REPRODUCTION

microRNA 376a regulates follicle assembly by targeting *Pcna* in fetal and neonatal mouse ovaries

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Abstract

In mammals, the primordial follicle pool, providing all oocytes available to a female throughout her reproductive life, is established perinatally. Dysregulation of primordial follicle assembly results in female reproductive diseases, such as premature ovarian insufficiency and infertility. Female mice lacking *Dicer1* (*Dicer*), a gene required for biogenesis of microRNAs, show abnormal morphology of follicles and infertility. However, the contribution of individual microRNAs to primordial follicle assembly remains largely unknown. Here, we report that microRNA 376a (miR-376a) regulates primordial follicle assembly by modulating the expression of proliferating cell nuclear antigen (*Pcna*), a gene we previously reported to regulate primordial follicle assembly by regulating oocyte apoptosis in mouse ovaries. miR-376a was shown to be negatively correlated with *Pcna* mRNA expression in fetal and neonatal mouse ovaries and to directly bind to *Pcna* mRNA 3′ untranslated region. Cultured 18.5 days postcoitum mouse ovaries transfected with miR-376a exhibited decreased *Pcna* expression both in protein and mRNA levels. Moreover, miR-376a overexpression significantly increased primordial follicles and reduced apoptosis of oocytes, which was very similar to those in ovaries co-transfected with miR-376a and siRNAs targeting *Pcna*. Taken together, our results demonstrate that miR-376a regulates primordial follicle assembly by modulating the expression of *Pcna*. To our knowledge, this is the first microRNA–target mRNA pair that has been reported to regulate mammalian primordial follicle assembly and further our understanding of the regulation of primordial follicle assembly.

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Introduction

Primordial follicles are composed of oocytes surrounded by several flattened granulosa cells, which are formed in fetal ovaries in humans and during the first few days after birth in rodents (Borum 1961, Baker 1963, Maheshwari & Fowler 2008). Once formed, the primordial follicles serve as a finite source of developing follicles that decrease in size with age. The reproductive aging process of women is dictated by a gradual and steady decrease in the quantity and quality of oocytes held within the follicles. By the time of menopause, the number of primordial follicles falls below 1000 (Faddy *et al.* 1992, Faddy & Gosden 1996, Broekmans *et al.* 2007). Thus, primordial follicle assembly is a critical process for female reproduction by determining the number of available oocytes. Disturbance of this process would lead to ovarian diseases, such as premature ovarian insufficiency and infertility (Sullivan & Castrillon 2011).

Primordial follicle assembly occurs, when oocytes are individually surrounded by squamous pre-granulosa cells (Pepling & Spradling 2001, Pepling 2006). Up to two thirds of the oocytes are lost before or shortly after birth (Pepling & Spradling 2001, Pepling 2006). Apoptosis has been proposed to be the major mechanism underlying oocyte loss (Coucouvanis *et al.* 1993, Pesce & De Felici 1994, De Pol *et al.* 1997, Pepling & Spradling 2001, Lobascio *et al.* 2007, De Felici *et al.* 2008, Rodrigues *et al.* 2009). The deletion of the anti-apoptotic gene *Bcl2* in mice reduces the number of both oocytes and primordial follicles at the sixth week *postpartum* but does not affect the number of primary and preantral follicles (Ratts *et al.* 1995, Flaws *et al.* 2001). On the contrary, targeted disruption of the apoptotic gene *Casp2*

significantly increases the number of primordial follicles at 4 days *postpartum* (dpp; Bergeron *et al*. 1998).

We recently found that proliferating cell nuclear antigen (*Pcna*), a key factor for DNA polymerase δ during DNA replication and repair, affects primordial follicle assembly by regulating oocyte apoptosis (Essers *et al*. 2005, Xu *et al*. 2011*a*). The expression of *Pcna* has been widely studied in ovaries of several arthropods and mammals (Oktay *et al*. 1995, Korfsmeier 2002, Musk-helishvili *et al*. 2005, Hutt *et al*. 2006, Balla *et al*. 2008, Picut *et al*. 2008, Zhang *et al*. 2010). For example, an increased *Pcna* expression during primordial follicle assembly has been observed in rats (Balla *et al*. 2008). The fluctuation of PCNA protein level was also observed during the development of fetal and neonatal mouse ovaries, with expression decreasing from 13.5 to 18.5 days postcoitum (dpc) and increasing from 18.5 dpc to 5 dpp (Xu *et al*. 2011*a*). During primordial follicle assembly, most ovarian cells are in a quiescent state with oocytes arrested in diplotene stage and pre-granulosa cells arrested at the G0 stage (Robker & Richards 1998). An earlier report showed that in quiescent cells, the level of *Pcna* is largely posttranscriptionally regulated (Chang *et al*. 1990). So this variation of *Pcna* expression we observed during ovarian primordial follicle assembly suggested that the level of this protein is highly regulated posttranscriptionally. microRNAs provide one possible posttranscriptional regulatory mechanism.

Over the past few years, microRNAs, small (∼22 nucleotides in length) non-coding RNA molecules, have emerged as a class of negatively regulatory factors that mainly regulate the turnover or translational efficiency of target mRNAs through base-pairing with their 3′ untranslated region (UTR) (Bartel 2009, Huntzinger & Izaurralde 2011). The small size of microRNAs, combined with their target recognition, provides microRNAs with the capacity and versatility to act as global gene regulators in a wide array of biological processes including cell proliferation and apoptosis (Bartel 2009, Krol *et al*. 2010, Huntzinger & Izaurralde 2011). Many microRNAs are present in ovaries around the time of primordial follicle assembly in mammals (Choi *et al*. 2007, Ahn *et al*. 2010, Tripurani *et al*. 2010, Huang *et al*. 2011, Torley *et al*. 2011, Zhang *et al*. 2011, 2013). Female mice lacking *Dicer1* (*Dicer*), a gene required for the biogenesis of vast majority of microRNAs, show abnormal follicle morphology (Lei *et al*. 2010, Baley & Li 2012). Thus, microRNAs probably play important roles during primordial follicle assembly. However, the contribution of individual microRNAs to primordial follicle assembly is largely unknown.

In this study, we found that miR-376a level was negatively correlated with that of *Pcna* mRNA in fetal and neonatal mouse ovaries. miR-376a has been demonstrated to directly bind to the 3′ UTR of *Pcna* mRNA. Transfection of miR-376a mimics into cultured ovaries at 18.5 dpc increased primordial follicle assembly by decreasing oocyte apoptosis via suppressing *Pcna* mRNA and protein expression. Our results indicate that miR-376a is a novel regulator of primordial follicle assembly that regulates oocyte apoptosis by modulating the expression of *Pcna*.

Materials and methods

Animals

ICR mice were purchased from the National Rodent Laboratory Animal Center (Shanghai Branch, China) and housed under controlled photoperiod conditions (lights on 0800–2000 h) and were supplied with food and tap water *ad libitum*. All animal studies were conducted in accordance with the Institutional Animal Care and Use Committee of University of Science and Technology of China. Fetal or neonatal mouse ovaries were obtained from the pregnant mice between 13.5 dpc and 5 dpp. The presence of a copulation plug in the noon after mating was designated as 0.5 dpc.

RNAi on cultured fetal mouse ovaries

The ovaries from 18.5 dpc mice were cultured as described (Shen *et al*. 2007). Fetal ovary RNAi was initiated as described (Wang & Roy 2006). Briefly, cultured ovaries were transfected with 200 nM siRNAs (Invitrogen) using Metafectene (Biontex, T020-1.0, Munich, Germany) following the supplier's instructions. Half of the medium was changed at the 48th hour after RNAi initiation with complete medium without siRNAs.

The sequence of *Pcna* siRNAs is 5′-GGCATTGCTAGAAATT-GAGAA-3′, targeting 930–950 nt of *Pcna* mRNA (Paddison *et al*. 2004), and the non-targeting control siRNA sequence is 5′-TTCTCCGAACGTGTCACG-3′ which has no homology to any known mouse mRNAs. All data were obtained from didymous ovaries (for two ovaries of a mouse, one was transfected with target siRNAs and the other with non-targeting control siRNAs).

Transfection of microRNA mimics

microRNA mimics are chemically synthesized, double-stranded RNAs that mimic mature endogenous microRNAs after transfection into cells. For the microRNA overexpression *in vitro*, cultured ovaries were transfected with microRNA mimics (200 nM, Genepharma, Shanghai, China). These microRNA mimics were delivered using Metafectene (Biontex) according to the manufacturer's protocol. Forty-eight hours after transfection, the medium was changed with replacement of half of the complete medium. Ovaries were harvested 96 h after transfection. All data were obtained from didymous ovaries (one ovary was transfected with microRNA mimics and the other with non-targeting siRNAs, which are random double-stranded sequence molecules that can mimic micro-RNAs and were validated not to produce identifiable effects on known microRNA function, as control).

Immunofluorescence and western blot assay

Immunohistochemistry and western blot assays were performed as described previously (Xu *et al*. 2011*a*).

Construction of luciferase reporter vectors

The sequence encoding the mouse *Pcna* mRNA 3′ UTR that contains the putative miR-376a binding site was amplified by PCR using the following two primers: forward primer 5′-GCTCTCTCGAGGCATTGCTAGAAATTGAGAAAACT-3′ and reverse primer 5′-GTCGAGCGGCCGCTGACTTTTCAAATTGT-TAACTTTA-3′. These two primers contain XhoI or NotI recognition sites at their 5′ end. The sequence encoding the mutant *Pcna* mRNA 3′ UTR that lacks the putative miR-376a binding ability was synthesized by PCR according to the published method (Higuchi *et al.* 1988). For mutation of the putative binding site (243–263 bp from the start of the 3′ UTR), the following pair of primers was employed in addition to the pair of primers used for amplification of WT 3′ UTR: 5′-GCTTTACAAATACTGGTGATTTTCATCATGCTTCTTGACG-3′ and 5′-CGTCAAGAAGCATGATGAAAATCACCAGTATTTG-TAAAGC-3′. These PCR products were digested with XhoI and NotI and cloned into the psiCHECK-2 luciferase reporter vector (Promega) at the corresponding restriction sites. The sequences of inserted fragments were verified by sequencing.

Luciferase assay

HEK 293T cell was transfected using Lipofectamine 2000 (Invitrogen, 11668) according to the manufacturer's instructions. In short, 10^5 cells were plated in a 24-well tissue culture plate the day before transfection. The luciferase reporter constructs (100 ng), together with miR-376a mimics (50 nM) (GenePharma) were incubated with 1.5 µl Lipofectamine 2000 and transfected into cells. After 6 h, the transfection solution was replaced with fresh medium. Cell lysates were produced 24 h after transfection and assayed using the Dual-Luciferase Reporter Assay System (Promega, E1910) following the manufacturer's instructions. The *Renilla* luciferase activity was normalized to that of the firefly luciferase.

Cell proliferation and apoptosis analysis

To assess cell proliferation, transfected mouse ovaries were cultured in medium with 100 nM bromodeoxyuridine (BrdU) (Sigma, B9285) added 24 h before ovary harvesting. The ovaries were fixed and embedded in paraffin and sectioned. BrdU-labeled cells in the sections were detected immunohistochemically using a mouse anti-BrdU MAB (NeoMarkers, MS-1058-P0, Fremont, CA, USA; 1:200) and imaged using a BX61 Olympus fluorescence microscope. Apoptotic cells were detected in sections by TUNEL assay according to the manufacturer's specifications. Ovarian sections were imaged and TUNEL assay-positive cells were counted manually using Image-Pro plus Software (Media Cybernetics, Rockville, MD, USA).

Real-time PCR for mRNAs

RNA isolation, RT-PCR, and real-time PCR were performed as described previously (Xu *et al.* 2011*a*). All PCR primers used were listed in Supplementary Table 1, see section on supplementary data given at the end of this article. For real-time PCR analyses, *C*T values of samples were normalized to the corresponding *C*T values of *Gapdh*. Quantification of the fold change in gene expression was determined by the comparative *C*T method.

Real-time PCR for microRNAs

For quantitative PCR analysis of microRNAs, pooled tissues or transfected ovaries were collected and total RNAs were extracted using TRIzol reagents (Invitrogen). For detecting and quantifying the expression of specific microRNAs, 1 µg RNA was reverse transcribed using RT Kit (ABI, 4366596, Carlsbad, CA, USA) with preformulated primers (ABI). Probe sets (ABI) were designed to perform real-time PCR amplification with products from RT reaction using TaqMan Universal PCR Master Mix (ABI, 4324018). For real-time PCR analyses, *C*T values of samples were normalized to the corresponding *C*T values of U6. Quantification of the fold change in microRNA expression was determined by the comparative *C*T method.

Locked nucleic acid *in situ* hybridization

Locked nucleic acid *in situ* hybridization (LNA–ISH) was performed on paraffin sections using LNA probes for miR-376a (Exiqon, Woburn, MA, USA). Ovaries were fixed in 10% formaldehyde for 20 min before paraffin embedding and 4 µm-thick paraffin sections were attached to microscope slides. Paraffin sections were incubated at 60 °C for 1 h. Following de-paraffinization in xylene, sections were re-hydrated in a series of graded ethanol/water solutions. After incubation in acetylation solution (acetic anhydride (Sigma, 320102; 0.25%) and triethanolamine (Sigma, T1377; 1.33%)) for 15 min, the slides were digested in proteinase K (Promega, V3021; 1:500) for 20 min and subsequently washed in PBS. Following rinse in PBS, the slides were de-hydrated in a series of graded ethanol/water solutions and incubated with hybridization buffer containing the digoxigenin-labeled LNA probe in an oven at 56 °C overnight. After washed with 5× SSC (saline-sodium citrate buffer) twice, 1× SSC twice, and 0.2× SSC twice, 15 min each at 55 °C, the slides were blocked with blocking buffer for 20 min and incubated with anti-digoxigenin Fab fragment (Roche, 11214667001; 1:500) overnight at 4 °C in a humid chamber. The visualization was performed using BCIP/NBT Liquid Substrate System (Sigma, B1911) in the dark. After stringent washes, the slides were mounted with coverslips using vectashield mounting medium.

Morphometric evaluation of oogenesis and folliculogenesis

To identify oocytes and follicles, ovarian sections were immunohistochemically labeled with an antibody-recognized MVH, a protein specifically expressed in germ cells (Fujiwara *et al.* 1994) and Hoechst 33342 for nuclei of ovarian cells. The sections were analyzed using an Olympus fluorescence microscope (BX61). All cells labeled by MVH antibodies (oocytes) were counted. Based on their localization, oocytes were designated as in primordial or primary follicles according to the previous reports (Meredith *et al.* 2000, Pepling & Spradling 2001).

Statistical analysis

For each ovary, oocytes or follicles were counted as previously reported (Chen *et al*. 2007, Nilsson *et al*. 2007, Reddy *et al*. 2008, Xu *et al*. 2011*a*, Kim *et al*. 2013). For real-time PCR and western blot analysis, two or three cultured ovaries per group were pooled to form one sample and repeated at least three times for statistical analysis. Student's *t*-test was used to compare means between two independent samples in real-time PCR analysis. In other statistical analyses, Tukey's multiple comparison test was used to compare means among multiple groups.

Results

miR-376a is negatively correlated with Pcna mRNA level in mouse ovaries in vivo during follicle assembly and can bind to the 3′ UTR of Pcna mRNA directly

To find microRNAs that may regulate mouse *Pcna* expression, the local package of miRanda v3.3a was used to predict the microRNAs that may target *Pcna* mRNA based on its 3′ UTR (Betel *et al*. 2008). The parameters used for the prediction process were listed in Supplementary Table 2, see section on supplementary data given at the end of this article. According to miRanda results, *Pcna* was potential target of 163 microRNAs (Supplementary Table 3). Among these microRNAs, 37 microRNAs were detected by our microarray chip assay in mouse ovaries during follicle assembly, and when 1.5-fold change in microRNA abundance was considered, only three microRNAs remained, of which only one microRNA, miR-376a, exhibited a negative correlation with *Pcna* mRNA level (H Zhang, X Jiang, Y Zhang, B Xu, J Hua, W Zheng and R Sun, unpublished data). To confirm the relationship between miR-376a and *Pcna* mRNA expression, the level of miR-376a and *Pcna* mRNA was measured in fetal and neonatal mouse ovaries by real-time PCR (Fig. 1A). miR-376a levels slightly increased from 13.5 to 16.5 dpc, then decreased, and reached a minimum in 3 dpp ovaries and increased again thereafter. miR-376a was also found to express very differently in various tissues, with much higher expression in ovary and testis of newborn and brain of adult mice than in other tissues (Fig. 1B). Furthermore, microRNA ISH experiments demonstrated that miR-376a was localized in cytoplasm of some oocytes in cysts at 16.5 dpc. At 1 dpp, miR-376a was mainly localized in cytoplasm of oocytes and granulosa cells in primordial follicles (Fig. 1C). This expression pattern in the developing ovaries and tissue specificity are consistent with a regulatory role of miR-376a in fetal and neonatal mouse ovaries.

To determine whether miR-376a can directly bind to the 3′ UTR of *Pcna* mRNA, a dual-luciferase reporter system was used as previously reported (Grentzmann *et al*. 1998). We constructed dual-luciferase reporter vectors by inserting the DNA sequence encoding the WT or mutant (which cannot bind miR-376a) 3′ UTR of *Pcna*

mRNA downstream the Renilla luciferase gene (Fig. 1D) and transfected them with miR-376a mimics into 293T cells respectively. A significant decrease in relative luciferase activity was observed in cells co-transfected with the WT constructs and miR-376a mimics, but not in cells co-transfected with the mutant constructs and miR-376a mimics (Fig. 1E). These results demonstrate that miR-376a can directly bind to the 3′ UTR of mouse *Pcna* mRNA.

miR-376a negatively regulates Pcna expression in cultured mouse ovaries

To determine whether miR-376a can regulate *Pcna* expression in cultured ovaries, we transfected miR-376a mimics into cultured 18.5 dpc mouse ovaries and measured *Pcna* mRNA and protein levels 96 h after transfection. An ∼20-fold increase in miR-376a level was observed in ovaries transfected with miR-376a mimics for 96 h, compared with those in control ovaries (Fig. 2A). This indicates a high efficiency of microRNA transfection in our fetal mouse ovary culture system. Of note, PCNA protein level as well as mRNA level was greatly decreased in ovaries transfected with miR-376a mimics, *Pcna* siRNAs or both together compared with controls (Fig. 2B, C and D). These results indicate that miR-376a is able to downregulate *Pcna* expression in cultured mouse ovaries.

miR-376a increases primordial follicle assembly by promoting oocyte survival around primordial follicle formation via downregulating Pcna expression

The effects of miR-376a on primordial follicle assembly in cultured ovaries were determined in cultured ovaries 96 h after transfection of miR-376a mimics or controls at 18.5 dpc (Fig. 3A). miR-376a mimic-transfected ovaries exhibited more primordial follicles than the controls; similar results were observed in ovaries transfected with *Pcna* siRNAs (Fig. 3A and B and Supplementary Fig. 1, see section on supplementary data given at the end of this article). To be noted, in the control group, there were fewer primordial follicles, in which oocytes were larger than those in miR-376a mimic-transfected ovaries, as reported in *Pcna* siRNA transfected ovaries (Xu *et al*. 2011*a*). We prolonged the culture to 6 days and found that there were still more primordial follicles in miR-376a mimic-transfected ovaries; however, the difference in the developing follicle index (the ratio of developing follicles:total follicles) between experimental and control ovaries decreased (Supplementary Fig. 2). We also employed miR-376a inhibitors in cultured ovaries; however, they had no obvious effect on primordial follicle assembly (Supplementary Fig. 3). To show whether the increase in primordial follicles in miR-376a mimic-transfected ovaries is caused by downregulation of *Pcna* expression, we co-transfected miR-376a mimics and *Pcna* siRNAs into 18.5 dpc ovaries and analyzed the
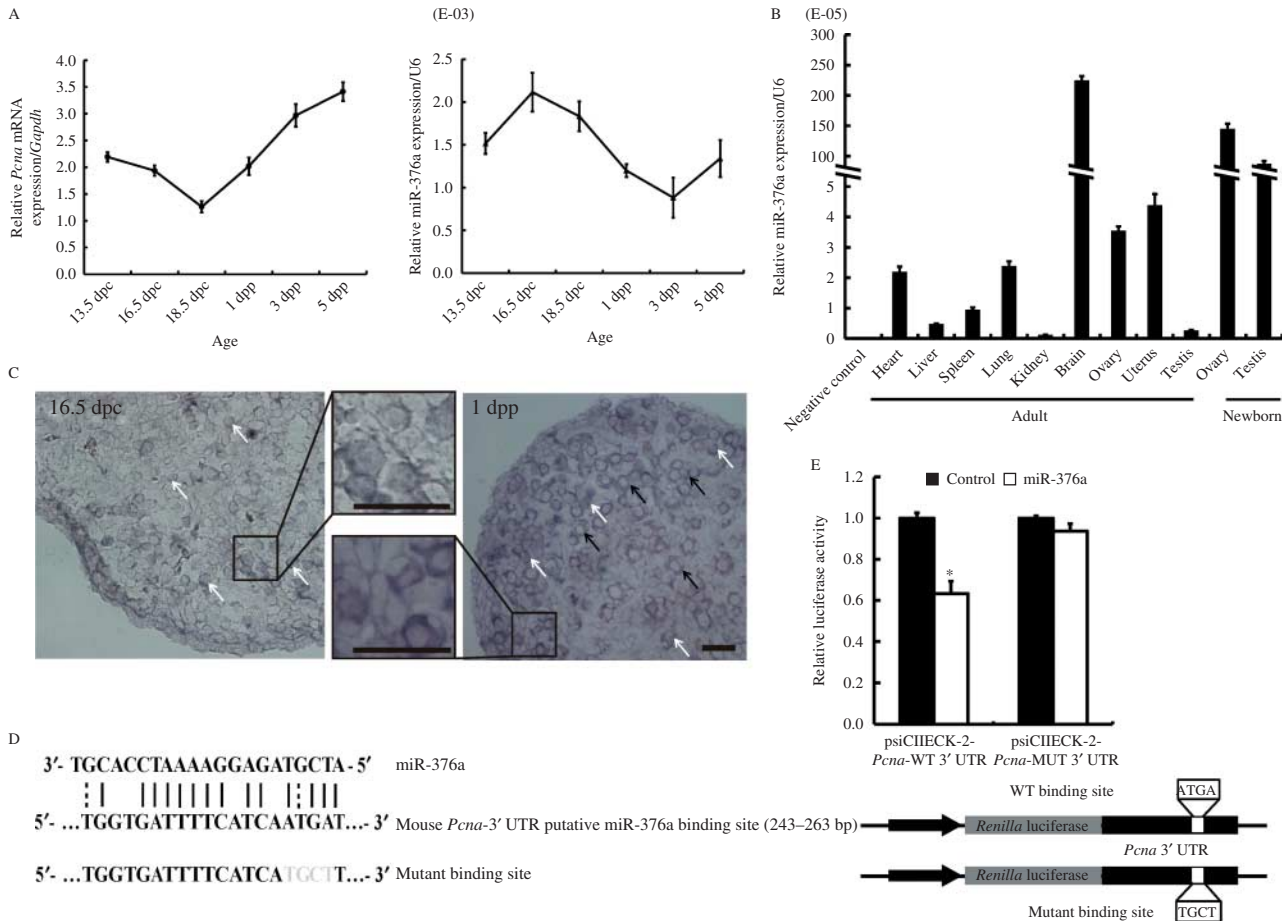
Figure 1 Expression and localization of miR-376a in fetal/neonatal ovaries and the direct binding of miR-376a to *Pcna* mRNA 3′ UTR. (A) miR-376a and *Pcna* mRNA levels were detected by real-time PCR in fetal and neonatal mouse ovaries respectively. U6 and *Gapdh* were used as internal control respectively. Data are presented as mean±s.e.m. (*n*=3). (B) miR-376a expression in various mouse organs, detected by real-time PCR, normalized to U6 expression. Negative control, no template control. Data are presented as mean±s.e.m. (*n*=3). (C) Expression and localization of miR-376a in fetal/neonatal mouse ovaries detected by *in situ* hybridization. White arrows, miR-376a-positive oocytes. Black arrows, miR-376a-positive somatic cells. Bar: 25 μm. (D) Mature miR-376a sequence and its putative binding site in the 3′ UTR of mouse *Pcna* mRNA. Dual-luciferase reporter constructs contain a DNA sequence encoding WT or mutant *Pcna* mRNA 3′ UTR, which is or is not able to bind miR-376a respectively. (E) Relative luciferase activity is measured 24 h after co-transfection of each of the constructs together with miR-376a mimics (50 nM) or controls in 293T cells. Data are presented as mean±s.e.m. (*n*=3). Differences between groups were considered significant when the Student's *t*-test gave a *P* value <0.05 (*).

primordial follicles 96 h after transfection. Indeed, results observed in ovaries after co-transfection were very similar to those in ovaries transfected with either miR-376a mimics or *Pcna* siRNAs alone (Fig. 3A and B and Supplementary Fig. 1). These results together with those mentioned earlier indicate that both miR-376a and *Pcna* siRNAs are functioning in the same pathway to regulate primordial follicle formation.

Increased primordial follicle number in neonatal mouse ovaries has been attributed to the increased number of surviving oocytes during follicle formation (Meredith *et al.* 2000). To understand whether the increased primordial follicles in miR-376a-transfected ovaries result from more surviving oocytes, we counted the oocytes in cultured ovaries every 24 h after miR-376a mimics transfection in 18.5 dpc ovaries (Fig. 3C).

Ovaries transfected with miR-376a mimics exhibited more oocytes at 72 and 96 h after transfection than the control ovaries (Fig. 3D). Similar results were observed in ovaries transfected with *Pcna* siRNAs, and importantly in those co-transfected with *Pcna* siRNAs and miR-376a mimics (Fig. 3D). These results indicate that transfection of miR-376a in mouse ovaries around the time of primordial follicle assembly causes the increase of surviving oocytes by decreasing the *Pcna* expression.

miR-376a overexpression reduces oocyte apoptosis in fetal mouse ovaries around primordial follicle assembly by suppression of Pcna expression

It has been established that *Pcna* promotes apoptosis of oocytes during primordial follicle assembly (Xu *et al.* 2011a).
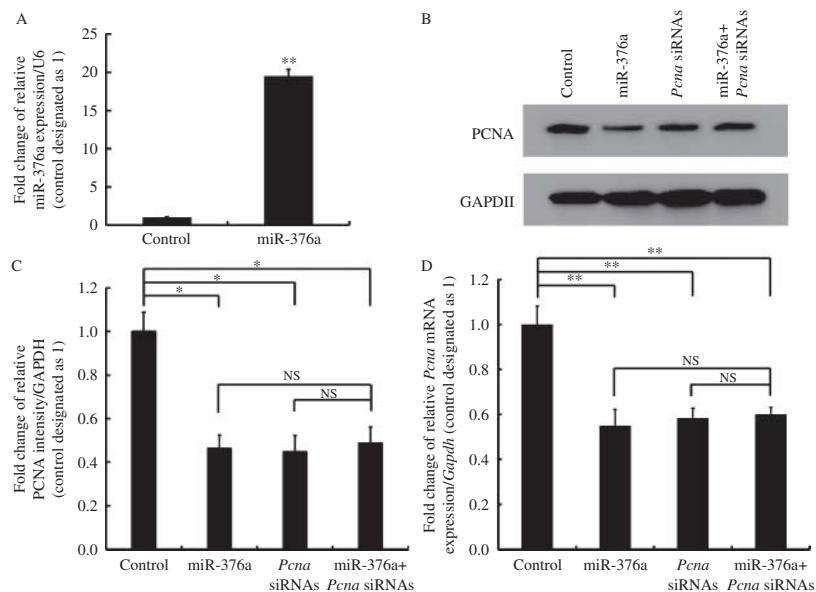
Figure 2 miR-376a overexpression downregulates *Pcna* expression in fetal mouse ovaries. (A) The overexpression efficiency of miR-376a in cultured fetal mouse ovaries was determined by real-time PCR 96 h after transfection. The expression of miR-376a was normalized to U6. The relative miR-376a expression in control ovaries was designated as 1. Data are presented as mean±s.e.m. (*n*=3). Differences between groups were considered highly significant when the Student's *t*-test gave a *P* value <0.01 (**). (B) PCNA protein levels in fetal mouse ovaries transfected with miR-376a mimics, *Pcna* siRNAs, both together or non-targeting microRNA mimics (Control) for 96 h were measured by western blot assays. GAPDH were used as an internal control. (C) Relative intensity of PCNA to GAPDH of the western blot bands. The data of control ovaries was designated as 1. Data are presented as mean±s.e.m. (*n*=3–9). Differences among groups were considered significant when the Tukey's multiple comparison test gave a *P* value <0.05 (*). (D) Relative *Pcna* mRNA expression levels in fetal mouse ovaries transfected with miR-376a mimics, *Pcna* siRNAs, both together or non-targeting microRNA mimics (control) for 96 h were detected by real-time PCR. The relative *Pcna* mRNA expression in control ovaries was designated as 1. Data are presented as mean±s.e.m. (*n*=3–9). Differences among groups were considered significant when the Tukey's multiple comparison test gave a *P* value <0.05. **P*<0.05 and ***P*<0.01.

To investigate whether the increased oocytes in ovaries caused by miR-376a transfection result from the decreased oocyte apoptosis mediated by downregulation of *Pcna* expression, TUNEL assays were performed on ovaries transfected with miR-376a mimics. The results showed that miR-376a mimics transfection apparently inhibited oocyte apoptosis (Fig. 4A) and statistical analysis revealed that the number of apoptotic oocytes in ovaries transfected with miR-376a mimics was significantly lower than in the controls (Fig. 4B). Additionally, we measured the expression levels of some genes that related to apoptosis and oocyte survival. mRNA levels significantly decreased for pro-apoptotic genes such as *Bax*, *Tnf*, and *Tnfrsf1b* (*Tnfr2*), increased for anti-apoptotic gene *Bcl2* and oocyte survival genes *Pard6a* (*Par6*), *Lhx8* in ovaries transfected with miR-376a, compared with the controls (Fig. 4C). Altogether, these results indicate that miR-376a is able to reduce oocyte apoptosis in fetal mouse ovaries during primordial follicle assembly.

A similar decrease in the number of apoptotic oocytes was observed in ovaries transfected with *Pcna* siRNAs, co-transfection of *Pcna* siRNAs and miR-376a mimics did not further decrease the number of apoptotic oocytes (Fig. 4A and B). This indicates that transfection of miR-376a decreases oocyte apoptosis by suppression of *Pcna* expression.

Overexpression of miR-376a slightly decreases somatic cell proliferation in mouse ovaries around primordial follicle assembly mediated by repression of Pcna expression

To determine whether miR-376a transfection induces a slight decrease in somatic cell proliferation as does *Pcna* siRNA transfection (Xu *et al.* 2011*a*), we measured cell proliferation in miR-376a mimic-transfected ovaries using BrdU incorporation as a marker of cell proliferation (Johnson *et al.* 2004). We counted the number of BrdU-positive somatic cells in ovaries every 24 h after transfection initiation (Fig. 5A and B). During the first 72 h when primordial follicles were formed actively, miR-376a mimic-transfected ovaries showed a slight but not significant decrease in the number of BrdU-positive somatic cells, when compared with the controls. From 72 to 96 h after transfection, the number of BrdU-positive cells increased apparently when compared with that in the first 72 h in miR-376a mimic-transfected ovaries. The BrdU-positive cells mainly localized in the area where the primordial follicle assembly was not active (Fig. 5A). A similar increase in somatic cell proliferation was observed in ovaries transfected with *Pcna* siRNAs, and co-transfection of *Pcna* siRNAs and miR-376a mimics did not further the influence on
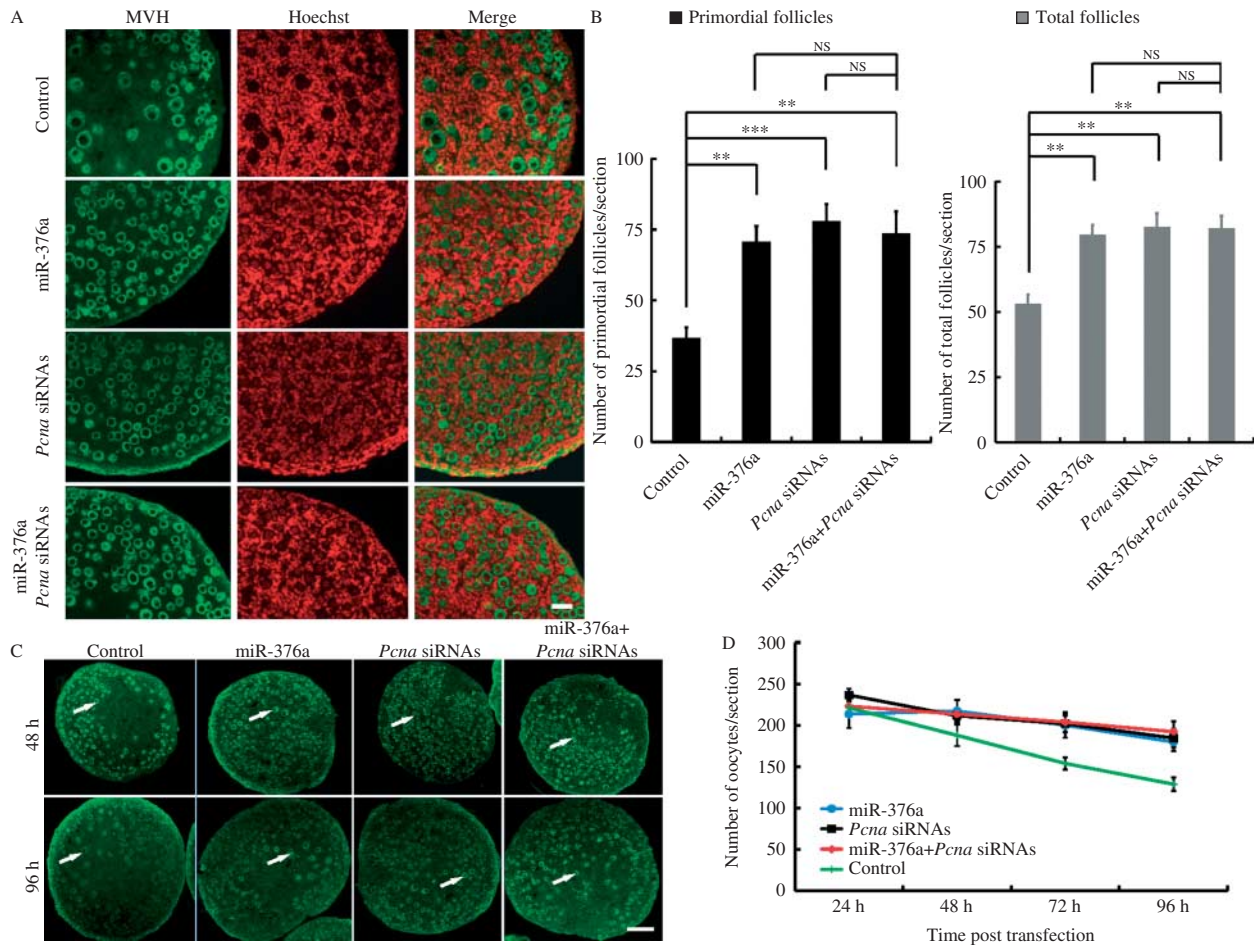
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Figure 3 miR-376a promotes primordial follicle assembly and oocyte survival by downregulating *Pcna* expression. (A) Follicles were detected immunohistochemically in sections of mouse ovaries 96 h after transfection with miR-376a mimics, *Pcna* siRNAs or both together. Ovaries transfected with non-targeting siRNAs are used as controls. MVH is a protein specifically expressed in germ cell cytoplasm. Bar: 25 μm. (B) Quantification of primordial and total follicles in the transfected and control ovaries. Each bar represents a mean ±s.e.m. of results from 16 ovaries from different animals of five independent experiments. Differences among groups were considered significant when the Tukey's multiple comparison test gave a *P* value <0.05. **P<0.01, and ***P<0.001. (C) Oocytes (arrow) were immunohistochemically detected in sections of ovaries every 24 h after transfection of 18.5 dpc fetal mouse ovaries with miR-376a mimics, *Pcna* siRNAs, or both together. Ovaries transfected with non-targeting siRNAs are used as the control. Oocytes are labeled with MVH antibodies (green). Bar: 100 μm. (D) Quantification of oocytes in the ovaries every 24 h after transfection of 18.5 dpc fetal mouse ovaries with miR-376a mimics, *Pcna* siRNAs, both together, or non-targeting siRNAs (control). Data are presented as mean ±s.e.m. (*n*=5).

somatic cell proliferation (Fig. 5A and B). Taken together, overexpression of miR-376a during primordial follicle assembly slightly decreases somatic cell proliferation that is not involved in primordial follicle formation.

Discussion

Previous reports have shown that female reproductive system-specific *Dicer1* conditional knockout or *Dicer1* general hypomorphic mutated mice are infertile due to the interruption of overall microRNA production (Murchison *et al.* 2007, Otsuka *et al.* 2008, Lei *et al.* 2010). Most recently, microRNA expression profiles have been established in ovaries in human (Zhang *et al.* 2011), mice (Choi *et al.* 2007, Ro *et al.* 2007,

Ahn *et al.* 2010, Zhang *et al.* 2013), and cattle (Tripurani *et al.* 2010, Huang *et al.* 2011) around the time of primordial follicle assembly. All these studies suggested important roles of microRNAs in the developing ovaries. However, little is known about the exact function of individual microRNAs and their targets in primordial follicle formation. Here, we found that during primordial follicle assembly, miR-376a can repress the expression of *Pcna* through directly binding to *Pcna* mRNA 3′ UTR. Overexpression of miR-376a results in more oocytes and primordial follicles in cultured mouse ovaries around primordial follicle assembly by reducing oocyte apoptosis through down-regulation of *Pcna* expression. This study demonstrates that miR-376a is a novel regulator of primordial follicle assembly in mouse.
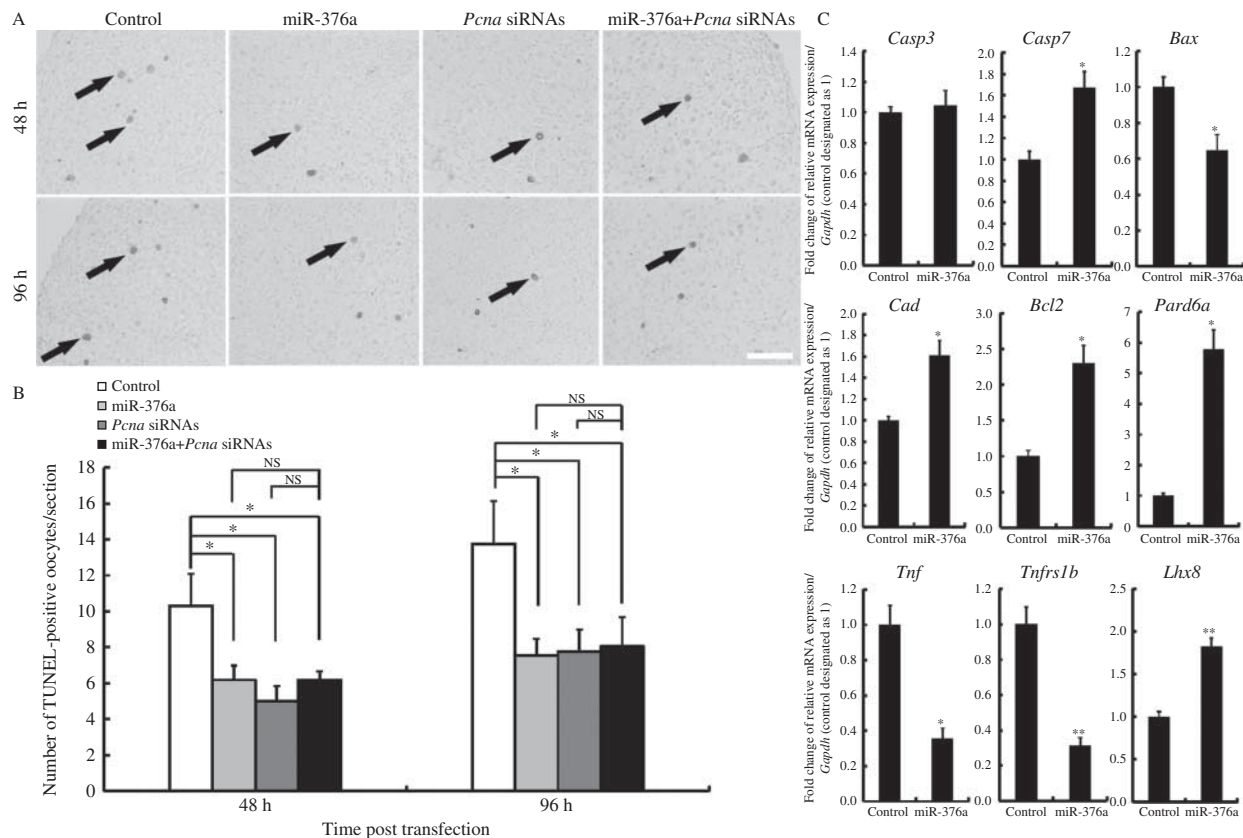
Figure 4 miR-376a alleviates the apoptosis of oocytes in fetal mouse ovaries by downregulating *Pcna* expression. (A) Apoptotic oocytes (arrow) were detected using TUNEL assay 48 and 96 h after transfection of 18.5 dpc fetal mouse ovaries with miR-376a mimics, *Pcna* siRNAs, or both together. Ovaries transfected with non-targeting siRNAs are used as controls. Bar: 100 μm. (B) Quantification of TUNEL-positive oocytes in ovaries transfected with miR-376a mimics, *Pcna* siRNAs, both together, or non-targeting siRNAs (control). Data are presented as mean±s.e.m. (*n*=3–9). Differences among groups were considered significant when the Tukey's multiple comparison test gave a *P* value <0.05 (*). (C) The mRNA expression of apoptosis- and oocytes' survival-related genes in 18.5 dpc fetal mouse ovaries transfected with miR-376a mimics or controls for 96 h was analyzed by real-time PCR. The relative expression was normalized to *Gapdh*. The relative mRNA expression levels in control ovaries were designated as 1. Data are presented as mean±s.e.m. (*n*=3). Differences between groups were considered significant or highly significant when the Student's *t*-test gave a *P* value <0.05 (*) or *P* value <0.01 (**).

The distinct expression profile of *Pcna* during the ovary development was found in fetal and newborn rats, with PCNA-positive oocytes observed decreasing after birth and increasing when primordial follicles start to assembly (Balla *et al*. 2008). In mice, during early ovary development, a fluctuant expression pattern was observed, i.e. decreasing expression from 13.5 dpc to 18.5 dpc and increasing expression from 18.5 dpc to 5 dpp (Xu *et al*. 2011*a*). The variable *Pcna* expression before and during primordial follicle assembly has also been detected by others in mice and rats (Muskhelishvili *et al*. 2005, Kerr *et al*. 2006). All these results indicate that the temporal expression of *Pcna* during primordial follicle assembly is tightly regulated. microRNAs have been shown to function as negative regulators of many genes during ovary development (Carletti *et al*. 2010, Xu *et al*. 2011*b*). The regulation of *Pcna* expression by microRNAs has been mentioned in several studies (Han *et al*. 2010, Sirotkin *et al*. 2010, Raschzok *et al*. 2011). For example, downregulation of *Dicer1* was associated

with enhanced expression of *Pcna* in human cancer cell lines (Han *et al*. 2010), and a total of 53 microRNAs were shown to most likely repress the expression of *Pcna* in cultured human granulosa cells (Sirotkin *et al*. 2010). However, these studies did not determine whether the *Pcna* expression is directly regulated by microRNAs because *Pcna* expression was always used as a cell proliferation marker in these reports and changes in its expression level might also be the accompanying effect of microRNAs on cell proliferation.

According to miRanda prediction (Betel *et al*. 2008), there is a putative binding site of miR-376a in *Pcna* 3′ UTR (Fig. 1D). Dual-luciferase activity assay confirmed that miR-376a could directly bind to *Pcna* 3′ UTR (Fig. 1E). We also found that over-expression of miR-376a in cultured 18.5 dpc ovaries could down-regulate PCNA protein and mRNA levels (Fig. 2B, C and D). The decrease in PCNA protein abundance in ovaries transfected with miR-376a may be a consequence of reducing *Pcna* mRNA abundance or a combination
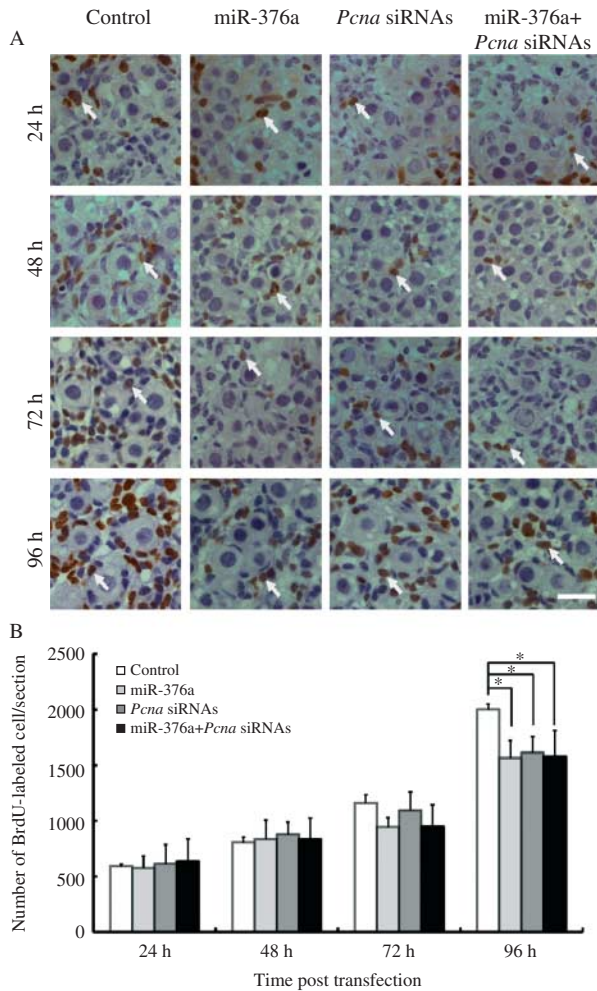
Figure 5 miR-376a slightly decreases somatic cell proliferation in fetal mouse ovaries by downregulating *Pcna* expression. (A) Proliferation of somatic cells were determined by BrdU-labeling assay in cultured ovaries every 24 h after transfection with miR-376a mimics, *Pcna* siRNAs, both together, or non-targeting siRNAs (control). Arrows, BrdU-positive somatic cells. Bar: 25 μm. (B) Quantification of BrdU-positive cells in ovaries transfected with miR-376a mimics, *Pcna* siRNAs, both together, or non-targeting siRNAs (control). Data are presented as mean±s.e.m. (n=3–9). Differences among groups were considered significant when the Tukey's multiple comparison test gave a *P* value <0.05 (*).

result of reduced mRNA abundance and directly suppressed translation. Several hypotheses have been proposed to delineate how microRNAs suppress gene expression (Omer *et al*. 2009, Huntzinger & Izaurralde 2011). In mammalian cells, it has been reported that the reduction in message RNA abundance results from accelerated deadenylation, which leads to rapid mRNA decay (Wu *et al*. 2006, 2010, Omer *et al*. 2009, Ricci *et al*. 2011). More recently, the direct repression of *Aplp2* mRNA's translation by miR-153 has also been showed (Liang *et al*. 2011). Although the mechanism underlying the decrease in PCNA protein abundance caused by miR-376a remained unknown, our results indicated that

miR-376a is able to suppress *Pcna* expression by directly binding to the 3′ UTR of its mRNAs.

The role of microRNAs during primordial follicle assembly is poorly understood and previous studies on microRNAs in the developing ovaries were largely limited to assessing their expression profiles. For example, a total of 398 known microRNAs and 30 novel microRNAs were found to be expressed in the newborn mouse ovary; however, the exact role of any of them in follicular development is unknown (Ahn *et al*. 2010). Here, we found that mouse fetal ovaries transfected with miR-376a exhibited more primordial follicles than the controls (Fig. 3B) and similar results were observed in ovaries transfected with *Pcna* siRNAs (Fig. 3A and B). Furthermore, co-transfection of miR-376a mimics and *Pcna* siRNAs did not further increase primordial follicles in ovaries (Fig. 3A and B). These results strongly indicate that miR-376a and *Pcna* siRNAs function in the same pathway to regulate primordial follicle formation. However, the transfection of miR-376a inhibitors had no obvious effect on primordial follicle assembly (Supplementary Fig. 3). This could be related to the low background concentration of miR-376a in the ovary. Therefore, the present observations demonstrate that miR-376a regulates primordial follicle assembly through regulating *Pcna* expression. This is the first microRNA–target mRNA pairs that clearly demonstrated to regulate mammalian primordial follicle assembly.

In female mammals, germ cell loss is a crucial event during primordial follicle assembly. In fetal and neonatal mouse ovaries, approximately two thirds of oocytes underwent cell death and only the surviving ones developed into primordial follicles (Pepling & Spradling 2001, Pepling 2006). Apoptosis has been proposed to be the major mechanism responsible for oocyte loss. Several genes, e.g. the *Bcl2* family genes (*Bcl2* and *Bax*) and Caspases (*Casp2* and *Casp4*) have been reported to be involved in the regulation of oocyte apoptosis (Ratts *et al*. 1995, Bergeron *et al*. 1998, Rucker *et al*. 2000, Flaws *et al*. 2001, Morita *et al*. 2001). Our previous studies have also demonstrated that *Pcna* contribute actively to oocyte loss by regulating apoptosis during primordial follicle assembly in mouse ovaries (Xu *et al*. 2011a). The expression of miR-376a in mouse ovaries, decreasing from 16.5 dpc to 3 dpp (Fig. 1A) indicates that this microRNA may be involved in oocyte loss through targeting *Pcna* during primordial follicle assembly. Moreover, microRNA ISH experiments demonstrated that miR-376a was localized in cytoplasm of some oocytes in cysts in 16.5 dpc ovaries in which primordial follicle formation has not occurred yet and in cytoplasm of oocytes and some granulosa cells of primordial follicles in 1 dpp ovaries (Fig. 1C). This different localization of miR-376a before and after primordial follicle assembly indicated that only the miR-376a–*Pcna* pairs present in oocytes is involved in the assembly of primordial follicles. Indeed, in cultured mouse ovaries, we found that

miR-376a transfection reduced oocyte apoptosis (Fig. 4A and B). This is consistent with the increased expression of anti-apoptotic genes and suppression of pro-apoptotic genes (Fig. 4C). Among the apoptosis-associated genes, *Casp3* expression was unchanged. This is consistent with former reports that oocyte death, initiated as a result of either developmental cues or pathological insults, was unaffected by the absence of *Casp3* (Matikainen *et al.* 2001). However, *Casp7*, a pro-apoptotic gene, increased after miR-376a transfection, suggesting a more complicated mechanism of oocyte apoptosis than anticipated. During primordial follicle pool foundation, the pro-survival genes, *Pard6a* and *Lhx8*, contribute to the maintenance of oocytes arrested at the diplotene stage (Choi *et al.* 2008, Wen *et al.* 2009). Increased expression of these oocyte pro-survival genes after miR-376a transfection is consistent with more surviving oocytes. Furthermore, as the culture time increased, there are still more primordial follicles in the miR-376a mimic-transfected ovaries, while the difference in the developing follicle index between experimental and control ovaries decreased, which rules out the possibility that the increased number of primordial follicles after miR-376a mimics transfection are resulted from the postponing oocyte apoptosis. Additionally, the lack of an additive effect on oocyte loss in fetal mouse ovaries after co-transfection of miR-376a mimics with *Pcna* siRNAs (Fig. 4A and B) indicated that miR-376a and *Pcna* function in the same pathway, and functionally confirmed that miR-376a reduced oocyte apoptosis through targeting *Pcna*.

In studies using cultured cells, some microRNAs involved in the regulation of apoptosis have been reported. For example, the miR-34a, b, c, and miR-214 were observed to promote apoptosis by increasing the expression of Caspases, *P21*, *P53*, and *Bax* (Corney *et al.* 2007, Gammell 2007, Zenz *et al.* 2009). miR-210 and miR-155 repressed apoptosis by decreasing the expression of pro-apoptotic Caspases (Wang & Lee 2009). However, all these data are obtained from experiments using cultured somatic cells. Our observations provide the first evidence for the existence of a new mechanism by which an individual microRNA regulates oocyte apoptosis in perinatal mouse ovaries.

In summary, we found that miR-376a functions as a negative regulator of *Pcna* by binding to *Pcna* mRNA 3′ UTR. Overexpression of miR-376a increased primordial follicle assembly by reducing oocyte apoptosis mediated by the downregulation of *Pcna* in perinatal mouse ovaries. To our knowledge, this is the first microRNA–target mRNA pairs that functions during folliculogenesis in mammalian ovaries and further our understanding of the regulation of primordial follicle assembly.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0508.

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