

RESEARCH REPORT

A trans-homologue interaction between reciprocally imprinted *miR-127* and *Rtl1* regulates placenta development

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ABSTRACT

The paternally expressed imprinted retrotransposon-like 1 (*Rtl1*) is a retrotransposon-derived gene that has evolved a function in eutherian placentation. Seven miRNAs, including *miR-127*, are processed from a maternally expressed antisense *Rtl1* transcript (*Rtl1as*) and regulate *Rtl1* levels through RNAi-mediated post-transcriptional degradation. To determine the relative functional role of *Rtl1as* miRNAs in *Rtl1* dosage, we generated a mouse specifically deleted for *miR-127*. The *miR-127* knockout mice exhibit placentomegaly with specific defects within the labyrinthine zone involved in maternal-fetal nutrient transfer. Although fetal weight is unaltered, specific *Rtl1* transcripts and protein levels are increased in both the fetus and placenta. Phenotypic analysis of single ($\Delta miR-127/Rtl1$ or *miR-127/ΔRtl1) and double ($\Delta miR-127/\Delta Rtl1$) heterozygous *miR-127*- and *Rtl1*-deficient mice indicate that *Rtl1* is the main target gene of *miR-127* in placental development. Our results demonstrate that *miR-127* is an essential regulator of *Rtl1*, mediated by a trans-homologue interaction between reciprocally imprinted genes on the maternally and paternally inherited chromosomes.*

KEY WORDS: Genomic imprinting, *Rtl1* (*Peg11*), *miR-127*, *Mir127*, Placenta development

INTRODUCTION

Mammalian genomic imprinting is an epigenetic process whereby genes are mono-allelically expressed in a parent-of-origin-specific manner (Ferguson-Smith, 2011). The imprinted gene cluster on mouse chromosome 12 contains four paternally expressed protein-coding genes and maternally expressed non-coding RNAs (Fig. 1A) (da Rocha et al., 2008). One of these paternally expressed genes, retrotransposon-like 1 (*Rtl1*; also known as *Peg11*), is derived from a Ty3/Gypsy-type retrotransposon that in eutherians has evolved a large conserved open reading frame (ORF) but has lost its long terminal repeats (LTRs), resulting in loss of the original retroviral promoter activity (Brandt et al., 2005; Youngson et al., 2005; Edwards et al., 2008).

The primary antisense *Rtl1* transcript (*Rtl1as*) is exclusively expressed from the maternally inherited *Rtl1* locus but in the

opposite direction to *Rtl1* (Fig. 1A) (Seitz et al., 2003). At least seven microRNAs (miRNAs) processed from *Rtl1as* are therefore perfectly complementary in sequence to *Rtl1* (Davis et al., 2005). Maternally inherited deletion of the differentially methylated imprinting control region for the locus (IG-DMR) causes a maternal-to-paternal epigenotype switch across the whole imprinted gene cluster (Lin et al., 2003). This is associated with repression of all the maternally expressed non-coding RNAs, including the miRNAs, and inappropriate activation of the usually paternally expressed protein-coding genes on the maternally inherited chromosome, resulting in a double dose. However, *Rtl1* mRNA levels increase 4.5-fold from both alleles, instead of the double dose expected from loss of imprinting (LOI). This suggests that the increase in *Rtl1* dosage in the mutant is the cumulative effect of both LOI and a failure to destabilise the now biallelically expressed transcript by the antisense miRNAs (Lin et al., 2003). Further evidence that these miRNAs can degrade *Rtl1* transcripts by the RNAi machinery *in vivo* came from the identification of both DROSHA and DICER cleavage products for each of the miRNAs (Davis et al., 2005). Previous work has shown that *Rtl1* gene deletion causes growth retardation of both the fetus and placenta and that removal of six of the seven miRNAs on *Rtl1as* leads to *Rtl1* overproduction and placentomegaly (Sekita et al., 2008).

Further findings indicate that *miR-127* on *Rtl1as* can be independently regulated in human cancer (Iorio et al., 2005; Lu et al., 2005), and that on its own *miR-127* may be the major contributor to *Rtl1* silencing in differentiating mouse embryonic stem cells (ESCs) (Claudio et al., 2009). These findings suggest that *miR-127* might play a prominent role in controlling *Rtl1* dosage during normal development. In order to clarify the biological significance of *miR-127*, we generated *miR-127* (*Mir127*) knockout mice and studied its impact on *Rtl1* transcript and protein levels and consequences for placental development.

RESULTS AND DISCUSSION

Maternal *miR-127* deletion induces placentomegaly

The schematic organisation of the imprinted *Rtl1* sense and antisense transcripts is shown in Fig. 1A. A 134 bp deletion removed *miR-127* upon maternal transmission ($\Delta miR-127$), while the same deletion when paternally transmitted ($\Delta Rtl1$) introduces a nonsense mutation in the third exon of *Rtl1*, resulting in premature translation termination of a normally transcribed mutant transcript (Fig. 1A; supplementary material Fig. S1B). Western blotting data showed no detectable RTL1 protein in $\Delta Rtl1$ conceptuses (supplementary material Fig. S1G), although *Rtl1* mRNA was stable (supplementary material Fig. S3A). All phenotypic analyses were carried out on the C57BL/6J background unless otherwise indicated.

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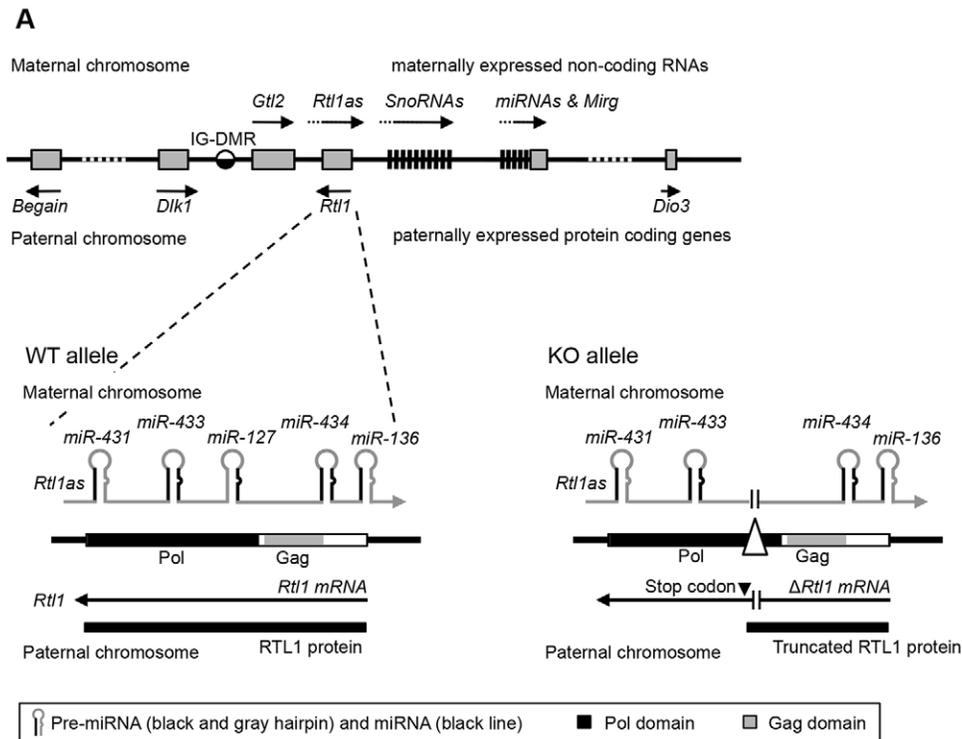
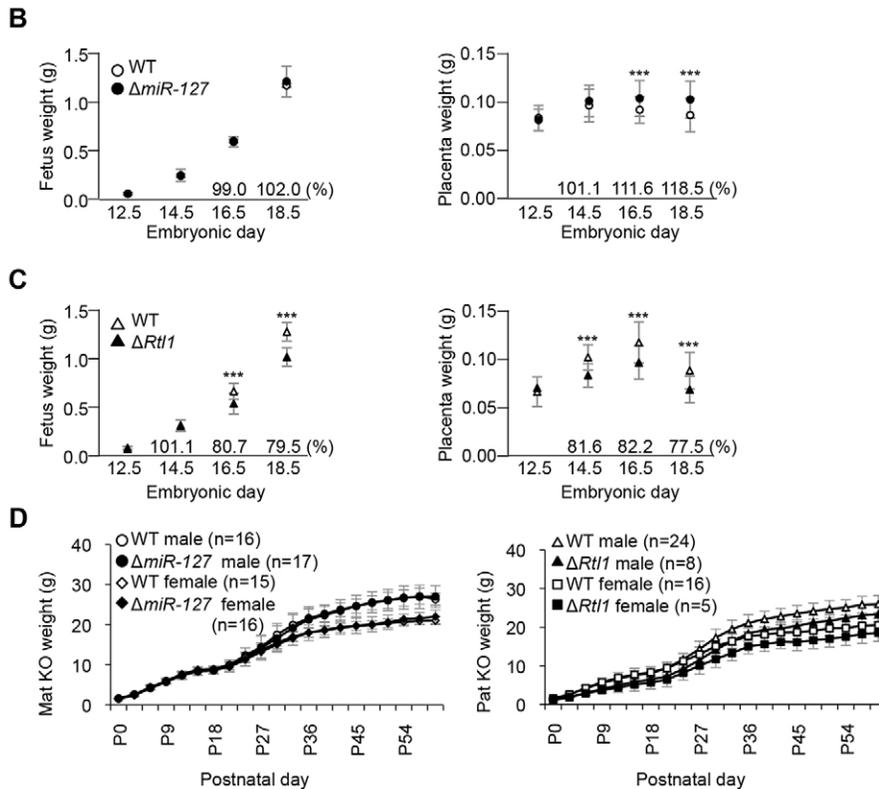


Fig. 1. Structure of the *Rtl1* locus and pre- and postnatal growth of *miR-127* and *Rtl1* knockout mice. (A) Schematic presentation of the mouse *Dlk1-Dio3* cluster. (Lower left) The WT *Rtl1* locus (exon 3). *Rtl1* is expressed from the paternal chromosome and *Rtl1as* is exclusively transcribed from the maternal chromosome. (Lower right) The knockout (KO) allele. The paternally transmitted deletion introduces an in-frame stop codon that results in premature termination of RTL1. The maternally transmitted deletion lacks *miR-127* expression. (B,C) Prenatal growth of Δ *miR-127* mice and Δ *Rtl1* mice, respectively. Left and right panels show embryonic and placental growth curves in mutant and WT littermates from E12.5 to E18.5. All embryos and placentas were collected from the N6 and N7 generation. (D) Postnatal growth curve of Δ *miR-127* (left) and Δ *Rtl1* (right) from birth to 2 months. Weights were measured every 3 days. Δ *Rtl1* mice were significantly smaller than WT. *** $P < 0.005$ (Student's *t*-test). Error bars indicate s.d.



Placentae were significantly overgrown in Δ *miR-127* mutants, which was first apparent at E16.5; placental weights were 111.6% and 118.5% compared with wild type (WT) at E16.5 and E18.5, respectively (Fig. 1B). By contrast, there was no effect of Δ *miR-127* on fetal weight during development (Fig. 1B). Previous work had shown that when six miRNAs, including *miR-127*, are deleted, mutant placental weights are 156% of WT values at E18.5, although fetal weights are not different (Sekita et al., 2008). These data

suggest that *miR-127* functions to suppress placental growth in pregnancy, although placentomegaly in Δ *miR-127* was milder than with the larger deletion encompassing six miRNAs. After birth, the Δ *miR-127* mice grew at comparable rates to WT and no lethality was observed either pre- or postnatally in these mice (Fig. 1D; supplementary material Tables S1 and S2).

Δ *Rtl1* mice showed prenatal growth retardation starting at E16.5; fetal weights were ~80% of WT (Fig. 1C). Mice have reduced wet

weight at birth (~70% of WT) and remain growth retarded into adulthood (Fig. 1D). Prenatally, the placenta is growth restricted from E14.5, prior to the onset of fetal growth restriction, suggesting a causal role for the placenta in the fetal growth phenotype (Fig. 1C). Prenatal lethality was not observed in $\Delta Rtl1$ but the majority of neonates died within 1 day of birth (supplementary material Tables S1 and S2). In situations in which $\Delta Rtl1$ newborns survived more than 2 days, animals survived to adulthood. The lethality of $\Delta Rtl1$ was not evident on a mixed 129aa and C57BL/6J background (supplementary material Table S1). The embryonic lethality we report differs from that associated with the previously reported larger deletion, where lethality occurred during gestation upon paternal transmission (Sekita et al., 2008), despite both mutants lacking the RTL1 protein.

$\Delta miR-127$ causes defects in the placental labyrinthine zone

Placental structure was analysed stereologically (Gundersen et al., 1988; Mandarim-de-Lacerda, 2003; Coan et al., 2004) upon both maternal and paternal transmission of the deletion at E18.5. In $\Delta miR-127$ the labyrinthine zone (Lz), which is the site of nutrient and gaseous exchange between the maternal and fetal blood supplies, was expanded (142.3% of WT; Fig. 2A,C). Conversely, the volume of the Lz was reduced in $\Delta Rtl1$ (64.7% of WT; Fig. 2B,D). In contrast to the Lz, the junctional zone, decidual basalis and chorion were all unaffected by $miR-127$ or $Rtl1$ deficiency.

Detailed structural analysis of the Lz showed that both the fetal capillaries (FCs) and the labyrinthine trophoblast (LT) were significantly increased in $\Delta miR-127$, with a non-significant trend for

expanded maternal blood spaces (MBSs) (Fig. 2E; supplementary material Fig. S2). Similar to the volume differences, the surface areas of FCs and MBSs were also extended in $\Delta miR-127$ (supplementary material Table S3). Moreover, the average length of FCs in the Lz was increased in $\Delta miR-127$, without a change in diameter. There was no effect of $miR-127$ deficiency on the thickness of the interhemal trophoblast membrane, where nutritional exchange takes place. These results suggest that $miR-127$ suppresses fetal capillarisation of the placental exchange region.

In $\Delta Rtl1$, placental abnormalities were observed in the same compartments affected by $miR-127$ deficiency, but with opposite phenotypes (Fig. 2F; supplementary material Fig. S2). These results suggest that $Rtl1$ supports FC elongation and that the two genes interact to regulate the same placental processes. The alterations in MBS and FC surface area would affect nutrient and oxygen supply to the fetus and contribute to the observed fetal growth restriction. The theoretical diffusion capacity (TDC) and specific diffusion capacity (SDC) are barometers for the potential ability of small molecules such as oxygen to transfer by passive diffusion from mother to fetus (Laga et al., 1973). The TDC and SDC values of the mutant placentae indicate that $\Delta miR-127$ mice have a higher diffusive capacity than WT and, conversely, that $\Delta Rtl1$ placentae have a reduced capacity (supplementary material Table S3). Although this is likely to contribute to the growth retardation of the $\Delta Rtl1$ fetuses, it is noteworthy that the $\Delta miR-127$ mutants are not growth enhanced. Previous work has proposed that $Rtl1$ cleaves an extracellular matrix (ECM) component resulting in a release of growth factors to promote hepatocarcinogenesis (Riordan et al., 2013). During

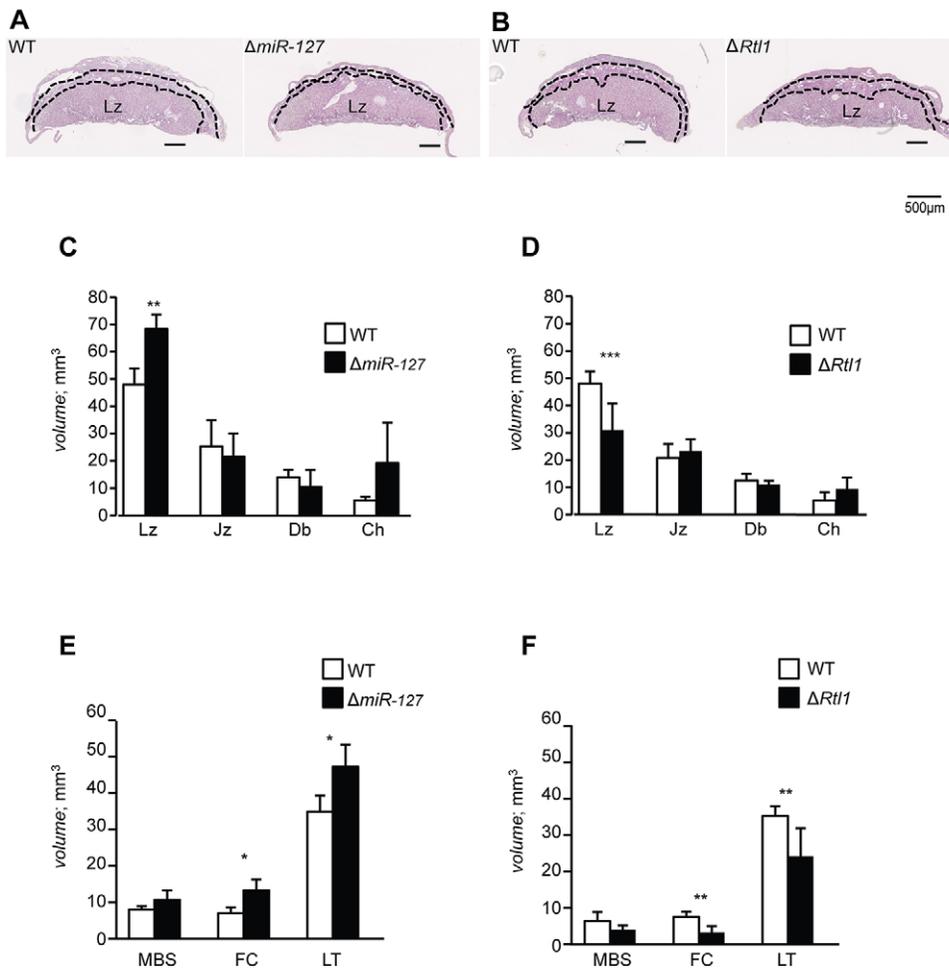


Fig. 2. Histological analysis shows abnormality in the labyrinthine zone in $\Delta miR-127$ and $\Delta Rtl1$. (A,B) Histological analysis of WT littermate and $\Delta miR-127$ or $\Delta Rtl1$, respectively. H&E-stained paraffin sections of E18.5 placentae. Dashed lines demarcate Lz (bottom layer), Jz (middle layer) and Db (top layer). Scale bars: 500 μ m. (C-F) The volumes of placental and labyrinthine compartments in WT, $\Delta miR-127$ and $\Delta Rtl1$. Lz, labyrinthine zone; Jz, junctional zone; Db, decidual basalis; Ch, chorion; MBS, maternal blood spaces; FC, fetal capillaries; LT, labyrinthine trophoblast. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ (Student's *t*-test). Error bars indicate s.d.

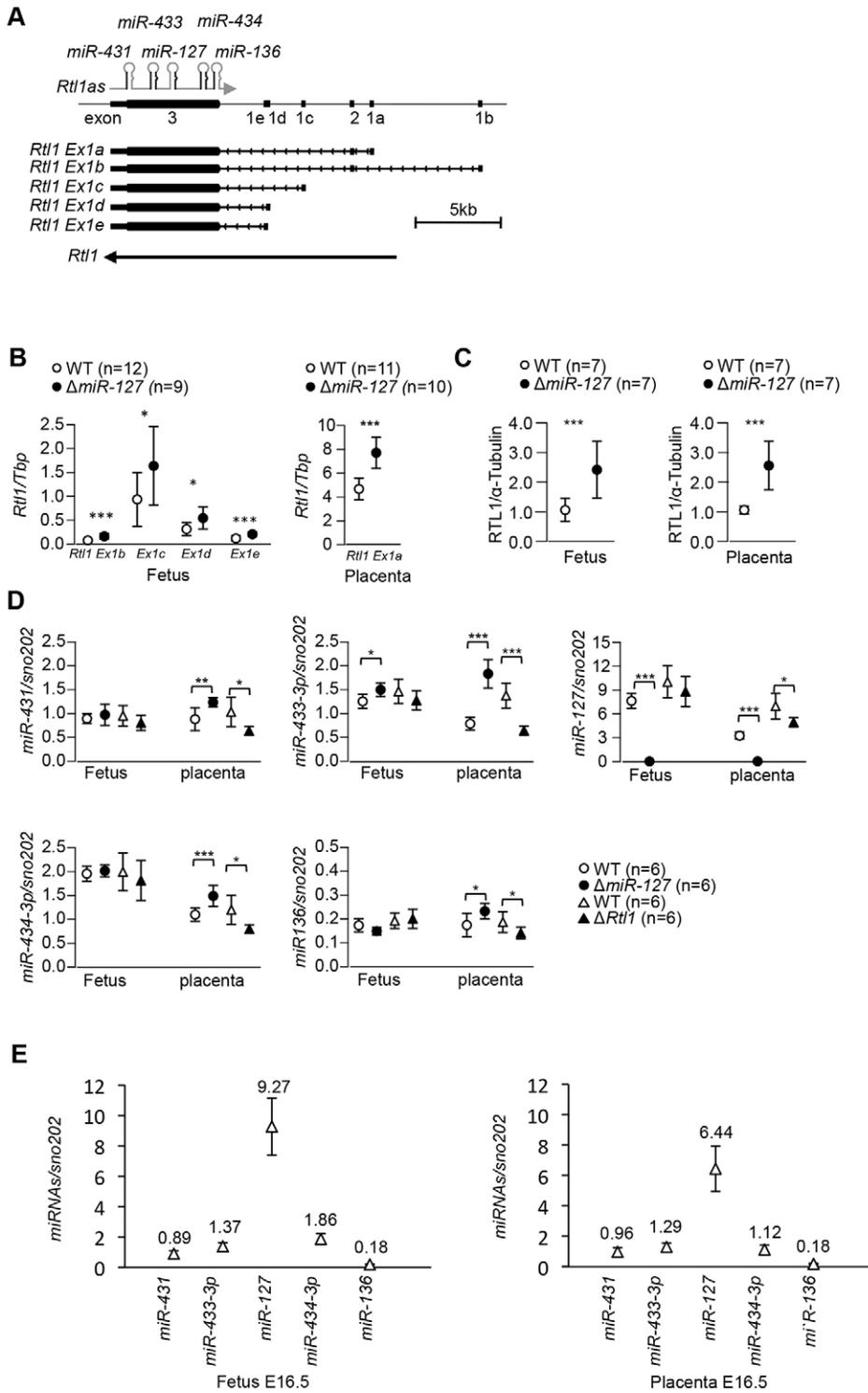


Fig. 3. Expression of *Rtl1* alternative transcripts and miRNAs in embryo and placenta at E16.5. (A) Structure of the mouse *Rtl1* locus. Alternative transcripts are transcribed from different leader exons. Exon 1s are named a-e. All alternative transcripts have a common exon 3 that has a conserved retrotransposon sequence. *Rtl1 Ex1a* and *Ex1b* also have a common exon 2. Exons are represented by a solid box. (B) Quantitative expression analysis for each alternative *Rtl1* transcript in $\Delta miR-127$ embryo and placenta at E16.5. (C) Western blotting for RTL1 normalised to α -tubulin in $\Delta miR-127$ embryo and placenta at E16.5. (D) miRNA expression is shown normalised to *snoRNA202*. (E) The relative expression of miRNAs. *miR-127* is the most abundant of the miRNAs in *Rtl1as*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ (Student's *t*-test). Error bars indicate s.d.

angiogenesis, the degradation of the basement membrane and ECM facilitates migration into the interstitial matrix and the formation of new capillaries (Jain, 2003). Since placental Lz RTL1 protein is expressed in the capillary endothelial cells (Sekita et al., 2008), we propose that RTL1 promotes cleavage of the basement membrane to progress the vascularisation of FCs.

All *Rtl1* isoforms are regulated by *miR-127*

cDNA screening previously revealed that *Rtl1* has two exons and a transcription start site located 5 kb upstream of the retrotransposon-

like sequences (Hagan et al., 2009), suggesting that *Rtl1* might be regulated by a host-derived promoter outside the retrotransposon. In order to clarify *Rtl1* transcription start sites by 5'RACE. One alternative leader exon was identified in E15.5 placenta (*Rtl Ex1a*) and three alternatives were identified in the E11 embryo (*Rtl Ex1b*, *Ex1d* and *Ex1e*) (Fig. 3A; supplementary material Fig. S4). All five *Rtl1* alternative transcripts, including the known *Rtl1 Ex1c* (GenBank: EU434918), contain a common large exon, namely exon 3, which contains the retrotransposon-derived ORF, and different small exons. All

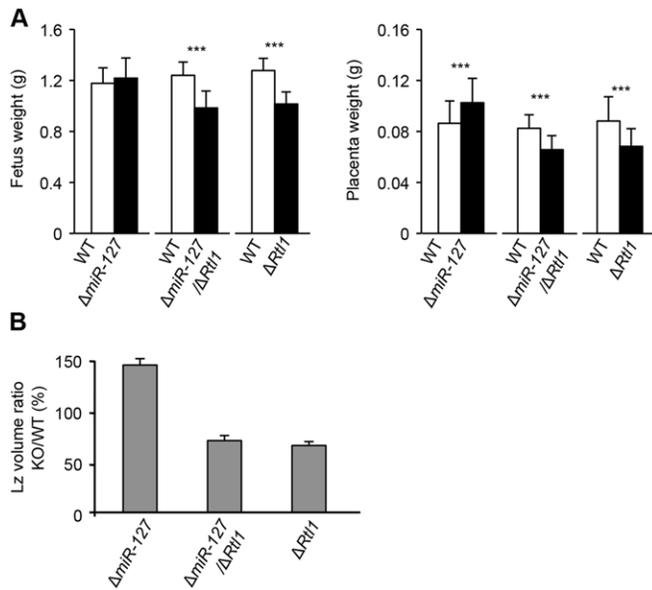


Fig. 4. $\Delta miR-127/\Delta Rtl1$ knockout mice are comparable to $\Delta Rtl1$. Double-heterozygous mice carrying both $\Delta miR-127$ and $\Delta Rtl1$ were born from heterozygous parents. (A) Fetal and placental weights at E18.5. (B) Volume ratio of the placental Lz at E18.5. *** $P < 0.005$ (Student's *t*-test). Error bars indicate s.d.

alternative exon 1s are spread over a 12 kb region, suggesting that they might be transcribed from different promoters. To address this, real-time RT-PCR was performed using alternative transcript-specific forward primers and a common reverse primer in exon 3. This showed that *Rtl1 Ex1c* is the most abundant transcript in E16.5 whole embryos (supplementary material Fig. S3B). The other *Rtl1* transcripts were also detectable in E16.5 embryos, but the *Rtl1 Ex1a* expression level was much lower (0.6% of total) than for the other four. Conversely, the most abundant mRNA in the placenta was *Rtl1 Ex1a*, which contributed more than 97% of total *Rtl1* expression compared with the others (supplementary material Fig. S3B).

In order to address whether all *Rtl1* transcripts were equivalently modulated by *miR-127*, we quantified *Rtl1* transcript levels in $\Delta miR-127$ embryos and placentae. Results showed that all alternative transcripts were significantly overexpressed (~1.7-fold of control) in E16.5 $\Delta miR-127$ embryos (Fig. 3B). *Rtl1 Ex1a* was significantly increased (1.7-fold) in $\Delta miR-127$ placentae (Fig. 3B). This is not an indirect effect caused by a disproportionate increase in the number of endothelial cells, since there is a similar increase of 70% in *Ex1a* expression when normalised to the endothelial cell marker *Pecam1* (*CD31*) (supplementary material Fig. S3C). Analysis of hybrid fetuses and placentae showed that all alternative transcripts are exclusively transcribed from the paternal chromosome in $\Delta miR-127$ (supplementary material Fig. S3D), indicating that the overexpression is not associated with LOI. Western blotting showed that RTL1 protein was significantly increased and in proportion to the increased level of the transcript in E16.5 $\Delta miR-127$ embryos and placentae (Fig. 3C; supplementary material Fig. S1H). Since deletion of six of the seven miRNAs results in only a 2.5-fold increase in *Rtl1* mRNA (supplementary material Fig. S3E), our findings indicate that, compared with the other miRNAs in the cluster, *miR-127* contributes a proportionately greater effect on *Rtl1* levels in placentae, and disruption of this repression causes placental overgrowth.

Consistent with its impact on *Rtl1* levels, *miR-127* is the most abundant miRNA generated from *Rtl1as* (Fig. 3E). We next determined whether the deletion of *miR-127* influences expression

of the neighbouring miRNAs to potentially impact *Rtl1* expression. As expected, *miR-127* was not detected in $\Delta miR-127$ embryos and placentae (Fig. 3D). In $\Delta miR-127$ fetuses, only *miR-433-3p* was upregulated, with no change in *miR-431*, *miR-434-3p* and *miR-136* expression (Fig. 3D). By contrast, all four miRNAs were significantly upregulated, with *miR-433-3p* the most induced, in $\Delta miR-127$ placentae. The same miRNAs that were upregulated in the placenta in the $\Delta miR-127$ mutant were downregulated in $\Delta Rtl1$ (Fig. 3D). Together, these results suggest that there might be a compensatory feedback mechanism involving RTL1 that acts specifically in the placenta to minimise the impact on *Rtl1* transcript levels. *miR-433* has its own promoter and thus might be more sensitive to this feedback mechanism (Song and Wang, 2008).

***Rtl1* is the main target gene of *miR-127* for placenta development**

Our data suggest *miR-127* can regulate placental growth through *Rtl1* repression. However, to address the possibility that other target genes of *miR-127* might also contribute to placental development, we generated double-heterozygous mice ($\Delta miR-127/\Delta Rtl1$) lacking both *Rtl1* and *miR-127*. If *Rtl1* is the main target of *miR-127* leading to repressed placental growth then the $\Delta miR-127/\Delta Rtl1$ mutant should show a similar phenotype to $\Delta Rtl1$. However, if *miR-127* has other targets contributing to this phenotype then the $\Delta miR-127/\Delta Rtl1$ mutant would be expected to have an intermediate phenotype between that seen in $\Delta Rtl1$ and that seen in $\Delta miR-127$. The $\Delta miR-127/\Delta Rtl1$ mutant mouse embryo and placental weight data show that they are similar to $\Delta Rtl1$ at E18.5 rather than to $\Delta miR-127$ (Fig. 4A). Histological analysis also showed that the extent and volume reduction of the placental Lz was the same in both $\Delta miR-127/\Delta Rtl1$ and $\Delta Rtl1$ (Fig. 2C and Fig. 4B). Detailed analysis of the Lz also determined that volumes and surface areas of the MBS, FC and LT and the TDC and SDC were similarly decreased in $\Delta Rtl1$ and $\Delta miR-127/\Delta Rtl1$ compared with WT (supplementary material Table S3). By contrast, these volumes were increased in $\Delta miR-127$. These striking similarities between $\Delta Rtl1$ and $\Delta miR-127/\Delta Rtl1$ placentae suggest that *miR-127* specifically acts upstream of *Rtl1* during placental development.

Comparative analysis of the genomic locus between eutherian, metatherian and protherian mammals suggests that miRNAs on *Rtl1as* evolved in eutherians along with the neofunctionalisation of RTL1 (Edwards et al., 2008). Marsupial mammals lack the miRNAs and have retained only remnants of the Ty3/Gypsy retrotransposon that evolved into *Rtl1* in eutherians. Hence, it is likely that *Rtl1as* miRNAs evolved as a host defence mechanism to suppress the activity of this retrotransposon-derived gene (Edwards et al., 2008). In particular, the reciprocally imprinted *miR-127* and *Rtl1*, which interact so effectively in trans, co-evolved to regulate placenta development.

MATERIALS AND METHODS

Generation of $\Delta miR-127/\Delta Rtl1$ mice

We generated a *miR-127* deletion construct that lacks 134 bp incorporating *miR-127* (chr12:109,592,803-109,592,936) (supplementary material Fig. S1A). The *miR-127* targeting construct was transfected into female 129SV ESCs and clones containing the targeting vector were selected (supplementary material Fig. S1C-E). After deletion of the neomycin resistance gene (supplementary material Fig. S1F), targeted ESCs were injected into blastocysts to make chimaeras and germline transmission confirmed. Animals were backcrossed to C57BL/6J for ten generations with consistent growth and viability phenotypes noted after N5 on this genetic background (supplementary material Table S1). Mice were subsequently maintained on a C57BL/6J genetic background. For further details, see the supplementary Materials and Methods.

Placental histology

Placentae from embryonic day (E) 18.5 conceptuses were dissected free of fetal membranes, weighed and bisected mid-sagittally. One half was fixed in 4% paraformaldehyde, paraffin embedded, sectioned, stained with Hematoxylin and Eosin (H&E) and the gross placental structure analysed. The other half was fixed in 4% glutaraldehyde, resin embedded, stained with Toluidine Blue and the structure stereologically assessed. Analyses were performed using the Computer Assisted Stereological Toolbox (CAST v2.0) program as previously described (Coan et al., 2004). Further details of placental histology are given in the supplementary Materials and Methods.

Rapid amplification of cDNA ends (5' RACE) and quantitative RT-PCR

5' RACE was performed using the First Choice RLM-RACE Kit (Ambion) following the manufacturer's protocol; 10 µg of total RNA from E11 fetus and E15.5 placenta was used as the starting material.

For real-time PCR, total RNA (10 µg) from whole embryos and placenta at E16.5 was treated with RQ1 RNase-free DNase (Promega). cDNA was synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit with random hexamers (Fermentas). Real-time RT-PCR assay for *Rtl1* was performed using alternative exon 1-specific forward primers and a common reverse primer on exon 3. TATA box binding protein (*Tbp*) and *Pecam1* (*CD31*; Wang et al., 2005) expression was used as an internal control.

For mature miRNA expression, we carried out real-time RT-PCR using TaqMan microRNA assays (Applied Biosystems). Additional details of 5' RACE and real-time RT-PCR are provided in the supplementary Materials and Methods.

Western blotting

Proteins were extracted from E16.5 embryos and placentae using RIPA buffer containing protease inhibitors (Complete, EDTA-free, Roche). RTL1 was detected by rabbit anti-RTL1 antibody (YZ2844) created in the C.L.S. laboratory, and then normalised by anti- α -tubulin (Sigma-Aldrich, T6199). Further details are given in the supplementary Materials and Methods.

Acknowledgements

We thank members of the A.C.F.-S. laboratory for discussions; and Neil Youngson, Simao Teixeira da Rocha, Sylvia Kocialkowski, Marika Charalambous and Marie Watkins for assistance and discussions during the course of this work.

Competing interests

The authors declare no competing or financial interests.

Author contributions

M.I. and A.C.F.-S. designed the study. M.I., A.N.S.-P., C.A.E., T.-H.L. and M.K. performed experiments. M.I., A.N.S.-P., S.E.A., T.K.-I., F.I., C.L.S. and A.C.F.-S. analysed and discussed the data. M.I., A.N.S.-P., B.T.A. and A.C.F.-S. wrote the manuscript.

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) and UK Medical Research Council (MRC) [MR/J001597/1] and EU FP7 Marie Curie Action 290123 (INGENIUM). This work was partly funded by a Australian Government National Health and Medical Research Council (NHMRC) CJ Martin Biomedical Fellowship to A.N.S.-P. Deposited in PMC for release after 6 months.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.121996/-/DC1>

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