The function of the imprinted transcription factor ASCL2 in mouse trophoblast development

by

Karen Jane Jacob

B.Sc.,The University of British Columbia, 2010

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies

(Medical Genetics)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2013

Abstract

The epigenetic phenomenon known as genomic imprinting which leads to the monoallelic expression of genes in a parent-of-origin dependent manner has been linked to the development and function of the placenta in mammals. The imprinted gene, *Ascl2,* codes for a transcription factor which is expressed from the maternal allele in the placenta and is required for its development. Mice that lack *Ascl2* expression from their maternal allele die around mid-gestation of placental failure. The effects of *Ascl2* can be studied *in vivo*, in mice that are *Ascl2*-deficient and, *in vitro* in trophoblast stem (TS) cells which provide an excellent model of early placental development. Here we compare the transcriptomes of *Ascl2*-deficient and wild-type E9.5 placentae and find that a total of 838 coding genes are significantly downregulated in the mutant placentae. These genes were deemed to be potential candidate targets of ASCL2. The downregulation of several genes from this list is verified by qRT-PCR and their location in the placenta investigated by *in situ* hybridization, verifying their overlap with *Ascl2* in the trophoblast. We also investigate the knock-out (KO) placental phenotype of one of these candidate target genes, *Hmga2*, and recognized a labyrinth phenotype in the *Hmga2*-KO. We also describe, for the first time, the establishment of *Ascl2*-deficient TS cells confirming that *Ascl2* is dispensable for TS cell establishment and maintenance. We find that *Ascl2*-deficient TS cells lack expression of several trophoblast cell lineage markers through differentiation suggesting they are unable differentiate into cells of the trophoblast lineage. We also find that *Ascl2* candidate gene expression in differentiating *Ascl2*-deficient TS cells is altered when compared to wild-type. These results provide important insight into the functional role of *Ascl2* in the development and differentiation of the cells of the trophoblast lineage.

Preface

The candidate (K. Jacob) performed all experiments except dissection of tissue for RNA-seq which was done by R. McGinnis. All experiments were done with the help of A. Bogutz.

RNA-seq was done at the Michael Smith Genome Sciences Center with normalization done by M. Bilenky.

Data mining and literature research to choose *Ascl2* target candidate genes was done by L. Lefebvre, A. Bogutz and K. Jacob.

Ethics approval was obtained from the Animal Care Committee at the University of British Columbia for mouse work (Protocol number A11-0293).

All *Hmga2*-knockout mouse breeding was done by the C. Eaves Lab at the BC Cancer Research Center.

L. Lefebvre and A. Bogutz conceived of the study.

Parts of Section 1.4 were published in the following review paper, and written concurrently with the thesis by the candidate (K. Jacob):

Jacob KJ, Robinson WP, Lefebvre L. Beckwith-Wiedemann and Silver-Russell syndromes: opposite developmental imbalances in imprinted regulators of placental function and embryonic growth. *Clinical Genetics*. PMID: 23495910 [Epub ahead of print], 2013

Table of contents

List of tables

List of figures

List of abbreviations

ABC avidin biotin complex
BSA bovine serum albumin
CpG cytosine-phosphate-guanine dinucleotide
C-TGC canal associated trophoblast giant cell
DAB diaminobenzidine
DAPI 4',6-diamindino-2-phenylindole
Del7^{AI} deletion allele of region between *Ascl2* and *Ins2*
DIG digoxigenin
DMR differentially methylated region
DNA deoxyribonucleic acid
E embryonic day
EPC ectoplacental cone
ExE extraembryonic ectoderm
FACS fluorescence-activated cell sorting
FGF4 fibroblast growth factor 4
FITC fluorescein isothiocyanate
GC trophoblast glycogen cells
H&E hematoxylin and eosin
IC imprinting center
IC1 imprinting centre 1
IC2 imprinting centre 2
ICM inner cell mass
IF immunofluorescence
IHC immunohistochemistry
IRES internal ribosome entry site
ISH *in situ* hybridization
IUGR intrauterine growth restriction
KI knock-in
KO knockout
M-MLV moloney murine leukemia virus
mRNA messenger ribonucleic acid
ncRNA noncoding RNA
OCT optimal cutting temperature
OPT optical projection tomography
PAS periodic acid Schiff
PBS phosphate buffered saline
PBS-T phosphate buffered saline-tween20
PCR polymerase chain reaction
PEF primary embryonic fibroblast
PFA paraformaldehyde
P-TCG parietal trophoblast giant cell
qRT-PCR quantitative reverse transcription polymerase chain reaction
RNA ribonucleic acid
RPKM reads per kilobase of transcript per million mapped reads

SpA-TGC spiral arter-associated TGC
S-TGC sinusoidal trophoblast giant cell
TE trophectoderm
TS trophoblast stem (cell)
TGC trophoblast giant cell
pUPD paternal uniparental disomy
UTR un-translated region
WT wild type

List of gene names

Ascl2 achaete-scute complex homolog 2
Atoh8 atonal homolog 8
Car2 carbonic anhydrase 2
Cdkn1c cyclin-dependent kinase inhibitor 1C
Cd81 CD81 antigen
Cdx2 caudal-type homeobox 2
Ceacam9 carcinoembryonic antigen-related cell adhesion molecule 9
Doxl1 Diamine oxidase-like protein 1
Eomes eomesodermin
Err-beta estrogen receptor beta
Fgfr2 fibroblast growth factor receptor 2
Gcm1 glial cells missing homolog 1
H19 H19 fetal liver mRNA
Hmga2 high mobility group AT-hook 2
Igf2 insulin growth factor 2
Igf2r insulin growth factor 2 receptor
Ins2 insulin 2
Kcnq1 potassium voltage-gated channel, subfamily Q, member1
Kcnq1ot1 KCNQ1 overlapping transcript 1
Lpl lipoprotein lipase
Mgp matrix Gla protein
Pappa2 pappalysin 2
Pcdh12 protocadherin 12
Phlda2 pleckstrin homology-like domain, family A, member 2
Ppia peptidylprolyl isomerase A
Prl3d1 prolactin family 3, subfamily d, member 1
Th tyrosin hydroxylase
Tpbpa trophoblast specific protein alpha
Tssc4 tumor-suppressing subchromosomal transferable fragment 4
Vgll1 vestigial like 1 homolog

Acknowledgments

First and foremost I would like to thank my family, especially my parents who risked everything to give my siblings and I a better life in Canada and who have provided me with unconditional love and support throughout my post-secondary endeavours.

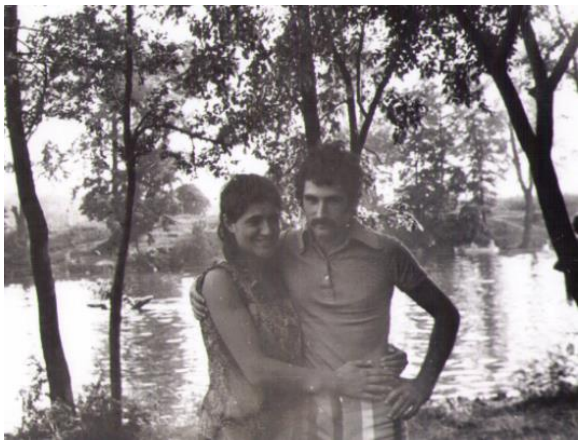
I would like to thank all of my lab members, past and present, especially Aaron Bogutz who is always there to answer my questions and my supervisor, Dr. Louis Lefebvre for taking me on as his graduate student.

I would also like to thank my friends in the Molecular Epigenetic Research Group who have provided me with guidance, support and friendship throughout my Master's degree.

Last but not least I would like to thank the members of my thesis committee, Dr. Diana Juriloff and Dr. Pamela Hoodless for their wisdom, patience and guidance.

Dedication

To Adrian and Aurelia Jacob



Bucharest, summer 1973

Chapter 1 Introduction

1.1 Genomic imprinting

The maternal and paternal mammalian genomes are not functionally equivalent, but complementary, contributing differently to embryonic and extra-embryonic development despite the fact that they contain the same genetic material. Both parental genomes are required for a mammalian embryo to survive to term. This was first demonstrated in 1984 with the construction of murine zygotes containing 2 full sets of chromosomes from parents of the same gender (1, 2). An embryo containing 2 maternal genomes, called a diploid gynogenetic embryo, goes through somewhat normal early development but has a severely underdeveloped placenta. In contrast, an embryo containing 2 paternal genomes, called a diploid androgenetic embryo, has a much more developed placenta but the embryo is severely underdeveloped. In both cases, development halts around mid-gestation. Based on these results, it was hypothesized that the mammalian genome contains some genes expressed only from a single parental allele. This phenomenon of unequal genomic contribution where genes are expressed differently depending on parent of origin is called genomic imprinting (1). To date, roughly 150 imprinted genes have been identified in the mouse.

Imprinted genes are generally found in clusters in the genome, a feature that allows them to be controlled together by one mechanism (reviewed in (3)). These clusters tend to contain important genes that when misregulated, can cause developmental problems leading to disease or death. Genomic imprinting is controlled epigenetically by parent-of-origin specific DNA methylation and histone modifications. Imprinting clusters harbour CpG islands that are differentially methylated on the maternal and paternal chromosomes. Some of these differentially

methylated regions (DMRs), known as imprinting centers (ICs) control parental-specific silencing and expression of multiple imprinted genes. For example, a 1Mb cluster of at least 12 imprinted genes lies on mouse chromosome 7F.5 (analogous to a region on chromosome 11p15.5 in humans, Figure 1.1). The imprinted cluster on mouse chromosome 7 is controlled by 2 ICs called IC1 (or H19DMR) and IC2 (or KvDMR1). IC1 is a DMR that lies just upstream of the gene H19 fetal liver mRNA (*H19*) and controls the parent-of-origin-specific expression of the most proximal genes in the domain: *H19*, insulin growth factor 2 (*Igf2*) and insulin 2 (*Ins2*) (4-6). It acts as a methylation sensitive insulator allowing differential access to an enhancer element on the differentially methylated parental alleles (7, 8). IC2 controls the more distal genes in the domain which include important maternally expressed genes including: achaete-scute complex homolog 2 (*Ascl2*), which encodes an essential transcription factor (9); cyclin-dependant kinase inhibitor 1C (*Cdkn1c*), which encodes a cyclin-dependant kinase inhibitor implicated in Beckwith-Weidemann Syndrome (10); and potassium voltage-gated channel, subfamily Q, member 1 (*Kcnq1*), which encodes a potassium channel implicated in Long QT syndrome (11). The IC2 DMR lies in an intron of *Kcnq1* and overlaps with the promoter of the non-coding RNA (ncRNA) KCNQ1 overlapping transcript 1 (*Kcnq1ot1*). *Kcnq1ot1* is only expressed from the unmethylated paternal chromosome, a process which leads to the silencing of at least 8 known protein coding genes *in cis* by the recruitment of proteins that establish repressive chromatin marks (12-15).

There are 2 prevailing hypotheses for the evolution of genomic imprinting in mammals. One hypothesis, called the ovarian time bomb hypothesis, maintains that imprinting evolved to prevent the trophoblast from becoming malignant which could happen if an unfertilized egg spontaneously began to develop in the mother. If the expression of important genes related to

growth of the trophoblast are absent from the egg, such as is the case when genes are imprinted, then it cannot differentiate into invasive trophoblast, giving the mother a selective advantage over those without imprinting (16). This hypothesis, however, does not adequately explain genes that are silenced in the paternal genome, and imprinted genes that are not involved in trophoblast development.

A more widely accepted hypothesis for the evolution of genomic imprinting in mammals is the parental conflict hypothesis (17). When a female is pregnant it is in her best interest to restrict the nutrients available to her fetuses and reserve energy for herself so she can have subsequent pregnancies to propagate her genome. For the male however, it is in his best interest for his fetuses to be as big and strong as possible despite the expense to the mother, as he can impregnate any number of females to propagate his genome. The hypothesis predicts that maternally expressed genes will restrict embryonic growth while paternally expressed genes will promote it. Many imprinted genes have been found to be consistent with this hypothesis. For example, the first imprinted gene to be discovered, *Igf2*, is paternally expressed and known to play a pivotal role in embryonic growth and development (18). Its overexpression in humans and in mice is associated with overgrowth while its perturbation is associated with intrauterine growth restriction (IUGR) (19-21). In contrast, the maternally expressed insulin growth factor 2 receptor (*Igf2r*) is known to restrict growth by negatively regulating *Igf2* (22). These examples demonstrate the contradictory effects of maternally and paternally expressed genes on development. It should be noted that although a growing number of imprinted genes fit the predictions of the conflict hypothesis, it does not explain all imprints. For example, a prediction of the hypothesis is that all paternal uniparental disomies (pUPD) where there are imprinted

genes on the chromosome would result in fetal overgrowth; however this does not seem to be the case as pUPD-6 has been found to be associated with fetal growth retardation in humans (23).

Mammalian embryos are unique in that they depend on their placenta to obtain nutrients from their mother. In theory, this makes the placenta a prime area of parental conflict as the mother tries to limit resource allocation to her young and the father tries to exploit it. In accordance with the parental conflict hypothesis it is therefore no surprise that many imprinted genes are expressed in the placenta (24).

1.2 The mouse placenta

The placenta is the first organ to develop in mammalian pregnancy, providing essential functions to sustain the development of the embryo. The placenta provides structural support in the uterus and is the site of gas, nutrient and waste exchange between mother and fetus. It also promotes the redirection of maternal resources like blood flow and immune functions to support the pregnancy (25). Without proper formation of the placenta both the developing embryo and mother may be at risk (26). Even if development of the embryo does not seem perturbed, a sub-optimal embryonic environment due to abnormal placentation has been linked to complications for the offspring later in life, such as cardiovascular disease and diabetes (27-29).

The structure of the placenta varies widely across mammalian species. For example, ruminants such as cattle, sheep and goats have what is called a cotyledonary placenta which consists of numerous small placentae (30). Carnivores such as cats and dogs typically have what is called a zonary placenta which forms a band that encircles the fetus (30). While the human and mouse placentae also have differences, they both have a single disk-shaped placenta with a single area of attachment called a discoid placenta (30). Anatomically, the human and mouse

placentae are similar, both with 3 physiologically distinct regions and several analogous cell types making the mouse placenta a worthy model for placental research. (For details on the similarities between mouse and human placentae see (28, 31, 32)).

The first differentiation event to occur in the murine conceptus takes place in the blastocyst when the trophectoderm segregates from the inner cell mass (ICM). The trophectoderm directly in contact with the ICM is termed the polar trophectoderm (polar TE), while the rest of the trophectoderm is termed the mural trophectoderm (mural TE). The TE goes on to differentiate further into the trophoblast cell lineages which make up the bulk of the placenta.

The mature mouse placenta consists of 3 distinct layers. The highly vascularized labyrinth layer lies on the fetal side of the placenta and is the site of fetal-maternal exchange. The spongiotrophoblast layer lies just above the labyrinth, and on the maternal side of the placenta, aiding in uterine implantation, is a monolayer of parietal trophoblast giant cells (P-TGCs). Glycogen cells, a transient cell type also populate the placenta, arising in the spongiotrophoblast around embryonic day (E) 12.5 and migrating past the P-TGC layer into the maternal decidua above (33). Another type of trophoblat giant cell (TGC), the spiral artery-associated giant cells (SpA-TCGs) also invades the decidua to line the maternal spiral arteries which bring blood into the placenta (34). (For a summary of mouse placental development see Figure 1.2).

Around the time of implantation, the cells of the mural TE stop dividing but continue to replicate their DNA by endoreduplication and contribute to the P-TCG layer, which has an important role in mediating the implantation process. These polyploid cells can reach up to 1000n in DNA content which gives them the capacity to secrete large amounts of proteins vital

to adaptation of the maternal physiology to pregnancy, including cytokines, hormones, cell adhesion molecules, vasodilators, anticoagulants, proteinases and extracellular matrix (25, 34).

The polar TE continues dividing to give rise to the extraembryonic ectoderm (ExE) and the ectoplacental cone (EPC) which are the diploid precursors to the chorion and the spongiotrophoblast, respectively. In addition, the polar TE also gives rise to some P-TGCs and to the other secondary TGC types. Around E8.5 the chorion makes contact with the allantois, a structure derived from the extraembryonic mesoderm, to form the labyrinth layer of the placenta. This event is called chorioallantoic attachment and is the beginning of the formation of the complicated vascular network of the placenta. Soon after attachment, the chorion forms branched villi providing a path for fetal vasculature from the allantois to invade the trophoblast. This network of vascularization continues to develop until the end of pregnancy, becoming increasingly complex. Lining the fetal blood vessels are 2 layers of multinucleated syncytiotrophoblast cells which are formed by the fusion of diploid chorionic trophoblast cells. The maternal blood vessels are lined by mononuclear trophoblast cells called sinusoidal TGCs (S-TGCs). This combination of cells in the labyrinth is known as the trilaminar trophoblast layer. The process of branching morphogenesis, vascularization of the placenta, and differentiation of chorion cells is crucial for proper nutrient and gas exchange between mother and fetus (32, 34).

The spongiotrophoblast layer, adjacent to the labyrinth, is derived from the EPC. The trophoblast cells that make up the mature spongiotrophoblast can be recognized by their expression of the gene trophoblast specific protein alpha *(Tpbpa)* (35). The function of the spongiotrophoblast remains unknown although it has been suggested to be involved in structural support of the placenta (31). It was previously assumed that glycogen cells, which also express *Tpbpa* (36), differentiate from cells of the spongiotrophoblast layer around E12.5; however they

are now thought to be their own distinct cell lineage with precursors arising in the EPC before its differentiation. Indeed, glycogen cell precursors have been found in the EPC as early as E7.5, marked by their expression of the gene protocadherin 12 (*Pcdh12*) (37). Trophoblast glycogen cells are named for their glycogen content which they begin accumulating at around E12.5. Through the latter half of gestation they migrate past P-TGC cell layer and settle near maternal vascular sinuses where they release their intracellular components. Their function is also unclear but it has been suggested that their glycogen stores may provide energy for parturition (37). Canal-associated TCGs (C-TGCs) that line the canals that bring maternal blood to the base of the labyrinth can also be found in the spongiotrophoblast and in the labyrinth (34).

1.3 Mouse trophoblast stem cells

The molecular aspects of placental development can be challenging to study *in vivo* when maternal tissue and blood are so closely intermingled within the fetal placenta, causing a considerable source of maternal contamination. Early placental development in particular can be challenging to study because of dissection difficulties and the small amount of material that can be obtained by dissection. As an alternative, placental development can be studied *in vitro* with the use of trophoblast stem (TS) cells. Self-renewing, pluripotent TS cells can be derived from the trophectoderm of the mouse blastocyst at E3.5 and maintained in the presence of fibroblast growth factor 4 (FGF4), heparin, and embryonic fibroblasts or embryonic fibroblast conditioned media (38). The addition of FGF4, heparin, and feeder cells mimics the signals provided *in vivo* by the ICM and the epiblast to the TS cell population which lies directly adjacent in the polar TE and later in the ExE. The signalling pathways required to maintain the TS cell state are not completely understood. Undifferentiated wild-type TS cells remain stable over many passages and have a normal euploid (2n) karyotype after upwards of 20 passages (38).

Upon removal of FGF4, heparin, and embryonic fibroblasts, TS cells begin to differentiate and may become any cell of the trophoblast lineage including TGCs, spongiotrophoblast cells, glycogen cells and syncytiotrophoblast cells of the trophoblast labyrinth, but they most readily differentiate into polyploid TGCs (38). It has been suggested that TS cells may default most readily to the TGC pathway *in vitro* because certain *in vivo* signals may be missing, such as those potentially coming from the extraembryonic mesoderm to signal labyrinth development. TS cells are able to contribute to the ExE, EPC and TGC layer of the placenta *in vivo* but not to tissue derived from the ICM (38). As TS cells differentiate they begin to lose expression of diploid ExE markers such as estrogen related receptor beta *(Err-β)*, caudal-type homeobox 2 (*Cdx2),* eomesodermin *(Eomes),* and fibroblast growth factor receptor 2 (*Fgfr2).* They begin to express various markers of the differentiated trophoblast cell lineages such as prolactin family 3, subfamily d, member 1 (*Prl3d1*), a P-TCG specific gene (34), *Tpbpa*, a spongiotrophoblast specific gene (38) and glial cells missing homolog 1 (*Gcm1*), a syncytiotrophoblast marker (39). Upon differentiation, TS cells also up-regulate the imprinted gene *Ascl2* which is expressed in the EPC and later the spongiotrophoblast and the labyrinth layer of the placenta (38).

1.4 *Ascl2* and mutational analysis of *Ascl2*-deficient conceptuses

The gene *Ascl2* (previously known as *Mash2*) is a mammalian homolog of the *Drosophila* achaete-scute gene (40). It encodes an imprinted, maternally expressed basic helix-loop-helix (bHLH) transcription factor that binds to E-box consensus sequences (CANNTG) in heterodimers with E-factor proteins to activate transcription (41, 42). *Ascl2* is expressed in all cells of the pre-implantation embryo and becomes restricted to the trophoblast around the time of implantation; however it is not required for blastocyst formation or for implantation (9). It is

highly expressed in the EPC and weaker in the chorionic ectoderm, which contribute to the spongiotrophoblast and the labyrinth layers of the mouse placenta respectively. After about E12, *Ascl2* expression becomes patchy and begins to decline in the mature placenta (43).

Knocking out *Ascl2* on the maternal allele has such a dramatic effect on the trophoblast lineages that the embryo does not survive past mid gestation, overcome by placental failure (9). The *Ascl2* mutant placenta has a defect in the P-TGC layer which becomes expanded in the mutant, and in the labyrinth layer, which is underdeveloped and does not have the highly vascularized appearance of a wild-type labyrinth. In addition, *Ascl2*-null placentae completely lack a spongiotrophoblast. This phenotype suggests that *Ascl2* is essential for proper differentiation of the trophoblast and it has been shown that it does this in a cell autonomous manner (9, 44). Even though *Ascl2*-null mice have defects in all 3 placental layers it is thought that the defects in the labyrinth and in the P-TGC layer are secondary effects of the spongiotrophoblast defect. It is hypothesized that the spongiotrophoblast may provide signals and/or structural support required for proper formation of the placenta (44). It has also been suggested that ASCL2 competes with another bHLH transcription factor in the trophoblast, HAND1, which is responsible for P-TGC formation, and in doing so ASCL2 prevents P-TGC formation. If this model is correct it could explain why the P-TGC population is expanded in the *Ascl2*-deficient placenta (45).

Mice with paternal uniparental disomy 7 (pUPD7) have a strikingly similar phenotype to that of the *Ascl2*-null. These mice do not survive past mid gestation and their placentae have no spongiotrophoblast layer and an expanded P-TGC layer (46). Expression of just *Ascl2* from a transgene is able to rescue these mice to late gestation and some to term, providing evidence that

Ascl2 is the only maternally imprinted gene on mouse chromosome 7 that causes early embryonic lethality (47).

Another mouse model, the *Ascl2*-knock in allele (*Ascl2*-KI, official name *Ascl2*$^{tm1.1Nagy}$ MGI:2155757) contains an internal ribosome entry site (IRES)-lacZ cassette in the 3' UTR of *Ascl2*. What was meant to be a bicistronic tool to measure parental-specific expression of *Ascl2* by X-gal staining in an otherwise wild-type embryo, causes a near loss of *Ascl2* functional gene product (48). It is currently unclear why the inserted IRES-lacZ cassette causes loss of functional *Ascl2* in this allele, but it has been suggested to be a matter of transcript instability (48). Although the knock-in (KI) allele is described as a hypomorph, the expression of *Ascl2* is so low that these mice exhibit the same phenotype as the *Ascl2*-null (48). The *Ascl2*-KI mouse was used as an *Ascl2*-null equivalent in the present study.

Demonstrating the importance of *Ascl2* gene dosage is the *Ascl2* hypomorph, the *Del*^{7AI} allele (official name *Del*$^{(7Ascl2\text{-}Ins2)1Lef}$; MGI 3662900). The *Del*^{7AI} allele contains a deletion spanning the DNA sequences in between the IC1 and IC2-regulated domains which is conserved in eutharians and contains short repeats and retrotransposons along with the gene for tyrosine hydroxylase (*Th*). This deletion does not disrupt imprinting in the region; however maternal inheritance of the deletion results in growth retarded pups (49). It was found that the deletion causes a 2-fold downregulation of *Ascl2* (at E9.5). The mechanism behind this change in gene expression is still unknown. As in the *Ascl2*-null, the placentae of these *Ascl2* hypomorphs display defects in all 3 placental layers, although the embryos survive to term (50). The placentae have a greatly reduced spongiotrophoblast layer, an expanded P-TGC layer, a disorganized and more highly vascularized labyrinth with an increase in trilaminar trophoblast layer cell types, as well as no glycogen cells (50). *Ascl2* is not imprinted in humans and so its stringent role in

human development may be questioned; however, the hypomorphic *Ascl2* mouse model demonstrates how abnormal dosage of this gene can have serious phenotypic effects on placental function and embryonic growth. Whether haploinsufficiency of the human orthologue causes phenotypic effects remains to be determined.

The lethality of *Ascl2*-null conceptuses can be rescued in chimeras between *Ascl2*-null embryos and wild-type tetraploid embryos, which almost exclusively contribute to the extraembryonic tissue (9). This suggests that despite *Ascl2*'s importance in extraembryonic development it seems to have no significant role in embryonic development. The only other place *Ascl2* has been shown to play a role is in the adult intestinal stem cell population in both mice and humans where it is not imprinted and controls the maintenance of these cells (51).

1.5 Thesis theme and objectives

The broad objective of my research is to elucidate the function of ASCL2 in the trophoblast lineage. As discussed above, ASCL2 is an essential transcription factor in development but what genes it targets remains unknown. In this thesis I will describe our efforts to identify potential target genes of ASCL2 in the placenta by comparing the transcriptomes of wild-type and *Ascl2*-KI placentae. One of these potential target genes (*Hmga2*) is studied in more depth in a placental context and the *Hmga2* knock-out (*Hmga2*-KO) placenta will also be examined. In addition, the development of an *in vitro* model, TS cells, is described and used to study the *Ascl2*-deficient phenotype, and the expression of the ASCL2 candidate target genes in more depth.

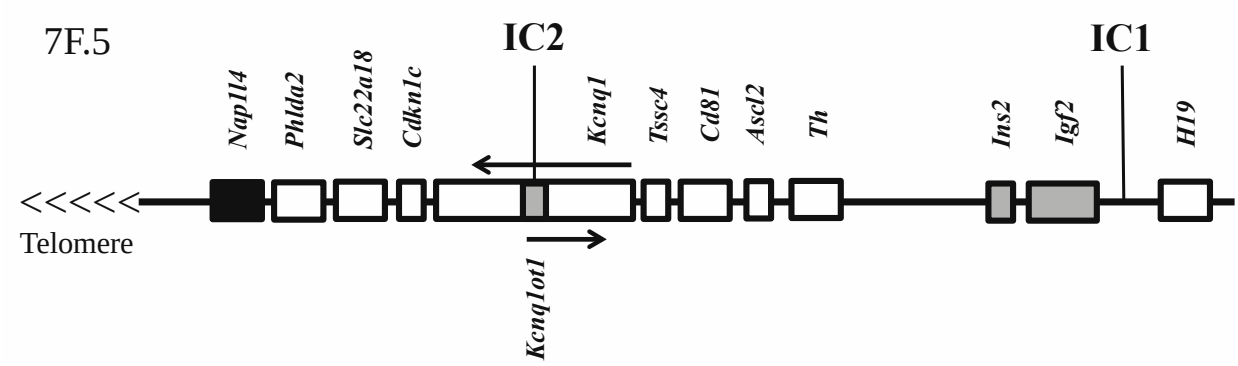


Figure 1.1 Imprinted region on distal mouse chromosome 7. Maternally expressed genes are shown in white, paternally expressed genes are shown in grey and non-imprinted genes are shown in black. Arrows indicate direction of transcription. (Based on Figure 1A in (52))

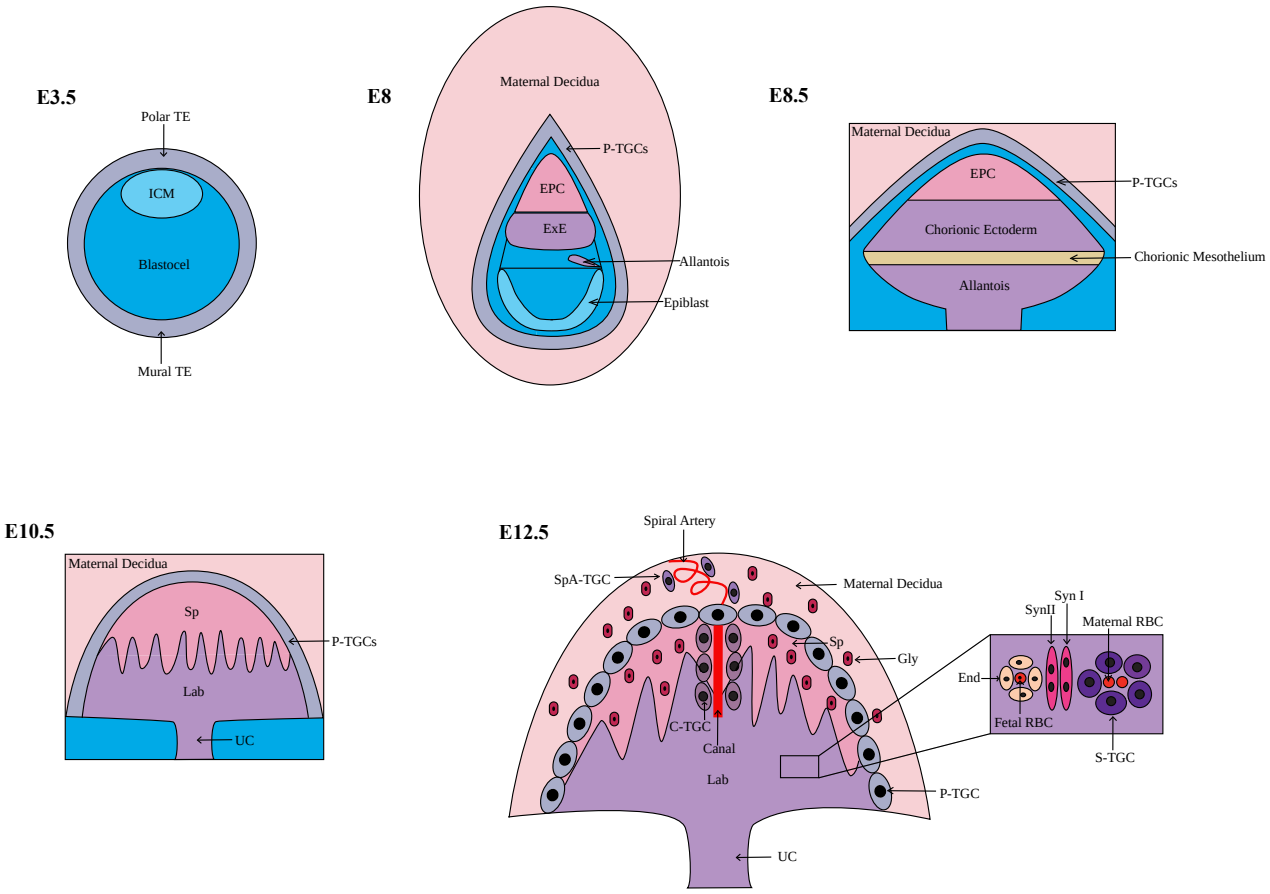


Figure 1.2 Mouse placental development. Placental development from blastocyst (E3.5) to maturity (E12.5). TE=trophectoderm, ICM=inner cell mass, EPC=ectoplacental cone, P-TCG=parietal trophoblast giant cell, EPC=ectoplacental cone, ExE=extraembryonic ectoderm, Sp=spongiotrophoblast, Lab=labyrinth, UC=umbilical cord, SpA-TGC=spiral associated trophoblast giant cell, C-TGC=canal-associated trophoblast giant cell, Gly=glycogen cell, End=fetal vascular endothelium, Syn I=syncytiotrophoblast layer I, Syn II=syncytiotrophoblast II, RBC=red blood cell, S-TGC=sinusoidal trophoblast giant cell (Based on Figure 1 in (32))

Chapter 2 Materials and methods

2.1 Mouse dissections and genotyping

Generation of the *Ascl2*-KI line and genotyping has been previously described (*Ascl2*$^{tm1.1Nagy}$; MGI:2155757) (48). Note that for heterozygotes the maternal allele is always stated first in this thesis. All work done with the *Ascl2*-KI lines was done on a CD-1 outbred background. The *Ascl2*-KI line was maintained by mating heterozygous males (+/*Ascl2*-KI) to wild-type females. *Ascl2*-KI/+ experimental placentae were obtained by mating heterozygous females (+/*Ascl2*-KI) to wild-type males. Mothers were sacrificed by carbon dioxide and cervical dislocation and conceptuses dissected out of the uterus at various developmental stages. All experiments were done according to certificate A11-0293 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.

Hmga2-KO placentae were obtained from the lab of Dr. Connie Eaves (Terry Fox Laboratories, Vancouver, BC). The *Hmga2*-KO mouse (53) is maintained on the C57BL/6 background.

Genotyping material from adult mice was obtained by ear punch. Genotyping material from conceptuses was obtained from the yolk sac, or by removing embryonic tissues with forceps from microscope slides after cryosectioning. DNA was extracted using the HotSHOT method as described (54). Mice were genotyped for the *Ascl2*-KI allele or the *Hmga2*-KO allele using PCR based on primers listed in Table 2.1.

2.2 RNA-seq and *Ascl2* candidate target gene selection

Tissue for RNA-seq was collected by Dr. Rosemary McGinnis. Conceptuses were split in half and embryos and yolk sacs removed. The remaining tissue (placenta with decidua attached) was submitted for RNA-seq. 3 E9.5 placentae with deciduae attached were pooled from each genotype (wild-type and *Ascl2*-KI/+). The E9.5 time point was chosen because it is just after the *Ascl2*-KI phenotype manifests and just before the embryos die.

RNA was extracted and libraries were constructed from mRNA as described (55). The Illumina Genome Analyzer IIx was used to carry out paired-end sequencing according to the recommended protocol (Illumina Inc., Hayward, CA). Reads per kilobase of transcript per million mapped reads (RPKM) normalization was done by Dr. Misha Bilenky at the Michael Smith Genome Sciences Center (Vancouver, BC). Genes were defined as significantly differing in expression between the 2 genotypes by the criteria: RPKM>0.025, N-reads>9, and fold change≥3.

Subsequent analysis was done on the genes that were significantly downregulated in the mutant compared to wild-type RPKM values. A microarray data set comparing mouse placental and decidual expression of genes at E9 (56) was mined using FlexArray, a software package that was developed by M. Blazejczyk and colleagues (Genome Quebec, Montreal) (57). Genes were not considered placental-specific unless they were expressed 2 fold or more in the placenta than the decidua. This list of placental-specific genes was then compared to the list of genes significantly downregulated in the mutant. In addition to data mining, literature research was conducted to select candidate genes that either had a known role in the placenta, were known to

be expressed in the placenta, or that seemed to have potential for placental involvement (Table 3.1).

2.3 RNA extraction, RT-PCR and qRT-PCR

For placentae, specimens were dissected as they had been for RNA-seq (see section 2.2) and yolk sacs taken for genotyping material. 3 biological replicates were used per genotype (not pooled), and technical triplicates done on all samples. Specimens were snap frozen on dry ice and stored at -80˚C for later use. RNA was extracted with Trizol (Invitrogen) and cDNA generated by M-MLV (Invitrogen).

For TS cells, one biological replicate was used for each genotype. 1mL of Trizol was added directly to a $10cm^2$ tissue culture dish for RNA extraction according to the manufacturer's protocol (Invitrogen), and cDNA generated by M-MLV (Invitrogen). Technical triplicates were done for all samples.

qRT-PCR primers for *Ppia* (58), *Phlda2*, *Tpbpa* and *Ascl2* (59) are previously described. All other primers for qRT-PCR were generated by the candidate (K.Jacob) using Primer Express 3.0 software. All primers used in this study are listed in Table 2.1. qRT-PCR results were analyzed with the LinRegPCR software using the $2^{-\Delta\Delta CT}$ method of analyzing relative gene expression (60, 61). Significance was determined by the Student's t-test (threshold set to $p<0.05$).

2.4 *In situ* hybridization (ISH) probe generation

Antisense and sense strand probes were designed and PCR amplified using standard conditions out of mouse CD1 cDNA. Primers were designed by the candidate (K. Jacob) using

Primer3 software, all PCR primers can be found in Table 2.1. PCR products were gel purified using QIAquick Gel Extraction Kit Protocol (Qiagen) and cloned into the vector pGEM-T (Promega). Probes were *in vitro* transcribed using T7, SP6 or T3 RNA polymerases (Roche) and DIG-labelled using 10X labelling mix (Roche). Probes were DNase digested and purified by lithium chloride precipitation. All ISH probes used in the present study were designed and prepared by the candidate (K.Jacob), except for the *Ascl2* probe which was obtained from the lab of Dr. Andras Nagy (Mount Sinai Hospital, Toronto, Ontario).

2.5 ISH

E9.5 conceptuses were removed from the uterus in RNase free PBS and fixed overnight in RNase-free 4% paraformaldehyde/1xPBS at 4˚C. The next day, samples were washed in RNase-free PBS and equilibriated in 30% sucrose (RNase free) overnight at 4˚C. Conceptuses were then incubated in OCT (Tissue-Tek) for half an hour before being embedded in OCT on dry ice and stored at -80˚C until sectioned. Blocks were sectioned at 12 microns on a Leica cryostat (model CM3050 S) and stored at -20˚C until used. ISH was carried out as described (59). Prehybridization was done for 4 hours at 60˚C and hybridization done at 55˚C overnight, except for the *Ascl2* probe which was hybridized at 65˚C. Consecutive sections of one conceptus per genotype were examined.

2.6 Immunohistochemistry (IHC)

E13.5 placentae were dissected in PBS and fixed overnight in 4% PFA/1x PBS. Placentae were then washed in PBS and put in 70% ethanol at 4˚C until used. Placentae were paraffin embedded and sectioned by Wax-It Histology Services Inc. (Vancouver, BC).

For detection of the laminin epitope, slides were deparaffanized, hydrated and blocked with 0.3% hydrogen peroxide for 30 minutes and then blocked with 5% goat normal serum, 0.5% BSA in PBS-T. Rabbit polyclonal anti-laminin (Sigma L9393) was incubated with the slides overnight at room temperature at a 1/50 dilution in serum blocking solution. The next day, the secondary biotinylated goat anti rabbit antibody (Jackson ImmunoResearch) was added at a dilution of 1/500 and incubated for 30 minutes. ABC (Vector) was added for 30 minutes and DAB for 1 minute. Sections were counterstained with hematoxylin and washed in Scott's Tap water solution (2% $MgSO_4$, 0.35% $NaHCO_3$ in distilled water) to help sharpen the contrast. Sections were then dehydrated and mounted with Entellan mounting medium (EM Science) under glass coverslips. Consecutive sections of one placenta per genotype were examined.

2.7 Immunofluorescence (IF)

Conceptuses or placentae were dissected in PBS at various stages and fixed in 4%PFA/1xPBS for 30 minutes to 2.5 hours (depending on size of the specimen) at 4˚C, shaking. Specimens were then equilibrated in 10% sucrose/1xPBS at 4˚C overnight. The next day specimens were equilibrated in 30% sucrose/1x PBS at 4˚C overnight. Specimens were then incubated in OCT (Tissue-Tek) for 30 minutes before being embedded in OCT on dry ice and stored at -80˚C until sectioned. Blocks were sectioned at 12 microns on a Leica cryostat (model CM3050 S) and stored at -20˚C until used.

For detection of the HMGA2 epitope, sections were warmed to room temperature, rinsed twice with PBS and permeabilized with 0.1% Triton-X 100 in PBS (Fisher). Sections were blocked with 4% donkey normal serum, 0.5% BSA in PBS-T. A rabbit polyclonal HMGA2 antibody (Cell Signalling) was diluted 1/400 in blocking solution and incubated for 30 minutes at

room temperature. Slides were washed again with PBS and incubated with a donkey anti rabbit labelled secondary antibody (Invitrogen) for one hour at room temperature. Sections were then washed with water and counterstained with phalloidin (1/400, Invitrogen) for 20 minutes at room temperature, washed again with water and counterstained with 4',6-diamindino-2-phenylindole (DAPI, 2µg/ml, Sigma) for 5 minutes at room temperature. Sections were washed with water and coverslips mounted on glass slides with Vectashield (Vector Labs). Consecutive sections of one conceptus or placenta per time point were examined.

2.8 Periodic acid Schiff (PAS) Stain

E13.5 placental paraffin sections were depariffinized, hydrated and oxidized in 0.5% periodic acid solution for 5 minutes. Slides were placed in Schiff Reagent for 15 minutes and then counterstained with hematoxylin. Slides were washed in Scott's tap water solution (2% $MgSO_4$, 0.35% $NaHCO_3$ in distilled water) to help sharpen the contrast. Sections were then dehydrated and mounted with Entellan mounting medium (EM Science) under glass coverslips. Consecutive sections of one placenta per genotype were examined.

2.9 Morphometric placental analysis

E13.5 placental paraffin sections were stained with hematoxylin and eosin (H&E) by Wax-it Histology Services Inc. (Vancouver, BC). 10x pictures of the sections were imaged on a Leica DMI6000B inverted microscope, captured with Openlab (Improvision) and stitched together in Adobe Photoshop. Placental layers were traced by hand in Adobe Illustrator and the area in pixels of each layer was calculated in Openlab.

2.10 Trophoblast stem (TS) cell derivation, expansion, differentiation

Wild-type and *Ascl2*-KI/+ TS cells were established from E3.5 blastocysts recovered from matings between +/*Ascl2*-KI females and wild-type males. In this study, a total of 15 TS cell lines were successfully established: 9 wild-type and 6 *Ascl2*-KI/+. The establishment, expansion and differentiation of trophoblast stem cells was done as previously described (38, 62). Cells were collected for genotyping and sexing by PCR (primers in Table 2.1). TS cell growth potential was determined by ability of the cells to keep expanding with minimal differentiation at a similar rate through multiple passages.

2.11 TS cell fluorescence-activated cell sorting (FACS)

TS cell differentiation was carried out over a period of 0-8 days with time points at day 0, 1, 2, 4, 6, and 8. TS cells were collected at each time point with 0.25% trypsin and vigorous pipetting to ensure detachment of all cells. Cells were plated at a confluency of approximately 30% on 60mm dishes and left overnight in TS conditioned media with 0.25ng/ml FGF4 and 1ug/ml Heparin. Time point 0 was taken the following day and differentiation initiated as described (63). Cells were collected, fixed in 70% ethanol and stored at -20˚C until the day of FACS analysis. Cells were prepared for FACS using propidium iodide {full protocol described in (63)}. Primary mouse embryonic fibroblasts were used as a diploid control. All data was gathered on a BD LSRII running BD FACS DIVA. Data was analyzed in FlowJo 9.5.

Purpose	Sequence (5'-3')	Name	Reference
Genotyping (M2KI)	CTTCACACGGCAGTTCTGTG	M2B	(64)
	TGAATGGGAAATGTGGTCCTTGG	M2G	(64)
	ACAGCAGGGTTCCCACACTGG	LacZ3'	This study
	GCTGTCAGGTTTGCACAAGG	726R	(64)
Genotyping (Hmga2KO)	ATTCTGGAGACGCAGGAAGA	Hmga2tg F	Jax
	TGCTCCTGGGAGTAGATTGG	Hmga2tg R	Jax
	CCCACTGCTCTGTTCCTTGC	Hmga2KO F	Jax
	GTGTCCCTTGAAATGTTAGGCG	Hmga2 KO R	Jax
qRT-PCR	TCCTGGTGGACCTACCTGCTT	Ascl2 qF1	(59)
	AGGTCAGTCAGCACTTGGCATT	Ascl2 qR1	(59)
	AGACAGCACCGCCCTAACAG	Ceacam9 qF1	This study
	CAGGCCCTGGTGATTGAAGA	Ceacam9 qR1	This study
	CAGATCCGCCGTCAGCAT	Pappa2 qF1	This study
	GGCGCAGGGTGGAGTTG	Pappa2 qR1	This study
	CCTCTCTATATTCCCTGTTCTTCATTG	Hmga2 qF3	This study
	GCAGTCCGAACCAAGATAATGC	Hmga2 qR3	This study
	ACACACTGCAGGACCGCTAGA	Car2 qF1	This study
	GGTCCGTTGTGCTTGCTGTA	Car2 qR1	This study
	CCGCTTCCCAATTACCTAATCTT	Doxl1 (Rik) qF1	This study
	GCACCTGATGGATTTTGGAGTAG	Doxl1 (Rik) qR1	This study
	CGCAAGGGAGCACAACAAG	Fgfbp1 qF3	This study
	CCTCCTCTGGTTGAGCACATCTG	Fgfbp1 qR3	This study
	CGCGTCTCCTCCGAGCTGTTTG	Ppia F	(58)
	TGTAAAGTCACCACCCTGGCACAT	Ppia R	(58)
	CGCTCCATTCATCTCTTCATTG	Lpl qF1	This study
	CTTGTTGATCTCATAGCCCAGATT	Lpl qR1	This study

Table 2.1. List of primers used in this study

Purpose	Sequence (5'-3')	Name	Reference
qRT-PCR	TGCGCACACACACAAATTCTC	Vgll1 qR1	This study
	CCCGCCAAGGAGCTGTTT	Phlda2 qF1	(59)
	CCTTGTAATAGTTGGTGACGATGGT	Phlda2 qR1	(59)
	GAA TGTGTCCTCCAAACCAACTG	Pl-1 qF3	This study
	CACATCTGCGGCCAAGATAAA	Pl-1 qR3	This study
	CAGCTTTGGACATCACAGGTACTT	4311 qF1	(59)
	TGCGCTTCAGGGACTATAGCA	4311 qR1	(59)
	TCCCTCGTCTTTGGCTGAAG	Cdx2 qF1	This study
	GGCTTGTTTGGCTCGTTACAC	Cdx2 qR1	This study
	GCAGAGGTGGCGAGCTAAAG	Mgp qF1	This study
	CGCAGGCCTCTCTGTTGATC	Mgp qR1	This study
	CTGGTGACCATCAGAAGCACTTA	Atoh8 qF2	This study
	CGTGGCTCTAGGAAGCTTGTCT	Atoh8 qR2	This study
ISH probe*	CAGATGAAGCCTGCGTGTTA	Hmga2 ISH F1	This study
	GCATTAGGCAAAAGGCTCAG	Hmga2 ISH R1	This study
	AGTATGGACGCTGCGAGAAT	Cdx2 ISH F2	This study
	AGGGGTGAAGTTGTCAGTGC	Pappa2 ISH R1	This study
	TGTATCTGCAGGCACTCAGG	Doxl1 ISH F1	This study
	CAGGGCTGTAGGAAATGGAA	Ceacam9 ISH R1	This study
	ACCACTGGGGATACAGCAAG	Car2 ISH F1	This study
	ACAGAGAGGCGGTCACACTT	Car2 ISH R1	This study
Sexing	GACTAGACATGTCTTAACATCTGTCC	Zfy1a	L. Lefebvre Lab
	CCTATTGCATGGACAGCAGCTTATG	Zfy1b	L. Lefebvre Lab

*For ISH probes, if a F or R primer is missing from the list, the matching qRT-PCR primer was used

Table 2.1 (continued). List of primers used in this study

Chapter 3 Results

3.1 Transcriptome analysis of wild-type and *Ascl2*-KI/+ placentae at E9.5

To investigate the function of *Ascl2* in the trophoblast lineage and how its expression affects other genes in the placenta, the transcriptomes of wild-type and *Ascl2*-KI/+ placentae at E9.5 with deciduae attached were examined by RNA-seq. We obtained 71.3% exonic reads for the wild-type sample and 71.8% exonic reads for the mutants. When compared, the distribution of RPKM values was similar between wild-type and mutant placentae (Pearson's correlation coefficient r=0.89024). A total of 108 coding genes were found to be significantly upregulated in the mutants, 838 coding genes found to be significantly downregulated in the mutants, and 21,191 coding genes did not significantly differ between the 2 genotypes (Figure 3.1).

To filter out genes that are expressed mainly in the maternal decidua and focus on the genes that are expressed in the placenta, a microarray comparing expression of genes in the placenta and the maternal decidua separately at E9 was examined (56). To elucidate possible targets of transcriptional activation of *Ascl2*, this information was compared to the 838 genes that were significantly downregulated in the *Ascl2* mutant by RNA-seq (Figure 3.2). This analysis revealed that most of the genes found to be downregulated in the mutant by RNA-seq are mainly decidually expressed. Approximately 20 of these genes were defined as placental based on the criteria that they are expressed at least 2 fold higher in the placenta than the decidua. Literature research was then carried out on the remaining genes and genes of interest were selected as candidate target genes of ASCL2 (Table 3.1). The genes initially chosen were: *Doxl1*, *Car2*, *Ceacam9*, *Hmga2*, *Vgll1*, *Lpl*, *Pappa2*, *Atoh8* and *Mgp*. Note that although the genes *Atoh8*, *Mgp* and *Vgll1* were not found to be expressed at least 2 fold or more in the placenta than the decidua,

they were chosen for other reasons which made them worthy of investigation. *Atoh8* is another bHLH transcription factor whose role in the trophoblast is not well characterized and therefore it was of interest to determine if it interacts with *Ascl2*, especially considering *Atoh8* has been shown to only be activated by other bHLH transcription factors (65). Although *Mgp* was found to have a very high decidual expression, its extreme fold downregulation found by RNA-seq in the mutant alone (275.554 fold) was thought to warrant its further investigation. *Vgll1* is expressed 1.043 fold higher in the placenta than the decidua, not making the 2-fold cut-off, however *Vgll1*'s expression pattern in TS cells through differentiation has been shown to follow *Ascl2*'s expression pattern (66) an attribute that might be expected of a gene that is directly turned on by *Ascl2*. Therefore we allowed this gene on our candidate list.

3.2 RNA-seq confirmation of candidate gene downregulation by qRT-PCR

To confirm the RNA-seq results, candidate gene expression was examined by qRT-PCR of 3 wild-type and 3 *Ascl2*-KI placentae with deciduae attached (dissected from a different litter than that of the RNA-seq, but in the same way). All selected candidate genes except *Mgp* and *Atoh8* were found to be significantly downregulated in the KI (Figure 3.3A). These 2 genes were dropped off the list of potential candidates and not examined further. The results confirmed that the expression of the remaining candidate genes (*Doxl1*, *Car2*, *Ceacam9*, *Hmga2*, *Vgll1*, *Lpl*, *Pappa2*) is indeed downregulated in the mutant placentae (Figure 3.3B).

3.3 Candidate gene ISH and IF in wild-type and *Ascl2*-KI E9.5 placenta

Several candidate genes examined at E9.5 by ISH (*Cdx2*, *Car2*, *Doxl1*, *Ceacam9*, *Pappa2*) and IF (*Hmga2*) showed overlapping expression with *Ascl2* in the placenta and showed obvious downregulation in the KI placenta (Figure 3.4A and B). Both *Cdx2* and *Doxl1* were

detected in the spongiotrophoblast where their expression overlaps *Ascl2'*s, while *Cdx2* and *Doxl1* expression in the mutant was not detected by ISH. *Pappa2* was detected globally in the labyrinth and the spongiotrophoblast; unfortunately, no KI staining of this gene was available for this study. *Car2* showed high expression in both the spongiotrophoblast and the labyrinth where it lines the fetal blood, and there were also faint speckles of detection in the decidua. The KI displayed detection of *Car2* only in the minimal labyrinth. *Ceacam9* was exclusively expressed in the spongiotophoblast and only a few spots remained in the mutant spongiotrophoblast. This result differs from previous findings that found *Ceacam9* exclusively expressed in TGCs (67, 68). HMGA2 was detected most strongly in the labyrinth lining fetal blood vessels in the wild-type placenta and was restricted to the chorionic plate in the KI.

3.4 HMGA2 is detected in the labyrinth and spongiotrophoblast of the mouse placenta

To our knowledge, the candidate gene *Hmga2* had never been examined in the placenta, therefore IF with an HMGA2 antibody was carried out in the wild-type mouse placenta at different developmental stages (Figure 3.5). At E8.5 HMGA2 detection was bright in the allanotois. At E9.5 and 10.5, expression was bright in the mesoderm-derived labyrinth and spotty in the labyrinth trophoblast and spongiotrophoblast. By E11.5 expression became weaker in the trophoblast but remained strong in the chorionic plate, and by E12.5 HMGA2 was fully restricted to the chorionic plate and the umbilical cord.

3.5 Examination of the *Hmga2*-KO placenta

Hmga2-KO mice display a pygmy phenotype with adults weighing about 40% of wild-type litter mates (53). E13.5 *Hmga2*-KO mouse placentae were sectioned, H&E stained, and examined for a potential developmental phenotype. There were no obvious gross phenotypic

abnormalities revealed by this staining in the *Hmga2*-KO placenta. Morphometric analysis of the sizes of the spongiotrophoblast and labyrinth layer revealed no obvious differences between wild-type and *Hmga2*-KO and no evidence to warrant more extensive morphometric analysis (Figure 3.6A). Glycogen cell content was also examined with glycogen cells still found in apparent normal patterning and normal levels in the mutant and heterozygote (Figure 3.6B). Lastly, to examine the vasculature of the labyrinth, sections were stained with laminin, a marker of fetal blood vessels (Figure 3.6C). This analysis revealed a placental labyrinth phenotype as the *Hmga2* heterozygotes showed less dense vascularization than wild-type and the *Hmga2*-KO showed an even more pronounced decrease in vascularization.

3.6 *Ascl2* is dispensable for TS cell derivation

Ascl2-deficient TS cells were established providing unlimited materials to study the lack of ASCL2 on early placental development *in vitro* with no sources of contamination. TS cells were established by mating females carrying the *Ascl2*-KI allele on the paternal chromosome with wild-type males, and collecting blastocysts at E3.5. Examination of the gross morphology of the 2 different genotypes revealed no obvious differences both in the stem cell state (Day 0) and through 8 days of differentiation (Figure 3.7).

KI cells were passaged over 20 times with stable ploidy and displayed no loss of growth potential (data not shown). The tetraploid mutant line (Line 9, see below) was also passaged upwards of 20 times with stable ploidy and no loss of growth potential (data not shown).

3.7 Giant cell differentiation dynamics appear similar in wild-type and KI TS cells

Because the KI mutant has an expanded giant cell layer *in vivo*, we sought to find out if *in vitro* mutant TS cells have a tendency to differentiate into TGCs sooner and more readily than wild-type cells. This prediction would be in line with the model suggesting that ASCL2 is a negative regulator of TGC formation (45). The nuclei of cells can be stained and discriminated by ploidy using FACS. Distinct peaks were visible for each ploidy and cells were considered to be giant cells if they had a ploidy of 8n or greater. Mouse primary embryonic fibroblasts and day 0 TS lines were used as controls and both showed peaks of only 2n and 4n, (4n representing cells in S-G2 phase). 2 mutant lines were examined: Line 9 and Line 10. The percentage of TGCs in each stage of differentiation was slightly lower in mutant Line 10 than the wild-type cells but the differentiation dynamics were not strikingly different between the 2 genotypes. The second mutant line (Line 9) showed no diploid peaks and was determined to be tetraploid. The tetraploid line also did not show an increased propensity to duplicate its DNA (Figure 3.8 A-D).

To determine if the ploidy state of Line 9 was just an anomaly or a true phenotype of the KI cells, 2 other mutant lines (Lines 14 and 15) were examined by FACS in their stem cell state. They were found to be diploid (Figure 3.8 E) for a total of 3 out of 4 KI lines found to be diploid. From this information, we reason that the ploidy state of Line 9 is likely an anomaly however more KI lines should be examined to make a solid conclusion.

3.8 *Ascl2*-KI cells display a different gene expression profile than wild-type cells

To examine the cell differentiation dynamics and transcriptional differences of the KI and wild-type cells, trophoblast cell lineage markers were examined by qRT-PCR in a wild-type (Line 3) and KI (Line 10) cell line through 8 days of differentiation (Figure 3.9). *Ascl2* showed the expected result with expression peaking shortly after differentiation is triggered, consistent with previous findings (38, 66). *Ascl2* is highly downregulated in the *Ascl2*-KI cells but not completely undetectable. This is as expected because the KI allele is in fact a hypomorph, not a complete knock-out.

The gene *Pcdh12* which marks glycogen cells is upregulated steadily over differentiation in wild-type cells reflecting the emergence of glycogen cell precursors in the placenta *in vivo* around E7.5 and their expansion in the latter half of gestation (33, 69). *Pcdh12* is almost completely undetectable in the mutant cells in their stem cell state and through differentiation suggesting that there are no glycogen cells or glycogen cell precursors formed in the mutant line.

The P-TGC specific gene *Prl3d1* is highly upregulated in the wild-type cells after 8 days of differentiation. In the mutant TS cells, this marker is downregulated substantially by comparison and is detectable again starting at day 4 of differentiation. This suggests that differentiation of the KI cells into P-TGCs is perturbed.

Tpbpa is exclusively expressed in the spongiotrophoblast and in glycogen cells in the placenta *in vivo*. In wild-type TS cells it is upregulated after 4 days of differentiation but is absent in the mutant cells likely reflecting the mutants lack of spongiotrophoblast *in vivo*.

Phlda2 is expressed in syncytiotrophoblast cells of the trophoblast labyrinth. It is upregulated, peaking at day 2 of differentiation and then falling. *Phlda2* expression in the mutant is very low in comparison to the wild-type levels suggesting that differentitation of the KI cells in to trophoblast labyrinth is perturbed.

Cdx2 expression is not significantly affected in the mutant, marking the stem cell state at day 0 in the KI and wild-type cells. It is sharply downregulated in both genotypes immediately upon differentiation and stays down throughout differentiation reflecting the loss of the stem cell state in both genotypes.

3.9 Candidate gene expression in TS cells over differentiation

To examine if candidate target genes were downregulated in the KI TS cells and to compare the expression patterns of the candidate target genes to that of *Ascl2*'s, their expression was measured in wild-type and *Ascl2*-KI/+ cells over 8 days of differentiation. In the wild-type TS cells, some of the candidate genes followed an expression pattern very similar to that of *Ascl2* (*Vgll1*, *Lpl*), or had a slightly delayed expression pattern (*Doxl1*, *Car2*, *Ceacam9*, *Pappa2*).

The expression of the candidate genes *Doxl1*, *Car2* and *Ceacam9*, were downregulated in mutant cells at all stages of differentiation. *Hmga2* was upregulated, *Vgll1* and *Lpl* mutant expression showed no particular pattern (Figure 3.10), and the expression of the candidate gene *Cdx2* remained the same (Figure 3.9).

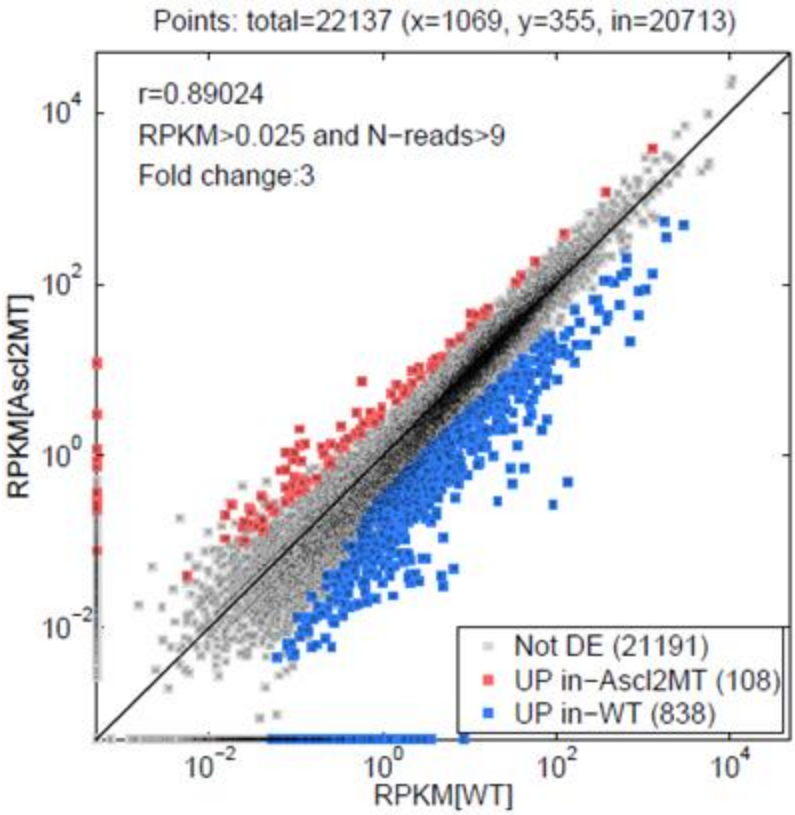


Figure 3.1 RNA-seq of wild-type and *Ascl2*-KI/+ placentae. The transcriptomes of wild-type and *Ascl2*-KI/+ placentae and deciduae were compared by RNA-seq. Red dots represent genes significantly upregulated in the mutant and blue dots represent genes significantly downregulated in the mutant by the set criteria (RPKM>0.025,N-reads>9, Fold change≥3) (x=genes on the x-axis [y=0], y=genes on the y axis [x=0], r=Pearson correlation coefficient, RPKM=reads per kilobase per million reads, N-reads=reads per gene, Not DE=Not Differentially Expressed, Ascl2MT=*Ascl2*-KI/+, WT=wild-type).

A

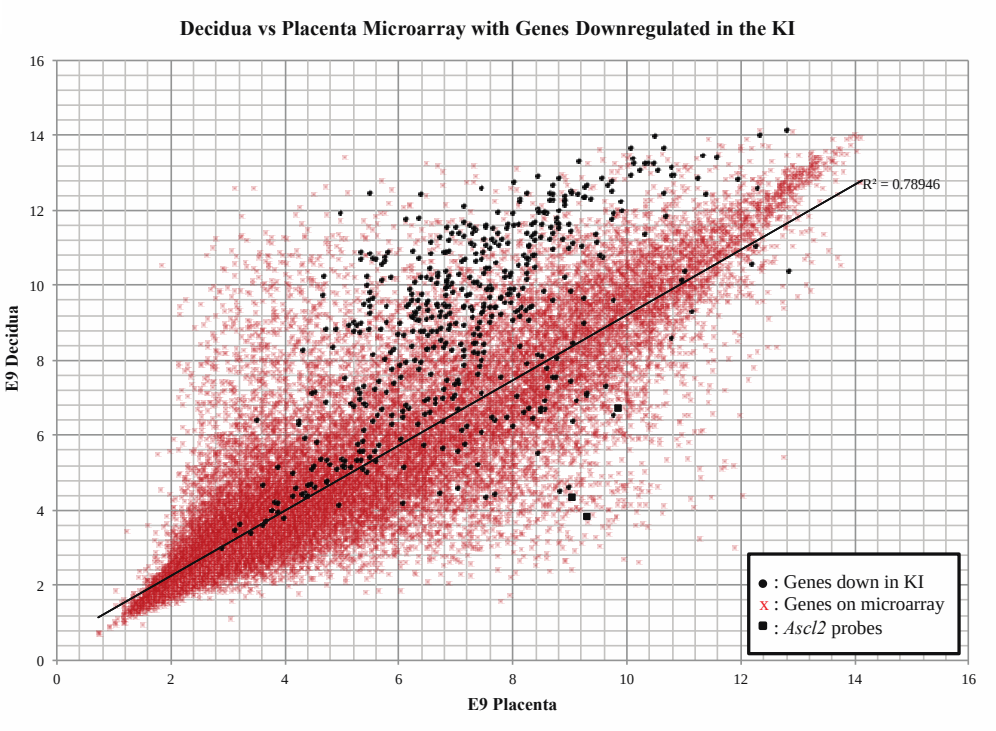


Figure 3.2 Genes downregulated in the *Ascl2*-KI/+ compared to a placental/decidual microarray. The genes found to be significantly downregulated in the mutant placentae and deciduae by RNA-seq are compared to a microarray examining the expression of genes in placentae and deciduae separately. According to this analysis the majority of genes found to be downregulated in the *Ascl2*-KI/+ are mainly decidually expressed**.**

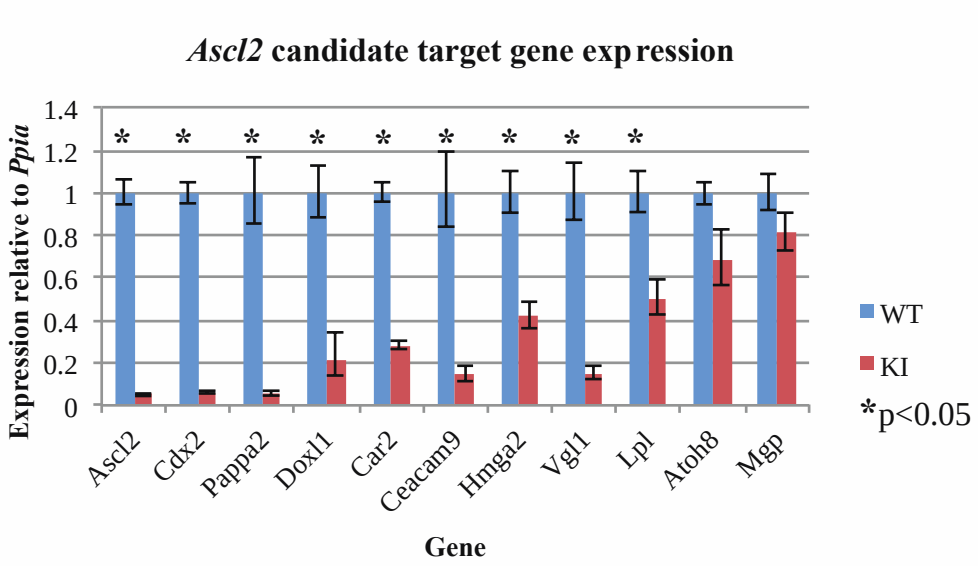
Gene symbol /name	Function	Known knockout phenotype?	Mouse placental expression previously shown?	Additional comments
Ascl2 (achaete-scute complex homolog 2)	Trophoblast development (9)	Embryonic lethality at E9.5 (9)	Yes, labyrinth and spongiotrophoblast (9)	-basic helix-loop-helix transcription factor
Cdx2 (caudal type homeobox 2)	Trophectoderm fate specification (70)	Lethality prior to gastrulation (71)	Yes, chorion and spongiotrophoblast (72)	-caudal-like homeodomain transcription factor
Pappa2 (pappalysin 2)	Cleaves insulin-like growth factor binding proteins (IGFBPs), activating IGF signalling pathways and playing a part in growth regulation (73-75)	Viable and fertile with reduced fecundity. Post natal growth retardation which is more pronounced in females (75)	Yes, most highly expressed in the spongiotrophoblast (76)	-*Pappa2* is upregulated in human preeclampsia placenta (77) -its relative, *Pappa*, is a known biomarker for human genetic disorder such as down syndrome and adverse pregnancy outcome such as preeclampsia
Doxl1/1600015I10Rik (Diamine oxidase-like protein 1)	n/a	n/a	Yes, expressed in the E9.5 placenta (78)	-has not been studied
Car2 (carbonic anhydrase 2)	Catalyzes the reversible hydration of carbon dioxide, believed to have a role in gas exchange and transport (79)	Growth retardation with renal tubular acidosis (80)	Yes, labyrinth, spongiotrophoblast, glycogen cells (81)	n/a

Table 3.1 Candidate genes – general information

Gene symbol /name	Function	Known Knockout phenotype?	Mouse placental expression previously shown?	Additional Comments
Ceacam9 (carcinoembr-yonic antigen-related cell adhesion molecule 9)	Immune system function, possibly plays a role in protecting the fetus from the maternal immune system (68)	No known phenotype (68)	Yes, a subset of primary and secondary giant cells (67, 68)	n/a
Hmga2 (high mobility group AT-hook 2)	Interacts with chromatin to regulate transcription (82)	Growth retardation and infertility (83)	n/a	n/a
Vgll1 (vestigial like 1 homolog)	Transcriptional co-activation (84)	n/a	Highly expressed in the *human* placenta (85)	-master regulator of wing development in *Drosophila* (86)
Lpl (lipoprotein lipase)	Lipoprotein metabolism and uptake (87)	Death within 2 days of birth due to chylomicron engorgement of capillaries, display hypertriglyceride-mia and reduced fat stores (88)	Detected in the syncytiotrophoblast of the *human* placenta (89)	n/a
Atoh8 (atonal homolog 8)	Transcriptional repressor, role in early embryogenesis, development of the pancreas (65), nervous system (90), kidney (91), retina and muscle (92)	Embryonic lethal, developmentally arrested around the time of gastrulation (65)	n/a	-basic helix-loop-helix transcription factor, has been found to only be activated by other bHLH transcription factors (65)
Mgp (matrix Gla protein)	Inhibits vascular calcification (93)	Lethal within the first 2 months of life due to arterial calcification leading to blood vessel rupture (93)	Found in *human* plancental microvasculature (94)	n/a

Table 3.1 (continued) Candidate genes – general information

A



B

Downregulation

Gene	By qRT-PCR	By RNA-seq
Ascl2	19.825*	33.831
Cdx2	16.961*	6.979
Pappa2	18.187*	6.922
Doxl1	5.103*	3.388
Car2	9.248*	4.68
Ceacam9	6.966*	9.963
Hmga2	2.374*	3.27
Vgll1	6.637*	4.104
Lpl	1.988*	2.719
Atoh8	1.349	8.287
Mgp	1.321	275.554

*p<0.05

Figure 3.3 Confirmation of candidate gene downregulation A. Expression of candidate genes was examined in E9.5 wild-type and the *Ascl2*-KI/+ placentae and deciduae by qRT-PCR. Technical triplicates were carried out on 3 wild-types and 3 mutants (n=3). Expression is relative to the housekeeping gene *Ppia* and wild-type expression normalized to 1. Error bars represent standard error of the mean. (WT=wild type, KI=*Ascl2*-KI/+). **B**. Comparison of the fold downregulation in the mutant found by qRT-PCR versus by RNA-seq on biological samples dissected the same way.

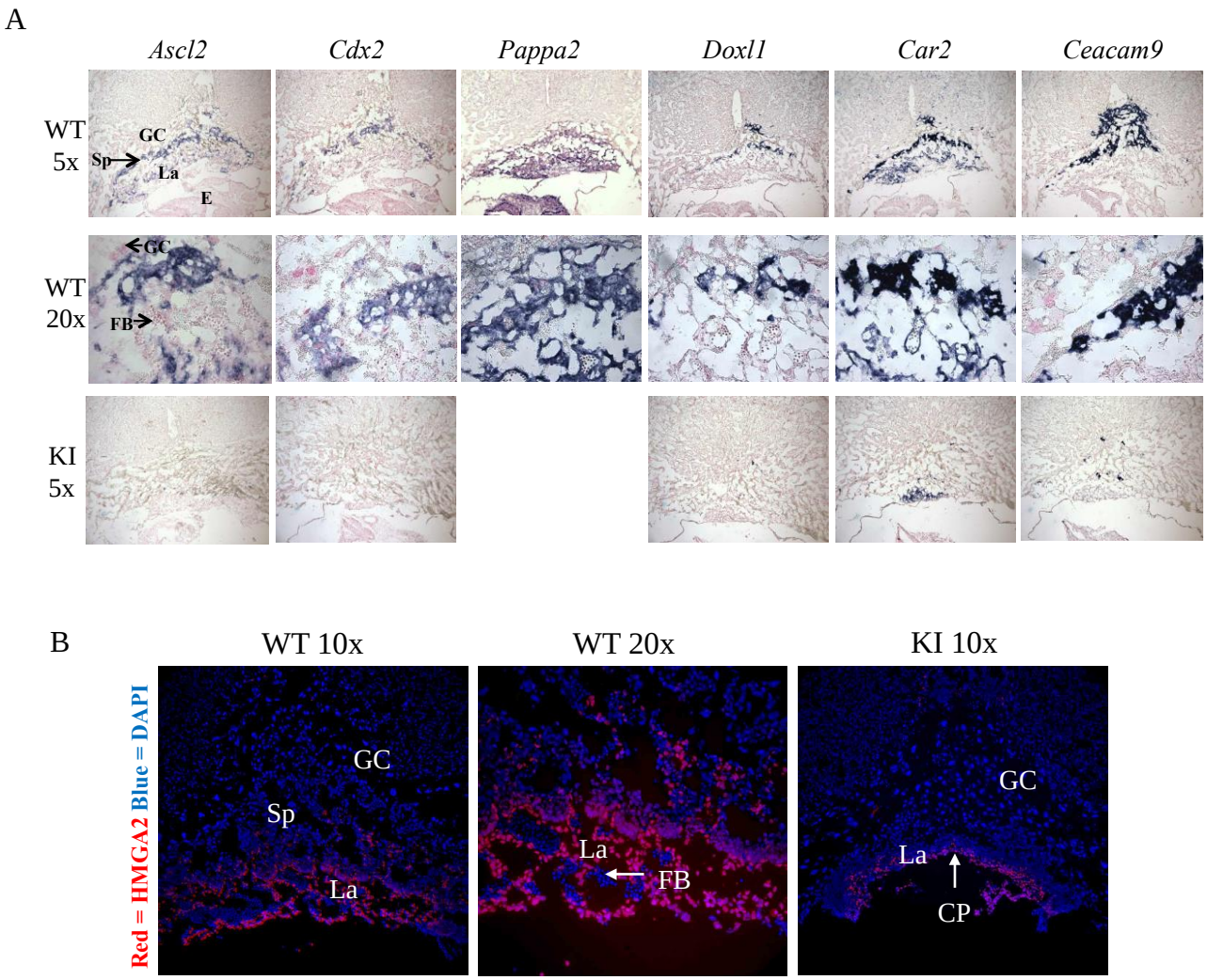


Figure 3.4 Location of candidate gene expression in E9.5 placenta. Candidate genes were examined by ISH (**A**) or IF (**B**). All genes show overlapping expression with *Ascl2* and all are downregulated in the KI. (GC=giant cell, Sp=spongiotrophoblast, La=labyrinth, E=embryo, FB=fetal blood, CP=chorionic plate, WT=wild-type, KI=*Ascl2*-KI/+).

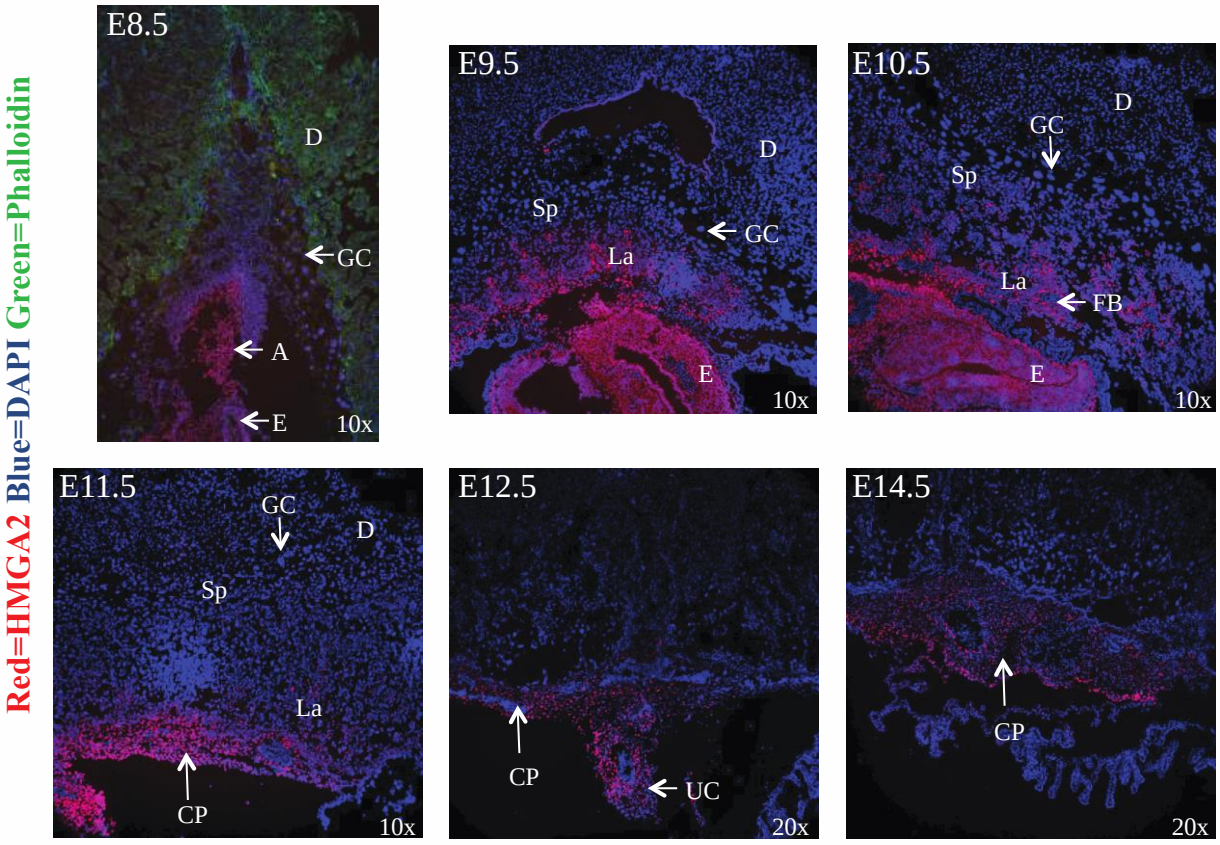
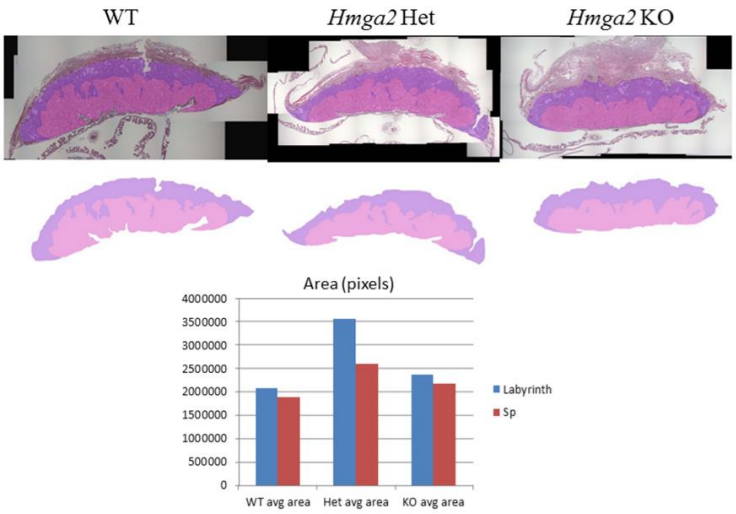
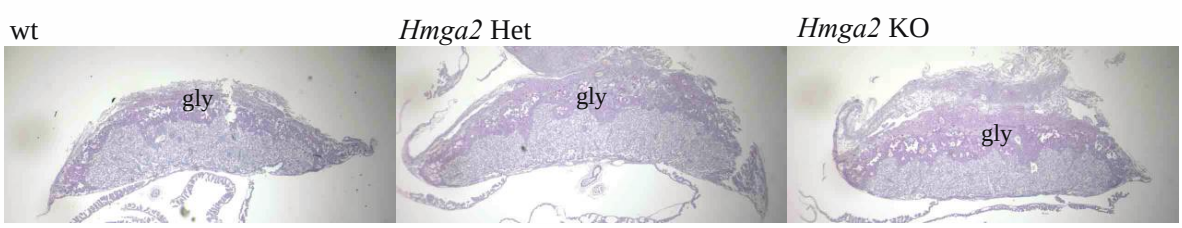


Figure 3.5. HMGA2 placental expression through development. The location of the HMGA2 (red) was examined in the placenta through development by immunofluorescence (IF) on cryostat sections. (A=allantois, E=embryo, D=decidua, GC=giant cell, Sp=spongiotrophoblast, La=labyrinth, CP=chorionic plate, UC=umbilical cord).

A



B



C

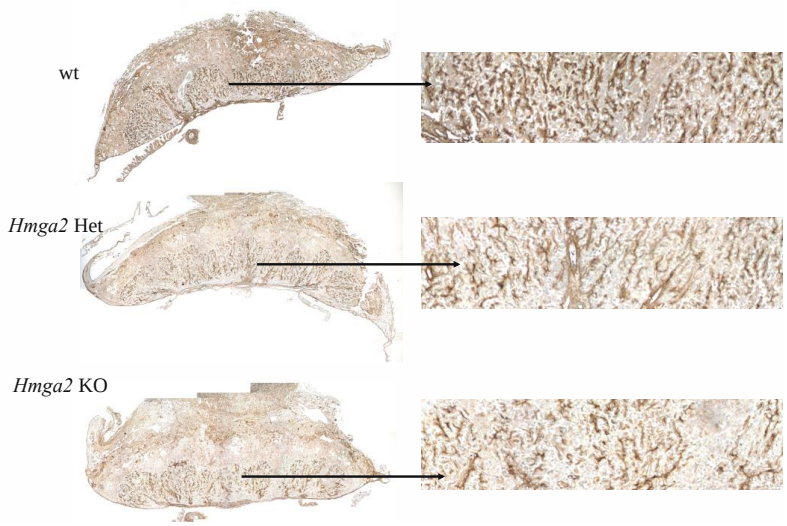


Figure 3.6 Preliminary analysis of E13.5 *Hmga2*-KO placental phenotype A. The labyrinth and the spongiotrophoblast layers of the different genotypes were compared by area in pixels. **B**. PAS staining showing glycogen cell location and content in the placentae **C**. Laminin staining reveals the fetal vasculature in the labyrinth (sp=spongiotrophoblast, gly=glycogen cell, wt= wild-type, *Hmga2* Het or Het=*Hmga2* heterozygote, *Hmga2* KO or KO=*Hmga2* knock-out).

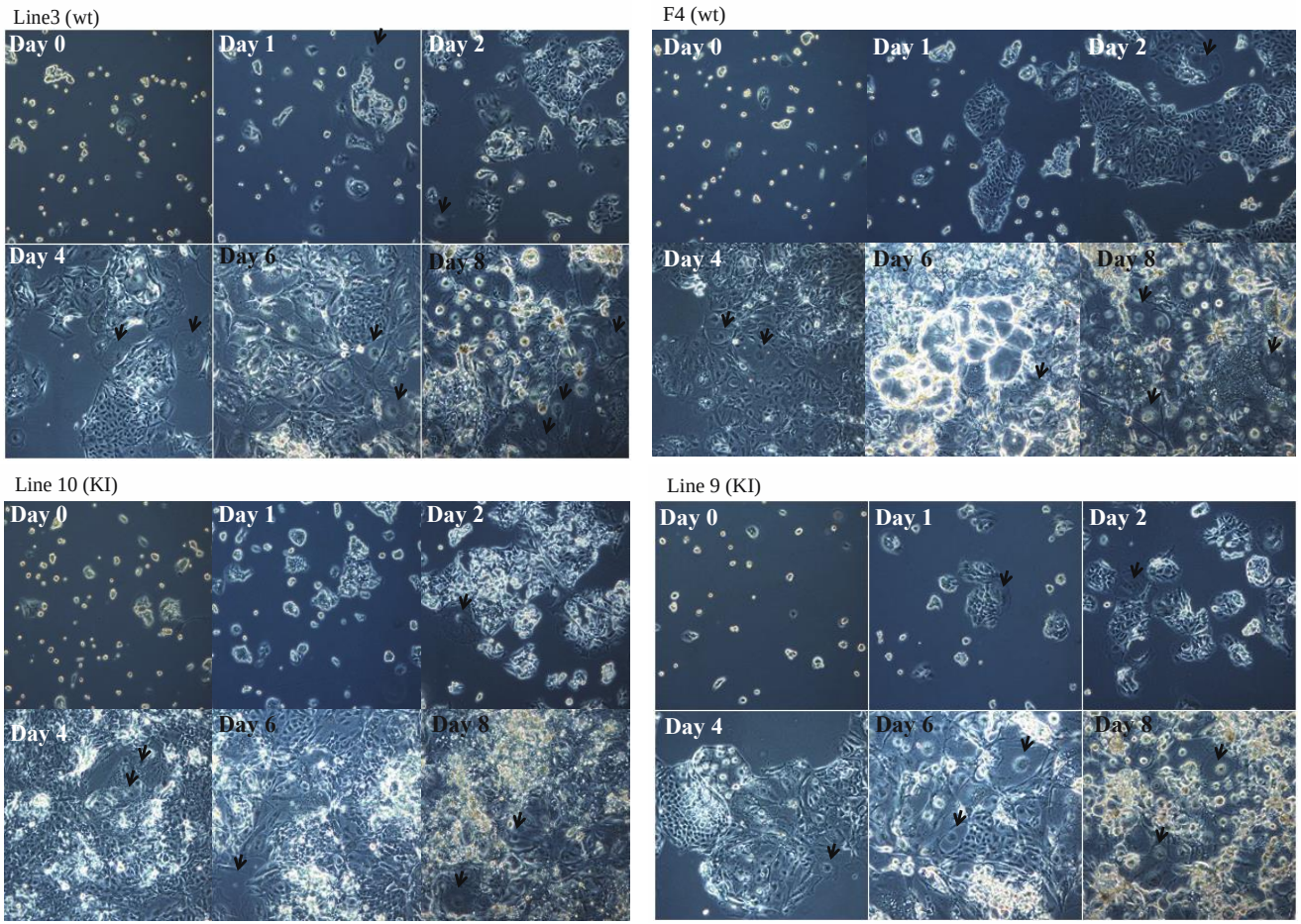


Figure 3.7 TS cell differentiation. TS lines were differentiated over 8 days. There are no obvious morphological differences between wild-type and *Ascl2*-KI/+ cells. Black arrows denote examples of giant cells, (wt=wild-type, KI=*Ascl2*-KI/+).

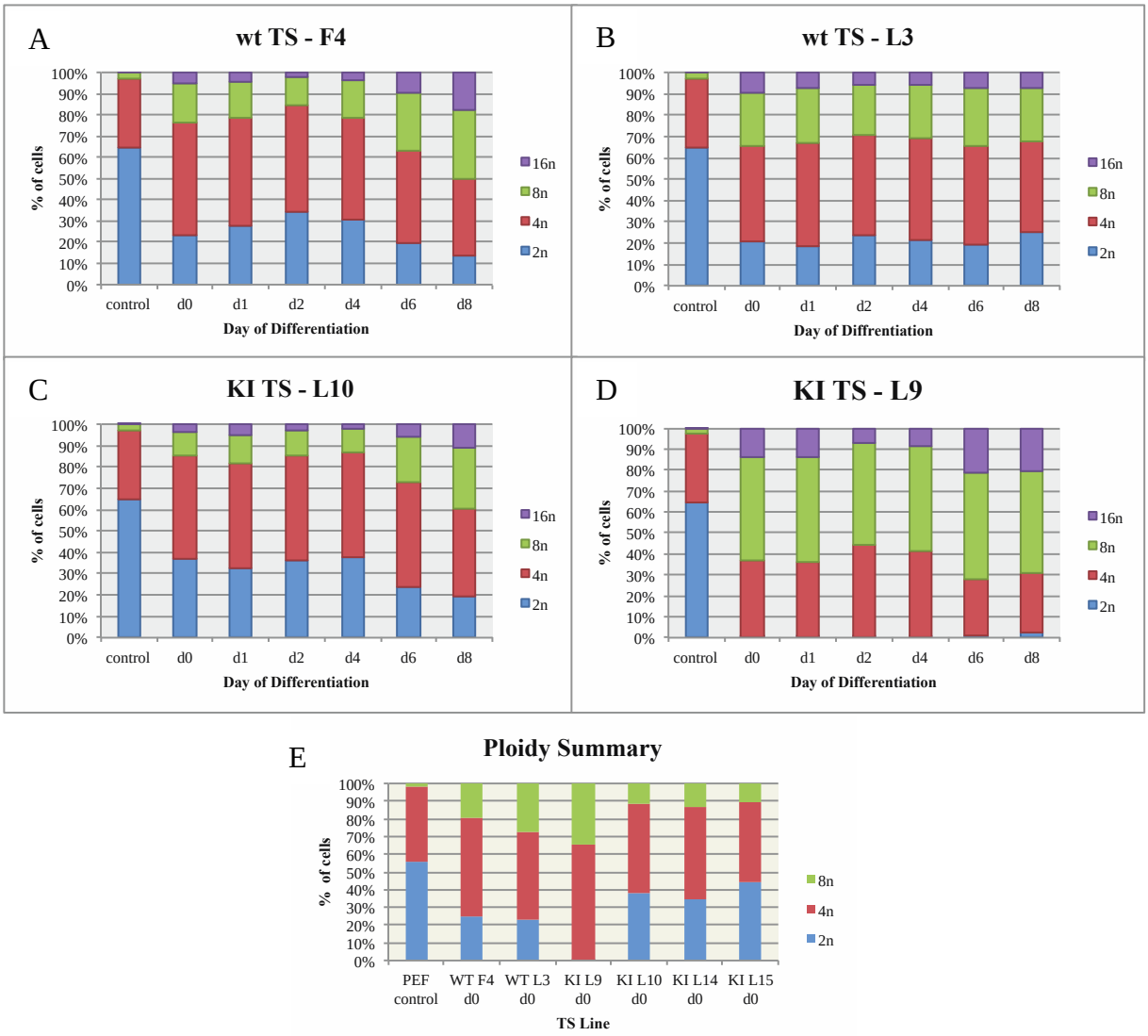


Figure 3.8. TS cell ploidy analysis. FACS analysis was done on 2 wild type lines (F4 and L3, [**A** and **B**]) and 2 *Ascl2*-KI/+ lines (L10 and L9, [**C** and **D**]) over 8 days of differentiation to analyze cells by ploidy. Cells with a ploidy of over 4n were considered TGCs. L9 shows no diploid cells. **E.** Compares the ploidy of 2 different wild-type lines (F4 and 3) and 4 different KI lines (9, 10, 14 and 15) at day 0 of differentiation (wt=wild-type, KI=*Ascl2*-KI/+, TS=trophoblast stem cells, L=line, PEF=primary embryonic fibroblast).

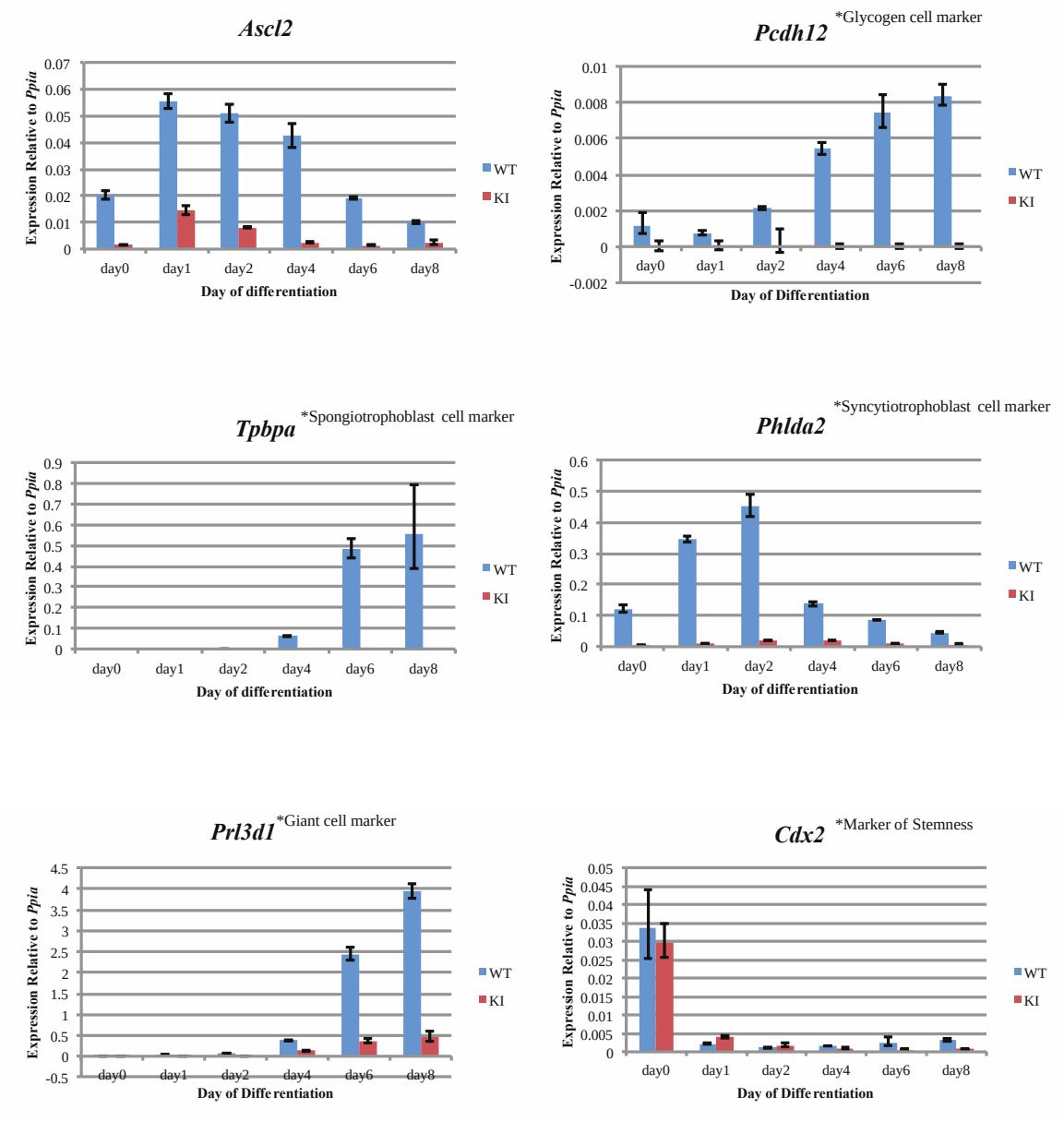


Figure 3.9. Expression of trophoblast cell markers over TS cell differentiation. Various markers of the trophoblast were examined over 8 days of differentiation in wild-type and mutant cells by qRT-PCR. Technical triplicates were carried out on one wild-type and one mutant line (n=1). Expression is relative to the housekeeping gene *Ppia*. Error bars represent standard error of the mean (WT=wild type, KI=*Ascl2*-KI/+).

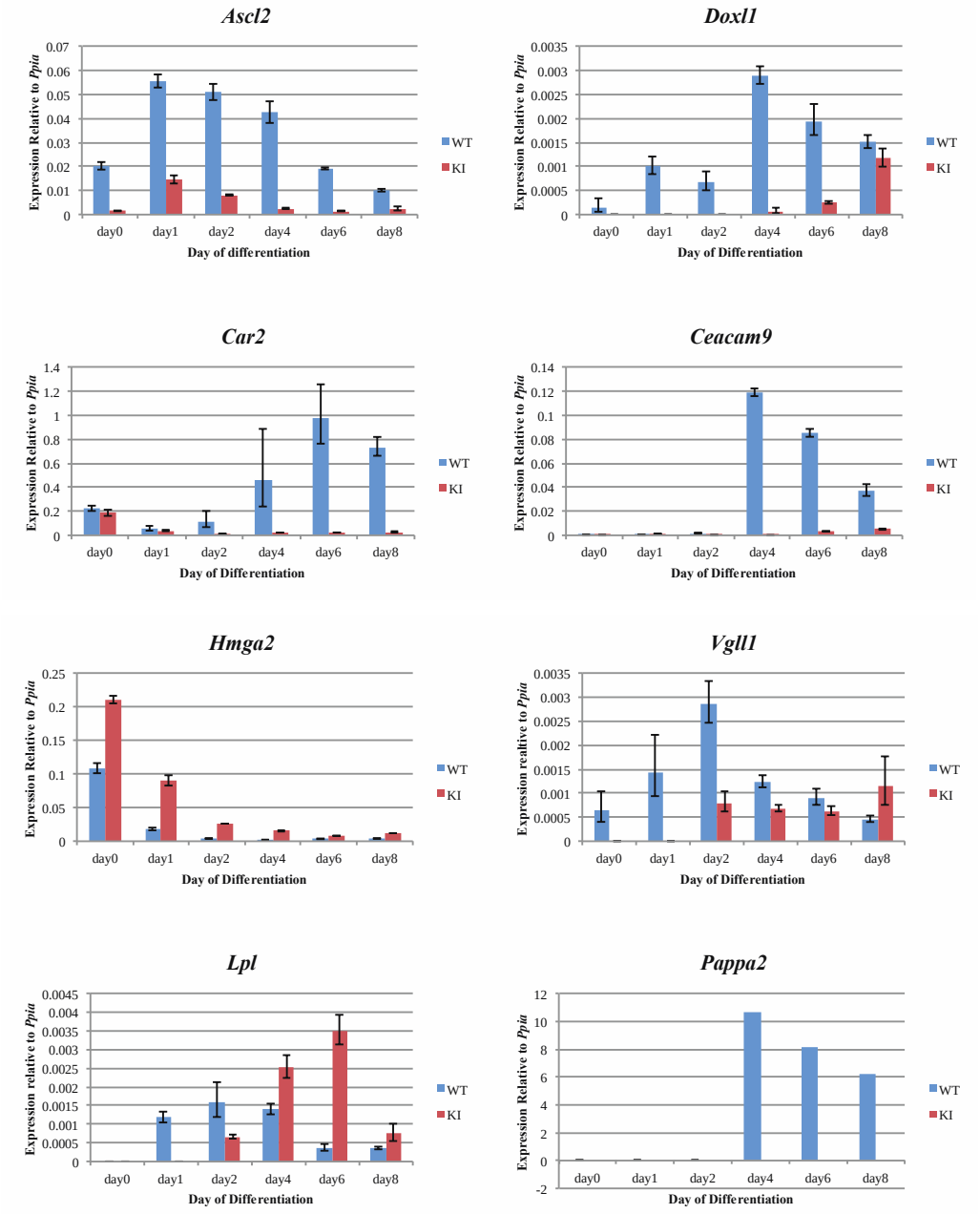


Figure 3.10. Candidate gene expression over TS cell differentiation. Expression of candidate ASCL2 target genes over 8 days of differentiation are compared to the expression of *Ascl2* in wild-type and mutant cell lines by qRT-PCR. Technical triplicates were carried out on one wild-type and one mutant line (n=1). Expression is relative to the housekeeping gene *Ppia*. Error bars represent standard error of the mean (WT=wild type, KI=*Ascl2*-KI/+).

Chapter 4 Discussion

The purpose of performing RNA-seq on wild-type and mutant placentae was to investigate the genes that have their expression in the placenta perturbed when *Ascl2* is taken out of the system. Focusing on the genes that are downregulated in the *Ascl2*-deficient placenta provides a starting point for determining which genes are turned on by this transcription factor in the wild-type placenta.

The decidual component of the placental samples submitted for RNA-seq was a source of maternal contamination. Since *Ascl2* is only expressed in the spongiotrophoblast and labyrinth trophoblast layers of the mouse placenta and is not secreted, genes directly affected by the absence of *Ascl2* are not expected to be outside the confines of *Ascl2* expression and therefore not in the decidua. However, a comparison of decidually expressed genes with genes significantly downregulated in the KI placenta revealed that an unexpected number of genes that are decidually expressed are also downregulated in the mutant. This suggests an interesting maternal response to the lack of *Ascl2* in the placenta and may be an exciting avenue of further research. Such an effect might reflect abnormal levels of secreted factors from the trophoblast which have an impact on decidual biology. The genes that were found to be significantly upregulated by RNA-seq are also very interesting. As the P-TGC layer is known to be expanded in placentae lacking *Ascl2* (9, 50), these could represent genes that are P-TGC specific or important for P-TGC development and function.

The genes that were downregulated in the KI and placentally expressed provided a list of *Ascl2* candidate target genes of which a select few were chosen to investigate further. The qRT-PCR experiments on E9.5 placentae with deciduae attached confirmed the downregulation found by RNA-seq of 8 out of the 10 chosen genes. The 2 genes that were not confirmed to be

significantly downregulated were *Atoh8* and *Mgp*, both of which were found to be decidual genes by our thresholds. The result that the gene *Mgp* was not found to be downregulated in the mutant by qRT-PCR is especially puzzling. *Mgp* was initially chosen as a candidate gene because the RNA-seq experiment showed an extremely high downregulation (~276 fold) in the mutant and because of its potentially interesting function involving calcification of placental vasculature (94). The fact that *Mgp* was not found to be downregulated in the mutant by qRT-PCR remains enigmatic but suggests that the maternal decidual response is variable between these mice.

The ISH of candidate genes in the wild-type E9.5 placenta revealed that all candidate genes examined have some overlapping expression with *Ascl2* in the spongiotrophoblast layer, the labyrinth layer, or both, and are excluded from the P-TGC population at this time point suggesting that they may indeed be directly regulated by ASCL2. Although the candidate target genes examined show expression in the same layer(s) as *Ascl2* we do not have confirmation by cell marker analysis that they are expressed in the same cell types. None of the candidates match the *Ascl2* expression pattern completely suggesting that they are not regulated by *Ascl2* alone. At E9.5, *Ascl2* is expressed in a *Cdx2* positive area in the spongiotrophoblast suggesting that this area has stem cell potential. Alternatively, *Cdx2* could have a secondary function in the mature placenta that has not been studied. Taken together, the ISH of the mutant placenta and the qRT-PCR results confirmed that most of the selected candidate target genes are downregulated in the mutant placenta. This is likely due to depletion of the tissues in the mutant placenta in the spongiotrophoblast and the labyrinth where the genes are expressed and we cannot conclude with this information alone that it is because they are direct targets of ASCL2.

The expression pattern of *Hmga2* in the placenta is a novel finding. The fact that *Hmga2* is most prominently expressed in the labyrinth and labyrinth precursors throughout development suggests that the *Hmga2*-KO mouse might have a labyrinth placental phenotype. *Hmga2*-KO mice have what is known as a "pygmy phenotype." By E15.5 there is a slight but significant reduction in body weight between KOs and wild-types. By adulthood, KOs have a body weight that is approximately 40% of wild-type littermates and adult heterozygotes have a body weight that is approximately 80% of wild-type littermates due to reduced growth trajectory (53). To our knowledge the placentae of these mice have never been examined to investigate if a placental phenotype contributes to their undergrowth. Nothing obvious was detected by our analysis of the KO placenta at E13.5. Replicates were not done of placental morphometric analysis or glycogen cell staining because preliminary results did not warrant further investigation and our materials were limited since we did not have this mouse in our facility. The most promising finding of these preliminary experiments was revealed by the laminin stain of the fetal vasculature. This stain revealed a possible difference in vascular density between the wild-type, heterozygote and KO placentae, with the heterozygote being less dense than the wild-type and the KO being less dense than the heterozygote.

In an attempt to quantify change in fetal vasculature in the *Hmga2*-KO placenta, dextran sulfate conjugated FITC was injected into E14.5 placentae through the umbilical cord and into the vasculature of the placentae to label it. A similar procedure is used for an established technique called vascular corrosion casting (95). After subsequent clearing of the tissue with BABB (a mixture of benzyl-alcohol and benzyl-benzoate), the vascular bed of the placentae was imaged by optical projection tomography (OPT) (96). The injections and clearing worked well

however, imaging of the placentae by OPT did not work well and we did not have time to explore other imaging techniques, although confocal microscopy might have worked better.

A phenotype is yet to be quantified in the *Hmga2*-KO placenta. It would be interesting to stain the placenta for labyrinth markers as well as do qRT-PCR for labyrinth markers to investigate any differences in this layer between KOs, heterozygotes, and wild-types. If the difficulties with imaging the FITC injected placentae were resolved, this would provide a powerful tool for imaging the labyrinth layer of any murine placenta and possibly reveal a labyrinth phenotype in the *Hmga2*-KO mouse.

Our attempt to study ASCL2 not only *in vivo* but also *in vitro* was successful with the derivation of TS cells from *Ascl2*-KI/+ mutant blastocysts. The finding that *Ascl2* is dispensable for TS cell derivation is not surprising given that ASCL2 has no known effects on early peri- and post-implantation development of the conceptus (9).

FACS analysis was carried out on the derived TS cells to examine their rate and tendency to differentiate into TGCs. The expectation was that KI cells would show a greater tendency to differentiate into TGCs because the *in vivo* phenotype of the KI mouse has an expanded P-TGC layer. However this was not the case; the TGC content was similar between the 2 genotypes and even slightly less in the KI lines examined. Several limitations could have confounded this experiment. Firstly TGCs adhere to the culture dish very tightly and it is difficult to know with certainty if a population of polyploid cells was left behind on the dish and excluded from analysis. Secondly, the FACS machine itself has limitations and cells of high ploidy (>16n) tend to be excluded from analysis. It would be informative to study TGC differentiation dynamics using a second method such as a nuclear stain followed by fluorescence microscope analysis.

Even though mutant Line 9 was found to be tetraploid by FACS analysis, these cells gave no indication of detriment; in fact Line 9 was one of the best growing derived lines with fast turnover and little differentiation in the stem cell state. The placenta is known to be a unique and adaptable organ that can adjust to different cellular insults that would be lethal if seen in the fetus, including chromosomal trisomy and chimeric androgenetic cells (97, 98). This tetraploid TS line is another example of the ability of placental cells to adapt and thrive in extraordinary situations.

By FACS and morphological analysis, the *Ascl2*-KI cells did not appear to be grossly different from wild-type cells, however transcriptionally they were found to be very different. All cell lineage markers examined by qRT-PCR were highly downregulated in the mutant when compared to wild-type except for *Cdx2* which stayed the same between the 2 genotypes, being downregulated at the initiation of differentiation as the cells lost their stem cell potential. These results suggest that the *Ascl2*-deficient TS cells are able to differentiate, losing *Cdx2* expression. However they do not become cells of the trophoblast lineage, but become some other unknown or abnormal cell types (Figure 4.1). Some of the mutant cells are perhaps staying in an intermediate multipotent progenitor state, unable to differentiate into the cells of the mature trophoblast. A slight increase in *Prl3d1* is seen in the mutant cells through differentiation indicating emergence of some P-TGCs however in comparison to wild-type, this upregulation is very minimal. The fact that the KI line shows a similar proportion of polyploid cells by FACS but shows highly downregulated expression of *Prl3d1* by qRT-PCR implies that KI cells are differentiating into polyploid cells that are not functionally P-TGCs. The discrepancy between the *in vivo* and *in vitro* P-TGC KI phenotype might be explained by the fact that the TS cells in culture are missing cues that the cells would have *in vivo* from the extraembryonic mesoderm

and the maternal decidua. There is certainly crosstalk between these structures and the trophoblast, for example, as discussed above, the lack of *Ascl2* in the placenta and possibly the lack of spongiotrophoblast itself has a substantial effect on the expression of genes in the decidua. It is possible that whatever triggers P-TGC expansion in the *Ascl2*-null/KI/*Del*^{7AI} mouse models is in fact a perturbation of signals from the decidua, for example, that are indirectly controlled by *Ascl2*. Since this cannot happen *in vitro*, the P-TGC phenotype is not the same.

The only cell type in the placenta to express the gene *Pcdh12* is the trophoblast glycogen cell (69). One of the major findings in the *Del7*^{AI} mouse line where *Ascl2* is expressed at about 50 percent of its normal level, is that their placentae are completely devoid of glycogen cells. This raises the question: is *Ascl2* required for the formation of the glycogen cell lineage? The lack of *Pcdh12* expression in the mutant TS cells provides evidence that the mutant cells are indeed unable to differentiate into glycogen cell precursors in culture. This suggests that *Ascl2* may be required for the formation of the glycogen cell lineage *in vivo*. It would be interesting to examine the KI mouse at a stage before its phenotype manifests and at the time of glycogen cell precursor formation (E7.5) to determine if glycogen cells are present in the EPC by staining for PCDH12.

Examining the *Ascl2* candidate target genes in TS cells during differentiation revealed that most of these genes including *Doxl1*, *Car2*, *Ceacam9* and *Pappa2* follow an expression pattern that is similar to *Ascl2* but delayed. This delay of days suggests that these genes may be indirect targets of ASCL2. The genes *Vgll1* and *Lpl* have expression patterns through differentiation that are more similar to *Ascl2*, peaking earlier than the other candidates and then coming down again. The genes that follow this pattern might be direct targets because it would be expected that a direct target of *Ascl2* would be upregulated and downregulated with the up

and downregulation of *Ascl2*. It is important to note, however, that if these candidate target genes are not targeted by ASCL2 alone then their expression patterns may vary from *Ascl2*'s even if they are direct targets.

Moreover, this experiment confirmed the downregulation of several candidate genes in the KI cells. For those genes that were not downregulated in the mutant, namely *Hmga2*, and *Lpl* and *Vgll1* in late differentiation, this may be because of obvious differences between *in vivo* and *in vitro* conditions, most notably the lack of the extraembryonic mesoderm and the decidua in culture as discussed above. However the expression of these genes in the mutant cells provides insight in to what the KI cells may be becoming as they differentiate, in other words, cells that express genes such as *Hmga2*, *Lpl*, *Vgll1*. The candidate gene *Hmga2* shows an interesting pattern of expression, peaking at day 0 and declining from there, an expression pattern that has been seen before in TS cells (66). This expression pattern seen in both the wild-type and KI cells suggests that *Hmga2* may have a role in TS cell maintenance.

The *Ascl2*-KI/+ TS cell lines are a valuable tool that can be continued to be used to investigate the gene *Ascl2* and the consequences of its absence in the trophoblast. These cells provide a controlled environment in which the trophoblast can be studied over time without contamination from blood and maternal tissue. These cell lines provide unlimited materials to carry out, for example, whole genome sequencing techniques such as RNA-seq of wild-type versus mutant TS cells, and ChIP-seq to find true direct targets of ASCL2.

In the present study we found that by our thresholds, the expression of a total of 946 genes is significantly perturbed in *Ascl2* mutant placentae and deciduae (108 upregulated, 838 downregulated). By our thresholds approximately 20 of these genes were determined to be

downregulated in the placenta alone making these genes potential candidate targets of the essential transcription factor ASCL2. A subset of these genes was shown to be expressed in the same tissue as *Ascl2* further suggesting that these genes may be controlled by ASCL2 but not proving evidence of any direct interactions. The candidate gene *Hmga2* was shown to have placental expression for the first time to our knowledge and preliminary experiments revealed that the placenta of the *Hmga2*-KO mouse has a placental labyrinth phenotype that may be contributing to the undergrowth of this mouse. We were also able to develop an *in vitro* model of early placental development with an *Ascl2*-deficiency showing that *Ascl2* is dispensable for TS cell derivation and expansion. These TS cells do not differentiate into the known trophoblast cell types as wild-type TS cells do, providing *in vitro* evidence that *Ascl2* is necessary for proper development and differentiation of the trophoblast placenta. The experiments conducted in this study need to be replicated on multiple samples; however the findings of the present study have great potential to contribute to our knowledge of mammalian placental biology and to be extrapolated to human reproductive medicine.

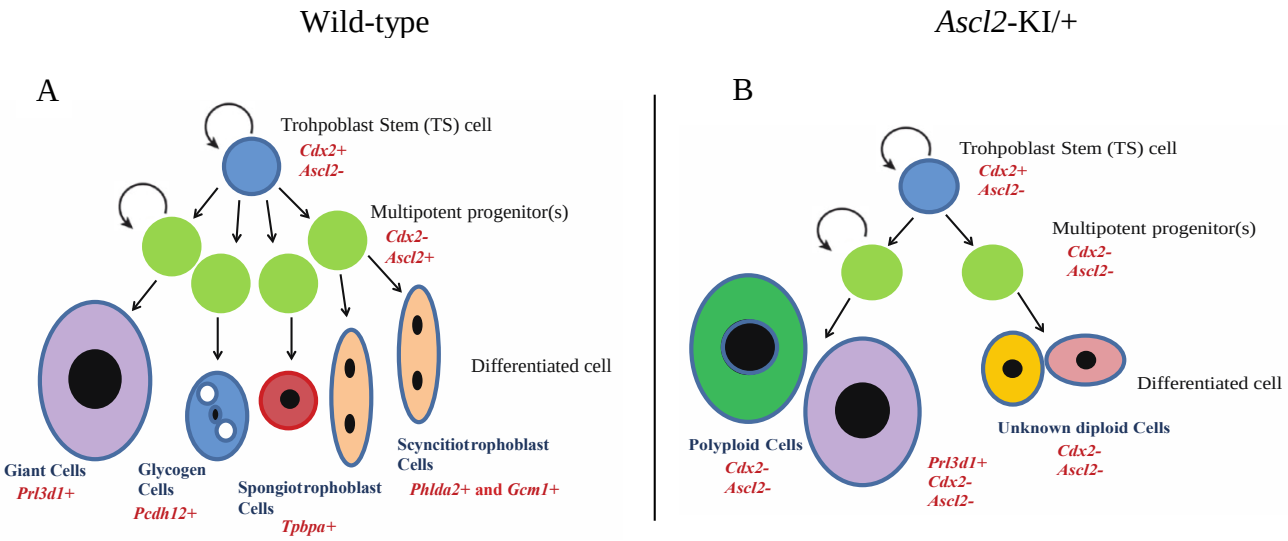


Figure 4.1 Models of TS cell differentiation. A. Wild-type TS cells start as *Cdx2* positive, *Ascl2* negative pluripotent cells that may differentiate into more specialized multipotent progenitors. These cells lose their *Cdx2* expression and become *Ascl2* positive, then terminally differentiate into the cells of the trophoblast lineage that express various marker genes. **B.** *Ascl2*-KI/+ TS cells also begin as *Cdx2* positive, *Ascl2* negative pluripotent cells and lose their *Cdx2* expression upon differentiation but do not gain *Ascl2* expression. It is unclear what these cells are becoming. Some of these cells may be stuck in the *Ascl2* negative multipotent progenitor cell state while others may be differentiating in to an unknown diploid cell type that is yet to be defined. Other cells are becoming polyploid but only a fraction of these polyploid cells are *Prl3d1* positive.

References

1. Surani MAH, Barton SC, Norris ML. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature. 1984;308(5959).

2. Mcgrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell. 1984;37(1).

3. Lewis A, Reik W. How imprinting centres work. Cytogenetic and Genome Research. 2006;113(1-4).

4. Bartolomei MS, Webber AL, Brunkow ME, Tilghman SM. Epigenetic mechanisms underlying the imprinting of the mouse H19-gene. Genes Dev. 1993;7(9):1663-7.

5. Thorvaldsen JL, Duran KL, Bartolomei MS. Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2. Genes Dev. 1998;12(23):3693-702.

6. Leighton PA, Ingram RS, Eggenschwiler J, Efstratiadis A, Tilghman SM. Disruption of imprinting caused by deletion of the H19 gene region in mice. Nature (London). 1995;375(6526):32-9.

7. Bell AC, Felsenfeld G. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature. 2000;405(6785):482-5.

8. Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature. 2000;405(6785).

9. Guillemot F, Nagy A, Auerbach A, Rossant J, Joyner AL. Essential role of mash-2 in extraembryonic development. Nature. 1994;371(6495).

10. Eggermann T. Silver-russell and beckwith-wiedemann syndromes: Opposite (epi)mutations in 11p15 result in opposite clinical pictures. Horm Res. 2009;71.

11. Crotti L, Celano G, Dagradi F, Schwartz PJ. Congenital long QT syndrome. Orphanet Journal of Rare Diseases. 2008;3:18.

12. Smilinich NJ, Day CD, Fitzpatrick GV, Caldwell GM, Lossie AC, Cooper PR, et al. A maternally methylated CpG island in KvLQT1 is associated with an antisense paternal transcript and loss of imprinting in beckwith-wiedemann syndrome. Proc Natl Acad Sci U S A. 1999;96(14):8064-9.

13. Mancini-DiNardo D, Steele SJS, Ingram RS, Tilghman SM. A differentially methylated region within the gene Kcnq1 functions as an imprinted promoter and silencer. Hum Mol Genet. 2003;12(3):283-94.

14. Fitzpatrick GV, Soloway PD, Higgins MJ. Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. Nat Genet. 2002;32(3).

15. Mancini-DiNardo D, Steele SJS, Levorse JM, Ingram RS, Tilghman SM. Elongation of the Kcnq1ot1 transcript is required for genomic imprinting of neighboring genes. Genes Dev. 2006;20(10):1268-82.

16. Varmuza S, Mann M. Genomic imprinting - defusing the ovarian time bomb. Trends in Genetics. 1994;10(4):118-23.

17. Moore T, Haig D. Genomic imprinting in mammalian development - a parental tug-of-war. Trends in Genetics. 1991;7(2).

18. Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, et al. Placental-specific IGF-II is a major modulator of placental and fetal growth. Nature. 2002;417(6892):945-8.

19. Eggenschwiler J, Ludwig T, Fisher P, Leighton PA, Tilghman SM, Efstratiadis A. Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the beckwith-wiedemann and simpson-golabi-behmel syndromes. Genes Dev. 1997;11(23).

20. Dechiara TM, Efstratiadis A, Robertson EJ. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor-ii gene disrupted by targeting. Nature. 1990;345(6270).

21. Dechiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor-ii gene. Cell. 1991;64(4).

22. Lau MMH, Stewart CEH, Liu ZY, Bhatt H, Rotwein P, Stewart CL. Loss of the imprinted Igf2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. Genes Dev. 1994;8(24).

23. Temple IK, Shield JPH. Transient neonatal diabetes, a disorder of imprinting. J Med Genet. 2002;39(12):872-5.

24. Lefebvre L. The placental imprintome and imprinted gene function in the trophoblast glycogen cell lineage. Reproductive Biomedicine Online. 2012;25(1):44-57.

25. Cross JC. IFPA 2004 award in placentology lecture - how to make a placenta: Mechanisms of trophoblast cell differentiation in mice - A review. Placenta. 2005;26:S3-9.

26. Cross JC. Trophoblast function in normal and preeclamptic pregnancy. Fetal and Maternal Medicine Review. 1996;8(02):57-66.

27. Barker DJP. In utero programming of chronic disease. Clin Sci. 1998;95(2):115-28.

28. Georgiades P, Ferguson-Smith AC, Burton GJ. Comparative developmental anatomy of the murine and human definitive placentae. Placenta. 2002;23(1):3-19.

29. Henriksen T, Clausen T. The fetal origins hypothesis: Placental insufficiency and inheritance versus maternal malnutrition in well-nourished populations. Acta Obstet Gynecol Scand. 2002;81(2):112-4.

30. Carter AM. Evolution of placental function in mammals: The molecular basis of gas and nutrient transfer, hormone secretion, and immune responses. Physiol Rev. 2012;92(4):1543-76.

31. Rossant J, Cross JC. Placental development: Lessons from mouse mutants. Nature Reviews Genetics. 2001;2(7):538-4.

32. Watson ED, Cross JC. Development of structures and transport functions in the mouse placenta. Physiology. 2005;20:180-93.

33. Coan PM, Conroy N, Burton GJ, Ferguson-Smith AC. Origin and characteristics of glycogen cells in the developing murine placenta. Developmental Dynamics. 2006;235(12).

34. Hu D, Cross JC. Development and function of trophoblast giant cells in the rodent placenta. Int J Dev Biol. 2010;54(2-3):341-54.

35. Lescisin KR, Varmuza S, Rossant J. Isolation and characterization of a novel trophoblast-specific cdna in the mouse. Genes Dev. 1988;2(12A):1639-46.

36. Teesalu T, Blasi F, Talarico D. Expression and function of the urokinase type plasminogen activator during mouse hemochorial placental development. Developmental Dynamics. 1998;213(1):27-38.

37. Bouillot S, Rampon C, Tillet E, Huber P. Tracing the glycogen cells with protocadherin 12 during mouse placenta development. Placenta. 2006;27(8):882-8.

38. Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J. Promotion of trophoblast stem cell proliferation by FGF4. Science. 1998;282(5396).

39. Natale DRC, Hemberger M, Hughes M, Cross JC. Activin promotes differentiation of cultured mouse trophoblast stem cells towards a labyrinth cell fate. Dev Biol. 2009;335(1):120-31.

40. Johnson JE, Birren SJ, Anderson DJ. 2 rat homologs of drosophila-achaete-scute specifically expressed in neuronal precursors. Nature. 1990;346(6287):858-61.

41. Johnson JE, Birren SJ, Saito T, Anderson DJ. Dna-binding and transcriptional regulatory activity of mammalian achaete-scute homologous (mash) proteins revealed by interaction with a muscle-specific enhancer. Proc Natl Acad Sci U S A. 1992;89(8):3596-600.

42. Guillemot F, Caspary T, Tilghman SM, Copeland NG, Gilbert DJ, Jenkins NA, et al. Genomic imprinting of Mash2, a mouse gene required for trophoblast development. Nat Genet. 1995;9(3).

43. Rossant J, Guillemot F, Tanaka M, Latham K, Gertenstein M, Nagy A. Mash2 is expressed in oogenesis and preimplantation development but is not required for blastocyst formation. Mech Dev. 1998;73(2).

44. Tanaka M, Gertsenstein M, Rossant J, Nagy A. Mash2 acts cell autonomously in mouse spongiotrophoblast development. Dev Biol. 1997;190(1):55-6.

45. Scott IC, Anson-Cartwright L, Riley P, Reda D, Cross JC. The HAND1 basic helix-loop-helix transcription factor regulates trophoblast differentiation via multiple mechanisms. Mol Cell Biol. 2000;20(2):530-41.

46. McLaughlin KJ, Szabo P, Haegel H, Mann JR. Mouse embryos with paternal duplication of an imprinted chromosome 7 region die at midgestation and lack placental spongiotrophoblast. Development. 1996;122(1).

47. Rentsendorj A, Mohan S, Szabo P, Mann JR. A genomic imprinting defect in mice traced to a single gene. Genetics. 2010;186(3).

48. Tanaka M, Puchyr M, Gertsenstein M, Harpal K, Jaenisch R, Rossant J, et al. Parental origin-specific expression of Mash2 is established at the time of implantation with its imprinting mechanism highly resistant to genome-wide demethylation. Mech Dev. 1999;87(1-2).

49. Lefebvre L, Mar L, Bogutz A, Oh-McGinnis R, Mandegar MA, Paderova J, et al. The interval between Ins2 and AscI2 is dispensable for imprinting centre function in the murine beckwith-wiedemann region. Hum Mol Genet. 2009;18(22).

50. Oh-McGinnis R, Bogutz AB, Lefebvre L. Partial loss of Ascl2 function affects all three layers of the mature placenta and causes intrauterine growth restriction. Dev Biol. 2011;351(2).

51. van der Flier LG, van Gijn ME, Hatzis P, Kujala P, Haegebarth A, Stange DE, et al. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell. 2009;136(5):903-12.

52. Jones MJ, Lefebvre L. An imprinted GFP insertion reveals long-range epigenetic regulation in embryonic lineages. Dev Biol. 2009;336(1):42-5.

53. Benson KF, Chada K. Mini-mouse - phenotypic characterization of a transgenic insertional mutant allelic to pygmy. Genet Res. 1994;64(1):27-33.

54. Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA, Warman ML. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). BioTechniques. 2000;29(1):52.

55. Morin RD, Bainbridge M, Fejes A, Hirst M, Krzywinski M, Pugh TJ, et al. Profiling the HeLa S3 transcriptome using randomly primed cDNA and massively parallel short-read sequencing. BioTechniques. 2008;45(1):81.

56. Knox K, Baker JC. Genomic evolution of the placenta using co-option and duplication and divergence. Genome Res. 2008;18(5):695-70.

57. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: Open software development for computational biology and bioinformatics. Genome Biol. 2004;5(10):R80.

58. Mamo S, Gal AB, Bodo S, Dinnyes A. Quantitative evaluation and selection of reference genes in mouse oocytes and embryos cultured in vivo and in vitro. Bmc Developmental Biology. 2007;7:14.

59. Oh-McGinnis R, Bogutz AB, Lee KY, Higgins MJ, Lefebvre L. Rescue of placental phenotype in a mechanistic model of beckwith-wiedemann syndrome. Bmc Developmental Biology. 2010;10:50.

60. Ramakers C, Ruijter JM, Deprez RHL, Moorman AFM. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett. 2003;339(1):62-6.

61. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-delta delta C) method. Methods. 2001;25(4):402-8.

62. Chiu S, Maruyama EO, Hsu W. Derivation of mouse trophoblast stem cells from blastocysts. Journal of visualized experiments : JoVE. 2010 (40).

63. Quinn J, Kunath T, Rossant J. Mouse trophoblast stem cells. Methods Mol Med. 2006;121:125-48.

64. Oh R, Ho R, Mar L, Gertsenstein M, Paderova J, Hsien J, et al. Epigenetic and phenotypic consequences of a truncation disrupting the imprinted domain on distal mouse chromosome 7. Mol Cell Biol. 2008;28(3):1092-103.

65. Lynn FC, Sanchez L, Gomis R, German MS, Gasa R. Identification of the bHLH factor Math6 as a novel component of the embryonic pancreas transcriptional network. Plos One. 2008;3(6):e2430.

66. Kidder BL, Palmer S. Examination of transcriptional networks reveals an important role for TCFAP2C, SMARCA4, and EOMES in trophoblast stem cell maintenance. Genome Res. 2010;20(4):458-72.

67. Finkenzeller D, Kromer B, Thompson J, Zimmermann W. Cea5, a structurally divergent member of the murine carcinoembryonic antigen gene family, is exclusively expressed during early placental development in trophoblast giant cells. J Biol Chem. 1997;272(50):31369-76.

68. Finkenzeller D, Fischer B, McLaughlin J, Schrewe H, Ledermann B, Zimmermann W. Trophoblast cell-specific carcinoembryonic antigen cell adhesion molecule 9 is not required for placental development or a positive outcome of allotypic pregnancies. Mol Cell Biol. 2000;20(19):7140-5.

69. Rampon C, Prandini MH, Bouillot S, Pointu H, Tillet E, Frank R, et al. Protocadherin 12 (VE-cadherin 2) is expressed in endothelial, trophoblast, and mesangial cells. Exp Cell Res. 2005;302(1):48-60.

70. Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, et al. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development. 2005;132(9):2093-102.

71. Chawengsaksophak K, James R, Hammond VE, Kontgen F, Beck F. Homeosis and intestinal tumours in Cdx2 mutant mice. Nature. 1997;386(6620):84-7.

72. Beck F, Erler T, Russell A, James R. Expression of cdx-2 in the mouse embryo and placenta - possible role in patterning of the extraembryonic membranes. Developmental Dynamics. 1995;204(3):219-27.

73. Overgaard MT, Boldt HB, Laursen LS, Sottrup-Jensen L, Conover CA, Oxvig C. Pregnancy-associated plasma protein-A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 proteinase. J Biol Chem. 2001;276(24):21849-53.

74. Boldt HB, Kjaer-Sorensen K, Overgaard MT, Weyer K, Poulsen CB, Sottrup-Jensen L, et al. The Lin12-notch repeats of pregnancy-associated plasma protein-A bind calcium and determine its proteolytic specificity. J Biol Chem. 2004;279(37):38525-31.

75. Conover CA, Boldt HB, Bale LK, Clifton KB, Grell JA, Mader JR, et al. Pregnancy-associated plasma protein-A2 (PAPP-A2): Tissue expression and biological consequences of gene knockout in mice. Endocrinology. 2011;152(7):2837-44.

76. Wang J, Qiu Q, Haider M, Bell M, Gruslin A, Christians JK. Expression of pregnancy-associated plasma protein A2 during pregnancy in human and mouse. J Endocrinol. 2009;202(3):337-45.

77. Nishizawa H, Pryor-Koishi K, Suzuki M, Kato T, Kogo H, Sekiya T, et al. Increased levels of pregnancy-associated plasma protein-A2 in the serum of pre-eclamptic patients. Mol Hum Reprod. 2008;14(10):595-602.

78. Shalom-Barak T, Zhang X, Chu T, Schaiff WT, Reddy JK, Xu J, et al. Placental PPAR gamma regulates spatiotemporally diverse genes and a unique metabolic network. Dev Biol. 2012;372(1):143-55.

79. SLY W, HU P. Human carbonic-anhydrases and carbonic-anhydrase deficiencies. Annu Rev Biochem. 1995;64:375-401.

80. Lewis SE, Erickson RP, Barnett LB, Venta PJ, Tashian RE. N-ethyl-N-nitrosourea induced null mutation at the mouse car-2 locus - an animal-model for human carbonic anhydrase-ii deficiency syndrome. Proc Natl Acad Sci U S A. 1988;85(6):1962-6.

81. Singh U, Sun T, Shi W, Schulz R, Nuber UA, Varanou A, et al. Expression and functional analysis of genes deregulated in mouse placental overgrowth models: Car2 and Ncam1. Developmental Dynamics. 2005;234(4):1034-45.

82. Reeves R, Beckerbauer L. HMGI/Y proteins: Flexible regulators of transcription and chromatin structure. Biochimica Et Biophysica Acta-Gene Structure and Expression. 2001;1519(1-2):13-29.

83. Zhou XJ, Benson KF, Ashar HR, Chada K. Mutation responsible for the mouse pygmy phenotype in the developmentally-regulated factor hmgi-C. Nature. 1995;376(6543):771-4.

84. Vaudin P, Delanoue R, Davidson I, Silber J, Zider A. TONDU (TDU), a novel human protein related to the product of vestigial (vg) gene of drosophila melanogaster interacts with vertebrate TEF factors and substitutes for vg function in wing formation. Development. 1999;126(21):4807-16.

85. Maeda T, Chapman DL, Stewart AFR. Mammalian vestigial-like 2, a cofactor of TEF-1 and MEF2 transcription factors that promotes skeletal muscle differentiation. J Biol Chem. 2002;277(50):48889-98.

86. Kim J, Sebring A, Esch JJ, Kraus ME, Vorwerk K, Magee J, et al. Integration of positional signals and regulation of wing formation and identity by drosophila vestigial gene. Nature. 1996;382(6587):133-8.

87. Braun JEA, Severson DL. Regulation of the synthesis, processing and translocation of lipoprotein-lipase. Biochem J. 1992;287:337-4.

88. Weinstock PH, Bisgaier CL, AaltoSetala K, Radner H, Ramakrishnan R, LevakFrank S, et al. Severe hypertriglyceridemia, reduced high density lipoprotein, and neonatal death in lipoprotein lipase knockout mice - knockout mice mild hypertriglyceridemia with impaired very low density lipoprotein clearance in heterozygotes. J Clin Invest. 1995;96(6):2555-68.

89. Lindegaard MLS, Olivecrona G, Christoffersen C, Kratky D, Hannibal J, Petersen BL, et al. Endothelial and lipoprotein lipases in human and mouse placenta. J Lipid Res. 2005;46(11):2339-46.

90. Inoue C, Bae SK, Takatsuka K, Inoue T, Bessho Y, Kageyama R. Math6, a bHLH gene expressed in the developing nervous system, regulates neuronal versus glial differentiation. Genes to Cells. 2001;6(11):977-86.

91. Ross MD, Martinka S, Mukherjee A, Sedor JR, Vinson C, Bruggeman LA. Math6 expression during kidney development and altered expression in a mouse model of glomerulosclerosis. Developmental Dynamics. 2006;235(11):3102-9.

92. Yao J, Zhou J, Liu Q, Lu D, Wang L, Qiao X, et al. Atoh8, a bHLH transcription factor, is required for the development of retina and skeletal muscle in zebrafish. Plos One. 2010;5(6):e10945.

93. Luo GB, Ducy P, McKee MD, Pinero GJ, Loyer E, Behringer RR, et al. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. Nature. 1997;386(6620):78-81.

94. Proudfoot D, Skepper J, Shanahan C, Weissberg P. Calcification of human vascular cells in vitro is correlated with high levels of matrix gla protein and low levels of osteopontin expression. Arteriosclerosis Thrombosis and Vascular Biology. 1998;18(3):379-88.

95. Whiteley KJ, Pfarrer CD, Adamson SL. Vascular corrosion casting of the uteroplacental and fetoplacental vasculature in mice. Methods Mol Med. 2006;121:371-92.

96. Sharpe J, Ahlgren U, Perry P, Hill B, Ross A, Hecksher-Sorensen J, et al. Optical projection tomography as a tool for 3D microscopy and gene expression studies. Science. 2002;296(5567):541-5.

97. Kalousek DK, Vekemans M. Confined placental mosaicism. J Med Genet. 1996;33(7):529-33.

98. Robinson WP, Lauzon JL, Innes AM, Lim K, Arsovska S, McFadden DE. Origin and outcome of pregnancies affected by androgenetic/biparental chimerism. Human Reproduction. 2007;22(4):1114-22.