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EXTRA-EMBRYONIC-SPECIFIC IMPRINTED EXPRESSION IS RESTRICTED TO DEFINED LINEAGES IN THE POST-IMPLANTATION EMBRYO

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Abstract

A subset of imprinted genes in the mouse have been reported to show imprinted expression that is restricted to the placenta, a short-lived extra-embryonic organ. Notably these so-called 'placental-specific' imprinted genes are expressed from both parental alleles in embryo and adult tissues. The placenta is an embryonic-derived organ that is closely associated with maternal tissue and as a consequence, maternal contamination can be mistaken for maternal-specific imprinted expression. The complexity of the placenta, which arises from multiple embryonic lineages, poses additional problems in accurately assessing allele-specific repressive epigenetic modifications in genes that also show lineage-specific silencing in this organ. These problems require that extra evidence be obtained to support the imprinted status of genes whose imprinted expression is restricted to the placenta. We show here that the extra-embryonic visceral yolk sac (VYS), a nutritive membrane surrounding the developing embryo, shows a similar 'extra-embryonic-lineage-specific' pattern of imprinted expression. We present an improved enzymatic technique for separating the bilaminar VYS and show that this pattern of imprinted expression is restricted to the endoderm layer. Finally, we show that VYS 'extra-embryonic-lineage-specific' imprinted expression is regulated by DNA methylation in a similar manner as shown for genes showing multi-lineage imprinted expression in extra-embryonic, embryonic and adult tissues. These results show that the VYS is an improved model for studying the epigenetic mechanisms regulating extra-embryonic-lineage-specific imprinted expression.

Keywords

genomic imprinting; placenta; yolk sac; non-coding RNA; insulator

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INTRODUCTION

In mammals, the maternal and paternal genomes contribute unequally to the developing embryo due to parental-specific or imprinted expression of a small number of genes (Barlow and Bartolomei, 2007; Cattanach and Kirk, 1985; Lyon and Glenister, 1977; McGrath and Solter, 1984; Surani et al., 1984). Imprinted genes mostly occur in clusters where a *cis*-acting Imprint Control Element (ICE) controls imprinted expression. The ICE is a gametic differentially methylated region (gDMR) established by the *de novo* methylase DNMT3A/3L complex in spermatogonia or oocytes and maintained in diploid cells on the same parental allele, by the DNMT1 maintenance methyltransferase (Bourc'his et al., 2001; Kaneda et al., 2004; Li et al., 1993). The unmethylated ICE acts as a promoter or activator of a long or macro non-protein-coding (nc) RNA (Koerner et al., 2009). In the *Igf2r* and *Kcnq1* clusters, the ICE controls expression of the *Airn* and *Kcnq1ot1* macro ncRNAs, which both silence imprinted protein-coding genes *in cis* (Mancini-Dinardo et al., 2006; Sleutels et al., 2002). In the *Igf2* cluster expression of the *H19* macro ncRNA is controlled by the ICE, but it plays no role in imprinted silencing. Instead the CTCF insulator protein binds the ICE and controls access to distal enhancers, thereby restricting *Igf2* expression to the paternal chromosome (Bell and Felsenfeld, 2000; Hark et al., 2000). Although imprinted expression of all genes in a cluster is controlled by the ICE or by the macro ncRNA it regulates, some genes show imprinted expression in embryonic, adult and extra-embryonic lineages (here referred to as multi-lineage (ML) imprinted expression), while others show imprinted expression only in the extra-embryonic lineages (here referred to as EXEL imprinted expression). Genes showing EXEL imprinted expression tend to be located further away from the ICE and their regulation has been suggested to be controlled or maintained by different downstream epigenetic factors compared to genes showing ML imprinted expression (Hudson et al., 2010).

Previous studies using the ectoplacental cone and its derivative the placenta as a model of EXEL imprinted expression (e.g. Green et al., 2007; Lewis et al., 2004; Umlauf et al., 2004) had two disadvantages. Firstly, the placenta is a complex organ that arises from multiple embryonic lineages and contains the trophectoderm-derived placental labyrinth, spongiotrophoblast and trophoblast giant cells and the epiblast-derived endothelial and fetal blood cells (Fig. 1A). If an imprinted gene is subject to tissue-specific silencing in some lineages and imprinted silencing in others (for example *Slc22a3* that is only expressed in placental labyrinth cells and *Ascl2* that is only expressed in spongiotrophoblast cells (Guillemot et al., 1994; Verhaagh et al., 2001), it will be difficult to identify repressive epigenetic marks specific to imprinted gene silencing (Fig. 1B). Secondly, the placenta is intermingled with maternally derived tissues such as the *decidua basalis* that interacts with the embryonic trophoblast to promote fetal-uterine interactions, and maternal blood in the labyrinth in the middle of the placenta (Fig. 1A). This intermingling leads to maternal contamination in placental preparations.

A number of genes also show imprinted expression in the extra-embryonic yolk sacs (summarized in Hudson et al., 2010) raising the possibility that this tissue could also provide an alternative model of EXEL imprinted expression. The embryo is surrounded by three bilaminar membranes that interface with the placenta (Nagy et al., 2003): the amnion (inner membrane), the visceral yolk sac (VYS; middle membrane) and the parietal yolk sac (PYS; the outer membrane that degenerates after 13.5 days post postcoitum (dpc) Fig. 1A). In most studies showing yolk sac imprinted expression the membrane displaying imprinted expression was not identified, but for *Ins2*, a gene showing imprinted expression in yolk sacs but not in the embryo, and for *Phlda2*, a gene that shows imprinted expression in yolk sacs and embryo, imprinted expression was localized to the VYS (Duvillie et al., 1998; Frank et al., 1999). Unlike the placenta, the VYS is a simple tissue with an outer layer of

primitive-endoderm-derived extra-embryonic visceral endoderm overlying a basement membrane, an inner layer of epiblast-derived visceral mesoderm, and blood islands that arise between the two layers. Importantly, and in contrast to the placenta, the VYS lacks contaminating maternal tissue, although it is the primary site of maternal immunoglobulin transfer in rodents (Mossman, 1991). Imprinted expression of *Ins2* and *Phlda2*, as well imprinted X inactivation is confined to the VYS endoderm layer, indicating that this cell lineage may be a good model for investigating EXEL imprinted expression (Duvillie et al., 1998; Frank et al., 1999; Sado et al., 2000; Takagi and Sasaki, 1975; West et al., 1977). A caveat of the VYS as a model is that the mesoderm layer may not show EXEL imprinted expression, but if necessary the VYS endoderm and mesoderm can be physically separated.

We show here that the *Slc22a2* and *Slc22a3* genes from the *Igf2r* imprinted gene cluster previously reported to have placental-specific imprinted expression (Zwart et al., 2001a), also show imprinted expression in the VYS endoderm layer. In addition, we demonstrate that restriction of EXEL imprinted expression to the VYS endoderm layer is found for other known and novel yolk sac imprinted genes. Previous studies have shown that the maintenance DNA methylation enzyme DNMT1 is required for multi-lineage imprinted expression, but not for genes showing EXEL imprinted expression when the placenta was analyzed (Green et al., 2007; Lewis et al., 2004; Umlauf et al., 2004). In contrast, we show here using $Dnmt1^{c/c}$ null mice that absence of DNA methylation in the VYS leads to increased expression of imprinted macro ncRNAs and decreased expression of imprinted mRNAs, as previously reported for genes showing imprinted expression in the embryo (Green et al., 2007; Seidl et al., 2006). This study shows that that EXEL imprinted expression is limited to a subset of primitive endoderm and trophectoderm derived lineages in the post-implantation embryo and identifies the VYS endoderm layer as an improved model for the analysis of the mechanisms regulating EXEL imprinted gene expression.

MATERIAL AND METHODS

Mice

Wild type mice, T-hairpin mice, *AirnT* mice and DR4 mice were maintained on a FVB/N background. $Dnmt1^{c/c}$ mice were on a C57BL/6J background (Weaver et al., 2010). For single nucleotide polymorphism (SNP) experiments reciprocal crosses were made between C57BL/6J and CAST/EiJ or between FVB/N and CAST/EiJ. Embryonic age days postcoitum (dpc) was timed from midnight of the night before the vaginal plug. Mice were humanely treated according to the guidelines of the European Union Council (86/609/EU) and Austrian regulations.

RNA/DNA analysis

Total RNA was extracted using TRI-reagent (Sigma-Aldrich T9424). DNA and RNA blots were performed according to standard procedures using probes listed in Supplementary Table 1. RT-PCR and real-time RT-qPCR: RNA was DNase1 treated using the DNA-free™ kit according to manufacturers instructions (Ambion). Reverse Transcription (RT) was performed using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas) according to manufacturers instructions. RT-PCR was performed according to standard procedures using the GoTaq® Flexi DNA polymerase (Promega) or the Long PCR Mix enzyme (Fermentas). Primers are listed in Supplementary Table 2. Real-time RT-qPCR was conducted with recommended cycling conditions on the ABI PRISM 7000 system using a Taqman mastermix or Mesa Green SYBR® mastermix (Eurogentec). Cycling conditions used were 2 minutes 50°C, 10 minutes 95°C, 40 cycles of 15 seconds 95°C and 1 minute 60°C. Taqman probes and primers were designed using PrimerExpress (Applied Biosystems) and are listed

in Supplementary Table 3. Relative quantification of RNA was calculated using the standard curve method according to the manufacturers protocol (Applied Biosystems).

Genotyping 8.5dpc T-hairpin embryos by *Igf2r* and *Airn* expression levels

Igf2r shows maternal-specific biased expression after E6.5 and thus it is reduced in an 8.5dpc Thp/+ embryo. *Airn* shows a more stringent paternal-specific expression and thus is absent from a +/Thp embryo (note the maternal allele is always written first: Mat/Pat). Individual 8.5dpc embryos were divided into embryo plus VYS and placenta plus PYS portions and genotyped as having a maternal Thp deletion (−/+) if the *Igf2r* expression level measured by qPCR (normalized to 18S rRNA) was less than 20% the mean level for the reciprocal paternal Thp cross (+/−) in both parts. Individual 8.5dpc embryos were genotyped as having a paternal Thp deletion (+/−) if *Airn* expression was less than 10% of the mean *Airn* expression levels for the reciprocal maternal cross. To reduce biological variation RNA from individual typed embryos was pooled for later analysis.

Separating visceral yolk sac layers

Three methods were tested. Visceral yolk sacs (VYS) dissected in DEPC treated PBS on ice were either incubated at 4°C for 2 hours in 1% DispaseII (Sigma D4693) in PBS, in 0.5% trypsin PBS or in 0.5% trypsin plus 2.5% pancreatin PBS at 4°C for 20 min, with Protector RNase Inhibitor (Roche) at the recommended concentration. After incubation, the VYS was transferred to DEPC treated PBS on ice and the layers separated using fine forceps under a dissecting microscope. Only the DispaseII protocol allowed the outer endoderm layer to be removed as a large continuous sheet of cells and this was used for the majority of preparations. The visceral mesoderm was dissected out together with the blood islands and the basement membrane. The yield from six 13.5dpc VYS was approximately 130μg visceral endoderm RNA and 30μg visceral mesoderm RNA.

Allele-specific expression analysis

RNA was prepared from 12.5dpc and 13.5dpc VYS (whole or separated layers), placenta and whole embryo from reciprocal crosses between homozygous *Mus musculus domesticus* (C57BL/6J or FVB/N) and *Mus musculus castaneus* (CAST/EiJ) was subject to RT-PCR. The SNP containing PCR product was gel purified and sequenced to evaluate parental allelic contribution. In cases where sequencing artifacts prevented evaluation of parental allelic contribution an RT-PCR based restriction fragment length polymorphism (RFLP) assay was performed (in duplicate). Gels were scanned using the Typhoon™ scanner and quantified using the ImageQuant™ software (GE Healthcare) to determine the ratio between the bands and detect allelic biases in expression (details in Fig. 4 legend). Primers and enzymes used for RFLP assays and sequencing are listed in Supplementary Table 2.

RESULTS

The placenta is contaminated by maternal tissue

Although the placenta is an embryonic-derived tissue it interfaces closely with maternal tissues that comprise the decidua and maternal blood vessels and blood (Fig. 1A, B). To test the extent of maternal contamination of placenta preparations we used DR4 mice that contain the antibiotic resistance genes for neomycin and puromycin at the *Dnmt1* locus, a randomly integrated hygromycin resistance transgene and a deletion of the X-linked *Hprt* gene conferring 6-Thioguanine resistance (Tucker et al., 1997). We crossed hemizygous DR4 females with wild type males and identified offspring that were wild type or hemizygous for the hygromycin resistance transgene by Southern blot using DNA from 13.5dpc embryonic head (Fig. 1C, right side). We then monitored levels of the hygromycin

resistance transgene DNA in the placenta and VYS from wildtype (WT) embryos and compared them to hemizygous (H) embryos. The dissections were performed by two dissectors at the same time who took care to remove the maternal decidua from the placenta. Despite this, the Southern blot shows faint hygromycin resistance transgene bands in wild type placenta that indicate maternal contamination, but no signal in wild type VYS or embryonic head (Fig. 1C). QPCR quantification showed that placental contamination depended on the dissector but was between 5–14% of the hemizygous level (Fig. 1D). This demonstrates that the placenta, but not the VYS, is contaminated by maternal tissue. To determine if decidua contamination of placenta may affect interpretation of imprinted expression we examined expression of known EXEL imprinted genes in the placenta and decidua from 12.5dpc embryos. *Osbpl5* showed approximately 20-fold higher expression in the decidua than placenta, *Tssc4* levels in decidua were similar to placenta, while *Slc22a3* previously shown by *in situ* hybridisation to be limited to the labyrinth layer of the placenta, showed levels in the decidua that were 0.3% of levels in the placenta (Fig, 1E, Verhaagh et al., 2001). This indicates that genes expressed in contaminating maternal tissues may affect the level of expression detected in placenta expressed genes, and particularly complicates analysis of genes like *Osbpl5* and *Tssc4* in placenta.

The visceral yolk sac endoderm expresses known imprinted genes

We next tested expression in the VYS of genes previously reported as showing placental-specific imprinted expression. We first examined expression of the *Slc22a2* and *Slc22a3* genes from the *Igf2r* imprinted gene cluster in all extra-embryonic tissues and in the maternal decidua. RNA blot analysis showed that the multi-lineage imprinted *Igf2r* gene was expressed in the amnion, VYS and PYS at 11.5dpc, 13.5dpc and 15.5dpc as well as in the placenta and maternal decidua at 13.5dpc and 15.5dpc (Fig. 2A). In contrast, *Slc22a2* and *Slc22a3* as well as *Plg*, a gene on the periphery of the cluster not reported to show imprinted expression, show a more restricted expression pattern. *Slc22a3* was expressed in the VYS at 11.5dpc, 13.5dpc and 15.5dpc, and as previously reported (Zwart et al., 2001a), in the placenta at 13.5dpc and 15.5dpc. *Slc22a2* expression was restricted to VYS at 11.5dpc, 13.5dpc and 15.5dpc (Fig. 2A). Note that *Slc22a2* expression was not detected in placenta by Northern blot as we previously reported (Zwart et al., 2001a), a difference likely due to contamination of placenta by VYS in the previous study. *Slc22a2* and *Slc22a3* expression in the VYS decreases with developmental age as previously described for *Slc22a3* in the placenta (Verhaagh et al., 2001). At all time points examined *Slc22a3* expression in the VYS was lower than *Slc22a2* indicating a primary function for the former in the placenta, and for the latter in the VYS. We confirmed by RT-qPCR that *Slc22a2* is predominantly expressed in the VYS compared to the amnion and PYS (Fig. 2B). *Slc22a3* showed a greater variation in relative expression that likely reflects its low expression in membranes, but this low expression mostly localized to the VYS (Fig. 2B). The VYS is composed of an outer layer of extra-embryonic visceral endoderm overlaying a basement membrane, and an inner layer of visceral mesoderm covering blood islands (Fig. 1A). To determine the sub-localization of genes we mechanically separated the VYS endoderm and mesoderm layers at 13.5 dpc after trypsin digestion. RT-PCR using the VYS endoderm marker *Afp* and mesoderm marker *Flk-1* confirmed that the separation had been successful (Fig. 2C). RT-qPCR of separated material showed that while *Igf2r* is expressed in both layers, *Slc22a2* and *Slc22a3* together with *Plg* are only expressed in the VYS endoderm layer (Fig. 2C).

We next wanted to determine the developmental onset of *Slc22a2* and *Slc22a3* expression. The visceral endoderm is present in the 7.5dpc embryo as a single epithelial layer overlying the ectoderm or epiblast that contributes to the embryo, where it is called the 'embryonic visceral endoderm'. It also overlies the extra-embryonic ectoderm, where it is called the 'extra-embryonic visceral endoderm' (Theiler, 1989). At least part of the embryonic visceral

endoderm contributes to the embryonic gut, while the extra-embryonic visceral endoderm contributes to the VYS, which is first identifiable as a membrane around E8.5 (Kwon et al., 2008). The placenta also arises at 8.5dpc with the fusion of the allantois and chorion (Watson and Cross, 2005). To determine the developmental onset of *Slc22a2* and *Slc22a3* expression, RNA was isolated from E7.5 whole embryos, while at E8.5 the embryo plus VYS was collected separately from the placenta plus PYS, and at E9.5 the embryo, the placenta plus PYS, and the VYS were collected separately (Fig. 2D). RT-qPCR analysis showed that *Igf2r* was continually expressed and increased 2–3 fold with developmental age, with the highest expression in 9.5dpc VYS. *Airn* ncRNA expression was similarly detected at all stages, although expression was relatively low at 7.5dpc and maximal in 9.5dpc VYS. *Slc22a2* expression was very low at 7.5dpc before being upregulated in 8.5dpc embryo plus VYS and at 9.5dpc was exclusively expressed in VYS, indicating that the expression at 8.5dpc was from the VYS component and not the embryo. We previously reported expression of *Slc22a3* in the 7.5dpc embryo using RT-PCR (Zwart et al., 2001c). Here using a quantitative assay we show that *Slc22a3* expression is relatively low at 7.5dpc before being upregulated 64-fold in 8.5dpc embryos plus VYS, and to a lesser extent in the 8.5dpc placenta plus PYS. At 9.5dpc, *Slc22a3* in the embryo alone was reduced compared to 8.5dpc embryo plus VYS, while the VYS expression was greatly upregulated, suggesting that most expression at 8.5dpc was from the VYS. In contrast, relative *Slc22a3* expression in the placenta plus PYS was similar between 8.5dpc and 9.5dpc. Lastly, *Plg* showed low expression at 7.5dpc, which was upregulated in 8.5dpc embryos plus VYS, but absent in placenta plus PYS. *Plg* was further upregulated in 9.5dpc VYS, but absent in embryo and placenta plus PYS, indicating that expression at 8.5dpc was from the VYS (Fig. 2D). In summary, we show that the *Slc22a2* and *Slc22a3*, genes previously reported to display placental-specific expression are also expressed in the VYS, specifically in the VYS endoderm layer.

The visceral yolk sac shows imprinted expression of *Slc22a2* and *Slc22a3*

We next tested if the *Slc22a2* and *Slc22a3* genes showed imprinted expression in VYS. We used T-hairpin (Thp) mice that have a 6Mb deletion spanning the entire *Igf2r* imprinted cluster, enabling us to examine expression from the maternal or paternal allele depending on which parent donated the deletion to the embryo (Barlow et al., 1991). Fig. 3A shows an RNA blot of 11.5dpc VYS where *Airn* is expressed only in samples containing a paternal allele (+/+ and −/+, note the maternal allele is always written first), while *Igf2r* is expressed only in samples containing a maternal allele (+/+ and +/−). Thus in VYS, *Airn* shows imprinted paternal-specific expression and *Igf2r* shows imprinted maternal-specific expression as previously described for placenta, embryonic and adult tissues (Barlow et al., 1991; Zwart et al., 2001a). At 11.5dpc both *Slc22a2* and *Slc22a3* were expressed only in samples containing a maternal allele, demonstrating that they show imprinted maternal-specific expression in VYS. *Plg* that flanks the *Slc22a3* genes was expressed from both the maternal and paternal allele (+/− compared to −/+) at reduced levels compared to the wild type (+/+), however, expression appeared to be stronger from the maternal allele suggesting a biased expression. We next used RT-qPCR to test if *Slc22a2* and *Slc22a3* imprinted expression is maintained later in development (Fig. 3B). At 16.5dpc *Slc22a2* showed full imprinted expression in VYS, which can be seen from the similar expression levels in samples only containing the maternal allele (+/−) and samples containing both alleles (+/+), while paternal-specific expression (−/+) remained very low. In contrast, *Slc22a3* expression from the maternal allele was reduced compared to the wild type and expression from the paternal allele was increased, although it was still lower than the maternal allele. This indicates that by 16.5dpc *Slc22a3* shows a loss of imprinted expression in the VYS. This result agrees with previous reports for the placenta, where *Slc22a3* imprinted expression is partly lost by 15.5dpc (Zwart et al., 2001a).

We also tested if *Slc22a2* and *Slc22a3* show imprinted expression from when they are first upregulated at 8.5dpc. Embryos from reciprocal Thp crosses were dissected as for Fig. 2D into embryo plus VYS and placenta plus PYS, genotyped according to *Igf2r* and *Airn* expression and pooled to reduce developmental variation (see Materials and Methods for details). In embryo plus VYS and in placenta plus PYS samples, *Airn* expression from the maternal allele (+/−) was 0.1% – 6.6% of wildtype littermates indicating that *Airn* is strongly repressed on the maternal allele by 8.5dpc (Fig. 3C). *Airn* expression from the paternal allele (−/+) was 100% of wildtype levels in placenta plus PYS as expected for a paternally expressed gene, but only 50% of wildtype levels in embryo plus VYS. The latter result likely indicates expression variation due to developmental delay in −/+ embryos that normally die between 13.5dpc – 16.5dpc, as *Airn* expression is more upregulated in embryo plus VYS than in placenta plus PYS between 7.5dpc – 8.5dpc (Fig. 2D). *Igf2r* showed the reciprocal expression pattern to *Airn*; with expression from the maternal allele (+/−) similar to wild type (+/+) littermates in both the embryo plus VYS and placenta plus PYS, while paternal expression (−/+) was less than 1% of wild type levels; showing that *Igf2r* is strongly repressed on the paternal allele by 8.5dpc in agreement with previous studies (Lerchner and Barlow, 1997; Szabo and Mann, 1995). *Slc22a2* and *Slc22a3* both behave as *Igf2r* in embryo plus VYS, where maternal expression (+/−) was the same as wildtype (+/+), but paternal expression (−/+) was low or undetectable at 8.5dpc. In addition, while *Slc22a2* was not expressed in placenta plus PYS, the *Slc22a3* gene was specifically expressed from the maternal allele. Thus both *Slc22a2* and *Slc22a3* show imprinted expression at E8.5 in the embryo plus VYS and *Slc22a3* also shows imprinted expression in the placenta plus PYS. The non-imprinted *Plg* gene showed similar levels of expression from the maternal (+/−) and paternal (−/+) alleles, indicating it is not showing an allelic bias at E8.5 (Fig. 3C).

Paternal repression of *Igf2r, Slc22a2* and *Slc22a3* in placenta, embryo and adult tissues is controlled by the *Airn* macro ncRNA (Sleutels et al., 2002). We used the *AirnT* allele that truncates *Airn* to 5% of its wildtype length, to test if *Airn* also represses genes in the VYS. Fig. 3D shows an RNA blot hybridized with *Slc22a2* and *18S rRNA* (top) and an RT-qPCR assay of *Slc22a2* and *Slc22a3* (bottom). 13.5dpc VYS carrying genotypes with and without the paternal or maternal allele in combination with the wildtype (−/+ and +/−) or the *AirnT* (−/*AirnT* and *AirnT*/−) allele were analyzed. The data shows that *Slc22a2* and *Slc22a3* are increased approximately 2 fold when *AirnT* is truncated on the paternal chromosome (compare +/*AirnT* with *AirnT*/+). Thus, *Airn* controls imprinted expression of *Slc22a2* and *Slc22a3* in VYS.

EXEL imprinted expression is restricted to the endoderm layer of the visceral yolk sac

The identification of *Slc22a2* and *Slc22a3* imprinted expression in the VYS endoderm prompted us to test if other genes previously reported as showing imprinted expression in the placenta, behave similarly. We used reciprocal crosses between C57BL/6J and CAST/EiJ mouse strains to obtain 12.5dpc embryos and assayed single nucleotide polymorphisms (SNPs) located in exons by restriction fragment length polymorphism (RFLP) or by sequencing. Representative examples are shown in Fig. 4A for *Osbpl5* and *Tssc4* from the *Kcnq1* cluster, *Pon3* from the *Peg10* cluster and the solo imprinted gene *Sfmbt2*. We included *Slc22a2* and *Slc22a3* as positive controls. For *Osbpl5, Tssc4* and *Pon3* assaying the SNP by sequencing was unsuccessful due to sequencing biases observed in the mixed RNA control so the RFLP approach was used. This showed that *Osbpl5, Tssc4* and *Pon3* only have a weak bias towards the maternal allele in VYS, that was present in reciprocal crosses. The RFLP assay also confirmed previous studies that these genes showed biallelic expression in the embryo and maternal-specific or biased expression in the placenta, with the caveat that the *Osbpl5* and *Tssc4* placental results are compromised by strong expression in the maternal decidua contained in the placenta samples (Fig. 1E; (Engemann et al., 2000;

Ono et al., 2003; Paulsen et al., 2000). Maternal-specific imprinted expression of *Sfmbt2, Slc22a2* and *Slc22a3* in VYS was confirmed by sequencing where only the maternal SNP was observed in reciprocal crosses (Fig. 4A). Thus this analysis shows clear EXEL imprinted expression for *Sfmbt2, Slc22a2* and *Slc22a3* in VYS, while *Osbpl5, Tssc4* and *Pon3* only show a weak biased allelic expression.

As *Slc22a2* and *Slc22a3* show imprinted expression in the VYS endoderm and are not expressed in the mesoderm layer (Fig. 2C), we considered if biallelic expression from the mesoderm layer could mask imprinted expression in total VYS to generate the weak biased allelic expression seen for *Osbpl5, Tssc4* and *Pon3*. We therefore separated the VYS layers to localise gene expression. Established protocols to separate the bilaminar VYS layers rely on trypsin or a trypsin/pancreatin mix (Nagy et al., 2003). However, we found this to be inefficient and time consuming so we developed a new protocol using the DispaseII protease that cleaves fibronectin and collagen (see Materials and Methods). This enabled easier and more rapid separation of the endoderm and mesoderm layers as intact sheets and a greater yield of RNA. We confirmed the efficiency of separation by showing that the VYS endoderm marker *Afp* was limited to the isolated endoderm and the VYS mesoderm marker *Flk-1* was limited to the isolated mesoderm, while *CypA* was found in both (Fig. 4B controls). We then assayed gene expression in the separated layers and the total VYS for *Osbpl5, Tssc4* and *Pon3* plus additional genes from the *Kcnq1* and *Peg10* imprinted clusters. Fig. 4B shows that *Osbpl5, Tssc4* and *Cd81* that are contained in the *Kcnq1* imprinted gene cluster, showed expression in both the VYS endoderm and mesoderm layers. Similarly, *Pon3 and Pon2* that both lie in the *Peg10* imprinted gene cluster were expressed in both endoderm and mesoderm layers. Thus it is possible that biallelic expression in the mesoderm layer would mask imprinted expression in total VYS for these genes. In contrast genes showing imprinted expression in total VYS, such as *Tfpi2* (Monk et al., 2008), *Sfmbt2, Slc22a2* and *Slc22a3*, are mainly expressed in the endoderm layer.

The expression of *Osbpl5, Tssc4* and *Pon3* in both VYS layers was consistent with our suggestion that their imprinted expression is masked in total VYS by biallelic expression in the mesoderm. To test this we used reciprocal crosses between FVB/N and CAST/EiJ mouse strains and separated the VYS layers to assay imprinted expression in endoderm and mesoderm. Expression of *Afp* restricted to VYS endoderm and *Flk-1* restricted to mesoderm indicated the separation was complete (Fig. 4C bottom). Using RFLP assays we assessed if there was any allelic bias in expression of *Osbpl5, Tssc4* and *Pon3* in the different layers and total VYS. Quantification of band intensity enabled a ratio of the FVB/N band divided by the CAST/EiJ band to be determined (F/C ratio, Fig. 4C). The reciprocal cross ratio was then calculated by dividing the FVB/N × CAST/EiJ ratio (maternal/paternal) by the CAST/EiJ × FVB/N ratio (paternal/maternal) for each gene and tissue (Fig. 4D) to control for technical and strain expression biases. If there were no allelic bias a value near to 1 would be expected, while a value greater than 1 would indicate a maternal bias and a value less than 1, a paternal bias. For *Osbpl5*, the mesoderm and total VYS had a value around 1.5 while in endoderm a clear maternal bias was apparent with a value around 14. Similarly, for *Tssc4* and *Pon3* the mesoderm value was near to 2, but a maternal bias was already apparent in the total VYS, and this became more pronounced in the isolated endoderm. For *Pon3*, ratios could not be determined in the endoderm as the gene is relatively weakly expressed there (Fig. 4B) and the paternal band could not be quantified. However, only the maternal band was clearly visible in the reciprocal crosses, indicating the endoderm shows maternally biased expression of *Pon3*. Together the results show that *Osbpl5, Tssc4* and *Pon3* display maternal biased expression in VYS endoderm that was absent in the mesoderm. The minor deviations from 1 in the mesoderm reciprocal cross ratio reflects experimental variation, as *Osbpl5*, a gene previously shown to be not imprinted in the embryo (Engemann et al., 2000),

showed a similar ratio around 2 in a C57BL/6J × CAST/EiJ reciprocal cross in embryo (data not shown),

DNA methylation regulates expression of imprinted genes in Visceral Yolk Sac

The localization of EXEL specific imprinted expression to the VYS endoderm indicates this cell lineage has some similarity to placental lineages that also show EXEL imprinted expression. Some genes showing EXEL imprinted expression in placenta were shown to maintain imprinted expression in the absence of DNA methylation, while other genes showing ML imprinted expression lose their imprinted expression (Green et al., 2007; Lewis et al., 2004). Therefore, we tested how loss of DNA methylation regulates imprinted expression in the VYS. First, we examined if the *Slc22a3* CpG island promoter shows parental-specific DNA methylation using a DNA blot on T-hairpin and wild type DNA digested with the methylation sensitive enzyme CfoI (and BssHII and HpaII not shown Fig. 5A). No DNA methylation was observed on the maternal or paternal allele of 11.5dpc placentas, or in wild type embryos, placenta and VYS at 11.5dpc and 16.5dpc (indicated by the absence of a 4kb band) consistent with our previous result in placenta (Zwart et al., 2001a). Next we confirmed that the ICE/*Airn* promoter displayed differential DNA methylation in VYS (Fig. 5B), by detecting an equal intense methylated and unmethylated band, as previously shown in the embryo and placenta (Stoger et al., 1993). Lastly, we confirmed the partial gain of DNA methylation on the silent paternal *Igf2r* promoter in VYS as previously reported for embryo and placenta (Fig. 5C; Stoger et al., 1993). The *Slc22a2* promoter lacks a CpG island and so was not examined for DNA methylation status (chr17:12,777,055-12,821,354; NCBI37/mm9 assembly).

To investigate how DNA methylation regulates genes showing EXEL or ML imprinted expression we used RT-qPCR to compare gene expression in 9.5dpc *Dmnt1* null ($Dnmt1^{c/c}$) embryos or VYS with wild type ($Dnmt1^{+/+}$) and heterozygote ($Dnmt1^{+/c}$) littermates. In the *Igf2r* cluster in both embryo and VYS, expression of the *Airn* ncRNA was increased by approximately 70 – 300%, while *Igf2r* expression was reduced by approximately 24 – 44% in the absence of DNA methylation (Fig. 5D). *Slc22a2* and *Slc22a3* were also reduced in VYS, while the non-imprinted *Plg* gene was unaffected by the loss of DNA methylation (Fig. 5D). In the *Igf2* cluster *H19* ncRNA was increased by approximately 160 – 170%, and *Igf2* was reduced by approximately 81 – 86%, in the absence of DNA methylation in both embryo and VYS (Fig. 5D). Lastly, in the *Kcnq1* cluster expression of the *Kcnq1ot1* ncRNA increased while expression of the protein-coding ML imprinted genes *Cdkn1c* and *Kcnq1* were reduced in the absence of DNA methylation in both the embryo and VYS (Fig. 5D). We did not examine the expression of EXEL specific genes from the *Kcnq1* cluster in $Dnmt1^{c/c}$ mice, because in the total VYS imprinted expression of these genes in the endoderm layer is masked by biallelic expression in the mesoderm, and we were not able to separate the VYS layers at this early developmental stage (Fig. 4). In summary, this data confirms previous reports that in the absence of DNA methylation, expression of the ML imprinted *Airn, H19* and *Kcnq1ot1* macro ncRNAs is activated, while expression of the ML imprinted *Igf2r, Igf2, Kcnq1* and *Cdkn1c* protein-coding genes is repressed. For the first time, we show that the expression of EXEL imprinted *Slc22a2* and *Slc22a3* protein-coding genes follow the same pattern and are repressed in the absence of DNA methylation. This indicates a similar role for DNA methylation in regulating ML and EXEL imprinted expression.

DISCUSSION

A large number of mouse and human genes have been identified that are considered to show imprinted expression only in extra-embryonic tissues (Hudson et al., 2010; Wagschal and Feil, 2006). Genes that only show extra-embryonic-lineage (EXEL) imprinted expression

are reported to respond differently to loss of DNA methylation and loss of the repressive histone modifying enzymes G9A and EED, compared to genes showing multi-lineage (ML) imprinted expression that is manifest in embryo, adult and extra-embryonic tissues (Lewis et al., 2004; Mager et al., 2003; Nagano et al., 2008; Terranova et al., 2008; Wagschal et al., 2008). Data from these studies was based on the analysis of the extra-embryonic placenta. The potential for the placenta to be contaminated with maternal tissue resulting in artefacts in interpreting imprinted gene expression has recently been highlighted (Proudhon and Bourc'his, 2010). We show here using DNA markers that maternal contamination of 5–14% occurs in the mid-gestation placenta. Maternal contamination will give the appearance of imprinted expression for genes that are more highly expressed in maternal tissues (such as the maternal decidua, blood cells and vessels) than in the placenta. For example, *Osbpl5* and *Tssc4*, shown here to have high levels of expression in the decidua, will show apparent increased expression from the maternal allele due to contamination with maternal tissues. Notably, the use of a reciprocal crosses from different mouse strains, will not detect maternal contamination in the placenta since the maternal polymorphism used to test for imprinted expression in embryonic tissue, is always shared with the mother (Proudhon and Bourc'his, 2010). To overcome the problem of maternal contamination of the placenta, it has been suggested that additional supporting evidence be obtained such as a function in placenta, or conserved imprinted expression in human and mouse placenta (Proudhon and Bourc'his, 2010). Confirmation of imprinted expression in the placenta can also be obtained from studies that show control by known epigenetic features such as the imprint control element (ICE) or the regulatory macro ncRNA (Mancini-Dinardo et al., 2006; Sleutels et al., 2003; Zwart et al., 2001a). We would also suggest that it is essential and perhaps simpler to first demonstrate, for example by *in situ* hybridisation, that expression of the gene arises from placenta and not contaminating maternal tissues (Verhaagh et al., 2001).

An additional problem with the use of the placenta is that this organ arises from multiple embryonic lineages and the imprinted gene under study may show imprinted silencing in one lineage and tissue-specific silencing in other lineages (Verhaagh et al., 2001). As a result, an analysis of whole placenta will not identify repressive epigenetic modifications specific to imprinted gene silencing nor determine the effect of their loss. In this situation, demonstration of re-expression of the silent parental allele of an imprinted gene can equally well arise from loss of tissue-specific silencing that affects both parental alleles (Lewis et al., 2004; Mager et al., 2003; Nagano et al., 2008; Terranova et al., 2008; Wagschal et al., 2008). An optimal organ for the analysis of repressive epigenetic modification that regulate EXEL imprinted expression is one where all lineages show imprinted expression, or one where the cell lineage showing imprinted expression can be isolated. We show here that the visceral yolk sac (VYS) offers an improved model for studying epigenetic mechanisms regulating EXEL imprinted expression that avoids problems associated with the placenta.

We first demonstrated that the *Slc22a2* and *Slc22a3* genes from the *Igf2r* imprinted gene cluster previously described as showing imprinted expression only in the placenta (Zwart et al., 2001a), show imprinted maternal-specific expression that is localized to the VYS endoderm layer. Imprinted expression of the *Slc22a2* and *Slc22a3* genes in the VYS is seen by 8.5dpc and while *Slc22a2* maintains full imprinted expression in later development, the *Slc22a3* gene shows only partial imprinted expression. A similar developmental loss of imprinted expression was also shown for *Slc22a3* in the placenta (Verhaagh et al., 2001; Zwart et al., 2001a). We also confirmed that VYS imprinted expression of *Slc22a2* and *Slc22a3* is controlled by the *Airn* ncRNA as previously demonstrated for imprinted expression in placenta and embryo (Sleutels et al., 2002). Imprinted expression of genes from other clusters, which were previously described as showing imprinted expression only in the placenta (*Osbpl5* and *Tssc4* from the *Kcnq1* imprinted cluster), or in an unspecified yolk sac (*Pon3* from the *Peg10* imprinted cluster) was also examined in VYS. In the total

VYS only a weak maternal expression bias could be detected for these genes, but when we separated the layers a clear maternal allele bias was seen in the endoderm layer only, while the mesoderm layer showed no bias. This illustrates that imprinted expression in a mixed tissue can be masked or appear less biased when imprinted expression is restricted to one cell type, and biallelic expression occurs in other cell types. This may lead to an underestimation of the number of genes showing imprinted expression in organs containing mixed cell types. The localization of *Osbpl5, Tssc4* and *Pon3* maternally biased expression to the VYS endoderm, together with full imprinted expression of *Slc22a2, Slc22a3* and *Sfmbt2*, indicates that EXEL imprinted expression is widespread in this cell lineage. The lack of maternal contamination together with the ability to isolate a pure cell population make this cell lineage an ideal model to study regulation of EXEL imprinted expression.

In separated VYS endoderm maternal biased allelic expression of *Osbpl5, Tssc4* and *Pon3* could be detected, but the biological significance of such incomplete imprinted expression remains unclear. *Slc22a2* and *Slc22a3* do show complete imprinted expression, but when they are deleted the phenotype is mild with no effect on viability (Jonker et al., 2003; Zwart et al., 2001b). There is currently no evidence that these genes display a dose-sensitive phenotype that could explain why EXEL imprinted expression was selected for during evolution. As imprinted genes usually occur in clusters controlled by a single ICE, it could be that selection for one or more dose sensitive genes also affects other nearby non-dose sensitive genes causing them to show imprinted or biased expression, the so-called “innocent bystander effect” (Miri and Varmuza, 2009). This effect may be more pronounced in extra-embryonic lineages due to a different epigenetic environment compared to the embryo.

The RFLP and SNP sequencing approaches used in this study and by others to assess imprinted expression have the disadvantage of being based on a non-quantitative RT-PCR, and being subject to potential RT, PCR, restriction enzyme and sequencing artefacts. These have to be controlled for especially when assaying genes showing weak biased imprinted expression. For some SNP sequencing assays we observed unequal peak heights resulting from inefficient incorporation of labelled ddNTPs, a known problem in dye-terminator sequencing. RNA high throughput sequencing uses reversible dye-terminators and likely suffers from similar artefacts. A recent study used RNA sequencing of C57BL/6J × CAST/EiJ reciprocal crosses to identify more than 1300 loci showing imprinted expression in the brain (Gregg et al., 2010). However, out of 885 genes analyzed 801 had SNPs that conflicted with each other, either by showing parental biased and biallelic expression, or the opposite parental bias. We suggest that in order to confirm that these are not sequencing artifacts the imprinted status of these genes needs to be confirmed by independent assays. Alternative SNP detection systems such as pyrosequencing and the Sequenom MassARRAY™ system may be more quantitative as they do not rely on labeled nucleotides.

A general loss of DNA methylation imprints and imprinted expression occurs in mouse embryos lacking the maintenance DNA methyltransferase *Dnmt1* (Hirasawa et al., 2008; Li et al., 1993). However, some studies report that genes in the *Kcnq1* cluster showing imprinted expression only in the placenta maintain imprinted expression in *Dnmt1* mutant embryos (Caspary et al., 1998; Lewis et al., 2004). These studies used non-quantitative RT-PCR to assay parental allelic expression differences between mutants deficient in *Dnmt1* and wild type littermates. However, other studies that quantitatively assay expression in mutant versus wild type embryos show deregulation of imprinted gene expression independent of whether imprinted expression is restricted to the placenta (Green et al., 2007; Weaver et al., 2010). Notably, a consistent theme in these quantitative assays is that pairs of genes in one cluster show the opposite pattern. For example, the *Airn* ncRNA is upregulated and *Igf2r* is repressed in *Dnmt1* null embryos (Seidl et al., 2006), while in VYS *Slc22a2* and *Slc22a3* are

additionally repressed as shown here. Similarly, the *Kcnq1ot1* ncRNA is upregulated, while genes with ML or EXEL imprinted expression such as *Phlda2*, *Cdkn1c* and *Ascl2* are all down regulated in both embryo and placenta (Green et al., 2007; Weaver et al., 2010). Upregulation of *Kcnq1ot1* and down regulation of *Cdkn1c* in the absence of functional *Dnmt1* was confirmed in VYS in this study. The opposite behavior of the *Airn* and *Kcnq1ot1* macro ncRNAs and the genes they repress (Mancini-Dinardo et al., 2006; Sleutels et al., 2002), is expected when loss of the ICE DNA methylation imprint leads to expression of the regulatory macro ncRNA from the normally silent maternal allele. The opposite response of the *H19* macro ncRNA/*Igf2* pair reflects their shared use of a *cis*-linked methyl-sensitive enhancer (Thorvaldsen et al., 1998), and this has also been suggested to explain the opposite behavior of the *Peg3/Zim1* pair (Weaver et al., 2010). Notably the upregulation of imprinted ncRNAs indicates the ICE gametic imprints directly regulate them, while the down regulation of imprinted protein-coding genes indicates they are downstream of the silencing effect mediated by the unmethylated ICE.

Thus the cumulative data plus results obtained here, shows that genes with ML or EXEL specific imprinted expression are regulated in embryo and extra-embryonic tissues by DNA methylation in a similar manner. The finding that some genes maintain imprinted expression in the placenta despite down regulation of expression could be explained in some cases by maternal contamination, which would have a greater impact on genes that are lowly expressed in the placenta itself. Alternatively, it is possible some genes maintain EXEL imprinted expression due to developmental timing effects that can be observed using different mouse mutants deficient for maternal *Dnmt3L* (that results in a failure to establish ICE methylation imprints in the oocyte), and *Dnmt1* null embryos (that may transiently maintain ICE DNA methylation due to a store of this enzyme in the oocyte). An example of the importance of developmental timing is the *Ascl2* gene that shows EXEL imprinted expression limited to the trophectoderm lineages and is repressed by the *Kcnq1ot1* ncRNA (Mancini-Dinardo et al., 2006). *Aslc2* maintains imprinted expression in *Dnmt1* null embryos, but shows a loss of imprinted expression in the absence of maternal *Dnmt3L* (Arima et al., 2006). Whether this behaviour of *Ascl2* indicates a general feature of EXEL imprinted expression in trophectoderm lineages will depend on further experiments that assess the epiblast, primitive endoderm and trophectoderm lineages for similarities and difference in imprinted gene silencing.

From this study and earlier studies in the placenta, EXEL specific imprinted expression has been localized to 3 different lineages and only in the post-implantation embryo: the ectoplacental cone/spongiotrophoblast lineage, the extra-embryonic ectoderm/labyrinth lineage and the VYS endoderm (Fig. 6). To date, no epiblast derived extra-embryonic lineage has been shown to display EXEL specific imprinted expression. Interestingly this lineage restriction pattern of EXEL imprinted expression is similar to the restriction of imprinted paternal-specific X-inactivation to the VYS endoderm, chorionic ectoderm and trophoblast, although imprinted X-inactivation already occurs in the pre-implantation embryo (Rastan and Cattanach, 1983). In parallel with imprinted X-inactivation the vast majority of EXEL genes show silencing of the paternal allele (Hudson et al., 2010). Using the improved separation technique that we developed, the VYS endoderm cell layer can be efficiently isolated allowing studies to be performed on a pure population of cells that show EXEL specific imprinted expression. Therefore, VYS and VYS endoderm present an improved model for studying the regulatory mechanisms controlling EXEL imprinted expression to identify the factors responsible for the larger number of genes showing EXEL imprinted expression.

Supplementary Material

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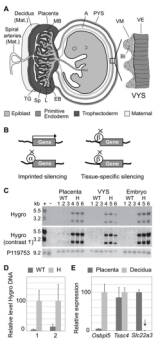

Figure 1. The placenta is contaminated with maternal tissues

(A) Diagrammatic representation of the 13.5dpc embryo with a close-up of the visceral yolk sac (VYS) showing the visceral mesoderm (VM) and extra-embryonic visceral endoderm (VE or VYS endoderm) layers. Different shades indicate each tissue as being of epiblast, primitive endoderm, trophectoderm or maternal origin (see shade key). A amnion, BI blood island, EB embryonic blood, L labyrinth, MB maternal blood, Sp Spongiotrophoblast, TG trophoblast giant cells.

(B) Genes can show imprinted-silencing or tissue-specific silencing that may be mediated by different epigenetic marks (α and β).

(C) DNA blot of +/+ (WT, 1–3) and Hygro/+ (H, 4–6) from placenta, VYS and embryonic head of 13.5dpc mouse embryos probed with hygromycin resistance transgene (Hygro) and loading control P119753.

(D) Quantification of maternal contamination of placenta for 2 different dissectors by real-time qPCR comparing hygromycin detected in +/+ (WT) and Hygro/+ (H) placentas. Values are normalized to Gapdh DNA levels, and then the Hygro/+ mean for each dissector set to 100. n = 9 in each case, error bars = 1 standard deviation.

(E) Real-time RT-qPCR of *Osbpl5*, *Tssc4*, and *Slc22a3* from 12.5dpc placenta and decidua samples (5 pooled samples). Values are normalized to *CypA* levels and the tissue showing the highest expression level for each gene set to 100. Error bars = 1 standard deviation.

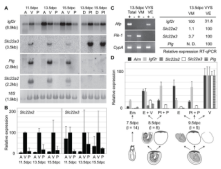

Figure 2. *Slc22a2* and *Slc22a3* expression is restricted to the endoderm of the visceral yolk sac

(A) RNA blot showing expression of *Igf2r*, *Slc22a3*, *Plg*, *Slc22a2* and *18S* in the amnion (A), visceral yolk sac (V), and parietal yolk sac (P) at 11.5dpc, 13.5dpc, and 15.5dpc, and at 13.5dpc and 15.5dpc for decidua (D) and placenta (Pl).

(B) Real-time RT-qPCR quantifying the expression levels of *Slc22a2* and *Slc22a3* in the amnion (A), visceral yolk sac (V) and parietal yolk sac (P) at 11.5dpc, 13.5dpc and 15.5dpc. Values normalized to the mean of *18S* and *Gapdh* expression, visceral yolk sac mean set to 100, standard deviation of biological replicates shown.

(C) RT-PCR showing expression of the extra-embryonic visceral endoderm (VE or VYS endoderm) marker *Afp* and visceral mesoderm (VM) marker *Flk-1* is restricted to these tissues in visceral yolk sac (VYS), while the ubiquitously expressed *CypA* is expressed in both tissues. Real time RT-qPCR shows that *Igf2r* is expressed in both VM and VYS endoderm, while *Slc22a2*, *Slc22a3* and *Plg* show expression largely restricted to the VYS endoderm of the VYS. Values normalized to *18S* expression, with the tissue showing the highest expression set to 100 for each gene. N. D. not detected.

(D) Real-time RT-qPCR showing the expression level of *Airn*, *Igf2r*, *Slc22a2*, *Slc22a3* and *Plg* expression at 7.5dpc (whole embryo, Em), 8.5dpc (embryo plus VYS (E + V) and placenta plus parietal yolk sac (Pl + P) collected separately) and 9.5dpc (embryo (E), placenta plus parietal yolk sac (Pl + P) and VYS (V) collected separately). Cartoons illustrating the embryo parts taken at each time point are shaded according to the cell lineage code in Fig. 1A (adapted from Theiler, 1989). One litter was pooled for each time point (litter size indicated). The data was normalized to *CypA* expression with the value for E9.5 VYS set to 100 for each gene. Standard deviation of 3 technical replicates is shown.

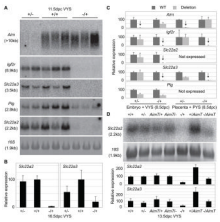

Figure 3. *Slc22a2* and *Slc22a3* show imprinted expression in visceral yolk sac

(A) RNA blot showing expression of *Airn, Igf2r, Slc22a3, Plg, Slc22a2* and *18S* in the visceral yolk sac (VYS) of T-hairpin paternal deletion (+/−), wild type (+/+) and maternal deletion (−/+) 11.5dpc mouse embryos.

(B) Real-time RT-qPCR of *Slc22a2* and *Slc22a3* at 16.5dpc in VYS of paternal deletion (+/−), wild type (+/+) and maternal deletion (−/+) mouse embryos. Values are normalized to *18S* expression and wild type set to 100. Standard deviation of 4 biological replicates is shown.

(C) Real-time RT-qPCR of *Igf2r, Airn, Slc22a2, Slc22a3* and *Plg* in paternal deletion cross (+/−) and maternal deletion cross (−/+) 8.5dpc embryos divided into embryo with VYS and placenta with parietal yolk sac (PYS). For each cross wild type (WT) and deletion littermates were assayed. Values are normalized to *CypA* and the wild type for each cross set to 100. Number of pooled embryos: paternal deletion cross WT 6, +/− 5, maternal deletion cross WT 6, −/+ 3. Standard deviation of 3 technical replicates on pooled tissues is shown.

(D) RNA blot of *Slc22a2* and *18S* and real-time RT-qPCR of *Slc22a2* and *Slc22a3* for wild type (+/+), paternal deletion (+/−), maternal *Airn* truncation (*AirnT*/+), maternal *Airn* truncation and paternal deletion (*AirnT*/−), maternal deletion (−/+), paternal *Airn* truncation (+/*AirnT*), and maternal deletion and paternal *Airn* truncation (−/*AirnT*) in 12.5dpc VYS. Values are normalized to *CypA* levels and wild type set to 100. Standard deviation of 3 technical replicates from pooled samples is shown.

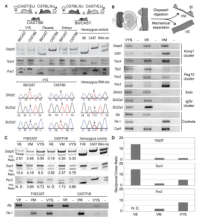

Figure 4. Extra-embryonic imprinted expression in the visceral yolk sac is restricted to the extra-embryonic visceral endoderm

(A) Genes previously reported to have extra-embryonic lineage (EXEL) specific imprinted expression were tested for imprinted expression in the visceral yolk sac (VYS). Strain-specific SNPs between C57BL/6J and CAST/EiJ were examined in a minimum of 2 biological replicates of 12.5dpc embryos from reciprocal crosses by a restriction fragment length polymorphism (RFLP) assay or by sequencing (representative examples shown). Homozygous RNA controls (detailed below) were analyzed separately and as an equal ratio mixed RNA was used as a control for the efficiency of reverse transcription, PCR and sequencing. Note that *Ospbpl5* shows unequal expression levels between the two strains.
(B) The two layers of the visceral yolk sac from FVB/N 12.5dpc embryos were separated mechanically following incubation in DispaseII. Successful separation was confirmed by RT-PCR for markers of visceral endoderm (*Afp*) and mesoderm (*Flk-1*). Localization of previously reported EXEL genes in total VYS (VYS), extra-embryonic visceral endoderm (VE or VYS endoderm), and visceral mesoderm (VM) is shown by RT-PCR.
(C) VYS layer separation into VE (Visceral endoderm) and VM (visceral mesoderm) was performed on 13.5dpc embryos from a FVB/N and CAST/EiJ reciprocal cross (tissues were pooled from 6 embryos before RNA preparation due to the low yield of separated tissues per embryo). The efficiency of the dissection was confirmed by an RT-PCR assay for *Afp* and *Flk-1*. An RFLP assay was used to assay parental allelic *Ospbpl5, Tssc4 and Pon3* expression in separated layers and in the total VYS. Homozygous RNA controls are detailed below. Gels were scanned using the Typhoon™ scanner and non-saturated bands quantified using the ImageQuant™ software (GE Healthcare). The F/C ratio (FVB/N divided by CAST/EiJ band intensity) for each lane is shown below each gel (note the band intensity ratio for *Pon3* in VE was not determined (N. D.) because the paternal band was too weak to quantify).
(D) Parental allelic expression displayed as a reciprocal cross ratio was calculated from the gels in Fig. 4C by dividing the F/C ratio of the FVB/N × CAST/EiJ cross by the F/C ratio of the CAST/EiJ × FVB/N cross and is displayed as a bar graph showing clear maternal biased expression of *Ospbpl5* and *Tssc4* (see text for details). Note: Unequal expression levels between strains can influence band intensity in RFLP assays and signal intensity at a SNP in a sequencing track. In the absence of strain bias, larger bands are more intense in RFLP assays due to increased ethidium bromide staining. A reciprocal cross ratio reduced the influence of these artefacts.
Homozygous RNA controls were adult kidney for *Tssc4, Osbpl5, Sfmbt2, Slc22a2* and adult heart for *Pon3* and *Slc22a3*. For each cross the female is written first. PCR primers and enzymes used for sequencing and RFLP assays are detailed in Supplementary Table 2.

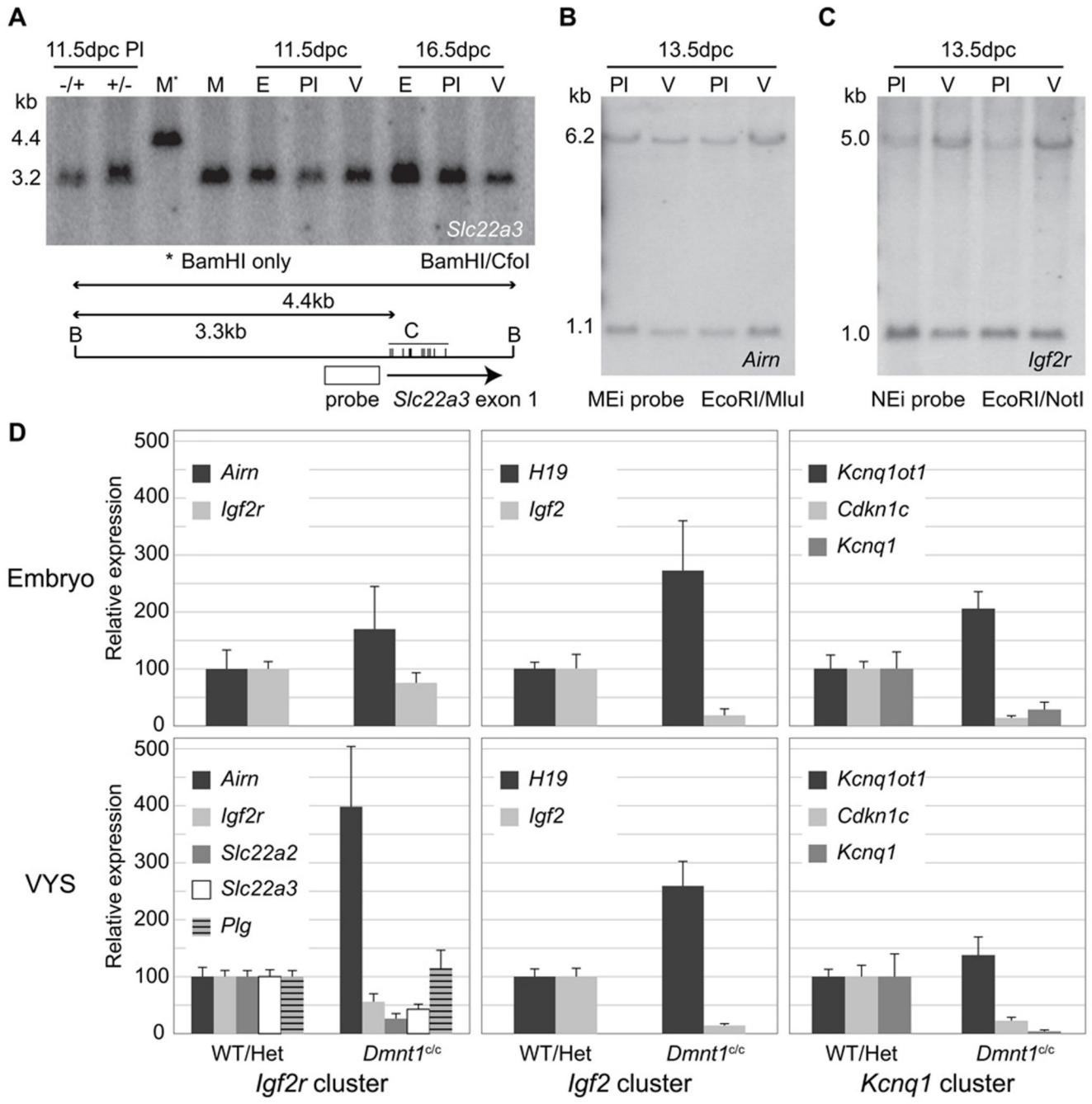

Figure 5. DNA methylation regulates imprinted expression in the visceral yolk sac

(A) The *Slc22a3* CpG island promoter does not show parental-specific methylation in visceral yolk sac (VYS). DNA blot using BamHI and the methyl sensitive CfoI. −/+ maternal deletion, +/− paternal deletion, M mouse embryonic fibroblasts, E embryo trunk, Pl placenta, V visceral yolk sac.

(B) Methylation blot of the *Airn* promoter using a EcoRI/MluI digest and the MEi probe. The methylated band is detected at 6.2kb and the non-methylated band at 1.1kb.

(C) Methylation blot of *Igf2r* promoter using a EcoRI/NotI digest and the NEi probe. The methylated band is detected at 5.0kb and the unmethylated band at 1.0kb.

(D) Real-time RT-qPCR of genes in the *Igf2r* imprinted cluster, *Igf2/H19* imprinted cluster and the *Kcnq1* imprinted cluster comparing expression in $Dnmt1^{+/+}$ and $Dnmt1^{+/c}$ (WT/Het, n = 6) with $Dnmt1^{c/c}$ (n = 6) in 9.5dpc embryos and VYS. Values are normalized to *Gapdh* levels with the WT/Het level set to 100 for each gene and tissue. The standard deviation of biological replicates is shown. *Slc22a2, Slc22a3* and *Plg* are not expressed in embryos.

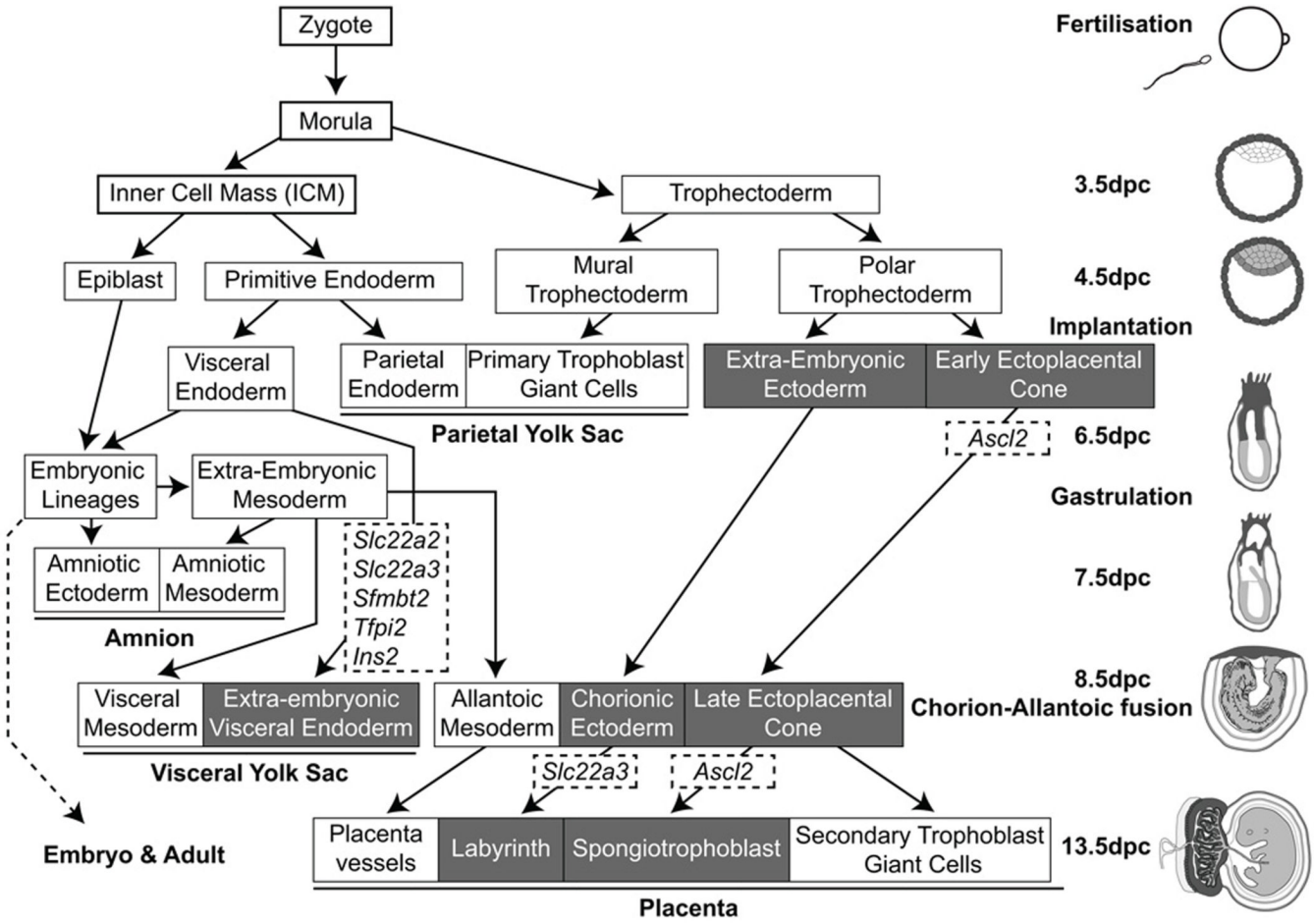

Figure 6. Extra-embryonic specific imprinted expression is limited to specific extra-embryonic lineages

The diagram shows the derivation of extra-embryonic cell lineages during mouse embryonic development. Extra-embryonic lineages that have been shown to display EXEL specific imprinted expression are highlighted in grey. Genes identified as showing full EXEL imprinted expression are boxed with a dotted line. Note that all embryonic and extra-embryonic cell lineages express genes such as *Igf2r* that show full ML imprinted expression. On the right side the stage of development coinciding with the origin of the cell lineage is shown together with cartoons representing embryos at that stage shaded according to the cell lineage code in Fig. 1A (adapted from Theiler, 1989). The cell lineage diagram is adapted from (Nagy et al., 2003).