterozygous DelTel7/IC2KO pups survive to term but it is unclear whether any placental abnormalities or defects in placental imprinted gene expression remain in the double mutants. Because of the importance of several IC2-regulated genes in placental function and development,
we present here a characterization of the rescued DelTel7/IC2KO placentae to determine whether they are comparable to their wild type litter mates and whether deletion of IC2 leads to a full rescue of the placental phenotype caused by the loss of maternal genes from the DelTel7 allele. Our assessment would have direct implications for BWS patients, since placental abnormalities usually accompany BWS fetuses (141,142,143). By examining the architecture, vasculature, and imprinted gene expression of the rescued DelTel7/IC2KO placentae we sought to determine whether it is possible to correct imprinting defects by restoring gene expression for a large number of genes in trans and thereby to further elucidate mechanisms of IC2-mediated epigenetic silencing.

2.2 Results

2.2.1 Loss of imprinted gene expression in DelTel7/+ placentae

The IC2 imprinted domain of distal Chr 7 contains at least several maternally expressed mRNA genes and spans almost 800kb. The entire domain is deleted in the DelTel7 allele, a truncation of Chr7 with a breakpoint upstream of Ins2 and deleting the last 2.7Mb of Chr7 (Figure 2.1A). We previously showed that maternal transmission of DelTel7 leads to abnormal placentation and embryonic lethality at E10.5, whereas paternal hemizygotes are viable (121). We show here that by immunohistochemistry for PHLDA2 (Figure 2.1B) as well as quantitative RT-PCR (qRT-PCR) for the imprinted genes Phlda2, Ascl2, and Cdkn1c, that E9.5 DelTel7/+ maternal hemizygous placentae are deficient for expression of these IC2-regulated genes. Placentae were assessed at E9.5, before the embryonic lethality of the DelTel7/+ embryos. We found only basal transcription levels of expression by qRT-PCR for these IC2-regulated genes in DelTel7/+ placentae (Figure 2.1C). Wild type levels were quite varied between samples,
Figure 2.1 Expression of IC2-regulated genes in DelTel7/+ at E9.5.

A) Diagram of distal mouse chromosome 7 (Chr 7) region showing the locations of the imprinting centres 1 (IC1) next to H19 and 2 (IC2) in intron 10 of the Kcnq1 gene, as well as the protein-coding genes known to be regulated by IC2 (Osbpl5, Phlda2, Cdkn1c, Kcnq1, Tssc4, Cd81, and Ascl2). This entire IC2-regulated cluster is deleted in the DelTel7 allele in which an array of telomere repeats (Tel array) was introduced distal of Ins2 [36].

B) PHLDA2 immunohistochemistry (IHC) on sections of wild type (+/+ ) and maternal hemizygous (DelTel7/+) placentae at E9.5. PHLDA2 IHC was performed on two placentae of each genotype. The PHLDA2 protein, localized to the labyrinth of the wild type placenta, is absent in DelTel7/+ mutants. Note the lack of spongiotrophoblast and expanded giant cell layer in the DelTel7/+ placenta. Scale bar: 1mm. sp: spongiotrophoblast, lab: labyrinth, Gi: giant cells.

C) qRT-PCR analysis of the imprinted genes Phlda2, Ascl2, and Cdkn1c, in E9.5 DelTel7/+ placentae reveals virtually no expression from the intact paternal allele. Placentae were assessed at E9.5 before the embryonic lethality of the DelTel7/+ observed at around E10.5. qRT-PCR was performed on five DelTel7/+ placentae and five wild type placentae with three technical replicates per individual placenta. Expression is relative to the reference housekeeping gene, Peptidylprolyl isomerase A (Ppia). The error bar is the standard deviation from five biological replicates, assayed in triplicates. p<0.02 for each qRT-PCR (t-test, unpaired, two-tailed).
suggesting dynamic changes in expression at this early developmental stage or the genetic heterogeneity present in these crosses on mixed background. Note the lack of spongiotrophoblast and the expanded giant cell layer in the DelTel7/+ placenta (Figure 2.1B), consistent with what has been published previously regarding the DelTel7/+ phenotype (121).

Other than the entire IC2 sub-domain, DelTel7 also deletes the very distal end of Chr7. This region contains close to 20 annotated genes. Unlike genes in the IC1 and IC2 domains, we find that most of these distal genes are not expressed in the placenta at significant levels (Figure A.1). The absence of an obvious phenotype in paternal hemizygotes for DelTel7 argues against haploinsufficiency effects in these mutants.

2.2.2 Germline transmission from DelTel7/IC2KO mice

IC2 is comprised of CpG-rich sequences within intron 10 of the *Kcnq1* gene and functions in gene silencing, at least in part, by serving as a promoter for the antisense ncRNA *Kcnq1ot1*. We previously showed that paternal transmission of the IC2KO allele can rescue the embryonic lethality phenotype of DelTel7/+ embryos (121). The rescued DelTel7/IC2KO mice are missing the genes that are normally maternally expressed (they are deleted in DelTel7) but expression of these genes is now supplied from the paternal allele, since the IC2KO allele no longer produces *Kcnq1ot1* (Figure 2.2A) (118). We confirmed the absence of *Kcnq1ot1* expression in DelTel7/IC2KO embryos and placentae by strand-specific RT-PCR (Figure 2.2B). Phenotypic analysis of these compound heterozygous mice therefore allows us to ask the following questions: (i) Are all the maternally-expressed genes required for normal placental development expressed from the IC2KO paternal allele? (ii) Is the methylated IC2 required in the regulation of maternally-expressed genes on the maternal allele in wild type embryos?
Figure 2.2 Structure of the DelTel7 allele and absence of *Kcnq1ot1* in rescued DelTel7/IC2KO embryos.

A) Simplified representation of the structure of distal Chr7 in the DelTel7/IC2KO rescued animals, revealing the DelTel7 truncation on the maternal allele and the absence of IC2 and lack of activation of *Kcnq1ot1* expression on the paternal allele. M=maternal; P=paternal. B) RT-PCR demonstrating lack of *Kcnq1ot1* expression in E9.5 DelTel7/IC2KO embryo and placenta.
We have previously shown that DelTel7/IC2KO pups survive to term, are recovered at the expected frequency and are indistinguishable from their wild type litter mates at birth (121). We now report that DelTel7/IC2KO males and females are fertile and pass on the mutant alleles at expected frequencies in their offspring. DelTel7/IC2KO males crossed with wild type (+/+ ) females produce litters of +/DelTel7 and +/IC2KO pups at roughly equal frequencies (Table 2.1). DelTel7/IC2KO females crossed with wild type males produce litters of only the IC2KO/+ genotype as expected, since the DelTel7/+ genotype is embryonic lethal (Table 2.1). Mice produced from these crosses do not possess any obvious developmental abnormalities and are able to survive to adulthood. These results demonstrate that the maintenance of imprinting is faithfully recapitulated in each generation and that there are no aberrant effects of transmitting either allele in the context of the compound heterozygotes.

2.2.3 DelTel7/IC2KO placentae exhibit apparently normal architecture

Because the DelTel7/+ genotype results in abnormal placentation, we investigated the histology of several DelTel7/IC2KO placentae compared to wild type litter mates to determine whether there were any abnormalities in placentation in the compound heterozygotes. Hematoxylin and eosin stain (H&E) revealed no apparent abnormalities between DelTel7/IC2KO and wild type placentae at E14.5, suggesting that the DelTel7/IC2KO placentae displayed normal placental architecture (Figure 2.3A and 2.2B). To document this in greater detail, we used in situ hybridization (ISH) and immunohistochemistry (IHC) to analyze the distribution of the several lineage markers in wild type and DelTel7/IC2KO placentae. Neither ISH for the placental lactogen-II gene Prl3b1 (Pl-II), a mid- to late gestation marker of the giant
Table 2.1 Transmission of the DelTel7 (Δ) and IC2KO alleles from compound heterozygotes.

p=0.000532 for crosses involving wild type males and rescued females using the chi-square ($\chi^2$) test and p=0.7324 for crosses involving rescued males with wild type females. The chi-square ($\chi^2$) test determines whether genotype ratios differs significantly from Mendelian ratios.

<table>
<thead>
<tr>
<th>male</th>
<th>female</th>
<th>progeny</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Δ het</td>
<td>IC2KO het</td>
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<tr>
<td>+/+</td>
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<td>Δ/IC2KO</td>
<td>+/+</td>
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Figure 2.3 Overall placental architecture of the DelTel7/IC2KO placentae at E14.5 by representative H&E and ISH.

A) H&E stain of DelTel7/IC2KO placentae (n=5) compared with wild type (+/+; n=5). Scale bar: 1 mm. B) Zoom-in of boxed regions in panel A. Scale bar: 0.5 mm. C) Analysis of Prl3b1 (Pl-II) expression by ISH of DelTel7/IC2KO placentae (n=5) compared with wild type (n=6). Scale bar: 0.25 mm. D) Tpbpa (4311) ISH of DelTel7/IC2KO placentae (n=4) compared with wild type (n=4) Scale bar: 0.25 mm.
cell layer, spongiotrophoblast, and mononucleated trophoblast cells of the labyrinth (Figure 2.3C) (144), nor ISH for Tpbpa (4311), a marker of the spongiotrophoblast layer (Figure 2.3D) (5) revealed any overall differences in these layers between the two genotypes.

To determine whether there was a defect in the generation of the glycogen cell (GC) lineage in the DelTel7/IC2KO placentae, both Periodic acid Schiff (PAS) stain and ISH for Protocadherin-12 (Pcdh12), a gene specifically expressed in GCs (10) were performed (Figure 2.4A and 4B). These analyses revealed no difference in the overall number or distribution of GCs between the two genotypes. For a detailed examination of the labyrinth, we performed IHC for laminin, a marker of the basement membrane in the labyrinth used for identifying fetal blood vessels (Figure 2.4C) (145) and ISH for the imprinted paternally expressed gene 1 (Peg1/Mest) which is also expressed in the fetal endothelial cells that line the fetal capillaries (Figure 2.4D). No abnormalities were observed in the cell lineages analyzed or in the fetal capillaries of the labyrinth of DelTel7/IC2KO placentae. Our results show that DelTel7/IC2KO placentae are phenotypically similar to wild type litter mates for the various representative markers analyzed.

To confirm the results of these marker analyses using an independent method, we also performed expression analysis for Tpbpa (4311) and Peg1 qRT-PCR and observed no difference between the two genotypes (Figure 2.5).

### 2.2.4 DelTel7/IC2KO placentae exhibit normal vasculature

Because we had no indication from our lineage marker analysis that the DelTel7/IC2KO placentae had a drastically abnormal phenotype, we explored the possibility that they may possess a subtle placental phenotype. One of the most common subtle placental phenotypes is an undervascularized labyrinth, otherwise known as a “small” labyrinth (12). Thus in addition to
A) Trophoblast glycogen cells are highlighted by periodic acid Schiff (PAS) staining of DelTel7/IC2KO placentae (n=4) compared with wild type (n=4). Scale bar: 1 mm. B) Detection of *Pcdh12*, a glycogen cell marker, by ISH on DelTel7/IC2KO placentae (n=4) compared with wild type (n=4). Scale bar = 0.25 mm. C) Extraembryonic mesoderm of the labyrinth layer detected by laminin IHC on DelTel7/IC2KO placentae (n=4) compared with wild type (n=4). Scale bar = 0.25 mm. D) *Peg1/Mest* ISH of DelTel7/IC2KO placentae (n=4) compared with wild type (n=4). Scale bar = 0.25 mm. GCs: glycogen cells; sp: spongiotrophoblast; lab: labyrinth.
Figure 2.5 Expression of placental marker in E14.5 placentae.

qRT-PCR analysis of the expression levels of the placental marker *Tpbpa* (4311) and *Peg1* in wild type (+/+) and rescued DelTel7/IC2KO (Δ/IC2KO) placentae at E14.5. Expression is relative to the reference housekeeping gene, *Peptidylprolyl isomerase A (Ppia)*. The error bar is the standard deviation from three biological replicates, assayed in triplicates. p>0.6 for each qRT-PCR (*t*-test, unpaired, two-tailed).
examining placental architecture, we also assessed whether the vascularization of the DelTel7/IC2KO placentae was perturbed using the vascular corrosion casting method (146). We found no apparent differences in the overall structure and number of capillaries in the fetal vasculature of DelTel7/IC2KO placentae compared to wild type litter mates at E14.5 (Figure 2.6A-D). Moreover, morphometric analysis of the average capillary diameter revealed no overall difference between DelTel7/IC2KO placentae and wild type placentae (Figure 2.6E), with both having an average of approximately 15 microns. This measure is in agreement with the previously documented average capillary diameter in wild type placentae (8).

2.2.5 DelTel7/IC2KO placentae exhibit proper expression of imprinted genes

To determine whether DelTel7/IC2KO placentae express the IC2-regulated genes from the paternal allele in a normal cell-type specific manner, we analyzed the expression of three key genes in the distal Chr 7 region strongly associated with placentation: *Phlda2*, *Ascl2*, and *Cdkn1c* (Figure 2.7). We chose to look at stages in which the expression level of each of the genes is high or at its highest according to our own observations as well as previously published work (86,117,147). IHC for *Phlda2* on E10.5 DelTel7/IC2KO placentae revealed exclusive expression in trophoblast cells of the labyrinth, consistent with wild type litter mates. ISH for *Ascl2* on DelTel7/IC2KO placentae revealed exclusive expression in cells of the spongiotrophoblast layer with some expression in glycogen cells (GCs), consistent with wild type litter mates. ISH for *Cdkn1c* on DelTel7/IC2KO placentae revealed high expression in GCs and low expression throughout other cell types in the labyrinth, as seen in wild type litter mates (Figure 2.7). The relative expression level of each of these genes was confirmed by quantitative RT-PCR (qRT-PCR). No statistically significant differences in expression levels between DelTel7/IC2KO and
Figure 2.6 Feto-placental vasculature of DelTel7/IC2KO placentae at E14.5.

A) Representative scanning electron micrographs of DelTel7/IC2KO fetal vasculature casts of whole placentae (n=4) compared with B) wild type litter mates (n=4). C) In-depth view of fetal capillaries of DelTel7/IC2KO compared with D) wild type. E) Morphometric analysis of fetal capillary diameters between DelTel7/IC2KO (Δ/IC2KO; 226 measurements from 4 placentae) and wild type (+/++; 144 measurements from 4 placentae) placentae. Scale bar for A) and B) =1 mm. Scale bar for C) and D) = 250 microns. Note that in B) the umbilical vein has been dissected away at the primary branch points.
Figure 2.7 Imprinted gene expression in DelTel7/IC2KO placentae.

The expression of three IC2-regulated genes was analyzed in wild type (+/+) and rescued (DelTel7/IC2KO) placentae on placental section (top) and by qRT-PCR on total placental RNA (bottom). A) Phlda2 expression analyzed by IHC on paraffin sections of wild type (n=4) and mutant (n=4) placentae at E9.5. The qRT-PCR was performed on E9.5 placental RNA; DelTel7/IC2KO and +/+. B) Ascl2 expression analyzed by ISH on frozen placental sections at E14.5; n=4 for DelTel7/IC2KO and wild type. Ascl2 expression by qRT-PCR at E14.5 on mutant placentae compared with +/-; C) Cdkn1c expression in DelTel7IC2KO placentae (n=9) at E14.5 compared with +/- (n=9). Cdkn1c expression by qRT-PCR at E14.5 on mutant placentae compared with +/-; Sense probes not shown. GCs=glycogen cells; lab=labyrinth; sp=spongiotrophoblast; Gi=trophoblast giant cells. The blue and brown stains show gene and protein expression, respectively. All scale bars: 0.25 mm. All qRT-PCRs at E9.5 were performed on five DelTel7/IC2KO placentae and five wild type placentae with three technical replicates per individual placenta. All qRT-PCRs at E14.5 were performed on three DelTel7/IC2KO placentae and three wild type placentae with three technical replicates per individual placenta. All expression results by qRT-PCR are shown normalized to the Ppia reaction and results are shown ± SD. First, the average from each placenta was calculated from technical replicates from the same cDNA. Then the SD was calculated from the average of biological replicates per genotype. For the three genes tested the differences between wild type and mutant placentae are not statistically significant (p>0.7). D) qRT-PCR of the other imprinted genes in the IC2 domain, Tssc4, Kcnq1 and Cd81, in E9.5 DelTel7/IC2KO placentae reveals no overall difference from wild type (p>0.2). qRT-PCR was performed as described above on five placentae of each genotype.
wild type placentae were observed for *Phlda2*, *Ascl2* and *Cdkn1c* at the stages analyzed (Figure 2.7A-C). Additionally, an examination of expression level of several of the other imprinted genes in the IC2 region (*Tssc4*, *Kcnq1*, and *Cd81*) by qRT-PCR at E9.5 revealed no significant differences between DelTel7/IC2KO and wild type placentae (Figure 2.7D).

2.3 Discussion

Placental abnormalities such as placentomegaly (143) are commonly observed in mothers carrying BWS fetuses. Mouse models of the BWS imprinted region such as the *Igf2* transgenic mouse model (107) and *Cdkn1c* null mouse models (113,114,115) which accurately recapitulate several phenotypic characteristics of the human disorder, are also accompanied by severe placental abnormalities, highlighting the importance of determining whether any placental abnormalities exist in the DelTel7/IC2KO mouse model. In these compound heterozygotes, IC2-regulated imprinted genes such as *Cdkn1c* are deleted from the maternal allele and can only be expressed from the paternal chromosome because of the IC2 deletion. Our studies have shown that rescuing the deleted IC2 imprinted domain results in appropriate placentation with respect to placental architecture, placental vasculature and imprinted gene expression. Our findings lead us to propose that rescuing an imprinting defect can also rescue the associated placental phenotype, which may have implications for BWS patients who have associated placental defects.

It has been postulated that mechanisms other than transcription and elongation of *Kcnq1ot1* may influence repression of the paternal distal Chr 7 genes. For example, *Cdkn1c* imprinting has previously been shown to be controlled by an element some distance away from the gene itself in a bacterial artificial chromosome (BAC) mouse model of *Cdkn1c*, which failed to reproduce appropriate *Cdkn1c* imprinting (117). In another study, a yeast artificial
chromosome (YAC) transgene containing the entire IC2 domain was shown to recapitulate faithful imprinting of most of the IC2-regulated genes (*Phlda2*, *Slc22a18*, *Kcnq1* and *Cdkn1c*), but loss of imprinting of *Ascl2* and *Tssc4* was observed when the YAC was paternally inherited (120). Both of these genes lie centromeric to IC2 and suggests that perhaps these genes require additional control elements beyond *Kcnq1ot1* transcription on the paternal allele that are absent on the YAC transgene. The results of our study differ from the above in that we have demonstrated that the absence of *Kcnq1ot1* transcription and/or deletion of other sequences in IC2 not only leads to full and normal expression of the paternal distal Chr 7 genes but to a full rescue of our DelTel7 placental phenotype, suggesting that this locus is the single most critical component in maintaining epigenetic silencing in this region. Since normal placental phenotype and placental expression of IC2 genes is observed in DelTel7/IC2KO conceptuses, our results also suggest that the normally methylated maternal allele of IC2 and its potential association with methyl-CpG-binding factors is not necessary in regulating the expression of the genes analyzed.

The placentomegaly resulting from maternal inheritance of a *Phlda2* null allele has been shown to be almost completely rescued by paternal inheritance of the same IC2KO allele used in this study (85), providing evidence of proper *Phlda2* expression levels from the paternal IC2KO allele. To the best of our knowledge, the DelTel7/IC2KO model is the first example of rescue of a large disrupted imprinted region, not just a single gene. We demonstrate that restoration of gene expression for a large number of genes *in trans* is possible and our work demonstrates the potential ability to rectify placental abnormalities caused by imprinting defects. Future studies include developing RNAi to knock down *Kcnq1ot1* during preimplantation stages in DelTel7 pregnant females to determine if this can rescue placental and embryonic phenotypes. Such experiments would suggest that it may be possible to therapeutically target the ncRNA as a step
towards aiding BWS patients with maternal defects at IC2 that result in maternal *Kcnq1ot1* reactivation.

The DelTel7 deletion offers an opportunity to assess the phenotypic consequences of loss of function for a cluster of maternally expressed genes on distal Chr7. Here we show that deletion of the *Kcnq1ot1* promoter and nearby sequences (IC2KO) on the paternal allele can fully rescue the placental phenotype associated with maternal inheritance of DelTel7. In the viable conceptuses, monoallelic expression of imprinted genes is reversed and is now provided by the non-silencing alleles on the paternal IC2KO chromosome. Our results show that all the genes required for normal placentation, missing in the DelTel7 allele are regulated by IC2 and the ncRNA *Kcnq1ot1* on the paternal allele during normal development. The methylated maternal allele of IC2 is not implicated in the regulation of these genes. Our work establishes the possibility of rescuing imprinted disorders such as BWS *in trans*, by interfering with the expression or the long-range silencing function of the regulatory ncRNA *Kcnq1ot1*.

### 2.4 Materials and methods

#### 2.4.1 Mice and genotyping

For all genotypes, the maternal allele is always given first. We previously presented the generation of the DelTel7 mouse line and genotyping of the DelTel7 allele (121). Genotyping of the IC2KO allele (KvDMR1) is as previously described (118). Females heterozygous for the deletion allele (+/DelTel7) maintained on the outbred CD-1 background were mated to +/IC2KO males on the C57BL/6 background and placentae at various developmental stages were dissected. All animal experiments were performed under certificate A07-0160 from the UBC.
Animal Care Committee and complied with the national CCAC guidelines to the ethical care and use of experimental animals.

2.4.2 RT-PCR and quantitative PCR

E9.5 and E14.5 placentae were dissected in cold PBS and snap frozen on dry ice. Placental RNA was extracted by Trizol (Invitrogen) and cDNA was synthesized using SuperScript II (Invitrogen). For Kcnq1ot1 detection, a gene specific primer for Kcnq1ot1 was designed (Kcnq1ot1 GSP 5’CACATACACACACCCAACCTCG 3’) and PCR was performed under standard conditions. Primers for the Ppia reaction have been previously described (148). Quantitative RT-PCR was performed in technical triplicate on five individual placental samples of each genotype at E9.5 and three samples at E14.5 (+/+ and DelTel7/IC2KO) for all genes analyzed. For Cdkn1c, forward primer (5’ GCGCAAAACGTCTGAGATGAG 3’) and reverse primer (5’ CAGCCGAAGCCCAGAGTTC 3’) were used. For Phlda2, forward primer (5’ CCCGCCAAGGAGCTGTTT 3’) and reverse primer (5’ CCTTGTAATAGTTGGTGACGATGGT 3’) were used. For Ascl2, forward primer (5’ TCCTGGTGACCTACCTGCTT 3’) and reverse primer (5’ AGGTCAGTCAGCACTTGGCATT 3’) were used. For Tssc4, forward primer (5’ ACGGGTGTCAGGTCGTATGG 3’) and reverse primer (5’ TGAGGGAGACGGTGTCAGAAG 3’) were used. For Kcnq1, forward primer (5’ AGAAGCAGGAGCAGGACTC 3’) and the reverse primer has been previously described (129). For Cd81, forward primer (5’ CTGGCTGGAGGCGTGATC 3’) and reverse primer (5’ TGGGTGCCGGTTTGTTTC 3’) were used. For 43I1, forward primer (5’ CAGCTTTGGAGCTACAGGCTATT 3’) and reverse primer (5’ TGGGTGCCGGTTTGTTC 3’) were used. For Peg1, forward primer (5’
TGTCCATCCCCATTCATTTT 3’) and reverse primer (5’ GAGTTCCAGCTGCCTGATTCC 3’) were used.

2.4.3 Immunohistochemistry

E9.5 and E14.5 placentae were dissected in cold PBS and fixed in fresh 4% paraformaldehyde overnight at 4°C. Paraffin-embedded sections were baked, cleared of paraffin and rehydrated by a series of xylene and ethanol washes. Antigen retrieval was done by microwaving slides in 1mM EDTA, pH 7.5. Endogenous peroxidase blocking was done in 0.3% hydrogen peroxidase for 30 minutes. Blocking was done with 5% goat normal serum (Vector Labs) in 1xTBS-T with 0.5% BSA for 30 minutes. To detect Phlda2 protein in the labyrinth layer, and to identify fetal capillaries of the labyrinth layer, a 1:1000 dilution of the Phlda2 antibody (gift from Ben Tycko) and a 1:25 dilution of rabbit polyclonal anti-laminin antibody (Sigma) were used, respectively. Incubation was done overnight at room temperature for Phlda2 antibody and 1 hour for laminin antibody. A 1:500 dilution of the biotinylated anti-rabbit IgG secondary antibody (Vector Labs) was used for 1 hour and after a series of TBS-T washes, the staining was performed with the Vectastain Elite ABC kit and DAB substrate (Vector Labs). Counterstain was done with hematoxylin (Sigma) for about 1 minute and the slides were dehydrated in a series of ethanol and xylene washes before mounting with Entellan mounting medium (EM Science) and coverslipped with glass.

2.4.4 In situ hybridization

Antisense and sense strand probes were DIG-labelled using 10X DIG labeling mix (Roche) and T7, SP6, or T3 RNA polymerases (Roche). Probes were digested with DNaseI and
purified by LiCl precipitation. Placentae at various stages were dissected in PBS and fixed in fresh 4% paraformaldehyde/1xPBS (RNase free) overnight at 4°C. The next day, they were washed in PBS and left overnight in 30% sucrose in PBS. They were allowed to equilibrate in OCT (Tissue-Tek) for half an hour at room temperature before embedding in OCT on dry ice. The blocks were stored at -80°C until ready to be used. Cryostat sections were cut at 10 microns and stored at -20°C until next day when hybridized. Sections were first thawed at 50°C, fixed in 4% PFA, treated with Proteinase K (final concentration of 40ug/ml) and acetylated in acetic anhydride to reduce background. Sections were then prehybridized in hybridization buffer (50% formamide, 5x SSC, 5x Denhardt’s, 0.25mg/ml Yeast tRNA) at 60°C for 3 hours before hybridization at 55°C overnight. The next day, non-specific probe was removed by a series of SSC washes and treated with RNase A before blocking for 1 hour in 1% blocking buffer (Roche) at 37°C. Sheep anti-DIG-alkaline phosphatase conjugated antibody (1:2000) (Roche). The BCIP (Roche) and NBT (Roche) reaction was used to detect the signal. After three 1xPBS washes, sections were fixed in a solution containing 3.7% formaldehyde, counterstained with nuclear fast red (Sigma) and dehydrated before mounting with Entellan mounting medium and coverslipping with glass. Multiple sections (between 16 and 36) were analyzed per genotype per marker.

2.4.5 Placental fetal vasculature casts

Fetal vascular corrosion casts of E14.5 placentae were generated by following the procedure previously described by Whiteley et al. (146). Vascular casts were analyzed by SEM and morphometric analysis of capillary diameter was carried out using Openlab software (Improvision).
2.5 Acknowledgements

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Chapter 3: Partial loss of $Ascl2$ function affects all three layers of the mature placenta and causes intrauterine growth restriction

3.1 Introduction

In regions of the mammalian genome subject to genomic imprinting, genes tend to be epigenetically regulated in clusters, suggesting that long-range mechanisms coordinate monoallelic expression during development (149,150). Distal mouse chromosome 7 (Chr 7), which shares homology with the Beckwith-Wiedemann Syndrome (BWS) region on human chromosome 11p15.5, is approximately 1 Mb in size and contains two imprinted clusters, each regulated by its own cis-acting differentially methylated region (DMR), commonly referred to as imprinting centre 1 and imprinting centre 2 (IC1 and IC2).

The distal end of the IC1 subdomain and the proximal end of the IC2 subdomain are separated by a distance of about 300kb. IC1 has been shown to control the maternally expressed $H19$ gene and paternally expressed $Igf2$ gene via an epigenetically controlled CTCF-binding insulator element (151,152). The mechanism of IC2-mediated epigenetic silencing of several genes both centromeric and distal to its position over a relatively broad area (over 800kb) is not as clear as is the case for IC1 and likely involves multiple components. Specifically, in the IC2 cluster of genes, at least eight protein-coding genes ($Ascl2$, $Tssc4$, $Cd81$, $Kcnq1$, $Cdkn1c$, $Slc22a18$, $Phlda2$, and $Oshpl5$) are known to be paternally silenced by the production and elongation of the large ncRNA $Kcnq1ot1$, transcribed from the unmethylated paternal IC2 (51,118,119). The current paradigm of imprinted regulation in this region proposes that $Kcnq1ot1$ transcription or the ncRNA itself is responsible for initiating and recruiting repressive

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H3K9me2, H3K9me3, and H3K27me3 specifically in the placenta to the paternal allele along the IC2 domain (57,58,59,71).

Even less is known about the large region between the IC1 and IC2 subdomains and information on this intervening region has only recently become available (122,153). It has been problematic to study because the entire region is repeat-rich and consists mostly of retroelements and tandem repeats, with one known gene, Tyrosine hydroxylase (Th) (153). Th-null mice die in utero but can be rescued by supplementing pregnant females with L-DOPA, the next metabolite in catecholamine biosynthesis pathway (154). To study whether the IC1-IC2 interval plays a role in regulating imprinting in this region, we previously generated a ~280kb deletion of the IC1-IC2 interval called the Del7A1 allele, with breakpoints 5’ of Ins2 in the IC1 sub-domain and 3’ of Ascl2 at the proximal end of the IC2 sub-domain (122). We reported that the Del7A1 deletion was viable upon both paternal and maternal inheritance, where it is recovered in Mendelian ratios, although Del7A1/+ pups were growth retarded at birth (122). Homozygous Del7A1/Del7A1 mice died in utero due to a lack of Th, consistent with the observed Th-null phenotype. This was demonstrated by showing that homozygous deletion mutants can be rescued to term by supplementing pregnant Del7A1 heterozygous females with L-DOPA during gestation (122,154). Otherwise, our analysis of the Del7A1 allele indicated that the deletion does not perturb acquisition or maintenance of imprinting marks at the flanking imprinting centres, regardless of parental inheritance (122).

Several genes in the distal Chr 7 imprinted domain have been implicated in placental development and the regulation of embryonic growth. These include Igf2 (155), Ascl2 (131), Cdkn1c (81,137), and Phlda2 (19,85,86). The achaete-scute complex homolog 2 gene Ascl2 codes for a basic helix-loop-helix (bHLH) transcription factor implicated in lineage specification.
in extra-embryonic tissues (18) as well as in the adult intestinal epithelium (156). Although \textit{Ascl2} expression in extra-embryonic tissue is imprinted with exclusive transcription from the maternal allele (131), it is biallelically expressed in adult LGR5-positive stem cells (156). During development, \textit{Ascl2} is first detected during preimplantation stages, with predominant expression in the trophectoderm cells of the blastocyst (157). Following implantation, \textit{Ascl2} transcripts are abundant in diploid cells of the ectoplacental cone and chorion (18,157). In the chorio-allantoic placenta, \textit{Ascl2} expression becomes restricted to spongiotrophoblast cells, with some patchy expression also detected in the labyrinth layer (157). The role of \textit{Ascl2} in the development of the extra-embryonic lineages was previously addressed by generating a null allele of \textit{Ascl2} by gene targeting (18). \textit{Ascl2}-null conceptuses die of placental failure at E10.0 (18,158). Mutant placentae show an absence of the spongiotrophoblast layer, as shown by loss of \textit{Tpbpa-} and \textit{Flt1-} positive cells as early as E7.5, as well as an expansion of the trophoblast giant cell layer (18,121).

Here we show that the \textit{Del7Al} allele, which is associated with low birth weights in maternal heterozygotes (82.6% of wild type), acts as a hypomorphic \textit{Ascl2} allele. Since the mutant embryos are viable, although growth retarded, we were able to assess the phenotypic consequences of reduced \textit{Ascl2} levels on placental development. Our results show that \textit{Ascl2} function is dosage-sensitive and that hypomorphs exhibit developmental abnormalities in the three main layers of the mature placenta.
3.2 Materials and methods

3.2.1 Mice and genotyping

Generation and genotyping of the Del7Al allele were previously described (122). The Del7Al allele (Del(7Ascl2-Ins2)1Lef; MGI:3662901) is a ~280kb deletion of the interval between Ins2 and Ascl2 (from NCBI m37 coordinates 149,868,122 to 150,148,458) obtained by Cre-loxP recombination in germ cells (122). The Ascl2-lacZ allele (Ascl2tm1.1Nagy; MGI:2155757) has been described previously (158). Although the Ascl2 open-reading frame is undisturbed in this allele, the introduction of an IRES-lacZ-pA cassette in the 3’UTR interferes with production of ASCL2, such that Ascl2-lacZ behaves as a null allele (158). Note that for heterozygous genotypes, the maternal allele is always given first. All animals in this study were maintained on the outbred CD-1 mouse background. All animal experiments were performed under certificate A07-0160 from the UBC Animal Care Committee and complied with the Canadian Council on Animal Care guidelines on the ethical care and use of experimental animals.

3.2.2 In situ hybridization

E9.5, E13.5, and E15.5 placenta were dissected in PBS and fixed in fresh 4% paraformaldehyde/1xPBS (RNase free) overnight at 4°C. For E9.5 placenta, entire conceptuses were fixed to ensure integrity of cryostat sections during the ISH procedure. Antisense and sense strand probes for Ascl2, Tphpa (4311), Pcdh12, Gcm1 and Cdkn1c were DIG-labeled and used for ISH on 10 micron cryostat sections for E13.5 and E15.5 placenta and 12 micron sections for E9.5 placenta as previously described (159). Nuclear fast red was used as a counterstain.
3.2.3 Immunohistochemistry

E7.5 conceptuses as well as E13.5 and E15.5 placentae were dissected in cold PBS and fixed in fresh 4% paraformaldehyde overnight at 4°C, paraffin-embedded, sectioned (12 microns) and subjected to immunohistochemistry as previously described, using the Vectastain Elite ABC kit and DAB substrate (Vector Labs) (159). To detect the PHLDA2 protein in the labyrinth layer, a 1:1000 dilution of the anti-PHLDA2 polyclonal antibody was used (160). ASCL2 IHC was performed with the Millipore clone 8F1 monoclonal antibody (Cat. #MAB4417) at a 1:50 dilution (156). To identify fetal capillaries of the labyrinth layer (161), a 1:25 dilution of rabbit polyclonal anti-laminin antibody (Sigma L9393) was used. Hematoxylin was used as a counterstain.

3.2.4 Periodic acid Schiff stain

E15.5 paraffin sections were deparaffinized, treated with 1% periodic acid for 5 minutes, washed in tap water, followed in distilled water. The sections were then covered in Schiff’s solution for about 15 minutes and rinsed in running tap water for about 15 minutes. Hematoxylin was used as a counterstain and Scott’s Tap water substitute was used to help sharpen the contrast. Sections were then dehydrated and mounted with Entellan mounting medium (EM Science) and coverslipped with glass.

3.2.5 DAPI stain

E15.5 paraffin sections were deparaffinized and treated with 4',6-diamidino-2-phenylindole (DAPI) (1:5000 in PBS) for 5 minutes. Sections were then washed with distilled water, mounted with Vectashield (Vector Labs) and coverslipped with glass.
3.2.6 Expression analysis by quantitative real-time RT-PCR

For total expression level analysis of IC1- and IC2-regulated genes, random-primed cDNA was generated from E9.5 or E15.5 placentae as previously described and cDNA was subject to qRT-PCR (159). qRT-PCRs were performed on three to four individual Del7AI/+ placentae, three +/Del7AI placentae and three to six wild type placentae with three technical replicates per individual placentae. All primer sequences for qRT-PCR analyses are available in Supplementary Table B.1.

3.2.7 Placental fetal vasculature casts

Fetal vascular corrosion casts of E14.5 placentae were generated by following the procedure previously described (146). Vascular casts were analyzed by scanning electron microscopy at the UBC Bioimaging Facility.

3.3 Results

3.3.1 Reduced placental and embryonic weights in maternal Del7AI/+ heterozygotes

We previously reported that the Del7AI allele is associated with a new imprinted phenotype characterized by a growth restriction of approximately 20% at birth and seen only in maternal Del7AI/+ heterozygous pups (122) (please refer to Figure B.4). To assess whether this postnatal phenotype reflects an in utero growth restriction, we measured placental and embryonic weights at E15.5 from reciprocal crosses between wild-type (CD-1 outbred strain) and Del7AI heterozygous mice. Consistent with the absence of a growth phenotype at birth, no difference was found between the embryonic or placental weights of the paternal +/Del7AI heterozygotes and their wild type littermates (Figure 3.1A). However upon maternal
Figure 3.1 Reduced placental and embryonic weights in maternal Del7Al heterozygotes at E15.5.

Scatterplots of individual placental and embryonic weights at E15.5 for cohorts of wild type and Del7Al heterozygous conceptuses obtained from reciprocal crosses. Bars show the average weight ± standard deviation for the given numbers of measurements (n). Results are shown following paternal (A) and maternal (B) transmission of the deletion. Differences in wild type and mutant means were evaluated using the two-tailed unpaired Student $t$ test (p values). Asterisks mark mutant results significantly different from those of their wild type littermates (p<0.05).
transmission of the deletion, we observed a significant (~28%) reduction in Del7AI/+ placental weights at E15.5 compared to their wild type littermates (Figure 3.1B). Weights of Del7AI/+ embryos were concurrently smaller than wild type littermates at this stage but to a lesser degree (~7%). Our results show that maternal inheritance of Del7AI is associated with growth defects during development and suggest that embryonic growth restriction occurs later than E15.5 as a consequence of a primary placental phenotype, as described previously for other imprinted mouse models of intrauterine growth restriction such as the Esx1 knock-out (96) and the placental-specific loss of Igf2 (68).

3.3.2 Both Ascl2 and Phlda2 are perturbed in maternal Del7AI/+ placentae

The imprinted phenotype associated with Del7AI suggests that the deletion interferes with the expression of a maternally expressed placental growth regulator during development. Of the currently annotated protein-coding genes deleted in the Del7AI allele (Ins2 Ensembl isoform Ins006, Gm6471, and Th) only Th is known to be expressed in the placenta, where it has been reported to be preferentially expressed from the maternal allele (89). However, whereas the embryonic lethality of Del7AI/Del7AI homozygotes can be pharmacologically rescued by provision of exogenous L-DOPA during pregnancy (the product of the reaction catalyzed by TH), such treatment has no effect on the growth restriction phenotype of Del7AI/+ heterozygotes (122) (please refer to Figure B.4). These observations suggest that the deletion might interfere with the expression of flanking imprinted genes, several of which have been implicated in placental biology such as Igf2 in the proximal IC1 subdomain (155), and Ascl2 (18), Cdkn1c (162), and Phlda2 (86) in the distal IC2 sub-domain. To address this possibility, we analyzed by quantitative reverse transcriptase PCR (qRT-PCR) the expression levels in E9.5 placentae of
imprinted genes located proximally (IC1 subdomain) or distally (IC2 subdomain) to the breakpoints of the Del7Al deletion (Figure 3.2A-B). In Del7Al/+ placentae, the gene immediately distal of the deletion breakpoint, Ascl2, was significantly lower in the maternal Del7Al/+ placentae compared to wild type littermates, with an overall reduction in expression levels of approximately two-fold (Figure 3.2C). In addition, we observed increased Phlda2 expression in maternal Del7Al/+ placentae compared to wild type littermates, also by approximately two-fold (Figure 3.2C). The same expression changes at Ascl2 and Phlda2 were also observed in E15.5 placentae (Figure B.1A). The rest of the genes analyzed in the IC2 or IC1 clusters, including Igf2, were unaffected in maternal Del7Al/+ placentae. Unlike what is observed for conceptuses inheriting the deletion maternally, we found no significant difference between +/-Del7Al placentae and wild type littermates for the IC1 and IC2 subdomain genes analyzed at this stage (Fig. 3.2C).

To confirm the status of Ascl2 and Phlda2 expression in maternal Del7Al/+ placentae using an independent method, we carried out in situ hybridization (ISH) and immunohistochemistry (IHC) on E9.5 and E13.5 Del7Al placentae, respectively. At E9.5, Ascl2 expression is highest in the spongiotrophoblast layer of the developing placenta, with weak expression also detected in the labyrinthine layer (157). We found a general decrease in Ascl2 levels within these placental layers in Del7Al/+ placentae. (Figure 3.3A). To ask whether the difference in Ascl2 expression was due to a general decrease in the number of spongiotrophoblast cells where Ascl2 is predominantly expressed, we performed ISH for Tpbpa (4311), a marker of the spongiotrophoblast layer at E9.5 (5). We found no decrease in the number of Tpbpa-positive cells in mutant placentae at this early stage (Figure 3.3B). We noted that Tpbpa levels appeared higher in some (but not all) mutant sections analyzed, but this expression difference was not found to be statistically significant by qRT-PCR analysis (Figure B.1C). As a control, we also
Figure 3.2 Total expression levels of IC1- and IC2-regulated imprinted genes in reciprocal heterozygous Del7AI placentae at E9.5.

A) Schematic representation of mouse chromosome 7 showing the breakpoints of the Del7AI allele relative to IC1 and IC2. DNA methylation status at the imprinting centres is shown by lollipops (white, unmethylated; black, methylated) on the maternal (M, black) and paternal (P, grey) homologues. B) Map of the IC1 and IC2 clusters showing selective maternally (black) and paternally (grey) expressed genes. C) qRT-PCR was performed on E9.5 placentae for the following genes: H19, Igf2, Ascl2, Tssc4, Cdkn1c, Phlda2 and Osbpl5. qRT-PCR was carried out as technical triplicates on three individual placentae (biological replicates) for each genotype and the error bars show the standard deviation for biological triplicates. Expression is relative to the reference housekeeping gene, Peptidylprolyl isomerase A (Ppia). Asterisks indicate mutant values significantly different from wild type values, using two-tailed unpaired Student t test (Ascl2, p=0.006; Phlda2 p=0.005).
performed ISH on another imprinted gene in the IC2 cluster, Cdkn1c, which is critical to placentation and expressed throughout the placenta at this stage, and did not observe any changes in levels or expression patterns (Figure 3.3C), consistent with our qRT-PCR results.

Although Phlda2 (also known as Ipl) is broadly expressed in the ectoplacental cone and chorion at E8.0 (2), it eventually becomes restricted to trophoblast cells of the labyrinthine layer such that by E10.5 it is predominantly expressed in syncytiotrophoblast layer I (SynT-I) with some lower levels in layer II (SynT-II) (13,86,160). At E13.5, we observed an overall increase in PHLDA2 protein expression by IHC in Del7AI/+ heterozygotes with a broader distribution of Phlda2-expressing foci throughout the labyrinth (Figure 3.3D). Our results show that Del7AI does not have a trans effect on the levels of Igf2 transcribed from the wild type paternal homologue, but rather affects the maternally expressed genes Ascl2 and Phlda2 of the IC2 sub-domain in cis.

3.3.3 Phlda2 upregulation is placental-specific and is also observed in Ascl2-null conceptuses

In light of our results showing misregulation of both Ascl2 and Phlda2 when the deletion is maternally inherited, we sought to distinguish between two possible mechanisms consistent with our observations. In the first model, the deletion could have direct although opposite effects on the expression levels of Ascl2 and Phlda2. According to the second model, Del7Al has a direct effect on Ascl2 levels, reducing them by 50%, and one of the consequences of this hypomorphic Ascl2 allele is an increase in Phlda2 levels in the labyrinth.

Unlike Ascl2 expression which is restricted to the extra-embryonic tissues, Phlda2 is expressed in the developing embryo and was isolated as a mesoderm-enriched cDNA clone from gastrulation stage embryos (2). To determine whether the deletion also affects this embryonic
Figure 3.3 Abnormal expression of the maternally expressed genes *Ascl2* and *Phlda2* in *Del7AI/+* placentae.

The expression patterns of *Ascl2* (A), *Tpbpa* (B) and *Cdkn1c* (C) were analyzed by ISH on frozen sections of wild type (+/+) and maternal deletion (*Del7AI/+*) placentae collected at E9.5. Multiple sections from two placentae of each genotype were assessed and representative sections are shown. Sense probes not shown. The blue stain shows gene expression. Nuclear fast red was used as the counterstain. D) Phlda2 IHC at E13.5. The brown stain shows protein expression. Scale bar: 0.5mm. sp: spongiotrophoblast layer, lab: labyrinthine layer, d: decidua.
expression of Phlda2, we carried out a Phlda2 qRT-PCR analysis on E13.5 Del7AI/+ embryos. We found no difference in Phlda2 expression levels between Del7AI/+ and wild type embryos (Figure 3.4A), suggesting that the Del7AI allele has no impact on embryonic Phlda2 levels. Since Phlda2 is expressed and imprinted in both the placenta and the embryo, these results show that the deletion does not have a global effect on Phlda2 levels, which are only upregulated in the placenta.

A prediction from the second model is that one would expect to observe an upregulation of Phlda2 in placentae deficient for Ascl2, before the manifestation of the lethality observed at E10.0 (18). To address this, we took advantage of an Ascl2-lacZ knock-in mouse line (Ascl2-lacZ allele, Ascl2\textsuperscript{tm1.1Nagy}; MGI: 2155757). Maternal inheritance of the Ascl2-lacZ allele (Ascl2\textsuperscript{lacZ/+} heterozygotes) results in midgestational lethality due to a lack of spongiotrophoblast formation (158), a phenotype similar to the one described for the original knock-out allele (18). Although it was unclear why the insertion of an IRES-lacZ reporter cassette in the 3'UTR of Ascl2 should generate a null allele (158), we found that Ascl2 mRNA levels are very low in Ascl2\textsuperscript{lacZ/+} placentae at E9.5 (Figure 3.4B). We performed qRT-PCR for Phlda2 on these mutant placentae and found a slight but significant increase (15%) in Phlda2 expression in the mutant placentae compared to wild type littermates (Figure 3.4B), establishing that loss of Ascl2 by itself can lead to altered Phlda2 levels in the placenta.

We next investigated whether this increase in Phlda2 expression in Ascl2-deficient placentae was paralleled by a general increase in syncytiotrophoblast markers at E9.5. We performed qRT-PCR for three syncytiotrophoblast markers: syncytin-A (Syna), a marker of syncytiotrophoblast layer I (13) required for syncytiotrophoblast formation by cell fusion (163); Glial cells missing homolog 1 (Gcm-1), a marker of syncytiotrophoblast layer II of the labyrinth
(11,13), and required for branching morphogenesis in the labyrinth following chorio-allantoic fusion (11); and Distal-less homeobox 3 (Dlx3), a pan-specific labyrinth trophoblast marker (13), required for normal morphogenesis of the labyrinthine layer (164). Although we observed a general decrease in these three markers in Ascl2-null placentae at E9.5, significant differences with wild type littermates were only detected for the syncytiotrophoblast layer I gene Syna (Figure 3.4C), a marker of the cell population where Phlda2 is predominantly expressed (86). Note that although the increase in Phlda2 levels at E9.5 is less pronounced in Ascl2-null (Figure 3.4B) than in hypomorphic Del7AI/+ placentae (Figure 3.2C), the concomitant decrease in Syna seen in Ascl2-null placentae (Figure 3.4B) is not observed in Del7AI/+ samples (Figure B.1B), suggesting a decrease in Phlda2-expressing cells in the null placentae.

Taken together, our analyses of Del7AI/+ embryos and Ascl2-null placentae suggest that Ascl2 operates upstream of Phlda2 in the placenta and that Del7AI acts primarily as an Ascl2 hypomorphic allele. Unlike what has been described for the phenotypes of the Ascl2 KO (18) and Ascl2-lacZ (158) alleles, maternal inheritance of Del7AI does not cause an embryonic lethality phenotype and therefore offered us the opportunity to study the consequences of reduced Ascl2 levels on the morphogenesis of the mature placenta.

3.3.4 Increased trophoblast giant cell numbers in Del7AI/+ heterozygotes

One of the phenotypic consequences of loss of Ascl2 is an increase in the number of parietal trophoblast giant cells making the giant cell layer of the mutant placentae before manifestation of the lethality at E10.0 (18). We found that reduced levels of Ascl2 in maternal Del7AI heterozygotes are also accompanied by a thicker giant cell layer, readily seen in
Figure 3.4 Dysregulation of Phlda2 in Ascl2-null placentae.

(A) Phlda2 qRT-PCR on E13.5 embryo was carried out on three technical replicates each from three individual Del7Al/+ embryos and three wild type littermate individual embryos (biological replicates) and the error bars shown are the standard deviation for biological replicates. Expression is relative to Ppia. No significant difference was found between the two genotypes (p=0.8). Gene expression analysis by qRT-PCR on total E9.5 placental RNA from Ascl2lacZ/+ (Ascl2-/-) mutants and wild type littermates (Ascl2+/+). Analysis is presented for (B) Ascl2 and Phlda2, as well as the labyrinth trophoblast markers Syna, Gcm-1, and Dlx3 (C). The results show a significant increase in Phlda2 expression in Ascl2-deficient placentae but not for the syncytiotrophoblast markers Gcm-1 and Dlx3. Syna was also downregulated in mutant placentae. Expression levels are relative to Ppia levels. Asterisks mark mutant levels statistically different from wild type levels (for Ascl2 p=0.0008; for Phlda2 p=0.002, and p=0.012 for Syna).
hematoxylin and eosin (H&E) stained sections (Figure 3.5A). At higher magnification, we observed a drastic thickening of the layer, which is usually a single cell layer in wild type placentae, but appears as a thick stack of 4 to 6 giant cells in the mutant placentae at E15.5 (Figure 3.5B). DAPI staining confirmed that these cells have large polyploid nuclei, a characteristic of endoreduplicating giant cells (Figure 3.5C).

3.3.5 Reduced spongiotrophoblast cell numbers and absence of trophoblast glycogen cells in mutant placentae

The main reported phenotype caused by \textit{Ascl2} deficiency (\textit{Ascl2} KO/+), is the absence of a spongiotrophoblast layer (also known as junctional zone) (18). Although at the stage of lethality (E10) this layer is mostly composed of spongiotrophoblast cells, past mid-gestation a second cell population, the trophoblast glycogen cells starts to populate this layer and eventually migrate into the decidua (6). We found that both of these cell types were severely affected in \textit{Del7AI/+} placentae.

In histological sections, the labyrinth appears directly juxtaposed to the expanded giant cell layer in the mutants, with no evidence of spongiotrophoblast or glycogen cells at E15.5, within the spongiotrophoblast layer or above the giant cell layer (Figure 3.5B). The trophoblast gene \textit{Tpbpa} is an early marker of spongiotrophoblast cells (5) but is eventually also expressed in trophoblast glycogen cells starting at E11.5 (165). We analyzed the expression of \textit{Tpbpa} by \textit{in situ} hybridization (ISH) on frozen sections of wild type and \textit{Del7AI/+} placentae at E15.5. In the wild type placenta, \textit{Tpbpa} is strongly expressed in a broad population of spongiotrophoblast cells overlying the labyrinth as well as in trophoblast glycogen cells within the spongiotrophoblast layer and within the decidua (Figure 3.6A). In the mutant placentae, only a thin layer of \textit{Tpbpa-
Overall morphology of wild type and Del7Al/+ placentae at E15.5 as revealed by hematoxylin and eosin (H&E) staining (A-B) and DAPI staining (C). Whereas the giant cell layer is hard to detect at lower magnification in wild type placentae (A), a thick layer is clearly visible in the mutants (arrowheads). At higher magnification, the single cell layer seen in wild type placentae (dotted line) is replaced by a 4 to 6 cell layer in the mutants (B). Also noticeable is the absence of clear spongiotrophoblast and trophoblast glycogen cells in proximity of the giant cell layer. DAPI staining readily identifies the giant polyploid nuclei of the cells stacked at the giant cell layer in the mutants (C). Scale bar: 500 μm (A); 100 μm (B-C). d: decidua; TGC: parietal trophoblast giant cells; SpT: spongiotrophoblast; GlyT: trophoblast glycogen cells; lab: labyrinth layer.
expressing spongiotrophoblast cells is detected, with no sign of glycogen cells. This conspicuous absence of glycogen cells was also confirmed by periodic acid Schiff (PAS) staining (Figure 3.6D), which detects glycogen producing cells (8), as well as by ISH for two other markers of trophoblast glycogen cells, Pcdh12 (166) (Figure 3.6B) and Cdkn1c (167) (Figure 3.6C). Cdkn1c is expressed at low levels in the labyrinth in both Del^7Al/+ and wild type placentae, but is most highly expressed in glycogen cells in the maternal decidua, where signal is detected in wild type but not in Del^7Al/+ placentae (Figure 3.6C). We investigated the spongiotrophoblast to labyrinth ratio and we found that although the labyrinth area of Del^7Al/+ placentae is similar to wild type, the spongiotrophoblast is reduced by about six-fold in the mutants.

Our results show that the correct dosage of Ascl2 levels is critical for proper development of the spongiotrophoblast layer and suggest that most of the weight loss observed in mutant placentae is attributable to a total absence of glycogen cells and a drastic decrease in the spongiotrophoblast cell population.

3.3.6 Dysmorphogenesis of the labyrinth layer in mutant placentae

Although Ascl2 is not expressed in the labyrinth layer of the mature placenta, we have shown that absence or decreased levels of Ascl2 lead to an upregulation of Phlda2, which is expressed at high levels in syncytiotrophoblast layer I (SynT-I) of the trilaminar labyrinthine trophoblast from E10.5 to E12.5, after which stage a sharp decrease in PHLDA2-positive cells is observed (86). To assess whether other trophoblast cell types of the labyrinth were also affected in Del^7Al/+ placentae at E15.5, we analyzed the expression of Placental lactogen-II (Prl3b1/Pl-II) and Gcm1 by ISH. At this stage, Prl3b1 is expressed in three different trophoblast populations: parietal trophoblast giant cells (the polyploid cells of the giant cell layer),
Figure 3.6 Abnormal spongiotrophoblast and glycogen cell phenotype in maternal Del7Al heterozygotes at E15.5.

A) Tpbpa, B) Pcdh12, and C) Cdkn1c ISH performed on E15.5 placentae. Multiple sections from two placentae of each genotype were assessed and representative sections are shown. Sense probes not shown. The blue stain shows gene expression. D) Periodic acid Schiff (PAS) stain revealing glycogen cells. Dark pink/purplish stain shows glycogen staining. Scale bar: 0.5 mm. d: decidua; TGC: parietal trophoblast giant cells; SpT: spongiotrophoblast; GlyT: trophoblast glycogen cells; lab: labyrinth layer.
spongiotrophoblast, as well as the mononucleated giant cells lining the maternal blood sinusoids in the labyrinth (sinusoidal trophoblast giant cells) (144). In the mutant placentae, expression of Prl3b1 is mostly seen in the thickened giant cell layer in the basal region, but also reveals a marked increase in the density of sinusoidal giant cells within the labyrinth (Figure 3.7A). A similar result was obtained for Gcm1 (Figure 3.7B), a marker of the labyrinth syncytiotrophoblast layer II (13). In light of the close functional and cellular interactions between the labyrinth trophoblast layers and the fetal vasculature, we also analyzed the architecture of fetal blood vessels in mutant placentae by immuno-staining for laminin, a marker of the basement membrane associated with fetal epithelial cells. Our results again highlight important defects in the structure of the labyrinth, characterized by a denser network of fetal blood vessels in the mutant labyrinth (Figure 3.7C). Note that despite these abnormalities in labyrinthine structures, the mutant placentae do not show evidence for increased thickness between the fetal and maternal circulations (Figure B.3).

To investigate further the defects in labyrinthine vasculature suggested by the differences in laminin stain as well as labyrinth trophoblast cell types, we performed fetal vasculature corrosion cast experiments (8,146). Both a lateral view and maternal view of the Del7Al/+ labyrinth revealed a greater degree of disorganization compared to wild type (Figure 7D-E). In the wild type placenta, the labyrinth is subdivided into functional units of capillary beds arranged in separate villous branches, each served by one arteriole and one venule. In the maternal Del7Al/+ heterozygotes, however, these subdivisions of the capillary branching were not clear and individual functional units could not be dissected from the mutant resin casts (Figure 3.7D). Observation of the maternal side of the fetal vasculature reveals this phenotype in an even more dramatic way (Figure 3.7E). In the wild type placenta, the discrete functional unit of capillaries
Figure 3.7 Dysmorphogenesis of the labyrinth layer in Del7Al/+ placentae.

ISH for (A) Prl3b1 and (B) Gcm1 on frozen sections of wild type and mutant placentae at E15.5. Multiple sections from two placentae of each genotype were assessed and representative sections are shown. Sense probes not shown. The blue stain shows gene expression. Prl3b1 is expressed in parietal trophoblast giant cells, spongiotrophoblast and mononucleated sinusoidal trophoblast giant cells of the labyrinth. In the mutants, the spongiotrophoblast staining is replaced by a thicker giant cell layer, and the labyrinth signal is denser. Gcm1 is a marker of the syncytiotrophoblast II of the labyrinth. As seen for Phlda2 (expressed in syncytiotrophoblast I) and Prl3b1, we observed a denser network of Gcm1-expressing cells in the mutant placenta. (C) IHC for laminin marks the basement membrane of the fetal blood vessels and revealed a denser network of capillaries in the mutant labyrinth. The brown stain shows protein expression. (D-E) Feto-placental vasculature of the maternal Del7Al heterozygotes. Representative scanning electron micrographs of E14.5 fetal vascular corrosion casts. (D) lateral view; (E) top view from maternal side. Four fetal vasculature casts were assessed per genotype. Scale bar: 1mm.
are clearly demarcated and separated by spaces filled by finger-like projections of the spongiotrophoblast as well as maternal blood space. In the mutants, this organization is lost and fetal blood vessels fill the entire diameter of the placenta. Our results suggest that sub-optimum levels of Ascl2 not only affect the spongiotrophoblast and trophoblast glycogen cells, but also lead to expansion of trophoblast cell types in the giant cell layer and labyrinth.

3.4 Discussion

We previously reported that maternal inheritance of a deletion of the ~280kb intergenic region between the two imprinted domains on distal Chr 7 is associated with reduced birth weights (122). Here we show that upon maternal inheritance of Del⁷AI, the expression levels of the genes in the IC1 and IC2 subdomains are unaffected with the exception of a decrease in Ascl2 expression. This is accompanied by a reduction in placental weight and a severe placental phenotype characterized by a reduced spongiotrophoblast, absence of trophoblast glycogen cells, expanded giant cell layer and a disorganized labyrinth. We also observed an increase in Phlda2 expression in Del⁷AI/+ placentae, which we propose is a secondary effect of decreased Ascl2 expression (see below).

We have previously demonstrated that inheritance of Del⁷AI does not affect the transcription of the ncRNA Kcnq1ot1 (122). The analysis of IC2-regulated genes presented here also argues against a global effect of the deletion on the entire cluster of imprinted genes. The mechanism whereby Ascl2 is decreased in Del⁷AI/+ heterozygotes is currently under investigation. One possibility is that the Del⁷AI allele deletes a placental-specific enhancer of Ascl2 expression on the maternal allele. However, to date no such enhancers have been identified.
in the IC1-IC2 interval, although an Ascl2 transgene rescuing the lethality of Ascl2 deficiency and encompassing ~60 kb of our deletion has recently been described (168).

The role of the spongiotrophoblast in the mouse placenta is unclear but it is essential for development, since a complete lack of spongiotrophoblast leads to midgestational lethality (18,121). Previous work on Ascl2 has established that it plays an important role in the formation of the spongiotrophoblast, together with a negative role on trophoblast giant cell differentiation from diploid ectoplacental precursors (131). Maternal inheritance of the Ascl2 null allele results in midgestational lethality due to a complete lack of spongiotrophoblast formation (18). The Del7Al allele gave us the opportunity to assess the phenotypic consequences in the mature placenta associated with suboptimal Ascl2 levels. We show that the dosage of Ascl2 levels is critical for normal placentation and that reduced Ascl2 results in a decreased spongiotrophoblast population, lack of trophoblast glycogen cells, an expanded giant cell layer, a reduction in placental size, and embryonic growth restriction. Nevertheless, these suboptimal Ascl2 levels can still result in viable pups, although growth retarded.

One of the drastic phenotypes described here is the disorganization of the fetal vasculature. Observed from the maternal side, the vascular bed is seen as a punctate structure with regularly spaced openings organizing the underlying network of fetal capillaries. In the mutants with a stunted spongiotrophoblast layer, this organization is lost and the labyrinth fills the entire placenta. Our results therefore suggest that finger-like projections of the spongiotrophoblast within the labyrinth play an important role, structurally and perhaps also functionally via the maintenance of an organized vascular network for proper feto-maternal exchanges. Although this aspect of the phenotype has not been studied in detail here, it is likely that the observed changes in fetal capillary beds and labyrinth trophoblast densities would impact
on the expansion of the maternal blood spaces. Alternatively, the observed labyrinth phenotype could also be a consequence of abnormal maternal blood supply and reduced perfusion of the uteroplacental unit, for instance via placental hypoxia.

The phenotype studied here describes a functional placenta with an apparent total loss of trophoblast glycogen cells. Both the developmental origin and function of this cell lineage is currently unknown (6). Based on the observation that these glycogen-rich vacuolated cells are first detected past midgestation within the spongiotrophoblast layer and that they express markers of spongiotrophoblast (such as \textit{Igf2} (169) and \textit{Tpbpa} (5)) it has been proposed that they may represent a specific subtype of spongiotrophoblast cells. However it has been shown that \textit{Pcdh12}, which is specifically expressed in trophoblast glycogen cells past mid-gestation (166), also labels a specific sub-population of cells within the ectoplacental cone at E7.5 (10). This led the authors to suggest that trophoblast glycogen cells and spongiotrophoblast might represent distinct trophoblast lineages both emerging from \textit{Ascl2}-positive ectoplacental cone (EPC) precursors. Our results clearly show that \textit{Ascl2} is required for formation of the trophoblast glycogen cell lineage. Whether this is mediated via a direct requirement for \textit{Ascl2} in precursors of the lineage or an indirect effect via EPC or spongiotrophoblast factors themselves dependent on \textit{Ascl2} will have to be addressed by lineage-specific knock out experiments.

With regards to the function of trophoblast glycogen cells during development, our results suggest that this trophoblast lineage is not required for parturition, as previously suggested (6), at least not within the context of mixed litters composed of wild type and mutant conceptuses, as studied here. It might be informative to analyze litters composed entirely of mutant conceptuses, an experiment which would require embryo transfers since the homozygous \textit{Del7Al/Del7Al} are not viable (122). However, it is tempting to suggest that the absence of
trophoblast glycogen cells documented in Del7AI/+ heterozygotes might contribute to the embryonic growth retardation phenotype observed. Although our results are suggestive of a correlation between absence of trophoblast glycogen cells and embryonic growth restriction, we cannot exclude the contributions of the abnormalities described in other placental trophoblast cell types to the embryonic phenotype. Therefore a definitive demonstration of the role of the trophoblast glycogen cells on embryonic growth is still lacking and would require the ablation of this specific lineage during development.

An intriguing aspect of our work is the finding that in addition to Ascl2, a second IC2-regulated gene, the imprinted gene Phlda2, is also affected in deletion heterozygotes. However the transcriptional effects at both genes is opposite such that Phlda2 is upregulated in the mutants. We considered the possibility that the deletion might have a direct effect on Phlda2, for instance via the removal of a silencer, but several of our results are not consistent with such a model. The first argument against this model is the structure of the allele itself. The deletion has a distal breakpoint less than five kilobase pairs away from Ascl2, whereas Phlda2 is located more than half a megabase away from this breakpoint. Furthermore, both Tssc4 and Cdkn1c, located in between Ascl2 and Phlda2 are not affected by Del7AI. Second, the deletion has no effect on the embryonic expression of Phlda2 and up-regulation is only observed in the placenta of Del7AI/+ mutants. Third, we showed that the levels of Phlda2 are also elevated in Ascl2-null placentae, suggesting that Ascl2 acts upstream of Phlda2 and exerts negative effects on Phlda2-expressing cells, the syncytiotrophoblast layer I. Finally, our analysis of markers for the other two trophoblast cell types of the labyrinth layer at E15.5 also suggests an increased cellularity in these lineages in the mature placenta. This would suggest that a decreased dosage in Ascl2 and the ensuing underdevelopment of the spongiotrophoblast have broader impacts on chorionic
ectoderm-derived lineages. Our results are consistent with a model proposing that, as for the trophoblast giant cell lineage, Ascl2 plays an inhibitory role on the differentiation of the trilaminar trophoblast cell lineages of the labyrinth and that normal development of the placenta requires an appropriate balance of positive and negative signals governing the ratio of spongiotrophoblast and labyrinth trophoblast precursors. Such a model could also help to explain previously published intriguing results on the effects of varying Phlda2 gene dosage. Phlda2 knockout mice are viable but their placentae are characterized by placentomegaly, an expanded spongiotrophoblast layer and increased number of trophoblast glycogen cells, but no fetal overgrowth (86). Although this has not been addressed by the authors, we propose that increased cellularity in the spongiotrophoblast layer of Phlda2-null placentae might be mediated via an increase in Ascl2 levels. Conversely, overexpression of Phlda2 from a BAC transgene leads to a reduced spongiotrophoblast layer and defects in trophoblast glycogen cells, changes which are accompanied by a reduction in Ascl2 levels (19). Together, these studies and our results suggest a previously unrecognized relationship between Ascl2 and Phlda2. Both are expressed in the ectoplacental cone (EPC) and extra-embryonic ectoderm in early development, two layers which are brought in close proximity with the collapse of the ectoplacental cavity at E8.0 (2,18). We have confirmed that ASCL2 and PHLDA2 are co-expressed in the EPC at E7.5 (Figure B.2), arguing against a direct repression of Phlda2 by ASCL2 at those early stages. Our results and those of others (19,85,86) could be reconciled by proposing that each of these two genes play positive roles in the maintenance of different precursor populations within these layers, and that reduced levels in one are accompanied by increased levels in the other, the proper balance assuring development of a fully functional placenta.
It has been proposed that elevated levels of PHLDA2 are associated with low birth weights (30,170). Although the ASCL2 gene might not be imprinted in human placenta (139), our results show that ASCL2 function is dosage sensitive and suggest that ASCL2 mutations could be involved in the etiology of these cases of intrauterine growth restriction (IUGR) associated with upregulation of PHLDA2. The Del7Al mouse line will provide a valuable model for the study of IUGR in the mouse, including its consequences on maternal physiology during pregnancy and postnatal adult phenotypes in growth retarded pups.

3.5 Acknowledgements

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Chapter 4: Partial loss of imprinting at Ascl2 and Tssc4 in paternal Del7Al heterozygotes

4.1 Introduction

Clustering of imprinted genes possibly evolved so that a common mechanism can efficiently and simultaneously regulate multiple genes, such as the use of single noncoding RNAs (ncRNAs) which can epigenetically silence several genes that are hundreds of kilobases away. Clusters of imprinted genes are found throughout the mammalian genome and can often include non-imprinted promoters, as is the case for the imprinted distal mouse chromosome 7 region. A gene-poor region of almost 300kb referred to as the IC1-IC2 interval lies between the well-known H19/Igf2/Ins2 imprinted cluster regulated by imprinting centre 1 (IC1; otherwise known as the H19 DMR) and the Kcnq1ot1 imprinted domain regulated by imprinting centre 2 (IC2; otherwise known as KvDMR1) (Figure 1.2). The majority of the IC1-IC2 interval contains retroelements and tandem repeats (153). One known protein-coding gene, Tyrosine hydroxylase (Th) (154), has previously been found in this interval and more recently, we have confirmed that this interval contains at least two other coding imprinted transcripts (88).

It is well known that epigenetic mechanisms including DNA methylation, histone modifications, and the action of ncRNAs allow the appropriate imprinting of genes, and several knockouts of imprinted genes, imprinting centres, and imprinted regions in the mouse have increased our understanding of the regulation of imprinting on distal mouse chromosome 7 (111,113,114,115,118,121,129). However we sought to learn whether the deletion, not of a specific imprinted gene or imprinting centre, but of a gene-poor region bringing two imprinted domains into closer proximity, would have an effect on the proper imprinting of genes flanking

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1 A version of this chapter will be submitted for publication. Rosemary Oh-McGinnis, Rita Ho, Andras Nagy and Louis Lefebvre: Partial loss of imprinting at Ascl2 and Tssc4 in paternal Del7Al heterozygotes.
the deletion breakpoints. To determine whether the IC1-IC2 interval plays any role in the regulation of imprinting in neighbouring imprinted domains, if any, we previously generated a ~280kb deletion of the IC1-IC2 interval called the Del7Al allele, spanning the 3’ end of Ins2 in the IC1 interval to the 5’ end of Ascl2 in the IC2 interval. Homozygous Del7Al/Del7Al mutants die at midgestation due to a lack of Th which is essential for viability (122). DNA methylation and allele-specific expression analyses at IC1 and IC2 revealed that the epigenetic marks are unperturbed upon either maternal or paternal inheritance of the Del7Al deletion, suggesting that the IC1-IC2 interval is not critical for the function of either of the imprinting centres (122). The Del7Al deletion was also found to be viable upon both paternal and maternal inheritance, although Del7Al/+ pups were growth restricted (122).

More recently, we described the phenotype of the Del7Al/+ in more detail which includes reduced placental and embryonic size, thin spongiotrophoblast, lack of trophoblast glycogen cells and expanded trophoblast giant cell (TGC) layer (23). We demonstrated that suboptimal maternal Ascl2 expression is the primary cause of the growth restriction and placental abnormalities in all three layers of the mature placenta in Del7Al/+ heterozygotes (23). Achaete-scute complex homolog 2 gene (Ascl2) is the most centromeric gene in the IC2 domain and is predominantly expressed in the spongiotrophoblast with some expression seen in the labyrinth (23,157,171). Ascl2-null mice die at midgestation due to placental abnormalities that include a lack of spongiotrophoblast and an expanded giant cell layer (18,131). Our hypomorphic Ascl2 allele provides new insight into the function of Ascl2 in later gestation and growth, since its role beyond being essential for spongiotrophoblast formation and suppressing giant cell formation was not previously known. Although the mechanism responsible for decreased Ascl2 expression
in Del7Al/+ heterozygotes is still unknown, a simple explanation of our results is that the sequences deleted in the Del7Al allele encompass an enhancer essential for full Ascl2 expression.

Along with a marked decrease in Ascl2 expression, we noted that Del7Al/+ heterozygotes also possess increased expression of another maternally expressed gene in the IC2 domain, Phlda2 (23). Pleckstrin homology-like domain family A member 2 (Phlda2, also known as Ipl) is expressed in the syncytiotrophoblast layer II of the labyrinth and its function is not completely clear. Phlda2 knockout mice have previously been shown to be viable but possess placentomegaly, an expanded spongiotrophoblast layer and reduced labyrinth (86). On the other hand, more recently, an extra dosage of Phlda2 has been shown to cause a variety of placental phenotypes including reduced placental size, lack of glycogen within glycogen cells and a thinner junctional zone (19). The mechanism by which irregular Phlda2 expression causes multiple phenotypes in the junctional zone is unknown, considering that it is not expressed in the spongiotrophoblast or thought to be responsible for the spongiotrophoblast lineage. Interestingly, Phlda2 transgenic mice that overexpress Phlda2 possess a decrease in Ascl2 expression (19). In addition, we have previously shown that Ascl2 levels impact Phlda2 levels in a reciprocal manner (23) and the potential relationship between these two genes requires further attention. Both Ascl2 and Phlda2 are known to be regulated by a large antisense ncRNA called Kcnq1ot1, which silences several protein-coding genes in its vicinity on the paternal allele. In fact, paternal inheritance of a knockout allele for IC2, which acts as a promoter for Kcnq1ot1 transcription, causes paternal activation of all of the genes on the entire IC2 domain and their expression in a biallelic manner (51,118,119).

Paternal inheritance of the Del7Al deletion has no obvious phenotype with respect to placental and embryonic size and the expression levels of the IC1- and IC2-regulated genes are
consistent with wild type at least at early stages (E9.5) (23). However, it was unknown whether the genes in the IC2 domain are appropriately expressed in an allele-specific manner in +/Del7Al heterozygotes nor whether these conceptuses possessed any subtle placental phenotype. We therefore hypothesized that the IC1-IC2 interval is required to keep the two imprinting clusters physically separated and that paternal inheritance of the Del7Al deletion could potentially result in a loss of imprinting (LOI) of one or more genes in the region.

4.2 Materials and methods

4.2.1 Mice and genotyping

Generation and genotyping of the Del7Al allele are previously described (122). The Ascl2-lacZ allele (Ascl2tm1.1Nagy; MGI:2155757), most commonly referred to as the Ascl2KI allele in this study, has been previously described (158). Note that for heterozygous genotypes, the maternal allele is always given first. Animals used in this study were on the CD-1 mouse background and a congenic line having distal MMU7 from Mus musculus castaneus on a CD-1 background at N3. All animal experiments were performed under certificate A07-0160 from the UBC Animal Care Committee and complied with the national CCAC guidelines to the ethical care and use of experimental animals. Weights of placentae and embryos were taken immediately upon dissection with as much of the liquid removed as possible before weighing.

4.2.2 In situ hybridization and immunohistochemistry

E13.5 and E15.5 placentae were dissected in PBS and fixed in fresh 4% paraformaldehyde/1xPBS (RNase free) overnight at 4°C. For E9.5 placentae, entire conceptuses were fixed to ensure integrity of cryostat sections during the ISH procedure. Antisense and sense
strand probes for \textit{Ascl2}, \textit{4311}, \textit{Pcdh12}, \textit{Cdkn1c}, \textit{Pl-II}, \textit{Gcm1}, and \textit{Igf2} were DIG-labeled and used for ISH on 10 micron cryostat sections for E13.5 and E15.5 placentae as previously described (159). Nuclear fast red was used as the counterstain. For \textit{Igf2}, probe sequence was obtained from www.genepaint.org (Accession number NM_010514; Entrez Gene ID 16002). IHC for laminin was done as previously described (23).

4.2.3 Allele-specific expression analysis and quantitative reverse transcriptase PCR

For allele-specific expression analysis of IC1- and IC2-regulated genes, random-primed cDNA was generated from E9.5 or E13.5 placentae as previously described (159) followed by RT-PCR and an informative restriction enzyme digest based on a CAST polymorphism. For \textit{Ascl2}, primers in2F1 and 726R followed by HpaII digest yielded a CAST-specific 313bp band and a CD-1-specific 217bp band. For \textit{Tssc4}, primers F1 and 35 followed by HaeIII digest yielded a CAST-specific 247bp band and a CD-1-specific 159bp band. For \textit{Cdkn1c}, primers p57S and p574 yielded a 364bp product and the CAST>CD-1 SNP was C>T at position 316. For \textit{Phlda2}, primers Ipl1 and Ipl R2 yielded a 578bp product and the CAST>CD-1 SNP was A>C at position 209. For \textit{Cdkn1c} and \textit{Phlda2}, direct sequencing of the PCR products followed by analysis using Phred software was done to determine if any \textit{Cdkn1c} and \textit{Phlda2} were detected from the paternal allele. For total expression level analysis, cDNA was subject to qRT-PCR. qRT-PCRs were performed on three E15.5 +/\textit{Del}^{7AI} placentae and three wild type littermate or three E13.5 \textit{Ascl2}^{KI}/\textit{Del}^{7AI} placentae and three wild type littermates with three technical replicates per individual placentae. All primer sequences for qRT-PCR and allele-specific analyses are available in Table C.1.
4.3 Results.

4.3.1 Partial loss of imprinting of Ascl2 and Tssc4 in paternal +/Del7Al heterozygotes

To first determine in a paternal versus maternal allele-specific manner whether loss of imprinting (LOI) of the genes in the IC2 cluster was occurring in paternal +/Del7Al placentae (Figure 4.1A), we crossed +/Del7Al males with females from our congenic line that has distal MMU7 from castaneus on a CD-1 background (C), to generate C/Del7Al progeny. We have previously shown that expression levels of the IC2-regulated genes in +/Del7Al heterozygotes were similar to wild type at E9.5 in early stages by qRT-PCR of total cDNA levels (23), but allele-specific analyses have not been performed and later stages have not been examined. To examine parent-of-origin specific expression using single nucleotide polymorphisms (SNPs), we performed either: 1) RT-PCR and allele-specific restriction enzyme digest or 2) direct sequencing of RT-PCR products and subsequent Phred analysis (a base-calling program that determines the quality of DNA sequence tracings) when a suitable restriction enzyme could not be found on E13.5 placentae. We found direct evidence for expression of the paternal allele of Ascl2 in paternal +/Del7Al placenta, indicating that Ascl2 is not properly silenced in +/Del7Al placenta (Figure 4.1B). We also noticed slight paternal derepression in the neighboring gene, Tssc4, although in the wild type context this gene is not tightly imprinted (Figure 4.1B) (78). Cdkn1c and Phlda2, which are located distal of IC2 (Figure 4.1A) showed no indication of LOI in either of these genes (Figure 4.1B). When maternal to paternal (M to P) ratios of the results from Figure 4.1B were assessed by ImageJ software, we confirmed that partial LOI was occurring in +/Del7Al for Ascl2 and Tssc4, but not for Cdkn1c and Phlda2 (Figure 4.1C). We also assessed total levels of gene expression at E15.5 by qRT-PCR and found a correlative increase in
Figure 4.1 Allele-specific and total expression for the IC2-regulated genes Ascl2, Tssc4, Cdkn1c, and Phlda2 in +/-Del7A1 placentae at E13.5 and E15.5.

A) Simplified map of the distal IC2 region on mouse chromosome 7. B) Restriction enzyme digest or direct sequencing (when informative SNP unavailable) for Ascl2, Tssc4, Cdkn1c, and Phlda2. Representative gels and sequence tracings are shown. Ascl2 and Tssc4 show slight paternal reactivation in CAST/Del7A1 E13.5 placentae. By direct sequencing of cDNA, no paternal Cdkn1c or Phlda2 expression was detected in CAST/Del7A1 E13.5 placentae. Nucleotide within black rectangle indicates the informative SNP. For Cdkn1c, the castaneus SNP is a C and the CD-1 SNP is a T. For Phlda2, the castaneus SNP is a C and the CD-1 SNP is an A. The reciprocal cross, +/-CAST, was done as a control and is not shown. C: castaneus; M: maternal; P: paternal. C) Maternal to paternal ratio (M to P ratio) is shown for each genotype taken from the gels and the direct sequencing data. For Ascl2 and Tssc4, ImageJ analysis was performed for two placentae per genotype. For Cdkn1c and Phlda2, Phred analysis was performed on the sequence peaks. n=4 for each genotype. Fading indicates that no paternal Ascl2 and Cdkn1c expression could be detected. D) qRT-PCR for each gene to assess total expression levels at E15.5 on +/-Del7A1 and wild type placentae on three technical replicates each from three biological replicates (individual placentae) for each genotype and the error bars shown are the standard deviation for biological replicates. Expression is relative to the reference housekeeping gene, Peptidylprolyl isomerase A (Ppia). Asterisk for Ascl2 and Tssc4 indicates a significance of p=0.004 and p=0.023, respectively. p=0.76 for Cdkn1c. p=0.099 for Phlda2.
total *Ascl2* and *Tssc4* expression in +/-Del7AI placentae, but not for *Cdkn1c* and *Phlda2* (Figure 4.1D). Allele-specific analysis for *H19* expression in the IC1 cluster has been previously shown not to be perturbed upon paternal inheritance of the Del7AI allele (122). These results demonstrate that although the paternal Del7AI heterozygotes have no observable phenotype, they show reactivation of *Ascl2* and to some extent, *Tssc4*, but not the remaining genes in the IC2 cluster examined in this study.

### 4.3.2 Partial rescue of maternal *Ascl2*KI with paternal inheritance of the Del7AI allele

Maternal inheritance of the *Ascl2* knock-in allele (*Ascl2*KI/+, also interchangeably referred to as *Ascl2*-lacZ) results in midgestational lethality which has been previously described (158). To provide genetic evidence for the Ascl2 LOI observed in paternal Del7AI heterozygotes further, we crossed +/-Ascl2KI females with Del7AI heterozygous males (Figure 4.2A-C). These experiments allowed us to determine if the small amount of paternal Ascl2 expression from the Del7AI allele could rescue the E10 lethality seen in conceptuses lacking a maternal copy of Ascl2. Indeed, we found a small percentage (~10%) of live pups that were recovered at birth which were of the *Ascl2*KI/Del7AI genotype (Figure 4.2B) and they usually survived to adulthood. We also represented our numbers obtained from breedings in terms of % observed/expected recovery of genotypes based on only the viable genotypes (Figure 4.2C). For instance, in Ascl2KI/Del7AI live pups, we found the observed/expected % was ~22.5% (Figure 4.2C). When +/-Ascl2 KO females were bred to Del7AI/+ males, two Ascl2 KO/Del7AI out of 63 (~3%) pups were recovered at birth (Figure 4.2B). Note that the main difference between the *Ascl2* KO and KI alleles is that while the *Ascl2* open reading frame (orf) has been deleted in the KO allele, the orf in the KI is not deleted and *Ascl2* is expressed at low levels, less than 10% of wild type levels (Chapter 3;
Figure 4.2 Midgestational lethality of maternally inherited Ascl2KI allele can be partially rescued by paternal inheritance of Del7AI.

A) Numbers of Ascl2KI/Del7AI embryos rescued in utero from dissections between E14.5 and E18.5 obtained from +/-Ascl2KI or +/-Ascl2 KO females crossed with +/- or Del7AI/+ males. Note that few Ascl2KI/+ and no Ascl2 KO/+ pups were recovered in utero. B) Numbers of Ascl2KI/Del7AI live pups rescued at birth from +/-Ascl2KI or +/-Ascl2 KO females crossed with +/- or Del7AI/+ males. The M2/M2 allele was used as a control to confirm that the PGK-neo itself is not the primary cause of interference with gene expression. C) Graphical representation of percentage observed over expected values from crosses rescued in utero (left) and live born (right). The X-axis represents the paternal alleles recovered in live progeny carrying a maternal Ascl2 allele and grey bars are values from crosses with Ascl2KI females and black bars are values from crosses with Ascl2 KO females. p<0.005 for all crosses for observed over expected values using the chi-square ($\chi^2$) test, with the exception of the in utero rescue for +/-Ascl2KI females crossed with Del7AI/+ males which gave a p=0.47.
Figure 3.4). Dissections between E14.5 and E18.5 from this rescue cross revealed that a larger percentage of Ascl2KI/Del7Al genotype was recovered, suggestive of perinatal death (Figure 4.2A). These experimental crosses provide direct evidence that the leaky paternal Ascl2 expression in +/Del7Al heterozygotes is at a level sufficiently high to rescue some pups to term that are missing the maternal copy of Ascl2. Taken together, this suggests that the combination of Ascl2KI allele which provides a small amount of Ascl2 and the paternal Del7Al may sometimes meet a threshold to rescue a small portion of pups (Figure 4.2B). The Del7Al allele was generated by targeting a PGK-loxP-neo-pA-loxP inserted ~4kb centromeric to Ascl2, referred to as the M2 allele (122). The M2 allele was thus used as a control to confirm that the PGK-neo itself which is present in both the Del7Al and M2 alleles is not the cause of interference with gene expression. When +/Ascl2KI females were bred to M2/M2 males, no significant difference was found by chi-square (χ²) analysis between this cross and when +/Ascl2KI females were bred to wild type males (Figure 4.2B-C).

Live born rescued Ascl2KI/Del7Al pups were growth-retarded and this growth retardation largely persists until weaning age (Figure 4.3A), a phenotype similar to what we previously described for Del7Al/+ pups, which were shown to have a marked decrease in Ascl2 expression (23). In light of the postnatal growth retardation we observed in Ascl2KI/Del7Al, we also measured placental and embryonic weights of Ascl2KI/Del7Al conceptuses at E15.5 and noticed a significant (~29%) reduction in placental size from their wild type littermates, consistent with an intrauterine growth restriction phenotype as previously described for the Del7Al/+ embryos (Figure 4.3B; (23)). Embryonic weights of Ascl2KI/Del7Al heterozygotes were also smaller than wild type littermates at this stage (~21%) (Figure 4.3C). We have previously shown that there is
Figure 4.3 Reduced placental weight and pre- and post-natal growth retardation of rescued Ascl2KI/Del^{7AI} heterozygotes.

A) Scatterplot of rescued live Ascl2KI/Del^{7AI} pups and their wild type litter mates at P0 (birth), P7 and P21 (time of weaning). n=7 for Ascl2KI/Del^{7AI} pups and n=14 for wild type littermates. Bars show the average weight ± standard deviation. p=7.9x10^-8 for P0 weights, p=6.5x10^-6 for P7 weights and p=0.001 for P21 weights. B) Scatterplot of E15.5 placental weights of Ascl2KI/Del^{7AI} and wild type littermates. n=10 for Ascl2KI/Del^{7AI} and n=25 for wild type littermates. Bars show the average weight ± standard deviation. p=0.0001 for placental weights. C) Scatterplot of E15.5 embryonic weights revealing weight difference between Ascl2KI/Del^{7AI} and +/+ littermates. n=10 for Del^{7AI}/+ and n=25 for wild type littermates. Bars show the average weight ± standard deviation. p=0.002 for embryonic weights.
no difference in placental and embryonic weights in $+/\text{Del}^{\text{7Al}}$ compared to wild type littermates (23).

4.3.3 *Ascl2KI/\text{Del}^{\text{7Al}}* placentae possess an expanded giant cell layer, thin spongiotrophoblast, and lack glycogen cells

Due to the decreased placental size of *Ascl2KI/\text{Del}^{\text{7Al}}* placentae, we performed histological analysis using a variety of informative markers at E15.5 compared with wild type and $+/\text{Del}^{\text{7Al}}$ littermates. Hematoxylin and eosin (H&E) stain revealed an expanded TGC giant layer in *Ascl2KI/\text{Del}^{\text{7Al}}*, also confirmed by DAPI stain, while $+/\text{Del}^{\text{7Al}}$ were indistinguishable from wild type littermates (Figure 4.4A-B). We next carried out ISH on E15.5 placentae for 4311 (*Tpbpa*), *Ascl2*, *Pcdh12*, and *Cdkn1c* in paternal $+/\text{Del}^{\text{7Al}}$ heterozygotes, *Ascl2KI/\text{Del}^{\text{7Al}}*, and wild type littermates (Figure 4.5A-D). $+/\text{Del}^{\text{7Al}}$ placentae were indistinguishable from wild type placentae with respect to spongiotrophoblast formation and glycogen cell biology (Figure 4.4A-E). Consistent with the E13.5 allele-specific *Ascl2* analysis, we noticed a slightly higher level of *Ascl2* expression in $+/\text{Del}^{\text{7Al}}$ placentae compared to wild type (Figure 4.5B), however this subtle increase does not seem to cause a noticeable placental phenotype in paternal $+/\text{Del}^{\text{7Al}}$ heterozygotes. *Pcdh12*, which is a marker exclusively expressed in glycogen cells (10), and *Cdkn1c*, which is often used as a marker for glycogen cell production (6) were normal in $+/\text{Del}^{\text{7Al}}$ placentae compared to wild type (Figure 4.4C-D). On the other hand, *Ascl2KI/\text{Del}^{\text{7Al}}* placentae, were severely abnormal and displayed a substantial reduction in the spongiotrophoblast (Figure 4.5A), as well as a lack of glycogen cells (Figure 4.5C-D). Little *Ascl2* expression was detected in *Ascl2KI/\text{Del}^{\text{7Al}}* placentae (Figure 4.5B). qRT-PCR analysis at E13.5 revealed *Ascl2* expression in *Ascl2KI/\text{Del}^{\text{7Al}}* was $\sim$11% that of wild type levels (Figure 4.6A). Periodic acid Schiff (PAS)
Figure 4.4 Trophoblast giant cell placental phenotype in \textit{Ascl2Kl/DeI}^{7Al} at E15.5.

A) Hematoxylin and eosin (H&E) staining and B) DAPI staining of wild type, +/DeI^{7Al} and \textit{Ascl2Kl/DeI}^{7Al} placentae at E15.5 both reveal an expanded giant cell layer in the \textit{Ascl2Kl/DeI}^{7Al}. +/DeI^{7Al} placentae were indistinguishable from wild type. Note in \textit{Ascl2Kl/DeI}^{7Al} placentae is the absence of clear spongiotrophoblast and trophoblast glycogen cells in proximity of the giant cell layer. DAPI staining readily identifies the giant polyploid nuclei of the cells stacked at the giant cell layer in the mutants. Scale bar: 0.5mm. d: decidua; TGC: parietal trophoblast giant cells; SpT: spongiotrophoblast; GlyT: trophoblast glycogen cells; lab: labyrinth layer. Dashed lines indicate TGC boundary.
Figure 4.5 Abnormal spongiotrophoblast and glycogen cell phenotype in Ascl2KI/Del7Al at E15.5.

A) 4311, B) Ascl2, C) Pcdh12, D) Cdkn1c ISH and E) Periodic acid Schiff (PAS) stain performed on E15.5 placentae. +/Del7Al placentae were indistinguishable from wild type, with the exception of increased Ascl2 expression in the spongiotrophoblast. Ascl2KI/Del7Al placentae revealed a near lack of spongiotrophoblast, glycogen cells, and Ascl2 expression. Multiple sections from two placentae of each genotype were assessed and representative pictures are shown. Sense probes not shown. The blue stain shows gene expression. Dark pink/purplish stain shows glycogen staining in (E). Scale bar: 0.5mm. SpT: spongiotrophoblast, TGC: trophoblast giant cells, lab: labyrinth, GlyT: glycogen cells, d: maternal decidua. Dashed lines indicate TGC boundary.
stain which stains for glycogen also did not show the presence of glycogen cells in $\text{Ascl2}^{\text{KI}}/\text{Del}^{7\text{Al}}$ (Figure 4.5E). The placental phenotype of the $\text{Ascl2}^{\text{KI}}/\text{Del}^{7\text{Al}}$ described thus far is virtually identical to the previously reported $\text{Del}^{7\text{Al}}/+\$ phenotype, which also possess suboptimal levels of $\text{Ascl2}$ (23), suggesting that $\text{Ascl2}^{\text{KI}}/\text{Del}^{7\text{Al}}$ conceptuses are hypomorphic for $\text{Ascl2}$.

### 4.3.4 $\text{Ascl2}^{\text{KI}}/\text{Del}^{7\text{Al}}$ placentae possess a labyrinthine phenotype but normal $\text{Phlda2}$ expression

We have previously reported that decreased $\text{Ascl2}$ levels in $\text{Del}^{7\text{Al}}/+\$ placentae is accompanied by increased $\text{Phlda2}$ expression, another IC2 regulated gene which is expressed in syncytiotrophoblast layer I of the labyrinth at around midgestation and could also partly contribute to the labyrinthine abnormalities previously observed (23,86). To determine the status of $\text{Phlda2}$ expression in $\text{Ascl2}^{\text{KI}}/\text{Del}^{7\text{Al}}$ placentae, we performed $\text{Phlda2}$ qRT-PCR on E13.5 $\text{Ascl2}^{\text{KI}}/\text{Del}^{7\text{Al}}$ placentae and found that $\text{Phlda2}$ expression was unchanged in $\text{Ascl2}^{\text{KI}}/\text{Del}^{7\text{Al}}$ compared to wild type (Figure 4.6B). The previously reported $\text{Del}^{7\text{Al}}/+\$ placental phenotype also included abnormal labyrinthine development, specifically, disorganized fetal vessels, overproliferation of syncytiotrophoblast cells, and increased numbers of mononucleated trophoblast cells (23). To determine if $\text{Ascl2}^{\text{KI}}/\text{Del}^{7\text{Al}}$ possessed any abnormalities in the labyrinth, we performed ISH and IHC for a few labyrinth specific markers at E15.5 compared with wild type and $+/\text{Del}^{7\text{Al}}$ littermates. In the labyrinth, *Placental lactogen-II* (*Prl3b1/Pl-II*) is expressed in the mononucleated giant cells lining the maternal blood sinusoids in the labyrinth (sinusoidal trophoblast giant cells) (144). We found a relative decrease in $\text{Prl3b1}$ stain in the labyrinth of $\text{Ascl2}^{\text{KI}}/\text{Del}^{7\text{Al}}$ compared with wild type, suggestive of a decreased density of sinusoidal giant cells (Figure 4.7A). Although initially ISH for $\text{Gcm1}$, a marker of the
Figure 4.6 Expression analysis of Ascl2, Phlda2 and Gcm1 in Ascl2KI/Del7Al at E13.5.

A) Ascl2, B) Phlda2 and C) Gcm1 qRT-PCR to assess total expression levels at E13.5 on Ascl2KI/Del7Al, +/-Del7Al and wild type placentae on three technical replicates each from three biological replicates (individual placentae) for each genotype and the error bars shown are the standard deviation for biological replicates. Expression is relative to Ppia. Asterisks for Ascl2 and Gcm1 indicates a significance of p=0.0003 and p=0.01, respectively.
Figure 4.7 Labyrinth phenotype in Ascl2KI/Del^{7Al} at E15.5.

ISH for (A) Prl3b1 and (B) Gcm1 and (C) IHC for laminin of wild type and mutant placentae at E15.5. +/Del^{7Al} placentae were indistinguishable from wild type. Multiple sections from two placentae of each genotype were assessed and representative sections are shown. Sense probes not shown. The blue stain shows gene expression. Prl3b1 is expressed in parietal trophoblast giant cells, spongiotrophoblast and mononucleated sinusoidal trophoblast giant cells of the labyrinth. In the mutants, the spongiotrophoblast staining is replaced by a thicker giant cell layer, and the labyrinth signal is denser. Gcm1 is a marker of the syncytiotrophoblast II of the labyrinth. C) IHC for laminin marks the basement membrane of the fetal blood vessels and revealed a denser network of capillaries in the mutant labyrinth. The brown stain shows protein expression. Scale bar: 0.5mm. SpT: spongiotrophoblast, TGC: trophoblast giant cells, lab: labyrinth, GlyT: glycogen cells.
syncytiotrophoblast layer II (13), did not appear remarkably different between *Ascl2*KI/Dei^7Al^
and wild type (Figure 4.7B), when we performed qRT-PCR for *Gcm1*, we found that the overall
levels were increased in the *Ascl2*KI/Dei^7Al^
mutants (Figure 4.6C), suggesting an increase in
labyrinthine trophoblast proliferation in the rescued mutants consistent with what we have
previously observed in Dei^7Al^/+ mutants (23). IHC for laminin, a marker of the basement
membrane associated with fetal vascular endothelium revealed that *Ascl2*KI/Dei^7Al^
possessed an
overdeveloped fetal vasculature compared with wild type (Figure 4.7C). In each case the +/Dei^7Al^
were indistinguishable from wild type (Figure 4.7A-C). Taken together, these results indicate a
labyrinthine phenotype in *Ascl2*KI/Dei^7Al^
similar to what has previously been reported for the
Dei^7Al^/+ phenotype, but solely attributable to substantially reduced *Ascl2* levels.

4.3.5 *Kcnq1ot1* spreading does not extend into the IC1 domain after paternal inheritance of
Dei^7Al^

The *Kcnq1ot1* ncRNA has been proposed to extend proximally past the *Ascl2* locus (M.
Mann, unpublished data). Our laboratory has recently obtained data suggesting that the action of
*Kncq1ot1* can extend as far as the *Ins2* locus which is ~300kb further than what has been
previously been suggested (88). Due to the close proximity of the IC1 domain to the IC2 domain
in the Dei^7Al^ heterozygotes, we considered the possibility that the IC1-IC2 interval is required to
act as a transcriptional boundary between the two domains and that the silencing *in cis* by the
*Kcnq1ot1* ncRNA on the paternal allele could extend beyond *Ascl2* to affect the paternally
expressed gene *Igf2*. To test whether the presence of the *Kcnq1ot1* ncRNA causes a decrease in
*Igf2* levels, qRT-PCR was performed on E13.5 +/Dei^7Al^ and wild type placentae. No difference
was found between the two genotypes (Figure 4.8A). ISH for *Igf2* on E15.5 placenta accordingly
Figure 4.8 *Kcnq1ot1* does not extend to the IC1 domain after paternal inheritance of *Del7Al*.

A) *Igf2* qRT-PCR on +/Del7Al E13.5 placental cDNA reveals no overall difference in expression level. Expression is relative to *Ppia*. Three technical replicates each from three biological replicates (individual placentae) for each genotype and the error bars shown are the standard deviation for biological replicates. B) *Igf2*ISH on +/Del7Al and wild type E13.5 placentae. Multiple sections from two placentae of each genotype were assessed and representative pictures are shown. Sense probes not shown. The blue stain shows gene expression. Scale bar: 0.5mm. sp: spongiotrophoblast, lab: labyrinth.
showed consistent levels and patterns of expression between paternal +/-Del7Al heterozygotes and wild type (Figure 4.8B). This data confirms that Kcnq1ot1-dependent silencing does not extend into the IC1 domain in +/-Del7Al heterozygotes and the only misregulated genes upon paternal inheritance of the Del7Al allele are within the IC2 domain.

4.4 Discussion

Here we report that paternal inheritance of a deletion of a ~280kb intergenic region between two imprinted domains leads to differences in allele-specific Ascl2 and Tssc4 imprinted gene expression. We show that upon paternal inheritance of Del7Al, the paternally inherited alleles of Ascl2 and Tssc4 subsequently become partially derepressed and paternal expression can be detected. The paternal Ascl2 expression is sufficiently high to rescue a small percentage of pups to term that have a mutated maternal copy of Ascl2. Finally, we show that these rescued Ascl2KI/Del7Al placentae exhibit abnormal characteristics that are similar to what we have previously reported under conditions of suboptimal Ascl2 levels in Del7Al/+ placentae, including placental and embryonic growth restriction, expanded TGC layer, reduced spongiotrophoblast, lack of glycogen cells, and abnormal labyrinthine development (23). We report that Ascl2KI/Del7Al placentae demonstrate a decrease in Prl3b1 levels compared to wild type and no change in Phlda2 expression compared to wild type, which differs from previous findings in Del7Al/+ placentae, where both Prl3b1 levels and Phlda2 expression are increased compared to wild type (23). The reason for this difference between the two genotypes is unclear. However, it should be noted that Phlda2 expression was assessed at different stages in the two genotypes.

Previous work describing an 800kb YAC of the IC2 domain has shown that imprinted expression of genes is appropriately recapitulated in these mice, with the exception of Ascl2 and
$Tssc4$ (120). Paternal inheritance of this YAC results in loss of $Ascl2$ and $Tssc4$ imprinting but has no phenotype, while maternal inheritance of the YAC results in growth retardation (120). The YAC extends roughly halfway into the IC1-IC2 interval and includes $Th$. The authors postulated that perhaps $Ascl2$ and $Tssc4$ imprinting requires additional centromeric control elements that were missing on their YAC. Our work likewise confirms that the presence of the entire IC1-IC2 interval is required for appropriate imprinting of the centromeric genes $Ascl2$ and/or $Tssc4$. Because the imprinted status of $Tssc4$ in the placenta is conflicting and at best shows leaky paternal expression (76,78,172) and the role that $Tssc4$ plays in the placenta, if any, has not been documented, we focused our efforts on the analysis of the $Ascl2$ locus in this study.

When $Ascl2$ levels are suboptimal, the resulting placental phenotype is severely abnormal including a reduced placental size, thin spongiotrophoblast, lack of glycogen cells and expanded giant cell layer, as we have previously shown (23) and now show in the $Ascl2$KI/$Del^{7Al}$ mice. We therefore reasoned that increased dosage of $Ascl2$ may potentially have an opposite effect on placental phenotype, such as an expanded spongiotrophoblast. However, we have confirmed in this study through several methods that $+/Del^{7Al}$ placentae are indistinguishable from wild type. We simply may not have a biologically significant increase in $Ascl2$ to cause a phenotype. A high copy $Ascl2$ transgenic model might allow us to address this question in more detail.

Although $Ascl2$KI/$Del^{7Al}$ placentae unlike $Del^{7Al}/+$ do not possess an increase in $Phlda2$ expression compared to wild type, the overlapping phenotype between the previously reported maternal $Del^{7Al}/+$ and $Ascl2$KI/$Del^{7Al}$ in this study provides additional support that $Ascl2$ levels are the primary cause of the placental phenotype observed in the three layers of the mature placenta.
The mechanism by which partial LOI occurs at the *Ascl2* and *Tssc4* loci in +/+*Del7Al* is unknown and is currently under investigation. IC2 function as well as *Kcnq1ot1* expression are unaffected in +/+*Del7Al* (122), eliminating a role for aberrant *Kcnq1ot1* expression in causing the paternal expression of *Ascl2* and *Tssc4*. We propose that the IC1-IC2 interval is required to maintain a transcriptional boundary between the two domains by not only acting as a “stuffer sequence” but by compartmentalizing the paternal and maternal chromosomes. This is supported by the observation that *Kcnq1ot1* does not extend into the IC1 domain in paternal +/+*Del7Al* heterozygotes (Figure 4.8). Consistent with this result is the observation that +/+*Del7Al* pups are phenotypically normal and one would expect that if they did possess lower *Igf2* levels due to *Kcnq1ot1* interference, they would be growth retarded (107). On the other hand, it could be argued that the two domains being brought into close proximity perturbs the proper chromosome architecture of the IC1 domain causing signals from the IC1 domain to affect part of the IC2 domain in +/+*Del7Al* heterozygotes. On the paternal allele, methylation at IC1 prevents CTCF binding, allowing *Igf2* promoters to access shared enhancers located downstream of H19 (151,152). Perhaps *Ascl2* and to some extent *Tssc4* can access these enhancers, albeit not with maximum efficiency. Perhaps repressive marks such as H3K9me2, H3K9me3 and H3K27me3 which are normally found along the paternal chromosome 7 at each of the IC2-regulated genes (57,58,59,71), are decreased in +/+*Del7Al*, which would suggest that the IC1-IC2 interval is required for acquisition of repressive marks and this loss may be in part responsible for the partial derepression of the genes closest to the deletion. Future studies include developing assays to perform chromatin immunoprecipitation for repressive histone marks and chromosome conformation capture in reciprocal *Del7Al* heterozygotes to determine the chromosomal interactions that occur, which interactions are lost (or gained) upon inheritance of the deletion,
and if any of these losses and/or gains are allele-specific. Such knowledge will give us a comprehensive overview of the intricate mechanisms at play in the imprinted distal mouse chromosome 7 region.

Distal mouse chromosome 7 shares homology with human 11p15.5, commonly referred to as the Beckwith-Wiedemann syndrome (BWS) region, an imprinted pre- and postnatal overgrowth disorder. Although the overwhelming majority of BWS cases is caused by increased \textit{IGF2} expression or lack of \textit{CDKN1C} expression or both of these situations, a rare number of BWS patients do not possess any deregulation of the imprinted genes on 11p15.5, and thus the epigenetic or molecular defect cannot be explained. For example, one case in which a family had no abnormalities in \textit{IGF2} or \textit{CDKN1C} expression, but possessed a frameshift mutation in the \textit{NLRP2} gene on chromosome 19 was shown to be the cause of BWS (173). In another example, in a few cases of Sotos syndrome, a pre- and postnatal overgrowth disorder which is caused by mutations in the \textit{NSD1} gene on chromosome 5, anomalies in 11p15 have been found (174). Conversely, \textit{NSD1} mutations in a cohort of BWS patients have also been observed (174). Thus perhaps large scale intrachromosomal and interchromosomal interactions with the 11p15 region can contribute to BWS. These observations in human and our work in mice shed light on the importance of studying intergenic neighbouring regions when assessing the status of regulation of imprinted genes.

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Chapter 5: Conclusion

5.1 Overall significance

The DelTel7/IC2KO project cleanly demonstrated that IC2 is the main regulator of genomic imprinting of the IC2-regulated genes in the mouse placenta. At the time this research was carried out, it was not known if additional factors on distal MMU7 other than IC2 and \textit{Kcnq1ot1} transcription could contribute to the regulation of imprinting in the placenta. Other factors might have included putative methyl binding proteins (MBDs) and tissue-specific differences. Tissue-specific differences in \textit{Cdkn1c} expression have been reported in mice with a truncation of \textit{Kcnq1ot1}, suggesting mechanisms other than \textit{Kcnq1ot1}-mediated silencing may regulate \textit{Cdkn1c} in those tissues, although in the placenta \textit{Cdkn1c} silencing appears \textit{Kcnq1ot1} dependent (51). Our data suggests that all of the IC2-regulated genes in the mouse placenta are silenced via an IC2-dependent mechanism.

The \textit{Del7Al} project revealed the consequences on imprinted regulation of IC2-regulated genes upon deleting a large region outside of an imprinted region on mouse chromosome 7. To the best of our knowledge, the \textit{Del7Al} study is the first example of a deletion made not to an imprinting centre or imprinted gene, but to a flanking non-imprinted region, that has disrupted the imprinting of neighbouring genes. Specifically, upon maternal inheritance of \textit{Del7Al}, we have made several key findings that have aided our understanding of the regulation of imprinting in the BWS imprinted region: 1) suboptimal \textit{Ascl2} expression and the accompanying phenotype; 2) a direct reciprocal relationship between the two imprinted genes, \textit{Ascl2} and \textit{Phlda2}. We have found that the \textit{Del7Al/+} is a hypomorph of \textit{Ascl2} and the second to report a relationship between the two imprinted genes, \textit{Ascl2} and \textit{Phlda2} (the first is (19)), although we are the first to establish that the two are linked and to suggest that part of the phenotype that has previously
been thought to occur from Phlda2 overexpression may more likely caused instead by decreased Ascl2 expression. Studying the paternal inheritance of Del7AI has allowed us to make several key findings that have aided our understanding of the regulation of imprinting in the BWS imprinted region: 1) the partial LOI of Ascl2 and Tssc4; 2) the partial rescue of a maternally inherited Ascl2KI allele by paternal inheritance of the Del7AI allele; 3) the remarkable phenotypic similarities these rescued Ascl2KI/Del7AI heterozygotes have to maternal Del7AI/+ heterozygotes, validating aberrant Ascl2 expression as the main culprit of the placental phenotype. In doing so, we also confirmed Ascl2 as being one of the most important genes in the imprinted distal MMU7 region for placental development.

5.2 Discussion of thesis research

The main strength of the DelTel7/IC2KO approach was the ability to study the IC2 domain in a hemizygous context. When the IC2KO allele was originally generated (118), the reciprocal wild type allele was present in +/-IC2KO and IC2KO/+ mutants, making it impossible to study the effects of the knockout allele in isolation. Biallelic expression of the IC2-regulated genes was observed in the +/-IC2KO mutants, whereas IC2KO/+ mutants had no abnormal phenotype (118). Our studies of DelTel7/IC2KO placentae definitively demonstrate that the levels of the IC2-regulated genes when the IC2KO allele is paternally inherited are sufficient in the absence of the maternal homologue to fully rescue the placental phenotype seen in DelTel7/+ heterozygotes.

The +/-DelTel7 genotype has no obvious abnormal phenotype. H&E stain also indicated that these placentae are normal. We also examined IC2KO/DelTel7 placentae by H&E and a variety of ISH placental marker analysis to determine if any irregularities were present in these
placentae, since it is unknown if any factor(s) such as MBDs bind the maternal methylated IC2, which would be absent in IC2KO/DelTel7 placentae (R. Oh-McGinnis and L. Lefebvre, unpublished data). We found no differences between these placentae and their wild type littermates.

One of the main weaknesses of the DelTel7/IC2KO project was the inability to obtain reliable placental and embryonic weight data to back up our findings due to the genetic background differences in the crosses. The +/-DelTel7 females were maintained on a CD-1 outbred background while the +/-IC2KO males were maintained on C57BL/6. Thus due to the genetic heterogeneity of the rescued placentae, it was difficult to determine if there was a size difference in DelTel7/IC2KO placentae compared to +/- littermates due to an insufficient number of samples. We can only hypothesize that due to the lack of any abnormalities in the DelTel7/IC2KO placentae by our marker analysis, placental size was unaffected or only minimally affected. Due to the lack of any difference in our histological studies, we also did not take our analysis further and assess whether nutrient transfer was impaired in DelTel7/IC2KO placentae, which is another critical indicator that would have supported our findings.

The main strength of the Del7AI project was the ability to study the consequence of deleting the IC1-IC2 interval in reciprocal hemizygotes. The next step required in the Del7AI project is follow-up studies to understand the mechanism of LOI at Ascl2 caused by loss of the IC1-IC2 interval. This is discussed in detail in Section 5.3. An issue of the Del7AI project is the discrepancy between the two manuscripts regarding Ascl2 expression and the relationship between Ascl2 and Phlda2 expression across all of the mutant genotypes (Del7AI/+, +/-Del7AI, Ascl2KI/Del7AI, Ascl2KI/+). The results from our studies have been summarized in Table 5.1. We did not initially observe an increase in Ascl2 expression at E9.5 by qRT-PCR in +/-Del7AI
heterozygotes in our first Del7Al manuscript (Figure 3.2). In our second manuscript, when we analyzed Ascl2 expression at E13.5 by allele-specific expression and ImageJ analysis and at E15.5 by qRT-PCR in +/-Del7Al (Figure 4.1), we found a subtle but significant increase in Ascl2 expression (Table 5.1). One possible explanation for the difference might be that Ascl2 is not downregulated properly in +/-Del7Al placentae. Even in the wild type plenta, Ascl2 expression varies depending on the stage and increases between E8.5 to E12.5 (where it is at its highest), then drops dramatically until embryos are at term (147). We also do not know why the difference in Phlda2 expression appears far less pronounced in the Ascl2KI/+ compared to Del7Al/+ (Figure 3.2, 3.4). However, preliminary gene microarray analysis on E9.5 Ascl2KI/+ placentae independently confirmed that Phlda2 is slightly increased compared with wild type. It would have been informative to compare Ascl2 and Phlda2 levels in Del7Al/+, +/-Del7Al, Ascl2KI/Del7Al, Ascl2KI/+ and wild type all at the same stage in the same qRT-PCR experiment. It is unclear why Ascl2KI/Del7Al do not exhibit any significant change in Phlda2 expression. This contrasts with our results from Del7Al/+ and Ascl2KI/+, where we observed a notable increase in Phlda2 expression (Figure 4.7; Table 5.1).

Our results from Chapters 3 and 4 have revealed that low levels of Ascl2 expression is compatible with survival, although the pups exhibit IUGR, which was not previously known. In fact, the lowest % of expression relative to wild type we observed that was compatible with survival was in the Ascl2KI/Del7Al at ~11% (Figure 4.6), while Del7Al/+ had ~57% Ascl2 expression that of wild type (Figure 3.1). The Ascl2KI/+ which do not survive, had ~4% Ascl2 expression relative to wild type (Figure 3.4).

In our hands, an increase, albeit slight, in Ascl2 does not result in an abnormal phenotype. Based on our observations in the Del7Al/+ as well as its role in the development of the
Table 5.1 \textit{Ascl2} and \textit{Phlda2} expression levels across genotypes.

Summary of \textit{Ascl2} and \textit{Phlda2} expression levels in \textit{Del}^{7AI}/+, \textit{Ascl2}KI/+, +/\textit{Del}^{7AI}, and \textit{Ascl2}KI/\textit{Del}^{7AI} with respect to wild type at the various stages analyzed in Chapters 3 and 4. Arrows with values indicate the relative fold change with respect to wild type controls.
spongiotrophoblast, we hypothesized that +/-Del7AI placentae might have a slightly expanded spongiotrophoblast. We observed no difference in placental size nor in spongiotrophoblast ratios of +/-Del7AI placentae compared to wild type littermates and. morphometric analysis of the spongiotrophoblast did not reveal any differences between the two genotypes (R. Oh-McGinnis and L. Lefebvre, unpublished data). Determining the placental phenotype of an Ascl2 transgenic line with elevated Ascl2 levels would be of interest; this has been generated by another group and is currently under investigation (Rosalind John, unpublished data). Finally, based on the inverse relationship between Ascl2 and Phlda2 in Del7AI/+, we postulated that +/-Del7AI could possess a slight decrease in Phlda2. In our hands, Phlda2 levels in +/-Del7AI placentae were similar to wild type (Figure 4.1; Table 5.1), suggesting that perhaps the slight increase in Ascl2 is not biologically significant to affect Phlda2 expression.

5.3 Future directions

The DelTel7/IC2KO project was essentially a follow-up project to the original description of the DelTel7 allele (121) and no additional experiments have been planned. Current studies being pursued by another member of the laboratory, Aaron Bogutz, include an assessment of other unique effects of the DelTel7 allele, such as the effect of parent-of-origin telomere shortening.

Several avenues of study can be taken for the Del7AI project. A few of the key questions that remain unanswered from the Del7AI project are:

1) Why is Ascl2 expression substantially reduced in Del7AI/+?

2) What is the function of Ascl2, particularly in the establishment of the trophoblast glycogen cell lineage?
3) What is the mechanism of partial LOI in +/Del7Al?

4) Does the IC1-IC2 interval play a role in regulating imprinting of the IC2 domain?

The precise cause of reduced Ascl2 expression in Del7Al/+ is unclear and could be investigated. As mentioned in the Discussion section (3.4) of Chapter 3, we cannot exclude with absolute certainty the possibility that we have deleted a putative enhancer(s) of Ascl2 or other factors that are responsible for Ascl2 expression. Future plans include a detailed bioinformatic analysis followed by genetic analysis of the IC1-IC2 region to determine this.

It may be useful to take advantage of an Ascl2 transgenic mouse line possessing an increased dosage of Ascl2 as a starting point to learn more about its regulation. Future plans include obtaining Ascl2 transgenic males (courtesy of Rosalind John) and mating them with Del7Al females to confirm whether the growth retardation observed in Del7Al/+ heterozygotes can be rescued solely by an increased dosage of Ascl2. This set of experiments would initially confirm that Ascl2 is the main culprit. However, the phenotype of Ascl2 transgenics is currently unknown, and could potentially complicate this experiment. We could also assess whether Ascl2 transgenics exhibit decreased Phlda2 expression, which would provide further insight into the intricate relationship that exists between Ascl2 and Phlda2.

The multiple roles that Ascl2 appears to have in the placenta has not been appreciated until now, including in particular, the profound impact that Ascl2 has on trophoblast glycogen cells. It suggests that Ascl2 plays a central role early on in the trophoblast glycogen cell lineage, which has never been previously reported. However, it is unknown how Ascl2 affects the trophoblast glycogen cell lineage. Current experiments are being carried out by another graduate student in the laboratory, Karen Jacob, to determine if Ascl2 is required in the formation of the trophoblast glycogen cell lineage and if so, how it functions to form trophoblast glycogen cells.
The IC1-IC2 interval has been postulated to function as a transcriptional boundary between the two imprinted subdomains (153). Partial LOI could be occurring in +/Del^{7Al} for a number of reasons, including spatial disorganization of the chromatin or loss of key elements in the IC1-IC2 interval. Based on our partial LOI results from Chapter 4, we can propose a model that might be tested in order to explain the evolution of the IC1-IC2 interval (Figure 5.1). In the wild type setting on the paternal allele, the presence of the 280kb IC1-IC2 interval may be sufficient to separate the two distinctive domains such that the most centromeric genes in the IC2 domain, *Ascl2* and *Tssc4*, are unaffected by transcription of the paternal IC1-regulated gene, *Igf2*. In the +/ Del^{7Al} setting, the IC2 domain is possibly brought closer into the active region of the nucleus such that *Ascl2* and *Tssc4* which lie at the boundary of the two regions are subsequently expressed at low levels, potentially due to access to *Igf2* enhancers or lower levels of repressive H3K9me3 and H3K7me3 accumulation near the periphery. Preliminary ChIP (chromatin immunoprecipitation) data suggests there is a significant decrease in H3K9me3 and H3K7me3 at *Ascl2* and *Tssc4* in E13.5 +/Del^{7Al} placentae compared to wild type and Del^{7Al}/+ placentae. However, we also noticed a decrease in H3K9me3 and H3K7me3 in the downstream IC2-regulated genes *Cdkn1c* and *Phlda2* in E13.5 +/Del^{7Al} placentae. ChIP experiments will be revisited in the future to confirm what is happening at these loci. Further support that the IC1-IC2 interval could act as a transcriptional boundary comes from experiments demonstrating that *Kcnq1ot1* establishes a nuclear domain that differs in the embryo and placenta (72). When 3-dimensional distances between *Kcnq1ot1* signal and the edge of the domain for *Cdkn1c* (located within the IC2 domain that is regulated by *Kcnq1ot1*), *Ascl2* (located at the edge of the domain, and *Igf2* (located outside the IC2 domain and is not regulated *Kcnq1ot1*) signals in both the embryo and placenta, was measured by RNA-DNA FISH, the positions of all three genes were
Figure 5.1 Model for the evolution of the IC1-IC2 interval.

Wild type situation is shown above and \textit{+/Del}^{7AI} situation is shown below. In the \textit{+/Del}^{7AI} setting, the lack of the IC1-IC2 interval brings the two domains into close proximity to each other. We propose that the IC2 domain is brought closer into the active region of the nucleus such that \textit{Tssc4} and \textit{Ascl2} which lie at the boundary of the two regions are subsequently expressed at low levels (dotted arrows), possibly due to lower levels of repressive H3K9me3 and H3K7me3 accumulation (hexagons) observed near the boundaries of the repressive and active domains. \textit{Kcnq1ot1} expression is unaffected in both situations. Grey genes are maternally expressed, black genes are paternally expressed and white genes are biallelic. Darkened region represents silent domain and white region represent active domain in the nucleus.
significantly different from each other in the placenta (72). Perhaps in our +/-Del7AI heterozygotes, the lack of the IC1-IC2 interval causes Ascl2 (and Tssc4) to shift just outside of the boundary of the Kcnq1ot1 nuclear domain.

Chromatin looping has been proposed as a mechanism by which CTCF (CCCTC-binding factor) boundary elements can segregate active and silent domains to achieve imprinted gene expression (175,176). This has been documented for IC1, where the separation of H19 and Igf2 into chromosomal loops is known to occur through the binding of CTCF (and possibly other proteins) on the unmethylated maternal allele (152,176,177). The binding of CTCF generates a boundary that prevents Igf2 promoters from gaining access to enhancers on the maternal allele. There is mounting evidence that CTCF not only mediates intra-chromosomal interactions (176) but also inter-chromosomal interactions (178,179). For example, IC1 has been found to colocalize with the Wsb1/Nf1 region on mouse chromosome 11, and omission of CTCF or deletion of the maternal IC1 inhibits this association, while deletion of the paternal IC1 has no effect (178), providing further evidence for the central role of CTCF binding sites and chromatin looping in the regulation of genomic imprinting. The Del7Al allele provides a unique opportunity to look specifically at the importance of chromosome looping in silencing, since imprinting is relaxed despite the production of Kcnq1ot1. Current attempts of chromosome conformation capture (3C) experiments at IC2 by another group have been unsuccessful but the reason for this technical difficulty is unclear (personal communication from N. Engel to L. Lefebvre). 3C followed by quantitative PCR (3C-qPCR) could be used to learn about the mechanism of partial LOI in the Del7Al. 3C-qPCR involves crosslinking interacting chromatin fragments followed by a restriction enzyme digest and intramolecular ligation of crosslinked fragments (177,180). qPCR is subsequently performed on the restriction fragments of interest to quantify chromatin loops.
between two segments that interact more often with each other than with neighbouring chromatin fragments (177,180). The relative crosslinking frequencies of the IC1-IC2 interval with neighbouring loci (including IC2) in +/Del7Al, Del7Al/+ and wild type placenta could then be compared to determine if the Del7Al deletion results in loss of chromosomal interactions, which would provide proof of the necessity for chromatin looping to maintain imprinting in the distal MMU7 region.
References


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Appendix A: Supplementary Material for Chapter 2

Figure A.1 Supplementary Figure. Expression of genes on distal mouse chromosome 7 in the placenta.

Four placental samples were collected from E15.5 male conceptuses from C57BL/6 mice. RNA was extracted from whole placenta and sent for microarray analysis at the McGill University and Genome Quebec Innovation Centre. Expression data was obtained from Illumina MouseRef-8 v2.0 Beadchip and pre-processed by the lumi normalization within the FlexArray software. IC1: Imprinting centre 1. IC2: Imprinting centre 2. *Ppia* and *Gapdh* are housekeeping genes. *Xist* and *Olfr541* are not expressed in male mouse placenta. *Olfr541* is located on chromosome 7, ~2Mb upstream of *Igf2*.
Figure B.1 Supplementary Figure. Expression of trophoblast markers in Del^{7AI}/+ mutant placentae.

(A) Ascl2, Phlda2 and Gcm1 expression levels were analyzed by qRT-PCR on E15.5 placental RNA. (B) Syna, Gcm1 and Dlx3, on E9.5 placental RNA, and (C) Tpbpa, on E9.5 placental RNA. The analysis was carried out on three technical replicates each from three individual Del^{7AI}/+ placentae and three wild type (+/+) littermate individual placentae (biological replicates) and the error bars shown are the standard deviation for biological replicates. Expression is relative to Ppia. Asterisks indicate statistically significant difference between wild type and mutants at E15.5 (Ascl2 p=0.0045; Phlda2 p=0.02; Gcm1 p=0.004).
Figure B.2 Supplementary Figure. Co-expression of ASCL2 and PHLDA2 in the ectoplacental cone at E7.5.

IHC using anti-ASCL2 and –PHLDA2 antibodies was performed on adjacent section of E7.5 conceptuses. Positive cells within the EPC were revealed using the colorimetric substrate DAB (brown). Scale bars are 400 μm (at 5x magnification) and 50 μm (at 40X).
Figure B.3 Supplementary Figure. Maintenance of trilaminar labyrinthine structure in Del⁷Al/+ mutant placentae.

Sections of E13.5 wild type (+/+) and mutant (Del⁷Al/+) placentae showing arrangement of the trilaminar labyrinth trophoblast. Each picture highlights the close association between fetal capillaries, filled with nucleated fetal red blood cells, and maternal blood spaces, filled with maternal erythrocytes.
Figure B.4 Supplementary Figure. Intrauterine growth restriction in maternal $Del^{7AI}$ heterozygotes.

Newborns from five litters obtained by crossing $+/Del^{7AI}$ heterozygous females to wild-type CD-1 outbred males and from six reciprocal crosses were weighted the morning of their delivery (p0) and genotyped. (A) Scatter plot showing the distribution of the weights at birth. The average weight of maternal heterozygous pups (1.42 g) is 17.4% smaller than that of their wild-type litter mates (1.72 g). Between the maternal heterozygotes and wild-type pups, $P = 1.82E-8$ (t-test). Number of animals analyzed: n = 58 wild-type, n = 18 $Del^{7AI}$/+, n = 34 $Del^{7AI}$/+ and n = 18 $Del^{7AI}$/+ treated with L-DOPA. (B–D) Body weight of mutant and wild-type litter mates at p0, p7 and p21 (time of weaning). Unlike the paternal heterozygotes ($+/Del^{7AI}$), maternal heterozygous pups ($Del^{7AI}$/+) do not catch up to their wild-type litter mates before weaning. This growth retardation is not rescued by addition of L-DOPA to the drinking water of pregnant females. Bars show the average weight+standard deviation. At each time point, $P < 0.001$ (t-test) between maternal heterozygotes and wild-types in C. 

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### Table C.1. Supplementary Table. Primers used for allele-specific analysis and qRT-PCR.

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