**Abstract:** Epigenetic regulation is responsible for transcriptional silencing of genes and parental imprinting. This study addresses the question whether microRNAs (miRNAs) could be affected by DNA methylation during morula-blastocyst transition. Mouse embryos were treated with/without a DNA methyltransferase inhibitor (5-aza-2′-deoxycytidine, 5-aza-dC, 10 nM–5 μM). Changes of miRNAs were analyzed by quantitative real-time (Q-PCR)-based megaplex pre-amp microRNA assays. Development from morula to blastocyst in mice was inhibited by 5-aza-dC in a dose-dependent manner (10 nM–5 μM), with half of the embryos arrested at morula stage when treated with levels of 5-aza-dC as low as 50 nM. In total, 48 down-regulated microRNAs and 17 up-regulated microRNAs (≥5-fold changes) were identified after 5-aza-dC treatment, including let-7e, mir-20a, mir-21, mir-34b, mir-128b and mir-452. Their predicted targets were selected based on software analysis, published databases and further confirmed by Q-PCR. At least eight targets, including dnmt3a, jagged 1, sp1, edg2, abcg4, numa1, tmsb10 and csf1r were confirmed. In conclusion, 5-aza-dC-modified microRNA profiles and identification of the microRNA’s targets during the morula to blastocyst stage in mice provide information that helps us to explore the relationship between fertility, microRNA regulation and epigenetic intervention.

**Key words:** blastocyst / DNA methylation / epigenetic / microRNA / morula

**Introduction**

Since microRNAs (miRNAs) were discovered as endogenous 22-nucleotide non-coding RNAs that regulate the expression of target genes by degradation of target mRNA transcripts and inhibition of mRNA translation (Meister and Tuschl, 2004), more than 1000 human miRNAs have been identified (Griffiths-Jones et al., 2008). Systematic analysis of the expression of miRNAs has shown that many miRNAs are expressed in a tissue-specific manner. The biogenesis and regulatory machinery of miRNAs play important roles in almost all the physiological and pathological processes (Lagos-Quintana et al., 2002; Gao et al., 2011). Although the functions and target genes of most miRNAs are still unknown, miRNAs are known to be involved in development, embryogenesis, neonatal birth, stem cell pluripotency, differentiation, organogenesis, growth control and apoptosis (Amanai et al., 2006; Tang et al., 2006; Cui et al., 2007; Byrne and Warner, 2008; Tzur et al., 2008; Renthal et al., 2010). Examples include mmu-mir-290, which is highly expressed during embryo development (Mineno et al., 2006), mmu-mir-296, which is potentially specific to embryonic stem cells (Suh et al., 2004), and mmu-mir-21 and mmu-mir-22, which are known as differentiation state-related miRNAs (Houbaviy et al., 2003).

Knowledge of how miRNAs are regulated in complex gene regulatory systems has attracted a lot of attention. Two major regulatory keys related to miRNA expression have been discovered in the past few years. One is the genetic regulation of miRNAs, which is similar to mRNA and involves the regulation of miRNAs by specific transcription factors or proteins that interact with the promoter (Meister and Tuschl, 2004; Ying et al., 2008). The other is evidence showing that epigenetic regulation (DNA methylation and histone acetylation) may play a significant role in regulating miRNA expression. A computer-assisted approach indicated that 46 potential miRNAs,
which are located in the human imprinted 14q32 domain, are embedded within a conserved CpG island locus that could be regulated by DNA methylation (Dong and Golden, 2008). Other studies have indicated that miRNA expression could be regulated by epigenetic regulation including miR-127 in human cancer cells and mouse embryos (Suberbielle et al., 2008; Cui et al., 2009). A further example consists of mir-34b/c and mir-124a, which have been shown to have a CpG island near their promoter region (Lujambio et al., 2008; Agirre et al., 2009).

Epigenetic mechanisms play diverse roles in development and have been shown to mediate the heritable repression or up-regulation of various genes in specific cell lineages (Ademokun and Turner, 2008). DNA methylation is able to regulate specific genes through a transcriptional repression mechanism that is mediated in part by methyl-CpG-binding proteins, and DNA methyltransferases (DNMTs) (Santos et al., 2002). Furthermore, DNA methylation has been shown to control the maternal and paternal imprinting genes, which are crucial to embryonic growth and development. It has been reported that >90% of genes expressed in embryos were found to exhibit CpG islands at their transcription start point (Ponger et al., 2001). During preimplantation development, a genome-wide loss of DNA methylation is observed after fertilization; methylation reaches its lowest level at the morula stage and this is followed by re-methylation after the blastocyst stage (Morgan et al., 2005; Shi and Wu, 2009). In this context, DNMT1 is involved in the maintenance of specific DNA methylation patterns during DNA replication, whereas, DNMT3a and DNMT3b exhibit de novo methyltransferase activity (Rahnama et al., 2006) and are highly regulated from oocyte to blastocyst (Santos et al., 2002; Barton et al., 2005; Christine et al., 2005; Ko et al., 2005; Morgan et al., 2005; Corry et al., 2009). All these findings pointed out towards DNA methylation serving as a key component during preimplantation development. However, due to the limitation of acquiring embryo samples, published studies exploring the role of epigenetic regulation of miRNA in the regulatory gene network during preimplantation embryo development are rare, even though this aspect of regulation could play a crucial role in controlling embryo proliferation and differentiation.

In the present work, we performed a Q-PCR-based Megaplex pre-amp miRNA assay aimed at characterizing miRNA expression profiles during the critical stages of embryo development during morula to blastocyst transition, while DNA methylation status is crucial for embryo development. Through integrating the expression patterns of miRNAs and their target genes via validation and reference-based predicting software, the potential functions and epigenetic regulation network of miRNAs in early mouse embryonic development were discovered.

Materials and Methods

Animal and embryo collection

This study was approved by the Institutional Review Board, the Animal Care and Use Committee in Taipei Medical University (Taipei, Taiwan), imprinting control region (ICR) mice (5 weeks old) were super oovulated by 5 IU pregnant mare’s serum globulin (CCP Co., Taiwan), which was followed 48 h later by 10 IU HCG (CCP Co.). Embryos (morula stage) were collected from oviduct and ovary after 66 h post HCG. Embryos were cultured in human tubal fluid medium (HTF medium; Irvine Scientific, Santa Ana, CA, USA) containing 10% of human plasma and 0.3% of bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) for 24 h with or without 5-aza-2’-deoxycytidine (5-aza-dC) treatment. Drug treatment

miRNA profiling of the ICR mouse embryos from morula to blastocyst with or without DNMT inhibitor (5-aza-dC, Sigma-Aldrich) treatment (n = 100 for each group) was carried out. The treatment concentration for miRNA profile and target gene validation is 10 nM. Embryos that have been treated 24 h with or without DNMT inhibitor were collected for total RNA extraction.

Immunofluorescence staining

For the detection of 5-methyl-cytosine (5-MeC), embryos were treated with 2 M HCl at room temperature for 30 min and subsequently neutralized for 10 min with 100 mM Tris/HCl buffer, pH 8.5, after Triton-X 100 permeabilization. After extensive washing with 0.05% Tween 20 in phosphate-buffered saline (PBS), embryos were blocked overnight at 4°C in 1% BSA, 0.05% Tween 20 in PBS. Anti-5-MeC antibodies (Merck KGaA, Darmstadt, Germany) were detected by a secondary antibody coupled with Alexa Fluor 594 (Molecular Probes, Inc.). DNA was stained with 3 μg/ml 4,6-diamidino-2-phenylindole (DAPI) in PBS (Sigma).

Q-PCR analysis of miRNA expression

miRNA expression profiling was performed using the Megaplex pre-amp miRNA assay according to the protocol from Applied Biosystems. Total RNA was extracted from the three groups of embryos (morula, blastocyst with or without 5-aza-dC) using TRIZOL (Life Technologies, Rockville, MD, USA) and a set of 450 mature form miRNAs were specifically amplified for individual miRNA TaqMan real-time Q-PCR analysis. The amplification procedures contain two steps including reverse transcription and preamp process. Reverse transcription condition was as follows: 40 cycles at 16°C for 2 min, 42°C for 1 min, 50°C for 1 s, then 85°C for 5 min and 4°C on hold. Preamp condition was as follows: 95°C for 10 min, 55°C for 2 min, 72°C for 2 min, then followed by 12 cycles at 95°C for 15 s, 60°C for 4 min, then on hold at 4°C. Quantification of the miRNAs was performed by TaqMan miRNA real-time Q-PCR assays (Applied Biosystems). Sn202 was used as an internal control of the miRNA expression. The PCR was run in a 7900HT Fast Real-Time PCR System (Applied Biosystems), PCR condition was as follows: 95°C for 10 min, followed by 50 cycles at 95°C for 15 s, 60°C for 1 min. SDS 2.3 software (Applied Biosystems) was used for the comparative ∆∆Ct analysis (Livak and Schmittgen, 2001). The experiments were performed three times in triplicate.

SYBR green real-time QRT-PCR

The expression levels of various predicted target genes was detected by real-time PCR on an ABI prism 7900 sequence detection system (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The relative expression level of the genes compared with that of GAPDH was defined as 

\[
\frac{\Delta C_{\text{gene}}}{\Delta C_{\text{GAPDH}}} = \frac{C_{\text{gene}} - C_{\text{GAPDH}}}{C_{\text{GAPDH}}}.
\]

The gene mRNA/gapdh mRNA ratio was calculated as 

\[
2^{-\Delta\Delta C} \times K
\]

in which K is a constant. Experiments were performed three times in triplicate. The primers used in this study are listed in supplementary data, Table SI.

miRNA targets prediction

The miRNA targets were predicted with some modifications from various comprehensive databases of experimentally supported miRNA targets.
software. These were as follows: pathway analysis for the mouse (http://140.120.213.10:8080/SpecificDB/mouse.html); MiRNAmap (http://mirmamap.mbc.nctu.edu.tw/); Miranda (http://www.micromap.org/miranda_new.html); clone gene convertor (http://idconverter.bioinfo.cncio.es/); and PicTar (http://pic.tar.mdc-berlin.de/). Metacore Pathway analysis software (GeneGo Inc., St. Joseph, MI, USA) was used to investigate the miRNA-predicted genes (https://portal.genego.com/cgi/data_manager.cgi) in order to identify the connections between the miRNAs and their downstream genes. The potential target genes were carefully examined and chosen by overlapping the PicTar and Miranda databases with the microarray data set from the morula to blastocyst stage (Hamatani et al., 2004). Genes were narrowed down further by selecting genes that had already shown to be important during embryo development. Putative target gene promoters were analyzed by UCSC genome database (http://genome.ucsc.edu/cgi-bin/hgGateway) to examine the CpG island location.

**Statistical analysis**

All experiments were performed in triplicate and analyzed by analysis of variance (Excel, Microsoft; Taipei, Taiwan) for significant differences. A 0.05 was considered statistically significant. Where appropriate, the data are presented as the mean ± SD.

**Results**

**Inhibition of DNA methylation by 5-aza deoxycytidine is able to alter early embryo development**

DNA methylation has been shown to play important roles in early embryo development, especially in the morula to blastocyst stage (Ponger et al., 2001; Morgan et al., 2005; Shi and Wu, 2009). To investigate the role of DNA methylation during early embryo development, embryos were collected at the morula stage and treated with the DNMT inhibitor 5-aza-dC. The effect of 5-aza-dC on embryos is concentration-dependent (10 nM–5 μM), whereas, high dosage (5 μM) may cause direct embryo toxicity (Supplementary data, Fig S1) and 1 μM 5-aza-dC was able to arrest embryo development (Fig. S1). To identify an optimal dose of 5-aza-dC that affected the regulation of embryo development without direct toxicity to the embryo, we titrated the dosage of 5-aza-dC to 10 and 50 nM. Figure 1A showed the control group was found to have an 84.1% success rate of development at 24 h (morula to blastocyst) and a 93.8% success rate from morula stage to blastocyst or hatching success rate of development at 24 h (morula to blastocyst) and a 93.8% success rate from morula stage to blastocyst or hatching success rate of development at 24 h (morula to blastocyst) and a 93.8% success rate from morula stage to blastocyst or hatching success rate of development at 24 h (morula to blastocyst). When treated with 10 and 50 nM 5-aza-dC, these values were 62.9 and 40.8% at 24 h and 72.7 and 41.3% at 48 h (Fig. 1A). Using 5-MeC antibody staining, we found that the DNA methylation status of 5-aza-dC-treated embryos was inhibited (Fig. 1B). Thus, under such low dosages of 5-aza-dC treatment, it is found that 5-aza-dC was able to inhibit embryo development from morula to blastocyst.

**Effects of 5-aza-dC-induced arrested development on miRNA expression profile**

Since morula to blastocyst was a critical time point for DNA re-methylation (Morgan et al., 2005; Shi and Wu, 2009), we tried to examine whether the dynamic regulation of miRNA expression profiles could be affected by the DNA methylation inhibitor, 5-aza-dC. Due to very limited amounts of total RNA in a single embryo, this was relatively difficult. We collected 100 embryos for each group and processed amplification assay specific for miRNAs. Although 10 nM 5-aza-dC treatment may cause 14.8% of embryo fragmentation (comparing with 1.5% in control group), only live embryos (morula and blastocyst) were collected for the miRNAs analysis.

Comparing the control group with the 10 nM 5-aza-dC-treated group, out of all the miRNAs screened, 48 were found to be down-regulated by an at least 5-fold change (e.g. mir-290, mir-96), while 17 miRNAs were up-regulated by at least 5-fold (e.g. mir-34b, mir-139, mir-34b) (Fig. 1C and Table. I). All 65 miRNAs showed significant differences (P < 0.05). Among these, several miRNAs that have been reported to play critical roles in early embryo development, differentiation, proliferation and apoptosis were identified (Table. II). These included: mir-21, mir-143, mir-290, mir-20a, let-7e and let-7g, which have been reported to involve in embryo implantation (Hu et al., 2008); mir-96, mir-296 and mir-34b, which may play a role in cell differentiation (Menino et al., 2006); mir-134, mir-138, mir-290, mir-296, mir-322, mir-452 and mir-128b, which are expressed during preimplantation development and might regulate embryo cell compaction and blastocyst differentiation (Yang et al., 2008); and mir-290, mir-302d, which have been shown to regulate eye, tail and neuron differentiation in mouse (Pasquinelli et al., 2005).

**Target prediction and validation of the 5-aza-dC-regulated miRNAs**

To clarify the possible functions of these 5-aza-dC-regulated miRNAs, we used several miRNA target gene prediction software approaches including PicTar, Miranda, MiRNAmap and pathway analysis of mouse to select potential miRNA target genes. By integrating the selected genes identified by the various pieces of software and morula/blastocyst-related gene expression profiles from published microarray data sets (Hamatani et al., 2004), hundreds of genes were selected after comparing the array data sets with our predicted target genes. Many of these miRNA target genes have been reported to be important in cell proliferation, differentiation and development (Table III) and some of these were selected for further validation.

According to the Q-PCR validation, the miRNAs showed a correlation with the putative eight selected targets (Fig. 1D). Among these, seven 5-aza-dC up-regulated miRNAs showed significant down-regulation of their target genes, viz. dnmt3a, csf1r, edg2, tmsb10, numa1, jag1 and sp1 and one 5-aza-dC down-regulated miRNA target, abcg4, showed significant up-regulation of its target gene.

To investigate the role of epigenetic regulation, we examined the presence of CpG islands in the possible promoter region of each of the target genes. Using the UCSC genome browser software, we were able to predict the presence and location of the CpG islands of our each target gene. Our results showed that among the eight target genes, only sp1 and numa1 have possible CpG islands located at the upstream promoter regions (Table III). The other six genes showed no signs of CpG islands at the upstream promoter regions. These results indicate that DNA methylation may not be directly responsible for regulation of these target genes.
**Discussion**

Previous studies showed dynamic changes in a series of epigenetic regulators (DNMT1, DNMT2, DNMT3a, DNMT3b, HD-1, SIN3a and SIN3b) which indicates epigenetic regulation may play an important role in early embryo development (Chen et al., 2005). In the present study, we focused on the dramatic changes in the epigenetic status that occur during the morula to blastocyst stages in order to investigate how miRNAs and their target genes are regulated by epigenetic modification and how this further affect embryo development. De novo DNA methylation by 5-aza-dC was inhibited during early mouse embryo development, and the miRNAs profiles and their potential target genes studied during the morula to blastocyst stages.
5-aza-dC has been widely used in studying DNA methylation on embryos (Rahnama et al., 2006; Weisenberger et al., 2004), although several reports have showed that during high dose (1 μM) or long-term treatment (48 h) it would cause potent teratogenic or mutagenic effect on preimplantation embryos (Patkin et al., 1998; Tsuji et al., 2009; Yu et al., 2009). In the present study, the concentration was titrated to a relatively lower dose (10 nM) and short treatment time (24 h), which showed no significant cytotoxic effects and may exhibit the minimum teratogenic or mutagenic effect, while still inhibiting DNA methylation.

By disrupting re-methylation of 5-methyl cytosine using 5-aza-dC, 65 miRNAs were identified that might be regulated by epigenetic modification during morula-blastocyst transition. A total of 48 miRNAs were down-regulated by the treatment of 5-aza-dC and 17 miRNAs up-regulated. In general, disrupting the methylation of DNA leads to promoting gene transcription. Although we can provide the evidence to show that 10 nM 5-aza-dC had no significant embryotoxicity within 24 h and DNA methylation is inhibited by 5-aza-dC under such condition. We still cannot explain the mechanisms of how the miRNAs were down-regulated upon 5-aza-dC treatment and suggest that these down-regulated miRNAs may be modulated by other up-regulated miRNAs or genes indirectly; however, the mechanisms are still unknown and need further investigation.

Many of these target genes and miRNAs had been previously reported to be involved in development (Hu et al., 2008; Yang et al., 2008). In our study, the 5-aza-dC-regulated miRNAs were able to regulate at least eight important genes that play crucial roles in embryo development. These eight genes are as follows. Abcg4 have been shown to have a role in CNS development (Bojanic et al., 2010). Tmsb10 might be involved in trophoblast development and implantation (Cammas et al., 2005). Numal is required for mitotic organization and spindle assembly, which is important in spindle-chromosome complex depletion of the embryo (Van Thuan et al., 2006). Csf1r regulates the signaling of cytokines in embryos and could control a range of broad functions related to the development, attachment and invasion of the blastocyst during implantation (Kauma, 2000). Edg2 is suspected of being involved in brain development (Contos et al., 2002). Dnmt3a, a putative target of mir-138, serves as one of the key component of de novo methylation and has been discussed extensively for its role in embryo development (Okano et al., 1999; Watanabe et al., 2002; Kaneda et al., 2004; Huntriss et al., 2004). Jagged 1, a ligand of notch signaling, which seems to be regulated by mir-124a, mir-34b/c and mir-449, contributes to cell–cell interaction and controls to formation of the trophoblast and the inner-cell mass in preimplantation development (Cormier et al., 2004). Finally, the transcriptional factor Sp1, which is a putative target of both mir-124a and mir-375, plays a critical role in development and has been proved to be essential to maintenance of many appropriate methylation patterns, since Sp1-binding sites are frequently located at CpG islands (Marin et al., 1997). Our results showed that expression of Sp1 was correlated with mir-124a and mir-375 during morula to blastocyst development. To further delineate this network of 5-aza-dC-regulated miRNAs and their target genes during embryo development, the data set was integrated using Metacore software (GeneGo bioinformatics software for pathway analysis, Inc., St. Joseph, MI, USA) (Ekins et al., 2007). Figure 2 shows the connections and functions of the 5-aza-dC-regulated miRNAs together with their target genes and the related pathways; this supports a complex network that is very important to preimplantation embryo development.

Using published data, we were able to conclude that seven important putative functions are controlled in part by the 5-aza-dC-regulated miRNAs. These include cell cycle, cell growth, development, differentiation, stem cell pluripotency and epigenetic regulation. It should be noted that many epigenetic-related genes (Hdac1, Hdac2, Hdac4, Dnmt3L and Id1) were found to be connected to our validated target genes, which indicates that the connectivity between miRNAs and epigenetic regulation is not just unidirectional (Corry et al., 2009; Geiman and Muegge, 2010; Weaver et al., 2009). It is possible that miRNAs and epigenetic regulation are concomitantly responsible for gene activation at different stages during development, and the interactivity and connectivity between miRNAs and the epigenetic changes may control the expression of imprinted genes and thus decide cell fate.

Preimplantation development is a complex process that includes not only genetic but also epigenetic factors. Recent studies (Ding et al., 2008, Tsuji et al., 2009) have focused on using 5-aza-dC on cloned embryos undergoing nuclear transfer in order to reprogram the somatic cells epigenetic status to improve embryo development. However, one group indicated that treatment with 5-aza-dC of mouse pronuclear embryos resulted in an inability to differentiate beyond the 8-cell stage (Yu et al., 2009). These contrasting findings demonstrated that only when there is a balanced and correct

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**Figure 1** The effects of 5-aza-dC on preimplantation development and miRNAs regulation. (A) Low dose (10–50 nM) 5-aza-dC treatment for 24 h at the morula stage shows that the development of the embryos was arrested. The right panel shows the embryo at morula stage (100 embryos for each groups) treated with (10–50 nM) 5-aza-dC for 14, 24 and 48 h. These photographs were used to identify the percentage of embryos that were at morula (arrested), that were undergoing lysis/fragment or that were at blastocyst (including hatching stage). (B) Immunofluorescense of 5-methyl cytosine during the morula-blastocyst transition. Embryos were collected at the morula stage, and treated with or without 10 nM of 5-aza-dC for 24 h. Then fixed and prepared for immunofluorescence evaluation. Embryos were stained for DNA with DAPI and Alexa Fluor 594 secondary antibody for 5-MeC and images captured using the Nikon C1 confocal microscopy. Lower panel: quantification of 5-MeC intensity. Intensity of nucleus 5-MeC were measured using MetaExpress software. Results represent means ± SD. *P < 0.05 is statistically significant. (C) Hierarchical clustering analysis of significantly changed miRNAs. A total of 65 miRNAs were selected from 450 screening miRNAs. Relative expression levels are indicated by a color code. Red indicates that the level of expression is higher than median, and green indicates that the level is lower than median. Three major clusters were identified (blue, black and pink). (D) Validation of the predicted targets of the miRNAs. One hundred embryos for each group were collected and treated with 5-aza-dC 10 nM for 24 h. Total RNA was extracted for SYBR green Q-PCR analysis, gapdh was used as the internal control in each group. Results represent means ± SD. *P < 0.05 is statistically significant.
Table I MicroRNAs profiles in mice embryo treated with or without 5-aza-dC (10 nM).

<table>
<thead>
<tr>
<th>Down-regulated microRNA</th>
<th>Fold change (5-aza/blastocyst)</th>
<th>Fold change (blastocyst/morula)</th>
<th>Fold change (5-aza/morula)</th>
<th>Up-regulated microRNA</th>
<th>Fold change (5-aza/blastocyst)</th>
<th>Fold change (blastocyst/morula)</th>
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<tr>
<td>mmu-miR-290</td>
<td>&lt;0.01</td>
<td>5571.63</td>
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<td>has-miR-210</td>
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<td>737.68</td>
<td>13295.30</td>
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Table I

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</table>

The expression profiles chosen from among the microRNAs were those with a ≥5-fold change in the 5-AZA/blastocyst group. We used Sno-202 as the internal control to normalize the expression level between the two groups. Experiments were repeated in triplicates. Fold change: 2^-DDCT of each group. SD, standard deviation of relative expression level in triplicates.

Table II Published microRNA related to embryo development.

<table>
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<tr>
<td>mir-96, mir-296, mir-34b</td>
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</tr>
<tr>
<td>mir-290, mir-302d</td>
<td>Regulate eye, tail and neuron differentiation</td>
<td>Pasquinelli et al. (2005)</td>
</tr>
</tbody>
</table>

Table III Potential targets of the 5-aza-dC-regulated microRNAs.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Target gene (Unigene No.)</th>
<th>Presence of CpG island at target gene promoter</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down-regulated miRNAs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-296, miR-32</td>
<td>Abcg4, ATP-binding cassette, sub-family G member 4 (Mm.101876)</td>
<td>No</td>
<td>Bojanic et al.</td>
</tr>
<tr>
<td>Up-regulated miRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-23a, miR-23b, miR-29b, miR-322, miR-381</td>
<td>Yy1, YY1 transcription factor (Mm.3868)</td>
<td>No</td>
<td>Affar el et al. (2006)</td>
</tr>
<tr>
<td>miR-449</td>
<td>Tmsb10, thymosin, beta 10 (Mm.3532)</td>
<td>No</td>
<td>Cammas et al. (2005)</td>
</tr>
<tr>
<td>miR-124a</td>
<td>Numa1, nuclear mitotic apparatus protein 1 (Mm.27259)</td>
<td>Yes</td>
<td>Van Thuan et al. (2006)</td>
</tr>
<tr>
<td>miR-124a, miR-375</td>
<td>Sp1, trans-acting transcription factor 1 (Mm.4618)</td>
<td>Yes</td>
<td>Marin et al. (1997)</td>
</tr>
<tr>
<td>miR-34c, miR-449</td>
<td>Csf1r, colony stimulating factor 1 receptor (Mm.795)</td>
<td>No</td>
<td>Kauma (2000)</td>
</tr>
<tr>
<td>miR-204, miR-211, miR-138</td>
<td>Edg2, endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2 (Mm.4772)</td>
<td>No</td>
<td>Contos et al. (2002)</td>
</tr>
<tr>
<td>miR-124a, miR-34b, miR-34c, miR-449</td>
<td>Dnmt3a, DNA methyltransferase 3A (Mm.5001)</td>
<td>No</td>
<td>Watanabe et al. (2002), Kaneda et al. (2004), Huntriss et al. (2004), Okano et al. (1999), Cormier et al. (2004)</td>
</tr>
<tr>
<td>miR-124a, miR-34b, miR-34c, miR-449</td>
<td>Jag1, jagged 1 (Mm.22398)</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>
epigenetic status does successful development occur. Furthermore, it also emphasizes the critical role of epigenetic regulation in embryo development. Different epigenetic interventions might cause very different results depending on the background status of the gene expression profiles under certain conditions.

Other studies showed that DGCR8- and DICER1-knockout mice could survive through preimplantation development (Bernstein et al., 2003; Suh et al., 2010). These results suggest that a lack of miRNA biogenesis would not interfere with embryo development. However, although DICER1 knockdown mice showed a lethal effect after E7.5 embryos (Bernstein et al., 2003), there was no evidence to show any abnormality or mutation at an earlier stage in preimplantation development. DGCR8-knockout mice showed normal preimplantation development, but since DGCR8-knockout mice could only block canonical biogenesis of miRNAs (Suh et al., 2010), biogenesis of certain miRNAs may have occurred. Interestingly, Suh et al. pointed out that early development of embryos might rely on maternal or paternal endogenous endo-siRNAs but not newly synthesis miRNAs and the transition stage from endo-siRNAs to miRNAs is still unclear. Our data showed a low expression pattern of miRNAs in the morula stage with dramatically higher expression in the blastocyst stage. Although early embryos might rely on the endo-siRNAs at the blastocyst stage, we do believe the miRNAs machinery might be turned-on after morula-blastocyst transition, and affect the development and differentiation of the embryos. Moreover, several important miRNAs may play a role during blastocyst differentiation (Yang et al., 2008), even if not important during preimplantation.

Although we have identified 5-aza-dC-regulated miRNAs and their potential targets from the morula to blastocyst stage, several issues still remain unclear. First, we cannot completely rule out the possibility

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**Figure 2** The hypothetical interactions between the miRNAs and their target genes together with related signaling pathways during preimplantation embryo development. Metacore pathway analysis of the validated miRNA and their target genes identified seven different functional pathways that were targets for miRNA control. The red boxes indicate the selected target genes. The green boxes indicate important genes that are already known to be related to embryo development. The green lines indicate positive effects between genes. The red line indicates negative effects between genes.
that some of the identified miRNAs are not regulated by changes in methylation status but are regulated by histone deacetylation or an off-target effect of 5-aza-dC. Second, the epigenetic regulatory mechanisms of miRNA and their targets together with their roles in embryo development are relatively unexplored and still need further investigation. Another limitation of this study is associated with the validation of the target proteins. The gene expression data set, we used does not confirm absolutely that these targets are only regulated by miRNAs and it is possible that some other factor(s) might alter protein expression rather than the miRNA.

In summary, by inhibiting de novo DNA methylation by 5-aza-dC and investigating the effect on the transcription of miRNAs, we identified some interesting candidate miRNAs that may be involved in early mouse embryonic development. Our novel findings showed the possible correlation and regulation between DNA methylation and miRNAs; which might interact with potential gene targets during morula to blastocyst transition. This preliminary study and our ongoing follow-up approach exploring how the interaction and network works in preimplantation development may be important and shed more light on the role of miRNAs in early embryo development and tissue differentiation. Ultimately this may help us to deal with some of the diseases associated with imprinted gene regulation.

Supplementary data
Supplementary data are available at http://molehr.oxfordjournals.org/.

Authors’ roles
L.-M.L. and C.-M.S. performed experimental studies. All authors contributed intellectual input toward the design, implementation and interpretation of results. L.-M.L. and H.-W.C. drafted the manuscript and all authors read and approved the final manuscript.

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Conflict of interest
None declared.

References


