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Meg3-DMR, not the *Meg3* gene, regulates imprinting of the *Dlk1-Dio3* locus

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ABSTRACT

The imprinted *delta like 1 homolog* (*DLK1*) - *thyroxine deiodinase type III* (*DIO3*) locus regulates development and growth. Its imprinting regulation involves two differentially methylated regions (DMRs), intergenic-DMR (IG-DMR) and maternally expressed gene 3-DMR (Meg3-DMR). In mice, a maternal deletion of the IG-DMR leads to LOI in the locus, proving that the IG-DMR is a cis-acting imprinting control region of the locus. However, the Meg3-DMR overlaps with the promoter, exon 1 and intron 1 of the Meg3 gene. Because deletion of the Meg3-DMR inactivates the *Meg3* gene, their roles in imprinting regulation of Meg3-DMR mice is unknown. Therefore, we generated two mouse models: $Meg3^{\Delta(1-4)}$ and $Meg3^{\Delta(2-4)}$, respectively targeting exons 1–4 and exons 2–4 of the *Meg3* gene. A maternal deletion of $Meg3^{\Delta(1-4)}$ caused embryonic death and LOI in both embryos and placentas, but did not affect methylation status of the IG-DMR. In contrast, mice carrying a maternal deletion of $Meg3^{\Delta(2-4)}$ were born normally and did not have LOI. These data indicate that it is the Meg3-DMR, not the *Meg3* gene, which regulates imprinting of the *Dlk1-Dio3* locus.

1. Introduction

Genomic imprinting is an epigenetic phenomenon in which expression of imprinted genes is parental-origin-dependent (Barlow and Bartolomei, 2014). An imprinted locus is formed by a cluster of imprinted genes in which their expression is tightly regulated by imprinting mechanisms. The *delta like 1 homolog* (*DLK1*) - *thyroxine deiodinase type III* (*DIO3*) locus is located on chromosome 14 in humans and on distal chromosome 12 in mice (da Rocha et al., 2008; Takada et al., 2000; Miyoshi et al., 2000). Balanced gene expression in the *DLK1-DIO3* locus plays a pivotal role in development and growth. Loss of imprinting (LOI) in this locus in humans leads to Kagami-Ogata syndrome (Ogata and Kagami, 2016; Kagami et al., 2010) or Temple syndrome (Ioannides et al., 2014). In mice, LOI of the locus causes developmental defects and premature death (Georgiades et al., 2000; Kumamoto et al., 2017). The locus is also essential in maintaining pluripotentiality of induced pluripotent stem cells (Stadtfeld et al., 2010).

The cis-elements controlling genomic imprinting are differentially methylated regions (DMRs) (Bartolomei and Ferguson-Smith, 2011). Two DMRs, intergenic DMR (IG-DMR) and maternally expressed gene 3-DMR (Meg3-DMR), are thought to play important roles regulating imprinting of the *DLK1-DIO3* locus (da Rocha et al., 2008; Kagami et al., 2010). Kagami et al. reported that a patient carrying a microdeletion overlapping with the IG-DMR showed LOI in the locus (Kagami et al., 2010). Similarly, Lin et al. demonstrated LOI in mice carrying a maternal deletion of the IG-DMR (ΔIG-DMR/+)(Fig. S3) (Lin et al., 2003, 2007). These data proved that the IG-DMR is an imprinting control region (ICR). The MEG3-DMR starts approximately 1.5 kb upstream of the MEG3 gene and extends into the MEG3 intron 1. We previously generated a Meg3-5kb mouse model, in which a 5-kb genomic DNA was targeted including a small portion of the *Meg3* promoter and exons 1 through 5 of the Meg3 gene (Fig. S3) (Zhou et al., 2010). The maternal deletion of the

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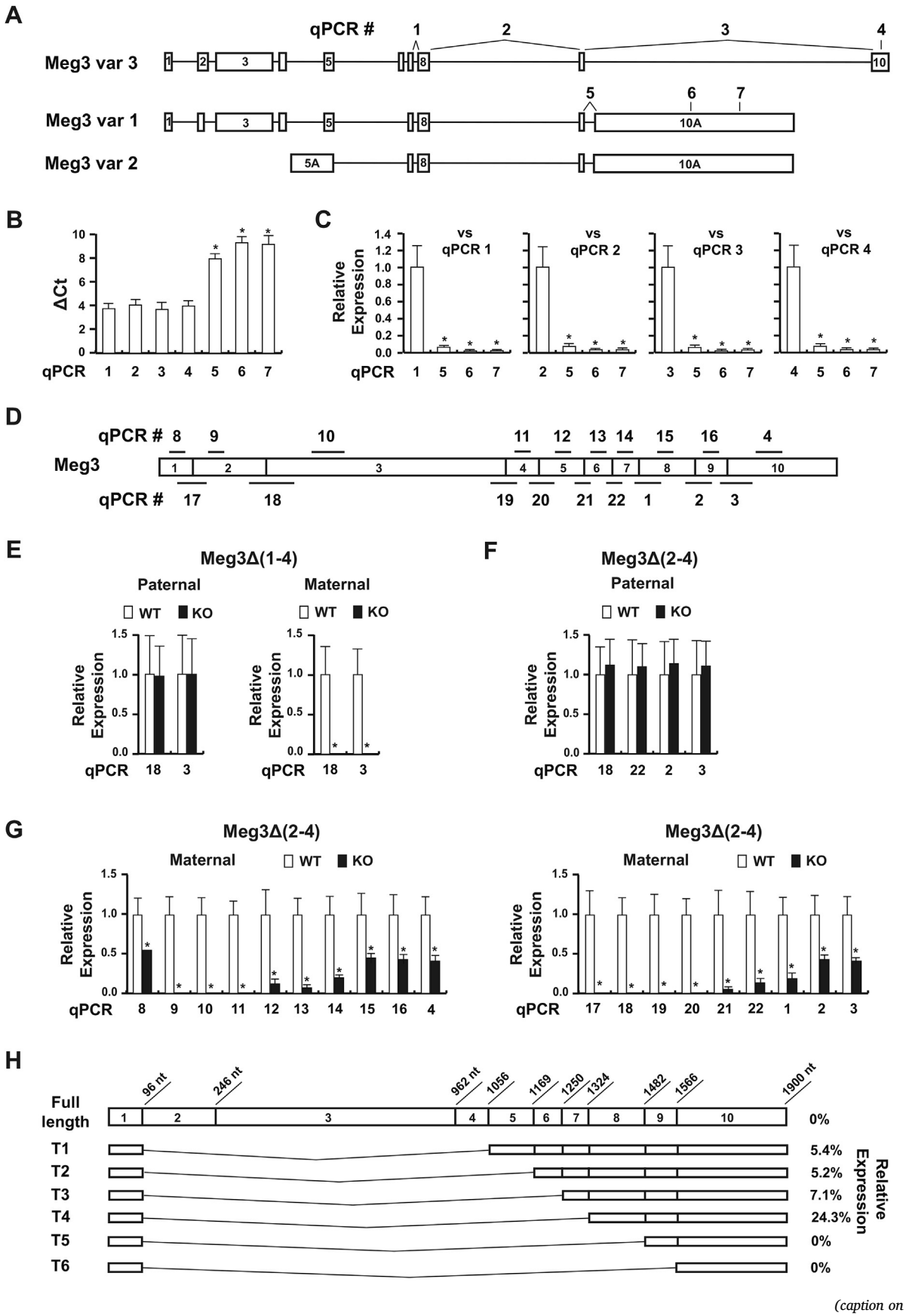

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targeted region resulted in LOI in the locus (Zhou et al., 2010), indicating that the targeted region is necessary for the imprinting regulation of the *Dlk1-Dio3* locus. In agreement, Kagami et al. reported that a patient with a microdeletion containing the MEG3-DMR had LOI in *DLK1-DIO3* locus (Kagami et al., 2010). Recently, Sanli et al. made deletions in the *Meg3* promoter or intron 1 in embryonic stem cells using CRISPR-Cas9 technique and found that these deletions caused LOI (Sanli et al., 2018). These data support the concept that the MEG3-DMR plays a role in imprinting regulation. However, a contradictory finding was reported by Takahashi et al., who generated a mouse model carrying an approximately 10-kb deletion including Meg3 exons 1 through 5 (designated as Meg3-10kb)(Fig. S3) (Takahashi et al., 2009). Mice carrying a maternal deletion of Meg3-10kb did not demonstrate LOI in the *Dlk1-Dio3* locus, although Meg3 expression was completely abolished. IG-DMR methylation was not affected by the Meg3-10kb deletion (Takahashi et al., 2009). These data imply that the Meg3-DMR does not play a significant role in imprinting regulation. Furthermore, because the Meg3-DMR overlaps with the *Meg3* gene promoter, any deletion in this region inactivates both Meg3-DMR and the *Meg3* gene. Therefore, it is still unknown as to whether the effects on imprinting in the *Dlk1-Dio3* locus were caused by lack of maternal Meg3-DMR, or Meg3 expression.

To address this key unanswered question, we created two mouse models, one with a deletion of exons 1 through 4 of the *Meg3* gene ($Meg3^{\Delta(1-4)}$) and the other with a deletion of exons 2 through 4 of the gene ($Meg3^{\Delta(2-4)}$). We show that LOI was only observed in embryos with a maternal $Meg3^{\Delta(1-4)}$ deletion ($Meg3^{\Delta(1-4)/+}$), not in those with a maternal deletion of $Meg3^{\Delta(2-4)}$ ($Meg3^{\Delta(2-4)/+}$). In addition, the maternal allele of the IG-DMR remains unmethylated in $Meg3^{\Delta(1-4)/+}$ embryos. Our data demonstrate that it is the Meg3-DMR, not the *Meg3* gene, that regulates imprinting, likely by regulating expression of maternally expressed genes.

2. Materials and methods

2.1. Mice

All animals used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital. They were housed at the animal facility of MGH Center for Comparative Medicine. Two strains of mice carrying deletions of the *Meg3* gene were created. $Meg3^{\Delta(1-4)}$ mice contain a deletion of exons 1 to 4 of the *Meg3* gene (Fig. S1)(Supplementary Data). To obtain this strain, a mouse strain carrying floxed exons 1–4 of the *Meg3* gene ($Meg3^{+/fl(1-4)}$) was created using services from inGenious Targeting Laboratories (iTL, Stony Brook, NY). To generate heterozygous mice carrying a deletion on the paternal allele ($Meg3^{+/\Delta(1-4)}$), male $Meg3^{+/fl(1-4)}$ mice were mated with female CRE transgenic mice (BALB/c-Tg(CMV-cre)1Cgn/J, Jackson Lab, Bar Harbor, ME). $Meg3^{\Delta(2-4)}$ mice carry a deletion of exons 2 through 4 of the *Meg3* gene (Fig. S2). To obtain this strain, a mouse strain carrying floxed exons 2–4 of the *Meg3* gene ($Meg3^{+/fl(2-4)}$) was created using services from Taconic (TaconicArtemis, Cologne, Germany). To generate heterozygous mice carrying a deletion on the paternal allele ($Meg3^{+/\Delta(2-4)}$), male $Meg3^{+/fl(2-4)}$ mice were mated with the female aforementioned CRE transgenic mice.

2.2. Gene expression

Expression of maternally and paternally expressed genes were detected by SYBR quantitative RT-PCR. Total RNAs were isolated from 11.5 dpc embryos and placentas using Qiagen RNeasy Mini Kit with on column DNase treatment (Qiagen, Germantown, MD). For lncRNAs and mRNAs, reverse transcriptions were performed using the ProtoScript® First Strand cDNA Synthesis Kit from New England Biolabs (Ipswich, MA). To detect Rtl1 RNA, the primer used in reverse transcription was 5′GATACTCAAACTTTTCTGA3'. The primer for Gapdh, 5′TTGAGTG AGTTGTCATATT3′, was included as the internal control. The primers used in qPCR are listed in Tables S1 and S2. To detect snoRNAs and miRNAs, reverse transcriptions were performed with the qScript mircoRNA DNA synthesis kit from Quantabio (Beverly, MA). The forward primers for small RNAs are listed in Table S2. The reverse primer was the PerfeCTa® Universal PCR Primer obtained from Quantabio. Quantitative PCRs were performed using PowerUp SYBR master mix from ThermoFisher Scientific (Grand Island, NY) per the manufacturer's instruction. Fold changes of gene expression in knockout mice over the WT mice were calculated by comparative Ct method described by Schmittgen et al. (Schmittgen and Livak, 2008) (Gao et al., 2015) and detailed in the technical note by Haimes et al. at Dharmacon of GE Healthcare (http://dharmacon.horizondiscovery.com/uploadedFiles/Resources/delta-cq-solaris-technote.pdf). Gapdh was used as the internal reference gene for lncRNAs and mRNAs. Sno202 was used as the reference for snoRNAs and miRNAs.

2.3. Bialleic expression

Chromosome-specific gene expression was determined by SNP analysis. In Dlk1 cDNA (NM_010052), nucleotide 1207 is a G in C57BL/6 and an A in DBA/2, respectively. In Rtl1 cDNA (NM_184109), nt2061 is a T in C57BL/6 and a C in NOD/ShLit. DBA/2J and NOD/ShLitJ mice were obtained from the Jackson Laboratory. Bialleic expression of Dlk1 in embryos was done as previously described (Zhou et al., 2010). Total RNA was isolated from 11.5 dpc embryos and placentas. Reverse transcriptions were done with the first strand cDNA synthesis kit from New England Biolabs using oligo dT as the RT primer for Dlk1 and Rtl1-RT (5′TCATCTCACTTTCCTTAAT3′) for Rtl1. The cDNA fragments containing SNPs for Dlk1 and Rtl1 were amplified by PCR. After gel purification, the fragments were sequenced to identify the SNPs. PCR primers for Dlk1 are 5′TCCTGAAGGTGTCCATGAAAGAGC3' (forward) and 5′AAGCAT AGCGTTCACTCGATTCCAC3' (reverse); for Rtl1 5′TCTGATACCCA CCTTAGGCTGGC3' (forward) and 5′GACGCCGCTTTGATCACTGTCTC3' (reverse).

Fig. 1. Meg3 expression in $Meg3^{\Delta(1-4)}$ and $Meg3^{\Delta(2-4)}$ mice. (A) There are three Meg3 transcript variants. Variant 3 contains 10 exons. Variants 1 and 2 contain a large alternative exon 10 (10A). Variant 2 has an alternative exon 5 (5A). Quantitative PCRs used to quantify their expression are indicated as numbers above each variant. (B) The SYBR based qRT-PCR was used to determine expression of the Meg3 variants in 11.5 dpc WT embryos (n=7). Gapdh was used as the internal reference gene. ΔCt values were presented as mean+/-SD. The ΔCt values from qPCR #5, 6 and 7 were compared with those from qPCR #1, 2, 3 and 4 using ANOVA multiple comparison tests. *$p<0.05$, considered to be statistically significant. (C) Relative expression levels of Meg3 variants 1 and 2 compared to all three variants (vs qPCR #1 and 2) or to variant 3 (vs qPCR #3 or 4). The values were calculated using $2^{-\Delta\Delta Ct}$ method as described in the Material and Methods. Student t-test was used to compare values against the reference qPCR. *$p<0.05$, considered to be statistically significant. (D) Detection of exons in Meg3 transcript was done by qPCR. For example, exon 1 was detected by qPCR #8; exons 1–2 by qPCR #17. Other exons were similarly detected by their respective qPCRs as indicated. (E) Relative expression levels of Meg3 detected with qPCR #3 and 18 in 11.5 dpc $Meg3^{\Delta(1-4)}$ embryos compared with their WT littermates. (F) Relative expression levels of Meg3 in 11.5 dpc paternal $Meg3^{\Delta(2-4)}$ embryos compared with their WT littermates. (G) Relative expression levels of Meg3 in 11.5 dpc maternal $Meg3^{\Delta(2-4)}$ embryos compared with their WT littermates. The levels of Meg3 transcripts detected by qPCR in KO embryos were normalized against the levels in their respective WT littermates, which were designated as 1. A minimum of 6 embryos for each genotype from at least 2 litters were used for gene expression analysis. Student t-test was used to compare values between KO mice and their WT littermates. *$p<0.05$, considered to be statistically significant. (H) Expression levels of predicted truncated Meg3 transcripts in $Meg3^{\Delta(2-4)/+}$ embryos. The percentage of each transcript in KO embryos compared to their WT littermates were deduced from data presented in (G).

2.4. DNA methylation analysis

Methlyation status of the IG-DMR was determined by bisulfite sequencing (Bisulfite Sequencing Services, Active Motif, Carlsbad, CA). A region of 500 nt long containing 35 CpG sites, located approximately 12.4 Kb upstream of the Meg3 gene, was selected for the analysis. Genomic DNAs were isolated from 11.5 dpc embryos, including 3 $Meg3^{\Delta(1-4)/+}$, 3 $Meg3^{+/\Delta(1-4)}$ and 3 $Meg3^{+/+}$, and their corresponding placentas. After bisulfite treatment, the converted genomic DNAs were amplified by PCR, processed into standard, barcoded Illumina sequencing libraries and sequenced in NextSeq 500 system (Illumia, San Diego, CA). Reads were analyzed using the bismark alignment program (v 0.7.7)

(http://www.bioinformatics.babraham.ac.uk/projects/bismark/). The mouse chr12 (mm10 assembly) was used as the reference sequence. Between 4.7 and 7.05 million reads were analyzed per sample.

3. Results and discussion

The mouse Meg3-DMR is approximately 3.5 kb long overlapping the promoter, exon 1 and intron 1 of the Meg3 gene. To clarify the role of the Meg3-DMR and *Meg3* gene in imprinting regulation of the Dlk1-Dio3 locus, two mouse models were generated. Part of the *Meg3* promoter as well as exons 1 through 4 of the Meg3 gene were deleted in $Meg3^{\Delta(1-4)}$ mice (Fig. S1). Exons 2 through 4 of the gene were deleted in $Meg3^{\Delta(2-4)}$ mice (Fig. S2). Mice carrying a paternal allele of $Meg3^{\Delta(1-4)}$ ($Meg3^{+/\Delta(1-4)}$) or $Meg3^{\Delta(2-4)}$ ($Meg3^{+/\Delta(2-4)}$) developed normally. Mice carrying a maternal allele of $Meg3^{\Delta(1-4)}$ ($Meg3^{\Delta(1-4)/+}$) died embryonically starting at 12.5 day post coitus (dpc). All $Meg3^{\Delta(1-4)/+}$ embryos died by 13.5 dpc (Table S1). In contrast, $Meg3^{\Delta(2-4)/+}$ embryos developed to full term and were born alive. Therefore, we used 11.5 dpc embryos to assess the effects of deletions on imprinting of the Dlk1-Dio3 locus.

There are three Meg3 transcript variants (Fig. 1A). Variant 3 (NR_027652.1) was first reported by Schuster-Gossler et al. (1998). The transcript contains all 10 exons. Variant 1 (NR_003633.3) and 2 (NR_027651.2) are derived from multiple sequence entries in NCBI. They contain a long alternative exon 10 (10A). Using quantitative PCR with primers targeting specific variant transcripts, we quantified their expression in WT embryos (Fig. 1B). The ΔCt values of PCR #1 and 2, detecting all three variants, were similar to those of PCR #2 and 4, detecting variant 3 only, suggesting that the variant 3 is the predominant transcript of all variants. In agreement, the ΔCt values of PCR #5, 6 and 7, detecting variants 1 and 2, were significantly higher than ΔCt values from other 4 qPCRs. Their expression levels were less than 6% of total Meg3 expression (Fig. 1C). Compared with the variant 3 levels, the combined expression of variants 1 and 2 were also very low (less than 6%) (Fig. 1C). These data suggest that the variant 3 is the main functional transcript of the Meg3 gene. Therefore, variant 3 was used as the functional product of the Meg3 gene.

To determine Meg3 expression in Meg3 KO embryos, we performed multiple qPCRs to quantify Meg3 transcripts with primers targeting specific exons as indicated in Fig. 1D. In $Meg3^{\Delta(1-4)/+}$ mice, no Meg3 transcripts were detected with PCR #3 and 18 (Fig. 1E). In $Meg3^{\Delta(2-4)/+}$ mice, no full-length Meg3 transcript was detected. Instead, multiple short truncated transcripts were detected in embryos, likely due to alternative RNA splicing. Their expression was very low compared to the level at which the full length Meg3 was expressed in the WT littermates (Fig. 1G). The highly possible transcripts were predicted (Fig. 1H). Among them, the most prominent transcript contained exons 1, 8, 9 and 10, which retains approximately 40% of the full-length Meg3 RNA (Fig. 1H). Previously, we have shown that deletion of less than a third of MEG3 RNA in the 5'-, 3′-end or middle of the molecule functionally inactivates MEG3 in p53 activation (Zhou et al., 2007). Therefore, the data suggest that the function of the Meg3 gene in $Meg3^{\Delta(2-4)/+}$ mice is most likely diminished. Because the function of Meg3 RNA has not been fully understood, however, our data could not rule out the possibility that the low-level expression of truncated Meg3 transcripts is enough to maintain imprinting of the Dlk1-Dio3 locus.

The mouse Dlk1-Dio3 domain contains nearly 60 known maternally expressed genes (MEGs) and 3 known paternally expressed genes (PEGs). We determined expression of 13 MEGs across the maternal locus in embryos and their corresponding placentas (Fig. 2A). In $Meg3^{\Delta(1-4)/+}$ embryos and placentas, expression of all MEGs was completely abolished compared to their WT littermates (Fig. 2B). Conversely, expression levels of Dlk1, Rtl1 and Dio3 were increased by 1.94, 3.24 and 2.36 fold, respectively, in $Meg3^{\Delta(1-4)/+}$ embryos (Fig. 2B). Increases in Dlk1 and Rtl1 expression were also observed in $Meg3^{\Delta(1-4)/+}$ placentas (Fig. 2B). However, the level of Dio3 expression was not changed in $Meg3^{\Delta(1-4)/+}$ placentas compared to WT placentas (Fig. 2B). Taking advantage of SNPs in the *Dlk1* and *Rtl1* genes among mouse strains, we found that both paternal and maternal alleles of the genes were expressed in $Meg3^{\Delta(1-4)/+}$ embryos and placentas (Fig. 3A). Therefore, the transcriptional activation of their respective maternal alleles plays a role in increase in expression of Dlk1 and Rtl1. In addition, the maternally expressed miR-127 and miR-136 are anti-sense to Rtl1, which have been suggested to down regulate Rtl1 transcript (Seitz et al., 2003). Therefore, lack of their expression is also likely to play a role in the increase in Rtl1 RNA levels in $Meg3^{\Delta(1-4)/+}$ embryos and placenta (Fig. 2B). These data demonstrated that deletion of the exons 1 through 4 of the Meg3 gene leads to LOI in the *Dlk1-Dio3 locus*.

Contrary to the findings in $Meg3^{\Delta(1-4)/+}$ mice, expression levels of all MEGs downstream of the *Meg3* gene in $Meg3^{\Delta(2-4)/+}$ embryos and placentas were comparable to their respective levels in WT littermates (Fig. 2C). There were no changes in expression of PEGs in $Meg3^{\Delta(2-4)/+}$ embryos and placentas (Fig. 2C). Furthermore, bialleic expression analysis indicated that the maternal alleles of *Dlk1* and *Rtl1* genes were not activated in $Meg3^{\Delta(2-4)/+}$ embryos and placentas (Fig. 3B). These data demonstrate that deletion of exons 2 through 4 does not result in LOI in the *Dlk1-Dio3* locus. Taken together, therefore, the LOI is caused by the deletion of the Meg3-DMR, not the *Meg3* gene.

We previously generated Meg3-5kb mice, in which exons 1–5 of the *Meg3* gene were deleted (Zhou et al., 2010). A maternal deletion of Meg3-5kb results in LOI in the *Dlk1-Dio3* locus, which is similar to the finding in $Meg3^{\Delta(1-4)/+}$. In mat-Meg3-5Kb mice, the IG-DMR is completely methylated (Zhou et al., 2010). We examined the methylation status of the IG-DMR core region in $Meg3^{\Delta(1-4)}$ mice (Fig. 4). The methylation status of IG-DMR in $Meg3^{\Delta(1-4)/+}$ embryos was very similar to those found in WT and paternal KO embryos, which were approximately 50% (Fig. 4B). There were also no obvious differences in methylation patterns among their respective placentas (Fig. 4C). These data indicate that deletion of the Meg3-DMR does not affect the methylation patterns of the IG-DMR. This raises the possibility that the mechanisms of LOI in these two mouse models are different. Because IG-DMR deletion results in LOI in the *Dlk1-Dio3* locus (Lin et al., 2003), it is very likely that the reason for LOI in mat-Meg3-5kb is methylation of the IG-DMR. This is further supported by the fact that mat-Meg3-5kb mice display identical phenotypes to ΔIG-DMR/+ mice, including skeletal muscle defects and death right after birth (Lin et al., 2003; Zhou et al., 2010). The Meg3-5kb mice were generated using a conventional recombination technology in which the targeted region was replaced by a *neomycin resistant* gene (Neo^r) cassette. The transcription of Neo^r is toward the IG-DMR (Zhou et al., 2010). It has been found that transcription-through is a common mechanism of DNA methylation in imprinted loci, such as the *Gnas* locus (Chotalia et al., 2009; Mehta et al., 2015) and the *Peg3* locus (Bretz and Kim, 2017). Therefore, it is most likely that IG-DMR methylation in the mat-Meg3-5kb mice is caused by transcription from the Neo^r cassette. Taken together, these data indicate that the underlying mechanisms for LOI in the *Dlk1-Dio3* locus between mat-Meg3-5kb mice and $Meg3^{\Delta(1-4)/+}$ mice are different: inactivation of IG-DMR in the former and inactivation of Meg3-DMR in the latter.

Lin et al. reported that methylation of the Meg3-DMR was significantly increased in ΔIG-DMR/+ mice (Lin et al., 2003)(Fig. S3). A similar

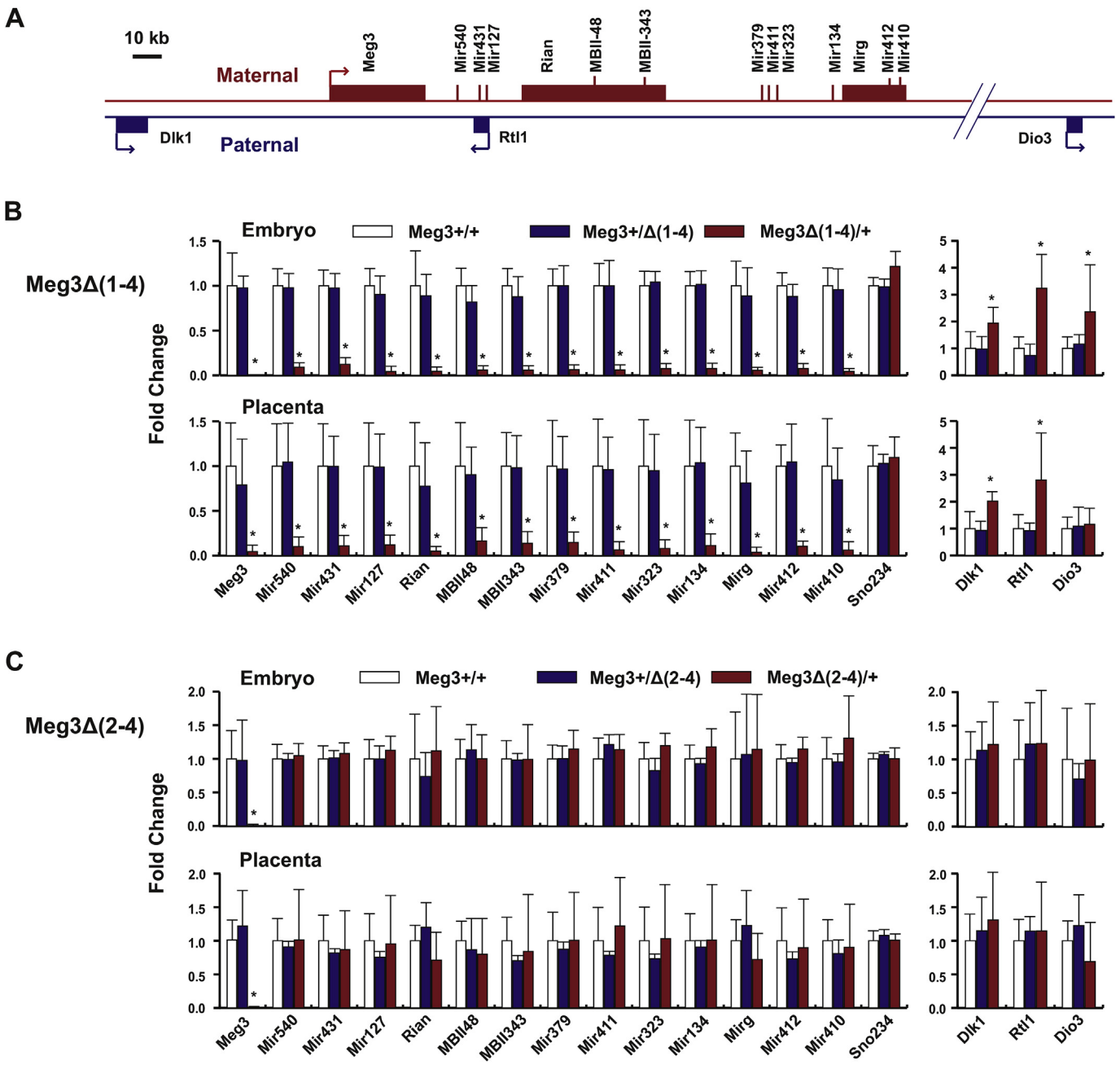

Fig. 2. Maternal deletion of Meg3$^{\Delta(1-4)}$, not Meg3$^{\Delta(2-4)}$, silences MEGs and increases PEG expression. (A) Schematic representation of locations for selected 14 MEGs and 3 PEGs in the Dlk1-Dio3 locus. (B) Relative expression levels of the selected genes in 11.5 dpc Meg3$^{\Delta(1-4)}$ embryos (upper panel) or placentas (lower panel) compared with their WT littermates. (C) Relative expression levels of the selected genes in 11.5 dpc Meg3$^{\Delta(2-4)}$ embryos (upper panel) or placentas (lower panel). Gene expression levels were determined by qRT-PCR. Sno202 was used as the internal reference gene for miRNAs and snoRNAs. Gapdh was the reference gene for lncRNAs and mRNAs. Sno234 is also included as an internal control gene. The expression levels for each gene in KO embryos and placentas were normalized against the levels in their respective WT littermates, which were designated as 1. For each genotype, a minimum of 7 embryos and their corresponding placentas from at least 2 litters were used for gene expression analysis. Student t-test was used to compare values between KO mice and their WT littermates. *$p<0.05$, considered to be statistically significant.

phenomenon was observed in a Kagami-Ogata patient carrying a microdeletion overlapping the IG-DMR (Kagami et al., 2010). Conversely, the methylation status of the IG-DMR is unaffected by a Meg3-DMR deletion as shown in Meg3$^{\Delta(1-4)/+}$ mice (Fig. 4) and in Kagami-Ogata patients with microdeletions containing the 5' part of the MEG3 gene (Kagami et al., 2010; Beygo et al., 2015). Considering that inactivation of either IG-DMR or Meg3-DMR results in LOI of the *Dlk1-Dio3* locus, these data suggest that imprinting regulation by IG-DMR is mediated by the Meg3-DMR. Luo et al. identified an element 10 kb upstream of the *Meg3* gene involved in regulating expression of MEGs (Luo et al., 2016). This element, known as the Meg3-proximal enhancer, contains binding sites for transcription factor ZFP281 (Luo et al., 2016; Wang et al., 2017). ZFP281 recruits AFF3, a component of RNA polymerase II elongation complex, to the enhancer on the maternal chromosome where the nearby IG-DMR is not methylated (Luo et al., 2016; Wang et al., 2017). This recruitment of AFF3 promotes transcription activation from the *Meg3* promoter (Luo et al., 2016). On the paternal chromosome, AFF3 is sequestered to the methylated IG-DMR via ZFP57 (Wang et al., 2017), which binds specifically to its methylated motifs (Quenneville et al., 2011). These data further indicate that the IG-DMR

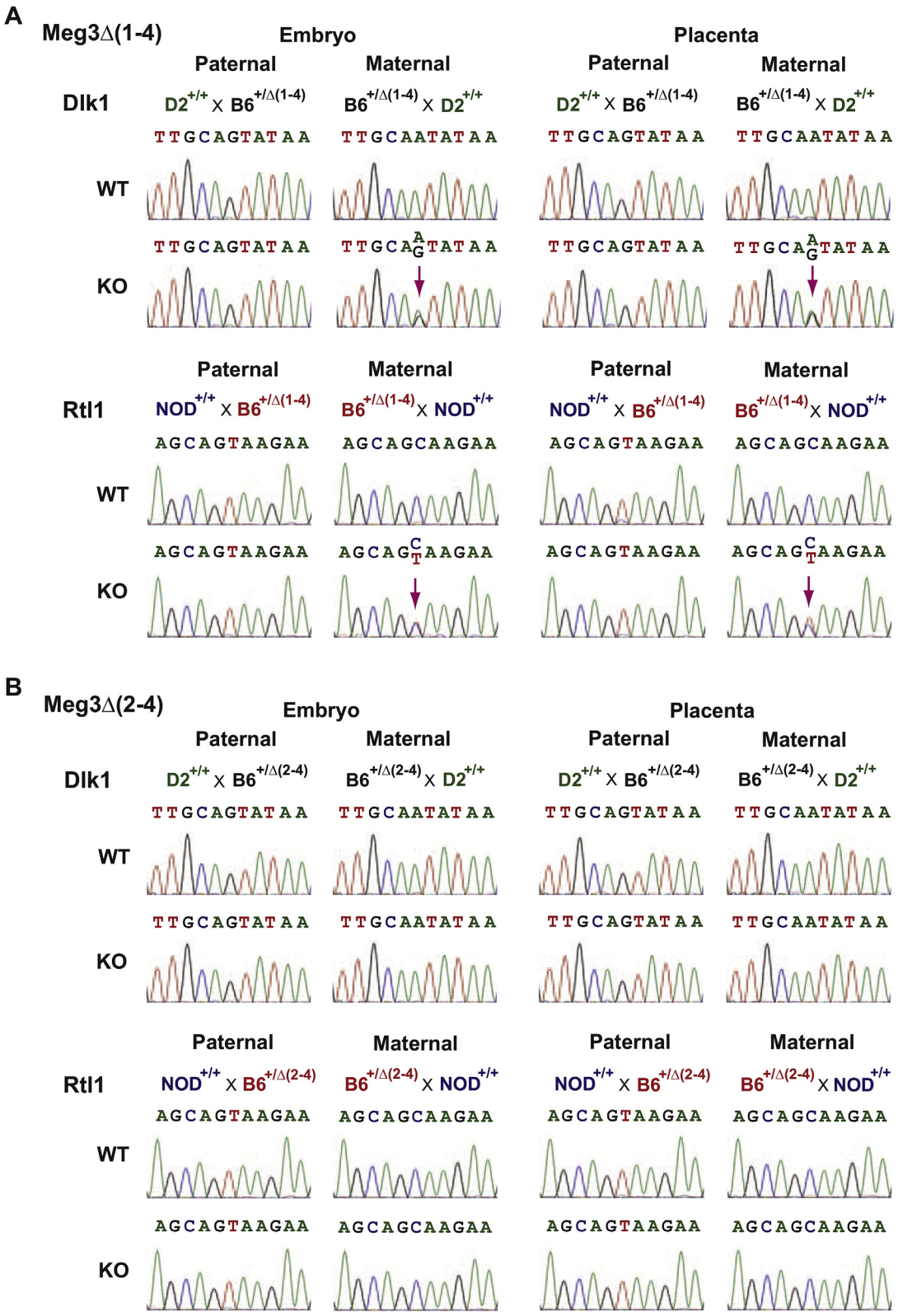

Fig. 3. Bialleic expression of Dlk1 and Rtl1. Bialleic expression of these two genes were observed in Meg3$^{\Delta(1-4)}$ mice (A), not in Meg3$^{\Delta(2-4)}$ mice (B). Chromosome-specific gene expression was determined by SNP. To detect SNP in Dlk1, Meg3$^{\Delta(1-4)}$ or Meg3$^{\Delta(2-4)}$ mice in C57BL/6 background (B6$^{\Delta(1-4)}$ or B6$^{\Delta(2-4)}$) were crossed with WT DBA/2J (D2$^{+/+}$) mice. To detect SNP in Rtl1, B6$^{\Delta(1-4)}$ or B6$^{\Delta(2-4)}$ mice were crossed with WT NOD/ShLit (NOD$^{+/+}$) mice. Total RNAs were extracted from embryos and placentas of 11.5 dpc. After reverse transcription, cDNA fragments containing SNPs for Dlk1 and Rtl1 were amplified by PCR, purified and sequenced. For each genotype, a minimum of 4 embryos and their corresponding placentas from at least 2 litters were used for SNP analysis. A purple arrow indicates detected bialleic expression.

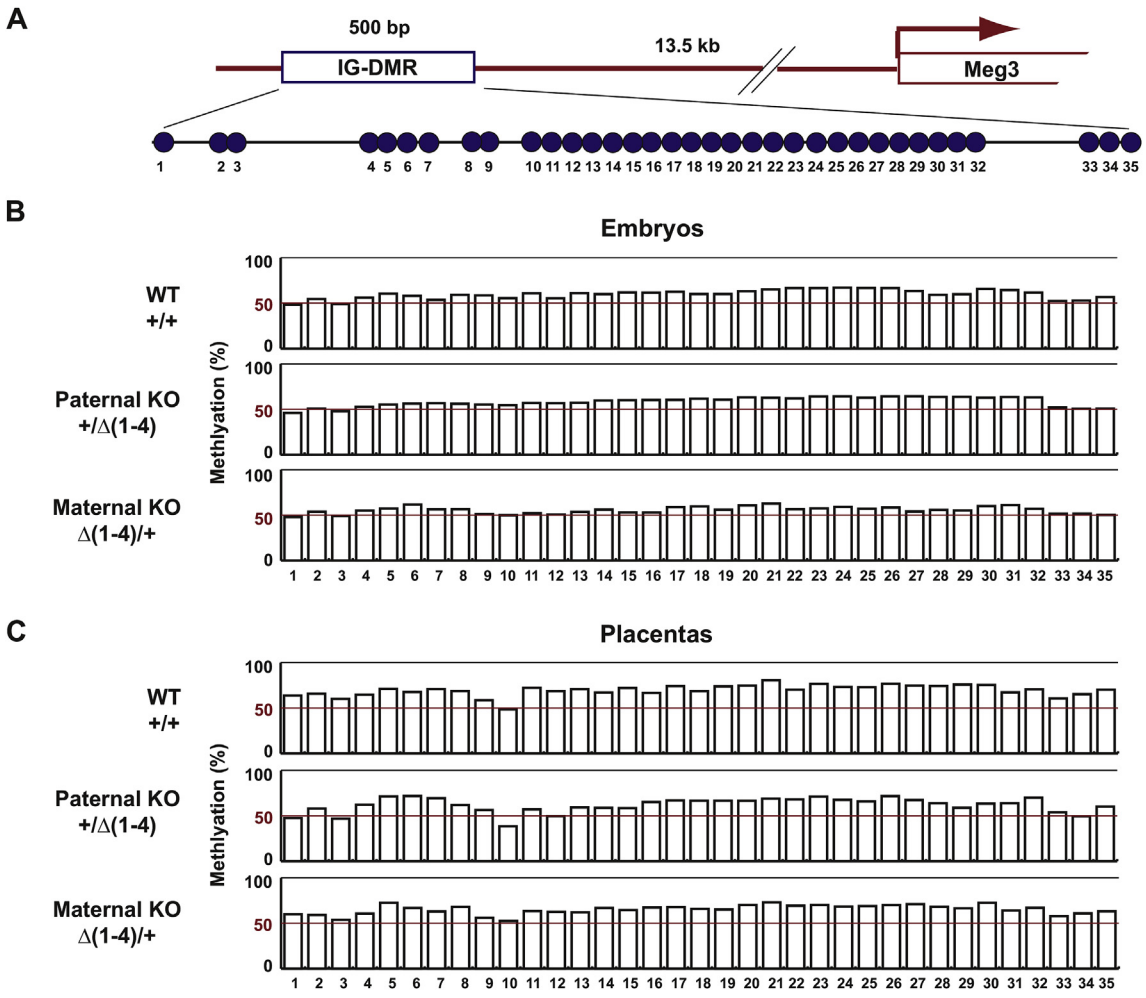

Fig. 4. Deletion of Meg3-DMR does not affect methylation status of IG-DMR. (A) Schematic representation of CpG locations (open circle) in the core region of IG-DMR. (B) Methylation status in embryos of WT, paternal KO (+/Δ(1–4)) and maternal KO (Δ(1–4)/+). (C) Methylation status in placentas of WT, paternal KO (+/Δ(1–4)) and maternal KO (Δ(1–4)/+). The values were derived from 3 WT, 3 $Meg3^{\Delta(1-4)/+}$ and 3 $Meg3^{+/\Delta(1-4)}$ embryos and their corresponding placentas.

regulates imprinting of the *Dlk1-Dio3* locus through Meg3-DMR.

Data from previous studies suggest that all maternally expressed genes in the *Dlk1-Dio3* domain are transcribed as one large transcript (Sanli et al., 2018; Luo et al., 2016; Tierling et al., 2006). Considering that all MEGs are downstream of the *Meg3* promoter, it is very likely that the *Meg3* promoter controls MEG expression. This is consistent with our finding that all MEG expression is silenced in $Meg3^{\Delta(1-4)/+}$ mice. The Meg3-DMR overlaps with the *Meg3* promoter. Because DNA methylation is a common mechanism in gene silencing, it is logical to hypothesize that the main function of the Meg3-DMR is to modulate *Meg3* promoter activity to control the parental origin-dependent expression of downstream MEGs. It turns on the *Meg3* promoter on the maternal chromosome; while it turns off the promoter on the paternal chromosome. Our results indicate that deletion of the *Meg3-DMR* inactivates the *Meg3* promoter which in turn silences expression of all MEGs (Schuster-Gossler et al., 1998), implying that the MEG products may play a role in suppression of PEG expression in the *Dlk1-Dio3* locus. Sanli et al. observed that Meg3 transcripts colocalized with the *Dlk1* gene on the maternal chromosome and the maternal *Dlk1* promoter was activated in $\Delta promoter^{-/-}$ and $\Delta intron1^{-/-}$ cells. They suggested that Meg3 RNAs play a role in regulation of Dlk1 expression (Sanli et al., 2018). However, all ES clones in the study contained deletions in either the *Meg3* promoter or *Meg3* intron 1, which overlap with the *Meg3-DMR*. Therefore, their data did not distinguish whether the effect was caused by lack of Meg3 expression or inactivation of *Meg3-DMR*. In contrast, data from our $Meg3^{\Delta(2-4)}$ mice demonstrated that the reduced Meg3 expression did not affect Dlk1 expression (Fig. 2), which is consistent with data from the Meg3-10kb mice by Takahashi et al. (2009). The Meg3 expression was completely abolished in mice carrying a maternal Meg3-10kb deletion. However, the Dlk1 expression did not increase at all in those KO mice compared with their WT littermates (Takahashi et al., 2009). Furthermore, no changes in Meg3-DMR methylation patterns were observed in $Meg3^{\Delta(2-4)/+}$ embryos compared with those in $Meg3^{+/\Delta(2-4)}$ and WT embryos (Fig. S4). Taken together, these data strongly indicate that Meg3 RNAs are not essential in imprinting regulation.

Anti-Rtl1 encodes seven miRNAs, such as miR-127 and miR-136, which are antisense to Rtl1 (Seitz et al., 2003; Davis et al., 2005). These miRNAs were shown to reduce Rtl1 RNA transcripts *in vivo* by RISC-mediated cleavage of RNAs (Davis et al., 2005). However, all Rtl1 RNAs were transcribed from the paternal allele in mice with the miR-127 deletion (Ito et al., 2015). These data suggest that these miRNAs are dispensable in Rtl1 imprinting. Approximately 80% of miRNAs in the *Dlk1-Dio3* locus were clustered between miR-379 and miR-410, which including miRNAs encoded by *Mirg* (Seitz et al., 2004). Labialle et al. reported that a maternal deletion of the miR-379/miR-410 cluster did not affect expression levels of Dlk1 and Dio3 (Labialle et al., 2014). In agreement, Gao et al. found that a smaller deletion between miR-379 and miR-544 did not affect the RNA levels of Dlk1, although the Dlk1 protein levels were increased by 1.6-fold in mice with a maternal deletion (Gao et al., 2015). These findings indicate that the vast majority of miRNAs in

the *Dlk1-Dio3* locus are not involved in inhibition of PEG transcription.

Gene silencing by transcription has been demonstrated in several imprinted loci (Kanduri, 2016). For example, In the *Igf2r* locus, the paternal allele of *Igf2r* is silenced by the continuous transcription of long non-coding RNA Airn (Santoro et al., 2013; Latos et al., 2012). The *Airn* promoter is inside a DMR located in the intron 2 of the *Igf2r* gene. The transcription of Airn over the *Igf2r* promoter blocks the transcription initiation from the promoter (Santoro et al., 2013). Similarly, the *Nesp* gene is transcriptionally silenced in the *Gnas* locus by the transcription of its anti-sense *Nespas* gene (Williamson et al., 2011). In the *Dlk1-Dio3* locus, the paternally expressed genes *Rtl1* and *Dio3* are downstream of the *Meg3*. Therefore, a likewise mechanism may be used by the Meg3-DMR to regulate the imprinting, which is to maintain an active transcription from the Meg3 promoter, whereby to silence Rtl1 and Dio3. This could be tested by inserting a polyadenylation (polyA) cassette before or after the Rtl1 gene, which stops transcriptions from the Meg3-DMR before or after the Rtl1 gene. This technique has been used to determine transcriptions of Airn or Nespas in imprinting regulation (Latos et al., 2012; Tibbit et al., 2015; Santoro and Pauler, 2013). Although the resultant RNA transcripts will contain full-length Meg3 RNAs, we expect that the maternal allele of the Rtl1 gene will be silenced in models with polyA inserted after the gene, not in models with poly A inserted before the gene. A caveat of this hypothesis does not explain how the transcription from the Meg3-DMR silences the Dlk1 expression. Because not all MEGs within the locus have been thoroughly investigated for their roles in regulating PEG expression, we speculate that *Dlk1* may be silenced by one or more of the MEG products, similar to the inhibition of *Slc22a3* by Airn lncRNA in the *Igf2r* locus (Nagano et al., 2008).

4. Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.07.005.

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