Placental imprinting of SLC22A3 in the IGF2R imprinted domain is conserved in therian mammals

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Abstract

Background: The eutherian IGF2R imprinted domain is regulated by an antisense long non-coding RNA, Airn, which is expressed from a differentially methylated region (DMR) in mice. Airn silences two neighbouring genes, Solute carrier family 22 member 2 (Slc22a2) and Slc22a3, to establish the Igf2r imprinted domain in the mouse placenta. Marsupials also have an antisense non-coding RNA, ALID, expressed from a DMR, although the exact function of ALID is currently unknown. The eutherian IGF2R DMR is located in intron 2, while the marsupial IGF2R DMR is located in intron 12, but it is not yet known whether the adjacent genes SLC22A2 and/or SLC22A3 are also imprinted in the marsupial lineage. In this study, the imprinting status of marsupial SLC22A2 and SLC22A3 in the IGF2R imprinted domain in the choriovitelline placenta was examined in a marsupial, the tammar wallaby.

Results: In the tammar placenta, SLC22A3 but not SLC22A2 was imprinted. Tammar SLC22A3 imprinting was evident in placental tissues but not in the other tissues examined in this study. A putative promoter of SLC22A3 lacked DNA methylation, suggesting that this gene is not directly silenced by a DMR on its promoter as seen in the mouse. Based on immunofluorescence, we confirmed that the tammar SLC22A3 is localised in the endodermal cell layer of the tammar placenta where nutrient trafficking occurs.

Conclusions: Since SLC22A3 is imprinted in the tammar placenta, we conclude that this placental imprinting of SLC22A3 has been positively selected after the marsupial and eutherian split because of the differences in the DMR location. Since SLC22A3 is known to act as a transporter molecule for nutrient transfer in the eutherian placenta, we suggest it was strongly selected to control the balance between supply and demand of nutrients in marsupial as it does in eutherian placentas.

Background

Genomic imprinting is a complex epigenetic process that leads to expression of a subset of genes in a parent-of-origin specific way [1, 2]. Amongst vertebrates, this phenomenon has been found in therian mammals (eutherians and marsupials), but there is no evidence for imprinting so far in monotreme mammals or non-mammalian vertebrates [3–6]. Since many imprinted genes function in the mammalian placenta, genomic imprinting is thought to have evolved in concert with mammalian placentation [7–12]. In this context, the ‘supply and demand’ theory suggests that imprinted genes in the placenta evolved to regulate the balance of nutritional interactions between mother and the fetus carrying father’s genes by controlling the supply of nutrients [13]. However, postnatal imprinting has been characterised in both marsupials and eutherians [14–17], so imprinting is involved not only in placentation, but also in postnatal growth and development. The question remains as to how and why genomic imprinting
evolved only in therian mammals amongst vertebrates. As imprinting must have evolved in the common ancestor of therian mammals, by comparing the characteristics of imprinting between marsupials and eutherians, we may be able to identify ancestral features of imprinting and understand how it evolved.

Imprinted genes of eutherians tend to be clustered in the genome [18, 19]. The grouping of imprinted genes within clusters allows sharing of common regulatory elements such as non-coding RNAs and differentially methylated regions (DMRs). When these regulatory elements control the imprinting status of clustered genes, they are known as imprinting control regions (ICRs). In eutherians, one of the best characterised imprinting clusters is the insulin-like growth factor 2 receptor (IGF2R) gene locus [20–26]. In mice, the Igf2r gene is a paternally imprinted (maternally expressed) gene, and its imprinting is regulated by an intrinsic CpG island, which is a DMR, and the antisense long non-coding (Inc) RNA, Airn [23, 25–28]. The DMR is established during gametogenesis and has differential epigenetic modifications between gametes [29, 30]. It acts as a promoter of the IncRNA, Airn [27]. Airn is a maternally imprinted (paternally expressed) gene and silences Igf2r expression on the paternal genome by transcriptional overlap [23]. This IncRNA also regulates the imprinting of a 10 + Mb region that includes two neighbouring genes in the placenta, the solute carrier family 22 members 2 and 3 (Slc22a2 and Slc22a3) [22, 28, 31] as well as seven distal genes more than 2 Mb away from the Igf2r locus [32]. Airn establishes epigenetic silencing of Slc22a3 by recruiting a histone modification enzyme, euchromatin histone methyltransferase 2 (EHMT2, also known as G9a) [31]. Although the exact mechanism by which Airn inactivates the paternal Slc22a2 is currently unknown, Airn may recruit histone 3 lysine 27 trimethylation (H3K27me3) and the polycomb repressive complex 2 (PRC2) to the gene locus [22]. Therefore, the DMR at the intrinsic CpG Island which controls Airn expression is the ICR of the Igf2r imprinted domain in mice [20, 25, 26]. Although the IncRNA-based mechanisms for establishing Igf2r imprinted domain in mice are well documented, imprinting of IGF2R and its neighbouring genes in other eutherian mammals is different from that of mice. In cows, IGF2R, AIRN, SLC22A2 and SLC22A3 are imprinted in their placentas [33]. Although there is no information about imprinting of SLC22A2 and SLC22A3, IGF2R is imprinted in sheep and dogs [34, 35]. In humans, genes of the IGF2R imprinted domain show an indicator of imprinting, but it is polymorphically imprinted in a subset of placentas [36]. In contrast, in pigs, the evidence is confused since in one study there is paternal IGF2R expression [37], in a second study there is maternal IGF2R expression [38] and in the third study there is bi-allelic expression [39]. SLC22A3 is not imprinted in their placentas [40]. Therefore, while the IGF2R imprinted domain is likely to have been present in the common ancestor of eutherian mammals it may not have been strongly selected in some species such as pigs. Whether the IGF2R imprinted domain evolved in a marsupial ancestor or developed by convergent evolution in marsupial lineages is currently unknown. Comparing the IGF2R gene locus between marsupials and eutherians would clarify its evolution.

The marsupial IGF2R gene is also imprinted [41–43]. However, until recently, it was assumed that IGF2R in marsupials lacked key regulatory features such as Airn and a DMR [41, 43] as there was no DMR at intron 2, the location of the mouse Igf2r DMR/ICR. However, we described a novel DMR in intron 12 which has a 687 bp antisense IncRNA, Antisense LncRNA in IGF2RDMR (ALID) [43]. The DMR location is totally different between eutherians (intron 2) [26, 30] and marsupials (intron 12) [43]. ALID is much shorter than mouse Airn [43], so the imprinting mechanism of IGF2R in marsupials may be different from that of mouse Igf2r. However, it is still possible that ALID may have an analogous mode of action in silencing the flanking genes SLC22A2/SLC22A3 in the placenta. If SLC22A2 and/or SLC22A3 are imprinted in the marsupial lineage, it would provide strong evidence that placenta-specific imprinting has been strongly selected for therian mammals, despite the differences in the DMR location.

In this study, the imprinting status of SLC22A2 and SLC22A3 in marsupial placentalas was investigated using a marsupial, the tammar wallaby (Macropus eugenii). The tammar has an epithelio-chorial placenta which consists of two regions, the avascular bilaminar omphalopleure (BOM) and the vascular trilaminar omphalopleure (TOM) separated by the terminal blood vessel, the sinus terminalis [44]. Marsupial orthologues of SLC22A2 and SLC22A3 were investigated by combining molecular experiments and analysis of tammar transcriptome data sets. Imprinting analysis was thereafter performed in the tammar placentas and other tissues to confirm its imprinting. We further evaluated protein localisation to determine conserved transporter function. Here, we report that SLC22A3 but not SLC22A2 is imprinted in the tammar placenta. By confirming SLC22A3 imprinting, this study revealed that the IGF2R imprinted domain has been strongly selected in marsupial mammals after either transposition or independent acquisition of the marsupial IGF2R DMR.
Results

Identification of marsupial SLC22A2

To characterise the imprinting cluster of the marsupial IGF2R gene locus, the marsupial orthologue of SLC22A2 was searched for using the wallaby genome database (Wallabase: https://wallabase.science.unimelb.edu.au/) comparing with mouse SLC22A2 (Accession number: NM_013667.3). 1693 bp of putative tammar SLC22A2 were identified. The putative tammar SLC22A2 was found in the vicinity of the tammar IGF2R gene. Furthermore, another SLC22A family gene, the SLC22A1 gene candidate, was found between the putative SLC22A2 and IGF2R, as seen in eutherians. Based on this conserved synteny, we considered the putative SLC22A2 as a candidate gene for our downstream analysis. While mouse and human SLC22A2 have 11 exons including 5’ and 3’ UTRs, the putative tammar SLC22A2 had 9 exons without a 3’ UTR (Fig. 1A). To characterise potential isoforms of SLC22A2 in placentas, full-length of tammar SLC22A2 transcripts were examined by 5’ and 3’ Rapid amplification of cDNA ends (RACE) reactions using BOM cdNA (Fig. 1B). While the 5’ RACE reaction produced a distinct single band, the 3’ RACE reactions produced multiple bands depending on primers used (Fig. 1B). By comparing three different 3’ RACE reactions, we confirmed that only the largest RACE product contained partial SLC22A2 with a poly-A tail and upstream polyadenylation signals (Fig. 1B). The identified full-length tammar SLC22A2 contained 11 exons and encoded 554 a.a (Fig. 1B). The identified tammar SLC22A2 had a high consensus with mouse SLC22A2 (similarity: 90%, identity: 79%) and human SLC22A2 (similarity: 88%, identity: 78%). The tammar SLC22A2 shared the major facilitator superfamily (MFS) domain with mouse Slc22a2 and human SLC22A2 (Fig. 1C).

The tammar SLC22A2 is not imprinted in placenta tissues

In order to identify potential single nucleotide polymorphism (SNP) sites, publically available tammar transcriptome data sets were analysed (SRA accession number: DRP001145). After analysing several adult tissues (testis, liver, lung, heart, spleen and brain), transcriptome data derived from liver were confirmed to have mapped-reads to the SLC22A2 exons which were confirmed by the RACE experiments. Fortunately, the liver transcriptome data had an informative SNP at the last exon of SLC22A2 (Fig. 2A). The C/G SNP was further confirmed by PCR using fetal gDNA followed by direct sequencing of the PCR products. After confirming the heterozygous C/G SNP in fetal gDNA, allelic expression analysis of SLC22A2 transcript was performed. In both BOM and TOM tissues, SLC22A2 clearly showed bi-allelic expression (Fig. 2B).

Identification of marsupial SLC22A3

To characterise the imprinting cluster of marsupial IGF2R gene locus, marsupial orthologue of SLC22A3 was searched for using the wallaby genome database (Wallabase: https://wallabase.science.unimelb.edu.au/) with mouse SLC22A3 (Accession number: NM_011395.2). 1692 bp of putative tammar SLC22A3 were identified. The putative tammar SLC22A3 was found next to the tammar SLC22A2 gene, as seen in eutherians. Based on this conserved synteny, we considered the putative SLC22A3 as a candidate gene for our downstream analysis. While mouse and human SLC22A3 have 11 exons including 5’ and 3’ UTRs, the putative tammar SLC22A3 had 11 exons without the 3’ UTR (Fig. 3A). To characterise potential isoforms of SLC22A3 in placentas, full-length of tammar SLC22A3 transcripts were examined by 5’ and 3’ RACE reactions using BOM cdNA (Fig. 3B). While the 5’ RACE reaction produced a distinct single band, the 3’ RACE reactions produced two different RACE products (Fig. 3B). After cloning and sequencing each RACE product, two different isoforms were confirmed. Both isoforms had poly-A tail and upstream polyadenylation signals (Fig. 3B). Although there were at least two isoforms based on the differences in the exon structure, all isoforms encoded the same 563 amino acids. The identified tammar SLC22A3 had a high homology with mouse Slc22a3 (similarity: 89%, identity: 81%) and human SLC22A3 (similarity: 91%, identity: 84%). The tammar SLC22A3 shared the MFS domain with mouse and human SLC22A3 (Fig. 3B and C).

The tammar SLC22A3 is imprinted in bilaminar placenta tissues

In order to identify potential SNP sites, published tammar transcriptome data sets were analysed. After analysing several adult tissues (testis, liver, lung, heart, spleen and brain), transcriptome data sets derived from heart and spleen were confirmed to have mapped-reads matched the SLC22A3 exons based on the RACE experiments. Fortunately, both heart and spleen transcriptome data had an informative SNP at the first exon of SLC22A3 (Fig. 4A). After confirming the T/G SNP by PCR reaction, allelic expression analysis of SLC22A3 transcripts was performed using the SNP information. Of the 20 biological replicates examined in this study, 3 samples had a clear heterozygous SNP. In BOM tissues (n = 3), SLC22A3 showed strongly skewed allelic expression with the signal intensities that differed between the two alleles more than fivefold and 2 out of the 3 animals showed clear...
Fig. 1 Identification of marsupial orthologue of SLC22A2 in the tammar. A Exon structure of mouse Slc22a2, human SLC22A2 and tammar putative SLC22A2. Black boxes and white boxes represent protein-coding exon and UTRs, respectively. B 5′ and 3′ RACE primers and RACE results. Full-length of tammar SLC22A2 encoding 554 a.a. was determined by RACE experiments. Asterisks indicate RACE product containing partial SLC22A2 sequences. 3′ RACE product contained poly-A signal (red-letters) and poly-A tail (green letters). C Protein alignment. Red-coloured highlight represents the major facilitator superfamily (MFS) domain of the tammar SLC22A2.
maternal expression as the mothers had homozygous SNP (Fig. 4B). The other animal also showed a strongly skewed allelic expression (Fig. 4B). However, since the mother of the animal was heterozygous, we could not conclude its parental origin-specific expression. The trilaminar placenta tissues of the same animals did not show the same strongly biased allelic expression as seen in the BOM tissue (Fig. 4C).

**SLC22A3 has variable expression in pouch young heart and spleen**

Since mouse Slc22a3 imprinting is only observed in the placenta [20], the tammar SLC22A3 may have a similar tissue-specific imprinting pattern. To confirm tissue-specific imprinting of SLC22A3 in the tammar, allelic expression of SLC22A3 was investigated using the tammar pouch young (PYs). Heart and spleen tissues derived from PYs were used for this analysis as the transcriptome data showed SLC22A3 expression in those tissues. After checking 12 animals, 6 animals had the heterozygous T/G SNP. In all of the 6 animals, SLC22A3 showed bi-allelic expression in heart (Fig. 5). In spleen, while two males had skewed expression with signal intensities that differed between the two alleles more than twofold, the other males and the three females showed bi-allelic expression (Fig. 5).

**SLC22A3 imprinting is not promoter DNA methylation dependent**

Given the imprinting of tammar SLC22A3 in the tammar placenta, we next analysed DNA methylation at its promoter to ask whether tammar SLC22A3 imprinting is DMR dependent or not. Based on the MethPrimer programme, we identified two CpG islands located over the putative promoter region of the tammar SLC22A3 (Fig. 6). Bisulphite sequencing of the CpG islands demonstrated that the majority of CpG sites were unmethylated in the entire region of the putative SLC22A3 promoter in both BOM and TOM tissues (Fig. 6).

**SLC22A3 is present in endodermal cells of the BOM and TOM**

To determine its conserved role in placental tissues, protein localisation of SLC22A3 in the BOM and TOM was examined (Fig. 7). SLC22A3 was localised at the luminal surface of endometrial tissues (Fig. 7B). SLC22A3 protein was detected in the endodermal cells of both BOM and TOM placental tissues (Fig. 7C).

**Discussion**

In the tammar placentas, SLC22A3 but not SLC22A2 was imprinted. As in the mouse, tammar SLC22A3 imprinting was evident in BOM placenta tissues but not in the other tissues examined in this study. We conclude that the
Fig. 3 Identification of marsupial orthologue of SLC22A3 in the tammar. A Exon structure of mouse Slc22a3, human SLC22A3 and tammar putative SLC22A3. Black boxes and white boxes represent protein-coding exon and UTRs, respectively. B 5' and 3' RACE primers and RACE results. Two isoforms of tammar SLC22A3 was determined by RACE experiments, and both encoded 563 a.a. Asterisks indicate RACE product containing partial SLC22A3 sequences. 3' RACE products contained poly-A signal (red-letters) and poly-A tail (green letters). C Protein alignment. Red-coloured highlight represents the major facilitator superfamily (MFS) domain of the tammar SLC22A3.
SLC22A3 imprinting in mammalian placentas evolved within the IGF2R imprinted domain in both marsupial and eutherian mammals.

SLC22A3 is a poly-specific organic cation transporter that transfers a wide variety of substrates and toxins across the cell membrane [45–47]. In Slc22a3 knockout mice, although there are no obvious placental and fetal growth defects, there are effects on placental transfer functions [48]. In concert with its transporter activity, mouse Slc22a3 is localised in the labyrinth layer of its chorio-allantoic placenta [49–51] where nutrient transfer from maternal blood occurs [52]. Mouse Slc22a3 is also present in the visceral endoderm of the yolk sac [51], where nutrient uptake into the embryo occurs [53]. In the tammar chorio-vitelline placenta, BOM appears to be responsible for a greater uptake of nutrients from uterine secretions than TOM [54–57]. SLC22A3 imprinting was evident in BOM tissues, suggesting that SLC22A3 imprinting is associated with uptake of nutrients from uterine secretions by the avascular BOM. Based on immunofluorescence, we confirmed that tammar SLC22A3 is localised in the endodermal layer of both BOM and TOM side of the tammar yolk sac (chorio-vitelline) placenta. The tammar placental endodermal cells are also derived from trophoblast, and in this and the mouse study [51] SLC22A3 expression in the endoderm cells of the yolk sac is conserved between mouse and the tammar. Furthermore, since a labyrinth layer marker, GCM1, is also present in the endodermal cell layer of the tammar placenta [56, 58], our data suggest that SLC22A3 is a conserved nutrient transporter in theian placentas and supports the previous suggestions [56, 59] that the endodermal cell layer functions as a centre of nutrient trafficking in the tammar placenta.
In the tammar placenta, the \( SLC22A3 \) imprinting was evident only in BOM tissues. However, it is currently unknown why only BOM tissues but not TOM tissues show imprinted \( SLC22A3 \) expression in the tammar placenta. In concert with their functional role in either nutrient transport (BOM) or respiration (TOM) [44, 57], BOM and TOM have different transcriptional profiles [56]. This suggests that BOM tissues are differentially transcriptionally regulated, resulting in imprinted expression of \( SLC22A3 \). Alternatively, other cells such as mesodermal cells and nucleated red blood cells in TOM tissues may express the \( SLC22A3 \) gene. In mice, \( Slc22a3 \) is highly expressed in the visceral endoderm and is present, albeit at low expression, in the mesoderm cells of the visceral yolk sac [51], so, it is possible that mesoderm cells masks \( SLC22A3 \) imprinted expression in the tammar TOM.

The mouse \( Igf2r \) imprinted domain is regulated by the lncRNA, \( Airn \) [20, 28, 31]. The \( Airn \) transcript recruits a histone modification enzyme, EHMT2, and adds an inactive histone-3 lysine-9 di-methylation at the \( Slc22a3 \) gene locus [31]. In addition, \( Airn \) recruits H3K27me3 to \( Slc22a2 \) and \( Slc22a3 \) to establish the broad imprinted domain in mice [22], and there is no promoter DNA methylation at either \( Slc22a2 \) or \( Slc22a3 \) in mice [20, 51], suggesting that both are regulated by histone modification-based imprinting. Similarly, human \( SLC22A2 \) and \( SLC22A3 \) also lack a promoter DMR in placenta [36]. Like the tammar, the cow has a chorio-epithelial placenta, and has \( SLC22A3 \) but not \( SLC22A2 \) with a DMR at the promoter region in its placenta [33]. Since \( AIRN \) is conserved across eutherians [33, 60], the bovine study indicates that \( SLC22A3 \) imprinting mechanism can be varied even within eutherian mammals. In our analysis, CpG islands at the putative promoter of the tammar
SLC22A3 lacked DNA methylation. This suggests that tammar SLC22A3 imprinting is not directly silenced by a DMR on its promoter as in mice and humans. In this context, the tammar IncRNA, ALID, is likely to have a similar function to Airn as this IncRNA is also expressed from a DMR which may be an ICR [43]. Further analysis of the interactions between EHMT2, PRC2 and ALID would be interesting in subsequent studies to determine potential function of ALID in silencing SLC22A3 in the tammar placenta.

In marsupials, the IGF2R DMR is located in intron 12 while the eutherian IGF2R DMR is located in intron 2 [26, 43, 61] (Fig. 8A). Based on this difference in genomic position, marsupial and eutherian IGF2R DMRs may have been translocated after the divergence of marsupials and eutherians (Fig. 8B and C), or they may have been acquired independently in each mammalian lineage after the marsupial–eutherian split (Fig. 8D) [43]. In either case, our study demonstrates that the SLC22A3 imprinting in placentas has been strongly selected in therian
mammals during mammalian evolution (Fig. 8). Since SLC22A3 is a poly-specific transporter in placental tissues that regulates placental transfer, this selection may have occurred to control the balance between supply and demand of nutrients.

**Conclusions**

By confirming imprinting of the SLC22A3 gene in a marsupial placenta, our data suggest the SLC22A3 imprinting has been strongly selected in both marsupials and eutherians the differences in the DMR locations. Our data further suggest that the control of nutrient transport in the placenta is a critical evolutionary function of genomic imprinting in marsupial, as well as in eutherian placentas.

**Material and methods**

**Animals**

Tammar wallabies (*Macropus eugenii*), of Kangaroo Island origin, were held in open grassy yards in our breeding colony at the University of Melbourne. Adults and pouch young (PY) were killed humanely as previously described [14, 55, 57]. Placentas from fetuses in the final third of gestation [day 19–25 days of the 26.5 day pregnancy (*n* = 20)] were collected post mortem from...
adult females and either snap frozen or fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH7.4), washed and stored in 100% methanol before histological analysis. PY aged between day 33 and day 81 after birth (n = 12) were dissected post mortem and heart and spleen snap frozen immediately. All animal experiments were approved by the University of Melbourne Animal Experimental Ethics Committees and followed the Australian National Health and Medical Research Council (2013) guidelines.

RNA extraction and cDNA synthesis
Snap-frozen placenta tissues consisting of the separated avascular bilaminar omphalopleure (BOM) and the vascular trilaminar omphalopleure (TOM) (see Fig. 7A), PY spleen and PY heart were used for RNA extraction using the GenElute Mammalian total RNA Miniprep Kit (Sigma-Aldrich, Missouri, USA) following the manufacturer’s instructions. The extracted RNA was treated with the DNA-free DNase treatment and removal kit (Thermo Fisher Scientific, Massachusetts, USA) to remove residual genomic DNA (gDNA). After confirming removal of gDNA by a Nanodrop (Thermo Fisher Scientific, Massachusetts, USA) and PCR using RNA as a template, either 200 ng of placenta RNA or up to 800 ng of PY spleen RNA or up to 400 ng of PY heart RNA were used as templates for cDNA synthesis using SuperScript IV First strand Synthesis System (Invitrogen, Carlsbad, USA).

5’ and 3’ rapid amplification of cDNA ends (RACE)
To determine the complete full sequence of marsupial SLCC22A2 and SLCC22A3, 5’ and 3’, rapid amplification of cDNA ends (RACE) experiments were performed using a SMARTer RACE 5’/3’ kit (Clontech, California, USA). The first RACE reactions were performed with BOM cDNA using SeqAmp DNA Polymerase (Clontech, California, USA) with gene specific primers (Additional file 1). The nested 5’ and 3’ RACE reactions were performed by GoTaq DNA polymerase (Promega, Wisconsin, USA) and the RACE products were cloned using pGEM-T Easy Vector (Promega, Wisconsin, USA) and Escherichia coli JM109 competent cells (Promega, Wisconsin, USA). Plasmids were extracted using Wizard Plus SV Minipreps DNA Purification System (Promega, Wisconsin, USA) and directly sequenced with M13 primers (Additional file 1).

Comparative analysis of therian SLCC22A2 and SLCC22A3
DNA sequences of human SLCC22A2, human SLCC22A3, mouse Slc22a2 and mouse Slc22a3 were obtained from NCBI (https://www.ncbi.nlm.nih.gov). Amino acid sequences retrieved from DDBJ/EMBL/GenBank/RefSeq database were used for generating alignment using CLC sequence viewer 8 (https://resources.qiagenbioinformatics.com/manuals/clcsquenceviewer/current/index.php?manual=CLC_Sequence_Viewer_vs_Workbenches.html). Accession numbers: Homo sapiens SLCC2A2, NM_003058.4; Mus musculus Slc22a2, NM_013667.3; H. sapiens SLCC2A3, NM_021977.4; M. musculus Slc22a3, NM_011395.2. Functional domains of deduced amino acid sequences were examined with the Prosite server (http://prosite.expasy.org).

Transcriptome analysis
To characterise the IGF2R imprinted domain as well as identifying informative single nucleotide polymorphisms (SNPs), tammar transcriptome data sets derived from various tissues (testis, liver, lung, heart, spleen and brain) were analysed. Publicly available tammar raw RNA-seq data sets (DRP001145) were downloaded from NCBI SRA (https://www.ncbi.nlm.nih.gov/sra). All RNA-seq reads were trimmed using TrimGalore! (v0.6.5) (https://github.com/FelixKrueger/TrimGalore) with default settings. The trimmed reads were aligned to the wallaby genome.v3 (https://wallabase.science.unimelb.edu.au) using HISAT2 (v2.1.0) [62] with a parameter—-rnamethodness FR to reflect the strandedness of sequenced RNA. The mapped reads were assigned to each strand by Samtools (v1.9) [63]. SNP sites were called using BCFtools (v1.9) and the output file was compared with the mapped reads on Integrative genome viewer (IGV) [64, 65].

Genomic DNA extraction
Snap-frozen BOM, TOM, PY tails and endometrial tissues were used for genomic DNA (gDNA) extraction. BOM and TOM were the fetal gDNA source and endometrium was the maternal gDNA source. PY tails were used for genotyping. DNA extraction was performed with Wizard Genomic DNA purification kit (Promega, Wisconsin, USA) following the manufacturer’s instructions.

Allelic expression analysis
Extracted gDNA was used as a template for PCR amplification. PCR reaction was performed using gene specific primers (Additional file 1) with Go-Taq polymerase (Promega, Wisconsin, USA) under the following cycle conditions: 95 °C 30 s, 65 °C 30 s, and 72 °C 1 min. To analyse sequences of the tammar SLCC22A2 and SLCC22A3 transcripts, strand-specific cDNA synthesis was performed using each gene specific reverse primer (Additional file 1). The synthesised cDNA was used as a template for PCR amplification with Go-Taq polymerase (Promega, Wisconsin, USA) under the following cycle conditions:
95 °C 30 s, 65 °C 30 s, and 72 °C 1 min. After performing gel electrophoresis, confirmed PCR products from gDNA and cDNA were extracted and directly sequenced by Sanger sequencing to confirm SNP sites and allele specific expression. We consider a biased expression as a strongly skewed expression when the signal intensities between the two alleles differed more than fivefold.

**Bisulphite sequencing**

Purified genomic DNA derived from the fetus used for allelic expression analysis was treated with sodium bisulphite solution using EpiMark Bisulfite Conversion kit (New England Biolabs, Massachusetts, USA). After the bisulphite treatment of the genomic DNA, 40 cycles of PCR were carried out using EpiTaq polymerase (Takara Bio, Shiga, Japan) with the primers (Additional file 1) designed by MethPrimer (https://www.urogene.org/methprimer/) [66]. The PCR products were cloned using a pGEM T-easy vector (Promega, Wisconsin, USA) and E. coli JM109 competent cells (Promega, Wisconsin, USA) and directly sequenced using M13 primers (Additional file 1). The sequence data were analysed by quantification tool for methylation analysis (QUMA) programme (http://quma.cdb.riken.jp) [67].

**Immunofluorescence (IF) staining**

PFA-fixed placenta samples (BOM and TOM at day 22 of gestation) were washed in 1× PBS and re-hydrated through an ethanol series before being embedded in paraffin. Embedded samples were serially sectioned at 5 μm and mounted on poly-lysine coated slides (ThermoFisher Scientific, Massachusetts, USA). The sections were de-waxed, rehydrated through decreasing concentrations of ethanol, and then incubated in 0.1% (v/v) Triton X-100 in 1× PBS (PBST) for 15 min at room temperature to permeabilise the tissue. Slides were boiled in Tris-EDTA (pH 8.0) for 20 min. The sections were treated with 0.3% (w/v) Sudan Black in 70% (v/v) EtOH solution for 15 min to reduce auto-fluorescent background. The Sudan Black treated sections were washed by 70% (v/v) EtOH and 1× PBS before blocking. Thereafter, the sections were incubated for 1 h with 10% (w/v) goat serum diluted in 1× PBS. After blocking, sections were incubated with primary antibody solution (Additional file 2) at 4 °C for 16 h. The following day, sections were washed three times with PBS and then incubated with fluorescent secondary antibodies (Additional file 2) for 1 h. The sections were washed three times with 1× PBS again and then incubated for 10 min with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Missouri, USA). DAPI-treated sections were mounted with fluorescence mounting solution. The controls for all treatments were no primary antibody and IgG isotype control antibodies. Images were collected on a Nikon A1R Confocal Laser Microscope System (Nikon, Tokyo, Japan).

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s13072-022-00465-4.

**Additional file 1.** Primers used for this study.

**Additional file 2.** Antibodies used for this study.

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**Author contributions**

TI, OWG, SS and MBR designed the research; TI performed experiments; TI and MBR collected the samples; TI, OWG, SS and MBR discussed the data, and TI and MBR wrote the paper. All authors read and approved the manuscript.

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**Availability of data and materials**

The datasets analysed during the current study are available in the NCBI SRA (https://www.ncbi.nlm.nih.gov/sra) as DRP001145.

**Declarations**

**Ethics approval and consent to participate**

Experimental procedures conformed to Australian National Health and Medical Research Council (2013) guidelines and were approved by the Animal Experimentation Ethics Committees of the University of Melbourne.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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50. Verhaagh S, Barlow DP, Zwart R. The extraneuronal monoamine transporter SLC22A3/Orct3 co-localizes with the Maoa metabolizing enzyme in mouse placenta. Mech Dev. 2001;100:127–30.

51. Hudson QJ, Seidl CM, Kulinski TM, Huang R, Warczok KE, Bittner R, et al. Extra-embryonic-specific imprinted expression is restricted to defined lineages in the post-implantation embryo. Dev Biol. 2011;353:420–31.

52. Woods L, Perez-Garcia V, Hembberger M. Regulation of placental development and its impact on fetal growth—new insights from mouse models. Front Endocrinol. 2018. https://doi.org/10.3389/fendo.2018.00570.

53. Bielinska M, Nanta N, Wilson DB. Distinct roles for visceral endoderm during embryonic mouse development. Int J Dev Biol. 1999;43:183–205.

54. Renfree MB, Tyndale-Biscoe CH. Intrauterine development after diapause in the marsupial Macropus eugenii. Dev Biol. 1973;32:28–40.

55. Renfree MB. Proteins in the uterine secretions of the marsupial Macropus eugenii. Dev Biol. 1973;32:41–9.

56. Guernsey MW, Chuong EB, Cornelis G, Renfree MB, Baker JC. Molecular conservation of marsupial and eutherian placentation and lactation. Elibe. 2017. https://doi.org/10.7554/eLife.27490.

57. Renfree MB. The composition of fetal fluids of the marsupial Macropus eugenii. Dev Biol. 1973;33:62–79.

58. Anson-Cartwright L, Dawson K, Holmyard D, Fisher SJ, Lazzarini RA, Cross JC. The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic plaet. Nat Genet. 2000;25:311–4.

59. Ager E, Suzuki S, Park A, Shaw G, Ishino F, Renfree MB. Insulin is imprinted in the placenta of the marsupial Macropus eugenii. Dev Biol. 2007;309:317–28.

60. Yotova I, Vlakovic IM, Pauler FM, Warczok KE, Ambros PF, Oshimura M, et al. Identification of the human homolog of the imprinted mouse air non-coding RNA. Genomics. 2008;92:464–73.

61. Stoger R, Kubiczk aR, Liu CG, Kafri T, Razin A, Cedar H, et al. Maternal-specific methylation of the imprinted mouse Igf2r locus identifies the expressed locus as carrying the imprinting signal. Cell. 1993;73:61–71.

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