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MicroRNA Let-7a and dicer are important in the activation and implantation of delayed implanting mouse embryos

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STUDY QUESTION: Does Let-7a have a functional role in modulating dicer expression to activate dormant mouse blastocysts for implantation?

SUMMARY ANSWER: Let-7a post-transcriptionally regulates dicer expression altering microRNA expression to affect the implantation competency of the activated blastocysts.

WHAT IS KNOWN ALREADY: The Let-7a microRNA is up-regulated during blastocyst dormancy and its forced-expression suppresses embryo implantation *in vitro* and *in vivo*. Dicer is a Let-7 target, which processes pre-microRNA to mature microRNA.

STUDY DESIGN, SIZE, DURATION: The effects on the expression of Let-7a and dicer in dormant blastocysts during the first 12 h after estradiol-induced activation, and the relationship between Let-7a and dicer in preimplantation embryos were determined. The effects on the microRNA expression and embryo implantation *in vivo* in dicer-knockdown mouse 5–8 cell embryos and dormant blastocysts at 1 h post estradiol activation were also studied.

PARTICIPANTS/MATERIALS, SETTING, METHODS: ICR female mice at 6 weeks of age were ovariectomized on Day 4 of pregnancy to generate the delayed implantation model. Mouse 5–8 cell embryos and/or dormant blastocysts at 1 h after estradiol injection were electroporated with dicer siRNA and Let-7a precursor or Let-7a inhibitor. At 48 h post electroporation, the Let-7a expression, dicer transcripts and proteins in the embryos were determined using qPCR and immunostaining/western blotting, respectively. All experiments were repeated at least three times.

MAIN RESULTS AND THE ROLE OF CHANCE: Estradiol injection down-regulated Let-7a and up-regulated dicer in the dormant blastocysts during the first 12 h post-activation. Dicer knockdown at 1 h post-activation of blastocysts suppressed EGFR expression, attenuated EGF binding and compromised implantation of the transferred embryos. Let-7a transcriptionally regulated dicer by binding to the 3′-UTR of dicer in trophoblast cells. Dicer knockdown in blastocysts suppressed mature Let-7a expression and compromised implantation.

LIMITATIONS, REASONS FOR CAUTION: Gain- and loss-of-function approaches were used by analyzing transient expressions of transfected microRNA modulators or genes. The consequence of the Let-7a-dicer interaction on pregnancy remains to be determined. The study used the mouse as a model and the applicability of the observed phenomena in humans warrants further investigation.

WIDER IMPLICATIONS OF THE FINDINGS: Our results indicate that the Let-7a-dicer interaction leads to differential microRNA expression in dormant blastocysts after estradiol activation. Because the expression pattern of Let-7a in human blastocysts is similar to that in mouse blastocysts, our observation that the Let-7a-dicer interaction has a role in regulating the implantation potential of the mouse blastocysts could be applicable to humans.

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Key words: dicer / microRNA / embryo implantation / dormant blastocyst / estradiol activation

Introduction

Implantation is initiated when a competent blastocyst attaches onto the receptive endometrium. The endometrium-embryo interaction and steroid hormones trigger the production of various factors in the endometrium to facilitate implantation. These factors include leukemia inhibitory factor, epidermal growth factor, transformation growth factor and insulin growth factors, which either induce blastocyst attachment and/or stimulate trophoblast migration and invasion during implantation. However, the regulatory processes leading to the implantation of the competent blastocysts are largely unknown.

MicroRNAs (miRNA) are small regulatory RNAs that modulate target protein expression. The miRNAs regulate biological activities in many cellular systems including in preimplantation embryos. They are generated by the transcription of primary miRNAs, which are then processed by Drosha/DGCR8 to form precursor miRNAs (Cullen, 2004; Kim and Nam, 2006; Macfarlane and Murphy, 2010). Upon Exportin-5 mediated translocation to the cytoplasm, the RNaseIII dicer cleaves the precursor miRNAs to mature miRNAs. The sense strand of the mature miRNA is then loaded to the RNA-induced silencing complex to form a target-specific microprocessor with argonaute-2, which binds to the 3′-untranslated region (3′-UTR) of its target gene to promote transcript degradation and/or translation inhibition.

The Lethal-7 (Let-7) miRNA family was first discovered as a developmental timing regulator in *Caenorhabditis elegans* (Esquela-Kerscher *et al.*, 2005). The patterns of Let-7 gene clustering and genomic positioning are highly conserved among species including drosophila, mouse and human, but not nematodes (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Bashir-ullah *et al.*, 2003; Liu *et al.*, 2012). In mouse embryos, Let-7 family is down-regulated during preimplantation embryo development (Suh *et al.*, 2010; Liu *et al.*, 2012) and in dormant blastocysts at 3 h post estradiol activation (Liu *et al.*, 2012). Our previous study demonstrated that Let-7a post-transcriptionally regulates integrin beta-3 expression to modulate blastocyst attachment and pregnancy (Liu *et al.*, 2012).

Like other miRNAs, the production of mature Let-7 is dicer-dependent. Dicer-knockout embryos have an aberrant microRNAome, and the defective mesoderm segregation during early gastrulation results in lethality at E6.5 (Murchison *et al.*, 2005; Luense *et al.*, 2009; Spruce *et al.*, 2010; Suh *et al.*, 2010). Similarly, conditional knockout of dicer in smooth muscle cells and brain cells causes aberrant miRNA expression and death of the transgenic mice at E14.5–15.5 (Pan *et al.*, 2011; McLoughlin *et al.*, 2012). However, the role of dicer during blastocyst implantation is not clear. On the other hand, dicer is a regulatory target of Let-7 in human cancer cells (Tokumaru *et al.*, 2008). Therefore, we postulated that the Let-7a-dicer interaction plays a role in blastocyst implantation. In this study, we determined the role of the Let-7a-dicer interaction in the delayed implantation mouse model. In this model, the implantation competency of the dormant blastocyst can readily be induced by estradiol injection (Paria *et al.*, 1993b). Our results showed that the Let-7a-dicer interaction is important in the implantation of the activated blastocysts. The phenomenon is also found in preimplantation embryos from normal pregnancy.

Materials and Methods

Embryo retrieval

The protocol was approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong (CULATR number: 2070-10). Imprinted-coding region (ICR; CD-1) female mice were superovulated as previously described (Cheong *et al.*, 2009). Preimplantation embryos at various developmental stages were retrieved by flushing either the oviduct or uterus of the pregnant ICR females with M2 medium (Lee *et al.*, 2009).

Dormant and estradiol-activated blastocysts were collected from uterine flushing fluid from the delayed implanting mice. Dormancy of the blastocysts was induced by ovariectomy of the superovulated pregnant mice on Day 4 before 8 a.m., followed by a progesterone injection (2 mg/mouse; i.p.) for 2 days consecutively. Dormant blastocysts were retrieved at 3 h after the progesterone injection. On Day 7, an estradiol injection (25 ng/mouse; i.p.) was given to activate the blastocysts, which were collected by flushing the uterus at 1, 3, 6 and 12 h post-activation. In some experiments, the activated blastocysts were retrieved and electroporated with siRNA at 1 h post estradiol activation. The embryos were then cultured in KSOMaa culture medium and lysed at 3 h post estradiol activation for expression analyses or allocated for an EGF binding assay at 6 h post estradiol activation.

Embryo transfection and embryo transfer

To study the effect of dicer on implantation, mouse embryos at the 5–8 cell stage were retrieved from the oviducts of the Day 3 pregnant females. They were randomly allocated to the experimental groups and were electroporated with 10 μM of either the dicer siRNA (SC-40490; Santa Cruz, MO, USA), Let-7a precursor (002478; Applied Biosystems, CA, USA) or Let-7a inhibitor (410017-00; Exiqon A/S, Vedbaek, Germany) as previously described (Liu *et al.*, 2012). The miRNA control (199004-00; Exiqon A/S, Vedbaek, Germany) or the siRNA control (D-001220-01-05, Thermofisher, Pittsburgh, PA, USA) were used as negative controls. Electroporation of the Cy3-labeled miRNA negative probe in 5–8-cell embryos demonstrated a high transfection efficiency, which could be detected as a red fluorescent signal in all the embryos immediately after the electroporation. The development of the electroporated embryos and the untreated control embryos was similar. The electroporated embryos were lysed using 2 M guanidinium isothiocyanate solution for total RNA extraction or lysed using 1 × SDS loading dye for western blotting analysis. A portion of the embryos was washed in 0.1% PVP/PBS (w/v) and the zonae pellucidae were removed by acid Tyrode's solution, followed by immediate fixing in 4% paraformaldehyde for immunostaining. For embryo transfer, blastocysts at 48 h post electroporation were transferred into the uterine horns of Day 3 pseudo-pregnant females. The number of implantation sites was determined at Day 8 after estradiol activation.

Immunostaining

Mouse embryos were immunostained using a previously described method (Lee *et al.*, 2004) that was modified. The fixed mouse embryos were

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incubated in 0.01 M sodium citrate buffer (pH 6.0) containing 0.05% Tween 20 (v/v) at 95°C for 15 min. After washing in PBS, the embryos were permeabilized in permeabilization buffer containing 0.05% Triton-X (v/v) and 0.5% Nonidet-40 (v/v) in PBS for 5 h. The embryos were then incubated with primary mouse monoclonal anti-dicer (ab14601, Abcam, MA, USA) or rabbit IgG (1:50) diluted in blocking solution containing 10% serum (v/v) and 3% BSA (w/v) in PBS overnight at 4°C. On the next day, the embryos were washed in washing buffer containing 2% serum (v/v), 0.6% BSA (w/v), 0.4% Triton-X (v/v) and 0.4% Nonidet-40 (v/v) in PBS and then incubated in either Cy3- or FITC-conjugated secondary antibody (1:100) diluted in blocking solution for an hour. After washing, the nuclei were counterstained with 25 ng/ml DAPI (1:1000) in PBS for an hour, then washed twice in washing buffer and once in PBS. The embryos were placed on poly-L-lysine coated confocal dishes and the confocal Z-stack images were captured using the LSM 700 inverted microscope at excitations of 488, 545 and 630 nm and at brightfield.

Cell culture and cell transfection

Human chorionic carcinoma JAr cells were seeded at 80 000 cells per well in 12-well plates and were transfected with 0.5 μM Let-7a precursor or inhibitor in OPTI-MEM (Invitrogen) for 48 h using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol. The cell lysates were harvested from the cell disruption buffer of the mirVana PARIS kit (Ambion, Applied Biosystems, Invitrogen, CA, USA) and used in the subsequent miRNA and protein expression analyses.

Dual luciferase assay

TargetScan (www.Targetscan.org) and miRanda (www.microrna.org/microrna/) predicted two Let-7 complementary sites (LCS) within the 3′-UTR of dicer, designated as LCS1 (upstream LCS) and LCS2 (downstream LCS). Three amplicons (full length, 429 bp; LCS1, 220 bp; and LCS2, 209 bp) were amplified with the PmeI-NotI recognition sites from the mouse uterine cDNA using the following designed primers (Invitrogen, CA, USA): F1: 5′-CAGGTTAACCAGAGTCACCAAAGCTGTG-3′, F2: 5′-CAGGTTTAACTTTTGCACTGTGACTATAAT-3′, R1: 5′-CAGGGGGCCGCGAGATGTGTGGGGAACGCGA-3′ and R2: 5′-CAGGCGGCCGCATGCAGGCAAAGGGAGGGAC-3′ in combinations of F1/R1, F1/R2 and F2/R1, respectively. The purified amplicons were cloned downstream of the renilla luciferase gene of the psiCHECK-2 vector to generate the wild-type reporter construct. The full length, LCS1 and LCS2 mutated reporter constructs were generated using the QuikChange II Site Directed Mutagenesis Kit (Stratagene, CA, USA) with the following designed primers (Invitrogen): Mutated LCS1: 5′-GGCCCCTCTTCACTTACACCTCGAGATCCCTCCCTTTGCCTGCAT-3′ and Mutated LCS2: 5′-TTTTGCACTGTGACTATAACTCGAGATAATTTACATTTAAAAATG-3′ according to the manufacturer's protocol. To perform the dual luciferase assay, JAr cells were co-transfected with either the Let-7a precursor (Ambion) or Let-7a inhibitor (Exiqon A/S, Vedbaek, Denmark) and one of the wild-type reporter constructs using the Lipofectamine 2000 transfection reagent. Transfection of the empty vector and the scramble control (Exiqon A/S) were used as negative controls. The transfection was repeated using the mutated reporter constructs. Cell lysates harvested at 48 h post-transfection were collected for analyses as previously described (Pang et al., 2010; Liu et al., 2012).

Reverse transcription–polymerase chain reaction

The miRNA and gene expression analyses were carried out using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), respectively. The miRNA probes for mature Let-7a (002478), Let-7e (002407), Let-7f (002478), miRNA-34a (000426), miRNA-181a (000480) and miRNA-16 (000391) (Applied Biosystems) were used. The TaqMan gene expression assay was used for human primary miRNA Let-7a (Hs03302546_pri), mouse primary miRNA Let-7a (Mm03306752_pri), mouse dicer (Mm00521722_m1) and mouse GAPDH (Mm99999915_g1) (Applied Biosystems).

Western blotting

Western blot analysis of the cell lysates was carried out according to the described protocol (Liu et al., 2012). To analyze the protein expression in mouse embryos, twenty embryos per lane were lysed in 10 μl 1× SDS loading dye. After denaturation at 95°C for 5 min, the proteins were resolved on a 12% SDS gel. The same blot was used for the analyses of different protein expressions with actin serving as the endogenous control. Primary mouse monoclonal antibodies against dicer (ab14601, Abcam, MA, USA), epidermal growth factor receptor (EGFR, E-8; sc-374607, Santa Cruz, CA, USA) and beta-actin (SC-47778, Santa Cruz) were used. The secondary antibodies were anti-rabbit IgG (NA-934, GE Healthcare, PA, USA) and anti-mouse IgG (NA-931, GE Healthcare) conjugated with horseradish peroxidase.

Blastocyst EGF binding assay

Early, mid and late mouse blastocysts retrieved from the uterine flushing fluid at Day 4.5, 5 and 5.5 after estradiol activation were incubated for 30 min with Alexafluor-488 conjugated epidermal growth factor (E13345, Invitrogen) in 20 μl of KSOMaa droplets. After washing, they were transferred onto a coverslip bottom confocal dish in PBS droplets for examination by confocal microscopy. The experiment was repeated with the dormant and activated blastocysts electroporated with dicer siRNA or siRNA control. The total green fluorescence intensities in the confocal microscopy images of the studied blastocysts were semi-quantitatively compared among the groups to determine the relative EGF binding ability.

Statistical analysis

All data were expressed as mean ± standard error of mean from at least three replicated experiments. The data were analyzed by the SigmaStat 2.03 software (Jandel Scientific, CA, USA) using one-way analysis of variance and Fisher's exact test where appropriate. A value of $P < 0.05$ was considered to define a statistically significant difference between groups.

Results

Expression of Let-7a and dicer in dormant and estradiol-activated blastocysts

The levels of primary and mature Let-7a were low in the Day 4 blastocysts (Fig. 1A). After induction of dormancy, the levels significantly increased ($P < 0.05$). Estradiol-induced activation suppressed Let-7a expression within 1-h post-activation. Subsequently, the primary Let-7a expression remained unchanged, whereas the mature Let-7a expression decreased further after estradiol activation.

Compared with the Day 4 blastocysts, the expression of dicer transcript was significantly reduced in the dormant blastocysts, but returned to that of the Day 4 blastocysts upon estradiol activation (Fig. 1B). Dicer immunoreactivity was detected in the trophectoderm and inner cell mass of Day 4 blastocysts (Fig. 2A). In contrast, dormant blastocysts did not express the dicer protein. Blastocysts treated with estradiol had higher dicer expression levels at the distal mural trophectoderm. Dicer expression increased towards the polar trophectoderm at 3 h post-activation and became detectable in the inner cell mass after 6 h

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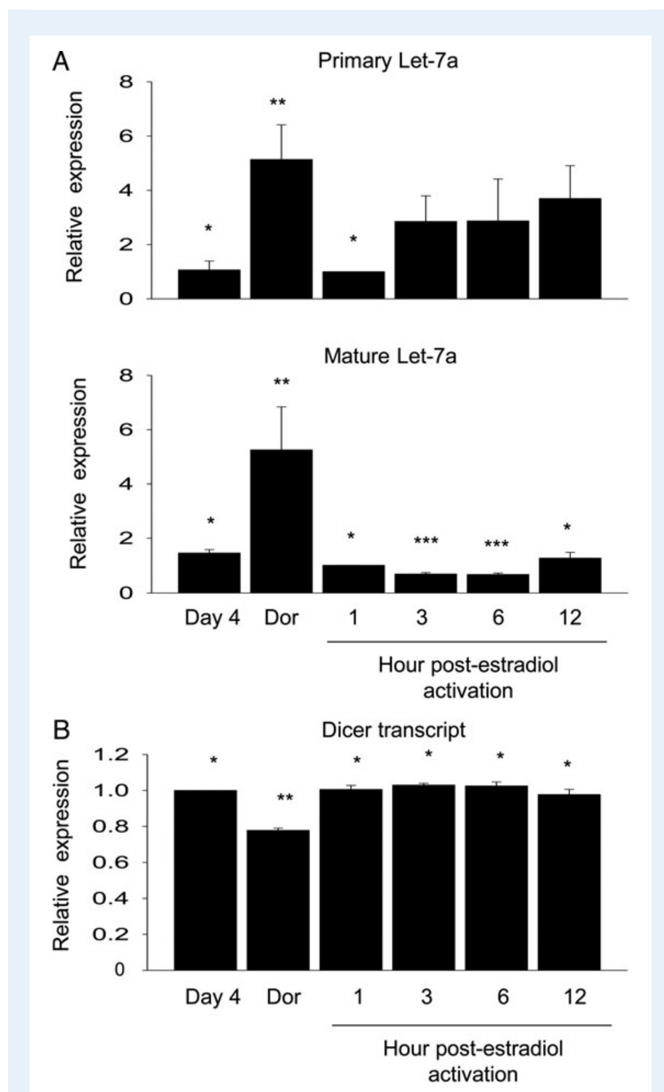

Figure 1 Expression of Let-7a and dicer transcript in dormant blastocysts during estradiol activation. Dormant (Dor) blastocysts (five embryos per reaction; $n = 5$) were retrieved from the uterine flushing fluid of delayed implanting mice on Day 7. Estradiol-activated blastocysts were retrieved at 1, 3, 6 and 12 h post-activation. (**A**) Dormant blastocysts had significantly higher primary (upper panel) and mature (lower panel) Let-7a expression. Estradiol activation suppressed primary and mature Let-7a expression in the blastocysts. (**B**) Estradiol activation increased but then did not temporally affect dicer transcript expression. The expression levels of primary and mature Let-7a and dicer transcript were relative to that of 1-h post estradiol activation. *-**, *-*** and **-*** denote statistically significant differences between groups ($P < 0.05$).

post-activation. Quantitative comparison of the dicer expression by fluorescence intensity of the stained blastocysts showed a gradual increase in the signal intensity after estradiol-induced activation (Fig. 2B).

Let-7a binds to the 3′-UTR of dicer to modulate dicer protein expression

The interaction between Let-7a and dicer in embryonic cells was confirmed by the dual luciferase assay in the trophoblast cells (JAr). The 3′-UTR of dicer contains two partial Let-7 complementary seeding (LCS1 and LCS2) regions (Fig. 3A). Luciferase reporter constructs of the two LCSs and their mutated forms are shown in Fig. 3B and C. At 48 h after co-transfection of the wild-type reporter construct of LCS1 with the Let-7a precursor, the luciferase signal was decreased by 50% ($P < 0.05$), whereas co-transfection with the Let-7a inhibitor increased the signal intensity by 2.2-fold ($P < 0.05$, Fig. 3D). Similarly for co-transfection with the wild-type reporter construct of LCS2, Let-7a precursor reduced the luciferase signal intensity by 40% ($P < 0.05$), whereas the Let-7a inhibitor increased the signal intensity by 1.4-fold ($P < 0.05$). Simultaneous co-transfection of Let-7a precursor with both wild-type reporter constructs lowered the luciferase intensity by 60% ($P < 0.05$), while co-transfection with Let-7a inhibitor increased the luciferase signal by 1.6-fold ($P < 0.05$). There were no changes in the signal intensity of the co-transfection of the mutated reporter constructs with the Let-7a precursor or inhibitor or with the miRNA control.

Dicer knockdown in the estradiol-activated dormant blastocysts alters microRNA expression and reduces EGF binding and implantation

The expression of the dicer transcript was significantly lower in dormant blastocysts compared with the estradiol-activated blastocysts at 1 h post-activation ($P < 0.05$, Fig. 4A). The dormant blastocysts at 1 h after estradiol activation were electroporated with either dicer siRNA or siRNA control. Dicer knockdown suppressed dicer transcript (Fig. 4A) and protein (Fig. 4B) expressions in the activated blastocysts after 3 h post-activation. This treatment did not affect the low expression of primary (Fig. 4C) and mature (Fig. 4D) Let-7a in the activated blastocysts.

The effects of dicer knockdown on other dormancy-related miRNAs in the estradiol-activated blastocysts were also examined. The expression of miRNA-34a was low in the activated blastocysts but was 20-fold higher ($P < 0.05$) in the dormant blastocysts (Fig. 4E). Transfection of dicer siRNA at 1 h post-activation did not affect the miRNA-34a expression after 3 h post-activation. In contrast, the expression of microRNA-181a, which was suppressed in the dormant blastocysts and was highly up-regulated in the activated blastocysts (Fig. 4F), was significantly suppressed in the dicer knockdown activated blastocysts (0.5-fold versus 3-fold in the untreated; $P < 0.05$). The siRNA control transfection had no such effect (4-fold in miRNA-181a versus 3-fold in the untreated; $P > 0.05$).

The effect of dicer on embryo implantation was further studied *in vitro* by assessing the EGF binding ability of the blastocysts. Dormant blastocysts did not express EGFR compared with the activated blastocysts as seen in the western blotting analysis (Fig. 5A). The untreated and the siRNA control electroporated blastocysts showed EGFR expression at 6 h post-activation, which bound EGF with comparable affinity ($P > 0.05$, Fig. 5B and C). Dicer knockdown in the estradiol-activated blastocysts suppressed EGFR expression (Fig. 5A) and attenuated the EGF binding affinity ($P < 0.05$, Fig. 5C). After embryo transfer, the dormant blastocysts had a lower implantation rate of 2 out of 14 compared with 11 out of 12 for the activated blastocysts ($P < 0.05$, Table I). The siRNA control electroporated blastocysts all successfully implanted with similar results to the estradiol-activated blastocysts. However dicer knockdown in the activated blastocysts compromised implantation

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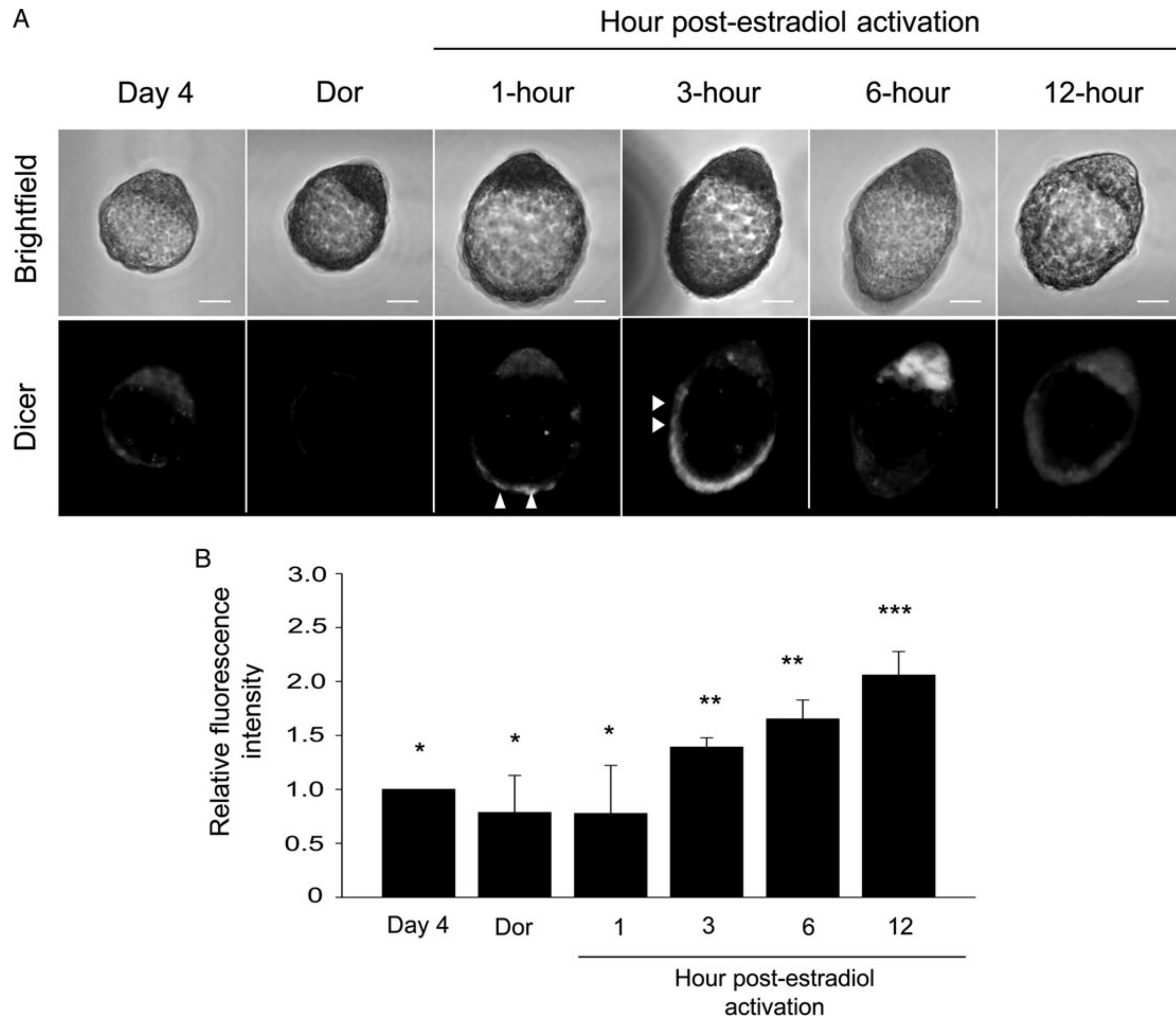

Figure 2 Spatiotemporal expression of dicer in blastocysts during the first 12-h of estradiol activation. (**A**) Dicer protein was detected in the Day 4 blastocysts. Dormant (Dor) blastocysts expressed lower levels of dicer protein. Estradiol activation up-regulated dicer expression in the mural trophectoderm of dormant blastocysts (white arrow heads) at 1 h post-activation and in the proximal trophectoderm (white arrow heads) at 3 h post-activation (×630 magnification, Bar = 30 μm). (**B**) Quantification of the relative fluorescence intensity among the dormant and the activated blastocysts. Dicer expression was lower in the dormant blastocysts when compared with the Day 4 blastocysts. Estradiol injection increased the expression of Dicer continuously in the first 12 h of activation. *-**, *-*** and **-*** denote significant difference between groups ($P < 0.05$).

of only 7 sites out of 13, which was significantly lower than 11 out of 12 for the activated blastocysts ($P < 0.05$, Table I).

Expression of Let-7a and dicer in preimplantation embryos

To determine whether the observed phenomena in the delayed implantation were relevant to the embryos in normal pregnancy, preimplantation embryos were collected from normal pregnant females. The expression of primary Let-7a was highest at the 2-cell stage and decreased at the blastocyst stages (Fig. 6A). Mature Let-7a was highly abundant in the oocytes. Similar to primary Let-7a, mature Let-7a decreased in the blastocysts (Fig. 6B). Dicer transcript expression was constant throughout the preimplantation period (Fig. 6C).

Reciprocal relationship between Let-7a and dicer in preimplantation embryos

Transfection of Let-7a precursor into 5–8-cell embryos did not affect the expression of primary Let-7a but significantly increased that of mature Let-7a by about 2-fold ($P < 0.05$, Fig. 7A) in the resulting blastocysts. Let-7a inhibition significantly suppressed the expression of primary and mature Let-7a by 40 and 20%, respectively ($P < 0.05$). Forced-expression of Let-7a significantly suppressed dicer transcript expression by 50% ($P < 0.05$). Inhibition of Let-7a did not affect dicer transcript expression (Fig. 7B). Immunostaining showed that forced-expression of Let-7a reduced dicer protein expression, whereas inhibition of Let-7a enhanced dicer protein expression in the mouse blastocysts (Fig. 7C).

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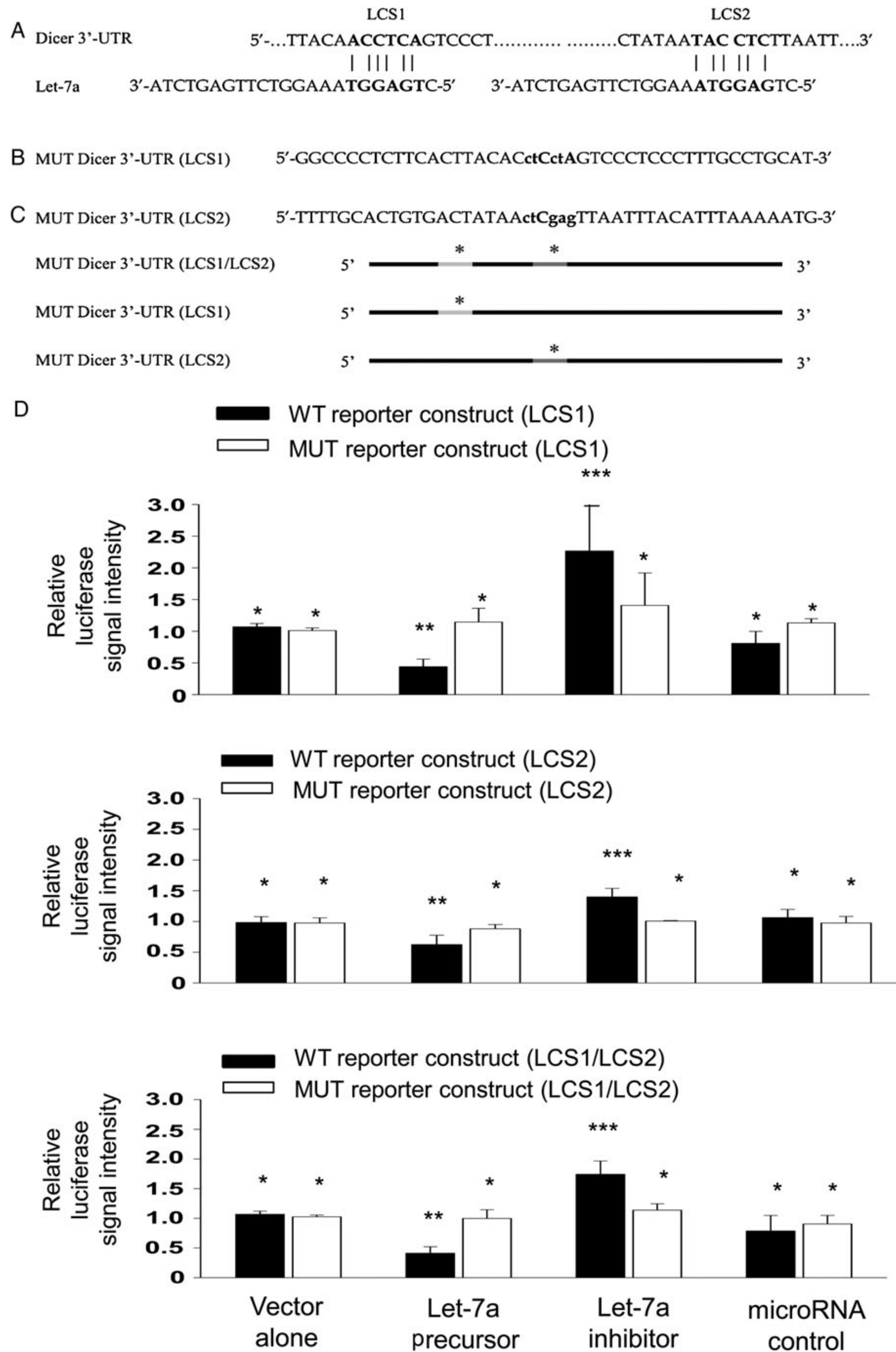

Figure 3 Let-7a targets dicer at the two LCSs within the 3′-UTR. (**A**) The 3′-UTR of dicer contains two mature Let-7a complementary sites (LCS) shown in bold. (**B**) The two LCS had point mutations (small letters) that generated the mutated dicer 3′-UTR reporter constructs. (**C**) Strategy for generating the mutated reporter constructs. Gray lines with asterisks indicate the mutated LCSs. (**D**) Dual luciferase assay was performed using the 48-h post-transfected cell lysates. Co-transfection of the WT-reporter construct for LCS1, LCS2 and both LCS1/LCS2 with either the Let-7a precursor or inhibitor altered the relative luciferase signal intensities. These changes were not detectable when the co-transfection was repeated with the mutated reporter constructs or with the miRNA control. *-**, *-*** and **-*** denote significant differences between groups ($P < 0.05$).

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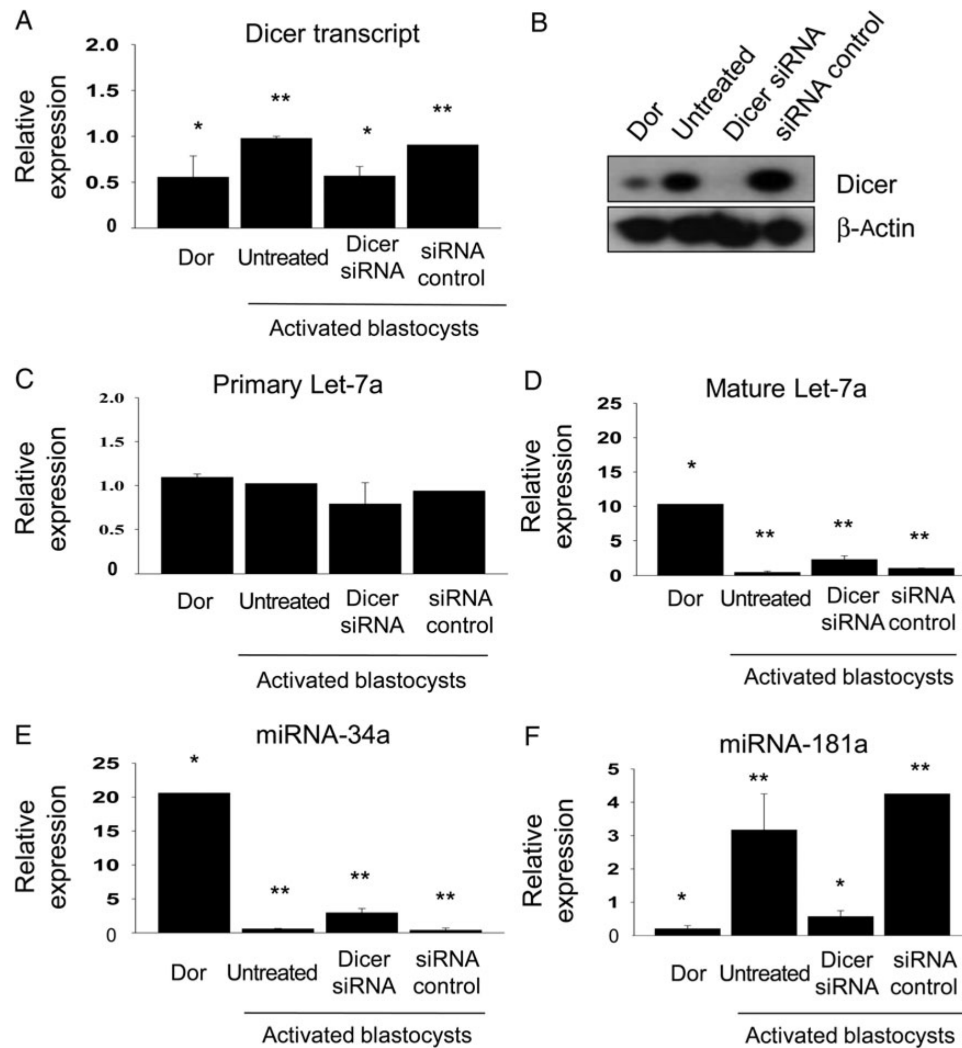

Figure 4 Suppressing dicer expression in the activated blastocysts alters the mature microRNA expression. The expression of dicer transcript (**A**) and protein (**B**) were lower in the dormant blastocysts (Dor) than in the activated blastocysts. (B) Dicer siRNA reduced the expression of dicer in the activated blastocysts. (**C**) Estradiol activation with or without dicer knockdown did not affect the primary Let-7a expression, (**D**) but lowered the expression of mature Let-7a. (**E**) The expression pattern of miRNA-34a was similar to that of mature Let-7a. (**F**) The expression of miRNA-181a was low in the dormant blastocysts and was increased after activation. Dicer siRNA suppressed the estradiol-induced increase in miRNA-181a. *-** denotes significant differences between groups ($P < 0.05$). Dicer protein was normalized by blastocyst number (30 embryos per lane) and against endogenous β-actin expression.

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Dicer knockdown alters microRNA expression and reduces blastocyst implantation

The role of dicer in blastocyst development was studied by electroporating dicer siRNA into 5–8 cell embryos. The siRNA treatment reduced dicer transcript expression by 60% ($P < 0.05$, Fig. 8A) and diminished the expression of dicer protein in mouse blastocysts (Fig. 8B and C). The decrease in dicer did not affect the expression of the primary Let-7a, but significantly down-regulated mature Let-7a and miRNA-34a levels ($P < 0.05$, Fig. 7D). Dicer knockdown resulted in a lower implantation rate of 6 out of 18 blastocysts compared with 14 out of 18 for the siRNA control transfected blastocysts ($P < 0.05$, Table II).

Discussion

The results showed that down-regulation of Let-7a during estradiol-induced activation of dormant blastocysts enhances dicer expression, and siRNA-mediated knockdown of dicer expression in the estradiol-activated blastocysts compromises implantation. An interaction between Let-7a and dicer in the embryos is evident by the reciprocal expression between Let-7a and dicer in the blastocysts, which is supported by the results of the 3′UTR luciferase assays in human trophoblast cells. The data are consistent with a continuous decrease in the expression of primary (Garcia-Lopez and del Mazo, 2012) and mature Let-7a (Suh *et al.*, 2010; Garcia-Lopez and del Mazo, 2012) during preimplantation embryo development, indicating that a low level of Let-7a is needed

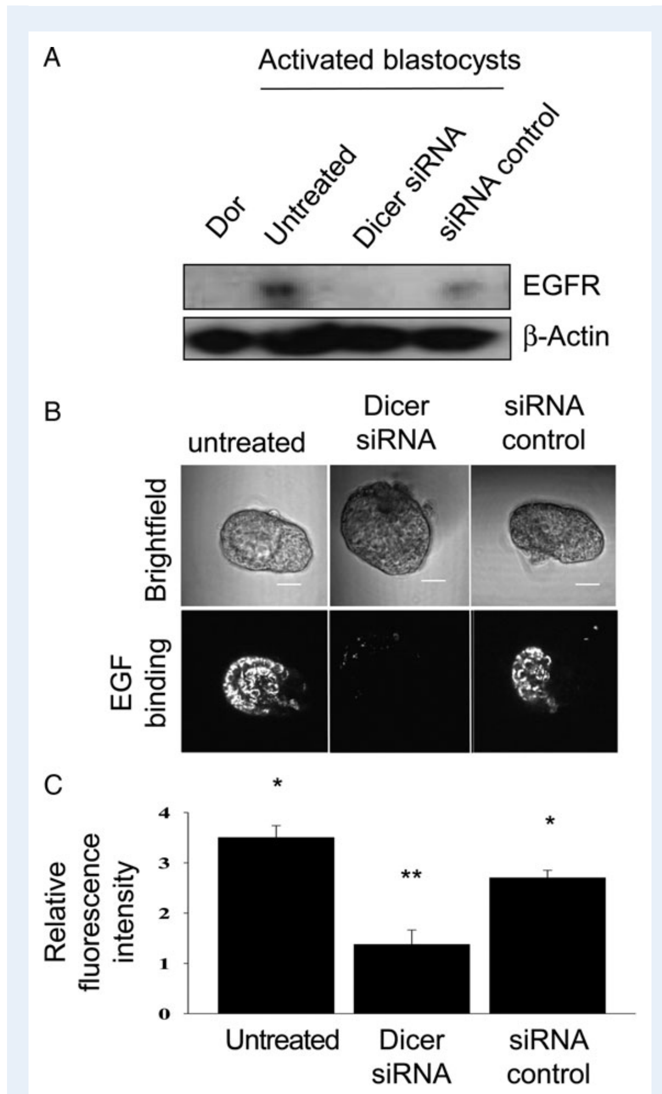

Figure 5 Dicer knockdown represses EGF binding ability of the activated blastocysts. One-hour post-activated blastocysts were electroporated with dicer siRNA or siRNA control. At 6 h post-activation, the embryos were retrieved for EGF binding assay. (**A**) EGF receptor (EGFR) was not expressed in Dormant (Dor) blastocysts and in the dicer knockdown of estradiol-activated blastocysts. Protein loading was normalized by blastocyst number (30 embryos per lane) and against endogenous actin expression. (**B**) Dicer knockdown reduced the binding of EGF in the activated blastocysts. (**C**) Plot of the relative fluorescence (EGF binding ability) of the untreated and the dicer siRNA or siRNA control transfected blastocysts at 6 h post-activation ($\times$630 magnification, Bar = 30 μm). *-** denotes significant differences between groups ($P < 0.05$).

during implantation (Liu *et al.*, 2012). The high level of mature Let-7a in the oocytes suggests a maternal origin of the miRNAs.

Molecules involved in degradation of miRNAs are detectable in the preimplantation embryos. One of them is endoribonuclease polyribonucleotide nucleotidytransferase I (PNPT1), which cleaves mature miRNA non-specifically (Zhang *et al.*, 2012). The PNPT1 transcript is up-regulated at the blastocyst stage (Zeng *et al.*, 2004). Our unpublished data show that PNPT1 transcript is up-regulated in the estradiol-activated blastocysts. Similarly, PNPT1 is up-regulated temporally by estradiol in the MCF-7 cells during the first 12 h after estradiol treatment (Klinge, 2001). Together with the low Let-7a expression, our data suggest that the estradiol-mediated increase in PNPT1 expression may suppress mature Let-7a expression in the activated blastocysts.

The RNA-binding proteins, Lin28A and Lin28B, are well-known regulators of Let-7 (Zhang *et al.*, 2012). The two Lin28 isoforms have similar functions, although they target different Let-7 species (Viswanathan *et al.*, 2008; Viswanathan and Daley, 2010; Nam *et al.*, 2011; Piskounova *et al.*, 2011). Lin28A transcript is up-regulated in blastocysts during preimplantation embryo development, whereas Lin28B transcript is up-regulated at the 8-cell stage and peaks at the blastocyst stage (Zeng *et al.*, 2004). Our unpublished data show that siRNA-mediated knockdown of both Lin28A and Lin28B increase Let-7a expression and suppress embryo implantation *in vivo*. This observation supports a regulatory role of Lin28 in the expression of Let-7a.

In the present study, dormant blastocysts had high primary and mature Let-7a expression. Estradiol-induced activation suppressed the expression of primary and mature Let-7a, although it did not continue to affect the expression of primary Let-7a. The data confirm our previous observation of a higher Let-7a expression in the dormant blastocysts compared with blastocysts at 3 h after estradiol activation (Liu *et al.*, 2012). The suppressive effect of estradiol on Let-7a in blastocysts is strong with the level of mature Let-7a dropping rapidly to the level of the Day 4 blastocysts upon activation, and remaining low during the first 12 h post-activation. In contrast, a lack of estradiol induces dormancy of the Day 4 blastocyst and up-regulates the primary and mature Let-7a expression in the delayed implantation model. Estradiol is known to affect miRNA expression, and the available evidence suggests that the estradiol action on miRNA expression is dependent on the cell-type. In the human breast cancer cells, MCF-7, estradiol stimulates Let-7a expression (Bhat-Nakshatri *et al.*, 2009) probably via the estrogen-responsive element in the promoter of Let-7a (Sha *et al.*, 2011). In the MCF-7 cells and the mouse proximal tubule derived mouse cortical tubule cell line (MCT), the estradiol-bound ERα interferes with Drosha-mediated miRNA processing, leading to a decrease in the Let-7a precursor expression (Yamagata *et al.*, 2009; Newman and Hammond, 2010). How estradiol regulates Let-7a expression in embryos warrants further investigation.

Dicer protein expression in the mouse blastocysts was suppressed during dormancy. Estradiol activation up-regulated the dicer protein initially in the distal mural trophectoderm, which extended towards the polar trophectoderm and the inner cell mass at 3 h post-activation. Dicer is primarily expressed in the cytoplasm (Barraud *et al.*, 2011) and resides in the nucleus where it processes small regulatory RNAs mediating gene silencing (Emmerth *et al.*, 2010; Barraud *et al.*, 2011). The presence of dicer immunoreactivity in the nuclei of the inner cell mass suggests that some dicer-dependent small regulatory RNAs may modulate the activation of the inner cell mass of the dormant blastocysts.

Forced-expression of Let-7a in blastocysts decreased dicer expression, whereas inhibition of Let-7a increased dicer expression. Forced-expression of let-7a reduced dicer transcripts and proteins in mouse blastocysts, indicating Let-7a transcriptionally regulated dicer in the embryos, which is in line with the observation in human carcinoma (Tokumaru *et al.*, 2008). Dicer is thus a target of Let-7a. Our data suggest that the two complementary sites within the 3′-UTR of dicer mediated the action of Let-7a. On the other hand, dicer knockdown

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Table I Dicer knockdown suppresses implantation of the activated blastocysts.

	Dormant	Activated		
		Untransfected	siRNA control transfected	Dicer siRNA transfected
No. of mice (*n*)	3	3	3	3
No. of embryos transferred in each mouse (total)	4, 4, 6 (14)	5, 4, 3 (12)	5, 4, 5 (14)	5, 4, 4 (13)
No. of implantation sites on Day 8 (total)	1, 1, 0 (2)[a]	4, 4, 3 (11)[b]	5, 4, 5 (14)[b]	2, 2, 3 (7)[a]

[a,b]Statistically significant differences ($P < 0.05$) between groups as determined by Fisher's exact test.

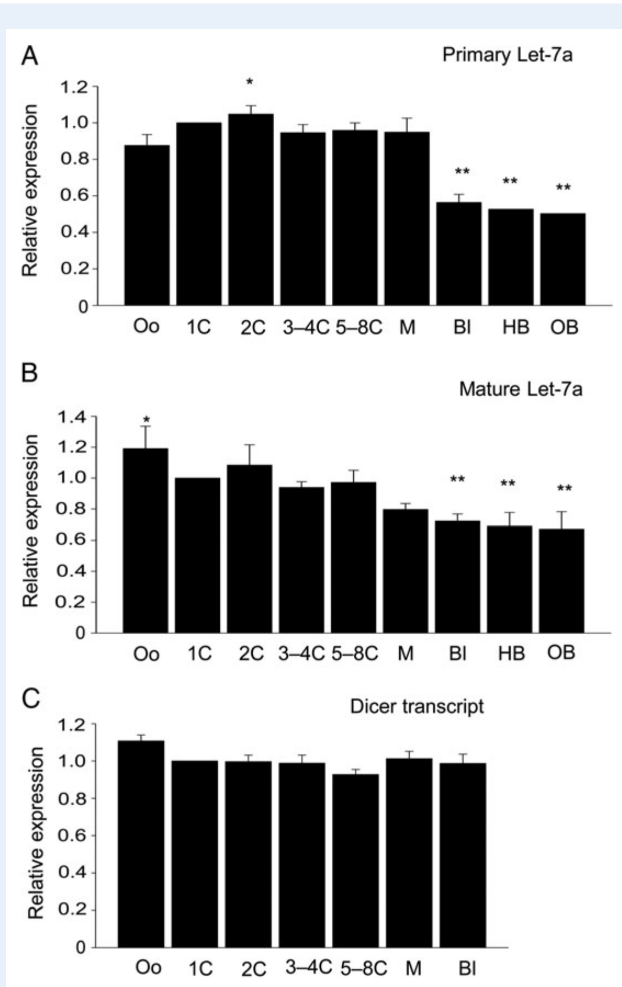

Figure 6 Expression of primary and mature Let-7a and dicer transcript in preimplantation embryos. *In vivo* developed mouse oocyte (Oo), preimplantation embryos at zygote (1C), 2-cells (2C), 3–4 cells (3–4C), 5–8 cells (5–8C), morula (M), blastocyst (Bl), hatching blastocyst (HB) stages and *in vitro* developed outgrown blastocyst (OB) were collected for qPCR analysis (five embryos per reaction; $n = 5$). (**A**) Primary Let-7a expression was highest at the 2-cell stage and was down-regulated at the blastocyst stage. (**B**) Mature Let-7a was originally expressed in the oocytes. The level dropped after fertilization and decreased gradually to a minimum in blastocysts. (**C**) Dicer transcript was constitutively expressed throughout the preimplantation period. The expression levels of primary and mature Let-7a and dicer transcript were relative to that of the zygote. *-** denotes significant differences between groups ($P < 0.05$).

suppressed mature Let-7a expression in the mouse blastocysts, probably due to reduced dicer-mediated maturation of Let-7a from the Let-7a precursor. The observation is consistent with the aberrant expression of miRNA in dicer-knockout embryonic stem cells and embryos (Tokumaru *et al.*, 2008). Our observations thus indicate a negative feedback mechanism via Let-7a-dicer interaction in the mouse blastocysts, which is also observed in the human carcinoma (Tokumaru *et al.*, 2008).

MicroRNAs are differentially expressed in dormant and estradiol-activated blastocysts (Liu *et al.*, 2012). Compared with the dormant blastocysts, activated blastocysts have low levels of Let-7a and miRNA-34a but high levels of miRNA-181a. This is consistent with the reported relative expression level of these miRNAs in dormant mouse blastocysts following estradiol activation (Liu *et al.*, 2012). Dicer has an important role in the regulation of mature miRNAs and dicer knockdown results in altered production of miRNAs. The mechanism for the reduced expression of miRNA-181a after dicer knockdown is not known. Aberrant miRNA expression has been reported in dicer-knockout embryonic stem cells (Kanellopoulou *et al.*, 2005). In mouse oocytes, dicer knockout leads to a differential microRNAome (Suh *et al.*, 2010) and disrupted chromosome spindle formation (Tang *et al.*, 2009) compared with wild-type oocytes. Our study demonstrated the role of miRNA during activation of dormant blastocysts.

Dicer knockdown in the activated blastocysts suppressed EGFR expression and reduced the EGF binding ability. EGFR is expressed in human (Chia *et al.*, 1995), rat (Aflalo *et al.*, 2007) and mouse embryos (Terada *et al.*, 1997; Cai *et al.*, 2003) and in the activated mouse blastocysts (Paria *et al.*, 1993b), but the dormant blastocysts do not express EGFR and lack EGF binding ability (Paria *et al.*, 1993a). Acquisition of EGF binding is a marker of activation of the dormant blastocysts (Paria *et al.*, 1993b). The binding of EGF modulates cell proliferation (Xian, 2007) and differentiation (Machida *et al.*, 1995). In mouse embryos, exogenous EGF stimulates trophoblast differentiation (Machida *et al.*, 1995), induces hatching (Chia *et al.*, 1995; Aflalo *et al.*, 2007) and facilitates trophoblast migration and invasion (Cai *et al.*, 2003). We showed that dicer knockdown diminished the implantation competency of the activated blastocysts, which would have been at least in part due to altering the EGF binding. The complete mechanism of how this is accomplished needs further investigation.

Dicer knockdown suppressed blastocyst implantation, which was also likely mediated by the reduction in miRNA expression. MiRNA-181a is one of the main up-regulated miRNAs in the activated blastocysts (Liu *et al.*, 2012). Dicer knockdown suppressed the miRNA in these embryos. The physiological role of miRNA-181a in the implantation of the activated blastocysts is not known and can only be speculated upon. One of the potential targets of miRNA-181a is

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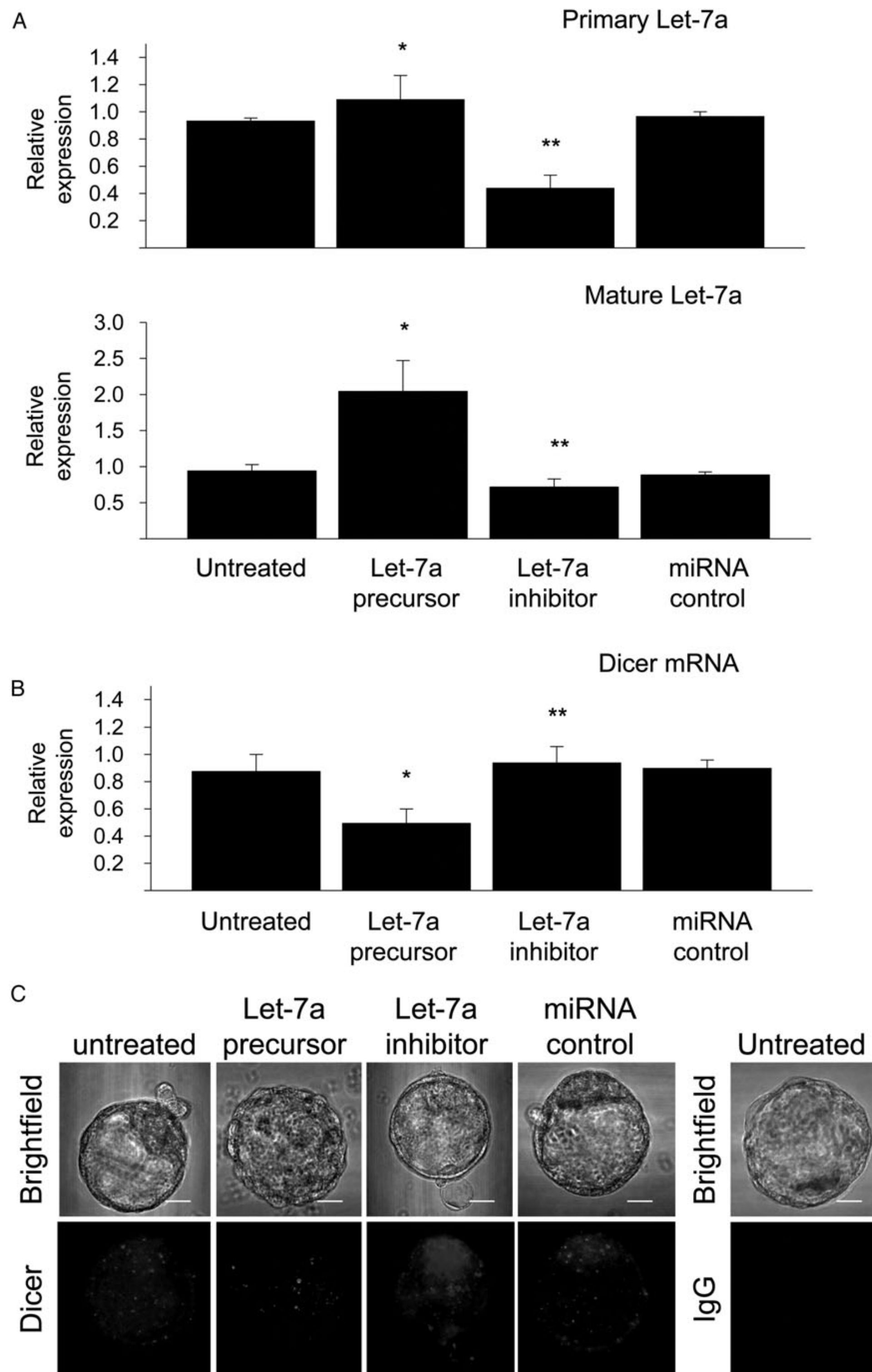

Figure 7 Let-7a modulates dicer transcription in mouse embryos. Mouse 5–8 cell embryos were electroporated with either Let-7a precursor or inhibitor and analyzed for the effect of Let-7a on dicer expression at 48 h post electroporation. (**A**) Forced-expression of Let-7a in mouse blastocysts did not affect the primary Let-7a expression but up-regulated mature Let-7a. Inhibition of Let-7a suppressed both primary and mature Let-7a expression. Let-7a forced-expression decreased dicer (**B**) transcript and (**C**) protein expression, whereas Let-7a inhibition increased dicer (B) transcript and (C) protein expression. MiRNA control transfection had no effect on dicer expression. ($\times$630 magnification, Bar = 30 μm). *-** denotes significant differences between groups ($P < 0.05$).

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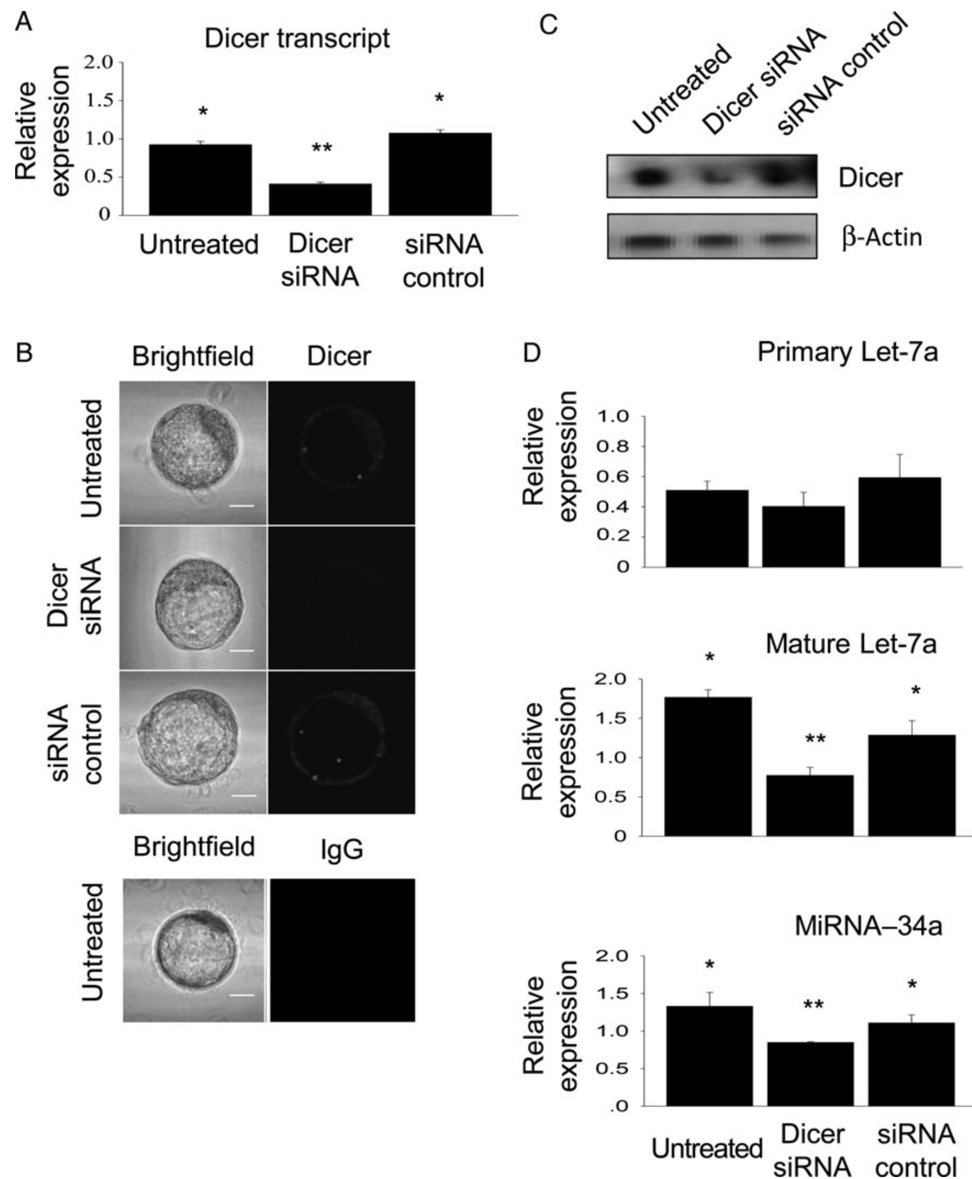

Figure 8 Loss of dicer attenuates blastocyst microRNA processing. Mouse 5–8 cell embryos were electroporated with dicer siRNA or siRNA control and analyzed for the effect of dicer at 48 h post electroporation. Dicer siRNA-mediated knockdown suppressed dicer (**A**) transcript and (**B** and **C**) protein expression. (B) Western blotting showed the lower expression of dicer protein in dicer siRNA treated sample. (C) Immunofluorescence staining showed decreased dicer protein expression in the dicer siRNA treated embryo. (**D**) Inhibiting dicer expression did not affect the primary Let-7a expression but did suppress mature Let-7a and miRNA-34a maturation ($\times 630$ magnification, Bar = 30 μm). *-** denotes significant differences between groups ($P < 0.05$).

olfactomedin-1 (Olfm1) (Targetscan; MicroRNA.org), which is expressed in the human JAr trophoblast cells (Kodithuwakku et al., 2012). Our unpublished data demonstrated that estradiol up-regulated miRNA-181a and suppressed Olfm1 transcripts during the first 12 h post-activation of the dormant blastocysts. In mouse embryos, the Olfm1 transcript was down-regulated during preimplantation embryo development (Zeng et al., 2004). Exogenous Olfm1 attenuates the attachment of trophoblastic spheroids (blastocyst surrogates) onto endometrial cells by interfering Wnt signaling (Kodithuwakku et al., 2012). The up-regulation of miRNA-181a may thus enhance embryo implantation by suppressing Olfm1. However, further investigations are needed to dissect the precise mechanism of how miRNA-181a facilitates embryo implantation.

This study demonstrated the importance of the Let-7a-dicer interaction in the regulation of blastocyst implantation in the delayed implantation mouse model. Specifically, estradiol treatment activated dormant blastocysts by down-regulating Let-7a and up-regulating dicer, which appeared to lead to the increase in EGF binding and production of implantation-related miRNAs such as miRNA-181a. Suppression of dicer expression compromised implantation. Further studies are needed to elucidate how the changes in dicer activity affect the expression of mRNAs and small RNAs, which will enable us to identify the

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Table II Loss of dicer in mouse embryos compromises implantation.

	siRNA control transfected	Dicer siRNA transfected
No. of mice (*n*)	4	4
No. of embryos transferred in each mouse (total)	5, 5, 4, 4 (18)	5, 5, 4, 4 (18)
No. of implantation sites on Day 8 (total)	4, 4, 3, 3 (14)[a]	2, 2, 1, 1 (6)[b]

[a,b]Statistically significant differences ($P < 0.05$) between groups as determined by Fisher's exact test.

factors that diminish or enhance the implantation competency of blastocysts. The expression of Let-7 family members in preimplantation human embryos also decrease from the zygote to the blastocysts as in mice (data not shown). However, the implantation process in mice and humans is different, and the applicability of the present observed phenomena in mice to humans requires further investigations.

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Authors' roles

A.W.Y.C., R.T.K.P., W.-M.L., K.S.A.K., K.-F.L. and W.S.B.Y. were involved in the study design and preparation of the manuscript. A.W.Y.C. carried out the experiments in this study. A.W.Y.C. and R.T.K.P. designed the reporter constructs and performed the dual luciferase assays. A.W.Y.C. analyzed the data, drafted the manuscript and had critical discussions with K.-.F.L. and W.S.B.Y.

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Conflict of interest

None declared.

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