Maternal microRNAs are essential for mouse zygotic development

Fuchou Tang,1,4 Masahiro Kaneda,1,4 Dónal O’Carroll,2 Petra Hajkova,1 Sheila C. Barton,1 Y. Andrew Sun,3 Caroline Lee,1 Alexander Tarakhovsky,2 Kaiqin Lao,3,6 and M. Azim Surani1,5

1Wellcome Trust/Cancer Research UK Gurdon Institute of Cancer and Developmental Biology, University of Cambridge, Cambridge CB2 1QH, United Kingdom; 2The Laboratory for Lymphocyte Signaling and the Laboratory of Molecular Immunology, The Rockefeller University, New York, New York 10021, USA; 3Advanced Research Technology, Applied Biosystems, Foster City, California 94404, USA

MicroRNAs (miRNAs) have important roles in diverse cellular processes, but little is known about their identity and functions during early mammalian development. Here, we show the effects of the loss of maternal inheritance of miRNAs following specific deletion of Dicer from growing oocytes. The mutant mature oocytes were almost entirely depleted of all miRNAs, and they failed to progress through the first cell division, probably because of disorganized spindle formation. By comparing single-cell cDNA microarray profiles of control and mutant oocytes, our data are compatible with the notion that a large proportion of the maternal genes are directly or indirectly under the control of miRNAs, which demonstrates that the maternal miRNAs are essential for the earliest stages of mouse embryonic development.

Results and Discussion

First, we decided to investigate if there is significant biogenesis of miRNAs in developing oocytes, and their inheritance in the zygote. We therefore examined expression of miRNAs in single cells during oogenesis by a real-time PCR-based miRNA expression profiling method that we recently developed (C. Chen et al. 2005; Tang et al. 2006a,b). We compared the miRNA expression profiles of growing oocytes obtained from females 15–16 d after birth (postnatal days 15–16 [P15–P16]) and at P20–P21, and of mature oocytes from adult females. This analysis revealed dynamic changes in miRNA expression during oogenesis [Fig. 1; Supplementary Table S1].

Next, we compared expression of miRNA in the mature oocyte with miRNAs in the zygote, which showed essentially the same miRNA expression pattern in both these cells [Fig. 1, Supplementary Tables S1, S2]. This observation indicates that the miRNAs we detected in the zygote are probably maternally inherited from oocytes and not transcribed in the zygote itself.

We then went on to determine if there are significant changes in miRNAs during early embryonic development. Indeed, we found that the miRNA expression profile of the zygote underwent dynamic changes during early embryonic development when examined through to the eight-cell stage [Fig. 1, Supplementary Table S2]. Notably, we found that the total amount of miRNA is down-regulated by 60% between one- and two-cell-stage embryos \(P < 0.001\) [Fig. 2A, Supplementary Table S3], suggesting that a very significant proportion of the maternally inherited miRNAs present in the zygote is probably actively degraded during the first cell division. Some of these miRNAs were selectively lost by >95% [Supplementary Table S2]. This is unexpected because there are no reports to show that miRNAs can be actively degraded in vivo under physiological conditions, although maternal miRNAs are globally degraded at this time [Hamatani et al. 2004]. We confirmed our findings of the loss of maternal miRNAs by RNA in situ hybridization with LNA probes, which has been shown to be able to detect mature miRNA expression [Supplementary Fig. S2C,D; Supplementary Table S4; Kloosterman et al. 2006]. Among the maternal miRNAs in the zygote, the most abundant are let-7 family miRNAs. They show dynamic regulation during oogenesis and early embryonic lethal at embryonic day 7.5 (E7.5) [Bernstein et al. 2003].

In this study, we have examined the role of miRNAs in the mouse oocyte. The mature oocyte contains a number of molecules that are manufactured during oocyte maturation and utilized during early stages of development before activation of the embryonic genome (Dean 2002). It is likely that miRNAs would also be present in the oocyte, but no information is yet available in the mouse. The purpose of this study was to determine if there is significant inheritance of maternal miRNAs in mammalian zygotes, and to investigate if they play a critical role in early mammalian development. We have investigated how the loss of Dicer affects synthesis of miRNA during oocyte maturation and their impact on mRNA and early development.

Keywords: Maternal microRNAs, Dicer, oocyte, zygote

*These authors contributed equally to this work.

Corresponding authors.

E-MAIL: as10021@mole.bio.cam.ac.uk; FAX 44-1223-334089.

E-MAIL: laokq@appliedbiosystems.com; FAX (650) 638-6343.

Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.418707.
Maternal miRNAs essential for zygotic development

The most abundant cluster is the miR-17-92 cluster. This miRNA cluster has been shown to be “oncomirs,” which is involved in cell proliferation (He et al. 2005; O’Donnell et al. 2005). Their abundance significantly increased during oogenesis and was inherited by the zygote and increased again after the two-cell embryo stage (Supplementary Fig. S3).

Further investigations showed that the total miRNA in a four-cell-stage embryo was ~2.2 times higher than the levels detected in a two-cell-stage embryo (Fig. 2A; Supplementary Table S3). This suggests that there is de novo expression of miRNAs between the two- and four-cell stages of development. Amongst the most significant miRNAs that are up-regulated in a four-cell-stage embryo are those from the miR-290 cluster, namely miR-290 to miR-295 (Houbaviy et al. 2003). Compared with the two-cell-stage embryos, these miRNAs were up-regulated by 15-fold and by 24-fold in four-cell- and eight-cell-stage embryos, respectively (Supplementary Fig. S4A,B; Supplementary Table S5). Thus, miRNAs from the miR-290 cluster are amongst the earliest to be expressed during early mouse embryonic development.

To determine if there are significant differences in miRNAs in individual blastomeres, we separated and compared the miRNA expression profiles of the two individual blastomeres from individual two-cell-stage embryos. We found that both blastomeres examined separately have essentially the same miRNA expression profile (Supplementary Fig. S5A; Supplementary Table S10). Furthermore, the total amount of miRNAs in the two blastomeres together was only ~40% of that detected in the zygote. This shows that the miRNA profiling method is reliable and confirms that a significant proportion of the miRNAs is degraded in embryos between the one- and two-cell stages. Similarly, the miRNA expression profiles of the four individual blastomeres from a four-cell-stage embryo are essentially the same (Supplementary Fig. S5B; Supplementary Table S10). These observations indicate that the individual blastomeres at two-cell and four-cell embryo stages have similar if not identical miRNA expression profiles.

Next, we asked if these maternally inherited miRNAs are functionally important for early development. To investigate this aspect, we used mice carrying the Dicer floxed conditional allele, where exon 23 of Dicer locus is flanked by two loxP loci (Yi et al. 2006). These mice with the floxed allele of Dicer were mated with Zp3-Cre transgenic mice, which express Cre recombinase under the control of Zona pellucida glycoprotein 3 promoter (Zp3) in the growing oocyte. We thus generated [Dicer^−/Flox, Zp3-Cre] animals. It is known that Zp3 expression is detected only in growing oocytes from about P5, which allowed us to delete Dicer specifically from maturing oocytes (de Vries et al. 2000). We would therefore expect loss of Dicer function in growing oocytes, and this would in turn block biogenesis of miRNAs at this stage. To establish how the loss of Dicer from the growing oocytes affects miRNAs in oocytes, we examined the miRNA profile in the mutant oocytes. We found that most, if not all, miRNAs were essentially lost from the oocytes lacking Dicer (Supplementary Fig. S6; Supplementary Table S1). We also found that the loss of the Dicer allele from oocytes rendered the females infertile.
To determine the reason for infertility and when the effect manifests itself, we recovered 11.5 embryos from control and mutant oocytes following the deletion of Dicer from the growing oocytes that were fertilized by sperm from wild-type males (Fig. 2B). We found that the control females had healthy two-cell-stage embryos as expected, but all of the mutant oocytes failed to proceed through the first cell division, and about half of them were fragmented (three litters, n = 28). Thus, maternal Dicer and miRNAs in the oocyte are crucial for the earliest stages of embryonic development.

To determine more precisely the stage at which the loss of Dicer from oocytes starts to affect oogenesis and embryonic development, we examined the expression of some of the key genes by single-cell cDNA analysis at three different stages during oogenesis (Rajkovic et al. 2004). We found that expression of a number of genes, including Oct4, Fragilis, Stella, C-mos, Bnc1, H2AX, H1foo, SCP3, Nobox, Gata4, and RFPL4, was unaffected in growing oocytes at P15–P16 (Supplementary Fig. S1). The overall morphology of the mutant oocytes was also indistinguishable from that of control oocytes at this stage. Next, we checked expression of the same genes in mature ovulated oocytes, which we obtained from [Dicer−/Fox, Zp3-Cre] females that were mated with vasectomized males. We again found that the Dicer mutant oocytes were morphologically indistinguishable from control oocytes in their appearance, maturity, and size. The overall numbers of ovulated mutant oocytes were also indistinguishable from those obtained from control females. However, we found that the Dicer mutant oocytes showed higher expression of C-mos and H2AX compared with the levels we found in control oocytes (Supplementary Fig. S1). In the E0.5 embryos, genes including H1foo and SCP3 also showed higher expression compared with the control. Therefore, mature oocytes lacking in Dicer, and consequently miRNAs, already showed an effect on mRNAs.

To determine if the miRNAs are involved in maturation of the oocyte, we also checked the chromosome and spindle organization (Lefebvre et al. 2002; Terret et al. 2004). We found that expression of a number of genes, including Oct4, Fragilis, Stella, C-mos, Bnc1, H2AX, H1foo, SCP3, Nobox, Gata4, and RFPL4, was unaffected in growing oocytes at P15–P16 (Supplementary Fig. S1). In the E0.5 embryos, genes including H1foo and SCP3 also showed higher expression compared with the control. Therefore, mature oocytes lacking in Dicer, and consequently miRNAs, already showed an effect on mRNAs.

To determine if the miRNAs are involved in maturation of the oocyte, we also checked the chromosome and spindle organization (Lefebvre et al. 2002; Terret et al. 2003). We found that compared with control oocytes, the Dicer mutant oocytes showed reduced and disorganized spindles, and the chromosomes were also not aligned altogether properly (Fig. 3). This indicates that loss of Dicer and miRNAs affects the spindle organization of mature oocytes.

To obtain more comprehensive information on how miRNAs affect embryonic development at the whole-genome scale, we prepared single-cell cDNAs from control and Dicer mutant oocytes for microarray analysis (Kurimoto et al. 2006). We found that compared with control oocytes, Dicer knockout oocytes showed an increase in the levels of many genes that are probably important during early development. The cDNA levels of more than one-third of the genes expressed in oocytes increased (>1.5-fold) after the loss of Dicer and miRNAs (Fig. 4; Supplementary Table S7). We confirmed the microarray data by quantitative PCR on single-cell cDNAs (Supplementary Table S8). This analysis shows that miRNAs that are expressed during oogenesis profoundly shape the gene expression profile of the mature oocyte. Since we did not find significant enrichment of predicted target genes of miRNAs amongst the expression-increased genes in the Dicer knockout oocytes, our findings are in agreement with a similar study in zebrafish, where the microarray data of Dicer mutant and wild-type embryos were also compared (Giraldez et al. 2006).

To further understand the molecular basis for the role of maternal miRNAs on early development, we compared our microarray data with the predicted target genes of maternal miRNAs using a bioinformatics approach. We searched the sequence complement to the “seed” region of miRNAs in the 3′ untranslated region (UTR) of all the 30,677 genes with defined 3′UTRs represented on the ABI chip. We found that the genes that have no target sites in their 3′UTRs for the 101 maternal miRNAs have
4.2-fold more chance to be coexpressed in oocytes with these miRNAs \(P = 2.2E-16\) [Supplementary Tables S9, S11]. This indicates that miRNAs expressed in the oocyte functionally shape the gene expression profile, which is in accordance with the reports that genes expressed in a particular tissue tend to avoid being the targets of coexpressed miRNAs in the same tissue [Farh et al. 2005; Stark et al. 2005; Sood et al. 2006]. By combining the analysis of the single-cell miRNA profile with single-cell cDNA microarrays, we confirmed at the resolution of single cells that some of the genes expressed in the oocyte had a strong tendency to avoid being the target sites of coexpressed miRNAs.

As it is known that miRNAs also apparently have an effect on the expression of repetitive elements [Fukagawa et al. 2004; Kanellopoulou et al. 2005], we examined the expression levels of IAP [intracisternal A particle element, an endogenous retrovirus], LINE1 [long interspersed nuclear element 1, a nonretrovirus retrotransposon], and MTs (mouse transcript; a nonautonomous retrotransposon), which are the most abundant repetitive elements in the mouse oocyte. However, quantitative real-time RT–PCR analysis showed no significant differences in their expression between Dicer mutant and control oocytes [Fig. 5A]. We also investigated the DNA methylation status of IAP and LINE1 by the bisulphite genomic sequencing method and found no significant differences between Dicer mutant and control oocytes [Fig. 5B]. Thus the phenotype of the Dicer mutant oocyte is due to the direct or indirect effects of the loss of miRNAs, not the derepression of repetitive elements.

In summary, we have generated a comprehensive miRNA expression profile for growing oocytes and embryos up to the eight-cell stage. Interestingly, unlike the observations in zebrafish and Xenopus [P. Y. Chen et al. 2005; Watanabe et al. 2005], we found abundant maternally inherited miRNAs in mouse zygotes, among which let-7 family miRNAs are the most abundant. It seems that sperm-borne miRNAs do not contribute significantly to miRNAs in the zygote [Amanai et al. 2006]. Furthermore, in zebrafish it was shown that maternal miRNAs are dispensable for early embryonic development, although the maternal Dicer is important because it is necessary for zygotic synthesis and expression of mir-430 [Giraldez et al. 2005, 2006]. In the mouse, while there is a significant global loss of maternal miRNAs between the one- and two-cell stages of development, de novo synthesis of miRNAs commences at the two-cell stage. This includes expression of mir-290 to mir-295, which are the first embryonic miRNAs to be detected. It is noteworthy that mir-290 to mir-295 are also specifically expressed in embryonic stem cells, which may suggest their association with pluripotency [Houbaviy et al. 2003]. We cannot formally rule out the possibility that the Dicer mutant phenotype is not solely due to loss of miRNAs because Dicer might directly play some unknown roles in chromatin formation. Amongst the genes that were detected at higher levels in the oocytes following the loss of Dicer, some of these effects may be due to secondary consequences of miRNA depletion. Nevertheless, this study provides evidence that the maternal inheritance of miRNAs is crucial for early mammalian development. The detailed analysis of the oocyte miRNAs and their impact on mRNAs we present here may help to elucidate their precise roles in early mouse development in the future.

**Materials and methods**

**Embryos and knockout mice**

Embryos before implantation were recovered from F1 [C57BL/6 × CBA] females mated with F1 male mice [Nagy et al. 2003]. Oocytes were isolated from F1 female mice. All the “mature oocytes” we mentioned in the text are ovulated mature oocytes.

The knockout mice carrying the Dicer floxed allele was described previously [Yi et al. 2006]. Basically, exon 23 of the Dicer locus was floxed by two loxP loci (referred to as Dicer\(^{\text{Flox}}\)). The Dicer\(^{\text{Flox/Flo}}\) mice were mated with Zp3-Cre transgenic mice, which express Cre recombinase under the control of the Zona pellucida glycoprotein 3 promoter [de Vries et al. 2000]. Then the [Dicer\(^{\text{Flox/Flo}}\), Zp3-Cre] female mice were mated with Dicer\(^{\text{Flox/Flo}}\) male mice. From this mating, we obtained [Dicer\(^{\text{Flox/Flo}}, \, \text{Zp3-Cre}\] mice, and following the deletion of the Floxed allele in the oocyte, we generate oocytes that are the null mutants for Dicer. The control mice we used are littermates with the genotype of [Dicer\(^{\text{Flox/Flo}}, \, \text{Zp3-Cre}\], Dicer\(^{\text{Flox/Flo}}\), or Dicer\(^{\text{Flox/Flo}}\).

The details of the microRNA expression profiling assay, RNA in situ hybridization by LNA probe, immunostaining, single-cell cDNA, real-time PCR, microarray gene expression procedures and analysis, and bisulphite genomic sequencing can be found in the Supplemental Material. The data of the single-oocyte cDNA microarray were deposited in GeneBank [http://www.ncbi.nlm.nih.gov/geo]. The accession number is GSE68806.
Acknowledgments

We thank Eric Miska, Anne McLaren, Kenneth Livak, Naoki Miyoshi, Katsumi Hayashi, Maria Elena Torres Padilla, Jie Na, David Adams, and James Smith for their helpful discussions and generous suggestions. We also thank W.N de Vries and B.B. Knowles for the Zp3-Cre transgenic mice. This work was supported by grants from the Wellcome Trust and BBSRC to M.A.S. M.K. is supported by the Japanese Society for the Promotion of the Science (JSPS).

References

Maternal microRNAs are essential for mouse zygotic development
Fuchou Tang, Masahiro Kaneda, Dónal O'Carroll, et al.

Genes Dev. 2007 21: 644-648
Access the most recent version at doi:10.1101/gad.418707

Supplemental Material
http://genesdev.cshlp.org/content/suppl/2007/03/09/21.6.644.DC1

References
This article cites 29 articles, 10 of which can be accessed free at:
http://genesdev.cshlp.org/content/21/6/644.full.html#ref-list-1

Open Access
Freely available online through the Genes & Development Open Access option.

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.

To subscribe to Genes & Development go to:
http://genesdev.cshlp.org/subscriptions

Copyright © 2007, Cold Spring Harbor Laboratory Press