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Dicer1 expression in preimplantation mouse embryos: Involvement of Oct3/4 transcription at the blastocyst stage

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Abstract

Dicer1, an RNAse III enzyme, is a key factor for the production of microRNAs involved in post-transcriptional gene silencing. To elucidate the roles of *Dicer1* and the microRNA pathway in early embryo development, we initially evaluated its gene expression in mouse oocytes and embryos *in vitro*. The transcript levels in GV stage oocytes steadily decreased up to the 2-cell embryo stage, and expression remained stable during morulae and blastocyst formation. DICER1 protein synthesis was additionally observed in mouse oocytes and early embryos. Silencing of mRNA expression by RNA interference (siRNA) did not inhibit development up to the blastocyst stage. Real-time RT-PCR experiments confirmed the decreased expression of selected transcription factors, including POU domain, class 5, transcription factor 1 (*Pou5f1*), SRY-box containing gene 2 (*Sox2*), and Nanog homeobox (*Nanog*). Moreover, POU5F1 protein expression was suppressed by *Dicer1* siRNA. The results suggest that *Dicer1* gene expression is associated with the levels of transcription factors, *Pou5f1*, *Sox2*, and *Nanog* which possibly regulate differentiation processes at the blastocyst stage.

Keywords: *Dicer1*; Mouse embryo; MicroRNA; Blastocyst; Transcription

MicroRNAs (miRNAs) generated from endogenous transcripts are single-stranded RNA sequences 19–25 nucleotides (nt) in length that form hairpin structures [1,2]. These miRNAs act as guide molecules by base-pairing with target mRNAs, and lead to transcriptional repression and/or mRNA cleavage. Mature animal miRNAs are about 22 nt in length, and are produced as a result of sequential processing by a series of RNase III-related enzymes, *Drosha* and *Dicer*. *Drosha* processes a pri-miRNA hairpin transcript of about 70 nt (pre-miRNA) from a longer precursor RNA [1]. Pre-miRNA is cleaved by a second Rnase III enzyme, *Dicer*, to yield the 22 nt mature miRNA [3–5]. Theoretically, targeted deletion of *Dicer* should yield mice deficient in all mature miRNAs. *Dicer* is also required for the processing of long double-stranded RNA or miRNA precursors into mature effective RNA molecules [6].

DICER proteins are involved in a variety of gene-silencing phenomena at the transcriptional, post-transcriptional, or translational level, depending on the organism. In *Caenorhabditis elegans*, *Dicer* is required for RNA interference and development [7–10]. Knock-down of *Dicer* with RNAi in a human cell line led to defects in both miRNA production and short hairpin RNA (shRNA)-mediated RNAi [11,12]. In addition, inactivation of *Dicer1* in the mouse germline results in cell death at day 7.5 and loss of multipotent stem cells [13].

miRNA machinery is implicated in maintaining the stem cell character, as well as the control of differentiation processes [14,15]. Conditional gene targeting of *Dicer1* in a mouse embryonic stem (ES) cell resulted in defective generation of miRNAs, in RNA interference, and in differentiation [14]. Homozygous *Dicer1* null ES cells could not be generated by sequentially targeting both alleles or by isolating cell lines from blastocysts of heterozygous intercrosses [14]. Furthermore, *Dicer* knockout mutant embryos

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produce *Pou5f1* mRNA, but have nonetheless significantly lower levels of POU5F1 protein [15]. ES cells are transient pluripotent cells that can be cultured *in vitro* without losing their ability to differentiate into various mouse tissues. Since ES cells are produced from the inner cell mass of the blastocyst, it is possible that *Dicer1* functions in blastocyst stage embryos. Previously, Svoboda et al. [16] demonstrated that targeted destruction of *Dicer* mRNA by injecting 1-cell embryos with double strand RNA resulted in a 50% increase in murine endogenous retrovirus-L and intracisternal A particles. This suggests that the microRNA system constrains expression of repetitive parasitic sequences in preimpantation embryos [16].

Pou5f1, also known as Oct3/4 [17], belongs to the class V family of POU proteins and mediates pluritropic control as a transcription factor [18,19]. *Pou5f1* mRNA and protein have been identified in the blastomeres of preimplantation embryos, in the inner cell mass (ICM) of blastocysts, in epiblasts and primordial germ cells, and in most germ cells [3,17,20]. Loss of *Pou5f1* in blastocysts leads to terminal differentiation of the inner cell mass into the trophoblast lineage [21], and a precise dose of *Pou5f1* is required for cell fate decisions [22]. Recent studies show that *Sox2* and *Nanog* interact with *Pou5f1* to regulate the transcriptional hierarchy that specifies ES cell identity [21,23,24].

Despite clear evidence of developmental regulation, limited information is currently available on the expression and functions of *Dicer1* genes during the early embryonic development in mammals. To elucidate the role of *Dicer1* in preimplantation embryos, we initially determined its mRNA and protein levels in mouse early embryos using real-time reverse transcription-polymerase chain reaction (RT-PCR) and immunocytochemistry. We then examined the possible role of these genes in oocyte maturation and preimplantation development using RNA interference analysis.

Materials and methods

Generation of mouse embryos. To obtain oocytes or fertilized embryos, 5-week-old B6D2 F1 female mice were superovulated by intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG, Sigma, St. Louis, MO), followed by 5 IU gonadotropin (hCG, Sigma) 48 h later. Experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. Germinal vesicle (GV) stage oocytes were collected 45 h after PMSG injection from the ovary by slicing. The medium for GV oocyte collection was M2 (Sigma) supplemented with 300 μM dibutyryl cyclic adenosine monophosphate (dbcAMP, Sigma) to inhibit germinal vesicle breakdown (GVBD) during collection. Unfertilized metaphase II eggs (MII) or one-cell (1C) embryos were collected from ampullae of superovulated females without mating or via mating within a day (20 h) after the hCG injection. Cumulus cells were removed with 0.1 mg/ml hyarulonidase (Sigma) by pipetting in M2 (Sigma) medium. Two-cell (2C), four-cell (4C), morula (MO), and blastocyst (BL) stage embryos were flushed out from oviducts or collected from the uterus at 40, 55, 82, or 96 h after hCG injection. Harvested embryos were washed in Ca^{2+}- and Mg^{2+}-free PBS, and either fixed with 4% formaldehyde (Sigma) for 20 min and stored at 4 °C or snap-frozen in liquid nitrogen and stored at –70 °C until use.

siRNA microinjection and in vitro culture. Zygotes were collected and denuded of cumulus cells. We purchase pre-designed siRNAs to silence mouse *Dicer1* (siRNA ID No., 173425, Ambion, Inc., Houston, TX, USA) or *Pou5f1* (siRNA ID No., 151960, Ambion), positive control (glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) siRNA, siRNA ID No. 407972, Ambion), and negative control (Cat. No. 4611G, Ambion). The siRNA was diluted with buffer (Ambion) to a final concentration of 100 μM, and stored at −20 °C. Approximately 10 pL of siRNA was

Table 1
List of primers used for real-time RT-PCR

Genes	GenBank Accession Nos.	Primer sequence	Annealing temperature (°C)	Base pairs
Dicer1	NM_148948	F: ggtggtctggcaggtgtact R: cctgaggctggttagctttg	60	272
Cdc42	NM_009861	F: ttgttggtgatggtgctgtt R: aatcctcttgccctgcagta	60	168
Cdh1	X06115	F: ttgaggagttgaatgctgac R: agctcgaactttccaagcag	55	485
Rhobtb2	AF420001	F: acccagatgatggtggacat R: ccaccggtgtttctcaaagt	60	195
Ilk	NM_010562	F: tgttgtgaagaaggtgctgaagg R: cagtgtgtgatgagggttgg	60	162
Tuba1	NM_011653	F: tcgtgatccacttccctctgg R: actggatggtacgcttggtc	60	239
Plat	NM_008872	F: gctgagtgcatcaactggaa R: gccacggtaagtcacacctt	60	243
Tie1	BC060182	F: caggcacagcaggttgtaga R: gtgccaccattttgacactg	60	160
Pou5f1	NM_013633	F: cgtggagactttgcagcctga R: ggcgatgtaagtgatctgctg	55	519
Nanog	AY278951	F: aagtacctcagcctccagca R: gtgctgagcccttctgaatc	60	163
Sox2	NM_011443	F: cacaactcggagatcagcaa R: ctccgggaagcgtgtactta	60	190
H2a	X16495	F: acaacaagaagacccgcatc R: cttggccttgtggtgactct	60	167
Globin (Rabbit)	X04751	F: gcagccacggtgtcgagtat R: gtgggacaggagcttgaaat	55	257

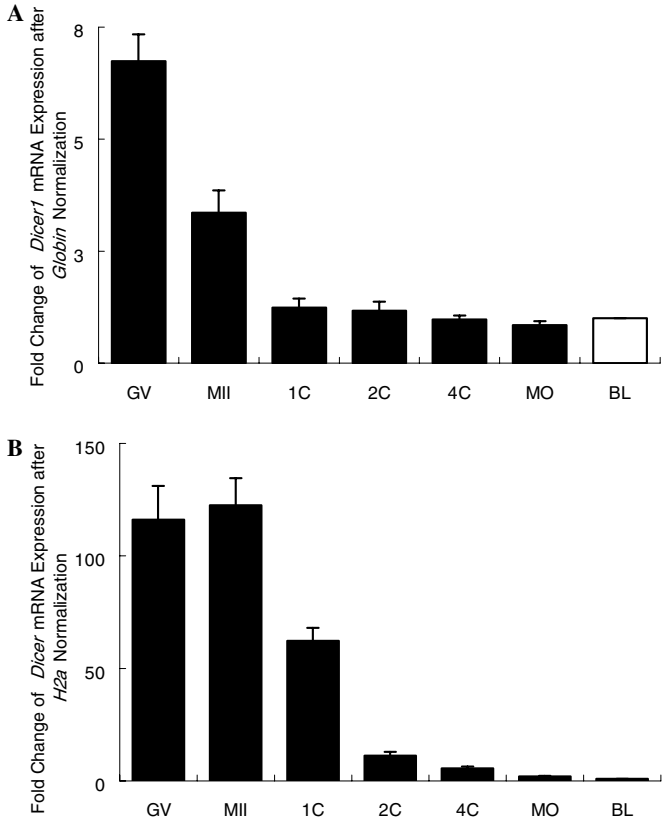

Fig. 1. Relative mRNA expression levels of *Dicer1* at various developmental stages of oocytes and embryos analyzed by real-time RT-PCR. Messenger RNA expression at the BL stage (□) was arbitrarily set as onefold. (A) Fold differences in mRNA expression from equivalent numbers of germinal vesicle (GV), metaphase II (MII), zygote (1C), 2-cell (2C), 4-cell (4C), morula (MO), and blastocyst (BL) stage oocytes or embryos after normalization to the external reference (rabbit *Globin* mRNA). (B) Fold differences in mRNA expression from equivalent numbers of GV, MII, 1C, 2C, 4C, MO, and BL stage oocytes or embryos after normalization to the internal reference (mouse *H2a*). Data are presented as means ± SEM of four separate experiments.

injected into the cytoplasm of GV stage oocytes or zygotes using an Eppendorf microinjector system (Eppendorf, Hamburg, Germany). Buffer or siRNA-microinjected oocytes or embryos were cultured in M16 (Sigma) medium supplemented with 0.4% BSA at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air to determine the rates of MII state oocyte or development to the blastocyst stage.

Immunofluorescence staining. Mouse embryos were fixed with 4% formaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 10 min. To determine the distribution of DICER1 or POU5F1, fixed embryos were incubated with mouse DICER1 (Abcam plc. Cat. # ab14601) monoclonal antibodies or anti-POU5F1 (Santa Cruz) for 1 h, followed by FITC-labeled secondary antibodies (Sigma). PI was used to stain nuclei. Slides were examined by laser scanning confocal microscopy using Leica DM IRB equipped with a krypton–argon ion laser for the simultaneous excitation of fluorescence for proteins and PI for DNA.

Real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR). Messenger RNA was extracted with the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway), according to the manufacturer's instructions. As the external reference, rabbit *Globin* mRNA (Sigma) was added at 0.1 pg per oocyte or embryo before extraction [25]. Initially, standard cDNA synthesis was achieved by reverse transcription of RNA using the oligo$(dT)_{12–18}$ primer and superscript reverse transcriptase (Invitrogen Co., Grand Island, NY). Real-time RT-PCR was performed using the 16 primer sets shown in Table 1 by DNA Engine OPTICOJ 2 (MJ research, USA) [26]. The relative quantification of gene expression was analyzed using the 2-ddCt method [27]. In all experiments, histone H2a (*H2a*) mRNA was employed as an internal standard and rabbit *Globin* mRNA as an external reference for the analysis of relative transcript levels of *Dicer1* in various developmental stages of oocytes and embryos.

Statistical analysis. The general linear model (GLM) procedure in the SAS program [28] was applied to analyze data from all experiments. Significant differences were determined using Tukey's multiple range test [29] and $P < 0.05$ was considered statistically significant.

Results

Expression of Dicer1 in mouse oocytes and embryos

The relative abundance of *Dicer1* transcripts was established by RT-PCR using the 2-ddCt method. Ten oocytes/embryos per treatment group were analyzed four

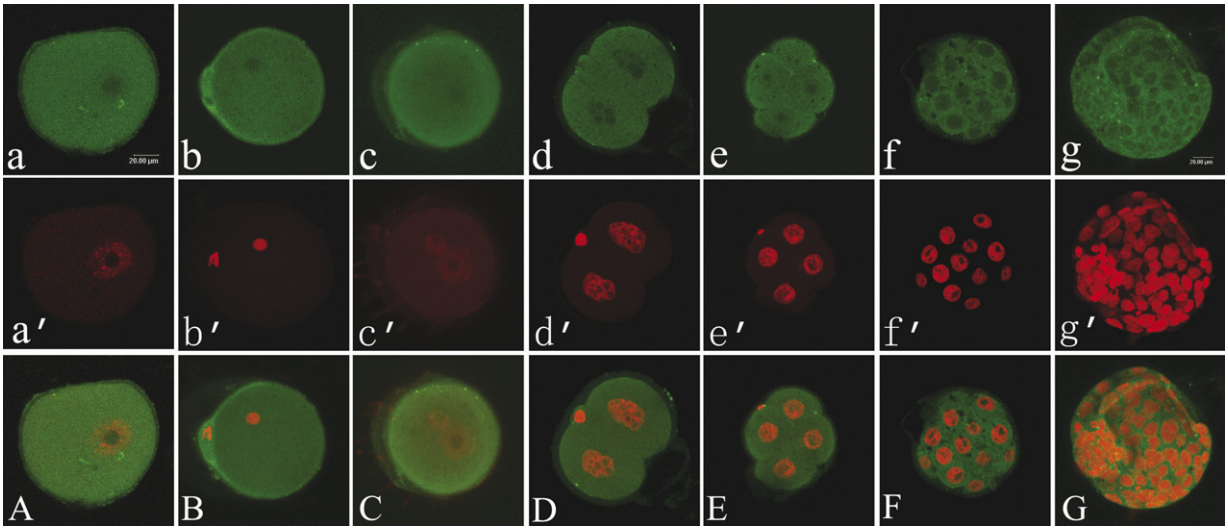

Fig. 2. (A) Laser scanning confocal microscopy images of DICER1 expression in mouse germinal vesicle (GV), metaphase II (MII), zygote (1C), 2-cell (2C), 4-cell (4C), morula (MO), and blastocyst (BL) stage oocytes or embryos. a–g: DICER1 protein (green); a′–g′: chromatin (red); A–G: DICER1 (green) and chromatin (red) merged images; a, a′ and A: b, b′ and B: c, c′ and C: d, d and D: e, e′ and E: f, f′ and F: GV, MII, 1C, 2C, 4C, or MO stage oocyte or embryo, respectively, 63×, Zoom 2; g, g′ and G: BL stage embryo, 40×, Zoom 1.5.

times with three replicates (Fig. 1). Samples were normalized using rabbit *Globin* mRNA as an external reference (Fig. 1A). To normalize the RT-PCR reaction efficiency and quantify *Dicer1* mRNA, *H2a* was applied as an internal standard (Fig. 1B). Following normalization to the *Globin* mRNA (Fig. 1A), the *Dicer1* transcript level was elevated in GV stage oocytes, decreased at the MII stage, and was further reduced after fertilization. When normalized to the internal reference (*H2a*), *Dicer1* mRNA expression was higher in MII stage oocytes (Fig. 1B). DICER1 proteins were detected in all oocytes and embryos (Fig. 2) by immunofluorescence staining using the primary antibody (Abcam plc. Cat. # ab14601) and FITC-labeled anti-mouse IgG (Sigma) secondary antibody.

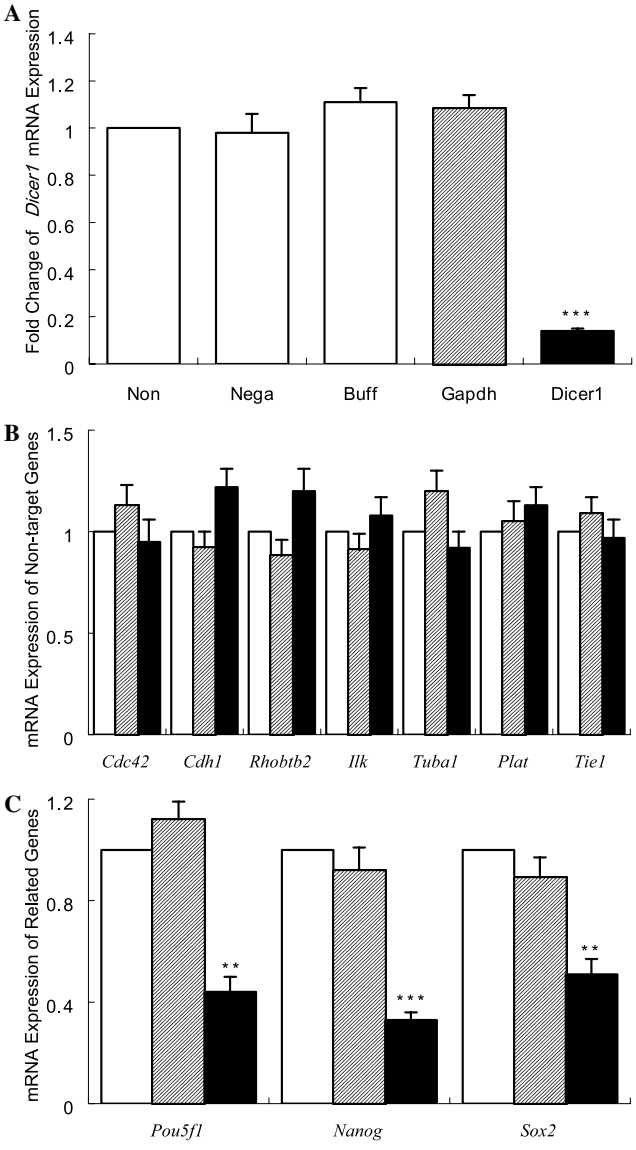

Fig. 3. Relative mRNA levels in blastocyst stage embryos were examined by real-time RT-PCR. (A) Relative mRNA levels of *Dicer1*. Zygotes injected with negative control (Nega), buffer (Buff), positive control (Gapdh, *Gapdh* siRNA), and *Dicer1* siRNA (Dicer1) or non-injected (Non) and *in vitro* cultured to the blastocyst stage. (B) Relative mRNA expression of non-target genes from buffer (control, □), *Gapdh* siRNA (▨), or *Dicer1* siRNA (■) injected group. (C) Relative mRNA expression of transcription related genes from buffer (control, □), *Gapdh* siRNA (▨), or *Dicer1* siRNA (■) injected group. Three replicates of 10 embryos each were assayed from each group and *H2a* was used as an internal standard. Statistically significant differences are indicated: $^{**}P < 0.01$, $^{***}P < 0.005$. Values are means ± SEM of four separate experiments.

Effects of each siRNA on target or non-target mRNA expression

As a control experiment, we injected zygotes with *Dicer1* siRNA, *Gapdh* siRNA (ID No. 407972, Ambion) as a positive control, a negative control (Cat. No. 4611G, Ambion), buffer only or left them untreated. The mRNA levels were then measured at the blastocyst stage by real-time RT-PCR. *Dicer1* siRNA-injected blastocysts displayed significant decrease in target mRNA, which were significantly lower than those in other groups ($P < 0.001$, Fig. 3A). The developmental ability of zygotes following *Gapdh* siRNA injection was comparable to those administered buffer only. Additionally, mRNA expression following the injection of siRNA dilution buffer (Buff) alone was similar to that of the negative control (Nega) and non-injected (Non) groups, which showed no reduction in *Gapdh* or *Dicer1* transcript levels. Similarly, the injection of *Dicer1* siRNA into GV stage oocyte significantly ($P < 0.05$) reduced (about 80%, data not shown) *Dicer1* mRNA expression and DICER1 protein levels compared to the negative controls, non-injected or buffer injected GV oocytes and the MII stage oocytes injected with *Gapdh* siRNA. In addition, immunofluorescent staining showed that siRNA injection reduced DICER1 protein levels in the blastocyst stage (Fig. 4a and b) compared to the control (Fig. 4A and B).

Several non-target genes, including *Dicer1*-related *Tie1* [30] and selected transcription factors, *Pou5f1*, *Nanog*, and *Sox2* were analyzed by real-time RT-PCR (Fig. 3B and C). Mouse *H2a* was used as an internal standard. Following *Dicer1* siRNA microinjection into zygotes, cell division cycle 42 homolog (*Cdc42*), E-cadherin (*Cdh1*), Rho-related BTB domain containing 2 (*Rhobtb2*), integrin linked kinase (*ILK*), tubulin, α1 (*Tuba1*), plasminogen activator, tissue (*Plat*), and tyrosine kinase receptor 1 (*Tie1*) transcript levels were not altered in BL stage embryos (Fig. 3B). However, *Pou5f1* ($P < 0.01$), *Nanog* ($P < 0.005$), and *Sox2* ($P < 0.01$) in BL were significantly down-regulated (Fig. 3C). POU5F1 protein synthesis was additionally suppressed (Fig. 4c and d) compared to the control (Fig. 4C and D). However, the *Dicer1* mRNA level was not affected, following *Pou5f1* siRNA injection (data not shown).

Effects of Dicer1 siRNA on mouse oocyte maturation and embryo development

Germinal vesicle stage oocytes microinjected with *Dicer1* siRNA displayed no differences from the buffer or *Gapdh* siRNA injected group with regard to first polar body extrusion (MII: *Dicer1*, 78.8 ± 6.9% vs. *Gapdh*, 73.2 ± 5.8% and buffer, 70.3 ± 6.5%). Similarly, development of

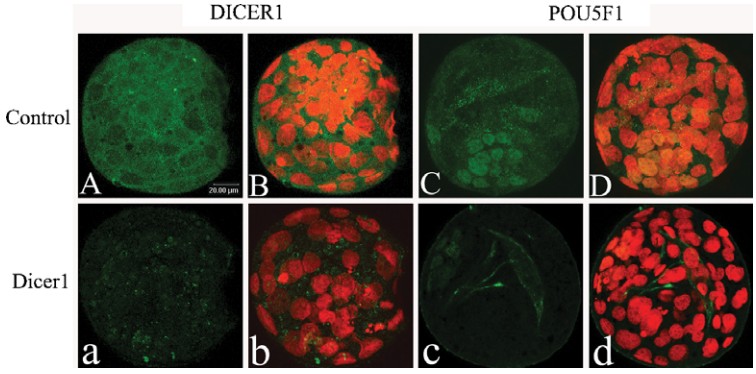

Fig. 4. (A) Laser scanning confocal microscopy images of DICER1 (A, B, and a, b) , POU5F1 (C, D, and c, d) protein expression in mouse blastocysts (40×, Zoom 1.5) after injection with either *Gapdh* (control, A–D) or *Dicer1* siRNA (a–d). Green, DICER1, or POU5F1 protein; red, chromatin.

siRNA-treated zygotes to the cleavage (2C, *Dicer1*, 96.8 ± 8.1% vs. *Gapdh*, 95.1 ± 8.3% and buffer, 98.1 ± 7.9%) and BL (*Dicer1*, 82.2 ± 9.3% vs. *Gapdh*, 83.2 ± 7.8% and buffer, 85.2 ± 8.9%) stages was not significantly different from the buffer or *Gapdh* siRNA injected zygotes.

Discussion

In the present study, we determined the expression patterns of *Dicer1* and its possible role in mouse preimplantation development. We initially demonstrated the presence of *Dicer1* mRNA in mouse preimplantation embryos using quantitative real-time RT-PCR and immunocytochemistry. Specifically, real-time RT-PCR results revealed elevated expression of *Dicer1* transcripts in GV oocytes and lower expression during oocyte maturation, which was further reduced up until the 2-cell stage embryo. The transcript level remained stable up to the morula and blastocyst stages. High expression of *Dicer1* in GV oocytes may be related to maternal mRNA expression. DICER1 protein synthesis was mainly observed in the cytoplasm of oocytes and during the early embryonic development. The reason for the slight differences between DICER1 transcript and protein levels is currently unknown but probably reflects supplementary controls at the post-transcription level. While adequate information is available on DICER protein levels in stem cells and day 7–17 mouse embryos [13], to our knowledge this is the first report about expression in preimplantation mouse embryos.

Dicer is essential for development of mice and zebrafish. In mice, at 7.5 days, *Dicer* mutant embryos appeared small and morphologically abnormal, although they were distinguishable because of differences between their embryonic and extraembryonic regions [15]. Target-selected inactivation of the *Dicer* gene in zebrafish was arrested at day 10 [31]. Moreover, *Dicer* mutant embryos had impaired angiogenesis, and had altered levels of angiogenesis regulators, such as *Tie1* [30]. In the present study, we show that specific silencing of *Dicer1* expression using double-stranded RNA does not influence oocyte maturation or early embryo development. Furthermore, the levels of early development related genes, such as *Cdh1* (cell compaction), *Cdc42* (polarity, blastocoel formation), *Rhobtb2*, and *Plat* (housekeeping gene) were not altered in *Dicer1* knockdown blastocysts. In agreement with earlier published results, [15,16], *Dicer1* gene expression is not required in developmental events up to the blastocyst stage, but may be involved in subsequent embryonic development following blastocoel formation.

Dicer1 is implicated in the RNAi machinery involved in maintaining the stem cell population during the early mouse development [15]. ES cells are transient pluripotent cells recovered from mammalian blastocysts that can be cultured *in vitro* without loss of their ability to contribute to all mouse tissues. Mouse ES cells contain *Dicer1* and express a substantial number of miRNAs, including some that are unique to ES cells [14]. Interestingly, *Dicer1*−/− embryos failed to express the stem cell marker, *Pou5f1*, and the primitive streak marker, brachyury (*T*) [15]. The transcription factors *Pou5f1*, *Sox2*, and *Nanog* have essential roles in early development, and are required for the propagation of undifferentiated ES cells in culture. In fact, *Pou5f1* has a substantial number of target genes, and collaborates with these genes to form regulatory and feed forward loops [32]. In the present study, we observe that specific gene silencing of *Dicer1* at the blastocyst stage reduces *Pou5f1*, *Sox2*, and *Nanog* expression. Moreover, *Pou5f1* silencing does not affect *Dicer1* gene regulation. This finding suggests that *Dicer1* expression at the blastocyst stage may be implicated in the differentiation via regulation of *Pou5f1*, *Sox2*, and/or *Nanog* gene levels.

In conclusion, our results demonstrate *Dicer1* expression in preimplantation mouse embryos. Expression of this gene is not essential for developmental events up to the blastocyst stage, but appears to be associated with the levels of transcription factors, *Pou5f1*, *Sox2*, and *Nanog* which possibly regulate gene transcription. Further analyses are required to determine the mechanisms involved in regulation of transcription factors and other functions at the blastocyst stage.

Acknowledgments

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References

[1] J. Han, Y.T. Lee, K.H. Yeon, Y.K. Kim, H. Jin, V.N. Kim, The Drosha-DGCR8 complex in primary microRNA processing, Gev. Dev. 18 (2004) 3016–3027.
[2] V. Ambros, B. Bartel, D.P. Bartel, C.B. Burge, J.C. Carrington, X. Chen, G. Dreyfuss, S.R. Eddy, S. Griffinths-Jones, M. Marshall, A uniform system for microRNA annotation, RNA 9 (2003) 277–279.
[3] E. Bernstein, A.A. Caudy, S.M. Hammond, G.J. Hannon, Role for a bidentate ribonuclease in the initiation step of RNA interference, Nature 409 (2001) 363–366.
[4] V. Ambros, The functions of animal microRNAs, Nature 431 (2004) 350–355.
[5] G. Meister, T. Tuschl, Mechanisms of gene silencing by double-stranded RNA, Nature 431 (2004) 343–439.
[6] M.A. Carmell, G.J. Hannon, RNase III enzymes and the initiation of gene silencing, Nat. Struct. Mol. Biol. 11 (2004) 214–218.
[7] A. Grishok, A.E. Pasquinelli, D. Conte, N. Li, S. Parrish, I. Ha, D.L. Baillie, A. Fire, G. Ruvkun, C.C. Mello, Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing, Cell 106 (2001) 23–34.
[8] R.F. Ketting, S.E. Fischer, E. Bernstein, T. Sijen, G.J. Hannon, R.H. Plasterk, Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*, Genes. Dev. 15 (2001) 2654–2659.
[9] S.W. Knight, B.L. Bass, A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*, Science 293 (2001) 2269–2271.
[10] R.C. Lee, V. Ambros, An extensive class of small RNAs in *Caenorhabditis elegans*, Science 294 (2001) 862–864.
[11] G. Hutvagner, J. McLachlan, A.E. Pasquinelli, E. Balint, T. Tuschl, P.D. Zamore, A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA, Science 293 (2001) 834–838.
[12] P.J. Paddison, A.A. Caudy, E. Bernstein, G.J. Hannon, D.S. Conklin, Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells, Genes. Dev. 16 (2002) 948–958.
[13] E.P. Murchison, J.F. Partridge, O.H. Tam, S. Cheloufi, G.J. Hannon, Characterization of Dicer-deficient murine embryonic stem cells, Proc. Natl. Acad. Sci. USA 102 (2005) 12135–12140.
[14] C. Kanellopoulou, S.A. Muljo, A.L. Kung, S. Ganesan, R. Drapkin, T. Jenuwein, D.M. Livingston, K. Rajewsky, Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing, Genes. Dev. 19 (2005) 489–501.
[15] E. Bernstein, S.Y. Kim, M.A. Carmell, E.P. Murchison, H. Alcorn, M.Z. Li, A.A. Mills, S.J. Elledge, K.V. Anderson, G.J. Hannon, Dicer is essential for mouse development, Nat. Genet. 35 (2003) 215–217.
[16] P. Svoboda, P. Stein, M. Anger, E. Bernstein, G.J. Hannon, R.M. Schultz, RNAi and expression of retrotransposons MuERV-L and IAP in preimplantation mouse embryos, Dev. Biol. 269 (2004) 276–285.
[17] H.R. Scholer, G.R. Dressler, R. Balling, H. Rohdewohld, P. Gruss, Oct-4: a germline-specific transcription factor mapping to the mouse *t*-complex, EMBO J. 9 (1990) 2185–2195.
[18] H.R. Scholer, T. Ciesiolka, P. Gruss, A nexus between Oct-4 and E1A: implications for gene regulation in embryonic stem cells, Cell 66 (1991) 291–304.
[19] A.K. Ryan, M.G. Rosenfeld, POU domain family values: flexibility, partnerships, and developmental codes, Genes. Dev. 11 (1997) 1207–1225.
[20] M.H. Rosner, M.A. Vigano, K. Ozato, P.M. Timmons, F. Poirier, P.W. Rigby, L.M. Staudt, A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo, Nature 345 (1990) 686–692.
[21] J. Nichols, B. Zevnik, K. Anastassiadis, H. Niwa, D. Klewe-Nebenius, I. Chambers, H. Scholer, A. Smith, Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4, Cell 95 (1998) 379–391.
[22] H. Niwa, S. Masui, I. Chambers, A.G. Smith, J. Miyazaki, Phenotypic complementation establishes requirements for specific POU domain and generic transactivation function of Oct-3/4 in embryonic stem cells, Mol. Cell. Biol. 22 (2002) 1526–1536.
[23] I. Chambers, D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie, A. Smith, Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells, Cell 113 (2003) 643–655.
[24] K. Mitsui, Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, M. Maruyama, M. Maeda, S. Yamanaka, The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells, Cell 113 (2003) 631–642.
[25] P.A. De Sousa, A. Caveney, M.E. Westhusin, A.J. Watson, Temporal patterns of embryonic gene expression and their dependence on oogenetic factors, Theriogenology 49 (1998) 115–128.
[26] X.S. Cui, X.Y. Li, Y.J. Jeong, J.H. Jun, N.H. Kim, Gene expression of cox5a, 5b, or 6b1 and their roles in preimplantation mouse embryos, Biol. Reprod. 74 (2006) 601–610.
[27] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[28] SAS User's Guide: Statistics, version 5. Cary, NC, SAS, 1985.
[29] R.G.D. Steel, J.H. Torrie, Principles and Procedures of Statistics, McGraw Hill Book Co., New York, 1980.
[30] W.J. Yang, D.D. Yang, S. Na, G.E. Sandusky, Q. Zhang, G. Zhao, Dicer is required for embryonic angiogenesis during mouse development, J. Biol. Chem. 280 (2005) 9330–9335.
[31] E. Wienholds, W.P. Kloosterman, E. Miska, E. Alvarez-Saavedra, E. Berezikov, E. de Bruijn, H.R. Horvitz, S. Kauppinen, R.H. Plasterk, MicroRNA expression in zebrafish embryonic development, Science 309 (2005) 310–311.
[32] L.A. Boyer, T.I. Lee, M.F. Cole, S.E. Johnstone, S.S. Levine, J.P. Zucker, M.G. Guenther, R.M. Kumar, H.L. Murray, R.G. Jenner, D.K. Gifford, D.A. Melton, R. Jaenisch, R.A. Young, Core transcriptional regulatory circuitry in human embryonic stem cells, Cell 122 (2005) 947–956.