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Expression patterns of long noncoding RNAs from *Dlk1-Dio3* imprinted region and the potential mechanisms of *Gtl2* activation during blastocyst development

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ABSTRACT

The function of long noncoding RNAs (lncRNAs) in cell differentiation and development have begun to be revealed in recent years. However, the expression pattern and mechanisms regulating lncRNAs are largely unknown during mammalian preimplantation development. LncRNAs expressed from *Dlk1-Dio3* imprinted region have been linked to pluripotency of induced pluripotent cells (iPSCs). In this study we show that these lncRNAs (Gtl2, Rian and Mirg) are first expressed at the morula stage and gradually restricted to the inner cell mass (ICM) as the embryo differentiates into the blastocyst. Analysis of DNA methylation at IG-DMR and Gtl2-DMR showed no change during preimplantation while the presence of the activating histone modification H3K4me3 increased significantly from 8-cell to blastocyst stage, which may explain the expression activation. Additionally, knockdown of transcription factors (Oct4, Sox2 and Nanog) in blastocyst reduced the expression of Gtl2, indicating pluripotency factors regulate transcription of these lncRNAs. This study provides the spatiotemporal expression and dynamic changes of lncRNAs from *Dlk1-Dio3* imprinted region in mouse preimplantation stage embryos and offers insight into the potential mechanisms responsible for *Gtl2* activation.

1. Introduction

Advances deep sequencing techniques have identified large numbers of lncRNAs that are expressed during preimplantation development [1,2]. Most of the lncRNAs expression restricted to precise stages in very narrow windows during early embryo cleavage [1,3]. The specific and dynamic expression of lncRNAs suggests diverse and important functions in early embryo development. However, little is documented about the expression patterns of individual lncRNAs and the mechanisms controlling their expression during early embryogenesis.

Gtl2 (also known as Meg3 in human), Rian and Mirg are three major maternal lncRNAs derived from imprinted *Dlk1-Dio3* locus. Recent studies on transcription factor-mediated induced pluripotent cells (iPSCs) suggest that the degree of activation of the maternally expressed lncRNAs from the *Dlk1-Dio3* locus positively correlates with the pluripotency level of individual iPSC lines, and may be a potential marker to distinguish embryonic stem cell-equivalent iPSCs from limited potency iPSCs [4]. Normal expression of the lncRNAs such as Gtl2 was shown to be crucial to the pluripotency of iPSCs, and iPSC lines that showed aberrant silencing of *Gtl2* (Gtl2^{off} iPSCs) contributed poorly to chimeras and failed to yield viable all-iPSC mice by tetraploid (4N) complementation. Conversely, iPSC lines with appropriate Gtl2 expression produced high-grade chimeras and all-iPSC mice [5,6]. Since iPSCs are similar to stem cells (ESCs) and ESCs are derived from inner cell mass (ICM) of blastocyst, understanding the normal expression patterns and mechanisms regulating the lncRNAs from *Dlk1-Dio3* domain *in vivo* during preimplantation development and blastocyst formation will enhance iPSC derivation and utility, and benefit the relative study on lncRNA function in early embryos.

In this paper, we demonstrated the lncRNAs from imprinted *Dlk1-Dio3* locus (Gtl2, Rian and Mirg) are first expressed at the morula and gradually restricted to the ICM of blastocyst during

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preimplantation development. Epigenetic analysis of IG-DMR and Gtl2-DMR showed that activating histone modification H3K4me3 increased significantly coinciding with expression of Gtl2, while methylation patterns showed no change. Additionally, Gtl2 is down-regulated in blastocyst with the knockdown of transcription factors (Oct4, Sox2 and Nanog).

2. Materials and methods

2.1. Collection of oocytes and embryos

All animal experiments were approved by the Rules for Animal Experiments 2011 published by the Chinese Government. For timed pregnancy, PMSG (10 IU) was administered by intraperitoneal injection into female mice aged 6–8 weeks. After 46–48 h, hCG (10 IU) was injected and females were mated with male mice. For expression assays, μChIP assay and microinjection experiments ICR mice were used. For DNA methylation and imprinting analysis, embryos from F1 generation of C57BL/6J (♀) and ICR (♂) were produced. Pregnant mice were euthanized at various time points to obtain embryos (Supplement Fig. 1A–G).

2.2. RNA preparation and qRT-PCR analysis

We collected at least 30 oocytes or embryos of each stage. After removing zona pellucida, we extracted RNA with PureLink® RNA Mini kit (Ambion). The extracted RNA was reverse transcribed by High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) with oligo(dT) primers. The qRT-PCR was performed with the SYBR Green PCR master mix kit (Applied Biosystems) following the manufacturer's instructions using 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s in a 7500 Fast Real- Time PCR system (Applied Biosystems). In order to avoid genomic DNA contamination, primers were designed to span introns or cross intron/exon boundaries (see Supplement Table 1). All qRT-PCR used *18s* as reference gene for normalization of gene expression.

2.3. In situ hybridization and RNA-FISH

RNA probes were generated as described previously [7]. In brief, Digoxigenin-labeled RNA probes were generated by direct *in vitro* transcription using the DIG RNA labeling kit (Roche). Oocytes or embryos were collected at appropriate time points. For ISH, samples were fixed in 4% paraformaldehyde and performed essentially based on described protocol [8]. For RNA-FISH on morula and blastocyst embryos, zona pellucida was removed and embryos were attached to glass slides. After whole-mount in situ hybridization with Digoxigenin-labeled RNA probes, anti-Digoxigenin-Rhodamine antibodies (Roche) were used for the fluorescence detection. Primers used for probe synthesis are listed in Supplement Table 2.

2.4. DNA methylation analysis

A minimum of 50 embryos form each stage were collected (2-cell, 4-cell, 8-cell and morula) and directly subjected to proteinase K digestion and bisulfite treatment using the EZ DNA methylation-direct kit (Zymo Research). Bisulfite-treated samples were amplified by nested PCR using ZymoTaq™ DNA Polymerase (Zymo Research). Primer sequences are listed in Supplement Table 3. PCR products were subcloned into pMD19 T-Vector. Plasmids were isolated from selected clones and sequenced by the ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, CA, USA) using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

2.5. μChIP assay

The μChIP assay was performed according to the established protocols [9]. In brief, embryos (approximately 1500 total cells per stage) were collected and cross-linked in PBS/20 mM Na-butyrate containing 1% formaldehyde. After incubation at room temperature for 8 min, the reaction was stopped by the addition of 125 mM glycine. The cross-linked chromatin was sonicated to shear chromatin into fragments to approximately 500 bp. Sheared chromatin was divided into groups for different histone modification detection. Each group was subjected to pull down with antibody-coated Dynabeads Protein A (Invitrogen). These antibodies include H3K9me3 (Abcam, ab8898), H3K27me3 (Cell Signaling, #9733), H3K4me3 (Cell Signaling Technology, #9751) and H3ac (Millipore, #06–599). Negative control was incubated with mouse IgG and input DNA without a primary antibody. The immunoprecipitated DNA was eluted from the beads after extensive washing. The qRT-PCR analysis was performed just as described above. Primers used for qPCR after ChIP are listed in Supplement Table 4.

2.6. Generation of shRNA lentiviral particles and microinjection

The complete details of the construction of pLKO.1 and producing lentiviral particles are described online (http://www.addgene.org/tools/protocols/plko/). In brief, the pLKO.1-puro plasmid was modified by replacing puromycin resistance gene to enhanced green fluorescent protein (EGFP) gene. Oligonucleotides encoding shRNA sequences (see Supplement Table 5) were ligated into the pLKO.1-EGFP vector, producing a final plasmid that expressed each shRNA. 293T cells were co-transfected with the shRNA plasmid, packaging plasmid pCMV-dR8.2 and envelope plasmid pCMV-VSVG using FuGENE 6 transfection reagent (Roche). The supernatant containing lentiviral particles was harvested and filtered through the 0.45 μm filter. The viral titer was determined by infecting 293T cells. We injected shRNA lentivirus into the perivitelline space of 1-cell embryos. Infected embryos were determined by GFP expression after 4 day culture in KSOM (Millipore). The efficiency of knockdown was assessed by qRT-PCR as described above and primers are provided in Supplement Table 5.

2.7. Statistics

Values are expressed as mean ± s.d. Three independent experiments were performed and each experiment was repeated in triplicate. Comparison between groups was tested using ANOVA module.

3. Results

3.1. Expression of lncRNAs during preimplantation embryo development

Previous studies have shown that the lncRNAs transcribed from *Dlk1-Dio3* region are crucial to development. For example, deletion of *Gtl2* results in perinatal death [10] or death within 4 weeks postpartum [11]. In addition, the temporal and spatial expression pattern of *Rian* [12] and *Mirg* [13] suggests their potential important roles in embryo development and organogenesis. However, all of these reports focus on post-implantation or postnatal development. Here, we found that the three lncRNAs are first expressed at the morula with increased expression in blastocysts by qRT-PCR (Fig. 1A), and no expression was detected in oocytes or 1-cell, 2-cell, 4-cell or 8-cell stage. In situ hybridization (ISH) using probes designed against each lncRNA confirmed the absence of early expression (Fig. 1B). Additionally, in morula the ISH signal seems

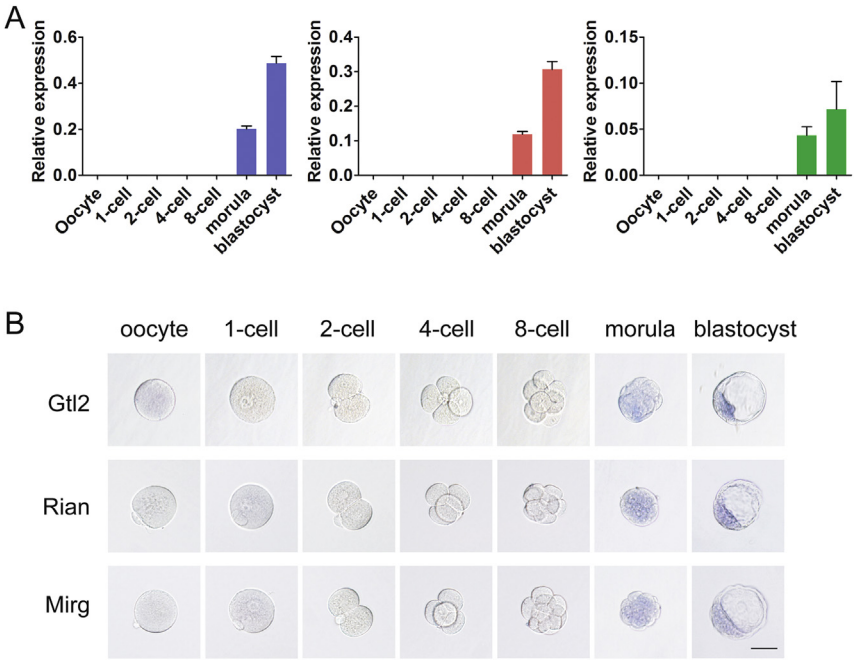

Fig. 1. Expression patterns of lncRNAs from *Dlk1-Dio3* imprinted region. (A) The expression of Gtl2, Rian and Mirg in preimplantation embryos assessed by qRT-PCR. The expression of each gene was normalized to levels of *18s* RNA. (B) Expression analysis of lncRNAs during preimplantation development by in situ hybridization in oocytes and early embryos of each stage. Scale bar, 50 μm.

uniformly distributed in blastomeres, but the signal is only detected in the ICM cells of blastocysts (Fig. 1B).

3.2. Dynamic expression of lncRNAs from morula to blastocyst

In order to more carefully assess the dynamic changes in expression, we detected the RNA of *Gtl2*, *Rian* and *Mirg* during blastocyst formation in detail by RNA fluorescence in situ hybridization (RNA-FISH). The results showed the signal of all the three lncRNAs appeared in early morula with fairly uniform RNA distribution in each blastomere (Fig. 2). However, with development to morula stage (16–32 cells), the signal in outer blastomeres became weaker compared to inner blastomeres (Fig. 2), and coincident with blastocoel cavitation, the expression of lncRNAs became asymmetric and restricted to the ICM cells in the early blastocyst. At blastocyst stage, the lncRNAs are largely restricted to the ICM cells (Fig. 2).

3.3. DNA methylation of IG-DMR and Gtl2-DMR during preimplantation development

The methylation status of two differentially methylated regions IG-DMR and Gtl2-DMR (Fig. 3A) correlate with the imprinted expression of the lncRNAs from *Dlk1-Dio3* region, and allele specific methylation has been shown to regulate allelic expression [14,15]. Hypermethylation of both the IG-DMR and the Gtl2-DMR was associated with reduced expression of lncRNAs in iPSCs with reduced potency [5,16]. Therefore we hypothesized that the methylation status of IG-DMR and/or Gtl2-DMR may correlate with the initial expression of Gtl2 during normal development. We

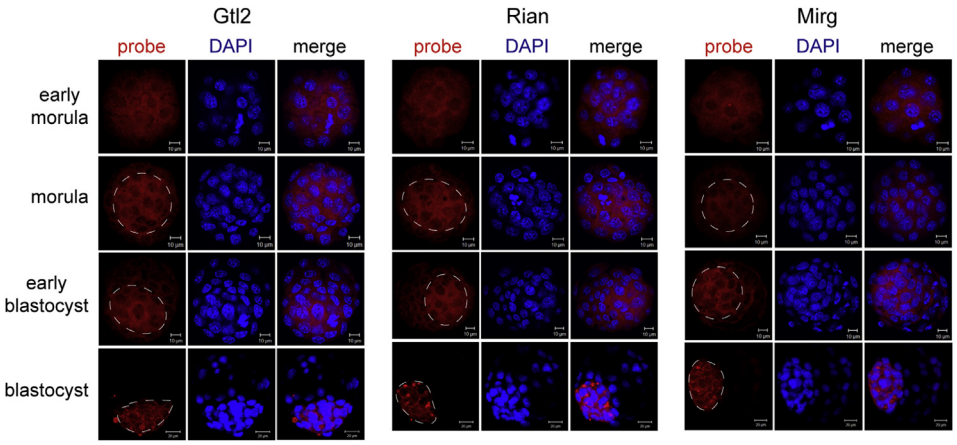

Fig. 2. Dynamic expression of Gtl2, Rian and Mirg by RNA-FISH from morula to blastocyst. After whole-mount in situ hybridization with Digoxigenin-labeled RNA probes, anti-Digoxigenin-Rhodamine antibodies were used for the fluorescence detection (red). DNA was counterstained with DAPI (blue). High Gtl2 expression area is delineated by a dashed line in morula, early blastocyst and blastocyst. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

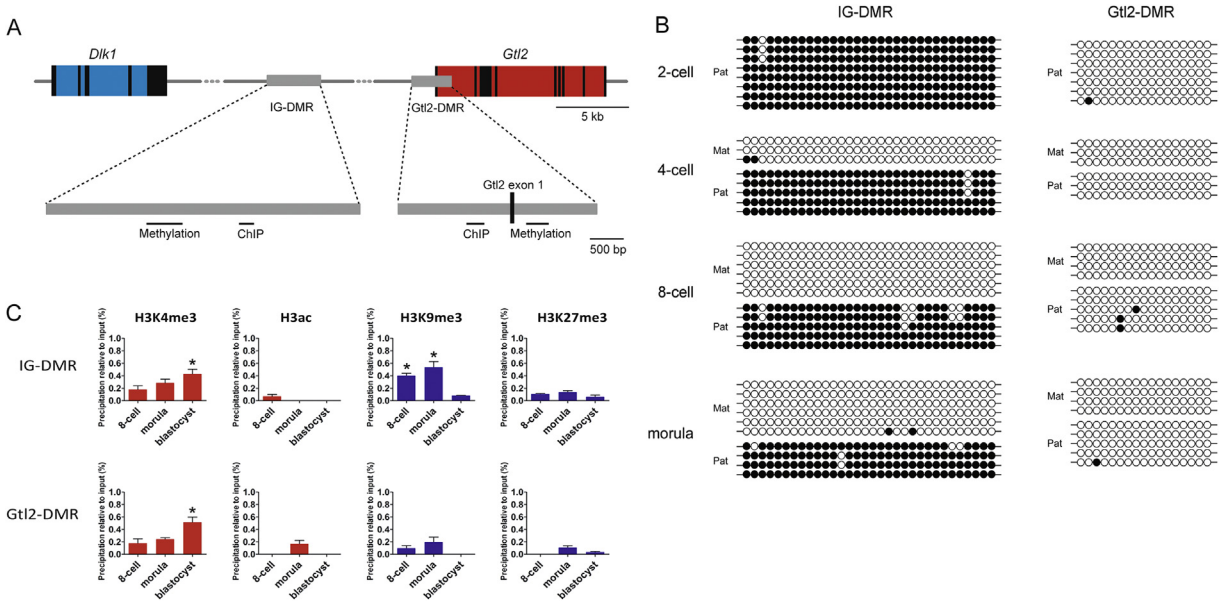

Fig. 3. DNA methylation patterns and histone modifications of IG-DMR and Gtl2-DMR. (A) Schematic representation of the *Dlk1-Gtl2* locus. *Dlk1* (blue rectangle) paternal expressed and *Gtl2* (red rectangle) maternal expressed are depicted. Gray boxes represent the DMR regions, and the areas detected with DNA methylation and histone modifications are designated with black lines. Black boxes represent the exons of each gene. (B) The analyzed regions of IG-DMR contained 32 CpGs. Gtl2-DMR contains 17 CpG dinucleotides. Each CpG dinucleotide is represented with a circle. Each row of circles represents an individual clone sequenced. Black and white circles represent methylated and unmethylated CpGs, respectively. (C) Histone modifications at IG-DMR and Gtl2-DMR from 8-cell to blastocyst. μChIP analysis of histone modifications in 8-cell stage, morula and blastocyst. Data minus the average value of IgG from each group and expressed as percent input chromatin (mean values ± s.d.). Significance is denoted by $^{*}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

examined methylation of the two DMRs (detected regions were shown in Fig. 3A) in 2-cell, 4-cell, 8-cell and morula by bisulfite sequencing analysis. Parental origin was distinguished by single-nucleotide polymorphisms (SNP) between the maternal and paternal alleles (see Supplement Fig. 2). Our results show that regardless of developmental stage, maternal IG-DMR is not methylated and paternal IG-DMR is hypermethylated. Furthermore, both the maternal and paternal alleles of Gtl2-DMR remain unmethylated throughout preimplantation development (Fig. 3B). These results indicate that the methylation status of the two DMRs remains unchanged during the specific developmental stages when the lncRNAs first become expressed. Therefore DNA methylation of these two DMRs is likely *not* involved in the activation of maternal *Gtl2*, *Rian* and *Mirg* transcripts in morula and blastocysts.

3.4. Histone modifications of IG-DMR and Gtl2-DMR from 8-cell to blastocyst

In order to further explore the epigenetic changes occurring with the onset of expression of the lncRNAs, we examined histone modifications at IG-DMR and Gtl2-DMR (detected regions were shown in Fig. 3A) in 8-cell, morula and blastocyst stage embryos by micro chromatin immunoprecipitation assay (μChIP). Two activating histone modifications H3K4me3, H3ac, and two repressive histone modifications H3K9me3, H3K27me3 were examined respectively in 8-cell stage, morula and blastocyst. The results showed H3K4me3 increased significantly both in IG-DMR and Gtl2-DMR from 8-cell to blastocyst (Fig. 3C), which is coincident with the increase of Gtl2 expression (Fig. 1). H3K9me3 maintained a relative high level in 8-cell stage and morula in IG-DMR, but decreased significantly in blastocyst. In contrast, H3K9me3 showed a low level in each stage at Gtl2-DMR. Additionally, H3ac and H3K27me3 were relatively low at all regions examined at all three stages (Fig. 3C).

3.5. The effect of pluripotency transcription factors on the expression regulation of the lncRNAs

Expression analysis above demonstrated Gtl2, Rian and Mirg are first transcribed at the morula stage and become restricted to the ICM cells at the blastocyst stage. Since the initial expression is coincident with ICM lineage specification, we hypothesized that pluripotency transcription factors (TFs) may be involved in regulation of expression. Therefore, we performed functional knockdown of Oct4, Sox2 and Nanog by lentiviral shRNA delivery to preimplantation embryos. These experiments were designed to determine if each TFs influenced the expression of Gtl2 in blastocysts. First, we generated highly infective shRNA lentiviral particles (see Supplement Fig. 3) and microinjected them into the perivitelline space of 1-cell embryos (see Supplement Fig. 1H). After culture to the blastocyst stage, we collected embryos and assessed the efficiency of gene knockdown as well as Gtl2 expression. As shown in Fig. 4, *Oct4*, *Sox2 and Nanog* transcript levels are each reduced by at least 70% after lentiviral infection. As predicted knock-down of any single TF resulted in significantly reduced expression of Gtl2 in blastocysts (Fig. 4), indicating that the Oct4/Sox2/Nanog pluripotency network positively regulates expression of Gtl2.

4. Discussion

Recent data from individual cells of preimplantation embryos by single cell RNA-seq revealed thousands of lncRNAs with specific expression during embryogenesis [1,3]. However, the expression patterns and mechanisms regulating these lncRNAs *in vivo* are not known. In this study we have detailed the expression pattern of lncRNAs Gtl2, Rian and Mirg, transcribed at the *Gtl2*/*Dlk1* imprinted locus. Proper regulation of these lncRNAs has been suggested as an indicator of iPSC quality. Each of the three lncRNAs show similar expression patterns: they are not present from zygote to 8-cell stage;

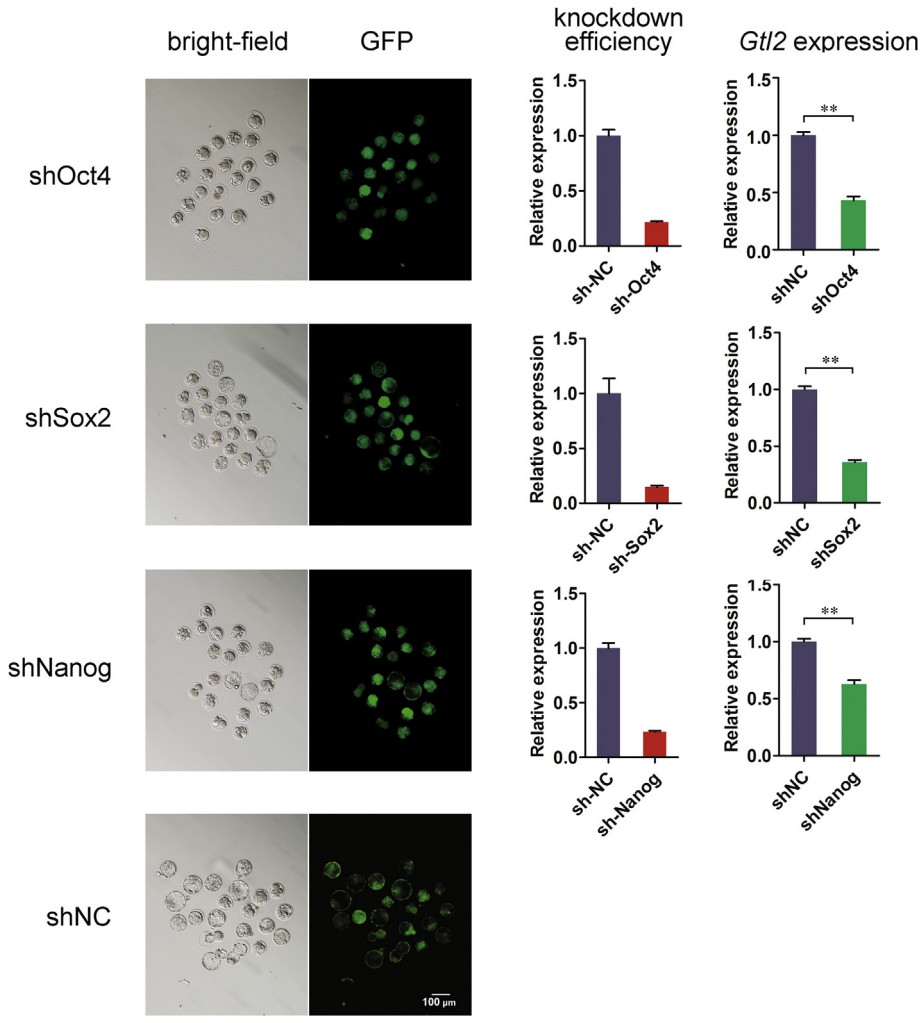

Fig. 4. Gtl2 expression after knockdown of Oct4, Sox2 or Nanog. Lentiviral shRNA delivery was achieved after infection by microinjection into the perivitelline space of 1-cell embryos. RNA was extracted from only GFP-positive embryos (indicating lentiviral expression) to assess knockdown efficiency and Gtl2 expression levels by qRT-PCR. RNA levels are normalized to *18s* levels and compared to levels in scrambled knockdown controls (shNC). Significance is denoted by $^{**}p < 0.01$.

are first expressed in morula, and transcript levels increase in blastocysts (Fig. 1). Importantly the expression becomes restricted to the ICM consistent with their potential function in cell fate allocation and/or pluripotency (Fig. 2). Consistent with our study, previous reports have documented the expression pattern of Gtl2 at the blastocyst stage in whole embryos [17], and also show Gtl2 expression only in the ICM of blastocyst [18]. Our results further define the expression patterns of the three lncRNAs both by examining each developmental preimplantation stage as well as identification of lineage specific expression in whole mount embryos.

Currently it is unknown what mechanism activates these lncRNAs after 8-cell compact morula. In iPSCs, hypermethylation of the IG-DMR and Gtl2-DMR can repress the transcription of *Gtl2* and results in reduced potency of cells [5,16]. However, our results show that the methylation patterns of IG-DMR and Gtl2-DMR do not change during preimplantation (Fig. 3B). IG-DMR maintains differential methylation (paternal hypermethylation and maternal hypomethylation) and Gtl2-DMR is not methylated on either allele, consistent with what has been shown in blastocysts [14,19], and confirming IG-DMR is resistant to the genome-wide DNA demethylation during preimplantation development [20,21]. These results indicate that DNA methylation is not involved in the activation of these lncRNA genes during preimplantation.

However, we do find that enrichment of activating H3K4me3 modification in IG-DMR and Gtl2-DMR increase significantly from the 8-cell stage to the morula and blastocyst stages (Fig. 3C). Similarly, in ESCs, H3K4me3 is also enriched at IG-DMR and Gtl2-DMR [22], which correlates with the activation of *Gtl2*. In iPSCs, $Gtl2^{off}$ iPSC lines show decreased H3K4me3 as well as acetylated H3 and H4 at the *Gtl2* promoter [5]. In contrast, the $Gtl2^{on}$ iPSC lines can be induced by treatment of ascorbic acid during reprogramming, which results in appropriate H3K4me3 and histone H3 acetylation at IG-DMR [16]. However, the *in vitro* results differ from midgestation embryos *in vivo*, where maternal specific histone acetylation was only detected at the Gtl2-DMR, but not at the IG-DMR [21]. In our preimplantation studies we also detected H3ac is specifically enriched at Gtl2-DMR in morula, but not in IG-DMR (Fig. 3C). As to repressive histone modifications, in our study the H3K27me3 always maintains relatively low levels in both DMRs in the stages we examined (Fig. 3C), which is similar to ESCs, iPSCs and midgestation embryos [16,22,23].

Another repressive histone modification, H3K9me3 is associated with imprinted alleles. Overlapping H3K9me3 and H3K4me3 enrichment is considered a common signature of imprinting control regions in ES cells [24]. The high levels of H3K9me3 at IG-DMR region in 8-cell and morula stages in our results might reflect the

specific inhibition on the paternal allele, coinciding with activation of imprinted expression of *Gtl2* in 8-cell compact morula (Supplement Fig. 4). Additionally, a recent study found that the short transcripts produced from the maternal IG-DMR are essential for the expression of lncRNAs in ESCs and blastocysts [18] indicating that an "transcriptionally active" status of IG-DMR is necessary for the expression of lncRNAs. This may explain the high levels of H3K4me3 in IG-DMR we found both in morula and blastocyst (where lncRNAs are most abundant). Similarly we only detected the short transcripts in morula and blastocyst, but not in the stages before 8-cell embryos (data not shown), consistent with our lncRNA expression data. In sum, we conclude that the enrichment of H3K4me3 in IG-DMR and Gtl2-DMR correlates with transcriptional activity of *Gtl2* during preimplantation. In the future, determining the proteins responsible for the epigenetic changes may further explain the activation of expression of *Gtl2* during morula/blastocyst stages.

Since expression results suggested a role for the lncRNA specifically in ICM, we determined the effect of loss of key pluripotency-associated TFs on Gtl2 expression. Knock-down of Oct4, Sox2 and Nanog (independently) each resulted in significant reduction of Glt2 (Fig. 4). These results suggest that ESC-specific TFs may be involved in the regulation of Gtl2 lncRNA expression. Some TF binding sites such as Oct4 and Klf4 were found in the *Gtl2* promoter [25], indicating potential direct binding to and regulation of *Gtl2* by Oct4. Consistent with this possibility, recent publications have revealed that lncRNAs have key roles in self-renewal and lineage differentiation [26,27] and are regulated by pluripotency-associated TFs [28,29]. Importantly, the expression of pluripotency-associated TFs is also influenced by lncRNAs [28], which reflects that lncRNAs also act in molecular network controlling pluripotency and differentiation. Further functional studies are required to determine the interaction between lncRNAs and pluripotency-associated TFs during preimplantation development.

In conclusion, we demonstrated that the lncRNAs from *Dlk1-Dio3* imprinted region are first expressed at the morula stage and become restricted to the ICM in blastocysts. We show significantly increased enrichment of activating histone modification H3K4me3 at the *Gtl2* promoter Gtl2-DMR and at the imprinting control region IG-DMR which may explain the activation of expression. Moreover, the core pluripotent TFs Oct4, Sox2 and Nanog are involved in regulation of *Gtl2*. Together, our results expand our understanding of the molecular regulation mechanism of Gtl2 and other lncRNAs during early embryogenesis.

Conflict of interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.126.

Transparency document

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