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DIPLOMARBEIT

Extension of imprinted silencing in the *Igf2r* cluster in extra-embryonic tissues

Verfasser

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I. ZUSAMMENFASSUNG

Geprägte Gene kommen in Gruppen vor, welche meist von einer makro-nicht-protein-kodierende (mnk) RNA reguliert werden. Die inneren Gene der Gruppe können von der mnk RNA überlappt werden und zeigen im gesamten Embryo und in extra-embryonalen Geweben geprägte Expression. Die äußeren Gene der Gruppe werden nicht von der mnk RNA überlappt und zeigen nur in extra-embryonalen Geweben eine geprägte Expression. Extra-embryonales Gewebe besteht aus unterschiedlichen Zelltypen und kann von mütterlichem Gewebe kontaminiert werden. Dadurch kann die genomische Prägung des untersuchten Gens durch biallelische Expression im kontaminierenden Gewebe maskiert werden.

Um Kontaminationen zu vermeiden, haben wir eine effiziente Methode entwickelt, um eine pure Population von viszeralem Endoderm (VE) aus dem Dottersack zu isolieren. VE ist somit eine gutes extra-embryonales Model-Gewebe um neue geprägte Gene zu finden. Die monoallelische “T-hairpin” Deletion, welche die gut charakterisierte geprägte *Igf2r* Gengruppe einschließt, wird genutzt um im VE nach neuen geprägten Genen zu suchen. In der Igf2r Gengruppe reguliert die mnk RNA Airn die Expression der geprägten Gene. Um zu testen, ob ein geprägtes Gen zu der *Igf2r* Gengruppe gehört, habe ich untersucht ob dieses von *Airn* reguliert wird. Dabei habe ich Wildtyp Embryonen mit Embryonen verglichen, welche eine gekürzte, nicht funktionelle Variante von *Airn* exprimieren. Mit diesem Ansatz habe ich 15 Gene getestet, wovon drei vorranging vom mütterlichen Allele exprimiert werden. Zusätzlich zeigen meine vorläufigen Daten, dass das in der Plazenta mütterliche geprägte Gen *Pde10a* von *Airn* reguliert wird. Zusammenfassend zeigen meine Ergebnisse, dass die *Igf2r* Gruppe nicht wie bisher angenommen 440Kb groß ist, sondern sich in extra-embryonalen Gewebe auf bis zu 4Mb ausbreitet.

I. ABSTRACT

Imprinted genes occur in clusters, several of which are regulated by a macro long non-protein-coding (lnc) RNA. Inner genes within the cluster can be overlapped by the macro lncRNA and show multi-lineage (ML) imprinted expression in the embryo and extra-embryonic tissues, while outer genes are not overlapped by the macro lncRNA and show extra-embryonic-lineage (EXEL) specific imprinted expression. Extra-embryonic tissues are a mixture of ML and EXEL cell types, so imprinted expression may be masked by biallelic expression in ML tissues if a whole organ is examined. We have developed an efficient method to isolate a pure population of visceral endoderm (VE), an EXEL cell type, providing a system to discover new imprinted genes as EXEL tissues show imprinted expression of both, ML and EXEL genes, and masking is avoided. Using this system I have taken advantage of the T-hairpin uniparental deletion to systematically check for the limits of the well-characterised *Igf2r* cluster. Imprinted genes can be definitively shown to be part of these *Igf2r* cluster by determining if they are regulated by the *Airn* macro lncRNA. This is done by comparing wildtype embryos with those containing a truncated and non-functional *Airn*. Using this system I have tested 15 genes and identified three genes showing biased imprinted expression in VE and shown that they are part of the *Igf2r* cluster. In addition, I have preliminary data confirming a recent report that *Pde10a* shows imprinted expression in placenta, and have shown that imprinted silencing is regulated by *Airn*. Taken together these results extend the known region showing imprinted expression in the *Igf2r* cluster from 440Kb up to 4Mb in extra-embryonic tissues.

II. INTRODUCTION

1. Genomic Imprinting

All mammals are diploid, which means that they have two homologous autosomal chromosomes, in which one copy comes from the mother and one from the father. Nearly all genes show biallelic expression whereas a minority (around 1%) show monoallelic expression, meaning that a gene is only expressed from one allele (Koerner et al., 2009). Monoallelic expression can arise randomly, for example by random X-inactivation in females (Lyon, 1961) or allelic exclusion shown by the B and T cell receptor genes (Mostoslavsky et al., 2004), but there is another class in which monoallelic expression is parental-specific. This means that a gene is only expressed from the paternal or from the maternal allele. Genes that show parental-specific expression are called imprinted genes (Figure 1). Imprinted expression is regulated by a process called genomic imprinting that is explained in the next section.

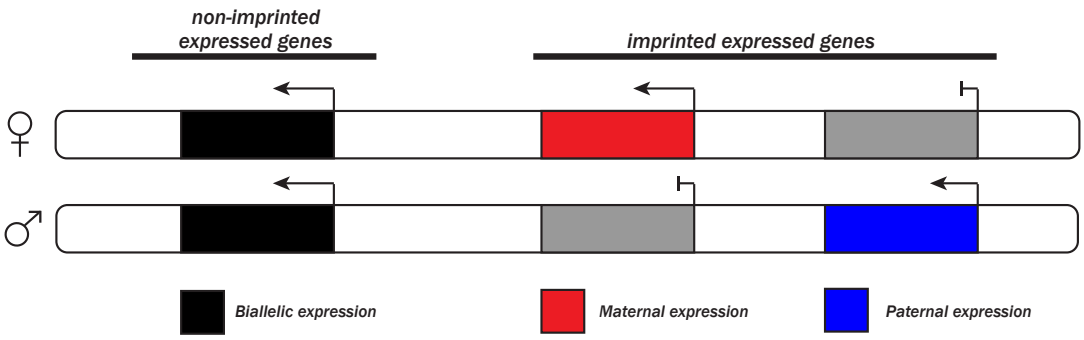


Figure 1 Non-imprinted expressed genes (black) show biallelic expression, whereas imprinted genes show parental-specific monoallelic expression, meaning that gene expression occurs only from the maternal allele (red) or only from the paternal allele (blue).

To date 150 genes have been verified as showing imprinted expression in mouse (Figure 2 (Williamson CM, 2012)). Some of those genes show imprinted expression in the embryo and/or adult tissues, whereas other genes show imprinted expression only in extra-embryonic tissues (Hudson et al., 2010).

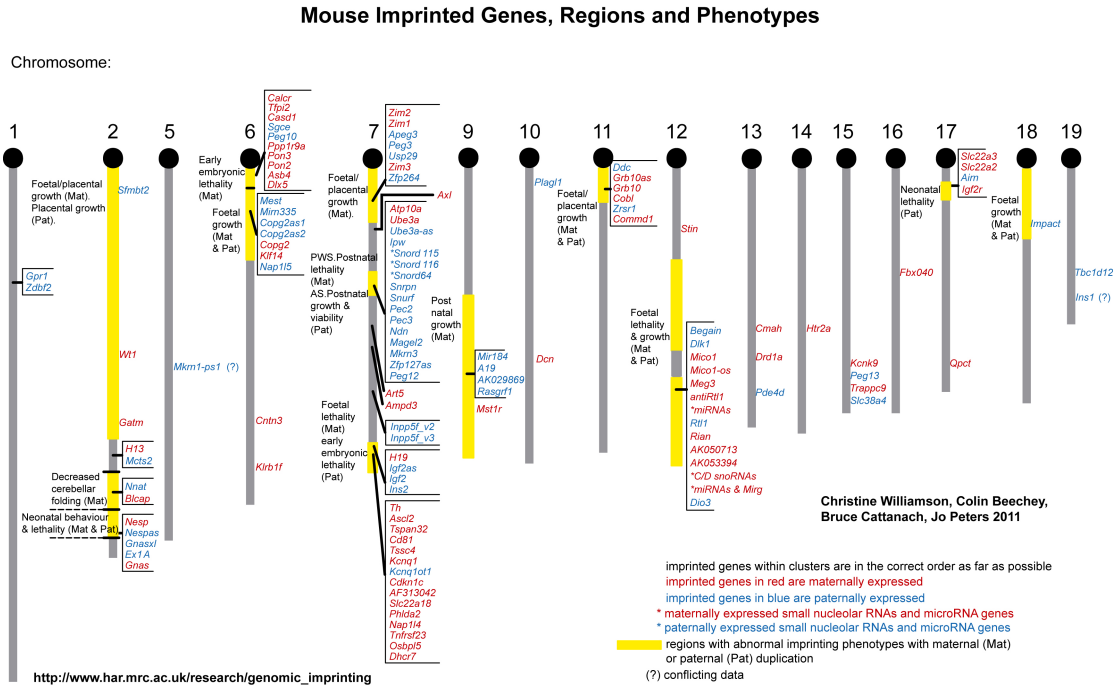


Figure 2 Overview of mouse imprinted genes, regions and phenotypes. All the mouse chromosomes are indicated in grey. Yellow bars show a natural deleted or duplicated region which induces an imprinted phenotype. Genes in red show maternal expression, whereas genes in blue show paternal expression. Figure taken from (Williamson CM, 2012).

1.1 The imprint control element (ICE) regulates imprinted gene expression

Genomic imprinting is regulated by a differentially DNA methylated region (DMR) called the imprint control element (ICE), where the unmethylated allele silences neighbouring imprinted genes in cis (Barlow, 2011). The DNA methylation mark of the ICE is set in the oocyte or spermatocyte by the de novo DNA methylation complex DNMT3A/3L, and maintained through cell division by the DNA methyltransferase DNMT1, which methylates the unmethylated “daughter” strand after DNA replication (Figure 3) (Bourc'his et al., 2001; Jia et al., 2007; Kaneda et al., 2004; Li et al., 1993). The ICE escapes the global demethylation that occurs in the pre-implantation embryo and is only reset in the primordial germ cells (PGCs) prior to the imprints being re-established during gametogenesis (Figure 3) (Morgan et al., 2005). The ICE is a CpG island, and while the majority of individual CpGs in the genome are methylated, outside of imprinted regions most CpG islands are unmethylated and form 60-70% of promoters in the genome (Antequera, 2003; Deaton and Bird, 2011). A minority of imprinted genes can become

methylated on their repressed promoter later in development, which may contribute to maintaining silencing, but is not necessary to establish imprinted silencing (Barlow, 2011).

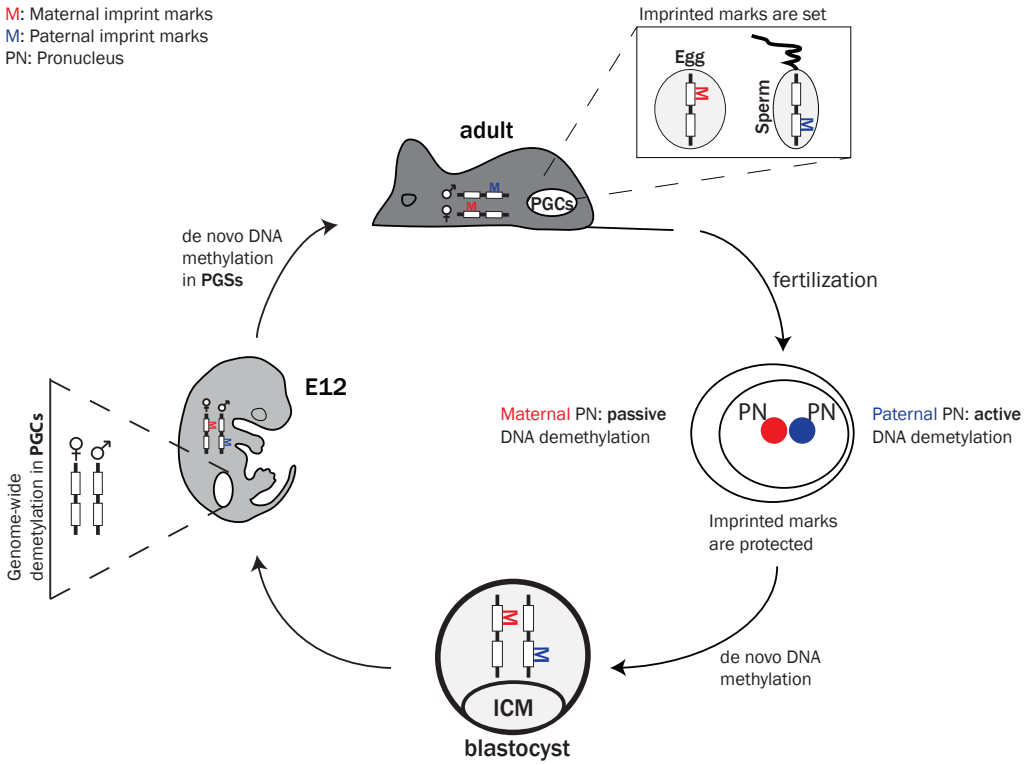


Figure 3 DNA methylation cycle. The first phase of DNA methylation reprogramming is during gametogenesis in a 12 day old embryo, where the DNA methylation marks are erased in primordial germ cells (PGCs), including the marks of the ICEs (M). The next step is a parental-specific de novo DNA methylation of the gametes, which starts shortly after birth. In this step the methylation imprint is established on the ICE. The second phase of DNA methylation reprogramming occurs during preimplantation, immediately after fertilization. Before the paternal and maternal pronucleus are fused together, the second round of demethylation starts. The paternal pronucleus (PN) in blue is actively demethylated whereas the maternal PN in red is passively. The imprinted methylation marks are protected from erasure in this stage. The second de novo methylation is needed for the blastocyst formation. (Morgan et al., 2005).

1.2 Imprinted genes occur mostly in clusters

Imprinted genes occur mostly in clusters in the genome, each controlled by a single ICE. To date sixteen imprinted clusters are known, which include 80% of all known imprinted genes (Barlow, 2011). The ICE has been defined by genetic deletion in seven imprinted clusters, where deletion leads to the loss of imprinted expression of all the genes in the cluster (Barlow, 2011). Known clusters contain between two and twelve imprinted genes including at least one macro long non-coding RNA, and can vary from the size from 80 to 4000 kb (Guenzl and Barlow, 2012).

1.3 Imprinted long non-coding RNAs

Long-non-coding RNAs (lncRNAs) are non-protein coding RNAs longer than 200 nt whose function does not depend on processing to smaller RNAs (Gingeras, 2007). Recently, a large number of lncRNAs have been discovered many of which have been associated with gene regulatory functions (Guttman and Rinn, 2012). The majority of lncRNAs so far described are large intergenic ncRNAs (lincRNAs) that are fully spliced and most are thought to act *in trans* to regulate genes (Guttman et al., 2011). In contrast, imprinted lncRNAs belong to a lncRNA sub-class known as macro lncRNAs which are inefficiently spliced with a relatively short half life, and a low level of conservation and many interspersed repeats (Guenzl and Barlow, 2012). For the *Igf2r*, *Kcnq1* and *Gnas* clusters truncation of the imprinted macro lncRNAs *Airn*, *Kcnq1ot1* and *Nespas* to less than 5% of the length, leads to loss of imprinted silencing in the whole cluster, showing that these macro lncRNAs are responsible for silencing (Mancini-Dinardo et al., 2006; Sleutels et al., 2002; Williamson et al., 2011). In these three examples, the promoter of the imprinted macro lncRNA is contained in the unmethylated ICE on the paternal allele.

In contrast, the imprinted lncRNA *H19* within the *Igf2* cluster is not involved in imprinted silencing of protein coding genes (Arney, 2003). The *H19* lncRNA shares features with lincRNAs rather than macro lncRNAs in that it is efficiently spliced, intergenic and the ICE does not act as its promoter (Bell and Felsenfeld, 2000; Brannan et al., 1990). In the *Igf2* cluster the DNA methylation mark on the ICE is present only on the paternal allele, which blocks the binding of the zinc finger protein CTCF. On the unmethylated maternal allele CTCF can bind and has insulator function preventing the interaction between distal enhancers and the *Igf2* promoter, and instead the enhancers activate *H19* expression. On the methylated paternal allele CTCF cannot bind and so the enhancers can interact and activate *Igf2* expression (Bell and Felsenfeld, 2000).

2. Genomic imprinting in extra-embryonic tissues

2.1 Extension of imprinted silencing in extra-embryonic tissues

Imprinted genes mainly occur in clusters, and in three tested cases are regulated by a macro lncRNA, under the control of an ICE (Barlow, 2011). In general, 'inner' genes within the cluster, which are closer to the ICE and in some cases overlapped by the regulating macro lncRNA, show multi-lineage (ML) imprinted expression in the embryo and extra-embryonic tissues. However 'outer' genes, which are further away from the ICE are not overlapped by the macro lncRNA, show only extra-embryonic-lineage (EXEL) specific imprinted expression (Hudson et al., 2011; Lewis et al., 2004; Terranova et al., 2008). *Igf2r* and *Airn* show imprinted expression in most tissues and are therefore good examples of ML specific genes (Barlow et al., 1991; Sleutels et al., 2002). However the genes *Slc22a2* and *Slc22a3* show only imprinted expression in some extra-embryonic tissues (Zwart et al., 2001) and are therefore EXEL specific genes (Figure 4) (Hudson et al., 2011).

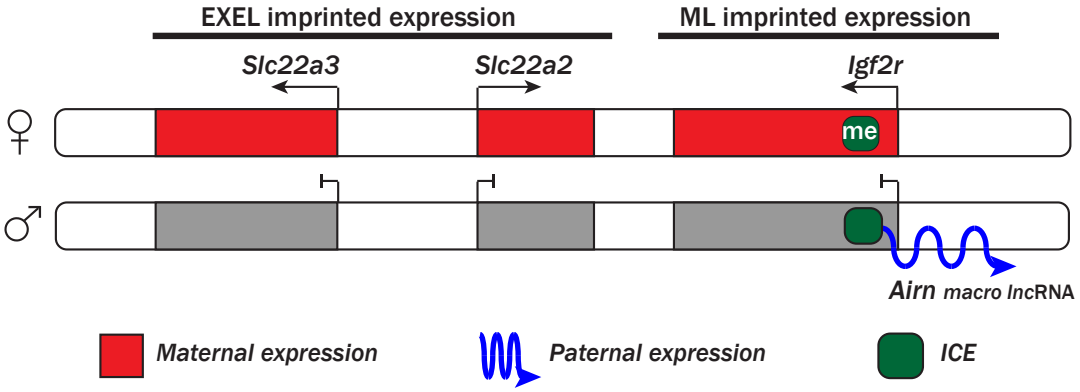


Figure 4 Genes showing extra-embryonic-lineage (EXEL) specific or multi-linage (ML) imprinted expression in the *Igf2r* cluster. *Igf2r* in red and *Airn* in blue show imprinted expression in almost all tissues and therefore show ML specific imprinted expression. However the genes *Slc22a2* and *Slc22a3* show only imprinted expression in extra-embryonic tissues and are therefore EXEL specific genes. The ICE in green, a DMR including the *Airn* promoter, which gains a methylation mark during oogenesis, controls expression of the macro lncRNA.

The only other clusters reported to show EXEL imprinted expression, the *Igf2*, *Kcnq1* and *Peg10* clusters, also show EXEL specific imprinted expression limited to 'outer' genes in the cluster (Hudson et al., 2010). This indicates that imprinted clusters may show an extension of imprinted silencing in extra-

embryonic tissues compared to embryonic and adult tissues. Epigenetic differences between extra-embryonic and embryonic tissues may account for why macro lncRNAs induce an extended domain of imprinted silencing in EXEL, but not ML tissues (Hudson et al., 2010).

2.2 Epigenetic differences between extra-embryonic and embryo tissues

Extra-embryonic tissues in mouse have a lifespan of 16 days, whereas embryonic tissues have to develop to an adult mouse, which can live up to two years. Extra-embryonic tissues are derived from the earliest differentiated cell types, forming in the preimplantation embryo between 3.5 and 4.5 days post coitum (dpc), while embryonic lineages do not differentiate until gastrulation at 6.5dpc in the postimplantation embryo (Rossant and Tam, 2002). Differences in the development, function and lifespan of embryonic and extra-embryonic tissues could have an effect on how gene expression is regulated (Hudson et al., 2010). Indications that this may be the case are the low global DNA methylation of extra-embryonic tissues compared to the embryo (Chapman et al., 1984), and reports of differences in chromatin modifications at some imprinted gene clusters (Pandey et al., 2008). It is known that extra-embryonic lineages tolerate polyploidy compared to embryonic tissues (Tarkowski et al., 1977) indicating that extra-embryonic tissues may be more tolerant to changes in gene dosage. In addition to showing EXEL imprinted expression, extra-embryonic tissues also show imprinted X-inactivation compared to random X-inactivation in embryonic lineages (Takagi and Sasaki 1975, Nature). Together these examples indicate that there are epigenetic differences between embryonic and extra-embryonic tissues that may be involved in regulating EXEL-specific imprinted expression.

2.3 Visceral Endoderm as an extra-embryonic imprinting model

Placenta was used in previous studies as a model tissue to study imprinted expression in extra-embryonic tissues (Green et al., 2007; Lewis et al., 2004). There are two drawbacks in using placenta as a model to study imprinted gene expression. First, placenta is an organ with a mixture of different tissues, therefore imprinted expression in one cell type can be masked by biallelic

expression in another cell type. Second, the placenta is contaminated with maternal tissues, namely decidua and blood vessels, which can cause a maternal bias (Figure 5A) (Hudson et al., 2011). A recent study examined genes reported to show placenta-specific imprinted expression failed to validate many of them revealing the extent of the maternal bias problem (Okae et al., 2011). Alternative extra-embryonic tissues to placenta are the membranes surrounding the embryo, the amnion, the visceral yolk sac (VYS) and the parietal yolk sack (PYS). Of these extra-embryonic tissues only the VYS has been reported to show EXEL imprinted expression (Hudson et al., 2010; Nagy et al., 2003) (Figure 5A). The VYS has two layers, the visceral endoderm (VE) and the visceral mesoderm (VM). Our lab has developed an efficient new method to separate the two layers and showed that EXEL imprinted expression is limited to the VE layer (Hudson et al., 2011) (Figure 5B).

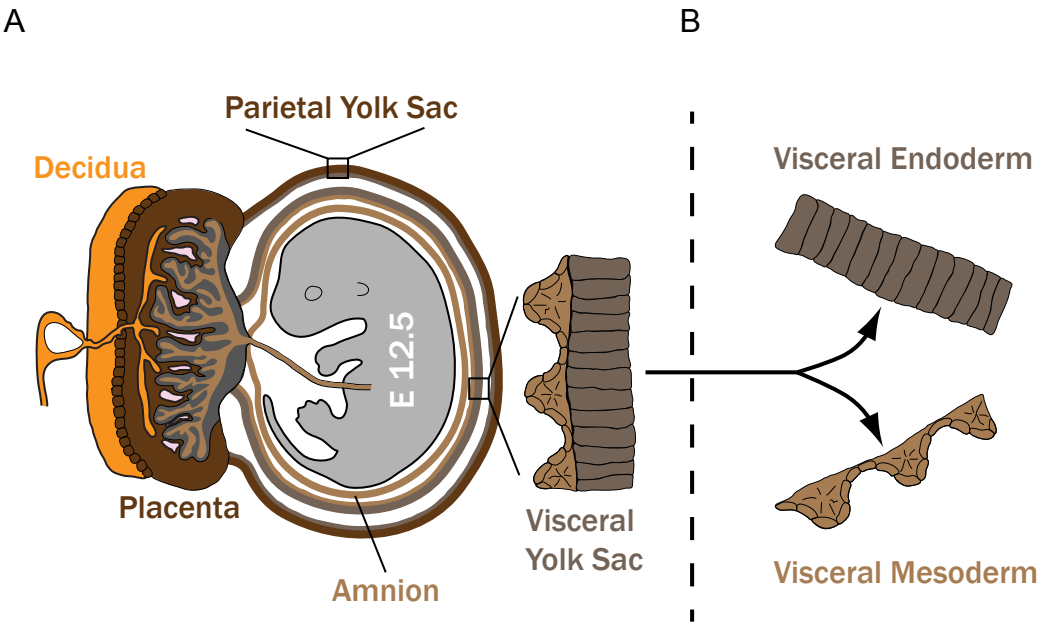


Figure 5 (A) The placenta is contaminated with maternal tissues (orange) namely, decidua and maternal blood vessels and blood. Alternative extra-embryonic tissues are the amnion, the visceral yolk sac (VYS) and the parietal yolk sack (PYS) which surround the embryo. (B) Isolation of a pure population of visceral endoderm was established by Dispase II digestion followed by mechanical separation of the yolk into visceral endoderm (VE) and visceral mesoderm (VM) (modified from (Hudson et al., 2011)).

The VE presents as an improved model of EXEL imprinted expression as, unlike placenta, it lacks maternal contamination and as pure population of cells; imprinted expression cannot be masked by biallelic expression in another cell type (Hudson et al., 2011).

3. The *Igf2r* Cluster

The *Igf2r* imprinted cluster spans 440Kb on mouse Chromosome 17 and contains the maternally expressed *Igf2r* (Insulin-like growth factor 2 receptor), *Slc22a2*, *Slc22a3* and the paternally expressed macro lncRNA *Airn* (antisense-*Igf2r*-RNA-non-coding) (Barlow et al., 1991; Zwart et al., 2001). (Figure 6).

3.1 Regulation of the *Igf2r* cluster

Igf2r is expressed only from the maternal allele (Barlow et al., 1991) in nearly all tissues and development stages except embryonic stem cells, testis and neurons (Lerchner and Barlow, 1997; Szabo and Mann, 1995a, b; Wang et al., 1994; Yamasaki et al., 2005). Imprinting of the *Igf2r* cluster is regulated by the ICE, a 3.7kb DMR in intron 2 of *Igf2r*. The DMR contains the 1.5kb CpG island promoter of the macro lncRNA *Airn*, which obtains methylation during oogenesis only on the maternal allele (Stoger et al., 1993). For that reason *Airn* is only expressed on the paternal allele (Lyle et al., 2000) and is responsible for silencing *Igf2r*, *Slc22a2* and *Slc22a3* in cis (Sleutels et al., 2002). Deletion of the unmethylated ICE and *Airn* promoter on the paternal allele shows the same phenotype as the truncated version of the macro lncRNA, namely biallelic expression of all parental-specific genes (Wutz et al., 2001). The *Igf2r* promoter itself is also a DMR and gains somatic methylation on the silenced paternal allele during differentiation, but this is not necessary for silencing of other genes in the cluster (Sleutels et al., 2003).

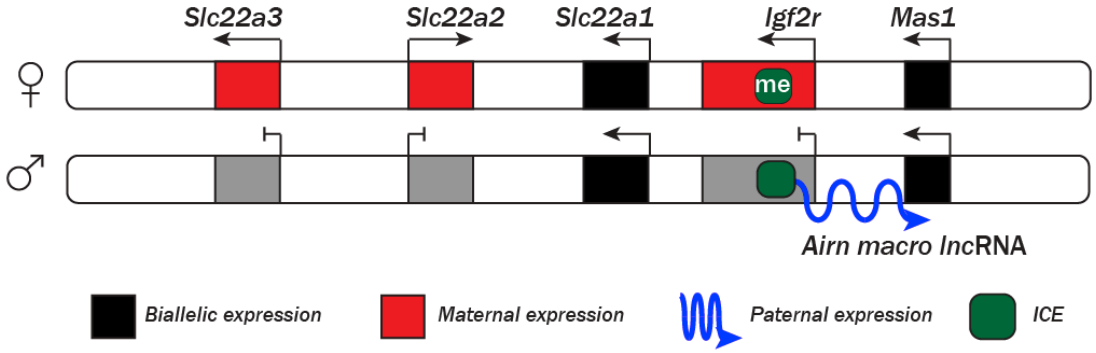


Figure 6 The *Igf2r* cluster in mouse: genes in red (*Slc22a3*, *Slc22a2* and *Igf2r*) show maternal expression, and are silenced by the paternal expressed macro lncRNA *Airn* in cis. *Igf2r* and *Airn* are expressed in nearly all tissues whereas the genes *Slc22a3* and *Slc22a2* are only expressed in extra-embryonic tissues. The ICE in green, a DMR including the *Airn* promoter, which gains a methylation mark during oogenesis, controls expression of the macro lncRNA. Genes in black (*Slc22a1*, *Mas1*) show biallelic expression.

Slc22a2 and *Slc22a3* show maternal-specific imprinted expression restricted to extra-embryonic tissues, with *Slc22a2* showing imprinted expression only in VYS, while *Slc22a3* shows imprinted expression in both VYS and placenta (Hudson et al., 2011; Zwart et al., 2001). Both genes show imprinted expression from 8.5 dpc, and while *Slc22a2* retains imprinted expression throughout embryonic development, *Slc22a3* losses imprinted expression at 15.5-16.5dpc (Hudson et al., 2011; Verhaagh et al., 1999; Wang et al., 2011). Truncation of *Airn* from 118kb to 3kb by inserting a polyadenylation signal induces re-expression of the genes *Slc22a2*, *Slc22a3* and *Igf2r* on the paternal allele, which demonstrates that *Airn* regulates allele-specific silencing (Sleutels et al., 2002). Subsequent work in our lab where *Airn* was truncated to different lengths indicated that only transcriptional overlap by *Airn* is required to silence *Igf2r*, indicating that silencing may occur by transcriptional interference (Latos et al unpublished). The EXEL genes may be silenced by a different mechanism as they are not overlapped by *Airn*. The Fraser lab demonstrated, that *Airn* associates with the *Slc22a3* promoter and with EHMT2 (Euchromatic histone-lysine N-methyltransferase 2) H3K9dimethylase. EHMT2 is required for imprinted expression of *Slc22a3* indicating that *Airn* may recruit EHMT2 to the promoter causing silencing (Nagano et al., 2008). However, this correlative data could also fit with other models of gene silencing, such as the recently proposed enhancer interference model (Pauler et al., 2012).

3.2 The T^{hp} -deletion

In 1962, a new deletion was found in The Jackson Laboratory in Maine, USA that causes the same phenotype as the brachyury mutation (T gene), namely a shorter tail length (Johnson, 1974). Mice showing this phenotype, caused by this deletion on Chr. 17 were named Hairpin-tail (T^{hp}). A indication of the T associated maternal effect (Tme) was the different litter sizes depending on if the T^{hp} deletion is inherited from the mother or from the father. Embryos, in which the T^{hp}-deletion is inherited from the mother, die after 15 days post conception (dpc), but if the T^{hp}-deletion is inherited from the father the embryos are viable and show the Hairpin-tail phenotype (Johnson, 1974). Later in 1991 the Tme phenotype was explained by the fact that the T^{hp}-deletion includes the *Igf2r* gene, which is only expressed from the maternal allele and therefore lethal for embryos carrying the deletion on the maternal allele (Barlow et al., 1991; Wang et al., 1994).

The deletion is approximately 5Mb long (Johnson, 1974) and contains 25 refseq genes including the well-studied imprinted *Igf2r* cluster described in the section above (Figure 7). Some genes close to the *Igf2r* cluster were already examined in different embryonic and extra-embryonic tissues for allele-specific expression (Barlow et al., 1991; Hudson et al., 2011; Zwart et al., 2001)

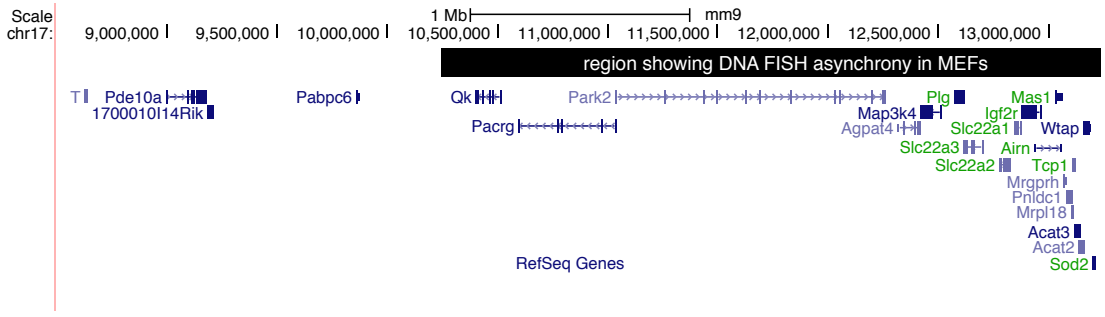


Figure 7 Overview of the T^{hp}-deletion. The approximately 5Mb long T^{hp}-region on mouse chromosome 17 contains 25 refseq genes (picture taken from UCSC genome browser). Genes in green are those genes that were already examined for allele-specific expression in different tissues. The black bar covers the region of DNA FISH asynchrony in MEFs, which is a feature of imprinted clusters (PhD thesis: (Koerner, 2010)).

DNA fluorescence in situ hybridization (FISH) asynchrony is a feature of imprinted clusters, but the mechanism and relevance to imprinted expression is not well understood (Simon et al., 1999). In dividing cells, DNA FISH can

show three possible results for a genomic probe. In the case in which the gene of interest is not yet replicated, two single spots (SS) are the expected result. When both alleles including the gene of interest are replicated, two double spots (DD) are expected. A third possible result can be seen in the case, if one allele is replicated and the other not yet. In this situation DNA FISH shows one single and one double spot (SD). A region shows DNA FISH asynchrony, if the SD ratio is between 30 and 40% (Gribnau et al., 2003). Martha Koerner showed in her PhD thesis that a 3Mb chromosomal region in the T^{hp}-deletion including the *Igf2r* cluster shows DNA FISH asynchrony in mouse embryonic fibroblasts (MEFs) (Koerner, 2010). She also showed that DNA FISH asynchrony did not result from DNA replication asynchrony, indicating that instead the DNA FISH asynchrony reflected chromatin differences between the maternal and paternal chromosomes. The fact that the DNA FISH asynchrony region is much larger than the known region showing imprinted silencing indicates that this larger region warrants further investigation to determine if imprinted silencing is also extended.

4. Aim of the study

Imprinted genes occur in clusters, most of which are regulated by a macro lncRNA, under the control of an ICE. In general it was shown, that genes closer to the ICE, within the cluster show ML imprinted expression. However genes further away from the ICE, show only imprinted expression in extra-embryonic tissues. *Igf2r* and *Airn* show imprinted expression in most tissues and are therefore good examples for ML specific genes. However the genes *Slc22a2* and *Slc22a3* show only imprinted expression in extra-embryonic tissues indicating that imprinted clusters show an extension of imprinted silencing in extra-embryonic tissues. To define the limits of imprinted silencing at the *Igf2r* cluster in extra-embryonic tissues in this thesis I take VE as a model extra-embryonic tissue and systematically check for parental allele-specific expression within the T^{hp}-region using reciprocal crosses of the T^{hp}-deletion mouse with WT. I then determine if genes showing maternal imprinted or biased expression are regulated by *Airn* using the *AirnT* mouse where *Airn* is non-functional.

III. RESULTS

1. Mouse crosses to test for imprinted expression

Existing mouse models enable parent allelic expression at the *Igf2r* cluster and regulation by *Airn* to be examined. T^{hp}-deletion mice can be used to examine allelic expression depending on from which parent the deletion is inherited, while the *AirnT* mouse with a truncation of *Airn* macro lncRNA can be used to determine if repression of paternal allele expression is dependent on *Airn* (Barlow et al., 1991; Sleutels et al., 2002).

1.1 The T^{hp}-mouse model to test for allele-specific expression

The T^{hp}-mouse (+/T^{hp}) is a good model to study allele-specific expression within the deletion by doing reciprocal crosses between a T^{hp} (+/T^{hp}) and a wildtype (+/+) mouse. In one cross, the mother is the +/+ and the father the +/T^{hp}-mouse and the genotype of the offspring can be +/+ or +/T^{hp} (Figure 8A) (Note: The maternal allele is always written on the left and the paternal allele is written on the right side of the slash). The +/T^{hp} embryos have the deletion on the paternal allele and are therefore viable. In this cross, maternal expression can be detected within the deletion. In the reciprocal cross the father is the +/+ and the mother the +/T^{hp}-mouse. The genotype of the offspring can be +/+ or T^{hp}/+ (Figure 8B). T^{hp}/+ embryos that have the deletion on the maternal allele die after 15dpc, because *Igf2r* is not expressed, but paternal expression can be detected at earlier stages within the deletion region. In +/+ embryos the expression of both alleles are detected.

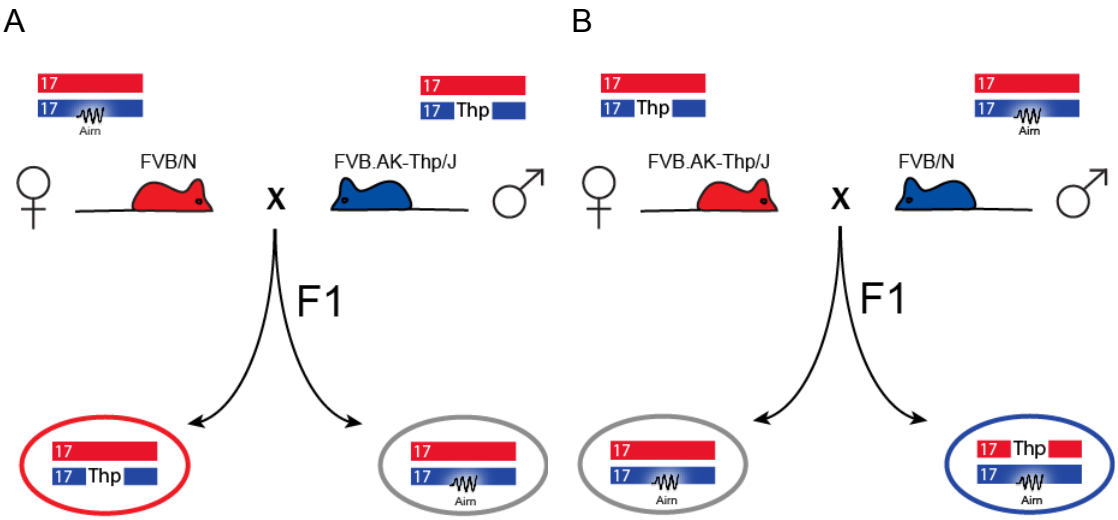


Figure 8 T^{hp}-mouse model to test for parental allele-specific expression. (A) The mother in red is the WT (+/+) and the father in blue the T^{hp}-mouse (+/T^{hp}). The genotype of the offspring from this cross can be +/+ or +/T^{hp}. In this case the T^{hp}-embryos have the deletion on the paternal allele and are therefore viable. (B) The father is the +/+ and the mother the +/T^{hp}-mouse. The genotype of the offspring from this cross can be +/+ or T^{hp}/+. In this case the T^{hp}-embryos have the deletion on the maternal allele and die after 15dpc. Note: The maternal allele is always written on the left and the paternal allele is written on the right side of the slash.

1.2 *AirnT*-mouse model to test for *Airn* regulation

Truncating the macro lncRNA *Airn* from 118Kb to 3Kb the genes *Slc22a2*, *Slc22a3* and *Igf2r* are re-expressed on the paternal allele, which demonstrates that *Airn* regulates allele-specific silencing in the *Igf2r* cluster (Sleutels et al., 2002). This *AirnT* truncation mouse can therefore be used to test if the macro lncRNA *Airn* regulates allele-specific expression of other genes in the *Igf2r* cluster (Figure 9). In this cross, the mother is a +T^{hp}-mouse and the father a +/*AirnT* mouse. The genotype of the offspring can be +/+, T^{hp}/+, +/*AirnT*, and T^{hp}/*AirnT*. If a maternally expressed gene is repressed on the paternal chromosome by *Airn*, it should be upregulated on the paternal allele in T^{hp}/*AirnT* embryos.

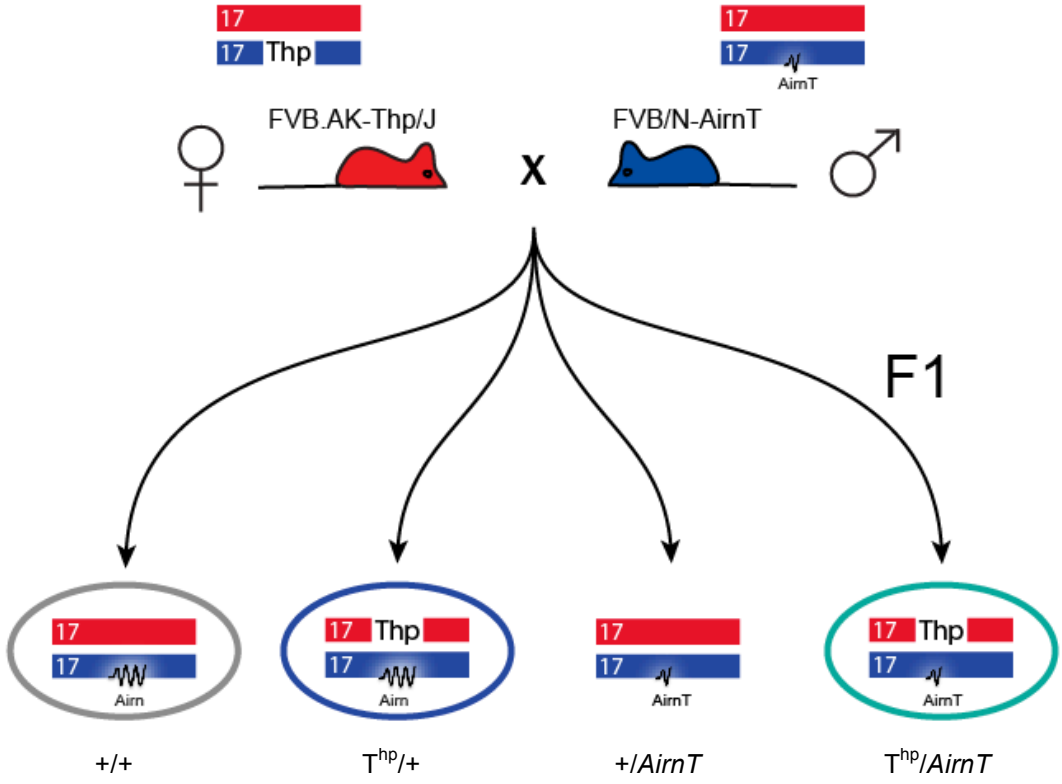


Figure 9 The *AirnT*-mouse (+/*AirnT*) model to test for *Airn* regulation. The mother in red is the +/T^{hp}-mouse and the father in blue, the +/*AirnT* mouse. The offspring from this cross can have the following genotypes: +/+, T^{hp}/+, +/*AirnT* and T^{hp}/*AirnT*. The circled genotypes (+/+, T^{hp}/+, and T^{hp}/*AirnT*) were examined in this study, while +/*AirnT* was not.

2. Genotyping of T^{hp} and *AirnT* crossings

We harvested 12.5 days post coitum (dpc) embryos from +/+ x +/T^{hp}, +/T^{hp} x +/+ and +/T^{hp} x +/*AirnT* crosses as described in the section above. The heads were taken for genotyping, and the embryo body, placenta and separated VE taken for RNA extraction as described in the methods. Before the samples could be used to test genes for allele-specific expression and for *Airn* regulation, a well-established Southern blot genotype assay was used to distinguish between the different alleles (Figure 10A). The methyl-sensitive restriction enzyme MluI was used to distinguish between maternal and paternal allele. Considering that the *Airn* promoter shows methylation only on the maternal allele (Stoger et al., 1993), MluI can only cut the unmethylated paternal allele. A MluI and EcoRI double-digest can distinguish the methylated maternal and unmethylated paternal allele by the fragment length, using a Southern blot assay (Figure 10B). The allele with the truncated version of *Airn* can be distinguished from the WT allele by an EcoRI restriction site, which is

only present on the *AirnT* allele, introduced by the targeting cassette. If the T^{hp} deletion is present on the allele, the probe cannot bind, because the fragment where the probe binds is within the deletion.

For the T^{hp} crosses (Figure 10C and 10D), both the methylated and unmethylated band can be seen in WT embryos. Alternatively, for $+/T^{hp}$ only the larger methylated band is present and for $T^{hp}/+$ only the smaller band can be seen. For the T^{hp}/*AirnT* cross (Figure 10E), for the WT alleles the methylated and unmethylated band are the same size as in Figures 10C and 10D, whereas the the *AirnT* allele makes the unmethylated band even smaller due to the extra EcoRI site.

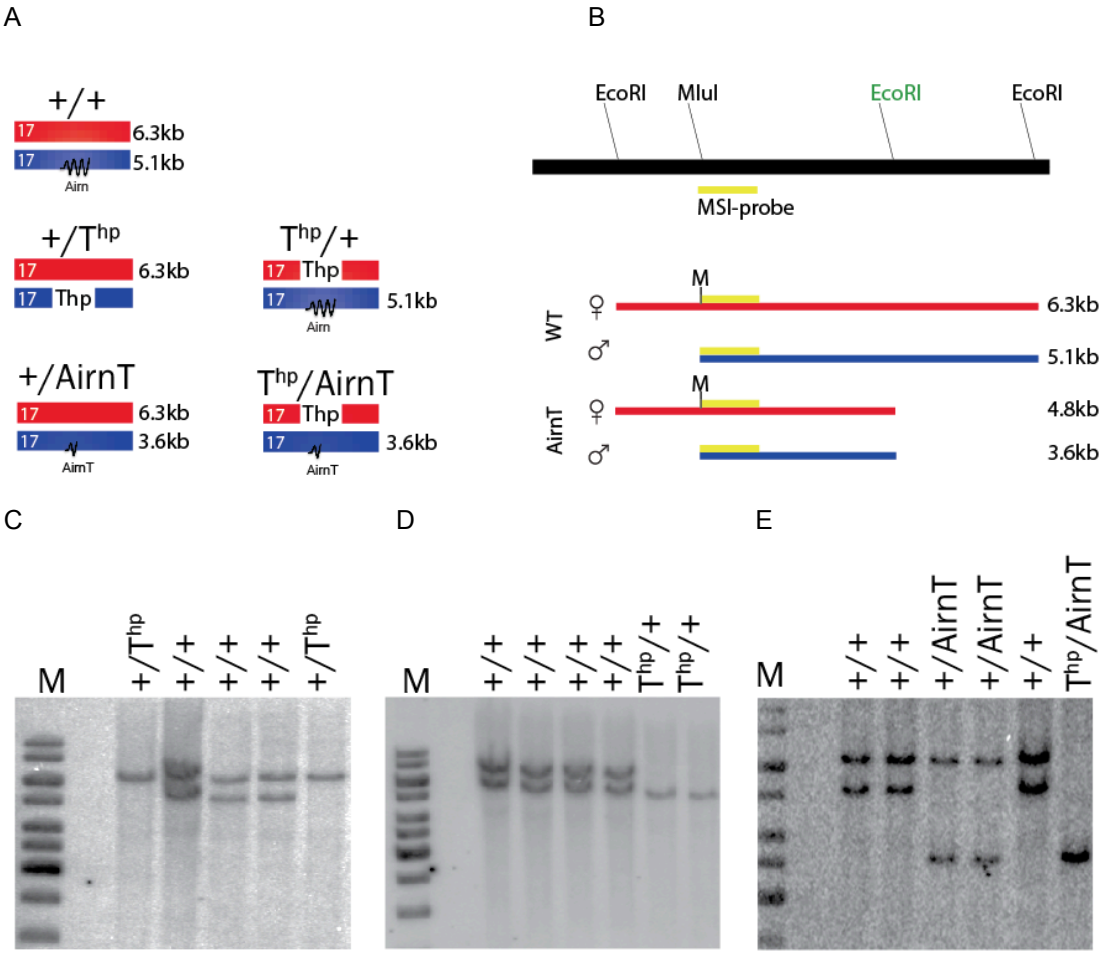


Figure 10 Genotyping of T^{hp} and *AirnT* crossings (A) All possible genotypes with fragment sizes. (B) Double digestion with the restriction enzymes MluI and EcoRI allow each genotype to be distinguished by different fragment length on a Southern blot. MluI can only cut if the restriction-site is not methylated. The yellow bar shows the detection probe (MSI-probe). The green EcoRI restriction side is only present if the allele contains the truncated version of *Airn*, as it was introduced by the targeting cassette. If the T^{hp}-deletion is present on the allele, the probe cannot bind. The red bar shows the maternal allele containing the methylation mark. The blue bar shows the paternal allele. The blots show genotyping of embryonic heads from the following crosses: (C) +/+ x $+/T^{hp}$, (D) $+/T^{hp}$ x +/+ (E) $+/T^{hp}$ x +/*AirnT.*

3. Visceral endoderm (VE) isolation

After isolating the 12.5 days old embryos, including extra-embryonic tissues from the mother, we separated the two layers of the VYS and collected the VE. To successfully isolate a pure population of VE, the VYS was digested with DispaseII and two layers mechanically separated as described in the methods. The quality of the separation was than verified by testing for the expression of VE and VM specific marker genes. For this analysis I prepared cDNA from VE according to the protocol given in the methods. The VYS was used as control, because it contains both VE and VM and therefore expresses both markers. The PCR results confirmed that the separation was successful, because all the samples express the VE marker gene *Afp*, but not the VM marker gene *Flk-1* (Figure. 11).

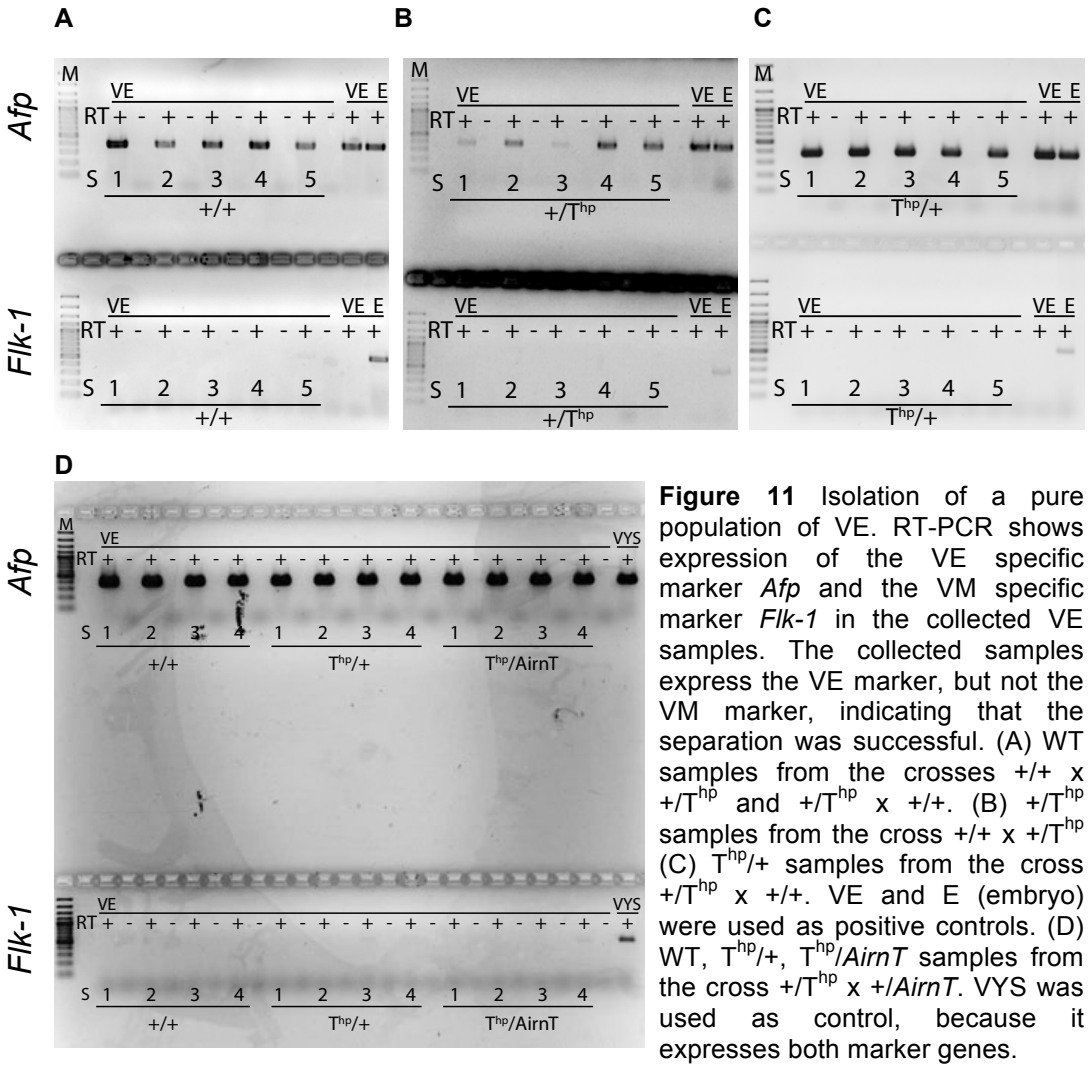


Figure 11 Isolation of a pure population of VE. RT-PCR shows expression of the VE specific marker *Afp* and the VM specific marker *Flk-1* in the collected VE samples. The collected samples express the VE marker, but not the VM marker, indicating that the separation was successful. (A) WT samples from the crosses +/+ x +/T^{hp} and +/T^{hp} x +/+. (B) +/T^{hp} samples from the cross +/+ x +/T^{hp} (C) T^{hp}/+ samples from the cross +/T^{hp} x +/+. VE and E (embryo) were used as positive controls. (D) WT, T^{hp}/+, T^{hp}/*AirnT* samples from the cross +/T^{hp} x +/*AirnT*. VYS was used as control, because it expresses both marker genes. RT+, RT-, with, without RT enzyme. M, 100bp DNA ladder. S, VE samples for each genotype.

4. Primer design for genes expressed in VE in the T^{hp}-region

To identify the expressed genes in VE within the T^{hp}-deletion, the RNA-sequencing data from the Barlow lab (Kulinski et al unpublished) was analysed by Cufflinks (Trapnell et al., 2010) and TopHat (Trapnell et al., 2009) to determine which genes are expressed in the VE. The cutoff of an expressed gene was arbitrarily set by an FPKM (fragments per kilobase of exon per million fragments mapped) of 3. Using this cutoff, 14 genes are expressed in VE shown in figure 12, covered with a green bar. For the known imprinted genes a Taqman assay was already designed in the lab, whereas for the others, I designed SYBR Green primers using Primer3 BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

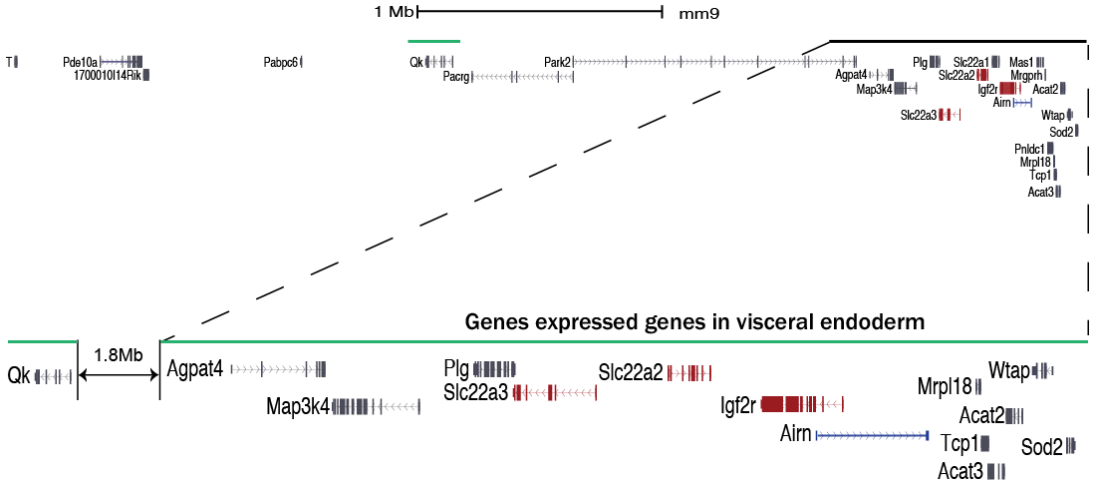


Figure 12 Expressed genes in VE within the T^{hp}-deletion. Genes in red are the known maternally expressed genes. The paternally expressed macro lncRNA *Airn* is shown in blue. All the genes, covered with a green bar are expressed in VE.

5. Allele-specific expression within the T^{hp}-deletion

To analyse allele-specific expression within the T^{hp}-deletion I used qPCR to examine expressed genes for each genotype from a reciprocal T^{hp}-cross. All the expressed genes in 12.5dpc VE were also examined in embryo for allele-specific expression. All the results were normalized to the housekeeper gene *Cyclophilin A*. Two categories of samples were used: 1, pooled samples, collected from reciprocal +/T^{hp} crosses, in which the RNA from individuals with the same genotype was pooled together and 2, individual samples collected from the reciprocal +/T^{hp} and the T^{hp}/*AirnT* crosses. The samples were

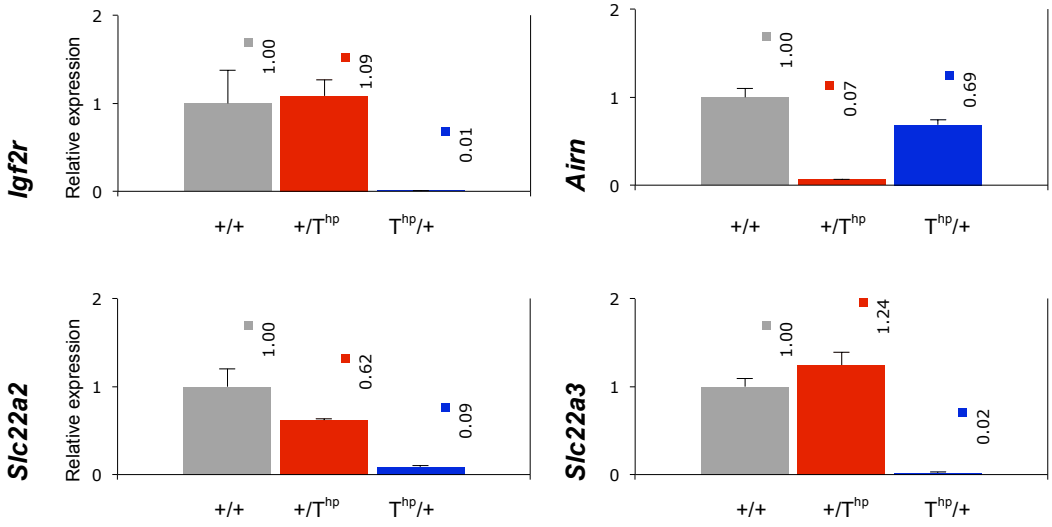
collected from different litters over a period of several months by myself and Quanah Hudson. Pooled samples average the biological variation of included samples, and may be sufficient to confidently distinguish large differences between genotypes, but do not allow for a statistical comparison. Individual samples enable the calculation of biological variation for a specific genotype and allow statistical tests to be performed.

5.1 Control genes show the expected allele-specific expression pattern in embryo and VE

To test the assay and the samples, I examined known imprinted genes as controls to confirm the expected expression pattern. All the control genes showed the expected allele specific expression pattern in VE (Figure 13 A) and in embryo (Figure 13 B) with pooled samples. The pooled samples contained three +/+, three +/T^{hp} and two T^{hp}/+ samples, collected from different litters.

The data confirm that the genes *Igf2r*, *Slc22a2* and *Slc22a3* are only expressed from the maternal allele whereas the macro lncRNA *Airn* is only expressed from the paternal allele. The error bar shows standard deviation of the technical variation.

A Pooled samples show expected allele specific expression in visceral endoderm



B Pooled samples show expected allele specific expression in embryo

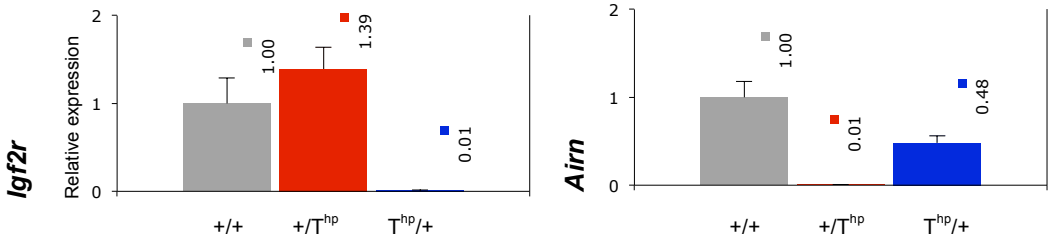
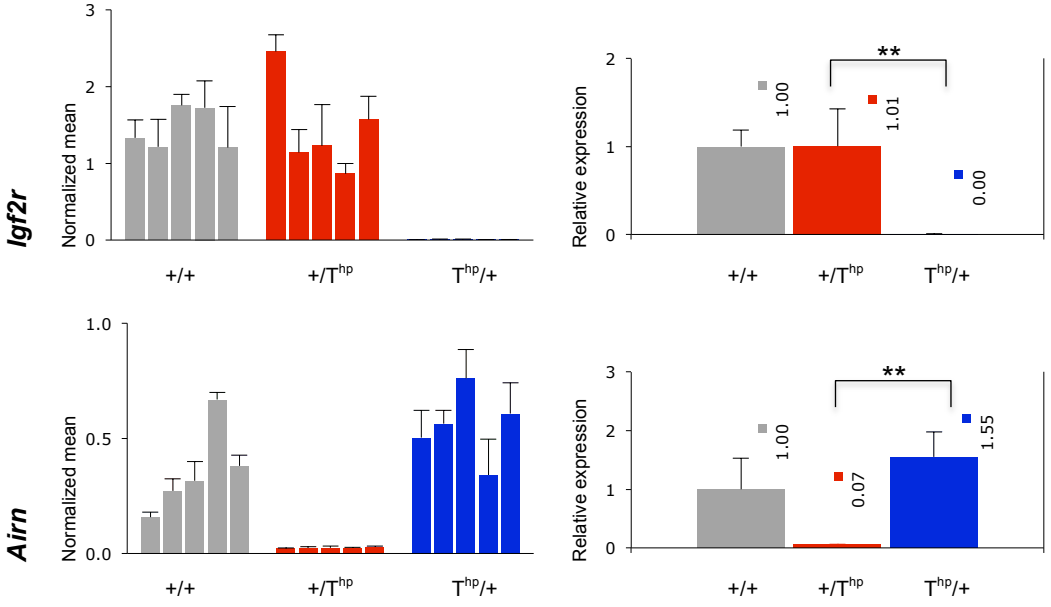
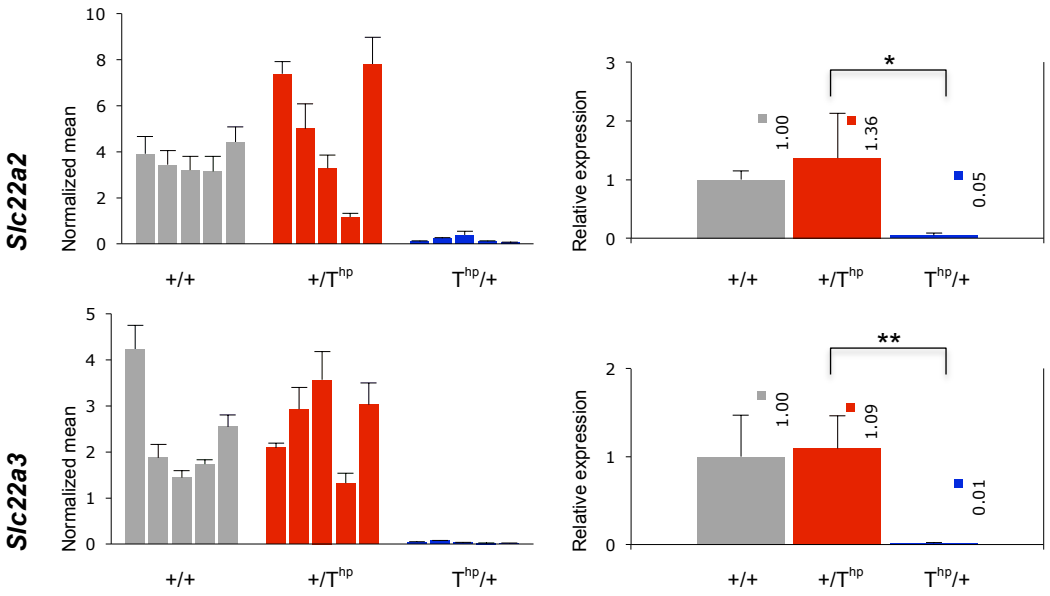


Figure 13 Pooled samples confirm allele specific expression of known imprinted genes within the T^{hp} deletion in 12.5dpc embryo and VE. Control genes show expected allele specific expression in VE (A) and embryo (B). Tissues have the T^{hp}-deletion either on the paternal allele (+/T^{hp}) or on the maternal allele (T^{hp}/+) or no deletion (+/+). The pooled samples consist of three +/+, three +/T^{hp} and two T^{hp}/+, collected from different litters. The grey bar shows expression from both alleles, the red bar shows expression only from the maternal allele and the blue bar shows expression only from the paternal allele. Maternal and paternal expression is shown relative to +/+ expression, which is set to one. All the results are normalized to the housekeeper gene *Cyclophilin A*.

As a biological replicate for the pooled results, I tested the same genes again with individual samples. Figure 14 shows gene expression from five individuals for the genotypes +/+, +/T^{hp} and T^{hp}/+, collected from different litters. All the control genes show the expected allele specific expression pattern with individual samples and confirm therefore the results from the pooled samples, in visceral endoderm (Figure 14 A) and in embryo (Figure 14 B).

A Individual samples show expected allele specific expression in visceral endoderm





B Individual samples show expected allele specific expression in embryo

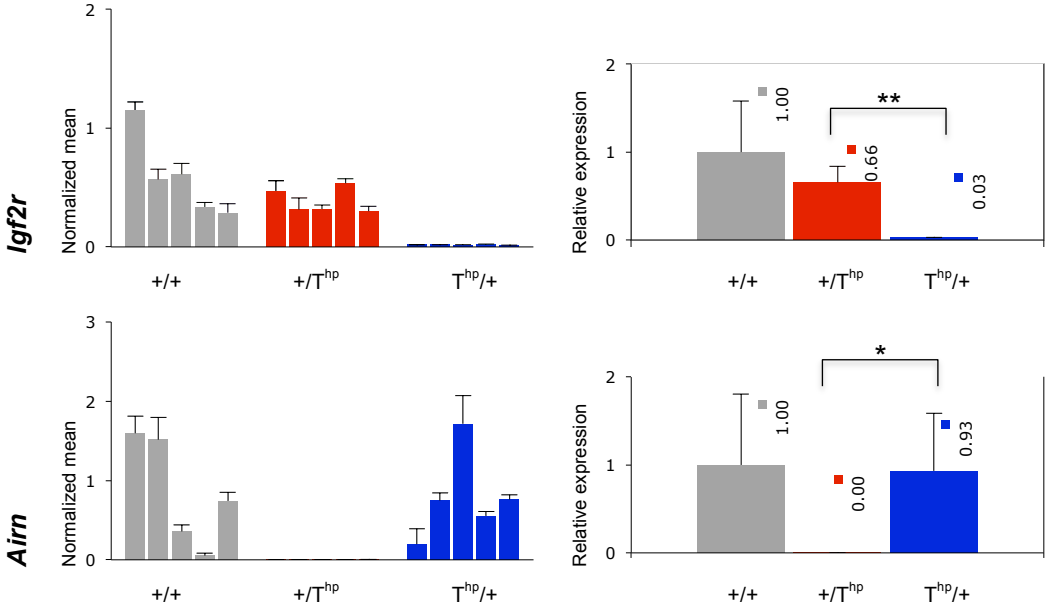


Figure 14 Individual samples confirm allele specific expression in 12.5 dpc embryo and VE. Control genes show expected allele specific expression in VE (A) and embryo (B). Tissues have the T^{hp}-deletion either in the paternal allele (+/T^{hp}) or in the maternal allele (T^{hp}/+) or no deletion (+/+). The grey bar shows expression from both alleles, the red bar shows expression only from the maternal allele and the blue bar shows expression only from the paternal allele. All the results are normalized to the housekeeper gene *Cyclophilin A*. On the left side are the normalized results for each individual for a specific genotype. The error bar shows the standard deviation of the technical variation. The mean of the individual results, for each genotype, is shown on the right side. Maternal and paternal expression are shown relative to +/+ expression, which is set to one. In this case the error bar shows standard deviation of the biological variation. The heteroscedastic T-test shows that the expression difference between maternal and paternal allele is statistically significant (*P=0.01-0.05, **P=0.001-0.01, ***P<0.001).

In figure 14, the graphs on the left side show the normalized results for each individual for a specific genotype. The error bar in these graphs shows the technical variation. The mean for each genotype is shown on the right. In this case the error bar shows the biological variation, by calculating the standard deviation from normalized individuals for each genotype. The variation in expression level between individuals for a specific genotype is quite high, but the mean of the individuals gives the expected result. The heteroscedastic T-test shows that the expression difference between the maternal and paternal allele is statistically significant.

Figure 15 shows the relative expression levels between VE and embryo for *Igf2r* and *Airn* from pooled and individual +/+ samples (Figure 15A) and for *Slc22a2* and *Slc22a3* only from pooled +/+ samples (Figure 15B). Interestingly *Igf2r* shows in both tissues approximately the same expression levels whereas the macro lncRNA *Airn*, which is responsible for silencing *Igf2r* on the paternal allele, shows only 6% expression in pooled and 21% expression in individual VE samples and yet is still able to silence. *Slc22a2* and *Slc22a3* do not show imprinted expression in embryo (Zwart et al., 2001), and at 12.5dpc *Slc22a2* is not expressed in the embryo, while *Slc22a3* shows a low level of expression relative to VE (Figure 15B).

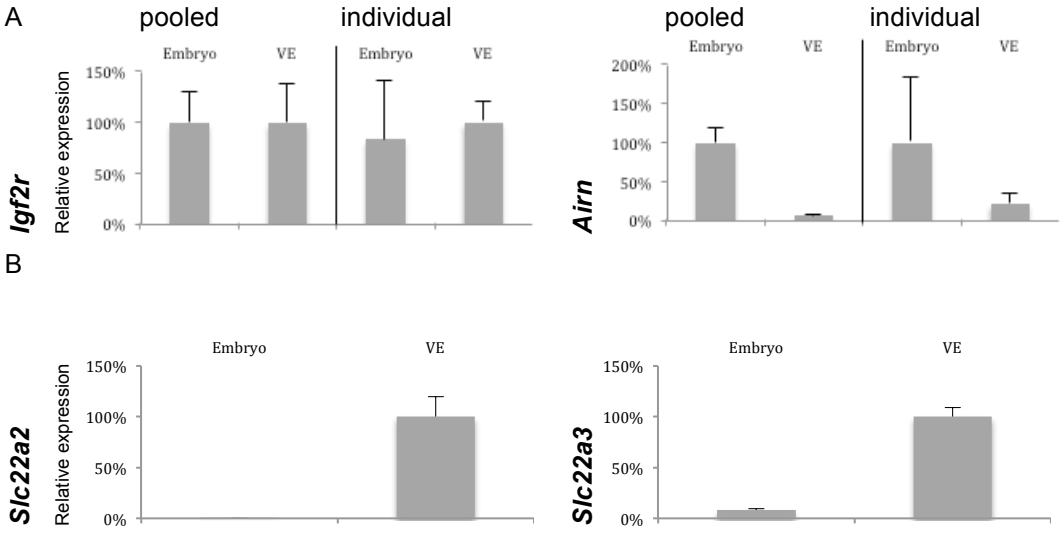


Figure 15 The relative expression level between embryo and VE of *Igf2r* and *Airn* (A), from pooled and individual +/+ samples and of *Slc22a2* and *Slc22a3* (B) from pooled +/+ samples. The error bar from the pooled samples shows the technical variation whereas the error bar from the individual samples shows the biological variation.

Additionally I tested the VE samples which I isolated from the +/T^{hp} x +/*AirnT* cross. Figure 16 shows gene expression from four individuals for each of the genotypes +/+, T^{hp}/+ and T^{hp}/*AirnT*, collected from different litters. The maternally expressed gene *Slc22a2* shows significant upregulation on the paternal allele to wildtype levels in T^{hp}/*AirnT* samples, confirming that the macro lncRNA *Airn* is responsible for silencing on the paternal allele. For *Airn* it was demonstrated again, that the expression occurs only from the paternal allele. In the T^{hp}/*AirnT* samples, *Airn* expression is greatly reduced due to truncation before the qPCR assay. The residual *Airn* detected maybe due to inefficient truncation in a minority of cases.

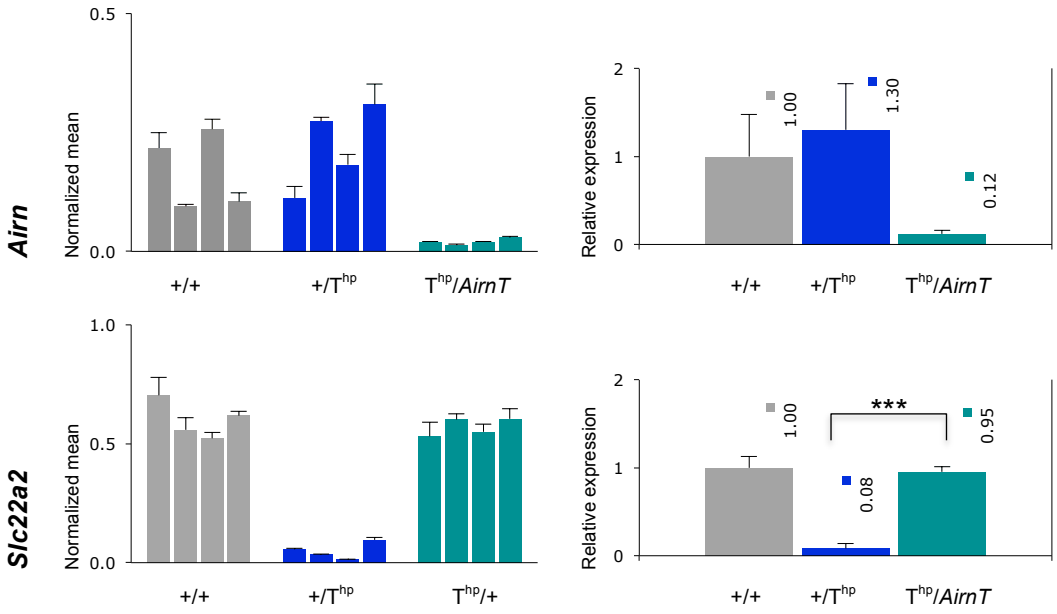


Figure 16 Regulation of *Slc22a2* by *Airn* confirmed in 12.5dpc VE. qPCR assay show that *Airn* is successfully truncated in VE, and that truncation leads to a significant upregulation of *Slc22a2* on the paternal allele in T^{hp}/*AirnT* compared to T^{hp}/+ samples (heteroscedastic T-test, *P=0.01-0.05, **P=0.001-0.01, ***P<0.001). All the samples are isolated from the +/T^{hp} x +/*AirnT* cross collected from different litters. Therefore the genotype from the individuals can be +/+, T^{hp}/+ and T^{hp}/*AirnT*. The grey bar shows expression from both alleles, the blue bar shows expression only from the paternal allele and the turquoise bar shows expression only from the paternal allele but with a truncated version of *Airn*. All the results are normalized to the housekeeper gene *Cyclophilin A*. On the left side are the normalized results for each individual for a specific genotype. The error bar in this table shows the technical variation. The average from the individuals results, for a specific genotype, is shown on the right side. Maternal and paternal expression are shown relative to +/+ expression, which is set to one with the error bar showing the standard deviation of the biological variation.

5.2 Genes showing biased allele specific-expression in VE

In the previous section I showed imprinted expression of known imprinted genes within the T^{hp}-region and their regulation by *Airn*, confirming the validity of my assay and the quality of the samples. I next systematically surveyed 10 genes within the T^{hp}-region that are expressed in VE for parental-allele specific expression and regulation by *Airn* in VE and embryo. In this section I describe the three biased genes that I detected in VE. I call a gene biased expressed, if the expression difference between maternal and paternal allele is significant and if the gene is regulated by the macro lncRNA *Airn*.

Acat3 (Acetyl-Coenzyme A acetyltransferase 3) shows the strongest parental specific bias in VE (Figure 17). Figure 17A demonstrates that *Acat3* is 36% less expressed in VE compared to embryo. *Acat3* shows maternal biased expression in pooled and individual VE samples (Figure 17B) whereas embryo pooled and individual samples show bialleic expression (Figure 17C). Additionally I could show that *Acat3* is significantly upregulated on the paternal allele from 15% to 57% in T^{hp}/*AirnT* samples, demonstrating that the macro lncRNA *Airn* is responsible for silencing on the paternal allele (Figure 17D).

The primer design for *Acat3* was complicated, because the sequence of the transcript shows 95% similarity to the upstream gene *Acat2* and the 3' UTR of *Acat3* overlaps with the 3' UTR of *Tcp1*. I was able to find a specific primer set which spans over an exon-exon junction. The specificity of the primers was confirmed by sequencing the qPCR product.

Acat3

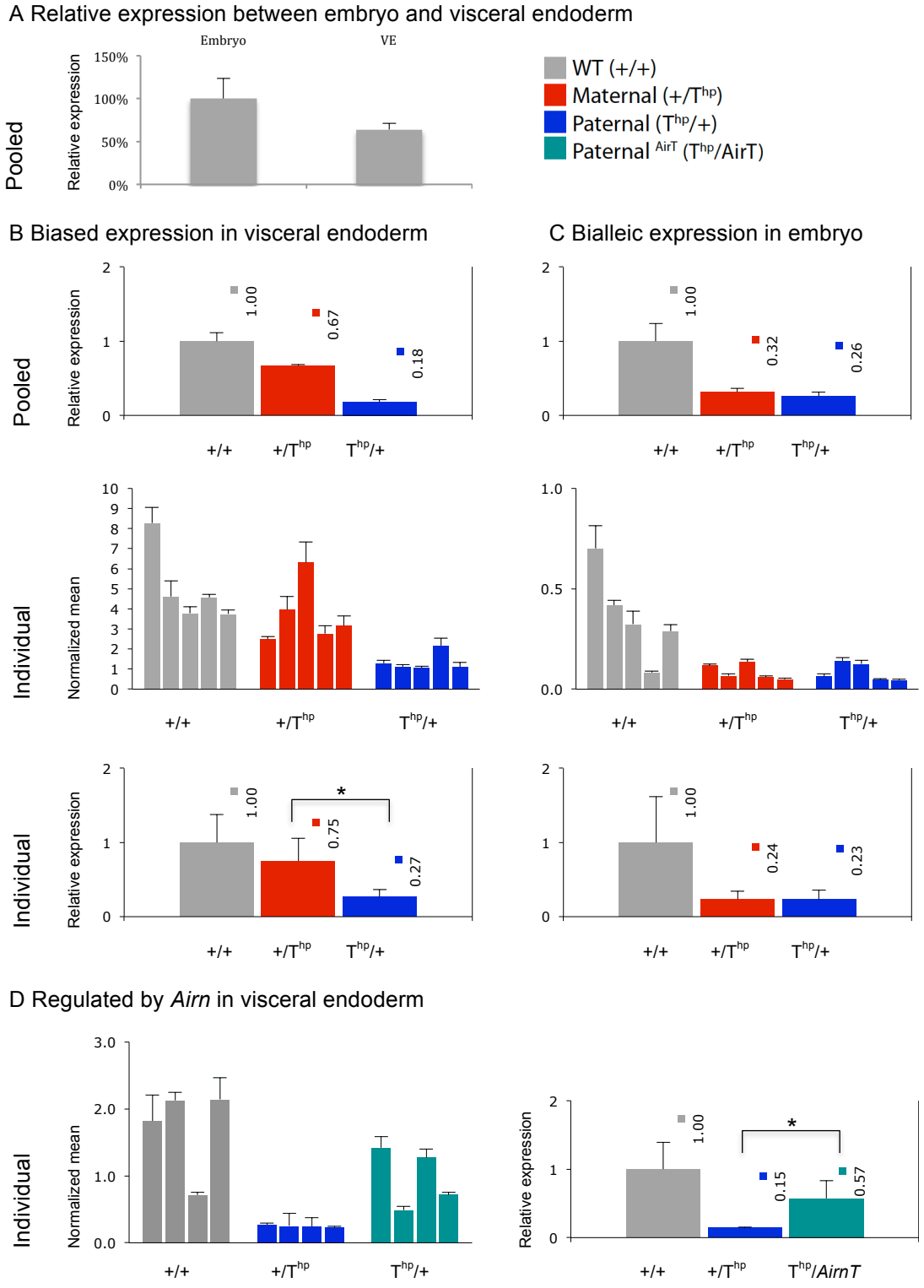
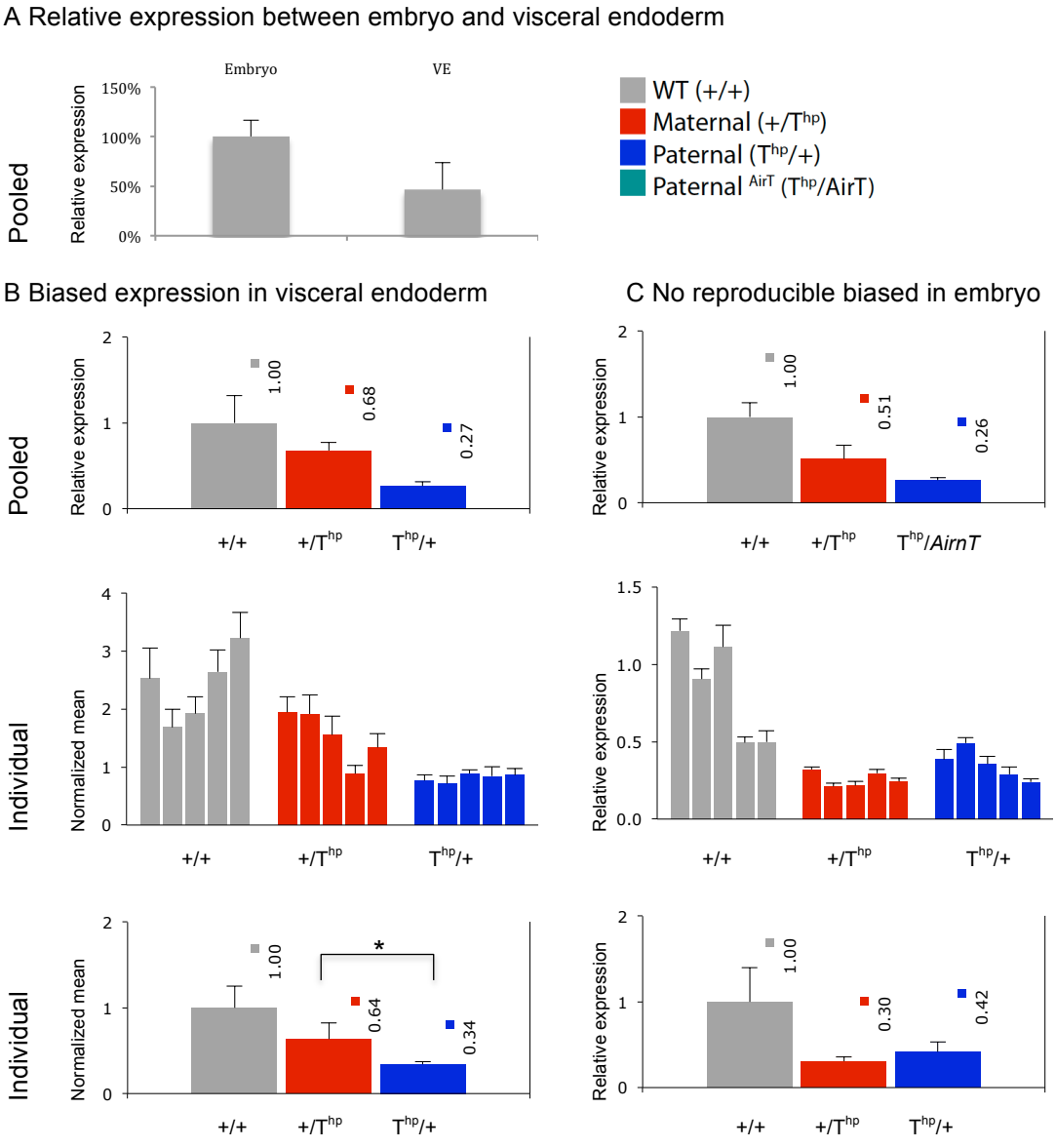


Figure 17 *Acat3* shows maternal biased expression and is regulated by *Airn* in 12.5dpc VE. (A) The relative expression between VE and embryo from pooled +/+ samples. *Acat3* shows biased expression in VE (B) and biallelic expression in embryo (C). (D) *Acat3* is significantly upregulated on the paternal allele in T^{hp}/*AirnT* samples. Details as in figure 13, 14, 15 and 16.

Sod2 (superoxide dismutase 2) shows parental specific bias in VE (Figure 18). Figure 18A demonstrates that *Sod2* is 54% less expressed in VE compared to embryo. *Sod2* shows maternal biased expression in pooled and individual VE samples (Figure 18B). In embryo, I could not reproduce the biased result that I found in pooled samples with the individual samples which showed biallelic expression (Figure 18C). In VE I could show that *Sod2* is significantly upregulated on the paternal allele from 36% to 59% in T^{hp}/*AirnT* samples compared to T^{hp}/+ samples, demonstrating that the macro lncRNA *Airn* is responsible for silencing on the paternal allele (Figure 18D).

Sod2



D Regulated by *Airn* in visceral endoderm

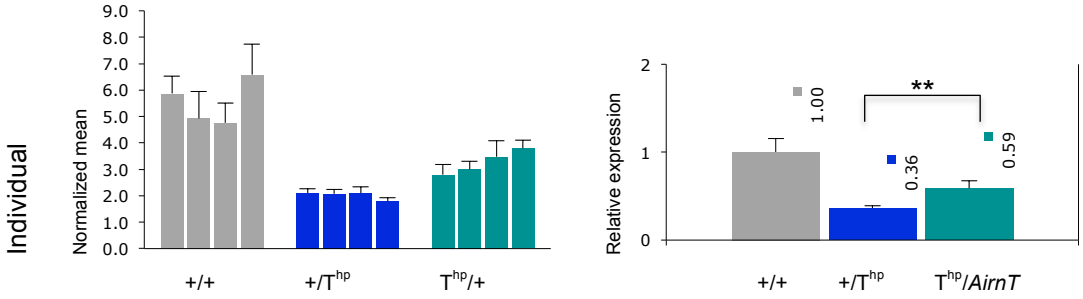
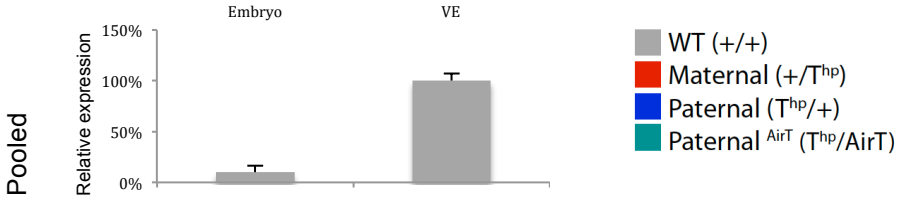


Figure 18 *Sod2* shows maternal biased expression and is regulated by *Airn* in 12.5dpc VE. (A) The relative expression between VE and embryo from pooled +/+ samples. *Sod2* shows biased expression in VE (B). (C) No reproducible biased in embryo samples, because the individual samples show biallelic expression. (D) The heteroscedastic T-test shows that *Sod2* is significantly upregulated on the paternal allele in T^{hp}/*AirnT* samples. Details as in figure 13, 14, 15 and 16.

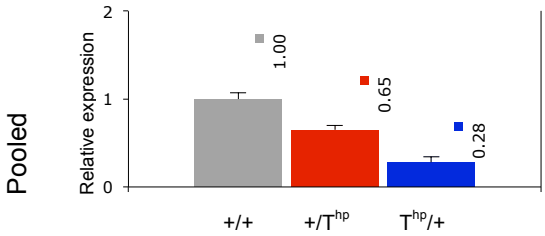
Plg (Plasminogen) shows parental specific bias in VE (Figure 19). In embryo only background expression was detected (Figure 19A). *Plg* shows maternal biased expression in pooled and individual VE samples (Figure 19B). In VE I could show that *Plg* is significantly upregulated on the paternal allele from 33% to 62% in T^{hp}/*AirnT* compared to T^{hp}/+ samples, demonstrating that the macro lncRNA *Airn* is responsible for silencing on the paternal allele (Figure 19C).

Plg

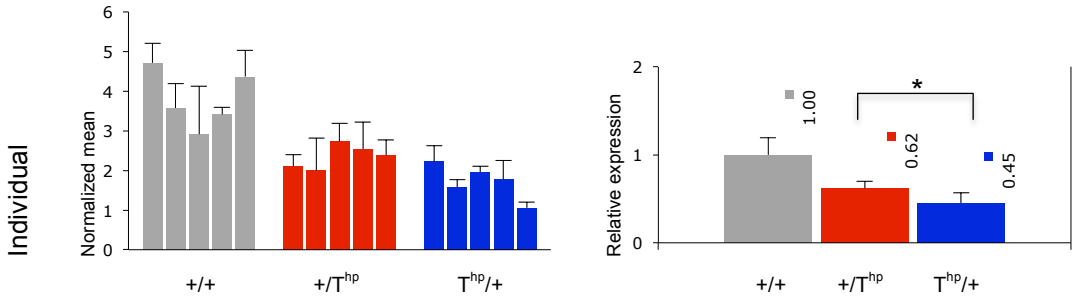
A Relative expression between embryo and visceral endoderm



B Biased expression in visceral endoderm



(Background expression in embryo)



C Regulated by *Airn* in visceral endoderm

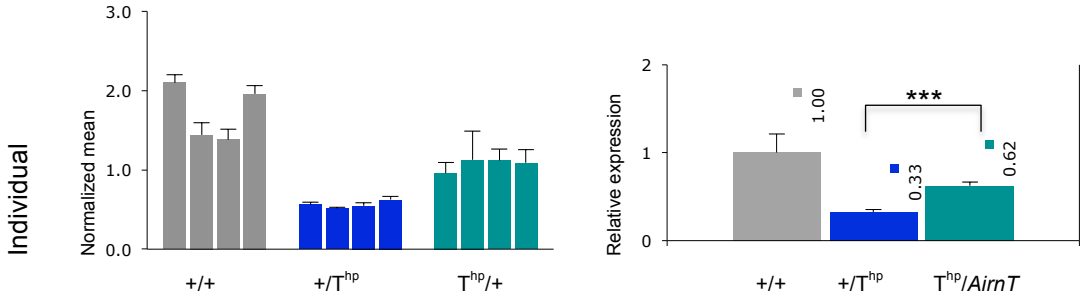


Figure 19 *Plg* shows maternal biased expression and regulated by *Airn* in VE. (A) The relative expression between VE and embryo from pooled +/+ samples. In embryo only background expression was detected (B) *Plg* shows biased expression in VE. (C) The heteroscedastic T-test shows that *Plg* is significantly upregulated on the paternal allele in T^{hp}/*AirnT* samples. Details as in figure 13, 14, 15 and 16.

5.3 Genes showing biallelic expression

All the other expressed genes in VE within the T^{hp}-deletion show biallelic expression and are not regulated by *Airn*. These genes show no significant difference between maternal and paternal expression levels and are also not upregulated on the paternal allele in T^{hp}/*AirnT* samples.

Map3k4 (Mitogen-activated protein 3 kinase 4) shows biallelic expression in embryo and VE (Figure 20). Figure 20A demonstrates that *Map3k4* is 42% less expressed in VE compared to embryo. I found biallelic expression in pooled and in individual VE samples (Figure 20B). In embryo, I found biallelic expression in pooled samples (Figure 20C). *Map3k4* shows also no significant upregulation on the paternal allele in T^{hp}/*AirnT* compared to T^{hp}/+ samples in VE (Figure 20D).

Map3k4

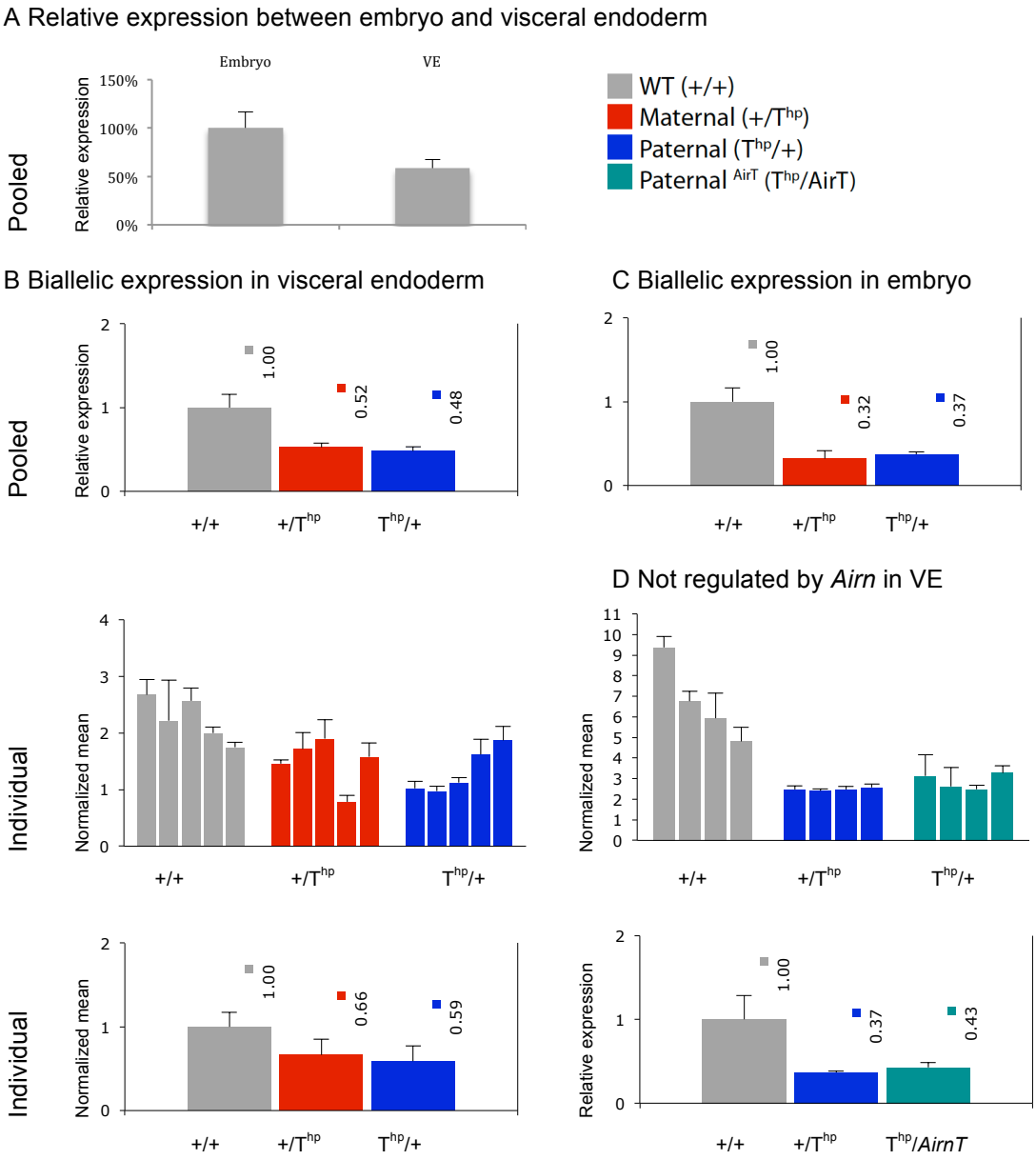


Figure 20 *Map3k4* shows biallelic expression and is not regulated by *Airn* in VE and embryo. (A) The relative expression between VE and embryo from pooled +/+ samples. *Map3k4* shows biallelic expression in pooled and individuals samples in VE (B) and embryo samples (C). (D) *Map3k4* is not regulated by *Airn*. Details as in figure 13, 14, 15 and 16.

Mrpl18 (Mitochondrial ribosomal protein L18) shows biallelic expression in VE and in embryo (Figure 21). Figure 21A demonstrates that *Mrpl18* is 44% less expressed in VE compared to embryo. I found biallelic expression in pooled and in individual VE samples (Figure 21B). In embryo, I found biallelic expression in pooled samples (Figure 21C). *Mrpl18* shows also no significant upregulation on the paternal allele in T^{hp}/*AirnT* compared to T^{hp}/+ samples in VE (Figure 21D).

Mrpl18

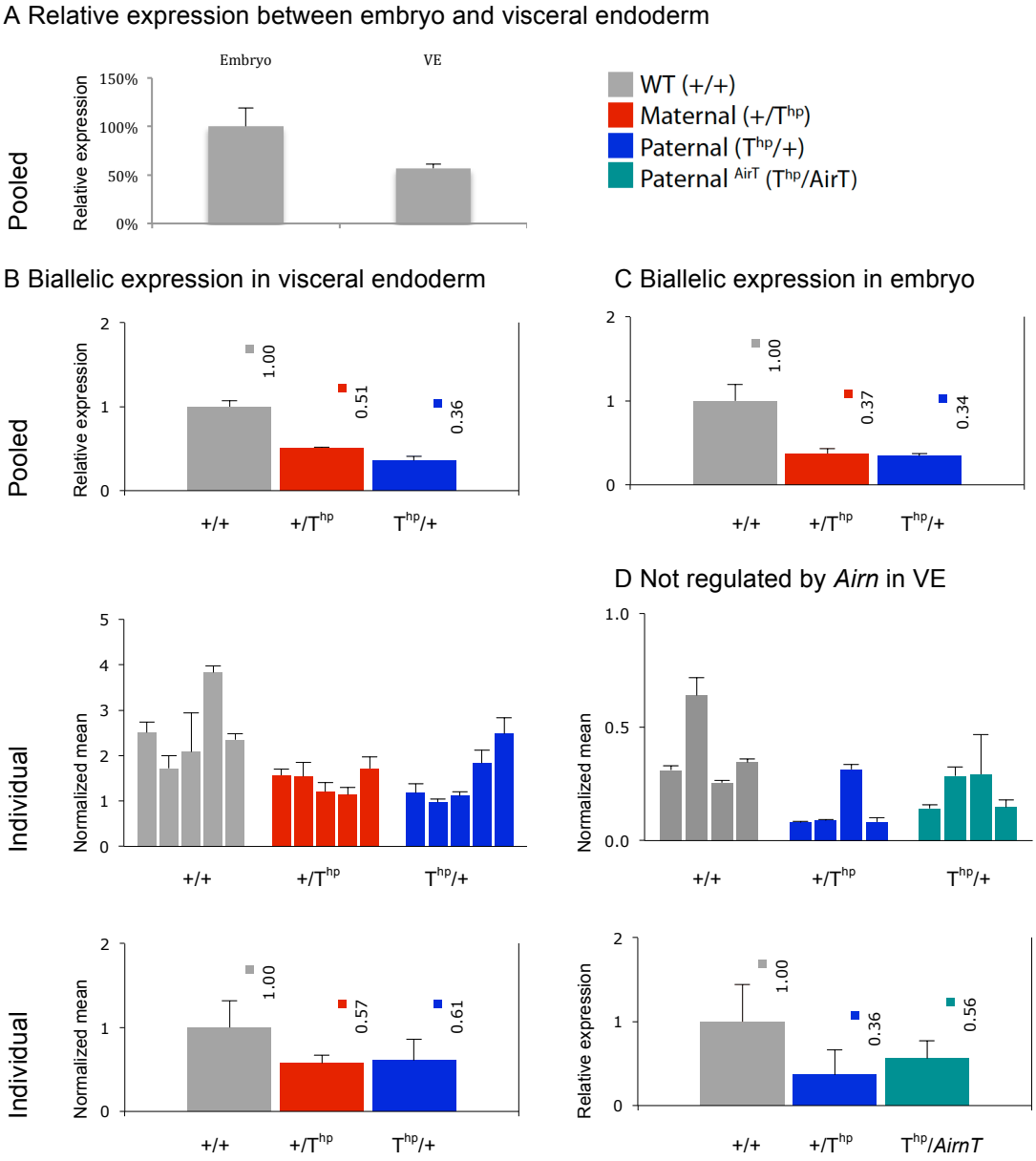


Figure 21 *Mrpl18* shows biallelic expression and is not regulated by *Airn* in VE & embryo. (A) The relative expression between VE and embryo from pooled +/+ samples. *Mrpl18* shows biallelic expression in pooled and individuals samples in VE (B) and embryo samples (C). (D) *Mrpl18* is not regulated by *Airn*. Details as in figure 13, 14, 15 and 16.

Tcp1 (T-complex 1) shows biallelic expression in embryo and in VE (Figure 22). Figure 22A shows that *Tcp1* is 57% less expressed in VE compared to embryo. I found biallelic expression in pooled VE samples (Figure 22B) and embryo (Figure 22C) samples. Additionally I could demonstrate that there is no significant upregulation on the paternal allele in T^{hp}/*AirnT* samples compared to T^{hp}/+ samples in VE (Figure 22D).

Tcp1

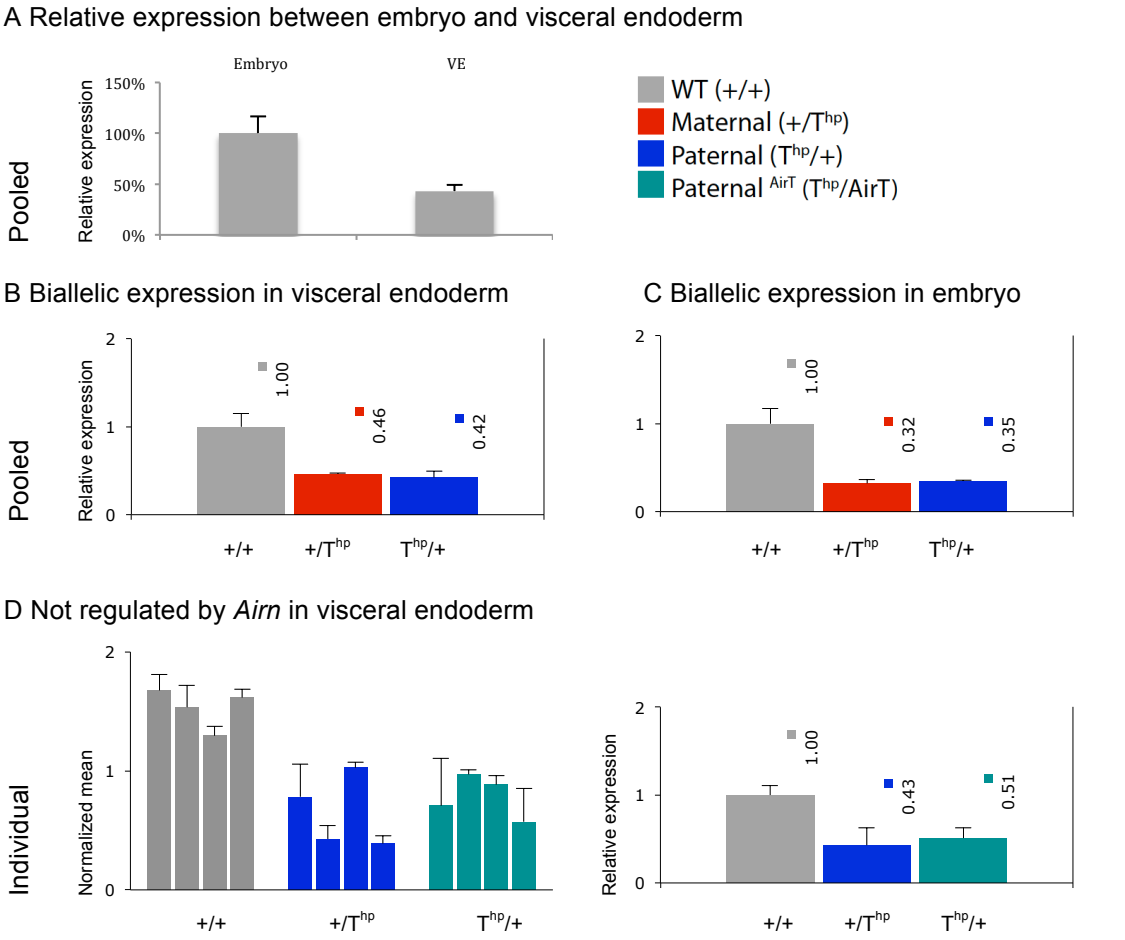
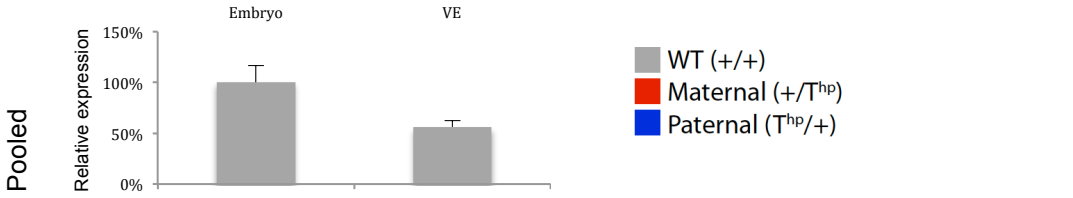


Figure 22 *Tcp1* shows biallelic expression and is not regulated by *Airn* in VE & embryo. (A) The relative expression between VE and embryo from pooled +/+ samples. *Tcp1* shows biallelic expression in pooled VE (B) and pooled embryo samples (C). (D) *Tcp1* is not regulated by *Airn*. Details as in figure 13, 14, 15 and 16.

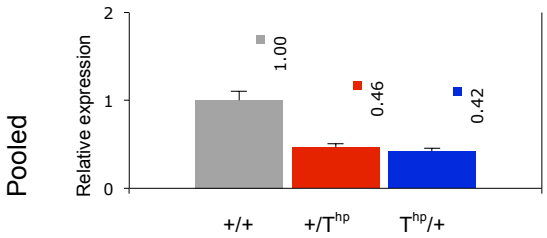
Wtap (Wilms' tumour 1-associating protein) shows biallelic expression in embryo and in VE (Figure 23). Figure 23A demonstrates that *Wtap* is 44% less expressed in VE compared to embryo. I found biallelic expression in pooled VE (Figure 23B) and embryo (Figure 23C) samples. For *Wtap*, I designed primers, which detect all three RefSeq annotated spliced variants.

Wtap

A Relative expression between embryo and visceral endoderm



B Biallelic expression in visceral endoderm



C Biallelic expression in embryo

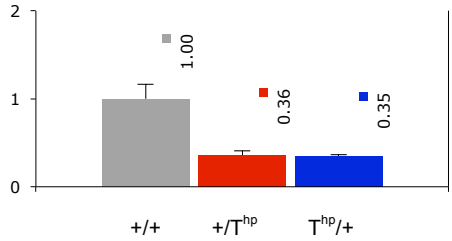
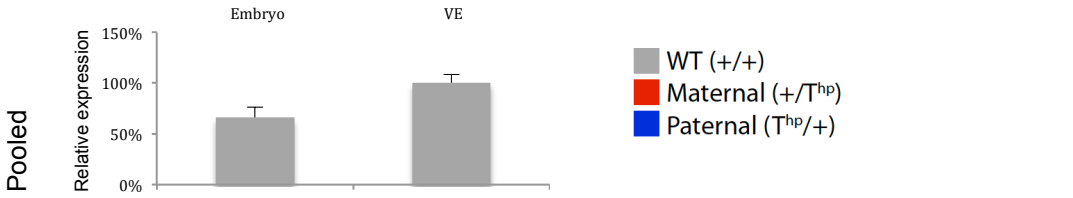


Figure 23 *Wtap* shows biallelic expression in visceral endoderm and embryo. (A) The relative expression between VE and embryo from pooled +/+ samples. *Wtap* shows biallelic expression in pooled VE (B) and embryo samples (C). Details as in figure 13, 14, 15 and 16.

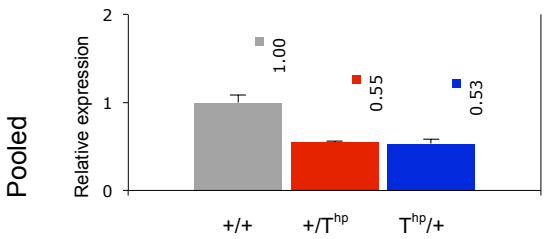
Agpat4 (1-acylglycerol-3-phosphate O-acyltransferase 4) shows biallelic expression in embryo and in VE (Figure 24). Figure 24A demonstrates that *Agpat4* is 44% less expressed in embryo compared to VE. I found biallelic expression in pooled VE (Figure 24B) and embryo (Figure 24C) samples.

Agpat4

A Relative expression between embryo and visceral endoderm



B Biallelic expression in visceral endoderm



C Biallelic expression in embryo

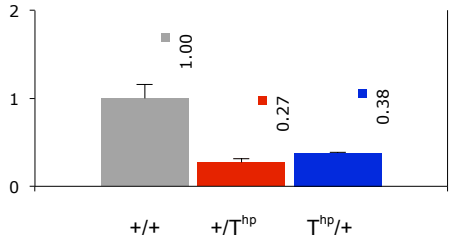


Figure 24 *Agpat4* shows biallelic expression in visceral endoderm and embryo. (A) The relative expression between VE and embryo from pooled +/+ samples. *Agpat4* shows biallelic expression in pooled VE (B) and pooled embryo samples (C). Details as in figure 13, 14, 15 and 16.

Acat2 (Acetyl-Coenzyme A acetyltransferase 2) shows biallelic expression in embryo and in VE (Figure 25). Figure 25A demonstrates that *Acat2* is 34% less expressed in embryo compared to VE. I found biallelic expression in pooled VE (Figure 25B) and embryo (Figure 25C) samples.

Acat2

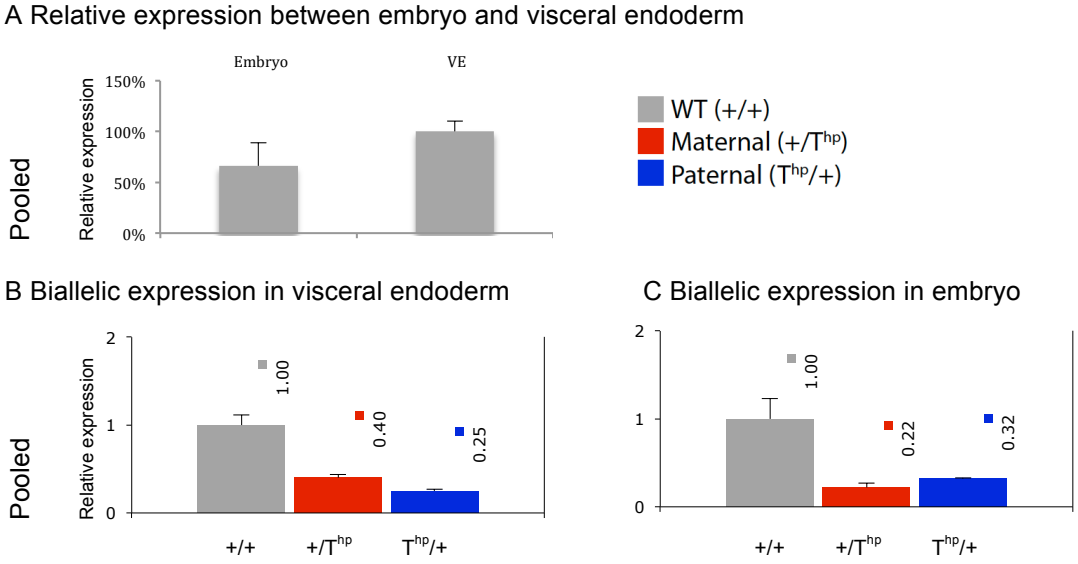
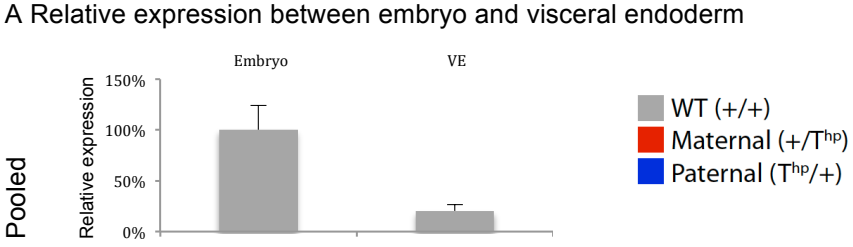


Figure 25 *Acat2* shows biallelic expression in visceral endoderm and embryo. (A) The relative expression between VE and embryo from pooled +/+ samples. *Acat2* shows biallelic expression in pooled VE (B) and embryo samples (C). Details as in figure 13, 14 15 and 16.

Qk (Quaking) shows biallelic expression in visceral endoderm and embryo (Figure 26). Figure 26A demonstrates that *Qk* is 80% less expressed in VE compared to embryo. *Qk* shows biased expression in pooled VE (Figure 26B) samples. I could not reproduce this result with individual samples (Figure 26 D), in which the maternal and paternal alleles show WT expression. In pooled embryo samples *Qk* shows biallelic (Figure 26C) expression.

Qk



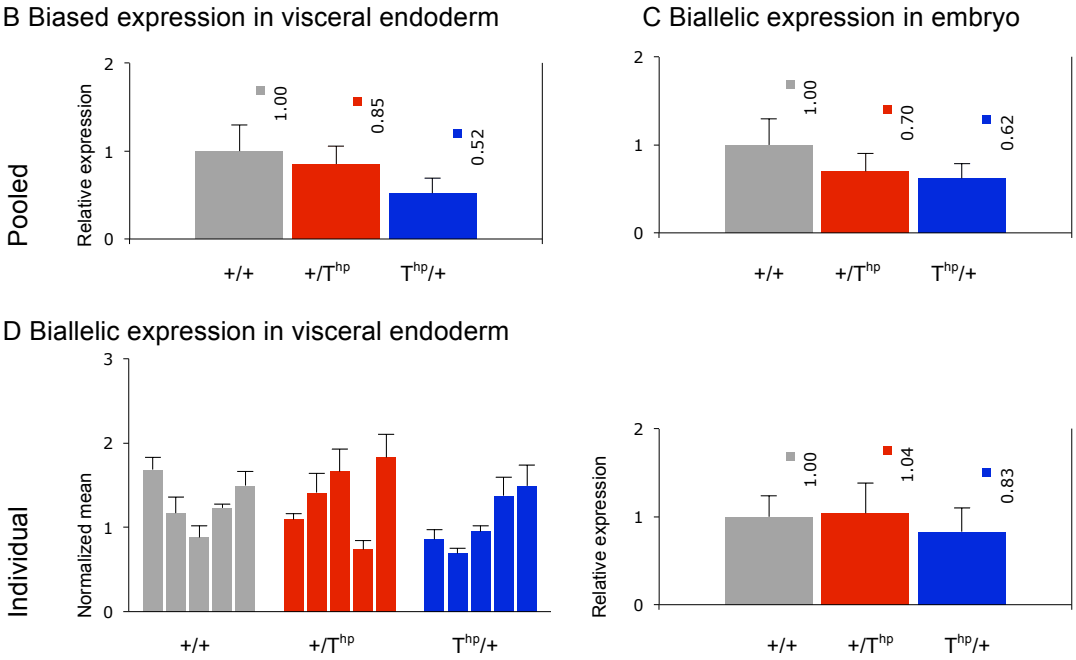


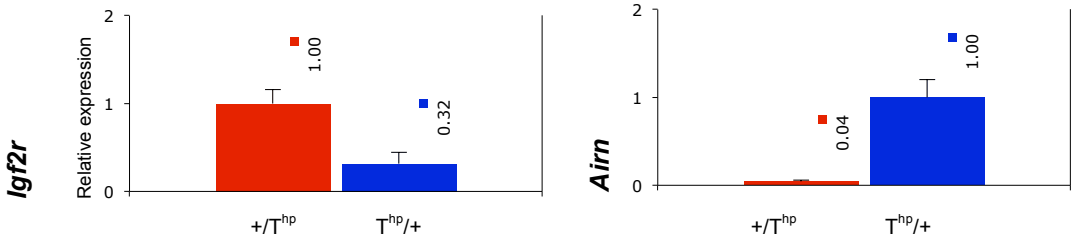
Figure 26 *Qk* shows biallelic expression in visceral endoderm and embryo. (A) The relative expression between VE and embryo from pooled +/+ samples. *Qk* shows biased expression in pooled VE (B) samples and biallelic expression in pooled embryo (C) samples. (D) In individual VE samples the maternal and paternal allele show WT expression. Details as in figure 13, 14 15 and 16.

From the 10 uncharacterised expressed genes within the T^{hp}-deletion, I found that *Acat3*, *Sod2* and *Plg* show significant higher expression levels from the maternal allele compared to paternal in VE. However embryo samples show biallelic expression. Additionally I could also show that the maternal bias of these three genes is regulated by the macro lncRNA *Airn* in VE. The other 7 expressed genes in VE, within the T^{hp}-deletion show biallelic expression. The discovery of novel imprinted genes extends the *Igf2r* cluster from 440kb to 640kb in visceral endoderm compared to 177kb in embryo.

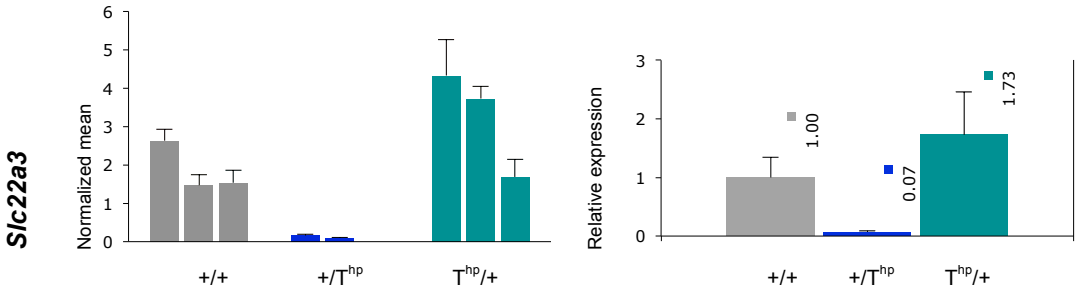
5.4 *Pde10a* is maternally imprinted in placenta and regulated by *Airn*

During my diploma, 5 novel imprinted expressed genes were described in placenta 17.5dpc, using an RNA sequencing approach to detect imprinted expression by comparing SNPs between two mouse strains (Wang et al., 2011). *Pde10a* (phosphodiesterase 10A) was one of those genes, which shows maternally imprinted expression in placenta. This gene is within the T^{hp}-region and 4Mb from *Airn*, but is not expressed in VE according to RNA-sequencing data from our lab (Kulinski et al unpublished) or in embryo according to my qPCR data (data not shown). However, I was interested to confirm that *Pde10a* shows imprinted expression in placenta, and test if imprinted silencing is regulated by *Airn*. To answer this question, we collected 12.5dpc placenta samples from different litters. There are again two categories of samples: pooled samples, which contain eight +/T^{hp} and six T^{hp}/+ samples collected from reciprocal +/T^{hp} crossings, and individual samples collected from the T^{hp}/*AirnT* crossing. To test these samples I confirmed imprinted expression (Figure 27A) and regulation by *Airn* for known imprinted genes in placenta (Figure 27B).

A Pooled samples show expected allele specific expression in placenta



B Individual samples confirm regulation by *Airn* in placenta



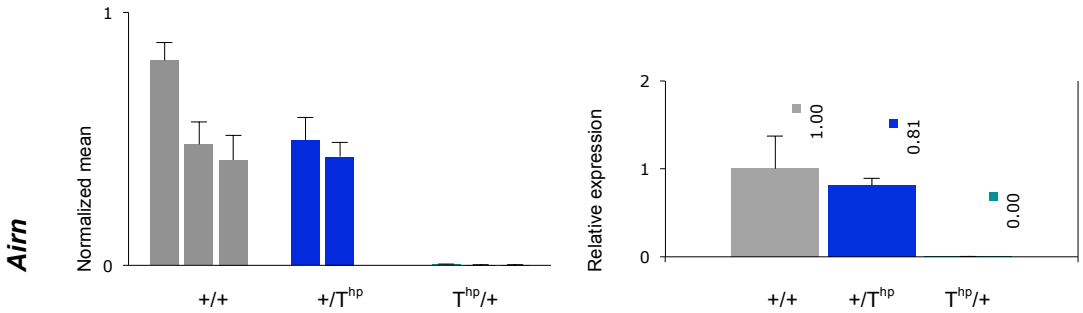
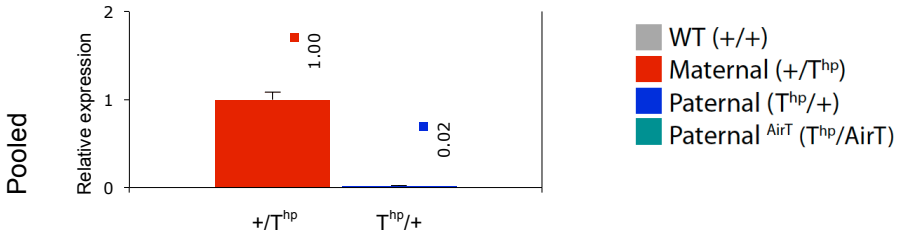


Figure 27 Pooled samples confirm allele specific expression of known imprinted genes within the T^{hp} deletion in 12.5dpc placenta. (A) Control genes show expected allele specific expression in placenta. The pooled samples consist of eight $+/T^{hp}$ and six $T^{hp}/+$, collected from different litters. (B) Regulation of *Slc22a3* by *Airn* was confirmed in 12.5dpc individual placenta samples. Details as in figure 13, 14, 15 and 16.

With pooled placenta samples I could confirm that *Pde10a* shows maternal imprinted expression (Figure. 28A). With individual samples carrying the truncated Airn on the paternal allele, I could show that *Pde10a* is upregulated to WT levels, demonstrating that the macro lncRNA *Airn* is responsible for silencing on the paternal allele (Figure 28B).

Pde10a

A *Pde10a* shows maternally imprinted expression in placenta



B Individual samples confirm regulation by *Airn* in placenta

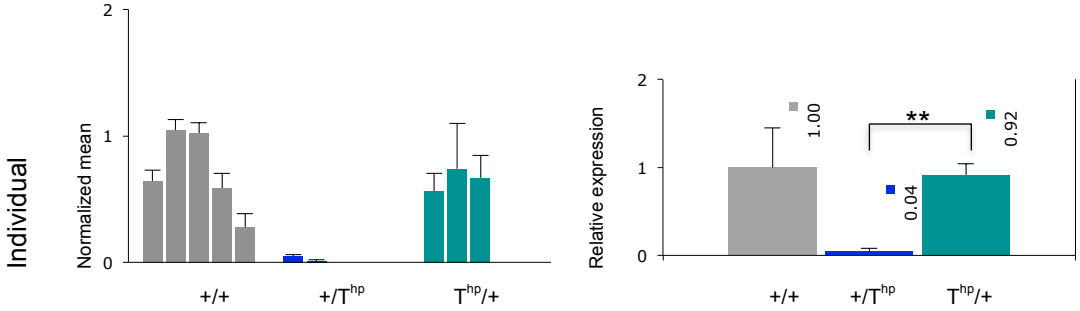


Figure 28 *Pde10a* shows maternally imprinted expression and is regulated by *Airn* in 12.5dpc placenta. (A) *Pde10a* shows maternal imprinted expression in pooled samples. (B) *Pde10a* is significantly upregulated on the paternal allele in T^{hp}/*AirnT* samples. Details as in figure 13, 14, 15 and 16.

In the introduction chapter I discussed the negative aspects for using placenta as a model to study imprinted gene expression. The placenta can be contaminated with neighbouring tissues, like decidua, endometrium, and parietal yolk sac (PYS). In figure 29 I compared the expression levels of *Pde10a* in placenta between all the neighbouring tissues. The result shows that *Pde10a* is expressed in all tested tissues. Tissue contamination from endometrium and PYS can occur if the placenta is not carefully dissected away from neighbouring tissues, while contamination from decidua can only be minimized and not eliminated by careful dissection. Therefore, contamination from neighbouring tissues may introduce a bias to the results.

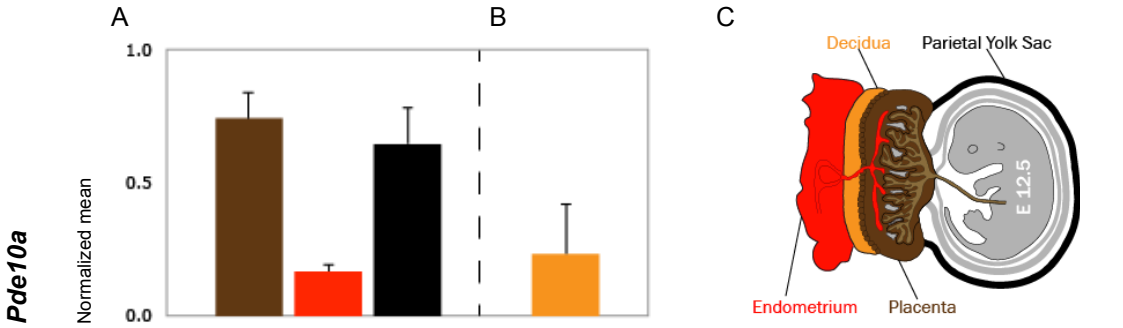


Figure 29 *Pde10a* is expressed in all experimentally tested neighbouring tissues. (A) Shows the expression of *Pde10a* in placenta (brown), endometrium (red), parietal yolk sac (black). The error bar shows the technical variation. (B) *Pde10a* expression in decidua from two biological replicates of two litters. The error bar shows minimum and maximum. (C) Shows an overview of the samples, which were tested.

My results indicate that *Pde10a* shows imprinted expression in placenta, and silencing is regulated by *Airn*, even though it is 4Mb away. Given the low number of individuals used in my experiments and the danger of contamination from neighbouring tissues, this preliminary result must be confirmed with further experiments.

IV. DISCUSSION

Imprinted genes occur in clusters and are under the control of an ICE (Barlow, 2011). In general, 'inner' genes within the cluster, which are closer to the ICE, show multi-lineage (ML) imprinted expression in the embryo and extra-embryonic tissues. However 'outer' genes, which are further away from the ICE show only extra-embryonic-lineage (EXEL) specific imprinted expression (Hudson et al., 2011). For the imprinted *Igf2r*, *Igf2*, *Kcnq1* and *Peg10* clusters, EXEL specific imprinted expression is limited to 'outer' genes in the cluster (Hudson et al., 2010), indicating an extension of imprinted silencing in extra-embryonic tissues. In this thesis I defined the limits of imprinted silencing at the *Igf2r* cluster, using 12.5 dpc VE as an extra-embryonic model tissue.

I found that the genes *Plg*, *Acat3* and *Sod2* show maternal biased expression in VE, defined by a significant expression difference between the maternal and the paternal allele and regulation by the macro lncRNA *Airn*. The discovery of those novel biased genes extends the *Igf2r* cluster to 640kb in VE compared to 177kb in embryo (Figure 30). Additionally, in preliminary experiments I could show that the maternally expressed gene *Pde10a* (Wang et al., 2011), is regulated by the macro lncRNA *Airn* in placenta, even though it is 4Mb upstream from the *Airn* promoter (Figure30).

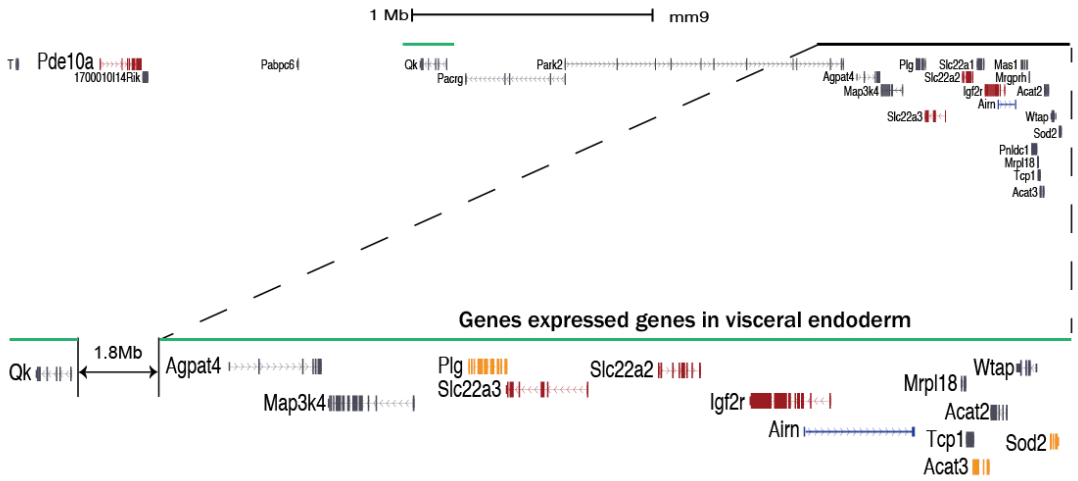


Figure 30 Summary of the results. Genes in red are the known maternally expressed genes in VE. The paternally expressed macro lncRNA *Airn* is shown in blue. All the genes, covered with a green bar are expressed in VE. Genes in orange are the novel maternally biased expressed genes in VE. *Pde10a* shows maternally imprinted expression and preliminary results indicate that it is regulated by *Airn* in placenta.

Martha Koerner showed in her Phd thesis, that a 3Mb chromosomal region in the T^{hp}-deletion shows DNA FISH asynchrony in MEFs (Koerner, 2010), an unexplained feature of imprinted clusters (Simon et al., 1999). The three maternally biased genes are within this region but *Pde10a* is not. However the 3Mb chromosomal region was found in MEFs, which represent embryonic tissues, and it is possible that the DNA FISH asynchrony region may be extended further in extra-embryonic tissues. This could be investigated in further studies to determine the extent of DNA FISH asynchrony in extra-embryonic tissues.

A number of imprinted gene knockout mice (KO) have no obvious imprinted phenotype, that is defined as a parental-allele specific effect in heterozygous mice. This indicates that their imprinted regulation may not be relevant for their function. Those genes are classified as “innocent bystanders” (Miri and Varmuza, 2009). For example, an *Igf2r* KO leads to a lethal phenotype at 16.5dpc (Wang et al., 1994), while its overexpression leads to loss of weight (Wutz et al., 2001). However, the KO of the neighbouring genes *Slc22a2* and *Slc22a3* has no obvious imprinted phenotype (Jonker et al., 2003) although it cannot be excluded that there may be a phenotype under certain conditions, which have not been reproduced in the lab.
In placenta, *Pde10a* (phosphodiesterase 10A) shows maternal imprinted expression. However it is highly expressed by medium spiny neuron of the striatum, where it has a major role in regulation. *Pde10a* KO mice show less exploratory activity when they were placed in a novel environment compared to wildtypes (Siuciak et al., 2006). Interestingly *Pde10a* KO mice weight less compared to wildtypes, whereas the weight reduction was only significant for females and not for males, indicating a gender effect (Siuciak et al., 2006). The zymogen *Plg* (Plamin) is released from the liver into the circulation, where it plays an important role in fibrinolysis and influences inflammatory cell migration (Plow et al., 1995). *Plg* KO mice showed increased levels of erythropoietin in both male and females, while only males had anemia (Okaji et al., 2012). The mitochondrial matrix protein *Sod2* (Superoxide dismutase 2) metabolizes the superoxide anion O_2^- to hydrogen peroxide (H2O2) and is therefore important to reduce the levels of reactive oxygen species (ROS)

(Weyemi et al., 2012). *Sod2* KO mice die within the first 10 days after birth, because ROS cannot be reduced (Li et al., 1995). (Li et al., 1995). For the gene Acetyl-Coenzyme A acetyltransferase 3 (*Acat3*) was suggested to regulate liver triglyceride (Sudbery et al., 2009). Unfortunately a KO mouse for the gene *Acat3* (Acetyl-Coenzyme A acetyltransferase 3) has not yet been produced, which would be important, to exclude an imprinted phenotype. In summary, no imprinted phenotype has yet been reported for the novel biased and imprinted genes in the *Igf2r* cluster.

1. How does long range silencing by *Airn* work?

1.1 RNA mediated silencing model

If the RNA product of the macro lncRNA *Airn* is responsible for allele-specific silencing, I would expect a weaker effect with distance, because the concentration of the RNA product would be expected to be high close to the *Airn* promoter and decreases with distance. A low concentration makes it less likely that the RNA product finds its target promoter. My results in VE are consistent with this hypothesis because the three biased expressed genes are incompletely silenced by *Airn* on the paternal allele.

Another indication for a distance effect is the fact that *Slc22a3* losses maternal-specific imprinted expression at 15.5-16.5dpc, while *Slc22a2*, which is closer to the macro lncRNA *Airn*, retains imprinted expression throughout embryonic development (Hudson et al., 2011; Verhaagh et al., 1999; Wang et al., 2011). This indicates, that the three maternally biased expressed genes may have shown a greater bias in imprinted expression earlier than 12.5dpc in development. Support for the RNA mediated silencing model comes from the finding, that *Airn* associates with the *Slc22a3* promoter and with EHMT2 (Euchromatic histone-lysine N-methyltransferase 2) H3K9dimethylase in placenta. EHMT2 is required for imprinted expression of *Slc22a3* indicating that *Airn* may recruit EHMT2 to the promoter causing silencing (Nagano et al., 2008). Under the model of Nagano my findings would fit in the following way: *Airn* may bring ETMH2 to the promoter of imprinted and biased genes and induces gene silencing with different efficiency (Figure 31). For the three

maternal biased genes the binding efficiency of the *Airn*/ETMH2 complex to their promoters may be less strong than for imprinted genes or, because they are further away from *Airn*, less *Airn* may reach their promoters.

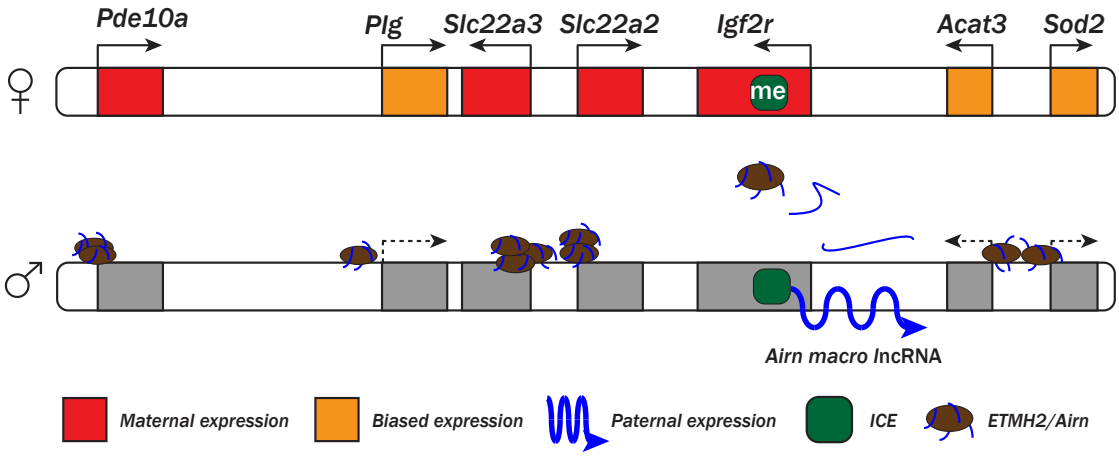


Figure 31 RNA mediated silencing for a linear distance model. Genes in red (*Slc22a3*, *Slc22a2*, *Igf2r* and *Pde10a*) show maternal expression, and are silenced from the paternally expressed macro lncRNA *Airn in cis*. Maternal biased genes (*Plg*, *Acat3* and *Sod2*) shown in orange are partially silenced by *Airn*. *Airn* causes silencing by recruiting the H3K9dimethylase EHMT2 to the promoters in cis. The ICE in green, a DMR including the *Airn* promoter, which gains a methylation mark during oogenesis, controls expression of the macro lncRNA *Airn*.

The result for *Pde10a* conflicts with a linear distance model, because *Pde10a* shows full imprinted expression throughout development, even though it is located 4Mb upstream from the *Airn* promoter, compared to *Slc22a3*, which loses imprinted expression in placenta later in development (Hudson et al., 2011; Verhaagh et al., 1999; Wang et al., 2011). The hypothesis that *Airn* has a weaker effect with distance does not fit for a linear distance model, but it may fit for a 3-dimensional distance model, where *Pde10a* may be close to the macro lncRNA *Airn*. Under this model, *Pde10a* and *Slc22a3* may be located close enough to the macro lncRNA *Airn* early in development to be silenced, but during development *Slc22a3* may increase in distance until by 15.5dpc the concentration of *Airn* product is too low to silence.

1.2 Enhancer transcriptional interference model

The correlative data from Nagano et al. could also fit with other models of gene silencing, such as the recently proposed enhancer interference model (Pauler et al., 2012) (Figure 32). Under this model the maternal allele forms active loops between enhancers within the *Airn* gene body and the promoters of imprinted and biased genes. The active loop formation induces high

expression levels on the maternal allele, whereas on the paternal allele transcription of the macro lncRNA *Airn* blocks the binding of transcriptional activators to the enhancer by transcription interference and inhibits the formation of active loops. For biased genes the interaction between their enhancer and their promoter is less strong or less frequent than for imprinted genes. The inactive promoters then attract the H3K9dimethylase EHMT2 and the polycomb complexes, which induce heterochromatin formation as a secondary step that locks in silencing.

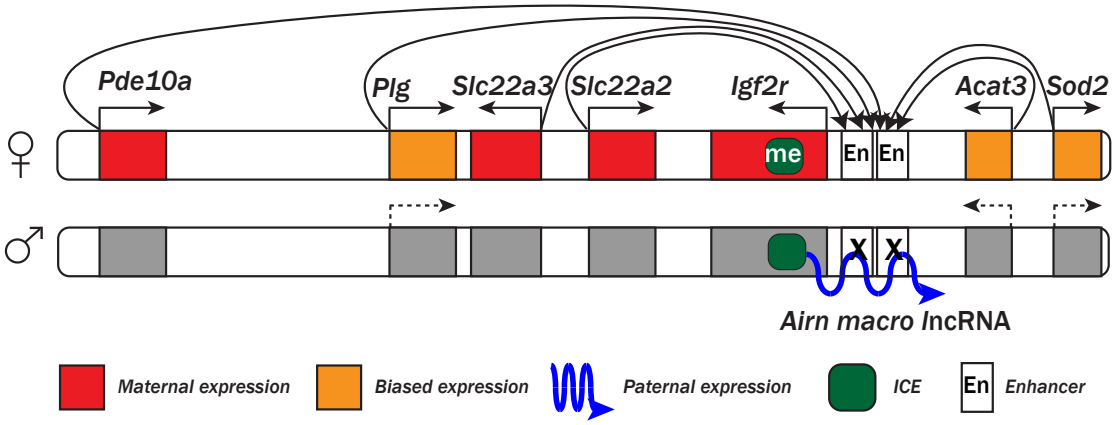


Figure 32 RNA mediated silencing for a linear distance model. Genes in red (*Slc22a3*, *Slc22a2*, *Igf2r* and *Pde10a*) show maternal expression, and are silenced on the paternal allele by the paternally expressed macro lncRNA *Airn* in cis. Maternal biased genes (*Plg*, *Acat3* and *Sod2*) shown in orange are partially silenced by *Airn*. The maternal allele forms active loops between the enhancers within the *Airn* gene body and the promoters of imprinted and biased genes. On the paternal allele the transcription of the macro lncRNA *Airn* blocks the binding of transcriptional activators to the enhancer by transcription interference and inhibits the formation of active loops. The ICE in green, a DMR including the *Airn* promoter, which gains a methylation mark during oogenesis, controls expression of the macro lncRNA.

2. Future directions

The results of my thesis show an extension of the *Igf2r* cluster in extra-embryonic tissues, indicating that other imprinted clusters may also show extended imprinted silencing in extra-embryonic tissues. To test for allele specific expression outside the T^{hp}-deletion and for the extension of other imprinted clusters, a genome wide approach is needed. For the genome-wide identification of imprinted genes I would RNA-seq 12.5dpc VE, of reciprocal mouse crosses to determine the size of imprinted clusters throughout the genome (Figure 33). This approach has been used by a number of studies to detect imprinted expression in embryo, brain and placenta (Babak et al., 2008; DeVeale et al., 2012; Gregg et al., 2010; Okae et al., 2011; Wang et al.,

2011; Wang et al., 2008). In these studies, relatively few novel reproducible imprinted genes were detected. This may be because of the tissue-specific nature of imprinted expression that in a complex tissue can lead to masking of imprinted expression in one cell type by biallelic expression in another cell type (Hudson et al., 2011). As VE is a homogenous tissue I would expect this problem would be overcome maximizing the detection of imprinted expression. The approach that I would take would be to cross two genetically distinct mouse strains, to maximize the number of SNPs between the crosses. I would collect VE from reciprocal crosses at 12.5dpc and perform RNA-sequencing. The reciprocal crosses enables imprinted expression to be distinguished from strain specific expression. Recently, a number of technical and statistical problems with detecting imprinted expression by RNA-seq have been highlighted, making a careful experimental and analysis design essential to minimize the number of false positives (DeVeale et al., 2012). To avoid this I would include biological replicates and implement the recommended statistical approaches taken by Deveale et al. to detect imprinted expression.

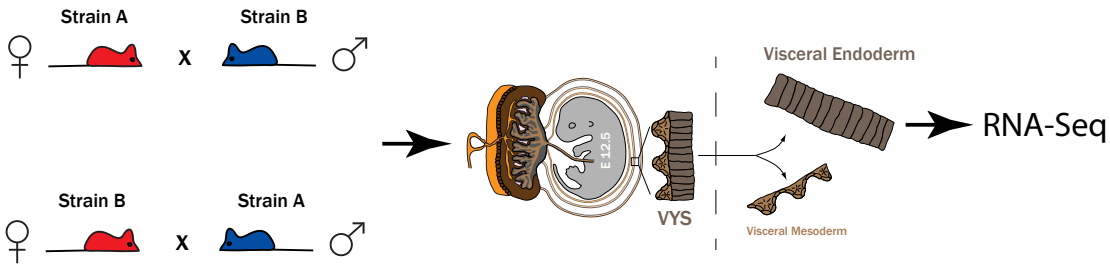


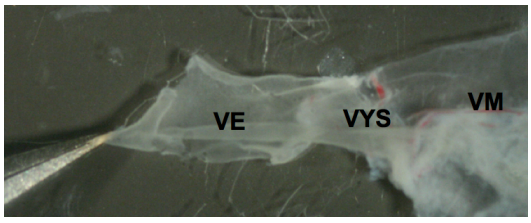
Figure 33 RNA-seq of reciprocal crosses for genome-wide identification of imprinted gene expression. Reciprocal mouse crosses of two genetically distinct strains (Strain A and Strain B). RNA-sequencing of the isolated 12.5 VE from both crosses.

V. MATERIALS AND METHODS

1. Methods

1.1 Tissue preparation

A pregnant mouse 12.5 days post coitum (dpc) was sacrificed and the embryos removed from the uterus. The VYS was then removed from around the embryo, taking care to separate it from the other membranes, the amnion and the PYS. Each VYS added to a well in a 24-well plate containing 1ml Dispase II in PBS with 0.1ul RNase inhibitor per ml and incubated for 2 hours at 4°C. The head of the embryo was transferred to lysis buffer for genotyping. The headless embryo and the placenta were snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. Special care was taken in dissecting the placenta to remove other attached tissues and to remove as much of the decidua as possible. After the incubation in Dispase II (Sigma-Aldrich, D46931G), the VYSs were transferred to PBS on ice and the VE and VM layers separated using forceps under the microscope. The VM layer also contains blood vessels and the basement membrane, whereas the VE is a pure population of epithelial cells (Figure 34). The quality of the VYS separation was verified by PCR of two marker genes, namely *Afp* for VE and *Flk-1* for VM. The VE was directly transferred into RNase free tubes containing 1ml TRI reagent for RNA isolation and stored at -80°C.



(Image provided by Tomasz Kulinski)

Figure 34 Separation of the VE from the VM of the VYS. After the enzymatic reaction the VYS was mechanical separated using forceps under the microscope. The VM contains blood vessels compared to the VE, which is a pure population of epithelial cells.

1.2 RNA isolation from tissues

Homogenization

Large tissue samples like embryo or placenta were transferred from -80°C directly into RNase free tubes containing 3ml TRI reagent. Samples were homogenized using a Polytron homogenizer (Kinematica AG) with 4-5 bursts of 1-2 seconds. 2ml homogenised sample was stored at -20°C as backup and 1ml was used to isolate RNA. Smaller tissue samples like VE were directly used for RNA isolation without homogenization.

RNA isolation

To each 1ml homogenized sample in TRI reagent 100µl BCP was added and mixed by shaking. Samples were incubated for 10 min at room temperature. Tubes were centrifuged at 12,000g for 15 min at 4°C. The clear upper phase was transferred to a 1.5ml RNase-free tube containing 500µl isopropanol (100%) and 1µl of Glycogen. Samples were mixed for 5 seconds and incubated again for 10 min at room temperature. Tubes were centrifuged at 12,000g for 8min at 4°C. The supernatant was removed with a pipette and 1 ml 75% EtOH was added for each sample. Tubes were centrifuged again at 7500°C for 5 min at 4°C. EtOH was removed with pipette and by *Airn*-drying the pellet. 100µl RNA storage solution (RSS) was added to placenta and embryo samples whereas to VE samples only 50µl were added. The RNA concentration of the samples was measured by Nanodrop®-1000 spectrophotometer. Samples in RSS were stored at -80° or precipitated by adding 0.1 volume 3M NaAc and 2.5 volume 100% EtOH and stored at -20°C.

1.3 First strand cDNA preparation

To remove the contaminating DNA from RNA samples, the DNA-Free kit from Ambion was used. For the first strand cDNA synthesis, the RevertAid kit from Fermentas was used. Both kits were used according to manufacturers instructions. cDNA was diluted 1:5 in water for use in PCR and qPCR.

1.4 Standard polymerase chain reaction (PCR)

Standard PCR was performed in a 25µl reaction containing 5µl of cDNA and 20µl of the following mastermix using the GoTaq kit from Promega:

H2O	6.9µl
5x Green Flexbuffer	5µl
$MgCl_2$ (25mM)	2.5µl
Betaine	4µl
Primer 1 (10µM)	0.5µl
Primer 2 (10µM)	0.5µl
dNTPs (10mM)	0.5µl
GoTaq	0.1µl

The thermocycling conditions were set as follows and run on the ptc-200 (MJ research) machine:

1. 95°C x 5min
2. 95°C x 30sec
   58°C x 30sec
   72°C x 1 min
   (cycle Nr.)
3. 72°C x 5min
4. 10°C x forever

The cycle number was adjusted to the expression levels of the gene and on the efficiency of the primers. For the VE marker *Afp* and for the VM marker *Flk-1* the cycle number was set to 25.

1.5 DNA isolation

Samples were incubated overnight at 55°C in 700µl Lysis buffer. 300µl saturated NaCl solution was added and tubes were mixed by hand. Tubes were spun for 10min at 13000rpm, at room temperature. The supernatant was transferred to a new tube containing 600µl isopropanol. Tubes were shaken by hand until the DNA was precipitated. Tubes were then centrifuged at 14000rpm for 7 min at room temperature. The supernatant was discarded and the remaining pellet was washed with 800µl 70% ethanol by shaking the tubes for 20 seconds. The tubes were again incubated for 15 min at room temperature and afterwards again centrifuged at 14000rpm for 7 min at room temperature. The ethanol was removed from the pellet, by pouring and pipetting. The pellet was resuspended in 80-150µl TE buffer (depending on the size of the pellet) and incubated overnight at 55°C for dissolving.

1.6 Southern blotting

Digestion of DNA

20µg genomic DNA was used for a double enzyme digestion assay with 2µl for each restriction enzyme (Mlul1 & EcorI) and appropriate buffers (Buffer O) in a 40µl reaction. Tubes were mixed well by vortexing and incubated in oven over night at 37°C.

Blotting

The digested DNA was mixed with 4µl 10x loading Dye and loaded on a 0,8% agarose in 1xTBE gel, together with 15µl 1kb ladder. Gel electrophoresis was conducted between 80-100V (depending on gel size) for 3.5 hours. The gel was stained with ethidium bromide for 1 hour and the DNA visualised under UV light and a photo taken using the UVsoloTS imaging system device. The double stranded DNA was denatured to single stranded DNA by incubating the gel for 30 min in denaturation solution twice. A plastic container was partially filled with denaturing solution and a glass plate placed on top to serve as the base for the blot. Three sheets of Whatmann paper were cut to the width of the gel extending long enough to dip into the buffer when soaked in denaturation solution and placed on the glass plate. The gel was placed upside down on the soaked Whatmann paper sheets and a hybond blotting membrane soaked in denaturation solution was placed on the top. The area around the gel was covered with plastic strips to prevent short-circuiting of the blot. The gel was covered with three Whatmann paper cut to the size of the gel, soaked in denaturation solution. 15 cm of dry paper towels were then placed on the top of the Whatmann paper with an additional glass plate with a 500 gram weight to hold everything in place. After 2 days the blot was dissembled and the membrane was neutralised with a 20mM Na_2HPO_4 solution.

Labeling the probe

20ng of unlabelled probe was diluted to a final volume of 14µl. The probe was denatured at 100°C for 5 min and placed on ice for 3 min. Afterwards 20µl LS, 6µl CTG mix, 1µl Klenow fragment an 2µl α^{32}P-dATP were added to the probe. The probe was incubated overnight at room temperature. The probe was diluted in 60µl TE buffer and cleaned with a Sephadex G-50 spin column

by centrifugation at 3000rpm for 3 min at room temperature to remove salt and nucleotides.

Hybridization

Approximately 150 ml Church buffer was used for pre-hybridizing the membrane for 30 min at 65°C in a hybridization tube. The labelled probe was hybridized again for 5 min at 100°C and placed on ice for 3 min. The hybridization buffer was discarded from the tube and replaced with 20ml Church buffer and the denaturized probe. The blot was hybridized under permanent rotation in an oven for 18 hours at 65°C.

Afterwards the membrane was washed twice with approximately 100 ml of 65°C Church wash buffer. The membrane was welded into a plastic foil, placed into a film cassette and covered with an phosphoimaging plate. The imaging plate was scanned on Typhoon 8600 Variable Mode Image scanner. The image was adjusted for contrast and brightness with the software imageJ.

1.7 qPCR assays

TaqMan and SYBRgreen assay were used to detect quantitative PCR products using the 7900HT Fast Real Time PCR machine with the Sequence detection system software, version 2.3 from Applied Biosystems. The qPCR was performed in a 25µl reaction containing 5µl of cDNA and 20µl of mastermix. The mastermix composition and thermocycling conditions are indicated below:

1.7.1 TaqMan

Kit: Taqman universal MasterMix Plus(uMM)

H2O	6.95µl
2x uMM Buffer	12.5µl
Primer 1 (100µM)	0.23µl
Primer 2 (100µM)	0.23µl
Probe (50mM)	0.1µl

Conditions of the cycles

1. 50°C x 2min
2. 95°C x 10min
3 95°C x 15sec
60°C x 1 min
(40 cycle Nr.)

Primers and probes

***Airn*-middle**
Forward: GACCAGTTCCGCCCGTTT
Reverse: GCAAGACCACAAAATATTGAAAAGAC
Probe: TACAAGTGATTATTAACTCCACGCCAGCCTCA

***Igf2r* exon 4-5**
Forward: GACTACTGCAGCCTGCAA
Reverse: AATCATGCTTCTGTAACTTGTCATCAA
Probe AAGCACCATGCGGCCTACGTGAA

Slc22a2
Forward: GGAAAATCGGTGCCAGTCTC
Reverse: AAGGGTTCAATTTCATGCCAGT
Probe: CTTCAGAGCCTGACGGCAGATGAGGA

Slc22a3
Forward: GAAATGCACGCTCATCCTTATG
Reverse: CAGGCGCATGACAAGTCCTT
Probe: TTGCTTGGTTCACGAGCGCCGT

Plg
Forward: GCGTGGCCAACCCTCAC
Reverse: GAAGTGCTGTCCGGTAAATCTTG
Probe: TGGCCCTGGCAAATCAGCCTTAGA

Sod2
Forward: TTAACGCGCAGATCATGCA
Reverse: GGCCAGAGCCTCGTGGTA
Probe: AAGCACCATGCGGCCTACGTGAA

QK
Forward: CGGGATGTAAAATAATGGTCC
Reverse: TGGGCTTGCCTCTATTTTGC
Probe: CAAAGGCTCAATGAGGGATAAAAAGAAGGAGG

cyclophilin
Forward: AGGGTTCTTCTTTTCACAGAATT
Reverse: GTGCCATTATGGCGTGTAAAGTC
Probe: TCCAGGATTCATGTGCCAGGGTGG

1.7.2 SYBRgreen

Kit: MESA GREEN qPCR MasterMix Plus for SYBR Assay I dTTP (eurogentec)

H2O	7.48µl
2x MESA Buffer	12.5µl
Primer 1 (100µM)	0.015µl
Primer 2 (100µM)	0.015µl

Conditions of the cycles

1. 50°C x 2min
2. 95°C x 10min
3 95°C x 15sec
60°C x 1 min
(40 cycle Nr.)
4 95°C x 15 sec
5 60°C x 15 sec

Primers

Gene	Forward primer	Reverse primer	Product length
Acat2	CAGACAGGACAAGGCTGAGAGCT	TTTCGTGGACAGGCACGGTGG	141
*Acat3**	AACTGCTAATGCAACAGGAATG	TGCAACAGAGAGAGCTGCAT	250
Agpat4	GCTGACTGCTACGTTCGGAGGA	GGGAGTCTCTGGGAAGACCCCT	144
Map3k4	GAGCGAGACGAGCCAGCGTAT	CTATGGGCCCCTGCTTAGAGGC	118
Mrpl18	TGACTTTGCCCAGGTCCCGTC	TGGTTGAGAGGGCTGCGAACC	104
Tcp1	AGATGGAGGGTCCTTTGTCCGTG	ATCCAAGCCAACTGGCCCAAAAGAA	128
Wtap	AGCAGGAGTCTGCACGCAGG	AGTTGGGCCACACTCGGCTG	127
Pde10a	CAGGATAGCCACAAAAGCCGACG	ATGAGCCGGGGTTGGCCTTC	111

All the primers are designed with the NCBI web tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and span an exon-exon junction.
*The specificity of the *Acat3* primer was additionally tested by sequencing the qPCR product.

1.8 Statistics

Pooled samples average the biological variation of included samples, and may be sufficient to confidently distinguish large differences between genotypes, but do not allow for a statistical comparison. Individual samples enable the calculation of biological variation for a specific genotype and allow statistical tests to be performed. By using the T-test I could verify, if the expression difference between the maternal, paternal or the *AirnT* allele is statistically significant (*P=0.01-0.05, **P=0.001-0.01, ***P<0.001). For this calculation, I used a heteroscedastic T-test (Two-sample unequal variance) with a two-tailed distribution.

2. Materials

2.1 Chemicals and consumables

100Bp ladder	Fermentas
1Kb ladder	Fermentas
2MM Whatmann paper	Whatmann
5x FoTag flexi buffer	Fermentas
α^{32}P-dATP	Hartmann Analytic
Buffer O	Fermentas
Betain	Sigma
Bromo chloropropane (BCP)	MRC
Boric acid	Applichem
Bovine serum albumin (BSA)	Fermentas
DispaseII	Sigma
DNA-Free	Ambion
dCTP	Bioron
dGTP	Bioron
dTTP	Bioron
dNTPs (10mM)	Fermentas
Disodium hydrogen phosphate ($NA_2^{HP}O_{4)}$	Merck
EDTA	Merk

Ethanol 96%	Merck
Ethidiumbromide	Applichem
EcorI	Fermentas
Glycogen	Ambion
Glycerol	Applichem
Hybond^{TM} XL membrane	Amersham
Hepes	Applichem
Hydrochloric acid (HCL)	Merck
Isopropanol	Merck
Klenow Fragment	Fermentas
Mesa Green qPCR Mastermix	Eurogentec
MluI1	Fermentas
$MgCl_2$ 25mM	Fermentas
ß-Mercapto ethanol	Invitrogen
Proteinase K	Applichem
qPCR Mastermix Plus	Eurogentec
RNase Inhibitor	Roche
RNA storage solution	Ambion
RevertAid	Fermentas
Sodium dodecyl sulphate (SDS)	Applichem
Sephadex^{TM} G-50	Amersham
Sodium Acetat (NaAc)	Ambion
Sodium hydroxide (NaOH)	Applichem
Tris	Applichem
TRI reagent	Sigma
Water for Embryo Transfer	Sigma
Xylenol orange	Sigma

2.2 Solutions and Media

5x TEN pH 9
- 250mM Tris pH9
- 100mM EDTA pH 8
- 200mM NaCL

Church buffer
- 20mM Na_2HPO_4
- 7% SDS
- 1mM EDTA

Church wash buffer:
- 1% SDS
- 20mM Na_2HPO_4

CTG mix:
- 100µM dCTP
- 100µM dTTP
- 100µM dGTP
- 2 mg/µl BSA

Denaturation solution
- 0.5M NaOH
- 1.5M NaCl

LS mix:
- 25ml 1M Hepes ph 6.6
- 1ml OL (50 units of hexamers dissolved in 1,6ml TE ph 8)
- 25ml TM (250mM Tris pH 9, 25mM $MgCl_2$, 50mM ß-mercapto ethanol)

Lysis Buffer
- 1x TEN ph 9
- 1% SDS
- 0.5mg/ml Proteinase K

TBE (10x stock)
- 108g Tris base
- 55g boric acid
- 40ml 0.5M EDTA ph 8.0

TE buffer
- 10mM Tris HCl pH 8.0
- 1mM EDTA

TAE (50x stock)
- 242g Tris base
- 57,1ml Acetic acid
- 100ml 0.5M EDTA ph 8.0

10x Loading Dye
- 0.125g 0,5% Xylenol orange
- 7.5ml 30% Glycerol
- 17.5ml 1xTAE

VI. Abbreviations

DMR	differentially methylated region
dpc	days post coitum
EXEL	extra-embryonic-lineage
FISH	fluorescence in situ hybridization
ICE	imprint control element
Kb	kilobase
KO	knockout
linc	large intergenic ncRNAs
lnc	long non-protein-coding
Mb	megabase
MEFs	mouse embryonic fibroblats
ML	multi-linage
PCR	polymerase chain reaction
PGCs	primordial germ cells
PYS	parietal yolk sack
qPCR	quantitative PCR
T^{hp}	hairpin-tail
Tme	T associated maternal effect
VE	visceral endoderm
VM	visceral mesoderm
VYS	visceral yolk sack

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Education and training

2007 – 2012 (August)	University of Vienna **Magister in Molecular Biology** **Second stage of degree (4 semesters):** → 2012 (August) Cell biology, molecular medicine, genetics/epigenetic **First stage of degree (6 semesters)** → completed in 2010 Biology, chemistry, biochemistry, cell biology, molecular biology
2005 - 2007 (study termination)	Vienna University of Technology **Mechanical Engineering – Economics**
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Research experience

Diploma thesis June 2011 to June 2012 (1year)	**CeMM** Center for Molecular Medicine: Barlow group Vienna (AUT) Project: **Characterization of novel imprinted genes in visceral endoderm**
Internship May 2011 (1.5 month)	**Max Planck Institute**: Pospisilik group Freibug (GER) Project: **Examination of chromatin modifiers in adipogenesis**
Internship February to March 2011 (2 months)	**IMBA**-Institute of Molecular Biotechnology: Penninger group, Vienna (Aut) Project: **Generation of induced pluripotent stem cells using nonviral minicircle**
Internship July to August 2010 (2 months)	**EURAC:** Andrew Hicks group, Bolzano (IT) Project: **Association between SNPs in the TMPRSS6 Gene and Hepcidin-expression in hepatocytes**
Internship February 2010 (1month)	**AKH-hospital**: Renate Fuchs group, Vienna (AUT) Project: **Entry of human rhinovirus 89 via ICAM-1 into epithelial cells requires an intact actin skeleton but does not depend on phosphoinositol-3-kinase**
Internship July to august 2009 (2 months)	**Department of Pathology:** Hospital, Eduard Egarter Vigl group, Bolzano (IT) Diagnosis of disease based on the analysis of bodily fluids such as blood and urine.
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